Phosphorylation of Tyrosine Residues 31 and 118 on Paxillin Regulates Cell Migration through an Association with CRK in NBT-II Cells

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Abstract. Identification of signaling molecules that regulate cell migration is important for understanding fundamental processes in development and the origin of various pathological conditions. The migration of Nara Bladder Tumor II (NBT-II) cells was used to determine which signaling molecules are specifically involved in the collagen-mediated locomotion. We show here that paxillin is tyrosine phosphorylated after induction of motility on collagen. Overexpression of paxillin mutants in which tyrosine 31 and/or tyrosine 118 were replaced by phenylalanine effectively impaired cell motility. Moreover, stimulation of motility by collagen preferentially enhanced the association of paxillin with the SH2 domain of the adaptor protein CrkII. Mutations in both tyrosine 31 and 118 diminished the phosphotyrosine content of paxillin and prevented the formation of the paxillin–Crk complex, suggesting that this association is necessary for collagen-mediated NBT-II cell migration. Other responses to collagen, such as cell adhesion and spreading, were not affected by these mutations. Overexpression of wild-type paxillin or Crk could bypass the migration-deficient phenotype. Both the SH2 and the SH3 domains of CrkII are shown to play a critical role in this collagen-mediated migration. These results demonstrate the important role of the paxillin–Crk complex in the collagen-induced cell motility.

Key words: cell migration • collagen • paxillin • tyrosine phosphorylation • Crk

Introduction

Cell migration is a fundamental aspect in numerous normal and pathological processes, including embryonic development, wound healing, inflammation, and metastasis of tumor cells (for reviews see Hay, 1995; Viebahn, 1995; Birchmeier et al., 1996; Gumbiner, 1996). Various factors in the cellular microenvironment participate in the regulation of cell migration. These include soluble growth factors and extracellular matrix (ECM)[1] proteins whose pivotal role has been clearly established using in vivo and in vitro model systems (Schor, 1994; Boyer et al., 1996; Brand-Saberi et al., 1996). Cell motility is also governed by the cell interpretation of external signals, which are transduced via multiple intracellular pathways. The motile process is finally executed by different biochemical events that modify the actin cytoskeleton and cell adhesion molecules (Ingber, 1993). Because of its wide implications for normal physiology and pathological conditions, considerable effort was directed towards the identification of intracellular signaling molecules that may control the dynamics of cell movement.

Induction of motility by the ECM is primarily associated with integrin-mediated cell adhesion. Integrins constitute a large family of α/β heterodimeric transmembrane receptors that mediate interaction of cells with many different ECM proteins (Hynes, 1992). The cytoplasmic tails of integrins interact with cytoskeletal-associated molecules providing a physical link between the ECM and the actin cytoskeleton. Ligation of integrins by the ECM initiates a cascade of intracellular signaling events involving the activation of tyrosine kinases and subsequent phosphorylation of multiple cytoskeleton-associated substrates (Clark and

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[1]*Abbreviations used in this paper:* P130P130Cas, Crk-associated substrate; ECM, extracellular matrix; FAK, focal adhesion kinase; FN, fibronectin; GFP, green fluorescent protein; LN, laminin; NBT-II, Nara Bladder Tumor II; PL, poly-L-lysine; SH, Src homology.

Brugge, 1995; Giancotti, 1997; Schoenwaelder and Burridge, 1999). These biochemical modifications ultimately bring about diverse biological responses, including cell survival, proliferation, and migration.

The interactions of cells with the ECM organize integrins into specialized adhesive structures, termed focal adhesions, which are sites where signaling molecules are highly enriched (Jockusch et al., 1995; Miyamoto et al., 1995; Burridge and Chrzanowska-Wodnicka, 1996). Integrin clustering at these adhesive structures is dependent on the proper assembly of the actin cytoskeleton that is regulated by the Rho family of GTPases (Keely et al., 1998; Mackay and Hall, 1998). Several signaling molecules that localize at focal adhesions after integrin stimulation have been implicated in cell motility; these include the nonreceptor focal adhesion kinase (FAK) and c-Src family kinases. FAK-deficient fibroblasts (Ilic et al., 1995) and Src kinase–inactivated epithelial cells (Rodier et al., 1995) show defective cell locomotion, whereas the overexpression of these kinases facilitates cell movement (Rodier et al., 1995; Cary et al., 1996; Gilmore and Romer, 1996). In addition, the protein tyrosine phosphatases Shp-2 (Yu et al., 1998) and PTP-PEST (Angers-Loustau et al., 1999) also have been implicated in the regulation of cell locomotion. A role for these tyrosine kinases and phosphatases in cell migration is also outlined by their substrates, including the adaptor protein p130Cas (Crk-associated substrate) that was recently shown to play a role in cell motility (Cary et al., 1998; Klemke et al., 1998). However, the involvement of other direct effectors of these kinases and phosphatases in cell motility remains to be determined.

Paxillin is a focal adhesion protein (Turner et al., 1990) that was originally identified as a major tyrosine-phosphorylated protein in cells transformed by the v-Src and v-Crk oncogenes (Glenney and Zokas, 1989; Birge et al., 1993). Paxillin becomes phosphorylated on tyrosine in response to various physiological stimulants including bombesin, PDGF, NGF, and angiotensin II (Zachary et al., 1993; Rankin and Rozengurt, 1994; Melamed et al., 1995; Turner et al., 1995), and in response to adhesion-mediated events that are associated with remodeling of the actin cytoskeleton (Burridge et al., 1992; Turner, 1998). Evidence from in vitro and in vivo studies has identified paxillin as a potential substrate for FAK (Bellis et al., 1995; Schaller and Parsons, 1995). These two proteins are coordinately phosphorylated on tyrosine upon cell stimulation, are localized in focal adhesions, and paxillin exhibits enhanced phosphorylation in FAK-overexpressing cells (for review see Hanks and Polte, 1997). Paxillin can also serve as a substrate for the protooncogene c-Abl in an integrin-dependent manner (Lewis and Schwartz, 1998). Several structural features identify paxillin as an adaptor protein capable of recruiting multiple signaling molecules (for review see Turner, 1998). It contains a proline-rich region that provides a potential binding site for the Src homology (SH) 3 domain of Src family members (Weng et al., 1993) and two pYXXP motifs that conform to consensus binding sites for the SH2 domain of Crk and the protein tyrosine kinase Csk (Birge et al., 1993; Sabe et al., 1994). The tyrosines at positions 31 and 118, in particular, have been shown to be involved in the binding of Crk (Schaller and Parsons, 1995). Paxillin also contains leucine rich motifs (LD repeats) that can selectively bind to vinculin, FAK (Brown et al., 1996), the FAK-related kinase Pyk2 (Salgia et al., 1996), the papillomavirus oncoprotein E6 (Tong et al., 1997), or to the recently identified p95PKL, or paxillin-kinase linker that links paxillin to the p21 GTPase–activated kinase PAK, and the guanine nucleotide exchange factor PIX (Turner et al., 1999). In the later case, it was proposed that a protein complex composed of paxillin in association with p95PKL/PAK/PIX is important for remodeling of the cytoskeleton. The ability of paxillin to interact with numerous proteins suggests that paxillin may mediate diverse signaling pathways. The functional significance of these different associations of paxillin is yet to be determined.

We have used the rat bladder carcinoma cell line Nara Bladder Tumor II (NBT-II) to study the factors that control ECM-mediated cell migration. NBT-II epithelial cells are converted to motile cells during a process known as the epithelium–mesenchymal transition (Boyer et al., 1996). The induction of cell dispersion is specific for collagens, whereas other ECM components such as fibronectin (FN) and laminin (LN) only allow cell adhesion and spreading (Tucker et al., 1990). Moreover, NBT-II cells can be induced to scatter after stimulation with soluble growth factors such as EGF and FGF (Boyer et al., 1989; Vallés et al., 1990). It has been shown that a crucial component in the scattering response of NBT-II cells to growth factors is the Src kinase activity (Rodier et al., 1995). However, this kinase activity is not necessary for the initial collagen response (Petit et al., 1999). This raises the question whether growth factors and ECM molecules utilize the same signaling pathways to induce motility. In a previous study, we demonstrated the importance of tyrosine phosphorylation for the collagen-induced migration of NBT-II cells (Petit et al., 1999). In this report we sought to identify the phosphotyrosinated molecules that are specifically regulated during the collagen-mediated motility response. We found that tyrosine phosphorylation of two major sites on the adaptor protein paxillin plays a central role in the collagen-induced migration of NBT-II cells.

Materials and Methods

Reagents and Antibodies

Rat collagen type I from Sigma Chemical Co. was prepared as described (Tucker et al., 1990). Fibronectin, laminin 1, and poly-L-lysine were obtained from Sigma Chemical Co. Antibodies against FAK (clone 2A7) used for immunoprecipitation and phosphotyrosine (clone 4G10) were obtained from Upstate Biotechnology. Antipaxillin (clone 349), anti-FAK (clone 77), anti-p130Cas (clone 21), and anti-Crk (clone 22) were obtained from Transduction Laboratories. The polyclonal anti-chicken paxillin was previously described (Bellis et al., 1995). Anti-p130Cas (C-20) used for immunoprecipitation was from Santa Cruz Biotechnology, Inc. Anti-GFP was obtained from Roche Molecular Biochemicals.

Constructions and Plasmids

cDNAs coding for chicken wild-type paxillin and the F118 mutant in the pcDNA3 expression plasmid were previously described (Turner and Miller, 1994; Bellis et al., 1995). PCR-based strategy was utilized to generate the F31 and the F31/118 mutants as follows. A first round of amplification was made with primers 5′ TAATACGACTCACTATAGGG 3′ (T7 promoter primer) and 5′ GGGTAGGAGAAAGGCGTTT 3,′ using either the wild-type or the F118 cDNAs as template. The resulting fragments were used as primers for a second amplification round with oligo-

nucleotide 5′ GGGGGCTGCTCACCTCCCC 3′, containing the SacI restriction site. The resulting products were digested with BamHI and SacI and the fragments obtained, containing the mutated codons, were used to replace the BamHI-SacI fragment of the full-length paxillin cDNA. The BamHI-EcoRI fragments corresponding to full-length paxillin F31 and F31/118 were subcloned into the pcDNA3 vector. GFP fusion proteins were generated by PCR amplification with primers 5′ GCGGCCGCGTTCATCAACGG 3′ and 5′ CGGAATTCCGAAGAGTTT 3′, containing the EcoRI restriction site, to eliminate the stop codon of paxillin. The resulting fragment was digested with EcoRI and SacII and used to replace the SacII-EcoRI fragment of the wt, F31, F118, or F31/118 constructs. The HindIII-EcoRI fragments corresponding to paxillin mutants lacking stop codons were cloned into pEGFP-N1 (CLONTECH Laboratories). The validity of all PCR-generated constructs was confirmed by automatic sequencing (ABI PRISM Dye Terminator cycle sequencing kit; Perkin Elmer). Plasmids encoding rat Crk-II, Crk-II (R38V), Crk-II (W169L), untagged wild-type p130Cas, and p130Cas in-frame deletion of its substrate domain (CASΔSD, amino acids 213–514) were provided by Dr. R.L. Klemke (The Scripps Research Institute, La Jolla, CA) and previously described (Matsuda et al., 1993, 1994; Mayer et al., 1995).

Cell Culture and Transfections

The rat bladder carcinoma NBT-II cell line, originally established by Toyoshima and colleagues (Toyoshima et al., 1971), was obtained from Prof. M. Mareel (University Hospital, Ghent, Belgium). The cells were grown in monolayers on tissue culture plastic in DME supplemented with 1% glutamine, 1% penicillin-streptomycin, and 10% heat-inactivated FCS (complete medium). Cells stably expressing the paxillin constructs were routinely grown in the presence of 400 μg/ml of active geneticin (G418, GIBCO BRL; Life Technologies). The cells were maintained at 37°C in a humidified atmosphere with 6% CO_2.

To generate clones stably expressing the different paxillin constructs, cells were transfected by the calcium phosphate method with 5 μg of the expression plasmid pcDNA3 encoding the various cDNAs. Stable clones were selected using complete medium containing 400 μg/ml active geneticin and screened by Western blotting.

Transient transfections were done using Lipofectamine (GIBCO BRL) following the manufacturer's instructions. In brief, for migration assays, NBT-II cells (1.7×10^5 cells/35-mm plate) were transfected with 8 μl of Lipofectamine and 2 μg of the expression vector, together with 0.2 μg of a reporter plasmid encoding green fluorescent protein (GFP) (pEGFP-C1; CLONTECH Laboratories). For immunoprecipitation experiments, 10 μg of expression vectors was transfected per 100-mm plate. Cells were incubated with the cDNAs for 5 h, washed, and analyzed 48 h later.

Immunofluorescence Microscopy

NBT-II cells were transiently transfected with the different paxillin-GFP constructs for 48 h as described. Transfected cells were trypsinized and seeded on collagen-coated dishes for 1 h at 37°C. The cells were fixed with 3.7% formaldehyde in PBS for 20 min, and permeabilized with 0.5% Triton X-100 in PBS for 2 min. GFP-transfected cells were visualized under a fluorescent Leica microscope. Images were acquired with the Photoshop software (Adobe Systems, Inc.) using a 63× objective.

Cell Adhesion and Cell Spreading Assays

Initial cell adhesion and cell spreading assays were done as described with minor modifications (Tucker et al., 1990). In brief, NBT-II cells were trypsinized (0.05% trypsin/EDTA), resuspended in complete medium, and washed three times in DME. For adhesion experiments, 5×10^5 cells were plated on small areas of bacterial petri dishes precoated with various concentrations of collagen and saturated with BSA. After incubation at 37°C for 1 h, nonadherent cells were removed, and the remaining attached cells were fixed with 3.7% formaldehyde in PBS. Cells were stained for 10 min with 5 mg/ml crystal violet and washed four times with H_2O. The incorporated crystal violet was released in 10% acetic acid and quantified as absorbance at 570 nm. To quantify cell spreading, fixed cells were counted under a phase-contrast microscope. Spreading was calculated as the percent cells that lost the round morphology. Each point represents duplicate areas and is expressed as the mean of at least three independent experiments ± SD. Nonspecific cell adhesion was measured on BSA-coated areas and subtracted from each point.

Immunoprecipitations and Western Blots

Cells growing in complete medium were trypsinized, resuspended in complete medium, and replated on plastic dishes precoated with poly-L-lysine (200 μg/ml), collagen (10 μg/ml), fibronectin (10 μg/ml), or laminin 1 (10 μg/ml). Because NBT-II cell migration requires serum components, it is systematically added in the medium. Consequently, the basal levels of tyrosine phosphorylation increase without modifying the pattern of phosphorylated proteins. After incubation at 37°C for the indicated times, cells were washed twice with ice-cold PBS. For Western blotting, total cellular proteins were lysed with NP-40 buffer (1% NP-40, 50 mM Tris, pH 8, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 100 mM sodium orthovanadate). For immunoprecipitation, Triton buffer (1% Triton X-100, 10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 100 mM sodium orthovanadate) was used. Cell lysates were centrifuged for 20 min at 14,000 rpm at 4°C. The cell lysates were incubated with the primary antibody for 2 h at 4°C on a rocking platform, followed by incubation with protein A–Sepharose precoupled with rabbit anti–mouse antibody, for an additional 1 h at 4°C. Bead-bound complexes were washed three times with cold lysis buffer and boiled with 1× SDS sample buffer.

For Western blot analysis of total cell lysates or immunoprecipitates, proteins were separated by SDS-PAGE under reducing conditions and electrophoretically transferred onto polyvinylidene fluoride membranes (Immobilon P; Millipore Corp.), blocked with 0.1% gelatin/0.1% Tween 20 in PBS, and subsequently incubated overnight at 4°C with the primary antibody. Membranes were washed and incubated with either anti–mouse or anti–rabbit IgG peroxidase conjugates. Proteins were visualized on Hyperfilm using enhanced chemiluminescence (ECL kit; Amersham Corp.). Images were acquired using Photoshop software. Protein concentration was determined using the Bradford protein assay (Bio-Rad Laboratories).

SH2 Binding Assays

The GST fusion protein containing the SH2 domain of rat c-Crk was a gift of Dr. R.B. Birge (The Rockefeller University, New York, NY) and was described (Birge et al., 1993). A cDNA construct encoding the SH2 domain of Grb2 fused with GST was provided by Dr. A. Ducruix (CNRS-UPR 9063, Gif/Yvette). GST fusion proteins were expressed in exponentially growing *Escherichia coli* cultures by addition of 1 mM isopropyl-β-thiogalactopyranoside. Bacterial lysates were incubated overnight at 4°C with glutathione-Sepharose 4B beads (Pharmacia Biotech Sverige). Samples were analyzed by Coomassie staining to ensure equal amount of GST fusion proteins. Cell lysates of transfected cells were prepared as for immunoprecipitation, and incubated with equal amount of GST fusion proteins bound to glutathione-Sepharose beads at 4°C for 2 h. Beads were washed three times with lysis buffer and resuspended in 1× SDS sample buffer. Protein complexes were subjected to Western blot analysis.

Cell Migration Assay

To assay for random cell migration, freshly trypsinized cells were plated at low density (10^5) on 35-mm collagen-coated bacterial petri dishes. The assay is done in complete medium to optimize the migration of NBT-II cells, as previously reported (Vallés et al., 1994). After 2 h, cells were placed on the motorized stage of a Leica inverted microscope equipped with a chamber providing a controlled temperature and CO_2 concentration, and a Princeton MicroMax CCD camera. Phase-contrast and fluorescent images were obtained and analyzed with the Metamorph software (Metamorph Imaging System; Universal Imaging Corp.) running on a PC workstation. The motility of individual cells was evaluated by tracking their movement over 12 h with images recorded every 4 min using the same software. The average speed (μm/h) of locomotion was calculated as the total track length divided by the number of hours recorded. For each experimental condition, 20–30 cells were analyzed. In transient transfections with GFP, only green fluorescent cells were followed.

Results

Paxillin and FAK Are Tyrosine-phosphorylated in NBT-II Cells Plated on Collagen

Sustained migrations of NBT-II cells are induced by fibrillar collagen, whereas other components of the ECM like

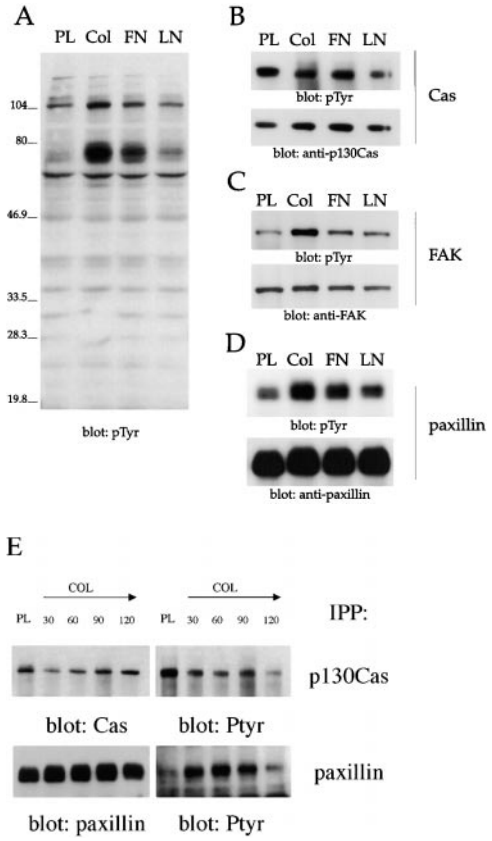

Figure 1. Adhesion of NBT-II cells on collagen induces tyrosine phosphorylation of FAK and paxillin. NBT-II cells were allowed to attach on either poly-L-lysine (PL), collagen-I (COL), fibronectin (FN), or laminin-1 (LN) for 2 h. (A) Total cellular lysates from stimulated cells were subjected to immunoblot analysis with antiphosphotyrosine antibody. Lysates prepared from cells stimulated by the different matrix molecules were immunoprecipitated with anti–p130Cas (B), anti–FAK (C), or antipaxillin (D) antibodies, and then immunoblotted with antiphosphotyrosine antibody or with the corresponding antibodies. (E) Time course of p130Cas and paxillin tyrosine phosphorylation. Total cellular lysates from cells stimulated with collagen for the indicated times were processed as above. The horizontal bars indicate the migration of molecular mass standards. The arrowheads point to the position of FAK and paxillin.

FN, vitronectin, and LN are permissive for adhesion and spreading. (Tucker et al., 1990). To identify cytoplasmic molecules that are tyrosine-phosphorylated in association with the persistent migratory phenotype induced by collagen, NBT-II cells were plated onto dishes coated with either collagen, FN, or LN and allowed to attach for 2 h in the presence of serum that is necessary for the migratory response. Cells plated onto PL served as control for nonintegrin-mediated adhesion. Antiphosphotyrosine immunoblot analyses of total cell extracts (Fig. 1 A) revealed proteins equally phosphorylated at a basal level on all matrices and on PL, in contrast to FN and LN, cell adhesion to collagen resulted in the significantly enhanced tyrosine phosphorylation of two prominent 70–80-kD and 120-kD molecular mass proteins (Fig. 1 A).

Various proteins were described to be tyrosine-phosphorylated after adhesion to matrix molecules, among them were p130Cas (Nojima et al., 1995), FAK, and paxillin (Burridge et al., 1992). To identify the proteins that are tyrosine-phosphorylated in NBT-II cells in response to collagen, immunoprecipitations were conducted with antibodies to p130Cas, FAK, and paxillin with lysates from cells plated on PL, FN, LN, and collagen, and analyzed for phosphotyrosine content. As shown in Fig. 1 B, the tyrosine phosphorylation of p130Cas was similar whether the cells were plated onto PL or after plating on the other ECM components. In contrast, the tyrosine phosphorylation of FAK increased after adhesion of NBT-II cells to collagen, but not after adhesion to FN or LN (Fig. 1 C). The tyrosine phosphorylation of paxillin was also higher on collagen, as compared with FN and LN (Fig. 1 D). The same blots were reprobed with the corresponding antibodies to verify that equal amounts of proteins were immunoprecipitated (Fig. 1). Kinetic analyses of cells on collagen verified that the tyrosine phosphorylation pattern of p130Cas is not modulated at earlier times points (Fig. 1 E). By immunodepletion experiments, we confirmed that paxillin was the prominent phosphorylated pp80 band apparent on the total lysate (data not shown), while other unidentified minor components with similar 120-kD migrations might be present. These results suggest that there is a correlation between the collagen-induced tyrosine phosphorylation of FAK and paxillin and the migratory behavior of NBT-II cells.

Cell Migration of NBT-II Cells Correlates with Phosphorylation of Paxillin on Tyrosine Residues 31 and 118

Since the phosphorylation of both FAK and paxillin increased in NBT-II cells on collagen, and FAK was already shown to be linked to cell migration in other systems (Ilic et al., 1995; Cary et al., 1996), we focused our study on paxillin. To investigate whether regulation of paxillin function by tyrosine phosphorylation was essential for the NBT-II collagen-mediated events, we employed tyrosine to phenylalanine mutants of the major phosphorylation sites Y31 and Y118. cDNAs encoding the full-length chicken paxillin and the F118 mutation have been described previously (Turner and Miller, 1994; Bellis et al., 1995). Using a PCR-based strategy, the F31 mutation was created, alone or in combination with the F118 mutation, as described in Materials and Methods. The resulting constructs were designated F31 and F31/118, respectively. Stable transfectants of NBT-II cells expressing the different mutants were generated. Individual clones were screened for paxillin expression by Western blotting with an antibody recognizing both species, and the levels of exogenous paxillin were verified by immunoprecipitation with an antibody specific to chicken paxillin (Bellis et al., 1995). The selected clones contained three- to fivefold higher paxillin levels as compared with parental ones (Fig. 2 A). In separate transient transfection experiments using GFP fusion forms of these mutants, we confirmed that the mutant paxillins displayed either less (F118-GFP or F31-GFP) or low (Fx-GFP) tyrosine phosphorylation in response to collagen (Fig. 2 B). The mutated proteins (Fig. 2 C, b–d) lo-

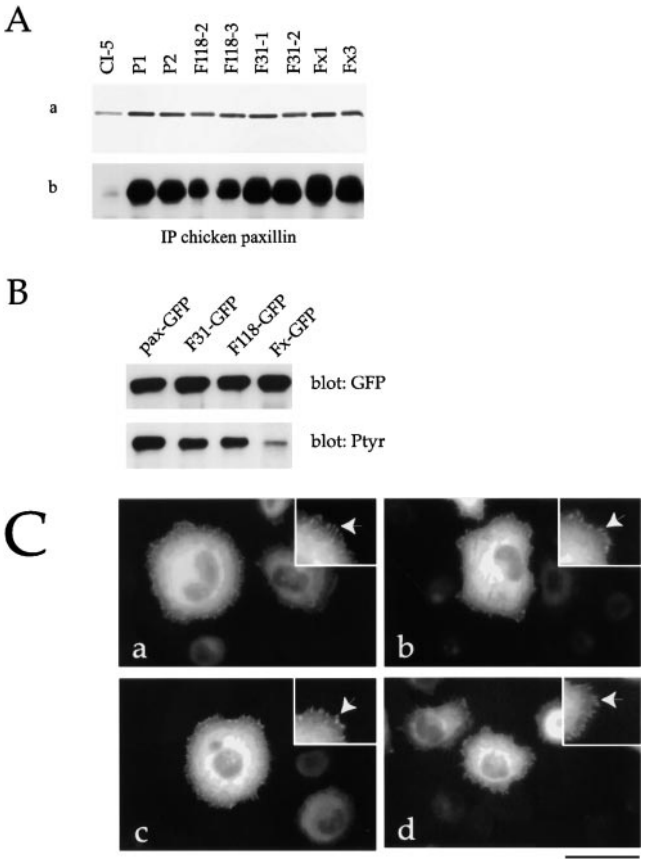

Figure 2. Expression and localization of wild-type and mutant forms of paxillin in NBT-II cells. (A) Equal amounts of total cell lysates from stable mock-transfected cells (clone C1), wild-type paxillin (clones P1 and P2), mutant F31 (clones F31-1 and F31-2), mutant F118 (clones F118-2 and F118-3), and double mutant F31/118 (clones Fx1 and Fx3) cells were (a) subjected to immunoblot analysis with antipaxillin antibodies or (b) immunoprecipitated with anti-chicken paxillin antibodies followed by antipaxillin immunoblots. (B) Lysates prepared from cells transiently transfected with the paxillin-GFP constructs and stimulated by collagen were immunoprecipitated with anti–GFP antibodies and immunoblotted with anti–GFP and antiphosphotyrosine (Ptyr) antibodies. (C) To localize the transfected paxillin forms, cells were transiently transfected with paxillin-GFP constructs for 48 h, allowed to spread on collagen-coated dishes for 1 h and then fixed (a, pax-GFP; b, F31-GFP; c, F118-GFP; and d, F31/118-GFP). Note that the GFP fluorescence localized to cell-substrate sites (arrows in inserts) in addition to a diffuse pattern in the cytoplasm. Bar, 20 μm.

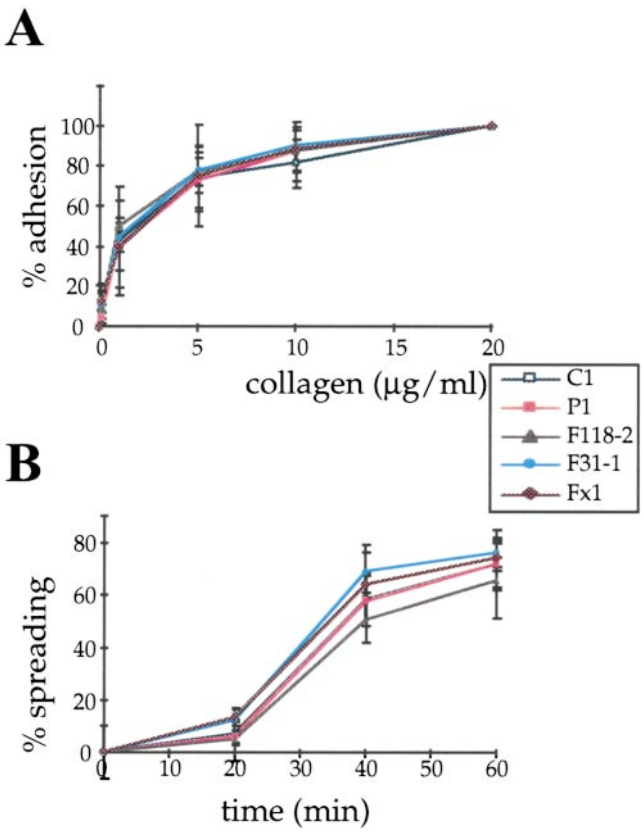

Figure 3. Adhesion and spreading of clones expressing wild-type and mutant forms of paxillin are similar on collagen. (A) Mock-transfected (clone C1) and cells stably transfected with wild-type (clone P1), or mutant forms of paxillin F31 (clone F31-1), F118 (clone F118-2), or F31/118 (clone Fx1) were allowed to adhere on plates coated with the indicated concentrations of collagen for 1 h at 37°C. Absorbance values corresponding to crystal violet uptake were determined for each point and expressed as percent adhesion. (B) Cell spreading was quantified by allowing cells to adhere on plates coated with 20 μg/ml collagen for the indicated times. Cell spreading was determined by calculating the percentage of spread cells at each time point. Values represent the mean of three independent experiments carried out in duplicate ± SD. Selected clones are representative of all the tested clones.

calized to cell–substrate adhesion sites similar to wild-type paxillin (Fig. 2 C, a).

Independent clones expressing paxillin mutants were used in standard adhesion and spreading assays. These experiments revealed rates of attachment on collagen for the different clones that did not differ to that of cells expressing the wild-type protein (Fig. 3 A). These results are in agreement with previous results showing that these mutations in paxillin do not interfere with the adhesion of CHO-K1 cells on fibronectin (Brown et al., 1998). The different clones also displayed comparable initial rates of spreading on collagen when compared with control mock-transfected cells (Fig. 3 B). Therefore, phosphorylation of tyrosine residues 31 and 118 on paxillin may not be directly involved in the regulation of cell adhesion or spreading of NBT-II cells on collagen.

To assess the migratory behavior of NBT-II cells expressing paxillin mutants on collagen, random migration assays were performed that measure the capacity of cells to move on a given substrate. The movement of individual cells was monitored at 4-min intervals for 12 h, and the migration rate was quantified as the total distance of migration by one cell divided by the time of recording. The results in Fig. 4 show that expression of the double mutations F31/118 (clones Fx1-3) resulted in a significant decrease in the migration of the cells on collagen (50–70% reduction for different clones) as compared with control (clones C1 and C2), whereas expression of the single mutations (clones F118-1, 2 and F31-1, 3) had a less pro-

nounced effect (Fig. 4 A). Overexpression of the wild-type construct produced a slight increase in migration of NBT-II cells (clones P1 and P2 compared with clones C1 and C2). Visualization of the tracks formed by individual cells clearly points to marked differences in the migratory behavior of these clones: a dramatic reduction in the persistence of locomotion for F31 and F31/118 mutants (Fig. 4 B, e–h) and less pronounced for F118 mutants (Fig. 4 B, d), as compared with control cells (Fig. 4 B, a). Moreover, although F31/118-transfected cells remained essentially stationary, they exhibited active membrane dynamics (data not shown), suggesting that the defect is specific to cell locomotion. Similar dominant-negative effects were obtained in transient transfections of the different constructs when coexpressed with GFP to visualize the transfected cells or with GFP-fusion proteins (Fig. 8; and data not shown). These results show that mutations in both tyrosine residues result in the strong reduction of cell motility and suggest that phosphorylation of these residues may be necessary for the collagen-induced migration of NBT-II cells. It should be noted that because of the high expression levels of endogenous paxillin, the dominant-negative effect of the mutant proteins was not able to completely abolish cell motility.

Crk Binding to Paxillin Is Impaired in F31/118-expressing Cells

Tyrosine residues 31 and 118 of paxillin reside within a YXXP motif that corresponds to an optimal Crk-SH2-binding sequence (Birge et al., 1993). When these residues are phosphorylated, they become effective binding sites for the adaptor protein Crk (Birge et al., 1993; Schaller and Parsons, 1995). To determine whether paxillin associates with Crk in NBT-II cells, cell lysates from parental cells adhering either to collagen or other nonpermissive substrates like PL, FN, and LN were immunoprecipitated with anti–Crk antibodies and immunoblotted after SDS-PAGE with antipaxillin antibodies (Fig. 5). As shown in Fig. 5 A, Crk and paxillin strongly associated in NBT-II cells upon collagen stimulation, although some interaction was also detectable on PL, FN, and LN. The associations on FN and LN are probably due to the small phoshorylation content of paxillin obtained on these substrates (Fig. 1) as a consequence of a brief scattering effect seen before cell contacts are reformed. Kinetics studies demonstrated an increase in paxillin–Crk association in a time-dependent manner after stimulation with collagen (Fig. 5 B). Conversely, Crk and p130Cas interactions although detectable on PL, decreased in time upon collagen stimulation. These results suggest that both p130Cas and paxillin can potentially associate with Crk in unstimulated NBT-II cells, but that upon motility induction by collagen, Crk will preferentially bind to paxillin.

To further define the molecular basis of the interaction between paxillin and Crk, we used the SH2 domain of CrkII (Crk-SH2) expressed as a GST fusion protein and immobilized on glutathione-Sepharose beads. Lysates of parental cells plated on collagen or PL were precipitated with GST-fusion proteins of Crk-SH2, Grb2-SH2, or GST alone, and the bound proteins were separated by SDS-PAGE and immunoblotted with antibodies to phosphotyrosine, paxillin, and p130Cas. As shown in Fig. 6, Crk-SH2 bound paxillin in the collagen-induced cell lysate and, to a much lesser extent, in the lysates from cells plated on PL. This fraction of paxillin that bound to Crk-SH2 in cells plated on PL could be due to the background levels of tyrosine-phosphorylated paxillin present in these cells (Fig. 6, left panel; see also Fig. 1 A). Paxillin failed to bind GST alone or the SH2 domain of Grb2 that belongs to the family of SH2/SH3-containing signaling molecules. Therefore, in NBT-II cells, paxillin appears to associate with the SH2 domain of Crk, and this interaction is increased after collagen stimulation. The results in Fig. 6 also show that the SH2 domain of Crk was able to bind p130Cas from both stimulated and unstimulated NBT-II cells. Taken together, these results support the notion that the full-length c-Crk preferentially binds to tyrosine-phosphorylated paxillin upon stimulation of cells with collagen. On the other hand, the SH2 domain of Crk can potentially bind to both p130Cas and paxillin.

Next, we tested the hypothesis that the inability of cells expressing mutant paxillin to move on collagen results from an altered association of paxillin with the SH2 domain of Crk. To this end, we analyzed the binding of Crk to the different paxillin mutants, under the experimental conditions used in the migratory assays. To discriminate between the exogenous mutants and the endogenous paxillin, the association between the SH2 domain of Crk and the paxillin mutants was examined in transient transfection experiments using the various GFP-paxillin constructs. The motile behavior of NBT-II cells transfected with GFP-paxillin constructs was the same as that of the nontagged paxillin constructs cotransfected with GFP (data not shown). Cell lysates of transiently transfected cells stimulated with collagen were incubated with GST fusion proteins of the Crk-SH2 or with GST alone, and precipitates were analyzed by Western blotting using antipaxillin antibodies (Fig. 7 A). As expected, the wild-type form of paxillin bound to the SH2 domain of Crk (Fig. 7 A). In cells expressing the F31/118-GFP construct (Fx-GFP), Crk binding was abolished, whereas cells expressing the constructs containing single mutations in paxillin (F118-GFP and F31-GFP) retained the capacity to bind to the SH2 domain of Crk. These different forms of

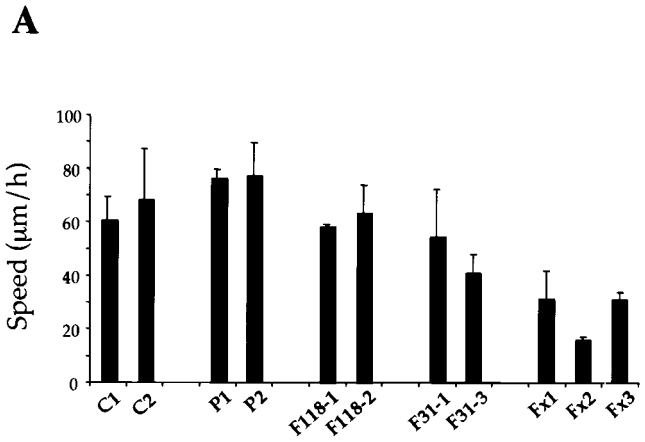

Figure 4 (continues on facing page).

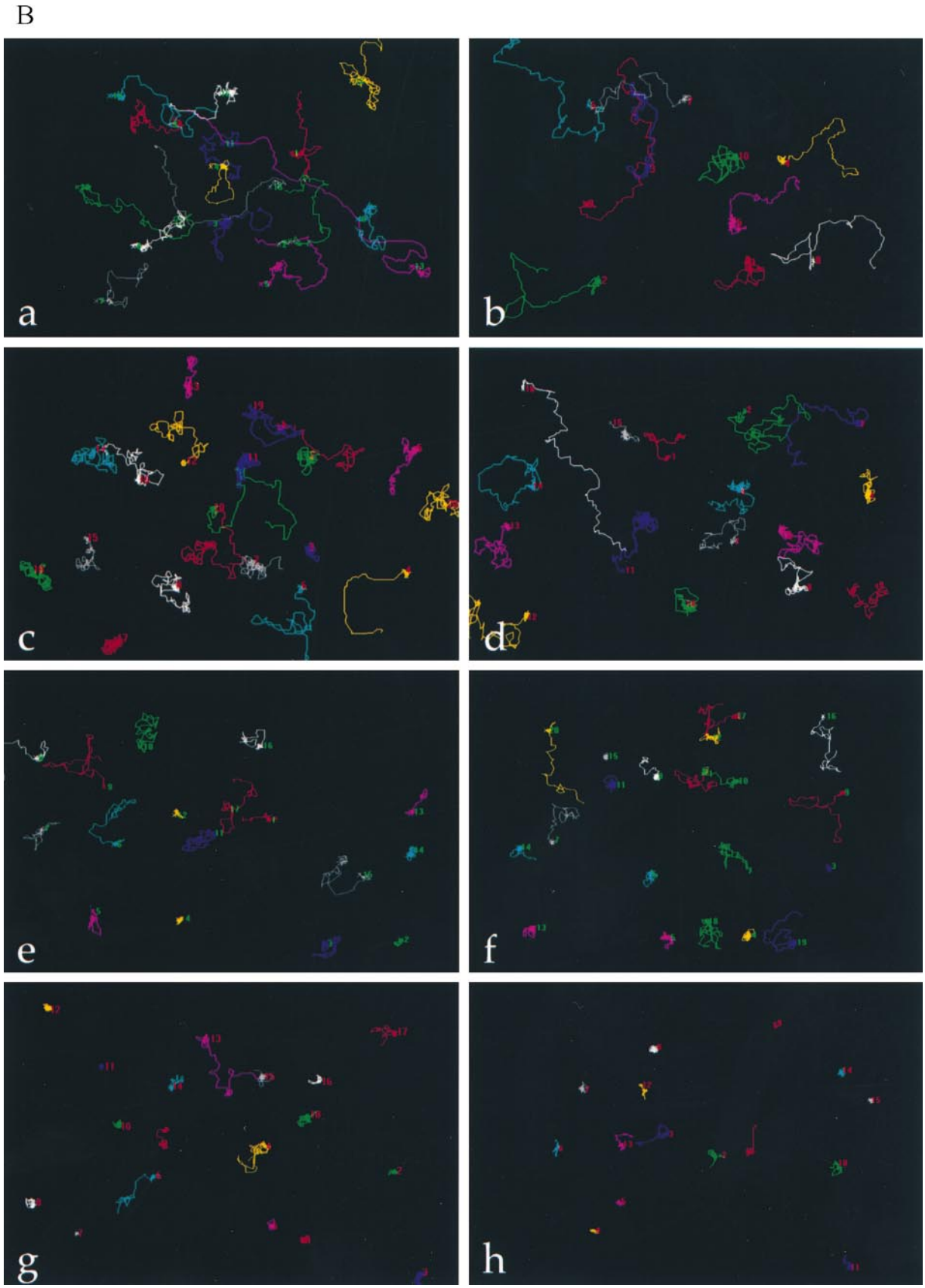

Figure 4. Random cell migration is reduced in cells expressing the F31/118 mutations on paxillin. Stable transfectants were allowed to attach on plates coated with 20 μg/ml of collagen for 2 h. Cell motility was evaluated by tracking individual cells for 12 h with a Leica inverted microscope connected to a computer using the Metamorph software. (A) Values obtained from measuring the tracks of cells are presented as micrometers per hour. Each bar represents the mean of at least three independent experiments ± SD. (B) Representative migration tracks formed by clones C1 (a), P1 and P2 (b and c), F31-1 and F31-3 (e and f), F118-1 (d), and Fx2 and Fx3 (g and h).

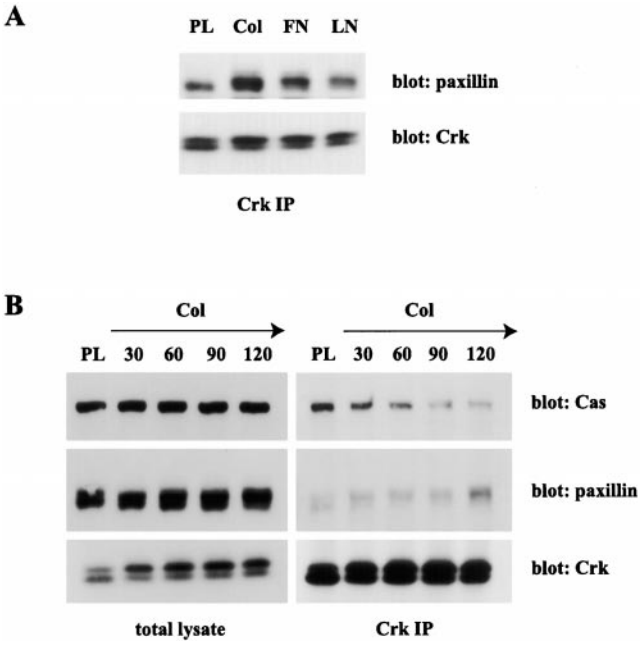

Figure 5. Adhesion on collagen enhances the association of CrkII with paxillin in NBT-II cells. (A) Total cell lysates prepared from cells plated on PL, collagen, FN, or LN for 2 h were immunoprecipitated with anti–Crk antibodies, subjected to SDS-PAGE and revealed with antipaxillin and anti–Crk antibodies. (B) Total cell lysates prepared from cells plated on either PL or collagen for 30, 60, 90, 120 min at 37°C were immunoprecipitated with anti–Crk antibodies. Total lysates (left panels) and immunoprecipitates (right panels) were subjected to SDS-PAGE and immunoblotted with antipaxillin, anti–p130Cas and anti–Crk antibodies. Note the presence of the CrkII protein doublet.

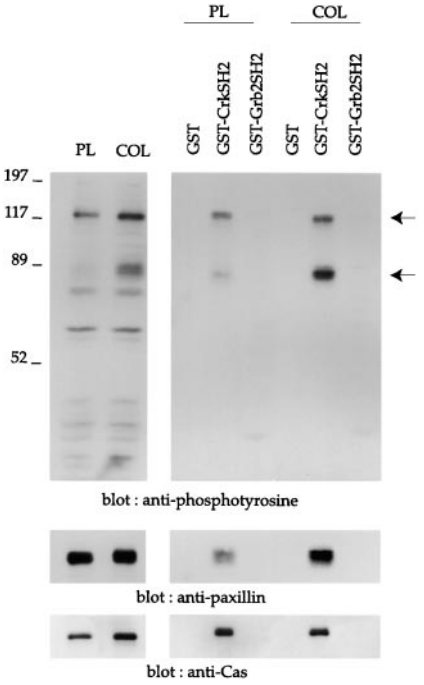

Figure 6. Plating on collagen increases the association of tyrosine-phosphorylated paxillin with GST-CrkSH2 in NBT-II cells. NBT-II cells were plated on either PL or collagen for 90 min at 37°C. Cell lysates were incubated for 2 h at 4°C with GST-CrkSH2, GST-Grb2SH2 fusion proteins or GST alone that were previously immobilized on glutathione-Sepharose 4B beads. Total cell lysates (left panel) and the bound proteins (right panel) were separated by SDS-PAGE and immunoblotted with antiphosphotyrosine, antipaxillin and anti–p130Cas antibodies. The arrows indicate the position of p130Cas (upper) and paxillin (lower).

paxillin failed to bind GST alone. Coimmunoprecipitation experiments were also done from intact cells transfected with either the wild-type paxillin-GFP or the mutant F31/118-GFP form. As shown in Fig. 7 B, only the transfected wild-type paxillin and not the double mutant form was capable of interacting with endogenous Crk. These results demonstrate that the paxillin F31/118 mutations can block the association of paxillin with the SH2 domain of endogenous Crk and, thus, may account for the reduced migration in these cells.

Involvement of Crk in the Collagen-induced Migration of NBT-II Cells

To further investigate the involvement of Crk in the collagen-induced migration, NBT-II cells were transfected with constructs encoding either the wild-type form of CrkII, or a point mutant of the SH2 domain together with a GFP plasmid, and only green fluorescent cells were analyzed for cell migration. The point mutation of Crk at residue 38 (arginine to valine, R38V) abolishes the function of the SH2 domain and prevents its binding to tyrosine-phosphorylated substrates, including paxillin (Matsuda et al., 1993; and our unpublished observations). The data in Fig. 8 show that transient expression of the wild-type CrkII resulted in an increase in cell migration, compared with cells expressing an empty vector. In contrast, the migration of cells overexpressing the R38V mutant of the CrkII SH2 was greatly impaired (Fig. 8). These results suggest that Crk is implicated in the collagen-mediated migration of NBT-II cells, and that it requires a functional SH2 domain.

Some adaptor proteins mediate their effect by coupling tyrosyl-phosphorylated proteins through their SH2 domain to downstream effector molecules using their SH3 domains. Since the SH3-(N) domain of Crk was shown to be involved in regulating the migration of COS cells (Klemke et al., 1998), we studied the role of CrkII SH3 domain in collagen-induced NBT-II cell migration. A point mutant of the SH3-(N) domain of CrkII (W169L) that cannot bind to effector molecules (Matsuda et al., 1994) was transfected in NBT-II. As shown in Fig. 8, expression of this mutant did not promote an increase in cell migration, unlike the wild-type CrkII, suggesting a role for the SH3-(N) domain in the mediation of NBT-II cell migration on collagen.

To further establish a link between paxillin and Crk in the collagen-induced migration of NBT-II, we examined whether Crk could bypass the motility-deficient phenotype observed in cells expressing the F31/118 mutations in paxillin. Stable NBT-II clones expressing the F31/118 mutations in paxillin were cotransfected with wild-type CrkII and GFP, and their migration on collagen was determined.

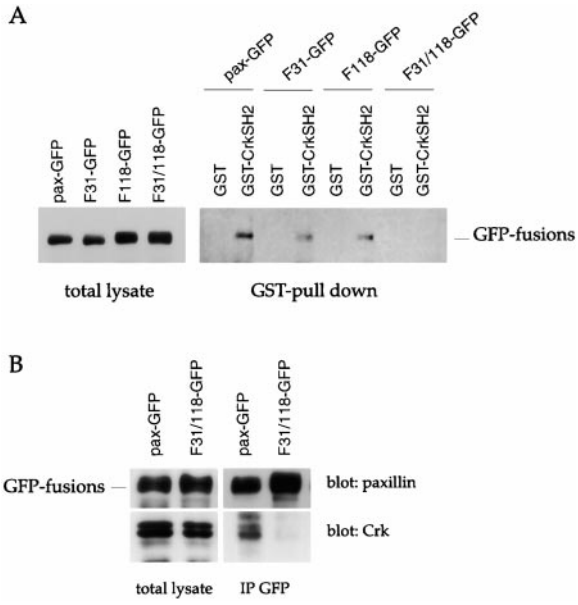

Figure 7. CrkII binding to paxillin is impaired in F31/118-transfected cells. (A) NBT-II cells were transiently transfected for 48 h with constructs encoding the different mutant forms of paxillin fused to GFP. Cells were stimulated on collagen for 90 min at 37°C, and cell lysates were incubated for 2 h at 4°C with GST-CrkSH2 fusion protein, or with GST alone, immobilized on glutathione-Sepharose 4B beads. Total cell lysates (left panel) and bound proteins (right panel) were separated by SDS-PAGE and immunoblotted with antipaxillin antibodies. (B) Cells transfected for 48 h with wild-type paxillin (pax-GFP) or the double mutant form (F31/118-GFP) were collagen stimulated, and the total cell lysates were incubated for 2 h with anti–GFP antibodies. After SDS-PAGE separation, immunoblots were revealed with anti-paxillin and anti–Crk antibodies.

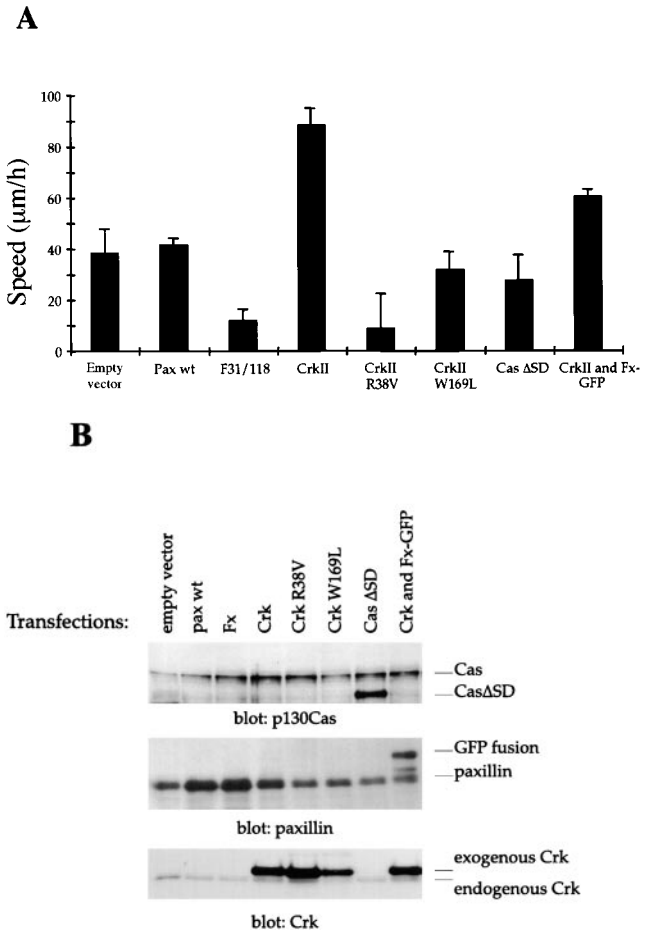

Figure 8. CrkII promotes cell migration of NBT-II cells on collagen. NBT-II cells were transiently transfected for 48 h with wild-type paxillin (Pax wt), CrkII, double paxillin mutants (F31/118), CrkII mutants (CrkII R38V and CrkII W169L), CAS deleted of its substrate domain (CasΔSD), together with a vector encoding GFP (10:1 ratio) or CrkII together with GFP-tagged F31/118 (Fx-GFP). (A) Cells were plated on collagen for 2 h at 37°C, and cell motility was evaluated by tracking individual green fluorescent cells for 12 h as described in Fig. 4. Values obtained from the quantification of the lengths of tracks are presented as μm/h. Each bar represents the mean of at least three independent experiments ± SD. (B) Lysates obtained from cells transfected with the different constructs were analyzed for expression of p130Cas, paxillin, and Crk with specific antibodies.

Expression of CrkII in such cells increased their rate of migration in two independent clones (Fig. 9). Transfection of wild-type paxillin into cells expressing the F31/118 paxillin mutation also could have rescued the dominant-negative effect (Fig. 9). In addition, transient cotransfection of wild-type CrkII and the F31/118-GFP construct showed that the double mutant form of paxillin could diminish the Crk-induced migration (Fig. 8). Taken together, these results indicate that wild-type CrkII is able to bypass the motility-deficient phenotype of the F31/118 paxillin mutations and demonstrate that CrkII acts downstream of paxillin.

Discussion

In this study, we provide evidence demonstrating that tyrosine phosphorylation of two major phosphorylation sites of the adaptor protein paxillin plays a central role in the collagen-induced migration of NBT-II cells. The rat bladder carcinoma NBT-II cell line represents an in vitro model for the study of factors that control the interconversion between the epithelial and mesenchymal states (Boyer et al., 1996). In these cells, stimulation by collagens through the α2β1 integrin results in the loss of epithelial features and the acquisition of mesenchymal properties including cell locomotion (Tucker et al., 1990; Vallés et al., 1996). In contrast, other components of the ECM such as FN or LN only allow cell adhesion and spreading. Therefore, we have used this dependence on the type of substrate to identify the signaling molecules that are activated during the migratory response of NBT-II cells.

We have previously shown the importance of tyrosine phosphorylation during the collagen-induced migration of NBT-II cells (Petit et al., 1999). Comparison of the tyrosine phosphorylation pattern of NBT-II cells stimulated with different ECM proteins revealed that both FAK and paxillin were specifically phosphorylated on tyrosine after collagen induction. FAK-tyrosine phosphorylation has been associated with cell migration in many cell types, and FAK-deficient fibroblasts exhibit a reduced motility (Cary

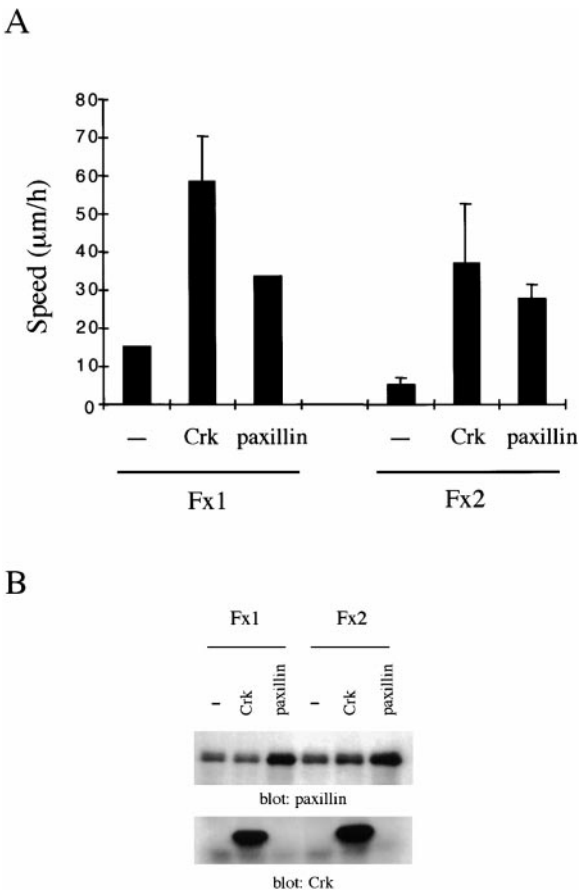

Figure 9. CrkII can rescue the migration deficiency of F31/118-transfected cells. Two stable transfected cell lines (clones Fx1 and Fx2) expressing the F31/118 mutations were transiently transfected for 48 h with wild-type CrkII or paxillin (Pax), together with a vector encoding GFP (10:1 ratio). (A) Cells were allowed to attach to collagen for 2 h at 37°C and cell motility determined as described in Fig. 4. Values obtained from the quantification of track paths are represented in micrometers per hour. Each bar represents the mean of at least three independent experiments ± SD. (B) Lysates obtained from cells transfected with the different constructs were analyzed for expression of paxillin and Crk with specific antibodies.

et al., 1996; Gilmore and Romer, 1996). Induction of FAK phosphorylation was also observed in NBT-II cells induced to scatter with EGF (Boyer et al., 1997). Therefore, FAK is a common target for growth factors and the ECM in the induction of NBT-II cell migration. Tyrosine phosphorylation of paxillin was shown to correlate with the migratory behavior of human melanoma cells on vitronectin (Aznavoorian et al., 1996) and with the invasive property of cervical and prostate cancers (Aprikian et al., 1997; McCormack et al., 1997). In NBT-II cells, stimulation with collagen greatly enhanced tyrosine phosphorylation of paxillin in contrast to cells seeded on other ECM components. This increased tyrosine phosphorylation on collagen was not due to differential spreading of cells on the various substrates, since time course analyses showed the same differences in tyrosine phosphorylation on these substrates (data not shown). Interestingly, paxillin was not phosphorylated on tyrosine after EGF-induced scattering of NBT-II cells (Boyer et al., 1997; Petit, V. and A.M. Vallés, unpublished results). Therefore, it appears that phosphorylation of paxillin on tyrosine is specific for the motile response induced by collagen.

To address the role of paxillin tyrosine phosphorylation in cell migration, we examined the consequences of overexpressing mutant forms of the major tyrosine phosphorylation sites Y31 and Y118. These tyrosine residues previously were shown to be phosphorylated upon adhesion to FN (Bellis et al., 1997). Our study demonstrates that phosphorylation of both Y31 and Y118 is required for an optimal motile response of NBT-II cells on collagen. Single mutations (F31 or F118) on paxillin produced a mild dominant-negative effect on migration with the strongest inhibitory effect obtained when phosphorylation on both tyrosine residues was eliminated. However, other responses to collagen such as cell adhesion and spreading were not affected by these mutations. Recent mutational analysis of paxillin identified the phosphorylation sites LIM2T403 and LIM3S481, rather than Y31 and Y118, as playing a role in these events (Brown et al., 1998). Moreover, correct targeting of paxillin to focal adhesions and to focal complexes does not depend on the phosphorylation of tyrosine residues Y31 and Y118 (Bellis et al., 1995; Brown et al., 1998; this work). These data suggest that the mutations on F31/118 in paxillin exert a dominant-negative effect by substituting for endogenous paxillin at active sites in the cell, thereby preventing the recruitment of specific cellular proteins necessary for the initiation of cell migration. These results also support the notion that phosphorylation of paxillin on distinct residues may serve as a major mechanism for modulating cellular functions like adhesion and migration.

It is well established that phosphorylated tyrosine residues provide docking sites for the interaction with proteins that possess SH2 domains (Koch et al., 1991). The tyrosine phosphorylation of paxillin at residues 31 and 118 creates binding sites for the SH2 domain of the adaptor protein Crk (Birge et al., 1993; Schaller and Parsons, 1995). We showed that the formation of the paxillin–Crk complex is required for the migration of NBT-II cells. In unstimulated cells, paxillin only weakly associates with CrkII. This association increases after collagen stimulation, when paxillin becomes highly phosphorylated and the cells are induced to move. When the cells overexpress the F31/118 mutant proteins, however, formation of the paxillin–Crk complex is prevented and consequently, cell locomotion is inhibited. Moreover, the decreased migration of NBT-II cells overexpressing F31/118 mutants can be rescued by overexpressing wild-type CrkII. Significantly, overexpression of the wild-type CrkII increases the rate of locomotion of NBT-II cells over that of control cells, but the mutant form of CrkII in which the SH2 domain is altered cannot. Taken together, these data support a role for paxillin–Crk complex in the collagen-induced cell migration. It should be noted that overexpression of paxillin did not significantly enhance the motile response to collagen, whereas Crk overexpression greatly stimulated it. This implies that the regulation of paxillin tyrosine phosphorylation by collagen is central in the control of the motile response. In addition, this suggests that Crk, but

not paxillin could be a limiting factor in the induction of cell motility in NBT-II cells. Residue Y118 of paxillin can also recruit the tyrosine kinase Csk via its SH2 domain (Sabe et al., 1995). Although the interaction between paxillin and Csk was not examined in the present study, we cannot exclude a role for this kinase in the collagen-mediated response since it has been described that Crk and Csk could compete for the binding to paxillin (Sabe et al., 1995).

Recently, the adaptor protein Crk was identified as a mediator of cell locomotion through association with p130Cas, another SH2-binding protein (Cary et al., 1998; Klemke et al., 1998). However, in NBT-II cells, the expression of a dominant form of p130Cas, CASΔSD, had only a slight inhibitory effect on random cell migration as compared with the paxillin double mutant (Fig. 8). Although p130Cas is expressed in NBT-II cells, its basal levels of tyrosine phosphorylation remain unchanged, regardless of ECM stimulation. Interestingly, p130Cas could be found associated with Crk in unstimulated NBT-II cells but the association decreased upon collagen stimulation. In contrast, the paxillin–Crk complex, which was detected before collagen stimulation, greatly increased with time. Whereas the paxillin–Crk complex augmented, that of CAS/Crk decreased after collagen stimulation. The selectivity of the paxillin–Crk complex in mediating NBT-II cell migration could be cell type–specific. For example, it has been shown that the activation of β1-integrin in a T cell line induced the association of CrkL with p110 HEF1, a p130Cas-like docking protein, whereas in a megakaryoblastic cell line, CrkL associated with p120 Cbl. While both p110 HEF1 and p120 Cbl are present in the two hematopoietic cell lines, CrkL associated preferentially with a single tyrosine phosphoprotein (de Jong et al., 1997). Alternatively, the choice between different target proteins like paxillin or p130Cas could depend on the type of inducing molecule. This is the case in serum-starved rat-1 fibroblasts where p130Cas, but not paxillin association to CrkII, is regulated by insulin (Nakashima et al., 1999). Stimulatory signals can also regulate the switch of one binding protein for another, as seen for Crk change in association from p130Cas to p120 Cbl upon EGF induction (Khwaja et al., 1996). While in other cases, the stimuli-dependent dephosphorylation of substrates like p130Cas promoted the dissociation from Crk and its association with other docking proteins (Sorokin and Reed, 1998). However, the two possibilities must not be mutually exclusive.

In the present study we show that the NH_2-terminal SH3 domain of Crk is also necessary for the Crk-induced promotion of migration in NBT-II cells as observed in COS cells (Klemke et al., 1998). Numerous cellular proteins have been recently identified on the basis of their interaction with the SH3-(N) domain of Crk, and are thus likely to be downstream effectors in this signaling pathway. Among these is the cellular protein DOCK180, which is a downstream target of integrin-mediated signaling (Hasegawa et al., 1996; Kiyokawa et al., 1998b). DOCK180 can bind directly and activate the small GTPase Rac1 (Kiyokawa et al., 1998a). Importantly, activation of Rac1 promotes the migration and invasion of mammary epithelial cells and the scattering of MDCK cells on collagen (Keely et al., 1997; Sander et al., 1998). Tyrosine phosphorylation–dependent paxillin–Crk complexes could possibly link integrin activation to downstream targets via their interaction with DOCK180.

Alterations in signal transduction pathways have been shown to contribute to initiation and progression of cancer. Specifically, the anchorage-independent growth of malignant cells has implicated changes in integrin-mediated signaling pathways (for reviews see Schwartz, 1997; Keely et al., 1998). For example, constitutive activation of Rho, on an integrin pathway, has been suggested to contribute to cell transformation (Schwartz et al., 1996). Likewise, disregulation of the integrin-linked kinase ILK, by overexpression, induces anchorage-independent cell growth and tumorigenicity (Wu et al., 1998). Similar effects are obtained with a constitutive active form of FAK (Wu et al., 1998). Interestingly, while paxillin phosphorylation by the c-Abl tyrosine kinase is integrin-dependent (Lewis and Schwartz, 1998), in *BCR/ABL*-transformed cells, paxillin was found phosphorylated and constitutively associated with the oncogenic variant $p120^{BCR/ABL}$ (Salgia et al., 1995). Moreover, it recently has been proposed that binding of a viral oncoprotein, the human papillomavirus HPV16 E6 gene product, to paxillin could disrupt its normal function and thereby contribute to neoplastic transformation (Tong and Howley, 1997; Vande Pol et al., 1998). Together, these findings support a model where promiscuous activation of integrin-dependent signaling by oncogenes acting on cytoskeletal proteins could provide a molecular basis for the aberrant behavior of transformed cells during the acquisition of invasive properties.

In conclusion, we have implicated the paxillin–Crk signaling pathway in the collagen-mediated motility response of NBT-II cells. We demonstrated that the collagen-induced phosphorylation of tyrosines 31 and 118 of paxillin is necessary for the interaction of paxillin with the adaptor protein CrkII. Inhibition of the formation of this complex by point mutations on either paxillin or CrkII resulted in reduced cell locomotion on collagen. These defects could be rescued by overexpression of wild-type paxillin. Moreover, over-repression of CrkII also restored the motile response, indicating that CrKII is downstream of paxillin in the signaling pathway. Finally, we demonstrated that the SH3-(N) domain of CrkII is necessary for the motile response to collagen probably by its interaction with downstream motility effector molecules. Therefore, the ability of paxillin to interact with various signaling molecules makes this adaptor protein a central element of regulation where different integrin signaling pathways could become integrated.

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