Cell and Heparin Binding in the Distal Long Arm of Laminin: Identification of Active and Cryptic Sites with Recombinant and Hybrid Glycoprotein

Uhna Sung,* Julian J. O'Rear,‡ and Peter D. Yurchenco*

*Departments of Pathology and ‡Molecular Genetics and Microbiology, Robert Wood Johnson Medical School, Piscataway, New Jersey 08854

Abstract. The long arm of laminin, which binds heparin and cells, consists of three polypeptides (A, B1, and B2) joined in a coiled-coil rod attached to a terminal A chain globule (G). Previously, we found that recombinant globular domain (rG) supported heparin and myoblast binding (Yurchenco, P. D., U. Sung, M. D. Ward, Y. Yamada, and J. J. O'Rear. 1993. *J. Biol. Chem.* 268:8356–8365). To further analyze long arm functions, we expressed the distal moiety of the mouse laminin A chain extending from the middle of the rod to the carboxyl terminus (rAiG). This larger glycoprotein, secreted by Sf9 insect cells infected with recombinant baculovirus, was intercalated in vitro into the corresponding disulfide-linked B chain segments of laminin fragment E8 (distal long arm rod and proximal globule). The hybrid molecule (B-rAiG) possessed a structure similar to laminin long arm as judged by electron microscopy and limited proteolysis. By joining rAiG with E8-B chains, the affinity of G domain for heparin decreased from that observed with rAiG and rG to one similar to native protein. HT1080 cells adhered to E8, rAiG, and B-rAiG, less well to rG, and not to denatured E8/B-rAiG, the A and B chain moieties of E8, or to a mixture of rG and E8-B chains. Cell adhesion to E8 and B-rAiG, in contrast to rAiG, was inhibited with antibodies specific for $\alpha 6$ and $\beta 1$ integrin chains. Since intercalation (*a*) restored a conformationally dependent $\alpha 6\beta 1$ integrin recognition site present in native protein, (*b*) inactivated a cryptic cell binding activity in the A chain, and (*c*) inhibited a heparin binding site present in proximal G domain, we conclude that biological activities of laminin are different from that of its isolated subunits.

LAMININ is a major glycoprotein of basement membranes which plays both architectural (Yurchenco et al., 1985, 1990, 1992, 1993) and cell-interactive roles. The latter functions, mediated by a variety of cell surface receptors, include regulation of cell adhesion, spreading, migration, growth, and differentiation (reviewed in Beck et al., 1990 and Mecham, 1991). The three subunit chains of laminin (A, B1 and B2) are bound together through disulfide and noncovalent linkages forming an asymmetrical, multidomain molecule with one long and three short arms. The long arm is composed of a rod-like coiled-coil (subdivided into domains I and II by an α-helical interruption of the B1 chain) derived from all three chains and representing the region of chain union, and a large distal globule (G domain) formed only by the COOH-terminal moiety of the A chain (Beck et al., 1990; Hunter et al., 1990, 1992; Paulsson et al., 1985). This terminal globule is further subdivided into five homologous subdomains, G1–G5, which have been recognized in negatively stained preparations of the long arm (Beck et al., 1990). The middle of the α-helical rod and the distal region of subdomain G3 (the latter in what appears to be a hinge region) contain protease-sensitive sites which can be cleaved by elastase (Beck et al., 1990) to generate fragment E8 (containing the distal half of the rod and the proximal portion of the G domain with a single disulfide link between the COOH-termini of the B1 and B2 chain segments) and fragment E3 (the distal end of the G domain starting at residue 2666).

The long arm has been found to play major cell-interactive roles, stimulating neurite outgrowth, promoting cell migration, and providing a crucial ligand for tubular differentiation in kidney development (Goodman et al., 1992; Klein et al., 1988; Sorokin, 1990). A variety of integrins ($\alpha 3\beta 1$, $\alpha 6\beta 1$, $\alpha 7\beta 1$), cell surface galactosyl-transferase, and other receptors have been reported to recognize this region of laminin (Aumailley et al., 1990; Begovic and Shur, 1990; Gehlsen et al., 1992; Kleinman et al., 1991; Kramer et al., 1991; Sonnenberg et al., 1990; von der Mark et al., 1991). The $\alpha 6\beta 1$ integrin has been shown to bind to fragment E8 (Aumailley et al., 1990; Sonnenberg et al., 1990) and antibodies to this fragment block adhesion of many cells to laminin. Antibodies specific for fragment E8 (Klein et al., 1988) and for the $\alpha 6$ integrin subunit (Sorokin et al., 1990) were observed to separately prevent the conversion of mesenchyme

to tubule in kidney organ culture. While both a sequence present in domain I of the A chain (IKVAV) and a sequence (RNIAEIIKDI) present in domain I of the B2 chain have separately been proposed as active sites for cell adhesion and neurite outgrowth (Liesi et al., 1989; Tashiro et al., 1989) it is difficult to reconcile these conclusions with the observation that several cell lines bind only to native laminin and E8 with nearly complete and irreversible loss of recognition following denaturation of substrate or limited proteolytic fragmentation (Deutzmann et al., 1990). Thus a specific conformation involving A and B chains, generally lost upon dissociation and reassociation, appears to be required for cell recognition and this has posed a special problem in the dissection and mapping of the E8 site.

The globular domain of the long arm possesses additional activities. First, this domain binds to heparin (Ott et al., 1982; Skubitz et al., 1991; Yurchenco et al., 1990, 1993) at sites present in the proximal and distal moieties of G domain. These sites may play a role in the modulation of laminin network formation and serve as anchorage sites for cell surface heparan sulfate proteoglycans. Second, there is evidence suggesting an $\alpha 3\beta 1$ integrin site is also present in the G5 subdomain (Gehlsen et al., 1992) although the distal E3 portion of G domain may play only a minor role for adhesion of some cell types (Sorokin et al., 1992).

Recently, genetic engineering has proved to be a valuable tool to generate extracellular matrix glycoproteins for the study of their functions (Fox et al., 1991; Nischt et al., 1991; Tsao et al., 1990; Yurchenco et al., 1993). One particularly useful method to express glycoproteins at high levels is the baculovirus expression system, which has been used to generate a variety of biologically active molecules with a typically high mannose N linked oligosaccharide content (Jarvis and Summers, 1989; Luckow and Summers, 1988). In a recent study using the baculovirus system, the biological roles of isolated recombinant G domain lacking upstream rod domain sequences were evaluated (Yurchenco et al., 1993). This glycoprotein (rG), rich in mannose, was found to possess an oval shape and size, β-rich structure, protease-resistant and -sensitive sites, and intrachain disulfide heterogeneity expected for native G domain. rG supported C2C12 murine myoblast cell adhesion and spreading (similar to that observed with fragment E8) and possessed at least two heparin binding sites (Yurchenco et al., 1993). From the studies of Deutzmann et al. (1990), there is evidence that at least several laminin long arm cell-recognition activities require rod domain as well as globular domain determinants. Given evidence to suggest that the rod and globule can cooperatively define cellular functions in laminin, which might also extend to heparin binding, we decided to evaluate cell and heparin interactions with G domain connected to its rodlike region.

It seemed likely that such a study could be initiated without first expressing all three chains of laminin. In particular, it has been reported that the triple coiled-coil of fragment E8 can be spontaneously reconstituted in vitro after complete dissocation of the three chains of E8 (Deutzmann et al., 1990; Hunter et al., 1990; Hunter et al., 1992). The denaturation required to dissociate the chains of E8 typically inactivates cell binding. However, as will be shown, this problem is circumvented if the A chain, as a recombinant glycoprotein, is never exposed to denaturing buffers. Such a recombinant approach then holds promise as means to further investigate the functions of this region of laminin.

Materials and Methods

DNA Constructions, Screening, Expression, and Purification of Recombinant Protein

Construction, transfection, and screening of recombinant baculovirus was performed essentially as previously described (Yurchenco et al., 1993). A modified baculovirus expression vector (AiG/pVL1392SS) encoding the rat fibronectin signal fused to the mouse laminin A chain (bp 5708–9520, corresponding to amino acid residues 1864 to the COOH terminus) was prepared by ligating a PstI cDNA fragment (laminin A chain bp 5708 to 6438 and present in AiG/pGEM [Yurchenco et al., 1993]) into the PstI cleavage site of AG/pVL1392SS (which encodes the fibronectin signal and mouse laminin G domain; Yurchenco et al., 1993). This was followed by linearization of the plasmid by partial digestion with PstI, and deletion of four base pairs between the fibronectin and laminin sequences using T4 DNA polymerase (Sambrook et al., 1989) to place the laminin nucleotide sequence in-frame with that of the fibronectin signal sequence. Cotransfection of Sf9 cells with AiG/pVL1392SS and baculovirus DNA was carried out by calcium phosphate precipitation as modified for insect cells and as described by Summers and Smith (1987). Recombinant virus was cloned by end point dilution and tissue culture medium was tested for protein production by Western analysis with E3-specific antibody (Yurchenco et al., 1993). A secreted recombinant protein (rAiG) was identified. For production of several mg of rAiG or the shorter recombinant G domain (rG), exponentially growing Sf9 cells, maintained in complete Grace's medium (GIBCO-BRL, Gaithersburg, MD), supplemented with 10% FCS (Upstate Biotechnology Inc., Lake Placid, NY) were infected with recombinant virus at an estimated multiplicity of infection of two to three, transferred to serum-free Sf900 medium (GIBCO-BRL) for generation of protein, and then grown for 63 to 72 hours in 400–600-ml/flask in several 1-liter micro-carrier spinner flasks (Bellco, Vineland, NJ) at 28°C with a rotation rate of ~100 rpm. The media were harvested by centrifugation to remove cells and debris and the protein purified as described in detail for rG (Yurchenco et al., 1993). Briefly, the medium supernatant was dialyzed against 50 mM NH_4HCO_3, pH 7.8, containing 5 mM EDTA, 0.5 mM PMSF in the cold and then passed down a heparin-Sepharose-4B affinity column (20 ml of beads) equilibrated in the same buffer. The bound protein was eluted with a 120-ml linear NaCl gradient in 50 mM Tris–HCl, pH 7.8, dialyzed against 150 mM Tris–HCl, pH 8.5 (5°C), containing 0.1 mM EDTA and 0.1 mM PMSF and passed down an 8–10-ml DEAE-Sephacel (Pharmacia Fine Chemicals, Piscataway, NJ) column equilibrated in 150 mM Tris–HCl buffer. The unbound fraction was bound to an HPLC TSK-gel heparin-5PW column (TosoHaas, Philadelphia, PA) and eluted with a programmed 0–1 M NaCl gradient in 3.8 ml. The rAiG peak, which eluted at a conductivity of ~32 mS, was dialyzed into TBS buffer (50 mM Tris–HCl, pH 7.4, 90 mM NaCl) with 0.1 mM EDTA, 0.1 mM PMSF), concentrated with Aquacide (Calbiochem-Novabiochem, San Diego, CA), and stored on ice or frozen.

Preparation of Laminin and Laminin Fragments

Laminin was isolated from lathyritic mouse EHS tumor and purified as described (Schittny and Yurchenco, 1990; Yurchenco et al., 1992). Elastase fragments E8 (distal long arm and proximal G domain), E3 (distal G domain), and E4 (B1 chain domains VI and V) were prepared by Sepharose-CLGB gel filtration and DEAE-5PW ion exchange chromatography and otherwise as described (Schittny and Yurchenco, 1990; Yurchenco et al., 1990). Laminin and fragments were stored in 50 mM Tris–HCl, pH 7.4, containing 90 mM NaCl (TBS) and 0.1 mM EDTA. The A chain moiety (E8-A) and the disulfide-linked B1-B2 chain moiety of elastase fragment E8 (E8-B) were separated from each other and purified by a modification of the procedure described by Hunter et al. (1990, 1992) as follows. E8 was denatured by dialyzing against 8 M urea, 20 mM Tris–HCl, pH 7.4, in the cold overnight, passed down a TSK-gel heparin-5PW (5 cm × 5 mm i.d., glass) HPLC column with a flow rate of 0.5 ml/min in the same buffer, and eluted with constant 1.0 M NaCl, E8-A bound to the column and eluted with high salt while E8-B passed through the column in the unbound fraction. The protein fractions were then exhaustively dialyzed into 50 mM Tris–HCl, pH 7.4, in the cold.

Intercalation of rAiG with the E8-B Chains

Purified rAiG and E8-B, each in 50 mM Tris-HCl, pH 7.9 (at 4°C), containing 0.2 mM EDTA, were mixed together, typically in a 1:3.5 molar or greater ratio (0.25 mg/ml rAiG and ≥0.4 mg/ml E8-B, final concentrations) at 4°-8°C (cold room). After incubation for 22 h in the cold with gentle shaking, the preparation was adjusted to 1 mM $CaCl_2$ and applied to a heparin-5PW column equilibrated in 50 mM Tris-HCl, pH 7.4 (at room temperature), containing 1 mM $CaCl_2$. The unassembled B chains passed through the column. The bound proteins were separated by applying a 0.0-1.0-M NaCl linear gradient in the same buffer and a single sharp peak that eluted at 0.33 M NaCl contained the hybrid glycoprotein, B-rAiG (in contrast to rAiG, which eluted at 0.4 M NaCl).

Antibodies and Other Reagents

Polyclonal affinity-purified and cross-absorbed rabbit antibodies specific for laminin fragments E8 and E3 were prepared as described (Yurchenco et al., 1993). E8-specific antibody reacted with both A and B chains (more strongly with the A chain). Monoclonal blocking antibody specific for the β1 integrin subunit (AIIB2; Hall et al., 1990), provided as rat ascites fluid, was a kind gift of Dr. Caroline Damsky (University of California, San Francisco, CA). Purification of AIIB2 was performed by ammonium sulfate precipitation at 33% saturated concentration (Hayashi et al., 1992). Monoclonal blocking antibody specific for the α6 integrin subunit (GoH3; Sonnenberg et al., 1990) was purchased from Amac Inc. (Westbrook, ME). Rat (Cappel, Durham, NC) and rabbit (Sigma Immunochemical, St. Louis, MO) IgG were obtained from commercial sources. Rabbit polyclonal antibody specific for subdomains G1-G3 (anti-rG70) was prepared by immunization with rG, affinity purification on an E8 column, and cross-absorption against an E3 column (Yurchenco et al., 1993). Antibody specific for the B chains of E8 (anti-E8-B) was prepared by adsorption of anti-E8 antibody (Yurchenco et al., 1993) on E8-B immobilized on Affie Prep 10 (Bio-Rad Labs, Cambridge, MA) and cross-absorption of the eluted protein against immobilized E8-A. Porcine intestinal heparin was purchased from Sigma Immunochemicals.

SDS-PAGE, Immunoblots, Carbohydrate Characterization, Protein Concentrations, and Elastase Digestion

(*a*) SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in 3.6 to 12% linear gradient gels (Laemmli, 1970) and stained with Coomassie brilliant blue R250. Molecular mass standards used were myosin (200 kD), β-galactosidase (116 kD), phosphorylase B (97 kD), bovine serum albumin (BSA, 66 kD), ovalbumin (43 kD) and carbonic anhydrase (31 kD). (*b*) *Staphylococcus aureus* Protein A (Pharmacia Fine Chemicals) was radio-iodinated by incubating 10 mg protein in 1 ml of 10 mM sodium phosphate, pH 7.4, 127 mM NaCl (PBS) with 10 mCi $Na^{125}I$ in the presence of 40 μg lactoperoxidase (Sigma Immunochemicals) and 0.0002% hydrogen peroxide for 30 min at room temperature and purified on a rabbit IgG-Sepharose CL4B affinity column (10 ml) in which Protein A was eluted in 0.2 M glycine-HCl, pH 2.0. (*c*) Proteins were electrophoretically transferred from the gels onto 0.2 or 0.45 μm nitrocellulose sheets (Schleicher & Schuell, Keene, NH) in 25 mM Tris, 192 mM glycine/20% (vol/vol) methanol at pH 8.3 at 10 to 11 Vh/cm by the Western blot method of Towbin et al. (1979). The membranes were then blocked with PBS containing 5% nonfat dried milk, 0.05% sodium azide, and 0.5% Tween 20 (blocking buffer), washed with PBS containing BSA (1 mg/ml), 0.05% Tween 20 and 0.02% sodium azide (washing buffer) and incubated with 5 to 10 μg/ml affinity-purified rabbit antibody in washing buffer for one to several hours. The membranes were again washed and then incubated with ^{125}I-protein A (~2×10^6 cpm/ml) in washing buffer for 1 h. The membranes were washed free of unbound protein A and dried. Autoradiograms were prepared by sandwiching Kodak x-Omat R film (Eastman Kodak, Rochester, NY) with plastic wrap-covered membranes between fluorescent intensifying screens and exposing at −80°C for several hours to several days. (*d*) For dot-immunoblots, protein samples were directly applied onto a 0.45-μm nitrocellulose membrane equilibrated with TBS using a 96-well vacuum manifold (GIBCO-BRL, Gaithersburg, MD). These membranes were then blocked, washed, and incubated with antibody and radio-iodinated protein A as described above. The sheets were washed, then either autoradiographed on x-ray film or cut into slices for quantitative determination of radioactivity (model 1271 gamma counter; LKB-Wallac, Turku, Finland). (*e*) Lectin blotting; for characterization of carbohydrate, protein was electrophoretically transferred onto nitrocellulose as described above and characterized using a lectin/enzyme immunoassay glycan differentiation kit (Boehringer Mannheim Biochemicals, Indianapolis, IN) according to the manufacturer's instructions. Briefly, the nitrocellulose, after treatment with blocking reagent, was incubated with lectin coupled to digoxigenin, washed, incubated with sheep anti-digoxigenin antibody (as Fab) coupled to alkaline phosphatase, washed again, and stained with 4-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate reagent. *Galanthus nivalis* agglutinin (GNA)[1] was used for the detection of terminal mannose and α(1-3), α(1-6), or α(1-2) linked to mannose. *Sambucus nigra* agglutinin (SNA) was used to recognize sialic acid linked α(2-6) to galactose and *Maackia amurensis* agglutinin (MAA) to identify sialic acid linked α(2-3) to galactose. Peanut agglutinin (PNA) was used to detect core galactose β(1-3) *N*-acetylgalactosamine in o-glycosidically linked chains, and *Datura stramonium* agglutinin (DSA) to recognize galβ-(1-4)GlcNAc. Deglycosylation was performed by *N*-glycosidase-F (New England Biolabs, Beverly, MA). Typically, 2-10 μg of protein was incubated with 1,000 U of *N*-glycosidase-F in TBS at 37°C for 16 to 22 h. The reaction was stopped by adding Laemmli solubilizing buffer and boiling. Sialic acid was removed by treatment of glycoprotein with *Clostridium perfringens* neuraminidase (ICN Biomedicals, Costa Mesa, CA; 0.5-1.5 U/mg), ~2 mU enzyme/pmol glycoprotein substrate (~10 nM substrate) in 1.5-ml aliquots at 37°C for 18 to 24 h. (*f*) Protein concentration was determined by absorbance at 280 nm or colorimetrically as previously described (Yurchenco et al., 1992, 1993). (*g*) Proteins were denatured by dialyzing into 8 M urea, 20 mM Tris-HCl, pH 7.4, overnight in the cold followed by redialysis into TBS containing 0.1 mM PMSF. (*h*) Elastase digestion of proteins was carried out at 37°C using a 1:50-enzyme:substrate mass ratio with digestion terminated by the addition of 2 mM PMSF. For a time-course elastase digestion, protein in 40-μl aliquots was mixed with 0.5 mg/ml BSA (as carrier protein) before incubation with elastase.

Electron Microscopy

Protein samples in 45% 0.15 M ammonium bicarbonate/acetate, pH 7.4/55% glycerol, were nebulized onto freshly cleaved mica discs. The discs were evacuated in a BAF500K freeze-etch unit (Bal-Tec, Middlebury, CT) at room temperature, cooled to 11°K, and replicated with 0.9 nm Pt/C (determined with a quartz crystal monitor) at an 8° angle on a rotating stage (100-120 rpm). The replicas were examined in a Philips 420 electron microscope at 80 kV.

Analysis of Heparin Binding by Heparin Affinity HPLC Chromatography

Protein samples were applied to a heparin-5PW HPLC column in 50 mM Tris-HCl, pH 7.4, containing 1 mM $CaCl_2$ at a 1-ml/min flow rate and eluted using a 0-1.0 M NaCl programmed linear gradient (0.5 M increase/h). The NaCl concentration of each fraction was determined by conductivity. The amount of protein was monitored by measuring the absorbance at 280 nm (300 mV/unit of absorbance), by direct quantitation of sample radioactivity in each fraction, or by dot blot assay with specific antibody and quantitation of bound ^{125}I-protein A radioactivity with a gamma counter.

Cells and Cell Attachment Assay

(*a*) Human fibrosarcoma HT1080 (ATCC No. CCL 121) cells were maintained in Dulbecco's modified Eagle's medium (DME; GIBCO-BRL) containing 10% FCS (Upstate Biotechnology Inc.) and 500 U/ml penicillin and 500 μg/ml streptomycin (GIBCO-BRL) in 5% CO_2 at 37°C. Confluent cells were harvested with 0.1% trypsin and 0.53 mM EDTA in 137 mM NaCl, 1.5 mM potassium phosphate, 2.7 mM KCl, 8.3 mM sodium phosphate, 0.2% phenol red for 2 min followed by the addition of 100 μg/ml soybean trypsin inhibitor (Sigma Immunochemicals) (Rapraeger et al., 1985). Cells were resuspended in DME containing 0.5% BSA (Sigma Immunochemicals) (previously heat-inactivated at 70°C for 1 h by the procedure of Goodman et al., 1987) with cells allowed to recover at 37°C for 20 min. Alternatively, cells were released with 5 mM EDTA in 1.25 mM NaCl, 4.8 mM KCl, 25 mM Hepes, 1.2 mM KH_2PO_4, and 5.6 mM glucose, pH 7.3 (Nurcombe et al., 1989). (*b*) For determination of cell adhe-

1. *Abbreviations used in this paper*: DSA, *Datura stramonium* agglutinin; GNA, *Galanthus nivalis* agglutinin; MAA, *Maackia amurensis* agglutinin; PNA, peanut agglutinin; SNA, *Sambucus nigra* agglutinin.

sion to protein substrates, protein samples were coated onto the wells of 96-well (half-area) tissue culture plates (Costar Corp., Cambridge, MA) at 0.7 μM in TBS containing 2 mM $CaCl_2$ according to the method of Lallier et al. (1992). In some experiments, the tissue culture wells were first treated with a nitrocellulose solution (1 cm^2/ml methanol) for 3 s. After incubation with protein solution in the cold overnight, the plates were washed with calcium- and magnesium-free Dulbecco's modified PBS (D-PBS) twice and then blocked with D-PBS containing 0.5% BSA at 37°C for 2 h. For the quantitation of bound cells, the cells were labeled the night before with methyl-[^{3}H]thymidine (10 μCi/ml; ~50 Ci/mmol) (Amersham Intl., Arlington Heights, IL) in complete medium. Cells were harvested as described above and plated into the protein-coated wells at ~2 × 10^4 cells/well. After incubation at 37°C in 5% CO_2 for 75 min (previously established condition for these cells; Goodman et al., 1987), the plates were washed with DME-BSA (0.5% heat-treated solution) twice to discard the unbound cells and the label in adherent cells extracted with 2% SDS in water and prepared for scintillation counting. The specific activity (dpm/cell) was determined from the cell density (counted with a hemocytometer) and the radioactivity in 10 0.1-ml aliquots as measured with a scintillation counter calibrated to convert cpm to dpm (model LS-6000IC; Beckman Instruments Inc., Palo Alto, CA). Each experimental value was determined from a group of 10 identically treated wells (n = 10) unless otherwise indicated. Cell adhesion was expressed as percent of total cells bound (or as percent bound relative to control) and error bars indicate the standard error of the mean. Antibody inhibition studies were carried out at a final antibody (or nonimmune IgG control) concentration of 10 μg/ml for monoclonal GoH3 and AIIB2, or 20 μg/ml for polyclonal anti-rG70 and anti-E8-B.

Results

Recombinant Glycoproteins and Fragments of Laminin

In this study we have evaluated two recombinant glycoproteins (rG and rAiG) in conjunction with proteolytic fragments of authentic laminin (E8, E3) as shown in Fig. 1.

Expression and Characterization of rAiG

The recombinant A chain encoding the E8 portion of domain I and the entire G domain (rAiG) was expressed in Sf9 insect cells as a secreted glycoprotein (Fig. 2 *A*). The principal species had an apparent molecular mass of ~160 kD as determined by SDS-PAGE under reducing conditions, larger than the predicted molecular mass of 133 kD for protein alone and larger than rG (120 kD) which lacks the α-helical sequences of domain I in the distal rod. Given 19 consensus sites for N-linked carbohydrate in the sequence (Sasaki et al., 1988) present in rAiG, it is likely that the difference in the predicted and observed size in rAiG is due to carbohydrate.

The carbohydrate linkages were probed by lectin blotting (Fig. 2 *B*). Both rAiG and rG were found to react strongly with GNA indicating the presence of mannose–mannose linkages which are characteristic of high mannose oligosaccharides and typical of glycoproteins secreted by insect cells (reviewed in O'Reilly et al., 1992). While high mannose oligosaccharides have been reported to be present in laminin (Arumugham et al., 1986; Fujiwara et al., 1988), such linkages were weakly detected in E8. rAiG also appeared to react weakly (but comparable to the levels in the control) with DSA (detects gal β1-3 GlcNAc) and, to a lesser extent, with MAA (detects sialic acid α1-3 gal). The presence of complex carbohydrate in addition to high mannose forms has also been reported for recombinant human plasminogen produced in Sf9 cells with baculovirus (Davidson et al., 1990). The A chain of E8, on the other hand, reacted strongly with MAA and DSA, but not with SNA (detects sialic acid α2-6

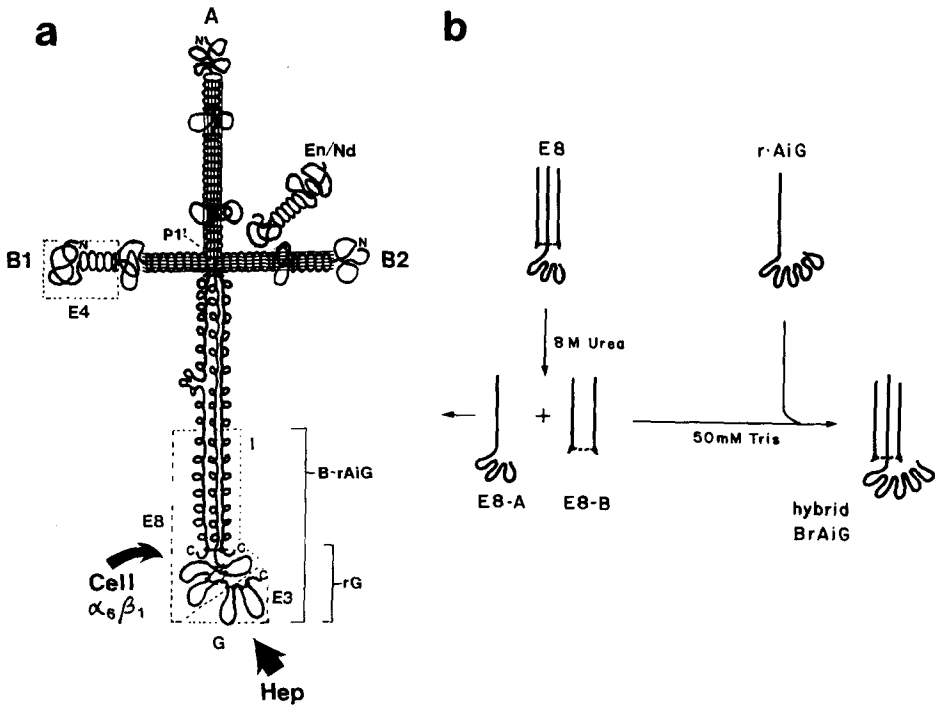

Figure 1. Laminin structure and scheme for the assembly of B-rAiG. a: B1, B2 and A chains of laminin are joined through noncovalent interactions in a coiled-coil along the length of the long arm. Disulfide bonds (*lines between pairs of dots*) at the proximal end (*all three chains*) and the distal end (one cystine between the B chains) of the long arm stabilize the structure. Entactin/nidogen (En/Nd) typically binds to the short arm of the B2 chain. Short arm complex P1' (containing another cell binding region; parallel hatched lines), short arm fragment E4, long arm fragment E8 (distal long arm and proximal G domain), long arm fragment E3 (distal G domain), and the regions corresponding to rG and B-rAiG are indicated. HT1080 cells (recognition mediated by α6β1 integrin) and heparin (Hep) bind to the distal long arm. The former activity is observed only in proteins in which A and B chains are intercalated. b: Intercalation of recombinant AiG with laminin E8-B chains to form a hybrid molecule. Authentic laminin elastase fragment E8 was dissociated into its A chain (E8-A) and disulfide-linked (*dotted line*) B1-B2 chain (E8-B) moieties in 8 M urea. These chains were separated by heparin affinity chromatography (only the A chain binds to heparin). The E8-B chains were then dialyzed into 50 mM Tris–HCl, pH 7.4, and mixed with the rAiG purified from Sf9 medium to form B-rAiG.

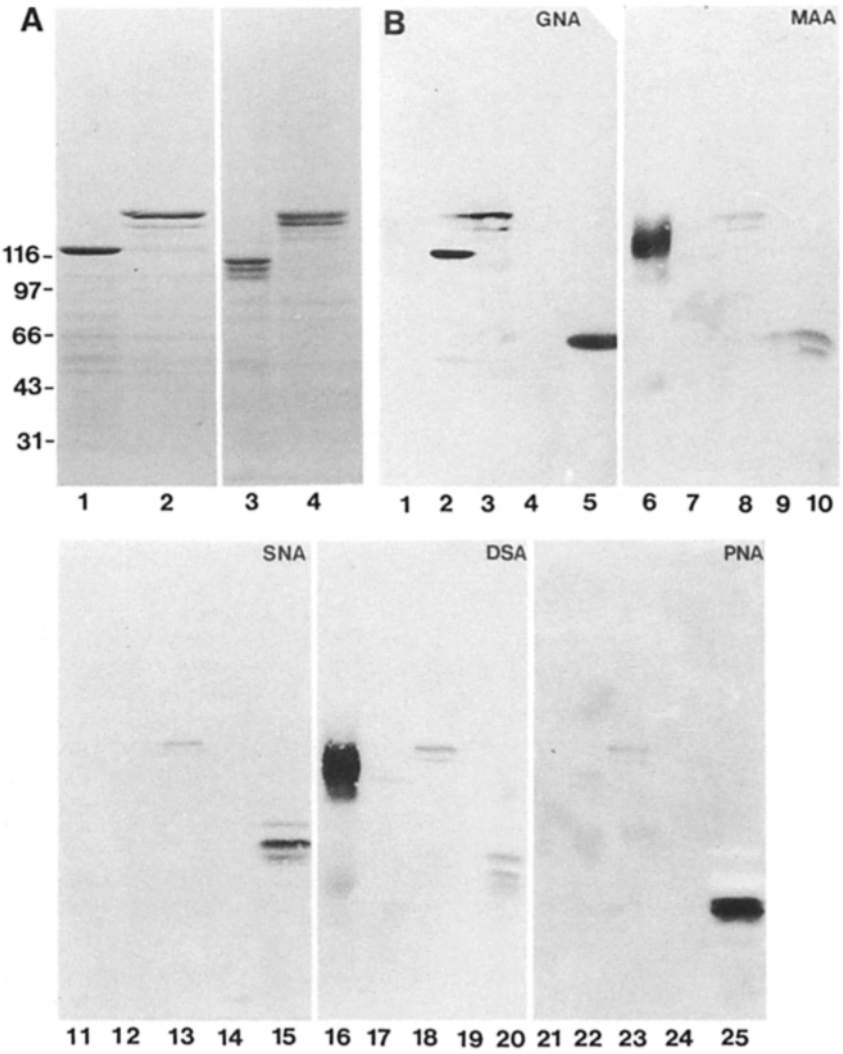

Figure 2. Comparison of rAiG with rG. A: Coomassie blue stained gels. Aliquots of purified rG (lanes *1* and *3*) and rAiG (lanes *2* and *4*) were analyzed by SDS-PAGE under reducing (lanes *1* and *2*) and nonreducing (lanes *3* and *4*) conditions. Under nonreducing conditions rG and rAiG have apparent molecular masses of 120 and 160 kD, respectively. The latter possesses minor 145-, 135-, and 120-kD bands. Under reducing conditions, the major bands of rG and rAiG are seen as a triplet and doublet respectively, reflecting intrachain disulfide heterogeneity. B: Lectin blotting. Aliquots (1.4 μg each of E8 (lanes *1, 6, 11, 16, 21*), rG (lanes *2, 7, 12, 17, 22*), rAiG (lanes *3, 8, 13, 18, 23*), myosin S1 (negative control, lanes *4, 9, 14, 19, 24*) and a mixture of transferrin, carboxypeptidase Y, fetuin and asialofetuin (positive controls, lanes *5, 10, 15, 20, 25*) were separated by SDS-PAGE under reducing conditions, transferred onto nitrocellulose membranes, and incubated with the following labeled lectins: GNA (lanes *1–5*), MAA (lanes *6–10*), SNA (lanes *11–15*), DSA (lanes *16–20*) and PNA (lanes *21–25*). rG and rAiG were found to react strongly with GNA (man-man) while the A chain of E8 reacted strongly with MAA (sialic acid α2–3 gal) and DSA (gal β1–4 GlcNAc).

gal). Given the strong PNA staining with standard and very weak staining with rAiG, we further concluded there was little or no gal β1-4 GlcNAc linkages in either E8 or rAiG. Thus, the predominant form of oligosaccharide in rAiG is high mannose. The sialic acid linkage specificity of E8 is consistent with a previous analysis of laminin carbohydrate structure (Knibbs et al., 1989). The B chains of E8 reacted only weakly with these lectins. This may be due to the low predicted carbohydrate content relative to E8-A (one and three consensus glycosylation sites in E8-B1 and E8-B2, respectively, as compared to 14 for E8-A) but may also reflect different linkages. Treatment of rAiG with *N*-glycosidase-F (data not shown), which removes N-linked oligosaccharide, caused an approximate 25 kD reduction in apparent molecular mass to about the predicted protein mass. This difference between apparent total and predicted protein molecular mass of rAiG is about half the difference (40–50 kD) found with the A chain of fragment E8 (Deutzmann et al., 1988) and is interpreted as evidence that the recombinant possesses less carbohydrate mass compared to its murine counterpart. This decreased fraction is similar to that observed with rG (Yurchenco et al., 1993) as well as other glycoproteins generated with the baculovirus system (Luckow and Summers, 1988), which nevertheless possess biological activity.

Disulfide and some size heterogeneity could be identified in preparations of rAiG. (*a*) The principal cause of variability appeared to be due to intrachain disulfide heterogeneity (Fig. 2 *A*), a characteristic shared with rG (Yurchenco et al., 1993). Under nonreducing conditions, the major 160-kD band migrated as a doublet and rG migrated as a triplet. This heterogeneity may be due to the presence of an odd number (15) of cysteines in G domain in which only an even number can be paired and in which no single combination of cysteine pairs is energetically favored. (*b*) While the largest band of rAiG has an apparent molecular mass of about 160 kD, there are also smaller minor species which react with A chain specific antibody with apparent molecular masses of 145, 135 and 120 kD under reducing conditions (Fig. 2 *A*). The minor 120-kD species comigrated with rG (reduced samples). Most preparations of rAiG had minor amounts of these smaller species (16% 145 kD and 4% 135 kD species in Fig. 2 *A*) while occasional preparations contained larger fractions of the smaller species. The size heterogeneity appears to be almost entirely a result of partial proteolysis at the NH_2-terminal end during culturing of infected Sf9 cells. The evi-

dence for this is as follows: (*a*) Treatment of rAiG containing smaller species with *N*-glycosidase to remove carbohydrate decreased the molecular weights to near that expected for protein alone, but had little effect on heterogeneity. (*b*) Treatment of rAiG or B-rAiG with elastase, followed by identification with antibody specific for the COOH-terminal moiety of G domain revealed that digestion converted the A chain triplet of rAiG and A chain doublet of B-rAiG to a single major band (rG50) under reducing conditions. Similar cell adhesion was observed for these varied preparations.

Assembly and Characterization of B-rAiG

A hybrid molecule, B-rAiG, was prepared by intercalating rAiG with isolated B chains (disulfide-linked) purified from laminin fragment E8 according to the scheme shown in Fig. 1 *b*. Purified rAiG (which has 25 more NH_2-terminal A chain amino acid residues than E8) was mixed with an excess of disulfide-linked B1 and B2 chains from E8 (E8-B). Use of a molar excess of B chains was found to improve the recovery of hybrid molecules, either because it increased the rate of intercalation (mass action effect) or because not all B chains were competent for the interaction. The assembled molecule could be purified and separated from unassembled A or B chains because it possesses a unique affinity, and hence unique salt elution concentration, for heparin. A new species was formed as shown by a shift in the elution profile from a heparin affinity column. The hybrid molecule was characterized by SDS-PAGE (Fig. 3 *A*), by immunoblotting (Fig. 3 *B*), and by EM (Fig. 4).

The purified molecule (B-rAiG) contained both the B1-B2 chain disulfide-linked dimer of E8 and rAiG as shown by SDS-PAGE (Fig. 3 *A*). Only the 160- to 135 kD-forms of rAiG intercalated into the B chains (the smaller 120-kD degradation product is identical in size to rG and lacks the heptad repeats of domain I). By inspection of the Coomassie blue stained gel (Fig. 3 *A*), the mass ratio of the A to B chain appeared greater for B-rAiG than for fragment E8. We examined this quantitatively by scanning the lanes for the two proteins (digitized scans not shown). For E8, the ratio of A to B chains was 1.0. For rAiG, the ratio of A to B chains was 1.54. This increase in ratio, however, is likely due to the differences in protein masses between E8-A and rAiG, and not a change in the expected 1:1 molar ratio of A to disulfide-linked E8-B chains, as follows. The E8-A glycoprotein chain has an apparent total molecular mass of 130 to 140 kD, but a calculated protein mass of only 85 kD (amino acid residues 1887-2665). It is more heavily glycosylated. The rAiG glycoprotein chain has an apparent total mass of 160 kD but a calculated protein mass of 133 kD. The mass ratio is therefore 1.56 (133/85) which is very similar to the Coomassie blue ratio (1.54) of rAiG over E8-A normalized to the B chains hybrid and E8 gel lanes (i.e., rAiG/E8-B divided by E8-A/E8-B), thus supporting the expectation of identical chain molar ratios for E8 and B-rAiG.

Fragment E8, rAiG, and B-rAiG were also compared in immunoblots prepared from nonreduced gels with antibodies specific for A chain subdomains G1–G3 (anti-rG70) and E8-B chains (Fig. 3 *B*). Anti-rG70 reacted with the E8-A chain and with rAiG in both isolated and hybrid protein. A doublet, reflecting disulfide heterogeneity, was observed with rAiG. Anti-B chain antibody reacted with the B chains of E8 and with the B chains in B-rAiG. Autoradiograms of

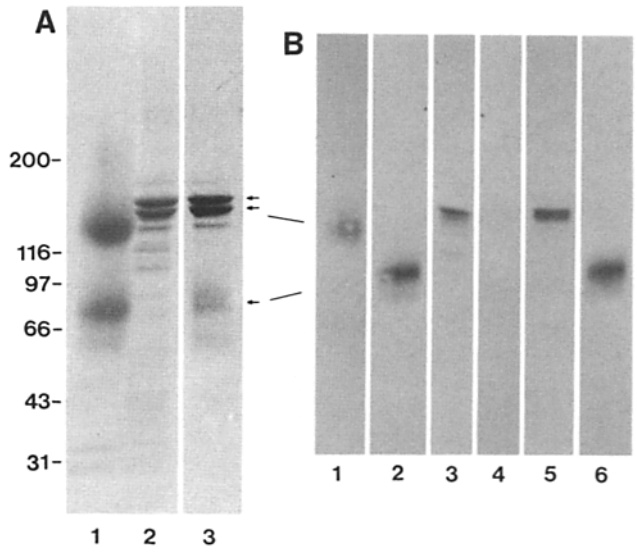

Figure 3. Characterization of B-rAiG: A: SDS–polyacrylamide gel electrophoresis of E8, rAiG and B-rAiG. Fragment E8 (lane *1*), rAiG (lane *2*) and the hybrid B-rAiG (lane *3*) were analyzed under nonreducing conditions and stained with Coomassie blue. B-rAiG consists of rAiG (*double arrows*) and the B chains (*single arrow*) of fragment E8. B: Fragment E8 (lanes *1–2*), rAiG (lanes *3–4*) and B-rAiG (lanes *5–6*) were subjected to SDS-PAGE under nonreducing conditions (2 μg load per lane in two parallel gels), electrophoretically transferred to nitrocellulose, and incubated with 10 μg/ml of anti-rG70 antibody (lanes *1*, *3*, and *5*) and 10 μg/ml of anti-E8-B antibody (lanes *2*, *4*, and *6*). Bound antibody was detected with radioiodinated protein A by autoradiography in which both nitrocellulose sheets were incubated identically and exposed to film together.

E8 and B-rAiG from two exposures were digitized and total band radioactivities were quantitated. The anti-rG70/anti-B chain ratios for E8 and B-rAiG were both 1:2.1. These data confirmed the chain identification of the bands in rAiG and B-rAiG and provided further evidence that the molar A/B chain ratios were the same in E8 and B-rAiG.

The molecular morphology (Fig. 4) was examined. Isolated rAiG possessed a globular morphology similar to that observed for rG (Yurchenco et al., 1993). The domain I extension could not be resolved. The B chain moiety (disulfide-linked B1 and B2 chains) of fragment E8 was observed to be a thin rod-like structure, about 50 nm long, but also with shorter lengths as well. We suspect that not all of the B chains are fully folded into a two-chain coiled-coil (Hunter et al., 1992) in the micrographs, and that the free polypeptide ends were below the effective resolution required to be visualized. When rAiG was mixed with the B chains and the new species purified and replicated, the newly formed species had a dramatically different shape compared to each separate component. A substantial rod attached to an oblong globule was observed. This ball and rod structure was not observed if the E8-B chains were mixed only with rG which lacks the domain I sequences (micrograph not shown). The B-rAiG structure was essentially identical in appearance to the distal half of the long arm of native laminin, and similar, but not identical, in appearance to authentic elastase fragment E8. The difference between B-rAiG and E8 is that the globule of the former species was a little larger, and more

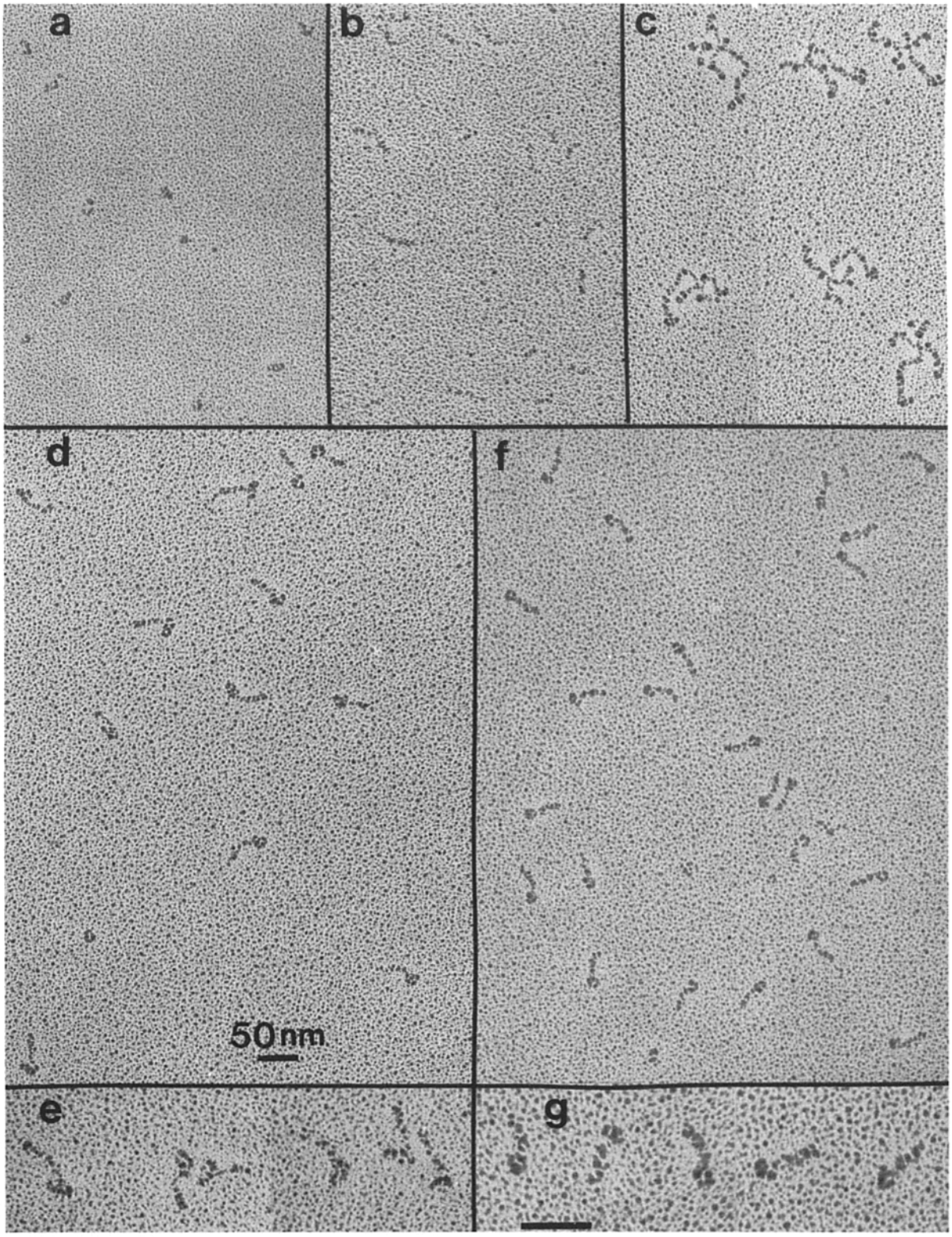

Figure 4. Electron microscopy. Low angle Pt/C rotary shadowed replicas of rAiG (*a*), fragment moiety E8-B (*b*), intact laminin (*c*), B-rAiG (*d* and *e*), fragment E8 (*f* and *g*). rAiG is seen as a compact structure with globular features. The E8-B chain moiety is a thin (and somewhat difficult to discern) short rod measuring up to 50 nm in length. B-rAiG, clearly different from its component parts (*a* and *b*), is seen as a rod with an oblong globule at one end. This structure is essentially identical to the distal half of the laminin long arm and is similar to fragment E8 except that the globule, as predicted, is slightly larger and more ellipsoid.

ellipsoid, as compared to the globule of the latter. The lengths of the rod domain and maximum diameter of the globular domain of E8 and B-rAiG were determined from prints at 288,000 magnification and the data plotted as histograms (Fig. 5). The average metal coated rod lengths (mean ± SD) were 29.6 ± 3.3 nm for E8 and 30.8 ± 3.8 nm for B-rAiG. The average maximal metal coated globular diameters were 13.9 ± 1.9 nm for E8, 18.8 ± 2.4 nm for B-rAiG and 19.6 ± 2.4 nm for intact laminin. Thus the rod portion was similar for E8 and B-rAiG while the globular domain was larger in B-rAiG, nearly identical to laminin. This was not surprising given that the globular domain of the hybrid B-rAiG is complete and contains subdomains G1–G5 while fragment E8 only contains subdomains G1–G3.

In a previous study (Yurchenco et al., 1993), rG was found to possess the expected secondary structure and restricted protease-sensitive site as the native G domain of laminin. In this study, we compared the elastase cleavage patterns of rAiG and B-rAiG to laminin G domain to further evaluate folding. A time-course elastase digestion of rG, rAiG, and B-rAiG was followed by immunoblotting using E8-specific and E3-specific polyclonal antibodies (Fig. 6). Cleavage of rAiG and B-rAiG into anti-E8 reacting fragments was different with respect to the A chain moiety. rAiG was rapidly converted into a 70-kD fragment nearly identical in size and possessing the same antibody reactivity to the previously described rG70 which contains globular subdomains G1–G3 (Yurchenco et al., 1993). Thus subdomains G1–G3 of rAiG are relatively protease resistant and the domain I portion protease sensitive. In contrast, B-rAiG was first cleaved into an intermediate 110-kD fragment (A chain origin, larger than the initial B chains from E8) before formation of the same 70-kD fragment observed with rAiG. This intermediate, present between 2 and ≥15 min of digestion, results from the rapid cleavage between subdomains G3 and G4 as seen with whole laminin and represents a region of the A chain protected from digestion because of an interaction with the B chains. The generation of a 50-kD fragment exclusively containing E3 epitopes was common to rAiG and B-rAiG (Fig. 6) and was expected given earlier findings with rG.

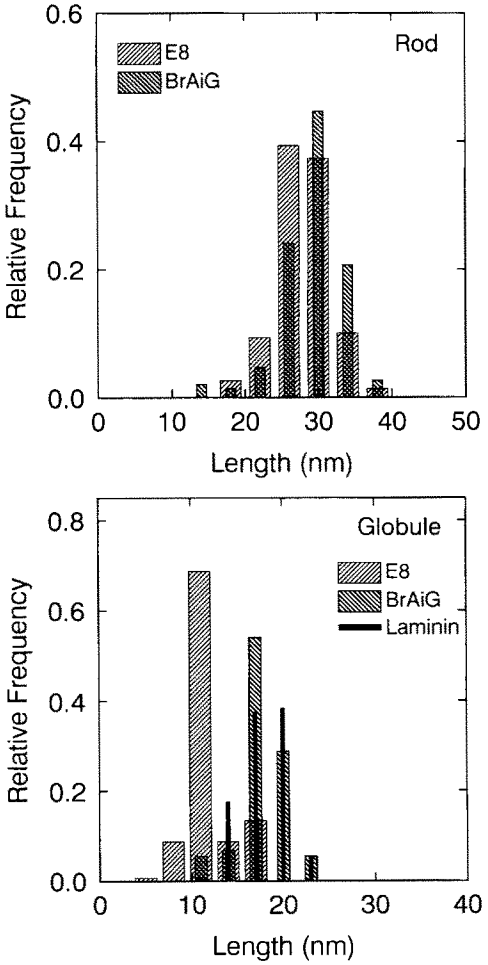

Figure 5. Morphometry of E8 and B-rAiG in electron micrographs. The lengths of the rod domains of E8 and B-rAiG and maximum diameter of the globular domains of these proteins and laminin were determined from prints and are shown as histograms. The average metal coated (1 nm Pt/C) rod lengths (mean ± SD, n = 150) were 29.6 ± 3.3 nm for E8 and 30.8 ± 3.8 nm for B-rAiG. The average maximal metal coated globular diameters were 13.9 ± 1.9 nm for E8 (n = 150), 18.8 ± 2.4 nm for B-rAiG (n = 150) and 19.6 ± 2.4 nm for intact laminin (n = 125). Thus the rod portion was similar for E8 and B-rAiG while the globular domain was larger in B-rAiG. The rods of E8 and B-rAiG also show a similar degree of length heterogeneity.

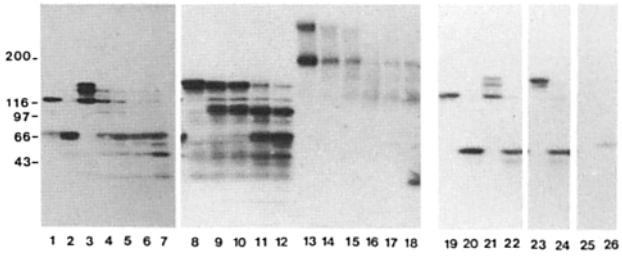

Figure 6. Limited elastase digestion of rG, rAiG, B-rAiG, and laminin. Proteins were incubated with elastase (37°C, enzyme/substrate ratio 1:50), electrophoresed (SDS-PAGE) under reducing condition, and transferred to nitrocellulose membranes. The membranes were incubated with either antibody specific for the A and B chains of E8 (lanes *1–18*) or with antibody specific for E3 subdomains G4-5 (lanes *19–26*). Immunostaining was detected by autoradiography following treatment with radioiodinated protein A. E8-specific antibody: Lanes *1–2*, rG incubated 0 and 15 min with elastase; lanes *3–7*, rAiG incubated 0, 2, 4, 8, 15 min with elastase; lanes *8–12*, B-rAiG incubated 0, 2, 4, 8, 15 min with elastase; lanes *13–18*, laminin incubated 0, 2, 4, 8, 15 and 30 min with elastase. E3-specific antibody: Lanes *19–20*, rG incubated 0 and 60 min with elastase; lanes *21–22*, rAiG incubated 0 and 15 min with elastase; lanes *23–24*, B-rAiG incubated 0 and 15 min with elastase; lanes *25–26*, laminin incubated 0 and 15 min with elastase. rG was converted from a 120 kD species to 70 kD species recognized by E8 antibody (rG70) and a 50-kD species recognized by E3 antibody (rG50). rAiG 160-, 145-, and 120-kD bands (latter band equivalent to rG) were also converted to a 70-kD species (and smaller bands by 15 min) recognized by E8 antibody (rG70) and a 50-kD species recognized by E3 antibody (rG50). The 160- and 145-kD bands of B-rAiG were converted to a ~110-kD intermediate recognized only by E8-specific antibody (indicating presence of a protected domain) followed by formation of the 70 kD species recognized by E8-specific antibody and the 50-kD species recognized by E3 antibody. Laminin was more slowly converted to 120 and 53 kD species recognized by E8 and E3 antibodies, respectively.

This fragment, rG50, is nearly identical to fragment E3 (except for some carbohydrate differences) and represents protease-resistant globular subdomains G4 and G5 (Yurchenco et al., 1993). These data support the conclusion that intercalation of the A chains into the B chains forms a protease-resistant, rodlike domain.

Heparin Binding

We used heparin affinity chromatography to measure the relative binding of native, recombinant and hybrid laminin proteins (Fig. 7). We found that rAiG bound to the heparin column the most avidly (identical to rG), requiring the highest salt concentration for elution. The hybrid B-rAiG, on the other hand, bound the column less avidly (Fig. 7). This difference demonstrated that intercalation of the B chains with rAiG substantially reduced the overall affinity for heparin. It also provided a means to identify and purify this new hybrid species. Previously, we have shown that there are two heparin binding regions in recombinant G domain with the proximal subdomains of rG70 binding more strongly than the distal subdomains of rG50 (Yurchenco et al., 1993). These relative affinities were reversed from that found with the corresponding E8 and E3 fragments such that rG70 bound to heparin more strongly than E8, and rG bound to heparin more strongly than intact laminin (Fig. 7, lower panel). To analyze these differences, B-rAiG was partially digested with elastase under conditions that maximized generation of the ~110-kD A chain intermediate described above. This mixture was evaluated by heparin affinity chromatography under the identical conditions used for rAiG with the fractions characterized in dot-blots using E3- and E8-specific antibodies (Fig. 7, lower panel). Nearly all of the protein eluted as two peaks, one at 40 min (0.19 M NaCl) and the other at 55 min (0.3 M NaCl). The first peak eluted at a salt concentration nearly identical to (and between) the elution positions of fragment E8 and fragment E8 treated with neuraminidase to remove sialic acid (E8d). This peak reacted only with E8-specific antibody and migrated, by SDS-PAGE, with the ~110-kD intermediate A chain band and B chains (which together form an E8-like molecule). The second peak eluted at a salt concentration nearly identical to the elution positions of neuraminidase-treated E3 (E3d). It was found to consist of a mixture of nearly intact B-rAiG (possessing the 140-kD but not the 160-kD band of rAiG) and E3-like rG50. Both bound to heparin more strongly than E8-like molecules and eluted at the identical higher salt concentration as pure intact B-rAiG (Fig. 7, compare upper and lower panels). Furthermore, in contrast to the E3-like moiety, the proximal E8-like moiety appeared to make little or no additional contribution to the overall affinity of B-rAiG. Thus intercalation of the B chains with the A chain to form the rod domain decreased the affinity of heparin to the proximal moiety of the long arm from the highest affinity to a low affinity of little consequence in overall binding. Overall, these results led to the conclusion that the only active heparin binding site of the long arm of laminin resides in subdomains G4–G5 and further that the relatively strong heparin binding activity identified in subdomains G1–G3 of rG and rAiG are cryptic in classical laminin due to an inhibiting contribution from the B chains upon formation of the rod domain.

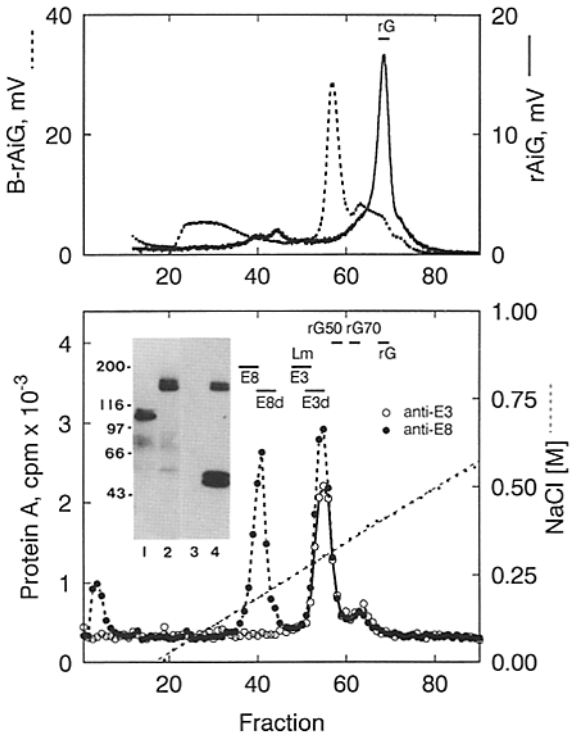

Figure 7. Relative heparin affinities. HPLC heparin affinity chromatography was used to compare the affinity of heparin for recombinant, hybrid and native protein. Samples were bound to the column in 50 mM Tris-HCl, pH 7.4, containing 1 mM $CaCl_2$ and eluted with identical linear 0.0 to 1.0 M NaCl gradients in the same buffer. One min fractions were collected. Upper Panel: rAiG (*solid line*) eluted at a salt concentration identical to rG but higher than B-rAiG (*short dashed line*). The elution position of rG is indicated with a bar. Lower Panel: B-rAiG was incubated with elastase for 10 min (at a 1:50-enzyme/substrate ratio in the presence of carrier BSA, 37°C with reaction terminated with PMSF) and the protein analyzed by heparin affinity chromatography. Each fraction was tested for the presence of E3 and E8 epitopes by dot-immunoblotting: bound radio-iodinated protein A was quantitated with a gamma counter. The peak which eluted at 40 min reacted only with anti-E8 antibody. The second peak (55 min) reacted with both anti-E3 and anti-E8 antibodies. The elution positions of E8, neuraminidase-treated E8 (E8d), E3, neuraminidase-treated E3 (E3d), rG50, rG70 and rG are indicated with bars from left to right. Inset, lower panel: Immunoblots of the 40 min peak (lanes *1* and *3*) and 55 min peak (lanes *2* and *4*) electrophoresed under nonreducing conditions and detected with E8 specific antibody (lanes *1* and *2*) and E3 specific antibody (lanes *3* and *4*). Positions of rAiG and E8-B chains indicated with arrows and molecular weight standards (in kD) indicated to the left. The 40 min peak contains only E8 epitopes consisting of a ~110-kD A chain band and the B chains of E8. The 55 min peak contains both E8 and E3 epitopes with the 145-kD recombinant A chain of B-rAiG and B chains of E8 as well as the rG50 band. This peak is interpreted as consisting of a mixture of nearly intact B-rAiG and E3-like rG50 elastase product.

HT1080 Cell Adhesion

Human HT1080 fibrosarcoma cells possess the laminin-specific $\alpha 6\beta 1$ integrin which selectively interacts with the E8 fragment (Aumailley et al., 1990; Sonnenberg et al., 1990). These cells have been observed to achieve up to 40 to 50% binding under saturating substrate coating conditions

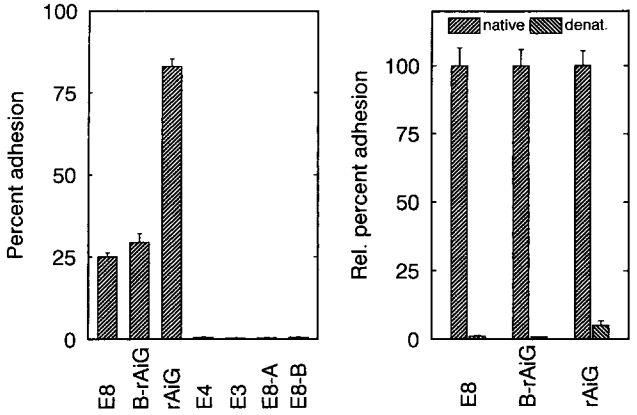

Figure 8. Adhesion of HT1080 cells to native, recombinant and hybrid protein substrates. Cells labeled with [^{3}H]thymidine were incubated for 75 min in 96-well plates coated with the indicated proteins and the percent binding determined from the fraction of radioactivity adherent to the plates following washing (n = 10). Left panel: Cells bound to fragment E8, B-rAiG and rAiG but not to fragments E4, E3 or the isolated A and B chain moieties of E8. Right panel: The effect of denaturing substrate with 8 M urea on relative cell adhesion was examined. Cell adhesion was greatly reduced on E8, B-rAiG, and rAiG.

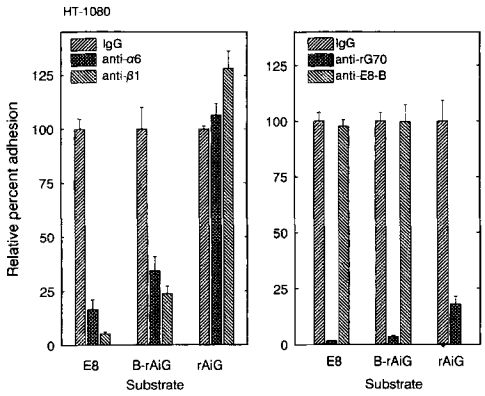

Figure 9. Antibody inhibition of HT1080 cell adhesion on native, recombinant and hybrid protein substrates. Left panel: Integrin binding. HT1080 cells were incubated with wells coated with E8, B-rAiG or rAiG as described above but in the presence of 10 μg/ml GoH3 (anti-α6 integrin blocking antibody), AIIB2 (anti-β1 integrin blocking antibody) or IgG control (n = 30 for each condition evaluated) GoH3 and AIIB2 inhibited adhesion to native fragment E8 and hybrid B-rAiG, but not to rAiG. Thus intercalation of the recombinant distal A chain (rAiG) with the B chain moiety of fragment E8 restored the α6β1 integrin-specific site and inhibited a non-β1 site present in rAiG. Right panel: Protein domains. HT1080 cells were incubated as above but in the presence of rabbit IgG (control), antibody specific for globular subdomains G1-3 (anti-rG70), and specific for the B chains of E8 (anti-E8-B). Binding of antibody to the proximal subdomains of G selectively blocked cell adhesion on all three substrates.

(Aumailley et al., 1990). We evaluated the adhesion of HT1080 cells to rAiG and B-rAiG in comparison to fragments E8, E4, and E3, and the A and B chain moieties of E8 (Fig. 8). B-rAiG and rAiG, like E8, supported cell adhesion. The fraction of cells which bound to B-rAiG was similar to that observed for fragment E8. Cell adhesion was generally highest for rAiG. HT1080 cells were inspected by inverted phase microscopy following adhesion of cells onto E8, rAiG, and B-rAiG. A "spread" morphology was observed to develop on all three of these substrates.

The A chain of E8, which was exposed to urea during the separation steps from the B chains, and the B chains alone, did not show any cell binding activity. Similarly fragments E4 and E3, used as negative controls, did not support cell binding. Adhesion to fragment E8, B-rAiG and rAiG were all found to be dependent upon substrate conformation since treatment of each of these components with 8 M urea resulted in a near-total loss of cell adhesion.

The integrin contributions to cell adhesion were then examined using specific monoclonal neutralizing antibodies (Fig. 9, *left panel*). Adhesion to fragment E8 was inhibited by over 80% with GoH3 (anti-α6) or AIIB2 (anti-β1), consistent with previously reported findings (Aumailley et al., 1990). Both GoH3 and AIIB2 antibody also each inhibited cell adhesion to B-rAiG by 70% and 80%, respectively. This inhibition by antibodies provided strong evidence that cell adhesion to B-rAiG is dependent on the α6β1 integrin. However, in contrast, neither GoH3 nor AIIB2 inhibited HT1080 adhesion to rAiG. This failure of inhibition indicates that the α6 and β1 integrin chains were not necessary for cell adhesion to rAiG and that an essential α6β1 cell recognition site was created by the joining of the separate subunits. A simple mixture of rG and B chains (see below) was in itself insufficient to support cell adhesion and therefore proper chain intercalation appears to be required for α6β1 recognition.

Cell adhesion was also evaluated in the presence of antibodies specific for the B chains of E8 (rod domain I) or globular subdomains G1–G3 (Fig. 9, *right panel*). Only the latter reagent inhibited adhesion of HT1080 cells to E8, B-rAiG as well as to rAiG. This is in contrast to C2C12 myoblast adhesion to laminin where both antibodies are found to separately block adhesion (Yurchenco, Ward and O'Rear, unpublished observations). The selective inhibition observed with HT1080 cells suggests that subdomains G1–G3 play an important role in both the α6β1 and the non-β1–mediated adhesion, although they do not exclude contributions from more distal sequences.

The binding of HT1080 cells to rG in comparison to E8 was also examined (Fig. 10). Cells adhered to rG, but less well in comparison to E8 (or rAiG). This binding was not inhibited by GoH3 (data not shown). When plastic wells were co-coated with an equimolar mixture of E8-B chains and rG, almost no cell binding could be detected. This result suggested that the α-helical sequences of rAiG, required for intercalation with B chains, were also required for part of the cell binding levels observed with rAiG.

Cell Adhesion in the Presence of Heparin

Since the long arm possesses both heparin and cell interactive sites, we questioned whether there might be an interrelationship between the two (Fig. 11). In the presence of 10 μg/ml heparin, cell adhesion on rAiG was almost completely inhibited, was partially inhibited on hybrid B-rAiG, and had little effect on fragment E8. Since rAiG and B-rAiG both possess the terminal moiety of G domain, while E8 does not, the data raise the interesting possibility that this region

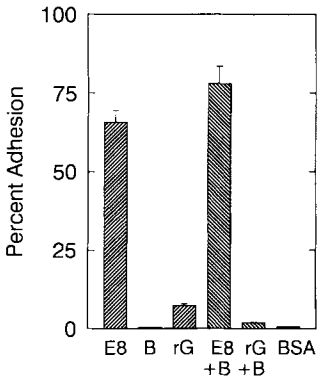

Figure 10. Adhesion of HT-1080 cells to rG and rG plus E8-B chains. Labeled cells were plated onto wells coated separately with E8, E8-B chains or rG (*upward diagonal cross-hatching*) and with E8, or coated with rG mixed with an equimolar amount of E8-B chains (*downward diagonal cross-hatching*). Cell binding to rG was decreased relative to E8 (and to rAiG) with almost no binding detected in the presence of B chains.

(G4–G5) can contribute a heparin-dependent modulating effect on cell recognition. The additional presence of a strong heparin binding site in the proximal portion of rAiG, which is inhibited in B-rAiG, might explain a more pronounced effect of heparin with this protein.

Discussion

In this study we have expressed a recombinant glycoprotein that can intercalate with authentic subunit fragments to produce a biologically active hybrid molecule and that provides insights into the relationships between laminin molecular structure and function. In particular, the synthesis of a multisubunit structure has resulted in the creation of an integrin cell recognition site not present in any of the component subunits as well as substantial inhibition of both a heparin-binding activity and a heparin-sensitive cell recognition activity present in one of the component subunits.

Recombinant Glycoprotein

Baculovirus-recombinant glycoprotein rAiG, consisting of the distal laminin A chain rod domain and entire globular domain, was secreted into the serum-free tissue culture medium by Sf9 insect cells. This glycoprotein, like the globular domain (rG) alone (Yurchenco et al., 1993), contained less mass of carbohydrate relative to the A chain of fragment E8. The carbohydrate of rAiG, with its 19 consensus sequence sites for N-glycosylation, consists mostly of high mannose oligosaccharide with a smaller fraction of complex linkages, given the detection of other residues. Carbohydrate has been found to affect the spreading of some cells and the neurite outgrowth of PC12 cells (Begovac and Shur, 1990; Chandrasekaran et al., 1991; Dean et al., 1990; Evans et al., 1993). For example, simple mannose-rich oligosaccharides have been proposed to play a central role in the spreading of B16 melanoma cells (Chandrasekaran et al., 1991) while an *N*-acetyl glucosamine substrate acceptor has been proposed to play a role in PC12 neurite outgrowth and the spreading of F9 and Swiss 3T3 cells (Evans et al., 1993). In the case of C2C12 myoblasts (Yurchenco et al., 1993), rG, which contains mannose-rich carbohydrate, supports cell spreading. For HT1080 cells, we have found that adhesion and spreading occurs on B-rAiG and, to a lesser extent, on rAiG. These new data reveal that, at least for some cells, spreading can occur on glycoprotein substrates with both complex and mannose-rich carbohydrates.

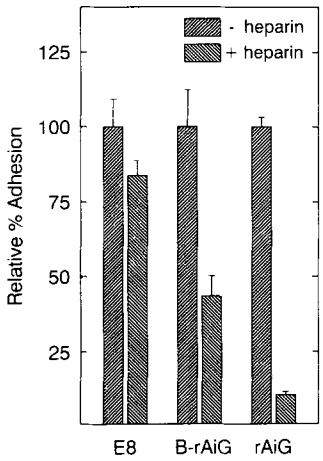

Figure 11. Effect of heparin on cell adhesion. HT1080 cells were incubated with wells coated with fragment E8, B-rAiG or rAiG in the absence (-) or presence (+) of 10 μg/ml heparin. Heparin inhibition was most pronounced with rAiG, but was also observed with B-rAiG.

Glycoprotein Structure

The hybrid molecule (B-rAiG) was formed by mixing recombinant A chain glycoprotein with the disulfide-linked B1 and B2 chain moiety of fragment E8. A specific assembly was demonstrated by the appearance of a species with a different heparin affinity, by the formation of a new molecular morphology, and by the development of A chain protease resistance. Specific subunit assembly, furthermore, required the presence of domain I sequences since rG did not form ball and rod structures. This folding of the three chains of the long arm was predicted because it had been previously found that the A chain of authentic fragment E8 can bind in vitro to the coiled B1-B2 chains to form a triple chain coiled-coil superhelix (Hunter et al., 1990, 1992). In particular, when the A, B1, and B2 chains were separated in urea (even with reduction to dissociate the B1 and B2 chains), and allowed to fold back by the removal of urea, they reassembled with recovery of a supercoiled structure indistinguishable from that of the native protein as measured by circular dichroism and electron microscopy (Hunter et al., 1990, 1992). Such treatment with chaotropic agents, however, does not generally lead to all molecules reforming monomers, and we have observed the globular ends of E8 attached together in dimers and trimers following reconstitution (electron micrographs not shown). These observations, coupled with the ability to refold purely monomeric and biologically active molecules with refolded E8-B chains, is interpreted as evidence that the difficulty in E8 refolding lies with the A chain moiety.

The hybrid B-rAiG glycoprotein was striking with respect to its molecular morphology and its similarity with the distal long arm of laminin. The protection of the A chain domain I sequences upon folding was furthermore demonstrated by the resistance to elastase digestion in which the formation of rG70-like and rG50 fragments, which encompass the G domain (Yurchenco et al., 1993) was significantly retarded. However, B-rAiG did not perfectly reproduce authentic laminin structure. The reduced resistance to proteolysis may reflect reduced stability or increased accessibility and may be due to differences in glycosylation. Nonetheless, the reconstituted structure was sufficiently correct to restore biological activity.

Heparin Binding

Recently, we have identified two potential heparin binding regions in the G domain of laminin (Yurchenco et al., 1993). The stronger one was found to be present in the first three subdomains (rG70) while the weaker one was localized in the last two subdomains (rG50). Recent data from proteolytic fragments of rG (Sung, O'Rear and Yurchenco, unpublished observations) indicate that the stronger heparin activity resides within subdomain G1 while the weaker one resides in subdomain G4. This inverse relationship of rG70 and rG50, relative to E8 and E3 was surprising and suggested that in intact laminin the proximal (rG70) site was somehow inhibited. While the absence of sialic acid in the recombinant glycoproteins produced a small increase in heparin affinity, this did not explain the major differences in laminin and rG binding. In the current study we evaluated the contribution of more upstream regions of laminin to heparin affinity. First, rAiG eluted at the same salt concentration as rG, indicating that the G domain sequence was likely the primary sequence required for heparin binding. Second, the hybrid glycoprotein B-rAiG, containing the B chains of E8, bound less strongly to heparin compared to rAiG or even to isolated subdomains G1–G3 (rG70). Partial digestion of B-rAiG with elastase led to the formation of an E8-like fragment and an E3-like fragment (rG50). The heparin affinity of these were very similar to their respective authentic fragments. The identical elution of rG50 and intact B-rAiG furthermore indicates that the stronger proximal binding region (G1–G3) plays little role in heparin binding once the B chains have intercalated with the A chain. Thus the B chains suppress binding to the adjacent proximal moiety of G domain. This effect might be steric in nature, for example, caused by a B chain extending over the heparin binding site. Alternatively, the inhibition could be due to a specific additional interaction of the distal rod domain with the G domain.

Cell Adhesion

The HT1080 human fibrosarcoma cell line was picked for evaluation because its $\alpha 6\beta 1$ integrin-specific adhesion to laminin has been well characterized (Aumailley et al., 1990; von der Mark et al., 1991). Binding of these cells to laminin fragment E8 has been shown to be dependent on conformation in which adhesion is lost if fragment E8 is treated with heat or urea and in which epitopes from both the A and B chains appear to be required for activity (Deutzmann et al., 1990). While HT1080 cells can also recognize the short arm region of laminin (fragment P1), this other interaction has been reported not to be observed in intact laminin (Nurcombe et al., 1989).

In this study we found that HT1080 cells recognize both the hybrid glycoprotein B-rAiG and the A chain glycoprotein rAiG; however, the two glycoproteins were recognized in substantially different ways. Binding was not observed to isolated E8-A or B chains or to fragment E3. Cells adhered to B-rAiG in a manner similar to fragment E8. Cell binding to rG, on the other hand, was low relative to E8 or rAiG. Cell binding to E8, B-rAiG, and rAiG was found to be destroyed by transient exposure of substrate to 8 M urea. Most important, however, was the observation that the cell recognition to active B-rAiG was inhibited by monoclonal antibodies GoH3 and AIIB2 which specifically neutralize the $\alpha 6$ and $\beta 1$ integrins, respectively. This activity was not restored following reassociation of authentic E8-A and E8-B chains following transient denaturation consistent with earlier observations that heat or exposure of protein to chaotropic agents irreversibly inactivates fragment E8 (Deutzmann et al., 1990.) The distinct activity was dependent on both A and B chains joining to form a rod and ball structure and was not seen with E8-B and rG mixtures that do not intercalate. These data then strongly suggest that an intimate and specific association of moieties of the three subunits of laminin in which a rod domain is formed led to restoration of a conformationally dependent cell adhesion site that is not present in any of the component subunit moieties in isolation.

While HT1080 cells also recognized rAiG in the absence of B chains, this latter interaction was not blocked either by GoH3 or by AIIB2. Thus adhesion to rAiG, in contrast to B-rAiG, is mediated by another cell surface binding macromolecule, a receptor which appears to lack both the $\alpha 6$ and the $\beta 1$ chain and which does not recognize E8 or B-rAiG. High levels of binding required the α-helical sequences from the rod domain of the A chain since lower levels were observed with cells plated onto rG alone. The non-$\alpha 6$, non-$\beta 1$ integrin receptor for rAiG also recognizes substrate conformation since treatment of rAiG with 8 M urea destroyed activity. This in turn suggests that the cell binding identified for this A chain recombinant glycoprotein also exists in authentic A chain but is lost upon the denaturing conditions required to separate the chains. The site of cell binding within rAiG is uncertain, but is in part dependent on globular subdomains G1–G3 given specific antibody inhibition. A question arising out of the distinctly different cell adhesion to rAiG is whether there are circumstances when the cryptic activity assumes biological importance. First, physiological degradation of laminin (turnover) might exposure the A chain cryptic sites. Second, the intercalation of variant B chains of laminin (e.g., B1s, B2t [Hunter et al., 1989; Kallunki et al., 1992]) might lead to exposure of otherwise cryptic sites.

We have drawn conclusions in this study with recombinant molecules that support and extend earlier studies carried out with proteolytic fragments of laminin (Deutzmann et al., 1990). Let us consider alternative hypotheses. First, it is useful to reiterate an underlying assumption made with in vitro cell binding studies, i.e., that the binding of protein substrates to plastic, which possesses no known specificity, is essentially stochastic without preferred protein binding sites. If not, one might hypothesize that different proteins attach to plastic by selected nonrandom domains and one might lose activity through artifact. One reason to suspect that the phenomena reported in this study are directly due to changes in the tertiary and quaternary structure of the substrate are that not only has an activity ($\alpha 6\beta 1$ recognition site) been restored, but also that new activities (heparin binding and a different type of cell binding) not present in E8 or E3 alone are found only in the isolated recombinant glycoprotein chain. Another reason is that the same phenomenon is observed when E8, rAiG, and B-rAiG is immobilized on nitrocellulose (data not shown), a polymer matrix with more hydrophilic adhesive properties. Given the assumption of random plastic immobilization, could a much more complex receptor mechanism, independent of protein folding, operate? For example, might not different receptors recognize the

separate A and B chains, activating a third receptor, the $\alpha 6\beta 1$ integrin, which then is identified in the binding assay? There are several arguments against such a complex model. First, no separate binding to E8-B chains can be detected. Second, cells do not adhere to mixtures of rG and E8-B chains, ruling out a hypothetical interaction stimulated by recognition of rAiG plus the B chains. The remaining NH_2-terminal α-helical sequences would seem the least likely to be exposed for cell interactions since they normally intercalate with the B chains with many residues buried in the coiled-coil (Beck et al., 1993).

Protein Folding and Function

The relationship between structure and some functions in laminin is proving not to be a simple relationship between linear sequence and activity, but rather a more complex one involving tertiary and quaternary structure as well. These studies, like those of Deutzmann et al. (1990) using a different experimental approach, raise questions about the strict use of small fragments or synthetic peptides to map biological activities in the absence of complementary studies with folded macromolecules (particularly when several subunits are involved) and suggest that a hierarchy of protein determinants can exist in extracellular matrix components. These higher ordered properties may be difficult to dissect because functional activities can be lost upon generation of increasingly smaller protein segments. In this study we have shown that it is possible to reconstitute laminin functions by the hybridization of authentic and recombinant glycoproteins, thereby opening a different avenue to investigate these functions. In evaluating cell and heparin interactions with these new reagents, we have found that some functions are activated and others are inhibited upon higher ordered protein assembly. Our results not only suggest that quaternary structure is very important for faithful reproduction of the long arm cell recognition activity, but also that a close association or intercalation of the COOH-termini of the B chains with the proximal subdomains of G creates a structure necessary for $\alpha 6\beta 1$ mediated binding and for inactivation of other cell and heparin binding sites, either directly or by induction of a conformational change in the G domain.

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