

Association of $\beta 1$ Integrin with Focal Adhesion Kinase and Paxillin in Differentiating Schwann Cells

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Schwann cells (SCs) differentiate into a myelinating cell when simultaneously adhering to an axon destined for myelination and basal lamina. We are interested in defining the signaling pathway activated by basal lamina. Using SC/sensory neuron (N) cocultures, we identified $\beta 1$ integrin and F-actin as components of a pathway leading to myelin gene expression and myelination (Fernandez-Valle et al., 1994, 1997). Here, we show that focal adhesion kinase (FAK) and paxillin are constitutively expressed by SCs contacting axons in the absence of basal lamina. Tyrosine phosphorylation of FAK and paxillin increases as SCs form basal lamina and differentiate. FAK and paxillin specifically coimmunoprecipitate with $\beta 1$ integrin in differentiating SC/N cocultures but not SC-only cultures. Paxillin coimmunoprecipitates with FAK and fyn kinase in differentiating SC/N cocultures. A subset of tyrosine-phosphorylated $\beta 1$ inte-

grin, FAK, and paxillin molecules reside in the insoluble, F-actin-rich fraction of differentiating cocultures. Cytochalasin D, an actin depolymerizing agent, decreases tyrosine phosphorylation of FAK and paxillin and their association with $\beta 1$ integrin and causes a dose-dependent increase in the abundance of insoluble FAK and paxillin complexes. Collectively, our work indicates that $\beta 1$ integrin, FAK, paxillin, and fyn kinase form an actin-associated complex in SCs adhering to basal lamina in the presence of axons. This complex may be important for initiating the process of SC differentiation into a myelinating cell.

Key words: Schwann cells; myelination; basal lamina; $\beta 1$ integrin; focal adhesion kinase; paxillin; tyrosine phosphorylation; signal transduction

Cell adhesion to extracellular matrix (ECM) regulates gene expression, motility, growth, differentiation, and survival of many cell types, including Schwann cells (SCs), the myelin-forming cell of the peripheral nervous system (Eldridge et al., 1987; Werb et al., 1989; Streuli et al., 1991; Fernandez-Valle et al., 1993; Lin and Bissell, 1993) (for review, see Lukashev and Werb, 1998). The biological effects of ECM are mediated in part by integrins, a large family of heterodimeric transmembrane receptors for ECM proteins (Hynes, 1992). Upon binding specific ligands, integrins cluster and stimulate tyrosine phosphorylation and recruitment of signaling and structural proteins to the plasma membrane at focal adhesions, cell-substratum contact sites (Clark and Brugge, 1995; Burridge and Chrzanowska-Wodnicka, 1996).

Focal adhesion kinase (FAK) and paxillin play critical roles in $\beta 1$ integrin-dependent signaling (for review, see Zachary and Rozengurt, 1992; Richardson and Parsons, 1995; Guan, 1997). FAK is an intracellular protein tyrosine kinase that rapidly autophosphorylates in response to $\beta 1$ integrin binding to ECM, growth factor receptor activation, and membrane depolarization (Burridge et al., 1992; Schaller et al., 1992; Siciliano et al., 1996). A direct $\beta 1$ integrin-FAK association is proposed based on results of *in vitro* peptide binding studies but has not been dem-

onstrated in cells (Schaller et al., 1995). FAK binds numerous signaling molecules, including Shc, Grb2, and src family kinases (Schlaepfer et al., 1999). FAK plays an essential role during development as FAK knock-out mice suffer lethal mesodermal defects (Ilic et al., 1995) (for review, see Ridyard and Sanders, 1999). Migration studies using FAK null cells suggest that FAK regulates focal adhesion turnover (Ilic et al., 1997). Focal adhesions also serve as nucleating sites for stress fibers. Disruption of actin polymerization with cytochalasin D (CD) inhibits FAK tyrosine phosphorylation but not its plasma membrane localization (Lipfert et al., 1992; Miyamoto et al., 1995). Paxillin is a 68 kDa adapter protein that localizes with $\beta 1$ integrin, FAK, vinculin, and src family kinases at focal adhesions (Bellis et al., 1995; Turner, 1998). It is phosphorylated after cell activation by ECM, growth factors, and neuropeptides, and during embryogenesis, metastasis, and wound repair (Turner, 1991; Mueller et al., 1992; Rozengurt, 1995; Chen et al., 1998). It contains multiple protein-protein binding motifs including Src homology 2 (SH2), SH3, four LD domain (leucine, aspartate rich), four double-zinc finger LIM domain [LIN-II, ISI-1, MEC-3 (homeodomain proteins)], and tyrosine and serine/threonine phosphorylation sites (Turner and Miller, 1994; Salgia et al., 1995). Paxillin binds FAK, vinculin, and a multitude of structural and signaling proteins and links integrin signaling with mitogen-activated protein kinase and c-Jun N-terminal protein kinase pathways (Turner and Miller, 1994; Brown et al., 1996; Tong et al., 1997).

SCs must synthesize and adhere to basal lamina to differentiate in response to axonal signals (Moya et al., 1980; Eldridge et al., 1987; Fernandez-Valle et al., 1993) (for review, see Bunge and Fernandez-Valle, 1995). In SC/sensory neuron (N) cocultures, SCs begin to assemble basal lamina immediately after ascorbate

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addition to the serum-containing medium and myelin 3–4 d later (Fernandez-Valle et al., 1998). Ascorbate is an essential cofactor for synthesis of triple helical collagen type IV, the scaffold for assembly of laminin–nidogen and heparan sulfate proteoglycan into basal lamina (Woodley et al., 1983; Aumailley et al., 1987). Purified laminin-1 induces SC myelination in the absence of ascorbate and is likely the “inducing” basal lamina component (Eldridge et al., 1989; Guenard et al., 1995). SCs express several laminin integrin receptors in a developmentally regulated manner. Undifferentiated and early differentiating SCs express $\alpha 1\beta 1$ and $\alpha 6\beta 1$ integrin, and myelinating SCs express $\alpha 6\beta 4$ integrin (Tawil et al., 1990; Einheber et al., 1993; Feltri et al., 1994; Niessen et al., 1994). $\beta 1$ integrin function-blocking antibody inhibits SC adhesion to nascent basal lamina and myelination in SC/N cocultures (Fernandez-Valle et al., 1994).

Previously, we found that low CD concentrations inhibit SC myelin gene expression and myelination, and we localized FAK to SC juxtamembrane regions at putative focal adhesions (Fernandez-Valle et al., 1997, 1998). Here, we provide evidence for formation of a $\beta 1$ integrin–FAK–paxillin–fyn kinase complex in SCs adhering to axons and basal lamina.

MATERIALS AND METHODS

Antibodies

Rabbit $\beta 1$ integrin polyclonal antibody was a gift from S. Carbonetto (McGill University, Montreal, Canada) (Tawil et al., 1990, 1993), and paxillin 165 antibody was a gift from Chris Turner (State University of New York, Syracuse, NY). Hamster $\beta 1$ integrin monoclonal antibody (catalog #09871D; PharMingen, San Diego, CA), FAK monoclonal antibody (catalog #F15020; Upstate Biotechnology, Lake Placid, NY), phosphotyrosine (TY-P) antibody (ICN, Costa Mesa, CA), Fyn kinase polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), paxillin and phosphotyrosine antibody conjugated to horseradish peroxidase (HRP) (Transduction Laboratories, Lexington, KY), normal rabbit serum (Sigma, St. Louis, MO), normal hamster IgG1 (PharMingen), normal mouse IgG1 (Sigma), and goat anti-mouse and goat anti-rabbit conjugated to HRP (Promega, Madison, WI) were purchased.

Tissue culture

Primary Schwann cell cultures. The Brookes method (Brookes et al., 1979) was used to isolate SCs from sciatic nerves of embryonic day 21 or newborn Sprague Dawley rats (Harlan Sprague Dawley, Indianapolis, IN). Briefly, sciatic nerves were removed and stripped of connective tissues. After collagenase (Worthington, Lakewood, NJ) and trypsin (Life Technologies, Rockville, MD) dissociation, isolated cells were placed on a 60 mm tissue culture dish (Corning, Corning, NY) in D10 medium consisting of DMEM containing 10% heat-inactivated fetal bovine serum (FBS) (HyClone, Logan, UT). On the following day, 10^{-5} M cytosine arabinoside (Sigma) was added for 5 d to eliminate fibroblasts. Residual fibroblasts were lysed by treatment with Thy 1.1 antibody (103-T1B; American Type Culture Collection, Manassas, VA), followed by guinea pig complement (Life Technologies). SCs were expanded in D10 plus 2 μ M forskolin (Sigma) and 20 μ g/ml pituitary extract (Bio-medical Technologies, Stoughton, MA) on poly-L-lysine-coated (200 μ g/ml; Sigma) 100 mm tissue culture dishes. SC cultures were passaged no more than four times before plating SCs onto sensory neuron cultures.

Sensory neuron cultures. Neurons were isolated from cervical dorsal root ganglia of Sprague Dawley rat embryos at 16 d of gestation by dissociation with trypsin. Cells were plated on poly-L-lysine- and laminin-coated (5 μ g/coverlip; Life Technologies) 11 mm German glass coverslips (Carolina, Burlington, NC) at a density of 0.8–1 ganglion per coverslip. The growth medium was Eagle’s MEM (EMEM) containing 5% human placental serum (a generous gift from Dr. R. Devon, University of Saskatchewan, Saskatchewan, Canada), 50 ng/ml nerve growth factor (a generous gift from Dr. P. Wood, University of Miami, Miami, FL), and 400 mM glucose. Non-neuronal cells were eliminated using one pulse of anti-mitotics (uridine–fluorodeoxyuridine, 10^{-5} M; Sigma). Purified neuron cultures were maintained in CB medium (EMEM containing 5% FBS, 50 ng/ml NGF, and 0.4% glucose) for 7–10 d before seeding

with SCs. Additional details of the culture procedure are provided by Kleitman et al. (1991).

SC/neuron cocultures. SCs were removed from culture dishes using trypsin, washed extensively in L-15 containing 10% serum. Approximately 100,000 SCs were seeded onto purified neuron cultures in CB medium and were maintained for 1 week to allow additional SC proliferation in response to axonal mitogens (Wood and Bunge, 1975; Marchionni et al., 1993; Morrissey et al., 1995). To initiate differentiation into myelin-forming cells, cultures were switched from CB (serum-only) to M (serum plus ascorbate) medium (EMEM containing 15% FBS, 50 ng/ml NGF, 50 μ g/ml ascorbate, and 0.4% glucose) and were grown for 4–21 d to allow myelination. Other cultures were maintained in CB medium as undifferentiated cultures.

Cytochalasin D treatment. CD-containing medium was prepared from a stock solution of 0.5 mg/ml in DMSO. Cultures were switched to M medium containing 0.25, 0.50, or 0.75 μ g/ml CD, or were maintained in CB. Some cultures were switched to M/D medium (M plus 0.075% DMSO). Cultures were fed every other day for 7–8 d before extraction.

Sudan black staining and quantitation

SC/N cultures were grown in serum-only medium for 21 d or in serum-plus ascorbate medium for 4, 7, 14, and 21 d. Cultures were fixed for 10 min in 4% paraformaldehyde in 0.1 M sodium phosphate buffer, rinsed several times in buffer, and osmicated in 1% osmium tetroxide–phosphate buffer for an additional 1 hr. Cultures were dehydrated in 25, 50, and 70% ethanol for 5 min each and stained with 0.5% Sudan black in 70% ethanol for 1 hr. Cultures were destained for 1–2 min in 70% ethanol, rehydrated in 50 and 25% ethanol followed by phosphate buffer, and then mounted in glycerin jelly. Myelin counts were determined at 400 \times magnification by sampling the same 20 coordinates in each culture and counting the number of myelin segments within a defined grid. The counts were adjusted to reflect the number of myelin segments in the total area of the coverslip. The adjusted numbers are an underrepresentation of the total myelin segments because of difficulty in accurately counting densely myelinated cultures. One culture at each time point from three separate experiments was quantitated, and the mean and SD are provided.

Cell extraction and immunoprecipitation

Cultures were rinsed three times on ice with PBS, pH 7.4 (Life Technologies). Cultures were extracted on ice for 30 min with cold TAN buffer consisting of 10 mM Tris-acetate, pH 8.0, 1.0% NP-40, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM *n*-ethylmaleimide, 2 μ g/ml aprotinin, 1 μ g/ml leupeptin, and 1 mM sodium orthovanadate (Sigma). After scraping, pooled cultures were sonicated for 15 min in an ice-water bath and centrifuged for 15 min at 14,000 rpm at 4°C. Protein concentrations were determined using DC protein assay kit (Bio-Rad, Hercules, CA). For immunoprecipitation, 100–400 μ g of total cell extracts were immunoprecipitated with normal mouse IgG1, hamster IgG1, rabbit IgG (1–2 μ g of antibody/100 μ g of protein), or preimmune rabbit serum for 2 hr, followed by an overnight incubation with protein A-Sepharose or protein G-agarose. The resulting supernatant was incubated with the indicated specific antibodies and were collected as above. Immune complexes were washed twice in TAN extraction buffer, centrifuged through a 60% sucrose cushion, and washed sequentially in solution A (10 mM Tris, pH 7.0, 1 mM EDTA, 0.5% Triton X-100, and 1 M NaCl), solution B (10 mM Tris, pH 7.0, 1 mM EDTA, 0.5% Triton X-100, 0.2 M NaCl, and 0.1% SDS), and solution C (10 mM Tris, pH 7.0, 1 mM EDTA, and 0.5% Triton X-100). Samples were solubilized in 2 \times SDS sample buffer containing 5% β -mercaptoethanol.

The soluble culture fractions were obtained by extraction in 100 μ l/coculture of CSK buffer (10 mM PIPES, pH 6.8, 0.5% Triton X-100, 50 mM NaCl, 300 mM sucrose, 2 mM MgCl₂, 2 μ g/ml aprotinin, 1 μ g/ml leupeptin, and 1 mM PMSF), as described by Plopper and Ingber (1993). The remaining insoluble fraction was extracted in 100 μ l/culture of TAN buffer. The extracts from 10–20 cultures were pooled and used for Western blot analysis and immunoprecipitations.

Gel electrophoresis and Western blotting

Immunoprecipitated samples were boiled at 100°C for 10 min. Equal protein was electrophoresed on 7.5 or 10% SDS-PAGE gels and transferred to nitrocellulose membranes. Membranes were stained with India Ink (1:1000) in TBS-T (20 mM Tris, pH 7.6, 137 mM NaCl, and 0.1% Tween 20) for 30 min to visualize protein bands. Membranes were blocked in TBS-T containing 5% dried milk, except membranes blotted

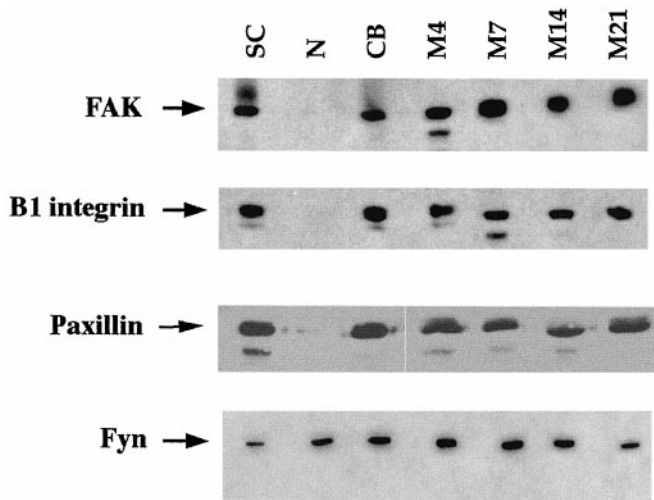


Figure 1. FAK, paxillin, and fyn kinase are constitutively expressed by Schwann cells. Western blot analysis of total protein from extracts of mitogen-stimulated SCs cultures, N cultures, undifferentiated SC/N cocultures grown in CB medium, and differentiating SC/N cocultures grown in M medium for the indicated days. Undifferentiated cocultures grown in CB medium do not contain basal lamina or myelin, but differentiating cocultures grown in M medium contain basal lamina and myelin. Thirty micrograms of protein was used to detect FAK, $\beta 1$ integrin, and fyn kinase, and 5 μ g of protein were used to detect paxillin. The bands were visualized using chemiluminescence. The result shows that equivalent amounts of FAK, $\beta 1$ integrin, paxillin, and fyn kinase are expressed by SCs under all conditions. Neurons, relative to SCs, express very low levels of $\beta 1$ integrin, FAK, and paxillin but substantial amounts of fyn kinase.

for phosphotyrosine were blocked in TBS-T containing 4% bovine serum albumin and 1% chicken ovalbumin. After blocking for 1–2 hr, the membranes were rinsed and incubated with primary antibody in TBS-T for 1 hr at the following dilutions: paxillin 1:10,000, FAK 1:500 or 1:1000, $\beta 1$ integrin 1:1000, and Fyn kinase 1:500. After incubation in primary antibody, membranes were rinsed in TBS-T and incubated with HRP-conjugated goat anti-mouse or goat anti-rabbit (Promega) at 1:15,000 antibody for 30 min. Membranes were rinsed in TBS-T and incubated with ECL reagents (Pierce, Rockford, IL). Controls for Western blotting consisted of identical lanes that were reacted with HRP-conjugated secondary antibody only.

RESULTS

SCs express FAK and paxillin in the absence of axon and basal lamina contact

Total protein extracts were prepared from primary cultures of SCs, N, and SC/N cocultures grown in serum-containing but ascorbate-lacking (CB) medium that does not support basal lamina formation or myelination, and serum plus ascorbate (M) medium that promotes basal lamina and myelin formation for 4–28 d. Western blot analysis was performed using equal protein for each culture type (Fig. 1). The expression pattern for FAK, paxillin, and $\beta 1$ integrin is very similar. The three proteins are expressed in all SC-containing cultures, and the level of expression does not vary substantially during SC differentiation. The abundance of paxillin in SCs is much greater than FAK or $\beta 1$ integrin because one-sixth as much protein was used in paxillin Western blots. Neuron-only cultures express negligible amounts of paxillin, FAK, and $\beta 1$ integrin compared with SCs. Fyn kinase is expressed in neurons, as well as in SCs, and its expression level does not change during SC differentiation. These results demonstrate that SCs do not require axon or basal lamina contact to synthesize FAK, paxillin, or $\beta 1$ integrin.

Representative cocultures were stained with Sudan black, and

Table 1. Quantitation of myelin segments in undifferentiated (CB) and differentiating (M) SC/N cocultures ($\times 10^3$)

CB	M4	M7	M14	M21
0	0.04 \pm 0	5.8 \pm 3	16.2 \pm 3	27.7 \pm 3

Values were adjusted to reflect the total number of myelin segments per coculture.

the number of myelin segments was counted to determine the number of terminally differentiated SCs (Table 1). Myelin abundance in differentiating SC/N cocultures grown in serum plus ascorbate medium increases over 700-fold during the time course. SCs grown with neurons in serum-only medium remain rounded and form few myelin segments.

FAK and paxillin are tyrosine-phosphorylated in response to basal lamina formation

Western blot analysis was performed using phosphotyrosine-HRP-conjugated antibody to identify the major tyrosine-phosphorylated proteins expressed by differentiating SCs (Fig. 2*A*, top panel). Two major bands are observed migrating at ~ 125 and 70 kDa in mitogen-stimulated SC cultures and differentiating SC/N cocultures. The 125 kDa band is also present in neuron-only cultures and undifferentiated SC/N cocultures, but its abundance is substantially lower in undifferentiated SC/N cocultures compared with differentiating SC/N cocultures. The 70 kDa band is not observed in neuron-only cultures or undifferentiated SC/N cocultures.

To determine whether these bands corresponded to FAK and paxillin, culture extracts were immunoprecipitated with FAK and paxillin antibody and were immunoblotted with anti-phosphotyrosine-HRP. Phosphorylated FAK is present in mitogen-stimulated SCs and in differentiating SC/N cocultures and is occasionally detected at very low levels in undifferentiated SC/N cocultures (Fig. 2*A*, middle panel). The abundance of phosphorylated FAK increases with time of differentiation up to 14 d and then appears to decrease. Similar results are obtained when phosphotyrosine immunoprecipitates are immunoblotted for FAK, except that FAK phosphorylation level remains high at 21 d (Fig. 2*A*, bottom panel). Phosphorylated paxillin is present in cultures of mitogen-stimulated SCs and increases with time in differentiating SC/N cocultures. Phosphorylated paxillin is not detected in neuron-only cultures or 14-d-old undifferentiated SC/N cocultures (Fig. 2*B*). This suggests that paxillin phosphorylation decreases in SCs contacting axons, because at the time of seeding onto neuron cultures, the mitogen-stimulated SCs contain phosphorylated paxillin. To confirm this observation, 7- and 21-d-old undifferentiated SC/N cocultures were extracted, and equal amounts of paxillin immunoprecipitates were immunoblotted for paxillin and phosphotyrosine. The level of paxillin tyrosine phosphorylation decreases with time of coculture (Fig. 2*C*). The results suggest that FAK and paxillin tyrosine phosphorylation is stimulated by basal lamina and coincides with the onset of SC differentiation.

Tyrosine-phosphorylated FAK, paxillin, and $\beta 1$ integrin localize to the cytoskeleton in differentiating SCs

Studies were conducted to determine the distribution of $\beta 1$ integrin, FAK, and paxillin in differentiating SCs. Soluble and insoluble fractions of 10-d-old differentiating SC/N cocultures were prepared and used in Western blot analysis. $\beta 1$ integrin and FAK are present in both the soluble and insoluble fractions (Fig. 3*A*). FAK is evenly distributed between both fractions, whereas the

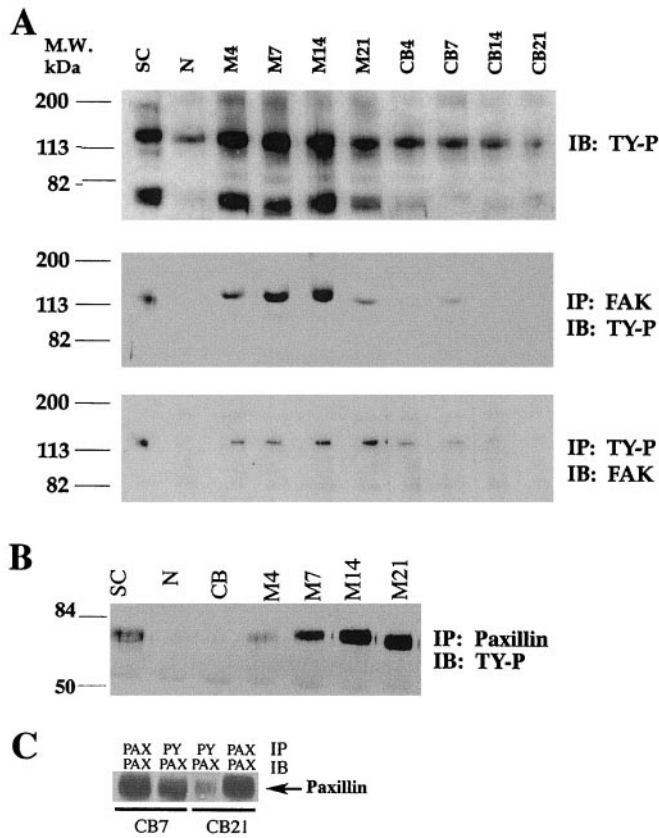


Figure 2. Tyrosine phosphorylation of FAK and paxillin is elevated in differentiating SC/N cocultures. *A, Top panel*, Thirty micrograms of total protein extracts from cultures of SC, N, and SC/N cocultures grown in CB or M medium for 4–21 d were subjected to 7.5% SDS-PAGE and were immunoblotted (IB) with HRP-conjugated TY-P. *Middle panel*, One hundred micrograms of total protein from the identical extracts were immunoprecipitated (IP) with 1 μ g of FAK antibody and were immunoblotted with HRP-conjugated TY-P antibody. *Bottom panel*, One hundred micrograms of the identical extracts were immunoprecipitated with 1 μ g of TY-P antibody and were immunoblotted with FAK antibody. *B*, Five micrograms of paxillin immunoprecipitates from SC-only (SC), neuron-only (N), 14-d-old undifferentiated (CB), and 4- to 21-d-old differentiating (M) SC/N coculture extracts were immunoblotted with HRP-conjugated TY-P antibody. *C*, Five micrograms of paxillin immunoprecipitates from 7- and 21-d-old undifferentiated cultures were immunoblotted with paxillin and TY-P antibody. The results show that FAK and paxillin become increasingly tyrosine-phosphorylated with time as increasing numbers of SCs synthesize basal lamina and myelin. The experiments were repeated a minimum of three times.

abundance of soluble $\beta 1$ integrin is $\sim 120\%$ greater than insoluble $\beta 1$ integrin. Soluble and insoluble fractions of 10-d-old differentiating SC/N cocultures were immunoprecipitated with phosphotyrosine antibody and were immunoblotted for $\beta 1$ integrin and FAK. $\beta 1$ integrin is not detected in phosphotyrosine immunoprecipitates from the soluble fraction but is present in phosphotyrosine immunoprecipitates from the insoluble fraction (Fig. 3B). $\beta 1$ integrin is present in the post-immunoprecipitated insoluble and soluble fractions. Therefore, only a small fraction of $\beta 1$ integrin molecules is confined to the insoluble compartment of differentiating SC/N cocultures. Tyrosine-phosphorylated FAK is present in both the insoluble and soluble fractions; however, the insoluble fraction contains 1.5- to 2-fold more FAK than the soluble fraction (Fig. 3C). Control immunoprecipitations with

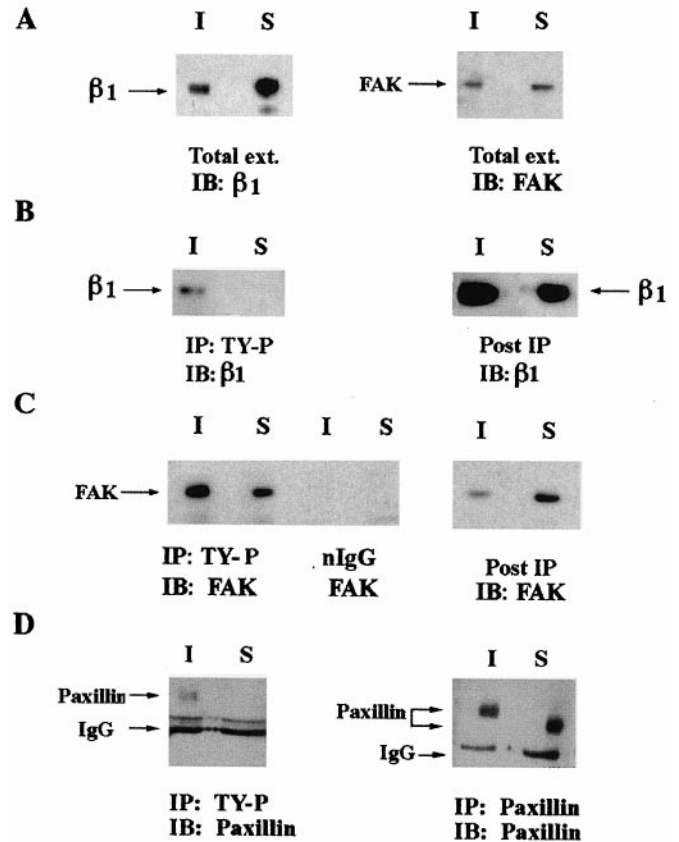


Figure 3. Tyrosine-phosphorylated $\beta 1$ integrin, FAK, and paxillin colocalize in the insoluble compartment of differentiating SC/N cocultures. *A*, Thirty micrograms of soluble (S) and insoluble (I) fractions of 10-d-old differentiating SC/N cocultures were immunoblotted (IB) with $\beta 1$ integrin antibody (left) and FAK antibody (right). *B*, Four hundred micrograms of soluble and insoluble fractions of 14-d-old differentiating SC/N cocultures were immunoprecipitated (IP) with anti-TY-P antibody and were immunoblotted (IB) with $\beta 1$ integrin antibody (left). Thirty micrograms of the post-immunoprecipitated extract were immunoblotted with $\beta 1$ integrin antibody (right). *C*, Four hundred micrograms of soluble (S) and insoluble (I) extracts of 14-d-old differentiating SC/N cocultures were immunoprecipitated with phosphotyrosine antibody and normal IgG and were immunoblotted with FAK antibody (left). Thirty micrograms of the post-immunoprecipitated extracts were immunoblotted for FAK (right). *D*, The soluble (S) and insoluble (I) fractions of 14-d-old differentiating SC/N cocultures were immunoprecipitated with paxillin and phosphotyrosine antibodies. Twenty micrograms of phosphotyrosine immunoprecipitate was immunoblotted with paxillin antibody (left). Five micrograms of paxillin immunoprecipitates (IP) were immunoblotted (IB) with paxillin antibody (right). The results indicate the tyrosine-phosphorylated $\beta 1$ integrin, FAK, and paxillin are bound to the cytoskeleton in differentiating SC/N cocultures.

normal IgG do not contain FAK. After immunoprecipitation, the remaining insoluble and soluble fractions were immunoblotted for FAK. The insoluble fraction is nearly depleted of FAK by one round of phosphotyrosine immunoprecipitation, whereas FAK is abundant in the soluble fraction and is most likely unphosphorylated. Tyrosine-phosphorylated paxillin is observed in the insoluble, but not the soluble, fraction of differentiating SC/N cocultures (Fig. 3D). Paxillin is present in both fractions, and tyrosine-phosphorylated insoluble paxillin migrates more slowly than soluble paxillin lacking tyrosine phosphorylation (Fig. 3D). Undifferentiated SC/N cocultures were not examined because FAK is not tyrosine-phosphorylated in this culture condition (Fig. 2B,C, CB lanes).

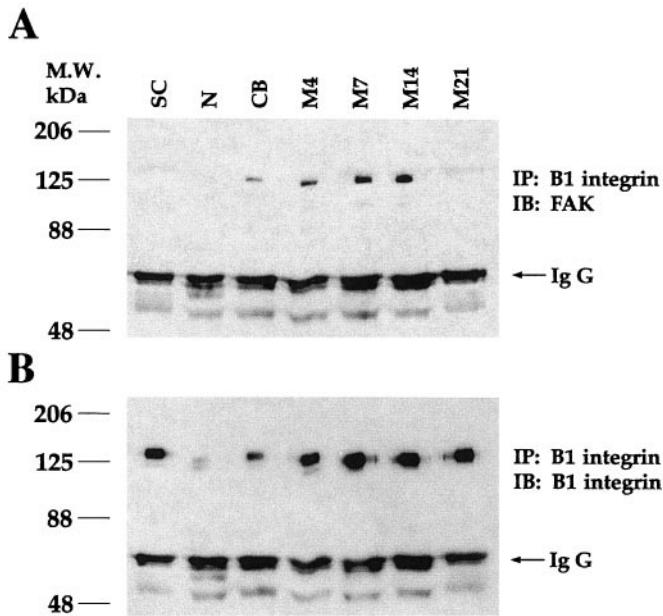


Figure 4. $\beta 1$ integrin antibody coimmunoprecipitates FAK. Two hundred micrograms of total protein extracts of SC, N, and undifferentiated SC/N cocultures grown for 14 d in CB medium and differentiating SC/N cocultures grown in M medium for 4–21 d (M4–M21) were immunoprecipitated (IP) with 1 μ g of hamster $\beta 1$ integrin antibody. The immunoprecipitates were divided in half, and 100 μ g of each sample was loaded onto two gels. The samples were resolved by 7.5% SDS-PAGE. $\beta 1$ immunoprecipitates were immunoblotted (IB) with FAK antibody in *A* and mouse $\beta 1$ integrin antibody in *B*. IgG from each sample is depicted by an arrow and shows equal loading in each lane. The results show that $\beta 1$ integrin and FAK coimmunoprecipitate in SC/N cocultures. This is a representative result of three similar experiments.

$\beta 1$ integrin antibody coimmunoprecipitates FAK from differentiating SC/N cocultures

Coimmunoprecipitation experiments were performed to determine whether FAK and $\beta 1$ integrin associated during SC differentiation. FAK is not detected in $\beta 1$ integrin immunoprecipitates from SC-only or neuron-only cultures (Fig. 4*A*). A small amount of FAK is detected in $\beta 1$ integrin immunoprecipitates from undifferentiated 14-d-old SC/N cocultures (CB). Increasing amounts of FAK are detected in $\beta 1$ integrin immunoprecipitates from 4-, 7-, and 14-d-old differentiating SC/N cocultures (M4–M14) grown in ascorbate-containing medium. FAK is not detected in the $\beta 1$ integrin immunoprecipitates from 21-d-old differentiating SC/N cocultures or when normal hamster IgG or protein A-Sepharose beads alone are used for immunoprecipitation (data not shown). Aliquots of the identical $\beta 1$ integrin immunoprecipitates were run simultaneously on a separate gel and were immunoblotted with anti- $\beta 1$ integrin (Fig. 4*B*). Equal loading of all samples is seen in the equivalent levels of IgG present in all lanes in both gels. Although both $\beta 1$ integrin and FAK are expressed in SC-only cultures (Fig. 1), FAK does not coimmunoprecipitate with $\beta 1$ integrin. This suggests that an FAK and $\beta 1$ integrin complex forms as SCs contact axons and becomes more abundant as SCs assemble basal lamina.

To determine whether $\beta 1$ integrin-associated FAK was tyrosine-phosphorylated, duplicate samples of extracts from 7-d-old differentiating SC/N cocultures were immunoprecipitated with a rabbit $\beta 1$ integrin antibody and were subjected to FAK and phosphotyrosine Western blot analysis. FAK is present in $\beta 1$

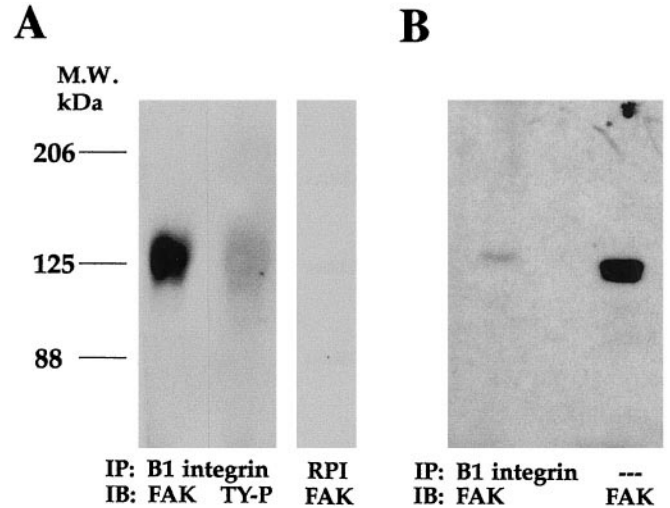


Figure 5. $\beta 1$ integrin-associated FAK is tyrosine-phosphorylated. *A*, Total extracts of differentiating SC/N cocultures (M7) were immunoprecipitated (IP) with rabbit $\beta 1$ integrin antiserum, were separated on 6% SDS-PAGE, and were immunoblotted (IB) with FAK antibody or HRP-conjugated TY-P antibody. RPI, Normal rabbit preimmune serum was used as a control. *B*, Rabbit $\beta 1$ integrin immunoprecipitates from 200 μ g of total extracts of 7-d-old differentiating (M7) SC/N cocultures (left lane) and 20 μ g of the identical total extract (right lane) were separated on 7.5% SDS-PAGE and were immunoblotted with FAK antibody. The results suggest that phosphorylated FAK associates with $\beta 1$ integrin and that only a very small fraction of FAK molecules associate with $\beta 1$ integrin.

integrin immunoprecipitates, as is an identically migrating phosphotyrosine-containing protein (Fig. 5*A*). Immunoprecipitations performed with normal rabbit serum do not contain FAK. This suggests that tyrosine-phosphorylated FAK and $\beta 1$ integrin associate. A measure of the extent to which FAK molecules associate with $\beta 1$ integrins was obtained by comparing the amount of FAK present in 20 μ g of total protein extract with the amount present in a $\beta 1$ integrin immunoprecipitation from 200 μ g of total protein. FAK Western blot analysis demonstrates that much less FAK is present in the $\beta 1$ integrin immunoprecipitate than in the total extract of 7-d-old differentiating SC/N cocultures (Fig. 5*B*). Quantitation of the blot and correction for protein loading suggests that $\sim 4\%$ of FAK molecules in the differentiating SC/N coculture at this time point associate with $\beta 1$ integrin.

Paxillin associates with $\beta 1$ integrin, FAK, and fyn kinase in differentiating SC/N cocultures

Reciprocal coimmunoprecipitation studies were performed to determine whether paxillin associated with $\beta 1$ integrin and to identify other paxillin-associated proteins in SC-only and SC/N coculture extracts. Paxillin is detected in large amounts in $\beta 1$ integrin immunoprecipitates of SC-only cultures, whether grown in the presence or absence of mitogens (Fig. 6*A*). The abundance of $\beta 1$ integrin-associated paxillin decreases dramatically when SCs are cocultured with axons in the absence of ascorbate but increases if SCs are allowed to differentiate for 10 d in ascorbate-containing medium (Fig. 6*A*). In the reciprocal coimmunoprecipitation using paxillin antibody, $\beta 1$ integrin is detected only in SCs grown in the absence of mitogens (Fig. 6*B*). FAK and paxillin reciprocally coimmunoprecipitate from SC-only cultures and differentiating SC/N cocultures but are not detected in undifferentiated SC/N cocultures (Fig. 6*C,D*). Paxillin and fyn kinase also reciprocally coimmunoprecipitate from SC-only and differentiat-

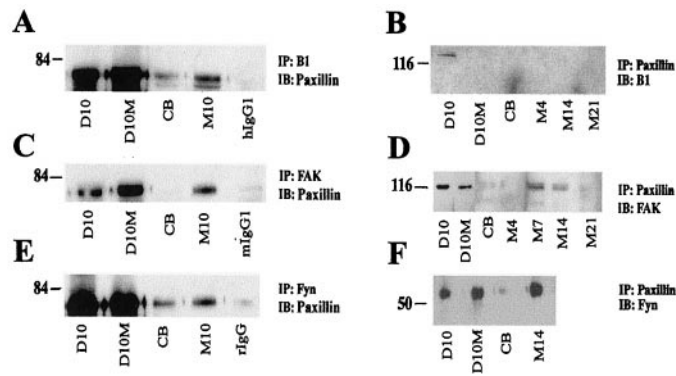


Figure 6. Paxillin interacts with β 1, FAK, and fyn kinase. Total protein extracts were prepared from SC cultures grown in the absence (*D10*) and presence (*D10M*) of mitogens, and undifferentiated (*CB*) and differentiating (*M*) SC/N cocultures grown for the indicated days. *A*, *C*, *E*, Equal amounts of protein from each culture type were immunoprecipitated (*IP*) with the indicated antibody and were immunoblotted (*IB*) with paxillin antibody. Controls include isotype-matched antibody for mouse (*mIgG*), hamster (*hIgG*), and rabbit (*rIgG*) antibodies. *B*, Equal amounts of total protein extracts from the various culture paradigms were immunoprecipitated (*IP*) with paxillin antibody and were immunoblotted (*IB*) with the indicated antibody. The results indicate that, in SCs cultured alone, paxillin interacts with β 1 integrin, FAK, and fyn kinase. In SCs cocultured with neurons, the association of paxillin with β 1 integrin, FAK, and fyn kinase is higher in cocultures containing basal lamina than in cocultures lacking basal lamina.

ing SC/N cocultures (Fig. 6*E,F*). Lesser amounts of paxillin–fyn complexes are observed in undifferentiated SC/N cocultures.

Cytochalasin D inhibits FAK and paxillin tyrosine phosphorylation and interaction with β 1 integrin

CD disrupts actin polymerization and inhibits FAK tyrosine phosphorylation and focal adhesion formation in other cells (Lipfert et al., 1992). When added to SC/N cocultures, CD inhibits myelin-specific gene expression and SC differentiation into myelinating cells (Fernandez-Valle et al., 1997). We investigated whether the inhibitory effect of 0.5 μ g/ml CD on SC differentiation was attributable to disruption of β 1 integrin interaction with FAK and paxillin. SC/N cocultures grown for 10 d in the absence of ascorbate and in ascorbate-containing medium with and without CD were extracted and immunoprecipitated with β 1 integrin and phosphotyrosine antibodies and were immunoblotted for FAK and paxillin. FAK and paxillin are present in β 1 immunoprecipitates of differentiating SC/N cocultures but are not detected in β 1 integrin immunoprecipitates from undifferentiated and CD-treated SC/N cocultures (Fig. 7*A*). Exposure to CD resulted in a significant decrease in phosphorylated FAK and paxillin compared with the levels present in undifferentiated and differentiating SC/N cocultures (Fig. 7*B*). The band below FAK is a nonspecific band recognized by the HRP-conjugated secondary antibody.

We also examined FAK and paxillin interactions in CD-treated SC/N cocultures. Extracts were prepared from soluble and insoluble fractions of undifferentiated, differentiating, and CD-treated cocultures and were immunoprecipitated for FAK and immunoblotted for paxillin (Fig. 8). Increasing amounts of paxillin coimmunoprecipitates with FAK as the CD concentration increases. The FAK–paxillin complex is found in the insoluble, actin-rich fraction of CD-treated cultures.

DISCUSSION

We use a well characterized *in vitro* myelination model to examine the possibility that FAK participates in signaling from β 1 integrins after basal lamina adhesion. We provide evidence for formation of a β 1 integrin–FAK–paxillin–fyn kinase complex and for FAK and paxillin tyrosine phosphorylation in differentiating SC/N cocultures. Moreover, we demonstrate that CD, shown previously to inhibit SC differentiation, inhibits β 1 integrin association with FAK and paxillin and tyrosine phosphorylation of both proteins. These findings support the role of FAK and paxillin as intermediates in β 1 integrin-dependent signaling in SCs adhering to basal lamina and axons.

FAK and paxillin are tyrosine-phosphorylated in differentiating SC/N cocultures

FAK and paxillin are constitutively expressed by isolated SCs (Fig. 1). Therefore SCs do not require axon or basal lamina contact to synthesize FAK and paxillin. The expression pattern for FAK, paxillin, and β 1 integrin are similar in SCs. Sensory neurons express β 1 integrin, FAK, and paxillin at extremely low levels compared with SCs. Only fyn kinase is expressed by sensory neurons at comparable levels to SCs. Previous immunogold labeling studies indicate that sensory neurons do not increase β 1 integrin and FAK expression after SC contact (Fernandez-Valle et al., 1994, 1998). Retinal and hippocampal neurons express FAK and proline-rich tyrosine kinase 2 (PYK2), an FAK family member, but the expression levels have not been compared with glial cells, as was done here (Burgaya et al., 1995; Grant et al., 1995).

There is a time-dependent increase in FAK and paxillin tyrosine phosphorylation in cocultures containing basal lamina until days 14–21. This is evident by comparing the levels of phosphorylated FAK and paxillin in *CB* lanes that represent cocultures lacking basal lamina with *M4–M21* lanes that represent cocultures containing basal lamina (Fig. 2). Because cocultures are maintained for 7 d in ascorbate-free medium to allow SC proliferation in response to axons, the basal FAK phosphorylation level at the time ascorbate is added is shown in *CB7* lanes. SCs begin to assemble basal lamina immediately after ascorbate supplementation and several days before initiating myelination, which increases slowly with time (Fernandez-Valle et al., 1998). The apparent decrease in FAK tyrosine phosphorylation at 21 d is consistent with the absence of FAK in β 1 integrin immunoprecipitations at 21 d shown in Figure 4*A*. The number of mature, myelinating SCs is likely greater than the number of newly differentiating SCs in 21-d-old cultures. Therefore, FAK and paxillin tyrosine phosphorylation begins during the time of rapid basal lamina formation and the onset of differentiation and may be involved in triggering differentiation rather than during active myelination or in maintenance of the myelinating phenotype.

FAK and paxillin are also phosphorylated in SCs grown in the presence of forskolin and pituitary extract used to stimulate SC proliferation before seeding on neuron cultures. Mitogens induce FAK and paxillin tyrosine phosphorylation in other cells (Rankin and Rozengurt, 1994) (for review, see Rozengurt, 1995). FAK is dephosphorylated upon SC contact with axons because very little FAK remains phosphorylated 4 d after seeding onto neurons (Fig. 2*A*, *CB4*). Paxillin appears to be slowly dephosphorylated because a small amount of phosphorylated paxillin is present in phosphotyrosine immunoprecipitates from 21-d-old cocultures lacking basal lamina (Fig. 2*C*). These results demonstrate that paxillin tyrosine phosphorylation increases with time in cocul-

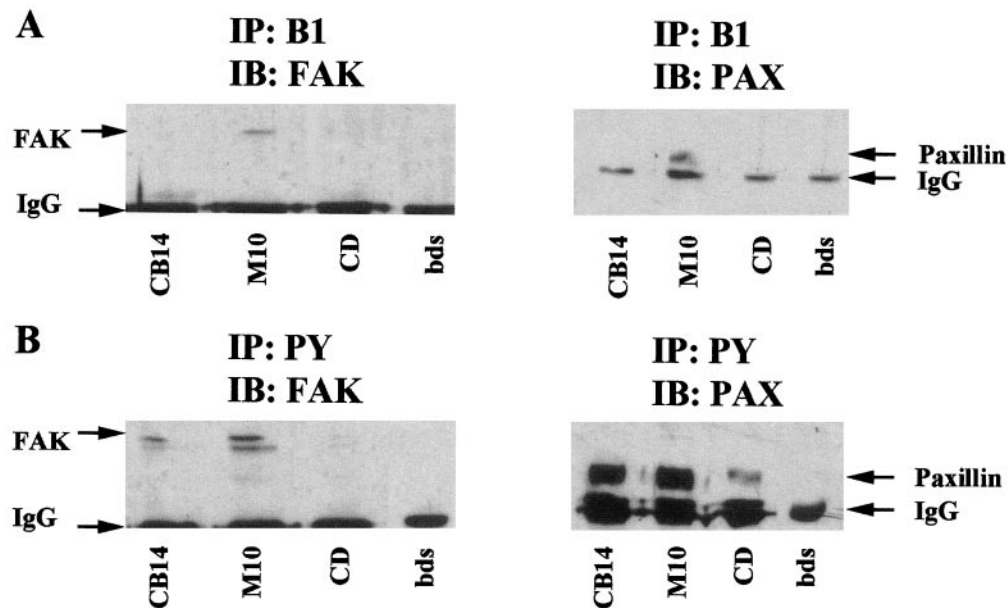


Figure 7. CD inhibits FAK and paxillin tyrosine phosphorylation and $\beta 1$ integrin association. SC/N cocultures were grown for 10 d in CB medium, M medium with and without 0.5 $\mu\text{g}/\text{ml}$ CD. Three hundred micrograms of total protein extracts for each culture condition were immunoprecipitated with $\beta 1$ integrin and phosphotyrosine antibody. Samples were electrophoresed on 10% SDS-PAGE and were immunoblotted with FAK and paxillin antibody. **A**, Two hundred micrograms of $\beta 1$ immunoprecipitate from each culture condition was immunoblotted with FAK antibody (*left panel*). Forty micrograms of $\beta 1$ immunoprecipitate from each culture condition was immunoblotted with paxillin antibody (*right panel*). **B**, Thirty-five micrograms of phosphotyrosine immunoprecipitates from each culture condition was immunoblotted with FAK (*left panel*) and paxillin (*right panel*) antibodies. $\beta 1$ integrin associates with FAK and paxillin in differentiating cocultures but not in CD-treated cocultures. FAK and paxillin tyrosine phosphorylation is lower in CD-treated cocultures than in differentiating cocultures.

tures forming basal lamina and myelin and decreases with time in cocultures that lack basal lamina and myelin.

A $\beta 1$ integrin–FAK–paxillin–fyn kinase complex forms in differentiating SCs

FAK and $\beta 1$ integrin do not coimmunoprecipitate from SC-only culture extracts, although both proteins are expressed and small amounts of tyrosine-phosphorylated FAK is present (Figs. 1, 2).

Coimmunoprecipitation of $\beta 1$ integrin and FAK has not been documented in isolated cells regardless of the substrate, ECM peptide, or integrin antibody used to activate cells. Here, $\beta 1$ integrin and FAK coimmunoprecipitate only when SCs are cocultured with neurons under conditions that promote basal lamina assembly. $\beta 1$ integrin mediates adhesion of newly forming basal lamina to the SC surface (Fernandez-Valle et al., 1994). It is likely that a $\beta 1$ integrin–FAK association is detected because SCs bind native basal lamina and differentiate.

Several controls indicate that the $\beta 1$ integrin–FAK coimmunoprecipitation is specific. First, the interaction is observed with two $\beta 1$ integrin function-blocking antibodies, a hamster monoclonal antibody, and a well characterized rabbit antiserum (Figs. 4, 5). Second, the amount of coimmunoprecipitated FAK increases with time for 14 d as the amount of differentiating SCs and phosphorylated FAK increases. Third, FAK is not immunoprecipitated by preimmune rabbit serum, normal hamster IgG, or protein A-Sepharose beads from differentiating SC/N cocultures (Fig. 5 and data not shown). Lastly, $\beta 1$ integrin antibody does not coimmunoprecipitate vinculin (data not shown), and silver-stained gels reveal only immunoglobulin bands and a band migrating at 130 kDa, consistent with $\beta 1$ integrin (data not shown). This indicates that focal adhesions are solubilized in our extraction buffer. In the reciprocal experiment, FAK immunoprecipita-

tion, and $\beta 1$ integrin immunoblotting, only weak positive results were obtained in 4- and 7-d-old differentiating cocultures (data not shown). This could be attributable to the small fraction of FAK molecules associated with $\beta 1$ integrin (Fig. 5B) or to epitope masking when FAK is bound to $\beta 1$ integrin. The small amount of FAK observed in $\beta 1$ integrin immunoprecipitates of 14-d-old undifferentiated SC/N cocultures is the greatest amount observed in five experiments (Fig. 4). It is likely that the FAK– $\beta 1$ integrin complex derives from differentiating SCs in the cocultures. We have localized FAK to the plasma membrane adjacent to basal lamina patches in differentiating and myelinating SCs (Fernandez-Valle et al., 1998). The abundance of $\beta 1$ integrin is higher in differentiating SCs than in myelinating SCs that preferentially express $\beta 4$ integrin (Einheber et al., 1993; Feltri et al., 1994; Fernandez-Valle et al., 1994). Axons are infrequently labeled in immunogold studies, and neurons express very low levels of FAK and $\beta 1$ integrin relative to SCs (Fig. 1).

Association of paxillin with $\beta 1$ integrin, FAK, and fyn kinase was much greater in differentiating (M) cocultures compared with undifferentiated (CB) cocultures (Fig. 6). The associations observed in undifferentiated cocultures could result from residual interactions occurring in the mitogen-treated SCs added to neuron cultures. The complex observed in mitogen-treated (D10M) and untreated (D10) SCs disassembles when SCs contact axons for an extended period of time in the absence of basal lamina. The undifferentiated cocultures are typically 14-d-old.

The respective binding domains on FAK and paxillin have been identified. The NH_2 terminus of paxillin contains LD domains involved in binding paxillin binding sequence 1 (PBS1) and PBS2 domains in the C terminus of FAK (Brown et al., 1996). Paxillin is proposed to target FAK to focal adhesions, but in other

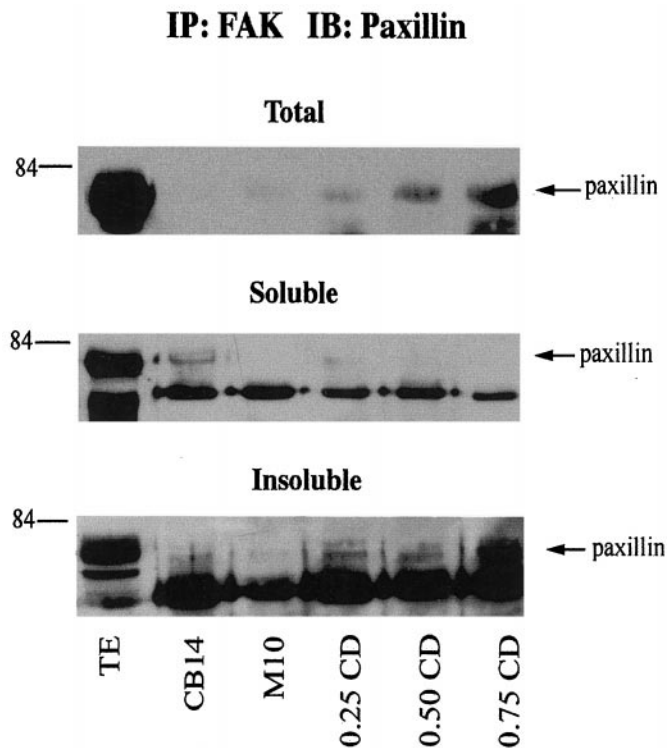


Figure 8. CD causes an increase in the abundance of insoluble FAK–paxillin complexes in SC/N cocultures. SC/N cocultures were grown for 10 d in CB medium, serum and ascorbate medium without CD (*M10*), and with 0.25, 0.50, and 0.75 $\mu\text{g/ml}$ CD. Equal protein from total, soluble, and insoluble culture extracts were immunoprecipitated with FAK antibody and were immunoblotted with paxillin antibody. *TE* lanes represent samples of the total, soluble, and insoluble extracts from *M10* cultures. An increasing amount of paxillin coimmunoprecipitates with FAK as CD concentration increases in SC/N cocultures. The FAK–paxillin complex is found in the insoluble fraction of the cocultures.

studies, FAK reaches focal adhesions in the absence of paxillin binding (Hildebrand et al., 1993; Tachibana et al., 1995). LIM domains in paxillin are involved in targeting paxillin to the plasma membrane, but the precise mechanism is unknown (Brown et al., 1998).

The actin cytoskeleton is essential for formation of a $\beta 1$ integrin complex in differentiating SCs

We demonstrate that a subset of tyrosine-phosphorylated $\beta 1$ integrin, FAK, and paxillin molecules binds the cytoskeleton in differentiating SC/N cocultures (Figs. 3, 8). Tyrosine-phosphorylated $\beta 1$ integrin and paxillin are found exclusively in the insoluble fraction, whereas tyrosine-phosphorylated FAK is more abundant in the insoluble than the soluble fraction. $\beta 1$ integrin is tyrosine-phosphorylated in v-src transformed cells (Hirst et al., 1986; Horvath et al., 1990). Soluble and insoluble paxillin differed in electrophoretic mobility, possibly because of tyrosine phosphorylation differences. Paxillin is also heavily phosphorylated on serine and threonine and is reported to shuttle from focal adhesions to a trans-Golgi–endosomal network (Brown et al., 1998; Norman et al., 1998).

CD inhibits FAK tyrosine phosphorylation and integrin-dependent signaling in other cells (Lipfert et al., 1992). CD, at 0.5 $\mu\text{g/ml}$, inhibits SC myelination *in vitro* but not adhesion to basal lamina or elongation (Fernandez-Valle et al., 1997). Here, we observe that CD inhibits tyrosine phosphorylation of FAK and

paxillin and coimmunoprecipitation of FAK and paxillin with $\beta 1$ integrin and causes an increase in the abundance of insoluble FAK–paxillin complexes (Figs. 7, 8). A small amount of FAK and paxillin coimmunoprecipitate from the soluble fraction of undifferentiated cocultures. This association is not detected when total culture extract is used (compare *CB* lanes in total and soluble fractions). This is likely a residual association occurring in the mitogen-treated SCs that are added to neuron cultures (Fig. 6*C,D*). An FAK–paxillin complex appears to become insoluble in differentiating (M) cocultures, although only low levels are observed compared with the amount in CD-treated cocultures. It is possible that FAK and paxillin require F-actin for targeting to the plasma membrane at developing focal adhesions. Src-related kinases bind both FAK and paxillin, and targeting of src kinase to the cell periphery requires F-actin (Fincham et al., 1996).

Our work, interpreted in the context of what is known about focal adhesion assembly, suggests that SC adhesion to nascent basal lamina by $\beta 1$ integrins leads to F-actin-dependent integrin aggregation and recruitment of FAK and paxillin. FAK autophosphorylates and recruits fyn kinase, which could bind and phosphorylate paxillin and activate further downstream signaling events. The precise function of this complex during SC differentiation is unknown. Techniques are being developed to produce stable cell lines of FAK null SCs for use in SC/N cocultures to further elucidate this pathway.

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