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Interactions of Phosphatidylinositol Kinase, GTPase-Activating Protein (GAP), and GAP-Associated Proteins with the Colony-Stimulating Factor 1 Receptor

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The interactions of the macrophage colony-stimulating factor 1 (CSF-1) receptor with potential targets were investigated after ligand stimulation either of mouse macrophages or of fibroblasts that ectopically express mouse CSF-1 receptors. In Rat-2 cells expressing the mouse CSF-1 receptor, full activation of the receptor and cellular transformation require exogenous CSF-1, whereas NIH 3T3 cells expressing mouse c-*fms* are transformed by autocrine stimulation. Activated CSF-1 receptors physically associate with a phosphatidylinositol (PI) 3′-kinase. A mutant CSF-1 receptor with a deletion of the kinase insert region was deficient in its ability to bind functional PI 3′-kinase and to induce PI 3′-kinase activity precipitable with antiphosphotyrosine antibodies. In fibroblasts, CSF-1 stimulation also induced the phosphorylation of the GTPase-activating protein (GAP)-associated protein p62 on tyrosine, although GAP itself was a relatively poor substrate. In contrast to PI 3′-kinase association, phosphorylation of p62 and GAP was not markedly affected by deletion of the kinase insert region. These results indicate that the kinase insert region selectively enhances the CSF-1-dependent association of the CSF-1 receptor with active PI 3′-kinase. The insert deletion mutant retains considerable transforming activity in NIH 3T3 cells (G. Taylor, M. Reedijk, V. Rothwell, L. Rohrschneider, and T. Pawson, EMBO J. 8:2029–2037, 1989). This mutant was more seriously impaired in Rat-2 cell transformation, although mutant-expressing Rat-2 cells still formed small colonies in soft agar in the presence of CSF-1. Therefore, phosphorylation of GAP and p62 through activation of the CSF-1 receptor does not result in full fibroblast transformation. The interaction between the CSF-1 receptor and PI 3′-kinase may contribute to c-*fms* fibroblast transformation and play a role in CSF-1-stimulated macrophages.

The macrophage colony-stimulating factor 1 (CSF-1) induces monocyte differentiation, is required for the survival of macrophages, and promotes mature macrophage cell functions (34, 39). The CSF-1 receptor (CSF-1R) is a transmembrane protein-tyrosine kinase encoded by the proto-oncogene c-*fms* (31). c-*fms* expression is restricted to hematopoietic cells of the monocyte lineage (49) and trophoblastic cells of the embryonic placenta (24, 25), suggesting that CSF-1 may be important in the function of extraembryonic tissue as well as in hematopoiesis.

The CSF-1R belongs to a class of receptorlike tyrosine kinases whose catalytic domains are characterized by a stretch of 60 to 110 amino acids in addition to the residues found in all tyrosine and serine/threonine-specific protein kinases. This kinase insert is located in the primary structure between the presumptive ATP-binding fold and the phosphotransfer region. The group of tyrosine kinases containing a kinase insert include the α- and β-type platelet-derived growth factor (PDGF) receptors (PDGFR) (16, 50), the basic fibroblast growth factor receptor (13), the *flt* tyrosine kinase (32), the product of the c-*kit* gene (51), which is allelic with mouse *W* developmental locus (2), and the *torso* protein responsible for formation of anterior and posterior terminal structures in the *Drosophila* embryo (33). Although the sequences of these kinase inserts are not closely related, it seems likely that they have similar functions, possibly related to the recognition of substrates for tyrosine phosphorylation. We have previously found that deletions within the kinase insert of the CSF-1R, or its oncogenic counterpart gp140$^{v\text{-}fms}$, do not affect in vitro kinase activity for the exogenous substrate poly(Glu-Tyr) (36). The insert is apparently exposed on the surface of the protein and hence well positioned to interact with substrates (36).

Recent work has identified several potential targets of activated tyrosine kinases, including p21^{ras} GTPase-activating protein (GAP) (6, 19), phospholipase Cγ (PLCγ) isoforms 1 and 2 (15, 18, 43, 44), phosphatidylinositol (PI) 3′-kinase (10, 46), and the protein-serine/threonine kinase Raf-1 (21, 22). Two of these proteins, GAP and PLCγ, each contain two copies of the SH2 domain implicated in protein-protein interactions of tyrosine kinases and their substrates (23; M. Moran, C. A. Koch, D. Anderson, C. Ellis, L. England, G. S. Martin, and T. Pawson, Proc. Natl. Acad. Sci. USA, in press); indeed, all four proteins physically associate with activated PDGFR (9). The kinase insert region of the β-PDGFR is required for the efficient binding of GAP (12) and PI 3′-kinase (3) to activated receptors and for PDGF-induced DNA synthesis (7). Autophosphorylation of Tyr-751 within the kinase insert of the human β-PDGFR is apparently important to these interactions, since its substitution with phenylalanine decreases the affinity of ligand-stimulated receptors for both GAP and PI 3′-kinases (11, 12). The CSF-1R also associates with PI 3′-kinase (41), but unlike both the PDGF and epidermal growth factor receptors, the CSF-1R does not phosphorylate or bind PLCγ or induce hydrolysis of conventional PI-4,5-P_2 (5). We have therefore investigated the interactions of activated CSF-1R with GAP

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and PI 3′-kinase and the contribution of the kinase insert to the biological and biochemical properties of activated CSF-1R.

MATERIALS AND METHODS

Cell culture. Rat-2 and NIH 3T3 cells expressing wild-type (wt) c-*fms* or the kinase insert deletion mutant Δc-*fms* were established by calcium phosphate transfection as previously described (36). Bone marrow macrophages were prepared as described by Tushinski et al. (39). BAC1.2F5 cells (courtesy of Richard Stanley) were cultured as detailed elsewhere (20). Assays for growth in soft agar were performed essentially as described by Weinmaster et al. (45). Where indicated, 10^{-8} M human recombinant CSF-1 (courtesy of Gordon Wong, Genetics Institute) was included in the soft agar culture medium without further supplementation during the growth period. Colonies were photographed 10 days after seeding. To examine monolayer morphology, cells were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and, where indicated, with 10^{-8} M CSF-1. Photographs were taken 48 h after addition of CSF-1.

Metabolic labeling and immunoprecipitation. Fibroblasts were metabolically labeled with [^{35}S]methionine and *fms* protein was immunoprecipitated with 4067-B3 anti-c-*fms* antibodies as previously described (36). BAC1.2F5 cells and bone marrow macrophages were similarly labeled in the absence of CSF-1. Immunoprecipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography.

Western immunoblotting. To examine in vivo tyrosine phosphorylation of CSF-1R or of GAP and associated proteins, subconfluent cultures of Rat-2, Rat-2 cells expressing mouse c-*fms* (R2C1), and Rat-2 cells expressing the kinase insert deletion mutant Δc-*fms* (R2ΔC) were incubated in DMEM supplemented with 0.5% FBS for 72 h to induce quiescence. Cells were stimulated for the indicated times with 10^{-8} M CSF-1, followed by detergent lysis. To examine GAP, p62, and p190 in parental or *fms*-expressing NIH 3T3 cells, cultures were grown to 90% confluence, followed by lysis in 1 ml of 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 EGTA–10 μg of aprotinin ml^{-1}–10 μg of leupeptin ml^{-1}–1 mM phenylmethylsulfonyl fluoride–200 μM sodium orthovanadate–10 mM pyrophosphate–100 mM sodium fluoride. Cell lysates were centrifuged for 30 min at 10,000 × *g*, and protein concentrations of the supernatants were determined with a Lowry protein assay kit (Sigma Chemical Co.). Equivalent amounts of protein in a 1-ml volume were incubated for 90 min at 4°C with either 4067-B3 antibodies or anti-GAP(171-448) antibodies (6) and 100 μl of 20% protein A-Sepharose. The immune complexes were washed three times with 20 mM HEPES (pH 7.5)–10% glycerol–0.1% Triton X-100–150 mM NaCl–1 mM sodium orthovanadate, heated in sodium dodecyl sulfate sample buffer, separated by gel electrophoresis, and immunoblotted with either anti-GAP(171-448) antibodies or affinity-purified antiphosphotyrosine antibodies. Polyclonal rabbit anti-GAP and antiphosphotyrosine antibodies were prepared as previously described (6).

Immunoprecipitation of PI kinase activity. Confluent plates of primary bone marrow macrophages or BAC1.2F5 cells were grown in the absence of CSF-1 for 18 h to induce quiescence. Fibroblastic cell lines were starved in DMEM containing 0.5% FBS for 72 h. Quiescent cells were stimulated with human recombinant CSF-1 at a final concentration of 10^{-8} M. After incubation at 37°C for the indicated time periods, cells were lysed in 1 ml of 20 mM Tris hydrochloride (pH 7.5)–150 mM NaCl–1% Nonidet P-40–5 mM EDTA–10 μg of aprotinin ml^{-1}–10 μg of leupeptin ml^{-1}–1 mM phenylmethylsulfonyl fluoride–200 μM sodium orthovanadate. Cell lysates were centrifuged for 30 min at 10,000 × *g*, and the supernatants were immunoprecipitated with either 4067-B3 antibodies or affinity-purified antiphosphotyrosine antibodies and protein A-Sepharose. The immunoprecipitates recovered from 10^6 cells were washed and assayed for in vitro PI kinase activity exactly as described by Fukui and Hanafusa (8). In some cases, the inhibitor 100 μM adenosine or 0.5% Triton X-100 was added to the assay buffer (46). The unlabeled lipid markers PI (Sigma) and PI-4-monophosphate (Boehringer Mannheim) were visualized by exposing the thin-layer chromatography plate to I_2 vapor. To determine the relative levels of PI kinase activity, PI-3-monophosphate spots were scraped from the plates and analyzed for ^{32}P by liquid scintillation counting.

RESULTS

The c-*fms* kinase insert is required for efficient transformation of Rat-2 fibroblasts. Expression of mouse c-*fms* in NIH 3T3 mouse fibroblasts induces cellular transformation, apparently as a result of autocrine stimulation of the CSF-1R by endogenous CSF-1 (27). We previously found that a mutant CSF-1R lacking 64 residues of the kinase insert (Δc-*fms*) was still able to induce morphological transformation of mouse NIH 3T3 fibroblasts, although such cells did not grow well in soft agar (36). Previous work has shown that the transforming activity of the human CSF-1R in NIH 3T3 cells is dependent on exogenously added human CSF-1, reflecting the fact that mouse CSF-1 does not bind to the human receptor (28). In addition, the ability of the feline CSF-1R to induce Rat-2 cells to form colonies in soft agar is dependent on added human CSF-1 (48). To investigate the biological function of the kinase insert region, we sought a system in which activity of the mouse CSF-1R could be induced by ligand and in which a clear distinction could be obtained between the wt c-*fms* and the Δc-*fms* mutant. Both criteria were fulfilled by Rat-2 fibroblasts.

Mammalian expression vectors containing either c-*fms* or Δc-*fms* cDNAs under the control of the simian virus 40 promoter were transfected into Rat-2 fibroblasts, and clones were selected for resistance to G418. Several cell lines were established which express levels of the c-*fms* or Δc-*fms* glycoproteins similar to those seen in the transformed NIH 3T3 cell lines (Fig. 1A). However, stable expression of c-*fms* or Δc-*fms* in Rat-2 cells did not induce morphological transformation or growth in soft agar, although cells expressing c-*fms* became somewhat more fusiform (Fig. 2). Addition of 10^{-8} M CSF-1 to the medium of Rat-2 cells expressing wt c-*fms* (R2C1) induced receptor autophosphorylation within 2 min (Fig. 1B), and a marked morphological transformation such that within 48 h the cells became highly fusiform, refractile, and overgrown (Fig. 2). In contrast, Rat-2 cells expressing Δc-*fms* (R2ΔC) retained an apparently normal phenotype after the addition of CSF-1 (Fig. 2), although they still underwent receptor autophosphorylation (Fig. 1B). Although the Δc-*fms* gene product has lost the autophosphorylation sites in the insert, it retains the autophosphorylation site at Tyr-807 (35) and might possess novel phosphorylation sites. The Δc-*fms* mutant was also impaired by fivefold in the induction of anchorage-independent growth in soft agar.

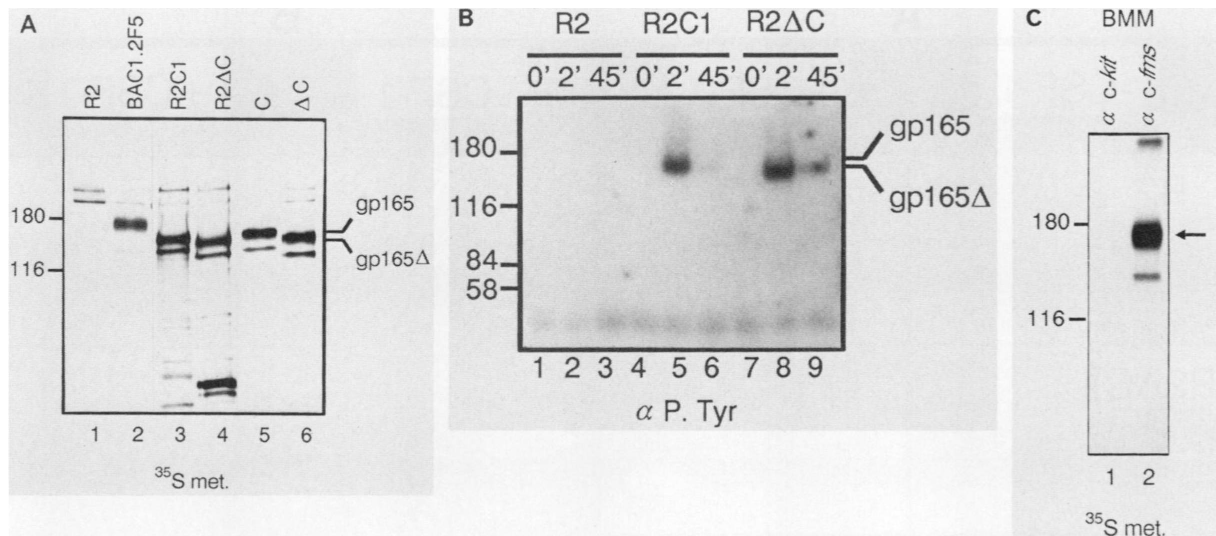

FIG. 1. Expression and tyrosine phosphorylation of wt and mutant CSF-1R. (A) Cells were labeled with [^{35}S]methionine for 2 h, and lysates were immunoprecipitated with anti-c-*fms* antiserum 4067-B3 as follows: Rat-2 parental cell line (lane 1); BAC1.2F5 macrophage cell line (lane 2); Rat-2 cells expressing wt mouse c-*fms* (lane 3); Rat-2 cells expressing Δc-*fms* (lane 4); NIH 3T3 cells expressing c-*fms* (lane 5); and NIH 3T3 cells expressing Δc-*fms* (lane 6). (B) Cell lysates were prepared from Rat-2 (R2) cells, Rat-2 cells expressing mouse c-*fms* (R2C1), or Rat-2 cells expressing the kinase insert mutant Δc-*fms* (R2ΔC). Cells were either unstimulated (lanes 1, 4, and 7) or stimulated with CSF-1 for 2 min (lanes 2, 5, and 8) or 45 min (lanes 3, 6, and 9). Lysates were immunoprecipitated with 4067-B3 antiserum. Immunoprecipitated c-*fms* proteins were electrophoresed through a 7.5% sodium dodecyl sulfate-polyacrylamide gel, transferred to nitrocellulose, and blotted with affinity-purified antiphosphotyrosine antibodies (α P. Tyr.) and ^{125}I-protein A. The mobilities of the mature c-*fms* (gp165) and Δc-*fms* (gp165Δ) glycoproteins are indicated. (C) [^{35}S]methionine-labeled primary bone marrow macrophage (BMM) cell lysates were immunoprecipitated with either anti-c-*kit* antiserum (26) (α c-*kit*) or with antiserum 4067-B3 (α c-*fms*). Mobilities of 180-, 116-, 84-, and 58-kDa molecular mass markers are indicated.

R2C1 cells formed anchorage-independent colonies in the presence of 10^{-8} M CSF-1 with an efficiency of approximately 10%, whereas R2ΔC cells formed much smaller colonies with an efficiency of 2% (Fig. 2). These observations were reproducible in each of four lines of both c-*fms*- and Δc-*fms*-expressing Rat-2 cells. These results indicate that Rat-2 fibroblasts provide a more stringent test of murine c-*fms* transforming activity than do NIH 3T3 cells. The Δc-*fms* mutant is clearly defective in CSF-1-dependent transformation of Rat-2 cells, although it retains the ability to induce modest anchorage-independent growth. The dependence of wt c-*fms* biological and kinase activity on added CSF-1 suggests that, in contrast to NIH 3T3 cells, Rat-2 cells do not synthesize sufficient levels of CSF-1 to fully stimulate the receptor.

Activated CSF-1R binds and stimulates PI kinase in macrophages. These data indicating a difference between the transforming potencies of wt c-*fms* and Δc-*fms* prompted us to examine the activities of the CSF-1R tyrosine kinase in fibroblasts and macrophages and the biochemical consequences of deleting the kinase insert region. A number of tyrosine kinases, including the CSF-1R, are reported to activate and associate with a PI 3'-kinase activity (1, 8, 14, 29, 41, 46, 47) tentatively assigned to an 85-kDa phosphotyrosine-containing protein (4, 10). Lysates of mouse macrophages, parental NIH 3T3 cells, or 3T3 cells expressing wt c-*fms* or Δc-*fms* were immunoprecipitated either with antiphosphotyrosine antibodies or with an anti-*fms* antiserum that recognizes the wild-type and mutant CSF-1 receptors with equivalent efficiency. These immunoprecipitates were then incubated in in vitro PI kinase assays, and the phosphorylated product was resolved by chromatography (Fig. 3A). Two minutes after addition of recombinant CSF-1 to BAC1.2F5 mouse macrophage cells (20), which express endogenous CSF-1R (Fig. 1A, lane 2), we observed a 50-fold increase in the PI kinase activity in antiphosphotyrosine immunoprecipitates (Fig. 3A, lanes 1 and 2). The PI kinase activity precipitable with anti-*fms* antibodies, and hence associated with the CSF-1R, increased 20-fold in a similar 2-min stimulation (Fig. 3A, lanes 3 and 4). Mouse primary bone marrow macrophages also demonstrated an elevation in CSF-1R-associated PI kinase activity after a 2-min stimulation with CSF-1, which returned to basal levels within 30 min (Fig. 3A, lanes 5 to 7). These cultured bone marrow cells were shown to express the CSF-1R but not the c-*kit* gene product (Fig. 1C) and to possess macrophage-specific markers (data not shown), consistent with a macrophage phenotype. The difference in the mobility of the CSF-1R in macrophages as compared with Rat-2 fibroblasts is presumably due to a difference in receptor processing between the two cell types, most likely involving the extent of glycosylation. These results are in agreement with those of Varticovski et al. (41) and in addition demonstrate that the association of PI 3'-kinase with the CSF-1R is likely a physiological response in macrophages stimulated with CSF-1.

The kinase insert region is required for the association of CSF-1R with active PI 3'-kinase in fibroblasts. Fibroblast cell lines were used to explore the possibility that the c-*fms* kinase insert is required for the interaction of the CSF-1R with PI kinase. In lysates of NIH 3T3 cells transformed by wild-type c-*fms*, the total PI kinase activity precipitable with antiphosphotyrosine antibodies was increased fivefold compared with that of parental NIH 3T3 cells (Table 1). In contrast, NIH 3T3 cells morphologically transformed by Δc-*fms* showed only a 1.7-fold elevation in precipitable PI kinase activity (Fig. 3A, lanes 8 to 10). Furthermore,

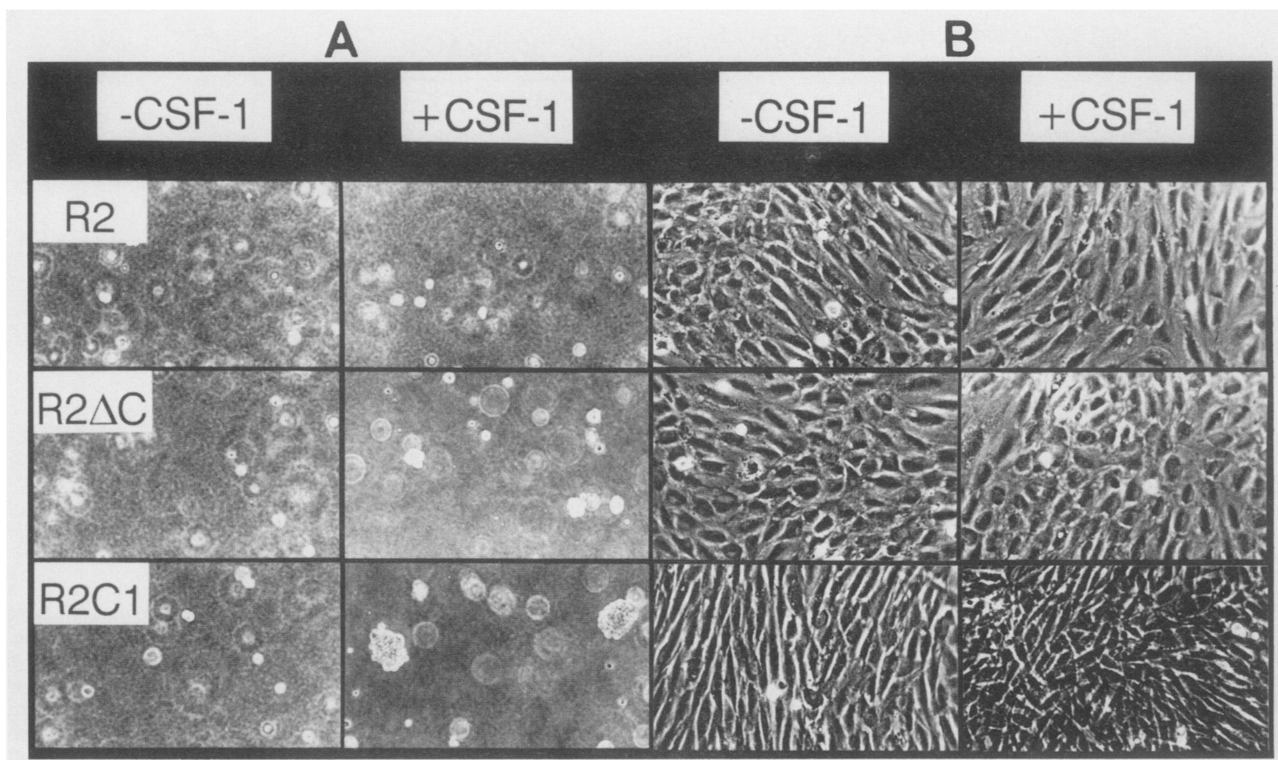

FIG. 2. Morphology and anchorage-independent growth of Rat-2 cells expressing wt or mutant CSF-1R. (A) Formation of colonies in soft agar in the absence (−CSF-1) or presence (+CSF-1) of human recombinant CSF-1. (B) Morphology of monolayer cells in the presence or absence of CSF-1. R2, Parental Rat-2 cells; R2ΔC, Rat-2 cells expressing Δc-*fms*; R2C1, Rat-2 cells expressing wt c-*fms*.

whereas substantial PI kinase activity coprecipitated with the wt CSF-1R in anti-*fms* immunoprecipitates, the amount of PI kinase activity associated with the mutant CSF-1R was reduced to 5% of wt levels (Fig. 3A, lanes 11 to 13; Table 1). A significant proportion of the PI kinase activity precipitable with antiphosphotyrosine antibody was associated with the CSF-1R. These results suggest that a PI kinase is constitutively complexed with the mouse CSF-1R in c-*fms*-transformed NIH 3T3 cells and that this association is largely dependent on an intact kinase insert region. Deletion of the kinase insert diminishes both the association of the CSF-1R with active PI kinase and the amount of PI kinase activity that can be recovered with antiphosphotyrosine antibodies. The PI kinase activity measured in these assays was inhibited by 0.5% Triton X-100 and was only slightly decreased by the addition of 100 μM adenosine (Fig. 3A, lanes 14 to 16) and hence is likely the PI 3′-kinase described by Whitman et al. (46).

We extended these observations to Rat-2 cells genetically engineered to overexpress wt c-*fms* or Δc-*fms* (Fig. 3B). Stimulation of starved R2C1 cells with 10^{-8} M CSF-1 for 2 min induced an 8.5-fold increase in PI kinase activity precipitable with antiphosphotyrosine antibodies (Fig. 3B, lanes 2 and 5). Since R2C1 cells appear to possess an elevated basal level of PI 3′-kinase activity (Table 1), the calculated induction of 8.5-fold probably underestimates the true level of induction of PI 3′-kinase activity by CSF-1. As in 3T3 cells, the PI 3′-kinase activity coprecipitated with the CSF-1R in anti-*fms* immune complexes after CSF-1 stimulation (Fig. 3B, lanes 8 and 11), suggesting a physical association between PI 3′-kinase and activated CSF-1R. In contrast, in R2ΔC cells stimulated with CSF-1 there was a severe reduction in the extent of complex formation between the mutant CSF-1R and active PI 3′-kinase, to approximately 15% of wt levels (Table 1), and only a modest (3.5-fold) increase in total PI 3′-kinase activity precipitable with antiphosphotyrosine antibodies. Hence, association of active PI 3′-kinase with the CSF-1R is a common feature of CSF-1-stimulated macrophages and fibroblasts and is greatly enhanced by the kinase insert region.

GAP and p62 are phosphorylated by CSF-1R lacking the kinase insert region. We have recently found that growth factors such as epidermal growth factor and PDGF as well as a variety of oncogenic tyrosine kinases induce the rapid tyrosine phosphorylation of GAP and two GAP-associated proteins, p62 and p190 (6). The activated PDGFR, in particular, also forms a physical association with GAP (12). This interaction of tyrosine kinases with GAP is of interest, since GAP also regulates $p21^{ras}$ and hence may serve to link tyrosine kinases to the *ras* signaling pathway. We therefore used the cell systems described above to investigate the phosphorylation of GAP, p62, and p190 in response to CSF-1. NIH 3T3 mouse fibroblasts or NIH 3T3 cells expressing wt c-*fms* or Δc-*fms* were lysed and immunoprecipitated with anti-GAP antiserum. Western blots were probed with either anti-GAP antiserum (Fig. 4A) or antiphosphotyrosine antibodies (Fig. 4B). Whereas the amount of precipitable GAP was similar in parental and *fms*-expressing cell lines, the activated CSF-1R induced a slight phosphorylation of GAP on tyrosine (Fig. 4B). The level of tyrosine-phosphorylated p62 immunoprecipitated with anti-GAP antibodies in a complex with GAP was increased in both c-*fms* and Δc-*fms* cell lines, although this increase was modest compared with that induced by other activated receptors. In addition, the level of p62 phosphorylation was similar in c-*fms*- and Δc-*fms*-expressing cells. No difference in p190

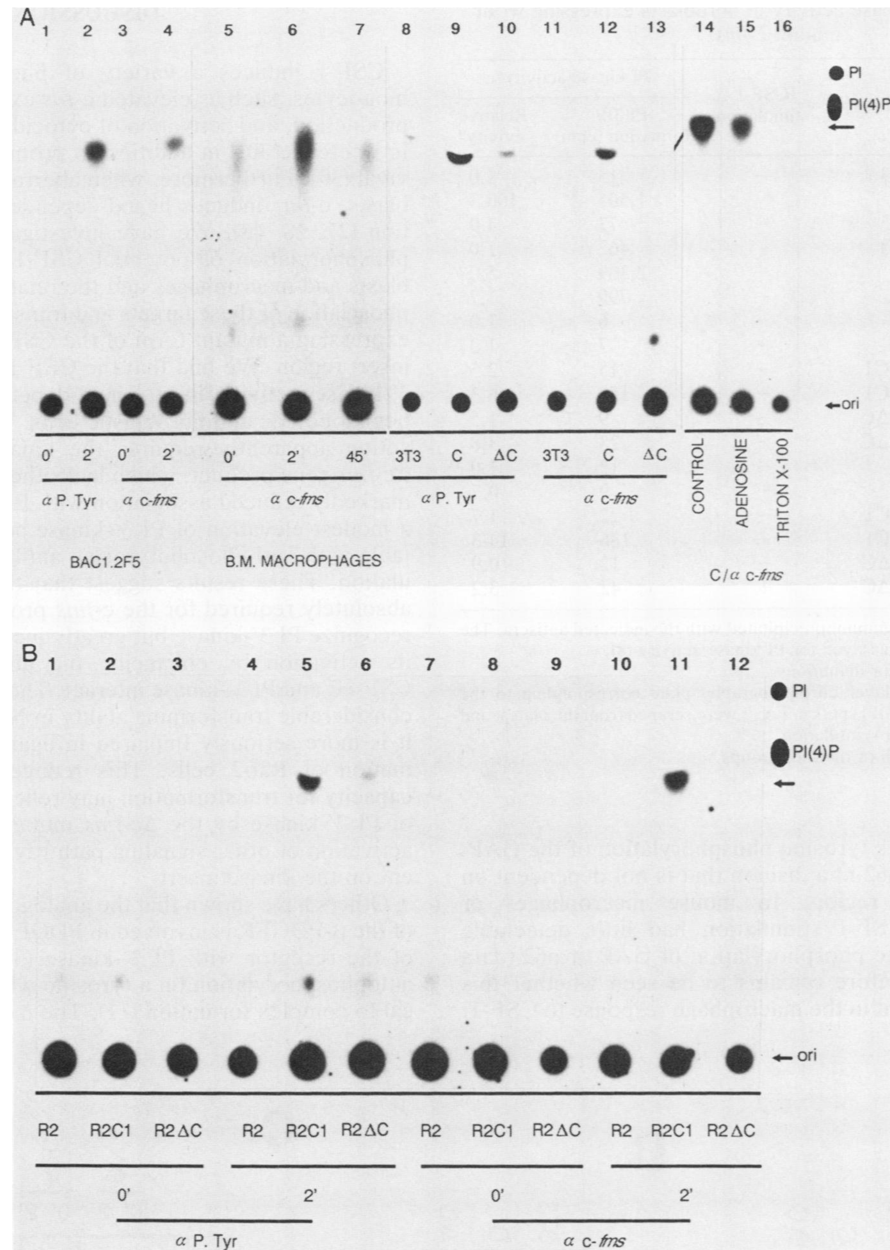

FIG. 3. Activation of PI 3'-kinase by the CSF-1R in macrophages and fibroblasts. Cells were lysed and immunoprecipitated with the indicated antisera, and the immune complexes were incubated in PI kinase assays. (A) BAC1.2F5 cells (lanes 1 to 4) or primary bone marrow (B.M.) macrophages (lanes 5 to 7) were stimulated with human recombinant CSF-1 (final concentration of 10^{-8} M) for the indicated time periods in minutes (0' represents no factor added). PI kinase activity was also tested in unstimulated NIH 3T3 fibroblasts (3T3; lanes 8 and 11) and in NIH 3T3 cells expressing either mouse c-*fms* (C; lanes 9, 12, and 14 to 16) or Δc-*fms* (ΔC; lanes 10 and 13). PI kinase activity was assayed in immune complexes obtained with either affinity-purified antiphosphotyrosine antibodies (α P. Tyr; lanes 1, 2, and 8 to 10) or antiserum 4067-B3 (α c-*fms*; lanes 3 to 7 and 11 to 16) and in the presence of the inhibitor 100 μM adenosine (lane 15) or 0.5% Triton X-100 (lane 16). (B) Confluent plates of Rat-2 fibroblasts (R2; lanes 1, 4, 7, and 10), Rat-2 cells expressing c-*fms* (R2C1; lanes 2, 5, 8, and 11), or Rat-2 cells expressing Δc-*fms* (R2ΔC; lanes 3, 6, 9, and 12) were starved in DMEM containing 0.5% FBS for 72 h; 10^{-8} M CSF-1 was added to resting cells for 2 min (lanes 4 to 6 and 10 to 12). Both stimulated and quiescent cells (lanes 1 to 3 and 7 to 9) were lysed and immunoprecipitated with either antiphosphotyrosine antibodies (α P. Tyr; lanes 1 to 6) or 4067-B3 antiserum (α c-*fms*; lanes 7 to 12). Positions of migration of the nonlabeled standards PI and PI-4-monophosphate [PI(4)P] are shown. The arrow indicates the migration of the major product of the lipid kinase assay. ori, Origin.

phosphorylation was observed. Similar results were obtained in CSF-1-stimulated Rat-2 lines expressing either wt or mutant c-*fms*. Within 2 min of stimulation with CSF-1, p62 became phosphorylated, to equivalent levels, in R2C1 and R2ΔC but not in parental R2 cells (Fig. 4C). No significant increase in phosphorylation of p190 or GAP was achieved upon CSF-1 stimulation. This result contrasts with the dramatic increase in phosphorylation of GAP, p190, and p62 observed in Rat-2 cells transformed with v-*src* (Fig. 4C, lane 1; 6). These results indicate that the activated CSF-1R

TABLE 1. PI 3'-kinase activity in fibroblasts expressing wt or mutant c-*fms*

Antiserum to[a]:	Cell line[b]	CSF-1 stimulation	PI kinase activity[c]	
			PI(3)P formation (cpm)	Relative activity[d]
c-*fms*	3T3	−	15	1.0
	C	−	1,505	100.3
	ΔC	−	73	4.9
Phosphotyrosine	3T3	−	462	1.0
	C	−	2,309	5.0
	ΔC	−	799	1.7
c-*fms*	R2	−	6	1.0
	R2	+	7	1.2
	R2C1	−	15	2.5
	R2C1	+	158	26.3
	R2ΔC	−	9	1.5
	R2ΔC	+	23	3.8
Phosphotyrosine	R2	−	13	1.0
	R2	+	9	0.7
	R2C1	−	22	1.7
	R2C1	+	186	14.3
	R2ΔC	−	12	0.9
	R2ΔC	+	42	3.2

[a] Cells were lysed and immunoprecipitated with the indicated antisera. The immune complexes were assayed for PI kinase activity (8).
[b] See legend to Fig. 3 for definitions.
[c] Regions on the thin-layer chromatography plate corresponding to the PI-3-monophosphate [PI(3)P] spots in Fig. 3 were scraped from the plates, and ^{32}P was counted by liquid scintillation.
[d] Compared within each of the four groups.

in fibroblasts induces tyrosine phosphorylation of the GAP-associated protein p62 in a fashion that is not dependent on the kinase insert region. In mouse macrophages or BAC1.2F5 cells, CSF-1 stimulation had little detectable effect on the tyrosine phosphorylation of GAP or p62 (data not shown). It therefore remains to be seen whether this pathway is significant in the macrophage response to CSF-1.

DISCUSSION

CSF-1 induces a variety of phenotypic alterations in monocytes, such as elevated c-*fos* expression (50), cytokine production, and activation of cytocidal properties (reviewed in reference 30), in addition to promoting macrophage survival (39). Furthermore, when aberrantly expressed in fibroblasts, c-*fms* induces ligand-dependent cellular transformation (27, 28, 48). We have investigated the activation and phosphorylation of potential CSF-1 targets in both fibroblasts and macrophages and the relationship between phosphorylation of these targets and transformation in fibroblasts expressing a mutant form of the CSF-1R lacking the kinase insert region. We find that the CSF-1R associates with a PI 3'-kinase activity in a ligand-dependent fashion in both hematopoietic and fibroblastic cells. This high-affinity interaction apparently requires the kinase insert region. The Δc-*fms* gene product, which lacks the kinase insert, shows a markedly reduced association with PI 3'-kinase and induces a modest elevation of PI 3'-kinase activity immunoprecipitable with anti-phosphotyrosine antibody upon CSF-1 stimulation. These results suggest that the kinase insert is not absolutely required for the c-*fms* protein-tyrosine kinase to recognize PI 3'-kinase but greatly increases the efficiency of its activation by enhancing the affinity with which the CSF-1R and PI 3'-kinase interact. The Δc-*fms* mutant retains considerable transforming ability in NIH 3T3 cells, although it is more seriously impaired in ligand-dependent transformation of Rat-2 cells. This reduced but still significant capacity for transformation may reflect a residual activation of PI 3'-kinase by the Δc-*fms* mutant or, more likely, the activation of other signaling pathways that are less dependent on the kinase insert.

Others have shown that the analogous kinase insert region of the β-PDGFR is involved in PDGF-dependent association of the receptor with PI 3'-kinase (3) and have implicated autophosphorylation on a tyrosine within the insert as critical to complex formation (11). The mouse CSF-1R becomes

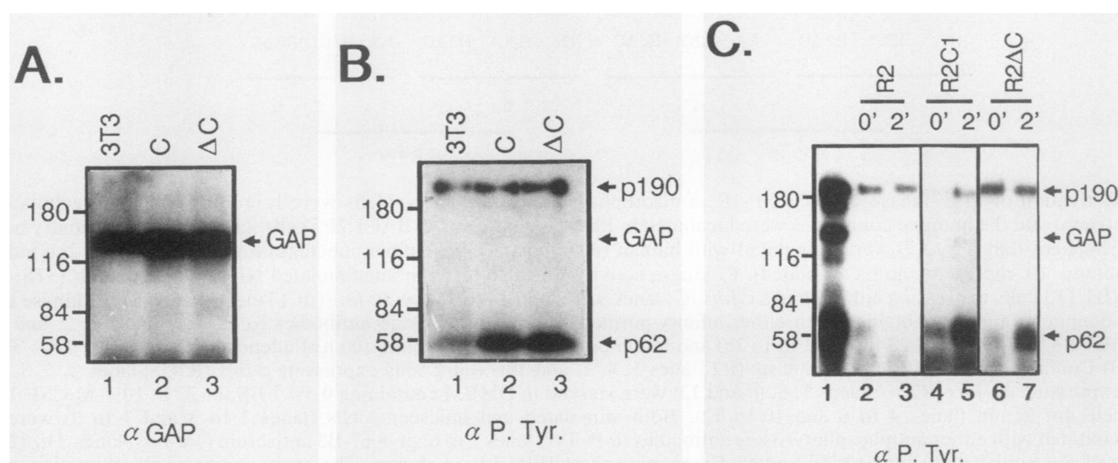

FIG. 4. Identification of phosphotyrosine-containing proteins in anti-GAP immunoprecipitations from fibroblasts expressing wt or mutant c-*fms*. Cell lysates were immunoprecipitated with anti-GAP(171-448) antiserum. Immunoprecipitates were separated by electrophoresis, and duplicate samples were blotted with anti-GAP antiserum (α GAP) (A) or antiphosphotyrosine antibodies (α P. Tyr.) (B) and ^{125}I-protein A. Immunoprecipitates were from normal NIH 3T3 cells (lane 1), NIH 3T3 cells expressing c-*fms* (lane 2), or NIH 3T3 cells expressing Δc-*fms* (lane 3). (C) Anti-GAP immunoprecipitates from unstimulated cells (lane 2, 4, and 6) or cells stimulated for 2 min with 5×10^{-8} M CSF-1 (lanes 3, 5, and 7) were blotted with antiphosphotyrosine antibodies (α P. Tyr.) and ^{125}I-protein A. Immunoprecipitates were from Rat-2 cells expressing v-*src* (6) (lane 1), normal Rat-2 cells (R2; lanes 2 and 3), Rat-2 cells expressing c-*fms* (R2C1; lanes 4 and 5), and Rat-2 cells expressing Δc-*fms* (R2ΔC; lanes 6 and 7). Mobilities of size markers are indicated.

autophosphorylated at two tyrosine residues within the insert, Tyr-697 and Tyr-706, although neither of these sites is within a sequence that is highly conserved with the β-PDGFR insert (35, 40). It is therefore possible that ligand binding induces a phosphorylation-dependent conformational change in the insert that promotes association, phosphorylation, and activation of PI 3'-kinase. An alternative possibility, which is not excluded by our results, is that the PI 3'-kinase is constitutively bound to the receptor but becomes active only upon ligand stimulation in a way that is dependent on an intact kinase insert. The ultimate fate of polyphosphoinositides phosphorylated in the D-3 position is not yet clear, although the strong relationship between PI 3'-kinase activation and DNA synthesis or transformation in a number of systems argues that they play an important role in signal transduction.

Other growth factor receptors associate with PLCγ and GAP as well as with PI 3'-kinase. The CSF-1R does not bind or phosphorylate PLCγ1 in fibroblasts or BAC1.2F5 cells (5) and is relatively weak in its phosphorylation of GAP. However, CSF-1 induces the tyrosine phosphorylation of a GAP-associated protein, p62, in fibroblasts. The evidence that GAP complex formation is important for signal transduction is as yet circumstantial, although the original identification of GAP as a regulator and potential target of $p21^{ras}$ make this an attractive hypothesis (17, 37, 38, 42). It is intriguing that in CSF-1-stimulated cells, the phosphorylation of p62 and its interaction with GAP are achieved with only modest tyrosine phosphorylation of GAP itself. It is therefore possible that the significant action of tyrosine kinases such as CSF-1R, as it relates to the GAP/$p21^{ras}$ signaling pathway, is not the tyrosine phosphorylation of GAP itself but rather the formation of GAP complexes with p62.

The inability of CSF-1R lacking the kinase insert to efficiently bind PI 3'-kinase correlates with a decreased transforming activity in fibroblasts, suggesting that PI kinase activation may contribute to c-*fms* transforming activity. Furthermore, PI kinase activation may be important to the physiological action of the CSF-1R in macrophages. However, using a variety of v-*src* mutants, Fukui and Hanafusa (8) have observed that association of PI 3'-kinase with $p60^{v\text{-}src}$ is not sufficient for transformation. Similarly, Roussel et al. have found that substitution of Tyr-809 within the catalytic domain of the human CSF-1R leads to a loss of CSF-1-induced transforming activity, without affecting association of the receptor with PI 3'-kinase (M. F. Roussel, S. A. Shurtleff, J. R. Downing, and C. J. Sherr, Proc. Natl. Acad. Sci. USA, in press). In this study, we found that although phosphorylation of the GAP complex may have a role in the biological activities of the CSF-1R, it is not by itself sufficient for full fibroblast transformation by c-*fms*. Indeed, there is as yet no direct functional evidence to implicate phosphorylation of GAP or p62 in the response to CSF-1. It is therefore likely that cellular transformation by tyrosine kinases such as $gp165^{c\text{-}fms}$ requires the activation of multiple signal transduction pathways or depends on a pathway that has yet to be defined.

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