Macrophage Colony-Stimulating Factor-Induced Tyrosine Phosphorylation of *c-fms* Proteins Expressed in FDC-P1 and BALB/c 3T3 Cells

PETER TAPLEY,^{†*} ANDRIUS KAZLAUSKAS, JONATHAN A. COOPER, AND LARRY R. ROHRSCHEIDER

Fred Hutchinson Cancer Research Center, 1124 Columbia Street, Seattle, Washington 98104

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The *c-fms* protein is a receptor for macrophage colony-stimulating factor (M-CSF) with intrinsic protein-tyrosine kinase activity. We investigated the tyrosine phosphorylation of murine *c-fms* proteins expressed from a retroviral vector in factor-dependent myeloid FDC-P1 cells and in BALB/c 3T3 fibroblasts transformed by the expression of the *c-fms* gene. FDC-P1 cells expressing *c-fms* were able to grow and differentiate in response to M-CSF. Their *c-fms* proteins were normally phosphorylated on serine and became phosphorylated on tyrosine residues contained in five tryptic peptides when the cells were exposed to M-CSF. A subset of these peptides was constitutively phosphorylated in BALB/c cells expressing *c-fms*, consistent with the production of M-CSF by these cells. All the peptides detected *in vivo* were also phosphorylated *in vitro*. These peptides were analyzed by susceptibility to proteases, comparison with synthetic peptides, and site-directed mutagenesis. The identities of four of the tryptic peptides were determined; they arise from three unique tyrosine phosphorylation sites. One major site of tyrosine phosphorylation at residue 697 accounted for two of the tryptic peptides. A second major site was identified at tyrosine residue 706. These two tyrosine phosphorylation sites are located within the tyrosine kinase insert region. Tyrosine 807, which has homology to the major autophosphorylation site of the p60^{src} tyrosine kinase, is a minor autophosphorylation site. Possible functional roles for these phosphorylations of the *c-fms* protein include interactions with substrate proteins, catalytic activity, and ligand-induced degradation.

Hemopoietic growth factors and their receptors play a central role in the control of hemopoiesis (23, 24, 26). Within the myeloid differentiation pathway, macrophage colony-stimulating factor (M-CSF or CSF-1) regulates the growth and differentiation of committed progenitor cells along the monocyte-macrophage lineage and stimulates the mature cell functions of macrophages (24). These effects are mediated by binding of M-CSF to a single class of high-affinity receptors that have been identified as the protein product of the *c-fms* proto-oncogene (36).

The *c-fms* protein is a member of a diverse group of transmembrane cell surface receptor proteins with intrinsic tyrosine kinase activity (10, 47). In intact cells, the tyrosine kinase activity of the *c-fms* protein is activated after M-CSF binding (5). Stimulation of the tyrosine kinase activity results in the phosphorylation of the *c-fms* protein plus several other cellular proteins which are thought to mediate the biological effects of M-CSF (5, 35). These substrate proteins have not been identified other than on the basis of their molecular weight. Recent studies have determined that the closely related platelet-derived growth factor receptor (PDGF-R) induces the phosphorylation of several proteins including the serine-threonine protein kinase *c-raf*, phospholipase C- γ , and a phosphatidylinositol-3 kinase (3, 9, 14, 25). It seems likely that the *c-fms* protein interacts with a similar group of substrates.

Human, murine, and feline *c-fms* cDNAs have been

isolated and found to encode cell surface receptor tyrosine protein kinases (4, 31, 44). These *c-fms* proteins are members of a group of growth factor receptors with tyrosine kinase activity that can be subdivided into three distinct classes on the basis of structural homologies (47). One class includes the epidermal growth factor receptor (EGF-R) and the HER2/*neu* protein. A second group is composed of the insulin receptor (INS-R) and the insulinlike growth factor 1 receptor, while the third class of receptors includes the *c-fms* protein, the α and β forms of the PDGF-R (21, 45), the *c-kit* protein (46), and the product of the *Drosophila torso* gene (38). This latter group of receptors is characterized by a catalytic protein kinase domain split by a stretch of 70 to 100 amino acids (the so-called kinase insert) and an extracellular domain organized into five immunoglobulinlike repeats. A recently identified receptor for basic fibroblast growth factor also shares some similarities with this receptor class (17).

One immediate consequence of ligand binding to the above tyrosine kinase receptors is tyrosine phosphorylation of the receptors, most likely by an autophosphorylation reaction. The locations of several of these tyrosine phosphorylations have been mapped on the INS-R (42, 43), EGF-R (7, 20), and PDGF-R (15). Biochemical and genetic techniques have been used to determine the effect of the above phosphorylations on receptor function. INS-R autophosphorylation is required for both sustained activation of the receptor tyrosine kinase activity and a biological response to insulin (8, 30, 41, 49). Autophosphorylation of the EGF-R does not greatly alter the activity of the receptor *in vitro* (1, 12), and the role of this autophosphorylation in EGF-R function *in vivo* is not clear. One group has reported that autophospho-

* Corresponding author.

[†] Present address: Division of Human Immunology, Institute of Medical and Veterinary Science, Box 14, Rundle Mall PO, Adelaide 5000, Australia.

rylation is important for biological activity (1), while others find that it is not (11). Autophosphorylation of the PDGF-R, a close relative of the *c-fms* protein, influences the function of this receptor in a novel manner (15). Phosphorylation of a tyrosine in the kinase insert region of the PDGF-R mediates the interaction of the receptor with several cellular proteins thought to be important for signal transduction, including a phosphatidylinositol kinase (3, 9).

The *c-fms* protein undergoes autophosphorylation both in vitro in the presence of $MnCl_2$ and in vivo after M-CSF binding; however, the location and significance of these phosphorylations has not been addressed. We report here the results of analysis of the sites of tyrosine phosphorylation of *c-fms* proteins expressed in FDC-P1 and BALB/c 3T3 cells. Two *c-fms*-expressing cell types were analyzed to check for effects specific for myeloid-lineage FDC-P1 cells versus the fibroblastic BALB/c cells.

MATERIALS AND METHODS

Materials. M-CSF was partially purified from supernatants of yeast cells expressing murine M-CSF (28) by ammonium sulfate fractionation followed by adsorption to DEAE-cellulose and elution with 0.15 M NaCl–0.03 M sodium phosphate buffer (pH 7.4). Peptides were synthesized with an Applied Biosystems peptide synthesizer and purified by high-pressure liquid chromatography. *Pseudomonas fragi*, *Staphylococcus aureus*, and *Flavobacterium meningosepticum* proteases were obtained from the sources described previously (15).

Cell lines. The murine *c-fms* cDNA and subcloning of this DNA into the pMZEN expression vector have been described previously (29, 31). Viral stocks from psi-2 NIH 3T3 packaging cells transfected with the pMZEN(*c-fms*) construct were then used to generate fibroblast BALB/c 3T3 (BALB/*c-fms* cells) and myeloid FDC-P1 [FD(*c-fms*) cells] cell lines expressing murine *c-fms* protein (28, 29). The cells were maintained in Dulbecco modified Eagle medium with 10% fetal calf serum [supplemented with 500 U of M-CSF per ml for the FD(*c-fms*) cells].

Metabolic labeling, immunoprecipitation, and in vitro kinase assays of *c-fms* protein. Metabolic labeling of BALB/*c-fms* and FD(*c-fms*) cells was at 37°C for 4 h in either methionine-free Dulbecco modified Eagle medium supplemented with 5% dialyzed calf serum and 100 μ Ci of [35 S]methionine (ICN Radiochemicals) per ml or phosphate-free Dulbecco modified Eagle medium supplemented with 5% dialyzed calf serum and 2 mCi of 32 P_i (ICN) per ml. FD(*c-fms*) cells were incubated in the absence of M-CSF for 3 h before labeling to upregulate receptor levels. The *c-fms* proteins were isolated from cells by immunoprecipitation from detergent cell lysates with a rabbit polyclonal antiserum directed against the cytoplasmic domain of the murine *c-fms* protein (antiserum 4599B) as described previously (29). In vitro kinase assays were performed with *c-fms* immunoprecipitates at 30°C for 10 min in the presence of [γ - 32 P]ATP (ICN) and 10 mM $MnCl_2$ at pH 5.2 as detailed previously (19). Synthetic peptides were phosphorylated with immune complex p60^{src} kinase (isolated by immunoprecipitation with monoclonal antibody 327 [18]) and [γ - 32 P]ATP as described previously (39).

Phosphoamino acid analysis and phosphopeptide mapping. Phosphoamino acids were analyzed as described previously (2). Phosphopeptide mapping was essentially as described by Kazlauskas and Cooper (15). Briefly, phosphorylated pro-

teins were isolated by sodium dodecyl sulfate-polyacrylamide, gel electrophoresis (SDS-PAGE), excised and eluted from the gel, trichloroacetic acid precipitated, performic acid oxidized for 1 h at 0°C, lyophilized, and digested with tosylphenylalanine chloromethylketone-trypsin (Miles Biochemicals). The tryptic digest products were then repeatedly washed with H₂O and separated on cellulose thin-layer chromatography (TLC) plates (E. Merck AG, Darmstadt, Federal Republic of Germany) at pH 1.9 (1.8 kV, 20 min) in 98% formic acid-glacial acetic acid-H₂O (45:155:1,800) followed by ascending chromatography in isobutyric acid-pyridine-glacial acetic acid-*n*-butanol-H₂O (325:25:15:10:14.5). Secondary digestion of the tryptic peptides was also as described previously (15). The molecular weights of the peptides were estimated by resolution on 27.5% polyacrylamide gels prepared exactly as described by Schuh and Brugge (34). The phosphopeptides were visualized by autoradiography at -70°C with intensifying screens. Relative levels of phosphorylation were estimated by scanning laser densitometry with an Ultrascan XL densitometer (LKB, Bromma, Sweden) and LKB 2400 Gelscan XL software.

Site-specific mutagenesis of *c-fms* cDNA. The template DNA for site-specific mutagenesis was a 3.7-kilobase *Eco*RI fragment containing the entire murine *c-fms* coding sequence (31) cloned into a modified Blue Scribe (Stratagene, San Diego, Calif.) vector, pVZ (pVZ was a gift from T. St. John, Fred Hutchinson Cancer Research Center). Mutagenesis was performed as described by Zoller and Smith (50), except that a single mutagenic primer was used for each reaction. The preparation and characterization of these mutants will be described in a subsequent publication. The 3.1-kilobase *Eco*RI-to-*Nde*I *c-fms* fragment from each mutant was purified and cloned into the *Eco*RI and *Nde*I sites in the polylinker of pZEN113 (pZEN113 was derived from pMZEN [29] by removal of an *Nde*I site within the vector and addition of a polylinker at the unique *Xho*I site). The resulting pZEN(*c-fms*) mutants DNAs were transfected along with a neomycin resistance gene into psi-2 cells by CaPO₄ precipitation. Cells expressing wild-type and mutant *c-fms* proteins were selected in 0.5 mg of G418 per ml, and the top 5% of *c-fms*-expressing cells was obtained by cell sorting with antibodies to the external domain of the *c-fms* protein (28, 29).

RESULTS

Previous reports have demonstrated that introduction of the murine *c-fms* gene in the pMZEN retroviral vector allows cells which do not normally express receptors for M-CSF to become M-CSF responsive. FDC-P1 cells are growth factor dependent and proliferate as undifferentiated myeloid cells that do not express the *c-fms* protein. However, if the murine *c-fms* protein is expressed in these cells, they will both proliferate and express some characteristics associated with differentiation in the presence of M-CSF (28). Expression of the murine *c-fms* protein in fibroblast BALB/c 3T3 cells results in an autocrine transformation of these cells due to endogenous M-CSF production and chronic stimulation of the receptor (29). The experiments described below characterized the M-CSF-induced tyrosine phosphorylation of the *c-fms* proteins expressed in the above cells to determine potential relationships to growth, differentiation, or transformation.

Phosphorylation of *c-fms* protein expressed in FDC-P1 and BALB/c 3T3 cells. Both immature (130 kilodaltons [kDa]) and

FIG. 1. Expression and phosphorylation of the *c-fms* protein in FDC-P1 cells. (A) FD(*c-fms*) cells were labeled with either [^{35}S]methionine (lanes 1 and 2) or $^{32}\text{P}_i$ (lanes 5, 6, and 7) in vivo before immunoprecipitations. Immunoprecipitates from unlabeled cells were labeled with [$\gamma\text{-}^{32}\text{P}$]ATP in vitro (lanes 3 and 4). *c-fms* proteins were immunoprecipitated with antiserum 4599B (lanes 1, 3, 5, 6, and 7) or control serum (lanes 2 and 4) and were resolved by SDS-7.5% PAGE as described in Materials and Methods. The samples in lanes 6 and 7 were from cells treated with 10,000 U of M-CSF per ml for 4 and 10 min, respectively, before lysis. An autoradiograph of the gel is shown. The mobilities of 200- and 97-kDa molecular mass markers are indicated by the arrows. (B) FD(*c-fms*) cells were labeled with $^{32}\text{P}_i$ in vivo and treated without (panel 1) or with (panel 2) 10,000 U of M-CSF per ml for 4 min before lysis and isolation of *c-fms* proteins as described in the legend to panel A. The 165-kDa *c-fms* proteins were then subjected to phosphoamino acid analysis as described in Materials and Methods. Autoradiographs of the separated phosphoamino acids are shown. The positions of phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y) standards are indicated. Also indicated is spot A, which migrates near the phosphotyrosine standard and has been previously shown to contain only phosphoserine (see reference 2).

mature (165 kDa) forms of the *c-fms* protein could be detected after metabolic labeling with [^{35}S]methionine in an FDC-P1 cell line infected with the ZEN(*c-fms*) retrovirus [FD(*c-fms*) cells] (Fig. 1). Both forms of the *c-fms* protein were phosphorylated in vitro (Fig. 1A, lane 3). This phosphorylation occurred exclusively on tyrosine residues (data not shown for *c-fms* from FDC-P1 cells but refer to Fig. 2B for *c-fms* from BALB/c cells). The *c-fms* protein was phosphorylated exclusively on serine residues in vivo before M-CSF stimulation and contained detectable phosphotyrosine only after M-CSF treatment (Fig. 1B). Tyrosine phosphorylation accounted for approximately 10% of the total phosphorylation after M-CSF treatment. A similar amount of M-CSF-dependent tyrosine phosphorylation of the murine *c-fms* protein has been observed previously in the simian virus 40-immortalized mouse macrophage cell line, BAC1.2F5 (5, 35).

The *c-fms* protein expressed in the BALB/*c-fms* cell line was also phosphorylated in vivo (Fig. 2). Overall levels of phosphorylation of *c-fms* protein in the intact cell were not markedly altered by M-CSF treatment and were slightly increased by incubation with the tyrosine phosphatase inhibitor sodium orthovanadate (Na_3VO_4). Phosphoamino acid analysis demonstrated that the *c-fms* protein was largely phosphorylated on serine, with trace amounts of phosphotyrosine which were elevated by Na_3VO_4 but not M-CSF treatment (Fig. 2; data not shown). These results are consistent with the observation that the *c-fms* protein expressed in this mouse fibroblast cell line is subject to chronic stimulation as a consequence of endogenous M-CSF production (29).

Tryptic phosphopeptide analysis of *c-fms* proteins. To characterize the tyrosine phosphorylation sites of the *c-fms* protein, we examined the phosphopeptides generated by trypsin digestion of the phosphorylated protein. The tryptic phosphopeptide maps of *c-fms* proteins expressed in FDC-P1 cells and labeled in vivo with $^{32}\text{P}_i$ are shown in Fig. 3.

FIG. 2. Phosphorylation of the *c-fms* protein in BALB/c 3T3 cells. (A) BALB/*c-fms* cells were labeled in vivo with $^{32}\text{P}_i$ for 4 h with (+) or without (–) 50 μM Na_3VO_4 and then incubated with (+) or without (–) 10,000 U of M-CSF per ml for 4 min as indicated (Materials and Methods). Cells were then lysed, *c-fms* protein was isolated by immunoprecipitation with antiserum 4599B (lanes 3 to 6), control immunoprecipitations were done with nonimmune sera (lanes 1 and 2), and both were resolved by SDS-7.5% PAGE as described in the text. An autoradiograph of the gel is shown. Lane 7 shows an immune complex kinase assay on *c-fms* proteins isolated with antiserum 4599B from unlabeled cells, phosphorylated with [$\gamma\text{-}^{32}\text{P}$]ATP, and resolved by SDS 7.5%-PAGE as described in Materials and Methods. (B) *c-fms* proteins from BALB/*c-fms* cells were labeled either in vitro with [$\gamma\text{-}^{32}\text{P}$]ATP or in vivo with $^{32}\text{P}_i$ as described above and subjected to phosphoamino acid analysis as detailed in Materials and Methods. Shown are autoradiographs of phosphoamino acids from the 130-kDa (panel 1) and 165-kDa (panel 2) forms of the proteins labeled in vitro and the 165-kDa forms of the *c-fms* proteins labeled in vivo in the absence (panel 3) or presence (panel 4) of Na_3VO_4 . The positions of phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y) standards are indicated.

FIG. 3. Tryptic phosphopeptide analysis of *c-fms* proteins from FDC-P1 cells. FD(*c-fms*) cells were labeled with $^{32}\text{P}_i$ in vivo and then incubated with or without 10,000 U of M-CSF per ml for 4 min before lysis and isolation of *c-fms* proteins with antiserum 4599B as described in Materials and Methods. The 165-kDa *c-fms* proteins were then resolved by SDS-7.5% PAGE and digested with trypsin as described in the text. The tryptic phosphopeptides were resolved on a cellulose thin-layer plate by electrophoresis at pH 1.9 and 1.8 kV for 20 min (in the horizontal dimension with the anode to the left) followed by ascending chromatography (in the vertical dimension). Autoradiographs of the tryptic peptide maps of the *c-fms* proteins from control (A, 2,000 cpm) and M-CSF-treated (B, 2,000 cpm) cells are shown. (C) Results of phosphoamino acid (PAA) analysis of some of the peptides resolved in panels A and B. The autoradiographs shown were exposed for 9 days at -70°C with intensifying screens. Peptides containing primarily phosphoserine (PS) are indicated by shading, and peptides containing primarily phosphotyrosine (PY) are not shaded.

Tryptic digestion of the *c-fms* protein from unstimulated FD(*c-fms*) cells resulted in a complex mixture of phosphopeptides composed almost exclusively of phosphoserine (Fig. 3A and C; data not shown). M-CSF induced the appearance of five phosphotyrosine-containing peptides, A, B, C, D, and E (Fig. 3B and C; data not shown). Scanning densitometry was used to determine that the distribution of tyrosine phosphorylation among these peptides was as follows: A, 33%; B, 17%; C, 22%; D, 8%; E, 20%. The phosphorylation of several other peptides, which did not contain detectable amounts of phosphotyrosine, also was altered by M-CSF treatment of the FD(*c-fms*) cells.

The *in vivo* sites of M-CSF-induced phosphorylation on the *c-fms* protein were compared with those phosphorylated *in vitro* (Fig. 4). Figure 4B shows a tryptic phosphopeptide map of *c-fms* proteins labeled *in vitro* with an immunoprecipitate prepared from BALB/*c-fms* cells with antiserum 4599B and labeled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Phosphorylation was detected in three major spots (peptides a, b, and c) and several minor sites (including peptides d and e). Densitometric analysis indicated the following distribution of radioactivity among these five peptides: a, 28%; b, 33%; c, 31%; d, 4%; e, 4%. A similar pattern and distribution of phosphopeptides was also observed after analysis of *in vitro*-labeled *c-fms* proteins from FD(*c-fms*) cells. The sites of *in vitro* phosphorylation were also not altered by either the pH of the kinase assay (19) (pH 7.2 versus 5.2) or when an alternate antiserum directed against the external domain of the *c-fms* protein (29) was used to immunoprecipitate the receptor before *in vitro* phosphorylation (data not shown).

Evidence that the phosphopeptides A, B, C, D, and E detected after *in vivo* labeling of *c-fms* protein and M-CSF treatment correspond to the peptides a, b, c, d, and e resulting from *in vitro* phosphorylation is provided in Fig. 4C. The *in vivo* and *in vitro* phosphopeptides comigrated after mixing before resolution in two dimensions on a cellulose thin-layer plate.

Figure 5A shows tryptic phosphopeptide map of *c-fms* proteins from BALB/*c-fms* cells after labeling *in vivo* with $^{32}\text{P}_i$. A complex pattern of approximately 15 to 20 phosphopeptides, which is reminiscent of the pattern observed in M-CSF-treated FD(*c-fms*) cells, was obtained. Phosphoamino acid analysis revealed that the majority of the peptides present contain phosphoserine (Fig. 5D; data not shown). This is consistent with the results of phosphoamino acid analysis of the intact protein (Fig. 2). Several phosphotyrosine-containing peptides were also detected, including the peptides labeled A, B, and C. Evidence that these three peptides correspond to the peptides a, b, and c phosphorylated *in vitro* (Fig. 5B) was provided by showing comigration after mixing the *in vivo* and *in vitro* tryptic phosphopeptides (Fig. 5C). There is no evidence for *in vivo* phosphorylation of peptides d and e in the BALB/*c-fms* cells, although phosphorylation of peptide d may be obscured by serine-containing phosphopeptides migrating in the same region of the map. The phosphorylation of these peptides was also difficult to detect *in vitro* in this experiment (Fig. 5B). This suggests that phosphorylation of the peptides d and e is cell-type specific.

Identification of three sites of autophosphorylation. The above data indicate that several tyrosine residues on the *c-fms* protein are phosphorylated both *in vitro* and *in vivo*. To characterize these residues, tryptic phosphopeptides from *c-fms* proteins phosphorylated *in vitro* were isolated after separation on cellulose TLC plates. These peptides

FIG. 4. Comparison of in vivo and in vitro phosphopeptide maps. FD(*c-fms*) cells were labeled with $^{32}\text{P}_i$ and treated with 10,000 U of M-CSF per ml for 4 min before lysis and immunoprecipitation of *c-fms* protein with anti-serum 4599B (Materials and Methods). The 165-kDa *c-fms* proteins from these cells, and also *c-fms* proteins from BALB/*c-fms* cells labeled in vitro with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, were isolated by SDS-7.5% PAGE and subjected to trypsin digestion and resolved on cellulose thin-layer plates as described in the legend to Fig. 3. Autoradiographs of the phosphopeptide maps of the *c-fms* protein from M-CSF-treated FD(*c-fms*) (A, 4,000 cpm), the in vitro-labeled *c-fms* protein from the BALB/*c-fms* cells (B, 800 cpm), and a mix of materials from these two sources (C, 2,000 cpm of A and 400 cpm of B) are shown. The autoradiographs were exposed for 4 days at -70°C . (D) Schematic representation of the peptides common to the *c-fms* proteins labeled in vivo and in vitro (hatched), peptides unique to the in vivo-labeled proteins (black), and peptides unique to the in vitro-labeled proteins (open).

were then subjected to both electrophoresis on a 27.5% polyacrylamide gel to estimate molecular weight and subcleavage with a panel of proteases of known specificity. The results of these analyses for peptides a, b, and d are summarized in Table 1. The deduced amino acid sequence of the murine *c-fms* protein exhibits 20 tyrosine residues within the cytoplasmic domain, and these would be contained in 13 potential tryptic peptides. From the susceptibility and resistance of the ^{32}P -labeled peptides to the proteases listed in Table 1, it was possible to determine the presence and absence of specific amino acids. Using this information, we were able to tentatively identify the tyrosine phosphorylation sites in peptides a and b as tyrosine residues 706 and 697, respectively.

To confirm the probable identities of peptides a and b, we synthesized peptides corresponding to portions of the *c-fms* protein including each of these phosphorylation sites. A peptide corresponding to the region of the *c-fms* protein including tyrosine 807 was also synthesized, as tyrosine residues homologous to this residue are phosphorylated in other growth factor receptors such as the PDGF-R (15) and INS-R (42, 43). We were subsequently able to demonstrate that tyrosine residue 807 was contained within peptide d as described below. The sequences of these peptides, Y706, Y697, and Y807, are shown in Table 1. To allow

direct comparison with the authentic peptides derived from the phosphorylated *c-fms* protein, the synthetic peptides were phosphorylated in vitro with p60^{src} kinase and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and then purified by electrophoresis on cellulose TLC plates. So far, we have been unable to phosphorylate these peptides with *c-fms* proteins contained in an immune complex.

The Y706 synthetic peptide corresponds to a portion of the *c-fms* protein including the tryptic peptide containing tyrosine 706 (Table 1). After trypsin digestion, the phosphorylated Y706 peptide comigrated with peptide a after electrophoresis at pH 1.9 followed by hydrophobic chromatography (Fig. 6A). The two species also comigrate after electrophoresis at pH 8.9 (data not shown). Further evidence for the identity of phosphopeptide a as that containing tyrosine 706 was provided by the demonstration that incubation of peptide a with thermolysin generated material comigrating with authentic phosphotyrosine (data not shown). This is consistent with the location of phosphotyrosine 706 as the N-terminal residue in the tryptic fragment PYVR, as thermolysin cleaves adjacent to aliphatic residues such as valine.

The Y697 synthetic peptide corresponds to the product expected to be generated by *F. meningosepticum* proline-specific protease digestion of the tryptic fragment of the

FIG. 5. Tryptic phosphopeptide analysis of *c-fms* proteins from BALB/*c-fms* cells. BALB/*c-fms* cells were labeled with $^{32}\text{P}_i$ before lysis and immunoprecipitation of *c-fms* protein with antiserum 4599B (Materials and Methods). The 165-kDa *c-fms* proteins from these cells, and also *c-fms* proteins from BALB/*c-fms* cells labeled in vitro with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, were isolated by SDS-7.5% PAGE and subjected to trypsin digestion and resolved on cellulose thin-layer plates as described in the legend to Fig. 3. Autoradiographs of the separated phosphopeptides from *c-fms* proteins labeled in vivo (A, 9,200 cpm) or in vitro (B, 400 cpm) or a mix of material from these two sources (C, 4,600 cpm of A and 200 cpm of B) are shown. The autoradiographs were exposed for 4 days for panel A and 8 days for panels B and C at -70°C with intensifying screens. (D) Results of phosphoamino acid (PAA) analysis of some of the peptides resolved in panels A and B. Peptides containing primarily phosphoserine (PS) are indicated by shading, peptides containing primarily phosphotyrosine (PY) are not shaded, and the hatched peptide contains primarily phosphoserine but also significant amounts of phosphotyrosine [PS(PY)].

c-fms protein containing tyrosine 697 (Table 1). Figure 6B shows comigration of the product generated by *F. meningosepticum* protease digestion of tryptic peptide b with the phosphorylated Y697 peptide. The two species also comigrate after electrophoresis at pH 8.9 (data not shown).

The Y807 synthetic peptide corresponds to the tryptic fragment of the *c-fms* protein containing tyrosine 807 (Table 1). Preliminary comparisons of the phosphorylated Y807 peptide and the unknown peptides c, d, and e indicated that peptide d may be equivalent to the Y807 peptide (data not shown). This possibility is supported by the demonstration that the tryptic peptide d and the phosphorylated Y807 peptide comigrated (Fig. 6C). The two species also comigrate after electrophoresis at pH 8.9 (data not shown). One unexpected property of peptide d was the cleavage by *F. meningosepticum* protease, which normally cleaves after proline residues (48). This susceptibility to the *F. meningosepticum* protease was, however, shared by both the peptide d derived from the *c-fms* protein and the synthetic peptide Y807 and therefore provides further evidence for their identity (data not shown). A tryptic phosphopeptide from the PDGF-R also has been reported to show a similarly unexpected sensitivity to this protease (15).

Confirmation of tyrosine phosphorylation sites by site-

directed mutagenesis. Mutations were made at the proposed phosphorylation sites by site-directed mutagenesis, changing the tyrosine (Y) residue to a phenylalanine (F) residue in each case. The presence or absence of the proposed phosphopeptide was then examined by two-dimensional mapping of tryptic fragments after phosphorylation of the wild-type and mutant *c-fms* proteins in vitro. The results in Fig. 7A show the tryptic phosphopeptides obtained from the wild-type *c-fms* protein. The five prominent phosphopeptides are labeled a to e. Mutant *c-fms* protein containing a phenylalanine at position 706 (F706) lacked phosphopeptide a but contained all others (Fig. 7B). Likewise, mutation of tyrosine to phenylalanine at position 807 (F807) resulted in the disappearance of phosphopeptide d only (Fig. 7C). When the single mutation F706 was combined with a mutation changing tyrosine 697 to a phenylalanine residue (F697/F706), then phosphopeptide a was absent as expected from the result with the single F706 mutant, and in addition, phosphopeptides b and c vanished (Fig. 7D). The triple mutant, F697/F706/F807, lacked all but the e phosphopeptide (Fig. 7E). These results confirm the identification of tyrosine phosphorylation sites at residues 697, 706, and 807 with phosphopeptides b, a, and d, respectively.

Phosphopeptide c is related to phosphopeptide b. The

TABLE 1. Protease susceptibility of tryptic phosphopeptides from the *c-fms* protein^a

Protease	Specificity ^b	Peptide		
		a	b	d
<i>P. fragi</i>	X-C ^c , D	—	+	ND ^d
<i>S. aureus</i> (V8)	E-X	—	+	—
<i>F. meningosepticum</i>	P-X	—	+	+ ^e
Chymotrypsin	F, W, Y-X P (not Y-X)	—	—	—
Thermolysin	X-I, L, V, M	+	+	+
Apparent molecular weight		<2,500	<2,500	<2,500

^a The *c-fms* protein was isolated by immunoprecipitation from BALB/*c-fms* cells, labeled in vitro, digested with trypsin, and resolved in two dimensions on a cellulose TLC plate as described in Materials and Methods. Phosphopeptides a, b, and d were located by autoradiography, scraped from the TLC plate, eluted, and subdigested with the above proteases. Digestion products were divided into aliquots, resolved by both thin-layer electrophoresis at pH 3.5 and ascending chromatography, and visualized by autoradiography. A positive score indicates that the mobility of the tryptic peptide was altered by further digestion with the indicated protease. The following are the amino acid sequences surrounding the tyrosine residues in peptides a and b and the tyrosine in a sequence homologous to the major autophosphorylation site at residue 416 in the p60^{src} protein (10):

The sequences of the synthetic peptides prepared to each of these sites are underlined, the arrowheads indicate the location of potential trypsin cleavage sites, and the superscript Ps indicate potential tyrosine phosphorylation sites.

^b The specificity of each protease, as defined by the N- and C-terminal amino acid residues on either side of the cleaved bond, is shown.

^c Cysteic acid.

^d ND, Not determined.

^e The predicted sequence for peptide d does not include a conventional cleavage site for the *F. meningosepticum* protease. However, both peptide d and the synthetic Y807 peptide were cleaved by this protease, possibly at the pY807-V808 bond (data not shown).

mutational analysis of *c-fms* phosphopeptides provided the interesting result that whenever tyrosine 697 was changed to a phenylalanine, both phosphopeptides b and c disappeared. A third, minor spot, migrating farther toward the cathode (the right in Fig. 7) but on the same line as phosphopeptides b and c, also disappeared. This could result from one mutation affecting multiple phosphorylation sites. However, the deduced amino acid sequence of the tryptic peptide containing the tyrosine 697 phosphorylation site contained three consecutive basic residues at the amino-terminal end of the peptide and could yield multiple tryptic fragments containing tyrosine 697. This latter possibility was tested by recovering phosphopeptides b and c from the cellulose thin-layer plate of wild-type *c-fms* and comparing their electrophoretic mobility before and after redigestion with two additional proteases. Redigestion of phosphopeptides b and c with either of two proteases known to cleave within the sequence of the b tryptic phosphopeptide generated new phosphopeptides of b and c that comigrated at pH 1.9 (Fig. 7F). Comigration also

was observed at pH 3.5 or pH 8.9 in one dimension, or when separations were performed in the standard two dimensions (data not shown). This indicates that phosphopeptide c also contains tyrosine residue 697 and is most likely identical to phosphopeptide b but with an additional lysine at the amino terminus.

DISCUSSION

Autophosphorylation sites in the kinase insert and kinase domains of the *c-fms* protein. The data presented here establish the presence of two autophosphorylation sites at residues 697 and 706 in the kinase insert region of the *c-fms* protein. These residues are major sites of phosphorylation both in vitro and in vivo. Tyrosine residue 807 is also phosphorylated, although at a somewhat lower level. This latter residue is located in the second region of the kinase domain and has homology to the p60^{src} residue 416 phosphorylation site which is conserved in many protein-tyrosine kinases.

The sequences surrounding each of the three phosphorylation sites showed limited similarity (Fig. 8). One conserved feature is the presence of an acidic amino acid three residues N terminal of the phosphorylated tyrosine. Two of the sites also have a second acidic residue seven residues N terminal of the phosphorylated tyrosine. The presence of acidic residues in these regions is characteristic of autophosphorylation sites on several protein-tyrosine kinases (13) and perhaps indicates sequence requirements of the *c-fms* protein-tyrosine kinase activity.

The autophosphorylation sites identified on the murine *c-fms* protein are all conserved in the human and feline *c-fms* proteins (4, 31, 44). The sites most closely resemble, in terms of their location in the primary sequence, the tyrosine phosphorylation sites identified on the PDGF-R (15). The kinase insert of the PDGF-R contains a phosphorylation site at residue 751. The kinase insert domains of the two receptors are, however, generally not closely conserved, and neither tyrosine 697 or 706 of the *c-fms* protein could be considered the exact homolog of tyrosine 751 of the PDGF-R. It will be of interest to determine whether tyrosine residues located in the insert regions of other related receptors such as the *c-kit* protein (46) and the candidate receptor for basic fibroblast growth factor (17) are also sites of autophosphorylation. The tyrosine phosphorylation site at residue 807 of the *c-fms* protein is homologous to the major autophosphorylation site of the PDGF-R at tyrosine 857 (15). The INS-R is also autophosphorylated on the residue corresponding to tyrosine 807, plus two other tyrosines in the same region of the protein (42, 43). The homologs of tyrosine 807 are also autophosphorylated in nonreceptor tyrosine kinases such as p60^{src} (27, 37) and the *fps* kinase (22 and references therein).

The autophosphorylation sites identified on the EGF-R and the closely related HER2/*neu* protein are located exclusively on the carboxy-terminal tails of these receptors (7, 20). Negative regulatory properties have been attributed to the C-terminal domain of the *c-fms* protein (32, 44), and the tyrosine residue at position 973 was originally hypothesized to be a site of phosphorylation (33). However, none of the tyrosine phosphorylation sites we identified in the *c-fms* protein are located in the C-terminal domain. We can also exclude the possibility that phosphopeptide e, or any of the remaining minor unidentified tyrosine phosphorylation sites, is located in the carboxy-terminal tail at residue 973. Exper-

FIG. 6. Comparison of authentic and synthetic phosphopeptides. Synthetic peptides were phosphorylated *in vitro* with p60^{src} kinase and isolated by thin-layer electrophoresis at pH 3.5 as described in Materials and Methods. The mobilities of the synthetic and authentic peptides were compared by the conditions described in the legend to Fig. 3. Autoradiographs of the maps of each peptide are shown. A 1:1 mix of these two samples is shown in the lower panel. (A) The mobility of phosphopeptide a (top panel) was compared with that of the phosphorylated tryptic digestion product of peptide Y706 (middle panel). (B) The mobility of the product of *F. meningosepticum* protease digestion of phosphopeptide b (top panel) was compared with that of the phosphorylated peptide Y697 (middle panel). (C) The mobility of phosphopeptide d (top panel) was compared with that of the phosphorylated peptide Y807 (middle panel). The dots in the photographs of panel B indicate the origin. The origin was not included in the photographs of panels A and C, but the arrowheads show the relative direction of that origin.

iments with a bacterially expressed C-terminal fragment of the *c-fms* protein phosphorylated with *v-src* kinase indicate that the tryptic fragment containing phosphotyrosine at residue 973 does not correspond to any of the unknown phosphotyrosine-containing peptides (unpublished observations).

Two of the major tyrosine autophosphorylation sites we identified are within the kinase insert region, yet this region can be deleted from both *v-fms* and the murine *c-fms* proteins without loss of the ability to transform fibroblasts (40). Phosphorylation of the tyrosines residing in peptides d and e was also greatly reduced by removal of the kinase insert (unpublished observations). One interpretation of these data is that extensive tyrosine phosphorylation of the *c-fms* protein is not required for *c-fms*-mediated transformation of fibroblasts. Alternatively, deletion of the entire kinase insert may mimic an effect normally induced by phosphorylation of this region. It is also important that the expression of the mutant *c-fms* protein lacking the kinase insert did not induce exactly the same phenotype as the normal *c-fms* protein. NIH 3T3 cells expressing the mutant *c-fms* protein formed smaller colonies in soft agar (40). The tyrosine phosphorylation of the *c-fms* protein may therefore

have significant effects on the efficiency of *c-fms* protein function in fibroblasts.

It is also possible that some of the functions mediated by autophosphorylation are specific for myeloid cells in which the *c-fms* protein is normally expressed and have effects on both the proliferation and differentiation of cells (28). The tyrosines in peptides d and e were more extensively phosphorylated in the myeloid FDC-P1 cells than the BALB/c 3T3 cells. This result also suggests that phosphorylation of these sites is more important for functions in myeloid cells.

Possible functional roles of tyrosine autophosphorylation. Possible functions of *c-fms* autophosphorylation are suggested by previous studies of the autophosphorylation of other tyrosine kinase growth factor receptors. Phosphorylation of a tyrosine in the kinase insert region of the PDGF-R is important for association with substrate proteins (15, 25). It is possible that the phosphorylation of tyrosine residues 697 and 706 facilitates or stabilizes the interaction of the *c-fms* protein with substrate proteins. There is also evidence that phosphorylation of residues homologous to tyrosine 807 is involved in the activation of the catalytic activity of tyrosine kinases such as the INS-R (41, 49), *fps* (22), and *src*

FIG. 7. Analysis of *c-fms* mutants and identification of phosphopeptide c. *c-fms* proteins were immunoprecipitated from psi-2 fibroblasts expressing wild-type (A), F706 (B) and F807 (C) single-mutant, F697/F706 double-mutant (D), or F697/F706/F807 triple-mutant (E) *c-fms* and labeled in vitro with [γ - 32 P]ATP. The 165- and 130-kDa *c-fms* proteins were isolated by SDS-7.5% PAGE and subjected to trypsin digestion, and tryptic phosphopeptides were resolved on cellulose TLC plates as described in the legend to Fig. 3. Arrows in panels B through E denote phosphopeptide(s) absent in each of the mutant *c-fms* proteins. (F) Phosphopeptides b and c were extracted from TLC plates and reanalyzed by electrophoresis at pH 1.9 (from left to right, as for the horizontal dimension of the two-dimensional maps) after further digestion with trypsin (lower pair of samples), *S. aureus* (V8) protease (middle pair), or *F. meningosepticum* (Fm) protease (upper pair).

(16) proteins. Another possibility is that phosphorylation of tyrosine residues 697, 706, and 807 provides a signal for endocytosis and degradation of the receptor after ligand binding. This hypothesis is consistent with the observation

that tyrosine kinase activity is required for ligand-induced degradation of the *c-fms* protein (6). These potential biological functions are currently being evaluated by expression of the site-directed mutants in both fibroblasts and myeloid cells.

FIG. 8. Autophosphorylation sites on the *c-fms* protein. The relative locations of the known tyrosine phosphorylation sites (Y697, Y706, and Y807) are shown on a schematic representation of the *c-fms* protein. Also indicated are the transmembrane domain (TM) and the lysine required for ATP binding (K614). The inset shows the sequences surrounding the three phosphorylation sites. The underlined residues indicate the sequences of the synthetic peptides prepared to test the identity of each site.

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