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Mutation of Tyr697, a GRB2-binding site, and Tyr721, a PI 3-kinase binding site, abrogates signal transduction by the murine CSF-1 receptor expressed in Rat-2 fibroblasts

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The receptor for the myeloid cell growth factor colony stimulating factor 1 (CSF-1) is a protein tyrosine kinase that is closely related to the PDGF receptor. Ligand binding results in kinase activation and autophosphorylation. Three autophosphorylation sites, Tyr697, Tyr706 and Tyr721, have been mapped to the kinase insert domain. Deletion of the entire kinase insert domain completely abrogates signal transduction by the CSF-1 receptor expressed in Rat-2 fibroblasts. To investigate the function of individual phosphorylation sites present in the CSF-1 receptor kinase insert domain, a number of phosphorylation site mutants were expressed in Rat-2 fibroblasts. Mutation of either Tyr697 or Tyr721 compromised signal transduction by the CSF-1 receptor. A mutant receptor, in which both Tyr697 and Tyr721 were replaced by phenylalanine, has lost all ability to induce changes in morphology or to increase cell growth rate in response to CSF-1. Tyr721 has been identified recently as the binding site for PI 3-kinase. Here we report that GRB2 associates with the CSF-1 receptor upon ligand binding. The phosphorylation on tyrosine of SHC and several other GRB2-associated proteins increased upon stimulation with CSF-1. Tyr697 was identified as a binding site for GRB2. We suggest that PI 3-kinase, GRB2 and some of the GRB2-associated proteins could play an important role in signal transduction by the CSF-1 receptor.

Key words: colony stimulating factor 1/signal transduction/tyrosine kinases

Introduction

A large number of growth and differentiation factors transduce their signals into the cell through receptor protein tyrosine kinases (RPTKs). It has been known for a long time that ligand binding results in activation of the cytoplasmic PTK domain, which is followed by substrate phosphorylation (Ullrich and Schlessinger, 1990; Schlessinger and Ullrich, 1992). The important substrates, however, have remained elusive until recently. The current consensus is that PTK receptors dimerize upon ligand binding leading to activation of the kinase domain and autophosphorylation. Following autophosphorylation receptors associate with a number of signal transducing proteins some of which have been identified, including PI 3-kinase, PLCγ and the Ras GTPase activating protein GAP120 (Cantley *et al.*, 1991; Pawson, 1992; Pawson and Gish, 1992; Schlessinger and Ullrich, 1992; Mayer and Baltimore, 1993). These proteins all contain Src homology 2 (SH2) domains that bind specific phosphorylated tyrosines within activated growth factor receptors and other cellular PTKs (Cantley *et al.*, 1991; Pawson, 1992; Pawson and Gish, 1992; Schlessinger and Ullrich, 1992; Mayer and Baltimore, 1993). The specificity of these interactions is determined by the SH2 domain and the sequence surrounding the phosphorylated tyrosine (Fantl *et al.*, 1992; Kazlauskas *et al.*, 1992; Reedijk *et al.*, 1992; Songyang *et al.*, 1993).

Colony stimulating factor 1 (CSF-1) is a growth factor that regulates differentiation, survival and renewal of macrophages (Stanley *et al.*, 1983). CSF-1 is also involved in the regulation of placental trophoblast proliferation during pregnancy (Pollard *et al.*, 1987, 1991). The receptor for CSF-1 was first identified as the cellular homolog of v-Fms, the transforming protein of the SM and HZ5 feline sarcoma viruses (Sherr *et al.*, 1985). The CSF-1 receptor is related to the α and β PDGF receptors (Yarden *et al.*, 1986; Matsui *et al.*, 1989), c-Kit, the stem cell factor (SCF) receptor (Qiu *et al.*, 1988) and Flk2/Flt3 (Matthews *et al.*, 1991; Rosnet *et al.*, 1991). Typically, members of the PDGF receptor family have an extracellular ligand-binding domain, consisting of five immunoglobulin repeats and a kinase domain, which is split into two parts by an insert of 80-100 residues, that is now commonly referred to as the kinase insert domain.

CSF-1 binding results in receptor dimerization followed by autophosphorylation (Sherr, 1991), which is similar to what has been found for other RPTKs. Several CSF-1 receptor autophosphorylation sites have been identified. Tyr807 (Roussel *et al.*, 1990; Tapley *et al.*, 1990; van der Geer and Hunter, 1990), which is located in the second half of the kinase domain, is a conserved residue in all PTKs (Hanks and Quinn, 1991), and is phosphorylated in most of them upon activation. Mutation of this residue reduces kinase activity and strongly affects the ability of the CSF-1 receptor to transduce signals into the cell (Roussel *et al.*, 1990; van der Geer and Hunter, 1991). Three autophosphorylation sites, Tyr697, Tyr706 and Tyr721, have been identified in the CSF-1 receptor kinase insert domain (Tapley *et al.*, 1990; van der Geer and Hunter, 1990; Reedijk *et al.*, 1992). Deletion of the CSF-1 receptor kinase insert has yielded different functional consequences in different systems. Deletion of the kinase insert from v-Fms does not affect its ability to transform NIH3T3 fibroblasts (Taylor *et al.*, 1989). In contrast, deletion of the kinase insert from the murine CSF-1 receptor decreases its ability to induce changes in morphology and to increase growth rate in response to CSF-1 in Rat-2 fibroblasts (Reedijk *et al.*, 1990). Nothing is known about the function of Tyr697. Mutation of Tyr706 can affect the induction of early response genes by CSF-1 in Rat-2 fibroblasts (van der Geer and Hunter, 1991). Tyr721 has been identified as the binding site for PI 3-kinase (Reedijk *et al.*, 1992). Although it has been established that the CSF-1 receptor kinase insert is essential for signal transduction in Rat-2 fibroblasts, no complete analysis of kinase insert

phosphorylation site mutants has been reported. Here we have investigated the role of kinase insert phosphorylation sites during signal transduction. In addition, we have tested whether the recently characterized SH2-containing adaptor proteins NCK, GRB2 and SHC (Chou *et al.*, 1992; Li *et al.*, 1992; Lowenstein *et al.*, 1992; Matuoka *et al.*, 1992; McGlade *et al.*, 1992; Meisenhelder and Hunter, 1992; Park and Rhee, 1992; Pelicci *et al.*, 1992; Rozakis-Adcock *et al.*, 1992) are involved in signalling by the CSF-1 receptor. The analysis of phosphorylation site mutants presented here shows that Tyr697 and Tyr721 play an important role in signalling by the CSF-1 receptor. Our data further show that the activated CSF-1 receptor associates with GRB2 and that several GRB2-associated proteins, including SHC, become tyrosine-phosphorylated in response to CSF-1. We have identified Tyr697 in the CSF-1 receptor kinase insert as a binding site for GRB2.

Results

Expression of wild type and phosphorylation site mutant CSF-1 receptors in Rat-2 fibroblasts

Three autophosphorylation sites, Tyr697, Tyr706 and Tyr721, have been mapped to the murine CSF-1 receptor kinase insert domain (Tapley *et al.*, 1990; van der Geer and Hunter, 1990; Reedijk *et al.*, 1992), and there is evidence suggesting that the kinase insert is important for signal transduction by the murine CSF-1 receptor in Rat-2 fibroblasts (Reedijk *et al.*, 1990). Here we have asked the question whether mutating the autophosphorylation sites in the kinase insert domain debilitates the CSF-1 receptor to the same extent as a complete deletion of the kinase insert. To investigate this a number of CSF-1 receptor phosphorylation site mutants were expressed in Rat-2 fibroblasts. Wild type (Wt), Met614 (a mutant that lacks kinase activity), Phe697, Phe706, Phe721, Phe697/721 and Phe697/706/721 mutant murine CSF-1 receptor cDNAs were expressed under the control of a CMV promoter from an MLV-based retroviral expression vector, containing a neomycin resistance gene as a selectable marker. Pools of G418-resistant Rat-2 fibroblasts expressing Wt and mutant CSF-1 receptors were used throughout all experiments.

To investigate whether these mutant receptors were able to respond to ligand binding, they were tested for their ability to autophosphorylate in the presence of CSF-1 *in vivo*. Blots of receptor immunoprecipitates from control and CSF-1-stimulated cells were probed with anti-phosphotyrosine (anti-P.Tyr) antibodies for the presence of P.Tyr. All the phosphorylation site mutants responded to CSF-1, showing an increase in P.Tyr content, whereas the kinase inactive Met614 mutant did not (Figure 1A). The same blot was stripped and probed with an anti-CSF-1 receptor polyclonal serum and all the mutant receptors were found to be expressed at approximately similar levels (Figure 1B). Since the kinase insert domain has been shown before to be dispensable for kinase activity (Taylor *et al.*, 1989), the differences in the relative level of autophosphorylation most likely reflect the absence of major phosphorylation sites in the mutant receptors. To show that the correct mutations had indeed been made, the different mutant receptors were isolated by immunoprecipitation from untreated cells, autophosphorylated *in vitro* in the presence of [γ-^{32}P]ATP (Figure 2A), and subjected to phosphotryptic peptide mapping (Figure 2B). Tryptic digestion gave rise to at least

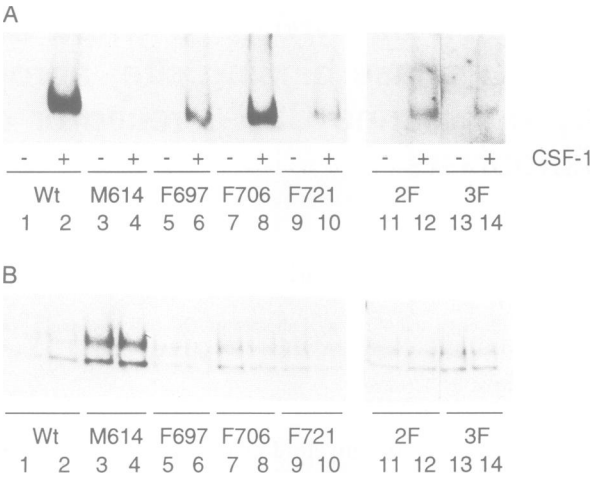

Fig. 1. Wt and mutant CSF-1 receptor autophosphorylation and expression level. Rat-2 fibroblasts stably expressing Wt and Met614, Phe697, Phe706, Phe721, Phe697/721 (2F) and Phe697/706/721 (3F) mutant CSF-1 receptors were grown to confluence and starved in 0.5% serum overnight. Receptors from control (lanes 1, 3, 5, 7, 9, 11 and 13) and CSF-1-stimulated cells (100 ng/ml for 3 min at 37°C, lanes 2, 4, 6, 8, 10, 12, 14) were immunoprecipitated, resolved by SDS–PAGE, transferred to Immobilon and probed with anti-P.Tyr antibodies using [^{125}I] protein A for detection (**A**). After exposure the blot was stripped in 60 mM Tris–Cl pH 6.8, 2% SDS, and 100 mM β-mercaptoethanol at 50°C, and probed with an anti-CSF-1 receptor antiserum (**B**).

six phosphopeptides that contain Tyr697 (marked b), and two phosphopeptides that contain Tyr706 (marked a). Tyr721 is represented by two major (marked d) and two minor phosphopeptides (marked d′). The phosphopeptide that contains Tyr807 is marked (c). The peptide marked (e) has not been identified as yet. Note that *in vitro* Tyr697 (peptides b) and Tyr706 (peptides a) are the major phosphorylation sites. The data are summarized in a schematic phosphopeptide map (Figure 2C).

In summary, the Wt and phosphorylation site mutant CSF-1 receptors used in this study were active *in vivo* and *in vitro* and expressed at comparable levels. Peptide mapping data showed that the correct changes had been introduced in these mutants.

Tyr697 and Tyr721 are essential for signal transduction by the CSF-1 receptor in Rat-2 fibroblasts

Rat-2 fibroblasts expressing Wt CSF-1 receptors show an increase in their growth rate and in the rate of transcription of a number of early response genes and change cell morphology in response to CSF-1 (Reedijk *et al.*, 1990; van der Geer and Hunter, 1991). Figure 3A shows that Rat-2 fibroblasts expressing Wt CSF-1 receptors grown to confluence in Dulbecco's modified Eagle's medium (DMEM) containing 1% fetal bovine serum (FBS) had an altered morphology after incubation in the presence of CSF-1 for the final 48 h, such that the cells became more elongated and spindle shaped and started to grow on top of each other in a criss-cross pattern. Rat-2 fibroblasts expressing kinase inactive Met614 mutant CSF-1 receptors did not show this response (Figure 3A). Cells that were grown to confluence in 10% serum were also included in this experiment to show that this change in morphology was not simply the result of an increase in cell density. The analysis of Rat-2 fibro-

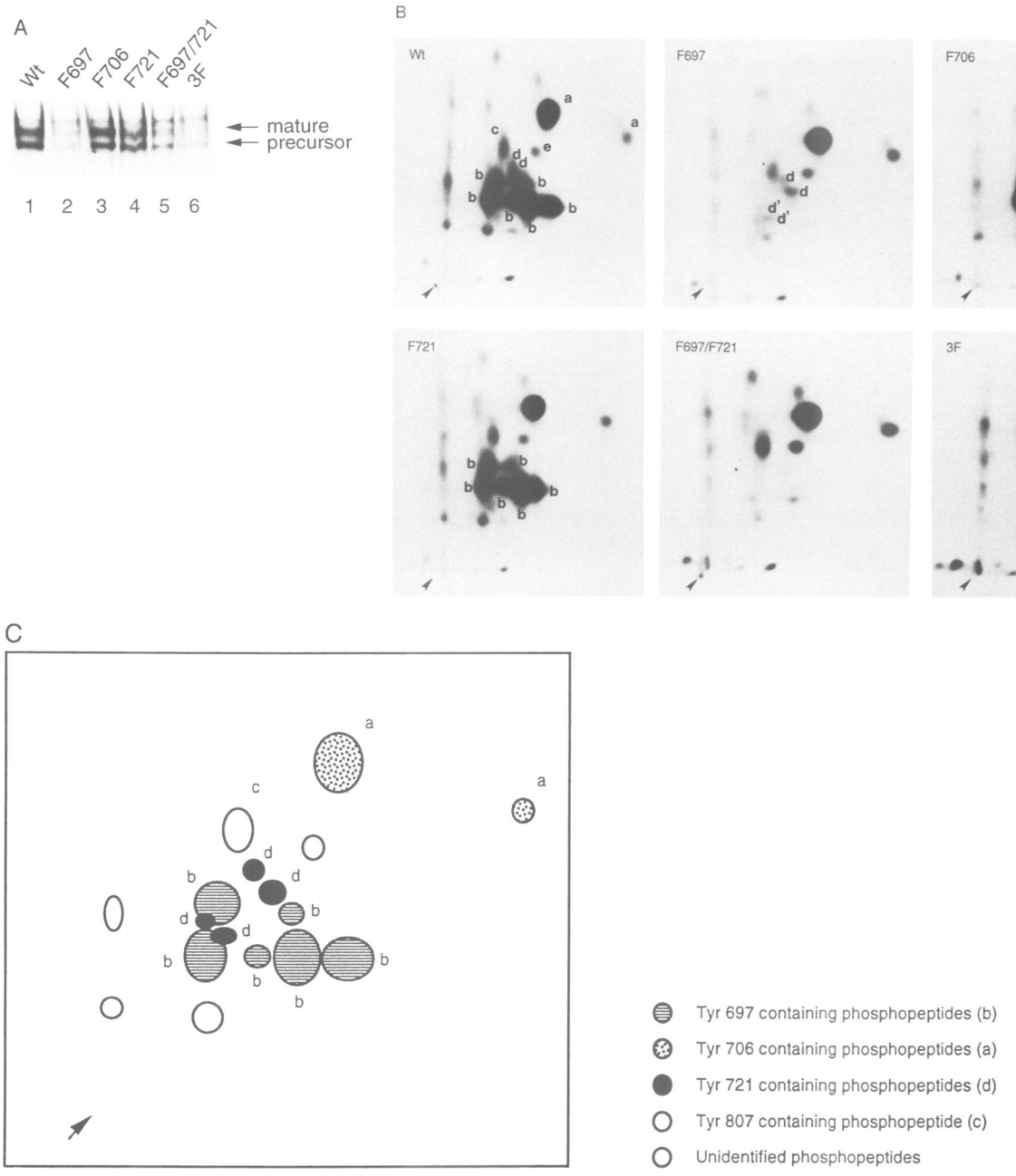

Fig. 2. Phosphopeptide maps of Wt and phosphorylation site mutant CSF-1 receptors. Wt and Phe697 (F697), Phe706 (F706), Phe721 (F721), Phe697/721 (F697/F721), and Phe697/706/721 (3F) mutant CSF-1 receptors were isolated by immunoprecipitation from untreated cells, phosphorylated *in vitro* in the presence of [γ-^{32}P]ATP, and resolved by SDS−PAGE (**A**). Two forms of the CSF-1 receptor were resolved representing a precursor form (precursor) and a mature cell surface form (mature). Proteins were digested with trypsin after transfer to Immobilon, and phosphopeptides were separated by electrophoresis at pH 1.9 and chromatography in two dimensions on thin layer cellulose plates (**B**). The origin is indicated with an arrowhead; the anode was on the left, and chromatography was in the vertical dimension. An overview of the data is shown in (**C**). Identification of the different phosphopeptides is partly based on earlier studies (Tapley *et al.*, 1990; van der Geer and Hunter, 1990, 1991; Reedijk *et al.*, 1992).

blasts expressing phosphorylation site mutants is shown in Figure 3B. Cells expressing Phe697 or Phe721 mutant CSF-1 receptors showed reduced ability to change cell morphology in response to CSF-1. The Phe697/721 and Phe697/706/721 mutant receptor-expressing cells showed essentially no response. In contrast, cells expressing Phe706 mutant CSF-1 receptors behaved like Wt receptor-expressing cells, as was shown before (van der Geer and Hunter, 1991).

To study CSF-1-induced changes in growth rate, Rat-2 fibroblasts expressing different CSF-1 receptor mutants were grown in medium containing 1% FBS in the absence or presence of CSF-1 (Figure 4). CSF-1 stimulated the growth rate of Rat-2 fibroblasts expressing Wt or Phe706 mutant CSF-1 receptors. Cells expressing kinase inactive Met614 mutant CSF-1 receptors showed no growth response (data not shown). Cells expressing Phe697 or Phe721 mutant receptors showed a greatly reduced response. CSF-1 did not stimulate the growth rate of either the Phe697/721 or the Phe697/706/721 mutant CSF-1 receptor-expressing cells (Figure 4). These data indicate that both Tyr697 and Tyr721 are involved in CSF-1 receptor signals that lead to altered morphology and increased growth rate.

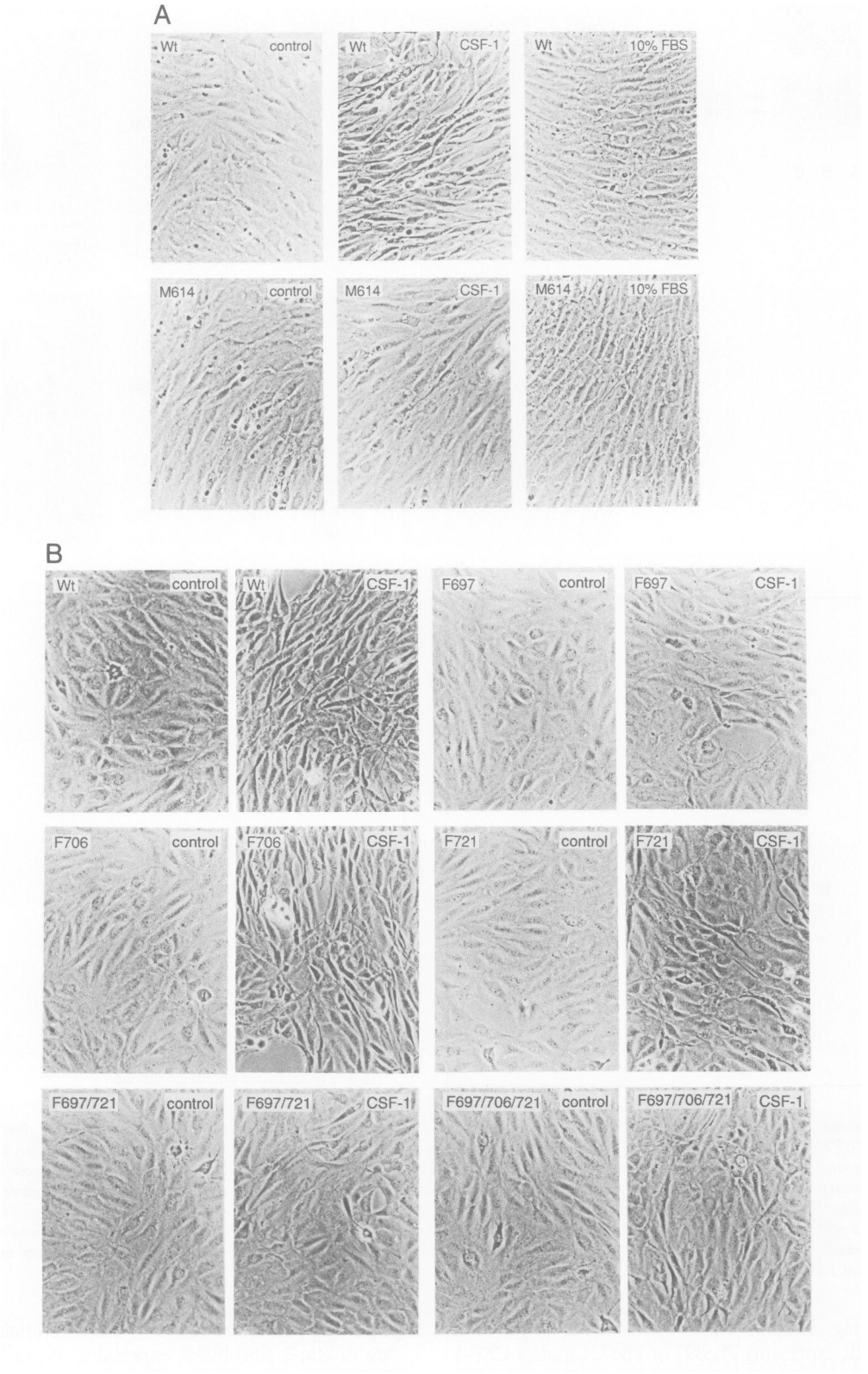

Fig. 3. Kinase insert phosphorylation site mutant CSF-1 receptors are affected in their ability to change the morphology of Rat-2 fibroblasts in response to CSF-1. Rat-2 cells expressing Wt or kinase inactive Met614 (M614) mutant CSF-1 receptors were seeded in 2.5 cm dishes and grown in DMEM containing 10% FBS. The medium was changed once and cells were left to reach confluence in 10% FBS or 1% FBS plus 20 mM HEPES pH 7.2 as indicated. CSF-1 (100 ng/ml) was added where indicated, and photographs were taken at 200-fold magnification two days later (**A**). In the same manner Rat-2 cells expressing Wt or phosphorylation site mutants Phe697, Phe706, Phe721, Phe697/721, Phe697/706/721 (3F) were tested for their morphological response to CSF-1 (**B**).

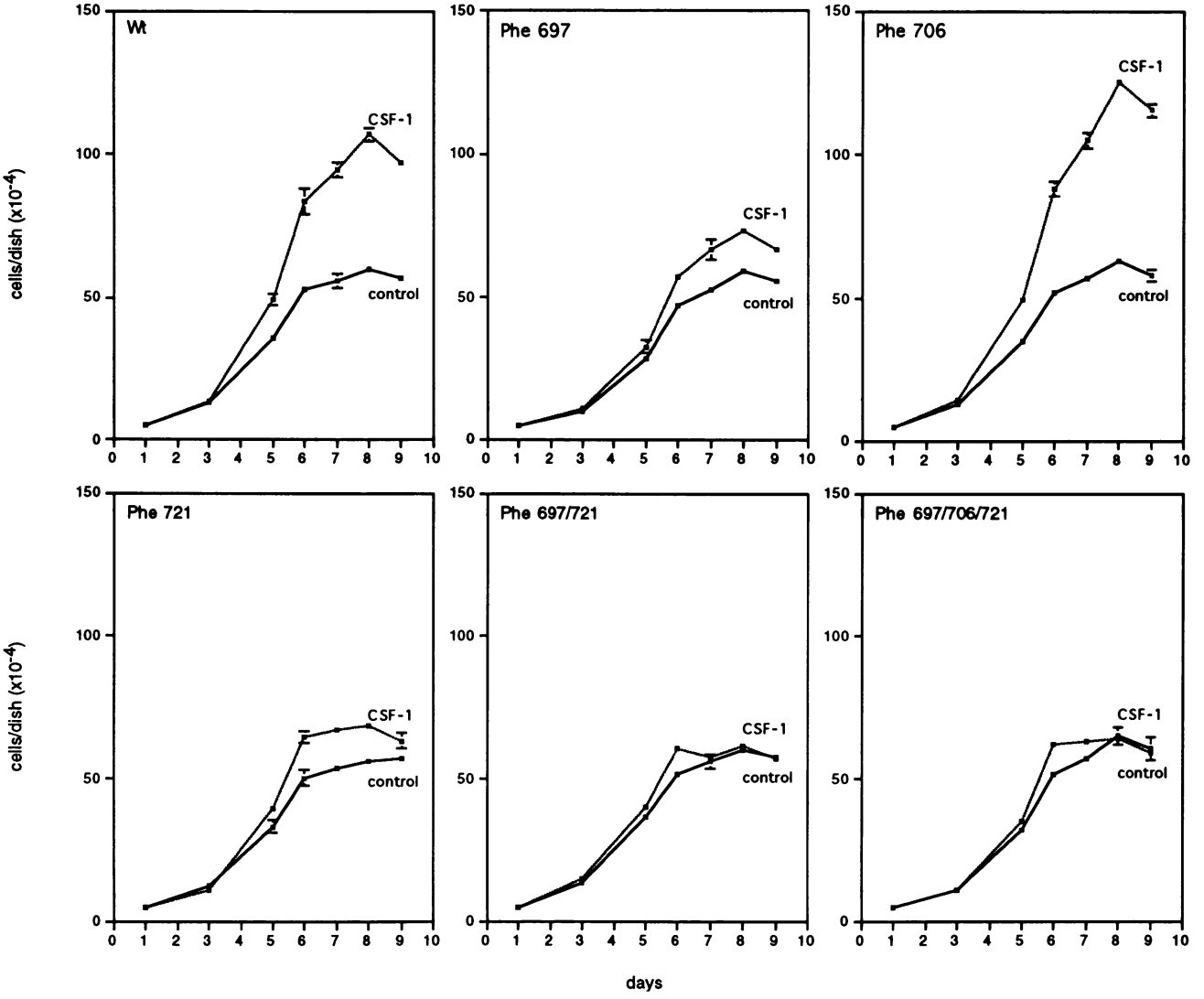

Fig. 4. Kinase insert phosphorylation site mutant CSF-1 receptors are affected in their ability to increase the growth rate of Rat-2 fibroblasts in response to CSF-1. Rat-2 cells expressing Wt or phosphorylation site mutants Phe697, Phe706, Phe721, Phe697/721, Phe697/706/721 CSF-1 receptors were seeded at 5×10^4 cells per 2.5 cm dish. The following day the medium was changed to DMEM, 1% FBS plus 20 mM HEPES, pH 7.2 (control) or DMEM, 20 mM HEPES pH 7.2, 1% FBS 100 ng/ml CSF-1 (CSF-1). The medium was changed every second day. Cell numbers were counted at days 1, 3 and 5–9. The results shown are the average of three independent cell number determinations. Standard deviations are indicated by vertical bars.

Association of the CSF-1 receptor with GRB2 and phosphorylation of SHC on tyrosine in response to CSF-1

GAP, PLCγ and PI 3-kinase associate with several growth factor RPTKs upon ligand binding, and are thought to be involved in signal transduction (Cantley *et al.*, 1991). However, neither GAP not PLCγ associates with, or becomes phosphorylated on tyrosine by the CSF-1 receptor upon binding of CSF-1 (Downing *et al.*, 1989; Reedijk *et al.*, 1990). In contrast, PI 3-kinase associates with the activated CSF-1 receptor (Varticovski *et al.*, 1989). We have previously identified Tyr721 as a PI 3-kinase binding site (Reedijk *et al.*, 1992). Although Phe697 mutant receptor-expressing cells show a similar phenotype to Phe721 CSF-1 receptor-expressing cells, Phe697 CSF-1 receptors bind PI 3-kinase in response to CSF-1 (Reedijk *et al.*, 1992). Therefore we sought to identify other targets that might bind to Tyr697 following phosphorylation.

A number of novel SH2-containing proteins have been cloned and characterized recently (Chou *et al.*, 1992; Li *et al.*, 1992; Lowenstein *et al.*, 1992; Matuoka *et al.*, 1992; Meisenhelder and Hunter, 1992; Park and Rhee, 1992; Pelicci *et al.*, 1992). They were shown to associate with, and in most cases were tyrosine phosphorylated by, activated RPTKs. We have investigated the phosphorylation status of NCK, GRB2 and SHC and their associated proteins in response to CSF-1. Immunoprecipitates from control and CSF-1-stimulated cells were analyzed by immunoblotting with anti-P.Tyr antibodies (Figure 5). Mature CSF-1 receptors became phosphorylated on tyrosine upon addition of CSF-1 (Figure 5, lanes 1 and 2). NCK immunoprecipitates contained a number of P.Tyr-containing proteins (130, 115, 75 and 70 kDa). The 47 kDa NCK polypeptide itself, however, was not phosphorylated on tyrosine in these cells, nor did stimulation with CSF-1 appear to change the phosphorylation status of any of the proteins present in NCK

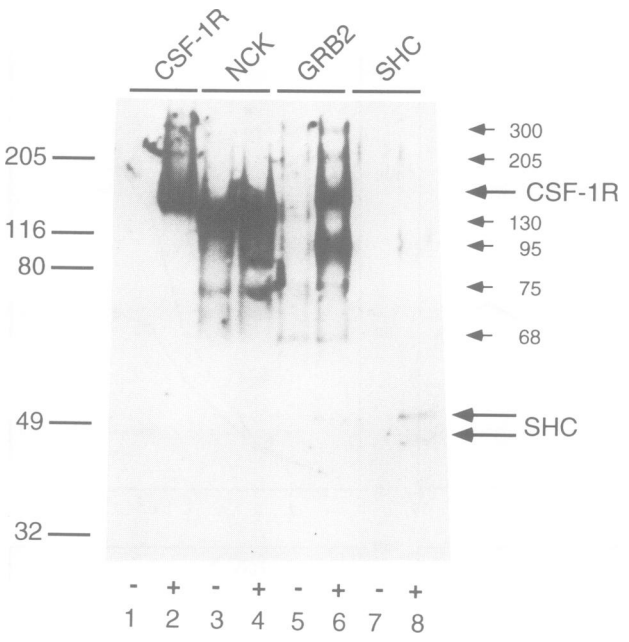

Fig. 5. Phosphorylation of GRB2-associated proteins on tyrosine in response to CSF-1. Cells expressing Wt CSF-1 receptors were grown to 90% confluence in DMEM containing 10% FBS and starved overnight in 0.5% serum. Control and CSF-1-stimulated (100 ng/ml for 3 min at 37°C) cells were lysed in NP40 lysis buffer and CSF-1 receptors (lanes 1 and 2), NCK (lanes 3 and 4), GRB2 (lanes 5 and 6) and SHC (lanes 7 and 8) immunoprecipitates obtained from control (lanes 1, 3, 5 and 7) and CSF-1-stimulated (lanes 2, 4, 6 and 8) cells were analyzed by immunoblotting with anti-P.Tyr antiserum. The positions of the CSF-1 receptor and SHC proteins are indicated with large arrows. The molecular weights of unidentified associated proteins are indicated with small arrows. The sizes of molecular weight markers are given on the left.

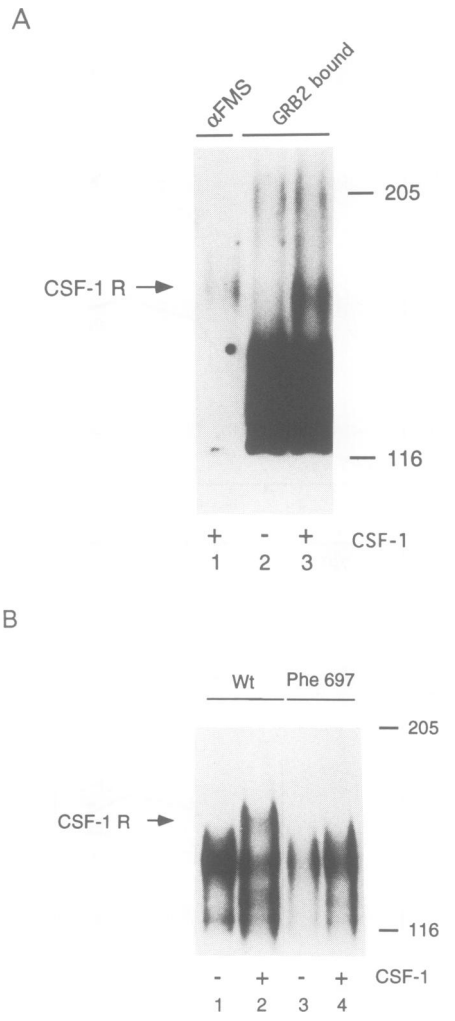

Fig. 6. Wt but not Phe697 mutant CSF-1 receptors associated with GRB2 *in vitro*. Rat-2 cells expressing Wt CSF-1 receptors were grown to 90% confluence in 10 cm dishes in 10% FBS and starved overnight in 0.5% serum. Control and CSF-1-stimulated cells (100 ng/ml) for 3 min at 37°C) were lysed in NP40 lysis buffer. CSF-1 receptors were immunoprecipitated using a polyclonal anti-CSF-1 receptor serum (αFMS). In parallel, NP40 lysates were incubated with recombinant GST−GRB2 immobilized on glutathione−Sepharose (GRB2 bound), and bound proteins were analyzed by SDS−PAGE on a 7.5% gel and immunoblotting with anti-P.Tyr antiserum. (**A**) CSF-1 receptor immunoprecipitates from CSF-1 stimulated cells (lane 1); GST−GRB2 Sepharose bound proteins from control and CSF-1-stimulated cells (lanes 2 and 3). The CSF-1 receptor is indicated by an arrow. (**B**) Lysates of control or CSF-1-stimulated Rat-2 cells expressing Wt or Phe697 mutant CSF-1 receptors were incubated with GST−GRB2 glutathione−Sepharose, and bound proteins were analyzed by immunoblotting with anti-P.Tyr antiserum. The CSF-1 receptor is indicated by an arrow. The sizes of molecular weight markers are given on the right.

immunoprecipitates (Figure 5, lanes 3 and 4). GRB2 immunoprecipitates obtained from resting cells also contained a number of P.Tyr-containing proteins with apparent sizes of ~300, 205, 130, 95, 75, 68, 66, 52 and 46 kDa. After stimulation with CSF-1 a new P.Tyr-containing protein was present in the GRB2 immunoprecipitates. The mobility of this protein during SDS−PAGE is identical to that of the CSF-1 receptor (compare Figure 5, lanes 2 and 6). In addition, the tyrosine phosphorylation of the 95, 75, 66, 52 and 46 kDa proteins was increased after stimulation with CSF-1 (Figure 5, lanes 5 and 6).

To test whether the autophosphorylated CSF-1 receptor binds directly to GRB2, NP40 lysates, obtained from control and CSF-1-stimulated cells expressing Wt CSF-1 receptors, were incubated with a bacterially expressed GST−GRB2 fusion protein bound to glutathione−Sepharose (Figure 6A). After several washes the bound proteins were resolved by SDS−PAGE and analyzed by immunoblotting with anti-P.Tyr antibodies. A 170 kDa protein that comigrated with the CSF-1 receptor bound to GST−GRB2, but only in lysates from CSF-1-stimulated cells (Figure 6A). To prove that this 170 kDa protein is the CSF-1 receptor, proteins were released from GST−GRB2 Sepharose by boiling in PBS, 1% SDS and 5 mM DTT and subjected to immunoprecipitation with anti-CSF-1 receptor serum. Immunoprecipitated proteins were detected by immunoblotting with anti-P.Tyr antibodies. Tyrosine phosphorylated CSF-1 receptors were detected in immunoprecipitates of proteins bound to GST−GRB2 from CSF-1 treated cell lysates (Figure 7A, lane 3).

SHC immunoprecipitates obtained from resting cells contained several P.Tyr-containing proteins (205, 95, 66, 52 and 46 kDa), (Figure 5, lanes 7 and 8). All of these, with the exception of the 205 kDa protein, showed increased phosphorylation upon stimulation with CSF-1. No tyrosine-phosphorylated CSF-1 receptors were present in SHC immunoprecipitates from CSF-1 stimulated Wt CSF-1 receptor-expressing cells. The 66, 52 and 46 kDa proteins, which were also present in the GRB2 immunoprecipitates,

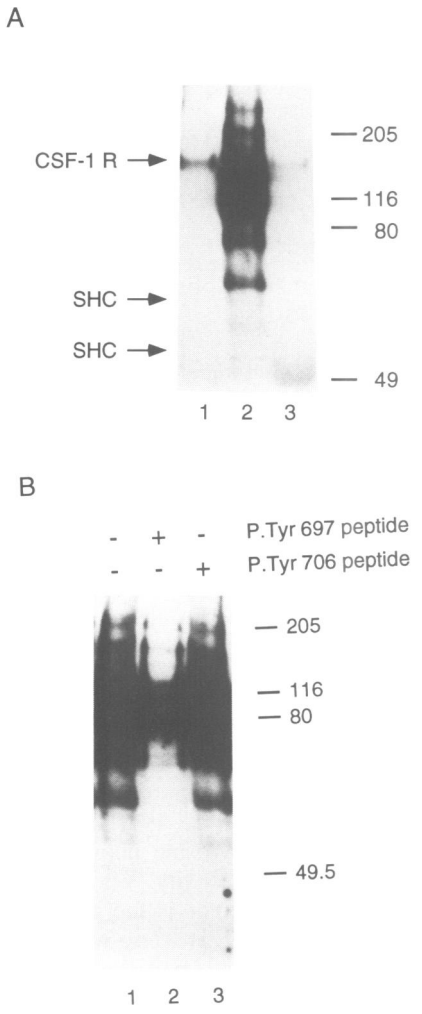

Fig. 7. Synthetic P.Tyr peptides block binding of P.Tyr-containing proteins to GRB2 *in vitro*. CSF-1 receptors immunoprecipitated with an anti-CSF-1 receptor serum and proteins bound *in vitro* to GST−GRB2 from CSF-1-stimulated Wt CSF-1 receptor-expressing Rat-2 cells were analyzed in parallel by SDS−PAGE and immunoblotting with an anti-P.Tyr antiserum. (**A**) CSF-1 receptor immunoprecipitates (lane 1), GRB2 bound proteins (lane 2) and CSF-1 receptor immunoprecipitate of proteins released from GST−GRB2 glutatione−Sepharose by boiling in phosphate-buffered saline containing 1% SDS, 5 mM DTT (lane 3). The positions of the CSF-1 receptor and SHC proteins are indicated by arrows. (**B**) Lysates obtained from CSF-1-stimulated Wt CSF-1 receptor expressing Rat-2 cells were incubated with GST−GRB2 glutathione−Sepharose in the absence (lane 1) or presence (lane 2, P.Tyr697; lane 3, P.Tyr706) of competing P.Tyr-containing synthetic peptides. The sizes of molecular weight markers are given on the right of both panels.

most likely represent the different forms of SHC (Pelicci *et al.*, 1992). SHC has been found to be phosphorylated on tyrosine in resting cells and its phosphorylation on tyrosine increases upon activation of the EGF receptor. Our data show that SHC is also phosphorylated on tyrosine upon activation of the CSF-1 receptor. In addition, a 95 kDa protein that coprecipitates with GRB2 and with SHC becomes phosphorylated on tyrosine in response to CSF-1.

The Phe697 mutant CSF-1 receptor fails to associate with GRB2 upon ligand binding

To investigate whether any of the tyrosine phosphorylation sites in the kinase insert domain are involved in the interaction with GRB2, the abilities of Wt and phosphorylation site mutant CSF-1 receptors to associate with GST−GRB2 *in vitro* were compared. Cell lysates obtained from control and CSF-1-stimulated cells were incubated with GST−GRB2 Sepharose. Bound proteins were analyzed by immunoblotting with anti-P.Tyr antibodies. As shown above, tyrosine-phosphorylated CSF-1 receptors obtained from CSF-1-stimulated cells expressing Wt receptors bound to GST−GRB2 Sepharose. In contrast, no tyrosine-phosphorylated Phe697 mutant receptors were bound to GST−GRB2 Sepharose, although other P.Tyr-containing proteins were detected in lysates of CSF-1-stimulated Phe697 mutant CSF-1 receptor-expressing cells (Figure 6B). To establish that Tyr697 can act as a binding site for the GRB2−SH2 domain, a chemically phosphorylated synthetic peptide corresponding to residues 691−703 of the CSF-1 receptor (P.Tyr697 peptide) was used in blocking experiments. Preincubation of the GST−GRB2 Sepharose with this phosphopeptide significantly reduced binding of most of the GRB2-binding proteins present in lysates of CSF-1-stimulated Wt CSF-1 receptor-expressing Rat-2 cells (Figure 7B). A phosphopeptide corresponding to residues 700−712 of the CSF-1 receptor, which contains a phosphorylated tyrosine at position 706, did not block the binding of P.Tyr-containing proteins to GRB2 (Figure 7B).

To confirm the *in vitro* binding data, GRB2 was immunoprecipitated from NP40 lysates obtained from control and CSF-1-stimulated cells expressing Wt or Phe697 mutant CSF-1 receptors. Immunoprecipitates were analyzed by immunoblotting with anti-P.Tyr antibodies. CSF-1 receptors were detected in GRB2 immunoprecipitates obtained from CSF-1-stimulated cells expressing Wt CSF-1 receptors (Figure 8, lane 4). In contrast, no Phe697 mutant CSF-1 receptors were detected in GRB2 immunoprecipitates obtained from CSF-1-stimulated cells (Figure 8, lanes 8 and 10). The Phe697 mutant CSF-1 receptor, however, was phosphorylated on tyrosine in response to CSF-1 in these cells (Figure 8, lane 6).

The data presented here indicate that the CSF-1 receptor associates with GRB2 upon ligand binding. Mutation of Tyr697 affects the ability of the receptor to bind GRB2 as well as its ability to transduce signals into the cell. This suggests that GRB2 binding to the CSF-1 receptor could be an important step during signal transduction.

Discussion

Understanding the mechanism of signal transduction by RPTKs has become a major challenge in molecular cell biology today. Upon ligand binding, RPTKs dimerize, autophosphorylate and activate their kinase domains (Ulrich and Schlessinger, 1990; Schlessinger and Ullrich, 1992). The most prominent substrate for activated RPTKs is usually the receptor itself, suggesting an important role for its autophosphorylation sites. Several autophosphorylation sites, which are detected after stimulation with CSF-1 *in vivo*, have been mapped in the CSF-1 receptor. Tyr807 (Roussel *et al.*, 1990; Tapley *et al.*, 1990; van der Geer and Hunter, 1990), which lies in the second half of the kinase domain, is conserved in all PTKs and is an autophosphorylation site in most (Hanks and Quinn, 1991). A Phe807 mutant murine CSF-1 receptor expressed in Rat-2 fibroblasts has reduced kinase activity *in vivo* and *in vitro*, and a decreased ability to induce morphological changes and increase growth rate

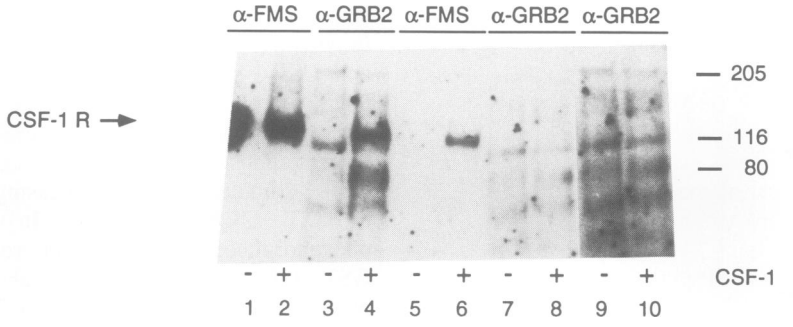

Fig. 8. Phe697 mutant CSF-1 receptors fail to associate with GRB2 in response to CSF-1 *in vivo*. Rat-2 cells expressing Wt and Phe697 mutant CSF-1 receptors grown to 90% confluence were starved overnight in 0.5% serum. CSF-1 receptor (αFMS) (lanes 1, 2, 5 and 6) and GRB2 (lanes 3, 4, 7 and 8) immunoprecipitates from control (lanes 1, 3, 5, 7 and 9) and CSF-1-stimulated cells (lanes 2, 4, 6, 8 and 10) were analyzed by immunoblotting with anti-P.Tyr antiserum. Lanes 9 and 10 show a five times longer exposure of lanes 7 and 8. The CSF-1 receptor is indicated with an arrow. The sizes of molecular weight markers are given on the right.

in response to CSF-1 (van der Geer and Hunter, 1991). A Gly807 mutant lacks kinase activity, implying that kinase activity is sensitive to conformational changes in this part of the protein (van der Geer and Hunter, 1991). Mutation of the equivalent Tyr, Tyr809, in the human CSF-1 receptor strongly reduces its ability to transform NIH3T3 fibroblasts and to induce c-*myc* RNA in the presence of CSF-1 (Roussel *et al.*, 1990, 1991). These data suggest that phosphorylation of Tyr807/809 is required for activation of the kinase domain, as is also true for other PTKs (Ellis *et al.*, 1986; Weinmaster and Pawson, 1986; Kmiecik and Shalloway, 1987; Piwnica-Worms *et al.*, 1987; Kazlauskas *et al.*, 1991). As a result it seems likely that the phenotypes resulting from mutation of Tyr807/809 are due to decreased phosphorylation of the receptor at other autophosphorylation sites.

Three other autophosphorylation sites, Tyr697, Tyr706 and Tyr721, lie in the kinase insert (Tapley *et al.*, 1990; van der Geer and Hunter, 1990; Reedijk *et al.*, 1992). Deletion of the kinase insert abrogates signal transduction in Rat-2 cells (Reedijk *et al.*, 1990). Conflicting results have been obtained with kinase insert deletion mutants expressed in NIH3T3 cells (Reedijk *et al.*, 1990; Shurtleff *et al.*, 1990; Choudhury *et al.*, 1991). No comprehensive study of the phenotype of kinase insert phosphorylation site mutants has been undertaken. Here we have shown that mutation of all three kinase insert tyrosine phosphorylation sites completely abrogates the ability of the CSF-1 receptor to alter cell morphology and increase growth rate in response to CSF-1 in Rat-2 cells. Mutation of either Tyr697 or Tyr721 results in a partial defect, whereas a double mutant displays the full phenotype. The phenotype of the CSF-1 receptor mutant lacking all the kinase insert phosphorylation sites is similar to that of the kinase insert deletion mutant in Rat-2 cells (Reedijk *et al.*, 1990). In contrast to the effects of mutating Tyr697 and Tyr721, Phe706 mutant receptors show unaltered ability to increase growth rate or change cell morphology (van der Geer and Hunter, 1991).

Ligand-induced RPTK autophosphorylation creates P.Tyr-containing sites for SH2 domain-containing proteins (Ullrich and Schlessinger, 1990; Cantley *et al.*, 1991; Pawson, 1992; Pawson and Gish, 1992; Schlessinger and Ullrich, 1992; Mayer and Baltimore, 1993). Two classes of SH2-containing proteins are known (Schlessinger and Ullrich, 1992); signal-transducing proteins that contain catalytic domains in addition to P.Tyr-binding SH2 domains (e.g. GAP and PLCγ), and proteins that contain nothing but SH2 and SH3 domains, which are believed to function as adaptor molecules and connect RPTKs to signal-transducing molecules that lack SH2 domains (e.g. GRB2, NCK and SHC). The true function of SH3 domains is not yet clear, although they bind specific proline-rich protein sequences and are involved in protein−protein interactions (Cicchetti *et al.*, 1992; Ren *et al.*, 1993).

PI 3-kinase is a heterodimer composed of an 85 kDa SH2-containing regulatory and a 110 kDa catalytic subunit (Carpenter *et al.*, 1990). Binding of p85 to P.Tyr activates PI 3-kinase activity (Backer *et al.*, 1992; Myers *et al.*, 1992), and it is suspected that tyrosine phosphorylation of PI 3-kinase also increases its activity. We have reported that Tyr721 in the CSF-1 receptor is the binding site for PI 3-kinase (Reedijk *et al.*, 1992). Here we have shown that Phe721 mutant receptors are partially defective in their signal-transducing capabilities in Rat-2 cells. This is similar to the debilitating effects of mutating both PI 3-kinase binding sites in the PDGF receptor on the induction of DNA synthesis by PDGF in some (Fantl *et al.*, 1992) but not all cell types (Yu *et al.*, 1991; Kazlauskas *et al.*, 1992). It is hard to determine how the inability to activate PI 3-kinase affects cellular responses because the role of PI 3-kinase in signal transduction is not understood. A yeast homolog of the PI 3-kinase catalytic subunit is involved in the regulation of vesicle transport during protein sorting (Herman *et al.*, 1992). This may imply that PI 3-kinase regulates events that take place at the plasma membrane in response to growth factors, like membrane ruffling or receptor downregulation. 3′ phosphoinositides produced by PI 3-kinase are poor substrates for PLC (Serunian *et al.*, 1989), but recent evidence shows that phosphatidylinositol 3,4,5-triphosphate, one of the possible products of PI 3-kinase, may regulate PKCζ (Nakanishi *et al.*, 1993).

GAP and PLCγ are common targets for activated RPTKs. However, neither GAP nor PLCγ binds the CSF-1 receptor upon activation (Downing *et al.*, 1989; Reedijk *et al.*, 1990), although the GAP-associated proteins p62 and p190 become tyrosine phosphorylated upon activation of the CSF-1 receptor (Reedijk *et al.*, 1990; Heidaran *et al.*, 1992). The c-Src protein tyrosine kinase and two other Src-family members have also been shown to be targets for activated RPTKs. c-Src, c-Yes and Fyn bind to the activated PDGF and CSF-1 receptors, and their PTK activity is stimulated (Ralston and Bishop, 1985; Gould and Hunter, 1988; Kypta *et al.*, 1990; Courtneidge *et al.*, 1993). Thus, Src family

members could play an important role in signal transduction by RPTKs. This idea is supported by the recent finding that kinase-inactive forms of Src and Fyn block PDGF-induced mitogenesis in NIH3T3 cells (Twamley-Stein *et al.*, 1993). However, based on the identification of a binding site for the c-Src SH2 domain (Songyang *et al.*, 1993), and on the identification of two tyrosines in the juxtamembrane domain of the PDGF β receptor that are important for association with c-Src (Mori *et al.*, 1993), it is unlikely that c-Src associates with the CSF-1 receptor through one of the autophosphorylation sites in the kinase insert domain. To identify other possible targets for the CSF-1 receptor we investigated NCK, GRB2 or SHC in CSF-1-stimulated cells. We find that GRB2 binds the activated CSF-1 receptor, and several lines of evidence suggest that Tyr697 in the kinase insert is a binding site for GRB2. GST−GRB2 binds Wt CSF-1 receptors in lysates of CSF-1-stimulated cells, but no P.Tyr-containing protein of the size of the CSF-1 receptor bound to GRB2 in lysates from CSF-1-stimulated Phe697 mutant CSF-1 receptor-expressing Rat-2 cells. A synthetic phosphopeptide based on the sequence around Tyr697 competed efficiently for the binding of several P.Tyr-containing proteins to GST−GRB2. In contrast, a synthetic peptide containing P.Tyr706 did not compete for binding to GST−GRB2. Furthermore, the synthetic P.Tyr697 peptide but not the P.Tyr706 peptide binds to GST−GRB2 after electrophoresis and transfer to Immobilon (data not shown). Finally, GRB2 precipitates from CSF-1-stimulated Wt CSF-1 receptor-expressing Rat-2 cells have a P.Tyr-containing protein that comigrates with the CSF-1 receptor during SDS−PAGE. This protein is absent from GRB2 precipitates from CSF-1-stimulated Phe697 mutant CSF-1 receptor-expressing cells. Tyr697 in the CSF-1 receptor is followed by the sequence Lys-Asn-Ile. Phosphopeptide binding studies show that the GRB2 SH2 domain selects P.Tyr residues followed by an Asn at position +2 (Songyang *et al.*, 1993).

Our results show that the CSF-1 receptor GRB2-binding site is important for CSF-1-induced fibroblast mitogenesis, implying that GRB2 binding activates a mitogenic signal pathway. GRB2 is a 24 kDa protein consisting of an SH2 domain located between two SH3 domains (Lowenstein *et al.*, 1992; Matuoka *et al.*, 1992). Genetic evidence from *Caenorhabditis elegans* and *Drosophila* indicates that GRB2 functions downstream of RPTKs and upstream of Ras (Olivier *et al.*, 1993; Simon *et al.*, 1993). The SH2 domain and both SH3 domains are required for its biological function (Clark *et al.*, 1992). GRB2 has also been shown to cooperate with c-Ras in the induction of DNA synthesis in quiescent fibroblasts (Lowenstein *et al.*, 1992). This cooperation is abrogated by mutations in the SH2 domain as well as in the SH3 domains. More recent biochemical studies have shown that GRB2 interacts with SOS, which is a nucleotide exchange factor for Ras, and that GRB2−SOS complexes associate with activated RPTKs, thus bringing SOS close to its target Ras on the plasma membrane (Buday and Downward, 1993; Egan *et al.*, 1993; Gale *et al.*, 1993; Li *et al.*, 1993; Rozakis-Adcock *et al.*, 1993). There is evidence that Ras activation is required for CSF-1 receptor signalling. Microinjection of the anti-Ras MAb 259 blocks v-Fms transformation of NIH3T3 cells (Smith *et al.*, 1986). Moreover, overexpression of the C-terminal catalytic domain of GAP blocks ligand-dependent transformation of NIH3T3 cells by the CSF-1 receptor (Bortner *et al.*, 1991). However, revertants of v-Ras-transformed NIH3T3 cells are still sensitive to transformation with v-Fms (Noda *et al.*, 1983; Yanagihara *et al.*, 1990). Our results suggest that GRB2 may link the CSF-1 receptor to Ras. We are currently studying the ability of the CSF-1 receptor to stimulate Ras GTP-loading and events such as MAP kinase activation that lie downstream of Ras.

GRB2 binding has not yet been shown to be critical for mitogenic signalling by any other RPTK. However, other members of the PDGF RPTK family contain a Tyr with an Asn at the +2 position within their kinase inserts: PDGF β receptor (Tyr683 and Tyr743), the SCF receptor (Tyr702) and Flk2/Flt3 (Tyr749) (Yarden *et al.*, 1986; Qiu *et al.*, 1988; Matthews *et al.*, 1991; Rosnet *et al.*, 1991). Tyr743 in the C-terminal half of the PDGF β receptor kinase insert overlaps with the GAP-binding site (Yarden *et al.*, 1986; Fantl *et al.*, 1992; Kashishian *et al.*, 1992). The PDGF α (Tyr993), CSF-1 (Tyr920) and SCF receptors (Tyr934) all contain a Tyr followed by an Asn at position +2 in the C-terminal tail (Rothwell and Rohrschneider, 1987; Qiu *et al.*, 1988; Matsui *et al.*, 1989). Tyr1068 and Tyr1086, both bona fide autophosphorylation sites in the C-terminal tail of the EGF receptor, are followed by Asn at position +2 (Downward *et al.*, 1984; Margolis *et al.*, 1989). Tyr1114 in the EGF receptor C-terminus is also followed by an Asn residue at position +2, but has not been shown to be phosphorylated in response to EGF (Margolis *et al.*, 1989). The presence of three potential binding sites may explain why GRB2 binds so strongly to the EGF receptor (Lowenstein *et al.*, 1992). The potential GRB2-binding sites in the PDGF and SCF receptors and in Flk2/Flt3 have not been identified as autophophorylation sites thus far, but GRB2 binds to autophosphorylated PDGF receptors via its SH2 domain *in vitro* (Lowenstein *et al.*, 1992).

We find that GRB2 interacts with a number of proteins that are tyrosine phosphorylated in CSF-1-stimulated Wt CSF-1 receptor-expressing Rat-2 cells. Among these proteins is SHC, which is a novel SH2-containing transforming protein that has been shown to associate with GRB2 (Lowenstein *et al.*, 1992; McGlade *et al.*, 1992; Pelicci *et al.*, 1992; Rozakis-Adcock *et al.*, 1992). SHC also binds activated EGF receptors, and is phosphorylated on tyrosine upon EGF receptor activation and in v-*src*- and v-*fps*-transformed cells. Since GRB2 binds tyrosine-phosphorylated SHC (Lowenstein *et al.*, 1992; Rozakis-Adcock *et al.*, 1992), GRB2 might bind to activated RPTKs through SHC. However, although we find that 30−40% of the tyrosine-phosphorylated CSF-1 receptors was associated with GRB2 in CSF-1-stimulated Wt CSF-1 receptor-expressing Rat-2 cells, activated CSF-1 receptors were not associated with SHC. However, in CSF-1-stimulated cells GRB2 is bound to tyrosine-phosphorylated SHC as well as several other unidentified proteins, some of which could be important for signal transduction.

Our data show that phosphorylation of Tyr697 in the CSF-1 receptor leads to GRB2 binding and activation of a GRB2-dependent pathway that may involve Ras, and that Tyr721 phosphorylation leads to the binding and activation of PI 3-kinase and a signal pathway triggered by 3′ phosphoinositides. (Since these are the only proteins known to bind to these two phosphorylation sites, we attribute the observed mutant phenotypes to the failure to bind GRB2 and PI 3-kinase, but one should bear in mind that the binding

of other SH2-containing proteins to one or both sites could contribute to the mutant phenotypes.) Why then do the Phe697 and Phe721 mutant CSF-1 receptors have similar phenotypes? Phosphorylation of both residues may regulate pathways that converge. Recent evidence suggests that PKCζ is required downstream of Ras in mitogenic signal transduction (Berra *et al.*, 1993), and since 3′ phosphoinositides are activators of PKCζ, this is a possible point of convergence. We do not know the mechanism of the CSF-1-induced morphological change, nor have we yet established how rapidly this occurs. The CSF-1 receptor could phosphorylate SH2-containing proteins that regulate the cytoskeleton, or else signal pathways activated by GRB2 and PI 3-kinase may affect the cytoskeleton. The diminished ability of the Phe697 and Phe721 mutant CSF-1 receptors to stimulate growth suggests that they may fail to induce genes normally induced by the CSF-1 receptor that are required for growth. We are currently investigating this possibility. In this regard, it is worth noting that the Phe706 mutant murine CSF-1 receptor is defective in the induction of c-*fos* in Rat-2 cells (van der Geer and Hunter, 1991), and that the Phe809 mutant human CSF-1 receptor is deficient in c-*myc* induction in NIH3T3 cells (Roussel *et al.*, 1991).

Based on the data presented here and similar work on other RPTKs, we propose that each of the CSF-1 receptor autophosphorylation sites triggers an independent signal pathway. Different pathways may cooperate to generate a full response to a mitogenic signal (e.g. those activated by phosphorylation of Tyr697 and Tyr721). The requirement for individual pathways may vary depending on the cell type, and ultimately it will be important to determine exactly which pathways are essential for CSF-1 receptor signalling in myeloid cells. It is likely that the existence of multiple receptor-activated cooperating signal pathways will be critical for obtaining appropriate cellular responses under physiological conditions where receptor numbers and ligand concentrations are much lower than those used experimentally.

Materials and methods

In vitro mutagenesis and CSF-1 receptor expression

For mutagenesis a 1164 bp *Pst*I fragment, residues 1817–2981 of the murine CSF-1 receptor cDNA (Rothwell and Rohrschneider, 1987), was inserted into M13mp18. Mutagenesis was carried out using the Amersham oligonucleotide-directed *in vitro* mutagenesis system (Amersham International plc, Amersham, UK), according to the manufacturer's manual. Mutant clones were screened by sequencing, and mutant *Pst*I fragments were reinserted into the murine CSF-1 receptor cDNA. The wild-type and mutant cDNAs were inserted into a MuLV-based retroviral expression vector (LNL–SLX CMV) that contains a neomycin resistance gene as selectable marker and a CMV promoter to drive expression of the inserted cDNA (Scharfmann *et al.*, 1991). Ψ-2 packaging cells were transfected, and selected for resistance to 400 μg/ml G418 in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum. Supernatants were filtered and used to infect Rat-2 rat fibroblasts. Cells were selected for expression of the neomycin resistance gene in DMEM containing 10% fetal bovine serum (FBS) and 400 μg/ml G418. Further analysis was done on pools of G418 resistant cells.

Antisera, immunoprecipitation and immunoblotting

For immunoprecipitation we used an anti-CSF-1 receptor C-terminal domain polyclonal rabbit antiserum (van der Geer and Hunter, 1991), anti-GRB2 polyclonal rabbit serum (#50) (Lowenstein *et al.*, 1992), anti-NCK polyclonal rabbit serum (#66) (Li *et al.*, 1992) and anti-SHC polyclonal rabbit serum (Pelicci *et al.*, 1992). For immunoprecipitations Rat-2 fibroblasts expressing Wt or mutant CSF-1 receptors were grown in DMEM containing 10% FBS to 90% confluence in 10 cm dishes. The day before cells were to be stimulated the medium was replaced by DMEM containing 20 mM HEPES pH 7.4, and the cells were starved for ~6 h. Cells were rinsed twice with ice cold phosphate-buffered saline, lysed in 1 ml ice cold RIPA buffer [10 mM sodium phosphate pH 7.0, 0.15 M NaCl, 0.1% SDS, 1% Nonidet P40 (NP40), 1% sodium deoxycholate, 2 mM EDTA, 50 mM NaF, 100 μM Na_3VO_4, 1% Trasylol (Mobay, New York, NY)] per dish and clarified by incubating with excess Pansorbin (Calbiochem, La Jolla, CA) for 1 h at 4°C and centrifugation at 10 000 *g* for 10 min at 4°C. For coprecipitation experiments cells were lysed in 1 ml NP40 lysis buffer (20 mM Tris–HCl pH 8.0, 1% NP40, 150 mM NaCl, 2 mM EDTA, 100 mM NaF, 400 μM Na_3VO_4, and 1% Trasylol per 10 cm plate and clarified by centrifugation at 10 000 *g* for 10 min at 4°C. Lysates were incubated with 5 μl polyclonal antiserum per ml lysate for 60 min at 4°C, followed by incubation for 60 min at 4°C with 25 μl recombinant protein A–Sepharose (Repligen, Cambridge, MA) on a rocker. Immune complexes were collected by centrifugation and were washed six times with RIPA or NP40 lysis buffer. Immunoprecipitates were denatured by boiling for 3 min in sample buffer (50 mM Tris–HCl pH 6.8, 10% glycerol, 2% SDS, 20% β-mercaptoethanol), and resolved by SDS–PAGE on a 10% gel unless indicated (Cooper and Hunter, 1981). For immunoblotting or phosphopeptide mapping proteins were electrophoretically transferred to Immobilon (Millipore Corporation, Bedford, MA) for 6 h at 40 V. Blots were probed with anti-P.Tyr (UBI, Lake Placid, NY) or anti-C-terminal CSF-1 receptor antibodies and [^{125}I] protein A as described previously (van der Geer and Hunter, 1991).

In vitro PTK reactions and phosphopeptide mapping

Immune complexes of murine CSF-1 receptors were washed three times with RIPA buffer, twice with 20 mM HEPES pH 7.4, and then incubated for 15 min at 37°C in 25 μl kinase buffer (20 mM HEPES pH 7.4, 10 mM $MnCl_2$ and 0.5 mM DTT) containing 25 μCi [γ-^{32}P]ATP (4500 Ci/mmol; ICN Radiochemicals, Irvine, CA). Following the autophosphorylation reaction the immune complexes were washed twice with RIPA buffer, boiled for 3 min in sample buffer, resolved by electrophoresis on a 7.5% polyacrylamide gel, electrophoretically transferred to Immobilon and exposed to X-ray film. CSF-1 receptor protein was digested with trypsin while bound to Immobilon (Luo *et al.*, 1990). Phosphopeptides were separated in two dimensions on 100 μm cellulose thin layer plates by electrophoresis at pH 1.9 (50 parts 88% formic acid; 156 parts glacial acetic acid; 1794 parts water) for 45 min a 1 kV, followed by ascending chromatography (65 parts isobutyric acid; 5 parts pyridine; 3 parts glacial acetic acid; 2 parts *n*-butanol; 29 parts water) (Scheidtmann *et al.*, 1982). Phosphopeptides were visualized by autoradiography.

Binding assays using GST–GRB2 fusion protein

A GST fusion protein containing full length human GRB2 was expressed from pGEX3X in *Escherichia coli* BL21 and purifed using glutathione–Sepharose as described (Lowenstein *et al.*, 1992). To test for binding to GRB2 *in vitro* cells were lysed in 1 ml NP40 lysis buffer per 10 cm dish, and incubated with 25 μl 50% GST–GRB2 immobilized on glutathione–Sepharose (Pharmacia, Uppsala, Sweden), containing ~1–5 μg of the fusion protein per ml of lysate. After incubation for 60 min at 4°C the Sepharose beads were washed four times with NP40 lysis buffer and twice with PBS. Bound proteins were released by boiling in SDS-sample buffer for 3 min and analyzed by SDS–PAGE and immunoblotting. Alternatively, bound proteins were released by boiling for 3 min in 100 μl phosphate-buffered saline containing 1% SDS and 5 mM DTT per 25 μl glutathione–Sepharose, the Sepharose beads were spun out and the supernatant was diluted 10-fold with RIPA buffer for subsequent analysis by immunoprecipitation and immunoblotting as described above.

Synthesis of phosphotyrosine-containing peptides

The individual N$^{\alpha}$ 9-*H*-fluorenylmethoxycarbonyl (Fmoc) protected amino acids used were obtained from Bachem, Inc. (Torrance, CA) with the exception of Fmoc-tyrosine, which was prepared in house. Peptides P.Tyr697 (Tyr-Ser-Glu-Gly-Asp-Ser-Ser-P.Tyr-Lys-Asn-Ile-His-Leu-Glu) and P.Tyr706 (Tyr-Ile-His-Leu-Glu-Lys-Lys-P.Tyr-Val-Arg-Arg-Asp-Ser-Gly) were synthesized using a solid phase-based Fmoc/Boc/*t*-Butyl approach in which the tyrosine to be phosphorylated was incorporated with an unprotected phenolic -OH group. Phosphorylation was accomplished post-synthetically by reacting the unprotected tyrosinyl -OH group of the resin-bound, fully assembled peptide with di-*t*-butyl *N,N*-diethylphosphoramidite followed by oxidation of the initially obtained P(III) species with *t*-butylhydroperoxide. The newly phosphorylated peptide was deprotected and cleaved from the resin support, and then purified by HPLC. Purity was assessed by both reverse phase HPLC and capillary zone electrophoresis. The structures of synthetic phosphopeptides were confirmed by mass spectral analysis. In

blocking experiments GST–GRB2 immobilized on glutathione–Sepharose was preincubated for 1 h at 4°C with 50 μM phosphopeptide in 20 mM HEPES, pH 7.2.

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Matuoka *et al.* [(1993) *EMBO J.*, **12**, 3467–3473] have recently reported that microinjection of anti-GRB2 antibodies into NRK rat fibroblasts inhibited EGF- and PDGF-induced DNA synthesis, consistent with an essential role for GRB2 downstream of the EGF and PDGF RPTKs in mitogenic signaling.