

The membrane topography of ecto-5'-nucleotidase in rat hepatocytes

Michael D. BARON,* Brian POPE† and J. Paul LUZIO*

*Department of Clinical Biochemistry, University of Cambridge, Addenbrooke's Hospital, Hills Road, Cambridge CB2 2QR, U.K., and †M.R.C. Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, U.K.

The transmembrane topography of the rat hepatocyte ectoenzyme 5'-nucleotidase was studied by the use of glycoprotein labelling and limited-proteolysis techniques. Comparison, by one-dimensional peptide mapping, of enzyme iodinated from outside the cell with that iodinated in the solubilized state showed that no additional iodination sites were revealed on solubilization. Incubation of newly synthesized enzyme in a microsomal membrane fraction with proteinase showed that the entire molecule of 5'-nucleotidase was protected from proteolysis. These data suggest that little, if any, of the 5'-nucleotidase molecule is present on the cytoplasmic side of the plasma membrane. No evidence was found for a previously proposed interaction between 5'-nucleotidase and actin, although the ability of preparations of 5'-nucleotidase to prevent inhibition of deoxyribonuclease I by actin was explained by minute traces of ATPase activity. Comparison of peptide maps of enzyme labelled by iodination or by methods specific for carbohydrate showed that in both cases predominantly one section of the molecule was labelled. It is proposed that the enzyme is a short-stalked integral membrane protein without a cytoplasmic domain in which about one-third of the molecule forms the accessible molecular surface.

INTRODUCTION

5'-Nucleotidase (EC 3.1.3.5) is an intrinsic membrane glycoprotein found in the plasma membrane of several cell types. It is an ectoenzyme, with its active site on the external face of the plasma membrane (De Pierre & Karnowsky, 1974; Newby *et al.*, 1975). Although no single physiological role has been assigned to the enzyme, several have been suggested, including nucleotide scavenging or production of adenosine as a 'local hormone' (Stanley *et al.*, 1982).

5'-Nucleotidase is widely used as a general plasma-membrane marker, although the enzyme appears to be concentrated in certain domains of polar epithelial (or epithelium-derived) cells, such as hepatocytes (Wisher & Evans, 1975) and intestinal epithelial cells (Gratecos *et al.*, 1978). In addition, up to 50% of the enzyme (depending on cell type) is found intracellularly, primarily in an endocytotic recycling pool (Stanley *et al.*, 1980; Widnell *et al.*, 1982), but also in lysosomes (Maguire & Luzio, 1985), in vesicles involved in the transcytosis of polymeric IgA (Mullock *et al.*, 1983) and in the Golgi apparatus (Farquhar *et al.*, 1974; Little & Widnell, 1975). It has been suggested that 5'-nucleotidase interacts with the cytoskeleton, either directly with actin (Mannherz & Rohr, 1978) or in an unspecified manner (Carraway *et al.*, 1979). Such an interaction would be extremely interesting, given the widespread distribution of the enzyme in subcellular compartments and its movement through nucleated cells on endocytic routes (Stanley *et al.*, 1980; Widnell, 1983). The studies reported, however, have relied on indirect evidence (Carraway *et al.*, 1979) or at best partially purified preparations of 5'-nucleotidase (Mannherz & Rohr, 1978). In addition, the only evidence that 5'-nucleotidase is a transmembrane protein, and therefore possesses a domain capable of interacting with intracellular actin, comes from studies on a cell line with aberrant insertion

of 5'-nucleotidase (Zachowski *et al.*, 1981). In order to investigate properly the mechanism by which enzyme circulates through the cell, and is segregated into different membrane domains, we have undertaken a study of the structure of the enzyme, its membrane topography and its interaction with cytoplasmic proteins.

Previous work in this laboratory has developed a method of immuno-purifying 5'-nucleotidase from rat liver (Bailey *et al.*, 1982) and shown that the enzyme is an integral membrane protein and normally exists as a homodimer of approx. 70 000- M_r (α -)subunits, each subunit possessing an enzymically active site (Bailey *et al.*, 1984). About 20% of the enzyme is found as a heterodimer of α - and β -subunits. The β -subunit has M_r approx. 38 000, and can be shown to be related to the α -subunit by Western blotting and peptide mapping (Bailey *et al.*, 1984).

We now report the use of peptide mapping and proteolytic cleavage to show the absence of a significant cytoplasmic portion of 5'-nucleotidase. In addition, we show that the enzyme has no direct interaction with actin, and previously reported effects on actin are due to contaminating ATPase activity.

EXPERIMENTAL

Materials

Proteinase V8 from *Staphylococcus aureus* was from Miles Laboratories. Proteinase K, bovine serum albumin (fatty acid-free), actin (from rabbit muscle), 5'-nucleotidase (from *Crotalus* sp. venom), neuraminidase, galactose oxidase, deoxyribonuclease I, firefly luminescence reagent (FLE50), salmon sperm DNA, collagenase (type I or IV) and Protein A-Sepharose were from Sigma Chemical Co. M_r standards for polyacrylamide-gel electrophoresis were from Bio-Rad Laboratories or Boehringer-Mannheim. Sulphobetaine 14 (Zwittergent 3-14) was from

Calbiochem-Behring. All radiochemicals were from Amersham International. Culture media were from Gibco. [$\alpha\beta$ -Methylene]ADP was from P-L Biochemicals. Iodogen was from Pierce and Warriner.

Antibodies

A non-inhibitory monoclonal murine antibody to rat liver 5'-nucleotidase (5NE5) and a rabbit antiserum to immuno-purified 5'-nucleotidase (209) have previously been reported (Bailies *et al.*, 1984). Monoclonal antibody was coupled to cellulose to make a solid-phase immunoabsorbent as previously described (Siddle *et al.*, 1981). A control immunoabsorbent (X63) was made by using IgG from the original myeloma line. An immunoglobulin fraction of 209 antiserum was made by Na_2SO_4 precipitation (Williams & Chase, 1967). Antiserum to rat secretory component (a fragment of the receptor for polymeric IgA) was a gift from Dr. J. Peppard.

5'-Nucleotidase

5'-Nucleotidase was purified from rat liver, assayed and iodinated essentially as previously described (Bailies *et al.*, 1982), except where otherwise described in the text.

Electrophoresis

Electrophoresis in polyacrylamide gels was by the method of Laemmli (1970), modified for one-dimensional peptide mapping by the method of Cleveland *et al.* (1977), as follows. EDTA (1 mM) was included in stacking-gel buffer, and gel slices were equilibrated with this buffer and overlaid with 30 μl of the same buffer containing 20% (v/v) glycerol and 0.05% (w/v) Bromophenol Blue, followed by 15 μl of buffer containing 10% (v/v) glycerol and proteinase at the indicated concentration. Proteins were electrophoresed into a 5 cm stacking gel at 70 V for approx. 90 min, power was turned off for 30 min, and then electrophoresis completed at 200 V. The resolving gel contained 17.5% (w/v) acrylamide and 0.25% (w/v) bisacrylamide.

Proteins were detected by fluorography with pre-flashed film (Laskey & Mills, 1975) or by autoradiography with Cronex Quanta or Lightning Plus intensifying screens (du Pont). Binding of ^{125}I -actin to protein in electrophoresis gels was by the method of Snabes *et al.* (1981).

Hepatocytes

Isolated rat hepatocytes were prepared essentially by the method of Berry (1974). Cells with improved maintenance of viability were obtained by further purification in a Percoll gradient (Dalet *et al.*, 1982). Cell viability was assessed by morphology under phase-contrast microscopy and by ability to exclude Trypan Blue.

Surface labelling

(a) With ^{125}I . Plastic scintillation vials were coated with iodogen (1,3,4,6-tetrachloro-3,6-diphenylglycoluril) by dissolving the iodogen in chloroform at 50 $\mu\text{g}/\text{ml}$, adding the required volume to the vial, and drying under a stream of N_2 . Vials were stored desiccated at 4 °C. Cells (6×10^7 – 10×10^7 ; > 98% viable) were washed free of serum albumin included in the isolation buffer and incubated in Hanks balanced salt solution (Paul, 1975) containing 20 mM-Hepes and 3 mM- CaCl_2 for 2½ h on ice with 200 μg of iodogen and 0.5 mCi of Na^{125}I . The cells

were then collected by centrifugation (100 g for 2 min) and washed three times with 5 mM-KI in Hanks balanced salt solution.

(b) With ^3H . Glycoproteins were labelled with NaB^3H_4 by a modification of the methods of Gahmberg & Anderson (1977). Sialic acid residues were oxidized by incubating 3×10^8 cells in 20 ml of buffer H (50 mM-sodium phosphate buffer, pH 7.5, containing 0.15 M- NaCl , 1 mM- CaCl_2 , 1 mM- MgCl_2 and 1% (w/v) bovine serum albumin) containing 1 mM- NaIO_4 for 10 min at 0 °C. Galactose residues were oxidized by incubating the same number of cells in buffer H with 40 units of neuraminidase and 40 units of galactose oxidase for 60 min at 4 °C. In both cases cells were washed three times in buffer H and resuspended to a total of 15 ml. Since NaBH_4 is rapidly hydrolysed at pH 7.5, cells were given four consecutive treatments with 5 mCi of NaB^3H_4 for 15 min at 4 °C. Cells were then washed once in buffer H and three times in PBS (50 mM-sodium phosphate buffer, pH 7.5, containing 0.15 M- NaCl).

Immunoabsorption of 5'-nucleotidase

Surface-labelled cells were suspended in PBS containing 1 mM-phenylmethanesulphonyl fluoride, disrupted by freeze-thawing and sonication, and a membrane pellet was prepared by centrifuging at 100 000 g for 1 h. This pellet was resuspended in PBS/phenylmethanesulphonyl fluoride and solubilized in 2% (w/v) sulphobetaine 14 for 20 min at 22 °C and 10 min at 37 °C. Insoluble material was removed by centrifugation (125 000 g for 30 min). To the supernatant was added $\frac{1}{20}$ vol. of 2% (w/v) gelatin and $\frac{1}{5}$ vol. of 5-fold concentrate of RIPEA [50 mM-Tris/HCl buffer, pH 7.5, containing 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, 0.1% SDS, 1 mM EDTA and 0.15 M- NaCl]. 5'-Nucleotidase was extracted by approx. 0.3 mg of solid-phase antibody 5NE5 per 10^8 cells, or by a similar amount of solid-phase X63 antibody, for 16 h at 4 °C. The immunoabsorbent was then washed once in RIPEA, twice in RIPEA containing 0.5 M- NaCl and twice in 10 mM-Tris/HCl buffer, pH 7.5, before being extracted with sample buffer (Laemmli, 1970) containing 2% SDS, for electrophoresis.

Protein labelling in primary cultured hepatocytes

Primary cultures were prepared essentially as described by Tanaka *et al.* (1978), and grown on 50 mm collagen-coated dishes for 2 days before use in labelling studies. Before labelling, cells were incubated for 2 h in methionine-free basal Eagle's medium supplemented with non-essential amino acids and containing 10% (v/v) dialysed foetal-calf serum, 0.03 μM -insulin and 10 μM -dexamethasone. Cells were then incubated in the same medium containing 0.125 μCi of [^{35}S]methionine/ml for 15 min. Cells were harvested in 2 ml of PBS per plate and disrupted with 70 strokes of a tight-fitting Dounce homogenizer. A microsomal fraction (Owen *et al.*, 1980) was prepared by centrifuging the broken cells at 1500 g for 10 min and centrifuging the resultant supernatant at 100 000 g for 1 h. Microsomal membranes were incubated for 1 h at 37 °C in PBS with or without 350 μg of proteinase K/ml. Proteolysis was stopped by the addition of bovine serum albumin to 10 mg/ml and phenylmethanesulphonyl fluoride to 2 mM.

Immunoprecipitation of proteins from microsomal membranes

5'-Nucleotidase and a control transmembrane protein, the receptor for polymeric IgA, were separately purified from membranes of pulse-labelled cells by using polyclonal antisera. Membranes were incubated with 64 μg of 209 antibody fraction/ml, 1/100 dilution of rabbit anti-(rat secretory component) antibody or control rabbit IgG, in the presence of 0.1% (v/v) Triton X-100, for 16 h at 4 °C. Membranes were then diluted 30-fold in PBS/phenylmethanesulphonyl fluoride and collected by centrifugation (125000 g for 45 min), solubilized in 2% (w/v) sulphobetaine 14 and centrifuged for 10 min at 120000 g to remove debris. The supernatants were then incubated for 2 h at 22 °C with 20 mg of protein A-Sephrose/ml. The protein A-Sephrose was then spun down, washed three times with PBS containing 0.05% (v/v) Tween 20, once with RIPEA, once more with PBS/Tween, and twice with 10 mM-Tris/HCl buffer, pH 7.5. Samples were then prepared for electrophoresis as described for the immunoadsorbent.

Other assays

ATP was measured by using the firefly luminescence assay (Addanki *et al.*, 1966). Deoxyribonuclease I was measured by the hyperchromicity assay (Blickstad *et al.*, 1978). The effect of 5'-nucleotidase on actin was determined by a modification of the method of Rohr & Mannherz (1979). Actin (50 $\mu\text{g}/\text{ml}$) was incubated with or without 5'-nucleotidase, in 100 μl of buffer (50 mM-Tris/HCl buffer, pH 7.5, containing 1 mM- CaCl_2 , 1 mM- MgSO_4 and 0.05% sulphobetaine 14). After incubation, 10 μl of 20 $\mu\text{g}/\text{ml}$ deoxyribonuclease I was added, and 50 μl of the resultant mixture was assayed for deoxyribonuclease I activity.

RESULTS

Cytoplasmic domain

Methods for demonstrating a cytoplasmic domain in an integral membrane protein have been discussed previously (Etemadi, 1980; Warren 1981); short of sequencing the entire molecule, there are two commonly used approaches, either labelling proteins or proteolytic cleavage of proteins, on one side of the membrane or the other. Both approaches have been used in the present study of 5'-nucleotidase.

Isolated hepatocytes (viability > 98%) were surface-labelled with ^{125}I and 5'-nucleotidase was isolated by immunoadsorption (Fig. 1). The iodination was performed at 0 °C in order to maintain the viability of the hepatocytes in the absence of bovine serum albumin, and for this reason iodogen was used rather than lactoperoxidase to catalyse the reaction. Under these conditions, some iodine penetrates the cells, but this was largely (88%) soluble in ice-cold trichloroacetic acid (10% (w/v)), and the specific radioactivity of iodine in proteins from the high-speed supernatant (crude cytoplasmic fraction) was less than 1/600th that of the membrane 5'-nucleotidase.

Cell-surface-labelled 5'-nucleotidase was compared with labelled purified 5'-nucleotidase, in which all iodination sites are accessible, by comparing the one-dimensional peptide maps produced by cleavage with V8 proteinase. Cleavage maps were prepared for labelled

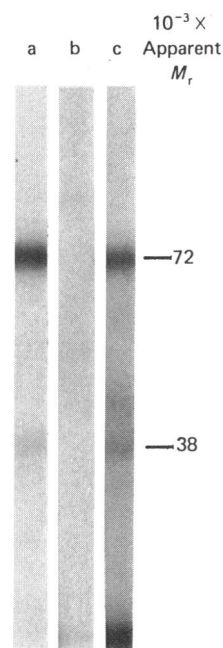


Fig. 1. 5'-Nucleotidase from surface-labelled hepatocytes

Solubilized membranes from ^{125}I -labelled cells were extracted with anti-5'-nucleotidase monoclonal antibody (lane a) or control IgG (lane b), and compared with ^{125}I -labelled purified 5'-nucleotidase (lane c) by polyacrylamide-gel electrophoresis as described in the Experimental section. Shown is the autoradiograph of the dried gel.

purified enzyme with the use of a range of proteinase concentrations (Fig. 2). In these gels, which contained a high concentration of acrylamide, the apparent M_r of the 5'-nucleotidase monomer (84000) was higher than that normally observed. This is probably due to anomalous migration of this glycoprotein, leading to a higher apparent M_r in the 17.5% acrylamide gel. Proteinase at 75 ng per track was used to produce maps of the two forms of labelled enzyme (Fig. 3). The two maps were identical, both in size of fragment produced and in relative amounts of label in each fragment, apart from small differences due to unavoidable interexperimental variability in the degree of proteolysis (compare Fig. 3 with Fig. 2, 75 ng track).

Proteolytic cleavage of the cytoplasmic domain of a membrane protein has been used to demonstrate transmembrane insertion (e.g. Owen *et al.*, 1980; Sutherland *et al.*, 1984; von Figura *et al.*, 1985). Treatment of newly synthesized microsomal proteins with proteinase K (Owen *et al.*, 1980) failed to cleave the 5'-nucleotidase (Fig. 4, lane a), indicating that the whole length of the protein was inaccessible in the vesicle and no cytoplasmic domain was present. The difference in M_r of the immature microsomal protein and the normal enzyme, about 5000, is probably due to the different amount and type of carbohydrate on the two forms of the enzyme.

When receptor for polymeric IgA was isolated after similar treatment with proteinase K, a decrease in M_r approx. 15000 was observed (Fig. 4), consistent with the cytoplasmic tail deduced from tryptic cleavage of membranes from whole rat livers labelled with cysteine

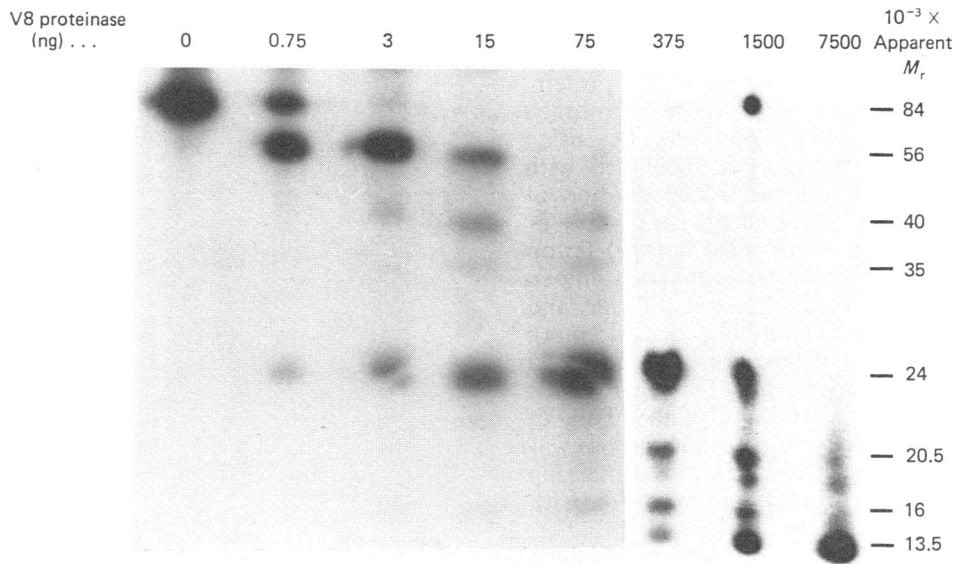


Fig. 2. V8 proteinase mapping of ^{125}I -labelled 5'-nucleotidase

^{125}I -labelled 5'-nucleotidase α -subunit was prepared by SDS/polyacrylamide-gel electrophoresis and maps were prepared as described in the Experimental section. Shown are the autoradiographs of two separate gels covering a range of proteinase concentrations.

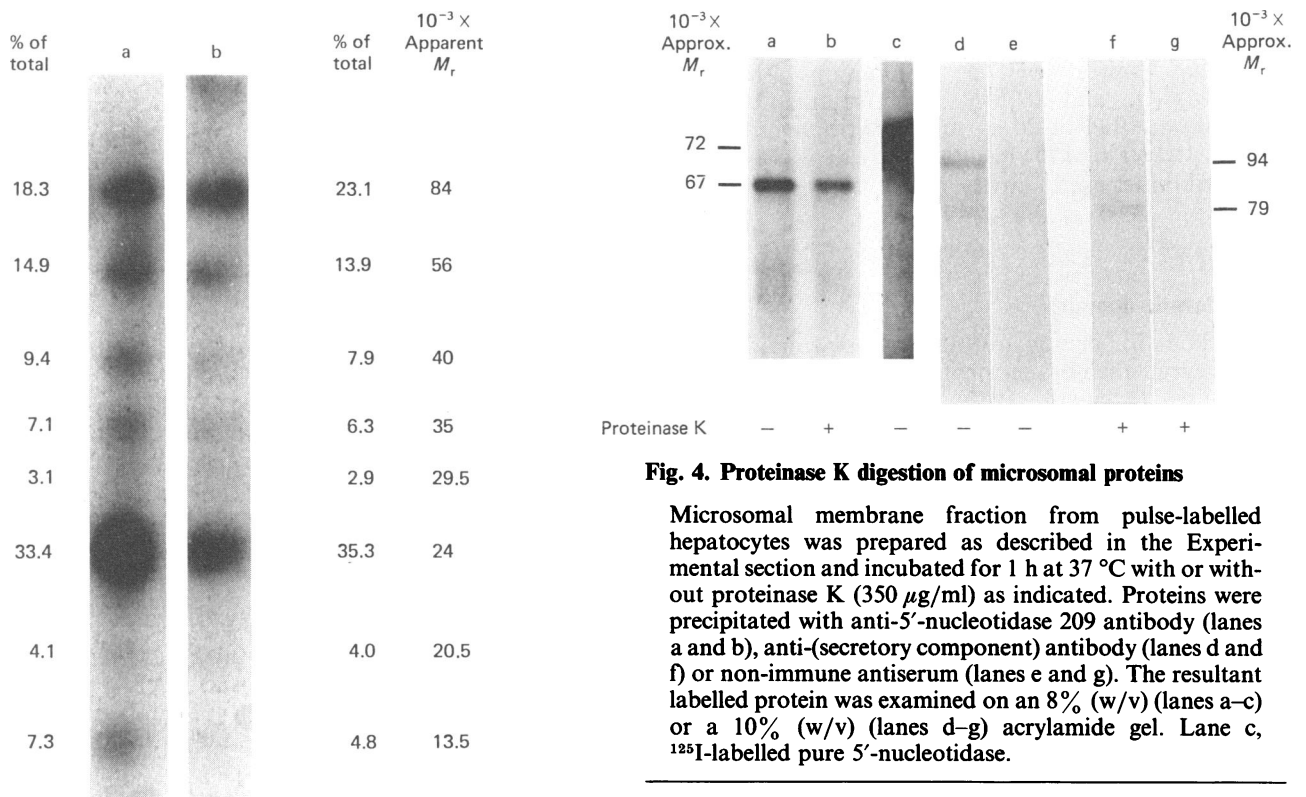


Fig. 3. Comparison of surface-labelled and labelled solubilized 5'-nucleotidase maps

5'-Nucleotidase, labelled after purification (lane a) or in intact cells (lane b), was prepared as indicated in Fig. 1 legend and excised from gels, and maps were prepared with 75 ng of V8 proteinase. The resulting autoradiogram was scanned and the amount of each fragment is expressed as a percentage of the total.

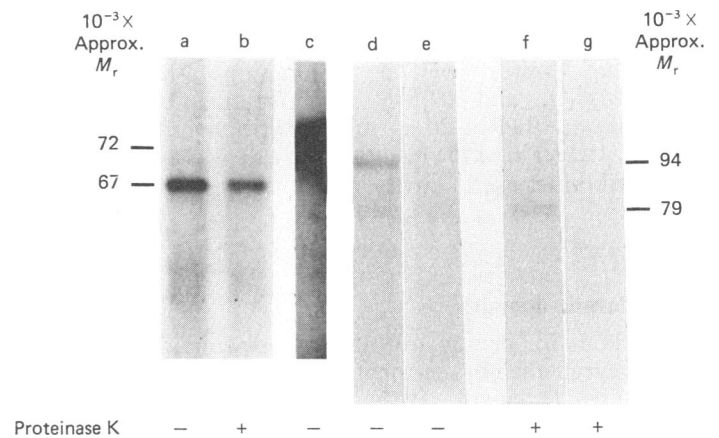


Fig. 4. Proteinase K digestion of microsomal proteins

Microsomal membrane fraction from pulse-labelled hepatocytes was prepared as described in the Experimental section and incubated for 1 h at 37 °C with or without proteinase K (350 $\mu\text{g}/\text{ml}$) as indicated. Proteins were precipitated with anti-5'-nucleotidase 209 antibody (lanes a and b), anti-(secretory component) antibody (lanes d and f) or non-immune antiserum (lanes e and g). The resultant labelled protein was examined on an 8% (w/v) (lanes a-c) or a 10% (w/v) (lanes d-g) acrylamide gel. Lane c, ^{125}I -labelled pure 5'-nucleotidase.

in vivo (Sztul *et al.*, 1985) and with the cytoplasmic tail of rabbit receptor for polymeric IgA deduced from the cDNA sequence (Mostov *et al.*, 1984).

Extracellular domain

Study of the cleavage maps (Fig. 2) of 5'-nucleotidase over a range of proteinase concentrations allows information to be derived about the order of cleavages and the relationship of the fragments to one another. From Fig.

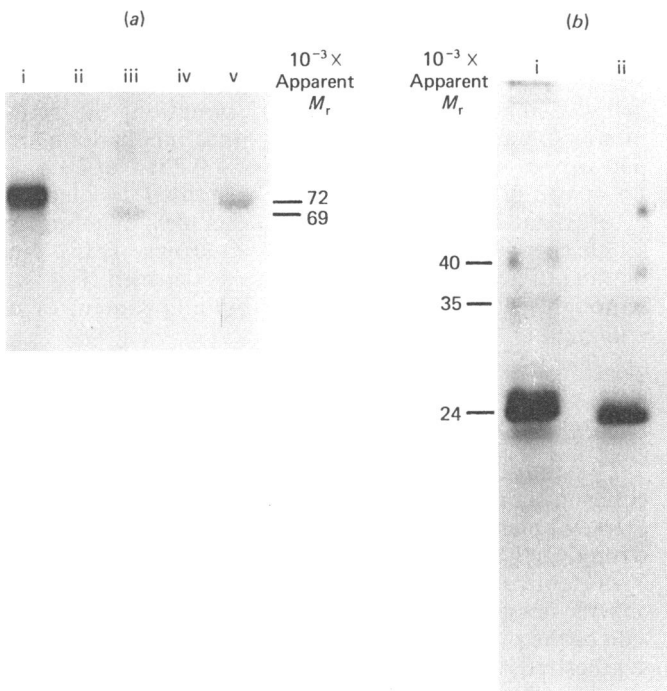


Fig. 5. Detection of carbohydrate in 5'-nucleotidase fragments

(a) Fluorograph of 2,5-diphenyloxazole-treated gel showing ^{125}I -labelled pure 5'-nucleotidase (lane i) and immunoprecipitates of hepatocytes ^3H -labelled on galactose residues (lanes ii and iii) or sialic acid residues (lanes iv and v). Solubilized membranes were extracted with anti-5'-nucleotidase monoclonal antibody (lanes iii and v) or control IgG (lanes ii and iv). (b) V8 proteinase maps of sialic acid-labelled (lane i) and galactose-labelled (lane ii) 5'-nucleotidase treated with 75 ng of V8 proteinase

2, plus other similar studies, it could be seen that the first cleavage produced a fragment of apparent M_r 56000 containing all the radioactive label. This radiolabelled 56000- M_r peptide was then cleaved to give 35000- M_r and 41000- M_r fragments, both of which were further cleaved to give a 24000- M_r peptide, followed by a mixture of 20500- M_r , 16000- M_r and 13500- M_r peptides. It is apparent that most of the radiolabel was found in one section of the protein, the smallest length resolved here being of M_r 13500, which was part of the 24000- M_r and 56000- M_r peptides. This phenomenon was not due to the iodination site in this region being relatively more reactive than others, as iodination of enzyme to specific radioactivities between 0.1 and 3 mol of ^{125}I /mol of enzyme produced the same pattern of label incorporation into each fragment (results not shown). We deduce, therefore, that this relatively short region of the molecule contains several accessible iodination sites.

Since 5'-nucleotidase is a glycoprotein, we have also established which of the V8-proteinase-cleavage fragments contained carbohydrate. Freshly isolated hepatocytes were labelled on either sialic acid or galactose residues, immuno-purified with solid-phase monoclonal antibody (Fig. 5a) and subjected to V8 proteinase cleavage as for the ^{125}I -labelled protein (Fig. 5b). Sialic acid removal (galactose-labelled protein) caused a decrease in apparent M_r of approx. 3000 (Fig. 5a), accounting for 60% of the difference in molecular mass of mature and immature forms of the enzyme (Fig. 4). The V8-proteinase-cleavage map showed that carbohydrate is found mostly in the same 24000- M_r fragment as the iodination sites.

Interaction with actin

It has previously been shown (Mannherz & Rohr, 1978) that 5'-nucleotidase prevents the inhibition of

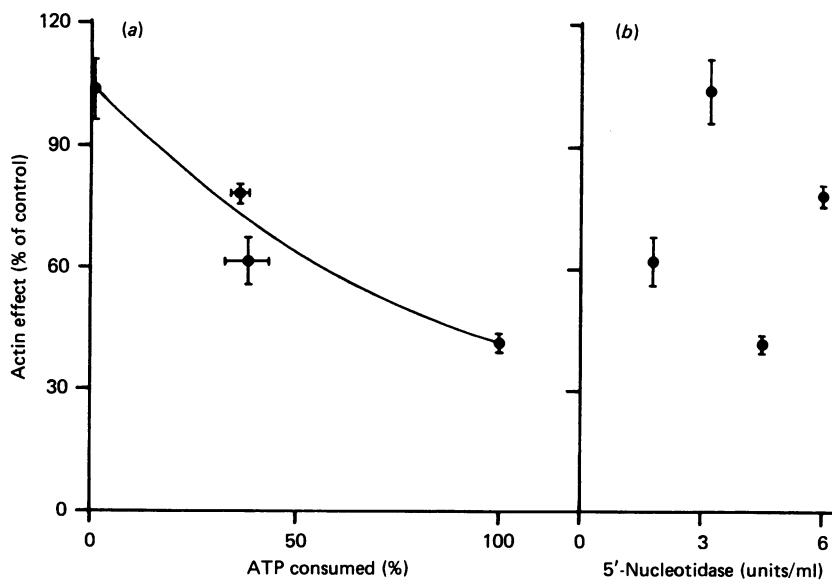


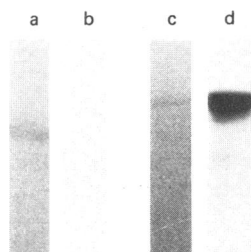
Fig. 6. Association between ATPase and actin suppression

Actin (0.05 mg/ml) was incubated alone or with four different preparations of 5'-nucleotidase for 30 min at 37 °C. The ability of the actin to inhibit deoxyribonuclease I and the amount of ATP remaining after incubation were determined, and actin activity (percentage of control) was plotted against percentage of ATP consumed (a) or 5'-nucleotidase activity (b). Actin alone (control) inhibited 35–40% of deoxyribonuclease I activity in different experiments. Bars show standard errors of the means for three experiments.

Table 1. Inhibition of 5'-nucleotidase by actin

5'-Nucleotidase activity was measured in 1.2×10^6 isolated rat hepatocytes (viability > 92%) or in purified enzyme (2 munits) in the presence or in the absence of actin in the monomeric (G) or the polymerized (F) state. Actin was dialysed against 50 mM-Tris/HCl buffer, pH 7.5, containing 0.1 mM-CaCl₂ and 50 μ M-ATP, with (F) or without (G) 100 mM-KCl and 0.2 mM-MgCl₂. 5'-Nucleotidase was determined spectrophotometrically (Ipata, 1967) in 50 mM-Tris/HCl buffer, pH 7.5, containing 20 mM- β -glycerophosphate, plus 0.1 mM-CaCl₂ and 0.25 M-sucrose (G) or 2 mM-MgSO₄ and 150 mM-NaCl (F). Results are expressed as percentages of the activity measured in the presence of an equal volume of dialysis buffer.

	Activity (% of control)	
	Purified enzyme	Whole cells
G-actin		
2 μ M	76.1	81.0
5 μ M	72.0	67.7
F-actin		
2 μ M	97.3	86.0
5 μ M	103.0	80.8

**Fig. 7. Actin overlay of 5'-nucleotidase**

Actin binding of 5'-nucleotidase (0.5 μ g) (lanes a and b) or gelsolin (0.3 μ g) (lanes c and d) was determined by the actin overlay technique (Snabes *et al.*, 1981). Lanes a and c, Coomassie Blue-stained gel; lanes b and d, autoradiographs of lanes a and c after incubation with ¹²⁵I-actin.

deoxyribonuclease I by actin, and it was suggested that this indicated direct interaction of the membrane enzyme with the cytoskeleton. We found that some preparations of purified rat liver 5'-nucleotidase did prevent deoxyribonuclease inhibition by actin, but that this effect correlated with a contaminating (approx. 0.0002%) ATPase activity in these preparations, rather than with 5'-nucleotidase activity (Fig. 6). In addition, no effect of 5'-nucleotidase on actin inhibition of deoxyribonuclease was found in the presence of 200 μ M-ATP (results not shown). F-actin has been reported to inhibit chick gizzard 5'-nucleotidase (Dieckhoff & Mannherz, 1985). Rat liver 5'-nucleotidase was only inhibited by G-actin (Table 1), and this inhibition was also elicited in intact cells, showing that it was not the result of an interaction at the cytoplasmic face of the plasma membrane. Actin overlay (Snabes *et al.*, 1981) is a highly sensitive method for the detection of actin-binding proteins, in which proteins are

subjected to SDS/polyacrylamide-gel electrophoresis, allowed to renature in the gel, and incubated with ¹²⁵I-actin. Although the concentration of actin used is below that required for filament formation, the actin monomers are in an F-like state, since incubations are performed in the presence of 0.2 M-NaCl and 1.0 mM-Mg²⁺; this method has been used to identify cytoskeletal attachment sites in other cell membranes (Schleicher *et al.*, 1984; Stratford & Brown, 1985). No binding of actin to 5'-nucleotidase was detected (Fig. 7), although gelsolin, a known actin-binding protein (Yin *et al.*, 1981), was labelled normally.

DISCUSSION

The study of the differential labelling of 5'-nucleotidase, either at all accessible sites (soluble enzyme) or at extracytoplasmic sites (cell-surface enzyme), though strongly suggesting the absence of a cytoplasmic end to 5'-nucleotidase, allows us to conclude only that the enzyme possesses no iodinated sites on the cytoplasmic side of the plasma membrane. The insensitivity of newly synthesized enzyme, the extracytoplasmic portion of which is protected by being inside the microsomal membrane, to proteinase K strongly suggests that there is no significant (i.e. no more than four or five amino acid residues) cytoplasmic domain in 5'-nucleotidase. We estimate that loss of more than five amino acid residues would give rise to an observable decrease in M_r in the gels used.

The major biochemical evidence in favour of a cytoplasmic domain is the putative interaction with the cytoskeleton. Such an interaction would be important in providing an attachment point for the cytoskeleton in the membrane, and a means of moving 5'-nucleotidase-containing vesicles through the cell. It has been shown that the effect of cytoskeletal drugs on inhibition of 5'-nucleotidase by concanavalin A (Carraway *et al.*, 1979) is not due to a direct interaction of enzyme and cytoskeleton (Craik *et al.*, 1985). We have found that the effects of 5'-nucleotidase on actin inhibition of deoxyribonuclease are due to contaminating ATPases, which has also been shown for chick gizzard 5'-nucleotidase (Dieckhoff & Mannherz, 1985). The ability of actin to inhibit deoxyribonuclease I is lost on incubation at 37 °C in the absence of ATP (results not shown), an effect that appears to be speeded up by the presence of ATPases (Gruda *et al.*, 1983) and to be related to loss of actin-bound nucleotide (Therien & Gruda, 1983; Dieckhoff & Mannherz, 1985). The interaction with F-actin proposed for this chick gizzard 5'-nucleotidase, associated with enzyme inhibition, does not occur with pure rat liver 5'-nucleotidase. This may be due to basic differences in these two enzymes, as antibodies against chick gizzard enzyme label only the endothelial cells in rat liver and not hepatocytes (Heidemann *et al.*, 1985), suggesting an absence of common antigenic determinants. The inhibition of 5'-nucleotidase activity caused by G-actin also occurs with enzyme located on the outside of intact cells, indicating that it is a direct effect on the extracytoplasmic portion of the enzyme, and not a normal physiological effect. There is therefore neither structural nor biochemical evidence for a cytoplasmic domain in the liver 5'-nucleotidase, although final confirmation will depend on cloning and sequencing the protein.

Short cytoplasmic domains are not unknown in plasma-membrane-located proteins [e.g. interleukin-2 receptor, 11 amino acid residues (Cosman *et al.*, 1984), and Sindbis-virion coat protein E1, two amino acid residues (Rice & Strauss, 1981)]. If the cell depends on the cytoplasmic domain of a protein to determine its location, then the signal must be very tightly encoded. However, evidence from hybrid proteins suggests that the cytoplasmic domain may not be, itself, sufficient for sorting. Alterations to the cytoplasmic domain of vesicular-stomatitis-virus G protein can prevent or inhibit its passage through the Golgi apparatus to the cell surface (Rose & Bergmann, 1983). However, exchange of its extracellular domain for the sequence of a secreted protein, growth hormone, also prevents passage of the protein beyond the Golgi apparatus (Guan & Rose, 1984), and *N*-linked glycosylation appears to be essential for correct insertion at the cell surface (Guan *et al.*, 1985). Information concerned with sorting 5'-nucleotidase in the liver cell may therefore exist in its extracytoplasmic domains.

5'-Nucleotidase is an integral membrane protein in that it normally requires detergent to be solubilized. Attempts to release the enzyme from membranes by proteolysis gave low yields (Bailyes, 1982), indicating that 5'-nucleotidase belongs to the group of short-stalked ectoenzymes identified by Kenny *et al.* (1983). However, release of the active enzyme has been achieved with phosphatidylinositol-specific phospholipase C (Low & Finean, 1978; Shukla *et al.*, 1980), suggesting an important role for phosphatidylinositol in anchoring the protein in the membrane. It is not yet clear whether the protein is itself inserted into the membrane, with a very small extension into the cytoplasm, similar to several microvillar peptidases (Kenny & Maroux, 1982; Kenny *et al.*, 1983), or the enzyme is anchored by a non-protein addition to one end, as has been suggested for the Thy-1 antigen (Williams & Gagnon, 1982; Low & Kincade, 1985).

The structure of the extracellular domain has also shown several interesting features, notably the presence of one section containing both the glycan chains and most of the iodinated sites in the molecule. This section may be that most exposed at the surface of the cell when the protein is inserted in the membrane. Since concanavalin A is a potent inhibitor of 5'-nucleotidase (Riordan & Slavik, 1974), it is probable that this section of the molecule also contains the active site. Since the molecule normally occurs as a non-covalently linked homodimer (Bailyes *et al.*, 1984), another part of the surface of the molecule must form the inter-subunit binding region. The α -subunit therefore contains at least three domains: the membrane anchor plus short stalk, the inter-subunit binding region, and the exposed surface, containing attached carbohydrate, most of the accessible iodination sites and the active site. This last domain is formed by a single section of the polypeptide chain, with an M_r of approx. 24000.

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