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Identification of Tyrosine 706 in the Kinase Insert as the Major Colony-Stimulating Factor 1 (CSF-1)-Stimulated Autophosphorylation Site in the CSF-1 Receptor in a Murine Macrophage Cell Line

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The receptor for colony-stimulating factor 1 (CSF-1) is a ligand-activated protein-tyrosine kinase. It has been shown previously that the CSF-1 receptor is phosphorylated on serine in vivo and that phosphorylation on tyrosine can be induced by stimulation with CSF-1. We studied the phosphorylation of the CSF-1 receptor by using the BAC1.2F5 murine macrophage cell line, which naturally expresses CSF-1 receptors. Two-dimensional tryptic phosphopeptide mapping showed that the CSF-1 receptor is phosphorylated on several different serine residues in vivo. Stimulation with CSF-1 at 37°C resulted in rapid phosphorylation on tyrosine at one major site and one or two minor sites. We identified the major site as Tyr-706. The identity of Tyr-706 was confirmed by mutagenesis. This residue is located within the kinase insert domain. There was no evidence that Tyr-973 (equivalent to Tyr-969 in the human CSF-1 receptor) was phosphorylated following CSF-1 stimulation. When cells were stimulated with CSF-1 at 4°C, additional phosphotyrosine-containing phosphopeptides were detected and the level of phosphorylation of the individual phosphotyrosine-containing phosphopeptides was substantially increased. In addition, we show that CSF-1 receptors are capable of autophosphorylation at six to eight major sites in vitro.

Colony-stimulating factor 1 (CSF-1) is a peptide growth factor which is involved in regulation of proliferation, differentiation, and survival of macrophages and their direct precursors (41). Recently CSF-1 has also been implicated in induction of trophoblast proliferation during pregnancy (31). The CSF-1 receptor has been identified as the cellular homolog of v-*fms* (40), the transforming gene of the SM-FeSV and HZ5-FeSV feline sarcoma viruses (1, 17). Both viruses cause fibrosarcomas in cats. cDNAs for CSF-1 receptors have been cloned from humans, mice, and, most recently, cats (9, 34, 50). Sequence comparison and in vitro mutagenesis studies indicate that the SM-FeSV v-*fms* gene has been activated as a result of one (35) or, at most, two (50) point mutations in the extracellular ligand-binding domain and a deletion of 50 amino acids at the C terminus of the protein that are replaced by 11 unrelated residues (9, 50). A conserved tyrosine residue close to the C terminus, which is deleted in v-*fms*, has been implicated in activation of the *fms* protein, because of the analogy with $pp60^{c\text{-}src}$, which is regulated by phosphorylation at a tyrosine six residues from the C terminus (2, 5, 24, 30). This hypothesis has been substantiated by analysis of mutants with point mutations at this site (36). Mutation of Tyr-969 to Phe in the human CSF-1 receptor enhances the effect of activating mutations elsewhere in the receptor, but there is no evidence that this residue is phosphorylated.

Several growth factor receptor genes, encoding ligand-activated protein-tyrosine kinases, have been cloned and sequenced in recent years (9, 25, 27, 45–47, 51). Stimulation of these receptors by their cognate ligands leads to activation of the cytoplasmic receptor-kinase domain. As a consequence, cellular substrates are phosphorylated on tyrosine and a cascade of events eventually leads to cell division or differentiation. After stimulation, growth factor receptors disappear from the cell surface, a process called receptor down regulation. This has been well documented for the CSF-1 receptor (10, 11, 49). Some growth factor receptors, when activated, can down regulate receptors for other growth factors that are expressed on the surface of the same cell, a process that is known as cross-modulation. Receptor activation and down regulation need to be tightly regulated. Abrogation of negative regulation can easily lead to uncontrolled cell proliferation and tumor formation, whereas failure to moderate receptor down regulation theoretically could lead to the presence of cell types that do not respond to certain stimuli. It is thought that both receptor activation and down regulation are controlled at least in part through phosphorylation events involving receptor as well as nonreceptor protein kinases and associated proteins.

The CSF-1 receptor is a ligand-activated protein-tyrosine kinase similar to the receptors for epidermal growth factor (EGF), insulin, and platelet-derived growth factor (PDGF). It contains an extracellular ligand-binding domain, a transmembrane domain, and an intracellular protein-kinase domain (9, 34, 50). Together with both the PDGF α and β receptors and c-*kit*, the CSF-1 receptor forms a separate group of protein-tyrosine kinase receptors (18, 52). This classification is based on the presence of a 77- to 107-amino-acid insert within the kinase domain, and on alignment of their kinase domains (18, 27, 52). The kinase insert is not highly conserved between these kinases, and at the moment there is no consensus about its function. The regulatory role of receptor phosphorylation has been intensively studied for the EGF receptor and insulin receptor, but until recently relatively little was known about phosphorylation of the receptors belonging to the PDGF receptor family. However, Kazlauskas and Cooper recently reported the identification and functional analysis of two autophos-

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phorylation sites in the PDGF β receptor (23), Tyr-857, located within the C-terminal half of the kinase domain, which is homologous to Tyr-416 in $pp60^{c\text{-}src}$, and Tyr-751, located within the kinase insert region (23). Here we present data on phosphorylation events that take place after stimulation of the BAC1.2F5 murine macrophage line with CSF-1. We studied the phosphorylation of the murine CSF-1 receptor in vivo and in vitro and identified Tyr-706 in the kinase insert as a major CSF-1-induced autophosphorylation site in vivo. In vitro the CSF-1 receptor autophosphorylates at six to eight major sites in addition to Tyr-706. In addition, we show that the CSF-1 receptor is phosphorylated constitutively on several serine residues in vivo.

MATERIALS AND METHODS

Cells. BAC1.2F5 cells (29) were obtained from R. Stanley, Albert Einstein College of Medicine, New York, N.Y. These cells were maintained in Dulbecco-Vogt modified Eagle medium supplemented with 15% fetal bovine serum and 25% mouse L-cell conditioned medium, which contains CSF-1. For our experiments, cells were grown to confluence, and the medium was then removed and replaced with Dulbecco-Vogt modified Eagle medium supplemented with 0.5% fetal bovine serum. After 16 to 20 h the cells were labeled, stimulated with CSF-1, lysed, and immunoprecipitated or, alternatively, lysed immediately, immunoprecipitated, and used for in vitro kinase reactions.

Biosynthetic labeling and stimulation with CSF-1. Cellular proteins were labeled with ^{32}P by incubation of cells for 4 h at 37°C in phosphate-free Dulbecco-Vogt modified Eagle medium containing 2.5 mCi of $^{32}P_i$ (ICN Radiochemicals Inc., Irvine, Calif.) per ml and supplemented with 0.5% fetal bovine serum. When cells were to be stimulated, recombinant human CSF-1 (kindly provided by Cetus Corp., Emeryville, Calif.) was added to the cells to a final concentration of 10,000 U/ml at the end of the labeling period.

Antiserum and immunoprecipitation. Cells were rinsed with ice-cold phosphate-buffered saline, lysed in 1 ml of ice-cold RIPA buffer (10 mM sodium phosphate [pH 7.0], 0.15 M NaCl, 0.1% sodium dodecyl sulfate [SDS], 1% Nonidet P-40, 1% sodium deoxycholate, 2 mM EDTA, 50 mM NaF, 100 μM Na_3VO_4, 1% Trasylol [Mobay, New York, N.Y.]), and clarified by incubation with excess Pansorbin (Calbiochem-Behring, La Jolla, Calif.) and centrifugation at 10,000 × *g*. A polyclonal anti-CSF-1 receptor serum, raised against a bacterially expressed 80-kilodalton (kDa) fragment containing the entire intracellular and part of the extracellular domain (16), was kindly provided by C. Sherr, St. Jude Children's Hospital, Memphis, Tenn. Lysates were incubated with this polyclonal antiserum (2 μl of serum per ml of lysate) for 1 h at 0°C, followed by a 1-h incubation with 25 μl of protein A-Sepharose (100 mg/ml; Pharmacia, Inc., Piscataway, N.J.) at 4°C on a rocking device. Immune complexes were collected by centrifugation and were washed six times with RIPA buffer. Proteins were boiled for 3 min in sample buffer (50 mM Tris hydrochloride [pH 6.8], 10% glycerol, 2% SDS, 20% β-mercaptoethanol), and analyzed on an SDS–7.5% polyacrylamide gel as described previously (6).

In vitro kinase reactions. Immune complexes of the murine CSF-1 receptor were washed twice with 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.4) and once with kinase buffer (20 mM HEPES [pH 7.4], 10 mM $MnCl_2$, 0.5 mM dithiothreitol) and were incubated for 15 min at 37°C in 25 μl of kinase buffer containing 20 μCi of [γ-^{32}P]ATP (4,500 Ci/mmol; ICN Radiochemicals). Reactions were stopped by the addition of 25 μl of 2× sample buffer. Reaction mixtures were boiled for 3 min and analyzed on 7.5% polyacrylamide gels.

Peptide mapping and phosphoamino acid analysis. CSF-1 receptor protein was isolated from the gel and subjected to digestion with trypsin as described previously (21). Phosphopeptides were separated in two dimensions on 100-μm cellulose thin-layer plates by electrophoresis at pH 1.9 (50 parts 88% formic acid, 156 parts glacial acetic acid, 1,794 parts water) for 35 min at 1 kV followed by ascending chromatography (65 parts isobutyric acid, 5 parts pyridine, 3 parts glacial acetic acid, 2 parts *n*-butanol, 29 parts water) (37). Peptides were visualized by autoradiography. For further analysis phosphopeptides were isolated from thin-layer plates and subjected to phosphoamino acid analysis, digestion with chymotrypsin or thermolysin, or manual Edman degradation (20). The phosphoamino acid content of proteins and peptides was determined as described previously (7).

Preparation and ^{32}P labeling of synthetic peptides. Tryptic peptides 44 (Tyr-Gln-Val-Arg) and 60 (Tyr-Val-Arg) were synthesized manually by using *t*-butyloxycarbonyl chemistry (42). These peptides were phosphorylated by incubation with $p130^{gag\text{-}fps}$, 10 mM MnCl, and 20 μCi of [γ-^{32}P]ATP in 20 mM HEPES (pH 7.4) at 37°C for 30 min. ^{32}P-labeled tryptic peptides were purified on thin-layer plates by electrophoresis at pH 1.9 in one dimension and chromatography in the second dimension as described above.

In vitro mutagenesis. A 1,164-base-pair *PstI-PstI* fragment, consisting of residues 1817 to 2981 of the murine CSF-1 receptor cDNA (34), was cloned into M13mp18. Positive clones were identified, and the orientation of the inserts was determined by means of conventional techniques (26). Oligonucleotides GAGAAGAAATTTGTGCGCAGG and GAC TCCAACTTTGTTGTCAAG for making Phe-706 and Phe-807 mutants, respectively, were synthesized on a model 8750 DNA synthesizer (Milligen/Biosearch, San Rafael, Calif.). Mutagenesis reactions were carried out by using an oligonucleotide-directed in vitro mutagenesis system (Amersham International plc, Amersham, United Kingdom) as specified by the manufacturer. Mutant clones were screened by sequencing. To check for additional mutations inadvertently introduced during the procedure, the complete mutant M13 clones were sequenced. The mutant *PstI-PstI* fragments were cloned back into the CSF-1 receptor cDNA, and the wild-type and mutant cDNAs were cloned into a murine leukemia virus-based retroviral expression vector containing a neomycin resistance gene as selectable marker (22).

CSF-1 receptor expression. PA12 amphotropic packaging cells (28) were transfected, by means of the calcium phosphate precipitation method (3), with the expression construct alone or with the expression construct containing the wild-type or either one of the mutant CSF-1 receptor cDNAs. After 48 h, supernatants containing amphotropic virus were used to infect Rat-2 cells. Infected cells were selected by growth in medium containing 400 μg of G418 per ml, and pools of resistant cells were used for immunoprecipitation to study the pattern of CSF-1 receptor autophosphorylation in vitro.

RESULTS

CSF-1 receptor autophosphorylates on several major sites in vitro. CSF-1 receptors can autophosphorylate on tyrosine when incubated in vitro with Mn^{2+} and [γ-^{32}P]ATP at 37°C

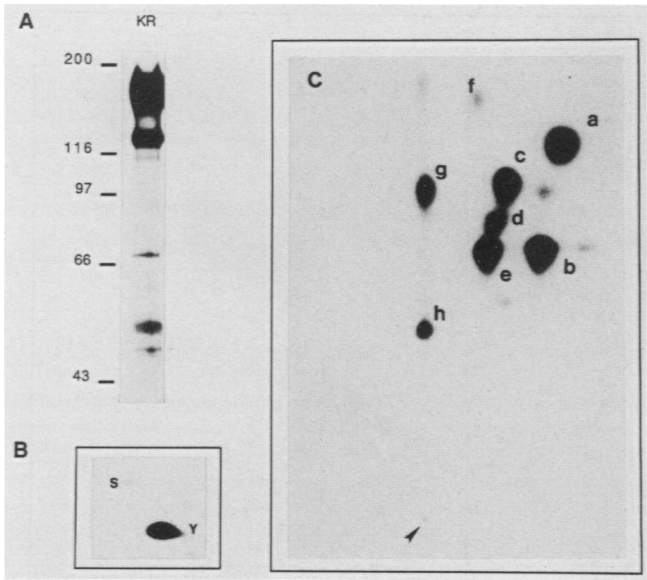

FIG. 1. Autophosphorylation of the murine CSF-1 receptor in vitro. BAC1.2F5 cells were grown to confluence, starved of CSF-1 for 20 h, and lysed in RIPA buffer. Receptors were isolated from one 6-cm dish of cells by immunoprecipitation and were incubated with [γ-^{32}P]ATP in kinase buffer as described in Materials and Methods. The reaction products were analyzed on an SDS–7.5% polyacrylamide gel. The gel was exposed for 1 h at −70°C. (A) Receptor protein was isolated from the gel and subjected to phosphoamino acid analysis and tryptic phosphopeptide mapping. (B) For phosphoamino acid analysis, 600 cpm was spotted and the plate was exposed for 10 days at −70°C with a intensifier screen. (C) For tryptic peptide mapping, 12,000 cpm of digest was spotted and phosphopeptides were separated by electrophoresis in the horizontal dimension with the anode on the left and ascending chromatography in the vertical dimension. The map was exposed for 40 h at −70°C with an intensifier screen. The origin is marked with an arrow, and major phosphopeptides are labeled a through h.

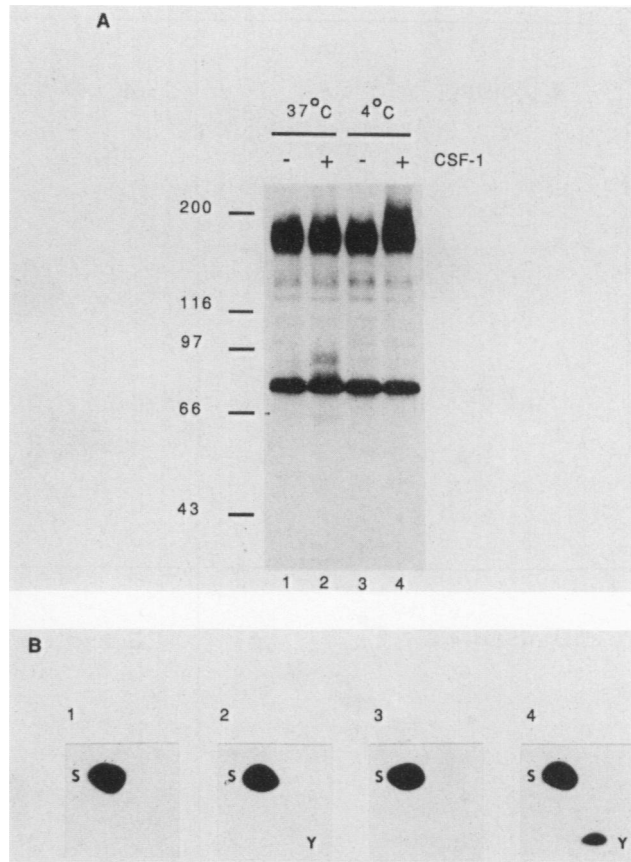

FIG. 2. CSF-1 receptor phosphorylation on tyrosine after stimulation with CSF-1. BAC1.2F5 cells were grown to confluence, starved of CSF-1 for 20 h, and labeled with $^{32}P_i$ for 4 h at 37°C. The cells were stimulated with CSF-1 at the end of the labeling for 2 min at 37°C (2) or for 30 min at 4°C (4) and were compared with control cells incubated under identical condition in the absence of CSF-1 (1, 3). CSF-1 receptors were isolated by immunoprecipitation and analyzed on an SDS–7.5% polyacrylamide gel. The gel was exposed for 1 h at −70°C with an intensifier screen (A). After exposure, receptor protein was isolated from this gel and subjected to phosphoamino acid analysis; 136 (panel 1), 89 (panel 2), 65 (panel 3), and 90 (panel 4) cpm were spotted and analyzed. Plates were exposed for 4 days at −70°C with an intensifier screen (B).

(33, 49). To investigate autophosphorylation of CSF-1 receptors in vitro, BAC1.2F5 cells were starved of CSF-1 for 20 h, to up regulate the number of CSF-1 receptors expressed on the cell surface, and lysed in RIPA buffer. Receptors were isolated by immunoprecipitation. The immune complexes were incubated with kinase buffer and [γ-^{32}P]ATP, and the reaction products were analyzed on a polyacrylamide gel (Fig. 1A). The 160- and 140-kDa bands are the mature receptor and its biosynthetic precursor, respectively. Most of the lower bands represent breakdown products of the receptor, as determined by peptide mapping (data not shown). Stimulation with CSF-1 prior to lysis did not result in a major increase in in vitro autophosphorylation (data not shown). Phosphoamino acid analysis showed that the receptor phosphorylated in vitro contains almost exclusively phosphotyrosine (Fig. 1B). Tryptic peptide maps of in vitro-labeled receptors show eight major phosphopeptides (peptides a through h) and several minor phosphopeptides (Fig. 1C). On the basis of further digestion with other proteases, manual Edman degradation, and the presence of different partial hydrolysis products generated during phosphoamino acid analysis, we conclude that peptides a through d are not directly related (peptides f through h were not analyzed [data not shown]). Our analysis does not exclude the possibility that peptide b is related to peptide e. All major phosphopeptides contain phosphotyrosine (P.Tyr) (data not shown). Peptides a through e were present in maps of the biosynthetic precursor and most of the breakdown products. We identified one 40-kDa breakdown product that contains peptides a through e. This indicates that all these peptides are contained within an approximately 360-amino-acid fragment of the receptor.

CSF-1 induces phosphorylation at several sites in vivo. To study CSF-1-induced receptor phosphorylation in vivo, BAC1.2F5 cells were starved of CSF-1 for 16 h, labeled for 4 h with $^{32}P_i$, and stimulated with 10,000 U of recombinant human CSF-1 per ml. In an initial experiment cells were stimulated with CSF-1 for 3 and 5 min at 37°C, and after these times the level of ^{32}P-labeled receptors was reduced to 80 and 40%, respectively, of that in control cells (data not shown). This is in agreement with studies reporting rapid down regulation of CSF-1 receptors after stimulation with CSF-1 or 12-*O*-tetradecanoylphorbol-13-acetate (11, 49). It has been recently found that receptors can be activated without being down regulated when stimulated with CSF-1 at 4°C (38). As a consequence, BAC1.2F5 cells were stimu-

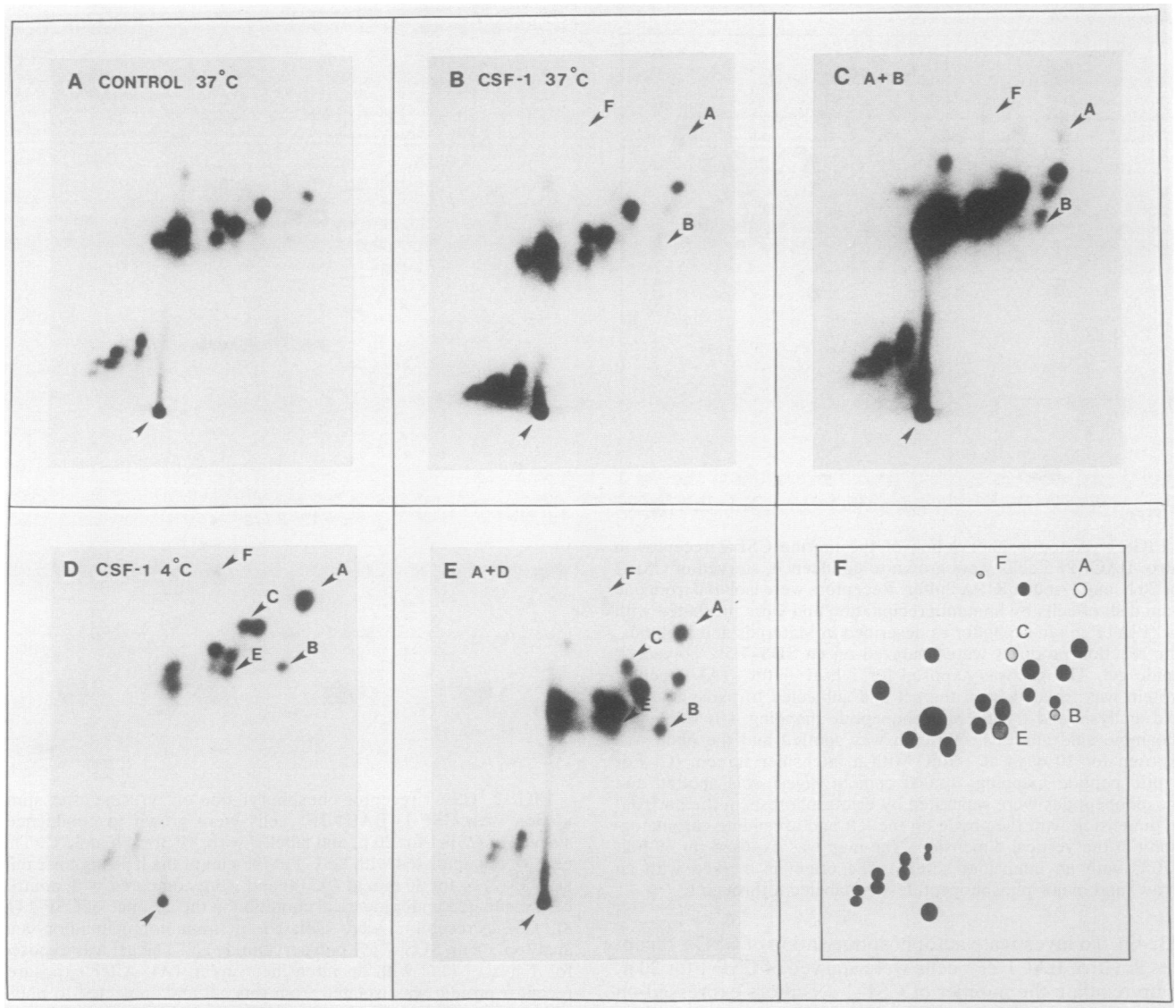

FIG. 3. Tryptic phosphopeptide maps of CSF-1 receptors labeled in vivo. Three confluent 9-cm dishes of BAC1.2F5 cells were starved of CSF-1 for 20 h and then labeled with $^{32}P_i$. One was used as a control, and the other two were stimulated with CSF-1 for 2 min at 37°C or for 30 min at 4°C. Receptors were isolated by immunoprecipitation and SDS-polyacrylamide gel electrophoresis and subjected to tryptic phosphopeptide mapping (A through E) as described in the legend to Fig. 1. Cells were incubated for 2 min at 37°C in the absence (panel A; 1,125 cpm spotted, exposed for 4 days at −70°C with intensifier screen) and presence (panel B; 1,325 cpm, 4 days) of CSF-1 or for 30 min at 4°C in the presence of CSF-1 (panel D; 1,025 cpm, 4 days). Panels C (2,450 cpm, 4 days) and E (2,325 cpm, 4 days) show mixes of the conditions in panels A and B and panels A and D, respectively. CSF-1-induced phosphopeptides are marked. Phosphopeptides were isolated from these maps and subjected to phosphoamino acid analysis. The data are summarized in panel F. Peptide A was induced by CSF-1 at both 37 and 4°C and contained P.Tyr (○). Peptide E was induced only at 4°C and contained P.Ser (●). Spots B and C were induced at both 37 and at 4°C (◍). These areas are most likely contain a mixture of phosphopeptides (see text). Peptide F never yielded enough radioactivity for further analysis (⊗). All other phosphopeptides were constitutively phosphorylated on serine (●). The increase in intensity of the P.Ser-containing peptides to the left of the origin following CSF-1 treatment is not reproducible.

lated for 2 min at 37°C or for 30 min at 4°C in subsequent experiments.

Figure 2A shows CSF-1 receptor immunoprecipitates from BAC1.2F5 cells that were labeled with $^{32}P_i$ for 4 h and stimulated with CSF-1 at 37°C for 2 min or at 4°C for 30 min. There were 10 and 15% increases in the amount of ^{32}P incorporated in the receptor after stimulation with CSF-1 at 37 and 4°C, respectively. The CSF-1 receptor immunoprecipitates from cells stimulated with CSF-1 at 37°C contained four bands between 60 and 90 kDa (Fig. 2A, lane 2). Comigrating bands were also seen in CSF-1 receptor precipitates from cells stimulated with 12-*O*-tetradecanoylphorbol-13-acetate (data not shown). These bands were not detected in immunoprecipitates of control cells or cells stimulated with CSF-1 at 4°C. Peptide mapping indicated that these proteins are not related to the CSF-1 receptor, but are indeed related to each other. Furthermore, only phosphoserine (P.Ser) and phosphothreonine (P.Thr) could be detected in these proteins (data not shown). These bands may be phosphoproteins that associate with the receptor after activation

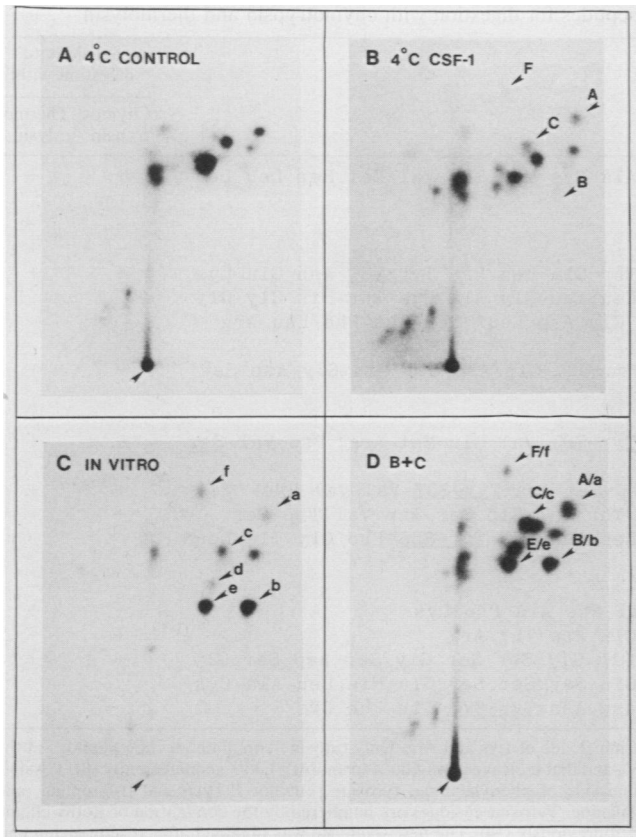

FIG. 4. Tryptic phosphopeptide maps obtained from CSF-1 receptors phosphorylated in vivo or in vitro contain several comigrating tryptic phosphopeptides. CSF-1 receptors phosphorylated in vivo by labeling cells with $^{32}P_i$ or phosphorylated in vitro by incubation with [γ-^{32}P]ATP in kinase buffer were isolated and subjected to tryptic phosphopeptide analysis as described in the legend to Fig. 1. (A) In vivo-labeled receptors, control (650 cpm, 10 days); (B) in vivo-labeled receptors, treated for 30 min with CSF-1 at 4°C (470 cpm, 10 days); (C) in vitro-phosphorylated receptors (220 cpm, 10 days); (D) mix of B and C (690 cpm, 10 days).

or, alternatively, proteins that are associated with the receptor and become phosphorylated upon stimulation with CSF-1, owing to activation of a protein-serine/threonine kinase. In the immunoprecipitate from cells stimulated at 4°C a 200-kDa phosphoprotein appeared above the regular CSF-1 receptor band (Fig. 2A, lane 4); it was not seen in the immunoprecipitates from cells stimulated with CSF-1 at 37°C or from control cells. This band represents a CSF-1 receptor form with a reduced mobility, as was determined by phosphopeptide mapping (data not shown). Interestingly, peptide A was the most prominent phosphopeptide in tryptic maps of this receptor form.

Receptor proteins were isolated from this gel and subjected to phosphoamino acid analysis and peptide mapping. Phosphoamino acid analysis showed that receptors are constitutively phosphorylated on serine and contain only trace amounts of P.Thr (Fig. 2B). P.Tyr was detected in the CSF-1 receptor only after stimulation with CSF-1 (Fig. 2B, panels 2 and 4). This is in agreement with previous reports (10). We estimate that P.Tyr accounts for not more than 1% of the total amount of ^{32}P incorporated in the receptor in vivo after CSF-1 stimulation at 37°C for 2 min (Fig. 2B). This increases to 10 to 15% when cells are stimulated with CSF-1 at 4°C for 30 min (Fig. 2B).

To investigate whether stimulation with CSF-1 induces phosphorylation at new sites, we performed tryptic phosphopeptide mapping on receptors isolated from ^{32}P-labeled cells (Fig. 3). In maps of CSF-1 receptors from unstimulated cells, 15 to 20 different peptides could be resolved (Fig. 3A). In CSF-1 receptor maps obtained from cells stimulated with CSF-1 at 37°C, two or three faint new phosphopeptides were detected (Fig. 3B and C, peptides A, B, and F). When cells were stimulated with CSF-1 at 4°C the intensity of the new phosphopeptides induced after stimulation at 37°C (Fig. 3D, peptides A, B, and F) was greater and additional new phosphopeptides were present (Fig. 3D and E, peptides C and E). To investigate which peptides contain P.Tyr, we isolated peptides from the thin-layer plates and subjected them to phosphoamino acid analysis. The results are summarized in a schematic representation (Fig. 3F). Peptide A, the major CSF-1-induced phosphopeptide, contained P.Tyr. Peptides B and C contained both P.Tyr and P.Ser (ratio, ca. 2:1) when cells were stimulated with CSF-1 at 4°C. However, when cells were stimulated at 37°C peptide B contained more P.Ser than P.Tyr, whereas peptide C contained only P.Ser. Peptide E, which is apparent only after stimulation at 4°C, contained only P.Ser; peptide F never yielded enough radioactivity for further analysis. In principle a single phosphopeptide, migrating at a certain position on a two-dimensional peptide map, cannot contain different relative amounts of two phosphoamino acids. Therefore, it is likely that what appears to be a single phosphopeptide actually is a mixture of phosphopeptides. Spots B and C therefore probably contain two phosphopeptides: one that is constitutively phosphorylated on serine and a second peptide that becomes phosphorylated on tyrosine when cells are stimulated with CSF-1. We can conclude that peptide A contains the major site of CSF-1-induced tyrosine phosphorylation in vivo, at both 37 and 4°C.

Peptide maps of in vivo- and in vitro-labeled receptor have several phosphopeptides in common. To find whether autophosphorylation in vitro occurs at the same sites that are phosphorylated in CSF-1-treated cells, ^{32}P-labeled CSF-1 receptors were isolated from control cells and cells stimulated with CSF-1 at 4°C and subjected to tryptic phosphopeptide analysis, and these maps were compared with those obtained from receptors phosphorylated in vitro (Fig. 4). Peptide A, the major P.Tyr-containing peptide induced by CSF-1 in vivo (Fig. 4B), comigrated with peptide a in the map of in vitro-labeled receptors (Fig. 4C and D). Furthermore, peptides b, c, and f, from the in vitro-labeled receptors, comigrated with three spots in maps of in vivo-labeled receptors (Fig. 4B, spots B, C, and F). Peptides d and e from the in vitro peptide maps migrated in a position similar to peptide E, which is phosphorylated after stimulation with CSF-1 at 4°C. Peptide E from in vivo maps, however, did not contain P.Tyr. Peptides g and h from in vitro maps had no counterparts in in vivo maps. We concentrated on identification of peptide A/a, since this seemed to be the major autophosphorylation site in vivo, at both 37 and 4°C, and in vitro.

The major site of autophosphorylation in vivo is tyrosine 706, located in the kinase insert region. The intracellular domain of the murine CSF-1 receptor contains 20 tyrosines distributed in 14 theoretical tryptic peptides (Table 1). The mobility of peptide A/a is compatible with the predicted mobilities of tryptic peptides 42, 44, 60, and 71 (Table 1). Since we were able to incorporate larger amounts of radioactivity in peptide A when the receptor was phosphorylated in vitro, this site was identified by using in vitro labeled

TABLE 1. Predicted sensitivity of tyrosine-containing tryptic peptides for digestion with chymotrypsin and thermolysin

Peptide sequence[a]	Sensitive to digestion with:	
	Chymotrypsin	Thermolysin
41. Gln Leu Pro Asp Glu Ser Leu Phe Thr Pro Val Val Val Ala Cys Met Ser Val Met Ser Leu Leu Val Leu Leu Leu Leu Leu Leu Leu Tyr-536 Lys	+	+
42. Tyr-538 Lys	−	−
44. Tyr-544 Gln Val Arg	−	+
47. Tyr-554 Glu Gly Asn Ser Tyr-559 Thr Phe Ile Asp Pro Thr Gln Leu Pro Tyr-569 Asn Glu Lys	+	+
57. Ile Met Ser His Leu Gly Gln His Glu Asn Ile Val Asn Leu Leu Gly Ala Cys Thr His Gly Gly Pro Val Leu Val Ile* Thr Glu Tyr-663 Cys Cys Tyr-666 Gly Asp Leu* Leu Asn Phe Leu Arg (Arg Lys)$^{\alpha}$		
58. (Arg Lys)$^{\alpha}$ Ala Glu Ala Met Leu* Gly Pro Ser Leu Ser Pro Gly Gln Asp Ser Glu Gly Asp Ser Ser Tyr-697 Lys	−	+
60. Tyr-706 Val Arg	−	+
61. (Arg)$^{\alpha/\beta}$ Asp Ser Gly Phe Ser Ser Gln Gly Val Asp Thr Tyr-721 Val Glu Met Arg$^{\gamma}$ Pro Val Ser Thr Ser Ser Ser Asp Ser Phe Phe Lys	+	+
68. (Ile Gly Asp Phe Gly Leu Ala Arg)$^{\beta}$ Asp Ile Met Asn Asp Ser Asn Tyr-807 Val Val Lys	+	+
70. Trp Met Ala Pro Glu Ser Ile Phe Asp Cys Val Tyr-829* Thr Val Gln Ser Asp Val Trp Ser Tyr-838 Gly Ile Leu Leu Trp Glu Ile Phe Ser Leu Gly Leu Asn Pro Tyr-853 Pro Gly Ile Leu* Val Asn Asn Lys	+	+
71. Phe Tyr-863 Lys	−	−
73. (Leu Val Lys)$^{\beta}$ Asp Gly Tyr-870 Gln Met Ala Gln Pro Val Phe Ala Pro Lys	+	+
74. Asn Ile Tyr-883 Ser Ile Met Gln Ser Cys Trp Asp Leu Glu Pro Thr Arg	+	+
77. (Arg)$^{\alpha/\beta}$ Asp Gln Asp Tyr-920 Ala Asn Leu Pro Ser Ser Gly Gly Ser Ser Gly Ser Asp Ser Gly Gly Gly Ser Ser Gly Gly Ser Ser Ser Glu Pro Glu Glu Glu Ser Ser Ser Glu His Leu Ala Cys Cys Glu Pro Gly Asp Ile Ala Gln Pro Leu Leu Gln Pro Asn Asn Tyr-973 Gln Phe Cys	+	+

[a] Theoretical tryptic peptides were generated by cutting the sequence at the C-terminal side of Lys and Arg. Only tyrosine-containing tryptic peptides in the intracellular domain are listed. Note that trypsin does not cut Lys-Pro and Arg-Pro (γ), and that it cleaves Lys-Glu/Asp and Arg-Glu/Asp inefficiently (β). Possible partial digestion products are marked (α). Chymotrypsin cleaves at the C-terminal side of phenylalanine, tyrosine (but not P.Tyr), and tryptophan, and thermolysin cleaves at the N-terminal side of leucine, isoleucine, valine, and phenylalanine. Tyrosine residues are numbered by the convention of Rothwell and co-workers (34). Residues marked with an asterisk differ from the sequence described previously (34). The new sequence was obtained after resequencing part of the murine CSF-1 receptor clone.

CSF-1 receptor. Peptide a was isolated from peptide maps and subjected to further digestion with trypsin, chymotrypsin, and thermolysin. It could not be digested with trypsin or chymotrypsin, but was sensitive to digestion with thermolysin (Fig. 5) (as will become clear below, the observed partial digestion may result from the inability of thermolysin to act as an exopeptidase adjacent to P.Tyr). This eliminates peptides 42 and 71, which lack a thermolysin site. Both peptides 44 and 60 contain a theoretical site for chymotrypsin cleavage, but the enzyme does not cut after P.Tyr. To gain further information, peptide a was subjected to four cycles of manual Edman degradation. After one round of degradation the label was released and comigrated with PTH-P.Tyr, indicating that the first residue in this peptide is P.Tyr (data not shown). Only two candidate peptides agree fully with the results described. These are peptides 44 and 60 (Table 1). Both peptides were synthesized by using a manual solid-phase procedure and phosphorylated by p130$^{gag\text{-}fps}$, a viral protein-tyrosine kinase (48). Phosphorylated peptides 44 and 60 were tested for comigration with peptide A/a isolated from maps of in vivo- and in vitro-labeled CSF-1 receptors. Peptide 60, but not peptide 44 (data not shown), comigrated with peptide A/a isolated from both in vivo- and in vitro-labeled peptide maps (Fig. 6). We conclude that Tyr-706, which is contained in peptide 60, is the major CSF-1-induced autophosphorylation site in the murine CSF-1 receptor in BAC1.2F5 cells in vivo.

Receptor mutagenesis and expression. To confirm our phosphopeptide mapping data, we used site-directed mutagenesis to change Tyr-706 into Phe. Wild-type and mutant receptor cDNAs were cloned into a murine leukemia virus-based retroviral expression vector (22), which was then used to transfect the PA12 packaging line. PA12 supernatants were used to infect Rat-2 fibroblasts, which were subsequently selected for G418 resistance. Pooled Rat-2 cells infected with virus containing the wild-type or mutant cDNA, but not cells infected with the virus alone, expressed mature and precursor CSF-1 receptors (data not shown). Wild-type as well as mutant receptors immunoprecipitated from RIPA lysates were able to autophosphorylate in vitro (data not shown), indicating that this mutation does not abrogate kinase activity. ^{32}P-labeled autophosphorylated receptors were gel purified and subjected to phosphoamino acid analysis and tryptic peptide mapping. Both wild-type and mutant receptors contained mainly P.Tyr (data not shown). Tryptic phosphopeptide maps showed that peptide a was present in the map of the wild-type receptors (Fig. 1C and 7A) but was absent from the map of the Phe-706 mutant (Fig. 7B), confirming our identification of peptide a as containing Tyr-706. Note that peptide x (Fig. 7) is not present in maps from in vitro-phosphorylated receptors expressed in BAC1.2F5 cells.

In addition, we mutated Tyr-807, which is homologous to Tyr-416 in pp60$^{c\text{-}src}$, to Phe. A tyrosine homologous to this residue is conserved in all protein-tyrosine kinases. Tryptic phosphopeptide maps of this mutant (data not shown) indicated that phosphopeptide c represented peptide 68 (Table 1), which contains Tyr-807.

DISCUSSION

In this report we have analyzed phosphorylation of CSF-1 receptors expressed in the simian virus 40-immortalized BAC1.2F5 murine macrophage cell line. Like other late-myeloid-series cells, BAC1.2F5 cells naturally express CSF-

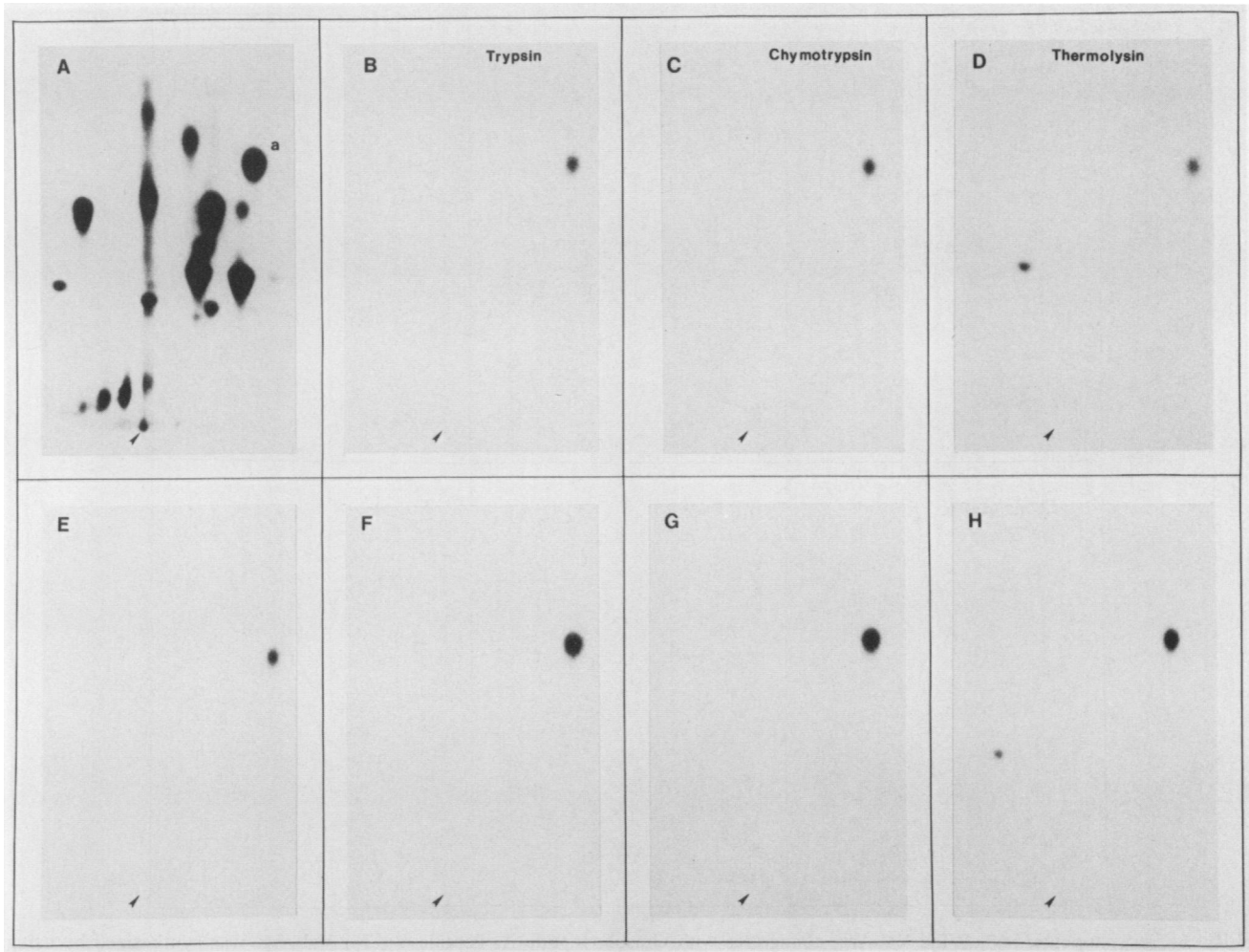

FIG. 5. Sensitivity of peptide "a" isolated from an in vitro tryptic phosphopeptide map to digestion with trypsin, chymotrypsin, and thermolysin. CSF-1 receptors were isolated by immunoprecipitation, labeled by incubation with [γ-^{32}P]ATP and kinase buffer, purified by SDS-polyacrylamide gel electrophoresis, and subjected to tryptic phosphopeptide mapping. Peptide a was isolated from this map and subjected to digestion with trypsin, chymotrypsin, and thermolysin. Reaction products were analyzed as described in the legend to Fig. 1. (A) Tryptic phosphopeptide map of whole receptor (8,000 cpm, 2 days); (E) peptide a; (B through D) peptide a digested with trypsin, chymotrypsin, and thermolysin, respectively; (F through H) mixes of E with B, C, and D, respectively. For panels B through H, 40 to 70 cpm was spotted and plates were exposed for 8 days at −70°C with an intensifier screen.

1 receptors on their cell surface and depend on the presence of CSF-1 for survival and continued proliferation (29). Analysis of receptors from ^{32}P-labeled cells indicated that CSF-1 receptors become phosphorylated on tyrosine only after stimulation with CSF-1. P.Tyr levels were maximally very low, never rising higher than 1% of the total phosphoamino acid content when cells were stimulated with CSF-1 at 37°C (Fig. 2B), similar to the report of Downing et al. (10). When cells were labeled and stimulated with CSF-1 in the presence of 50 μM orthovanadate, an inhibitor of P.Tyr phosphatases, the P.Tyr level did not increase more than three times above the level with CSF-1 alone (data not shown). After stimulation with CSF-1 at 4°C the P.Tyr level increased to as much as 10 to 15% of the total phosphoamino acid content. It is known that CSF-1 receptors are normally rapidly down regulated after stimulation with CSF-1 or 12-*O*-tetradecanoylphorbol-13-acetate at 37°C (11, 49). This down regulation is impaired when cells are stimulated with CSF-1 at 4°C (38). In our experiments the ability to down regulate CSF-1 receptors seems to be inversely correlated with the appearance of P.Tyr in the receptor. We speculate that the P.Tyr content of the receptor is kept low by receptor down regulation rather than by the action of P.Tyr phosphatases. There might even be a causal relation between receptor phosphorylation on tyrosine and receptor down regulation. This is consistent with the observation that kinase-negative mutants of the CSF-1 receptor (11), the EGF receptor (19), and the insulin receptor (4) fail to down regulate in response to ligand. In contrast, it has been shown that kinase-negative PDGF β receptors are still capable of down regulation in response to PDGF (13). We are testing whether CSF-1 receptor autophosphorylation is required for down regulation by using the tyrosine phosphorylation site mutants described in this paper.

Peptide mapping and in vitro mutagenesis experiments showed that Tyr-706 is the major residue phosphorylated in vivo in the CSF-1 receptor in response to CSF-1. Since this residue is also phosphorylated in immunoprecipitates in vitro, it is most likely that phosphorylation at this site is due to autophosphorylation. Tyr-706 is located in the N-terminal

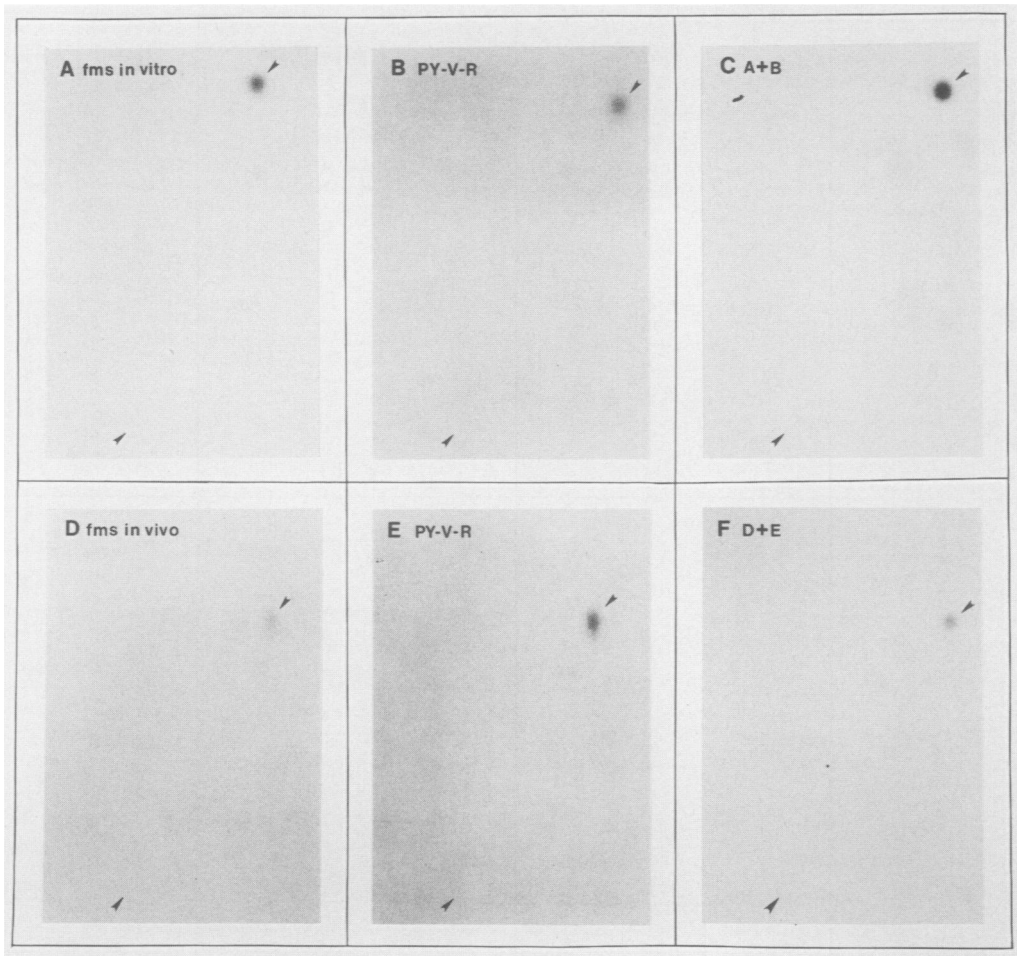

FIG. 6. Phosphopeptide 60, P.Tyr-Val-Arg, comigrates with phosphopeptide A. Peptide 60, Tyr-Val-Arg, was synthesized by using a manual solid-phase procedure, phosphorylated by incubation with p130$^{gag\text{-}fps}$, [γ-^{32}P]ATP, and kinase buffer, and purified by separation in two dimensions on a thin-layer plate. Peptides A/a were isolated from tryptic phosphopeptide maps from in vivo- and in vitro-labeled CSF-1 receptors. For in vivo maps the cells were stimulated with CSF-1 at 37°C. The two-dimensional mobilities of phosphopeptides A/a and P.Tyr-Val-Arg were compared. (A) Peptide a isolated from a phosphopeptide map of in vitro-labeled receptor (60 cpm, 3 days); (B) peptide 60, P.Tyr-Val-Arg (50 cpm, 3 days); (C) mix of A and B (110 cpm, 3 days); (D) peptide A isolated from an in vivo phosphopeptide map (20 cpm, 12 days); (E) peptide 60, P.Tyr-Val-Arg (25 cpm, 12 days); (F) mix of D and E (45 cpm, 12 days).

part of the CSF-1 receptor kinase insert domain (Fig. 8). This tyrosine is conserved in the CSF-1 receptors from humans, mice, and cats (9, 34, 50). This residue, however, is not conserved in c-*kit* (32), the PDGF β receptor (51), or the recently cloned PDGF α receptor (27). The sequence around Tyr-706 (HLEKKYVRRDS) is not homologous to known sites of tyrosine phosphorylation, including Tyr-751 (EDES VDYVPML), in the PDGF β receptor, and, indeed, it is uncharacteristically basic.

In addition to Tyr-706, we mutated Tyr-807, which is homologous to Tyr-416 in pp60$^{c\text{-}src}$ and is conserved in all protein-tyrosine kinases (18) (Fig. 8). Tryptic phosphopeptide maps of Phe-807 mutant receptors labeled in vitro lacked peptide c (results not shown), a major phosphopeptide seen in receptors phosphorylated in vitro (Fig. 1C). This indicates that Tyr-807 is a major autophosphorylation site in vitro. In addition, our tryptic phosphopeptide maps indicate that this residue is phosphorylated when cells are stimulated with CSF-1 at 4°C (Fig. 3A, D, and E and Fig. 4). It is possible that Tyr-807 becomes phosphorylated when BAC1.2F5 cells are stimulated at 37°C but that the steady-state level is too low to allow detection in our experiments.

Whether receptor phosphorylation occurs in *trans* within a receptor dimer or via a true intramolecular reaction remains to be determined. In this context it is also worth noting that in contrast to the situation with the PDGF receptor, for which in vivo phosphorylation at Tyr-857 (the Tyr-416 homolog) is favoured over phosphorylation of Tyr-751 in the kinase insert, phosphorylation of the kinase insert site in the CSF-1 receptor predominates over that of the Tyr-416 equivalent site.

Recently two autophosphorylation sites were mapped in the PDGF β receptor (23). One site, Tyr-857, is located within the kinase domain (Fig. 8) and is homologous to Tyr-416 in pp60$^{c\text{-}src}$ (23), a residue that has been conserved in all protein-tyrosine kinases (18). This residue is autophosphorylated in most of the kinases that have been studied thus far, with the exception of the EGF receptor. Autophosphorylation of this residue is important for activation of the insulin receptor (12) and has also been implicated in activation of pp60$^{c\text{-}src}$ kinase activity (2, 24, 30). The function of autophosphorylation at this residue in other receptor protein kinases is not well characterized. Kazlauskas and Cooper report that mutation of this residue has no effect on receptor

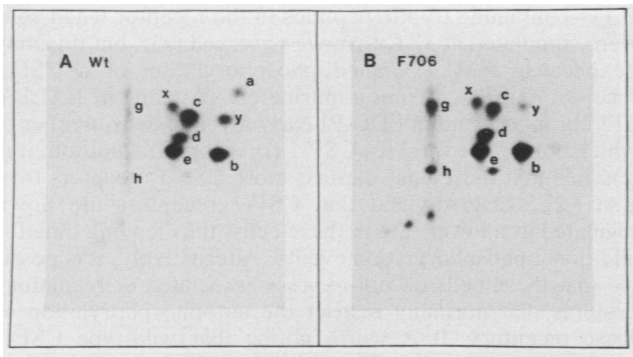

FIG. 7. In vitro phosphorylation of Phe-706 mutant CSF-1 receptors. Wild-type and Phe-706 mutant CSF-1 receptors were expressed in Rat-2 cells. Receptors were isolated by immunoprecipitation and used in in vitro phosphorylation reactions as described in the legend to Fig. 1. ^{32}P-labeled receptors were gel purified and subjected to phosphopeptide mapping. (A) Wild-type receptors (550 cpm, 28-day exposure); (B) Phe-706 mutant receptors (1,030 cpm, 8-day exposure). Peptide y, which is prominent in these maps, was much weaker in the maps shown in Fig. 1 and 4. The reason for its variable intensity is unknown.

kinase activity in vitro or the ability of activated receptor to associate with other proteins in vivo (23). In contrast, Fantl et al. find that mutation of this residue impairs both the rapid PDGF-induced change in receptor conformation and PDGF-induced DNA synthesis in vivo and inhibits PDGF-induced substrate phosphorylation in vitro (15). The second autophosphorylation site in the PDGF β receptor, Tyr-751, is located within the kinase insert domain (Fig. 8) (23). Tyr-751 is not conserved in the CSF-1 receptor (9, 34, 50). However, it is conserved in the PDGF α receptor (27) and in c-*kit* (32).

The function of the kinase insert domain in the PDGF receptor is not well understood, but two studies addressing possible functions of the PDGF receptor kinase insert have been conducted recently. First, Escobedo and Williams reported that a deletion of 83 amino acids from the kinase insert domain yields PDGF β receptors with normal ligand-binding properties and normal kinase activity (14). Although cells that express these mutant receptors show most of the immediate responses that are described for stimulation with PDGF, two major defects were reported. There was no increase in PDGF receptor-associated phosphatidylinositol kinase activity, and the induction of DNA synthesis and cell division was greatly reduced compared with what was found with wild-type receptors (8, 14). Mutation of Tyr-751 in the PDGF β receptor does not affect most of the early responses to PDGF (23). However, it reduces PDGF-induced association of the receptor with a phosphatidylinositol kinase (8) and three other proteins that have been found to coprecipitate with the receptor after stimulation with PDGF (23). Although effects on PDGF-induced DNA synthesis and proliferation were not determined in the initial report (23), more recent studies with the Phe-751 mutant receptor suggests that it is partially defective in eliciting a mitogenic response (A. Kazlauskas and J. A. Cooper, personal communication).

Kinase insert deletions have also been made in v-*fms* and

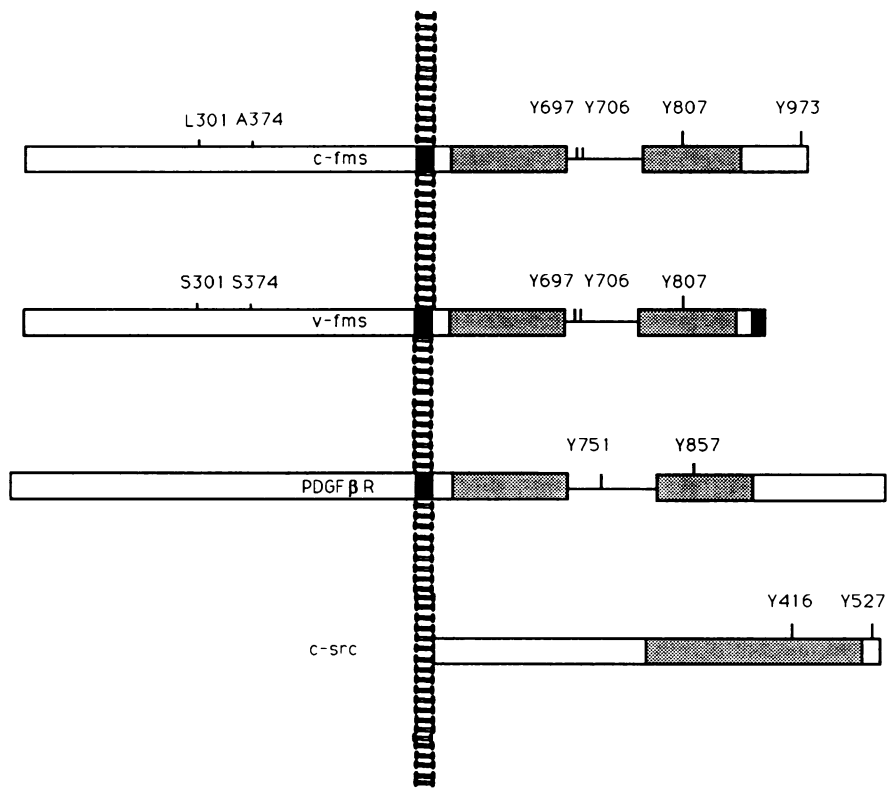

FIG. 8. Schematic representation of the structures of CSF-1 receptor, v-*fms* protein, PDGF receptor, and c-*src* protein. The positions of phosphorylation sites and other residues mentioned in the text are indicated. Symbols: ▦, kinase domain; ——, kinase insert region; ■, the 11 new residues present at the C terminus of v-*fms*. Residues in the *fms* genes are numbered by the convention of Rothwell and co-workers (34). Phosphorylation sites in v-*fms* are assigned on the basis of homologies.

the murine CSF-1 receptor. These mutations do not appear to affect the kinase activity or, in contrast to what has been found for the PDGF β receptor, the ability to transduce signals by either v-*fms* or the CSF-1 receptor (44). v-*fms* lacking a kinase insert domain is still able to transform fibroblasts, and CSF-1 receptors lacking this domain are still able to sustain CSF-1-dependent colony formation in soft agar (44). This supports the hypothesis that the autophosphorylation site in the kinase insert domain might play a role in negative regulation of the CSF-1 receptor.

It has been speculated that phosphorylation of Tyr-969 plays a role in negative regulation of the CSF-1 receptor. This residue is located at the C terminus of the CSF-1 receptor (Fig. 8), a position similar to Tyr-527 in pp60$^{c\text{-}src}$ (39). Phosphorylation of Tyr-527 in pp60$^{c\text{-}src}$ inhibits its kinase activity (2, 5, 24, 30), and it is thought that pp60$^{c\text{-}src}$ is activated by dephosphorylation at this residue. Both Tyr-527 in pp60$^{c\text{-}src}$ and Tyr-969 in the CSF-1 receptor are deleted in the respective viral transforming proteins. Analysis of the transforming potential of several *fms* mutants indicates that changing Tyr-969 into a phenylalanine does not by itself fully activate the transforming potential of the *fms* protein (35, 36). However, changes at this position seem to enhance the effect of activating mutations elsewhere within the *fms* protein (35, 36). This result supports the hypothesis that Tyr-969 is involved in negative regulation. If the analogy with pp60$^{c\text{-}src}$ regulation were true, one would expect to find this residue phosphorylated before stimulation with CSF-1 and dephosphorylated after stimulation. Thus far, P.Tyr has been detected in the CSF-1 receptor only after activation with CSF-1. This indicates that phosphorylation on tyrosine takes places as a consequence of activation rather than being constitutive in order to negatively regulate kinase activity, as observed for pp60$^{c\text{-}src}$. Tyr-973 in the murine CSF-1 receptor, which is homologous to Tyr-969 in the human CSF-1 receptor (9, 34), is contained within a 60-amino-acid tryptic peptide. We have preliminary evidence that the series of phosphopeptides in the lower left corner of the in vivo phosphopeptide maps (Fig. 3 and 4) represent multiple phosphorylated forms of this tryptic peptide. The fact that phosphoamino acid analysis of these peptides obtained from in vivo-labeled receptors has so far yielded only P.Ser supports the idea that Tyr-969 is not phosphorylated within the cell. This in turn suggests that Tyr-969 must have some other essential negative regulatory function that cannot be substituted by phenylalanine.

Murine CSF-1 receptors become highly phosphorylated on tyrosine when subjected to autophosphorylation in vitro. Eight major and several minor phosphopeptides could be resolved on two-dimensional tryptic peptide maps. All the major phosphopeptides contained P.Tyr. Analysis of receptor breakdown products indicate that peptides a through e are contained within an approximately 360-amino-acid fragment of the CSF-1 receptor. Whether this fragment represents the minimal active kinase domain and these phosphorylations are due to autophosphorylation or whether this phosphorylation is a consequence of transphosphorylation by intact receptors is not clear. Peptides a, b, c, and f comigrate with peptides that become phosphorylated in vivo (Fig. 4C and D, peptides A, B, C, and F). Although all of these peptides become phosphorylated when cells are stimulated with CSF-1 at 4°C, phosphorylation on tyrosine seems to be limited to peptides A and B after stimulation at 37°C.

In a parallel study, Tapley et al. have also examined CSF-1-stimulated CSF-1 receptor phosphorylation. They were able to induce significantly higher levels of P.Tyr and P.Tyr-containing tryptic peptides in the receptor when cells were stimulated at 37°C than we observed (43), but this may be because they examined phosphorylation of a CSF-1 receptor expressed from a murine cDNA clone in BALB/c 3T3 fibroblasts and in FDC-P1 early myeloid cells, neither of which normally expresses CSF-1 receptors. In addition, it is possible that these lines express more CSF-1 receptors than BAC1.2F5 cells do and that CSF-1 receptors are down regulated at a lower rate in these cells, thus leaving time for additional phosphorylation events. Alternatively, it is possible that these cells do not express associated or regulatory proteins that normally restrict the autophosphorylation of these receptors. It is worth noting that wild-type CSF-1 receptors expressed in Rat-2 cells phosphorylated in vitro contain one tryptic phosphopeptide which is not seen when receptors are expressed in BAC1.2F5 cells (compare Fig. 1C and 7A). Tapley et al. identified Tyr-697, Tyr-706, and Tyr-807 as the major CSF-1-stimulated phosphorylation sites (43). Tyr-697 is located within the kinase insert domain just upstream of Tyr-706 (Fig. 8). From a comparison of our peptide maps with theirs, we can determine that our tyrosine phosphorylation site in peptide E/e corresponds to Tyr-697. The tyrosine phosphorylation sites in peptide B/b, d, and F/f remain unidentified.

In conclusion, our studies and those of Tapley et al. have identified three phosphorylation sites in the murine CSF-1 receptor. Two of these three sites are located within the kinase insert domain (Fig. 8). Further analysis of the phosphorylation site mutants described in this study, currently in progress, should shed some light on the function of autophosphorylation in the CSF-1 receptor. In addition, we hope to find some clues to the function of the kinase insert domain.

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