A MONOCLONAL ANTIBODY INHIBITING RAT LIVER 5′-NUCLEOTIDASE

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1. Introduction

5′-Nucleotidase (EC 3.1.3.5) is an intrinsic glycoprotein of the plasma membrane. Inhibitory antisera have been used in defining it as an ectoenzyme [1], to investigate its movement during endocytosis and membrane re-cycling [2], and to assess its physiological function [3]. The enzyme has been extensively purified [4,5], but not to homogeneity. Antisera raised to 5′-nucleotidase preparations therefore contain antibodies to other proteins and this has hindered their use for immunological localisation and other studies. We have prepared a monoclonal antibody which avoids such difficulties, and used this in the characterization of highly purified, solubilized 5′-nucleotidase. The results indicate that this antibody should be of value in purifying the enzyme further, and in studying its subcellular distribution and circulation.

2. Experimental

2.1. *Preparation of monoclonal antibody*

Male Balb/c mice were immunized with partially purified rat liver 5′-nucleotidase (step 8 from [4], spec. act. 30 units/mg; 8 μg protein subcutaneously in Freund's adjuvant, followed after 4 months by 15 μg protein intravenously in phosphate-buffered saline). Spleen cells were fused with myeloma cells (P3-NSI/1-Ag4-1) by using polyethylene glycol [6–8]. Culture supernatants were tested initially for binding to partially purified liver plasma membranes [8]. Those identified as positive in this assay were subsequently tested for binding activity with ^{125}I-labelled 5′-nucleotidase, and for inhibition of enzyme activity. Only one cell line was positive in all these assays (coded 5N 4-2) and this was grown to high density and used for the production of tumours in mice.

Abbreviations: SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis, SB14, sulphobetaine 14

2.2. *Preparation of immunoadsorbents*

Antibody 5N 4-2 was purified from ascites tumour fluid by precipitation with 40% saturated $(NH_4)_2SO_4$ and DEAE-cellulose chromatography [9]. The antibody was coupled to diazocellulose [10] to produce an immunoadsorbent containing 290 μg protein/mg cellulose.

Sheep anti-rabbit IgG (SαR) and sheep anti-mouse IgG (SαM) sera were obtained following immunization with 1 mg IgG (Koch-Light or Miles) in Freund's adjuvant at monthly intervals. Immunoglobulin fractions of these sera were obtained by Na_2SO_4 precipitation [9] and coupled to diazocellulose [10].

Rabbit anti-(rat liver 5′-nucleotidase) serum was as in [2]. An adsorbent containing this antibody was prepared by incubating 10 mg SαR adsorbent with 0.1 ml rabbit antiserum for 2 days at 4°C.

All adsorbents were stored in 50 mM veronal buffer (pH 8) containing 1 mg bovine serum albumin/ml [10], at 4°C, and washed in this buffer before use.

2.3. *5′-Nucleotidase preparations and assay*

Rat isolated hepatocytes [11], plasma membranes [12,13] and purified 5′-nucleotidase [4,5] were prepared as described.

5′-Nucleotidase activity was measured by radioassay [1,2], except in the experiment to define the type of inhibition of enzyme by monoclonal antibody. In this experiment spectrophotometric assay [14] at different substrate concentrations was used.

2.4. *Preparation and use of iodinated 5′-nucleotidase*

The purest available enzyme (spec. act. ~200 units/

mg, [5]) was used for iodination by the Iodogen method [15]. Protein (3–6 μg) in 0.1 ml 100 mM Tris–HCl buffer (pH 7.5) containing 2 mM $MgCl_2$ and 0.1% SB14 (Calbiochem) [5], was reacted with 0.5–1 mCi $Na^{125}I$ (Radiochemical Centre, Amersham) for 2–20 min in the presence of 10 μg Iodogen (Pierce Chemical Co.). Iodinated protein was separated from unreacted iodide on a small column of Sephadex G-25 (Pharmacia). The final specific activity was 0.02–0.15 mCi/μg.

As a screening test, 10 μl cell culture medium was incubated with ^{125}I-5′-nucleotidase (10^5 cpm) in 100 μl 25 mM veronal buffer (pH 8) containing 0.1% SB14, 1 mM $MgCl_2$ and 0.5 mg bovine serum albumin/ml. After 1 h, 0.5 mg SαM immunoadsorbent was added in the same buffer and the incubation was continued for a further 1 h. The immunoadsorbent was then centrifuged, washed twice with veronal buffer and counted for radioactivity bound.

Binding of iodinated enzyme to immunoadsorbents containing mouse monoclonal antibody or rabbit antiserum was performed using adsorbent (0.01–1 mg) and iodinated enzyme (10^6 cpm) in 200 μl veronal buffer as above. After 1 h the immunoadsorbent was centrifuged and washed twice in veronal buffer and then once in 100 mM Tris–HCl (pH 7.5).

2.5. *Electrophoresis*

Reduced and alkylated [16] protein samples were analysed by SDS–PAGE [17] on slab gels containing 10% (w/v) acrylamide with 0.27% (w/v) *N,N′*-methylenebisacrylamide. Gels were stained for protein [18] and scanned on a densitometer. Each track was then cut into 2 mm slices for radioactive counting.

3. Results

3.1. *Interaction of antibody with cell membranes and purified 5′-nucleotidase*

The initial screening assays of one cell fusion identified 9 sub-cultures which contained antibody binding to rat liver plasma membranes. These culture fluids were further tested for their ability to bind to radioiodinated, purified, solubilized 5′-nucleotidase, and for their ability to inhibit 5′-nucleotidase enzyme activity. Only one subculture (coded 5N 4-2) was positive in these assays and the antibody-secreting cells were subsequently cloned. The nature of the liver membrane antigens recognised by other subcultures is unknown, but these were presumably present as impurities in the enzyme preparation used for immunization. Antibody 5N 4-2 was shown to be an IgG_1 by the binding of labelled enzyme–monoclonal antibody complex to immunoadsorbents containing rabbit antibody specific for mouse heavy chain subclasses.

Antibody 5N 4-2 was shown to bind to isolated rat hepatocytes, but not to rat erythrocytes, in binding assays similar to that employing plasma membranes. The antibody in either tissue-culture medium or ascites tumour fluid inhibited 5′-nucleotidase activity in intact cells, plasma membrane preparations, or purified enzyme preparations (fig.1). The maximum inhibition was 40–60% in different experiments, and half-maximum inhibition of ~0.5 mU enzyme occurred at similar antibody concentrations in all cases (fig.1). The antibody had no effect on the activity of 5′-nucleotidase in homogenates of mouse or human liver.

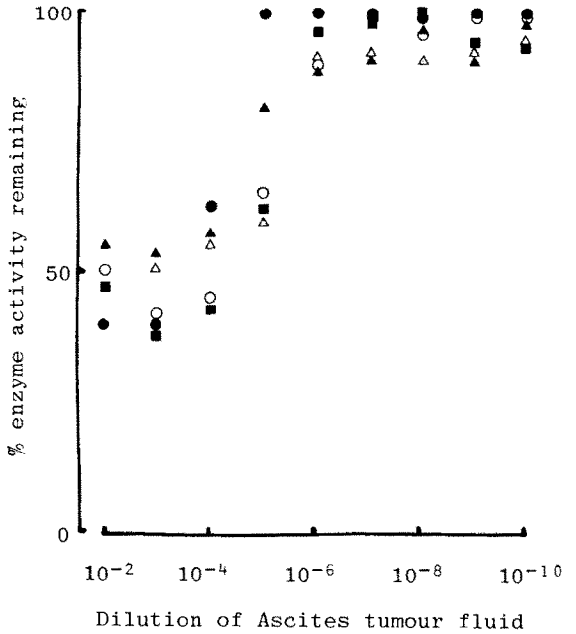

Fig.1. Inhibition of 5′-nucleotidase activity by monoclonal antibody. Portions of enzyme (50 μl) were incubated with diluted ascites tumour fluid (50 μl) for 1 h at 37°C before addition of radioassay cocktail for 30 min at 37°C [1]. Enzyme and tumour fluid were diluted with 50 mM Tris–HCl buffer (pH 8) containing 1 mg bovine serum albumin/ml. Hepatocyte cell surface activity [11] (●, 0.38 mU enzyme/tube); plasma membrane [12] (▲, 0.69 mU); plasma membrane [13] (■, 0.50 mU); purified enzyme [4] (○, 0.47 mU); highly purified enzyme [5] (△, 0.61 mU). Each point represents the mean of 2 determinations.

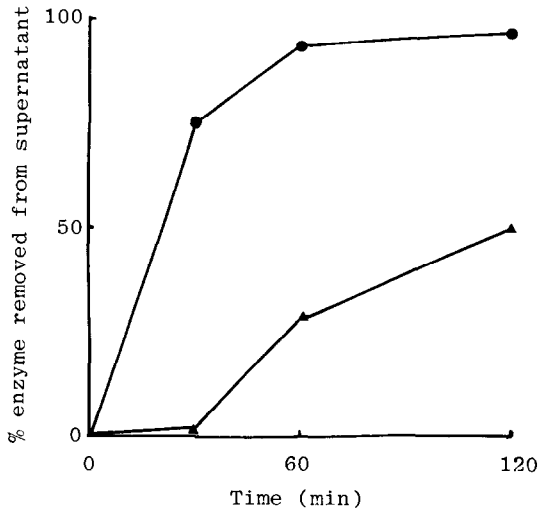

Fig.2. Binding of 5′-nucleotidase activity to monoclonal antibody immunoadsorbent. Portions (500 μl, 8.7 mU) of purified solubilized enzyme [5] were incubated with immunoadsorbent at 20°C. They were centrifuged 8500 × *g* for 2 min at the times shown and 50 μl portions of the supernatant assayed for remaining 5′-nucleotidase activity. Each point represents the mean of 2 determinations. (●) 0.01 mg immunoadsorbent; (▲) 0.001 mg immunoadsorbent. At the 60 min time point with 0.01 mg immunoadsorbent 50% of the initial enzyme activity was found in the immunoadsorbent pellet. When incubated with 0.1 mg immunoadsorbent <2% of the enzyme remained in the supernatant after 30 min at 20°C.

The inhibition of 5′-nucleotidase was further investigated by testing the effect of ascites tumour fluid on partially purified enzyme [4] at different substrate concentrations. The partial inhibition was shown to be due to a decrease in V_{max} of the enzyme, with no change in the K_m for AMP.

An immunoadsorbent containing antibody 5N 4-2 bound essentially all the enzyme activity from a purified, solubilized preparation of 5′-nucleotidase (fig.2). Enzyme activity was detected in the immunoadsorbent pellet, the amount of activity measured being ~50% of that initially present before binding to antibody.

3.2. *Binding of iodinated 5′-nucleotidase to mouse monoclonal antibody and rabbit antiserum*

The purest available preparation of 5′-nucleotidase was labelled with radioactive iodine to a specific activity corresponding to 0.6–4.7 mol I/70 000 g protein (5′-nucleotidase being M_r ~70 000 on SDS–PAGE). The radioactivity in such preparations was at least 90% precipitable by trichloroacetic acid (10% w/v)

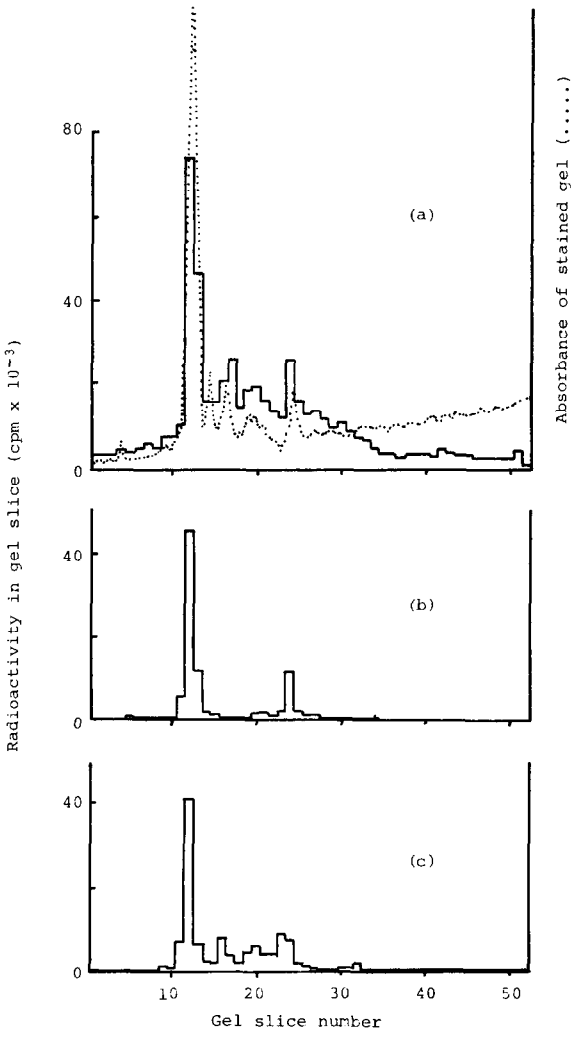

Fig.3. Binding of iodinated 5′-nucleotidase to antibody. Purified, solubilized enzyme [5] was radioiodinated to spec. act. 0.04 mCi/μg, and 1270 × 10^3 cpm was incubated with 0.1 mg of 5N 4-2 adsorbent or 0.5 mg rabbit antibody adsorbent. After washing, the radioactivity bound to the adsorbents was 148 × 10^3 cpm (5N 4-2) or 220 × 10^3 cpm (rabbit antiserum). Corresponding non-immune mouse IgG or rabbit serum adsorbents non-specifically bound 6 × 10^3 cpm and 18 × 10^3 cpm, respectively, under the same conditions. The material bound to immunoadsorbents, and the equivalent amount of labelled enzyme, was applied to polyacrylamide slab gels under denaturing conditions. After fixation and staining, each track was cut into 2 mm slices, which were counted for radioactivity. The counts recovered in each track were: 543 × 10^3 cpm for the enzyme preparation (a); 100 × 10^3 cpm for 5N 4-2 adsorbent pellet (b), 142 × 10^3 cpm for rabbit antiserum adsorbent pellet (c). Also shown (a) is a densitometer scan of the stained gel for a track containing 24 μg trichloroacetic acid-precipitated purified enzyme.

and 70% insoluble in chloroform/methanol (2:1, v/v) [18]. The iodinated preparations retained >90% of the original enzyme activity, and all this activity bound to 5N 4-2-immunoadsorbent.

The maximum specific binding of iodinated enzyme to immunoadsorbents containing monoclonal antibody 5N 4-2 or rabbit antiserum was 10–12% or 17–19%, respectively (8 expt) of the total radioactivity. This was not changed by using longer incubation times, or greater amounts of immunoadsorbents, or by varying the specific radioactivity of the enzyme preparation over an 8-fold range.

The distribution of radioactivity in the labelled enzyme preparation and in the material bound to immunoadsorbents was investigated by using SDS–PAGE. Native enzyme stained for protein showed one major band (app. M_r ~70 000), and 4 minor bands (M_r ~61 000, 55 000, 47 000 and 38 000) (fig.3a). Radioactivity was incorporated into each of these bands and to a lesser extent was found in other regions of the gel without visible protein staining (fig.3a). The major protein band contained 24% of the radioactivity recovered in the gel track, but ~60% of the protein staining material. Monoclonal antibody 5N 4-2 significantly bound only 70 000 and 38 000 M_r material, of which the former accounted for 63% of the recovered radioactivity in the gel track (fig.3b). In contrast, rabbit antiserum bound material in each of the discrete bands of the enzyme preparation, of which the main component was again at 70 000 M_r, accounting for 42% of the recovered radioactivity in the gel track (fig.3c).

The washed immunoadsorbent bound 11.7% and 17.3% of the total radioactivity, for 5N 4-2 and rabbit antiserum, respectively, in the experiment of fig.3. These values correspond to ~17% and 25%, respectively, of the iodinated protein, assuming this to be represented by that 70% of the total radioactivity which is insoluble in chloroform/methanol. The SDS–PAGE profiles show that only 63% and 42% of the adsorbent-bound radioactivity, for 5N 4-2 and rabbit antiserum respectively, migrates with the major protein peak, which is assumed to be 5′-nucleotidase. It therefore appears from the binding and gel data for both types of antibody that 5′-nucleotidase constitutes a maximum of 11% of the iodinated protein in the enzyme preparation.

4. Discussion

Antisera to rat liver 5′-nucleotidase, raised in rabbits, have been useful reagents in studying the properties and function of the enzyme in rat cells [1–3]. However, these antisera are limited in their application because, having been raised against impure enzyme preparations, they react with several different protein components in addition to 5′-nucleotidase, (fig.3). We have now obtained from a mouse myeloma hybrid a monoclonal antibody (IgG_1 subclass) to 5′-nucleotidase. This antibody binds to and partially inhibits the enzyme at all stages of purity from intact cells to ~7000-fold purified (fig.1,2). The antibody does not inhibit the enzyme from mouse or human liver and does not bind to rat erythrocytes which contain no 5′-nucleotidase on their cell surface.

The maximum inhibition of rat 5′-nucleotidase by monoclonal antibody was ~50% (fig.1) and reflected a decrease in V_{max} of the enzyme. In contrast, rabbit antisera inhibit the rat enzyme almost completely [2]. There are however many precedents for partial inhibition of enzyme activity by antibodies [20]. The fact that 5′-nucleotidase binds completely to antibody-containing immunoadsorbent (fig.2) suggests that the partial inhibition is not accounted for by heterogeneity of the enzyme such that only a subfraction interacts with antibody.

The best available preparation of 5′-nucleotidase, purified 7000-fold, was thought to be at least 50% pure [5], as judged from the profile of protein staining after SDS–PAGE. However, the binding of a fully active iodinated enzyme preparation to antibody immunoadsorbents indicated a radiochemical purity of ≤11% (fig.3). It is not certain that either protein staining or radioactive incorporation will be a direct measure of protein mass for the different components of the enzyme preparation. The calculation of radiochemical purity is also subject to some uncertainty because of the incomplete recovery of total and adsorbent-bound radioactivity after SDS–PAGE (fig.3). The discrepancy may be due to several factors, including losses during sample preparation and transfer, deiodination of protein by dithiothreitol [21] and removal of material such as free iodide or iodinated phospholipid [22] during fixation and staining of gels.

Rabbit antiserum bound all the main components of the iodinated enzyme preparation (fig.3), confirming the presence of multiple antibodies to impurities as well as to 5′-nucleotidase itself. Surprisingly, mono-

clonal antibody 5N 4-2 also bound two components from the labelled enzyme preparation. The nature of the minor band bound to 5N 4-2 (M_r ~38 000) is unknown. This could be an impurity which happens to cross-react with the antibody or a fragment of the 5′-nucleotidase molecule which contains the appropriate antigenic determinant. Alternatively, this might represent a subunit or other tightly associated protein complexed with 5′-nucleotidase throughout purification and binding to immunoadsorbents, and only separated by treatment with SDS and dithiothreitol.

In conclusion, the results presented illustrate that monoclonal antibodies of mouse origin may be raised against specific components of rat cell membranes, and used in the characterization of such components during purification. The antibody to 5′-nucleotidase should be of value for analysing the subcellular distribution of the enzyme and its circulation through the cell [2]. Antibody-containing immunoadsorbents should be useful in the further purification of 5′-nucleotidase, and in the isolation of membrane subfractions containing the enzyme [10,23].

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