Binding of hepatic lipase to heparin: identification of specific heparin-binding residues in two distinct positive charge clusters

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Abstract **The interaction of hepatic lipase (HL) with heparan sulfate is critical to the function of this enzyme. The primary amino acid sequence of HL was compared to that of lipoprotein lipase (LPL), a related enzyme that possesses several putative heparin-binding domains. Of the three putative heparin-binding clusters of LPL (*J. Biol. Chem.* 1994.** 269: **4626–4633; *J. Lipid Res.* 1998.** 39: **1310–1315), one was conserved in HL (Cluster 1; residues Lys 297–Arg 300 in rat HL) and two were partially conserved (Cluster 2; residues Asp 307–Phe 320, and Cluster 4; residues Lys 337, and Thr 432–Arg 443). Mutants of HL were generated in which potential heparin-binding residues within Clusters 1 and 4 were changed to Asn. Two chimeras in which the LPL heparin-binding sequences of Clusters 2 and 4 were substituted for the analogous HL sequences were also constructed. These mutants were expressed in Chinese hamster ovary (CHO) cells and assayed for heparin-binding ability using heparin-Sepharose chromatography and a CHO cell-binding assay. The results suggest that residues within the homologous Cluster 1 region (Lys 297, Lys 298, and Arg 300), as well as some residues in the partially conserved Cluster 4 region (Lys 337, Lys 436, and Arg 443), are involved in the heparin binding of hepatic lipase. In the cell-binding assay, heparan sulfate-binding affinity equal to that of LPL was seen for the RHL chimera mutant that possessed the Cluster 4 sequence of LPL. Mutation of Cluster 1 residues of HL resulted in a major reduction in heparin binding ability as seen in both the cell-binding assay and the heparin-Sepharose elution profile. These results suggest that Cluster 1, the N-terminal heparin-binding domain, is of primary significance in RHL. This is different for LPL: mutations in the C-terminal binding domain (Cluster 4) cause a more significant shift in the salt required for elution from heparin-Sepharose than mutations in the N-terminal domain (Cluster 1).**—Sendak, R. A., D. E. Berryman, G. Gellman, K. Melford, and A. Bensadoun. **Binding of hepatic lipase to heparin: identification of specific heparin-binding residues in two distinct positive charge clusters. *J. Lipid Res.* 2000.** 41: **260–268.**

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Hepatic lipase (HL) is synthesized primarily by hepatocytes (1–3) and is secreted into the sinusoidal space where it binds to both the capillary endothelium and hepatocytes (4). In concert with lipoprotein lipase (LPL), HL plays several major roles in plasma lipoprotein metabolism (5). Hepatic lipase has both triglyceride hydrolase and phospholipase activity. In vivo inhibition of HL activity by intravenous injections of anti-HL antibodies results in the accumulation of intermediate density lipoproteins and an increase in high density lipoprotein (HDL), cholesterol, and phospholipids (6–8). Similarly, patients with hepatic lipase deficiency exhibit increased plasma triglyceride, cholesterol, intermediate density lipoproteins, and HDL when compared to control subjects (9, 10). In a report on HL-deficient mice produced by homologous recombination, the main change compared to control mice was an increase in large HDL with no major change in apoB-containing lipoproteins (11). A recent report (12) on the phenotype of young (10–14 weeks of age) homozygous (−/−) HL-deficient mice demonstrated a role of mouse HL in the formation of dense LDL as well as in the metabolism of HDL. It has been postulated that HL also modifies the cholesterol to phospholipid ratio of HDL thus facilitating transfer of cholesterol to liver cells (13). In addition to these roles in lipid metabolism that are dependent on the catalytic activity of the enzyme, HL has been shown to enhance the uptake of lipoproteins by lipoprotein receptors (5). This stimulation is not contingent on catalytic activity of the enzyme but is highly dependent on the presence of heparan sulfate proteoglycans on the cell surface (14–18).

The binding of HL to heparan sulfate proteoglycans (HSPGs) is an important interaction, which may be neces-

Abbreviations: HL, hepatic lipase; LPL, lipoprotein lipase; CHO, Chinese hamster ovary; HDL, high density lipoprotein; HSPG, heparan sulfate proteoglycan; RHL, rat hepatic lipase; cLPL, chicken lipoprotein lipase; ELISA, enzyme-linked immunosorbent assay.

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sary for many of the metabolic tasks that the lipase performs. To date, the regions of HL that are essential for the binding of the enzyme to heparin have not been identified. The heparin-binding regions of lipoprotein lipase (LPL), on the other hand, have been extensively studied (19–22). LPL and HL are members of the triglyceride lipase superfamily that share extensive sequence homology, but differ significantly in substrate specificity, optimal hydrolysis conditions, cofactor requirements, and heparin-binding affinity. Several studies have been carried out that described the construction of chimeric lipases in which the N- and C-terminal domains of HL and LPL were exchanged (23–25). These studies revealed that HL and LPL each possess unique functional domains that can be localized to the N- or C-terminal domains of the proteins, further demonstrating the structural basis for the functional differences of these enzymes.

Putative heparin-binding regions of LPL were predicted in the primary amino acid sequence of the enzyme based on comparison with consensus heparin-binding sequences of other heparin-binding proteins (26) and in the tertiary structure based on molecular modeling (27). Four regions of positive charge were identified: these have been subjected to site-directed mutagenesis revealing that they may play a role in the heparin binding of the protein. Cluster 1 (avian sequence numbering): Arg 281, Lys 282, and Arg 284 (19–21) and Cluster 4: Lys 321, Arg 405, Arg 407, Arg 409, and Lys 416 (22) were shown to be involved in heparin binding while conflicting results were obtained for Cluster 2: residues 296–302 (20, 21). Cluster 3 (residues 147–151, human numbering) was not found to play a role in heparin binding (20). Alignment of the sequences of LPL and HL reveals that Cluster 1 is conserved between the two lipases while Clusters 2 and 4 are only partially conserved.

In this study, we have systematically analyzed the potential heparin-binding regions of rat HL (RHL) to identify those sequences involved in this important interaction. In addition, we have analyzed the putative LPL heparin-binding sequences that are poorly conserved in HL to characterize the basis for the difference in heparin-binding affinity between these two evolutionarily conserved proteins.

MATERIALS AND METHODS

Reagents and enzymes

Restriction enzymes and molecular biology reagents were purchased from New England Biolabs, MBI Fermentas, or Gibco BRL Life Technologies. Previously described procedures were used to purify native RHL (28) and chicken LPL (cLPL) (29) used for control experiments.

Construction of rat hepatic lipase heparin-binding mutants

Alignment of the RHL and cLPL primary sequences (30, **Table 1** and **Table 2**) allowed for comparison of the putative heparin-binding regions of LPL with the analogous sequences in RHL. We have identified discrepancies between the published cDNA sequence of RHL (31) and the results of our recent sequencing efforts. Both strands of the same clone isolated by Sensel et al. (31) were re-sequenced at the Cornell BioResource Center on PE-Biosystems 377 automated DNA sequencers. The published sequence reads: 5′ ^{994}ATC GGC CAC GTC^{1005} 3′, while our most recent sequence reads: 5′ ^{994}GAT CGG CCA CGC^{1005} 3′. In addition, we found that the codon for Gly 11 is GGG (instead of GGA), amino acid 256 (AAC, Asn) is actually Lys (AAA), and amino acid 448 (ATG, Met) is actually Ile (ATA). The revised amino acid sequence, the nucleotide changes, as well as the three positive charge clusters studied are indicated on Table 1.

The full-length RHL cDNA was previously cloned into pcDNA3 to create pcDNA3/RHL (28). Several RHL mutants of Clusters 1, 2, and 4 were constructed (**Table 3**). The polymerase chain reaction in combination with the overlap extension method was used to construct the substitution mutants HB-A, HB-B, HB-C, HB-E, and the chimeric mutants RHL2LPL and RHL4LPL (32). The mutagenic primers are listed in **Table 4**. The flanking oligonucleotides used had the sequences 5′ ^{574}AC AGGGCTGGACCCTGCAGGA^{594} 3′ and 5′ ^{574}CTCATCTGCT CTTTTGCTTCT^{1506} 3′. The final PCR product containing the mutation was subcloned into pcDNA3/RHL using *Eco*N I and *Esp*3IA in the case of RHL4LPL and RHL2LPL, and *Bsg* I in the case of HB-A, HB-B, HB-C, and HB-E. The region containing the mutated fragment was sequenced. HB-F and HB-G were constructed using the QuikChange™ Site-Directed mutagenesis kit (Stratagene) according to the manufacturer's protocol. The entire cDNA of these mutants was sequenced to confirm the absence of any misincorporated bases by the polymerase.

Cell culture and protein expression

Chinese hamster ovary (CHO) cells (American Type Culture Collection, CCL 61) were cultured in Ham's F12 medium (Gibco BRL Life Technologies) supplemented with 5% fetal bovine serum, 2 mm l-glutamine, and 10 mm HEPES, pH 7.2. Cells were maintained in a humidified incubator at 5% CO_2. The CHO cells were stably transfected with the RHL DNA constructs using Lipofectamine™ according to a previously described procedure (22).

Lipase mass and activity measurements

Sandwich ELISAs with affinity-purified immunoglobins were used to measure the mass of cLPL (33) and RHL (34). In its most sensitive form, the standard curve for the RHL ELISA ranged from 0.02 ng to 1 ng with an OD_{490} of 1.05 at 1 ng (r^2 = 0.999). The cLPL ELISA standard curve spanned from 0.01 to 1 ng with an OD_{490} of 1.27 at 1 ng (r^2 = 0.999). Catalytic activity of RHL constructs was determined using a ^{3}H-labeled triolein substrate with a specific activity of 500,000 cpm/μmol oleic acid (35).

Heparin-Sepharose chromatography of wild-type RHL, LPL, and RHL heparin-binding mutants

Heparin-Sepharose chromatography was carried out using a 1-ml HiTrap Heparin-Sepharose column (Pharmacia) at 4°C in an FPLC system (Pharmacia). The column was equilibrated in buffer A (0.1 m NaCl, 1 mm decyl-sucrose (Calbiochem), 10 mm phosphate, pH 7). Medium was incubated for 5 h in four 75-cm^2 flasks containing cells of a given construct at 80–90% confluency. The medium, containing heparin (Sigma, 5 units/ml), was centrifuged at 3,000 *g* for 15 min and loaded onto the HiTrap column at a rate of 0.5 ml/min. The matrix was washed with buffer A (5 ml) and the lipase was eluted with a 40 ml linear gradient from buffer A to buffer B (2 m NaCl, 1 mm decyl-sucrose, 10 mm phosphate, pH 7). The flow rate was 0.5 ml/min and either 40 or 80 fractions were collected. The column was then washed with 5 ml of buffer B and equilibrated in buffer A. Three runs were completed for each mutant construct and the fractions were assayed for lipase by ELISA. A fourth chromatography was carried

TABLE 1. Revised rat hepatic lipase sequence

	cgc	atg	gga	aat	cac	ctc	caa	atc	tcc	gtt	tcc	atg	gtg	ctg	tgc	
	Atc	ttt	atc	cag	tca	agt	gcc	TGT	GGA	CAA	GGC	GTG	GGA	ACA	GAG	
								C	*G*	*Q*	*G*	*V*	*G*	*T*	*E*	*8*
88	CCC	TTT	GGG	AGA	AAC	CTT	GGA	GCT	ACT	GAA	GAA	AGG	AAA	CCG	TTA	
	P	*F*	*G*	*R*	*N*	*L*	*G*	*A*	*T*	*E*	*E*	*R*	*K*	*P*	*L*	*23*
133	CAG	AAG	CCA	GAG	ATC	AGA	TTC	CTG	CTC	TTC	AAA	GAT	GAA	AGT	GAC	
	Q	*K*	*P*	*E*	*I*	*R*	*F*	*L*	*L*	*F*	*K*	*D*	*E*	*S*	*D*	*38*
178	CGC	CTG	GGT	TGT	CAG	CTC	AGA	CCT	CAG	CAC	CCG	GAA	ACA	CTG	CAG	
	R	*L*	*G*	*C*	*Q*	*L*	*R*	*P*	*Q*	*H*	*P*	*E*	*T*	*L*	*Q*	*53*
223	GAG	TGT	GGC	TTC	AAC	AGC	TCC	CAT	CCA	CTT	GTC	ATG	ATC	ATC	CAC	
	E	*C*	*G*	*F*	*N*	*S*	*S*	*H*	*P*	*L*	*V*	*M*	*I*	*I*	*H*	*68*
268	GGG	TGG	TCG	GTG	GAT	GGC	TTG	CTA	GAA	ACC	TGG	ATC	TGG	AAG	ATA	
	G	*W*	*S*	*V*	*D*	*G*	*L*	*L*	*E*	*T*	*W*	*I*	*W*	*K*	*I*	*83*
313	GTG	GGT	GCC	CTG	AAG	TCC	CGA	CAG	TCC	CAA	CCC	GTG	AAC	GTG	GGA	
	V	*G*	*A*	*L*	*K*	*S*	*R*	*Q*	*S*	*Q*	*P*	*V*	*N*	*V*	*G*	*98*
358	TTA	GTG	GAC	TGG	ATC	TCC	CTG	GCA	TAC	CAG	CAC	TAT	GCT	ATT	GCC	
	L	*V*	*D*	*W*	*I*	*S*	*L*	*A*	*Y*	*Q*	*H*	*Y*	*A*	*I*	*A*	*113*
403	GTG	CGC	AAC	ACC	CGT	GTT	GTG	GGC	CAG	GAG	GTG	GCT	GCT	CTT	CTC	
	V	R	N	T	R	V	V	G	Q	E	V	A	A	L	L	128
448	CTA	TGG	CTG	GAG	GAA	TCT	ATG	AAG	TTT	TCT	CGG	AGC	AAA	GTT	CAC	
	L	*W*	*L*	*E*	*E*	*S*	*M*	*K*	*F*	*S*	*R*	*S*	*R*	*V*	*H*	*143*
493	TTA	ATT	GGG	TAC	AGC	CTG	GGA	GCA	CAC	GTT	TCA	GGA	TTC	GCA	GGC	
	L	*I*	*G*	*Y*	*S*	*L*	*G*	*A*	*H*	*V*	*S*	*G*	*F*	*A*	*G*	*158*
538	AGC	TCC	ATG	GGT	GGG	AAG	CGC	AAG	ATC	GGA	AGA	ATC	ACA	GGG	CTG	
	S	*S*	*M*	*G*	*G*	*K*	*R*	*K*	*I*	*G*	*R*	*I*	*T*	*G*	*L*	*173*
583	GAC	CCT	GCA	GGA	CCT	ATG	TTT	GAG	GGA	ACT	TCC	CCC	AAT	GAG	CGC	
	D	*P*	*A*	*G*	*P*	*M*	*F*	*E*	*G*	*T*	*S*	*P*	*N*	*E*	*R*	*188*
628	CTT	TCT	CCA	GAT	GAT	GCC	AAT	TTT	GTG	GAT	GCT	ATT	CAT	ACC	TTT	
	L	*S*	*P*	*D*	*D*	*A*	*N*	*F*	*V*	*D*	*A*	*I*	*H*	*T*	*F*	*203*
673	ACC	AGG	GAG	CAC	ATG	GGT	CTG	AGT	GTG	GGC	ATC	AAA	CAG	CCC	ATT	
	T	*R*	*E*	*H*	*M*	*G*	*L*	*S*	*V*	*G*	*I*	*K*	*Q*	*P*	*I*	*218*
718	GCC	CAC	TAT	GAC	TTC	TAC	CCC	AAC	GGG	GGC	TCC	TTC	CAG	CCT	GGC	
	A	*H*	*Y*	*D*	*F*	*Y*	*P*	*N*	*G*	*G*	*S*	*F*	*Q*	*P*	*G*	*233*
763	TGC	CAC	TTC	CTG	GAG	CTC	TAC	AAA	CAC	ATT	GCA	GAG	CAT	GGC	TTA	
	C	*H*	*F*	*L*	*E*	*L*	*Y*	*K*	*H*	*I*	*A*	*E*	*H*	*G*	*L*	*248*
808	AAT	GCC	ATA	ACC	CAG	ACC	ATC	AAA	TGT	GCC	CAT	GAG	CGT	TCT	GTG	
	N	*A*	*I*	*T*	*Q*	*T*	*I*	***K***	*C*	*A*	*H*	*E*	*R*	*S*	*V*	*263*
853	CAC	CTC	TTC	ATT	GAC	TCC	TTG	CAA	CAC	AGC	AAC	CTG	CAG	AAC	ACA	
	H	*L*	*F*	*I*	*D*	*S*	*L*	*Q*	*H*	*S*	*N*	*L*	*Q*	*N*	*T*	*278*
898	GGC	TTC	CAG	TGC	AGC	AAC	ATG	GAC	AGC	TTC	AGT	CAG	GGT	CTA	TGT	
	G	F	**Q**	C	S	N	M	D	**S**	F	S	Q	G	L	C	293
						Cluster 1										
943	CTG	AAC	TGC	AAG	AAG	GGC	CGT	TGC	AAC	AGT	CTG	GGC	TAT	GAC	ATC	
	L	*N*	*C*	*K*	*K*	*G*	*R*	*C*	*N*	*S*	*L*	*G*	*Y*	*D*	*I*	*308*
								Cluster 2								
988	CGC	AGG	GAT	CGG	CCA	CGC	AAG	AGC	AAG	ACA	CTC	TTC	CTC	ATC	ACC	
	R	*R*	*D*	*R*	*P*	*R*	*K*	*S*	*K*	*T*	*L*	*F*	*L*	*I*	*T*	*323*
1033	CGA	GCC	CAG	TCC	CCC	TTC	AAA	GTT	TAT	CAT	TAC	CAG	TTC	AAG	ATC	
	R	*A*	*Q*	*S*	*P*	*F*	*K*	*V*	*Y*	*H*	*Y*	*Q*	*F*	*K*	*I*	*338*
1078	CAG	TTC	ATC	AAT	CAA	ATG	GAG	AAG	CCA	ATA	GAG	CCT	ACT	TTT	ACC	
	Q	*F*	*I*	*N*	*Q*	*M*	*E*	*K*	*P*	***I***	*E*	*P*	*T*	*F*	*T*	*353*
1123	ATG	ACA	CTG	CTG	GGG	ACA	AAA	GAA	GAA	ATA	AAG	AAA	ATT	CCC	ATC	
	M	T	L	L	G	T	K	E	E	I	K	K	I	P	I	368
1168	ACC	CTG	GGC	GAA	GGA	ATT	ACC	AGC	AAT	AAA	ACC	TAT	TCC	TTA	CTT	
	T	*L*	*G*	*E*	*G*	*I*	*T*	*S*	*N*	*K*	*T*	*Y*	*S*	*L*	*L*	*383*
1213	ATC	ACA	CTG	GAC	AAA	GAC	ATC	GGC	GAG	TTG	ATC	ATG	CTC	AAG	TTC	
	I	*T*	*L*	***D***	*K*	*D*	*I*	*G*	*E*	*L*	*I*	*M*	*L*	*K*	*F*	*398*
1258	AAG	TGG	GAA	AAC	AGC	GCA	GTG	TGG	GCC	AAT	GTC	TGG	AAC	ACA	GTG	
	K	*W*	*E*	*N*	*S*	*A*	*V*	*W*	*A*	*N*	*V*	*W*	*N*	*T*	*V*	*413*
1303	CAG	ACC	ATA	ATG	CTA	TGG	GAC	ACA	GAG	CCT	CAC	TAC	GCG	GGC	CTC	
	Q	*T*	*I*	*M*	*L*	*W*	*D*	*T*	*E*	*P*	*H*	*Y*	*A*	*G*	*L*	*428*
										Cluster 4						
1348	ATT	CTG	AAG	ACC	ATC	TGG	GTC	AAA	GCT	GGA	GAG	ACG	CAG	CAA	AGA	
	I	***L***	*K*	*T*	*I*	*W*	*V*	*K*	*A*	*G*	*E*	*T*	*Q*	*Q*	*R*	*443*
1393	ATG	ACA	TTT	TGC	CCT	GAT	AAT	GTG	GAT	GAT	CTC	CAG	CTT	CAC	CCC	
	M	*T*	*F*	*C*	*P*	*D*	*N*	*V*	*D*	*D*	*L*	*Q*	*L*	*H*	*P*	*458*
1438	ACC	CAG	GAG	AAA	GTC	TTC	GTG	AAA	TGT	GAC	CTG	AAG	TCA	AAA	GAC	
	T	*Q*	*E*	*K*	*V*	*F*	*V*	*K*	*C*	*D*	*L*	*K*	*S*	*K*	*D*	*473*
1483	TGA	aga	agc	aaa	aga	gca	gat	gag	tca	aga	gac	cca	agc	aca	aaa	
	stop															
	taa	ata	gac	tat	tct	tta	tct	gta	atg	gtt	gcc	tta	ttc	gga	agc	

Nucleotides and amino acids that were found to be different from those published (31) are in boldface. The potential heparin-binding clusters are underlined.

TABLE 2. Alignment of chicken LPL (cLPL) and rat HL (RHL) sequences

	Cluster 1	Cluster 2		Cluster 4
cLPL	^{281}RKNR^{284}	^{291}KVNRVRTKRNTKMYLKTRAQM^{311}	^{321}K	^{405}RVRVKSGETQKK^{416}
RHL	^{297}KKGR^{300}	^{307}DIRRDRPRKSKTLFLITRAQS^{327}	^{337}K	^{432}TIWVKAGETQQR^{443}

Residues that were previously implicated in heparin binding of LPL are underlined; evidence for Cluster 2 is conflicting (19–22).

out where both enzyme mass and catalytic activity were measured. For this run, buffer A contained 2 mm decyl sucrose. The specific activity for each mutant for a given HL peak was calculated (**Table 5**) by taking the average and standard deviations of specific activities of fractions with an activity equal or larger than 0.1 μmol/ml per h. Conductivity was determined using a conductivity meter (Radiometer, Copenhagen). Three 40-fraction runs and three 80-fraction runs were selected at random and assayed for conductivity. The average results were converted to NaCl concentration using a standard curve ($r^2 = 0.998$) and were used to plot the data sets. To determine peak salt concentration for each mutant, the fraction at which the peak lipase value was detected in the individual runs was assayed by conductivity. The average and standard deviation of these three values were calculated for each mutant.

Native purified RHL was run on the column under the same conditions to verify that the recombinant RHL produced in CHO cells possessed the same affinity for heparin-Sepharose. Native purified chicken LPL was also run on the column for comparison with the RHL/LPL chimeric mutants.

A control Sepharose column was constructed. One gram of CNBr-activated Sepharose (Pharmacia) was suspended in 1 mm HCl, washed with 300 ml of HCl, then with 200 ml of 0.1 m sodium carbonate, pH 8.3. The matrix was then incubated with 20 ml of 1 m ethanolamine, pH 8.0, for 2 h at room temperature. The Sepharose was then washed three times consecutively with 0.5 m NaCl, 0.1 m acetate, pH 4, then 0.5 m NaCl, 0.1 m Tris-HCl, pH 8.0. Purified native RHL was run on the Sepharose column under the same conditions used for heparin-Sepharose chromatography.

Cell binding assay

A cell-binding assay was used to quantify changes in the equilibrium binding affinity of the lipase constructs for cell surface HSPGs (16). CHO cells transfected with wild-type cLPL (19), RHL, RHL2LPL, RHL4LPL, HB-A, HB-B, HB-C, HB-E, HBF, and HB-G were cultured on 60-mm dishes. Fresh medium (3 ml) was added to eight dishes per construct when the transfected cells reached ~90% confluency. After a 4-h incubation, the dishes were placed on ice at 4°C for 2 h. The medium was removed and saved; two dishes were pooled per sample yielding 4 samples/construct. The dishes were then washed with 3 ml of ice-cold PBS, and the bound lipase was eluted with medium containing 100 units heparin/ml. The "bound" and "free" lipase samples were assayed for mass. The cells were harvested and assayed for DNA content using a previously described procedure (36).

RESULTS

Construction of RHL heparin-binding mutants

Several mutants were made to characterize the regions in hepatic lipase that are responsible for the binding of the enzyme to heparin and heparan sulfate. The mutations were of two types: substitution mutants in which putative heparin-binding residues of RHL were mutated to Asn, and chimera mutants which involved the substitution of previously identified heparin-binding sequences of LPL for the corresponding regions of RHL. The substitution of a polar amino acid for a basic residue is a conservative one and therefore would most likely preserve the relative solvent exposure of the residue, minimizing the chance of a disruption in structure of the enzyme. The mutants were assayed for heparin-binding ability using heparin-Sepharose chromatography and a cell surface HSPG binding assay, and for enzymatic activity to verify that the overall structure was not disrupted by the mutations.

Heparin-Sepharose chromatography of lipase constructs

Heparin-Sepharose chromatography has been used in several studies to demonstrate a loss in heparin affinity of site-directed mutants of LPL (19–22). The salt concentration required for elution of the protein from the column matrix provides a measure of the affinity of the lipase for heparin. Dimeric LPL elutes at ~1.2–1.3 m NaCl; elution of heparin-binding mutants at lower salt concentration indicates a loss in affinity for heparin relative to the wild-type protein (19, 22). The high-affinity binding of proteins to heparin is due to specific interactions between heparin and precisely arranged residues within a binding region on the protein (37). As a control we constructed a

TABLE 3. Heparin-binding mutants of RHL

Cluster	Mutant	Wild-type	Mutant
1	HB-A	^{297}KGR^{300}	^{297}NKGR^{300}
1	HB-B	^{297}KKGR^{300}	^{297}KNGR^{300}
1	HB-C	^{297}KKGR^{300}	^{297}KKGN^{300}
1	HB-E	^{297}KKGR^{300}	^{297}NNGN^{300}
2	RHL2LPL	^{307}DIRRDRPRKSKTLFLITRAQS^{327}	^{307}KVNRVRTKRNTKMYLKTRAQM^{327}
4	RHL4LPL	^{431}KTIW^{434}	^{431}QKIR^{434}
4	HB-F	^{337}K ^{432}TIWVKAGETQQR^{443}	^{337}N ^{432}TIWVKAGETQQR^{443}
4	HB-G	^{337}K ^{432}TIWVKAGETQQR^{443}	^{337}K ^{432}TIWVNAGETQQN^{443}

Mutated residues are underlined.

TABLE 4. Mutagenic primers used in construction of hepatic lipase mutants

Mutant	Oligonucleotide
HB-A	^{940}TGT CTG AAC TGC AAT AAG GGC CGT TGC AAC^{969}
HB-B	^{943}CTG AAC TGC AAG AAT GGC CGT TGC AAC AGT^{972}
HB-C	^{949}TGC AAG AAG GGC AAT TGC AAC AGT CTG GGC^{978}
HB-E	^{946}AAC TGC AAC AAC GGC AAT TGC AAC AGT CTG^{975}
RHL2LPL	^{967}AAC AGT CTG GGC TAT AAA GTC AAC AGG GTC CGC ACC AAA AGG AAC ACG AAA ATG TAC CTC AAA ACC CGA GCC CAG ATG CCC TTC AAA GTT TAT^{1059}
RHL4LPL	^{1339}GCG GGC CTC ATT GTG CAG AAG ATC CGG GTC AAA GCT GGA GAG ACG^{1383}
HB-F	^{1060}CAT TAC CAG TTC AAC ATC CAG TTC ATC^{1086}
HB-G	^{1354}AAG ACC ATC TGG GTC AAC GCT GGA GAG ACG CAG CAA AAC ATG ACA TTT TGC CCT GAT^{1410}

Mutated bases are underlined. Primers are written in a 5′ to 3′ direction. A primer that encoded for the reverse complement of each of the sequences listed above was also utilized.

Sepharose column with matrix derivatized with ethanolamine. RHL eluted from such a column at 0.35 M NaCl.

Site-directed mutagenesis of cluster 1 of cLPL (Arg 281–Arg 284) was previously shown to disrupt heparin binding of the enzyme. This region is conserved in RHL, therefore, substitution mutants of RHL were made in which the basic residues in the region Lys 297–Arg 300 were singly substituted (HB-A, HB-B, HB-C), or entirely replaced (HB-E) with Asn. These mutations lowered the affinity of RHL for heparin-Sepharose (**Fig. 1A**, Table 5) implying that Cluster 1 is involved in the binding of RHL to heparin. The single substitution mutants HB-A and HB-B showed a modest decrease in the salt required for elution, while HB-C eluted at a much lower salt concentration (0.48 M NaCl) than wild-type RHL (0.70 M NaCl). The mutant in which all residues were substituted for Asn (HB-E), eluted at 0.44 M salt, which is much lower than the value for wild-type RHL (0.70 M NaCl).

TABLE 5. Heparin-Sepharose elution data and enzymatic activity of lipase constructs

Construct	Heparin-Sepharose Elution Position	Enzymatic Activity[a]
	NaCl (M)	*μeq fatty acid/mg/h*
Wild-type		
HL	0.70 ± 0.01	13.7 ± 3.1
LPL	1.30 ± 0.01	10.5 ± 0.5[b]
Cluster 1 mutants		
HB-A	0.64 ± 0.01	7.3 ± 1.2
HB-B	0.63 ± 0.01	8.2 ± 0.5
HB-C	0.48 ± 0.01	7.9 ± 1.1
HB-E	0.44 ± 0.01	9.0 ± 1.4
Cluster 2 mutant		
RHL2LPL	*	*
Cluster 4 mutants		
RHL4LPL	0.90 ± 0.01	7.5 ± 1.4
HB-F	0.61 ± 0.02	16.9 ± 2.5
HB-G	0.57 ± 0.02	8.7 ± 1.6

Heparin-Sepharose chromatography was used to assess the heparin-binding ability of the constructs as described in Materials and Methods. The salt concentration of the peak elution for each mutant was determined in three individual chromatograms and the average and standard deviation of these measurements are reported.

[a] Media samples were chromatographed on heparin-Sepharose columns. The specific activity of RHL peaks (see Figs. 1 and 2) was calculated by taking the average and standard deviation of specific activities of fractions with activity greater than 0.1 μeq/ml/h. RHL2LPL secreted very low levels of enzyme and measurements of enzyme mass or activity after chromatography were not possible (*).

[b] The activity of wild-type LPL was determined in a separate assay and was reported previously (ref. 22, Table 3).

A chimeric mutant was constructed in which Cluster 2 of LPL was substituted for the corresponding region in RHL. Several attempts to transfect RHL2LPL into CHO cells failed to produce stable transformants secreting catalytically active enzyme. In addition, the transfected cells secreted very low amounts of enzyme protein. CHO cells transfected with wild-type RHL secreted 16.5 ng/h per 75-cm^2 flask, whereas the corresponding value for RHL2LPL was 0.6. Accordingly we cannot draw any conclusions concerning the function of Cluster 2 in heparin binding.

Mutations in the Cluster 4 region of LPL were shown to affect heparin-binding (22). This region is partially conserved in RHL. The conserved residues (Lys 337, Lys 436, and Arg 443) were mutated to determine whether they are involved in the heparin binding of RHL. A substitution mutant in which Lys 337 (corresponding to Lys 321 in LPL) was mutated to Asn (HB-F), and one in which Lys 436 and Arg 443 (corresponding to Lys 409 and Lys 416 in LPL) were both changed to Asn (HB-G), were constructed. These mutants both eluted at a lower salt concentration from heparin-Sepharose than wild-type RHL (Fig. 1B, Table 5) indicating that these conserved residues may be important in the binding of RHL to heparin. Part of Cluster 4 is not conserved in RHL, namely Thr 432–Trp 434. This region (residues 431–434) was mutated to that of LPL in the chimera mutant RHL4LPL (Table 3). This mutant showed an increased affinity for heparin-Sepharose, relative to wild-type RHL, as reflected by a higher salt concentration required for elution from the affinity matrix (**Fig. 2**, Table 5).

Equilibrium binding of lipase constructs to cell-surface heparan sulfate

Heparin-Sepharose chromatography provides a measure of the relative affinity of different site-directed mutants for heparin, but yields no direct information regarding how the equilibrium binding constant of the enzyme has been affected by these mutations. An equilibrium binding experiment described by Berryman and Bensadoun (19) gives a direct measure of this effect. Briefly, after a me-

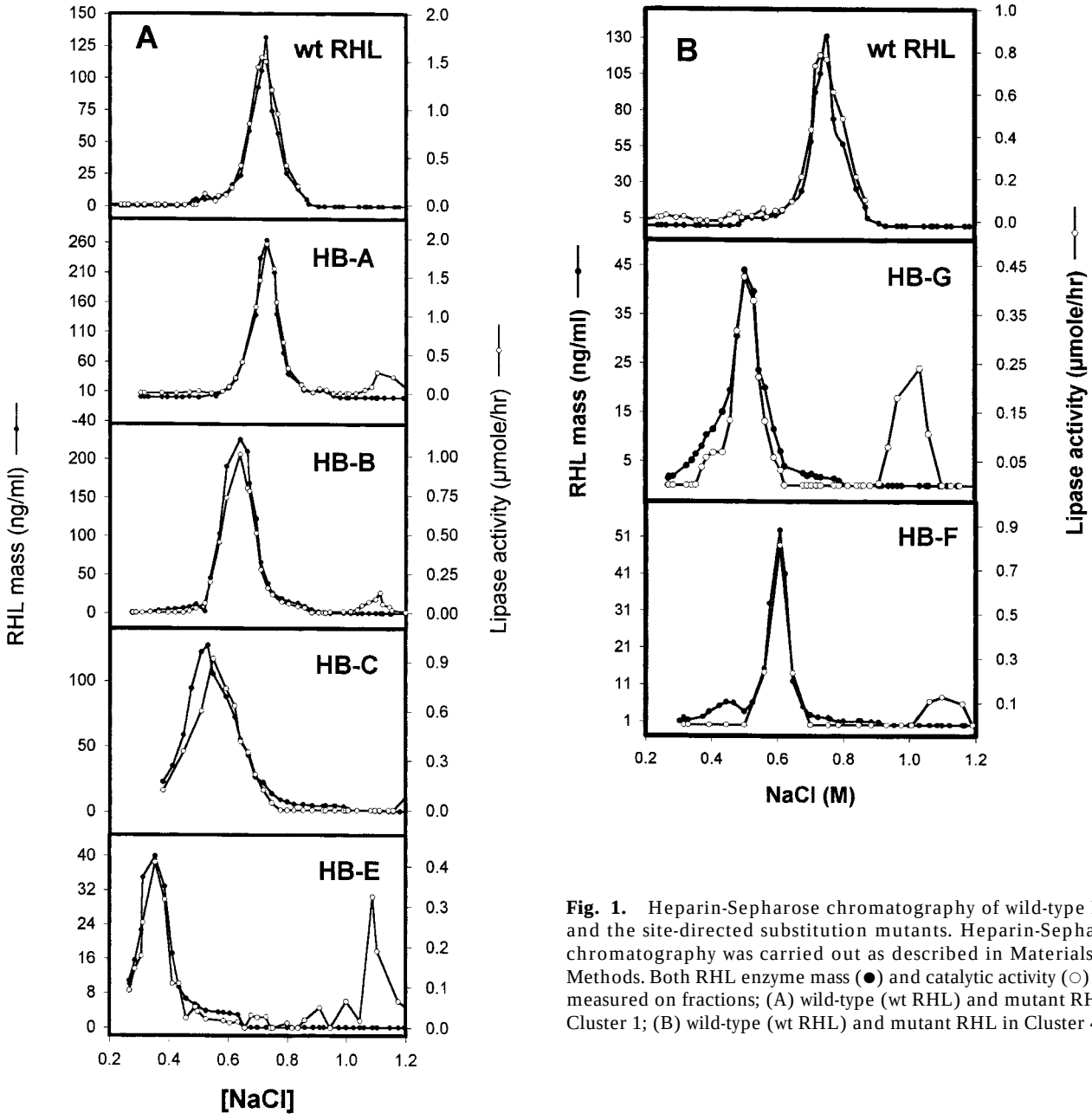

Fig. 1. Heparin-Sepharose chromatography of wild-type RHL and the site-directed substitution mutants. Heparin-Sepharose chromatography was carried out as described in Materials and Methods. Both RHL enzyme mass (●) and catalytic activity (○) were measured on fractions; (A) wild-type (wt RHL) and mutant RHL in Cluster 1; (B) wild-type (wt RHL) and mutant RHL in Cluster 4.

dium change transfected CHO cells are permitted to secrete lipase for 4 h, then the cells are incubated at 4°C during which time the lipase partitions between the bound (cell surface) and free (medium) states. It has been shown previously that HSPGs are primarily responsible for the binding of LPL to CHO cells (38) and this is most likely the case for hepatic lipase as well. The enzyme concentrations are low (5 to 3 times less than K_d) so this simple relationship holds:

$$B = n K F$$

where B is the bound enzyme, n is the number of binding sites on the CHO cell surface, K is the affinity constant, and F is the free enzyme concentration. Because n, the number of binding sites for RHL on the cell surface, is not expected to change from construct to construct, the ratio of bound to free enzyme is proportional to the affinity constant.

We measured the bound to free ratios for all RHL mutants (**Table 6**). Mutation of all basic residues in Cluster 1 in HB-E reduced the affinity of HL for the CHO cell surface dramatically. Single point mutations of basic residues in cluster 1 had major effect on affinity. Mutation of arg 300 (HB-C) caused the greatest decrease in binding affinity when compared to mutations of Lys 297(HB-A) and Lys 298(HB-B). The chimera mutant RHL4LPL demonstrated an increase in equilibrium binding as compared to wild-type RHL. In fact, this mutant showed a cell-binding affinity that was similar to that of wild-type LPL.

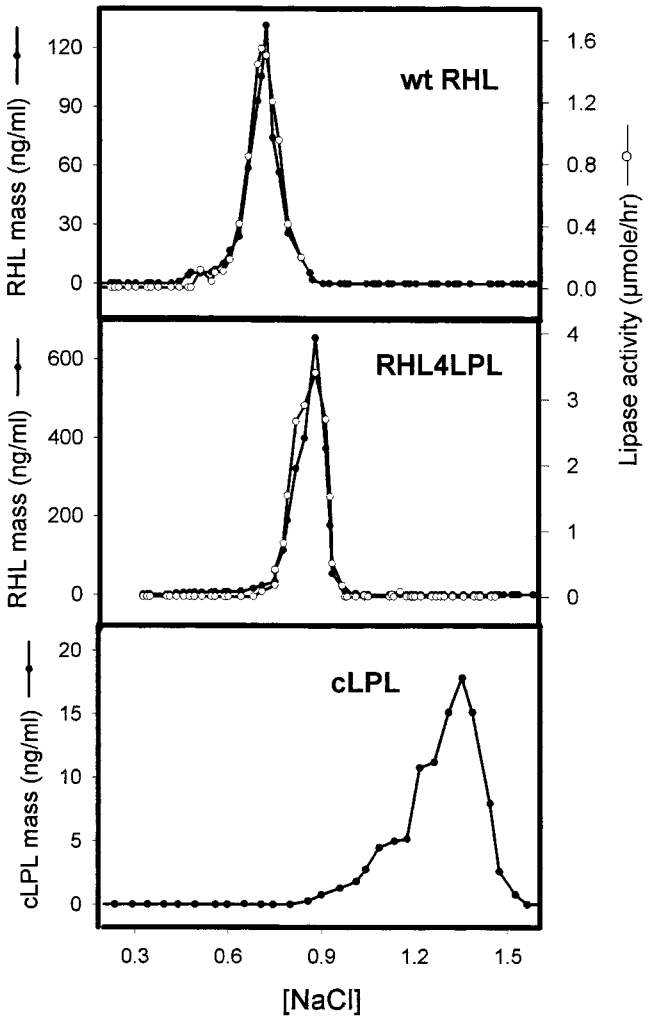

Fig. 2. Heparin-Sepharose chromatography of wild-type RHL, wild-type LPL and the Cluster 4 chimeric mutant RHL4LPL. Heparin-Sepharose chromatography was carried out as described in Materials and Methods. Both RHL enzyme mass (●) and catalytic activity (○) were determined on wild-type RHL (wt RHL) and RHL4LPL. A chromatogram of cLPL is included for comparison.

TABLE 6. Equilibrium binding of lipase constructs to Chinese hamster ovary cells

Construct	[Bound]/[Free]
	(ng/dish)/(100 μg DNA) / *ng/ml*
LPL	8.87 ± 1.04
HL	6.20 ± 1.39
HB-A	1.10 ± 0.09
HB-B	1.03 ± 0.12
HB-C	0.22 ± 0.10
HB-E	0.14 ± 0.03
HB-F	0.65 ± 0.18
HB-G	0.57 ± 0.17
RHL2LPL	0.87 ± 0.18
RHL4LPL	11.49 ± 1.10

Transfected cells secreted lipase for 4 h at 37°C and were then incubated at 4°C for 2 h. The medium was removed (Free) and the bound lipase (Bound) was eluted with a heparin wash (100 U heparin/ml of medium). The free and bound lipase were quantified by ELISA. Cell DNA was quantified by a fluorimetric assay. The results were used to normalize the binding data. Data are expressed as the mean ± standard deviation (n = 4).

Enzyme activity of RHL constructs

The enzymatic activity of the constructs was assayed as an indicator of any global changes in the tertiary structure of the mutants (Table 5). All of the mutants possessed activity with the exception of RHL2LPL. RHL2LPL was secreted at very low levels, and enzyme activity could not be measured reliably. In some of the activity profiles (Fig. 1), a peak is seen eluting at about 1.1 m NaCl. This is most likely due to endogenous LPL that CHO cells are known to secrete (39).

DISCUSSION

Several site-directed mutants of hepatic lipase were constructed to identify regions of the protein involved in heparin binding. These mutant constructs were expressed in CHO cells and evaluated for heparin-binding ability using heparin-Sepharose chromatography and a cell-based binding assay. Site-directed mutagenesis was used to confer increased or decreased heparin binding of various HL mutants. A limitation of this method is that there exists a possibility that the mutation causes a loss of function by altering the structure of the protein. Indicators such as preservation of enzymatic activity suggest that the overall structure has not been perturbed, but one cannot state with certainty that this is the case. Despite this limitation, in the absence of information such as X-ray data, site-directed mutagenesis is a useful tool for identifying potential structural features of proteins. The results of these experiments define many of the putative residues that could be involved in heparin binding of the protein, and allow for a comparison with the structurally related enzyme LPL.

The RHL mutant constructs, with the exception of RHL2LPL, exhibited enzymatic activities that were similar to that of wild-type RHL (Table 5). This suggests that for mutants of Cluster 1 and 4, the overall structure of the lipase was not affected by the mutations. Berryman et al. (28) saw a similar preservation of activity subsequent to epitope-tagging of the lipase. The stability of RHL is in contrast to that of LPL, which was shown to be much more sensitive to mutation, as reflected by large reductions in enzymatic activity, in similar studies (19–22). The greater stability of RHL, as compared to LPL, indicates that it is a superior candidate for future structure/function studies.

Cluster 1 of LPL (Arg 281–Arg 284, avian sequence numbering) was previously proposed to be involved in heparin binding (19–21). This region is conserved in RHL (Table 2). Mutation of the basic residues of this cluster resulted in a reduced heparin binding ability of RHL. This was reflected by a shift to lower salt concentration required for elution from a heparin-Sepharose column; 0.44 m NaCl for the triple mutant HB-E versus 0.70 m NaCl for wild-type RHL. RHL eluted from the control column at

0.35 M NaCl. HB-E eluted at a higher salt concentration (0.44 M NaCl) suggesting that other residues in addition to Cluster 1 might be involved in the heparin-RHL interaction. The effect of this triple mutation on the equilibrium affinity constant of RHL for cell surface HSPGs was shown in a cell-based binding assay (Table 6). This experiment indicated a major decrease in affinity of the lipase for heparan sulfate; the [bound]/[free] values for HB-E and RHL were 0.14 ± 0.03 and 6.20 ± 1.39 (ng/dish)/(ng/ml)/100 mg DNA, respectively. Both of these measures of heparin affinity suggested that Cluster 1 is a significant heparin-binding region of RHL.

Cluster 2 of LPL has previously been subjected to site-directed mutagenesis yielding conflicting results as to which specific residues are involved in heparin binding, but suggesting that, overall, this region has importance in the heparin-LPL interaction (20, 21). We constructed a chimeric mutant, RHL2LPL, in which the sequence of LPL within the vicinity of Cluster 2 has been substituted for the corresponding region in RHL. CHO cells transfected with this construct secreted very low levels of enzyme and therefore no conclusion can be drawn on the significance of Cluster 2 in heparin binding.

Cluster 4 of LPL has been shown to be involved in the heparin binding of LPL (22). This cluster is partially conserved in RHL (Table 2). Mutation of the conserved residues in this cluster (Lys 337, Lys 436, Arg 443) in the mutants HB-F and HB-G resulted in a loss in heparin-binding ability based on heparin-Sepharose elution data (Table 5, Fig. 1B) and cell binding data. This suggests that these residues are involved in the heparin binding of RHL and that they may account for the residual heparin binding seen in the mutant HB-E (Fig. 1A). The non-conserved residues in this region (residues 431–434) were mutated to the corresponding LPL residues in the RHL4LPL chimera (Table 3). This mutant exhibited enhanced binding to heparin-Sepharose (Table 5, Fig. 2) and cell surface HSPGs (Table 6), as compared to wild-type RHL. Production of an intact Cluster 4 region in RHL4LPL resulted in cell surface binding similar to that of LPL.

These data have provided a broad picture of putative regions in HL that may be required for binding to heparin. This interaction requires residues in Cluster 1 (Lys 297–Arg 300) and Cluster 4 (Lys 337, Lys 436, and Arg 443). HL, which possesses a lower affinity for heparin than does LPL, does not have a fully intact Cluster 4 heparin-binding region. The biological significance of the different heparin-binding affinities of LPL and HL is not clear. Fromm et al. (37) demonstrated that heparin, with its closely spaced charge density, interacts with high affinity with peptides containing closely spaced basic residues, while heparan sulfate, with its more widely spaced charge clusters, binds most tightly to peptides with widely spaced basic residues. This indicates that the positioning of basic residues within the tertiary structure of a protein dictates whether that protein will interact with a given heparan sulfate chain. The differences in affinity for heparin seen between LPL and HL may indicate that the HSPG binding sites of these proteins in vivo differ. Evidence in support of this theory includes a rat liver perfusion study, which demonstrated that, in normal rat liver, additional perfused HL was not capable of binding to sites within the liver, while additional perfused LPL was capable of binding (40). This suggests that at least some of the binding sites of HL and LPL within the liver are not interchangeable. Additional experiments to determine the specificity of HL and LPL for particular HSPGs would be of interest to further characterize the binding of these enzymes to HSPGs in vivo.

In summary, we have produced site-directed mutants of HL and assessed these constructs for their ability to bind heparin and heparan sulfate. Our results suggest that all of the basic residues in Cluster 1 (Lys 297–Arg 300) and some of the residues in Cluster 4 (Lys 337, Lys 436, and Arg 443) are involved in the heparin binding of HL. The lack of a completely intact Cluster 4 region, as compared to LPL, is responsible for the lower heparin-binding affinity seen for HL.

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