The Effect of Activating Mutations on Dimerization, Tyrosine Phosphorylation and Internalization of the Macrophage Colony Stimulating Factor Receptor

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Oncogenic activation of the macrophage colony stimulating factor (M-CSF) receptor (c-Fms) requires mutation or truncation of the carboxyl terminus and specific amino acid substitutions in or near the fourth immunoglobulin (Ig)-like loop in the extracellular domain. Using a murine c-Fms system, we investigated the effect of C-terminal truncation, substitutions at amino acids 301 and 374 in the fourth Ig-like loop of the extracellular domain, or the combined mutations on individual steps in receptor activation. The mutations at amino acids 301 and 374 were necessary, but not sufficient, for receptor dimerization in the absence of M-CSF. Only receptors with a truncated C-terminus as well as the extracellular domain mutations dimerized efficiently in the absence of M-CSF, suggesting that the C-terminus of c-Fms also regulates receptor oligomerization. Truncation of the C-terminus alone did not cause receptor dimerization and did not activate the kinase enzymatic activity. Thus, truncation of the C-terminus did not activate receptor monomers *in cis*. Receptors with both a truncated C-terminus and the extracellular domain mutations underwent ligand-independent aggregation, transphosphorylation, and phosphorylation of cellular proteins, followed by rapid internalization and degradation. These results suggest that M-CSF binding to c-Fms initiates activation by inducing conformational changes in both the cytoplasmic C-terminal domain and the fourth Ig-like loop of the extracellular domain, leading to the formation of stable receptor dimers.

INTRODUCTION

The *c-fms* proto-oncogene encodes the receptor for macrophage colony stimulating factor (M-CSF or CSF-1) (Sherr *et al.*, 1985). The M-CSF receptor (c-Fms) is expressed on cells of the monocyte/macrophage lineage and is important for the proliferation and maturation of cells along this differentiation pathway (Tushinski, *et al.*, 1982; Stanley *et al.*, 1983; Wiktor-Jedrzejczak *et al.*, 1990). c-Fms is a member of the class III receptor tyrosine kinase (RTK) family that includes the α and β platelet-derived growth factor receptors (PDGF-R) and the stem cell factor receptor (c-Kit) (Ullrich and Schlessinger, 1990). Each of these receptors is an integral membrane glycoprotein with an extracellular (ligand binding) domain composed of five immunoglobulin (Ig)-like loops. The cytoplasmic domain contains a tyrosine-specific protein kinase that is interrupted by the so called

kinase insert region that is dispensable for kinase enzymatic activity (Taylor *et al.*, 1989) but seems to be important for recognition of certain substrate proteins (Downing *et al.*, 1991; Reedijk *et al.*, 1992).

In the absence of M-CSF, the normal c-Fms protein has little or no tyrosine kinase activity and remains, apparently unaltered, on the cell surface for several hours (Rettenmier *et al.*, 1987; Carlberg *et al.*, 1991). When M-CSF binds, the receptor is activated, and the tyrosine kinase activity of the receptor sets in motion a cascade of cellular signals leading to proliferation. Many of the molecular steps in this receptor activation have been elucidated: 1) binding of M-CSF (a homodimeric ligand) results in dimerization of the receptor (Li and Stanley, 1991); 2) dimerization apparently facilitates receptor autophosphorylation, which is thought to occur primarily (perhaps exclusively) by an intermolecular

(*trans*) mechanism (Ohtsuka *et al.*, 1990); 3) receptor autophosphorylation allows the binding of certain substrate molecules via SH2-phosphotyrosine interactions (Shurtleff *et al.*, 1990; McGlade *et al.*, 1992; Reedijk *et al.*, 1992); 4) tyrosine phosphorylation of substrate molecules by the receptor initiates a cascade of signals for cell proliferation; and 5) activated receptors are rapidly internalized via coated pits and vesicles and are targeted to the lysosome, where both receptor and ligand are degraded (Manger *et al.*, 1984; Guilbert and Stanley, 1986).

Other members of the RTK family are apparently activated by a very similar mechanism, including the PDGF-R (Heldin *et al.*, 1989; Li and Schlessinger, 1991), c-Kit (Blume-Jensen *et al.*, 1991; Lev *et al.*, 1992), and the epidermal growth factor receptor (EGF-R) (Yarden and Schlessinger, 1987).

Oncogenic versions of the c-Fms protein are encoded by two different feline sarcoma viruses (FeSV) (McDonough *et al.*, 1971; Besmer *et al.*, 1986). These v-Fms proteins transform fibroblasts in culture and cause M-CSF-independent proliferation of hematopoietic cells (Sarma *et al.*, 1972; Wheeler *et al.*, 1986). Introduction of v-Fms-containing cells into animals leads to a variety of neoplasms (Heard *et al.*, 1987). Whereas the kinase activity of c-Fms is tightly regulated by M-CSF binding, the kinase activity of v-Fms is constitutively active. Comparison of the amino acid sequences of the feline c-Fms protein and the v-Fms protein encoded by Susan-McDonough FeSV (SM-FeSV) reveals that the v-Fms protein differs by nine single amino acid substitutions. In addition, the 50 carboxy-terminal amino acids of Fms are deleted and replaced with 14 unrelated amino acids (Woolford *et al.*, 1988). Construction of chimeric c-Fms/v-Fms molecules and site-directed mutagenesis has shown that an altered C-terminus is essential for oncogenic activity (Roussel *et al.*, 1987, 1988; Woolford *et al.*, 1988). Mutation of the carboxyl terminus alone, however, results in a Fms protein that is only slightly transforming and not oncogenic (Roussel *et al.*, 1988; Woolford *et al.*, 1988). Mutation of amino acids 301 and 374 (in feline c-Fms) or mutation of amino acid 301 (in human c-Fms) is also required to generate a fully oncogenic c-Fms protein (Roussel *et al.*, 1988; Woolford *et al.*, 1988).

Because the constitutive activation of c-Fms requires mutations in both the extracellular and cytoplasmic domains, it seemed possible that receptors with mutations in only a single domain, although not fully transforming, might mimic part of the normal activation mechanism. For instance, activating mutations in the extracellular domain might stabilize receptor dimers in the absence of M-CSF. A single amino acid change within the transmembrane region of the *neu* proto-oncogene protein apparently activates the protein by causing constitutive dimerization (Weiner *et al.*, 1989). On the other hand,

extracellular domain mutations might act to relieve the requirement for receptor dimerization so that activation of the kinase domain may occur in receptor monomers.

The carboxy-terminal region of Fms has been described as a negative regulatory domain because deletion or mutation of this region is necessary for oncogenic activation (Roussel *et al.*, 1987, 1988; Woolford *et al.*, 1988). The C-terminal domains of some other receptor (Qiu *et al.*, 1988; Haley *et al.*, 1989) and nonreceptor (Cooper and King, 1986) tyrosine kinases are also involved in regulating the activity of the kinase domains. The kinase activity of *src* is inhibited by the phosphorylation of a C-terminal tyrosine (Tyr527) (Cooper and King, 1986). Binding of the phosphorylated tyrosine to the SH2 domain of the same molecule may inhibit substrate binding (Roussel *et al.*, 1991), whereas deletion or mutation of Tyr527 results in a constitutively active *src* kinase. The mechanism by which the C-terminal domains of receptor tyrosine kinases regulate their kinase activity is unknown, however. It has been suggested that unphosphorylated tyrosines in the C-terminus of the EGF-R may interact with the active site of the receptor and inhibit substrate binding. Upon ligand binding, autophosphorylation of these sites may cause the C-terminal domain to move out of the active site, thus allowing substrate binding (Ullrich and Schlessinger, 1990). Although the C-terminal region of c-Fms that is missing in v-Fms contains one tyrosine residue, there is as yet no evidence that this tyrosine is phosphorylated either in the absence or presence of ligand (Tapley *et al.*, 1990; Myles, personal communication). Furthermore, the C-terminal domains of different classes of RTKs have little or no homology to one another and may therefore have entirely different functions (Ullrich and Schlessinger, 1990).

To better understand the role of the extracellular domain and the C-terminal domain of Fms in receptor activation, we have examined the properties of receptors with activating mutations in the extracellular domain only, truncation of the C-terminus only, or receptors with both kinds of mutation.

MATERIALS AND METHODS

Materials

Recombinant murine M-CSF was produced in SF9 insect cells from a baculovirus vector and partially purified on diethylaminoethyl-cellulose (Wang and Rohrschneider, 1993). Conditioned medium from WEHI-3B cells growing in Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated fetal bovine serum (FBS) was used at 2% as a source of IL-3, without further purification. Rabbit polyclonal antiserum 5679 to the extracellular domain of murine c-Fms was obtained after multiple subcutaneous injections of New Zealand White rabbits with Rab-9 fibroblasts (ATCC CRL 1414) expressing pZen c-fms S301, S374, Δ 3'. This antiserum recognizes wild-type and mutant receptors equally well. The preparation of rabbit polyclonal antisera 4062 and 4069 against the kinase domain of c-Fms was described previously (Rohrschneider *et al.*, 1989). Monoclonal antibody

to phosphotyrosine was obtained from Upstate Biotechnology (UBI 05-321, Lake Placid, NY). S-NHS-biotin and the cross-linkers DTSSP and BS³ were obtained from Pierce (Rockford, IL). Streptavidin-biotinylated horseradish peroxidase (ABC-HRP) and reagents for enhanced chemiluminescence (ECL) were from Amersham (Arlington Heights, IL). Prestained high molecular weight marker proteins (14–200 kDa) were obtained from GIBCO (Grand Island, NY). A 340-kDa unstained marker (α_2 -macroglobulin) was obtained from Boehringer-Mannheim (Indianapolis, IN).

Mutagenesis and Expression of c-Fms in Cells

The starting material for mutagenesis was a 3.7 kilobase (kb) *Eco*RI fragment containing the entire coding sequence for murine c-Fms (Rothwell and Rohrschneider, 1987) cloned into the *Eco*RI site of pVZ, a modified Stratagene Bluescribe vector (La Jolla, CA). Oligonucleotide-directed mutagenesis was performed with single-stranded DNA template using the method of Zoller and Smith (1984). The c-fms $\Delta 3'$ mutant was made by creating a stop codon at amino acid 930, which is the point at which v-fms (SM-FeSV) and c-fms sequences diverge (Woolford *et al.*, 1988). Amino acids Leu301 and Ala374 were each changed to serine. The 3.1-kb *Eco*RI to *Nde* I c-fms fragment from each mutant was gel purified and cloned into pZEN113 plasmid as described (Tapley *et al.*, 1990).

pZEN c-fms plasmids were transfected into $\psi 2$ cells along with a neomycin resistance plasmid using CaPO_4 precipitation. Transfected cells were selected by growth in 0.5 mg/ml Geneticin (GIBCO) and then sorted by flow cytometry using a rabbit antibody (5679) against the extracellular domain of c-Fms. Sorted $\psi 2$ cells were used to infect FDC-P1 cells by cocultivation as described previously (Rohrschneider and Metcalf, 1989). The infected FDC-P1 cells were sorted by flow cytometry to obtain wild-type and mutant c-Fms-expressing cells (FDC-fms) with similar levels of cell-surface c-Fms protein. Rat-2 cells were infected with filtered $\psi 2$ cell-conditioned medium for several days, and then c-Fms-expressing cells were selected by fluorescence cell sorting.

FDC-fms cells were maintained in DMEM 10% FBS containing 2% WEHI-3B cell conditioned medium (WCM) as a source of IL-3. Rat-2 cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS).

Biotinylation of c-Fms Proteins

To label cell-surface receptors, cells were washed with phosphate-buffered saline (PBS) and resuspended at about 1×10^7 cells/ml in ice-cold PBS. S-NHS-biotin was added to 200 $\mu\text{g}/\text{ml}$ dimethyl sulfoxide (DMSO) freshly diluted from a 10 mg/ml stock. The cells were incubated on ice for 30 min and then washed twice in ice-cold DMEM 10% FCS.

Assays for Internalization and Degradation of c-Fms

c-Fms internalization was assayed as described previously (Carlberg *et al.*, 1991) by immunoprecipitation of receptors remaining on the cell surface at timepoints after M-CSF stimulation. FDC-fms cells were biotinylated and then incubated for 10 min on ice in the absence or presence of 2500 U/ml M-CSF. The cells were then transferred to 37°C, and aliquots were collected at timepoints after M-CSF addition, pelleted, and resuspended in ice-cold DMEM 10% FCS, 0.02% NaN_3 . Antiserum 5679 (1:100) was incubated with the intact cells on ice 1 h. After washing three times in ice-cold PBS to remove unbound antiserum, the cells were lysed in RIPA buffer (0.1% SDS, 1% deoxycholate, 1% Triton X-100, 150 mM NaCl, 20 mM Tris pH 7.3, 20 mM ethylenediaminetetraacetic acid (EDTA), 50 mM NaF, 2 mM $\text{C}_2\text{H}_5\text{IO}_2\text{Na}$, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 200 μM Na_3VO_4) (Gentry *et al.*, 1983) and immunoprecipitated. To measure c-Fms degradation, biotinylated cells were incubated on ice in the

absence or presence of M-CSF as above and then lysed in RIPA buffer. c-Fms proteins were immunoprecipitated from cell lysates with antiserum 5679. The samples were electrophoresed on reducing 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, and the proteins were electroblotted to nitrocellulose as described previously (Gliniak and Rohrschneider, 1990). The nitrocellulose blots were incubated 1 h with Block solution (1% bovine serum albumin, 1% ovalbumin, 150 mM NaCl, 25 mM Tris(hydroxymethyl)aminomethane [Tris] pH 8.0, 0.5% Tween 20) and then incubated with ABC-HRP (diluted 1:250 in Block) for 2–4 h. The blots were washed 4×15 min with Rinse solution (150 mM NaCl, 25 mM Tris pH 8.0, 0.5% Tween 20) and then biotinylated c-Fms proteins were detected using ECL according to the manufacturer's instructions.

In Vitro Kinase Assays

In vitro kinase assays were performed as described previously (Woolford *et al.*, 1988) using immunoprecipitated receptors from unstimulated cells. To measure Fms kinase activity toward an exogenous substrate, activated enolase (1.67 $\mu\text{g}/\text{sample}$) was included in the reaction mix. Kinase reactions were performed at 25°C for the indicated times.

Autophosphorylation of c-Fms Proteins

FDC-fms cells were incubated 16 h in DMEM 10% FCS with or without 1000 U/ml M-CSF. The cells were washed with ice-cold PBS and lysed in NP-40 lysis buffer (0.5% NP-40, 50 mM NaCl, 10 mM Tris-base, 30 mM Na_2PO_4 , 50 mM NaF, 2 mM $\text{C}_2\text{H}_5\text{IO}_2\text{Na}$, 5 μM ZnCl_2 , 1 mM PMSF, and 200 μM Na_3VO_4 pH 7.3). The lysates were clarified by centrifugation. c-Fms proteins were immunoprecipitated with antibody 5679 as described above. The proteins were electroblotted to nitrocellulose, and the blots were incubated 16 h at 4°C with antibody to phosphotyrosine (0.025 $\mu\text{g}/\text{ml}$ in Block). The blots were washed 4×15 min in Rinse and then incubated for 1 h with peroxidase-conjugated goat anti-mouse secondary antibody (Boehringer-Mannheim) diluted 1:16 000 in Block. The blots were washed 4×15 min in Rinse and then phosphotyrosine-containing proteins were detected using ECL. After detecting phosphotyrosine, the blots were stripped of antibodies by washing for 2 h with eight changes of 0.1 M glycine-HCl pH 2.5. The amount of c-Fms protein in each lane was determined by incubating the blots for 1 h with c-Fms antibody 4599 (diluted 1:10 000 in Block). The blots were washed as above, incubated 1 h with HRP-conjugated secondary antibody and detected by ECL. To help quantitate levels of phosphotyrosine and receptor by ECL, a sample of wild-type c-Fms protein (from cells stimulated on ice with M-CSF) was immunoprecipitated with the other samples. After elution with SDS-gel buffer, this sample was serially diluted, and the dilution series was electrophoresed and immunoblotted in parallel with the other samples. Bands produced by ECL were recorded with a Sharp JX-320 scanner (Mahwah, NJ) and quantified using Optimas software (BioScan Corporation, Edmond, WA). Integrated optical densities were calculated for each band and then the relative levels of phosphotyrosine on short and long ECL exposures could then be determined by comparison with the dilution series.

Detection of Phosphotyrosine-containing Proteins in Cell Lysates

FDC-fms cells were incubated 16 h at 37°C in DMEM 10% FCS with or without 1000 U/ml M-CSF. The cells were harvested, washed in PBS, and lysed in 0.5 ml of NP-40 lysis buffer. The lysates were clarified by centrifugation (15 000 rpm $\times$ 15 min); protein contents were measured using a BioRad (Richmond, CA) protein assay and equalized. Lysates ($\sim 2 \times 10^4$ cell equivalents/sample) were run on a 7.5% SDS-PAGE gel. After transferring the proteins to nitrocellulose,

the blots were probed first with antibodies to phosphotyrosine and then stripped and reprobed with antibody 4599 to c-Fms.

Assays for c-Fms Dimerization

To detect cell surface c-Fms dimers, $\sim 1 \times 10^6$ FDC-*fms* cells were biotinylated and resuspended in ice-cold DMEM 10% FCS with or without M-CSF (10 000 U/ml). After 1 h on ice the cells were washed twice in ice-cold PBS and then lysed in 100 μ l of NP-40 lysis buffer. To cross-link receptor dimers, 1 μ l of BS³ or DTSSP (from a 100 mM stock in DMSO) was added immediately after lysis. After 30 min on ice, the reaction was quenched by addition of 10 μ l of 1 M glycine pH 8.0. The samples were clarified by centrifugation (15 000 rpm $\times$ 15 min), and the protein contents of the samples were adjusted to give approximately equal levels of cell-surface receptors. c-Fms proteins were immunoprecipitated with antiserum 4069 (1:100), which recognizes the kinase domain of c-Fms. (Identical results were obtained using antiserum 5679 against the extracellular portion of the receptor). After 1 h on ice, the immune complexes were stringently washed (3 washes in RIPA buffer, 1 wash in 1 M MgCl₂, 10 mM Tris pH 7.3, and 2 washes in 10 mM Tris pH 6.8, 10 mM EDTA). The immunoprecipitated proteins were electrophoresed on 3–15% gradient SDS-PAGE gels and electroblotted to nitrocellulose. Nitrocellulose blots were probed with antibody to phosphotyrosine as described above. To detect cell-surface-labeled c-Fms proteins, the phosphotyrosine blots were then stripped of antibodies, incubated again with Block for 1 h, and incubated 2–4 h with ABC-HRP. The blots were washed in Rinse and biotinylated c-Fms proteins were detected using ECL. To detect both cell surface and cytosolic c-Fms proteins, the phosphotyrosine blots were stripped, incubated with Block solution, and then incubated 1 h with c-Fms antibody 4599 (1:10 000 in Block). The blots were washed as above, incubated 1 h with HRP-conjugated secondary antibody, and detected by ECL.

RESULTS

Transforming Potential of Activating Mutations in Murine c-Fms

The transforming mutations (Leu301 to Ser301, Ala374 to Ser374, and C-terminal truncation at amino acid 930) were made individually or in combination in the murine c-*fms* gene and introduced into the ψ 2 packaging cell line by CaPO₄ transfection. Because the oncogenic potential of these mutations was originally assayed in Rat-2 cells (Woolford *et al.*, 1988), ψ 2 viral supernatants were first used to infect Rat-2 fibroblasts. The effects of these mutations in the murine c-*fms* gene were similar to the effects in feline or human c-*fms* (Roussel *et al.*, 1988; Woolford *et al.*, 1988), except that each of the individual activating mutations in murine c-*fms* caused a phenotypic transformation of Rat-2 cells even in the absence of added M-CSF (Table 1A). This was most likely because of the weak cross-reactivity we have observed between the M-CSF produced in Rat-2 cells and the introduced murine receptor (Chen and Myles, personal communication).

Individually, both Ser301 and Ser374 were activating mutations in murine c-*fms*, but Ser374 caused a more pronounced transformed phenotype than Ser301. When combined, these 2 mutations were only slightly more transforming than Ser374 alone (unpublished data). In subsequent experiments, only the double mutant c-*fms*

Table 1. Colony-forming activity of c-Fms proteins

A. Soft agar assay on Rat-2 cells expressing c-Fms proteins^a

Rat-2 cells expressing	Growth factor added	Colonies/plate
Uninfected	—	0, 0
	M-CSF	0, 0
c-Fms WT	—	0, 0
	M-CSF	92, 89
c-Fms Δ 3'	—	53, 26
	M-CSF	121, 103
c-Fms S301, S374	—	92, 102
	M-CSF	128, 77
c-Fms S301, S374, Δ 3'	—	111, 101
	M-CSF	112, 148

B. Soft agar assay on FDC-P1 cells expressing c-Fms proteins^b

FDC-P1 cells expressing	Growth factor added	Colonies/plate (% of the number of colonies in GM-CSF)
Uninfected	—	0, 0
	M-CSF	0, 0
	GM-CSF	100, 100
c-Fms WT	—	0, 1
	M-CSF	45, 34
	GM-CSF	100, 100
c-Fms Δ 3'	—	3, 4
	M-CSF	118, 82
	GM-CSF	100, 100
c-Fms S301, S374	—	3, 7
	M-CSF	93, 61
	GM-CSF	100, 100
c-Fms S301, S374, Δ 3'	—	69, 72
	M-CSF	123, 98
	GM-CSF	100, 100

^a Rat-2 cells expressing approximately equal levels of wild-type or mutant c-Fms were plated in soft agar with or without 1000 U/ml M-CSF. Colonies of >50 cells were counted after 10 d and are shown as total colonies in two different experiments.

^b FDC-P1 cells expressing approximately equal levels of wild-type or mutant c-Fms were assayed for colony formation in soft agar medium containing no hematopoietic growth factors (—), 1000 U/ml M-CSF, or 200 U/ml GM-CSF. For each cell line, equal aliquots of cells were plated in the different factors. Colonies of >50 cells were counted after 7 d. The colony numbers from two separate experiments are normalized to the number of colonies in the GM-CSF plates.

S301, S374 was used to test the effect of mutations in the fourth Ig-like loop of the extracellular domain. The C-terminal truncation mutant, c-*fms* Δ 3', also caused a transformed phenotype in Rat-2 cells (Table 1A). The transformed phenotype caused by c-Fms Δ 3' was indistinguishable from that caused by c-Fms 3'V, a mutant in which the 74 C-terminal amino acids of c-Fms were replaced with the last 41 amino acids of v-Fms (unpublished data). Therefore, activation resulted from the loss of the normal C-terminus of c-Fms, rather than addition of the unique v-Fms amino acids.

Because c-Fms is normally expressed in myeloid cells, the $\psi 2$ cells were used to infect the factor-dependent murine myeloid cell line FDC-P1. These cells do not express the endogenous *c-fms* gene and do not produce M-CSF. FDC-P1 cells were sorted by flow cytometry to obtain populations of cells expressing uniformly high levels of wild-type or mutant c-Fms (Figure 1). The level of c-Fms S301, S374, $\Delta 3'$ on the cell surface was always lower than that of the other receptors, even after repeated sorting.

The FDC-*fms* cells were assayed in soft agar to compare M-CSF-dependent and factor-independent growth. As described previously (Rohrschneider and Metcalf, 1989), the parental FDC-P1 cell line required IL-3 or GM-CSF for growth and did not respond to M-CSF. Upon infection with *c-fms*, the cells acquired the ability to grow in M-CSF. FDC-P1 cells expressing the wild-type receptor grew well in M-CSF but exhibited little or no factor-independent growth (Table 1B). Cells expressing receptors with activating mutations formed two- to three-fold more colonies in M-CSF than cells expressing wild-type c-Fms. c-Fms S301, S374 and c-Fms $\Delta 3'$ each stimulated only a little factor-independent growth, whereas c-Fms S301, S374, $\Delta 3'$ stimulated many factor-independent colonies. Thus, as shown previously for feline c-Fms (Woolford *et al.*, 1988), truncation of the normal C-terminus of the murine receptor or point mutations at amino acids 301 and 374 in the extracellular domain each cause a partially transformed phenotype in infected FDC-P1 cells. Receptors with mutations in both the extracellular domain and the C-terminus are considerably more transforming than receptors with either mutation alone.

Receptor Internalization

Wild-type c-Fms has a half-life of 2–4 h on the cell surface in the absence of M-CSF. Stimulation of cells with M-CSF causes the rapid (<1 min) internalization of ligand-bound receptors (Rettenmier *et al.*, 1987; Carlberg *et al.*, 1991). In cells expressing v-Fms, the fraction of total receptor that is present on the cell surface is low even in the absence of M-CSF, suggesting that v-Fms may internalize constitutively (Manger *et al.*, 1984; Wheeler *et al.*, 1986). Because the level of c-fms S301, S374, $\Delta 3'$ on the cell surface was also lower than that of the other receptors, we investigated the possibility that this mutant internalizes constitutively.

Like v-Fms, c-Fms proteins with mutations at amino acids 301 or 374 are inefficiently transported from the endoplasmic reticulum to the Golgi complex (Anderson *et al.*, 1984) and therefore the appearance of the mature form of the protein on the cell surface is delayed relative to the wild-type protein (Roussel *et al.*, 1988; Woolford *et al.*, 1988). Because of this processing defect, we were unable to use metabolic labeling of receptors to follow

Figure 1. Expression of c-Fms proteins. FDC-*fms* cells were incubated 1 h on ice with antibody 5679 (1:200 in DMEM 10% FCS), washed twice, and incubated 1 h on ice with fluorescein isothiocyanate-conjugated secondary antibody. Cells were then analyzed by flow cytometry for cell-surface expression of c-Fms proteins.

receptor internalization (Carlberg *et al.*, 1991). Instead, cell-surface proteins on FDC-P1 cells were labeled on ice with biotin. Internalization in the presence or absence of M-CSF was measured by immunoprecipitation of receptors remaining on the cell surface at timepoints after transferring the cells to 37°C. As shown in Figure 2A, wild-type c-Fms remained fairly stable on the cell surface in the absence of M-CSF. Addition of M-CSF resulted in the rapid internalization of cell-surface-labeled receptors. Neither c-Fms $\Delta 3'$ nor c-Fms S301, S374 was internalized more rapidly than the wild-type receptor in the absence of M-CSF. However, c-Fms S301, S374, $\Delta 3'$ was internalized nearly as rapidly in the absence of M-CSF as in its presence. Thus, c-Fms S301, S374, $\Delta 3'$ internalizes in the absence of ligand, and this constitutive internalization requires both the activating mutations in the extracellular domain and truncation of the C-terminus.

It was shown previously that a c-Fms mutant lacking the kinase insert region (c-Fms Δ KI) is not downregulated after M-CSF stimulation. c-Fms Δ KI is rapidly internalized after ligand binding but is recycled to the cell surface rather than being degraded, so that a high level of receptors on the cell surface is maintained (Carlberg *et al.*, 1991). Unlike c-Fms Δ KI, c-Fms S301, S374, $\Delta 3'$ was degraded, in the absence and presence of M-CSF, with kinetics similar to the M-CSF-stimulated wild-type receptor (Figure 2B). The ligand-independent internalization and degradation of this mutant is therefore responsible for its constitutively low cell-surface expres-

sion. This means that the transforming activity of c-Fms S301, S374, $\Delta 3'$ did not result from a failure of the receptor to downregulate.

A trivial explanation for the constitutive internalization of c-Fms S301, S374, $\Delta 3'$ is that the cells expressing this mutant produced M-CSF. To test this, conditioned medium from FDC-*fms* S301, S374, $\Delta 3'$ cells growing in DMEM 10% FCS was concentrated 10-fold and used in a soft agar assay. Conditioned medium from these cells was not able to support the growth of murine bone marrow cells, although the positive controls indicated that colonies would have been seen with less than 0.2 U/ml M-CSF (unpublished data). We were also unable to detect M-CSF mRNA from FDC-*fms* S301, S374, $\Delta 3'$ on Northern blots (unpublished data).

Receptor Tyrosine Kinase Activity

The mechanism by which the c-Fms C-terminus inhibits receptor kinase activation in the absence of ligand is unknown. If the C-terminus of Fms regulates kinase activity by blocking or interacting with the kinase catalytic domain, then receptors with a truncated C-terminus might show an increase in specific kinase activity. To test this, in vitro kinase assays were performed using wild-type and mutant receptors. c-Fms proteins were

Table 2. Autophosphorylation of c-Fms proteins in vitro

	Exp. 1	Exp. 2
c-Fms WT	100	100
c-Fms $\Delta 3'$	113	130
c-Fms S301, S374	62	110
c-Fms S301, S374, $\Delta 3'$	127	115

FDC-*fms* cells were metabolically labeled with [35 S] methionine, and then unstimulated c-Fms proteins were immunoprecipitated and used in in vitro kinase assays. The ratio of incorporated 32 P to [35 S]-labeled receptor is shown as a percentage of that obtained for c-Fms WT.

immunoprecipitated from unstimulated cells and rigorously washed in ionic detergents. Tyrosine phosphorylation of c-Fms was quantified and normalized to the amount of receptor present in each lane. Table 2 shows that autophosphorylation of the mutant c-Fms proteins was not significantly greater than that of the wild-type receptor in in vitro assays. To measure in vitro kinase activity toward an exogenous substrate, enolase was included in the kinase reaction mix. A timecourse of enolase phosphorylation showed that neither truncation of the C-terminus alone nor the extracellular domain mutations alone increased the kinase activity of

Figure 2. (A) Internalization of c-Fms proteins. The disappearance of biotinylated receptor from the cell surface is shown after 0–60-min incubation at 37°C in the absence or presence of M-CSF. (B) Degradation of c-Fms WT and c-Fms S301, S374, $\Delta 3'$. The loss of cell-surface-labeled receptors from whole cell lysates is shown after 0–60-min incubation at 37°C in the absence or presence of M-CSF. The graphs show the amount of c-Fms protein present in each lane relative to the 0 timepoint, which is shown as 100%.

Figure 3. Phosphorylation of enolase in vitro. Wild-type or mutant receptors from ^{35}S -labeled unstimulated cells were immunoprecipitated, stringently washed, and used in in vitro kinase assays. Enolase (1.67 μg) was added to each sample, and reaction mixes were incubated at 25°C for 0.25, 1, 4, or 16 min. ^{32}P -enolase counts were normalized for the amount of ^{35}S -labeled receptor (from duplicate lanes without ^{32}P). ■, c-Fms WT; □, c-Fms $\Delta 3'$; ●, c-Fms S301, S374; ○ c-Fms S301, S374, $\Delta 3'$.

Fms in vitro (Figure 3). The kinase activity of c-Fms S301, S374, $\Delta 3'$ was somewhat greater than that of the wild-type receptor. The increased activity of this mutant might be because of preexisting receptor dimers or other conformation changes.

Because immunoprecipitated receptors are active in in vitro kinase assays even in the absence of M-CSF (Rettenmier *et al.*, 1985), we also compared tyrosine phosphorylation of c-Fms proteins in vivo. Cells expressing wild-type or mutant c-Fms were incubated in the absence or presence of 1000 U/ml of M-CSF for 16 h. Under these conditions of M-CSF stimulation, wild-type and mutant receptors were similarly downregulated and therefore more easily compared. After im-

munoprecipitation of c-Fms proteins, Western blots were used to detect receptor phosphotyrosine and total c-Fms protein. The ratio of phosphotyrosine to the mature c-Fms band was determined for each receptor (Figure 4, Table 3). In the absence of M-CSF, phosphotyrosine on the wild-type receptor was barely detectable (Figure 4). Neither c-Fms $\Delta 3'$ nor c-Fms S301, S374 had significantly higher levels of receptor phosphotyrosine in the absence of M-CSF, however c-Fms S301, S374, $\Delta 3'$ exhibited about a 10-fold increase in basal receptor phosphotyrosine relative to the wild-type receptor. This level of phosphotyrosine was similar to the level of autophosphorylation on wild-type receptors growing continuously in M-CSF (Table 3, Figure 4). In the continuous presence of M-CSF, the level of receptor phosphotyrosine on c-Fms $\Delta 3'$ and c-Fms S301, S374 was also similar to that of wild-type c-Fms, whereas c-Fms S301, S374, $\Delta 3'$ was somewhat more highly tyrosine phosphorylated.

Similar results were obtained when the steady-state levels of phosphotyrosine on cellular proteins were compared. When cells expressing wild-type c-Fms were incubated 16 h in the absence of M-CSF, the level of phosphotyrosine on cellular proteins was very low (Figure 5). Cells expressing c-Fms $\Delta 3'$ or c-Fms S301, S374 also had very low levels of phosphotyrosine in the absence of M-CSF. (The level of phosphotyrosine in cells expressing S301, S374 appears to be slightly higher in Figure 5 because somewhat more lysate was loaded in these lanes. Note the higher level of c-Fms protein in the bottom panel.) However, in cells expressing c-Fms

Table 3. Tyrosine phosphorylation levels of c-Fms proteins in vivo

	Stimulation conditions	
	A	B
c-Fms WT	1.0	11.2
c-Fms $\Delta 3'$	0.9	10.6
c-Fms S301, S374	1.1	11.2
c-Fms S301, S374, $\Delta 3'$	10.1	16.4

The ratio of phosphotyrosine to the 165-kDa band of c-Fms protein was determined as described in MATERIALS AND METHODS and is shown relative to that obtained for c-Fms WT in no M-CSF. (A) Cells maintained 16 h at 37°C in medium without M-CSF. (B) Cells maintained 16 h at 37°C in 1000 U/ml M-CSF. The experiment was repeated three times with very similar results. The values shown are for the experiment shown in Figure 4.

Figure 4. Relative phosphotyrosine levels in c-Fms proteins. FDC-*fms* cells were incubated 16 h in the absence or presence of 1000 U/ml M-CSF. c-Fms proteins were immunoprecipitated and immunoblotted with antibody to phosphotyrosine. The blots were then stripped and reprobed with antibody to c-Fms.

Figure 5. Tyrosine-phosphorylated proteins in lysates of FDC-*fms* cells. FDC-P1 cells expressing wild-type or mutant c-Fms were incubated 16 h at 37°C in the absence or presence of 1000 U/ml M-CSF. Cell lysates were electrophoresed and immunoblotted with antibody to phosphotyrosine. Bottom: The relative level of c-Fms protein in each lane was determined by reprobing the blot with antibody to c-Fms. Neg, uninfected FDC-P1 cells.

S301, S374, $\Delta 3'$, the level of phosphotyrosine in the absence of M-CSF was as high as in the presence of M-CSF. No unique phosphoproteins were seen in cells expressing the mutant receptors, however several cellular proteins were more highly tyrosine phosphorylated in the cells expressing c-Fms S301, S374, $\Delta 3'$. We therefore found no evidence that truncation of the C-terminus alone increased the tyrosine kinase activity of c-Fms in the absence of M-CSF. A substantial increase in kinase activation was seen only in receptors that had both the extracellular domain mutations and a truncated C-terminus.

Receptor Dimerization

Li and Stanley (1991) observed that M-CSF stimulation leads to the formation of covalently linked c-Fms dimers. It seemed possible that c-Fms proteins with activating mutations in the extracellular domain might be able to dimerize without M-CSF stimulation. Although we expected dimers of wild-type c-Fms to be tyrosine phos-

phorylated, we wanted to be able to detect dimers of mutant c-Fms proteins even if they were not phosphorylated. We therefore used a method similar to that of Li and Stanley that would allow us to see nonphosphorylated as well as tyrosine-phosphorylated receptor dimers. FDC-*fms* cells were stimulated on ice with a saturating concentration of M-CSF then lysed and immunoprecipitated with antibodies to c-Fms. Using Western blots to detect either c-Fms or receptor phosphotyrosine, only the monomeric 165-kDa band was seen (unpublished data). Covalently linked c-Fms dimers were detected only when the intact cells were incubated with an antibody (5679) that recognizes the extracellular domain of Fms. Unfortunately, use of this antibody on intact cells also resulted in covalently linked dimers of wild-type c-Fms in cells that had not been stimulated with M-CSF (unpublished data). It was not clear why covalently linked dimers were detected after binding of an antibody and not by binding of M-CSF.

We subsequently used chemical cross-linking to detect c-Fms dimers in M-CSF-stimulated or unstimulated cells. Ligand-stimulated receptor aggregation probably occurs at the cell surface before receptor endocytosis. Therefore, we initially looked for c-Fms aggregates present on the cell surface in cells that were incubated at 4°C to prevent receptor internalization. Cells expressing wild-type or mutant c-Fms were biotinylated to label cell-surface proteins and then incubated for 1 h on ice in the absence or presence of a saturating concentration (10,000 U/ml) of M-CSF. The cells were lysed in NP-40 and immediately cross-linked by addition of BS³. We were unable to cross-link c-Fms proteins efficiently by adding BS³ or other cross-linkers to the intact cells, regardless of whether they were previously biotinylated. This suggested that chemical cross-linking of c-Fms proteins occurred primarily between adjacent cytoplasmic domains. c-Fms proteins were immunoprecipitated and then immunoblotted with antibodies to phosphotyrosine. The blots were then stripped to remove phosphotyrosine antibodies, and cell-surface receptors were detected using a HRP-conjugated ABC complex. As shown in Figure 6A, nearly all of the wild-type, cell-surface-labeled receptors could be cross-linked into a high molecular weight complex (400–450 kDa) in the presence of a saturating level of M-CSF, whereas in the absence of M-CSF no high molecular weight complexes were detected. In agreement with other studies (Li and Schlessinger, 1991; Li and Stanley, 1991), all of the phosphotyrosine was present on the aggregated form of the receptor (Figure 6B). Perhaps surprisingly, no dimers of c-Fms $\Delta 3'$ or c-Fms S301, S374 were detected in the absence of M-CSF, although these receptors were completely aggregated in the presence of M-CSF. Most of the cell-surface c-Fms S301, S374, $\Delta 3'$ was also apparently in the monomer form in the absence of M-CSF, but phosphotyrosine blots re-

Figure 6. Dimerization of cell-surface c-Fms proteins detected after chemical cross-linking of M-CSF-stimulated or unstimulated cells. FDc-*fms* cells were biotinylated and then treated with or without 10 000 U/ml M-CSF for 1 h on ice. The fraction of c-Fms S301, S374, Δ3' on the cell surface was lower than that of the other receptors. Therefore, more cells expressing this mutant were used to load approximately equal numbers of cell-surface receptors. Cross-linker was added immediately after cell lysis, and then c-Fms proteins were immunoprecipitated and immunoblotted with antibody to phosphotyrosine. The blot was then stripped and cell-surface (biotinylated) proteins were detected by ECL. The exposure time for 5 B was 1 min.

vealed that some of the c-Fms S301, S374, Δ3' was present in a high molecular weight complex and was activated. To better compare aggregation of the tyrosine-phosphorylated receptors in the absence of M-CSF, longer exposures were needed. Figure 7 shows another experiment comparing mutant receptors after cross-linking in the absence of M-CSF. Although blots for biotinylated proteins again showed that most of the cell-surface receptors in the absence of M-CSF were monomeric, longer exposures of phosphotyrosine blots revealed that c-Fms S301, S374, Δ3' had a significant increase in receptor aggregation compared to the wild-type receptor. Interestingly, on such long exposures, a small amount of aggregated receptor could be seen with mutant S301, S374 as well.

Although blots against phosphotyrosine and against biotin cannot be directly compared, it appeared that in the absence of M-CSF, c-Fms aggregates were more easily detected with antibodies to phosphotyrosine. The phosphotyrosine blots in Figures 6 and 7 would have detected tyrosine phosphorylated receptors present in an intracellular compartment as well as cell-surface re-

ceptors. For comparison therefore, we also immunoblotted with antibodies to c-Fms to detect total cellular receptors (Figure 8).

FDc-*fms* cells were stimulated with 10 000 U/ml M-CSF for 1 h on ice (Figure 8A, the same conditions used in Figures 6 and 7) or incubated with or without 1000 U/ml M-CSF for 16 h at 37°C (Figure 8, B and C, the same conditions used in Figures 4 and 5). The cells were then lysed and immediately cross-linked. The immunoblots of total c-Fms protein detected not only the mature (165 kDa) form of c-Fms, but also the immature intracellular Fms precursor of ~135 kDa. (The receptors with C-terminal truncations were ~5 kDa smaller.) In the presence of M-CSF (Figure 8, A and B), both wild-type and mutant receptors formed complexes of 400–450 kDa. These bands corresponded in size to the cross-linked bands seen on blots of cell-surface protein or phosphotyrosine (compare Figure 6 and Figure 8). The immunoblots with antibodies to c-Fms also revealed a second, smaller cross-linked band (260–280 kDa) of c-Fms S301, S374 and c-Fms S301, S374, Δ3'. These 260–280 kDa bands were not seen when detecting cell-surface receptors or phosphotyrosine (Figures 6, 7, and 8D). The percentage of total receptor present in the 260–280 kDa bands was not altered by M-CSF stimulation. Further, when cell-surface-labeled receptors were stimulated on ice with M-CSF and then allowed to internalize at 37°C, no biotinylated or tyrosine-phosphorylated receptors could be chased into the 260–280 kDa bands (unpublished data). This suggested that these bands

Figure 7. Dimerization of cell-surface c-Fms protein detected by cross-linking of unstimulated cells. Phosphotyrosine and cell-surface c-Fms were detected as described in Figure 5. The exposure time for 6 B was 10 min.

Figure 8. Dimerization of total c-Fms proteins detected by cross-linking of M-CSF stimulated or unstimulated cells. Unlabeled FDc-*fms* cells were (A) incubated 16 h without M-CSF and then stimulated for 1 h on ice with 10 000 U/ml M-CSF or (B) incubated 16 h at 37°C with 1000 U/ml M-CSF or (C) incubated 16 h at 37°C with no M-CSF. The cells were lysed, cross-linked, and then c-Fms proteins were immunoprecipitated and immunoblotted with antibodies to c-Fms. (D) Blot C was stripped and reprobed with antibody to phosphotyrosine.

probably consisted of dimerized intracellular receptor precursors rather than activated receptors that had internalized.

When the cells were stimulated with a saturating amount of M-CSF on ice (as in Figure 6), most of the wild-type, $\Delta 3'$, and S301, S374 c-Fms proteins could be cross-linked as dimers (Figure 8A). This would be expected if most of the c-Fms protein in these cells was exposed on the cell surface at the time M-CSF was added. A smaller percentage of the total c-Fms S301, S374, $\Delta 3'$ was aggregated under the same conditions (Figure 8A). This was likely because of the constitutive downregulation of the cell-surface c-Fms S301, S374, $\Delta 3'$. (The total amount of aggregated c-Fms S301, S374, $\Delta 3'$ in Figure 6 was greater, because more cells expressing this mutant were used to load approximately equal numbers of cell-surface receptors in each lane.)

When cells were growing continuously at 37°C in M-CSF, wild-type and mutant receptors were similarly downregulated (Figure 8B). Under these conditions, 20–25% of the mature wild-type, $\Delta 3'$, and S301, S374 c-Fms proteins could be cross-linked into a complex of 400–450 kDa. The percentage of c-Fms S301, S374, $\Delta 3'$ in a 400–450 kDa complex was only slightly higher. In the absence of M-CSF (Figure 8C), no dimers were observed with c-Fms WT or $\Delta 3'$. A cross-linked complex of 400–450 kDa was easily visible with c-Fms S301, S374, $\Delta 3'$, but only a trace amount of this complex was detectable with c-Fms S301, S374. The amount of 400–450 kDa cross-linked c-Fms S301, S374, $\Delta 3'$ was similar to the amount of aggregated c-Fms WT in cells that were growing continuously in M-CSF. The results with c-Fms immunoblots again showed that only c-Fms S301, S374, $\Delta 3'$ formed the 400–450 kDa activated complex efficiently in the absence of M-CSF, suggesting that although the mutations at amino acids 301 and 374 contributed to M-CSF-independent receptor aggregation, truncation of the C-terminus was also required for efficient ligand-independent receptor complex formation.

The cross-linked bands of 260–280 kDa did not appear to play a role in receptor activation because they neither appeared on the cell surface nor contained phosphotyrosine (Figures 6, 7, and 8D). However, because these cross-linked bands were formed by c-Fms S301, S374 as well as by c-Fms S301, S374, $\Delta 3'$, we investigated the possibility that mutations at amino acids 301 and 374 caused aggregation of these receptors in an intracellular compartment. Unstimulated cells expressing c-Fms WT or c-Fms S301, S374, $\Delta 3'$ were labeled for 15 min with [35 S]methionine and then chased for 4 h with excess unlabeled medium. At timepoints after the 35 S-pulse, aliquots of cells were lysed and cross-linked. Figure 9A shows that unstimulated c-Fms WT could be cross-linked into a band of 280 kDa early after synthesis. The amount of the 280-kDa band was proportional to the amount of immature (135 kDa) c-Fms at each timepoint. This was also true for c-Fms S301, S374, $\Delta 3'$, but in this mutant both the immature (130 kDa) band and the 260-kDa cross-linked bands had a much longer half-life. These results suggested that dimerization of immature c-Fms proteins in an intracellular compartment normally occurs during synthesis (Rose and Doms, 1988). The mutations at amino acids 301 and 374 may lead to retention of these proteins in the endoplasmic reticulum (ER) but are not required for formation of receptor aggregates in this compartment.

The size of the 260–280-kDa bands suggested that they may consist of receptor homodimers. Alternatively, these bands could contain c-Fms bound to another protein such as a chaperone protein in the ER (Georgopoulos, 1992). To address this question, [35 S]methionine-labeled c-Fms S301, S374, $\Delta 3'$ cells were lysed and treated with a reversible cross-linker, DTSSP. The 260-kDa band was cut out, and the cross-linking was reversed by treatment with dithiothreitol (DTT). When the 260-kDa band was electrophoresed under reducing conditions, the only major band was a protein of 130 kDa (Figure 9B). c-Fms immunoblots confirmed that this

Figure 9. (A) Timecourse of appearance of 260–280-kDa cross-linked bands. Unstimulated FDC-*fms* cells were labeled for 15 min with 200 μ Ci/ml of [35 S]methionine and then chased for 4 h with medium containing an excess of unlabeled methionine. At timepoints after the 35 S-pulse, aliquots of cells were lysed and cross-linked with BS 3 as described in MATERIALS AND METHODS. c-Fms proteins were immunoprecipitated and electrophoresed on a 7.5% SDS-PAGE gel. An 35 S exposure of the gel is shown. (B) Reversible cross-linker used to examine 260- and 450-kDa bands. Cells expressing c-Fms WT or S301, S374, $\Delta 3'$ were labeled for 4 h with 200 μ Ci/ml [35 S]methionine. The cells were then stimulated for 1 h on ice with 10 000 U/ml M-CSF, lysed, and cross-linked with DTSSP. Immunoprecipitated receptors were electrophoresed under nonreducing conditions on a 3–15% gradient SDS-PAGE gel. 35 S-labeled bands corresponding to the 260-kDa band from c-Fms S301, S374, $\Delta 3'$ and the 400–450-kDa band from c-Fms WT were cut out and the cross-linking was reversed by soaking the gel slices in sample buffer containing 100 mM DTT. The proteins were run again on a 3–15% gradient gel. An 35 S exposure of the result is shown.

protein was c-Fms (unpublished data). Therefore, the 260–280-kDa cross-linked bands appeared to be simple homodimers of the immature form of the receptor.

Different results were obtained when the 400–450-kDa complex was examined using a reversible cross-linker. When the 400–450-kDa band from M-CSF-stimulated c-Fms WT was reduced with DTT, this complex was seen to contain a number of proteins in addition to the 165-kDa c-Fms band (Figure 9B). Because c-Fms proteins were cross-linked after cell lysis, proteins that were closely associated with Fms after ligand stimulation might also become cross-linked into a complex with Fms. The 85-kDa subunit of phosphatidylinositol 3-kinase (PI3-kinase) has been shown to bind to ligand-activated c-Fms via the SH2 domain of p85 and Tyr721 of c-Fms (McGlade *et al.*, 1992; Reedijk *et al.*, 1992). To determine whether proteins involved in c-Fms signaling were cross-linked into a complex with Fms, cross-linked immunoprecipitated receptors from M-CSF-stimulated or unstimulated FDC-*fms* WT were immunoblotted with antibodies to phosphotyrosine, c-Fms, or the p85 subunit of PI3-kinase (Figure 10A). The cross-linked complex detected by antibody to phosphotyrosine was a broad band extending from $\sim$ 330 kDa to greater than 500 kDa (Figure 10A, left). When the same material was blotted with antibody to c-Fms, two broad bands (not clearly separated) were seen (Figure 10A, center). The smaller band was $\sim$ 330–380 kDa, and the larger band smeared upward from $\sim$ 400 kDa. When blotted with antibody to p85, only the higher molecular weight

part of the complex (>400 kDa) was detected (Figure 10A, right).

To confirm that p85 was specifically cross-linked into a complex with Fms, c-Fms WT and c-Fms Y721F were stimulated with M-CSF and cross-linked with DTSSP. When the immunoprecipitated receptors were run under nonreducing conditions and immunoblotted with antibody to phosphotyrosine, almost all of the phosphotyrosine was present in a complex of >330 kDa (Figure 10B, left). When electrophoresed in the presence of DTT, a number of additional tyrosine-phosphorylated proteins were released from the complexes (Figure 10B, center). The phosphotyrosine blot in the center was then stripped and reprobed with antibody to p85. The p85 subunit of PI3-kinase was easily detectable in the reduced complex from c-Fms WT but was not found in the complex from c-Fms Y721F (Figure 10B, right). These results demonstrated that the high molecular weight cross-linked complexes also contained other proteins that associate with c-Fms after ligand stimulation.

DISCUSSION

Generation of a fully transforming c-Fms protein requires mutations in both the extracellular and C-terminal domains (Roussel *et al.*, 1988; Woolford *et al.*, 1988). Substitutions of serine for Leu301 and Ala374 in the fourth Ig-like loop of the extracellular domain led to partial activation of the murine c-Fms protein.

Figure 10. Detection of p85 subunit of PI3-kinase in a high molecular weight complex with c-Fms. (A) FDC-*fms* WT cells were treated 1 h on ice with or without 10 000 U/ml M-CSF, lysed, and cross-linked with BS³. c-Fms proteins were immunoprecipitated under stringent conditions and electrophoresed on a 3–15% gradient SDS-PAGE gel. The proteins were transferred to nitrocellulose and immunoblotted with antibody to phosphotyrosine (left), c-Fms (center), or the p85 subunit of PI3-kinase (right). The dark streaks at the bottom of the center and right panels are because of cross-reactivity between rabbit antibody from the immunoprecipitation and anti-rabbit secondary antibody from the Western blot. (B) FDC-*fms* WT and Y721F cells were stimulated 1 h on ice with 10 000 U/ml M-CSF, then lysed and cross-linked with DTSSP. Half of the cross-linked immunoprecipitated material was run under nonreducing conditions (left) and half was run under reducing conditions (center). The proteins were immunoblotted with antibody to phosphotyrosine (left and center). The center blot was then stripped and reprobed with antibody to the p85 subunit of PI3-kinase (right). The dark bands at the bottom of the right panel are because of cross-reactivity between antibodies from immunoprecipitation and from the Western blot.

Previous studies have shown that substitution of the v-Fms C-terminus for the normal c-Fms tail is also a partially activating mutation (Roussel *et al.*, 1988; Woolford *et al.*, 1988). Our results indicated that this partial activation was because of loss of the normal c-Fms C-terminus rather than to the presence of new v-Fms sequences. Because these mutations identify important regulatory domains, receptors with these mutations were used to clarify the role of each of these domains in receptor activation.

c-Fms WT, c-Fms $\Delta 3'$, and c-Fms S301, S374 all required M-CSF stimulation to undergo rapid endocytosis and degradation in lysosomes. In contrast, the fully transforming mutant c-Fms S301, S374, $\Delta 3'$ was internalized and degraded nearly as rapidly in the absence of M-CSF as in its presence. Because c-Fms S301, S374, $\Delta 3'$ was continuously internalized and degraded, even in the absence of M-CSF, the level of receptors on the cell surface was constitutively low. This indicates that

the transforming activity of this mutant is not because of a failure of the receptor to downregulate, as described for an internalization-defective EGF-R mutant (Wells *et al.*, 1990).

That deletion or mutation of the c-Fms C-terminus is an activating mutation has been known for some time (Roussel *et al.*, 1987, 1988; Woolford *et al.*, 1988), however the mechanism by which this domain regulates the receptor kinase activity has not been elucidated. We investigated the possibility that deletion of the C-terminus increases the kinase enzymatic activity of the receptor. Using in vitro kinase assays to measure receptor autophosphorylation or phosphorylation of an exogenous substrate, we found no increase in the kinase activity of c-Fms $\Delta 3'$. Assays for receptor autophosphorylation or phosphorylation of cellular proteins in vivo also showed no increase in the kinase activity of c-Fms $\Delta 3'$. Only the receptor with mutations in the extracellular domain as well as a truncated C-terminus (c-Fms S301, S374, $\Delta 3'$) exhibited increased tyrosine kinase activity in the absence of M-CSF. Therefore, truncation of the C-terminus did not activate receptor monomers in *cis*. It remains possible that the C-terminus regulates Fms activity by other means. For instance, a conformational change in the C-terminus might regulate access to substrate binding sites or control interactions with another regulatory protein. However, any such regulation must occur only after receptor dimerization.

Although Li and Stanley (1991) found disulfide-linked dimers of c-Fms after M-CSF stimulation, we were unable to detect M-CSF-specific c-Fms dimers without using cross-linkers. The reason for this discrepancy is unknown. It is possible that disulfide bond exchange occurred during some part of Li and Stanley's protocol, but this exchange would have involved only preexisting M-CSF-stimulated dimers. Alternatively, disulfide linkage of receptor dimers may occur in the BAC1.2F5 cell line used by Li and Stanley but not in our FDC-P1 cells.

It is likely that c-Fms dimerization and activation normally occurs on the cell surface immediately after M-CSF binding. When cells expressing wild-type or mutant c-Fms were stimulated with a saturating concentration of M-CSF on ice, nearly all of the cell surface-labeled receptors could be cross-linked into a tyrosine phosphorylated high molecular weight complex. Because the cross-linker was added after cell lysis, other cellular proteins closely associated with Fms after M-CSF stimulation could also become cross-linked into a complex with Fms. Experiments using a reversible cross-linker confirmed that the high molecular weight complexes contained a number of other tyrosine phosphorylated proteins. No single protein in the reduced complex appeared to be present at equimolar levels with c-Fms. However, in most of our experiments using cross-linkers, the 400–450-kDa complexes actually extended from ~ 330 to >500 kDa. The efficiency of cross-linking

was probably not equally high for all proteins in the activated complex, so a heterogeneous mixture of aggregates was obtained. The smallest of these c-Fms aggregates could have been simple homodimers of the 165-kDa c-Fms protein, whereas the larger aggregates were probably c-Fms dimers cross-linked to one or more other cellular proteins. Using a reversible cross-linker, we demonstrated that the 85-kDa subunit of PI3-kinase was present in the activated complex from c-Fms WT but not from c-Fms Y721F. This suggests that the high molecular weight cross-linked aggregates may represent functional signaling complexes.

In addition to the 400–450 kDa cross-linked bands, c-Fms aggregates of 260–280 kDa were also observed in c-Fms S301, S374 and c-Fms S301, S374, $\Delta 3'$. These cross-linked bands were detectable only when immunoblotting with antibody to c-Fms and were not seen when immunoblotting biotinylated (cell-surface) proteins or phosphotyrosine. Experiments using a reversible cross-linker suggested that these intracellular aggregates were simple homodimers of immature c-Fms proteins. Dimerization of c-Fms precursors was not a result of the mutations at amino acids 301 and 374, because a 280-kDa cross-linked band was also seen in c-Fms WT at times early after synthesis. Fms proteins with mutations at amino acids 301 and 374 are inefficiently exported from the ER (Anderson *et al.*, 1984; Nichols *et al.*, 1985), and therefore the immature dimerized form of these mutants was easily detectable after cross-linking. Although c-Fms precursors may be aggregated in the ER, our evidence suggests that Fms proteins are transported to the plasma membrane as monomers, because in the absence of M-CSF, no dimers could be cross-linked on the cell surface. Because the 260–280-kDa bands contained no phosphotyrosine, these intracellular c-Fms dimers must differ in some way from the activated dimers formed on the cell surface.

Our data suggests that the activating mutations in the extracellular domain are required to help stabilize receptor dimers in the absence of M-CSF. Activated high molecular weight complexes of unstimulated c-Fms S301, S374, $\Delta 3'$ were clearly visible using antibodies to phosphotyrosine or to c-Fms. Activated complexes of c-Fms S301, S374 were also detected but at a much lower level. In contrast, no aggregates of wild-type or $\Delta 3'$ c-Fms were seen in the absence of M-CSF. Most of the identified activating mutations in c-Fms fall in or near the fourth Ig-like loop in the extracellular domain (Roussel *et al.*, 1988; Woolford *et al.*, 1988; van Daalen Wetters, *et al.*, 1992). The fourth and fifth Ig-like loops are not part of the M-CSF binding domain (Wang and Rohrschneider, 1993), and receptors with point mutations in this region are able to bind and respond to M-CSF (Roussel *et al.*, 1988). The fourth Ig-like loop is the only one of five not stabilized by disulfide bonds. M-CSF binding to Fms may therefore induce a confor-

mational change involving the fourth Ig-like region that facilitates dimerization.

Although the extracellular domain mutations were required for dimerization in the absence of M-CSF, a receptor with only these mutations (c-Fms S301, S374) formed activated complexes very inefficiently. In contrast, unstimulated c-Fms S301, S374, $\Delta 3'$ formed activated high molecular weight complexes as efficiently as M-CSF-stimulated c-Fms WT (when comparing steady-state levels at 37°C). We were initially surprised to find that high molecular weight complexes of c-Fms S301, S374, $\Delta 3'$ were not apparent in blots for cell-surface proteins. However, this result simply reflects the fact that c-Fms S301, S374, $\Delta 3'$ is rapidly internalized in the absence of M-CSF. There is evidence that activated dimers are preferentially internalized (Li and Stanley, 1991). If dimers are rapidly internalized as soon as they form on the cell surface, then most of the receptors detectable on the cell surface at steady state would be those that had not yet dimerized. Our assays for internalization demonstrated that c-Fms S301, S374 is not internalized in the absence of M-CSF and, like wild-type c-Fms, unstimulated receptors accumulate on the cell surface. Therefore, if c-Fms S301, S374 formed aggregates (even unphosphorylated aggregates) in the absence of M-CSF, these complexes should have been readily apparent in blots for cell surface protein or c-Fms. Other evidence suggests that the S301, S374 receptors were not activated in the absence of M-CSF. The level of receptor autophosphorylation and the level of tyrosine phosphorylation on cellular proteins in cells expressing this mutant were also very low.

These results suggest an important role for the C-terminus of Fms as well as the extracellular domain in receptor dimerization. Only in receptors with both a deleted C-terminus and the extracellular domain mutations could a significant percentage of the receptors be cross-linked into a high molecular weight complex in the absence of M-CSF. In the wild-type receptor therefore, both the fourth Ig-like loop in the extracellular domain and the C-terminus may provide conformational barriers to receptor dimerization. Upon M-CSF binding, conformational changes in both the extracellular domain and the C-terminus may occur, allowing the formation of stable receptor dimers.

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