## Expression of the neural cell adhesion molecule in human brain tumours

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Cell–cell interactions are believed to play a key role during development and maintenance of tissue structures and organization. Cell-surface glycoproteins have been identified that appear to be involved in cell–cell recognition. One of the best characterized proteins is the neural cell adhesion molecule (NCAM) [1]. This molecule comprises a family of glycoproteins coded for by a single gene. In the mouse brain, three main isoforms of 180, 140 and 120 kDa (in their desialo form) have been described. The two larger forms are transmembrane glycoproteins, while the smallest isoform is linked to the membrane via a glycosyl-phosphatidylinositol anchor. A particular feature of the molecule is the presence of long chains of polysialic acid. The degree of polysialylation is developmentally regulated. Embryonic NCAM is heavily polysialylated, whereas adult NCAM is not. In addition, the expression of NCAM is also developmentally regulated. It is widely expressed during embryogenesis in all three germ layers, but becomes restricted to tissues derived from the neuro-ectoderm in the adult.

Recently, we have shown that monoclonal antibodies ERIC-1 (raised against human retinoblastoma cell line) and UJ13A (raised against human foetal brain) recognize different epitopes on human NCAM [2, 3]. We have utilized ERIC-1 to investigate the expression of NCAM in human brain and primary brain tumours.

Tissue samples were lysed in 0.1 M-Tris/HCl, pH 6.8, 10% (v/v) glycerol, 2% (v/v) SDS, 2 mM-phenylmethanesuphonyl fluoride, 2 mM-EDTA, leupeptin (10 $\mu$g/ml) and 8% (v/v) $\beta$-mercaptoethanol. Proteins were separated by SDS/polyacrylamide-gel electrophoresis and transferred to nitrocellulose membrane. Immunoblotting was performed with ERIC-1 as described previously [2].

Immunoblotting of foetal brain showed a smear of 120–200 kDa. This smearing is characteristic of embryonic NCAM and is due to the presence of polysialic acid residues. Neuraminidase treatment resolved the smear into two major bands of 180 and 140 kDa, and a minor band of 120 kDa. Immunoblotting of brain samples of different ages (16 weeks to 30 years) showed that as development proceeds, the expression of the 180 kDa isoform decreases, whereas that of the 120 kDa isoform increases. In contrast, the expression of the 140 kDa isoform appeared to be fairly constant at all stages of development. Adult human brain was also found to contain a 170 kDa isoform which has not been observed in mouse and chicken brain in addition to the three bands described above.

NCAM expression in different areas of adult brain was also studied. White matter from the cerebellum and cerebrum was found to express the 120 kDa isoform only, whereas the grey matter contained 180, 140 and 120 kDa proteins. These results are in agreement with those reported recently by Bhat & Silberberg [4]. In addition, the meninges was found to express the 140 and 120 kDa isoforms.

Since UJ3A reacts with a number of embryonic and brain tumours, a corollary is that NCAM is present on these tissues. We have investigated NCAM expression in primary brain tumours to assess whether abnormal/altered expression contributes to the malignant phenotype. Differences in NCAM isoforms present in any particular tumour were noted. Oligodendroglioma and Grade II astrocytoma were shown to express 180, 140 and 120 kDa isoforms, whereas medulloblastoma, juvenile astrocytoma, schwannomas and eppendymomas expressed only 140 and 120 kDa proteins. Gliomas, on the other hand, expressed 180 and 120 kDa proteins. In addition, the meningioma was found to express two isoforms of NCAM, one of 120 kDa and the other 155 kDa. At present, it is not known whether the occurrence of 155 kDa isoform is due to differences in primary amino acid sequence or to post-translational modifications.

Cell–cell interactions mediated by molecules such as NCAM are thought to play a key role in embryogenesis and development. These cell–cell interactions may also be important in tumourigenesis. The significance of the observations described here with regard to cell–cell recognition and tumour biology, however, remains unclear but can now be addressed using a combination of biochemical and molecular approaches.

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## The use of peptide mimetics and proton magnetic resonance to define actin-binding sites on the myosin head

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Force production in muscle resides in the interaction between actin, myosin and ATP. It is readily acknowledged that the energy-transducing component of myosin is contained within the head of the myosin molecule [1]. This fragment of the molecule is easily isolated by proteolytic digestion from the parent molecule [2] and is referred to as subfragment 1 (S1). When chymotrypsin is used as the protease, S1 consists of a single heavy chain (95 kDa) and associated alkali light chain (20 kDa). A series of studies [3, 4] using different proteolytic enzymes has shown that the S1 heavy chain can be cleaved at two additional points, resulting in three fragments: *N*-25 kDa, 50 kDa and 20 kDa-*C*, that are suggested to represent domains within the myosin head. Experiments in which myosin is chemically crosslinked to actin by a 'zero length' carbodi-imide reaction have reported

that both the 50 kDa and 20 kDa domains of S1 come into close contact with actin in the acto–S1 complex [5, 6].

The macroscopic movements associated with muscle contraction must be based upon specific molecular motions. Definition of the molecular mechanisms therefore entails a description of how the components of the organized protein assembly dock with their substrates/partners, then interact and transmit information. In order to understand this process, we are exploiting the fact that myosin and actin can be cleaved into smaller, function-related segments that are more easily handled than the parent molecule and can be subsequently analysed for their dynamic and conformational features. The feasibility of this 'dissective' protocol, which has been extended to the use of synthesized peptides, is amply demonstrated by our earlier work on this system [7, 8]. In this report we show how this approach has helped to delineate precisely the regions on the heavy chain of S1 that interact with actin.

The first indication of the region of the 20 kDa *C*-terminal domain of S1 that interacts with actin came from experiments using whole S1 labelled at Cys-705 (in the 20 kDa domain) with a nitroxyl spin label [7]. This spin-labelled S1 perturbed resonances in the $^{1}$H-n.m.r. spectrum of the *N*-terminal region of actin, indicating the close spatial proximity of these two regions in the actomyosin complex. This region of the S1 heavy chain is also one of the most highly conserved among myosins from *Dictyostelium discoideum* [9] to rat [10]. Thus, a series of peptides from this region were synthesized by the conventional solid-phase route (Fig. 1) and tested for their ability to interact with actin by a number of different methods. Although it was not expected that the peptides would assume their 'S1-structure' in solution, it was anticipated that the sequences contained sufficient information to allow them to adopt this structure in the presence of a suitable template, namely actin. This was borne out by subsequent analysis.

Using $^{1}$H-n.m.r. to monitor binding to actin all of the peptides containing the region 687–725 showed selective spectral broadening when actin was titrated into them. The flanking regions, Y763, Y726 and Y727, acted as controls and no significant spectral perturbations were seen with these peptides, strongly suggesting that a specific interaction was occurring in the intervening region. Within the interacting peptides, broadening occurred at selective sites, possibly indicating that the peptides were adopting some secondary structure in the bound form. The n.m.r. exchange rate was intermediate-slow exchange, suggesting an affinity in the region $K_b \sim 10^5$ M$^{-1}$.

Further indication of the involvement of this region of the 20 kDa domain in actin interaction has come from using the peptides as inhibitors of the actin-activated MgATPase of S1 and of force production in skinned fibres. All of the peptides containing the region 687–725 acted as mixed inhibitors of the acto–S1 ATPase activity with $K_i$ values ranging from 5 $\mu$M (for Y630) to 400 $\mu$M (for Y762) [11]. The flanking regions were non-inhibiting and again acted as suitable controls.

One of the peptides, Y629, was also labelled at Cys-705 with *N*-ethyl-[2,3-$^{14}$C]maleimide [12] and used in a direct binding co-sedimentation assay with F-actin [13]. The binding was hyperbolic and 1 mol of peptide bound per mol of actin with a dissociation constant of $1.41 \times 10^{-5}$ M, which agrees very well with the $K_i$ value of $1.34 \times 10^{-5}$ M obtained in solution assays. Addition of S1 to these binding assays caused displacement of the peptide, indicating the specificity of interaction.

In collaboration with Professor J. C. Ruegg (University of Heidelberg) we have also tested the ability of these peptides to influence force generation in rabbit psoas fibres chemically skinned with 1% (v/v) Triton X-100 [14]. The various peptides, at concentrations ranging from 20 to 200 $\mu$M, were added to the bath containing the fibres, which were then activated by $Ca^{2+}$. All the peptides in the 687–725 region were inhibitory to force development to some degree at maximal activating concentrations of $Ca^{2+}$ (pCa = 4.5). However, at submaximal [$Ca^{2+}$] (pCa = 5.5) two differing effects were apparent. Peptides from the *N*-terminal end of this region (Y762, Y668, Y669, Y629 and Y608) caused an increase in force development (up to 75%, $n = 6$) and thus a leftward shift in the pCa–force relationship, whereas peptides from the *C*-terminal end (Y670 and Y630) remained only inhibitory to force development. Thus, it appears that this peptide region is spanning two functional domains in actin: one that is involved solely in force production and one that is involved in $Ca^{2+}$ sensitivity. It has been known for some time that crossbridges (myosin heads) attaching to actin increase the $Ca^{2+}$ sensitivity of the contractile system [15]. Here, we propose that a region of the myosin heavy chain, 687–715, that is involved in actin binding may also be involved in $Ca^{2+}$ sensitivity regulation.

The region of the 50 kDa domain that interacts with actin was thought to be on the *C*-terminal 6 kDa end based upon chemical-crosslinking studies [5], from experiments with (i) a 30 kDa thrombin-cleaved fragment that contained the whole of the 20 kDa domain and 10 kDa of the 50 kDa domain [16], and (ii) a 26 kDa formic acid-cleaved fragment [17]. The latter two fragments exhibited ATP-sensitive binding to actin that does not occur with the 20 kDa domain. Two peptides from the *C*-terminal end of the 50 kDa domain were synthesized and one of these was found to bind to actin by $^{1}$H-n.m.r. Close observation of the perturbed resonances suggested that binding occurring at the *C*-terminal end of a pep-

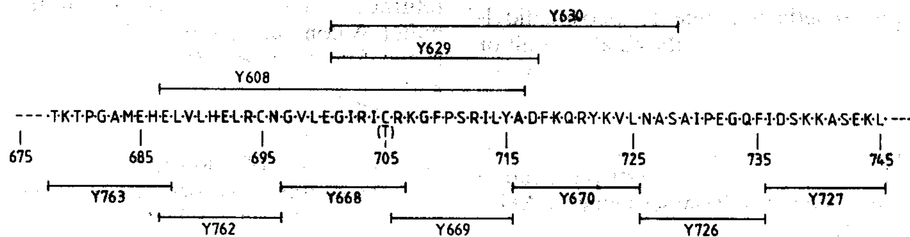


Fig. 1. *Peptides synthesized from the region 675–745 of the 20 kDa domain of rabbit fast skeletal myosin S1*

The lengths of the various peptides synthesized are shown by bars. They were synthesized by the conventional solid-phase route by Alta Bioscience, University of Birmingham, and were purified by semi-preparative h.p.l.c. on Vydac C-18 columns (25 cm × 2.2 cm) with 300 Å pore size. Their purity and the concentration of the peptide solutions were determined by amino acid analysis.

tide containing residues 585–614, around 610–614, although we cannot be sure whether or not binding is occurring *C*-terminal to this. Further studies along the lines shown above for the 20 kDa domain site will be necessary to delineate this 50 kDa site precisely. Thus, two sites on the S1 heavy chain and one on the alkali light chain [7] are involved in interaction of myosin with actin.

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# Solubilization and purification of lipid methyltransferase

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## *Introduction*

Lipid methyltransferase (LMTase; phosphatidylethanolamine methyltransferase; EC 2.1.1.17) catalyses the *S*-adenosylmethionine (SAM)-dependent methylation of phospholipids, a reaction that is associated with hormone and neurotransmitter action [1]. A theory on the involvement of phospholipid methylation in biological signal transmission has been proposed [2]. Despite the importance of LMTase, misunderstandings about kinetic data [3] and the purification and structure of this enzyme, an integral membrane protein, exist. Kinetic data have been presented for a number of tissues and interpreted in terms of two enzymes, but data from the purified or partially purified enzyme suggested that one enzyme is involved in the methylation of phospholipids [4, 5]. Pajares *et al.* [5] purified a 25 kDa protein monomer which existed as a 50 kDa dimer. The partially purified protein existed as a 300 kDa aggregate with a lipid to protein ratio of 0.4. McBride *et al.* [6] partially purified a 200 kDa protein with two 50 kDa subunits. Others [4] have reported the purification of an 18.3 kDa protein that catalysed the conversion of phosphatidylethanolamine to phosphatidylcholine. There is no consensus yet about the catalytic unit or its relationship to the membrane-bound enzyme. In previous experiments, 0.3% (w/v) CHAPS [5, 6] or 0.7% (v/v) Triton X-100 [4] were used in solubilizing rat liver microsome LMTase. Here we report the solubilization of rat liver microsome LMTase using 0.9% (w/v) CHAPS and its purification by anion-exchange and *S*-adenosylhomocysteine (SAH)-Sepharose affinity chromatographies.

## *Results and discussion*

*Enzyme preparation and solubilization.* Fresh rat liver was homogenized in 4 vol. of 25 mM-Tris/HCl (pH 7.4) containing 0.25 M-sucrose and 0.1 mM-phenylmethanesulphonyl fluoride (PMSF). The homogenate was centrifuged (15 000 ***g*** for 30 min) and the resultant supernatant was spun at 100 000 ***g*** for 1 h to obtain microsomal pellets. The microsomes were solubilized by stirring for 45 min at 4°C in buffer A (25 mM-Tris/HCl, pH 8.35/50 mM-mercaptoethanol/0.1 mM-PMSF) containing 0.9% (w/v) CHAPS, then diluted to 0.3% (w/v) CHAPS using buffer A. Solubilized enzyme was obtained after centrifugation (100 000 ***g*** for 1 h) and used in subsequent purification steps.

*Purification and enzyme assay.* Anion-exchange chromatography using Whatman DEAE-52 cellulose resin was performed with varying gradient procedures using buffer A and buffer A plus 1 M-NaCl on a fast protein liquid chromatography system. In all cases, unbound and bound peaks of activities were obtained. The DEAE-52-bound activity was desalted using Pharmacia Sephadex-G15 mini-columns and used for SAH-affinity chromatography.

Epoxy-activated Sepharose 6B was used to synthesize an SAH–Sepharose affinity matrix using recommended procedures [7]. SAH–Sepharose was packed into glass column and equilibrated with buffer A. DEAE-52 purified enzyme was applied on to the column at a flow rate of 0.08 ml/min in buffer A containing 50 mM-NaCl; the column was washed with the same buffer until the absorbance at 280 nm returned to the baseline. Bound proteins were eluted with buffer A containing 1 M-NaCl and 1 ml fractions were collected for enzyme assay. Methylation was initiated by the addition of 20 $\mu$l of [$^3$H-*methyl*]SAM (100 mmol/l, 0.5 $\mu$Ci, 15 Ci/mmol) cocktail to 400 $\mu$l aliquots from each fraction. After incubation at 37°C for 30 min, the reaction was terminated using 2 ml of chloroform/methanol/2 M-HCl (6:3:1, by vol.); the mixture was vortexed, centrifuged and the lipid-rich chloroform extract washed with 0.5 M-KCl in 50% (v/v) methanol. The chloroform layer was dried under $N_2$ and the amount of activity incorporated into lipids was determined by scintillation counting. Enzyme activity after the SAH-affinity step was measured in the presence of phosphatidylethanolamine (0.8 mM), phosphatidyl-*N*-monomethylethanolamine (0.4 mM) and phosphatidyl-*N,N*-dimethylethanolamine (0.08 mM), applied as liposomes in buffer A.

Abbreviations used: LMTase, lipid methyltransferase; SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine; PMSF, phenylmethanesulphonyl fluoride.