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Functional Role of GTPase-Activating Protein in Cell Transformation by pp60$^{v\text{-}src}$

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Morphological transformation of NIH 3T3 cells was observed following coexpression of a portion of the *ras* GTPase-activating protein (GAP) comprising the amino terminus (*GAP-N*) and a mutant of v-*src* (*MDSRC*) lacking the membrane-localizing sequence. Cells expressing either of these genes alone remained nontransformed. Coexpression of *GAP-N* with *MDSRC* did not alter the subcellular localization, kinase activity, or pattern of cellular substrates phosphorylated by the *MDSRC* product. In contrast to SHC, phospholipase C-γ1, and the p85 α phosphatidylinositol 3′-kinase subunit, the endogenous *GAP* product (p120^{GAP}) was highly tyrosine-phosphorylated only in cells transformed by wild-type v-*src*. Furthermore, for transformation induced by wild-type v-*src* as well as by coexpression of *MDSRC* and *GAP-N*, a strict correlation was observed between cell transformation, elevated tyrosine phosphorylation of p62, p190, and a novel protein of 150 kDa, and complex formation between these proteins and p120^{GAP}. As with cells transformed by wild-type v-*src*, the *MDSRC* plus *GAP-N* transformants remained dependent on endogenous Ras. The results suggest that tyrosine phosphorylation and complex formation involving p120^{GAP} represent critical elements of cell transformation by v-*src* and that complementation of the cytosolic v-*src* mutant by *GAP-N* results, at least in part, from the formation of these complexes.

The v-*src* product of Rous sarcoma virus was the first protein-tyrosine kinase (PTK) to be identified, and v-*src* is commonly used to probe the mechanism of cellular transformation by PTKs (18, 38). Since the products of oncogenes such as v-*src* represent activated versions of normal cellular proteins implicated in cellular signalling, results obtained with oncogenes often have implications for physiologic signal transduction pathways, especially those associated with mitogenesis (4). Many features of downstream signalling appear to be shared by receptor PTKs, such as those for platelet-derived and epidermal growth factors, and nonreceptor PTKs, such as the v-*src* product. Observations made with v-*src* may therefore be relevant to other PTKs.

The v-*src* product is a 60-kDa membrane-associated phosphoprotein (pp60$^{v\text{-}src}$) (38) whose PTK activity, which is located near the C terminus of pp60$^{v\text{-}src}$, is required for transformation. pp60$^{v\text{-}src}$ associates with membranes because its N terminus is myristylated; disruption of its N-terminal myristylation signal leads to a cytosolic protein that is nontransforming (5, 19). pp60$^{v\text{-}src}$ contains two other conserved domains, designated Src homology regions 2 and 3 (SH2 and SH3, respectively), that lie between the membrane localization sequence and the kinase domain (39). SH2 and SH3 domains have been shown to function in the regulation of protein-protein interactions during signal transduction (23). SH2 domains are present in a wide variety of cytoplasmic signalling molecules, including certain PTKs, phospholipases, and phosphotyrosine phosphatases (40). SH3 domains are often found in conjunction with SH2 domains, although they are also present in proteins that lack SH2 domains.

In cells with activated PTKs, a large number of proteins are phosphorylated on tyrosine (4, 18). However, the functional significance of many of these phosphorylations remains unknown, and the substrates that are critical for mitogenesis have not been fully elucidated. It is well established that mitogenesis induced by physiologic activation of receptor PTKs or by constitutively active PTKs such as pp60$^{v\text{-}src}$ requires cellular Ras (13, 46), which is a guanine nucleotide-binding protein first identified as the viral oncoprotein of several transforming retroviruses. Ras possesses an intrinsic GTPase activity and cycles between an active GTP · Ras form and inactive GDP · Ras form (27), with receptor activation by growth factors inducing a rapid increase in the proportion of GTP · Ras (43). In fibroblasts, this increase is secondary to stimulation of guanine nucleotide exchange on Ras, although Ras does not appear to be a physiologic substrate for PTKs (2, 32, 53). Phosphorylation of SHC, which is a recently identified substrate for PTKs, may help mediate the increase in GTP · Ras, perhaps by stimulating an exchange factor (41, 42).

The GTPase-activating protein (GAP), which is a widely expressed 120-kDa molecule (p120^{GAP}) that is predominantly cytosolic, is another substrate for PTKs, including pp60$^{v\text{-}src}$ (12, 33). GAP was initially identified by its ability to inactivate GTP · Ras to GDP · Ras by accelerating the intrinsic GTPase activity of Ras (48). This enzymatic activity of p120^{GAP} has been localized to its C-terminal end (29). The N terminus of p120^{GAP} contains one SH3 domain and two flanking SH2 domains.

GAP, via its enzymatic activity, has been implicated as a major negative regulator of Ras, and overexpression of full-length GAP or its C-terminal catalytic domain can induce morphologic reversion of v-*src*-transformed cells (9, 37). Other evidence suggests that GAP may in addition represent a downstream target of Ras, although GAP has thus far not been directly implicated in cell transformation

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(17, 28). Interference with GAP SH3 function either by antibodies directed against the SH3 domain of GAP or by an SH3 peptide led to inhibition in *Xenopus* oocytes of germinal vesicle breakdown induced by injected Ras or by insulin, whose ability to induce germinal vesicle breakdown depends on endogenous Ras, but not by progesterone, which induces germinal vesicle breakdown by a Ras-independent mechanism (10). These results support a model in which the interaction of GTP · Ras with the C terminus of GAP activates an N-terminal function of GAP that requires the SH3 domain (30).

In cells stimulated by growth factor-activated receptor PTKs or transformed by oncogenic PTKs, a proportion of $p120^{GAP}$ undergoes phosphorylation at tyrosine 460, which lies immediately downstream from the second SH2 domain (26, 34). Furthermore, in such cells, $p120^{GAP}$ has been shown to form intermolecular complexes, via its SH2 domains, with activated receptor PTKs and with v-*src* and c-*src* products, as well as with cellular proteins of 62 kDa (termed p62) and 190 kDa (p190) (1, 12, 21, 22, 33). The GAP-p62 and GAP-p190 complexes appear to be distinct, as a substantial portion of the former is membrane associated, while the latter is exclusively cytoplasmic (36). The genes encoding p62 and p190 have been molecularly cloned. p62 is a protein with multiple tyrosine phosphorylation sites and nucleic acid-binding properties (51). p190 contains a putative GTP-binding domain, a region that acts as a GAP for members of the rho GTPase family, which are related to Ras and regulate cellular actin microfilaments, and a segment with homology to a factor reported to repress transcription (44, 45). The biological roles of GAP-containing complexes are unknown, although the p190-bound GAP has somewhat lower GTPase-stimulating activity than uncomplexed GAP (36).

To probe the biological function of GAP in signal transduction involving PTKs, we have examined the phenotypes induced by the stable expression in NIH 3T3 cells of a premature termination mutant of *GAP* encoding the SH2 and SH3 domains and of a v-*src* gene product that has been rendered nontransforming by mutation of its myristylation/membrane-localizing sequence. Surprisingly, we found that these two genes cooperated to induce stable morphological transformation, that this phenotype correlated specifically with the formation of intermolecular complexes involving GAP, p62, p190, and an unidentified protein, and that the transformation was dependent upon endogenous Ras. These observations suggest that GAP, via protein-protein interactions, serves a critical role in transformation by oncogenic PTKs.

MATERIALS AND METHODS

Construction of plasmids, cell culture, and transfections. The *GAP-N* gene, encoding the first 456 amino acids of the human type I GAP, was constructed by deletion of sequences from an *Nco*I site at nucleotide 1175 to the 3′ end of the gene and replacement of the deleted region with a polymerase chain reaction (PCR)-amplified fragment comprising 312 bp of *GAP* with an artificial TGA and *Eco*RI site inserted. The sequence of the PCR fragment was verified directly by dideoxynucleotide sequencing. The resulting fragment was cloned into the previously described Harvey vector (52) as an *Eco*RI fragment to yield plasmid pJDC215. The wild-type v-*src* gene (Schmidt-Ruppin A strain) was also introduced into the Harvey vector as an *Eco*RI fragment to yield plasmid pJDC173. The *MDSRC* gene, identical in sequence to the previously described XD315 mutant (5), was constructed by PCR with *pfu* DNA polymerase (Stratagene) and oligonucleotides designed to replace codons 2 to 15 of v-*src* with codons for the amino acids DLG while retaining the remainder of the wild-type v-*src* sequence. The PCR product was cloned as an *Eco*RI fragment into the Harvey vector to yield plasmid pJDC186. The c-*ras*-encoding plasmid pBW1631 (50) and the previously described LTR-c-*raf* (47) were used for transfections of these genes.

Transfections were carried out as described previously (49), and foci were scored 12 to 15 days later. For v-*src*, 100 ng of DNA was used; for *MDSRC* and *GAP-N*, 1,000 ng (or 500 ng of each when cotransfected) was used; for c-*ras*, 600 ng was used; and for c-*raf*, 400 ng was used. Cell lines transformed by coexpression of *MDSRC* and *GAP-N* were derived by the cloning of individual foci of transformation. Cell lines expressing v-*src*, *MDSRC*, or *GAP-N* singly were isolated by cotransfection of these plasmids (1,000 ng) with 10 or 20 ng of pSV2neo and selection with 600 μg of G418 per ml for 14 days. Individual G418-resistant colonies were then cloned.

Cell labeling, immunoprecipitation, in vivo guanine nucleotide-binding analysis, and kinase assays. Labeling of cells with ^{35}S-amino acids, cell lysis, and immunoprecipitation with anti-Src serum from a tumor-bearing rabbit (TBR) or with anti-GAP A serum were carried out as described previously (6). Labeling of cells with [^{3}H]myristic acid was carried out as described before (8); immunoprecipitation from lysates of these cells was done as for the ^{35}S-labeled cells. Cell fractionation and immunoprecipitation from cells labeled with ^{35}S-amino acids were done as described previously (6). In vivo guanine nucleotide binding was tested as described before (7) except that cells were plated at a density of $8.0 \times 10^4/cm^2$ and serum starved for 6 h prior to and throughout the 10-h labeling period.

For kinase assays, cells were plated at 5×10^6 per 100-mm dish, and the next day, the cells were lysed in 1 ml of C-type lysis buffer (CLB; 20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM $MgCl_2$, 2 mM EDTA, 10% [vol/vol] glycerol, 1% Nonidet P-40, 16 μg of aprotinin per ml, 10 μg of leupeptin per ml, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 100 mM NaF, 10 mM sodium PP_i). The lysates were clarified by centrifugation at $10,000 \times g$ for 10 min at 4°C in a microcentrifuge and frozen at −70°C. The protein concentration of an aliquot from each lysate was determined by the bicinchoninic acid assay (Pierce); the lysates were then thawed, and portions of each lysate containing 350 μg of protein were subjected to immunoprecipitation with 1 μg of monoclonal antibody 327 (Oncogene Science) for 2 h at 4°C. The immunoprecipitates were washed twice with CLB and once with kinase buffer (KB; 50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.4], 5 mM $MgCl_2$, 5 mM $MnCl_2$, 150 mM NaCl, 100 μg of bovine serum albumin per ml), resuspended in 30 μl of KB containing 330 nM ATP and 10 μCi of [γ^{32}P]ATP (3,000 Ci/mmol; New England Nuclear), and incubated at 30°C for 20 min. After the reaction, the immunoprecipitates were washed three times with CLB and analyzed on a 10% polyacrylamide gel.

Western blotting (immunoblotting) of cell lysates and immunoprecipitates. Western blotting of cell lysates with anti-GAP A or polyclonal rabbit antiphosphotyrosine serum was carried out as follows. Cells were plated at 5×10^6 per 100-mm dish and lysed the following day in 1 ml of lysis buffer (20 mM Tris-HCl [pH 7.4], 100 mM NaCl, 5 mM $MgCl_2$, 1% Nonidet P-40, 0.5% sodium deoxycholate, 16 μg of aprotinin per ml, 10 μg of leupeptin per ml, 1 mM

phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 100 mM NaF, 10 mM sodium PP_i). The lysates were clarified, and protein concentrations were determined as for the kinase assays. Protein from each lysate (75 μg) was electrophoresed in an 8 or 10% polyacrylamide gel and electroblotted onto an Immobilon membrane (Millipore). The membranes were blocked overnight at 4°C with 3% bovine serum albumin, incubated with a 1:1,000 dilution of antibody for 2 h at room temperature, washed, and incubated with 10 μCi of ^{125}I-labeled goat anti-rabbit immunoglobulin G (IgG) antiserum (ICN) for 1 h at room temperature.

The presence of GAP, SHC, phospholipase C-γ1 (PLCγ1), and the p85 α subunit of phosphatidylinositol 3′-kinase (PI3′K) in antiphosphotyrosine immunoprecipitates was assayed in the same manner except that 350 μg of each lysate was subjected to immunoprecipitation with 1 μg of the monoclonal antiphosphotyrosine antibody IgG2BK (UBI). The immunoprecipitates were electrophoresed and immunoblotted, and the filter was probed with rabbit antiserum to the protein of interest and then with ^{125}I-labeled goat anti-rabbit IgG antiserum as above.

Tyrosine phosphorylation of GAP-associated proteins was assayed in a similar manner. Cells were lysed in CLB, an equal portion of each lysate (not frozen) was subjected to immunoprecipitation with the anti-GAP N-terminal monoclonal antibody B4F8 (Santa Cruz Biochemicals), and the immunoblot was analyzed with the rabbit polyclonal antiphosphotyrosine antiserum and ^{125}I-labeled goat anti-rabbit IgG antiserum. For the analysis of proteins bound to p120^{GAP} but not to p58$^{GAP\text{-}N}$, immunoprecipitation was carried out with RH6-2A rabbit anti-GAP (catalytic region) serum, and the immunoblot was probed with the IgG2BK monoclonal and then with ^{125}I-labeled rabbit anti-mouse IgG antiserum.

Microinjection of neutralizing Ras antibody. The microinjection experiments were carried out as described previously (46). Briefly, cells were plated on coverslips at 20% confluency. The next day, discrete areas of cells were microinjected with a 5% cell volume of antibody Y13-259 solution (12 mg/ml). After 18 to 21 h, the cells were pulse-labeled with [^{3}H]thymidine, fixed with paraformaldehyde, and stained with biotin-conjugated antibody against rat IgG and then with avidin-Texas red. Autoradiography was performed in parallel with fluorescence microscopy so that the injected cells could be distinguished. Multiple experiments were carried out for each of the lines, and the percentages of injected and uninjected cells that became labeled with [^{3}H]thymidine were calculated.

RESULTS

Myristylation-defective v-*src* and GAP N terminus cooperate to transform NIH 3T3 cells. To investigate the biological activity of the GAP amino terminus, a premature termination mutant of *GAP* (designated *GAP-N*) was constructed, *GAP-N*, which encodes the first 456 codons of the human cDNA, includes both SH2 domains as well as the lone SH3 but not tyrosine 460, which is a substrate for TPKs (26). When *GAP-N* was placed in a retrovirus expression vector and transfected into NIH 3T3 cells, no transformation was observed in several independent experiments (Fig. 1).

These results suggested that expression of the SH2/SH3 region of GAP by itself was insufficient to transform NIH 3T3 cells. However, since the N terminus of GAP forms complexes in response to TPKs, it seemed possible that GAP might serve a positive biological role in TPK-dependent transformation. To establish such a function for the N terminus of GAP, we coexpressed *GAP-N* with an active but nontransforming PTK. Since disruption of the myristylation signal of v-*src* severely impairs its transforming function without diminishing the kinase activity of its encoded product, a myristylation-defective form of v-*src* (*MDSRC*) was constructed by deletion of codons 2 to 15 from the wild-type sequence. The *MDSRC* mutant is identical to the previously described XD315, which was found to be transformation defective in chicken embryo fibroblasts (5).

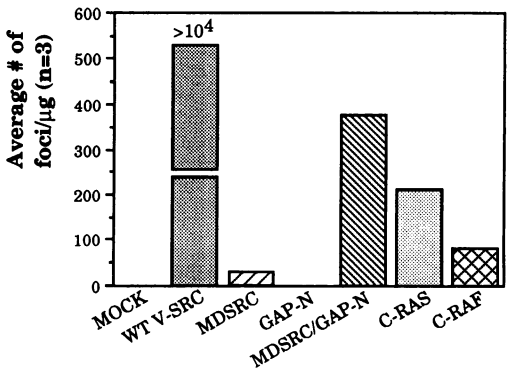

FIG. 1. Myristylation-defective v-*src* (*MDSRC*) and the GAP amino terminus (*GAP-N*) cooperate to induce focal transformation of NIH 3T3 cells. Transfections were carried by the calcium phosphate method with 100 to 1,000 ng of plasmid DNA, and focus formation was scored 12 to 15 days later. In the three experiments, the increase in foci per microgram obtained when the *MDSRC*-carrying plasmid was cotransfected with the *GAP-N*-carrying plasmid (in a 1:1 ratio) compared with that after transfection with *MDSRC* alone ranged between 5- and 19-fold. For the mock sample, only carrier DNA was used. All of the constructs were expressed from murine retrovirus expression vectors: the Harvey vector for the wild-type (WT) v-*src*, *MDSRC*, and *GAP-N* genes, pGV16 for c-*ras*, and LTR-c-*raf* for c-*raf*.

When *MDSRC* was cloned into a retrovirus vector and transfected into NIH 3T3 cells, it yielded only a very low level of transformation, as expected: approximately 30 foci per μg of DNA, compared with more than 10,000 foci per μg for wild-type v-*src* (Fig. 1). However, when the *MDSRC* plasmid was mixed in a 1:1 ratio with the *GAP-N* plasmid, a dramatic increase (more than 10-fold in repeat experiments) in the number of transformed foci, to an efficiency that was higher than with c-*ras* or c-*raf*, was seen (Fig. 1). After replating, cell lines derived from the foci induced by cotransfection of *MDSRC* and *GAP-N* retained a transformed morphology, while those induced by *MDRSRC* alone did not. The lines transfected with *MDSRC* plus *GAP-N* (designated fd1, fd3, and fd5; Fig. 2, panels 6 to 8) displayed a rounded, refractile shape that resembled that of v-*src*-transformed NIH 3T3 cells (Fig. 2, panel 3) more than that of v-*ras*-transformed NIH 3T3 cells (Fig. 2, panel 2). As with wild-type v-*src*-transformed NIH 3T3 cells, the transformed morphology of the fd lines was most apparent at high cell densities.

To obtain cells that stably expressed *GAP-N* and *MDSRC* individually, NIH 3T3 cells were cotransfected with pSV2neo and *MDSRC* or *GAP-N*, and independent G418-resistant colonies were isolated and expanded into cell lines. As expected, lines transfected with *MDSRC* alone (designated MDSRC 1 and 2; Fig. 2, panels 4 and 5) or *GAP-N* alone (designated GAP-N 1 and 2; Fig. 2, panels 9 and 10) were not

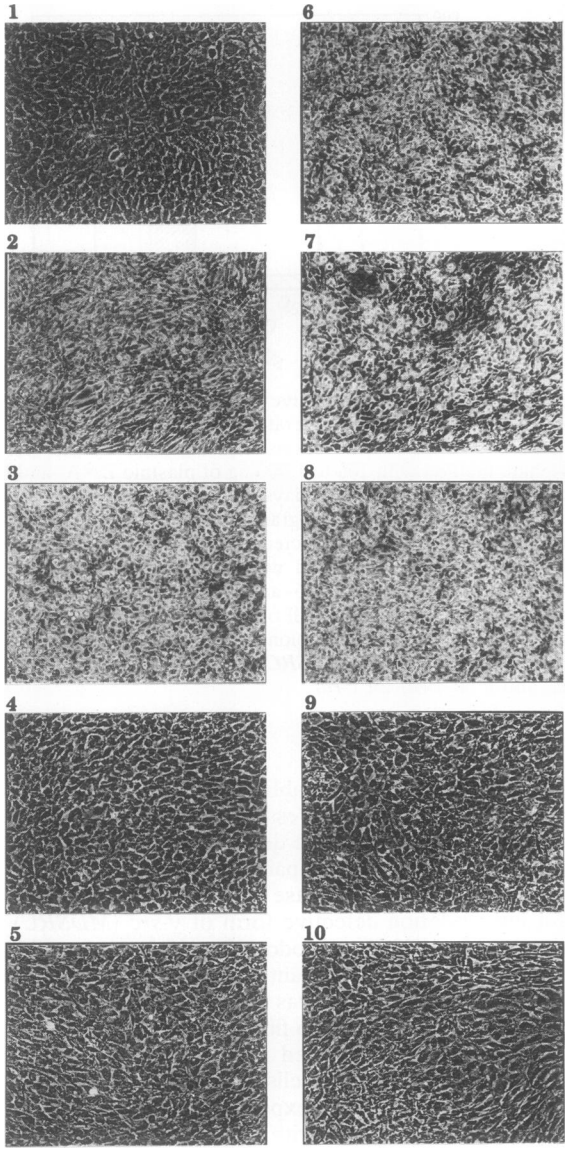

FIG. 2. Cellular morphology of control lines and NIH 3T3 cell lines expressing *MDSRC* and *GAP-N* singly or in combination. Panels: 1, NIH 3T3; 2, v-*ras*-transformed NIH 3T3; 3, v-*src*-transformed NIH 3T3; 4, MDSRC 1; 5, MDSRC 2; 6, fd1; 7, fd3; 8, fd5; 9, GAP-N 1; 10, GAP-N 2. Cells were plated at 5×10^5 per 60-mm dish 4 to 5 days prior to microscopy. Magnification, ×85.

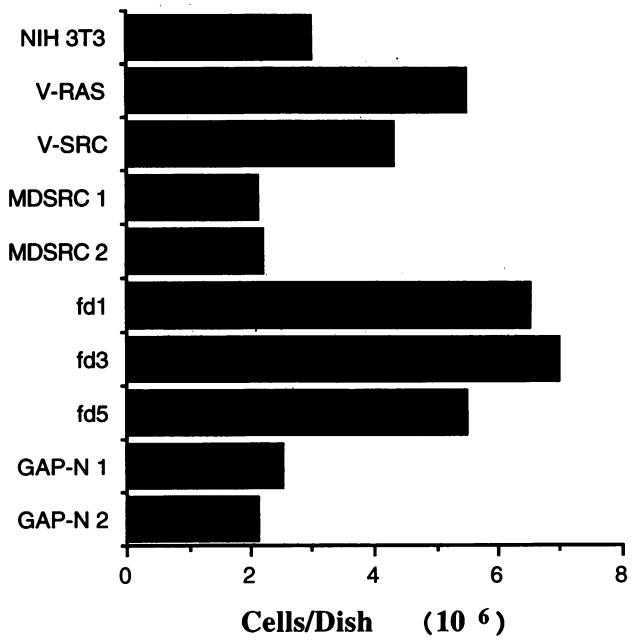

FIG. 3. Density-independent growth of cells transformed by cotransfection of *MDSRC* and *GAP-N*. Cells were plated at 3×10^5 per 35-mm dish on day 0, duplicate dishes of each line were trypsinized, and cells were counted every 2 days, starting at day 3 postplating through day 13. Consistent differences in cell numbers for the various lines were noted for days 5 to 13 postplating. Results are shown only for day 9.

transformed morphologically and resembled NIH 3T3 cells (Fig. 2, panel 1). When the growth of the different lines was assayed, the nontransformed lines displayed density-dependent growth inhibition, in contrast to cells transformed by v-*ras*, v-*src*, or *MDSRC* plus *GAP-N*, which grew to higher cell densities (Fig. 3).

Expression of the *GAP-N* and *MDSRC* protein products. The expression of the proteins encoded by *MDSRC* and *GAP-N* was assessed for the cell lines described above (Fig. 4). To determine the level of the *MDSRC* product, designated $pp58^{MDSRC}$, cells were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine, and the cell extracts were subjected to immunoprecipitation with a TBR serum that is specific for $pp60^{v\text{-}src}$. As expected, the TBR serum immunoprecipitated $pp60^{v\text{-}src}$ from cell lysates of the wild-type control v-*src* line (Fig. 4A, lane 3). No *src* product was detected in lysates of normal NIH 3T3 cells or the v-*ras*-transformed line (Fig. 4A, lanes 1 and 2), since the serum does not cross-react with endogenous mouse $pp60^{c\text{-}src}$. The levels of $pp58^{MDSRC}$ in the MDSRC 1 and 2 lines (Fig. 4A, lanes 4 and 5), which were nontransformed, were comparable to that of wild-type $pp60^{v\text{-}src}$ in the v-*src*-transformed cells. The fd1, fd3, and fd5 lines, derived from cotransfection of *MDSRC* and *GAP-N*, also expressed $pp58^{MDSRC}$ (Fig. 4A, lanes 6 to 8). However, the level of $pp58^{MDSRC}$ in the three fd lines was somewhat lower than that found in the nontransformed MDSRC lines, ruling out the possibility that the focus-derived lines might have been transformed because they had higher levels of *MDSRC* expression. When the same experiment was carried out with [^{3}H]myristic acid-labeled cells, the wild-type $pp60^{v\text{-}src}$ product was detected (Fig. 4B, lane 2), while no product was detected in lysates of NIH 3T3 cells (Fig. 4B, lane 1) or in lysates of the cell lines expressing $pp58^{MDSRC}$ (Fig. 4B, lanes 3 to 7). This result confirms the absence of bound myristic acid on the *MDSRC* product and is consistent with the type of mutation present in *MDSRC*.

The level of *GAP-N* product in the lines was examined by immunoblotting cell lysates with a polyclonal anti-GAP serum raised against the N terminus of GAP (Fig. 4C). Since this serum also recognizes the full-length, endogenous $p120^{GAP}$ present in the cell lysates, the full-length protein serves as an internal control. Indeed, the level of $p120^{GAP}$ appeared to be virtually identical in all 10 of the cell lines (Fig. 4C). In contrast, the *GAP-N* product was detected only in lysates from the lines transfected with *GAP-N*, namely fd1, fd3, and fd5 (Fig. 4C, lanes 6 to 8) and GAP-N 1 and 2

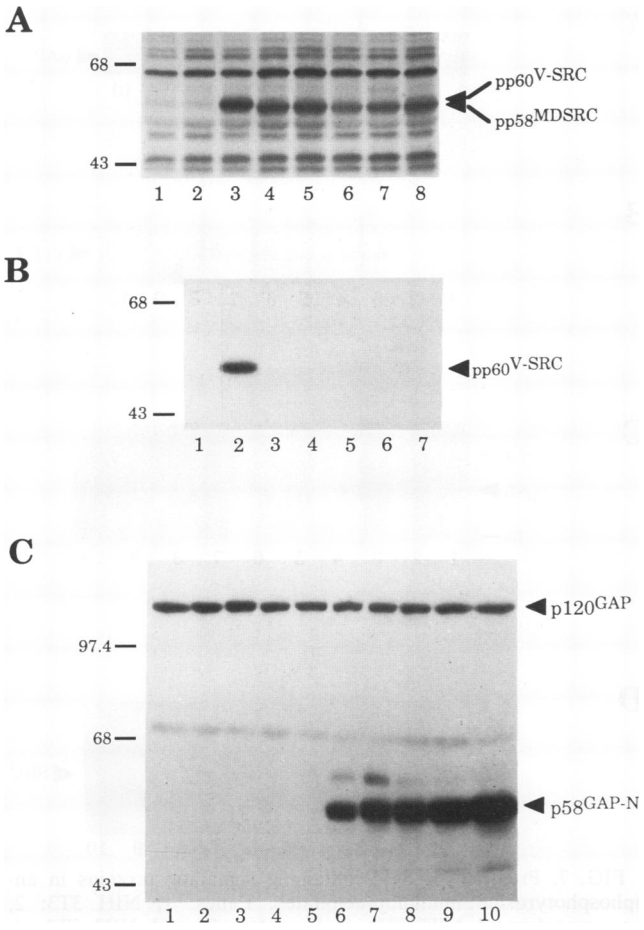

FIG. 4. Expression of Src and GAP proteins in NIH 3T3 cell lines. (A) Expression of pp60$^{v\text{-}src}$ and pp58^{MDSRC} as determined by metabolic labeling with [^{35}S]methionine and [^{35}S]cysteine followed by cell lysis and immunoprecipitation with TBR serum. Cell lines: lane 1, NIH 3T3; lane 2, v-*ras*-transformed NIH 3T3; lane 3, v-*src*-transformed NIH 3T3; lane 4, MDSRC 1; lane 5, MDSRC 2; lane 6, fd1; lane 7, fd3; lane 8, fd5. Immunoprecipitates were analyzed on a 10% polyacrylamide gel. (B) Myristylation of pp60$^{v\text{-}src}$ and pp58^{MDSRC} proteins as analyzed by radiolabeling with [^{3}H]myristic acid and immunoprecipitation of the cell lysates with TBR serum. Cell lines: lane 1, NIH 3T3; lane 2, v-*src*-transformed NIH 3T3; lane 3, MDSRC 1; lane 4, MDSRC 2; lane 5, fd1; lane 6, fd3; lane 7, fd5. (C) Expression of endogenous p120^{GAP} and p58$^{GAP\text{-}N}$ as assayed by immunoblotting. Cell lines: lane 1, NIH 3T3; lane 2, v-*ras*-transformed NIH 3T3; lane 3, v-*src*-transformed NIH 3T3; lane 4, MDSRC 1; lane 5, MDSRC 2; lane 6, fd1; lane 7, fd3; lane 8, fd5; lane 9, GAP-N 1; lane 10, GAP-N 2. Unlabeled cell lysates were prepared, and protein (100 μg) from each lysate was separated on a 10% polyacrylamide gel and transferred to a filter. The filter was incubated with anti-GAP A antiserum, washed, and incubated with ^{125}I-labeled goat anti-rabbit IgG antiserum (ICN). The positions of molecular size standards are shown at the left (in kilodaltons).

(Fig. 4C, lanes 9 and 10). The migration of the *GAP-N* product was somewhat slower than predicted; from the observed migration, the truncated GAP protein was designated p58$^{GAP\text{-}N}$. As was the case for the *MDSRC* product, the level of p58$^{GAP\text{-}N}$ in the transformed fd1, fd3, and fd5 lines was lower than in the pSV2neo-coselected, nontransformed GAP-N 1 and 2 lines.

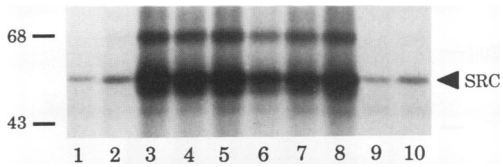

FIG. 5. In vitro kinase activity of Src proteins present in the different cell lines: lane 1, NIH 3T3; lane 2, v-*ras*-transformed NIH 3T3; lane 3, v-*src*-transformed NIH 3T3; lane 4, MDSRC 1; lane 5, MDSRC 2; lane 6, fd1; lane 7, fd3; lane 8, fd5; lane 9, GAP-N 1; lane 10, GAP-N 2. Unlabeled cell lysates were prepared and immunoprecipitations were carried out with the Src-specific monoclonal antibody 327, which recognizes pp60$^{c\text{-}src}$, pp60$^{v\text{-}src}$, and pp58^{MDSRC}. The immunoprecipitates were washed and incubated with [γ-^{32}P]ATP for 20 min at 30°C, washed, and analyzed on a 10% polyacrylamide gel. The arrowhead denotes the approximate migration position of the Src proteins, which all undergo autophosphorylation in this assay. The positions of molecular size standards are shown at left (in kilodaltons).

These findings further strengthen the interpretation that transformation induced by cotransfection of *MDSRC* and *GAP-N* is the result of genuine synergy between their encoded products and suggest that coexpression of the *GAP-N* and *MDSRC* products did not lead to an increase in the stability of either protein. In addition, the fact that the transformed cell lines derived from cotransfection of both genes express both proteins rules out several trivial explanations of the data (e.g., carrier effects of cotransfection and increased uptake of *MDSRC*). Subcellular fractionation of the various lines indicated that pp58^{MDSRC} and p58$^{GAP\text{-}N}$ were both cytosolic, as expected, whether expressed separately or together in the same cell, and immunoprecipitation of one protein did not coprecipitate the other protein (data not shown). These data rule out the possibility that the cooperation between these proteins might have resulted from relocalization of pp58^{MDSRC} to the cell plasma membrane when it is expressed with p58$^{GAP\text{-}N}$ and argue against a stable association between p58$^{GAP\text{-}N}$ and pp58^{MDSRC}.

Tyrosine phosphorylation of cellular proteins in the cell lines. To assess the in vitro kinase activity of the wild-type and mutant Src proteins, unlabeled cell lysates were prepared and subjected to immunoprecipitation with Src-specific monoclonal antibody 327, which recognizes the products of c-*src*, v-*src*, and *MDSRC* (25). The immunoprecipitates were washed and incubated with [γ-^{32}P]ATP, and the autokinase reaction products were analyzed by polyacrylamide gel electrophoresis (Fig. 5). In immunoprecipitates from NIH 3T3 cells, the v-*ras*-transformed line, and the two GAP-N lines, which express c-*src* but no v-*src* products, only a low level of Src autokinase activity was detected, corresponding to the endogenous pp60$^{c\text{-}src}$ (Fig. 5, lanes 1, 2, 9, and 10). The positive-control v-*src*-transformed line (Fig. 5, lane 3) exhibited a strongly phosphorylated 60-kDa product, while the MDSRC 1 and 2 lines and the fd1, fd3, and fd5 lines all exhibited a slightly faster-migrating product that also was strongly phosphorylated (Fig. 5, lanes 4 to 8). A second phosphorylated band migrating at 68 kDa was observed in all lines expressing pp60$^{v\text{-}src}$ or pp58^{MDSRC}; the identity of this phosphoprotein is not known. There was a good correlation between the level of pp60$^{v\text{-}src}$ or pp58^{MDSRC} expressed in the cell and the amount of autophosphorylated product obtained in these kinase reactions (compare lanes 3 to 8 in Fig. 3A with the same lanes in Fig. 5). These data demonstrate that the *MDSRC* product retains protein kinase activity and that

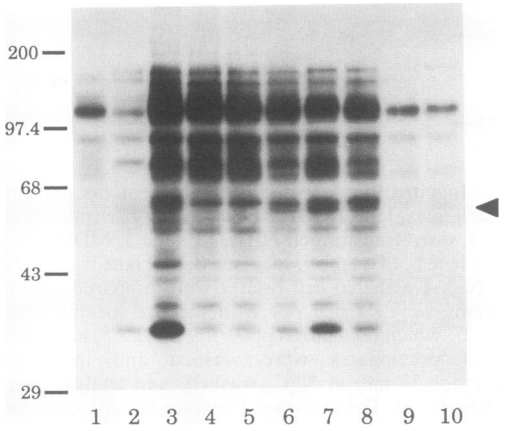

FIG. 6. Phosphotyrosine-containing proteins present in lysates of the cell lines, as detected by immunoblotting with antiphosphotyrosine antibodies. Cell lines: lane 1, NIH 3T3; lane 2, v-*ras*-transformed NIH 3T3; lane 3, v-*src*-transformed NIH 3T3; lane 4, MDSRC 1; lane 5, MDSRC 2; lane 6, fd1; lane 7, fd3; lane 8, fd5; lane 9, GAP-N 1; lane 10, GAP-N 2. Protein (75 μg) from each lysate was separated on an 8% polyacrylamide gel and transferred to a filter. The filter was incubated with rabbit polyclonal antiphosphotyrosine antiserum (ICN), washed, and incubated with ^{125}I-labeled goat anti-rabbit IgG antiserum. The arrowhead marks the migration position of the transformation-specific 62-kDa protein. The positions of molecular size standards are shown at left (in kilodaltons).

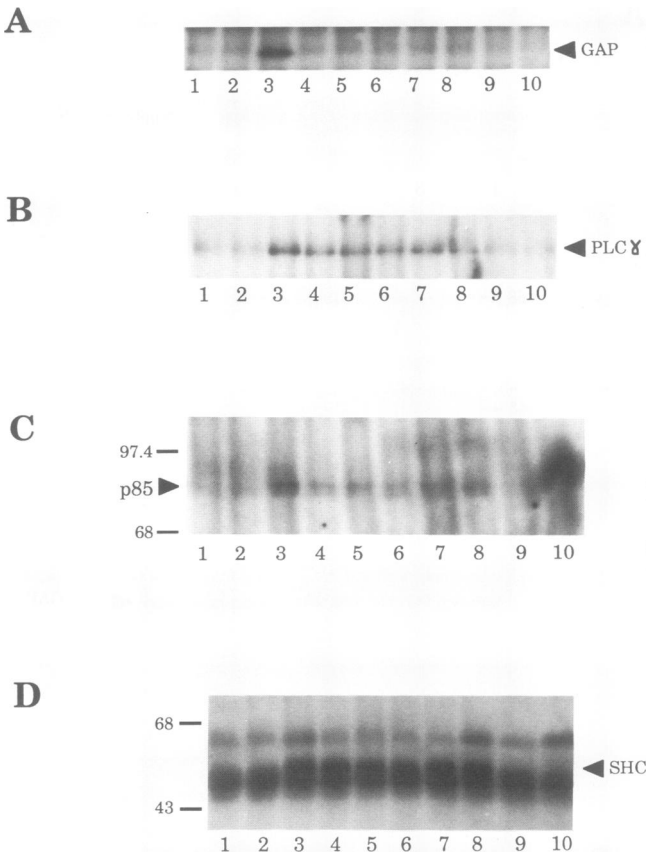

FIG. 7. Presence of SH2-containing signalling proteins in antiphosphotyrosine immunoprecipitates. Lanes: 1, NIH 3T3; 2, v-*ras*-transformed NIH 3T3; 3, v-*src*-transformed NIH 3T3; 4, MDSRC 1; 5, MDSRC 2; 6, fd1; 7, fd3; 8, fd5; 9, GAP-N 1; 10, GAP-N 2. Portions of cell lysates from each line containing approximately 350 μg of protein were subjected to immunoprecipitation with a monoclonal antiphosphotyrosine antibody. The immunoprecipitates were run on 8% (A to C) or 10% (D) polyacrylamide gels and transferred to filters. The filters were probed with rabbit antisera specific for (A) GAP (a gift of F. McCormick), (B) PLCγ1 (UBI), (C) the p85 α subunit of PI3′K (UBI), or (D) SHC (a gift of T. Pawson). Arrowheads mark the migration positions of the different products. Only the 52-kDa SHC product can be visualized, as the 46-kDa product is lost in the background band because of the immunoglobulin heavy chain that is present in every lane.

this activity is not significantly altered by coexpression of p58$^{GAP\text{-}N}$ in the same cell.

Phosphorylation of proteins on tyrosine residues in cells expressing pp60$^{v\text{-}src}$, pp58^{MDSRC}, or p58$^{GAP\text{-}N}$ was assayed in two different types of experiments. First, lysates were prepared from unlabeled cells and subjected to immunoblotting with a rabbit polyclonal antiphosphotyrosine serum (Fig. 6). NIH 3T3 cells, the v-*ras*-transformed line, and the two GAP-N lines contained relatively few tyrosine-phosphorylated proteins (Fig. 6, lanes 1, 2, 9, and 10); a single band migrating at approximately 110 kDa was the most prominent. In contrast, cells transformed by wild-type v-*src* contained a large number of immunoreactive bands, ranging in molecular mass from 36 to 150 kDa (Fig. 6, lane 3). Virtually all of these proteins were also tyrosine phosphorylated in the MDSRC 1 and 2 lines (Fig. 6, lanes 4 and 5) and the fd1, fd3, and fd5 lines (Fig. 6, lanes 6 to 8), although some were significantly less immunoreactive. For example, proteins of 45 and 36 kDa were less prominent in cells expressing pp58^{MDSRC} than in the v-*src*-transformed line. Of all the proteins detected in this assay, a single band at 62 kDa (Fig. 6, arrowhead) showed a strict correlation with transformation. This protein, which probably represents GAP-associated p62 (see below), appeared only in cells transformed by v-*src* and in the three fd lines (Fig. 6, lanes 3 and 6 to 8).

Several signalling proteins that contain SH2 or SH3 domains are also substrates for tyrosine phosphorylation in cells transformed by oncogenic PTKs (40). These include GAP, the SHC protein, PLCγ1, and the p85 α subunit of PI3′K. Since antisera that recognize these proteins are now available, their tyrosine phosphorylation in the lines could be evaluated.

Phosphorylation of these proteins was investigated by combined immunoprecipitation and Western blotting assays, in which antiphosphotyrosine immunoprecipitates from unlabeled cell extracts were run on gels, transferred to filters, and probed with antibodies against the specific product (Fig. 7). Surprisingly, the level of the 52-kDa SHC product, the 145-kDa PLCγ1, and p85-α in the antiphosphotyrosine immunoprecipitates was higher in all lines expressing pp60$^{v\text{-}src}$ or pp58^{MDSRC} (Fig. 7B, C, and D, lanes 3 to 8) than in the NIH 3T3, v-*ras*-transformed, and GAP-N lines (Fig. 7B, C, and D, lanes 1, 2, 9, and 10). Thus there was no correlation between tyrosine phosphorylation of these proteins and cell transformation. A different profile was noted for p120^{GAP}, significant levels of which appeared only in the antiphosphotyrosine immunoprecipitates from the wild-type v-*src*-transformed line (Fig. 7A, lane 3). The negative results obtained for p120^{GAP} with the fd lines indicate that a high level of GAP tyrosine phosphorylation is not required for their transformation. The converse experiment, in which the substrate was immunoprecipitated and then probed with antiphosphotyrosine antibodies, was performed for GAP and SHC with similar results (Fig. 8 and data not shown). We

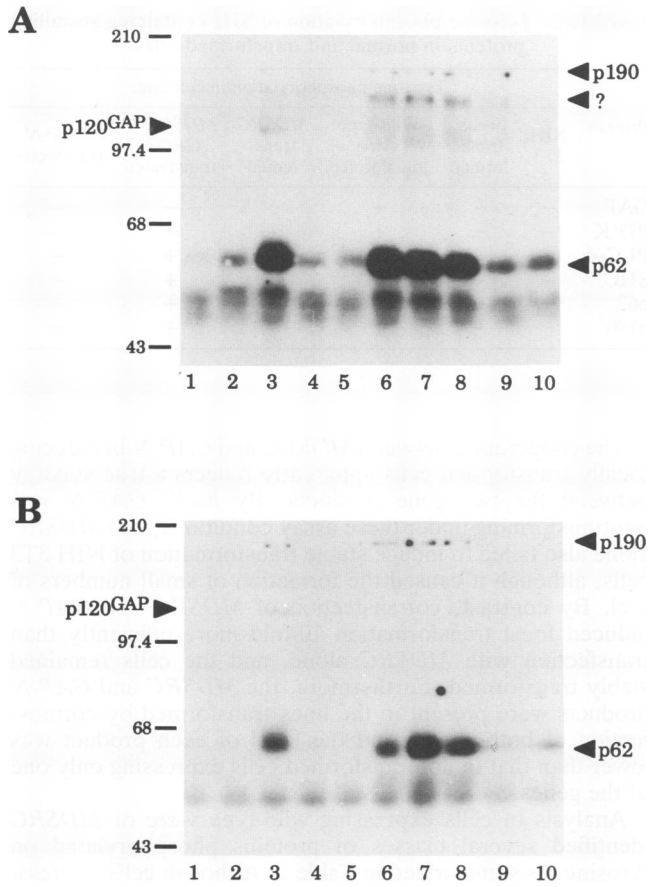

FIG. 8. Tyrosine phosphorylation of p120^{GAP} and GAP-associated proteins. Lanes: 1, NIH 3T3; 2, v-*ras*-transformed NIH 3T3; 3, v-*src*-transformed NIH 3T3; 4, MDSRC 1; 5, MDSRC 2; 6, fd1; 7, fd3; 8, fd5; 9, GAP-N 1; 10, GAP-N 2. (A) Portions of cell lysates from each line containing 350 μg of protein were subjected to immunoprecipitation with monoclonal antibody B4F8 (Santa Cruz Biochemicals), which recognizes an epitope in the GAP N terminus. The immunoprecipitates were run on an 8% polyacrylamide gel and transferred to a filter. The filter was probed with rabbit polyclonal antiphosphotyrosine antiserum, washed, and incubated with ^{125}I-labeled goat anti-rabbit IgG antiserum. (B) The same experiment was carried out except that immunoprecipitation was done with rabbit anti-GAP RH62A serum, which recognizes p120^{GAP} but not p58$^{GAP\text{-}N}$, and the filter was probed with a monoclonal antiphosphotyrosine antibody and then with ^{125}I-labeled rabbit anti-mouse IgG antiserum. Arrowheads mark the migration positions of p120^{GAP} and the GAP-associated p190, p62, and an unknown 150-kDa protein (?). The positions of molecular size standards are shown at left (in kilodaltons).

conclude that membrane association of the v-*src* product is required for tyrosine phosphorylation of p120^{GAP} and that tyrosine phosphorylation of SHC, PLCγ1, and PI3′K, even in the same cell, is insufficient for stable transformation.

The ability of p120^{GAP} to form intermolecular complexes in the various cell lines was examined by immunoprecipitation with a monoclonal antibody that recognizes the N terminus of GAP followed by immunoblotting with antiphosphotyrosine antibodies (Fig. 8A). In accordance with the previous assay, p120^{GAP} was tyrosine phosphorylated in the v-*src*-transformed line, while little if any GAP was detected in the fd lines (Fig. 8A, lane 3). Although small amounts of GAP-associated p62 were detected in most of the lines,

TABLE 1. In vivo GTP binding of p21^{ras} in cell lines expressing *MDSRC* and *GAP-N*[a]

Cells	Mean % GTP bound ± SE
NIH 3T3	4.8 ± 0.2
v-*ras* transformed	77.4 ± 1.3
v-*src* transformed	10.9 ± 1.0
MDSRC 1	4.7 ± 0.1
MDSRC 2	5.2 ± 0.3
fd1	11.5 ± 0.2
fd3	10.4 ± 0.7
fd5	12.9 ± 2.1
GAP-N 1	4.5 ± 0.6
GAP-N 2	5.9 ± 0.2

[a] Cell lysates were prepared from [^{32}P]phosphate-labeled cells, and immunoprecipitation and thin-layer chromatography were carried out as described in the text. After scanning with AMBIS, background correction and correction for phosphate content were done before the values were calculated. Values represent means from two independent experiments ± standard error.

much higher levels of tyrosine phosphorylation of this protein were observed in immunoprecipitates from the fd1, fd3, and fd5 lines as well as those from the v-*src*-transformed (Fig. 8A, line lanes 6 to 8 and 3). A similar pattern was observed for GAP-associated p190, although it was much less striking than for p62. The relative faintness of the p190 band probably results from its being less highly tyrosine phosphorylated than p62, as reported by others (36), and from poor recognition by the antiphosphotyrosine antibodies used. As expected, no tyrosine phosphorylation of p58$^{GAP\text{-}N}$ was detected, consistent with this protein's lacking the known phosphorylation site on p120^{GAP} (tyrosine 460). A third protein of approximately 150 kDa was also detected in the same lines (Fig. 8A, arrowhead labeled ?). Similar results were obtained when the polyclonal anti-GAP N terminus serum was used (data not shown). These data therefore demonstrate a correlation between the formation of GAP complexes and cell transformation.

Surprisingly, the GAP-p62 and GAP-p190 complexes were also detected in the v-*src*-transformed and fd lines when an antibody against the GAP catalytic region that does not recognize p58$^{GAP\text{-}N}$ was used for immunoprecipitation (16) (Fig. 8B, lanes 3 and 6 to 8). This result implies that, in cells expressing pp58^{MDSRC} and p58$^{GAP\text{-}N}$, a significant portion of the complexes involve p120^{GAP}. Together with the fact that tyrosine phosphorylation of all the other substrates examined failed to correlate with transformation, these data strongly imply that the molecular basis for the cooperation between *MDSRC* and *GAP-N* includes the formation of signalling complexes involving p120^{GAP}.

Cells transformed by *MDSRC* plus *GAP-N* require endogenous Ras activity. To determine whether the proportion of GTP · Ras was altered in the fd cells, the lines were metabolically labeled with $^{32}PO_4$ and lysed, and the lysates were subjected to immunoprecipitation with a Ras monoclonal antibody, after which the levels of GTP and GDP in the immunoprecipitates were analyzed by thin-layer chromatography (Table 1). Consistent with previous results (15), the proportion of GTP · Ras in the v-*src*-transformed cells was about twice that in NIH 3T3 cells. While the GTP · Ras levels in the nontransformed GAP-N lines and MDSRC lines were similar to those in NIH 3T3 cells, the levels in the fd lines, which were transformed by cotransfection of *GAP-N* and *MDSRC*, were elevated and resembled those of GTP · Ras in the v-*src* transformants (Table 1).

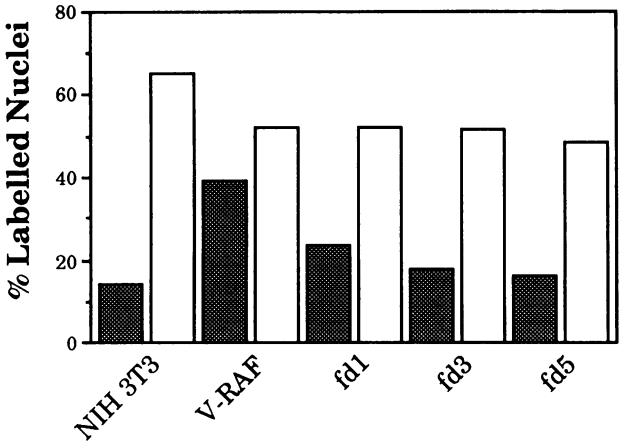

FIG. 9. Dependence of cell lines transformed by *MDSRC* and *GAP-N* on endogenous Ras for the induction of DNA synthesis. Assays were conducted as described in the text. For each cell line, the experiment was carried out three to six times. The total number of cells injected with monoclonal antibody 259 (shaded bars) assayed for each line ranged from 81 to 347, while the number of uninjected cells (open bars) ranged from 291 to 718. The graph depicts the percentages of injected and uninjected cells from each line that became labeled with [^{3}H]thymidine 18 to 21 h postinjection.

These results suggested that, with respect to GTP · Ras, transformation in the fd cells was similar to that in v-*src*-transformed cells, which are known to depend on endogenous Ras for their transformation. To examine the Ras dependence of the fd1, fd3, and fd5 lines, they were microinjected with neutralizing anti-Ras monoclonal antibody 259 and monitored for subsequent DNA synthesis (Fig. 9). In cells transformed by v-*raf*, which lies downstream of *ras*, 40% of the injected cells and 50% of the uninjected cells underwent DNA synthesis. By contrast, the three fd cell lines all displayed significant inhibition by antibody 259, similar to that of control NIH 3T3 cells, indicating that they require endogenous Ras function (Fig. 9), as do cells transformed by wild-type v-*src* (46). These data suggest that the transformation-associated formation of GAP complexes is an event that occurs upstream of *ras* or by an independent pathway that does not obviate the requirement for Ras activity.

DISCUSSION

We have demonstrated that a transformation-defective, cytosolic mutant product of v-*src* can stably transform cells when it is coexpressed with a premature termination mutant of GAP encoding the amino-terminal SH2 and SH3 domains. These experiments were initiated to determine whether the N terminus of GAP, which has been shown biochemically to be involved in protein-protein interactions, serves a biological role in TPK transformation. The *GAP-N* construction was designed to terminate after the second SH2 domain but before the known tyrosine phosphorylation site at amino acid 460, so that effects due to the SH2 and SH3 domains were not confused with effects due to the presence of phosphotyrosine. Since SH2 domains are known to function primarily, although not exclusively, through interaction with phosphorylated tyrosine residues, we chose to express the *GAP-N* construct in the presence of an active tyrosine kinase (pp58^{MDSRC}) that was severely impaired for transformation.

TABLE 2. Tyrosine phosphorylation of SH2-containing signalling proteins in normal and transformed lines

Protein	Tyrosine phosphorylation in cell line:					
	NIH 3T3	v-*ras* transformed	Wild-type v-*src* transformed	*MDSRC* transfected	*MDSRC* + *GAP-N* transfected	*GAP-N* transfected
GAP	–	–	+	–	–	–
PI3′K	–	–	+	+	+	–
PLCγ1	–	–	+	+	+	–
SHC	–	–	+	+	+	–
p62	–	–	+	–	+	–
p190	–	–	+	–	+	–

The cooperation between *MDSRC* and *GAP-N* in inducing focally transformed cells apparently reflects a true synergy between the two gene products. By itself, *GAP-N* was nontransforming under these assay conditions, and *MDSRC* alone also failed to induce stable transformation of NIH 3T3 cells, although it caused the formation of small numbers of foci. By contrast, cotransfection of *MDSRC* and *GAP-N* induced focal transformation 10-fold more efficiently than transfection with *MDSRC* alone, and the cells remained stably transformed. Furthermore, the *MDSRC* and *GAP-N* products were present in the lines transformed by cotransfection of both genes, and the level of each product was lower than that in nontransformed cells expressing only one of the genes.

Analysis of cells expressing wild-type v-*src* or *MDSRC* identified several classes of proteins phosphorylated on tyrosine, as summarized in Table 2. Although cells expressing *MDSRC* alone remained nontransformed, many proteins in the lines were tyrosine phosphorylated, as described earlier for avian cells (20). The SH2-containing proteins SHC, PLCγ1, and the p85 α subunit of PI3′K were also all recovered in antiphosphotyrosine immunoprecipitates from nontransformed lines expressing *MDSRC*, suggesting that their tyrosine phosphorylation is insufficient for transformation of NIH 3T3 cells. However, these substrates may contribute to the transformed phenotype, and activation of these targets might account, at least in part, for the reported ability of cytosolic mutants of v-*src* (including one identical to *MDSRC*) to retain mitogenicity for chicken neuroretina cells (3). In contrast to these other proteins, p120^{GAP} was found to be highly tyrosine phosphorylated only in cells transformed by wild-type v-*src*. The fact that little if any tyrosine phosphorylation of p120^{GAP} is detected in the transformed fd lines suggests either that tyrosine phosphorylation of p120^{GAP} is not critical for v-*src* transformation or that p58^{GAP} can substitute functionally for tyrosine-phosphorylated p120^{GAP}.

The requirement for Src protein to be membrane localized in order to induce GAP tyrosine phosphorylation represents an intriguing result, since p120^{GAP} itself is primarily cytosolic. Furthermore, the PLCγ1 product, which is primarily membrane bound, can be tyrosine phosphorylated by both membrane-bound and cytosolic Src proteins. These findings suggest that other parameters in addition to subcellular localization and the presence of SH2 and/or SH3 domains play a role in determining the steady-state level of tyrosine phosphorylation for a given substrate.

With the exception of the tyrosine phosphorylation of p120^{GAP}, the cells transformed by *MDSRC* plus *GAP-N* closely resembled v-*src* transformants in other respects.

Specifically, the formation of stable complexes between GAP and phosphotyrosine-containing proteins and an increase in GTP · Ras were limited to the transformed cells, and the *MDSRC* plus *GAP-N* transformants remained dependent on endogenous Ras, as reported previously for v-*src* transformants. The absence of these phosphotyrosine protein complexes with GAP and the low levels of GTP · Ras in the nontransformed MDSRC cells and the GAP-N cells strongly suggest that the complexes and increased GTP · Ras levels serve a positive function in the transformants.

The transformation-associated complexes noted here were formed between GAP and three tyrosine-phosphorylated proteins, namely p62, p190, and an unknown 150-kDa protein (p150). Complexes of GAP-p62 and GAP-p190 have been identified in TPK-transformed cells and after ligand activation of receptor TPKs (12, 33). Their formation is known to depend on the SH2 domains of GAP, but it has not been possible until now to assign a biological role to their formation. By themselves, the genetic data presented here could imply that endogenous p120^{GAP} serves a positive or negative function in v-*src* transformation, since p58$^{GAP\text{-}N}$ might, in principle, contribute to transformation by mimicking or competing with a function of p120^{GAP}. However, the similarity of the v-*src* and the *MDSRC* plus *GAP-N* transformants strongly suggests that p58$^{GAP\text{-}N}$ mimics rather than antagonizes a function of p120^{GAP} and that GAP-associated complexes represent a positive component of the mitogenic or transforming signalling pathway of TPKs. Previous analyses of v-*src* SH2/SH3 mutants have suggested that tyrosine phosphorylation of GAP and p62 may be important for v-*src*-dependent mammalian cell transformation (24, 35). In the *MDSRC* plus *GAP-N* transformants, p58$^{GAP\text{-}N}$ allows the formation of complexes in the absence of GAP phosphorylation. Others have demonstrated that the N terminus of GAP, when expressed in CHO or NIH 3T3 cells, increased gene expression from certain promoters and that this effect was blocked by expression of a dominant inhibitory mutant of *ras*, although the formation of GAP complexes was not analyzed (31).

The ability of p58$^{GAP\text{-}N}$ plus pp58^{MDSRC} to induce complex formation suggests that this process requires adequate concentrations of accessible SH2/SH3 domains and phosphorylated substrates. The p58$^{GAP\text{-}N}$ in the cell should raise the concentration of SH2/SH3 domains, allowing GAP complexes to form in the presence of the TPK activity provided by pp58^{MDSRC}. This process may result from binding of the SH2 domains of p58$^{GAP\text{-}N}$ to phosphorylated residues on the associated proteins, protecting them from the action of phosphatases. While the complexes may initially occur with p58$^{GAP\text{-}N}$, the fact that many of the steady-state complexes involve p120^{GAP} suggests a dynamic process in which associations form, break up, and reform. Others have recently provided in vitro evidence that even strong interactions between SH2 domains and tyrosine-phosphorylated peptides are dynamic because of a relatively high dissociation rate (14).

It seems likely that GAP complex formation may regulate several pathways, including Ras. The dependence of the fd lines on coexpression of *MDSRC* and *GAP-N* for increased Ras · GTP levels (and transformation) demonstrates that their gene products cooperate to bring this about. In spite of the participation of GAP in downstream signalling by Ras (10), the findings that the fd lines displayed an increase in GTP · Ras levels and remained dependent on endogenous Ras make it likely that GAP complex formation participates in the formation of GTP · Ras.

One possibility is that GAP complexes induce a decrease in the effective catalytic activity of GAP, which would lead to an increase in GTP · Ras levels (36). Alternatively, GAP complex formation might be associated, directly or indirectly, with an increase in stimulated guanine nucleotide exchange on Ras. Consistent with this latter possibility, we and others have presented evidence that stimulated exchange, rather than changes in GAP catalytic activity, is primarily responsible for the increase in Ras · GTP levels that occurs after activation of receptor TPKs in fibroblasts (2, 32, 53). Furthermore, the Grb-2/Sem-5 protein, an "adaptor" molecule that functions in the Ras pathway between TPKs and Ras-specific exchange factors, has recently been shown to form complexes with p62 (43a). The SHC protein has been implicated as a link between pp60$^{v\text{-}src}$ and Sos, which is a Ras-specific guanine nucleotide exchange factor (11). The Grb-2/Sem-5 protein, SHC, and the exchange factor are recruited as a complex to the plasma membrane, and Ras · GTP levels are elevated in v-*src*-transformed cells. Our data indicate that tyrosine phosphorylation of SHC in the *MDSRC*-transfected lines is insufficient to activate Ras · GTP, suggesting that the *MDSRC*-expressing cells lack a putative signal or binding site for plasma membrane association of the SHC complex.

At least two mechanisms may be involved in the activation of GAP as a signal transducer. One, which is represented by the formation of GAP complexes as described here, is induced by TPKs. In addition to possible effects on the level of GTP · Ras, the GAP-p62 and GAP-p190 complexes may serve other signalling functions as well, for example, by altering interactions between proteins and RNA or DNA (p62) or by influencing the cytoskeleton (p190). The possible functions of the previously unidentified 150-kDa protein may be elucidated by further characterization. The second mechanism would be mediated by the interaction of GTP · Ras with the catalytic region of p120^{GAP} (10). The cooperation between *MDSRC* and *GAP-N* provides a transformation assay with which to assess the biological role of this region of GAP in mammalian cells and the potential significance of its interactions with other macromolecules.

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