Tyrosine phosphorylation of the v-Fms oncogene product is required for its association with a 55-kDa protein

Tyrosine autophosphorylation of the v-Fms oncogene product results in the formation of high affinity binding sites for cellular proteins with Src homology 2 (SH2) domains that are involved in various signal cascades. Tryptic digestion of the autophosphorylated v-Fms and of its cellular counterpart, the feline c-Fms polypeptide, gave rise to at least six common major phosphopeptides, four of which have been characterized previously. Employing site-directed mutagenesis and phosphopeptide mapping of in vitro phosphorylated glutathione S-transferase v-Fms fusion proteins as well as full-length v-Fms molecules expressed in various cells, we show here that Tyr543 of the juxtamembrane domain and Tyr696 of the molecules expressed in various cells, we show here that ferasev-Fmsfusionproteinsaswellasfull-lengthv-Fms

polyacrylamide gel electrophoresis; GT, glutathione

ptermed PTP-1C), SH2 containing protein tyrosine phosphatase; PAGE, GST, glutathione

sociate specifically with an as yet undefined 55-kDa cel-

known SH2 domain-containing cellular proteins but as-

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The v-fms oncogene of feline sarcoma virus encodes a modi-

ified receptor tyrosine kinase that differs from the cellular re-

ceptor for the colony-stimulating factor 1 (CSF-1, also termed

M-CSF)1 only in seven amino acid positions and in the C-

terminal sequence (1–4). Both proteins are known to share

overall structural similarities with the platelet-derived growth

factor (PDGF) receptor (5, 6). The c- and v-Fms molecules

contain a large extracellular domain that binds CSF-1 and a

cytoplasmic tyrosine kinase domain, split by an insertion of

approximately 70 amino acids termed the kinase insert (KI)

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Tyrr579 and Tyr581, provide the binding sites for polypeptides

in the v-Fms molecule is thought to have gained biochemical

properties that are observed with the c-Fms polypeptide only

transiently upon binding to CSF-1 (3, 7). Activation of the

tyrosine kinase leads to autophosphorylation of the cytoplasmic

domain of the Fms molecule at multiple sites. The newly

formed phosphotyrosine residues constitute binding sites for

Src homology 2 (SH2) domain-containing cytoplasmic proteins

that may participate in the control of mitogenic pathways, cell

metabolism, or cell morphology. The entire cytoplasmic domain

of v-Fms (408 amino acids) contains 18 tyrosine residues that

are conserved among human, mouse, feline, and chicken c-Fms.

All c-Fms proteins, however, contain an additional tyrosine

residue at the C-terminal end (3, 8, 9).2 Three tyrosine phos-

phorylation sites in the KI domain, Tyr543, Tyr558, and Tyr581

(reverse to Tyr697 in the mouse c-Fms), and Tyr579 in the second kinase (K2) domain, have been mapped previously (10–17). Tyrosine phosphorylation sites of Fms-related tyrosine kinase receptors and the corresponding proteins binding to such sites have been studied extensively (18). For the PDGF β receptor, nine tyrosine phosphorylation sites have been mapped, two of which are located in the JX domain (19, 20). These two tyrosine phosphorylation sites, Tyr579 and Tyr581, provide the binding sites for polypeptides belonging to the Src family (19, 20) and Shc (21). Although it has been suggested that the corresponding tyrosine, Tyr558, in the Fms JX domain provides a similar function (22), neither phosphorylation of this residue nor the potential regulatory role for downstream cascades have been studied.

In this paper, we show that Tyr543 in contrast to Tyr558 is

indeed a major autophosphorylation site of the v-Fms polypep-

dide. We demonstrate that cellular proteins including the

growth factor receptor bound protein 2 (Grb2) and p85 and

p110 subunits of phosphatidylinositol 3′-kinase. In contrast,
fusion proteins containing the juxtamembrane domain

phosphorylated on Tyr543 fail to bind any of the known SH2 domain-containing cellular proteins but associate specifically with an as yet undefined 55-kDa cellular protein that by itself is phosphorylated on tyrosine.

The v-fms oncogene of feline sarcoma virus encodes a modified receptor tyrosine kinase that differs from the cellular receptor for the colony-stimulating factor 1 (CSF-1, also termed M-CSF)1 only in seven amino acid positions and in the C-terminal sequence (1–4). Both proteins are known to share overall structural similarities with the platelet-derived growth factor (PDGF) receptor (5, 6). The c- and v-Fms molecules contain a large extracellular domain that binds CSF-1 and a cytoplasmic tyrosine kinase domain, split by an insertion of approximately 70 amino acids termed the kinase insert (KI) region (Fig. 1). Furthermore, a segment of 35 amino acids, termed the juxtamembrane (JX) domain, separates the membrane spanning domain from the first tyrosine kinase domain (Fig. 1). Through two of the amino acid substitutions in the extracellular domain and the replacement of the C terminus, the v-Fms molecule is thought to have gained biochemical properties that are observed with the c-Fms polypeptide only transiently upon binding to CSF-1 (3, 7). Activation of the tyrosine kinase leads to autophosphorylation of the cytoplasmic domain of the Fms molecule at multiple sites. The newly formed phosphotyrosine residues constitute binding sites for Src homology 2 (SH2) domain-containing cytoplasmic proteins that may participate in the control of mitogenic pathways, cell metabolism, or cell morphology. The entire cytoplasmic domain of v-Fms (408 amino acids) contains 18 tyrosine residues that are conserved among human, mouse, feline, and chicken c-Fms. All c-Fms proteins, however, contain an additional tyrosine residue at the C-terminal end (3, 8, 9).2 Three tyrosine phosphorylation sites in the KI domain, Tyr543, Tyr558, and Tyr581 (corresponding to Tyr697, Tyr706, and Tyr721 in the mouse c-Fms), and Tyr579 in the second kinase (K2) domain, have been mapped previously (10–17). Tyrosine phosphorylation sites of Fms-related tyrosine kinase receptors and the corresponding proteins binding to such sites have been studied extensively (18). For the PDGF β receptor, nine tyrosine phosphorylation sites have been mapped, two of which are located in the JX domain (19, 20). These two tyrosine phosphorylation sites, Tyr579 and Tyr581, provide the binding sites for polypeptides belonging to the Src family (19, 20) and Shc (21). Although it has been suggested that the corresponding tyrosine, Tyr558, in the Fms JX domain provides a similar function (22), neither phosphorylation of this residue nor the potential regulatory role for downstream cascades have been studied.

In this paper, we show that Tyr543 in contrast to Tyr558 is indeed a major autophosphorylation site of the v-Fms polypeptide. We demonstrate that cellular proteins including the growth factor receptor bound protein 2 (Grb2) and p85 and p110 subunits of phosphatidylinositol 3′-kinase bind specifically to recombinant proteins containing the KI domain but not to those containing the JX domain. A fusion protein containing this latter domain binds a yet undefined 55-kDa cellular protein in a Tyr543 phosphorylation-dependent manner.

EXPERIMENTAL PROCEDURES

Cells and Antibodies—Mouse NIH 3T3 cells, wt-v-Fms cells (NIH 3T3 cells expressing the wild type v-fms gene), or Y696F-, Y705F-, or Y907F-v-Fms cells (expressing mutant v-Fms proteins in which a single tyrosine in position 696, 705, or 807, respectively, was replaced by phenylalanine) were grown in Dulbecco modified Eagle’s medium supplemented with 10% fetal calf serum. FDCP-1Mac11 cells (23) were maintained in Dulbecco modified Eagle’s medium supplemented with 10% fetal calf serum and WEHI3BD-conditioned medium as a source of interleukin-3 at a concentration that stimulated optimal cell growth.

* This work was supported by Deutsche Forschungsgemeinschaft Grants Ta-111/1-3, SFB272 B4, and HIFSP. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ This work is part of the thesis submitted as partial fulfillment for the Ph.D. degree.

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1 The abbreviations used are: CSF-1, colony-stimulating factor 1; GST, glutathione S-transferase; JX, juxtamembrane; KI, kinase insert; PDGF, platelet-derived growth factor; GAP, GTPase-activating protein; PI, phosphatidylinositol; SH2, Src homology 2; SHP-1 (previously termed PTP-1C), SH2 containing protein tyrosine phosphatase; PAGE, polyacrylamide gel electrophoresis; GT, glutathione.

2 H. Beug, personal communication.
An anti-v-Fms antiserum was used as described previously (24). Monoclonal antibodies against phosphotyrosine (4G10, Shc, and the p85 subunit of PI 3’ kinase were purchased from Upstate Biotechnology Incorporated (Lake Placid, NY). Polyclonal cross-reactive antibody that bound to the SH2 domains of both the p85 and p85 subunits of PI 3’ kinase, and monoclonal antibodies against Grb2 or Nck were from Transduction Laboratory (Lexington, KY). A polyclonal antibody against the p110 subunit of PI 3’ kinase was from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-est antibody was kindly provided by S. A. Courtneidge (SUGEN, Redwood City, CA), monoclonal antibody against p120Ras GTPase-activating protein (GAP) was provided by S. J. Parsons (University of Virginia Health Science Center, Charlottesville, VA) (25). Antibody against protein tyrosine phosphatase, SHP-1, was provided by A. Ulrich (Munich, FRG).

**RESULTS**

**Six Major phosphopeptides Are Common in the v-Fms and Feline c-Fms—**The cytoplasmic domain of v-Fms contains 18 tyrosine residues in positions identical with those in the feline c-fms molecule. The latter contains, however, an additional tyrosine residue, Tyr977, at the C-terminal end. This tyrosine was suggested to regulate tyrosine kinase activity (28). To compare the autophosphorylation sites of v-Fms and feline c-Fms, we isolated the two proteins from cell lysates by immunoprecipitation labeled with anti-species-specific immunoglobulin G (IgG, ICN). Bound radioactivity was quantified with a model BAS1000 bio-imaging analyzer (Fuji Photo Film Co., Kanagawa, Japan).

**Kinase Assays, Phosphoamino Acid Analyses, and Tryptic Peptide Mapping—**Kinase assays, phosphoamino acid analyses, and tryptic peptide mapping were performed as described previously (24, 27). Fms-specific immune complexes were incubated for 20 min at room temperature with 3 μCi of [gamma-32P]Pi (Amersham Buchler) in the presence of 10 mM MnCl2 and analyzed by SDS-PAGE. For phosphoamino acid analyses, material was eluted from the gels and hydrolyzed for 2 h in 6 M HCl at 110 °C. Samples were analyzed by two-dimensional electrophoresis (24). For tryptic peptide mapping, material was digested with trypsin (300 µg/ml) for 20 h. Products were analyzed by two-dimensional electrophoresis and chromatography, as described (27).

A compilation of published data on Fms autophosphorylation sites involves Fms molecules from several animal species (10–17) and suggests that by analogy, Tyr696, Tyr705, Tyr720, and Tyr807 of the v-Fms molecule (numbering according to Ref. 3) should also be phosphorylated. Experimentally, however, this has been proven only for Tyr705 (16) and Tyr807 (16, 17). We first wanted to assess the role of Tyr696. For this purpose, we expressed mutant v-Fms proteins in mouse NIH 3T3 cells in which Tyr696, Tyr705, or Tyr807 were individually replaced by phenylalanines. Mutant and wild type v-Fms molecules were isolated and phosphorylated as above and analyzed by tryptic peptide map-
In agreement with previous studies, spots e or c were absent from samples lacking Tyr705 or Tyr807, respectively. Upon mutation of Tyr696, spots b and b9 disappeared. These data show that Tyr696 of v-Fms is indeed a major autophosphorylation site. Three major phosphopeptides designated a, d, and f and four minor phosphopeptides, designated g, h, i, and j remain to be assigned to phosphorylation of specific sites.

The JX Domain of v-Fms Contains a Major Autophosphorylation Site—To define additional tyrosine phosphorylation sites, we incubated recombinant GST fusion proteins containing various C-terminal v-Fms sequences in autophosphorylation assays together with wild type v-Fms-containing immune complexes (Fig. 4). Three of the fusion proteins, GST-JX-Fms, GST-KI-Fms, and GST-K2-Fms, became phosphorylated on tyrosine, whereas GST-CT-Fms (containing Tyr921) and GST were no substrates for the v-Fms kinase. The level of phosphorylation of GST-K2-Fms was about 5-fold lower than observed with GST-JX-Fms and GST-KI-Fms (data not shown). We have shown previously that the second kinase domain of v-Fms contains Tyr807 as a major phosphorylation site, yielding spot e upon phosphopeptide mapping (17). GST-KI-Fms (containing Tyr662, Tyr665, Tyr705, and Tyr720) yielded four phosphopeptides, migrating in positions b, b’, e, and f in Fig. 5C. The deletion of Tyr662 and Tyr665 did not alter this pattern of phosphopeptides (data not shown), suggesting that these residues are not phosphorylated. A replacement of Tyr720 by phenylalanine (GST-Y720F-KI-Fms) resulted in the disappearance of spot f (data not shown). Together, these data indicate that material underlying spots b and b’ provides phosphotyrosines Tyr696 and Tyr705 and that spot f represents a peptide containing Tyr720. Importantly, tryptic peptide maps from GST-JX-Fms revealed one major and one minor phosphopeptide that co-migrated with peptides a and g derived from autophosphorylated full-length v-Fms (Fig. 5, A and B). This suggests that the juxtamembrane domain of the Fms tyrosine kinase also contains autophosphorylation sites.
Tyrosine Phosphorylation of Juxtamembrane Domain of v-Fms

Tyr\textsuperscript{543} in the JX Domain Is a Major Autophosphorylation Site—Which tyrosine residue of the JX domain is phosphorylated? The GST-JX-Fms contains 5 tyrosine residues at amino acid positions 537, 543, 553, 558, and 563 and could potentially yield three Fms-specific fragments upon exhaustive trypsin digestion. The first fragment contains Tyr\textsuperscript{543}, the second contains Tyr\textsuperscript{543} and Tyr\textsuperscript{558}, and the third contains tyrosine residues Tyr\textsuperscript{537}, Tyr\textsuperscript{543}, Tyr\textsuperscript{558}, and Tyr\textsuperscript{563}. We generated mutant GST-JX-Fms fusion proteins in which Tyr\textsuperscript{537} or Tyr\textsuperscript{543} were individually replaced by phenylalanine residues and used them as substrates for the v-Fms tyrosine kinase. Whereas GST-Y537F-JX-Fms was phosphorylated to the same extent as the GST-wt-JX-Fms (Fig. 6, A and B, lanes 2), the Y543F point mutation completely abolished phosphorylation (Fig. 6, A and B, lanes 3), suggesting that Tyr\textsuperscript{543} is indeed one of the main phosphorylation targets of the v-Fms tyrosine kinase. To further support that phosphorylation of Tyr\textsuperscript{543} correlates with the presence of spot a, we compared the phosphorylation pattern full-length wt-v-Fms and Y543F-v-Fms as derived from transfected NIH 3T3 cells. Fig. 6 (C and D) shows that the mutant protein exhibited unchanged tyrosine kinase activity but specifically lacked spot a in phosphopeptide analyses.

In Vivo Phosphorylated GST-KI-Fms Fusion Protein Associates with Grb2 and the p110 and p85 Subunits of PI 3′ Kinase, Whereas GST-JX-Fms Protein Associates with p55—To examine whether tyrosine phosphorylation of the JX domain generates novel binding sites for cellular proteins, we purified GST, GST-JX-Fms, and GST-KI-Fms from E. coli strain TKX-1, which expresses the active tyrosine kinase Elk. Fig. 7B shows that GST-Fms proteins from this strain indeed contained photophosphorylation (lanes 4 and 6), as demonstrated by photophosphorylase activity. Control preparations of the same proteins from E. coli strain DH5\textalpha; lacked photophosphorylation (Fig. 7B, lanes 3 and 5). According to the results for v-Fms fusion proteins with cellular proteins, several SH2 domain-containing proteins including PI 3′ kinase (14), Grb2 (15), members of Src-family (29), and p120RasGAP (17) were shown to bind to the activated receptor molecule. In hematopoietic cells, p150, a protein with an as yet unknown function, was shown to associate with c-Fms (30). Furthermore, SHP-1 (previously termed PTP-1C; Refs. 31 and 32) appears to be one of the major substrates of the c-Fms tyrosine kinase in these cells (33). For these reasons, we employed cell lysates from the premyeloid cell line FDCP-1Mac11 (25) and NIH 3T3 cells to study binding of cellular proteins to the above phosphorylated GST-Fms fusion proteins. In agreement with previous work (14), the p85 subunit of PI 3′ kinase bound to phosphorylated GST-KI-Fms (GST-pY-KI-Fms, Fig. 7C). In addition, the adapter protein Grb2 was detected in the GST-pY-KI-Fms fraction (15). No other proteins such as Shc, SHP-1, Nck, p120RasGAP, or members of the Src-family were

Fig. 6. Tyr\textsuperscript{543} is a tyrosine phosphorylation site of the v-Fms tyrosine kinase. A, GST-JX-Fms (lane 1), GST-JX-Y537F-Fms (lane 2), or GST-JX-Y543F-Fms (lane 3) were purified by GT-agarose. Samples were analyzed by 11% SDS-PAGE and visualized by Coomassie Blue staining. B, purified GST-Fms fusion proteins were used as substrates for in vitro kinase assays using v-Fms tyrosine kinase as detailed in Fig. 4. C, cell lysates from wild type- (lane 1) or Y543F-v-Fms-expressing NIH 3T3 cells (lane 2) were subjected to Fms-specific immunoprecipitation and in vitro kinase phosphorylation reaction. Products were analyzed by SDS-PAGE using 7.5% gels and autoradiography. D, tryptic peptide mapping of wild type- (panel 1) and Y543F-v-Fms molecules (panel 2). The arrowhead marks a phosphopeptide that is missing in the Y543F-v-Fms sample.

Fig. 7. Binding of Grb2 and PI 3′ kinase to in vivo phosphorylated GST-KI-Fms. GST, GST-JX-Fms, and GST-KI-Fms fusion proteins were isolated by binding to GT-agarose beads from E. coli strains TKX1 (TK) or DH5\textalpha; (DH) as phosphorylated or nonphosphorylated proteins, respectively. A and B, analysis of agarose bead fractions with various GST fusion proteins (50 \textmu;g each) were incubated with cell lysates from 10\textsuperscript{5} resting NIH 3T3 or FDCP-1Mac11 cells as detailed under “Experimental Procedures” and analyzed by SDS-PAGE followed by immunoblotting using monoclonal antibodies against Grb2, She, p85 of PI 3′ kinase, p120RasGAP, Nck, or polyclonal antibodies against SHP-1 (PTP-1C), or the C-terminal sequence of the Src (CST). Cell lysates (50 \textmu;g of protein) from NIH 3T3 or FDCP-1Mac11 were blotted as positive controls (Cr).
were analyzed by SDS-PAGE and phosphotyrosine immunoblotting.

containing in vivo FDCP-1Mac11 cells as indicated. Bound protein was analyzed by SDS-PAGE and phosphotyrosine immunoblotting.

Tyr537 or Tyr543 were replaced by phenylalanine residues. In employed the two GST-JX-Fms fusion proteins in which either tyrosine phosphorylation of the Fms-specific segment, we em-

ployed the two GST-JX-Fms fusion proteins in which either

antibody (Fig. 7

because it was not reactive with a Shc-specific monoclonal

species did not represent the p55 subunit of PI 3

lanes 1

lanes 1

lanes 3

lanes 1

lanes 1

lanes 3

lanes 1

lanes 2

lanes 2

lanes 1

lanes 2

lanes 1

lanes 2

FIG. 8. Phosphorylation of Tyr543 of the v-Fms protein is a prerequisite for association with a 55-kDa cellular protein. A, a 55-kDa cellular protein binds to the JX domain of v-Fms. GT-agarose beads precharged with GST (lanes 1), or tyrosine-phosphorylated GST-JX-Fms (lanes 2), or GST-KI-Fms (lanes 3) were incubated overnight with lysates from metabolically Tran[35S]-labeled NIH 3T3 or FDC-P1Mac11 cells. Washed beads were analyzed by 11% SDS-PAGE. Closed circles mark proteins that bind specifically to GST-JX-Fms, and open circles indicate those binding to GST-KI-Fms. B, p55 is distinct from the p55 subunit of PI 3’ kinase. Cell lysates of v-Fms-transformed NIH 3T3 and FDCP-1Mac11 cells were incubated with precharged GT beads as described above. Proteins eluted from the beads were analyzed by immunoblotting using antibodies against p110, the SH2 domain of p85, or p55 of PI 3’ kinase. Cr, control cell lysate; lanes 1, GST; lanes 2, GST-JX-Fms; lanes 3, GST-KI-Fms. C and D, binding of p55 to recombinant GST-JX-Fms requires phosphorylation of Y543. C, GT-agarose beads precharged with GST-JX-Fms (lane 1), GST-Y537F-JX-Fms (lane 2), or GST-Y543F-JX-Fms (lane 3) from E. coli TKX-1 were analyzed by 11% SDS-PAGE and phosphotyrosine immunoblotting. D, precharged beads were incubated with metabolically Tran[35S]-labeled lysates from FDC-P1Mac11 cells. Bound proteins were analyzed by SDS-PAGE (7.5% gels). The closed circle marks a 55-kDa protein that specifically bound to GST-JX-Fms and GST-Y537F-Fms but not to GST-Y543F-Fms. E and F, p55 contains phosphotyrosine. E, agarose beads precharged with recombinant tyrosine-phosphorylated GST-JX-Fms (lanes 1) or GST-Y543F-Fms (lanes 2) were incubated with cell lysates from v-Fms-transformed NIH 3T3 (Fms 3T3) or CSF-1-stimulated FDCP-1Mac11 cells as indicated. Bound protein was analyzed by SDS-PAGE and phosphotyrosine immunoblotting. F, NIH 3T3 cell lysates containing in vivo expressed wt-v-Fms (lane 1) or Y543F-v-Fms (lane 2) were subjected to Fms-specific immunoprecipitation. Immune complexes were analyzed by SDS-PAGE and phosphotyrosine immunoblotting.

detected in this fraction. Furthermore, none of these proteins bound to the GST-pY-JX-Fms (Fig. 7C).

To learn whether additional cellular proteins associated with the individual GST-pY-Fms proteins, we next employed metabolically labeled cell lysates from the same cells. As expected, p85 and p110, the two subunits of PI 3’ kinase, were found to associate with GST-pY-KI-Fms (Fig. 8, A, open circles, and B). Neither of the two proteins were found in the eluate from GST-pY-JX-Fms, again underscoring the specificity of binding to the KI domain. Instead, two proteins with molecular masses of 55 and 80 kDa, respectively, were detected in the GST-pY-JX-Fms-bound fraction (Fig. 8A, closed circles). The 55-kDa species did not represent the p55 subunit of PI 3’ kinase (34), because it was not detected with a cross-reactive antibody that bound to the SH2 domains of both the p85 and p55 subunits (Fig. 8B). Furthermore, p55 was clearly distinct from Shc, because it was not reactive with a Shc-specific monoclonal antibody (Fig. 7C). It should be noted that similar negative results were obtained with cell lysates from v-Fms-transformed NIH 3T3 cells and from CSF-1-stimulated c-Fms overexpressing FDCP-1Mac11 cells (data not shown).

To further examine whether this binding correlated with tyrosine phosphorylation of the Fms-specific segment, we employed the two GST-JX-Fms fusion proteins in which either Tyr537 or Tyr543 were replaced by phenylalanine residues. In agreement with the in vitro phosphorylation data (Fig. 6), the wild type and the GST-Y537F-JX-Fms fusion proteins contained phosphotyrosine when isolated from E. coli TKX-1 (Fig. 8C, lanes 1 and 2). In contrast, the level of tyrosine-phosphorylation was reduced by more than 80% in GST-Y543F-JX-Fms (Fig. 8C, lane 3), suggesting that Tyr543 is also a major phosphorylation site for Elk tyrosine kinase. Wild type GST-JX-Fms and the two mutants were incubated with radiolabeled FDCP-1Mac11 cell lysates. As shown in lane 3 of Fig. 8D, the point mutation of Tyr543 abolished the binding of p55, but not of p80. In contrast, GST-Y537F-pY-Fms continued to bind p55. Together, this is strong evidence that Tyr543 indeed constitutes a phosphorylation-dependent binding site for p55. Additional evidence for the specificity of interaction between p55 and the Fms tyrosine kinase would be provided, if p55 by itself was also phosphorylated on tyrosine. To address this issue, we analyzed the eluate from GST-JX-Fms-agarose beads by phosphotyrosine immunoblotting. Fig. 8E shows that p55 from v-Fms-transformed NIH 3T3 or CSF-1-stimulated FDCP-1Mac11 cells is indeed phosphorylated on tyrosine. Furthermore, we performed co-immunoprecipitation studies using wild type v-Fms and Y543F-v-Fms expressing cells. As shown in Fig. 8F, phosphotyrosine containing the 55-kDa protein was co-immunoprecipitated with the wild type v-Fms (lane 1) but not with the Y543F-v-Fms mutant polypeptide (lane 2).

**DISCUSSION**

Generally, tyrosine phosphorylation sites in growth factor receptors serve two purposes: (i) to control the state of activity of the kinase and (ii) to create binding sites for downstream signal transduction molecules, which in many cases also are substrates for the kinase.

In this paper, we show for the first time that Tyr543 in the JX
Tyrosine Phosphorylation of Juxtamembrane Domain of v-Fms

domain of the v-Fms tyrosine kinase represents a major auto-phosphorylation site, and we demonstrate that phosphorylation at this site is a prerequisite for binding of a cellular protein both in premyeloid cells and in fibroblasts. Furthermore, we show that Tyr506, Tyr507, and Tyr709 of the KI domain and Tyr1007 of the second kinase domain are phosphorylated. No phosphorylation was observed with mutant proteins that contained the C-terminal segment of v-Fms including Tyr252. The above conclusions are supported by phosphopeptide analyses involving mutant v-Fms proteins derived from (i) mammalian cells, (ii) various bacterial strains as tyrosine-phosphorylated GST fusion proteins, or (iii) recombinant proteins labeled in vitro by tyrosine kinase reaction.

Regarding the mapping of tyrosine phosphorylation sites, the PDGβ receptor has been characterized in greater detail. Nine tyrosine residues including Tyr759 and Tyr761 in the JX domain, Tyr786, Tyr740, Tyr741, and Tyr721 of the KI domain, Tyr1007, in the second kinase domain, and Tyr1009 and Tyr1021 in the C-terminal end domain have been mapped as autophosphorylation sites (18). Three phosphorylation sites of the v-Fms molecule involving Tyr786, Tyr740, and Tyr1007 reside in regions that exhibit significant sequence homology with the corresponding Tyr746, Tyr740, and Tyr852, containing regions of the PDGβ receptor, thus indicating that these three residues might serve similar functions in the two receptor molecules.

The KI domain of the PDGβ receptor associates with Grb2, p85 of PI 3′ kinase, Nck, and p120RasGAP (35–40), and Tyr716, Tyr740, and Tyr741 were shown to specifically bind Grb2 and PI 3′ kinase (36, 37). These residues are located at positions corresponding to Tyr796 and Tyr797 in the KI domain of the v-Fms tyrosine kinase. We show that in agreement with this similarity, both molecules bound tightly to the GST-pK-I-Fms protein in vitro.

Interestingly, binding of p85 was paralleled by stoichiometric binding of p110, the catalytic subunit of PI 3′ kinase. This finding is in line with the notion that binding of p110 to the KI domain is mediated through the p85 subunit. The efficiency of p85 binding, however, varied drastically depending on the source of p85. Whereas p85 from FDC-P1Mac1 cells bound nearly quantitatively, less than 5% or 20% of p85 from nontransformed or v-Fms-transformed NIH 3T3 cells, respectively, associated with the GST fusion protein. The significance of this finding is unclear but may depend on cell culture conditions. It has been shown previously that growth factor-induced tyrosine phosphorylation of p85 may alter the affinity of its SH2 domains for phosphoryrosine residues (41).

Our finding that the JX domain of v-Fms fails to bind members of the Src tyrosine kinase family is in contrast to results obtained with the PDGβ receptor that can bind Src, Fyn, or Yes (19, 20) and contradicts co-immunoprecipitation studies performed with human c-Fms (22). Neither the GST-JX-Fms fusion protein used in this study nor a cross-reactive peptide-performed with human c-Fms (22). Neither the GST-JX-Fms fusion protein used in this study nor a cross-reactive peptide-performed with human c-Fms (22). Neither the GST-JX-Fms fusion protein used in this study nor a cross-reactive peptide-performed with human c-Fms (22). Neither the GST-JX-Fms fusion protein used in this study nor a cross-reactive peptide-performed with human c-Fms (22). Neither the GST-JX-Fms fusion protein used in this study nor a cross-reactive peptide-performed with human c-Fms (22). Neither the GST-JX-Fms fusion protein used in this study nor a cross-reactive peptide-performed with human c-Fms (22). Neither the GST-JX-Fms fusion protein used in this study nor a cross-reactive peptide-performed with human c-Fms (22). Neither the GST-JX-Fms fusion protein used in this study nor a cross-reactive peptide-performed with human c-Fms (22). Neither the GST-JX-Fms fusion protein used in this study nor a cross-reactive peptide-performed with human c-Fms (22). Neither the GST-JX-Fms fusion protein used in this study nor a cross-reactive peptide-performed with human c-Fms (22). Neither the GST-JX-Fms fusion protein used in this study nor a cross-reactive peptide-performed with human c-Fms (22). Neither the GST-JX-Fms fusion protein used in this study nor a cross-reactive peptide-performed with human c-Fms (22). Neither the GST-JX-Fms fusion protein used in this study nor a cross-reactive peptide-performed with human c-Fms (22). Neither the GST-JX-Fms fusion protein used in this study nor a cross-reactive peptide-performed with human c-Fms (22). Neither the GST-JX-Fms fusion protein used in this study nor a cross-reactive peptide-performed with human c-Fms (22). Neither the GST-JX-Fms fusion protein used in this study nor a cross-reactive peptide-performed with human c-Fms (22). Neither the GST-JX-Fms fusion protein used in this study nor a cross-reactive peptide-performed with human c-Fms (22). Neither the GST-JX-Fms fusion protein used in this study nor a cross-reactive peptide-performed with human c-Fms (22). Neither the GST-JX-Fms fusion protein used in this study nor a cross-reactive peptide-performed with human c-Fms (22). Neither the GST-JX-Fms fusion protein used in this study nor a cross-reactive peptide-performed with human c-Fms (22). Neither the GST-JX-Fms fusion protein used in this study nor a cross-reactive peptide-performed with human c-Fms (22). Neither the GST-JX-Fms fusion protein used in this study nor a cross-reactive peptide-performed with human c-Fms (22). Neither the GST-JX-Fms fusion protein used in this study nor a cross-reactive peptide-performed with human c-Fms (22). Neither the GST-JX-Fms fusion protein used in this study nor a cross-reactive peptide-performed with human c-Fms (22). Neither the GST-JX-Fms fusion protein used in this study nor a cross-reactive peptide-performed with human c-Fms (22). Neither the GST-JX-Fms fusion protein used in this study nor a cross-reactive peptide-performed with human c-Fms (22). Neither the GST-JX-Fms fusion protein used in this study nor a cross-reactive peptide-performed with human c-Fms (22). Neither the GST-JX-Fms fusion protein used in this study nor a cross-reactive peptide-performed with human c-Fms (22). Neither the GST-JX-Fms fusion protein used in this study nor a cross-reactive peptide-performed with human c-Fms (22). Neither the GST-JX-Fms fusion protein used in this study nor a cross-reactive peptide-performed with human c-Fms (22). Neither the GST-JX-Fms fusion protein used in this study nor a cross-reactive peptide-performed with human c-Fms (22).