

The Arg92Cys colipase polymorphism impairs function and secretion by increasing protein misfolding

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Abstract Colipase is essential for efficient fat digestion. An arginine-to-cysteine polymorphism at position 92 of colipase (Arg92Cys) associates with an increased risk for developing type-2 diabetes through an undefined mechanism. To test our hypothesis that the extra cysteine increases colipase misfolding, thereby altering its intracellular trafficking and function, we expressed Cys92 colipase in HEK293T cells. Less Cys92 colipase is secreted and more is retained intracellularly in an insoluble form compared with Arg92 colipase. Nonreducing gel electrophoresis suggests the folding of secreted Cys92 colipase differs from Arg92 colipase. Cys92 colipase misfolding does not trigger the unfolded protein response (UPR) or endoplasmic reticulum (ER) stress. The ability of secreted Cys92 colipase to stimulate pancreatic triglyceride lipase (PTL) is reduced with all substrates tested, particularly long-chain triglycerides. The reaction of Cys92 colipase with triolein and Intralipid has a much longer lag time, reflecting decreased ability to anchor PTL on those substrates. Our data predicts that humans with the Arg92Cys substitution will secrete less functional colipase into the duodenum and have less efficient fat digestion. Whether inefficient fat digestion or another property of colipase contributes to the risk for developing diabetes remains to be clarified.—Xiao, X., M. R. Ferguson, K. E. Magee, P. D. Hale, Y. Wang, and M. E. Lowe. **The Arg92Cys colipase polymorphism impairs function and secretion by increasing protein misfolding.** *J. Lipid Res.* 2013. 54: 514–521.

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In Western diets, triglycerides constitute 95% of dietary fats and provide 30–40% of total caloric intake. Prior to absorption, triglycerides must be hydrolyzed to free fatty acids or monoglycerides by the concerted action of lipases (1). The majority of fatty acids are released in the duodenum by pancreatic triglyceride lipase (PTL). Because

many constituents present in intestinal lumen, including bile salts, phospholipids, cholesterol esters, dietary proteins, and carbohydrates, inhibit PTL, another pancreatic protein, colipase, is required for PTL activity (2).

Hence, colipase plays an important role in dietary fat digestion. Colipase is predominantly expressed in the exocrine pancreas and, to a lesser degree, in the stomach and intestine. Newly synthesized colipase contains a 17 amino acid signal peptide and a 5 amino acid propeptide at the N-terminus. The propeptide is cleaved from procolipase to form colipase and releases a pentapeptide named enterostatin (3). Enterostatin is a satiety factor that reduces voluntary fat intake in animals (4). Mature colipase is a 10 kDa protein with minimal secondary structure. Five disulfide bonds determine the folding of colipase and stabilize the loops or fingers of colipase (5) (Fig. 1). The limited secondary structure protects colipase from surface denaturation and accounts for its high stability in adverse environments (5). Models of colipase function include a lipid-binding surface on the tips of the loops and a PTL-binding surface on the opposite surface. Colipase anchors PTL on the emulsion surface and stabilizes the active conformation of PTL (6).

Analysis of genomic sequence data identified a polymorphism in codon 109 of human colipase gene, resulting in an arginine-to-cysteine substitution at position 92 (Arg92Cys) of procolipase. Several studies have reported that the Arg92Cys substitution in colipase increased the odds risk for developing type-2 diabetes in two independent Caucasian populations (7, 8). The authors speculated that the polymorphism contributes to increased susceptibility for type-2 diabetes by influencing postprandial serum triglyceride levels or by altering lipoprotein metabolism. No direct evidence supports either of these hypotheses.

Abbreviations: Arg92, arginine 92; Arg92Cys, arginine-to-cysteine substitution at position 92; Cys92, cysteine 92; ER, endoplasmic reticulum; NaTDC, sodium taurodeoxycholate; PDI, protein disulfide isomerase; PTL, pancreatic triglyceride lipase; UPR, unfolded protein response; Xbp1, X-box binding protein-1.

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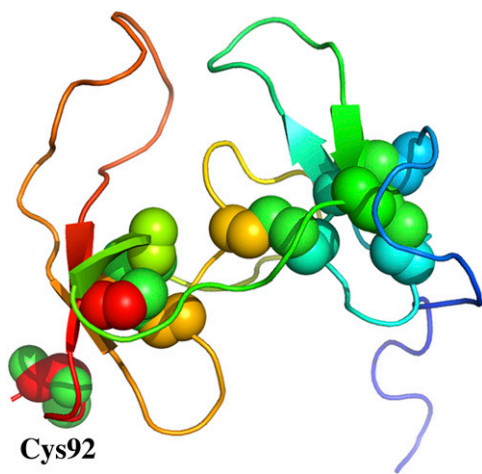


Fig. 1. Schematic representation of human colipase protein. The α -carbon backbone is shown as a ribbon structure. The cysteines that form disulfide bonds and Cys92 are displayed as spheres. The position of Cys92 is labeled. The structure was drawn with PyMOL Molecular Graphics System, Version 1.4, Schrodinger, LLC, using the Protein Data Bank files 1LPB for porcine colipase.

Furthermore, analysis of the colipase structure does not explain how the Arg92Cys substitution might affect colipase function. The residue does not participate in the adsorption of colipase to an interface or in the binding of colipase to PTL (9). Because Arg92 is located near the C-terminus of colipase in an unstructured, highly mobile region, we hypothesized that the extra cysteine residue in Cys92 colipase might result in mispaired cysteine residues and alter the structural conformation and function of colipase. We characterized the biochemical properties of Cys92 colipase using purified recombinant protein from *P. pastoris* yeast (10). Recombinant Cys92 colipase had decreased function against long-chain triglycerides and was less stable on storage at 4°C compared with Arg92 colipase, but we found no evidence of aberrant disulfide bonds. A major concern with our previous study was that we may have selected against improperly folded Cys92 colipase by purifying secreted Cys92 colipase. To address this possibility, we expressed Cys92 colipase in HEK293T cells by transient transfection and characterized its synthesis and secretion from the cells and assayed the function of secreted, unpurified Cys92 colipase. The knowledge obtained from the present study sheds additional light on the physiological consequences of the Arg92Cys polymorphism on the metabolism of dietary fats and the development of type-2 diabetes.

MATERIALS AND METHODS

Construction of colipase plasmids

The full-length cDNA of human colipase was amplified by PCR using the cDNA previously obtained (3) and the following primers: 5'-TCACCATGGAGAAGATCCT GATCCTCCTG-3' and 5'-GTCTCACT GCTTGGAGCG TCCAGCGTC-3'. The amplified cDNA was cloned into mammalian protein expression vector

pcDNA3.3 Topo TA (Invitrogen, Carlsbad, CA). Substitution of Arg92 with Cys92 was accomplished by site-directed mutagenesis using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The sequences of all plasmid DNA constructs were verified by dideoxynucleotide sequencing.

Culture and transfection of HEK293T cells

HEK293T cells were cultured in DMEM supplemented with 10% FBS. Twenty-four hours prior to transfection, cells were harvested by trypsinization and seeded at 2×10^6 cells in 6-well plates, about 50% confluence. The cells were transfected with 1.65 μ g of plasmid DNA (pcDNA3.3, pcDNA3.3 TOPOTA containing Arg92 or Cys92 colipase) using 5 μ l of Fugene 6 in 100 μ l of Opti-MEM I Reduced Serum Medium (Invitrogen) according to the manufacturer's manual (Roche Applied Science, Indianapolis, IN). Samples were collected 72 h after transfection unless stated otherwise. The amount of DNA, Fugene 6, and medium were adjusted proportionately for transfections in 10 cm dishes. Forty-eight hours after transfection, conditioned media were withdrawn, and the cells were switched to Opti-MEM I Reduced Serum Medium for 24 h. Conditioned media were collected for further analysis.

Sample collection and preparation

The conditioned media and attached cells were harvested at indicated time points after transfection. The pelleted cells were lysed in 200 μ l of NP40 lysis buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, and 5% glycerol) with EDTA Free Complete Protease Inhibitor Cocktail (Roche), followed by 15,000 *g* for 15 min centrifugation at 4°C. The protein concentration of the supernatant, referred to as the soluble cell lysate, was determined by Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). The pellets were washed twice with ice-cold PBS, and then resuspended with 100 μ l of NP40 lysis buffer and 2 \times Laemmli sample buffer (125 mM Tris HCl, pH 6.8, 4% SDS, 20% glycerol). The pellets were sonicated 3 \times 10 s with 15 s intervals on ice. The sample was boiled at 95°C for 10 min. Alternately, whole cell lysates were prepared by lysing pelleted cells with 200 μ l of 1 \times Laemmli sample buffer, followed by sonication and boiling.

For cells transfected in 10 cm dishes, approximately 20 ml of conditioned media from duplicate transfections was thoroughly dialyzed and lyophilized. The powder was reconstituted in 500 μ l of 25 mM Tris-HCl, pH 8.0. The samples were centrifuged at 15,000 *g* for 3 min, and the supernatants were stored at 4°C.

Pulse-chase experiments

Twenty-four hours posttransfection, cells were harvested and reseeded on collagen coated 24-well culture plates and incubated until 90–100% confluence (\sim 48 h). The cells were incubated in 3 \times 333 μ l/well of pulse medium (methionine-free DMEM supplemented with 250 μ Ci/ml of S^{35} methionine, MP Biomedicals, Santa Ana, CA) for 60 min and switched to 3 \times 333 μ l/well of chase medium (DMEM only) for 0, 30, 60, 120, 180, or 240 min. Samples from each three wells were collected at the indicated time points. Culture medium was subjected to 1,000 *g* for 5 min to remove debris. The cells were lysed with 1 ml of NP40 lysis buffer supplemented with protease inhibitors. Cell lysates were obtained after centrifugation at 15,000 *g* for 15 min. The S^{35} methionine incorporation was measured by TCA scintillation counting. Immunoprecipitations were carried out using 200 μ l of medium or cell lysate and 10 μ l of a rabbit anti-human procolipase antibody. The eluted samples were resolved by 18% SDS-PAGE, and dried gels were subjected to autoradiography.

Colipase activity assay

Colipase assays were performed as described (11). The assays were 5 min unless noted otherwise. Each substrate was emulsified with assay buffer [1 mM Tris-HCl, pH 8.0, 2.0 mM CaCl₂, 150 mM NaCl, and 4 mM sodium taurodeoxycholate (NaTDC)] in a final volume of 15 ml. Purified recombinant human colipase and PTL were prepared as described previously (12, 13). The activity of colipase was expressed in international lipase units. One unit corresponds to one micromole of fatty acid released per minute. When different volumes of sample were assayed, we adjusted the samples to the same volume by adding conditioned medium from mock-transfected cells.

Thermal stability assay

Aliquots (200 μ l) of fresh conditioned media from HEK293T cells expressing Arg92 or Cys92 colipase 72 h posttransfection were heat treated for various lengths of time. Colipase activity in the aliquot was determined against tributyrin prior to heat treatment. After heat treatment, the remaining colipase activity was assayed immediately using tributyrin as substrate. The data is expressed as a percentage (remaining activity/starting activity \times 100).

Binding assay

The binding assays were done by published protocols (11, 12, 14). Substrate (0.5 ml) was added to 14.5 ml of binding buffer (50 mM Tris-HCl, pH 8.0, 2 mM CaCl₂, 150 mM NaCl, and 4 mM NaTDC). Concentrated medium containing expressed colipase was added so that each assay included an equimolar ratio of colipase (0.5 μ g) to PTL (2.5 μ g). The colipase in the medium was estimated by immunoblot. Purified colipase was used as a standard.

Western blot analysis

Aliquots of culture medium or cell lysate were analyzed by 4–20% SDS-PAGE. The separated proteins were transferred onto Immobilon-FL PVDF membranes (Millipore, Billerica, MA). For colipase, membranes were incubated with a rabbit polyclonal antibody against human procolipase, followed by a goat-anti rabbit IgG 680 secondary antibody (LI-COR, Lincoln, NE). The blots were reincubated with a mouse polyclonal anti-GAPDH antibody (Cell Signaling, Danvers, MA) and then with a goat-anti mouse IgG 800 secondary antibody. For detection of endoplasmic reticulum (ER) stress markers, a rabbit polyclonal antibody against BiP, GRP94, PDI, or ERp57 (Cell Signaling) was used as primary antibody. α -tubulin, which was used for normalizing sample loading, was detected with a mouse polyclonal antibody.

Semiquantitative RT-PCR

Total RNA was isolated using TRIzol (Invitrogen) and reverse-transcribed with M-MLV reverse transcription kit (Ambion). Semiquantitative measurements were performed by PCR of X-box binding protein-1 (Xbp1) cDNA and its spliced form using the following primers: 5'-CCT TGT AGT TGA GAA CCA GG-3' and 5'-GGG CTT GGT ATA TAT GTG G-3'. GAPDH served as an endogenous control, with the primers 5'-GTC-CAC TGG CGT CTT CAC CA-3' and 5'-GTG GCA GTG ATG GCA TGG AC-3'. PCR products were resolved by 2% agarose gels and visualized by ethidium-bromide staining.

Statistics

Comparisons were conducted with GraphPad Prism 5 (La Jolla, CA). Pairwise comparisons were done by *t*-test. Multiple comparisons were carried out using one-way ANOVA. *P* < 0.05 was considered significant.

In our previous study, we expressed Cys92 and Arg92 colipase in *Pichia pastoris*. The activity of Cys92 colipase in the medium was consistently < 5% of that of Arg92 colipase. Although variability in transfection efficiency could explain this finding, the observation also raises the possibility that Cys92 colipase was improperly folded and retained in the yeast. To determine the effect of Arg92Cys polymorphism on intracellular trafficking, we expressed Arg92 and Cys92 colipase in transfected HEK293T cells. We measured the presence of colipase in the conditioned medium by activity assay using 20 μ l of medium, 2 μ g of PTL, and tributyrin emulsified in 4 mM NaTDC. Preliminary assays with varying amounts of medium containing colipase demonstrated that PTL was in excess in this assay. The activity in the medium of both colipase variants increased linearly with time (Fig. 2A). Over time, the activity of Cys92 remained roughly half that of Arg92 colipase at each time point. We next determined the dose response of increasing amounts of conditioned medium collected 72 h posttransfection (Fig. 2B). Both Arg92 and Cys92 restored bile-salt-inhibited PTL to a similar maximal activity (15.4 \pm 0.6 U for Arg92 versus 14.8 \pm 0.8 U for Cys92). Two times more Cys92-conditioned medium was needed to restore half-maximal activity to bile-salt-inhibited PTL (133 \pm 15 μ l for Cys92 versus 45 \pm 9 μ l for Arg92).

Either decreased function or decreased secretion or both of Cys92 colipase could explain the decreased activity. To distinguish between these mechanisms, we determined the amount of colipase present in the conditioned medium and retained inside the cells 72 h posttransfection. The amount of Cys92 colipase in the conditioned medium was 35% lower than that of Arg92 colipase when assayed by protein immunoblot. The amount of Cys92 colipase retained inside the cells was twice that of Arg92 colipase (Fig. 2C, D). The reduced amount of Cys92 colipase in the medium was confirmed by GelCode Blue staining after the separation of concentrated conditioned medium by SDS-PAGE (Fig. 2E). The band density of Cys92 colipase was \sim 20% lower than that for Arg92 colipase, which is consistent with the results determined by immunoblot (Fig. 2F). These results suggest that decreased secretion contributes in part to the decreased ability of Cys92 colipase in the conditioned medium to restore activity to inhibited PTL.

We next performed a pulse-chase experiment to determine whether trafficking of Cys92 colipase was impaired. There was a slight but statistically significant delay in the movement of Cys92 out of the cells compared with Arg92 colipase (Fig. 3A). The half-life for Arg92 colipase was 77 min compared with 120 min for Cys92 colipase. Concomitantly, there was a delay in the appearance of Cys92 in the medium compared with Arg92 colipase (Fig. 3B). The half-time for appearance in the medium was 8.7 min for Arg92 colipase compared with 120 min for Cys92 colipase. The results are consistent with a delay in the processing or trafficking of Cys92 colipase.

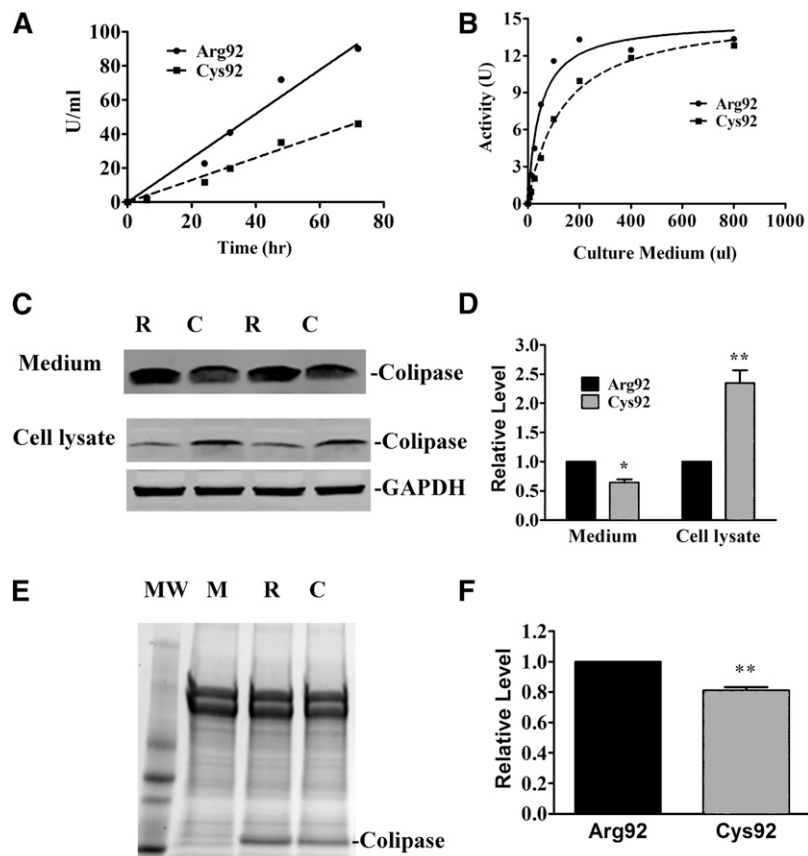


Fig. 2. The expression of Arg92 and Cys92 colipase in HEK293T cells. Cells were transiently transfected to express Arg92 and Cys92 colipase. Samples were collected 72 h posttransfection unless noted otherwise. A: The time course of the activity of Arg92 and Cys92 colipase in the conditioned medium to reactivate inhibited PTL against tributyrin in 4 mM NaTDC. B: Colipase dependence of PTL for Arg92 and Cys92 colipase. The activity of PTL against tributyrin with varying volumes of conditioned medium. C: Immunoblot of Arg92 and Cys92 colipase in conditioned medium and total cell lysate. D: Quantification of the relative levels of Arg92 and Cys92 colipase in the medium and cells. The mean and SD of six replicates is presented. E: SDS-PAGE GelCode Blue staining of concentrated, conditioned media containing Arg92 or Cys92 colipase. The image of gel staining was the representative of four replicates. F: The quantification analysis of band density through densitometry. C, Cys92; R, Arg92. Significant difference: * $P < 0.05$; ** $P < 0.01$.

To determine whether Cys92 colipase also has decreased function, we performed assays with physiological substrates (trioctanoin, triolein, or Intralipid). The decreased ability of Cys92 colipase to restore PTL function against these substrates was statistically significant compared with the function of Arg92 colipase (Table 1). With long-chain triglyceride substrates triolein and Intralipid, there was a statistically significant increase in the lag time prior to the burst phase for Cys92 colipase compared with Arg92 colipase, even after normalizing the amount of Cys92 and Arg92 colipase added to the assay (Fig. 4 and Table 1). These results suggest that Cys92 colipase is less efficient at anchoring PTL to the substrates.

Since an important function of colipase is to anchor PTL on the lipid emulsion surface, we measured the ability of Cys92 colipase to facilitate PTL adsorption to various lipid emulsions. Assays were done with equimolar concentrations of colipase and PTL in the presence of 4 mM NaTDC. We included an incubation of PTL without colipase to control for noncolipase-mediated adsorption of PTL to the substrate interface. The ability of Cys92 colipase

to anchor PTL to the various lipid emulsions was decreased significantly compared with Arg92 colipase (Fig. 5). The lower percentage of PTL adsorbed to triolein and Intralipid is a function of the incubation time of these assays and reflects the delayed adsorption suggested by the long lag time for these substrates. Thus, the decreased ability of Cys92 colipase to restore PTL activity is, in part, secondary to ineffective formation of the colipase-PTL complex on the lipid emulsion.

The decreased function of Cys92 colipase, the increased intracellular retention, and the presence of an additional cysteine residue raises the possibility that a significant fraction of Cys92 colipase folds incorrectly, perhaps as a consequence of mismatched intermolecular or intramolecular disulfide bonds. We approached this question by SDS-PAGE separation of the proteins in conditioned medium and in the supernatant and pellet of centrifuged cell lysates under reducing and nonreducing conditions. Colipase was detected by protein immunoblot (Fig. 6). Under reducing conditions, the medium samples had a single predominant band of identical mobility for both colipase

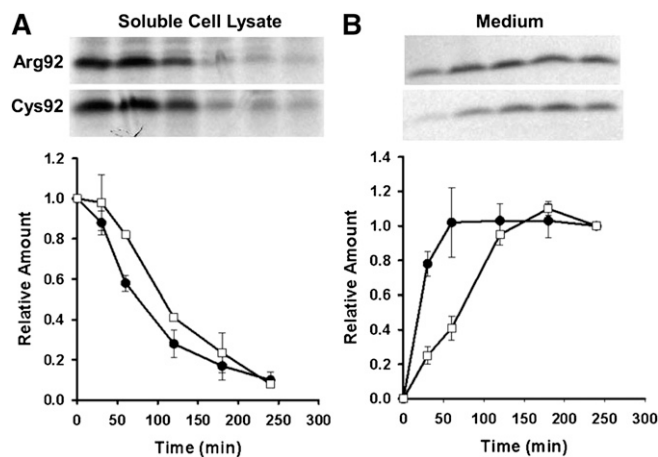


Fig. 3. Pulse-chase of Arg92 and Cys92 colipase. HEK293T cells were transfected with plasmids encoding Arg92 or Cys92 colipase. The cells were metabolically labeled with S^{35} methionine for 60 min and chased for 0 to 240 min. Timed samples were collected from the medium and cell lysates. Colipase was immunoprecipitated and analyzed by SDS-PAGE and autoradiography. **A:** Representative autoradiographs of S^{35} -colipase in the cell lysate and kinetics of its disappearance from the cells. **B:** Representative autoradiographs of S^{35} -colipase in the medium and kinetics of its appearance in the medium. Samples were collected at 0, 30, 60, 120, 180, and 240 min. Value in the graphs are presented as mean \pm SD of four experiments. Filled circles, Arg92; open squares, Cys92.

variants. The patterns differed under nonreducing conditions. A single major band and a minor faster migrating band were detected in the Arg92 colipase medium. Similar bands were present in the Cys92 sample, but the distribution of the two bands was roughly equal. In addition, a slower migrating band around 20 kDa was detected in the Cys92 colipase samples. Because the samples separated under reducing conditions did not have this band, it likely represents intermolecular disulfide bonding between two molecules of Cys92 colipase. A similar pattern was detected for the soluble fraction of the cell lysates. Under reducing conditions, both soluble colipase variants migrated as a single band at a slightly higher molecular weight compared with colipase in the medium, consistent with a signal peptide. Without a reducing agent, the soluble intracellular Arg92 colipase has a pattern similar to that seen in the medium, with a more prominent slower migrating band slightly larger than 15 kDa and a minor faster migrating

band slightly smaller than 15 kDa. Both bands are present in the soluble intracellular Cys92 sample, but as observed in the medium, the faster migrating band was a larger fraction of the two bands. Analysis of insoluble intracellular proteins did not detect any Arg92 colipase in this fraction. In contrast, Cys92 colipase was present in the insoluble proteins fraction. It migrated as a single band of the same size under reducing and nonreducing conditions. The results suggest that processing of Cys92 colipase differs from that Arg92 colipase in ways that lead to decreased secretion rates and retention of insoluble protein in the cell.

If Cys92 colipase has altered structural conformation, we predicted Cys92 would have decreased thermal stability. We tested this hypothesis by determining the temperature stability of Arg92 and Cys92 colipase in the conditioned medium from transfected cells. Seventy-two hours post-transfection, aliquots of conditioned medium were incubated for 1 or 2 h at temperatures ranging from 45 to 85°C at 10°C intervals. When assayed with tributyrin, the function of both Arg92 and Cys92 colipase was marginally reduced by incubation at 45°C but abolished for both variants by incubation at 85°C (**Fig. 7**). Incubation at temperatures between these ranges showed a statistically significant difference at 55°C, 65°C, and 75°C for the 1 h incubation ($P < 0.001$) and at 55°C and 65°C for the 2 h incubation ($P < 0.001$). Results with the unpurified colipases differ from those of the purified Cys92 and Arg92 colipase, which retained about 70% of full function after incubation at 85°C. Although the mechanism for this difference is not clear from these studies, the difference between Arg92 and Cys92 colipase suggests the Arg92Cys substitution alters the structure of colipase.

Our observation that insoluble Cys92 colipase accumulates in the cell raises the possibility that the variant is proteotoxic. The resulting stress could adversely affect acinar cell function. With that in mind, we measured various pathways of the ER stress response. The expression of Cys92 colipase in HEK293T cells did not elevate the protein level of protein disulfide isomerase (PDI) or ERp57 (another thiol-disulfide oxidoreductase) (**Fig. 8A**) (15–17). Cys92 expression did not increase the levels of BiP or GRP94 (major ER molecular chaperones) and did not activate the splicing of Xbp1 mRNA, an indicator of the

TABLE 1. Colipase activity in the conditioned medium from HEK293T cells expressing Arg92 or Cys92 colipase

Substrate	Maximal Activity (U)		Lag Time (min)	
	Arg92	Cys92	Arg92	Cys92
Tributyrin	109 \pm 1.6 ^a	86 \pm 3.2 ^b	ND	ND
Triolein	68 \pm 1.4 ^a	51 \pm 3.2 ^b	ND	ND
Triolein	38 \pm 2.7 ^a	26 \pm 3.3 ^b	8 \pm 1.3 ^a	16 \pm 2.1 ^b
Intralipid	14 \pm 0.8 ^a	8.5 \pm 1.0 ^b	41 \pm 18 ^a	184 \pm 32 ^b

Each assay contained 2 μ g of PTL and 20 μ l of the conditioned medium from cells expressing Arg92 or Cys92 colipase (<200 ng of colipase), except for the assays against Intralipid, which contained 10 μ g of PTL and 100 μ l of the conditioned medium. The activity levels were normalized for relative protein amounts based on immunoblot analysis. The values shown are mean \pm SD of three or more determinations from separate transfection experiments. ND, none detected.

^{a,b} Significantly different ($P < 0.001$) from significant superscript values for each set of activity and lag time measurements.

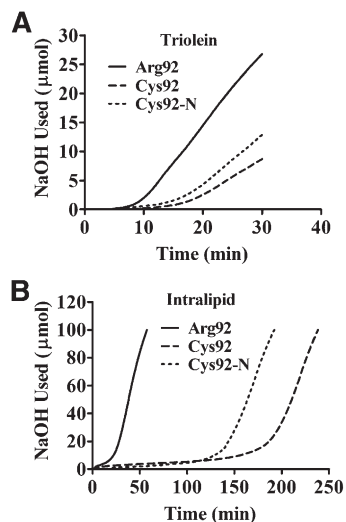


Fig. 4. Lag phase of Arg92 and Cys92 colipase to restore inhibited PTL activity against triolein and Intralipid. Kinetics of PTL against triolein and Intralipid in 4 mM NaTDC was examined using the prolonged pH-stat assay in the presence of Arg92 and Cys92 colipase from concentrated conditioned medium 72 h posttransfection. A: Representative continuous tracings of pH-stat assays with triolein. B: Representative continuous tracings of pH-stat assays with Intralipid. At least triplicate determinations were done for each assay. Components of the assay are listed in the legend. Cys92-N is Cys92 colipase medium with the colipase protein amount normalized to that in Arg92 conditioned medium. The statistical analysis of multiple assays is presented in Table 1.

unfolded protein response (UPR) (Fig. 8). These results suggest that the concentration of intracellular Cys92 colipase does not stimulate the UPR and cause increased ER stress.

DISCUSSION

The present study increases our knowledge about how the Arg92Cys substitution affects colipase function. There are two notable differences between this study and our previous report on purified recombinant Cys92 colipase (10). Purified Cys92 colipase had normal function against

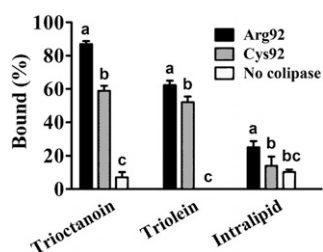


Fig. 5. Adsorption of PTL to lipid emulsions mediated by Arg92 and Cys92 colipase. The assay was done as described in Materials and Methods. A total of 2.5 μ g of PTL was added to each tube along with approximately 0.5 μ g of colipase from concentrated conditioned medium 72 h posttransfection at an equimolar ratio of colipase:PTL. The values shown are mean \pm SD of triplicate determinations. For each substrate, bars with different letters are significantly different by *t*-test ($P < 0.05$).

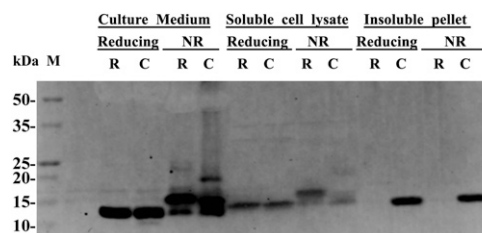


Fig. 6. Western blot analysis of Arg92 and Cys92 colipase under reducing and nonreducing conditions. HEK293T cells were transiently transfected to express Arg92 or Cys92 colipase. Samples were collected 72 h posttransfection and prepared with (reducing condition) or without β -mercaptoethanol (nonreducing condition). Protein samples were resolved by 4–20% SDS-PAGE and immune-blotted using a rabbit polyclonal antibody against human procolipase. The blot was the representative of at least eight replicates. Blots were overexposed to evaluate minor bands. C, Cys92 colipase; M, molecular weight marker; R, Arg92 colipase. Analyzed were 0.3% of the medium, 3.8% of the soluble fraction, and 7.5% of the insoluble fraction.

tributyrin, whereas Cys92 colipase expressed in HEK293T cells had lower function with tributyrin. Additionally, the temperature stability of the purified Cys92 and Arg92 colipases did not differ significantly. In contrast, Cys92 in conditioned medium was significantly less stable than Arg92 in conditioned medium. A feasible explanation for these differences is that the Cys92 colipase expressed in yeast went through several purification steps, which may have excluded misfolded protein from the final product. That possibility is supported by the observation that nonreducing SDS-PAGE gave a single band of the same migration for purified Arg92 and Cys92 colipase rather than the two or three species of each colipase present in conditioned medium.

Expression in HEK293T cells allowed us to examine the trafficking and function of Cys92 colipase. The results support the conclusion that the Arg92Cys substitution affects colipase folding. First, Cys92 colipase was secreted into the medium at a slower rate and in smaller amounts compared with Arg92 colipase. Second, the banding patterns under nonreducing SDS-PAGE differed between Cys92 and Arg92 colipase. Both the secreted and soluble intracellular colipase variants migrated as two predominant bands. The slower migrating band was predominant for Arg92 colipase, whereas the faster migrating band was a much larger fraction of Cys92 colipase. The observation that both colipases collapse into one faster migrating species after reduction suggests that differences in disulfide bond formation are responsible for the presence of the two bands observed in nonreducing gels.

The third observation supporting an effect of Cys92 on colipase structure comes from our observation that a sizeable fraction of intracellular Cys92 colipase was insoluble, whereas little to no Arg92 colipase was insoluble. The observation that the migration of insoluble Cys92 colipase was the same regardless of the reducing conditions suggests that the protein aggregated because disulfide bonds failed to form rather than because intramolecular disulfides formed. Finally, the thermal stability of Cys92 was significantly decreased. As this was not seen with purified

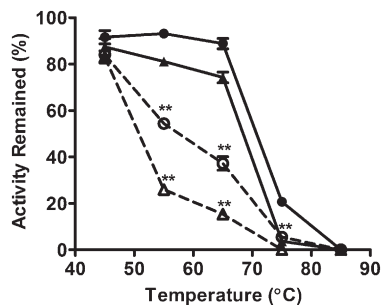


Fig. 7. Thermal stability of Arg92 and Cys92 colipase in the conditioned medium. Seventy-two hours posttransfection, conditioned medium from transfected HEK293T cells expressing Arg92 or Cys92 colipase were collected. Activity in the medium was determined immediately after harvesting the medium, and aliquots were subjected to heat incubation at the indicated temperatures for 1 or 2 h. The remaining colipase activity after heat incubation was assayed using tributyrin as substrate and expressed as a percentage (remaining activity/starting activity \times 100). The values shown are mean \pm SD of triplicate determinations. Circle, 1 h incubation; triangle, 2 h incubation; filled symbols and solid line, Arg92 colipase; open symbols and dashed line, Cys92 colipase. ** $P < 0.001$ Arg92 colipase compared with Cys92 colipase by Student *t*-test.

Cys92 colipase, the increased thermal denaturation might occur because colipase interacts abnormally with proteins or lipids in the medium.

Because the overall structure of colipase is dictated by five disulfide bonds, any alteration in the pairing of specific cysteine residues would disrupt the normal conformation of colipase (5). The location of Cys92 near the carboxyl-terminus of the protein suggests it could form aberrant disulfide bonds with Cys49, Cys61, Cys69, or Cys87. All but Cys49 are within 10 Å of Cys92. Mispairing of these

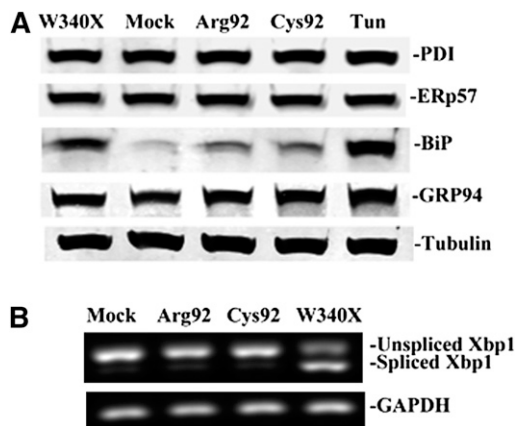


Fig. 8. The cellular response to the expression of Arg92 and Cys92 colipase in HEK293T cells. Seventy-two hours after transfection, cells expressing mock, Arg92, or Cys92 colipase were harvested. A: Immunoblot analysis of major ER stress markers and molecular chaperons. Positive controls included cells transfected to express PLRP2 W340 \times mutant protein and cells treated with 10 μ g/ml tunicamycin (Tun) for 12 h prior to sample harvest. The blot was the representative of at least four replicates. B: Semiquantitative RT-PCR analysis of Xbp1 mRNA splicing. PCR products were resolved by 2% agarose gels and visualized by ethidium-bromide staining. Cells transfected to express PLRP2 W340 \times served as positive control. The image was one of duplicates.

cysteine residues would likely alter the structure of the fourth finger (the 70–80 loop) without destroying colipase function. The fourth finger and the carboxyl-terminus display a high diversity of structure in the NMR structure of porcine colipase, indicating that a stable structure is not required for colipase function (5). The observation that proteolytic cleavage between positions 79 and 80 of the fourth finger, which can occur during purification of porcine colipase, does not destroy the function of colipase supports this possibility (18). In fact, the cleavage alters the function of colipase in much the same way as does the Cys92 substitution. Both can restore PTL to full activity when in molar excess, and both exhibit a prolonged lag time with triolein and Intralipid (18).

Another possible explanation for the decreased function of Cys92 colipase arises from older studies of colipase isolated from pancreatic secretions or pancreatic tissue. Multiple forms of colipase ranging from 84 to 101 amino acids were isolated. Removal of 5 amino acids, enterostatin, from the N-terminus or multiple residues from the C-terminus accounts for the different sizes (19). As residues were cleaved from the C-terminus, the lag time with Intralipid decreased. The shortest lag time was observed with forms of colipase that were cleaved proximal to Arg92. These findings suggest that residues in the C-terminus affect colipase function, perhaps by influencing anchoring of PTL to the substrate interface. The Arg92Cys substitution may alter the conformation in the C-terminus of colipase and thereby alter function.

The altered function of Cys92 colipase could increase risk for type-2 diabetes by causing fat malabsorption or by moving fat digestion further down the small intestine. Studies with tetrahydrolipstatin (Orlistat), a lipase inhibitor that causes fat malabsorption in humans, improved postprandial serum lipid profiles and insulin sensitivity and reduced risk for developing type-2 diabetes (20, 21). A similar effect would be expected if Cys92 colipase leads to fat malabsorption. Given that Cys92 colipase has good function after a lag time, dietary fat digestion may not be impaired; rather, fat digestion may be moved distally. The presence of fatty acids in the lower small intestine may alter enteroendocrine hormone secretion, which may modify metabolism or may blunt the satiety response mediated by signals in the upper intestine.

Alternatively, another unknown function of colipase may contribute to the elevated risk for developing type-2 diabetes associated with the Arg92Cys polymorphism. The decreased secretion of Cys92 colipase would also decrease the level of enterostatin. Although the role of enterostatin in humans remains unclear, enterostatin clearly has effects on feeding behavior and metabolism in other animals (4). Recent reports have shown that colipase can be expressed in the liver, intestine, stomach, brain, and adipose tissue (22–25). Colipase gene expression was greatly upregulated in the skeletal muscle of diabetic human subjects and in the liver and other tissues, including adipose tissue, of mice stressed by living in constant darkness (24, 26). Also, colipase-deficient mouse pups have decreased survival (27). Pup loss is not secondary to fat malabsorption. On a

C57/Bl6 background, mating of heterozygous mice resulted in no births of colipase-deficient pups, suggesting colipase is required for in utero survival on this background (unpublished data). Taken together, these observations suggest that colipase has functions aside from its role in PTL lipolysis and enterostatin signaling. It is plausible that the Arg92Cys substitution has a greater effect on these other functions of colipase. The delineation of the mechanism by which Cys92 colipase increases risk for type-2 diabetes will require additional study.^{5,6}

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