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to Fibronectin at Implantation: Intracellular Calcium Transients and Vesicle Trafficking in Primary Trophoblast Cells

Jun Wang, Linda Mayernik, and D. Randall Armant[1]

C. S. Mott Center for Human Growth and Development, Departments of Anatomy and Cell Biology, and Obstetrics and Gynecology, Wayne State University School of Medicine, Detroit, Michigan 48201

Accumulating evidence indicates that the endometrial extracellular matrix (ECM) modulates trophoblast adhesion during mouse blastocyst implantation. In previous studies of adhesion-competent mouse blastocysts, we have demonstrated that integrin-mediated fibronectin (FN)-binding activity on the apical surface of trophoblast cells is initially low, but becomes strengthened after embryos are exposed to FN. In the present study, we have examined whether the ligand-induced upregulation of trophoblast adhesion to FN is mediated by integrin signaling. The strengthening of adhesion to FN required integrin ligation, which rapidly elevated cytoplasmic-free Ca^{2+}. Chelation of intracellular Ca^{2+} using BAPTA-AM, or inhibition of the Ca^{2+}-dependent proteins, protein kinase C or calmodulin, significantly attenuated the effect of FN on binding activity. Furthermore, direct elevation of cytoplasmic Ca^{2+} levels with ionomycin upregulated FN-binding activity, demonstrating that Ca^{2+} signaling is required and sufficient for strong adhesion to FN. Ca^{2+} signaling may induce protein trafficking, a known requirement for ligand-induced upregulation of FN-binding activity. Indeed, intracellular vesicles accumulated in adhesion-competent blastocysts, but were absent after exposure to either FN or ionomycin. These findings suggest that, during implantation, contact between peri-implantation blastocysts and FN elevates intracellular Ca^{2+}, which strengthens trophoblast adhesion to ECM through protein redistribution. © 2002 Elsevier Science (USA)

Key Words: **ovum implantation; blastocyst; trophoblast; fibronectin; integrins; extracellular matrix; trafficking; cell adhesion; Ca^{2+} signaling; signal transduction.**

INTRODUCTION

Blastocyst implantation in mice and humans depends on the interaction of differentiated, adhesion-competent trophoblast cells with extracellular matrix (ECM) components of the receptive uterus. Newly formed mouse trophoblast cells comprise a transporting epithelium, the trophectoderm, with nonadhesive apical surfaces devoid of most integrins, except $\alpha_v\beta_3$ (Hierck *et al.*, 1993; Sutherland *et al.*, 1993; Schultz *et al.*, 1997). Acquisition of adhesion-competence by trophoblast cells occurs at the late blastocyst stage and is characterized by a change in both adhesive behavior and the onset of motility (Chavez, 1984; Bevilacqua and Abrahamsohn, 1988). Trophoblast phenotype conversion is marked by apical accumulation of additional integrin receptors for fibronectin (FN) and strong FN-binding activity on the surface of intact blastocysts (Schultz and Armant, 1995; Schultz *et al.*, 1997). Once the adhesive stage is achieved, trophoblast cells are able to adhere and migrate on ECM substrates (Armant *et al.*, 1986a,b; Carson *et al.*, 1988; Kao *et al.*, 1988; Sutherland *et al.*, 1988; Yelian *et al.*, 1993) or penetrate three-dimensional structures formed by ECM (Wordinger *et al.*, 1991; Armant and Kameda, 1994).

FN-binding activity on the outer surface of the adhesion-competent blastocyst is upregulated approximately fourfold by exposure to either immobilized or soluble FN-120 (Schultz and Armant, 1995), a proteolytic fragment that contains the central cell binding domain of FN and includes the Arg-Gly-Asp (RGD) integrin recognition sequence (Pierschbacher *et al.*, 1981). The ligand-mediated upregula-

[1] To whom correspondence should be addressed. Fax: (313) 577-8554. E-mail: D.Armant@wayne.edu.

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tion of FN-binding activity occurs on gestation day (GD) 7 after blastocyst culture from either GD3 or GD4 in serum-free medium devoid of growth factors (Schultz and Armant, 1995; Schultz *et al.*, 1997; Wang *et al.*, 1998). However, if embryos are either retained in the uterus until GD5, or stimulated with bioactive uterine proteins, FN-binding activity is detected on GD6 (Wang *et al.*, 1998, 1999, 2000), close to the time *in utero* when the blastocyst first contacts the endometrial basement membrane (Blankenship and Given, 1992). Developmental regulation of FN-binding activity coincides with the initiation of trophoblast outgrowth, suggesting that increased trophoblast–FN interaction occurs prior to the initiation of trophoblast cell migration (Schultz and Armant, 1995). Upregulation of FN-binding activity depends on neither transcription nor protein synthesis, but is blocked by disruption of intracellular protein trafficking or actin microfilament integrity (Schultz and Armant, 1995). Therefore, it appears that intracellular signaling initiated by the ligation of FN receptors strengthens the adhesion of trophoblast cells to FN and may also influence subsequent trophoblast development required for ECM invasion and further trophoblast differentiation.

Intracellular signaling mediated by integrins plays an important role in the regulation of cell proliferation, adhesion, migration, and invasion (Hynes, 1992; Burridge and Chrzanowska-Wodnicka, 1996; Aplin *et al.*, 1998). The RGD sequence contained within several ECM ligands appears to be capable of initiating intracellular signals, including the activation of phospholipase C (PLC) to mobilize intracellular Ca^{2+} stores (Somogyi *et al.*, 1994), and recruitment of protein tyrosine kinases (PTKs) to the sites of ligand binding (Miyamoto *et al.*, 1995). In addition, activation of β_1- and β_3- integrins by exposing cells to FN, vitronectin, or anti-integrin antibodies causes Ca^{2+} influx and activation of the Na-H antiporter (Schwartz *et al.*, 1991a,b; Leavesley *et al.*, 1993; Schwartz, 1993). These signaling phenomena comprise the "outside-in" intracellular signaling cascades initiated by ligation of integrins (Hynes, 1992; Damsky and Werb, 1992; Schwartz and Ingber, 1994; Burridge and Chrzanowska-Wodnicka, 1996; Aplin *et al.*, 1998). Furthermore, integrin activity can be regulated by "inside-out" signaling pathways initiated by integrins, cytokines, or other factors (Hynes, 1992; Schwartz, 1993). However, the roles of these signaling pathways in the ligand-induced upregulation of trophoblast adhesion to FN are not yet clear. In the present report, we have investigated the role of intracellular Ca^{2+} signaling during ligand-mediated upregulation of integrin activity in mature (GD7) blastocysts.

MATERIALS AND METHODS

Production and Culture of Mouse Embryos

Mouse embryos were generated from superovulated 5- to 8-week-old female NSA or MF1 mice (Harlan Sprague Daly, Indianapolis, IN) mated with B6SJLF1/J males (Jackson Laboratory, Bar Harbor, ME). Embryos were collected by flushing either the oviducts on GD3 or the uterus on GD4 (where GD1 is the day of the vaginal plug) with M2 medium (Sigma Chemical Company, St. Louis, MO), as previously described (Wang *et al.*, 1998). All experiments were conducted by using adhesion-competent blastocysts produced by culturing embryos to GD7 in Ham's F10 medium containing 4 mg/ml BSA, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (all from Sigma) at 37°C in a 5% CO_2/air incubator.

FN-Binding Activity

FN-binding activity was upregulated by exposing blastocysts to either 50 μg/ml FN-120 for 1 h or immobilized FN-120 for 3 h, as previously described (Schultz and Armant, 1995; Wang *et al.*, 1998). Some blastocysts were exposed to immobilized (50 μg/ml) mouse type 1 laminin (Collaborative Research, Bedford, MA), entactin/nidogen (a gift from Dr. Albert Chung, University of Pittsburgh), synthetic hexapeptides, or proteolytic fragments of FN (Life Technologies, Inc., Gaithersburg, MD). Blastocysts were also treated for 1 h at 37°C with 10 μg/ml of monoclonal antibody against mouse α_5 (5H10-27, rat IgG), α_v (H9.2B8, hamster IgG), β_1 (9EG7, rat IgG), or β_3 (Hmβ3, hamster IgG) integrin subunit extracellular domains (BD PharMingen, San Diego, CA). In some experiments, the blastocysts were treated at 37°C for 1 h before and during exposure to FN-120 with the following inhibitors: 0.25 μM calphostin C, 10 μM W7, 10 μM W5, 10 μM BAPTA-AM, 200 μM brefeldin A (all from Calbiochem La Jolla, CA). None of the inhibitors cause a decrease in baseline (prior to upregulation) FN-binding activity.

FN-binding activity was assayed, as previously described (Schultz and Armant, 1995), using 1.0-μm fluorescent green polystyrene microspheres (Bang's Laboratories, Carmel, IN) coated with FN-120. The fluorescence intensity of the bound microspheres was quantified over the abembryonic pole of each blastocyst by using computer-based image analysis, as described by Schultz and Armant (1995). Baseline FN-binding activity, determined by exposing embryos to BSA in place of FN-120, was subtracted from all values. Data are reported as the mean change (Δ) in FN-binding activity $\pm$ SEM.

Intracellular Ca^{2+} Measurements

For estimation of the intracellular Ca^{2+} concentration, blastocysts loaded for 1 h with 5 μM fluo-3 acetoxymethyl ester (fluo-3-AM; Molecular Probes, Inc., Eugene, OR) were subjected to epifluorescence microscopy and image analysis, as previously described (Wang *et al.*, 1998). Embryos were imaged individually at 37°C in 5-μl drops of Ham's F10 with or without 50 μg/ml FN-120 on petri dishes flooded with mineral oil. To monitor Ca^{2+} mobilization, embryos were briefly illuminated every 0.5–2 min for fluorescence imaging. All data presented depict single embryos that are representative of a minimum of 10 embryos for each treatment.

Electron Microscopy

Blastocysts were fixed at room temperature for 3 h in Karnovsky's fixative, consisting of 1% formaldehyde and 1% gluteraldehyde (Polysciences, Warrington, PA) in 0.1 M sodium phosphate buffer, pH 7.2, containing 2% sucrose and 0.15% NaCl (310 mOsm). Following fixation, embryos were stained in 1% uranyl acetate (Ted Pella, Redding, CA) for 30 min, washed free of

precipitate, and transferred to 8-μl drops of water on the surface of a petri dish. A 1% solution of low-melting point agarose (Sigma) was maintained at 37°C on a hot plate while the water drop was carefully removed from the embryos by mouth pipette and replaced with agarose solution. The agarose drops were left to solidify for 30 min and dehydrated through a series of ethanol (50–100%) and embedded in Poly/Bed 812 Resin (Polysciences). Semi-thin sections were stained with 1% Toluidine blue in 1% sodium borate. Thin sections were prepared by using a diamond knife (Diatome, Biel, Switzerland) and an Ultracut E Microtome (Reichert, Germany), collected on copper- or nickel-coated 200 mesh grids and stained with 2% uranyl acetate and 0.4% lead citrate (Ted Pella). Thin sections were viewed on a Philips 100 (Mahwah, NJ) transmission electron microscope.

Morphometric Analysis

The cytoplasm of mural trophoblast cells was photographed randomly, with an average of five micrographs/blastocyst sampled. Micrographs were uniformly printed to a final magnification of 20,000× and analyzed for vesicle density. Only vesicles that were completely included within each micrograph were counted. An average of two to three embryos per treatment group and three trophoblast cells per embryo were analyzed. A second morphometric analysis was conducted to compare total vesicle area in mural trophoblast cells of blastocysts treated with brefeldin A to control embryos. Micrographs (10/group) were placed on a lightbox and digitized by using a Sony video camera module (Model XC-77CCD, Tokyo, Japan) with a 55-mm F-2.8 Nikon (Tokyo, Japan) lens. The diameter of each vesicles was traced on-screen with a computer mouse and vesicle area was calculated in μm^2 by the MCID-M4 system (Yelian *et al.*, 1993). The areas of all vesicles completely included within each micrograph were totaled and the average ± SEM for each treatment group was determined.

Statistical Analysis

All experiments were repeated at least three times. Values reported for FN-binding activity were obtained by using at least 15 blastocysts per treatment group. Differences in the FN-binding activity between treatment groups were tested for significance by using a one-way ANOVA and the Bonferroni/Dunn posthoc test.

RESULTS

Upregulation of FN-Binding Activity Is Ligand-Specific

We have compared the known ability of FN-120 to upregulate FN-binding activity with that of other immobilized proteins or peptides in order to determine the specificity of this induction (Fig. 1A). As expected, the nonadhesive protein, α_1-acid glycoprotein, failed to upregulate FN-binding activity beyond baseline levels (BSA). The nonadhesive hexapeptide GRADSP was ineffective, while exposure to GRGDSP stimulated FN-binding activity ($P <$ 0.05, compared with BSA), but to a degree significantly less than FN-120. Other proteolytic fragments of FN, FN-40, FN-45, and FN-50, which do not contain the RGD sequence (Pierschbacher and Ruoslahti, 1984; Ruoslahti, 1988), each failed to upregulate FN-binding activity. Laminin, which contains a cryptic RGD sequence that is not required for mouse trophoblast adhesion (Armant, 1991; Yelian *et al.*, 1993; Sutherland *et al.*, 1993), was unable to upregulate FN-binding activity. Entactin, which interacts with trophoblast cells through an RGD site (Yelian *et al.*, 1993), effectively upregulated FN-binding activity. These results

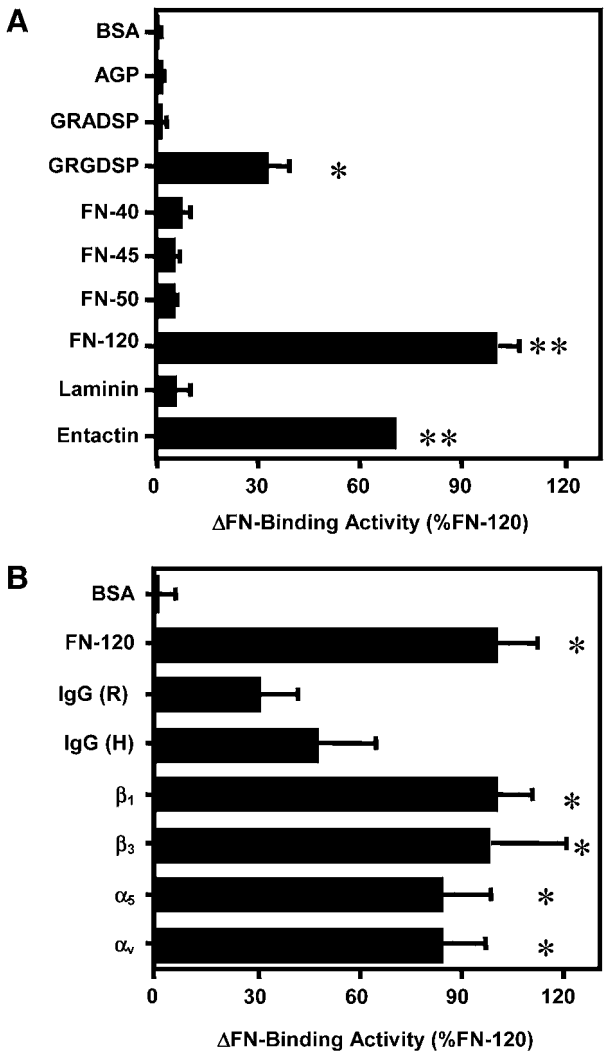

FIG. 1. Integrin regulation of FN-binding activity. ΔFN-binding activity was assayed as detailed in the Materials and Methods. Baseline FN-binding activity is represented by nontreated blastocysts (BSA). Blastocysts were cultured (A) for 3 h on surfaces coated (50 μg/ml) with the indicated ligands, or (B) for 1 h with 50 μg/ml FN-120 or 10 μg/ml of the indicated antibodies against α_5, α_v, β_1, or β_3. In (B), nonimmune rat (R) or hamster (H) IgG (IgG) served as negative controls. FN-binding activity, normalized to the FN-120-stimulated group, is reported as mean ± SEM of at least 15 embryos assayed in 3 separate experiments. AGP, α_1-acid glycoprotein. *, $P < 0.05$, compared with BSA; **, $P < 0.05$, compared with BSA and GRGDSP.

demonstrate that the interaction of trophoblast cells with the RGD integrin recognition sequence (Ruoslahti, 1988) in FN or other proteins will activate the FN adhesion system. It is not necessary that the activating ligand be immobilized; we previously reported that FN-binding activity is optimally stimulated by treatment for 1 h with 50 μg/ml soluble FN-120 (Schultz and Armant, 1995).

Upregulation of FN-Binding Activity Is Mediated by β_1 and β_3 Integrins

The requirement of an RGD recognition site indicated the involvement of integrins in the process of upregulating FN-binding activity. Antibodies recognizing integrins can induce integrin clustering, which initiates an intracellular signaling cascade that alters integrin affinity for its ligand (Frelinger *et al.*, 1991; Mould *et al.*, 1995). To investigate the roles of β_1 and β_3 integrins in mediating the effect of FN-120 on trophoblast adhesiveness, we determined whether activation of potential target integrins with specific antibodies could upregulate FN-binding activity. A 1-h incubation with monoclonal antibodies recognizing the α_5, β_1, α_v, or β_3 integrin subunits each significantly increased FN-binding activity, similar to treatment with FN-120 (Fig. 1B). These data suggest that FN-120-induced upregulation of FN-binding activity is mediated by FN-binding integrins of the β_1 and β_3 classes.

Integrin Ligation Elevates Intracellular Ca^{2+}

Integrin binding to ECM components induces intracellular Ca^{2+} signaling in several cell systems (Pardi *et al.*, 1989; Ng-Sikorski *et al.*, 1991; Smith *et al.*, 1991; Pelletier *et al.*, 1992; Schwartz, 1993; Berk *et al.*, 1995; Sjaastad *et al.*, 1996). Since Ca^{2+} is an important second messenger that mediates cellular activities in preimplantation embryos (Stachecki *et al.*, 1994a,b; Wang *et al.*, 1998, 2000), we suspected its role in the regulation of FN-binding activity. To examine this hypothesis, we have monitored the intracellular Ca^{2+} concentration in blastocysts contacting immobilized FN-120. Contact with FN-120 elevated intracellular Ca^{2+} within minutes and appeared to spread across the cellular field as a wave, moving from cell to cell (Fig. 2A). While Ca^{2+} signaling continued within the embryo for at least 30 min, it occurred for a much briefer time within individual cells. Intracellular Ca^{2+} levels remained low throughout control embryos retained on BSA-coated plates. The estimated intracellular Ca^{2+} concentration averaged over the entire embryo increased after contacting FN-120 from a baseline of approximately 210 nM to over 400 nM, and returned to baseline after 30–60 min (Fig. 2B). Intracellular Ca^{2+} concentrations remained low in embryos contacting BSA.

To demonstrate a functional role for free cytoplasmic Ca^{2+} in the upregulation of FN-binding activity, blastocysts were treated before exposure to FN-120 with 10 μM BAPTA-AM for 1 h to chelate intracellular Ca^{2+}, as described previously (Stachecki and Armant, 1996). In embryos loaded with BAPTA-AM, FN-120 failed to upregulate FN-binding activity (Fig. 2C), presumably due to a requirement for intracellular Ca^{2+} signaling. Chelation of intracellular Ca^{2+} did not reduce baseline FN-binding activity, demonstrating that Ca^{2+} mobilization functions specifically in the strengthening of cell adhesion. This hypothesis is further supported by the ability of pharmacologically induced Ca^{2+} transients to generate inside-out signaling. Exposure of blastocysts to 5 μM ionomycin rapidly upregulated FN-binding activity to the same extent as a much longer incubation with FN-120 (Fig. 2C). These experiments demonstrate that the elevation of intracellular Ca^{2+} is required and sufficient for the upregulation of FN-binding activity.

Requirements Downstream to Ca^{2+}

Protein kinase C (PKC) and calmodulin activities both depend on Ca^{2+} (Kishimoto *et al.*, 1980; Means and Redman, 1980; Sasaki and Hidaka, 1982). To investigate their possible roles in the inside-out signaling pathway that upregulates FN-binding activity, blastocysts were treated with calphostin C, an inhibitor of PKC, or W7, an inhibitor of calmodulin (Kobayashi *et al.*, 1989). Both Calphostin C and W7 blocked the upregulation of FN-binding activity when blastocysts were exposed to either FN-120 or ionomycin (Figs. 3A and 3B). The less active structural analogue of W7, W5, was without effect. The inhibitors did not reduce baseline adhesion to FN, suggesting that PKC and calmodulin are required specifically for transducing signals that strengthen integrin-mediated adhesion. These findings support the central role of intracellular Ca^{2+} in this pathway, and indicate that downstream signaling pathways transduced by PKC and calmodulin are required to strengthen the activity of integrins on the apical surface of trophoblast cells.

Schultz *et al.* (1995), using brefeldin A, found that upregulation of FN-binding activity requires active protein trafficking. Intracellular Ca^{2+} can trigger the cortical reaction during oocyte activation (Ducibella, 1991) and induces exocytosis in a number of cell types (Guo *et al.*, 1996; Kiraly-Borri *et al.*, 1996). Requirements for both calmodulin (Chamberlain *et al.*, 1995; Peters and Mayer, 1998) and PKC (Chen *et al.*, 1999; Quetglas *et al.*, 2000; Lan *et al.*, 2001) in protein trafficking have been implicated. To determine whether protein trafficking operates downstream to Ca^{2+} during upregulation of FN-binding activity, embryos treated with 200 μM brefeldin A were exposed to 0 or 5 μM ionomycin for 5 min, as in Fig. 3B. Brefeldin A treatment alone (-3.05 ± 15.02) did not significantly reduce baseline activity (0 ± 15.27), but prevented the increase in adhesion induced by ionomycin (1.9 ± 17.63, $P < 0.05$, compared to ionomycin only, 99.93 ± 23.21). Therefore, it appears that protein trafficking is required downstream to Ca^{2+} signaling in the upregulation of FN-binding activity.

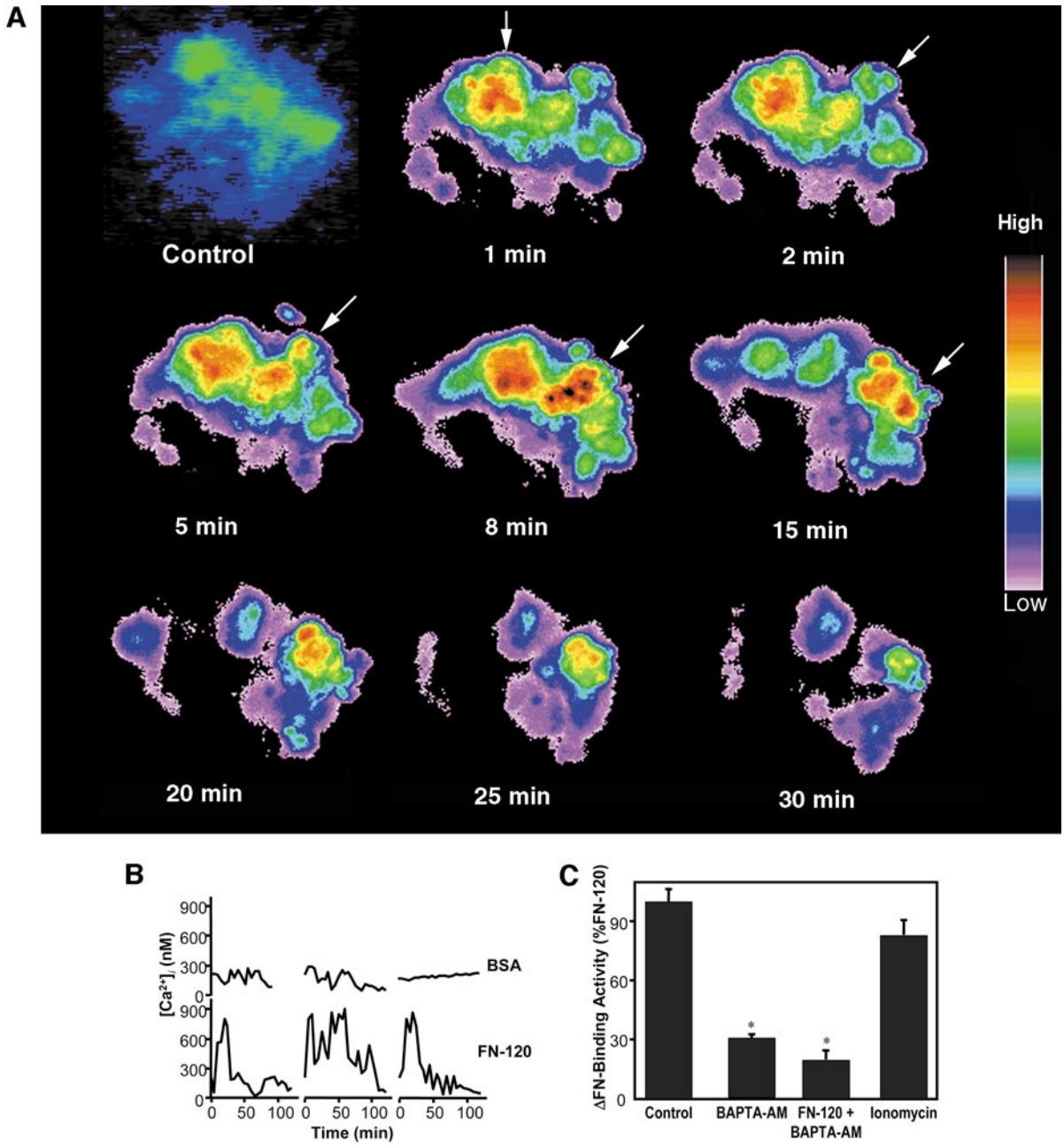

FIG. 2. Intracellular Ca^{2+} signaling. (A) The relative levels of intracellular Ca^{2+} in a typical blastocyst loaded with fluo-3-AM are indicated in pseudocolor representing fluorescent intensity according to the color bar. A control blastocyst contacting only BSA is shown for reference. The time after the experimental blastocyst was transferred to a petri dish coated with FN-120 is indicated below its images. Arrows indicate cells that displayed elevated Ca^{2+} levels. (B) The intracellular Ca^{2+} concentration was estimated after exposure to BSA or immobilized FN-120. Each graph depicts the average concentration integrated over an entire representative embryo. At least 10 embryos in each group produced similar responses in three separate experiments. (C) Blastocysts were preloaded for 1 h with 10 μM BAPTA-AM where indicated. ΔFN-binding activity was assayed as in Fig. 1 after exposure for 1 h to 50 μg/ml FN-120 (Control, FN-120 + BAPTA-AM), or treatment for 5 min with 5 μM ionomycin (Ionomycin). Treatment with BAPTA-AM only (BAPTA-AM) did not reduce the baseline FN-binding activity. *, $P < 0.05$, compared with control.

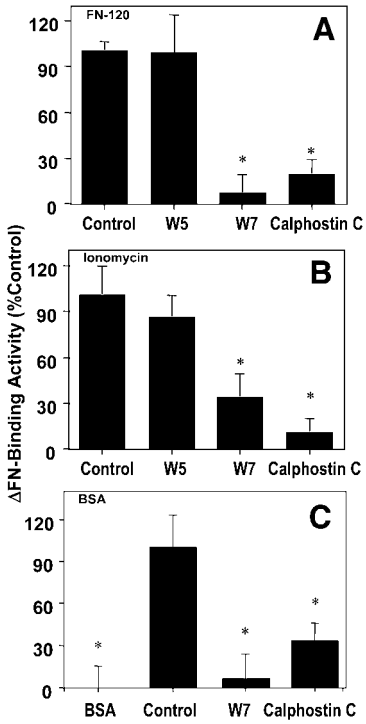

FIG. 3. Inhibition of PKC and calmodulin during upregulation of FN-binding activity. Blastocysts were incubated for 1 h without (Control, BSA) or with 0.25 μM calphostin C, 10 μM W7, or 10 μM W5, as indicated on the abscissa. They were then cultured in the continued presence of the same inhibitors with (A) 50 μg/ml FN-120 for 1 h, (B) 5 μM ionomycin for 5 min or (C) BSA for 1 h. In (C), a control blastocyst exposed to FN-120 is shown for reference. ΔFN-binding activity was then assayed, as in Fig. 1. *, $P < 0.05$, compared with control.

Cytological Changes during Upregulation of FN-Binding Activity

Based on the apparent role of protein trafficking in regulating trophoblast adhesion to FN, we have used transmission electron microscopy to examine the cytological changes induced by integrin signaling. A dramatic accumulation of intracellular vesicles occurred in cultured blastocysts during the period between GD6 and GD7 (Figs. 4A and 4B). Large numbers of vesicles were observed on GD7 in mural trophoblast cells, but not in polar trophoblast cells or the inner cell mass (data not shown). The abundance of vesicles dramatically decreased after GD7 blastocysts were exposed to immobilized FN-120 (Fig. 4C), suggesting that integrin-mediated signaling induced a rapid change in vesicle dynamics. A morphometric evaluation of vesicle populations within the cytoplasm of mural trophoblast cells confirmed a significant increase between GD6 and GD7, followed by a significant decrease after the blastocysts were incubated on immobilized FN-120 for 3 h (Fig. 5A). These results are consistent with previous findings suggesting that the upregulation of FN-binding activity depends on intracellular trafficking (Schultz and Armant, 1995). Indeed, when trafficking was inhibited by using brefeldin A, the vesicles were retained within the cytoplasm after treatment with FN-120, although they fused into larger vesicles (Fig. 4D). Brefeldin A-treated embryos contained a reduced number of vesicles after incubation on immobilized FN-120 (Fig. 5A); however, when the total area occupied by vesicles was determined, it was not significantly different from nontreated blastocysts cultured to GD7 (Fig. 5B).

Vesicle Trafficking Induced by FN-120 Is Stimulated by Intracellular Ca^{2+}

To investigate the role of Ca^{2+} signaling in FN-120-induced vesicle trafficking, blastocysts were treated on GD7 with 0.1% ethanol to directly elevate the concentration of intracellular Ca^{2+} (Stachecki *et al.*, 1994a). Changes in cytoplasmic vesicles were visualized by using electron microscopy and subjected to morphometric analysis. Ethanol treatment diminished the number of intracellular vesicles similarly to FN-120 exposure (Fig. 5A).

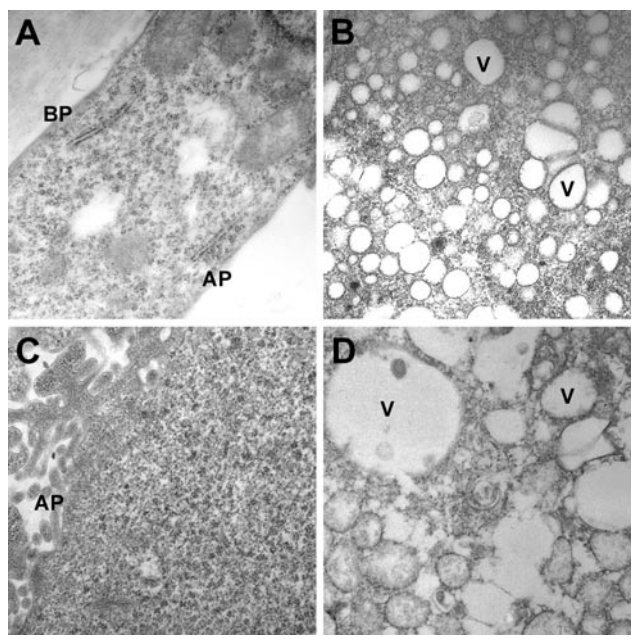

FIG. 4. Cytological changes associated with blastocyst differentiation and upregulation of FN-binding activity. Transmission electron micrographs are shown of mural trophoblast cells in blastocysts cultured to GD6 (A), GD7 (B), GD7 and incubated for 3 h on FN-120 (C), or GD7 and incubated on FN-120 in the presence of 400 μM brefeldin-A (D). Vesicles (V), the apical plasma membrane (AP), and the basolateral plasma membrane (BP) are indicated. Magnification: 20,000×.

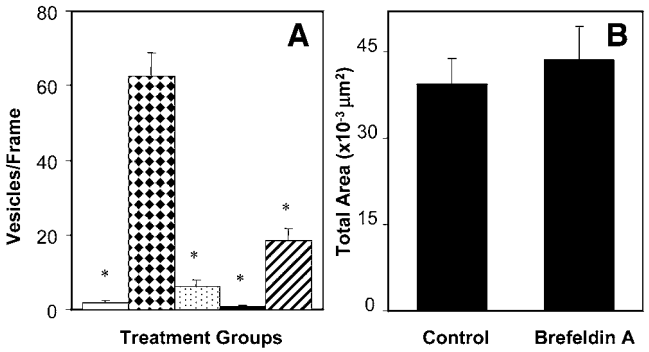

FIG. 5. Morphometric evaluation of vesicle population during development. (A) Blastocysts cultured to GD6 (open bar) revealed relatively low numbers of vesicles when compared with blastocysts cultured to GD7 (diamond bar). After 3 h of incubation on FN-120 (stippled bar), vesicle number was near that of blastocysts cultured to GD6. Blastocysts on GD7 treated with 0.1% ethanol revealed very few vesicles within the cytoplasm of mural trophoblast cells (solid bar). When incubated on immobilized FN-120 in the presence of 400 μM brefeldin-A (hatched bar), the abundance of vesicles was at an intermediate level. *, $P < 0.05$ compare with GD7 untreated. (B) Vesicle area was calculated (as detailed in Materials and Methods) in blastocysts cultured to GD7 or blastocysts pretreated with 400 μM brefeldin A and cultured on FN-120 for 3 h in the presence of 400 μM brefeldin A. The mean area of 10 frames/group revealed no significant difference between the 2 groups, better representing the effects of brefeldin-A on inhibiting vesicle exocytosis.

DISCUSSION

This study addressed questions raised by the ability of FN to stimulate the binding activity of its own receptor on the apical surface of trophoblast cells. Based on the results presented here, it can be concluded that FN initiates intracellular signaling through its ligation of β_1- and β_3-class integrins. This report provides the first direct evidence that trophoblast behavior during implantation is regulated by intracellular Ca^{2+} signaling in response to proteins residing in the endometrial ECM. Ca^{2+} signaling plays a critical role during preimplantation development by mediating autocrine and paracrine growth factor signaling (Wang *et al.*, 1998, 2000; Emerson *et al.*, 2000). Integrin-mediated intracellular Ca^{2+} signaling has been reported in other cell systems. Signal transduction stimulated by fluid shear stress in endothelial cells involves an interaction between integrins present in focal adhesion complexes and the Ca^{2+}-dependent kinases, PKC and Ca^{2+}-calmodulin-dependent protein kinase (Berk *et al.*, 1995). Consistent with these findings, we observed intracellular Ca^{2+} mobilization that induced by FN-120 was both necessary and sufficient for strengthening trophoblast adhesion to FN. This signaling pathway may ultimately be responsible for protein trafficking and cytoskeletal reorganization that is required for the upregulation of FN-binding activity (Schultz and Armant, 1995). Integrin signaling induced by FN, as well as other ECM components, may regulate subsequent trophoblast differentiation. Indeed, trophoblast contact with ECM alters protein secretion and the expression of specific genes, including those encoding matrix metalloproteinases, urokinase-type plasminogen activator, integrins, and other adhesive proteins (Bischof *et al.*, 1991; Zhang *et al.*, 1996; Zhou *et al.*, 1997).

The ligand-induced upregulation of FN-binding activity was mediated specifically by interaction between the RGD recognition sequence of FN and integrins on the apical surface of adhesive trophoblast cells. This finding is consistent with an earlier observation that either FN or a hexapeptide containing the RGD sequence recruit intracellular signaling factors to focal adhesion complexes (Miyamoto *et al.*, 1995), suggesting that the RGD sequence is the major interactive unit in FN for induction of intracellular signaling. Other fragments of FN lacking RGD were ineffective, as was type-1 laminin, which interacts with trophoblast cells through its E8 domain rather than an RGD site (Armant, 1991; Sutherland *et al.*, 1993). Laminin, however, is able to upregulate trophoblast binding of microspheres coated with E8 peptide (J. F. Schultz and D.R.A., unpublished observation), suggesting that cross talk does not occur between the laminin and FN adhesive systems. Entactin, which mediates trophoblast adhesion through an RGD site (Yelian *et al.*, 1993), effectively upregulated FN-binding activity. This result and our observation that FN upregulates entactin-binding activity on the surface of blastocysts (L.M. and D.R.A., unpublished observation) indicate that RGD-recognizing integrins may be activated through similar intracellular signaling pathways. Binding of β_1- or β_3-class integrins with specific monoclonal antibodies was as effective as receptor ligation by FN-120. Schultz and Armant (1995) demonstrated that integrins containing α_5, α_v, β_1, and β_3 subunits mediate FN-binding activity. It now appears that the same integrins that eventually mediate strong adhesion to FN initially transduce outside-in signals to upregulate their own binding activity.

The Ca^{2+}-dependent signaling protein, calmodulin (Means *et al.*, 1991; Lu and Means, 1993), was required to achieve strong FN-binding activity. The relevant downstream targets of calmodulin in peri-implantation blastocysts are not presently known. Calmodulin is capable of both increasing and decreasing integrin-mediated adhesion to FN. The binding activity of $\alpha_5\beta_1$ requires a calmodulin-dependent serine/threonine phosphatase, calcineurin (Pomies *et al.*, 1995), while activation of Ca^{2+}-calmodulin-dependent protein kinase II reduces $\alpha_5\beta_1$ integrin affinity (Bouvard *et al.*, 1998). In addition to modulating integrin affinity, calmodulin could promote protein trafficking (Chamberlain *et al.*, 1995; Peters and Mayer, 1998) to recruit additional integrins or other molecules that regulate adhesion to FN.

The PKC family of serine-threonine kinases includes three isoforms (α, β, γ) activated by elevation of intracellular Ca^{2+} (Livneh and Fishman, 1997). Integrin ligation

recruits and activates PKC (Vuori and Ruoslahti, 1993; Miyamoto *et al.*, 1995), suggesting that PKC can participate in outside-in integrin signaling. During blastocyst adhesion, PKC activity was required downstream to Ca^{2+} signaling, demonstrating its contribution to the inside-out signaling pathway that strengthens trophoblast adhesion to FN. Like calmodulin, PKC can influence integrin activity, as well as promote protein trafficking. Integrin activity could be regulated by PKC through direct phosphorylation of β_1 integrins, as previously reported (Schwartz *et al.*, 1995). The proposed requirement for PKC in protein trafficking (Chen *et al.*, 1999; Quetglas *et al.*, 2000; Lan *et al.*, 2001), including recycling of integrin receptors in the plasma membrane (Ng *et al.*, 1999), suggests another putative function for PKC during upregulation of FN-binding activity, which requires protein trafficking (Schultz and Armant, 1995).

Cytosolic free Ca^{2+} regulates exocytosis in many cell types, including rat chromaffin cells (Guo *et al.*, 1996) and endocrine cells (Kiraly-Borri *et al.*, 1996). Both calmodulin and PKC, shown here to regulate FN-binding activity, modulate critical steps in the exocytotic machinery of neurons (Chen *et al.*, 1999; Quetglas *et al.*, 2000). Previous work suggests that intracellular protein trafficking is required during upregulation of FN-binding activity (Schultz and Armant, 1995). Indeed, there was a dramatic increase in cytosolic vesicles in mural trophoblast cells between GD6 and GD7, which then disappeared immediately exposure to either FN-120 or ionophore. Whether these vesicles translocate to the apical plasma membrane, trafficking molecules that enhance the affinity of FN receptors, is not known. An alternative hypothesis is that the vesicles represent endocytosis that is terminated by FN exposure. However, the ability of brefeldin A to prevent the FN-induced decrease in vesicle presence strongly argues in favor of an exocytotic pathway. Recent findings from our laboratory reveal that, while there is no change in surface expression of the integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$ after exposure to FN-120, $\alpha_{IIb}\beta_3$ localization shifts from intracellular to the apical surface of trophoblast cells (U. K. Rout, J.W., and D.R.A., unpublished observation). Because $\alpha_{IIb}\beta_3$ also recognizes FN-120, its translocation may represent a critical trafficking event that strengthens FN-binding activity. Studies are in progress to determine whether the observed changes in vesicle content correlate with trafficking of α_{IIb} or other proteins that may influence adhesion to FN.

We have demonstrated that, prior to implantation, trophoblast cells undergo a program of differentiation to achieve an adhesion-competent state (Schultz *et al.*, 1997; Wang *et al.*, 1998, 2000). Once adhesion-competent, trophoblast cells can undergo upregulation of FN-binding activity by exposure to ligand (Schultz and Armant, 1995). We now provide direct evidence that an intracellular signaling cascade is activated upon binding of FN to β_1- and β_3-class integrins, suggesting that the function of growth factors in regulating trophoblast invasion and early placenta formation may be augmented by uterine ECM components. As in the case of several growth factors that advance preimplantation development, integrins modulate the developmental program of trophoblast cells by elevating cytoplasmic-free Ca^{2+}. It has not been determined conclusively whether FN binding is strengthened by increased integrin affinity or recruitment of additional integrin receptors. Competitive inhibition experiments suggest the latter (Schultz and Armant, 1995), although rigorous kinetic studies of FN binding have not been conducted.

Our data, together with previous reports that the expression of integrins is actively regulated during placentation or invasion of ECM *in vitro* (Damsky *et al.*, 1994; Kilburn *et al.*, 2000), reveal the critical role played by trophoblast integrins in early development. Although we found that FN and laminin appear to operate independently in the regulation of their respective receptors, the possibility remains that FN-induced signaling impacts trophoblast adhesion to laminin and other components of the ECM later in development. The ability of trophoblast cells to mobilize different adhesion mechanisms depending on the composition of the immediate ECM milieu attests to the remarkable plasticity of these cells as they encounter a dynamic, diverse microenvironment during implantation.

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