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# Ligand and Protein Kinase C Downmodulate the Colony-Stimulating Factor 1 Receptor by Independent Mechanisms

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**The turnover of the colony-stimulating factor 1 receptor (CSF-1R), the c-*fms* proto-oncogene product, is accelerated by ligand binding or by activators of protein kinase C (PKC), such as the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA). The mechanisms of ligand- and TPA-induced downmodulation were shown to differ by the following criteria. First, in cells in which PKC was downmodulated, CSF-1R reexpressed at the cell surface remained sensitive to ligand but was refractory to TPA-induced degradation. Second, a kinase-defective receptor containing a methionine-for-lysine substitution at amino acid 616 at its ATP-binding site failed to undergo ligand-induced downmodulation but remained responsive to TPA. Following CSF-1 stimulation, no intermediates of receptor degradation could be immunoprecipitated with polyvalent antisera to CSF-1R. In contrast, TPA induced specific proteolytic cleavage of the receptor near its transmembrane segment, resulting in the release of the extracellular ligand-binding domain from the cell and the generation of an intracellular fragment containing the kinase domain. Two-dimensional phosphopeptide mapping demonstrated no new sites of phosphorylation in response to TPA in either the residual intact receptor or the intracellular proteolytic fragment. Therefore, PKC appears not to trigger downmodulation by directly phosphorylating the receptor but, rather, activates a protease which recognizes CSF-1R as a substrate.**

The macrophage colony-stimulating factor (CSF-1) is a lineage-specific hematopoietic growth factor which governs the proliferation and survival of mononuclear phagocytes and their committed bone marrow progenitors (29, 30; C. J. Sherr and E. R. Stanley, *in* M. B. Sporn and A. B. Roberts, ed., *Peptide Growth Factors and Their Receptors*, in press). CSF-1 mediates these effects by binding to a high-affinity cell surface receptor (CSF-1R), which is encoded by the c-*fms* proto-oncogene and is a member of a family of growth factor receptors with intrinsic tyrosine-specific protein kinase activity (27). Human CSF-1R is a transmembrane glycoprotein consisting of a 512-amino-acid extracellular ligand-binding domain, a single 25-amino-acid transmembrane segment, and a 435-amino-acid intracellular kinase domain (9). Binding of CSF-1 to its receptor initiates a cascade of rapidly occurring events, including the phosphorylation of the receptor on tyrosine, tyrosine phosphorylation of intracellular substrates, and the internalization and degradation (downmodulation) of ligand-receptor complexes (10, 16, 21, 27, 32–34). It is assumed that phosphorylation of certain physiologic substrates by the receptor kinase triggers the mitogenic response.

Turnover of CSF-1R is also greatly accelerated by exposure of receptor-bearing cells to activators of protein kinase C (PKC), such as the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (5, 14, 15, 32), which transmodulates the receptor in the absence of ligand. In contrast to CSF-1R, the v-*fms*-encoded oncogene product of the McDonough strain of feline sarcoma virus fails to downmodulate in response to TPA (23, 32). A chimeric receptor containing the first 308 amino acids of the extracellular domain of human CSF-1R fused to the remaining extracellular, transmembrane, and complete kinase domain of the v-*fms* gene product was found to be degraded in response to ligand but not TPA, suggesting that these compounds induce receptor turnover through independent mechanisms (23). We now show that ligand- but not TPA-induced receptor degradation requires the intrinsic tyrosine kinase activity of CSF-1R. In contrast, transmodulation of CSF-1R by PKC was shown to occur by a novel mechanism involving activation of a protease that specifically cleaves the receptor and releases its ligand-binding domain from the cell.

## MATERIALS AND METHODS

**Preparation of cell lines expressing mutant CSF-1R[M616].** Oligonucleotide-directed mutagenesis was used to construct a human CSF-1R cDNA containing a methionine-for-lysine substitution at amino acid 616 (CSF-1R[M616]). Mutagenesis was performed as described previously (24) by using a bacteriophage M13 kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Briefly, the mutation was programmed in gapped duplexes formed between a linearized double-stranded M13mp9 vector and an excess of circular single-stranded M13 DNA that contained a 995-base insert (*Sal*I to *Bam*HI) representing the 3′ end of c-*fms* cDNA (9). Following mutagenesis, the insert was resequenced in its entirety by dideoxy-chain termination, using the universal M13 primer and three additional oligonucleotide primers corresponding to different coding sequences within the c-*fms* cassette. The mutated insert was reassembled into complete c-*fms* cDNA, inserted into a feline sarcoma virus McDonough strain-based plasmid vector (25), and transfected into NIH 3T3 cells by the calcium phosphate precipitation technique (24, 26). A cell line was established by cotransfecting 50 ng of plasmid encoding CSF-1R[M616] with 50 ng of pSV2*neo* (28) and selecting transformants in G418 (Geneticin; GIBCO Laboratories, Grand Island, N.Y.). G418-resistant populations were fractionated by fluorescence-activated cell sorting with monoclonal antibodies (MAbs) specific for human CSF-1R epitopes (2, 23, 26a). Sorted populations were used for studies without further subcloning. Assays for

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colony formation in agar were performed as previously described (25).

**Cell labeling, immunoprecipitation, and immune complex kinase reactions.** The procedures for metabolic labeling with L-[$^{35}$S]methionine or $^{32}P_i$ (1, 10), immunoprecipitation, polyacrylamide gel electrophoresis in sodium dodecyl sulfate (1), and the immune-complex kinase assay (26) are described in detail in the references cited. For receptor turnover experiments, transfected NIH 3T3 cells expressing human CSF-1R or CSF-1R[M616] were treated with a saturating concentration ($1 \times 10^{-8}$ M) of purified human recombinant CSF-1 (lot 150 no. 2, provided by Peter Ralph, Cetus Corp., Emeryville, Calif.) or with TPA (Sigma Chemical Co., St. Louis, Mo.) before being subjected to detergent lysis. TPA was prepared as a $1 \times 10^{-2}$ M stock solution in dimethyl sulfoxide and diluted in culture medium to a final concentration of $5 \times 10^{-7}$ M. Cell lysates prepared at various times after CSF-1 or TPA treatment were immunoprecipitated with a polyvalent rabbit antiserum to a recombinant v-*fms*-encoded polypeptide (12). In experiments in which the release of receptor fragments into the medium was analyzed, immunoprecipitation was performed with rat MAbs that react specifically with extracellular epitopes of human CSF-1R (2, 26a). In control experiments, the soluble diacylglycerol *sn*-1,2-dioctanoylglycerol ($diC_8$) (Sigma), prepared in dimethyl sulfoxide, was added at a final concentration of $2 \times 10^{-4}$ M to the cell cultures for 30 min prior to cell lysis. To activate PKC through a physiologic pathway, we added purified porcine platelet-derived growth factor (ICN Biomedicals, Inc., Costa Mesa, Calif.) at 10 U/ml to CSF-1R-bearing NIH 3T3 cells that were growth arrested by overnight incubation in medium containing 0.1% fetal calf serum.

**Two-dimensional phosphopeptide mapping and phosphoamino acid analysis.** $^{32}$P-labeled proteins separated by polyacrylamide gel electrophoresis were localized by autoradiography, eluted from the gel, precipitated with trichloroacetic acid, oxidized with performic acid, and digested with trypsin, as described by Cooper et al. (8). The digested products were lyophilized and then suspended and relyophilized three more times with 800, 400, and 200 μl of water in sequence. The samples were suspended in 1% ammonium carbonate (pH 8.9) and spotted onto cellulose thin-layer plates. Peptides were separated in two dimensions by electrophoresis at pH 8.9 in 1% ammonium carbonate or suspended and run at pH 1.9 in acetic acid-formic acid-water (30:10:160 by volume) for 25 min at 1 kV and then subjected to chromatography in *n*-butanol–pyridine–acetic acid–water (70:65:50:25 by volume). $^{32}$P-labeled peptides were detected by autoradiography on presensitized film with intensifying screens at −70°C. Phosphoamino acid analysis was carried out as previously described (20).

## RESULTS

**PKC is not required for ligand-induced turnover of CSF-1R.** To examine receptor turnover, NIH 3T3 cells expressing human CSF-1R were metabolically labeled for 15 min with [$^{35}$S]methionine and then chased for 90 min in the presence of the unlabeled precursor. During the chase period, the immature form of the c-*fms*-encoded glycoprotein, gp130$^{c\text{-}fms}$, undergoes modification of its N-linked oligosaccharide chains to yield the mature plasma membrane-associated form of CSF-1R, gp150$^{c\text{-}fms}$ (21, 22). In the absence of ligand, the mature receptor has a half-life of 3 to 4 h (21, 23, 33). However, when saturating concentrations of human recombinant CSF-1 were added for different periods after the chase period and CSF-1R molecules immunoprecipitated from cell lysates were resolved on polyacrylamide gels, the receptor was rapidly degraded (Fig. 1A). Addition of TPA in lieu of CSF-1 also resulted in accelerated CSF-1R turnover, with kinetics similar to those observed with the ligand.

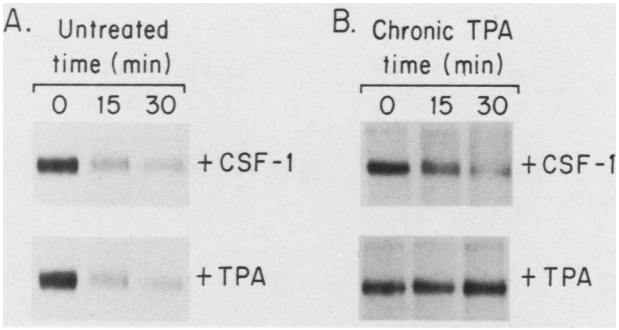


FIG. 1. Downmodulation of CSF-1R after chronic TPA treatment. (A) NIH 3T3 cells expressing human CSF-1R were metabolically labeled for 15 min with 200 μCi of [$^{35}$S]methionine per ml and incubated for an additional 90 min in medium containing a 100-fold excess of unlabeled methionine. Parallel cultures were then stimulated with a saturating concentration ($1 \times 10^{-8}$ M) of human recombinant CSF-1 or with $5 \times 10^{-7}$ M TPA for the times indicated above the panels. The cells were lysed with detergent in buffer containing protease inhibitors, and the CSF-1R glycoproteins were immunoprecipitated with rabbit antiserum to a recombinant v-*fms*-encoded polypeptide and resolved on denaturing polyacrylamide gels. Radiolabeled products were detected by autoradiography of the dried gels. (B) Cells were incubated for 24 h in culture medium containing $5 \times 10^{-7}$ M TPA before being radiolabeled and treated with CSF-1 or additional TPA as indicated above. Exposure times for autoradiography were 24 h.

To determine whether PKC activity might play a role in ligand-induced receptor turnover, PKC was itself downmodulated by treating cells expressing CSF-1R with $5 \times 10^{-7}$ M TPA for 24 h (3, 4, 35). The cells were then treated with CSF-1, or additional TPA was added to a final concentration of $1 \times 10^{-6}$ M. Following chronic TPA exposure, CSF-1R reexpressed at the cell surface remained sensitive to rapid ligand-induced receptor degradation (Fig. 1B). In contrast, further addition of TPA failed to induce receptor degradation. Since PKC activity is not required for ligand-induced receptor turnover, CSF-1 and PKC must downmodulate CSF-1R through independent mechanisms.

**Tyrosine-specific kinase activity is necessary for ligand- but not PKC-induced receptor turnover.** By analogy to results obtained with the insulin (6, 11) and epidermal growth factor receptors (13, 17), elimination of lysine in the predicted ATP-binding site (Lys-616) of CSF-1R should render the receptor devoid of phosphotransferase activity. We therefore used site-directed mutagenesis to prepare a mutant c-*fms* gene that contained a methionine-for-lysine substitution at codon 616. The wild-type and mutant receptor genes were inserted into retroviral vectors and cotransfected with pSV2*neo* into NIH 3T3 cells. After selection of cells with G418, fluorescence-activated cell sorting with a MAb directed to human CSF-1R was used to enrich for cells expressing c-*fms*-encoded epitopes at their surface. Immunoprecipitation of receptors from cells metabolically labeled with [$^{35}$S]methionine demonstrated expression of CSF-1R[M616] at levels approaching those of the wild-type re-

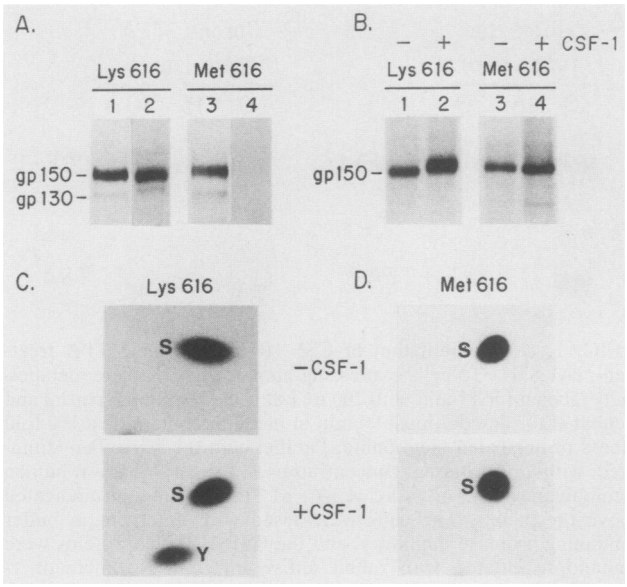


FIG. 2. CSF-1R[M616] lacks tyrosine-specific protein kinase activity. (A) Cells expressing wild-type CSF-1R (lanes Lys 616) or a mutant receptor (lanes Met 616) were metabolically labeled for 15 min with either 100 or 200 μCi of [$^{35}$S]methionine per ml, respectively, chased for 1 h with excess unlabeled methionine, and lysed in detergent, and the immunoprecipitated receptors were electrophoretically separated on denaturing polyacrylamide gels (lanes 1 and 3). Receptors were immunoprecipitated from lysates of parallel unlabeled cultures, and the washed immune complexes were incubated in an in vitro kinase reaction with [$\gamma$-$^{32}$P]ATP (lanes 2 and 4). Immunoprecipitated receptors were detected by autoradiography of the dried gels. The position of the immature (gp130) and mature (gp150) CSF-1R glycoproteins are indicated in the left-hand margin. (B) Cells labeled with 1 mCi of $^{32}P_i$ per ml for 2 h were lysed in the absence of the growth factor (lanes 1 and 3) or were stimulated with $1 \times 10^{-8}$ M human recombinant CSF-1 for 5 min prior to lysis (lanes 2 and 4). The immunoprecipitated receptors were separated on denaturing gels and detected by autoradiography. The exposure time for autoradiography was 2 h. The receptor glycoproteins were eluted, hydrolyzed in acid, and subjected to two-dimensional phosphoamino acid analysis (panels C and D). The positions of phosphoserine (S) and phosphotyrosine (Y) were determined with ninhydrin-stained standards. The exposure time for autoradiography was 24 h for panels C and D.

ceptor (Fig. 2A, lanes 1 and 3). When incubated in immune complex kinase assays, the wild-type CSF-1R underwent efficient autophosphorylation (lane 2), whereas CSF-1R[M616] could not be labeled (lane 4). Similarly, the wild-type receptor was able to phosphorylate the heterologous substrate poly(Glu-Tyr), whereas CSF-1R[M616] was devoid of phosphotransferase activity, being unable to phosphorylate poly(Glu-Tyr) at either 0.3 or 10 μM ATP (data not shown).

Like wild-type CSF-1R, the mature cell surface form of the mutant receptor could be metabolically labeled with $^{32}P_i$ in either the presence or absence of CSF-1 (Fig. 2B). In the absence of ligand, both receptors were phosphorylated only on serine (Fig. 2C and 2D). When cells were treated with a saturating concentration of purified recombinant CSF-1 for 5 min before lysis, the wild-type receptor was phosphorylated on tyrosine (Fig. 2C), but CSF-1R[M616] was not (Fig. 2D). Therefore, by these biochemical criteria, NIH 3T3 cells expressing CSF-1R[M616] lacked ligand-induced tyrosine-specific protein kinase activity.

To determine whether CSF-1R[M616] had biologic activity, vectors containing the wild-type or mutated c-*fms* alleles were cotransfected into mouse NIH 3T3 cells together with a second retroviral vector containing the human CSF-1 gene. Under these conditions, the wild-type c-*fms* gene induces foci of transformed cells through an autocrine mechanism (25). As expected, no foci were seen with CSF-1R[M616], even though the transfected cells expressed high levels of receptor epitopes at their surface and secreted CSF-1 into the medium (data not shown). Similarly, NIH 3T3 cells expressing CSF-1R[M616] alone failed to form colonies in soft agar in the presence of exogenously added CSF-1, whereas cells expressing the wild-type receptor were highly responsive (25). Therefore, mutation of lysine 616 generated a kinase-defective receptor that was no longer capable of transducing CSF-1-dependent mitogenic signals.

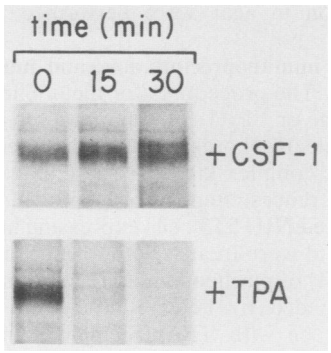


FIG. 3. Turnover of CSF-1R[M616] in response to ligand or TPA. NIH 3T3 cells expressing CSF-1R[M616] were metabolically labeled for 15 min with [$^{35}$S]methionine and incubated for an additional 90 min in medium containing a 100-fold excess of unlabeled methionine. Following the chase period, parallel cultures were incubated in medium containing $1 \times 10^{-8}$ M recombinant human CSF-1 or $5 \times 10^{-7}$ M TPA for the indicated periods. The cells were lysed, and the immunoprecipitated receptors were separated on denaturing polyacrylamide gels. Matched autoradiographic exposures are shown.

To examine the effect of elimination of kinase activity on receptor turnover, cells expressing CSF-1R[M616] were metabolically labeled with [$^{35}$S]methionine and exposed to CSF-1 or TPA for various times, and the receptors were immunoprecipitated and analyzed on denaturing gels. Unlike CSF-1R, the mutant receptor failed to undergo accelerated ligand-induced receptor turnover (Fig. 3). The inability of CSF-1 to downmodulate CSF-1R[M616] could not be attributed to the loss of high-affinity ligand-binding sites, since equilibrium binding assays performed with $^{125}$I-labeled human recombinant CSF-1 revealed similar $K_d$s for both the wild-type and mutant receptors. In contrast to addition of ligand, addition of TPA resulted in the rapid degradation of CSF-1R[M616] (Fig. 3). Thus, the intrinsic tyrosine kinase activity of CSF-1R, although essential for efficient ligand-induced receptor degradation, was not required for downmodulation by TPA.

**PKC transmodulates CSF-1R by inducing specific proteolytic cleavage.** In the absence of ligand, CSF-1R is phosphorylated exclusively on serine and has a half-life of 3 to 4 h. Addition of CSF-1 results in a nearly twofold increase in phosphate incorporation into the receptor followed by rapid receptor degradation (10, 16, 23, 24, 27, 34). When $^{32}P_i$-labeled NIH 3T3 cells expressing human CSF-1R were stimulated for various periods with ligand and the lysates were immunoprecipitated with polyvalent antiserum to CSF-1R, no intermediates of receptor degradation could be iden-

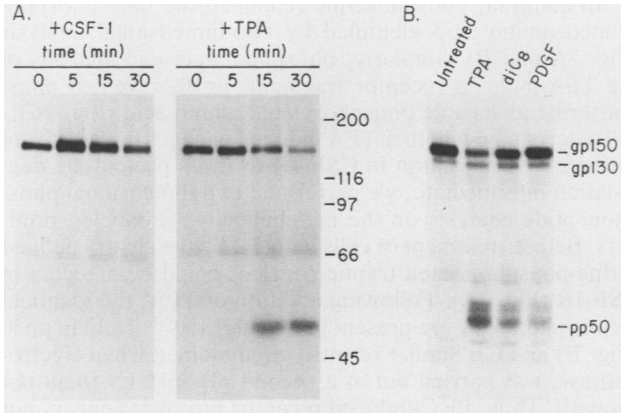


FIG. 4. TPA induces proteolytic cleavage of CSF-1R. (A) NIH 3T3 cells expressing CSF-1R were metabolically labeled with 1 mCi of $^{32}P_i$ per ml for 2 h and then stimulated with $1 \times 10^{-8}$ M human recombinant CSF-1 or $5 \times 10^{-7}$ M TPA for the times indicated (top of figure). The cells were lysed, immunoprecipitated with a polyvalent rabbit anti-receptor serum, and resolved on denaturing polyacrylamide gels. Radiolabeled products were detected by autoradiography of the dried gels for 45 min. (B) Parallel unlabeled cell cultures expressing CSF-1R were either left untreated or stimulated for 30 min with $5 \times 10^{-7}$ M TPA, $2 \times 10^{-4}$ M $diC_8$, or 10 units of purified porcine platelet-derived growth factor (PDGF) per ml, and the immunoprecipitated products were incubated in an in vitro kinase reaction with [$\gamma$-$^{32}$P]ATP (200 Ci/mmol). Labeled proteins were separated on denaturing gels and detected by autoradiography of the dried gels for 90 min. The positions of the immature (gp130) and mature (gp150) CSF-1R glycoproteins and the proteolytic degradation intermediate (pp50) are indicated in the right-hand margin.

tified (Fig. 4A). In contrast, addition of TPA resulted in receptor turnover which was associated with the specific immunoprecipitation of a 50-kilodalton (kDa) phosphoprotein (pp50) (Fig. 4A). The kinetics of the appearance of pp50 were similar to the rate of loss of the phosphorylated cell surface form of CSF-1R, suggesting that the polypeptide might represent an intermediate of receptor degradation. When cells expressing CSF-1R were treated with the solvent dimethyl sulfoxide at concentrations identical to those present during TPA addition, no receptor degradation was seen, nor was pp50 detected (data not shown). The generation of pp50 in response to TPA was seen in independently derived NIH 3T3 cell lines expressing different levels of human CSF-1R, including cells expressing various mutants of the receptor, such as CSF-1R[M616] and CSF-1R[S301], an oncogenic c-*fms* gene product (24). In addition, TPA induced the appearance of a similar phosphoprotein in the CSF-1-dependent murine macrophage cell line BAC1.2F5, indicating that the formation of pp50 was not limited to expression of receptors outside their normal context (data not shown).

Immune complex kinase assays performed on receptor-containing immunoprecipitates from TPA-treated cells also revealed the presence of the 50-kDA polypeptide (Fig. 4B). Phosphoamino acid analysis confirmed that pp50 labeled in vitro contained only [$^{32}$P]phosphotyrosine. Generation of pp50 was also induced by other activators of PKC, including the soluble diacylglycerol $diC_8$ and platelet-derived growth factor (Fig. 4B). The ability of pp50 to be specifically immunoprecipitated and phosphorylated on tyrosine in immune complexes suggested that pp50 was either a fragment derived from CSF-1R or a coprecipitating protein that served as a substrate for the intact receptor kinase. To distinguish

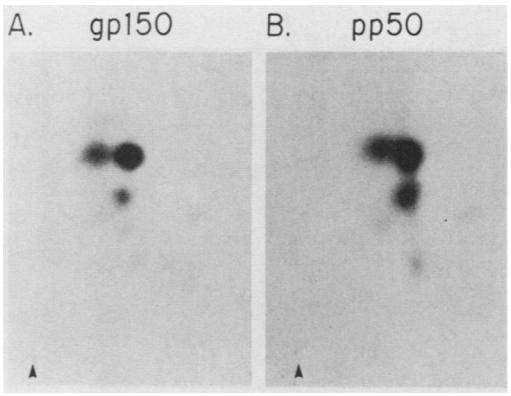


FIG. 5. Two-dimensional phosphopeptide maps of intact gp150$^{c\text{-}fms}$ and pp50 phosphorylated in an immune complex kinase reaction. In vitro-labeled proteins (Fig. 4B) were eluted from gel slices and subjected to trypsin digestion. Peptides were spotted on cellulose-coated thin-layer plates (origin indicated by the arrow) and separated by electrophoresis at pH 1.9 from left (anode) to right followed by ascending chromatography. The exposure time for autoradiography was 24 h.

between these possibilities, we performed two-dimensional tryptic phosphopeptide analysis on CSF-1R and pp50 labeled with [$^{32}$P]ATP in immune complexes. Identical phosphotyrosine-containing peptides were generated from both polypeptides (Fig. 5), demonstrating that pp50 is a proteolytic fragment of CSF-1R. Since 50 kDa is the predicted size of the complete intracellular tyrosine kinase domain of CSF-1R, TPA appears to induce proteolytic cleavage of the receptor at a specific site(s) near its transmembrane segment.

To determine whether PKC induces the cleavage of receptors expressed at the cell surface, cells were metabolically labeled for 15 min with [$^{35}$S]methionine and then chased for 90 min in the presence of the unlabeled precursor to maximize the number of radiolabeled mature gp150$^{c\text{-}fms}$ molecules. The cells were then stimulated with either CSF-1 or TPA, and cell lysates were immunoprecipitated with polyvalent antiserum and analyzed on denaturing polyacrylamide gels. In parallel, the culture medium was immunoprecipitated with a MAb specific for an extracellular epitope of human CSF-1R. Ligand-induced receptor downmodulation neither generated intracellular intermediates of receptor degradation nor yielded immunoprecipitable receptor fragments in the culture medium (Fig. 6A). In contrast, TPA-induced receptor degradation generated the 50-kDa intracellular polypeptide and, in addition, yielded a 100-kDa immunoprecipitable polypeptide in the culture medium corresponding in size to the extracellular ligand-binding domain of CSF-1R (Fig. 6B). Virtually identical results were observed when MAbs that react to other human CSF-1R epitopes were used (data not shown). Therefore, PKC transmodulates CSF-1R by inducing its proteolytic cleavage, thereby releasing the extracellular ligand-binding domain from the cell and generating an intracellular fragment representing the complete kinase domain.

**TPA does not induce new sites of CSF-1R phosphorylation.** In the absence of ligand, CSF-1R is phosphorylated exclusively on serine residues (Fig. 2). The protein kinase(s) responsible for phosphorylating the receptor has not been identified, and so PKC might potentially be involved. However, following the activation of PKC by TPA, no increase in phosphate incorporated into the receptor was detected (Fig.

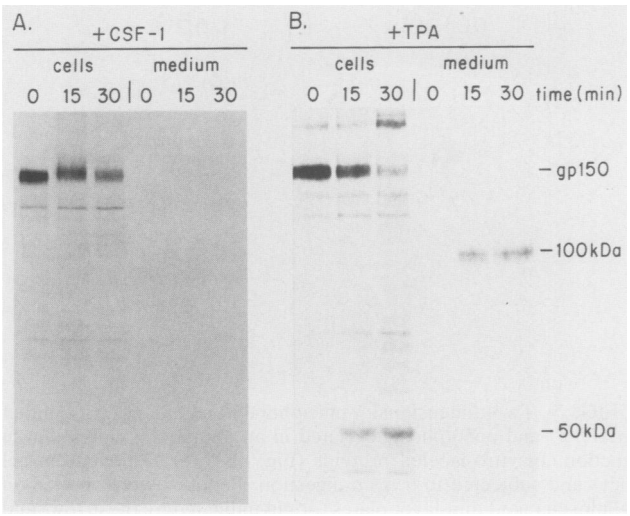


FIG. 6. TPA induces the release of the CSF-1R extracellular ligand-binding domain from the cell. Cells expressing CSF-1R were metabolically labeled for 15 min with [$^{35}$S]methionine and incubated for an additional 90 min in medium containing a 100-fold excess of unlabeled methionine. Following the chase, cultures were treated with $1 \times 10^{-8}$ M human recombinant CSF-1 (A) or $5 \times 10^{-7}$ M TPA (B) for the indicated times. Cell lysates were immunoprecipitated with polyvalent rabbit antiserum to a recombinant v-*fms*-encoded polypeptide. The culture medium was immunoprecipitated with a rat MAb specific for an epitope in the extracellular domain of human CSF-1R. The precipitated products were separated on denaturing gels. The electrophoretic mobilities of the mature CSF-1R (gp150) and the proteolytic degradation intermediates (100 kDa and 50 kDa) are indicated in the right-hand margin. Matched autoradiographic exposures are shown.

4). In addition, phosphoserine remained the only phosphorylated amino acid identified by two-dimensional analysis (Fig. 7A and B). Similarly, phosphoamino acid analysis of the TPA-induced receptor fragment, pp50, revealed phosphoserine as its sole phosphorylated amino acid (Fig. 7C).

To determine whether TPA induced changes in the sites of serine phosphorylation in CSF-1R or in its proteolytic degradation intermediate, we performed two-dimensional phosphopeptide analysis on the metabolically $^{32}$P-labeled products. Before treatment of cells with TPA, five clearly defined serine-phosphorylated tryptic peptides could be detected in CSF-1R (Fig. 7A). Following addition of TPA, the identical tryptic peptides were present in intact gp150$^{c\text{-}fms}$ and in pp50 (Fig. 7B and C). Similar results were obtained when electrophoresis was carried out at a second pH (pH 1.9 [data not shown]). Thus, PKC-induced receptor turnover appears not to be associated with direct receptor phosphorylation. Instead, downmodulation must result from the PKC-induced activation of a protease that recognizes CSF-1R as a substrate.

## DISCUSSION

Addition of CSF-1 or TPA to mononuclear phagocytes (10, 14–16, 32) or to mouse NIH 3T3 cells expressing a transduced human CSF-1 receptor (21, 23) induces receptor internalization and the rapid degradation of ligand-receptor complexes. Thus, the cellular machinery necessary to compartmentalize and degrade CSF-1R appropriately downmodulates the receptor in response to these extracellular signals, even when the receptor is introduced into cells in which it is not normally expressed. Downmodulation of CSF-1R by its ligand requires receptor tyrosine kinase activity, since a CSF-1R ATP-binding-site mutant (CSF-1R[M616]) which lacked phosphotransferase activity in vitro and in vivo failed to undergo ligand-induced degradation. This result was

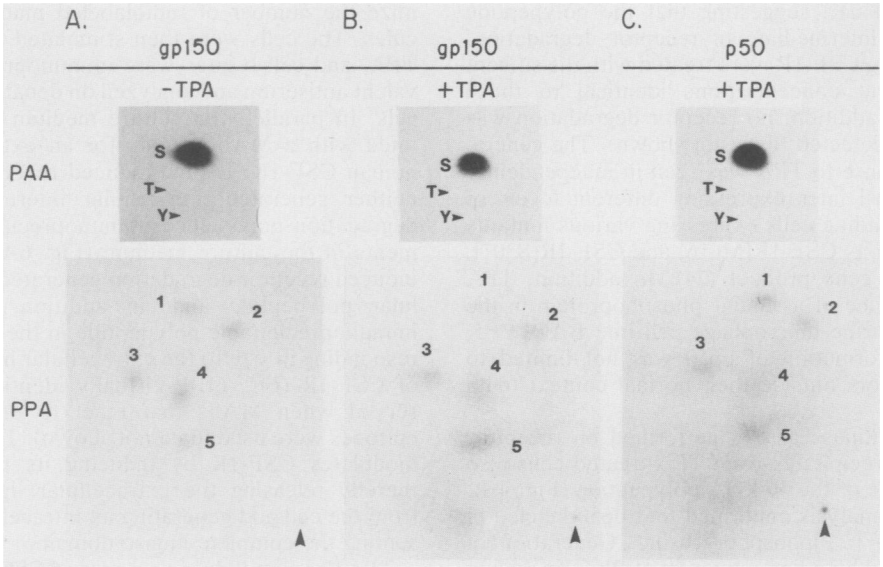


FIG. 7. Phosphoamino acid analysis and phosphopeptide maps of gp150$^{c\text{-}fms}$ and pp50 from TPA-treated cells. Proteins metabolically labeled with $^{32}P_i$ (Fig. 4A) were eluted from gel slices, and half of each sample was subjected to two-dimensional phosphoamino acid analysis (PAA). The positions of phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y) were determined from ninhydrin-stained standards. A portion of the remaining half of each sample was subjected to two-dimensional phosphopeptide analysis (PPA). Peptides were separated on cellulose-coated thin-layer plates by electrophoresis at pH 8.9 from left (anode) to right followed by ascending chromatography. The origin is indicated by the arrow. A spot corresponding to free phosphate remained on the plate but was cropped from the panels shown. The exposure times for autoradiography were 24 h for phosphoamino acid analysis and 20 days for the phosphopeptide maps.

expected in light of similar conclusions previously drawn from studies with analogous epidermal growth factor and insulin receptor mutants (6, 11, 13, 17). Each of these kinase-defective receptors, including CSF-1R[M616], also fails to propagate mitogenic signals by its respective ligand, demonstrating the critical role of tyrosine kinase activity in mediating the proliferative response.

Transmodulation of CSF-1R by phorbol esters does not involve tyrosine kinase activity, since the turnover of unstimulated receptor molecules lacking phosphotyrosine could be accelerated by TPA. Moreover, CSF-1R[M616] remained as sensitive as the wild-type receptor to TPA-induced degradation, despite its lack of phosphotransferase activity. The effects of phorbol esters on CSF-1R are presumed to require PKC and can be duplicated by the soluble diacylglycerol $diC_8$ and, in NIH 3T3 cells, by other physiological inducers of PKC, such as platelet-derived growth factor. Following chronic exposure of receptor-bearing cells to TPA and downmodulation of PKC activity, CSF-1R reexpressed at the cell surface remained responsive to CSF-1 but was refractory to TPA. Furthermore, a chimeric receptor containing a portion of the human CSF-1R extracellular domain fused to the remainder of the v-*fms* oncogene product was previously shown to be downmodulated by CSF-1 but not by TPA (23). Collectively, these results indicate that PKC transmodulates CSF-1R by a mechanism different from that used by the ligand.

Degradation of CSF-1R in response to TPA was associated with the specific immunoprecipitation of a 50-kDa intracellular fragment which contained all of the in vitro tyrosine-labeled tryptic phosphopeptides detected in the intact receptor (Fig. 5). Similarly, the fragment contained all of the metabolically labeled serine-containing phosphopeptides (Fig. 7), indicating that it could be derived only from the receptor intracellular domain. The size of this fragment (50 kDa) suggests that it originates from cleavage of CSF-1R at a site near its unique membrane-spanning segment. A reciprocal fragment of 100 kDa appeared with the same kinetics and was precipitated from the culture supernatants by using a MAb specific for an extracellular epitope of human CSF-1R (2, 26a). Similar results have been obtained with other MAbs directed to different epitopes in the receptor extracellular domain. This demonstrates that proteolytic cleavage of CSF-1R in response to TPA treatment releases the CSF-1R extracellular ligand-binding domain from the cells.

Specific proteolytic cleavage of CSF-1R at sites near its membrane-spanning segment could be mediated by proteolytic activity induced by PKC or, alternatively, by an active protease that recognizes a PKC-phosphorylated form of the receptor as a specific substrate. Since PKC regulates epidermal growth factor receptor ligand binding affinity and turnover by directly phosphorylating the receptor at threonine 654, close to the membrane-spanning segment (7, 18, 19), we initially favored the latter hypothesis. However, following TPA treatment, no increase in phosphate incorporation into CSF-1R was observed, nor were changes in the phosphoamino acid content of CSF-1R detected. More importantly, no new sites of serine phosphorylation could be identified by two-dimensional mapping of tryptic phosphopeptides, either in the residual intact receptor or in the pp50 fragment generated after TPA treatment. We cannot formally exclude the presence of new minor sites of CSF-1R phosphorylation in response to TPA treatment, since all phosphopeptides may not have been quantitatively recovered or adequately resolved. However, similar results were obtained under alternative conditions for peptide separation. Therefore, all evidence suggests that PKC does not directly phosphorylate CSF-1R.

We suggest that activation of PKC induces a protease that recognizes CSF-1R as a substrate and releases its ligand-binding domain from the cell. Under these conditions, receptor transmodulation might not only abrogate the ability of the cell to respond to CSF-1 but also solubilize receptor fragments that retain the potential to bind to, and competitively inhibit stimulation by, the extracellular ligand. Many physiologic activators of PKC would be expected to induce transmodulation of CSF-1R by this novel mechanism. Since macrophage activators, such as bacterial lipopolysaccharides, and other hematopoietic growth factors, such as interleukin-3 and granulocyte-macrophage CSF, have been suggested to downmodulate CSF-1R (15, 31), it will be of interest to determine whether specific proteolytic cleavage of CSF-1R at the same site(s) also occurs in response to these agents.

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