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v-Crk-induced cell transformation: changes in focal adhesion composition and signaling

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SUMMARY

v-Crk is an oncogene product in which a viral Gag sequence is fused to a cellular Crk sequence. It contains one SH2 and one SH3 domain. To gain insight into the molecular mechanisms underlying v-Crk-induced cell transformation, we studied the subcellular localization and molecular interactions of v-Crk in v-Crk-transformed NIH-3T3 cells. Our results show that v-Crk specifically localizes to focal adhesions where it induces protein tyrosine phosphorylation. Subcellular fractionation studies indicated that a significant amount of v-Crk is present in the cytoskeletal cell fraction, a fraction that includes focal adhesions. Tyrosine phosphorylated proteins, including p130^{CAS}, were also predominantly found in the cytoskeletal fraction. We show that v-Crk induces a translocation of p130^{CAS} to the cytoskeleton, which is accompanied by hyperphosphorylation of this protein. Mutational analyses showed that a functional v-Crk SH2 domain is required for the localization of v-Crk in focal adhesions. Functional v-Crk SH2 and SH3 domains were both found to be required for the observed increase in tyrosine phosphorylation of focal adhesion proteins and for the translocation and hyperphosphorylation of p130^{CAS}. v-Crk immunoprecipitation studies revealed that cytoskeleton-associated v-Crk interacts with both p130^{CAS} and an unidentified tyrosine kinase. These findings suggest the formation of a focal adhesion-located complex consisting of v-Crk, a tyrosine kinase and p130^{CAS}, which may lead to the hyperphosphorylation of p130^{CAS}. These specific and localized signaling events may represent initial steps in the process of v-Crk-induced cell transformation.

Key words: Cytoskeleton, Focal adhesion, Paxillin, p130^{CAS}, SH2, SH3, v-Crk, Tyrosine phosphorylation

INTRODUCTION

The viral oncogene product v-Crk was originally isolated as the transforming protein of the avian sarcoma virus CT10. The v-Crk protein consists of a viral Gag sequence fused to cellular Crk sequences containing an Src homology (SH) 2 and an SH3 domain (Mayer et al., 1988). These domains are involved in the control of complex formation of signaling proteins (Koch et al., 1991; Musacchio et al., 1992; Mayer and Baltimore, 1993). The v-Crk protein belongs to the family of adaptor proteins (Moran et al., 1990; Pawson and Gish, 1992), like Grb2/Ash/Sem-5 (Lowenstein et al., 1992; Matuoka et al., 1992; Clark et al., 1992) and Nck (Lehman et al., 1990). The c-Crk proteins are the cellular homologues of v-Crk and are expressed in virtually all cells. This family of proteins includes Crk II, comprised of an N-terminal SH2 domain followed by two SH3 domains (Matsuda et al., 1992; Reichman et al., 1992); Crk I, truncated after the N-terminal SH3 domain, thereby most closely resembling v-Crk (Reichman et al., 1992); and CrkL, a Crk-like protein with one SH2 and two SH3 domains that shares about 60% homology with Crk II (ten Hoeve et al., 1993). Little is known yet about the function of the c-Crk proteins, but the existence of an oncogenic counterpart and their composition of SH2 and SH3 domains suggest an important role for the Crk proteins in cellular signaling events. In addition to the viral Gag sequence, the v-Crk protein is truncated after the N-terminal SH3 domain and contains point mutations in both its SH2 and SH3 domains. All of these alterations appear to contribute to the oncogenic potential of this protein (Reichman et al., 1992).

Although the v-Crk protein does not possess any intrinsic kinase activity, at least three proteins, paxillin, p110 and p130^{CAS}, are highly phosphorylated on tyrosyl residues in CT10-transformed chicken embryo fibroblasts, an event that has been correlated with cell transformation (Mayer and Hanafusa, 1990a). v-Crk has also been shown to associate with these proteins via its SH2 domain (Mayer and Hanafusa, 1990b; Matsuda et al., 1990, 1991; Birge et al., 1992, 1993; Sakai et al., 1994a,b; Schaller and Parsons, 1995). The lack of a tyrosine kinase domain in v-Crk suggests that v-Crk cooperates with cellular protein tyrosine kinase activity. It has been demonstrated that the tyrosine kinase c-Abl binds to the

Crk SH3 domain and is able to phosphorylate p130^{CAS} in vitro (Feller et al., 1994; Ren et al., 1994; Mayer et al., 1995). This makes c-Abl a candidate kinase responsible for the increased phosphotyrosine levels in v-Crk-transformed cells.

Adhesion of mammalian cells to components of the extracellular matrix (ECM) is mediated by specialized structures of the cytoskeleton, the focal adhesions (Abercrombie and Dunn, 1975; Izzard and Lochner, 1976; Heath and Dunn, 1978; for reviews see Burridge et al., 1988; Jockush et al., 1995). In these cellular structures, receptors for ECM components, the integrins, are concentrated and connected to actin stress fibers via several structural focal adhesion proteins like talin, vinculin, α-actinin, and paxillin (Lazarides and Burridge, 1975; Burridge and Connel, 1983; Schliwa and Potter, 1986; Turner et al., 1990; Hynes, 1992; Schwartz et al., 1995). Adhesion of cells to the ECM plays an essential role in the regulation of cell proliferation. Without the formation of focal adhesions most mammalian cell types fail to proliferate despite the presence of growth factors and nutrients (Stoker et al., 1968; Guadagno and Assoian, 1991; Han et al., 1993; for recent reviews see Clark and Brugge, 1995; Schwartz et al., 1995). Although the intracellular signals induced by integrins are at present undefined, it is thought that they are mediated by proteins that are recruited to focal adhesion structures upon cell adhesion. Among these are focal adhesion kinase (FAK), c-Src, Grb2 and mSOS1 (Schlaepfer et al., 1994; Schaller and Parsons, 1994). Recently, it has been shown that p130^{CAS} is present in focal adhesions of cells spread on the ECM component fibronectin (Petch et al., 1995), suggesting that p130^{CAS} may also be recruited to focal adhesions upon cell adhesion.

In addition to protein recruitment, increased protein tyrosine phosphorylation of for instance FAK, paxillin, tensin and recently also p130^{CAS} has been shown to occur upon adherence of cells to ECM components (Burridge et al., 1992; Guan and Shalloway, 1992; Clark and Brugge, 1995; Nojima et al., 1995; Petch et al., 1995; Richardson and Parsons, 1995; Vuori and Ruoslahti, 1995). Remarkably, increased tyrosine phosphorylation of paxillin and p130^{CAS} has also been observed in v-Crk- and v-Src-transformed cells (Glenney and Zokas, 1989; Matsuda et al., 1990; Birge et al., 1993; Sakai et al., 1994a), suggesting that these oncoproteins may be involved in the same signal transduction pathways as the integrins. This implies that v-Crk and v-Src may cause cell transformation by interfering with integrin mediated signal transduction. Interestingly, v-Src, the oncogenic counterpart of the non-receptor tyrosine kinase c-Src, and also v-Abl, have been reported to be present in focal adhesion structures (Rohrschneider, 1980; Rohrschneider and Najita, 1984; van Etten et al., 1994), which may explain the increase in tyrosine phosphorylation of focal adhesion proteins. However, so far nothing is known about the localization of v-Crk, or about the mechanism by which the v-Crk adaptor protein induces an increase in protein tyrosine phosphorylation in vivo.

In this paper, we have investigated the localization of v-Crk, and the role of this localization in the increase in protein phosphotyrosine levels. NIH-3T3 cells were stably transfected with wild-type v-Crk cDNA, or with v-Crk SH2 and SH3 loss of function mutants. Localization of the v-Crk proteins was investigated by both immunolocalization and subcellular fractionation. The same approach was used to study the localization of tyrosine phosphorylated proteins. Our results demonstrate that v-Crk is located in focal adhesion structures in an SH2 domain-dependent manner. Elevated phosphotyrosine levels were also found predominantly in cytoskeletal focal adhesion structures, and required both the v-Crk SH2 and SH3 domains. Our data suggest that v-Crk may contribute to cell transformation by recruiting proteins to focal adhesions and by stimulating tyrosine kinase activity at these specific sites, thereby changing both focal adhesion composition and focal adhesion signaling.

MATERIALS AND METHODS

Cell lines

NIH 3T3 fibroblasts were transfected with the expression vector pMEX-neo containing the cDNA of v-Crk, or the indicated v-Crk mutants, using the calcium-phosphate precipitation method (Graham and van der Ab, 1973). Transfected cells were selected and maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Paisley, UK) supplemented with 7.5% fetal calf serum (Gibco, Paisley, UK) and 400 μg/ml G418. Colonies were isolated and screened for expression of the v-Crk proteins by immunoblotting and immunofluorescence. To assay anchorage-independent growth, cells expressing the v-Crk proteins were trypsinized and 10,000 cells were plated per 6 cm plastic dish in 2 ml of DMEM containing 10% fetal calf serum and 0.375% agar (Difco, Detroit, MI, USA) on a layer of 2 ml of the same medium containing 0.5% agar. The plates were incubated at 37°C for three weeks and colonies were scored.

Antibodies

The monoclonal antibody against the Gag fusion of v-Crk, mAb 3C2, has been previously described (Potts et al., 1987). The monoclonal antibodies against phosphotyrosine (PY20) and p130^{CAS} were purchased from Transduction Laboratories, Lexington, KY, USA; the monoclonal antibody against paxillin was from Zymed, San Francisco, USA; Cyanine Cy3- and fluorescein-conjugated secondary anti-mouse antibody and secondary horseradish peroxidase-coupled anti-mouse antibody were purchased from Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA.

Construction of v-Crk mutants

The v-Crk SH2 mutants R273N and H294R were generated by oligonucleotide-directed mutagenesis and subcloned into pBluescript (pCT10) (Mayer and Hanafusa, 1990a). Histidine at position 294 was changed to arginine (H294R) and arginine at position 273 was changed to asparagine (R273N). These amino acid changes were generated because the arginine at position 273 of the molecule is conserved in all known SH2 domains, except protein tyrosine phosphatase 1C, and is located within the highly conserved FLV**R**XS hexapeptide. Likewise, the histidine at position 294 (β-D4 position) is invariant in most SH2 domains and is located at the C-terminal boundary of the motif. The v-Crk SH3 mutant was made by ligation of an *Sph*I 8mer linker to *Bgl*II-cut, end-filled pCT10 DNA (Mayer and Hanafusa, 1990a). A 1.8 kb *Alw*N1 fragment containing the entire coding region of the v-Crk SH2 or SH3 mutants was subcloned from pCT10, and ligated into *Bam*HI linearized pMEXneo vector, using *Bam*HI adaptors (5′ GATCAAG 3′ plus 5′ GATCCCT 3′). For transformation the DH5α bacterial strain was used, and random colonies were picked and used for maxipreps. All constructs were verified by DNA sequencing.

Immunofluorescence

Cells grown on glass coverslips were washed twice with phosphate buffered saline (PBS, pH 7.2), and fixed for 30 minutes at room temperature in freshly prepared 3% formaldehyde (Polysciences Inc.,

Warrington, PA, USA) in PBS. Fixed cells were washed twice with PBS and permeabilized in 0.2% Triton X-100 in PBS for 5 minutes at room temperature. Cells were rinsed and subsequently incubated in 50 mM glycine in PBS for 10 minutes. Cells were washed with PBS containing 0.2% gelatine (PBG) and incubated with primary antibodies. Primary antibodies were used at the following dilutions: PY20 1:200, anti-paxillin 1:200 and anti-Gag 1:400 in PBG. Phalloidin-FITC (Sigma) was diluted 1:10, 000 in PBG. Incubation was for 60 minutes at room temperature. After washing with PBG, cells were incubated with fluorescein-conjugated anti-mouse antibody (dilution 1:200), or Cy3-conjugated anti-mouse antibody (dilution 1:500) for double-labeling experiments with phalloidin-FITC, in PBG for 45 minutes at room temperature. After rinsing in PBG the preparations were mounted in Mowiol 4-88 (Hoechst, Frankfurt, FRG) supplemented with 0.1% paraphenylene diamine and examined with a Leitz Orthoplan microscope.

Cell fractionation

Cell fractionation was performed as previously described (van Bergen en Henegouwen et al., 1989; Kaplan et al., 1994) with some minor modifications. Briefly, cell lines expressing v-Crk proteins were plated on 10 cm diameter tissue culture dishes (Costar, Cambridge, MA, USA). The next day cells were chilled on ice for 15 minutes and then washed twice with 5 ml ice-cold PBS. Cells were subsequently lysed for 2 minutes in 500 μl of cytoskeleton stabilizing (CSK) buffer (10 mM Pipes, pH 6.8, 0.3 M sucrose, 0.5% Triton X-100, 100 mM KCl, 1 mM $CaCl_2$, 2.5 mM $MgCl_2$, with the following phosphatase inhibitors: 1 mM Na_3VO_4, 1 mM Na_2MoD_4, 10 mM NaF and the following protease inhibitors: 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF and 1 mM benzamidine). Cells were briefly washed (<5 seconds) in 500 μl CSK buffer and combined with the lysate, resulting in a final volume of 1 ml; this fraction is referred to as the detergent-soluble fraction. For immunoprecipitation, the cytoskeletal fraction was lysed in 1 ml RIPA buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 10 mM Na_2HPO_4 and phosphatase and protease inhibitors as described above) for 5 minutes on ice, after which the material was scraped off the dish. Lysates were centrifuged at 14,000 ***g*** at 4°C for 10 minutes to clear cell debris and transferred to fresh tubes before being immunoprecipitated as described below. When cell fractions were used for immunoblotting, cells were plated on 6-well tissue culture plates and proteins of the detergent-soluble fraction were precipitated with 10% (w/v) tri-chloro-acetic acid (TCA), washed once with ice-cold acetone, air dried, and solubilized in 80 μl sample buffer (60 mM Tris-HCl, pH 6.8, 10% glycerol, 45 mM dithiothreitol, 80 mM sodium dodecylsulfate). The cytoskeletal proteins were directly solubilized in 80 μl sample buffer. When necessary, protein samples prepared from v-Crk or v-Crk mutant cell lines were normalized for equal amounts of v-Crk protein.

Immunoprecipitation and immunoblotting

For immunoprecipitation, both cell fractions were diluted twice with RIPA buffer. Lysates were precleared by incubating for 1 hour at 4°C with 40 μl of a 50% slurry of Protein A-Sepharose, after which the beads were pelleted. Subsequently, 2 μg of PY20 antibody or 2 μg anti-Gag antibody, was added to the precleared cell lysates, and incubated overnight at 4°C. Protein A-Sepharose was added as described above and incubated for 1 hour at 4°C. Beads were washed three times with 1 ml of ice-cold RIPA buffer, boiled in sample buffer, and loaded onto SDS-polyacrylamide gels. After electrophoresis, proteins were transferred to PVDF membrane (Immobilon, Bedford, USA) and blots were blocked in 5% BSA in TBST (10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.01% Tween-20) when PY20 was used, and in 2% milk powder (Protifar, Nutricia, Zoetermeer, The Netherlands) in PBST (PBS with 0.01% Tween-20) for the other antibodies, for 1 hour at 37°C. Subsequently, blots were incubated with primary antibody (PY20, 1:10,000; anti-Gag, 1:5,000; anti-$p130^{CAS}$, 1:2,000; anti-paxillin, 1:10,000) in 0.5% BSA in TBST for PY20 and in 0.2% milk powder in PBST for the others, for 1 hour at room temperature. After extensive washing, blots were incubated for 1 hour at room temperature with secondary horseradish peroxidase (HRP) coupled antibodies diluted 1:10,000 in the same buffers as used for the primary antibodies. Proteins were detected using the chemiluminescence procedure as described by the manufacturer (Dupont, Boston, USA).

Immune complex kinase assay

Immunoprecipitations using the anti-Gag antibody were performed as described above. After washing with RIPA buffer, the immunoprecipitates were washed twice with 1 ml kinase buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% β-mercaptoethanol, 0.1% Tween-20, 5 mM $MnCl_2$). Kinase reactions were carried out in 50 μl kinase buffer containing 1 mM Na_2MoD_4 and 5-10 μCi [γ-^{32}P]ATP for 60 minutes at 30°C. Kinase reactions were stopped by adding sample buffer and incubation for 5 minutes at 95°C. Phosphorylated proteins were analyzed by SDS-PAGE and autoradiography (overnight). Alkali treatment of gels was done with 1 M NaOH at 55°C for 2 hours.

RESULTS

v-Crk localizes to focal adhesions

The localization of v-Crk was investigated both by immunolocalization and cell fractionation. For these experiments v-Crk cDNA, under the control of the murine sarcoma virus long terminal repeat (MSV-LTR) promoter, was stably transfected into NIH-3T3 fibroblasts. Geneticin (G418) resistant colonies were examined for v-Crk expression using a monoclonal antibody against the Gag portion of v-Crk, which allows the discrimination between c-Crk and v-Crk. Twenty-four independent clones expressing both high and low levels of the v-Crk protein, were labeled with the anti-Gag antibody and studied by immunofluorescence microscopy. In all clones examined, v-Crk staining was seen as bright spots at the cell bottom, most likely reflecting focal adhesions, together with nuclear and cytoplasmic staining (Fig. 1A). Control NIH-3T3 fibroblasts did not show any immunoreactive labeling (data not shown). To ascertain that the focal adhesion-like labeling pattern seen with the anti-Gag antibody is equivalent to focal adhesion staining, we double-labeled v-Crk expressing cells with phalloidin-FITC, which stains the actin stress fibers, and anti-Gag or anti-paxillin. Paxillin is a well known focal adhesion protein (Turner et al., 1990). Focal adhesions were clearly visible in paxillin labeled v-Crk expressing cells (Fig. 1C), and the punctate staining pattern was similar to that found in anti-Gag labeled cells. When Fig. 1A and C are compared to Fig. 1B and D, it is clear that the bright spots seen with anti-Gag and anti-paxillin labeling, are located at the ends of actin stress fibers, thereby reflecting focal adhesion structures.

Focal adhesions are specialized structures that connect integrins to microfilaments (Heath and Dunn, 1978; Burridge et al., 1988). In this respect, focal adhesions might be considered as specialized parts of the cytoskeleton. The cytoskeleton can be isolated as the detergent-insoluble fraction, and has previously been shown to contain microfilaments, intermediate filaments, and the focal adhesion proteins FAK, v-Src, c-Src, paxillin, vinculin, talin and α-actinin (Brown et al., 1976; Lenk et al., 1977; Capco and Penman, 1983; van Bergen en Henegouwen et al., 1992; Kaplan et al., 1994; and data not shown). The extraction was

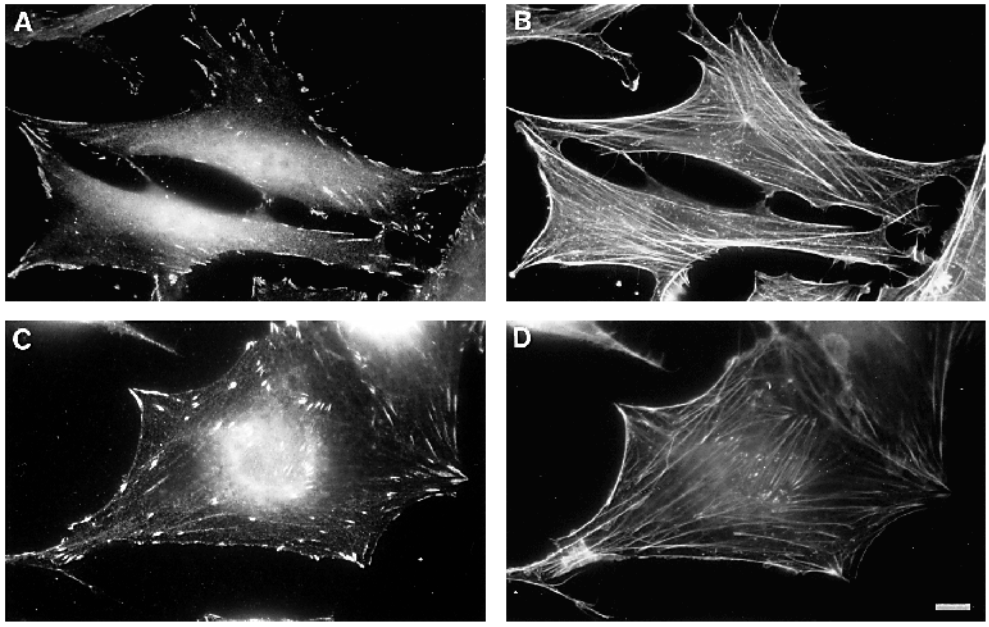

Fig. 1. v-Crk localizes to focal adhesions. NIH-3T3 fibroblasts expressing v-Crk were plated on glass coverslips in serum-containing medium. Cells were fixed and double-stained with a monoclonal antibody against the viral Gag protein (A), or a monoclonal antibody against paxillin (C), and phalloidin-FITC (B and D). Bar, 1.5 μm.

performed at 4°C, resulting in the absence of tubulin due to depolymerization of microtubules at this temperature (Lenk et al., 1977; van Bergen en Henegouwen et al., 1992). To examine whether v-Crk co-fractionates with the cytoskeleton, we dissected v-Crk expressing cells into two fractions, the cytoskeletal (detergent-insoluble) and the detergent-soluble fraction (Brown et al., 1976; Lenk et al., 1977; van Bergen en Henegouwen et al., 1989; Kaplan et al., 1994). As shown in Fig. 2A, v-Crk is present in both cell fractions. The same results were obtained with two independent clones (data not shown). Paxillin was also found in both the cytoskeletal and the detergent-soluble fraction (Fig. 2B).

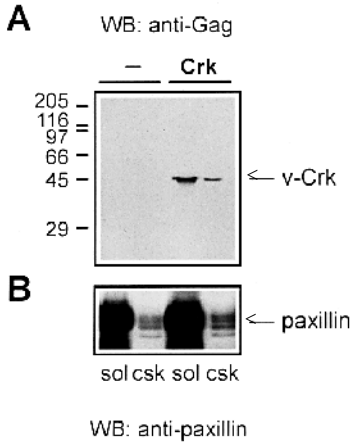

Fig. 2. v-Crk is present in the detergent-soluble and the cytoskeletal fraction of v-Crk expressing NIH-3T3 fibroblasts. NIH-3T3 fibroblasts (–) and fibroblasts expressing v-Crk (Crk) were fractionated into the detergent-soluble (sol) and the detergent-insoluble cytoskeletal fraction (csk) as described in Materials and Methods. Proteins were separated on 12% SDS-PAGE and blotted onto PVDF membrane. For immunodetection of v-Crk and paxillin the anti-Gag antibody (A) or a monoclonal antibody against paxillin (B) were used. Molecular mass standards are indicated in kDa.

SH2-dependent localization of v-Crk in focal adhesions

In order to examine whether the SH2 and/or the SH3 domain of v-Crk is required for the focal adhesion localization, v-Crk mutated in either the SH2 (SH2a and b) or the SH3 domain (SH3) was stably transfected into NIH-3T3 fibroblasts, and cells were used for immunolocalization and cell fractionation studies. Both SH2 mutants contain a single amino acid substitution at well-conserved residues (Mayer and Hanafusa, 1990a). The SH3 mutant has an in frame linker insertion of five amino acids (Mayer and Hanafusa, 1990a) (Fig. 3). From each transfection, 12 independent clones expressing high levels of mutant v-Crk protein were selected, using immunoblotting and immunofluorescence (data not shown). Immunoblotting showed that the expression levels of the SH2 mutants were significantly lower than that of v-Crk and the v-Crk SH3 mutant. Immunofluorescence studies revealed that only a subpopulation of cells highly express the v-Crk SH2 mutants, while other cells do not express these mutants at all, or at very low levels. We also observed this phenomenon in PC12 cells transfected with the v-Crk SH2 mutants. A possible explanation may be that the v-Crk SH2 mutants are dominant negative for growth.

In all clones examined, anti-Gag immunolabeling demonstrated the presence of the v-Crk SH3 mutant in focal adhesions (Fig. 4D). Cells expressing high levels of the v-Crk SH2 mutant, however, did not show v-Crk labeling at these

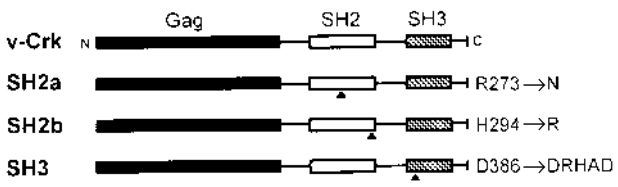

Fig. 3. Schematic representation of v-Crk mutants. The viral Gag sequence, the SH2, and the SH3 domain are indicated. Amino acid changes in the various mutants are indicated on the right in the single-letter amino acid code.

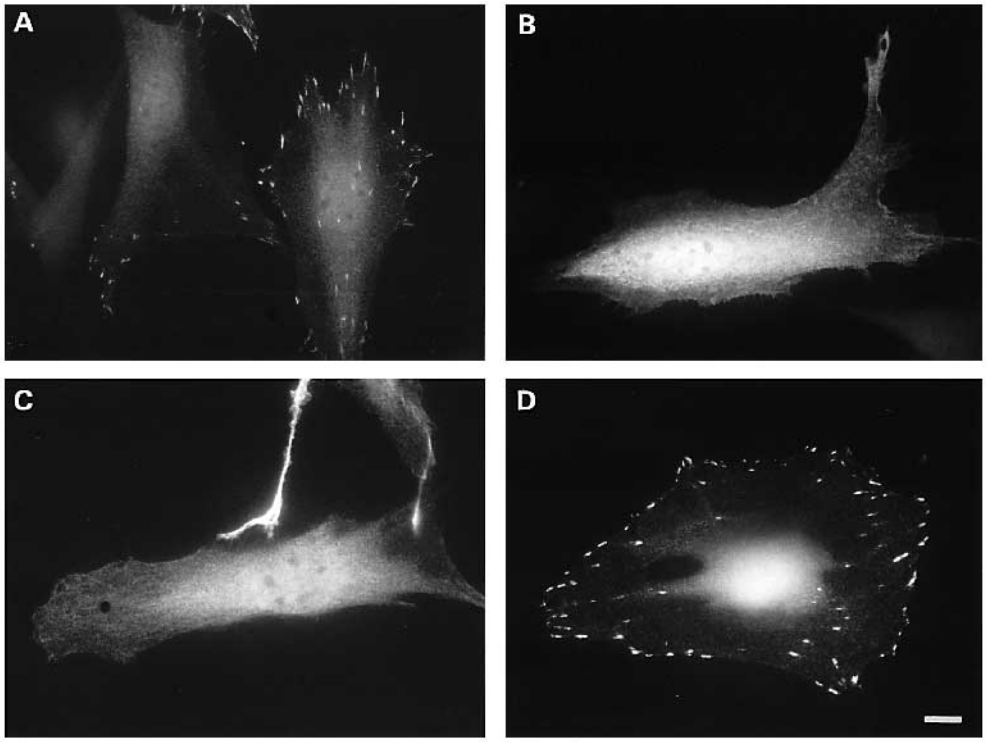

Fig. 4. SH2-dependent localization of v-Crk in focal adhesions. NIH-3T3 fibroblasts expressing either v-Crk (A), SH2a (B), SH2b (C), or SH3 (D) were plated on glass coverslips in serum-containing medium. Cells were fixed and immunolabeled with the anti-Gag antibody. Bar, 1.5 μm.

sites, despite the fact that these cells still contain focal adhesion structures as detected by paxillin staining (data not shown). Instead, they exclusively showed nuclear and cytoplasmic staining (Fig. 4B and C), demonstrating that a functional SH2 domain is essential for focal adhesion localization. These data correlated well with the cell fractionation data. v-Crk and SH3 mutant v-Crk were found both in the cytoskeletal and in the detergent-soluble fraction, whereas the SH2 mutants were exclusively present in the detergent-soluble fraction (Fig. 5A). The latter result also demonstrates that the presence of v-Crk in the cytoskeletal fraction is not due to non-specific binding to cytoskeletal proteins, which might possibly be induced by overexpression of the v-Crk protein. Paxillin was found to be present in the both the detergent-soluble and the cytoskeletal cell fraction of all v-Crk-expressing cell lines (Fig. 5B). Taken together, these results demonstrate that the presence of v-Crk in focal adhesions requires a functional v-Crk SH2 domain.

v-Crk induces an increase in tyrosine phosphorylation of focal adhesion proteins

CT10 infection of chicken embryo fibroblasts causes cell transformation concomitant with an increase in tyrosine phosphorylation of a specific set of proteins, including paxillin and p130^{CAS} (Mayer and Hanafusa, 1990a). In order to gain more insight in the role of focal adhesion localization of v-Crk in these processes, phosphotyrosine levels of focal adhesion proteins in cells expressing v-Crk, or the v-Crk mutants, were investigated. Before this, we studied the transforming capacity of v-Crk by studying cell morphology and anchorage-independent cell growth of NIH-3T3 fibroblasts transfected with v-Crk, or the v-Crk mutants. Cells expressing v-Crk showed a spindle and refractile cell morphology (Fig. 6) and formed colonies in soft agar (25.6±1.8% colony forming ability), indicating that v-Crk is transforming when transfected into NIH-3T3 fibroblasts. SH2 and SH3 mutant expressing cells, however, were morphologically identical to control cells (Fig. 6) and did not show anchorage-independent growth, consistent with previous data obtained from CT10 infection of chicken embryo fibroblasts (Mayer and Hanafusa, 1990a).

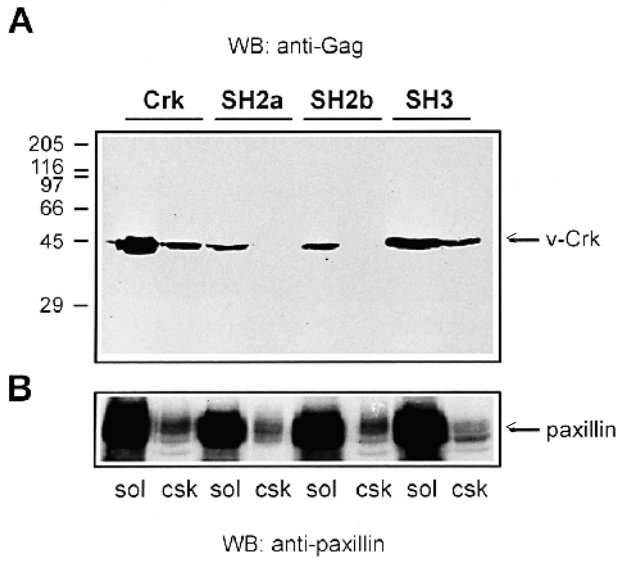

Fig. 5. SH2-dependent localization of v-Crk in the cytoskeletal fraction. NIH-3T3 fibroblasts expressing v-Crk (Crk), or the v-Crk mutants (SH2a, SH2b and SH3) were fractionated as described in Materials and Methods. Detergent-soluble (sol) and cytoskeletal (csk) proteins, were separated on 12% SDS-PAGE and blotted onto PVDF membrane. The anti-Gag antibody (A) or a monoclonal antibody against paxillin (B) were used for immunodetection of the v-Crk proteins and paxillin. Molecular mass standards are indicated in kDa.

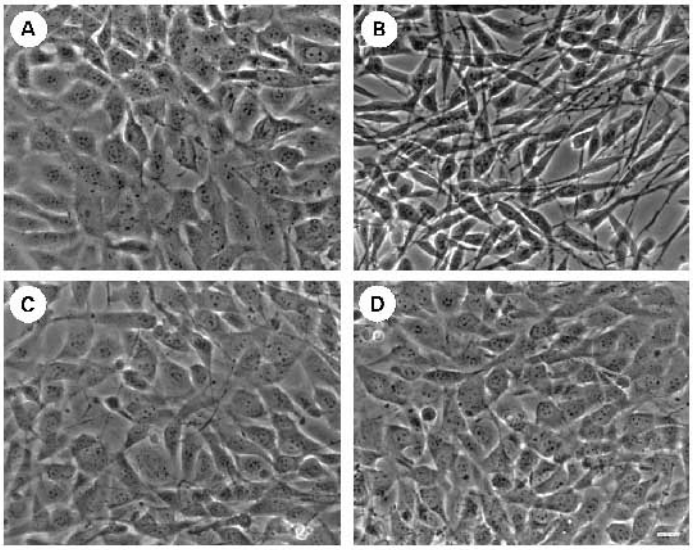

Fig. 6. Morphological alteration of v-Crk expressing fibroblasts. NIH-3T3 cells (A) and fibroblasts expressing v-Crk (B), the v-Crk SH2a mutant (C), or the v-Crk SH3 mutant (D) were analyzed by phase contrast microscopy. Bar, 10 μm.

Tyrosine phosphorylation of focal adhesion proteins was investigated using the anti-phosphotyrosine antibody PY20 for immunofluorescence and immunoprecipitation studies. Two independent clones of v-Crk and mutant v-Crk expressing cells were used. Immunofluorescence revealed a much stronger labeling of focal adhesions in v-Crk-transfected NIH-3T3 fibroblasts, reflecting higher phosphotyrosine levels in focal adhesions, when compared to non-transfected cells (Fig. 7). In contrast to v-Crk, cells expressing SH2 and SH3 mutant v-Crk did not show an increase in tyrosine phosphorylation at these sites (data not shown). These observations were confirmed by cell fractionation. Tyrosine phosphorylated proteins were immunoprecipitated from both the detergent-soluble and the cytoskeletal cell fraction and detected on a western blot using the PY20 anti-phosphotyrosine antibody. As shown in Fig. 8A, tyrosine phosphorylation is increased in the detergent-soluble fraction of v-Crk transfected cells, but a major increase in tyrosine phosphorylation is observed in the cytoskeletal fraction. Both fractions showed increased tyrosine phosphorylation of several proteins. The two major phosphorylated proteins have an apparent molecular mass of 68 kDa (p68) and 130 kDa (p130). The SH2 and SH3 mutant expressing cells, however, did not show elevated phosphotyrosine levels in the cytoskeletal nor in the detergent-soluble fraction (Fig. 8A), indicating that v-Crk-induced protein tyrosine phosphorylation requires both v-Crk SH2 and SH3 domains to be functional. It is interesting to note that, while the v-Crk SH3 mutant localizes to focal adhesions, it does not appear to increase tyrosine phosphorylation of focal adhesion proteins. This suggests that the v-Crk SH3 domain may be responsible for the activation of a tyrosine kinase in focal adhesions.

v-Crk induces a redistribution of p130^{CAS} to the cytoskeletal fraction

Adaptor proteins have been shown to recruit signal transduction proteins to their site of action (Pawson and Gish, 1992; Bar-Sagi et al., 1993). We were interested to know whether the v-Crk adaptor protein is able to recruit signaling proteins to the cytoskeletal cell fraction. Since p68 and p130 were the most prominent phosphotyrosine containing proteins found in v-Crk expressing NIH-3T3 cells (Fig. 8A), we investigated whether v-Crk induces translocation of these proteins to the cytoskeleton.

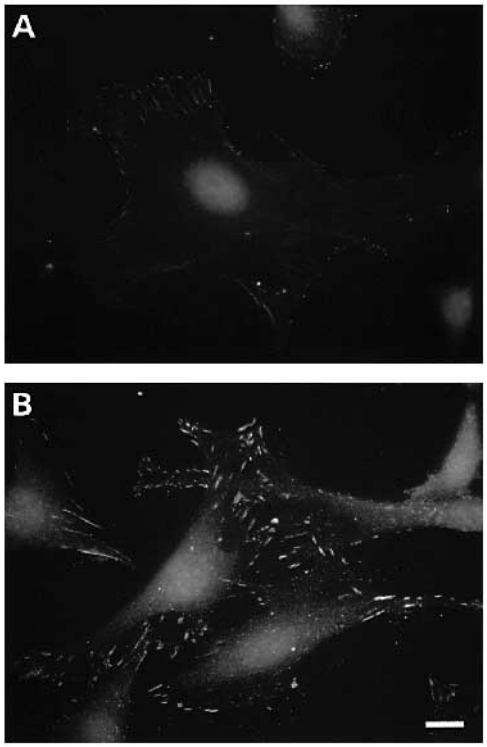

Fig. 7. Increased protein phosphotyrosine levels in focal adhesions of v-Crk expressing cells. NIH-3T3 fibroblasts (A) and fibroblasts expressing v-Crk (B) were fixed and immunolabeled with the monoclonal PY20 antibody to visualize tyrosine phosphorylated proteins. Exposure times and development of both films and micrographs were identical. Bar, 3.0 μm.

In order to test whether the tyrosine phosphorylated proteins p68 and p130 were paxillin and p130^{CAS}, the anti-phosphotyrosine blot (Fig. 8A) was stripped and reprobed with monoclonal anti-paxillin and anti-p130^{CAS} antibodies. The 68 kDa and 130 kDa tyrosine phosphorylated proteins could indeed be identified as paxillin and p130^{CAS} (Fig. 8B and C). Tyrosine phosphorylated p130^{CAS} was present in the detergent-soluble fraction, but even more abundantly in the cytoskeletal fraction of v-Crk-transformed cells (Fig. 8C). This is in contrast to phosphorylated paxillin, which was predominantly found in the detergent-soluble fraction (Fig. 8B). Interestingly, not only the amount of tyrosine phosphorylated p130^{CAS} protein was elevated in the cytoskeletal fraction of v-Crk expressing cells, but also the number of phosphorylation sites (hyperphosphorylation) was increased, as can be seen by the mobility shift of p130^{CAS} (Fig. 8C).

The increase in the amount of tyrosine phosphorylated p130^{CAS} protein in the cytoskeletal fraction can be explained by an increase in phosphorylation of p130^{CAS} that is already present in this cell fraction, but also by a v-Crk-induced translocation of p130^{CAS} to cytoskeletal focal adhesion struc-

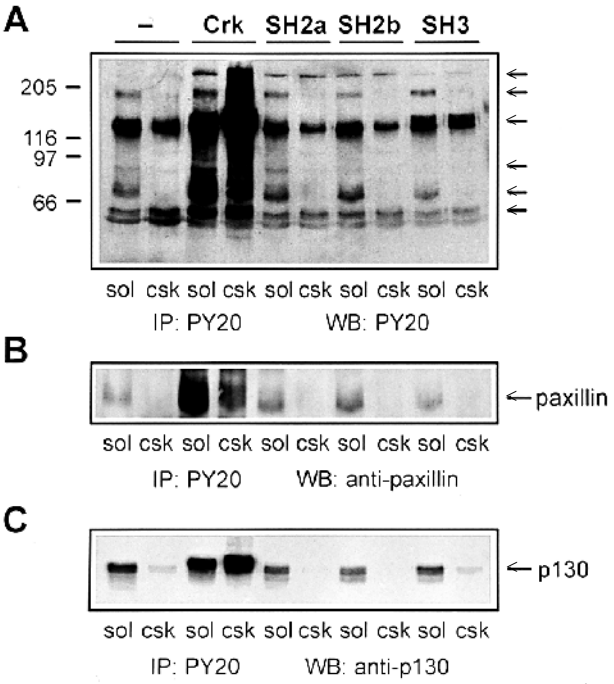

Fig. 8. Increased protein phosphotyrosine levels in the detergent-soluble and the cytoskeletal fraction of v-Crk expressing cells. (A) NIH-3T3 fibroblasts (–) and fibroblasts expressing v-Crk (Crk), or the v-Crk mutants (SH2a, SH2b and SH3), were fractionated as described in Materials and Methods. Phosphotyrosine containing proteins were immunoprecipitated from detergent-soluble (sol) and cytoskeletal (csk) cell fractions with the monoclonal PY20 antibody. Proteins were separated on 6% SDS-PAGE, blotted, and phosphotyrosine containing proteins were detected using the PY20 antibody. Proteins showing increased phosphotyrosine levels are indicated by arrows. The immunoblot shown in A was stripped and reprobed with a monoclonal antibody against paxillin (B) or a monoclonal antibody against p130^{CAS} (C). Molecular mass standards are indicated in kDa.

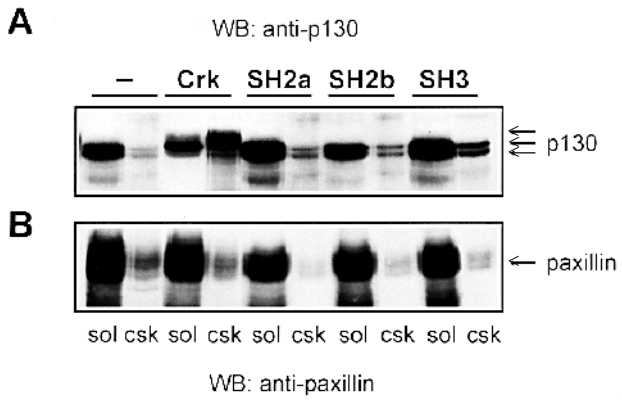

Fig. 9. p130^{CAS}, and not paxillin, is redistributed to the cytoskeletal cell fraction. NIH-3T3 fibroblasts (–) and fibroblasts expressing v-Crk (Crk) or the v-Crk mutants (SH2a, SH2b and SH3) were fractionated as described in Materials and Methods. Detergent-soluble (sol) and cytoskeletal (csk) proteins were separated on 6% SDS-PAGE and blotted. The monoclonal anti-p130^{CAS} (A) and anti-paxillin (B) antibodies were used for immunodetection.

tures. To test whether v-Crk is able to recruit p130^{CAS} or paxillin to the cytoskeleton, we examined p130^{CAS} and paxillin distribution in cytoskeletal and detergent-soluble fractions of v-Crk and mutant v-Crk expressing cells. The anti-p130^{CAS} antibody recognized three distinct bands, which is in agreement with previous data (Sakai et al., 1994a). In Fig. 9 it is shown that p130^{CAS} and paxillin are present in both the cytoskeletal and the detergent-soluble fraction of control, v-Crk and v-Crk mutant expressing cells. However, the cytoskeletal fraction of v-Crk expressing cells contained a much higher amount of p130^{CAS} than the cytoskeletons of control cells and cells expressing the v-Crk mutants (Fig. 9A). In v-Crk-transformed cells, almost 60% of total p130^{CAS} protein was associated with the cytoskeleton, whereas only 7% was present in the cytoskeletal fractions of control and v-Crk mutant expressing cells. Such a translocation was not observed for paxillin (Fig. 9B). These data demonstrate that v-Crk induces a dramatic redistribution of p130^{CAS} from the detergent-soluble to the cytoskeletal fraction, in a v-Crk SH2- and SH3 domain-dependent manner.

Additionally, p130^{CAS} present in the cytoskeletal fraction of cells expressing v-Crk was hyperphosphorylated (Fig. 9A, see also Fig. 8A and C). A shift of p130^{CAS} to the middle band was seen in the detergent-soluble fraction of v-Crk expressing cells, demonstrating that there is also an increase in tyrosine phosphorylation of detergent-soluble p130^{CAS}, which is in agreement with the results shown in Fig. 8. This increase was, however, not as high as the increase found in the cytoskeletal fraction, implying that specifically cytoskeleton-associated p130^{CAS} is hyperphosphorylated.

Binding of the v-Crk SH2 domain to cytoskeletal proteins

The SH2-dependent localization of v-Crk in focal adhesions suggests that v-Crk and the v-Crk SH3 mutant associate with tyrosine phosphorylated proteins in the focal adhesion. To investigate this, we immunoprecipitated v-Crk or the v-Crk mutants from both detergent-soluble and cytoskeletal cell fractions and studied co-precipitation of the two major tyrosine phosphorylated proteins paxillin and p130^{CAS}. In Fig. 10 it is shown that both paxillin and p130^{CAS} co-precipitate with cytoskeleton-associated v-Crk of v-Crk-transformed cells. In contrast to paxillin, p130^{CAS} also co-precipitated with soluble v-Crk, although to a much lesser extent. Both v-Crk SH2 mutants did not associate with paxillin or p130^{CAS}, as was to be expected. Although we could detect a little tyrosine phosphorylated p130^{CAS} in the cytoskeletal fraction of control cells and cells transfected with the v-Crk SH3 mutant (Fig. 8C), we did not detect co-precipitation of paxillin or p130^{CAS} with the v-Crk SH3 mutant in the cytoskeletal cell fraction. Only a very small amount of p130^{CAS} associated with the v-Crk SH3 mutant immunoprecipitated from the soluble fraction (Fig. 10). These data show that v-Crk binds to paxillin and p130^{CAS} primarily in the cytoskeletal cell fraction of v-Crk-transformed cells. The identity of the tyrosine phosphorylated focal adhesion protein that associates with the v-Crk SH3 mutant remains unclear.

Cytoskeleton-associated v-Crk co-precipitates protein tyrosine kinase activity

It has previously been shown that tyrosine kinase activity is present in v-Crk immunoprecipitates prepared from CT10

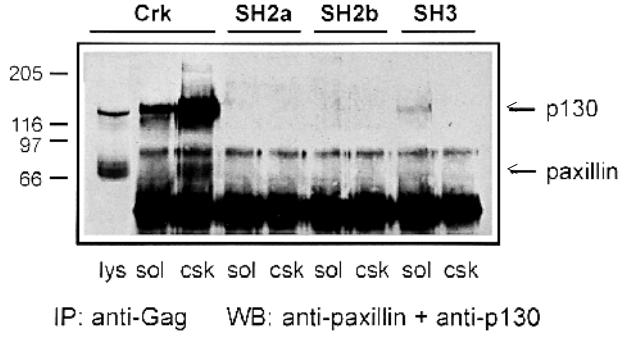

Fig. 10. Binding of v-Crk to cytoskeletal proteins. Fibroblasts expressing v-Crk (Crk) or the v-Crk mutants (SH2a, SH2b and SH3) were fractionated as described in Materials and Methods. The v-Crk proteins were immunoprecipitated from detergent-soluble (sol) and cytoskeletal (csk) cell fractions using the anti-Gag antibody. Proteins were separated on 10% SDS-PAGE and blotted onto PVDF membrane. Monoclonal anti-paxillin and anti-p130^{CAS} antibodies were used simultaneously for immunodetection of paxillin and p130^{CAS}. A total cell lysate (lys) of v-Crk-expressing fibroblasts was used as a positive control for immunodetection of paxillin and p130^{CAS}. The bands at the bottom of the blot represent the immunoglobulins used for immunoprecipitation of the v-Crk proteins. Molecular mass standards are indicated in kDa.

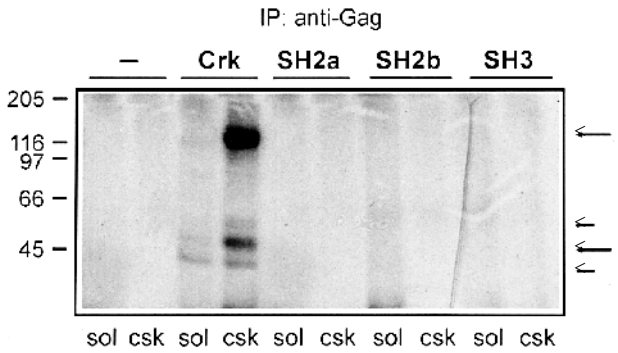

Fig. 11. Cytoskeleton associated v-Crk co-precipitates protein tyrosine kinase activity. NIH-3T3 fibroblasts (–) and fibroblasts expressing v-Crk (Crk) or the v-Crk mutants (SH2a, SH2b and SH3) were fractionated as described in Materials and Methods. The v-Crk proteins were immunoprecipitated from both detergent-soluble (sol) and cytoskeletal (csk) cell fractions using the anti-Gag antibody. Immune complex kinase assays were performed as described in Materials and Methods. Phosphorylated proteins were analyzed by 10% SDS-PAGE and autoradiography (overnight) of the alkali treated gel. Major phosphorylated proteins are indicated by large arrows, minor phosphorylated proteins by small arrows.

infected chicken embryo fibroblasts (Mayer and Hanafusa, 1990b). As we found that increased protein tyrosine phosphorylation in v-Crk-transformed cells is primarily found in focal adhesions and the cytoskeletal fraction, we next studied whether v-Crk-associated tyrosine kinase activity is also mainly present in the cytoskeletal fraction. To investigate this v-Crk, or the v-Crk mutant proteins, were immunoprecipitated from detergent-soluble and cytoskeletal cell fractions using the anti-Gag antibody. Immunoprecipitates were incubated with [γ-^{32}P]ATP, proteins were separated on SDS-PAGE, and the gel was alkali treated to study protein tyrosine phosphorylation. Autoradiography showed tyrosine phosphorylation of several endogenous substrates in v-Crk immunoprecipitates prepared from the cytoskeletal fraction of v-Crk-transformed cells (Fig. 11). These substrates have apparent molecular masses of 44, 47, 58 and 130 kDa. Phosphorylation of the 44, 47 and 130 kDa proteins was also found in the detergent-soluble fraction of these cells, although to a much lesser extent (Fig. 11). No phosphorylation of endogenous proteins could be detected in v-Crk immunoprecipitates prepared from SH2 and SH3 mutant v-Crk expressing cells (Fig. 11), indicating that both the v-Crk SH2 and SH3 domains are necessary for inducing phos-phorylation of endogenous proteins. p47 and p130 have been identified previously by partial proteolytic mapping, and appeared to have essentially identical maps to p47 v-Crk and the 130 kDa Crk-associating-substrate p130^{CAS} (Mayer and Hanafusa, 1990b). In conclusion, our data show that cytoskeleton-associated v-Crk co-precipitates protein tyrosine kinase activity.

DISCUSSION

In this paper we demonstrate that the viral oncogene product v-Crk is associated with the cytoskeleton of v-Crk expressing mouse fibroblasts. Immunofluorescence studies revealed that v-Crk is present in a specialized part of the cytoskeleton that interacts with the extracellular matrix: the focal adhesion. We have shown that the presence of v-Crk in focal adhesions depends on a functional SH2 domain. Mutation of the SH3 domain did not disrupt the association of v-Crk with focal adhesions. This is in contrast to previous data, where SH3 domains, instead of SH2 domains, were found to be responsible for targeting signaling molecules to specific subcellular locations (Bar-Sagi et al., 1993). Preliminary data also show the presence of c-Crk in focal adhesion structures, although to a much lesser extent, suggesting a function for both cellular and viral Crk in focal adhesion signaling.

It has been shown previously that v-Crk causes an increase in protein tyrosine phosphorylation (Mayer et al., 1988; Mayer and Hanafusa, 1990a). Our data show that mainly cytoskeletal proteins become tyrosine phosphorylated. Immunofluorescence studies revealed that the tyrosine phosphorylated proteins are predominantly found in focal adhesion structures. Several cytoskeletal proteins showed elevated phosphotyrosine levels, in particular paxillin and p130^{CAS}. It has previously been reported that increased tyrosine phosphorylation of paxillin and p130^{CAS} leads to more binding sites for the v-Crk SH2 domain (Sakai et al., 1994a; Mayer et al., 1995; Schaller and Parsons, 1995). By immunoprecipitation studies we showed that cytoskeleton-associated v-Crk interacts with both paxillin and p130^{CAS}. These proteins have previously been shown to be present in focal adhesions (Turner et al., 1990; Petch et al., 1995). Together, these data suggest that v-Crk may be present in focal adhesions by binding to tyrosine phosphorylated paxillin and p130^{CAS} via its SH2 domain. As we have shown that the presence of v-Crk in focal adhesions requires a functional v-Crk SH2 domain, but not a functional SH3 domain, we also expected the v-Crk SH3 mutant to bind to tyrosine phosphorylated focal adhesion proteins via its intact SH2 domain. Although we could detect the presence of a little tyrosine phosphorylated paxillin and p130^{CAS} (Fig. 8B and C) in the cytoskeletal fraction of control cells and cells transfected

with the v-Crk mutants, we were not able to detect paxillin or p130^{CAS} in immunoprecipitates of the v-Crk SH3 mutant, nor any other tyrosine phosphorylated proteins (data not shown). Possibly, tyrosine phosphorylation of cytoskeletal proteins in v-Crk SH3 mutant immunoprecipitates is below detection level. The identity of the focal adhesion protein(s) that is (are) responsible for binding the v-Crk SH3 mutant to focal adhesions is currently under investigation.

The specific mechanism by which v-Crk increases tyrosine phosphorylation of focal adhesion proteins remains an elusive question. Our data clearly show that increased phosphotyrosine levels in focal adhesion structures requires both v-Crk SH2 and SH3 domains to be functional, implying an important role for both domains in protein tyrosine phosphorylation. It has been suggested that v-Crk, by binding phosphotyrosyl residues via its SH2 domain, may inhibit dephosphorylation of proteins (Birge et al., 1993). This would cause a shift in the phosphorylation/dephosphorylation equilibrium state, leading to increased phosphotyrosine levels (Birge et al., 1992, 1993). However, we have shown that the v-Crk SH3 mutant, which contains a functional SH2 domain, does not induce an increase in protein tyrosine phosphorylation. This result suggests that inhibition of dephosphorylation is not the main mechanism for increasing phosphotyrosine levels of focal adhesion proteins.

It is more likely that v-Crk, since it lacks intrinsic kinase activity, co-operates with cellular, focal adhesion located, tyrosine kinases. Indeed, our data show the presence of protein tyrosine kinase activity in v-Crk immunoprecipitates prepared from the cytoskeletal cell fraction of v-Crk-transformed cells. Although several endogenous substrates were found to be present in v-Crk immunoprecipitates, we could not detect phosphorylation of paxillin, despite the fact that it co-precipitated with v-Crk. However, we did find increased tyrosine phosphorylation of paxillin in v-Crk-transformed cells. This indicates that the tyrosine kinase responsible for paxillin phosphorylation does not associate with v-Crk.

The question that remains is which tyrosine kinase(s) is involved in the v-Crk-induced changes in tyrosine phosphorylation of focal adhesion proteins. At least three tyrosine kinases, c-Src, focal adhesion kinase (FAK), and c-Abl, are potential candidates because these kinases have been shown to be present in focal adhesions (Schaller et al., 1992; Kaplan et al., 1994; van Etten et al., 1994). In addition, they all have been shown to associate with p130^{CAS} (Kanner et al., 1991; Mayer et al., 1995; Polte and Hanks, 1995). In contrast to c-Src and c-Abl, FAK is known to phosphorylate paxillin (Schaller and Parsons, 1995), and may therefore be responsible for the increase in paxillin phosphorylation in v-Crk-transformed cells. We were unable to detect the presence of FAK in v-Crk immunoprecipitates (data not shown), consistent with our data showing that the tyrosine kinase that phosphorylates paxillin does not associate with v-Crk. On the other hand, c-Src and c-Abl have been shown to phosphorylate p130^{CAS} in vitro (Kanner et al., 1991; Sakai et al., 1994a; Mayer et al., 1995). Unlike c-Src, c-Abl has been found in Crk immunoprecipitates, and has been shown to directly associate with the v-Crk SH3 domain (Feller et al., 1994; Ren et al., 1994; Mayer et al., 1995). This kinase has also been shown to phosphorylate the c-Crk II protein (Feller et al., 1994). Preliminary data showed that a GST-c-Crk II fusion protein is an excellent substrate for the tyrosine kinase that is bound to cytoskeleton-associated v-Crk (data not shown). This suggests a role for c-Abl in the increase in tyrosine phosphorylation of focal adhesion proteins. Unfortunately, we were not able to show the presence of c-Abl in v-Crk immunoprecipitates, possibly due to the fact that NIH-3T3 cells express c-Abl at very low levels (van Etten et al., 1994). Together, these data suggest that c-Abl may be responsible for the hyperphosphorylation of cytoskeleton-associated p130^{CAS} in v-Crk-transformed cells. Further experiments will be required to determine whether c-Abl tyrosine kinase activity is essential for v-Crk-induced p130^{CAS} hyperphosphorylation and transformation.

In addition to tyrosine kinase activity, cytoskeleton-associated v-Crk precipitated the tyrosine kinase substrate p130^{CAS}. Furthermore, v-Crk appeared to cause a redistribution of p130^{CAS} to the detergent-insoluble cytoskeleton, and hyperphosphorylation of specifically this cytoskeleton-associated p130^{CAS}. Preliminary data show the presence of v-Crk, but also hyperphosphorylated p130^{CAS} in focal adhesions isolated from v-Crk-transformed cells, in contrast to control cells (data not shown). Together, these data point to a novel mechanism for p130^{CAS} hyperphosphorylation, involving a specific subcellular compartment, the focal adhesion. In this compartment, v-Crk may induce the formation of a ternary complex with p130^{CAS} and a tyrosine kinase, in which the v-Crk SH2 domain binds p130^{CAS}, and the SH3 domain a tyrosine kinase. This may then result in the hyperphosphorylation of cytoskeleton-associated p130^{CAS}. This model is consistent with the model presented by Mayer and co-workers (Mayer et al., 1995) in which the Crk SH2 domain is required to promote hyperphosphorylation of p130^{CAS} by the c-Abl tyrosine kinase in vitro.

The elevated phosphotyrosine levels of focal adhesion proteins correlated well with cell transformation, suggesting a role for focal adhesion signaling in cellular transformation. Recruitment of cellular proteins to focal adhesions, as we have shown for p130^{CAS}, and increased tyrosine phosphorylation of focal adhesion proteins, is not only detected in v-Crk-transformed cells, but also when cells are allowed to adhere to ECM components, like fibronectin, via their integrin receptors (Schlaepfer et al., 1994; Miyamoto et al., 1995). Protein phosphorylation is one of the earliest events detected in response to integrin stimulation. Increased phosphorylation of for instance FAK, paxillin and tensin has been shown in cells spread on fibronectin, as recently reviewed (Clark and Brugge, 1995; Richardson and Parsons, 1995). Also, an increase in tyrosine phosphorylation of p130^{CAS} upon adherence of cells to the ECM has recently been reported (Nojima et al., 1995; Petch et al., 1995; Vuori and Ruoslahti, 1995). In addition, recruitment of c-Src, Grb2, and mSOS1 to focal adhesion sites of cells grown on fibronectin has been demonstrated (Schlaepfer et al., 1994). Kinases like FAK and MAP-kinase, but also the small G-protein Ras, are activated upon integrin stimulation, suggesting that integrin-mediated signaling is using the Ras pathway (Schlaepfer et al., 1994; Morino et al., 1995). v-Crk is believed to signal through the Ras and Rap1 pathway since it binds the guanine-nucleotide exchange factors mSOS1 and C3G (Knudsen et al., 1994; Feller et al, 1995; Gotoh et al., 1995). Taken together, these data show that v-Crk transformation and integrin receptor stimulation both lead to increased tyrosine phosphorylation, recruitment of specific proteins, and activation of kinases in focal adhesions. This raises the possibility that the viral oncogene product v-Crk induces cell transforma-

tion because of its ability to bypass or disrupt integrin-mediated signal transduction.

Recently, it has been shown that hyperphosphorylation of p130^{CAS} is critical for cell transformation by ornithine decarboxylase, c-Ha-Ras and v-Src (Auvinen et al., 1995). Treatment of transformed cells with tyrosine kinase inhibitors like herbimycin A, genistein or tyrphostin resulted in reversion of the transformed phenotype, as did expression of anti-sense p130^{CAS} mRNA. Additionally, p130^{CAS} is constitutively hyperphosphorylated in v-Crk-transformed cells independent of cell adhesion (Nojima et al., 1995; Petch et al., 1995; Vuori and Ruoslahti, 1995). This is in contrast to non-transformed cells which only show hyperphosphorylation of p130^{CAS} upon cell adhesion. This raises the intriguing possibility that v-Crk may induce cell transformation by causing constitutive hyperphosphorylation of cytoskeleton-associated p130^{CAS}, thereby bypassing the need for integrin activation. This would then culminate in an integrin-independent activation of focal adhesion signal transduction pathways, which may finally lead to cell proliferation. Our data show that for the elucidation of the mechanism of v-Crk transformation, it will be important to focus future experiments on changes in focal adhesion signaling in v-Crk-transformed cells.

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