Vol. 150, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1988 Pages 476-482

IDENTIFICATION OF AN ACID-LIPASE IN HUMAN SERUM WHICH IS CAPABLE OF SOLUBILIZING GLYCOPHOSPHATIDYLINOSITOL-ANCHORED PROTEINS

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Received December 7, 1987

SUMMARY: A lipase has been identified in human serum which can convert the membrane form of the variant surface glycoprotein of Trypanosoma brucei to a water soluble form. The conversion can be monitored by loss of [^{3}H] myristic acid incorporated into the diacylglycerol of the glycophosphatidylinositol membrane anchor of the protein, but does not lead to the exposure of the antigenic determinant in the polar head group of the glycolipid. The serum lipase is a glycoprotein, and is optimally active at pH 5.4. Treatment at 62° for one hour does not inactivate the enzyme, which is inhibited by chelating agents.

The variant surface glycoprotein (VSG) of the African trypanosome is the best characterized example of a class of membrane proteins anchored to the plasma membrane through a complex glycophosphatidylinositol covalently attached to the C-terminus of the protein (1,2). One characteristic of members of the family is that they can be solubilized, either directly from the membrane, or indirectly from detergent micelles, through the action of a subclass of phosphatidylinositol-specific phospholipase C, with enhanced specificity for the glycophosphatidylinositol substrate.

To date, three such enzymes have been characterized, from Trypanosoma brucei (3-5), Staphylococcus aureus (6), and rat liver plasma membranes (7). The normal role of these enzymes is not understood. It is assumed that VSG is the natural substrate of so-called VSG lipase of trypanosomes, and that the rat liver enzyme is responsible for the production from an insulin-sensitive glycophosphatidylinositol precursor of an inositolphosphate glycan which can act as an effector for some of the metabolic effects of insulin (8).

Here we report the presence in human serum of a new class of lipase which can solubilize VSG, as monitored by phase separation and by release of biosynthetically incorporated [^{3}H] myristate, but without exposure of the CRD. The enzyme has been partially purified and characterized.

0006-291X/88 $1.50

MATERIALS AND METHODS

A. Assay for VSG solubilization.

The assay is a modified form of the previously described phase separation assay (9). Membrane form VSG (mfVSG; 1.0 mg ml^{-1}), prepared as described (10), was biotinylated with biotinyl-ε-aminocaproic acid N-hydroxysuccinimide (Biotin-X-NHS Calbiochem; 0.1 mg ml^{-1}) for 2 hours in 0.1 M $NaHCO_3$, 0.05% n-octyl-β-D-glucopyranoside (nOG), pH 8.3. Following dialysis into 10 mM Tris.HCl, 150 mM NaCl (TBS), supplemented with 0.05% nOG, pH 7.4, biotinylated mfVSG (mfVSG-B; 100 µg) was complexed with [^{125}I] streptavidin (^{125}I-S, 5 µg; 100 mCi.μM^{-1}) by incubation for 30 minutes at room temperature. Following 3 cycles of phase separation (11) in 4% TX114 in TBS, the detergent phase containing pure complex (mfVSG-B-S) was diluted 50-fold with water to give a substrate preparation containing approximately 2000 cpm μl^{-1} in 0.2% TX114. In the assay, 10 µl of substrate and 10 µl of enzyme were mixed with 10 µl of buffer (150 mM). Following incubation for 30 min. at 30°C, reaction was terminated by the addition of 300 µl of ice-cold 2% TX114 in TBS.Phases were then immediately induced to separate by incubation for 5 minutes at 37°C, and the radioactivity counted in both phases. Maximally, 65% of total counts were found in the aqueous phase, with a background of about 8% in the absence of enzyme. The same maximum was observed with both VSG lipase and the human serum enzyme. The remaining 35% of counts are associated with a non-VSG contaminant (not shown).

B. Partial purification of the human enzyme

Normal human serum (NHS; 50 ml) was dialyzed at 4°C against 100 volumes of 10 mM sodium acetate, pH 5.4, and the precipitated euglobulins removed by centrifugation (10,000 g, 30 min., 4°C). The supernatant was dialyzed against 50 mM Tris HCl, pH 7.8, and was applied to a column (2.5 x 40 cm) of DEAE-Sephacel (Pharmacia). The column was eluted (20 $ml.h^{-1}$) with a 0-0.3 M gradient of NaCl in the same buffer. Fractions (10 ml) were assayed as described above. Active fractions were pooled, and submitted to precipitation with ammonium sulphate. Most of the activity (90%) was recovered in the range of 30%-75% of saturation. The proteins precipitated by 75% ammonium sulphate were resuspended in TBS (15 ml) containing 0.02% NaN_3, and were dialyzed overnight against the same buffer. A sample was eluted (10 $ml.h^{-1}$) from a column (2.5 x 70 cm) of Sephacryl S-300 (Pharmacia) equilibrated with the dialysis buffer. Fractions (5 ml) were collected and assayed. Fractions 40-44 were pooled, and a sample was applied to a column (1 x 10 cm) of wheat germ agglutinin - Sepharose 6MB (Pharmacia) equilibrated in 10 mM Tris.HCl, pH 7.4, 0.5 M NaCl, 0.02% NaN_3, 0.2 mM phenylmethylsulfonylfluoride. Bound glycoproteins were eluted by addition to the buffer of 100 mg.ml N-acetyl-O-glucosamine. Fractions (5 ml) were collected and assayed.

Analytical scale isoelectric focussing was performed to determine the pI of the enzyme. An aliquot (20 µl) of the activity pooled from the S300 column was mixed with 10% glycerol (50 µl) and pH 4-6 Ampholine (5 µl, LKB) prior to focussing for 1 h at 150 V, and overnight at 400 V, at 4°C, on a 7.5% acrylamide, 0.02% bisacrylamide slab gel, containing 3.5% (v/v) pH 3.5-10 Ampholine, plus 0.5% (v/v) pH 4-6 Ampholine. A group of pI standards from Pharmacia was focussed in parallel. Following focussing, the standards track was stained with Coomassie Blue, and the track to which active enzyme was applied was sliced into 0.5 cm slices. Each gel slice was placed in a 1.5 ml Eppendorf tube, rinsed twice with 50 mM Tris acetate, pH 5.4 (0.5 ml), and was then macerated in the same buffer (80 µl). Following incubation overnight with mfVSG-B-S (20 µl), an aliquot (50 µl) was removed and submitted to phase separation

C. Preparation of [^{3}H]myristate-labelled mfVSG.

Trypanosomes (1 x 10^8) of variant MITat 1.6, isolated from infected rat blood by passage over DEAE-cellulose, were preincubated in Dulbeccos Modified Eagles Medium (DMEM; 5 ml) containing fatty acid free bovine serum albumin

(BSA; Sigma) for 30 min. at 30°C. The trypanosomes were then incubated in 250 µCi of [9, 10(n)-^{3}H] myristic acid (50 Ci mmol^{-1}; Amersham U.K.) added in ethanol (20 µl). Cells were washed in DMEM (5 ml), resuspended in TBS (20µl) and 4% SDS (20 µl) at 100 °C was added. The sample was boiled for 1 min. to inactivate endogenous phospholipase. After cooling, 2.5% Triton X100 (120 µl) was added. A total of 4.9 x 10^6 cpm of TCA precipitable material was present in the final volume of 160 µl.

D. Effect of lipase on mfVSG.

Samples of MITat 1.6 mfVSG, and of MITat 1.6 [^{3}H] myristate mfVSG (9 µl of the above lysate, equal to 5 x 10^6 cells and equivalent to 5 µg mfVSG) each in a volume of 60 µl were incubated with 60 µl of active, or 60 µl heat inactivated (100°C, 2 min.) enzyme. The enzyme preparations were either VSG lipase, prepared as described (9), or the human serum lipase from the S300 column. After 2 h at 30°C, 2 M Tris . HCl, pH 7.4 (60µl) was added to each reaction. Samples were phase separated following the addition of ice-cold 2% Triton X114 in TBS, and aqueous and detergent phases equalized for volume and detergent concentration. The VSG was immunoprecipitated from each sample by incubation for 2 h on ice, firstly with affinity purified rabbit anti-M Tat 1.6 VSG (100 µg), and subsequently with a 10% suspension (200 µl) of fixed S. aureus (Cowans strain A). Adsorbed complexes were washed twice in 50 mM Tris . HCl, pH 8.0, 0.15 M NaCl, 2% Triton X100, once in TBS, and were eluted by boiling in 80 µl of SDS gel sample buffer containing 10% (v/v) 2-mercaptoethanol. Samples were resolved on 10% polyacrylamide gels containing 4 M urea (12), and were visualized either by autoradiography or, following transfer to nitrocellulose (13), by staining with 0.1% Ponceau red, and by blotting with affinity purified anti-CRD (12), using [^{125}I] protein A for detection of bound antibody.

RESULTS AND DISCUSSION

Figure 1A shows clearly that conversion by the enzyme in NHS is pH-dependent, with an optimum at around pH 5.4. This pH was optimal for more than 10 samples of NHS tested (not shown). It should be noted that the enzyme is still significantly active at physiological pH in a serum sample diluted 1 to 80. Furthermore, the conventional procedure for heat inactivation of all sera (57°C, 20 min.) has no effect on the activity of the enzyme (Figure 1B). The thermal resistance of the enzyme is the same when tested at pH 7.0 and a pH 5.4 (data not shown).

Figure 2 summarizes the progress made in purifying the enzyme. The enzyme binds to DEAE cellulose at pH 7.8 (Figure 2A), consistent with the measured isoelectric point of approximately 4.7 (Figure 2D). Comparison with the elution profiles for serum albumin and IgG on gel filtration, suggests a solution molecular weight somewhere around 140 kD. The enzyme is clearly a glycoprotein, since it binds to wheat germ agglutinin-Sepharose (Figure 2C). The enzyme has not been purified to homogeneity, since the most pure preparation still shows 7-10 bands on a stained SDS gel.

Figure 3 shows a clear difference between VSG lipase and the nature of the enzyme activity in human serum. VSG lipase is a phospholipase C, and its effect can be seen in three ways. Firstly, there is a difference in the mobility of substrate and product on SDS polyacrylamide gel electrophoresis, vis-

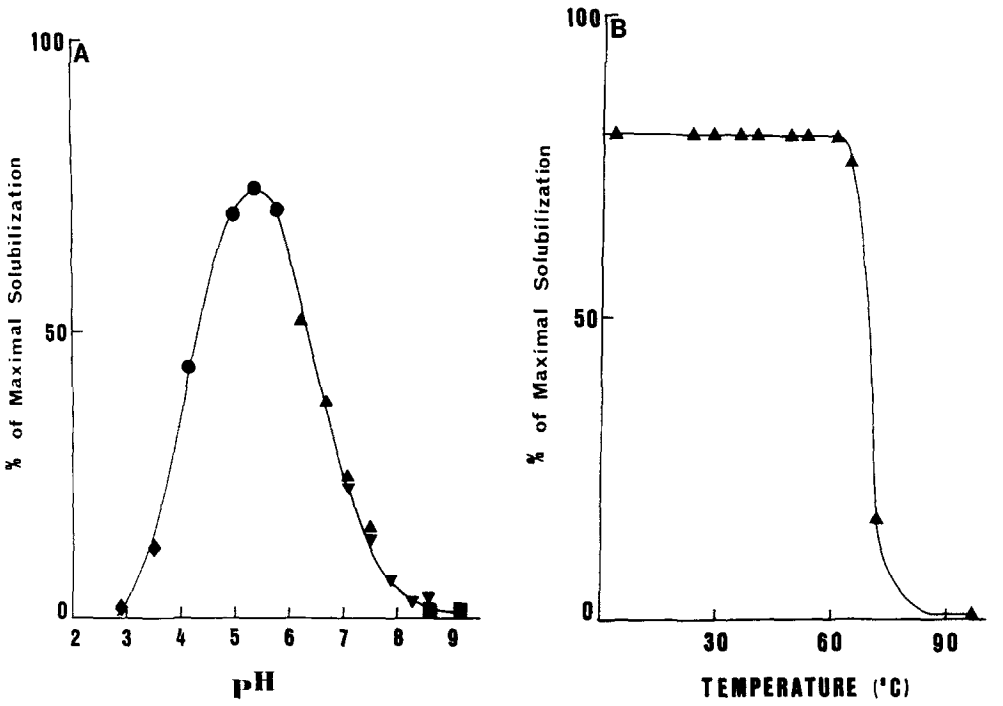

Figure 1. Measurement of pH optimum and thermal resistance of the human serum enzyme. The pH optimum of the enzyme (Figure 1A) was determined using a 1/80 dilution of normal human serum, which produced 80% of maximal solubilization. Buffers used to cover the pH range were glycine.HCl (Figure 1A, ◆); tris acetate (●); tris maleate (▲); tris.HCl (▼) and glycine.OH (■). Thereafter, one unit of enzyme was defined as the amount of enzyme necessary to solubilize 50% of the substrate in 30 minutes at 30°C, in 50 mM Tris acetate, pH 5.4. Thermal resistance (Figure 1B) was measured by preincubating NHS diluted 1.80 in TBS for 1 h at the indicated temperature. Aliquots (10 µl) were then added to 150 mM Tris-acetate buffer, pH 5.4 (10 µl), and reaction was started by the addition of mfVSG-B/^{125}I-S (10 µl). After 30 minutes incubation at 30 °C, the reaction was terminated and percent solubilization assessed as described.

ible as an apparent increase in molecular weight of the product (12) (Figure 3A; compare tracks 1a and 2d). Secondly, the lipase releases [^{3}H] myristate from mfVSG, in the form of dimyristylglycerol (13,14). This effect is apparent on autoradiography of substrate and product (Figure 3C; compare tracks 1a and 2d). Thirdly, release of diacylglycerol exposes the CRD on sVSG (12) (Figure 3B; compare tracks 1a and 1d). In contrast, the serum enzyme produces the same mobility shift on SDS gels (Figure 3A; compare 3a with 1a and 2d). It also leads to the loss of incorporated [^{3}H] myristate (Figure 3C; compare tracks 3a and 3d, with tracks 1a and 1d). The enzyme clearly alters the hydrophobic character of the substrate, since the product partitions in the aqueous layer following phase separation (Figure 3A; compare tracks 3a and 4d). However, the serum enzyme does not expose the CRD (Figure 3B; compare tracks 1a and 3a). The enzyme is, therefore, likely to be a phospholipase of a different class, perhaps a phospholipase D or A. It will be necessary to characterize the released hydrophobic entity to determine with certainty the specificity of the enzyme.

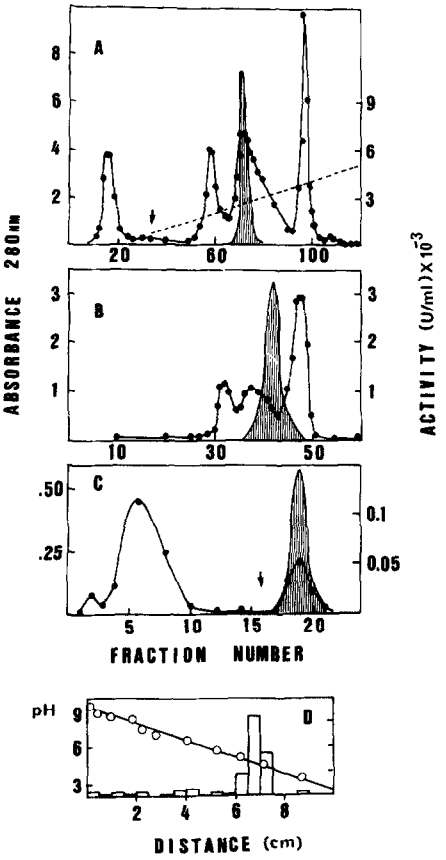

Figure 2. Partial purification and characterization of the enzyme. The enzyme was subjected to ion exchange on DEAE cellulose (Figure 2A), gel filtration on Sephacryl S-300 (Figure 2B), affinity chromatography on wheat germ agglutinin-Sepharose (Figure 2C), and analytical isoelectric focussing. In each case, the shaded area shows pooled active fractions. In Figure 2A, the arrow indicates the point at which elution with a salt gradient (0-0.3 M NaCl, dashed line) was initiated. In Figure 2B, the arrow indicates the point at which elution with N-acetyl-D-glucosamine was initiated. In Figure 2D, the pH standard curve was generated through the use of the following standards: amyloglucosidase, pI 3.5; soybean trypsin inhibitor, pI 4.55; α-lactoglobulin A, pI 5.2; bovine and human carbonic anhydrases, pI 5.85 and 6.55 respectively; horse myoglobins, pI 6.85 and 7.35; lentil lectins of pI 8.15, 8.45 and 6.65; and trypsinogen, pI 9.30.

The enzyme also differs from VSG lipase in the effects of different inhibitors (Table 1). VSG lipase is stimulated by dithiothreitol and strongly inhibited by PCMPSA (4,10). there is no evidence for any metal ion requirement. In contrast, the serum enzyme is relatively insensitive to PCMPSA, is slightly inhibited by dithiothreitol, and is very sensitive to the chelating agents EDTA, EGTA, and O-phenanthrolene. Inhibition by EGTA suggests the enzyme is calcium-dependent, but at present, attempts to reverse the inhibition with calcium or other divalent cations have yielded inconsistent results. Addition of some metal ions, notably nickel, produces moderate inhibition.

The normal role of this serum enzyme remains to be established. The enzyme is present in serum of several other species including fetal calf (data

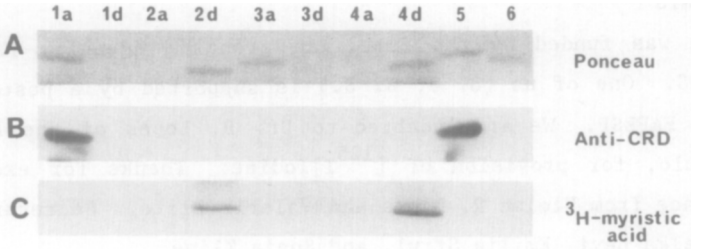

Figure 3. Effects of serum lipase on mfVSG. Figure 3A shows a Ponceau red stain of immunoprecipitated VSG following treatment with either VSG lipase or serum enzyme. Figure 3B shows an anti-CRD blot of the same gel. Figure 3C is an autoradiogram of immunoprecipitated VSGs, following the use of [^{3}H] myristate -- mfvSG as substrate. In each case, tracks 1 and 2 refer to the use of active (1) or heat-inactivated (2) VSG lipase, and tracks 3 and 4 to the use of active (3) or heat inactivated (4) serum enzyme. Tracks 5 and 6 contain unlabelled standards of sVSG and mfVSG respectively. Tracks labelled a correspond to the aqueous phase, and d to the detergent phase following phase separation in Triton X114.

not shown). It is unlikely to be a conventional calcium-dependent phosphatidylinositolspecific phospholipase C, since mfVSG is not a substrate for this enzyme (A. Gurnett, P. Wightman and M. J. Turner, unpublished). Further characterization will allow the specificity of the enzyme to be determined more precisely, and may therefore provide a clue to the natural substrate.

Table 1. Effect of different reagents on the activity of serum enzyme

Reagent	Concentration (mM)	Relative Activity	
		NHS	Purified Enzyme
None	--	100	100
p-Chloromercuriphenyl-sulphonic acid	10	34	72
	0.1	57	93
Dithiothreitol	10	68	56
	0.1	83	107
EDTA	10	0	0
EGTA	10	8	5
o-phenanthroline	10	3	0
$ZnCl_2$	10	98	100
	1	86	85
$CaCl_2$	10	82	66
	1	80	105
$MgCl_2$	10	99	111
	1	79	88
$NiCl_2$	10	47	33
	1	33	72
LiCl	10	86	113
	1	81	111
Phenylmethylsulphonyl-fluoride	1	90	113
Benzamidine	10	75	113

ACKNOWLEDGEMENTS

This work was funded by the Brazilian granting agencies FAPESP, FINEP, CNP, and FIPEC. One of us (B. J. U. S.) is supported by a postgraduate studentship from FAPESP. We are indebted to Dr. D. Lopes of the Ludwig Institute, Sao Paulo, for provision of [^{125}I]Iodine. Thanks for excellent technical assistance from Eloise R. Lopes and Valeria Brito. Secretarial help was provided by Malka Levi, Emilia Strul, and Sonia Kline.

NOTE

In the course of preparation of this manuscript, a paper appeared describing the characterization of a glycophosphatidylinolitol-specific phospholipase D from human serum (15). The activity described here differs from that in reference 15 in sensitivity to heat treatment, and in pH optimum.

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