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A polymorphism in the gene encoding procolipase produces a colipase, Arg92Cys, with decreased function against long-chain triglycerides

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Abstract

Type 2 diabetes mellitus is a multifactorial and polygenic disorder with increasing prevalence. Recently, a polymorphism in the gene encoding procolipase, a cysteine for arginine substitution at position 92, was associated with type 2 diabetes in two human populations. Because procolipase plays a critical role in dietary fat metabolism, polymorphisms that affect the function of procolipase could influence the development of type 2 diabetes. We hypothesized that the Arg92Cys polymorphism has functional consequences. To test our hypothesis, we expressed recombinant cysteine 92 (Cys92) procolipase in a yeast expression system and compared the function and stability of purified Cys92 with that of the more common arginine 92 (Arg92) procolipase. Cys92 fully restored the activity of bile-salt inhibited lipase with short- and medium-chain triglycerides but only had 50% of Arg92 function with long-chain triglycerides. After storage at 4°C, Cys92 lost the ability to restore pancreatic triglyceride lipase activity with medium- and long-chain triglycerides. The loss of function correlated with the inability of Cys92 to anchor lipase on an emulsion surface and oxidation of the cysteine. No detectable degradation or intramolecular disulfide formation occurred in Cys92 after storage. Our findings demonstrate that the Arg92Cys polymorphism decreases the function of Cys92 colipase. This change may contribute to the development of type 2 diabetes.

Supplementary key words

lipase; type 2 diabetes; digestion; recombinant protein; mutagenesis

Pancreatic colipase has a central role in dietary fat digestion (1). Dietary fats, of which triglycerides constitute > 95%, provide 30–40% of the total caloric intake in the Western diet. Before uptake into the enterocyte and transport into the circulation, fatty acids must be released from triglycerides by lipases. Fatty acid release begins with lipolysis in the stomach and proceeds in the upper duodenum, where the majority of fatty acids are released by pancreatic triglyceride lipase (PTL). Many other constituents found in intestinal chyme, such as bile salts and phospholipids, inhibit PTL. In vitro, colipase restores activity to PTL in the presence of these inhibitors (2). In vivo, reports of colipase deficiency in humans and in mice support the role of colipase in dietary fat digestion. Both colipase-deficient humans and mice have fat maldigestion and malabsorption (1, 3).

The mRNA for human colipase encodes preprocolipase, a 112 amino acid protein with a molecular mass of 11.6 kDa (4). The first 17 amino acids constitute a signal peptide.

Removal of the signal peptide results in the formation of procolipase. Procolipase is the secreted form, which is converted to colipase by proteolytic cleavage, probably by trypsin, of an N-terminal pentapeptide. The peptide, called enterostatin, has been implicated in the control of voluntary fat intake (5). Berger et al. (6) proposed that enterostatin restricts fat intake by preventing the overconsumption of fat.

Recently, Lindner et al. (7) reported an association between a new polymorphism in the colipase gene and type 2 diabetes mellitus in two independent populations. They found a novel nonsynonymous single nucleotide polymorphism in codon 109 of the colipase gene, which results in an arginine-to-cysteine substitution at position 92 of procolipase. Logistic regression analysis showed a statistically significant association of the arginine-to-cysteine genotype with type 2 diabetes in two separate populations; one population had an odds ratio of 3.75, and the other had an odds ratio of 4.86. The relationship of fat metabolism with the pathophysiology of type 2 diabetes mellitus and the role of colipase in fat metabolism led the authors to speculate that the polymorphism might contribute to increased susceptibility of type 2 diabetes through changes in postprandial serum triglyceride levels or by altering lipoprotein pathways.

One mechanism through which cysteine 92 (Cys92) colipase might affect lipid metabolism is through alterations in the function or stability of the protein. The more common arginine 92 (Arg92) colipase contains 10 cysteine residues that form five disulfide bonds. These bonds stabilize the structure of colipase and form the loops or fingers of colipase that contribute to the lipid binding function of colipase (Fig. 1) (8, 9). The addition of an extra cysteine residue may lead to an altered conformation of colipase, producing changes in function or stability. We sought to determine whether Cys92 colipase has altered function or stability compared with Arg92 colipase. Detailed knowledge about the function of Cys92 colipase may provide supporting evidence for its possible role in type 2 diabetes mellitus and may offer a clue to the pathophysiology of type 2 diabetes mellitus.

METHODS

Construction of Cys92 colipase

All manipulations of DNA were done by standard methods unless noted otherwise (10). A substitution of Arg92 with Cys92 was accomplished by site-directed mutagenesis using the Quick-Change II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The primers were 5'-CATGACGCTGGATGCTCCAAGCAG-3' and 5'-CTGCTT-GGAGCAATCCAGCGTCATG-3', and the template was human colipase cDNA cloned into pPIC9, as described previously (11). Amplification was with 18 cycles in a Robocycler (Stratagene). The amplified product was transformed into *Escherichia coli* as described by the manufacturer. Plasmid DNA was prepared from single colonies with the Qiagen Spin MiniPrep Kit according to the directions (Qiagen, Valencia, CA). The presence of the desired base changes and the absence of unwanted mutations were confirmed by dideoxynucleotide sequencing of both strands of the entire cDNA insert.

Production and purification of Arg92 and Cys92 colipase

The Arg92 and Cys92 plasmids were transformed into *Pichia pastoris* strain GS115 and expressed as described previously (11). The cells were removed by centrifugation at 3,000 *g* for 5 min, and the medium was concentrated using a Labscale TFF System over a Pellicon XL Biomax 5 membrane (Millipore, Bedford, MA). The sample was diluted 1:1 with 0.1 M NaPO₄ containing 2 M ammonium sulfate, and the pH was adjusted to 7.0. The sample was applied to two 5 ml HiTrap Phenyl HP columns (Amersham Biosciences, Uppsala, Sweden)

connected in tandem and equilibrated in 50 mM NaPO₄ containing 1 M ammonium sulfate, pH 7.0. Chromatography was controlled with an AktaExplorer system (Amersham Biosciences). The column was washed with the equilibration buffer until the optical density at 280 nm (OD₂₈₀) returned to baseline, and the bound colipase was eluted with a 20 column volume gradient from 1 to 0 M ammonium sulfate in the phosphate buffer. Fractions containing colipase were identified by monitoring the OD₂₈₀ and by activity assay as described below. The fractions were pooled and concentrated over an Amicon Ultra-15 5,000 MWCO centrifuge filter according to the parameters described by the manufacturer (Millipore). The buffer was exchanged by gel filtration over a Superdex 75 HR 10/30 column equilibrated in 50 mM Tris-Cl, pH 8.0, and 150 mM NaCl. Fractions containing colipase were pooled and concentrated over the centrifugation filter as above. The concentration of colipase was determined by ultraviolet light spectrophotometry at OD₂₈₀ and an extinction coefficient of E_{1%} = 3.0. The homogeneity of the purified colipase was confirmed by SDS-PAGE and staining with GelCode Blue according to the manufacturer's instructions (Pierce, Rockford, IL) (12).

Activity and adsorption assays

Activity against tributyrin, tricaprylin, and Intralipid was determined in the pH Stat as described previously (8). For each assay, 0.5 ml of the substrate was added to 14.5 ml of 1 mM Tris-HCl, pH 8.0, 2.0 mM CaCl₂, 150 mM NaCl, and 4 mM sodium taurodeoxycholate. The final concentration of the substrate was 310 mM for tributyrin and 200 mM for tricaprylin. Adsorption to tributyrin was measured by a centrifugation assay, and adsorption to Intralipid was measured by an ultrafiltration method as described previously (8, 11). For these assays, 0.5 ml of substrate was added to 14.5 ml of 50 mM Tris-Cl, pH 8.0, 2 mM CaCl₂, 150 mM NaCl, and 4 mM sodium taurodeoxycholate. Recombinant human PTL was prepared as described previously and used in all assays (13).

Immunoblot analysis

Proteins were subjected to SDS-PAGE on a Mini-Gel apparatus using an 18% Ready Gel (Bio-Rad, Hercules CA). They were transferred to an Immobilon-P membrane in 25 mM Tris, 192 mM glycine, and 10% methanol. Nonspecific binding was blocked with 5% nonfat dry milk in TBST (20 mM Tris-Cl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20), incubating for 1 h at room temperature. The membrane was next incubated with a rabbit polyclonal antiserum against human colipase diluted 1:2,000 in TBST for 1 h at room temperature or overnight at 4°C. The blot was washed with TBST three times for 15 min each and then incubated with a 1:250,000 dilution of a goat anti-rabbit IgG coupled to horseradish peroxidase (Pierce) in TBST for 1 h at room temperature. The membrane was washed with TBST as above and developed with SuperSignal WestFemto Maximum Sensitivity Substrate (Pierce) and exposed to X-ray film.

Cysteine quantitation

Ellman's reagent (Pierce) was dissolved in reaction buffer (0.1 M sodium phosphate, pH 8.0, containing 1 mM EDTA) at a concentration of 4 mg/ml. For each sample, a glass tube containing 50 µl of Ellman's reagent and 2.5 ml of reaction buffer was prepared. A total of 250 µl of Arg92, fresh Cys92, or stored Cys92 at 0.4 mM in reaction buffer was added to each tube. A control of reaction buffer was included. After incubation at room temperature for 15 min, the optical density at 412 nm was read and the amount of cysteine was calculated using an extinction coefficient for 2-nitro-5-thiobenzoic acid of 14,150 M⁻¹ cm⁻¹. The lower limit of detection for this amount of sample would be ~0.01 mol cysteine/mol colipase.

Statistics

Comparisons were done with the software package SigmaStat (SPSS, Chicago, IL). Pairwise comparisons were done by *t*-test. Multiple comparisons were done by one-way ANOVA followed by pairwise multiple comparisons with the Holm-Sidak method. $P < 0.05$ was considered significant.

RESULTS

To determine the effect of the Arg92Cys substitution on colipase function, we first measured the ability of Cys92 and Arg92 colipase to reactivate sodium taurodeoxycholate-inhibited PTL over a range of colipase concentrations in the presence of excess tributyrin and a constant amount of PTL. The titration curves for each colipase were virtually superimposable (Fig. 2). From these data, we determined the concentration of colipase that restored half-maximal activity to PTL and the concentration that restored maximal activity to PTL using a rectangular hyperbola function. The nearly identical concentrations of 210.5 ± 6.3 for Arg92 and 249.6 ± 28.5 for Cys92 to restore half-maximal activity indicate that the two colipases activated PTL with similar efficiency. Furthermore, each colipase formed competent complexes with PTL, as indicated by their identical maximal activity, $4,347 \pm 71$ for Arg92 colipase and $4,449 \pm 113$ for Cys92 colipase, suggesting that each colipase effectively anchors PTL at an oil-water interface.

To directly test whether Cys92 has altered ability to anchor PTL to the substrate surface, we measured the ability of each colipase to anchor PTL at an oil-water interface. We measured adsorption with a centrifugation assay that rapidly separates the organic lipid phase from the aqueous phase. Each assay contained different molar ratios of colipase to PTL added to an emulsion of tributyrin in 4.0 mM sodium taurodeoxycholate. In this assay, the amount of PTL adsorbed varied with the molar ratio of colipase (Fig. 3). More than 90% of the added PTL was adsorbed in the presence of either Arg92 or Cys92 colipase. There was no significant difference in the ability of the two colipases to anchor PTL to a tributyrin emulsion at any molar ratio, a finding that confirms the previous results.

We next determined the function of both colipases with other substrates. In an earlier study, we had demonstrated that amino acid substitutions in colipase could differentially affect colipase function depending on the substrate (8). We tested activity against trioctanoin, a medium-chain triglyceride, and against Intralipid, an emulsion of phospholipids and triolein, an 18 carbon monounsaturated triglyceride. Trioctanoin and triolein have limited solubility in water and present more hydrophobic substrates than tributyrin. Intralipid provides a complex interface that more closely mimics dietary emulsion particles. Both substrates were emulsified with 4.0 mM sodium taurodeoxycholate. The specific activities (U/mg) of Arg92 and Cys92 against trioctanoin were not significantly different, $7,880 \pm 500$ and $7,970 \pm 375$, respectively. The specific activities against Intralipid (triolein) were $1,110 \pm 76$ for Arg92 and 620 ± 68 for Cys92 ($P = 0.001$). The decreased function against the more complex substrate suggests that Cys92 colipase may have decreased function against dietary emulsions in vivo as well.

Another property that could be altered by the polymorphism is protein stability. The main stabilizing forces of the colipase conformation are the disulfide bonds. We tested the effect on protein stability of adding an additional cysteine to colipase in several ways. First, we incubated Arg92 and Cys92 colipase at 65°C or 85°C for 120 min and measured the residual function in the standard PTL assay with various substrates and 4 mM sodium taurodeoxycholate (Table 1). There was no difference detected between the two colipases at either temperature or with the different substrates. Both Arg92 and Cys92 colipase were

stable at 65°C, and each lost ~70% of their activity at 85°C. Clearly, the Arg92Cys substitution does not significantly affect the temperature stability of colipase.

During the course of these studies, we stored portions of both Arg92 and Cys92 colipase at 4°C, conditions in which Arg92 colipase retains function for many months. One month after we had isolated Cys92, we repeated the assays against trioctanoin and discovered that the preparation no longer had any function. We then measured the activity of the same preparation against tributyrin and Intralipid. The stored Cys92 retained the ability to reactivate PTL when the substrate was tributyrin but not when we assayed with Intralipid. In fact, with tributyrin, the activity was similar for the two colipases (Table 2). Over the same time period, Arg92 retained full function with all substrates. We repeated this experiment with freshly isolated recombinant Cys92. Initially, the function of this protein against tributyrin and trioctanoin was indistinguishable from that of Arg92 and ~50% of activity with Intralipid, just like the first preparation. When we stored the newly prepared Cys92 at 4°C for 1 month, this preparation also lost activity against trioctanoin and triolein while retaining function with tributyrin (Table 2).

To determine the mechanism for the decreased activity of freshly prepared Cys92 and the lost function of stored Cys92 colipase against Intralipid, we measured the ability of both Cys92 colipases and Arg92 colipase to anchor PTL on emulsions of Intralipid using a previously described method (8). Adsorption was measured at various molar ratios of colipase to PTL. Under these conditions, both Arg92 colipase and freshly prepared Cys92 colipase showed a concentration-dependent anchoring of lipase. Importantly, the ability of Cys92 colipase to anchor PTL was decreased significantly compared with that of Arg92 (Fig. 4). Although some PTL adsorbed to the emulsion in the presence of stored Cys92, the amount of PTL adsorbed to the emulsion was independent of the stored Cys92 colipase concentration and remained constant throughout the tested range. Given the lack of concentration dependence and the lack of PTL activity in the presence of stored Cys92 colipase, the adsorption seen at each concentration most likely represents nonspecific adsorption of PTL or adsorption of an inactive colipase/PTL complex. These results suggest that Cys92 affects the ability of this colipase isoform to anchor lipase onto the emulsion surface.

We next tested three potential explanations for the loss of function by Cys92: oxidation of the cysteine, degradation of the protein, and the formation of intermolecular disulfide bonds during storage. We measured the oxidation of cysteine in freshly prepared Cys92 and Cys92 stored at 4°C for 1 month using Ellman's reagent. The values of cysteine for Arg92, fresh Cys92, and stored Cys92 colipase were <0.01, 0.97, and <0.01 mol cysteine/mol colipase, respectively. These results indicate that the free cysteine in Cys92 either oxidized with time or formed an intermolecular disulfide bond.

We tested for the degradation and formation of intermolecular disulfide bonds by running aliquots of Arg92 and Cys92 colipase on an 18% polyacrylamide gel with and without reducing agent. After electrophoresis with reducing agent, the proteins were transferred to a membrane and the proteins were detected by immunoblot with a polyclonal antibody against human colipase (Fig. 5A). Each protein was present as a single band, without any evidence of smaller fragments. Furthermore, Cys92 and Arg92 migrated at the same position. No additional bands were present on GelCode Blue-stained gels (data not shown). In the absence of reducing agents, when the gel was stained with GelCode Blue, both Cys92 and Arg92 colipases migrated at the same position and no larger molecular weight species were detected (Fig. 5B). These findings indicate that there was no significant degradation or aggregation of Cys92.

We then determined whether the addition of 1 mM EDTA and 1 mM dithiothreitol to the purification buffers and storage buffer improved the activity of freshly prepared Cys92 against Intralipid. One preparation was tested, and three replicate assays were done. The specific activity of Cys92 isolated under these conditions was 605 ± 53 , a value not significantly different from that of Cys92 prepared without EDTA and dithiothreitol. This result and the presence of detectable cysteine in freshly prepared Cys92 suggest that oxidation does not explain the decreased activity against Intralipid.

DISCUSSION

In this study, we examined the function of a naturally occurring polymorphism of human colipase and found that the polymorphism affects the function of colipase depending on the substrate and on storage. Freshly prepared Cys92 colipase has normal activity against short- and medium-chain triglycerides but has 2-fold less function against a long-chain triglyceride emulsified in phospholipid and bile salt. After storage at 4°C, Cys92 colipase loses its ability to restore activity to bile salt-inhibited PTL when the substrate is medium- or long-chain triglyceride. It retains activity against the short-chain substrate. The mechanism for the lost function appears to be oxidation of the single cysteine, which decreases the ability of the stored Cys92 colipase to anchor PTL onto the surface of Intra-lipid emulsions.

The location of Cys92 in the structure of colipase suggests that it is likely to alter the structure of the fourth and last finger of colipase (residues 68–88). The importance of this region in the adsorption of colipase to an interface is supported by two pieces of evidence. First, the tip of the loop contains residues isoleucine 75, valine 76, isoleucine 79, and phenylalanine 84, which contribute to the large hydrophobic plateau formed by colipase and the lid domain of PTL. Egloff et al. (14) proposed that the interaction of the colipase/PTL complex takes place through residues in this plateau. Second, Rugani et al. (15) demonstrated that cleavage of the bond between isoleucine 79 and serine 78 by elastase did not cause separation of the two peptides but did decrease the function and lipid binding properties of the modified colipase. If this loop contributes to lipid binding, then any disruption in the orientation of the hydrophobic residues in the loop relative to the other hydrophobic residues in colipase and PTL could greatly affect the ability of colipase to anchor PTL at an oil-water interface. The discoordinate effect of the Cys92 polymorphism on function with medium- and long-chain substrates compared with short-chain substrates suggests that residues in the fourth finger of colipase play a greater role in anchoring PTL to these substrates, perhaps because a larger hydrophobic surface is required for interactions with these lipids.

Another potential explanation arises from our work on the $\beta 5'$ loop of PTL formed by residues 405–414 of the C-terminal domain (16, 17). Although the colipase does not contact the $\beta 5'$ loop, increased hydrophilicity in the loop clearly impaired the interaction between colipase and PTL, particularly in the presence of bile salt micelles (16, 17). Our observations and a neutron diffraction study of the colipase/PTL complex that showed a bile salt micelle binding to the fourth finger of colipase and the $\beta 5'$ loop of PTL suggested that a third partner, bile salt micelles or lipid interface, influences the binding of colipase and PTL (18). If so, alterations in the fourth finger of colipase, as might occur in Cys92 colipase, could affect the formation of the ternary complex and decrease the interaction between colipase and PTL, as we observed.

Our finding of decreased function for Cys92 compared with Arg92 on long-chain triglycerides suggests several possible connections between the polymorphism and type 2 diabetes. The decreased function of Cys92 may well move fat digestion farther down the small intestine than normally occurs. Delayed digestion and release of fatty acids farther

down the intestine may alter food intake through a mechanism that might include the action of gut hormones (19–26). In the case of Cys92 colipase, the decreased function may be enough to limit the satiety response originating in the upper intestine by producing lower concentrations of fatty acids.

Of course, it remains possible that procolipase may have unknown functions yet to be elucidated. Although colipase expression is maximal in the exocrine pancreas, it is expressed in the stomach and in the small intestine (27, 28). Additionally, procolipase can be expressed ectopically under particular conditions. For example, mice kept in the dark express the gene encoding procolipase in their liver and other tissues, including adipose tissue (29). The role of colipase in these tissues is unclear, and it may influence metabolism through functions unrelated to dietary fat digestion.

Acknowledgments

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Abbreviations

Arg92	arginine 92
Cys92	cysteine 92
OD₂₈₀	optical density at 280 nm
PTL	pancreatic triglyceride lipase

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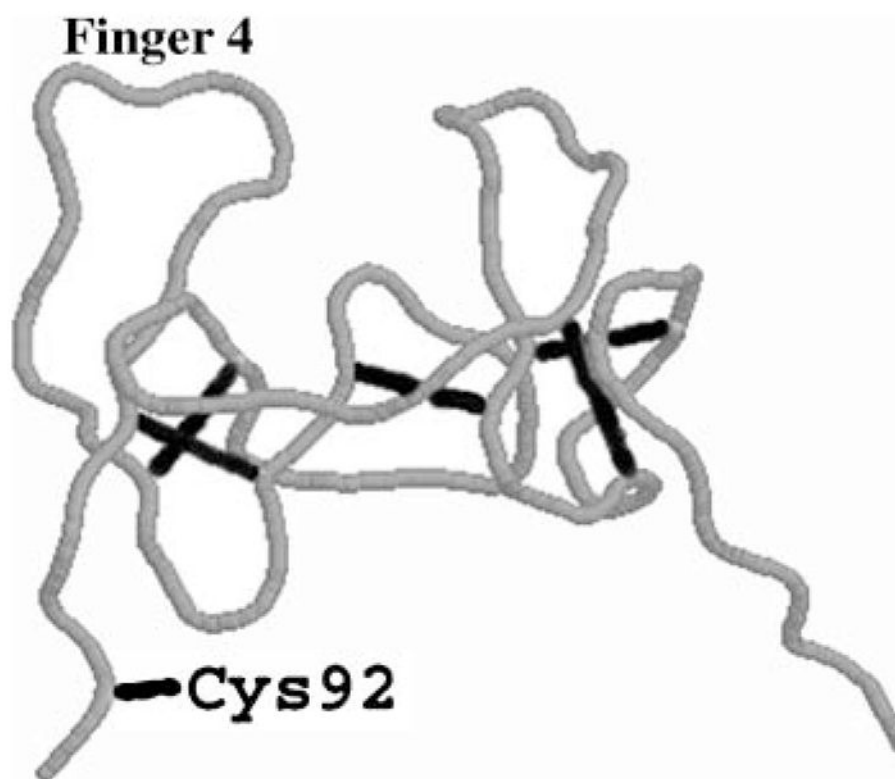


Fig. 1. Schematic representation of the structure of human colipase. The α -carbon backbone is shown in light gray, and the disulfide bonds are indicated in black. The position of cysteine 92 (Cys92) is labeled.

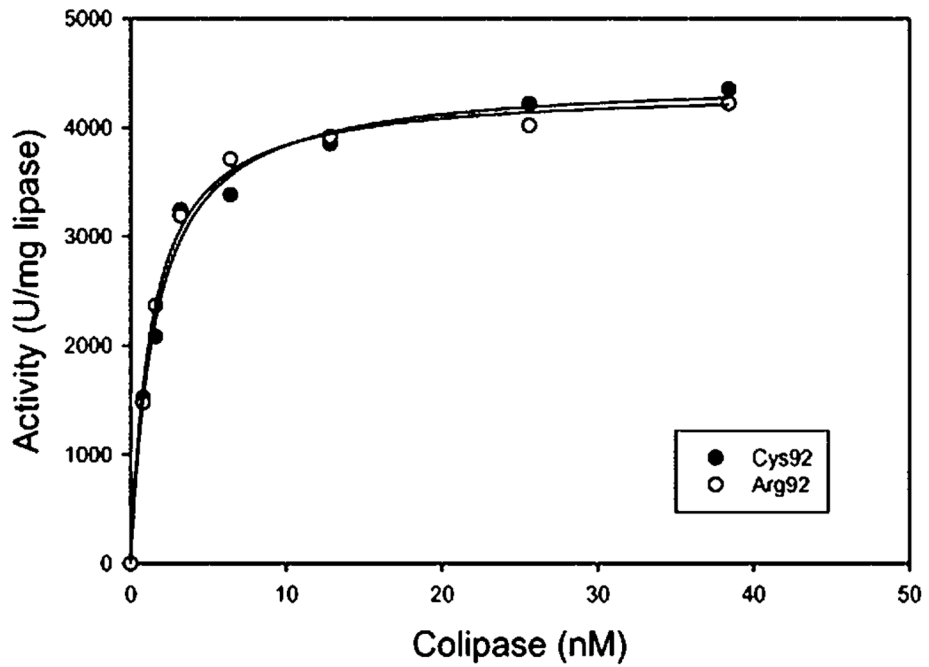


Fig. 2. Colipase dependence of lipase for arginine 92 (Arg92) and Cys92 colipase. The activity of pancreatic triglyceride lipase (PTL) against tributyrin with various concentrations of colipase is presented. Each assay contained 1 μ g of human PTL, 310 nM tributyrin, and 4 mM sodium taurodeoxycholate.

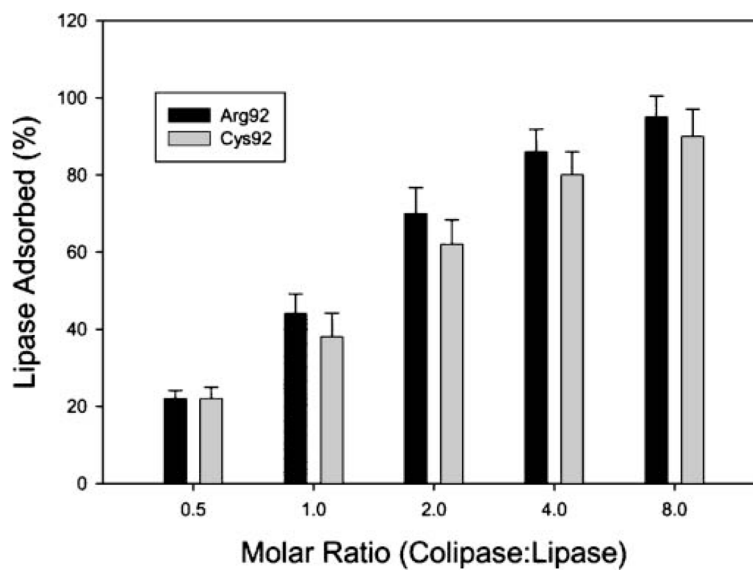


Fig. 3. Adsorption of PTL to tributyrin emulsions mediated by Arg92 and Cys92 colipase. All assays were done as described in Methods. A total of 2.5 μg of PTL was added to each tube along with varying amounts of colipase. The values are expressed as percentages of the total added PTL determined from the amount of PTL remaining in the supernatant after incubation with tributyrin in the absence of colipase. The values shown are averages \pm SD of three determinations.

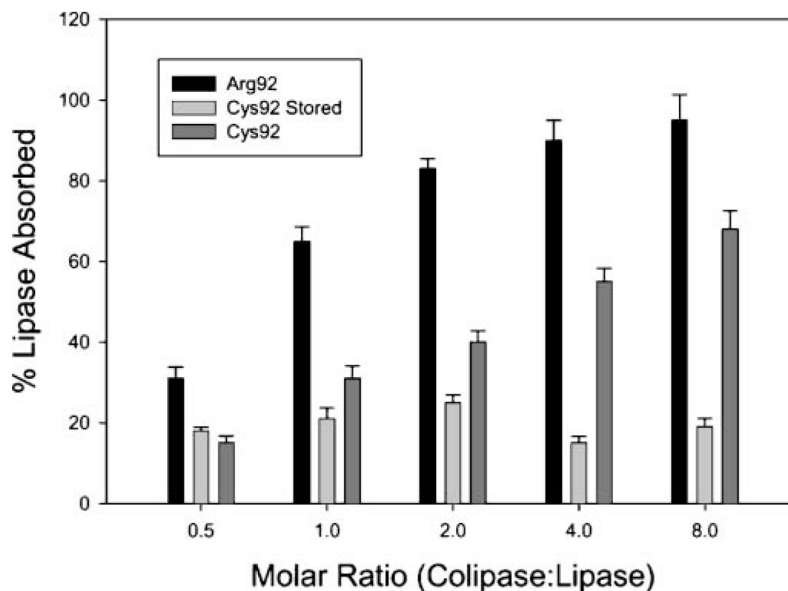


Fig. 4. Adsorption of PTL to Intralipid emulsions mediated by Arg92, fresh Cys92, and stored Cys92 colipase. The assay was done as described in Methods. A total of 2.5 μ g of PTL was added to each tube along with varying amounts of colipase. The values are expressed as percentages of the total added PTL determined from the amount of PTL remaining in the supernatant after incubation with Intralipid in the absence of colipase. The values shown are averages \pm SD of three determinations. The data at each colipase-to-lipase ratio were analyzed by one-way ANOVA followed by pairwise multiple comparisons with the Holm-Sidak method. There was a significant difference among the groups ($P = 0.0001$) at all molar ratios. In the 0.5 molar ratio group, Arg92 was significantly different from the other two samples, which were not different from each other. At every other ratio, the ability of each colipase to anchor PTL differed significantly from the other two.

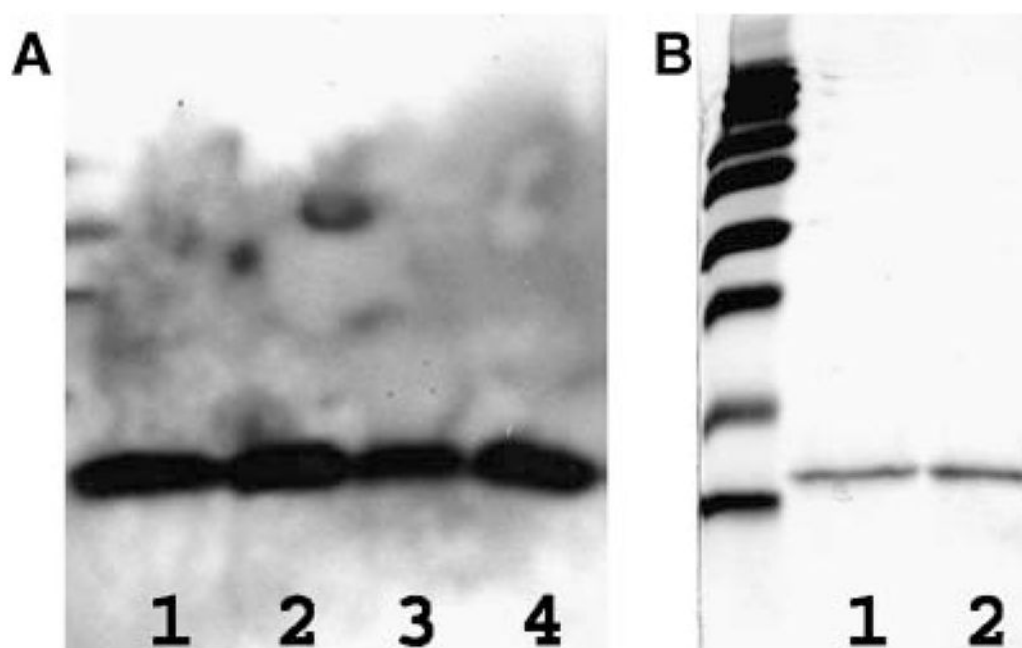


Fig. 5. SDS-PAGE analysis of Arg92 and Cys92 colipase. A: Samples were prepared with β -mercaptoethanol. After separation, the proteins were transferred to an Immobilon membrane and detected by immunoblot analysis as described in Methods. Lanes 1 and 2 contain two different samples of Arg72 colipase; lane 3 contains freshly isolated Cys92 colipase; lane 4 contains stored Cys92. B: Nonreducing electrophoresis of Arg92 and Cys92 colipase. Four micrograms of each protein was subjected to SDS-PAGE and detected with GelCode Blue. Lane 1, Arg92; lane 2, stored Cys92.

TABLE 1

Effect of temperature on activity

Temperature	Tributyrin		Triolein		Intralipid	
	Arg92	Cys92	Arg92	Cys92	Arg92	Cys92
65°C	110 ± 4	103 ± 11	102 ± 8	99 ± 7	95 ± 11	102 ± 10
85°C	74 ± 2	70 ± 3	68 ± 3	72 ± 4	65 ± 4	73 ± 5

Arg92, arginine 92; Cys92, cysteine 92. Values are expressed as percentages of the activity of unheated colipase. Each assay contained 1 µg of pancreatic triglyceride lipase (PTL) and 100 ng of colipase, except for the Intralipid assays, which contained 10 µg of PTL and 1 µg of colipase. The value at 65°C is significantly different from the value at 85°C for each pair by *t*-test.

TABLE 2

Change in activity of Cys92 colipase with storage

Substrate	Sample 1			Sample 2		
	Arg92 Fresh	Arg92 Stored	Cys92 Fresh	Cys92 Stored	Cys92 Fresh	Cys92 Stored
Tributyrin	15,260 ± 536	14,000 ± 230	14,760 ± 215	15,000 ± 190	13,480 ± 412	12,860 ± 247
Trioctanoin	7,730 ± 550	7,880 ± 500	7,970 ± 375	ND ^a	7,670 ± 453	ND
Intralipid	1,090 ± 85	1,110 ± 76 ^{b,c}	620 ± 68 ^b	ND	675 ± 82 ^c	ND

Each assay contained 1 µg of PTL and 100 ng of colipase, except for the Intralipid assays, which contained 5 µg of PTL and 500 ng of colipase. Activity is given as µmol fatty acid release/min/mg colipase.

^aND, none detected.

^b*P* = 0.001.

^c*P* = 0.003.