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The role of kinase activity and the kinase insert region in ligand-induced internalization and degradation of the c-*fms* protein

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Molecular steps in endocytosis and degradation of the c-*fms* protein were analyzed by following the fate of mutated c-*fms* molecules after M-CSF binding. A mutant c-*fms* protein lacking tyrosine kinase activity was rapidly internalized after M-CSF binding but not degraded. Another mutant c-*fms* molecule that lacked most of the kinase insert region was similarly internalized after M-CSF binding and also not degraded. This indicates that the signal for internalization is separate from that directing degradation of the receptor. It has been shown previously that a c-*fms* mutant in which the kinase insert domain is deleted retains tyrosine kinase activity but lacks two major sites of autophosphorylation. The degradation step therefore requires both kinase activity and the kinase insert region whereas the internalization step is independent of these factors. The major sites of tyrosine autophosphorylation within the kinase insert region were next mutated to determine whether autophosphorylation in the kinase insert region of c-*fms* might be the signal that triggers degradation of internalized receptors. These mutant receptors were still rapidly degraded in response to M-CSF. Therefore, ligand-induced degradation of c-*fms* may require tyrosine phosphorylation of a protein other than the c-*fms* receptor itself and the kinase insert region may be necessary for recognition of this substrate.

Key words: c-*fms* proto-oncogene/M-CSF/M-CSF receptor/receptor mediated endocytosis/tyrosine phosphorylation

Introduction

The c-*fms* proto-oncogene encodes the receptor for macrophage colony stimulating factor (M-CSF or CSF-1) (Sherr *et al.*, 1985) and is expressed primarily in the monocyte/macrophage lineage of hematopoietic cell development but also in trophoblast cells of the developing embryo and placenta (Byrne *et al.*, 1981; Stanley *et al.*, 1983; Müller *et al.*, 1983; Regenstreif and Rossant, 1989). The c-*fms* protein is a member of a class of growth factor receptors that contain a cytoplasmic domain with tyrosine-specific protein kinase activity. The kinase domain of c-*fms* is interrupted by a sequence of ~70 amino acids (the kinase insert domain) (Coussens *et al.*, 1986; Rothwell and Rohrschneider, 1987; Woolford *et al.*, 1988) that is not required for kinase activity (Taylor *et al.*, 1989; Shurtleff *et al.*, 1990; Reedijk *et al.*, 1990). This interrupted kinase domain defines a subclass of receptor tyrosine kinases that includes the PDGF receptors and the c-*kit* protein, with the FGF-type receptors and the *torso* gene product from *Drosophila* (Sprenger *et al.*, 1989) showing more distant relatedness (reviewed in Ullrich and Schlessinger, 1990). Two other subclasses of tyrosine kinase-containing growth factor receptors exhibit distinct structural features and are represented by the epidermal growth factor (EGF) receptor and the insulin receptor.

The tyrosine kinase class of growth factor receptors educe signals for growth through ligand-dependent activation of the cytoplasmic tyrosine kinase domain. Binding of ligand to the extracellular portion of the receptor results in the rapid tyrosine phosphorylation of a number of cellular proteins, including the receptor itself (Ullrich and Schlessinger, 1990). Ligand binding also results in the rapid loss of growth factor receptors from the cell surface. This receptor downregulation may provide a mechanism for attenuation of signals for proliferation (Wells *et al.*, 1990). The mechanism of receptor downregulation has been more closely studied in the receptors for insulin and for EGF. After EGF binds, the EGF receptors cluster in clathrin coated pits, are rapidly internalized, transported to lysosomes, and degraded with a half-time for degradation of 40–50 min (Carpenter and Cohen, 1976, 1979; Schlessinger, 1986). Kinase-inactive mutant EGF receptors are internalized after ligand binding, but not degraded (Honegger *et al.*, 1990). Instead, these mutant receptors are recycled to the cell surface (Felder *et al.*, 1990; Honegger *et al.*, 1987). Although EGF receptor kinase activity is required for degradation, the receptor itself does not need to be phosphorylated on tyrosine (Honegger *et al.*, 1988; Chen *et al.*, 1989). The insulin receptor is rapidly internalized after ligand binding, but unlike the EGF receptor, the majority of insulin receptors recycle intact to the cell surface (Ey *et al.*, 1978). Efficient internalization of the insulin receptor requires the receptor tyrosine kinase activity (McClain *et al.*, 1987; Russell *et al.*, 1987), but not receptor autophosphorylation (Backer *et al.*, 1989). The cellular trafficking of these two receptors is therefore regulated somewhat differently, but the mechanisms by which a cell determines whether to internalize, degrade, or recycle a particular receptor are incompletely understood.

Previous studies with the c-*fms* protein have shown that in the absence of M-CSF, this receptor is relatively stable and turns over with a half-life of 3–4 h (Rettenmier *et al.*, 1987). Upon M-CSF binding, the receptor is rapidly internalized via clathrin coated pits and vesicles (Manger *et al.*, 1984) and targeted to lysosomes where both the receptor and ligand are degraded (Guilbert and Stanley, 1986). The kinase activity of the receptor is necessary for its eventual degradation in the lysosome since it has been shown that a kinase-inactive mutant of human c-*fms* is not degraded after M-CSF stimulation (Downing *et al.*, 1989). However, it is not known whether the kinase activity is necessary for

internalization of the c-*fms* protein. Nor has it been determined whether receptor autophosphorylation is the signal for degradation, or whether degradation requires the phosphorylation of another protein by c-*fms*. Therefore, to explore the mechanisms that regulate these steps in the M-CSF receptor, we have examined both the internalization and degradation of normal M-CSF receptors and mutant receptors lacking kinase activity or the kinase insert region.

Results

The c-*fms* protein is normally expressed in cells of the monocyte/macrophage lineage where it mediates the M-CSF-dependent growth and differentiation of these cells (Metcalf, 1984). In order to study the normal functions of the receptor in a relatively natural context, we have introduced murine c-*fms* genes into the factor-dependent murine myeloid cell line FDC-P1. These undifferentiated cells do not express an endogenous c-*fms* gene, but when infected with a retroviral vector containing murine c-*fms*, the cells acquire the ability to proliferate and to differentiate in response to murine M-CSF (Rohrschneider and Metcalf, 1989). Wild type or mutant c-*fms* genes were introduced into FDC-P1 cells by cocultivating the FDC-P1 cells with ψ-2 packaging cells expressing transfected c-*fms* genes (Rohrschneider and Metcalf, 1989). FDC-P1 cells expressing high levels of c-*fms* were selected by cell sorting using an antibody to the external domain of the c-*fms* protein.

FDC-P1 cells expressing the c-*fms* protein were metabolically labeled for 2 h in the absence of M-CSF to obtain a high concentration of labeled receptors on the cell surface. The cells were then stimulated with a saturating concentration of murine M-CSF, and aliquots of cells were removed at intervals and assayed for internalization or for degradation of the c-*fms* protein. Internalization was measured by immunoprecipitation of receptors remaining on the surface of intact cells at various times after M-CSF addition, using polyclonal antibodies directed against the extracellular domain of the c-*fms* protein. Binding of M-CSF to c-*fms* on intact cells was found to interfere somewhat with antibody binding. To compensate for this effect, '0 min' samples were not stimulated with M-CSF at 37°C but treated with M-CSF on ice in the presence of sodium azide prior to addition of the primary antiserum. Therefore at all time points the M-CSF was bound to the receptor before the antibody.

Degradation was measured by immunoprecipitation of the total population of labeled receptors from cell lysates at time points after M-CSF addition. M-CSF did not interfere with binding of the primary antiserum to the c-*fms* protein in the detergent lysates. Very similar results were obtained regardless of whether immunoprecipitations of cell lysates were performed using polyclonal antibodies against the cytoplasmic domain of c-*fms* or against the extracellular domain.

In the absence of M-CSF, the wild type murine c-*fms* protein was stably expressed on the cell surface of FDC-P1 cells with a half-life of >2 h. Addition of saturating levels

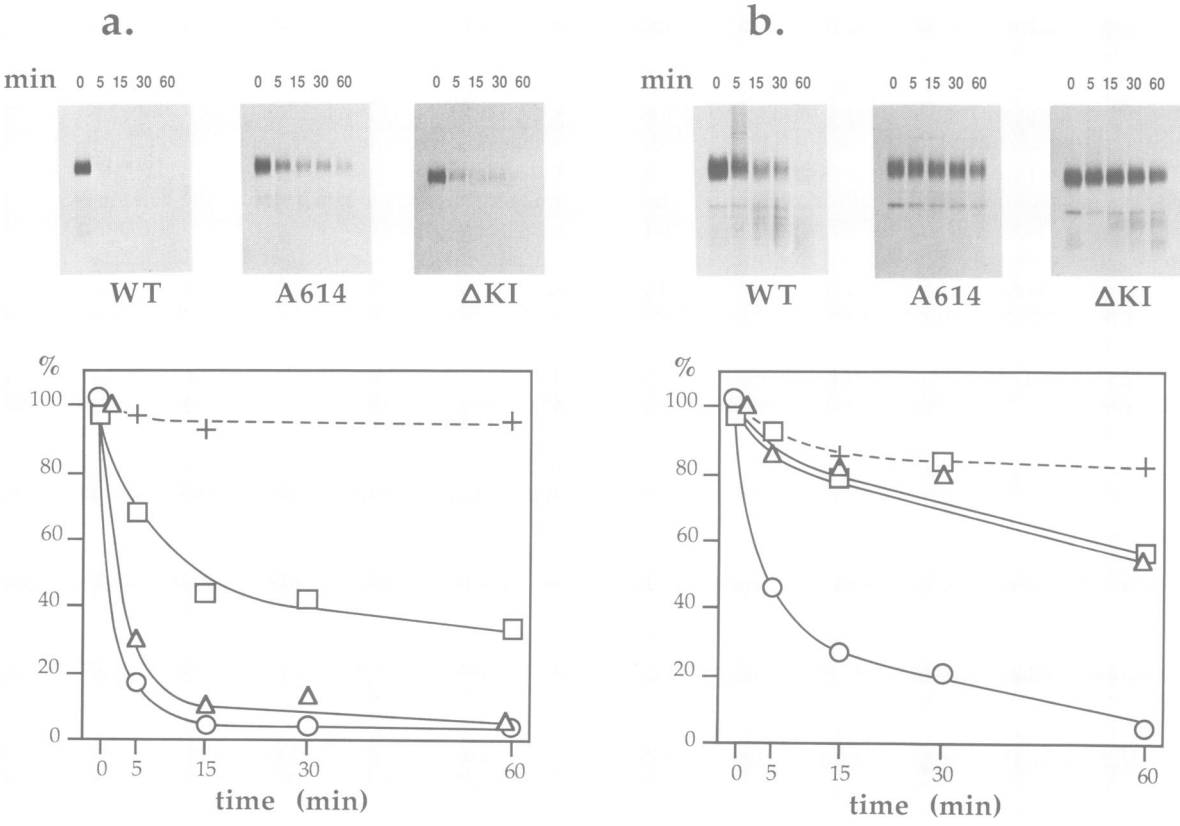

Fig. 1. M-CSF-stimulated internalization (**a**) and degradation (**b**) of c-*fms* wt, c-*fms*A614, and c-*fms*ΔKI in FDC-P1 cells. A graph showing the relative amount of receptor at each time point after M-CSF addition was generated by densitometric scanning of the 165 kd c-*fms* bands in the autoradiographs shown. The rate of receptor loss in the absence of ligand (+) was not significantly different for wild type and mutant c-*fms*, and is shown as the average value for all three receptors. Each experiment was performed at least three times with very similar results. (○) c-*fms* wt, (□) c-*fms*A614, (△) c-*fms*ΔKI.

of M-CSF to the medium caused the rapid internalization and degradation of the receptors (Figure 1). The half-time for degradation of the protein was decreased to ~5 min in the presence of M-CSF (Figure 1b). This result is very similar to that obtained for the wild type human c-*fms* protein in NIH 3T3 cells (Downing *et al.*, 1989) or in a macrophage cell line (Downing *et al.*, 1988).

A kinase-defective c-*fms* mutant was generated by replacing lysine 614, which forms part of the ATP-binding site in the kinase domain of murine c-*fms*, with alanine. Cells expressing c-*fms*A614 produced a 165 kd receptor that had no detectable tyrosine kinase activity (unpublished data). As shown previously for a similar human c-*fms* mutant, the rates of synthesis and degradation of the kinase-defective receptors were identical to that of the wild type receptor in the absence of exogenous ligand (Downing *et al.*, 1989, and our unpublished data). As shown for the human kinase-defective mutant in NIH 3T3 cells (Downing *et al.*, 1989), the murine c-*fms*A614 receptor in FDC-P1 cells was resistant to degradation after ligand binding (Figure 1b). The half-time for degradation of c-*fms*A614 in FDC-P1 cells was 70–90 min in the presence of M-CSF. However, this mutant receptor was still fairly rapidly internalized in the presence of M-CSF (Figure 1a). Thus, in FDC-P1 cells, internalization of ligand-bound receptors occurred in the absence of receptor kinase activity, but degradation of internalized receptors (or targeting of internalized receptors to the lysosome) required either tyrosine phosphorylation of the receptor or tyrosine phosphorylation of another protein by c-*fms*.

A murine c-*fms* protein lacking the kinase insert domain (c-*fms*ΔKI) has been characterized in NIH 3T3 cells (Taylor *et al.*, 1989). This receptor has tyrosine kinase activity and can transform NIH 3T3 cells. The c-*fms*ΔKI receptor, when introduced into FDC-P1 cells, stimulated M-CSF-dependent proliferation and must therefore be able to phosphorylate at least some of the normal cellular substrates (unpublished data). In FDC-P1 cells, c-*fms*ΔKI was rapidly internalized in response to M-CSF (Figure 1a), but this receptor was as deficient in ligand-stimulated degradation as the kinase-defective mutant c-*fms*A614 (Figure 1b). A pulse–chase experiment demonstrated that the apparent difference in degradation rates in FDC-P1 cells was not due to differences in the rates of receptor synthesis or processing between wild type and ΔKI receptors (Figure 2).

To determine whether the defect in ligand-induced degradation of these c-*fms* mutants was limited to FDC-P1 cells, wild type and mutant c-*fms* genes were also introduced into rat-2 fibroblasts. Internalization and degradation of the c-*fms* mutant receptors in rat-2 cells were assayed by immunoprecipitation of proteins from unlabeled cells, followed by immunoblotting. As in FDC-P1 cells, both receptors specified by the kinase-defective mutant c-*fms*A614 and the kinase-active mutant c-*fms*ΔKI were internalized after ligand binding, but were not degraded (Figure 3). Very similar results were obtained when c-*fms* proteins were detected by [^{35}S]methionine labeling rather than immunoblotting. These results in FDC-P1 cells and in rat-2 cells demonstrated that internalization and degradation required two distinct and separate steps. Kinase activity was not needed for internalization and, although it was necessary for ligand-stimulated degradation, receptor kinase activity *per se* was not sufficient for this degradation.

Two of the major sites of tyrosine autophosphorylation in c-*fms* (tyr 697 and tyr 706) lie within the kinase insert region that has been deleted from c-*fms*ΔKI (Tapley *et al.*, 1990). The third identified site, tyr 807, is within the kinase domain, in a position homologous to tyr 416 of the *src* protein. Since the c-*fms*ΔKI receptor has tyrosine kinase activity towards exogenous substrates, but lacks two major sites of autophosphorylation, it seemed possible that phosphorylation of the c-*fms* protein at one or both of these tyrosines might be the signal that triggers degradation of internalized receptors. Alternatively, the c-*fms*ΔKI receptor might be unable to phosphorylate a cellular protein that mediates ligand-stimulated degradation.

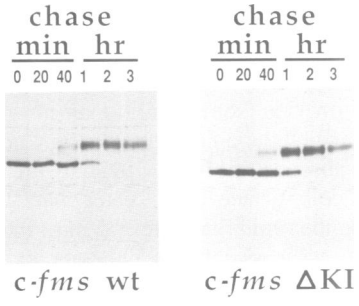

Fig. 2. Rate of synthesis and processing of c-*fms* wt and c-*fms*ΔKI in FDC-P1 cells. Cells were labeled for 20 min with [^{35}S]methionine, and then chased with excess unlabeled methionine for 0–3 h, prior to lysis and immunoprecipitation.

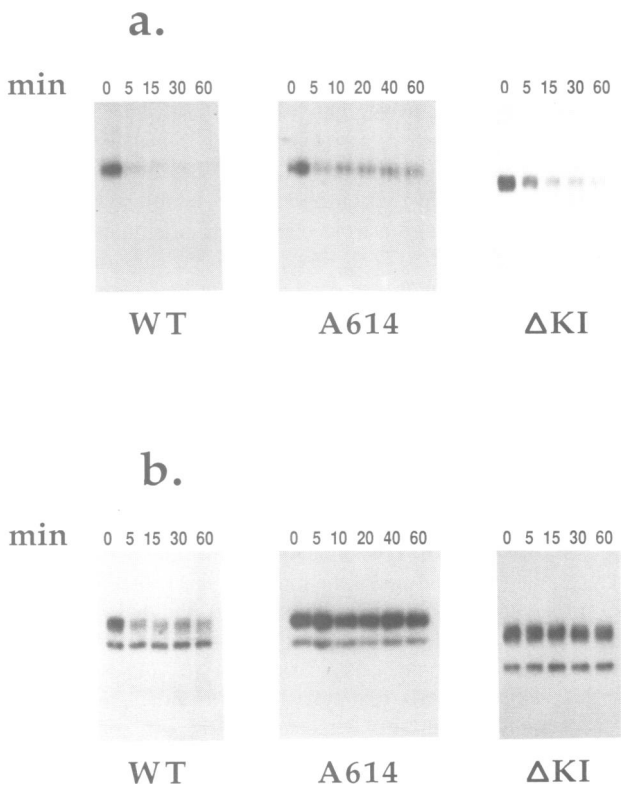

Fig. 3. M-CSF-stimulated internalization (**a**) and degradation (**b**) of c-*fms* wt, c-*fms*A614, and c-*fms*ΔKI in rat-2 cells. The autoradiographs show the amount of cell surface (a) or total (b) cellular receptor remaining at time points after M-CSF addition. Each experiment was performed twice with similar results.

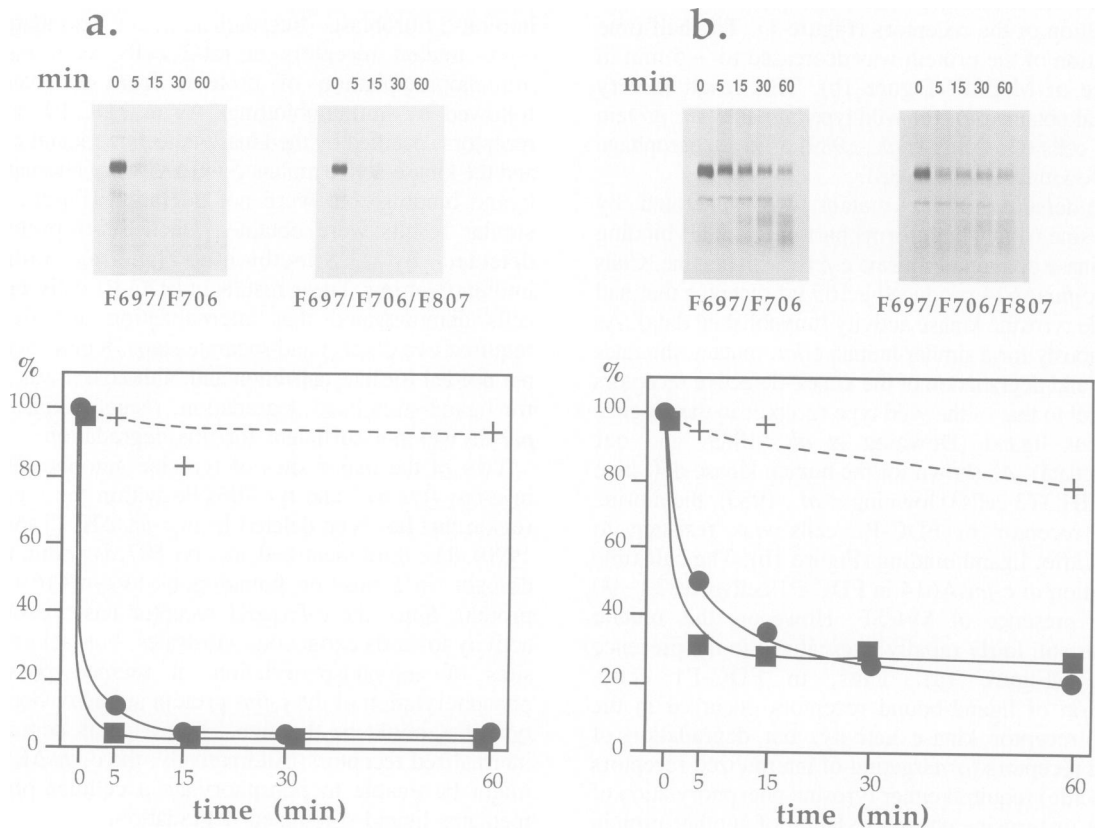

Fig. 4. M-CSF-stimulated internalization (**a**) and degradation (**b**) of c-*fms*F697/F706 and c-*fms*F697/F706/F807 in FDC-P1 cells. A graph showing the relative amount of receptor remaining at each time point after M-CSF addition (from densitometric scanning of the above autoradiographs) is shown. The loss of receptor in the absence of ligand (+) is shown as the average of the two receptors. These experiments were performed twice with very similar results. (●) c-*fms*F697/F706, (■) c-*fms*F697/F706/F807.

To distinguish between these possibilities, oligonucleotide-directed mutagenesis was used to alter the identified sites of tyrosine autophosphorylation of c-*fms*. In c-*fms*F697/F706, the two autophosphorylatable tyrosines in the kinase insert region were changed to phenylalanines. In c-*fms*F697/F706/F807, all three identified sites of tyrosine phosphorylation were altered. Receptors from both c-*fms*F697/F706 and c-*fms*F697/F706/F807 phosphorylated cellular proteins and stimulated M-CSF-dependent proliferation of FDC-P1 cells (P.Tapley, in preparation). When these mutant receptors were tested for M-CSF-dependent receptor degradation, both double and triple mutant receptors were internalized and degraded with the same kinetics as the wild type receptor (Figure 4). Thus, ligand-induced receptor degradation does not require phosphorylation of tyrosines 697, 706 or 807. Tryptic phosphopeptide analysis of the mutant c-*fms* proteins in FDC-P1 cells detected only two additional sites and these contained only a fraction of the phosphotyrosine found in the three major sites at tyrosines 697, 706, and 807 (Tapley *et al.*, 1990). This suggests that tyrosine phosphorylation of the receptor itself is not necessary for ligand-induced degradation. Rather, phosphorylation of some exogenous substrate by c-*fms* may be required to trigger degradation of internalized receptors. The c-*fms*ΔKI receptor, although it has kinase activity, may be unable to recognize or interact with some substrate that targets the internalized receptors to lysosomes.

A kinase-defective EGF receptor is also internalized, but not degraded after ligand stimulation. Instead, this mutant is rapidly recycled to the cell surface (Honegger *et al.*, 1987). We therefore investigated whether wild type and mutant c-*fms* proteins are recycled after internalization. To look for receptor recycling, FDC-P1 cells expressing wild type c-*fms* or c-*fms*ΔKI receptors were stimulated with saturating levels of M-CSF to allow receptor internalization, and then washed to remove M-CSF from the medium. In the absence of M-CSF, any recycled receptors should accumulate on the cell surface. As shown in Figure 5, both the wild type c-*fms* and c-*fms*ΔKI proteins were rapidly cleared from the cell surface after M-CSF stimulation. The c-*fms*ΔKI receptors rapidly reappeared on the cell surface when exogenous M-CSF was removed, whereas the wild type receptors did not.

Discussion

These results provide some additional insight into the mechanism by which the c-*fms* protein is downregulated by M-CSF. In the absence of M-CSF, the half-life of the c-*fms* protein on the cell surface was greater than 2 h. M-CSF binding induced the rapid (half-life ≈5 min) internalization and degradation of the receptor-ligand complex. Our results show that a kinase-inactive receptor (c-*fms*A614) was also internalized upon ligand binding, although the rate of internalization of this mutant receptor was somewhat slower than that of the wild type receptor. The reason for this difference in rate is unknown. A kinase-inactive EGF

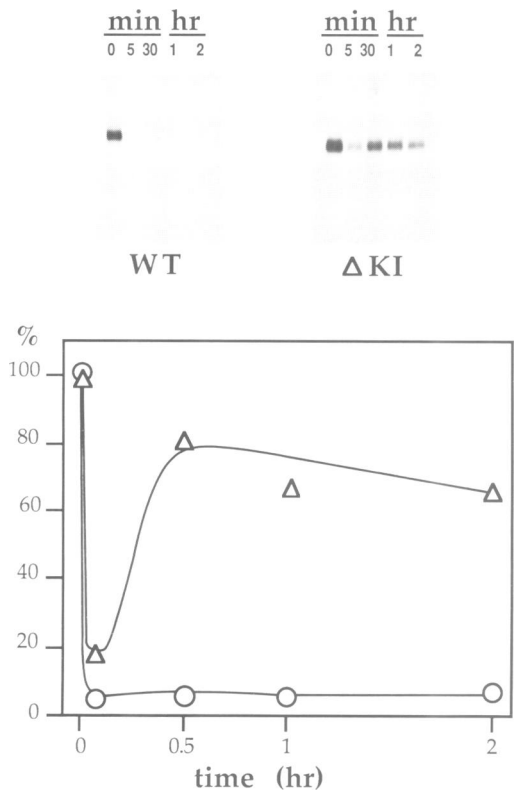

Fig. 5. Recycling of receptors c-*fms* wt and c-*fms*ΔKI to the cell surface after internalization in FDC-P1 cells. A graph showing the relative amount of receptor at each time point plotted against incubation time with M-CSF is shown. The experiment was repeated three times with similar results. (○) c-*fms* wt, (△) c-*fms*ΔKI. The amount of c-*fms* protein in each lane was quantitated by densitometric scanning of the autoradiographs. The values shown have been normalized to the amount of labeled receptor present at each time point in the absence of M-CSF.

receptor is also internalized somewhat more slowly than the kinase-active receptor (Felder *et al.*, 1990). The kinase activity of these receptors may influence, perhaps indirectly, the rate of internalization. Nevertheless, our data indicate that the binding of M-CSF to the external domain was sufficient to induce fairly rapid receptor internalization, and receptor tyrosine kinase activity was not required for this step.

In contrast, degradation of internalized receptors was shown to require both the kinase activity of the receptor and the kinase insert domain. The requirement for receptor kinase activity could mean either that receptor autophosphorylation is a necessary signal for degradation, or that phosphorylation of another cellular protein on tyrosine is necessary for degradation. A c-*fms* mutant receptor that has tyrosine kinase activity but lacks the kinase insert domain was also resistant to degradation after M-CSF treatment. Because two major sites of tyrosine autophosphorylation (Y697 and Y706) lie within the kinase insert domain that is deleted in this mutant (Tapley *et al.*, 1990), it seemed possible that phosphorylation of one or both of these sites could be the necessary signal for targeting receptors to the lysosomes. However, when mutant receptors lacking two or three major sites of autophosphorylation (c-*fms*F697/F706 and c-*fms*-F697/F706/F807) were tested, these mutant receptors were internalized and degraded as efficiently as the wild type receptor. Although degradation of the c-*fms* protein does not require autophosphorylation at tyrosines 697, 706 or 807, it remains possible that phosphorylation at another minor site could be a necessary signal for receptor degradation. However, since the c-*fms*F697/F706/F807 receptor in FDC-P1 cells contains only a fraction of the phosphotyrosine found in the wild type receptor (unpublished data) and yet is degraded just as rapidly, it seems most likely that degradation of internalized receptors does not require phosphorylation of the receptor itself. The receptor kinase activity would then be necessary for phosphorylation of one or more cellular proteins.

The pattern of internalization and degradation of the c-*fms* protein appears very similar to that of the EGF receptor, a structurally distinct member of the tyrosine kinase class of growth factor receptors. Kinase-active and kinase-inactive EGF receptors are both internalized after ligand binding (Honegger *et al.*, 1987) and then sorted into different endosomal compartments in multivesicular bodies (Felder *et al.*, 1990). The kinase-defective EGF receptors recycle to the cell surface, while the kinase-active receptors are targeted to lysosomes and degraded (Honegger *et al.*, 1990; Felder *et al.*, 1990). Although degradation of the EGF receptor requires the receptor kinase activity (Honegger *et al*, 1987), degradation does not require tyrosine phosphorylation of the EGF receptor itself (Honegger *et al*, 1988; Chen *et al*, 1989). Degradation of the PDGF receptor is also independent of receptor autophosphorylation (A.Kazlauskas, personal communication). It is possible therefore that degradation of all of these receptors requires the *trans*-phosphorylation of a common cellular component. Comparison of the cellular proteins phosphorylated by the wild type and ΔKI c-*fms* proteins may help identify the substrates involved in degradation of the receptor.

It has been shown that a kinase-defective c-*fms* mutant receptor can be phosphorylated in *trans* by a kinase-active c-*fms* protein (Ohtsuka *et al.*, 1990). When kinase-defective and kinase-active c-*fms* proteins are co-expressed, the degradation of the kinase-defective receptors is somewhat accelerated (Ohtsuka *et al.*, 1990). This result is consistent with either of two mechanisms for ligand-induced receptor degradation. *Trans*-phosphorylation of the kinase-defective receptor could signal its degradation, or movement of the kinase-defective receptor into a complex with the enzymatically competent receptor could facilitate its degradation. Our results suggest that the latter mechanism is more likely. Phosphorylation of an unknown cellular protein by the kinase-active receptors may therefore induce degradation of both active and inactive receptors.

The degradation of M-CSF-stimulated c-*fms* proteins required the kinase insert region in addition to the kinase activity. There are at least two possible interpretations for the requirement of this region in the degradation step. The deletion of the kinase insert region from the c-*fms* molecule could leave the remainder of the kinase domain with a conformation that was either resistant to proteolysis or resistant to mechanisms that transfer these molecules to lysosomes. Although we cannot exclude this possibility, the majority of data indicate that the c-*fms*ΔKI receptors are surprisingly normal in most respects (Taylor *et al.*, 1989; Shurtleff *et al.*, 1990). The tyrosine kinase activity is retained and the ΔKI receptors still stimulate mitogenesis and induce autocrine transformation, although at a somewhat reduced

level compared to the wild type receptors. A second possibility is that the ΔKI region is needed for substrate recognition of a cellular target molecule that transfers the c-*fms*/M-CSF complex from the multivesicular body to the lysosomes for degradation. The phosphorylation of tyrosines 697 and 706 within the kinase insert region would not be necessary for this interaction because c-*fms*F697/F706 mutant receptors were internalized and degraded with the same efficiency as wild type receptors. The tyrosine phosphorylation of the cellular protein, however, would be needed for the transfer to lysosomes and degradation. This latter mechanism would be similar to that reported for the EGF receptor, but involving a potential role for the kinase insert region.

Materials and methods

Materials

Murine M-CSF was partially purified as previously described (Rohrschneider *et al.*, 1989) from supernatants of yeast cells expressing a recombinant M-CSF gene. Rat polyclonal antibodies #322 and #323 to the extracellular domain of the murine c-*fms* protein, and rabbit polyclonal antibody #4599B to the cytoplasmic domain of c-*fms* were prepared as described in Rohrschneider *et al.* (1989).

c-fms mutants

Murine c-*fms*ΔKI was kindly provided by G.Taylor and M.Reedijk (Taylor *et al.*, 1989). The kinase-defective mutant c-*fms*A614 was made by site-directed mutagenesis using the method of Zoller and Smith (1984), as previously described (Tapley *et al.*, 1990). In this mutant, lysine 614, which forms part of the ATP-binding site, was changed to alanine. The phosphorylation site mutants, c-*fms*F697/F706 and c-*fms*F697/F706/F807, were prepared by the same method, changing the autophosphorylatable tyrosines to phenylalanines. The c-*fms* mutant DNA fragments were cloned into the pZEN113 vector and transfected into the ψ-2 packaging cell line as described previously (Tapley *et al.*, 1990).

Cell lines

FDC-P1 cells were maintained in Dulbecco's modified Eagle's medium (DME) supplemented with 10% fetal calf serum (FCS) and 4% WEHI-3B cell-conditioned medium as a source of Multi-CSF. About 36 h prior to assays, FDC-P1 cells were transferred to DME + 10% FCS supplemented with 20% L cell-conditioned medium (a source of M-CSF) to allow expression of c-*fms* proteins. (In the presence of Multi-CSF, c-*fms* proteins are downmodulated. Gliniak and Rohrschneider, 1990.) About 12 h before assays, the cells were transferred to DME + 10% FCS without hematopoietic growth factors, to allow c-*fms* proteins to accumulate on the cell surface (upregulation). Rat-2 cells were maintained in DME + 10% FCS with no hematopoietic growth factors.

Pulse–chase analysis

Cells were incubated overnight in medium without M-CSF to upregulate receptor levels, and transferred to methionine-free medium for 30 min before labeling for 20 min with 200 μCi/ml of [^{35}S]methionine. The cells were washed, and incubated in medium containing excess unlabeled methionine for 0–3 h. Aliquots of cells were removed at various times after labeling, lysed and immunoprecipitated as described above, using a rabbit polyclonal antiserum against the cytoplasmic domain of the c-*fms* protein.

Measurement of receptor internalization and degradation

FDC-P1 cells expressing c-*fms* proteins were incubated overnight in the absence of M-CSF to upregulate receptor levels, and then metabolically labeled for 2 h with 50 μCi/ml of [^{35}S]methionine. The labeled cells were washed and stimulated for 0–60 min at 37°C with 2500 U/ml of M-CSF. Aliquots of untreated and M-CSF-stimulated cells were collected at time points after M-CSF addition and pelleted at 2500 r.p.m. for 1 min. To measure internalization of cell surface receptors, cells were resuspended in ice-cold medium with 0.02% sodium azide, and antiserum #322 was incubated with the intact cells for 1 h on ice. In order to compensate for any inhibition of antibody binding by M-CSF, samples that were not stimulated with M-CSF at 37°C were allowed to bind 2500 U/ml M-CSF on ice in the presence of 0.02% sodium azide for 10 min, prior to incubation with the primary antiserum. M-CSF-stimulated and unstimulated cells were then washed, lysed in RIPAE detergent and immunoprecipitated as previously described (Gentry *et al.*, 1983) using a rabbit anti-rat secondary antibody. To measure degradation of receptors in FDC-P1 cells, aliquots of untreated and M-CSF-stimulated cells were collected at the indicated time points, lysed, and immunoprecipitated with antiserum #322 as described above. During these experiments, the cells remained in the continuous presence of M-CSF, so that newly synthesized receptors or recycling receptors that reached the cell surface bound M-CSF and were rapidly internalized again.

Rat-2 cells expressing c-*fms* were maintained in medium without hematopoietic growth factors and assayed as described above, except that the cells were unlabeled. The c-*fms* proteins were immunoprecipitated with antiserum #322 and detected by immunoblotting using antiserum #4599B as previously described (Gliniak and Rohrschneider,1991).

To measure recycling of c-*fms* proteins to the cell surface after M-CSF stimulation, FDC-P1 cells were labeled with 200 μCi/ml of [^{35}S]methionine for 15 min, washed, and incubated for 90 min at 37°C with medium containing an excess of cold methionine to chase the labeled receptor to the cell surface. The cells were then stimulated with M-CSF as described above, except that after a 5 min incubation with M-CSF to allow receptor internalization, the cells were extensively washed to remove M-CSF from the medium. The cells were then incubated at 37°C and aliquots of cells were removed at the indicated time points. Cell surface receptors were immunoprecipitated as described above, using antiserum #322.

Proteins were separated on 7.5% SDS–polyacrylamide gels, and detected by autoradiography of the dried gels. The c-*fms* protein in each lane was quantitated by whole band analysis using the Visage 2000 video densitometry and analysis system (BioImage Co., Ann Arbor, MI).

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Note added in proof

Tyrosine 721 in the kinase insert domain of c-*fms* lies within a putative consensus sequence for phosphatidylinositol (PtdIns) 3-kinase binding [Cantley *et al.* (1991) *Cell*, **64**, 281–302]. Association of the receptor with PtdIns 3-kinase would presumably require phosphorylation of this tyrosine. Although tyrosine 721 has not been identified as a major site of autophosphorylation in c-*fms*, we cannot exclude the possibility that phosphorylation of this tyrosine occurs and is necessary for degradation. We are currently testing this possibility by further site-directed mutagenesis.