Functional characterization of the distal long arm of laminin: Characterization of Cell-and heparin binding activities

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Abstract

Basement membrane laminin is a multidomain glycoprotein that interacts with itself, heparin and cells. The distal long arm plays major cell and heparin interactive roles. The long arm consists of three subunits (A, B1, B2) joined in a coiled-coil rod attached to a terminal A chain globule (G). The globule is in turn subdivided into five subdomains (G1-5). In order to analyze the functions of this region, recombinant G domains (rG, rAiG, rG5, rGΔ2980-3028) were expressed in *Sf9* insect cells using a baculovirus expression vector. A hybrid molecule (B-rAiG), consisting of recombinant A chain (rAiG) and the authentic B chains (E8-B)was assembled *in vitro*. The intercalation of rAiG into E8-B chains suppressed a heparin binding activity identified in subdomain G1-2. By the peptide mapping and ligand blotting, the relative affinity of each subdomain to heparin was assigned as G1> G2= G4> G5> G3, such that G1 bound strongly and G3 not at all. The active heparin binding site of G domain in intact laminin appears to be located in G4 and proximal G5. Cell binding was examined using fibrosarcoma cells. Cells adhered to E8, B-rAiG, rAiG and rG, did not bind on denatured substrates, poorly bound to the mixture of E8-B and rG. Anti-$\alpha 6$ and anti-$\beta 1$ integrin subunit separately blocked cell adhesion on E8 and B-rAiG, but not on rAiG. Heparin inhibited cell adhesion on rAiG, partially on B-rAiG, and not on E8. In conclusion, 1) There are active and cryptic cell and heparin binding activities in G domain. 2) Triple-helix assembly inactivates cell and heparin binding activities and restores $\alpha 6 \beta 1$ dependent cell binding activities.

Introduction

Laminin is a major glycoproteins of basement membranes which plays both architectural and cell interactive roles. The latter functions, mediated by a variety of cell surface receptors, regulate cell adhesion, spreading, migration, and differentiation. Laminin is an asymmetrical cruciform heterotrimer consisting of an A, a B1, a B2 (alternatively, called $\alpha 1$, $\beta 1$, $\gamma 1$) (Fig.1). The long arm cinsists of a rod-like coiled-

coil structure, assembled from all three subunit chains, and a large distal globule (G domain), projecting the A chain end from the rod. The distal globule is further subdivided into five homologous subdomains, G1-G5. The middle of the α-helical rod and the distal region of subdomain G3 contain protease sensitive sites which can be cleaved by elastase to generate fragment E8 (containing the distal half of the rod and the proximal portion of the G domain) and E3 (the distal end of the G domain starting at residue 2666).

The long arm has been found to play a major cell-interactive roles, stimulating neurite outgrowth, cell attachment, and promoting migration. A variety of integrins ($\alpha 6 \beta 1$ in many cell types, $\alpha 6 \beta 4$ in keratinocytes, $\alpha 7 \beta 1$ in myoblasts) and non-integrin cell surface proteins (including galactosyl transferase) have been reported to interact with the E8 region of laminin. Antibody specific for fragment E8 blocks epithelial polarization in kidney development. Laminin receptor $\alpha 6 \beta 1$ is prominently expressed in development of chick retina. Antibody against the $\alpha 6$ subunit of integrin prevents the conversion of mesenchyme to tubule in developing kidney. There is increasing evidence that cell-adhesion supporting activity of fragment E8 is conformation-dependent. Denaturation of E8 with urea or heating irreversibly destroyed its activity (Goodman et al. 1987, 1991).

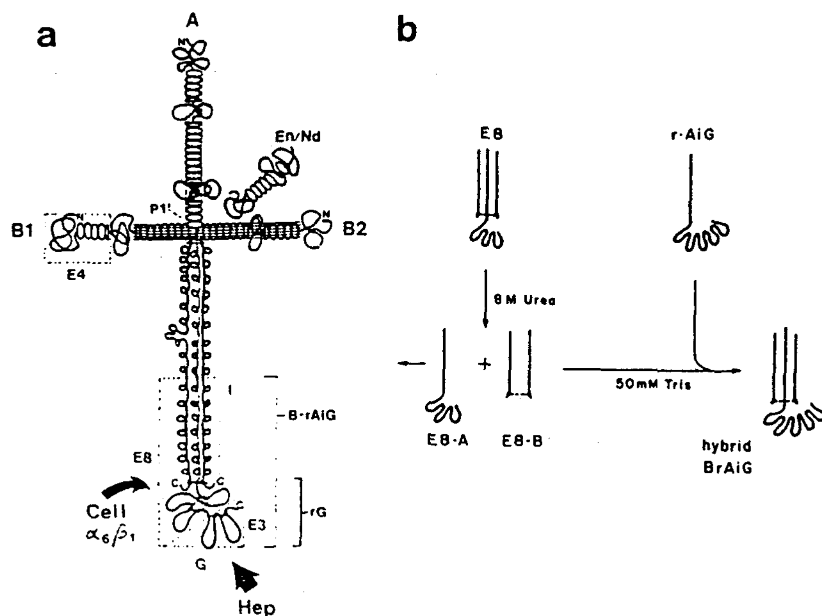


Fig. 1.A. Laminin structure and scheme for the assembly of B-rAiG. a. Laminin structure, laminin long arm fragments E8 and E3, recombinant G domain constructs. b. Hybrid assembly by the intercalation of recombinant AiG with laminin E8-B chains.

Fragmentation of E8, in which most of the rod-region or globular domain was cleaved, inactivated the substrate with respect to support adhesion or spreading (Deutzmann et al. 1990). Thus a specific conformation involving A and B chains appears to be required for cell recognition and this has posed a special problem in the dissection and mapping of the E8 site.

Some cells can recognize the E3 region. Heparan sulfate proteoglycans, $\alpha 3 \beta 1$ integrin, and other glycoproteins have been proposed as E3-recognizing cell surface proteins. The E3 region has been known to possess a major heparin binding activity of laminin. This function may play a role in the modulation of laminin network formation (Yurchenco et al. 1990) and in anchoring to cell surface or basement membrane heparan sulfate proteoglycans. Although the functions of heparan sulfate proteoglycans in basement membrane and on cell surface are partially understood, they are thought to be important regulators of cell behavior in development.

In this study, recombinant A chain glycoproteins were expressed and an A chain recombinant glycoprotein was intercalated into the B chains of laminin fragment E8. The hybrid glycoprotein and recombinant glycoproteins were used for the characterization of the functions of the distal end of the long arm. By this approach, we identified that the activities identified in laminin are different from that of its isolated subunits.

Methods, Results and Discussions

The recombinant G domains (rG, rAiG, rG5, rGΔ2980-3028) were expressed in Sf9 insect cells infected by recombinant baculovirus . The proteins were found in media, as secreted proteins and glycosylated with simple mannose. The observed molecular weights of rG and rAiG were 120 kD and 160 kD on SDS-PAGE, larger than the expected size 107kD and 133kD, respectively. It is likely that these differences are due to carbohydrates. rG5 migrated with a size of 25kD and rGΔ2980-3028 was observed as a 115kD band. The recombinant proteins were purified as described before (Yurchenco et al. 1993).

A hybrid molecule, B-rAiG, was prepared by intercalating rAiG with the isolated B chains *in vitro* (Fig.1). The triple helix structure is energetically stable and can be reconstituted *in vitro* after complete dissociation of the three chains (Hunter et al. 1992). Recombinant AiG (rAiG), which has never been exposed to denaturing reagent, was intercalated into the B chains, which were stripped from fragment E8. The hybrid molecule (B-rAiG), containing authentic B1-2 chains and rAiG, possessed a ball and rod shape morphology and showed a similar biochemical property as observed in the laminin long arm of authentic laminin (Sung et al. 1993).

Heparin binding affinity of recombinant G domains and the hybrid (B-rAiG) were compared to that of authentic laminin and its fragments. Heparin affinity HPLC

chromatography was used and the relative affinity of proteins was deduced from the eluting salt concentrations. First, intact laminin and fragment E3 eluted at the same salt concentration from the column. Fragment E8 only weakly bound to the column (Fig.2). This result was consistent to the previous reports that E3 is the major heparin binding fragment of laminin. However, denatured E8 or the A chain of E8 required a much higher concentration of salts to elute from the column, indicating that the A chain alone binds to heparin with a strong affinity, but is suppressed in E8 and intact laminin. rG and rAiG bind to the heparin column with the same affinity. They bind to heparin with a stronger affinity, comparing to fragments rG50 (subdomain G4-5) and rG70 (subdomain G1-3). rG70 eluted at a higher salt concentration from the column, comparing to rG50. As expected, triple-helix assembly suppressed the heparin affinity of rAiG (Fig.3). B-

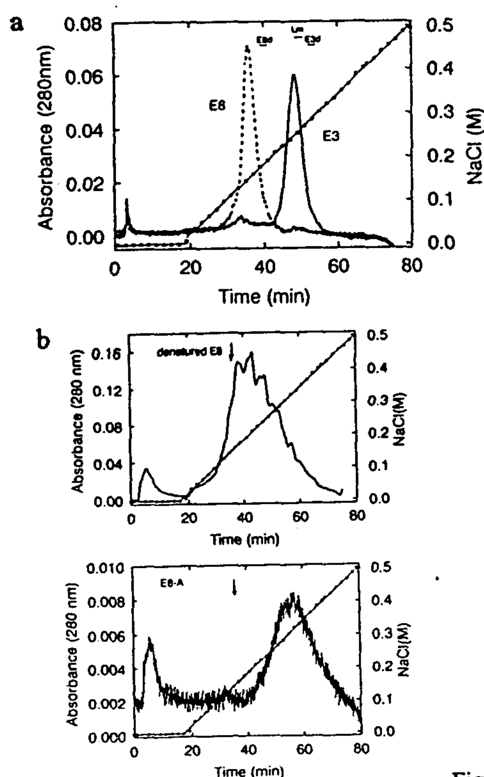


Fig.2.

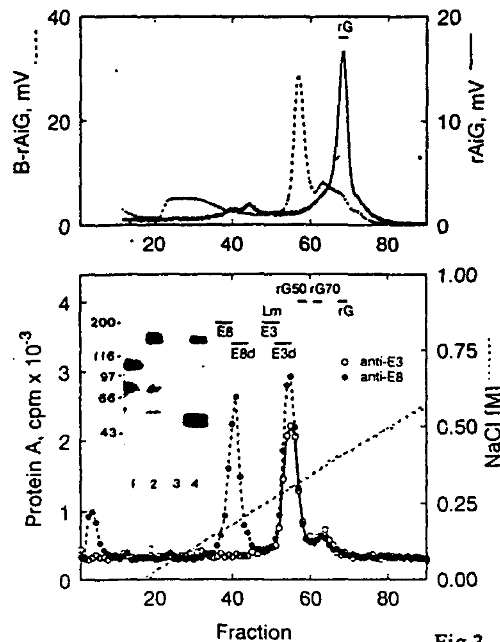


Fig.3.

Fig.2.a. Comparison of heparin binding affinities of laminin, E3, and E8. b. Effect of denaturation and chain separation.

Fig.3. Relative heparin binding affinities of recombinant, hybrid, and native proteins.

Top: rG (bar), rAiG (solid line), and B-rAiG (dashed line). Bottom: Heparin binding affinity of elastase cleaved B-rAiG. inset: anti-E8 (1,2), anti-E3 (3,4). The 40 min peak (1,3) contains the "E8-like" fragment and the 55 min peak (2,4) contains rG50 ("E3-like" recombinant fragment).

rAiG eluted at a significantly lower concentration of salt than rAiG or rG alone. The affinity of B-rAiG to heparin was same as that of rG50. B-rAiG was partially digested with elastase and applied to heparin column. The peak, eluted at the same salt concentration as the intact molecule, contained rG50. The new peak, eluted at a much lower concentration of salt, contained the B chains and a 110 kD band. The B chains and the 110 kD band were interpreted to form an "E8-like" protein, based on the size and antigenicity. These results also support that the heparin-binding activity of subdomain G1-3 was suppressed by the intercalation of the A chain into the B chain moieties.

Heparin binding affinity of each subdomain was further evaluated by peptide mapping and western-type ligand blotting (Fig.4). The affinities of deletion mutant G domains were also compared. The relative affinity of each subdomain was identified as G1> G2= G4> G5> G3, such that G1 bound strongly and G3 not at all (Fig.5). The active heparin binding site of laminin was located in the region encompassing subdomain G4 and the proximal end of G5. The distal end of G5, previously proposed as the heparin binding site of laminin, was turned out to be not the case.

Human HT1080 fibrosarcoma cells possess the laminin specific $\alpha6\beta1$ integrin which selectively interacts with the E8 fragment. The adhesion-promoting activities of authentic, recombinant, and hybrid molecules were evaluated using this cell lines. B-rAiG and rAiG, like E8, supported the cell attachment (Fig.6). The A chain and B chains of E8

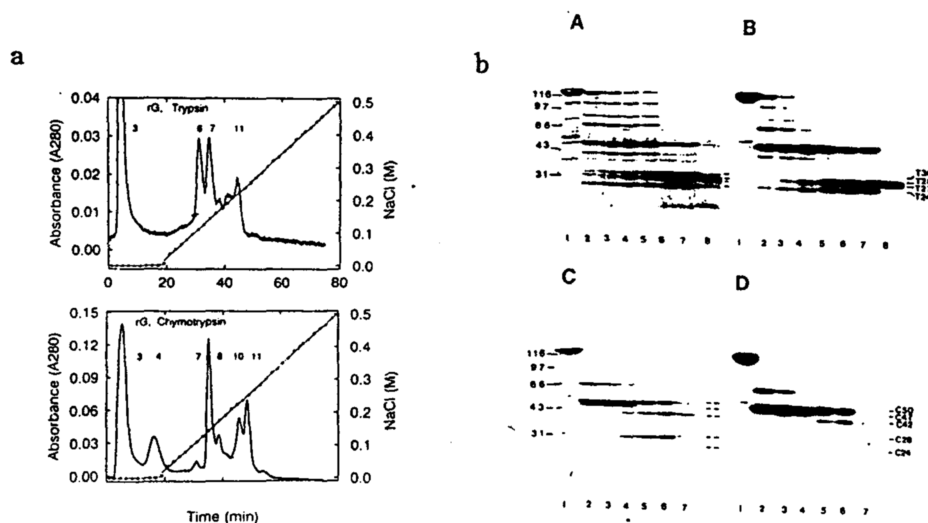
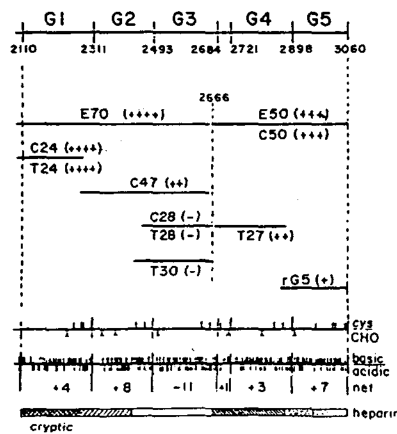
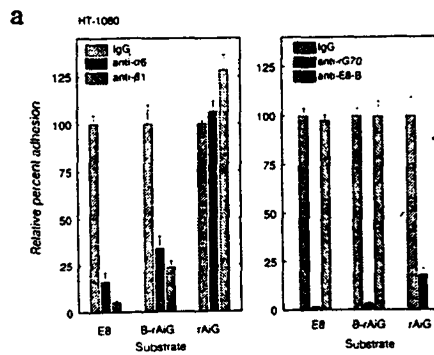


Fig.4.a.Heparin binding to tryptic and chymotrypuc fragments of rG. b. Ligand blotting of trypsin and chymotrypsin digested rG with radioactive heparin.

did not support the adhesion. Neither the denatured molecules nor the mixture of recombinant A chain and the B chains of E8 did support cell-adhesion. Cell adhesion of B-rAiG, like E8, was blocked by antibody against $\alpha 6$ and $\beta 1$ integrin subunits. None of the antibodies blocked the cell adhesion on rAiG. The antibody against G domain blocked the adhesion on all substrates (E8, B-rAiG, rAiG), but the anti-B chain antibody did not have any effect.

In summary, the relationship between structure and function of laminin is rather complex, therefore should include the discussion of its tertiary and quaternary structure. The denatured proteins lost its cell-interactive roles and showed a change in heparin binding. The cell and heparin binding functions identified in subunit chains (rG, rAiG) were different from that of the intact molecules (B-rAiG, E8, laminin). A quaternary structure formation restored $\alpha 6 \beta 1$ dependent cell binding activity, but suppressed





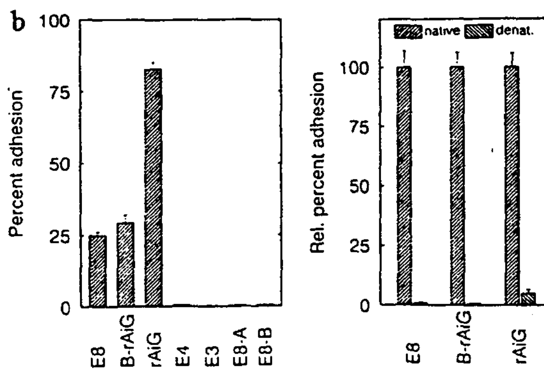


Fig.5.

Fig.6.

Fig.5. Summary of the heparin binding affinity of G domain.

Fig.6. a. Adhesion of HT1080 cells to native, recombinant, and hybrid proteins. b. Antibody inhibition of HT1080 adhesion on native, recombinant, and hybrid proteins.

non-integrin dependent cell binding activity. The supramolecular assembly also inactivated a heparin binding activity present in proximal G domain. In this study, we have shown that it is possible to reconstitute the functions by *in vitro* assembly of the supramolecule, thereby opening a new avenue to study these functions.

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