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Ligand-Induced Phosphorylation of the Colony-Stimulating Factor 1 Receptor Can Occur through an Intermolecular Reaction That Triggers Receptor Down Modulation

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Ligand-induced tyrosine phosphorylation of the human colony-stimulating factor 1 receptor (CSF-1R) could involve either an intra- or intermolecular mechanism. We therefore examined the ability of a CSF-1R carboxy-terminal truncation mutant to phosphorylate a kinase-defective receptor, CSF-1R[met 616], that contains a methionine-for-lysine substitution at its ATP-binding site. By using an antipeptide serum that specifically reacts with epitopes deleted from the enzymatically competent truncation mutant, cross-phosphorylation of CSF-1R[met 616] on tyrosine was demonstrated, both in immune-complex kinase reactions and in intact cells stimulated with CSF-1. Both in vitro and in vivo, CSF-1R[met 616] was phosphorylated on tryptic peptides identical to those derived from wild-type CSF-1R, suggesting that receptor phosphorylation on tyrosine can proceed via an intermolecular interaction between receptor monomers. When expressed alone, CSF-1R[met 616] did not undergo ligand-induced down modulation, but its phosphorylation in cells coexpressing the kinase-active truncation mutant accelerated its degradation.

The binding of human colony-stimulating factor 1 (CSF-1) to the extracellular segment of its receptor (CSF-1R) results in a transmembrane signal that stimulates the intrinsic tyrosine kinase activity of the intracellular receptor domain (for a recent review, see reference 37a). The transduction of extracellular signals through this mechanism triggers phosphorylation of the receptor on tyrosine and leads to the phosphorylation of cellular substrates, some of which relay mitogenic signals to the cell nucleus. Mutated oncogenic forms of CSF-1R provide sustained signals for cell growth in the absence of ligand (34, 34a, 35, 41), demonstrating that CSF-1 per se is not required for mitogenic activity. In contrast, a kinase-defective human CSF-1R mutant (CSF-1R[met 616]), containing a methionine-for-lysine substitution at its ATP-binding site, lacks tyrosine kinase activity and fails to stimulate cell growth, even though it binds CSF-1 with high affinity (11). Therefore, activation of the receptor kinase, either by the ligand or through critical mutations, is required for mitogenesis.

Ligand-stimulated phosphorylation of CSF-1R on tyrosine could potentially result from an intramolecular conformational change that activates the kinase in *cis* or, alternatively, from CSF-1-induced receptor aggregation and *trans*-phosphorylation of receptor subunits. The latter mechanism may be more likely, since experiments performed with the epidermal growth factor receptor (EGF-R) and the B-type platelet-derived growth factor receptor (PDGF-R) revealed that ligand binding induces receptor dimerization, leading to tyrosine phosphorylation of the receptors and enhanced kinase activity (5, 19, 42, 43). We therefore examined whether a kinase-defective CSF-1R mutant could serve as a substrate for an enzymatically competent receptor kinase, both in vitro and in intact cells, to determine whether phosphorylation of CSF-1R on tyrosine can also proceed via an intermolecular mechanism.

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MATERIALS AND METHODS

Construction of a C-terminally truncated receptor, CSF-1R[ΔC13]. An in-frame termination codon was introduced after codon 959 in human CSF-1R cDNA to generate a truncation mutant lacking 13 carboxy-terminal amino acids. Briefly, the human CSF-1R cDNA (c-*fms* proto-oncogene [38]) in a pBR322-based expression plasmid (9) was digested with *Eco*RV, which recognizes a unique site 42 nucleotides 5′ to the authentic termination codon. A palindromic synthetic oligonucleotide (ATCTAGAT) containing an *Xba*I recognition site including a TAG termination codon was self-annealed and inserted into the plasmid by blunt-end ligation. Recovered supercoiled plasmid DNAs were screened by *Xba*I digestion, CSF-1R cDNA was excised with *Bam*HI, and the coding sequences were inserted into a feline sarcoma virus-based vector (35). The resulting plasmid was amplified in bacteria, and extracted supercoiled DNA was purified on cesium chloride gradients, recovered after gel filtration, and precipitated with ethanol to remove residual salt prior to transfection into mammalian cells.

Transfection of NIH 3T3 cells and generation of cell lines. Transfection was performed by the calcium phosphate method as described in detail elsewhere (34, 36). Cells expressing CSF-1R[ΔC13] were established by cotransfecting 50 ng of the expression plasmid together with 50 ng of pSV2*neo* (39) and selecting transformants in G418 (geneticin; GIBCO Laboratories, Grand Island, N.Y.). G418-resistant populations were fractionated by fluorescence-activated cell sorting with a monoclonal antibody that reacts specifically to an extracellular epitope in human CSF-1R (2). Sorted populations were used without further subcloning. Cells expressing a kinase-defective receptor containing a methionine-for-lysine substitution at the ATP-binding site (CSF-1R[met 616]) were prepared as previously described (11). To obtain cells coexpressing both CSF-1R mutants, 50 ng of each vector was cotransfected with 50 ng of pSV2*neo* DNA and transformants selected in G418 were subjected to

fluorescence-activated cell sorting to obtain a receptor-positive population. Individual cells were seeded at limiting dilution into 96-well microdilution dishes, and clones derived from single cells were grown and characterized for coexpression of mutant receptors (see Results). For all cell lines used, the levels of cell surface expression of the human c-*fms*-encoded gene products, as determined by flow cytometry, accurately reflected the levels of receptor biosynthesis determined by metabolic labeling procedures.

Preparation of antipeptide serum reacting to the CSF-1R C terminus. A peptide corresponding to the carboxy-terminal 23 amino acids of human CSF-1R was synthesized by the solid-phase method of Merrifield (27), and its composition was verified by amino acid analysis. The peptide was covalently coupled to keyhole limpet hemocyanin (Calbiochem-Behring, La Jolla, Calif.) with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (29). New Zealand White rabbits were immunized subcutaneously with 0.1 mg of the keyhole limpet hemocyanin-peptide conjugate in 0.5 ml of phosphate-buffered saline emulsified with 0.5 ml of complete Freund adjuvant. Booster injections were given at 3-week intervals with 30 μg of the coupled peptide in incomplete Freund adjuvant. The antiserum used in these studies (here designated anti-C-ter serum) was obtained after the third immunization.

Sequential immunoprecipitation. To examine the ability of CSF-1R[ΔC13] to phosphorylate CSF-1R[met 616] in vitro, detergent lysates from cells expressing the individual receptors were mixed and immunoprecipitated with a polyvalent rabbit antiserum to a recombinant v-*fms*-encoded polypeptide (14, 38), and the immune complexes were incubated in a kinase reaction mixture with [γ-^{32}P]ATP for 20 min at 28°C as described previously (36). The washed complexes were then boiled for 10 minutes in 50 mM Tris buffer (pH 7.4) containing 0.5% sodium dodecyl sulfate and 5 mM dithiothreitol, and the denatured proteins released from solubilized immune complexes were diluted with 10 volumes of ice-cold Nonidet P-40 lysis buffer (10). The resulting solutions were reprecipitated with either the same polyvalent antiserum or antipeptide serum.

Other analytical methods. The procedures for the metabolic labeling of cells with L-[^{35}S]methionine (1) or $^{32}P_i$ (10), immunoprecipitation, gel electrophoresis in denaturing polyacrylamide gels (1), the immune-complex kinase assay (36), phosphoamino acid analysis (31), and two-dimensional mapping of tryptic phosphopeptides (32) are described in detail in the references cited.

RESULTS

Cross-phosphorylation of CSF-1R in immune complexes. We examined the ability of an enzymatically competent receptor to phosphorylate a kinase-defective receptor (CSF-1R[met 616]) in vitro. To provide a means of unambiguously identifying CSF-1R[met 616] in the presence of another CSF-1R species, we deleted the C-terminal 13 amino acids of the wild-type receptor and used an antiserum directed to the deleted residues to specifically immunoprecipitate CSF-1R[met 616]. NIH 3T3 cells expressing either wild-type CSF-1R or the truncated variant, CSF-1R[ΔC13], were metabolically labeled with [^{35}S]methionine for 15 min and then incubated for 90 min in medium containing excess unlabeled methionine. During the chase period, the immature 130-kilodalton CSF-1R glycoprotein (gp130) undergoes modification of its asparagine-linked oligosaccharide chains to yield the mature plasma membrane-associated form (gp150).

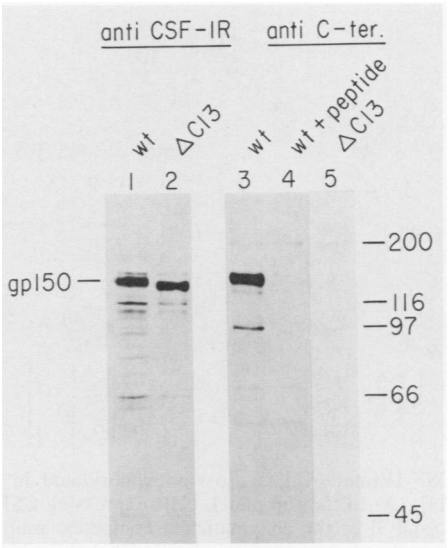

FIG. 1. CSF-1R antipeptide serum specifically immunoprecipitates wild-type CSF-1R but not CSF-1R[ΔC13]. Cells expressing wild-type CSF-1R (lanes 1, 3, and 4) or mutant CSF-1R[ΔC13] (lanes 2 and 5) were metabolically labeled for 15 min with 200 μCi of [^{35}S]methionine per ml, incubated for an additional 90 min in medium containing a 100-fold excess of unlabeled methionine, and lysed with detergent in a buffer containing protease inhibitors. Receptor glycoproteins were immunoprecipitated with either a rabbit antiserum to a recombinant v-*fms*-encoded polypeptide (anti-CSF-1R) or a rabbit antipeptide serum raised against the carboxy-terminal 23 amino acids of CSF-1R (anti-C-ter) and resolved on denaturing polyacrylamide gels. Free peptide at 15 μM was added to the cell lysate prior to immunoprecipitation (lane 4). Radiolabeled products were detected by autoradiography of the dried gels for 24 h. The position of the mature (gp150) CSF-1R glycoprotein is indicated in the left-hand margin. Molecular masses (in kilodaltons) of known proteins are indicated in the right-hand margin.

Cell lysates immunoprecipitated with a polyvalent antiserum to a recombinant *fms*-encoded polypeptide (anti-CSF-1R) contained CSF-1R[ΔC13] in amounts similar to those in cells expressing the wild-type receptor (Fig. 1, lanes 1 and 2). As expected, the truncated receptor exhibited a slightly faster electrophoretic mobility in denaturing polyacrylamide gels due to the loss of its 13 C-terminal amino acids. Like the anti CSF-1R serum, an antiserum prepared to the C-terminal 23 amino acids of CSF-1R (anti-C-ter) efficiently precipitated the wild-type receptor, but was blocked by preincubation with excess free peptide (Fig. 1, lanes 3 and 4). Although the antiserum was raised to a 23-mer, anti-C-ter serum failed to precipitate CSF-1R[Δ13], indicating that the antibodies reacted to epitopes within the distal C terminus of the wild-type receptor (Fig. 1, lane 5).

The kinase activity of CSF-1R in immune complexes is not stimulated by CSF-1 (31, 38). Previous results demonstrated that CSF-1R[met 616] was devoid of kinase activity in immune complexes, was not phosphorylated on tyrosine in intact cells after binding CSF-1, and could not induce cell proliferation (11), whereas CSF-1R[ΔC13] is active as a kinase. The truncation in CSF-1R[ΔC13] abrogates a negative regulatory effect mediated by the receptor C-terminal tail, thereby facilitating an increased mitogenic response to CSF-1 (35). However, the mutant receptor does not stimulate ligand-independent cell growth, nor do its rates of synthesis, turnover, or ligand-induced down modulation differ from those of the wild-type receptor (37a). Metaboli-

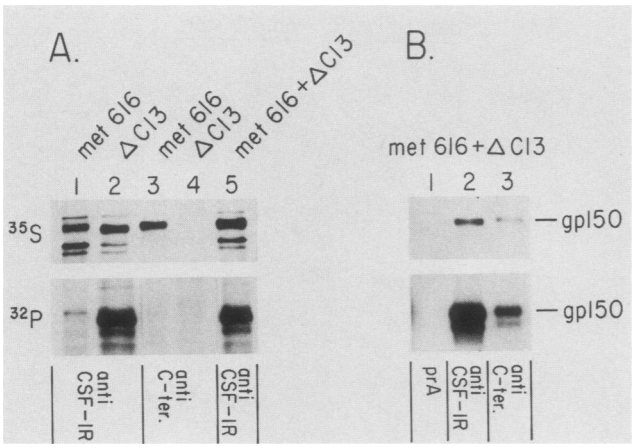

FIG. 2. CSF-1R[met 616] is cross-phosphorylated in vitro by CSF-1R[ΔC13]. (A) In the top panel, cells expressing CSF-1R[met 616] (lanes 1 and 3) or the enzymatically competent mutant CSF-1R[ΔC13] (lanes 2 and 4) were metabolically labeled for 15 min with 200 μCi of [^{35}S]methionine per ml, chased for 90 min in medium containing a 100-fold excess of unlabeled methionine, and lysed in detergent. Receptor glycoproteins were then immunoprecipitated with the antiserum indicated beneath the panel from lysates obtained from either individual cell lines (lanes 1 to 4) or a mixture containing equal volumes of the two lysates (lane 5). Labeled receptors were separated on denaturing polyacrylamide gels and detected by autoradiography. The exposure time for autoradiography was 24 h. In the bottom panel, receptors were immunoprecipitated from lysates of matched unlabeled cultures, and the washed immune complexes were incubated in an in vitro kinase reaction with [γ-^{32}P]ATP. Labeled proteins were separated in denaturing polyacrylamide gels and detected by autoradiography of the dried gels for 2 h. (B) ^{35}S- or ^{32}P-labeled receptor glycoproteins were eluted from triplicate immune complexes identical to those in panel A, lanes 5, by boiling in 0.1 ml of buffer containing 0.5% sodium dodecyl sulfate and 5 mM dithiothreitol. The eluted receptors were diluted to 1 ml with lysis buffer, and receptors were reprecipitated with either protein A-Sepharose alone (prA) or the antisera indicated below the lanes. Labeled proteins were separated on denaturing acrylamide gels and detected by autoradiography. The position of the mature (gp150) CSF-1R glycoprotein is indicated in the right-hand margin. The exposure time for autoradiography was 2 days for the top panel (^{35}S-labeled glycoproteins) and 1 day for the bottom panel (^{32}P-labeled glycoproteins).

cally labeled CSF-1R[met 616] was immunoprecipitated with either anti-CSF-1R or anti-C-ter serum (Fig. 2A, top, lanes 1 and 3) but, as previously shown, was devoid of phosphotransferase activity in immune complexes (Fig. 2A, bottom, lanes 1 and 3). In contrast, CSF-1R[ΔC13] was precipitated by anti-CSF-1R serum but not by anti-C-ter serum (Fig. 2A, top, lanes 2 and 4) and was readily phosphorylated in the immune-complex kinase reaction (Fig. 2A, bottom, lane 2). When cell lysates containing equivalent amounts of the two receptors were mixed, immunoprecipitated with anti-CSF-1R, and incubated in an immune-complex kinase reaction, receptor phosphorylation was again detected (Fig. 2A, bottom, lane 5).

To determine whether CSF-1R[met 616] was a substrate for the CSF-1R[ΔC13] kinase in mixed immune complexes, receptors labeled in vivo with [^{35}S]methionine or in vitro with [γ-^{32}P]ATP (Fig. 2A, lanes 5) were released from denatured immunoprecipitates, and the CSF-1R molecules were reprecipitated with either anti-CSF-1R or anti-C-ter serum and separated on gels (Fig. 2B). Under the conditions of receptor release, the initial precipitating antibodies were

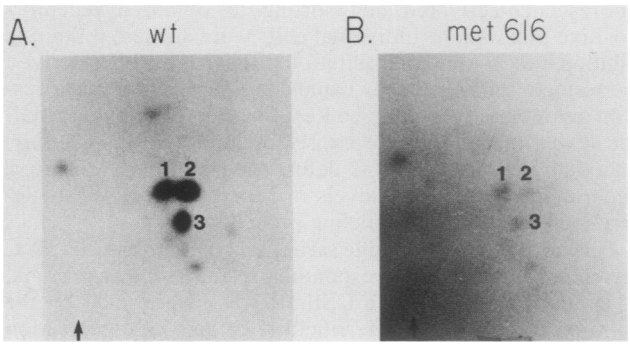

FIG. 3. Two-dimensional phosphopeptide maps of wild-type CSF-1R and CSF-1R[met 616] cross-phosphorylated in an immune-complex kinase reaction. Wild-type CSF-1R (A), labeled in an immune-complex kinase reaction, and CSF-1R[met 616] (B), cross-phosphorylated by the enzymatically active receptor mutant (Fig. 2B, lane 3, bottom), were eluted from gel slices and digested with trypsin. Peptides were spotted on cellulose-coated thin-layer plates (origins indicated by arrows) and separated by electrophoresis at pH 1.9 from left (anode) to right followed by ascending chromatography. The exposure times for autoradiography were 24 h (panel A) and 10 days (panel B).

denatured and were no longer able to recombine with the labeled receptors (Fig. 2B, lanes 1). In contrast, readdition of anti-CSF-1R serum resulted in efficient reprecipitation of the receptor molecules (Fig. 2B, lanes 2). Specific immunoprecipitation of [^{32}P]CSF-1R[met 616] with anti-C-ter serum established that it had been phosphorylated in *trans* by CSF-1R[ΔC13] in mixed immune complexes. As expected, CSF-1R[met 616] made up about half of the reprecipitated receptors (Fig. 2B, top, compare lanes 2 and 3) and was labeled proportionally with ^{32}P (Fig. 2B, bottom, lanes 2 and 3).

Control experiments confirmed that denatured CSF-1R[ΔC13] molecules, like the native protein (Fig. 1), were not reprecipitated by the antipeptide serum, nor was there any detectable kinase activity in anti-C-ter immunoprecipitates from mixed lysates, confirming that CSF-1R[met 616] was not contaminated with CSF-1R[ΔC13]. Honegger et al. indicated that kinase-defective and enzymatically competent EGF-R could associate in solution, particularly in the presence of EGF, and that the heterocomplexes could be precipitated with antibodies specific for only one of the two species (24). Using their lysis buffers adjusted with concentrations of purified human recombinant CSF-1 up to 8 nM, immunoprecipitation with anti-C-ter serum of CSF-1R[met 616] from mixed lysates did not yield immune complexes containing kinase-active CSF-1R[ΔC13]. Thus, under both our lysis conditions and theirs, CSF-1R molecules did not associate to form stable complexes in dilute solution, even in the presence of CSF-1.

Two-dimensional phosphoamino acid analysis confirmed that phosphotyrosine was the only radiolabeled amino acid in CSF-1R[met 616] after *trans*-phosphorylation by CSF-1R[ΔC13] in vitro (data not shown). When two-dimensional tryptic phosphopeptide analysis was performed, the peptide map obtained with CSF-1R[met 616] was nearly identical to that generated from the wild-type receptor (Fig. 3), which also undergoes phosphorylation in vitro only on tyrosine (31, 38). Three major tryptic phosphopeptides detected in the wild-type receptor (Fig. 3A, numbers 1, 2, and 3) were also observed in the *trans*-phosphorylated mutant (Fig. 3B). In addition, several minor peptides, which accounted for less

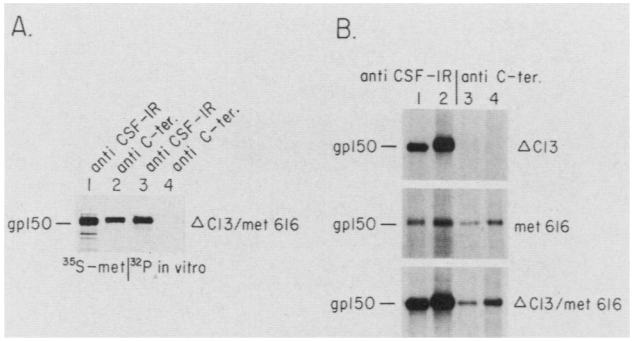

FIG. 4. CSF-1 induces cross-phosphorylation of CSF-1R[met 616] by CSF-1R[ΔC13] in intact cells. (A) Cells coexpressing CSF-1R[ΔC13] and CSF-1R[met 616] were metabolically labeled for 15 min with [^{35}S]methionine, incubated for 90 min in medium containing an excess of unlabeled precursor, and lysed with detergent, and the receptors were immunoprecipitated with the antisera indicated above the panel (lanes 1 and 2). Receptor glycoproteins from parallel unlabeled plates were immunoprecipitated with the indicated sera, and the washed immune complexes were incubated in an in vitro kinase reaction with [γ-^{32}P]ATP. Labeled receptors were separated on denaturing polyacrylamide gels and detected by autoradiography (lanes 3 and 4). The position of the mature CSF-1R glycoprotein (gp150) is indicated in the left-hand margin. The exposure times were 24 h for lanes 1 and 2 and 1 h for lanes 3 and 4. (B) Cells expressing the individual receptor mutants or coexpressing both receptors (ΔC13/met 616) were metabolically labeled with 1 mCi of $^{32}P_i$ per ml for 2 h and then stimulated with a saturating concentration of human recombinant CSF-1 for 5 min prior to lysis (lanes 2 and 4) or lysed prior to growth factor treatment (lanes 1 and 3). Receptors immunoprecipitated with the sera indicated above the panel were separated on denaturing polyacrylamide gels. The mature cell surface forms of the various receptors (indicated to the left of the panel) were eluted and subjected to phosphoamino acid analysis (see Fig. 5) or two-dimensional phosphopeptide analysis (see Fig. 6). The exposure time for autoradiography was 24 h.

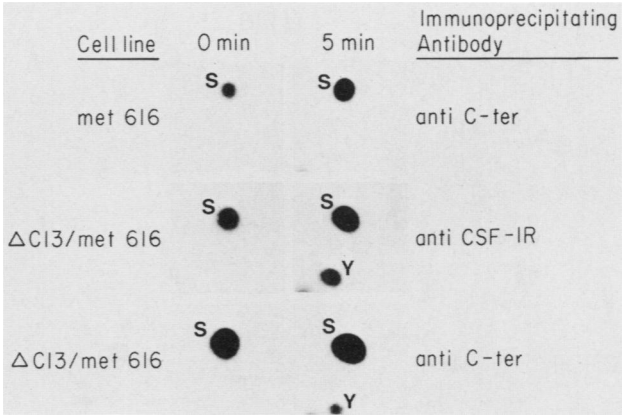

FIG. 5. Phosphoamino acid analysis of in vivo-labeled CSF-1R mutants from cells stimulated by CSF-1. Proteins metabolically labeled with $^{32}P_i$ were eluted from gel slides (Fig. 4B), hydrolyzed in acid, and subjected to two-dimensional phosphoamino acid analysis. The positions of phosphoserine (S) and phosphotyrosine (Y) were determined from ninhydrin-stained internal standards. The exposure time for autoradiography was 5 days for met 616 and ΔC13/met 616 immunoprecipitated with the antipeptide serum (anti-C-ter) and 24 h for ΔC13/met 616 immunoprecipitated with the polyvalent rabbit anti-receptor serum (anti-CSF-1R).

than 5% of the total incorporated radioactivity, were also detected in wild-type CSF-1R. Several of the latter spots were also observed in the maps of CSF-1R[met 616], whereas others could not be resolved, owing to the lower levels of phosphate incorporated into the mutant molecules. Therefore, CSF-1R[met 616] was phosphorylated in *trans* by CSF-1R[ΔC13], and the tyrosine-phosphorylated tryptic peptides corresponded to the major phosphorylated peptides observed in vitro with wild-type CSF-1R.

CSF-1 induces receptor cross-phosphorylation in vivo. To test whether ligand-induced cross-phosphorylation of CSF-1R also occurs in intact cells, individual NIH 3T3 clones cotransfected with both receptors were analyzed for CSF-1R expression by metabolic labeling and in vitro kinase reactions. Figure 4 shows results with a representative clone. Polyvalent anti-CSF-1R serum precipitated polypeptides that underwent efficient phosphorylation in immune complexes, consistent with expression of CSF-1R[ΔC13] in these cells (Fig. 4A, lanes 1 and 3). Similarly, immunoprecipitation with the antipeptide serum detected [^{35}S]methionine-labeled kinase-defective CSF-1R[met 616] molecules in the same cell lysates (Fig. 4A, lanes 2 and 4). From the proportion of metabolically labeled receptors precipitated with anti-C-ter serum, we estimated that these cells coexpressed approximately equal amounts of both CSF-1R mutants.

Cells expressing the individual mutants or coexpressing both were next metabolically labeled with $^{32}P_i$ and incubated with saturating concentrations of human recombinant CSF-1 (1 nM) for 5 min before lysis and receptor precipitation. In the absence of ligand stimulation, the mature cell surface form of CSF-1R[ΔC13] was phosphorylated (Fig. 4B, top, lanes 1) exclusively on serine residues. Addition of CSF-1 to cells expressing CSF-1R[ΔC13] resulted in a nearly twofold increase in the total phosphate incorporated into the receptor (Fig. 4B, top, lane 2), which corresponded to its phosphorylation on tyrosine (data not shown). In contrast, binding of CSF-1 to cells expressing CSF-1R[met 616] resulted in a minimal increase in phosphate incorporation into the receptor (Fig. 4B, middle, lanes 1 and 2), with no detectable tyrosine phosphate (Fig. 5). The basis for the ligand-induced increase in serine phosphate remains unclear. As shown above, anti-C-ter serum selectively precipitated only the CSF-1R[met 616] mutant (compare Fig. 4B, top and middle, lanes 3 and 4). When cells coexpressing both mutants were stimulated with CSF-1, a significant increase in phosphate incorporation was detected not only in the total receptor population (Fig. 4B, bottom, lanes 1 and 2) but also in kinase-defective CSF-1R[met 616] after its selective immunoprecipitation with anti-C-ter serum (Fig. 4B, bottom, lanes 3 and 4). Phosphoamino acid analysis of the eluted receptors showed that after coexpression with CSF-1R[ΔC13] and ligand stimulation, CSF-1R[met 616] was phosphorylated on tyrosine (Fig. 5). Thus, the kinase-defective mutant served as an in vivo substrate for the ligand-activated CSF-1R tyrosine kinase.

To again determine whether cross-phosphorylation involved the same tyrosine residues as those phosphorylated in the wild-type receptor, two-dimensional tryptic phosphopeptide analysis was performed on the metabolically ^{32}P-labeled products. Wild-type receptors recovered from unstimulated cells yielded five phosphoserine-containing peptides (Fig. 6A). Following CSF-1 stimulation, five new tyrosine-phosphorylated tryptic peptides were identified (Fig. 6D, major spots labeled A to D and minor spot labeled X in Fig. 6F). The same major phosphotyrosine-containing peptides were detected in CSF-1R[met 616] after phosphorylation in vivo by CSF-1R[ΔC13] (Fig. 6E). Because the conditions for two-dimensional separation of phosphoserine-

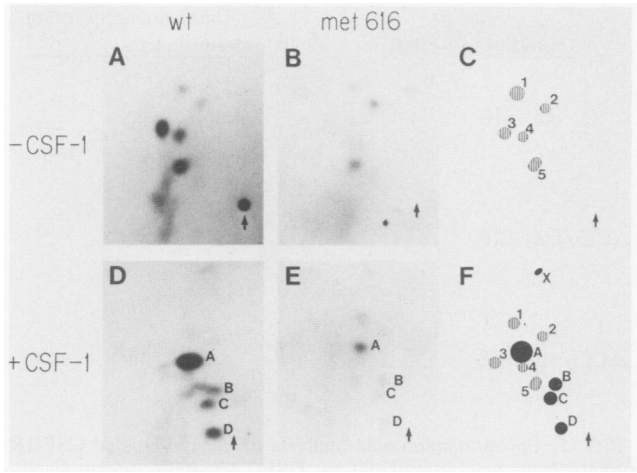

FIG. 6. Phosphopeptide maps of in vivo-labeled CSF-1R mutants from CSF-1-treated cells. Proteins metabolically labeled with $^{32}P_i$ (Fig. 4B) were eluted from gel slices, digested with trypsin, and spotted on cellulose-coated thin-layer plates (origin indicated by arrows). The peptides were separated by electrophoresis at pH 8.9 from left (anode) to right followed by ascending chromatography. (A and D) Results with wild-type CSF-1R in the absence (panel A) or presence (panel D) of CSF-1, respectively. (B and E) Parallel results with CSF-1R[met 616] phosphorylated in *trans*. All labeled receptors were immunoprecipitated with anti-C-ter serum. Individual peptide spots were eluted from the plates (panels A and D) and subjected to two-dimensional phosphoamino acid analysis to determine their phosphoamino acid composition. (C and F) Composite drawings showing the phosphoserine-containing (numbers) and CSF-1-induced phosphotyrosine-containing (letters) peptides. Spots labeled A to D were the major phosphotyrosine-containing tryptic peptides, whereas X was a minor tyrosine phosphorylated peptide.

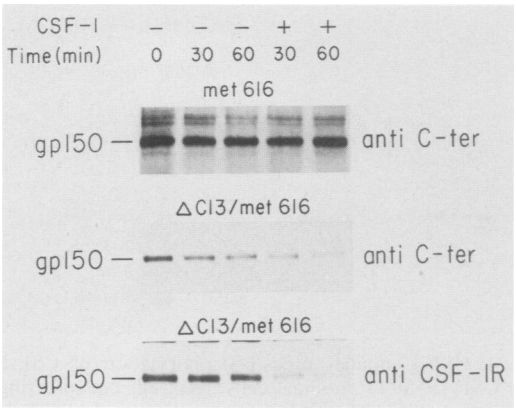

FIG. 7. Coexpression of CSF-1R[met 616] and CSF-1R[ΔC13] facilitates ligand-induced down modulation of the kinase-defective receptor. Cells expressing either CSF-1R[met 616] alone or coexpressing both mutant receptors were metabolically labeled for 15 min with [^{35}S]methionine and incubated for an additional 90 min with medium containing a 100-fold excess of unlabeled methionine. Parallel cultures were then either left untreated (−) or stimulated with 0.1 nM human recombinant CSF-1 (+) for the times indicated above the lanes. The cells were lysed, and receptors were immunoprecipitated with the antisera indicated at the right-hand margin and separated on denaturing polyacrylamide gels. Radiolabeled products were detected by autoradiography of the dried gel. The positions of the mature CSF-1R glycoproteins (gp150) are indicated in the left-hand margin. The exposure time for autoradiography was 24 h.

and phosphotyrosine-containing peptides labeled in vivo (Fig. 6) differed from those used to resolve peptides phosphorylated in vitro (Fig. 3), the different maps cannot be directly compared. However, studies with the wild-type receptor have indicated that two of the three major sites of in vitro phosphorylation (Fig. 3, peptides 2 and 3) correspond to peptides A and B in the fingerprints shown in Fig. 6. If the autoradiographic exposure of the in vitro labeled products is prolonged, spots corresponding to the in vivo-labeled peptides C and D can also be detected, whereas no counterpart to spot 1 (Fig. 3) has been observed after phosphorylation in vivo. Thus, the patterns of in vivo phosphorylation of CSF-1R differed partially from those obtained in immune complexes. Because the phosphotyrosine-containing peptides recovered from CSF-1R[met 616] *trans*-phosphorylated in intact cells remained identical to those in the wild-type receptor, the results indicate that receptor phosphorylation on tyrosine can proceed via an intermolecular mechanism.

Tyrosine phosphorylation of CSF-1R[met 616] correlates with its down modulation. The intrinsic tyrosine kinase activity of CSF-1R is required for efficient ligand-induced down modulation (11). We therefore examined whether cross-phosphorylation of CSF-1R[met 616] would also accelerate its turnover. Cells coexpressing both mutant receptors were metabolically labeled with [^{35}S]methionine and exposed to CSF-1 for various times, and the receptors were then immunoprecipitated from cell lysates and analyzed on gels. When expressed alone, CSF-1R[met 616] molecules did not undergo detectable turnover in 60 min, in either the presence or absence of ligand (Fig. 7, top). This result differs from that obtained with the wild-type receptor, which has a half-life of ca. 3 h in the absence of ligand but shows greatly accelerated turnover ($t_{1/2}$ = 15 min) in the presence of saturating concentrations of CSF-1 (11). When CSF-1R[met 616] was coexpressed with the kinase-active mutant, its turnover was appreciably accelerated, in both the presence or absence of ligand (Fig. 7, middle).

Although significant ligand-induced degradation of CSF-1R[met 616] was observed in the presence of CSF-1R[ΔC13], its down modulation was somewhat less efficient than that observed for the total receptor pool (Fig. 7, bottom). A possible explanation was that only some of the CSF-1R[met 616] molecules formed complexes in which cross-phosphorylation on tyrosine occurred. In agreement with this interpretation, the undegraded CSF-1R[met 616] glycoproteins that remained 60 min after CSF-1 stimulation lacked detectable phosphotyrosine (negative data not shown). Similar results were obtained with a series of independently derived clones that expressed different levels of each mutant receptor. In general, when the quantity of CSF-1R[ΔC13] exceeded that of CSF-1R[met 616], the proportion of kinase-defective molecules that underwent down modulation was greater than when the ratios of expression were reversed. Thus, the ligand-dependent tyrosine phosphorylation of the kinase-defective CSF-1R mutant in *trans* correlated with its down modulation, and all tyrosine-phosphorylated forms were degraded.

DISCUSSION

Binding of CSF-1 to cells expressing its receptor induces phosphorylation of CSF-1R on tyrosine followed by the rapid internalization and degradation of ligand-receptor complexes. The fact that a kinase-defective CSF-1R mutant can be phosphorylated in *trans* by an enzymatically active receptor kinase, both in immune complexes and in intact

cells stimulated by CSF-1, demonstrates that "autophosphorylation" of the receptor can involve bimolecular interactions between different receptor subunits. Control experiments indicated that the data obtained with intact cells were not due to an association of receptor monomers after lysis in dilute solution, in either the presence or absence of CSF-1. Under these conditions, the antipeptide serum specifically precipitated only CSF-1R[met 616] and did not coprecipitate the enzymatically competent truncation mutant from lysates containing both receptor mutants. Both in vitro and in vivo, the phosphorylation of CSF-1R[met 616] occurred on the same major tyrosine-containing tryptic peptides as those phosphorylated in the wild-type receptor, even though some sites of in vitro and in vivo phosphorylation differed from one another. Although the specificity of the kinase was retained when the reactions proceeded bimolecularly, the data do not directly address whether this is the normal mechanism of receptor phosphorylation or whether wild-type receptors can autophosphorylate in *cis*.

Intermolecular interactions between integral transmembrane receptors would require ligand-induced lateral movement within the plane of the membrane. It has been proposed that such receptors are in monomer-dimer equilibrium and that the activation of the receptor kinase depends on the ability of the ligand to aggregate the receptors and shift the equilibrium toward dimers (5, 42, 43). In support of this model, Scatchard analysis of EGF binding to its receptor reveals the presence of both high- and low-affinity binding sites, with the latter present in excess. When solubilized from the membrane, EGF-R was resolved into a major monomer and minor dimer fraction after sedimentation in sucrose gradients (5) or separation in nondenaturing gels (42, 43), and the solubilized dimers exhibited a significantly higher affinity for ligand (5, 42, 43). Addition of EGF to solubilized receptor monomers induced dimerization and increased their phosphorylation state, and the dimers could be covalently stabilized by chemical cross-linking (5, 7, 13, 42, 43). Although the phosphorylation of EGF-R dimers was not appreciably stimulated by ligand, their kinase activity was increased compared with that of monomers (5, 42, 43). Together, these findings suggest that EGF induces phosphorylation by promoting the aggregation of receptor subunits. Similarly, insulin receptor halves (α, β) are unable to undergo autophosphorylation if their association into complete molecules (α_2, β_2) is prevented (6). Purified PDGF-Rs in solution also undergo ligand-induced dimerization, leading to an increase in their kinase activity (19), and the ability of A-type and B-type PDGF-Rs to form both homo- and heterodimers underlies their differential responses to multiple forms of PDGF (18, 37).

CSF-1, like PDGF, is a dimeric glycoprotein which might be assumed to readily invoke receptor aggregation. However, only a single class of high-affinity binding sites has been observed on receptor-bearing cells (16, 17), and chemical cross-linking studies performed with ^{125}I-labeled CSF-1 bound to intact cells at 4°C have not provided clear evidence for dimer formation (28). These results may reflect the relatively small numbers of binding sites on cells expressing CSF-1R, as well as the inefficiency of chemical cross-linking of receptor-ligand complexes on intact cells. However, cross-phosphorylation of CSF-1R in response to ligand binding is most consistent with the formation of enzymatically active receptor aggregates.

Are there physiologic consequences of CSF-1R phosphorylation on tyrosine, or does this process simply reflect the ability of the receptor to serve as its own substrate after ligand activation? For several tyrosine kinases, including c-*src* (8, 25, 30), v-*fes*/*fps* (26, 40), and the insulin receptor (12, 20, 33, 44), their phosphorylation on tyrosine at specific sites in their kinase domains results in altered phosphotransferase activity. In contrast, variable effects have been demonstrated for EGF-R (3, 4, 23). Tyrosine phosphorylation might also indirectly attenuate ligand-induced signals by targeting receptors for degradation. Indeed, we (11) and others (15, 22) found that the intrinsic tyrosine kinase activity of CSF-1R and EGF-R was required for their efficient ligand-induced degradation. However, when the major sites of tyrosine phosphorylation in EGF-R were substituted by other residues, the receptors underwent phosphorylation at secondary sites and were still down modulated by ligand, suggesting that "autophosphorylation" at specific sites does not provide a down-modulatory signal (21). In the present studies, we found that *trans*-phosphorylation of a kinase-defective form of CSF-1R preceded its degradation and that the extent of receptor down modulation directly correlated with its degree of phosphorylation on tyrosine. Either the ligand-induced movement of a kinase-defective CSF-1R subunit into a complex with an enzymatically competent receptor must facilitate its internalization and degradation, or CSF-1R phosphorylation on tyrosine might initiate receptor down modulation. Regardless of the mechanism, the data are most consistent with the concept that coexpression of enzymatically active and kinase-defective forms of CSF-1R in the same cell leads to the ligand-induced phosphorylation and down modulation of those homodimers and heterodimers that contain at least one enzymatically active subunit.

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