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The SH2 and SH3 domains of pp60^{src} direct stable association with tyrosine phosphorylated proteins p130 and p110

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Transformation of chicken embryo cells with the tyrosine kinase oncogene *src* results in the tyrosine phosphorylation of numerous cellular proteins. We have recently generated monoclonal antibodies to individual tyrosine phosphorylated cellular *src* substrates, several of which are directed to the phosphotyrosine-containing proteins p130 and p110. These proteins form stable complexes with activated variants of pp60^{src}. Mutagenesis of the *src* homology domains (SH2 and SH3) of activated pp60^{src} resulted in *src* variants with altered association with p130 and p110. Analysis of these variants showed that the SH3 domain was required for association of p110, while the SH2 domain contained residues necessary for the formation of the ternary complex involving p130, p110 and pp60^{src}. Both the tyrosine phosphorylation status and pp60^{src} association of p130 and p110 appeared to correlate, in part, with the extent of cell transformation. Biochemical analysis demonstrated that p130 and p110 were substrates of both serine/threonine and tyrosine kinases. In addition, p130 was redistributed from the nucleus to cellular membranes upon *src* transformation, whereas p110, which normally co-localized with cytoskeletal elements, was observed in adhesion plaques (podosomes) in *src* transformed cells. These data indicate that tyrosine phosphorylation of two different phosphoproteins may play a role during *src* transformation either by directing their interaction with pp60^{src}, by redirecting subcellular distribution or both.

Key words: phosphotyrosine/protein complexes/*src* homology domain/transformation/tyrosine kinase

Introduction

The importance of tyrosine phosphorylation of cellular proteins during transformation by protein tyrosine kinase (PTK) oncogenes has been indicated by both genetic and biochemical studies (Jove and Hanafusa, 1987; Parsons and Weber, 1989; Cooper, 1990). Specifically, the *src* oncogene of the Rous sarcoma virus (RSV) encodes a 60 kd membrane-associated PTK, pp60^{src} (Parsons and Weber, 1989), the activation of which induces the tyrosine phosphorylation of 20-30 cellular proteins (Hamaguchi *et al.*, 1988; Kamps and Sefton, 1988; Linder and Burr, 1988; Glenney and Zokas, 1989; Kanner *et al.*, 1989a,b; Reynolds *et al.*, 1989a). Mutations in *src* that alter its elevated kinase activity and prohibit the concomitant tyrosine phosphorylation of specific cellular protein substrates are transformation-defective (Parsons and Weber, 1989). Although the initial detection and partial characterization of cellular *src* substrates has relied on the use of antibodies to phosphotyrosine (anti-pTyr) (Kamps and Sefton, 1988; Glenney *et al.*, 1988), we have recently reported the isolation and characterization of monoclonal antibodies (mAbs) to eight individual tyrosine phosphorylated protein targets (Kanner *et al.*, 1990). These reagents allow a more complete biochemical analysis of the individual substrates, as well as ultimately, their identification.

The regulation and specificity of interaction between oncogene-encoded PTKs or growth factor receptor PTKs and their cellular protein substrates has been studied in several experimental systems. For example, stimulation of cells with platelet-derived growth factor (PDGF) results in the direct association of tyrosine phosphorylated forms of GTPase activating protein (GAP), phospholipase C-γ (PLC-γ), and raf-1 with the autophosphorylated PDGF receptor (Kazlauskas and Cooper, 1989; Kaplan *et al.*, 1990; Kazlauskas *et al.*, 1990; Morrison *et al.*, 1989, 1990; Ullrich and Schlessinger, 1990). In addition, the PTK oncogene product pp60^{src} forms stable complexes with two cellular substrates, tyrosine phosphorylated proteins p130 and p110 in chicken cells (Reynolds *et al.*, 1989b) or a single phosphotyrosine-containing protein pp120 in mammalian cells (Lau, 1986, 1989). Initial studies of the chicken p130/p110/pp60^{src} complex have shown that the interaction of these phosphoproteins requires both the activation of the PTK activity of pp60^{src} and the structural integrity of two regions of *src* designated the *src* homology domains SH2 (B/C box) and SH3 (A box) (Reynolds *et al.*, 1989b). The SH2 and SH3 domains comprise regions identified in other oncogenes (e.g. v-*crk*, v-*fgr*, v-*abl* and v-*fps*), in several cytoskeletal-associated proteins (e.g. myosin heavy chain, yeast actin binding protein and α-spectrin) and in cellular enzymes (e.g. PLC-γ and GAP) (Mayer *et al.*, 1988; Pawson, 1988; Stahl *et al.*, 1988). It has been suggested that these common domains function to promote specific protein-protein interactions (Pawson, 1988). Anderson *et al.* (1990) have demonstrated that bacterially expressed SH2 domains from GAP, PLC-γ and *src* mediate stable association with mammalian growth factor receptors.

Monoclonal antibodies to p130 and p110 were previously generated during the development of specific reagents to individual phosphotyrosine-containing proteins in *src* transformed chicken embryo (CE) cells (Kanner *et al.*, 1990). Here we report on the characterization of the individual components of the p130/p110/pp60^{src} complex, detailing the structural requirements within the SH2 and SH3 domains of pp60^{src} for the association of p130 and p110.

Analysis of the subcellular localization of p130 and p110 in normal and *src* transformed cells demonstrated that both p130 and p110 appear to be redistributed upon cellular transformation. The data indicate that both the association with pp60^{src} and the tyrosine phosphorylation status of p130 and p110 correlates with their subcellular compartmentalization. Finally, different forms of the proteins and their complexes appear to relate directly to the transformed state of the cell.

Results

Monoclonal antibodies define pp60^{src}-associated phosphoproteins p130 and p110

Immunoprecipitation of pp60$^{v\text{-}src}$ or activated variants of pp60$^{c\text{-}src}$ from chicken embryo (CE) cells results in the co-immunoprecipitation of two tyrosine phosphorylated proteins, p130 and p110 (Reynolds *et al.*, 1989b). Both p130 and p110 are detected by immunoblotting of pp60^{src} immune complexes with anti-pTyr, by *in vitro* kinase assays, as well as by metabolic labeling of CE cells (Reynolds *et al.*, 1989b). By using monoclonal antibodies (mAbs) to the individual components of the p130/p110/pp60^{src} complex (Kanner *et al.*, 1990), the binding activities and biochemical characteristics of the three phosphoproteins were directly examined. To demonstrate the specificity of the mAbs to the individual pp60^{src}-associated proteins, immunoprecipitates of pp60^{src}, p130 and p110 were prepared from *src*-transformed CE cells [using an activated variant of c-*src* containing a substitution of Tyr527 with Phe (Cartwright *et al.*, 1987; Kato *et al.*, 1987; Kmiecik and Shalloway, 1987; Piwnica-Worms *et al.*, 1987; Reynolds *et al.*, 1987)], resolved by SDS−PAGE and immunoblotted with anti-pTyr (Figure 1A). The three major phosphotyrosine-containing proteins p130, p110 and pp60^{src} were detected in the pp60^{src} immunoprecipitate using mAb EC10 (Parsons *et al.*, 1984) (lane 1). Two additional proteins of ~85 kd and 70 kd were also observed, although these appear to be breakdown products of p110. Immunoprecipitation of p130 with mAb 4F4 resulted in the co-immunoprecipitation of a low, but detectable amount of p110 (lane 2). Finally, tyrosine-phosphorylated p110 was immunoprecipitated with mAb 4C3 (lane 3). The absence of pp60^{src} in immunoprecipitates of p130 or p110 suggests that the mAbs may react preferentially with uncomplexed forms of their respective proteins under non-denaturing conditions, the epitopes recognized by mAbs 4F4 and 4C3 are masked by complex formation or that the amount of pp60^{src} associated with p130 and p110 is low.

In order to show that the two major phosphoproteins detected in immunoprecipitates of pp60^{src} were immunoreactive with mAbs to p130 and p110, immunoprecipitates of pp60^{src} were immunoblotted with mAbs to p130, p110 or pp60^{src} (Figure 1B). The anti-p130 mAb 4F4 detected a single 130 kd polypeptide species in an immunoprecipitate of pp60^{src} (lane 4). Similarly, p110 was detected in the pp60^{src} immunoprecipitate using the mAb 4C3 (lane 5), as was pp60^{src} using the anti-*src* mAb 327 (lane 6). Immunoprecipitates of p130 or p110 immunoblotted with their respective mAbs resulted in the detection of the individual proteins (see Figure 8). To estimate the amount of tyrosine phosphorylated p110 either associated with pp60^{src} or remaining uncomplexed in *src*-transformed cells, immunoprecipitates prepared with anti-*src*, anti-p110 and anti-pTyr were probed with anti-p110 mAb. Comparison of p110 in each case showed that ~10−15% of p110 was tyrosine phosphorylated and that an equivalent portion of p110 was pp60^{src}-associated (data not shown). Taken together, the above results clearly document the specificity of the mAbs to the individual components of the p130/p110/pp60^{src} complex, and show that significant amounts of p130 and p110 are associated with pp60^{src}.

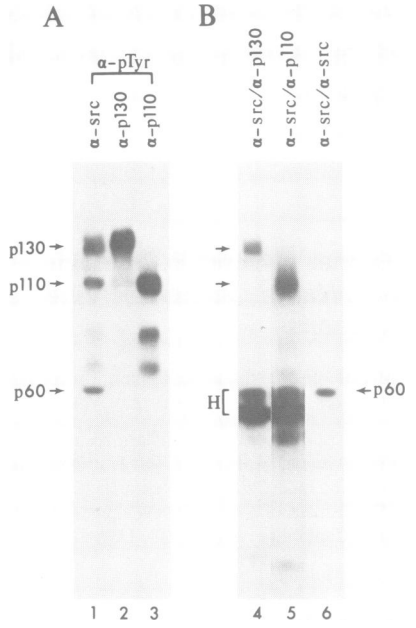

Fig. 1. Specificity of mAbs to p130 and p110. (**A**) Tyrosine phosphoproteins were immunoprecipitated from 500 μg total cell protein of 527F transformed CE cells: lane 1, pp60^{src} (mAb EC10); lane 2, p130 (mAb 4F4); lane 3, p110 (mAb 4C3). Proteins were immunoblotted with affinity purified rabbit anti-pTyr. (**B**) Immunoprecipitates of pp60^{src} were immunoblotted with anti-p130 mAb 4F4 (lane 4), anti-p110 mAb 4C3 (lane 5) or anti-pp60^{src} mAb 327 (lane 6). Immunoblots probed with mAbs to p130 and p110 were developed with [^{125}I]anti-mouse Ig (lanes 4 and 5) or with [^{125}I]mAb 327 (lane 6). H, immunoglobulin heavy chain.

Serine, threonine and tyrosine phosphorylation of p130 and p110

To demonstrate the presence of phosphotyrosine on p130 and p110, immunoprecipitates of p130 and p110 were prepared from $^{32}P_i$-labeled CE cells, and the individual labeled proteins were isolated from SDS−polyacrylamide gels and subjected to two-dimensional phosphoamino acid analysis (Figure 2). Analysis of p130 (panel A) and p110 (panel B) from untransformed CE cells showed that both proteins are phosphorylated predominantly on serine residues, and to a lesser extent on threonine residues. In addition, longer exposure of the TLC plate revealed that p130 contained a low, but detectable amount of phosphotyrosine. In CE cells transformed by 527F, an increase in the level of phosphotyrosine was observed in p130 (panel C), and phosphotyrosine was detected in p110 (panel D). In order to determine the pattern of phosphorylation of p130 and p110 in complex with pp60^{src}, immunoprecipitates of pp60^{src} were prepared from 527F cells, isolated by SDS−PAGE, and individual bands containing co-immunoprecipitated p130 and p110 were subjected to phosphoamino acid analysis. A similar pattern of amino acid phosphorylation was observed

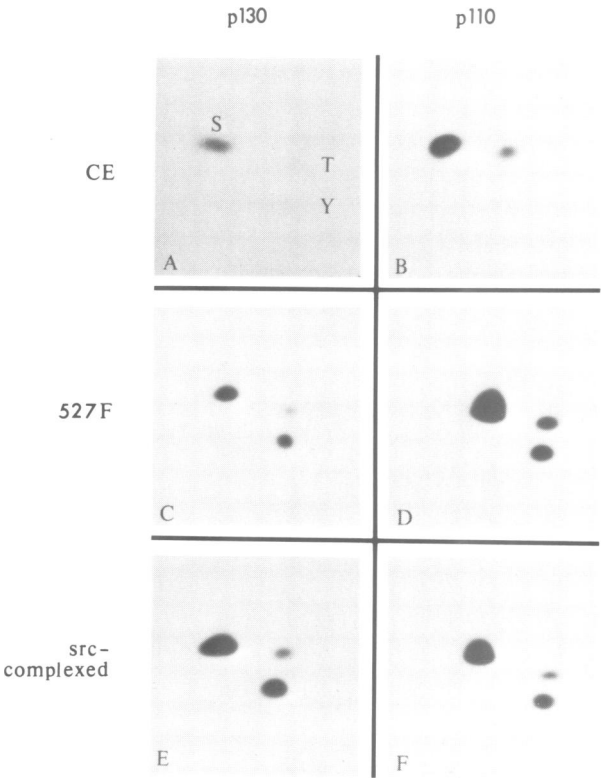

Fig. 2. Phosphoamino acid analysis of p130 and p110. CE cells and 527F transformed CE cells were labeled with $^{32}P_i$ and p130 and p110 were immunoprecipitated with mAbs 4F4 and 4C3, respectively, or co-immunoprecipitated with pp60^{src} using mAb EC10. Proteins were extracted from SDS–polyacrylamide gels and processed for two-dimensional phosphoamino acid analysis as described in Materials and methods. Panel **A**, p130 from CE; panel **B**, p110 from CE; panel **C**, p130 from 527F; panel **D**, p110 from 527F; panel **E**, p130 from immunoprecipitates of pp60^{src} from 527F; panel **F**, p110 from immunoprecipitates of pp60^{src} from 527F. S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine. TLC plates were exposed to X-ray film for 1 day, except panel A, which was exposed for 5 days.

for both p130 (panel E) and p110 (panel F) in the p130–p110–pp60^{src} complex as that seen in the individual analyses from *src*-transformed CE cells. Thus, both p130 and p110 are substrates of serine/threonine and tyrosine kinases, while p130 appears to be phosphorylated on tyrosine to a limited extent in CE cells.

Mapping of p130 and p110 association with the src homology domains of pp60^{src}

In a previous report, we demonstrated that mutations in the *src* homology domains (SH2 and SH3) of activated pp60^{src} affected the association of p130 and p110 with pp60^{src} in *src* immune complexes (Reynolds *et al.*, 1989b). These results indicated that a mutation in the SH3 domain ablated the stable association of p110, whereas neither phosphoprotein was observed in immune complexes of *src* containing a mutation in the SH2 region. Since most of these analyses were performed by immunoblotting with anti-pTyr, the possibility as to whether tyrosine phosphorylation was required for the binding of p130 and p110 to pp60^{src} was not addressed directly. In addition, we could not ascertain whether uncomplexed p130 and p110 were tyrosine phosphorylated in *src* variant infected cells. To determine more precisely the regions in the SH2 and SH3 domains of pp60^{src} involved in modulating the association of p130 and p110 with pp60^{src}, and to demonstrate the presence or absence of unphosphorylated forms of p130 and p110 bound to pp60^{src} mutants, ten deletion variants were constructed in the *src* homology domains of the 527F variant (Figure 3).

The residues deleted in the SH2 and SH3 variants of *src* represent residues shared by other tyrosine kinases (e.g. v-*fps*, v-*abl* and v-*fgr*) and other proteins with SH2/SH3 domains (e.g. GAP, PLC-γ, v-*crk* and α-spectrin). Thus, these *src* variants provide a means to study the interactions of p130 and p110 with activated pp60^{src} and analyze the potential relevance of these common domains. Three deletion variants in the SH3 domain were generated with (i) a 4 amino

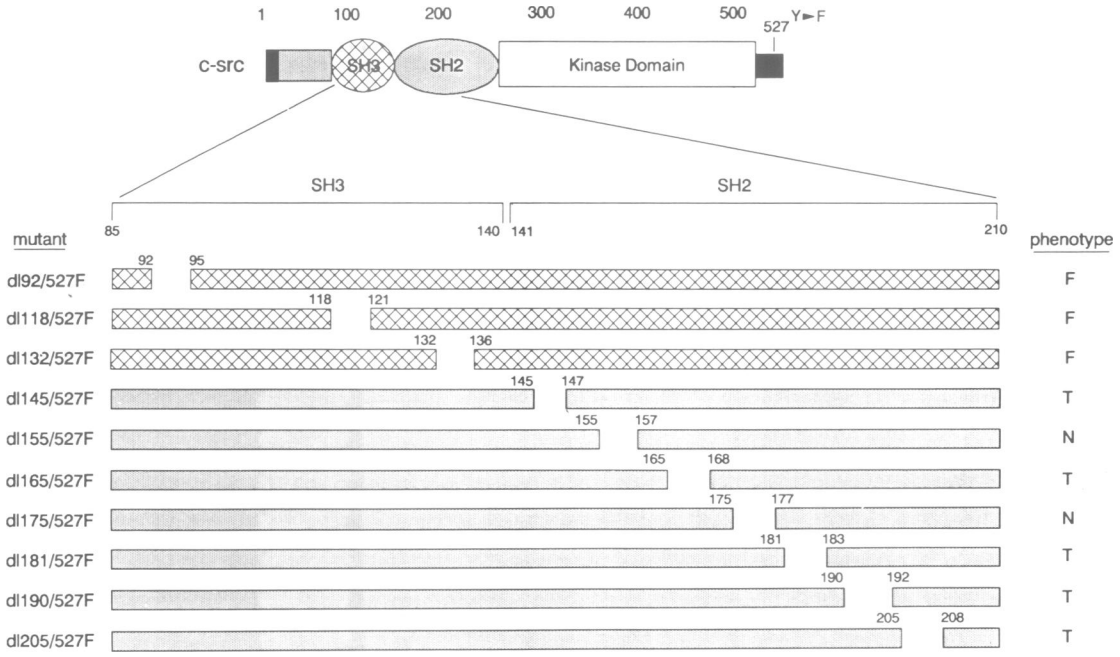

Fig. 3. SH2 and SH3 variants of 527F. Deletion variants were generated as described in Materials and methods. The numbers on either side of the break in the bars denote the boundaries of residues deleted from the protein. Phenotypes were determined by transfection of each variant in CE cells and assessment of morphology by light microscopy after 9 days (examples shown in Figure 4). Cell morphology: N, normal; F, fusiform; T, transformed.

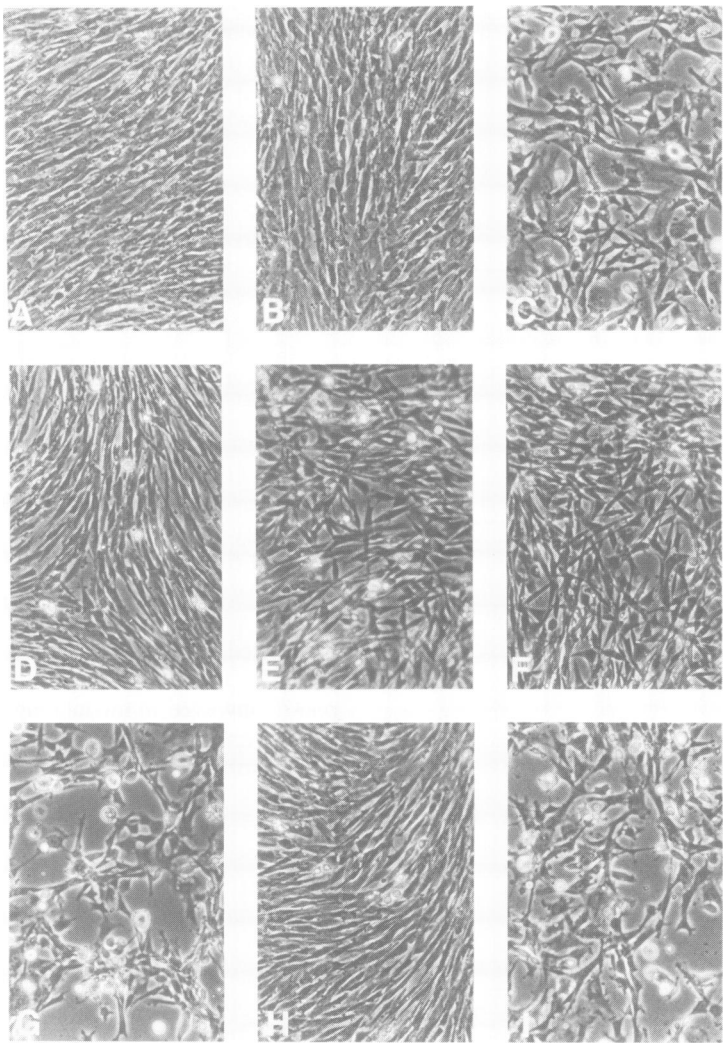

Fig. 4. Cellular morphology of CE cells transfected with *src* variants. Morphology was determined after 9 days by light microscopy. Panel **A**, CE cells; panel **B**, c-*src*; panel **C**, 527F; panel **D**, 2A/527F; panel **E**, dl92/527F; panel **F**, dl132/527F; panel **G**, dl145/527F; panel **H**, dl155/527F; panel **I**, dl205/527F.

acid deletion of residues 92–95 (Potts *et al.*, 1988), dl92/527F; (ii) a 4 amino acid deletion of residues 118–121, dl118/527F; and (iii) a 5 amino acid deletion of residues 132–136, dl32/527F (Figure 3). CE cells (Figure 4A) expressing these SH3 variants exhibited a fusiform morphology, characterized by spindle-shaped cells with a disorganized pattern of growth (Figure 4, panels E and F). Seven deletion variants in the SH2 domain were isolated and contained (i) a 3 amino acid deletion of residues 145–147, dl145/527F; (ii) a 3 amino acid deletion of residues 155–157, dl155/527F; (iii) a 4 amino acid deletion of residues 165–168, dl165/527F; (iv) a 3 amino acid deletion of residues 175–177, dl175/527F; (v) a 3 amino acid deletion of residues 181–183, dl181/527F; (vi) a 3 amino acid deletion of residues 190–192, dl190/527F; and (vii) a 4 amino acid deletion of residues 205–208, dl205/527F. Variants dl155/527F and dl175/527F were defective for transformation when expressed in CE cells (panel H), whereas all of the other variants with SH2 deletions induced a phenotype (panels G and I) indistinguishable from that of 527F (panel C). A double mutant of 527F with an additional mutation at residue 2 (Gly → Ala) results in an unmyristylated non-transforming variant, 2A/527F (Reynolds *et al.*, 1989a). These cells were slightly elongated but contact inhibited (panel D).

To determine the level of pp60^{src} expression encoded by the various SH2 and SH3 variants, whole cell lysates were subjected to SDS–PAGE and pp60^{src} was detected by immunoblotting with mAb 327 (Figure 5A). Total phosphotyrosine-containing proteins were detected by immunoblotting with anti-pTyr (Figure 5B). Expression of pp60^{src} was apparent in all of the infected CE cells (panel A), and was highest in cells expressing the non-transforming variants of *src*, including c-*src* (lane 2) and the unmyristylated variants 2A/C (lane 3) and 2A/527F (lane 4). The deletion variant dl118/527F exhibited an interesting alteration in mobility possibly due to changes in the conformation of the protein because of the deletion of two Trp residues (Trp119 and 120) (lane 7). The pattern of tyrosine phosphorylated proteins in normal CE cells included a single species of 125 kd (Figure 5B, lane 1). Overexpression of either non-transforming c-*src* or 2A/C did not alter the pattern of tyrosine phosphorylation, except for the appearance of the tyrosine phosphorylated pp60^{src} (lanes 2 and 3). However, expression of any of the 527F-containing variants resulted in an increase in the tyrosine phosphoryl-

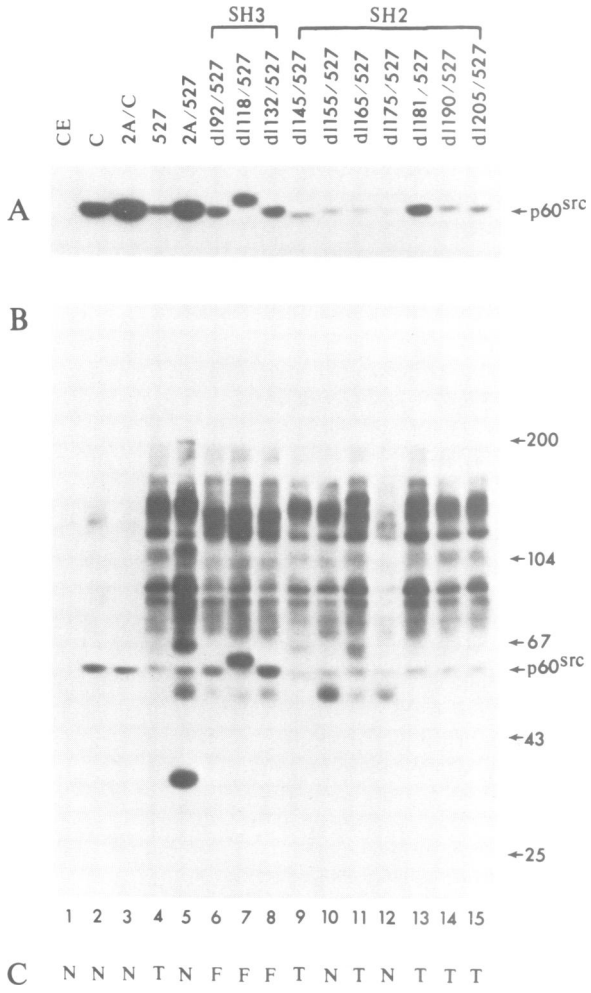

Fig. 5. Expression and tyrosine kinase activity of *src* variants in CE cells. (**A**) CE cells transfected with individual *src* variants were lysed with hot SDS−PAGE sample buffer, and 25 μg total cellular proteins were subjected to SDS−PAGE and immunoblotting with [^{125}I]mAb 327. Lane 1, CE cells; lane 2, c-*src*; lane 3, 2A/C; lane 4, 527F; lane 5, 2A/527F; lane 6, dl92/527F; lane 7, dl118/527F; lane 8, dl132/527F; lane 9, dl145/527F; lane 10, dl155/527F; lane 11, dl165/527F; lane 12, dl175/527F; lane 13, dl181/527F; lane 14, dl190/527F; lane 15, dl205/527F. (**B**) An identical immunoblot as in panel (A) was probed with anti-pTyr. Mol. wt markers are in kd. (**C**) Phenotypes of *src* variants: N, normal; F, fusiform; T, transformed.

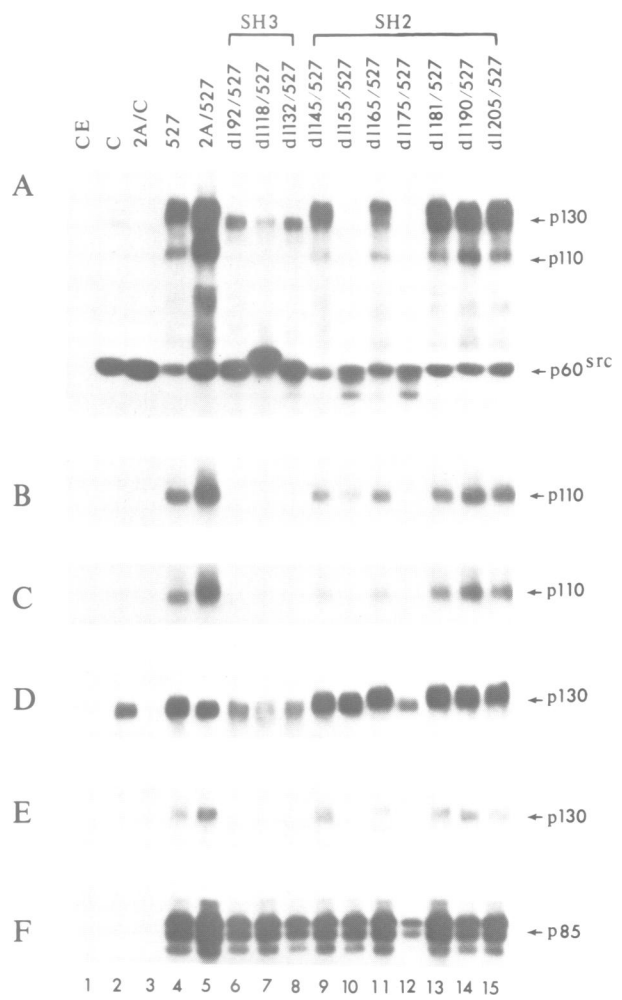

Fig. 6. Mapping of p130 and p110 to the SH2 and SH3 domains of pp60^{src}. (**A**) CE cells expressing the indicated *src* variants (as in Figure 5) were lysed in modified RIPA buffer and pp60^{src} was immunoprecipitated with mAb EC10 and immunoblotted with affinity purified anti-pTyr. (**B**) Immunoprecipitates of p110 using mAb 4C3 were immunoblotted with anti-pTyr. (**C**) Immunoprecipitates of pp60^{src} were immunoblotted with anti-p110 mAb 4C3. (**D**) Immunoprecipitates of p130 using mAb 4F4 were immunoblotted with anti-pTyr. (**E**) Immunoprecipitates of pp60^{src} were immunoblotted with anti-p130 mAb 4F4. (**F**) Immunoprecipitates of p85 were prepared using mAb 1H1 (Kanner *et al.*, 1990) and immunoblotted with anti-pTyr.

ation of cellular proteins (lanes 4−15). Comparison of the phenotype of CE cells expressing the different variants (Figure 5C) with the patterns of tyrosine phosphorylated proteins showed that all *src* variants containing the 527F mutation induced the tyrosine phosphorylation of multiple substrates, although an increase in the total phosphotyrosine content of the cells did not appear to correlate exclusively with transformation. Infection of CE cells with transforming and non-transforming SH2 variants (Figure 5, lanes 9−15) resulted in most cases in a reduced level of pp60^{src} expression. However, the lower expression of pp60^{src} did not strictly correlate with the loss of the transformed phenotype (e.g. dl145/527F and dl165/527F) or a decrease in the extent of tyrosine phosphorylation of cellular proteins. The exception to this observation was dl175/527F, the steady state level of which appeared to be significantly reduced. These results were consistent with previous observations that a similar mutation in v-*src* results in an unstable protein (Wang and Parsons, 1989). Taken together, the data indicate that expression of two SH2 and three SH3 variants in CE cells results in both phenotypic alterations and an increase in the level and complexity of tyrosine phosphorylation of cellular substrates.

In order to map more precisely the residues in the SH2 and SH3 regions of *src* involved in promoting stable association with p130 and p110, pp60^{src} immunoprecipitates prepared from CE cells expressing each of the ten *src* homology domain variants were immunoblotted with the individual mAbs to p130 and p110 or anti-pTyr. Immunoblotting of pp60^{src} immune complexes with anti-pTyr (Figure 6A) revealed the presence of tyrosine phosphorylated pp60^{src} in all of the infected cells, while the appearance of p130 and p110 in these immune complexes was clearly affected in cells expressing different variants. Tyrosine phosphorylated forms of both p130 and p110 were observed in 527F and 2A/527F, but p110 was not detected

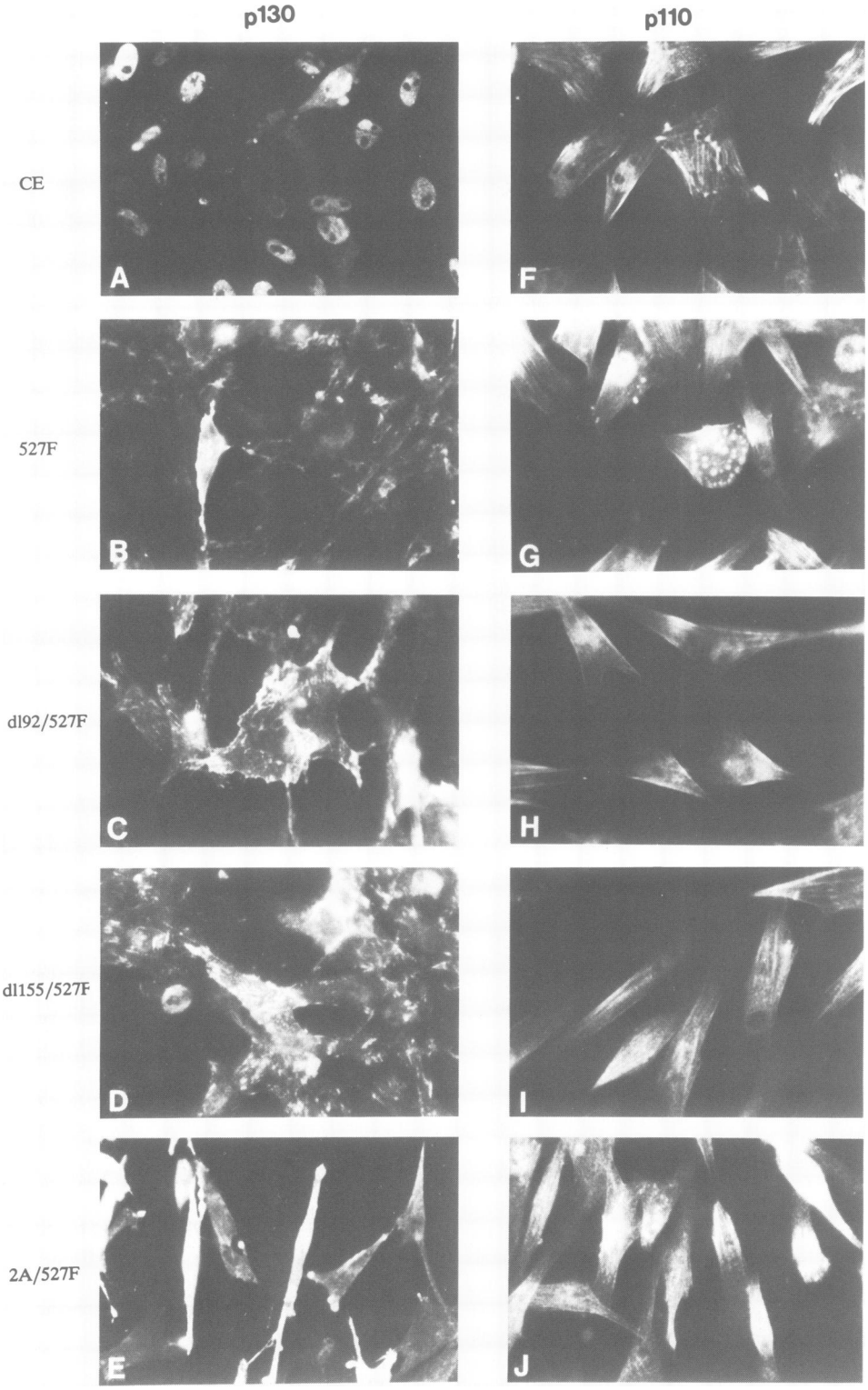

Fig. 7. Indirect immunofluorescence of p130 and p110. CE cells (panels **A** and **F**) were infected with the transforming *src* variant 527F (panels **B** and **G**), the SH3 variant dl92/527F (panels **C** and **H**), the transformation defective SH2 variant dl155/527F (panels **D** and **I**) or the non-myristylated variant 2A/527F (panels **E** and **J**). The cells were fixed as described in Materials and methods and stained for p130 with mAb 4F4 (left panels) or for p110 with mAb 4C3 (right panels).

in immunoprecipitates of the SH3 variants (lanes 6–8). In addition, the mobility of p130 was altered in the SH3 variants. Two SH2 variants dl155/527F (lane 10) and dl175/527F (lane 12) exhibited greatly reduced amounts of co-immunoprecipitated tyrosine phosphorylated p130 and p110, whereas none of the other SH2 deletions influenced association of p130 or p110 with pp60^{src}. The lack of association of phosphotyrosine-containing p130 and p110 with pp60^{src} encoded by dl155/527F and dl175/527F correlates with the transformation defective phenotype of these variants.

To determine whether p110 was tyrosine phosphorylated

in CE cells expressing SH2 and SH3 variants, p110 was immunoprecipitated from whole cell lysates and immunoblotted with anti-pTyr (Figure 6B). This analysis showed that p110 was not tyrosine phosphorylated in cells expressing any of the SH3 variants, but it was tyrosine phosphorylated to substantial levels in the SH2 variant dl155/527F (lane 10), and to a lesser extent in cells infected with dl175/527F (lane 12). These results indicate that tyrosine phosphorylation of p110 may be a co-requisite for association with pp60^{src}. However, the results with dl155/527F indicate that tyrosine phosphorylation *per se* may be insufficient for complex formation. To ascertain whether unphosphorylated forms of p110 were associated with the *src* homology variants, immunoprecipitates of pp60^{src} were immunoblotted with the anti-p110 mAb 4C3. As shown in panel C, p110 was not detected in pp60^{src} immune complexes in the SH3 mutants, nor in the transformation defective SH2 variants dl155/527F and dl175/527F. Thus, the association of p110 with activated pp60^{src} required the structural integrity of the entire SH3 domain and two regions of SH3.

A similar analysis performed on p130 revealed that total p130 was tyrosine phosphorylated to a greater extent in CE expressing c-*src* or any of the activated variants of *src* compared with the level observed in uninfected cells or cells expressing 2A/C (Figure 6D). Further, p130 was not complexed with the SH2 variants dl155/527F and dl175/527F (panel E, lanes 10 and 12). In cells expressing the SH3 variants, the amount of p130 associated with pp60^{src} was significantly decreased when anti-p130 was used to immunoblot pp60^{src} immune complexes (panel E, lanes 6–8). However, similar immune complexes probed with anti-pTyr revealed a protein of ~125 kd. It is unclear whether this component of the complex is related to p130. Hence, the association of tyrosine phosphorylated p130 with pp60^{src} was ablated by two specific deletions in the SH2 domain, while alterations in SH3 significantly reduced the association of p130 detected by mAb 4F4.

To assess total PTK activity of the *src* variants, p85, a substrate of oncogene-encoded tyrosine kinases (Kanner *et al.*, 1990), was immunoprecipitated with mAb 1H1 (Kanner *et al.*, 1990) and immunoblotted with anti-pTyr (Figure 6F). The PTK activity of the different activated variants appeared to be similar, with the exception of the unstable variant dl175/527F which was reduced by ~60–70% (lane 12). The results with immunoprecipitated p85 correlated closely with the kinase activity observed by total tyrosine phosphoprotein analysis in cell lysates (Figure 5B).

Subcellular distribution of p130 and p110

To determine the subcellular distribution of p130 and p110 directly, we examined CE cells in an indirect immunofluorescence assay (Figure 7). Uninfected CE cells stained with the p130-specific mAb 4F4 showed a nuclear staining pattern (panel A). Nuclear fluorescence was observed with culture supernatants, partially purified mAbs or ascites fluid, but not with secondary antibody (rabbit anti-μ chain) in the absence of mAb 4F4 (data not shown). Transformation of CE cells with 527F resulted in a redistribution of p130 to the cytoplasmic membrane (panel B). This immunofluorescence staining pattern was also observed in cells partially transformed by the SH3 variants (Figure 7C) as well as in the transformation defective SH2 variants (panel D). These data indicate that both the activation of the *src* PTK and the concomitant increase in tyrosine phosphorylation of pp60^{src}-associated p130 correlated with the cellular membrane localization of p130 and a reduction in nuclear staining. Finally, p130 was observed in the cytosol of CE cells expressing the unmyristylated transformation defective *src* variant 2A/527F (panel E).

Analysis of the subcellular localization of p130 was also performed by fractionation of hypotonically lysed CE cells, followed by immunoprecipitation and immunoblotting of p130 (Figure 8A). A low, but detectable amount of p130 was observed in the nuclear fraction of uninfected CE cells (lane 1). In 527F transformed cells, p130 was detected both in the membrane fraction and in the cytosol (lanes 5 and 6). In contrast, in CE cells expressing 2A/527F, virtually all of the p130 was detected in the cytosol (lane 9). These results correlate with the redistribution of p130 observed in the indirect immunofluorescence assay (Figure 7).

Immunofluorescence staining of p110 revealed a different pattern of subcellular localization in comparison with p130. In uninfected CE cells, p110 appeared associated with cytoskeletal elements (Figure 7F), as indicated by a staining pattern similar to that exhibited by actin stress cables (Reynolds *et al.*, 1989a). A partial redistribution of p110 was observed upon transformation of the cells with 527F (panel G), such that the staining appeared in dense, punctate clusters in some cells, perhaps in focal adhesions or podosomes. Interestingly, the redistribution of p110 was not complete, in that many cells exhibited residual staining of stress fibers. In cells transformed by dl92/527F (SH3 variant, panel H) and in cells expressing the transformation defective, PTK activated variants dl155/527F and 2A/527F (panels I and J), the normal staining pattern of p110 was observed. Thus, the redistribution of p110 appeared to correlate with transformation in cells in which p110 was associated with activated pp60^{src}.

Subcellular fractionation analysis of p110 showed that in CE cells it was present mainly in the cytosolic compartment, perhaps reflecting a salt-sensitive interaction with cytoskeletal structures (Figure 8B, lane 3). However, upon transformation by 527F, p110 was partially redistributed (~50%) to the cellular membrane fraction (lane 5). In CE cells expressing 2A/527F, the p110 fractionation pattern was similar to that observed in uninfected cells (lanes 7–9). We conclude from the above analyses that the intracellular distribution of p110 in *src*-transformed cells correlated both with its state of tyrosine phosphorylation and its stable association with pp60^{src}.

Discussion

The roles and identities of cellular proteins that become tyrosine phosphorylated in response to a variety of cell stimuli are largely unknown. However, diverse cellular activities involve the tyrosine phosphorylation of protein substrates, including ligand stimulation of receptor PTKs (Kazlauskas and Cooper, 1989; Kumjian *et al.*, 1989; Molloy *et al.*, 1989; Morrison *et al.*, 1989, 1990; Wahl *et al.*, 1989; Kaplan *et al.*, 1990; Kazlauskas *et al.*, 1990; Margolis *et al.*, 1990; Ullrich and Schlessinger, 1990; Kanner *et al.*, 1991), agonist-stimulated secretion (Ely *et al.*, 1990), platelet activation (Ferrell and Martin, 1988, 1989; Golden and Brugge, 1989), cell cycle changes (Morla and Wang, 1986; Draetta *et al.*, 1988) and lymphocyte activation

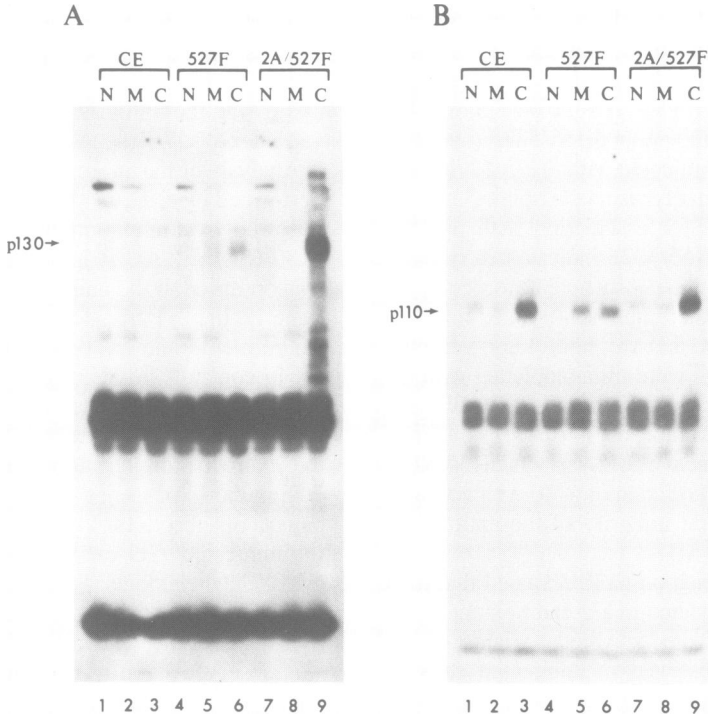

Fig. 8. Subcellular fractionation analysis of p130 and p110. CE cells (lanes 1–3), 527F transformed CE cells (lanes 4–6) and CE cells expressing 2A/527F (lanes 7–9) were lysed in hypotonic buffer followed by Dounce homogenization. The lysates were separated by differential centrifugation into a nuclear (N) fraction by low-speed centrifugation (P1), and a membrane (M) fraction (P100) and cytosolic (C) fraction (S100) by ultracentrifugation. (**A**) Immunoprecipitates of p130 using mAb 4F4 and (**B**) immunoprecipitates of p110 using mAb 4C3 were prepared from each fraction. The proteins were immunoblotted with their respective mAbs. The bands at ~50 kd and ~25 kd are the immunoglobulin heavy and light chains respectively.

(Campbell and Sefton, 1990; Gold *et al.*, 1990; June *et al.*, 1990). The most dramatic increase in total tyrosine phosphorylation of cellular proteins occurs upon the expression of oncogene-encoded PTKs (Hamaguchi *et al.*, 1988; Kamps and Sefton, 1988; Linder and Burr, 1988; Morrison *et al.*, 1988; Glenney and Zokas, 1989; Reynolds *et al.*, 1989a,b; Kanner *et al.*, 1990; Kozma *et al.*, 1990; Matsuda *et al.*, 1990; Mayer and Hanafusa, 1990) such as *src*. In this report, we have characterized two *src* substrates, tyrosine phosphorylated proteins p130 and p110, that form stable complexes with activated variants of pp60^{src} through the *src* homology domains SH2 and SH3.

Analysis of *src* variants with structural alterations in the SH2 and SH3 domains provides insight into the sequences that are necessary for the formation of stable complexes between p130, p110 and pp60^{src}. Several structural and functional prerequisites appear to be necessary, but perhaps not sufficient for complex formation. First, activation of the *src* PTK is required. Uninfected CE cells do not contain detectable complexes between p130, p110 and endogenous pp60$^{c\text{-}src}$. Mutations which inactivate *src* PTK activity block both p130 and p110 tyrosine phosphorylation and pp60^{src} association (data not shown). Stable complex formation also requires the structural integrity of part of the SH2 domain of *src*. Sequences within and including residues 155–165 and 175–180 appear particularly important for complex formation. Mutations in the region 170–180, which includes the highly conserved FLVRES sequence, exhibit an unusual host-range phenotype (Vederame *et al.*, 1989; Hirai and Varmus, 1990a,b). The region between residues 155 and 180 also influences the oncogenic activation of c-*src* (Parsons and Weber, 1989; Hirai and Varmus, 1990a,b; O'Brien *et al.*, 1990), e.g. point mutations within this region (Parsons and Weber, 1989; Hirai and Varmus, 1990a,b; O'Brien *et al.*, 1990) as well as deletion of residues 165–168 (H.-C.R.Wang and J.T.Parsons, unpublished observations) activate the oncogenicity of c-*src*. In the latter case, such activation leads to tyrosine phosphorylation of p130 and p110 and stable p130–p110–pp60^{src} complex formation. Three separate mutations in the SH3 domain significantly reduce the stable association of p110 with pp60^{src} and appear also to reduce pp60^{src}-associated p130. The residues deleted in these *src* variants are highly conserved among the SH3 regions of the *src* family kinases, v-*crk* and GAP (Mayer *et al.*, 1988; Pawson, 1988; Stahl *et al.*, 1988). Sequences within these regions of *src* thus appear to influence both positive and negative interactions between p130, p110 and pp60^{src}.

The association of p110 with pp60^{src} in transformed cells appears to correlate with the redistribution of p110 to focal adhesions or podosomes. Although not all p110 appears to be redistributed (either in immunofluorescence assays or by subcellular fractionation), the amount of p110 in 527F-transformed CE cells that is stably associated with pp60^{src} (10–15%) correlates well with the approximate amount of p110 that is tyrosine phosphorylated. In addition, in *src*-transformed CE cells, a significant fraction of p110 is stably associated with cellular membranes, presumably through its interaction with pp60^{src}. Since p130 and p110 were both tyrosine phosphorylated and associated with the non-transforming unmyristylated variant 2A/527F, these events are not sufficient for mediating transformation. Instead, we suggest that because p130 and p110 are abundant proteins, substantial pools of soluble p130 and p110 exist, and thus

functional interactions are dictated by both stable complex formation and correct intracellular localization.

The correlation between *src* structure, p130–p110–pp60^{src} complex formation and cellular transformation led to several interesting possibilties. In SH3 variants, the cells exhibit a fusiform morphology, and p110 is neither tyrosine phosphorylated nor stably associated with pp60^{src}. These observations suggest that both tyrosine phosphorylation of p110 and complex formation with membrane-associated pp60^{src} are prerequisites for complete transformation, i.e. the characteristic rounded cell phenotype observed in RSV or 527F transformed cells. Analysis of transformation defective SH2 variants (dl55/527F and dl175/527F) revealed reduced tyrosine phosphorylation of p110 and an absence of complex formation. While our data do not distinguish between p110 and p130 binding to the same or different pp60^{src} molecules it is possible that binding of tyrosine phosphorylated p130 with pp60^{src} influences stable p110 binding, in that the SH2 domain stabilizes p130 binding and SH3 residues facilitate the association of p110. This possibility is prompted by the observation that p110 can be detected in immunoprecipitates of p130 by direct Western immunoblotting (S.B.Kanner, unpublished observations). Although the converse experiment does not reveal co-immunoprecipitation of p130 with p110 using the anti-p110 mAb, perhaps the p110 epitope is masked by the association of p130. Thus, we speculate that p130 and p110 may interact to contribute to *src* activity, either by stabilizing the active conformation of pp60^{src} or by contributing to the co-localization of *src* with its substrates.

Immunofluorescence localization of p130 in uninfected cells indicated that p130 is in the nucleus. Although this observation is somewhat provocative, several features of the anti-p130 mAb lead us to conclude that it preferentially detects the tyrosine hyperphosphorylated forms of p130: (i) direct immunoblotting of cell lysates (data not shown) or immunoprecipitation of p130 from uninfected CE cells versus *src* transformed cells clearly showed an increase in the amount of p130 detected in *src* transformed cells (see Figure 8A); (ii) phosphatase treatment of immunoprecipitates of p130 from *src* transformed cells showed both a decrease in the level of phosphotyrosine in p130 and a concomitant loss of detection of the protein in a direct immunoblot (R.R.Vines and J.T.Parsons, unpublished observations); (iii) immunoprecipitation of p130 from $^{32}P_i$-labeled cells showed a limited level of immunoprecipitable p130 in normal versus *src* transformed cells (Figure 2); and yet, (iv) phosphotyrosine did not block the activity of mAb 4F4 (Kanner *et al.*, 1990). Collectively, these studies have led us to speculate that mAb 4F4 is specific for tyrosine phosphorylated forms of p130. Thus, the form of p130 associated with the nucleus in normal CE cells may be hypophosphorylated on tyrosine.

The interactions of tyrosine phosphorylated proteins have been correlated with functional cellular activities. Specifically, activation of receptor PTKs such as the PDGF receptor coincides with the association of tyrosine phosphorylated forms of PLC-γ, GAP and raf-1 (Kazlauskas and Cooper, 1989; Kumjian *et al.*, 1989; Morrison *et al.*, 1989, 1990; Kaplan *et al.*, 1990; Kazlauskas *et al.*, 1990). Further, tyrosine phosphorylation of GAP during oncogene-encoded PTK transformation or by receptor PTK stimulation results in the association of two tyrosine phosphorylated proteins p64 and p190 (Ellis *et al.*, 1990; Moran *et al.*, 1990; Bouton *et al.*, 1991). Perhaps the SH2 and SH3 domains in GAP, PLC-γ and α-spectrin facilitate their interaction with other cellular proteins. For example, it has been demonstrated recently that the same phosphotyrosine containing proteins bound to GAP in immune complexes bind to SH2 domain—containing polypeptides generated in bacteria (Moran *et al.*, 1990). The formation of protein–protein complexes may thereby facilitate the transduction of signals for cell growth and differentiation. The mechanisms involved in such cellular changes may include alteration of the activity of multi-component complexes, subcellular redistribution of complexes or both.

Finally, several investigators have demonstrated that mutations in the SH2 and SH3 domains of *src* induce a variety of changes in the activity of normal and activated pp60^{src} molecules (Potts *et al.*, 1988; DeClue and Martin, 1989; Vederame *et al.*, 1989; Wang and Parsons, 1989; Wendler and Boschelli, 1989; Hirai and Varmus, 1990a,b; O'Brien *et al.*, 1990). These alterations involve host-range specificity, transformation defectiveness, temperature sensitivity and cell phenotypic changes. The SH2 and SH3 domains are clearly relevant for modulating the activity of pp60^{src} and perhaps that of other proteins harboring these common domains. In light of these observations, our current results suggest that potentially critical SH2/SH3 dependent interactions of pp60^{src} with cellular proteins may be necessary for *src* to alter the normal state of a cell. The identity and function of p130 and p110 will ultimately facilitate our understanding of their interactions with pp60^{src} and establish the relevance of tyrosine phosphorylation of these proteins in altering cell growth and morphology.

Materials and methods

Cells, viruses and plasmids

Primary chicken embryo (CE) cells were prepared from 10-day-old chicken embryos (Spafas, Norwich, CT) as previously described (Reynolds *et al.*, 1987). Construction and transfection of plasmids bearing c-*src* gene variants in a non-permuted RSV clone were performed as described previously (Reynolds *et al.*, 1987, 1989a).

Monoclonal antibodies, immunoprecipitation and immunoblotting

Anti-p130 mAb 4F4 (IgM) was generated as described previously (Kanner *et al.*, 1990), and partially purified from tissue culture supernatants by ammonium sulfate precipitation. The mAb to p110 (4C3; IgG_{2b}) generated previously (Kanner *et al.*, 1990) was purified from ascites fluid by protein A–Sepharose chromatography. Anti-pp60^{src} mAbs EC10 (Parsons *et al.*, 1984) and 327 (Lipsich *et al.*, 1983) were purified from ascites fluid as described above. Immunoprecipitation of individual proteins with mAbs was performed as described (Kanner *et al.*, 1989b, 1990, 1991). Immunoblotting was performed as described previously using 5 μg/ml mAb in milk buffer (Kanner *et al.*, 1990) or 2 μg/ml affinity purified rabbit anti-pTyr (Kamps and Sefton, 1988; Kanner *et al.*, 1989a,b; Reynolds *et al.*, 1989a).

Phosphoamino acid analysis

Uninfected or infected CE cells were labeled with 2 mCi/ml $^{32}P_i$ for 6–11 h, rinsed with phosphate-buffered saline (PBS) and lysed in modified RIPA buffer containing proteinase and phosphatase inhibitors as previously described (Kanner *et al.*, 1989b; Reynolds *et al.*, 1989a). Proteins were immunoprecipitated with mAbs and extracted from the gel slice with 50 mM ammonium bicarbonate, 0.1% SDS and 5% β-mercaptoethanol (Kanner *et al.*, 1988). Extracted proteins were TCA precipitated, washed in ethanol and hydrolyzed in 5.7 M HCl at 110°C for 1.5 h under nitrogen, prior to two-dimensional thin layer electrophoresis (Kanner *et al.*, 1989b). Authentic phosphoamino acids were co-electrophoresed and identified by ninhydrin staining.

Isolation and characterization of mutations within the SH2 and SH3 domains of src

For mutagenesis of the SH2 and SH3 domains, the c-*src*/527F gene was excised from pRL527F and cloned into the single-stranded coliphage vector

M13mp18 between the *Hin*dIII and *Kpn*I sites (Reynolds *et al.*, 1987, 1989a,b). Mutagenesis was carried out by using mutant oligonucleotides (24 or 30mers) to introduce specific mutations as described previously (Zoller and Smith, 1984; Wang and Parsons, 1989). The mutations were confirmed by dideoxy DNA sequencing, and the mutated *src* genes were recloned into the pRL vector (Reynolds *et al.*, 1987). The deletion variant encoded *src* proteins with deletions of 3, 4 or 5 amino acids within the SH2 or SH3 domains are denoted by the position of the first amino acid of the deletion: dl92, deletion of YESR; dl118, deletion of WWLA; dl132, deletion of IPSNY; dl145, deletion of AEE; dl155, deletion of RRE; dl165, deletion of PENP; dl175, deletion of RKS; dl181, deletion of KGA; dl190, deletion of DFD; dl205, deletion of CKLY.

Immunofluorescence microscopy

Cells were seeded and grown overnight on glass coverslips, washed with PBS and fixed for 5–20 min with 3% paraformaldehyde as described previously (Reynolds *et al.*, 1989a). The cells were permeabilized with 0.4% Triton X-100, washed with PBS and incubated with 150 μl of mAb [10 μg/ml of 4F4 (anti-p130) or 4C3 (anti-p110)] for 60 min at room temperature. For immunostaining with mAb 4C3, the cells were washed and then incubated with affinity purified rabbit anti-mouse immunoglobulin G for 60 min, washed and then incubated with fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit immunoglobulin G (5 μg/ml, Jackson ImmunoResearch Labs, West Grove, PA) for 60 min. Cells reacted with mAb 4F4 were washed and incubated with FITC-conjugated rabbit anti-mouse immunoglobulin M (5 μg/ml). Cells were visualized on a Leitz Orthoplan fluorescence microscope.

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