Structural differences between plasma-membrane 5'-nucleotidase in different cell types as evidenced by antibodies

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Antibodies raised against bovine 5'-nucleotidase inhibit this enzyme as well as 5'-nucleotidase from other bovine tissues, showing common structure(s) between these proteins. However, an IgG fraction directed against the glucidic moiety of the liver enzyme did not cross-react with the enzyme from lymphocyte or caudate nuclei, a clear indication that within the same species the 5'-nucleotidase differs from one cell type to another. In addition, immunoblots after electrophoresis show that the previous antibodies recognize 5'-nucleotidase from human, mouse or chicken origin. However, only human 5'-nucleotidase activity can be inhibited by the antibodies. Thus at least three groups of antigenic determinants must exist on the 5'-nucleotidase: one related to the glucidic moiety of the glycoprotein whose binding inhibits the enzyme activity, another related to the catalytic site, as its binding also led to enzyme inhibition, and a last one of structural nature. It seems that the third group of determinant is common to many species, whereas the second one is more restricted.

INTRODUCTION

5'-Nucleotidase is an integral membrane glycoprotein found in a variety of mammalian cells (Hayman & Crumpton, 1972; De Pierre & Karnovsky, 1974). The enzyme spans the membrane bilayer (Zachowski *et al.*, 1981a), and the catalytic site is generally located at the external face of the plasma membrane (Gurd & Evans, 1974; Trams & Lauter, 1974; Stanley *et al.*, 1980). Numerous studies have shown that this enzyme is probably implicated in a variety of biological processes, such as vasodilatation (Berne & Rubio, 1979), cellular proliferation (Carson & Seegmiller, 1976) and immuno-suppression (Fisher *et al.*, 1976).

We have previously demonstrated a heterogeneity in the glucidic moiety of the 5'-nucleotidase from several bovine cell types: whereas the glycosylation of lymphocyte enzyme is of a high-mannose type (Meflah *et al.*, 1984a), that of caudate nuclei is complex (Meflah *et al.*, 1984b). Furthermore, the interactions of the liver plasma-membrane 5'-nucleotidase with lectins indicate a probable hybrid-type glycosylation (Harb *et al.*, 1983).

In the present study anti-5'-nucleotidase antibodies were used to compare the structure of enzyme in plasma membranes isolated from several tissues. We have confirmed that the glucidic moiety of the enzyme differs from one cell to another, as it represents a major antigenic determinant. Furthermore an interspecies cross-reactivity clearly indicates that some structural domains remained unchanged between non-related species.

EXPERIMENTAL

Materials

Ultrogel AcA-22 and *Staphylococcus aureus* Protein A were purchased from IBF Pharmindustrie (Villeneuve-La Garenne, France). *Staphylococcus aureus* Protein A-Sephrose 4B was obtained from Pharmacia (Uppsala,

Sweden). Goat anti-(rabbit IgG) antibody conjugated with peroxidase was obtained from Nordic Immunology (Tilburg, The Netherlands).

Preparation of antibodies

A 10 µg portion of purified enzyme (Harb *et al.*, 1983) was directly immobilized on 1 ml of Ultrogel AcA-22 previously activated with glutaraldehyde by the procedure of Ternynck & Avrameas (1972). Rabbits were injected and 'boosted' three times over a 6-week period with this immobilized enzyme in an aluminium hydroxide (10 mg/ml)/magnesite (10 mg/ml) suspension. The blood was collected from the ear marginal vein. The IgG fraction was purified by affinity chromatography on a Protein A-Sephrose 4B column: after elution by 0.2 M-glycine/HCl buffer, pH 2.8, the IgG preparation was quickly neutralized and dialysed for 24 h against 110 mM-NaCl/40 mM-Tris/HCl buffer, pH 7.4. Three different IgG fractions were isolated by using 0.1 M-sodium citrate buffer at pH 5, 4 and 3 (Ey *et al.*, 1978). An antibody fraction against 5'-nucleotidase was purified on enzyme linked to Ultrogel AcA-22.

Purification of plasma membranes

Plasma membranes were purified as described by Emmelot *et al.* (1964) for bovine, mouse and human liver, by Jones & Matus (1974) for bovine caudate nuclei, by Kidwai *et al.* (1973) for bovine skeletal muscle and by Ferber *et al.* (1972) for bovine and human lymphocytes.

Assays

For the immune inhibition assays, the plasma membranes (2 mg/ml) were solubilized by 0.4% Sulphobetaine 14 as described by Harb *et al.* (1983) and preincubated for 1 h, in the absence or the presence of sugars, with different concentrations of antibodies at 37 °C in 0.4 ml of 110 mM-NaCl/40 mM-Tris/HCl buffer, pH 7.4 (buffer A). The 5'-nucleotidase activity was then

determined by the method of Zachowski *et al.* (1981b). The proteins were assayed as described by Lowry *et al.* (1951), with bovine serum albumin as standard.

Immunoblotting

Plasma-membrane proteins were submitted to an electrophoresis on polyacrylamide gel in presence of sodium dodecyl sulphate (Laemmli, 1970) and then transferred on to nitrocellulose sheets (Towbin *et al.*, 1979). After transfer, the sheets were incubated with buffer B (buffer A supplemented with 3% bovine serum albumin and 0.2% Tween 20) for 1 h at 40 °C and overnight at 4 °C. Each sheet (four bands) was then gently shaken for 16 h at room temperature with 1 mg of IgG dissolved in 75 ml of buffer B. After being washed with the same buffer without bovine serum albumin, the nitrocellulose sheets were incubated with goat anti-(rabbit IgG) antibody conjugated with peroxidase (1/500) for 1 h at room temperature. After being washed five times with buffer A containing 0.2% Tween 20, the immune complexes were located by incubating the sheets with 0.1% 3,3'-diaminobenzidine plus 0.01% H_2O_2 in 50 mM-Tris/HCl buffer, pH 7.6.

Ultracentrifugation

Density-gradient ultracentrifugation [5–20% (w/v) sucrose, containing 0.2% Sulphobetaine 14] was performed at 40000 rev./min (280000 *g* at r_{av} , 10.9 cm) for 20 h at 4 °C in a Beckman SW 41 rotor.

Determination of the inhibition constant

A total 5'-nucleotidase activity of 400 nmol/h was incubated with various amounts of antibodies: 10 to 20 μ g of IgG purified on a Protein A-Sepharose column or 1 to 2 μ g of IgG purified on a 5'-nucleotidase-Ultrogel AcA-22 column. For each condition, the initial velocity of 5'-AMP hydrolysis was determined in a substrate range of 10–1000 μ M. Subsequently, data were plotted in accordance with Lineweaver & Burk (1934) (i.e. a double-reciprocal plot of velocity versus substrate concentration), which allows the determination of the inhibition constant (K_i) and of the nature of the inhibition. We estimated M_r about 150000 for the IgG. The values are means $\pm$ S.E.M.

RESULTS AND DISCUSSION

Production and specificity of the antibodies against 5'-nucleotidase

After immobilization of the 5'-nucleotidase, the catalytic activity was recovered in the gel and no activity was detected in the supernatant, indicating a total binding of the enzyme. The enzyme activity was not altered by storage of the gel during 6 weeks at 4 °C. The immunization of three rabbits by 10 μ g of immobilized enzyme was effective for the production of antibodies against 5'-nucleotidase. The 5'-nucleotidase activity of the different bovine plasma membranes was strongly inhibited by the antiserum produced or the immunoglobulin fraction purified on a Protein A-Sepharose column. In contrast, no inhibition was observed with the non-immune rabbit serum (control), or with the immunoglobulin fraction that was not retained on the Protein A-Sepharose column. As expected, no immunoreactivity was observed with other plasma-membrane

enzymes: alkaline phosphatase or $Na^+ + K^+$ -dependent ATPase. Moreover, gel-diffusion (Ouchterlony, 1958) or immunoelectrophoresis (Laurell, 1966) techniques did not allow us to observe a precipitation line between antibodies and 5'-nucleotidase from different cell types or purified enzyme.

Characterization of the immune complex

Antibodies produced against 5'-nucleotidase were often characterized by their inhibitory power of the 5'-nucleotidase activity (Riemer & Widnell, 1975; Fisher *et al.*, 1976; Zachowski *et al.*, 1981a). In addition, polyclonal or/and monoclonal antibodies have been used to study the structure of rat liver 5'-nucleotidase (Bailey *et al.*, 1984) and its subcellular localization (Stanley *et al.*, 1983; Matsuura *et al.*, 1984). In order to determine the nature of the immune complex, we have examined the sedimentation profile of the purified 5'-nucleotidase preincubated with antibodies.

The sedimentation coefficient of the purified 5'-nucleotidase is about 7 S. When the enzyme was incubated with IgG, we observed an inhibition of the activity peak and heavy 5'-nucleotidase peaks appeared, indicating the presence of immune complexes at 11.3 S and 14.3 S. Moreover, the addition of Protein A precipitates these immune complexes, thus proving that the enzyme and the antibodies produce soluble immune complexes. As the localization of the enzyme on the gradient was determined by its hydrolytic activity, it happens that immune complexes can occur without totally masking the enzymic activity, although completely inhibited complexes can exist. However, in this last case, our method does not allow us to reveal these structures.

Reactivity of 5'-nucleotidase from different bovine cell types with antibodies

Preincubation of the solubilized bovine plasma membranes (liver, lymphocytes, caudate nuclei) with antibodies produced an inhibition of 5'-nucleotidase activity (Fig. 1). A systematic study of the influence of various amounts of antibodies on the enzyme initial velocity in the presence of different AMP concentrations was performed (see the Experimental section). The Lineweaver-Burk plots show that the inhibition is of a non-competitive nature. It has been noted that the inhibition constants were lower for caudate nucleus ($0.18 \pm 0.04 \mu$ M, $n = 3$) and lymphocyte membranes ($0.40 \pm 0.02 \mu$ M, $n = 3$) than for hepatocyte membranes ($0.57 \pm 0.03 \mu$ M, $n = 3$). The IgG also inhibited the 5'-nucleotidase from bovine skeletal-muscle membranes. The fact that the specific activity of this enzyme was much lower than that observed in the other bovine tissues prevented us from further characterizing this reaction. Moreover, the column of immobilized 5'-nucleotidase enables us to obtain a highly purified IgG fraction against 5'-nucleotidase. The K_i values of this IgG fraction for the enzyme from different bovine tissues are roughly 8–10-fold lower than those reported above.

When plasma-membrane proteins were electrophoresed in the presence of sodium dodecyl sulphate and transferred on to nitrocellulose, the incubation of the sheets with antibodies revealed a unique band at M_r 70000 corresponding to the subunit of the 5'-nucleotidase (Harb *et al.*, 1983) (Fig. 2a). The fact that the antibodies react specifically with the 5'-nucleotidase from all bovine

Fig. 1. Effect of IgG on 5'-nucleotidase activity

Fractions of the different solubilized membranes such as to ensure the same 5'-nucleotidase activity (400 nmol/h) were incubated with different concentrations of IgG, as described in the Experimental section. Each point represents the mean of triplicate assays. The random error was about $\pm 2\%$. ▲, Liver; □, lymphocytes; ●, caudate nuclei.

Fig. 2. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and immunoblotting of 5'-nucleotidase from different plasma membranes with antibodies

Portions (50 μg) of bovine plasma membranes (a) or other species (b) were electrophoresed, transferred on to nitrocellulose and incubated with IgG as described in the Experimental section. Track 1, bovine liver; track 2, bovine lymphocytes; track 3, bovine caudate nuclei; track 4, bovine skeletal muscle; track 5, mouse liver; track 6, chicken liver; track 7, human liver; track 8, human lymphocytes. Arrowheads indicate the positions of M_r standards: ferritin (220000); catalase (60000); lactate dehydrogenase (36000); ferritin (18500).

cell types implies the existence of a high degree of similitude between the structural antigenic determinants of these enzymes.

Effect of sugars on the immune inhibition assay

The different plasma membranes were preincubated with a concentration of antibodies that inhibited 75–80% of the 5'-nucleotidase activity. In a first set of experiments, we added to these assays a mixture of sugars (*N*-acetyl-D-glucosamine, α -L-fucose, D-galactose, methyl α -D-mannoside and β -*N*-acetylneuraminic acid). The sugars were chosen assuming that the glycan borne by 5'-nucleotidase is of hybrid nature (Harb *et al.*, 1983). This mixture (50 mM of each sugar) decreased by about $30 \pm 2\%$ (mean $\pm$ S.E.M., $n = 5$) the IgG inhibitory power against the liver membrane 5'-nucleotidase. This phenomenon was not observed with the lymphocytes or the caudate-nuclei enzyme.

In a second set of experiments, we tested the effect of each of the monosaccharides on the inhibition assay. It was clearly apparent (Table 1) that only β -*N*-acetylneuraminic acid partially restores the liver 5'-nucleotidase activity, suggesting that an IgG fraction can specifically recognize the glucidic moiety of the liver enzyme.

The IgG fractions eluted at pH 5, 4 and 3 from the Protein A–Sephadex column represented respectively 12, 76 and 12% of the bound IgG. The inhibition of the enzyme activity by the IgG eluted at pH 5 and 4 was partially reversed in the presence of β -*N*-acetylneuraminic acid (Table 1). However, when the enzyme was inhibited by the IgG fraction eluted at pH 3, not only β -*N*-acetylneuraminic acid, but also D-galactose and α -L-fucose, partially restored the enzyme activity. Thus, although all IgG fractions recognized the glucidic moiety of the liver 5'-nucleotidase, the IgG fraction eluted at pH 3 seems to have a wider spectrum of reactivity towards the glycan. Moreover, when these experiments were performed with lymphocytes or caudate-nuclei enzyme, no effect of the different sugars was detected on the 5'-nucleotidase inhibition by these IgG fractions.

Antigenic cross-reactivity of 5'-nucleotidase from different species

The antibodies against bovine liver 5'-nucleotidase recognize the enzyme from human, chicken and mouse,

Table 1. Effect of sugars on the liver plasma-membrane 5'-nucleotidase inhibition by IgG eluted at different pH values

Sugar	Inhibition (%)			
	Whole IgG	IgG (pH 5)	IgG (pH 4)	IgG (pH 3)
None	70 $\pm$ 3	80 $\pm$ 2	80 $\pm$ 2	67 $\pm$ 3
<i>N</i> -Acetylneuraminic acid	46 $\pm$ 2	56 $\pm$ 1	55 $\pm$ 1	33 $\pm$ 1
Methyl α -D-mannoside	70 $\pm$ 1	80 $\pm$ 2	78 $\pm$ 2	61 $\pm$ 2
D-Galactose	70 $\pm$ 2	80 $\pm$ 1	76 $\pm$ 3	50 $\pm$ 1
α -L-Fucose	70 $\pm$ 2	80 $\pm$ 1	80 $\pm$ 2	46 $\pm$ 2
<i>N</i> -Acetyl-D-glucosamine	70 $\pm$ 1	80 $\pm$ 2	77 $\pm$ 3	63 $\pm$ 2

Solubilized liver plasma membranes were preincubated at 37 °C with 75 μg of each of different IgG fractions in the absence or the presence of different sugars (20 mM) in 0.4 ml of buffer A. After 1 h, the enzymic assays were started by adding 1 mM-AMP. Controls (plasma membrane + sugar) were performed under the same conditions. Values are means $\pm$ S.E.M. ($n = 3$).

Table 2. Effect of IgG on 5'-nucleotidase from different species

Plasma membranes were incubated at 37 °C in 0.4 ml of buffer A with different IgG concentrations. After 1 h, the enzymic reactions were started by 1 mM-AMP addition. The values represent the inhibitions as percentages of the controls without IgG. The control values were 244 nmol/h for human liver, 172 nmol/h for human lymphocytes, 336 nmol/h for mouse liver and 50 nmol/h for chicken liver. Values are means $\pm$ S.E.M. ($n = 2$).

Source	IgG ...	Inhibition (%)		
		35 μ g	70 μ g	350 μ g
Human liver		24 $\pm$ 2	48 $\pm$ 3	78 $\pm$ 2
Human lymphocyte		20 $\pm$ 2	35 $\pm$ 2	66 $\pm$ 3
Mouse liver		0	0	0
Chicken liver		0	0	0

as shown by immunodetection (Fig. 2b): in fact, as with the bovine enzyme, we detected a simple band corresponding to the subunit of the 5'-nucleotidase (Harb *et al.*, 1983). However, only the human enzyme is inhibited by these antibodies (Table 2). These results indicate that the antigenic determinants near the catalytic site are probably different in chicken and mouse enzymes, in spite of the cross-reactivity observed by immunodetection. It is obvious that the different 5'-nucleotidases exhibit some differences in their structure.

CONCLUSION

In the present study, we have shown a cross-reactivity between the 5'-nucleotidase from different bovine tissues. This implies the existence of a high degree of structural similitude among these different molecules. However, our results show that the carbohydrate microheterogeneity of the 5'-nucleotidase from different tissues (Harb *et al.*, 1983; Meflah *et al.*, 1984a,b) seems to be involved in the glycoprotein immunogenicity: in fact, we observed that an IgG fraction recognized only the hybrid-type glycan but not the complex or the simple one.

Alhadeff & Andrew-Smith (1984) suggested that β -N-acetylneuraminic acid is implicated in the antigenic determinant of α -L-fucosidase. Although we observe that this sugar is implicated in the antigenic recognition of the liver 5'-nucleotidase, it clearly appears that other sugars may be competitors for the immune-inhibition reaction in the presence of the IgG fraction eluted at pH 3. Moreover, it seems that, in spite of the presence of these monosaccharides in the glycan of caudate-nuclei or liver 5'-nucleotidases, the recognition of these glycans by the antibodies is not the same, as the binding to liver 5'-nucleotidase can be reversed by addition of sugars, this addition being ineffective on the caudate-nuclei assay. These observations suggest that the spatial conformation of the glycan is probably implicated in the antigenic recognition. Moreover, the enzyme structure can be divided into at least three domains: one (the glycan) that might be tissue-specific within a given species, a second one (non-glucidic in nature) that might be common to

several species and that is related to the catalytic activity, and a third one shared by many species but without link to the catalytic site.

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