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Short communication

Possible Molecular Chaperones for Lipoprotein Lipase in Endoplasmic Reticulum

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Studies in adipocytes indicate that secretion of active lipoprotein lipase (LPL) was strictly regulated by a quality control system in the endoplasmic reticulum (ER). However, there has been no report about the ER chaperones participating in the folding and assembly of LPL. Many chaperones are known to bind unfolded proteins and dissociate from them through the ATP-hydrolyzing reaction. In this study, putative ER chaperones for LPL were determined by affinity chromatography using denatured LPL as an affinity ligand and elution with ATP. BiP, grp94, calreticulin, and another 50 kDa K-D-E-L protein in the ER of rat adipose tissue were bound to denatured LPL and eluted by ATP. Calnexin was bound to denatured LPL; however, it was not eluted by ATP but by acetic acid. These results indicate that, at least, BiP, grp94, calreticulin, calnexin, and the unidentified 50 kDa protein might act as putative chaperones for the proper folding and assembly of LPL in ER.

Keywords: Chaperones, Endoplasmic reticulum, Lipoprotein lipase.

Introduction

The products of stress genes, stress proteins, or heat shock proteins are a family of peptides characterized by a sequence that is highly conserved along the evolutionary scale (Lindquist & Craig, 1988). Their functions are essential to cell survival not only in the presence of environmental stressors but also under physiological conditions. Stress proteins have been defined as molecular chaperones (Georgopoulos & Welch, 1993; Hendrick & Hartl, 1993), and thought to temporarily stabilize unfolded or partially-folded structures and to maintain them in a state competent for subsequent folding and assembly (Hendrick & Hartl, 1993; Hartl *et al.*, 1994). They also assist the translocation or the delivery of newly synthesized proteins to the proper intracellular targets or along the secretory pathways (Bruneau & Lombardo, 1995; Zhang *et al.*, 1997).

Many proteins have been thought to act as molecular chaperones in the endoplasmic reticulum (ER). One major ER protein that clearly functions as a chaperone is a glucose-regulated protein BiP/grp78 (Lee *et al.*, 1981), an hsp70 ER homologue (Gething & Sambrook, 1992). The grp94, the hsp90 homologue in the ER (Sorger & Pelham, 1987), calnexin which reacts with glycoproteins (Ware *et al.*, 1995), and calreticulin, the primary Ca^{2+}-binding protein in the ER (Michalak *et al.*, 1992; Nigam *et al.*, 1994), have also been proposed to function as chaperones. Also, an important molecule in the ER protein folding is protein disulfide isomerase (PDI), which is essential for establishing proper disulfide bonds in newly translocated polypeptides (Noiva & Lennarz, 1992).

Lipoprotein lipase (LPL) is an acylglycerol hydrolase (EC 3.1.1.34) which hydrolyzes the triacylglycerol component of lipoproteins into two fatty acids and monoacylglycerol (Bensadoun, 1991). It plays a central role in body triacylglycerol metabolism, and the deficiency or dysfunction of LPL has been found in association with the pathogenesis of hypertriglyceridemia (Eckel, 1989).

LPL is produced by parenchymal cells of extrahepatic tissues and transferred to capillaries (Braun & Severson, 1992). Active LPL is a noncovalent dimer of identical subunits (Garfinkel *et al.*, 1983) having two N-linked oligosaccharide chains per subunit (Masuno *et al.*, 1991; Ben-Zeev *et al.*, 1994). Studies in adipocytes revealed that inhibition of glycosylation or oligosaccharide processing in ER resulted in the synthesis of LPL being inactive and retained in ER as an aggregate (Masuno *et al.*, 1991; Park

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et al., 1995; 1996). Inhibition of ER-Golgi protein transport also produced the inactive LPL (Park *et al.*, 1997).

Since ER is the initial quality control site in the secretory pathway where membranous and secretory proteins are folded and assembled (Hammond & Helenius, 1994; Zhang *et al.*, 1997), retention of unfolded or misfolded LPL might evoke the response of various ER-resident chaperones. The molecular chaperones exhibit strict structural specificity to discriminate the properly folded and misfolded proteins. Synthetic peptides bind to BiP, an ER-resident chaperone, with varying affinity *in vitro* (Fourie *et al.*, 1994), and the different affinities of ER chaperones to ligand proteins were reported in mammalian cells (Nigam *et al.*, 1994). However, there is no report yet about the ER chaperones participating in the folding of LPL. In this study, we tried to identify the possible ER chaperones for LPL folding by affinity chromatography using denatured LPL as a ligand and eluting with ATP.

Materials and Methods

Purification of LPL LPL was purified from post-heparin plasma of rats according to the procedures of Jackson and McLeans (1991). Normal fasting rats were given 100 units heparin per kg, and blood was collected after 15 min. After removing the cells by low-speed centrifugation, post-heparin plasma was mixed with an equal volume of 10 mM potassium phosphate–0.4 M NaCl–20% glycerol (pH 6.8). One ml packed gel of heparin-Sepharose CL-6B (Pharmacia) per 10 ml of diluted serum was added, and the mixture was incubated at 4°C for 2 h with gentle mixing. Heparin-Sepharose was poured into the column, washed with equilibration buffer (10 mM Tris·HCl, 1 mM EDTA, 10% glycerol, pH 7.4), and eluted by a gradient of equilibration buffer with 0.3 to 2 M NaCl. LPL-containing fractions were pooled, desalted using CentriPrep 30 (Amicon), and applied to a second round of heparin-Sepharose column chromatography. Pooled fractions showing LPL activity were concentrated using CentriPrep 30 and stored at −70°C. Purified LPL showed a single band of 57 kDa on SDS-PAGE (Fig. 1).

LPL activity was measured as described previously (Park *et al.*, 1997). A stock triacylglycerol emulsion containing 5 mCi of tri[9,10(n)-^{3}H]oleoylglycerol (Amersham), 1.13 mmole of trioleoylglycerol, 60 mg of 1-α-phosphatidylcholine (bovine liver), and 9 ml of glycerol was prepared according to the method of Nilsson-Ehle and Schotz (1976). Before assay, 1 vol of the stock emulsion, 19 vol of 3% BSA in 0.2 M Tris·HCl buffer (pH 8.1), and 5 vol of heat-inactivated fasted rat serum (heated at 60°C for 30 min) were mixed and incubated for 15–30 min. For assay, 100 μl of this activated substrate mixture was added to a same amount of enzyme solution and incubated at 37°C for 60 min. Released fatty acids were extracted and its radioactivity was measured. One unit of lipolytic activity represents the release of 1 μmole of fatty acid/min.

Preparation of affinity column Purified LPL was coupled to Affi-Prep 10 (Bio-Rad) according to the manufacturer's instruction. Slurry was washed with 30–40 vol of cold 10 mM sodium acetate (pH 4.5, coupling buffer) and 0.5 mg of proteins per ml of gel was added. The proteins were dialyzed against the coupling buffer to remove smaller amine-containing contaminants. After gentle mixing at room temperature for 1 h, remaining active esters were blocked by the addition of 0.1 ml of 1 M ethanolamine (pH 8.0) per ml of slurry. Coupled gel was washed with 0.5 M NaCl, transferred to a column and rinsed with phosphate buffered saline (PBS) until no protein was eluted. The binding efficiency calculated from the protein concentrations before and after coupling was over 90%.

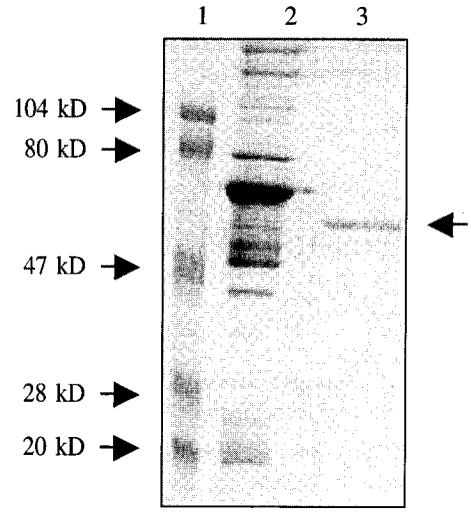

Fig. 1. SDS-PAGE of purified lipoprotein lipase from rat post-heparin plasma. SDS-polyacrylamide gel electrophoresis was performed on 10% resolving and 3% stacking gels and stained with Coomassie blue. Lane 1, molecular weight marker; lane 2, whole rat post-heparin plasma (300-fold dilution); lane 3, purified lipoprotein lipase.

Preparation of ER fractions The ER fraction from rat adipose tissue was prepared as described by Nigam *et al.* (1994). Dissected intra-abdominal adipose tissue were washed twice with cold PBS, and homogenized with 2 vol of homogenizing buffer (20 mM Tris, 150 mM NaCl, 250 mM sucrose, 1 mM dithiothreitol, pH 8.0). Unbroken cells, nuclei, mitochondria, and other large organelles were removed by centrifugation at 20,000 $\times$ *g* for 30 min. The postmitochondrial supernatant was spun again at the same condition to eliminate residual contamination by these organelles. The microsomal fraction was then precipitated by centrifugation at 100,000 $\times$ *g* for 1 h. Microsomal pellets were suspended in homogenizing buffer and washed twice with the same buffer. Finally, the microsomal fraction was suspended in homogenizing buffer at the protein concentration of 10 mg/ml and kept frozen at −70°C in aliquots.

Affinity chromatography Possible ER chaperones were partially purified by affinity chromatography using denatured proteins as the affinity ligands and eluting with ATP (Nigam *et al.*, 1994). LPL attached to the Affi-Gel were denatured by incubation in a 6 M urea, 1 M mercaptoethanol solution for 30 min. Approximately 5 mg of purified rat adipose tissue ER fractions were solubilized in a buffer of 20 mM Tris, 75 mM

NaCl, 125 mM sucrose, 1 mM dithiothreitol, 2.5 mM EDTA, 1 mM EGTA, 0.5% Triton X-100 (pH 8.0), and applied to the affinity column. The column was washed first with elution buffer (20 mM Tris, 150 mM NaCl, 1 mM dithiothreitol, pH 8.0) containing 2.5 mM EDTA and 1 mM EGTA (flow-through), and then with elution buffer containing 2 mM $MgCl_2$ and 0.5 mM $CaCl_2$ (pre-elution). Putative ER chaperones were eluted with elution buffer containing 2 mM $MgCl_2$, 0.5 mM $CaCl_2$, and 1 mM ATP (ATP elution). Proteins remaining on the column after the ATP elution were eluted with 100 mM acetic acid (pH 2.5) (acid elution) (Nigam *et al.*, 1994). The proteins from different fractions were precipitated with 10% trichloroacetic acid, washed with acetone, and separated using SDS-PAGE (Laemmli, 1970).

SDS-PAGE and Western blotting Samples were mixed with a double-strength electrophoresis sample buffer (62.5 mM Tris, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.001% bromophenol blue, pH 6.8), and boiled for 5 min. Proteins were separated by SDS-PAGE with 10% resolving and 3% acrylamide stacking gels (Laemmli, 1970), and stained with Coomassie blue or transferred to nitrocellulose membrane. The membranes were blocked with 2% BSA, reacted overnight with primary antibody for BiP, calnexin, or calreticulin, and with alkaline phosphatase-conjugated secondary antibody for 2 h. Color was developed in BCIP/NBT solution. Antibodies for BiP, calnexin, and calreticulin were purchased from StressGen Biotechnologies Corp. (Canada).

Results and Discussion

A specific property of many chaperones in both bacterial and eukaryotic cells is their ability to bind selectively to unfolded polypeptides and dissociate from them through an ATP-hydrolyzing reaction, which is coupled to protein folding (Rothman, 1989; Nigam *et al.*, 1994). Consequently, affinity chromatography on denatured protein columns and elution by ATP has proven to be a highly selective method of purifying the major chaperones from bacteria (Sherman & Goldberg, 1991) or eukaryotic cells (Nigam *et al.*, 1994). In this study, the possible ER chaperones for LPL were purified by affinity chromatography using denatured LPL as an affinity ligand and ATP as an eluate.

To prepare the LPL affinity column, LPL was purified from rat post-heparin plasma using heparin-Sepharose column chromatography. LPL has a specific high affinity for heparin (Bensadoun, 1991), and active LPL was usually eluted between 1.2 and 1.6 M NaCl. After a second round of heparin-Sepharose column chromatography, purified LPL showed a single band of 57 kDa on SDS-PAGE (Fig. 1). Purified LPL was used to prepare the affinity column for the identification of ER chaperones.

Putative ER chaperones were determined by affinity chromatography using denatured LPL as an affinity ligand and eluting with 1 mM ATP in the presence of 2 mM Mg^{2+} and Ca^{2+} (Nigam *et al.*, 1994). LPL was coupled to Affi-Prep 10 and denatured with urea and mercaptoethanol. After applying the rat adipose tissue ER lysate to the column, unbound proteins were washed out until no proteins were eluted out (Fig. 2, lane 3). Then, the bound proteins were eluted with ATP (Fig. 2, lane 4), and unreleased proteins were finally eluted with acid (Fig. 2, lane 5).

As it was impossible to identify the specific chaperone bands in protein-stained SDS-PAGE gels, immunoblottings with anti-BiP, anti-calreticulin, and anti-calnexin antibodies were tried. The major bands detected by anti-BiP antibody were BiP of 78 kDa, grp94 of 94 kDa, and a protein at 50 kDa in the ER lysate. Anti-calreticulin and anti-calnexin antibodies detected the major bands at 55 and 88 kDa, respectively (data not shown). Corresponding bands were indicated as arrows in Fig. 3.

Most of the BiP, grp94 (Fig. 3, Panel A), and calreticulin (Fig. 3, Panel B) were bound to denatured LPL and eluted with 1 mM ATP. An unidentified 50 kDa protein was also detected by the anti-BiP antibody to bind and be released by ATP (Fig. 3, Panel A). The monoclonal antibody for BiP was prepared against the synthetic peptide comprised of residues K-S-D-K-D-E-L, the carboxyl terminal sequence of rat BiP (Huovila *et al.*, 1992), suggesting that the 50 kDa protein is one of the K-D-E-L proteins. Nigam *et*

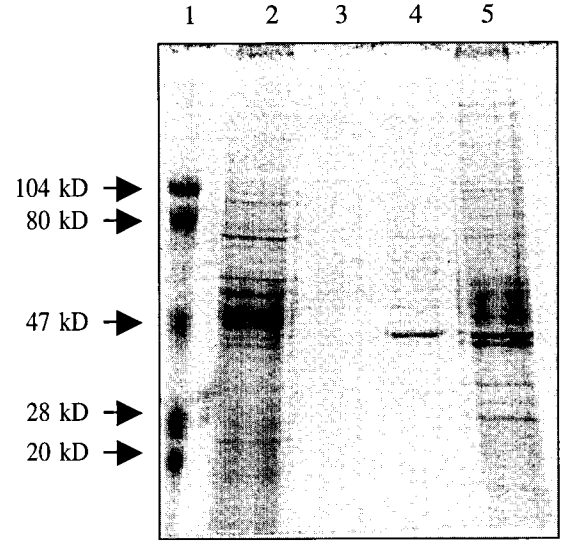

Fig. 2. ATP-dependent release of solubilized ER proteins from a denatured LPL column. Purified rat adipose tissue ER extracts were applied to the urea-denatured LPL column, washed with buffer extensively, and then eluted with 1 mM ATP as described under Materials and Methods. The materials that remained bound after the ATP elution was released with 100 mM acetic acid (pH 2.5). Each fraction was separated by SDS-PAGE and stained with Coomassie blue. Lane 1, molecular weight marker; lane 2, flow-through (unbound fraction, applied one-fifth of 5 bed vol); lane 3, wash (before ATP addition, applied last bed vol in 5 bed vol of wash); lane 4, ATP-eluted proteins (applied 5 bed vol of elution); lane 5, acid-eluted proteins (applied 5 bed vol).

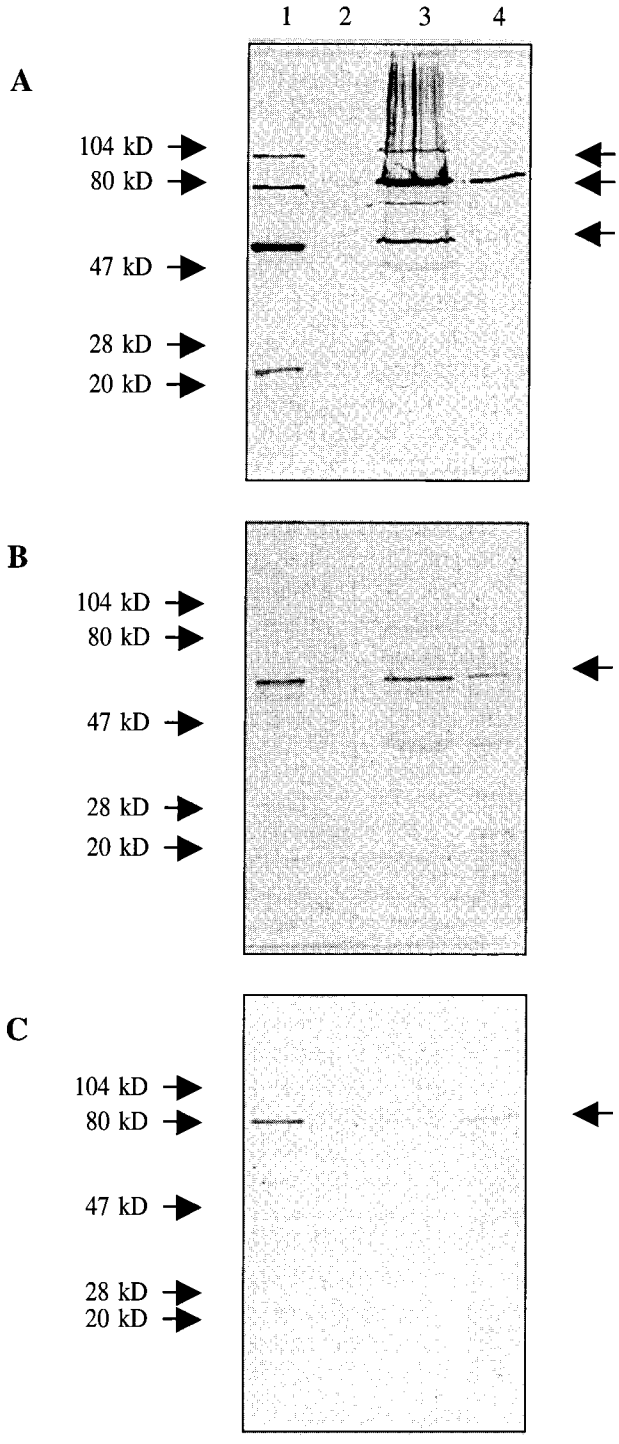

Fig. 3. Identification of putative ER chaperones interacting with denatured LPL column. Purified rat adipose tissue ER extracts were applied to the urea-denatured LPL column, washed, and eluted with ATP and acetic acid as described in the legend of Fig. 2. After separation by SDS-PAGE and transfer to nitrocellulose membrane, proteins were identified using chaperone-specific antibodies. Panel A, anti-BiP; panel B, anti-calreticulin; panel C, anti-calnexin antibodies. Each panel contains flow-through (lane 1), wash (lane 2), ATP-eluted (lane 3), and acid-eluted (lane 4) proteins.

al. (1994) reported the presence of a 50 kDa protein in rat liver, which binds to denatured histone and is released by ATP. The amino terminal sequence of this protein was about 80% identical to that of the mature form of the golden hamster protein P5 (Chaudhuri *et al.*, 1992). They can bind Ca^{2+} and contain two internal thioredoxin motifs (Peter *et al.*, 1992). The coordinate binding and elution of these luminal proteins suggests that they may function in the folding and assembly of LPL. The identification of this protein needs further study.

Calnexin was bound to the LPL ligand, but not eluted with ATP. Bound calnexin was eluted out in the acid elution fraction (Fig. 3, Panel C). Calnexin, another Ca^{2+} binding protein in the ER, has been found to act as a chaperone, especially for the N-linked glycosylated proteins (Bergeron *et al.*, 1994; Ware *et al.*, 1995). Although calnexin preferentially interacts with monoglucosylated oligosaccharides (Ware *et al.*, 1995), some unglycosylated proteins have also been found to interact with calnexin (Margolese *et al.*, 1993; Nigam *et al.*, 1994; Rajagopalan *et al.*, 1994), suggesting that N-linked glycans are not the only site of interaction. The participation of ATP in calnexin action is controversial in contrast to other chaperones (Bergeron *et al.*, 1994). Although not specifically released by ATP, calnexin can bind to denatured LPL and might still function as a chaperone, but may require additional factors for dissociation from unfolded proteins, as was found for groEL (Ellis & van der Vies, 1991).

Our results indicate that, at least, BiP, grp94, calreticulin, calnexin, and the unidentified 50 kDa K-D-E-L protein might act as putative chaperones for the proper folding and assembly of LPL. It was reported that BiP and grp94 were concomitantly induced under stress conditions (Kozutsumi *et al.*, 1988), and strong homologies existed between the promoter of BiP and the 5′-flanking regions in the genes for grp94, calreticulin, and PDI (McCauliffe *et al.*, 1992; Tasanen *et al.*, 1992). Recent study using *in situ* cross-linking suggested that the resident ER chaperones including BiP, grp94, calreticulin, calnexin, and other proteins exist as a network of weak association (Tatu & Helenius, 1997). It may serve as a complex, mixed-bed affinity matrix that transiently adsorbs incompletely folded and assembled proteins for quality control. Different proteins might react with a same network of ER chaperones instead of single chaperone molecules.

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