Apolipoprotein(a) and Plasminogen Interactions with Fibrin: A Study with Recombinant Apolipoprotein(a) and Isolated Plasminogen Fragments†

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Received December 20, 1991; Revised Manuscript Received April 8, 1992

ABSTRACT: Lipoprotein(a) [Lp(a)], but not low-density lipoprotein (LDL), was previously shown to impair the generation of fibrin-bound plasmin [Rouy et al. (1991) *Arterioscler. Thromb. 11*, 629–638] by a mechanism involving binding of Lp(a) to fibrin. It was therefore suggested that the binding was mediated by apolipoprotein(a) [apo(a)], a glycoprotein absent from LDL which has a high degree of homology with plasminogen, the precursor of the fibrinolytic enzyme plasmin. Here we have evaluated this hypothesis by performing comparative fibrin binding studies using a recombinant form of apo(a) containing 17 copies of the apo(a) domain resembling kringle 4 of plasminogen, native Lp(a), and Glu-plasminogen (Glu1–Asn791). Attempts were also made to identify the kringle domains involved in such interactions using isolated elastase-derived plasminogen fragments. The binding experiments were performed using a well-characterized model of an intact and of a plasmin-digested fibrin surface as described by Fleury and Anglés-Cano [(1991) *Biochemistry 30*, 7630–7638]. Binding of r-apo(a) to the fibrin surfaces was of high affinity (K_d = 26 ± 8.4 nM for intact fibrin and 7.7 ± 4.6 nM for plasmin-degraded fibrin) and obeyed the Langmuir equation for adsorption at interfaces. The binding to both surfaces was inhibited by the lysine analogue AMCHA and was completely abolished upon treatment of the degraded surface with carboxypeptidase B, indicating that r-apo(a) binds to both the intrachain lysines of intact fibrin and the carboxy-terminal lysines of degraded fibrin. As expected from these results, both r-apo(a) and native Lp(a) inhibited the binding of Glu-plasminogen to the fibrin surfaces. The concentration of r-apo(a) that inhibited the binding of 1 μM plasminogen to degraded fibrin by 50% was 44 nM. Surprisingly, the binding of r-apo(a) to carboxy-terminal lysines was not inhibited by the isolated plasminogen kringle 4, suggesting lack of interaction of its highly homologous counterpart [r-apo(a) kringle 4–repeat 17] with the lysines of fibrin. The binding was, however, inhibited by the plasminogen fragment kringle 1–3, suggesting a functional similarity of other kringle 4-like domains in r-apo(a) with the binding subsite of kringle 1. Therefore, at least in this case, a parallelism cannot be established between structural homology and function. These results demonstrate the functional heterogeneity of the kringle 4-like domains of apo(a), at least in terms of fibrin binding. Mini-plasminogen exerted no effect on the binding, suggesting that kringle 5 of r-apo(a) is not directly involved in this interaction. Taken together, these results are in complete agreement with present knowledge concerning the binding of the kringles of plasminogen to fibrin and clearly indicate that the plasminogen-like behavior of apo(a) may explain the perturbation of fibrinolysis caused by the Lp(a) particle.

The primary structure of human plasminogen and apolipoprotein(a) has been established by amino acid sequence analysis and cDNA cloning (Petersen et al. 1990; Forsgren et al., 1987; Mc Lean et al., 1987; Eaton et al., 1987; Kratzin et al., 1987). Both proteins contain structures termed kringles and a catalytic domain including the protease triad His-Asp-Ser. Kringles are sequences of 80–90 amino acids arranged in a triple-loop tertiary structure rigidly stabilized by three disulfide bridges (Patthy et al., 1984). These structures are not identical in apo(a) and plasminogen. Kringle domains of plasminogen, designated 1–5, differ from each other and are connected to the protease domain by a sequence containing the activation cleavage site Arg561–Val562. Cleavage of this peptide bond by plasminogen activators results in the transformation of the zymogen into the fibrinolytic enzyme plasmin (Robbins et al., 1967). Apo(a) contains multiple tandem repeats of a kringle that shares 61–75% homology with kringle 4 of plasminogen, followed by a single copy of plasminogen kringle 5 and a protease domain that shares 94% homology with the corresponding domain of plasminogen. However, the arginine residue of the activation cleavage site in plasminogen has been replaced by serine in apo(a), a substitution that impairs the generation of plasmin-like activity by activators (Mc Lean et al., 1987). Since the kringle 4 domain of plasminogen contains subsites with high affinity for lysine which function as binding sites for exposed surface lysine residues in fibrin (Trexler et al., 1982; Hochschwender & Laursen, 1981; Petros et al., 1989; Mulichak & Tulinsky, 1990), apo(a) may bind to the lysine sites available for plasminogen on the fibrin surface, thereby interfering with the fibrinolytic process. The existence of such a potential pathophysiological mechanism and its relevance to the development of thrombosis are strengthened by the fact that apo(a) is the specific glycoprotein of lipoprotein(a) [Lp(a)],[1] a lipoprotein particle which is

† This work was supported by INSERM; D.R. is a recipient of a scholarship from the Fondation pour la Recherche Médicale. M.L.K. is supported by the Medical Research Council of Canada. V.F. is a recipient of a scholarship from the Ministère de la Recherche et de la Technologie.
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[1] Abbreviations: r-apo(a), recombinant apolipoprotein(a); Lp(a), lipoprotein(a); Glu-plasminogen, native human plasminogen with N-terminal glutamic acid; t-PA, tissue-type plasminogen activator; 6-AHA, 6-aminohexanoic acid; AMCHA, *trans*-4-(aminomethyl)cyclohexanecarboxylic acid; VPL, dansylvalyl-L-phenylalanyl-L-lysine chloromethyl ketone; EDTA, ethylenediaminetetraacetic acid; DFP, diisopropyl fluorophosphate; SDS, sodium dodecyl sulfate; LDL, low-density lipoprotein.

0006-2960/92/0431-6333$03.00/0

considered to be an independent risk factor for coronary artery disease [see Scanu and Fless (1990) for a review]. Lp(a) may therefore represent a link between thrombosis and atherosclerosis.

The effect of Lp(a) on the fibrinolytic system has been studied by several groups (Hajjar et al., 1989; Harpel et al., 1989; Gonzalez-Gronow et al., 1989; Loscalzo et al., 1990; Leerinck et al., 1991; Rouy et al., 1991). However, the existence of an antifibrinolytic effect of Lp(a) and the specific mechanism underlying such an effect are still a matter of debate. We have shown that, in a plasma millieu, high levels of Lp(a) may decrease the generation of plasmin by tissue-type plasminogen activator on the surface of fibrin and that Lp(a) binds to the surface of fibrin during ongoing plasminogen activation. Since Lp(a) was eluted from the surface of fibrin with 6-AHA, and LDL [a particle lacking the apo(a) glycoprotein] was not specifically bound to fibrin during the activation, it was concluded that the binding of Lp(a) was mediated by interactions between apo(a) and the lysine residues of intact and degraded fibrin. In the latter case the binding was related to the unveiling of new C-terminal lysine residues by plasmin as indicated by the disappearance of the binding after treatment of the degraded surface with carboxypeptidase B (Anglès-Cano et al., 1990).

Recently we have characterized the differential binding of Glu-plasminogen to lysine side chains exposed in intact fibrin and to carboxy-terminal lysines appearing on the surface of fibrin upon degradation by plasmin (Fleury & Anglès-Cano, 1991). The fibrin surface model used in such studies can now be applied to the study of apo(a)–fibrin interactions. Furthermore, problems of partial denaturation and misfolding which encumber studies using apo(a) derived from Lp(a) by disulfide reduction may be avoided by use of recombinant apo(a).

Such a recombinant form has been recently expressed in mammalian cells (Koschinsky et al., 1991). The recombinant molecule contains 17 kringle 4-like units and has a predicted M_r of 250 000 but migrates in reducing SDS–PAGE at ~500 000. Carbohydrate composition and isoelectric focusing analyses indicate that, as is the case for plasma-derived (Lp(a), the recombinant protein is highly glycosylated and negatively charged. The r-apo(a) retains lysine binding properties similar to those reported for Lp(a) (Eaton et al., 1987) and binds to macrophages by a plasminogen-independent pathway (Zioncheck et al., 1991).

Availability of pure r-apo(a) and the fibrin model system has allowed us to perform comparative binding studies using r-apo(a), plasminogen, and various elastase-derived plasminogen fragments. Our results indicate that r-apo(a) binds both to fibrin and to degraded fibrin surfaces with high affinity and can also compete with plasminogen for binding to fibrin surfaces. Such binding can be inhibited by lysine analogues, thereby indicating that the lysine affinity sites present in the kringle domains of apo(a) mediate the process.

Materials and Methods

Chemicals and Reagents. Materials were purchased from the following sources: ethylenediaminetetraacetic acid (EDTA), bovine serum albumin, poly(ethylene glycol), M_r ~20 000, and Tween 20 from Serva (Heidelberg, Germany); Ultrogel AcA 44 and DEAE-Trisacryl from IBF (Villeneuve-La-Garenne, France); lysine- and CH-Sepharose 4B, Sephadex G-100, and PD-10 Sephadex G-25 (medium grade) columns from Pharmacia (Uppsala, Sweden); bis(acrylamide), ammonium persulfate, and *N,N,N′,N′*-tetramethylethylenediamine from Bio-Rad Laboratories (Richmond, CA); poly(vinyl chloride) U-shaped microtitration plates and plate sealers from Dynatech (Saint Cloud, France); $Na^{125}I$ from Amersham International (Amersham, Buckinghamshire, U.K.); chromogenic substrate methylmalonylhydroxyprolylarginine *p*-nitroanilide (CBS 1065) and aprotinin from Diagnostica Stago (Asnières, France); benzamidine, 6-aminohexanoic acid (6-AHA), and diisopropyl fluorophosphate (DFP) from Aldrich (Beerse, Belgium); *trans*-4-(aminomethyl)cyclohexanecarboxylic acid (AMCHA), *p*-nitrophenyl *p*-guanidinobenzoate, and DFP-treated carboxypeptidase B [EC 3.4.17.2] from Sigma (St. Louis, MO); dansylvalyl-L-phenylalanyl-L-lysine chloromethyl ketone (VPL) from France Biochem (Meudon, France); bovine thrombin [EC 3.4.21.5] from Hoffmann-La Roche (Basel, Switzerland); porcine pancreatic elastase, plasmin, and aprotinin from Boehringer (Mannheim, Germany). All other chemicals were of the best reagent grade commercially available.

Buffers. Buffer A was 0.05 M sodium phosphate buffer, pH 7.4, containing 0.08 M NaCl. Buffer B was 0.05 M sodium phosphate buffer, pH 6.8, containing 0.08 M NaCl. Assay buffer was buffer A containing 2 mg of bovine serum albumin/mL and 0.01% (v/v) Tween 20. Binding buffer was buffer B containing 4 mg of bovine serum albumin/mL, 0.01% (v/v) Tween 20, and 2 mM EDTA. Mass buffer, a buffer that provided the mass action effects on the competitive nonspecific adsorption of plasminogen or r-apo(a) to the fibrin surfaces, was binding buffer containing 40 mg of bovine serum albumin/mL. All other buffers were prepared as described in the text.

Proteins and Lipoproteins. (*A*) *Recombinant Apolipoprotein(a).* A recombinant form of apo(a) was purified from stably transfected human embryonic kidney cells by affinity chromatography on lysine–Sepharose 4B as previously described (Koschinsky et al., 1991). The protein concentration was determined by the method of Bradford (1976). This recombinant protein has been shown to mimic the behavior of plasma-derived lipoprotein(a) (Koschinsky et al., 1991).

(*B*) *Glu-plasminogen* was purified from DFP-treated fresh-frozen human plasma by affinity chromatography on lysine–Sepharose 4B (Deutsch & Mertz, 1970) in the presence of aprotinin, followed by ion-exchange chromatography on DEAE-Trisacryl and gel filtration on Ultrogel AcA 44. All procedures were performed at 4 °C. No contaminant plasmin activity was detected by incubating the plasminogen with the chromogenic substrate CBS 1065 (1.5 mM final concentration) for 48 h at 37 °C. The concentration of the plasminogen preparations was measured spectrophotometrically in buffer A using $E^{1\%}_{1cm}$ = 16.8 at 280 nm (Wallen & Wiman, 1972).

(*C*) *Fibrinogen* was purified from fresh-frozen human plasma supplemented with proteinase inhibitors (100 KIU/mL aprotinin, 2 mM DFP, 1 μM VPL, 0.2 M 6-AHA, 10 μM *p*-nitrophenyl *p*-guanidinobenzoate, 4 mM benzamidine, and 0.5 unit/mL hirudin, final concentrations) by glycine precipitation according to Kazal et al. (1963) with minor modifications. The purified fibrinogen was free of von Willebrand factor, plasminogen, fibronectin, and factor XIII as determined by an enzyme-linked immunosorbent assay specific for these proteins. Protein concentration was determined by measuring the absorbance in buffer A at 280 nm using $E^{1\%}_{1cm}$ = 15.1 (Blombäck & Blombäck, 1956).

(*D*) *Lipoprotein(a) and low-density lipoproteins* were purified as previously described (Rouy et al., 1991; Chapman et al., 1988).

Protein Radioiodination. Glu-plasminogen and recombinant apolipoprotein(a) were radiolabeled with $Na^{125}I$ according

to the Iodogen method of Fraker and Speck (1978) with the following modifications. Protein (10 μg in 0.2 M phosphate buffer, pH 7.4) and 0.5 mCi of the radioisotope were added (20-μL final volume) to a conical plastic centrifuge vial (Eppendorf) precoated with 10 μg of Iodogen (Pierce Chemical Co., Rockford, IL) according to the manufacturer's instructions. After an iodination time of 4 min at 4 °C [15 min for apo(a)], the labeled protein was separated from free $Na^{125}I$ by gel filtration on a PD-10 Sephadex G-25 (medium grade) column. The specific activities obtained were 20 nCi/ng of Glu-plasminogen and 57 nCi/ng of r-apo(a). The integrity of radiolabeled proteins was verified by SDS-polyacrylamide gel electrophoresis of radioiodinated proteins performed under reducing conditions [2-mercaptoethanol, 5% (v/v) final concentration] on 0.75 mm thick, 4% stacking, and 10% (w/v) polyacrylamide separating slab gels according to Laemmli (1970). After electrophoresis, the gels were stained with Coomassie Blue.

Preparation and Analysis of the Fibrin Surfaces. Intact fibrin and plasmin-degraded fibrin surfaces were prepared and characterized as previously described (Anglés-Cano, 1986; Fleury & Anglés-Cano, 1991) with the following modifications. Plasmin-degraded fibrin was prepared by incubating 25 nM plasmin in assay buffer for 30 min at 37 °C. The intact fibrin surface was treated with 50 μg/mL DFP-treated carboxypeptidase B in 0.05 M HEPES, pH 7.5, containing 0.5 M NaCl and 10 μM $ZnCl_2$, for 2 h at 37 °C, to eliminate the possible existence of carboxy-terminal lysines.

Binding of Recombinant Apolipoprotein(a) and Plasminogen to Fibrin Surfaces. Solutions of either Glu-plasminogen (0–10 μM) or r-apo(a) (0–200 nM) with different specific radioactivities were prepared in mass buffer containing 1 mM benzamidine by addition of a trace amount [6 nM Glu-plasminogen or 0.5 nM r-apo(a), final concentration] of the ^{125}I-labeled protein. In parallel experiments varying amounts of a competitive ligand, AMCHA or plasminogen fragments, were added. These mixtures (50 μL/well) were incubated for 18 h at 4 °C with the intact or modified fibrin surfaces. The supernatant was collected, the surface washed three times with binding buffer, and the extent of binding determined by counting the radioactivity of the wells in a γ-radiation counter. The amount of protein bound to the surface of intact or degraded fibrin was calculated by dividing the radioactivity of each well by the molar radioactivity (molar concentration/total dpm) of plasminogen or r-apo(a) in each of the solutions. Nonspecific binding was determined from the amount of radioactivity bound in the presence of an excess of unlabeled ligand (ratio 1:500) or 0.2 M 6-AHA.

Analysis of Binding Data. The fibrin/r-apo(a) dissociation constant (K_d) was calculated on the assumption of single-site binding. The fraction of protein bound to the fibrin surfaces was plotted versus ligand concentration and fitted to the Langmuir isotherm (De Marco et al., 1982; Fleury & Anglés-Cano, 1991)

$$[\text{Fn·r-apo(a)}] = [\text{Fn}_0]\frac{K[\text{r-apo(a)}]}{1 + K[\text{r-apo(a)}]} \quad (1)$$

where $[Fn_0]$ represents the total number of fibrin binding sites, [Fn·r-apo(a)] the number of molecules of r-apo(a) adsorbed on fibrin, and K the association constant. The total binding sites for r-apo(a) in the fibrin surfaces and the affinity of the adsorption were evaluated by using the linearized expression of eq 1:

$$\frac{1}{[\text{Fn·r-apo(a)}]} = \frac{1}{[\text{Fn}_0]} + \frac{1}{K[\text{Fn}_0][\text{r-apo(a)}]} \quad (2)$$

The competitive binding between plasminogen and r-apo(a) obeys modifications of the Langmuir isotherm:

$$[\text{Fn·Pg}] = [\text{Fn}_0]\frac{K[\text{Pg}]}{1 + K[\text{Pg}] + K'[\text{r-apo(a)}]} \quad (3)$$

where K' is the association constant of r-apo(a).

Preparation of Elastase-Degraded Plasminogen Fragments. Digestion of plasminogen with porcine pancreatic elastase was performed according to Sottrup-Jensen et al. (1978) with the following modifications. To 100 mg of purified Glu-plasminogen were added 1 mg of aprotinin and 0.45 mg of porcine pancreatic elastase. The solution in 0.3 M carbonate buffer, pH 8.3, was incubated at room temperature for 3.5 h with stirring. The reaction was stopped by addition of 2 mM final concentration DFP and incubated for an additional 18 h at 4 °C. To separate the resulting fragments, the solution was chromatographed on a Sephadex G-100 column. Three main peaks were obtained. Peak I, containing mainly undigested plasminogen, was discarded. Peaks II and III were further purified by affinity chromatography on lysine–Sepharose and eluted with 0.01 M 6-AHA. The different fractions were electrophoresed in a 10% polyacrylamide gel in the presence of SDS under both reducing and nonreducing conditions. The effluent of peak II migrated as a single band of 39 000 daltons and was referred to as mini-plasminogen. The eluate of the same peak contained two subfractions, which migrated with M_r values of 33 000 and 39 000; this fraction was referred to as kringle 1–3. The flow-through of peak III was discarded. The fraction of the same peak eluted from the lysine–Sepharose column had an M_r of 10 000 and corresponded to plasminogen kringle 4. The functional integrity of isolated kringle 4 was verified by determining its ability to inhibit Glu-plasminogen binding to both intact and degraded fibrin surfaces (data not shown). The mini-plasminogen was further purified by chromatography on aprotinin–Sepharose to remove contaminant proteolytic activity. Aprotinin–Sepharose was prepared by coupling 10 mg of purified aprotinin to 9 mL (3 g) of activated CH-Sepharose according to the specifications of the manufacturer. The fraction obtained had no amidolytic activity as determined by chromogenic assay using the substrate CBS 1065. The three isolated fragments and the Glu-plasminogen were dialyzed against buffer A to remove 6-AHA. Concentrations of the plasminogen fragments were calculated using the above M_r values and the following absorption coefficients: E_{280} (Vali & Patthy, 1982) for kringle 4 (3.1×10^4 M^{-1} cm^{-1}), mini-plasminogen (8.9×10^4 M^{-1} cm^{-1}), and kringle 1–3 (8×10^4 M^{-1} cm^{-1}).

Results

Binding of Recombinant apo(a) to Fibrin Surfaces. Incubation mixtures containing 4 nM ^{125}I-r-apo(a) and a range of unlabeled r-apo(a) concentrations (from 0 to 200 nM) prepared in mass buffer were incubated (50 μL per well) overnight with the fibrin surfaces; fibrin-bound ^{125}I-r-apo(a) was separated from unbound material by aspiration of the supernatant. The wells were washed with mass buffer, and radioactivity was counted in a γ-radiation counter. Free radioactivity was determined by subtracting bound from total counts. Nonspecific binding in the presence of 0.2 M 6-aminohexanoic acid was subtracted to yield values for specific binding. The latter was calculated from the total concentration of protein and the radioactivity added by dividing the radioactivity of each well by the molar radioactivity of protein in each of the solutions containing the radioactive probe. The results are shown in Figure 1A. As observed, the binding was specific and saturable. B_{max}, the maximum binding, and K_d values were

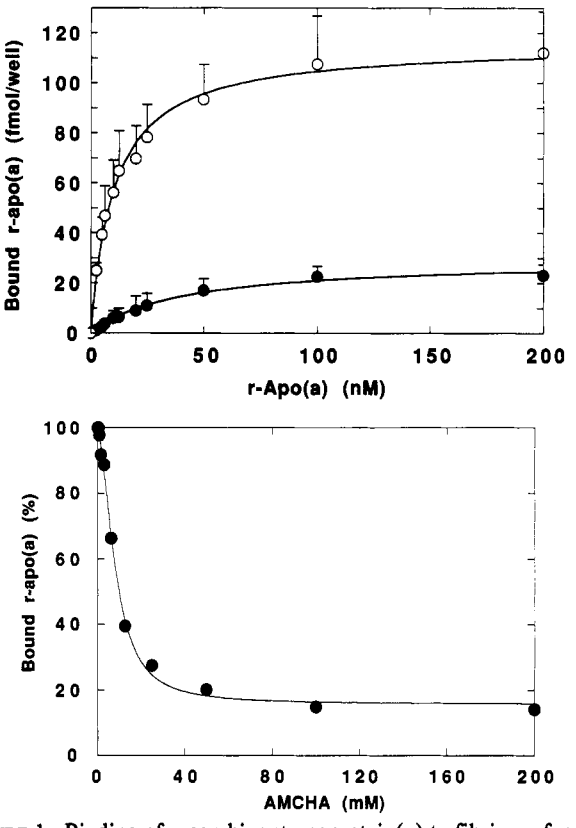

FIGURE 1: Binding of recombinant apoprotein(a) to fibrin surfaces: effect of lysine analogues. (A, top) Various concentrations of r-apo(a) containing a trace amount of radiolabeled protein as a tracer were incubated with fibrin (●) and plasmin-degraded fibrin (○). The wells were washed, and radioactivity was counted in a γ-radiation counter. The specific binding was calculated by dividing the bound radioactivity by the molar radioactivity of each of the solutions. The binding obeys the Langmuir equation; a plot of bound r-apo(a) vs the equilibrium r-apo(a) concentration yields a characteristic hyperbolic curve, and data were fitted to the Langmuir equation (eq 1). Results are expressed as bound femtomoles per well as a function of the concentration of incubated r-apo(a). The results are means ± SD of five independent experiments performed in duplicate. (B, bottom) In order to investigate the lysine specificity of the binding, a constant amount (5 nM) of unlabeled r-apo(a) containing a trace amount of radiolabeled probe (0.3 nM) was incubated with a range of concentrations of the lysine analogue *trans*-4-(aminomethyl)cyclohexanecarboxylic acid (AMCHA, 0–200 mM) on plasmin-degraded fibrin. After washing, the wells were counted as before. Maximum bound radioactivity was taken as 100%, and the relative radioactivities observed are plotted versus the inhibitor concentration. The IC_{50} (the concentration of AMCHA that decreased the maximum bound amount by 50%) was 10 mM.

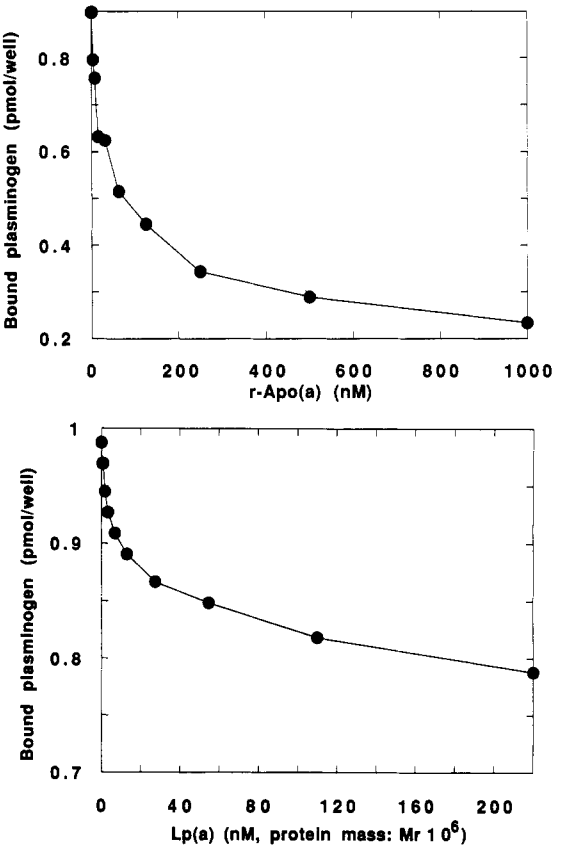

FIGURE 2: Inhibition of plasminogen binding by r-apo(a) and purified Lp(a). A constant amount of 1 μM Glu-plasminogen containing a trace amount of radiolabeled plasminogen (5 nM) was incubated with various concentrations of either r-apo(a) (A, top) or purified Lp(a) (B, bottom). Incubation was conducted overnight. The wells were washed, and radioactivity was counted in a γ-radiation counter. The bound plasminogen was calculated by dividing the bound radioactivity by the molar radioactivity of the initial solution. The results are expressed as picomoles bound per well of plasminogen, as a function of the added inhibitor.

calculated using eq 2, the linearized transformation of eq 1. For the intact fibrin surface, the B_{max} and K_d values were 26.2 ± 2.2 fmol/well and 26 ± 8.4 nM, respectively, and for the modified fibrin surface the respective values were 114.8 ± 14.4 fmol/well and 7.7 ± 4.6 nM. The increase of B_{max} on the plasmin-treated surface was due to the appearance of new C-terminal lysines as indicated by the dramatic reduction of binding following treatment of the surface with carboxypeptidase B (data not shown). Accordingly, the binding of r-apo(a) to the fibrin surfaces was specifically abolished by AMCHA, a lysine analogue that interacts most strongly with plasminogen kringle 4 ($K_a \sim 159$ mM^{-1}; Petros et al., 1989), as shown in Figure 1B (IC_{50} = 10 mM for degraded fibrin).

Inhibition of the Binding of Plasminogen to the Fibrin Surfaces by r-apo(a). The specific binding of plasminogen to intact fibrin and to plasmin-degraded fibrin surfaces was characterized previously (Fleury & Anglés-Cano, 1991). Briefly, incubation mixtures prepared in mass buffer and containing ~4 nM ^{125}I-Glu-plasminogen and a range of unlabeled Glu-plasminogen (from 0 to 20 μM) were incubated with the fibrin surfaces. Specific binding was determined as before, and data were fitted to the Langmuir equation. B_{max} and K_d values obtained were similar to those previously reported. Due to its structural homology with plasminogen, r-apo(a) interfered with the binding of the fibrinolytic proenzyme to the modified fibrin surface with an IC_{50} = 180 nM for a plasminogen concentration of 1 μM (Figure 2A). A similar effect was observed with purified native human lipoprotein(a) (Figure 2B) while LDL was without effect on the binding (data not shown). To determine the inhibition constant, plasminogen binding experiments were performed in the presence of various concentrations of r-apo(a). Recombinant apo(a) decreased the binding of plasminogen in a concentration-dependent manner; by plotting the reciprocal of the maximum amount bound against the r-apo(a) concentration, an estimation of the inhibition was calculated: K_i = 44 nM (data not shown).

Effect of Various Plasminogen Fragments on the Binding of r-apo(a) to the Fibrin Surfaces. By using elastase-derived fragments of plasminogen, we attempted to evaluate which fragment(s) specifically interfere(s) with the binding of r-apo(a) to the fibrin surfaces. Apo(a) is known to possess two types of kringle corresponding to plasminogen kringles 4 and 5 which may interact with the two types of binding sites that have been described on the surface of fibrin and to which plasminogen can bind: intrachain lysines present within intact

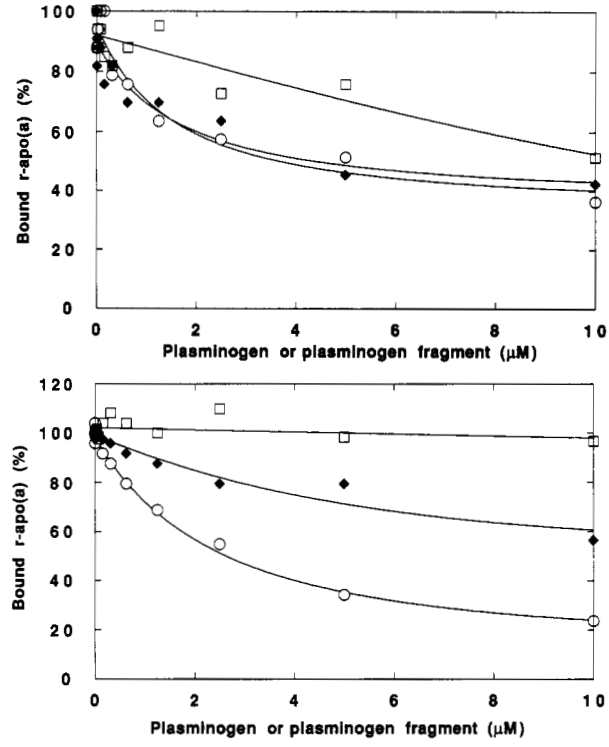

FIGURE 3: Effect of elastase-derived plasminogen fragments on the binding of r-apo(a) to intact and plasmin-degraded fibrin. A constant amount of 10 nM r-apo(a) containing a trace amount of radiolabeled r-apo(a) (0.3 nM) was incubated with various concentrations of either Glu-plasminogen or one of the three main plasminogen fragments: kringle 1–3, kringle 4, and mini-plasminogen. Mini-plasminogen did not modify the binding of r-apo(a) (data not shown). Incubation was conducted overnight. The wells were washed, and radioactivity was counted in a γ-radiation counter. The bound r-apo(a) was calculated by dividing the bound radioactivity by the molar radioactivity of the initial solution. The results are expressed as a percentage of total bound r-apo(a), as a function of the added inhibitor. (A, top) Intact fibrin; (B, bottom) plasmin-degraded fibrin. Symbols: (○) native Glu-plasminogen; (◆) fragment kringle 1–3 containing the first three kringles of plasminogen; (□) fragment kringle 4 containing only kringle 4 of plasminogen.

fibrin and C-terminal lysines present on partially degraded fibrin. Three different plasminogen fragments were used: fragment kringle 1–3, fragment kringle 4, and mini-plasminogen (kringle 5 + protease domain). In the experiment shown in Figure 3, a mixture containing a constant concentration (10 nM) of r-apo(a) and various amounts of these fragments was incubated with either intact fibrin (Figure 3A) or plasmin-degraded fibrin (Figure 3B). Native Glu-plasminogen and fragment kringle 1–3 decreased the binding of r-apo(a); the IC_{50} values for both plasminogen and fragment kringle 1–3 on intact fibrin were 5 μM, and 3 μM and ~10 μM, respectively, on degraded fibrin. Interestingly, the effect of kringle 4, which has the highest degree of homology with the kringle domains of apo(a), was limited to inhibition of r-apo(a) binding to the intact fibrin surface (IC_{50} >10 μM), a finding which is in agreement with the low affinity of kringle 4 to fibrin (K_d = 164 μM; Lucas et al., 1983). When mini-plasminogen was used as a competitor, minimal (<10%) inhibition of r-apo(a) binding was observed (data not shown).

Discussion

Fibrinolysis is a surface-controlled process leading to the plasmin-catalyzed proteolysis of fibrin. The adsorption of plasminogen to fibrin and the surface dynamics of fibrin and plasminogen transformation during this process have been well characterized (Rouy & Anglés-Cano, 1990; Fleury & Anglés-Cano, 1991). It is now generally accepted that the newly exposed carboxy-terminal lysine residues generated by plasmin at the surface of fibrin increase the binding of plasminogen, facilitate its transformation to plasmin, and accelerate clot dissolution (Suenson et al., 1984; Tran-Thang et al., 1984). We have previously shown that high concentrations of Lp(a), but not LDL, may interfere with this process by occupying binding sites for plasminogen via a mechanism that involves interactions between lysine residues of fibrin and Lp(a) (Anglés-Cano et al., 1990; Rouy et al., 1991). In the present study we have clearly demonstrated that apo(a) mediates this process. A recombinant form of apo(a) was used for these studies to avoid problems resulting from partial denaturation and misfolding of apo(a) derived by disulfide reduction from the plasma Lp(a) particle. Previous studies (Koschinsky et al., 1991; Zioncheck et al., 1991) have shown that r-apo(a) possesses physicochemical properties similar to those reported for plasma-derived Lp(a).

In this study, we provide the first quantitative characterization of the binding of Lp(a) to fibrin surfaces and show that the kringle 4-like domains of apo(a) are functionally heterogeneous at least in terms of fibrin binding. Moreover, we demonstrate the competitive behavior of apo(a) with respect to plasminogen for fibrin binding.

Our results show that apo(a) binds both to intact fibrin and to degraded fibrin surfaces in a saturable and specific manner. The binding obeys the simple Langmuir equation for adsorption at interfaces indicating single-site binding and a single association–dissociation mechanism. The equilibrium association–dissociation constant and B_{max} values were derived from the linearized expression of the Langmuir equation (Fleury & Anglés-Cano, 1991). The binding involves the lysine residues of the fibrin surfaces as shown by the ability of AMCHA, a ligand that interacts most strongly with kringle 4, to inhibit binding and by the lack of binding of r-apo(a) to carboxypeptidase B-treated degraded fibrin surfaces. These binding properties have been previously described for plasminogen (Fleury & Anglés-Cano, 1991). Furthermore, we show that plasminogen and r-apo(a) compete for the same binding sites on fibrin. Recombinant apo(a) displayed a slight difference between the affinity for the intact fibrin as compared to the degraded fibrin surface (K_d ~26 nM vs 8 nM, respectively). This difference was similar to that observed for Glu-plasminogen (K_d 0.99 ± 0.17 μM vs 0.66 ± 0.22 μM). However, the absolute K_d values of Glu-plasminogen and r-apo(a) differed by 2 orders of magnitude, indicating a higher affinity of r-apo(a) for fibrin surfaces. Indeed, r-apo(a) efficiently inhibited the binding of Glu-plasminogen to degraded fibrin (K_i = 44 nM). Although the total number of binding sites for r-apo(a) in fibrin (B_{max}) and the fractional number of sites per molecule of fibrin increased 5-fold after plasmin digestion, it was less pronounced than the 9-fold increase observed for Glu-plasminogen (Fleury & Anglés-Cano, 1991). This phenomenom most probably reflects steric hindrance due to differences in the molecular masses of Glu-plasminogen (M_r ~93 000) and r-apo(a) (M_r ~ 500 000 by SDS–PAGE).

Discrepancies in the dissociation constants of Glu-plasminogen and r-apo(a) for the fibrin surfaces are less comprehensible. The recombinant apo(a) we have used contains 17 plasminogen kringle 4-like repeats and includes copies of each kringle sequence variant based on the cDNA sequence reported by McLean et al. (1987). In particular, its kringle 17 is a copy of the kringle corresponding to kringle 37 of the published apo(a) cDNA. This kringle contains most of the aromatic amino acids of the hydrophobic trough as well as charged residues (Asp57 and Arg71) which have been implicated as

ionic centers of the lysine binding subsite region of plasminogen kringle 4. As shown by others (Sottrup-Jensen et al., 1978; Markus et al., 1981; Wu et al., 1988), substantial information concerning kringle–fibrin interactions can be obtained using elastase-derived fragments of plasminogen. It was therefore tempting to perform comparative studies using isolated plasminogen kringle 4. Because of the comparative analysis described above, we expected that the isolated kringle 4 of plasminogen would be the best competitor of the r-apo(a) binding. Figure 3 shows unequivocally that this is not the case, indicating clearly that binding of r-apo(a) to carboxy-terminal lysines is not dependent on kringle 17, which is the most similar to plasminogen kringle 4 with respect to the organization of residues implicated in lysine binding. This suggests that other kringle 4 domains in apo(a) must function in lysine binding. Indeed, eight repeats of kringle 4 with an identical amino acid sequence and seven other copies of kringle 4 with amino acid substitutions are present in the r-apo(a). In agreement with this hypothesis, it was found that the elastase-derived fragment kringle 1–3 efficiently inhibited the binding of r-apo(a) (IC_{50} = 5 μM for fibrin and 10 μM for degraded fibrin). This suggests that the hydrophobic trough harboring the ionic center of the lysine binding subsite in one or more of the kringles of apo(a) is composed of amino acids which generate a binding site functionally analogous to that found in plasminogen kringle 1. The sequence arrangement of kringles with such a subsite in the tertiary structure of the protein may enhance the affinity of r-apo(a) for the carboxy-terminal lysine residues, thus explaining the differences in affinity compared with plasminogen and the observation that plasma levels of Lp(a) lower than those of plasminogen may impair fibrinolysis (Rouy et al., 1991). Finally, it was found that mini-plasminogen has no effect on the binding of r-apo(a), indicating that the kringle 5 domain of r-apo(a) is not involved in fibrin binding. Our results are in complete agreement with our present knowledge concerning the interaction of plasminogen kringle domains with lysine residues (i.e., kringle 1 contains a high-affinity binding site, whereas kringle 4 has lower affinity and kringle 5 does not bind to lysine–Sepharose).

Conclusions. Considered together, our present data constitute the first demonstration of the functional characterization of the plasminogen-like kringles of apo(a). We have shown that r-apo(a) binds specifically to fibrin and that this binding increases upon treatment of the fibrin surface with plasmin. It was further shown that intrachain lysines of intact fibrin and carboxy-terminal lysines of degraded fibrin are involved in this interaction. The binding of r-apo(a) is of high affinity and interferes efficiently with the binding of plasminogen to the new binding sites unveiled by plasmin digestion of the fibrin surface. Binding of the native Lp(a) particle through this mechanism may impair clot lysis, may favor the accumulation of cholesterol in thrombi at sites of vascular injury, and may therefore represent an important link between thrombosis and atherosclerosis.

ACKNOWLEDGMENTS

We are indebted to Patrick Grailhe (U.143) for purifying human fibrinogen and to Laurence Hervio and Fabienne Nigon (U.321) for their help in the purification of human Lp(a) and LDL.

Registry No. Lys, 56-87-1; plasmin, 9001-90-5.

REFERENCES

Anglés-Cano, E. (1986) *Anal. Biochem. 153*, 201–210.

Anglés-Cano, E., Fleury, V., Rouy, D., & Chapman, J. (1990) *Circulation 82* (Suppl. III), III-92.

Blombäck, B., & Blombäck, M. (1956) *Ark. Kemi 10*, 415–443.

Bradford, M. (1976) *Anal. Biochem. 72*, 248–252.

Chapman, M. J., Laplaud, P. M., Luc, G., Forgez, P., Bruckert, E., Goulinet, S., & Lagrange, D. (1988) *J. Lipid Res. 29*, 442–458.

De Marco, A., Hochschwender, S. M., Laursen, R. A., & Lliñas, M. (1982) *J. Biol. Chem. 257*, 12716–12721.

Deutsch, D. G., & Mertz, E. T. (1970) *Science 170*, 1095–1096.

Eaton, D., Fless, G., Kohr, W., McLean, J., Xu, Q., Miller, C., Lawn, R., & Scanu, A. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 3224–3228.

Fleury, V., & Anglés-Cano, E. (1991) *Biochemistry 30*, 7630–7638.

Forsgren, M., Raden, B., Israelsson, M., Larsson, K., & Heden, L. O. (1987) *FEBS Lett. 213*, 254–260.

Fraker, P. J., & Speck, J. C. J. (1978) *Biochem. Biophys. Res. Commun. 80*, 849–857.

Gonzalez-Gronow, M., Edelberg, J. M., & Pizzo, S. V. (1989) *Biochemistry 28*, 2374–2377.

Hajjar, K. A., Gavish, D., Breslow, J. L., & Nachman, R. L. (1989) *Nature 339*, 303–305.

Harpel, P. C., Gordon, B. R., & Parker, T. S. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 3847–3851.

Hochschwender, S. M., & Laursen, R. A. (1981) *J. Biol. Chem. 256*, 11166–11171.

Kazal, L. A., Amsel, S., Miller, O. P., & Tocantins, L. M. (1963) *Proc. Soc. Exp. Biol. Med. 113*, 989–994.

Koschinsky, M. L., Tomlinson, J. E., Zioncheck, T. F., Schwartz, K., Eaton, D. L., & Lawn, R. M. (1991) *Biochemistry 30*, 5044–5051.

Kratzin, H., Armstrong, V. W., Niehans, M., Hischmann, N., & Siedel, D. (1987) *Biol. Chem. Hoppe-Seyler 368*, 1533–1544.

Laemmli, U. K. (1970) *Nature 227*, 680–685.

Leerinck, C. B., Gimpel, J. A., Kortland, W., Bouma, B. N., & Van Rijn, H. J. M. (1991) *Fibrinolysis 5*, 233–238.

Loscalzo, J., Weinfeld, M., Fless, G. M., & Scanu, A. M. (1990) *Arteriosclerosis 10*, 240–245.

Lucas, M. A., Fretto, L. J., & McKee, P. A. (1983) *J. Biol. Chem. 258*, 4249–4256.

Markus, G., Camiolo, S. M., Sottrup-Jensen, L., & Magnusson, S. (1981) *Prog. Fibrinolysis 5*, 125–128.

McLean, G., Tomlinson, J., Kuang, W., Eaton, D., Chen, E., Fless, G., Scanu, A., & Lawn, R. (1987) *Nature 330*, 132–137.

Mulichak, A. M., & Tulinsky, A. (1990) *Blood Coagulation Fibrinolysis 1*, 673–679.

Patthy, L., Trexler, M., Vali, Z., Banyai, L., & Varadi, A. (1984) *FEBS Lett. 171*, 131–136.

Petersen, T. E., Martzen, M. R., Ichinose, A., & Davie, E. W. (1990) *J. Biol. Chem. 265*, 6104–6111.

Petros, A., Ramesh, V., & Lliñas, M. (1989) *Biochemistry 28*, 1368–1376.

Robbins, K. C., Summaria, L., Hsieh, B., & Shah, R. J. (1967) *J. Biol. Chem. 242*, 2333–2342.

Rouy, D., & Anglés-Cano, E. (1990) *Biochem. J. 271*, 51–57.

Rouy, D., Grailhe, P., Nigon, F., Chapman, J., & Anglés-Cano, E. (1991) *Arterioscler. Thromb. 11*, 629–638.

Scanu, A. M., & Fless, G. M. (1990) *J. Clin. Invest. 85*, 1709–1715.

Sottrup-Jensen, L., Claeys, H., Zajdel, M., Petersen, T. E., & Magnusson, S. (1978) *Prog. Chem. Fibrinolysis Thrombolysis 3*, 191–209.

Suenson, E., Lutzen, O., & Thorsen, S. (1984) *Eur. J. Biochem. 140*, 513-522.
Tran-Thang, C., Kruithof, E. K. O., & Bachmann, F. (1984) *J. Clin. Invest. 74*, 2009-2016.
Trexler, M., Vali, Z., & Patthy, L. (1982) *J. Biol. Chem. 257*, 7401-7406.
Vali, Z., & Patthy, L. (1982) *J. Biol. Chem. 257*, 2104-2110.
Wallen, P., & Wiman, B. (1972) *Biochim. Biophys. Acta 257*, 122-134.
Wu, H. L., Chang, B. I., Wu, D. H., Chang, L. C., Gong, C. C., Lou, K. L., & Shi, G. Y. (1990) *J. Biol. Chem. 265*, 19658-19664.
Zioncheck, T. F., Powell, L. M., Rice, G. C., Eaton, D. L., & Lawn, R. M. (1991) *J. Clin. Invest. 87*, 767-771.

The Type II Isoform of Bovine Brain Protein L-Isoaspartyl Methyltransferase Has an Endoplasmic Reticulum Retention Signal (...RDEL) at Its C-Terminus†

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Received March 9, 1992

ABSTRACT: Bovine brain is known to contain two major isoforms of protein L-isoaspartyl methyltransferase (PIMT), an enzyme that facilitates repair of atypical L-isoaspartyl peptide bonds in proteins. Although the two isoforms can be separated by anion-exchange chromatography, they appear to have similar, if not identical, substrate specificities in vitro. The more basic type I isoform has been extensively characterized, and its complete sequence has been reported. The present study was undertaken in an attempt to understand the structural and functional uniqueness of the more acidic type II isoform. Electrospray mass spectrometry of the intact enzymes revealed that the type II isoform is approximately 43 amu heavier than the type I isoform. Cyanogen bromide cleavage followed by HPLC with on-line mass analysis revealed that the type II isoform contains a unique C-terminal fragment which is 43 amu heavier than the corresponding fragment from the type I isoform. Amino acid composition analysis and direct sequencing of this fragment indicate that the type II isoform ends in the sequence ...RDEL, while the type I is known to end in ...RWK. Since ...RDEL, like ...KDEL, serves as an effective endoplasmic reticulum retention signal, we propose that the type II isoform serves to repair damaged proteins within the endoplasmic reticulum or, perhaps, within some other specialized compartment of the cell. Comparison of the protein sequences of the two bovine brain isoforms to DNA sequences for rodent PIMT reported by others suggests that the type II isoform may be produced by splicing within the codon for Arg_{224}.

Protein L-isoaspartyl methyltransferase (PIMT,[1] E.C. 2.1.1.77), an enzyme that transfers the active methyl group from *S*-adenosyl-L-methionine (SAM) to the α-carboxyl of L-isoaspartyl residues in peptides and proteins, has been found in a wide variety of organisms, including several vertebrate and invertebrate animals, bacteria, a plant, and a fungus (O'Connor & Clarke, 1985; Johnson et al., 1991a; Fu et al., 1991). PIMT is unusual in that it has extremely broad substrate specificity, and natural polypeptides are typically methylated with a low stoichiometry by the purified enzyme (Clarke, 1985). The low stoichiometries of methylation are consistent with the view that isoaspartyl residues are found only in atypical subpopulations of polypeptides that have undergone spontaneous isomerization of aspartate residues and/or spontaneous deamidation of asparagine residues [reviewed in Johnson et al. (1990) and Ota and Clarke (1990)].

A normal, α-carboxyl-linked aspartate residue is one of the products that follows the spontaneous demethylation of the labile α-carboxyl methyl ester (Johnson et al., 1987a). The in vitro methylation of an isoaspartate-containing form of age-damaged calmodulin by purified bovine brain PIMT, coupled with spontaneous demethylation, has been shown to restore a substantial amount of its functional activity (Johnson et al., 1987b). It has been proposed that, in vivo, PIMT may play an important part in the repair of proteins that have been damaged by spontaneous isoaspartate formation (Aswad, 1984; Murray & Clarke, 1984).

† This work was supported in part by Grant NS-17269 from the U.S. Public Health Service.

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[1] Abbreviations: PIMT, protein L-isoaspartyl methyltransferase; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; βME, β-mercaptoethanol; PMSF, phenylmethanesulfonyl fluoride; SAH, *S*-adenosyl-L-homocysteine; SAM, *S*-adenosyl-L-methionine; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; CNBr, cyanogen bromide; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*'-2-ethanesulfonic acid: TEMED, *N*,*N*,*N*',*N*'-tetramethylethylenediamine; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; ER, endoplasmic reticulum.

0006-2960/92/0431-6339$03.00/0