IMMUNOLOCALISATION WITHIN NEURAL TISSUE OF MADM - A NOVEL METALLOPROTEASE WITH A POTENTIAL INTEGRIN-BINDING DOMAIN

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by

Sally A. Mitchell B.Sc. Hons. (Bristol)
MRC Toxicology Unit
University of Leicester

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ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

SALLY A. MITCHELL.

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ABSTRACT

A metalloprotease previously isolated from bovine brain was shown to be a member of a family which includes both mammalian proteins and snake venom metalloproteases containing a potential integrin-binding (disintegrin) domain. The bovine enzyme was named MADM for Mammalian-Disintegrin Metalloprotease (Howard *et al.*, 1996; *Biochemical Journal* **371**; 45-50). The present studies were undertaken to generate antibodies reactive with the rat MADM homologue and to use these for immunolocalisation of the protein in rat central nervous system (CNS).

A truncated rat MADM homologue was initially isolated from a commercial brain cDNA library, and the sequence encoding the mature protein was completed using the technique of 5'-RACE (rapid amplification of cDNA ends). It was found that the rat MADM sequence had a very high degree of identity at both the nucleic acid and the amino acid level with bovine MADM, possibly implying a critical function for this protein.

An antigenicity plot of the mature rat protein was used to design peptides for anti-MADM antibody production. Although the level of immunogenicity was generally low, two specific polyclonal antisera were produced in rabbits, and used in Western blotting experiments to demonstrate the presence of MADM protein in a range of rat tissues and cultured cell lines. Within adult rat CNS, the MADM protein was localised by immunohistochemistry to microglial cells of the white matter. The patterns of expression of β1, β2, β3, and β4-integrin subunits within the CNS were also examined, with the intention of identifying potential candidates for MADM's cognate integrin; only β2 integrins have been shown to co-localise with MADM to microglia.

Ramified microglia of normal adult rat brain were shown to express low levels of MADM, and the protease was not demonstrated to be upregulated by reactive microglia either during early postnatal development or in the vicinity of a cerebral stab wound. Fluorescent confocal microscopy used to examine MADM expression by isolated microglia demonstrated that *in vitro*, MADM was found intracellularly, and could not be induced to move to a cell surface location even on microglia activated by cytokines; this intracellular localisation is in direct contradiction of the structural evidence pointing to a transmembrane position for MADM. However, MADM was found to be expressed on the surface of cultured mouse NS0 myeloma cells, a result which may reflect a facet of the immortalisation process, or may imply that MADM is dependent on specific integrin expression for its surface localisation.

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This thesis is dedicated to my mother, for giving me her whole-hearted support along an often lonely path.

ABBREVIATIONS

A	Absorbance
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
ADAM	A Disintegrin And Metalloprotease containing mammalian enzyme
BBB	Blood-brain barrier
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bp	base pairs
BSA	Bovine serum albumin
BSE	Bovine spongiform encephalopathy
CAM	Cell adhesion molecule
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonatedihydrate
CNBr	Cyanogen bromide
CNS	Central nervous system
con A	concanavalin A
cpm	counts per minute
DAB	Diaminobenzidene
DEPC	Diethylpyrocarbonate
DMF	Dimethylformamide
DMSO	Dimethylsulphoxide
dpm	disintegrations per minute
DTT	Dithiothreitol
EAE	Experimental autoimmune encephalomyelitis
EAP-1	Epididymal Apical Protein-1
EBSS	Earle's balanced salt solution
ECM	Extracellular matrix
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbance assay
ER	Endoplasmic reticulum
FITC	Fluorescein isothiocyanate
g	Relative centrifugal force
G3PDH	Glyceraldehyde-3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
GM-CSF	Granulocyte/monocyte-colony stimulating factor
HORF	Human open reading frame
HRP	Horseradish peroxidase
HTL	High titre lysate
IAP	Integrin-associated protein
Ig	Immunoglobulin
IHC	Immunohistochemistry

IL	Interleukin
IPTG	Isopropylthio-β-D-galactoside
IR	Internal repeat
ISH	*In-situ* hybridisation
kb	kilobases
kbp	kilobase pairs
kDa	kiloDalton
kg	kilogram
KLH	Keyhole limpet hemocyanin
LPS	Lipopolysaccharide
M	Molar
MADM	Mammalian disintegrin-metalloprotease
MAG	myelin-associated glycoprotein
MBP	Myelin basic protein
MBS	3-maleimidobenzoic acid N-hydroxy succinimide ester
MCS	Multiple cloning site
MDBK	Madin Darby bovine kidney cells
MDC	Metalloprotease, Disintegrin, Cysteine-rich enzyme
MHC	Major histocompatibility complex
mg	milligram
ml	millilitre
mm	millimetre
mM	milliMolar
mmol	millimole
μg	microgram
μl	microlitre
μM	micromolar
MMP	Matrix metalloprotease
MOPS	3-(N-Morpholino)propanesulfonic acid
Mr	Relative molecular mass
MS	Multiple sclerosis
MT-MMP	Membrane-type matrix metalloprotease
NBT	Nitroblue tetrazolium
ng	nanogram
NGF	Nerve growth factor
nm	nanometre
NS	Nervous system
nt	nucleotide
O.D.	Optical density
ORF	open reading frame
PA	Plasminogen activator
PAI	Plasminogen activator inhibitor
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pfu	plaque forming units
PLP	Proteolipid protien
pmol	picomole
poly-A	Polyadenylation
RACE	Rapid Amplification of cDNA Ends
RGD	Arg-Gly-Asp
rpm	revolutions per minute
RT	Room temperature
RT-PCR	Reverse transcription-polymrase chain reaction
SCR	Short consensus repeat
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SSC	Saline buffered sodium citrate
TBS	Tris buffered saline
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-Tetramethylethylenediamine
TGF	Transforming growth factor
TIMP	Tissue inhibitor of metalloproteases
TM	Transmembrane
TNF	Tumour necrosis factor
TPA	12-ortho-tetradecanoyl-phorbol-13-acetate
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
UTS	Untranslated sequence
UV	Ultraviolet

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CHAPTER 1. INTRODUCTION

1.1 The central nervous system (CNS)

Although the basic cellular processes of the CNS are not unique, the interactions between cell types, such as synaptic contacts between neurones and myelin sheaths around axons are peculiar to this tissue. These specialisations and those allowing sequestration of the CNS from the outer environment (the blood-brain barrier and the absence of lymphatics) become major issues in considerations of normal and disease processes in the nervous system.

Brain has often been considered as an immunologically privileged organ, not normally accessible to leukocyte traffic. This is in part due to the presence of the blood-brain-barrier (BBB), consisting of specialised microvasculature and surrounding astrocytes, which restricts the exchanges between blood and brain. However, more recent studies have revealed that activated leukocytes can cross into the CNS, at very low levels under normal conditions, in much higher numbers during neuropathological disorders like multiple sclerosis (MS) or viral infection, and within brain parenchyma can interact with CNS cells (reviewed by Couraud, 1994). The recruitment of circulating leukocytes into inflammatory lesions in any tissue requires adhesion to vascular endothelium, followed by migration between endothelial cells into the underlying tissues. This is complicated in the CNS by the existence of a specialised microvasculature, characterised by the presence of a continuous network of complex tight junctions, and expression of asymmetric transport systems and specific enzymes. This BBB is under the control of surrounding astrocytes, and limits exchange between the blood and brain of soluble substances such as hormones, growth factors, cytokines and immunoglobulins (Pardridge, 1988).

CNS parenchyma is made up of nerve cells and their dendrites and axons, all closely enveloped by glial cells (oligodendrocytes and astrocytes). In addition the CNS parenchyma contains blood vessels, macrophages (pericytes) and microglial cells.

Neurones can be excitatory, inhibitory, or modulatory in their effect; motor, sensory or secretory in their function. This is reflected in the large variation of

dendritic and axonal outgrowth (reviewed by Raine, 1994). Specialisation is also evident at axonal terminals, where a wide variety of junctional complexes (synapses) exist. The axon emerges from a neurone as a slender thread and frequently does not branch until it nears its target. In contrast to dendrites and the soma, the axon is myelinated, increasing its efficiency as a conducting unit. Myelin, a spirally wrapped membrane, is laid down in segments by oligodendrocytes in the CNS, separated by the nodes of Ranvier, or naked regions of axon, where Na^+ channels are concentrated and the action potential propagates (Doyle and Colman, 1993).

The afferent components of neurones, dendrites, are frequently arranged about the neuronal soma in a stellate fashion. Axons and dendrites emerging from different neurones intercommunicate by means of specialised junctional complexes known as synapses, and either with electrical or a variety of chemical signals (Raine, 1994).

In the CNS there are also three groups of **glial cells** - astrocytes and oligodendrocytes (the macroglia) which are of ectodermal origin, and the microglia which are of mesodermal origin, and which invade the CNS during embryonic and early post-natal life via the vasculature and the meninges. Glial cells differ from neurones in that they possess no synaptic contacts and retain the ability to divide throughout life, particularly in response to injury (reviewed by Raine, 1994).

Astrocytes play an important role in brain metabolism, and have traditionally been divided into protoplasmic and fibrous subgroups. Although these two groups could represent opposite ends of a spectrum, it has been suggested that the two groups may arise from different progenitors, and that the progenitor of the fibrous astrocyte is the same as that of the oligodendrocyte (Raff *et al.*, 1983). Protoplasmic astrocytes are frequently located in grey matter in relation to capillaries, while the fibrous astrocyte occurs in white matter. Desmosomes and gap junctions occur between adjacent astrocytic processes and the outer membranes of astrocytes adjacent to blood vessels possess a specialised thickening.

A major role for astrocytes is concerned with repair. Subsequent to trauma, astrocytes proliferate and become fibrous; this state of gliosis may be total, in which case all other components are lost, leaving a glial scar, or it may be a generalised response occurring against a background of regenerated or normal CNS parenchyma

(Chiang *et al.*, 1994).

Another putative role of the astrocyte is its involvement in transport mechanisms and in the BBB system, but studies seem to indicate that astrocytic end-feet provide little resistance to the movement of molecules in blood vessels, and that blockage of material passing into the brain occurs instead at the endothelial cells lining the vessels (Reese and Karnovsky, 1967). Finally it is believed that astrocytes are responsible for the regulation of local pH levels and local ionic balances.

Oligodendrocytes are myelin-producing cells in the CNS, and as with the astrocytes, are highly variable, differing in location, morphology and function. The three broad classes of oligodendrocytes correspond to satellite, intermediate and myelinating. Satellite oligodendrocytes are restricted to grey matter, are closely linked to the surface of neurones and are assumed to play a role in neuronal maintenance. Intermediate forms are regarded as potential satellite or myelinating oligodendrocytes. Myelinating oligodendrocytes are capable of producing many internodes of myelin simultaneously. However, they possess a slow mitotic rate and poor regenerative capacity (Pfeiffer *et al.*, 1993). The oligodendrocyte is potentially highly vulnerable to immune-mediated damage since it shares with the myelin sheath many molecules with known affinities to elicit specific T and B cell responses which lead to its destruction, for example, myelin basic protein (MBP), proteolipid protein (PLP), and myelin-associated glycoprotein (MAG) (Bernard and Kerlero de Rosbo, 1992). Damage to only a few oligodendrocytes can be expected to produce an appreciable area of primary demyelination, and in most CNS diseases in which myelin is a target, oligodendrocytes are known to be among the most vulnerable elements and the first to degenerate.

Oligodendrocytes are derived from precursor cells that, in response to specific growth factors, divide, migrate in the developing white matter, and eventually myelinate axons (Doyle and Coleman, 1993). Oligodendrocytes originate as neuroectodermal cells of the subventricular zones, and must migrate and mature into post-mitotic myelin-producing cells in distinct phenotypic stages characterised by proliferative capacity, migratory abilities and morphological changes (Warrington and Pfeiffer, 1992).

The **microglial** cell is the resident macrophage of the CNS. However, microglia are usually in a less activated state than their peripheral tissue counterparts (Thomas, 1992) and are seen as ramified (resting) microglia, constituting between 5-20% of the neuroglial population, though less numerous in white than in grey matter (Lawson *et al.*, 1990). During normal wear and tear, as well as during the resorbtion and remodelling of fibre tracts that characterise brain development, CNS components degenerate and apoptose and microglia phagocytose the debris (Fujimoto *et al.*, 1989). In a number of disease instances such as trauma, microglia are known to be stimulated to become activated and phagocytic, and migrate to the area of injury where they engage in the removal of cellular and matrix debris (Kaur *et al.*, 1987). There is evidence for a role for microglia in a wide range of conditions: in MS and experimental autoimmune encephalomyelitis (EAE), microglia are thought to play a major role in myelin destruction and antigen presentation (Woodroofe *et al.*, 1986; Hayes *et al.*, 1987); they appear to be prominently involved in such pathological processes as acquired immunodeficiency syndrome (Williams *et al.*, 1994a), prion diseases (Betmouni *et al.*, 1996), and the degenerative disorders Alzheimer's and Parkinson's disease (reviewed by Banati and Graeber, 1994 and Barron, 1995).

It is known that the activation state and differentiation of microglia *in vitro* can be influenced by the presence of various matrix proteins: fibronectin appears to influence microglia towards a resting, ramified state, and laminin towards activation and migration (Chamak and Mallat, 1991). Microglia express class II MHC upon activation (Hickey and Kimura, 1988), and are also producers of a number of cytokines e.g.: IL1 (Giulian *et al.*, 1986); TNF (Sawada *et al.*, 1989); IL6 (Frei *et al.*, 1989); NGF (Mallat *et al.*, 1989), with known effects upon T-cells as well as astrocytes (Giulian and Lachman, 1985). Added to this is the ability of activated microglia to secrete matrix metalloproteases, suggesting that these enzymes may also have important functions in brain parenchyma during inflammatory states (Colton *et al.*, 1993; Yamada *et al.*, 1995a).

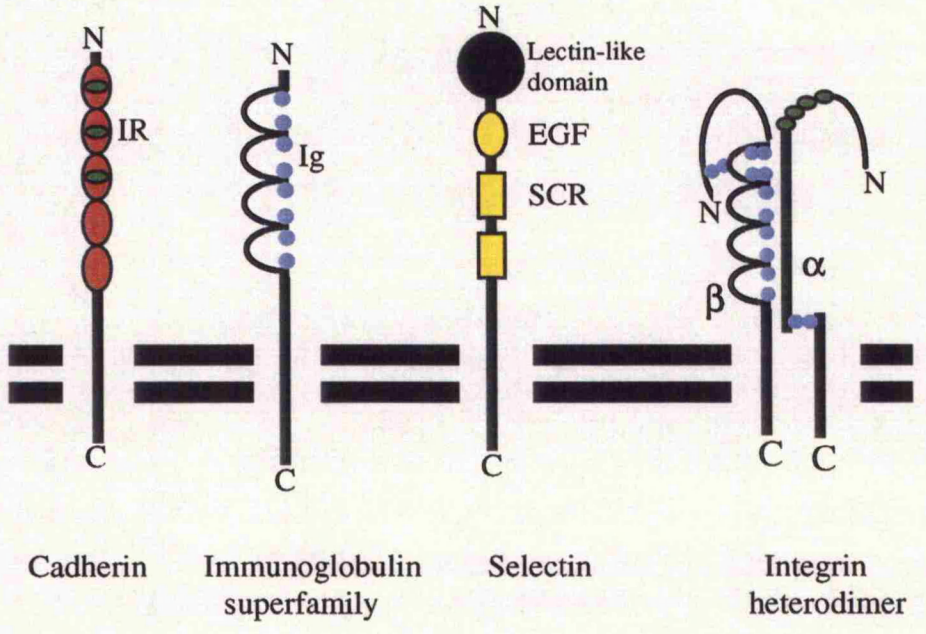
Despite the fact that in the adult CNS mature neurones do not divide, and that the system would appear with the presence of the blood-brain barrier to be essentially a closed system, this tissue is not static and many types of cell-cell and cell-extracellular

matrix (ECM) interactions are continually going on. Further, in the developing and diseased CNS, other processes such as cell migration and extracellular proteolysis have major roles to play. Study of the proteins mediating these processes is thus of great interest, not least because they may constitute targets for therapeutic intervention in various neuropathological conditions.

1.2 Cell Adhesion Molecules (CAMs)

There are 4 major families of cell adhesion receptor molecules: the cadherins, the immunoglobulin superfamily, the selectins and the integrins (figure 1.1). Many CAMs were first discovered in neural tissue, and much of the analysis of these large glycoproteins has been made in neural cells and during development of the nervous system.

Figure 1.1 Schematic of the four major classes of cell adhesion molecules
IR is an internal repeat sequence which may contain a calcium-binding motif; Ig is an immunoglobulin domain; EGF is an epidermal growth factor motif; SCRs are short consensus repeats; disulphide-bonded cysteines are shown as blue dots; calcium binding sites are shown as green filled circles. Not to scale.



The cadherins are calcium dependant cell-cell adhesion molecules which are present on most cells (Takeichi, 1988). There are a number of different types known (Suzuki *et al.*, 1991) and they mediate homophilic adhesion between cells. Indeed cells expressing different cadherins will segregate from one another to form separate aggregates (Takeichi *et al.*, 1990).

The immunoglobulin superfamily are a large family of molecules including nerve cell adhesion molecule (NCAM) and also many which are found on lymphocytes (ICAM, VCAM etc.). The members of this family are typified by immunoglobulin repeat sequences, and often also by fibronectin type III repeats which are also found in cytokine receptors (Bazan, 1990) and in several ECM proteins. Some Ig super family adhesion receptors are thought to be homophilic, particularly those expressed in the CNS e.g.: NCAM in vertebrates (Cunningham *et al.*, 1987), although it is also known that NCAM binds to heparan sulphate proteoglycans (Cole, 1986).

Selectins are expressed on blood and endothelial cells and three types (L-, E-, and P-selectin) are known (Bevilacqua, 1991). Selectins typically mediate heterotypic interactions between/among blood and endothelial cells during such processes as leukocyte adhesion before infiltration of the CNS and migration to sites of inflammation. Generally cells of the normal or developing CNS have not been shown to express selectins, although L-selectin-positive cells have been found in the brains and spinal cords of EAE mice (Dopp *et al.*, 1994).

Integrins are a large family of heterodimeric proteins that always consist of an α subunit coupled non-covalently to a β subunit. These large dimers have an extensive extracellular region with several calcium binding sites, a transmembrane region, and a small intracellular tail that attaches to the cytoskeleton in conjunction with several accessory proteins such as talin and α-actinin (reviewed by Jones, 1996). At least nine β subunits have been described so far, and each of these associates with a specific subset of the 16 (so far) α subunits (Hynes, 1992). Some mediate cell-cell interactions by binding to Ig superfamily members on other cells (Springer, 1990), while many others mediate cell-matrix interactions. In addition to mediating cell-cell and cell-ECM interactions during cell adhesion and migratory events, the integrin family of

receptors also function as transmembrane signal inducers (Hynes, 1992). Many integrins recognise the Arg-Gly-Asp (RGD) region as a binding site, a common sequence found in ECM molecules including fibronectin, vitronectin, various collagens and laminin. Integrins mediate many cell-matrix interactions during cell adhesion to basement membranes, other extracellular matrices, and also during cell migrations. Most cells express several integrins and are therefore able to adhere to several adhesive ECM proteins.

Integrin functions have been shown to be regulated on several levels. Both fibronectin and laminin receptors are dynamically regulated on neurones and seem likely to be important in regulating changes in neuronal behaviour in vivo (reviewed by Reichardt and Tomaselli, 1991). Growth factors and oncogenes have been shown to regulate integrin subunit expression in vitro. $\alpha 1$ is strongly induced by NGF on PC12 pheochromocytoma cells (Rossino *et al.*, 1990), as are the cell adhesion molecules L1 and NCAM (Prentice *et al.*, 1987).

1.3 ECM and extracellular proteolysis

The ECM is a multifunctional complex of proteins and proteoglycans, assembled in a highly organised manner that contribute to providing the structural integrity of an organism. The ECM is important in many biological processes such as development (Reichardt and Tomaselli, 1991), tumour invasion (Mignatti and Rifkin, 1993), and tissue remodelling (Woessner, 1991). Cell-ECM interactions influence cell proliferation, differentiation, adhesion and migration.

Various factors are involved in maintaining the integrity of the ECM. The proteolytic activities of matrix metalloproteases (MMPs) and plasminogen activators (PAs) and their inhibitors are one such factor. In the CNS, proteolysis of the ECM is involved in neuronal cell migration in the developing cerebellum and in neurite outgrowth (Moonen *et al.*, 1982). Likewise in pathological conditions such as brain tumour growth and invasion in the development of astrocytomas and meningiomas, leukocyte infiltration and trafficking in inflammatory diseases such as MS and viral encephalitis, and in nerve demyelination, matrix-degrading proteases and their inhibitors have been implicated (reviewed by Romanic and Madri, 1994).

MMPs are a group of zinc dependant enzymes that degrade molecules of the ECM such as proteoglycans, glycoproteins and various types of collagen (Matrisian, 1990). They are defined by the following criteria: they are able to degrade at least one component of the ECM; they bind a zinc ion which is required for enzymic activity; they are secreted as inactive pro-forms which require activation; proteolytic activity of MMPs is inhibited by specific tissue inhibitors of metalloproteases (TIMPs); they share regions of amino acid similarity (Nagase et al., 1992).

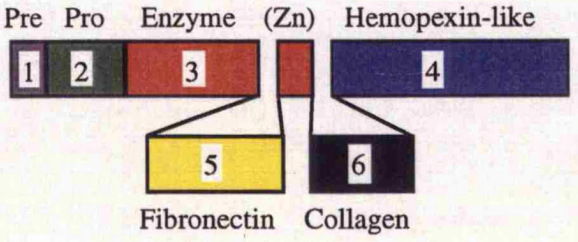
There are 3 broad families of MMPs, based on substrate specificity (reviewed by Alexander and Werb, 1991): interstitial collagenases, gelatinases and stromelysins (see table 1.1). **Collagenases** degrade interstitial or fibrillar collagen: collagen type I, found in bone, tendon and skin; collagen type II, present in cartilage and vitreous humour; collagen type III, associated with foetal skin, placenta and elastic tissues such as aorta (reviewed by Weiss, 1984). Collagen is extremely resistant to proteolysis, so collagenases are essential for initiating fibrillar collagen breakdown to form gelatin, which can then be further degraded by other enzymes (Alexander and Werb, 1991). **Gelatinases** degrade collagen type IV (a component of basement membranes) and type V (pericellular collagen) as well as gelatin (denatured collagen) and elastin. **Stromelysins** degrade many components of the ECM including proteoglycans, fibronectin, laminin and gelatins. Stromelysin degrade macromolecules to give soluble products.

MMPs are composed of several functional protein domains (table 1.1) and analysis of the structure of their genes suggests that members of this family have evolved by gene-duplication and exon-shuffling (Matrisian, 1992). MMPs and their inhibitors have been localised in cells of the CNS (Apodaca *et al.*, 1990). Most MMP genes are not constitutively expressed, and transcriptional activation may occur as a result of agents including cytokines (e.g.: TGFβ), phorbol esters and oncogene expression (Matrisian *et al.*, 1985; Angel *et al.*, 1987). Finally, extracellular factors such as cell-cell and cell-ECM interactions can also affect MMP synthesis and secretion. It has been demonstrated that engagement of various integrin receptors by their ligands induces MMP expression (Werb *et al.*, 1990). Adhesive and migratory events during development, metastasis and inflammation have been shown to correlate with changes

Table 1.1 Matrix metalloproteases (adapted from Matrisian, 1992)

A. Domain structure of MMPs

Predomain 1 is a signal sequence; prodomain 2 contains the residues which maintain MMPs in an inactive state; enzyme domain 3 includes the residues which bind the catalytic zinc; the hemopexin-like domain (4) is found in all MMPs with the exception of MMP-7. In MMP-2 and -9, the catalytic domain is interrupted by a fibronectin-like domain (5), and MMP-9 also possesses a domain (6) which has homology to type V collagen.



B. Subfamilies of MMPs

MATRIXIN	SUBSTRATES	DOMAINS
Collagenases:		
MMP-1 (54.1 kDa) (fibroblast collagenase)	Collagens I, II, III, VII, X	1, 2, 3, 4
MMP-5 (53.4 kDa) (neutrophil collagenase)	Collagens I, II, III	1, 2, 3, 4
Gelatinases:		
MMP-2 (73.9 kDa) (gelatinase A)	Gelatins, collagens V, IV, and elastin.	1, 2, 3, 4, 5
MMP-9 (78.4 kDa) (gelatinase B)	Gelatins, collagens IV, V, and elastin.	1, 2, 3, 4, 5, 6
Stromelysins:		
MMP-3 (54 kDa) (proteoglycanase)	Proteoglycans, fibronectin, laminin, gelatins, collagens III, IV, V, IX.	1, 2, 3, 4
MMP-7 (27.7 kDa) (matrilysin, pump-1)	Proteoglycans, fibronectin, gelatins, collagen IV, elastin.	1, 2, 3

in ECM synthesis and deposition, integrin expression and protease-inhibitor expression (Chen, 1992).

MMP activity is also regulated at the post-transcriptional level. The active protease is inhibited by specific tissue inhibitors of matrix metalloproteases (TIMPs) which are secreted by the same cell types (Stricklin and Welgus, 1983; Stetler-Stevenson *et al.*, 1989). The MMPs are initially secreted as inactive pro-forms which require extracellular proteolytic activation (Springman *et al.*, 1990). In the case of collagenase (MMP-1) and stromelysin (MMP-3) this appears to be achieved by plasminogen activators (Woessner, 1991). Pro-MMP-2 appears to be activated by a novel membrane-type metalloprotease (MT-MMP, Sato *et al.*, 1994).

Other enzymes involved in matrix degradation are the plasminogen activators (PAs) (Vassalli *et al.*, 1991). These are serine proteases, which degrade plasminogen to plasmin, itself another serine protease capable of degrading a broad range of molecules including fibrin, fibronectin, laminin and MMP precursors. Thus PAs and plasmin can act to amplify a cascade of ECM proteolysis via MMP activation (He *et al.*, 1989). PAs, like MMPs, are regulated by specific inhibitors PAI-1 and -2, as well as at the level of transcription. Phorbol esters, cytokines, glucocorticoids and growth factors have all been shown to affect PA and PAI gene expression (Waltz *et al.*, 1993). It has also been suggested that the low levels of PAs found in the mature cerebellum may play a role in the processing of growth factors and neurotransmitters, independent of their effects on the ECM (Verrall and Seeds, 1989).

Elastase is another serine protease which may also be important in the CNS. Microglia in culture have been shown to secrete elastase or an elastase-like protease into culture media, as assessed by Western blotting (Nakajima *et al.*, 1992). It is possible that microglia-derived elastase could play a role in degrading macromolecules in pathological lesions, in morphogenesis during brain development, in regulation of neurite growth or regeneration through degradation of ECM, as well as in normal tissue turnover of the extracellular environment.

1.4 Cell-cell and cell-ECM interactions in the CNS

The most elaborate use of cell-surface and extracellular matrix molecules in cellular targeting is seen in the developing nervous system. The highly specific

synaptic connections characteristic of the nervous system arise as a result of guided cell migration and axon growth. Studies have revealed tremendous diversity in the molecules that regulate the development of cells in the nervous system. These include chemotrophic factors (e.g.: NGF), cell adhesion molecules such as cadherins, Ig superfamily molecules, integrins (selectins have not so far been shown to be present in developing NS) and molecules of the ECM (laminin and fibronectin). Each class of molecule has now been shown to influence major steps in the development of the NS, including neuronal survival, migration, axonal growth and guidance, synapse formation, and glial differentiation (reviewed by Reichardt and Tomaselli, 1991). Where the NS distinguishes itself from other developing tissues is in the diversity of expression of such molecules. For example, the majority of known Ig superfamily members are expressed in the NS (reviewed by Grumet, 1991), as are the majority of known cadherins (Suzuki *et al.*, 1991), as well as many members of the integrin family (Grumet, 1991). Both neuronal cells and glial cells are guided as they migrate, as are growing axons, by many of these adhesive molecules. Molecules such as NCAM and the cadherins not only appear to be involved in holding the cells of the NS together, but also serve as substrata that promote the outgrowth of neurites. Molecules such as L1 are widely distributed on axons and appear to play a widespread role in axon bundling or fasciculation. Many other Ig superfamily members are more restricted in their distribution, and probably mediate the specific fasciculation of certain groups of axons (reviewed by Hynes and Lander, 1992).

The adhesion receptors implicated earliest in development are the cadherins. In the mouse embryo, E-cadherin plays a key role in compaction, a cell-cell adhesion event leading to polarisation of the cells of the early blastocyst (Takeichi, 1988, 1990). N-cadherin is first expressed at gastrulation, and differential expression of cadherins plays a causal role in the segregation of tissues.

It is also clear that cell-matrix adhesion mediated by integrins plays a role in cell migration during gastrulation and neural crest migration, while the ECM protein fibronectin is strongly expressed in areas where this cell migration occurs (Thiery *et al.*, 1989). The alpha 6 integrin subunit is only weakly expressed in the adult CNS, but may play an important role in embryogenesis. The interaction between $\alpha 6$ and its ligand laminin seems to be essential for cell differentiation in the foetus, and for

subsequent maintenance of tissue integrity in the adult. α6 is found in the nerve cells of the developing embryo, but not in the nerve cells of the adult organism (Terpe *et al.*, 1994), and may be involved in the migration of nerve cells or axonal outgrowth, possibly guided by laminin (Baier and Bonhoeffer, 1991).

Protease action is also important in the developing brain. During the development of the cerebellum, granule neurones migrate from the cerebellar surface, through a Purkinje cell monolayer, and finally differentiate to form the mature internal granule layer (Soreq and Miskin, 1983). Various investigations have demonstrated, both in vivo and in vitro, that PAs are produced and secreted by granule neurones and contribute to the cells ability to migrate inward (Verrall and Seeds, 1989). PAs and MMPs have also been shown to be secreted by foetal astrocytes, possibly facilitating astrocyte migration during development (Apodaca *et al.*, 1990).

There are many similarities between the events occurring during development and those which occur during disease processes. Inhibition of cadherins expressed by cells in culture can convert these cells to an invasive, migratory phenotype (Behrens *et al.*, 1989). Thus cells expressing cadherins adhere to one another, while those that lose the expression separate as single migratory cells. Loss of specific integrins can also produce reduced adhesion to ECM, and can thus contribute to malignant transformation (Plantefaber and Hynes, 1989).

Brain macrophages, including the resident microglial cells and the monocytes infiltrating the BBB, are implicated in the generation of the intra-CNS immune response. Adhesion proteins belonging to the β1, β2 integrin and Ig superfamily participate in many immune and inflammatory functions of macrophages. Thus the αLβ2/ICAM-1 interaction is involved in antigen presentation, whereas receptors for complement (CR3, CR4) are implicated in phagocytosis (Hynes, 1992). Adhesion molecules also have a major role in the adhesion of monocytes to either neurones and astrocytes or to endothelial cells and thus in the control of mononuclear cell migration through the BBB and CNS parenchyma (Sloan *et al.*, 1992). The expression of these molecules on CNS cells is still poorly understood, with only scant data available (Akiyama and McGeer, 1990; Birsdall *et al.*, 1992), although it is known that cytokine activation of microglia by IL1α and TNFα causes increased expression of molecules such as ICAM-1 and VCAM-1 (Sebire *et al.*, 1993).

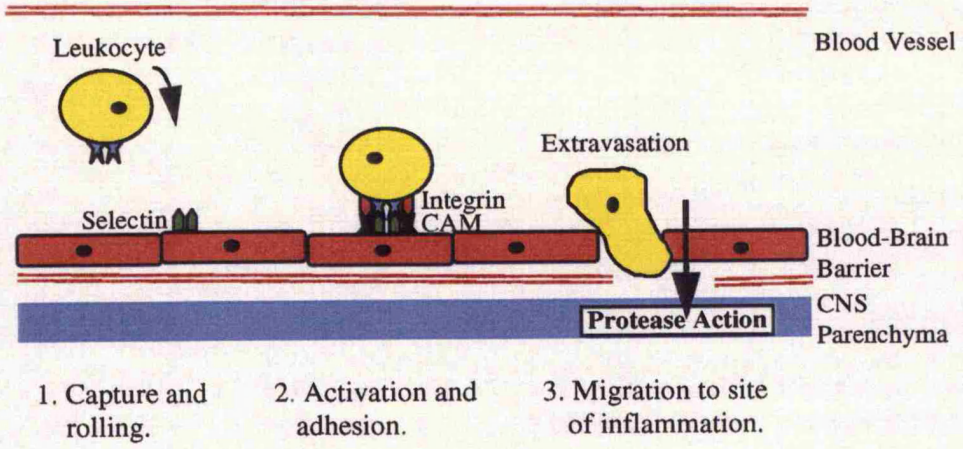
Activated microglia are also known to produce MMPs, such as MMP-9. It has been shown that MMP-9 is present in cerebrospinal fluid obtained from MS patients (Gijbels, 1992) and also in fluid obtained from mice with EAE (Gijbels, 1993), although it is unknown whether microglia are the source of this protease in vivo. It is possible that MMP-9 is important in glioma invasion of brain parenchyma by degrading the basement collagen of blood vessels, but other possible functions include the potential activation of other matrix-degrading enzymes (Reith and Rucklidge, 1992) and turnover in angiogenesis following brain injuries such as stroke. MMP-2 has been localised to white matter microglia in vivo (Yamada *et al.*, 1995a), and it has been suggested that MMP-2, itself under the control of MT-MMP which is also found in white matter microglia (Yamada *et al.*, 1995b), may be a processing enzyme for the degradation of β-amyloid protein in the formation of senile plaques (Bignami *et al.*, 1994). Integrins have also been localised to reactive microglia in Alzheimer's disease tissue (McGeer *et al.*, 1991).

The local invasion of brain tissue by tumours such as gliomas has also been associated with matrix-degrading proteinases that participate in proteolytic breakdown of the ECM. It has been shown that glioma cells secrete an array of MMPs and PAs that can facilitate growth and invasion (Apodaca *et al.*, 1990). Protease inhibitors are also secreted, in various amounts, by glioma cells (Halaka *et al.*, 1983); the balance between ECM-degrading proteases and their inhibitors is one of the determining elements in the extent of tumour growth and invasion (Mignatti *et al.*, 1986).

Leukocyte infiltration into the CNS during inflammatory disease such as MS requires both adhesion events and proteolysis to be successful (figure 1.2). Initially, through the action of selectins expressed by vessel endothelial cells, circulating leukocytes transiently attach to endothelium (Bevilacqua, 1993), and roll along, sampling the local environment. If they encounter appropriate activation signals, this weak interaction is strengthened and stabilised by binding of αLβ2 (LFA-1) and α4β1 (VLA-4) expressed on the leukocyte to ICAM-1 and VCAM-1 respectively, expressed by endothelium (Springer, 1994). Finally the leukocytes flatten and migrate through the endothelium following chemotactic signals present in the CNS parenchyma (reviewed by Hartnung *et al.*, 1995). It is proposed that the extravasation and subsequent migration is facilitated by secretion of MMPs and PAs by the leukocytes,

resulting in degradation of the basement membrane and interstitial matrix of the brain. Leukocytes such as T-cells, neutrophils and monocytes have been demonstrated to secrete MMP-2 and MMP-9 which could perform this function (Montgomery *et al.*, 1993). It has also been proposed that in inflammation and metastasis, infiltrating leukocytes and activated brain tissue secrete matrix-degrading proteases that contribute to the breakdown of the BBB as well as cause damage to other areas of the CNS (Rosenberg *et al.*, 1993).

Figure 1.2 Leukocyte extravasation during disease processes in the CNS
A process involving selectins, CAMs, integrins and proteases.



1.5 Background to the project

A metalloprotease which degrades myelin basic protein in brain myelin membrane preparations was isolated from bovine brain and partially characterised (Glynn *et al.*, 1987; Chantry *et al.*, 1989). This enzyme had biochemical and enzymic properties distinct from previously described mammalian metalloproteases. A monoclonal antibody raised against this purified enzyme was used to examine bovine CNS tissue sections. The antibody appeared to detect the enzyme in oligodendrocytes but not in neurones or astrocytes (Chantry *et al.*, 1992), raising the possibility that this enzyme could be involved in some aspect of the myelination process.

Figure 1.3 Domain and sequence similarities between bovine MADM and the snake venom haemorrhagic proteins

The amino acid sequence is aligned with that of Jararhagin (Paine *et al.*, 1992) and HR1B (Takeya *et al.*, 1990) with gaps introduced to maximise alignment. The initial alignment was performed with the GCG programme PILEUP, then refined by hand. Extended zinc-binding site is in bold red, disintegrin-binding sequence in bold blue, and conserved residues in pink. Diagrammatic representation of the sequence alignment highlights the different domains.

```
MADM  MVLLRVLILL LSWVAGLGGQ YGNPLNKYIR HYEGLSYDVD SLHQKHQRAK RAVSHEDQFL   60
JAR   ---------- ---------- ---------- ---------- -----ATRPK GAVQPKYEDA

MADM  -RLDFHAHGR HFNLRMKRDT SLFSEEFR-- --VETSNAVL DYDTSH---I YTGHIYGEEG  112
JAR   MQYEFKVNGE PVVLHLEKNK GLFSKDYSEI HYSPDGREIT TYPPVEDHCY YHGRI-ENDA

MADM  SLAMGLLLME DLKDSFRLMV ARFYVEPAER YIKDRTLPFH SVIYHEDDIK YPHKYGPQGR  172
JAR   DSTASISACN GLKGYFKLQR ETYFIEP--- ---------- --------LK LPDSEAHAVF

MADM  CADHSVFERM RKYQMTGVEE VTQTPQEKHA INGPELLRKK RTTVAEKNTC QLYIQTDHLF  232
JAR   -KYE-NVEKE DEAPKMCG-- VTQNWKSYEP IKKASQLAFT A----EQQRY DPYK-----Y
HR1B                                              EQRFP RRY-------

MADM  FKYYGTRE-A VIAQISSHVK AIDT-IYQTT DFSG------ IRNISFMVKR IRINTTADEK  284
JAR   IEFFVVVDQG TVTKNNGDLD KIKARMYELA NIVNEIFRYL YMHVALVGLE IWSNGDKITV
HR1B  IKLAIVVDHG IVTKHHGNLK KIRKWIYQLV NTINNIYRSL NILVALVYLE IWSKQNKITV

MADM  DPTNPFRFPN IGVEKFLELN SEQNHDDYCL AYVFTDRDFD DGVLGLAWVG APSGSSGGIC  344
JAR   KPDVDYTLNS FAEWRKTDLL TRKKHDN--- AQLLTAIDFN GPTIGYAYIG S-------MC
HR1B  QSASNVTLDL FGDWRESVLL KQRSHD--C- AQLLTTIDFD GPTIGKAYTA S-------MC

MADM  EKSKLYSDGK KKSLNTGIIT VQNYGSHVPP KVSHITFAHE VGHNFGSPHD SGTECTPGES  404
JAR   HPKR------ ------SVGI VQDYSPI--N LVVAVIMAHE MGHNLGIHHD TGS-CSCGDY
HR1B  DPKR------ ------SVGI VQDYSPI--N LVVAVIMTHE MGHNLGIPHD GNS-CTCGGF

MADM  K-NLGQKENG NYIMYARATS GDKLNNNKFS LCSIRNISQV LEKKRNNCFV -ES------G  456
JAR   PC-------- --IMGPTIS- --NEPSKFFS NCSYIQCWDF IMNHNPECII NEPLGTDIIS
HR1B  PC-------- --IMSPMIS- --DPPSELFS NCSKAYYQTF LTDHKPQCIL NAPSKTDIVS

MADM  QPICGNGMVE QGEECDCGYS DQCKDECCYD ANQPEGKKCK LKPGKQCSPS QGPCCTAHCA  516
JAR   PPVCGNELLE VGEECDCGTP ENCQNECC-- ----DAATCK LKSGSQC--G HGDCCE-QCK
HR1B  PPVCGNELLE AGEECDCGSP ENCQYQCC-- ----DAASCK LHSWVKC--E SGECCD-QCR

MADM  FKSKTEKCRD -DSDCAKEGI CNGITALCPA SDPKPNFTDC NRHTQVCING QCAGSICEKH  575
JAR   FSKSGTECRA SMSECDPAEH CTGQSSECPA DVFHKNGQPC LDNYGYCYNG NCPIMYHQCY
HR1B  FRTAGTECRA AESECDIPES CTGQSADCPT DRFHRNGQPC LYNHGYCYNG KCPIMFYQCY

MADM  GLEECTCASS DG---KD-DK ELCHVCCMK- --KMEPST-- --------CA STGSVQWN--  616
JAR   ALFGADVYEA EDSCFKDNQK GNYYGYCRKE NGKKIPCAPE DVKCGRLYCK DNSPGQNNPC
HR1B  FLFGSNATVA EDDCFNNNKK GDKYFYCRKE NEKYIPCAQE DVKCGRLFCD -NKKY---PC

MADM  KYFLGRTIT- ---LQPGSPC NDFRGYCDVF MRCRLVDADG P                      653
JAR   KMFYSNDDEH KGMVLPGTKC ADGKV-CSNG H-CVDV-ATA Y
HR1B  HYNYSEDLDF -GMVDHGTKC ADGKV-CSNR Q-CVDV-NEA Y
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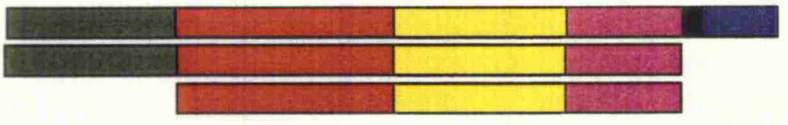


■, pre-prosequence (residues 1-213 of MADM); ■ enzyme domain (residues 214-455); □, disintegrin domain (residues 456-550); ■ cysteine-rich region (residues 551-653); ■, transmembrane domain; ■ cytoplasmic tail.

1.5.1 Isolation of the bovine brain metalloprotease cDNA clone

Purified bovine metalloprotease was digested with trypsin, the peptides separated by HPLC and N-terminally sequenced. Seven tryptic peptide sequences were obtained and redundant oligonucleotide probes were designed from two of these. These probes were used to screen a λgt10 bovine brain cDNA library (done by P.Glynn and S.Griffiths). A 0.9kbp fragment was isolated and subcloned into the pUC9 vector before sequencing. This fragment turned out to encode the N-terminus of the mature protein, as well as three of the seven tryptic peptides. The fragment terminated with an *Eco*RI site, and had neither a polyadenylation signal or a poly-A tail, suggesting a truncated clone.

This 0.9kbp truncated clone was used to rescreen the library (L.Howard), and a clone was isolated which contained an insert of 2.4kbp. On digestion with *Eco*RI the insert yielded fragments of 1.5 and 0.9kbp. This cDNA insert was excised and inserted into the pUC18 vector at *Hin*dIII and *Bam*HI sites. When sequenced, the 5'-end of this cDNA was seen to be identical to the 0.9kbp clone isolated by P.Glynn.

1.5.2 Homology of this novel bovine enzyme to other proteins

A search of the GenBank database in February 1993 showed the deduced protein sequence of the enzyme to be novel, but also to have significant homology (23.5% identity over the region of overlap) with Jararhagin, a metalloprotease present in the venom of the pit viper *Bothrops jararaca* (Paine *et al.*, 1992). Jararhagin and other closely related snake venom proteins have a four domain structure (Kini and Evans, 1992): a preprosequence; an enzyme domain including a catalytic zinc-binding site; an integrin-binding domain known as the disintegrin domain; and a cysteine-rich C-terminal domain (figure 1.3).

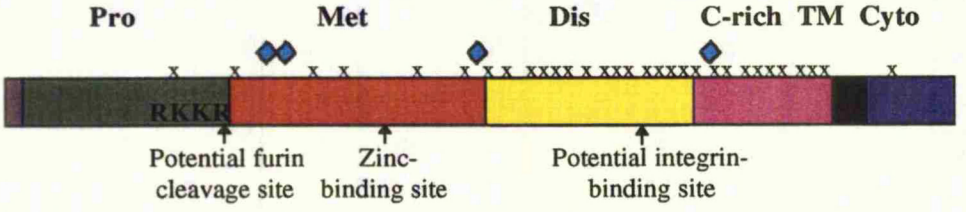
The region of Jararhagin with the greatest similarity to the new bovine brain metalloprotease was the disintegrin domain (36.6% identity), indicating the presence of such a domain in the bovine protein. For this reason the bovine protein was named **MADM** for Mammalian Disintegrin-Metalloprotease.

Unlike Jararhagin, however, MADM contained 6 domains, of which the first four were similar to those of the snake venom proteins, but with the addition of a potential transmembrane domain and a cytoplasmic tail (Howard *et al.*, 1996).

1.5.3 Domain structure of the bovine MADM protein

The 2.4kbp cDNA clone isolated by L.Howard had 16 nucleotides of untranslated sequence (UTS) at the 5'-end, 130 nucleotides of UTS at the 3'-end and contained a long open reading frame encoding a protein of 748 amino acids in length. The N-terminal of the mature protein, as determined by Edman sequencing, is found at residue Thr 214 and the mature enzyme has a predicted molecular weight of 59.2kDa. There are also 4 potential glycosylation sites, of which at least one (Asn 278) is known to be modified (figure 1.4).

Figure 1.4 Schematic diagram showing the domain structure and important features of bovine MADM including the 6 domains, conserved cysteine residues and potential glycosylation sites.



Pro = pro-domain, thought to be cleaved before secretion by furin-like proteases in the golgi.
Met = metalloprotease domain containing extended zinc-binding sequence.
Dis = disintegrin domain containing putative integrin binding site.
C-rich = domain containing many conserved cysteine residues, important for conformation.
TM = putative transmembrane domain.
Cyto = short cytoplasmic domain, rich in proline and basic residues.

x = conserved cysteine residue.
◆ = potential glycosylation site.

The first domain of the MADM protein is a **prepropolypeptide** of 213 residues, initiated by a typical eukaryotic signal sequence to direct co-translational import of the nascent polypeptide into the endoplasmic reticulum (ER). The long prosequence is analogous to that found in matrix metalloproteases and some of the snake venom proteins; in these proteins a conserved cysteine residue is seen in this domain, which interacts with a catalytic zinc moiety in the protease domain and maintains the enzyme in an inactive state. Removal of the prosequence, or conformational changes in this region result in the release of the cysteine-zinc interaction leading to protein activation via the "cysteine-switch" mechanism (Grams *et al.*, 1993). MADM does have a single cysteine (residue 173) in the prosequence which could conceivably be involved in keeping MADM in a latent form.

However, the prosequence of MADM terminates with a potential furin cleavage site (figure 1.4) which, if used, would result in the loss of the whole of the prosequence, and would thus negate the need for MADM activation via a cysteine-switch-type mechanism. The presence of a signal sequence and a potential transmembrane domain implies that MADM will be present at the cell surface; the protein will therefore move through the ER and the Golgi before being transported to the cell surface either directly (constitutive secretion) or via secretory granules (regulated secretion). During this process the proprotein is exposed to a number of proteolytic enzymes, including the furins and furin-like proteases, which are serine proteases, in the Golgi (Halban and Irminger, 1994; Smeekens, 1993). The standard cleavage site for furin has the sequence **-K/R-X-X-R-⇓**. This sequence is shared by a variety of secreted proteins such as: Anthrax toxin (RKKR, Klimpel *et al.*, 1992); Complement pro-C3 (RRRR, Misumi *et al.*, 1991); β-nerve growth factor (RSKR, Bresnahan *et al.*, 1990); Rat pro-insulin II (KSRR and RQKR, Vollenweider *et al.*, 1992; Vollenweider *et al.*, 1993); Von Willebrand factor (RSKR, KSKR, RSDR, van de Ven *et al.*, 1990; Wise *et al.*, 1990).

The tetrabasic motif RKKR at the end of MADM's prosequence is a typical cleavage site for such enzymes, so MADM is likely to be presented at the cell surface with its mature N-terminus exposed, and thus will presumably be active. This is quite unlike the majority of the matrix metalloprotease family which are secreted complete with a prosequence, as latent pro-forms.

Interestingly, the sequence of a recently described membrane-bound matrixin (MT-MMP) also has a potential furin-cleavage site (RRKR) immediately upstream of its mature N-terminus (Sato *et al.*, 1994). In addition, the anthrax toxin lethal factor has a furin-cleavage site identical to that found in MADM (RKKR) and has recently been shown to contain a zinc-binding metalloprotease domain (Klimpel *et al.*, 1994), whilst several members of the astacin family have a furin-cleavage site upstream of the mature N-terminus (Shimell *et al.*, 1992; Lepage *et al.*, 1992).

Cleavage by furin proteases is a feature of proteins transported to the cell surface via the constitutive pathway i.e. secreted shortly after synthesis rather than translocated to granules for storage until secretion in response to a stimulus (Halban and Irminger, 1994). This therefore implies that MADM is constitutively secreted as an active protease, raising interesting questions about its regulation and possible substrates.

The second domain of MADM is the **catalytic domain**, containing an extended zinc-binding site HEXXHXXGXXH, indicating that the enzyme is a zinc metalloprotease and confirming the conclusions of Chantry *et al.*, 1988 and 1989. By analogy with those metalloproteases which have been examined by X-ray crystallography, the three His residues chelate the catalytic zinc moiety in the active site (Bode *et al.*, 1992; Gomis-Ruth *et al.*, 1993). The first two His residues are on the same side of an α-helix that terminates with the Gly residue, facilitating a tight turn. The third His is thus held opposite the first two, forming a histidine-lined pocket in which the zinc is held. The Glu following the first His is a catalytic base which anchors a water molecule next to the zinc; the proximity of the zinc moiety further polarises the water, facilitating hydrolysis of the scissile bond.

Outside of the extended zinc-binding site, MADM has little homology with other metalloproteases, or snake venom proteins, which may indicate that the active sites accommodate different protein substrates.

The amino acid 12 residues away from the first zinc ligand can be used to classify metalloproteases into families, as shown in table 1.2 (Jiang and Bond, 1992). For members of the astacin family, this residue is Glu; for matrixins it is Ser; serralysins have Pro; members of the snake venom protein family have Asp in this position.

MADM, a bovine protein, has an Asp residue, indicative of a member of the snake family of proteins, rather than the astacin/matrixin families more commonly associated with mammals.

Table 1.2 Classification of metalloproteases into families
Strictly conserved residues shown in bold. Residues diagnostic of a given family: snake venom metalloproteases **D**; astacin-like metalloproteases **E**; serralysins **P**; matrixins **S**.

MADM [1]	**HE**VG**H**NF**G**SP**H**D	
Jarahagin [2]	**HE**MG**H**NL**G**IH**H**D	
HR1B [3]	**HE**MG**H**NL**G**IP**H**D	
Astacin [4]	**HE**LM**H**AI**G**FY**HE**	
Meprin α (mouse) [4]	**HE**IL**H**AL**G**FF**HE**	[1] Howard *et al.*, 1996
Serralysin [4]	**HE**IG**H**AL**G**LS**HP**	[2] Paine *et al.*, 1992
Erwinia [4]	**HE**IG**H**AL**G**LN**HP**	[3] Takeya *et al.*, 1990
MMP-2 (human) [3]	**HE**FG**H**AM**G**LE**HS**	[4] From Bode *et al.*, 1993
MT-MMP (human) [5]	**HE**LG**H**AL**G**LE**HS**	[5] From Sato *et al.*, 1994

The third domain of the MADM protein is the potential integrin-binding domain (**disintegrin domain**). Disintegrins are a family of small, soluble, cysteine-rich. non-enzymatic proteins initially characterised in *crotalid* and *viperid* snake venom (Bjarnason and Fox, 1989; Dennis *et al.*, 1989) by their ability to bind to integrins competitively and inhibit platelet aggregation (Gould *et al.*, 1990).

Snake venom disintegrins are synthesised as a large multi-domain precursor molecule, which also contains a metalloprotease domain (Kini and Evans, 1992). By binding specific cell surface integrins, the disintegrin domain is able to prevent platelet aggregation, whilst the metalloprotease domain destroys the ECM of vascular endothelium, thus leading to uncontrollable haemorrhage (Takeya *et al.*, 1990).

Examination of these snake venom disintegrins has shown that the large number of cysteine residues in this domain are conserved, and are involved in extensive disulphide bonding resulting in the formation of a flexible hairpin loop, with the residues at the tip interacting with integrins (Saudek *et al.*, 1991). Most disintegrins are not integrin-specific, and as well as blocking GPIIb-IIIa (Lu *et al.*, 1994) they can

inhibit the adhesive function of many other integrins. Barbourin is one exception, it contains a conservative amino acid substitution from RGD at the tip of the hairpin to a KGD tripeptide which confers a unique high specificity for GPIIb-IIIa on Barbourin (Scarborough *et al.*, 1993).

More than 25 disintegrin haemorrhagic proteins have now been isolated, and the primary structures determined. Many snake disintegrins contain the RGD tripeptide which represents a common integrin recognition site (Ruoslahti and Pierschbacher, 1987); however, a significant proportion of known integrin ligands, including some disintegrins, have residues other than RGD. Indeed, the substitution KGD for RGD in Barbourin suggests that RGD is not the only sequence able to bind to integrins. Jarahagin has the sequence MSEC while the venom protein HR1B (from the venom of *Trimeresurus flavoviridis*) has the sequence ESEC; in both of these the cysteine residue is putatively unpaired (Hite *et al.*, 1994). In the same location, MADM has DSDC, a sequence with obvious similarity, and all 15 cysteine residues of the disintegrin domain of Jararhagin are completely conserved with those in the same region of MADM.

The fourth domain of MADM is a **cysteine-rich domain** as seen in snake venom precursor molecules (Takeya *et al.*, 1993). This domain is relatively rich in cysteine residues, although those of the snake and those of MADM do not align exactly (figure 1.3). The precise function of this domain is unknown, although it is possibly important in structure and conformation of the protein.

This cysteine-rich domain leads, via a 19 amino acid segment, to the **transmembrane domain** which is composed of 24 hydrophobic and non-polar amino acids forming an α-helical structure.

Finally, there is a C-terminal **cytoplasmic tail** of 51 residues in length, containing 23.5% basic amino acids (Arg and Lys) and 23.5% Pro, which would disrupt an α-helical structure. The high proline content is a possible indicator of SH3 binding domains. At first glance, the cytoplasmic tail of MADM does not appear to contain such a site, which has a consensus sequence of RPLPXXP; however, a recent paper by Weskamp and Blobel (1996) suggests that the consensus sequence may be flexible

enough to take in a sequence such as RRRPPQP which is contained in the MADM cDNA.

1.6 MADM function and substrates

Very little is known about potential substrates for the MADM enzyme. The initial observation of metalloprotease activity was specific cleavage of myelin basic protein (MBP) at the Pro73-Gln74 bond (Groome *et al.*, 1988). However, since sequence data suggests an extracellular location for MADM, whereas MBP is a cytoplasmic protein, cleavage of MBP by MADM is almost certainly an *in vitro* artefact, and MBP is unlikely to be a physiologically relevant substrate. In addition, while MBP is a myelin-specific protein, MADM has been shown to be present at low levels in a wide variety of different tissues (Chantry and Glynn, 1990).

Members of the matrixin, astacin and snake venom protein families are known to degrade extracellular matrix components (Alexander and Werb, 1991; Bjarnason and Fox, 1994; Kaushal *et al.*, 1994) so it seemed conceivable that ECM proteins could be substrates for MADM. However, MADM failed to release TCA-soluble ^{14}C from ^{14}C-acetylated native or denatured type I collagen at levels higher than negative controls. On a zymogram, bovine kidney MADM failed to generate zones of clearance in gelatin (denatured type I collagen), and there was also no degradation of type IV collagen (L.Howard, Ph.D. thesis).

So MADM has no activity against fibrillar (type I) collagen or basement membrane (type IV) collagen; nor does it degrade denatured type I collagen (gelatin). All members of the matrixin family whose substrates have been characterised are able to degrade at least one of these substrates (Alexander and Werb, 1991; Nagase and Salveson, 1993). This suggests that the enzyme is not matrixin-like in its activity, in line with the available sequence data, and it is therefore unlikely that MADM is directly involved in ECM degradation.

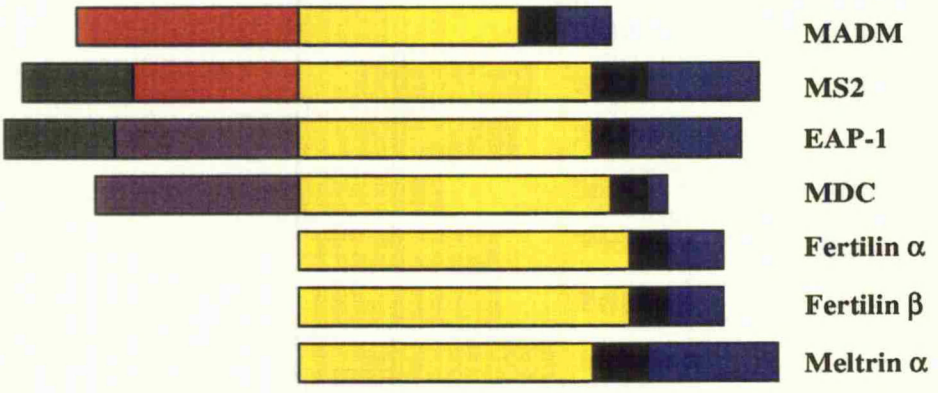
1.7 The ADAM family of mammalian disintegrin-metalloproteases

Since the isolation and cloning of MADM, a number of other mammalian proteins have been described which also have this arrangement of a metalloprotease and a disintegrin-like domain (fig 1.5) and there are others on the databases, still

unpublished (e.g.: HORF: isolated from a human male myeloblast cell line; GenBank D14665, submitted March 1993).

Among these other mammalian proteins, MADM has the greatest similarity (21.6% identity) to **MS2**. MS2 is a 3kb cDNA with an ORF of 2.48kb, cloned from murine monocytes. Like MADM, MS2 has a signal peptide, an extracellular region containing a metalloprotease and a putative disintegrin domain, a transmembrane domain and a basic/proline-rich cytoplasmic tail region (Yoshida *et al.*, 1990). MS2 encodes a mature protein which possesses all the residues required to bind a catalytic zinc moiety. This implies that MS2 should have metalloprotease activity, but to date this is unproven. In contrast, MADM was originally isolated actually as an active protease.

Figure 1.5 Mammalian disintegrin-metalloproteases: the domain structure of the mature polypeptides
MS2 (Yoshida *et al*, 1990); EAP-1 (epididymal apical protein; Perry *et al*, 1992); MDC (Emi *et al*, 1993); fertilin β (Blobel *et al*, 1992). Mature polypeptides which lack some of the critical residues (HEXXHXXGXXH) required for catalytic-site zinc-binding are shown with a metalloprotease-like domain. (See also figure 1.7).



, N-terminal domain; , Metalloprotease; , Metalloprotease-like domain (catalytically inactive); , Disintegrin/cysteine-rich domain; , Transmembrane helix; , Cytoplasmic domain.

Epididymal apical protein (**EAP-I**) is an 89 kDa transmembrane protein found in the epididymal epithelium of both rat and monkey (Perry *et al.*, 1992). It is unlikely to be an active protease since it lacks the critical active site E residue required for catalytic activity.

The human **MDC** gene was isolated from a cosmid clone containing part of chromosomal region 17q21.3. The corresponding cDNA was subsequently isolated from a human cerebellar cDNA library, and encodes a 524 amino acid protein with the same basic four domain structure as Jararhagin. The gene itself was isolated from the chromosomal region 17q21.3, where a tumour suppressor gene involved in breast and ovarian cancers is thought to be present; the MDC gene has shown rearrangements in two breast cancers (Emi *et al.*, 1993). A longer, more abundant isoform of MDC (769 amino acids) which contains transmembrane and cytoplasmic domains was subsequently isolated (Katagiri et al., 1995); it is this isoform which is depicted in figure 1.5. The transcript of MDC is found in the CNS as well as endocrine and reproductive tissues including mammary gland, adrenal gland, thyroid, pancreas, testis and ovary. Restriction mapping of genomic clones using cDNA probes and sequence comparisons between cDNA and genomic clones indicated that MDC consists of more than 20 relatively small exons, the majority of which are spread over a genomic region of around 20kb (Katagiri *et al.*, 1995). This data implies considerable potential for alternative splice forms within this family.

Another mammalian protein is **fertilin** (originally named PH-30, due to its localisation to the posterior head domain on guinea-pig sperm), an integral membrane polypeptide consisting of an αβ heterodimer (Blobel *et al.*, 1992). Fertilin α contains a disintegrin domain, TM sequence and a putative fusion sequence in its mature form. Fertilin β also contains an epidermal growth factor (EGF) repeat sequence of 29 residues. Both fertilin α and β lack a metalloprotease domain in their mature forms, although the fertilin α cDNA sequence does encode a potentially active zinc-binding site which is cleaved before protein secretion. Fertilin has been implicated in sperm-egg fusion, since it has been shown that the β subunit of fertilin uses its disintegrin domain to bind to a specific integrin in the ovum plasma membrane via a TDE tripeptide sequence, and that this binding step is required for sperm-egg fusion (Myles *et al.*, 1994). Peptide analogues of portions of the disintegrin domain were also able to

bind to the egg plasma membrane and effectively inhibit fusion from occurring. So these data indicated that fertilin β was able to bind, via its disintegrin domain, to a cell surface molecule; this molecule was subsequently identified as the integrin α6β1 (Almeida *et al.*, 1995).

Cyritestin (**cy**steine-**ri**ch, **testi**cular) was cloned from mouse cDNA and appears to encode a protein with comparable properties/function to fertilin β. This too has an EGF repeat. However, it seems unlikely that cyritestin has any metalloprotease activity, since it has no amino acid arrangement capable of metal binding at the relevant site (Heinlein *et al.*, 1994).

Many other testis sequences have been detected which contain disintegrin domains: a monkey homologue of cyritestin, known as testis MDC I (**tMDC I**, Barker *et al.*, 1994); **tMDC II**, a fertilin homologue (Perry *et al.*, 1994); and **tMDC IV** isoforms (Perry *et al.*, 1995b) which are also expressed in the liver.

Meltrin α is a recent addition to the family, revealed through the work of Yagami-Hiromasa and co-workers (1995). The authors propose that the meltrins are new candidates for possible participants in myoblast fusion, which is a prerequisite of the formation of multinucleate muscle fibres or myotubes. The genes for meltrins α, β, and γ were isolated by searching in muscle cells for genes encoding proteins with sequence homology to the fertilin proteins, as the latter have been implicated in sperm-and-egg binding and fusion (Blobel *et al.*, 1992). The meltrin α precursor, like that of fertilin α, contains metalloprotease and disintegrin domains, although as a mature protein the metalloprotease domain is cleaved. Meltrin α also has a region similar to fertilin α's putative fusion peptide, and its expression, unlike that of meltrin β and γ, is restricted to skeletal muscle and bone where multinucleate osteoclasts reside. Expression of meltrin α in myoblasts begins at about the same time that fusion starts, while fusion can be inhibited by adding antisense oligonucleotides to decrease levels of meltrin α. It seems likely that fusion occurs following the co-operation of meltrin α with integrins, or other membrane proteins such as cadherins or N-CAMs which have been implicated in myoblast fusion.

Figure 1.6 Sequences of the disintegrin domain
Conserved residues are highlighted in red, and the putative integrin-binding motif is marked ****. Potentially important differences in the MADM sequence are marked in blue.

```
Jar (1)                  CGNELLEVGEECDCGTPENCQNE---CC------DAATCKLKSGSQ
HR1B (2)                 CGNELLEAGEECDCGSPENCQYQ---CC------DAASCKLHSWVK
MADM (3)                 CGNGMVEQGEECDCGYSDQCKDE---CCYDANQPEGKKCKLKPGKQ
MS2 (4)                  CGNLFVEHGEQCDCGTPQDCQNP---CC------NATTCQLVKGAE
Monkey fertilin α (5)    CGNGVVEDTEECDCGS--AC-HLD-PCC------DP-TCTLKEHAE
Metargidin (6)           CGNMFVEPGEQCDCGFLDDCV--D-PCC------DYFTCQLRPGAQ
Meltrin α (7)            CGNGYVEEGEECDCGEPEECTNR---CC------NATTCTLKPDAV
Rat EAP-1 (8)            CGNKKVDEGEECDCGPVQECTNP---CC------DAHKCVLKPGFT
Monkey tMDC I (9)        CGNGILEPTEQCDCGYRKACTF--KKCC------NPADCTLIGFAE
Monkey tMDC II (10)      CGNGLLEGGEECDCGNKDNCTH--KLCC------DALTCRLKDNAQ
MDC (11)                 CGNGFVEAGEECDCGSVQECSRAGGNCC------K--KCTLTHDAM
Guinea pig fert. β (12)  CGNNRVEQGEDCDCGSQEECQD---TCC------DAATCRLKSTSR
Monkey fertilin β (13)   CGNAKLEAGEECDCGTQQNCFLLGAKCC------DTATCRFKAGSN

                                                        ****
Jar                      C--GHGDCC--EQCKFSKSGTECRAS-MSECDPAEHCTGQSSECP
HR1B                     C--ESGECC--DQCRFRTAGTECRAA-ESECDIPESCTGQSADCP
MADM                     CSPSQGPCCT-AHCAFKSKTEKCRD--DSDCAKEGICNGITALCP
MS2                      C--ASGTCC--HECKVKPAGEVCRLS-KDKCDLEEFCDGRKPTCP
Monkey fertilin α        C--SHGLCC-LD-CTFRRKGFLCRPT-QDECDLPEYCDGSSAECP
Metargidin               C-ASDGPCC-QN-CKLQPAGWQCRLP-TDDCDFPEFCNGTSASCP
Meltrin α                C--AHGQCC--EDCQLKPPGTACRGS-SNSCDLPEFCTGTAPHCP
Rat EAP-1                C--VEGECC--ESCQMKKEGVICRPA-KNECDISEVCTGYSPECP
Monkey tMDC I            C--GSGPCCNNKTCTIYARGHVCRKS-IDMCDFPEYCNGTSEFCV
Monkey tMDC II           C--GSGDCCS-KDCKFKPANTICRKSVDVECDFTEFCNGSYPYCQ
MDC                      C--SDGLCC--RRCKYEPRGVSCREA-VNECDIAETCTGDSSQCP
Guinea pig fert. β       C--AQGPCC--NQCEFKTKGEVCRES-TDECDLPEYCNGSSGACQ
Monkey fertilin β        C--AEGPCC-EN-CLFMSQERVCRPS-FDECDLPEYCNGTSASCP
```

Figure 1.7 Sequences of the extended zinc-binding site
Critical and conserved residues are highlighted in red.

```
Active protease consensus  M-HE--H--G--HD

Jar (1)                    MAHEMGHNLGIHHD
HR1B (2)                   MTHEMGHNLGIPHD
MADM (3)                   FAHEVGHNFGSPHD
MS2 (4)                    MAHELGHNLGMSHD
Monkey fertilin α (5)      MVHELGHNLGIQHD
Metargidin (6)             IAHELGHSLGLDHD
Guinea pig fert. α (12)    LVHELGHNLGIRHD
Meltrin α (7)              LAHELGHNFGMNHD
Monkey EAP-1 (8)           MAHQLGHNLGMQHD
Rat EAP-1 (8)              MAHQLGHSLGMRHD
Monkey tMDC I (9)          MAQLLGINLGLTYD
Monkey tMDC II (10)        IVQLLGLNLGLTYD
MDC (11)                   LAQTLGQNLGMMWN
Guinea pig fert. β (12)    LVQLLSVSMGIAYD
Monkey fertilin β (5)      LAQLLSLSMGIPYD
Monkey tMDC IV (13)        TTQTLMRSMGVEYD
```

(1) Paine *et al.*, 1992
(2) Takeya *et al.*, 1990
(3) Howard *et al.*, 1996
(4) Yoshida *et al.*, 1990
(5) Perry *et al.*, 1995a
(6) Kratzschmar *et al.*, 1996
(7) Yagami-H. *et al.*, 1995
(8) Perry *et al.*, 1992
(9) Barker *et al.*, 1994
(10) Perry *et al.*, 1994
(11) Emi *et al.*, 1993
(12) Blobel *et al.*, 1992
(13) Perry *et al.*, 1995b

More recently, initial analysis of a novel cellular disintegrin from a human mammary epithelial cell line has revealed the sequence of a 110kDa glycoprotein, which contains an RGD sequence in its disintegrin domain - the first of these mammalian proteins to do so (Kratschmar *et al.*, 1996).

This protein, named **metargidin**, has been shown to be present in a large variety of human tissues. It also has the extended zinc-binding site, and, in common with another novel, widely-expressed, disintegrin-metalloprotease known as **MDC9** (Weskamp *et al.*, 1996), it has two proline-rich sequences within the cytoplasmic tail which the authors suggest may act as cytoplasmic SH3 ligands. If active, these motifs could suggest an intracellular signalling role (as well as an integrin-binding/metalloprotease role) for this type of protein. The initial biochemical characterisation of MDC9 has revealed that its proline-rich sequences can bind the SH3 domain of src, and Weskamp and co-workers (1996) have therefore proposed a role for MDC9 of interacting with the cytoskeleton or with intracellular signalling molecules.

Recently, a paper has put forward a new nomenclature for the whole group of mammalian disintegrin-metalloproteases. In a mini-review, Wolfsberg *et al.* (1995) propose to name the whole family **ADAM** for proteins containing **A D**isintegrin **A**nd **M**etalloprotease domain. They describe 11 full length members of the ADAM family, and designate MADM as ADAM 10. This research group has also reported the cloning, sequencing and characterisation of mouse homologues to fertilin α and β, as well as 5 additional sequence-similar cDNAs from guinea pig and mouse testis (Wolfsberg *et al.*, 1995).

Members of the ADAM family express a variety of residues at their putative integrin binding sites, implying interactions with a variety of integrins. The precise integrin ligands are not known for any of these proteins except for fertilin β which interacts with $\alpha 6\beta 1$ on the oocyte (Almeida *et al.*, 1995).

However, MADM differs from other ADAMs in a variety of ways which may affect its ability to bind to integrins (figure 1.6). MADM contains a six amino acid insert (residues 489-494 of the bovine sequence) which is not present in the other sequences, and it has a single amino-acid deletion immediately preceding the putative

integrin-binding site. Following this integrin-binding sequence, the mammalian disintegrins all have a cysteine residue which is believed to be unpaired. Next to this, MADM has an alanine residue, but all of the other mammalian disintegrins together with Jararhagin have an aspartic acid in this position. Alanine is a non-conservative substitution which may interfere with the ability of MADM to bind integrins. Similarly, two residues further on, MADM has glycine in a position occupied by Glu in the other enzymes which may render the disintegrin region inactive. However, many cysteine residues, and a number of glycine residues are highly conserved between all the mammalian disintegrins (fig 1.6).

As mentioned previously, the protease domains of the mammalian disintegrins also contain significant sequence variations, but these differences fall into two distinct categories (figure 1.7). The majority of the proteins (EAP, cyritestin and homologues, MDC) do not contain a viable zinc-chelating site within the protease domain of the peptide. Of those that do, only MS2, Metargidin and MADM have it expressed in the mature protein, and of these two, only MADM has been proven to display any proteolytic activity (Chantry *et al.*, 1989).

Except for the fertilin heterodimer, and meltrin, the biological functions of the ADAMs are unknown.

Wolfsberg *et al.* (1993) conducted phylogenetic analysis on the pro-, met- and cysteine-rich domains of EAP-1, fertilin, cyritestin and 6 snake proteins, and concluded that all of these proteins derived from a multidomain progenitor gene. This can be reasonably extrapolated to MADM, which would seem to have diverged early on from other members of the family, a conclusion drawn by Rawlings and Barrett (1995) after a similar sequence analysis.

It has proved difficult to detect MADM mRNA, to examine transcript sizes (L.Howard); however, transcripts have been detected by Northern blotting for other ADAMs. Several are specific to the male reproductive tract (fertilin, EAP-1, tMDC I and II, cyritestin), while MS2 is specific to macrophages and macrophage-derived cell lines. Transcription of EAP-1 has been shown to be regulated by the presence/absence of androgens (Perry *et al.*, 1992; Walker *et al.*, 1990). Cyritestin is not expressed in mouse testicular cells until 15 days post-natally, suggesting possible developmental regulation of expression (Heinlein *et al.*, 1994). Transcription of MS2 is substantially

enhanced by exposing macrophages to phorbol esters or cytokines (Yoshida *et al.*, 1990). So MADM, by analogy, may also be upregulated in some way, possibly during a disease process or during development.

From all the evidence so far considered, it is apparent that members of the ADAM family vary substantially in terms of mature protein structure, abundance, distribution, control of biosynthesis, and, presumably, function. They have the ability to perform multipotential functions in cell-cell and cell-matrix interactions (Weskamp and Blobel, 1994), providing indirect evidence that similar interactions may be mediated by the MADM protein. The common linking feature is the presence of the disintegrin domain, therefore it would be very interesting to determine to what extent integrin-binding capability and specificity has been retained.

1.8 Possible interactions with integrins

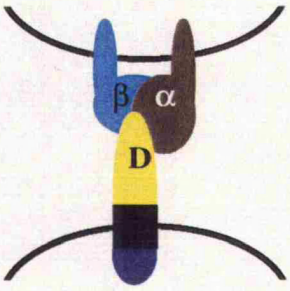
The supposed ligands of these disintegrin proteins, the integrins, have been previously mentioned, in section 1.2. They are often represented as transmembrane links between the ECM and the cell cytoskeleton, although they are also involved in cell-cell interactions. Twenty heterodimers are currently known, formed by the non-covalent association of an α with a β subunit, and these heterodimers are expressed in a cell-type specific manner.

There are at least 3 potential mechanisms by which an integrin could interact with a disintegrin, as shown in figure 1.8. In the first of these, a disintegrin on one cell simply recognises and binds to a disintegrin on another cell, thus allowing cell-cell interactions. Such a mechanism has been postulated for the disintegrin proteins fertilin and meltrin α. However, it is worth considering that these proteins are precisely the ones which lack a metalloprotease domain in their mature form. It is significant that while over-expression of a meltrin-a construct lacking the metalloprotease domain promoted myoblast fusion, over-expression of the construct containing this domain inhibited fusion (Yagami-Hiromasa *et al.*, 1995). In the case of disintegrins such as MADM and MS2, which retain an active metalloprotease domain, such a cell-cell interaction could be inhibited by the presence of an extra protein domain.

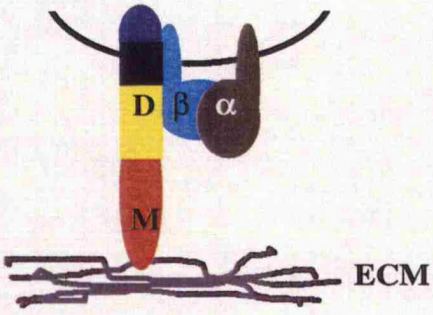
So we must consider two other potential interactions, both of which involve the disintegrin region of one of these proteins binding to an integrin on the same cell. This

type of interaction could permit the localisation of the metalloprotease domain in the vicinity of the substrate, which could potentially be a constituent of the ECM. Or, via an integrin-CAM interaction, the metalloprotease could be positioned to cleave membrane-bound proteins on other cells.

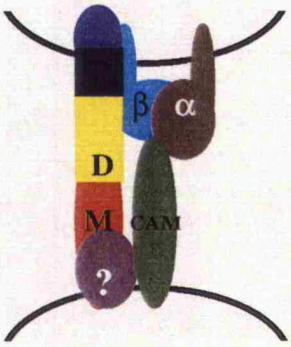
Figure 1.8 Possible disintegrin-integrin interactions
Schematic diagram to show potential mechanisms of interaction between disintegrin proteins and integrins.



1. Cell-cell recognition via integrin-disintegrin interactions e.g. Fertilin, Meltrin-α.



2. Integrin-disintegrin binding allows positioning of metalloprotease for substrate degradation.



3. Integrin binding to CAM on another cell may allow disintegrin to locate protease domain in position to cleave other cell surface proteins.

1.9 Aims of the project

A monoclonal antibody designated CG4 was raised to MADM purified from bovine brain (Chantry and Glynn, 1990). On bovine CNS tissue sections a preliminary study with CG4 appeared to localise MADM to oligodendrocytes (Chantry *et al.*, 1992); this suggested that MADM might play a role in myelinogenesis either during development or following demyelination. Thus, the overall aims of this project, supported by the Multiple Sclerosis Society, were to further characterise MADM with particular relevance to these processes.

However, the range of further studies on MADM in bovine systems is clearly limited, compared with those possible in an animal such as the rat. Bovine brain is difficult to obtain since the outbreak of bovine spongiform encephalopathy in British cattle, whereas rat brain is easily acquired. Also rats would be a useful species to study a role for MADM in MS, since the rat model EAE is available, or in myelinogenesis which has been extensively studied in rats. Furthermore, early biochemical studies have shown that comparable amounts of MADM, as assessed by degradation of MBP (section 1.5), are present in rat as in other mammalian brain tissues (Chantry *et al.*, 1988).

It was decided to pursue an immunohistochemical study of MADM in normal adult rat brain and compare this with the distribution of various integrin subunits; this might provide clues about candidates for MADM's putative cognate integrins in the CNS. This could subsequently be extended to examine MADM expression in developing brain and in experimentally induced pathological conditions.

The first requirement for the project was an antibody reactive with rat MADM. Two monoclonal antibodies reactive to MADM were previously generated in this laboratory: CG4 was raised against MADM protein isolated from bovine brain (Chantry and Glynn, 1990); M-N was raised to a synthetic peptide, TTVAEKNTC, corresponding to the first 9 residues of the mature bovine protein. Both CG4 and M-N were completely specific for bovine MADM by Western blotting assays and did not cross-react with the rat or human homologues. Since MADM is present at very low levels in the brain (comprising less than 0.01% of total brain protein) it would not be practical to isolate sufficient pure protein from rat brain for immunisation protocols.

The method chosen was therefore to clone the rat MADM homologue from a rat brain cDNA library, using the bovine sequence as a probe, and use the deduced protein sequence of the cDNA clone to design antigenic peptides. These peptides could then be linked to a carrier protein, and inoculated into a range of species to produce a variety of antibodies for different purposes, but primarily for IHC of brain sections.

The layout of this thesis is therefore as follows: chapter 3 describes the cloning and sequencing of rat MADM cDNA; chapter 4 describes the raising and characterisation of antibodies to peptides of the deduced rat MADM protein; chapter 5 describes the immunolocalisation of MADM in rat neural cells using these antibodies. Each of these chapters 3-5 is presented in a combined results and discussion format, where immediately obvious comparisons and conclusions are drawn. Finally, the wider implications of these findings, particularly of the immunolocalisation studies, are discussed in depth in chapter 6.

CHAPTER 2. EXPERIMENTAL PROCEDURES

All materials were obtained from the Sigma Chemical Company (Poole, U.K.) unless otherwise specified.

2.1 ISOLATION OF A CLONE FROM A λ cDNA LIBRARY

This section describes the procedures used during the screening of a λ cDNA library using bovine MADM cDNA as a probe, and the isolation of a single clone containing an insert of homologous rat cDNA. All the methods detailed in this section are essentially those decribed by Sambrook *et al.* (1989).

2.1.1 The library

The library used to isolate cDNA clones was a rat brain cDNA library (5'-stretch) bought from Clontech Laboratories (Cambridge Bioscience, Cambridge, UK). This library is both oligo(dT)-primed and random-primed using mRNA isolated from an adult male Sprague-Dawley rat. The cloning vector is λgt10, and the cloning site is *Eco*RI. The host strain of bacteria used for library selection is *E. coli* C600 *Hfl*.

2.1.1.1 Solutions used during library screening

All solutions were prepared using deionised water and were immediately sterilised by autoclaving. Once sterile, they were stored at room temperature until required.

SM buffer: 0.1M NaCl; 8mM $MgSO_4.7H_2O$; 50mM Tris/HCl pH 7.5; 0.01% Gelatin.

20x SSC: 175.32g l^{-1} NaCl; 88.20g l^{-1} Trisodium Citrate.

L-Broth: 10g l^{-1} Bactotryptone; 5g l^{-1} Bacto-yeast extract; 10g l^{-1} NaCl; pH 7.0.

L-Agar: L-Broth containing 15g l^{-1} Bactoagar; pH 7.0.

Top-Agar: L-Broth containing 10g l^{-1} Bactoagar; pH 7.0.

Bactotryptone, Bacto-yeast extract and Bactoagar were bought from DIFCO (East Molesey, UK).

50x Denhardt's solution: 10g l^{-1} Ficoll; 10g l^{-1} polyvinylpyrrolidone; 10g l^{-1} bovine serum albumin; filter sterilised.

2.1.1.2 Preparation of competent cells

The first step was to produce an accurate library titre. To do this, competent cells were required. Ten microlitres of C600 *Hfl* were added to 10ml of L-broth + 0.2% maltose and grown up overnight at 37°C with shaking in a 50ml tube. (Maltose induces the maltose operon leading to expression of the gene *lamB* which encodes the bacteriophage λ receptor.)

E. coli are most efficiently infected by bacteriophage whilst in the log phase of growth (Sambrook *et al.*, 1989). To ensure cells were in this phase an aliquot (0.5ml) of overnight culture was added to 25ml L-broth + 0.2% maltose and shaken for approximately 2 hours at 37°C. The O.D.600 was then measured against L-broth, and the cells pelleted at 400 xg for 5 minutes. The cells were resuspended in Vml of cold 10mM $MgSO_4$, where V = (O.D.600/0.5) x 7.5. Following resuspension, the cells were placed on ice for at least 2 hours.

2.1.1.3 Library titration

Using the given titre of the library, dilutions were made of the λgt10 in order to give approximately 10, 100 and 1000 plaque forming units (pfu) per plate. One hundred microlitres of each dilution was added to 100μl of resuspended cold cells and incubated at 37°C for 15 minutes. Then 5ml of top-agar was added, the mixture swirled and poured onto an L-agar plate. When set, the plates were incubated at 37°C overnight. An accurate titre was calculated by counting plaques.

2.1.2 Production of a radiolabelled probe

2.1.2.1 Solutions and materials required

11.1x PCR Buffer: 45mM Tris/HCl (pH 8.8); 11mM Ammonium Sulphate; 4.5mM $MgCl_2$; 6.7mM β-mercaptoethanol; 4.4mM EDTA (pH 8.0); 1mM dATP; 1mM dCTP; 1mM dGTP; 1mM dTTP; 113μg ml^{-1} BSA (molecular biology

grade). Use 0.9µl per 10µl reaction. Ultrapure dNTPs are from Pharmacia Biotech (St. Albans, UK).

PCR reaction mix: 50ng cDNA template; 50ng primer A; 50ng primer B; 0.9µl 11.1x buffer; 5.9µl H_2O; 0.2µl Taq polymerase (1 unit); deionised water to produce a total reaction volume of 10µl. Taq polymerase is from Perkin Elmer (Applied Biosystems, Warrington, UK).

2.1.2.2 Production of probes by PCR

The probe used for the first round of screening was the full protein coding region of the bovine MADM cDNA. The polymerase chain reaction (PCR) technique was used to produce this DNA from template and primers supplied by Linda Howard (figure 3.1). All the required reagents were assembled in a 0.5ml microtube, and overlaid with a drop of mineral oil. Thermal cycling then commenced, using a DNA thermal cycler 480 (Perkin Elmer) and the following sequence: 95°C for 1 minute 20 seconds; 55°C for 1 minute; 70°C for 1 minute; 32 cycles. This was followed by 1 minute at 67°C, and 10 minutes at 70°C, before cooling of the completed reaction to 4°C. The mineral oil was removed at this stage.

A second probe, used in screening in conjunction with the first probe, was also produced by PCR. This probe corresponded to the disintegrin region only of bovine MADM (figure 3.1), and was used to specifically detect clones which had a disintegrin-containing insert. Both probes were purified from the completed PCR reaction mix by agarose gel electrophoresis.

2.1.2.3 Agarose gel electrophoresis of DNA

The choice of agarose concentration used to make the gel was determined by the size of the DNA fragments to be examined (see table 2.1).

Agarose was dissolved in 97ml water, 2ml 50 x running buffer (1.8M Tris; 1.5M sodium dihydrogen phosphate; 50mM EDTA). Thin agarose gels were poured as follows: a well-forming comb was held in bulldog clips over a clean glass plate on a level platform such that the teeth were approximately 1mm from the glass surface. Agar was carefully poured onto the plate until it was covered; the gel remained on the plate by surface tension. After the gel had set, the comb was

carefully removed without tearing the wells. The gel was submerged in a gel tank containing 1x running buffer, to which had been added 0.5μg/ml ethidium bromide, with the wells of the gel at the cathode end of the tank.

DNA samples were prepared by the addition of 10 x DNA loading buffer (50% sucrose; 0.1M EDTA; 0.2% bromophenol blue). These samples and the DNA standards were loaded into the wells of the gel. Electrophoresis was performed at constant voltage (80V) until the dye-front had travelled approximately 75% the length of the gel. The ethidium bromide-stained DNA was visualised by placing the gel on a UV light box.

2.1.2.4 Purification of probe DNA

The ethidium bromide-stained band was excised from the agarose gel and purified using the Geneclean II kit (Stratagene, Cambridge, UK), following the protocol supplied with the kit. The DNA yield was then quantified by spectrophotometry.

Table 2.1 Range of separation of linear DNA molecules in gels containing different amounts of agarose. (Taken from Sambrook *et al.*, 1989).

Amount of agarose in gel (% [w/v])	Efficient range of separation of linear DNA molecules (kb).
0.3	5-60
0.6	1-20
0.7	0.8-10
0.9	0.5-7
1.2	0.4-6
1.5	0.2-3
2.0	0.1-2

2.1.2.5 Quantifying nucleic acid by spectrophotometry

The concentration of double-stranded (ds) DNA, single-stranded (ss) DNA or RNA was determined by measuring the absorbance of a dilution of the nucleic acid

solution in a quartz cuvette at 260nm. An aliquot of the nucleic acid was diluted using ultraclean water (normally 251-fold, i.e. 2µl was added to 500µl water). The spectrophotometer was zeroed at 260nm using ultraclean water, and the absorbance (A) of the diluted nucleic acid solution was then measured.
Nucleic acid concentration was then determined as follows (Sambrook *et al*, 1989):

A solution of double stranded DNA of concentration **50**µg ml^{-1} will have an A_{260nm} of 1.0.
Therefore **dsDNA** concentration (µg ml^{-1}) = A_{260} x dilution x 50.

A solution of ssDNA *e.g.* primers, or RNA of **40**µg ml^{-1} will have an A_{260nm} of 1.0.
Therefore **ssDNA** or **RNA** concentration (µg ml^{-1}) = A_{260} x dilution x 40.

Comparison of absorbance values at 260 and 280nm of a nucleic acid solution provides an estimate of the purity. A 260/280 ratio of approximately 1.8 for DNA and of 2.0 for RNA indicates a protein free preparation; ratios considerably lower than these indicate protein contamination. A contaminated sample can be cleaned by phenol:chloroform extraction (as described in Sambrook *et al.*, 1989) or by using a Geneclean II kit as described in section 2.1.2.4.

2.1.2.6 Radiolabelling procedure

Both probes were radiolabelled using α^{32}P-dCTP and the Amersham Megaprime System (Amersham, Little Chalfont, UK). The procedure is that recommended by the manufacturer. The labelled probe is then purified from unincorporated radionucleotide using Pharmacia NAP-5 columns. The reaction mix is loaded onto a column and subsequently eluted with 200µl fractions of sterile water. Each fraction is tested for radioactivity using the Easicount 2000 benchcounter (Scotlab, Strathclyde, UK). The earliest radioactive fractions contain the purified probe, while later fractions containing unincorporated nucleotides are discarded. The

probe is then denatured by boiling for at least 5 minutes before adding to any hybridisation solution.

2.1.3 First round screening

Competent cells were made as for library titration (see section 2.1.1). Two hundred microlitres of cells were added to 200µl λ in SM buffer, having previously diluted the λ to give approximately 50,000 pfu per plate. This mixture was incubated at 37°C for 15 minutes, then 15ml top-agar was added before pouring onto a 150mm diameter L-agar plate which had been previously made and dried. Seventeen plates were produced in this way.

As a negative control, 200µl cells were added to 200µl SM buffer, then treated as above. When set, all 18 plates were incubated inverted overnight at 37°C.

The following day, all the plates were placed in the cold room for 2 hours before filter lifting. Filters (Hybond-N+ nucleic acid transfer membranes) were bought from Amersham. Each plate was overlaid with a filter for a period of 2 minutes. During this time, both plate and filter were numbered and marked in such a way that they could be easily aligned later. Each filter was then treated with (a) 0.5M NaOH + 1.5M NaCl for 5 min; (b) 0.5M Tris/HCl pH 7.5 for 5 min; (c) 2xSSC for 2 min; and finally allowed to air dry. At this stage, a positive control was also produced: 5µl of a 1000x dilution of the DNA used to make the probe was dotted onto a filter; this filter was subsequently treated in the same way as the other 18 filters. After air drying, all filters were crosslinked with UV irradiation.

After the filter lift, each plate was inverted over chloroform for 1 minute, then sealed with Nescofilm and stored at 4°C.

2.1.3.1 Prehybridisation and hybridisation

The filters were prehybridised (in a solution of 20% formamide; 5x Denhardt's solution; 1% SDS; 6xSSC) at 42°C (in a Techne hybridisation oven) for a period of at least 4 hours. This was followed by hybridisation at 42°C overnight, with the radiolabelled probe (see section 2.1.3) at a concentration of 10^6 counts ml^{-1} of prehybridisation solution.

2.1.3.2 Washing protocol

Following hybridisation, filters were initially washed twice in 6xSSC, 0.5% SDS at 42°C for 15 minutes. Subsequent washes were in 2xSSC, 0.1% SDS at 45°C for 15 minute intervals, monitoring by Geiger counter until the negative control registered very low or no counts on the filter. The filters were then wrapped in saranwrap, and subjected to autoradiography overnight with an intensifying screen at -80°C.

2.1.3.3 Detection and treatment of positive plaques

After the autoradiographs were developed, positive signals were matched up to plaques on the plates. These positive plaques were then excised from the plate and placed in 1ml SM buffer + 100µl chloroform. These plaque extracts were then vortexed and stored at 4°C. The filters and plates were subsequently discarded.

2.1.4 Second and subsequent rounds of screening

Plaque extract was diluted 50,000x and 200,000x. To 200µl extract was added 200µl competent cells, and the mixture was incubated and plated out with top-agar as described in section 2.1.3. However, filter lifts and hybridisation were carried out in duplicate, with one set of filters being probed with the full length bovine cDNA, and the other set with cDNA corresponding to the disintegrin region only. Plaques which were positive with both probes were excised and stored as before.

Screening continued through a third and fourth round until single λ clones were isolated. The plaque extract of selected clones was then used to make a high titre lysate for subsequent work.

2.1.5 Generation of a High Titre Lysate (HTL)

Competent cells were prepared as in section 2.1.1. Three hundred microlitres of plaque extract was added to 300µl cells, and incubated at 37°C for 15 minutes. Following incubation, 5ml of L-Broth containing 5mM $CaCl_2$ was added, and the mixture was agitated at 37°C overnight.

The following day, 200µl chloroform was added. Shaking continued at 37°C for 30 minutes, then the lysate was centrifuged at 2,000 xg for 10 minutes at room temperature. The supernatant containing λ was divided into ten 0.5ml aliquots and 50µl chloroform was added to each tube before storage of HTL's at 4°C.

2.1.6 Small scale preparation of λ DNA

This was done with the Qiagen Lambda Mini Kit, according to the protocol supplied (Qiagen, Dorking, UK). The DNA obtained by this method was resuspended in 20μl water and quantified according to section 2.1.2.5.

2.1.7 Examination of the insert contained in the λgt10 clone

The DNA obtained in section 2.1.6 was digested with the restriction enzyme *Eco*RI (3 units of enzyme per microgram of DNA), in order to excise the insert. The products of the digest were then analysed on a 1% agarose gel against a 1kb ladder standard (from Gibco BRL, Paisley, UK) in order to check the size of the excised insert.

2.2 SUBCLONING

The protocols and techniques described in this section are those which were used to purify the insert from the lambda clone isolated in section 2.1, and to ligate this insert into a new vector to allow the cDNA to be sequenced and analysed.

2.2.1 Production of microgram quantities of bacteriophage λ DNA

The λ clones which, when digested with *Eco*RI, appeared to contain an insert of approximately 2.4kb (figure 2.1) were selected for subcloning. The first stage was to isolate a larger amount of the λ DNA than could be obtained by the Qiagen mini-prep method.

Day 1: Competent cells were produced (see section 2.1.1.2 for method). Two aliquots of 10^{10} cells were spun down (OD_{600} of 1 = 5 x 10^8 cells ml^{-1}; Linda Howard, personal communication) and resuspended in 3ml 10mM $MgSO_4$. They were then placed on ice for at least 2 hours. Each aliquot was infected with 75μl HTL and incubated at 37°C for 15 minutes. Then 500ml prewarmed L-Broth + 5mM $CaCl_2$ was added and incubated at 37°C overnight.

Day 2: 10ml chloroform was added, and the culture incubated at 37°C for a further 30 minutes before being chilled in ice water. DNAase and RNAase were added to a final concentration of 1μg ml^{-1} followed by incubation at room temperature for 30 minutes. NaCl was added to a concentration of 1M and the mixture left on ice for 1 hour before centrifugation at 11,000 xg for 10 minutes at 4°C. PEG 8000 was added to the supernatant to a concentration of 10% and left on ice for 1 hour followed by centrifugation at 11,000 xg for 10 minutes at 4°C. The supernatant from this spin was discarded and the pellet resuspended in 8ml SM buffer. To this suspension was added 8ml chloroform, and the mixture vortexed and centrifuged. The aqueous layer was retained and caesium chloride added to 0.5g ml^{-1} before storage at 4°C overnight.

Day 3: Caesium chloride gradients were constructed, comprising a bottom layer of 2ml of 1.70g ml^{-1} caesium chloride; a 1.5ml middle layer of 1.50g ml^{-1}; and a 1.5ml top layer of 1.45g ml^{-1}. The tube was marked between the 1.5g ml^{-1} and 1.45g ml^{-1} layers to facilitate visualisation of the phage later on. Six millilitres of λ suspension was carefully layered onto the top of the gradient, which was then centrifuged at 15,000 xg for 2 hours at 4°C in a swing-out rotor.

The tube was placed against a black background and illuminated from above. The lambda phage, which appeared as a bluish band at the interface of the 1.5g ml^{-1} and 1.45g ml^{-1} layers, was collected and placed in sterile dialysis tubing. It was then dialysed against 1000x volume of 10mM $MgCl_2$; 10mM NaCl; 50mM Tris/HCl (pH 8.0) for 1 hour at room temperature. The buffer was renewed and the lambda dialysed for a further 1-2 hours.

After dialysis, the λ was transferred to a 50ml tube, to which EDTA, proteinase K and SDS were added, to give final concentrations of 20mM, 50μg ml^{-1} and 0.5% respectively. The solution was incubated at 65°C for 1 hour before being extracted with phenol:chloroform. The resulting aqueous phase was dialysed overnight in 1000x volume of 10mM Tris/HCl (pH 8.0).

Day 4: The λ DNA was removed from the dialysis tubing and placed in a 50ml tube. Sodium acetate solution (pH 7.0) was added to a final concentration of 0.3M, together

with 2 volumes of ethanol. This solution was left overnight at -20°C to bring about the precipitation of the DNA.

Day 5: The solution was centrifuged at 15,000 xg for 1 hour at 4°C. The pellet of DNA was air-dried, followed by resuspension in 500µl water and quantification.

2.2.2 pBluescript

The vector chosen for subcloning was Bluescript SK- (figure 2.1), and its host strain XL1-Blue (from Stratagene). These strains allow blue-white colour selection for bluescript plasmids containing inserts when plated on L-agar plates containing 50µg ml^{-1} ampicillin; 0.5mM IPTG; 40µg ml^{-1} X-Gal. Colonies containing plasmids with no inserts, allowed to grow at 37°C for 12-18 hours, will turn blue; colonies with plasmids containing inserts will remain white. Further enhancement of the blue colour can be obtained by placing the plates at 4°C following overnight growth at 37°C.

2.2.3 Subcloning into pBluescript

Day 1: Ten micrograms of the λ DNA containing the insert of interest, purified in section 2.2.1, was digested by the restriction enzyme *Eco*RI. This digest was separated on an agarose gel and the insert bands excised. The vector (pBluescript) was also digested with *Eco*RI. A small amount was analysed on an agarose gel to check linearisation. The agarose bands containing the insert were purified using the Geneclean II kit (see section 2.1.2.4) as was the digested vector.

A 10ml culture of XL1-blue cells in L-Broth was incubated overnight at 37°C with shaking.

Day 2: 2ml of the overnight XL1-blue culture was added to 20ml fresh L-Broth, and the cells grown until the OD_{600} = 0.5 (ca. 2-3 hours). At this point the cells were pelleted, and resuspended in 10ml cold 50mM $CaCl_2$, before being left on ice for 20 minutes. The cells were then recentrifuged and resuspended in 2ml cold 50mM $CaCl_2$. Competent cells were aliquoted into 200µl fractions and left on ice.

The ligation mixes were then assembled (see table 2.2). To each mix was added 1μl (corresponding to 1U) T4 DNA ligase, 2μl T4 ligase 5x reaction buffer (Gibco BRL) and water to a final volume of 10μl. A negative control for ligation was also made, consisting of vector alone + buffer, ligase, and H_2O. Each ligation mix was incubated at 16°C for at least 4 hours. Half of the ligation mix (5μl) was used for the transformations, and the other half was stored at 4°C in case a further transformation was necessary.

Five microlitres of ligation mix were added to 200μl cells. The cells were then heat-shocked at 42°C for 90s, before being cooled on ice. A negative control for transformation was also made, consisting of buffer alone + cells. L-Broth (800μl) was then added to allow expression of the ampicillin resistance gene on the plasmid. Cells were then pelleted, and resuspended in 200μl L-Broth. Each cell suspension was split into 2 aliquots of 50μl and 150μl which were then spread onto selection plates (L-Agar plates containing 50μg ml^{-1} ampicillin; 0.5mM IPTG; 40μg ml^{-1} X-Gal). Once dried, these plates were incubated inverted at 37°C overnight.

Table 2.2 Relative molar ratios of the ligation mixes used during subcloning
For 2.98kb vector and 2.4kb insert:

vector : insert	molar ratios
100ng : 80ng	1 : 1
100ng : 240ng	1 : 3
100ng : 27ng	3 : 1

Day 3: The selection plates were placed in the cold room for approximately 2 hours to allow full colour development. The plates spread with the ligation negative control were examined to ensure all colonies growing were blue; the transformation negative control plates were checked that they had no colony growth. White colonies were then picked from the remaining plates, together with one blue colony as a control.

The next stage was to verify that the white colonies did have insert-containing plasmids.

2.2.4 Confirmation of successful subcloning

2.2.4.1 Isolation of DNA from subcloned colonies

To each colony picked from the selection plates was added 5ml L-Broth + 50μg ml^{-1} ampicillin, and the cultures incubated at 37°C overnight with shaking. The following day, a glycerol stock was made of all possible subclones (500μl cells + 500μl glycerol). DNA was then isolated from 3ml of each colony culture using the Wizard Miniprep system (from Promega, Southampton, UK), following the protocol supplied.

The eluted DNA was quantified as described in section 2.1.2.5.

2.2.4.2 Confirmation by restriction digestion and Southern blotting

The purified DNA (2-3μg) was digested with *Eco*RI, and the digest analysed on an agarose gel to check for presence of the insert. The blue colony was used as the vector standard. Ethidium bromide staining was used to disclose DNA bands on the gel. Subsequently, this gel was prepared for Southern blotting by placing it in 0.2M HCl for 15 minutes, then rinsing it in distilled water. The gel was agitated in denaturation buffer (0.5M NaOH; 1.5M NaCl) for 30 minutes at room temperature, then rinsed again in distilled water. Finally the gel was agitated in neutralisation buffer (1.5M Tris/HCl pH 7.5; 1.5M NaCl) for 15 minutes, rinsed in distilled water and blotted onto Hybond-N+ nitrocellulose (from Amersham) using standard capillary action (as described in Sambrook *et al.*, 1989). After blotting, the DNA was fixed to the membrane by UV crosslinking, then prehybridised (section 2.1.3.1) at 50°C for at least 4 hours.

A radiolabelled probe was made (section 2.1.2.5) using some of the insert DNA purified in section 2.2.3. This probe was then added to the prehybridisation mix at a concentration of 10^6 counts ml^{-1} of prehybridisation solution, and the filter hybridised overnight at 50°C. The following day, the filter was washed, initially using 2xSSC, 0.1% SDS solution at 50°C for 15 minute intervals, monitoring with a Geiger counter. The stringency of the washing was then increased, by decreasing the concentration of SSC to 0.5x and raising the temperature to 55°C. After washing, the filter was subjected to autoradiography at -80°C with intensifying

screens. The resulting film was used to verify the identity of the inserts produced by digestion of the sub-clones.

2.2.4.3 Confirmation by PCR

The *Eco*RI site of pBluescript, into which the insert was cloned, is flanked by M13 priming sites (see figure 2.1). It was therefore possible to perform PCR on the DNA purified from the subclones using the Universal primer as the forward primer, together with the M13 reverse primer (see section 2.1.2.2 for PCR reaction details). The completed PCR reactions were run out on a 2% agarose gel, and ethidium bromide-stained to disclose the bands. Clones containing the insert were expected to produce bands of approximately 2.4kb in size.

This gel was then Southern blotted and autoradiographed in exactly the same way as the gel in section 2.2.4.2, using the same radiolabelled probe, to confirm the identity of the bands.

2.2.5 Large scale plasmid prep

The Qiagen midi-prep system was used in order to produce a larger amount of DNA for sequencing from sub-clones containing our insert. The procedure followed is that supplied with the kit. The DNA produced by this method is of a high quality, suitable for sequencing.

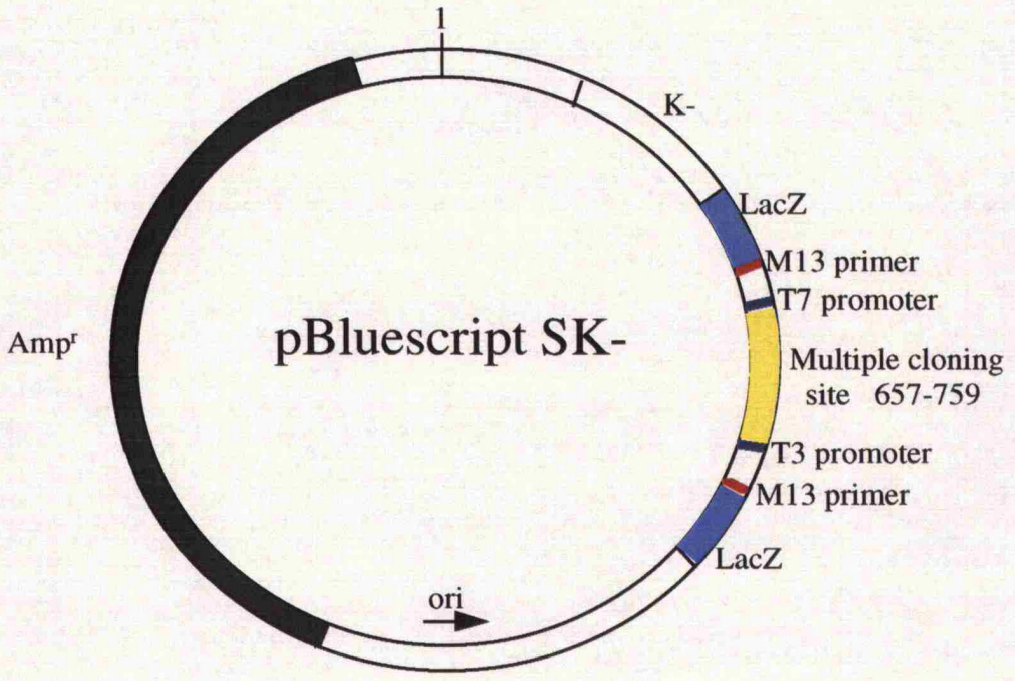
2.3 DNA Sequencing

2.3.1 Sequencing Protocol

2.3.1.1 Reagents

Terminator premix: 1.58μM A-DyeDeoxy; 94.74μM T-DyeDeoxy; 0.42μM G-DyeDeoxy; 47.37μM C-DyeDeoxy; 78.95μM dITP; 15.79μM dATP; 15.79μM dCTP; 15.79μM dTTP; 168.42mM Tris/HCl (pH 9.0); 4.21mM $(NH_4)_2SO_4$; 42.1mM $MgCl_2$; 0.42 units μl^{-1} AmpliTaq thermostable DNA polymerase, supplied by Applied Biosystems (a division of Perkin Elmer, Warrington, UK).

Figure 2.1 Schematic representation of the cloning vector pBluescript SK-
The SK- polylinker contains 21 unique restriction sites, including EcoRI, and is flanked by T3 and T7 promoter sequences for transcription, as well as M13 priming sites. The ampicillin resistance (amp^r) gene allows selection on antibiotic containing media, and LacZ allows blue/white colony selection.



2.3.1.2 Procedure

Sequencing was done with the PRISM Ready Reaction DyeDeoxyTerminator Cycle Sequencing Kit and protocol (Applied Biosystems).

The following reagents were mixed in a 0.5ml thin-walled microtube:

terminator premix	9.5µl
template (1µg dsDNA)	5.0µl
primer (for dsDNA)	3.2pmol
distilled water	Xµl to a total of 20µl

This reaction mix was overlaid with 1 drop of mineral oil, and subjected to thermal cycling: 96°C for 30s; 50°C for 15s; 60°C for 4 minutes; total 25 cycles, followed by a rapid thermal ramp to 4°C and hold. Eighty microlitres of water was added to the completed reaction and the oil removed. Terminators were extracted by adding 100µl phenol:water:chloroform (68:18:14), vortexing and centrifuging (14,000 xg, room temperature). The upper aqueous layer was saved and re-extracted with a further 100µl phenol mixture. Extension products were precipitated by adding 15µl 2M sodium acetate pH 4.5 and 300µl 100% ethanol, incubating for 15 minutes on ice and then centrifuging at 14,000 xg for 15 minutes at room temperature. The pellet was washed with 70% ethanol and allowed to air-dry, before being submitted for sequencing on the ABI automated sequencer at the protein and nucleic acid chemistry laboratory (PNACL, University of Leicester).

2.3.2 Primers used in sequencing

Initially the primers used for the first sequencing run were the Universal primer in one direction, and the M13 reverse primer on the opposite strand (fig. 2.2a). Sequencing then continued simultaneously on both strands (figure 2.2b), with primers being designed to the elucidated sequence (figure 3.7). The DNA was sequenced at least 3 times in each direction in order to firmly identify each nucleotide.

Primers were made PNACL, University of Leicester, and were supplied in 1ml ammonia solution. Before use, the primer was dried using a Savant Speed-vac Plus system (supplied by Life Sciences International [UK] Ltd., Basingstoke, UK) to remove the ammonia, then resuspended in 500µl water. Then a NAP-5 column (Pharmacia) was equilibrated with 3ml water, before loading on the resuspended DNA

and allowing it to run through. Elution of the DNA was achieved by adding 1ml water to the column and collecting the run off. The DNA was then quantified as described in section 2.1.2.5.

Figure 2.2a Schematic of the initial sequencing strategy for the subcloned insert showing the positions of the M13 priming sites relative to the insert.

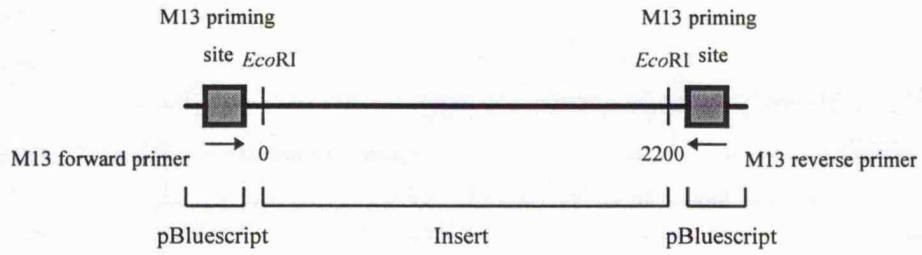
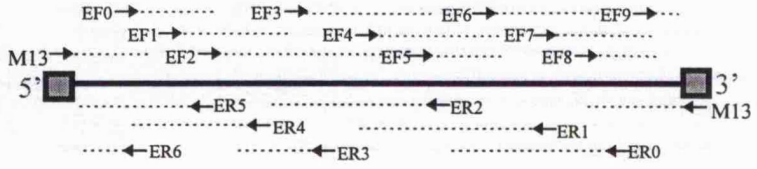


Figure 2.2b Schematic of clone E sequencing to show relative primer positions and sequence deduced with each primer. Each section of DNA was sequenced at least 3 times on each strand.



2.3.3 Analysis of DNA Sequence

This was done using the MacVector (Apple Macintosh) and GCG programs (PC).

2.3.4 5'-RACE

Once it became obvious that the clone isolated from the lambda library was truncated at the 5' end, it was decided to use the technique of 5'-RACE as a faster alternative to rescreening the original library. mRNA was extracted from rat brain tissue by the Dynabead® method (Dynal, Bromborough, UK) and stored at -80°C (see section 2.4). Three gene-specific, antisense primers and one gene-specific, sense

primer were designed to the truncated 5' end of clone E (figure 3.7) and purified as described in 2.1.2. Synthesis of cDNA by reverse transcription and PCR amplification were performed according to the instructions supplied with the Clontech 5'-RACE kit (figure 2.3). However, 6μg poly-A+ RNA was used for the initial stage, instead of the recommended 2μg, since it was thought that MADM mRNA was present only at very low levels.

The cDNA produced was mixed with 2μl sample buffer (50% Sucrose; 0.1M EDTA, pH 8.0; 0.2% Bromophenol blue) and loaded on to a 1.8% agarose gel, containing 0.5μg ml^{-1} ethidium bromide in 0.5x TBE buffer (5.4g l^{-1} Tris; 2.75g l^{-1} Boric acid; 0.001M EDTA, pH 8.0). Electrophoresis was performed in 0.5x TBE buffer for 1-2 hours at 100V. The bands were then visualised on a UV transilluminator. The PCR reaction using the anti-sense PCR primer and the sense PCR primer produced a band of known size, thus confirming the success of the 5'-RACE technique. The reaction using the anti-sense primer and the Anchor primer (supplied by Clontech) produced longer bands encoding the 5' end, which were excised and sequenced using a nested anti-sense sequencing primer (figure 2.3).

2.4 RNA AND NORTHERN BLOTTING

2.4.1 Materials used in RNA Preparation and Blotting

It is very important when using RNA that all equipment and chemicals are RNAase-free. This is facilitated by making up all solutions with diethyl pyrocarbonate (DEPC)-treated water, and using sterile tubes, tips etc.

DEPC-treated Water: 1ml DEPC per 1 litre of water, mixed and autoclaved.

2.4.2 Isolation of mRNA by the Dynal Method

The brain was removed from an adult Fischer rat using aseptic procedures. A piece of brain tissue weighing 200μg was used to prepare mRNA with the Dynabeads® mRNA Direct kit (Dynal). The mRNA is bound to 25 nucleotide-long chains of deoxy-thymidylate covalently attached to superparamagnetic, polystyrene beads, and purified according to the large-scale isolation protocol provided with the kit. The mRNA produced was quantified by spectrophotometry (see section 2.1.2.5).

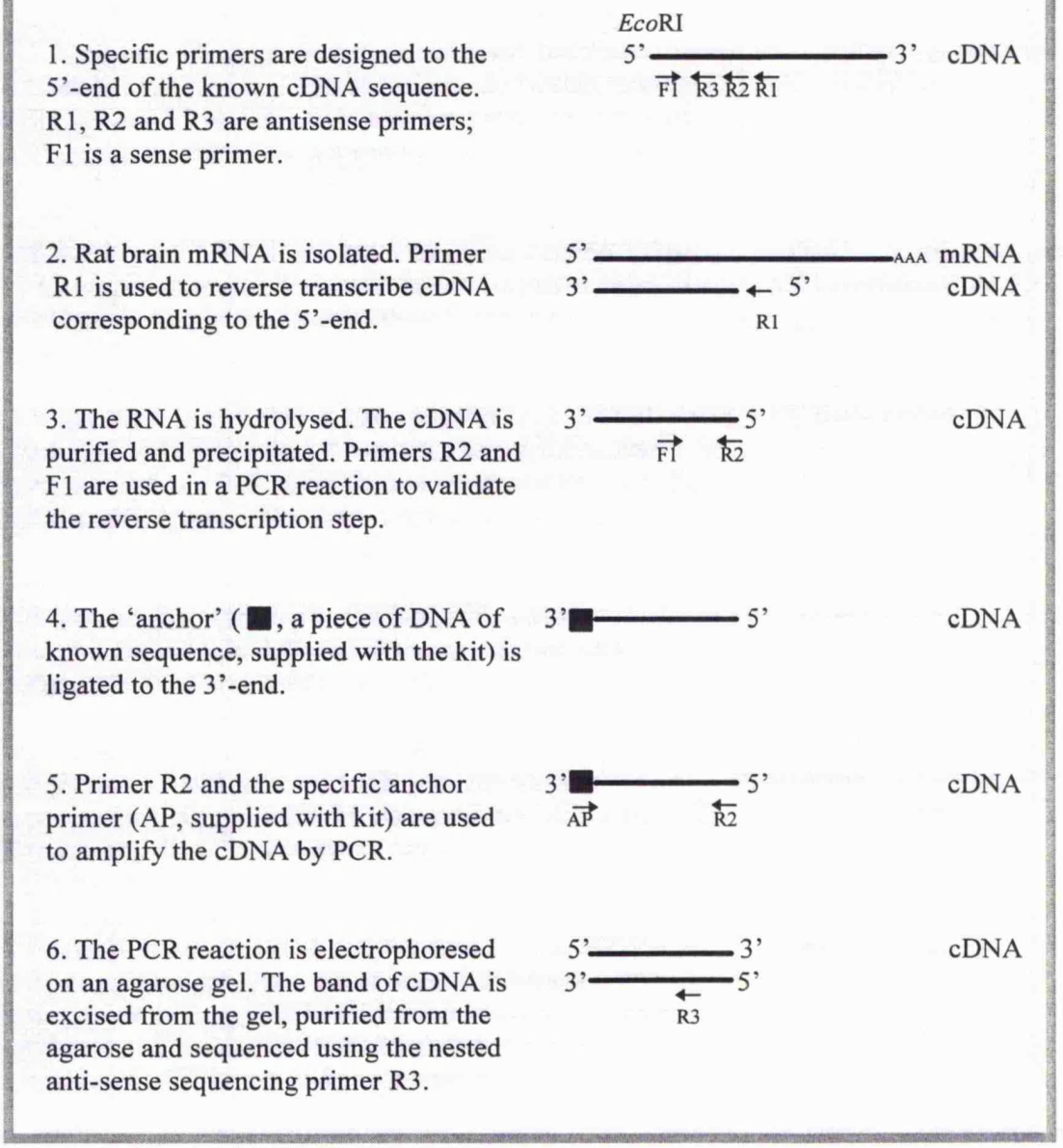
2.4.3 Electrophoresis of mRNA

2.4.3.1 Solutions required

RNA loading buffer: 50% glycerol; 1mM EDTA; 0.2% xylene cyanole; 0.2% bromophenol blue.

5xMOPS buffer: 0.1M MOPS pH 7.0; 40mM sodium acetate; 5mM EDTA pH 8.0. The yellow colour is produced by autoclaving.

Figure 2.3 A schematic of the 5'-RACE protocol
Adapted from the protocol provided with the Clontech 5'-RACE kit.



2.4.3.2 Sample preparation

mRNA (up to 5μg) was made up to 5μl in DEPC-treated water and mixed with 2μl 5x MOPS buffer, 3.5μl formaldehyde, and 10μl formamide. The solution was then heated to 65°C for 15 minutes, followed by a brief spin in a microfuge before the addition of 2μl RNA loading buffer and 1μl ethidium bromide (for later visualisation) to each sample. The RNA was then loaded into the middle wells of the gel, with RNA size markers (Gibco BRL) in the flanking lanes.

2.4.3.3 Gel preparation

Agarose (1.12g) was dissolved in 70ml DEPC-treated water in an RNase-free flask. Once cooled to 50°C, 20ml formaldehyde and 22ml 5x MOPS buffer were added and the gel poured in an RNAase-free tray and allowed to set in a fume-hood for 2 hours before use.

The gel was pre-run for 5 minutes in 1x MOPS buffer at 35V before loading of the samples. Electrophoresis was performed in 1x MOPS buffer at 22V, room temperature, overnight, attached to a recirculation pump.

2.4.4 Northern Blotting and Hybridisation

The gel was soaked in 0.05M NaOH for 20 minutes, rinsed in DEPC-treated water, and then equilibrated in 20xSSC for 45 minutes. The mRNA contained within the gel was transferred onto a nitrocellulose filter by capillary action (Sambrook *et al*, 1989).

After fixing the RNA by UV crosslinking, the filter was briefly exposed to UV light in order to check that the transfer had been successful, and so that the size markers could be visualised and their positions marked on the filter with a soft pencil. The filter was then placed in prehybridisation buffer (50% formamide, 5x SSPE, 2x Denhardt's solution, 0.1% SDS) at 45°C for at least 2 hours, before addition of a radiolabelled probe to a concentration of 10^6 counts ml^{-1} of prehybridisation solution.

2.4.4.1 Hybridisation to a specific MADM probe

The DNA used as a probe was cDNA produced by PCR using pBluescript clone E as template and primers ER0 and EF0, purified by the Geneclean method. This cDNA corresponded to the truncated rat MADM enzyme, and was used to detect

expression of rat MADM transcripts in rat tissues. The hybridisation of this probe to the mRNA was carried out at 45°C overnight, followed by washing in a solution of 2x SSC, 0.1% SDS solution at 45°C for 15 minute intervals. The wash was then made more stringent by decreasing the concentration of SSC to 0.5x and increasing the temperature of the wash to 50°C, then in steps of 5°C to 65°C. This procedure was monitored with a Geiger counter until the area of nitrocellulose above the wells, with no bound RNA to hybridise, showed no counts present. The filter was then sandwiched in Saranwrap and autoradiographed at -80°C with intensifying screens.

2.4.4.2 Checking RNA integrity with G3PDH

The integrity of the mRNA was checked by hybridising a control cDNA probe to the mRNA on the filter. The probe used was human glyceraldehyde 3-phosphate dehydrogenase (G3PDH) cDNA (from Clontech), which specifically hybridises to the mRNA product of the ubiquitously expressed housekeeping gene G3PDH. This probe strongly cross-hybridises to rat G3PDH mRNA at 1.4kb, thus providing a good test of the mRNA quality (figure 3.13). The hybridisation and washes were carried out in exactly the same way as for the specific probe. The blot was not usually stripped of the specific probe before probing with G3DPH, since the expected sizes of the specific bands (>2.4kb) were such that they did not interfere with subsequent G3PDH binding.

2.4.4.3 Removal of a hybridised probe

If it was necessary to remove a hybridised probe, this was done by boiling the filter in a solution of 0.05x SSC, 0.01M EDTA (pH 8.0), 0.1% SDS for 15 minutes. This step was then repeated with a fresh batch of boiling elution buffer. It was very important not to allow the filter to dry at any stage during hybridisation, washing and exposure to X-ray film, or the probe would have become irreversibly bound. Following elution of the probe, the filter was rinsed briefly in 0.01xSSC at room temperature, then sandwiched between two sheets of saranwrap and exposed to X-ray film to ensure that all the G3PDH probe had been removed.

2.5 PROTEIN METHODS

2.5.1 Isolation of glycoprotein fractions

2.5.1.1 Reagents

Medium A: 10mM Tris/HCl (pH 7.4); 0.02% sodium azide.

Con A buffer: 50mM Tris/HCl (pH 7.4); 100mM NaCl; 2mM $MgCl_2$; 2mM $CaCl_2$; 0.02% sodium azide.

Elution Buffer: 0.5M methyl-α-D-mannopyranoside in Con A buffer containing 0.3% CHAPS.

2.5.1.2 Preparation from tissue (Howard and Glynn, 1995)

Approximately 1g of frozen or fresh rat tissue was homogenised in 10 volumes of cold Medium A. The homogenate was subjected to centrifugation at 15,000 xg in a for 30 minutes. The resulting pellet was resuspended in 10ml of Medium A and recentrifuged. The washed pellet was then resuspended in 5ml of Medium A, and 5ml of 2% Triton X-100 in Concanavalin (Con) A buffer was added. This mixture was rotated for 30 minutes at room temperature, then centrifuged at 40,000xg for 30 minutes. The clear supernatant was transferred to a clean tube and the pellet discarded. To the supernatant was added 0.3ml Con A-Sepharose, followed by rotation at 4°C overnight.

The following day, the Sepharose was spun down and the supernatant discarded. The pellet was resuspended in 25ml Con A buffer containing 0.1% Triton X-100 and spun down before being washed sequentially with 25ml Con A buffer (detergent-free) and 25ml Con A buffer containing 0.3% CHAPS. The glycoprotein was then eluted from the sepharose by addition of at least 3ml elution buffer and rotation for 1 hour at room temperature; the sepharose pellet was then spun down and discarded. The glycoprotein containing supernatant was stored at -20°C in 0.5ml fractions.

2.5.1.3 Preparation from cells in culture

Culture media was aspirated from 3x 180cm^2 flasks of confluent cells, and the cell sheet was washed with PBS to remove serum. Then 10ml of Con A buffer

containing 1% Triton X-100 was added to each flask, and the cells scraped off with a rubber policeman. These cells were then pooled in a 50ml tube and rotated for 2 hours at room temperature. The cells were then spun down at 15,000 xg for 15 minutes at 4°C, the supernatant transferred to a clean tube and 0.3 ml of Con A-Sepharose added. From this stage the method is identical to that used for preparation of tissue glycoprotein.

2.5.1.4 Determining concentrations of glycoproteins (Bradford, 1976)

A BSA standard of 1mg ml^{-1} was pipetted in 2, 5, 10, 15, 20 and 25µl quantities into tubes in order to produce a standard curve. The glycoprotein samples were pipetted in 5, 10, 20, and 50µl quantities; a reference tube containing no protein was also prepared. To all these tubes was added 1ml of the BioRad dye reagent (BioRad, Hemel Hempstead, UK) which had been diluted 1:5 with water. The tubes were all vortexed and the absorbance of each at 595nm was measured. The absorbance of the reference sample was used to zero all the other measurements, and a standard curve of the BSA measurements was constructed, from which the concentrations of the other glycoprotein samples could be calculated.

2.5.2 One-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the procedure of Laemmli (1971) using the mini-gel apparatus from BioRad.

2.5.2.1 Assembly of gel apparatus

Gel plates (7.5 or 8.5 x 10cm) were thoroughly cleaned with methanol, and the gel cassettes assembled according to the manufacturer's instructions using 1.5mm spacers.

2.5.2.2 Gel solutions

Solution A - 30% w/v acrylamide; 0.8% w/v bis-acrylamide
(purchased as a premixed solution from Scotlab).

Solution B - 1.5M Tris/HCl (pH 8.8).

Solution C - 10% SDS.

Solution D - 0.5M Tris/HCl (pH 6.8).

Solution E - 10% w/v ammonium persulphate.

TEMED - N,N,N',N'-tetramethylethylene diamine.

2.5.2.3 Running gel preparation

The choice of acrylamide concentration depends on the molecular weight range of the proteins being studied, therefore glycoproteins were usually examined on SDS-7.5% or 10% PAGE. The required volumes of the above solutions (ml) for 8 gels are shown in table 2.3. Solutions A, B and water were mixed in a Buchner flask, and deaerated under vacuum for 10 minutes. Solution C was added and polymerisation initiated by the addition of solution E and TEMED. Gel solution was poured into the cassette to a level approximately 20mm below the top of the smallest plate. A small amount of butan-2-ol was layered over the top of the gel to exclude air and increase the speed of polymerisation. Once the gels had set, the butan-2-ol was washed off with distilled water, and any excess water absorbed with tissue.

Table 2.3 Composition of SDS-PAGE running gels showing amounts (ml) of each reagent required at the two most commonly used concentrations.

	7.5%	10%
Water	39	32
Solution A	20	27
Solution B	20	20
Solution C	0.8	0.8
Solution E	0.4	0.4
TEMED	0.06	0.06

2.5.2.4 Stacking gel preparation

A 15 lane-forming comb was inserted into the cavity above each running gel. The 5% stacking gel was prepared by mixing 28ml water, 8.5ml solution A and 12.5ml solution D in a Buchner flask, and degassing for 10 minutes. Solution C (500µl) was then added and polymerisation initiated by adding 500µl solution E and 50µl TEMED. The stacking gels were then poured rapidly, taking care to avoid trapping air bubbles around the comb, and allowed to set.

2.5.2.5 Sample preparation

Ten microlitres of 10% sample buffer (5g sucrose; 50mg Bromophenol Blue; 100µl 2M Tris; made up to 50ml with 10% SDS) containing 1% DTT were mixed into 40µl of glycoprotein sample. The mixtures were then boiled for approximately 5 minutes in order to reduce the proteins, and centifuged briefly before being loaded onto the gel.

2.5.2.6 Gel running conditions

5x SDS-PAGE Running Buffer: 15g l^{-1} Tris; 72g l^{-1} Glycine; 5g l^{-1} SDS.

The combs were removed, and the lanes filled with 1x SDS-PAGE running buffer. Samples (30µl) were loaded into the wells using a 100µl Hamilton syringe, or gel loading tips. In some lanes 10µl Novex protein size markers (R&D Systems Europe Ltd, Abingdon, UK) were loaded. The gel was clamped into an electrophoresis cell, the upper reservoir filled with 1x running buffer, and the lower reservoir filled with enough 1x running buffer to cover the bottom of the gel.

Gels were run at 200V constant voltage until the dye front of bromophenol blue was at or near the bottom of the gel. Gel cassettes were disassembled and the proteins within were transferred to a nitrocellulose membrane for western blotting, or were stained with Coomassie blue.

2.5.3 Western Blotting

2.5.3.1 Reagents

Western Blotting Buffer: 9g Tris; 43g glycine; 2.4 litres of water; 600ml methanol.

Ponceau S stain: 0.25% Ponceau S in 1% acetic acid.

Tris-Buffered Saline (TBS): 50mM Tris/HCl (pH 7.4); 0.9% NaCl.

Alkaline Phosphatase substrate: 0.1M NaCl, 5mM $MgCl_2$, 0.1M Tris/HCl pH 9.5 (5ml); 0.825mg BCIP dissolved in 100% Dimethylformamide (50µl); 1.65mg nitroblue tetrazolium dissolved in 70% DMF (50µl).

2.5.3.2 Transfer of SDS-PAGE resolved protein onto nitrocellulose

(Towbin *et al.*, 1979).

A plastic tray was filled with blotting buffer, and in this an SDS gel was placed on top of a sheet of nitrocellulose. The gel and membrane were then sandwiched between 2 sheets of 3MM filter paper, and placed between 2 sponges in a plastic holder. The holder and its contents were placed inside a BioRad transfer cell filled with blotting buffer, such that the nitrocellulose sheet was between the gel and the anode. Blotting was performed at 65V for 1 hour.

2.5.3.3 Blot staining, incubations with antisera and visualisation

The original gel was discarded and the blot immersed in Ponceau S stain for 1 minute, then washed in distilled water. The positions of each lane and of the protein size markers were marked using a pencil, and the blot completely destained using distilled water.

The blot was then incubated in 3% Marvel in TBS for 30 minutes at 37°C, or at 4° C overnight, to block non-specific binding of antibodies. The primary antibody was diluted appropriately (see section 2.8.4) in 3% Marvel in TBS, and incubated with the blot in a heat-sealed bag at room temperature for at least 1 hour with agitation. The blot was washed sequentially with (a) cold tap water; (b) 0.005% Tween 20 in TBS; (c) cold tap water; (d) distilled water.

Alkaline-phosphatase conjugated secondary antisera was purchased from Sigma, and used at a dilution of 1:250 in 3% Marvel in TBS. Incubations and washes were performed as for the primary antisera. Substrate was then added and the blot incubated until bands of a dark purple colouration appeared. This reaction was terminated by washing the blot in distilled water.

2.5.3.4 Staining and destaining of SDS-PAGE gels

The gel was placed into a dish containing 0.25% Coomassie blue-R dye in 50% methanol/10% acetic acid for 30 minutes. It was then initially destained in 50% methanol/10% acetic acid for 1 hour, then transferred to 25% methanol/10% acetic acid until the protein bands could be visualised.

2.6 CELL CULTURE TECHNIQUES

2.6.1 Cell lines cultured (obtained from ECACC, Porton Down, Salisbury).

33B: rat nervous tissue/oligodendroglioma, grown in DMEM supplemented with 10% foetal bovine serum (FBS).

C6: rat glial tumour cell line, grown in Hams F10 or F12 media supplemented with 15% horse serum and 2.5% FBS.

L6-G8-C5: rat skeletal muscle myoblast, grown in DMEM supplemented with 2mM Glutamax and 10% FBS.

NS0: mouse myeloma, grown in RPMI 1640 supplemented with 10% FBS, 0.1% gentamycin and 2mM Glutamax.

Y0: Rat myeloma, grown in RPMI 1640 supplemented with 10% FBS and 2mM Glutamax.

2.6.2 Materials used in cell cuturing

Antibiotics: Penicillin-streptomycin mix; gentamycin sulphate.

Freezing Mixture: Culture media containing up to 90% appropriate serum + 10% cryoprotectant (DMSO or glycerol).

2.6.3 Resuscitation of cells from liquid nitrogen

Ampoules containing frozen cells were removed from liquid nitrogen and left at room temperature for 2-3 minutes, before being transferred to a 37°C waterbath until thawed. Prior to opening, the ampoule was wiped with a tissue soaked in 70% alcohol. The contents of the ampoule were then transferred to a sterile tube containing 9ml of appropriate prewarmed media, and centrifuged for 5 minutes at 80g.

After aspiration of supernatant, the cells were resuspended in 10ml of prewarmed media, transferred to a 25cm^2 sterile plastic flask and incubated for 2-3 days at 37°C with 5% CO_2.

2.6.4 Passaging cells

2.6.4.1 Suspension cultures (NS0 and Y0)

The media was swirled gently to completely suspend cells, and transferred to a sterile tube. This suspension was then centrifuged at 200 xg for 5 minutes to pellet the cells. Following aspiration of the supernatant, the cells were resuspended in a total of 2ml of culture media. Twenty microlitres of cells were mixed with 20µl of trypan blue, and counted on a haemocytometer to determine the total number of viable cells present in the resuspended pellet. This total, together with the recommended seeding density of the cells (given on the data sheet provided with the cells) allowed the calculation of the number of flasks which could be seeded and cultured, and of the volume of cells which needed to be added to fresh media in each of these flasks. After successful resuscitation, cells were cultured in 10ml of media (75cm^2 flasks) or 25ml media (180cm^2 flasks).

2.6.4.2 Adherent cultures (33B, C6 and L6-G8-C5)

Once the cells had reached confluence the media was aspirated, and the cell sheet washed with PBS before addition of trypsin/EDTA (1ml per 25cm^2 flask and 2.5ml per 75cm^2 flask). The cells were then incubated at 37°C for 5-10 minutes or until the cells detached from the flasks. Then 5ml of serum-containing media was added to each flask, and the cells transferred to a sterile tube before centrifugation at 80 xg for 5 minutes. These cell pellets were resuspended in a total of 2ml of culture medium, and counted and seeded in the same way as the suspension cultured cells.

2.6.5 Freezing cells

Cells were harvested in the same way as for routine subculture (section 2.6.4), and viable cells counted. The cells were then sedimented at 80 xg for 5 minutes, and the pellet resuspended in freezing mixture (section 2.6.2) to give a cell density of

$4x10^6$ cells ml^{-1}. One millilitre aliquots of this mixture were dispensed into freezing tubes, and the cells were slowly frozen at -80°C overnight before being transferred to liquid or gaseous phase nitrogen.

2.7 Antibody Production

2.7.1 Preparation of peptide

Peptides were selected from an antigenicity plot constructed from the mature rat MADM protein sequence (figure 4.1) using the protein toolbox in the MacVector programme.

2.7.1.1 Peptides: 10mg was dissolved in 1ml of 10mM HCl. Each dissolved peptide was extracted with 5ml ether to remove contaminating ethanedithiol. The purified peptide was recovered in the aqueous phase, and the absorbance of a sample diluted 10^{-3} was measured at 205nm. For a peptide concentration of 1mg ml^{-1} the absorbance at 205nm is 30.

2.7.1.2 Activation and purification of the carrier protein (after Harlow and Lane, 1988).

Carrier protein: Keyhole Limpet Hemocyanin (KLH, 10mg per peptide) dissolved in PBS to a concentration of 10mg ml^{-1}.

Activator: MBS (3-maleimidobenzoic acid N-hydroxysuccinimide ester, 2.5mg per 10 mg of KLH) dissolved in dimethylformamide (DMF) to a concentration of 25mg ml^{-1}.

To each 1ml aliquot of KLH solution was added 100µl of MBS in DMF, and the mixture rotated for 1-2 hours at room temperature. The activated KLH was separated from the free MBS using NAP-5 columns (Pharmacia). Approximately 350µl was loaded onto each column (i.e. 3 columns were required to purify the carrier to be coupled to a single peptide), and the activated carrier was eluted with sequential 200µl aliquots of PBS. Fractions were selected manually - those containing active carrier eluted early and were frothy.

2.7.1.3 Coupling a peptide to activated KLH

The frothy eluate derived from 10mg original KLH was mixed with 1ml purified peptide, and the pH was adjusted to 7.4 by the addition of 0.1 volume of 0.25M sodium phosphate, pH 6.5. The mixture was then rotated overnight at room temperature. The following day, the volume of the mixture was made up to 4ml with PBS, and then stored at -20°C in 1ml aliquots.

2.7.2 Immunisation of rabbits

All injections and bleeds were carried out by the staff of the BioMedical Services department, University of Leicester.

2.7.2.1 Protocol for immunisation

To 1ml of carrier-coupled peptide was added 1ml of complete Freund's adjuvant. This mixture was made into an emulsion and two rabbits (New Zealand whites, young adult females) were injected at multiple subcutaneous sites with 1ml each. Approximately one month after the first injection this process was repeated with 1ml peptide added to 1ml incomplete Freund's adjuvant, and again one month later.

2.7.2.2 Test bleeds and terminal bleeds

Blood was sampled 10 days after the third injection and evaluated by examining MADM detection by the isolated sera on a Western blot of purified glycoprotein. Those animals with a good response to MADM were immediately sacrificed by a terminal bleed, and all other animals were culled. The whole blood of the best responders was subjected to centrifugation, and the sera carefully aspirated before being frozen at -20°C in aliquots.

2.7.2.3 KLH adsorption of R70

This was done to remove reactivity to the carrier molecule.

The first step was to prepare KLH-coupled Sepharose. CNBr-activated Sepharose (3g) was placed in a Falcon tube and 50ml 1mM HCl added. It was allowed to swell for 10 minutes at room temperature, and then pelleted by centrifugation. The

gel was washed three times with 50ml 1mM HCl, and once with water. All the liquid was then carefully aspirated and the wet gel was weighed. Meanwhile, 10mg of KLH was dissolved in 10ml of water. This was added to the wet gel to a final concentration of 1mg KLH per 1g of gel. Thirty millilitres of freshly made 0.1M $NaHCO_3$/0.5M NaCl solution was added to the tube, and the mix rotated overnight at 4°C.

The following day the gel was pelleted by centrifugation and resuspended in 40ml 0.1M $NaHCO_3$/0.5M NaCl/0.2M glycine (to block any remaining free CNBr sites). After rotation for 2 hours at room temperature, the gel was again pelleted. It was then washed once in 0.1M Na acetate/0.5M NaCl pH 4.0; once with 0.1M $NaHCO_3$/0.5M NaCl; and three times with TBS. Finally, the KLH-coupled sepharose was resuspended in 20ml TBS containing 0.02% sodium azide, and stored at 4°C until required.

To adsorb serum, 1ml of packed KLH-sepharose gel was added to 1ml serum diluted 1:10 with TBS, and the mixture rotated overnight at 4°C. The gel was then spun down and discarded, and the adsorbed sera stored at -20°C in 0.5ml fractions.

2.7.3 Immunisation of chickens

A pre-immune egg was required from each hen before beginning inoculations.

2.7.3.1 Protocol for immunisation

This was based on the method described by Gassmann *et al.*, 1990).

To 1ml of KLH-coupled peptide was added 1ml PBS and 1ml complete Freund's adjuvant. This was mixed to an emulsion and split between 3 Warren Studler hens, 0.5ml being injected into each of 2 sites in the breast muscle. This was repeated 12 and 20 days later. Eggs were collected and tested beginning 6 days after the final injections.

2.7.3.2 IgY preparation from chicken eggs

The egg yolk was carefully separated from the white, and washed briefly in distilled water before removal of the yolk skin. Approximately 15ml of Buffer K

(10mM potassium phosphate pH7.2; 100mM NaCl) was added per egg and mixed in gently. Thirty millilitres of Buffer K containing 7% PEG 6000 was added per egg, and the mixture stirred at room temperature for 30 minutes.

Following centrifugation for 10 minutes at 14000g and 4°C, the supernatant was filtered through a nylon gauze, before addition of solid PEG 6000 to a final concentration of 12%. Once the PEG had dissolved, the mixture was centrifuged as before. This time the supernatant was discarded and the pellet resuspended in Buffer K (10ml per egg). An equal volume of Buffer K containing 24% PEG 6000 was added and mixed thoroughly. This was again followed by centrifugation and the supernatant discarded. The remaining pellet was resuspended in Buffer K (5ml per egg) then placed into dialysis tubing and dialysed overnight against Buffer K at 4°C.

The following day, the dialysate was centrifuged as before to remove any floating precipitate. Following quantitation of the protein (IgY) content by the Bradford assay, the dialysate was frozen at -20°C in 1ml fractions. This protocol typically produced 70mg IgY per egg, which could be detected by electrophoresis of 10μg IgY protein on an SDS-PAGE gel, blotting this gel, and probing the blot with an alkaline phosphatase-conjugated anti-IgY antibody (Sigma) and the NBT/BCIP substrate.

The IgY was tested for anti-MADM activity both by Western blotting against brain preparations, using the alkaline phosphatase-conjugated anti-IgY as a secondary antibody and by ELISA (figure 4.8). For the ELISA assay, purified bovine MADM (4μg/ml in TBS, from Dr. Paul Glynn) or KLH (4μg/ml in TBS) was adsorbed overnight to the plastic wells of an ELISA plate. The following day, the wells were washed three times with tap water and once with distilled water before blocking with a 3% solution of Marvel (100μl per well) at 37°C for 30 minutes. The washing stages were then repeated, the IgY and control antigens added at a range of dilutions and the plates incubated at room temperature for 1-2 hours. After further washes, the secondary peroxidase-conjugated antibodies were added and the plates were incubated for 1-2 hours at room temperature. Finally, after another set of washes, the substrate was added: to 10ml of 0.1M citrate buffer pH4.0 was added 10mg of ABTS and 5μl hydrogen peroxide; 100μl was added to each well and the

plates were incubated for 30 minutes at room temperature before reading the absorbance at 405nm.

2.7.4 Monoclonal antibody production in mice (Harlow and Lane, 1988)

2.7.4.1 Immunisation protocol

To 100µl of peptide-KLH conjugate was added 100µl of complete Freund's adjuvant. This was injected subcutaneously into a young adult female Balb/c mouse. Approximately 2 weeks later, 100µl conjugate was mixed with 100µl incomplete adjuvant and used to inoculate the mouse; this was repeated 38 days after the first injection.

2.7.4.2 Selection of best responders

Approximately 48 days after the first injection, a test bleed was taken from the tail vein of each mouse. The resultant blood was centrifuged to pellet the red blood cells and allow aspiration of the serum. This serum was tested on a Western blot for its ability to detect MADM. Any non-responders were culled; only the best-responders were given an intravenous booster shot of 100µl peptide-conjugate, approximately 2 months after beginning immunisations. Three days after this, the mice were killed by a cardiac bleed-out, and the spleens removed aseptically. The sera obtained from these terminal bleeds was frozen at -20°C for use as a positive control later, when testing hybridomas obtained from the spleen cells.

2.7.4.3 Fusion of splenocytes and myeloma cells

Each harvested spleen was placed in 10ml serum-free RPMI 1640 media (supplemented with 2mM glutamine, 0.1% gentamycin and 1% HEPES buffer) on ice. The cells were then dissociated by being forced through a 50 mesh screen (supplied with the cell dissociation kit, Sigma), and washed in serum-free media. Approximately 50µl of the splenocyte suspension was combined with 450µl red blood cell lysis buffer and mixed. Then 10µl of this solution was mixed with 10µl of trypan blue, and a viable cell count made on a haemocytometer.

For a viable fusion at least 50 million viable splenocytes are required. These were combined in a 4:1 ratio with NS0 mouse myeloma cells (cultured as detailed in

section 2.6), which had been previously harvested and placed in serum-free media. Any remaining spleen cells, and cells from other spleens not required at the time were frozen and stored in liquid nitrogen. The mixture of splenocytes and NS0 cells for fusion was pelleted by centrifugation and the medium discarded. The pellet was resuspended by gentle tapping, and 1ml of PEG solution (50% w/v, average molecular mass 1450) was added over a period of 1 minute, accompanied by gentle stirring. After a further minute of stirring, 10 ml of pre-warmed serum-free medium was added over a period of 5 minutes, still stirring, to reform the cell membranes. The fused cells were then pelleted by centrifugation and resuspended in 150ml hybridoma medium consisting of RPMI 1640 supplemented with 2mM glutamine, 0.1% gentamycin, 1% HEPES buffer, 15% foetal bovine serum, 0.1% OPI supplement and 15% Doma Drive (Immune Systems Ltd, Bristol, UK). Approximately 150µl of the cell suspension was placed into each well of ten 96-well plates, and incubated overnight at 37°C with 5% CO_2. The following day, 100µl of drug-selection medium (hybridoma medium containing 0.1% azaserine and 1.4% hypoxanthine) was added to each well. Medium was replenished as required with drug-selection medium until the appearance of the hybridoma colonies.

2.7.4.4 Selection of positive hybridoma colonies

All wells containing a hybridoma colony, as assessed by inverted stage microscopy, were tested for anti-MADM monoclonal antibody production. Such tests were initially carried out by using a small quantity of the conditioned culture media as a probe for the rat and bovine MADM proteins on a Western blot. The conditioned media from colonies identified as positive from Western blots was also tested by ELISA, the method was that detailed in section 2.7.3.2. Purified bovine MADM (from Paul Glynn) was diluted with TBS to 4µg/ml, adsorbed overnight at 4°C to the plastic wells of an ELISA plate, blocked with 3% Marvel at 37°C for 30 minutes, and probed with a range of antibodies and sera. These included CG4, an antibody known to recognise native bovine MADM; hybridoma conditioned media; rabbit anti-MADM polyclonal antiserum; and normal mouse IgG as a background indicator. A peroxidase-conjugated secondary antibody and ABTS

substrate was used to detect binding to MADM by measuring the absorbance at 405nm (figure 4.12).

2.7.4.5 Cloning of the antibody producing colony

Once confirmed, positive colonies were expanded, and a large quantity of cells (ca. 50 million) were frozen in liquid nitrogen as a back-up. Once this had been done, the drug-selection was gradually removed from the cells, by gradually replenishing media with hybridoma media containing hypoxanthine but no azaserine. After the cells had been growing in azaserine-free media for approximately 2 weeks, the hybridoma was single-cell cloned by limiting dilution. After 14 days, the wells containing a colony grown from a single cell were selected and grown up into small flasks. The culture media was retested for antibody production, and positive clones underwent a second round of limiting dilutions, to ensure that the final cell line was the result of a single hybridoma cell. After a final test of the resultant colony for a high antibody titre in the media, the cell line was grown in bulk to produce a large quantity of the monoclonal antibody.

2.7.4.6 Addition of antibody to NS0 cells in culture

NS0 cells were grown as detailed in section 2.6. On day 0 the cells were seeded in 6 well plates at 10^5 viable cells ml^{-1}, and to the growth media was added one of the following: R70 antiserum to a final concentration of 1:50, R70 antiserum to 1:250, preimmune rabit serum to 1:50, preimune serum to 1:250, and a TBS control. For each experiment, each well was duplicated x4, and cell counts were averaged.

In the first experiment the media was not replaced or replenished for the duration of the timecourse (up to 96 hours after seeding); 25µl of cell suspension was withdrawn from each well at various times, mixed 1:1 with trypan blue, and the number of viable cells was counted using a haemocytometer (figure 4.13 panel A).

In the second experiment the media, containing antiserum/control substance, was replaced with fresh solutions every 24 hours, at which time a viable cell count was also made (figure 4.13 panel B).

2.8 IMMUNOHISTOCHEMISTRY

2.8.1 Cutting and treating sections

Slides were precoated with a gelatine substrate, in a fume hood. Half a gram of chrome alum was added to 500ml of a 1% gelatine solution, and the mixture stirred thoroughly. A single crystal of thymol was added, dissolved, and the solution filtered through 3MM paper (Whatmann). Slides were dipped into this solution for 20 seconds, then allowed to dry overnight, before being stored (shelf life 6 months at room temperature).

Small blocks of fresh rat brain were covered in Tissue-Tek embedding medium (Raymond A. Lamb, London, UK) and snap-frozen on cork in isopentane at -40°C, then stored in liquid nitrogen until required. Sections were cut from these blocks using a cryostat machine (Slee, London, UK) to a thickness of 10μm and allowed to adhere to the gelatine-coated slides for 1 hour at room temperature. The sections were then fixed in ice-cold acetone for 10 minutes, and allowed to air-dry before being frozen at -80°C with desiccant until required. Before use, these slides were allowed to equilibrate to room temperature.

2.8.2 Single staining protocol

Slides were rinsed in TBS and the sections covered in a 1% H_2O_2 solution and rocked gently at room temperature for 15 minutes to remove endogenous peroxidase activity. The sections were then rinsed once more in TBS before blocking with a 10% solution of BSA in TBS and incubation in a humidity chamber at room temperature for 30 minutes. The blocking agent was then drained from the slide and 100-200μl primary antibody, suitably diluted (see section 2.8.4) in blocking agent, was added to cover the section. Incubation was either for 1-2 hours at room temperature or overnight at 4°C. The slides were then drained and given 3 washes in TBS for 5 minutes each wash.

The secondary antibody (usually swine anti-rabbit or rabbit anti-mouse biotin-conjugate) was added at a concentration of 1:400 in TBS (figure 2.4), and the slides incubated for 30 minutes at room temperature. This was followed by another set of 5 minute washes before addition of the StreptABComplex (DAKO Ltd., High Wycombe, UK), a streptavidin-horse-radish peroxidase conjugate, and incubation for

a further 30 minutes. The sections were given a final wash, then the substrate was added. Generally this was Sigma Fast DAB (diaminobenzidine), VectaVIP or VectaRed (Vector Laboratories Ltd., Peterborough, UK) made up according to the manufacturers' instructions. The reaction was allowed to proceed for up to 5 minutes at room temperature, then stopped by rinsing in distilled water. The sections were then counterstained in Mayer's Haematoxylin or Methyl Green, before being dehydrated by passing through a series of IMS baths of increasing concentration, placed in xylene, and finally mounted with coverslips in DPX mountant. It is important in the case of methyl green not to keep reusing the counterstain since this results in fibrous blotches and elongation of the nuclear staining which detracts from the specific stain.

2.8.3 Double staining protocol

The method used is an adaptation of one detailed by van der Loos and co-workers (1992). Using the first primary antibody (a monoclonal), the sections were treated exactly as for single staining, using a biotinylated anti-mouse secondary antibody, followed by the Streptavidin-HRP conjugate, being particularly careful to wash thoroughly between additions. After addition of the first substrate, DAB, the sections were rinsed well in distilled water and reblocked with BSA. Then the second of the primary antibodies (a rabbit polyclonal) was added, and the whole staining procedure repeated using a biotinylated anti-rabbit secondary and Streptavidin-HRP (figure 2.4). The second substrate used is VectorVIP.

It is very important to include appropriate single negative and double negative control sections for each set of antibodies used.

After completion of the staining, the sections were washed once more in distilled water before being dehydrated and mounted as in section 2.8.2.

2.8.4 Antibodies used and their working dilutions

Anti-b1 integrin: (Serotec, Oxford, UK) a monoclonal antibody used at 1:100.

Anti-b3: (Chemicon, Harrow, UK) a monoclonal antibody used at 1:250.

Anti-b4: (Chemicon) a monoclonal antibody used at 1:200.

Anti-GFAP: (DAKO Ltd.) a monoclonal antibody to glial fibrillary acidic protein was used at 1:500 to detect astrocytes.

OX42: (Serotec) a monoclonal antibody to the rat homologue of the complement receptor CR3 (αMβ2 integrin) found on microglia. Used at 1:80 (cells) or 1:150 (sections).

R70: a polyclonal antisera made to peptide 3 of MADM. Commonly used at a concentration of 1:75 (cells) or 1:200 (sections).

R93: a polyclonal antisera made to peptide 5 of MADM. Used as R70.

14E: (a gift from Dr. Jia Newcombe) a monoclonal antibody (hybridoma supernatent) used to detect oligodendrocytes, used at 1:15.

2.9 MADM Expression on Cultured Cells

The expression of MADM by both cells in culture and primary brain cultures was examined by flourescent confocal microscopy, both in a resting state, and after stimulation of the cells by a range of compounds.

2.9.1 Primary culture of microglia

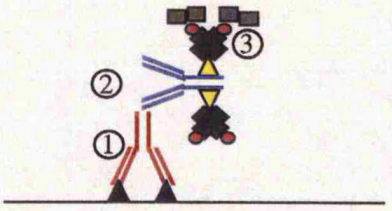
Microglia were isolated from 30 day old rat brains and cultured as detailed by Woodroofe and Cuzner (1995), except that instead of culturing in plastic chamber slides, the cells were plated out and maintained on poly-L-lysine coated glass slides (from Sigma). The cells were allowed to grow for at least 5 days before being used further.

For immediate staining, the media was removed from the cells, and the slides were rinsed in PBS and allowed to air-dry.

For stimulation of the cells, the compound of interest was added at appropriate dilutions to produce activation and upregulation of surface antigens (Loughlin *et al.*, 1991; Loughlin *et al.*, 1993). Control slides were also produced. After incubation at 37°C for 48 or 72 hours, the supernatant was removed, and the cells rinsed in PBS before air-drying.

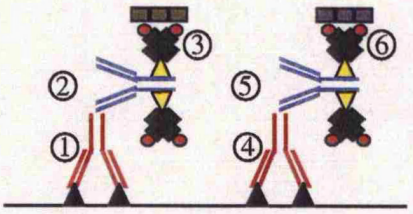
Figure 2.4 Schematic of immunohistochemical staining
Cartoon diagrams to show how (a) individual antigens are visualised, and (b) how 2 antigens can be visualised om the same section by sequential staining.

a: Schematic of single staining

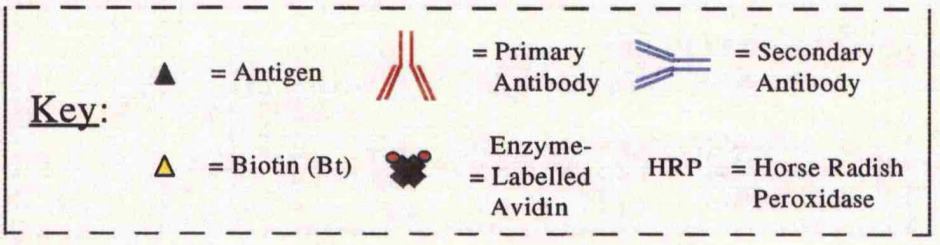


① Monoclonal antibody/ Polyclonal antisera
② Bt-anti mouse antibody/ Bt-anti rabbit antibody
③ Streptavidin-HRP complex followed by DAB (■) or VIP (■)

b: Schematic of double staining



① Monoclonal antibody
② Bt-anti mouse antibody
③ Strep-HRP complex followed by DAB (■)
④ Polyclonal antisera
⑤ Bt-anti rabbit antibody
⑥ Strep-HRP complex followed by VIP (■)



2.9.2 Compounds used to stimulate microglia

Lipopolysaccharide (LPS, Sigma; stored at -20°C as10mg ml^{-1} in EBSS) was used at 1μg per ml of culture media. **Interleukin 3** (Gibco; stored at -20°C as 10^5 U ml^{-1}in 1% BSA/PBS) was used at 1U per ml of media. **Interleukin 4** (Gibco; stored at 4°C as 10^3 U ml^{-1}in 0.1% BSA/PBS) was used at 50U per ml of media. **Granulocyte-macrophage colony-stimulating factor** (GM-CSF, Gibco; stored at -20°C as 10^3 U ml^{-1}in 1% BSA/PBS) was used at 1U per ml of media.
EBSS, 1% BSA/PBS and 0.1% BSA/PBS were used as control substances.

2.9.3 NS0 cells

NS0 cells were cultured as described in section 2.6. On the day of staining, cells taken from a single T75 flask were washed twice in PBS and resuspended in PBS to give a final volume of 1ml. An aliquot of this suspension was counted using a haemocytometer and trypan blue, and the cells diluted to give a final concentration of viable cells of 0.5 million per ml of suspension.

2.9.4 Fluorescence microscopy using acetone-fixed cells

For the production of permeablised cells, an aliquot of 100μl of the cell suspension was spread evenly onto a poly-L-lysine coated glass slide and allowed to air dry. The air-dried cells were fixed in ice-cold acetone for 5 minutes, then dried thoroughly before addition of a blocking solution of 10% BSA in TBS. After blocking for 5 minutes at 37°C, the slides were washed twice by immersion in distilled water and once with TBS. The primary antibody (diluted in 10% BSA) was then added, and the slides incubated for 2 hours at room temperature. Following another round of washing, the secondary antibody (biotinylated anti-rabbit or anti-mouse diluted 1:400 in TBS) was added and the slides incubated for a further 90 minutes, again at room temperature.

For the last stage of the staining, the slides were thoroughly washed once more and a solution of streptavidin-fluorescein isothiocyanate (FITC, Amersham) was added at a concentration of 1:100 in TBS. Incubation was for 75 minutes at room temperature

in the dark, before a final washing stage and air-drying the slides for mounting in fluorescence mountant with cover slips of thickness 0.

The cells on the slides were visualised by excitiation of the fluorescein at 490nm to produce emission at 525nm. Confocal microscopy was performed by Andy Hubbard, of the MRC Toxicology Unit, Leicester University.

2.9.5 Fluorescence microscopy using live (non-permeablised) cells

Aliquots of cells (250µl) were dispensed into 1.5ml microtubes. Two hundred and fifty microlitres of TBS blocking solution containing 0.02% sodium azide and 10% BSA was added to each tube, and the cells incubated with rotation for 30 minutes at room temperature. The cells were pelleted by centrifugation at 1000 xg for 1 minute, and resuspended in a solution of the primary antibody appropriately diluted in 0.5ml TBS blocking solution. The cells were then rotated overnight at 4°C, followed by three washes in TBS containing 0.02% azide. The secondary biotin-conjugated antibody was added, diluted 1:250 in TBS/azide, and the cells rotated at room temperature for 1 hour. Finally, the fluorescein-streptavidin conjugated antibody was added, diluted 1:100 in TBS/azide, and the cells rotated at room temperature for a further 60 minutes. The cells were washed thoroughly three times and resuspended in 50µl of TBS/azide, before submitting them to Andy Hubbard for confocal microscopy.

CHAPTER 3. THE NUCLEIC ACID AND DEDUCED AMINO ACID SEQUENCE OF RAT MADM cDNA

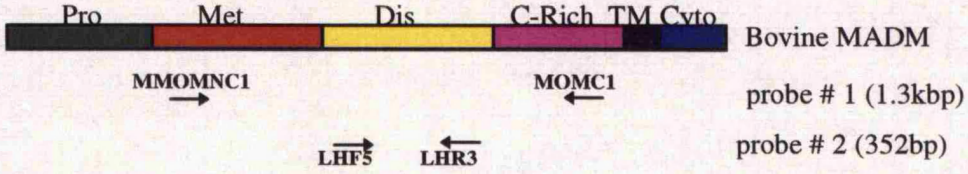
3.1 Results and discussion

3.1.1 Library Screening

The first major aim of this Ph.D. project was to clone the cDNA sequence of the rat MADM homologue. A commercially available rat brain cDNA library was screened as detailed in section 2.1.2. Four rounds of screening were carried out, utilising as probes PCR products corresponding to bovine MADM cDNA sequence. Initially the probe used was a 1.3kbp fragment which corresponded to the first three domains of the mature bovine protein coding region, and was generated by PCR using primers MMOMNC1 and MOMC1, provided by L.Howard (Fig 3.1).

Over 1 million plaques were examined on this first round of filter lifting, and just 10 positive plaques were obtained. This could imply either that rat MADM is a clone at low abundance within the library, or that the bovine sequence is not very similar to the rat MADM sequence and hybridises poorly even under low stringency conditions, an assumption not borne out by later evidence.

Figure 3.1 Production of probes for library screening
Probes were produced by PCR, with bovine MADM cDNA as a template, using primers supplied by L.Howard.

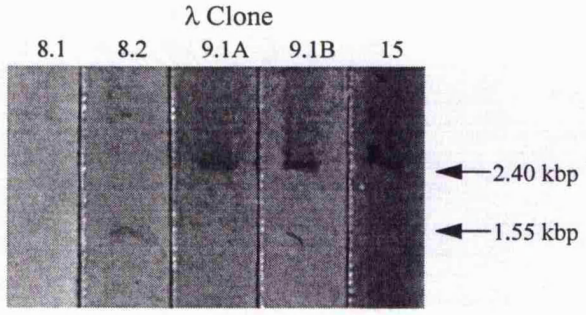


The next three rounds of filter lifting were done in duplicate, using both the probe to the mature protein coding region and also a 352bp probe corresponding to the disintegrin region only (also generated by PCR, using primers LHF5 and LHR3, from L. Howard). By this method, it was hoped to screen out clones encoding

metalloproteases other than disintegrin-metalloproteases. Of the original 10 candidates, only 5 (λ's 8.1, 8.2, 9.1A, 9.1B and 15) came through this stage, possibly indicating that the rest of the clones either contained DNA encoding other metalloproteases or that they were false positives.

After pure clones had been obtained for these 5 positives, the DNA was extracted. This was then digested with *Eco*RI and analysed by agarose gel electrophoresis. This allowed an estimation of the size of the DNA inserts contained within these clones (figure 3.2). Of the 5 positives, three (λ9.1A, 9.1B and λ15) contained inserts close to the expected size for a rat homologue of the original bovine MADM cDNA clone (ca. 2.4kb), one (λ8.2) contained an insert of approximately 1.55kb, and λ8.1 appeared to contain no insert at all, although the reason for this is unclear. The insert of 1.55kb was thought probably to be truncated, therefore it was discarded. Clone 9.1A was chosen to be examined further by subcloning and sequencing.

Figure 3.2 Positive inserts excised from λgt10
*Eco*R1 restriction digestion of the λ DNA extracted from 5 positive clones identified by library screening. λ8.1 has no apparent insert; the insert of λ8.2 is probably truncated; λ9.1A, 9.1B and 15 all contain an insert of approximately the expected size for a rat MADM homologue.



3.1.2 Subcloning

It was decided to clone the positive insert from λ9.1A into the vector pBluescript. Compared to pUC, into which the bovine MADM cDNA was cloned, this vector has the advantage of containing T3 and T7 promoters (fig. 2.1) which could be very useful since the insert could be transcribed from either promoter depending upon the

orientation. The vector pBluescript also contains M13 priming sites for the initial reactions when sequencing the insert.

Three molar ratios of vector to insert (3:1, 1:1, 1:3) were chosen for ligation (see table 2.2). Ligation was into an *Eco*RI site, and the insert was cut out from the λ clone with the same enzyme, leading to a straight-forward sticky-ends procedure. Twenty-three positive white colonies were selected and replated on 90mm L-agar plates containing ampicillin to ensure that a discrete colony could be picked for each subclone and grown up for DNA extraction. Some of this extracted DNA was digested with *Eco*RI and analysed by agarose gel electrophoresis, to ensure that the subclones contained an insert of around 2.4kb. Out of all 23 positives, only 9 appeared to contain an insert; all the others had no insert and were therefore assumed to have been false positives.

A subclone containing an insert of the expected size was chosen for further work (subclone E). An *Eco*RI digest of this clone was run on an agarose gel, blotted and probed with the disintegrin probe (figure 3.3). This showed that subclone E appeared to contain the disintegrin domain. As a confirmation, clone E was also subjected to PCR of the whole pBluescript cloning site using M13 forward and reverse primers (figure 3.4) and was again shown to contain an insert of approximately the expected size. This gel was also blotted and probed with the disintegrin probe, and hybridisation of the probe to the PCR product was seen (fig. 3.4).

3.1.3 Sequencing clone E

The primers used for initial sequencing of clone E were the M13 forward and reverse primers. Vector sequence and *Eco*RI cloning sites were quickly found, followed by sequence which was not vector-associated and therefore belonged to the insert. However, neither a poly-A tail nor a realistic polyadenylation consensus signal sequence was found.

The sequencing then continued, with primers designed to both strands from the previously elucidated sequence. The rat sequence appeared to begin at a point corresponding to nucleotide 915 in the bovine sequence. Just 5' to bovine MADM cDNA position 915 there is an internal *Eco*RI site and it seemed probable that the rat cDNA clone had also been interrupted by an *Eco*RI site.

Figure 3.3 pBluescript clone E assessed by restriction digestion and blotting
Lane A is the restriction digest of pBluescript clone E, electrophoresed on an agarose gel. Lane B is the autoradiograph produced from a blot of this agarose gel, probed with a PCR product corresponding to the disintegrin region of bovine MADM.

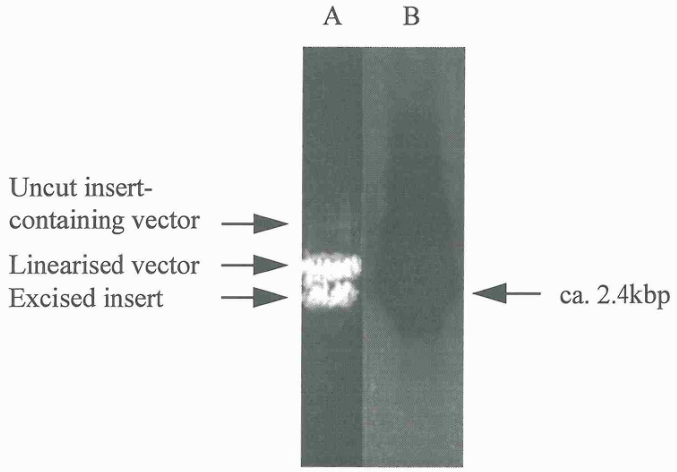
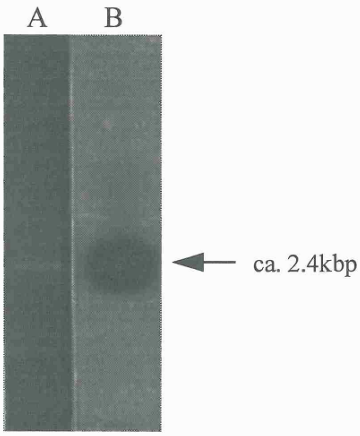


Figure 3.4 Confirmation of disintegrin-containing insert by PCR and blotting
Lane A is the product of a PCR reaction using pBluescript clone E as template and M13 primers, electrophoresed on an agarose gel. Lane B is the autoradiograph produced from the blot of this gel, probed with the same disintegrin region cDNA used for the autoradiograph in figure 3.3.



The first 1.4kb of 5' sequence of clone E could be translated to an unbroken open reading frame which is almost identical to the deduced bovine MADM protein sequence throughout the metalloprotease, disintegrin, TM and proline-rich cytoplasmic domains (figure 3.5).

Figure 3.5 Comparison of the deduced protein sequences of bovine MADM and rat brain clone E

The catalytic site of the metalloprotease domain is indicated in red, the putative integrin binding site in blue, and the potential transmembrane sequences in purple. The stop codons are indicated by •. Non-identical residues are highlighted in green.

```
Clone E       -----EQNHDDYCLAYVFTDRDFDDGVLGLAWVGAPSGSSGGICEKSKLYSDGKKKSLNTGIITV
Bovine MADM   LELNSEQNHDDYCLAYVFTDRDFDDGVLGLAWVGAPSGSSGGICEKSKLYSDGKKKSLNTGIITV 365

Clone E       QNYGSHVPPKVSHITFAHEVGHNFGSPHDSGTECTPGESKNLGQKENGNYIMYARATSGDKLNNN
Bovine MADM   QPYGSHVPPKVSHITFAHEVGHNFGSPHDSGTECTPGESKNLGQKENGNYIMYARATSGDKLNNN 430

Clone E       KFSLCSIRNISQVLEKKRNNCFVESGQPICGNGMVEQGEECDCGYSDQCKDECCFDANQPEGKKC
Bovine MADM   KFSLCSIRNISQVLEKKRNNCFVESGQPICGNGMVEQGEECDCGYSDQCKDECCYDANQPEGKKC 495

Clone E       KLKPGKQCSPSQGPCCTAQCAFKSKSEKCRDDSDCAKEGICNGFTALCPASDPKPNFTDCNRHTQ
Bovine MADM   KLKPGKQCSPSQGPCCTAHCAFKSKTEKCRDDSDCAKEGICNGITALCPASDPKPNFTDCNRHTQ 560

Clone E       VCINGQCAGSICEKYDLEECTCASSDGKDDKELCHVCCMKKMAPSTCASTGSLQWNKQFNGRTIT
Bovine MADM   VCINGQCAGSICEKHGLEECTCASSDGKDDKELCHVCCMKKMEPSTCASTGSVQWNKYFLGRTIT 625

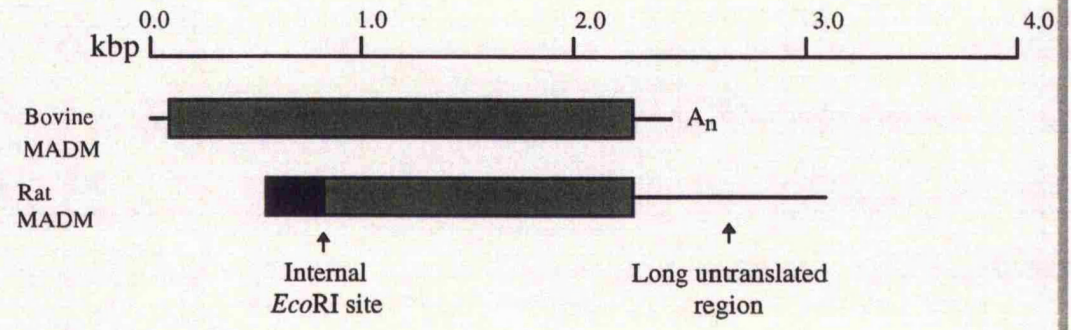
Clone E       LQPGSPCNDFRGYCDVFMRCRLVDADGPLARLKKAIFSPQLYENIAEWIVAHWWAVLLMGIALIM
Bovine MADM   LQPGSPCNDFRGYCDVFMRCRLVDADGPLARLKKAIFSPELYENIAEWIVAYWWAVLLMGIALIM 690

Clone E       LMAGFIKICSVHTPSSNPKLPPPKPLPGTLKRRRPPQPIQQPQRQRPRESYQMGHMRR•
Bovine MADM   LMAGFIKICSVHTPSSNPKLPPPKPLPGTLKRRRPPQPIQQPQRQRPRESYQMGHMRR•       749
```

There is also a high degree of identity between the 118 bases of the two nucleotide sequences immediately following the stop codons, right up to the beginning of the poly-A tail of the bovine clone. However, the further 660 nucleotides which constitute the 3' end of clone E had no counterpart in the sequence of the bovine MADM clone, and no open reading frame was found in this area. Aligning the 5' end of clone E with nucleotide 915 of bovine MADM cDNA indicated that rat MADM cDNA had a relatively long (ca.780bp) 3' untranslated region (figure 3.6).

Figure 3.6 Arrangement of bovine MADM cDNA and rat MADM cDNA sequence

Protein coding and untranslated sequences are indicated by bars and single lines respectively. Green bars show sequence obtained from lambda library clones (bovine, 2.4 kbp; rat clone E, 2.1 kbp). The black section shows additional sequence obtained by PCR (5'-RACE).



3.1.4 Obtaining more 5' sequence for rat MADM cDNA

To obtain more of the protein coding sequence of rat MADM, 5'-RACE (rapid amplification of cDNA ends) was performed. Reverse transcription of the 5' end could be performed on mRNA isolated from rat brain tissue, using a primer made to 5' sequence of the partial clone. After second strand cDNA synthesis, the product could be amplified by PCR and sequenced (a modification of the method of Frohmann *et al*, 1988) (fig 2.3). Primers were therefore designed to the truncated 5' end of clone E (figure 3.7) and RACE was attempted. The products produced were sequenced, and shown to encode approximately 300bp of the missing 5' region of rat MADM, including an *Eco*RI restriction site. Homology to the deduced bovine MADM protein sequence was observed, including the putative furin cleavage site (figure 3.8).

Figure 3.7 Rat cDNA sequence (deduced from clone E and 5'-RACE) and the primers used in sequencing. The diagram shows primers (sense primers in red and antisense in blue) and the direction of each primer (shown by the arrow). The *Eco*RI restriction site is in bold.

```
GGTCCTGAGCTCCTGAGGAAAAAGCGCACAACTCTGCCTGAGAGAAATACTTGTCAGCTCTATATCCAGACAGAT   75
SAMF2→
CACCTGTTCTTTAAATCCTATGGAACACGAGAAGCTGTGATTGCTCAGATATCCAGTCATGTTAAAGCAATTGAT  150
          ←SAMR6                              ←SAMR5
GCAATTTACCAGACTACAGACTTCTCCGGAATCCGTAACATCAGCTTTATGGTGAAACGCATAAGAATCAATACA  225
                                ←SAMR4
ACATCTGATGAAAAAGATCCTACAAATCCTTTCAGGTTCCCAAATATTGGTGTGGAGAAGTTCCTGGAGTTGAAT  300

TCTGAGCAGAATCATGATGACTACTGCTTGGCCTATGTCTTCACGGACCGGGATTTTGATGATGGTGTTCTTGGT  375
    SAMF1→
CTGGCATGGGTTGGAGCACCTTCAGGAAGCTCTGGAGGAATATGTGAGAAATCCAAGTTGTATTCAGATGGCAAG  450
←SAMR2                ←SAMR3
AAGAAGTCATTGAACACGGGCATCATTACTGTTCAGAACTATGGCTCTCACGTGCCTCCCAAAGTCTCTCATATT  525
  ←SAMR1      EF0→
ACTTTTGCTCATGAAGTTGGACATAACTTTGGATCTCCACATGATTCTGGAACAGAGTGCACTCCAGGAGAGTCT  600

AAGAACTTAGGCCAAAAAGAAAATGGCAATTACATCATGTATGCGAGAGCAACGTCTGGGGACAAACTTAACAAC  675
                                  EF1→          ←ER6
AACAAATTTTCACTCTGCAGCATTAGGAACATAAGCCAAGTGCTTGAGAAGAAGAGGAACAACTGTTTTGTTGAA  750

TCTGGCCAGCCTATCTGTGGAAATGGGATGGTGGAGCAAGGGGAAGAGTGTGACTGTGGCTACAGTGACCAATGC  825
          ←ER5 EF2→
AAAGATGAGTGCTGCTTCGATGCAAACCAGCCAGAGGGGAAGAAATGCAAGCTGAAGCCTGGGAAGCAGTGCAGT  900
                                                      ←ER4
CCAAGTCAAGGACCCTGCTGTACAGCACAGTGTGCATTCAAGTCAAAGTCTGAGAAATGTCGGGATGATTCTGAC  975

TGTGCAAAGGAAGGGATTTGCAATGGCTTCACAGCCCTTTGCCCAGCATCTGATCCCAAGCCCAACTTTACAGAC 1050

TGTAACAGGCATACACAAGTGTGCATTAATGGGCAATGTGCAGGTTCTATCTGTGAGAAGTATGACTTGGAGGAG 1125
                                                  EF3→
TGCACCTGTGCCAGCTCTGATGGCAAAGATGATAAGGAATTATGTCATGTTTGCTGCATGAAGAAAATGGCCCCA 1200
          ←ER3
TCAACTTGTGCCAGTACAGGCTCTTTGCAGTGGAACAAGCAGTTCACTGGTCGGACTATCACTCTGCAGCCAGGC 1275

TCTCCATGTAATGACTTCAGAGGCTACTGTGATGTTTTCATGCGGTGCAGATTAGTAGATGCTGATGGCCCTCTA 1350

GCTAGGCTGAAAAAAGCAATTTTTAGTCCACAACTCTATGAAAACATTGCTGAGTGGATTGTGGCTCACTGGTGG 1425
                                                          EF4→
GCAGTACTGCTTATGGGAATTGCCCTGATCATGCTAATGGCTGGATTTATCAAGATTTGCAGTGTTCACACTCCA 1500

AGTAGTAATCCAAAGTTGCCTCCTCCTAAACCACTTCCAGGCACTTTAAAGAGGAGGAGACCGCCACAGCCCATT 1575
                                                  EF5→
CAGCAGCCCCAGCGTCAGAGGCCCCGGGAGAGTTATCAAATGGGACACATGCGACGCTGATGCAGCTTTTGCCTT 1650
                                                  ←ER2
GGTTCTTCCTAGTGCCTACAGTGGGAAAACTTCACTCCAAAGAGAAACCTGTTAAGTCATCATCTGCAAATGAAA 1725

CCCTCACAGTTAATAGTTGAAGAAAAAATGGCAAGAGATCATGTCCTCAGACCAGGTGGAATTACCTAAAATTTA 1800
                                  EF6→
AAGCCTGAAAATTCCAATTTTTTTGGGGGGGGTGGGATGGAAAAGGAACCCAATTTTCTTATGGACAGATATTTTT 1875
```

```
AACCTAATGGCACAAAGTCTTAGAATATTATTATGTGCCCTGTGTTCCCTGTTCTTCGTTGCTGCATTTTCTTCA  1950

CTTGCAGACAGACTTGGCTCTCGATAAACTTTTATCACAAATTGAAATATATTTTTTTCAACTGCCAATCTAGGC  2025
                                                                  EF7→

TGGGAGGCTCGACCACCTCAACATTGGATACATCACTTGCCAATGTACATACCTTGTAATATGCAGACATGTATT  2100
                                        ←ER1

TCTTACGTACACTGTACTTCTCTGTGCAATTTGTAAACAGAAATTGCAATATGGATGTTTCTTTGTATAATAAAT  2175
 EF8→

TTTTCCGCTCTTAATGAAAAATTACTGTTTAATTGACATACTCAGGATAACAGAGAATGGTGGTGTGCAGTGGTC  2250
                                                    ←ER0

CAGGATTCTGTAATGCTTCATGCAGGCCATTTTGAAATGAGAATTGAAAACCCTTTTTCTTATGGTGGAGTTGGT  2325
                                                    EF9→

TCTTACATGCATCCTTTTCCTGTTCCATGGCTTCTGTGAGTAGGAGAGGCTGTGTTGAAGAGCTCAGCTGGTGGC  2400

TTACCCG
←SAMR0
```

Actual Primer Sequences

SAMF1	GCAGAATCATGATGACTACTGCTTGGCC	**SAMR0**	GGGTAAGCCACCAGCTGAGCTC
SAMF2	GGTCCTGAGCTCCTGAGG	**SAMR1**	GACTTCTTCTTGCCATCTGAATACAAC
EF0	CACGGGCATCATTACTGTTCAG	**SAMR2**	AGAGCTTCCTGAAGGTGCTCCAACCCA
EF1	GCGAGAGCAACGTCTGGGGAC	**SAMR3**	GCCAGACCAAGAACACCATCATC
EF2	GTGGAAATGGGATGGTGG	**SAMR4**	CGGATTCCGGAGAAGTCTGT
EF3	GAAGTATGACTTGGAGGAGTGC	**SAMR5**	GCAATCACAGCTTCTCGTGTTCC
EF4	GCTCACTGGTGGGCAGTACTGC	**SAMR6**	GATTTAAAGAACAGGTGATCTG
EF5	GGAGACCGCCACAGCCCATTCAGCA	**ERO**	CTGCACACCACCATTCTCTG
EF6	GGCAAGAGATCATGTCCTCAGACCAGG	**ER1**	TTGGCAAGTGATGTATCCAATG
EF7	CTGCCAATCTAGGCTGGGAGGCTCG	**ER2**	CATCAGCGTCGCATGTGTCCC
EF8	CTTACGTACACTGTACTTCTCTGTGC	**ER3**	GCCATCAGAGCTGGCACAGGTGC
EF9	GGTGGAGTTGGTTCTTACATGCATCC	**ER4**	ACTGCACTGCTTCCCAGGCTTC
		ER5	CCACAGATAGGCTGGCCAGATTC
		ER6	GTCCCCAGACGTTGCTCTCGC

For 5'-RACE groups of primers were used as follows:

GROUP A) SAMR1, SAMR2, SAMR3 AND SAMF1
GROUP B) SAMR4, SAMR5, SAMR6 AND SAMF2

Where R1/R4 are used for cDNA synthesis; R2/R5 are used for PCR in conjunction with the anchor primer in the kit; F1/F2 are used as PCR control primers; and R3/R6 are used as sequencing primers.

Figure 3.8 Nucleotide sequence obtained by 5'-RACE, and its amino acid translation

The putative furin cleavage site RKKR is shown in pink; sequence which overlaps that obtained from clone E is underlined. The EcoR1 restriction site is marked in bold

.

```
GGTCCTGAGCTCCTGAGGAAAAAGCGCACAACTCTGCCTGAGAGAAATACTTGTCAGCTCTATATCCAGACAGAT   75
GlyProGluLeuLeuArgLysLysArgThrThrLeuProGluArgAsnThrCysGlnLeuTyrIleGlnThrAsp

CACCTGTTCTTTAAATCCTATGGAACACGAGAAGCTGTGATTGCTCAGATATCCAGTCATGTTAAAGCAATTGAT  150
HisLeuPhePheLysSerTyrGlyThrArgGluAlaValIleAlaGlnIleSerSerHisValLysAlaIleAsp

GCAATTTACCAGACTACAGACTTCTCCGGAATCCGTAACATCAGCTTTATGGTGAAACGCATAAGAATCAATACA  225
AlaIleTyrGlnThrThrAspPheSerGlyIleArgAsnIleSerPheMetValLysArgIleArgIleAsnThr

ACATCTGATGAAAAAGATCCTACAAATCCTTTCAGGTTCCCAAATATTGGTGTGGAGAAGTTCCTGGAGTTGAAT  300
ThrSerAspGluLysAspProThrAsnProPheArgPheProAsnIleGlyValGluLysPheLeuGluLeuAsn

TCTGAGCAGAATCATGATGACTACTGCTTGGCCTATGTCTTCACGGACCGGGATTTTGATGATGGTGTTCTT     372
SerGluGlnAsnHisAspAspTyrCysLeuAlaTyrValPheThrAspArgAspPheAspAspGlyValLeu
```

3.1.5 Detailed examination of the rat MADM sequence

3.1.5.1 Comparing rat, bovine and human MADM cDNA sequences

The combined clone E and 5'-RACE nucleic acid sequence, translated using the MacVector programmes for Apple Macintosh, contains the complete coding region for mature rat MADM as well as 27bp encoding 5 residues of pro-sequence and a putative furin cleavage site at the 5'-end, and an untranslated region at the 3' end of the clone (figure 3.9). Rat MADM cDNA is highly homologous to both the bovine and human MADM cDNA sequences (figure 3.10, L.Howard and X-H.Lu) with around 89.7% identity at the nucleotide level over the region of overlap. In addition, the rat clone has a long (approximately 780bp) 3'-untranslated region (see figure 3.6), which is not echoed in either the bovine or the human sequence, and there is no realistic polyadenylation signal sequence or poly-A+ tail contained within this region. There does appear to be a polyadenylation signal sequence (AAUAAA, residues 2169-2174) which is the exact consensus polyadenylation/cleavage signal (Sheets et al, 1990). However, this is more than 200 bases from the 3'-end of the clone and seems unlikely to have been used in this particular case.

Figure 3.9 Total available rat MADM cDNA and the protein encoded by the ORF

The protein coding region is shown in capital letters, 3'-UTS is in lower case.
The extended zinc-binding site is highlighted in red, the putative integrin-binding site in blue and the potential transmembrane domain in purple. Also on this figure, the putative furin cleavage site is in pink, while the internal *Eco*RI site and polyadenylation sequence are shown in bold.

```
GGTCCTGAGCTCCTG AGGAAAAAGCGCACA ACTCTGCCTGAGAGA AATACTTGTCAGCTC TATATCCAGACAGAT   75
GlyProGluLeuLeu ArgLysLysArgThr ThrLeuProGluArg AsnThrCysGlnLeu TyrIleGlnThrAsp

CACCTGTTCTTTAAA TCCTATGGAACACGA GAAGCTGTGATTGCT CAGATATCCAGTCAT GTTAAAGCAATTGAT  150
HisLeuPhePheLys SerTyrGlyThrArg GluAlaValIleAla GlnIleSerSerHis ValLysAlaIleAsp

GCAATTTACCAGACT ACAGACTTCTCCGGA ATCCGTAACATCAGC TTTATGGTGAAACGC ATAAGAATCAATACA  225
AlaIleTyrGlnThr ThrAspPheSerGly IleArgAsnIleSer PheMetValLysArg IleArgIleAsnThr

ACATCTGATGAAAAA GATCCTACAAATCCT TTCAGGTTCCCAAAT ATTGGTGTGGAGAAG TTCCTGGAGTTGAAT  300
ThrSerAspGluLys AspProThrAsnPro PheArgPheProAsn IleGlyValGluLys PheLeuGluLeuAsn

TCTGAGCAGAATCAT GATGACTACTGCTTG GCCTATGTCTTCACG GACCGGGATTTTGAT GATGGTGTTCTTGGT  375
SerGluGlnAsnHis AspAspTyrCysLeu AlaTyrValPheThr AspArgAspPheAsp AspGlyValLeuGly

CTGGCATGGGTTGGA GCACCTTCAGGAAGC TCTGGAGGAATATGT GAGAAATCCAAGTTG TATTCAGATGGCAAG  450
LeuAlaTrpValGly AlaProSerGlySer SerGlyGlyIleCys GluLysSerLysLeu TyrSerAspGlyLys

AAGAAGTCATTGAAC ACGGGCATCATTACT GTTCAGAACTATGGC TCTCACGTGCCTCCC AAAGTCTCTCATATT  525
LysLysSerLeuAsn ThrGlyIleIleThr ValGlnAsnTyrGly SerHisValProPro LysValSerHisIle

ACTTTTGCTCATGAA GTTGGACATAACTTT GGATCTCCACATGAT TCTGGAACAGAGTGC ACTCCAGGAGAGTCT  600
ThrPheAlaHisGlu ValGlyHisAsnPhe GlySerProHisAsp SerGlyThrGluCys ThrProGlyGluSer

AAGAACTTAGGCCAA AAAGAAAATGGCAAT TACATCATGTATGCG AGAGCAACGTCTGGG GACAAACTTAACAAC  675
LysAsnLeuGlyGln LysGluAsnGlyAsn TyrIleMetTyrAla ArgAlaThrSerGly AspLysLeuAsnAsn

AACAAATTTTCACTC TGCAGCATTAGGAAC ATAAGCCAAGTGCTT GAGAAGAAGAGGAAC AACTGTTTTGTTGAA  750
AsnLysPheSerLeu CysSerIleArgAsn IleSerGlnValLeu GluLysLysArgAsn AsnCysPheValGlu

TCTGGCCAGCCTATC TGTGGAAATGGGATG GTGGAGCAAGGGGAA GAGTGTGACTGTGGC TACAGTGACCAATGC  825
SerGlyGlnProIle CysGlyAsnGlyMet ValGluGlnGlyGlu GluCysAspCysGly TyrSerAspGlnCys

AAAGATGAGTGCTGC TTCGATGCAAACCAG CCAGAGGGGAAGAAA TGCAAGCTGAAGCCT GGGAAGCAGTGCAGT  900
LysAspGluCysCys PheAspAlaAsnGln ProGluGlyLysLys CysLysLeuLysPro GlyLysGlnCysSer

CCAAGTCAAGGACCC TGCTGTACAGCACAG TGTGCATTCAAGTCA AAGTCTGAGAAATGT CGGGATGATTCTGAC  975
ProSerGlnGlyPro CysCysThrAlaGln CysAlaPheLysSer LysSerGluLysCys ArgAspAspSerAsp

TGTGCAAAGGAAGGG ATTTGCAATGGCTTC ACAGCCCTTTGCCCA GCATCTGATCCCAAG CCCAACTTTACAGAC 1050
CysAlaLysGluGly IleCysAsnGlyPhe ThrAlaLeuCysPro AlaSerAspProLys ProAsnPheThrAsp

TGTAACAGGCATACA CAAGTGTGCATTAAT GGGCAATGTGCAGGT TCTATCTGTGAGAAG TATGACTTGGAGGAG 1125
CysAsnArgHisThr GlnValCysIleAsn GlyGlnCysAlaGly SerIleCysGluLys TyrAspLeuGluGlu

TGCACCTGTGCCAGC TCTGATGGCAAAGAT GATAAGGAATTATGT CATGTTTGCTGCATG AAGAAAATGGCCCCA 1200
CysThrCysAlaSer SerAspGlyLysAsp AspLysGluLeuCys HisValCysCysMet LysLysMetAlaPro

TCAACTTGTGCCAGT ACAGGCTCTTTGCAG TGGAACAAGCAGTTC ACTGGTCGGACTATC ACTCTGCAGCCAGGC 1275
SerThrCysAlaSer ThrGlySerLeuGln TrpAsnLysGlnPhe ThrGlyArgThrIle ThrLeuGlnProGly

TCTCCATGTAATGAC TTCAGAGGCTACTGT GATGTTTTCATGCGG TGCAGATTAGTAGAT GCTGATGGCCCTCTA 1350
SerProCysAsnAsp PheArgGlyTyrCys AspValPheMetArg CysArgLeuValAsp AlaAspGlyProLeu
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```
GCTAGGCTGAAAAAA GCAATTTTTAGTCCA CAACTCTATGAAAAC ATTGCTGAGTGGATT GTGGCTCACTGGTGG 1425
AlaArgLeuLysLys AlaIlePheSerPro GlnLeuTyrGluAsn IleAlaGluTrpIle ValAlaHisTrpTrp

GCAGTACTGCTTATG GGAATTGCCCTGATC ATGCTAATGGCTGGA TTTATCAAGATTTGC AGTGTTCACACTCCA 1500
AlaValLeuLeuMet GlyIleAlaLeuIle MetLeuMetAlaGly PheIleLysIleCys SerValHisThrPro

AGTAGTAATCCAAAG TTGCCTCCTCCTAAA CCACTTCCAGGCACT TTAAAGAGGAGGAGA CCGCCACAGCCCATT 1575
SerSerAsnProLys LeuProProProLys ProLeuProGlyThr LeuLysArgArgArg ProProGlnProIle

CAGCAGCCCCAGCGT CAGAGGCCCCGGGAG AGTTATCAAATGGGA CACATGCGACGCtga tgcagcttttgcctt 1650
GlnGlnProGlnArg GlnArgProArgGlu SerTyrGlnMetGly HisMetArgArg

ggttcttcctagtgc ctacagtgggaaaac ttcactccaaagaga aacctgttaagtcat catctgcaaatgaaa 1725
ccctcacagttaata gttgaagaaaaaatg gcaagagatcatgtc ctcagaccaggtgga attacctaaaattta 1800
aagcctgaaaattcc aatttttttgggggg gtgggatggaaaagg aacccaattttctta tggacagatattttt 1875
aacctaatggcacaa agtcttagaatatta ttatgtgccctgtgt tccctgttcttcgtt gctgcattttcttca 1950
cttgcagacagactt ggctctcgataaact tttatcacaaattga aatatatttttttca actgccaatctaggc 2025
tgggaggctcgacca cctcaacattggata catcacttgccaatg tacataccttgtaat atgcagacatgtatt 2100
tcttacgtacactgt acttctctgtgcaat ttgtaaacagaaatt gcaatatggatgttt ctttgtataataaat 2175
ttttccgctcttaat gaaaaattactgttt aattgacatactcag ataacagagaatggt ggtgtgcagtggtcc 2250
aggattctgtaatgc ttcatgcaggccatt ttgaaatgagaattg aaaaccctttttctt atggtggagttggtt 2325
cttacatgcatcctt ttcctgttccatggc ttctgtgagtaggag aggctgtgttgaaga gctcagctggtggct 2400
tacccg                                                                       2406
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It is possible however, that this signal could be used in alternative 3'-processing, a post-translational mechanism postulated for other proteins (I-Hong Hsu *et al*, 1990). Differential transcript heterogeneity has been shown in various multidrug-resistant cell lines to be generated by alternative usage of different poly(A) addition signals in the 3'-UTS of mdr1a transcripts. A similar pattern has been reported for the human aromatase P450 gene (Toda *et al.*, 1989). It is therefore possible that a variation in MADM transcripts could be found in different tissues/cell lines.
The nucleotide and derived protein sequence was submitted to EMBL in February 1995, and allocated the accession number Z48444.

3.1.5.2 Protein comparisons

Overall there is ca. 97% identity at the amino acid level between the deduced mature protein sequences for rat and bovine MADM (figure 3.11). Such a high level of conservation across species suggests a critical function for the MADM protein. This is a substantially greater degree of interspecies conservation than that observed for other members of the ADAM family: rat and monkey EAP-I exhibit a 72% sequence identity overall, 77% in the disintegrin domain (Perry *et al.*, 1992); guinea-pig fertilin shows only 40% sequence similarity to mouse cyritestin (Heinlein *et al.*, 1994). The N-terminal of the rat protein does however differ significantly from the bovine sequence; the anti-bovine MADM monoclonal antibody M-N was raised to the sequence TTVAEKNTC, whereas the equivalent rat sequence reads TTLPERNTC. The three mismatches in the middle of this sequence may provide the answer as to why this monoclonal antibody was unable to recognise rat MADM on Western blots.
However, rat MADM does contain a full metalloprotease consensus sequence (HEXXHXXGXXH) identical to bovine MADM, as does human MADM (figure 3.11). The residue immediately following the last H is a D, as expected, thus confirming MADM as a member of the snake venom family of proteases (table 1.4). The disintegrin domain is also largely identical, particularly at the putative integrin binding site, DSDC, and all of the important cysteine residues in this region are conserved.

Figure 3.10 Comparison of rat MADM cDNA to the bovine and human MADM cDNA sequences.

Rat cDNA sequence is shown in red, bovine sequence in blue and human sequence in black. Sequence forming an open reading frame is in upper case, untranslated regions in lower case. Potential polyadenylation sequences are in bold type.

```
BOV                          GGCGGCGG CACGGAAGAT GGTGTTGCTG AGAGTGTTAA TTCTGCTCCT   48
BOV CTCCTGGGTT GCGGGGCTGG GAGGTCAGTA TGGAAATCCT TTAAATAAAT ACATTAGACA TTATGAAGGA  118
BOV TTATCATATG ATGTGGATTC ATTACACCAA AAACACCAGC GTGCCAAAAG AGCAGTATCA CATGAGGACC  188
BOV AGTTTTTACG GCTAGATTTT CATGCTCATG GAAGACATTT CAACCTTCGA ATGAAGAGGG ATACTTCCCT  258
BOV TTTCAGTGAG GAGTTTAGGG TGGAAACATC AAATGCAGTA CTGGATTATG ATACTTCTCA TATTTACACT  328
BOV GGACATATTT ATGGTGAAGA AGGAAGTTTA GCCATGGGTC TGTTATTGAT GGAAGATTTG AAGGATTCAT  398
BOV TCAGACTCAT GGTGGCACGA TTTTATGTTG AACCAGCAGA GAGATATATT AAAGACCGAA CTCTGCCATT  468
BOV TCACTCTGTC ATTTATCATG AAGATGATAT TAAGTATCCC CATAAATATG GTCCACAGGG GCGTTGTGCA  538
BOV GATCATTCAG TGTTTGAAAG AATGAGGAAG TACCAGATGA CTGGTGTAGA AGAAGTAACA CAGACACCTC  608

RAT                          GGTCCTGAGC TCCTGAGGAA AAAGCGCACA ACTCTGCCTG AGAGAAATAC   50
BOV AAGAAAAACA TGCTATTAAT GGTCCAGAAC TCCTGAGGAA AAAACGTACA ACTGTAGCTG AAAAAAATAC  678

RAT TTGTCAGCTC TATATCCAGA CAGATCACCT GTTCTTTAAA TCCTATGGAA CACGAGAAGC TGTGATTGCT  120
BOV CTGTCAGCTT TATATTCAGA CCGATCATCT GTTCTTTAAA TATTACGGAA CACGAGAAGC TGTGATGCCT  748

RAT CAGATATCCA GTCATGTTAA AGCAATTGAT GCAATTTACC AGACTACAGA CTTCTCCGGA ATCCGTAACA  190
BOV CAGATATCCA GTCATGTTAA AGCAATTGAC ACAATTTACC AGACAACAGA CTTCTCTGGA ATCCGTAACA  818
HUM                                CC AGACAACAGA CTTCTCCGGA ATCCGTAACA   32

RAT TCAGCTTTAT GGTGAAACGC ATAAGAATCA ATACAACATC TGATGAAAAA GATCCTACAA ATCCTTTCAG  260
BOV TCAGTTTCAT GGTGAAACGC ATAAGAATCA ACACAACTGC TGATGAGAAG GACCCTACAA ATCCATTCCG  888
HUM TCAGTTTCAT GGTGAAACGC ATAAGAATCA ATACAACTGC TGATGAGAAG GACCCTACAA ATCCTTTCCG  102

RAT GTTCCCAAAT ATTGGTGTGG AGAAGTTCCT GGAGTTGAAT TCTGAGCAGA ATCATGATGA CTACTGCTTG  330
BOV TTTTCCAAAT ATTGGTGTGG AGAAGTTTCT GGAGCTGAAT TCTGAGCAGA ATCATGATGA CTACTGTTTG  958
HUM TTTCCCAAAT ATTAGTGTGG AGAAGTTTCT GGAATTGAAT TCTGAGCAGA ATCATGATGA CTACTGTTTG  172

RAT GCCTATGTCT TCACGGACCG GGATTTTGAT GATGGTGTTC TTGGTCTGGC ATGGGTTGGA GCACCTTCAG  400
BOV GCCTACGTTT TCACAGATCG AGATTTTGAT GATGGTGTCC TTGGTCTGGC GTGGGTTGGA GCACCTTCAG 1028
HUM GCCTATGTCT TCACAGACCG AGATTTTGAT GATGGCGTAC TTGGTCTGGC TTGGGTTGGA GCACCTTCAG  242

RAT GAAGCTCTGG AGGAATATGT GAGAAATCCA AGTTGTATTC AGATGGCAAG AAGAAGTCAT TGAACACGGG  470
BOV GAAGCTCTGG AGGAATATGT GAAAAAAGTA AGCTCTATTC AGATGGTAAG AAGAAGTCTT TAAACACTGG 1098
HUM GAAGCTCTGG AGGAATATGT GAAAAAAGTA AACTCTATTC AGATGGTAAG AAGAAGTCCT TAAACACTGG  312

RAT CATCATTACT GTTCAGAACT ATGGCTCTCA CGTGCCTCCC AAAGTCTCTC ATATTACTTT TGCTCATGAA  540
BOV AATTATTACT GTTCAGAACT ATGGGTCTCA CGTACCCCCC AAAGTCTCTC ACATTACGTT TGCTCATGAA 1168
HUM AATTATTACT GTTCAGAACT ATGGGTCTCA AAAGTCTCTC ACATTACTTT TGCTCACGAA GTTGGACATA  402

RAT GTTGGACATA ACTTTGGATC TCCACATGAT TCTGGAACAG AGTGCACTCC AGGAGAGTCT AAGAACTTAG  610
BOV GTTGGACATA ACTTTGGATC TCCGCATGAT TCTGGAACAG AGTGCACTCC AGGAGAATCT AAGAATTTAG 1238
HUM TGTACCTCCC ACTTTGGATC CCCACATGAT TCTGGAACAG AGTGCACACC AGGAGAATCT AAGAATTTGG  452

RAT GCCAAAAAGA AAATGGCAAT TACATCATGT ATGCGAGAGC AACGTCTGGG GACAAACTTA ACAACAACAA  680
BOV GACAAAAAGA AAATGGCAAC TACATCATGT ATGCAAGAGC AACATCTGGG GACAAACTTA ACAACAATAA 1308
HUM GTCAAAAAGA AAATGGCAAT TACATCATGT ATGCAAGAGC AACATCTGGG GACAAACTTA ACAACAATAA  522

RAT ATTTTCACTC TGCAGCATTA GGAACATAAG CCAAGTGCTT GAGAAGAAGA GGAACAACTG TTTTGTTGAA  750
BOV ATTCTCACTC TGTAGTATTA GAAATATAAG TCAAGTTCTT GAGAAGAAGA GAAACAACTG TTTTGTTGAA 1378
HUM ATTCTCACTC TGTAGTATTA GAAATATAAG CCAAGTTCTT GAGAAGAAGA GAAACAACTG TTTTGTTGAA  592

RAT TCTGGCCAGC CTATCTGTGG AAATGGGATG GTGGAGCAAG GGGAAGAGTG TGACTGTGGC TACAGTGACC  820
BOV TCTGGCCAAC CTATTTGTGG AAATGGGATG GTAGAACAAG GTGAAGAATG TGATTGTGGT TATAGTGACC 1448
HUM TCTGGCCAAC CTATTTGTGG AAATGGAATG GTAGAACAAG GTGAAGAATG TGATTGTGGC TATAGTGACC  662

RAT AATGCAAAGA TGAGTGCTGC TTCGATGCAA ACCAGCCAGA GGGGAAGAAA TGCAAGCTGA AGCCTGGGAA  890
BOV AGTGTAAAGA CGAGTGCTGC TACGATGCAA ATCAGCCGGA GGGGAAAAAA TGCAAGCTGA AACCTGGAAA 1518
HUM AGTGTAAAGA TGAATGCTGC TTCGATGCAA ATCAACCAGA GGGAAGAAAA TGCAAACTGA AACCTGGGAA  732

RAT GCAGTGCAGT CCAAGTCAAG GACCCTGCTG TACAGCACAG TGTGCATTCA AGTCAAAGTC TGAGAAATGT  960
BOV GCAGTGCAGT CCAAGTCAAG GTCCCTGTTG TACAGCACAT TGTGCATTCA AGTCAAAAAC TGAAAAGTGT 1588
HUM GCAGTGCAGT CCAAGTCAAG GTCCTTGTTG TACAGCACAG TGTGCATTCA AGTCAAAGTC TGAGAAGTGT  802
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RAT CGGGATGATT CTGACTGTGC AAAGGAAGGG ATTTGCAATG GCTTCACAGC CCTTTGCCCA GCATCTGATC 1030
BOV CGGGATGATT CAGACTGTGC AAAAGAAGGA ATATGTAATG GCATCACAGC TCTCTGCCCA GCCTCTGATC 1658
HUM CGGGATGATT CAGACTGTGC AAGGGAAGGA ATATGTAATG GCTTCACAGC TCTCTGCCCA GCATCTGACC  872

RAT CCAAGCCCAA CTTTACAGAC TGTAACAGGC ATACACAAGT GTGCATTAAT GGGCAATGTG CAGGTTCTAT 1100
BOV CTAAACCGAA CTTCACAGAC TGTAATAGAC ATACGCAAGT GTGCATTAAT GGGCAATGTG CAGGTTCTAT 1728
HUM CTAAACCAAA CTTCACAGAC TGTAATAGGC ATACACAAGT GTGCATTAAT GGGCAATGTG CAGGTTCTAT  942

RAT CTGTGAGAAG TATGACTTGG AGGAGTGCAC CTGTGCCAGC TCTGATGGCA AAGATGATAA GGAATTATGT 1170
BOV CTGTGAGAAA CATGGCTTGG AGGAGTGTAC CTGTGCCAGT TCTGATGGCA AAGATGATAA GGAATTATGC 1798
HUM CTGTGAGAAA TATGGCTTAG AGGAGTGTAC GTGTGCCAGT TCTGATGGCA AAGATGATAA AGAATTATGC 1012

RAT CATGTTTGCT GCATGAAGAA AATGGCCCCA TCAACTTGTG CCAGTACAGG CTCTTTGCAG TGGAACAAGC 1240
BOV CATGTCTGCT GTATGAAGAA GATGGAGCCA TCAACTTGTG CCAGTACAGG GTCTGTGCAG TGGAACAAGT 1868
HUM CATGTATGCT GTATGAAGAA AATGGAGCCA TCAACTTGTG CCAGTACAGG GTCTGTGCAG TGGAGTAGGC 1082

RAT AGTTCACTGG TCGGACTATC ACTCTGCAGC CAGGCTCTCC ATGTAATGAC TTCAGAGGCT ACTGTGATGT 1310
BOV ACTTCCTTGG TCGAACTATC ACCCTGCAAC CTGGATCCCC ATGCAATGAT TTTAGAGGCT ACTGTGATGT 1938
HUM ACTTCAGTGG TCGAACCATC ACCCTGCAAC CTGGATCCCC TTGCAACGAT TTTAGAGGTT ACTGTGATGT 1152

RAT TTTCATGCGG TGCAGATTAG TAGATGCTGA TGGCCCTCTA GCTAGGCTGA AAAAAGCAAT TTTTAGTCCA 1380
BOV TTTCATGCGG TGCAGATTAG TAGATGCTGA TGGTCCTGTA GCGAGGCTTA AAAAAGCAAT TTTCAGTCCA 2008
HUM TTTCATGCGG TGCAGATTAG TAGATGCTGA TGGTCCTCTA GCTAGGCTTA AAAAAGCAAT TTTTAGTCCA 1222

RAT CAACTCTATG AAAACATTGC TGAGTGGATT GTGGCTCACT GGTGGGCAGT ACTGCTTATG GGAATTGCCC 1450
BOV GAGCTCTATG AAAACATAGC TGAATGGATT GTGGCTTACT GGTGGGCAGT ATTACTTATG GGAATTGCCC 2078
HUM GAGCTCTATG AAAACATTGC TGAATGGATT GTGGCTCATT GGTGGGCAGT ATTACTTATG GGAATTGCTC 1292

RAT TGATCATGCT AATGGCTGGA TTTATCAAGA TTTGCAGTGT TCACACTCCA AGTAGTAATC CAAAGTTGCC 1520
BOV TGATCATGTT AATGGCTGGT TTTATTAAGA TATGCAGTGT ACACACTCCA AGTAGTAATC CAAAGTTGCC 2148
HUM TGATCATGCT AATGGCTGGA TTTATTAAGA TATGCAGTGT TCATACTCCA AGTAGTAATC CAAAGTTGCC 1362

RAT TCCTCCTAAA CCACTTCCAG GCACTTTAAA GAGGAGGAGA CCGCCACAGC CCATTCAGCA GCCCCAGCGT 1590
BOV TCCTCCTAAA CCTCTTCCAG GCACTTTAAA GAGGAGAAGA CCTCCCCAGC CCATTCAACA GCCCCAGCGT 2218
HUM TCCTCCTAAA CCACTTCCAG GCACTTTAAA GAGGAGGAGA CCTCCACAGC CCATTCAGCA ACCCCAGCGT 1432

RAT CAGAGGCCCC GGGAGAGTTA TCAAATGGGA CACATGCGAC Gctgatg-ca gcttttgcct tggttcttcc 1659
BOV CAGAGGCCCC GGGAGAGTTA TCAGATGGGA CACATGAGAC Gttaact-ca gcttttgcct tggttcttcc 2287
HUM CAGCGGCCCC GAGAGAGTTA TCAAATGGGA CACATGAGAC Gctaactgca gcttttgcct tggttcttcc 1502

RAT tagtgcctac agtgggaaaa cttcactcca aagagaaacc tgttaagtca tcatctgcaa atgaaaccct 1729
BOV tagtgcctac aatgggaaaa cttcactcca aagagaaacc tattaaatca tcatccccaa atgaaaccct 2357
HUM tagtgcctac aatgggaaaa cttcactcca aagagaaacc tattaagtca tcatctccaa actaaaccct 1572

RAT cacagttaat agttgaagaa aaaatggcaa gagatcatgt cctcagacca ggtggaatta cctaaaattt 1799
BOV cacaaataac agtagaaaaa aaaaaaaa                                             2385
HUM cacaagtaac agttgaagaa aaaatggcaa gagatcatat cctcagacca ggtggaatta cttaaatttt 1642

RAT aaagcctgaa aattccaatt ttttggggg ggtgggatgg aaaaggaacc caatttttctt atggacagat 1869
HUM aaagcctgaa aattccaatt t----ggggg tgggaggtgg aaaaggaacc caatttttctt atgaacagat 1708

RAT atttttaacc taatggcaca aagtcttaga atattattat gtgccctgtg ttccctgttc ttcgttgctg 1939
HUM atttttaact taatggcaca aagtcttaga atattattat gtgccccgtg ttccctgttc ttcgttgctg 1778

RAT cattttcttc acttgcagac agacttggct ctcgataaac ttttatcaca aattgaaata tatttttttc 2009
HUM cattttcttc acttgcaggc aaacttggct ctcaataaac ttttcggaat tc                    1830

RAT aactgccaat ctaggctggg aggctcgacc acctcaacat tggatacatc acttgccaat gtacatacct 2079
RAT tgtaatatgc agacatgtat ttcttacgta cactgtactt ctctgtgcaa tttgtaaaca gaaattgcaa 2149
RAT tatggatgtt tctttgtata ataaattttt ccgctcttaa tgaaaaatta ctgtttaatt gacatactca 2219
RAT ggataacaga gaatggtggt gtgcagtggt ccaggattct gtaatgcttc atgcaggcca ttttgaaatg 2289
RAT agaattgaaa acccttttttc ttatggtgga gttggttctt acatgcatcc ttttcctgtt ccatggcttc 2359
RAT tgtgagtagg agaggctgtg ttgaagagct cagctggtgg cttacccg                         2407
```

Figure 3.11 Comparisons of Bovine/Rat/Human MADM protein sequences
Non-identical residues are shown in green, and positions of the extended zinc-binding site (HEV....PH) in red, the putative integrin-binding site (DSDC) in blue and the transmembrane helix (WIV....GFI) in purple. Stop codons are indicated by •.

```
BOVINE   MVLLRVLILLLSWVAGLGGQYGNPLNKYIRHYEGLSYDVDSLHQKHQRAK    50

BOVINE   RAVSHEDQFLRLDFHAHGRHFNLRMKRDTSLFSEEFRVETSNAVLDYDTS   100

BOVINE   HIYTGHIYGEEGSLAMGLLLMEDLKDSFRLMVARFYVEPAERYIKDRTLP   150

BOVINE   FHSVIYHEDDIKYPHKYGPQGRCADHSVFERMRKYQMTGVEEVTQTPQEK   200

BOVINE   HAINGPELLRKKRTTVAEKNTCQLYIQTDHLFFKYYGTREAVIAQISSHV   250
RAT          GPELLRKKRTTLPERNTCQLYIQTDHLFFKSYGTREAVIAQISSHV

BOVINE   KAIDTIYQTTDFSGIRNISFMVKRIRINTTADEKDPTNPFRFPNIGVEKF   300
HUMAN            QTTDFSGIRNISFMVKRIRINTTADEKDPTNPFRFPNISVEKF
RAT      KAIDAIYQTTDFSGIRNISFMVKRIRINTTSDEKDPTNPFRFPNIGVEKF

BOVINE   LELNSEQNHDDYCLAYVFTDRDFDDGVLGLAWVGAPSGSSGGICEKSKLY   350
HUMAN    LELNSEQNHDDYCLAYVFTDRDFDDGVLGLAWVGAPSGSSGGICEKSKLY
RAT     LELNSEQNHDDYCLAYVFTDRDFDDGVLGLAWVGAPSGSSGGICEKSKLY

BOVINE   SDGKKKSLNTGIITVQPYGSHVPPKVSHITFAHEVGHNFGSPHDSGTECT   400
HUMAN    SDGKKKSLNTGIITVQNYGSHVPPKVSHITFAHEVGHNFGSPHDSGTECT
RAT     SDGKKKSLNTGIITVQNYGSHVPPKVSHITFAHEVGHNFGSPHDSGTECT

BOVINE   PGESKNLGQKENGNYIMYARATSGDKLNNNKFSLCSIRNISQVLEKKRNN   450
HUMAN    PGESKNLGQKENGNYIMYARATSGDKLNNNKFSLCSIRNISQVLEKKRNN
RAT     PGESKNLGQKENGNYIMYARATSGDKLNNNKFSLCSIRNISQVLEKKRNN

BOVINE   CFVESGQPICGNGMVEQGEECDCGYSDQCKDECCYDANQPEGKKCKLKPG   500
HUMAN    CFVESGQPICGNGMVEQGEECDCGYSDQCKDECCFDANQPEGRKCKLKPG
RAT     CFVESGQPICGNGMVEQGEECDCGYSDQCKDECCFDANQPEGKKCKLKPG

BOVINE   KQCSPSQGPCCTAHCAFKSKTEKCRDDSDCAKEGICNGITALCPASDPKP   550
HUMAN    KQCSPSQGPCCTAQCAFKSKSEKCRDDSDCAREGICNGFTALCPASDPKP
RAT     KQCSPSQGPCCTAQCAFKSKSEKCRDDSDCAKEGICNGFTALCPASDPKP

BOVINE   NFTDCNRHTQVCINGQCAGSICEKHGLEECTCASSDGKDDKELCHVCCMK   600
HUMAN    NFTDCNRHTQVCINGQCAGSICEKYGLEECTCASSDGKDDKELCHVCCMK
RAT     NFTDCNRHTQVCINGQCAGSICEKYDLEECTCASSDGKDDKELCHVCCMK

BOVINE   KMEPSTCASTGSVQWNKYFLGRTITLQPGSPCNDFRGYCDVFMRCRLVDA   650
HUMAN    KMDPSTCASTGSVQWSRHFSGRTITLQPGSPCNDFRGYCDVFMRCRLVDA
RAT     KMAPSTCASTGSLQWNKQFTGRTITLQPGSPCNDFRGYCDVFMRCRLVDA

BOVINE   DGPLARLKKAIFSPELYENIAEWIVAYWWAVLLMGIALIMLMAGFIKICS   700
HUMAN    DGPLARLKKAIFSPELYENIAEWIVAHWWAVLLMGIALIMLMAGFIKICS
RAT     DGPLARLKKAIFSPQLYENIAEWIVAHWWAVLLMGIALIMLMAGFIKICS

BOVINE   VHTPSSNPKLPPPKPLPGTLKRRRPPQPIQQPQRQRPRESYQMGHMRR•   748
HUMAN    VHTPSSNPKLPPPKPLPGTLKRRRPPQPIQQPQRQRPRESYQMGHMRR•
RAT     VHTPSSNPKLPPPKPLPGTLKRRRPPQPIQQPQRQRPRESYQMGHMRR•
```

The rat transmembrane domain has one non-conservative substitution (Y to H), as does the human clone, while the cytoplasmic domain is identical between the 3 species.

However, the differences characterising bovine MADM as distinct from other ADAMs are also conserved in rat MADM (section 1.7), further emphasising MADM's early divergence within the reprolysin family (Rawlings and Barrett, 1995). A six amino acid insert, residues 281-286 of the available rat sequence, is not found in other reprolysin sequences, and there is a single amino acid deletion immediately preceding the putative integrin-binding site. Immediately following the unpaired cysteine residue of the integrin-binding sequence, reprolysins contain an aspartic acid. However, MADM contains instead an alanine, a non-conservative substitution which may interfere with binding ability. Two residues further on is another substitution which may render the disintegrin domain inactive - MADM contains a glycine where other reprolysins have a conserved Glu. Yet, as is the case with bovine MADM, the rat sequence does contain many of the highly conserved cysteine and glycine residues conserved in all mammalian disintegrins.

3.1.6 Northern blotting

This was undertaken to examine MADM transcript sizes, and to investigate possible alternative splicing of the MADM gene. However, mRNA detection can be difficult if the gene is transcribed at very low levels, and this could well be the case for MADM, judging by the low number of clones isolated from the cDNA library.

mRNA was isolated from adult rat brain and kidney using Dynabeads® in the mRNA Direct kit (Dynal), and subjected to electrophoresis. The contents of the gel were capillary blotted on to charged nitrocellulose and probed with cDNA produced by PCR using primers EF0 and ER0 (figure 3.7) and corresponding to the truncated rat MADM clone E.

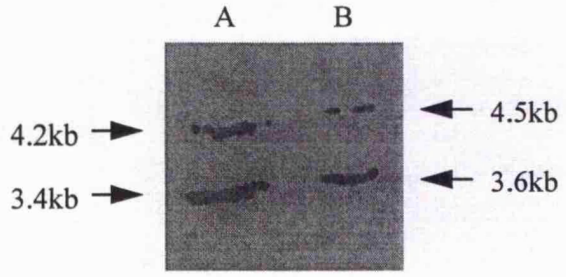
The resulting bands, as visualised by autoradiography, were extremely faint, further proof that MADM is a rare transcript. However, both rat brain and rat kidney showed evidence of 2 transcripts (Fig. 3.12). This result is in line with data obtained from human cell lines, which also contain 2 transcripts (of approximately 3.2 and 4.5kb; Howard *et al.*, 1996). The transcripts found in rat brain were estimated to be 3.4 and

4.2kb in size, while those found in kidney seemed slightly larger, ca. 3.6 and 4.5kb; however, this apparent size difference between tissues could equally be interpreted as a slight gel distortion. In each case, the lower MW transcript appeared to be slightly predominant. The transcript size of 3.4kb in brain is compatible with the length of the rat MADM clone isolated from the brain cDNA library.

The differences in transcript sizes within each tissue is probably too large to be attributable to differential 3'-processing - it would seem to be more likely that this is due to alternative splicing of the RNA. By analogy with MDC (Katagiri *et al.*, 1995), there is the potential for the MADM gene to consist of many small exons and introns.

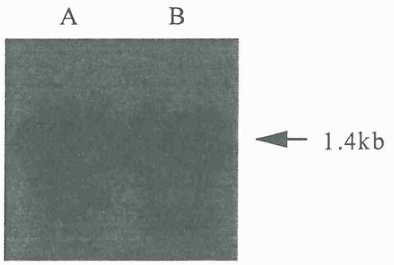
However, the differences between tissues are much smaller: the transcripts found in the brain samples would appear to be 200-300 bases smaller than those isolated from kidney. This figure correlates well with the potential for 3'-processing, since use of the internal polyadenylation signal sequence could produce transcripts which were at least 200 bases short.

Figure 3.12 Detection of rat MADM transcripts by Northern blotting
mRNA (10μg) was run on a formaldehyde-agarose gel, blotted, and probed with a PCR product corresponding to the truncated rat MADM clone E. The autoradiograph was obtained after 9 days exposure at -80°C. Lane A: brain; B: kidney.



Probing the same blot with the G3PDH housekeeping cDNA confirmed that the method and the blot were viable, with undegraded mRNA present on the blot. Lane loading was also seen to be equal (fig. 3.13). However, the bands of G3PDH were much stronger than those of MADM, as expected for an abundant transcript.

Figure 3.13 G3PDH verification of the Northern blot
The mRNA blot from figure 3.12 probed with G3PDH housekeeping cDNA to verify equal lane loading and RNA integrity. The autoradiograph was obtained after 18 hours exposure at -80°C. An abundant transcript at ca. 1.4kb is expected.



3.2 Summary

This chapter has described the successful isolation and sequencing of a truncated rat MADM homologue from a commercial brain cDNA library and completion of the sequence encoding the mature protein by 5'-RACE. This sequence had a very high degree of identity at the amino acid level (ca. 97%) and also at the nucleotide level (ca. 89.7%) with both bovine and human MADM. It would now be possible to utilise the deduced amino acid sequence in further work towards the goal of localising MADM by immunohistochemistry.

CHAPTER 4. GENERATION OF ANTIBODIES REACTIVE TO RAT MADM PROTEIN

4.1 Results and Discussion

4.1.1 Selection of MADM peptides for antibody production

Using the deduced protein sequence of mature rat MADM, an antigencity plot was produced (figure 4.1) and from this 7 regions of probable antigenicity spread along the length of the mature protein were identified. The 7 peptides (labelled 0-6) of between 11 and 17 residues all terminated in a C-terminal cysteine residue to facilitate coupling to the carrier molecule KLH (figure 4.2).

The conjugates were used to inoculate a range of species: rabbits, for polyclonal antisera; mice, for monoclonal antibodies; chickens, for polyclonal IgY. The anti-MADM reactivity was assessed by screening on Western blots of brain preparations. By this criterion the peptides appeared to be fairly non-immunogenic since only a minority of the immunised animals generated detectable antibody (table 4.1).

The lack of immunogenicity of the peptides in mice and rabbits was not entirely unexpected, due to the enormous conservation already found in the mammalian MADM homologues, implying a critical function for this protein. For this reason chickens were used, since it was hoped that in this phylogenetically distant species mammalian epitopes would provoke a much bigger immune reaction. Unfortunately, antibody production in chickens also proved very difficult.

Figure 4.1 Antigenicity plot produced for the mature rat MADM protein
Created by MacVector, using the protein toolbox. Regions above the axis are predicted to be exposed at the protein surface. The putative transmembrane (TM) sequence and the relative positions of the peptide sequences are indicated.

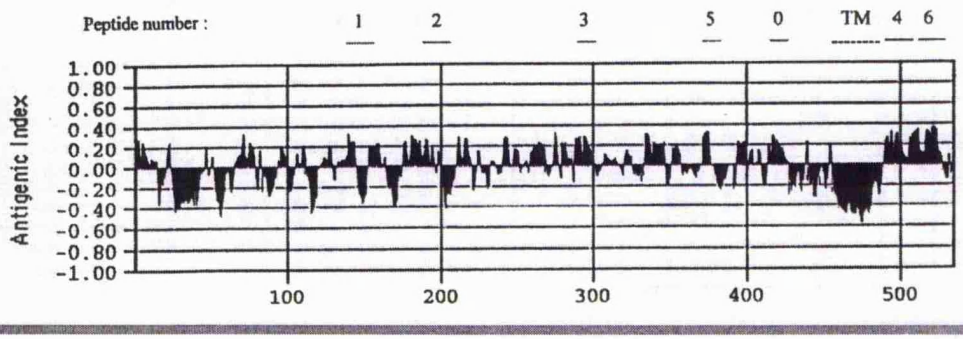


Figure 4.2 Positions of the peptides used to make antibodies to rat MADM
The peptides shown here were made for us by Nigel Groome at Oxford Brookes University, then conjugated to the carrier protein KLH before being injected into a range of animal species. Peptide sequences are shown in bold red and underlined, while the zinc-binding site, putative integrin-binding site and transmembrane region are highlighted in blue. The stop codon is shown by *.

```
RAT       GPELLRKKRTTLPERNTCQLYIQTDHLFFKSYGTREAVIAQISSHV   50
RAT   KAIDAIYQTTDFSGIRNISFMVKRIRINTTSDEKDPTNPFRFPNIGVEKF  100
RAT   LELNSEQNHDDYCLAYVFTDRDFDDGVLGLAWVGAPSGSSGGICEKSKLY  150
RAT   SDGKKKSLNTGIITVQNYGSHVPPKVSHITFAHEVGHNFGSPHDSGTECT  200
RAT   PGESKNLGQKENGNYIMYARATSGDKLNNNKFSLCSIRNISQVLEKKRNN  250
RAT   CFVESGQPICGNGMVEQGEECDCGYSDQCKDECCFDANQPEGKKCKLKPG  300
RAT   KQCSPSQGPCCTAQCAFKSKSEKCRDDSDCAKEGICNGFTALCPASDPKP  350
RAT   NFTDCNRHTQVCINGQCAGSICEKYDLEECTCASSDGKDDKELCHVCCMK  400
RAT   KMAPSTCASTGSLQWNKQFTGRTITLQPGSPCNDFRGYCDVFMRCRLVDA  450
RAT   DGPLARLKKAIFSPQLYENIAEWIVAHWWAVLLMGIALIMLMAGFIKICS  500
RAT   VHTPSSNPKLPPPKPLPGTLKRRRPPQPIQQPQRQRPRESYQMGHMRR*   549
```

Table 4.1 Results of inoculations for each peptide

PEPTIDE #0 = GRTITLQPGSPC	=	**R45** (weak polyclonal).
PEPTIDE #1 = EKSKLYSDGKKKC	=	Both of these peptides have been injected into rabbits, mice and chickens, but have
PEPTIDE #2 = GESKNLGQKENGC	=	failed to induce detectable antibodies.
PEPTIDE #3 = FDANQPEGKKC	=	**R70** (strong polyclonal); **R71** (weak polyclonal). No immune response in mice.
PEPTIDE #4 = DTPSSNPKLPPPKC	=	**R91** (weak polyclonal).
PEPTIDE #5 = ASSDGKDDKELC	=	**R93** (strong polyclonal); **M7** (strong responder, spleen cells used to produce monoclonal antibody secreting cell line **6F9**); **M21** and **M22** (spleen cells frozen down for future fusions if required).
PEPTIDE #6 = RPPQPIQQPQRQRPREC=		No response in mice or chickens.

KEY: R = rabbit; M = mouse.

4.1.2 Rabbit polyclonal antisera to MADM

Twelve rabbits were immunised (2 per peptide, peptides #0-5), and 5 positive sera were eventually obtained (table 4.1). However, 3 of these sera had only weak anti-MADM responsiveness and were therefore of limited use. Two sera, R70 (made to peptide #3) and R93 (to peptide #5), were strongly positive for anti-MADM activity, and these have been the most frequently used antisera for subsequent work. The sera were initially analysed by assaying for MADM detection on Western blots of glycoprotein preparations (see figure 4.3). Sera R70 and R93 reacted with a broad polypeptide band of approximately the apparent Mr (62kDa) expected for MADM. R71 reacted weakly with the same polypeptide, while R69, R91 and R92 did not react with MADM under these conditions. In addition to anti-MADM reactivity, a number of extra immunoreactive bands were observed in some samples. A strong band at ca.56kDa was observed in lanes probed with R69 and R71, and more weakly with R70. This may be carrier associated reactivity, as also may be the case for the bands at ca.60kDa seen in the lanes probed with R93. R71 was placed in storage as a back-up serum, while R70 and R93 were treated further for use in various applications. Having 2 distinct anti-MADM sera, made to different peptides would be useful as an internal control when considering staining patterns later in immunohistochemistry.

It was interesting to note that the immunogenicity of the peptides did seem to show a slight correlation with the status of the C-terminal cysteine residue: peptides which ended naturally in a cysteine (#0, 3, 5) produced more reactivity in the immunised animals than the peptides which had an extra cysteine added to the sequence for conjugation to the carrier, although this trend may simply have been coincidental. No correlation between sequence conservation and lack of immunogenicity was determined; indeed, peptide 5, the most immunogenic of all the 7 peptides, was identical in all three mammalian protein sequences examined (figure 3.11).

R70 and R93 antisera were adsorbed with KLH coupled to CNBr-activated sepharose. The results of this procedure are shown in figure 4.4. It can be seen that a number of bands, presumably carrier-associated, were removed from the treated samples, including the 60kDa band in lane C which was previously observed in figure 4.3, associated with the untreated R93 antiserum.

Figure 4.3 Assay of anti-MADM activity of rabbit sera on Western blots
Glycoproteins (7.5μg) were run on a 7.5% SDS-PAGE gel, blotted and probed with sera (1:100). Lane A: bovine kidney glycoprotein; lane B: rat kidney glycoprotein.

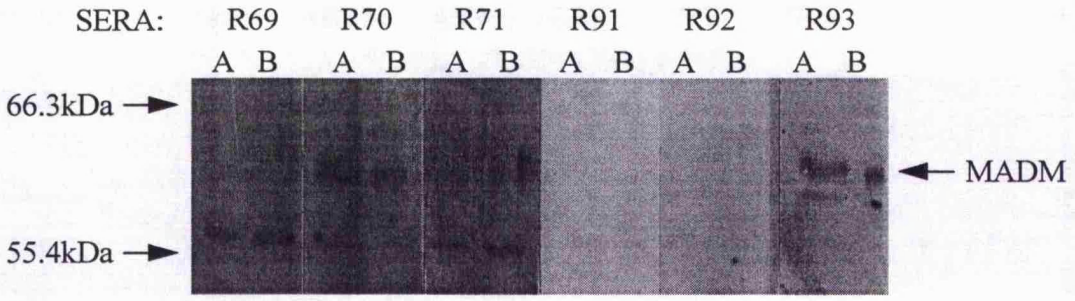
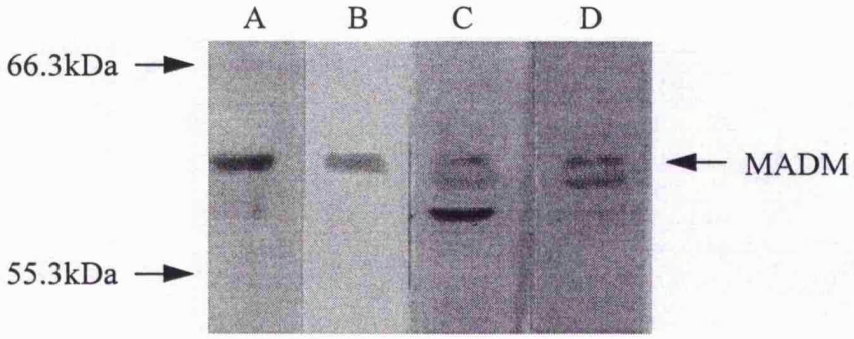


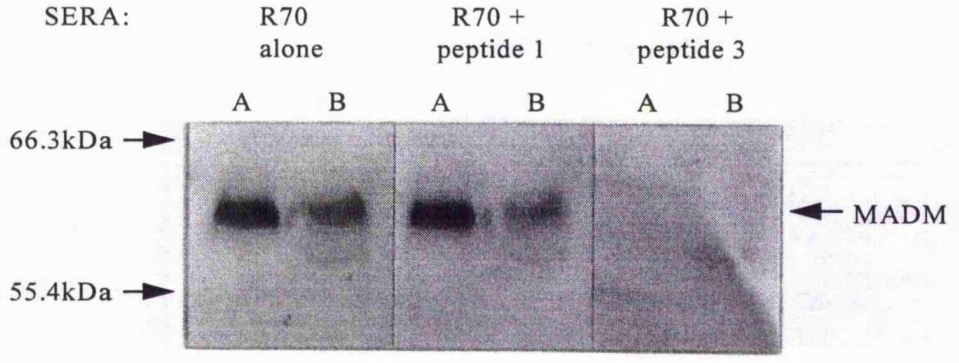
Figure 4.4 Results of KLH-adsorption of R70 and R93 antisera
Rat brain glycoproteins (9μg) were run on an 7.5% SDS-PAGE gel, blotted and probed with the sera shown below (all diluted 1:100). Lane A, original R70 antiserum; lane B, R70 after KLH-adsorption; lane C, R93 antiserum; lane D, R93 after KLH-adsorption.



On these blots R93 appeared to detect a doublet band at ca.61.5 and 62kDa, whereas R70 detected only a single band of MADM protein. It was unclear whether the doublet actually represents 2 forms of MADM protein or whether this observation was an artefact of the serum or of the glycoprotein sample used.

It was necessary to check the specificity of the antisera before using them on sections for immunohistochemistry. This was done by undertaking blocking experiments on Western blots, with the peptide (#3) used to raise R70, to demonstrate that the R70 antiserum was indeed reacting to a MADM epitope (figure 4.5). The R70 antiserum detected MADM as a band at ca. 62kDa on blots of both rat and bovine brain preparations (the apparent difference in intensity of the two bands was not due to a lower reactivity to bovine MADM, but to differences in lane loading). Peptide #3 completely blocked all detectable anti-MADM activity, while the control peptide #1 did not have this effect. This result strongly implies that the R70 antiserum is specific to the peptide #3 MADM epitope. A similar result was obtained for R93 using peptide #5 to block (data not shown).

Figure 4.5 Blocking R70 activity on Western blots
KLH-adsorbed R70 was used to detect MADM on a Western blot of a 7.5% SDS-PAGE gel, either with no treatment, following overnight 4°C incubation with peptide #1 (4μg ml^{-1}), or following identical incubation with peptide #3 (4μg ml^{-1}). Lane A, rat kidney glycoprotein (9μg); lane B, bovine kidney glycoprotein (7.5μg).

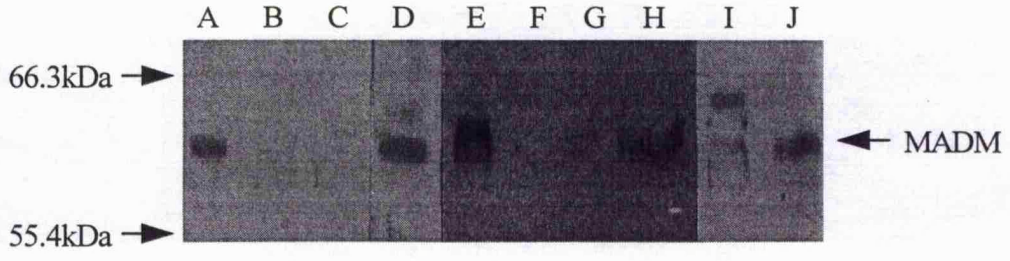


The KLH-adsorbed R70 serum was used to test a variety of rat cells and tissues for evidence of MADM expression (figure 4.6). A wide range of tissues, as well as all cell lines so far tested appeared to express the MADM protein, but not at levels higher

than those found in brain; other tissues had a much lower level of expression, or no detectable MADM at all (liver, lane B, and lung, lane C, respectively). It was not possible to test epididymis or heart since the glycoprotein recovery from these tissues is extremely low. On this blot, R70 appeared to strongly detect a doublet band in the brain glycoprotein (lane E), with the upper band being predominent. Of the other rat tissues, testis (lane A) contained a relatively strong single MADM band, and kidney (lane D) had a strong MADM band at 62kDa and a very weak band at ca.63.5kDa.

The cell lines showed a much more varied staining pattern. NS0 cells (lane H) contained a very strong MADM band, which may well be a doublet. Y0 cells (lane G) showed a similar pattern, but very much weaker signal, with the upper band of the two being predominent, as in the brain sample. 33B cells (lane J) had a single band at approximately the same intensity as that seen in the testis sample. C6 cells (lane F) appeared to express no MADM protein at all, while L6-G8-C5 cells (lane I) had a MADM band as well as a much higher Mr band at ca.65kDa, which was not seen in any other sample.

Figure 4.6 MADM detection in rat tissues and cells
Glycoprotein samples (7.5μg) were run on a 10% SDS-PAGE gel, blotted and probed with KLH-adsorbed R70 antiserum diluted 1:100. Lanes A-E are rat tissue samples, F-J are cell line samples. Lane A: testis; B: liver; C: lung; D: kidney; E: brain; F: C6 cells (rat glial tumour); G: Y0 cells (rat myeloma); H: NS0 cells (mouse myeloma); I: L6-G8-C5 cells (skeletal muscle myoblast); J: 33B cells (oligodendroglioma).



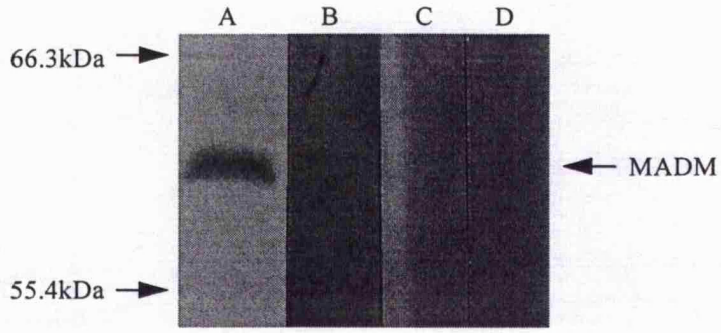
4.1.3 Attempts to generate chicken IgY antibodies to MADM

Since the peptides proved to be fairly non-immunogenic in rabbits and mice (section 4.1.1) chickens were used in an attempt to produce anti-MADM IgY. This was done following a methodology paper, which detailed an immunisation protocol for producing chicken antibodies to a highly conserved mammalian protein (Gassmann *et al*, 1990). The idea is that such a mammalian protein will be more highly immunogenic in the phylogenetically distant birds; in addition, egg yolk is a rich and inexpensive source of specific polyclonal antibodies. It was found that ca. 70mg of IgY could be purified from a single chicken egg, although it was unknown how much of the antibody would be anti-MADM specific if the inoculations were successful.

Initially 3 chickens were inoculated with a combination of peptides #1 and #2. However, at the end of the procedure, these chickens appeared to be displaying signs of an adverse reaction, and were no longer producing eggs due to stress. Other chickens inoculated at the same time by another group, using the same protocol but different peptides, had no such response and continued to produce eggs throughout the procedure (T.Gant; personal communication). This could imply that the MADM peptides were responsible, although a precise reason is unclear. Eventually, our chickens were test bled to determine whether they had produced antibodies to the peptides. Sera isolated from this blood showed no detectable anti-MADM activity when assayed by the Western blot method (figure 4.7).

Despite this disappointing result, more chickens were inoculated, with a different peptide (#6, corresponding to a cytoplasmic epitope). No adverse reaction was noted in these chickens, but there was still no anti-MADM activity in the IgY produced (table 4.1) as assessed by Western blot (data not shown). An ELISA assay was done on this IgY to determine whether any antibodies at all had been produced as a result of the inoculations, by testing for antibodies to epitopes on both MADM and on the carrier protein, KLH; a sample of mouse serum containing known anti-MADM activity was used as a positive control in this experiment (figure 4.8).

Figure 4.7 Assay of anti-MADM activity in chicken sera by Western blot
Brain glycoprotein samples (9μg) were run on a 7.5% SDS-PAGE gel, blotted and probed with the following: Lane A, R70 serum (positive control at 1:100); lane B, chicken #6 serum (1:100); lane C, chicken #7 serum (1:100); lane D, chicken #8 serum (1:100).

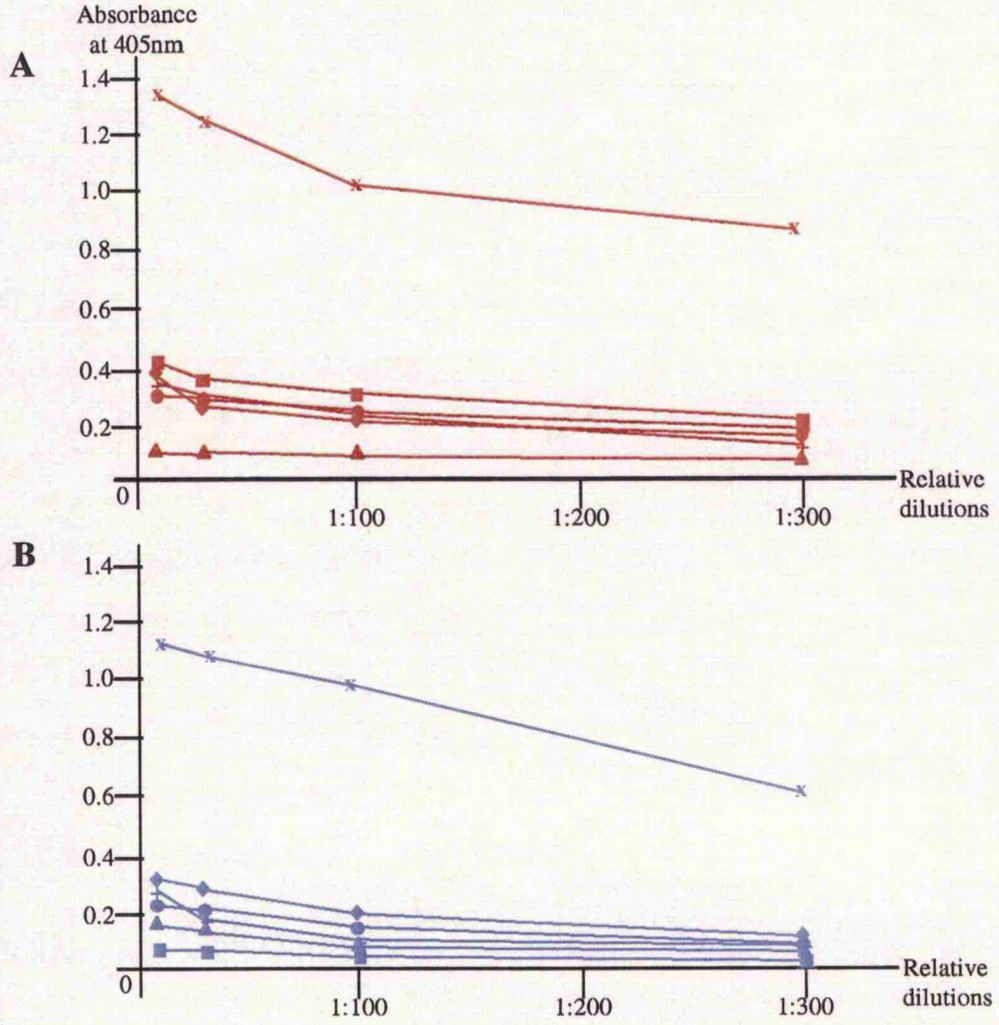


None of the inoculated chickens produced antibodies to either MADM or KLH, a surprising result since KLH is normally highly immunogenic. This is a very unusual situation which cannot be completely explained, assuming the chickens were immunologically normal to begin with. The alkaline phosphatase-conjugated secondary antibody has been shown to detect IgY by Western blotting (section 2.7.3.2) confirming that the technique was viable.

4.1.4 Production of a mouse monoclonal antibody to MADM

In the initial experiment, 7 mice were inoculated - 2 mice with peptide #2, 3 mice with peptide #3, and 2 mice with peptide #5. Peptides #2 and #3 produced no detectable anti-MADM response; however, both mice immunised with peptide #5 produced a good response (see figure 4.9). Because of this, 2 more mice were inoculated with peptide 5, found to be seropositive by Western blotting against brain glycoprotein (data not shown), and their splenocytes frozen in liquid nitrogen as a back-up, in case a hybridoma colony proved difficult to establish from the available spleen cells. As well as the MADM band at ca.62kDa, another band can be seen in lanes B and C at approximately 63kDa. This band may be carrier associated, or it may be due to cross-rectivity of epitopes on another protein.

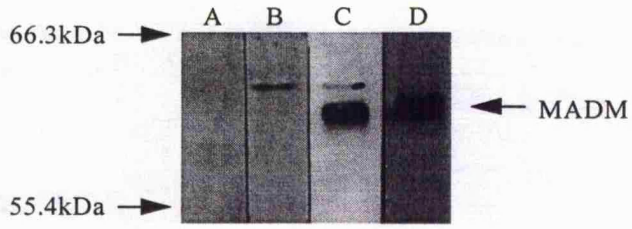
Figure 4.8 ELISA assay of chicken IgY
Panel A: reactivity to purified bovine MADM; panel B: reactivity to KLH.
Mouse serum from inoculated mouse was used as a positive control for reactivity to both MADM and KLH; normal mouse serum was bought from Sigma; IgY was isolated from eggs taken from pre-immune chickens and chickens immunised with the peptide #6-KLH conjugate.



KEY: x, serum from mouse inoculated with peptide5-KLH conjugate; +, normal mouse serum; ■, chicken #11 IgY; ▲, chicken #12 IgY; ◆, chicken #13 IgY; ●, pre-immune chicken IgY.

The starting concentration of the IgY preparations was 5mg ml^{-1}.

Figure 4.9 Results of some of the mice sera tested on Western blots of rat glycoprotein
Rat brain glycoprotein (9μg) was run on a 7.5% SDS-PAGE gel, blotted and probed with sera (diluted 1:100) from mice inoculated with the following MADM peptides: lane A, peptide #2; lane B, peptide #3; lane C, peptide #5; lane D, R70 rabbit serum positive control.



For reasons which remain unclear, the first fusion attempted was a failure, with no hybridoma colonies being produced. However, following a second fusion of splenocytes from mouse #7 (immunised with peptide #5) to NS0 myeloma cells, a total of 29 hybridoma colonies were detected from a total of 960 wells. When assayed on Western blots, only one of these colonies (#6F9) appeared to be secreting anti-MADM antibody (data not shown). The high level of antibody production by this colony can be seen in figure 4.10. The media collected had been conditioned for 24 hours by cells at a density of 1 $x10^5$ cells/ml

In addition to rat MADM, the 6F9 antibody was also shown to detect the bovine and human homologues on Western blots (figure 4.11). Although the antiserum appeared to detect a single rat band, there was a doublet detected in the bovine sample, and a wide band, which may also be a doublet, in the human sample.

The conditioned media collected from hybridoma colony 6F9 was tested for reactivity to purified bovine MADM adsorbed onto ELISA plates. The media was used at varying concentrations from 1:100 to 1:2500, and compared to the detection sensitivities of other anti-MADM antibodies (CG4, a bovine-specific monoclonal antibody raised to purified bovine MADM; R70 antisera, raised to MADM synthetic peptide #3, as well as normal mouse IgG and unconditioned hybridoma media at

Figure 4.10 Evaluation of the mouse monoclonal antibody 6F9
Rat kidney glycoprotein (9μg) was run on 7.5% SDS-PAGE, blotted and probed with the following: lane A, R70 serum (positive control at 1:100); lane B, mouse #7 serum (1:100); lane C, 6F9 conditioned medium (1:100); lane D, 6F9 conditioned medium (1:200); lane E, 6F9 conditioned medium (1:400); lane F, unconditioned hybridoma medium (negative control).

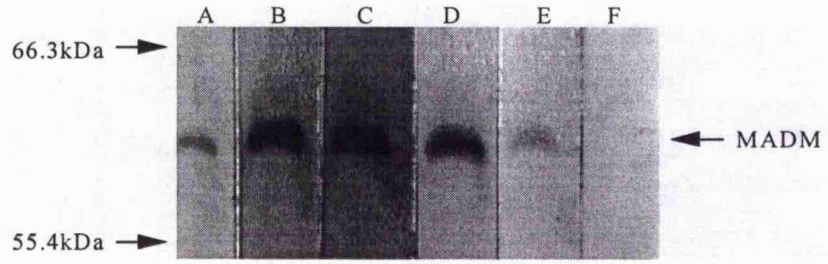
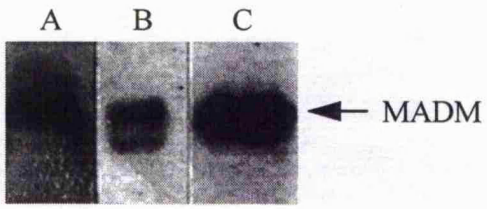


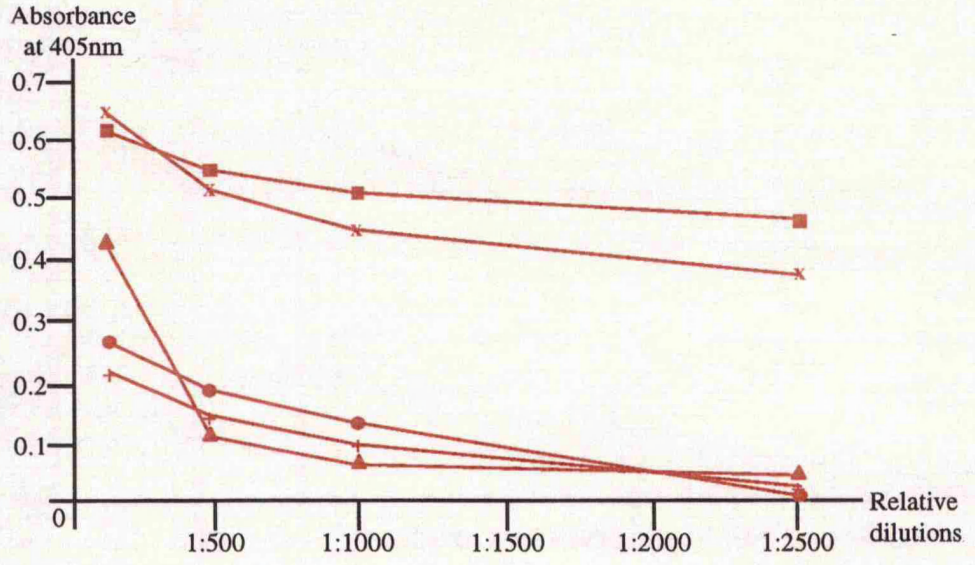
Figure 4.11 Detection of rat, bovine and human MADM by 6F9
Glycoprotein samples (7.5μg) were run on a 7.5% SDS-PAGE gel, blotted and probed with 6F9 conditioned medium diluted 1:200. Lane A, rat kidney glycoprotein; lane B, bovine kidney glycoprotein; lane C, human A549 cell line glycoprotein.



similar dilutions, used to provide background readings. The results (shown in figure 4.12) indicate that in this assay the 6F9 monoclonal antibody had an activity against MADM protein well above background levels at all dilutions, whereas R70, beyond the initial dilution of 1:100, had no such activity. This makes the 6F9 antibody easily comparable to CG4 in terms of its sensitivity, whilst seemingly having the advantage of being able to detect rat and human MADM protein in addition to bovine.

The hybridoma colony 6F9 was then cloned, to yield a pure monoclonal antibody-producing cell line. However, it proved to be very unstable and repeatedly lost antibody production. The azaserine drug selection was removed from the media; this was done over a period of several days, whilst leaving the hypoxanthine in place. The cells were then passed through 1 round of limiting dilution, and the best clones resulting from this were grown and retested for antibody production. The antibody was isotyped as an IgG1 and the conditioned media was tested for immunohistochemistry but preliminary results were relatively poor compared to those obtained with the rabbit sera R70 and R93 (data not shown). Since the 6F9 cells proved very difficult to keep alive and in antibody production, further attempts to obtain a mouse monoclonal antibody for immunohistochemistry were abandoned.

Figure 4.12 Results of the ELISA assay on anti-MADM antibodies
CG4 is purified IgG; 6F9 is conditioned medium collected from the hybridoma in culture; R70 is the rabbit polyclonal antiserum; normal mouse IgG was bought from Sigma; unconditioned medium is taken from the stock used to culture the hybridoma cell line.



KEY: x, CG4 monoclonal antibody; ■, 6F9 conditioned medium; ▲, R70 polyclonal antiserum ●, normal mouse IgG; +, unconditioned medium.

4.1.5 Anti-MADM antiserum and myeloma cells

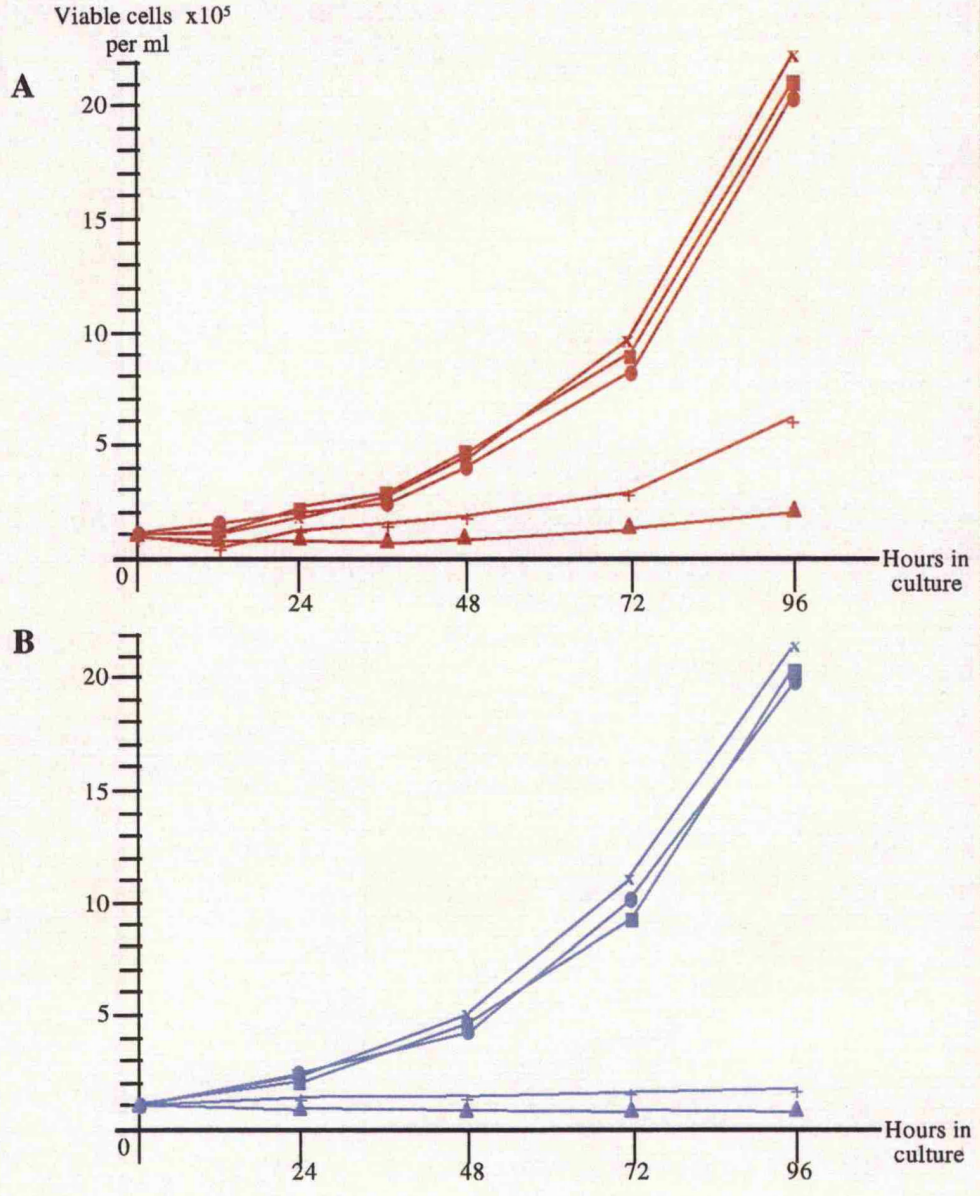
The reasons behind the failure of the 6F9 hybridoma cell line to continue high levels of antibody production were difficult to understand, until it was noticed that glycoprotein samples isolated from the NS0 cell line actually contained a large amount of MADM protein (figure 4.6). It was therefore possible that the hybridoma cell line, which is composed of NS0-fused cells, was producing autoantibodies directed against its own surface proteins and that these were somehow detrimental to hybridoma growth, survival or function.

Evidence that MADM was indeed expressed on the surface of the NS0 cells is presented in chapter 5. In addition, an experiment was set up to determine whether exogenous anti-MADM antiserum would alter the growth pattern of NS0 cells in culture. R93 antiserum (raised to the same MADM peptide, #5, as the 6F9 monoclonal antibody) was added at two concentrations (1:50 and 1:250) to the culture media; preimmune serum from rabbit #93 was used as a control.

The results of these experiments are shown in figure 4.13, and appear to support the idea that MADM is present on the surface of NS0 cells, and that its interaction with a specific antibody can have a pronounced effect on cell survival. In the first experiment (panel A), cells were grown for 96 hours after being added to media containing either antiserum or a control, and a viable cell count was done at various time intervals. The results clearly show that the addition of R93 antiserum to the growth media prevented an increase in the cell population, either by inhibiting growth, or by inducing a cell killing process such as apoptosis. However, after some days in culture, the effect began to wear off, possibly as the concentration of antibody became limiting.

In the second experiment (panel B) media containing antiserum or a control was replaced with fresh solutions every 24 hours for 5 days, at which times viable cell counts were also calculated. As before, the preimmune serum had no effect and the cells grew as strongly as those of the TBS control. Again, R93 had a population growth inhibitory effect, but this time it was maintained as the antibody was replenished each day and did not therefore become limiting.

Figure 4.13 Effect of anti-MADM antiserum on the growth of NS0 cells
Panel A shows results of cells grown with one initial addition at time 0 of antiserum; panel B shows results when media and antiserum are replenished daily.



KEY: ▲, R93 antiserum 1:50; +, R93 antiserum 1:250; ■, pre-immune serum 1:50; ●, pre-immune serum 1:250; **x**, TBS.

It is possible that the interaction of the antibody with MADM on the cell surface may cause an apoptosis-type event, either directly, by a mechanism such as intracellular signalling by MADM's cytoplasmic domain, or perhaps indirectly by blocking a MADM-integrin interaction. It is already known that preventing or disrupting integrin-ligand binding can cause a cell to apoptose (Mason *et al.*, 1996). It has also been shown that haemorrhagic snake venom disintegrins are able to induce an apoptosis-like effect *in vitro* (Araki *et al.*, 1993), again, possibly through the medium of integrin interaction.

Previous attempts at monoclonal antibody production in this laboratory were also problematic: four fusions were carried out before the CG4 monoclonal antibody specific to bovine MADM was isolated (Chantry and Glynn, 1990). This antibody was made to the purified protease, and it is possible that positive self selection allowed antibody production only to a species specific epitope, *i.e.*: a sequence where the bovine protein differed significantly from human, rat and, presumably, mouse MADM protein sequence.

4.2 Summary

This chapter has described the selection of peptide sequences from an antigenicity plot of the deduced rat MADM protein sequence, and the inoculation of a range of species with peptide-carrier conjugates for antibody production. The level of immunogenicity is low, probably due to the high level of conservation of MADM homologues between species, but 2 MADM-specific polyclonal antisera were produced in rabbits, and used to demonstrate the presence of MADM protein in a range of rat tissues and cultured cell lines.

Chickens, used as a phylogenetically distant host, failed to generate detectable antibodies to MADM. A mouse monoclonal antibody was finally produced, but the hybridoma cell line (6F9) producing the antibody was difficult to maintain, repeatedly dying or losing antibody production after just a few days in culture. It was shown that addition of R93 antisera to NS0 cells (i.e. the fusion partner in the hybridoma) in culture inhibits the growth of these cells. Thus the 6F9 hybridoma cells may inhibit their own growth by secreting an antibody against a surface autoantigen.

CHAPTER 5 INVESTIGATION OF MADM EXPRESSION IN RAT CNS

5.1 Results and discussion

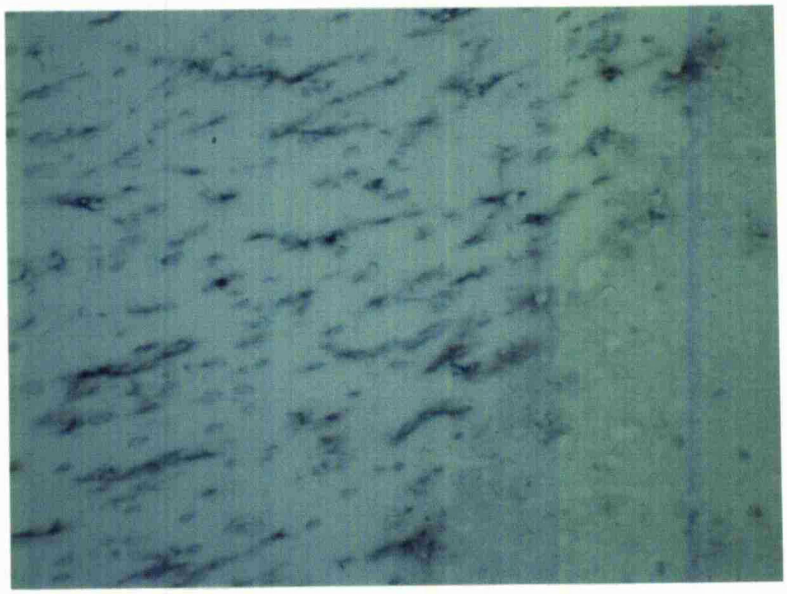
5.1.1 Immunolocalisation of MADM in normal adult rat CNS

This work was carried out using R70 and R93, the two polyclonal antibodies produced to 2 different MADM peptides. Both of these sera work well on frozen acetone-fixed rat brain sections, with a low level of background staining which could be blocked with a 10% solution of BSA in TBS. Both of these antisera were also tested for their reactivity on tissue fixed with formal-acetic acid, paraformaldehyde, methanol, and paraffin wax-embedded. No staining was detected on tissue treated by any of these methods.

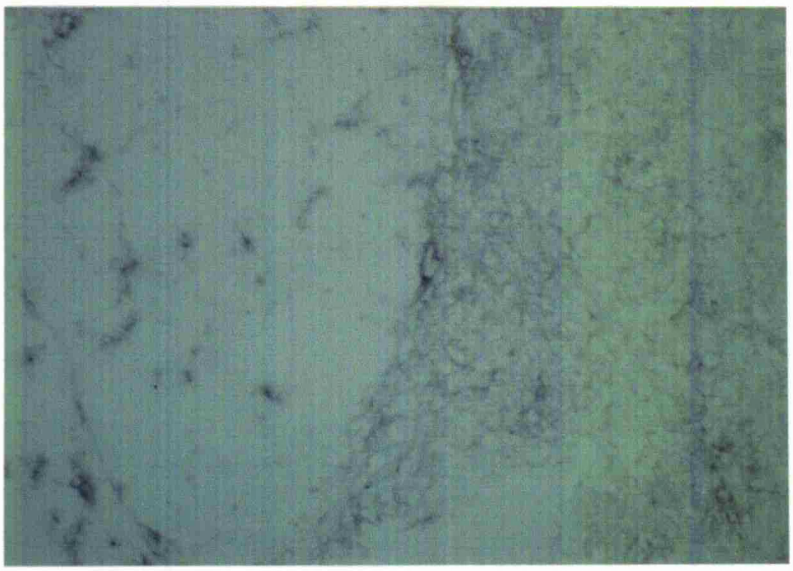
Using the R70 antiserum, sections from various areas of CNS were assessed. These sections included cerebral cortex, cerebellum, brain stem, and spinal cord. As shown in figure 5.1, initial staining localised MADM to a glial-type cell, with short stubby processes; rather more than half of these R70-positive cells showed evidence of a distinctive triskelion morphology. The majority (ca. 95%) of MADM expression also seemed to be restricted to cells found in white matter, an observation which was consistent throughout the CNS. In grey matter the occasional R70-positive cell was found, often in close proximity to a blood vessel. In figure 5.1A, MADM staining can be seen in purple, with a background of greeny-blue counterstain. Most of the MADM specific staining was observed on the left side of the picture, in the white matter area; grey matter (on the right side) showed little specific staining. Panel B of this figure shows MADM staining in the brainstem, with white matter on the left showing purple glial cell staining; the grey matter on the right has a higher level of background staining than is seen in the previous picture. Panel C is a longitudinal section of spinal cord, with grey matter at the top and white matter below; most staining is restricted to small glial cells in the white matter, but there is some background staining seen in the grey matter. Panel D is a region of the cerebellum showing white matter and a granular layer separated by molecular layers. Again, the majority of specific anti-MADM staining (visualised in red) was located in the white matter region, with a small amount being seen in the molecular layers.

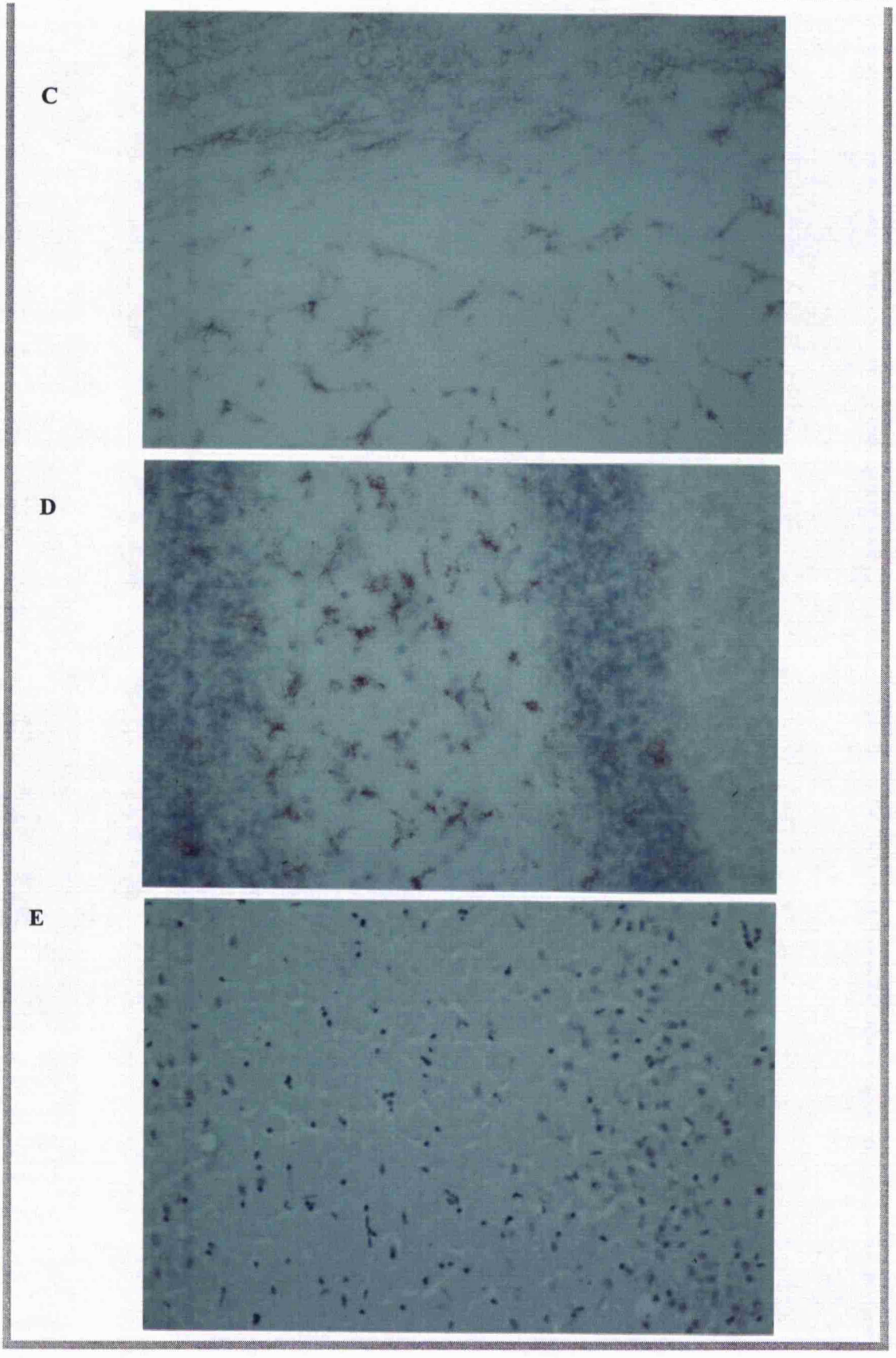
Figure 5.1 MADM visualisation with R70 antiserum
Immunostaining with R70 antiserum (diluted 1:200) in neurologically normal rat brain tissue.
A, Cerebral cortex: MADM visualised in purple, counterstain is methyl green (50x).
B, Brain stem: MADM visualised in purple, no counterstain (50x).
C, Spinal cord: MADM visualised in purple, no counterstain (50x).
D, Cerebellum: MADM visualised in red, counterstain is haematoxylin (50x).
E, Cerebellum: R70 antisera preincubated with peptide#3 (4μg ml^{-1}) overnight at 4°C, counterstain is methyl green (50x).

A



B




C
D
E

The staining produced with the R70 antiserum could be completely blocked by preincubating the antiserum with the specific peptide (#3), as seen in figure 5.1E. This picture shows an area of cerebellum, similar to that in panel D, with the white matter, granular and molecular layers, but in which all traces of anti-MADM reactivity were blocked. This result argues that the staining seen on these sections of rat CNS is specific to sites of MADM localisation.

The morphology and the distribution of the cells stained by the R93 antisera (figure 5.2A) was identical to that of R70, further evidence for MADM expression by a glial cell, with restriction to white matter. This staining could also be blocked, as shown in figure 5.2B, by preincubation of the R93 serum with its specific peptide #5.

Figure 5.2 Blocking MADM-specific staining with peptide
Cryostat sections of rat cerebral cortex (x50). Counterstained with methyl green; MADM staining visualised in purple, using the following:
A, R93 antiserum (1:200).
B, R93 (1:200) preincubated with peptide #5 (4μg ml^{-1}).

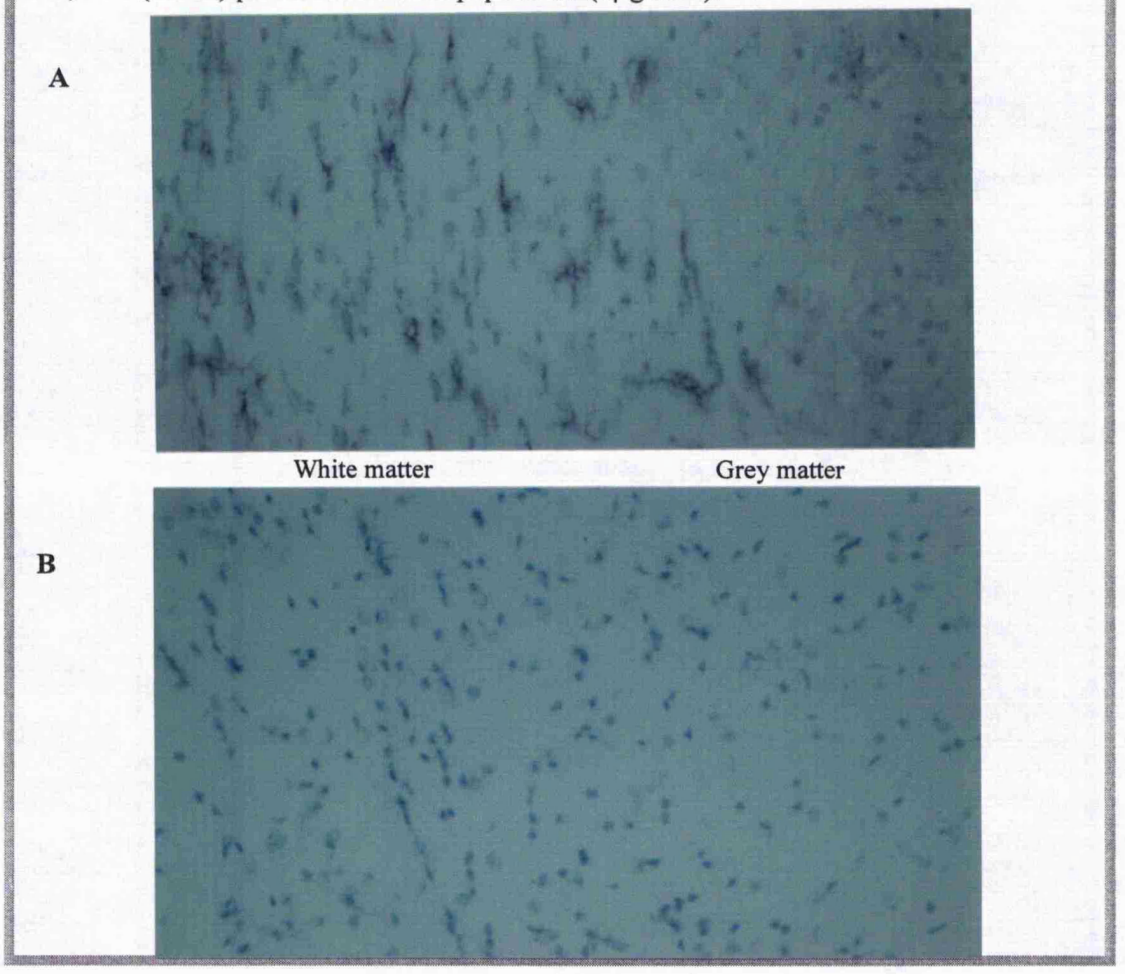


Table 5.1 shows the four cell types found in the CNS, and the antibody markers used to visualise them on frozen acetone-fixed sections. Since MADM appears to be present on a cell with glial morphology, attention was focused on the three glial cell species.

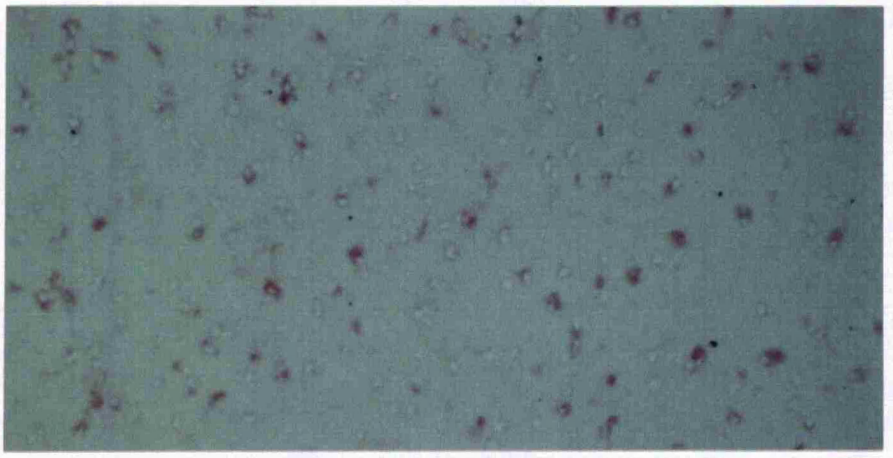
Table 5.1 Cell types found within the CNS and their specific antibody markers

Cell Type	Function	Antibody Marker
Neurones	Conduct nerve impulses	Neurofilament
Oligodendrocytes	Myelinating cells of the CNS	14E
Astrocytes	Support neurones	anti-GFAP
Microglia	CNS resident macrophages	OX42

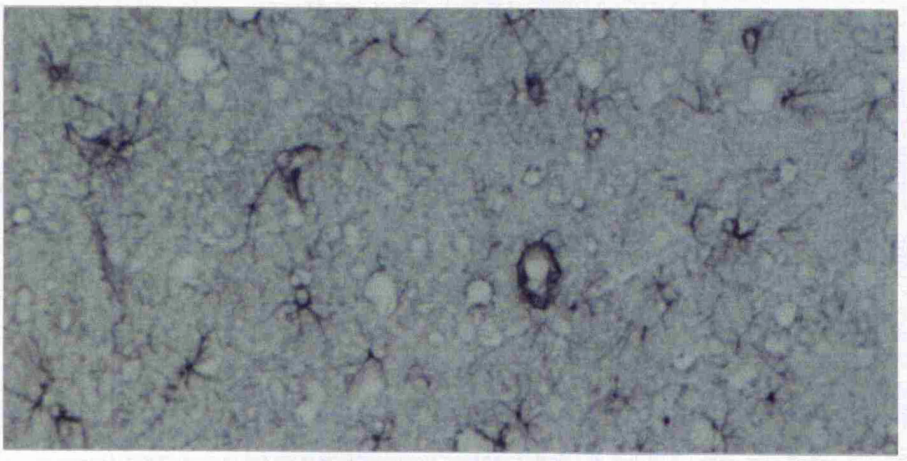
Figure 5.3 shows examples of each type of glia, stained with a specific antibody. The oligodendrocyte antibody marker 14E stained only in the perinuclear area, making it difficult to compare cell morphologies (figure 5.3A); however, oligodendrocytes are small cells with long, thin processes (Pfieffer *et al.*, 1993), rather than the short stubby processes found on the MADM stained cells. Astrocytes visualised with the anti-glial fibrillary acidic protein (anti-GFAP) antibody (figure 5.3B) appeared larger than oligodendrocytes, with many branched processes, and were very abundant throughout both the white and grey matter of the CNS. Microglia (figure 5.3C), stained with the OX42 antibody (specific to the $\alpha M\beta 2$ integrin), had a distinctive shape with a few short processes bearing few branches. These microglial cells were very similar to those cells stained by the anti-MADM antisera, both in terms of relative size and morphology, although their distribution is somewhat different. OX42-positive microglia are spread throughout both white and grey matter in the CNS, whereas MADM-expressing cells appeared to be mainly confined to white matter areas, possibly constituting a subpopulation of microglial cells.

Figure 5.3 Glial cell staining: Sections of rat cerebral cortex, magnification x50.
A: Oligodendrocytes in white matter, visualised with the 14E antibody (1:15) and the Fast Red chromogen.
B: Astrocytes in white matter (left) and grey matter (right), visualised with anti-GFAP (1:500) and Vector Purple.
C: Microglia in white matter (left) and grey matter (right), visualised with OX42 (1:150) and DAB.

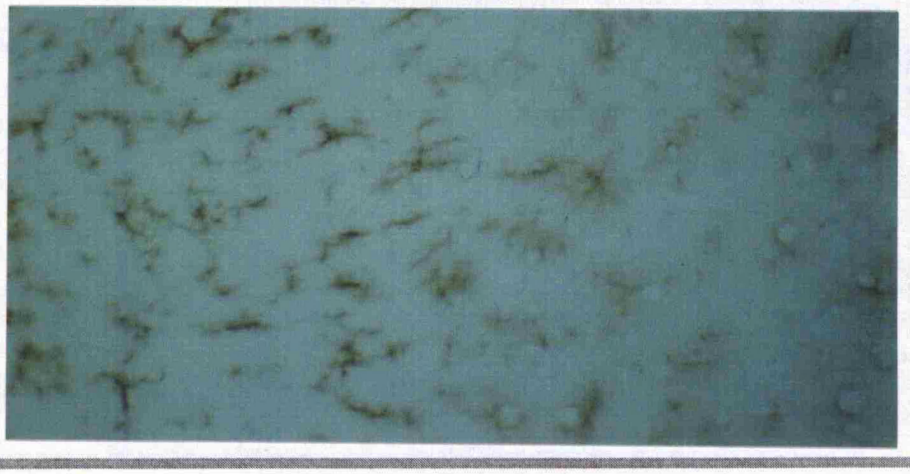
A



B



C



However, of these OX42-positive microglial cells in the white matter, approximately 90% also appear to be MADM-positive.

To confirm a microglial localisation for MADM, double immunostaining experiments were done (figures 5.4 and 5.5). There was a high degree of coincidence of OX42 staining with both of the anti-MADM antisera in white matter areas, implying that both R70 and R93 were identifying MADM expressed by white matter microglial cells.

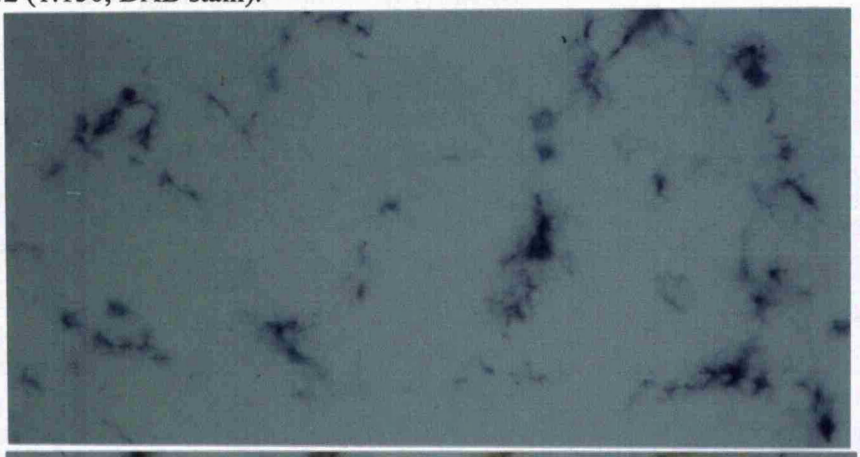
Various modes of putative interactions between MADM and integrins were discussed in section 1.8, with some preference for a model in which MADM and its cognate integrins are expressed on the same cell. The distribution of integrin beta-subunits in rat brain was now examined to compare with that of MADM (figure 5.6). Results show that while β2 (panel B) was obviously expressed on the same cells as MADM (since OX42 is binding to a beta 2 heterodimer), the other beta subunits did not seem to be expressed on microglia. Beta 1 (panel A) appeared mainly restricted to vessel epithelia; β 3 (panel C) was found mainly on neurones in grey matter and long chains of oligodendrocytes in white matter, and β 4 (panel D) was also found on neurones and on satellite oligodendrocytes.

These data are fairly compatible with published data on integrin expression in the CNS, although most work has concentrated on human tissue and cells, and therefore comparisons should be made with care. It is commonly agreed that the β1 integrin subunit is expressed by vessel epithelia in rat brain (Grooms *et al.*, 1993); however, in the human CNS, β1 integrins have also been shown to be expressed by astrocytes (Malek-Hedayat and Rome, 1994) and by oligodendrocytes (Milner and ffrench-Constant, 1994), results which are not immediately obvious in the rat brain shown in figure 5.6A. Human brain microglia have been shown to constitutively express β2 integrins (Akiyama and McGeer, 1990), but data on the expression of the β3 integrin subunit in the CNS is very scant, only having been found on cultured human cells of the oligodendrocyte lineage (Milner and ffrench-Constant, 1994). The expression of the β4 subunit in the CNS is slightly contradictory, with one report ruling out expression in the human CNS entirely (Sonnenberg *et al.*, 1990), while other workers report that astrocytes in some areas do express β4 chains (Paulus et al., 1993).

However, this same group also reports that neurones are negative for b4 expression; it can be seen therefore that this data is not easily comparable with the rat brain data presented in figure 5.6D.

Figure 5.4 Double staining with R93 and OX42
Sections of rat cerebrum (magification x100), counterstained with methyl green, and specifically stained with the following:
A, R93 (1:200) visualised in purple;
B, R93 (1:200, purple stain) and OX42 (1:150, brown DAB stain);
C, OX42 (1:150, DAB stain).

A



B

C

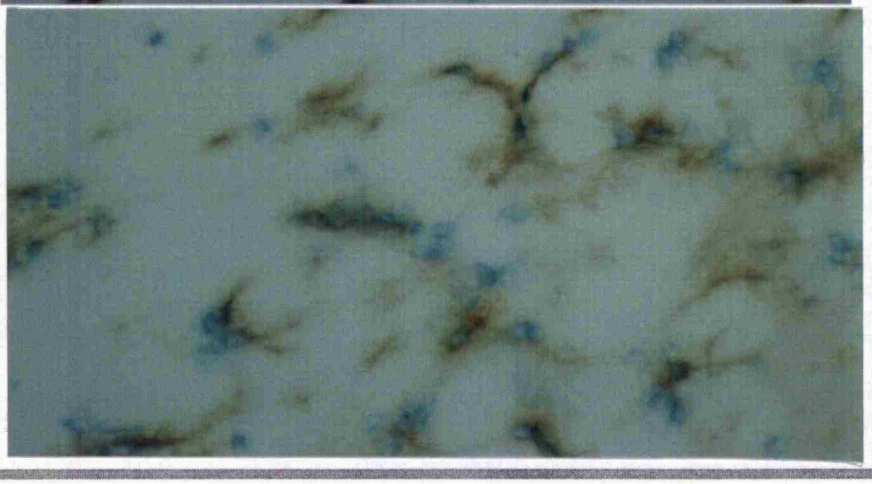
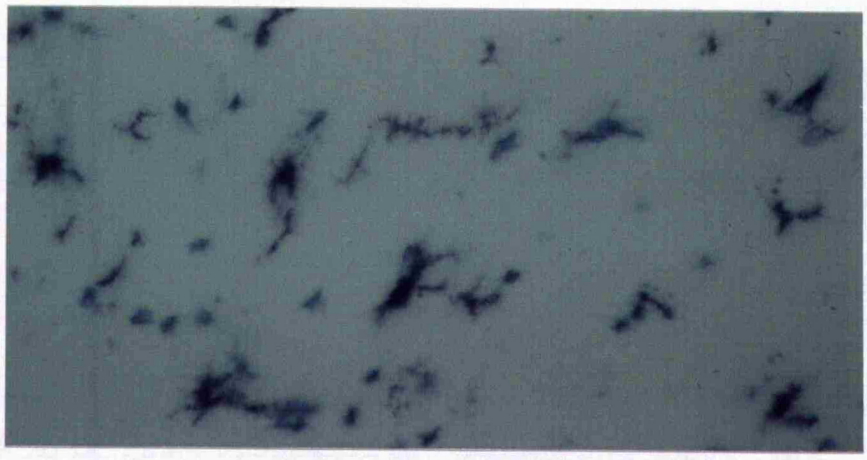
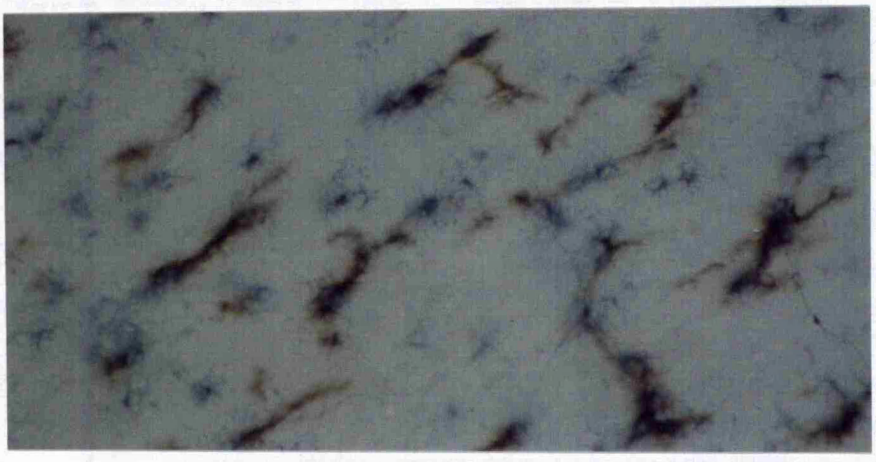


Figure 5.5 Double labelling with R70 and OX42
Rat cerebral cortex (magification x100), counterstained with methyl green, and specifically stained with the following:
A, R70 (1:200) visualised in purple;
B, R70 (1:200, purple stain) and OX42 (1:150, brown DAB stain) with slightly 'fibrous' counterstain (section 2.8.2);
C, OX42 (1:150, DAB stain).

A



B



C

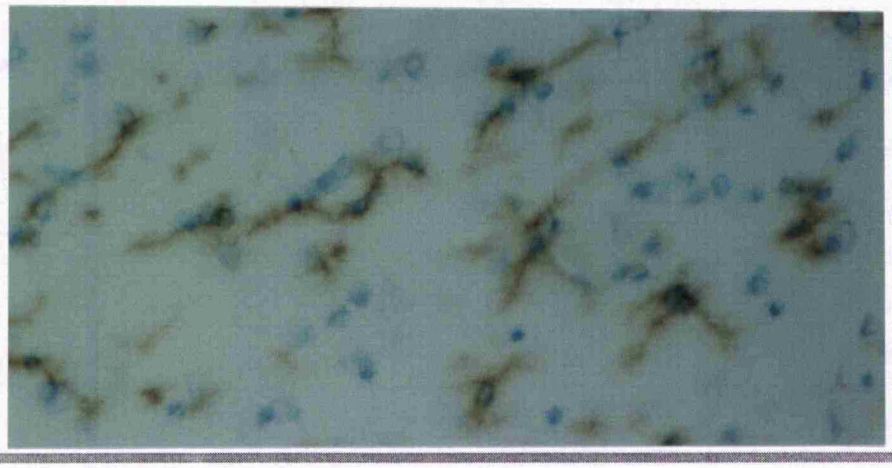
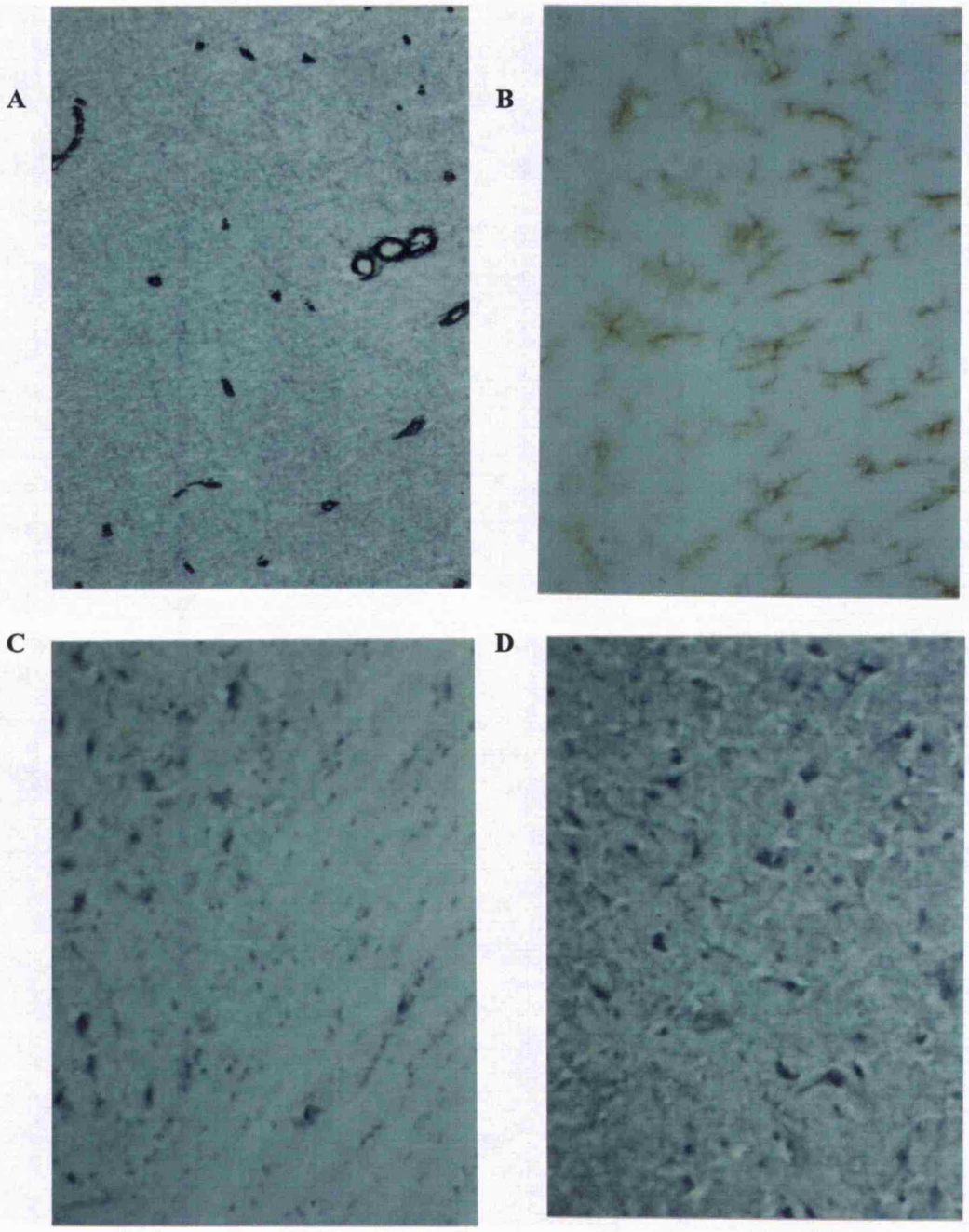


Figure 5.6 Localisation of beta integrin subunits on sections of rat brain
Sections of rat cerebrum (grey matter on the left of the picture, white matter on the right) stained with the following:
A, anti-β1 integrin (1:100), visualised with nickel-enhanced DAB (x25);
B, anti-β2 integrin (OX42, 1:150), visualised with DAB (x50);
C, anti-β3 integrin (1:250), visualised with nickel-enhanced DAB (x50);
D, anti-β4 integrin (1:200), visualised with nickel-enhanced DAB (x50).



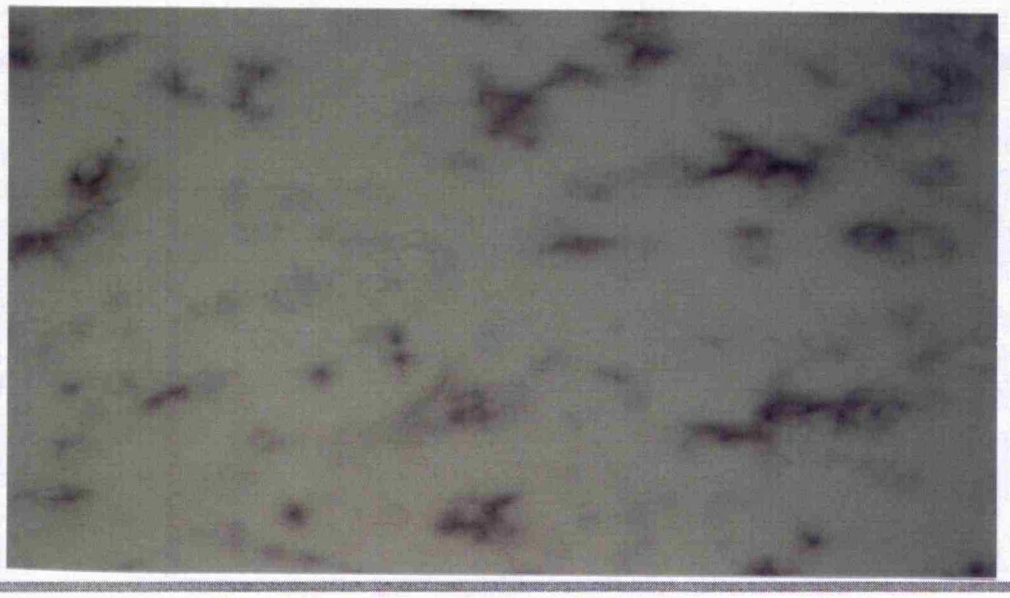
5.1.2 MADM expression in rat brain during development and injury

MADM is very difficult to detect on normal resting cells in the adult brain, requiring the use of a great deal of amplification during the staining process in order to visualise it successfully. This implies that MADM is a low abundance antigen on such cells, unlike integrins which are relatively abundant and are comparatively easily visualised. Such data raises the question of whether MADM is downregulated on normal cells, and whether it might be found to be upregulated during occasions when microglia might be expected to be active. To examine this possibility, an examination was made of CNS tissue taken from developing animals, and also of brain sections from animals which had undergone a stab injury to the cerebrum.

Developing brain

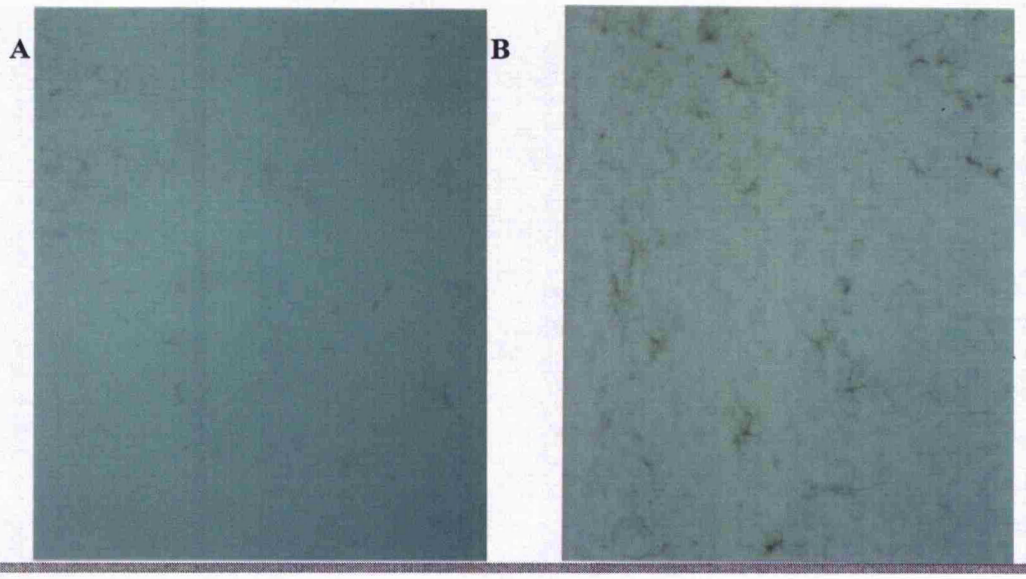
Brains were removed from 2-day and 20-day old rats. Cryostat sections were taken from each brain, and stained with R93. In the case of the 20 day old brain, MADM was expressed at the same low levels seen in adult brain (as judged by titration of the antibody until all staining disappeared) and, as in the adult brain, was restricted to white matter areas (figure 5.7).

Figure 5.7 MADM expression in the brain of a 20 day old rat
Cerebral white matter of a 20 day old rat, stained with vector purple.
Magnification x100.



However, in the brain from a 2-day old animal, there was no MADM staining detectable, even in areas where there is OX42 staining (figure 5.8). This result suggests that the presence of MADM may be related to the presence of white matter in the brain - the developing brain of a 2 day old rat contains very little white matter as myelination is in its early stages, while at 20 days myelination is proceding at a maximal rate (Morell et al, 1994).

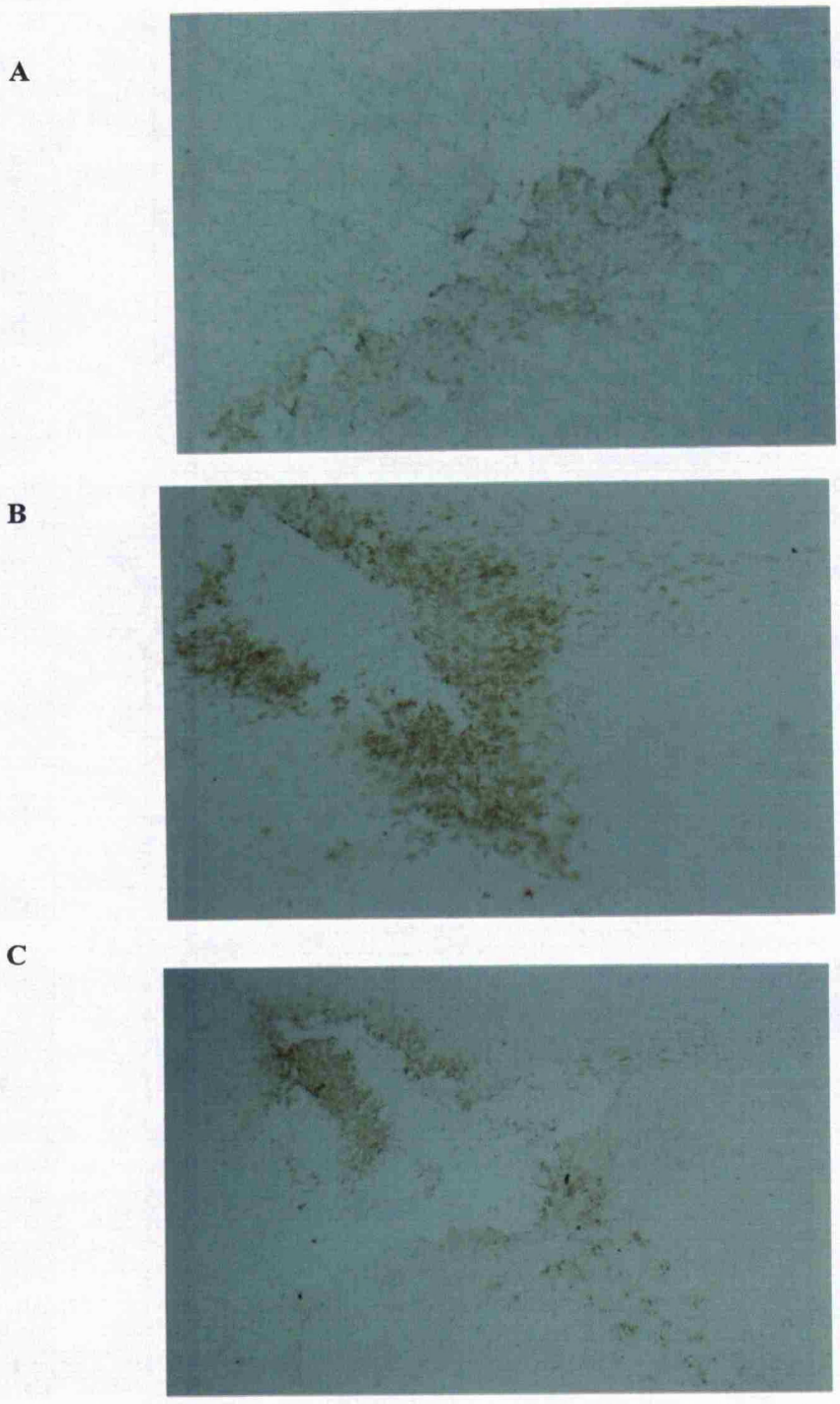
Figure 5.8 MADM expression in the brain of a 2 day old neonate
Cerebrum from a 2 day old neonate, counterstained with methyl green. Magnification x 25.
A: stained with R93 (1:200), visualised with Vector Purple.
B: stained with OX42 (1:150), visualised with DAB.



Stabbed brain

In the sections of cerebrum injured by a stab wound, the microglia are upregulated at all times examined (days 2, 3 and 5 after injury, figure 5.9); the OX42 antibody could be titrated to 1:800 until normal staining was abolished but microglia around the wound site, both in the grey and the white matter, showed increased levels of the antigen, a characteristic of microglial activation.

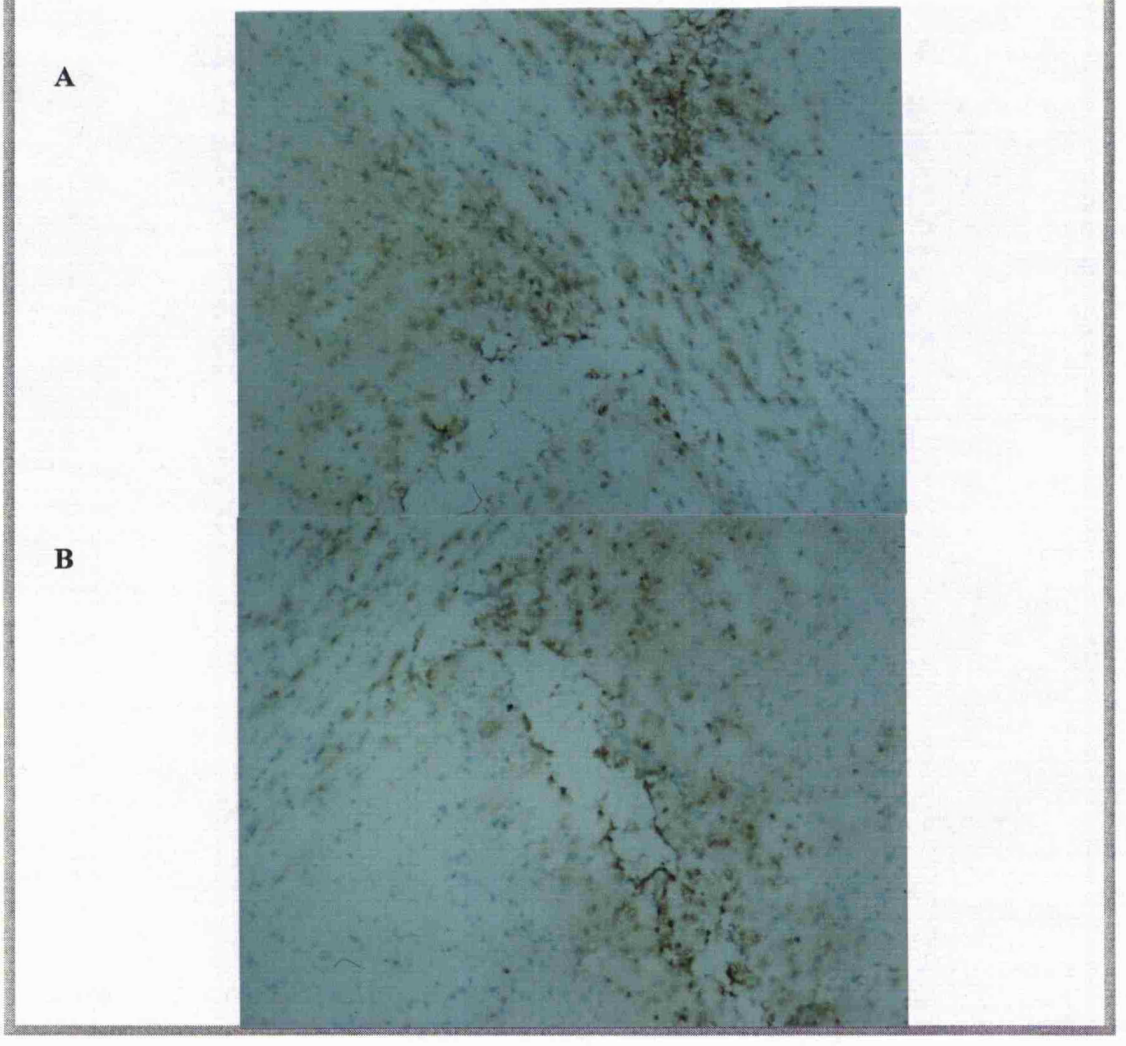
Figure 5.9 OX42 is upregulated at the site of a stab wound
Sections of cerebral cortex stained with OX42 (1:800) and visualised with DAB. Counterstain is methyl green.
A, day 2 post-injury. Magnification x6.25.
B, day 3 post-injury. Magnification x12.5.
C, day 5 post-injury. Magnification x6.25.



At day 2 after injury (figure 5.9A), the microglial activation, as assessed by OX42 antigen upregulation, was just begining around the site of the wound. At day 3 (panel B), a lot of activation was seen around the wound and in unaffected tissue bordering the injury. By day 5 (panel C) the activation was beginning to subside, probably due to removal of much of the debris associated with the wound.

ED1, a marker of active microglia, was also expressed by cells surrounding the wound site (figure 5.10). The stained cells were probably a mixture of macrophages, which have migrated in from the blood, as well as upregulated microglia. Again, activation was seen in both white and grey matter around the injury.

Figure 5.10 ED1 expression at the site of a stab wound
Cerebral sections taken from the site of the stab wound, and stained with ED1 (1:400), visualised with DAB and counterstained with methyl green. Magnification x25.
A, day 3 post-injury; B, day 5 post-injury.



In contrast, MADM did not appear to be upregulated on these activated microglia - when the R93 antisera is titrated to 1:600, all the staining rapidly disappears, including that around the stab, even at 3 days post-injury when activation levels would appear to be at their highest (figure 5.11). Normal staining (with the antiserum used at 1:150) is unaffected, with MADM being detected at the usual low levels in white matter areas (data not shown).

Figure 5.11 MADM expression at the site of a stab wound (3 days)
Cerbral cortex, stained with R93 (1:600), counterstained with methyl green. Magnification x25.

5.1.3 Expression of MADM by cells *in vitro*

Since increased levels of MADM were not detected in either developing brain, or at a site of brain injury, it was decided to see whether it was possible to upregulate MADM on microglia *in vitro* using cytokines or agents such as lipopolysaccharide. Also, use of microglia in culture would allow investigation of whether MADM is expressed on the cell surface of both normal and activated cells, and a comparison with a known surface antigen of microglia, the αMβ2 integrin (*i.e.*: the OX42 antigen).

Microglia were isolated from the brains of 30 day old rats, and were cultured for 5-7 days. Both fixed (with acetone) and unfixed (i.e. unpermeablised) cells were then probed with R93 antiserum to determine whether MADM was expressed either inside or on the surface of the cells. Unfixed, non-permeablised cells showed no staining above background levels (figure 5.12), which implied that MADM was not expressed on the surface of these cells. In contrast, OX42 staining was obviously localised to a surface protein, being detected at high levels both on non-permeablised cells (figure 5.12C), and also on permeablised cells (figure 5.13D). Staining fixed, permeablised cells with R93 antiserum confirmed that most, if not all of the MADM expressed by microglial cells was intracellular (figure 5.13A). The staining indicated that MADM was present at quite high levels, and much of the protein appeared to be concentrated in an area close to the nucleus, rather than near the cell membrane. This result was somewhat unexpected as MADM's domain structure (section 1.4.3) predicts a type I plasma membrane glycoprotein with the R93 epitope exposed outside the cell. The specificity of this staining is demonstrated in figure 5.13B, where the R93 antiserum was preincubated with peptide #5, and all fluorescence at levels above background was abolished. It is worth noting that this image was obtained using a higher laser intensity than that used for panels A and D in order to pick out the positions of the cells. A phase contrast picture showing the morphology of the isolated microglia under culture conditions (panel C) identified the patch of brighter fluorescence in panel B as due to non-specific staining of debris.

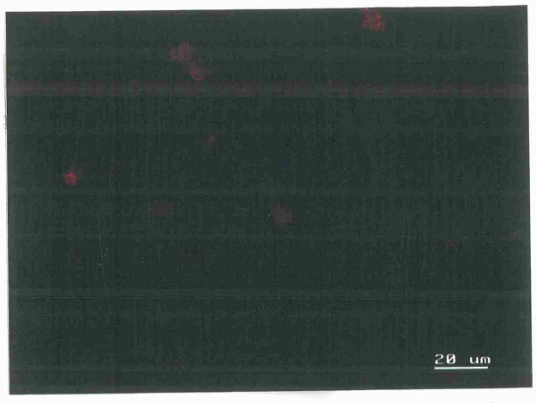
As a comparison to the microglial cell staining, three cell lines were also examined for MADM expression. The cell line NSO, a mouse myeloma cell line was chosen, since western blot data indicated that it contained relatively high levels of MADM when compared to brain (figure 4.6). The rat equivalent of this cell line, Y0 was also chosen, as well as cell line C6, which, by Western blotting, appeared to have no MADM and could thus be used as a negative control.

Non-permeablised NS0 cells stained with R93 were highly fluorescent, compared to the pre-immune serum negative control (figure 5.14). This staining was also blocked by preincubation of the antiserum with peptide #5 (figure 5.14C). Thus, in contrast to microglia, NS0 cells expressed relatively large amounts of MADM on the

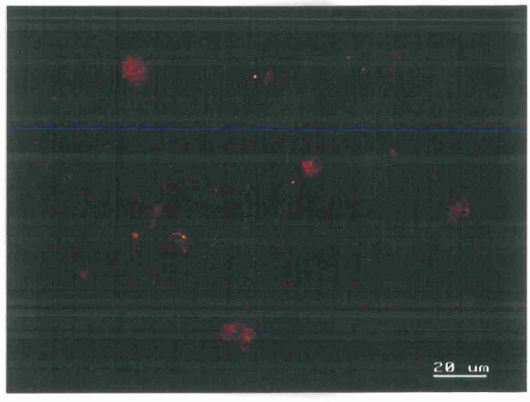
cell surface. A high magnification view of a single slice through cells stained with R93 showed the surface staining very well (figure 5.15).

Figure 5.12 MADM is not expressed on the surface of resting microglial cells
Primary cultures of microglia were stained to show surface expression of antigens with the following:
A, pre-immune sera from rabbit #93, diluted 1:150, as a negative control;
B, R93 immune sera, 1:150;
C, OX42 monoclonal antibody, 1:150.

A



B



C

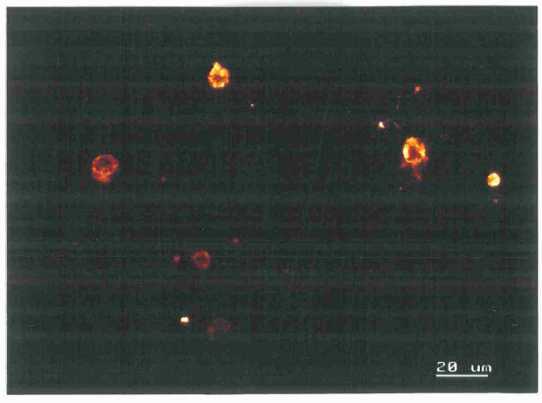


Figure 5.13 Comparison of MADM and αMβ2 localisation on permeablised microglia

Primary cultures of microglia were fixed by immersion in acetone, and then stained with the following:

A, R93 antiserum, diluted 1: 150; B, R93 preincubated with peptide #5 (4μg ml^{-1}) overnight at 4°C; D, OX42 antibody, diluted 1:150. C is a phase contrast image of the cells stained in panel B.

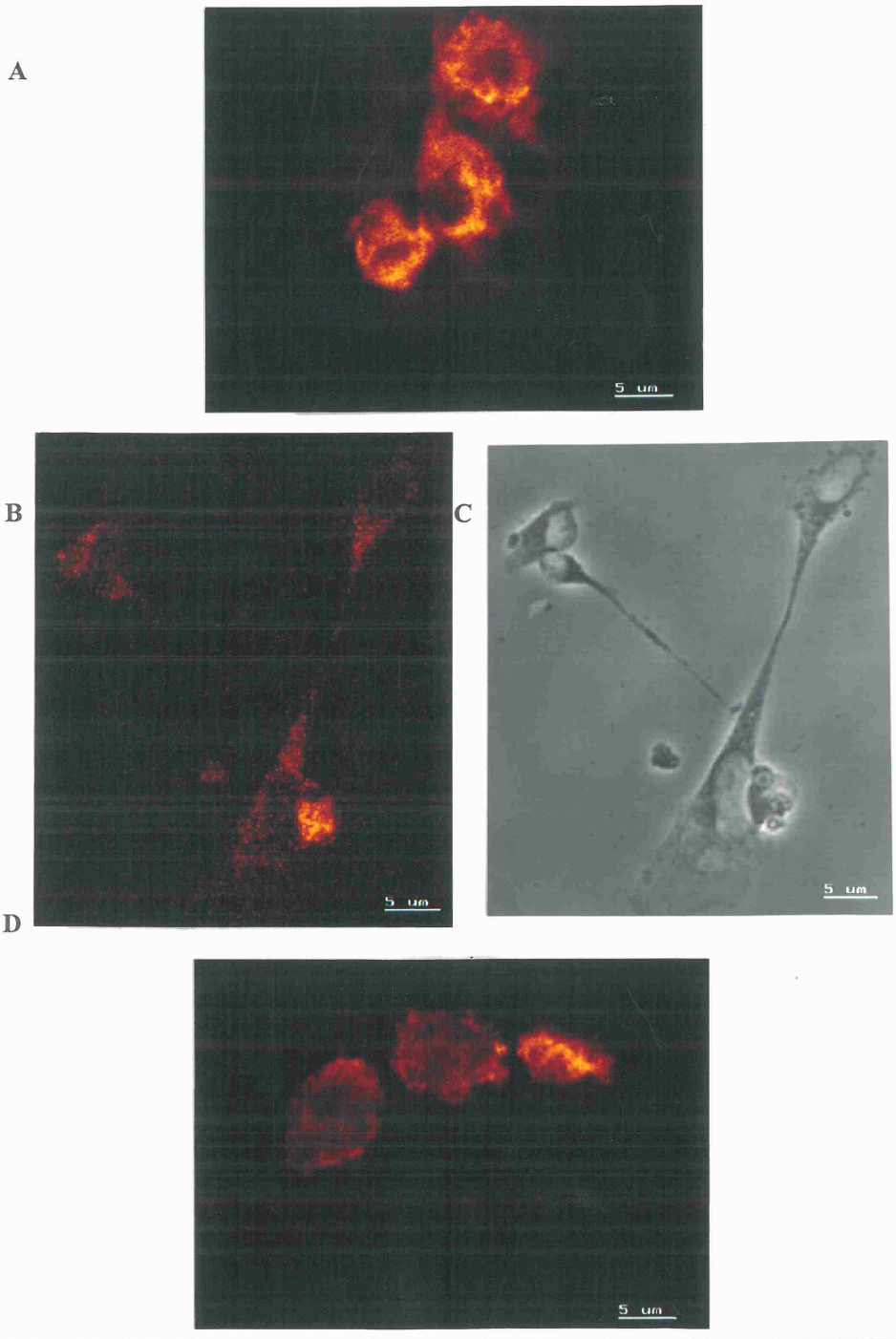
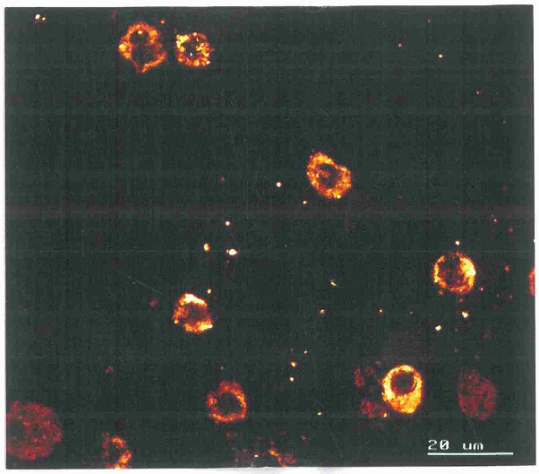
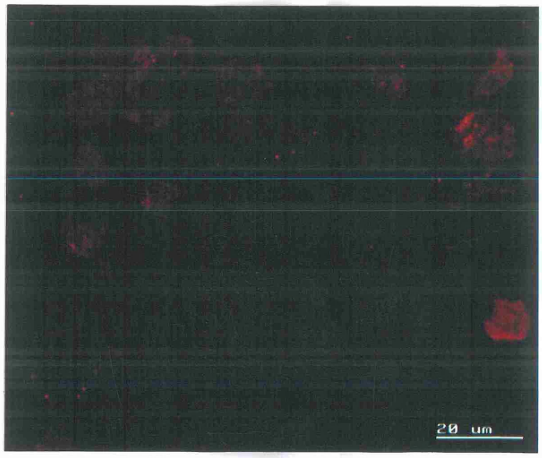


Figure 5.14 Surface staining of NSO cells with the R93 antiserum
Non-permeablised cells were stained to examine surface expression of the MADM protein. Composite confocal images are shown.
A, stained with R93 antiserum, 1:150;
B, pre-immune serum negative control, 1:150;
C, R93 antiserum preincubated with peptide #5 (4μg ml^{-1}) overnight at 4°C.

A



B



C

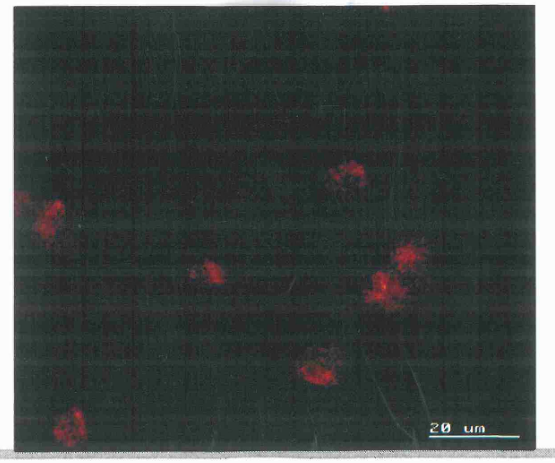
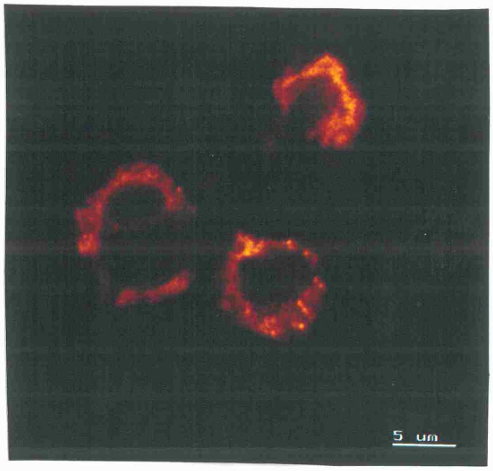


Figure 5.15 A higher power view of surface stained NSO cells
A single slice through cells stained with R93 (1:150) to show staining which is clearly limited to the cell surface.

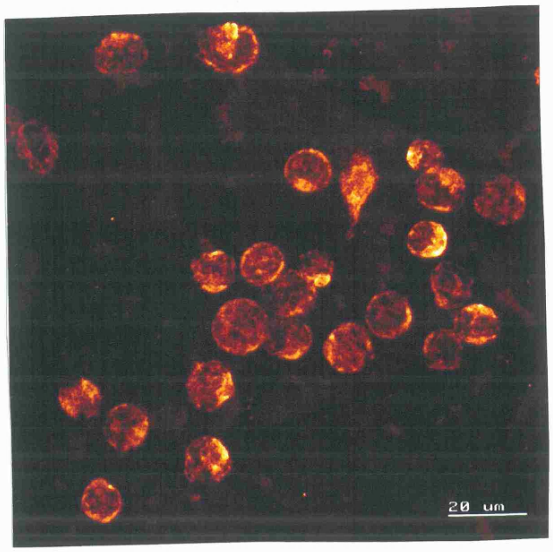


Acetone-fixed NSO cells also showed bright staining with R93 (figure 5.16). The rat myeloma cell line Y0, which by Western blotting analysis (figure 4.5) had less MADM than NS0, showed less intense immunocytochemical staining with R93 (figure 5.17). Finally, the C6 glioma cells, apparently devoid of MADM by Western blotting analysis, appeared negative by R93 immunocytochemistry (figure 5.18).

Although these results help to confirm why the monoclonal anti-MADM antibodies proved so difficult to produce, since MADM would have been effectively acting as an autoantigen on the NS0-fused cells (section 4.1.4), other questions remain. Why do these cells express MADM on the cell surface, while resting microglia do not? It is possible that the answer to this question may reflect the activation state of each set of cells. NS0 cells are an actively growing tumour cell line, and have also been immortalised, both of which aspects may well lead them to upregulate existing surface antigens, as well as express new ones. It is therefore important to look at the levels and location of the MADM protein in microglial cells which have been activated by cytokines and other compounds, in order to make a comprehensive comparison.

Figure 5.16 Staining for MADM on acetone-fixed NSO cells
Cells which had been fixed by immersion in acetone were stained with the following:
A, R93 antiserum, 1:150;
B, pre-immune serum, 1:150, negative control.

A



B

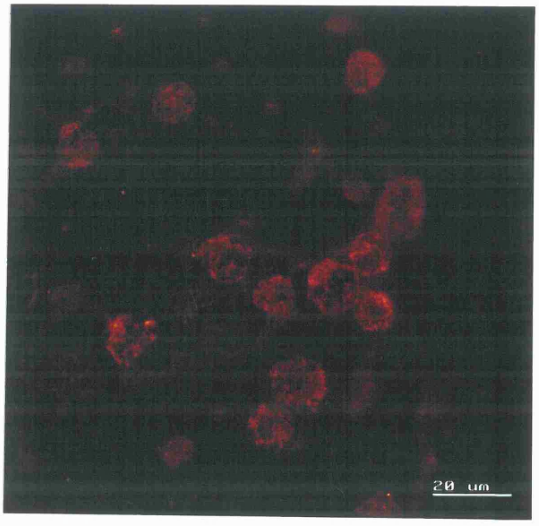
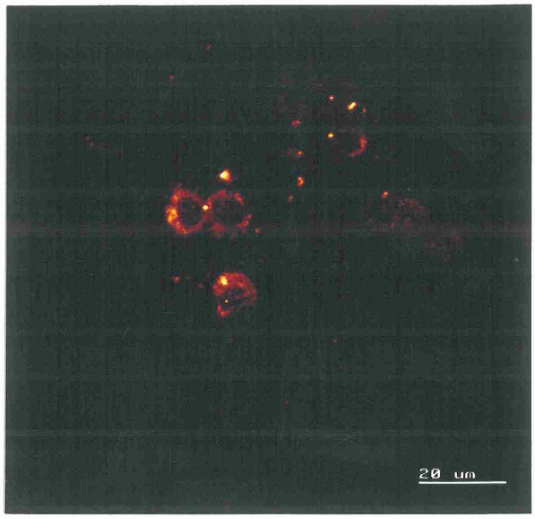


Figure 5.17 Expression of MADM on acetone-fixed Y0 cells
Cells which had been fixed by immersion in acetone were stained with the following:
A, R93 (1:150).
B, pre-immune serum (1:150) as a negative control.

A



B

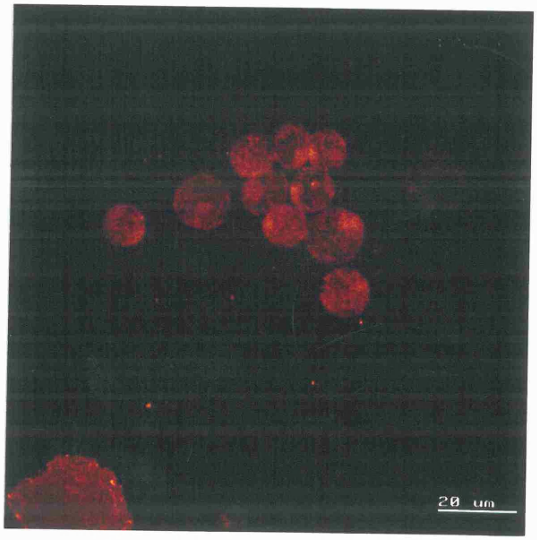
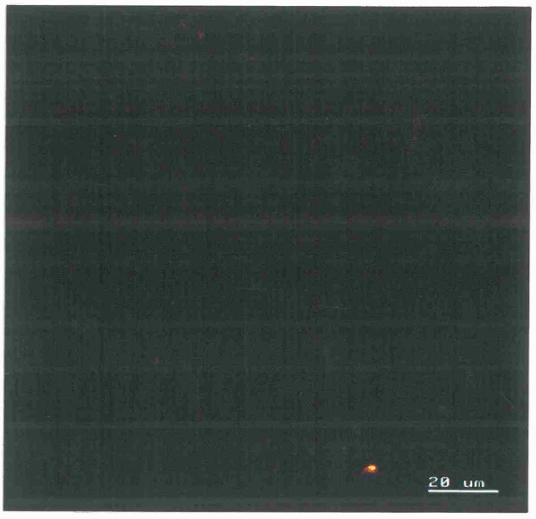


Figure 5.18 C6 glioma cells do not contain detectable MADM
Cells were permeablised by immersion in acetone, and then stained with R93 antiserum (1:150).



5.1.4 Activation of microglia

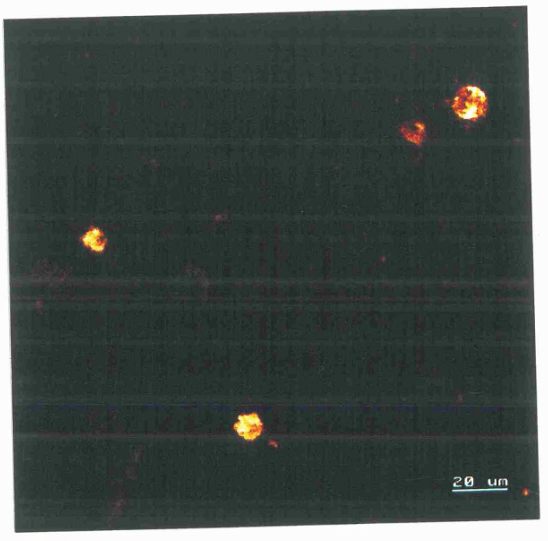
After 5 days, one of the following cytokines/activating agents was added to the microglial culture medium: lipopolysaccharide (LPS), granulocyte/monocyte colony-stimulating factor (GM-CSF), interleukin-3 (IL-3) or interleukin-4 (IL-4). After a further 48 or 72 hours the cells were washed and stained for examination by confocal microscopy. The cells were not permeablised as these experiments were intended to discover whether microglia activated by any of these agents would express MADM as a surface antigen, in contrast to microglia in the resting state. As well as R93 antiserum, cells were stained with OX42 which had already been shown to be expressed at the surface (figure 5.12).

The results of these experiments showed that none of the activating agents used caused microglia to express MADM as a surface antigen, either 48 or 72 hours after exposure. The addition of LPS to the culture medium did appear to activate the microglia, as assessed by both a morphological change - the cells became more

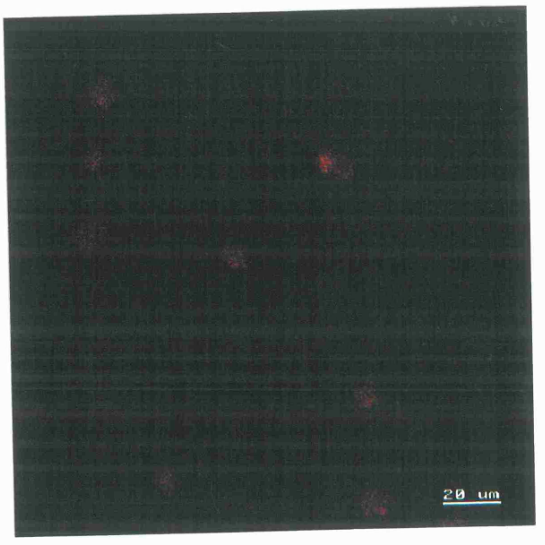
rounded and less adherant - and also by a slight increase in the intensity of the OX42 staining (figure 5.19). Despite this apparent activation, there was no detectable MADM expressed at the cell surface by these cells. The results for cells stimulated with GM-CSF, IL-3 and IL-4 were virtually identical to those produced by LPS (data not shown).

Figure 5.19 MADM is not upregulated on LPS-stimulated microglial cells
Cells which had been stimulated with LPS for 72 hours were stained with the following:
A, OX42 (1:150).
B, R93 (1:150).

A



B



5.2 Summary

This chapter has described the localisation of the MADM protein by immunohistochemistry to white matter microglial cells in the rat CNS. It was shown that β2 integrins appear to be the only candidates for a cognate integrin present on the same cell, and that MADM was not upregulated on microglia either in 2 day old developing rat brain or at the site of a stab injury. Fluorescence confocal microscopy demonstrated that in microglia *in vitro*, MADM appears to be found inside the cells and cannot be upregulated to cell surface expression by the addition of various cytokines/activating agents to the culture media. In contrast, MADM was found to be expressed as a surface antigen by immortalised myeloma cells in culture.

6. GENERAL DISCUSSION

A metalloprotease isolated from bovine brain myelin membrane preparations was shown to be a type I integral membrane protease, similar to a number of snake venom metalloproteases. The protein contained a potential integrin-binding domain, and was named MADM for Mammalian Disintegrin-Metalloprotease. This thesis has reported the cloning of a rat MADM homologue, antibody production and localisation of this novel enzyme in white matter microglial cells of the CNS.

This localisation of MADM to a subpopulation of microglia in the white matter would appear to be a contradiction of previously published preliminary immunohistochemical results using the CG4 monoclonal antibody, in which MADM was reported to be expressed by oligodendrocytes in bovine CNS sections (Chantry et al., 1992). However, much of the data in the earlier study are still valid, and the rest is open to reinterpretation in the light of the results presented in this thesis. Both studies have shown that MADM is predominantly localised to white matter areas of the CNS. This study has shown by means of double immunolabelling of sections that the cells stained in CNS white matter are microglia. The study by Chantry and co-workers (1992) alleged that the staining was found in the cytoplasm of small process-bearing cells, with diffuse myelin staining. The cells indicated in the relevant photographs of that publication could as easily be microglia as oligodendrocytes; the diffuse myelin staining may have been due to generalised background staining, such as was seen in the present studies when using serum to block tissue sections (data not shown), but which was completely removed by blocking sections with a solution of BSA instead. The CG4 immunoreactivity seen in the grey matter may have been due to non-specific nuclear staining, which could also have been eliminated by the use of a different blocking agent. Since without double staining it can be difficult to definitively identify a particular cell type, it is therefore entirely possible that the pattern of staining was misinterpreted in the earlier study.

The distribution of MADM immunoreactivity predominantly to white matter microglia is particularly interesting. Such a preferential localisation to microglia of the white matter has also been shown for MMP-2 in human brain (Yamada *et al.*, 1995a), and for the c-met proto-oncogene product, a receptor for hepatocyte growth factor

(Yamada *et al.*, 1994), as well as for the MMP-2 activating enzyme, MT-MMP, a membrane matrix metalloprotease with some similarities to MADM.

Microglia are potentially the most migratory cells in the adult CNS, particularly in pathological conditions. As mentioned in section 1.1, a role for microglia has been implicated in a wide range of conditions including demyelinating disease (Woodroofe *et al.*, 1986) and prion diseases, (Williams *et al.*, 1994b), and it has been shown that microglial cells are extremely sensitive to ionic changes in the brain microenvironment (Gehrmann et al., 1993). Cultured microglia have been shown to secrete MMP-2 and MMP-9, which are capable of degrading extracellular matrix components and would thus help microglia progress through the tissue to sites of injury or infection (Colton *et al.*, 1993; Gottschall *et al.*, 1995).

During microglial activation, many molecules are upregulated, including the Fc receptor (Loughlin *et al.*, 1992); the secretion of matrix metalloproteases increases (Gottschall *et al.*, 1995); activated microglial cells show striking *de novo* expression of major histocompatibility complex class II antigens (Gehrmann *et al.*, 1993); in scrapie, microglia showed increased staining of surface antigens such as leukocyte-common antigen and the type 3 complement receptor (the αMβ2 integrin; Williams *et al.*, 1994b).

Any speculation on possible functions of MADM in microglia must take account of the present findings that MADM is found intracellularly in these cells, and could not be induced to shift to surface expression by the methods used. In the original immunohistochemical study of MADM localisation in bovine peripheral nerve using the CG4 monoclonal antibody, MADM-specific staining appeared to be cytoplasmic: in the outer ring of cytoplasm of myelinating Schwann cells, in the cytoplasm of Schmitt-Lanterman incisures, and in the cell bodies of non-myelinating Schwann cells (Chantry *et al.*, 1992). A single, more recent experiment using the R70 antiserum appeared to confirm this localisation in rat sciatic nerve, although this work was not continued (data not shown). In Schwann cells, integrins have been localised to areas where MADM appears to be expressed; the α6β4 heterodimer has been shown to be present not only in the outer plasma membrane of the myelinating Schwann cell but also in the Schmitt-Lanterman clefts (Niessen et al., 1994). The myelin-associated glycoprotein (MAG) is also found in these clefts (Einheber *et al.*, 1993). Thus, by

light microscopy, 3 membrane glycoproteins (α6β4, MAG and MADM) appear to reside in a predominantly cytoplasmic region of the Schwann cell membrane process - an observation for which there is currently no explanation.

If MADM is an intracellular protein in microglia and Schwann cells, despite the secretory signal sequence and the potential transmembrane domain, it must be assumed that it is residing either in a compartment of the Golgi apparatus, or in storage vesicles. The confocal microscopy staining of microglia shown in chapter 5 appears to show MADM in a band around the nucleus, possibly indicative of storage in the Golgi, although this is a very speculative assumption; in Schwann cells there is no evidence to favour either Golgi or vesicle storage, even if the cytoplasmic staining is assumed not to be artefactual. It would seem likely, however, that furin cleavage will have occurred, and that MADM will be present in the mature form; it is entirely possible that catalytically-active MADM is present in microglia and macrophages, since epitopes of MBP resulting from the specific cleavage by MADM at the Pro73-Gln74 bond can be immunostained within both microglia and macrophages around active or chronic plaques in MS brain tissue (Li et al., 1993). However, it is subject for further speculation as to whether a small amount of MADM is expressed on the surface of these cells and cleaves extracellular MBP, fragments of which are subsequently phagocytosed, or whether MBP (soluble or associated with myelin) is first phagocytosed and only then comes into contact with MADM within an intracellular compartment.

Further, if MADM is an intracellular antigen, how could a potential interaction with an integrin occur? The association may occur only after transport of MADM to the cell surface, in response to a (as yet undiscovered) signal, placing MADM in position to cleave its substrate (also unknown). Alternatively, the initial interaction may be an intracellular one, with MADM and its cognate integrin associating in the Golgi or in a storage vesicle; in this scenario, the transport of MADM to the cell surface could conceivably be dependant upon the movement and upregulation of the integrin heterodimer in response to signals. The presence of integrins inside cells has been shown: many cell lines have been shown to synthesize an excess of precursor β1 integrin in the endoplasmic reticulum (ER) which are retained in a pool of free subunits (Akiyama and Yamada, 1987). This pool is not stable, but is regulated by

growth and differentiation factors (Heino *et al.*, 1989) and may decrease during malignant transformation (Koivisto *et al.*, 1994). Similar models have been described for other integrin subunits, including αV integrins (Sheppard *et al.*, 1992).

If MADM does associate with integrins expressed on the same cell, then αMβ2 is currently the only obvious candidate for a potential cognate integrin, since microglia do not appear to express other integrin subtypes. The expression of the β2 integrin by microglia appears to be under the control of cytokine signals - αMβ2 has been shown to be upregulated in scrapie (Williams *et al.*, 1994b). Macrophages, the cell type to which microglia are most closely related, have also been shown to express αVβ3 (the vitronectin receptor), an integrin which appears to play a role in apoptosis (Savill *et al.*, 1990; Cox *et al.*, 1994). αVβ3 is known to be able to associate with a membrane-bound protein (IAP, integrin-associated protein) which may subsequently affect the binding of the integrin to its ligand (Lindberg *et al.*, 1993); αVβ3 also interacts with MMP-2, facilitating directed cellular invasion (Brooks *et al.*, 1996). Macrophages may well express MADM, since microglia are cells of the macrophage lineage; in addition, MADM has been detected in the promonocytic cell line U937 (Howard *et al.*, 1996) and in infiltrating macrophages in lesions of MS brain (J.Newcombe, personal communication). Macrophages are known to express high levels of the ADAM family member MS2 (Yoshida et al., 1990). Both metargidin and MDC9 are also potentially expressed by macrophages, since RNA transcripts of both are found at relatively high levels in the spleen (Kratschmar *et al.*, 1996; Weskamp *et al.*, 1996).

If MADM needs to bind to its integrin ligand intracellularly in order to be transported to the cell surface, we could speculate that perhaps microglia do not express the specific integrin required and thus MADM in these cells remains internal. It would be interesting to discover whether MADM can be detected on the surface of αVβ3-expressing macrophages and is associated with this integrin.

Alternatively, the results of the work on NS0 cells may provide the clue to MADM's expression. Unlike isolated microglia, these cells are immortalised and actively dividing in culture, and for these reasons alone may be able to express MADM at the cell surface. However, it is also possible that it is the integrins which they express which may be responsible. Although no specific data are available on the integrin complement of NS0 cells, it can reasonably be expected to be similar to that

of other myeloma cell lines, expressing β1 integrins including α4β1 and α5β1 (Hata et al., 1993), and some β2 integrins (Kim *et al.*, 1994).

In conclusion then, although the experimental work detailed in this thesis has discovered a great deal about this novel protein, there is still much that remains to be elucidated. There are still few clues to MADM's function and substrates, either in brain or elsewhere, despite the implications of the unusually high levels of sequence conservation between mammalian species. Certainly in brain, a major role in myelogenesis appears to have been ruled out, although there is still scope for a potential role in antigen cleavage and presentation.

However, very recently another MADM homologue named *kuz* has been identified, this time in *Drosophila*, with an essential role early in embryogenesis deciding the fate of cells involved in neurogenesis (Rooke *et al.*, 1996). With 43% amino acid identity overall to bovine MADM, this homologue is also very highly conserved and provides further reasoning against the idea of MADM being some sort of vestigial relic of a lost or redundant process.

Although future investigations of the MADM family of proteins should not necessarily be limited to the CNS, by extrapolation from the *kuz* data it now seems important to look closely at mammalian MADM expression much earlier in neural development than was achieved in this study. The expression of MADM by other cell types should also be examined, particularly in the light of the myeloma cell data; macrophages particularly should be studied since expression of MADM by this cell type could account for the widespread distibution seen in tissues throughout the body. The question of MADM association with integrins also remains to be determined: a result here could have wider implications in our understanding of the ways in which MADM expression and localisation may be regulated.

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