Protein Kinase C Regulates the Recruitment of Syndecan-4 into Focal Contacts

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Cell surface heparan sulfate proteoglycans have been implicated as co-receptors facilitating cell adhesion and growth factor binding. Recent studies on the role of a family of transmembrane heparan sulfate proteoglycans, syndecans, in cell adhesion has identified one member, syndecan-4, to be present within focal contacts. The current study investigates the mechanisms regulating the association of syndecan-4 with focal contacts based upon its immunolocalization with vinculin in quiescent, serum-stimulated, and 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced cultures. In quiescent cells, syndecan-4 did not localize to focal contacts. However, activation of protein kinase C by TPA or serum induces the active recruitment of syndecan-4 into focal contacts. This induction preferentially localizes syndecan-4 to focal contacts behind the leading lamella, the subnuclear region, and along the trailing edge of migratory cells. Focal contacts in either freshly adhered cells or in the leading lamellae of migrating cells did not stain for syndecan-4. In addition to the observed subcellular distribution and recruitment, syndecan-4 was observed to co-localize with endogenously synthesized fibronectin fibrils within focal contacts as well as with fibrils present in the matrix. These findings suggest that protein kinase C activation results in syndecan-4 recruitment to focal contacts and its association with sites of matrix deposition.

INTRODUCTION

Syndecans are transmembrane proteoglycans in which both heparan sulfate and chondroitin sulfate glycosaminoglycan chains are attached to the extracellular domain of the core protein. To date, four family members have been cloned from several different species. The main characteristic shared by the family members is the high homology of the cytoplasmic domain of the core protein and the conservation of four tyrosine residues within that domain. Many, if not all, functions of syndecans are attributed to the interaction of the glycosaminoglycan chains with a large number of biological molecules. These include extracellular matrix molecules, growth factors, growth factor receptors, cell adhesion molecules, protease inhibitors, degradative enzymes, lipolytic enzymes, nu-

clear proteins, and viral coat proteins (Bernfield *et al.*, 1992).

Functionally, syndecans are described as co-receptors that either facilitate or inhibit the interaction of ligands with their high affinity signaling receptors (Elenius and Jalkanen, 1994). For example, cell surface heparan sulfate proteoglycans (HSPGs)¹ have been shown to modulate the interaction of bFGF and amphiregulin with their signaling receptors (Olwin and Rapraeger, 1992; Guimond *et al.*, 1993; Johnson and Wong, 1994). In interactions with extracellular matrix molecules, one class of cell surface HSPGs (i.e., syndecans) are proposed to function as co-receptors with

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¹ Abbreviations used: CEF, chicken embryo fibroblasts; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; HSPG, heparan sulfate proteoglycan; PBS, phosphate-buffered saline; PKC, protein kinase C; TCA, trichloroacetic acid; 12-*O*-tetradecanoylphorbol 13-acetate; TRITC, tetramethyl rhodamine isothiocyanate.

integrins during cell adhesion, cell spreading, focal contact formation, actin polymerization, and migration (Woods *et al.*, 1986, 1993; Woods and Couchman, 1992, 1994; Couchman *et al.*, 1988; Sanderson *et al.*, 1992; Carey *et al.*, 1994a,b; Liebersbach and Sanderson 1994; Yoneda *et al.*, 1995).

Although the above studies either directly or indirectly identify potential roles for syndecans, these functions are neither applicable to all cell types nor restricted to only cell surface HSPGs. In the case of growth factors, perlecan, an extracellular matrix proteoglycan, can promote bFGF activity to a greater extent than that observed for cell surface HSPG (Aviezer *et al.*, 1994). In cell adhesion assays cell surface HSPGs appear to be required for rat hepatocytes and HT 1080 cells to spread (Hansen *et al.*, 1994; Yoneda *et al.*, 1995) whereas capillary endothelial cells, vascular smooth muscle cells, human fibroblasts, and Chinese hamster ovary cells deficient in glycosaminoglycan synthesis are able to spread on either RGD peptides or the cell binding domain of fibronectin but are not able to form actin stress fibers or focal contacts (LeBaron *et al.*, 1988; Burridge *et al.*, 1992; Woods and Couchman 1992). Therefore, the ability of cell surface HSPGs to regulate cell spreading, actin polymerization, and focal contact formation appears to be dependent on both the cell type and experimental conditions. The cell and assay dependence may reflect the ability of cells to mediate spreading, actin organization, and focal contact formation along alternate pathways or to regulate signaling downstream from HSPGs. One potential downstream event is the activation of protein kinase C (PKC) by HSPG. This is based on the ability of 12-*O*-tetradecanoylphorbol 13-acetate (TPA) to mimic the requirement of the heparin binding domain of fibronectin in the formation of focal contacts (Woods and Couchman *et al.*, 1992).

The activation of PKC can occur in cells plated on the cell binding domain of fibronectin (Chang *et al.*, 1993) or through integrin clustering (Vuori and Ruoslahti, 1993). These findings suggest that cell surface HSPGs are not required for PKC activation. If PKC activation is independent of HSPGs then the role of PKC in mediating HSPG signaling may be through the regulation of HSPG's aggregation or association with focal contacts normally regulated by heparin-binding domains. The major HSPG identified in focal contacts is syndecan-4 (Woods and Couchman, 1994).

To study the role of PKC in mediating the association of HSPG with focal contacts, we examined the regulation of syndecan-4 association with focal contacts in newly adhered, quiescent, serum-stimulated, and TPA-induced chicken embryo fibroblasts (CEF) plated on fibronectin. These studies indicated the following: 1) PKC regulates syndecan-4 recruitment into focal contacts; 2) syndecan-4 association with focal contacts is a late event during adhesion; 3) syndecan-4

associates with a sub-population of focal contacts localized behind the leading lamella, in the subnuclear region and the trailing edge of migratory cells; and 4) the association of syndecan-4 with focal contacts correlates with fibronectin deposition, suggesting its involvement in matrix assembly.

MATERIALS AND METHODS

Cell Culture

Primary CEF were obtained from 8-day dorsal skins as described in Baciú *et al.* (1994). CEFs were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 250 µg/ml streptomycin, and 250 U/ml penicillin (Life Technologies, Gaithersburg, MD) in a humidified atmosphere of 10% CO₂ at 37°C. Cells were seeded at a density of 0.5×10^6 cells in 25 cm² flasks (Costar, Cambridge, MA) and upon attaining confluency they were trypsinized and reseeded at the original density. Subconfluent CEFs in the third to sixth passage were used in all experiments.

Cell Plating and Treatments

Cells were trypsinized with 0.05% trypsin/0.53 mM EDTA (Life Technologies) and the trypsin was quenched with complete medium. Quenched cells were washed once in 0.5% FBS/DMEM and resuspended to a density of 1.25×10^4 cells/ml in either serum-free DMEM, 0.5% FBS/DMEM, or 10% FBS/DMEM as indicated in figure legends. Two hundred microliters of diluted cells were plated per well of an eight-chamber glass slide (Lab-TEK, Nunc, Naperville, IL) precoated with fibronectin to give a cell density of 2.5×10^3 cells per cm². Plated cells were incubated in a humidified incubator before treatment or fixation. Pre-coating of the glass slides was performed with human fibronectin at 100 µg/ml (Collaborative Biomedical Products, Bedford, MA) for 1 h, followed by blocking for 30 min with 1% heat-inactivated bovine serum albumin in phosphate-buffered saline (PBS). Slides were washed three times with PBS before cell plating. TPA was solubilized in DMSO at 100 µg/ml (Sigma, St. Louis, MO) and stored at -80°C. TPA was diluted with PBS before the addition to cells. The final concentration of the DMSO was 0.1%. This concentration of DMSO was used in control cultures. For experiments in which either cycloheximide (Sigma) or calphostin C (LC Laboratories, Woburn, MA) was used, the cells were pre-treated with each compound for 2 h before the addition of TPA or serum.

Cell Fixation, Antibody Staining, and Analysis

Cells were fixed according to the procedure of Woods and Couchman (1994). Before the addition of primary antibodies the slides were blocked with 1% normal goat serum for 30 min. Three primary antibodies were used. CS-4-E was generated in rabbits immunized with a synthetic peptide that covers a unique amino acid sequence of the ectodomain of chicken syndecan-4 as described by Baciú *et al.* (1994). Affinity-purified CS-4-E was used at a concentration of 20 µg/ml. Specificity of CS-4-E was verified by the ability of the immunizing peptide to block all staining. Peptide inhibitions were done by pre-incubating CS-4-E with a 10-fold molar excess of the immunizing peptide before staining. The vinculin (VN 3-24) and fibronectin (B3/D6) monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD, and the Department of Biological Sciences, University of Iowa, Iowa City, IA, under contract N01-HD-2-3244 from the National Institute of Child Health and Human Development. All monoclonal antibodies were obtained from ascites fluid and used at a 1/100 dilution. To visualize primary antibody staining, the slides were incubated with a 1/75

dilution of tetramethyl rhodamine isothiocyanate (TRITC)-conjugated anti-rabbit and fluorescein isothiocyanate (FITC)-conjugated anti-mouse antibodies (Pierce, Rockford, IL) for 30 min at room temperature. FITC-phalloidin (Molecular Probes, Eugene, OR) was used for actin staining according to the manufacturer's instructions. After washing three times with PBS, coverslips were mounted using Gelmount (Biomedica, Foster City, CA).

Antibody staining was observed using epifluorescence with a Nikon Microphot-FXA microscope (Nikon, Melville, NY). Of the two emission channels used during double labeling experiments, there was no evidence of bleed-through from one channel to the other when single labeling experiments were done. Quantification of cells considered positive for the inclusion of syndecan-4 in focal contacts was determined by examining slides at 400 $\times$ magnification in a grid pattern to prevent scoring the same cell twice. More than 100 cells were scored per data point. Localization to focal contacts was based upon co-staining with vinculin. Cells characterized as negative for syndecan-4 in focal contacts are shown in Figure 3, A and C. Cells considered positive for syndecan-4 association in focal contacts are shown in Figure 3, E and G. These morphological criteria formed the basis for quantifying cells for the presence or absence of syndecan-4 in focal contacts.

Determination of Protein Synthesis

CEFs in 0.5% FBS/DMEM were plated at 2×10^5 cells per well in Corning 12-well tissue culture plates. Following a 14- to 16-h incubation period, cells were treated with various cycloheximide concentrations ranging from 25 μ g/ml to 1 μ g/ml for 2 h. At that time TPA was added to a final concentration of 100 ng/ml and the cells were labeled with 250 μ Ci/ml Trans³⁵S-mix (Dupont, Boston, MA). DMSO at 0.1% was used as a solvent control. After addition of label, cells were incubated for an additional 2.5 h. At the end of the labeling period cells were washed with PBS and lysed with PBS/1% NP40 (containing 1 mM EDTA, 1 mM EGTA, 0.1 ng/ml aprotinin, 10 μ g/ml leupeptin, 10 μ g/ml pepstatin, and 0.2 mg/ml [4-(2-aminoethyl)-benzenesulfonyl fluoride, HCl]; Calbiochem, San Diego CA) for 20 min on ice. Cell layers were scraped, centrifuged for 10 min at 15,000 $\times$ g, and the supernatants were collected. Total cellular protein was determined using the BCA protein assay (Pierce, Rockford, IL). Protein synthesis was determined by measuring [³⁵S]methionine incorporation into trichloroacetic acid (TCA) precipitable material. Briefly, 10–20 μ l of cell supernatant was spotted onto microporous glass fiber filters (Millipore, Bedford, MA) and allowed to dry. Filters were then immersed for 10 min in ice-cold 10% TCA with gentle agitation and rinsed twice with 10% ice-cold TCA followed by two washes with acetone and drying. Radioactivity incorporated into TCA precipitate was determined in a Beckman LS6000IC scintillation counter (Beckman, Fullerton, CA) using EcoLite aqueous liquid scintillation fluid (ICN, Costa Mesa, CA).

RESULTS

Syndecan-4 Localizes to Maturing Focal Contacts

Syndecan-4 in CEFs plated on fibronectin and cultured for 14–16 h in 10% FBS/DMEM co-localizes with vinculin, confirming its inclusion within focal contacts (Figure 1A). The specificity of the anti-syndecan-4 antibodies (CS-4-E) used in these studies was demonstrated by the ability of the immunizing peptide to block completely the staining for syndecan-4 (Figure 1B) without affecting the detection of vinculin (Figure 1C). In cells determined to be migratory based on their morphology, syndecan-4 does not co-distribute uniformly with vinculin. At the distal end of lead-

ing lamella and cell periphery, syndecan-4 staining is not detectable in vinculin-positive focal contacts (Figure 1, D and E). Syndecan-4 was observed to co-localize with vinculin (Figure 1, A, D and E) at sites proximal to the leading edge of the lamella as well as in the subnuclear region and the trailing edge of cells. Within these regions of the cell, focal contacts are fewer in number and are likely to represent more stable and specialized adhesions. In nonmigratory cells, syndecan-4 co-localizes with vinculin throughout the cell as well as at its periphery. In addition to the differential cellular distribution, syndecan-4 was also observed to be deposited into the extracellular matrix (Figures 1, A, D, and E).

These findings suggest that the inclusion of syndecan-4 in focal contacts may be a late event in focal contact formation. To establish the association of syndecan-4 with maturing as opposed to newly forming focal contacts, CEFs were plated in the presence and absence of serum on fibronectin-coated slides and examined for the co-localization of syndecan-4 with vinculin at various time points. At 1 h, 59% of the cells plated in the absence of serum were positive for vinculin staining and by 2 h 100% of the cells were positive. In the presence of serum, 100% of the cells plated were positive for vinculin at all time points examined. In contrast, syndecan-4 association with vinculin in serum-free cultures occurred in only 7.6% and 9.3% of the cells at 2 and 3 h, respectively, with maximal levels of association occurring by 9 h (Figure 2, solid bars). In the presence of serum, syndecan-4 association with focal contacts was 30% and 27% at 2 and 3 h, respectively, with maximal levels occurring by 6 h (Figure 2, hatched bars). Continued culturing of cells in serum-free or serum-depleted media resulted in the loss of syndecan-4 from focal contacts. However, in the presence of serum no change in syndecan-4 staining was observed (see below). These data indicate that the inclusion of syndecan-4 in focal contacts is a late event during cell adhesion relative to vinculin and that serum enhances its association and maintenance with focal contacts. These observations support the previous conclusions that syndecan-4 is associated with maturing focal contacts.

Serum Causes Syndecan-4 To Be Included into Focal Contacts of Quiescent Cells

To study the assembly of syndecan-4 into focal contacts and potential signaling pathways regulating its association with focal contacts we examined the distribution of syndecan-4 in serum-stimulated and serum-starved cells. Previous studies have indicated that focal contacts formed under serum-deficient conditions are less well formed and represent an earlier stage in focal contact formation and that the addition of serum induces their maturation (Thom *et al.*, 1979).

Figure 1. Distribution of syndecan-4 in proliferating CEFs. CEFs were plated on glass slides coated with human fibronectin and cultured in the presence of 10% FBS in DMEM for 14–16 h. Cells were fixed and prepared for indirect immunofluorescence for syndecan-4 and vinculin using the antibodies CS-4-E and VN-3-24, respectively. Syndecan-4 staining was identified using TRITC-conjugated goat anti-rabbit IgG (rhodamine). Vinculin staining was identified using FITC-conjugated goat anti-mouse IgG (fluorescein). Nuclear staining was done using Hoechst 33258. (A) Triple exposure showing syndecan-4 (rhodamine), vinculin (fluorescein), and nuclear (Hoechst 33258) staining. Co-localization of vinculin and syndecan-4 is indicated by the yellow color due to co-fluorescence of fluorescein and rhodamine. (B and C) Inhibition of CS-4-E staining with the immunizing peptide. Syndecan-4 staining (B) and vinculin staining (C) of the same cell. (D and E) Syndecan-4 (D) and vinculin (E) staining showing vinculin, but not syndecan-4, in the leading edge (distal region) of the lamella (large arrow), co-localization of syndecan-4 and vinculin (small arrow) in the proximal region of the lamella, and deposition of syndecan-4 but not vinculin in the matrix (open arrow). Photographs were taken with a 40 $\times$ objective using Kodak Ectachrome 400DX elite film.

For serum-starvation experiments, CEFs were plated at 2.5×10^3 cells/cm² on fibronectin-coated glass slides for 14–16 h in DMEM containing 0.5% FBS. Under these conditions, [³H]thymidine incorporation is inhibited by 90% as compared with cells cultured in the presence of 10% FBS for the same period. In serum-starved cells, focal contacts are present as indicated by vinculin staining (Figure 3B), although syndecan-4 staining is observed to be in small diffuse clusters adjacent to focal contacts (Figure 3, A and B) or diffusely spread over the cell surface (Figure 3C). This lack of association of syndecan-4 with focal contacts in serum-starved cells is in stark contrast to the strong punctate staining observed in cells cultured in the presence of 10% serum (Figure 1). The absence of syndecan-4 from focal contacts in quiescent cells (Figure 3, A and C) and in freshly adhered cells (Figure 2) suggests that syndecan-4 inclusion in focal contacts is not necessary for their formation or maintenance. However, the ability of serum to affect both the rate of recruitment (Figure 2) and maintenance (Figure 1A) of syndecan-4 in focal contacts indicates that syndecan-4

association with the matrix is regulated, in part, by factors present in serum.

To examine the ability of serum to induce syndecan-4 inclusion in focal contacts, quiescent cells were stimulated with 10% FBS/DMEM. Upon serum stimulation a re-organization of actin fibers (Figure 3, compare D with H) as well as the inclusion of syndecan-4 in focal contacts is observed (Figure 3, compare A with E and C with G). As in cells cultured in the presence of serum, syndecan-4 localizes behind the leading lamellae and in subnuclear regions as well as along the trailing edge. Additionally, syndecan-4 was observed to be present at the end of actin stress fibers (Figure 3, G and H). Thus, the localization of syndecan-4 to focal contacts can be regulated by serum in quiescent cells.

Quantitative analysis of the rate of syndecan-4 association with focal contacts indicates that in a population of quiescent cells, 13% of the cells have syndecan-4 localized to focal contacts (Figure 4). When quiescent cells are stimulated with 10% FBS/DMEM and analyzed at 1 and 2.5 h after treatment, an increase in syndecan-4-positive cells is observed. By 1 h

Figure 2. Inclusion of syndecan-4 in focal contacts during cell adhesion. Subconfluent CEFs were trypsinized and plated onto fibronectin-coated glass slides in either the presence (hatched bars) or absence of serum (solid bars). At the indicated time points cells were fixed and co-stained for syndecan-4 and vinculin. At least 100 cells were scored for either the presence or absence of syndecan-4 in focal contacts for each time point. Error bars represent standard deviations; $n = 3$.

the percentage of syndecan-4-positive cells rose to 23% and by 2.5 h 50% of the cells stained for syndecan-4 in focal contacts (Figure 4). Occasionally, as much as 70% of the cells were observed to be syndecan-4 positive. Examination of cultures up to 7 h indicated no further increase in the percentage of positive cells beyond that observed at 2.5 h. The inability to observe greater than 50–70% of syndecan-4-positive cells may reflect a differential response of individual cells to serum stimulation. In contrast to the late association of syndecan-4 with focal contacts, actin reorganization occurred within 30 min. This rate of actin reorganization is similar to that seen in Swiss 3T3 cells (Ridley and Hall, 1992). These results suggest that syndecan-4 association with focal contacts is part of the maturation and not the initiation of focal contact formation.

TPA Stimulates Syndecan-4 Inclusion into Focal Contacts of Quiescent Cells

The observation that PKC activation is associated with focal contact formation as well as the immunolocaliza-

tion of PKC to these structures (Jaken *et al.*, 1989; Woods and Couchman, 1992; Vuori and Ruoslahti, 1993) suggests that PKC may also be involved in the regulation of syndecan-4 association with focal contacts. To test this possibility, serum-starved cells were treated with TPA and analyzed for the presence of syndecan-4 in focal contacts. Analysis of syndecan-4 association with focal contacts induced by 50, 100, and 150 ng/ml of TPA demonstrates a dose-dependent response (Figure 5A), with 100 ng/ml of TPA achieving levels similar to those observed with serum stimulation. The time course of syndecan-4 inclusion into focal contacts with 100 ng/ml of TPA follows a pattern similar to that observed with serum stimulation in that the initial association with focal contacts occurs by 1 h post induction (Figure 5B). Both the subcellular distribution of syndecan-4 and the reorganization of actin stress fibers as a result of TPA treatment are similar to the results obtained in serum-induced cells (Figure 6). After stimulation with 100 ng/ml of TPA, syndecan-4 co-localizes with focal contacts as well as induces the reorganization of actin filaments (Figure 6, E–H). DMSO alone had no effect on syndecan-4 association with focal contacts. As in quiescent cells, syndecan-4 distribution in DMSO-treated cells was either clustered at sites of cell attachment or diffusely spread over the cell surface (Figure 6, A–D).

Inclusion of Syndecan-4 into Focal Contacts Occurs through the Activation of PKC

The association of syndecan-4 with focal contacts as a result of TPA treatment suggests that the PKC pathway may be involved in the inclusion process. To investigate this possibility we used calphostin C, a specific inhibitor of PKC. The calphostin C levels used were well below the IC_{50} values that inhibit other cellular kinases including cAMP-dependent protein kinase, protein tyrosine kinase, and calcium/calmodulin-dependent protein kinase (Kobayashi *et al.*, 1989). Cells were pre-incubated for 2 h with inhibitor before the addition of TPA (Figure 7A) or serum (Figure 7B). Calphostin C inhibited both TPA- and serum-induced inclusion of syndecan-4 into focal contacts by 50% at 0.5 μ M and by 100% at 1.5 μ M. These results indicate that the inclusion of syndecan-4 into focal contacts occurs through the activation of PKC.

The Inclusion of Syndecan-4 into Focal Contacts Is Independent of Protein Synthesis and Occurs through Recruitment

The inclusion of syndecan-4 in focal contacts may occur by de novo protein synthesis and deposition or by recruitment of pre-existing proteoglycans from either the cell surface or from intracellular pools. To address the potential role of protein synthesis, cells were pre-treated with cycloheximide (12.5 μ g/ml) for

Figure 3. Inclusion of syndecan-4 into focal contacts and reorganization of actin stress fibers by serum. Quiescent CEFs were either not treated (A–D) or treated with 10% FBS/DMEM (E–H) for 2.5 h before fixation. Cells were co-stained for either syndecan-4 and vinculin (A, B, E, and F) or syndecan-4 and actin (C, D, G, and H). Arrowheads indicate clustering of syndecan-4 at focal contacts in quiescent cells (A and B), small arrows indicate localization of syndecan-4 at the ends of actin bundles (G and H), and large arrows indicate focal contacts from which syndecan-4 is absent (E and F). Open arrows indicate incorporation of syndecan-4 in the matrix. Note that in quiescent cells the majority of focal contacts do not contain syndecan-4. However, upon serum stimulation syndecan-4 becomes included within focal contacts.

2 h before TPA stimulation. Under these experimental conditions cycloheximide inhibits protein synthesis by at least 95% as measured by the incorporation of [35 S]methionine into TCA precipitable material. The pre-treatment of cells with cycloheximide did not significantly inhibit the inclusion of syndecan-4 into focal contacts (Figure 8). These results indicate that the TPA induction of syndecan-4 recruitment into focal contacts is either from pre-existing cell surface molecules or from intracellular pools.

Syndecan-4 Recruitment into Focal Contacts Coincides with Fibronectin Deposition

Cell surface HSPGs have been found in association with fibronectin at points of cell adhesion (Woods *et al.*, 1984) and more recently focal contacts have been characterized as sites of fibronectin assembly (Dzamba *et al.*, 1994). These observations, coupled with the localization of syndecan-4 to maturing focal contacts, suggest a possible relationship between syndecan-4 and fibronectin deposition. To determine if syndecan-4 is associated with fibronectin at focal contacts, we co-stained CEFs for syndecan-4 and avian fibronectin in proliferating, quiescent, and TPA-induced cells plated on human fibronectin. In proliferating CEFs, syndecan-4 co-localizes with avian fibronectin in focal contacts (Figure 9, A and B, large arrows) as well as with extracellular fibrils in cell tracks (Figure 9, A and B, open arrows). In serum-

starved cells neither syndecan-4 nor avian fibronectin were observed in subcellular structures resembling focal contacts (Figure 9, C and D). TPA stimulation of serum-starved CEFs induces the co-localization of syndecan-4 with fibronectin in subcellular structures similar to that observed in proliferating cells (Figure 9, E and F). These results suggest that the recruitment of syndecan-4 into focal contacts coincides with sites of fibronectin deposition. Whether syndecan-4 is directing the deposition of fibronectin or fibronectin is directing the recruitment of syndecan-4 is not known.

DISCUSSION

In the present study we characterized the potential mechanism regulating the association of syndecan-4 with focal contacts. Focal contacts were identified from the pattern of vinculin staining as described by Geiger (1979) and Burridge and Feramisco (1980). From these studies we conclude that syndecan-4 associates with mature or maturing focal contacts and that PKC activation can induce syndecan-4 localization to focal contacts. The observation that the response to PKC activation can take place in the absence of protein synthesis indicates that the inclusion of syndecan-4 in focal contacts is through an active recruitment process. In addition to its localization to focal contacts, syndecan-4 was observed to correlate with sites of fibronectin assembly and deposition into the matrix.

Figure 4. Time course of serum-induced inclusion of syndecan-4 in focal contacts. Quiescent cells were stimulated with 10% FBS/DMEM and then fixed and stained for syndecan-4 and vinculin at 0, 1.0, and 2.5 h. Cells were observed by epifluorescence at 400 $\times$. At least 100 cells were scored for the presence or absence of syndecan-4 in focal contacts for each time point. Error bars represent standard deviations; n = 3.

Focal contact formation has been suggested to occur in at least two discrete stages (Burridge *et al.*, 1992). Events related to the first stage can be mediated by integrin activation and clustering. These events include PKC and focal adhesion kinase activation

(Chang *et al.*, 1993; Vuori and Ruoslahti, 1993) as well as the aggregation of vinculin, talin, paxillin, tensin, filamin, and α -actinin at sites of cell matrix interaction (Miyamoto *et al.*, 1995). The second stage is dependent on the heparin binding domain of fibronectin and results in the formation of focal contacts as determined by interference reflection microscopy (Woods *et al.*, 1993). The mechanism by which the heparin binding domain of fibronectin mediates focal contact formation has been proposed to occur through the activation of PKC (Woods and Couchman, 1992). This proposal is based on the ability of TPA, and thus the activation of PKC, to overcome the dependence of focal contact formation on the heparin binding domain of fibronectin as well as on the ability of PKC inhibitors to mimic the effect of heparitinase I and III treatment in blocking focal contact formation (Woods and Couchman 1992; Woods *et al.*, 1993). The major HSPG identified in focal contacts, and thus implied to regulate HSPG-mediated PKC activation during cell adhesion, is syndecan-4 (Woods and Couchman, 1994). However, the observation that syndecan-4 is not localized to focal contacts during early stages of adhesion to fibronectin suggests that the direct association of syndecan-4 with focal contacts may not be required to influence their assembly or activation of PKC.

Whereas cell surface HSPG may be involved in the activation of PKC (Woods and Couchman, 1992), the current study has established that a result of PKC activation is the recruitment of syndecan-4 into focal contacts. If syndecan-4 is the HSPG responsible for PKC activation (Woods and Couchman, 1994), then the observation that PKC regulates the association of

Figure 5. TPA induction of syndecan-4 inclusion in focal contacts. (A) Quiescent CEFs were treated with either 0.1% DMSO as a solvent control or with 50, 100, or 150 ng/ml of TPA. They were then fixed, stained for syndecan-4 and vinculin after 2.5 h of treatment, and scored. (B) Quiescent CEFs were stimulated with 100 ng/ml of TPA for 0.5, 1.0, or 2.5 h. They were then fixed, stained for syndecan-4 and vinculin, and scored. Error bars represent standard deviations; n = 3.

Figure 6. Inclusion of syndecan-4 in focal contacts and reorganization of actin stress fibers by TPA. Quiescent CEFs were either treated with 0.1% DMSO (A–D) or 100 ng/ml TPA (E–H) for 2.5 h before fixing. Cells were co-stained for either syndecan-4 and vinculin (A, B, E, and F) or syndecan-4 and actin (C, D, G, and H). Arrowheads (A and B) indicate clustering of syndecan-4 at focal contacts in quiescent cells, small arrows (G and H) indicate localization of syndecan-4 at ends of actin bundles, and large arrows (E and F) indicate focal contacts from which syndecan-4 is absent. Note that in DMSO-treated cells the majority of focal contacts do not contain syndecan-4. However, upon TPA treatment syndecan-4 becomes included within focal contacts.

syndecan-4 with focal contacts implicates the existence of a positive feedback loop involving both inside-out and outside-in signaling pathways through PKC. The identification of a positive feedback loop involving PKC during HeLa cell spreading on gelatin supports this hypothesis (Chun and Jacobson, 1993).

The mechanism by which PKC regulates syndecan-4 recruitment is not known. The effect of PKC activation may ultimately result in the direct modification of the cytoplasmic domain of syndecan-4 by phosphorylation or by the modulation of cellular proteins with the cytoplasmic domain. To date, phosphorylation of pu-

tative phosphorylation sites in syndecan family members has not been demonstrated. Modulation of syndecan-4 by cellular proteins may occur by removing blocking proteins or by activating proteins that associate with the cytoplasmic domain. Association of the cytoplasmic domain with blocking proteins could occur in a manner analogous to that proposed for the cytoplasmic domains of α_1 integrin subunits, which block recruitment of β_1 and β_3 integrin subunits to focal contacts (LaFlamme *et al.*, 1992; Ylänne *et al.*, 1993). Whatever mechanisms regulate syndecan-4 recruitment into focal contacts, it is likely to involve the sequence KPIYK of the cytoplasmic domain. This sequence is homologous to the integrin β_1 subunit sequence NPIYK, which is required for focal contact localization (Reszka *et al.*, 1992).

The recruitment of syndecan-4 into focal contacts is likely to influence cell behavior through several mechanisms. The presence of heparan sulfate glycosaminoglycan chains on syndecan-4 in focal contacts may provide a molecular scaffold for matrix protein assembly and recruitment of other cell surface receptors and/or their ligands. The association of other cell surface receptors with syndecans in focal contacts has not been directly demonstrated, but the ability of the FGF receptor and NCAM to bind heparan sulfate (Kallapur and Akeson, 1992; McKeehan and Kan, 1994) suggests that these molecules are likely candidates for such interaction. Additionally, the ability of bFGF and heparin to form "a concerted

Figure 9. Co-localization of syndecan-4 with fibronectin in proliferating, quiescent, and TPA-induced cells. Proliferating (A and B), or quiescent cells treated with either DMSO (C and D) or 100 ng/ml of TPA (E and F) for 2.5 h, were fixed and co-stained for syndecan-4 and avian fibronectin. Open arrows indicate co-localization of syndecan-4 and avian fibronectin (A and B) deposited into the matrix. Large arrows indicate co-localization of syndecan-4 and fibronectin at cell borders (A, B, E, and F).

bridge mechanism" (Pantoliano *et al.*, 1994) for receptor binding and dimerization provides a secondary mechanism for the association of cell surface receptors with syndecans in focal contacts. The cytoplasmic domain may also be involved in the assembly of intracellular proteins associated with the cytoskeleton.

In cells plated in the presence of serum, 60–70% of the cells demonstrate pronounced syndecan-4 staining in focal contacts. In the remaining 30–40% of the cells, staining for syndecan-4 was not closely associated with focal contacts but resembled the distribution seen in serum-starved cells. The observation that a subpopulation of cells in a proliferating culture does not stain for syndecan-4 in focal contacts may reflect heterogeneity within the population of primary cells used in the studies.

The observation of syndecan-4 deposition into the matrix suggests that this may be the cell surface proteoglycan involved in fibronectin deposition (Woods *et al.*, 1984, 1988). Examination of fibronectin staining before and after TPA induction demonstrated that fibronectin co-localizes with syndecan-4 in focal contacts. These results suggest that the induction of syndecan-4 recruitment into focal contacts by TPA is associated with matrix deposition. However, syndecan-4

may not be the only member of the syndecan family associated with the assembly of fibronectin because syndecan-1 was also observed to be associated with endogenously synthesized fibronectin (Yamagata *et al.*, 1993). The role of syndecan-4 in matrix deposition is only correlative at the present time and studies are currently being conducted to more directly address the role of syndecan-4 in regulating cell surface binding and matrix deposition of fibronectin.

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REFERENCES

- Aviezer, D., Hecht, D., Safran, M., Eisinger, M., David, G., and Yayon, A. (1994). Perlecan, basal lamina proteoglycan, promotes basic fibroblast growth factor-receptor binding, mitogenesis, and angiogenesis. *Cell* 79, 1005–1013.
- Baciú, P.C., Acaster, C., and Goetinck, P.F. (1994). Molecular cloning and genomic organization of chicken syndecan-4. *J. Biol. Chem.* 269, 696–703.
- Bernfield, M., Kokenyesi, R., Kato, M., Hinkes, M.T., Spring, J., Gallo, R.L., and Lose, E.J. (1992). Biology of the syndecans: a family of transmembrane heparan sulfate proteoglycans. *Annu. Rev. Cell Biol.* 8, 365–393.
- Burridge, K., and Feramisco, J.R. (1980). Microinjection and localization of a 130K protein in living fibroblasts: a relationship to actin and fibronectin. *Cell* 19, 587–595.
- Burridge, K., Turner, C.E., and Romer, L.H. (1992). Tyrosine phosphorylation of paxillin and pp125^{FAK} accompanies cell adhesion to extracellular matrix: a role in cytoskeletal assembly. *J. Cell Biol.* 119, 893–903.
- Carey, D.J., Stahl, R.C., Cizmeci-Smith, G., and Asundi, V.K. (1994a). Syndecan-1 expressed in Schwann cells causes morphological transformation and cytoskeletal reorganization and associates with actin during cell spreading. *J. Cell Biol.* 124, 161–170.
- Carey, J.D., Stahl, R.C., Tucker, B., Bendt, K.A., and Cizmeci-Smith, G. (1994b). Aggregation-induced association of syndecan-1 with microfilaments mediated by the cytoplasmic domain. *Exp. Cell Res.* 214, 12–21.
- Chang, Z.-L., Beezhold, D.H., Personius, C.D., and Shen, Z.-L. (1993). Fibronectin cell-binding domain triggered transmembrane signal transduction in human monocytes. *J. Leukocyte Biol.* 53, 79–85.
- Chun, J.-S., and Jacobson, B.S. (1993). Requirement for diacylglycerol and protein kinase C in HeLa cell-substratum adhesion and their feedback amplification of arachidonic acid production for optimum cell spreading. *Mol. Biol. Cell* 4, 271–281.
- Couchman, J.R., Austria, R., Woods, A., and Hughes, R.C. (1988). Adhesion-defective BHK cell mutant has cell surface heparan sulfate proteoglycan of altered properties. *J. Cell. Physiol.* 136, 226–236.
- Dzamba, B.J., Bultmann, H., Akiyama, S.K., and Peter, D.M. (1994). Substrate-specific binding of the amino terminus of fibronectin to an integrin complex in focal adhesions. *J. Biol. Chem.* 269, 19646–19652.
- Elenius, K., and Jalkanen, M. (1994). Function of the syndecans: a family of cell surface proteoglycans. *J. Cell Sci.* 107, 2975–2982.
- Geiger, B. (1979). A 130K protein from chicken gizzard: its localization at the termini of microfilament bundles in cultured chicken cells. *Cell* 18, 193–205.
- Guimond, S., Maccarana, M., Olwin, B.B., Lindahl, U., and Rapraeger, A.C. (1993). Activating and inhibitory heparin sequences for FGF-2 (Basic FGF): distinct requirements for FGF-1, FGF-2 and FGF-4. *J. Biol. Chem.* 268, 23906–23914.
- Hansen, L.K., Mooney, D.J., Vacanti, J.P., and Ingber, D.E. (1994). Integrin binding and cell spreading on extracellular matrix act at different points in the cell cycle. *Mol. Biol. Cell* 5, 967–973.
- Jaken, S., Leach, K., and Klauck, T. (1989). Association of type 3 protein kinase C with focal contacts in rat embryo fibroblasts. *J. Cell Biol.* 109, 697–704.
- Johnson, G.R., and Wong, L. (1994). Heparan sulfate is essential to amphiregulin-induced mitogenic signaling by the epidermal growth factor receptor. *J. Biol. Chem.* 269, 27149–27154.
- Kallapur, S.G., and Akeson, R.A. (1992). The neural cell adhesion molecule (NCAM) heparin binding domain binds to cell surface proteoglycans. *J. Neurosci. Res.* 33, 538–548.
- Kobayashi, E., Nakano, H., Morimoto, M., and Tamaoki, T. (1989). Calphostin C (UCN-1028C), a novel microbial compound, is a highly potent and specific inhibitor of protein kinase C. *Biochem. Biophys. Res. Commun.* 159, 548–553.
- LaFlamme, S.E., Akiyama, S.K., and Yamada, K.M. (1992). Regulation of fibronectin receptor distribution. *J. Cell Biol.* 117, 437–447.
- LeBaron, R.G., Esko, J.D., Woods, A., Johansson, S., and Hook, M. (1988). Adhesion of glycosaminoglycan-deficient Chinese hamster ovary cell mutants to fibronectin substrata. *J. Cell. Biol.* 106, 945–952.
- Liebersbach, B.R., and Sanderson, R.D. (1994). Expression of syndecan-1 inhibits cell invasion into type I collagen. *J. Biol. Chem.* 269, 20013–20019.
- McKeehan, W.L., and Kan, M. (1994). Heparan sulfate fibroblast growth factor receptor complex: structure-function relationships. *Mol. Reprod. Dev.* 39, 69–82.
- Miyamoto, S., Akiyama, S.K., and Yamada, K.M. (1995). Synergistic roles for receptor occupancy and aggregation in integrin transmembrane function. *Science* 267, 883–885.
- Olwin, B.B., and Rapraeger, A. (1992). Repression of myogenic differentiation by aFGF, bFGF, and K-FGF is dependent on cellular heparan sulfate. *J. Cell. Biol.* 118, 631–639.
- Pantoliano, M.W., Horlick, R.A., Springer, B.A., Van Dik, D.E., Tobery, T., Wetmore, D.R., Lear, J.D., Nahapetian, A.T., Bradley, J.D., and Sisk, W.P. (1994). Multivalent ligand-receptor binding interactions in the fibroblast growth factor system produce a cooperative growth factor and heparin mechanism for receptor dimerization. *Biochemistry* 33, 10229–10248.
- Reszka, A.A., Hayashi, Y., and Horwitz, A.F. (1992). Identification of amino acid sequences in the integrin β 1 cytoplasmic domain implicated in cytoskeletal association. *J. Cell Biol.* 117, 1321–1330.
- Ridley, A.J., and Hall, A. (1992). The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* 70, 389–399.
- Sanderson, R.D., Sneed, T.B., Young, L.A., Sullivan, G.L., and Lander, A.D. (1992). Adhesion of B lymphoid (MPC-11) cells to type

- I collagen is mediated by the integral membrane proteoglycan, syndecan. *J. Immunol.* 148, 3902–3911.
- Thom, D., Powell, A.J., and Rees, D.A. (1979). Mechanisms of cellular adhesion. VI. Role of serum glycoproteins in fibroblast spreading on glass. *J. Cell Sci.* 35, 281–305.
- Vuori, K., and Ruoslahti, E. (1993). Activation of protein kinase C precedes $\alpha 5 \beta 1$ integrin-mediated cell spreading on fibronectin. *J. Biol. Chem.* 268, 21459–21462.
- Woods, A., and Couchman, J.R. (1992). Protein kinase C involvement in focal adhesion formation. *J. Cell Sci.* 101, 277–290.
- Woods, A., and Couchman, J.R. (1994). Syndecan-4 heparan sulfate proteoglycan is a selectively enriched and widespread focal adhesion component. *Mol. Biol. Cell* 5, 183–192.
- Woods, A., Couchman, J.R., Johansson, S., and Höök, M. (1986). Adhesion and cytoskeletal organization of fibroblasts in response to fibronectin fragments. *EMBO J.* 5, 665–670.
- Woods, A., Höök, M., Kjellén, L., Smith, C.G., and Rees, D.A. (1984). Relationship of heparan sulfate proteoglycans to the cytoskeleton and extracellular matrix of cultured fibroblasts. *J. Cell Biol.* 99, 1743–1753.
- Woods, A., Johansson, S., and Höök, M. (1988). Fibronectin fibril formation involves cell interactions with two fibronectin domains. *Exp. Cell Res.* 177, 272–283.
- Woods, A., McCarthy, J.B., Furcht, L.T., and Couchman, J.R. (1993). A synthetic peptide from the COOH-terminal heparin-binding domain of fibronectin promotes focal adhesion formation. *Mol. Biol. Cell* 4, 605–613.
- Yamagata, M., Saga, S., Kato, M., Bernfield, M., and Kimata, K. (1993). Selective distributions of proteoglycans and their ligands in pericellular matrix of cultured fibroblasts: implications for their roles in cell-substratum adhesion. *J. Cell Sci.* 106, 55–65.
- Ylänne, J., Chen, Y., O'Toole, T.E., Loftus, J.C., Takada, Y., and Ginsberg, M.H. (1993). Distinct functions of integrin α and β subunit cytoplasmic domains in cell spreading and formation of focal adhesions. *J. Cell Biol.* 122, 223–233.
- Yoneda J., Saiki, I., Igarashi, Y., Kobayashi, H., Fujii, H., Ishizaki, Y., Kimizuka, F., Kato, I., and Azuma, I. (1995). Role of the heparin-binding domain of chimeric peptides derived from fibronectin in cell spreading and motility. *Exp. Cell. Res.* 217, 169–179.