Metabolism of Activated Complement Component C3 Is Mediated by the Low Density Lipoprotein Receptor-related Protein/α2-Macroglobulin Receptor*

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Complement component 3 (C3) and α2-macroglobulin evolved from a common, evolutionarily old, ancestor gene. Low density lipoprotein-receptor-related protein/α2-macroglobulin receptor (LRP/α2MR), a member of the low density lipoprotein receptor family, is responsible for the clearance of α2-macroglobulin-protease complexes. In this study, we examined whether C3 has conserved affinity for LRP/α2MR. Ligand blot experiments with human 125I-C3 on endosomal proteins show binding to a 600-kDa protein, indistinguishable from LRP/α2MR by the following criteria: it is competed by receptor-associated protein (the 39-kDa receptor-associated protein that impairs binding of all ligands to LRP/α2MR) and by lactoferrin and *Pseudomonas* exotoxin, other well known ligands of the multifunctional receptor. Binding of C3 is sensitive to reduction of the receptor and is Ca2+-dependent. All these features are typical for cysteine-rich binding repeats of the low density lipoprotein receptor family. In LRP/α2MR, they are found in four cassettes (2, 8, 10, and 11 repeats). Ligand blotting to chicken LR8 demonstrates that a single 8-fold repeat is sufficient for binding. Confocal microscopy visualizes initial surface labeling of human fibroblasts incubated with fluorescent labeled C3, which changes after 5 min to an intracellular vesicular staining pattern that is abolished in the presence of receptor-associated protein. Cell uptake is abolished in mouse fibroblasts deficient in LRP/α2MR. Native plasma C3 is not internalized. We demonstrate that the capacity to internalize C3 is saturable and exhibits a Kd value of 17 nM. After intravenous injection, rat hepatocytes accumulate C3 in sedimentable vesicles with a density typical for endosomes. In conclusion, our ligand blot and uptake studies demonstrate the competence of the LRP/α2MR to bind and endocytose C3 and provide evidence for an LRP/α2MR-mediated system participating in C3 metabolism.

LRP/α2MR† is a member of the low density lipoprotein (LDL) receptor family, which includes the LDL receptor, LRP/α2MR, megalin (gp330), the very low density lipoprotein receptor, apolipoprotein E receptor 2, and LR8B (1). A gene closely related to that of LRP/α2MR was identified in *Caenorhabditis elegans*, showing that LRP/α2MR is an evolutionarily old molecule (2). LRP/α2MR was discovered by its homology to structures of complement components (3), and it was suggested that it might function together with the LDL receptor as receptor for apoE-containing lipoproteins executing the mass transport of chylomicron remnants (4). Later, LRP/α2MR was shown to be identical with the α2MR (5, 6). In recent years, a plethora of new ligands were reported, and LRP/α2MR was classified as a multifunctional receptor (1, 7). Members of the LDL receptor family all consist of the same basic structural components: (i) a class of cysteine-rich repeats of approximately 40 amino acids, which are also present in the terminal complement components and are therefore referred to as complement type repeats (this sequence is believed to be responsible for binding of ligands); (ii) a second class of cysteine-rich repeats like those present in the epidermal growth factor precursor; (iii) an epidermal growth factor precursor homologous domain containing TWTD motifs, (iv) a single membrane-spanning segment; and (v) a cytoplasmic domain that contains one or more NPXY motifs, which are responsible for coated pit-mediated endocytosis (8, 9). The ligand binding repeats are found in various members of the LDL receptor family assembled in clusters harboring 2–11 repeats, thereby varying affinity for different ligands. The study of the avian equivalents of members of the LDL receptor family profoundly refined the knowledge about the physiologic function of this receptor class as apoE is not expressed in avians. Similar to mass transport of chylomicron remnants into liver, LR8 on chicken oocyte membranes was found to mediate mass transport of egg yolk components into developing oocytes (1, 10).

Human α2M is a macromolecular protease scavenger consisting of four subunits with an M_r of 180,000 each. It is an acute phase protein inhibiting a wide range of proteases by forming a complex with them. Proteases cleave the molecule at the “bait region,” leading to the hydrolysis of the internal thioester followed by an extensive conformational transition of the α2M molecule. The conformational change results in the entrapment of the protease. Simultaneously, recognition sites are exposed enabling the α2M molecule to interact with its specific cell surface receptor, LRP/α2MR (11). The resulting complex is then internalized by LRP/α2MR-mediated endocytosis. The transformation (“activation”) of the α2M molecule can also be

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§ The abbreviations used are: LRP/α2MR, low density lipoprotein receptor-related protein/α2-macroglobulin receptor; α2M, α2-macroglobulin; C3, complement component 3; DIC, denaturant-induced cleavage; GST, glutathione S-transferase; HRP, horseradish peroxidase; LDL, low density lipoprotein; PAGE, polyacrylamide gel electrophoresis; RAP, receptor-associated protein.
induced by a direct nucleophilic attack in a process in which the thioester bond is hydrolyzed without initial alteration of the bait region. This form of αM is also recognized by LRP/α2MR (5, 6).

Complement component 3 (C3) is a key molecule of the complement system. The classical and the alternative pathway lead to the formation of a convertase that cleaves C3 to C3a (molecular mass, 9 kDa) and C3b (178 kDa), resulting in the hydrolysis of the thioester bond. The subsequent dramatic conformational change in the molecule is a pivotal step in the complement activation (12). The classical pathway is activated by antibody-antigen reactions. In the alternative pathway, the initial reaction and its conformational consequences are not as clearly defined as in the classical pathway. Initial complement deposition on surfaces is thought to arise from spontaneously activated C3 (C3*). The inherent instability of the internal thioester in the native C3 molecule spontaneously and continuously generates small amounts of fluid-phase and bound C3* ("tick over" activation). A number of proteins activated C3* from triggering unnecessarily the amplification loop of the complement system. The spontaneous hydrolysis rate of the thioester bond in vitro, under physiologic conditions, is estimated to amount to approximately 1%/h. In adult humans, approximately 240 mg of C3* have to be removed continuously per day by a system with high capacity.

Similar to in vivo tick over C3, activated C3* can be produced in vitro by treatment of native C3 with methylamine or with chaotropic agents or by slowly freezing and thawing of the in vitro cleavage of activated C3* (C3*). The inherent instability of the internal thioester in the native C3 molecule spontaneously and continuously generates small amounts of fluid-phase and bound C3* ("tick over" activation). A number of proteins activated C3* from triggering unnecessarily the amplification loop of the complement system. The spontaneous hydrolysis rate of the thioester bond in vitro, under physiologic conditions, is estimated to amount to approximately 1%/h. In adult humans, approximately 240 mg of C3* have to be removed continuously per day by a system with high capacity.

Similar to in vivo tick over C3, activated C3* can be produced in vitro by treatment of native C3 with methylamine or with chaotropic agents or by slowly freezing and thawing of the protein and is referred to as C3(N)* (N stands for nuleophiles, such as methylamine, ammonia, or water (13)). C3 and αM belong to a group of evolutionarily related thioester-containing proteins (14, 15). In the horseshoe crab, a single protein called Limulus α-macroglobulin complement-like protein possesses functional properties of both sides of the superfamily, namely, protease inhibition and participation in a hemolytic system (16). Although with the evolutionary development, thioester proteins exhibited marked functional divergence, structural homologies persist. This led us to examine whether LRP/α2MR is capable of binding and mediating endocytosis of certain forms of C3*.

We found that LRP/α2MR bound C3* in ligand blots. Very likely, the cysteine-rich complement type repeats were involved. Other ligands of LRP/α2MR, such as lactoferrin, receptor-associated protein (RAP), and Pseudomonas exotoxin, competed with C3*. Confocal microscopy demonstrated that fluorescent-labeled C3* but not native C3 was taken up into cells and visualized with an anti-C3 antibody. Reduced native C3 without DIC treatment, because it is similar to the proteolytically generated C3b. For analysis, the samples were boiled in the presence of 3% SDS without reducing reagents at 95 °C for 15 min and then reduced in electrophoresis sample buffer with 0.1 mM dithiothreitol at 95 °C for 5 min. After separation on SDS-polyacrylamide gels, were stained with Coomassie Brilliant Blue. Alternatively, they were blotted on nitrocellulose sheets and detected with the antibody against C3. Reduced native C3 without DIC treatment gave two bands and electrophoretic mobility values of 115 (un-cleaved α-chain) and 75 (β-chain), whereas after DIC treatment, it showed an additional band of 40 kDa resulting from the cleavage of the reactive α-chain. We used C3 preparations in which the 40-kDa band was absent or minor; upon three cycles of freezing in liquid nitrogen and thawing, these preparations developed as described (19). To study the difference in cell uptake between native and activated C3, we used fresh human serum and serum that was freeze/thaw cycled three times to activate C3.*

**Antibodies and Subcellular Fractions** — For the LRP/α2MR antibody, polyclonal rabbit anti-LRP/α2MR antiserum was obtained from rabbits immunized with LRP/α2MR from rat liver endosomal fractions purified with a glutathione S-transferase (GST)-RAP affinity column. Recombinant GST and rat 39-kDa fusion protein (designated GST-RAP) were produced following the procedure of Herz et al. (20). Subcellular fractions (eosine membranes from chicken, plasma membranes, and endosomes from rat liver) were prepared essentially as described (10, 21, 22).

**Materials** — We obtained chemicals from Sigma and Na2HPO4 for protein iodination from NEN Life Science Products. Bovine lactoferrin was produced following the procedure of Herz et al. (20). Subcellular fractions (eosine membranes from chicken, plasma membranes, and endosomes from rat liver) were prepared essentially as described (10, 21, 22).

**Cell Culture and Microscopy** — Fibroblasts were maintained in monolayer and used for experiments as described previously (22, 24). Cells grown on coverslips (Nunc) were incubated with BODIPY-labeled C3* (2 μg/ml) for the indicated times and observed in a Zeiss Axiovert 135 microscope with fluorescence equipment. Experiments were duplicated with unlabeled C3*, and localization was detected with anti-C3 antibody (DAKO) and visualized with a fluorescein isothiocyanate-labeled second antibody. Where indicated, cell nuclei were stained with bisbenzimid dyes (Hoechst 33258).

**Electrophoresis and Blotting** — Controls with preparation of C3 were performed on 4–15% Phast-gels with SDS-buffer strips according to the manufacturer's protocols (Pharmacia Biotech). Immunodetection of endosomal fractions and L8 preparations was done using 4–12% SDS gradient gels in a Bio-Rad apparatus followed by transfer to nitrocellulose according to the manufacturer's protocols. Immunodetection was done essentially as described (23) using HRP-conjugated secondary antibodies (Bio-Rad) and the ECL reagent (Amerham Pharmacia Biotech) according to the specifications of the manufacturer. ECL signal was quantitated by a Bio-Rad Fluor-S MultiImager. For quantification of bound radioactive ligands, counting was performed in a Packard Instant Imager (Packard Canberra, Vienna, Austria).

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Complement Component C3* Binds to LRP/α₂MR

RESULTS

Binding Proteins for C3*—We reasoned from the inherent genetic relationship of C3 and α₂M that LRP/α₂MR may bind C3. Thus, we performed ligand blotting experiments using liver subcellular fractions to screen for proteins binding C3* with high affinity. For ligand overlays, we used C3 treated to favor production of thioester cleaved forms (C3*) as tested by DIC–C3* visualization by Packard Instant Imager. C3* bound by proteins of chicken oocyte membrane extract (OM) was immunodetected by overlay with anti-C3 followed by anti-rabbit HRP-coupled antibody and visualized by the ECL procedure. The approximate positions of 100 kDa (rap-insensitive binding and chicken LR8) and 600 kDa (RAP-sensitive binding to LRP/α₂MR) are shown in the left lane.

All results are one representative of experiments performed at least twice.

Analysis of Endosomal Proteins—We next determined C3* internalization mediated by LRP/α₂MR on the cellular level. Human fibroblasts, which express internalization-competent LRP/α₂MR, were grown in chamber slides. We incubated the cells with fluorescent-labeled C3* either 60 min at 4 °C to demonstrate plasma membrane binding, or 30 min at 37 °C for uptake into cells. In the plasma membrane labeling experiment, an even surface distribution of the labeled ligand was seen (Fig. 3B). Exposing cells for 30 min caused a distinct vesicular pattern to be exhibited (Fig. 3C). In confocal planar section series (Fig. 3A), the late stages of endocytosis were clearly located in the midsections of the cell, with the perinuclear compartments positive for C3* fluorescence. In the presence of RAP (Fig. 3D), intracellular perinuclear distribution of fluorescent labeled C3* was completely abolished. To further demonstrate the competence of LRP/α₂MR we studied the uptake of C3* in the mouse fibroblasts deficient in LRP/α₂MR. In contrast to wild type cells (Fig. 3, LRP−), the mutant fibroblasts do not internalize C3. This experiment proved on a genetic level that LRP/α₂MR mediates uptake of C3*.

Analysis of Cell Uptake—Using the mouse fibroblasts, we determined the kinetic parameters of uptake of C3* by LRP/α₂MR.

Addition | EDTA | DTT | PE | RAP | LF
---|---|---|---|---|---
Optical Density | 0.45 | 0 | 0.05 | 0 | 0.1 | 0.11

Fig. 2. Binding of 125I-C3* to endosomal proteins in the presence of inhibitors and competitors. Rat liver endosomal fractions (100 μg of protein per lane) were separated by 4–12% gradient SDS-PAGE (nonreducing) and blotted onto nitrocellulose membranes. Individual nitrocellulose strips were subjected to ligand blot analysis with radiolabeled C3* alone (2 × 10^6 cpd/ml) and with the indicated additions: EDTA, 10 mM EDTA during incubation and washing; DTT, preincubation with 100 mM dithiothreitol for 1 h at 37 °C; PE, 1 mg/ml Pseudomonas exotoxin during incubation with C3*; RAP, 100 nM GST-RAP; LF, 1 μg bovine lactoferrin. Autoradiographs were quantitated by a Packard Instant Imager.

medium was replaced by medium containing biotinylated C3*. After 3 h, equilibrium of uptake and elimination was reached, determined by receptor activity. Cells were washed three times and lyzed, the content of C3 detected on slot blots using HRP-conjugated anti-biotin antibody (Vector) with ECL reagent (Amersham Pharmacia Biotech) and quantitated by Packard Instant Imager. This experiment proved on a genetic level that LRP/α₂MR may bind C3*. When RAP was added, binding disappeared in the same way as when EDTA was added.

Examination of Specificity of Binding—Specific features of the binding were tested by varying cofactors, inhibitors, and competitors specific for LRP/α₂MR (Fig. 2). Equal amounts of endosomal protein were examined by ligand blotting, and the amount of C3 that bound to LRP/α₂MR was quantitated by densitometric scanning of the ECL signal. When Ca^{2+} was removed by addition of EDTA (10 mM) to the incubation buffer, binding was abolished. Again, complete inhibition of binding was achieved when RAP was added to the incubation mixture.

Treatment of the endosomal proteins bound to the nitrocellulose with dithiothreitol before incubation with C3* reduced the binding signal to 10% of normal. This feature was extensively described to be due to the reduction of cysteine disulfide bridges that are crucial for maintaining the tertiary structure of the ligand binding domains of members of the LDL receptor family. In addition, Pseudomonas exotoxin, which is one of the ligands described for LRP (26), reduces the binding to 20% of normal. Lactoferrin, which is another example of an immediate immune response molecule that was also shown to be a ligand of LRP/α₂MR (22, 23, 27, 28), competed as efficient as Pseudomonas exotoxin.

Cell Uptake of C3*—We next determined C3* internalization mediated by LRP/α₂MR in the presence of inhibitors and competitors. Rat liver endosomal fractions (100 μg of protein per lane) were separated by 4–12% gradient SDS-PAGE (nonreducing) and blotted onto nitrocellulose membranes. Individual nitrocellulose strips were subjected to ligand blot analysis with radiolabeled C3* alone (2 × 10^6 cpd/ml) and with the indicated additions: EDTA, 10 mM EDTA during incubation and washing; DTT, preincubation with 100 mM dithiothreitol for 1 h at 37 °C; PE, 1 mg/ml Pseudomonas exotoxin during incubation with C3*; RAP, 100 nM GST-RAP; LF, 1 μg bovine lactoferrin. Autoradiographs were quantitated by a Packard Instant Imager.

mammalian very low density lipoprotein receptor. We have previously shown that this receptor was able to bind some typical ligands of LRP/α₂MR (25). Using chicken oocyte membrane extracts, LR8 (95 kDa) was detected as the sole binding protein for C3*. When RAP was added, binding disappeared in the same way as when EDTA was added.

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for 3 h, and the cell-associated C3 transferred to nitrocellulose. It was quantitated with an anti-biotin antibody that was labeled with HRP. A linear signal in the concentration range between 0.5 and 5 ng was accomplished with ECL signal detection in the Bio-Rad Fluor-S MultiImager. Unspecific binding was determined accordingly but using LRP-deficient fibroblasts and subtracted from the total binding measured in LRP+ fibroblasts. In Fig. 4, it can be seen that specific uptake of C3* into LRP+ fibroblasts is saturable. At saturation, approximately 4 ng of C3 per 10⁵ cells were taken up within a 3-h incubation period (40,000 molecules/cell/h). In Fig. 4, inset, we show Scatchard analysis of these data, giving a K_D of 17 nM (mean, 15 ± 3).

Our initial hypothesis was that a conserved structural similarity between α_M and C3 determines the ability of C3* to be recognized by the LRP/α_MR. To validate this suggestion, we added a 10-fold molar excess of methylamine-activated α_M to some of the incubation mixtures and measured C3* uptake. This addition resulted in an inhibition of uptake of C3* of 75%. The same reduction was found with the addition of a 50-fold molar excess of GST-RAP (not shown).

**Cell Uptake of Native and Activated C3**—In plasma, C3 is an abundant protein occurring with a concentration of roughly 1–2 mg/ml. In the experiment described above, we determined that saturation of LRP/α_MR-mediated uptake occurs above 50 nM. We thus incubated LRP+ fibroblasts with fresh serum diluted to this concentration of C3 for 45 min at 37 °C and determined intracellular C3 by immunofluorescence techniques as described. In Fig. 5, it can be seen that incubation with native C3 does not result in detectable uptake. We then activated the serum by three cycles of freezing in liquid nitrogen and thawing at 40 °C. In DIC assays, this treatment notably inhibited the appearance of the 40-kDa fragment. Uptake experiments with this activated form of C3* resulted in considerable intracellular localization. The addition of RAP again prohibited this uptake, strongly indicating that C3 has to be activated to allow uptake into cells mediated by LRP/α_MR.

**Liver Uptake of Intravenously Injected C3*—**These experiments were done to determine whether uptake of C3* in vivo followed the kinetics of liver uptake seen with other ligands of LRP/α_MR. Furthermore, we wanted to find out what molecular weight form of C3 accumulates in endosomes. In vivo turnover experiments with ¹²⁵I-C3*, we found a considerable fraction of C3* to be removed from the plasma and taken up within 20 min into the liver (not shown). In Fig. 6, we show the results of liver uptake experiments with a bolus injection of 250 μg of unlabeled C3* and a saline-injected rat (untreated). The intracellular distribution of C3* recovered in liver subcellular fractions (40,000 × g, vesicles) was analyzed by zonal rotor density gradient centrifugation. In Fig. 6, top panel, the density gradient profile is given in g/ml. Immunodetection of C3 in the density gradient fractions after injection of saline (untreated) and a mass bolus of unlabeled C3* are shown in the bottom two panels. Clearly, two different localization patterns of C3* were seen in immunoblot analysis of the density gradient fractions with anti-C3 polyclonal antibody detecting rat and human C3. In animals injected with saline, we detected immunoreactive material at the top of the gradient (density, 1.05 g/ml), corresponding to endogenous rat C3. This material was not particle-bound, indicating that it was likely native C3 of extracellular origin. Some C3-positive material was located in endosomes, which is in accordance with the hypothesis that endogenous tick over reaction in the rat produces a form of C3* that is taken up by endocytosis. In rats injected with C3*, immunopositive material accumulated markedly in the 1.12 g/ml density range. These subcellular fractions have been previously characterized to contain predominantly endosomes (21). We also demonstrated that these fractions exhibit the highest concentration of LRP/α_MR, whereas RAP is located preferentially in fractions with higher density (23). C3 in endosomal fractions was sedimentable by centrifugation, verifying that it was enclosed in vesicles (not shown). The molecular weight of the immunopositive material was indistinguishable from the injected material, indicating that a processing prior to uptake did not significantly alter migration in gel electrophoresis.

**DISCUSSION**

C3 and α_M evolved from a common ancestor in which two specific functions, protease trapping and cell lysis, were combined, as described in the arthropod Limulus polyphemus (16). The thioester bonds in complement component C3 and the protease inhibitor α_M have traditionally been thought of as fulfilling the dual roles of mediating covalent attachment and
maintaining a thermodynamically unfavorable conformational state of the native (nonactivated) protein. The thus stabilized conformation keeps binding sites buried, to be uncovered for further reaction only after activation. The removal of the activated ancestral molecule that was not consumed by the complement cascade reactions was presumably performed by an evolutionarily old receptor. We speculated that LRP/α2MR, which is expressed in mammals, as well as C. elegans, might have preserved affinity not only for α2M but for C3α as well. Whether this feature is archaic or was preserved because of physiologic benefits will be an interesting task to determine. Our experiments clearly demonstrate that C3α specifically binds to LRP/α2MR immobilized on nitrocellulose and is internalized by LRP/α2MR in cell culture. In serum, C3 is present in concentrations over 1 g/liter, whereas in ligand binding experiments, concentrations as low as 2 μg/ml gave a positive signal. However, we never observed binding of C3α using total fresh serum in the ligand blot experiments. Thus, we believe that although transition of C3 from the native to the activated form continuously takes place, these forms are rapidly removed and consequently in a very low steady state concentration in serum.

The data are therefore consistent with the hypothesis that a C3b-like form (C3α), which was activated in an activation process similar to the tick over process, is recognized by LRP/α2MR and is rapidly removed from the circulation, much like activated α2M.

Our experiments also demonstrate that type A-like cysteine-rich repeats, which constitute the ligand-binding domain of the LDL receptor family, are responsible for the high affinity of LRP/α2MR to C3α. These ligand binding repeats are found in various members of the LDL receptor family assembled in 2–11-fold clusters, thereby varying binding parameters for different ligands. Interestingly, we could demonstrate that the described function does not require more than one cluster because LR8, a chicken homologue of the very low density lipoprotein receptor bearing eight repeats, was also able to bind C3α. ApoE, a ligand of LRP/α2MR in mammals, is not expressed in birds. C3, in contrast, is present and functional in these animal species as well (29). Thus, our findings might help to refine our view about evolutionary aspects about structure/function relationship of members of this receptor family.

Although a vast number of data exist, not all details of conformational changes in the activation of native C3 are known. To this end, it is reasonable to assume an activation process similar to that occurring in rats that were injected 250 μg of C3α 20 min prior to removal of the livers were separated by 4–12% SDS-PAGE under nonreducing conditions and blotted onto nitrocellulose. C3 in these fractions was detected by anti-C3 antibody and visualized by a second antibody (anti-IgG) coupled to HRP and development by an ECL system until saturation of the strongest signal was achieved.

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Finally, an interesting, newly discovered function of C3 could involve LRP/α2MR in C3 metabolism. It was recently reported that chylomicrons stimulate C3 synthesis in adipocytes by a factor of 100. In this situation, C3 is cleaved to C3a- and C3b-like portions. The first is used in the processing of the acylation stimulation protein governing fatty acid removal from the plasma; the latter has no known function and is likely to be removed (30, 31). Indeed LRP/α2MR is present on adipocytes, and it is tempting to infer its participation in this metabolic reaction.

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