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Identification and Characterization of a Novel Cytoskeleton-Associated pp60^{src} Substrate

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Transformation of cells by the *src* oncogene results in elevated tyrosine phosphorylation of two related proteins, p80 and p85 (p80/85). Immunostaining with specific monoclonal antibodies revealed a striking change of subcellular localization of p80/85 in *src*-transformed cells. p80/85 colocalizes with F-actin in peripheral extensions of normal cells and rosettes (podosomes) of *src*-transformed cells. Sequence analysis of cDNA clones encoding p80/85 revealed an amino-terminal domain composed of six copies of a direct tandem repeat, each repeat containing 37 amino acids, a carboxyl-terminal SH3 domain, and an interdomain region composed of a highly charged acidic region and a region rich in proline, serine, and threonine. The multidomain structure of p80/85 and its colocalization with F-actin in normal and *src*-transformed cells suggest that these proteins may associate with components of the cytoskeleton and contribute to organization of cell structure.

Transformation of cells by tyrosine kinase oncogenes leads to alterations of cell shape, cellular metabolism, growth control, and gene expression (16, 31, 54). A substantial body of evidence indicates that many if not all of these changes are a direct result of the tyrosine kinase activity of the oncogene product (reviewed in references 31, 54, and 66). Rous sarcoma virus (RSV) encodes an enzymatically activated, 60-kDa tyrosine protein kinase, pp60$^{v\text{-}src}$ (6, 15, 44). However, the product of the normal cellular homolog of *src*, pp60$^{c\text{-}src}$, is enzymatically down regulated and does not induce significant alterations in cellular growth or changes in cell morphology when overexpressed in rodent or avian cells (30, 53, 67). Oncogenic activation of c-*src* and concomitant activation of tyrosine kinase activity can be achieved by mutation of the regulatory site of tyrosine phosphorylation, Tyr-527 (11, 40, 58, 63). Thus, expression of pp60$^{v\text{-}src}$ or activated forms of the c-*src* protein pp60^{527F} results in efficient cellular transformation and the increased tyrosine phosphorylation of approximately 15 to 30 cellular proteins (27, 33, 45, 61).

Genetic studies have shown that structural perturbation of several different domains of pp60^{src} leads to alterations in the pattern of tyrosine phosphorylation of specific cellular proteins and accompanying changes in morphological phenotypes (reviewed in references 31 and 54). For example, mutation of the site of myristylation (e.g., Gly-2 to Ala) of pp60$^{v\text{-}src}$ or pp60^{527F} blocks cellular transformation (9, 32, 61) and the tyrosine phosphorylation of a 120-kDa cellular protein (35, 45, 61). pp60^{src} contains two regions that share amino acid sequence similarity with other nonreceptor tyrosine protein kinases (55) and regulatory proteins such as phospholipase C-γ, Crk, and GTPase-activating protein (GAP) (70, 73, 76). Structural alterations within these regions alter or abolish the transforming activity of *src* (18, 28, 51, 59, 77, 79) and lead to alterations in the tyrosine phosphorylation of two pp60^{src}-binding proteins of 130 and 110 kDa (37, 62). These observations indicate that the interaction of pp60^{src} with its cellular substrates is governed by a complex process involving correct intracellular distribution as well as domain-mediated interactions with cellular proteins.

Whereas recent experiments have shown that tyrosine phosphorylation of some cellular proteins appears to direct stable protein-protein interactions (10, 56), knowledge of how tyrosine phosphorylation of cellular proteins leads to reorganization of cellular structures and/or the modulation of the functional activity of components of growth regulatory pathways is just now beginning to emerge. For example, many components of the cytoskeleton appear to be tyrosine phosphorylated in RSV-transformed cells, including vinculin, talin, ezrin, calpactin, paxillin, and fibronectin receptor (reviewed in reference 8). Yet it is unclear how modifications of such proteins influence the global alterations in structure of the cytoskeleton common to transformed cells. Still other proteins that are tyrosine phosphorylated in RSV-transformed cells appear to play a role in regulation of growth control or metabolism, including epidermal growth factor receptor (78), GAP and GAP-associated proteins (4, 22), mitogen-activated protein kinase (64), and phosphatidylinositol kinase (10). For a number of these proteins, tyrosine phosphorylation appears to contribute directly to alterations in functional activity (10, 60, 64, 71).

We have previously described the isolation of a panel of monoclonal antibodies (MAbs) with specificity for proteins that become tyrosine phosphorylated in cells expressing activated forms of the *src* protein (36). Among these antibodies, several are directed toward immunologically related proteins, the major species having apparent molecular weights of 80 and 85 kDa. In this report we describe the properties of these proteins (referred to as p80/85) in normal and *src*-transformed cells and the sequence of a cDNA clone encoding p80/85. In normal cells, the p80/85 proteins are phosphorylated on serine and threonine; in cells expressing activated forms of pp60^{src}, they become heavily phosphorylated on tyrosine. Immunofluorescence analysis reveals a striking change of subcellular localization of p80/85 upon

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transformation. In normal chicken cells, immunostaining with MAbs revealed a punctate staining within the cytoplasm and staining of peripheral cell extensions. The colocalization of p80/85 and actin in peripheral extensions suggests that p80/85 may associate with elements of the cytoskeleton in normal cells. In transformed chicken cells, p80/85 colocalized with actin in rosettelike structures representative of membrane contact sites with the substratum (17, 48, 72). Such structures, referred to as rosettes or podosomes, are characteristic of cells transformed by tyrosine kinase oncogenes and contain a variety of cytoskeletal proteins in addition to F-actin, such as α-actinin, vinculin, talin, and fimbrin (reviewed in reference 8). Analysis of the deduced amino acid sequence of a cDNA clone encoding p80/85 revealed a unique structural organization. The amino-terminal half of the protein is composed of six copies of a direct tandem repeat, 37 amino acids in length. The carboxy-terminal portion contains an SH3-like domain, characteristic of cytoskeleton-associated proteins, and is separated from the repeat by a region of charged amino acids and a region rich in Pro, Ser, and Thr. The multiple-domain structure of p80/85 and its colocalization with cytoskeleton-associated proteins suggest that these proteins may contribute to the organization of the cytoskeleton.

MATERIALS AND METHODS

Cells, viruses, and plasmids. Primary chicken embryo (CE) cells were prepared from 10-day embryos (SPAFAS, Norwich, Conn.). Chicken cells were transfected with plasmid DNA containing c-*src* gene mutants in a nonpermuted RSV clone as described previously (61) and maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. COS cells were transfected with either a control plasmid, pSV.25, an expression vector containing a simian virus 40 (SV40) origin of replication, early promoter, and a cDNA clone of p80/85, or pCMV.25, an expression vector containing the cytomegalovirus promoter and enhancer, an SV40 origin, and the p80/85 cDNA clone 25. Transfected COS cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum for 60 h before immunostaining or lysis.

Immunoprecipitation and Western immunoblotting. Immunoprecipitates of p80/85 were prepared by lysis of cells in modified RIPA buffer containing proteinase and phosphatase inhibitors as previously described (61), followed by incubation of 500 μg of cellular protein with 5 μg of protein A-purified MAb (4F11 or 1H1) at 0°C for 1 h (37, 61). Immunocomplexes were recovered by the addition of 50 μl of protein A-Sepharose beads (Pharmacia, Piscataway, N.J.) that were previously preincubated with 5 μg of affinity-purified rabbit anti-mouse immunoglobulin G (IgG) (Jackson ImmunoResearch Laboratories, West Grove, Pa.), followed by gentle agitation for 1 h. The beads were washed in modified RIPA buffer three times and then once in TN buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl). The proteins were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and immunoblotting with 5 μg of anti-p80/85 MAb (4F11, 1H1, or 1H3) or affinity-purified rabbit phosphotyrosine (pTyr) antibodies per ml as described previously (36, 61, 62).

Metabolic labeling, subcellular fractionation, and phosphoamino acid analysis. CE cells were labeled with $^{32}P_i$ (Dupont, NEN Research Products, Boston, Mass.) as previously described (35). Subcellular fractionation of CE cells was carried out by differential centrifugation of cell lysates as previously described (37, 61). Two-dimensional phosphoamino acid analysis of p80/85 was performed as previously described (36).

Immunofluorescence microscopy. Cells were seeded and grown overnight on glass coverslips, washed with phosphate-buffered saline, and fixed for 5 to 20 min in 3% paraformaldehyde as described previously (61). The cells were permeabilized with 0.4% Triton X-100, washed with phosphate-buffered saline, and incubated with 150 μl of primary antibody (10 μg of MAb 4F11 or 1H3 per ml) for 60 min at room temperature. After washing, the cells were further incubated with affinity-purified rabbit anti-mouse IgG for 60 min, washed, and then incubated with fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG (5 μg/ml; Jackson ImmunoResearch Laboratories) for 60 min. For double-immunostaining experiments, cells were incubated first with MAb 4F11, washed, and incubated with rabbit anti-mouse IgG and then incubated for 60 min with Texas red-conjugated donkey anti-rabbit IgG (5 μg/ml) and nitrobenzoxadiazole-phalloidin (0.16 μg/ml; Molecular Probes, Inc., Eugene, Ore.). Samples were visualized with a Leitz Orthoplan fluorescence microscope.

Identification of lambda gt11 cDNA clones, DNA sequence analysis, and computer-aided analysis of DNA and protein sequences. A lambda gt11 CE cDNA library (Clontech, San Diego, Calif.) was screened with anti-p80/85 MAbs. An appropriate dilution of the library was plated on *Escherichia coli* Y1090, and plates were incubated at 42°C for 3.5 h. Plates were then incubated with isopropyl-β-D-thiogalactopyranoside (IPTG)-saturated nitrocellulose filters at 37°C for 3.5 h. The filters were removed and incubated with anti-p80/85 MAbs. Antibody binding was scored by incubation with alkaline phosphatase-conjugated rabbit anti-mouse IgG (Jackson ImmunoResearch Laboratories), and the color reaction was developed by using alkaline phosphatase substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma, St. Louis, Mo.). Phage from positive plaques were purified by three rounds of plaque purification. The cDNA inserts were isolated and subcloned into Bluescript (Stratagene, La Jolla, Calif.). Smaller DNA fragments generated by restriction enzyme digestion of the initial cDNA inserts were isolated and subcloned into Bluescript plasmid. DNA sequencing was done by the dideoxy sequencing procedure (65), using T7 DNA polymerase (Pharmacia) and [^{35}S]dATP (1,350 Ci/mmol; New England Nuclear). DNA and amino acid sequences were analyzed by using the sequence analysis software DB System (R. Staden), the GCG package of the University of Wisconsin Genetics Computer Group (19), and FASTA (57).

Northern (RNA) blotting. Total cellular RNA was purified from CE fibroblasts by the guanidinium isothiocyanate-cesium chloride method (14). Poly(A)$^+$ RNA was prepared by oligo(dT)-cellulose chromatography (1). Ten micrograms of total cellular RNA of 1.7 μg of poly(A)-containing RNA was electrophoresed on a 1.2% formaldehyde-agarose gel, transferred to a nylon membrane, and hybridized with a ^{32}P-labeled, nick-translated 2.0-kb *Bam*HI-*Eco*RI fragment of p80/85 cDNA (see Fig. 6).

Nucleotide sequence accession number. The nucleotide sequence of clone p85.25 has been given the GenBank accession number M73705.

RESULTS

Biochemical characterization of p80/85. Previous experiments (36) have led to the identification and characterization

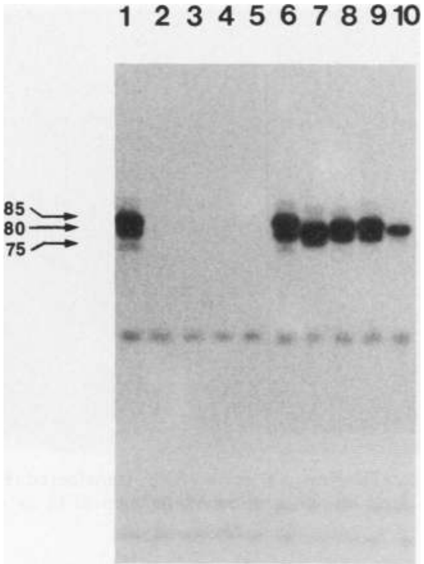


FIG. 1. Species cross-reactivity of p80/85 MAbs. Cell extracts, prepared from either 527F- or RSV-transformed cells, were immunoprecipitated with the p80/85 MAb 1H3 (lanes 1 to 5) or 4F11 (lanes 6 to 10). The resulting immune complexes were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-pTyr serum as described in Materials and Methods. Lanes: 1 and 6, 527F-infected CE cells; 2 and 7, RSV-transformed NIH 3T3 cells; 3 and 8, RSV-transformed Rat-1 cells; 4 and 9, RSV-transformed hamster 10W cells (34); 5 and 10, RSV-transformed human osteosarcoma cell line (HOS) (42).

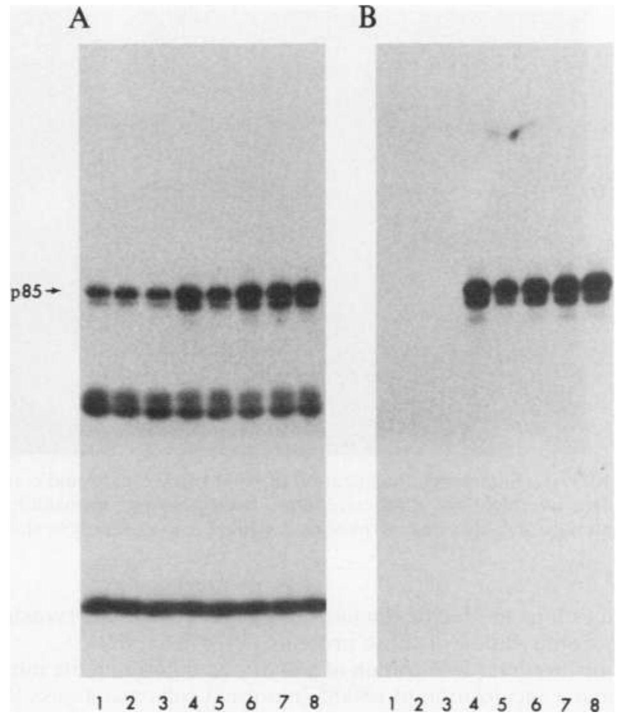


FIG. 2. Expression and tyrosine phosphorylation of p80/85 in CE cells infected with transforming and nontransforming *src* variants. (A) Detection of p80/85 in CE cells following immunoprecipitation and immunoblotting with MAb 1H1. CE cells (lane 1) were transfected with nonpermuted RSV viral vectors encoding c-*src* (lane 2), 2A/C (lane 3), 527F (lane 4), 2A/527F (lane 5), *dl*92 (lane 6), *dl*92/527F (lane 7), and *dl*155/527F (lane 8). The bands present at approximately 50 and 25 kDa are the IgG heavy and light chains, respectively. (B) Tyrosine phosphorylation of p80/85 in CE cells expressing activated *src* variants. An immunoblot identical to that shown in panel A was probed with anti-pTyr serum as described in Materials and Methods.

of several MAbs that react with a group of immunologically related, tyrosine-phosphorylated proteins of 80 to 85 kDa. As a first step in the characterization of these proteins, we determined the reactivity of several different MAbs with respect to proteins in cells of avian, rodent, and human origin. As shown in Fig. 1, MAb 1H3 immunoprecipitated multiple polypeptides (the major species being 80 and 85 kDa) from extracts of RSV-transformed CE cells (lane 1) but did not react with similar proteins from extracts of RSV-transformed rodent or human cells (lanes 2 to 5). In contrast, MAb 4F11 (and MAb 1H1; data not shown) immunoprecipitated identical polypeptides of 80 to 85 kDa from extracts of RSV-transformed CE cells and polypeptides of approximately 80 kDa from extracts of RSV-transformed rodent and human cells (lanes 7 to 10). The reactivity pattern observed with MAbs 1H3 and 4F11 defines at least two separate epitopes within the p80/85 proteins, one that is avian specific and another that is apparently conserved in similar proteins of other species. Since each of the major polypeptides observed in CE cells contained at least two different MAb epitopes, these polypeptides appear to be structurally very similar. For simplicity, we will refer to these proteins as p80/85.

Since structural alterations of specific domains of $pp60^{src}$ have been shown to alter the extent of tyrosine phosphorylation of the $pp60^{src}$ substrates p120, p110, and p130 (35, 37, 61, 62), we sought to determine whether alterations of specific domains of $pp60^{src}$ altered the tyrosine phosphorylation of p80/85. Cells were infected with viruses encoding c-*src* or different transforming and nontransforming variants of c-*src* (37, 61), including 2A/c-*src* (unmyristylated mutant of c-*src*), 527F (activated, transforming variant of c-*src*), 2A/527F (unmyristylated, nontransforming mutant of 527F), *dl*92c-*src* (a weakly transforming SH3-deletion mutant of c-*src*), *dl*92/527F (an SH3-deletion mutant of 527F that induces a fusiform morphology), and *dl*155/527F (a nontransforming SH2-deletion mutant of 527F). Cell extracts were prepared from variant-infected cells and immunoprecipitated with MAb 1H1, and the immune complexes were resolved by SDS-PAGE and transferred to nitrocellulose. Western blot analysis with MAb 1H1 revealed similar amounts of p80/85 in the various immune complexes (Fig. 2A). Parallel Western blot analysis using pTyr antibodies showed little detectable tyrosine-phosphorylated p80/85 in extracts of CE cells or in cells infected with c-*src* or the unmyristylated form of c-*src*, 2A/c-*src*. In contrast, tyrosine-phosphorylated p80/85 was readily observed in immune complexes from cells infected with activated c-*src* mutant 527F and unmyristylated mutants of 527F and 2A/527F, with SH3-deletion variants *dl*92c-*src* and *dl*92/527F, and with the SH2-deletion variant *dl*155/527F (Fig. 2B). These results indicated that p80/85 was efficiently tyrosine phosphorylated only in cells expressing *src* variants encoding an activated tyrosine kinase. Structural alterations of $pp60^{src}$ that influence membrane association or alteration of the SH2 and SH3 domains did not reduce the tyrosine phosphorylation of p80/85. This is in marked contrast to the tyrosine phosphorylation of other *src* substrates (e.g., p120, p110, and p130) wherein

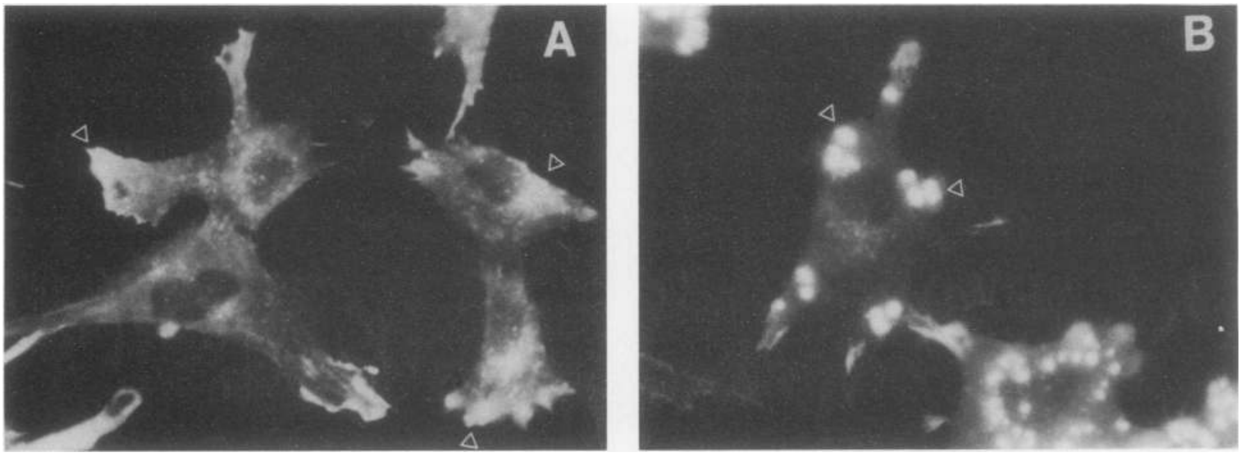


FIG. 3. Fluorescent localization of p80/85 in CE cells and c-*src* 527F-transformed cells. Normal (A) and 527F-transfected (B) cells were grown overnight on glass coverslips, fixed with paraformaldehyde, permeabilized, and immunostained with MAb 4F11 as described in Materials and Methods. Arrowheads denote areas of intense staining.

alterations in specific domains of pp60^{src} reduce the tyrosine phosphorylation of these proteins (35, 37, 61, 62).

Intracellular localization of p80/85. To determine the intracellular localization of p80/85 in normal cells and assess the effect of transformation on the intracellular distribution of p80/85, we used antibodies to p80/85 to immunostain CE cells and cells transformed with 527F (Fig. 3). CE cells exhibited an intense staining at the periphery of cells as well as a punctate staining within the cytoplasm, indicating association with plasma membranes and possibly endoplasmic reticulum (arrowheads in Fig. 3A). CE cells transformed with 527F exhibited a strikingly different pattern of staining,

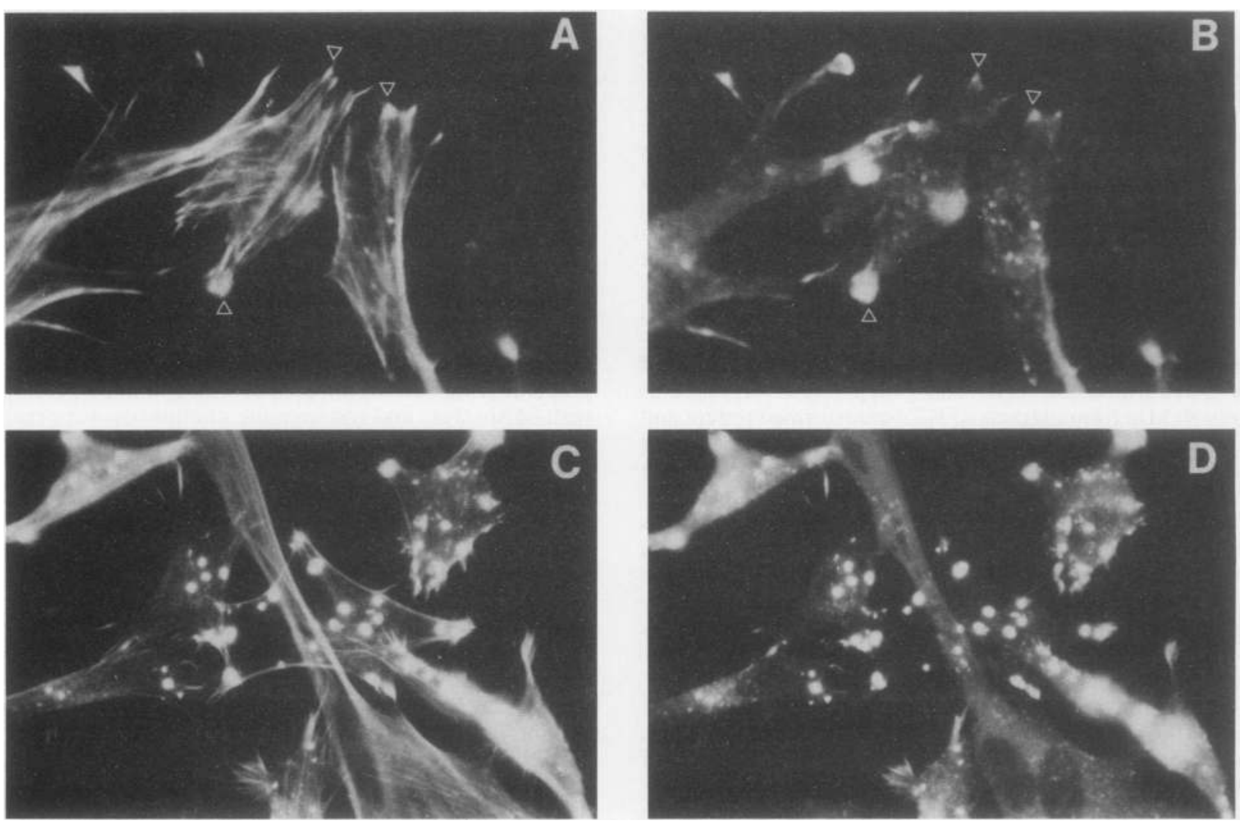


FIG. 4. Coimmunostaining of p80/85 and F-actin in CE cells and c-*src* 527F-transformed cells. CE cells and CE cells transformed with 527F were grown on glass coverslips and processed as described for Fig. 3. (A and C) Detection of F-actin in cells incubated with nitrobenzoxadiazole-phalloidin; (B and D) identical cells incubated with p80/85 MAb 4F11, rabbit anti-mouse antibody, and Texas red-conjugated donkey anti-rabbit antibody. The arrowheads denote coincident areas of immunostaining in the respective preparations.

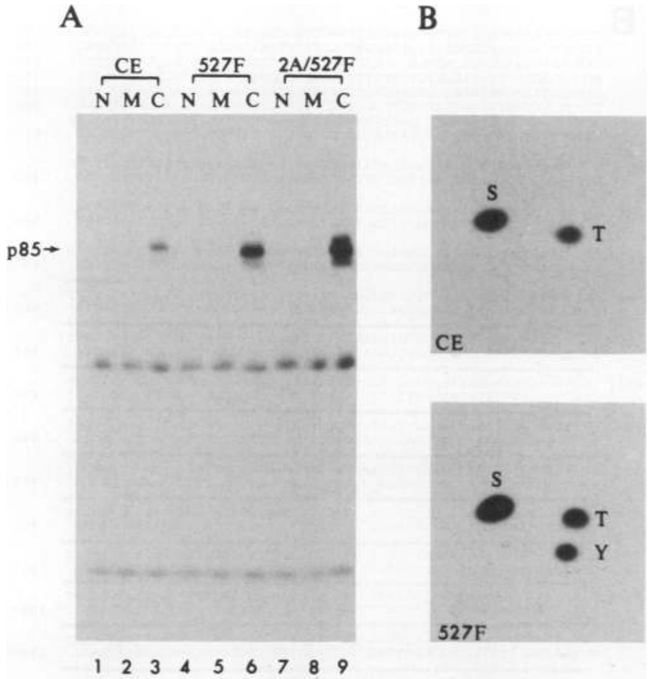


FIG. 5. Subcellular fractionation and phosphoamino acid analysis of p80/85. (A) Subcellular fractionation of p80/85 was carried out following hypotonic lysis and Dounce homogenization of CE cells (lanes 1 to 3), cells infected with 527F (lanes 4 to 6), and cells infected with 2A/527F (lanes 7 to 9). Individual fractions were immunoprecipitated with MAb 1H1, subjected to SDS-PAGE, and immunoblotted with MAb 1H1. Nuclear (N), membrane (M), and cytosolic (C) fractions were generated as described in Materials and Methods. (B) Phosphoamino acid analysis of p80/85. CE cells and 527F-transformed CE cells, as indicated, were labeled with $^{32}P_i$. Immune complexes of p80/85 were prepared as described in Materials and Methods, subjected to SDS-PAGE, extracted, acid hydrolyzed, and analyzed by two-dimensional thin-layer electrophoresis. Authentic phosphoamino acids were coelectrophoresed and identified by ninhydrin staining. S, phosphoserine; T, phosphothreonine; Y, pTyr.

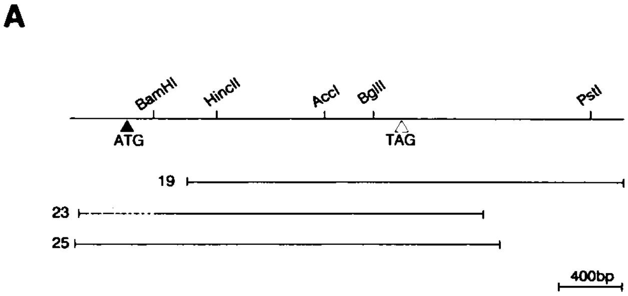
most of the p80/85 being localized to sites of membrane-substratum contact termed rosettes or podosomes (17, 48, 72). Cells infected with the unmyristylated, nontransforming variant 2A/527F exhibited a pattern similar to that of CE cells, although fewer areas of distinct focal staining were observed (data not shown).

Because of the characteristic pattern of p80/85 staining exhibited by cells transformed with 527F, double-label immunofluorescence was used to localize both p80/85 and F-actin, a component of focal adhesions in normal cells and rosettes/podosomes in RSV-transformed cells. CE cells exhibited a characteristic array of actin stress filaments (Fig. 4A) when stained for F-actin; however little p80/85 staining was evident in stress filaments themselves. Instead, colocalization of p80/85 and actin was observed at or near the ends of actin filaments (arrowheads in Fig. 4A and B). The colocalization of actin and p80/85 in these cells is consistent with p80/85 being associated with elements of the cytoskeleton and may indicate that it is present at or near sites of actin filament attachment. When cells transformed with 527F were stained for F-actin and p80/85, costaining of rosettes/podosomes was clearly evident. The virtually quantitative colocalization of p80/85 and actin in rosettes/podosomes, structures enriched for cytoskeleton-associated proteins, is further evidence for the association of p80/85 with elements of the cytoskeleton.

The interaction of p80/85 with subcellular components was further addressed by biochemical subcellular fractionation experiments. CE cells or cells infected with 527F or 2A/527F were homogenized in hypotonic buffer, and the extracts were subjected to differential centrifugation to yield a low-speed fraction enriched for nuclei and nuclear membranes, a high-speed (100,000 × g) fraction enriched for cellular membranes, and a supernatant fraction containing cytosolic proteins. Each fraction was immunoprecipitated with MAb 1H1, and the immune complexes were isolated, analyzed by SDS-PAGE, and immunoblotted with either MAb 1H1 (Fig. 5A) or pTyr antibodies (data not shown). In each instance, p80/85 was recovered in the cytosolic fraction, indicating that under the conditions used to fractionate the extracts, p80/85 was not stably associated with nuclei or cellular membranes.

To confirm the tyrosine phosphorylation of p80/85, phosphoamino acid analysis of p80/85 was carried out with ^{32}P-labeled CE and 527F-infected cells. p80/85 from uninfected CE cells was phosphorylated on both serine and threonine residues, whereas in 527F- and 2A/527F-infected cells, p80/85 contained phosphoserine, phosphothreonine, and pTyr (Fig. 5B). Thus, p80/85 proteins appear to be substrates for both serine/threonine and tyrosine kinases.

Isolation of cDNA clones encoding p80/85. To identify cDNA clones encoding p80/85, a CE lambda gt11 cDNA library was screened with four MAbs, two reactive only with chicken p80/85 and two that exhibited species cross-reactivity. Eight positive clones were identified, subcloned, and characterized by restriction enzyme mapping. The eight cDNAs fell into three distinct groups of cDNA inserts. Restriction mapping of representative clones from each of the three groups, clones 19, 23, and 25, revealed the presence of an overlapping region in all three clones (Fig. 6A). The complete sequence of clone 25 was obtained by sequence analysis of smaller DNA fragments generated by restriction enzyme digestion. In addition, limited sequencing of clones 19 and 23 confirmed the sequence of overlapping regions of these clones. As shown in Fig. 6B, clone 25 was 2,460 bp in length and contained a long open reading frame beginning at nucleotide 383 and terminating at nucleotide 2071 (TAG amber stop codon). The open reading frame encoded a putative polypeptide of 563 amino acids (predicted molecular weight of 63,300). The demarcation between the coding and noncoding sequence was supported by nucleotide sequence composition analysis using GCG Program Testcode and CodonPreference. Inspection of the sequence revealed a second in-frame ATG located 30 bp downstream from the first in-frame ATG. While the first ATG conformed to the canonical Kozak sequence, matching at positions −6, −3, and −1 (41), it is not known which of the putative start codons is used. Sequence analysis of clone 25 revealed a 382-bp 5′ leader sequence containing translation stop codons in all three reading frames. That these codons function to prevent translational initiation from an upstream start codon was indicated by the observation that extracts from bacteria expressing the lambda gt11 clone 25 contained an approximately 70- to 80-kDa peptide that was reactive with MAb 4F11 in a Western immunoblot, indicating the absence of a β-galactosidase–p85 fusion protein. Clone 25 contains 439 bp 3′ of the putative amber stop codon. Analysis of other cDNA clones suggested a substantially longer 3′ untranslated region (Fig. 6A). When cDNAs (clone 25 or 19) were nick



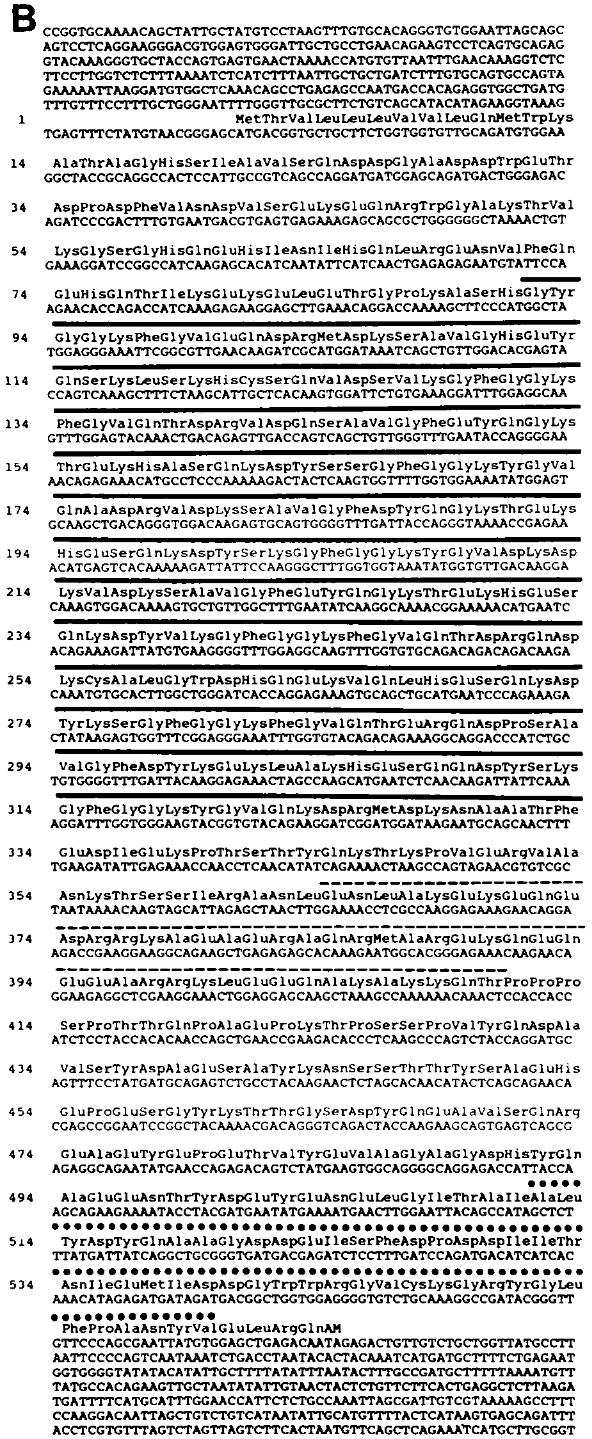


FIG. 6. p80/85 cDNA structure and sequence. (A) Restriction enzyme map of p80/85 cDNAs. The structures of cDNA clones isolated by screening a lambda gt11 cDNA library with p80/85 MAbs were determined by restriction enzyme digestion as described in Materials and Methods. Based on sequence analysis of individual clones (see text), the putative translational start site and stop site were located and are indicated by arrowheads. The broken line denotes nucleotides present in clone 23 and not in the other p80/85 clones. The origin of these nucleotides was not determined. (B) Nucleotide sequence and predicted amino acid sequence of clone p85.25. The internal tandem repeats are overlined, the predicted long α-helical region is indicated by dashes, and the SH3 motif is indicated by dots. Numerical positions of the nucleotides are shown at the right, and the numerical positions of amino acid residues are indicated at the left.

translated and used to probe a Northern blot of either total or poly(A)-containing CE RNA, a major RNA species of approximately 4 kb (and a minor species of 5.5 kb) was detected (Fig. 7).

Clone 25 encodes proteins of 80 and 85 kDa. To verify that clone 25 encoded a component of the p80/85 proteins, the cDNA insert of clone 25 was subcloned into expression vectors containing either the SV40 (pSV.25) or cytomegalovirus (pCMV.25) promoter-enhancer and an SV40 origin of replication. COS cells were transfected with either pSV.25 or a control plasmid lacking the clone 25 insert. Sixty hours posttransfection, the cells were immunostained with either a chicken-specific MAb (1H3; Fig. 8A and B) or a species-cross-reactive p80/85 MAb (4F11; Fig. 8C). A significant

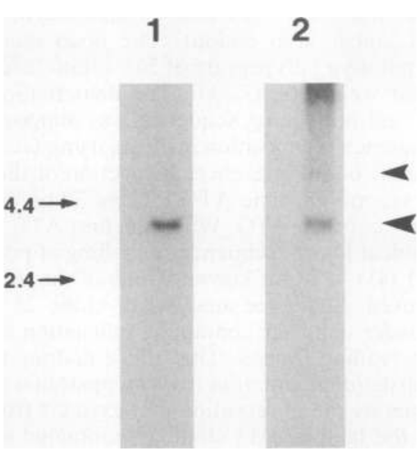


FIG. 7. Northern blot analysis of p80/85. Total RNA (lane 1) and poly(A)$^+$ RNA (lane 2) from CE cells were hybridized with a 2.0-kb *Bam*HI-*Eco*RI fragment of p80/85 cDNA as described in Materials and Methods. Relative sizes of RNAs are indicated in kilobases at the left. The large and small arrowheads indicate the major band hybridizing with the p80/85 probe and the minor band, respectively.

number (greater than 5%) of the COS cells transfected with pSV.25 (or pCMV.25; data not shown) exhibited intense staining within the cytoplasm and at cell-substratum contact sites (Fig. 8A). In contrast, cells transfected with the control DNA showed no such staining (Fig. 8B). Cells stained with the species-cross-reactive antibody (4F11) exhibited intense staining at sites of peripheral extensions (Fig. 8C), reflecting the presence of endogenous monkey p80/85. In a parallel analysis, cells were transfected with pCMV.25 and 70 h

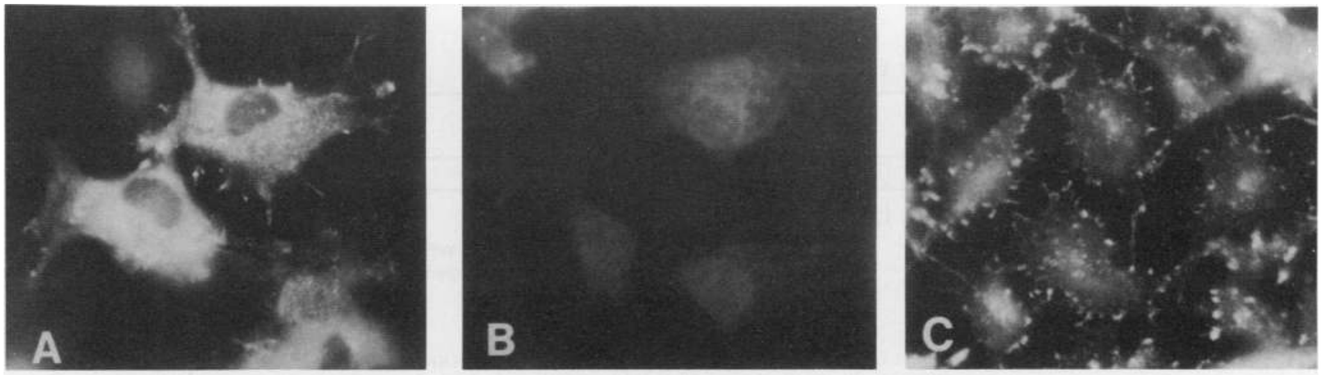


FIG. 8. Expression of pSV.25 in transfected COS cells as detected by indirect immunofluorescence. COS cells (C) and COS cells transfected with pSV.25 (A) or a control plasmid (B) were cultured for 60 h and immunostained with a chicken-specific MAb against p80/85 (1H3; A and B) or with a cross-species-reactive anti-p80/85 MAb (4F11; C) as described in Materials and Methods.

posttransfection, cell lysates were subjected to SDS-PAGE and immunoblotted with the chicken-specific MAb 1H3 to confirm the presence of immunologically related p80/85 polypeptides (Fig. 9). Lysates from cells transfected with vector DNA or cells transfected with pCMV.AS (the clone 25 insert in reverse orientation) contained little detectable p80/85 (Fig. 9, lanes 2 and 5). In contrast, pCMV.25-transfected cell lysates contained two major species that were reactive with MAb 1H3 (lanes 3 and 4). These proteins comigrated with the authentic p80/85 proteins of CE cells (lane 6). These data confirmed that the cDNA clone p85.25 encodes both the p80 and the p85 proteins observed in normal and transformed CE cells.

Predicted structural features of p80/85. The predicted molecular size of the protein product encoded by the longest open reading frame of clone 25 is 63.3 kDa, suggesting that additional posttranslational modifications or protein folding contributes to the greater molecular size as determined by SDS-PAGE. The p80/85 protein is relatively rich in Gln, Lys, and Gly residues and has a predicted isoelectric point of 5.3. The amino-terminal 16 amino acids comprise a hydrophobic region; however, the results of cell fractionation experiments (Fig. 5) indicate that this region does not anchor p80/85 in cellular membranes. Analysis of the deduced amino acid sequence revealed several interesting structural features (Fig. 10A). First, the amino-terminal half of the protein contains six complete internal repeats arranged in tandem fashion. The repeat region starts at amino acid residue 91 and extends to about residue 325 (Fig. 6B). The first six repeats consist of 37 amino acids, whereas the seventh is degenerate, containing only 15 to 17 residues. The amino acid sequence of the repeat units and a derived consensus sequence is shown in Fig. 10B. Secondary structure analysis using the Chou-Fasman or Garnier-Osguthrope-Robson algorithms suggests that the tandem repeats exhibit a general helix-turn-helix structure. The repeated region is highly hydrophilic. No amphipathic helix or coiled coil could be recognized by inspection of the amino acid sequence. A search of the available data bases (National Biochemical Research Foundation protein data base and GenBank nucleotide data base) using either the consensus repeat or individual repeat sequences revealed no sequence similarity with other known proteins (see Discussion). However, a search of the protein data base for proteins with sequence similarity to other regions of p80/85 revealed a second interesting structural feature, an SH3 motif, present at the C terminus (Fig. 6B and 10C). The SH3 motif has been identified in several nonreceptor tyrosine kinases (55), cytoskeleton-associated proteins including fodrin and α-spectrin (50), yeast actin-binding protein 1 and isoforms of myosin (21), and NADPH oxidase-associated proteins (43), as well as in proteins and enzymes thought to play a role in signal transduction (e.g., GAP, Crk, and phospholipase C-γ) (49, 70, 73, 76). The SH3 motif of p80/85 shares approximately 50% sequence identity with the SH3 region of pp60^{src}. Its location within p80/85 parallels that of the SH3 motifs of cytoskeleton-associated proteins such as myosin 1 and yeast actin-binding protein 1 (21). Two remaining features of the p80/85 sequence deserve comment (Fig. 10A). C terminal of the repeated domain is a predicted helical region of 50 to 60 residues, many of which are charged amino acids. Adjacent to the helical region is a sequence of 18 to 20 residues rich in Pro, Ser, and Thr. Such a region may provide a bend or kink in the p80/85 proteins. Potential tyrosine phosphorylation sites (XE/DYX) were identified within each of the copies of the repeated region and immediately upstream of the SH3 domain. A myosine heavy-chain kinase phosphorylation site (KXXS) (5, 38) was identified in repeats 2, 3, and 5 (Fig. 10B), and a CDC2-like phosphorylation site (XKSPX) (38) was found in the proline-rich region (Fig. 6B). Whether any of these sites are phosphorylated in vivo remains to be determined.

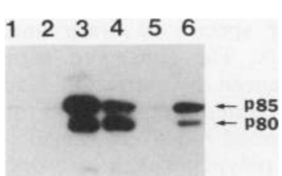
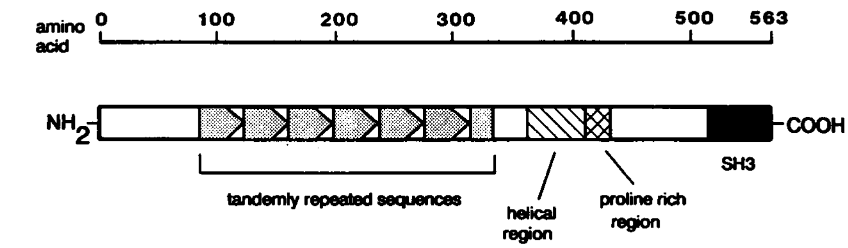


FIG. 9. Immunoprecipitation of p80/85-related proteins from transfected COS cells. COS cells were transfected with pCMV vectors as described in the text, and cell lysates were subjected to SDS-PAGE and immunoblotted with the chicken-specific p80/85 MAb 1H3. Lanes: 1, COS cells; 2, COS cells transfected with pCMV vector DNA; 3 and 4, COS cells transfected with pCMV.25 DNA; 5, COS cells transfected with pCMV.AS DNA (clone 25 in the reverse orientation); 6, CE cells.

A



B

```
repeat.1 : GYGGKFGVEQDRMDKSAVGHEYQSKLSKHCSQVDSVK
repeat.2 : -F------QT--V-Q----F---G-TE--A--K-YSS
repeat.3 : -F---Y--QA--V------FD--G-TE--E--K-YS-
repeat.4 : -F---Y--DK-KV------F---G-TE--E--K-Y--
repeat.5 : -F------QT--Q--C-L-WDH-E-VQL-E--K-YKS
repeat.6 : -F------QTE-Q-P----FD-KE--A--E--Q-YS-
repeat.7 : -F---Y--QK-----N-ATF-DIE-

consensus: GFGGKYGVQ-DR-DKSAVGFEYQ-K--KH-SQ-DY--
                (F)          (D)

human HS1: GFGG-YGVE-DR-DKSAVG--YKGEVEKH-SQ-G---
```

C

p85 :	ALYDY	Q	A	AG	DDEISF	DPD	D	I	I	TN	I	EMI	D	DG	WW	RGV	C	KGRY..	G	LF	P	A	NYV	ELRQ.
plc2 :	--F--	K	-	QR	E--LT-	TKS	A	-	-	Q-	V	- Kq	-	G-	--	--.	D	Y-GKkq	L	W-	-	S	---	-EMIN
myoH :	----F	A	-	EN	P--LT-	NEG	A	V	V	-V	-	N.Ks	N	PD	--	E-E	L	N..Gqr	-	V-	-	-	S--	--IP-
fodrin :	-----	-	E	KS	PR-VTM	KKG	-	-	L	-L	L	N.St	N	KD	--	KVE	V	ND-Q..	-	FV	-	-	A--	K.KLD
c-fgr :	-----	E	-	RT	E-DLT-	TKG	E	K	F	HI	L	N.Nt	E	GD	--	EAR	S	LSSGkt	-	CI	-	S	---	A.PVD
c-fyn :	-----	E	-	RT	E-DL--	HKG	E	K	F	QI	L	N.Ss	E	GD	--	EAR	S	LTTGet	-	YI	-	S	---	A.PVD
gag-crk :	--F-F	K	G	ND	-GDLP-	KKG	-	-	L	KI	R	D.Kp	E	EQ	--	NAE	D	MD.Gkr	-	MI	-	V	P--	-.KC-
gag-yes :	-----	E	-	RT	T-DL--	KGG	E	R	F	QI	-	N.Nt	E	GD	--	EAR	S	IATGkt	-	YI	-	S	---	A.PAD
c-src :	-----	E	S	RT	ETDL--	KKG	E	R	L	QI	V	N.Nt	E	GD	--	LAH	S	LSTGqt	-	YI	-	S	---	A.PSD
hck :	-----	E	-	IH	HEDL--	QKG	-	Q	M	VV	L	-.Es	.	GE	--	KAR	S	LATRke	-	YI	-	S	---	A.-VD
abpl :	-E---	D	-	-E	-N-LT-	VEN	-	K	-	I-	-	-.Nv	-	-D	--	L-E	L	EKDGsk	-	--	-	S	---	.....
spectrin :	-----	-	E	SA	PR-VTM	KKG	-	-	L	-L	L	N.St	N	KD	--	KVE	V	ND-Q..	-	FV	-	-	A--	.....
c-abl :	----F	V	-	S-	-NTL-I	TKG	E	K	L	RV	L	GYNh	N	GE	-C	EAQ	T	kn....	-	QG	W	V	PSN	V.ITP
hsl :	-----	-	G	E-	S--L--	---	-	V	-	-D	-	--v.	-	E-	--	--R	-	H-HF..	-	--	-	-	---	KLLE

FIG. 10. Structural features of p80/85. (A) Diagram of p80/85 structure. The internal repeats are indicated by the dotted region, and the SH3 motif is shown by the black region. The predicted long α-helix and the adjacent Pro-, Ser-, and Thr-rich region are denoted by the striped and cross-hatched boxes, respectively. (B) Alignment of the amino acid sequences comprising the seven repeated units, obtained by using the MBIR program ss2align. Positions of aligned identical amino acids are denoted by dashes. The consensus sequence is derived from identical amino acid residues present in more than five of the seven repeats. The consensus sequence of the repeat region of human erythropoietic cell protein HSl is shown for comparison. (C) Comparison of the amino acid sequences of the SH3 regions of p80/85 with the SH3 regions of other proteins, using MBIR program ss2align. The boxed amino acid residues are identical or conserved in more than half of the proteins compared with the aligned residues in p80/85. p80/85 sequences are compared with those of phospholipase C-γ (plc2), *Acanthamoeba* myosin IB heavy chain (myoH), human α-fodrin (fodrin), c-Fgr, c-Fyn, Gag-Crk, Gag-Yes, c-Src, Hck, yeast actin-binding protein 1 (abpl), chicken α-spectrin, c-Abl, and human erythropoietic cell protein HSl (hsl). Aligned identical amino acids are represented by dashes, aligned nonidentical residues are shown in uppercase letters, unaligned residues are in lowercase letters, and gaps in the alignment are denoted by dots.

DISCUSSION

The p80/85 proteins described in this report are composed of two major, related polypeptide species and are present in a wide variety of vertebrate species. The striking alteration in intracellular distribution of p80/85 observed in RSV-transformed cells and the colocalization of these proteins with F-actin in peripheral extensions of normal cells and podosomes of RSV-transformed cells indicate that p80/85 may be associated with elements of the cytoskeleton.

Structural relatedness of p80/85 polypeptides. Immunoprecipitation or direct Western blotting of p80/85 from CE cells revealed two major immunologically related peptides as well as several minor species of lower and higher apparent molecular weights. The conserved nature of the p80/85 proteins is evidenced by detection using MAb 4F11 of immunologically related proteins of 75 to 80 kDa in normal and RSV-transformed rodent or human cells and immunologically related polypeptides in drosophila tissue culture cells (80). Expression of cDNA clone 25 in COS cells yielded both an 80- and a 85-kDa protein which reacted with the chicken-specific antibody 1H3. In vitro phosphorylation of immune complexes containing either COS cell p80/85 or CE cell p80/85 with pp60^{src} followed by CNBr cleavage and SDS-PAGE yielded identical patterns of phosphorylated peptides (data not shown). These observations demonstrate that clone 25 encodes the two major forms of p80/85 and are consistent with both forms of the protein being encoded by the same mRNA. The multiple polypeptides observed in CE cells, however, do not appear to be derived from differential phosphorylation. Treatment of p80/85 immune complexes with potato acid phosphatase or tyrosine phosphatase did not significantly alter the mobility of the p80/85 polypeptides on SDS-gels (75a). These observations lead us to conclude that the difference in migration of p80 and p85 may reflect proteolytic processing or the use of alternative sites of translational initiation. The latter possibility is strengthened by the observation that in a 1-h pulse with [^{35}S]methionine followed by either a 1-, 2-, or 6-h chase, no alteration in the ratio of labeled p80 and p85 was observed.

Cellular distribution of p80/85. Immunostaining of p80/85 in normal CE cells revealed that the proteins are present at the periphery of the cell, colocalizing with the ends of the actin stress fibers but not the fibers themselves (Fig. 3 and 4). Previous studies have shown that the sites of attachment of cells to the substratum are rich in a variety of well-characterized cytoskeletal components, including actin, α-actinin, fimbrin, vinculin, talin, and tensin (8). Indeed, the cytoskeletal matrix of cells is replete with proteins of 70 to 85 kDa, including radixin, an 82-kDa polypeptide found in cell-to-cell adherens junctions (74), an unidentified 82-kDa polypeptide localized to sites of cell substratum adhesion (3), and paxillin (70 kDa), a vinculin-binding protein present in focal adhesions (75). The latter is tyrosine phosphorylated in CE cells transformed with RSV (25). It is not clear at present whether one or more of these proteins is structurally related to the p80/85 family of proteins. The p80/85 proteins described here, however, are unrelated to the p85 subunit associated with PI3 kinase (23, 52, 69) or ezrin (26). Preliminary biochemical evidence supports the idea that p80/85 is associated with elements of the cytoskeleton (23a). Like pp60^{src}, a substantial fraction of p80/85 is found associated with detergent-resistant subcellular structures composed mostly of cytoskeletal proteins (7, 24).

Upon transformation by RSV, avian and rodent cells exhibit abnormal adhesions to the substratum, termed rosettes or podosomes (17, 48, 72). Like focal adhesions, rosettes or podosomes are regions of the cell surface in close contact with the underlying substratum. Coimmunostaining with antibodies to p80/85 and F-actin indicated that in *src*-transformed cells, p80/85 proteins are components of podosomes. Podosomes appear to be unique to cells transformed by the tyrosine kinase oncogenes *src*, *abl*, *fps*, and *yes*, and in addition to containing cytoskeletal proteins normally found in focal adhesions, these structures also have been shown to contain the tyrosine kinase oncoproteins (8). Thus, it is not surprising that p80/85 is a target for pp60^{src}. The role of podosomes in transformation is not known. It has been suggested that such structures may be important in mediating the activation of extracellular proteases and degradation of extracellular matrix by transformed cells (12). Indeed, it has been suggested that the presence of pp60^{src} in these structures is indicative of a role for tyrosine kinases in this activation process (13).

Tyrosine phosphorylation of p80/85. An important consideration in the analysis of p80/85 proteins is how (or whether) tyrosine phosphorylation of these proteins plays a role in altering the cellular morphology or growth properties of the transformed cells. The extent of p80/85 tyrosine phosphorylation (10 to 30% of total p80/85, as judged by immunoprecipitation with pTyr antibody) is similar in cells expressing transforming variants of pp60^{src} or cells infected with either unmyristylated, nontransforming variants of *src* or transformation-defective SH2 variants. In contrast, the striking redistribution of p80/85 occurs only in fully transformed cells. These observations argue that tyrosine phosphorylation of p80/85 may not be sufficient to trigger alteration of the cytoskeletal structures and that redistribution of p80/85 is a direct consequence of other events in transformation. However, additional points must be considered before dismissing a role of p80/85 tyrosine phosphorylation in cellular changes. Additional structural modifications, e.g., serine or threonine phosphorylation of p80/85 or tyrosine phosphorylation of other cellular proteins, may be necessary to initiate changes in the cytoskeleton. Alternatively, the p80/85 proteins may exist in several compartments within the cell, and the redistribution of unmyristylated pp60^{src} (or SH2-defective pp60^{src}) may prevent the tyrosine phosphorylation of the proper pool of cytoskeleton-associated p80/85 molecules. Preliminary experiments have indicated that p80/85 proteins are tyrosine phosphorylated by other members of the *src* tyrosine kinase family (NIH 3T3 cells transformed with activated *src*, *fgr*, and *fyn* [65a]), in response to stimulation of normal cells with the growth factor EGF (37b, 46a), and in platelets stimulated with thrombin (45a). In contrast, activation of p56^{lck} by binding of CD4 or CD3 antibodies, while stimulating the tyrosine phosphorylation of proteins of approximately 75 to 80 kDa, did not lead to increased p80/85 tyrosine phosphorylation (33a). Indeed, we have noticed significantly reduced expression of p80/85 in B cells and T cells (37a). Thus, tyrosine phosphorylation of p80/85 may depend on activation of some, but not all, tyrosine kinases and in addition reflect differences in tissue-specific expression of these proteins. Clearly, additional experimentation is warranted before one can assign a functional role to p80/85 tyrosine phosphorylation and its contributions to cellular growth regulation and transformation.

Structure of p80/85. The deduced amino acid sequence of p80 reveals the presence of several distinguishable sequence motifs (Fig. 10), including a repeated-sequence domain composed of six internal tandem repeats, a predicted α-helical region, a Pro-, Ser-, and Thr-rich region, and an SH3 domain. The mosaic nature of p80 indicates that it may have evolved by duplication as well as insertion of common structural units. Thus, it provides another example for a growing number of mosaic proteins which contain similar structural units yet exhibit diverse cellular functions (2, 20). Internal tandem repeats appear to be a feature of some membrane-associated proteins and cellular proteins involved in cell cycle control or differentiation. Such repeat sequence motifs can be divided into several groups. A 33-amino-acid ankyrinlike repeat is found not only in ankyrin but also in Notch, Lin-12, Glp-1, CDC10, SW14, SW16, and Bcl3 (46). A second repeat motif, the 34-amino-acid knob-and-hole motif, has been observed in CDC23, Nuc2, CDC16, SSN6, and SK13 (29, 68). Finally, the cytoskeletal protein spectrin contains multiple copies of a 106-amino-acid repeat (47). A comparison of the amino acid sequence of the p80/85 repeat with the other repeat motifs revealed sequence similarity only with HS1 (see below). However, it is noteworthy that internal tandem repeats are often conserved in proteins of different species, present in various copy numbers, and are present in proteins of diverse cellular functions. It has been suggested that each repeat motif may represent a basic structural and functional unit that could be involved in mediating interactions with other cellular proteins or that these domains may facilitate interactions among similar domains of the same protein (46). It is interesting to speculate that in different cells or tissues, the strength or specificity of such interactions mediated by repeated sequence motifs could be readily modulated by addition or deletion of copies of the repeats.

The C terminus of p80/85 is composed of an SH3 motif, which is conserved in nonreceptor tyrosine kinases, and several cytoskeletal-associated proteins and proteins involved in cellular signalling (Fig. 10C). A phylogenetic analysis of the various SH3 regions, which is based on the similarity of the amino acid sequences as determined by the computer algorithm MULTALIGN, showed that the SH3 motifs can be grouped into two subfamilies. One subfamily is present in nonreceptor tyrosine kinases, Crk, and myeloid kinase, while the other is found in p85, HS1, myosin heavy chain, yeast actin-binding protein 1, fodrin, α-spectrin, and phospholipase C-γ (Fig. 10). It is interesting that this phylogenetic division of the SH3 motifs coincides with the putative function of the proteins, one group being composed of mostly protein kinases and the other being composed of structural proteins. Thus, the different subfamilies of SH3 motifs may encode different biological functions. The SH3 motif may function as a binding region to cytoskeleton, as indicated by its presence in various cytoskeleton-binding proteins. It will be interesting to investigate the nature of possible interactions of p80/85 with pp60^{src}, other members of the *src* family of kinases, or tyrosine kinase receptors.

Kitamura et al. (39) have reported the sequence of HS1, a cDNA expressed only in cells of hematopoietic lineage. The overall structure of HS1 is very similar to that of p85. HS1 contains three copies of an internal tandem repeat which bears substantial sequence similarity (70 to 75% sequence identity) with the repeat region of p85 (Fig. 10B). HS1 also contains a C-terminal SH3 motif (Fig. 10C); however, the amino acid sequences between these two landmarks are only 20% identical with the corresponding region of p85. Based on the similarity in amino acid sequence and structural arrangement, HS1 could either be a human homolog of p85 or a member of a highly related gene family. The fact that we have been unable to detect a comparable level of expression of p80/85 in B cells and T cells (37a) suggests that p80/85 and HS1 are members of a gene family and not homologs of the same gene product in different organisms.

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