The Basement Membrane Glycoprotein Entactin Promotes Cell Attachment and Binds Calcium Ions*

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Mouse entactin derived from the extracellular matrix of M1536-B3 cells and from insect cells infected with a recombinant virus containing entactin sequences were shown to promote the attachment of mouse mammary tumor, human melanoma, and other cells. The cell attachment was inhibited by antibodies against mouse entactin but not by anti-fibronectin or anti-laminin antibodies. On a weight basis entactin was as effective as laminin in promoting the attachment of mouse mammary tumor cells. The attachment of cells to entactin was in part mediated by the integrin recognition RGD peptide sequence. This was demonstrated by the cell attachment properties of peptides derived from entactin which contained this sequence. Furthermore, the peptide RGDs could inhibit the attachment of mouse mammary tumor cells to entactin to approximately 60% of control. It is suggested that additional cell recognition sequences may be present in entactin. The direct binding of calcium ions to entactin was observed. It is probable that the binding sites reside in peptide sequences located toward the NH2 terminus region of entactin. This conclusion was supported by the demonstration that synthetic peptides, containing potential calcium binding sequences derived from entactin, bound calcium. In addition, a recombinant peptide containing the amino-terminal 330 amino acids of entactin also bound calcium ions. The significance of these properties of entactin is discussed.

The structure and biological functions of the basement membrane have been reviewed in detail in the past several years (1, 2). The basement membrane is a continuous sheet of extracellular matrix that underlies and makes intimate contact with epithelial and endothelial cells, as well as with muscle, fat, and neural tissues. Information to the cells is transferred through these contacts via membrane receptors specific for one or more of its molecular components, which include type IV collagen (3), laminin (4, 5), entactin (6-8), and heparan sulfate proteoglycans (9-11). The organization, properties, and interactions of the molecular constituents are compatible with the biological function of a particular basement membrane. Rapid and significant advances have been made in understanding basement membrane function by analysis of the biological properties of each component, and in particular studies on laminin have been revealing and rewarding (12-17). On the other hand, very little is known about the functions of entactin and its role in the assembly and behavior of basement membranes. Entactin, a 150-kDa sulfated glycoprotein, was first discovered in the extracellular matrix synthesized by the mouse cell line M1536-B3 (18). A similar protein was shown to be present in mouse Reichert's membrane (19). Several years later a degraded form obtained from Englebreth-Holm-Swarm (EHS)1 tumors was mistaken identified as a new basement membrane protein and hence named nidogen (20). The primary structures of mouse (21, 22) and human (23) entactin have been determined by a combination of amino acid and cDNA sequencing. The 85% sequence identity between the mouse and human molecules and the virtual identity of the partial rat sequence (24) with that of the corresponding segment from mouse indicate that the molecular structure is highly conserved. Entactin forms a tight stoichiometric complex with laminin, and images from rotary-shadowed specimens have revealed that the dumbbell-shaped entactin binds via one of its globular termini to a short arm of the cruciform laminin in the proximity of the intersection of the arms. It has been suggested that this binding occurs through the carboxyl end of entactin (25, 43).

The predicted secondary structure of entactin which is consistent with the physical image obtained by electron microscopy has several additional interesting features. The NH2-terminal 639 amino acid residues which are predicted to fold into a 70-kDa globular domain are separated from the carboxyl terminus globular domain by a cysteine-rich region which provides the rodlike connection (21). Most of the cysteines in the rodlike domain are organized into an EGF-like repeat pattern (~40 residues long). The amino terminus segment of the molecule (residues 15-26 and 250-261) and two of the EGF-like repeat units contain potential calcium binding sequences (21). The central domain also contains one copy of the Arg-Gly-Asp (residues 672-674) integrin recognition sequence in one of the several EGF-like cysteine-rich homology repeats.

The biological function of entactin has been an enigma. Several lines of evidence suggest, however, that it is important in the organization and normal function of basement membranes. Indirect immunolocalization of tissues with antisera raised against entactin derived either from M1536-B3 or EHS tumor extracellular matrix has shown that the molecule appears early in embryogenesis and persists through adulthood (26-29). The attachment of epithelial (30, 31) and other cells (22) in vitro is promoted by entactin. Finally, its strong

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1 The abbreviations used are: EHS, Englebreth-Holm-Swarm; EGF, epidermal growth factor; DME, Dulbecco's modified Eagle's; ECM, extracellular matrix; PBS, phosphate-buffered saline; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate.

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interaction with laminin in the extracellular matrix, together with the demonstration of its association with type IV collagen and fibronectin in solid phase binding assays (8), indicates that it may be involved in basement membrane and extracellular matrix assembly.

In this paper the biological properties of entactin have been investigated with both an insect cell-derived recombinant molecule and that derived from mouse cells. Particular emphasis was placed on the cell and calcium binding potentials of the molecule. In addition, synthetic peptides from entactin-derived sequences have been shown to mimic these biological properties and to suggest the assignment of distinct functional domains in the molecule.

MATERIALS AND METHODS

Cells—M1536-B3, a parietal endoderm-like mouse cell line (18), MM7 006082 mouse mammary tumor cells (American Type Culture Collection), and human melanoma M21 cells (the generous gift of Dr. David Cheres. Scripps Clinic and Research Foundation. La Jolla, CA) were maintained in Dulbecco’s modified Eagle’s (DME) medium supplemented with 10% fetal calf serum. The insect cell line Spodoptera frugiperda (Sf21) was grown in Grace’s insect cell medium supplemented with 10% fetal calf serum (32).

Purification of Entactin—Entactin was purified from M1536-B3 ECM preparations. The M1536-B3 ECM was prepared as described previously (4) and extracted overnight with PBS containing 4 μg/ml aprotinin. Undissolved particles were removed from the extract by centrifugation at 1000 × g in a Beckman J212 centrifuge. The supernatant (~1 mg/ml solution) was resolved by high pressure liquid chromatography on a Zorbax GF 450 gel filtration column (Du Pont, Wilmington, DE) at a flow rate of 0.7 ml/min. Fractions enriched for entactin were pooled, dialyzed against 3 × 2-liter distilled water, concentrated in a vacuum centrifuge, and rechromatographed under similar conditions to obtain pure entactin. The presence of contaminating laminin in the entactin preparations was tested by indirect immunostaining with anti-laminin antibodies.

Isolation of Entactin from Insect Cells—The recombinant baculovirus, 14-6 containing the entire entactin cDNA sequence and isolated as described earlier (31), was used to infect SF9 cells according to the instruction manual (Biosystems, Foster City, CA). Antibody Preparation—Antibodies against mouse entactin and laminin derived from M1536-B3 extracellular matrix have been described earlier (4).

Cell Attachment Assays—Cell monolayers, radioactively labeled with [3H]thymidine (2.5 μCi/ml) for 12 h were detached by treatment with 0.05% trypsin, 0.02% EDTA in PBS, harvested by centrifugation, and resuspended in serum-free DME medium containing 0.05% BSA. Aliquots of the cell suspension were plated on Petri dishes (35 mm) coated with synthetic peptides, ECM extract, or purified entactin to a final concentration of ~1 × 10^5 cells/plate. The plates were incubated for 2-4 h at 37 °C in an atmosphere of 100% humidity, 5% CO_2 in air, then washed twice with 1 ml of PBS and once with 0.5 ml of trypsin to remove unattached cells. Attached cells were dislodged by incubation with 1 ml of trypsin, transferred to scintillation vials, and mixed with scintillation fluid, Formula 963 (Du Pont, Wilmington, DE). The associated radioactivity was determined with a Beckman LS1800 liquid scintillation counter. Percent attachment was calculated as follows: (cpm in coated plate – cpm in BSA-coated plate) / (total cpm/plate) × 100.

The procedure for coating of plastic Petri dishes (35-mm Falcon) with synthetic peptides has been described earlier (21). To coat Petri dishes with intact proteins, these were first coated with 0.1 ml of nitrocellulose (Schleicher and Schuell) dissolved in 100% methanol (1 × 5-cm strip in 6 ml) and air-dried. Entactin or whole ECM extract in a final volume of 1 ml was added. The plates were incubated and oven-dried overnight at 4 °C followed by blocking of nonspecific binding sites with 3% BSA in PBS for an additional 24 h period. Nitrocellulose treated dishes coated with BSA alone served as the negative control.

In order to demonstrate the attachment of cells directly to entactin from M1536-B3 ECM or from infected insect cells, the proteins in the extract were separated on SDS-containing polyacrylamide gels and then blotted to Immobilon P membrane (Millipore Corp., Bedford, MA) as described in the next section. The membrane was blocked with a solution of 0.5% BSA and then exposed to a cell suspension (~1 × 10^5 cells/ml) for 2 h as described elsewhere (33). The cells were fixed with 3% paraformaldehyde in PBS and stained with 2% crystal violet in a gentle black and barely staining cells where they were attached to the membrane.

Electrophoretic Protein Transfer—Proteins separated by SDS-polyacrylamide gel electrophoresis (34) were electrophoretically transferred to Immobilon filters according to the method of Towbin et al. (35). For staining with antibodies, the filters were washed once in TBS buffer containing 0.05% Tween 20 (TBST) and then incubated with 3% BSA in TBST overnight at 4 °C or for 2 h at room temperature to block nonspecific binding sites. The primary antibody was added to the BSA containing TBST at a dilution of 1:500 to 1:2000 and incubated at room temperature for 2 h with gentle shaking. The filters were rinsed three times in TBST. For each wash the filters were soaked in 0.02 M Tris-HCl, pH 9, 0.1 M NaCl, and 0.005 M MgCl_2 (AP buffer) once, and incubated with 55 μl of 5% nitroblue tetrazolium in 70% dimethyl formamide and 16.5 μl of 5% 5-bromo-4-chloro-3-indolyl phosphate in 100% dimethyl formamide/5 ml of AP buffer (alkaline phosphatase substrate). To stop the reaction, the filters were soaked in 0.02 M Tris-HCl, pH 8, and 0.005 M EDTA and dried immediately.

Calcium Binding—To test for the binding of "Ca⁺⁺" by different proteins, a method developed by Maruyama et al. (36) was used. Filters were first blocked as described above and electrophoretically transferred to Immobilon filters. The filters were washed three times in 60 mM KCl, 5 mM MgCl_2, and 10 mM imidazole HCl, pH 6.8, for 1 h, incubated in 10 min in the same buffer containing 1 mM/liter of "CaCl_2", and then rinsed twice in distilled water to remove "CaCl_2" that was nonspecifically bound. "CaCl_2" binding by specific protein bands was detected by exposure to Kodak SB-5 film for 2-3 days. All manipulations were carried out at room temperature. To test for the binding of "Ca⁺⁺" by synthetic peptides, solutions of the peptides were directly applied to nitrocellulose filters, air-dried, and tested by the assay described above.

RESULTS

Isolation of Entactin from M1536-B3 Extracellular Matrix—To ensure that the promotion of cell attachment by entactin...
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was not due to contamination by small amounts of laminin, samples of entactin purified (Fig. 1A) as described above were immunostained with polyclonal anti-laminin antiserum. The results shown in Fig. 1B demonstrate that this was indeed the case. The anti-laminin antiserum failed to detect any laminin in the entactin preparation (Fig. 1B, lane 5). Most importantly, the anti-entactin antiserum did not show any cross-reactivity with laminin (Fig. 1B, lanes 6 and 7). This anti-entactin antiserum was used in all the cell adhesion assays described in the following paragraphs. The recombinant entactin isolated from infected Sf9 cells, as expected, was also free of laminin as shown in Fig. 1C (lane 8).

Inhibition of Cell Attachment to Entactin-containing ECM—The extracellular matrix isolated from M1536-B3 cells contains entactin tightly complexed to laminin. This complex supported the attachment of MMT and M21 cells (not shown here). In further quantitative studies with the MMT cell line it was shown to promote cell attachment in a dose- and time-dependent manner (Fig. 2, A and B). In these experiments the data were corrected for the background attachment to dishes coated with bovine serum albumin alone. This background was generally in the range of 2–10% of the maximum of the matrix-stimulated attachment. The inhibition of cell attachment to M1536-B3 ECM by antisera to either laminin or entactin (Fig. 3) suggested that both molecules were involved. It is noteworthy that the polyclonal antibodies to either the A or B chains of laminin inhibited cell attachment in contrast to the monoclonal antibodies Lam I (37) and Lam V (38). Two separate preparations of anti-entactin antibodies inhibited cell attachment by approximately 40%. To resolve the contribution of each component to cell attachment, experiments were carried out with purified laminin and entactin preparations.

Attachment of MMT Cells to Laminin and Entactin—As shown in Fig. 4, either laminin or entactin separately enhanced the attachment of MMT cells in a dose-dependent manner. These results demonstrate that at similar quantities, with this particular cell line, the two molecules were equally effective. A comparison with the intact matrix (data not shown) indicated that it was an order of magnitude more effective in promoting cell adhesion than either laminin or entactin. The specificity of cell attachment was further supported by the effects of anti-entactin antiserum on attachment to entactin of either MMT (Fig. 5) or human melanoma M21 cells (see Ref. 31). Neither anti-laminin nor anti-fibro-

Fig. 1. Isolation of entactin. A, entactin was purified from the M1536-B3 ECM as described in the text. Purified samples were tested on SDS-polyacrylamide gels and stained with 0.1% silver nitrate as shown here. Lane 1, M1536-B3 ECM; lane 2, isolated laminin; lane 3, isolated intact entactin and its 130-kDa degradation product; lane 4, 130 kDa and smaller degradation products of entactin. B, laminin and entactin purified from M1536-B3 ECM were immunostained with polyclonal anti-laminin and anti-entactin antisera. Lanes 1 and 2 were stained with 0.1% Amido Black. Lanes 3-5 and lanes 6-8 were immunostained with anti-laminin and anti-entactin antisera, respectively. Lane 1, molecular mass standards: myosin (200 kDa), β-galactosidase (116 kDa), bovine albumin (66 kDa); lanes 2, 3, and 6, ECM; lanes 4 and 7, isolated laminin; lanes 5 and 8, isolated entactin. C, crude extracts of 14-6/Sf9 and uninfected Sf9 cells were immunostained with anti-laminin and anti-entactin antisera, respectively. Lane 1, molecular mass standards: myosin (200 kDa), β-galactosidase (116 kDa), phosphorlase b (97 kDa), bovine albumin (66 kDa); lanes 2, 3, and 6, ECM; lanes 4 and 7, isolated laminin; lanes 5 and 8, isolated entactin; lanes 5-7 and lanes 8-9 were immunostained with anti-entactin and anti-laminin antisera, respectively. Lane 1, molecular mass standards: β-galactosidase (116 kDa), phosphorlase b (97.4 kDa), bovine albumin (66 kDa); lanes 2, 7, and 9, M1536-B3 ECM; lanes 3 and 5, Sf9 cell extract; lanes 4, 6, and 8, partially purified entactin from 14-6/Sf9 cell extract.

Fig. 2. Cell attachment to M1536-B3 ECM. Approximately 1–2 × 10^5 [H]thymidine-labeled MMT 090562 cells were added to plates coated with 1 ml of 0.2–20 µg/ml ECM (A) as described under "Materials and Methods." Levels of nonspecific binding, usually in the range of 2–10% of maximum adhesion, were subtracted from the experimental values. Each data point represents the mean of two observations (±S.D.). The kinetics of cell adhesion to ECM are shown in B. Cells were incubated in plates coated with 1 ml of 5 µg/ml ECM. Cell attachment was assayed as in A at different intervals. The data points represent the mean (±S.D.) of three separate measurements except for those that are shown without error bars.
FIG. 3. Effect of antisera on cell attachment to M1536-B3 ECM. Attachment of MMT cells to ECM (1 ml of 5 μg/ml) was determined as described in the text. The antisera used were anti-lam A, a polyclonal antiserum against laminin A and anti-lam B, a polyclonal antiserum against laminin B (B1 + B2); anti-ent MV3 and anti-ent are two separate polyclonal antisera against entactin from M1536-B3 ECM. Lam I and Lam V are two monoclonal antibodies against the laminin B and laminin A subunits, respectively. Attachment in the absence of any antiserum was taken as 100%. The data represent the mean (±S.D.) of six to eight measurements obtained from three separate assays, except for the Lam I and Lam V results, which consist of the mean (±S.D.) of two observations.

FIG. 4. Cell adhesion to purified entactin and laminin. Recombinant entactin used in these assays was purified from 14-6/Sf9 cell extracts, and laminin was purified from M1536-B3 ECM as described in the text. Attachment of MMT cells to plates coated with different concentrations of entactin (1 ml of 0.75-15 μg/ml) and laminin (1 ml of 0.4-20 μg/ml) was assayed as for Fig. 2. The data represent the mean (±S.D.) of two (entactin) and four (laminin) independent observations.

FIG. 5. Effect of antiserum on cell attachment to entactin. Attachment of MMT cells to entactin (~10 μg/plate) was assayed in the presence of different antisera (1:50 dilution in DME medium) as described for Fig. 3. Attachment in the absence of any antiserum was taken as 100%. The data shown with error bars represent the mean (±S.D.) of multiple observations as indicated in the figure.

FIG. 6. Attachment of MMT cells to transblots of M1536-B3 ECM and 14-6/Sf9 cell extracts. MMT cells were incubated with transfer blots of 14-6/Sf9 cell extracts resolved by SDS-polyacrylamide gel electrophoresis for 90 min at 37 °C as described in the text. Lanes 1 and 2 were stained with Amido Black. Lanes 3 and 4 were immunostained with the polyclonal anti-entactin antiserum. Lanes 5-7 were incubated with cells. Lanes 1, 3, and 5, uninfected Sf9 cell extract; lanes 2, 4, and 6, 14-6/Sf9 cell extract; lane 7, recombinant entactin band excised from 14-6/Sf9 cell extract resolved by SDS-polyacrylamide gel electrophoresis and re-electrophoresed.

Calcium Binding to Entactin—The primary structure of entactin has revealed the presence of several potential calcium binding sequences, of which two reside in the amino-terminal globular domain. As shown in Fig. 10A, peptide E3, representing the first putative Ca²⁺ binding sequence, binds “Ca²⁺,” and somewhat unexpectedly, the two RGD-containing pep-
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**FIG. 7.** Dose-dependent cell attachment to synthetic peptides and its inhibition with RGDS. [H]Thymidine-labeled MMT cells were plated on Petri dishes coated with the synthetic peptides E1 (SIGFRGDGQTC) as shown in A or E5 (CYIG-THGCDSNAACRFPGPTQFTCECSIGFRGDGQT) as shown in B. Attachment levels are presented as a percent of radioactivity added per plate. Each data point represents the mean ±S.D. of eight observations from 4-day experiments for E1 and four observations from 2-day experiments for E5. Effects of the RGDS peptide on cell attachment to E1 and E5 are shown in C. MMT cells were added to Petri dishes coated with 1 ml of 10 µg/ml E1 or E5. Increasing amounts of the RGDS peptide dissolved in DME medium were added to the Petri dishes to final concentrations in the range of 20–200 µg/ml, and cell adhesion was assayed as before. The results represent the mean (±S.D.) of four observations.

**FIG. 8.** Effect of RGDS peptide on cell attachment to entactin. Attachment of MMT cells to entactin (10 µg/plate) was assayed in the presence of increasing concentrations of RGDS (20–200 µg/ml final concentration) as described for Fig. 7C. The percent attachments shown represent the mean (±S.D.) of two observations except for the data points at 100 and 150 µg/ml RGDS which constitute the mean (±S.D.) of three observations.

**FIG. 9.** Phase contrast micrographs of MMT cells attached to synthetic peptides and entactin. Amino acid sequences of the RGD-containing synthetic peptides E1 and E5 are described in the legend to Fig. 8. Cell attachment assay was performed as for Fig. 2. The results of MMT cells plated on Petri dishes coated with BSA (A), 20 µg/plate E1 (B) or E5 (C), and 15 µg/plate recombinant entactin (D) are shown here. Bar represents 100 µm.

tides E1 and E5 also bound 45Ca2+. Direct binding of Ca2+ to entactin, both from M1536-B3 ECM and 14-6/Sf9 cells was also shown on electrophoretic blots (Fig. 10B, lanes 4 and 5). It is noteworthy that the laminin B subunits but not the A subunits bound 45Ca2+ (Fig. 10B, lane 4). The recombinant baculovirus 2-2 which produces a peptide of molecular mass of ~54,600 kDa corresponding to the first 300 amino acids of entactin bound 45Ca2+ (Fig. 10C). This fragment included both putative calcium binding sequences from the NH2-terminal globular domain of entactin.

**DISCUSSION**

The recent publication of the primary structure of entactin (21) has enabled us to examine more closely two of its biological properties. The presence of the integrin recognition RGD sequence in a cysteine-rich EGF precursor homology repeat and potential calcium binding sequences in the amino-terminal segment of the molecule provided clues to potential functions of the molecule. In addition, the availability of a recombinant form of entactin, free of laminin, in substantial amounts allowed us to test for the presence of these properties.

The first suggestion that entactin could enhance cell attachment was provided by the partial inhibition of the attachment of mouse mammary tumor cells to the extracellular matrix of the endodermal cell line M1536-B3 by antibodies against entactin. It was found that entactin derived from either the matrix, representing the natural species, or from the recombinant baculovirus-infected insect cells by itself promoted cell adhesion. The specificity was supported by the observation that cell attachment was inhibited by anti-entactin antiserum but not by antibodies against either fibronectin or laminin. The direct attachment of cells to entactin in transfer blots of M1536-B3 ECM and 14-6/Sf9 cell extract resolved by SDS-polyacrylamide gel electrophoresis provided additional evidence supporting the cell adhesive potential of entactin. Although MMT cells were studied most extensively, other cells such as human melanoma and ras-transformed 3T3 cells could utilize entactin as a substrate for attachment.
Entactin, a protein of the extracellular matrix, binds calcium through its RGD sequences, which are known for their role in cell adhesion. The calcium binding properties of entactin were demonstrated in intact molecules as well as in peptides derived from its NH₂ terminus region. Previous work has shown that the RGD sequence in entactin can indeed serve as a recognition signal for cell attachment.

The calcium binding properties of entactin were demonstrated with intact molecules as well as peptide sequences derived from its NH₂ terminus region. Previous work has shown that the laminin-entactin complex undergoes polymerization in response to calcium ions (45, 46). There is some evidence which suggests that the globular domain of entactin at the carboxyl end of the molecule binds to laminin (25, 43), thus leaving the NH₂ terminus region free to interact with other molecules. Furthermore, the isolation of the extracellular matrix from M1536-B3 cells in intact form requires the presence of calcium ions, and the extraction of soluble forms of matrix from EHS tumors is facilitated by EDTA (4, 43). These observations taken together clearly suggest that calcium ions may play a key role in the configuration of the extracellular matrix. The free calcium binding amino terminus segment of entactin in the laminin-entactin complex could form a special type of bridge between adjacent complexes. Extracellularly, calcium ion concentrations could regulate such entactin-mediated cross-linking of preformed complexes. Additionally, this region could provide binding sites in the extracellular matrix for other calcium binding molecules or even serve as a reservoir for calcium ions in tissues.

It is well documented that entactin is exquisitely sensitive to proteases and that the protease-sensitive sites reside both in the carboxyl- and amino-terminal segments of the molecule (8, 47). The proposed bridging function of entactin in the extracellular matrix would provide a protease-sensitive site for breaching the basement membrane in such processes as metastasis and tissue reorganization. It is to be noted that a characteristic feature of metastatic cells is their potential to synthesize metal ion-dependent proteases (48).

Interestingly, the RGD sequence containing peptides also displayed calcium binding activities in our assays. The entactin RGD sequence is located in one of the EGF-type repeats. Some of the proteins of the coagulation and complement pathways containing such repeat structures have been shown to bind Ca²⁺, and this is believed to be associated with the presence of β-hydroxylated aspartate and asparagine residues in the EGF-like domains (49). The RGD-containing EGF-like repeat of entactin does not contain the proposed consensus sequence for β-hydroxylation Ca²⁺ binding. Furthermore, whether this segment or any of the EGF-like repeats actually bind Ca²⁺ must be demonstrated. However, an earlier study on the ECM protein thrombospondin reported the binding of calcium by its RGD sequences (50). Whether the RGD sequence of entactin indeed binds calcium with possible effects on its cell binding property remains to be studied. A calcium-related overall conformational transition of the RGD-containing rodlike domain could affect the cell binding property of entactin.
entactin. Alternatively, binding of calcium at the RGD site could be involved in stabilizing the RGD site in a cell surface receptor compatible conformation as suggested for thrombospondin (50).

The results described represent an attempt to define the biological functions of entactin. The cell attachment and calcium binding functions provide the basis for further exploration of specific cell surface receptors and the role of calcium in the organization and function of basement membranes.

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REFERENCES

1. Timpl, R., and Dziedzic, M. (1986) Int. Rev. Exp. Pathol. 29, 1-113
2. Martin, G. R., and Timpl, R. (1987) Annu. Rev. Cell Biol. 3, 57-86
3. Bornstein, P., and Sage, H. (1980) Annu. Rev. Biochem. 49, 957-1003
4. Chung, A. E., Jaffe, R., Freeman, I. L., Vergnes, J.-P., Braginski, J. E., and Carlin, B. E. (1979) Cell 16, 277-287
5. Timpl, R., Rohde, H., Robey, P. G., Rennard, S. I., Foidart, J.-M., and Martin, G. R. (1979) J. Biol. Chem. 254, 9933-9937
6. Carlin, B., Jaffe, R., Bender, B., and Chung, A. E. (1981) J. Biol. Chem. 256, 5209-5214
7. Hogan, B. L. M., Taylor, A., Kurkinen, M., and Couchman, J. R. (1982) J. Cell Biol. 95, 197-204
8. Dziedzic, M., Paulsson, M., and Timpl, R. (1985) EMBO J. 4, 2513-2518
9. Kowar, Y. S., and Farquhar, M. G. (1979) J. Cell Biol. 81, 137-153
10. Hassell, J. R., Kimura, J. H., and Hascall, V. C. (1986) Annu. Rev. Biochem. 55, 539-567
11. Ruoslabti, E. (1988) Annu. Rev. Cell Biol. 4, 229-255
12. Engel, J., Odermatt, E., Engel, A., Madri, J. A., Furthmayr, H., Rohde, H., and Timpl, R. (1981) J. Mol. Biol. 150, 97-120
13. Paulsson, M., Deutzmann, R., Timpl, R., Dalzoppo, D., Odermatt, E., and Engel, J. (1986) EMBO J. 4, 309-316
14. Sasaki, M., Kato, S., Kohno, K., Martin, G. R., and Yamada, Y. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 933-939
15. Sasaki, M., and Yamada, Y. (1987) J. Biol. Chem. 262, 17111-17117
16. Sasaki, M., Kleinman, H. K., Huber, H., Deutzmann, R., and Yamada, Y. (1988) J. Biol. Chem. 263, 10530-10544
17. Durkin, M. E., Bartos, B. B., Liu, S.-H., Phillips, S., and Chung, A. E. (1988) Biochemistry 27, 5198-5204
18. Chung, A. E., Freeman, I. L., and Braginski, J. E. (1977) Biochem. Biophys. Res. Commun. 79, 859-863
19. Inoué, S., LeBlond, C.-P., and Laurie, G. W. (1983) J. Cell Biol. 97, 1524-1537
20. Timpl, R., Dziedzic, M., Fujisawa, S., Nowack, H., and Wick, G. (1986) Eur. J. Biochem. 157, 455-465
21. Durkin, M., Chakravarti, S., Bartos, B. B., Liu, S.-H., Friedman, R., and Chung, A. E. (1988) J. Cell Biol. 107, 2749-2756
22. Mann, K., Deutzmann, R., Aumailley, M., Timpl, R., Rainondi, L., Yamada, Y., Pan, T.-C., Conway, D., and Chu, M.-L. (1989) EMBO J. 8, 65-72
23. Nagayoshi, T., Sanborn, D., Hickok, N. J., Olsen, D. R., Fazio, M. J., Chu, M.-L., Knowlton, R., Deutzmann, R., Timpl, R., and Utito, J. (1983) DNA 8, 581-594
24. Durkin, M. E., Carlin, B. E., Vergnes, J., Bartos, B., Merlie, J., and Chung, A. E. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 1570-1574
25. Mann, K., Deutzmann, R., and Timpl, R. (1988) Eur. J. Biochem. 178, 71-80
26. Laurie, G. W., LeBlond, C.-P., Martin, G. R., and Chung, A. E. (1988) Am. J. Anat. 189, 463-481
27. Wu, T.-C., Wan, Y.-J., Chung, A. E., and Damjanov, I. (1983) Dev. Biol. 100, 496-505
28. Warburton, M. J. P., Monaghan, S. A., Ferns, S. A., Rudland, P. S., Persunghe, N., and Chung, A. E. (1984) Exp. Cell Res. 152, 479-504
29. Dziedzic, M., and Timpl, R. (1985) Dev. Biol. 111, 372-382
30. Chakravarti, S. (1989) The Role of Entactin in Basement Membranes. Ph.D. thesis, University of Pittsburgh
31. Tsao, T., Hsieh, J.-C., Durkin, M. E., Wu, C., Chakravarti, S., Dong, L.-J., Lewis, M., and Chung, A. E. (1990) J. Biol. Chem. 265, 5188-5191
32. Summers, M. D., and Smith, G. E. (1987) A Manual for Baculovirus Vectors and Insect Cell Procedures, Texas A&M University, College Station, TX
33. Hayman, E. G., Engvall, E., A’Hearn, E., Barnes, D., Pierschbacher, M., and Ruoslahti, E. (1982) J. Biol. Chem. 255, 20-23
34. Lammli, U. K. (1970) Nature 227, 680-685
35. Towbin, H., Stahelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4550-4554
36. Maruyama, K., Mikawa, T., and Ebashi, E. (1984) J. Biochem. (Tokyo) 95, 511-519
37. Chung, A. E., Jaffe, R., Bender, B., Lewis, M., and Durkin, M. (1988) Lab. Invest. 59, 576-581
38. Simons, A. J., and Boynton, G. W., LeBlond, C.-P., Martin, G. R., and Chung, A. E. (1985) Lab. Invest. 59, 576-581
39. Chung, A. E., Friedman, R., and Chung, A. E. (1988) Biochemistry 27, 8703-8707
40. Timpl, R., Johansson, S., van Delden, V., Oberbäumer, I., and Höck, M. (1983) J. Biol. Chem. 258, 8922-8927
41. Graf, J., Iwamoto, Y., Sasaki, M., Martin, G. M., Kleinman, H. K., Robey, F. A., and Yamada, Y. (1987) Cell 48, 999-996
42. Charonis, A. S., Tsilibraki, E. F., and Furthmayr, H. (1985) J. Cell Biol. 100, 1843-1853
43. Sabin, T. P., McCarthy, J. B., Charonis, A. S., and Furthmayr, H. (1985) J. Cell Biol. 100, 7184-7193
44. Paulsson, M., Aumailley, M., Deutzmann, R., Timpl, R., Beck, K., and Engel, J. (1987) Eur. J. Biochem. 166, 11-19
45. von der Mark, K., and Kühl, U. (1985) Biochim. Biophys. Acta 829, 147-160
46. Yurchenco, P., Tsilibraki, E., Charonis, A., and Furthmayr, H. (1985) J. Biol. Chem. 260, 8736-8744
47. Paulsson, M. (1988) J. Biol. Chem. 263, 5425-5430
48. Paulsson, M., Deutzmann, R., Dziedzic, M., Nowack, H., Timpl, R., Weber, S., and Engel, J. (1986) Eur. J. Biochem. 156, 476-483
49. Liotta, L. A. (1986) Cancer Res. 46, 1-7
50. Rees, D. J. G., Jones, I. M., Handford, P. A., Aker, S. J., Esnouf, M. P., Smith, K. J., and Dowle, O. G. (1996) EMBO J. 7, 2053-2061
51. Lawner, J., Weinstein, R., and Hynes, R. O. (1988) J. Cell Biol. 107, 2061-2061
The basement membrane glycoprotein entactin promotes cell attachment and binds calcium ions.
S Chakravarti, M F Tam and A E Chung

J. Biol. Chem. 1990, 265:10597-10603.