p80/85 Cortactin Associates with the Src SH2 Domain and Colocalizes with v-Src in Transformed Cells*

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Heidi Okamura‡§ and Marilyn D. Resh¶

From the Cell Biology and Genetics Program, Sloan-Kettering Institute, New York, New York 10021 and the §Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544

Expression of oncogenic variants of pp60src leads to dramatic changes in cytoskeletal organization characteristic of transformation. Activated Src associates with the cytoskeletal matrix, resulting in tyrosine phosphorylation of specific cytoskeletal substrates. We have previously shown that stable association of Src with the cytoskeletal matrix is mediated by the Src SH2 domain in a phosphotyrosine-dependent interaction. In this report, we demonstrate that one of the cytoskeletal binding partners of Src is p80/85 cortactin. The association was observed in lysates of transformed cells but was not seen in normal fibroblasts. The interaction could be reconstituted in vitro using transformed cell extracts and a glutathione S-transferase (GST) fusion protein containing the Src SH2 domain but not with GST-Src SH3 or with GST-Src SH2 containing a point mutation in the FLVRES sequence. Confocal microscopy revealed that cortactin redistributed and colocalized with v-Src and a Src SH3 deletion mutant in transformed cells. However, in cells expressing a Src SH2 deletion mutant, the redistribution of cortactin and colocalization with Src did not occur. Furthermore, biochemical fractionation of transformed cells indicated that a significant increase in cortactin distribution to the cytoskeletal fraction occurred, which correlated with a shift in the tyrosine-phosphorylated form of the protein. Cortactin fractionated from cells expressing kinase-defective or myristylation-defective Src mutants did not exhibit this shift. These data suggest a molecular mechanism by which tyrosine phosphorylation of cortactin and association with the Src SH2 domain influence the cytoskeletal reorganization induced in Src-transformed cells.

The v-Src oncogene mediates cellular transformation by encoding a tyrosine kinase which exhibits elevated levels of catalytic activity. Interaction of pp60Gsrc with the plasma membrane is essential for achieving transformation and results in the tyrosine phosphorylation of a number of membrane and cytoskeletal proteins. It is thought that tyrosine phosphorylation of key cytoskeletal substrates leads to the dramatic changes in morphology, cytoskeletal reorganization, and decreased adhesive properties characteristic of transformed cells (for review, see Refs. 1 and 2). Consistent with this hypothesis, pp60Gsrc and its nontransforming cellular homolog, pp60c-src differ in their ability to bind to the cytoskeletal matrix (3, 4). When expressed in fibroblasts, v-Src and oncogenic mutants of c-Src stably associate with the cytoskeletal matrix, whereas nontransforming Src variants, including wild-type c-Src, do not. These observations suggest that it is not only the phosphorylation of cytoskeletal proteins, but also the association of activated Src with specific cytoskeletal substrates that is important for the changes observed upon transformation.

We have recently shown that stable association of Src with proteins in the cytoskeletal matrix is mediated by the Src SH2 domain in a phosphotyrosine-dependent interaction (5). Several proteins, which are tyrosine-phosphorylated in transformed cells, are candidate binding partners for activated Src in the cytoskeletal matrix (6–9). One of these potential cytoskeletal binding partners is p80/85 cortactin. Cortactin is an F-actin binding protein that possesses several interesting structural features, including an SH3 domain, a proline-rich region, and a series of tandem internal repeats that serves as the actin binding domain (10, 11). Cortactin is normally phosphorylated on serine and threonine, but it becomes tyrosine-phosphorylated in response to growth factor stimulation or upon transformation by activated Src (11–13). In nontransformed cells, cortactin has been shown to transiently associate with c-Src in response to thrombin activation in platelets (14) and in response to FGF-1 in fibroblasts (15). In oncogenic cells, the cortactin locus is amplified and overexpressed in certain human breast and squamous cell carcinomas (16), while in mice the inappropriate expression of cortactin appears to be associated with the transformation of plasma cells (17). Based on these observations, it has been suggested that cortactin may play an important role in the transduction of mitogenic signals, cytoskeletal organization, and cell adhesion.

In this report, we demonstrate that v-Src and cortactin form a complex in Rous sarcoma virus-transformed cells that is dependent on the structural integrity of the Src SH2 domain. The association was most readily observed in the cytoskeletal matrix-enriched fraction of the cell and correlated with the tyrosine phosphorylation of cortactin. The interaction of pp60Gsrc and cortactin could be reconstituted in vitro by incubating cytoskeletal matrix extracts from transformed cells with GST fusion protein containing the Src SH2 domain, but it was not observed with a GST Src SH2 domain containing a point mutation in the FLVRES sequence. Confocal microscopy revealed that cortactin redistributed from the cell periphery to colocalize with wild-type v-Src in transformed fibroblasts. However, in cells expressing a Src SH2 deletion mutant, the redistribution of cortactin and colocalization with Src did not occur. Upon transformation, a significant increase in cortactin distri-

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¶ A Rita Allen Foundation Scholar and an Established Scientist of the American Heart Association. To whom correspondence should be addressed: Cell Biology and Genetics Program, Sloan-Kettering Inst., 1275 York Ave., Box 143, New York, NY 10021. Tel.: 212-639-2514; Fax: 212-717-3317; E-mail: m-resh@ski.mskcc.org.

The abbreviations used are: GST, glutathione S-transferase; CEF, chicken embryo fibroblast; PAGE, polyacrylamide gel electrophoresis.
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bution to the cytoskeletal fraction was observed that could be attributed to a shift by the tyrosine-phosphorylated form of the protein. In contrast, cortactin fractionated from cells expressing a kinase-defective Src mutant did not exhibit this shift, consistent with its lack of tyrosine phosphorylation. Taken together, these data demonstrate a functional role for the tyrosine phosphorylation of cortactin. Our results provide direct evidence for the interaction of tyrosine-phosphorylated cortactin with v-Src and suggest a molecular mechanism by which this association influences the cytoskeletal reorganization induced in Src-transformed cells.

MATERIALS AND METHODS

Cells, Plasmids, Antibodies, and Virus—Chicken embryo fibroblasts (CEF) were prepared from 10- or 11-day-old embryos (SPAFAS, Inc., Norwich, Conn.). The plasmid pSR-GEM and the Src SH2 and SH3 deletion constructs have been described previously (5). Virus containing the kinase-defective mutant K295M was a kind gift of R. Jove (University of Michigan). NY315 virus, Src antibody 873 has been described previously and is immunoreactive with both pp60-src and pp60v-src (18). Monoclonal antibody 4F11 (9) against p80/85 cortactin was generously provided by J. T. Parsons (University of Virginia).

Generation of GST Fusion Proteins—Sequences encoding the v-Src SH2 (residues 148–251), SH3 (residues 79–147), and SH3-SH2 domains were generated by polymerase chain reaction amplification using the v-Src plasmid pSR-GEM as template and the appropriate primers. Similarly, generation of the R175K SH2 domain was achieved by polymerase chain reaction chain reaction amplification of a pSR-GEM plasmid containing the R175K mutation (5). All constructs were verified by DNA sequencing. The polymerase chain reaction products were cloned into the BamHI and EcoRI sites of the pGEX-2T vector. GST fusion protein expression and purification were performed as described previously (15). Fusion proteins were eluted with glutathione and dialyzed. Protein concentrations were determined by BCA protein assay (Pierce Chemical Co.). GST fusion proteins containing the SH2 domain of Abl, Crk, Nck, and phospholipase Cγ (20–22) were generously provided by H. Hanafusa and S. Feller (Rockefeller University). The GST-Fps SH2 fusion protein (23) was provided by R. Feldman (University of Maryland), and the GST-P13 kinase SH2 fusion protein (24) was provided by Y. Fukui (University of Tokyo).

Immunoprecipitation and Immunoblotting—CEF were washed twice with phosphate-buffered saline containing 1 mM sodium vanadate and disrupted on ice with lysis buffer (150 mM NaCl, 50 mM HEPES (pH 7.5), 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride). After clarification, approximately 500 μg of cell lysate was incubated with polyclonal anti-Src antibody 873. The immune complexes were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membrane (Millipore) as described previously (20). Blots were probed with monoclonal anti-p80/85 cortactin and detected with the ECL chemiluminescence system (Amersham Corp.).

Cell Lysate Preparation and Binding to GST Fusion Protein—Cytoskeletal matrix-enriched fractions were prepared by extracting cells (10 mM Tris (pH 7.2), 140 mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.05% SDS, 1 mM EDTA, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride) by clarifying, and used for binding experiments. Unless otherwise indicated, 3 μg of GST fusion protein was preadsorbed to glutathione-Sepharose beads and incubated with 500 μg of cellular protein for 3 h at 4 °C with constant rocking. The beads were washed 4 times with modified RIPA, and the bound proteins were eluted by boiling in SDS sample buffer. Samples were subjected to electrophoresis through a 8% polyacrylamide gel, and immunoblotted as described above with monoclonal antibody against p80/85 cortactin.

Immunodiffuson Microscopy—CEF expressing the appropriate Src mutants were seeded onto glass coverslips, grown overnight, and processed as described previously (20). For double staining experiments, the primary antibody solution consisted of a 1:2000 dilution of monoclonal anti-Src and 10 μg/ml monoclonal anti-p80/85 in phosphate-buffered saline + 10% calf serum. The secondary antibody solution contained a 1:75 dilution of fluorescein-conjugated goat anti-rabbit (Boehringer Mannheim) and 5 μg/ml of Texas Red-conjugated goat anti-mouse (Molecular Probes Inc.) in phosphate-buffered saline + 10% calf serum. Cells were visualized with a 100× objective using a Nikon fluorescence microscope coupled to a Molecular Dynamics laser scanning confocal microscope.

Cortactin Fractionation—Normal CEF or cells expressing the appropriate Src construct were fractionated by incubating in 1% Triton X-100 buffer as described above. After solubilizing the Triton-insoluble fraction with RIPA buffer, all fractions were adjusted to equivalent detergent concentrations. Fractions were immunoprecipitated with anti-cortactin antibody, subjected to SDS-PAGE, and immunoblotted with either anti-cortactin or phosphotyrosine, as described above. Blots were stripped by incubating in 62.5 mM Tris (pH 6.8), 2% SDS, 100 mM β-mercaptoethanol at 70 °C for 30 min with constant agitation. Blots were then blocked overnight before reprobing with primary antibody and processed as above.

RESULTS

pp60v-src Forms a Complex with p80/85 Cortactin—pp60v-src, but not pp60src, stably associates with the cytoskeletal matrix (3, 4) in an interaction mediated by the Src SH2 domain (5). To identify proteins in the cytoskeleton that interact with Src, pp60v-src was immunoprecipitated from lysates of normal CEF or CEF expressing pp60v-src. The immune complexes were resolved by SDS-PAGE and then Western immunoblotted with antibodies to cytoskeletal proteins that are putative Src substrates. Using this method, we identified p80/85 cortactin as an interacting protein. Immunoblotting with anti-cortactin antibody revealed a weak signal in Src immunoprecipitates isolated from whole cell lysates of v-Src-transformed CEF (Fig. 1, lane 2), but not from those of normal cells (lane 1). A stronger cortactin signal was observed in Src immunoprecipitates obtained from a cytoskeleton-enriched fraction of transformed cells (lane 3). Therefore, p80/85 cortactin appears to form a complex in vivo with pp60v-src but not pp60src.

The Association with Cortactin Requires an Intact Src SH2 Domain in Vitro—Previously, we demonstrated that the Src SH2 domain was required for association with the cytoskeletal matrix. We next determined whether this region of Src was specifically responsible for its interaction with cortactin. Since cortactin possesses a proline-rich region, it was also possible that the protein interacted with Src through the Src SH3 domain. To more directly assess potential interactions between the Src homology domains and cortactin, GST fusion proteins containing the Src SH2 and/or SH3 domains were employed. Cellular protein from the cytoskeleton-enriched fraction of Rous sarcoma virus-transformed CEF was incubated with GST.
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The Src SH2 Domain Binds Cortactin Obtained from Transformed Cells But Not from Normal CEF—Since the Src SH2 domain is required for binding to cortactin, it is likely that a phosphotyrosine residue(s) on cortactin is involved in the association. However, there have been several reports of SH2 domains involved in phosphotyrosine-independent interactions (25–28). In normal CEF, cortactin is phosphorylated only on serine and threonine, whereas in Src-transformed cells cortactin also becomes tyrosine-phosphorylated (11). To verify the involvement of a phosphotyrosine-dependent interaction, fusion proteins were incubated with cell lysate from the cytoskeleton-enriched fraction of uninfected cells in parallel with lysate from transformed cells (Fig. 3). None of the GST fusion proteins bound cortactin from normal cell lysate (lanes 1–3), whereas the GST-Src SH2 fusion bound the cortactin obtained from transformed cells (lane 7). These experiments were also conducted using whole cell lysates from normal and v-Src-transformed cells, with identical results (data not shown). To further confirm that the cortactin-Src interaction observed in lysates from transformed cells is dependent upon SH2-phosphotyrosine association, competition was performed using a phosphopeptide containing the optimal sequence for binding to the Src SH2 domain, pYEEI (22). Simultaneous addition of pYEEI and the cellular lysate to GST-Src SH2 effectively abolished association of the fusion protein with cortactin from transformed cells (Fig. 4).

Interaction of Cortactin with Other SH2 Domain-containing Proteins—To investigate whether cortactin association is limited to the Src SH2 domain, a panel of GST-SH2 fusions from other proteins was utilized. 5 μg of each SH2 fusion protein was adsorbed to glutathione-Sepharose beads and incubated with 500 μg of cellular protein from transformed CEF. As shown in Fig. 5, GST-Src SH2 and GST-Abl SH2 bound similar levels of cortactin. GST-Crk SH2 and GST-Nck SH2 also bound cortactin, although with lower efficiency than the GST-Src and -Abl SH2 domains. In contrast, GST-Fps SH2, GST-Pi3 kinase SH2, and GST-phospholipase C-γ SH2 bound cortactin very poorly. These results suggest that specificity of cortactin association is maintained in vitro and that other SH2 domain-containing proteins may interact with cortactin in vivo.

Redistribution and Colocalization of Cortactin with pp60<sup>src</sup> Requires the Src SH2 Domain in Vivo—Based on the finding that cortactin binds to the v-Src SH2 domain in vitro, it was of interest to determine whether the v-Src SH2 domain influences cortactin localization in vivo. Colocalization studies of cortactin

Association of cortactin with Src homology domains. 3 μg of GST fusion protein containing various Src homology domains were bound to glutathione-Sepharose beads and then incubated with approximately 500 μg of cellular protein from the cytoskeleton-enriched fraction of v-Src transformed cells. Bound proteins were analyzed by SDS-PAGE and immunoblotting with cortactin antibody. GST fusion proteins consisted of GST alone (lane 1), GST-Src SH3/SH2 (lane 2), GST-Src SH3 (lane 3), GST-Src SH2 (lane 4), or GST-Src SH2(R175K) point mutation (lane 5). As a control, an aliquot of lysate from the cytoskeleton-enriched fraction was run in parallel (lane 6).

Interaction of cortactin with other SH2 domain-containing proteins. Approximately 500 μg of protein from the cytoskeleton-enriched fraction of Src-transformed cells was incubated with 5 μg of GST fusion protein containing the SH2 domains of Src, Abl, Crk, Fps, Nck, phosphatidylinositol 3-kinase (PI3K) (C-terminal), and phospholipase C-γ (PLC) (N-terminal). An aliquot of lysate from Src-transformed cells was run alongside as a control. Bound proteins were analyzed by SDS-PAGE and immunoblotting with anti-cortactin antibody.

Fig. 2. Association of cortactin with Src homology domains. 3 μg of GST fusion protein containing various Src homology domains were bound to glutathione-Sepharose beads and then incubated with approximately 500 μg of cellular protein from the cytoskeleton-enriched fraction of v-Src transformed cells. Bound proteins were analyzed by SDS-PAGE and immunoblotting with cortactin antibody. GST fusion proteins consisted of GST alone (lane 1), GST-Src SH3/SH2 (lane 2), GST-Src SH3 (lane 3), GST-Src SH2 (lane 4), or GST-Src SH2(R175K) point mutation (lane 5). As a control, an aliquot of lysate from the cytoskeleton-enriched fraction was run in parallel (lane 6).

Fig. 3. Interaction of cortactin with other SH2 domain-containing proteins. Approximately 500 μg of protein from the cytoskeleton-enriched fraction of Src-transformed cells was incubated with 5 μg of GST fusion protein containing the SH2 domains of Src, Abl, Crk, Fps, Nck, phosphatidylinositol 3-kinase (PI3K) (C-terminal), and phospholipase C-γ (PLC) (N-terminal). An aliquot of lysate from Src-transformed cells was run alongside as a control. Bound proteins were analyzed by SDS-PAGE and immunoblotting with anti-cortactin antibody. GST fusion proteins contained GST alone (lanes 1 and 5), GST-Src SH3 (lanes 2 and 6), or GST-Src SH2 (lanes 3 and 7) were incubated in parallel with lysates from normal CEF (lanes 1-3) or Src-transformed CEF (lanes 5-7). Bound proteins were separated by SDS-PAGE and immunoblotted with anti-cortactin antibody. Aliquots of lysate from normal (lane 4) and transformed cells (lane 8) were run alongside as controls.

Fig. 4. Competition of Src SH2-cortactin binding with pYEEI peptide. GST-Src SH2 fusion protein bound to glutathione beads was incubated with cellular protein from transformed cells without peptide (lane 1), or in the presence of 25 μM (lane 2) and 75 μM (lane 3) pYEEI peptide.

Fig. 5. Interaction of cortactin with Src homology domains. GST fusion proteins containing GST alone (lanes 1 and 5), GST-Src SH3 (lanes 2 and 6), or GST-Src SH2 (lanes 3 and 7) were incubated in parallel with lysates from normal CEF (lanes 1-3) or transformed CEF (lanes 5-7). Bound proteins were separated by SDS-PAGE and immunoblotted with anti-cortactin antibody. GST fusion proteins containing GST alone (lanes 1 and 5), GST-Src SH3 (lanes 2 and 6), or GST-Src SH2 (lane 4) efficiently bound p80/85 cortactin, while GST alone (lane 1) or GST-Src SH3 (lane 3) failed to bind. In addition GST-SH2(R175K) (lane 5), which contains a point mutation in the phosphotyrosine binding pocket of the Src SH2 domain, failed to bind efficiently. Titration of GST-Src SH2, by incubating increasing amounts of fusion protein with a constant level of cellular protein, revealed concentration-dependent binding. Cortactin association could be detected using as little as 0.1 μg of GST fusion protein, and appeared to reach saturation at 3 μg of GST-Src SH2 (data not shown).

In addition, we were able to observe direct association in vitro of purified, phosphorylated cortactin with purified Src SH2 domain. GST-p80 cortactin fusion protein (10) expressed in Escherichia coli was purified, and approximately 5 μg of fusion protein were attached to glutathione-Sepharose beads. The bound GST-cortactin fusion protein was phosphorylated in vitro with purified Src, washed extensively, and then cleaved with thrombin to remove the GST moiety. The phosphorylated cortactin fragment was isolated and tested for its ability to associate with GST-Src SH2. Cortactin protein purified and phosphorylated in this manner was able to associate with the Src SH2 domain, while nonphosphorylated cortactin purified and assayed the same way did not appear to interact. Together, these results indicate that the interaction between Src and cortactin is specific and requires an intact Src SH2 domain.
and various Src mutants were undertaken using immunofluorescence and confocal microscopy. In normal CEF, cortactin binds to the ends of F-actin, exhibiting an intense staining in the peripheral extensions of the cell (Ref. 11 and Fig. 6A). Upon transformation, cortactin redistributes (11) from the cell periphery to podosomes, sites of membrane-substratum contact containing high levels of cytoskeletal proteins (29, 30). In cells transformed by wild-type v-Src, redistribution of cortactin resulted in the colocalization of both cortactin (Fig. 6C and D) and Src (Fig. 6D) to podosomes. In contrast, in cells expressing an SH2 deletion mutant of v-Src, colocalization of v-Src and cortactin did not occur, and no redistribution of cortactin into podosomes was observed. Cells transfected with the Src SH2 deletion mutant exhibited cortactin staining similar to that seen in nontransfected cells with localization predominantly at the cell periphery (Fig. 6E), while Src distribution was primarily perinuclear (Fig. 6F). Deletion of the SH3 domain does not impair the ability of Src to induce morphological transformation. Cells expressing the Src SH3 deletion mutant were examined by confocal microscopy and also found to exhibit colocalization of cortactin and Src proteins (Fig. 6, G and H).

Tyrosine Phosphorylation of Cortactin Results in Increased Cytoskeletal Binding—Approximately 10–30% of the cortactin population becomes tyrosine-phosphorylated upon Src transformation or growth factor stimulation (11). The effect of this tyrosine phosphorylation is unknown since it does not appear to alter F-actin binding, an apparent function of cortactin in normal cells (14). We therefore tested the possibility that tyrosine phosphorylation of cortactin was involved in regulating its subcellular distribution to the cytoskeleton. Cells were extracted with a 1% Triton X-100 buffer (3,5) and were separated into Triton-soluble and -resistant fractions to determine the ability of cortactin to associate with the detergent-insoluble cytoskeletal matrix. Each fraction was immunoprecipitated with anti-cortactin and then immunoblotted with either anti-cortactin or anti-phosphotyrosine antibodies. Although there was an appreciable amount of cortactin associated with the cytoskeletal fraction in normal CEF, the majority was present in the soluble fraction (Fig. 7A). In cells transformed by v-Src, however, there was a significant shift in cortactin distribution to the detergent-resistant cytoskeletal matrix fraction (Fig. 7A). Likewise, fractionation of cells transformed by an SH3 deletion mutant also showed cortactin to be distributed predominantly in the cytoskeletal matrix. In contrast, mutations within other domains of the v-Src protein prevented cortactin relocalization to podosomes. The distribution of cortactin fractionated from cells expressing a kinase-defective Src mutant (K295M) resembled that seen in uninfected cells, with the majority of cortactin partitioning with the soluble fraction (Fig. 7A). A similar result was obtained with cells expressing a nonmyristylated, cytosolic mutant of v-Src, NY315 (data not shown). Mutations within the SH2 domain of Src also appeared to interfere with cortactin redistribution. Fractionation of cells expressing a Src SH2 deletion mutant showed that the majority of cortactin was detergent soluble, similar to the distribution of cortactin in normal cells. Cortactin isolated from cells expressing a Src SH2 domain point mutant, R175K, was also
found predominantly in the soluble fraction, although the difference in cortactin distribution was not as dramatic as that from cells expressing the Src SH2 deletion mutant.

The immunoblot was stripped and reprobed with anti-phosphotyrosine antibodies to determine whether the increase in cytoskeletal distribution was attributable to tyrosine phosphorylation. Cortactin from normal CEF or from CEF expressing the kinase-defective Src contained no phosphotyrosine (Fig. 7B). As expected, cortactin isolated from v-Src-transformed cells, as well as from cells expressing a Src SH3 deletion mutant, did contain phosphotyrosine, and this population fractionated almost exclusively with the cytoskeletal fraction (Fig. 7B). Although the vast majority of cortactin from cells infected with the Src SH2 domain mutants was found in the soluble fraction, there were low levels of tyrosine-phosphorylated cortactin that remained in the cytoskeletal fraction. A similar result was obtained with the nonmyristylated v-Src mutant NY315, with tyrosine-phosphorylated cortactin fractionating with the cytoskeletal matrix. Taken together, these results strongly suggest that Src-dependent phosphorylation of cortactin results in redistribution of cortactin to the detergent-insoluble cytoskeletal matrix.

**DISCUSSION**

Interaction between v-Src and Cortactin—The stable association of transforming variants of Src with the cytoskeletal matrix is consistent with the hypothesis that tyrosine phosphorylation of key cytoskeletal substrates contributes to the striking changes in morphology, adhesion, and cytoskeletal organization observed in oncogenesis. We have previously demonstrated that pp60^src^ interacts with the cytoskeletal matrix through its SH2 domain in a phosphotyrosine-dependent interaction (5). Here, we show that p80^c-src^ cortactin is one of the cytoskeletal protein binding partners of v-Src. Interaction between Src and cortactin was observed in vivo by coprecipitation from the cytoskeletal matrix-enriched fraction of transformed cells, and in vitro using GST fusion proteins. Several lines of evidence indicate that formation of the complex is mediated by binding of phosphotyrosine residues on cortactin to the Src SH2 domain. The association was not observed, in vitro or in vivo, when lysate from nontransformed cells was utilized, correlating with the lack of tyrosine-phosphorylated cortactin in these cells. Using GST fusion proteins, the cortactin binding site was mapped to the Src SH2 domain. No interaction between Src and cortactin occurred when the Src SH2 domain contained a point mutation in the phosphotyrosine-binding FLVRES sequence. Cortactin did not associate with a GST-Src SH3 fusion protein, even though a proline-rich region is present within cortactin. Moreover, the cortactin-Src SH2 domain interaction could be abolished by addition of the tyrosine-phosphorylated peptide, pYEEI, which binds specifically to the Src SH2 domain.

Although tyrosine phosphorylation of cortactin is dependent on Src kinase activity (Fig. 7B), it is not known whether cortactin is a direct Src substrate. Purified c-Src can phosphorylate a GST-cortactin fusion protein in vitro (data not shown), but the sites of phosphorylation have not been mapped and compared with the sites phosphorylated in vivo. Alternatively, another tyrosine kinase may phosphorylate cortactin. pp60^src^ may phosphorylate and thereby activate another tyrosine kinase, which then phosphorylates cortactin and enables association with Src. In fact, a tyrosine kinase different from Src is associated with cortactin, but it appears inactive in nontransformed cells. Determination of the kinase directly responsible for cortactin tyrosine phosphorylation, as well as identification of the phosphorylation sites themselves, awaits further study.

Tyrosine-phosphorylated Cortactin Localizes to the Detergent-insoluble Matrix—Tyrosine phosphorylation of cortactin does not appear to influence its actin binding properties (10), and the functional consequences of this modification were not previously known. Our data suggest that tyrosine phosphorylation of cortactin serves to regulate its localization. In normal, untransformed cells, cortactin did not contain detectable phosphotyrosine, and the majority of the protein partitioned with the detergent-soluble fraction. In cells expressing wild-type v-Src or an SH3 Src deletion, cortactin became tyrosine-phosphorylated, and a significant increase in cortactin distribution to the Triton-insoluble cytoskeletal fraction was observed. Immunoblotting with anti-phosphotyrosine antibodies demonstrated that the tyrosine-phosphorylated cortactin population partitioned almost exclusively with the detergent-insoluble cytoskeleton (Fig. 7B). Two other v-Src mutants with active kinase domains (R175K and NY315) induced low levels of cortactin tyrosine phosphorylation, and this population of cortactin was localized to the detergent-resistant fraction as well (Fig. 7, Table I).

What does tyrosine-phosphorylated cortactin bind to in the matrix? Our data suggest that one of the binding partners is the v-Src SH2 domain. Confocal microscopy revealed that cortactin does not relocate in cells expressing Src SH2 deletion or point mutants. However, the v-Src SH2 domain is probably not the only binding partner for cortactin. Cortactin contains nearly 30 tyrosine residues, some of which, if phosphorylated, would lie within potential "consensus" sequences for binding to Src, Nck, or 3BP2 SH2 domains (22, 23). In vitro binding assays with the SH2 domains of other proteins suggest that cortactin can also associate with other tyrosine kinases like Abl or with bridging proteins like Crk or Nck (Fig. 5). Yamanashi et al. recently showed that HS1, a cortactin-like protein, becomes tyrosine-phosphorylated and binds to the Lyn SH2 domain (32). In addition, the presence of both an SH3 domain and a proline-rich region in cortactin further suggests that cortactin plays an important role in creating a nucleation site for the clustering of other proteins in the cytoskeletal matrix. Interestingly, it has also recently been demonstrated that tyrosine phosphorylation of β1 integrin results in an altered subcellular localization as compared with the nonphosphorylated form.

**TABLE I**

| Construct | Cell morphology | Src localization | Cortactin localization | Cortactin tyrosine phosphorylation | Src Triton X-100 | Cortactin Triton X-100 |
|-----------|-----------------|-----------------|------------------------|-----------------------------------|------------------|------------------------|
| CEF       | Flat            | Cell periphery  | —                      | Soluble                           | Soluble          | Soluble                |
| v-Src     | Rounded         | Podosomes       | Podosomes              | ++                                 | Resistant        | Resistant              |
| dll SH3   | Rounded         | Podosomes       | Podosomes              | ++                                 | Resistant        | Resistant              |
| dll SH2   | Fusiform        | Cytoplasm/perinuclear | Cell periphery      | +/−                                 | Soluble          | Soluble                |
| K295M     | Flat            | Punctate cytoplasm | Cell periphery (more diffuse) | —                                  | Intermediate    | Soluble                |
| NY315     | Flat            | Cytoplasm       | Cell periphery         | +                                  | Soluble          | Soluble                |

2 H. Wu, and J. T. Parsons, personal communication.
Cortactin, a 120 kDa protein (35, 36) that is associated with actin stress fibers (37). Recently, it was demonstrated that activated c-Src associates with pp110 and pp125FAK isolated from nontransformed cells is autophosphorylated or is the result of interaction between v-Src and cortactin. In the first instance (Model 1), cortactin localization to podosomes would be independent of v-Src localization in podosomes. In Model 2, redistribution of cortactin into podosomes would require the presence of v-Src in podosomes. Our data, summarized in Table I, support the second model. Cells expressing SH2 domain mutants of Src are partially transformed yet do not exhibit redistribution of cortactin to podosomes, emphasizing the importance of Src localization for this event. The cortactin redistribution is dependent on tyrosine phosphorylation of cortactin and an intact v-Src SH2 domain. Tyrosine phosphorylation of cortactin alone, however, is not sufficient for cortactin redistribution as illustrated by the NY315 Src mutant. In all instances, the bulk of the cortactin redistributes to the podosomes only when v-Src is also present in podosomes.
p80/85 cortactin is not the only protein that interacts with Src in the cytoskeletal matrix. Previous reports have established that activated forms of Src stably interact with a 110-kDa protein (35, 36) that is associated with actin stress filaments (37). Recently, it was demonstrated that activated c-Src also stably interacts with pp125FAK (38), a tyrosine kinase associated with focal adhesions (31). Unlike cortactin, however, the association of activated Src with pp110 and pp125FAK does not appear to be dependent on Src kinase activity (37, 38). pp125FAK isolated from nontransformed cells is autophosphorylated on tyrosine and is able to efficiently bind to the Src SH2 domain (38). It is therefore possible that Src is initially recruited to the cytoskeletal matrix through an interaction with pp125FAK. As the functional significance of such Src-cytoskeletal associations are determined, it will become possible to elucidate the mechanism by which Src mediates the various cellular changes characteristic of transformation.