Polyamines Are Essential for Cell Transformation by pp60^{v-src}: Delineation of Molecular Events Relevant for the Transformed Phenotype

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Abstract. Ornithine decarboxylase (ODC), the key enzyme of polyamine biosynthesis, becomes upregulated during cell proliferation and transformation. Here we show that intact ODC activity is needed for the acquisition of a transformed phenotype in rat 2R cells infected with a temperature-sensitive mutant of Rous sarcoma virus. Addition of the ODC inhibitor α-difluoromethyl ornithine (DFMO) to the cells (in polyamine-free medium) before shift to permissive temperature prevented the depolymerization of filamentous actin and morphological transformation. Polyamine supplementation restored the transforming potential of pp60^{v-src}. DFMO did not interfere with the expression of pp60^{v-src} or its in vitro tyrosine kinase activity. The tyrosine phosphorylation of most cellular proteins, including *ras* GAP, did not either display clear temperature- or DFMO-sensitive changes. A marked increase was, however, observed in the tyrosine phosphorylation of phosphatidylinositol 3-kinase and proteins of 33 and 36 kD upon the temperature shift, and these hyperphosphorylations were partially inhibited by DFMO. A DFMO-sensitive increase was also found in the total phosphorylation of calpactins I and II. The well-documented association of GAP with the phosphotyrosine-containing proteins p190 and p62 did not correlate with transformation, but a novel 42-kD tyrosine phosphorylated protein was complexed with GAP in a polyamine- and transformation-dependent manner. Further, tyrosine phosphorylated proteins of 130, 80/85, and 36 kD were found to co-immunoprecipitate with pp60^{v-src} in a transformation-related manner. Altogether, this model offers a tool for sorting out the protein phosphorylations and associations critical for the transformed phenotype triggered by pp60^{v-src}, and implicates a pivotal role for polyamines in cell transformation.

Ornithine decarboxylase (ODC),[1] which catalyzes the formation of putrescine and CO_2 from ornithine, is the rate-controlling enzyme in the biosynthesis of polyamines. ODC and polyamines have been implicated to play a pivotal role in cell proliferation and to contribute to the development of cancer (28, 48, 61). Mitogenic stimulation of normal cells triggers a rapid, transient increase in ODC activity, whereas cell transformation by chemical carcinogens (22), viruses (21, 25, 35) and oncogenes (34, 59, 60) appears to be accompanied by a constitutive activation of ODC and loss of the cell cycle-related fluctuations in the enzyme activity. To assess the significance of the ODC induction for the transformation process, we decided to examine the effects of α-difluoromethyl ornithine (DFMO), a highly specific and irreversible inhibitor of ODC (44), on different parameters of transformation in a rat fibroblast cell line (2R) having a temperature-sensitive Rous sarcoma virus (RSV) mutant integrated into the genome (63).

The transforming gene of RSV, v-*src*, encodes a 60-kD phosphoprotein, pp60^{v-src}, which is a tyrosine-specific protein kinase (17). pp60^{v-src} is localized to the inner face of the plasma membrane and other intracellular membranes, and represents the prototype of nonreceptor tyrosine kinases. The transforming potential of pp60^{v-src} is dependent on its intrinsic tyrosine kinase activity suggesting that phosphorylation of proteins on tyrosine is the key event in the process of transformation (for reviews see references 11, 30, 56). A number of proteins are known to become phosphorylated on tyrosine in v-*src*-transformed cells (36), but it is not yet clear which of these proteins are direct substrates of pp60^{v-src} and which phosphorylations are essential for transformation. The critical substrates of pp60^{v-src} have been supposed to reside in the vicinity of the plasma membrane, because mutants of pp60^{v-src} deficient in myristoylation and membrane anchoring have lost their transforming capability (10, 30, 56).

Recently, it has become evident that the critical targets of pp60^{v-src} may also be cytosolic proteins. Activation of growth factor receptor tyrosine kinases by their ligands, including receptors for PDGF, EGF and colony-stimulating growth factor (CSF-1) is known to trigger an association of a specific set of cytosolic signal transduction proteins, like *ras* GTPase-activating protein (GAP), phosphatidylinositol (PI) 3-kinase, phospholipase Cγ1 (PLC γ1) and Raf-1 with the receptors (1, 10, 18, 41). All these signal transducers, ex-

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1. *Abbreviations used in this paper:* DFMO, α-difluoromethyl ornithine; GAP, GTPase-activating protein; MAP, mitogen-activated protein; ODC, ornithine decarboxylase; RSV, Rous sarcoma virus.

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cept for c-Raf, share sequences called SH2 (*src* homology region 2), which are involved in the association of the proteins with the receptors or each other (1). Likewise, the SH2 domain of pp60$^{v\text{-}src}$ appears to be critical for the binding of its substrates (29, 38, 45). Regarding the signal transducers, it is known that PI 3-kinase associates with pp60$^{v\text{-}src}$ (19), and that this binding most likely occurs through the SH2 domain of pp60$^{v\text{-}src}$ (10). It has also been shown that GAP and GAP-associated proteins become tyrosine phosphorylated in v-*src*-transformed cells (16). Thus, it can be hypothesized that activation of v-*src* triggers recruitment of a series of cytosolic signal transduction molecules to the plasma membrane, and that constitutive activation of these signal transducers is responsible for cell transformation by pp60$^{v\text{-}src}$.

In this study, we show that the inhibition of polyamine biosynthesis by DFMO results in blocking of transformation of rat fibroblasts by v-*src*, despite the fact that the *src* kinase activity and the tyrosine phosphorylation of the majority of proteins remains unchanged. A transformation-associated increase was, however, observed in the tyrosine phosphorylation of the PI 3-kinase subunit(s) and 33–36-kD proteins, though the rise in the phosphorylation of the smaller subunit of PI 3-kinase was only slightly inhibited by DFMO. Further, we found that DFMO interferes with the tyrosine phosphorylation and association of a previously unidentified 42-kD protein with GAP, whereas the complex formations of GAP with the tyrosine phosphorylated proteins p190 and p62 do not dictate the induction of transformation. Finally, we show that some tyrosine phosphorylated proteins become physically associated with the activated pp60$^{v\text{-}src}$ (or a complex containing pp60$^{v\text{-}src}$) in a polyamine-dependent and transformation-related manner.

Materials and Methods

Cell Culture

The rat 2R fibroblast cell line carrying the ts339 *src* gene of RSV-B77 and the parental Rat-1 cell line (63) were routinely maintained at 37°C in RPMI-1640 medium containing 5% FCS. For experiments, the medium was replaced by RPMI-1640 supplemented with 5% horse serum depleted of polyamines by extensive dialysis.

Assay of Ornithine Decarboxylase

ODC activity was measured by determining the release of $^{14}CO_2$ from L-[1-$^{14}CO_2$]ornithine (34).

Analysis of ODC mRNA

Total cellular RNA was fractionated on 1% agarose/formaldehyde gels, transferred to Hybond *N* filters and hybridized with nick-translated pODC16 as previously described (34).

Metabolic Labeling and Immunoprecipitation Assays

For ^{32}P-labeling, cells were cultured with phosphate-free medium containing 5% dialyzed horse serum (prewarmed to the appropriate temperature) for 30–45 min, and then labeled with [^{32}P]ortophosphate (0.6–3.5 mCi/5 ml) for 3–6 h. For [^{35}S]methionine labeling, cells were preincubated in methionine-free MEM (or MEM containing 5% of the normal methionine concentration) for 30–45 min and labeled for 1–3 h with 100–200 μCi/ml [^{35}S]methionine. Cells were then washed with PBS, detached by trypsin, washed again with PBS and lysed in 0.5 ml 50 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM PMSF, 100 mM NaF and 1 mM sodium ortovanadate. Lysates were centrifuged at 15,000 *g* for 10 min at 4°C, and the supernatants were preadsorbed with nonimmune sera/protein A Sepharose, whereafter they were incubated for 1.5–3 h with the specific antibodies or nonimmune sera. Protein A Sepharose beads were then added and the incubation continued for 1 h. In the case of mAbs, either protein A Sepharose beads precoated with 10 μg affinity-purified rabbit anti-mouse immunoglobulin antibodies or agarose beads with covalently conjugated goat anti-mouse IgG (Sigma Chemical Co., St. Louis, MO) were used. The resulting immunocomplexes were washed four to five times with the lysis buffer or sequentially with 0.5% Triton X-100 - 0.05% SDS, 0.5% Triton X-100 - 150 mM NaCl, 0.05% Triton X-100 - 0.5 M NaCl, 0.05% Triton X-100 - 150 mM NaCl and H_2O.

Assay of Src Kinase Activity

The kinase activity of pp60$^{v\text{-}src}$ was measured by an immune complex assay using the heavy chain of IgG as the substrate essentially as described earlier (47) with minor modifications. In brief, 5×10^6 cells were lysed in 0.5 ml 50 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM PMSF, 100 mM NaF and 1 mM sodium ortovanadate, and nuclei removed by centrifugation. pp60$^{v\text{-}src}$ was immunoprecipitated from the cleared cell lysates by incubating with 10 μl monoclonal anti-v-*src* antibody mAb 327 for 2 h, and collecting the immunocomplexes by addition of 50 μl rabbit anti-mouse IgG-Agarose. The pellets were washed four times with the lysis buffer and once with the assay buffer (20 mM Na_2HPO_4/NaH_2PO_4, pH 6.5, 100 mM KCl, 10 mM $MgCl_2$, 0.1% NP-40), resuspended in 100 μl of the assay buffer, and the reaction was started by adding 20 μCi of γ-^{32}P ATP (sp act 3,000 Ci/mmol, 10 mCi/ml) and cold ATP to a final concentration of 2 μM. The samples were incubated for 10 or 30 min at 25°C with gentle shaking every 10 min. Thereafter, the samples were washed with buffer A (47), resuspended in Laemmli's sample buffer and electrophoresed on 8% SDS-PAGE.

Western Blotting

The proteins resolved by SDS-PAGE were electrophoretically transferred on nitrocellulose. Immunoblotting with anti-phosphotyrosine antibodies was performed essentially as described by Kamps and Sefton (36). The nitrocellulose filters were incubated in blocking buffer (3% BSA, fraction V [Sigma Chemical Co.] in TN buffer [10 mM Tris, pH 7.2, 0.9% NaCl]) for 4 to 16 h. The filters were then incubated with affinity-purified polyclonal or monoclonal antibodies in blocking buffer for 2–4 h. The filters were rinsed twice with TN buffer, once with TN buffer containing 0.05% Nonidet P-40, and twice again with the TN buffer. The rabbit antibodies were detected by incubating the filters for 1 h with ^{125}I-protein-A (Amersham Corp. [Buckinghamshire, UK], 1 μCi/ml) in 10 ml of blocking buffer. For detection of mAbs, in turn, ^{125}I-labeled sheep anti-mouse IgG (Amersham Corp.) was used. The filters were washed with the TN buffer, and TN buffer containing 0.05% Nonidet P-40 as described above. Filters were exposed to Kodak XAR-2 film with an intensifying screen at −70°C.

For detection of GAP and PI 3-kinase, the immunoblot analyses were performed as above, using the appropriate rabbit polyclonal antisera and ^{125}I-protein-A or ^{125}I-labeled donkey anti-rabbit IgG (Amersham Corp., 1 μCi/ml) as a probe. All the analyses were verified by at least three independent experiments.

Antibodies

Affinity-purified polyclonal rabbit anti-v-*src* antibodies (52) were kindly provided by R. L. Erikson. Monoclonal anti-v-*src* antibody mAb 327 was from Oncogene Science Inc. (Manhasset, NY) and mAb 2–17 and the synthetic src peptide 2–17 from Quality Biotech Inc. (Camden, NJ). Affinity-purified rabbit anti-phosphotyrosine antibodies (36) were kindly donated by B. Sefton, and the monoclonal anti-phosphotyrosine antibodies 4G 10 and PT-66 were from Upstate Biotechnology, Inc. (Lake Placid, NY) and Sigma Chemical Co., respectively. Rabbit polyclonal anti-GAP antiserum (16) was generously given by T. Pawson, antiserum to tensin (13) by L. B. Chen and antibodies to calpactins I and II (65) by J. Glenney. Rabbit polyclonal antibodies to cdc 2 kinase (raised against the conserved carboxy terminal peptide of human cdc2) were kindly provided by D. Beach. Rabbit polyclonal anti-PI 3-kinase antiserum was from Upstate Biotechnology, Inc.

Results

Induction of Ornithine Decarboxylase by v-src

We and others have previously found that induction of transformation of chick embryo fibroblasts by infection with RSV

is associated with a marked increase in the activity of ODC (21, 25, 35). To study the significance of ODC induction for transformation we used here a rat fibroblast cell line (2R) that has a ts mutant LA339 derived from RSV B77 stably integrated into its genome (63). These cells are phenotypically normal when grown at the restrictive temperature (39.5°C) and become transformed at the permissive temperature (35°C).

Fig. 1 shows the induction of ODC activity in the 2R cells after shift from the restrictive temperature to the permissive one. The activity of ODC remained at low levels until 18–24 h, rose then sharply and persisted at high levels for at least 2 d. It is notable that no increase in ODC activity was observed after switching cultures of the parental normal Rat-1 fibroblasts from 39.5 to 35°C (data not shown). The basal ODC activity and the rise in ODC activity in response to the permissive temperature in the 2R cells could be totally blocked by treatment with DFMO, an irreversible inhibitor of ODC. No induction of ODC was either observed after the simultaneous addition of DFMO and putrescine, the reaction product of ODC (Fig. 1).

The transformation-associated increase in ODC activity could largely be accounted for a rise in the ODC mRNA content. This rise was not inhibited by DFMO (Fig. 1, *inset*). In addition, a two- to threefold increase in the half-life of ODC was seen 24 h after the cell transfer to the permissive temperature (data not shown). The mechanisms of the increase in ODC activity in response to v-*src* activation thus appear to be similar to those found in the c-Ha-*ras* oncogene-transformed NIH 3T3 cells (34).

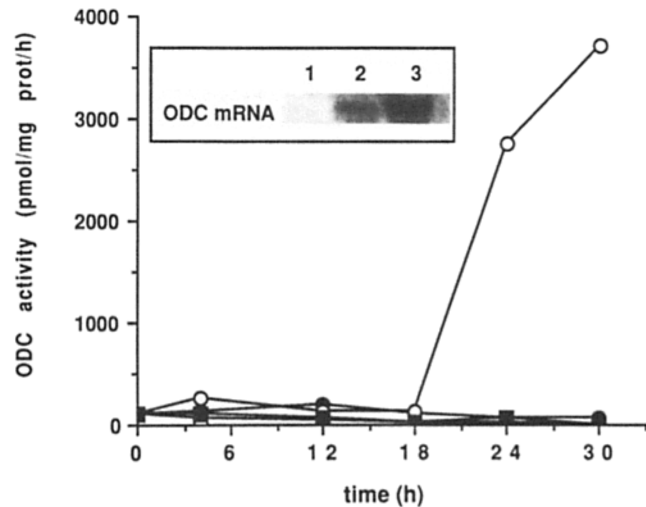


Figure 1. Time course of induction of ODC activity and the changes in ODC mRNA content in 2R cells after transfer to permissive temperature. (●) Cells grown at 39.5°C; (○) cells shifted from 39.5 to 35°C; (□) cells transferred to 35°C in the presence of DFMO; (■) cells shifted down to 35°C in the presence of DFMO and putrescine. (*Inset*) Northern blot analysis of ODC mRNA. (Lane *1*) Cells grown at 39.5°C; (lane *2*) cells shifted to 35°C; (lane *3*) cells shifted to 35°C in the presence of DFMO. 2R cells were cultured in RPMI-1640 medium supplemented with 5% FCS for 4 d to subconfluency. Thereafter, the cells were fed with fresh RPMI-1640 medium containing 5% dialyzed horse serum (prewarmed to 39.5°C), 2 mM DFMO and 20 μM putrescine were added to the indicated cultures, and the appropriate cultures were transferred to a 35°C incubator. The activity of ODC was determined using a 50 μM ornithine concentration. Cytoplasmic RNA was prepared from cells kept at the indicated temperatures for 24 h, electrophoresed (20-μg aliquots) on a 1% gel containing 6% formaldehyde, transferred to Hybond-*N* membrane and hybridized to a nick-translated mouse ODC cDNA probe. Control hybridization to the glyceraldehyde-3-phosphate dehydrogenase probe showed that its mRNA levels were slightly higher (≤twofold) in cells grown at 35°C than at 39.5°C, and unaffected by DFMO.

DFMO Inhibits v-src-induced Transformation

The 2R cells, which displayed a regular epithelioid morphology when grown at 39.5°C (Fig. 2 *a*), acquired typical features of neoplastic transformation (fusiform shape, growth in multilayers and subsequent rounding) within 24–36 h after transfer to 35°C (Fig. 2 *b*). Notably, this morphological transformation at 35°C was prevented in the presence of DFMO when the cells were cultured in polyamine-free medium (Fig. 2 *c*). The addition of putrescine counteracted the effect of DFMO and allowed cell transformation to occur (Fig. 2 *d*). DFMO did not affect the morphology of cells grown at the restrictive temperature.

The transformation was also reflected in the organization of the cytoskeleton. When grown at the restrictive temperature the 2R cells displayed a normal organized cytoskeleton of polymerized, filamentous actin (Fig. 2 *e*), while transformation at the permissive temperature for 36 h resulted in disappearance of the bundles (Fig. 2 *f*). In concert with the morphological findings, a normal filamentous organization of stress fibers was preserved in DFMO-treated cells cultivated at 35°C (Fig. 2 *g*), while addition of putrescine together with DFMO led to disintegration of the actin cables (Fig. 2 *h*).

DFMO Does Not Interfere with v-src Expression

Because inhibition of polyamine synthesis may interfere with protein synthesis (33), we investigated whether the block of transformation by DFMO was due to an inhibition of the expression of $pp60^{v\text{-}src}$. Fig. 3 shows the results of this study. Analyses of the synthesis of $pp60^{v\text{-}src}$ at 12, 18, and 24 h by [^{35}S]methionine pulse-labeling (1–3 h), immunoprecipitation and SDS-PAGE revealed that the expression of $pp60^{v\text{-}src}$ was increased at about 18 h after the temperature shift. However, this increase in $pp60^{v\text{-}src}$ synthesis was relatively small (and not always detected), suggesting that the temperature-sensitivity of the mutant v-*src* action is dependent on other mechanisms. In any case, DFMO did not inhibit the synthesis of the v-*src* oncoprotein at any of the time points studied (Fig. 3 *a*). The steady state levels of $pp60^{v\text{-}src}$ as determined by Western blotting were also found to be unaffected by DFMO (Fig. 3 *b*). It is also important to note that DFMO did not significantly affect the rate of total protein synthesis or DNA synthesis for 30–36 h of culture (data not shown).

As the transforming capability of $pp60^{v\text{-}src}$ is dependent on its intrinsic tyrosine kinase activity (17, 56), we examined the possible effect of DFMO treatment on the protein kinase activity of $pp60^{v\text{-}src}$ in the cells by an immune complex assay using the heavy chain of IgG as the substrate (47). However, treatment of the cells with DFMO for 24 h did not cause any inhibition of the in vitro Src kinase activity, which neither appeared to be temperature-sensitive, in agreement with earlier findings (A. Catling and E. K. Parkinson, personal communication). The autophosphorylation of $pp60^{v\text{-}src}$ was not affected by DFMO either (Fig. 4). In vitro labeling of the

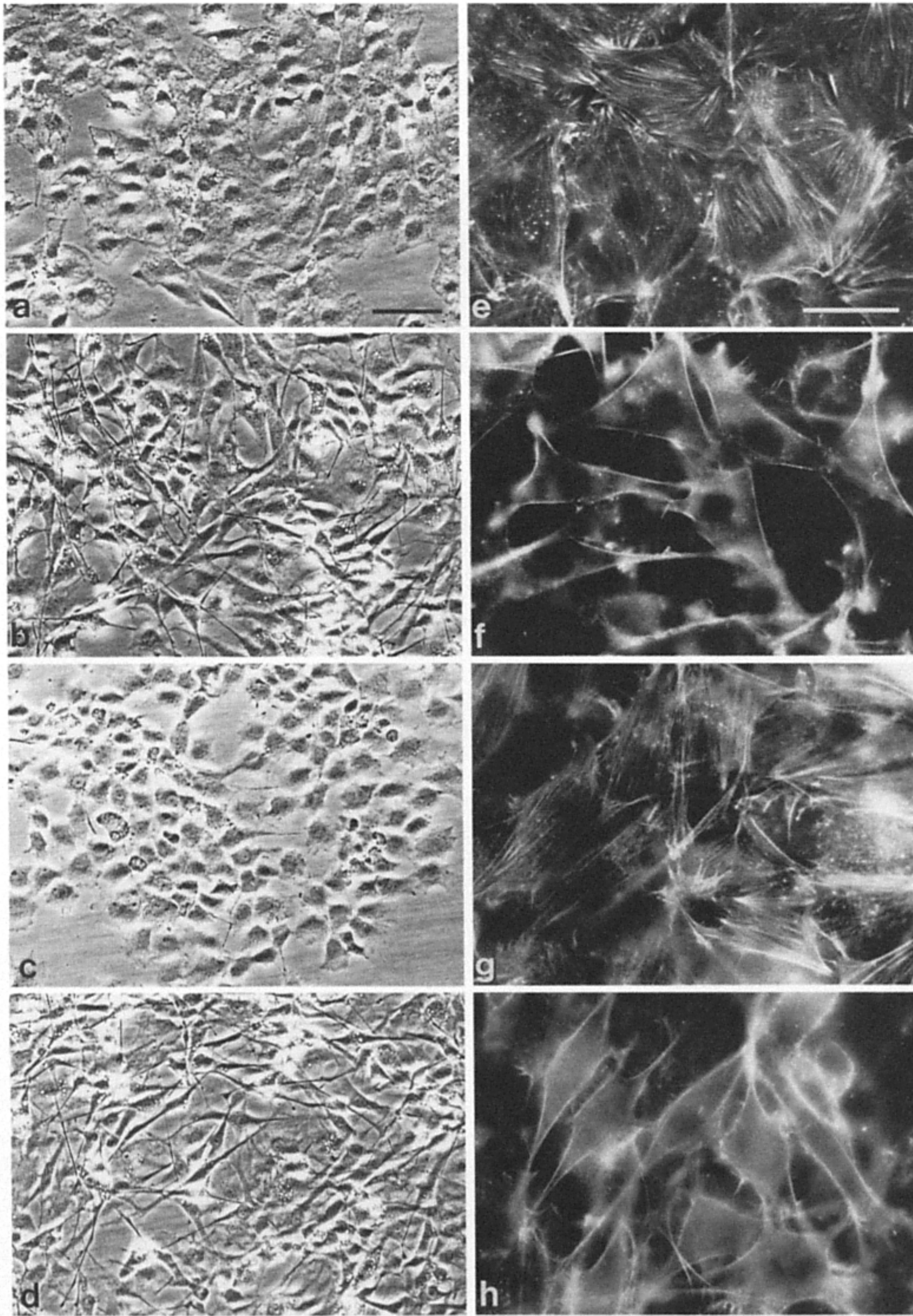
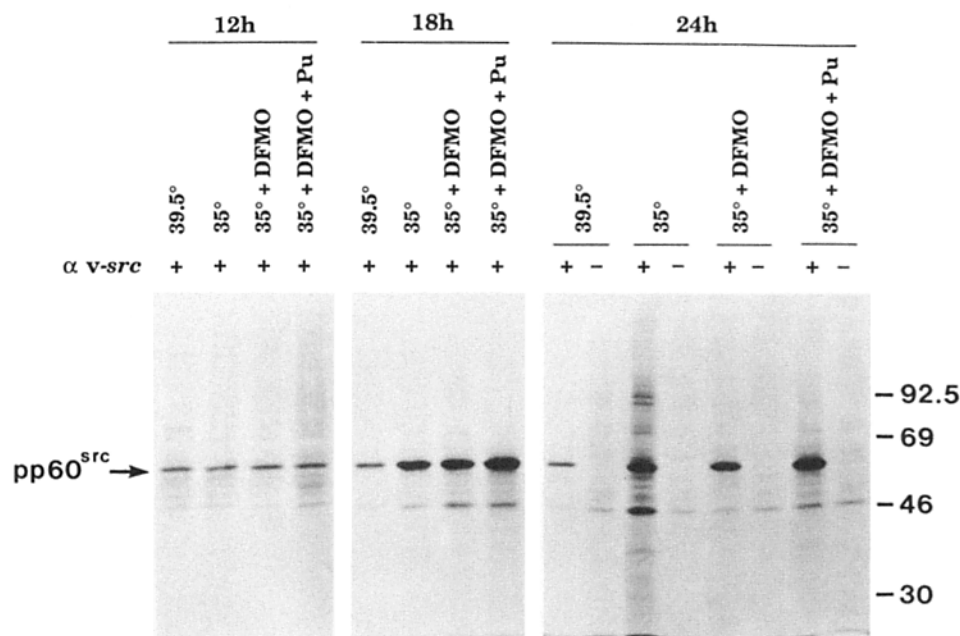


Figure 2. Effect of DFMO on the morphology and actin filament organization of the 2R cells after the temperature shift. (*a–d*) Phase contrast microphotographs of cell morphology; (*e–h*) immunofluorescence analysis of the actin microfilament organization. (*a* and *e*) Cells grown at 39.5°C; (*b* and *f*) cells shifted to 35°C; (*c* and *g*) cells shifted to 35°C in the presence of DFMO; (*d* and *h*) cells transferred to 35°C in the presence of DFMO and putrescine. The cells were cultured and exposed to DFMO and putrescine as described in the legend of Fig. 1. At 36 h after the temperature shift the cells were photographed in phase contrast microscopy. For visualization of actin the cells were fixed with paraformaldehyde, permeabilized with 0.05% NP-40 detergent and stained with rhodamine-conjugated phallacidin. Bars: (white) 20 μm; (black) 40 μm.

cell lysates with [γ-^{32}P] ATP also revealed that DFMO did not inhibit the pp60$^{v\text{-}src}$ kinase-catalyzed phosphorylations of the major 190-kD, 125–130-kD and 70–74-kD proteins and minor 60-kD (pp60$^{v\text{-}src}$) and 52-kD proteins described previously (36, 58) (data not shown). Although these proteins may not all represent the in vivo substrates of pp60$^{v\text{-}src}$ (36), it can be concluded that the *src* kinase stays active in the DFMO-treated cells.

DFMO Treatment Interferes with the In Vivo Protein Tyrosine Phosphorylation of Selected Substrates

Tyrosine phosphorylation of proteins with apparent M_r of 130–135 kD (27, 40), 120 kD (53), and 95 kD (40) has recently been found to correlate with v-*src*-induced transformation. It was thus of interest to see whether the inhibition of transformation of the 2R cells by DFMO was associated with changes in tyrosine phosphorylation of such proteins. Our Western blot analysis with affinity-purified antibodies to phosphotyrosine revealed that there were multiple proteins phosphorylated on tyrosine already at the restrictive temperature and that the temperature shift did not affect much the pattern of tyrosine phosphorylated proteins at 24 h of culture, which agrees with the earlier findings (7). However, we found that the phosphotyrosine content of some proteins was



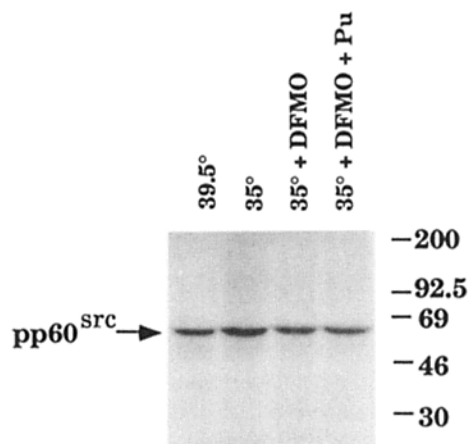


Figure 3. Effect of DFMO on the expression of pp60$^{v\text{-}src}$ in 2R cells. (*a*) Synthesis of pp60$^{v\text{-}src}$. The cells were cultured and treated with DFMO and putrescine for the indicated times as described in the legend to Fig. 1. The cells were preincubated for 30 min in minimal essential medium (MEM) containing 5% of the normal methionine concentration, and pulse-labeled with [^{35}S]methionine (100 μCi/ml) for 1 h. Detergent lysates (10 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride) of the cells were immunoprecipitated with normal rabbit serum (−) or affinity-purified polyclonal antibody to pp60$^{v\text{-}src}$, and the precipitates analyzed on 8% SDS-PAGE. The migration of the marker proteins ([^{14}C]methylated standards from Amersham Corp.) is shown on the right. (*b*) Steady state levels of pp60$^{v\text{-}src}$. The cells were treated as indicated for 24 h. Equal amounts of detergent lysates of the cells were then subjected to 8% SDS-PAGE and Western blotting, using the mAb p60$^{v\text{-}src}$ antibody (mAb 327) and ^{125}I-labeled sheep anti-mouse IgG (Amersham Corp.) as a probe.

increased after shift of the cells from the restrictive temperature to the permissive one. In repeated experiments, proteins with apparent molecular weights of 36 and 33 kD showed the most distinctive increase of tyrosine phosphorylation. This increase was, at least partially, sensitive to DFMO treatment (Fig. 5). The 36-kD protein is likely to be calpactin I (see below). The possibility that the 33-kD tyrosine phosphorylated protein might have been p34^{cdc2}, the central regulator of mitosis, was excluded by immunoprecipitation analysis of the cell lysates with specific antibodies to cdc2 kinase (unpublished data).

Polyamines Modulate the Phosphorylation/ Associations of Some Signaling Proteins

Ellis et al. (16) have recently found that the phosphorylation of *ras* GAP, the molecular weight of which is ~120 kD, correlates with transformation in rat cells expressing a mutant of v-*src* temperature sensitive for the kinase activity. To find out whether GAP was similarly phosphorylated on tyrosine in 2R cells, we harvested the cells at 20 h after the temperature shift (initial phase of transformation), precipitated the cell lysates with a specific antiserum to GAP and performed a Western blot analysis with affinity-purified antiphosphotyrosine antibodies. As illustrated in Fig. 6 *a*, in 2R cells GAP was phosphorylated on tyrosine already at the restrictive temperature, and shift of the cells to the permissive temperature resulted only in a minor increase in its tyrosine phosphorylation. Probing of a parallel filter with the anti-GAP antibodies revealed that similar and readily detectable amounts of GAP were precipitated from the different cell extracts. Interestingly, an additional anti-GAP-reactive protein of 50 kD (migrating below the IgG heavy chains) was found to coprecipitate in a transformation-specific manner (Fig. 6 *b*).

In v-*src* transformed cells, GAP is known to form independent complexes with two tyrosine-phosphorylated proteins of 62 and 190 kD (6, 16, 46), which have been suggested to be associated with transformation (6, 16). Studies with the

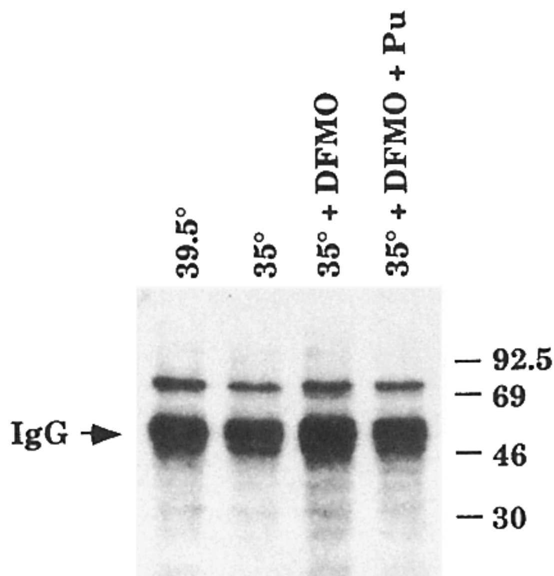


Figure 4. Treatment with DFMO does not interfere with the in vitro kinase activity of pp60$^{v\text{-}src}$. The cells were treated as indicated for 24 h. Lysates of the cells were immunoprecipitated with the monoclonal anti-p60$^{v\text{-}src}$ antibody (mAb 327) and the resulting immunocomplexes assayed for the kinase activity as described in Materials and Methods. The reaction products were separated on 8% SDS-PAGE. The autoradiogram shows the amount of radioactive phosphate incorporated into the heavy chain of the immunoglobulin. The migration of the molecular mass markers (Rainbow, Amersham Corp.) is indicated on the right.

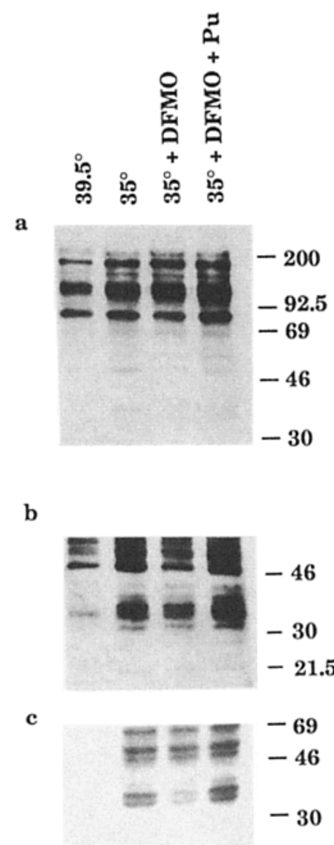


Figure 5. Anti-phosphotyrosine immunoblot analysis of proteins in 2R cells with or without DFMO treatment. (*a*) The cells were cultured and exposed to DFMO as in Fig. 1 for 24 h and then the cells were lysed in 0.5 ml 50 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM PMSF, 100 mM NaF and 1 mM sodium ortovanadate. Equivalent amounts of cellular proteins were resolved by SDS-PAGE and subjected to Western blotting using affinity purified antibodies to phosphotyrosine as described in Materials and Methods. The phosphotyrosine-containing proteins were detected by incubation with ^{125}I-protein A. The autoradiogram was exposed for 4 h. A shorter exposure revealed that the 120–130-kD broad band consists of at least two proteins and that the tyrosine phosphorylation of a 120-kD protein changed in part in a transformation-related manner. *b* represents a longer exposure from the lower part of the same gel. (*c*) Lower molecular weight tyrosine phosphorylated proteins from another experiment. Relative molecular mass markers (Rainbow, Amersham Corp.) are indicated in kilodaltons. Similar results were obtained in over 20 independent experiments.

v-*src* mutants having alterations in the SH2 domain have, however, indicated that the tyrosine phosphorylation of p190 does not correlate with transformation (45). Our anti-GAP immune complexes also revealed the presence of the tyrosine phosphorylated proteins p190 and p62/64 (doublet) in 2R cells (Fig. 6 *a*). It is notable, however, that both the p190 and the most prominently tyrosine-phosphorylated protein p62/64 were not only associated with GAP in the transformed cells, but also in the untransformed cells grown at nonpermissive temperature. The anti-GAP precipitates also contained additional tyrosine phosphoproteins. Among these proteins, the most prominent was a 57-kD protein, which appeared to be heavily tyrosine phosphorylated irrespective of the state of transformation. Intriguingly, a minor tyrosine phosphorylated protein with a relative molecular mass of 42 kD was found to be associated with GAP in a transformation-specific manner (Fig. 6 *a*). The protein(s) with an approximate molecular weight of 150–160 kD was found to show interexperimental variations in its phosphorylation, and could be a breakdown product of p190 (see reference 46) or a novel GAP-binding protein. Recently, Davis et al. (13) presented an attractive hypothesis that the actin-binding protein tensin, possessing an SH2 domain, might serve as a nucleus around which the signaling molecules are gathered. As the antibodies to tensin further recognize two closely related proteins of 150 and 200 kD (13), this prompted us to look at whether the GAP-associated proteins p150 and p190 comprised tensin. However, our blottings of the anti-GAP immunoprecipitates with antibodies to tensin have repeatedly given negative results.

Transformation by v-*src* appears also to be associated with an elevation of PI 3-kinase activity. The precise role played by the PI-3 kinase which phosphorylates phosphatidylinositols at D-3 position of the inositol ring is still unknown, but it has been suggested to be involved in the regulation of cell signaling (10) and reorganization of the actin filaments (15). Here we found that the tyrosine phosphorylation of the smaller, regulatory subunit (85 kD) of PI 3-kinase (10) was markedly increased upon shift of the 2R cells from the nonpermissive to permissive temperature and that DFMO slightly inhibited this increase (a 30–40% inhibition after normalization to the amounts of p85 immunoprecipitated) (Fig. 7). Moreover, a tyrosine phosphorylated protein of ~110 kD was coprecipitated with the regulatory subunit of PI 3-kinase in a polyamine- and transformation-dependent manner (Fig. 7).

Further support for polyamines promoting the phosphorylation and interaction of proteins was obtained in studies on calpactins I and II. Calpactins are a family of phospholipid- and Ca^{2+}-binding proteins that associate with cytoskeleton (12), and calpactin I (also called p36) is one of the major substrates of pp60$^{v\text{-}src}$. Fig. 8 shows that the total phosphoryation of both calpactin I and calpactin II changed in a transformation-specific manner in the 2R cells. The immunoprecipitates from transformed cells displayed several additional phosphoproteins as compared with the normal cells, and treatment with DFMO reduced the amount of the co-

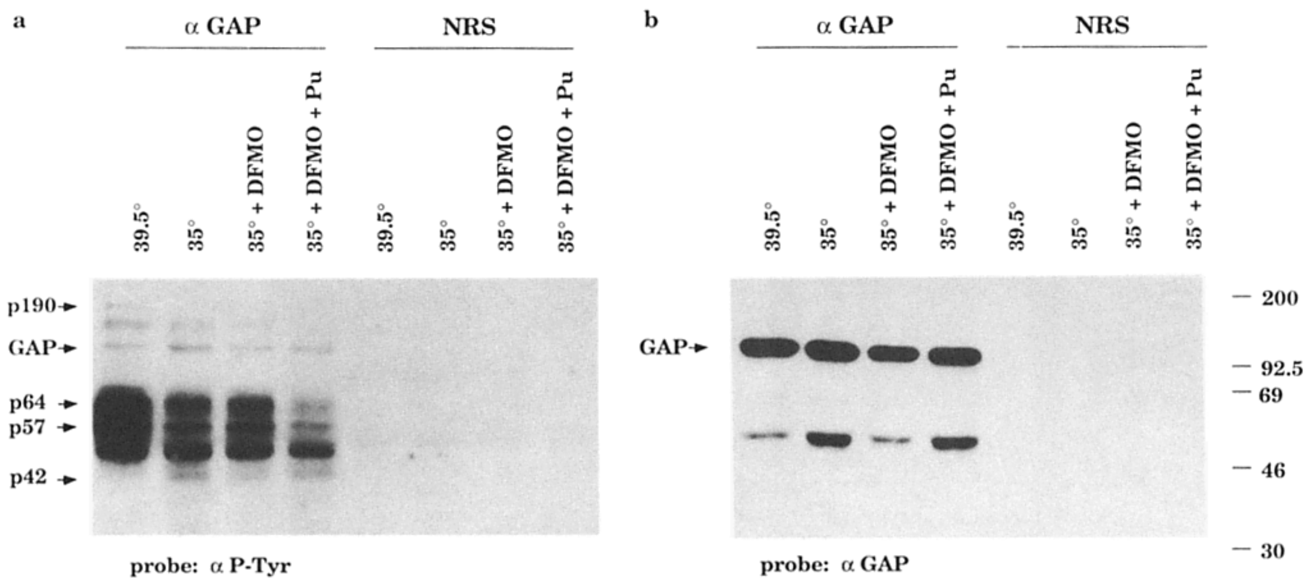


Figure 6. Tyrosine phosphorylation of GAP and GAP-associated proteins in cells grown at the restrictive and permissive temperatures with or without DFMO. The cells were treated as indicated for 20 h, and GAP was immunoprecipitated from the cell lysates with rabbit anti-GAP antibodies. The immunocomplexes were then subjected to SDS-PAGE and the resolved proteins were transferred to nitrocellulose and immunoblotted either with affinity-purified anti-phosphotyrosine antibodies (*a*) or anti-GAP antiserum (*b*), using ^{125}I-labeled protein A for detection. The position of GAP is indicated by an arrow. As seen, slightly less GAP is immunoprecipitated from the DFMO-treated cells, which must be taken into consideration in evaluating the data. The bands with *Mr* 50–55 kD arise from immunoglobulin heavy chains (but may, of course, also comprise other proteins). Note that in *b*, the immunoprecipitates of transformed cells contain an additional 50-kD protein reactive with the anti-GAP antibodies. Similar results were obtained in six repeated experiments.

precipitating proteins (Fig. 8). It should be noted here that, in several repeated SDS-PAGE analyses no appreciable increase in the overall phosphorylation of proteins was observed in v-*src* transformed cells and that DFMO did not interfere with the total protein phosphorylation (data not shown). A distinct 60-kD phosphoprotein, which might be pp60$^{v\text{-}src}$, was enriched in the precipitates obtained with both anti-calpactin antibodies (Fig. 8). In addition, many

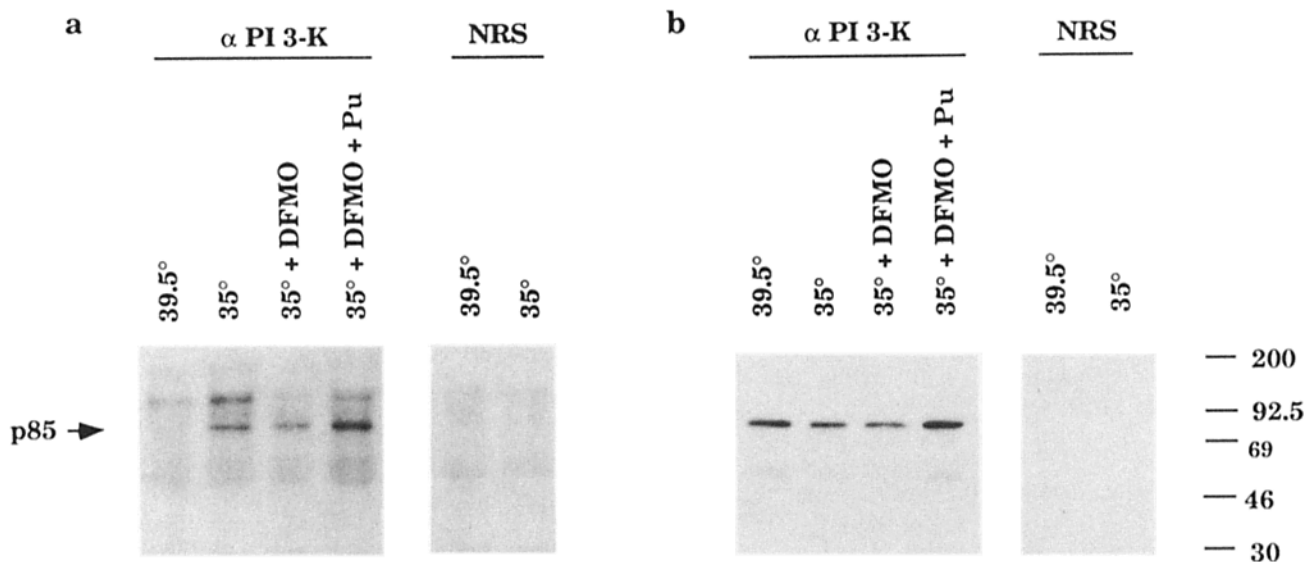


Figure 7. Tyrosine phosphorylation of the smaller subunit of phosphatidylinositol 3-kinase is increased in response to v-*src*-induced transformation. The cells were treated as indicated for 24 h, and PI 3-kinase was immunoprecipitated from the cell lysates with rabbit anti-rat PI 3-kinase antiserum. The immunoprecipitates were washed five times with the lysis buffer and subjected to SDS-PAGE. The fractionated proteins were transferred to nitrocellulose and blotted either with monoclonal anti-phosphotyrosine antibodies (4G 10) (*a*) and anti-PI 3-kinase antiserum (*b*), using ^{125}I-labeled sheep anti-mouse IgG and ^{125}I-labeled protein A, respectively, for detection. The position of the smaller subunit (85 kD) of PI 3-kinase is indicated by an arrow. As seen, there is somewhat less PI 3-kinase protein in the DFMO lane, which should be taken into consideration in evaluating the data. Note that a 110-kD tyrosine phosphorylated protein, that could be the catalytic subunit of PI 3-kinase, is co-precipitated in a transformation-specific manner.

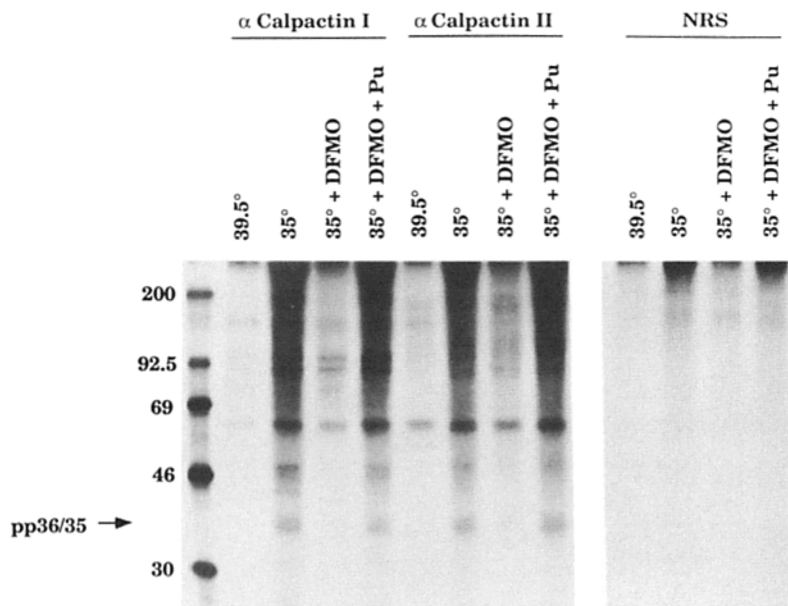


Figure 8. The phosphorylation of calpactin I and II shows transformation-related changes. The cells were labeled with [^{32}P]orthophosphate as described in Materials and Methods. Equal amounts of radiolabeled cell lysates (counts/min) were immunoprecipitated with specific antibodies to calpactin I and II, and normal rabbit serum, whereafter the immunocomplexes were subjected to SDS-PAGE, and the gel was autoradiographed.

other of the proteins coprecipitating with the antisera to calpactin I and II are common, but some are specific ones. That several proteins coprecipitate with antibodies to calpactins I and II has also been observed in other studies (31, 55), but the identities of the proteins are not known. One possibility is that they are membrane/cytoskeletal proteins, with which the calpactins are known to associate.

pp130 Associates with pp60$^{v\text{-}src}$ in a Transformation-related Manner

Having established that GAP forms multiple complexes, we asked whether GAP associates with the activated pp60$^{v\text{-}src}$, and if so, whether polyamines are needed for the interaction. Lysates from cells cultured at 39.5 and 35°C without or with DFMO were immunoprecipitated with mAbs to v-*src* peptide, the precipitated proteins resolved by SDS-PAGE and analyzed by Western blotting with antiserum to GAP. As illustrated in Fig. 9, GAP was coprecipitated with pp60$^{v\text{-}src}$ after shifting the cells from the nonpermissive to permissive temperature and treatment with DFMO abolished the coprecipitation of GAP with Src. However, the amounts of GAP co-immunoprecipitated were very low, $<$0.5% of the total GAP content (data not shown).

We have also investigated whether, in analogy to the situation with the ligand-activated PDGF and EGF receptors, several key signaling molecules might become associated with the activated pp60$^{v\text{-}src}$. The best studied such a molecule is PLC γ1. We have, however, failed to detect any significant amounts of PLC γ1 in the anti-pp60$^{v\text{-}src}$ immunoprecipitates by immunoblotting analyses (data not shown). In concert with this, it has been found lately that PLC γ1 does not become appreciably tyrosine phosphorylated in cells transformed by pp60$^{v\text{-}src}$ (62, our unpublished data).

In chicken embryo cells, it has been found that pp60$^{c\text{-}src}$ associates with two major tyrosine phosphorylated proteins of 130 and 110 kD, and that these associations, which depend on the SH2 and SH3 domains, respectively, correlate in part with transformation (38). In rat 2R cells, our immunoblotting analyses of the anti-pp60$^{v\text{-}src}$ immunoprecipitates with anti-phosphotyrosine antibodies likewise revealed three major tyrosine phosphorylated proteins, with apparent molecular masses of 130, 110, and 60 kD (pp60$^{v\text{-}src}$ itself) and additional fainter bands (Fig. 10). The abundant tyrosine phosphorylated proteins p130 and p110 were not specific to transformed cells, because an association with pp60$^{v\text{-}src}$ was

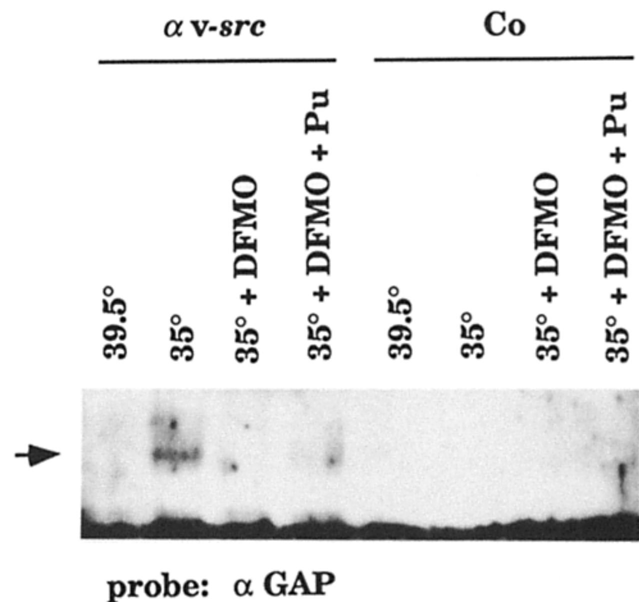


Figure 9. GAP is co-immunoprecipitated with pp60$^{v\text{-}src}$ in a DFMO-sensitive manner. The cells were cultured with or without DFMO at the indicated temperatures for 24 h. The proteins from cell lysates were immunoprecipitated with the monoclonal anti-p60$^{v\text{-}src}$ antibody (mAb 2–17). The immunoprecipitates were washed five times with the lysis buffer, whereafter the bound proteins were resolved by SDS-PAGE, transferred to nitrocellulose and blotted with anti-GAP antiserum using ^{125}I-labeled donkey anti-rabbit IgG (Amersham Corp., 1 mCi/ml) for detection. Although putrescine did not fully reverse the effect of DFMO in this experiment this was confirmed in another experiment. A similar result was obtained using ^{125}I-protein-A as a probe. The low molecular weight bands of 50 kD at the bottom are derived from immunoglobulin heavy chains.

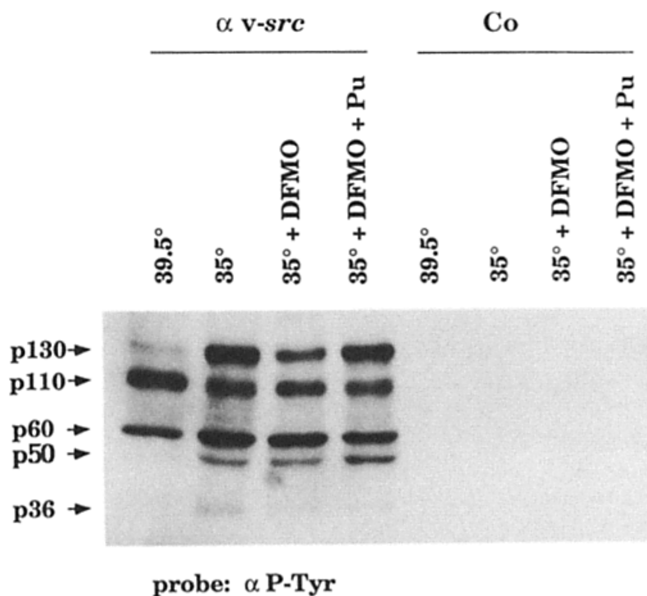


Figure 10. Association of tyrosine phosphorylated proteins with pp60$^{v\text{-}src}$. The cells were cultured as indicated for 24 h, whereafter the cell lysates were immunoprecipitated with the monoclonal anti-p60$^{v\text{-}src}$ antibody (mAb 2–17). Immunoprecipitates were then fractioned by SDS-PAGE, and analyzed by immunoblotting with affinity-purified anti-phosphotyrosine antibodies, using ^{125}I-labeled protein A as a probe.

also seen in untransformed cells. However, the association of pp130, but not of pp110, was increased in response to v-*src* activation in a transformation-related manner. Further, it can be seen in Fig. 10 that the tyrosine phosphorylated proteins with relative molecular masses of ∼80–85 kD (minor doublet band) and 36 kD (tentatively identified as calpactin I by immunoblotting) showed a transformation-dependent association with pp60$^{v\text{-}src}$. That these tyrosine phosphorylated proteins were specifically associated with pp60$^{v\text{-}src}$ was ascertained by using two Src-specific monoclonal antibodies, mAb 2–17 (with peptide blocking) and 327, and by immunoprecipitations with several irrelevant monoclonal antibodies (e.g., the anti-Ras mAb Y13-259) (data not shown) and normal rabbit serum.

Discussion

Transformation by pp60$^{v\text{-}src}$ Requires Polyamines

The data presented here show that ODC plays a crucial role in the v-*src*-induced transformation process of rat 2R fibroblasts. The fact that the amount of ODC mRNA is increased implies that v-Src tyrosine kinase does not directly regulate ODC, but that the v-Src initiated signal has to go into the nucleus to trigger ODC expression. Indeed, our transient transfection assays employing ODC gene promoter-chloramphenicol acetyltransferase constructs indicate that the transcription of the ODC gene is increased (both in the absence or presence of DFMO) upon v-*src* activation in R2 cells, although the magnitude of this transcriptional increase appears to be less than the rise in ODC mRNA (unpublished data). However, the fact that the cells showed transformation in the presence of DFMO and putrescine without activation of ODC indicates that it is not the rise in ODC per se, but the sufficient availability of polyamines which is essential for neoplastic transformation. Of the different polyamines, putrescine appears to accumulate most during the transformation process (3, 35, and references therein). We and others have previously reported that DFMO does not interfere with the transformation of chick embryo fibroblasts infected with RSV (3, 35), but under those complex culture conditions DFMO did not fully block the increase in cellular polyamines. Here we found that it is essential to use polyamine-free cell culture conditions to see the inhibitory effect of DFMO on transformation, as mammalian cells have an active transport system for polyamines which is activated in response to growth stimulation, oncogene activation and inhibition of polyamine biosynthesis (3, 48), and counteracts the polyamine-depleting effect of DFMO.

Polyamines Modulate the Phosphotyrosine Content of Some Proteins

A large number of proteins, 40–50 bands, has been shown to become phosphorylated on tyrosine in v-*src*-transformed cells (36). Therefore, it has proved difficult to identify which of these phosphorylations are necessary and sufficient for triggering of cell transformation. As we found DFMO to block transformation of 2R cells by the ts339 v-*src* mutant, it enabled us to evaluate the role of protein tyrosine phosphorylation in the transformation process. Brady et al. (7) have found that infection of NIH 3T3 cells with the ts339 *src* gene cloned into a retroviral expression vector resulted in an elevation of tyrosine phosphoproteins, but they failed to see any difference in the amount or pattern of the tyrosine phosphoproteins in the cells grown at the permissive or restrictive temperature. However, we found that the ts339 *src* mutant, the tyrosine kinase activity of which is not temperature-sensitive in vitro, caused an increase in tyrosine phosphorylation of a few proteins upon shift of the 2R cells down to the permissive temperature. The basis for the different results might be that the pp60$^{v\text{-}src}$ encoded by the ts339 *src* has only a subtly altered specificity to exclude only a limited number of critical substrates at the restrictive temperature (7), which makes their detection harder. This is, however, an advantage in attempts to identify the true transformation-relevant targets.

Our Western blottings with affinity-purified anti-phosphotyrosine antibodies showed that the tyrosine phosphorylation of the vast majority of proteins does not correlate with transformation, which corrobates the findings on pp60$^{v\text{-}src}$ mutants defective in membrane anchoring and transformation (37). We found, however, that the tyrosine phosphorylation of proteins with apparent molecular masses of 33, 36, and 110 kD (a protein coprecipitating with antibodies to the 85-kD subunit of PI 3-kinase) and in part that of the regulatory subunit (p85) of PI 3-kinase correlated with transformation. Tyrosine phosphorylation of several proteins have previously been suggested to be transformation-relevant (23, 36, 53), but the PI 3-kinase subunit(s) and 33-kD protein are novel in this respect. However, the tyrosine phosphorylation of the p85 subunit of PI 3-kinase alone is not likely to be sufficient for transformation, as the inhibition of transformation by DFMO was only associated with a 30–40% reduction in the p85 tyrosine phosphorylation. It remains interesting to see whether our tyrosine phosphorylated 33 kD protein could be the cyclin-dependent kinase-2 (p33^{cdk2}) involved in the con-

trol of G1/S phase transition or perhaps the recently identified 32-kD "receptor" for the myristoylated pp60$^{v\text{-}src}$ (51) (although there is no evidence yet for the latter one being a tyrosine phosphorylated protein).

Cytoskeletal Rearrangements May Be Regulated by Polyamine-dependent Phosphorylations

Our studies revealed that the total phosphorylation of calpactin I (p36) was changed in a transformation-specific manner in the 2R cells. Whether the 36-kD protein displaying transformation-dependent tyrosine phosphorylation changes in the anti-phosphotyrosine immunoblottings represents calpactin I is currently under investigation. Previously, the tyrosine phosphorylation of calpactin I has been found to correlate with *src*-induced transformation in many studies, though not in all cases (37). We found also that the overall phosphorylation of calpactin II changed in a transformation-related manner. There is no indication that calpactin II would become tyrosine phosphorylated in v-*src* transformed cells (T. Hunter, personal communication), and thus it is not likely a component of the transformation-specific tyrosine-phosphorylated 36 kD band. However, it should be kept in mind that calpactin II is an identified substrate for the EGF receptor tyrosine kinase (55) that becomes activated upon v-*src* transformation (62). The physiological function of calpactins is still unsettled, but they are supposed to be important for cytoskeletal organization. As they bind to actin, spectrin and membrane phospholipids (e.g., phosphatidyl inositols) and show submembranous localization, it is possible that they control the interactions between the plasma membrane and cytoskeleton (32 and references therein).

Loss of the filamentous actin is one of the earliest morphological changes in pp60$^{v\text{-}src}$-transformed cells (5, 57). As the depolymerization of actin was prevented by DFMO treatment, it is tempting to speculate that polyamines might play a role in the organization of the actin filaments through their effect on the phosphorylation of calpactins and other proteins associating with, or being integral components of the cytoskeleton. In favor of this idea, we have found that the phosphorylation of vinculin, which is a substrate for pp60$^{v\text{-}src}$ (57) also changes in a polyamine- and transformation-dependent manner (our unpublished data). Moreover, our recent results indicate that the treatment with DFMO interferes with the tyrosine phosphorylation of a number of proteins present in the cytoskeletal fraction (our unpublished data). That the assembly of cytoskeleton is regulated by tyrosine phosphorylation is also evidenced by recent studies with inhibitors of tyrosine kinases (43). In addition, there are cytoskeletal phosphotyrosine phosphatases, which may regulate the integrity of cytoskeleton (24, 64).

Because polyamines are intracellular cations, which show a rapid increase upon induction of cell growth and transformation, and display a high affinity for acidic phospholipids, there is a possibility that polyamines (like Ca^{2+}) might regulate the association of calpactins and other substrates of pp60$^{v\text{-}src}$ with the membrane phospholipids and cortical cytoskeleton. Hence, polyamines could be involved in the regulation of the linkage of the cytoskeleton to the plasma membrane. In any case, the present data clearly demonstrate that the organization of the stress fibers is directly or indirectly controlled by the changes in ODC and polyamine levels. Moreover, there are also previous studies suggesting a role for polyamines in the organization of actin network (4, 49).

Does the Assembly of Signaling Complexes Need Polyamines?

*Ras*GAP, which regulates the intrinsic GTPase activity of p21^{ras} (reviewed in 26), is known to become associated with other proteins upon activation of normal and oncogenic tyrosine kinases (46). In v-*src* transformed cells, the majority of GAP complexes with p190 in the cytosol, and a minor fraction associates with p62 and is translocated to the plasma membrane (16, 46). Ellis et al. (16) found that GAP becomes phosphorylated both on tyrosine and serine residues upon induction of transformation of rat-2 cells by v-*src*. Most of the phosphorylation is, however, found on serine residues, and only 3–4% of GAP is tyrosine phosphorylated in these v-*src* transformed rat cells (46). In 2R cells, GAP was found to be tyrosine phosphorylated already at the restrictive temperature, but showed an additional small increase in phosphorylation upon the temperature downshift. The significance of this minor increase for transformation is, however, hard to envision, unless it serves as a trigger for subsequent phosphorylations. In support of this possibility, we have found that the total phosphorylation of GAP is increased substantially more than its tyrosine phosphorylation in the cells upon transformation (unpublished data).

The phosphorylation of p62/64 on tyrosine and its coincident association with GAP have earlier been found to correlate with transformation by pp60$^{v\text{-}src}$ (6, 16). However, we found repeatedly that p62/64 (consisting of at least two forms of the same protein or two separate proteins) was highly tyrosine phosphorylated and complexed with GAP also in untransformed 2R cells grown at the restrictive temperature. This indicates that the tyrosine phosphorylation and association of p62 alone is not sufficient for transformation. Similarly, the tyrosine phosphorylation of the new 57-kD GAP-associating protein, which may be identical to the 55-kD protein recently described (8), did not appear to correlate with transformation. Our immunoprecipitation analyses disclosed, however, a novel GAP-associated protein of 42 kD in size, which displayed a transformation-dependent change in its tyrosine phosphorylation and might thus be relevant for transformation. Studies are underway to see whether this protein could be a component of the mitogen-activated protein (MAP) kinase cascade. Our recent studies indicate that the tyrosine phosphorylation of MAP2 kinase correlates with the v-*src* induced transformation (unpublished data).

Our results further indicate that GAP may become associated with the activated pp60$^{v\text{-}src}$ (or a complex containing pp60$^{v\text{-}src}$) in a DFMO-sensitive and transformation-related manner. Recently, Brott et al. (9) have likewise found GAP to associate with pp60$^{v\text{-}src}$ and with normal p60$^{c\text{-}src}$ as well. However, only the immunoprecipitates of pp60$^{v\text{-}src}$ appeared to catalyze tyrosine phosphorylation of GAP (9). Thus, these data could give a reason to hypothesize that a stable association with, and constitutive phosphorylation of GAP by pp60$^{v\text{-}src}$ is a prerequisite for transformation. This does not, however, look probable as only a very small fraction (<0.5%) of GAP is coprecipitated with pp60$^{v\text{-}src}$ under the cell lysis conditions used (unpublished data). Moreover, in converse experiments we have repeatedly failed to see any

significant amounts of pp60$^{v\text{-}src}$ in the anti-GAP immunoprecipitates (unpublished data), in agreement with a recent study on v-*src* transformed NIH 3T3 cells (50).

We found the tyrosine phosphorylated proteins of 130, 80/85 and 36-kD (apparently calpactin I) to physically associate with pp60$^{v\text{-}src}$ in a manner correlating with transformation. Similarly, a tyrosine phosphoprotein(s) of 130 kD has also previously been reported to associate with activated pp60$^{v\text{-}src}$ and implicated to be important for transformation (38, 53, 54). We have identified our pp130 protein as the same protein as described by J. T. Parsons and co-workers (38) (our unpublished data). What are then the novel 80–85-kD protein(s) coprecipitating with Src? One candidate might be the 85-kD subunit of PI 3-kinase, as we found it to show transformation-related changes in tyrosine phosphorylation and the PI 3-kinase activity is known to co-precipitate with pp60$^{v\text{-}src}$ (19).

Immunoprecipitation analyses of the p85 subunit of PI 3-kinase also revealed a tyrosine phosphorylated protein of ~110 kD that was specifically coprecipitated in a polyamine-dependent and transformation-related manner. Recently, a protein of similar size has also been found to coprecipitate with p85 in BALB/c 3T3 fibroblasts stimulated with PDGF (39). It is highly possible that the 110-kD protein represents the larger, catalytically active subunit of PI 3-kinase (39). Thereby, the formation of an active PI 3-kinase complex (or its tyrosine phosphorylation-promoted interactions) could be relevant for transformation. Moreover, it has been found that the levels of PI 3-kinase products correlate with transformation (42).

In conclusion, the data presented in this study indicate that polyamines are essential for the transforming activity of pp60$^{v\text{-}src}$. Tyrosine phosphorylation of some protein(s) must evidently be important in the signaling process leading to cell transformation by pp60$^{v\text{-}src}$, but the results with 2R cells show that the tyrosine phosphorylation per se of most of the proteins, including *ras*GAP and the GAP-associated proteins p190 and p62/64 is not relevant for the transformed state. Our study revealed, however, some proteins, whose tyrosine phosphorylation and/or association with other proteins correlated with transformation, like that of the regulatory (and tentatively the catalytic) subunit of PI 3-kinase, 33–36-kD proteins, the *ras*GAP-associated p42 and the pp60$^{v\text{-}src}$-associated p130. The fact that the blocking of cell transformation by DFMO was not associated with a total inhibition of the tyrosine phosphorylation/association of these proteins could be explained by a requirement for a certain threshold level of phosphorylation for transformation. However, it is perhaps more likely that polyamine depletion also interferes with other steps further downstream in the signaling pathway. In future experiments, it will be of particular interest to see whether the polyamines also affect the serine/threonine phosphorylation of the phosphotyrosine proteins studied and thereby contribute to the observed changes in protein associations, mediated by the SH2/SH3 domains (1, 14, 20) or unknown protein motifs. Nevertheless, our recent studies with another ts RSV mutant, the Rat-1 ts LA29 cells, which, in contrast to the 2R cells, display a marked temperature-dependent increase in protein phosphorylation on tyrosyl residues, disclose that DFMO can quite effectively inhibit the protein tyrosine phosphorylation (2). Finally, our data lend support to the idea that polyamines control either directly or indirectly (by modulating the phosphorylation of appropriate proteins, like PI 3-kinase and calpactins), the actin filament rearrangements.

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