The Binding Activity of the Macrophage Lipoprotein(a)/Apolipoprotein(a) Receptor Is Induced by Cholesterol via a Post-translational Mechanism and Recognizes Distinct Kringle Domains on Apolipoprotein(a)*

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George A. Keesler‡, Brent R. Gabel‡, Cecilia M. Devlin‡, Marlys L. Koschinsky§†, and Ira Tabas¶

From the ¶Departments of Medicine, and Anatomy & Cell Biology, Columbia University College of Physicians and Surgeons, New York, New York 10032 and the ¶Department of Biochemistry, Queen’s University, Kingston, Ontario K7L 3N6, Canada

Elevated plasma levels of lipoprotein(a) (Lp(a)) can be a risk factor for atherosclerosis, and the interaction of Lp(a) with cholesterol-loaded macrophages (foam cells) in atheromata may be important in Lp(a)-induced atherogenesis. We have previously shown that cultured macrophages are loaded with cholesterol, they acquire the ability to internalize and lysosomally degrade Lp(a) via interaction between a novel cell-surface receptor activity and the apolipoprotein(a) (apo(a)) moiety of Lp(a). Herein we explore the cell-surface binding of recombinant apo(a) (r-apo(a)) by foam cells. Whereas the induction of degradation of r-apo(a) by cholesterol loading of macrophages depended on new protein synthesis, the induction of binding of r-apo(a) did not. Furthermore, J774 macrophages bound r-apo(a) in a cholesterol-regulatable and specific manner but degraded r-apo(a) poorly. Thus, the binding and internalization/degradation functions of the receptor activity are distinct. To explore which domains on r-apo(a) interact with the foam cell receptor, we conducted a series of competitive and direct binding and degradation experiments using 12 r-apo(a) constructs that differed in their content of specific kringle subtypes. These data, as well as complementary data with anti-apo(a) monoclonal antibodies, indicated that the region centered around kringle type IV, subtypes 6-7 (KIV_6-7) is important in receptor binding. Remarkably, a cholesterol-induced receptor activity with similar structural specificity was also found on Chinese hamster ovary cells. In conclusion, the foam cell Lp(a)/apo(a) receptor consists of a cholesterol-regulatable binding activity and a short-lived component necessary for internalization or lysosomal degradation; the binding activity interacts with a distinct region of apo(a) that is different from that involved in competition for plasminogen binding.

Lp(a) is an LDL-like lipoprotein in which the apoB-100 moiety of LDL is covalently attached to a glycoprotein called apo(a) (1, 2). Apo(a) consists of multiple domains called kringle, which are regions of protein folds each stabilized by three disulfide bonds (3). Apo(a) shares 80% homology with another kringle-containing protein, plasminogen (3). Although the physiological role of Lp(a) is not known, elevated levels of this lipoprotein in certain human populations are often associated with increased risk for atherosclerotic coronary artery disease and stroke (4). Furthermore, Lp(a) transgenic mice (5, 6) and, in one report (7, cf. Ref. 8), apo(a) transgenic mice have been found to have accelerated atherosclerosis.

The mechanism of Lp(a)-induced atherosclerosis is not known. Several groups of investigators have postulated that the ability of Lp(a) to compete for plasminogen binding sites on cells is important in certain potentially atherogenic processes, such as decreased fibronectin (9–11) and increased smooth muscle cell proliferation (12). Another possible clue to the potential atherogenicity of Lp(a) comes from the observation that Lp(a) and apo(a) are often physically in contact with cholesterol-loaded macrophages (foam cells) (13), which are prominent components of atherosclerotic lesions (14–16). In previous work, we have demonstrated that mouse peritoneal and human monocyte-derived macrophages have a receptor activity that can bind, internalize, and lysosomally degrade Lp(a) (17, 18). Importantly, the receptor activity is induced by cholesterol loading (17) and down-regulated by interferon-γ (19), suggesting possible roles in atherosclerosis and inflammation. The receptor recognizes the apo(a) moiety of Lp(a) and interacts similarly with Lp(a) and a 17-kringle recombinant apo(a) construct (17, 18, 20). We have shown that this foam cell receptor is distinct from the LDL receptor, the scavenger receptor, the LDL receptor-related protein, and plasminogen receptors (17, 18); furthermore, antibodies against the macrophage MAC-1 receptor do not block interaction of foam cells with apo(a).2

To further elucidate how the foam cell Lp(a)/apo(a) receptor interacts with apo(a), the present study was designed to determine which sites on apo(a) bind to the receptor. By conducting a series of competitive and direct binding and degradation experiments using 12 recombinant apo(a) constructs and two

1 The abbreviations used are: Lp(a), lipoprotein(a); apo(a), apolipoprotein(a); BSA, bovine serum albumin; CHO, Chinese hamster ovary; DMEM, Dulbecco’s modified Eagle’s medium; K, kringle; LDL, low density lipoprotein; LPDS, lipoprotein-deficient serum; MOPS, 3-(N-morpholino)propanesulfonic acid; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; r-apo(a), recombinant apo(a); TBS, Tris-buffered saline; VLDL, very low density lipoprotein; bp, base pairs.

2 G. A. Keesler and I. Tabas, unpublished data.
anti-apo(a) monoclonal antibodies, we show that the region centered around KIV_{6-7} is important in receptor binding. Furthermore, additional studies revealed that the receptor consists of distinct binding and internalization/degradation activities. Finally, we took advantage of a CHO cell line that could be cholesterol loaded to show that this cell type also has a cholesterol-inducible receptor that recognizes the KIV_{6-7} region of apo(a).

EXPERIMENTAL PROCEDURES

Materials—The Falcon tissue culture plasticware used in these studies was purchased from Fisher. Tissue culture media and reagents were obtained from Life Technologies, Inc., and fetal bovine serum was purchased from Gemini Bioproducts (Calabasas, CA). Lipoprotein-deficient serum (LPDS) was prepared from fetal calf serum by preparative ultracentrifugation (density, 1.21 g/ml). Na\textsuperscript{125}I (carrier free) was obtained from New England Nuclear, by immunoblot analysis, but neither had been further characterized before this report.

Antibodies and Immunoaffinity Chromatography—Monoclonal antibody 12C11 raised against the apo(a) moiety of human Lp(a) was purchased from Perimmune, Inc. (Rockville, MD). Monoclonal antibody 8B4 raised against human Lp(a) was generously provided by Dr. Gunther Fless, University of Chicago. These monoclonal antibodies were previously shown to recognize Lp(a) and apo(a), but not plasminogen. Two days prior to an experiment, 200 \mu g of Affi-Gel-10 matrix according to the manufacturer's instructions (Bio-Rad, Briefly, 200 \mu g of each antibody was reacted with 1 ml of Affi-Gel-10 in 100 mM MOPS buffer, pH 7.5, for 3 h at 4 \textdegree C on a rotating wheel. After 3 h, the matrix was centrifuged in a microcentrifuge and the supernatant was removed. All remaining active sites on the matrix were blocked by the addition of 100 mM ethanolamine, pH 7.4, by incubating for 1 h at 4 \textdegree C. Matrix not linked to antibody (control matrix) was made by incubating the Affi-Gel-10 with ethanolamine only. The control and antibody-conjugated matrices were then washed extensively with NET buffer (150 mM NaCl, 20 mM Tris-HCl, 1 mM EDTA, 1.0% Triton X-100, 0.1% SDS, pH 7.4). Each 125K-kringle construct (6.0 \mu g, 100–800 cpm/ng) was first incubated with control matrix for 1 h at 4 \textdegree C, after which the initial pre-clearing step, the matrix was pelleted, and aliquots of the supernatant, which contained greater than 90% of each of the 125I-recombiant kringle peptides, were incubated overnight at 4 \textdegree C with each of the two antibody-conjugated matrices. The matrices were then washed extensively in NET buffer with an SDS concentration of 1.0%, followed by several washes in NET buffer containing 1.5 mM NaCl. The matrices were re-equilibrated in the original NET buffer (0.1% SDS and 150 mM NaCl), and antibody-125K-kringle complex was dissociated by the addition of NET buffer containing 50 mM glycine HCl, pH 2.5; the eluate was neutralized using NaOH. Recovery of radioactivity during this procedure was greater than 90%.

Lipoproteins and Non-lipoprotein Cholesterol—LDL (density, 1.020–1.063 g/ml) from fresh human plasma and \beta-VLDL (density, >1.006 g/ml) from the serum of cholesterol-fed rabbits (22) were isolated by preparative ultracentrifugation (23). Acetylated LDL was prepared by treating LDL with acetic anhydride as described by Goldstein and Coughlin (24). All lipoprotein preparations were stored under argon at 4 \textdegree C and used within 4 weeks. Lp(a) was iodinated using the iodicmonochloride method, as described previously (17). Non-lipoprotein cholesterol was added to media from a 20 mg/ml stock in ethanol; the final concentration of cholesterol (also added to control wells) was 0.25%.

Cells—All cells were maintained in a 37 \textdegree C tissue culture incubator with a 5% CO\textsubscript{2} atmosphere, 77% air microbubbles. J774A.1 macrophages (25) and 16-mm dishes in DMEM containing 10% fetal calf serum as described (26). CHO-mSRAI cells, which were transfected with a murine macrophage scavenger receptor (class AI) cDNA (27), were generously provided by Dr. Monty Krieger (Massachusetts Institute of Technology). These cells were grown in monolayer culture in Ham's F12 medium containing 10% fetal bovine serum.

For the indicated others, the cells were washed twice with phosphate-buffered saline (PBS) 1 h after plating and then incubated for 2 days in DMEM, 10% LPDS alone or containing the indicated sources of cholesterol. Four hours before the beginning of the binding or degradation assay, the cells were washed twice with PBS and then incubated with DMEM containing 0.2% BSA in order to remove residual surface-bound lipid.

Cloning of Recombinant Apo(a) Constructs—The recombinant apo(a) (r-apo(a)) derivatives used to identify the domains in apo(a) that mediate binding to the macrophage receptor are shown schematically in Fig. 1. All of the apo(a) derivatives illustrated were assembled in the expression vector pRK5, which contains the cytomegalovirus (CMV) promoter and SV40 termination sequences (28). The construction and expression of r-apo(a) derivatives 12K, 6K, KIV\textsubscript{p}, KIV\textsubscript{p}+p, KIV\textsubscript{p}−p, and KIV\textsubscript{p}−p has also been described elsewhere (29). For the construction of (KIV\textsubscript{p})\textsubscript{b}, the cDNA clone \textsubscript{b} (3) was partially digested with BamHI. A fragment encoding four copies of kringle IV subdomain 5 was isolated and ligated into the single-site type II 2 expression construct (30) that had been digested with BamHI. An expression plasmid encoding apo(a) kringle IV subtype 10 followed by the kringle V and protease domain sequences was generated as follows: a construct encoding single kringle IV subtype 10 (30) was digested with Av\textsubscript{II}; the resultant 580-bp fragment was replaced with a 1,673-bp Av\textsubscript{II} fragment isolated from pRK5ha6(3), which contains the latter 22 bp of kringle IV subtype 10, followed by sequences encoding the kringle V and protease domains. The resultant expression construct was designated pRK5ha6V\textsubscript{p}−. An expression plasmid encoding apo(a) kringle IV subtypes 6–7 was constructed using a three-part ligation. A 735-bp EcoRI/AuI fragment (encoding the signal sequence followed by the complete sequence of KIV\textsubscript{p}−p (see above) with EcoRI and AuI. In order to obtain the latter 17 bp of kringle IV subtype 7 followed by a stop codon, we digested an expression plasmid encoding single kringle IV subtype 7 with AuI and SafI, the latter construct was used for assembling cationic oligonucleotides as described previously (30). The EcoRI/AuI fragment and the AuI/SafI fragment were ligated into pRK5 that had been digested with EcoRI and SafI; the resulting expression plasmid was designated pRK5ha6V\textsubscript{p}−.

For the construction of a plasmid encoding kringle subtypes 5–8, we utilized the pRK5ha6 construct, which has been previously described (29). We digested the latter plasmid with HindIII and AuI; this released a 1,509-bp fragment that encodes the latter 242 bp of kringle IV subtype 5, followed by the complete sequence of kringle IV subtype 6, and the first 325 bp of kringle IV subtype 7. In order to obtain the latter 17 bp of kringle IV subtype 7 followed by the complete sequence of kringle IV subtype 8 and a stop codon, we utilized PCR to generate a 385-bp fragment spanning this region; the 5’ PCR primer was designed to anneal to a sequence 5’ to the AuI site in kringle IV subtype 7, while the 3’ PCR primer was designed to anneal to the 3’ end of the kringle IV subtype 8 sequence and contained a stop codon and SafI restriction site. The HindIII/AuI fragment and the AuI/SafI-digested PCR product were ligated into pRK5ha6 that had been digested with HindIII and SafI; the resultant expression construct was designated pRK5ha6V\textsubscript{p}−.

In order to construct an expression plasmid encoding apo(a) kringle IV subtypes 6–8, we utilized a three-part ligation as follows. An expression plasmid encoding KIV\textsubscript{p}−p (see above) was digested with EcoRI and AuI, giving rise to a 735-bp fragment that encoded the signal sequence, followed by the complete sequence of KIV\textsubscript{p}−p and the first 325 bp of KIV\textsubscript{p}−p. In order to obtain the final 17 bp of KIV\textsubscript{p}−p, followed by the complete sequence encoding KIV\textsubscript{p}−p with a stop codon at the 3’ end, we digested the plasmid encoding KIV\textsubscript{p}−p with SmI and SafI. This fragment (380 bp) and the 735-bp EcoRI/AuI fragment (above) were ligated into pRK5 that had been digested with EcoRI and SafI. The final expression plasmid was designated pRK5ha6V\textsubscript{p}−.

Expression, Purification, and Oligodization of r-Apo(a) Derivatives—The apo(a) derivatives shown in Fig. 1 were used to stably transfect human embryonic kidney cells (293 cells) (31) by the method of calcium
phosphate co-precipitation (32). Briefly, 10 μg of each expression plasmid was co-transfected with 1 μg of plasmid encoding the neomycin resistance gene (33). Stable transfectants were selected by cultivating cells in the presence of the antibiotic G418 (800 μg/ml) (Life Technologies, Inc.) as described previously (20). Stable transfectants expressing the various apo(a) derivatives were identified by enzyme-linked immunoassorbent assay as described previously (29).

All of the apo(a) derivatives, with the exception of KIV_{1}, were purified by affinity chromatography on lysine-Sepharose (Pharmacia Biotech Inc.) columns (29). The apo(a) derivative (KIV_{2}) was purified by immunoaffinity chromatography using an anti-apo(a) polyclonal antibody, raised in rabbits (34), immobilized on Affi-Gel (see above). Briefly, conditioned medium (OptiMEM; Life Technologies, Inc.) from 293 cells expressing (KIV_{2}) was applied to a 5-ml immunoaffinity column, and the column was washed with 5 volumes of Tris-buffered saline (TBS; 25 mM Tris-HCl, pH 7.4, 135 mM NaCl), followed by 10 volumes of TBS containing 1 mM NaCl and final 5 volumes of TBS. Specifically bound protein was eluted with 100 mM glycine, pH 2.3, and associated 125I radioactivity was determined by gamma counting. The KIV_{1} subtypes, including eight copies of KIV2 (17 KIV domains) surface binding of 125I-r-apo(a) to macrophages and foam cells.

**Cholesterol-induced Lipoprotein(a)/Apolipoprotein(a) Receptor**

**FIG. 1. Construction of apo(a) expression plasmids.** The uppermost diagram, labeled 17K, illustrates the organization of the 17-kDa apo(a) (PRK5ha17), which was derived from the published apo(a) cDNA sequence (20). The organization of the apo(a) derivatives is shown relative to 17K. In all cases, open boxes are used to designate the kringle repeats of identical amino acid sequence (i.e., kringle IV subtype 2), and hatched boxes represent kringle units that contain amino acid differences relative to KIV_{2}; the 10 subtypes of kringle IV sequences are indicated above the 17-kDa kringle. The signal sequence is designated by the narrow closed box to the left of the first kringle IV of each derivative. The position of the free cysteine in apo(a) kringle IV subtype 9 is shown with a bar. The constructs designated 12K and 6K each contain a hybrid kringle, which represents a fusion of kringle IV subtype 1 and kringle IV subtype 2 (for the 12K derivative) or kringle IV subtype 1 and kringle IV subtype 5 (for the 6K derivative). Details of the constructions of all of the apo(a) expression plasmids are provided under “Experimental Procedures.”

The data depicted by the diagonal-hatched bars verify our previous results mentioned above, namely that 125I-17K degradation is induced in mouse peritoneal macrophage foam cells and that the induction is blocked by treatment of the foam cells with the protein synthesis inhibitor, cycloheximide (18). Binding of 125I-17K (cross-hatched bars) is also induced in these foam cells, but, remarkably, the induction of binding is not inhibited by cycloheximide treatment. These data suggest that the receptor consists of at least two distinct functional components: a binding component that is post-translationally induced by cellular cholesterol loading, and a relatively short-lived component that mediates internalization and degradation of the ligand. Note that the internalization and lysosomal degradation of 125I-acetylated LDL, which enters cells via the scavenger receptor (38), is not blocked by cycloheximide (18), indicating that cycloheximide is not simply a nonspecific inhibitor of receptor internalization or lysosomal degradation of endocytosed ligands.

In previous unpublished work, we made the intriguing finding that the J774 murine macrophage cell line, unlike mouse peritoneal and human monocyte-derived macrophages, degraded very little 125I-17K or 125I-Lp(a), and the degradation was not induced by cholesterol loading. This point was confirmed by the data depicted by the diagonal-hatched bars in Fig. 2B. At the initial time of this finding, we concluded that J774 macrophages lacked the Lp(a)/apo(a) receptor. Our new data, however, prompted us to examine 125I-17K binding to these cells. As shown by the cross-hatched bars in Fig. 2B,
binding of $^{125}$I-17K was substantial and was induced by cholesterol loading (cross-hatched bars). Furthermore, as with mouse peritoneal macrophages, cholesterol-mediated induction of binding was not inhibited by cycloheximide under conditions where protein synthesis was inhibited by $>95\%$. These data confirm the distinct natures of the binding and internalization activities of the receptor and suggest that J774 macrophages may be selectively lacking the internalization component.

The Foam Cell Lp(a)/Apo(a) Receptor Recognizes Distinct Kringles on Apo(a)—The first series of experiments to determine the receptor-binding site on apo(a) assayed the ability of several unlabeled r-apo(a) constructs to compete for $^{125}$I-17K binding to J774 macrophage foam cells. These constructs and their nomenclature are shown in Fig. 1. 12K includes a KIV1–KIV2 hybrid kringle, three full copies of KIV2, and one copy each of the remaining KIV subtypes (12 KIV domains in all), plus kringle V and the protease domain. 6K includes a KIV1–4 domains and differ in the number of the remaining KIV subtypes. The last five constructs shown in Fig. 1 have no KIV1–4 domains and differ in the number of the remaining KIV subtypes. The last four constructs in Fig. 1 contain only specific KIV subtypes and no KV or protease domains.

In contrast, the more truncated forms lacking KIV6 through KIV9, as well as the repeating KIV2 construct (KIV2$_r$), were relatively poor competitors or did not compete at all. In particular, the greatest loss of competitive inhibition was noted in constructs lacking KIV6 and KIV7. These data can be interpreted in two ways, either specific kringle domains in the area of KIV6–7 region are important for receptor binding or the total number of kringles is critical.

The goals of the next series of experiments were to confirm the competitive binding data in Fig. 3 with direct and competitive $^{125}$I-ligand degradation studies as well as to distinguish between the two interpretations of the data in Fig. 3 mentioned above. In Fig. 4A, unloaded mouse peritoneal macrophages, shown by the cross-hatched bars, and cholesterol-loaded mouse peritoneal macrophages (foam cells), shown by the solid bars, were incubated directly with $^{125}$I-labeled 6K in the absence or presence of excess unlabeled 6K or 17K and assayed for $^{125}$I-ligand degradation. Consistent with the competitive binding data in Fig. 3, $^{125}$I-6K was degraded by the macrophages, and degradation was induced 3-fold by cholesterol loading. Both unlabeled 6K or 17K competed well for receptor activity in the cholesterol-loaded macrophages. To determine the relative importance of specific kringles versus number of kringles, we tested the ability of unloaded macrophages and foam cells to degrade $^{125}$I-KIV$_{5–8}$ (Fig. 4B). The data clearly show that this 4-kringle construct was recognized by the foam cell receptor and was competed by both unlabeled ligands to compete for $^{125}$I-lipoprotein(a) degradation by foam cells: inhibition (relative to unloaded macrophages) by unlabeled Lp(a), 17K, and KIV$_{5–8}$ was 79.1, 74.6, and 63.8%, respectively. To further define the receptor-binding domain, we examined the interaction of macrophages with $^{125}$I-KIV$_{6–8}$ (Fig. 4C). Degradation of this ligand was induced greater than 7-fold by cholesterol loading. Although in this particular experiment the absolute level of degradation was less than that seen with $^{125}$I-6K or $^{125}$I-KIV$_{5–8}$, in another
experiment the levels of degradation between $^{125}$I-KIV$_6$-$8$ and $^{125}$I-17K were similar (data not shown). Furthermore, adding 20-fold excess unlabeled KIV$_6$-$8$ and 17K instead of 10-fold excess inhibited $^{125}$I-KIV$_6$-$8$ degradation to values similar to or less than the unloaded macrophages value (data not shown).

Consistent with the competitive binding data in Fig. 3, the degradation of $^{125}$I-KIV$_6$-$7$ (Fig. 6D) was considerably less than those of the three other ligands in Fig. 6, A-C; in addition, there was no statistically significant induction of $^{125}$I-KIV$_6$-$7$ degradation in foam cells, and unlabeled 17K failed to compete. Finally, $^{125}$I-(KIV$_2$)$_5$ was poorly degraded by macrophages or foam cells (Fig. 4E), also consistent with the competitive binding data in Fig. 3. Thus, the failure of some of the ligands to compete for receptor binding in Fig. 3 cannot be explained simply by their having a small number of kringles. From these data, we conclude that the foam cell receptor recognizes a domain in the KIV$_5$-$8$ region. Recall that deletion of KIV$_5$ or KIV$_8$ had only minor effects on the ability of r-apo(a) constructs to compete for receptor binding (Fig. 3). Thus, the data in Fig. 4 most likely indicate that the KIV$_6$-$7$ domain is most critical but needs to be presented to the receptor in the context of a larger sequence.

Given the importance of the data in Fig. 4, we conducted a set of experiments with a similar set of labeled and unlabeled ligands, except cell-surface binding was assayed. Fig. 5, A–C, shows that both $^{125}$I-6K and $^{125}$I-KIV$_6$-$8$, but not $^{125}$I-(KIV$_2$)$_5$, bound specifically and in a cholesterol-regulatable manner to J774 macrophages. Although there were trends toward slight specific binding of $^{125}$I-KIV$_6$-$7$ (Fig. 6D), none of the differences shown are statistically different. Thus, these binding data are entirely consistent with the mouse peritoneal degradation data in Fig. 4, and, as expected, similar cell-surface binding data were obtained with mouse peritoneal macrophages (not shown). Furthermore, consistent with the data in Fig. 2A, the

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**Fig. 3.** Ability of various r-apo(a) constructs to competitively inhibit the binding of $^{125}$I-17K by cholesterol-loaded J774 macrophages. Monolayers of J774 macrophages were incubated for 2 days in DMEM, 10% LPDS containing 25 μg of acetyl-LDL/ml. The cells were then washed twice with PBS, incubated at 37 °C for 4 h in DMEM, 0.2% BSA, washed again with PBS, and cooled on ice for 30 min. The 3-h binding assay was conducted using 10 nM $^{125}$I-17K alone (No Competitor) or in the presence of the indicated unlabeled ligands. The amount of unlabeled ligand used was 100 nM based upon number of KIV domains relative to $^{125}$I-17K, as follows: (17 = the number of KIV domains in the unlabeled competitor) × 100 nM (e.g. 100 nM unlabeled 17K and 1.7 μM KIV$_{10}$ were used).

**Fig. 4.** Degradation of various $^{125}$I-r-apo(a) constructs by unloaded and cholesterol-loaded mouse peritoneal macrophages. Monolayers of mouse peritoneal macrophages were incubated for 2 days in DMEM, 10% LPDS alone (cross-hatched bars) or containing 25 μg of acetyl-LDL/ml (solid bars). The cells were then washed twice with PBS, incubated at 37 °C for 4 h in DMEM, 0.2% BSA, washed again with PBS, and cooled on ice for 30 min. The 3-h degradation assay was conducted using 10 nM of the indicated $^{125}$I-ligands alone (No Compet.) or in the presence of the indicated unlabeled ligands. The amount of unlabeled ligand used was 100 nM based upon number of KIV domains relative to the $^{125}$I-ligand, as described in the legend to Fig. 3.
degradation of 125I-KIV5–8 by cholesterol-loaded mouse peritoneal macrophages was dependent upon new protein synthesis (80.3 ± 6.1 versus 39.5 ± 0.4 pmol degraded per 3 h/mg of DNA, in the absence and presence of cycloheximide, respectively), whereas the binding of this specific ligand was not dependent upon new protein synthesis (28.7 ± 0.5 versus 31.6 ± 2.9 pmol bound per 3 h/mg of DNA, in the absence and presence of cycloheximide, respectively).

Finally, we utilized two anti-apo(a) monoclonal antibodies raised against apo(a) or Lp(a) (see "Experimental Procedures"). Based upon immunoaffinity data, we found that both antibodies recognized 17K and (KIV2)5 to a similar extent (Table I). In contrast, whereas 8B4 recognized 6K and KIV5–8 very well, 12C11 interacted poorly with these structures. Thus, 8B4, but...
not 12C11, recognizes the region of apo(a) that we postulate interacts with the foam cell receptor (see above). The effect of these two antibodies on $^{125}$I-17K degradation and binding by mouse peritoneal foam cells is shown in Fig. 6. Antibody 8B4 inhibited both degradation (A) and binding (B), whereas 12C11 inhibited these processes hardly at all. These data further support the conclusion that the foam cell receptor recognizes a domain in the KIV$_{5-8}$ region.

**Ligand Dose Relationship and Scatchard Analysis of $^{125}$I-KIV$_{5-8}$ Cell-surface Binding to Mouse Peritoneal Macrophages**—Based upon the data presented above, we conducted a binding study with $^{125}$I-KIV$_{5-8}$ to determine the affinity and number of sites of the cholesterol-inducible receptor on macrophages. The data in Fig. 7 display total (open squares), specific (closed circles), and nonspecific (open diamonds) binding data; there was very little nonspecific binding, and the specific and total binding curves showed a tendency toward saturation at the concentrations of $^{125}$I-KIV$_{5-8}$ used. Scatchard analysis (inset) of these data was consistent with a single class of binding sites with an affinity of $2.5 \times 10^{-10}$ M and a number of sites equal to approximately $2 \times 10^{5}$ per cell. Whether a lower affinity, higher capacity site would be evident at higher concentrations of ligand cannot be determined from these data.

**CHO Cells Have an Apo(a) Receptor Activity with Similar Properties as the Macrophage Receptor**—To determine if cells other than macrophages have an apo(a) receptor similar to that described above, it was necessary to work with a cell type that could be loaded with substantial amounts of cholesterol. For this purpose, we utilized CHO-mSRAII cells, which are CHO cells that have been transfected with a murine macrophage scavenger receptor (class AII) cDNA (27). When incubated with acetyl-LDL, these cells accumulate large amounts of free and esterified cholesterol (39). As shown in Fig. 8A, unloaded cells bound relatively little $^{125}$I-17K, but cells loaded with cholesterol bound an amount of ligand similar to or greater than that bound by cholesterol-loaded macrophages (see previous figures). The $^{125}$I-17K was not simply sticking to surface-bound acetyl-LDL: the cells were routinely incubated overnight in the absence of acetyl-LDL prior to the binding assay (Fig. 8), and even when fucoidin was included in this overnight incubation to displace any residual cell-surface acetyl-LDL (40), there was no decrease in $^{125}$I-17K binding (data not shown). Furthermore, as shown in the inset to Fig. 8A, $^{125}$I-17K binding was further enhanced by increasing the free cholesterol content of the cells (by incubation with acetyl-LDL plus the ACAT inhibitor 58035; see Ref. 39). These data are similar to our previous findings in macrophages (17). Cholesterol-loaded CHO cells also degrade $^{125}$I-17K (Fig. 8B, solid bars) as well as the more

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**Table I**

Interaction of $^{125}$I-r-apo(a) constructs with anti-apo(a) monoclonal antibodies

| $^{125}$I-r-apo(a) construct | Amount $^{125}$I-r-apo(a) pelleted by mAb:Affi-Gel complex |
|-------------------------------|----------------------------------------------------------|
| 17K                           | 69                                                       |
| (KIV)$_{2}$$_{1}$             | 55                                                       |
| 6K                            | 88                                                       |
| KIV$_{5-8}$                   | 84                                                       |

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**Fig. 7. Ligand dose relationship and Scatchard analysis of $^{125}$I-KIV$_{5-8}$ cell-surface binding to mouse peritoneal macrophages.** Mouse peritoneal macrophages were incubated for 2 days in DMEM, 10% LPDS, containing 5 $\mu$g of $\beta$-VLDL/ml. The cells were then washed three times in PBS and incubated for 3 h in DMEM, 0.2% BSA to rid the cells of residual surface-bound $\beta$-VLDL. The cells were then washed twice with cold DMEM, 0.2% BSA, 20 mM HEPES, placed on ice in this medium for 30 min, and finally incubated at 4°C for 3 h with the indicated concentrations of $^{125}$I-KIV$_{5-8}$. The closed circles display total binding, and the open diamonds display binding observed in the presence of excess unlabeled ligand (nonspecific binding); specific binding (closed circles) was calculated by subtracting the nonspecific value from the total value at each ligand dose. Inset, Scatchard analysis of the specific binding data. Free refers to the concentration of $^{125}$I-KIV$_{5-8}$ in the medium (fmol/ml); the percent of ligand added to the wells that bound to the cells varied from 0.8 to 2.5%. Linearity was determined by linear regression analysis ($r = 0.98$). The $K_d$ (affinity) and $B_0$ (number of binding sites) calculated from the Scatchard analysis are shown above the regression line.
specific ligand, 125I-KIV$_6$–8 (Fig. 8B, cross-hatched bars). Thus, CHO cells, like macrophages, have a cholesterol-inducible receptor activity that mediates the binding and degradation of specific recombinant apo(a) constructs.

**DISCUSSION**

The original goal of this study was to determine the domains on apo(a) that are recognized by the foam cell Lp(a)/apo(a) receptor. In this regard, the combined binding, degradation, and monoclonal antibody data indicate that a region centered around KIV$_6$ and KIV$_7$ is critical, with flanking kringles probably necessary for proper conformation of these kringles or for optimal “presentation” of this region to the receptor (Figs. 3–6). Analysis of our initial 125I-r-apo(a) cell-surface binding data (Fig. 2), however, revealed other important properties of the receptor that were not evident from our previous work (17, 18), which used solely the ligand degradation assay. In particular, we obtained evidence that the binding and internalization/degradation functions of the receptor are distinct. It is the binding function that is induced by cholesterol, and this induction does not require new protein synthesis.

One hypothesis to explain these data is that the receptor exists in a relatively inactive conformation in the plasma membrane of unloaded macrophages. Cholesterol loading of macrophages enriches the plasma membrane with cholesterol (41), and this is known to affect the conformation and function of a wide variety of plasma membrane proteins (42–47). Thus, cholesterol loading may change the conformation of the Lp(a)/apo(a) receptor to a more active form. Alternatively, cholesterol loading may affect the trafficking of the receptor to the plasma membrane, as was recently found for a glycosylphosphatidylinositol-anchored protein that is localized to cholesterol-rich domains of the plasma membrane (48). Since inhibition of protein synthesis blocks lysosomal degradation of r-apo(a) but not binding or lysosomal degradation of other receptor ligands, we conclude that there is a separate short-lived component of the Lp(a)/apo(a) receptor that is somehow necessary for internalization or delivery of ligand to lysosomes. Precedents for this idea include the mediation of G protein-coupled receptor internalization by β-arrestins (49) and the requirement for receptor ubiquitination in the internalization of certain yeast plasma membrane receptors (50, 51). Interestingly, J774 macrophages can bind r-apo(a) in a cholesterol-regulatable (Fig. 2B) and specific (Fig. 5) manner, but these cells degrade r-apo(a) poorly (Fig. 2B). Thus, these cells may be lacking the putative internalization component. Proof of these ideas awaits molecular identification of the binding and internalization/degradation activities of the receptor.

The most important issue surrounding the foam cell Lp(a)/apo(a) receptor is its physiological function and possible pathophysiological roles. Its regulation by cholesterol may indicate importance in atherosclerosis, and its regulation by interferon-γ (36) may suggest a role in inflammation. Along these lines, we have previously postulated that the receptor may function to focally clear Lp(a) and apo(a) in atherosclerotic and inflammatory lesions (18, 36), but this idea awaits experimental testing. Moreover, the fact that the receptor is present in species without Lp(a) (i.e., mice) suggests the presence of at least one other ligand, and the finding that the receptor is present on CHO cells (Fig. 8) may also indicate a broader function. Perhaps one clue for receptor function is revealed by its affinity ($K_D = 2.5 \times 10^{-11}$ M), which is quite high compared with most other mouse macrophage receptors whose affinities have been published. For example, mouse peritoneal macrophage receptors for the Fc moiety of IgG (52), acetyl-LDL (53), and mannoside albumin (54) have affinities of $1.5 \times 10^{-8}$ M, $7.5 \times 10^{-9}$ M, and $9.5 \times 10^{-9}$ M, respectively. Two of the few reported recep-
tors with affinities similar to the one described here include the receptor for platelet activating factor (55) and the receptor for granulocyte macrophage-colony stimulating factor (56), both of which mediate cell signaling responses. Whether the high affinity nature of the cholesterol-inducible apo(a) receptor indicates a role in signal transduction remains to be explored.

To address these and other issues, our future goal is to identify the binding domain of the receptor by expression cloning. In this regard, we may be able to take advantage of the presence of the receptor on CHO cells (above), which are well-suited for somatic cell genetic strategies. Once cloned, studies utilizing targeted disruption of the receptor gene in mice should help shed light on receptor function. Cloning will also facilitate molecular interaction studies, including the identification of other ligands for the receptor and of the putative internalization component suggested by the data in this report.

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