The EMBO Journal vol.12 no.3 pp.943-950, 1993

Activation of Src family kinases by colony stimulating factor-1, and their association with its receptor

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Communicated by S.A.Courtneidge

The receptor for the macrophage colony stimulating factor-1 (CSF-1R) is a transmembrane glycoprotein with intrinsic tyrosine kinase activity. CSF-1 stimulation promotes the growth of cells of the macrophage lineage and of fibroblasts engineered to express CSF-1R. We show that CSF-1 stimulation resulted in activation of three Src family kinases, Src, Fyn and Yes. Concomitant with their activation, all three Src family kinases were found to associate with the ligand-activated CSF-1 receptor. These interactions were also demonstrated in SF9 insect cells co-infected with viruses encoding the CSF-1 receptor and Fyn, and the isolated SH2 domain of Fyn was capable of binding the CSF-1R *in vitro*. Analysis of mutant CSF-1Rs revealed that the 'kinase insert' (KI) domain of CSF-1R was not required for interactions with Src family kinases, but that mutation of one of the receptor autophosphorylation sites, Tyr809, reduced both their binding and enzymatic activation. Because fibroblasts expressing this receptor mutant are unable to form colonies in semi-solid medium or to grow in chemically defined medium in the presence of CSF-1, the Src family kinases may play a physiological role in the mitogenic response to CSF-1.

Key words: colony stimulation factor/CSF-1 receptor/protein tyrosine kinases/Src family kinases/src homology-2 (SH2) domains

Introduction

Colony stimulating factor-1 [CSF-1, also known as macrophage (M)-CSF] stimulates the proliferation, differentiation and survival of cells of the mononuclear phagocyte lineage (Sherr and Stanley, 1990). The receptor for CSF-1 (CSF-1R) is a transmembrane glycoprotein with an intrinsic, ligand-responsive protein tyrosine kinase (PTK) activity, which is encoded by the cellular homologue of the retroviral oncogene v-*fms* (Sherr *et al.*, 1985). The CSF-1 receptor is more closely related to the α and β receptors for platelet-derived growth factor (PDGF), the c-*kit* proto-oncogene product (the receptor for Steel factor), and fibroblast growth factor (FGF) receptors than to members of other PTK receptor subfamilies (Hanks *et al.*, 1988). Even though CSF-1R expression is normally restricted to macrophages and monocytes, it can function in other cell types. For example, mouse NIH-3T3 fibroblasts engineered to express human CSF-1 receptors form colonies in agar in response to human CSF-1, proliferate continuously in chemically defined medium containing human CSF-1 as their only exogenous growth factor, and undergo transformation by an autocrine mechanism when cotransfected with vectors encoding human CSF-1 (Roussel *et al.*, 1987; Roussel and Sherr, 1989). These results imply that the CSF-1 receptor couples to a similar signal transduction machinery in macrophages and fibroblasts.

Ligand activation of growth factor receptors with PTK activity often results in their association with, and activation of, a number of cytoplasmic effector proteins that are thought to play important roles in signal transduction (reviewed in Cantley *et al.*, 1991). Among the proteins that have been shown to bind directly to activated PDGF and/or epidermal growth factor (EGF) receptors are phospholipase C-γ1 (PLC-γ1) (Kumjian *et al.*, 1989; Margolis *et al.*, 1989; Meisenhelder *et al.*, 1989; Wahl *et al.*, 1989; Morrison *et al.*, 1990), the p21*ras* GTPase activating protein GAP (Molloy *et al.*, 1989; Kaplan *et al.*, 1990; Kazlauskas *et al.*, 1990), phosphatidylinositol 3-kinase (PI3-K) (Kaplan *et al.*, 1987; Kazlauskas and Cooper, 1989), the SHC gene product (Pelicci *et al.*, 1992), GRB-2/*sem*-5 (Lowenstein *et al.*, 1992), and other tyrosine kinases of the Src family (Kypta *et al.*, 1990; Twamley *et al.*, 1992). These interactions require Src homology-2 (SH2) domains in the targeted cellular proteins (Anderson *et al.*, 1990), which in turn bind to phosphorylated tyrosine residues and their flanking sequences on the activated receptors (Fantl *et al.*, 1992; Kashishian *et al.*, 1992; Mohammadi *et al.*, 1992; Rönnstrand *et al.*, 1992; Rotin *et al.*, 1992).

In contrast to the PDGF and EGF receptors, the effector proteins that bind to the CSF-1 receptor are not well characterized. Ligand-activated CSF-1R associates with the 85 kDa subunit of PI3-K (Varticovski *et al.*, 1989) via sequences in its 'kinase insert' (or KI) domain (Reedijk *et al.*, 1990; Shurtleff *et al.*, 1990), requiring phosphorylation of Tyr723 in human or Tyr721 in murine CSF-1R (Reedijk *et al.*, 1992). However, binding of PI3-K is not required for CSF-1-stimulated cell growth, because a v-*fms* oncoprotein from which the KI domain has been deleted (ΔKI) can still transform rat fibroblasts (Taylor *et al.*, 1989), and NIH-3T3 or rat-2 cells expressing a similar deletional variant of human CSF-1R grow in response to CSF-1 (Reedijk *et al.*, 1990; Shurtleff *et al.*, 1990). Neither PLC-γ1 nor p21*ras* GAP associates with the activated CSF-1 receptor (Downing *et al.*, 1989; Reedijk *et al.*, 1992). That other downstream effectors exist is underscored by another CSF-1 receptor mutant, in which Tyr809 (a major phosphorylation site homologous to Tyr416 in Src) was replaced by phenylalanine. In spite of displaying wild-type levels of kinase activity, binding to and activating PI3-K, and selectively inducing some immediate early response genes, this CSF-1R mutant fails to induce cell proliferation

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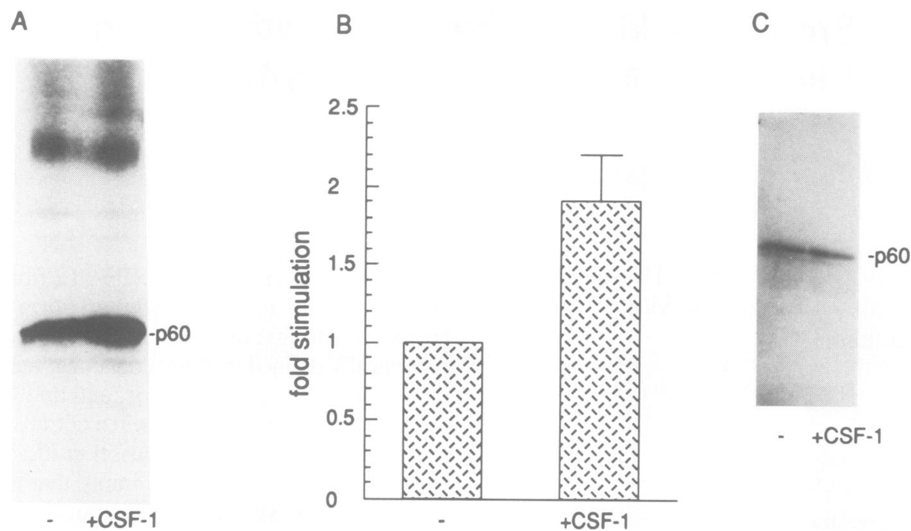

Fig. 1. Activation of Src family kinases by CSF-1. Src family kinases were immunoprecipitated from RIPA lysates of either quiescent (−) or CSF-1-stimulated (+CSF-1) NIH-3T3 cells expressing wild-type human CSF-1R, using the anti-cst.1 antibody. Immunocomplexes were assayed by either kinase assay or immunoblotting. (**A**) Kinase assay. The position of the autophosphorylated Src family kinases is marked (p60). (**B**) Kinase assay. Enolase was added to the kinase reactions as an exogenous substrate. Depicted is the fold stimulation of counts incorporated into enolase after CSF-1 stimulation, compared with the unstimulated level, which was set to 1. The values represent the mean of six independent experiments, and the error bar shows the standard deviation. (**C**) Immunoblot. Immunoprecipitates were resolved by SDS−PAGE, transferred to nitrocellulose, and probed with anti-cst.1 antibodies. The position of the autophosphorylated Src family kinases is marked (p60).

in response to CSF-1 (Roussel *et al.*, 1990, 1992). Thus, binding of PI3-K to CSF-1R appears neither necessary nor sufficient for mitogenesis, implying that an as yet unidentified component of the signal transduction machinery, dependent on Tyr809, is crucial for this response.

The Src family of tyrosine kinases comprises at least eight members, most of whose expression is restricted to certain cell types, but some of which (Src, Fyn and Yes) are more ubiquitous (Cooper, 1988). Recent examples directly implicating Src kinases in signal transduction include the association of Lck with CD4 and CD8 and its enzymatic activation upon CD4−CD8 cross-linking (Rudd *et al.*, 1988; Veillette *et al.*, 1988), the association of Fyn with the T cell receptor (Samelson *et al.*, 1990), and the interaction of Src with the FcγRII receptor (Huang *et al.*, 1992). These transmembrane receptors lack intrinsic PTK activities, and the Src family kinases can thus be considered to act as signalling subunits. Src family kinases also associate with the ligand-activated PDGF receptor tyrosine kinase (Kypta *et al.*, 1990). In this case, the proteins bind directly to the PDGF-R PTK domain, leading to phosphorylation and enzymatic activation of the Src kinases (Twamley *et al.*, 1992). Binding requires the SH2 domain of the Src family members, which interacts directly with phosphotyrosine residues in PDGF-R (Twamley *et al.*, 1992). These associations do not require phosphotyrosines within the KI domain of PDGF-R (Courtneidge *et al.*, 1991; and S.A.Courtneidge and J.A.Cooper, unpublished observations), but mutation of Tyr857, a major autophosphorylation site analogous in position and context to Tyr809 of CSF-1R, significantly reduces binding (Courtneidge *et al.*, 1991).

The similarities in overall structure and the properties of the PDGF and CSF-1 receptors mutated at their equivalent phosphorylation sites prompted us to investigate whether the activated CSF-1 receptor might also associate with Src family kinases. We report here that three members of the Src family kinases, Src, Fyn and Yes, bind to, and are activated by, ligand-stimulated CSF-1R. Their binding is mediated via their SH2 domains, and Tyr809 of CSF-1R contributes to these associations.

Results

CSF-1 activates Src family tyrosine kinases

We studied interactions between CSF-1R and Src family kinases in NIH-3T3 cells engineered to express high levels of human CSF-1 receptors (Roussel *et al.*, 1987). In spite of the fact that mouse fibroblasts secrete CSF-1, the murine growth factor fails to bind with high affinity to the human receptor and does not activate its tyrosine kinase, thereby precluding autocrine growth. To test whether CSF-1 could activate Src family kinases, quiescent CSF-1R bearing cells were stimulated with ligand, and Src, Fyn and Yes were immunoprecipitated from cell lysates using an antibody, anti-cst.1, previously shown to recognize all three proteins via their common C-terminal sequences. These were then tested both for autophosphorylation and for their ability to phosphorylate an exogenous substrate. Both the autophosphorylation of the Src family kinases (Figure 1A) and their ability to phosphorylate enolase (Figure 1B) increased ~2-fold following treatment of the cells with physiological doses of CSF-1 (3000 units/ml; 1 unit = 0.44 fmol). This was due to an increase in the specific activity of the kinases, since immunoblotting of similar immunoprecipitates showed that the levels of the proteins did not change during stimulation (Figure 1C). The degree of activation was the same as that previously reported to occur following PDGF stimulation, where only that fraction of the Src family kinases that associates with the receptor (~5%) becomes activated (Kypta *et al.*, 1990). Therefore, the 2-fold stimulation observed in total cell lysates reflects a significantly greater activation of a small percentage of molecules. The same is likely to be true for their activation by CSF-1 (see below).

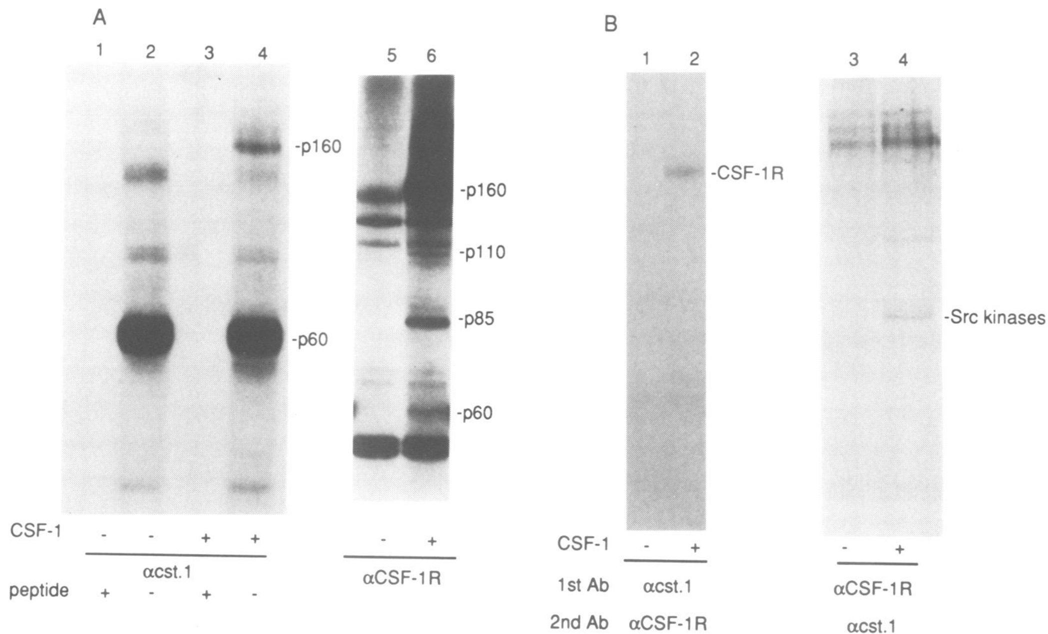

Fig. 2. Association of the CSF-1 receptor with Src family kinases. (**A**) Kinase assay of immunoprecipitates made with anti-cst.1 pre-blocked with specific peptide (lanes 1 and 3), anti-cst.1 (lanes 2 and 4), and a rat monoclonal anti-CSF-1R antibody (lanes 5 and 6), and LB lysates of quiescent (lanes 1, 2 and 5) or CSF-1-stimulated (lanes 3, 4 and 6) cells. (**B**) Reprecipitation analysis of products detected by kinase assay in anti-cst.1 (lanes 1 and 2), or anti-CSF-1R (lanes 3 and 4) immunocomplexes from unstimulated cells (lanes 1 and 3) and CSF-1-stimulated cells (lanes 2 and 4), using anti-CSF-1R (lanes 1 and 2) and anti-cst.1 (lanes 3 and 4). The human CSF-1R, previously described to have a molecular weight of 150 kDa, has a migration in the gel system used in these analyses of 160 kDa, and is therefore referred to as p160 throughout this manuscript.

Association between Src family kinases and the activated CSF-1 receptor

Given the similarities in activation of Src family kinases by PDGF and CSF-1, we next asked whether they formed physical complexes. Indeed, we detected such associations by kinase assay (Figure 2A). In anti-cst.1 immunoprecipitates, the predominant bands detected were the autophosphorylated Src family kinases themselves (the increase in autophosphorylation observed after CSF-1 stimulation is here obscured by the overexposure of the gel required to see the associated CSF-1R). After CSF-1 stimulation of intact cells and immunoprecipitation with anti-cst.1, a phosphorylated protein of ~160 kDa was also detected. This band was not seen when the antibody was pretreated with a specific competing peptide prior to addition of lysate. Given the similarity between the mobility of this band and the CSF-1 receptor itself (Figure 2A), we suspected that this protein represented activated CSF-1R. When immunoprecipitates formed under the same conditions were labelled in an *in vitro* kinase assay, dissociated with SDS, and subjected to a second round of immunoprecipitation, the 160 kDa protein was reprecipitated with antibodies to CSF-1R. The receptor was only detected in anti-cst.1 immunoprecipitates from CSF-1-stimulated cells, but not in those from quiescent cells (Figure 2B). These results clearly demonstrate that ligand stimulation of CSF-1R induced a physical association between the Src family kinases and the activated CSF-1 receptor.

Kinase assays of immunoprecipitates using antibodies specific for CSF-1R revealed the presence of several CSF-1 receptor-associated bands that were only detected upon ligand stimulation (Figure 2A). None of these were observed when the receptor was immunoprecipitated from unstimulated cells. The major bands had molecular weights of 110, 85 and 60 kDa, and were recovered using four different antibodies specific for CSF-1R. Reprecipitation analysis showed that the 85 kDa band was the p85 regulatory subunit of PI3-K (data not shown), whereas the 110 kDa band may be the catalytic subunit. The 60 kDa band was reprecipitated with the anti-cst.1 antibodies (Figure 2B), again demonstrating that Src family kinases were associated with the activated CSF-1 receptor. No 60 kDa bands were detected in association with CSF-1R isolated from quiescent cells, suggesting that these interactions were transient and ligand dependent.

In order to estimate the stoichiometry of these complexes, we performed immunoblotting analyses. Immunoprecipitates prepared with anti-cst.1 and antibodies to CSF-1R were separated on denaturing polyacrylamide gels, transferred to nitrocellulose, and probed with antibodies specific for the CSF-1 receptor. Following CSF-1 stimulation, but not before, we detected the CSF-1 receptor in the anti-cst.1 immunoprecipitates (Figure 3A). Since the antibodies were used in excess, a comparison of the amount of receptor immunoprecipitated in Src family and CSF-1R immunocomplexes could be made (see Figure 3 legend). At the concentration of CSF-1 used in this experiment (8000 units/ml), ~2–3% of total CSF-1 receptors formed complexes with the Src family kinases. We also probed similar filters with antibodies to phosphotyrosine, in which case only the activated receptors would be detected (Figure 3B). Using this approach, we estimated that 5% of activated receptors were associated with the Src family kinases. This is approximately the same stoichiometry previously reported for the association of Src family kinases with the activated PDGF receptor (Kypta *et al.*, 1990).

To address which of the Src family kinases expressed in NIH-3T3 cells were associated with the activated CSF-1 receptors, we performed similar experiments using antibodies specific for each Src family member (recognizing

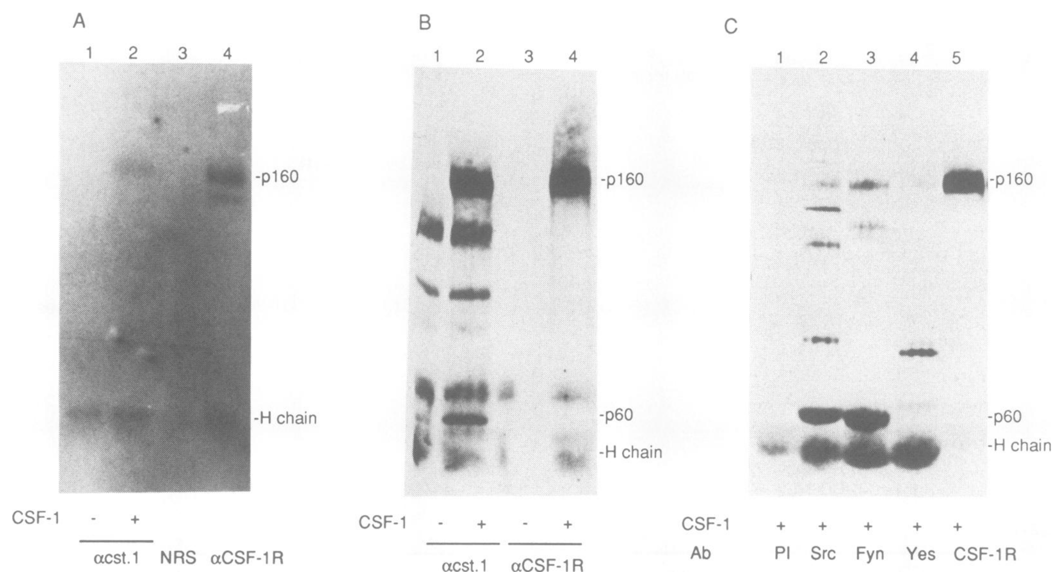

Fig. 3. Stoichiometry of association of the CSF-1 receptor with Src family kinases. (**A**) Immunocomplexes made from lysates of quiescent (lane 1) and CSF-1-stimulated (lanes 2–4) cells, and anti-cst.1 (lanes 1 and 2), normal rabbit serum (lane 3) and rabbit anti-CSF-1R (lane 4), were resolved by SDS–PAGE, transferred to nitrocellulose, and probed with antibodies specific for the CSF-1R. The positions of the CSF-1R (p160) and the immunoglobulin heavy chain (H chain) are marked. Twenty times more lysate was used in lanes 1 and 2 compared with lanes 3 and 4. (**B**) Immunocomplexes made from lysates of quiescent (lanes 1 and 3) and CSF-1-stimulated (lanes 2 and 4) cells, and anti-cst.1 (lanes 1 and 2) or rabbit anti-CSF-1R (lanes 3 and 4), were resolved by SDS–PAGE, transferred to nitrocellulose, and probed with antibodies specific for phosphotyrosine. The positions of the CSF-1R (p160), Src family kinases (p60) and the immunoglobulin heavy chain (H chain) are marked. Twenty times more lysate was used in lanes 1 and 2, compared with lanes 3 and 4. (**C**) Immunocomplexes made from lysates of CSF-1-stimulated cells, and pre-immune serum (PI, lane 1), or antibodies specific for Src (lane 2), Fyn (lane 3), Yes (lane 4) and the CSF-1 receptor (lane 5), were resolved by SDS–PAGE, transferred to nitrocellulose, and probed with antibodies specific for phosphotyrosine. The positions of the CSF-1R (p160), Src family kinases (p60) and the immunoglobulin heavy chain (H chain) are marked. Note that the Yes protein has a slightly slower migration than Src and Fyn. Ten times more lysate was used in lanes 1–4, compared with lane 5.

sequences in their respective unique domains). These results demonstrated that Src, Fyn and Yes were all associated with the activated CSF-1 receptor (Figure 3C).

The association of Fyn with the CSF-1 receptor is specific, and involves the Fyn SH2 domain

An insect cell expression system was used to study the interaction between CSF-1R and the Src family kinases. We concentrated on Fyn, which we previously showed to be active when expressed in SF9 cells (Twamley *et al.*, 1992). In Figure 4, we show the analysis of insect cells infected with baculovirus vectors encoding the EGF receptor, the CSF-1 receptor, or co-infected with each of the receptors and Fyn. Co-association of Fyn and the CSF-1 receptor was detected using antibodies specific for either protein. In contrast, no association was detected between the EGF receptor and Fyn, even though EGF-R was active, as judged by its capacity to autophosphorylate and its ability to associate with the p85 component of PI3-K (data not shown). The very faint p180 band detected in the anti-Fyn immunoprecipitate (lane 4) was not reproducibly observed, and we believe represents a low degree of non-specific association of the EGF-R with immune complexes.

The association of Fyn with the activated PDGF receptor requires the Fyn SH2 domain. To test whether its binding to the activated CSF-1 receptor was similar, we made use of glutathione *S*-transferase (GST) fusion proteins containing portions of Fyn. Figure 5 shows that an SH2-containing protein (GST–SH2FYN) was able to associate with activated CSF-1 and PDGF receptors *in vitro*, whereas neither was able to associate with an SH3-containing construct (GST–SH3FYN). It seems reasonable to assume

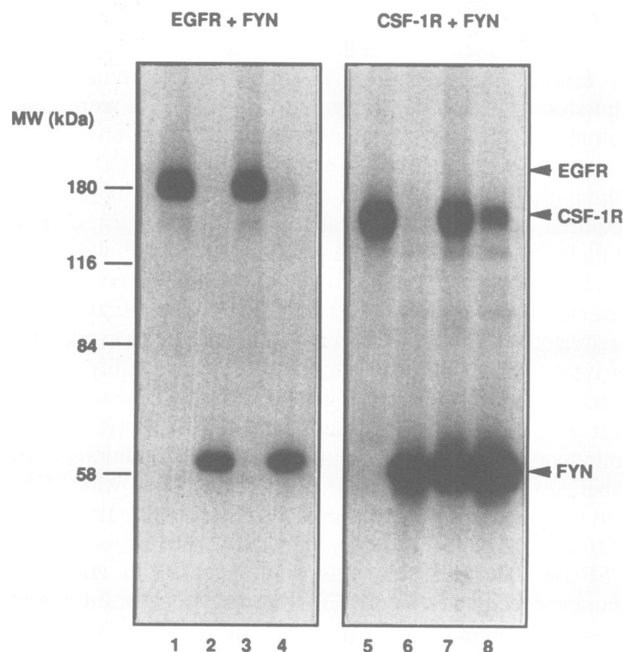

Fig. 4. Association of Fyn and the CSF-1 receptor in insect cells. Sf9 cells were infected with viruses expressing the EGF receptor (lane 1) the CSF-1R (lane 5) or Fyn (lanes 2 and 6), or co-infected with viruses expressing the EGF receptor and Fyn (lanes 3 and 4) or the CSF-1R and Fyn (lanes 7 and 8), and lysates immunoprecipitated with antibodies specific for the EGF receptor (lanes 1 and 3), Fyn (lanes 2, 4, 6 and 8) or the CSF-1R (lanes 5 and 7), and kinase assays performed. The positions of the EGF receptor, the CSF-1R, Fyn and molecular weight standards are marked.

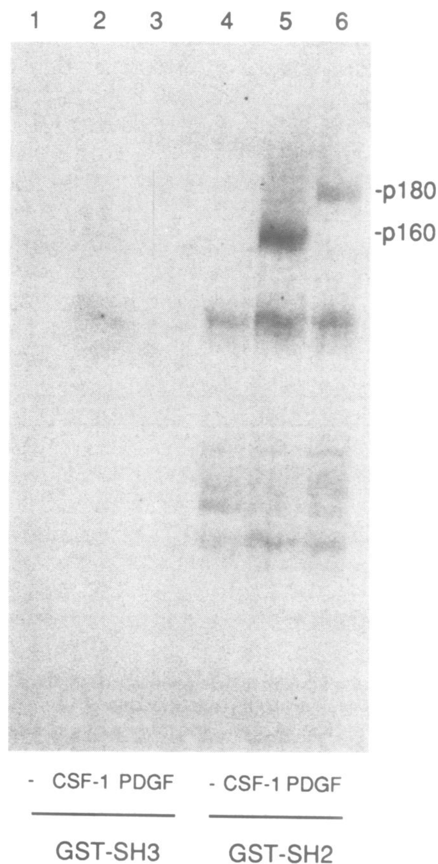

Fig. 5. The SH2 domain of Fyn mediates the association with the CSF-1 and PDGF receptors. Lysates from quiescent cells (lanes 1 and 4), or cells stimulated with either CSF-1 (lanes 2 and 4) or PDGF (lanes 3 and 6), were incubated with glutathione–Sepharose saturated with either GST–SH3FYN (lanes 1–3) or GST–SH2FYN (lanes 4–6). After washing, SDS–PAGE and transfer to nitrocellulose, blots were probed with antibodies specific for phosphotyrosine. The positions of the activated CSF-1R (p160) and PDGF receptor (p180) are marked.

that the SH2 domain is also responsible for the association of the Src family kinases with the activated CSF-1 receptor *in vivo*.

A mutant CSF-1 receptor is defective in its association with, and activation of, Src family kinases

We next tested two CSF-1 receptor mutants, to ask whether the ability of the CSF-1 receptor to associate with and/or activate Src family kinases might potentially be linked to their ability to transduce mitogenic signals. The kinase insert deletion mutant, CSF-1R(ΔKI) is unable to associate with PI3-K, but fibroblasts that express it can grow in response to CSF-1 (Shurtleff *et al.*, 1990). In contrast, CSF-1R (Phe809), while still able to associate with the PI3-K, is unable to transduce mitogenic signals in response to the growth factor (Roussel *et al.*, 1990). The CSF-1R (ΔKI) receptor, and the CSF-1R (Phe809) were expressed to the same level in these cells as wild-type CSF-1R (data not shown). Quantitative analysis (as described for Figure 3) revealed that ~3–5% of activated CSF-1R (ΔKI) associated with Src family kinases after 5 min of stimulation with CSF-1 (Figure 6). This was the same stoichiometry of association that we detected between wild-type CSF-1 receptors and the Src family kinases. However, the association of the CSF-1R (Phe809) with these kinases was significantly reduced (Figure 6): a comparison of lanes 6 and 8 shows that although the receptor was activated, and phosphorylated on tyrosine, very few of these activated molecules were able to associate with Src family kinases. From several experiments, we estimated that the latter receptor mutant showed a 2- to 5-fold reduction in its ability to associate with Src family kinases, as compared with wild-type CSF-1R. Similar results were obtained when the mutants were tested by *in vitro* kinase assays (data not shown).

We also tested the ability of CSF-1 to activate Src family kinases in cells containing mutant receptors (Figure 7). Stimulation of their kinase activity was observed in cells bearing the CSF-1R (ΔKI) mutant, although it was somewhat reduced (30–50%) compared with cells expressing wild-type receptors. In the cells expressing CSF-1R (Phe809), however, no detectable stimulation of Src family kinases was observed, either by measuring autophosphorylation (data not shown) or enolase phosphorylation (Figure 7). This was not due to the inability of the cells to respond to growth factors, because addition of PDGF resulted in the expected increase in overall Src family kinase activity. We conclude that the CSF-1R (Phe809) mutant is defective in its ability to associate with and activate Src family kinases, whereas the ΔKI mutant is not.

Discussion

When NIH-3T3 fibroblasts expressing the human CSF-1 receptor were stimulated with human recombinant CSF-1, the activity of Src family kinases rapidly increased. A transient association between the Src family kinases (Src, Fyn and Yes) and the activated CSF-1 receptor was concomitantly observed. The increase in kinase activity was due to a change in the intrinsic activity of the Src family kinases, and not that of the associated receptor, because it was detected when cells were lysed and immunoprecipitates were prepared in RIPA buffer, conditions under which the CSF-1 receptor tyrosine kinase is inactive. Between 2 and 5% of the total Src family proteins in cells bound to ligand-activated CSF-1R, similar to the stoichiometries of their binding to the activated PDGF receptor (Kypta *et al.*, 1990). In the latter case, activation occurs in conjunction with phosphorylation of the Src family proteins themselves, and it is possible that these phosphorylations contribute directly to their increased enzymatic activity (Twamley *et al.*, 1992). In preliminary experiments, we have observed phosphorylation of Fyn at similar sites when co-expressed in insect cells with the CSF-1 receptor (G.M.Twamley and S.A.Courtneidge, unpublished observations), raising the possibility that activation of Fyn by CSF-1 and PDGF occurs via a similar mechanism.

In fibroblasts, the three Src family kinases expressed (Src, Fyn and Yes) associated with the activated CSF-1 receptor. Experiments using a CSF-1 dependent murine macrophage cell line, BAC1.2F5 (which expresses Src and Fyn), and the anti-cst.1 antibody, have yielded similar results (data not shown). In addition, macrophages also express other members of the Src family, including Hck and Fgr (Cooper, 1988), which are not recognized by the anti-cst.1 antibody, and it remains possible that these kinases might also associate with CSF-1R.

An impetus for examining possible associations of the Src

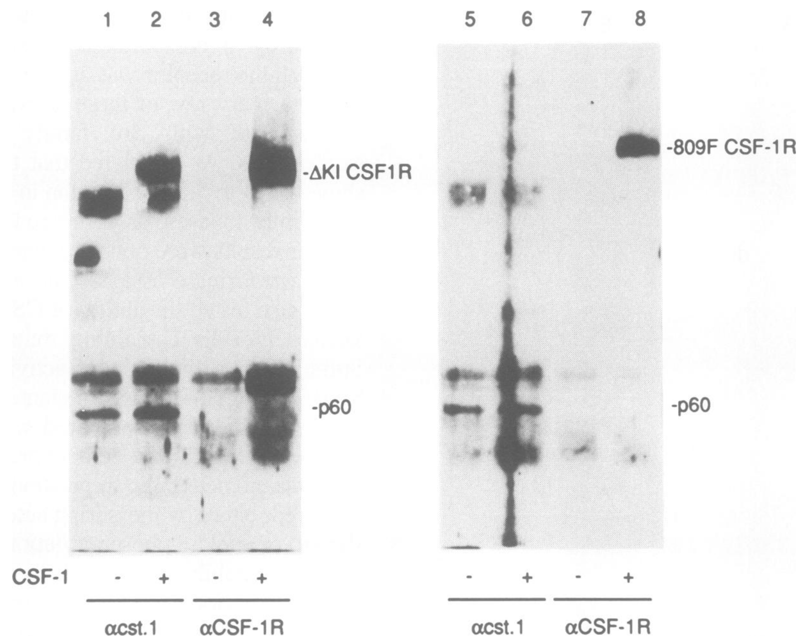

Fig. 6. Association of mutant CSF-1 receptors with Src family kinases. Cells containing either the CSF-1R (ΔKI) (lanes 1–4) or the CSF-1R (Phe809) (lanes 5–8), were either left quiescent (odd lanes) or stimulated with CSF-1 (even lanes). Src family kinases (lanes 1, 2, 5 and 6) and CSF-1R (lanes 3, 4, 7 and 8) were immunoprecipitated, resolved by SDS–PAGE, transferred to nitrocellulose, and probed with antibodies specific for phosphotyrosine. The positions of the CSF-1R and the Src family kinases (p60) are marked. Ten times more lysate was used for the anti-cst.1 immunoprecipitates compared with those made with anti-CSF-1R.

family kinases with the activated CSF-1 receptor was the similarity in phenotype of equivalent mutants of the PDGF and CSF-1 receptors. Cells expressing the PDGF-R (Phe857) mutant grow less well than wild-type in response to PDGF (Kazlauskas *et al.*, 1991), and among other defects, the compromised receptor associates only weakly with Src family kinases (Courtneidge *et al.*, 1991). Likewise, CSF-1R (Phe809), even though it remains able to associate with PI3-K, is unable to transmit a mitogenic signal. We show here that this mutant is also defective in its ability to associate with, and enzymatically activate, Src family kinases. This result raises the possibility that these properties are critical for mitogenic signalling through the CSF-1 receptor. Enforced expression of c-*myc* in cells bearing CSF-1R (Phe809) can resensitize these cells to the mitogenic effects of the growth factor (Roussel *et al.*, 1992), suggesting perhaps that one function of activated Src family kinases is to initiate signals that culminate in c-*myc* transcription. If this is the case, co-expression of CSF-1R (Phe809) together with an activated Src family kinase might also allow such cells to proliferate in the presence of CSF-1.

Does Tyr809 represent a binding site for the Src family kinases? If it does, then there must be more than one receptor binding site, since mutation of this residue reduces, but does not completely eliminate, binding. Either the binding sites are redundant, or the inability of the mutant receptor to undergo phosphorylation at Phe809 might reduce its phosphorylation at other sites, although mapping of tryptic phosphopeptides does not support the latter interpretation (Roussel *et al.*, 1990). We are therefore currently attempting to identify other potential binding site(s) in CSF-1R, using a combination of direct phosphopeptide binding assays and site-directed mutagenesis. This approach seems warranted, because not all tryptic phosphopeptides detected in the ligand-stimulated CSF-1 receptor have as yet been assigned (Downing *et al.*, 1991).

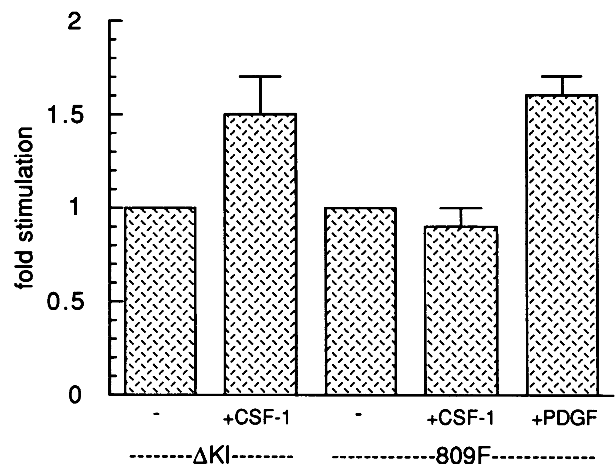

Fig. 7. Activation of Src family kinases by mutant CSF-1 receptors. Src family kinases immunoprecipitated from RIPA lysates of cells expressing CSF-1R (ΔKI) or CSF-1R (Phe809) were tested in kinase assays using enolase as substrate. Depicted is the fold stimulation of counts incorporated into enolase after CSF-1 treatment, compared with the unstimulated level, which was set to 1. The values represent the mean of three to five independent experiments, and the error bars show the standard deviation in each case. Student's *t*-test shows that the ΔKI +CSF-1 is significantly different from ΔKI −CSF-1, whereas the 809F +CSF-1 does not differ significantly from the untreated control.

Phenotypic analysis of mutant mice has suggested similarities in animals defective in Src and CSF-1R signalling. Both targeted disruption of c-*src* (Soriano *et al.*, 1991) and naturally occurring mutations that inactivate the mouse CSF-1 gene (the *op*/*op* phenotype) (Wiktor-Jedrzejczak *et al.*, 1990; Yoshida *et al.*, 1990) lead to osteopetrosis, due to the failure of osteoclasts to remodel bone. Although both syndromes involve the same target cells, they are not equivalent. The *op*/*op* mice exhibit a decreased

capacity to form osteoclasts, whereas in Src-deficient mice, the defect does not affect osteoclast number but rather the intrinsic functional capacity of the mature cells to resorb bone (P.Soriano, personal communication). CSF-1 exerts pleiotropic effects on cells of the mononuclear phagocyte lineage by supporting differentiation, survival, proliferation and effector functions, depending in part on the state of maturation of the target cells (Sherr and Stanley, 1990). Perhaps Src family kinases do not engage CSF-1R in all stages of macrophage development, but rather act to transduce signals only in more mature, differentiated cells. Alternatively, because several members of the Src family associate with the activated CSF-1 receptor, each might transduce different signals, with Src being specifically involved in osteoclasts.

Materials and methods

Antibodies

Antibodies that recognize Src family kinases have been described previously (Courtneidge and Smith, 1984; Kypta *et al.*, 1988, 1990). The peptide used to block the anti-cst.1 binding represents amino acids 527–533 in the C-terminal domain of c-Src (Courtneidge and Smith, 1984). Antibodies to phosphotyrosine were purchased from Upstate Biotechnology Incorporated (UBI), and used according to the manufacturer's recommendations. For CSF-1 receptor immunoprecipitations, we used polyclonal rabbit antisera (Furman *et al.*, 1986) and rat monoclonal antibodies (Ashmun *et al.*, 1989), as previously described. Immunoblotting of the CSF-1 receptor was performed with a polyclonal rabbit antiserum to murine CSF-1R, generously provided by Dr Larry Rohrschneider.

Growth factors

Recombinant human CSF-1 was the kind gift of Dr Steven Clark (Genetics Institute, Cambridge, MA) and was used at 1000–8000 units/ml as indicated in the text and figure legends. Porcine PDGF (BB homodimer) was purchased from UBI and used at 20 ng/ml. Stimulation of quiescent cells with the growth factors was conducted at 37°C for 5 min prior to cell lysis.

Cell lines

The NIH-3T3 cells expressing wild-type and mutant human CSF-1 receptors have been described previously (Roussel *et al.*, 1987, 1990; Shurtleff *et al.*, 1990). Cells were maintained in DMEM containing 10% fetal calf serum (FCS). They were growth arrested at confluence for 2 days and then incubated overnight in serum-free DMEM supplemented with 5 μg/ml of insulin and 5 μg/ml of transferrin prior to stimulation with growth factors.

Baculoviruses and insect cell infections

Construction of a recombinant baculovirus expressing Fyn, and infection of insect cells has been described in Twamley *et al.* (1992). A baculovirus vector encoding CSF-1R was generously provided by Dr D.Morrison. Infections, lysis of cells, and analysis of the expressed proteins was carried out as described in Otsu *et al.* (1991); Twamley *et al.* (1992).

GST fusion proteins

DNA fragments suitable for subcloning the SH2FYN (residues 148–246 of human Fyn) and SH3FYN (residues 86 and 139 of human Fyn) domains were obtained by polymerase chain reaction. The oligonucleotides used as primers contained *Nde*I sites (the 5′ oligos) and *Bam*HI sites (the 3′ oligos). SH3FYN domain: 5′-AGAATTCCCATATGACGGGGACCTTGCGT-ACGAGAGG-3′ and 5′-CAAGGATCCTTACTGGATAGAGTCAACT-GGAGCC-3′; SH2FYN domain: 5′-AGATTCCCATATGGCTCCAGTT-GACTCTATCCAGGC-3′ and 5′-CAAGGATCCTTAATCGGTAAGC-CTTGGCATCCC-3′.

Following the PCR reactions, the DNA products were digested with *Nde*I and *Bam*HI, and ligated into pGEX-2T (Pharmacia, modified by V.Baldin to contain an *Nde*I site) that had been similarly digested. The recombinant plasmid was introduced into *Escherichia coli*, and the bacterial transformants analysed for the presence of the insert. GST fusion proteins were expressed after induction with 1 mM IPTG and purified from bacterial lysates using glutathione–Sepharose as described (Kaelin *et al.*, 1991). For binding analyses, 5 μl of glutathione–Sepharose beads saturated with either GST–SH3FYN or GST–SH2FYN were incubated with cell extracts (100 μl of a 500 μg/ml solution) for 1 h at 4°C. The beads were then washed four times in LB (20 mM Tris, pH 7.5, 150 mM CaCl, 1% NP-40, 1% aprotinin, 20 μM leupeptin, 100 μM sodium orthovanadate) and once in TBS (20 mM Tris, pH 7.5, 150 mM NaCl, 100 μM sodium orthovanadate) and the bound proteins were eluted with SDS.

Other biochemical analyses

Methods for immunoprecipitation of proteins, kinase assays and SDS–PAGE have all been described before (Kypta *et al.*, 1990; Twamley *et al.*, 1992). Briefly, cells were rinsed twice with cold TBS and then lysed by scraping into LB, except for the experiments shown in Figures 1 and 7, where cells were lysed in RIPA buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1% aprotinin, 20 μM leupeptin, 100 μM sodium orthovanadate). Lysed cells were transferred to microfuge tubes, vortexed, incubated 10 min and then centrifuged for 10 min at 10 000 *g* to remove insoluble material. Lysates were incubated with antisera for 60 min and then centrifuged for 10 min before transfer into microfuge tubes containing 10 μl *Staphylococcus aureus* (for kinase assays) or 10 μl protein A–Sepharose (for immunoblotting experiments) and incubation for 30 min. All incubations and centrifugations were at 4°C. Immune precipitates were washed four times with LB or RIPA as appropriate and once with TBS. Kinase assays were performed in 20 μl of 20 mM HEPES pH 7.5, 10 mM $MnCl_2$, containing 2–10 μCi [γ-^{32}P]ATP (3000–5000 Ci/mmol) for 4–10 min at 30°C. In the experiments shown in Figure 2A, the gels were treated with 1 M KOH at 55°C for 1 h after electrophoresis and before autoradiography to remove background due to serine phosphorylation. In the reprecipitation experiments shown in Figure 2B, the kinase assays were stopped in TBS, and after centrifugation, the *S.aureus* pellet was resuspended in 0.5% SDS and boiled for 2 min. *Staphylococcus aureus* was removed by centrifugation, the supernatant was diluted 5-fold with RIPA buffer lacking SDS, and immunoprecipitations were carried out with the indicated antibodies.

For immunoblotting experiments, transfer of proteins to nitrocellulose (BA85, Schleicher and Schuell) was performed using a semi-dry apparatus according to the manufacturer's instructions (Millipore). Following incubation with the indicated antibodies, blots were probed with ^{125}I-labelled protein A (Amersham, 1.11 GBq/mg) in the case of rabbit antibodies, and horseradish peroxidase-coupled anti-mouse antibodies followed by the ECL detection reagent (Amersham) in the case of the anti-phosphotyrosine antibodies.

Acknowledgements

We thank Charles J.Sherr and Giulio Draetta for helpful suggestions and criticisms of the manuscript, Larry Rohrschneider for the generous gift of CSF-1 receptor antibodies, Dr Steven Clark for recombinant CSF-1, Dr Deborah Morrison for the baculovirus encoding CSF-1R, Angelika Heber and Carol Bockhold for maintenance of cell lines, Veronique Baldin for the pGEX-2T vector, and our colleagues for advice and encouragement. M.F.R. is supported in part by NIH grant CA-56819 and gratefully acknowledges support from ALSAC of St Jude Children's Research Hospital.

References

Anderson,D., Koch,C.A., Grey,L., Ellis,C., Moran,M.F. and Pawson,T. (1990) *Science*, **250**, 979–982.

Ashmun,R.A., Look,A.T., Roberts,W.M., Roussel,M.F., Seremetis,S.T., Ohtsuka,M. and Sherr,C.J. (1989) *Blood*, **73**, 827–837.

Cantley,L.C., Auger,K.R., Carpenter,C., Duckworth,B., Graziani,A., Kapeller,R. and Soltoff,S. (1991) *Cell*, **64**, 281–302.

Cooper,J.A. (1990) In Kemp,B.E. (ed.), *Peptides and Protein Phosphorylation*. CRC Press Inc., Boca Raton, FL, pp. 104–113.

Courtneidge,S.A. and Smith,A.E. (1984) *EMBO J.*, **3**, 585–591.

Courtneidge,S.A., Kypta,R.M., Cooper,J.A. and Kazlauskas,A. (1991) *Cell Growth Diff.*, **2**, 483–486

Downing,J.R., Margolis,B.L., Zilberstein,A., Ashmun,R.A., Ullrich,A., Sherr,C.J. and Schlessinger,J. (1989) *EMBO J.*, **8**, 3345–3350.

Downing,J.R., Shurtleff,S.A. and Sherr,C.J. (1991) *Mol. Cell. Biol.*, **11**, 2489–2495.

Fantl,W., Escobedo,J.A., Martin,G.A., Turck,C.W., del Rosario,M., McCormick,F. and Williams,L.T. (1992) *Cell*, **69**, 413–424.

Furman,W.L., Rettenmier,C.W., Chen,J.H., Roussel,M.F., Quinn,C.O. and Sherr,C.J. (1986) *Virology*, **152**, 432–445.

Hanks,S.J., Quinn,A.M. and Hunter,T. (1988) *Science*, **241**, 42–52.

Huang,M.M., Indik,Z., Brass,L.F., Hoxie,J.A., Schreiber,A.D. and Brugge,J.S. (1992) *J. Biol. Chem.*, **267**, 5467–5473.

Kaelin,W.G., Pallas,D.C.J., DeCaprio,J.A., Kaye,F.J. and Livingston,D.M. (1991) *Cell*, **64**, 521–532.
Kaplan,D.R., Whitman,M., Schaffhausen, B., Pallas,D.C., White,M., Cantley,L. and Roberts,T.M. (1987) *Cell*, **50**, 1021–1029.
Kaplan,D.R., Morrison,D.K., Wong,G., McCormick,F. and Williams,L.T. (1990) *Cell*, **61**, 125–133.
Kashishian,A., Kazlauskas,A. and Cooper,J.A. (1992) *EMBO J.*, **11**, 1373–1382.
Kazlauskas,A. and Cooper,J.A. (1989) *Cell*, **58**, 1121–1133.
Kazlauskas,A., Ellis,C., Pawson,T. and Cooper,J.A. (1990) *Science*, **247**, 1578–1581.
Kazlauskas,A., Durden,D.L. and Cooper,J.A. (1991) *Cell Regul.*, **2**, 413–425.
Kumjian,D.A., Wahl,M.I., Rhee,S.G. and Daniel,T.O. (1989) *Proc. Natl Acad. Sci. USA*, **86**, 8232–8236.
Kypta,R.M., Hemming,A. and Courtneidge,S.A. (1988) *EMBO J.*, **7**, 3837–3844.
Kypta,R.M., Goldberg,Y., Ulug,E.T. and Courtneidge,S.A. (1990) *Cell*, **62**, 481–492.
Lowenstein,E.J., Daly,R.J., Batzer,A.G., Li,W., Margolis,B., Lammers,R., Ullrich,A., Skolnik,E.Y., Bar-Sagi,D. and Schlessinger,J. (1992) *Cell*, **70**, 431–442.
Margolis,B., Rhee,S., Felder,S., Mervic,M., Lyall,R., Levitzki,A., Ullrich,A., Zilberstein,A. and Schlessinger,J. (1989) *Cell*, **57**, 1101–1107.
Meisenhelder,J., Suh,P.-G., Rhee,S.G. and Hunter,T. (1989) *Cell*, **57**, 1109–1122.
Mohammadi,M., Dionne,C.A., Li,W., Li,N., Spivak,T., Honegger,A.M., Jaye,M. and Schlessinger,J. (1992) *Nature*, **358**, 681–684.
Molloy,C.J., Bottaro,D.P., Fleming,T.P., Marshall,M.S., Gibbs,J.B. and Aarsonson,S.A. (1989) *Nature*, **342**, 711–714.
Morrison,D.K., Kaplan,D.R., Rhee,S.G. and Williams,L.T. (1990) *Mol. Cell. Biol.*, **10**, 2359–2366.
Otsu,M., Hiles,I., Gout,I., Fry,M.J., Ruiz-Larrea,F., Panayotou,G., Thompson,A., Dhand,R., Hsuan,J., Totty,N., Smith,A.D., Morgan,S.J., Courtneidge,S., Parker,P.J. and Waterfield,M.D. (1991) *Cell*, **65**, 91–104.
Pelicci,G., Lanfrancone,L., Grignani,F., McGlade,J., Cavallo,F., Forni,G., Nicoletti,I., Grignani,F., Pawson,T. and Pelicci,P.G. (1992) *Cell*, **70**, 93–104.
Reedijk,M., Liu,X. and Pawson,T. (1990) *Mol. Cell. Biol.*, **10**, 5601–5608.
Reedijk,M., Liu,X., Van der Geer,P., Letwin,K., Waterfield,M.D., Hunter,T. and Pawson,T. (1992) *EMBO J.*, **11**, 1365–1372.
Rönnstrand,L., Mori,S., Arridsson,A.-K., Eriksson,A., Wernstedt,C., Hellman,U., Claesson-Welsh,L. and Heldin,C.-H. (1992) *EMBO J.*, **11**, 3911–3926.
Rotin,D., Margolis,B., Mohammadi,M., Daly,R.J., Daum,G., Li,N., Fischer,E.H., Burgess,W.H., Ullrich,A. and Schlessinger,J. (1992) *EMBO J.*, **11**, 559–567.
Roussel,M.F. and Sherr,C.J. (1989) *Proc. Natl Acad. Sci. USA*, **86**, 7924–7927.
Roussel,M.F., Dull,T.J., Rettenmier,C.W., Ralph,P., Ullrich,A. and Sherr,C.J. (1987) *Nature*, **325**, 549–552.
Roussel,M.F., Shurtleff,S.A., Downing,J.R. and Sherr,C.J. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 6738–6742.
Roussel,M.F., Cleveland,J.L., Shutleff,S.A. and Sherr,C.J. (1992) *Nature*, **353**, 361–363.
Rudd,C.E., Trvillyan,J.M., Dasgupta,J.D., Wong,L.L. and Schlossmann,S.F. (1988) *Proc. Natl Acad. Sci. USA*, **85**, 3277–3281.
Samelson,L.E., Phillips,A.F., Luong,E.T. and Klausner,R.D. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 4358–4362.
Sherr,C.J. and Stanley,E.R. (1990) In Sporn,M.B. and Roberts,A.B. (eds), *Peptide Growth Factors and Their Receptors*. Springer-Verlag, Heidelberg, pp. 667–698.
Sherr,C.J., Rettenmier,C.W., Sacca,R., Roussel,M.F., Look,A.T. and Stanley,E.R. (1985) *Cell*, **41**, 665–676.
Shurtleff,S.A., Downing,J.R., Rock,C.O., Hawkins,S.A., Roussel,M.F. and Sherr,C. (1990) *EMBO J.*, **9**, 2415–2421.
Soriano,P., Montgomery,C., Geske,R. and Bradley,A. (1991) *Cell*, **64**, 693–702.
Taylor,G.R., Reedijk,M., Rothwell,V., Rohrschneider,L. and Pawson,T. (1989) *EMBO J.*, **8**, 2029–2037.
Twamley,G., Hall,B., Kypta,R. and Courtneidge,S.A. (1992) *Oncogene*, **7**, 1893–1901.
Varticovski,L., Druker,B., Morrison,D., Cantley,L. and Roberts,T. (1989) *Nature*, **342**, 699–702.
Veillette,A., Bookman,M.A., Horak,E.M. and Bolen,J.B. (1988) *Cell*, **55**, 301–308.
Wahl,M., Nishibe,S., Suh,P.-G., Rhee,S.,G. and Carpenter,G. (1989) *Proc. Natl Acad. Sci. USA*, **86**, 1568–1572.
Wiktor-Jedrzejczak,W., Bartocci,A., Ferrante Jr,A.W., Ahmed-Ansari,A., Sell,K.W., Pollard,J.W. and Stanley,E.R. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 4828–4832.
Yoshida,H., Hayashi,S.I., Kunisada,T., Ogawa,M., Nishikawa,S., Okamura,H., Sudo,T., Shultz,L.D. and Nishikawa,S.I. (1990) *Nature*, **345**, 442–444.

Received on November 11, 1992; revised on December 11, 1992