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Tyrosine 807 of the v-Fms Oncogene Product Controls Cell Morphology and Association with p120RasGAP

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Expression of the v-*fms* oncogene of feline sarcoma virus in fibroblasts causes surface exposure of an activated receptor tyrosine kinase, v-Fms, that is autophosphorylated at multiple sites within its cytoplasmic domain. Cellular proteins interacting with this part of v-Fms modulate the mitogenic activity and morphology of the cells. We show here that the tyrosine residue in position 807 (Y-807) of the v-Fms molecule constitutes a major autophosphorylation site. The replacement of this residue by phenylalanine (Y807F mutation) allowed us to functionally dissect v-Fms-specific mitogenic and morphogenic cascades. Cells expressing the mutant v-Fms molecule resembled wild-type (wt) v-Fms-transformed (wt-v-Fms) cells in terms of [^{3}H]thymidine uptake rates and activation of the Ras/Raf-1 mitogenic cascade. Such cells showed, however, a flat morphology and contained intact actin cables and fibronectin network. Our studies indicate that the v-Fms molecule controls cell morphology by a cascade that involves a direct interaction with p120RasGAP and p190RhoGAP: (i) in contrast to wt v-Fms molecules, the Y807F v-Fms protein failed to associate with and phosphorylate p120RasGAP; (ii) tight complexes between p120RasGAP and p190RhoGAP as well as detectable RhoGAP activity were present exclusively in wt-v-Fms cells; and (iii) p190RhoGAP was dispersed throughout the cytoplasm of wt-v-Fms cells, whereas its distribution was restricted to perinuclear regions of cells expressing the mutant v-Fms gene.

The v-*fms* oncogene of feline sarcoma virus encodes a modified receptor tyrosine kinase that differs only in seven amino acid positions and in the C-terminal sequence from the cellular receptor for macrophage colony-stimulating factor (M-CSF) (14, 43, 44, 55). Both proteins contain a large extracellular domain that binds M-CSF and a cytoplasmic tyrosine kinase domain, split by an insertion of approximately 70 amino acids, termed the kinase insert region. Through two of the amino acid substitutions in the extracellular domain and the replacement of the C terminus, the v-Fms molecule is thought to have gained biochemical properties that are observed with the c-Fms polypeptide only transiently upon binding of M-CSF (39, 55): the tyrosine kinase is activated, which leads to autophosphorylation of the cytoplasmic domain at multiple sites. The newly formed phosphotyrosine residues constitute binding sites for SH2 domain-containing cytoplasmic proteins that may participate in the control of mitogenic pathways, cell metabolism, or cell morphology. Whereas c-Fms molecules are internalized and inactivated within minutes after binding of the growth factor (3, 31), v-Fms molecules remain at the plasma membrane with a half-life of about 4 h (46). Thus, v-Fms molecules continue to produce the spectrum of Fms-specific signals which cause the dramatic enhancement of the mitogenic activity and the morphological changes, i.e., breakdown of actin filaments and the fibronectin network, generally observed with transformed cells.

A series of studies performed on a variety of receptor tyrosine kinases, including the epidermal growth factor and platelet-derived growth factor (PDGF) receptors, led to the identification of proteins that acted uniformly irrespective of the receptor in the transduction of the mitogenic signal (21, 49). In nontransformed cells, a protein complex that contained the growth factor receptor-bound protein, GRB2, and the nucleotide exchange factor, Sos, was detected (2, 8, 12, 22–24, 41). This protein complex binds via the SH2 domain of GRB2 to phosphotyrosine residues of the activated tyrosine kinase, allowing Sos to convert membrane-associated Ras to its GTP-bound form, which subsequently binds and activates the serine/threonine kinase Raf-1. Following a cascade of reactions in which additional serine/threonine kinases become activated, mitogen-activated protein kinase finally induces transcription of a series of immediate-early transcription factors, including c-Jun and c-Fos.

In comparison, much less is known about proteins controlling the transduction of signals leading to depolymerization and reorganization of the cytoskeleton. It was reported that phosphotyrosine residues of the kinase insert domains of the PDGF β receptor and the M-CSF receptor constituted binding sites for p85, the regulatory subunit for phosphatidylinositol-3′ kinase (PI-3′ kinase) (5, 18, 19, 35, 52). The presence of such binding sites was reported to be a prerequisite for PDGF-induced membrane ruffling and chemotaxis (29, 53), but the precise mechanism by which PI-3′ kinase contributes to the control of the cytoskeleton remains to be established.

A second protein that has been shown to interact with both the PDGF and M-CSF receptors is p120RasGAP. Although this protein was originally suggested to control the mitogenic activity of cells by a direct interaction with GTP-bound Ras (7, 25, 30), removal of the p120RasGAP binding site from the

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kinase insert domain of the PDGF β receptor had no consequences for the β-receptor-mediated mitogenic activity (11, 17). With respect to the M-CSF receptor, it was shown that M-CSF induces an immediate tyrosine phosphorylation of p120RasGAP (9, 16, 34), but a p120RasGAP binding site has yet not been mapped on the M-CSF receptor.

The N-terminal domain of p120RasGAP contains two SH2 domains and a single SH3 domain. In addition to binding to the PDGF β receptor, p120RasGAP stably associates with p62, an RNA-binding protein (9, 33, 54), and p190RhoGAP, a protein that possesses GTPase-activating protein (GAP) activity toward the small guanine nucleotide-binding proteins in the Rho/Rac family (42). Conversion of Rho into the GTP-bound form is thought to be required for the formation of actin stress fibers, whereas GTP-bound Rac causes membrane ruffling (36, 37). It has been suggested that an association of p120RasGAP with receptor tyrosine kinases causes an activation of p190RhoGAP, thereby downregulating Rho (26).

In a preliminary study performed in psi2 cells (48), we showed that a replacement of tyrosine 807 (Y-807) of v-Fms by phenylalanine (Y807F mutation) had little effect on its mitogenic activity but prevented the v-Fms-specific morphologic alterations. The molecular mechanisms underlying these effects were, however, not investigated. Here, we show that the Y807F mutation of v-Fms produced similar results in NIH 3T3 cells. The mutation has no detectable effects on the Fms-specific tyrosine kinase or on the mitogenic activities. In addition, we show for the first time that wild-type (wt) v-Fms molecules directly associate with p120RasGAP and that this association is abolished by the Y807F mutation. In contrast to wt v-Fms-transformed NIH 3T3 (wt-v-Fms) cells, NIH 3T3 cells expressing the Y807F mutant (Y807F-v-Fms cells) contained only traces of the p120RasGAP-p190RhoGAP complex, lacked detectable RhoGAP activity, and contained p190RhoGAP exclusively in perinuclear regions. We conclude that the v-Fms-induced degradation of the cytoskeleton can be uncoupled from its mitogenic potential. Phosphorylation of Y-807 is required for stimulation of a p120RasGAP-mediated signaling cascade that involves activation of p190RhoGAP and ultimately leads to a breakdown of the cytoskeleton.

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MATERIALS AND METHODS

Cells. Mouse NIH 3T3 cells, wt-v-Fms cells, and Y807F-v-Fms cells were grown in Eagle's Dulbecco modified minimum essential medium supplemented with 0.5 to 10% fetal calf serum (FCS).

Antibodies. The production and characterization of monoclonal and polyclonal antibodies 6F2 and p23 against p120RasGAP and antibodies 8C10 and p27 against p190RhoGAP will be described elsewhere (4). The anti-Raf-1 antibody was kindly provided by Ulf Rapp (University of Würzburg, Würzburg, Germany). A combination of anti-v-Fms antisera, binding to either the extracellular or the cytoplasmic domain of v-Fms, was used as described previously (45, 46, 47a). Each of these sera precipitated >98% of the v-Fms molecules when applied individually in serial immunoprecipitation reactions. Monoclonal antibodies against phosphotyrosine (4G10) and against the p85 subunit of PI-3′ kinase were purchased from Upstate Biotechnology Incorporated (Lake Placid, N.Y.). The monoclonal antibody against Ras (Y13-259) was from Oncogene Science (Uniondale, N.Y.). Species-specific anti-immunoglobulin G (IgG) was from Sigma (Munich, Germany).

Plasmid constructions and transfection procedures. Generation of the Y807F mutation and transfection of eukaryotic cells were performed as described previously (48). For generation of glutathione *S*-transferase (GST) fusion proteins containing C-terminal v-Fms sequences representing either the kinase insert domain (residues 617 to 759) or the kinase domain (residues 757 to 944 with or without the Y807F mutation), we used the pGex system (Pharmacia, Freiburg, Germany) and the protocols provided by the manufacturer. A pGex clone encoding a GST-RhoA fusion protein was kindly provided by Alan Hall (London, England).

Binding of p120RasGAP to GST–v-Fms fusion proteins. Strain TKX-1 (Stratagene, La Jolla, Calif.) was used for the isolation of phosphorylated GST–v-Fms (residues 757 to 944) fusion proteins with or without the Y807F mutation. Production and evaluation of phosphorylated GST fusion proteins were performed as recommended by the manufacturer. The corresponding nonphosphorylated molecular species were obtained from *Escherichia coli* DH5α. Purified GST fusion proteins (50 μg) were bound for 1 h at 4°C to glutathione-agarose beads (40 μl of slurry; Pharmacia) suspended in binding buffer (50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.5], 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EDTA, 1% Trasylol, 100 μg of leupeptin per ml, 1 mM phenylmethylsulfonyl fluoride, 200 μM sodium orthovanadate, 10 mM sodium pyrophosphate, 100 mM sodium fluoride). The precharged beads were incubated for 4 h at 4°C with lysates from 10^7 NIH 3T3 cells in 2 ml of binding buffer. The beads were washed five times with binding buffer, and pellets were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and immunoblotting with monoclonal antibodies against p120RasGAP.

Immunoprecipitation and Western blot (immunoblot) analyses. For coprecipitation experiments, cells were incubated for 24 h in the presence of 0.5% FCS. Sodium orthovanadate (100 μM, final concentration) was added 30 min prior to lysis. A total of 10^7 cells were lysed in cell lysis buffer (20 mM Tris-HCl [pH 8.0], 1% Nonidet P-40, 150 mM NaCl, 2 mM EDTA, 1 mM NaF, 400 μM Na_3VO_4, 1% Trasylol). Equal amounts of cleared lysates were incubated with the corresponding antibodies and protein A-Sepharose 6B (Pharmacia) or, alternatively, *Staphylococcus aureus* Cowan 1. Immune complexes were analyzed by SDS-PAGE using 7.5% gels. For identification of proteins by immunoblotting, proteins were transferred onto Immobilon-P sheets (Millipore, Bedford, Mass.) by a semidry blotting technique. Bound antibody was visualized by incubation of blots in 3 ml of 20 mM Tris-HCl (pH 7.6) containing 137 mM NaCl and 2 μCi of ^{125}I-labeled anti-species-specific IgG (Amersham Buchler, Braunschweig, Germany). Bound radioactivities were quantified with a model BAS1000 bioimaging analyzer (Fuji Photo Film Co., Kanagawa, Japan).

Ras loading assay. Subconfluent cells were deprived of FCS for 24 h and then labeled for 6 h with $^{32}P_i$ (0.5 mCi/ml) in phosphate-free Dulbecco modified minimum essential medium supplemented with 0.5% phosphate-free FCS. Cells were rinsed with ice-cold phosphate-buffered saline (PBS) and lysed in Ras lysis buffer (50 mM HEPES [pH 7.4], 1% Triton X-100, 100 mM NaCl, 5 mM $MgCl_2$, 1 mg of bovine serum albumin (BSA) per ml, 1% Trasylol, 100 μg of leupeptin per ml, 100 mM benzamidine, 10 μg of soybean trypsin inhibitor per ml). Lysates were centrifuged for 3 min at 14,000 × *g*, and supernatants were brought to 500 mM NaCl–0.5% deoxycholate–0.05% SDS. Excess free nucleotides were removed by incubation with activated charcoal. Ras protein was immunoprecipitated by the addition of 3 μg of rat monoclonal anti-Ras antibody Y13-259, immobilized on protein A-Sepharose beads via rabbit anti-rat IgG. Triplicate samples in which the Y13-259 antibody was omitted were used as negative controls. The immune complexes were washed eight times with washing buffer containing 50 mM HEPES (pH 7.4), 500 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100, and 0.005% SDS. Bound nucleotides were eluted by incubation for 20 min at 68°C with 2 mM EDTA–2 mM dithiothreitol–0.2% SDS. Nucleotides were separated by thin-layer chromatography on polyethyleneimine-cellulose plates in 1 M KH_2PO_4 (pH 4.0). After exposure to X-ray film (Kodak, Rochester, N.Y.), GTP and GDP spots were quantified by densitometer scanning.

Tyrosine kinase assay, phosphoamino acid analyses, and tryptic peptide mapping. Kinase assays, phosphoamino acid analyses, and tryptic peptide mapping were performed as described previously (46, 47, 47a).

RhoGAP assay. RhoGAP assays were performed as described previously (13). A GST-RhoA fusion protein was isolated by adsorption to glutathione-Sepharose 4B beads (Pharmacia). RhoA was released by thrombin cleavage. Two hundred nanograms of the Rho protein was incubated for 10 min at 30°C with [γ-^{32}P]GTP (10 mCi/ml, 3,000 Ci/mmol; Amersham Buchler) in 20 μl of preloading buffer (20 mM Tris-HCl [pH 7.5], 25 mM NaCl, 0.1 mM dithiothreitol, 5 mM EDTA). GTP binding was then terminated by the addition of 5 μl of 100 mM $MgCl_2$ (13). To study GAP activity, GTP-bound Rho was incubated for 10 min in preloading buffer containing 1 mg of BSA per ml, 1 mM GTP, and equal amounts of p120RasGAP or p190RhoGAP immunoprecipitates. The reaction was stopped by the addition of 200 μl of stopping buffer (50 mM Tris-HCl [pH 7.5], 0.1 mM dithiothreitol, 5 mM $MgCl_2$). After centrifugation, the amount of radiolabeled GTP bound to RhoA was determined by filtration through nitrocellulose and liquid scintillation counting of the washed and dried filters.

Immunofluorescence. Cells were grown on glass coverslips to subconfluency and deprived of FCS for 24 h. Cells were fixed for 20 min in 3% paraformaldehyde, permeabilized for 4 min with 0.4% Triton X-100, and sequentially incubated (40 min at room temperature for each step) with the primary mouse monoclonal antibody (10 μg/ml in PBS), goat anti-mouse IgG, 8% rabbit serum, and finally fluorescein isothiocyanate (FITC)-conjugated rabbit anti-goat IgG. The coverslips were washed three times with PBS between treatments and antibody incubation steps. All antibodies and sera were preabsorbed with methanol-fixed NIH 3T3 cells, except for the monoclonal antibodies against p120RasGAP (6F2) and p190RhoGAP (8C10) (4). Procedures for visualizing actin cables were described previously (46). FITC-labeled phalloidin and FITC-conjugated rabbit anti-goat IgG were purchased from Sigma.

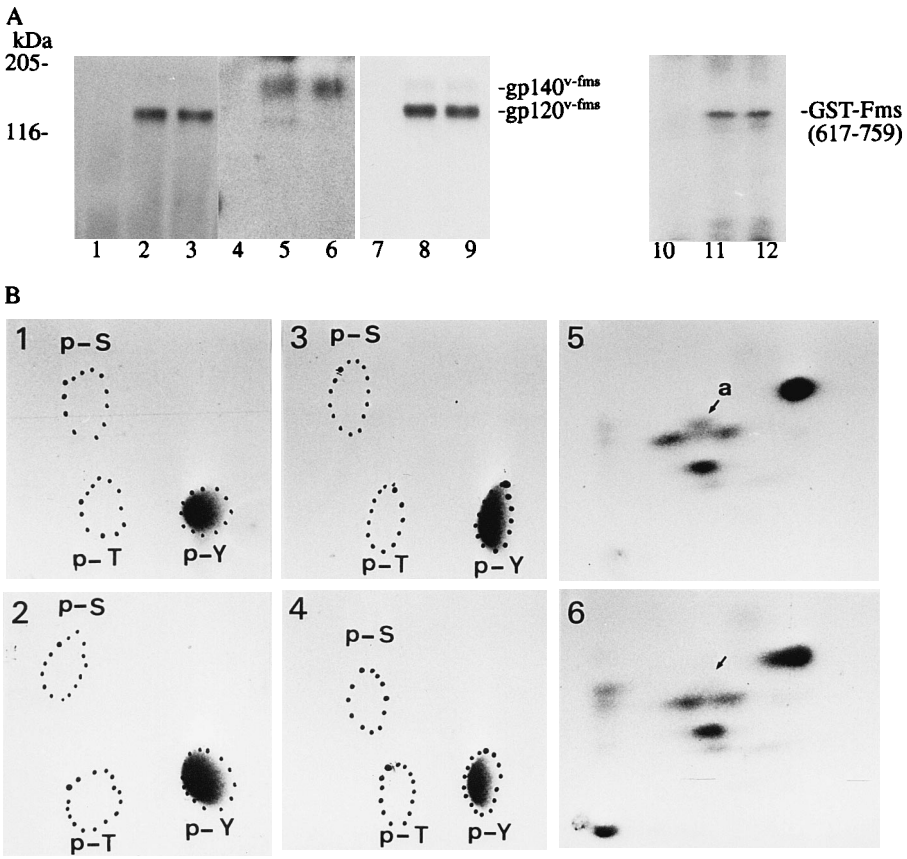

FIG. 1. The Y807F mutation has no effect on v-Fms-specific tyrosine kinase activity in vivo or in vitro. (A) Comparison of expression rates and tyrosine kinase activities of Y807F v-Fms and wt v-Fms. Cell lysates were prepared from cultures (5×10^6 cells each) of nontransformed NIH 3T3 cells (lanes 1, 4, 7, and 10), wt-v-Fms cells (lanes 2, 5, 8, and 11), and Y807F-v-Fms cells (lanes 3, 6, 9, and 12). Aliquots of cell lysates were subjected to immunoprecipitation with an Fms-specific antiserum and analyzed by SDS-PAGE and immunoblotting for the levels of Fms expression with an Fms-specific antiserum (lanes 1 to 3) or in vivo autophosphorylation with a phosphotyrosine-specific antibody (lanes 4 to 6). Alternatively, kinase reactions were performed, and samples were analyzed by SDS-PAGE and autoradiography to determine the in vitro tyrosine kinase activity by autophosphorylation (lanes 7 to 9) or the activity toward an exogenous GST-Fms (residues 617 to 759) fusion protein (lanes 10 to 12). (B) Comparative phosphoamino acid analyses and tryptic peptide maps of wt v-Fms and Y807F v-Fms derivatives. Material underlying the $gp140^{v\text{-}fms}$ bands (Fig. 1A, lanes 8, 9, 11, and 12) was recovered, and phosphoamino acid analyses (panels 1 to 4) and tryptic peptide mapping (panels 5 and 6) were performed. Panels 1, 3, and 5 show the results obtained with the wt v-Fms polypeptide; panels 2, 4, and 6 show the corresponding results obtained with the Y807F v-Fms polypeptide. p-S, phosphoserine; p-T, phosphothreonine; p-Y, phosphotyrosine.

RESULTS

Replacement of tyrosine 807 of v-Fms by phenylalanine does not affect the protein's kinase activity. Expression of the v-*fms* gene in a variety of mammalian fibroblasts causes cell transformation, characterized by a drastic enhancement of the proliferation rate as well as a breakdown of the fibronectin network and actin cables. In this study, we wanted to study the biological role of a particular autophosphorylation site, Y-807, located within the second half of the kinase domain of the v-Fms molecule. The presence of a tyrosine residue in this position is highly conserved among all tyrosine kinases (15), and it is autophosphorylated in all those that possess it except for the epidermal growth factor receptor.

In a previous report, we showed that a mutation of Y-807 had little effect on the v-Fms-specific mitogenic potential in mouse psi2 cells, whereas morphological transformation was impaired (48). Available data regarding mutations of this autophosphorylation site in the murine and human c-Fms proteins diverge significantly with respect to the resulting tyrosine kinase activity and the Fms-specific mitogenic activity. Whereas Roussel and coworkers (40) reported that a Y-809 mutation of the human c-Fms protein (corresponding to Y-807 in v-Fms) had no influence on the tyrosine kinase activity but abolished the M-CSF-induced mitogenic potential in mouse NIH 3T3 cells, the same mutation of the murine c-*fms* gene, studied in Rat2 cells, reduced both the tyrosine kinase and the mitogenic activity (50).

To analyze the molecular basis for the effects of the Y807F mutation, we established NIH 3T3 cell clones that constitutively expressed either the wt v-*fms* gene (wt-v-Fms cells) or the Y807F v-*fms* gene (Y807F-v-Fms cells). We next performed a series of control experiments with 10 different clones of each cell type to determine whether the expression levels and the Fms-specific tyrosine kinase activities were altered. No differences were detected between 10 independent Y807F-v-Fms cell clones, making it unlikely that the observed effects were due to cellular mutations during selection of the clones. The results obtained with representative clones of each cell type allow the following conclusions (Fig. 1). First, immunoblotting with an anti-v-Fms antibody showed that wt-v-Fms cells and Y807F-v-Fms cells expressed similar levels of the intracellular v-Fms-protein $gp120^{v\text{-}fms}$ and of the plasma membrane-associated $gp140^{v\text{-}fms}$ species (Fig. 1A, lanes 2 and 3). Second, immunoblotting with a monoclonal antibody against phosphoty-

TABLE 1. Doubling times of wt-v-Fms and Y807F-v-Fms cells

FCS concn (%)	Doubling time[a] (h)		
	NIH 3T3 cells	wt-v-Fms cells	Y807F-v-Fms cells
5	23	18	19
0.5	No growth	24	28

[a] Triplicate sister cultures (10^5 cells of each type per 5-cm-diameter dish) were prepared in complete medium containing either 5 or 0.5% FCS. Viable cell counts were determined at various time intervals. Doubling times were calculated from cell counts averaged from the three separate experiments.

rosine indicated that the two cell lines contained similar levels of phosphorylated gp140$^{v\text{-}fms}$ (lanes 5 and 6), suggesting that the two v-Fms-specific tyrosine kinases were equally active. Third, v-Fms-specific immune complexes from either cell line exhibited similar in vitro tyrosine kinase activities, as judged by autophosphorylation (lanes 8 and 9) or phosphorylation of an exogenous GST fusion protein containing the v-Fms kinase insert domain (lanes 11 and 12). Fourth, phosphoamino acid analyses of autophosphorylated v-Fms derivatives (Fig. 1B, panels 1 and 2) or of the exogenous substrate phosphorylated by either wt v-Fms (panel 3) or Y807F v-Fms (panel 4) revealed phosphotyrosine as the only phosphoamino acid, thus excluding the possibility that immune complexes contained contaminating serine/threonine kinases. Finally, to verify that the genetically engineered mutation of the v-*fms* gene was indeed stable, we performed tryptic phosphopeptide mapping. The results indicated that the Y807F v-Fms protein lacked one tyrosine phosphorylation site, resulting in the disappearance of a phosphopeptide designated a (compare panels 5 and 6).

Together, our data demonstrate unambiguously that the Y807F v-Fms protein is similar in expression levels and tyrosine kinase activity to the wt v-Fms protein.

The Y807F mutation has no significant effect on the Fms-induced mitogenic activity. We then compared the growth properties of Y807F-v-Fms cells and wt-v-Fms cells at various serum concentrations. Only small differences in the proliferation rates were observed with 5% FCS (Table 1). Even in the presence of 0.5% serum, Y807F-v-Fms cells exhibited only a slightly prolonged doubling time in comparison with wt-v-Fms cells, resulting in a 3.4-fold increase in cell number within 2 days. In comparison, wt-v-Fms cells showed a fourfold increase over this time period. Furthermore, at 0.5% serum, [^{3}H]thymidine incorporation of Y807F-v-Fms cells was about 96% of that of wt-v-Fms cells (Fig. 2A). Together, these data demonstrate that the mitogenic activity of Y807F-v-Fms cells was not significantly less than that of wt-v-Fms cells.

It has been well established that GTP-bound Ras, formed in response to activation of a receptor tyrosine kinase, is a prerequisite for transduction of the mitogenic signal to the serine/threonine kinase Raf-1 (6). To measure Ras loading with GTP, Y807F-v-Fms cells were incubated for 24 h in the presence of 0.5% FCS and then labeled with $^{32}P_i$ for 6 h. We then determined the ratio of GTP- to GDP-bound Ras (Fig. 2B). Again, wt-v-Fms and Y807F-v-Fms cells produced very similar results: in both instances, about 20% of the Ras-associated radiolabel was recovered as GTP (Fig. 2B, lanes 2 and 3). In contrast, in resting cells, less than 3% was GTP-bound Ras (Fig. 2B, lane

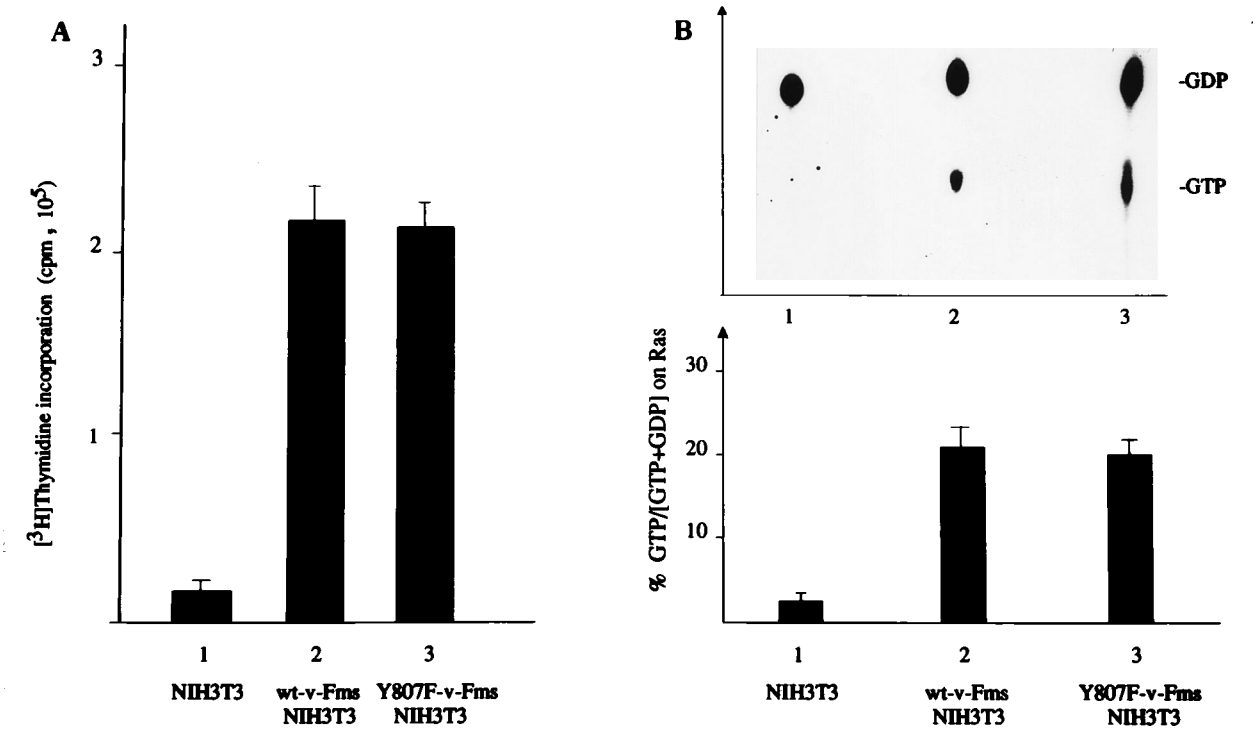

FIG. 2. [^{3}H]thymidine incorporation and Ras activation in wt-v-Fms and Y807F-v-Fms cells. (A) [^{3}H]thymidine incorporation of Y807F-v-Fms cells resembles that of wt-v-Fms cells. Sister cultures (5×10^4 cells per well) were starved for 48 h in medium containing 0.5% FCS before [^{3}H]thymidine (100 μCi/ml) was added for 6 h. Trichloroacetic acid-precipitable radioactivity was determined and expressed as the mean value of six independent experiments. (B) Ras is similarly activated in wt-v-Fms and Y807F-v-Fms cells. Sister cultures of normal NIH 3T3 cells (lane 1), wt-v-Fms cells (lane 2), and Y807F-v-Fms cells (lane 3) were grown for 24 h in medium containing 0.5% FCS. Cells were metabolically labeled for 6 h with $^{32}P_i$. Cell lysates were subjected to immunoprecipitation with a monoclonal antibody against Ha-Ras; Ras-bound nucleotides were extracted and analyzed by thin-layer chromatography as detailed in Materials and Methods. Results are expressed as percent bound GTP averaged from triplicate samples.

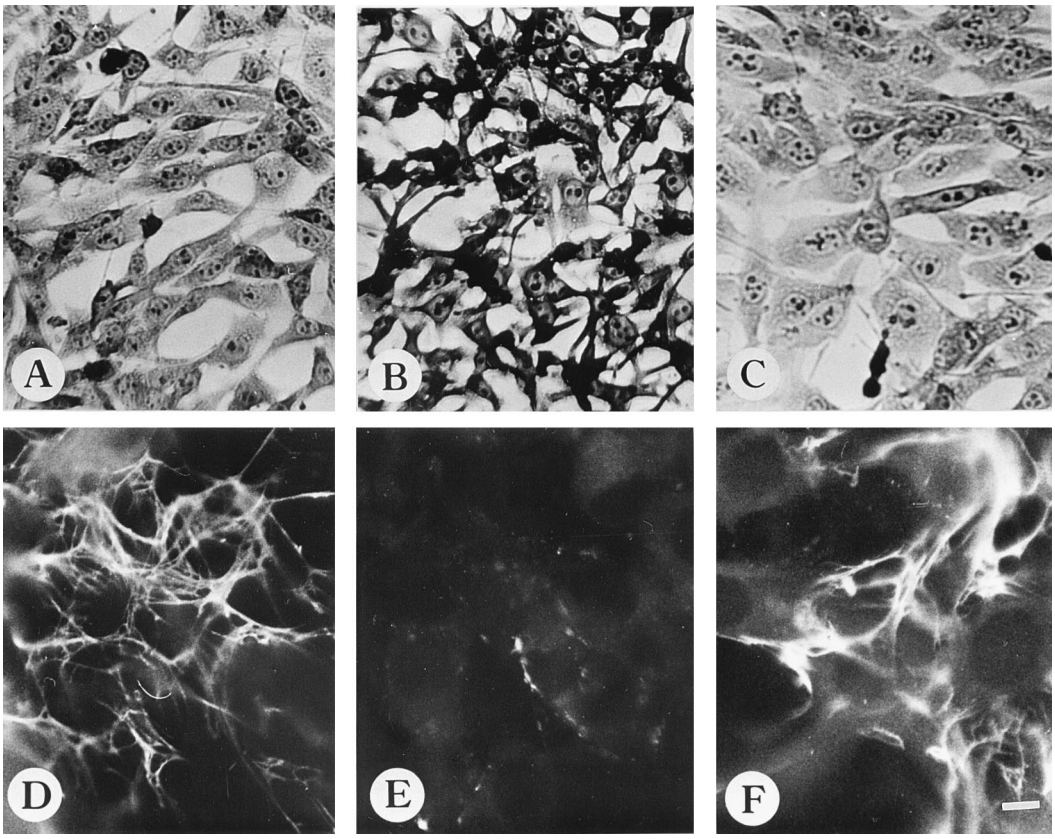

FIG. 3. Y807F-v-Fms cells retain the morphology of nontransformed cells. Nontransformed mouse NIH 3T3 cells (A and D), wt-v-Fms cells (B and E), and Y807F-v-Fms cells (C and F) were chemically fixed 3 days after replating and visualized by Giemsa staining (A to C) or indirect immunofluorescence using an antifibronectin antibody (D to F). The bar represents 10 μm.

1). We further confirmed these findings by mobility shift assays of Raf-1 due to hyperphosphorylation commonly used as a marker for activation of Raf-1. In both cell types, Raf-1 had a lower mobility than Raf-1 from resting cells (data not shown), indicating that the Ras/Raf-1 mitogenic cascade was indeed activated by Y807F v-Fms.

The Y807F mutation of v-Fms abolishes the protein's ability to alter cell morphology. We noticed that all 10 Y807F-v-Fms cell clones differed from wt-v-Fms cells, as they retained a flat morphology commonly observed with normal NIH 3T3 cells (compare Fig. 3A and C). Furthermore, Y807F-v-Fms cells contained an intact fibronectin network (Fig. 3F) and actin cables (Fig. 4C). In contrast, wt-v-Fms cells showed the expected transformed cell morphology (Fig. 3B) and lacked a fibronectin network (Fig. 3E) as well as actin cables (Fig. 4B). We noted that the fibronectin network of Y807F-v-Fms cells

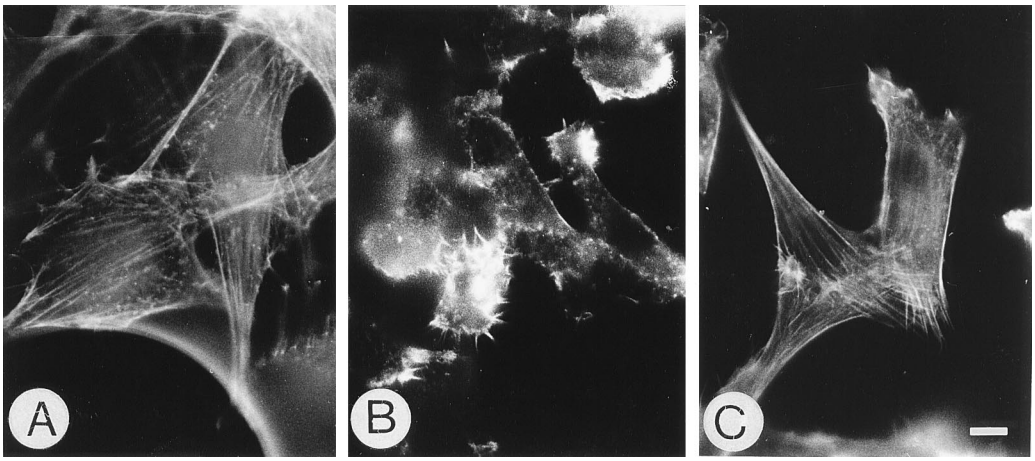

FIG. 4. Y807F-v-Fms cells retain actin cables. Nontransformed mouse NIH 3T3 cells (A), wt-v-Fms cells (B), and Y807F-v-Fms cells (C) were fixed and visualized by FITC-labeled phalloidin to stain actin cables. The bar represents 10 μm.

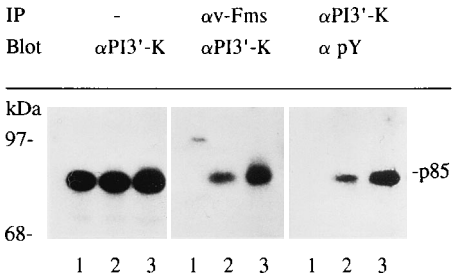

FIG. 5. The Y807F mutation does not abolish association of v-Fms molecules with PI-3′ kinase. Cells were maintained for 24 h in medium containing 0.5% FCS. Equal amounts of cell lysates from nontransformed mouse NIH 3T3 cells (lanes 1), wt-v-Fms cells (lanes 2), and Y807F-v-Fms cells (lanes 3) were subjected to immunoprecipitation (IP) with antibodies as indicated and analyzed by SDS-PAGE. Proteins were identified by immunoblotting (Blot) with specific antibodies. αv-Fms, anti-v-Fms-specific rat serum; αPI3′-K, anti-PI-3′ kinase; αpY, monoclonal antibody against phosphotyrosine (4G10).

reproducibly appeared to be more condensed than that of normal cells. At this moment, however, we have no explanation for this finding.

We conclude that autophosphorylation of the v-Fms molecule at Y807 is required for the induction of cell morphological changes.

Y807F v-Fms molecules associate with PI-3′ kinase. Two lines of evidence in the literature link PI-3′ kinase to receptor tyrosine kinase-mediated signaling. First, PI-3′ kinase associates via its p85 subunit with the M-CSF and PDGF receptors (5, 10, 18, 19, 40, 52). Binding was shown to involve Y-721 of the M-CSF receptor (35, 51) and both Y-740 and Y-751 of the PDGF β receptor (11, 17, 20). In both instances, the p85 subunit becomes phosphorylated on tyrosine in response to treatment of cells with the corresponding growth factor. Second, the substitution of tyrosine residues identified as binding sites reduced the Fms-specific mitogenic potential (51) and prevented PDGF-induced ruffling and chemotaxis (53). We therefore investigated whether the observed inability of the Y807F v-Fms kinase to induce changes in cell morphology correlated with a reduced binding or phosphorylation of PI-3′ kinase (Fig. 5). Interestingly, association and phosphorylation were reproducibly greater in Y807F-v-Fms cells (lanes 3) than in wt-v-Fms cells (lanes 2). The significance of this finding is unclear.

wt v-Fms associates directly with p120RasGAP, and this association is prevented by the Y807F mutation. Previous studies showed that both v- and c-Fms phosphorylated p120RasGAP on tyrosine (9, 16, 34), although stable complexes between these molecules had not been demonstrated. To study whether v-Fms and p120RasGAP molecules indeed formed such complexes, we used sodium orthovanadate, an inhibitor of tyrosine phosphatases, shown previously to prolong the half-life of phosphotyrosines of v-Fms molecules in vivo (47a). Initial experiments indicated that similar levels of p120RasGAP are present in NIH 3T3 cells and cells expressing either the wt or Y807F v-Fms protein (Fig. 6A, lanes 1 to 3). With an antiserum against v-Fms, p120RasGAP was coimmunoprecipitated exclusively from lysates derived from wt-v-Fms cells (lane 5), not from normal NIH 3T3 cells (lane 4) or from Y807F-v-Fms cells (lane 6). We next determined whether gp140$^{v\text{-}fms}$ was detectable in immune complexes generated with anti-p120RasGAP antibodies. For this purpose, we had to perform in vitro kinase assays and to analyze the products by autoradiography, since we could not convincingly identify the Fms polypeptide by immunoblotting. Again, only the wt-v-Fms cells produced detectable levels of gp140$^{v\text{-}fms}$ (Fig. 6B, lane 5), whereas immune complexes from NIH 3T3 cells (lane 4) or Y807F-v-Fms cells (lane 6) were negative. The phosphopeptide migrating with a molecular mass of 120 kDa in lane 5 likely constitutes phosphorylated p120RasGAP, as judged from its slightly enhanced electrophoretic mobility in comparison to gp120$^{v\text{-}fms}$.

To further investigate whether Y-807 was the binding site for p120RasGAP, we used recombinant GST fusion proteins containing residues 757 to 944 of wt v-Fms or of Y807F v-Fms, fused to the C terminus of GST. Phosphorylation of these fusion proteins was achieved by expression in *E. coli* TKX1, which produces an active Elk tyrosine kinase (35). Nonphosphorylated fusion proteins were obtained from standard *E. coli* cells. Although the Fms-specific portion in the fusion protein encompassed eight tyrosine residues, in vitro tyrosine kinase assays with full-length wt v-Fms kinase and subsequent tryptic peptide maps revealed only a single spot which migrated in the position of spot a in panel 5 of Fig. 1B (data not shown). Similar amounts of fusion proteins (Fig. 7A) were then bound to glutathione-agarose beads and used to study binding of p120RasGAP from NIH 3T3 cell lysates. Binding of p120RasGAP was exclusively observed with a phosphorylated fusion protein containing the wt v-Fms sequence (Fig. 7B, lane 2), whereas no binding was observed with the same fusion protein in its nonphosphorylated form (lane 3) or with the corresponding Y807F mutant proteins (lanes 4 and 5). Together, these data again support the idea that Y807 of v-Fms constitutes the p120RasGAP association site.

p120RasGAP-p190RhoGAP complexes are exclusively found in wt-v-Fms cells. The association of p120RasGAP with p190RhoGAP is well documented (1, 9, 16, 27, 28, 32, 42), and the contribution of such complexes in the control of cell morphology has been proposed (26). Figure 8A (lanes 1 to 3) shows that the three cell types used in this study contain similar levels of p190RhoGAP. To determine whether p120-p190

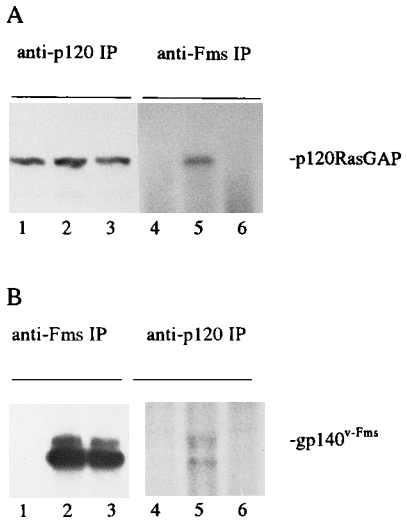

FIG. 6. wt-v-Fms molecules form a complex with p120RasGAP, and this interaction is abolished by the Y807F mutation. Equal amounts of cell lysates from nontransformed mouse NIH 3T3 cells (lanes 1 and 4), wt-v-Fms cells (lanes 2 and 5), and Y807F-v-Fms cells (lanes 3 and 6) were subjected to immunoprecipitation (IP) with antibodies as indicated. (A) Proteins present in the immune complexes were identified after SDS-PAGE by immunoblotting with an anti-p120RasGAP antibody. (B) Immune complexes were further incubated with an anti-v-Fms specific rat serum and washed, and an in vitro kinase reaction was performed. Samples were analyzed by SDS-PAGE and autoradiography.

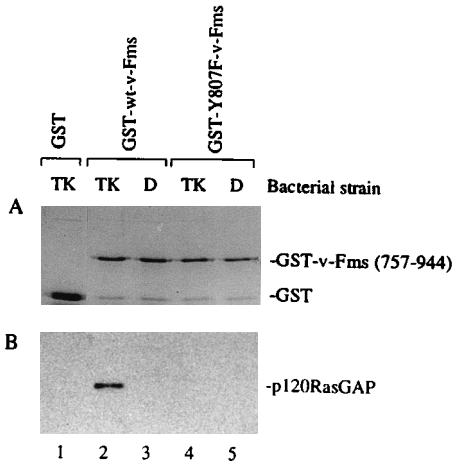

FIG. 7. Binding of p120RasGAP to recombinant GST–v-Fms fusion proteins. (A) GST, GST–wt v-Fms, and GST–Y807F v-Fms fusion proteins were produced in bacterial *E. coli* TKX1 (TK) or DH5α (D) to generate phosphorylated or nonphosphorylated molecular species, respectively. Equal amounts of protein were bound to glutathione-agarose beads, and aliquots of the beads were analyzed by SDS-PAGE and Coomassie brilliant blue staining. (B) Precharged beads were incubated with cell lysates derived from 10^7 resting NIH 3T3 cells as detailed in Materials and Methods and analyzed by SDS-PAGE followed by immunoblotting with monoclonal antibody 6F2.

complexes were detectable and affected by the Y807F mutation, we performed immunoprecipitations with an anti-p120RasGAP antibody and determined the presence of p190RhoGAP by immunoblotting with a monoclonal anti-p190 antibody. Interestingly, p190RhoGAP was precipitable with the p120 antiserum only from wt-v-Fms cells, whereas normal cells or Y807F-v-Fms cells contained no or significantly less

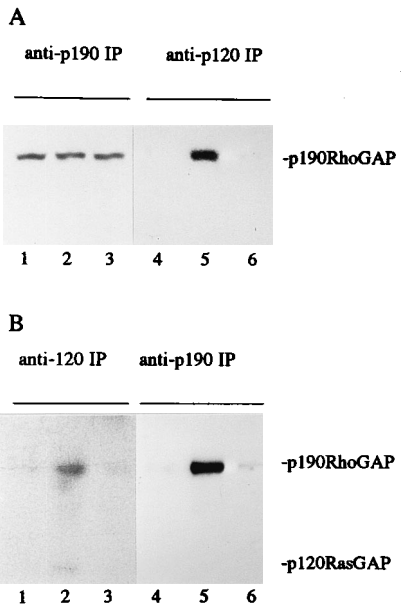

FIG. 8. p120RasGAP and p190RhoGAP form complexes in wt-v-Fms cells. Equal amounts of cell lysates from nontransformed mouse NIH 3T3 cells (lanes 1 and 4), wt-v-Fms cells (lanes 2 and 5), and Y807F-v-Fms cells (lanes 3 and 6) were subjected to immunoprecipitation (IP) with antibodies as indicated. Proteins present in the immune complexes were identified after SDS-PAGE by immunoblotting with an anti-p190RhoGAP antibody (A) or a monoclonal antibody against phosphotyrosine (B).

TABLE 2. RhoGAP activity in p120RasGAP- and p190RhoGAP-specific immunoprecipitates from various cell lines

Immune serum	Activity[a] (mean ± SE)			
	NIH 3T3 cells	wt-v-Fms cells	Y807F-v-Fms cells	Buffer control
Negative control IgG	82 ± 11	85 ± 8	85 ± 5	83 ± 9
Anti-p120RasGAP	78 ± 9	52 ± 7	84 ± 10	86 ± 6
Anti-p190RhoGAP	75 ± 7	42 ± 13	88 ± 4	87 ± 9

[a] Determined after 10 min of incubation with GTP-labeled recombinant RhoA. Values are expressed as percent bound GTP remaining (n = 6) as detailed in Materials and Methods.

coprecipitable p190. The analysis of such immune complexes with a monoclonal antibody against phosphotyrosine revealed that p120 was only poorly phosphorylated in comparison with p190 (Fig. 8B, lane 2). This finding is not surprising, considering that phosphorylation of p120 is not required for its binding to p190, since a point mutation or a deletion of the tyrosine phosphorylation site in p120 has no consequences for binding (26, 32). The notion that the degree of p190RhoGAP phosphorylation was drastically enhanced in wt-v-Fms cells was further supported by immunoprecipitations with an anti-p190RhoGAP antibody (Fig. 8B, lanes 4 to 6). The absence of phosphorylated p120RasGAP in such p190 immune complexes (lane 5) is not understood. Whereas our data clearly show that p120-p190 complexes form preferentially in wt-v-Fms cells and that p190 becomes phosphorylated on tyrosine, the nature of the kinase is unclear, and it is possible that another tyrosine kinase acting downstream from Fms is involved in this reaction.

p190RhoGAP activity is absent in Y807F-v-Fms cells. To determine whether the complex formation of p190RhoGAP with p120RasGAP specifically seen in wt-v-Fms cells but not in Y807F-v-Fms cells was paralleled by distinct RhoGAP activities, we generated immune complexes with anti-p120RasGAP or anti-p190RhoGAP and tested them for the ability to enhance the GTPase activity of recombinant RhoA in vitro (13, 26). As expected, no significant GAP activity was observed with immune complexes from resting NIH 3T3 cells, whereas both types of precipitates from wt-v-Fms cells increased in the hydrolysis of RhoA-bound GTP about threefold (Table 2). In contrast, precipitates from Y807F-v-Fms cells lacked detectable RhoGAP activity, again supporting the idea that phosphorylation of Y-807 is indeed essential for a Fms-induced formation of active p190RhoGAP.

Distinct subcellular distribution of p190RhoGAP in Y807F-v-Fms cells and in wt-v-Fms cells. RhoA functions in its GTP-bound form at the plasma membrane in the generation of stress fibers and focal adhesions, causing the reorganization of the cytoskeleton after mitogenic stimulation (36). The experiments described so far showed that p190RhoGAP was differentially phosphorylated and activated in wt-v-Fms and Y807F-v-Fms cells. We next investigated whether p190RhoGAP and p120RasGAP had an altered subcellular distribution in normal NIH 3T3 cells, wt-v-Fms cells, and Y807F-v-Fms cells. For this purpose, we used monoclonal and polyclonal antibodies (4), both of which produced essentially the same results (Fig. 9 and data not shown). In all three cell types, p120RasGAP was distributed throughout the cytoplasm (Fig. 9A, C, and E). p190RhoGAP was detected in perinuclear regions of resting NIH 3T3 cells (Fig. 9B). By contrast, in wt-v-Fms cells, p190 was dispersed into the cytosol (Fig. 9D). As shown above, this distribution correlates with the formation of p120-p190 complexes and with the tyrosine phosphorylation of p190 in these

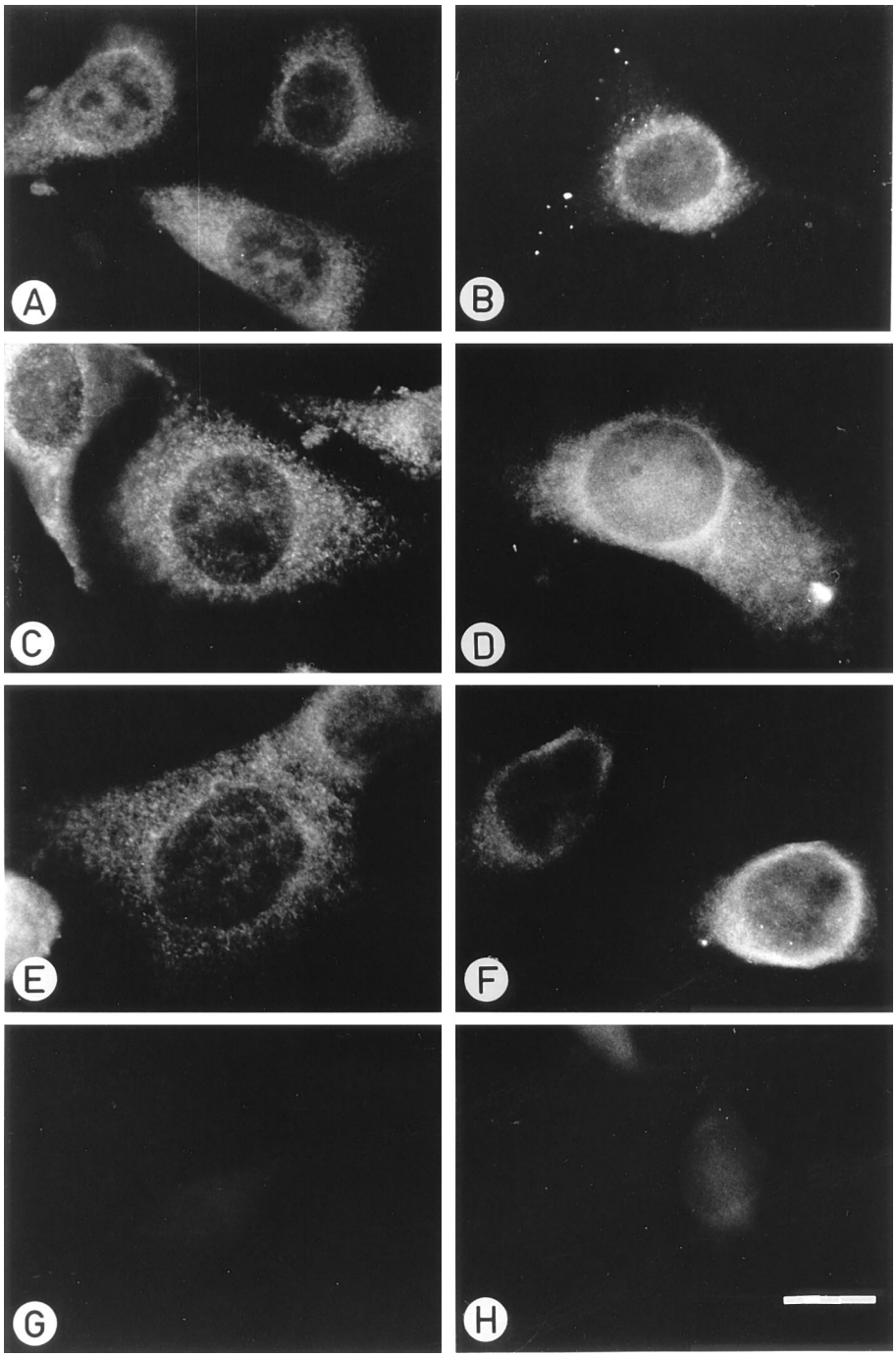

FIG. 9. Spreading of p190RhoGAP into the cytoplasm of wt-v-Fms cells is abolished by the Y807F mutation. Nontransformed mouse NIH 3T3 cells (A, B, G, and H), wt-v-Fms cells (C and D), and Y807F-v-Fms cells (E and F) were maintained for 24 h in medium containing 0.5% FCS, chemically fixed, and visualized by indirect immunofluorescence using monoclonal antibodies against p120RasGAP (A, C, and E) and against p190RhoGAP (B, D, and F). To demonstrate the specificity of the antibodies, the same staining protocol was used but either anti-p120RasGAP (panel G) or anti-p190RhoGAP (panel H) was omitted. The bar represents 10 μm.

cells. In Y807F-v-Fms cells kept under low serum, p190 was trapped again in perinuclear regions (Fig. 9F). It is intriguing to speculate that p190RhoGAP, activated through phosphorylation and/or binding of p120RasGAP (26), blocks RhoA activity at the plasma membrane, thus preventing the reorganization of the cytoskeleton. This model would agree with our findings that a mutant v-Fms molecule that fails to associate with p120RasGAP fails to induce transformation-specific morphological changes.

DISCUSSION

Some viral oncogenes, such as v-*fms*, v-*kit*, and v-*erbB*, encode activated tyrosine kinases that are structurally related to cellular growth factor receptors. The M-CSF receptor, encoded by the c-*fms* gene, exhibits tyrosine kinase activity only upon dimerization caused by binding of M-CSF. Prior to rapid internalization and degradation, autophosphorylation of the receptor molecules at multiple sites generates phosphotyrosine

residues that constitute specific binding sites for cellular effector proteins with SH2 domains. These may transduce a variety of mitogenic, biochemical, or morphogenic signals in a highly regulated and strictly ligand-induced manner (21, 49). In contrast, the v-Fms-specific tyrosine kinase encoded by feline sarcoma virus is constitutively activated and, although autophosphorylation apparently involves the same tyrosine residues, forces cells into the transformation-specific phenotype, characterized by a drastically enhanced mitogenic activity and a depolymerization of the cytoskeleton.

We report here that Y807F-v-Fms cells, expressing a mutant v-Fms molecule with a single tyrosine-to-phenylalanine mutation at position 807, resembled wt-v-Fms cells in in vivo and in vitro tyrosine kinase activity and in overall mitogenic activity, as further determined by thymidine incorporation and estimation of levels of activated Ras and Raf-1 molecules. The morphology of Y807F-v-Fms cells, however, remained the same as in nontransformed cells, as judged by the presence of actin filaments and the fibronectin network. We demonstrate that wt-v-Fms molecules form tight complexes with p120RasGAP, whereas the Y807F-v-Fms molecules fail to do so. We propose that the interaction between wt v-Fms and p120RasGAP or with some yet undefined cellular protein(s) triggers the release of p190RhoGAP from a perinuclear pool by an unknown mechanism, resulting in the formation of complexes between p120RasGAP and p190RhoGAP and the simultaneous phosphorylation of the latter on tyrosine. Phosphorylation and/or complex formation with p120RasGAP enhances the p190-specific RhoGAP activity, facilitating GTP hydrolysis of GTP-bound RhoA and its inactivation.

These findings have implications for the understanding of individual signaling cascades originating from the wt-v-Fms molecule that lead to cell transformation. First, our data allow the conclusion that the mitogenic activation of the Ras/Raf-1 cascade by v-Fms occurs independently of a morphogenic signaling cascade and that these two cascades can be uncoupled from each other. Second, they directly link the transformation-specific morphological alterations induced by wt v-Fms molecules to the interaction with p120RasGAP and p190RhoGAP.

A series of control experiments performed in this study exclude the possibility that the observed effects can be attributed either to different expression levels or to a reduced tyrosine kinase activity. The Y807F v-Fms tyrosine kinase activity was unaltered in vivo, as judged by autophosphorylation and phosphorylation of PI-3′ kinase, and unaltered in vitro, as determined again by autophosphorylation or phosphorylation of an exogenous GST fusion protein. This leaves us with the conclusion that the above-mentioned effects induced by the removal of a particular autophosphorylation site must be ascribed to an altered interaction of the mutant v-Fms molecule with specific cellular proteins.

Several observations suggest that the interactions with those proteins that are likely to initiate the mitogenic cascade, GRB2 and Sos, are not affected by the Y807F mutation: the binding site for GRB2 has been mapped to Y-697 of the c-Fms protein (51), and the corresponding site was not altered in this study. Furthermore, Ras, acting downstream from GRB2 and Sos (2, 8, 12, 22, 24, 41), was activated in Y807F-v-Fms cells to levels similar to those observed in wt-v-Fms cells. Indeed, the mutant cells showed no significant difference in their overall growth properties (Table 1 and Fig. 2A).

The most surprising effect caused by the Y807F mutation of v-Fms in this study was the loss of the ability of the mutant molecule to induce detectable transformation-specific morphologic changes. This fact suggests that the v-Fms-specific activation of the Ras/Raf-1 signaling cascade does not automatically cause the v-Fms-specific depolymerization of the cytoskeleton.

What other cellular effector molecules interacting with wt v-Fms but not with Y807F v-Fms could be involved in the control of the cytoskeleton? We showed here that the p85 subunit of PI-3′ kinase associated even more strongly with the mutant than with the wt Fms protein, and in addition, the level of its phosphorylation was clearly enhanced in Y807F v-Fms cells. The consequences of this enhanced binding, however, remain enigmatic. In addition to containing an SH3 domain in the N-terminal region and two SH2 domains in the C-terminal region, p85 contains an intervening RhoGAP domain. Experiments designed to demonstrate a RhoGAP activity in immunoprecipitates generated with a v-Fms- or p85-specific antiserum were unsuccessful (data not shown). At this stage, we cannot exclude, however, that the enhanced interaction between p85 and Y807F-v-Fms molecules contributes in some unknown way to the establishment of an intact cytoskeleton.

Another striking difference observed between wt v-Fms and Y807F v-Fms concerned their opposite abilities to form a complex with p120RasGAP and to phosphorylate this species in tyrosine. This association was observed exclusively with wt v-Fms molecules and required the presence of sodium orthovanadate, i.e., conditions that stabilize phosphotyrosine residues of v-Fms (47a). Do these findings imply that the Y807 autophosphorylation site of v-Fms constitutes the p120Ras GAP binding site? In the PDGF β receptor, the p120RasGAP binding site has been mapped to Y-771, located in the kinase insert domain (11, 20). This is clearly different from findings for the Fms molecules that lack a tyrosine residue in the corresponding position: the bacterially expressed mouse c-Fms kinase insert domain failed to bind p120RasGAP (35), and a deletion of the entire domain of mouse c-Fms did not affect tyrosine phosphorylation of p120RasGAP in NIH 3T3 cells (34). These reports exclude the possibility that p120RasGAP binding occurs within the kinase insert domain of Fms and suggest that Y-807 could indeed be a good candidate. This possibility is further supported by our experiments with a bacterially expressed and phosphorylated GST–v-Fms fusion protein containing the second half of the kinase domain (amino acid residues 759 to 944). This fusion protein contained eight Fms-specific tyrosine residues and was capable of binding p120RasGAP, whereas a corresponding fusion protein carrying the Y807F mutation could not. Furthermore, the wt v-Fms fusion protein was phosphorylated in vitro by the intact wt v-Fms tyrosine kinase only at a single position, and tryptic peptide maps indicated that phosphorylation involved Y-807. Together, these data provide strong additional evidence that Y-807 of v-Fms indeed constitutes the p120RasGAP binding site.

As a consequence of the Y807F mutation, p190RhoGAP acquired a subcellular distribution in perinuclear regions, normally seen only in resting cells that were maintained for some time in the absence of serum factors. What mechanism(s) cause p190 to accumulate in these regions, and what mechanisms allow it to diffuse throughout the cytosol when cells are exposed to growth factors? The observation that the intracellular distribution of p190 changes in response to a single amino acid substitution of v-Fms which affects neither the Fms-specific tyrosine kinase nor the cellular Ras activity suggests that neither of the two activities alone can be the reason for the altered distribution of p190RhoGAP. It is intriguing to speculate that Fms-bound or -primed p120RasGAP triggers a signal causing the redistribution of p190RhoGAP. In this respect, it would be important to identify the intracellular location where the p120-p190 complexes are formed. We have repeat-

edly failed to see significant amounts of p190RhoGAP in anti-v-Fms immunoprecipitates, suggesting that ternary complexes between v-Fms, p120, and p190 either do not exist or are too unstable or short-lived. Moran et al. (28) reported that v-*src*-transformed cells contained the majority of p120-p190 complexes in the cytosol and only a minor population in the membrane-associated fraction. The molecular principles underlying the formation of p120-p190 complexes remain to be unraveled. Our current working hypothesis rests on a direct interaction between the Y-807 autophosphorylation site and an SH2 domain of p120RasGAP, thereby allowing subsequent binding of p190RhoGAP. Binding of p190 to the wt v-Fms–p120 complex would result in tyrosine phosphorylation of p190 through either v-Fms itself or yet another undefined tyrosine kinase acting downstream from Fms. Tyrosine phosphorylation of p190 generates an alternative binding site(s) for the SH2 domains of p120, thereby stabilizing the p120-p190 complex. Simultaneously, such complex formation would reduce the p120-specific RasGAP activity toward p21Ras (28) and activate the RhoGAP activity of p190 (26), thus facilitating inactivation of Rho. In line with these data, McGlade et al. (26) reported that the association of p190 with an N-terminally truncated p120RasGAP variant (residues 1 to 445, containing two SH2 domains and one SH3 domain but lacking the Ras GTPase-activating domain) is accompanied by the presence of RhoGAP activity in the immune complex. The same authors (26) showed that expression of the N-terminal domain of p120RasGAP caused drastic effects on the cytoskeleton, including a disruption of stress fibers, a reduction in focal contacts, and an impaired ability to adhere to fibronectin without alteration of DNA synthesis and the rate of cell doubling. The authors demonstrated that the truncated p120RasGAP species retained the ability to associate with p190RhoGAP and suggested that the observed effects resulted from the activity of p190RhoGAP. In line with this hypothesis, microinjection of p190RhoGAP into NIH 3T3 cells inhibited Rho-mediated stress fiber formation (38). Effects similar to those caused by an active p190RhoGAP are generally observed in Fms-transformed cells.

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