The Low Density Lipoprotein Receptor-related Protein/α2-Macroglobulin Receptor Binds and Mediates Catabolism of Bovine Milk Lipoprotein Lipase*

David A. Chappell†‡, Glenna L. Fry†, Michelle A. Wakin‡‡, Per-Henrik Iverius§, Suzanne E. Williams¶**, and Dudley K. Strickland||

From the †Department of Internal Medicine, University of Iowa College of Medicine, Iowa City, Iowa 52242, the ‡Department of Internal Medicine, Veterans Affairs Medical Center, University of Utah, Salt Lake City, Utah 84148, and the §Biochemistry Laboratory, American Red Cross, Rockville, Maryland 20855

Lipoprotein lipase (LPL), the major lipolytic enzyme involved in the conversion of triglyceride-rich plasma lipoproteins to remnants, was found to compete with binding of activated α2-macroglobulin (α2M) to the low density lipoprotein receptor-related protein (LRP)/α2-macroglobulin receptor. Bovine milk LPL displaced both 125I-labeled α2M and 39-kDa α2M receptor-associated protein (RAP) from the surface of cultured mutant fibroblasts lacking LDL receptors with apparent $K_d$ values at 4 °C of 6.8 and 30 nM, respectively. Furthermore, LPL inhibited the cellular degradation of 125I-α2M at 37 °C. Because both α2M and RAP interact with LRP, these data suggest that LPL binds specifically to this receptor. This was further supported by observing that an immunoprecipitation-polyclonal antibody against the cytoplasmic carboxyl-terminal 11 amino acids of LRP conjugated to keyhole limpet hemocyanin (KLH) thought that lipoprotein remnant clearance occurs via a specific receptor that is distinct from the LDL receptor (4). The identity of the remnant receptor remains unknown. However, recently the low density lipoprotein receptor-related protein (hereafter referred to as LRP), which is identical to the α2-macroglobulin (α2M) receptor (5, 6), became a candidate for a remnant receptor because of its high sequence homology to the LDL receptor (5) and its ability to mediate cellular catabolism of apoE-enriched β-migrating very low density lipoproteins (β-VLDL); these are abnormal, remnant-like particles produced in cholesterol-fed rabbits (7).

Strickland et al. (6) discovered that LRP is identical to the α2M receptor. This was a surprising observation because no previous relationship between α2M and lipoprotein metabolism was suspected. α2M can be activated to bind LRP by a variety of proteases and primary amines (8). When bound to its receptor, activated α2M (α2M*) is known to enter cells via clathrin-coated pits and undergo degradation in lysosomes (9, 10). In addition to α2M* and apoE-enriched β-VLDL, other ligands for LRP include the 39-kDa receptor-associated protein (RAP) and Pseudomonas exotoxin A (6, 11). The 39-kDa RAP is particularly important because it is cosynthesized and copurifies with LRP and thus appears to act as an endogenous cellular ligand that can inhibit binding of other molecules to LRP (12-14).

Recently, the possibility that LPL might be involved in lipoprotein remnant recognition by LRP was suggested by Beisiegel et al. (15). These investigators chemically cross-linked 125I-LPL to a protein resembling LRP in size in intact cells. Also, addition of LPL to incubation media markedly increased surface binding of β-VLDL by cells at 4 °C (15). The effect of LPL on β-VLDL binding was observed in mutant fibroblasts that could not express LDL receptors but which expressed LRP normally. In the current studies, we investigated the ability of LPL to bind and compete for ligands binding to LRP both on cultured fibroblasts lacking LDL receptors and in a solid-phase assay using highly purified LRP.

**EXPERIMENTAL PROCEDURES**

Proteins—Human α2M was purified by polyethylene glycol precipitation of plasma followed by zinc chelate and size-exclusion chromatography as described (14). α2M was activated by incubation in buffer containing 200 mM methylamine as described (14). LRP was isolated from human placenta by affinity chromatography (16) followed by heparin-Sepharose chromatography to remove the 39-kDa RAP which copurifies with LRP. A rabbit polyclonal antibody to purified LRP was prepared and isolated by affinity chromatography over LRP conjugated to Sepharose as described (11). Another rabbit polyclonal antibody against the cytoplasmic carboxyl-terminal 11 amino acids of LRP conjugated to keyhole limpet hemocyanin (KLH)
was used as a control. The peptide comprised of the carboxyl-terminal 11 amino acids of LRP was synthesized by a t-Boc-benzyl protection strategy using a T-bag method and conjugated to KLH as described (5, 17). The 39-kDa RAP used in ligand binding assays was produced as a fusion protein with glutathione-S-transferase (GST) in an E. coli expression system utilizing human placental RAP cDNA (14). In initial studies, the intact fusion protein (RAP-GST) was used (M, 70,000). Subsequently, thrombin digestion at a cleavage site in the fusion protein was performed to generate recombinant RAP (M, 39,000) (14).

LPL was isolated by heparin-Sepharose chromatography of bovine milk as described (18) and stored at −20 °C in buffer containing 10 mM sodium phosphate (pH 7.4) and 50% glycerol. Because native LPL is dimeric (M, 101,000) it was used in calculations of binding constants (20). Human apoCII, which activates LPL's enzymic activity (1), was saturated with either apoCIII (Mr < 10,000) or phenylalanine (Mr > 240,000) for exclusion and ion-exchange chromatography (21). Human LDL (d = 1.02−1.05 g/ml) and VLDL (d < 1.006 g/ml) were isolated from normal subjects as described (22). Proteins were iodinated to specific activities of 1,000 to 3,000 cpm/ng using IODOBEADS (Pierce Chemical Co.) or ENZYM-BeaDS (Bio-Rad) as described (14, 23).

Free l25I was removed from l25I-RAP-GST by dialysis at 4 °C in buffer containing 10 mM sodium phosphate (pH 7.4) and 50% glycerol.

Binding Assays—Mutant skin fibroblasts that are incapable of expressing LDL receptors (24) were purchased from the NIGMS Human Genetic Mutant Cell Repository (GM00486A), Camden, NJ. In DNA transfection experiments, normal human foreskin fibroblasts or NRK cells purchased from the American Type Culture Collection, Rockville, MD were used. Cells were grown to confluence in 25- or 35-mm plastic wells, and binding of l25I-ligands to cells at 4 °C for 3 h or 37 °C for 5 h was determined using previously described methods (25). Some binding assays at 37 °C were performed in the presence of lipoprotein-deficient serum (LPDS) at 4 mg of protein/ml, whereas other assays at 37 °C and all assays at 4 °C were performed in buffer containing 4 mg/ml BSA. Surface-bound ligands were measured using a modification of the method of Van Leuven et al. (26) as radioactivity released by incubation of cells for 1 h at 4 °C in phosphate-buffered saline (pH 7.4) containing 50 μg/ml trypsin (GIBCO), 50 μg/ml proteinase K (Sigma), and 5 mM sodium EDTA. During this incubation, the cells detached from the well. The detached cells were pelleted in microfuge tubes by centrifugation for 90 s, washed once in protein-free buffer, and recentrifuged. Radioactivity in the supernatant fluid and the cell pellet was measured separately. This method, which gave results comparable to those using 10 mg/ml heparin for 125I-LPL release, was used because heparin did not adequately displace surface-bound 125I-α1-M* (data not shown). Degraded ligands were measured as trichloroacetic acid-soluble radioactivity.

RESULTS

Mutant fibroblasts that are incapable of expressing LDL receptors were used so that data interpretation would not be confounded by the possibility that binding to LDL receptors contributed to the results. Fig. 1A shows that bovine milk LPL displaced 125I-α1-M* from the surface of fibroblasts at 4 °C; the average Kd for two separate experiments was 6.8 nM. In control assays, LPL displaced 125I-α1-M* bound with a Kd of 0.37 nM (data not shown). Qualitatively similar results were obtained when the ability of LPL to inhibit 125I-α5-M* binding to normal fibroblasts or NRK cells was studied (data not shown). LPL also inhibited cellular degradation of 125I-α2-M* at 0.7 nM with an average EC50 of 15 ± 1.3 nM in three separate experiments of which Fig. 1B is representative. In control experiments, neither normal LDL nor VLDL at concentrations up to 100 μg of protein/ml inhibited binding or degradation of 125I-α2-M* (data not shown).

The assay in Fig. 1B was performed in the presence of LPDS at 4 mg of protein/ml. Preparation of LPDS involved activation of coagulation with thrombin which is also known to activate α1M (29). LPDS was heat-inactivated prior to use but, nevertheless, could have contained thrombin-activated α1M. In addition, LPDS contains apoCII, the cofactor for LPL's enzymic activity (1). However, results obtained when BSA was substituted for LPDS in the incubation buffer were similar (data not shown) which suggests that neither α1M nor apoCII present in LPDS affected the outcome of the assay. Also, addition of purified apoCII in 5-fold molar excess to the LPL concentration did not affect LPL binding affinity at 4 °C (data not shown).

LPL displaced 125I-RAP-GST from the surface of fibroblasts lacking LDL receptors (Fig. 2A); the average Kd from two separate experiments at 4 °C was 30 nM. Fig. 2B shows the ability of various proteins to compete for 125I-LPL binding. By homologous ligand displacement, LPL bound with an average Kd of 11 nM (n = 2). RAP-GST competed for 125I-LPL binding with an average Kd of 2 nM (n = 2) which is comparable to the affinity of RAP for purified LRP (14). In contrast, α5M, native α1M (not shown), and GST competed poorly (Fig. 2B). RAP-GST also inhibited degradation of 125I-LPL at 37 °C (data not shown). Thus, LPL and RAP appeared to compete for the same cell-surface-binding sites. As dis-
was bound to sites that were not blocked by the antibody. In contrast to LRP, we studied its binding to highly purified receptors using a solid-phase assay. Fig. 3, the amounts corresponding to 100% for surface binding, uptake, and degradation of 125I-LPL were 64, 65, and 55 ng/well, respectively, and 3.0, 1.5, and 14 ng/well, respectively, for 125I-a2M. Thus, relative to the amounts of the ligands degraded, much more 125I-LPL was present on the surface or intracellularly than was the case for 125I-a2M. The possibility that binding to slowly internalized cell-surface proteoglycans contributed to 125I-LPL catabolism is addressed under “Discussion.”

In other experiments, denaturation of 125I-LPL by boiling for 30 min reduced degradation by about 10-fold as compared to native LPL. However, the degradation remaining could still be blocked by the antibody to LRP. Also, denaturation reduced but did not eliminate the ability of LPL to compete for 125I-a2M degradation (data not shown). Qualitatively similar results to those presented above were obtained in other assays in which lower antibody concentrations were tested (data not shown).

To more definitively establish that LPL bound specifically to LRP, we studied its binding to highly purified receptors using a solid-phase assay. Fig. 4A shows that 125I-LPL exhibited high affinity, saturable binding to LRP-coated wells at 4°C in contrast to binding to BSA-coated wells. The average Kd from three separate assays was 18 ± 5 nM which is close to the value of 11 nM obtained in cell-surface assays. a2M partially displaced 125I-LPL binding with a Kd at 4°C of 7 nM (Fig. 4B), whereas RAP partially displaced 125I-LPL with a Kd of 3 nM (Fig. 4C). Denaturation of LPL by boiling for 30 min reduced but did not destroy binding to LRP (data not shown).

**DISCUSSION**

Beisiegel et al. (15) showed that LPL can be cross-linked in intact cells to a protein resembling LRP in size. The current studies establish that LPL binds directly to LRP and further indicate that LPL can enter cells and be degraded via an LRP-mediated process. LPL inhibits binding to cell surfaces by two previously described ligands for LRP, a2M, and RAP and competes for intracellular degradation of a2M. Degradation of LPL can be blocked by a polyclonal antibody against LRP. Furthermore, LPL displays saturable, high affinity binding to purified LRP in a solid-phase assay. Denaturation of LPL by boiling reduces but does not destroy its binding to LRP. Several important aspects of the binding interaction remain to be determined, including the binding stoichiometry and the sites of molecular contact.

It appears that LRP contributes only a fraction of the cell-surface-binding sites available to LPL. Although LPL can displace a2M binding to cells, the reverse is not true despite the fact that a2M can partially compete for LPL binding to purified LRP in a solid-phase assay (see Figs. 1A, 2B, and 4B). Further evidence that binding of LPL to LRP is only a portion of the total LPL bound to the cell surface comes from studies using a polyclonal antibody to LRP. The majority of intracellular degradation of LPL can be blocked by the antibody without significantly diminishing surface binding or uptake (Fig. 3). Also, unlike a2M, 39-kDa RAP can completely displace cell-surface binding of LPL and vice versa (Fig. 2). Apparently, LPL and RAP bind cell-surface sites that cannot bind a2M.

One possible explanation for the data is that both LPL and RAP, which are heparin-binding proteins (1, 12, 30), bind to cell-surface proteoglycans under our assay conditions. If so, LPL and RAP might compete for proteoglycan attachment sites that cannot bind a2M. Preliminary, unpublished studies suggest that a2M does not bind heparin-Sepharose as strongly as does LPL. Also, digestion of fibroblasts with a mixture of heparinase and heparitinase can reduce LPL binding to the cell surface by up to 70% without affecting a2M binding.2

Detailed data on RAP and a2M binding to cell-surface proteoglycans are unavailable, but studies by other investigators indicate that LPL binds proteoglycans on endothelial cells with a Kd of 140–560 nM (31, 32). A 220-kDa proteoglycan on the surface of endothelial cells has been identified that mediates slow internalization of surface-bound LPL (28% in 1 h) (32, 33). Cheng et al. (31) estimated that there are 107 LPL-binding sites/endothelial cell; this is a large number in comparison to the 106 high affinity sites/cell (Kd = 0.2 nM) and 6 × 104 low affinity sites/cell (Kd = 100 nM) for a2M binding to fibroblasts (34). In consideration of the currently available data, it is plausible that binding of LPL to both cell-surface proteoglycans and LRP is important in determining...
the catabolic fate of LPL in cells. Conceivably, proteoglycans are involved in presenting LPL to LRP. This scheme is analogous to the recently described interaction of urokinase/plasminogen activator inhibitor type-1 complexes with LRP in which the complexes appear to first bind urokinase-type plasminogen activator receptors, and then bind to LRP (35).

A major, unanswered question is whether LPL bound to triglyceride-rich lipoproteins in vivo can provide a recognition site for lipoprotein clearance via hepatic receptors as originally proposed by Felts et al. (36) or, more specifically, via LRP as suggested by recent data. Normally, LPL circulates in the picomolar range in plasma and almost exclusively bound to lipoproteins (37, 38). After intravenous heparin, LPL's concentration increases about 10-fold into the nanomolar range (37), although still somewhat lower than the $K_D$ of LPL binding to LRP at 4°C (18 nM). In vivo, LPL is rapidly cleared by the liver, which is also the major site for LPL's concentration increases about 10-fold into the nanomolar range (37), although still somewhat lower than the $K_D$ of LPL binding to LRP at 4°C (18 nM). In vivo, LPL is rapidly cleared by the liver, which is also the major site for lipoprotein uptake into cells. Although also potentially relevant to lipoprotein clearance, the present studies did not test LPL binding to LDL receptors or whether hepatic lipase, another lipolytic enzyme with major effects on lipoprotein catabolism (1), might also bind to LRP. Further studies are needed to address these possibilities.

Acknowledgments—We thank Arthur Spector and Larry Tobacman for helpful discussions. We acknowledge the Protein Structure Facility at the University of Iowa College of Medicine for synthesis of the peptide comprising the carboxy-terminal 11 amino acids of LRP.

REFERENCES

1. Nilsson-Ehle, P., Garfinkel, A. S., and Schotz, M. C. (1980) Annu. Rev. Biochem. 49, 667-698

2. Bergman, E. N., Havel, R. J., Wolfe, B. M., and Bohmert, T. (1971) J. Clin. Invest. 50, 1831-1839

3. Steichenhofer, F. H., Malloy, M. J., Kane, J. P., and Havel, R. J. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 1838-1843

4. Kita, T., Goldstein, J. L., Brown, M. S., Watanabe, Y., Hornick, C. A., and Havel, R. J. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 3063-3067

5. Hara, J., Hansano, U., Rognes, S., Myklebost, O., Gaussevich, H., and Stanley, K. K. (1988) EMBO J. 7, 4119-4127

6. Strickland, D. K., Ashcom, J. D., Williams, S., Burgassa, W. H., Miliorni, M., and Argraves, W. S. (1990) J. Biol. Chem. 265, 17401-17404

7. Kowal, R. C., Herz, J., Goldstein, J. L., Esser, V., and Brown, M. S. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 5810-5814

8. Sottrup-Jensen, L. (1989) J. Biol. Chem. 264, 11539-11542

9. Williamman, M. C., Masicki Field, P. R., and Fustr, I. R. (1979) J. Cell Biol. 82, 614-625

10. Kaplan, J., and Nielsen, M. L. (1979) J. Biol. Chem. 254, 7223-7228

11. Konhans, M. Z., Morris, R. E., Thompson, M. R., Fitzgerald, D. J., Strickland, D. K., and Saelinger, C. E. (1992) J. Biol. Chem. 267, 12420-12425

12. Moestrup, S. K., and Gliemann, J. (1991) J. Biol. Chem. 266, 14011-14017

13. Reina, M., Rossl, J., Loko, D. K., Ho, Y. K., and Brown, M. S. (1991) J. Biol. Chem. 266, 21232-21238

14. Williams, S. E., Ascho, J. D., Argraves, W. S., and Strickland, D. K. (1992) J. Biol. Chem. 267, 9035-9040

15. Beissig, U., Weber, W., and Bengtsson-Olivecrona, G. D. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 8342-8345

16. Ashcom, J. D., Tiller, S. E., Dickerson, K., Craven, J. L., Argraves, W. S., and Strickland, D. K. (1990) J. Cell Biol. 110, 1041-1048

17. Houghton, R. A., DeGrave, S. T., Bray, M. K., Hoffman, S. R., and Frizzell, N. D. (1986) BioTechniques 4, 521-535

18. Van Roon, L. H., and Oosten-Lindquist, M. A. (1986) J. Biol. Chem. 251, 7791-7795

19. Garfinkel, A. S., Kemenyi, E., Ben-Zeev, O., Nikazy, J., James, S. J., and Schotz, M. C. (1983) J. Lipid Res. 24, 771-780

20. Wion, K. L., Kirchgeissner, T. G., Losic, A. J., Schrott, M. C., and Lawr, M. B. (1987) Science 235, 1638-1641

21. Jackson, R. L., and Toddsworth, G. (1996) Methods Enzymol. 128, 286-297

22. Havel, R. J. Ed. H. A., and Bradman, J. H. (1965) J. Clin. Invest. 34, 1324-1332

23. Markwell, M. K. (1982) Anal. Biochem. 128, 472-478

24. Hoib, H. H., Brown, M. S., Russell, D. W., Davidson, J., and Goldstein, J. L. (1987) N. Engl. J. Med. 317, 734-747

25. Chappell, D. A., Fry, G. L., Warknitz, M. A., and Berns, J. J. (1992) J. Biol. Chem. 267, 270-279

26. Van Roun, L. H., Rasmussen, J. J., and Van Den Bergh, G. (1979) J. Biol. Chem. 1015, 5155-5160

27. Mswon, P. J. (1988) Methods Enzymol. 92, 543-576

28. Deoane, D. L., and Mwnon, P. J. (1976) Am. J. Physiol. 235, E97-E102

29. Steiner, P., Bhattacharyya, P., and Strickland, D. K. (1966) Biochemistry 24, 293-3001

30. Strickland, G. K., Ashcom, J. D., Williams, S., Batfe, E., Beire, E., McTighe, K. J., Batfe, J. P., and Argraves, W. S. (1991) J. Biol. Chem. 266, 13364-13369

31. Chen, C. F., Oxana, G. M., Benedouin, A., and Rosenberg, R. D. (1986) J. Biol. Chem. 261, 12988-12998

32. Saxena, U., Klein, M. G., and Goldberg, J. L. (1999) J. Biol. Chem. 266, 12680-12685

33. Saxena, U., Klein, M. G., and Goldberg, J. L. (1991) J. Biol. Chem. 266, 17018-17021

34. Diekman, B. R., Willingham, M. C., and Pastan, I. (1981) J. Biol. Chem. 256, 3454-3456

35. Nygren, A., Peterson, C. M., Miller, B., Johnson, P. H., Moestrop, S. K., Blute, T. L., Eberoll, M., Thersen, H. C., Munch, M., Andreasen, P. A., and Gliemann, J. (1982) J. Biol. Chem. 267, 14543-14546

36. Felts, J. M., Rube, K., and Crane, T. (1976) Biochem. Biophys. Res. Commun. 66, 1477-1483

37. Kern, P. A., Martin, R. A., Carty, J., Goldberg, I. J., and Ong, M. J. (1990) J. Lipid Res. 31, 17-26

38. Goldberg, I. J., Kandel, J. J., Blum, C. B., and Ginsberg, H. N. (1986) J. Biol. Chem. 261, 1525-1530

39. Willrans, L., Peterson, G., Olivecrona, G., and Bengtsson-Olivecrona, G. (1985) Biochem. Biophys. Acta 795, 513-524

40. Fuch, A., Shifman, M. A., and FCsso, S. V. (1982) Biochim. Biophys. Acta 716, 151-157

41. Vilaro, S., Ramirez, I., and Llobera, M. (1986) Biochem. J. 236, 273-278