High Affinity Binding, Endocytosis, and Degradation of Conformationally Modified Albumins

POTENTIAL ROLE OF gp30 AND gp18 AS NOVEL SCAVENGER RECEPTORS*

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Scavenger receptors interact with a variety of modified proteins, mediate their endocytosis and degradation, and may play an important role in protein catabolism and pathogenic processes such as atherosclerosis, aging, and diabetes. Many scavenger receptors have been detected kinetically but few such binding proteins have actually been identified. Recently, we found that two membrane-associated proteins, gp30 and gp18, interact more avidly with albumins conformationally modified by chemical processes rather than by surface adsorption or by fibroblasts was to colloidal gold particles than with native albumin. In this study, we show that gp30 and gp18 behave similarly to other known scavenger receptors. Competition studies indicate a similar ligand binding profile to other known scavenger receptors. Polyanionic molecules (dextran sulfate, fucoidan, polyglutamic acid, polyinosinic acid, heparin) and modified albumins such as formaldehyde-treated or maleylated albumin (Mal-bovine serum albumin) competed with albumin conjugated to colloidal gold particles (A-Au) for the blotting of gp30 and gp18. A-Au and Mal-bovine serum albumin bound cultured endothelial cells with high affinity. Modified and native albumins were each internalized, but only modified albumins were then released degraded. Inhibition studies revealed that only the same molecules that were effective in blocking A-Au blotting of gp30 and gp18, also inhibited A-Au degradation. Addition of the lysosomotropic agent chloroquine resulted in more than 70% inhibition of degradation. Differential processing of A-Au by cultured smooth muscle and endothelial cells along with fibroblasts was observed in a manner consistent with gp30 and gp18 expression. Cumulatively, these results suggest that gp30 and gp18 may mediate the high affinity binding, endocytosis, and degradation of conformationally modified albumins but not native albumin.

Selective cellular internalization of macromolecules is mediated by specific cell surface receptors that cluster within vesicular carriers which provide a delivery system to intracellular organelles such as lysosomes. This basic cellular process is known as receptor-mediated endocytosis and has been defined through examining the interactions with cells of various macromolecules including LDL (1). It has become clear that ligands such as LDL when modified by a variety of chemical processes cease to be recognized by the LDL receptor and interact preferentially with a new set of modified LDL receptors for degradation within lysosomes (2). Receptor-mediated endocytosis of a variety of modified proteins including LDL and albumin has been collectively termed "scavenger function" (2-14). This process has been identified in many cells including macrophages, fibroblasts, endothelial cells, and smooth muscle cells (2, 4-7, 9, 11, 15, 16) and appears to be necessary for the physiological removal from tissues of proteins that are modified by processes such as glycation or oxidation.

In the last decade, several different scavenger receptors have been studied extensively in terms of their role in protein catabolism and their possible link to atherosclerosis, aging, and diabetes (2, 4-8). Although kinetic binding experiments indicate that cells can express a number of distinguishable scavenger receptors (3, 5-9, 17, 18), each with different ligand specificities, few binding proteins have been actually identified and well characterized as scavenger receptors (2, 11, 19, 20). Recently, we demonstrated that modified albumins interact very avidly with two cell membrane-associated glycoproteins, gp30 and gp18 (21). Albumins that are conformationally modified either by surface absorption to colloidal gold particles (A-Au) or by treatment with formaldehyde or maleic anhydride (Mal-BSA) bind gp30 and gp18 with much greater affinity than native albumin (21). In this study, we provide evidence that gp30 and gp18 may act as scavenger receptors that mediate high affinity surface binding resulting in rapid internalization and lysosomal degradation of conformationally modified albumins. The cellular processing of modified albumins is characterized by defining the ligand binding specificity and by assessing the kinetics of equilibrium surface binding, internalization, and degradation. Comparisons with other known scavenging receptors are made.

EXPERIMENTAL PROCEDURES

Materials—Reagents and other supplies were obtained from the following sources: fetal calf serum, and phosphate-buffered saline

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1 The abbreviations used are: LDL, low density lipoprotein; PBS, phosphate-buffered saline; BSA, bovine serum albumin; MDA-LDL, malondialdehyde-treated LDL; Mal-BSA, maleylated bovine serum albumin; Ac-LDL, acetylated LDL; Fm-BSA, formaldehyde-treated BSA; RFC, rat fat capillary; DMEM, Dulbecco's modified Eagle's medium.

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particles with a mixture of "I-BSA and BSA (usually 1:100 ratio) as per the usual procedure (21). Washed and then incubated for 30 min at 4 °C with 1 ml of 251-A-AuX-100 and 1% SDS and finally scraped from the wells and pipetted. No radioactive ligand was processed as described previously (24). Briefly, cell monolayers plated onto 6-well trays, or 35-mm dishes were extensively blocked overnight with 1-2 mg/ml of rabbit or bovine immunoglobulins and then blotted with the A-Au complexes as in Ref. 21. For cellular processing assays, the cells were plated onto 6-well trays at 5 x 10⁴-5 x 10⁵ cells per well in 2 ml per well growth medium for 3 min at 12 °C as in our past work (21, 25). Before lung excision, the lung vasculature was perfused for 3 min at 12 °C with a protease inhibitor mixture containing 400 μg/ml benzamidine, 10 μg/ml leupeptin, 19 μg/ml pepstatin A, 65 μg/ml aprotinin, 10 μg/ml chymostatin, 100 μg/ml P-phenanthroline, and 350 μg/ml phenylmethanesulfonyl fluoride.

Lung Perfusion-Male albino rats (Sprague-Dawley, 200-250 g) were anesthetized with an intraperitoneal injection of 50 mg/kg ketamine and 10 mg/kg xylazine (33 mg/kg). Following tracheostomy and median thoracotomy, each animal was ventilated using a small animal respirator. The pericardium was opened and 0.5 ml of DMEM containing 30 μM freshly prepared nitroprusside (as a vasodilator) and 500 units of heparin (as an anticoagulant) was injected into the right ventricle. After removing the thymus to fully expose the pulmonary artery, a catheter was inserted through the right ventricle into the pulmonary artery, where it was fastened snuggly by a ligature. After cutting the left atrium to provide an outflow port, the pulmonary vasculature was flushed free of blood with DMEM at 18-20 mm of mercury for 3 min per heart. After perfusion, the slice of lung was then incubated at 37 °C for 3 min in 2 ml of DMEM. The specific activity ranged from 5 to 8 Ci/μg. "I-A-Au was created by incubating the colloidal gold particles with "I-protein (pellet) for 2 h at 4 °C (32). After centrifugation and removal of excess unlabeled Mal-BSA (1 mg/ml) or for "I-A-Au to excess A-Au (AAu = 20), ~40 μg/ml), quantitation of specific binding was performed as in Ref. 24.

Cell-associated Binding, Uptake, and Degradation Assay at 37 °C-Confluent RFC monolayers were washed extensively at 37 °C with DMEM (2X, 1 min; 1X, 10 min; 3X, 1 min) before incubation at 37 °C for various times with "I-ligand in the presence or absence of potential competitors. All of the medium from each well of cells was removed and saved. The cells were immediately washed with DMEM at 4 °C (3X, 1 min) and then lysed with PBS containing 5% Triton X-100 and 1% SDS. The lysates were collected and counted using a Beckman Gamma 5000B counter. In order to determine the extent of ligand degradation by the cells, the saved medium from each well was subjected to 10% trichloroacetic acid precipitation after the addition of BSA to a final concentration of 10 mg/ml. The addition of BSA was optional for "I-A-Au, because it did not significantly affect the final precipitation. The trichloroacetic acid-soluble counts were considered to be degradation products from cellular processing of the radioactive ligand, whereas the trichloroacetic acid-insoluble counts (pellet) represented the undegraded ligand remaining in the medium. A control ("I-protein in medium without exposure to the cells) was subjected to trichloroacetic acid precipitation in order to define the background of the assay which was then subtracted from the total samples. As shown in Fig. 2, our assumptions in using this assay for estimating degradation of the radiolabeled ligand seem reasonable, because when this assay was performed at 4 °C, the apparent degradation of "I-A-Au detected in the trichloroacetic acid-soluble counts was negligible, especially when compared to the radioactivity detected after incubation with the cells at 37 °C.

RESULTS

In our recent work (21), ligand blotting was used to demonstrate that gp30 and gp18 interact specifically with modified albums such as A-Au and Mal-BSA. They are expressed by many cells and tissues and interact more avidly with various modified albums than with native albumin. Scavenger receptors also interact preferentially with modified albums and mediate endocytosis and degradation of their ligands (4-8, 12, 17). In this study, the ligand specificity of gp30 and gp18 was compared with other scavenger receptors. We examined the kinetics of the binding, internalization, and degradation of modified albums by cultured endothelial cells. Chloroquine-sensitive lysosomal degradation of modified albums was demonstrated and then inhibited by a variety of molecules, all of which interacted with gp30 and gp18. Ligand processing is also examined in other cell types including fibroblasts and smooth muscle cells.

Inhibition of A-Au Binding to gp30 and gp18 with Ligands of Scavenger Receptors-Some of the modified albums (Mal-BSA and Fm-BSA), that were shown recently to interact specifically with gp30 and gp18 (21), are also known to interact with scavenger receptors located on the surface of a variety of cells (2, 4-7, 9, 11, 15-17). Mal-BSA interacts with a 220-260-kDa scavenger receptor (3, 8, 11) that also binds various modified LDL including acetylated LDL (Ac-LDL) and MDA-LDL. Fm-BSA interacts with another scavenger receptor, a 125-kDa glycoprotein that recognizes several modified forms of albumin (4, 10, 17, 19). We tested a variety of molecules, that are known to interact with these two well-characterized scavenger receptors (see Table I for list and a summary of ligand specificity), for their ability to compete with A-Au blotting of gp30 and gp18. Some of the strips from our competition experiments are shown in Fig. 1 and all of the competition results are presented quantitatively in Fig. 2. Several molecules known to bind to the 260-kDa scavenger receptor also appeared to interact with gp30 and gp18 by significantly reducing A-Au binding. Fucoidan and Mal-BSA...
that of scavenger receptors. Gp30 and gp18 may represent two novel scavenger receptors that recognize not only some of the ligands for the other known scavenger receptors but also a different and unique set of ligands.

**Time Course of RFC Binding, Uptake, and Degradation of Modified Albumins**—If gp30 and gp18 are scavenger receptors for modified albumins, then cells expressing gp30 and gp18 such as the cultured endothelial cells isolated from rat epidymal fat pads (RFC) (21) should be able to bind, internalize, and degrade their ligands. Initially, recognition of A-Au by RFC cells was studied by performing binding and degradation assays at 4 and 37 °C. The RFC cells were incubated with [125I]A-Au for various times ranging from 5 to 90 min and a mass balance was performed by following the radioactivity in the degraded fraction of the media (trichloroacetic acid-soluble counts), in the degraded fraction of the media (trichloroacetic acid-insoluble counts, pellet) and in the cellular fraction (cell-associated counts). Fig. 3 shows the time course for the cell association and degradation of A-Au by RFC cells at both 4 and 37 °C. The results are presented relative to the total [125I]-A-Au added to the cells in the assay. At 4 °C, the interaction of A-Au with RFC cells reaches equilibrium after 20–30 min and is expected to represent only surface binding of A-Au to the RFC cell monolayers. As expected at 4 °C, there is negligible degradation of A-Au which indicates a lack of ligand internalization and processing by the cells at this temperature. At 37 °C, the detected A-Au associated with the cells is about 3–4-fold greater than that observed at 4 °C, probably because it represents both surface-bound and internalized A-Au. The RFC cells clearly degrade A-Au at 37 °C and appear to begin releasing degraded products into the media after a short initial lag period of only about 10–20 min. After 90 min, about 25% of the total [125I]-A-Au in the medium has been degraded by the cells. This significant depletion of the ligand in the medium may explain the decreasing amount of [125I]-A-Au associated with the cell after a peak association at 20 min. For example, after 40 min, greater than one-third of the total A-Au incubated with cells is either associated with

![Fig. 1. Competition of A-Au blotting of gp30 and gp18 with molecules known to interact with scavenger receptors.](image)

significantly decreased the observed binding of A-Au to gp30 and gp18 by greater than 90%, whereas other macromolecules including Ac-LDL, LDL, and MDA-LDL did not appear to have a significant effect on A-Au binding. Some ligands recognized by the 125-kDa scavenger receptor also interacted with gp30 and gp18. Fm-BSA and the lectin concanavalin A effectively competed with more than 90% of the A-Au binding to gp30 and gp18, whereas glycated BSA did not. In addition, several polyanionic molecules (dextran sulfate, polyglutamic acid, polyinosinic acid, and heparin), which have been shown to interact with one or both types of scavenger receptors, were tested. All of these polyanions were able to inhibit about 90% or more of the binding of A-Au to gp30 and gp18. Other molecules, which did not compete significantly, included BSA, dimerized BSA, orosomucoid, transferrin, and ovalbumin. From these data, it appears that gp30 and gp18 have a distinct ligand binding profile that in general terms is very similar to

![Table 1: Comparison of the ligand binding behavior of gp30 and gp18 with known scavenger receptors](image)

**Table 1**

| Competitors          | Ac-LDL        | Modified            | Glycated BSA | A-Au |
|----------------------|---------------|---------------------|--------------|------|
| LDL and modified forms | LDL           | +                   | +            | +    |
|                      | Ac-LDL        | +                   | +            | +    |
|                      | MDA-LDL       | -                   | -            | ND   |
| Albumin and modified forms | Mal-BSA      | +                   | +            | +    |
|                      | BSA           | -                   | -            | ND   |
|                      | Fm-BSA        | -                   | +            | +    |
|                      | Glycated BSA  | +                   | +            | +    |
|                      | A-Au          | -                   | +            | +    |
| Miscellaneous        | Fucoidan      | +                   | +            | +    |
|                      | Dextran sulfate | +                | +            | +    |
|                      | Polyglutamic acid | -                | +            | +    |
|                      | Polyinosinic acid | +                 | +            | +    |
|                      | Heparin       | +                   | +            | +    |
|                      | Con A         | ND                  | +            | +    |
|                      | Transferrin   | ND                  | ND           | ND   |
|                      | Orosomucoid   | ND                  | ND           | ND   |

*The abbreviations used are: RAGE, receptors for advanced glycosylation end products; ND, not determined.

![Fig. 2. Quantitation of competition of A-Au binding to gp30 and gp18 with various molecules including many known to interact with scavenger receptors.](image)
the cells or degraded. Similar results were attained using $^{125}$I-Mal-BSA.

Analysis of Surface Binding of Modified Albumins—Modified albumin binding to the surface of RFC cells was assessed in vitro at 4 °C using confluent monolayers that were washed extensively and then incubated with $^{125}$I-A-Au or $^{125}$I-Mal-BSA. Before quantitating binding, we performed a series of experiments not only to define the optimal binding conditions but also to verify that the binding does indeed adhere to established rules governing receptor-ligand interactions.

Assessment of Binding Equilibrium Kinetics—The time required for achieving equilibrium of $^{125}$I-A-Au and $^{125}$I-Mal-BSA binding to the RFC monolayers was examined at 4 °C. Both Mal-BSA and A-Au binding equilibrated quite quickly after just 20–30 min (data for A-Au shown as part of Fig. 3; other data not shown). This is similar to the equilibrium time for native albumin binding to RFC cells (15–20 min) (22). An incubation time of at least 20 min was used for all other binding assays.

Competition of $^{125}$I-modified Albumin Binding with Unlabeled Albumins—Binding assays were conducted using $^{125}$I-Mal-BSA (0.2–0.3 μg/ml) mixed with various amounts of unlabeled Mal-BSA, BSA, and A-Au. As shown in Fig. 4, the detected radioactivity decreased significantly as the molar excess of unlabeled Mal-BSA and A-Au increased; however, BSA did not compete. It appears that the binding of $^{125}$I-modified albumins can be effectively competed with unlabeled modified albumins but not native albumin. Competition binding assays using $^{125}$I-A-Au yielded similar results with unlabeled modified but not native albumin competing (data not shown). All of these competition studies for binding to the RFC cell surface are very consistent with our previous ligand blotting studies done on gp30 and gp18 (21). The concentration at which 50% competition is achieved, provides a reasonable estimation of the overall binding affinity and was very similar for blotting and cell surface binding assays with a range of 3–7 μg/ml.

Analysis of Binding Data—Since the competition studies shown above indicate that Mal-BSA and A-Au interact very similarly with the RFC cell surface, we focused primarily on one of these modified albumins to analyze in more detail the binding parameters. The data collected from both direct and competition binding studies were combined, quantitated, and analyzed as in our past work (24). Scatchard analysis of the data is shown in Fig. 5. The nonlinear, concave upward shape of the binding data suggests that two populations of binding sites exist and/or binding is negative cooperative in nature. Assuming two binding components, we used linear regression analysis to analyze the data. Both the high and moderate affinity binding components provided an excellent linear fit to the data. The apparent equilibrium binding constants ($K_d$) were calculated from the linear regression equations. For the moderate affinity binding, the $K_d$ was 0.98 μM (65.0 μg/ml) with a maximum number of binding sites ($B_{max}$) of 135 ng/10^6 cells (1.23 x 10^6 binding sites/cell or on a per area basis, 156 μg/m²). After subtraction of the moderate affinity component, the $K_d$ for the higher affinity binding was 48 nM (3.2 μg/ml), while $B_{max}$ was 13 ng/10^6 cells (120,000 binding sites/cell or 13 μg/m²). Scatchard analysis of binding studies with 125I-A-Au also revealed high affinity binding with a $K_d$ of 4.2 μg/ml. Although a moderate affinity component was clearly present, it could not be fully evaluated because concentrations as high as 1 mg/ml (used in the Mal-BSA assays to assess nonspecific binding) are not attainable in prac-
tical terms because the colloidal gold solution became essentially a packed "slurry."

Relative Affinity of Radiolabeled and Unlabeled Ligand—The process of radiolabeling proteins may change their binding behavior. Therefore, the effect of radiiodination of A-Au and Mal-BSA on its ability to bind to the cell monolayer was assessed by examining the relative affinity of labeled and unlabeled ligand as in our previous work (24). The mean affinity ratio for labeled to unlabeled ligand was 1.05 ± 0.14 (n = 4) for A-Au and 0.93 ± 0.11 (n = 4) for Mal-BSA. Therefore, the "hot" and "cold" ligands bind to the cell surface very similarly which indicates that the radiiodination did not alter significantly the ligand binding behavior.

Reversibility of Binding—Fig. 6 shows the dissociation curves for \( ^{125}\text{I}-\text{A-Au} \) and \( ^{125}\text{I}-\text{Mal-BSA} \) bound to the RFC cell surface. The dissociation was the same in the absence of presence of unlabeled ligand which suggests that the binding is not competitive in nature. About 50 and 70% of the total detected radioactivity is reversed in about 60 min for \( ^{125}\text{I}-\text{A-Au} \) and \( ^{125}\text{I}-\text{Mal-BSA} \), respectively.

A-Au Is Internalized to a Pronase-resistant Compartment—In order to be certain that the modified albumins were indeed internalized by the RFC cells as part of the degradative process, we performed Pronase-protection assays. The usual binding assay was performed at 4 and 37 °C, and the A-Au bound at the cell surface was removed by Pronase digestion. The RFC cells were incubated with \( ^{125}\text{I}-\text{A-Au} \) for 30 min and after the usual washing, they were incubated with 0.25 mg/ml Pronase with 2 mM EDTA in PBS or just 2 mM EDTA in PBS (as a control for removing the cells from the plastic). After scraping the cells into vials, they were sedimented centrifugally and washed once with PBS by resuspension. Direct comparison of the radioactivity \( (^{125}\text{I}-\text{A-Au}) \) detected from the Pronase-treated cells versus that from the control cells revealed that as expected, less A-Au was associated with the Pronase-treated cells than with the control cells. Almost all of the cell-associated A-Au at 4 °C was removed by Pronase treatment with a mean of 7.0 ± 0.4% (n = 3) remaining after digestion, whereas at 37 °C a mean of 72.8 ± 14.2% (n = 6) of the A-Au associated with the cells was resistant to Pronase digestion when compared to the control-treated cells. At 4 °C, A-Au should only be able to interact with the cell surface and should not be internalized. At 37 °C, detected cell-associated A-Au is expected to be composed of both surface-bound and internalized components. This latter component should be resistant to Pronase digestion. Our experiments are consistent with these expectations and show that: (i) Pronase digests almost all of the A-Au bound at the cell surface at 4 °C and therefore, is indeed effective in removing surface-bound A-Au, and (ii) about 25% of the cell-associated A-Au at 37 °C is bound at the cell surface where it is sensitive to Pronase digestion, whereas the vast majority is not. This Pronase-resistant fraction of about 75% must be in a Pronase-protected compartment, most likely residing within the cell. These data are very consistent with the 4 versus 37 °C binding data shown in Fig. 3. The cell-associated A-Au at 37 °C is generally three to four times greater than that found at 4 °C. Cumulatively, these data show that, under the conditions of the assay, about one-quarter of the A-Au associated with the cells at 37 °C resides on the cell surface and is accessible to Pronase digestion, whereas the vast majority of A-Au is present within the cells and is protected from Pronase digestion.

Internalized Modified but Not Native Albumin Is Released (Exocytosed) and Degraded—The RFC cell monolayers were incubated at 37 °C with \( ^{125}\text{I}-\text{BSA}, ^{125}\text{I}-\text{Mal-BSA}, \) or \( ^{125}\text{I}-\text{A-Au} \), washed, and then subjected to the Pronase digestion as described above. After washing the cells twice with cold DMEM by sedimentation-resuspension, they were incubated in suspension with DMEM at 37 °C. At the times indicated in Fig. 7, the cells were pelleted, and the media and pellet were saved. The media were subjected to trichloroacetic acid precipitation in order to assess the amount of degraded and nondegraded albumins released by the cells. Fig. 7 shows that for the modified albumins (Mal-BSA and A-Au), greater than 80% of the internalized albumins (as assessed by its resistance to Pronase digestion) was released within 60 min from the cells primarily in degraded form. Only 5% of the original internalized ligand was undegraded, whereas 75–80% was degraded. Conversely, native BSA was released from the cells much more slowly and primarily in undegraded form. Greater than 97% of the released BSA was not degraded and little if any degraded BSA was detected. These data indicate that native and modified albumins are all internalized by these cultured endothelial cells but are processed quite differently by the cells. Modified albumin once inside the cells is rapidly degraded and exocytosed, whereas native albumin is exocytosed more slowly apparently in native form.

Effect of Chloroquine on Cellular Processing and Degradation of A-Au—We attempted to pharmacologically block lysosomal degradation of A-Au by using chloroquine. RFC cell monolayers were incubated with 50–100 μM chloroquine for 30 min before coincubation with \( ^{125}\text{I}-\text{A-Au} \) for 30 min. The cells were then processed as per the usual assay for determining cell association and degradation. Fig. 8 shows that chloroquine successfully inhibited greater than 70% of the observed degradation of A-Au by the RFC cells when compared to the controls done identically without chloroquine. The treatment with chloroquine caused greater amounts of A-Au to be associated with the cells, probably as a result of accumulation in the lysosomal/endoosomal compartments within the cell. These results show that chloroquine, which inhibits lysosomal enzymes by increasing the lysosomal pH, did interfere significantly with the degradation and processing of A-Au.

![Fig. 6. Reversibility of \(^{125}\text{I}-\text{A-Au} \) and \(^{125}\text{I}-\text{Mal-BSA} \) surface binding. After exposure to \(^{125}\text{I}-\text{A-Au} \) (OD_{515}= 0.02) or \(^{125}\text{I}-\text{Mal-BSA} \) (0.1 μg/ml) as per the usual binding protocol, the RFC cell monolayers were incubated at 4 °C with DMEM alone or in the presence of the same unlabeled ligand (A-Au (OD_{145}= 0.5) or Mal-BSA (1 mg/ml)). After the indicated incubation time (abscissa), the fluid was aspirated and the cells were solubilized and counted for radioactivity. Detected \(^{125}\text{I}-\text{A-Au} \) and \(^{125}\text{I}-\text{Mal-BSA} \) is expressed as a percentage of the mean of the control wells at time zero. Since the results were indistinguishable in the presence or absence of cold ligand, the results were combined and the means values calculated (n ≥ 6 for all points).](image-url)
Molecular Inhibition of Cellular Processing of A-Au—After washing as per the usual protocol, the RFC monolayers at 37 °C were preincubated for 10 min with various potential binding inhibitors, and then 125I-A-Au was added. After 1 h, the cell media were removed and analyzed for degradation products. The potential inhibitors were chosen for their ability to recognize gp30 and gp18 (as assessed in Fig. 1 and 2) and/or other scavenger receptors (see Table I). BSA was tested as a control for the native form of the protein; it is a weak competitor for A-Au binding to gp30 and gp18 (21). Fig. 9 shows that Mal-BSA, Fm-BSA, heparin, and polyglutamic acid significantly inhibited degradation of A-Au when compared to the control. Ovalbumin, native BSA, glycated BSA, dimeric BSA, and LDL did not have any effect. Ac-LDL appeared to have a small effect by decreasing degradation by 20-25%. This molecular inhibition profile for degradation of A-Au is very similar to the ligand binding profile from the competition studies for A-Au binding to gp30 and gp18 (Fig. 3). In this survey all of the molecules that inhibited A-Au degradation significantly competed with A-Au blotting of gp30 and gp18. The molecules that did not compete with A-Au blotting of gp30 and gp18 did not significantly inhibit degradation. These results coupled with the ligand blotting studies suggest a role for gp30 and gp18 as surface receptors mediating the endocytosis and degradation of A-Au.

Differential Cellular Processing of A-Au by Fibroblasts, Endothelial, and Smooth Muscle Cells—The processing of A-Au by different cell types associated with the vascular wall was examined by incubating cell monolayers with 125I-A-Au for up to 90 min at 37 °C and performing a mass balance for the 125I-A-Au as discussed for Fig. 3. Fig. 10 shows that the RFC were able to bind, internalize, and degrade A-Au to a greater degree than the NRK-F and to a much greater degree than the A-10 cells. Slightly more A-Au appears to be associated with the RFC cells than NRK-F, whereas clearly 70% less A-Au was associated with the A-10 cells. NRK-F and A-10 cells degraded about 30 and 75%, respectively, less A-Au than the RFC cells. Although the time course for maximal association of A-Au with the cells is very similar for all three cell types, it appears that both the NRK-F and A-10 cells had...
protein. Average period of only about 10-20 min. Cumulatively, these results were detected in the medium. The RFC cells had a lag period of about 30-40 min before degradation indicates scavenger receptors recognizing various modified proteins (2-10, 15-19). Since at this time there appears to be an assortment of "scavenger receptors" and only two of these proteins have been sequenced (13,14,26), the structural relationship among these receptors is unclear. However, from a functional perspective, they all appear to interact preferentially with endogenous proteins that are modified in some way that ultimately results in their endocytosis via specific scavenger receptor has a trimeric structure of disulfide-linked 70-kDa subunits that appear to have two variant forms. Cross-competition experiments (7) indicate that maleylated albumin and oxidized LDL not only bind to this Ac-LDL receptor but also interact each with at least one other population of kinetically distinct receptors which have yet to be identified and isolated as specific proteins.6 This group of scavenger receptors has been the focus of intensive study because of their potential role in the development of atherosclerosis and coronary heart disease (27). MDA-LDL, which possibly results from platelet production, and oxidized LDL, which is modified by vascular cells including endothelial cells, smooth muscle cells, and even monocytes/macrophages, not only ultimately result in the cholesterol ester accumulation within macro-

DISCUSSION

Many cells including macrophages/monocytes, endothelia, and fibroblasts have been reported to express a number of distinguishable scavenger receptors recognizing various modified proteins (2-10, 15-19). Since at this time there appears to be an assortment of "scavenger receptors" and only two of these proteins have been sequenced (13,14,26), the structural relationship among these receptors is unclear. However, from a functional perspective, they all appear to interact preferentially with endogenous proteins that are modified in some way that ultimately results in their endocytosis via specific scavenger receptors. With this criteria in mind, it appears that gp30 and gp18 are likely candidates for these other binding site(s) of Mal-BSA.

FIG. 10. Comparison of the cellular processing of A-Au by NRK-F, A-10, and RFC cells. The indicated cells were incubated with 125I-A-Au at 37 °C and processed as for experiments presented in Fig. 3. The amount of 125I-A-Au associated with the cells (A) or degraded by the cells (B) is given after normalization to the total cell protein. Average of duplicates is given for each time point.

a fairly longer lag period of about 30-40 min before degradation was detected in the medium. The RFC cells had a lag period of only about 10-20 min. Cumulatively, these results are quite consistent with the significantly lower expression of gp30 and gp18 observed for the A-10 cells relative to both the NRK-F and RFC cells (21).

Our data implicates gp30 and gp18 as scavenger receptors recognizing various modified proteins (2-10, 15-19). Since at this time there appears to be an assortment of "scavenger receptors" and only two of these proteins have been sequenced (13,14,26), the structural relationship among these receptors is unclear. However, from a functional perspective, they all appear to interact preferentially with endogenous proteins that are modified in some way that ultimately results in their endocytosis via specific scavenger receptors. With this criteria in mind, it appears that gp30 and gp18 are likely candidates for these other binding site(s) of Mal-BSA. 

Our data implicates gp30 and gp18 as likely candidates for these other binding site(s) of Mal-BSA.
phages and cause foam cell formation but also may contribute to the development of atherosclerosis by inducing monocyte adhesion to arterial endothelium and stimulating monocyte differentiation into resident macrophages of the vascular wall (for review, see Ref. 27). The Ac-LDL scavenger receptor appears also to be present on the surface of other cell types including endothelium and under certain conditions, fibroblasts, and smooth muscle cells (16).

So far, several scavenger receptors for modified proteins have been identified kinetically on the surface of endothelium (3, 4, 8-10, 15, 17). One population of receptors recognizes both Ac-LDL and maleylated albumin and appears to consist of a 250-kDa Ac-LDL binding protein which was partially purified from rat liver membranes and may represent the endothelial cell equivalent of the Ac-LDL receptor discussed above (3). Another one recognizes maleylated albumin but not Ac-LDL (8). It has only been identified kinetically and has not been isolated as a specific distinct protein. From the available data, it appears likely that gp30 and gp18 mediate this binding, especially since they are indeed expressed in the liver (21). A third scavenger receptor was first characterized kinetically using formaldehyde-treated albumin (9, 17) and then identified as a 125-kDa protein complex by ligand blotting (19). It also interacts with various proteins modified with formaldehyde and other aliphatic aldehydes (10) as well as with nonenzymatically glycosylated proteins including albumin (4). A similar modified protein receptor may also be present on the surface of monocytes/macrophages, especially with regard to its ability to recognize glucose-modified proteins (4, 5). Very recently, endothelial receptors for advanced glycosylation end products of proteins (nonenzymatically glycosylated proteins such as glycated BSA) have been purified and sequenced (20, 26). One receptor was lactoferrin (80 kDa), and another one was a novel 35-kDa protein whose primary structure differs significantly from the Ac-LDL receptor.

Table I shows a comparison of the ligand binding specificity of gp30 and gp18 with that of the receptors for advanced glycosylation end products and the 125- and 260-kDa scavenger receptors. gp30 and gp18 appear to be two new distinct scavenger receptors that differ in their ligand specificity from other previously identified scavenger receptors. In addition, it is unlikely that gp30 and gp18 are subunits or degradation products of the scavenger receptors of higher molecular weight. Unlike gp30 and gp18 (21), the 260-kDa receptor is not normally expressed in brain tissue (13). Furthermore, both the 260- and 125-kDa scavenger receptors require intact subunit complexes for ligand binding so that upon reduction the individual subunits cannot be blotted (3, 8, 17, 19), gp30 and gp18 are blotted similarly under both nonreducing and reducing conditions (28). One study reports that two hepatic proteins of 35 and 15 kDa bind both Ac-LDL and Mal-BSA similarly to the 260-kDa scavenger; however, these interactions occur only after protein disulfide bond reduction (29). It appears that gp30 and gp18 are novel and distinct scavenger receptors interacting with their own unique set of modified proteins and polyanions.

The observation that scavenger receptors including gp30 and gp18 are blocked by polyanions such as polyinosinic acid suggests that they may share some structural similarities. Perhaps the basic structure is such that it creates an appropriate electrostatic cleft or microenvironment which is recognized by these polyanions. This common structure could then be modified to render each receptor its own specificity. Although previous work suggests that conformational changes in albumin by maleylation (12, 21) or by surface absorption to gold particles may expose scavenger recognition domains not normally accessible in the native protein, electrostatic effects apparently also play an important role in the scavenger receptor binding, because polyanionic molecules inhibit these interactions. In addition, at least for gp30 and gp18, high salt concentrations and low pH diminish A-Au binding significantly (21). Speculation about a common structural motif must await a detailed examination of the molecular structure for each of the scavenger receptors.

**Potential Role of gp30 and gp18 in Endocytosis and Degradation—** Many of the ligands that interact with gp30 and gp18 also potentially interact with other known scavenger receptors. Because other scavenger receptors could contribute to endothelial cell endocytosis and degradation of A-Au, we performed degradation inhibition assays using ligand(s) that do not interact with gp30 and gp18 but are recognized by other scavenger receptors. Ac-LDL interacts specifically with the Ac-LDL receptor which may be present on some endothelial cells including RFC cells (we actually use Ac-LDL to isolate the RFC cells because of their specific ability to internalize it (22, 25)). As shown in Fig. 9, Ac-LDL had only a small effect on A-Au degradation which suggests that A-Au was primarily internalized and degraded via a distinct molecular mechanism that does not involve the Ac-LDL receptor. Furthermore, we have also attempted to use radioiodinated Ac-LDL to detect possible interaction with gp30 and gp18 in a more sensitive and direct manner than the competition studies. However, we were unable to detect any direct interactions by blotting (data not shown). The other known scavenger receptors are also unlikely mediators of the observed binding and degradation of A-Au because they clearly differ in their ligand specificity (see Table I). For instance, neither of these two scavenger receptors interacts with Mal-BSA which clearly inhibits both A-Au binding to gp30 and gp18 and A-Au degradation by the RFC cells. The same set of molecules that compete with A-Au blotting of gp30 and gp18 also inhibited A-Au degradation by the RFC cells, whereas other molecules exclusively recognized by the other scavenger receptors (but not gp30 and gp18) did not have a significant effect.

Current evidence suggesting a direct role of gp30 and gp18 in mediating the endocytosis and degradation of conformationally modified albumins includes: (i) ligands recognized by these other scavenger receptors such as Ac-LDL and glycated BSA do not compete with modified albumin binding to gp30/18 on blots or to the degradation of the modified albumin, (ii) the same ligands that inhibit binding to gp30/18 on blots inhibit degradation by the cells, and (iii) the binding affinity to the blots is quite similar to the high affinity binding at the cell surface. Furthermore, the blots themselves provide evidence for this interaction. In fact, ligand blotting was the methodology used to identify past scavenger receptors (2, 19). These results suggest a role for gp30 and gp18 as cellular receptors; however, further investigation will be necessary to substantiate this hypothesis. We are beginning to attempt to isolate gp30 and gp18 for antibody production which may prove useful in delineating the specific functions of these molecules.

**Functional Implications—** Scavenger receptors as a group have several common features: (i) preferential recognition of modified proteins over native proteins; (ii) location at cell surfaces to mediate ligand binding; (iii) cellular internalization of the ligand-receptor complex via vesicular carriers; (iv) intracellular lysosomal degradation of their ligands; and (v) polyanion inhibition of binding and degradation. Perhaps, the role of these receptors is to recognize old, damaged, or dele-

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3 J. E. Schnitzer and J. Bravo, unpublished observations.
terious proteins for selective degradation or even for specific protein catabolism. Since many cells express these scavenger receptors, each cell type may work in its specialized environment to remove these aberrant proteins. Endothelial cells and specialized macrophages such as the Kupffer cells of the liver may clear modified plasma proteins from the circulation, whereas fibroblasts may play a role in tissue removal of proteins at the level of the basement membrane and interstitium. Activated monocytes, which express more scavenger receptors as they differentiate to become macrophages, may remove damaged proteins at sites of inflammation and, under pathological stimuli, become residents of the arterial wall to form foam cells (27). Rapid removal of cytotoxic proteins such as oxidized LDL by macrophages may serve to minimize local vascular damage (27). Very recent work (30) showed that subarachnoid macrophages can endocytose A-Au via coated vesicles, ultimately for delivery to lysosomes. It was suggested that this receptor-mediated process may serve "a cleansing function" following blood-brain barrier disruption during inflammatory reactions. Since macrophages do express gp30 and gp18, it is likely that they mediate this A-Au endocytosis. Macrophage removal of cytotoxic proteins may also serve a protective function necessary to maintain the integrity of the endothelial cell barrier in order to control excess tissue exudation and even thrombosis.

Relationship to Albumin-binding Proteins—Albumins, that are conformationally modified either by surface absorption to colloidal gold particles or by treatment with formaldehyde or maleic anhydride, not only bind gp30 and gp18 with much greater affinity than native albumin (21) but also are degraded by pathological stimuli, become residents of the arterial wall to as oxidized LDL by macrophages may serve to minimize local inflammatory reactions. Since macrophages do express gp30 and gp18, it is likely that they mediate this A-Au endocytosis. Macrophage removal of cytotoxic proteins may also serve a protective function necessary to maintain the integrity of the endothelial cell barrier in order to control excess tissue exudation and even thrombosis.

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