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PURIFICATION OF 5'-NUCLEOTIDASE FROM HUMAN PLACENTA AFTER RELEASE FROM PLASMA MEMBRANES BY PHOSPHATIDYLINOSITOL-SPECIFIC PHOSPHOLIPASE C

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SUMMARY: 5'-Nucleotidase was purified >1000-fold from human placenta by treatment of plasma membranes with S. aureus phosphatidylinositol-specific phospholipase C and affinity chromatography on Con A Sepharose and AMP-Sepharose. The resulting enzyme had a specific activity of >5000 μmol/hr/mg protein and a subunit molecular weight of 73,000. Goat antibodies against 5'-nucleotidase inhibited enzyme activity and detected 5'-nucleotidase after Western blotting. These antibodies also recognized a soluble form of 5'-nucleotidase and residual membrane-bound 5'-nucleotidase which could not be released by phosphatidylinositol-specific phospholipase C treatment, suggesting that the three forms of the enzyme are structurally related. The soluble 5'-nucleotidase may be derived from the membrane-bound form by the action of an endogenous phospholipase C. The structural basis for the inability of some of the membrane-bound 5'-nucleotidase to be released by phosphatidylinositol-specific phospholipase C is unknown.

5'-Nucleotidase (E.C.3.1.3.5) catalyzes the dephosphorylation of purine and pyrimidine ribo- and deoxyribonucleoside monophosphates to their corresponding ribo- and deoxyribonucleosides (1). This enzyme has been used as a plasma membrane marker for many years (2) and was first thought to be a conventional integral membrane protein since it could only be solubilized by treatment of membranes with a variety of ionic and non-ionic detergents including Triton X-100 and sodium deoxycholate. However, in 1978, it was reported that 5'-nucleotidase could be released from the plasma membranes of several tissues by treatment with purified phosphatidylinositol (PI)-specific phospholipase C (3), suggesting that PI was involved in the attachment of 5'-nucleotidase to the membrane. Thus, 5'-nucleotidase is a member of a diverse group of proteins which can be released from membranes by treatment with PI-specific phospholipase C (4). In some cases inositol has been found to be covalently attached to the

ABBREVIATIONS: PI, phosphatidylinositol; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonylfluoride; Con A, concanavalin A; SDS PAGE, polyacrylamide gel electrophoresis with sodium dodecyl sulfate; BSA, bovine serum albumin.

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released protein, suggesting that the mode of membrane attachment is by covalent linkage to PI. This group of proteins includes alkaline phosphatase (3), acetylcholinesterase (5,6), murine Thy-1 (7,8), the T cell-activating protein (9), the rat alloantigen RT 6.2 (10), decay accelerating factor (11), the variant surface glycoprotein from Trypanosoma brucei (12), and the predominant surface glycoprotein, p63, of Leishmania (13).

Although the precise physiologic function of 5'-nucleotidase is unknown, this enzyme is of particular interest because it is a maturation marker for human T and B lymphocytes (14,15) and its expression is reduced in lymphocytes from patients with a variety of immunodeficiencies (16-20). The development of antibodies specific for 5'-nucleotidase to allow studies of surface expression of the enzyme by immunofluorescence during normal lymphocyte ontogeny and in patients with immunodeficiency diseases has been hampered by the difficulty in isolating sufficiently pure enzyme from lymphocytes. Therefore, we decided to purify the enzyme from a tissue high in 5'-nucleotidase activity (human placenta) and to take advantage of the relatively selective release of 5'-nucleotidase from the plasma membrane by PI-specific phospholipase C. We used our purified placental 5'-nucleotidase to produce a goat antiserum which cross reacts with lymphocyte 5'-nucleotidase and is suitable for immunofluorescence (manuscript in preparation).

METHODS

Purification of 5'-nucleotidase from human placenta

Fresh whole placenta was washed with cold PBS to remove as much blood as possible and then homogenized in 50 g aliquots for 2 min at 4°C in a Waring blender in 80 ml of 0.01 M Tris, pH 7.4, 250 mM sucrose, 1 mg iodoacetamide/ml, 1.0 mM benzamidine, 0.25% ε-aminocaproic acid, and 10 µg phenylmethylsulfonylfluoride (PMSF)/ml. All additional procedures were carried out at 4°C unless otherwise specified. The homogenate was centrifuged at 3000 rpm in a Sorvall RT6000 for 20 min to remove fibrous material and the supernatant crude extract was concentrated 5- to 10-fold by vacuum dialysis. Aliquots of the concentrated crude extract were layered over 3.5 ml cushions of 38% sucrose in 0.01 M Tris, pH 7.4, 1.0 mM $MgCl_2$, and 1.0 mM $CaCl_2$ and centrifuged for 2 hr at 32,500 rpm in a SW41 rotor. The plasma membrane bands were dialyzed against 0.01 M Tris, pH 7.4 and then centrifuged at 100,000 x g for 1.5 hr. The resulting 100,000 x g pellet was treated with proteinase K at 200 µg/ml for 60 min at room temperature to release alkaline phosphatase from the membranes since it would otherwise later be released by PI-specific phospholipase C and contaminate the 5'-nucleotidase. The reaction was terminated with 2.0 mM PMSF. The treated membranes were centrifuged at 100,000 x g for 1.5 hr. The pellet was treated with S. aureus PI-specific phospholipase C at 3 µg/ml for 45 min at 37°C. After centifugation at 100,000 x g for 1 hr, the majority of 5'-nucleotidase was found in the supernatant. 5'-nucleotidase was further purified by Con A Sepharose affinity chromatography and elution with 0.3 M α-methyl mannoside in 0.05 M Tris, pH 7.4, 1.0 M NaCl, 1.0 mM $MgCl_2$, and 1.0 mM $CaCl_2$. Finally, the dialyzed Con A Sepharose eluate was passed over an AMP Sepharose column (in the presence of 0.02 M β-glycerophosphate to prevent the binding of residual alkaline phosphatase) and 5'-nucleotidase activity was eluted with 25 mM AMP in

0.01 M Tris, pH 7.4. 5'-nucleotidase activity was determined by measuring the conversion of [^{14}C] IMP to [^{14}C] inosine as previously described (17).

Production of 5'-nucleotidase antiserum in a goat

A goat was immunized with purified 5'-nucleotidase (pooled AMP-S eluates from four placentas) in complete Freund's adjuvant at 0, 3, and 6 weeks (doses: 100, 100, and 150 μg). By the seventh week, the antiserum inhibited 5'-nucleotidase activity at dilutions >1:10,000. The antiserum was absorbed on insolubilized normal human serum proteins and on a Sepharose 4B column to which 20 mg of human placental proteins depleted of 5'-nucleotidase (Con A Sepharose and AMP-Sepharose effluents) had been coupled.

SDS polyacrylamide gel electrophoresis and Western blotting

Aliquots of fractions from the purification of placental 5'-nucleotidase were subjected to SDS PAGE under reducing conditions (21). The protein bands were visualized with either Coomassie blue or silver staining. From other gels, the separated proteins were transferred to nitrocellulose by Western blotting (22). After blocking with 1% BSA, 5'nucleotidase was localized by incubation with absorbed 7 wk antiserum (1:1000) followed by [^{125}I] rabbit anti-goat IgG and autoradiography.

RESULTS AND DISCUSSION

Purification of 5'-nucleotidase from human placenta. In preliminary experiments, aliquots of placental plasma membranes were treated with increasing concentrations of S. aureus PI-specific phospholipase C for 45 minutes at 37°C. The plasma membranes were then centrifuged for 1 hr at 100,000 x g and the amount of 5'-nucleotidase activity released into the supernatant was determined. The release of 5'-nucleotidase from plasma membranes was dose dependent and reached a maximum at approximately 1 μg/ml (Figure 1). The percentage of 5'-

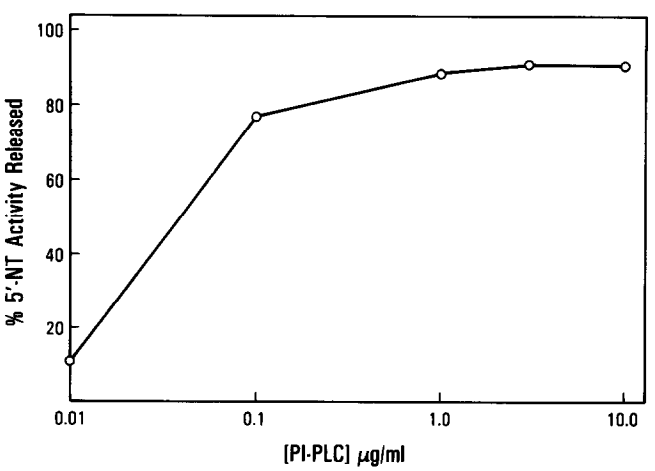

Figure 1. Release of 5'-nucleotidase from plasma membranes by treatment with PI-specific phospholipase C. Aliquots of placental plasma membranes were treated with the indicated concentrations of PI-specific phospholipase C for 45 min at 37°C. The membranes were then centrifuged for 1 hr at 100,000 x g and the amount of 5'-nucleotidase activity released into the supernatant was determined.

Table 1. Purification of 5'-Nucleotidase from Human Placenta

FRACTION	TOTAL ACTIVITY (μmoles/hr)	μMOL/HR/MG	% RECOVERY	FOLD PURIFICATION
Crude extract	27,000	3.85	100	1.0
Plasma membranes	12,200	20.3	44	5.3
100,000 x g ppt.	9,550	29.9	35	7.8
Proteinase K ppt.	7,540	46.0	27	12
PI-PLC supn't.	6,060	338	22	88
Con A-S eluate	4,210	2,480	15	640
AMP-S eluate	2,990	5,250	11	1360

nucleotidase activity released by PI-specific phospholipase C treatment varied from placenta to placenta and ranged from 70-90%. For large scale purifications, PI-specific phospholipase C was used at 3 μg/ml.

5'-nucleotidase was purified from human placenta as outlined in METHODS. Typical results from a single placenta are shown in Table 1. The average recovery was approximately 200 μg (10%) with >1000-fold purification. The most significant loss of activity occurred during the isolation of plasma membranes since about 25% of the total 5'-nucleotidase activity in the crude extract is present in a non-membrane bound form. Aliquots of each fraction were subjected to SDS PAGE in order to assess the purity. The final AMP-S eluate gives a single major band of molecular weight 73,000 upon staining with either Coomassie blue or silver (Figure 2). The degree of purification achieved by this method

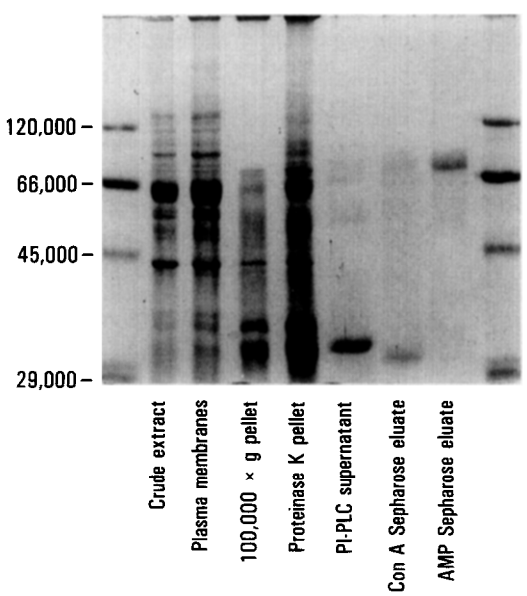

Figure 2. SDS PAGE of fractions from the purification of placental 5'-nucleotidase. Aliquots of fractions from the purification of placental 5'-nucleotidase were subjected to SDS PAGE under reducing conditions. The protein bands were stained with Coomassie blue.

Table 2. Inhibition of 5'-Nucleotidase Activity by Absorbed 7-week Antiserum

ENZYME SOURCE	UNITS OF ENZYME ACTIVITY[a] ENZYME ALONE	+ ANTISERUM	% INHIBITION
Placenta			
Membranes treated with PI-specific phospholipase C	3.85	0.30	92
Detergent treated membranes (enzyme resistant to PI-specific phospholipase C)	1.03	0.13	87
Soluble fraction	1.86	0.23	86
Peripheral lymphocytes			
Human T cells	3.92	0.26	93
Human B cells	2.17	0.10	95

[a]One unit of enzyme activity catalyzes the hydrolysis of nmol IMP/hr.

and the molecular weight of the protein are similar to those obtained by other investigators (1,23). The release of 5'-nucleotidase from plasma membranes by PI-specific phospholipase C rather than by conventional detergent treatment offers several advantages. First, a seven-fold increase in specific activity is achieved since not many proteins are released by this treatment. Second, the enzyme is released in a completely water-soluble form, eliminating the need for detergents in the buffers during subsequent purification steps. That the released 5'-nucleotidase is water-soluble suggests that the hydrophobic domain responsible for anchoring the protein into the membrane (presumably 1,2-diacylglycerol) has been cleaved by the action of PI-specific phospholipase C.

Ability of polyclonal goat anti-5'-nucleotidase to recognize the three major forms of human placental 5'-nucleotidase and human lymphocyte ecto-5'-nucleotidase. The absorbed polyclonal anti-5'-nucleotidase antiserum was tested for the ability to recognize the three major forms of human placental 5'-nucleotidase. At a dilution of 1:100, the absorbed antiserum inhibited 5'-nucleotidase activity from membranes treated with PI-specific phospholipase C, detergent-solubilized membranes (i.e., activity resistant to PI-specific phospholipase C), and the soluble fraction to a similar degree (86-92%) (Table 2). AMP-Sepharose eluates from plasma membranes treated with PI-specific phospholipase C, detergent solubilized membranes, and the soluble fraction were subjected to SDS PAGE. The proteins were then transferred to nitrocellulose by Western blotting. The 5'-nucleotidase bands were localized with absorbed anti-5'-nucleotidase antiserum (at 1:1000) followed by [^{125}I] rabbit anti-goat IgG and autoradiography. The antiserum recognized 5'-nucleotidase from all three sources (Figure 3) and furthermore, the molecular weights from all three sources were similar.

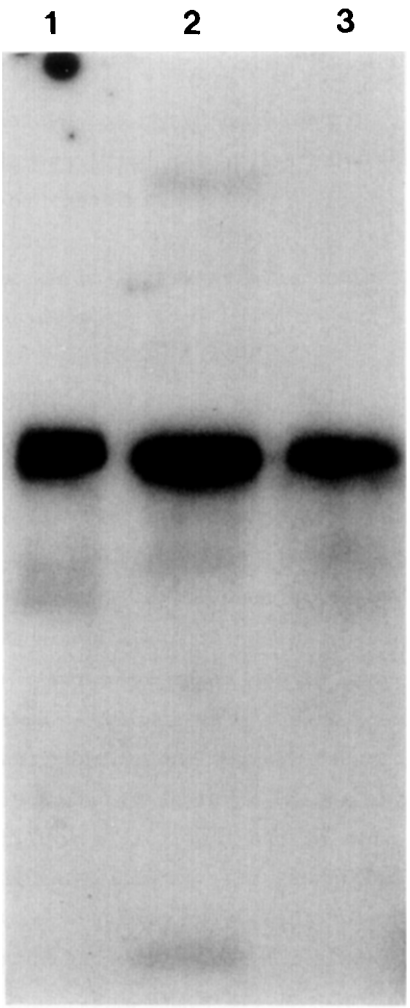

Figure 3. Detection of 5'-nucleotidase after SDS PAGE and Western blotting by goat antibodies. AMP-S eluates were prepared from plasma membranes solubilized with PI-specific phospholipase C (Lane 1), the soluble fraction (Lane 2), and PI-specific phospholipase C resistant enzyme solubilized with 1% Triton X-100 + 2% deoxycholate (Lane 3). The three preparations were subjected to SDS PAGE under reducing conditions and transferred to nitrocellulose by Western blotting. 5'-nucleotidase was localized as described in METHODS.

The absorbed anti-5'-nucleotidase antiserum also inhibited enzyme activity on purified human T and B lymphocytes (Table 2). Immunofluorescence studies using $F(ab')_2$ fragments of IgG isolated from the absorbed antiserum plus biotinylated $F(ab')_2$ rabbit anti-goat IgG and Fl-avidin provided additional evidence that the antiserum recognizes human lymphocyte ecto-5'-nucleotidase. The percentages of total T cells, T cell subsets, and total B cells showing specific immunofluorescence agreed well with previous results utilizing a histochemical stain to detect 5'-nucleotidase positive lymphocytes (15,24,25). Furthermore, when fluorescent positive and negative populations were prepared using the

fluorescence activated cell sorter, only the Fl^+ population had 5'-nucleotidase activity.

As demonstrated by both enzyme inhibition and Western blotting, antibodies against placental 5'-nucleotidase releasable by PI-specific phospholipase C also recognize soluble enzyme, as well as enzyme resistant to PI-specific phospholipase C treatment. These three forms of human placental 5'-nucleotidase appear to be structurally related and to have very similar molecular weights. It is not known why some proteins are anchored into the membrane via covalent linkage to PI, rather than by hydrophobic interactions between the lipid bilayer and hydrophobic amino acid side chains, but it has been speculated that the function of such proteins requires that they have the capacity to be released from the cell rapidly by the action of an endogenous phospholipase C (26). Thus, the soluble form of 5'-nucleotidase may be derived from the membrane-bound form by the action of a placental PI-specific phospholipase C or other enzyme that degrades this unique anchoring structure (27). Inositol analysis of cytoplasmic 5'-nucleotidase will be required to confirm this hypothesis. The structural basis for the inability of some of the membrane-bound 5'-nucleotidase to be released by PI-specific phospholipase C is unknown. However, forms of other PI-anchored proteins resistant to PI-specific phospholipase C have been observed (6,8,11). Some of the enzyme may be physically inaccessible to added PI-specific phospholipase C or perhaps some of the inositol is modified such that it is no longer a substrate for S. aureus enzyme. An understanding of the factors which control the release of 5'-nucleotidase from membranes may lead to further insight into the function of this enzyme and the regulation of its expression in lymphocytes.

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