Ligand Affinity of the 67-kD Elastin/Laminin Binding Protein Is Modulated by the Protein's Lectin Domain: Visualization of Elastin/Laminin-Receptor Complexes with Gold-tagged Ligands

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**Abstract.** Video-enhanced microscopy was used to examine the interaction of elastin- or laminin-coated gold particles with elastin binding proteins on the surface of live cells. By visualizing the binding events in real time, it was possible to determine the specificity and avidity of ligand binding as well as to analyze the motion of the receptor–ligand complex in the plane of the plasma membrane. Although it was difficult to interpret the rates of binding and release rigorously because of the possibility for multiple interactions between particles and the cell surface, relative changes in binding have revealed important aspects of the regulation of affinity of ligand–receptor interaction in situ.

Both elastin and laminin were found to compete for binding to the cell surface and lactose dramatically decreased the affinity of the receptor(s) for both elastin and laminin. These findings were supported by in vitro studies of the detergent-solubilized receptor. Further, immobilization of the ligand–receptor complexes through binding to the cytoskeleton dramatically decreased the ability of bound particles to leave the receptor. The changes in the kinetics of ligand-coated gold binding to living cells suggest that both laminin and elastin binding is inhibited by lactose and that attachment of receptor to the cytoskeleton increases its affinity for the ligand.

CELL surface receptors for extracellular matrix components are important for cell movement and attachment, matrix assembly, and recognition and interpretation of matrix-associated signals that play a significant role in directing and maintaining cellular differentiation and homeostasis (4, 15). For these reasons, much effort has been directed at defining the structure and binding properties of protein families that mediate the interaction of cells with the extracellular matrix.

As matrix receptors become better characterized, it is clear that their mechanism of interaction with cell- or matrix-associated ligands is extremely complex. Several cell–matrix and cell–cell receptors, for example, have multiple ligand binding sites; one that recognizes peptide sequences in proteins, such as the Arg-Gly-Asp recognition sequence common to many integrins (19), and a second site that shows significant homology with lectin-like proteins (1, 6, 22). The precise function of the lectin domain is unclear, but its very existence suggests that interactions with carbohydrate may influence the biological properties of the receptor. In a few cases the influence of carbohydrate on receptor binding has been documented (2, 10, 11), although a clear picture of the mechanism whereby the lectin-binding site influences receptor function is not yet available.

Functional characterization of the 67-kD elastin/laminin receptor suggests that it has both protein and carbohydrate binding domains (11). In addition to interacting with elastin, this 67-kD protein also binds to laminin with an affinity approximately equal to that of elastin (nanomolar binding coefficient) and is similar, if not identical, to the 67-kD laminin receptor that is thought to mediate the metastatic properties of tumor cells (18). A striking feature of this receptor is that its affinity for elastin or laminin is greatly influenced by its lectin domain: in the absence of carbohydrate, the receptor binds to elastin or laminin with high affinity, whereas in the presence of galactoside sugar, little, if any, binding occurs.

To examine how the carbohydrate-binding site influences the functional properties of the elastin/laminin receptor, we used video-enhanced microscopy to study the binding of elastin- or laminin-coated gold particles to receptors on live cells. The gold particles behave as individual ligand molecules and can be used to predict both the location and binding properties of the receptor. The results of this study confirm the biochemical finding that carbohydrate decreases the affinity of the receptor for protein ligand and reveal that the receptor–ligand complex switches rapidly from free diffusion within the plasma membrane to directed transport along microfilaments.
Materials and Methods

Preparation of Elastin- and Laminin-coated Gold Particles

Gold particles (40 nm, EY Laboratories, Inc., San Mateo, CA) were incubated for 30 min at 4°C with 2 mg/ml laminin purified from Engelbreth-Holm-Swarm tumor as described by Kleinman et al. (14) or 1 mg/ml alpha- or etna-elastin peptides (Elastin Products Co., St. Louis, MO). Both ligands were dissolved in PBS. BSA was added to the ligand-gold suspension to block remaining protein binding sites and the gold particles were washed free of unbound ligand by centrifugation (10,000 g for 15 min), resuspended in PBS containing 2 mg/ml BSA to block remaining binding sites, washed with PBS by repeated centrifugation, and resuspended in one-tenth of the original volume of the gold suspension with bath sonication. Particles were used within 24 h of preparation.

Cell Culture and Video-enhanced Microscopy

Fibroblasts from the ligamentum nuchae of a late gestation bovine fetus were cultured from tissue explants (16) and plated after trypsinization on polylysine-treated glass coverslips in DMEM with 10% calf serum. After 2-3 d the coverslips were washed with and incubated in Hepes-buffered MEM without serum or phenol red indicator dye and with 2 mg/ml BSA. The elastin- or laminin-coated gold particles were sonicated to dissociate aggregates, diluted 1:4 with Hepes-buffered MEM and added to the cells. The coverslip was sealed and the cells observed by video-enhanced differential interference contrast (DIC) microscopy (23-25). Images were recorded on video tape. Particle binding to the cell surface was assessed by dividing the video screen into a 2 x 2 cm grid and following the position and binding time of particles within each square of the grid.

Results

Quantitation of Particle Binding to Individual Cells

Fibroblasts from the ligamentum nuchae of a late gestation bovine fetus are easily maintained in culture and have been shown to stably express the 67-kD elastin receptor in vitro (17). The rationale for using this cell type for the studies described in this report is the presence on the cell surface of a large number of high-affinity sites (2 x 10^6 binding sites per cell, K_d = 8 nM) that bind elastin with relatively little ligand internalization (27). Furthermore, the ligamentum nuchae contains essentially a single cell type so that by studying cells from this tissue we are ensured of sampling a homogeneous cell population. Because the elastin receptor interacts with laminin, it is also important to note that the ligamentum fibroblast produces elastin but no laminin.

Quantification of particle binding was performed on single FCL-270 cells, 2-3 d after plating on glass cover slips. After washing the cells to remove serum proteins, elastin- or laminin-coated gold particles were added to the culture medium (final concentration of gold particles was 3 x 10^10 particles/ml) and their binding to the cell surface was viewed and recorded using video-enhanced DIC microscopy. The number of particles that bound per unit area was determined from the video tape by freezing the image on a video screen every few seconds and counting the number of particles that were associated with a defined area of the cell surface. Maximal binding of both elastin- and laminin-coated particles was obtained after 30-50 min, which is in rough agreement with the time required to obtain saturation with radiolabeled tropoelastin (27). Fig. 1 is a representative video micrograph showing resolution of the video images for both elastin- and laminin-coated gold particles bound to the cell surface.

To assure appropriate comparisons between different cells in each experiment, the number of bound particles was determined after at least 40 min for several cells exposed to an equivalent concentration of ligand-coated particles. The binding to individual cells was surprisingly uniform for both elastin and laminin particles (Fig. 2), suggesting that the number of elastin and laminin receptors is similar from cell to cell and that valid estimates of ligand binding can be obtained as long as comparisons are made on cells from the same cell population at similar times after the addition of ligand-coated particles. The number of bound particles is in reasonable agreement with estimates of the number of bound radiolabeled ligand under similar conditions.

Specificity of Particle Binding

By definition, receptor-mediated binding requires specific recognition of ligand by the receptor. Specificity is generally established by demonstrating that binding of a marker-containing ligand is reduced in the presence of high concentrations of unlabeled ligand, and, similarly, that an unrelated protein does not interfere with ligand recognition. To demonstrate that particle binding to the surface of FCL cells was specific, elastin-coated particles were added in medium containing soluble elastin peptides. Fig. 3 shows that soluble peptides effectively inhibited particle binding to the cell. Similarly, beads coated with BSA, a protein that does not bind to the elastin receptor or to any other cell surface receptor on the fibroblast, demonstrated no binding.

Effects of Carbohydrate on Elastin Binding

Our earlier characterization of the elastin/laminin receptor predicts that galactoside sugars should interfere with elastin-particle binding to FCL cells. To test this prediction, elastin-coated beads were added to cells in medium containing 30 mM lactose. Fig. 4 shows that, in the presence of sugar, only half as many particles bound to the cell surface. To ensure that this diminished binding did not result from factors extrinsic to the elastin receptor, maltose, a disaccharide that does not interact with the elastin receptor, was added in place of lactose with elastin-coated particles. In contrast to decreased particle binding observed with lactose, maltose had no effect (Fig. 4). A second control was to eliminate the possibility that lactose was producing a general inhibition of all cell surface binding events. For these experiments we chose to determine whether lactose altered binding of con A-coated particles to con A binding sites (i.e., mannos specific) on the ligament cell surface. Although fewer con A beads bound to the cell, as seen in Fig. 4, there was no alteration in con A particle binding in the presence of lactose. These results suggest that decreased elastin particle binding in the presence of lactose is a specific effect of the disaccharide on the elastin receptor and not a global inhibition of surface binding.

Elastin and Laminin Interact with the Same Receptor

In addition to interacting with elastin, the elastin receptor has been shown to bind to laminin in vitro (16) with an affinity that approximates elastin binding. When laminin-coated beads were incubated with FCL cells, binding similar to that for elastin was observed (Fig. 5). Binding was

The Journal of Cell Biology, Volume 113, 1991 188
Figure 1. Video micrographs of FCL-270 cells showing the binding of ligand-coated particles to lamellipodial regions of the cells. Particles coated with BSA failed to bind (A) and served as a control. B and C show binding of laminin- and elastin-coated particles, respectively. Bar, 2 μm.
Figure 2. Comparison of relative particle binding to multiple cells. Video images of FCL-270 cells incubated with elastin- or laminin-coated particles were analyzed by dividing the video screen into a 2 x 2 cm grid (corresponding to an area of 2 x 2 μm on the cell surface) and counting the number of particles bound per unit area. After a 30-min incubation to obtain equilibrium, particle counts were made every 30 s for each individual cell. Shown is the mean ± SD of 20 different determinations for three cells exposed to elastin particles and two cells incubated with laminin-coated particles.

hibited when soluble laminin was added to the incubation medium, thereby supporting specific binding.

To determine whether laminin-coated particles were interacting with the elastin receptor, elastin peptides were included in the incubation buffer as a competing ligand. Fig. 5 shows that the decrease in laminin-particle binding in the presence of elastin peptides was equivalent to the low level of binding observed with soluble laminin. In contrast, BSA had no effect on binding of the laminin-coated gold. Further evidence that laminin is interacting with the elastin receptor was demonstrated by a diminution of laminin-coated particle binding in the presence of lactose. As with elastin, laminin-particle binding was approximately 50% less in the presence of lactose (Fig. 5). Maltose, as a control, induced a slight enhancement of binding (not shown).

The Presence of Galactoside Sugars Decreases the Binding Time of Elastin and Laminin-coated Particles

The observation that 50% of the elastin- or laminin-coated particles remain associated with the cell surface in the presence of lactose was surprising given that lactose has been shown to dramatically decrease the ability of the receptor to interact with elastin or laminin affinity columns (11, 17). When the videotapes of the particle binding experiments were examined closely, however, it became evident that those beads that remained associated with the cell surface in the presence of lactose bound for much shorter times than in the absence of sugar. The histogram in Fig. 6 shows that in the presence of lactose the majority of laminin-coated particles bind to the cell for only 3-9 s, whereas in the absence of lactose, binding times of >100 s were observed. Many of the particles which release in the presence of lactose rebind to the surface and some were followed through five to eight rebinding events before they were lost to the medium. Particles that bind for intermediate periods of 27 and 86 s are also evident in the presence of lactose. A similar alteration in particle binding time was observed for elastin beads in the presence of lactose (not shown). Interestingly, no intermediate binding times were detected for elastin particles.

Receptor Diffusion and Ligand Transport

When the motion of surface bound elastin- or laminin-coated particles was determined, two distinct modes of movement were observed. Immediately after binding, the particles...
demonstrated random diffusion followed by directed transport towards the nucleus along microfilaments. The diffusional properties of the elastin- and laminin-coated particles appeared to be similar, with a calculated diffusion coefficient of $1 \times 10^{-7}$ cm$^2$/s. With increasing time after binding, both the diffusional and rotational motion of the surface-bound particles would end abruptly and the particles would begin to be transported back towards the nucleus of the cell. Interestingly, the transition from random diffusion to directional transport seemed to be associated with particles becoming aligned with visible stress fibers in the cell (see Fig. 1 C), suggesting that coupling of the receptor to the cytoskeleton may be responsible for this change in motion.

Fig. 7 documents the movement of an elastin-coated particle along a stress fiber over a period of $\sim$30 min. Midway through this sequence, a second particle can be seen to bind to a neighboring stress fiber and begin rearward transport. Interestingly, both particles move with comparable velocity, suggesting that a similar motor is driving their movement along the cytoskeleton.

In the presence of lactose, both the lateral and rotational diffusional properties of the beads that bind to the cell surface appear accentuated. Fewer particles undergo the transition from random diffusion to rearward transport, probably due to a shorter residence time for the particle on the cell surface. It is important to note, however, that those particles that do make this transition to the transported state lose their sensitivity to lactose and remain tightly bound to the cell surface. We were not able to release the bound particles with the addition of lactose.

**Discussion**

Our understanding of receptor–ligand interactions primarily stems from biochemical studies designed to provide quantitative analysis of the specificity and affinity of macromolecular associations. These kinds of measurements, however, represent an average of the total binding events and do not assess changes in a single molecule's behavior. Much more could be learned if the association of ligand with receptor could be "visualized" in real time on living cells where the binding properties and movements of the receptor-ligand complex through the plasma membrane could be related directly to aspects of cellular ultrastructure or membrane fluidity. Using video-enhanced microscopy to examine the binding properties of ligand-coated gold particles, we were able to visualize and quantify alterations in affinity of the elastin/laminin receptor as well as analyze the motion of the receptor-ligand complex in the plane of the plasma membrane of live cells.

**Kinetics of Particle–Receptor Interactions: Negative Cooperativity and Competitive Binding**

In earlier studies we showed that the carbohydrate binding properties of the 67-kD elastin/laminin receptor greatly influence the receptor's ability to interact with protein ligand in vitro (11, 17, 18). Since elastin is not a glycoprotein and, hence, can not directly interact with a lectin site on the receptor, we proposed that the affinity of the receptor for elastin was diminished in the presence of appropriate carbohydrate. We have now shown by these elastin-gold binding studies that the receptor in situ has a decreased affinity for elastin in the presence of lactose. The total time that each particle remains associated with the cell surface decreases dramatically compared with the control. This is in contrast to the behavior of particles added together with excess soluble elastin peptides where total binding is reduced to only a few particles per cell, but the duration of particle binding is equivalent in the presence or absence of peptides.

If we consider particle binding in terms of a ligand–receptor interaction, the properties of receptor-bound particles in the presence of lactose and elastin peptides are precisely those predicted for negative cooperativity and competitive inhibition, respectively. Because the ligand in our study is a particle that is difficult to describe in terms of concentration, the association rate constant cannot be easily determined. With video images, however, it is possible to establish when a particle binds to a receptor and to define the stability of the receptor–ligand interaction in terms of the length of time that the particle remains bound.

For cells treated with lactose, the average binding time for elastin- or laminin-coated particles is dramatically reduced compared with controls, suggesting a lactose-induced increase in the dissociation constant and an overall reduction in receptor affinity. This is typical of negative cooperativity where binding of one ligand to a receptor site reduces the affinity of an adjacent site (3) and is in agreement with predictions of elastin receptor function (17). We have observed that a released particle often rebinds to the membrane several times in the presence of lactose, indicating that receptors rapidly reassociate with ligand after ligand dissociation. This provides a partial explanation for why the total number of bound particles remains relatively high ($\sim$50% of control) in the presence of carbohydrate. It is important to consider that even though receptor affinity is reduced in the presence of lactose, the total number of binding sites on the cell is unchanged. The binding, even though reversible, will concentrate ligand in the region of the receptor.

When soluble elastin peptides are present in cell culture medium the number, but not the duration, of elastin and laminin particle binding is reduced. These results fit the prediction for a competitive inhibitor which has the effect of reducing the number of binding sites available to a particle
Figure 7. Retrograde transport along microfilaments. Video micrographs taken over a period of 32 min showing the retrograde transport of elastin-coated gold particles on the surface of fibroblasts. The circled particle is initially diffusing, then attaches to the cytoskeleton (at ~8 min) and tracks rearward (13 and 20 min). Particles noted by the arrow and triangle are steadily tracking rearward from their time of notation. The particle marked by the triangle came on to the cell at ~18 min and rapidly attached to the cytoskeleton. Bar, 2 μm.
but does not alter either the association or dissociation constant. Therefore, ligand that binds to a receptor in the presence of a competitive inhibitor should do so with no change in receptor affinity. Our experimental observations for binding of elastin- or laminin-coated particles in the presence of excess soluble elastin peptides are consonant with this prediction.

**Competition between Elastin and Laminin for the Same Receptor**

Biochemical studies have demonstrated that the 67-kD receptor can interact with laminin or elastin with equal affinity (21) but it has not been known whether or not other receptors would be active in situ or if the same behavior would exist in a living cell. The demonstration in this current study of inhibition of elastin particle binding by soluble laminin, as well as inhibition of laminin particle binding by soluble elastin, suggests that there is no difference in binding properties of either ligand. Furthermore, decreases in the duration of laminin particle binding in the presence of lactose reinforce the notion that receptor affinity for laminin, like elastin, is reduced in the presence of carbohydrate, providing even more evidence for the equivalency of binding for both ligands. It is interesting to note that while the duration of laminin particle binding in the presence of lactose showed the same dramatic decrease as seen for elastin-particles, several intermediate binding times were evident when interactions with the high-affinity receptor was reduced by carbohydrate. There are two likely explanations for this, one being that the intermediate values represent binding of laminin to other receptors that are lactose-insensitive but of lower affinity than the 67-kD receptor. Although the nature of these other receptors is unknown, several members of the integrin receptor family have been shown to interact with laminin (8, 9, 13), and, hence, are likely candidates. That the binding sites might be integrins would also be consistent with the absence of intermediate binding times observed for elastin-particle binding in the presence of lactose since elastin does not interact with any of the known integrins. A second possibility is that the laminin–gold particles are binding to several receptors. The multivalent nature of the ligand–gold particle complexes poses this problem, which is probably more serious for those particles which bind to the surface for longer times. We are producing monovalent ligand–gold complexes to test between these possibilities.

**Receptor Movement on the Plasma Membrane and Interactions with the Cytoskeleton**

In addition to providing a “visualization” of receptor–ligand binding properties, the power of the video-enhanced, particle-binding technique was particularly evident when the fate of the receptor–ligand complex was followed in real time. Our results demonstrate that both elastin- and laminin-coated particles undergo an initial random diffusion upon binding to the cell surface followed by a sudden switch to directed transport back towards the cell nucleus. In this respect, the behavior of the particles is similar to that observed previously for con A-coated particles bound to the surface of macrophages (24) and suggests that rearward transport is due to attachment of the receptor to the cytoskeleton. In agreement with this possibility is the observation that those elastin- and laminin-coated particles that show retrograde motion are most often aligned over visible stress fibers (Figs. 1 and 7). Since actin filaments in the lamellipodial region of the cell are known to be moving rearward (12, 26), receptors associated with the actin cytoskeleton would be transported towards the rear of the cell.

In addition to providing a motor for directed transport, attachment of the receptor to the cytoskeleton suppresses its ability to interact with lactose such that binding of elastin- or laminin-coated particles is no longer inhibited by carbohydrate. It is not known, however, whether the mechanism involves inactivation of the carbohydrate domain or an uncoupling of the regulatory elements linking the carbohydrate and protein binding sites. The possibility that the cytoskeleton can regulate ligand–receptor interactions may have important implications for the mechanism of ligand-induced effects.

Whether the receptor interacts directly or indirectly with intracellular actin is still uncertain, since opinions differ as to whether the 67-kD receptor is an integral or peripheral membrane protein. Initial characterization of the laminin receptor suggested that it was an integral membrane protein (7, 20), although the protein encoded by a recently characterized cDNA for the receptor does not contain a typical transmembrane domain (21, 28). Our own characterization of the 67-kD elastin receptor suggests that it is a peripheral, not an integral membrane protein and we have proposed that it interacts specifically with one or more transmembrane proteins to form a functional receptor complex (18). In this model, the transmembrane component would, of necessity, mediate the interactions between the 67-kD protein and the cytoskeleton. Whether the transmembrane subunit binds directly to intracellular actin, or interacts via talin as has been postulated for the integrin receptors (5), remains to be established. It is also not known whether the possible multivalent nature of the ligand–gold complexes facilitates cytoskeletal attachment through cross-linking of receptors.

In summary, our results suggest the following sequence of receptor–ligand interactions: elastin or laminin binds to a receptor that is initially uncoupled from the cytoskeleton and undergoing random diffusion within the plane of the membrane. The presence of bound ligand imparts information to the receptor, either directly or through cross-linking of receptors, that enables or directs its association with cytoskeletal elements where its diffusional and rotational properties become restricted and various cytoskeletal motors move the receptor–ligand complex to appropriate sites on the cell surface. In addition, linkage with the cytoskeleton provides informational signals that inactivate the carbohydrate binding domain on the receptor, thereby locking in the protein ligand until another signal elicits its release. Although each step in this sequence needs to be confirmed independently, these studies of ligand–gold binding to the surfaces of living cells reinforce the concept of bidirectional signaling, through the receptor, between the extracellular and intracellular compartments. They also confirm that the lectin properties of the 67-kD protein are important for its normal function as a laminin- and elastin-binding protein.

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