Analysis of the Proteolytic Activity of a Recombinant Form of Apolipoprotein(a)†

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ABSTRACT: We have analyzed the proteolytic activity of a recombinant form of apolipoprotein(a) [r-apo(a)]. A mutant 17-kringle form of r-apo(a) was engineered that contained a serine to arginine substitution which reinstates the tissue-type plasminogen activator (tPA) cleavage site in the protease domain of r-apo(a). The mutant form of r-apo(a) was cleaved by tPA as determined by SDS−PAGE and fluorography and by Western blot analysis. However, tPA cleavage did not result in an active protease as both wild-type r-apo(a) and the mutant, either free or incorporated into r-Lp(a) particles, were uniformly inactive against a variety of chromogenic serine protease tripeptide substrates. To assess whether the large number of kringle IV repeats present in apo(a) inhibits proteolytic activity, we generated truncated forms of the Ser→Arg mutant containing one or 10 kringle IV repeats. These truncated versions of r-apo(a) were susceptible to cleavage by tPA but were inactive against the plasmin substrate S-2251. Treatment of the Ser→Arg mutant of the 17-kringle r-apo(a) with tPA and diisopropylfluorophosphate (DFP) did not result in modification of the mutant protease domain by DFP. Finally, we incubated r-apo(a) or r-Lp(a) particles formed *in vitro* with purified human LDL; no degradation of LDL was observed after 16 h at 37 °C. The results of this study suggest that one or more of the substitutions present in the protease domain of apo(a), in addition to the Arg→Ser substitution, render apo(a) proteolytically inactive.

Lipoprotein(a) [Lp(a)][1] has been identified as an independent risk factor for coronary heart disease, myocardial infarction, and infarct artery patency (Rhoads *et al.*, 1986; Loscalzo, 1990; Rosengren *et al.*, 1990; Scanu & Fless, 1990; Moliterno *et al.*, 1993; Dahlen, 1994). However, the mechanism by which Lp(a) mediates its pathogenic effects is poorly understood at present. Structurally, Lp(a) is very similar to low density lipoprotein (LDL), but is distinguished by the presence of the unique glycoprotein moiety apolipoprotein(a) [apo(a)], which is attached to the apolipoproteinB-100 (apoB-100) component of LDL by a single disulfide bridge (Brunner *et al.*, 1993; Koschinsky *et al.*, 1993). The primary sequence of apo(a) has been demonstrated to share extensive homology with the serine protease zymogen plasminogen (McLean *et al.*, 1987). Plasminogen consists of five distinct kringle motifs (denoted kringles I−V), followed by a protease domain containing the His−Asp−Ser catalytic triad characteristic of serine proteases. Apo(a) contains multiply-repeating copies of a sequence similar to plasminogen kringle IV, followed by sequences corresponding to the kringle V and protease domains of plasminogen.

Plasminogen is converted to the active serine protease plasmin by activators such as tissue-type plasminogen activator (tPA) and urokinase plasminogen activator (uPA) which cleave the Arg560−Val561 bond of plasminogen to generate the active two-chain plasmin structure (Robbins *et al.*, 1967; Wiman, 1977). In apo(a) the corresponding arginine residue has been replaced by a serine (Eaton *et al.*, 1987; McLean *et al.*, 1987), a substitution that has generally been considered to render apo(a) proteolytically inactive. Indeed, it has been reported that treatment of either Lp(a) or apo(a) with plasminogen activators does not result in plasmin-like activity (Eaton *et al.*, 1987; McLean *et al.*, 1987; Salonen *et al.*, 1989). However, there have been reports that both Lp(a) purified from plasma, and apo(a) obtained upon reduction of the Lp(a) molecule, exhibit amidolytic activity against artificial chromogenic substrates (Salonen *et al.*, 1989; Chulkova, 1990; Jauhiainen *et al.*, 1991). In these studies, Lp(a) and apo(a) exhibited specificity towards arginine residues rather than lysine; the side chain of the latter amino acid is specifically recognized by plasmin. Lp(a) and apo(a) have also been reported to be able to proteolytically degrade fibronectin (Salonen *et al.*, 1989). Additionally, it has been reported that apo(a), either free (Chulkova & Tertov, 1993), or in the context of the Lp(a) particle (Pursiainen *et al.*, 1994b), is capable of proteolytically degrading LDL.

In the present study, the proteolytic activity of a well-characterized recombinant apo(a) [r-apo(a)] species (Koschinsky *et al.*, 1991) was analyzed. This r-apo(a) derivative has been shown to bind to fibrin (Rouy *et al.*, 1992) and fibronectin (van der Hoek *et al.*, 1994), and can be used to form r-Lp(a) complexes with purified human LDL (Koschinsky *et al.*, 1993). Further, we sought to determine the effect of reinstatement of the tPA cleavage site into the protease domain of r-apo(a). Our results with both r-apo(a) and the mutant containing the tPA cleavage site indicate that apo(a) does not exhibit plasmin-like activity and is not proteolytically active against a wide variety of chromogenic serine protease substrates. Additionally, r-apo(a) or recombinant Lp(a) particles (r-Lp(a); containing 17-kringle r-apo(a) and

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[1] Abbreviations: Lp(a), lipoprotein(a); apo(a), apolipoprotein(a); r-apo(a), recombinant apo(a); tPA, tissue-type plasminogen activator; apoB-100, apolipoproteinB-100; LDL, low density lipoprotein; CM, conditioned medium; DFP, diisopropylfluorophosphate; PMSF, phenylmethylsulfonyl fluoride.

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purified human LDL) do not degrade LDL as has been previously reported. Finally, we show that the lack of proteolytic activity of apo(a) is not a consequence of the large number of kringle IV repeats present in the molecule. Our data clearly demonstrate that mutations within the protease domain other than the arginine to serine substitution render apo(a) proteolytically inactive.

EXPERIMENTAL PROCEDURES

Construction of Apo(a) Expression Vectors. A recombinant apo(a) expression plasmid that encodes 17 kringle IV-like domains as well as the kringle V and protease-like domains (pRK5ha17; Koschinsky *et al.*, 1991) was utilized as the parental plasmid for all subsequent constructions (see Figure 1). An 841 bp *Eco*RV/*Xba*I fragment was isolated from pRK5ha17 and cloned into M13Tg130 (Amersham) for site-directed mutagenesis. A 17-mer oligonucleotide (5′CCT ACA ATC CTT CCA GG 3′) containing a G→C substitution (bold) was utilized to perform site-directed mutagenesis by the method of Kunkel *et al.* (1987). This nucleotide substitution results in the replacement of the serine at position 4 308 in the published apo(a) amino acid sequence (McLean *et al.*, 1987) with an arginine. The fragment containing the mutation was ligated into pRK5ha17, which had been digested with *Eco*RV and *Xba*I, for subsequent expression studies. The 10-kringle r-apo(a) expression plasmid pRK5ha10 was constructed by fusion of a 385 bp *Eco*RI/*Hha*I fragment derived from pRK5ha17, which contains sequences encoding the 5′-untranslated sequence, the signal sequence, and the first 291 bp of kringle IV repeat #1, to a 4147 bp *Hha*I/*Eco*RI fragment containing the latter 51 bp of kringle IV repeat #28, followed by sequences corresponding to kringle IV repeats #29−#37 and the kringle V and protease domains. In order to create the Ser→Arg substitution in this construct, the plasmid was digested with *Eco*RV and *Xba*I and the resultant 841 bp fragment replaced with the mutant fragment generated above. The expression plasmid pRK5ha1/37, encoding a fusion of kringle #1 and #37 followed by the kringle V and protease domains, was constructed by fusion of a 273 bp *Eco*RI/*Pst*I fragment derived from pRK5ha17, which contains sequences encoding the 5′ untranslated sequence, the signal sequence, and the first 179 bp of kringle IV repeat #1, to a 1238 bp *Pst*I fragment (obtained by digestion of pRK5ha17) containing the last 163 bp of kringle IV repeat #37, followed by the kringle V and protease domains. In order to create the Ser→Arg substitution in this construct, the plasmid was digested with *Eco*RV and *Xba*I and the resultant 841 bp fragment replaced with the mutant fragment generated above.

All constructs described above were verified by DNA sequence analysis.

Transient Transfection and Metabolic Labeling of Cells. Human embryonic kidney cells (293 cell line; Graham *et al.*, 1977) were cultured in 100-mm dishes in minimal essential medium (MEM; GIBCO/BRL) supplemented with 10% fetal calf serum (FCS; ICN). Cells were transfected by the method of calcium phosphate coprecipitation (Graham & van der Eb, 1973) using 10 μg DNA per 100-mm dish of the expression plasmids shown in Figure 1. The precipitate was left on the cells for 8 h, after which time the medium containing the precipitate was removed and fresh medium was added. For determination of the proteolytic activity of r-apo(a), serum free medium (supplemented with 40 μg/mL insulin (Sigma)) was added to the transfected cells (5 mL/dish) and subsequently collected after a 48-h incubation period. For fluorographic visualization of tPA-cleaved fragments of r-apo(a) species corresponding to pRK5ha17Δser, pRK5ha10Δser, and pRK5ha1/37Δser, transfected cells were allowed to recover for 36 h in MEM containing 10% FCS prior to metabolic labeling (see below).

For metabolic labeling, transfected cells were preincubated for 45 min in Met/Cys-depleted MEM (GIBCO/BRL) supplemented with 2 mM glutamine in the absence of fetal calf serum. [^{35}S]Cys (ICN) was then added (50 μCi/mL of media). Conditioned medium (CM) from cultured cells was collected 3.5 h post labeling and clarified by brief centrifugation.

Treatment of Transfected Cell CM with tPA. CM from metabolically labeled transfected cells was incubated in the absence or presence of 4 μg of recombinant tPA (Activase; Genentech, Inc.) for 1 h at 37 °C. The reactions were then incubated overnight at 4 °C with 10 μg of an apo(a)-specific polyclonal antibody raised in rabbits. Protein A−Sepharose (Pharmacia) was then added, and the samples were incubated at 4 °C for an additional 2−3 h. At this time, the Sepharose was pelleted by brief centrifugation at 16 000*g* and washed three times with RIPA buffer (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1% Triton X-100, 20 mM EDTA, 1% sodium deoxycholate, 0.1% SDS) containing 0.5 M NaCl, followed by a final wash with TE (10 mM Tris-HCl pH 7.5, 1 mM EDTA). Laemmli sample buffer (Laemmli, 1970) was added to each sample; dithiothreitol (DTT) was added to a final concentration of 10 mM in order to reduce samples where required. Samples were boiled for 5 min, and solubilized proteins were separated by SDS−PAGE using 12% gels. Gels were then treated for 15 min with Enlightning (Dupont), dried at 60 °C under vacuum, and exposed to film.

In order to compare the efficiency of tPA cleavage of the r-apo(a) species corresponding to the pRK5ha17Δser expression plasmid with that of plasminogen, either 1 mL of CM from unlabeled transfected cells (containing ~1 μg/mL r-apo(a) as determined by ELISA) or plasminogen (0.25 μg in 1 mL PBS) was incubated in the absence or presence of tPA (2 μg) for 1 h at 37 °C. Mixtures were concentrated 20-fold using a Centricon 30 microconcentrator (Amicon); Laemmli sample buffer and DTT (to a final concentration of 10 mM) were added, and samples were separated by SDS−PAGE using 12% gels. Bands were visualized by Western blot analysis as previously described (Koschinsky *et al.*, 1990); following transfer of the gel to nitrocellulose, the blot was incubated with a polyclonal antibody raised in rabbits against human apo(a) (20 μg/mL), followed by the addition of a goat anti-rabbit secondary antibody conjugated to alkaline phosphatase for visualization (400 ng/mL; Bio-Rad).

Assessment of the Proteolytic Activity of r-Apo(a) Species. Unlabeled CM harvested from transfected cells was dialyzed overnight against two changes of PBS. For treatment of CM harvested from cells transfected with pRK5ha17Δser, tPA was coupled to CNBr-activated Sepharose CL-4B (Pharmacia); 50 μL of this resin (80 μg/mL tPA) was incubated with 1 mL of dialyzed CM for 12 h at 37 °C, at which time the tPA-Sepharose was removed by centrifugation. Dialyzed CM (800 μL) or tPA-Sepharose-treated dialyzed CM corresponding to cells transfected with pRK5ha17 or pRK5ha17Δser, respectively, was incubated with a variety of chromogenic tripeptide substrates (200−300 μM) (Kabi).

The absorbance at 405 nm was monitored at 30 s intervals and compared to that of tPA-Sepharose-treated or untreated mock-transfected cell CM, as well as mock-transfected cell CM containing plasminogen (1 μg in 800 μL; CalBiochem) and treated with tPA-Sepharose. In a similar fashion, dialyzed CM harvested from cells transfected with pRK5ha1/37 and pRK5ha10 was incubated with the chromogenic tripeptide substrate S-2251 (Kabi; 300 μM final concentration); CM harvested from cells transfected with pRK5ha1/37Δser and pRK5ha10Δser was treated with tPA-Sepharose as described above for pRK5ha17Δser prior to incubation with S-2251. In order to assess the amidolytic activity of r-apo(a) in the context of the Lp(a) particle, Lp(a) was reconstituted from purified 17-kringle r-apo(a) and LDL (see below); the reconstituted Lp(a) was incubated with the substrates S-2251, S-2765, and S-2222 as described above.

Protein Purification. The wild-type 17-kringle form of recombinant apo(a) was purified as previously described, using lysine-Sepharose affinity chromatography (Koschinsky *et al.*, 1991). Recombinant apo(a) present in CM harvested from pRK5ha17Δser-transfected cells was purified by immunoaffinity chromatography as previously described, using an apo(a)-specific monoclonal antibody coupled to Affigel (BioRad) (Koschinsky *et al.*, 1990). For purification of human LDL, solid $(NH_4)_2SO_4$ was added to 10 mL of plasma to 20% saturation at room temperature. The solution was stirred for 1 h at 4 °C and then centrifuged at 2 200*g* for 20 min at 4 °C. The resulting pellet was dissolved in a minimal volume of 20 mM HEPES pH 7.4, 1.0 M NaCl and subjected to gel filtration chromatography over Sepharose CL-4B (Pharmacia; 100 cm × 2 cm column); the column was developed with this same buffer. LDL-containing fractions were pooled and dialyzed against 20 mM HEPES at pH 7.4, with 0.15 M NaCl. The purity and integrity of all protein preparations was assessed by SDS−PAGE and silver staining (Merril *et al.*, 1981).

Determination of the Incorporation of [^{3}H]DFP into tPA-Cleaved Mutant r-Apo(a). Five micrograms of r-apo(a) (purified as described above) corresponding to the pRK5ha17Δser expression plasmid was incubated in the absence or presence of 2 μg of tPA at 37 °C for 1 h, in a total reaction volume of 15 μL. At this time, the reactions were diluted to 1 mL with PBS, and 30 μL of [^{3}H]DFP (10 μCi/μL; NEN) was added; samples were subsequently incubated for an additional 3 h at 37 °C. Samples were then concentrated 20-fold using a Centricon 30 microconcentrator (Amicon). Laemmli sample buffer was added, and samples (nonreduced) were analyzed by SDS−PAGE using a 4−15% polyacrylamide gradient gel. The gel was treated with En^{3}Hance (NEN), dried at 60 °C under vacuum, and exposed to film.

Assessment of the Proteolytic Degradation of LDL by r-Lp(a) and Plasmin. LDL (40 μg/mL) (purified as described above) was incubated for 16 h at 37 °C with either purified 17-kringle r-apo(a) or plasmin (4 μg/mL and 1.4 μg/mL, respectively) or in the absence of any additional protein. Under these conditions, the r-apo(a) is quantitatively incorporated into r-Lp(a) particles, as was assessed by Western blot analysis of an aliquot of the reaction using an anti-apo(a) polyclonal antibody raised in rabbits. At the conclusion of the incubation period, an equivalent volume of 2× Laemmli sample buffer was added in order to terminate the reactions. Control reactions were terminated immediately after the addition of either r-apo(a) or plasmin. Samples were boiled for 5 min, and a volume of the reactions corresponding to 1 μg of LDL was subjected to SDS−PAGE using a 2.5−15% polyacrylamide gradient gel. Resolved proteins were visualized by silver staining (Merril *et al.*, 1981).

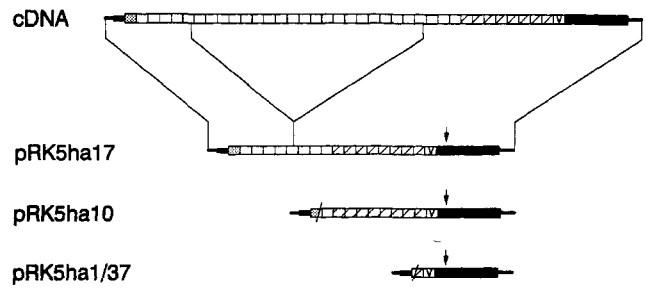

FIGURE 1: Organization of recombinant apo(a) expression plasmids used in this study. All constructs contain the 5′ untranslated region and signal sequence, followed by varying numbers of kringle IV−like repeats, the kringle V−like (V) and the apo(a) protease-like domain (solid bar). Stippled and hatched boxes indicate kringles that contain amino acid substitutions relative to the major repeat kringle, which is indicated with open boxes. The arrows indicate the location of the Ser→Arg substitution that restores the tPA cleavage site in pRK5ha17Δser, pRK5ha10Δser, and pRK5ha1/37Δser. A slashed line indicates a hybrid kringle sequence as described in the text.

RESULTS

Cleavage of Apo(a) Ser→Arg Mutants by tPA. The construction and expression of a 17-kringle form of recombinant apo(a) [r-apo(a)] has been previously described (Koschinsky *et al.*, 1991). Figure 1 shows derivatives of the r-apo(a) expression vector pRK5ha17 (Koschinsky *et al.*, 1991) that were generated for use in this study. These include truncated versions of the construct (pRK5ha10 and pRK5ha1/37), which contain 10 kringle IV repeats and one kringle IV repeat, respectively. A serine to arginine substitution at amino acid position 4 308 of the published apo(a) sequence, which restores the tPA cleavage site, was introduced in these constructs to generate pRK5ha17Δser, pRK5ha10Δser, and pRK5ha1/37Δser, respectively.

The expression of r-apo(a) corresponding to pRK5ha17 and truncated versions of this plasmid, as well as the respective Ser→Arg mutants, was detected by immunoprecipitation of [^{35}S]Cys-labeled transfected cell conditioned medium (CM) using an apo(a)-specific polyclonal antibody. Treatment of metabolically labeled proteins corresponding to pRK5ha17Δser, pRK5ha10Δser, and pRK5ha1/37Δser with tPA (4 μg/mL) prior to immunoprecipitation and SDS−PAGE (under reducing conditions) resulted in the appearance in all cases of a distinct band migrating at ~25 kDa (Figure 2A−C). This band is lacking in both the respective wild-type r-apo(a) species as well as in the non-tPA-treated Ser→Arg mutants, and corresponds in size to the apo(a) protease domain. These data clearly demonstrate that reinstatement of the tPA cleavage site by the Ser→Arg substitution in r-apo(a) allows proteolytic processing of these species by tPA. In Figure 2C, an additional band migrating at ~35 kDa is present in the lane containing tPA-treated CM harvested from cells transfected with pRK5ha1/37Δser. This band (not detectable for the larger r-apo(a) species in Figure 2A,B) corresponds in size to the amino-terminal portion of the tPA-cleaved 1/37Δser protein. Based on the data shown in Figure 2, it is clear that the tPA cleavage of Ser→Arg mutants of r-apo(a) was not quantitative. This is not

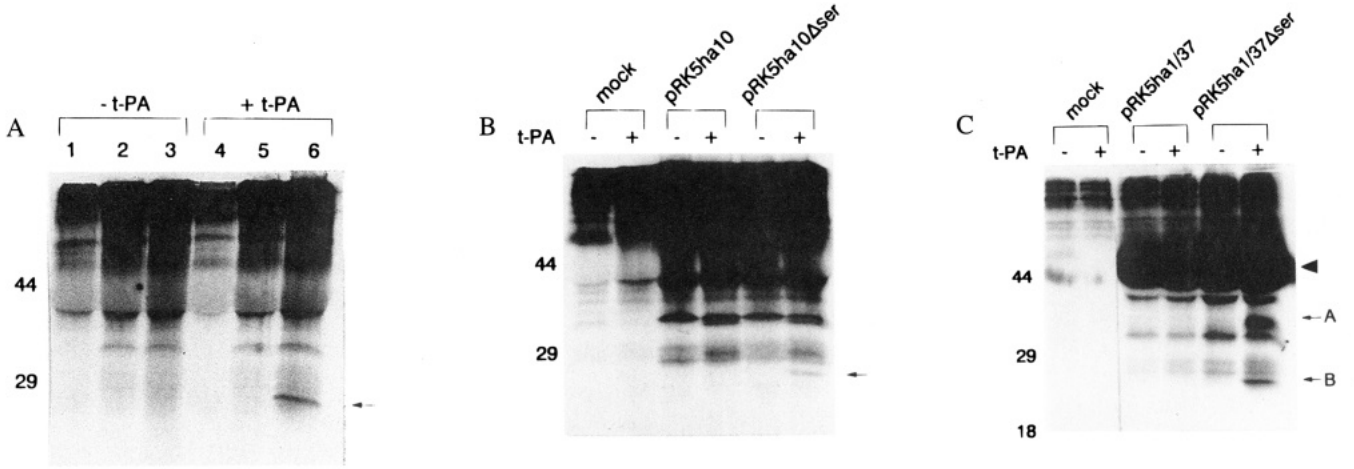

FIGURE 2: Expression and tPA cleavage of recombinant forms of apo(a). 293 cells were transiently transfected with either pRK5ha17, pRK5ha10, or pRK5ha1/37 as well as the corresponding Ser→Arg mutants. Following a 36 h recovery period in MEM containing 10% FCS, cells were metabolically labeled as described in Experimental Procedures. Labeled CM (1 mL) was incubated in the absence (−) or presence (+) of 4 μg tPA at 37 °C for 1 h and subsequently immunoprecipitated using an anti-apo(a) polyclonal antibody. Samples were reduced with 10 mM DTT and resolved by SDS−PAGE using 12% gels. Gels were treated with Enlightning (Dupont), dried, and exposed to film. (Panel A) Lanes 1 and 4: CM harvested from mock-transfected cells. Lanes 2 and 5: CM harvested from cells transfected with pRK5ha17. Lanes 3 and 6: CM harvested from cells transfected with pRK5ha17Δser. Arrow: Apo(a) protease domain released upon reduction. (Panel B) CM harvested from mock-transfected cells or cells transfected with pRK5ha10 or pRK5ha10Δser. Arrow: apo(a) protease domain released upon reduction. (Panel C) CM harvested from mock-transfected cells or cells transfected with pRK5ha1/37 or pRK5ha1/37Δser. Arrows: apo(a) amino terminal domain (A) and protease domain (B) released upon reduction; arrowhead: position of uncleaved r-apo(a). For all panels, the positions of molecular mass standards are indicated to the left of the fluorograms. Note that for the 17- and 10-kringle r-apo(a) variants (Mr ~525 000 and ~290 000, respectively), the uncleaved species and the N-terminal fragments of the cleaved species could not be visualized on these 12% polyacrylamide gels.

unexpected as activation of plasminogen by tPA is relatively inefficient in solution, and requires fibrin as a cofactor for maximal efficiency (Hoylaerts *et al.*, 1982).

We determined by Western blot analysis that tPA cleavage of equimolar amounts of transiently expressed pRK5ha17Δser and purified plasminogen results in approximately equivalent levels of the carboxyl-terminal fragment released upon reduction (see Figure 3). The polyclonal antibody used in this experiment was raised against 17-kringle r-apo(a), and has been found to cross-react with plasminogen (M.L.K., unpublished results). The smaller size of the apo(a) protease domain relative to the plasminogen light chain likely reflects a nine amino acid deletion present in the protease domain of apo(a) (McLean *et al.*, 1987) (see below).

Analysis of the Proteolytic Activity of Wild-Type r-Apo(a) and r-Apo(a) Containing the Ser→Arg Substitution. A variety of chromogenic tripeptide substrates (S-2222, S-2238, S-2251, S-2266, S-2288, S-2302, S-2366, S-2390, S-2444, S-2484, S-2586, and S-2765) were used to assay the activity of CM collected from cells transfected with either pRK5ha17 or pRK5ha17Δser as described in Experimental Procedures. None of these substrates, which are specific for a variety of serine proteases, were observed to be cleaved by wild-type r-apo(a) or the mutant r-apo(a) treated with tPA-Sepharose. Of note is the absence of activity of tPA-Sepharose-treated mutant r-apo(a) against the chromogenic substrates S-2251 and S-2765. S-2251 is a plasmin-specific substrate and was effectively cleaved when tPA-Sepharose, at levels used in the preceding experiments, was added to purified plasminogen (ΔA_{405} = 0.0225/min; data not shown). The uniform inactivity of both r-apo(a) species against this panel of chromogenic tripeptide substrates suggests that the Ser→Arg substitution is not the only change in the apo(a) protease domain which results in the absence of proteolytic activity.

To examine the effect of multiple kringle repeats on the potential proteolytic activity of r-apo(a), two truncated

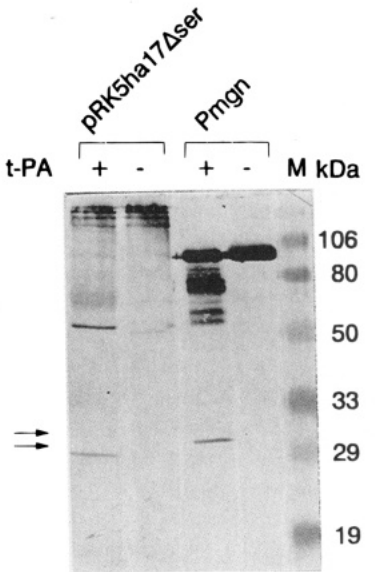

FIGURE 3: Analysis of the efficiency of tPA cleavage of r-apo(a) and plasminogen. Plasminogen (0.25 μg) or CM harvested from pRK5ha17Δser-transfected cells [containing 1 μg/mL r-apo(a)] were incubated in the absence (−) or presence (+) of tPA (2 μg) for 1 h at 37 °C. Samples were concentrated, reduced with 10 mM DTT in Laemmli sample buffer and separated by SDS−PAGE using a 12% gel. Bands were visualized by Western blot analysis as described in Experimental Procedures. Arrows indicate the positions of the protease domains released upon reduction. Prestained low-range molecular mass standards (BioRad) are shown in the far right lane.

versions of pRK5ha17 (pRK5ha10 and pRK5ha1/37) and mutant forms in which the tPA cleavage site was reinstated (pRK5ha10Δser and pRK5ha1/37Δser) were constructed (see Figure 1). As shown in Figure 2 (panels B and C; see above), r-apo(a) species corresponding to both pRK5ha10Δser and pRK5ha1/37Δser were cleaved by tPA. However, both truncated forms of r-apo(a), as well as their counterparts containing the tPA cleavage site, were found to be inactive

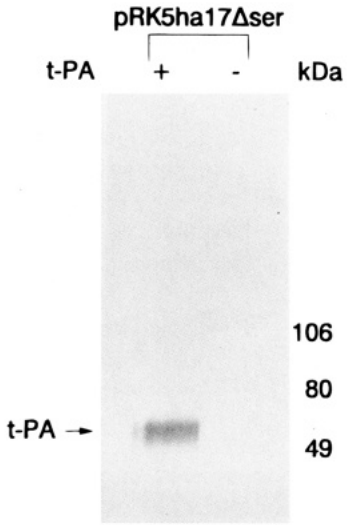

FIGURE 4: Determination of the incorporation of [^{3}H]DFP into tPA-cleaved r-apo(a). Purified protein corresponding to the expression plasmid pRK5ha17Δser (5 μg) was incubated in the absence (−) or presence (+) of tPA for 1 h at 37 °C. Samples were diluted to 1 mL with PBS and incubated with 30 μCi of [^{3}H]DFP for an additional 3 h at 37 °C. Following 20-fold concentration, samples (nonreduced) were resolved by SDS−PAGE using a 4−15% polyacrylamide gradient gel. The gel was treated with En^{3}Hance (Dupont), dried, and exposed to film. The position of the tPA containing incorporated [^{3}H]DFP is shown with an arrow. The positions of the molecular mass standards are shown to the right of the fluorogram.

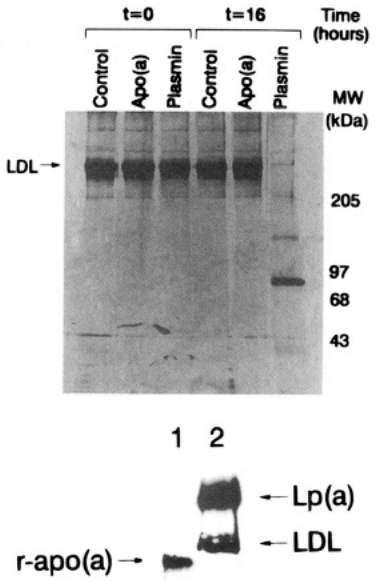

FIGURE 5: Proteolytic degradation of LDL by plasmin but not r-Lp-(a). (Panel A) Purified LDL (80 μg/mL) was incubated with either plasmin or r-apo(a) at a molar ratio of 20:1 (LDL:r-apo(a) or plasmin) for 16 h at 37 °C. Control reactions contained only LDL. Incubations were terminated at the indicated time by the addition of an equal volume of 2× Laemmli sample buffer. The samples were boiled, and volumes corresponding to 1 μg of LDL were subjected to SDS−PAGE using a 2.5−15% polyacrylamide gradient gel. Resolved proteins were visualized by silver staining. The position of untreated LDL is indicated to the left of the gel while the positions of molecular mass standards are indicated on the right. (Panel B) An aliquot of the r-apo(a)/LDL reaction was subjected to SDS−PAGE using a 3−12% polyacrylamide gradient gel (nonreducing conditions) followed by Western blot analysis using a polyclonal antibody raised in rabbits against r-apo(a) (lane 2); lane 1 contains an amount of purified r-apo(a) equivalent to that present in the reaction aliquot. The positions of uncomplexed r-apo-(a), r-Lp(a), and LDL (visualized due to cross-reactivity with the anti-apo(a) antibody) are indicated.

against S-2251 following treatment or mock treatment with tPA-Sepharose. These results suggest that the absence of plasmin-like activity in apo(a) is not the result of structural constraints imposed by the presence of multiple kringle repeats.

Incorporation of [^{3}H]DFP. It has previously been demonstrated that purified Lp(a) does not incorporate the irreversible serine protease inhibitor DFP (McLean *et al.*, 1987; Jauhiainen *et al.*, 1991), suggesting that the active site serine is either inaccessible owing to steric constraints or is not sufficiently nucleophilic. Purified protein corresponding to the tPA cleavage site mutant pRK5ha17Δser was either mock-treated or treated with tPA and examined for its ability to incorporate [^{3}H]DFP. As shown in Figure 4 (lane 1), [^{3}H]-DFP was incorporated into tPA but not into r-apo(a) corresponding to pRK5ha17Δser. No [^{3}H]DFP incorporation was observed in the absence of tPA, as expected (Figure 4, lane 2).

Assessment of the Proteolytic Degradation of LDL by r-Lp-(a) and Plasmin. LDL (40 μg/mL) was incubated for 16 h at 37 °C in the absence or presence of either purified r-apo-(a) or plasmin (at a molar ratio of approximately 20:1). Control reactions (in which no incubation at 37 °C was performed) were terminated immediately after the addition of either r-apo(a) or plasmin. All samples were analyzed by SDS−PAGE using a 2.5−15% polyacrylamide gradient gel, and protein bands were visualized by silver staining (Figure 5A). The formation of r-Lp(a) species upon incubation of r-apo(a) and LDL is quantitative under these conditions, and was verified by Western blot analysis of an aliquot of the reaction (Figure 5B). The lower molecular weight band apparent in lane 2 of the blot corresponds to LDL and was visualized due to cross-reactivity of the anti-apo(a) polyclonal antibody with LDL (M.L.K., unpublished results).

Although LDL is efficiently degraded by plasmin following 16 h incubation at 37 °C, no degradation of LDL was observed in the presence of r-Lp(a) under these conditions. The pattern of degradation of LDL by plasmin is similar to that reported by Steele (1979). We have also tested the proteolytic activity of the r-Lp(a) formed by incubation of r-apo(a) and LDL against the chromogenic peptide substrates S-2251, S-2765, and S-2222. No amidolytic activity attributable to the Lp(a) particle was observed.

DISCUSSION

We have examined the proteolytic activity of a recombinant form of apo(a) [r-apo(a)] containing 17 plasminogen kringle IV-like repeats followed by the kringle V and protease domains. We have also analyzed mutant derivatives of this protein for proteolytic activity. Specifically, we have determined the effect of a serine to arginine substitution corresponding to amino acid position 4 308 in the published cDNA sequence (McLean *et al*, 1987) which reinstates the tPA cleavage site in the protease domain of the 17-kringle r-apo(a). This substitution resulted in tPA cleavage of the corresponding mutant r-apo(a) at this position, which is analogous to tPA cleavage of the Arg560−Val561 bond in plasminogen. Interestingly, this amino acid substitution and subsequent processing by tPA did not result in protease activity of the resultant r-apo(a) derivative, even towards the specific plasmin substrate S-2251. A possible explanation for this lack of activity could be structural constraints

imposed by the large number of kringle repeats present in the 17-kringle form of r-apo(a). Therefore, we constructed truncated versions of pRK5ha17Δser that varied in the number of kringle IV motifs (see Figure 1). Recombinant apo(a) species corresponding to pRK5ha10Δser and pRK5ha1/37Δser (containing 10 and 1 apo(a) kringle IV domains, respectively) were cleaved by tPA but again were found to be inactive against the plasmin substrate S-2251. These data demonstrate that the lack of plasmin-like activity observed with r-apo(a) corresponding to pRK5ha17Δser was not due to the large number of kringle repeats present in this protein. Previous reports have suggested that plasma-derived Lp(a) and apo(a) obtained by reduction of Lp(a) have intrinsic serine protease activity with a side-chain specificity for arginine, rather than for lysine as would be expected on the basis of the homology of apo(a) and plasminogen (Salonen *et al.*, 1989; Chulkova, 1990; Jauhiainen *et al.*, 1991). We were unable to detect amidolytic activity of either the 17-kringle r-apo(a) or the 17-kringle Ser→Arg mutant against a variety of chromogenic serine protease substrates. These substrates included S-2765 and S-2222, which were previously reported to be hydrolyzed by both Lp(a) as well as apo(a) obtained by reduction of purified Lp(a) (Salonen *et al.*, 1989; Jauhiainen *et al.*, 1991). The amidolytic activities previously demonstrated with S-2765 and S-2222 were obtained using purified Lp(a) at a concentration of approximately 200 μg/mL, and a substrate concentration of 1.14 mM. More recently, this same group has examined the amidolytic activity of purified r-apo(a) (identical to the 17-kringle construct used in our study) (Pursiainen *et al.*, 1994a,b). Interestingly, no amidolytic activity was observed in these studies, although the concentration of apo(a) used was considerably lower (3.5−15.0 μg/mL) than that used in their previous study (Jauhiainen *et al.*, 1991), and is comparable to that which we have employed in our own investigation.

Recent reports have suggested that apo(a) purified from plasma Lp(a) (Chulkova & Tertov, 1993) as well as reconstituted Lp(a) formed from LDL and a 17-kringle form of r-apo(a) (Pursiainen *et al.*, 1994b) are able to degrade LDL. However in the present study we detected no degradation of LDL by an r-Lp(a) particle consisting of purified r-apo(a) and LDL (Figure 5). The degradation of LDL by Lp(a) or r-apo(a) which has been previously reported may result from the copurification of small amounts of contaminating protease(s). This is plausible, given the small amount of degradation of LDL (~1.5%) by r-Lp(a) that was reported by Pursiainen and co-workers (1994b) over an incubation period of 24 h at 37 °C using a large (~35-fold) molar excess of r-apo(a). It has been reported that human LDL purified from plasma by differential ultracentrifugation possesses a protease-like activity, which could be inhibited by addition of the serine protease inhibitor PMSF (Krishnaiah & Wiegandt, 1974). As such, it is also possible that previous reports of degradation of LDL by r-apo(a) or reconstituted Lp(a) may reflect a protease activity attributable to the LDL preparation itself. In our study, which differed from previous reports in that we purified LDL from plasma by gel filtration chromatography, we were unable to detect amidolytic activity of r-Lp(a) particles (formed *in vitro* from purified LDL and the 17-kringle r-apo(a) species) against a variety of chromogenic tripeptide protease substrates. These included the Factor Xa-specific substrate S-2765, which has previously been reported to be cleaved by r-Lp(a) particles formed from the 17-kringle r-apo(a) species (Pursiainen *et al.*, 1994a). Again, the amidolytic activity observed by these workers may reside in the LDL moiety.

Our results suggest that other alterations in the protease domain of apo(a) (in addition to the arginine to serine substitution at amino acid position 4 308) render apo(a) inactive. This is significant, since it has been suggested that polymorphisms may exist in the apo(a) gene that reinstate the tPA cleavage site (MBewu & Durrington, 1990). Our data demonstrating the uniform inactivity of r-Lp(a) and r-apo(a) (both wild-type and tPA-cleaved mutant forms) against both protein and artificial peptide substrates strongly suggest that even if such polymorphisms exist, the apo(a) protease domain would still be inactive upon cleavage by plasminogen activators.

The inability of the mutant protein corresponding to pRK5ha17Δser to incorporate DFP in the absence or presence of tPA suggests a potentially nonfunctional active site. This could be the consequence of (1) inaccessibility of the active site serine or (2) reduced nucleophilicity of the active site serine. A comparison of the primary structures of the protease domains of plasminogen and apo(a) (Figure 6) reveals that residues that are important in maintaining the specificity pocket and charge relay system have not been substituted in apo(a) (McLean *et al.*, 1987), suggesting that the side-chain specificity and activity should be unaffected. However, there is a nine amino acid deletion in apo(a) corresponding to residues 716−724 in plasminogen (Figure 6); this deletion falls within a region that appears to be conserved among a number of serine proteases (Furie *et al.*, 1981). Based on a modeled active site of plasmin (using the coordinates for elastase; Figure 7), the loop between residues Cys168 and Cys182 in elastase that is also present in plasminogen (between Cys709 and Cys725; see Figure 6) is not present in apo(a) due to the 9 amino acid deletion. Based on the model shown in Figure 7, this loop in plasmin appears to provide hydrophobic support to Trp760 (Trp215 in elastase), a residue that is functionally important in all serine proteases (Bode *et al.*, 1989). Partial deletion of this loop in apo(a) may collapse the active site as a result of unfavourable hydrophobic contacts with solvent molecules. The subtitution of Gly727 in plasminogen for a glutamic acid residue at the corresponding position in apo(a) (see Figure 6) might also render apo(a) proteolytically inactive. This glycine residue in plasminogen (present in an analogous position in a number of serine proteases) is quite close to the S1 residue Asp734 of plasminogen (Asp189 in elastase; see Figure 7). As such, substitution of this glycine for a bulky, charged residue such as glutamic acid might severely inhibit the proteolytic activity of apo(a). It has been previously reported that the replacement of an alanine with a threonine close to the active site histidine residue in plasminogen results in inactive plasmin species (Miyata *et al.*, 1982; Ichinose *et al.*, 1991). This suggests that substitutions in amino acids near the active site residues may affect proton transfer, such that the normal catalytic cycle cannot occur. It is of interest to note that there are a number of amino acid substitutions in the apo(a) protease domain in close proximity to the active site His and Asp residues (see Figure 7).

Additional evidence to support the apparent inactivity of the apo(a) protease domain is provided by a comparison of the human and rhesus monkey apo(a) sequences (Tomlinson *et al.*, 1989). Both human and rhesus apo(a) contain the

```
                                                    *
apo(a)   SIVGGCVAHPHSWPWQVSLRTRFGKHFCGGTLISPWEVLTAAHCLKKSSR          4357
pmgn     RVVGGCVAHPHSWPWQVSLRTRFGMHFCGGTLISPWEVLTAAHCLEKSPR          609
                                                    *

                                          *
apo(a)   PSSYKVILGAHQEVNLESHVQEIEVSRLFLEPTQADIALLKLSRPAVITD          4407
pmgn     PSSYKVILGAHQEVNLEPHVQEIEVSRLFLEPTRKDIALLKLSSPAVITD          659
                                          *

apo(a)   KVMPACLPSPDYMVTARTECYITGWGETQGTFGTGLLKEAQLLVIENEVC          4457
pmgn     KVIPACLPSPNYVVADRTECFITGWGETQGTFGAGLLKEAQLPVIENKVC          709

                                    *
apo(a)   NHYKYI---------CAEHLARGTDSCQGDSGGPLVCFEKDKYILQGVTS          4498
pmgn     NRYEFLNGRVQSTELCAGHLAGGTDSCQGDSGGPLVCFEKDKYILQGVTS          759
                                    *

apo(a)   WGLGCARPNKPGVYARVSTFVTWIEGMMRNN                           4529
pmgn     WGLGCARPNKPGVYVRVSRFVTWIEGVMRNN                           790
```

FIGURE 6: Alignment of apo(a) and plasminogen (pmgn) protease domains. The cleavage site for tPA and uPA is indicated with an arrow, and corresponds to the Arg560–Val561 bond in plasminogen. Amino acid substitutions are indicated by bold type, and the members of the catalytic triad are indicated with asterisks. Dashes correspond to a nine amino acid deletion in the apo(a) sequence (see text for details). Numbering of the amino acid residues of apo(a) (McLean *et al.*, 1987) and plasminogen (Castellino & Powell, 1981) is indicated to the right.

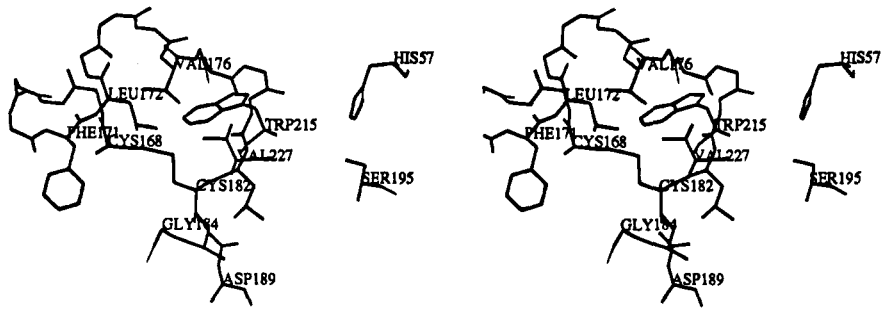

FIGURE 7: Stereo diagram of the modeled active site of plasmin based on the crystal structure of porcine pancreatic elastase (Bode *et al.*, 1989). The numbering system is that used for chymotrypsin, where the members of the catalytic triad are His57, Asp102, and Ser195. Note in particular the loop structure bounded by Cys168 and Cys182, which encompasses the nine amino acid deletion in the apo(a) protease-like domain (see text for details).

mutation that replaces the plasminogen activator cleavage site arginine with a serine. The deletion of 9 amino acids which characterizes the protease domain in human apo(a) is not present in rhesus monkey apo(a). However, the catalytic triad of rhesus monkey apo(a) has undergone considerable changes. The active site histidine and serine residues have been replaced by cysteine and asparagine, respectively. Although the proteolytic activity of rhesus apo(a) has not been investigated, these changes likely render rhesus monkey apo(a) proteolytically inactive as site-directed mutagenesis of members of the catalytic triad of trypsin and subtilisin dramatically reduces their protease activity (Higaki *et al.*, 1987; Carter & Wells, 1988).

The absence of proteolytic activity of apo(a) may be significant from an evolutionary perspective, since it is clear that apo(a) has arisen by duplication of the plasminogen gene. Since the true biological role of Lp(a) remains unclear, loss of proteolytic activity may be important for the as yet undefined biological function of this molecule. Furthermore, the lack of plasmin-like activity of Lp(a) would serve to amplify the prothrombotic effects of this lipoprotein, which have been proposed to reside in the ability of Lp(a) to compete with the normal fibrinolytic functions of plasminogen.

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