Sites within the 39-kDa Protein Important for Regulating Ligand Binding to the Low-Density Lipoprotein Receptor-Related Protein†

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ABSTRACT: A 39-kDa protein copurifies with the low-density lipoprotein receptor-related protein/α_2-macroglobulin receptor (LRP) and inhibits the binding and/or cellular uptake of ligands by this receptor. We recently utilized glutathione *S*-transferase (GST)−39-kDa fusion protein constructs to demonstrate that constructs encoding amino-terminal residues 1−114 and carboxy-terminal residues 115−319 of the 39-kDa protein independently bind to purified LRP and to LRP on hepatoma cells with similar affinities as the full-length GST−39-kDa protein ($K_d \sim 8-10$ nM). These regions, however, inhibit ligand binding to LRP differently: GST/1−114 inhibits both tissue-type plasminogen activator (t-PA) and α_2-macroglobulin−methylamine (α_2M*) binding whereas GST/115−319 only potently inhibits t-PA binding. Four domains, containing residues 18−24 and 100−107 within amino-terminal constructs and residues 200−225 and 311−319 within carboxy-terminal constructs, are required for inhibition of ligand binding. In the present study, we generated additional 39-kDa protein constructs to precisely define residues within each domain required for inhibition of t-PA and α_2M* binding to LRP. The potential importance of these residues in mediating direct binding both to purified LRP and to LRP on hepatoma cells was examined. Within amino-terminal residues 1−114, alanine 103 and leucine 104 are required for inhibition of t-PA and α_2M* binding. These residues, however, are not required for binding either to purified LRP or to LRP on hepatoma cells. Within domain 18−24, arginine 21 is required for inhibition of t-PA and α_2M* binding as well as for the direct binding of amino-terminal constructs to LRP. Within carboxy-terminal domains 200−225 and 311−319, leucine 222 and leucine 319 are both required for inhibition of t-PA binding. Deletion of leucine 319 changes the ligand specificity from inhibition of t-PA binding to inhibition of α_2M* binding. Thus, leucine 319 is not required for direct binding to LRP whereas leucine 222 is required for high-affinity binding to LRP.

The low-density lipoprotein receptor-related protein/α_2-macroglobulin receptor (LRP)[1] as a cell-surface glycoprotein that functions as a hepatic clearance receptor for several circulating plasma proteins. LRP is synthesized as a ~600-kDa single-chain precursor protein and is cleaved in the *trans* Golgi into two subunits of 515 and 85 kDa which remain noncovalently associated on the cell surface. The 515-kDa subunit contains 15 epidermal growth factor repeats and 31 ligand binding complement-type repeats. The 85-kDa subunit contains seven epidermal growth factor repeats, a transmembrane domain, and two NPXY internalization sequences essential for rapid endocytosis of the ligand−receptor complex through coated pits [reviewed in Krieger and Herz (1994)].

LRP binds a diverse array of ligands, including α_2-macroglobulin−methylamine (α_2M*) (Moestrup & Gliemann, 1991; Williams et al., 1992), β-migrating very low density lipoproteins complexed with either apolipoprotein E (apoE/BVLDL) (Beisiegel et al., 1989; Kowal et al., 1989) or lipoprotein lipase (LPL/βVLDL) (Willnow et al., 1992; Nykjaer et al., 1993), uncomplexed LPL (Nykjaer et al., 1993; Beisiegel et al., 1991), lactoferrin (Willnow et al., 1992), *Pseudomonas* exotoxin A (Kounnas et al., 1992), tissue-type plasminogen activator (t-PA) (Bu et al., 1992a), urokinase-type plasminogen activator (u-PA) (Kounnas et al., 1993), complexes of t-PA and u-PA with plasminogen activator inhibitor type-1(PAI-1) (Bu et al., 1993; Orth et al., 1992; Nykjaer et al., 1992), and a 39-kDa protein which copurifies with LRP (Ashcom et al., 1990; Strickland et al., 1991). This 39-kDa protein, also termed receptor-associated protein (RAP), potently inhibits the binding and/or cellular uptake of all ligands by LRP and may regulate LRP activity *in vivo* (Bu et al., 1992a, 1993; Kounnas et al., 1993; Orth et al., 1992; Nykjaer et al., 1992; Herz et al., 1991; Warshawsky et al., 1993a; Moestrup et al., 1991).

Competition binding experiments between LRP-specific ligands suggest LRP contains both independent and partially overlapping ligand binding domains. For example, the finding that α_2M* and t-PA do not cross-compete with one

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[1] Abbreviations: LRP, low-density lipoprotein receptor-related protein/α_2-macroglobulin receptor; t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator; PAI-1, plasminogen activator inhibitor type-1; α_2M*, α_2-macroglobulin−methylamine; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; βVLDL, β-migrating very low density lipoprotein(s); apo E, apolipoprotein E; LPL, lipoprotein lipase; GST, glutathione *S*-transferase; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; K_d, equilibrium dissociation constant.

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another for LRP binding, as shown by cross-linking and immunoprecipitation experiments, implies these ligands bind to independent sites on LRP (Bu et al., 1992a). Hussain et al. (1991) reported that α_2M* and apoE/βVLDL partially cross-compete for binding to LRP, but only at high concentrations, suggesting the competition results from steric hindrance at neighboring but not identical sites. The observation that degradation of t-PA−PAI-1 complexes by fibroblasts is not inhibited by LPL/βVLDL, apoE/βVLDL, lactoferrin, or α_2M* implies that t-PA−PAI-1 complexes bind to a site on LRP separate from the other ligands (Willnow et al., 1992). The binding sites for α_2M* and lactoferrin are also separate since these ligands do not inhibit one another's degradation in fibroblasts (Willnow et al., 1992). Cross-competition binding experiments with lactoferrin, LPL/βVLDL, and apoE/βVLDL suggest that these ligands bind to three distinct, but partially overlapping sites on LRP (Willnow et al., 1992). Recently, it was reported that α_2M* (100 nM) inhibits ^{125}I-LPL (25 pM) binding by 50% whereas LPL (100 nM) inhibits ^{125}I-α_2M* (20 pM) binding by 85−90% (Nykjaer et al., 1993). These results may suggest that LPL binds to multiple sites on LRP and shares about half of these sites with α_2M*. Most recently, the binding of the 39-kDa protein (RAP), u-PA−PAI-1 complexes, and α_1-macroglobulin light chain was localized to a 624 amino acid region of LRP containing a cluster of 8 complement-repeats (Moestrup et al., 1993). Whether these ligands bind to distinct sites within this cluster is not currently known.

It is intriguing that the 39-kDa protein inhibits the binding and/or cellular uptake of all ligands by LRP. The mechanism(s) by which the 39-kDa protein inhibit(s) ligand interactions with LRP is (are) not clear. LRP on hepatoma cells has approximately 6 times as many 39-kDa protein binding sites as t-PA (ladonato et al., 1993) or α_2M* (Bu et al., unpublished observation) binding sites. This may suggest each independent ligand binding domain on LRP can also bind the 39-kDa protein. Binding of the 39-kDa protein may sterically block ligand binding or may induce a local conformational change in LRP such that ligand binding is abolished. To examine whether different regions of the 39-kDa protein could bind to LRP and to define domains on the 39-kDa protein required for inhibition of t-PA and α_2M* binding, we previously generated a series of glutathione *S*-transferase (GST) fusion protein constructs encoding different regions of the 39-kDa protein (Warshawsky et al., 1993b). We reported that constructs encoding amino-terminal residues 1−114 and carboxy-terminal residues 115−319 each bind to purified LRP and to LRP on hepatoma cells with affinities similar to the full-length GST−39-kDa protein (K_d values of ~8−10 nM). These regions, however, bind to separate sites on LRP and regulate ligand binding differently: Amino-terminal regions inhibit both t-PA and α_2M* binding whereas carboxy-terminal regions also potently inhibit t-PA binding (Warshawsky et al., 1993b, 1994). Inhibition of t-PA and α_2M* binding requires domains containing residues 18−24 and 100−107 within amino-terminal constructs whereas inhibition of t-PA binding by carboxy-terminal constructs requires domains containing residues 200−225 and 311−319 (Warshawsky et al., 1993b). In the present study, we have defined five residues on the 39-kDa protein within domains 18−24, 100−107, 200−225, and 311−319 required for inhibition of t-PA and α_2M* binding to LRP. We also investigated whether these residues were required for binding to purified LRP and compared the relative binding affinities between constructs that contained residues critical for inhibition of ligand binding with constructs that had these residues deleted. The inhibitory effects of various constructs on the binding of ^{125}I-labeled 39-kDa protein and ^{125}I-labeled amino- and carboxy-terminal 39-kDa protein constructs to hepatoma cells were also examined.

EXPERIMENTAL PROCEDURES

Materials. Single-chain recombinant human t-PA expressed in Chinese hamster ovary cells was supplied by Genentech (Lot 9124AX). α_2-macroglobulin was purified from human plasma and activated with methylamine (to yield α_2M*) (Warshawsky et al., 1993b). Human placental LRP was purified by α_2M*−Sepharose chromatography (Ashcom et al., 1990) followed by heparin−agarose chromatography to remove the 39-kDa protein that copurifies with LRP. Affinity-purified anti-39-kDa protein IgG and antibodies directed against human placental LRP were described previously (Warshawsky et al., 1993b; Bu et al., 1993). Carrier-free sodium [^{125}I]iodide and Hyperfilm-MP were purchased from Amersham. Iodogen was from Pierce. Enzymobeads were from Bio-Rad. Glutathione−agarose, heparin−agarose, and reduced glutathione were from Sigma. Goat anti-rabbit IgG was from Boehringer Mannheim. The pGEX2T vector was purchased from Pharmacia. Taq DNA polymerase and 10× PCR buffer were from Promega. Oligonucleotides were synthesized in the Washington University School of Medicine Protein Chemistry facility. Tissue culture media were from GIBCO/BRL.

Plasmid Construction. The pGEX2T expression vector, which produces a fusion protein between glutathione *S*-transferase (GST) and the target protein, was used to generate GST−39-kDa protein constructs. Polymerase chain reaction (PCR) amplification of the rat 39-kDa cDNA encoding specific sequences of the 39-kDa protein was performed as described previously (Warshawsky et al., 1993b). The PCR primers used are shown in Table 1 or listed previously (Warshawsky et al., 1993b). Each forward primer contains a *Bam*HI site (single underline) while each reverse primer has a TGA stop codon immediately after the coding segment followed by an *Eco*RI site (boldface). Five base overhangs were included at the free ends of the *Bam*HI and *Eco*RI sites to facilitate binding of restriction enzymes. PCR products were electrophoresed on agarose, excised, and gel-purified using Geneclean II or Mermaid kits (Midwest Scientific). The PCR products were then digested with *Bam*HI and *Eco*RI, and the resultant fragments were gel-purified again and ligated to gel-purified *Bam*HI- and *Eco*RI-cut pGEX2T for 16−18 h at 16 °C. Ligation reactions were used to transform *Escherichia coli* DH5α-competent cells. Plasmids of the obtained colonies were isolated and subjected to restriction analysis. The DNA sequences of the constructs were verified by dideoxy sequencing (Sanger et al., 1977) using the Sequenase 2.0 kit (United States Biochemical) and were identical to the coding sequence of the 39-kDa protein (Pietromonaco et al., 1990) except construct GST/1−114 (del 103,104) which had a single base change at amino acid 10 where ATG (Met) was changed to ATT (Ile). The primers used to sequence the constructs were 5′-CAAATTGATAAGTACTTGAAAT-3′ and 5′-TGCATGTGTCAGAGGTTTTCA-

Table 1: Polymerase Chain Reaction Primers Used for Cloning 39-kDa Protein Constructs

construct	forward primers	reverse primers
GST/18−114	see Warshawsky et al. (1993b)	see Warshawsky et al. (1993b)
GST/18−104	see Warshawsky et al. (1993b)	ACGATGAATTCTCAGAGGGCGTTGCTGTGCAC
GST/18−103	see Warshawsky et al. (1993b)	ACGATGAATTCTCAGGCGTTGCTGTGCACTGT
GST/18−102	see Warshawsky et al. (1993b)	ACGATGAATTCTCAGTTGCTGTGCACTGTCTG
GST/18−100	see Warshawsky et al. (1993b)	see Warshawsky et al. (1993b)
GST/(RMEK)21−107	CGCGTGGATCCCGCATGGAGAAGCTGAACCAG	see Warshawsky et al. (1993b)
GST/22−107	CGCGTGGATCCATGGAGAAGCTGAACCAGCTA	see Warshawsky et al. (1993b)
GST/23−107	CGCGTGGATCCGAGAAGCTGAACCGCTATGG	see Warshawsky et al. (1993b)
GST/24−107	see Warshawsky et al. (1993b)	see Warshawsky et al. (1993b)
GST/(AAAA)Δ21−107	CGCGTGGATCCGCCGCCGCCGCCCTGAACCAGCTATGGGAG	see Warshawsky et al. (1993b)
GST/(AAEK)Δ21−107	CGCGTGGATCCGCCGCCGAGAAGCTGAACCAGCTA	see Warshawsky et al. (1993b)
GST/(RMMA)Δ21−107	CGCGTGGATCCCGCATGGCCGCCCTGAACCAGCTATGG	see Warshawsky et al. (1993b)
GST/212−319(HNEL)	CGCGTGGATCCACTGAGTTTGAAGAGCCCCGA	see Warshawsky et al. (1993b)
GST/222−319	CGCGTGGATCCCTGTGGGACCTGGCTCAGTCT	see Warshawsky et al. (1993b)
GST/223−319	CGCGTGGATCCTGGGACCTGGCTCAGTCTGCC	see Warshawsky et al. (1993b)
GST/224−319	CGCGTGGATCCGACCTGGCTCAGTCTGCCAAC	see Warshawsky et al. (1993b)
GST/212−318	CGCGTGGATCCACTGAGTTTGAAGAGCCCCGA	ACGATGAATTCTCACTCATTGTGCCGAGCCCT
GST/212−317	CGCGTGGATCCACTGAGTTTGAAGGAGCCCCGA	ACGATGAATTCTCAATTGTGCCGAGCCCTTGA
GST/Δ319(AAAA)	CGCGTGGATCCACTGAGTTTGAAGAGCCCCGA	ACGATGAATTCTCAGGCGGCGGCGGCCCGAGCCCTTGAGACCCT
GST/212−Δ319(HNEA)	CGCGTGGATCCACTGAGTTTGAAGAGCCCCGA	ACGATGAATTCTCAGGCCTCATTGTGCCGAGCCCT
GST/212−Δ319(HNED)	CGCGTGGATCCACTGAGTTTGAAGAGCCCCGA	ACTGATGAATTCTCAGTCCTCATTGTGCCGAGCCCT
GST/212−Δ319(HNEAL)	CGCGTGGATCCACTGAGTTTGAAGAGCCCCGA	ACGATAATTCTCAGAGGGCCTCATTGTGCCGAGCCCT
GST/212−Δ319(HNL)	CGCGTGGATCCACTGAGTTTGAAGAGCCCCGA	ACGATGAATTCTCAGAGATTGTGCCGAGCCCTTGA
GST/1−114(del 103, 104)	see Warshawsky et al. (1993b)	ACGATGAATTCTCAGTCCCCCAGCTCATCCTTGGGTCTGTTCATT GTTGCTGTGCACTGTCTG

3′, corresponding to nucleotides 818−839 and 992−1012 in the pGEX2T vector, respectively.

Expression and Purification of Recombinant GST Fusion Proteins. Recombinant proteins were purified to homogeneity by one-step glutathione−agarose affinity chromatography as described previously (Warshawsky et al., 1993b). Briefly, *E. coli* harboring the expression plasmids were grown at 37 °C to an optical density of 0.4−0.5 at 600 nm. Expression was induced by the addition of isopropyl β-D-galactoside to a final concentration of 0.01%, and the cultures were grown for 4−6 h at 30 °C. Cultures were harvested by centrifugation and resuspended in PBS containing 1% (v/v) Triton X-100, 1 μM pepstatin, 2.5 μg/mL leupeptin, and 0.2 mM phenylmethanesulfonyl fluoride. Cells were disrupted by sonication and centrifuged. After incubation of the supernatant overnight with glutathione−agarose beads, the beads were washed with PBS and 50 mM Tris-HCl at pH 8.0. Bound GST fusion proteins were eluted with 5 mM reduced glutathione in 50 mM Tris-HCl at pH 8.0. Eluates were dialyzed against PBS or 50 mM Tris-HCl at pH 8.0. The purity of the fusion proteins was confirmed by SDS−PAGE and staining with Coomassie brilliant blue. The absolute yield of several of the GST fusion proteins per liter of culture was low owing to formation of inclusion bodies. However, approximately 1−15 mg of soluble fusion proteins was obtained per liter of culture from the various constructs. Proteins were stable for at least 2 weeks at 4 °C.

Protein Iodinations. t-PA (Bu et al., 1992b), α_2M* (Warshawsky et al., 1993b), and 39-kDa protein constructs (Warshawsky et al., 1994) were iodinated as described previously. Goat anti-rabbit IgG was iodinated using the Iodogen method (Bu et al., 1992b). Specific activities were generally 5−10 μCi/μg of protein.

Ligand Binding Assays. Rat hepatoma MH_1C_1 cells were cultured as described previously (Bu et al., 1992b). Cells were seeded into 12 well-dishes 1 or 2 days prior to assay. Ligand binding buffer for t-PA was PBS containing 0.2 mM $CaCl_2$ and 10 mM ε-amino-*n*-caproic acid. Ligand binding buffer for α_2M* was Dulbecco's modified Eagle's medium containing 6 mg/mL bovine serum albumin and 5 mM $CaCl_2$. Ligand binding buffer for 39-kDa protein constructs was PBS containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$. Cell monolayers were washed twice on ice with the corresponding prechilled ligand binding buffer. Binding was initiated by adding binding buffer containing 4 nM ^{125}I-t-PA, 50 pM ^{125}I-α_2M*, or the indicated concentrations of ^{125}I-39-kDa protein constructs in the absence or presence of various competitors. After incubation for 2 h at 4 °C, cells were washed 3 times with 4 °C ligand binding buffer. Cells were lysed in 62.5 mM Tris-HCl, pH 6.8, containing 0.2% (w/v) SDS and 10% (v/v) glycerol. The radioactivity of cell lysates was determined in a Packard gamma counter; 100% binding was determined in the absence of any competitor protein. Nonspecific binding (typically less than 10% of total binding) was determined in the presence of the indicated concentrations of excess unlabeled ligand. The data are presented as the average of duplicate determinations from the indicated number of independent experiments (generally two to five) wherein the standard deviation is less than 10% of the mean.

Ligand Blotting. Purified human placental LRP (nonboiled, nonreduced) was subjected to 6% SDS−PAGE and transferred to nitrocellulose paper. Individual strips (containing ~0.2 μg of LRP/strip) were blocked for 1 h in 50 mM Tris-HCl, 150 mM NaCl, and 5% non-fat dry milk at pH 7.5 (blocking buffer). Strips were then incubated overnight at 4 °C with blocking buffer containing anti-LRP antiserum (1:500 dilution), 50 nM samples of various unlabeled GST−39-kDa protein constructs, or 20 nM aliquots of the indicated ^{125}I-GST−39-kDa protein constructs in the absence or presence of 1 μM unlabeled competitors. Binding of anti-LRP antibody was detected with ^{125}I-goat anti-rabbit IgG (1 × 10^6 cpm/mL). Binding of the unlabeled GST−39-kDa protein constructs to LRP was detected by incubating the strips with 1 μg/mL affinity-purified anti-39-kDa protein IgG followed by incubation with ^{125}I-goat anti-rabbit IgG (1 × 10^6 cpm/mL). Strips incubated with ^{125}I-GST−39-kDa protein constructs were washed with Tris-buffered saline and placed directly on film. Strips were exposed to film at room temperature for varying lengths of time.

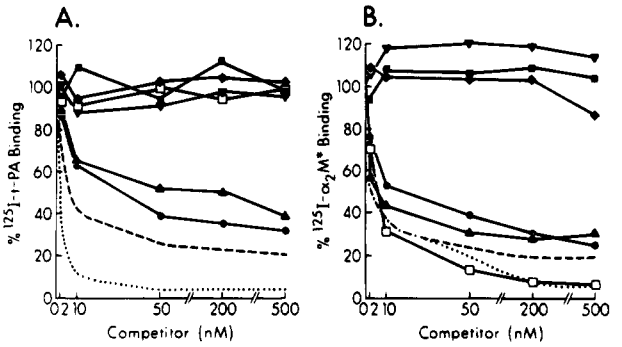


FIGURE 1: (Panel A) Inhibition of ^{125}I-t-PA binding to rat hepatoma MH_1C_1 cells by amino-terminal constructs. Cells were incubated for 2 h at 4 °C with 4 nM ^{125}I-t-PA in the absence or presence of increasing concentrations of 39-kDa protein constructs. Each symbol represents the average of duplicate determinations from the indicated number (*n*) of independent experiments. Nonspecific binding, determined in the presence of 500 nM unlabeled t-PA, has been subtracted from each point. 100% binding was determined in the absence of competitor protein. The constructs used were GST/1−319 (•••) (*n* = 2), GST/1−114 (- - -) (*n* = 3), GST/18−114 (●) (*n* = 2), GST/18−104 (▲) (*n* = 5), GST/18−103 (□) (*n* = 4), GST/18−102 (♦) (*n* = 4), GST/18−100 (▼) (*n* = 3), and GST/1−114 (del 103,104) (■) (*n* = 2). (Panel B) Inhibition of ^{125}I-α_2M* binding to MH_1C_1 cells. The inhibition of 50 pM ^{125}I-α_2M* binding was performed as described above in panel A. Nonspecific binding, determined in the presence of 50 nM unlabeled α_2M*, has been subtracted from each point. 100% binding was determined in the absence of competitor protein. The constructs used were GST/1−319 (•••) (*n* = 2), GST/1−114 (- - -) (*n* = 4), GST/18−114 (●) (*n* = 1), GST/18−104 (▲) (*n* = 2), GST/18−103 (□) (*n* = 3), GST/18−102 (♦) (*n* = 3), GST/18−100 (▼) (*n* = 2), and GST/1−114 (del 103,104) (■) (*n* = 2).

RESULTS

Residues Required for Inhibition of Ligand Binding within the Amino Terminus of the 39-kDa Protein. We previously demonstrated (Warshawsky et al., 1993b) that amino-terminal residues 1−114 of the 39-kDa protein, generated as a fusion protein with glutathione *S*-transferase (GST/1−114), inhibited both ^{125}I-t-PA and ^{125}I-α_2M* binding to LRP on rat hepatoma MH_1C_1 cells similarly to the full-length protein, GST/1−319 (see Figure 1). Domains containing residues 18−24 and 100−107 within the amino terminus were both required for this inhibition (Warshawsky et al., 1993b).

To further define residue(s) within domain 100−107 required for inhibition of ligand binding to LRP, a construct terminating at residue 104 was generated. Figure 1A shows that this construct, GST/18−104, effectively inhibits ^{125}I-t-

Table 2: Summary of 39-kDa Protein Constructs[a]

construct	K_i values (nM)		% binding inhibited at 500 nM competitor		K_d values (nM) (direct binding)
	t-PA inhibition	α_2M* inhibition	t-PA	α_2M*	
GST/1−319	3[b]	2[b]	95[b]	95[b]	8.1[c]
GST/1−114	8[b]	4[b]	75[b]	75[b]	9.7[c]
GST/18−114	~20	~5	65	70	
GST/18−104	Y50	~5	55	65	
GST/18−103	none	~5	none	95	
GST/18−102	none	none	none	none	
GST/18−100	none	none	none	none	
GST/(RMEK)21−107	8	15	80	70[d]	15[d]
GST/22−107	~200	>200	55	40[d]	
GST/23−107	≫500	none	none	none[d]	no binding[d]
GST/24−107	≫500	none	none	none[d]	
GST/(AAAA)Δ21−107	>500	>500	40	20[d]	
GST/(AAEK)Δ21−107	≫500	>500	20	25[d]	
GST/(RMAA)Δ21−107	~200	~200	65	50[d]	
GST/1−114(del 103, 104)	none	none	none	none	45[d]
GST/115−319	2	none[b]	95	none[b]	8.9[c]
GST/212−319(HNEL)	5	none	65	none	
GST/222−319	20	none[d]	60	none[d]	8.1[d]
GST/223−319	>500	none	40	none[d]	
GST/224−319	>500	none[d]	35	none[d]	>500[d]
GST/225−319	none[b]	none[b]	none[b]	none[b]	
GST/212−318	none	~200	none	60	
GST/212−317	none	none	none	none	
GST/212−Δ319(AAAA)	none	>500	none	35	
GST/212−Δ319(HNAL)	5	none	45	none	
GST/212−Δ319(HNEA)	none	>500	none	30	
GST/212−Δ319(HNED)	none	none	none	none	
GST/212−Δ319(HNEAL)	none	~500	none	50	
GST/212−Δ319(HNL)	none	~500	none	50	

[a] The K_i values for inhibition of ^{125}I-t-PA and ^{125}I-α_2M* binding to MH_1C_1 cells by GST−39-kDa protein constructs are indicated. The percent inhibition of ligand binding by 500 nM of the construct is also shown. These values are the average of duplicate determinations from 2−6 separate experiments. Equilibrium dissociation constants (K_d) for selected ^{125}I-labeled 39-kDa protein constructs were determined from saturating binding experiments. Values are derived from Scatchard analysis of specific binding and are the average of duplicate determinations from a single binding assay. [b] See Warshawsky et al. (1993b). [c] See Warshawsky et al. (1994). [d] Data not shown.

PA binding to MH_1C_1 cells, although the apparent K_i value[2] for inhibition (~50 nM) was increased and the extent of inhibition by 500 nM GST/18−104 was slightly less than that observed with either 500 nM GST/18−114 or 500 nM GST/1−114 (Table 2). Nevertheless, these results implied residues within 100−104 were important and constructs terminating at residues 103, 102, and 100 were generated. As seen in Figure 1A, the constructs GST/18−103, GST/18−102, and GST/18−100 do not inhibit ^{125}I-t-PA binding and suggest L104 is required to inhibit binding within the amino terminus.

We next examined the inhibition of ^{125}I-α_2M* binding by these same constructs. Figure 1B demonstrates that GST/18−104 inhibits ^{125}I-α_2M* binding similarly to the constructs GST/18−114 and GST/1−114. Whereas GST/18−103 did not inhibit ^{125}I-t-PA binding (Figure 1A), GST/18−103 inhibits ^{125}I-α_2M* binding essentially identically to the full-length protein, GST/1−319. GST/18−103 inhibits ^{125}I-α_2M* binding more potently and to a greater extent than GST/18−104. This may suggest that L104 has an inhibitory effect on the inhibition of ^{125}I-α_2M* binding or that GST/18−103 binds with a higher affinity to LRP than GST/18−104. Figure 1B also shows that the constructs GST/18−102 and GST/18−100 do not inhibit ^{125}I-α_2M* binding. Thus, A103 within the amino terminus appears to be important for inhibition of ^{125}I-α_2M* binding.

[2] Apparent K_i is defined as the concentration of competitor which inhibits 50% of radioligand binding as determined by inspection.

To confirm the importance of A103 and L104 within the full amino terminus (i.e., residues 1−114), a construct encompassing residues 1−114 in which A103 and L104 were deleted was generated. This construct, GST/1−114 (del 103,104), inhibits neither ^{125}I-t-PA binding (Figure 1A) nor ^{125}I-α_2M* binding (Figure 1B).

Using a similar strategy, we sought to define residue(s) important for inhibition of ligand binding within domain 18−24 of the 39-kDa protein. Figure 2A demonstrates that GST/21−107 inhibits ^{125}I-t-PA binding identically to GST/1−114 (K_i values of 8 nM). However, when R21 was removed, the apparent K_i value for inhibition of ^{125}I-t-PA binding by the resultant construct, GST/22−107, increased to ~200 nM. In addition, whereas GST/21−107 and GST/1−114 each inhibited 75−80% of ^{125}I-t-PA binding at 500 nM competitor, 500 nM GST/22−107 inhibited ~55% of binding. Figure 2A also shows that GST/23−107 and GST/24−107 do not inhibit ^{125}I-t-PA binding, suggesting that M22 is also important for inhibiting ^{125}I-t-PA binding. In Figure 2B, the effect of changing all or some of the amino acids within residues 21−24 to alanine was examined. When residues 21−24 were changed from RMEK to all alanines, the resulting construct, GST/(AAAA)Δ21−107, inhibited 40% of ^{125}I-t-PA binding at 500 nM competitor, and the apparent K_i value for inhibition was >500 nM (Table 2). Mutation of R21M22 to alanines resulted in the construct GST/(AAEK)Δ21−107, which was essentially noninhibitory (K_i value ≫500 nM). Changing E23K24 to alanines while

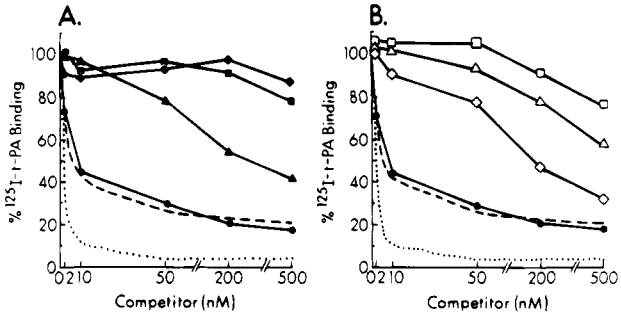


FIGURE 2: Inhibition of ^{125}I-t-PA binding to rat hepatoma MH_1C_1 cells requires R21 of the 39-kDa protein. Cells were incubated for 2 h at 4 °C with 4 nM ^{125}I-t-PA in the absence or presence of increasing concentrations of 39-kDa protein constructs. Each symbol represents the average of duplicate determinations from the indicated number (n) of independent experiments. Nonspecific binding, determined in the presence of 500 nM unlabeled t-PA, has been subtracted from each point. 100% binding was determined in the absence of competitor protein. (Panel A) The constructs used were GST/1−319 (•••) (n = 2), GST/1−114 (- - -) (n = 2), GST/21−107 (●) (n = 6), GST/22−107 (▲) (n = 4), GST/23−107 (■) (n = 3), and GST/24−107 (♦) (n = 3). (Panel B) The constructs used were GST/1−319 (•••) (n = 2), GST/1−114 (- - -) (n = 2), GST/(RMEK)21−107 (●) (n = 6), GST/(AAAA)Δ21−107 (△) (n = 3), GST/(AAEK)Δ21−107 (□) (n = 3), and GST/(RMAA)Δ21−107 (◇) (n = 3).

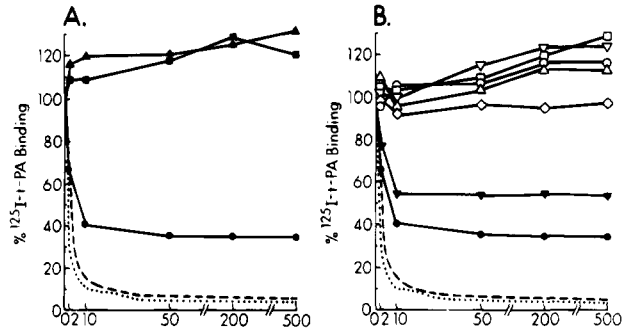


FIGURE 3: Inhibition of ^{125}I-t-PA binding to rat hepatoma MH_1C_1 cells by carboxy-terminal constructs. Cells were incubated for 2 h at 4 °C with 4 nM ^{125}I-t-PA in the absence or presence of increasing concentrations of 39-kDa protein constructs. Each symbol represents the average of duplicate determinations from the indicated number (n) of independent experiments. Nonspecific binding, determined in the presence of 500 nM unlabeled t-PA, has been subtracted from each point. 100% binding was determined in the absence of competitor protein. (Panel A) The constructs used were GST/1−319 (•••) (n = 2), GST/115−319 (- - -) (n = 2), GST/212−319 (●) (n = 5), GST/212−318 (▲) (n = 5), and GST/212−317 (■) (n = 3). (Panel B) The constructs used were GST/1−319 (•••) (n = 2), GST/115−319 (- - -) (n = 2), GST/212−319(HNEL) (●) (n = 5), GST/212−Δ319(HNAL) (▽) (n = 4), GST/212−Δ319-(AAAA) (○) (n = 2), GST/212−Δ319(HNEA) (△) (n = 3), GST/212−Δ319(HNED) (□) (n = 3), GST/212−Δ319(HNEAL) (▽) (n = 3), and GST/212−Δ319(HNL) (◇) (n = 3).

retaining R21M22 resulted in the construct GST/(RMAA)Δ21−107, which inhibited 65% of ^{125}I-t-PA binding at 500 nM. Thus, E23K24 within the amino terminus are important for high-affinity inhibition of ^{125}I-t-PA binding.

The same constructs were used to define residue(s) within the 18−24 region required for inhibition of ^{125}I-α_2M* binding. Essentially identical results seen for inhibition of ^{125}I-t-PA binding were obtained (Table 2). Thus, R21 of the 39-kDa protein is required within the amino terminus for inhibition of both ^{125}I-t-PA and ^{125}I-α_2M* binding to hepatoma cells, and E23K24 are required for high-affinity inhibition.

Residues Required for Inhibition of Ligand Binding within the Carboxy Terminus of the 39-kDa Protein. We previously reported that GST/115−319 inhibited ^{125}I-t-PA binding to hepatoma cells and that domains containing residues 200−225 and 311−319 were both required for this inhibition (Warshawsky et al., 1993b). To define more precisely those essential amino acid(s) within residues 200−225, the constructs GST/212−319 and GST/222−319 were made and found to inhibit ^{125}I-t-PA binding similarly with approximate K_i values of 5−20 nM (Table 2). Since GST/225−319 did not inhibit ^{125}I-t-PA binding (Warshawsky et al., 1993b), this implied residues within 222−225 were important, and the constructs GST/223−319 and GST/224−319 were generated. These constructs inhibit ^{125}I-t-PA binding poorly with K_i values >500 nM (Table 2). Thus, L222 within the carboxy terminus of the 39-kDa protein is important for inhibition of ^{125}I-t-PA binding.

To define residue(s) within domain 311−319 required for inhibition of ^{125}I-t-PA binding, residues 318 and/or 319 were deleted from the construct GST/212−319. As seen in Figure 3A, the resulting constructs GST/212−317 and GST/212−318 did not inhibit binding, demonstrating L319 is required for inhibition of ^{125}I-t-PA binding within the carboxy terminus. The effect of changing L319 to A319 or D319 was then examined. Neither these constructs [GST/212−Δ319(HNEA) and GST/212−Δ319(HNED)] nor a construct in which residues 315−319 were changed from HNEL to alanines [GST/212−Δ319(AAAA)] inhibited ^{125}I-t-PA binding (Figure 3B). Changing E318 to A318 generated the construct GST/212−Δ319(HNAL) which still inhibited ^{125}I-t-PA binding, although not as potently or as effectively as the parent construct GST/212−319(HNEL) (Figure 3B and Table 2). To examine whether the position of L319 was important, two additional constructs were generated. In the first, an alanine was inserted between residues 318 and 319, and in the second, E318 was deleted. Figure 3B demonstrates that these constructs, GST/212−Δ319(HNEAL) and GST/212−Δ319(HNL), do not inhibit ^{125}I-t-PA binding. It is not apparent at present why a slight increase in ^{125}I-t-PA binding is seen with several of the competitors [e.g., GST/212−318, GST/212−317, GST/212−Δ319(AAAA), GST/212−Δ319(HNEA), GST/212−Δ319(HNED), GST/212−Δ319(HNEAL)].

The observation that deletion of L104 from GST/18−104 (yielding GST/18−103) increased its potency with respect to inhibition of ^{125}I-α_2M* binding (Figure 1B) prompted us to examine whether deletion, mutation, and/or changing the positions of L222 and L319 would alter the ability of carboxy-terminal constructs to inhibit ^{125}I-α_2M* binding. The results are shown in Figure 4; 500 nM GST/212−318 inhibited 60% of ^{125}I-α_2M* binding whereas GST/212−319, GST/212−317, GST/212−Δ319(HNAL), and GST/212−Δ319(HNED) at concentrations ranging from 2 to 500 nM were noninhibitory. Constructs in which the position of L319 was changed [GST/212−Δ319(HNEAL) and GST/212−Δ319(HNL)] inhibited 50% of ^{125}I-α_2M* binding at 500 nM competitor. Constructs in which L319 was changed to alanine [GST/212−Δ319(AAAA) and GST/212−Δ319-(HNEA)] inhibited ~30% of ^{125}I-α_2M* binding at 500 nM competitor. Since the extent of inhibition of ^{125}I-α_2M* binding by these constructs had not yet reached a plateau at 500 nM (Figure 4), it is probable that further inhibition of

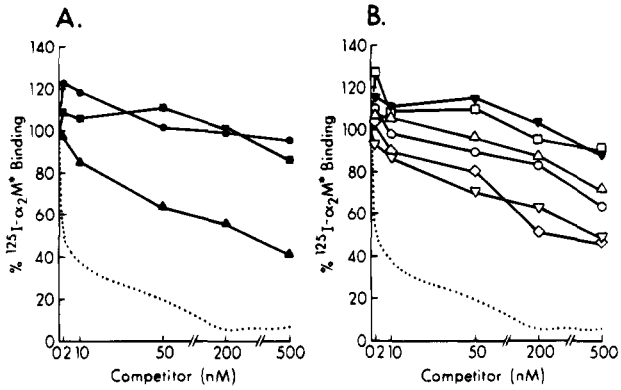


FIGURE 4: Effect of deletion, mutation, and/or position of L319 on inhibition of ^{125}I-α_2M* binding. Cells were incubated for 2 h at 4 °C with 50 pM ^{125}I-α_2M* in the absence or presence of increasing concentrations of 39-kDa protein constructs. Each symbol represents the average of duplicate determinations from the indicated number (n) of independent experiments. Nonspecific binding, determined in the presence of 50 nM unlabeled α_2M*, has been subtracted from each point. 100% binding was determined in the absence of competitor protein. (Panel A) The constructs used were GST/1−319 (•••) (n = 2), GST/212−319 (●) (n = 5), GST/212−318 (▲) (n = 3), and GST/212−317 (■) (n = 3). (Panel B) The constructs used were GST/1−319 (•••) (n = 2), GST/212−Δ319(HNAL) (▼) (n = 4), GST/212−Δ319(AAAA) (○) (n = 4), GST/212−Δ319(HNEA) (△) (n = 5), GST/212−Δ319(HNED) (□) (n = 2), GST/212−Δ319(HNEAL) (▽) (n = 3), and GST/212−Δ319(HNL) (◇) (n = 3).

binding would be seen at competitor concentrations >500 nM. GST/222−319, GST/223−319, and GST/224−319 did not inhibit ^{125}I-α_2M* binding at concentrations up to 500 nM (Table 2).

Binding of GST−39-kDa Protein Constructs to LRP. To examine whether residues of the 39-kDa protein which were required for inhibition of ^{125}I-t-PA and ^{125}I-α_2M* binding to LRP were required for direct binding to LRP, the ability of various constructs to bind to purified LRP immobilized on nitrocellulose was examined. Binding affinities of selected constructs to LRP on hepatoma cells as well as inhibitory effects of various unlabeled constructs on ^{125}I-GST/1−114, ^{125}I-GST/115−319, and ^{125}I-GST/1−319 binding to hepatoma cells were also determined.

In Figure 5, purified LRP was subjected to SDS−PAGE and transferred to nitrocellulose. LRP-containing nitrocellulose strips were incubated with anti-LRP antibody or with 50 nM of the indicated unlabeled GST−39-kDa protein constructs. Binding of GST−39-kDa protein constructs to LRP was detected with anti-39-kDa protein antibody followed by ^{125}I-goat anti-rabbit IgG. As seen, anti-LRP antibody recognizes both the 515-kDa and the 85-kDa subunits of LRP. All of the GST−39-kDa protein constructs examined bound to the 515-kDa subunit of LRP except GST/22−107 and GST/23−107. GST alone also did not bind to purified LRP. These results demonstrate that A103, L104, L222, and L319, which were required for the inhibition of ^{125}I-t-PA and/or ^{125}I-α_2M* binding to LRP, are not required for binding to purified LRP. R21, however, is required for binding to LRP on ligand blots. In addition, the degree of GST/1−114 binding is apparently less than that of GST/115−319.

In Figure 6, the ability of amino- and carboxy-terminal GST−39-kDa protein constructs to inhibit ^{125}I-GST/1−114, ^{125}I-GST/115−319, and ^{125}I-GST/1−319 binding to LRP on MH_1C_1 cells was examined. The K_i values for inhibition of ^{125}I-GST/1−114 binding by amino-terminal constructs (Figure 6A) are as follows: GST/1−114, ~10 nM; GST/18−104, ~25 nM; GST/18−103, ~70 nM; GST/18−102, ~70 nM; GST/1−114 (del 103,104), ~40 nM; GST/21−107, ~10 nM; GST/22−107, >100 nM; GST/23−107, >1 μM; GST/24−107, ≫1 μM. Thus, in agreement with the ligand blotting data (Figure 5), A103 and L104 are not required for binding to LRP since GST/1−114 (del 103,104) inhibits ^{125}I-GST/1−114 binding, although its K_i value is increased ~4-fold when compared to GST/1−114. GST/1−114 (del 103,104) binds specifically to MH_1C_1 cells with a K_d value of 45 nM, which is ~4-fold reduced affinity as GST/1−114 (Table 2). Constructs in which R21 is removed (GST/22−107, GST/23−107, and GST/24−107) do not bind to purified LRP (Figure 5) and inhibit ^{125}I-GST/1−114 binding to hepatoma cells poorly.

To further investigate the importance of R21 in mediating direct binding to LRP, the abilities of unlabeled GST/22−107 and GST/23−107 to alter ^{125}I-GST/21−107 binding to LRP on hepatoma cells and to purified LRP were examined. Figure 7A demonstrates that 1 μM GST/21−107 inhibits 90% of ^{125}I-GST/21−107 binding (5 nM) to hepatoma cells and the K_i value for inhibition is ~15 nM. GST/22−107 inhibits ^{125}I-GST/21−107 binding with a K_i value >1 μM, and GST/23−107 does not inhibit binding at concentrations ranging from 10 to 1000 nM. Figure 7B shows that GST/22−107, GST/23−107, and GST alone do not inhibit ^{125}I-GST/21−107 binding to purified LRP immobilized on nitrocellulose whereas GST/21−107, GST/1−114, and GST/115−319 do inhibit binding. Together these observations further suggest that R21 is required for binding to LRP on hepatoma cells. Therefore, the binding affinities of GST/21−107 and GST/23−107 were directly compared by performing saturation binding experiments on MH_1C_1 cells. The ^{125}I-GST/21−107 and ^{125}I-GST/23−107 used for these experiments are shown in Figure 7C and migrate as single bands on SDS−PAGE with apparent molecular masses of approximately 38 and 36 kDa, respectively. We found that ^{125}I-GST/21−107 binds specifically to MH_1C_1 cells over the concentration range of 1−40 nM. Nonspecific binding accounted for ~12% of total binding, and saturation of specific binding was observed at concentrations in excess of 20 nM (data not shown). Scatchard analysis (Scatchard, 1949) of the binding data yielded a K_d value of 15 nM (Table 2). In contrast, ^{125}I-GST/23−107 (examined ≤70 nM) bound with extremely low affinity to MH_1C_1 cells, and no discernible K_d was appreciated from the data (data not shown).

We previously demonstrated that GST/1−114, GST/12−107, and GST/18−114 inhibited ~40% of ^{125}I-GST/115−319 binding at concentrations of 500 nM (Warshawsky et al., 1994). In Figure 6B, we investigated whether A103, L104, and R21 were required for inhibition of ^{125}I-GST/115−319 binding. As seen, the amino-terminal constructs GST/1−114, GST/18−104, and GST/21−107 inhibit 20−35% of ^{125}I-GST/115−319 binding at concentrations of 1 μM. It is difficult to discern whether the slight inhibitions of binding by 1 μM GST/18−102 and GST/1−114 (del 103,104) are significant. However 1 μM GST/23−107 does not inhibit ^{125}I-GST/115−319 binding. Although high inhibitor concentrations were required to see inhibition of binding, these results are fully consistent with the notion that A103 and

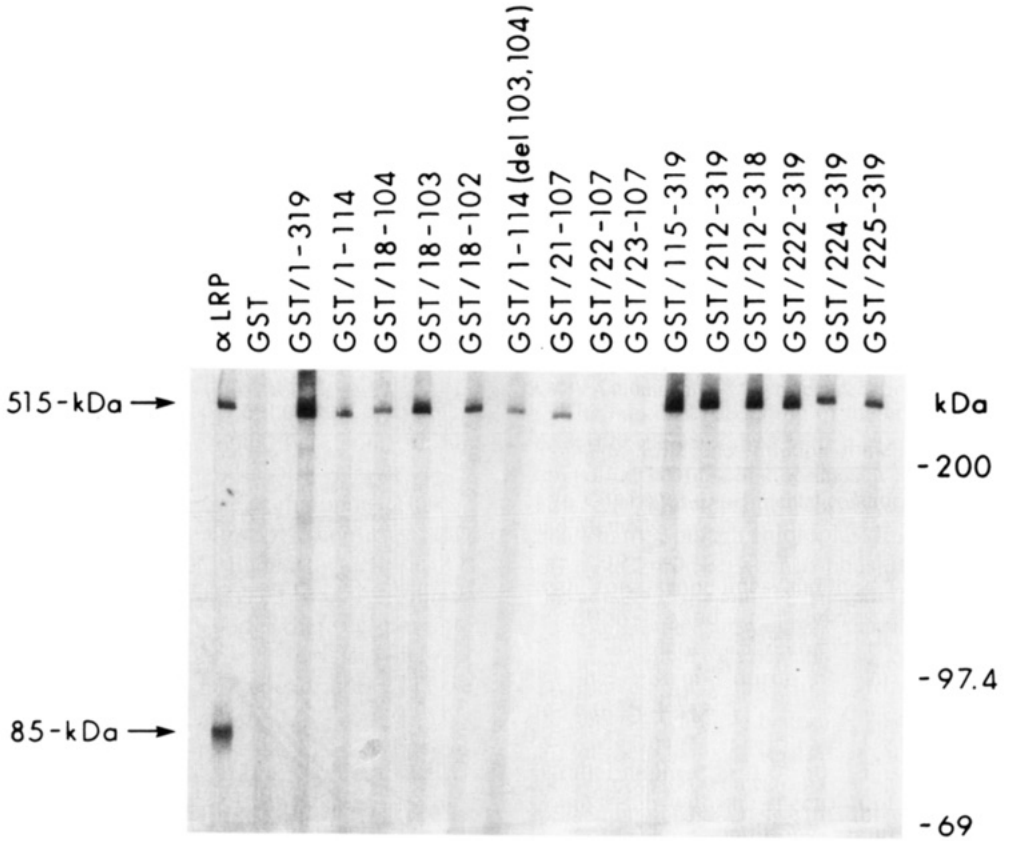


FIGURE 5: Binding of GST−39-kDa protein constructs to purified LRP. Purified human placental LRP was subjected to 6% SDS−PAGE and transferrred to nitrocellulose. Individual strips (containing ~2 μg of LRP/strip) were incubated with a 50 nM aliquot of the indicated GST−39-kDa protein constructs, 50 nM GST alone, or a 1:500 dilution of anti-LRP antiserum. Binding of GST−39-kDa protein constructs was detected with affinity-purified anti-39-kDa protein IgG followed by ^{125}I-goat anti-rabbit IgG. Binding of LRP antibody was detected with ^{125}I-goat anti-rabbit IgG. The positions of the 515-kDa and 85-kDa subunits of LRP are indicated on the left. Molecular mass markers, in kilodaltons, are indicated on the right. Strips were exposed to film 8 h prior to developing.

L104 are not required for binding to LRP whereas R21 is required.

GST/115−319 and GST/200−319 inhibited 80−90% of ^{125}I-GST/1−114 and ^{125}I-GST/115−319 binding to MH_1C_1 cells at 500 nM competitor (Warshawsky et al., 1994). Therefore, we investigated whether L222 and L319 were required within carboxy-terminal constructs to inhibit ^{125}I-GST/1−114 and ^{125}I-GST/115−319 binding to hepatoma cells. As seen, GST/212−318 inhibits ^{125}I-GST/1−114 binding (Figure 6D) essentially identically to GST/212−319 (K_i values ~8 nM). GST/212−319, GST/212−318, and GST/212−Δ319(HNEA) also inhibit ^{125}I-GST/115−319 binding similarly (Figure 6E), with K_i values of 150−200 nM. Together these results demonstrate L319 is not required for inhibition of ^{125}I-GST/1−114 or ^{125}I-GST/115−319 binding. Deletion of L222, however, increases the apparent K_i values for inhibition of both ^{125}I-GST/1−114 binding (GST/222−319, K_i ~ 50 nM, versus GST/224−319, K_i > 200 nM) (Figure 6D) and ^{125}I-GST/115−319 binding. In addition, 1 μM GST/222−319 inhibits ~60% of ^{125}I-GST/115−319 binding whereas GST/223−319 and GST/224−319 are essentially noninhibitory (Figure 6E). These results suggest that L222 may be important for high-affinity binding to LRP. Thus, the binding affinities of ^{125}I-GST/222−319 and ^{125}I-GST/224−319 to MH_1C_1 cells were directly compared. We found that K_d values for ^{125}I-GST/222−319 and ^{125}I-GST/224−319 binding were 8.1 nM and >500 nM, respectively (Table 2). In addition, 1 μM unlabeled GST/222−319 inhibited ~90% of ^{125}I-GST/222−319 (5 nM) binding to MH_1C_1 cells with a K_i value of ~10 nM whereas the K_i value for inhibition by GST/224−319 was >1 μM (data not shown). Unlabeled GST/224−319 also inhibited ^{125}I-GST/224−319 (15 nM) binding poorly, with a K_i value >1 μM (data not shown). Thus, L222 is important for high-affinity binding to LRP on hepatoma cells.

Figure 6C,F demonstrates the ability of amino- and carboxy-terminal constructs to inhibit the binding of the full-length ^{125}I-GST−39-kDa protein to MH_1C_1 cells. Both GST/1−114 and GST/115−319 inhibit ^{125}I-GST/1−319 binding in dose-dependent manners. However, 1 μM GST/115−319 inhibits ~70% of binding whereas GST/1−114 inhibits ~35% of binding. Inhibition of ^{125}I-GST/1−319 binding by an equimolar mixture of GST/1−114 and GST/115−319 gave a pattern of inhibition essentially identical to inhibition by GST/115−319 alone (data not shown). Figure 6C,F also shows that A103, L104, and L319 are not required for inhibition of ^{125}I-GST/1−319 binding since GST/1−114 (del 103,104) *versus* GST/1−114 and GST/212−318 *versus* GST/212−319 inhibit binding similarly. R21 and L222, however, are required for inhibition of ^{125}I-GST/1−319 binding since constructs lacking these residues were noninhibitory.

DISCUSSION

In this study, GST−39-kDa fusion protein constructs were utilized to define specific amino acid residues on the 39-kDa protein required for inhibition of t-PA and α_2M* binding to LRP on rat hepatoma MH_1C_1 cells. The deletion, insertion, and mutation of single amino acid residues within

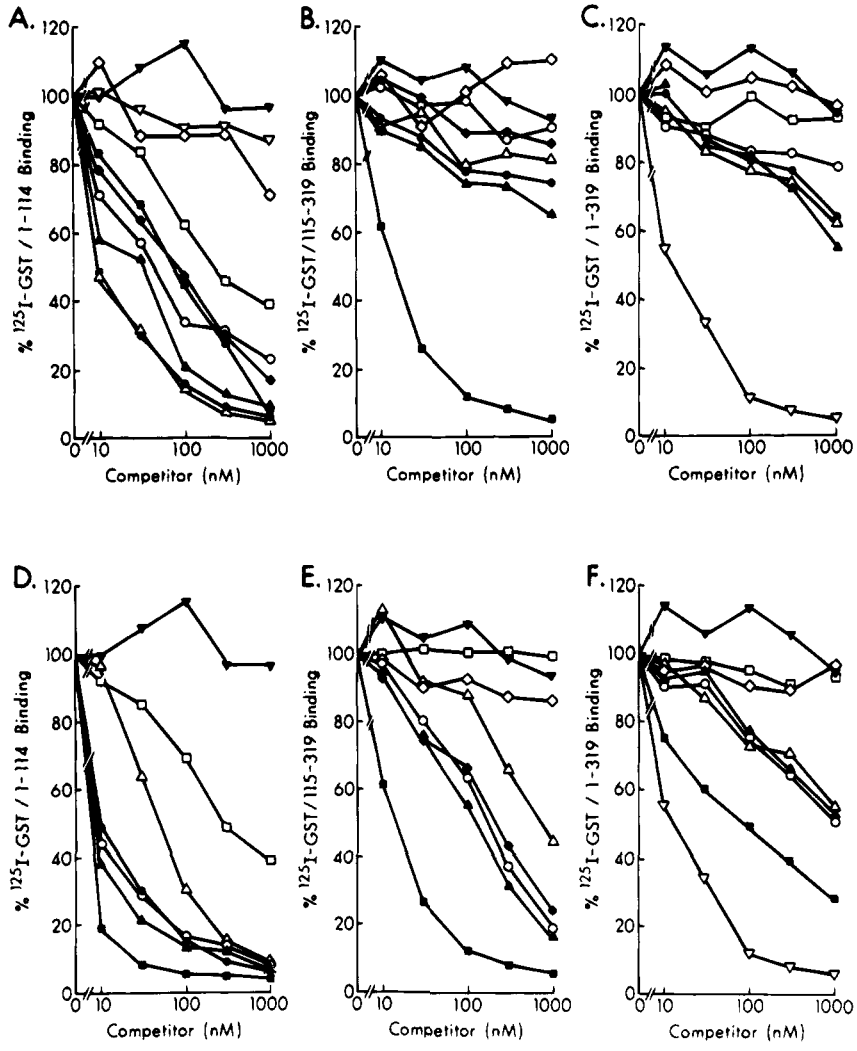


FIGURE 6: MH_1C_1 cells were incubated for 2 h at 4 °C with 5 nM ^{125}I-GST/1−114 (panels A and D), 5 nM ^{125}I-GST/115−319 (panels B and E), or 5 nM ^{125}I-GST/1−319 (panels C and F) in the absence or presence of increasing concentrations of unlabeled amino-terminal (panels A−C) or carboxy-terminal (panels D−F) GST−39-kDa protein constructs. The GST−39-kDa protein constructs used were as follows: panel A, GST/1−114 (●), GST/18−104 (▲), GST/18−103 (■), GST/18−102 (♦), GST/1−114 (del 103,104) (○), GST/21−107 (△), GST/22−107 (□), GST/23−107 (◇), GST/24−107 (▽), and GST (▼); panel B, GST/115−319 (■), GST/1−114 (●), GST/18−104 (▲), GST/18−102 (♦), GST/1−114 (del 103,104) (○), GST/21−107 (△), GST/23−107 (◇), and GST (▼); panel C, GST/1−319 (▽), GST/1−114 (●), GST/18−104 (▲), GST/1−114 (del 103,104) (○), GST/21−107 (△), GST/22−107 (□), GST/23−107 (◇), and GST (▼); panel D, GST/1−114 (●), GST/115−319 (■), GST/212−319 (▲), GST/212−318 (○), GST/222−319 (△), GST/224−319 (□), and GST (▼); panel E, GST/115−319 (■), GST/212−319 (▲), GST/212−Δ319(HNEA) (♦), GST/212−318 (○), GST/222−319 (△), GST/223−319 (◇), GST/224−319 (□), and GST (▼); panel F, GST/1−319 (▽), GST/115−319 (■), GST/212−319 (▲), GST/212−318 (○), GST/222−319 (△), GST/223−319 (◇), GST/224−319 (□), and GST (▲). 100% binding was determined in the absence of any competitor proteins. Each symbol represents the average of duplicate determinations from one or two separate experiments.

these constructs also allowed the identification of specific residues required for interaction with LRP to inhibit t-PA but not α_2M* binding and vice versa. The abilities of these constructs to bind to purified LRP, the binding affinities of selected constructs to LRP on hepatoma cells, and the inhibitory effects of the constructs on the binding of ^{125}I-labeled amino-terminal, carboxy-terminal, and full-length 39-kDa protein were examined. The major conclusions from these studies are illustrated in Figure 8 and are summarized in Table 2.

Within amino-terminal residues 1−114 of the 39-kDa protein, we previously reported domains containing residues 18−24 and 100−107 were both required for inhibition of t-PA and α_2M* binding to LRP on MH_1C_1 cells (Warshawsky et al., 1993b). Herein we found that R21 was required within domain 18−24 for the inhibition of both t-PA and α_2M* binding. R21 was also required for the binding of amino-terminal constructs to LRP. Within domain 100−107, L104 was required for inhibition of t-PA binding since GST/18−104 effectively inhibited binding whereas GST/18−103 did not. Inhibition of α_2M* binding required A103 because GST/18−103 inhibited binding but GST/18−102 did not. A construct encompassing amino-terminal residues 1−114 in which A103 and L104 were deleted did not inhibit t-PA or α_2M* binding. Thus, A103 and L104 are absolutely required within amino-terminal residues 1−114 for inhibition of t-PA and α_2M* binding. These residues, however, are not required for binding to LRP although deletion of residues 103 and 104 reduced the affinity of GST/1−114 binding ~4-fold. Together these results are consistent with the notion that R21 mediates amino-terminal construct binding to LRP whereas A103 and L104 sterically block t-PA and α_2M* binding.

Within carboxy-terminal residues 115−319 of the 39-kDa protein, domains containing residues 200−225 and 311−319 were both required for inhibition of t-PA binding

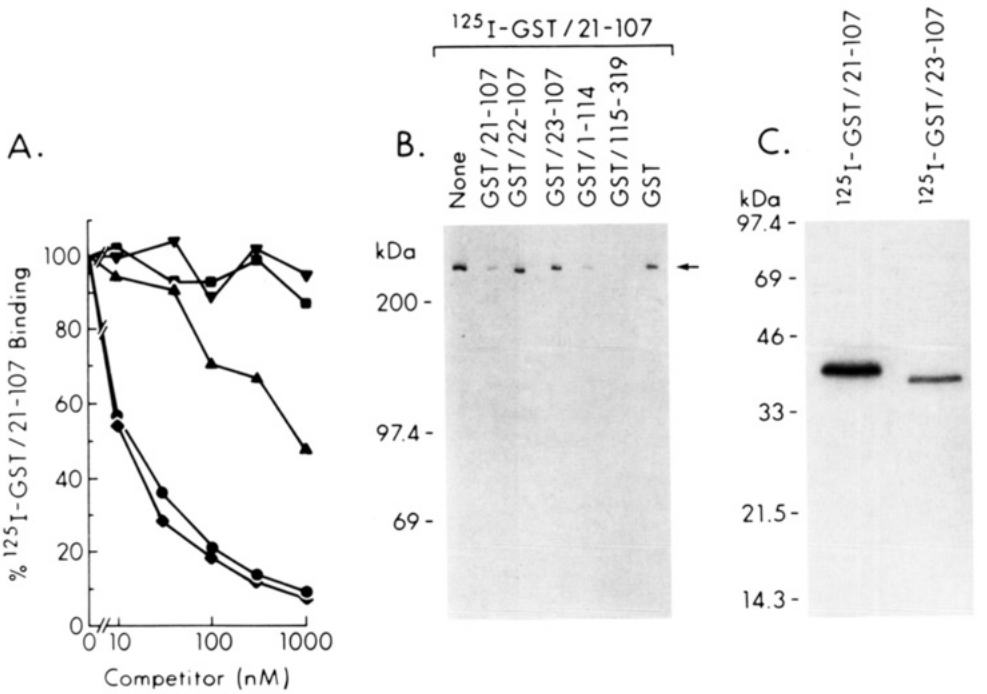


FIGURE 7: (Panel A) MH_1C_1 cells were incubated for 2 h at 4 °C with 5 nM ^{125}I-GST/21−107 in the absence or presence of increasing concentrations of unlabeled GST/21−107 (●), GST/22−107 (▲), GST/23−107 (■), GST/1−114 (♦), and, as a negative control, GST (▼). 100% binding was determined in the absence of any competitor proteins. Each symbol represents the average of duplicate determinations from a single experiment. (Panel B) Purified human placental LRP was subjected to 6% SDS−PAGE and transferred to nitrocellulose. Individual strips (containing ~2 μg of LRP/strip) were incubated with 20 nM ^{125}I-GST/21−107 in the absence (None) or presence of 1 μM samples of the indicated unlabeled competitors. The position of the 515-kDa subunit of LRP is indicated with an arrow. Molecular mass markers, in kilodaltons, are indicated on the left. Strips were exposed to film 6 h prior to developing. (Panel C) ~10 000−20 000 cpm of ^{125}I-GST/21−107 and ^{125}I-GST/23−107 were subjected to 12.5% SDS−PAGE. The gel was dried and exposed to film for 18 h. Molecular mass markers, in kilodaltons, are indicated on the left.

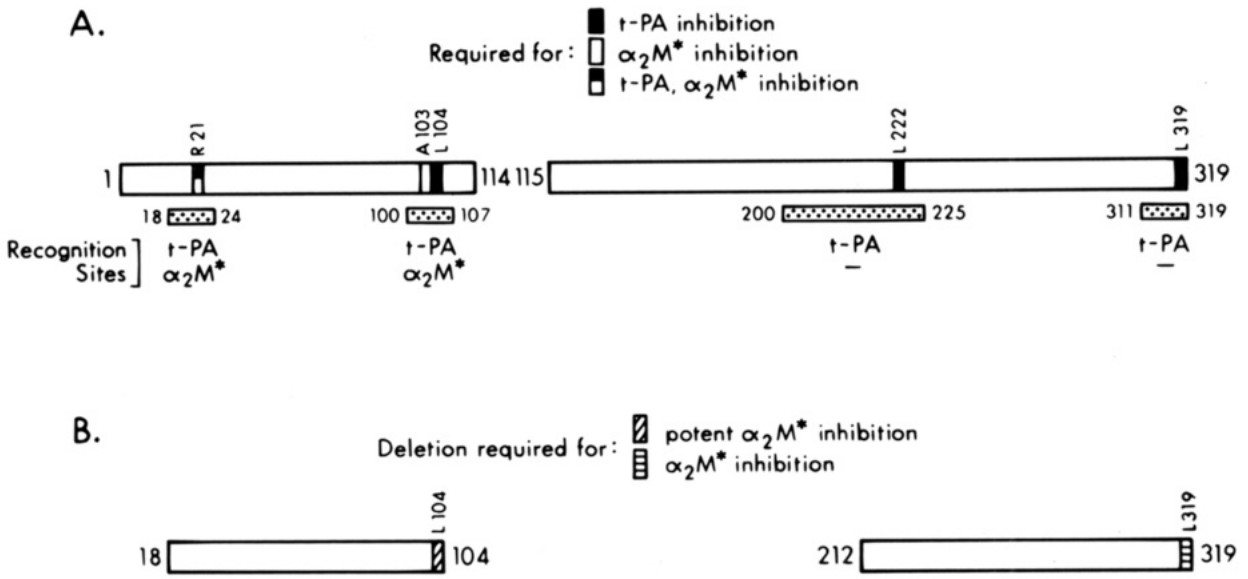
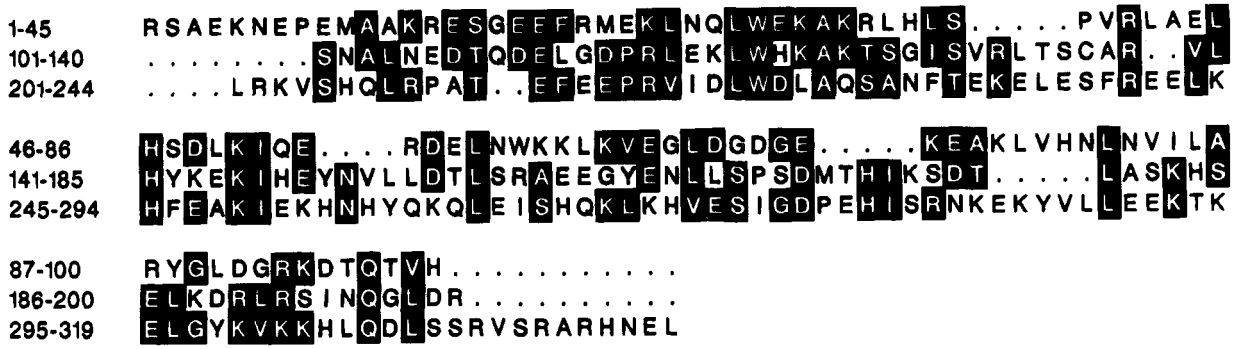


FIGURE 8: (A) Amino acid residues of the 39-kDa protein required for the inhibition of t-PA and α_2M* binding to LRP on MH_1C_1 cells. Recognition sites which define domains of the 39-kDa protein required for inhibition of t-PA and α_2M* binding are indicated. The identification of amino acids within each of the recognition sites required for inhibition of t-PA, α_2M*, or t-PA and α_2M* binding is indicated. (B) Amino acid residues of the 39-kDa protein whose deletion or position results in inhibition of α_2M* binding. Deletion of L104 from residues 18−104 increases the potency and extent of α_2M* inhibition. Deletion or altering the position of L319 results in α_2M* inhibition.

(Warshawsky et al., 1993b). In the current report, we found that L222 was required for inhibition within domain 200−225 since GST/222−319 inhibited binding but GST/223−319 was essentially noninhibitory. L222, however, was not required for direct binding to purified LRP but was required for high-affinity binding to LRP on MH_1C_1 cells. L319 was also required for inhibition of t-PA binding because constructs in which L319 was deleted or mutated did not inhibit binding. The position of L319 was important since the insertion of an additional amino acid (alanine) between E318 and L319 or the deletion of E318 generated constructs which did not inhibit t-PA binding. Upon deletion of L319 or alteration of its position, the ligand specificity of the 39-kDa protein changed from inhibition of t-PA binding to



Residues	101-200	201-319
1-100	26/12/38*	16/15/31
101-200	-	26/12/38

Overall: 13/8/21

*Identical/Similar/Both

FIGURE 9: Sequence alignment of residues 1−100, 101−200, and 201−319 of the 39-kDa protein. (Top) Identical residues and residues with similar chemical and physical properties are in blackened. (Bottom) The percent of residues that are identical/similar/ or identical plus similar (both) between residues 1−100 and 101−200, 1−100, and 201−319 (overall) is indicated.

inhibition of α_2M* binding. This suggested L319 was not required for direct binding to LRP. Indeed, constructs lacking L319 bind to purified LRP and inhibit the binding ^{125}I-GST/1−319, ^{125}I-GST/1−114, and ^{125}I-GST/115−319 to MH_1C_1 cells essentially identically to constructs containing L319.

Table 2 lists the extents of inhibition of ligand binding by 500 nM aliquots of the indicated GST−39-kDa protein constructs, the apparent K_i values for inhibition of ligand binding, and K_d values for the binding of selected constructs to LRP. As seen, the extents of inhibition vary, although at present it is not clear why. For example, inhibition of t-PA binding by GST/115−319 plateaus between 50 and 500 nM competitor where 95% of t-PA binding is maximally inhibited. Inhibition of t-PA binding by GST/212−319 also plateaus between 50 and 500 nM competitor. However, GST/212−319 maximally inhibits 60% of t-PA binding (Figure 3). One explanation for these findings is that these constructs bind to the same site(s) on LRP but there are twice as many GST/115−319 binding sites as GST/212−319 binding sites. If all of the GST/115−319 binding sites inhibited t-PA binding, then saturating concentrations of GST/212−319 would be predicted to inhibit ~50% of t-PA binding whereas saturating concentrations of GST/115−319 would completely inhibit t-PA binding. Alternatively, it is possible that steric hindrance accounts for the varying extents of inhibition. For example, residues 115−212 may participate to sterically limit t-PA binding.

The observations that residues every ~100 amino acids along the 39-kDa protein (i.e., residues 21, 103, 104, 222, and 319) were required for inhibition of ligand binding and/or direct binding to LRP and that isolated fragments of the 39-kDa protein can independently bind to LRP raised the possibility that the 39-kDa protein may contain an internal triplication. Therefore, amino acid residues 1−100, 101−200, and 201−319 of the 39-kDa protein were aligned, and the result is shown in Figure 9. Identical and similar amino acids are shown in *boldface*. The percent of identical/similar amino acids, allowing for gaps, is as follows: residues 1−100 and 101−200, 26%/12%; residues 1−100 and 201−319, 16%/15%; residues 101−200 and 201−319, 26%/12%. Overall there is 21% identity and similarity between residues 1−100, 101−200, and 201−319. Only L222, which is required for inhibition of t-PA and α_2M* binding, is conserved between all three regions. The overall homology, albeit rather low, may explain why mutiple regions of the 39-kDa protein can independently bind to LRP. The lack of conservation of residues critical for inhibition of ligand binding may explain the differential regulation of ligand binding. The observation that the carboxy terminus of the 39-kDa protein is a more effective competitor of the binding of the full-length protein than the amino terminus (Figure 6C,F) and binds more effectively to isolated LRP (Figure 5) may suggest that the carboxy terminus is the predominant region that binds LRP while the amino terminus may function to inhibit ligand binding.

In summary, we have defined five residues within the 39-kDa protein required for inhibition of t-PA and α_2M* binding to LRP and have examined whether these residues were also required for binding to LRP. R21 is required for both t-PA and α_2M* inhibition presumably because it mediates the binding of amino-terminal constructs to LRP. A103 and L104 are required for t-PA and α_2M* inhibition but are not required for binding to LRP. L222 and L319 are both required for t-PA inhibition, but only L222 is required for high-affinity binding to LRP. Future site-directed mutagenesis of these residues will be required to establish whether these are the only critical residues or whether there are additional, as yet undefined residues of the 39-kDa protein required for inhibition of ligand binding to LRP. Ultimately, elucidation of the crystal structure of the 39-kDa protein will be useful in localizing additional important residues within

the three-dimensional structure of the protein.

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