Recombinant Entactin Promotes Mouse Primary Trophoblast Cell Adhesion and Migration Through the Arg-Gly-Asp (RGD) Recognition Sequence

Frank D. Yelian,* Nancy A. Edgeworth,* Li-Jin Dong,‡ Albert E. Chung,‡ and D. Randall Armant*§

*C. S. Mott Center for Human Growth and Development, Department of Obstetrics and Gynecology, and §Department of Anatomy and Cell Biology, Wayne State University School of Medicine, Detroit, Michigan; and ‡Department of Biological Science, University of Pittsburgh, Pittsburgh, Pennsylvania

Abstract. In vitro culture of mouse blastocysts during the period coinciding with implantation has revealed that primary trophoblast cells can adhere and migrate in serum-free medium when provided with certain extracellular matrix components, including fibronectin and laminin. Tightly associated with laminin is the glycoprotein, entactin, that may play an important role in basement membrane assembly and cell attachment. Mouse blastocysts were studied using this in vitro model to determine whether entactin was capable of mediating trophoblast invasive activity. Although entactin has never been shown to promote cell migration, we report here that recombinant entactin supported blastocyst outgrowth in a dose-dependent manner, with a maximal effect at 20–50 μg/ml. The ability of trophoblast cells to adhere and migrate on entactin was specifically inhibited by anti-entactin antibody, but not by antibodies raised against laminin. The synthetic peptide, Gly-Arg-Gly-Asp-Ser-Pro, that contains the Arg-Gly-Asp (RGD) integrin recognition site, reversibly inhibited entactin-mediated blastocyst outgrowth in a dose-dependent manner, but had no effect on laminin-mediated outgrowth. The synthetic peptide, Gly-Phe-Arg-Gly-Asp-Gly-Gln, that comprises the actual RGD-containing sequence within entactin, promoted trophoblast outgrowth when immobilized on the substratum. Furthermore, a mutated recombinant entactin, altered to contain a Glu in place of Asp at the RGD site, provided no trophoblast cell adhesive activity. We conclude that entactin promotes trophoblast outgrowth through a mechanism mediated by the RGD recognition site, and that it may play an important role during invasion of the endometrial basement membrane at implantation.

PREIMPLANTATION development of the mouse embryo culminates with the formation of a blastocyst that hatches from its zona pellucida and is capable of implanting within the uterine wall. Trophoblast cells derived from the trophectoderm of the blastocyst play a critical role in embryo implantation and subsequent placenta formation. After a short period of trophoblast attachment to the uterine epithelium, the epithelial cells slough in the area adjacent to the embryo, allowing the trophoblast cells to adhere to and penetrate the underlying basement membrane (Schlafke and Enders, 1975). In the mouse, invasion by the trophoblast cells continues into the decidua where these cells adhere to and degrade the extracellular matrix that surrounds the cells comprising the stroma of the endometrium (Yagel et al., 1988). Most of the glycoproteins and proteoglycans of the endometrial basement membrane and extracellular matrix have been identified by immunohistochemical or biochemical methods, and include hyaluronic acid, heparan sulfate proteoglycan, type IV collagen (COL-IV)[1], fibronectin, laminin and entactin (Wartiovaara et al., 1979; Leivo et al., 1980; Wu et al., 1983; Wewer et al., 1985; Kisalus et al., 1987; Carson et al., 1987). The function of extracellular matrix components during embryo implantation has been investigated in vitro by determining the adhesive properties of mouse primary trophoblast cells derived from blastocysts cultured in serum-free medium on surfaces precoated with extracellular matrix (Armant et al., 1986*a*). Using this model of trophoblast invasiveness, several investigations have demonstrated that fibronectin, laminin, vitronectin, various collagen forms, thrombospondin and hyaluronic acid are all capable of promoting trophoblast cell adhesion and migration in vitro (Armant et al., 1986*a*,*b*; Carson et al.,

Address correspondence to Dr. D. R. Armant at C. S. Mott Center for Human Growth and Development, Department of Obstetrics and Gynecology, Wayne State University School of Medicine, 275 E. Hancock Avenue, Detroit, MI 48201.

1. *Abbreviations used in this paper*: COL-IV, type IV collagen; GFRGDGQ, Gly-Phe-Arg-Gly-Asp-Gly-Gln; GRADSP, Gly-Arg-Ala-Asp-Ser-Pro; GRGDSP, Gly-Arg-Gly-Asp-Ser-Pro; RGD, Arg-Gly-Asp; RGE, Arg-Gly-Glu.

1987, 1988; Sutherland et al., 1988; O'Shea et al., 1990). The adhesive interaction of trophoblast cells with these matrix proteins induces the secretion of specific new proteins by the embryos (Nieder, 1990), as well as a host of other biochemical changes (Sherman et al., 1981). In vitro outgrowth culture of mouse blastocyst-derived trophoblast cells has therefore proven to be an important method for investigating the biochemical basis of primary trophoblast cell differentiation during mouse embryogenesis.

Immunohistochemical studies show entactin to be ubiquitous to basement membrane (Wu et al., 1983; Martinez-Hernandez and Chung, 1984; Dziadek and Timpl, 1985; Timpl and Dziadek, 1986), and, along with laminin, COL-IV, and heparan sulfate proteoglycan, it is a major basement membrane component (Chung and Durkin, 1990). Entactin is a sulfated glycoprotein of M_r 158 kD that forms a tight noncovalent complex with the laminin B2 chain and also binds to COL-IV (Chung and Durkin, 1990). The sulfated basement membrane glycoprotein, nidogen, has been shown to be identical to entactin after the cloning and sequencing of both proteins (Durkin et al., 1988; Mann et al., 1989). Both mouse and human entactin primary structures have been determined by cDNA sequencing and were found to be 85% identical (Durkin et al., 1988; Nagayoshi et al., 1989). Structurally, entactin consists of two globular domains at either end with a central rodlike connecting domain (Chung and Durkin, 1990; Fox et al., 1991). There are six EGF-like cysteine-rich repeats throughout the protein. The second EGF repeat, located in the central rodlike domain, has an RGD sequence that is considered to be responsible for cell binding activity (Durkin et al., 1988; Chakravarti et al., 1990). There are two potential calcium binding sites at the NH_2-terminus, as well as a site for binding COL-IV (Chung and Durkin, 1990; Fox et al., 1991). In the COOH-terminal globule are Tyr-Trp-Thr-Asp repeats involved in binding to laminin (Carlin et al., 1983; Dziadek et al., 1985; Mann et al., 1988; Fox et al., 1991). Entactin can thus bind to laminin and COL-IV at its opposite ends, whereas the central rodlike domain remains relatively free for cellular interactions (Chung and Durkin, 1990). This structural organization indicates that entactin may play an important role in basement membrane assembly (Chung and Durkin, 1990; Fox et al., 1991). Laminin and COL-IV are each capable of independent polymerization through mass action-driven self-assembly (Yurchenco et al., 1992), however entactin can also participate in the assembly process when all three components are present, possibly by acting as a bridging molecule to stabilize or modulate the basement membrane structure (Chung and Durkin, 1990; Fox et al., 1991; Yurchenco et al., 1992). Because of the very tight association between entactin and laminin, entactin has mainly been studied in an isolated complex with laminin. However, Tsao et al. (1990) have inserted the cloned entactin gene into a baculovirus expression vector, providing recombinant entactin, free of laminin or other contaminating proteins, for investigating its biological activity. The recombinant entactin retains the structural and functional features found in entactin purified from native extracellular matrix sources (Tsao et al., 1990).

Studies of mouse mammary tumor, human melanoma, and other cells have shown that entactin promotes epithelial cell attachment (Mann et al., 1989; Tsao et al., 1990; Chakravarti et al., 1990). Recently, entactin has been shown to possess chemotactic activity for neutrophils as well (Senior et al., 1992). During mouse preimplantation development, entactin is first synthesized at the morula stage (Dziadek and Timpl, 1985) and by late blastocyst is localized along the trophectoderm cells on the surface facing the blastocoel (Wu et al., 1983). During later embryogenesis, it appears in various basement membrane structures, including the Reichert's membrane, but not in the migrating trophoblast cells (Wu et al., 1983; Dziadek and Timpl, 1985; Sternberg and Kimber, 1986; Paulsson et al., 1985). The decidual tissue adjacent to the implanted embryo also contains entactin (Wu et al., 1983), and synthesizes entactin mRNA between gestational days 4 and 7 during early postimplantation development (Farrar and Carson, 1992). However, whether trophoblast cells are able to use entactin for cell-substratum adhesion during the initial invasion of the endometrial basement membrane is not known. Therefore, the present study was conducted using the in vitro blastocyst outgrowth culture system to determine whether recombinant entactin could support mouse trophoblast cell adhesion and migration, and to elucidate the mechanism of trophoblast-entactin cell recognition.

Materials and Methods

Materials

Recombinant entactin was produced by inserting a cDNA clone encoding mouse entactin into *Autographa californica* multiple nuclear polyhidrosis virus that was subsequently expressed in *Spodoptera frugiperda*, Sf9, insect cells (Tsao et al., 1990). The protein was purified by SDS gel electrophoresis and excision of the entactin band from the gel (Durkin et al., 1988). A mutated form of recombinant entactin, RGE-EN, containing an alteration in its primary sequence from RGD to Arg-Gly-Glu (RGE) at amino acids 672–674 was produced using entactin cDNA and the Sf9 expression system, as detailed by Senior et al. (1992). Murine laminin from Englebreth-Holm-Swarm tumor basement membrane was purchased from Collaborative Research Inc. (Bedford, MA). Fibronectin was purified from bovine plasma as previously described (Armant, 1991). Peptides, Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) and Gly-Arg-Ala-Asp-Ser-Pro (GRADSP), were purchased from Calbiochem Corp. (La Jolla, CA). The peptide, Gly-Phe-Arg-Gly-Asp-Gly-Gln (GFRGDGQ), was prepared using a peptide synthesizer (Applied Biosystems Inc., Foster City, CA). Anti-entactin antiserum was raised by immunizing rabbits with purified entactin (Carlin et al., 1981). Affinity purified anti-laminin antibody was purchased from Sigma Chemical Co. (St. Louis, MO). HBSS and CMRL 1066 medium were obtained from Gibco Laboratories (Grand Island, NY). All other biochemicals used were of the highest quality available.

Embryo Collection and Culture In Vitro

Female non-Swiss albino (NSA) mice (Colony 202, Harlan Sprague-Dawley, Indianapolis, IN) age 8–10 wk were superovulated by intraperitoneal injection of 5 IU pregnant mare serum gonadotropin, followed by 5 IU hCG 48 h later. The mice were paired individually with B6SJL/J males (Jackson Laboratory, Bar Harbor, ME) immediately after hCG injection and allowed to mate overnight. Blastocysts were obtained on the fourth day of gestation (day 1 = day of vaginal plug) by removing the uterine horns and flushing them with prewarmed HBSS. The embryos were washed free of debris and cells by transfer with a micropipette through several drops of HBSS containing 4 mg/ml BSA. The embryos were then cultured in small drops of CMRL 1066 medium supplemented with 2 mg/ml sodium lactate, 60 μg/ml sodium pyruvate, 0.3 mg/ml glutamine, 4 mg/ml BSA, 100 U/ml penicillin and 0.1 mg/ml streptomycin on plastic petri dishes flooded with water-extracted (1:10) light mineral oil (Aldrich Chem. Co., Inc., Milwaukee, WI) to prevent evaporation.

Trophoblast Outgrowth Culture System

The embryos were cultured in medium on plastic petri dishes (Falcon 1008,

Falcon Labware, Oxnard, CA) precoated with proteins or synthetic peptides prepared in HBSS and incubated overnight at 37°C as small (5 μl) drops under a layer of light mineral oil. The substratum was washed three times with HBSS containing 4 mg/ml BSA to remove unbound protein or peptides. The precoated plastic was then blocked by incubation overnight at 37°C with HBSS containing 20 mg/ml BSA to prevent any proteins present during subsequent steps from binding to the surface. Finally, serum-free CMRL 1066 medium with or without competitive synthetic peptides or antibodies, was added and pre-equilibrated 2 h in a CO_2 incubator before introducing one embryo to each drop. Antibodies and peptides used for outgrowth inhibition experiments included the synthetic peptides, GRGDSP and GRADSP, at final concentrations from 10 to 250 μg/ml, anti-entactin antiserum at dilutions of 1:10 to 1:40, and anti-laminin antibody at 500 μg/ml.

Blastocysts were cultured for 24 h on BSA-coated plates after their collection from the uterus to allow hatching while preventing adhesion to the substratum. They were then cultured individually for an additional 72 h on precoated surfaces. Trophoblast cell outgrowth was observed by noting the appearance of a trophoblast monolayer around the embryo and the disappearance of the spherical blastocyst morphology using a Leitz (Wetzlar, Germany) Fluorovert FU microscope with Hoffman modulation contrast optics. Embryos were video taped once every 24 h during in vitro culture and the area of each trophoblast outgrowth was measured by morphometry using a computer-based image analysis system (MCID-BRS2, St. Catherines, ON) interfaced with the microscope through a Dage 72 videocamera (Dage-MTI, Inc., Michigan City, IN). The perimeters of the blastocyst outgrowths were traced using a computer mouse and converted to area in μm^2 based on prior calibration of the system with a stage micrometer.

All experiments were repeated at least three times. The percentage of embryos achieving outgrowth was determined using pooled data from repeated experiments. The inhibitory effects of various antibodies and synthetic peptides were analyzed by Chi square. P values <0.05 were considered statistically significant.

Results

Recombinant Entactin Promotes Trophoblast Outgrowth

Previous studies from this laboratory and others have demonstrated that mouse embryos cultured from day 4 of gestation in serum-free medium attach and form trophoblast outgrowths between 24 and 48 h when cultured on surfaces precoated with fibronectin, laminin, vitronectin, or collagen (Armant et al., 1986*a*,*b*; Sutherland et al., 1988; Carson et al., 1988). Entactin is tightly associated with laminin, as well as COL-IV and fibronectin, in the basement membrane (Chung and Durkin, 1990) and can promote the attachment of several cell types in vitro (Mann et al., 1989; Tsao et al., 1990; Chakravarti et al., 1990). Therefore, we have investigated the ability of mouse blastocysts to outgrow on entactin-coated plates using recombinant entactin. The recombinant entactin supported mouse primary trophoblast cell attachment and migration in a dose dependent manner (Fig. 1). Blastocyst outgrowth was first detectable at a precoating concentration of 5 μg/ml and the maximal number of embryos outgrew at 20–50 μg/ml entactin (Fig. 1 *a*), similar to results obtained using the adhesive glycoproteins, fibronectin, laminin and vitronectin (Armant et al., 1986*a*, *b*). The area occupied by the outgrowing embryos increased continuously during the 72 h period of monitoring as the trophoblast cells migrated outward (data not shown), again with a dose response that leveled off between 20 and 50 μg/ml (Fig. 1 *b*). An example of trophoblast outgrowth obtained using surfaces precoated with recombinant entactin is compared with outgrowths on fibronectin and laminin in Fig. 2 (*a–c*). Trophoblast cells spread and migrated readily on entactin, but expansion of the outgrowth cell mass was slower than that obtained with either fibronectin or laminin. The trophoblast giant cells adhering to entactin demonstrated extensive cell migration and the elaboration of filopodia, which is characteristic of their behavior on other extracellular matrix components.

Anti-Entactin Antibody Inhibits Entactin-Mediated Outgrowth

To test the specificity of entactin-mediated trophoblast cell adhesion and migration, anti-entactin antibody was added to the medium during culture on plates precoated with BSA, fibronectin, laminin or entactin and the percent of cultured embryos that had begun to outgrow by 72 h was determined. Anti-entactin antibody significantly inhibited trophoblast outgrowth on entactin, but had no effect on either fibronectin- or laminin-mediated cell migration (Fig. 3). Precoating with BSA did not support blastocyst outgrowth, as previously reported (Armant et al., 1986*a*), and adhesion factors like fibronectin and vitronectin that may have been present in the antiserum were unable to bind to the surface and promote outgrowth following our blocking procedure with BSA (Fig. 3). We also found that an antibody raised against laminin had

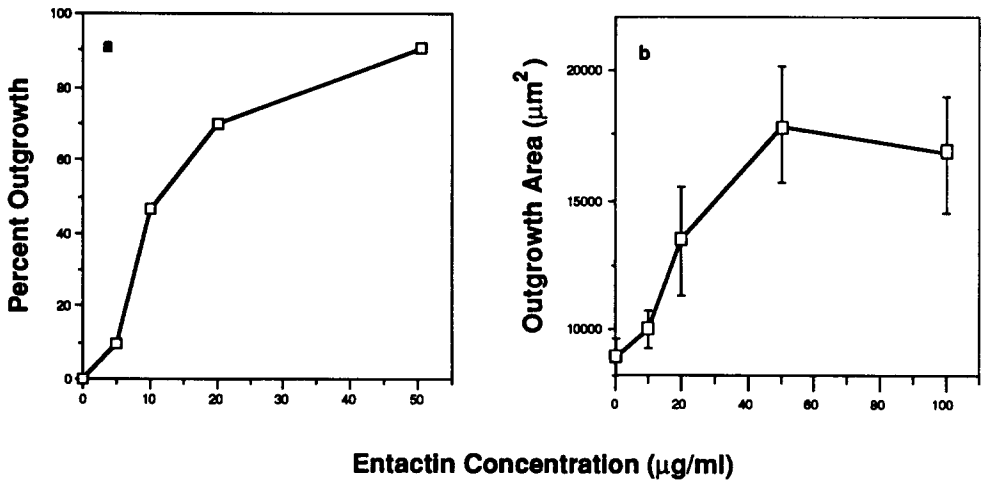

Figure 1. Recombinant entactin promotes mouse trophoblast outgrowth. Plastic petri dishes were precoated with 0–100 μg/ml of recombinant entactin. Peri-implantation embryos were cultured on the entactin substrata for 72 h at 37°C before determining (*a*) the percent having initiated outgrowth (N = 20–41 embryos), or (*b*) the average trophoblast outgrowth area (N = 10 embryos). The area indicated in *b* at 0 μg/ml reflects the area occupied by nonadherent blastocysts cultured on BSA-coated surfaces. Error bars are SEM.

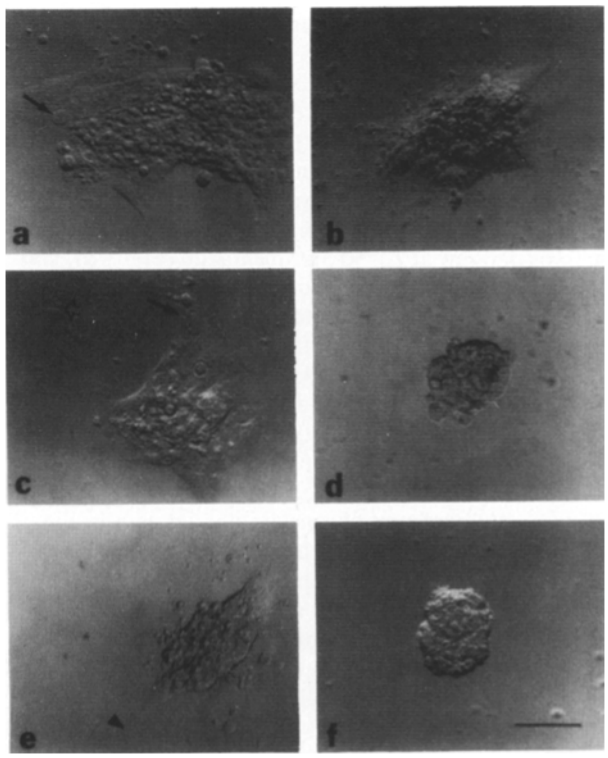

Figure 2. The morphology of trophoblast outgrowths. Embryos were cultured on dishes coated with 25 μg/ml fibronectin (*a*), 25 μg/ml laminin (*b*), 50 μg/ml recombinant entactin (*c* and *d*), 200 μg/ml of the synthetic peptide, GFRGDGQ (*e*), or 50 μg/ml of mutant RGE-EN (*f*). The medium in *d* has been supplemented with 200 μg/ml of the synthetic peptide, GRGDSP. Features found that were typical of differentiated trophoblast cells cultured in vitro included giant nuclei with prominent nucleoli (*arrows*), long cortical extensions (*arrowheads*) and cell migration away from the embryo mass (*open arrow*). Bar, 100 μm.

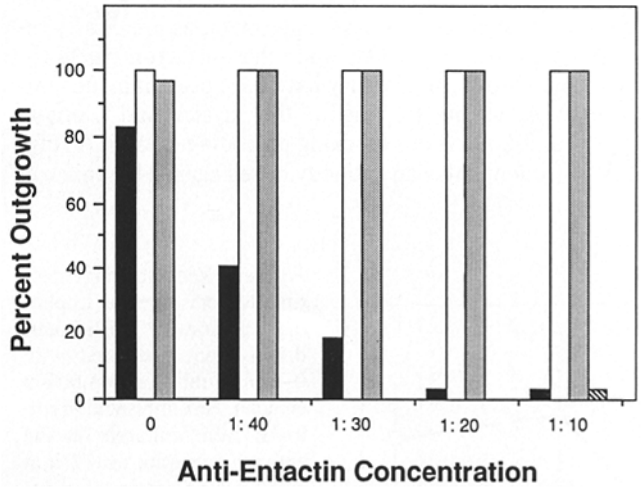

Figure 3. The effect of anti-entactin antibody on extracellular matrix-mediated trophoblast outgrowth. Petri dishes were precoated overnight with 25 μg/ml entactin (*solid bars*), 20 μg/ml fibronectin (*open bars*), 20 μg/ml laminin (*stippled bars*) or 20 mg/ml BSA (*striped bars*), and then blocked by overnight incubation with 20 mg/ml BSA. The culture medium was supplemented with the indicated concentrations of anti-entactin antiserum. After 72 h of subsequent culture, the percent outgrowth was determined. These results were pooled from four separate experiments. N = 21–101 embryos.

no effect on entactin-mediated outgrowth, although it significantly inhibited cell migration mediated by laminin. Of 21 embryos cultured in medium containing antilaminin on plates precoated with entactin, 85.7% outgrew, however none outgrew among 40 embryos similarly cultured on laminin-coated surfaces.

Synthetic RGD-Peptides Inhibit Entactin-Mediated Outgrowth

It has been shown that the RGD sequence in a number of extracellular matrix proteins, including fibronectin, vitronectin, and collagen, plays a critical role in cell adhesion (Ruoslahti and Pierschbacher, 1986; Hynes, 1992). Because entactin contains an RGD sequence that has been shown to be functional in cell adhesion (Durkin et al., 1988; Chakravarti et al., 1990), we have determined the role of this domain in mouse blastocyst outgrowth on entactin using competitive inhibition experiments with a synthetic peptide, GRGDSP, that contains the RGD recognition signal. Our results (Fig. 4) demonstrated that 100 μg/ml GRGDSP significantly inhibited blastocyst outgrowth on entactin, but had no effect on laminin-mediated adhesion, whereas the control peptide, GRADSP, which contains the conservative substitution of alanine for glycine, had no detectable inhibitory activity. The ability of blastocysts to develop normally on laminin in the presence of GRGDSP, which corroborated an earlier finding (Armant et al., 1986*b*), demonstrated that the peptide was not embryo toxic and revealed the biochemical distinction between trophoblast cell recognition of these two extracellular matrix proteins. An example of the inhibitory effect of GRGDSP on outgrowth formation, depicted in Fig. 2 *d*, illustrates the lack of interaction with the substratum by the embryo; cell attachment and spreading were undetectable. Embryos inhibited from outgrowing for 3 d by culture in medium containing as much as 250 μg/ml

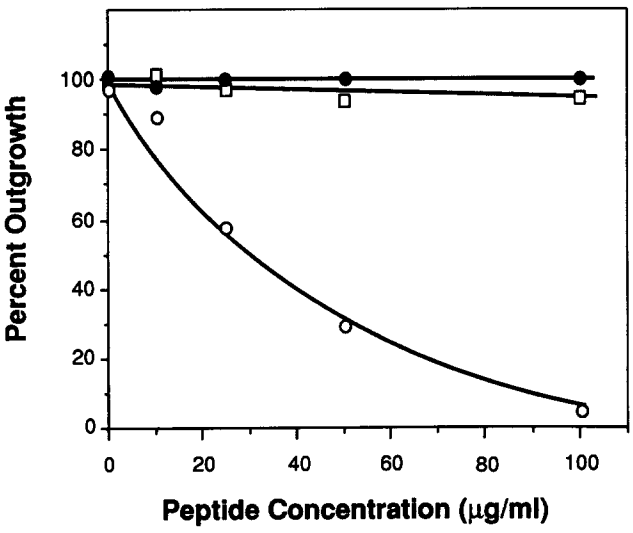

Figure 4. The effect of RGD-containing synthetic peptides on entactin- and laminin-mediated trophoblast outgrowth. Embryos were cultured on dishes precoated with either 50 μg/ml entactin (*open symbols*) or 20 μg/ml laminin (*closed circles*). The medium contained 0–100 μg/ml of the synthetic peptides, GRGDSP (*circles*) or GRADSP (*squares*). The percent of blastocysts outgrowing after 48–72 h of in vitro culture is shown. N = 30–66 embryos.

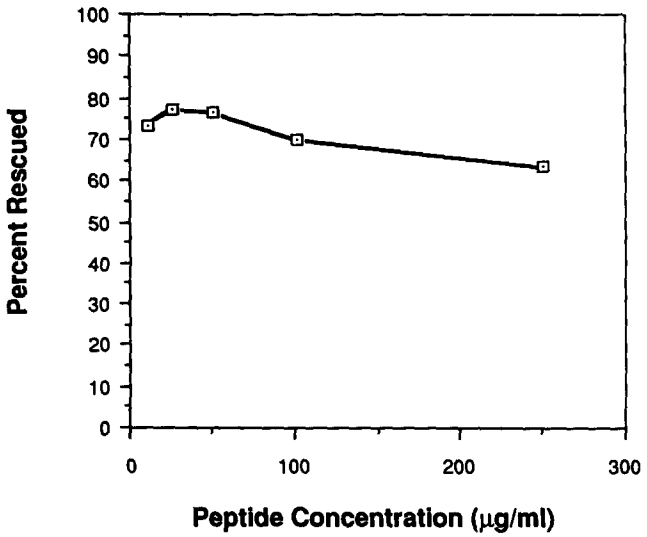

Figure 5. Rescue of blastocysts inhibited from outgrowing by the synthetic peptide, GRGDSP. Blastocysts inhibited from outgrowing for 3 d by addition of peptide GRGDSP (10–250 μg/ml) to the culture medium were washed free of peptide and transferred to entactin-coated plates containing fresh medium without peptide. The percent embryos forming outgrowths 24 h later is shown. The retained ability of blocked embryos to outgrow after peptide removal demonstrated the competitive nature of the inhibition. N = 21 embryos.

GRGDSP were found to remain viable and capable of producing trophoblast outgrowths within 24 h when transferred to entactin-coated plates containing fresh medium without peptide (Fig. 5). These experiments indicate that, as with fibronectin and vitronectin (Armant et al., 1986*b*), trophoblast cells adhere to and migrate on entactin-coated plates through their ability to recognize an RGD sequence.

Peptides GFRGDGQ and GRGDSP Promote Cell Outgrowth

To more specifically test the role of the cell recognition site of entactin in blastocyst outgrowth, we directly coupled the peptides, GRGDSP and GRADSP to petri plates, as well as the actual RGD-containing sequence of entactin, GFRGDGQ, and determined their ability to support blastocyst outgrowth. The percent of blastocysts outgrowing on these peptides after 72 h of culture is shown in Table I. Both of the RGD-containing peptides, GRGDSP and GFRGDGQ, promoted blastocyst attachment and outgrowth at rates of 92 and 78.5%, respectively, a difference that was not significant ($P > 0.05$). The control peptide, GRADSP, did not promote trophoblast cell migration. The appearance of the trophoblast cells outgrowing on GFRGDGQ (Fig. 2 *e*) was very similar to their appearance on the intact protein (Fig. 2 *c*).

Table I. Mouse Trophoblast Outgrowth on the Synthetic Peptides, GFRGDGQ, GRGDSP and GRADSP, and on Wild-type or Mutant Recombinant Entactin

Peptide or protein	Precoat concentration	Number of embryos	Number of outgrowths	Outgrowth rate
Recombinant entactin	50 μg/ml	40	36	90%
GFRGDGQ	200 μg/ml	42	33	78%
GRGDSP	200 μg/ml	51	47	92%
GRADSP	200 μg/ml	46	0	0%
Mutant (RGE) entactin	100 μg/ml	59	0	0%

Dishes were precoated overnight with peptides or proteins at the indicated concentrations. Embryos were then cultured on the precoated dishes in CMRL 1066 medium for 72 h to determine the percent undergoing trophoblast outgrowth.

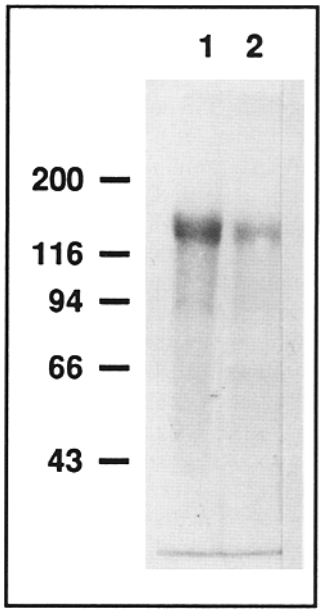

Figure 6. SDS-gel electrophoresis of recombinant entactin and mutant RGE-EN. Purified recombinant wild-type (lane *1*) or mutant (lane *2*) entactin were analyzed by SDS gel electrophoresis on 7.5% acrylamide gels in the presence of 5% β-mercaptoethanol, according to Laemmli (1970). A major protein band of ~ M_r 150 kD was visible in each lane after staining with Coomassie blue R250. The migration of molecular weight marker proteins is indicated to the left with their M_r in kD.

Mutant RGE-EN Does Not Promote Trophoblast Outgrowth

A mutated form of recombinant entactin, RGE-EN, was prepared containing the single conservative substitution of glutamate for aspartate at amino acid 674. The wild-type recombinant entactin and RGE-EN migrated identically on SDS gels (Fig. 6). However, dishes precoated with RGE-EN at concentrations as high as 100 μg/ml failed to support any trophoblast cell adhesion or spreading (Table I, Fig. 2 *f*), demonstrating the central importance of this site within the entactin molecule in mediating trophoblast cell adhesion and migration.

Discussion

Blastocyst outgrowth on entactin was demonstrated using recombinant entactin and a specific anti-entactin antibody to block entactin-mediated adhesion. Antilaminin antibody did not interfere with outgrowth on entactin, verifying that the recombinant protein was not contaminated with laminin. Although entactin has been associated with adhesive and chemotactic activity in a number of cells (Tsao et al., 1990; Chakravarti et al., 1990; Senior et al., 1992), this is the first report of cell migration on a homogeneous entactin preparation. Perris et al. (1989) have reported that avian neural crest cells do not migrate on entactin, however, like mouse trophoblast cells, they migrate on laminin through an interaction that is not disrupted by peptides containing the RGD sequence. Entactin, like the other adhesive basement mem-

brane proteins (Armant et al., 1986*a*; Carson et al., 1988; Sutherland et al., 1988), exhibited optimal blastocyst outgrowth promoting activity when precoated at concentrations in the range of 20 to 50 μg/ml, raising the question of the respective roles of these proteins in vivo. On a molar basis, entactin was essentially as effective as laminin, COL-IV or fibronectin, although cell migration, measured as outgrowth area, was somewhat less on entactin than that obtained using laminin or fibronectin. It therefore remains unclear whether entactin provides a significant stimulus for trophoblast invasion of the basement membrane, or serves strictly in a structural role. Elucidation of the spatial organization of entactin and other glycoproteins within the basement membrane and their accessibility to the invading trophoblast cells may help to resolve this question.

To better understand the role of entactin during implantation, we investigated the mechanism of trophoblast-entactin adhesion during blastocyst outgrowth culture. Previous studies (Durkin et al., 1988; Chakravarti et al., 1990) have shown that entactin-mediated cell adhesion involves the RGD recognition sequence found in the second cysteine-rich repeat. An RGD sequence was first identified as the cell recognition site of fibronectin (Pierschbacher and Ruoslahti, 1984), and has since been associated with cell adhesion mediated by a number of other extracellular matrix proteins (Ruoslahti and Pierschbacher, 1986; Hynes, 1992), including entactin (Durkin et al., 1988). Therefore, we used an RGD-containing synthetic peptide in experiments to competitively inhibit trophoblast outgrowth on entactin. The peptide, GRGDSP, reversibly inhibited nearly all outgrowth at a concentration of 100 μg/ml, whereas a control peptide, GRADSP, was ineffective at that concentration. Mouse mammary tumor cells adhering to entactin were inhibited ~60% by a synthetic peptide containing the RGD sequence (Chakravarti et al., 1990), whereas the inhibition that we report here using trophoblast cells was nearly complete. This difference in inhibitory effectiveness suggests that any additional cell recognition sequences that might be present within the entactin molecule may play a very minor role in the adhesion of trophoblast cells, as compared to other entactin-binding cells. A synthetic peptide composed of seven amino acids comprising the actual RGD-containing region of entactin was capable of supporting trophoblast outgrowth, indicating that this region of the protein possesses the essential structural requirements for directing trophoblast adhesive activity. The RGD epitope of fibronectin, GRGDSP, was also very effective in supporting blastocyst outgrowth, as we have previously reported (Armant et al., 1986*b*). The central importance of the RGD epitope of entactin in blastocyst outgrowth was clearly established using recombinant RGE-EN. The conservative substitution of a glutamate for aspartate at amino acid 674 of the recombinant protein rendered it completely inactive in supporting trophoblast outgrowth. Although other domains of the glycoprotein may participate in directing cell attachment, the RGD epitope of entactin is clearly essential for adhesive activity. Therefore, it is primarily this domain of entactin that is responsible for interacting with trophoblast cells. This interaction may differ somewhat in vivo because the recombinant protein is undersulfated and underglycosylated (Tsao et al., 1990), and native entactin in the basement membrane is always complexed with laminin. Outgrowth on laminin, however, proceeded unperturbed in the presence of up to 100 μg/ml of the synthetic peptide, GRGDSP, indicating that laminin does not interact with trophoblast cells through an RGD epitope. Mouse primary trophoblast cells recognize the E8 domain of laminin (Armant, 1991), which is near the COOH-terminus and far removed from the only RGD sequence present in mouse laminin (Sasaki et al., 1988). These differences in trophoblast recognition of entactin and laminin, along with the lack of inhibition of outgrowth on laminin by anti-entactin, demonstrate that trace amounts of entactin that may have contaminated our laminin preparation did not contribute to laminin-mediated cell adhesion. Although laminin and entactin exist as a very tight complex in native basement membrane, their binding to trophoblast cells appears to be quite independent and may serve distinct functions in vivo.

The apparent central role of the RGD site in entactin to its function in trophoblast adhesion, provides evidence that these cells adhere to entactin using a member of the integrin family of heterodimers. The RGD sequence motif is used by several integrin receptors, but not all, in binding to extracellular matrix glycoproteins (Hynes, 1992). It has been proposed that trophoblast outgrowth on a number of extracellular matrix components, including fibronectin, laminin, vitronectin and COL-IV, is regulated by integrins (Armant et al., 1986; Sutherland et al., 1988; Armant, 1991), although the identity and developmental expression of integrins in early mouse embryos remain to be determined. The behavior reported here of trophoblast cells cultured on recombinant entactin again suggests the involvement of integrins. Identification of an entactin receptor has not been conclusively made for any of the cell types that attach to entactin. However, Dedhar et al. (1992) recently provided evidence that the entactin receptor of human PC-3 prostate carcinoma cells is the $\alpha 3\beta 1$ integrin, although the RGD recognition site of entactin appears not to be involved in that interaction. In addition to entactin, $\alpha 3\beta 1$ has been shown to bind to several extracellular matrix components, including fibronectin, collagen and laminin (Hynes, 1992). Binding between $\alpha 3\beta 1$ and fibronectin appears to be mediated through recognition of RGD, while binding to the long arm of laminin and to COL-IV does not involve this recognition sequence (Elices et al., 1991). The reported behavior of $\alpha 3\beta 1$ is consistent with our characterization of trophoblast adhesion on fibronectin and laminin (Armant et al., 1986*b*; Armant, 1991), and its presence on outgrowth trophoblast cells might explain their ability to adhere to several extracellular matrix components. Inconsistency regarding the reported noninvolvement of an RGD recognition site in binding between $\alpha 3\beta 1$ and COL-IV (Elices et al., 1991) or entactin (Dedhar et al., 1992), and the adhesive behavior of trophoblast cells on these substrates in the presence of RGD-containing peptides may reflect differences among cell types. The coexpression of other integrins can apparently alter the binding characteristics of $\alpha 3\beta 1$ (Elices et al., 1991; Dedhar et al., 1992), and it remains quite possible that receptors other than $\alpha 3\beta 1$ are responsible for entactin-mediated adhesion and migration of mouse primary trophoblast cells. Indeed, trophoblast cells likely express several integrins during outgrowth, perhaps in response to the composition of the basement membranes or extracellular matrices that they encounter. The mouse trophoblast receptor for entactin may

therefore be among a group of extracellular matrix receptors that are coordinately expressed as the blastocyst differentiates to its invasive phenotype.

We have used an in vitro model of primary trophoblast cell invasiveness and recombinant entactin to demonstrate for the first time that entactin provides an adhesive substrate for these cells through the RGD integrin-binding site located in its second EGF-like repeat. Because entactin is a major component of the endometrial basement membrane and the peridecidual extracellular matrix, these results indicate that entactin, along with other adhesive glycoproteins, could play an important role during implantation in directing the initial trophoblast invasion, as the embryo contacts the basement membrane and begins to penetrate the endometrium.

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