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) proteins whose pivotal role has been clearly established using in vivo and in vitro model systems (Schörr, 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
Parsons, 1995). Paxillin also contains leucine rich motifs shown to be involved in the binding of Crk (Schaller and Rosines, 1995). These two proteins are coordinately phosphorylated in FAK-overexpressing cells (for review see Turner, 1998). It contains a proline-rich region capable of recruiting multiple signaling molecules (for review see Brown et al., 1996; Cary et al., 1996; Gilmore and Romer, 1996). In addition, the protein tyrosine phosphatases Shp-2 (Y u et al., 1998) and PTP-PEST (A ngers-Loustau et al., 1999) also have been implicated in the regulation of cell motility. 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 (G lenney and Z okas, 1989; Bir ge 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; R ankin and R ozengurt, 1994; M elamed et al., 1995; Turner et al., 1995), and in response to adhesion-mediated events that are associated with remodeling of the actin cytoskeleton (B urridge et al., 1992; Turner, 1998). E vidence from in vitro and in vivo studies has identified paxillin as a potential substrate for FAK (B ellis 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 H anks and Polte, 1997). Paxillin can also serve as a substrate for the protooncogene c-A Bl in an integrin-dependent manner (L ewis 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 (W eng et al., 1993) and two pY X P motifs that conform to consensus binding sites for the SH2 domain of Crk and the protein tyrosine kinase Csk (B irge et al., 1993; S abe 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 (B rown et al., 1996), the F AK-related kinase Pyk2 (Sal gia et al., 1996), the papillomavirus oncoprotein E6 (T ong et al., 1997), or to the recently identified p95PKL, or paxillin-kinase linker that links paxillin to the p21 GTPase-activated kinase PA K, and the guanine nucleotide exchange factor PIX (T urner 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 (B oyer et al., 1996). The induction of cell dispersion is specific for collagen, whereas other ECM components such as fibronectin (FN) and laminin (LN) only allow cell adhesion and spreading (T ucker et al., 1990). Moreover, NBT-II cells can be induced to scatter after stimulation with soluble growth factors such as EGF and FGF (B oyer et al., 1989; Va llés 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 (R odier 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 (T ucker et al., 1990). Fibronectin, laminin 1, and poly-L-lysine were obtained from Sigma Chemical Co. Antibodies against FAK (clone 2A 7) used for immunoprecipitation and phosphotyrosine (clone 4G10) were obtained from U pstate B iotechnology. A ntipaxillin (clone 349), anti-FAK (clone 77), anti-p130Cas (clone 21), and anti-Crk (clone 22) were obtained from Transduction L aboratories. The polyclonal anti-chicken paxillin was previously described (B ellis et al., 1995). A nt-p130Cas (clone 77) used for immunoprecipitation was from Santa Cruz Biotechnology, Inc. A nt-GFP was obtained from Roche M olecular B iochemicals.

Constructions and Plasmids
cDNA s coding for chicken wild-type paxillin and the F118 mutant in the pcDNA3 expression plasmid were previously described (Turner and M iller, 1994; B ellis 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' T AATACGACTCACTATAGGG 3' and 5' TAATACGACTCACTATAGGG 3' using either the wild-type or the F118 cDNA s as template. The resulting fragments were used as primers for a second amplification round with oligo-
nucleotide 5'-GGGGGCTGCTCACCC-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-Sacl fragment of the full-length paxillin cDNA. The BamHI-EcoRI fragments corresponding to full-length paxillin Fl31 and F31/118 were subcloned into the pcDNA3 vector. GFP fusion proteins were generated by PCR amplification with primers 5'-GGGCGCCTGCTACCACTCCGC-3' and 5'-GGGAAATTCGAGAAGTTTGC-3', containing the EcoRI restriction site, to eliminate the stop codon of paxillin. The resulting fragment was digested with EcoRI and SacI and used to replace the Sacl-EcoRI fragment of the wt, F31, F118, or F31/118 constructs. The HindIII-EcoRI fragments corresponding to paxillin mutants replacing the SacII-EcoRI fragment of the wt, F31, F118, or F31/118 constructs. 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-I (R368), Crk-II (W169L), untagged wild-type p130Cas, and p130Cas in in-frame deletion of its substrate domain (CA SAD5, 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; M ayer et al., 1995).

**Cell Culture and Transfections**

The rat bladder carcinoma NBT-II cell line, originally established by Toyoshima and colleagues (Toyoshima et al., 1986), 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). The rat bladder carcinoma NBT-II cell line, originally established by Toyoshima and colleagues (Toyoshima et al., 1986), 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 aminopterin (complete medium). Cells stably expressing the paxillin constructs were routinely grown in the presence of 400 μg/ml aminopterin (complete medium). Cells stably expressing the paxillin constructs were routinely grown in the presence of 400 μg/ml aminopterin (complete medium). Cells stably expressing the paxillin constructs were routinely grown in the presence of 400 μg/ml aminopterin (complete medium). Cells stably expressing the paxillin constructs were routinely grown in the presence of 400 μg/ml aminopterin (complete medium). Cells stably expressing the paxillin constructs were routinely grown in the presence of 400 μg/ml aminopterin (complete medium). Cells stably expressing the paxillin constructs were routinely grown in the presence of 400 μg/ml aminopterin (complete medium). Cells stably expressing the paxillin constructs were routinely grown in the presence of 400 μg/ml aminopterin (complete medium). Cells stably expressing the paxillin constructs were routinely grown in the presence of 400 μg/ml aminopterin (complete medium). Cells stably expressing the paxillin constructs were routinely grown in the presence of 400 μg/ml aminopterin (complete medium). Cells stably expressing the paxillin constructs were routinely grown in the presence of 400 μg/ml aminopterin (complete medium). Cells stably expressing the paxillin constructs were routinely grown in the presence of 400 μg/ml aminopterin (complete medium). Cells stably expressing the paxillin constructs were routinely grown in the presence of 400 μg/ml aminopterin (complete medium). Cells stably expressing the paxillin constructs were routinely grown in the presence of 400 μg/ml aminopterin (complete medium).

**Immunoprecipitation 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 tyrrosine phosphorylation increase without modifying the pattern of phosphorylated proteins. A fter 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 PMSE, 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 PMSE, 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 in 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/0.2% 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; A mersham Corp.). Images were acquired using Photoshop software. Protein concentration was determined using the Bradford protein assay (Bio-Rad Laboratories).

**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, cells were plated on small areas of bacterial petri dishes precoated with various concentrations of collagen and saturated with BSA. A fter 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 H2O. The incor- porated 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 per- cent cells that lost the round morphology. Each point represents duplicate areas and is expressed as the mean of at least three independent experiments ± SD. Non-specific cell adhesion was measured on BSA-coated areas and subtracted from each point.

**Results**

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

Sustained migrations of NBT-II cells are induced by fibrilar collagen, whereas other components of the ECM like...
Figure 1. A 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.

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. cDNA's 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-
localized 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 on 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 phosphorylation 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 phosphoryrosine, 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 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

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**Figure 4 (continues on facing page).**

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A
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).
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. 7B, 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.
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 et al., 1996).
CrkII can rescue the migration deficiency of F31/118-specific antibodies. Constructs were analyzed for expression of paxillin and Crk with SD. (B) Lysates obtained from cells transfected with the different track paths are represented in micrometers per hour. Each bar represents the mean of at least three independent experiments as described in Fig. 4. Values obtained from the quantification of to attach to collagen for 2 h at 37°C with a vector encoding GFP (10:1 ratio). (A) Cells were allowed 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.

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.

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, CA SAD5, had only a slight inhibitory effect on random cell migration as compared with the paxillin double mutant (Fig. 8). Although p130Cas as 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 CA S/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 p1-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 as 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, 1999). However, the two possibilities must not be mutually exclusive.

In the present study we show that the NH₂-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, deregulation 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 p120BCR/ABL (Salgia et al., 1995). Moreover, it recently has been proposed that binding of a viral oncoprotein, the human papillomavirus HPV 16 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 oncoproteins 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.
