# Insulin Promotes Shedding of Syndecan Ectodomains from 3T3-L1 Adipocytes: A Proposed Mechanism for Stabilization of Extracellular Lipoprotein Lipase†

Ofer Reizes,*,‡,§ Olga Goldberger,‡ April C. Smith,|| Zhiqiang Xu,|| Merton Bernfield,‡,⊥ and Perry E. Bickel*,||

*Division of Newborn Medicine, Children's Hospital, Boston, Massachusetts 02115, and Division of Endocrinology, Metabolism and Lipid Research, Department of Medicine and Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri 63110*

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ABSTRACT: Syndecans are a family of four transmembrane heparan sulfate proteoglycans that act as coreceptors for a variety of cell-surface ligands and receptors. Receptor activation in several cell types leads to shedding of syndecan-1 and syndecan-4 ectodomains into the extracellular space by metalloproteinase-mediated cleavage of the syndecan core protein. We have found that 3T3-L1 adipocytes express syndecan-1 and syndecan-4 and that their ectodomains are shed in response to insulin in a dose-, time-, and metalloproteinase-dependent manner. Insulin responsive shedding is not seen in 3T3-L1 fibroblasts. This shedding involves both Ras-MAP kinase and phosphatidylinositol 3-kinase pathways. In response to insulin, adipocytes are known to secrete active lipoprotein lipase, an enzyme that binds to heparan sulfate on the luminal surface of capillary endothelia. Lipoprotein lipase is transported as a stable enzyme from its site of synthesis to its site of action, but the transport mechanism is unknown. Our studies indicate that shed adipocyte syndecans associate with lipoprotein lipase. The shed syndecan ectodomain can stabilize active lipoprotein lipase. These data suggest that syndecan ectodomains, shed by adipocytes in response to insulin, are physiological extracellular chaperones for lipoprotein lipase as it translocates from its site of synthesis to its site of action.

The syndecans are a family of four closely related transmembrane heparan sulfate proteoglycans (HSPGs;[1] syndecan-1, syndecan-2, syndecan-3, and syndecan-4) that are regulated in a tissue-specific and developmental stage-specific manner (*1*−*3*). Essentially, all adherent cells express syndecans on their surfaces (*2*). Via their HS chains, the syndecans bind many extracellular proteins, including growth factors, proteinases, anti-proteinases, matrix proteins, and others (*4*−*7*). As a result, syndecan proteins can function as receptors or co-receptors for these extracellular ligands.

Syndecan ectodomains are shed into the extracellular space by metalloproteinase-mediated cleavage of their core proteins near the plasma membrane (*8*, *9*). The shedding site in syndecan-1 was mapped to an extracellular juxtamembrane site 9 amino acids from the plasma membrane (*9*). Shedding is promoted by receptor activation of intracellular signaling pathways. The soluble syndecan ectodomains can compete for the same extracellular ligands bound by cell-surface syndecans (*10*−*12*). In this way, syndecan shedding can play an important role in the regulation of cellular interactions with extracellular effector proteins (*13*−*15*).

Adipose tissue is the major depot for long-term storage of energy as triglycerides. Adipocytes synthesize and secrete lipoprotein lipase (LPL), a glycoprotein enzyme that is rate-limiting for the hydrolysis of circulating triglycerides (*16*, *17*). LPL acts at the luminal surface of the capillary endothelium to release free fatty acids from triglyceride-rich lipoproteins. These free fatty acids then translocate to the adipocyte, where they are re-esterified into triglycerides. How LPL is released from adipocytes, traverses the extracellular space to the endothelium, and then translocates across the endothelial cell is not known. The instability of LPL activity has led to the proposal that an extracellular chaperone for LPL, thought to be a heparan sulfate (HS) fragment, may guide it to its ultimate site of action (*16*, *18*).

The role of insulin in the synthesis and release of LPL from skeletal muscle and adipose tissue has been extensively investigated as a physiological mechanism, as reviewed by Bensadoun (*16*). Details of this role differ across experimental systems, but overall, the data suggest that insulin enhances the release of enzymatically active LPL enzyme

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* To whom correspondence should be addressed: Procter and Gamble Pharmaceuticals, Inc., Health Care Research Center, Mason, OH 45040. Telephone: (513) 622-4462. Fax: (513) 622-1195. E-mail: reizes.o@pg.com (O.R.); Division of Endocrinology, Metabolism and Lipid Research, Department of Medicine and Department of Cell Biology and Physiology, Washington University School of Medicine, Campus Box 8127, St. Louis, MO 63110. Telephone: (314) 747-3973. E-mail: pbickel@im.wustl.edu (P.E.B.).

‡ Children's Hospital.

§ Current address: Procter and Gamble Pharmaceuticals, Mason, OH 45040.

|| Washington University School of Medicine.

⊥ Deceased.

[1] Abbreviations: CM, conditioned medium; FCS, fetal calf serum; GPI, glycosylphosphatidylinositol; HS, heparan sulfate; HRP, horseradish peroxidase; LPL, lipoprotein lipase; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PI 3-kinase, phosphotidylinositol 3-kinase; PG, proteoglycan; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; WAT, white adipose tissue.

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from adipocytes by an unknown mechanism. High-carbohydrate meals, which stimulate insulin secretion, significantly increase LPL levels in adipose tissue (*17*), and infusion of insulin during a euglycemic clamp results in a dose- and time-dependent increase in adipose tissue LPL activity (*20*).

Both adipocytes and endothelial cells contain a pool of LPL that is released rapidly upon exposure of the cells to heparin (*21*−*23*). LPL dimers, which are the catalytically active form of the enzyme, bind heparin and HS with high affinity (*24*−*26*). Because heparin has a 6000-fold greater affinity for the active dimeric LPL than for inactive LPL monomers, heparin treatment of adipocytes likely releases primarily active LPL (*26*). HSPGs have been implicated in the binding, uptake, degradation, and recycling of LPL in the capillary endothelium and in adipocytes (*27*−*30*). The importance to lipid metabolism of the interaction of LPL with HSPGs has been established in a mouse model. Lutz et al. created transgenic mice that expressed mutant human LPL defective in heparin binding and bred those mice onto an LPL null background (*31*). The mutant LPL was unstable at 37 °C, and the mice had high postprandial free fatty acids and abnormal targeting of lipids to tissues.

Because LPL is stabilized by HS and is transported from adipocytes to the endothelium, we hypothesized that HSPGs may be released from adipocytes in complex with LPL. Thus, we analyzed the HSPGs in the conditioned media (CM) from insulin-stimulated 3T3-L1 adipocytes. We have found that 3T3-L1 adipocytes express syndecan-1 and syndecan-4 on their surfaces, that they shed soluble syndecan ectodomains in response to insulin in a dose-, time-, and metalloproteinase-dependent manner, and that shed syndecan ectodomains bind LPL and stabilize its activity. We propose that insulin-stimulated syndecan synthesis and shedding of syndecan ectodomains from adipocytes and formation of syndecan ectodomain−LPL complexes regulate triglyceride metabolism by modulating the transport of active LPL from its site of synthesis to its site of action.

## EXPERIMENTAL PROCEDURES

*Materials.* Bovine LPL, insulin, dexamethasone, 3-isobutyl-1-methylxanthine, wortmannin, L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin, and soybean trypsin inhibitor were purchased from Sigma (St. Louis, MO). PD98059 was purchased from Calbiochem (La Jolla, CA). Heparin lyases I and III and chondroitinase ABC were purchased from Seikagaku America, Inc. (Rockville, MD). BB1101 was from British Biotech Pharmaceuticals, Ltd. (Oxford, U.K.).

*Immunochemicals.* Antibodies that recognize the mouse syndecan-1 ectodomain [monoclonal antibody (mAb) 281-2], the mouse syndecan-4 ectodomain (mAb Ky 8.2), a lyase-generated epitope on HSPG core proteins (anti-stub mAB 3G10), L-selectin (mAb MEL-14, BD PharMingen, San Diego, CA), and mouse LPL (chicken polyclonal raised against bovine LPL) have been described previously (*32*−*37*). Horseradish peroxidase (HRP)-coupled secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA) or Amersham Life Sciences (Little Chalfont, U.K.).

*Cell Culture.* 3T3-L1 mouse fibroblasts (American Type Culture Collection, Rockville, MD) were propagated and differentiated into adipocytes as described (*38*).

*Syndecan Ectodomain Shedding Assays.* 3T3-L1 preadipocytes and adipocytes at confluence in 10 cm plates were washed 3 times in serum-free DME. The cells were then incubated in serum-free DME with or without additives, and the CM were collected, as indicated in the captions of the figures. Each CM sample was cleared of cellular debris by centrifugation at 3000 rpm in a Centra CL2 clinical centrifuge (IEC, Needham Heights, MA) and supplemented with Tween-20 to a final concentration of 0.3%. Samples were assayed for syndecan-1 and syndecan-4 ectodomains by dot blot as described (*39*) using Immobilon-N cationic PDVF membranes (Millipore Corp., Bedford, MA). We have previously published data demonstrating the reproducibility and reliability of this assay for syndecan ectodomain shedding (*39*).

*Preparation of Soluble HSPG Core Ectodomains.* 3T3-L1 adipocytes were incubated overnight in serum-free medium in the presence or absence of 1 $\mu$M insulin. A total of 1 mL of each CM was concentrated to 50 $\mu$L in a Centricon-10 unit (Amicon, Inc., Beverly, MA) and further processed as described in the caption of Figure 1, essentially according to the protocol of David et al. (*35*).

*Immunoblotting.* After 1 h of incubation in a blocking buffer that consisted of TBS/T (10 mM Tris-HCl at pH 7.4, 150 mM NaCl, and 0.3% Tween 20) supplemented with 3% nonfat dry milk, membranes were incubated with primary and HRP-coupled secondary antibodies diluted in blocking buffer. Membranes were washed in TBS/T 3 times after each antibody incubation. Bound antibodies were detected by enhanced chemiluminescence (ECL, Amersham Life Science, Inc., Arlington Heights, IL). Exposed radiographic films were scanned, and the images were processed with Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA). Quantitative densitometric analysis was performed using NIH Image 1.62. For the quantitative dot blots, a standard curve was generated by dot-blotting known quantities of purified murine syndecan-1 and syndecan-4 ectodomains.

*Cell-Surface Trypsinization.* 3T3-L1 preadipocytes and adipocytes at confluence in 10 cm plates were washed twice in phosphate-buffered saline (PBS) supplemented with 5 mM ethylenediaminetetraacetic acid (EDTA) and then scraped on ice into 1 mL of the same buffer. Cell-surface trypsin digestion was performed as previously described (*39*), and the trypsinates were assayed for syndecan-1 ectodomains by dot blot.

*Immunoprecipitation of Syndecan/LPL Complexes.* Four 10 cm plates of 3T3-L1 adipocytes were washed in serum-free DME 3 times, and the media were replaced with serum-free DME that contained 100 nM insulin. After 18 h, the CM samples were collected, pooled into one tube, cleared of cellular debris by centrifugation in a clinical centrifuge, and treated with protease inhibitors (Complete Tablet, Roche Molecular Biochemicals, Indianapolis, IN). Three 13 mL aliquots were incubated overnight at 4 °C with either 200 $\mu$L of negative control beads (anti-L-selectin mAb Mel-14, coupled to sepharose) or 200 $\mu$L of a 1:1 mixture of anti-syndecan-1 and anti-syndecan-4 beads (mAb 281-2 and mAbs Ky8.2, respectively, coupled to sepharose) with constant mixing. The anti-syndecan beads were added to the CM in the presence or absence of 150 $\mu$g/mL heparin. After overnight mixing, the beads were collected by 20 s of centrifugation in a clinical centrifuge, washed 2 times with

TBS, and boiled in 4× Laemmli sample buffer. Samples were resolved by 7.5% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and transferred to nitrocellulose. The blot was blocked in TBS supplemented with 1% yeast extract and incubated with chicken anti-LPL antibody followed by an HRP-conjugated goat anti-chicken secondary antibody. Detection and postprocessing was as described above.

*$^{35}SO_4$ Labeling of Syndecan-1*. Eight 10 cm plates of 3T3-L1 adipocytes were incubated overnight in the presence or absence of insulin. The next morning, cells were washed and $^{35}SO_4$ (125 $\mu$Ci/plate) was added in low sulfate media to the cells for a 20 min labeling period in the presence or absence of insulin. Cells were washed rapidly and incubated for an additional 0, 10, 30, or 60 min in media containing 300 $\mu$M cycloheximide in the presence or absence of insulin. At the indicated times, CM and cells were collected and subjected to immunoprecipitation as described above.

*Purification of HSPGs from 3T3-L1 CM*. CM (100 mL) were collected, adjusted to 7 M urea, 100 mM sodium acetate at pH 4.5, and 0.35 M NaCl, with protease inhibitors (Complete Tablet), and purified using a Q sepharose column (Amersham Pharmacia Biotech, Piscataway, NJ). HSPGs were eluted from the column in 2 M NaCl and concentrated with 3 volumes of 95% ethanol containing 1.3% potassium acetate. Samples were dissolved in TBS buffer and quantified by the BCA assay; varying amounts of purified HSPGs were incubated with LPL as described below.

*Assay for Stabilization of LPL Activity by Adipocyte-Derived HSPGs.* LPL (Sigma, St. Louis, MO) was incubated in a buffer containing TBS for 16 h at 4 °C with BSA, heparin, syndecan-1 ectodomain, or HSPGs purified from adipocyte CM. LPL activity assays were performed according to established procedures (*40*). Briefly, the assay components included 10 mg of triolein (Sigma, St. Louis, MO), 3.3 $\mu$Ci $^{14}$C glycerol trioleate, and 0.6 mg of soybean L-α-phosphatidylcholine, type III-S (Sigma, St. Louis, MO). Fresh LPL or LPL that had been incubated as above were added to the assay buffer (total volume of 0.2 mL) and incubated with shaking at 37 °C for 1 h. Reactions were stopped by adding 3.2 mL of methanol/$CHCl_3$/heptane followed by the addition of 1 mL of 0.1 M potassium carbonate−borate buffer at pH 10.5. Samples were vortexed and centrifuged, and 1 mL was counted in a scintillation counter. The fresh LPL prior to incubations had a specific activity of 55 milliunits/$\mu$g, similar to that previously reported (*40*).

*Immunohistochemical Analysis.* FVB mice were anesthetized by intraperitoneal injection of ketamine and xylazine and perfused with 4% paraformaldehyde. The mice were then sacrificed by carbon dioxide asphyxiation, and white adipose tissue (WAT) was removed and postfixed overnight at 4 °C. Subsequently, the tissue was dehydrated and embedded in paraplast media (Kendall Healthcare Co., Mansfield, MA). A total of 10 $\mu$m sections were prepared and stored at 4 °C. Sections were rehydrated, blocked in PBS with 10% goat serum, and then incubated with 5.6 $\mu$g/mL anti-syndecan-1 mAb (mAb 281-2) or, as a negative control, mAb Mel-14. Antibody binding was detected using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA), according to the instructions of the manufacturer. Procedures involving mice were approved by the animal care committee of Children's Hospital, Boston, MA, where these studies were performed.

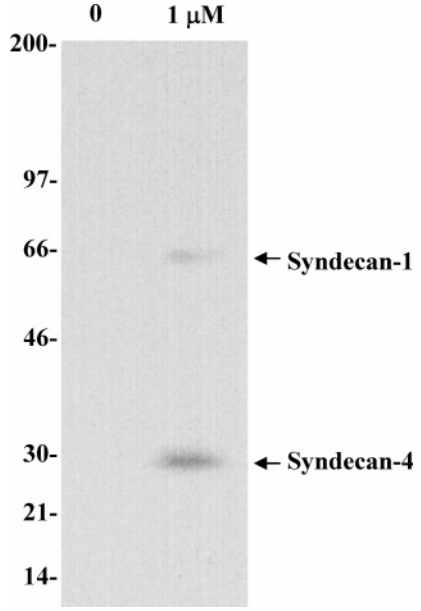


FIGURE 1: Soluble syndecan-1 and syndecan-4 ectodomains are present in the CM of insulin-stimulated 3T3-L1 adipocytes. 3T3-L1 adipocytes were incubated overnight in either the absence or presence of 1 $\mu$M insulin. CM were cleared, concentrated, and then digested with heparin lyases I and III and chondroitinase ABC. Digested samples were resolved by 3.5−15% SDS−PAGE, transferred to cationic PDVF membrane, and blotted with mAb 3G10, which specifically recognizes a lyase-generated epitope on HSPGs. Soluble HSPGs of a core protein size characteristic of syndecan-1 and syndecan-4 are indicated by arrows.

*Other Methods.* Protein assays were performed with the BCA assay (Pierce, Rockford, IL). Statistical analysis by one-way ANOVA with Dunnett's post-test was performed using GraphPad InStat version 3.01 for Windows (GraphPad Software, San Diego, CA, www.graphpad.com).

## RESULTS

*HSPGs Are Shed by Adipocytes in Response to Insulin.* We used a pan-selective HSPG antibody to detect the presence of proteoglycans (PGs) in CM from insulin-stimulated 3T3-L1 adipocytes. 3T3-L1 adipocytes were incubated overnight under serum-free conditions in the presence or absence of 1 $\mu$M insulin. These CM were concentrated and lyase-treated to convert soluble PGs to core proteins identifiable by their characteristic mobility on SDS−PAGE. PG core proteins were detected by immunoblotting with mAb 3G10, which recognizes an epitope generated by heparin lyase treatment (*35*). No HSPG core proteins were detected in the CM without insulin. However, medium conditioned by insulin-stimulated adipocytes contained core proteins corresponding to syndecan-1 and syndecan-4 ectodomains (Figure 1). Other HSPG core proteins were not detected on the blot.

*Messenger RNAs for Syndecan-1 and Syndecan-4 Are Expressed in Adipose Tissue.* We evaluated the expression of syndecan-1 and syndecan-4 in adipose tissue by Northern analysis. The RNA species of the expected sizes on a northern blot of mouse tissues was detected using radiolabeled cDNAs for syndecan-1 and syndecan-4 (Figure 2A). The relative abundance of syndecan-1 RNA in adipose tissue was noteworthy because it is not usually expressed in cells of mesenchymal origin after terminal differentiation (*41*).

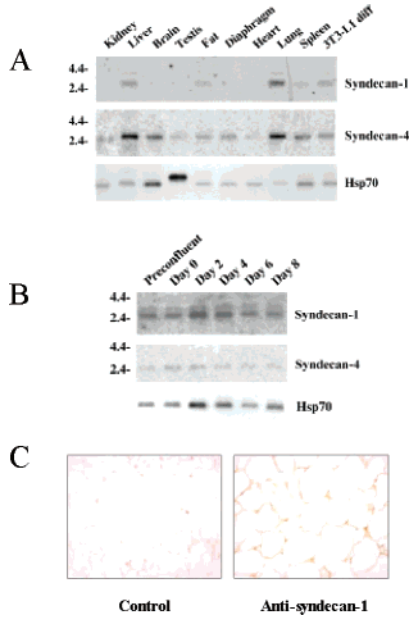


FIGURE 2: (A and B) Syndecan-1 and syndecan-4 are expressed in adipose tissue and 3T3-L1 cells. Poly(A)+ RNA isolated from the indicated tissues (1 $\mu$g each) (A) and from 3T3-L1 cells (0.5 $\mu$g each) at sequential stages of adipocyte differentiation (B) were subjected to formaldehyde-agarose gel electrophoresis and transferred to nylon membranes. ("3T3-L1 diff" in A refers to day 8 adipocytes.) These membranes were hybridized with radiolabeled DNA probes that corresponded to the coding sequences of syndecan-1 and syndecan-4. The same membranes were probed for HSP70 to demonstrate the loading of RNA. (C) Syndecan-1 is expressed by WAT adipocytes. Mouse WAT was collected and subjected to immunohistochemical staining for syndecan-1 (right panel) or for a negative control protein, L-selectin, a leukocyte-specific cell adhesion molecule (left panel), as described in the Experimental Procedures.

Several studies have shown that syndecan-4 is expressed in adipose tissue.

3T3-L1 cells are a well-characterized mouse fibroblast cell line that can be induced to differentiate into adipocytes by established protocols (*42*). To investigate whether syndecan-1 and syndecan-4 expression changes during adipocyte differentiation, we probed a northern blot of 3T3-L1 cells at varying stages of adipogenesis (Figure 2B). The abundance of neither syndecan RNA changed significantly from the undifferentiated state (preconfluent) to the fully differentiated state (day 8), but there was a transient increase in the expression of both syndecans early in the differentiation program (day 2). These Northern blot data are similar to previously reported gene chip data that reflect expression patterns for syndecan-1 and syndecan-4 during 3T3-L1 adipogenesis (*43*). Also, Landry et al. have reported an increase in syndecan-4 mRNA and protein between day 0 and day 1 in 3T3-F442A cell differentiation into adipocytes (*44*).

*Murine Adipocytes Express Syndecan-1 Protein*. To confirm that adipocytes in adipose tissue express syndecan proteins, we performed immunohistochemistry on wild-type FVB mouse WAT with a mAb to syndecan-1 and with a negative control antibody of the same isotype and species. Consistent with the results from 3T3-L1 adipocytes, specific immunoreactivity for syndecan-1 was present in WAT adipocytes (Figure 2C).

*Syndecan-1 and Syndecan-4 Are Shed from 3T3-L1 Adipocytes in an Insulin-Dependent Manner*. To confirm that

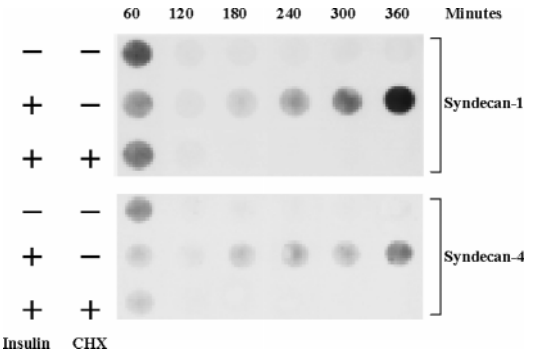


FIGURE 3: Insulin stimulates shedding of syndecan-1 and syndecan-4 ectodomains from 3T3-L1 adipocytes in a process that depends upon new protein synthesis. 3T3-L1 adipocytes were washed and then incubated in serum-free media in the presence or absence of 1 $\mu$M insulin, with or without 300 $\mu$M cycloheximide. CM were collected and replaced every 30 min for 360 min total. Each sample was processed as described in the Experimental Procedures and applied to a cationic PDVF membrane via a dot blot apparatus. The membrane was then immunoblotted with specific monoclonal antibodies for syndecan-1, stripped, and reblotted for syndecan-4. Only data for the hourly time points are shown.

the syndecan-1 and syndecan-4 proteoglycans were indeed shed from 3T3 cells, we performed a dot blot assay with mAbs specific for syndecan-1 and syndecan-4 (Figure 3). 3T3-L1 adipocytes were washed to remove components of fetal calf serum (FCS) and incubated in serum-free media with or without supplemental insulin (1 $\mu$M). The CM were then collected and replaced every 30 min for a total of 360 min. After the withdrawal of serum, syndecan-1 and syndecan-4 ectodomains continued to shed into the media for the first 60 min whether insulin was present or not. After 120 min, the rate of shedding had decreased to the lowest point under both conditions. However, after 180 min, in the presence but not the absence of insulin, both syndecan-1 and syndecan-4 ectodomains reappeared in the CM. This insulin-dependent syndecan shedding required new protein synthesis because it was prevented by co-incubation with cycloheximide, an inhibitor of eukaryotic protein synthesis.

At doses as high as 1 $\mu$M, insulin may activate not only its own receptor but also the insulin-like growth factor (IGF-1) receptor. Therefore, we tested the effect of varying the concentration of insulin from 0 to 1 $\mu$M on syndecan ectodomain shedding (Figure 4A). Half-maximal ectodomain shedding was observed at ~10 nM insulin. These data are consistent with those observed for other insulin actions in 3T3-L1 adipocytes that are mediated specifically by the insulin receptor, such as insulin-stimulated glucose transport (*38*). A concentration of 10 nM insulin exceeds physiological levels in intact animals and the doses necessary to achieve half-maximal effects in freshly isolated rodent adipocytes, but 3T3-L1 adipocytes are relatively insulin-resistant compared with these other experimental models. Shedding of syndecan-4 was also promoted by insulin, but the dose response appeared to be shifted to the right compared with that of syndecan-1.

Two major signaling cascades downstream of the insulin receptor are the Ras-MAP kinase pathway and the phosphotidylinositol 3-kinase (PI 3-kinase) pathway (*19*, *45*). We used specific inhibitors of these two pathways, PD98059 (MEK inhibitor) and wortmannin (PI 3-kinase inhibitor), at

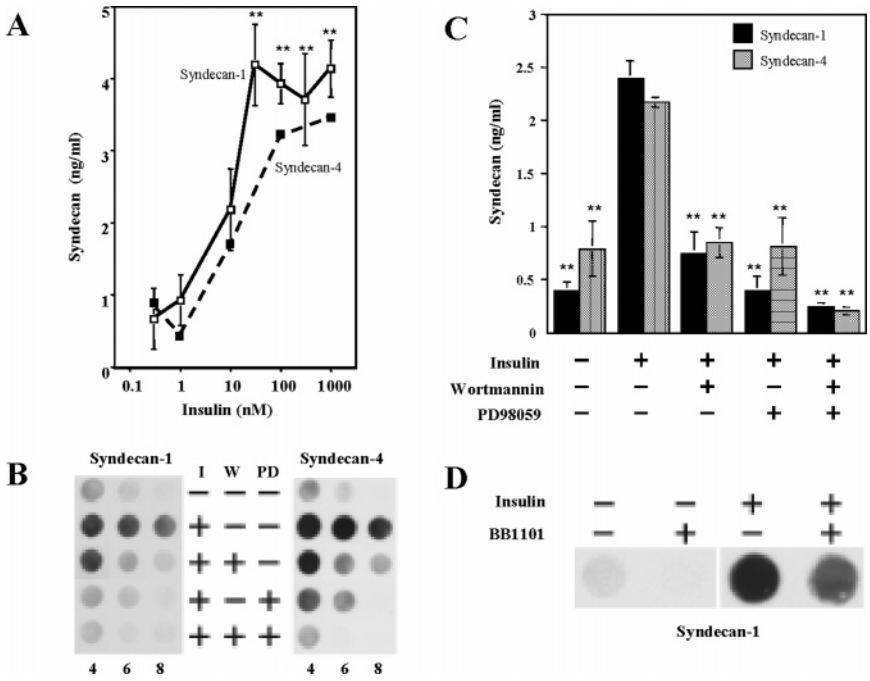


FIGURE 4: (A) Insulin-stimulated shedding of syndecan-1 and syndecan-4 ectodomains is dose-dependent. 3T3-L1 adipocytes were washed and then incubated in serum-free media supplemented with the indicated concentrations of insulin for 12 h. These media were aspirated and discarded; the cells were washed; and the identical media were replaced. After 4 h of incubation, the media were collected and cleared of insoluble debris and 1.0 mL samples were analyzed by dot blot. Assays were performed in triplicate for syndecan-1 (□) and in duplicate for syndecan-4 (■). Data are represented ±SEM for syndecan-1. The mean value for no added insulin was 0.40 ± 0.35 ng/mL. The amount of syndecan-1 for each concentration of insulin was compared to the amount present in the sample with no added insulin. One-way ANOVA with Dunnet's post-test was performed. Significantly increased syndecan-1 (**, $p < 0.01$) was shed at 30 nM insulin and above. (B and C) Inhibitors of insulin-signaling pathways attenuate insulin-stimulated shedding of syndecan-1 and syndecan-4. 3T3-L1 adipocytes were washed and then incubated in serum-free media supplemented with the following compounds as indicated: 100 nM insulin (I); 100 nM wortmannin (W), an inhibitor of PI 3-kinase; and 30 $\mu$M PD98059 (PD), a MEK inhibitor. Triplicate 1.0 mL samples were processed for each condition. The media were collected; the cells were washed; and identical fresh media were added every 2 h for a total of 8 h. Supplements were freshly diluted from stock at each time point. After collection, each sample was cleared of insoluble debris and analyzed by dot blot and densitometry. Representative dot blot data for each condition at the 4, 6, and 8 h collection points are presented in B. Densitometric data for the triplicate samples corresponding to the third 2 h collection period (i.e., the media collected from hours 4 to 6 of the experiment) are represented in C as ±SEM. The amount of shed syndecan-1 or syndecan-4 for each condition was compared to the amount shed in the presence of insulin and the absence of wortmannin and PD98059. Significant differences (**, $p < 0.01$) were identified by one-way ANOVA with Dunnett's post-test. (D) Insulin-stimulated shedding of syndecan-1 from adipocytes is attenuated by a metalloproteinase inhibitor. 3T3-L1 adipocytes were washed and then incubated for 12 h in serum-free media supplemented or not with 100 nM insulin and/or 10 $\mu$M BB1101, a peptide hydroxamate inhibitor of metalloproteinases. These media were aspirated and discarded; the cells were washed; and the identical media were replaced. After 4 h of incubation, the media were collected, cleared of insoluble debris, and analyzed by dot blot.

known effective doses (*46*, *47*) to determine whether either pathway is necessary for full insulin-stimulated syndecan ectodomain shedding. 3T3-L1 adipocytes were incubated in serum-free media that contained insulin alone or in combinations with these inhibitors, as indicated in Figure 4B. CM were collected and replaced every 2 h for a total of four 2 h intervals prior to analysis by dot blot assay. Inhibition of the Ras-MAP kinase pathway resulted in significant reductions in insulin-stimulated syndecan-1 and syndecan-4 shedding compared with insulin alone. For example, PD98059 reduced insulin-stimulated shedding of syndecan-1 and syndecan-4 by 83 and 63%, respectively, during the third 2 h interval after serum withdrawal (Figure 4C). Wortmannin also inhibited insulin-stimulated shedding of syndecan ectodomains, but the onset of the effect was later (by 6 h as opposed to 4 h for PD98059) and the extent of inhibition during the third 2 h interval was slightly less (69%) for syndecan-1 and approximately the same (61%) for syndecan-4. The degree of maximal inhibition of syndecan-1 and syndecan-4 ectodomain shedding, which was observed during the fourth 2 h interval for both, was greater for PD98059 than for wortmannin at the doses tested. The addition of both wortmannin and PD98059 resulted in further inhibition of insulin-stimulated shedding during the third collection period but not subsequently.

*An Inhibitor of Metalloproteinases Attenuates the Effect of Insulin on Syndecan Ectodomain Shedding*. Regulated shedding of syndecan ectodomains from mammary epithelial and endothelial cells involves a membrane-associated metalloproteinase (*8*). We used compound BB1101, a peptide hydroxamate inhibitor of matrix metalloproteinases, to assess whether insulin-stimulated syndecan ectodomain shedding from 3T3-L1 adipocytes likely involves such proteases. Coincubation of 3T3-L1 adipocytes with both insulin and BB1101 inhibited shedding by 50% compared with insulin alone (Figure 4D). BB1101 also inhibited basal shedding of syndecan-1 in the absence of insulin.

*Insulin-Stimulated Syndecan Shedding Is Differentiation-Dependent*. LPL synthesis and secretion are acquired by 3T3-L1 adipocytes early in their differentiation (*24*). Insulin-stimulated syndecan ectodomain shedding might also be acquired during this differentiation if it is a mechanism of LPL transport from the adipocyte to the endothelium. We compared 3T3-L1 fibroblasts with 3T3-L1 adipocytes with

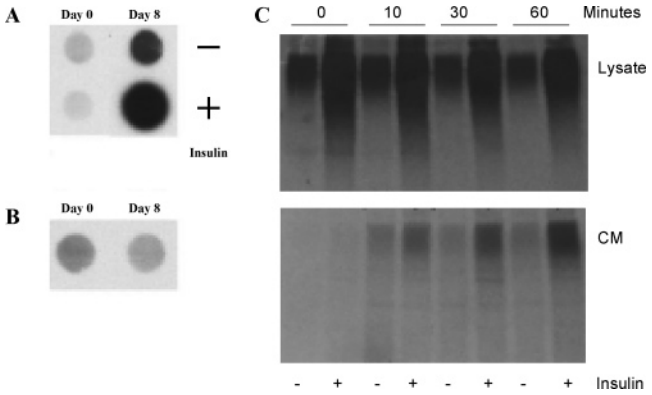


FIGURE 5: (A) Insulin-stimulated shedding of syndecan-1 is acquired in adipogenesis. 3T3-L1 cells at days 0 and 8 of adipocyte differentiation were washed and then incubated in serum-free media with or without 100 nM insulin for 15 h. These media were aspirated and discarded; the cells were washed; and the identical media were replaced. After 4 h of incubation, the media were collected, cleared of insoluble debris, and analyzed by dot blot. (B) Cell-surface syndecan-1 expression does not differ significantly between 3T3-L1 preadipocytes and adipocytes. 3T3-L1 cells at days 0 and 8 of adipocyte differentiation were washed in PBS/5 mM EDTA. Cell-surface syndecan-1 was removed from the intact cells by incubation in TPCK-treated trypsin at 4° for 5 min followed by the addition of 10 $\mu$g of soybean trypsin inhibitor. The cells were pelleted by centrifugation, and the trypsinates were applied to a cationic PDVF membrane via a dot blot apparatus and assayed for the presence of syndecan-1. (C) Insulin stimulates the synthesis and shedding of syndecan-1. 3T3-L1 adipocytes were incubated overnight in the presence or absence of insulin. The next morning, cells were washed and incubated with $^{35}SO_4$ ± insulin for 20 min to label the HS chains on the syndecan. Cells were then washed free of the label; 300 $\mu$M cycloheximide was added; and cells were incubated in the presence or absence of insulin for 60 min. At the indicated time (0, 10, 30, and 60 min), the CM were removed and cells (cell lysates) were lysed in immunoprecipitation buffer. For the "0 min" time point, media were added to the cells and immediately removed. The cell lysates and CM were immunoprecipitated with the syndecan-1 mAb (281-2). Immunoprecipitates were analyzed on a 5−20% gradient gel and visualized by autoradiography.

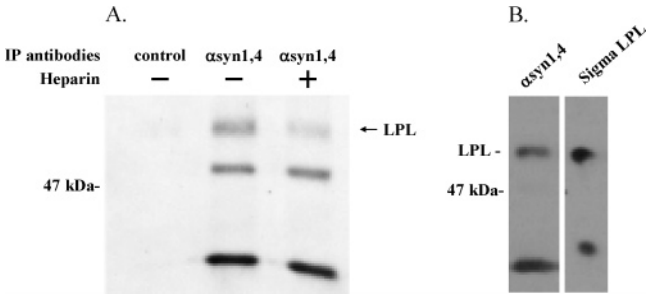


FIGURE 6: Soluble syndecan-1 and/or syndecan-4 ectodomains shed from insulin-stimulated adipocytes exist in a heparin-sensitive complex with LPL. (A) CM from 3T3-L1 adipocytes incubated overnight with 1 $\mu$M insulin were cleared by centrifugation and mixed with either negative "control" beads (rat monoclonal Ig2a antibody specific for L-selectin coupled to sepharose) or specific "αsyn1,4" beads (rat monoclonal Ig2a antibodies specific for syndecan-1 and syndecan-4 coupled to sepharose) in the absence or presence of 150 $\mu$g/mL heparin. After an overnight incubation, the immunoprecipitates were washed with TBS/T, resolved by 7.5% SDS−PAGE, and blotted onto nitrocellulose membranes. (B) Immunoprecipitated LPL and Sigma bovine LPL were analyzed on SDS−PAGE and immunoblotted with LPL antibodies as described above. 3T3-L1 LPL, coimmunoprecipitated with the syndecans, migrates at an equivalent molecular size as the Sigma bovine LPL monomer with an apparent molecular weight of 55 kDa.

respect to their level of insulin-stimulated shedding of cell-surface syndecan-1. Only the adipocytes responded to insulin with increased shedding of the syndecan-1 ectodomain (Figure 5A), even though 3T3-L1 fibroblasts expressed nearly equivalent amounts of syndecan-1 mRNA (Figure 2) and cell-surface syndecan-1 (Figure 5B). Further, insulin-responsive syndecan ectodomain shedding begins as early as 48 h into adipogenesis (data not shown).

Using a pulse-chase labeling experiment, we determined that insulin stimulates syndecan synthesis as well as syndecan shedding (Figure 5C). The data show that in the presence of insulin there is a large increase in syndecan synthesis compared to in its absence (cell lysates). As expected, the cellular pool of labeled syndecan declines during the chase period. In contrast, the shed labeled syndecan-1 (supernatant) appears at 10 min and continues to increase in the presence of insulin, whereas, in the absence of insulin, the shed syndecan-1 appears at 10 min and decreases during the time of incubation. These data indicate that there is indeed an increase in shedding in the presence of insulin as well as an increase in syndecan synthesis.

*Shed Syndecan Ectodomains Are Bound to Active Lipoprotein Lipase.* On the basis of the kinetics of shedding, we wondered whether syndecans could mediate the transport of adipocyte LPL to the endothelium. We used specific monoclonal antibodies to immunoprecipitate syndecan-1 and syndecan-4 ectodomains from the conditioned medium of insulin-stimulated 3T3-L1 adipocytes and assayed for the presence of LPL in the immunoprecipitate by western blot using an anti-LPL antibody (Figure 6). Three immunoreactive polypeptides were detected, but no bands were detected when the immunoprecipitation was performed with an irrelevant mAb of the identical species and isotype. The upper band was the expected molecular size of the LPL monomer and migrated at a molecular size equivalent to bovine LPL (Figure 6B). The lower molecular weight bands are either LPL degradation products, which are commonly observed because of the instability of active LPL (*24*), or possibly irrelevant proteins with cross-reactive epitopes. In parallel, the syndecan immunoprecipitation was performed in the presence of heparin, which preferentially displaces the active LPL dimer. In the presence of heparin, nearly all of the upper band fails to immunoprecipitate, thereby suggesting that this protein represents active LPL bound to the HS chains of soluble syndecan ectodomains.

*Shed Syndecan Ectodomains Stabilize LPL Activity in Vitro.* Because of the reported ability of heparin and HS to stabilize LPL activity in solution, we hypothesized that the HS moieties of syndecan ectodomains shed from adipocytes in response to insulin might stabilize LPL activity as well. To test this hypothesis, we first purified HSPGs from the CM of serum-starved 3T3-L1 adipocytes in the presence and absence of 100 nM insulin. Although the HSPGs purified in this manner are heterogeneous, syndecan-1 and syndecan-4 ectodomains are the most abundant HSPGs present in 3T3-L1 adipocyte CM (Figure 1). The purified HSPGs were then incubated at 4 °C for 16 h with commercially available LPL at various doses of the HSPGs. After incubation of bovine LPL with the purified HSPGs, residual LPL activity was measured for each HSPG dose and compared with the LPL activity prior to incubation. The CM derived from insulin-treated adipocytes contained 6-fold greater syndecan-1 ectodomain protein than untreated cells, as assessed

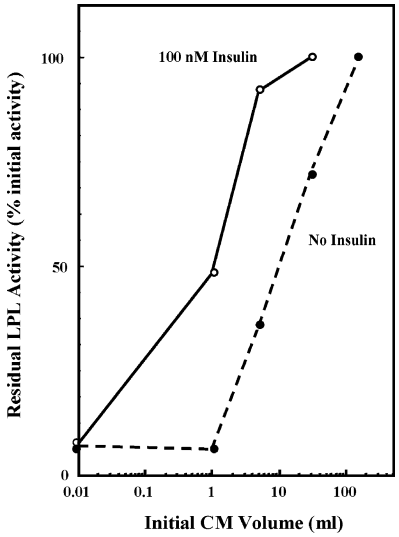


FIGURE 7: HSPGs purified from adipocyte CM stabilize LPL activity. CM were collected after overnight incubation in the presence and absence of 100 nM insulin under serum-free conditions. The soluble HSPGs in the CM were partially purified using Q sepharose chromatography, concentrated by ethanol precipitation, resolubilized, and quantified by the BCA assay. Six-fold increased quantities of HSPGs were recovered from CM in the presence of insulin compared with in its absence. Varying concentrations of syndecan-1 and syndecan-4 HSPGs were incubated with Sigma LPL at 4 °C overnight, and the following day, the residual LPL activity was assayed as described in the Experimental Procedures. The residual LPL activity for each condition (○, 100 nM insulin; ●, no insulin) is expressed as the percent of the experimentally determined activity of Sigma LPL prior to incubation, i.e., the "initial LPL activity". Residual LPL activity is plotted as a function of the original volume of CM used to purify each dose of HSPG. The experiment shown is representative of three independent experiments.

by quantitative dot blot (data not shown). We observed a dose-dependent stabilization of LPL activity by the HSPGs regardless of whether the HSPGs were from insulin-treated or untreated adipocytes (Figure 7A) (*24*). The HSPGs purified from the two sources were equipotent when equal concentrations of each HSPG were used. However, when LPL activities were expressed as functions of the initial CM volumes from which each dose of HSPG was purified, stabilization of LPL activity was greater for the HSPGs purified from the CM of insulin-treated adipocytes compared with that purified from the CM of untreated adipocytes. These data suggest that insulin treatment of adipocytes does not alter the intrinsic ability of the shed syndecan ectodomains to stabilize LPL but rather regulates the rate of shedding. Syndecan-1 ectodomains purified from a nonadipocyte source were also able to stabilize LPL activity in a dose-dependent manner, whereas even up to a $10^4$-fold greater concentration of bovine serum albumin had no ability to stabilize LPL activity (data not shown).

## DISCUSSION

In this study, we have explored a potential function of syndecans in triglyceride metabolism. We find that syndecan-1 and syndecan-4 are expressed by adipocytes in adipose tissue and by 3T3-L1 adipocytes and that they are shed as soluble ectodomains in the extracellular space by insulin-stimulated 3T3-L1 adipocytes. Syndecan-1 and syndecan-4 expression in adipogenic cell lines has been reported previously (*43*), but to our knowledge, the present study is the first identification of syndecan-1 expression by adipocytes in adipose tissue. Insulin-stimulated syndecan ectodomain shedding is acquired early during adipocyte differentiation, coincident with the onset of LPL expression. The insulin effect on shedding is seen at insulin concentrations that are consistent with those at which other physiological actions of insulin occur in 3T3-L1 adipocytes, involves the Ras-MAP kinase and the PI-3 kinase pathways, and is inhibited by metalloproteinase inhibitors. Finally, the syndecan ectodomains shed in response to insulin bind via their HS chains to active dimeric LPL, and the interaction of LPL with the shed ectodomains stabilizes LPL activity.

The present data extend our prior findings that syndecan ectodomain shedding is a highly regulated process involving physiological mediators, intracellular signaling pathways, and metalloproteinase activity and confirm that shedding of the syndecan-1 and syndecan-4 ectodomains from adipocytes is regulated similarly. The effective insulin concentration is in the range to suggest that syndecan ectodomain shedding is a normal response to the hormone, but it is unknown by what mechanism insulin regulates this shedding. The fact that cycloheximide prevents insulin-stimulated syndecan ectodomain shedding suggests that the regulation requires de novo protein synthesis. Whether the protein synthesis is required to replenish the syndecan pool or to provide a necessary factor (e.g., a protease) for shedding remains unknown. Using a pulse-chase experiment, we were able to show that expression of syndecan-1 as well as syndecan-1 shedding is induced by insulin (Figure 5C). It is likely that the initial burst of syndecan shedding following serum withdrawal depletes the cell-surface syndecan-1 and syndecan-4, thus requiring new protein synthesis that is stimulated by insulin, although, as evident in the pulse-chase experiment, syndecan-1 shedding is also induced by insulin.

The data are consistent with the role of the adipocyte in triglyceride metabolism. 3T3-L1 fibroblasts do not demonstrate insulin-stimulated syndecan ectodomain shedding. This capability is acquired because these cells differentiate into adipocytes, which, unlike fibroblasts, robustly synthesize triglycerides from fatty acid and glycerol substrates. Active LPL bound to syndecans at the adipocyte cell surface would be a ready reservoir of enzyme to respond to metabolic demands. For example, following a meal, plasma levels of triglyceride-rich lipoproteins and insulin both increase. Increased insulin would cause increased shedding of syndecan ectodomain−LPL complexes and lead to enhanced transport of active LPL to the capillary endothelium, where it can hydrolyze triglycerides to release the free fatty acids needed for cellular metabolism.

These data suggest that LPL is bound to the HS chains of syndecan-1 and syndecan-4 on adipocyte cell surfaces and that activation of the insulin receptor and its downstream signaling pathways leads to metalloproteinase-induced shedding of the ectodomains. Alternatively, the association of LPL with syndecan ectodomains may occur only after shedding of the ectodomains into the extracellular space. The syndecan ectodomain−LPL complex is soluble and can stabilize the enzyme during its transport through interstitial spaces to the capillary endothelium. Our data do not clarify how the complex traverses the endothelial cell to reach the luminal cell surface, the site of LPL action on chylomicrons

and very low density lipoprotein (VLDL). The transcytosis of LPL across endothelial cells is dependent upon cell-surface proteoglycans and the VLDL receptor (*53*). Therefore, it is attractive to speculate that the soluble syndecan ectodomain "hands off" LPL to endothelial HSPGs on the abluminal plasma membrane followed by transcytosis of the HSPG−LPL complex to the luminal surface.

Although insulin-induced shedding of the syndecan−LPL complex appears to be physiologically relevant, we do not yet know its biogenesis. LPL, itself a glycoprotein, could be captured within the secretory pathway by syndecans. Alternatively, it could associate with syndecans only after it is itself secreted. Also, it remains to be determined whether the syndecan−LPL complex is shed from the adipocyte cell surface or forms between soluble syndecan ectodomains and active LPL in the extracellular space. Because LPL is rapidly released from adipocytes upon exposure to heparin, it is likely that the syndecan−LPL complex is present on the adipocyte cell surface, but our data do not directly address this issue. Regardless of the exact mechanism of its biogenesis, formation and transport of the syndecan−LPL complex links several aspects of lipid metabolism. Our model complements an emerging appreciation of the importance of syndecans in lipoprotein metabolism. In transfected Chinese hamster ovary cells, syndecans have been shown to mediate LPL-dependent binding, uptake, and degradation of methylated LDL (*49*).

Several independent mechanisms may mediate LPL transport from the adipocyte to the endothelium, which would allow for distinct responses to different metabolic demands. For instance, a pool of cell-surface LPL is bound to glycosylphosphatidylinositol (GPI)-anchored HSPGs in cultured rat heart cells (*50*) and in adipocytes (*30*). However, treatment of adipocytes with phosphatidylinositol-specific phospholipase C (PIPLC) releases only about 25% of both total HS chains and LPL (*30*). LPL itself has been reported as linked via a GPI anchor to the adipocyte cell surface and released by insulin action (*48*). Incubation of BFC-1$\beta$ adipocytes with VLDL leads to LPL release in a lipolysis-dependent fashion (*51*). HS oligosaccharides that result from the activity of an endothelial heparanase may act as chaperones for LPL (*52*). Finally, insulin stimulation of cultured endothelial cells results in the dose-dependent release by those cells of an unidentified factor that promotes the appearance of active LPL in the CM of 3T3-L1 adipocytes (*53*). It is not known whether endothelial syndecans are shed in response to insulin. Our data and that of other labs cited above suggest that the increase in adipose tissue LPL activity in response to insulin likely is the integrated result of several molecular mechanisms. The relevance of these mechanisms in vivo will require careful analyses of mouse models.

In summary, we report here for the first time that insulin regulates syndecan synthesis and the proteolytic shedding of syndecan ectodomains from at least one terminally differentiated cell type, the adipocyte, and that the soluble syndecan ectodomains bind LPL and stabilize LPL activity via their HS side chains. Because of the ability of syndecans to bind numerous growth factors, including fibroblast growth factor (FGF) proteins, the role of insulin in syndecan ectodomain shedding may regulate processes other than LPL delivery. For example, FGF proteins may play a role in early adipocyte differentiation (*54*, *55*). Insulin-regulated shedding of syndecan ectodomain/FGF complexes in principle could modulate the FGF-signaling pathway by altering the availability of ligand. Whether such mechanisms are important for adipocyte ontogeny or function remains for future investigations.

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