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Tyrosine 569 in the c-Fms Juxtamembrane Domain Is Essential for Kinase Activity and Macrophage Colony-Stimulating Factor-Dependent Internalization

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The receptor (Fms) for macrophage colony-stimulating factor (M-CSF) is a member of the tyrosine kinase class of growth factor receptors. It maintains survival, stimulates growth, and drives differentiation of the macrophage lineage of hematopoietic cells. Fms accumulates on the cell surface and becomes activated for signal transduction after M-CSF binding and is then internalized via endocytosis for eventual degradation in lysosomes. We have investigated the mechanism of endocytosis as part of the overall signaling process of this receptor and have identified an amino acid segment near the cytoplasmic juxtamembrane region surrounding tyrosine 569 that is important for internalization. Mutation of tyrosine 569 to alanine (Y569A) eliminates ligand-induced rapid endocytosis of receptor molecules. The mutant Fms Y569A also lacks tyrosine kinase activity; however, tyrosine kinase activity is not essential for endocytosis because the kinase inactive receptor Fms K614A does undergo ligand-induced endocytosis, albeit at a reduced rate. Mutation of tyrosine 569 to phenylalanine had no effect on the M-CSF-induced endocytosis of Fms, and a four-amino-acid sequence containing Y-569 could support endocytosis when transferred into the cytoplasmic juxtamembrane region of a glycophorin A construct. These results indicate that tyrosine 569 within the juxtamembrane region of Fms is part of a signal recognition sequence for endocytosis that does not require tyrosine phosphorylation at this site and that this domain also influences the kinase activity of the receptor. These results are consistent with a ligand-dependent step in recognition of the potential cryptic internalization signal.

The c-*fms* proto-oncogene encodes the receptor for the macrophage colony-stimulating factor (M-CSF, CSF-1) (44). This receptor is expressed in cells of the monocyte-macrophage lineage and is required for their survival, proliferation, and differentiation to the mature phenotype (37). Association of M-CSF with its receptor facilitates the rapid autophosphorylation of several tyrosine residues on the receptor's cytoplasmic domain (47, 50) as well as the phosphorylation of a wide range of intracellular substrates (26, 36, 54). Coincident with these initial events of M-CSF stimulation, the receptor is rapidly internalized, having a half-life on the cell surface in the order of minutes (7, 34). Studies on the transforming v-Fms protein suggest that its internalization occurs via clathrin-coated pit-mediated endocytosis (29). Phosphorylation of the receptor on any of its principal tyrosine phosphorylation sites is not prerequisite for rapid receptor endocytosis (7). In fact, even eliminating the receptor's kinase activity by mutating an essential lysine at amino acid 614 of the ATP-binding site does not completely block this internalization activity (7).

In other receptors, a short consensus internalization sequence within the cytoplasmic domain is required for association of the receptor with clathrin-coated pit adaptor molecules (48). This association and subsequent internalization may occur constitutively (e.g., low-density lipoprotein [LDL] and transferrin receptors [16]) or may require ligand binding (e.g., platelet-derived growth factor [PDGF], epidermal growth factor [EGF], and insulin receptors [31, 49]). The majority of receptors characterized thus far possess an essential tyrosine within this internalization signal (12, 19, 25, 27). In a substantial proportion of these receptors, this tyrosine may be substituted with phenylalanine (1, 5, 12, 13, 32); therefore, phosphorylation of this residue is not required for endocytosis. In addition to a characteristic tyrosine, internalization signals tend to reside proximal to the cytoplasmic face of the plasma membrane (12). In fact, studies on deletion mutants of the transferrin receptor have shown that the essential tyrosine may be as close as 10 amino acids from the membrane before endocytosis is inhibited (11). Structural studies on a variety of receptors indicate that the conserved tyrosine (phenylalanine) is an integral component of a β-turn motif (1, 2, 11, 14).

The c-Fms protein has a domain arrangement characteristic of a family of tyrosine kinase receptors, including those for stem cell factor (Kit ligand) (35a), Flt3/Flk2 (30a, 39a), and PDGF (10, 30, 53). Specifically, the cytoplasmic domain contains a two-part kinase domain which is divided by a kinase insert. Adjacent to the carboxy-terminal end of the kinase domain is a serine/glycine/glutamate-rich region of purported negative regulatory function (6, 40, 52). Proximal to the plasma membrane is a stretch of 50 amino acids of unknown function termed the juxtamembrane domain. Within this domain are six tyrosine residues; two of these residues, Y-536 and Y-538, are immediately adjacent to the transmembrane domain within the putative stop-transfer sequence. The remaining four tyrosines Y-544, Y-554, Y-559, and Y-569, are distal to the stop transfer sequence, and on the basis of analogous sequences in other receptors internalized via coated pits, they are candidates for part of an internalization signal.

Our interest is to study the potential role of receptor endocytosis in c-Fms signal transduction. In this report, we describe the mutagenesis of the juxtamembrane domain both by deleting successive tyrosines and by substituting tyrosines with alanine. We find that this region of the protein is exquisitely sensitive to mutation in terms of affecting its in vitro

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kinase activity as well as its capacity to undergo ligand-induced endocytosis.

MATERIALS AND METHODS

Materials. *Escherichia coli* CJ236 was purchased from Bio-Rad, and *E. coli* NM522 was purchased from Pharmacia. Both *E. coli* strains were cultured in 2× YT (41); 34 μg of chloramphenicol per ml was added to CJ236 cultures for F-factor selection.

T4 DNA ligase, T4 DNA polymerase, T4 polynucleotide kinase, and restriction endonucleases were from either GIBCO-BRL, New England Biolabs, or Boehringer Mannheim Biochemicals. Sequenase was from United States Biochemical.

[^{35}S]methionine was purchased from ICN Biomedicals; [^{32}P]ATP was purchased from DuPont NEN. Deoxynucleoside triphosphates (dNTPs) were from Pharmacia, and ATP was from Boehringer Mannheim.

Partially purified murine M-CSF was prepared as described by Wang et al. (51). Interleukin-3 is contained in conditioned medium from WEHI-3B cells grown in Dulbecco modified Eagle medium (DME) supplemented with 10% fetal bovine serum (FBS). This medium (WCM) is routinely used at 2% without further purification.

Fetal bovine and calf sera were from HyClone. Geneticin (G418) and methionine-free DME were obtained from GIBCO-BRL.

Radioimmunoprecipitation assay (RIPA) buffer is 1% Triton X-100–0.1% sodium dodecyl sulfate (SDS)–20 mM EDTA–1% aprotinin–1% deoxycholic acid in phosphate-buffered saline (PBS) (pH 7.2). Nonidet P-40 (NP-40) lysis buffer is 20 mM Tris (pH 7.2)–50 mM NaCl–0.5% NP-40. $MgCl_2$-Tris buffer is 1 M $MgCl_2$–10 mM Tris (pH 7.5). Pansorbin was prepared from formaldehyde-inactivated *Staphylococcus aureus* and was blocked with 5% each bovine serum albumin and ovalbumin in RIPA buffer prior to use.

All c-Fms antibodies were produced in our laboratory. 4599B (38) and 5674 (6) are rabbit polyclonal antisera with activities against the cytoplasmic and extracellular c-Fms domains, respectively; tumor-bearing rat serum contains antibodies with specificity for the c-Fms extracellular domain. Fluorochrome-labeled secondary antibodies for immunofluorescence were purchased from Jackson ImmunoResearch Laboratories, Inc.

All other reagents used in this study were obtained from Sigma Chemical Company.

Construction of deletion and point mutations in the c-Fms juxtamembrane domain. Site-directed mutagenesis was performed essentially as originally described (55), using a uracil-containing template prepared from *E. coli* CJ236 supernatants (23).

The murine c-Fms cDNA has been cloned into a pBluescript (Stratagene) vector modified in the multiple cloning site (pVZ) (52). Single-stranded pVZ murine c-Fms was isolated from *E. coli* CJ236 superinfected with 10^7 PFU of M13K07 per ml and cultured overnight in 2× YT (41) supplemented with 34 μg of chloramphenicol, 70 μg of kanamycin, and 200 μg of ampicillin per ml.

Oligonucleotides were prepared in house on an Applied Biosystems model 380 A/B DNA synthesizer and were used without further purification. The sequences of the oligonucleotides were as follows: Δ544–550, 5′ CAAGCAGAAGCCGA AGATCGAGAGATACGAAGGC 3′; Δ544–555, 5′ CAAG CAGAAGCCGAAGGGCAATAGCTACACCTTC 3′; Δ544–565, 5′ CAAGCAGGAAGCCGAAGCAGTTGCCCTACAA TGAG 3′; Δ544–576, 5′ CAAGCAGAAGCCGAAGCGGAA CAACCTGCAGTTTGG 3′; Δ577–586, 5′ GAGAAGTGGG AGTTCCCTGGAGCCGGTGCCTTTGGG 3′; Δ570–586, 5′ CCTACTCAGTTGCCCTACGGAGCCGGTGCCTTTGGG 3′; Δ560–586, 5′ CGAAGGCAATAGCTACGGAGCCGGT GCCTTTGGG 3′; Δ555–586, 5′ GATCATCGAGAGATACG GAGCCGGTGCCTTTGGG 3′; Y544A, 5′ GAAGCCGAAG GCCCAGGTGCGC 3′; Y554A, 5′ CATCGAGAGAGCTGA AGGCAATAG 3′; Y559A, 5′ GGCAATAGCGCCACCTT CATTG 3′; Y569A, 5′ CAGTGCCCGCCAATGAGAAG 3′; Y569F, 5′ CAGTTGCCCTTTAATGAGAAG 3′; and K614A, 5′ GGTGGCTGTGGCGATGCTAAAG 3′.

Oligonucleotides were phosphorylated on the 5′ end in a buffer containing 100 μM Tris (pH 8.0), 10 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM ATP, and 5 U of T4 polynucleotide kinase. A typical 10-μl annealing reaction mixture contained 8 pmol of phosphorylated oligonucleotide and 1 μg of single-stranded DNA template in 20 mM Tris (pH 7.4)–2 mM $MgCl_2$–50 mM NaCl. Samples were heated to 70°C for 5 min and slowly cooled to room temperature. The following reagents were then added sequentially: (i) 1 μl of 10× synthesis buffer containing 5 mM each dNTP, 10 mM ATP, 100 mM Tris (pH 7.4), 50 mM $MgCl_2$, and 20 mM dithiothreitol; (ii) 2 U of T4 DNA ligase; and (iii) 1 U of T4 DNA polymerase. The mutagenesis reaction was transformed into *E. coli* NM522 and plated on 2× YT agar containing ampicillin. Single-stranded template was prepared from single colonies picked into 2 ml of 2× YT supplemented with ampicillin, kanamycin, and M13K07. Mutants were identified by single-strand dideoxy sequencing (42). Double-strand plasmids were isolated on $CsCl_2$ gradients, and the c-*fms* cDNA was subcloned as a 3.1-kb *Not*I-*Nde*I fragment into the pZen113 retroviral expression vector (39).

Construction of c-Fms–glycophorin A chimeras. A chimeric c-Fms–glycophorin A molecule was constructed by fusion of the c-Fms extracellular and transmembrane domains and the glycophorin A cytoplasmic domain. DNA for these constructs was mutagenized by the protocol described above and manipulated as follows. Site-directed mutagenesis was used to incorporate a *Bam*HI restriction site between the codons for amino acids 535 and 536 in pVZ c-Fms and amino acids 94 and 95 in pBS GPA (22). The respective oligonucleotide sequences were c-Fms 535BamHI536 (5′ CTGTTGCTGCTCTTGGATCCG TACAAGTACAAGCAG 3′) and GPA 94BamHI95 (5′ TAT CAGTCGGCGAATCGGATCCCCGTAAGAAATTAA 3′).

From these constructs, the c-*fms* *Not*I-*Bam*HI DNA fragment and the *Bam*HI-*Nde*I GPA fragment were ligated into the *Not*I-*Nde*I sites of pZen113 to create pZen113 c-Fms535$^-$GPA95$^+$.

To create c-Fms–glycophorin A chimeras into which alternate four-amino-acid sequences were cloned, single-stranded pBS GPA 94BamHI95 was used as the template. The oligonucleotides used to create mutations in the glycophorin A cytoplasmic domain were GPA QLPY (5′ GGGGAGAGGTT TGTAAGGCAATTGGCTTTTCTTTAT 3′), GPA QLPA (5′ GGGGAGAGGTTTGGCAGGCAATTGGCTTTTCTTTAT 3′), GPA NPVY (5′ GGGGAGAGGTTTGTAAACAGGG TTGCTTTTCTTTAT 3′), GPA GNSY (5′ GGGGAGAGG TTTGTAAGAATTTCCGCTTTTCTTTAT 3′), and GPA V106Y (5′ GAGAGGTTTGTAATCAGATGG 3′).

Production of cell lines. The ψ-2 packaging cell line was transfected with 10 μg of each pZen113 c-Fms construct and 1 μg of RSV-Neo coprecipitated with $CaPO_4$. The cells were cultured in DME–10% calf serum at 37°C in 7.5% CO_2. After 18 to 24 h, Geneticin was added to 500 μg/ml to select for stable retroviral integrants. After approximately 2 weeks in

culture, c-Fms-expressing cells were selected by fluorescence-activated cell sorting (FACS) by staining with a primary tumor-bearing rat serum containing antibodies against the c-Fms extracellular domain and a secondary phycoerythrin-conjugated anti-rat antibody. Equivalent levels of c-Fms expression were obtained by setting the same sort gate for each cell line.

The murine myeloid cell line FDC-P1 was infected with c-Fms retrovirus by cocultivation of 5×10^5 ψ-2 cells and 1×10^5 FDC-P1 cells in medium containing DME–10% FBS–2% WCM. After approximately 64 h in coculture, the nonadherent FDC-P1 cells were transferred to DME–10% FBS–2% WCM. c-Fms expressors were obtained by two successive FACS sorts as described above for the ψ-2 cell lines.

Rat-2 cell lines were infected with conditioned medium collected from the relevant ψ-2 c-Fms cell line. The retroviral supernatants were clarified by centrifugation (10 min at 1,500 rpm) and sterilized by filtration through a 0.22-μm-pore-size Millipore filter. After 3 days in culture, the Rat-2 fibroblasts were washed free of unadsorbed retrovirus and cultured without further selection in DME–10% calf serum.

Radioimmunoprecipitation and in vitro kinase assays. FDC-P1 cells (5×10^5) cultured in DME–10% FBS–2% WCM were washed twice with ice-cold methionine-free DME, suspended in 2 ml of methionine-free DME–10% dialyzed FBS, and transferred to a 6-cm-diameter plate containing 200 μCi of [^{35}S]methionine. After 2 h at 37°C in 7.5% CO_2, cells were collected by centrifugation and washed with DME–10% FBS. Cell lysates were prepared by suspending the cell pellet in ice-cold buffer containing 0.5% NP-40 and precleared with 40 μl of Pansorbin. The lysates were incubated at 4°C with an antibody against the extracellular domain of c-Fms (antibody 5674). The immune complexes were adsorbed to Pansorbin, washed twice with 500 μl of RIPA buffer, and washed once each with 500 μl of $MgCl_2$-Tris buffer and 500 μl of NP-40 lysis buffer. Proteins were resolved on SDS–7.5% polyacrylamide gels (25), fixed in destain, soaked sequentially in En^{3}Hance (NEN) and water, dried, and autoradiographed.

The in vitro kinase activities of the various c-Fms mutants were assayed by the method of Lyman and Rohrschneider (28). The Pansorbin-adsorbed immune complexes were split into two equivalent aliquots. One was washed once with 500 μl of 10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 5.5)–100 mM NaCl–1% aprotinin. These pellets were then suspended in 25 μl of 20 mM succinate (pH 5.5)–10 mM $MnCl_2$–20 μCi of [γ-^{32}P]ATP. After 10 min at 30°C, the Pansorbin was washed once with 500 μl of RIPA buffer and once with 500 μl of NP-40 lysis buffer. Both the ^{35}S- and ^{32}P-labeled pellets were suspended in SDS-polyacrylamide gel electrophoresis sample buffer containing 250 mM EDTA and electrophoresed on SDS–7.5% polyacrylamide gels.

Internalization assays. The time-dependent disappearance of c-Fms from the cell surface was measured by two methods. In the first (see also reference 7), 5×10^6 FDC-P1 cells were metabolically labeled with [^{35}S]methionine as described above. The cells were washed with and suspended in DME–10% FBS. Immature protein was chased to the cell surface by incubation at 37°C for 30 min or longer, depending on the particular cell line. At desired times following the addition of M-CSF (5,000 U/ml), internalization was quenched by transferring an aliquot of 5×10^5 cells to a microcentrifuge tube containing 1 μl of 20% NaN_3 and put on ice. The cells were collected by centrifugation and suspended in 0.5 ml of ice-cold DME–10% FBS–0.1% NaN_3. Rabbit antiserum containing antibodies to the c-Fms extracellular domain (i.e., 10 μl of antibody 5674) was added to the intact cells, and the mixture was incubated on ice for 60 min. Cells were sedimented, washed once with 500 μl of ice-cold PBS, and lysed with 500 μl of RIPA buffer. Lysates were adsorbed to 40 μl of Pansorbin, incubated on ice for 30 min, and washed as described for radioimmunoprecipitation. Proteins were electrophoresed on SDS–7.5% polyacrylamide gels, which were dried and exposed to film.

In the second method, Rat-2 fibroblasts expressing the various c-Fms mutants were treated with M-CSF and stained with fluorescent antibodies for visualization by confocal fluorescence microscopy. Cycloheximide was used to block new protein synthesis and eliminate the large intracellular pool of Fms precursors in mutant-infected Rat-2 cells, and chloroquine blocked the transfer of Fms from endosomes to lysosomes for degradation (25). The treatment time with cycloheximide was sufficient to chase most precursors to the mature Fms form, as judged from previous radiolabeled pulse-chase experiments (data not shown). Fibroblasts grown in DME–10% calf serum were trypsinized from confluent plates and replated at ~1/20 cell density on 12-mm-diameter no. 1 glass coverslips (Bellco) in six-well plates. After ~48 h of incubation, coverslips were transferred to a 24-well plate. Chloroquine was added to 50 μM, and the cells were incubated for 3 h at 37°C. In experiments in which cycloheximide was used, it was added at 10 μg/ml for 6 h at 37°C. M-CSF was then added for 30 min at 37°C. The cells were fixed for 6 min with −20°C methanol and then rinsed three times in succession by submersion for 5 min each time in room temperature IF-PBS. Twenty microliters of 1:200-diluted rabbit antibody 4599B (with specificity for the c-Fms cytoplasmic domain) was layered onto the coverslips, which were then incubated in a humidifying chamber for 45 min at 37°C. After three washes with IF-PBS, 20 μl of the secondary antibody, DTAF anti-rabbit (1:300), was incubated with the fixed cells for 45 min at 37°C. The coverslips were washed three times with IF-PBS and mounted with elvanol (20 g of polyvinyl alcohol [Monsanto 20-30], 80 ml of PBS heated to 70°C to dissolve; cool and add 40 ml of glycerol) containing the antioxidant 1,4-diazabicyclo[2.2.2]octane (DABCO) at 26 mg/ml (pH adjusted to 8.6 with HCl). Fluorescence was visualized by confocal microscopy.

Quantification of internalization and kinase assays. Radioactivity on internalization and kinase experiments was quantified on a Molecular Dynamics model 425 PhosphorImager, and data were analyzed with Molecular Dynamics ImageQuant software. For internalization, net counts per minute in the mature c-Fms band for each time point was determined by subtracting background counts in an equivalent section of an unloaded lane. Each time point was normalized to the $t = 0$ min point and was expressed as a percentage of that value. The kinase activity was expressed as the ratio of ^{32}P incorporated to ^{35}S counts per minute for a given sample. Net values were determined as described for internalization experiments, and each ratio was normalized to the value determined for the wild-type protein.

RESULTS

Mutations in the c-Fms juxtamembrane domain. A set of nested internal deletion mutations in the c-Fms juxtamembrane domain was designed in light of work on other receptors and in accordance with the fine-structure analysis of the transferrin receptor (8, 11). Specifically, it was found that the maximum rate of internalization of the transferrin receptor required a minimum 10-amino-acid separation between the membrane and an essential cytoplasmic tyrosine within an internalization signal motif. Amino acids at positions −2 and −3 relative to this tyrosine were also required for internaliza-

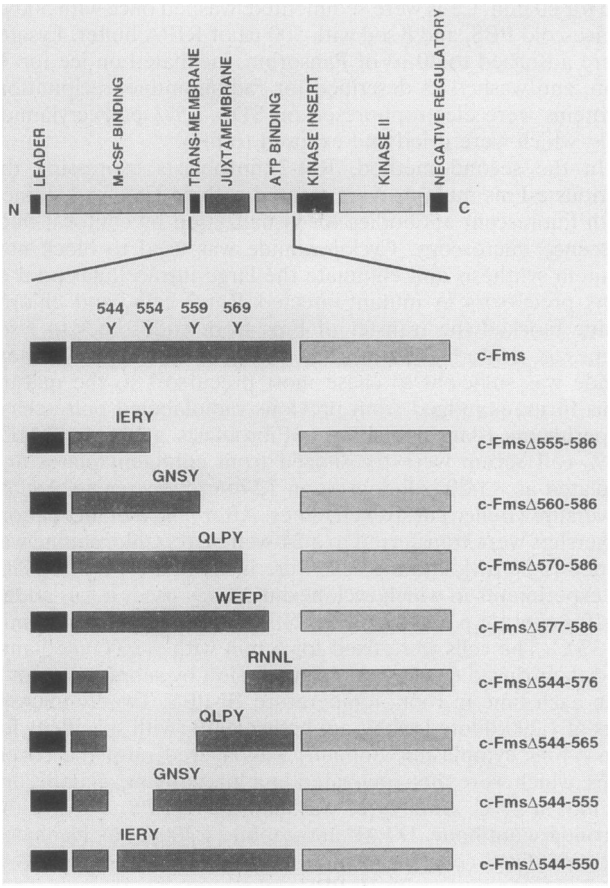

FIG. 1. Site-directed point mutations and nested internal deletion mutations within the murine c-Fms juxtamembrane domain. Each of the four tyrosines in the juxtamembrane domain was mutated to alanine with the exception of Y-569, which was mutated to both alanine and phenylalanine. The deletion mutations were designed in accordance with the structure of the transferrin internalization signal (11). Specifically, two groups of mutations were constructed: (i) successive truncations from a fixed residue (586) proximal to the amino-terminal junction of the kinase domain and proceeding toward the transmembrane domain (each successive truncation removed amino acids immediately downstream of a tyrosine) and (ii) successive truncations from a fixed amino acid (544) seven residues to the carboxy-terminal side of the transmembrane domain (each successive deletion removed up to the third amino acid to the amino-terminal side of a tyrosine residue). In this way, a 10-amino-acid spacer was fashioned between the membrane and each tyrosine.

tion. The c-Fms protein has four tyrosine residues within its 50-amino-acid juxtamembrane domain which could contribute to an internalization signal, and their potential role in this process was tested by deletion analysis. Internal deletions were made in two sets (Fig. 1) and constructed such that the seven amino acids proximal to the membrane were retained in each mutant. In the first set, all deletions started at and included amino acid 586 and extended progressively further upstream into the juxtamembrane region. Each deletion terminated at the carboxy-terminal side of one of the tyrosine residues (Y-554, Y-559, and Y-569) or at amino acid 577. Deletions in the second set started at and included amino acid 544 and extended up to but did not include the amino acid at position −3 relative to each of the respective tyrosines Y-554, Y-559, and Y-569. These overlapping sets of deletion mutants covered most of the juxtamembrane region.

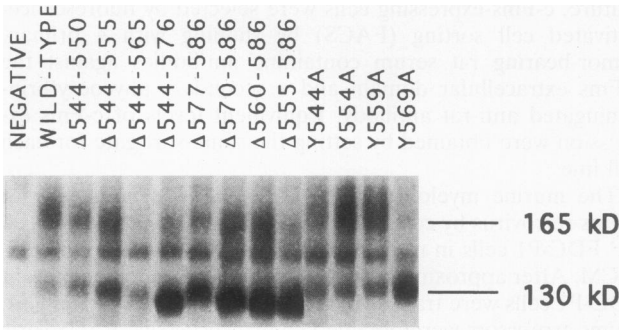

FIG. 2. Expression of mutant c-Fms proteins in FDC-P1 cells. Each of the c-*fms* mutations created in pVZ c-Fms was subcloned as a full-length c-DNA into the pZen113 retroviral vector and stably expressed in FDC-P1 cells. Shown is an autoradiograph of lysates from 5×10^5 cells radiolabeled with [^{35}S]methionine and immunoprecipitated with polyclonal antiserum 5674, containing antibodies with specificity for the extracellular domain. 130 kD refers to the immature, cytoplasmic form of c-Fms, while 165 kD refers to the mature cell surface form.

In addition to the internal deletion mutants, each of the four tyrosines (i.e., Y-544, Y-554, Y-559, and Y-569) was substituted with alanine (Fig. 1). The more conservative substitution to phenylalanine was not made initially because in a large percentage of internalization signals, phenylalanine can substitute for the essential tyrosine (13).

Expression of c-Fms mutants in FDC-P1 cells. To assess the effect of each juxtamembrane domain mutation within a biologically relevant cell line, the mutants were expressed in FDC-P1 cells, a factor (i.e., granulocyte-macrophage colony-stimulating factor or interleukin-3)-dependent myeloid cell line (38). FDC-P1 cells do not express endogenous c-*fms* but will proliferate in response to M-CSF if c-*fms* is expressed exogenously. FDC-P1 cells were infected with retroviral c-*fms* constructs packaged in ψ-2 cells. A population of cells expressing high levels of c-Fms was then obtained by two successive FACS sorts using a constant gate for all mutants. Expression was confirmed by radioimmunoprecipitation from ^{35}S-labeled cell lysates with antibody 5674 (Fig. 2). This analysis revealed that all mutants expressed the mature 165-kDa cell surface form of Fms as expected from the cell-sorting selection method. The largest difference in expression level, however, was observed with the immature 130-kDa cytoplasmic form of Fms. Accumulation of the 130-kDa species was apparent with mutations that altered sequences around or including Y-569 (i.e., Δ544–576, Δ577–586, Δ570–586, Δ560–586, Δ555–586, and Y569A). This finding suggests that these mutants are defective in protein processing. An unusually high proportion of the Y569A mutant Fms molecules was also detected within the endoplasmic reticulum network (see below and Fig. 6E) by immunofluorescence analysis.

In vitro kinase activities of c-Fms mutants. The in vitro kinase assay measures the autophosphorylation of immunoprecipitated receptors. It therefore approximates the inherent kinase activity of the cytoplasmic domain. Previous experiments from our laboratory have shown that Fms kinase activity is not essential for receptor internalization, although a kinase-inactive receptor does internalize with diminished kinetics compared with the wild-type protein (7). The internal deletion mutants described in this report were made by removing segments of protein within the juxtamembrane domain. On the basis of studies which describe the boundaries of the kinase

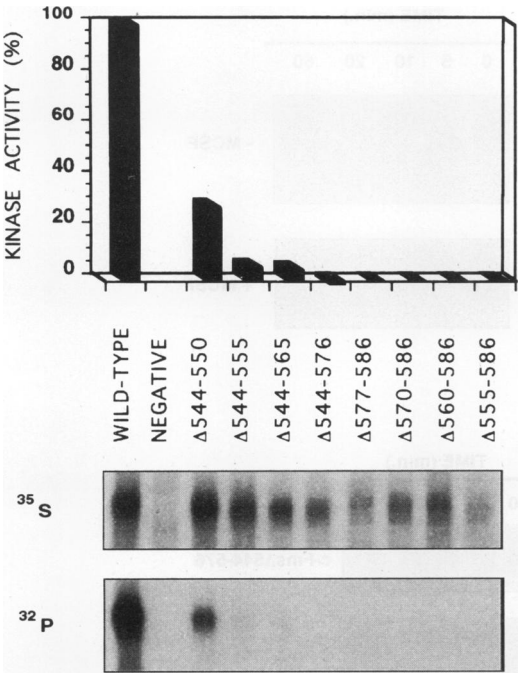

FIG. 3. Kinase activity of c-Fms internal deletion mutants. FDC-P1 cells (5×10^5) were metabolically radiolabeled with [^{35}S]methionine for 2 h; cell surface receptors were selectively immunoprecipitated with polyclonal antisera, and half of the immunoprecipitate was autophosphorylated in the immune complex. The labeled products were electrophoresed on an SDS–7.5% polyacrylamide gel, and quantification was performed on a PhosphorImager for ^{32}P; ^{35}S was detected by autoradiography. Kinase activity is expressed as the ratio of ^{32}P to ^{35}S counts and is normalized to the wild-type activity.

domains of other tyrosine kinases (17), we predicted that this section of the protein would not be essential for its kinase activity. However, analysis of each mutant receptor's in vitro kinase activity (Fig. 3) revealed that successive truncations from the transmembrane domain toward the kinase domain resulted in a parallel decrease in in vitro autophosphorylation. Further, all deletions which started at amino acid 586 and removed incremental sections from the carboxy-terminal end of the juxtamembrane domain eliminated c-Fms kinase activity altogether. Mutations beginning at amino acid 544 had decreasing kinase activity as the deletion extended toward the carboxy-terminal end of the juxtamembrane region. Thus, c-Fms Δ544–550 had approximately 30% of wild-type activity, and Δ544–555 and Δ544–565 had 6 and 4%, respectively, of wild-type activity. The largest deletion, Δ544–576, had no detectable in vitro kinase activity. All of the deletion mutants Δ577–586, Δ570–586, Δ560–586, and Δ555–586 were entirely kinase inactive. Similar analysis of point mutations Y544A, Y554A, and Y559A revealed wild-type kinase activity (data not shown), while Y569A was kinase inactive (see Fig. 5A).

Internalization of wild-type and mutant c-Fms. The results in Fig. 4 show the internalization of wild-type (Fig. 4A) and mutant (Fig. 4B to D) c-Fms from the surface of FDC-P1 cells. In the absence of M-CSF, the wild-type c-Fms receptor was stably expressed on the cell surface, having a half-life of greater than 1 h. Addition of M-CSF resulted in the rapid endocytosis of the receptor; under the conditions of these experiments, more than 80% of the receptor was depleted from the cell surface within the first 5 min after M-CSF addition (Fig. 4A).

Analysis of the deletion mutants Δ544–550, Δ544–555, Δ544–565, and Δ544–576 revealed a decrease in the rate of internalization which roughly paralleled each receptor's relative kinase activity (Fig. 4B). Likewise, juxtamembrane domain deletions which included amino acids adjacent to the kinase domain and removed segments toward the transmembrane domain were completely devoid of M-CSF-dependent receptor internalization activity (Fig. 4C). This loss of internalization correlated directly with the absence of receptor kinase activity. The half-life of these receptors in the presence of M-CSF was indistinguishable from that of the wild-type receptor in the absence of M-CSF.

c-Fms juxtamembrane tyrosine-to-alanine point mutants were analyzed similarly. Mutations at Y-544, Y-554, and Y-559 had no effect on c-Fms M-CSF-dependent internalization. In contrast, c-Fms Y569A showed no ligand-stimulated internalization and was depleted from the cell surface at a rate comparable to that of the wild-type protein in the absence of M-CSF (Fig. 4D).

Like c-Fms K614A, c-Fms Y569A is kinase inactive; however, only c-Fms Y569A is entirely internalization defective. Although internalization of the kinase-inactive c-Fms mutant K614A is accelerated by ligand binding, the rate of ligand-stimulated endocytosis of the mutant is considerably slower than that of wild-type c-Fms (7). These data suggest that the receptor's kinase activity must be important for regulating the rate of internalization rather than being absolutely required for internalization. With the mutants described here, we found a tight correlation between the receptor's kinase activity and capacity for rapid internalization. We therefore wished to compare the kinetics of internalization of the kinase-inactive, internalization-competent mutant K614A with that of the kinase-inactive, internalization-defective mutant Y569A. Figure 5A shows that neither protein had detectable in vitro autophosphorylation activity. Comparison of the internalization kinetics for the K614A and Y569A c-Fms mutants is shown in Fig. 5B. The Y569A mutant was absolutely stable on the cell surface even after 2 h with M-CSF. In contrast, the amount of c-Fms K614A remaining on the cell surface at 2 h was 40% of the starting level. Thus, there was a detectable difference in the c-Fms internalization between the K614A and Y569A mutants.

Analysis of c-Fms internalization by immunofluorescence. Because of the low rate of internalization observed with c-Fms K614A, we wished to compare the internalization of c-Fms wild type, K614A, and Y569A in an additional system. We therefore chose to express these c-Fms receptors in Rat-2 fibroblasts and, by immunofluorescence, assay for the receptor's subcellular localization under a number of conditions. Figure 6 shows the results of such an experiment. Rat-2 cells expressing the wild type, Y569A, and K614A were treated for 3 h with the lysosomotropic drug chloroquine to enrich for endosomes by inhibiting the transfer of their contents into lysosomes. To one set of cells, cycloheximide was added to block protein synthesis and chase cytoplasmic Fms to the cell surface. After incubation for 6 h with cycloheximide, M-CSF was added and the cells were incubated for 30 min at 37°C (Fig. 6A to C). To another set of cells, no cycloheximide was added prior to addition of M-CSF (Fig. 6D to F).

In experiments that included the protein synthesis inhibitor cycloheximide (Fig. 6A to C), cells expressing the wild-type c-Fms protein displayed a prominent pattern of punctate cytoplasmic staining adjacent to the nucleus characteristic of endosomal staining. There was a relatively low level of plasma membrane staining, indicating that the majority of cell surface receptor was internalized under these conditions. In contrast

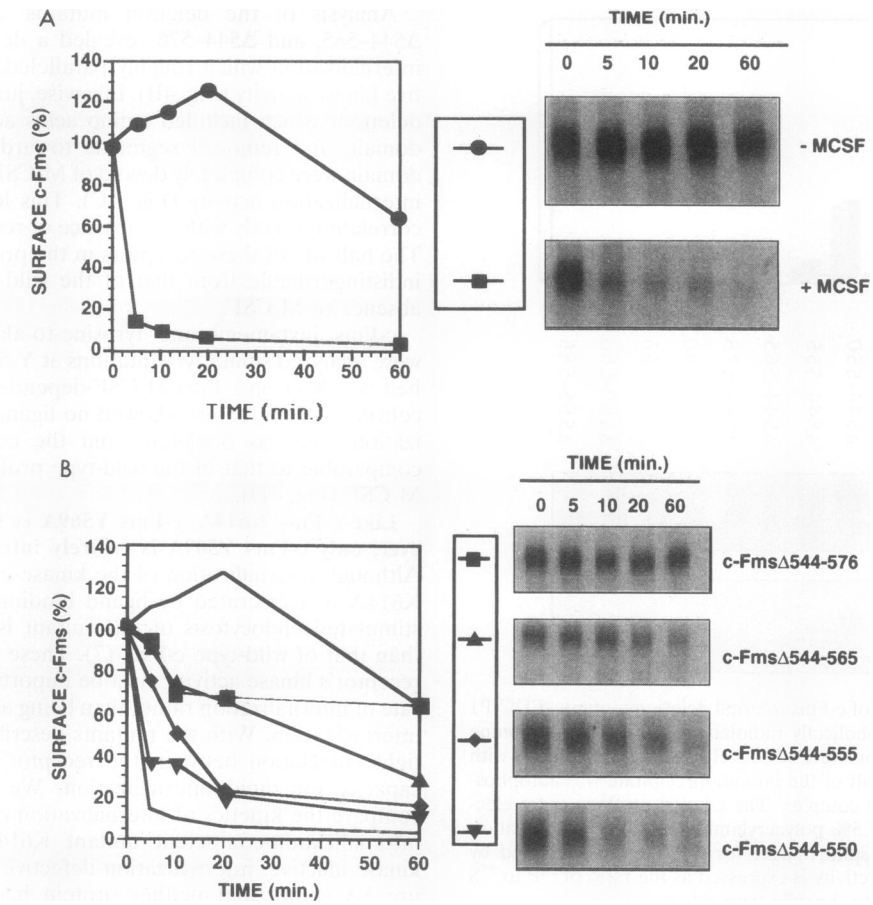

FIG. 4. (A) Internalization of wild-type c-Fms in FDC-P1 cells. FDC-P1 cells (5×10^6) expressing wild-type c-Fms were radiolabeled with [^{35}S]methionine, and internalization assays were performed as described in Materials and Methods. The autoradiogram or PhosphorImager analysis of the radiolabel in Fms (right) and graphical analysis of the cell surface-expressed Fms (left) are shown. Counts per minute in all time points were expressed as a percentage of the $t = 0$ min value. (B to D) Internalization of c-Fms internal deletion (B and C) and tyrosine point mutants (D) within the juxtamembrane region. Only the M-CSF-stimulated internalization was measured for the mutant Fms molecules, and each graph is overlaid with the wild-type data from panel A for comparison (no symbols).

to the wild-type receptor, c-Fms Y569A was expressed primarily on the cell surface and very little endosomal staining was detectable. The pattern of c-Fms K614A staining was clearly distinct from that of c-Fms Y569A in that the former mutant was localized to a very high degree within the endosomal compartment. The Y569A mutant differed from the wild type, however, in that there remained a high level of cell surface expression. In the absence of M-CSF stimulation, wild-type Fms was located primarily on the cell surface with little or no endosomal staining and looked similar to the cells expressing Fms Y569A shown in Fig. 6B. Absence of M-CSF stimulation did not alter the distribution of either Fms Y569A or K614A in the Rat-2 cells (data not shown). Hence, the K614A mutant was capable of endocytosis albeit with diminished kinetics, while the Y569A protein was virtually devoid of internalization activity.

Figure 6D to F show the results of an analogous experiment in which protein synthesis is not blocked with cycloheximide prior to staining. Figures 6D and F are the results for the wild-type and K614A c-Fms, respectively. The pattern of staining for these two proteins was indistinguishable from that in Fig. 6A and C. In contrast, the staining pattern for c-Fms Y569A (Fig. 6E) was dramatically different from that obtained in the absence of cycloheximide (Fig. 6B). In the absence of cycloheximide, c-Fms Y569A was found primarily within the Golgi and endoplasmic reticulum network rather than on the cell surface. This dramatic accumulation in the cytoplasm was consistent with the results in Fig. 2, demonstrating a processing defect unique to the Y569A protein.

c-Fms Y569F has kinase activity and is rapidly internalized. Studies on the internalization of other receptors have revealed that the essential tyrosine in a large number of these molecules can be replaced with phenylalanine to yield a receptor with equivalent internalization activity. We therefore determined the effect of replacing the tyrosine at position 569 of Fms with phenylalanine. Figure 7A shows in vitro kinase activity of the Y569F mutant compared with that of the wild-type receptor. In contrast to Y569A, Y569F had a high level of in vitro tyrosine kinase activity and internalized with kinetics similar to that of the wild-type protein (Fig. 7B).

The region around Y-569 can function as an internalization signal in a heterologous system. Our results suggested that tyrosine 569 may have an essential role in c-Fms endocytosis beyond its function in the receptor's kinase activity. We were therefore interested in probing the amino acid sequence surrounding Y-569 (i.e., QLPY) to determine whether it might mediate receptor endocytosis when transplanted into a suitable sequence context. Interestingly, the QLPY sequence is not

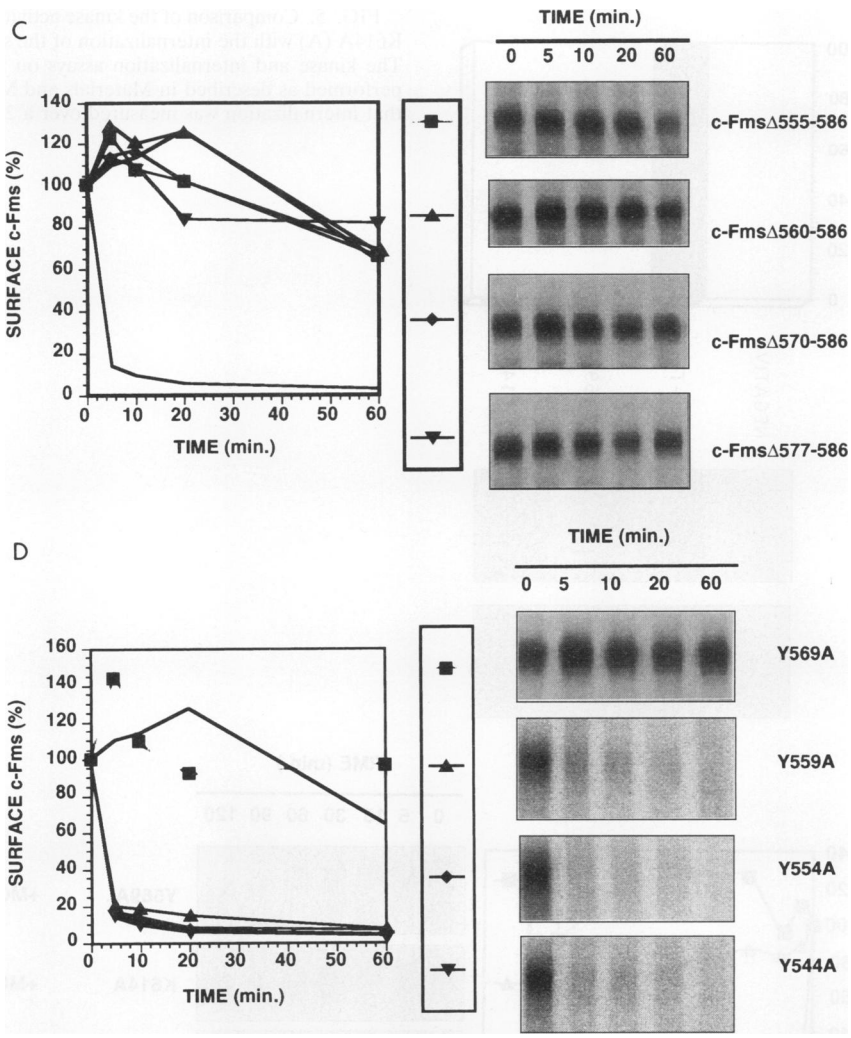

FIG. 4—*Continued.*

sufficient for internalization in the context of the wild-type Fms because a large carboxy-terminal deletion mutant expressing only the juxtamembrane domain in the cytoplasm did not internalize in the absence or presence of M-CSF (data not shown). One possibility regarding the role of the receptor's kinase activity in endocytosis is to alter the conformation of the juxtamembrane domain such that the internalization sequence is recognized by the endocytosis machinery.

Ktistakis et al. (21) have shown that the erythrocyte transmembrane protein glycophorin A internalizes constitutively when valine 106 is substituted with tyrosine. The tyrosine substitution creates a sequence favorable for assuming a tight β-turn structure analogous to that of the LDL receptor internalization sequence. We wished to extend these observations by creating a c-Fms–glycophorin A chimera in which the c-Fms extracellular and transmembrane domains are fused to the glycophorin A cytoplasmic domain (Fig. 8A). In the glycophorin A cytoplasmic domain, we substituted tyrosine for valine 106. We also substituted the QLPY or GNSY sequences corresponding to c-Fms amino acids 566 to 569 or 556 to 559, respectively, or the LDL receptor sequence NPVY for the normal PSDV sequence in glycophorin A. The Y569A equivalent QLPA was created to test the importance of Y-569 in this background.

Internalization was measured as the loss of ^{35}S-labeled receptor from the cell surface at various times after M-CSF addition (Fig. 8B). Consistent with the observations of Ktistakis et al. (21), the c-Fms–glycophorin A chimera did not internalize unless valine 106 was mutated to tyrosine. An equivalent level of internalization activity was obtained when the amino acids corresponding to glycophorin A 103 to 106 (PSDV) were substituted with the QLPY and NPVY sequences from c-Fms and the LDL receptor, respectively. Further, the chimera with the QLPY sequence internalized equally well in the presence or absence of M-CSF. Mutation of tyrosine to alanine in the QLPY sequence abolished this internalization activity. Paradoxically, the GNSY sequence, which we predicted from the mutagenesis studies would not confer internalization proficiency, also resulted in an equivalent level of endocytosis activity. Significantly, however, secondary structure analysis of this sequence predicted that it would fold into a β turn. Therefore, it is plausible that in the glycophorin A sequence context, it is also able to adopt a suitable internalization signal conformation. These results do not prove that the QLPY sequence is the internalization signal for c-Fms but rather indicate that it could, in the correct context, direct internalization.

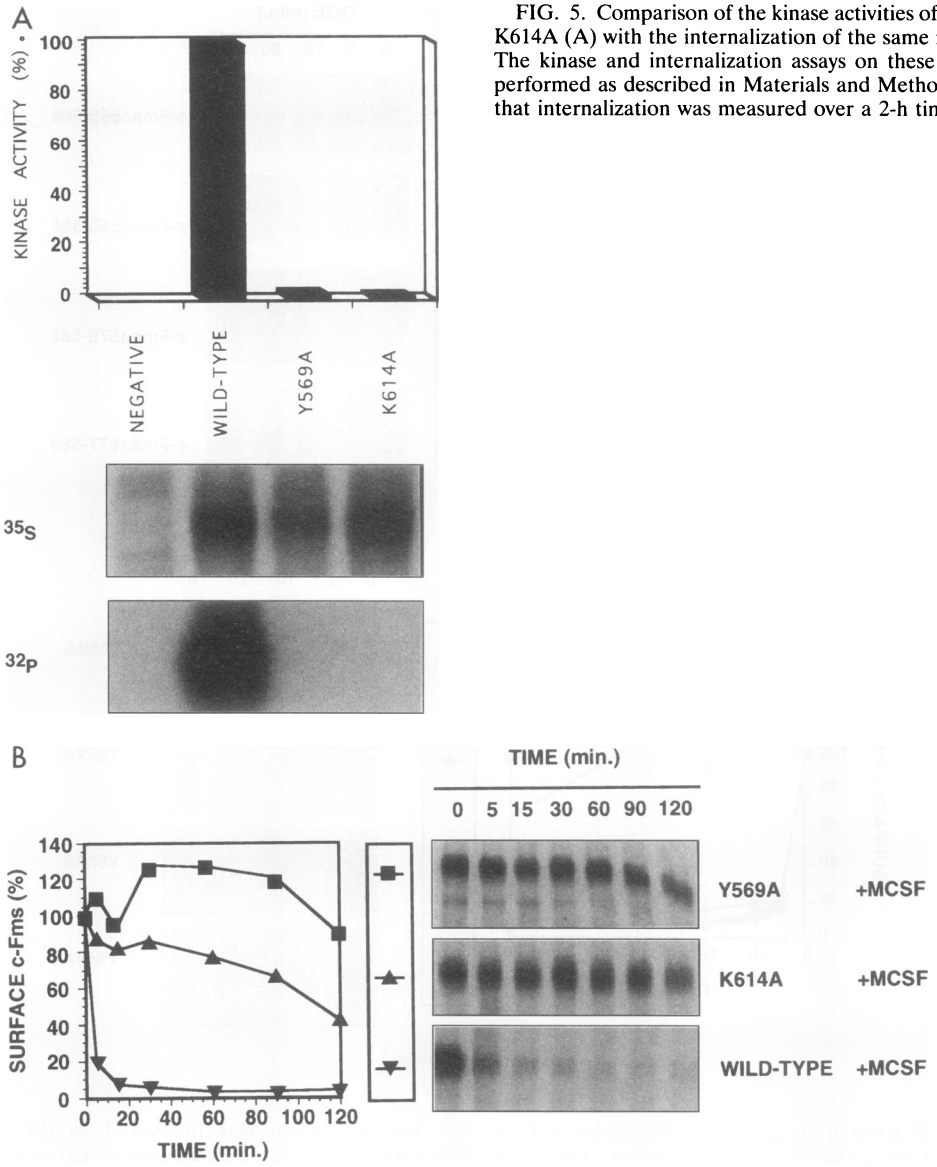

FIG. 5. Comparison of the kinase activities of Fms Y569A and Fms K614A (A) with the internalization of the same mutant receptors (B). The kinase and internalization assays on these c-Fms mutants were performed as described in Materials and Methods with the exception that internalization was measured over a 2-h time course.

DISCUSSION

The receptor signal for endocytosis has been described for receptors which internalize either constitutively or in a ligand-dependent manner. In both types of receptors, a short four- to six-amino-acid sequence localized proximally to the cytoplasmic face of the plasma membrane serves as a signal directing internalization via clathrin-coated pits. These signal sequences adopt a β-turn configuration and have been described structurally for the LDL, lysosomal acid phosphatase, and transferrin receptors (2, 11, 14). An equivalent sequence has been described for the insulin receptor which requires ligand binding for endocytosis. However, an analogous structure has not been described for any of the c-Fms-like receptor tyrosine kinases (i.e., c-Kit, and α and β PDGF, and Flt3/Flk2 receptors). In contrast to these receptors, studies on the EGF and polymeric immunoglobulin receptors have implicated a negatively charged carboxy-terminal tail region as performing an essential function in endocytosis (3, 9). c-Fms has both a negatively charged carboxy terminus and a juxtamembrane region, with sequences in each that could represent internalization signals. Our previous results, however, indicate that a carboxy-terminal deletion mutant internalizes at the same rate as the wild-type receptor (6), and we therefore focused on potential internalization signal sequences within the juxtamembrane region.

Site-directed mutagenesis was used to identify a region within the 50-amino-acid juxtamembrane segment of c-Fms required for its M-CSF-dependent internalization. Four internal deletion mutants, each starting seven amino acids from the transmembrane-juxtamembrane domain junction and removing successive tyrosines toward the carboxy-terminal end of the domain, had decreasing M-CSF-dependent internalization ability paralleling each mutant receptor's in vitro kinase activity. Four additional deletions all started at the juxtamembrane-kinase domain junction and removed successive amino acid segments toward the amino-terminal end of the juxtamembrane domain. These mutations completely abolished both kinase and internalization activities of the receptors. Point

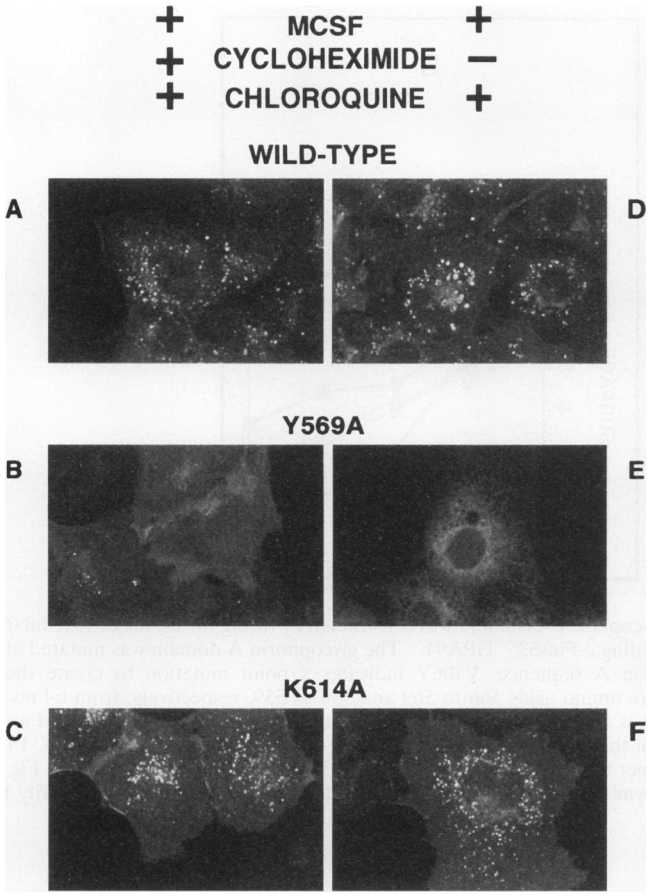

FIG. 6. Internalization of c-Fms wild type, Y569A, and K614A measured by immunofluorescence. Indirect immunofluorescence was used to detect Fms in fixed and permeabilize Rat-2 cells. All samples were treated with chloroquine (3 h prior to M-CSF stimulation) to block transfer of Fms from endosomes to lysosomes for degradation, and samples shown in panels A to C received cycloheximide 6 h before M-CSF treatment (to chase Fms precursors to the cell surface). All cells were stimulated with 2000 U of murine M-CSF per ml 10 min before fixation.

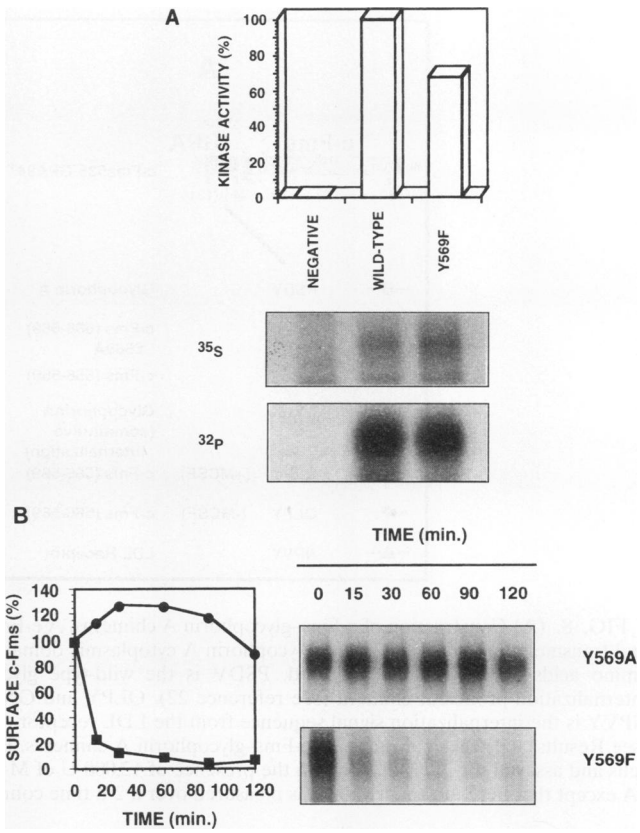

FIG. 7. (A) Kinase activity of Y569F; (B) comparison of the M-CSF-dependent internalization of Y569A and Y569F. The kinase and internalization assays on these c-Fms mutants were performed as described in Materials and Methods with the exception that internalization was measured over a 2-h time course.

mutants Y544A, Y554A, and Y559A within the juxtamembrane domain all had normal internalization as well as wild-type in vitro kinase activities. In contrast, c-Fms Y569A was defective in both of these activities. The amino acid sequence surrounding Y-569 was therefore a candidate internalization signal; however, the receptor kinase activity was also destroyed by changing the tyrosine to alanine.

One interpretation of these results is that the receptor kinase activity is absolutely essential for internalization. This is not the case, however, since previous results have demonstrated that upon M-CSF stimulation, the kinase inactive K614A Fms is internalization competent but is not degraded (7). K614A Fms did internalize at a slower rate than wild-type Fms, suggesting that the receptor kinase activity increases the rate of endocytosis. Our present results confirmed this finding by indirect immunofluorescence analysis and demonstrated that in contrast to K614A Fms, the Y569A Fms was stable on the cell surface after M-CSF stimulation in the same assay. These results suggest that the sequence surrounding amino acid Y-569 of the juxtamembrane region plays a dual role in influencing both internalization and the tyrosine kinase activity of the cytoplasmic domain.

Alignment of kinase domain sequences from 65 protein kinases (38 protein serine/threonine kinases and 27 protein tyrosine kinases) has demonstrated that the first highly conserved residue among all 65 kinases corresponds to L-586 of the murine c-Fms sequence (17). It was surprising to find, therefore, that deletions in c-Fms amino terminal to the juxtamembrane-kinase domain junction had a profound effect on receptor kinase activity. Specifically, c-Fms Δ544–550 had only 30% of the wild-type in vitro kinase activity, while activities of Δ544–555 and Δ544–565 had only 6 and 4%, respectively, of wild-type in vitro kinase activity, and Δ544–576 had no detectable kinase activity. Thus, while the juxtamembrane domain is not strictly speaking part of the kinase domain, these two domains must be closely interconnected. Mutations in the juxtamembrane region affect kinase activity, and conversely, mutations in the kinase domain affect internalization.

One can imagine a functional role of the kinase domain in regulating the accessibility to the internalization signal within the juxtamembrane region. Unlike the transferrin and LDL receptors, Fms accumulates on the cell surface and does not rapidly internalize until stimulated with M-CSF. Therefore, the ligand-dependent internalization signal must be nonfunctional, or cryptic, in the unstimulated receptor molecule. M-CSF stimulation could induce a conformational change in the cytoplasmic juxtamembrane domain such that a cryptic internalization signal is exposed and recognized by the internaliza-

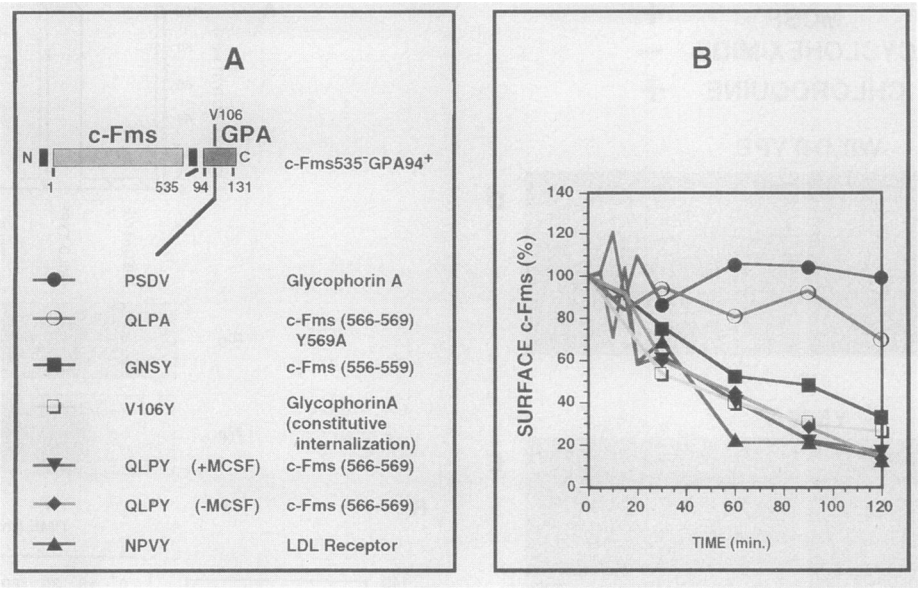

FIG. 8. (A) Construction of c-Fms–glycophorin A chimeras. A c-Fms–glycophorin A chimera was constructed by fusing the c-Fms extracellular and transmembrane domains to the glycophorin A cytoplasmic domain, creating c-Fms535$^-$GPA94$^+$. The glycophorin A domain was mutated at amino acids 103 to 106 as indicated. PSDV is the wild-type glycophorin A sequence. V106Y indicates a point mutation to create the internalization-proficient chimera (see reference 22). QLPY and GNSY are amino acids 566 to 569 and 556 to 559, respectively, from c-Fms; NPVY is the internalization signal sequence from the LDL receptor. QLPA is equivalent to the Y569A mutation described for full-length c-Fms (see Results). (B) Internalization of c-Fms–glycophorin A chimeras. Each of the c-Fms–glycophorin A chimeras was stably expressed in FDC-P1 cells and assayed for internalization in the presence of 12,000 U of M-CSF per ml (except for QLPY −M-CSF) as described in the legend to Fig. 4A except that here internalization was measured over a 2-h time course. (Symbols for the <30-min time points have been eliminated for clarity.)

tion machinery, presumably the adaptor molecules that couple the receptor to the clathrin-coated pits for endocytosis (15, 35). This mechanism seems plausible to prevent internalization of unstimulated receptors. M-CSF binding would induce either dimerization and/or another conformational change revealing the internalization signal sequence. The conformational change could be mediated by the receptor dimerization event or by the receptor tyrosine kinase activity. The existence of a cryptic internalization signal is supported by the observation that c-Fms does not internalize rapidly in the absence of M-CSF. Further, a c-Fms mutant truncated at the beginning of the kinase domain (amino acid 586) does not internalize in the presence or absence of M-CSF (unpublished observation). However, when sequences from the juxtamembrane region are inserted into a favorable background (within the glycophorin A cytoplasmic domain), they can serve as an internalization signal.

Several roles for receptor kinase activity in facilitating endocytosis are possible. Previous data with the kinase inactive Fms K614A from this laboratory suggest that tyrosine kinase activity per se is not required for endocytosis (7); however, the kinase inactivating mutation itself could have caused a conformational change affecting the recognition of the internalization signal. Fms K614A did internalize, but its rate was diminished compared with the wild-type Fms rate. Therefore, the kinase activity is not absolutely essential, but autophosphorylation of tyrosines within the cytoplasmic domain could induce a more favorable conformation for endocytosis, perhaps by permitting the β-turn configuration around Y-569. Such a kinase-dependent conformational change has been detected in the cytoplasmic domain of the PDGF receptor after PDGF binding (20). Autophosphorylation also could create binding sites on Fms for internalization molecules. Sorkin and Carpenter (44a) recently reported that EGF receptor autophosphorylation is necessary but not sufficient for association with α-adaptins and coated-pit assembly. Another possible function for the Fms kinase activity in facilitating endocytosis is that Fms could phosphorylate and activate a molecular component of the endocytic machinery. This role in endocytosis has not been described for Fms; however, the recent demonstration that the EGF receptor can phosphorylate the clathrin light-chain LCa suggests that this is a possibility (33).

Overall, our results complement studies by others (4, 43), who have reported internalization of the kinase inactive insulin receptor, and Sorkin et al. (46), who found that the kinase activity of the β PDGF receptor promoted its internalization activity although it was not absolutely required. Also, autophosphorylation of the EGF receptor at three tyrosine residues within the carboxy-terminal tail reduced but did not abolish internalization of these receptors (18, 45). Thus, an emerging view is that the kinase activity plays a regulatory role in receptor endocytosis. Interestingly, alignment of the c-Fms, c-Kit, and α and β PDGF receptor juxtamembrane sequences reveals an absolute conservation of the QLPY sequence in these four receptors (Fig. 9).

One of our interests in studying c-Fms internalization is to assess the potential role of receptor endocytosis in modulating specific elements of the signal transduction cascade. The experiments described here represent the initial steps in determining the role of signals transmitted with or without Fms endocytosis, and additional mutations in the region of Y-569 will be produced in an attempt to obtain receptors defective in internalization only.

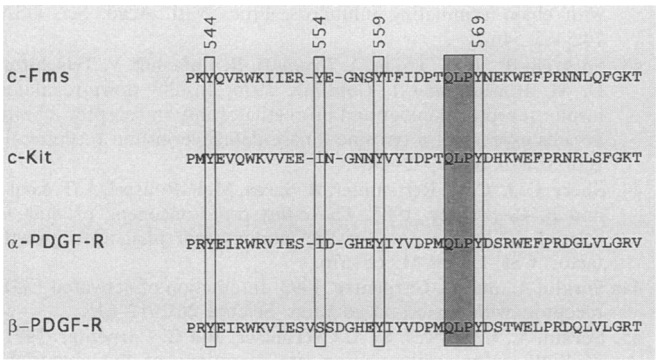

FIG. 9. Alignment of the juxtamembrane domain amino acid sequences of c-Fms, c-Kit, and the α and β PDGF receptors. The clear boxes indicate the four juxtamembrane domain tyrosines studied in this report. The shaded box indicates the three-amino-acid sequence QLP that together with Y-569 is entirely conserved in these four receptors.

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