Tyrosine Phosphorylations In Vivo Associated with *v-fms* Transformation

DEBORAH K. MORRISON,¹ PHILIP J. BROWNING,² MORRIS F. WHITE,^{3,4} AND THOMAS M. ROBERTS^{1*}

Dana-Farber Cancer Institute and Department of Pathology,¹ Research Division, Joslin Diabetes Center,³ and Department of Medicine,⁴ Harvard Medical School, Boston, Massachusetts 02115, and National Institutes of Health, Bethesda, Maryland 20892²

Received 28 July 1987/Accepted 9 October 1987

The role of tyrosine-specific phosphorylation in *v-fms*-mediated transformation was examined by immunoblotting techniques together with a high-affinity antibody that is specific for phosphotyrosine. This antiphosphotyrosine antibody detected phosphorylated tyrosine residues on the gp140^{*v-fms*} molecule, but not gp180^{*v-fms*} or gp120^{*v-fms*}, in *v-fms*-transformed cells. This antibody also identified a number of cellular proteins that were either newly phosphorylated on tyrosine residues or showed enhanced phosphorylation on tyrosine residues as a result of *v-fms* transformation. However, the substrates of the *v-fms*-induced tyrosine kinase activity were not the characterized pp60^{*v-src*} substrates. The phosphorylation of some of these cellular proteins and of the gp140^{*fms*} molecule was found to correlate with the ability of *v-fms/c-fms* hybrids to transform cells. In addition, immunoblotting with the phosphotyrosine antibody allowed a comparison to be made of the substrates phosphorylated on tyrosine residues in various transformed cell lines. This study indicates that the pattern of tyrosine phosphorylation in *v-fms*-transformed cells is strikingly similar to that in *v-sis*-transformed cells.

Phosphorylation of proteins on tyrosine residues provides an ideal mechanism to elicit major changes in cellular growth and metabolism. Several growth factor receptors, such as those for platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulin, and macrophage-specific colony-stimulating factor (CSF-1), have an associated tyrosine kinase activity as assayed in vitro (5, 37). In each case, binding of the ligand stimulates the tyrosine kinase activity of the receptor. In this manner the tyrosine kinase activity may function to transmit an exogenous signal to multiple cellular targets by catalyzing phosphorylation on tyrosine residues and thereby activating growth-regulatory pathways. Interestingly, a number of retroviruses which induce continuous cellular proliferation encode transforming proteins with an associated tyrosine kinase activity (6, 32). If the proliferation of cells is controlled by the transient activation of tyrosine kinase activity, then constitutive activation of this enzymatic activity may explain how these oncogene products cause the unlimited growth of cells in culture. *v-fms*, the transforming gene of the McDonough strain of feline sarcoma virus (SM-FeSV), is an example of such an oncogene with an associated tyrosine kinase activity (3, 22). Furthermore, the *v-fms* cellular homolog (*c-fms*) is biochemically, structurally, and functionally related, if not identical, to the tyrosine kinase receptor for CSF-1 (7, 10, 33).

The *v-fms* gene was formed by an in-frame insertion of *c-fms* sequences into *gag* sequences derived from feline leukemia virus (10, 12, 14). The primary translation product is a fusion protein which is glycosylated cotranslationally in the endoplasmic reticulum to yield a 180-kilodalton (kDa) glycoprotein, gp180^{*v-fms*} (1, 2). This molecule is proteolytically cleaved to form an amino-terminal *gag* fragment (p55^{*gag*}) and a carboxy-terminal *v-fms*-encoded glycoprotein (gp120^{*v-fms*}) (4, 30, 36). Some of the gp120^{*v-fms*} molecules are transported through the Golgi complex for further carbohy-

drate processing before translocation to the plasma membrane, where they are detected as mature gp140^{*v-fms*} (1, 2). The gp140^{*v-fms*} is oriented at the cell surface with its glycosylated amino-terminal domain outside the cell and its carboxyl-terminal domain in the cytoplasm, with the two domains separated by a single 25-amino-acid membrane-spanning region (26). Cell surface expression of the mature gp140^{*v-fms*} is essential for cellular transformation (24, 29). The gp140^{*v-fms*} retains an intact extracellular ligand-binding domain, so that cells transformed by *v-fms* express glycoproteins at the cell surface which will bind CSF-1 (31, 33). This observation raised the possibility that *v-fms* transformation might reflect retroviral transduction of a CSF-1 receptor gene into mesenchymal cells that synthesize the CSF-1 growth factor. Although fibroblasts susceptible to transformation by *v-fms* do produce CSF-1, *v-fms*-mediated transformation does not depend on an exogenous source of the growth factor, and neutralizing antibodies to CSF-1 do not affect the transformed phenotype (31). In addition, expression of the *c-fms* gene in fibroblasts does not induce transformation (28). These findings indicate that *v-fms* transformation is not simply a result of autocrine stimulation, but rather is mediated by a CSF-1-independent mechanism.

The cytoplasmic carboxy-terminal domain of both *v-fms* and *c-fms* shows amino acid sequence homology with pp60^{*v-src*} and other enzymes of the tyrosine kinase gene family (10, 14). Genetic analysis demonstrates that the tyrosine kinase activity of pp60^{*v-src*} and pp60^{*c-src*} and the state of the tyrosine phosphorylation of these proteins in vivo correlate with their ability to transform cells (8, 18, 20, 25). However, such a correlation has not been established for the *v-fms* product. Immune complexes containing *v-fms*-coded molecules exhibit an associated tyrosine-specific protein kinase activity that phosphorylates all three *v-fms* glycoproteins in vitro (3, 29, 33). In previous reports examining the phosphorylation state of gp140^{*v-fms*} and gp120^{*v-fms*} in vivo, Woolford et al. found trace amounts of phosphotyrosine (P-Tyr) in both proteins (40), while Tamura et al. detected phosphory-

* Corresponding author.

lated tyrosine residues predominantly in the gp140^{v-fms} molecule (35). *v-fms*-transformed cells do not show an increase in total cellular P-Tyr, nor have cellular proteins with increases in P-Tyr content been identified in these cells by isotopic labeling techniques (3, 27, 35). In addition, the correlation between the phosphorylation states in vitro and in vivo of the *v-fms* glycoproteins and the ability of *v-fms* to elicit the transformed phenotype have not been examined. Therefore, the role of the *fms*-associated tyrosine kinase activity in *v-fms* transformation is unclear.

To elucidate the role of tyrosine phosphorylation in *v-fms*-mediated transformation, we examined the phosphorylation of proteins on tyrosine residues in cells transfected with SM-FeSV-based vectors or infected with murine retroviral vectors encoding the *v-fms* gene or various *v-fms/c-fms* hybrid genes (7; Browning, unpublished results). To detect proteins phosphorylated on tyrosine residues in the *v-fms*-transformed cells, we used an antibody that specifically recognizes P-Tyr (19, 38). These studies were conducted by immunoblot analysis of lysates prepared from the *fms*-producing cell lines. This technique allows the identification of P-Tyr-containing proteins that might not be detected by isotopic labeling and two-dimensional gel analysis. Furthermore, this procedure is more advantageous than methods involving immunoprecipitation, in which the anti-P-Tyr antibody may not be able to recognize conformationally hidden P-Tyr residues. Results from our study indicate that the tyrosine phosphorylation in vivo of the gp140^{fms} and certain cellular proteins correlates with the transforming potential of *v-fms*.

MATERIALS AND METHODS

Cell culture. NIH-3T3 and mink epithelial cell lines were maintained in Dulbecco modified Eagle medium supplemented with 10% (vol/vol) newborn bovine serum.

Antisera. The murine anti-*fms* monoclonal antibody was a gift from V. Rothwell (Fred Hutchinson Cancer Research Center, Seattle, Wash.), and the rat anti-*v-fms* monoclonal antibody was obtained from Oncogene Sciences. Antivinculin, antitalin, and anticalmodulin antibodies were provided by L. B. Chen (Dana-Farber Cancer Institute). Anti-p36 (lipocortin II) was obtained from B. Pepinsky (Biogen Research Corporation, Cambridge, Mass.). Murine anti-*c-src* monoclonal antibody (GD11) was a gift from S. Parsons (University of Virginia, Charlottesville).

Construction of vectors. Plasmids containing the SM-FeSV-based vectors (SM-FeSV-*v-fms*, SM-FeSV-*v/c-fms*, and SM-FeSV-*v/c/v-fms*) were generated as previously described (7). The pLJ-*fms*-expressing vectors were constructed by inserting the *gag-v-fms*, *gag-v/c-fms*, and *gag-v/c/v-fms* sequences obtained from the SM-FeSV plasmids into the *Bam*HI cloning site of the pLJ vector (21).

Establishment of *fms* cell lines. To generate mink epithelial lines expressing either the *v-fms* gene or hybrid *v-fms/c-fms* genes, an SM-FeSV vector containing the appropriate proviral genome was cotransfected with a pSV2neo plasmid into CCL64 cells by the calcium phosphate method (39). The recipient cells were selected in medium containing G418 (34), and drug-resistant lines were obtained. NIH-3T3 cell lines expressing either the *v-fms* gene or one of the *fms* hybrid genes were generated by using the pLJ vectors as described previously (25). All cell lines were screened for the presence of *fms*-encoded proteins by immunoblot analysis with a murine anti-*v-fms* monoclonal antibody.

Immunoblot analysis. For cytoplasmic protein extracts, 2×10^6 cells were rinsed twice with cold phosphate-buffered

saline (PBS) and lysed in 400 μ l of lysis buffer (20 mM Tris, pH 8.0, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40) containing 1 mM phenylmethylsulfonyl fluoride, aprotinin (0.15 U/ml), 10 mM EDTA, and 1 mM sodium vanadate at 4°C for 10 min. Insoluble material was removed by centrifugation at 4°C for 15 min at $10,000 \times g$. Total protein content of the lysate was determined by the Bio-Rad Laboratories protein assay. Gel sample buffer (70 μ l; 33% glycerol, 0.3 M dithiothreitol, 6.7% sodium dodecyl sulfate [SDS], and bromophenol blue) was added, and the extracts were heated to 100°C for 5 min prior to polyacrylamide gel electrophoresis (PAGE).

Lysates (150 μ g/cm of lane) were analyzed by SDS-PAGE with either 7.5 or 15% polyacrylamide. Proteins were electrophoretically transferred from the gel onto a 0.2- μ m nitrocellulose filter (Schleicher & Schuell) in a buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol at 0.4 mA for 4 h at 4°C. Residual binding sites on the filter were blocked by incubating the nitrocellulose in TBS (10 mM Tris, pH 8.0, 150 mM NaCl) containing 2% bovine serum albumin for 1 h at 37°C. The blots were then washed twice for 5 min in TBST (TBS with 0.05% Tween-20) at 25°C. All subsequent reactions were done at 25°C. Blots were incubated overnight with either anti-P-Tyr antibody or anti-*fms* monoclonal antibody diluted 1:7,000 in TBST. The primary antibody was removed, and the blots were washed three times for 5 min each in TBST. To detect antibody reactions, the blots were incubated for 45 to 90 min with either anti-rabbit immunoglobulin G (IgG) or anti-mouse IgG (alkaline phosphatase conjugated) diluted 1:2,000 in TBST, washed three times in TBST, and then placed in a buffer containing 100 mM Tris, pH 9.5, 100 mM NaCl, 5 mM MgCl₂, 330 μ g of 5-bromo-4-chloro-3-indolyl phosphate per ml, and 150 μ g of Nitro Blue Tetrazolium per ml for 5 to 60 min. Enzymatic color development was stopped by rinsing the filters in deionized water.

Immunoprecipitation and protein kinase assays. For *v-fms* immunoprecipitation, 2×10^6 cells were washed twice with cold PBS and lysed in 1 ml PBS-TDS buffer (10 mM sodium phosphate, pH 7.2, 154 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) containing 1 mM phenylmethylsulfonyl fluoride, aprotinin (0.15 U/ml), and 1 mM sodium vanadate at 4°C for 10 min. Insoluble material was removed by centrifugation at 4°C for 15 min at $10,000 \times g$. Cell lysates were normalized for protein content and immunoprecipitated overnight at 4°C with a rat monoclonal antibody which recognizes the 5' external domain of the *v-fms* proteins (Oncogene Science). Sepharose-linked protein A beads (Sigma Chemical Co.) coated with goat anti-rat IgG antibody were used to collect the antigen-antibody complexes. The immunoprecipitates were washed and analyzed for kinase activity as described previously (29). The proteins were then separated electrophoretically on a 7.5% polyacrylamide-SDS gel, and the phosphoproteins were visualized by fluorography.

For *c-src* immunoprecipitations, 2×10^6 cells were washed once in methionine-free medium and incubated in 1.5 ml of methionine-free medium supplemented with 1 mCi of [³⁵S]methionine per ml and 2 mM glutamine for 2 h at 37°C. Cells were lysed in PBS-TDS and normalized for protein content as described above. Two identical samples of each lysate were immunoprecipitated with anti-*c-src* monoclonal antibody for 1 h at 4°C. Immune complexes were collected on Sepharose-linked protein A beads. Immunoprecipitates were washed and either analyzed directly by SDS-PAGE or assayed for kinase activity. In vitro kinase assays

with exogenously added, acid-denatured enolase were performed as described previously (25).

RESULTS

Tyrosine phosphorylation of the gp140^{v-fms} molecule. To detect residues on the *v-fms* glycoproteins that are phosphorylated on tyrosine in vivo, immunoblot experiments were conducted with an affinity-purified anti-P-Tyr antibody which is specific for tyrosine residues. Mink epithelial cells transfected with SM-FeSV-*v-fms* were analyzed in these experiments. Cytoplasmic cell lysates were prepared, and the extracted proteins were separated by SDS-PAGE and transferred electrophoretically to nitrocellulose filters. First, to determine that the *v-fms* gene was being expressed in this cell line, the blot was probed with a monoclonal antibody specific for the *v-fms* glycoproteins. In this experiment the blot was cut so that half of the lane was probed with the anti-*fms* antibody (Fig. 1A, lane 2) and the remaining half of the lane was probed with the anti-P-Tyr antibody (Fig. 1A, lane 1). The *v-fms* monoclonal antibody recognized proteins at 180, 140, and 120 kDa, indicating that the *v-fms* gene products were being expressed in these cells (Fig. 1A, lane 2). When the lysate was probed with the anti-P-Tyr antibody, only a protein at 140 kDa was detected whose migration was identical to that of one of the *v-fms* proteins (Fig. 1A, lane 1). (The diffuse appearance of the gp140^{v-fms} band was thought to be due to the nature of terminal sugar modifications.)

To confirm that the 140-kDa protein detected by the anti-P-Tyr antibody was gp140^{v-fms}, *v-fms* proteins were directly immunoprecipitated from the mink *v-fms* cell line with anti-*v-fms* antibody. The immunoprecipitated proteins were then separated by SDS-PAGE, transferred to a nitrocellulose filter, and probed with either anti-P-Tyr antibody (Fig. 1B, lane 1) or anti-*v-fms* antibody (Fig. 1B, lane 2). The anti-*v-fms* antibody detected three proteins at 180, 140, and

120 kDa (gp180^{v-fms}, gp140^{v-fms}, and gp120^{v-fms}) in the *v-fms* immunoprecipitate (Fig. 1B, lane 2), while the anti-P-Tyr antibody only recognized a 140-kDa protein (gp140^{v-fms}) in the *v-fms* immunoprecipitate (Fig. 1B, lane 1). Identical experiments with NIH-3T3 *v-fms*-expressing cells were performed and confirmed these results (data not shown). Therefore, our findings agree with those of Tamura et al., who used isotopic labeling techniques to demonstrate phosphorylation in vivo of the gp140^{v-fms} molecule on tyrosine residues (35). However, our experiments did not detect phosphorylated tyrosine residues in vivo on the gp120^{v-fms} or the gp180^{v-fms} glycoprotein.

Transforming potential of *v-fms/c-fms* hybrid proteins correlates with tyrosine phosphorylation of gp140^{fms}. To determine the role of tyrosine phosphorylation of the gp140^{fms} molecule in *fms*-mediated transformation, cell lines expressing *v-fms/c-fms* hybrid proteins were generated. SM-FeSV-based vectors were obtained that contained chimeric feline *v-fms* and human *c-fms* genes (Fig. 2A). In the *v/c-fms* hybrid construct, the entire cytoplasmic domain of the *v-fms* protein was replaced with the corresponding coding sequence of the *c-fms* gene by using a common *PvuII* site located just 3' to the transmembrane region. In the *v/c/v-fms* hybrid construct, a *PvuII*-*BglI* fragment encoding the *c-fms* tyrosine kinase domain was used to replace the corresponding *v-fms* coding sequences. Therefore, the *v/c/v-fms* hybrid gene contains the *v-fms*-encoded carboxy terminus, while the *v/c-fms* construct contains the *c-fms*-encoded carboxy terminus. The difference between these two carboxy termini is that the 40 terminal amino acids of the *c-fms* gene product are replaced in the *v-fms* gene with 11 unrelated codons of unknown origin (7, 10). NIH-3T3 and mink epithelial cell lines expressing the *v-fms/c-fms* hybrids were generated. Cell lines were studied which expressed the *fms* proteins at approximately equal levels as determined by immunoblot analysis with an anti-*v-fms* antibody.

The transforming potential of the *v-fms/c-fms* hybrid cell lines was determined by focus formation and soft-agar growth assays. NIH-3T3 and mink cell lines containing the *v/c/v-fms* construct appeared morphologically transformed and formed colonies in 0.3% agar as efficiently as *v-fms*-expressing cell lines (Fig. 2B). However, NIH-3T3 and mink cell lines expressing the *v/c-fms* hybrid gene appeared morphologically identical to control cells and did not form colonies in soft-agar assays (Fig. 2B). The *v/c/v-fms* hybrid gene construct did induce focus formation on a monolayer of NIH-3T3 cells, while the *v/c-fms* hybrid gene plasmid did not (Fig. 2B). Based on these observations, the *v/c-fms* gene appeared to be nontransforming while the *v/c/v-fms* gene had a transforming potential equivalent to that of the *v-fms* gene in the transformation parameters assayed, suggesting that there are mutations in the extreme carboxy terminus of the *v-fms* gene which are necessary for the transformed phenotype.

To determine whether there was a correlation between transformation potential and phosphorylation of the gp140^{fms} molecule on tyrosine residues, cell lysates were prepared from the mink and NIH-3T3 cell lines expressing the *v-fms*, *v/c-fms*, and *v/c/v-fms* gene products and analyzed by immunoblot analysis with the anti-P-Tyr antibody. A distinct 140-kDa band was detected by the anti-P-Tyr antibody in the *v/c/v-fms* lines (Fig. 3A and B, lanes 3) which migrated identically to the 140-kDa band recognized in the *v-fms* lines (Fig. 3A and B, lanes 4). However, no proteins of the same mobility as gp140^{v-fms} were recognized by the anti-P-Tyr antibody in either the control (Fig. 3A, lane 1) or

FIG. 1. Detection of phosphorylated tyrosine residues in vivo on the *v-fms* glycoproteins. (A) Cytoplasmic cell lysates were prepared from the mink *v-fms* cell line. The proteins were electrophoretically separated on a 7.5% polyacrylamide-SDS gel and transferred onto a nitrocellulose filter. In this experiment, half of the lane was probed with the anti-*fms* antibody (2) and the remaining half of the lane was probed with the anti-P-Tyr antibody (1). (B) *v-fms* proteins were directly immunoprecipitated from the mink *v-fms* cell line, electrophoretically separated on a 7.5% polyacrylamide-SDS gel, and transferred to a nitrocellulose filter. The immunoprecipitated *v-fms* proteins were probed with either anti-P-Tyr antibody (lane 1) or anti-*v-fms* antibody (lane 2). The arrows indicate the gp140^{v-fms} recognized by the anti-P-Tyr antibody in the mink *v-fms* cell line. Substrate development time for the blots was 5 min.

FIG. 2. *v-fms/c-fms* hybrid constructs and their transforming potential. (A) *v-fms*, *v/c-fms*, and *v/c/v-fms* genes. The *v/c-fms* and *v/c/v-fms* genes were constructed as described in the text. Cross-hatched area, *c-fms* sequences; TM, transmembrane region; P, *Pvu*II site; B, *Bgl*I site. (B) Transforming potential of the *v-fms*, *v/c-fms*, and *v/c/v-fms* genes. NIH-3T3 and mink cell lines expressing each of the constructs were generated as described in Materials and Methods. For the growth in soft agar assay, NIH-3T3 and mink cell lines expressing *v-fms*, *v/c-fms*, and *v/c/v-fms* proteins were seeded as single cells into 0.3% agar. Cells which grew to >4 cell colonies were scored as positives in this assay. For the focus-forming assay, NIH-3T3 cells were infected with retroviral stocks lacking *fms* sequences (pLJ) or infected with retroviruses encoding *v-fms*, *v/c-fms*, and *v/c/v-fms*. Cells were trypsinized 24 h postinfection, and one-half of the cells were grown in the absence of any drug selection (a). The other half were grown in the presence of G418 (400 μ g/ml) (b). After 2 weeks, cells were fixed and stained with 0.2% methylene blue, and the foci and drug-resistant colonies were counted. For cells infected with the pLJ vector alone, 48 drug-resistant colonies were counted, while no foci formed in the absence of drug selection.

FIG. 3. Proteins in the *v-fms*, *v/c-fms*, and *v/c/v-fms* cell lines recognized by the anti-P-Tyr antibody. To detect high-molecular-weight proteins phosphorylated on tyrosine residues, proteins from NIH-3T3 (A) and mink (B) cell lines were separated electrophoretically on 7.5% polyacrylamide-SDS gels and transferred to nitrocellulose filters, and control (lane 1), *v/c-fms* (lane 2), *v/c/v-fms* (lane 3), and *v-fms* (lane 4) cell lines were probed with the anti-P-Tyr antibody. (C) To detect low-molecular-weight proteins phosphorylated on tyrosine residues, proteins from NIH-3T3 cells (lane 1), NIH-3T3 *v/c-fms* (lane 2), NIH-3T3 *v/c/v-fms* (lane 3), NIH-3T3 *v-fms* (lane 4) and mink *v-fms* (lane 5) cell lines were separated on a 15% polyacrylamide-SDS gel, transferred to a nitrocellulose filter, and probed with the anti-P-Tyr antibody. The large arrows indicate gp140^{v-fms}, and the small arrowheads indicate the P-Tyr proteins present in both the *v-fms* and *v/c/v-fms* cell lines. Molecular size markers are shown to the left (in kilodaltons). Substrate development time for the blots was 5 min.

v/c-fms NIH-3T3 (Fig. 3A, lane 2) cell lines. There was one protein in the 140-kDa range of the control and *v/c-fms* mink cell lines (Fig. 3B, lanes 1 and 2), but the migration of this protein was not identical to that of gp140^{*v-fms*}. These findings were confirmed by immunoprecipitating the *v-fms* proteins from these cell lines and then probing the immunoprecipitates with anti-P-Tyr antibody (data not shown).

Protein kinase assays were then performed *in vitro* to determine whether the *v/c-fms* gene products had functional associated tyrosine kinase activity. No significant difference was detected in the ability of the hybrid proteins to be phosphorylated *in vitro* when cell lines expressing *v/c-fms* (Fig. 4, lanes 2 and 6) or *v/c/v-fms* (Fig. 4, lanes 3 and 7) glycoproteins were assayed. The *fms* proteins were phosphorylated on tyrosine residues, as determined by phosphoamino acid analysis (data not shown). The *v/c-fms* molecules were slightly larger than the *v/c/v-fms* or *v-fms* proteins, reflecting the presence of additional amino acids in the *c-fms* carboxyl terminus. This analysis indicates that phosphorylation of the gp140^{*fms*} protein on tyrosine residues *in vivo* as detected by the anti-P-Tyr antibody, rather than tyrosine phosphorylation of the *fms* molecules as observed *in vitro*, correlates with the transformation potential of the gene.

Changes in protein tyrosine phosphorylation in *fms*-transformed cells. To elucidate the role of oncogene-associated tyrosine kinase activities in mediating transformation, it is important to identify their cellular substrates in intact cells. To date, no substrates for the *v-fms* oncogene have been detected. To address this question, we examined the *v-fms* and the *v-fms/c-fms* hybrid NIH-3T3 and mink cell lines by immunoblot analysis with the anti-P-Tyr antibody. While the anti-P-Tyr antibody did recognize some bands in both NIH-3T3 and mink control cells, we identified distinct proteins that either were newly phosphorylated on tyrosine residues or showed enhanced phosphorylation on tyrosine residues as a result of *fms* transformation (Fig. 3A, B, and C). In the NIH-3T3 *v-fms* cell line, changes in the P-Tyr content of proteins, as detected by the anti-P-Tyr antibody, were observed at 200, 185, 140 (gp140^{*fms*}), 110 to 115, 92, 75 to 85, 50, 41, and 30 kDa (Fig. 3A and C, lanes 4 versus lanes 1). To determine whether any of the proteins recognized by the anti-P-Tyr antibody correlated with *fms* transformation ability, the staining pattern of the *v-fms* (Fig. 3A and C, lanes 4) and *v/c/v-fms*-transformed NIH-3T3 cells (Fig. 3A and C,

FIG. 4. *In vitro* tyrosine kinase activities of *v-fms*, *v/c-fms*, and *v/c/v-fms* cell lines. Control NIH-3T3 (lane 1), NIH-3T3 *v/c-fms* (lane 2), NIH-3T3 *v/c/v-fms* (lane 3), NIH-3T3 *v-fms* (lane 4), mink control (lane 5), mink *v/c-fms* (lane 6), and mink *v/c/v-fms* (lane 7) cell lines were assayed for tyrosine kinase activity *in vitro* as described previously (29). Proteins were separated electrophoretically on 7.5% polyacrylamide-SDS gels, and the phosphoproteins were detected by autoradiography. The results in each lane are derived from quantitative immunoprecipitation of lysates containing equal amounts of protein. Molecular size markers are expressed in kilodaltons.

FIG. 5. Specificity of the anti-P-Tyr antibody. Proteins from the NIH-3T3 *v-fms* cell line were separated electrophoretically on a 7.5% polyacrylamide-SDS gel and transferred to a nitrocellulose filter. Blots were probed with the anti-P-Tyr antibody in the presence of 40 mM P-Tyr (lane 1) or 40 mM P-Ser (lane 2). Molecular size markers are expressed in kilodaltons. Substrate development time for the blots was 5 min.

lanes 3) was compared with that of the transformation-defective *v/c-fms* NIH-3T3 cell line (Fig. 3A and C, lanes 2). Proteins of 200, 185, 140 (gp140^{*fms*}), 115, 75 to 85, 50, 41, and 30 kDa were present in *v-fms*- (Fig. 3A and C, lanes 4) and *v/c/v-fms*-transformed NIH-3T3 cells (Fig. 3A and C, lanes 3) but not in the transformation-defective *v/c-fms* NIH-3T3 cell line (arrowheads in Fig. 3 indicate the proteins which correlated with transformation). In analysis of the mink cell lines, it was apparent that the control mink epithelial cells had a very different background pattern of phosphorylated tyrosine proteins than control NIH-3T3 cells, with the mink cell line showing more P-Tyr-containing proteins. However, in the mink *v-fms* cell lines, changes in the P-Tyr content of proteins were detected by the anti-P-Tyr antibody and were observed at 200, 140 (gp140^{*fms*}), 100, 75 to 85, 50, 41, and 30 kDa (Fig. 4B, lane 4 versus lane 1, and Fig. 4C, lane 5), all of which correlated with transformation (Fig. 3B, lanes 1 to 4, and Fig. 3C). Thus, changes in the P-Tyr content of proteins which correlated with *fms* transformation in both the NIH-3T3 and mink cell lines were detected at 200, 140 (gp140^{*fms*}), 75 to 80, 50, 41, and 30 kDa. While some of the bands recognized by the anti-P-Tyr antibody were faint, we found their detection to be reproducible.

The specificity of the anti-P-Tyr antibody was confirmed by adding either P-Tyr or phosphoserine (P-Ser) to the primary antibody mixture. The addition of 40 mM P-Tyr to the primary antibody mixture prevented detection of all bands by the anti-P-Tyr antibody in the NIH-3T3 *v-fms* cell line (Fig. 5, lane 1), while the addition of P-Ser (Fig. 5, lane 2) at the same concentration had no effect. These results indicate that the anti-P-Tyr antibody did specifically recognize P-Tyr-containing proteins.

Substrates of *v-fms*-induced tyrosine kinase activity are not the characterized pp60^{*v-src*} substrates. In an attempt to determine the identity of the proteins detected by the anti-P-Tyr antibody in the *v-fms*-transformed cell lines, we performed

FIG. 6. p36 (lipocortin II) is not a substrate of the *v-fms*-induced tyrosine kinase activity. Protein lysates from control NIH-3T3 (lanes 1), NIH-3T3 *v-src* (lanes 2), NIH-3T3 *v-fms* (lanes 3), and mink *v-fms* (lanes 4) cell lines were either immunoprecipitated with anti-p36 antibody before analysis (A) or analyzed directly (B) with anti-P-Tyr. Proteins were separated on a 10% polyacrylamide-SDS gel and transferred to a nitrocellulose filter. The substrate development time for these blots was 5 min.

immunoblot analysis with both the anti-P-Tyr antibody and antibodies to various cellular proteins. In these experiments the lysates from *v-fms*-transformed lines were first immunoprecipitated with an antibody which specifically recognized a known protein. The immunoprecipitated proteins were then separated by SDS-PAGE and transferred to a nitrocellulose filter before being probed with the anti-P-Tyr antibody. For these experiments, we obtained antibodies directed against proteins whose level of phosphorylation on tyrosine residues changes after *v-src* transformation (9, 11, 13, 16). One example of this analysis is presented in Fig. 6A for anti-P-Tyr and anti-p36 (lipocortin II) antibodies. p36 immunoprecipitates from *v-src*-transformed NIH-3T3 cells (Fig. 6A, lane 2) were recognized by the anti-P-Tyr antibody, while p36 immunoprecipitates from control NIH-3T3 (Fig. 6A, lane 1), NIH-3T3 *v-fms* (Fig. 6A, lane 3), and mink *v-fms* cell lines (Fig. 6A, lane 4) were not. For comparison, Fig. 6B shows total cell lysates of the same cell lines probed with anti-P-Tyr. Again, only in *v-src*-transformed NIH-3T3 cells was a 36-kDa protein recognized by the anti-P-Tyr antibody (Fig. 6B, lane 2). In similar experiments, we determined that talin, p81 (ezrin), vinculin, calmodulin, and tensin were not detected by the anti-P-Tyr antibody in *v-fms*-transformed cells. However, the anti-P-Tyr antibody used for these studies did recognize vinculin, talin, and p81 (ezrin) in *v-src*-transformed NIH-3T3 cells (data not shown).

To determine whether *v-fms* transformation had an effect on the tyrosine kinase activity of pp60^{c-src}, we examined the ability of pp60^{c-src} from control and *v-fms*-transformed cells to phosphorylate enolase in vitro. In this experiment, NIH-3T3 cells and NIH-3T3 *v-fms* cells were labeled with [³⁵S]methionine, and cell lysates were prepared, normalized for protein content, and immunoprecipitated with an anti-*c-src* monoclonal antibody. Immunoprecipitates were analyzed directly by SDS-PAGE (Fig. 7A), or acid-denatured enolase was added and kinase assays were performed (Fig. 7B). Immunoprecipitates from control and *v-fms*-transformed NIH-3T3 cells contained equivalent amounts of pp60^{c-src} (Fig. 7A), and *v-fms* transformation did not alter the kinase activity of pp60^{c-src} (Fig. 7B). In addition, the state of tyrosine phosphorylation in pp60^{c-src} molecules in vivo did not change in *v-fms*-transformed cells, in that no

phosphate was detected on Tyr-416 in pp60^{c-src} molecules (H. Piwnicka-Worms, unpublished observations).

Comparison of proteins recognized by anti-P-Tyr in various transformed cell lines. To determine whether the proteins recognized by the anti-P-Tyr antibody in *v-fms*-transformed cells were shared by other transformed cells, we compared the pattern of phosphorylated tyrosine bands in activated *c-Ha-ras*, *v-src*, *v-fms*, and polyomavirus middle T cell lines (Fig. 8A). Compared with control NIH-3T3 cells (Fig. 8A, lane 1), only an additional band at ~41 kDa was detected in activated *c-Ha-ras*-transformed cells (Fig. 8A, lane 5), while in polyomavirus middle T-transformed cells, a group of bands was detected at 50 to 60 kDa (Fig. 8A, lane 3). We determined that pp60^{c-src} and the polyomavirus middle T protein (56 kDa) were two of the proteins recognized by the anti-P-Tyr in the polyomavirus middle T-transformed cells (data not shown). The pattern of proteins detected in *v-src*-transformed cells (Fig. 8A, lane 4) was strikingly different from the *v-fms* (Fig. 8A, lane 2) and other transformed cell lines, with many bands being detected by the antibody. (Due to the high degree of staining in the *v-src* cell lysate, the development time of the blot presented in Fig. 8A was only 2 min versus 5 min for all other blots shown. In addition, all experiments were done with excess antibody, so that substrate development time was the only experimental parameter which changed.)

Next, the pattern of phosphorylated tyrosine protein in *v-fms*- (Fig. 8B, lane 9) and *v-sis*- (Fig. 8B, lane 8) transformed cells was compared with that of NIH-3T3 cells treated with PDGF for various times (Fig. 8B, lanes 1 to 7). The proteins which showed a change in tyrosine phosphorylation in response to PDGF stimulation were similar to those reported by Morla and Wang (23). Changes in tyrosine phosphorylation were detected at >200, 180, 120, 100, 80 to 85, 75, 55 to 60, 45 to 43, and 41 kDa. Staining was strongest at 5 min after PDGF treatment and decreased during the 5-h period assayed. All phosphorylated tyrosine proteins induced by PDGF, except for the 43-kDa protein, appeared to be present in the *v-sis*-transformed cells but stained with different intensities. In the *v-sis* cell line, additional proteins were detected by the anti-P-Tyr antibody which were not detected in PDGF-treated cells (bands at 140, 130, 65, and 80 to 85 kDa). When comparing *v-sis*- (Fig. 8B, lane 8) and *v-fms*- (Fig. 8B, lane 9) transformed cells, proteins detected

FIG. 7. Tyrosine kinase activity of pp60^{c-src} in *v-fms*-transformed cells as assayed in vitro. (A) Total levels of pp60^{c-src} present in NIH-3T3 (lane 1) and NIH-3T3 *v-fms* (lane 2) cell lines were determined by first labeling the cellular proteins with [³⁵S]methionine and then immunoprecipitating the pp60^{c-src} molecules with the GD11 antiserum. The precipitated proteins were separated on a 10% polyacrylamide-SDS gel. The [³⁵S]methionine gel was enhanced and exposed for 2 days. (B) pp60^{c-src} molecules immunoprecipitated from control NIH-3T3 (lane 1) and NIH-3T3 *v-fms* (lane 2) cell lines were assayed for their ability to phosphorylate acid-denatured enolase as described previously (25). Proteins were separated electrophoretically on a 10% polyacrylamide-SDS gel, and the phosphoproteins were detected by autoradiography.

FIG. 8. Proteins recognized by the anti-P-Tyr antibody in various transformed or PDGF-treated cell lines. (A) Cytoplasmic cell lysates were prepared from NIH-3T3 (lane 1), activated c-Ha-ras (lane 5), v-src (lane 4), polyomavirus middle T (lane 3), and v-fms (lane 2) cell lines. The extracted proteins were separated electrophoretically on 7.5% polyacrylamide-SDS gels and transferred to nitrocellulose filters. The blotted proteins were then probed with the anti-P-Tyr antibody. (B) Cytoplasmic cell lysates were prepared from NIH-3T3 cells untreated (lane 1) or stimulated with PDGF for 5 min (lane 2), 15 min (lane 3), 30 min (lane 4), 1 h (lane 5), 2 h (lane 6), or 5 h (lane 7) and v-sis (lane 8) and v-fms (lane 9) cell lines. The extracted proteins were separated electrophoretically on 7.5% polyacrylamide-SDS gels and transferred to nitrocellulose filters. The blotted proteins were then probed with the anti-P-Tyr antibody. (C) Cytoplasmic cell lysates from NIH-3T3 (lane 1), v-fms (lane 2), and v-sis (lane 3) cell lines were separated electrophoretically on 15% polyacrylamide-SDS gels and transferred to nitrocellulose filters. The blotted proteins were then probed with the anti-P-Tyr antibody. Molecular size markers (lane M in panel C, left to panels A and B) are expressed in kilodaltons. The substrate development time was 2 min (A) or 5 min (B and C).

by the anti-P-Tyr antibody above 100 kDa were different, but below 100 kDa virtually all of the bands recognized by the anti-P-Tyr antibody were common to the two cell lines, based on the results of one-dimensional gel analysis. This correlation also extended to the low-molecular-weight proteins (Fig. 8C, lanes 2 and 3).

DISCUSSION

Using immunoblotting techniques together with a high-affinity antibody specific for P-Tyr, we made the following observations regarding the role of tyrosine-specific phosphorylation in v-fms-mediated transformation. The phosphorylation of the gp140^{fms} molecule correlated with the ability of v-fms/c-fms hybrids to transform cells. v-fms transformation resulted in the phosphorylation of a number of cellular proteins on tyrosine residues. Phosphorylation of some of these cellular proteins also correlated with the ability of v-fms/c-fms hybrids to transform cells.

By virtue of its associated tyrosine kinase activity, the fms oncogene is related to the largest group of oncogenes thus far identified (*src*, *abl*, *yes*, *fps/fes*, *ros*, *fgr*, *erbB*, *trk*, *ret*, and *met*) (5, 6, 32). For several of these oncogenes, genetic analysis suggests that the tyrosine kinase activity is essential to the ability of the oncogenes to transform cells (18). Tyrosine kinase activity has also been demonstrated as a property of several growth factor receptors (PDGF receptor, insulin receptor, EGF receptor, and CSF-1 receptor) (18). Binding of the appropriate ligand stimulates the tyrosine

kinase activity of these receptors. Therefore, constitutive activity and/or altered substrate specificity of the tyrosine kinases may be an important component leading to transformation and/or growth factor-independent growth. Previous to this report, it has been difficult to determine the role of the fms-associated tyrosine kinase activity in v-fms-mediated transformation. However, the experimental techniques used in this report have suggested a role for tyrosine phosphorylation in v-fms-mediated transformation.

In contrast to previous reports in which phosphorylated tyrosine residues were detected in vivo on gp140^{v-fms} and gp120^{v-fms} (35, 40), we found that only the fully processed gp140^{v-fms} was recognized by the anti-P-Tyr antibody in v-fms-transformed cell lines. It could be argued that the gp120^{v-fms} and the gp180^{v-fms} do contain phosphorylated tyrosine residues that are not recognized by this antibody. However, even if this were the case, there was still a unique phosphorylated tyrosine site found only on the mature gp140^{v-fms}. It is interesting that only fms molecules which are expressed on the cell surface were phosphorylated so that they could be detected by the anti-P-Tyr antibody. Genetic analysis of fms mutants indicates that cell surface expression of the glycoprotein is obligatory for the transformed phenotype (24, 29). Thus, it appears that both the phosphorylation of gp140^{fms} on tyrosine residues and cell surface expression of fms molecules are necessary for fms-mediated transformation. Current technology is not sufficient to measure the percentage of gp140^{v-fms} which is phosphorylated on tyrosine. However, if we assume that the

affinities of the anti-*v-fms* and anti-P-Tyr antibodies are roughly equal, then the data in Fig. 2 suggest that the percentage is sizable.

We have also shown a strong positive correlation between the transforming ability of *v-fms/c-fms* hybrids and the presence of P-Tyr in the gp140 form of the *fms* molecule. In this analysis, two *v-fms/c-fms* hybrid genes were studied. The *v/c/v-fms* construct encoded a protein containing a *v-fms* external domain, transmembrane region, and carboxy terminus with a *c-fms* tyrosine kinase domain. Cells expressing *v/c/v-fms* proteins were morphologically transformed and formed foci in focus formation assays. The gp140^{*v/c/v-fms*}, like the gp140^{*v-fms*}, was phosphorylated on tyrosine residues in intact cells. Therefore, substitution of the *c-fms* tyrosine kinase domain into the *v-fms* gene did not affect the transforming potential of the *v-fms* protein (Fig. 2 and 3) (P. Browning, unpublished results). Very different results were obtained for another *v-fms/c-fms* hybrid cell line. The *v/c-fms* construct generated a *fms* protein containing the *v-fms* extracellular domain and transmembrane region while most of the cytoplasmic domain was encoded by the *c-fms* gene. In *v/c-fms* cell lines, the three *fms* forms were detected, and the *v/c-fms* glycoproteins had fully functional associated tyrosine kinase activity as assayed in vitro. However, evidence from the transformation parameters analyzed here and elsewhere (7) indicates that the *v/c-fms* hybrid is nontransforming. These data suggest that the *c-fms* cytoplasmic domain has markedly affected the transforming potential of the *v-fms* protein. The main structural difference between the *v-fms* and *c-fms* proteins is found in the cytoplasmic carboxy domain in that the terminal 40 amino acids of the *c-fms* protein are replaced by 11 different amino acids in the *v-fms* protein (7, 10). As a result of retroviral transduction, this pattern of carboxy-terminal divergence and deletion also occurs in several other oncogenes and their respective proto-oncogenes, including *c-* and *v-ros*, *c-* and *v-src*, *c-* and *v-fgr*, *c-* and *v-yes*, and the EGF receptor and *v-erbB* (5, 17). In all cases, including *fms*, the carboxy termini of the cellular cognates contain tyrosine residues which may play important roles in regulating its tyrosine kinase activity. Experimentation has shown that mutation of Tyr-527 to Phe-527 in pp60^{*c-src*} activates the molecule as a tyrosine kinase and as a transforming agent (8, 20, 25). Data about the mutation of an equivalent carboxy-terminal tyrosine of *c-fms* (Tyr-969) to phenylalanine have shown that this change increases the transforming potential of the molecule but is not itself sufficient to induce transformation, indicating that other alterations are required (28).

It is significant that the *v/c-fms* hybrid is an example of a *fms* product that was nontransforming, yet is expressed on the cell surface and has an associated tyrosine kinase activity, as observed in vitro. By immunoblot analysis, the gp140^{*v/c-fms*} was not recognized by the anti-P-Tyr antibody, implying that the phosphorylated tyrosine residue found in gp140^{*v-fms*} and gp140^{*v/c/v-fms*} is not present in the *v/c-fms* molecule. Therefore, tyrosine phosphorylation of the gp140^{*fms*} in vivo is a better indicator of transformation ability than *fms*-associated tyrosine kinase activity assayed in vitro. The site(s) of tyrosine phosphorylation in the gp140^{*fms*} molecules in intact cells is unknown. However, the phosphorylated tyrosine residue recognized by the anti-P-Tyr antibody in gp140^{*v-fms*} and gp140^{*v/c/v-fms*} was not Tyr-969, since this residue is not present in the truncated *v-fms* carboxy terminus. In addition, when Tyr-969 is present, as it was in gp140^{*v/c-fms*}, it is apparently not phosphorylated, since the gp140^{*v/c-fms*} was not detected by the anti-P-Tyr

antibody. This is distinctly different from pp60^{*c-src*}, where the analogous residue, Tyr-527, is phosphorylated in vivo and could be detected by this anti-P-Tyr antibody (H. Piwnicka-Worms, unpublished results).

Our findings indicate that as a result of *v-fms* transformation, there are distinct cellular proteins that either are newly phosphorylated on tyrosine residues or show enhanced phosphorylation on tyrosine residues. While the overall background staining in the control NIH-3T3 and mink cells was very different, the anti-P-Tyr antibody recognized a similar group of proteins whose phosphorylation state changed in these cell lines after *v-fms* transformation even though they were from different species (mouse and mink) and cellular origin (fibroblast and epithelial). The phosphorylation of many of the proteins correlated with *fms*-mediated transformation. Although some of the proteins recognized by the anti-P-Tyr antibody showed only slight increases in P-Tyr content, they may represent critical substrates. The increased P-Tyr content of proteins in *v-fms*-transformed cells suggests that they are substrates for the *fms* tyrosine kinase, but it is possible that they are phosphorylated by another protein tyrosine kinase activated as a result of *v-fms* transformation.

We also observed that the number of proteins which underwent new or increased phosphorylation on tyrosine residues in *v-fms*-transformed cell lines were few in comparison with *v-src*-transformed cells. By our analysis, many of the known *v-src* phosphorylated tyrosine substrates did not show changes in their phosphorylation state in *v-fms* cell lines. However, since no target for the *v-src* protein kinase activity has been described whose phosphorylation on tyrosine residues could account for the distortion of cellular proliferation that underlies transformation, the possibility remains that *v-src* and *v-fms* share critically important substrates. The data presented here do suggest that phosphorylation of gp140^{*fms*} and a number of cellular proteins on tyrosine residues plays a role in transformation by the *fms* oncogene. The importance of these phosphorylations will be elucidated after identification of cellular substrates whose biochemical function is modulated by tyrosine phosphorylation.

From this study it appears that all transformed cells do not exhibit the same pattern of proteins phosphorylated on tyrosine residues, suggesting that these proteins are not simply phosphorylated on tyrosine residues as a result of constitutive cell proliferation. However, it is interesting that the pattern of tyrosine phosphorylation in *v-fms*- and *v-sis*-transformed cells was very similar, indicating potentially common substrates in these two transformed cell lines. The *v-sis* oncogene is thought to mediate transformation, at least in part, by constitutively activating the PDGF receptor (15). The published sequence analysis indicates that the tyrosine kinase domain of the PDGF receptor and the *c-* and *v-fms* genes are highly homologous, with each containing an interrupted tyrosine kinase domain (41). Perhaps this structural homology between the *v-fms* protein and the PDGF receptor results directly in the similar pattern of phosphorylated tyrosine substrates observed in *v-fms*- and *v-sis*-transformed cells. Alternatively, *v-fms* and *v-sis* may also increase tyrosine phosphorylation of proteins by mechanisms independent of PDGF growth-stimulatory pathways. Finally, the fact that each oncogene and activated receptor seemed to have a distinct pattern of P-Tyr-containing bands suggests that the immunoblotting techniques described here may be of use in measuring tyrosine kinase function in tissue samples as well.

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