Lipoprotein lipase stored in adipocytes and muscle cells is a cryptic enzyme

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Abstract The status of lipoprotein lipase (LPL) has been examined in different cell types (adipose, skeletal muscle, and heart muscle cells) and different tissues (adipose, muscle, and cardiac tissues) from mouse, rat, and human. Cell and secreted activities were compared in cycloheximide-, heparin-treated cells present in culture. A gross underestimation of cell LPL activity was found; excess of LPL over substrate and/or apolipoprotein C-II was excluded as well as inhibition by cell component(s) or detergent molecules used to disrupt membrane structures in the cell lysates. Unmasking of LPL activity occurred upon dilution: the higher the concentration of LPL, the higher were the dilution factor and the concentration of heparin required to reach a plateau of activity. This maximal value was found to be identical to that determined in the secretion medium, indicating that the cell LPL activity can be determined in toto. The unmasking effect of dilution upon LPL activity was extended to adipose, muscle, and cardiac tissues from rat and to adipose tissues from mouse and human. In agreement with previous results (Vannier et al., 1989, *J. Biol.* **264:** 13199–13205), our results are in favor of LPL as being cryptic within the cell. A model is proposed, in which potentially active LPL molecules are present as aggregates in various membrane compartments. It is concluded that the determination of the pool size of catalytically active cell LPL has to be estimated in vitro under the appropriate conditions described herein. —**Pradines-Figuères, A., C. Vannier, and G. Ailhaud.** Lipoprotein lipase stored in adipocytes and muscle cells is a cryptic enzyme. *J. Lipid Res.* 1990. **31:** 1467–1476.

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Lipoprotein lipase (LPL) is secreted by parenchymal cells in tissues of mesodermal origin (adipose and muscle tissues), but it functions on plasma apolipoprotein C-II-containing lipoproteins once it has been transferred to the luminal surface of endothelial cells where it is bound. The activity of LPL in tissues has been examined in numerous studies with respect to diet and hormonal status, and it is now recognized that physiological stimuli direct triglycerides as well as cholesterol utilization by parenchymal cells by means of a control of LPL expression (for reviews, see refs. 1–3). Hence, both the determination of LPL activity and the elucidation of the mechanisms involved in its synthesis and secretion have attracted much interest (3).

Our studies have been focused on delineating the status of secretory protein for LPL in Ob17 adipose cells (4–6). These investigations have shown that cell LPL appears localized in cisternae of the Golgi complex and in Golgi-derived compartments. This localization has also been observed in rat and human adipose cells (7, 8). An approach based on the drug-induced perturbation of the intracellular transport and/or glycosylation of the protein led to the demonstration that the functional maturation of LPL is an intracellular event taking place in the proximal Golgi and requiring glycosylation (5, 6). Similar observations on the importance of post-translational events in the expression of catalytically active LPL have been extended to the rat enzyme from adipose tissue and myocardiac cells (9–11) although the trimming and processing of the oligosaccharide chains appear not to be necessary for guinea pig LPL to become catalytically active (12). Recent studies on the biosynthesis and turnover of LPL in 3T3-F442A cells (13) have demonstrated that LPL is synthesized in the endoplasmic reticulum as an inactive monomer (M_r = 55,500). It is then transported to the Golgi apparatus where the maturation of its two N-oligosaccharide side chains takes place. LPL is subsequently stored in vesicles that appear to belong to a regulated pathway of secretion as a homodimer only (subunit of M_r = 58,000), with which the enzymatic activity is associated. Apart from this fully active form, no intracellular accumulation of an inactive precursor form of LPL could be detected (13).

Heparin has long been known to induce the rapid secretion of LPL from adipocytes (14, 15). To study LPL secretion we have devised a perifusion system by means of

Abbreviations: LPL, lipoprotein lipase, DME, Dulbecco's modified Eagle's; PMSF, phenylmethylsulfonyl fluoride; apoC-II, apolipoprotein C-II.

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which the actual rate of secretion of active LPL, and therefore the size of the releasable pool, can be accurately measured (16). The secretion potential μ, taken as the ratio of total releasable activity (or antigen) to inital cellular activity (or antigen) was determined (17). This was shown in cells treated with heparin and cycloheximide to be equal to 1 for LPL antigen but significantly greater than 1 for LPL activity assayed under standard conditions. No LPL was actually degraded within the cells. A dramatic enhancement of the intracellular activity was induced by a mere dilution of the detergent-treated cell lysates with no change in LPL antigen. The total intracellular activity reached a plateau at a value that became identical to that obtained in the medium of cells exposed to heparin and cycloheximide. The existence of an inhibitor of LPL activity was excluded, as well as that of an increase in the molecular activity of LPL during its secretion, before or after exposure to heparin. These results indicated a systematic underestimation of the intracellular activity of LPL and suggested that LPL is present within intracellular cisternae in a cryptic state resulting from the condensation of potentially active molecules, and also that this potential activity can be fully unmasked in vitro. The present report indicates that the results obtained in 3T3-F442A cells can be extended to adipose tissue of various species as well as to skeletal muscle cells and cardiac cells, and that the systematic underestimation of intracellular LPL activity is likely due to the fact that the enzyme is present, at least in part, under a cryptic state in all LPL-containing tissues. A model for LPL intracellular aggregation involving sulfated glycosaminoglycans is discussed, which takes these and previous results into account.

MATERIALS AND METHODS

Cell culture

Mouse 3T3-F442A cells (18) were obtained through the courtesy of Dr. H. Green (Boston, MA) and were routinely cultured as previously described (17). Briefly, cells were plated at 2×10^3 cells/cm^2 in Dulbecco's modified Eagle's (DME) medium containing 200 units penicillin/ml, 50 μg streptomycin/ml, 33 μM biotin, 17 μM pantothenate, and 7.5% (v/v) fetal bovine serum (Flow Laboratories, Bethesda, MD). At confluence, 5 days after seeding, cells were exposed to 17 nM insulin and 2 nM triiodothyronine in the same medium. This complete medium (termed differentiation medium) was changed every other day. Differentiated, LPL synthesizing-cells were used between days 5 and 11 after confluence.

Stromal-vascular cells from rat epididymal adipose tissue were cultured and differentiated in chemically defined medium, containing 850 nM insulin, 0.2 nM triiodothyronine, and 130 nM transferrin, as described by Deslex, Negrel, and Ailhaud (7). Differentiated cells were used 5 or 7 days after seeding, as indicated.

Heart muscle cells were isolated from 2-day-old rats and cultured according to Chajek, Stein, and Stein (19). Excised hearts were minced, and a cell suspension was obtained by serial digestions with 1 μg of trypsin per ml in Dulbecco's modified Eagle's medium at 37°C. After centrifugation (2000 rpm, 5 min), the cell pellet was dispersed in DME medium containing 10% (v/v) fetal bovine serum and 10% (v/v) heat-inactivated horse serum (defined as complete growth medium). The cell suspension was filtered through an 80-μm nylon screen and plated at 37°C under a CO_2/air atmosphere for 90 min to allow fibroblasts to attach. The suspension was then transferred to 35-mm culture dishes at an average density of 2×10^6 cells per dish. Complete growth medium was changed every other day, and the cells were used at day 12 after seeding.

Rat skeletal muscle cells were prepared as described for heart cells except that at confluence, complete growth medium was replaced with DME medium containing 5% (v/v) fetal bovine serum. The cells were used 9 days after seeding.

Animals

Six-week-old male OF-1 mice and 5-week-old male Wistar rats were obtained from Iffa-Credo (Lyon, France). They were fed ad libitum on a chow diet and killed between 9 and 11 AM by cervical dislocation before immediate tissue removal.

Human adipose tissue

Human adipose tissue was obtained (Hôpital Pasteur, Nice, France) at the time of elective abdominal surgery. Tissue was immersed at room temperature in DME medium and immediately transported to the laboratory.

Preparation of cell lysates

The procedure to obtain cell lysates in Triton X-114 has already been described (4, 17) and was used with minor modifications. In the experiments reported below, cells were solubilized at 0–2°C for 2 h with 5 mM sodium barbital, pH 7.4, 150 mM NaCl, and 1 M glycerol (buffer A) containing 0.2% (w/v) Triton X-114 and a cocktail of protease inhibitors (PMSF 5×10^{-4} M, aprotinin 4 μg/ml, pepstatin 4 μg/ml). A constant ratio of 50 μl of solubilization buffer per cm^2 of cultured cells was maintained throughout all experiments. After heat treatment at 30°C to pellet Triton X-114 (20), the detergent-depleted aqueous phase (<0.008% Triton X-114), which will be referred to as initial sample of cell lysate, was used for LPL assays. The lysates could be stored at −20°C before assaying LPL activity.

Membranes of the post-nuclear supernatant containing all the latent LPL activity were obtained as described elsewhere (5). They were solubilized as above using the same ratio of volume to area of cultured cells.

Unless otherwise indicated, all dilutions of the initial samples of cellular LPL were made in buffer A containing 3 μg/ml heparin.

Preparation of tissue lysates

Skeletal and cardiac muscles were minced and first homogenized with a Polytron homogenizer (20 sec, low setting), then with a Potter-Elvehjem homogenizer (20 strokes) at 0°C in buffer A containing 0.4% (w/v) Triton X-114 and the same cocktail of protease inhibitors as above, using 2 ml of buffer per gram of wet tissue. Homogenates were then lysed under gentle stirring for 1 h at 4°C. The same procedure was followed for adipose tissue except that the Polytron step was omitted and the temperature was raised to 25°C during homogenization. In both cases, solubilized tissues were first centrifuged for 20 min at 10000 *g* to remove lipids, cell debris, and insoluble material. The supernatant was treated at 30°C as described for cells. After heat treatment, lipid-free, detergent-depleted lysates (defined as initial samples of tissue lysates) could be stored at −20°C until use.

Preparation of secreted enzyme

Secretion of LPL was carried out under conditions previously described (16, 17). Two protocols were used. In the first protocol, a batch-wise secretion was used for the preparation of LPL samples intended for immediate assays (see legends of figures) or for affinity chromatography on heparin-Ultrogel (17). For this purpose, cells were washed with DME medium at 37°C and further incubated in the differentiation medium supplemented with 3 μg/ml heparin at the same temperature for 30 to 60 min. This medium was then collected and rapidly chilled at 0°C. It was then diluted 1.5-fold with buffer A to stabilize the enzymatic activity. The final volume of the diluted secretion medium was 100–150 μl per cm^2 of cultured cells. The medium was maintained at 0°C or stored at −70°C until use.

In the second protocol, the continuous flow technique was used for the accurate determination of active LPL secreted from the cells (disposed in a perifusion chamber) as already described (16). With this procedure LPL activity, rapidly stabilized by continuous dilution of the effluent medium and collected every 10 min with cold buffer A, was assayed in the diluted fractions. The activity was calculated from the measured activity as indicated elsewhere (16, 17).

Purification of LPL on heparin-Ultrogel

LPL from secretion media or cell extracts (detergent-depleted aqueous phase) was allowed to bind to heparin-Ultrogel (2 ml packed beads in 10 ml enzyme sample) for 2 h at 4°C in buffer A containing 0.3 M NaCl instead of 0.15 M NaCl. The beads were then collected by low speed centrifugation (3000 *g*, 5 min) and washed with the same buffer. LPL was eluted with a final concentration of 1.5 M NaCl final concentration in 5 mM sodium barbital, pH 7.4, 1 M glycerol. The eluate, which routinely contained more than 90% of the LPL protein present in the load, could be stored at −20°C with no loss of activity. It was diluted 10-fold in 5 mM sodium barbital, pH 7.4, containing 1 M glycerol just before use.

Enzymatic assay of LPL

The conditions for the enzymatic assay have been previously described (4). LPL activity is defined as the apolipoprotein C-II-dependent hydrolysis of tri[9,10-^{3}H]oleoyl glycerol (370 TBq/mol). Routinely, a constant volume of 90 μl of the diluted samples was used per assay for both the secreted and cellular enzyme. The stimulation factor of LPL activity brought about by apolipoprotein C-II (apoC-II) (5 μg/ml) and defined also as activity ratio, ranged from 5 to 7 for both cellular and secreted LPL. One LPL unit hydrolyzes one μequivalent of ester bond per minute at 30°C.

In this report, for the sake of clarity: *i*) LPL activity assayed in serial dilutions of either cell lysates or secretion media, and not corrected for dilution, is referred to as measured activity and is expressed as mU/ml of diluted sample; *ii*) LPL activity obtained after correcting the measured activity for dilution is referred to as apparent activity and is expressed as mU/ml or as mU/dish. Maximal apparent activity corresponds to actual (e.g., total) activity when its value does not vary any more upon dilution of the sample. In other words, the actual activity refers to the value obtained upon dilution in the presence of 3 μg/ml of heparin when reaching the plateau of activity. All assays were performed in duplicate and the replicates did not differ by more than 10%.

Material

Tri[9,10-^{3}H]oleoyl glycerol was obtained from the Radiochemical Centre (Amersham, France). Heparin-Ultrogel A4R was from IBF (Société Chimique Pointet-Girard, Villeneuve la Garenne, France). Cycloheximide, heparin, and Triton X-114 were products of Sigma. Homogenous apoC-II was a kind gift from Dr. P. Puchois (SERLIA, Institut Pasteur, Lille, France).

RESULTS

As the first stage of this study the effect of LPL concentration on the determination of its enzymatic activity was investigated. For this, LPL activity was assayed in serial dilutions of cell lysates or samples of secreted enzyme

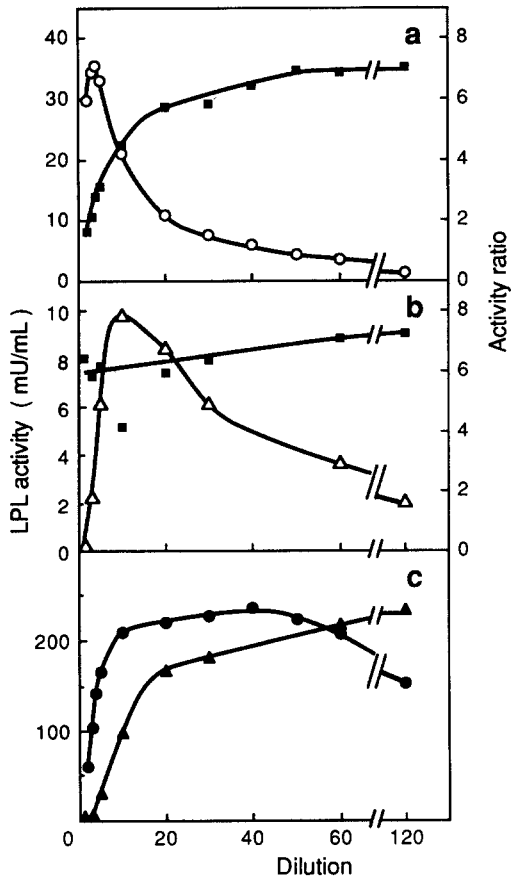

Fig. 1. Effect of sample dilution on measured and apparent activities of secreted and cell LPL. Two parallel sets of 9-day post-confluent 3T3-F442A cells were used to prepare concentrated samples of secreted LPL purified by heparin-Ultrogel chromatography and intracellular LPL present in intial samples of cell lysate, as described in Materials and Methods. a and b. Measured LPL activity (○, △) and activity ratio (■) are plotted as a function of dilution in samples of secreted (a) and cell (b) LPL. LPL activity, expressed as milliunits per ml of diluted sample is not corrected for dilution. c. Apparent activity of secreted (●) and cell (▲) LPL after correcting for dilution the activity values of panels a and b.

prepared from 3T3-F442A cells (**Fig. 1**). To allow a direct comparison of the respective effects of dilution on the enzyme from both origins to be made, preliminary assays were performed to adjust LPL at the same concentration in initial, concentrated samples (236 and 235.2 mU/ml total activity for secreted and cell LPL, respectively). The results show a striking difference between the secreted (Fig. 1a) and cell enzyme (Fig. 1b). In the case of secreted LPL (obtained after purification by heparin-Ultrogel), the measured activity was maximal at low dilutions, i.e., at high concentrations of LPL, and decreased thereafter as a function of dilution. As expected, the curve giving the measured activity of secreted LPL as a function of dilution was hyperbolic in the range of a 4- to 60-fold dilution (Fig. 1a). The results obtained at lower dilutions (1- to 4-fold), which show an apparent lack of effect of dilution, were caused by a high percentage of substrate being hydrolyzed (up to 20%) and the LPL reaction rates were not proportional to time under these conditions. In agreement with this interpretation is the observation that the activity ratio (or stimulation factor by apoC-II) was decreased severely by increasing enzyme concentration (4- to 1-fold dilution) but remained in the range of 5 to 7 above a 10-fold dilution. In the case of cell LPL (Fig. 1b), and in contrast to the secreted enzyme, there was actually an increase in the measured activity by increasing dilution from 1- to 10-fold. Under these conditions, the percentage of hydrolyzed substrate was below 2%, and the activity ratio remained similar, ranging from 6 to 7. The consequences of the different behavior of secreted and cell LPL as a function of dilution are well illustrated in Fig. 1c. After correction for dilution of the measured activity, the curves show that the apparent activity of the secreted enzyme was not significantly affected by dilutions ranging from 4- to 60-fold. By contrast, the apparent activity of cell LPL increased upon dilution and reached a plateau above a 60-fold dilution. It had been shown previously that this maximal activity of cell LPL corresponds to the total and thus to the actual cell activity (17). To exclude the possibility that important changes in the affinity for apoC-II might explain part of the differences between the secreted and cell enzyme, the experiments of **Fig. 2** were performed. As shown, using similar activities of secreted and cell enzyme the apparent affinity for apoC-II was approximately 2-fold higher for the secreted enzyme, but the activity ratio was identical in both cases. Taken together, the results of Figs. 1 and 2 indicate that the concentration-dependent effect observed for the cell LPL could not be attributed to an excess of LPL over substrate and/or apoC-II.

Parallel experiments were also performed to exclude the artifactual interferences of cell and/or operational factors in the assay. This was of importance because: *i*) the assay

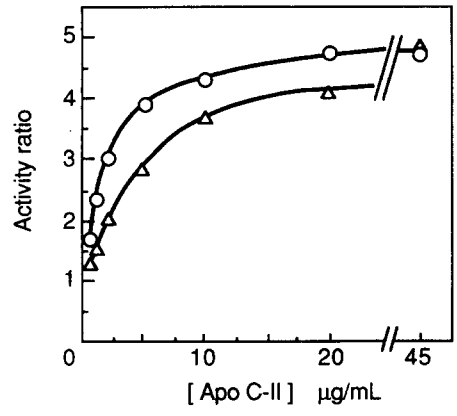

Fig. 2. Effect of apoC-II on secreted and cell LPL activity. Samples of secreted activity (○, 16.2, mU/ml) and cell activity (△, 20.1 mU/ml) were assayed in the presence of apoC-II at increasing concentrations. Results are expressed as the activity ratio (see Materials and Methods).

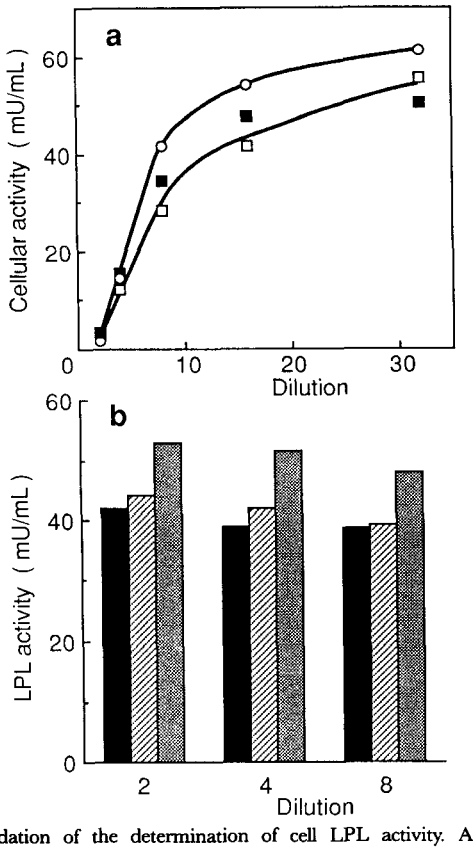

Fig. 3. Validation of the determination of cell LPL activity. A cell lysate (detergent-depleted aqueous phase) was prepared from 9-day post-confluent 3T3-F442A cells as described in Materials and Methods. a. A lipid-free extract was obtained by centrifuging an aliquot of the cell lysate at 8000 *g* for 20 min at 4°C. LPL activity was assayed in the cell lysate (○) and in the lipid-free extract (■, □) after serial dilution in buffer A alone (○, ■) or in buffer A containing 0.008% (w/v) Triton X-114 (□). The apparent activity is plotted after correction for dilution. b. LPL was first affinity-purified from 4 ml of cell lysate by heparin-Ultrogel chromagraphy (see Materials and Methods) then used as a 10-fold dilution of the eluate (800 μL) in 5 mM sodium barbital, pH 7.4, 1 M glycerol. The unretained fraction (4 ml) from heparin-Ultrogel chromatography was collected and used as a 2-fold dilution in the same buffer to adjust the concentration of NaCl to 150 mM. Enzymatic activity of purified cell LPL was assayed after a 2- to 8-fold dilution obtained by adding to 1 ml of the purified LPL fraction either 1 to 7 ml of buffer A (black bars) or 1 to 7 ml of buffer A containing 3 μg/ml heparin (dotted bars). In the third experiment (hatched bars), 1 ml of unretained fraction was added to 1 ml of the purified LPL fraction (dilution 2) and the mixture was further diluted by adding 2 (dilution 4) to 6 ml (dilution 8) of buffer A. Thus, at each dilution, the enzyme amounts to be assayed were identical. Apparent activities are plotted after correction for dilution.

was routinely performed using trioleoyl glycerol micelles stabilized with Triton X-100 as a substrate [0.015% (w/v) final concentration in the assay]; *ii*) cell triglycerides may lead to an isotopic dilution of the added substrate; and *iii*) Triton X-114 present in the lysate [0.008% (w/v) in the initial sample], like any detergent of this series, is a potential inhibitor of LPL. The results of **Fig. 3a** indicate that neither Triton X-114, when maintained for each dilution of cell lysate at a concentration of 0.008%, nor lipids present in the cell lysate could significantly interfere with the assay. Consistent with this observation is the fact that Triton X-114 added at concentrations up to 0.03% (w/v) to cell lysates or secretion media did not inhibit LPL activity (not shown). Moreover, the activity of LPL purified from a cell lysate was neither activated by heparin, as already demonstrated in a previous work (4), nor inhibited by a component present in the LPL-depleted cell lysate (Fig. 3b). Thus it appears in the latter case that no endogenous cell inhibitor of the enzyme can be recovered in LPL-depleted cell lysates. Furthermore, it must be stressed that, in Fig. 3b, there is a negligible dilution of cell lysate when passed through the heparin-Ultrogel column. The fact that addition of purified LPL, recovered from the column, to the LPL-depleted cell lysate does not bring any decrease in LPL activity indicates also that no artifactual aggregation of LPL takes place under these conditions.

Taken together, these results demonstrate that the activity of cell LPL is grossly underestimated under conditions that maintain the proportionality of the assay of secreted LPL. In contrast to the secreted enzyme, a very low activity, if any, can be detected in concentrated cell lysates (no or low dilution). Since Triton X-114 disrupts all membrane structures (5), the interpretation is that the activity of intracellular LPL is nil at high concentrations and becomes progressively unmasked as the concentration of LPL molecules decreases.

As already reported (16, 17) the determination of the actual size of the potentially active LPL pool is only possible when this activity is assayed in highly diluted cell lysates. The results of **Fig. 4** show clearly that the maximal activity of cell LPL, which can be determined upon extensive dilution of cell extracts, is in excellent agreement with the heparin-releasable LPL activity as determined in cycloheximide-treated cells using the continuous flow method (16, 17). This maximal activity, which corresponds to the plateau of activity shown in Fig. 4b, was only attained when the concentration of cellular proteins was maintained below 40 μg/ml ($\sim 3.5 \times 10^4$ cells per ml after dilution).

It has been previously suggested that an underestimation of cell LPL activity could also occur in cell lysates of rat stromal-vascular cells (21). It was thus of interest to study the effect of dilution on cell lysates prepared from various mesodermal cells known to synthesize and export LPL. Experiments were performed as described in the legends of Figs. 1 and 4. **Table 1** summarizes results obtained with rat cultured cells. Clearly, the enzymatic activity of LPL increases with dilution and, as already noted with 3T3-F442A cells (17), heparin produces a further increase in the maximal activity. As for the 3T3-F442A cells, the results in Table 1 show clearly that the maximal apparent activity of cell LPL (e.g., actual activity) after correction for dilution was almost identical in cyclohexemide-, heparin-treated cells to the activity of secreted

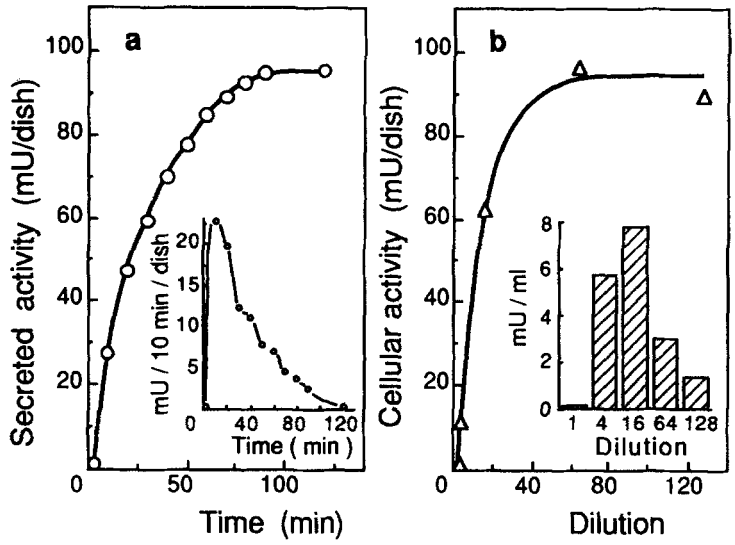

Fig. 4. Determination of total releasable and cells pools of active LPL. Two parallel sets of 7-day post-confluent 3T3-F442a cells were used. a. Kinetics of LPL secretion (first set of cells) in the presence of 3 μg/ml heparin and 15 μM cycloheximide. The curve was calculated (see Materials and Methods) from the secretion rate measured every 10 min (ΔU/Δt: see ref. 16). Inset: variation of ΔU/Δt as a function of time of exposure to the secretion medium. b. Apparent cell LPL activity (second set of cells) not exposed to the secretion medium. Cell lysates were prepared and diluted as described in Materials and Methods. The apparent activity, expressed in mU/dish, was obtained by correcting for dilution the measured activity of the diluted samples. Inset: measured LPL activity of the same samples (e.g., not corrected for dilution).

LPL (after correction for inactivation). This indicates unambiguously that, for these various cell types originating from rat, the cell LPL activity can be determined in toto or that all active enzyme molecules escape intracellular degradation and are recovered in an active state in the secretion medium. This dilution-induced increase of LPL activity could be extended to human, mouse, and rat adipose tissues as well as to rat cardiac and muscle tissues (**Table 2**). Therefore, this phenomenon was not confined to cells in culture, excluding the possibility that it could be the result of conditions in vitro, but rather appears to be a general phenomenon.

To investigate further the relation between LPL concentration in cell lysate and its apparent activity, the requirement for heparin was first more precisely delineated using 3T3-F442A cells. This was performed by assaying LPL activity in a serial dilution of the cell lysate in the presence of increasing concentrations of the glycosaminoglycan. The results in **Fig. 5** were obtained from experiments in which the apparent activities were normalized relative to the percent of the activity determined at the highest concentration of heparin and then plotted as a function of heparin concentration. Enzyme dilution was required to unmask LPL activity at any concentration of heparin. However, as revealed by a shift of one order of magnitude in the concentration of heparin required for a half-maximal effect ($\leq$0.2 μg/ml compared to 2 μg/ml), it is clear that the lower the dilution of LPL the higher was the concentration of heparin required to bring the apparent activity to its maximal value. These results are consis-

TABLE 1. Apparent activity of cell LPL in lysates from rat LPL-synthesizing cells in culture

Dilution	Adipose Tissue Stromal-Vascular Cells Day After Plating			Skeletal Muscle Cells Day after Plating		Heart Muscle Cells Day After Plating
	5 (a)	7 (b)		9 (c)		12 (d)
	+	−	+	−	+	+
1	8.1	0.9	4.6	0	3.6	23.1
2	96.7	26.9	46	19.8	57.2	35.8
4	100	46.8	63	56.5	85.6	61.6
8				58.9	100	80
16				57.8	89.7	94.2
32	78	67.4	100			100

These experiments are identical to that of Fig. 4b. Cells were cultured for the indicated time and then lysed according to the protocols given in Materials and Methods. Serial dilutions of the initial samples of lysates were made in buffer A containing (+) or not (−) 3μg/ml (stromal vascular cells) or 30 μg/ml (muscle cells) heparin. Apparent LPL activity was determined as described in the legend to Fig. 4b. Results are expressed as percent of maximal activity obtained at the plateau in the presence of heparin. Maximal activities were: (a) 10.7, (b) 20.5, (c) 4.74, and (d) 1.9 mU/dish. In each case, parallel experiments were performed by exposure of the cells at 37°C to culture media (see Materials and Methods) containing 15 μM cycloheximide and 3 μg (or 30 μg, see above) heparin per ml. Secretion media (0.5 ml per 35-mm dish) were then collected every 10 min for 80 min. Measured activity of secreted LPL was then corrected for inactivation. The total secreted activities corresponding to (b), (c), and (d) were found to be 19.8, 4.7, and 2.0 mU/dish, respectively.

TABLE 2. Apparent activity of LPL in lysates from tissues of different origin

Dilution	Muscle Rat (a)	Heart Rat (b)	Adipose Tissue Rat (c)	Adipose Tissue Mouse (d)	Adipose Tissue Human (e)
1	11.8		0.53	1.62	
2	62.52	36.75	18.56	27.6	
4	87.83	76.8	72.11	84.06	25.4
8	100	100	100	100	76.9
16	88.8	93.9	88.94	93.1	100
32					88.8

Tissue lysates were prepared as described in Materials and Methods. Apparent LPL activity was determined as described in the legend to Table 1 in serial dilutions of the initial samples of lysates in heparin-containing buffer A. Results are expressed as percent of maximal activity obtained at the plateau. Maximal activities were (a) 166, (b) 43.6, (c) 286, (d) 271, and (e) 25 mU per gram wet tissue.

tent with our previous report (17) and, since heparin is not an activator per se of LPL activity (see Fig. 3), they strongly favor the hypothesis of an interaction in the cells of LPL molecules, directly or indirectly, with components structurally related to heparin (17).

Moreover, it is of interest to note in the case of stromal-vascular cells (Table 1), that the ongoing differentiation taking place between day 5 and day 7 after plating (which is accompanied by a 2-fold increase in the LPL intracellular content) contributed to a shift of the curve towards higher dilutions. It is thus suggested that the degree of underestimation of this activity may increase as a consequence of an increase in the specific activity of LPL in lysates and not only with increasing lysate concentration. Since in these experiments it was essential to maintain at a constant value the ratio of lysate volume to cell culture area or cell number, these observations raised the possibility that the concentration of LPL molecules within intracellular compartments, and not only its operationally imposed concentration in lysates, might explain the unmasking effect of dilution. To explore further the relationships between the cell LPL content and the dilution-induced phenomenon, 3T3-F442A cells were first depleted of any activity and enzyme content by cycloheximide and heparin treatment, then allowed to restore their intracellular enzyme content after drug removal (4). Cell lysates were prepared at various times of the repletion phase and the effect of dilution on the apparent activity of LPL was determined. As shown in **Fig. 6**, the dilution factor required to determine accurately the total activity of LPL increased with the cell LPL content. After 15 and 60 min of repletion, 10% and 50% of the initial cell activity were recovered; this was sufficient to shift the dilution factor from 4 to 64. Interestingly enough, the highest value corresponded to that determined for untreated control cells. Since there was no change in the concentration of cell lysates in these experiments (1.2 mg of protein per ml or 1.4×10^6 cells per ml), the results strongly suggest that LPL concentration itself, which is the only variable parameter, is directly responsible for the magnitude of the underestimation in the enzyme activity of the cells.

DISCUSSION

In this report we have analyzed extensively the conditions for determining LPL activity in cell lysates from mesodermal cells in culture as well as from LPL-containing tissues. The results show that, when using enzyme concentrations compatible with the proportionality of the assay, the enzymatic activity of LPL from cellular origin is determined with a systematic underestimation. No

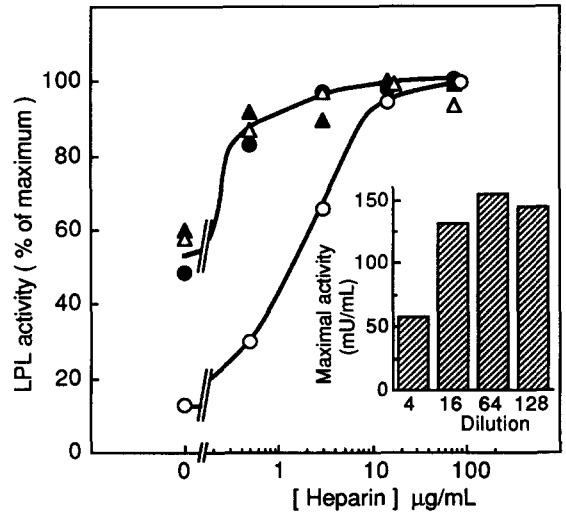

Fig. 5. Effect of heparin on the apparent activity of cell LPL. Membranes of the postnuclear supernatant from 9-day post-confluent 3T3-F442A cells were prepared and lysed as described in Materials and Methods. LPL activity was then assayed in the detergent-depleted phase after a 4-(○), 16-(●), 64-(△), or 128-fold (▲) dilution in buffer A containing heparin at the indicated concentrations. Results are expressed as percent of the maximal value obtained at each dilution for the apparent activity of cell LPL. Inset: maximal apparent activity of cell LPL at various dilutions. Activities (expressed as mU/ml for the four dilutions of initial sample after correction for dilution) define 100% in each of the four curves in the main figure, respectively.

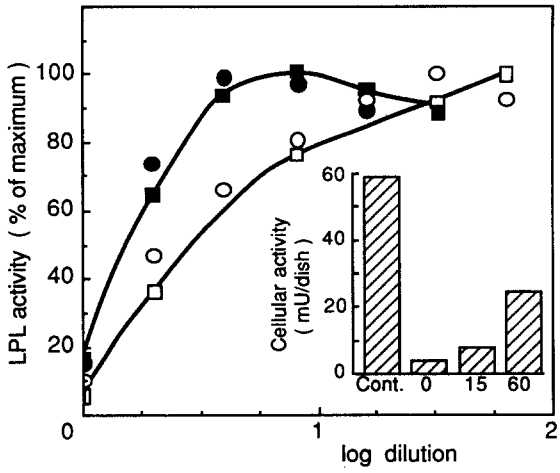

Fig. 6. Effect of intracellular LPL content on its apparent enzymatic activity. 3T3-F442A cells in 60-mm dishes were used at day 7 after confluence. Cells were first exposed at 37°C to the differentiation medium containing 15 μM cycloheximide and 3 μg/ml heparin. After 2 h (time 0 of the curves), they were washed three times in fresh differentiation medium to remove both agents and to initiate the repletion of the intracellular stores of LPL (4). Cells were then maintained for up to 60 min under these conditions. Cell lysates were preapared as described in Materials and Methods from repleting cells at time 0 (●), 15 (■), and 60 min (○), and from control untreated cells (□). LPL activity was assayed after serial dilution with buffer A containing 3 μg/ml heparin. The apparent activity of cell LPL is plotted as a function of dilution and is expresssed as percent of the maximal value for each time point. Inset: maximal values of activities of cell LPL in control cells (Cont) and in repleting cells at time 0, 15, 60 min.

similar observation could be made when secreted LPL was assayed. Two sets of evidence support the view that this underestimation is a result of a limited access of substrate molecules to the catalytic site of the enzyme: *i*) the activity ratio of apoC-II-stimulated over basal triacylglycerol-hydrolase activities of LPL remains constant when both are assayed over a wide range of enzyme concentrations, demonstrating that concentration of apoC-II is not rate-limiting in the assay; and *ii*) no inhibition of LPL from cell lysates, either by cell components (unknown inhibitor(s) or lipids), or by detergent molecules necessarily present in the cell lysates to disrupt membrane structures (4), could be observed.

This underestimation in the determination of LPL activity in cell lysates leads in turn to an erroneous determination of the actual size of the intracellular pool of active enzyme. The present results demonstrate that this can be overcome by merely performing the enzymatic assay in highly diluted samples. Under these conditions only, the actual activity present in these lysates, defined by the maximal and constant value obtained by serial dilution, can be accurately known. This conclusion is supported by the fact that in 3T3-F442A cells the actual activity of the cell enzyme is equal to the activity of LPL pool that is released under heparin stimulation in the absence of protein synthesis (Table 1). Our previous studies on LPL secretion had demonstrated that the accurate determination of this releasable activity could be made using a continuous flow method in order to take into account the inactivation of LPL (16). Recently, we have provided compelling evidence for LPL activity being cryptic within the cell, whereas the existence of a stored inactive precursor form of the enzyme in 3T3-F442A cells was excluded (17). In agreement with these conclusions, the present results indicate that the additional effects of dilution and heparin in unmasking enzyme activity are not confined to 3T3-F442A cells. These conclusions have been extended to primary cultures of skeletal muscle cells, heart muscle cells, and stromal-vascular cells of adipose tissue from rat (Table 1). Importantly enough, this phenomenon also occurs with skeletal and cardiac muscle and adipose tissues, thus favoring the conclusion that LPL has the general status of a cryptic enzyme. The physiological implications of this statement are obvious and should lead to a reevaluation of the pool size of potentially active LPL in these various tissues by assaying cell LPL under proper conditions. Clearly, under such conditions, catalytically active LPL molecules present in the different compartments of the tissues are all assayed. It is thus concluded that LPL molecules present at the surface of endothelial cells and those already secreted, which are not likely to exhibit an increase of activity upon dilution (Fig. 1c), should represent a low proportion of the total LPL molecules. If it were not so, the dilution-induced phenomenon should not be observed. However, this conclusion might depend upon the nutritional status of the animals. For instance, the proportion of active LPL molecules present at the endothelial cell surface could be increased post-prandially, whereas the proportion of potentially active (cryptic) LPL molecules could be decreased. In that case, it is predicted that dilution-induced phenomenon could be of lower magnitude.

Although the relationships between intracellular transport and functional maturation have received less attention in muscle and heart than in adipocytes, the present findings strengthen our previous proposals (17). First, it is suggested that the existence of a putative reservoir of inactive LPL precursor as well as a secretion-coupled activation of LPL are unlikely, in contrast to proposals made several years ago (22, 23). This conclusion is supported by the fact that nearly identical activities are present both in the secretion medium of cycloheximide-heparin-treated cells, in which LPL degradation is abolished, and in the cells before drug treatment. Should this putative reservoir of inactive LPL be present, then the secreted activity would be significantly higher than the cell activity, and this is not observed. However, it cannot be ruled out that heparin could stimulate the secretion of active LPL molecules only and that inactive LPL molecules of this putative reservoir would escape detection by activity measurements; this has clearly been shown not to be the case in 3T3-F442A cells (17). Second, in Golgi-derived vesicles

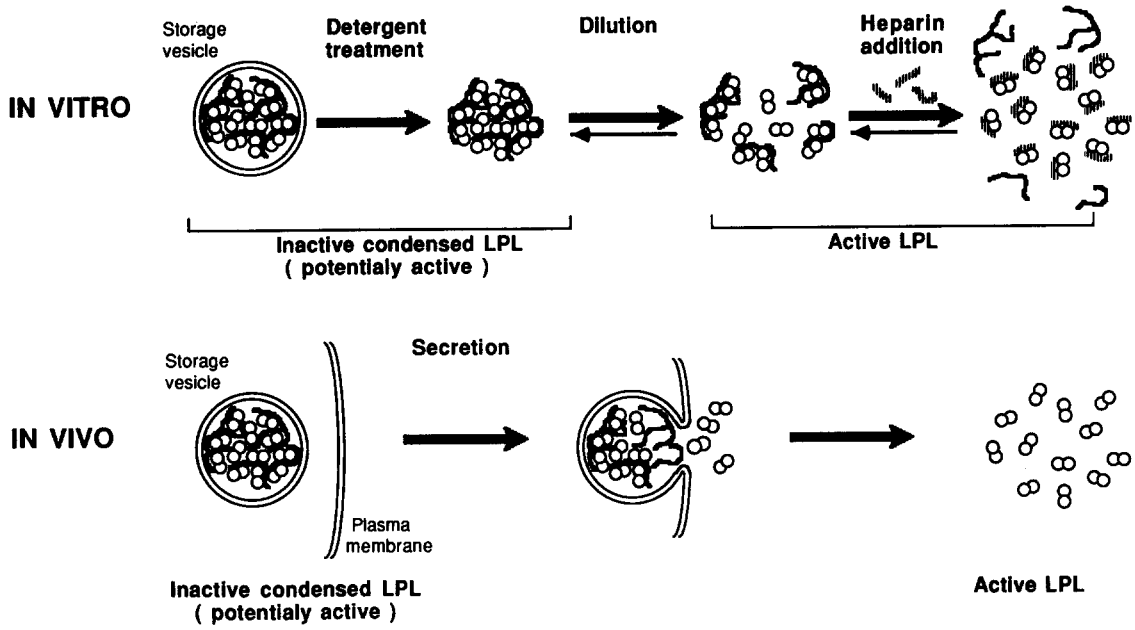

Fig. 7. Scheme illustrating the hypothesis of the condensation of cell LPL. Three kind of molecules are represented: LPL subunits associated as homodimers (white circles), sulfated glycosaminoglycans (black thick lines), and heparin (dashed thick lines). The upper part of the figure summarizes the sequence of events that leads in vitro to the "activation" of LPL upon solubilization of binary condensates that may be formed within an intracellular compartment. The lower part shows the exocytosis of LPL in vivo. Heparin (or related molecules) may or may not be involved in this latter process and is not represented.

where they are stored, potentially active LPL molecules (4) are very likely present under a condensed or aggregated form. A recent ultrastructural study shows in mouse myocytes the presence of LPL molecules within membrane structures only, including Golgi stacks, transport vesicles, and secretion vesicles (24), in agreement with its localization in adipose cells as seen by immunofluorescence microscopy (4, 5). All available data obtained with cells maintained in a defined hormonal environment allow the tentative model for LPL storage within intracellular vesicles to be proposed (**Fig. 7**). In this model it is assumed that, during their packaging into vesicles of the regulated pathway (17), LPL molecules interact with sulfated glycosaminoglycans or proteoglycans. Presumably this kind of heterophilic combination, which may take place in the *trans* Golgi and *trans* Golgi network (25, 26), results in the condensation of enzyme molecules into aggregates. Two lines of evidence support this view. *i*) As indicated by the present work, the progressive accumulation of LPL within the cell by itself constitutes the factor responsible for masking functional catalytic sites. This suggests that, at some intracellular level, the crypticity of activity is acquired via an enzyme concentration-dependent process. *ii*) The concentration of heparin that is effective in unmasking enzymatic activity increases as a function of that of LPL in lysates. This finding, coupled to the absence of any direct stimulatory effect of the sulfated glycosaminoglycan on the catalytic efficiency of the enzyme, can be explained merely by the displacement, equivalent to a solubilization process, of LPL molecules from their binding sites within the aggregate. In agreement with this interpretation of a direct interaction of LPL with intravesicular binding sites is the fact that heat-inactivated LPL is able to mimic heparin (17). In agreement with the predictions of the model, the results show that disrupting the vesicle membrane with detergent does not bring any increase in LPL activity. In vitro, by a mere dilution in the presence of heparin, the complete recovery of LPL homodimers can be obtained as a consequence of both the mass action law and the disruption of interactions occurring between LPL molecules and the sulfated glycosaminoglycans. This might well correspond to the in vivo situation where exocytosis, i.e., going from a small intravesicular volume to a large extracellular volume, would lead to an equivalent solubilization of LPL molecules, therefore explaining the apparent "activation" of LPL. Nevertheless, the model indicates that, in vitro, by virtue of its affinity for the enzyme, heparin remains bound to free active LPL homodimers (2). Regarding this point, depending upon the animal species, LPL has been reported to be active as a homodimer (13, 27, 28) or even as a monomer (29); it is of interest that heparin is able to decrease very significantly the rate of inactivation of LPL (2). It is tempting to speculate that this stabilization would be obtained by means of both the formation of a dimeric form and the interaction of this active form with sulfated glycosaminoglycans; whether such an interaction does take place in vivo is not known. It cannot be excluded that heparin-like molecules secreted by endothelial cells could play such a role in vivo.

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