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Colony stimulating factor-1 (CSF-1) stimulates temperature dependent phosphorylation and activation of the RAF-1 proto-oncogene product

Manuela Baccarini, David M.Sabatini, Harald App[1], Ulf R.Rapp[1] and E.Richard Stanley

Department of Developmental Biology and Cancer, Albert Einstein College of Medicine, Bronx, NY 10461 and [1]Laboratory of Viral Carcinogenesis, National Cancer Institute, Frederick, MD 21701, USA

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The serine/threonine kinase RAF-1 is phosphorylated in intact macrophages in response to CSF-1 at 37°C The augmented phosphorylation of RAF-1 and a concomitant increase in RAF-1 associated serine/threonine kinase activity are kinetically later events than CSF-1 induced protein tyrosine phosphorylation. Furthermore, phosphoamino acid analysis of RAF-1 reveals the presence of phosphoserine, trace amounts of phosphothreonine but no phosphotyrosine and the phosphorylated RAF-1 does not react with anti-phosphotyrosine antibodies. In contrast to CSF-1 induced protein tyrosine phosphorylation, RAF-1 phosphorylation and activation are temperature dependent and do not occur at 4°C. Furthermore, coprecipitation experiments failed to reveal any non-covalent association of RAF-1 with the CSF-1 receptor. Thus, while RAF-1 is not a direct substrate for the CSF-1 receptor tyrosine kinase *in vivo*, its temperature dependent phosphorylation and activation represent an intriguing aspect of the CSF-1 response.

Key words: CSF-1/PDGF/growth factor receptors/RAF-1 kinase/signal transduction

Introduction

Colony stimulating factor-1 (CSF-1) is a growth factor that selectively mediates the survival, proliferation and differentiation of cells of the mononuclear phagocytic lineage (reviewed in Stanley *et al.*, 1983). Its action is mediated via cell surface receptor identical to the c-*fms* proto-oncogene product (Sherr *et al.*, 1985; Guilbert and Stanley, 1986; Roussel *et al.*, 1987; Yeung *et al.*, 1987). The CSF-1 receptor (CSF-1R) is a member of a family of tyrosine kinase receptors which includes the A and B isoforms of the platelet derived growth factor (PDGF) receptor (Yarden *et al.*, 1986), the c-*kit* proto-oncogene product (Yarden *et al.*, 1987) and the receptor for basic fibroblast growth factor (Lee *et al.*, 1989).

At 37°C within 30 s of addition of CSF-1 to macrophages, the tyrosine phosphorylation of an array of cytosolic and membrane-bound proteins can be demonstrated by immunoprecipitation of ^{32}P-labeled proteins (Sengupta *et al.*, 1988) or Western blotting with an anti-phosphotyrosine antibody (Downing *et al.*, 1988). Phosphorylation of these proteins also occurs, with slower kinetics, at 4°C, a condition under which internalization of the ligand–receptor complex is prevented (Sengupta *et al.*, 1988). The identity of these tyrosine-phosphorylated proteins is still unknown, but many of them correspond in size to proteins whose tyrosine phosphorylation has been shown to be increased in fibroblasts after PDGF treatment (Frackelton *et al.*, 1984) or transformation with the v-*fms* oncogene (Morrison *et al.*, 1988a).

Recently, the product of the proto-oncogene RAF-1 has been identified as one of the proteins phosphorylated on tyrosine in response to PDGF (Morrison *et al.*, 1988b, 1989). RAF-1 is a 74 kd serine/threonine kinase localized primarily in the cytosol (Molders *et al.*, 1985; Gastl *et al.*, 1986; Moelling *et al.*, 1984; Schultz *et al.*, 1988). It is phosphorylated *in vivo* in serine and threonine, but, in contrast to its transforming counterpart, v-*raf*, it does not have demonstrable autophosphorylating activity (Schultz *et al.*, 1988). This is consistent with a mechanism for oncogenic activation of RAF-1 that involves amino-terminal truncation and release of the molecule from the negative regulation of its kinase domain (Bonner *et al.*, 1986). In normal cells, activation of the RAF-1 protein may be achieved by enzymatic modification of the amino-terminal domain or, alternatively by the binding of regulatory ligands to the same region.

In this paper, we show that RAF-1 is not among the proteins that are rapidly phosphorylated on tyrosine residues following CSF-1 stimulation of macrophages *in vivo*. CSF-1 induces phosphorylation of RAF-1 on serine and threonine residues exclusively. The increased phosphorylation is accompanied by an increase in RAF-1 associated serine/threonine kinase activity as measured in an *in vitro* immune complex kinase assay. Experiments on the PDGF response, carried out in parallel, yielded similar results, indicating that these phenomena represent a common aspect of the response to these two growth factors. Interestingly, in contrast to protein tyrosine phosphorylation, neither phosphorylation nor activation of RAF-1 are observed when cells are incubated with CSF-1 at 4°C.

Results

Kinetics of the CSF-1 induced RAF-1 phosphorylation at 37°C and 4°C

To investigate the kinetics of RAF-1 phosphorylation in the CSF-1 response, CSF-1 starved BAC1.2F5 cells were preincubated with [^{32}P]orthophosphate and [^{3}H]leucine for 3 h at 37°C and then stimulated with 13.2 nM CSF-1 for 10 s, 30 s, 1.5 min, 5 min and 15 min at 37°C. RAF-1 was isolated by immunoprecipitation with a rabbit polyclonal antiserum from whole cell lysates and analyzed by SDS–PAGE. Figure 1a, lane 1, shows that the ^{32}P-labeled RAF-1 immunoprecipitated from unstimulated cells ran as a 72/74 kd doublet. CSF-1 stimulation resulted in an increased ^{32}P incorporation into both RAF-1 proteins, maximum incorporation being observed 5 min after stimulation (Figure 1a, lanes 2–6; and Figure 1c). Further-

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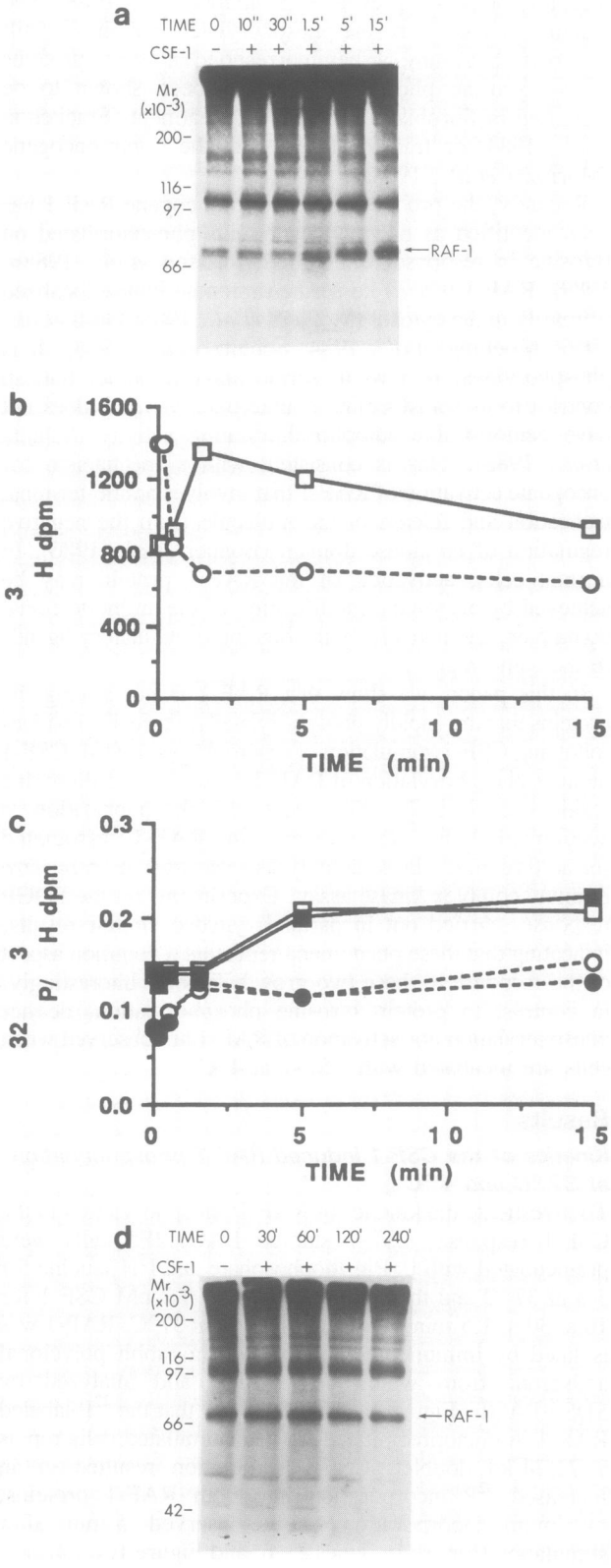
more, accumulation of the 74 kd form of the protein as well as an increase in the apparent molecular weight of the doublet were observed with time of stimulation.

The individual $^{3}H/^{32}P$-labeled RAF-1 bands shown in Figure 1a were excised, dissolved and counted in a liquid scintillation counter programmed to determine d.p.m. in dual labeled samples. The ^{3}H d.p.m. recovered in the 72 kd or 74 kd RAF-1 bands with time of stimulation are shown in Figure 1b. The sum of the ^{3}H d.p.m. in each band at any time point was relatively constant, although there was a slight decrease in the total amount of RAF-1 protein recovered by immunoprecipitation with time of stimulation. In unstimulated cells, ~60% of the total ^{3}H d.p.m. incorporated into the RAF-1 protein was recovered in the 72 kd band. CSF-1 stimulation rapidly decreased this percentage to 35% after 1.5 min of stimulation.



The change in the ratio of [^{32}P]orthophosphate and [^{3}H]leucine incorporated into each of the bands with time is shown in Figure 1c. ^{32}P incorporation in the 72 kd band increased sharply in the first 1.5 min after CSF-1 stimulation to approximately the level of incorporation in the 74 kd band, which remained constant during the same period. This sharp increase in phosphorylation of the 72 kd band was temporally correlated with the 72–74 kd shift (Figure 1b). The 74 kd band was further phosphorylated after 1.5 min, consistent with a further decrease in its mobility, as noted in Figure 1a. These results suggested that the difference in mobility between the 72 kd and 74 kd bands is due to a difference in incorporated phosphate.

Morrison *et al.* (1988b) attributed the altered RAF-1 migration pattern following PDGF stimulation to phosphorylation, reporting that phosphatase treatment of the RAF-1 immune complexes increased RAF-1 mobility. Treatment of the RAF-1 immune complexes from CSF-1 stimulated cells with calf intestine alkaline phosphatase (CIAP) (Figure 2A and B) as well as with potato acid phosphatase (data not shown) abolished the CSF-1 induced shift in RAF-1 molecular weight (Figure 2A), and caused almost complete dephosphorylation of ^{32}P-labeled RAF-1 immunocomplexes (Figure 2B). The presence of phosphatase inhibitors impaired dephosphorylation and abrogated the shift in molecular weight. Interestingly, when BAC1.2F5 cells were stimulated with CSF-1 at 4°C for a prolonged time, under conditions in which phosphorylation of proteins on tyrosine was observed (Sengupta *et al.*, 1988), neither increased phosphorylation of the RAF-1 protein, nor alterations in its migration pattern were seen (Figure 1d). The failure to alter the electrophoretic mobility of RAF-1 by stimulation with CSF-1 at 4°C can be better appreciated in the immunoblot shown in Figure 6A and D, in which RAF-1 immunoprecipitates from unstimulated cells as well as from cells stimulated at 4°C (60 min) or at 37°C (5 min) were run side by side and immunoblotted with anti-RAF-1 antisera.

RAF-1 is not phosphorylated on tyrosine in response to growth factor stimulation

A series of experiments were carried out to determine whether RAF-1 is a candidate substrate of the CSF-1R kinase *in vivo*. Phosphoamino acid analysis of the ^{32}P-labeled

Fig. 1. Changes in RAF-1 phosphorylation and electrophoretic mobility following CSF-1 stimulation of BAC1.2F5 cells. Dual labeled ([^{32}P]orthophosphate/[^{3}H]leucine) BAC1.2F5 cells were stimulated with 13.2 nM human recombinant CSF-1 at 37°C (**a**) or at 4°C (**d**) for different times prior to lysis in 1% Triton X-100. RAF-1 molecules were immunoprecipitated with the anti-RAF-1 (SP63) rabbit polyclonal antiserum and the immune precipitates subjected to 7.5% SDS–PAGE. After autoradiography, RAF-1 bands were excised and counted in a liquid scintillation counter. (**b**) ^{3}H d.p.m. recovered in the 72 (○) and in 74 (□) kd RAF-1 bands at different times after stimulation with CSF-1 at 37°C. (**c**) Ratio of [^{32}P]orthophosphate/ [^{32}H]leucine incorporation in the 72 (○) and 74 (□) kd RAF-1 bands at different times after stimulation with CSF-1 at 37°C. Open and closed symbols represent duplicates.

RAF-1 doublet from unstimulated cells indicated that it was phosphorylated exclusively in serine (Figure 3, left panel). The ^{32}P-labeled RAF-1 doublet isolated from cells stimulated *in vivo* with CSF-1 at 37°C for 5 min exhibited increased phosphorylation in serine and, to a much lesser extent, threonine, but no phosphorylation on tyrosine (Figure 3, middle and right panels). Phosphoamino acid analysis of the RAF-1 band immunoprecipitated from cells incubated with CSF-1 at 4°C for 60 min was indistinguishable from the phosphoamino acid analysis of RAF-1 from unstimulated cells (data not shown).

Since previous reports (Morrison *et al.*, 1988b, 1989) claimed that tyrosine phosphorylation of RAF-1 was a consequence of growth factor stimulation in the highly related PDGF receptor (PDGFR) system, it was possible that our inability to demonstrate tyrosine phosphorylation of RAF-1 following *in vivo* stimulation of BAC1.2F5 cells with CSF-1 was due to the instability of the small amounts of phosphotyrosine during the preparation of samples for phosphoamino acid analysis. We therefore determined whether tyrosine-phosphorylated RAF-1 could be recovered from cell lysates by immunoaffinity chromatography with an anti-phosphotyrosine antibody coupled to Sepharose 4B beads (anti-PY beads). BAC1.2F5 cells were metabolically labeled with [^{32}P]orthophosphate and stimulated with CSF-1 (13.2 nM) for 5 min at 37°C or for 90 min at 4°C. After lysis, the phosphotyrosine-reactive proteins were purified by immunoaffinity chromatography and subjected to SDS−PAGE as previously described (Sengupta *et al.*, 1988). Under the conditions used, 50% of the c.p.m. recovered in the CSF-1R from the total lysate of ^{32}P-labeled cells were recovered in the anti-PY eluate from cells incubated with CSF-1 at 4°C for 60 min and 60% in the anti-PY eluate from cells incubated with CSF-1 at 37°C for 5 min. Since ~50% of CSF-1Rs are expressed on the cell surface after upregulation (M. Baccarini, Wei Li and E.R.Stanley, unpublished observation), it appears that the vast majority of the cell surface CSF-1Rs bind the anti-PY beads. A faint band of ~74 kd could be discerned in the anti-PY eluate from CSF-1 stimulated cells (Figure 4a, middle and right lanes, arrowhead). In order to establish whether these protein bands were related to RAF-1, we immunoprecipitated half of each anti-PY eluate (anti-PY reactive proteins) and unabsorbed fraction with anti-RAF-1 antibodies and subjected them to 5−15% SDS−PAGE. Figure 4b shows that RAF-1 molecules could be readily recovered from the unabsorbed fraction. RAF-1 from BAC1.2F5 cells stimulated with CSF-1 at 37°C (right lane) showed the expected shift in apparent molecular weight, indicating that hyperphosphorylated RAF-1 molecules do not bind to the anti-PY−Sepharose beads. Furthermore, the recovery of RAF-1 in the unabsorbed fraction obtained from either unstimulated or CSF-1 stimulated cells was 100% of the RAF-1 recovered by immunoprecipitation from whole cell lysates (data not shown). Consistently, RAF-1 could not be immunoprecipitated from the anti-PY eluate (Figure 4c), while the PY-containing band of ~74 kd was completely recovered in the supernatant of the RAF-1 immunoprecipitation (Figure 4d, middle and right lane, arrowhead; the lanes in Figure 4d are broader due to the high NP-40 concentration in the sample).

Our failure to demonstrate tyrosine phosphorylation of RAF-1 after CSF-1 stimulation prompted us to examine whether tyrosine phosphorylation of RAF-1 could be stimulated by human recombinant PDGF in NIH3T3 cells overexpressing RAF-1 (c-raf* cells). c-raf* cells were therefore metabolically labeled with [^{32}P]orthophosphate and stimulated with recombinant PDGF (50 ng/ml) for 5 min at 37°C. Lysates from stimulated and unstimulated cells were subjected to immunoaffinity chromatography on an anti-PY antibody−Sepharose and both the unabsorbed fraction and the anti-PY eluate immunoprecipitated with anti-RAF-1 antibodies and subjected to SDS−PAGE. Figure 5 shows that the result obtained with CSF-1 stimulated BAC1.2F5 cells was reproduced with PDGF stimulated c-raf* cells. The 73 kd band from the anti-PY eluate (Figure 5a, lane 2), previously claimed (Morrison *et al.*, 1988b) to be identical with RAF-1 on the basis of similaries in the peptide maps, could not be immunoprecipitated with the SP63 antiserum against RAF-1 (Figure 5c, right lane) but was recovered in the supernatant of the immunoprecipitation (Figure 5d, right lane), whereas RAF-1 could be efficiently precipitated by the same antiserum from the unabsorbed fraction of the anti-PY−Sepharose beads (Figure 5b). As with the CSF-1 stimulation, the increase in RAF-1 phosphorylation and its associated electrophoretic mobility shift could be observed after PDGF stimulation. The results shown in Figures 4 and 5 were repeated using an antiserum (anti-v-*raf* 30 kd) directed against a different carboxy-terminal peptide of the RAF-1 molecule (data not shown), indicating that the failure to immunoprecipitate RAF-1 from the anti-PY eluate was not due to poor recognition by the particular antiserum used. Use of a mouse monoclonal anti-PY antibody (Morrison *et al.*, 1988b) to immunoprecipitate the PY-containing proteins prior to RAF-1 immunoprecipitation also yielded the results shown in Figures 4 and 5 (data not shown), indicating that two different anti-PY antibodies were unable to recognize RAF-1. We were also unable to demonstrate the presence of RAF-1 by Western blotting the anti-PY reactive proteins with anti-RAF-1 antisera. While Western

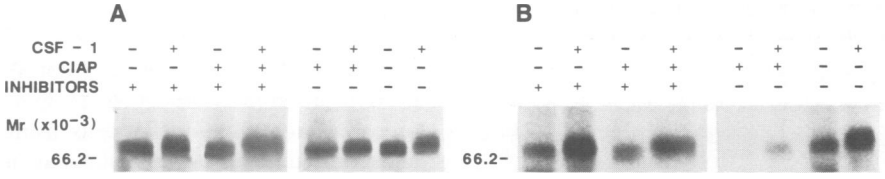


Fig. 2. Treatment with calf intestine alkaline phosphatase (CIAP) abolishes the CSF-1 induced change in RAF-1 electrophoretic mobility. BAC1.2F5 cells were labeled with [^{32}P]orthophosphate prior to stimulation with human recombinant CSF-1 (13.2 nM, 5 min, 37°C). Cell lysates normalized for protein content were subjected to immunoprecipitation with the rabbit anti-RAF-1 (SP63) antiserum. Constant volumes of immunoprecipitates were incubated in assay buffer in the presence or absence of phosphatase inhibitors or assay buffer containing CIAP in the presence or absence of phosphatase inhibitors, subjected to 7.5% SDS−PAGE and transferred to PVDF membrane. **A**, RAF-1 immunoblot; **B**, autoradiogram obtained by exposing the blot shown in A for 5 h at −70°C.

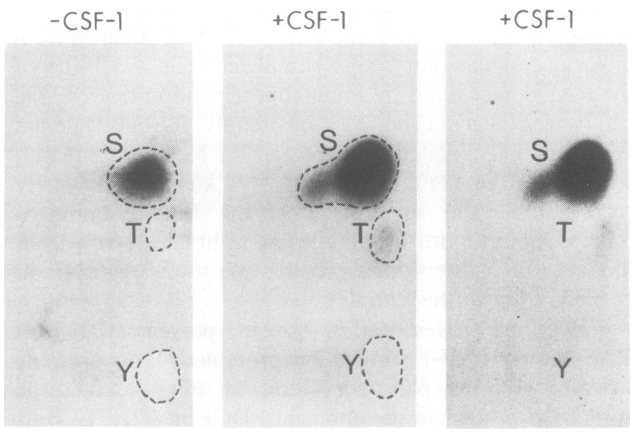


Fig. 3. Phosphoamino acid analysis of RAF-1 bands from CSF-1 stimulated or control cells. RAF-1 immunoprecipitates were prepared from 2 × 10^6 ^{32}P-labeled cells incubated for 5 min in the absence (−CSF-1) or presence of CSF-1 (13.2 nM, 37°C; +CSF-1) and subjected to 7.5% SDS−PAGE. The RAF-1 bands were excised and processed for phosphoamino acid analysis as described in Materials and methods. The total recovered material (left panel: 324 Cerenkov c.p.m.); middle and right panels: 540 Cerenkov c.p.m. was subjected to 2-D electrophoresis in each case. Autoradiography exposure time: 6 days. S, serine; T, threonine, Y, tyrosine. In the right panel, the outlines of the phosphoamino acid standards have been eliminated to improve visualization of the phosphothreonine spot.

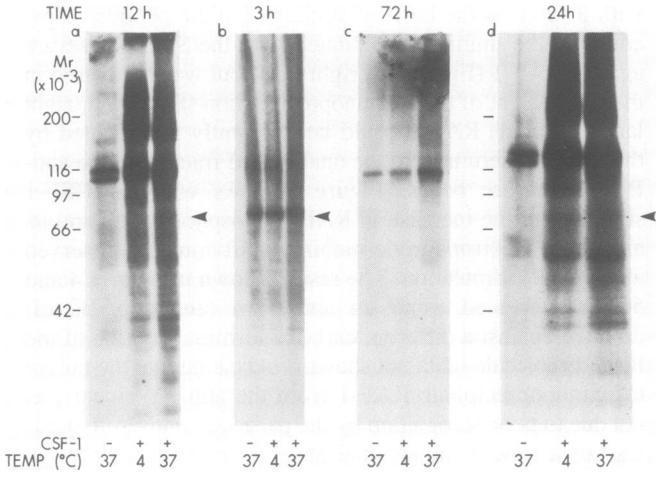


Fig. 4. RAF-1 is not one of the proteins phosphorylated on tyrosine following CSF-1 stimulation of BAC1.2F5 cells. ^{32}P-labeled BAC1.2F5 cells were stimulated with 13.2 nM human recombinant CSF-1 at 4°C for 90 min (middle lanes) or at 37°C for 5 min (right lanes). Left lanes contain immunoprecipitates from control cells. Cell lysates were incubated with monoclonal anti-phosphotryosine antibody coupled to Sepharose 4B (anti-PY beads) and the anti-phosphotyrosine reactive proteins eluted competitively by incubation in 1 mM phenylphosphate. Half of each anti-PY eluate (**a**), anti-RAF-1 (SP63) immunoprecipitates from half of each anti-PY unreactive fraction (**b**), anti-RAF-1 (SP63) immunoprecipitates from half of each anti-PY eluate (diluted to 1 ml in lysis buffer) (**c**), and the supernatants of the immunoprecipitations shown in (c) (**d**), were analyzed by 5−15% gradient SDS−PAGE. Arrowheads in (a) and (d) indicate the position of a 73 kd phosphotyrosine reactive protein and the arrowhead in (b) the position of RAF-1. The autoradiogram exposure time is shown at the top of each section.

blots of RAF-1 immunoprecipitates from stimulated and unstimulated cells probed with a rabbit polyclonal anti-PY antiserum (W.K.Shum and E.R.Stanley, unpublished results) revealed a faint band of ~74 kd, the same band was still

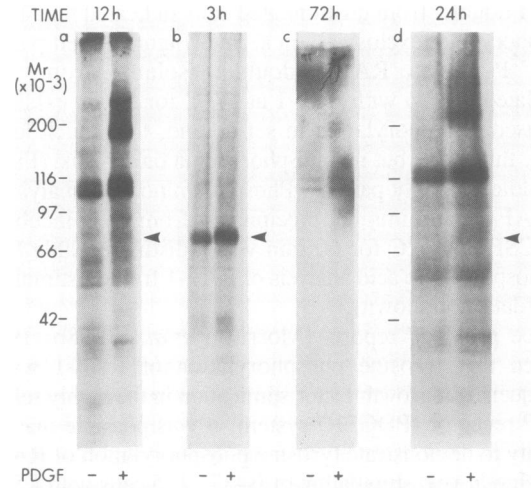


Fig. 5. RAF-1 is not one of the proteins phosphorylated on tyrosine following PDGF stimulation of NIH3T3 (c-raf*) cells. ^{32}P-labelled c-raf* cells were stimulated with 50 ng/ml human recombinant PDGF at 37°C for 5 min (right lanes). Left lanes contain immunoprecipitates from control cells. Cell lysates were chromatographed on anti-PY beads. Half of each anti-PY eluate (**a**), anti-RAF-1 immunoprecipitates from half of each anti-PY unreactive fraction (**b**), anti-RAF-1 (SP63) immunoprecipitates from half of each anti-PY eluate diluted to 1 ml of lysis buffer (**c**) and the supernatants of the immunoprecipitations shown in (c) (**d**), were analyzed by 5−15% gradient SDS−PAGE. Arrowheads and autoradiogram exposure times are as indicated in Figure 4.

observed when the blots were probed in the presence of excess phosphotyrosine (2 mg/ml, data not shown). These results indicate that RAF-1 is not one of the proteins that are rapidly phosphorylated on tyrosine in intact cells in response to either CSF-1 or PDGF.

Failure of RAF-1 to coprecipitate with either the CSF-1R or the PDGFR

RAF-1 has been claimed to associated with the PDGFR in a growth factor dependent manner (Morrison *et al.*, 1989). We sought to establish whether a growth factor dependent association between the RAF-1 protein and either the CSF-1R or the PDGFR could be demonstrated in spite of the lack of demonstrable RAF-1 tyrosine phosphorylation. Receptor and RAF-1 immunoprecipitates were therefore isolated from either control or growth factor stimulated cells, blotted and probed with anti-RAF-1 antisera (Figure 6). Figure 6A shows an anti-RAF-1 (SP63) antiserum control immunoblot of anti-v-*raf* 30 kd immunoprecipitates isolated from control and growth factor stimulated cells. Approximately equal amounts of RAF-1 were recovered from all cell lines and growth factor stimulation at 37°C but not at 4°C caused accumulation of the 74 kd form of the protein as well as an increase in the apparent molecular weight of the doublet. In Figure 6B, CSF-1R or PDGFR immunoprecipitates from control and growth factor stimulated cells were blotted and probed with the anti-RAF-1 (SP63) antiserum. Murine CSF-1R immunoprecipitates were prepared from BAC1.2F5 cells using a goat polyclonal antiserum directed against the purified murine CSF-1R (A.W.Ferrante, Y.G.Yeung and E.R.Stanley, unpublished results) (lanes 1−3). Murine PDGFR immunoprecipitates were prepared from c-raf* cells using a rabbit antiserum directed against the murine PDGF β-type sequence 739−757 (Kumjian *et al.*, 1989) (lanes 4 and 5). Human PDGFR immunoprecipitates were prepared from NIH3T3 cells

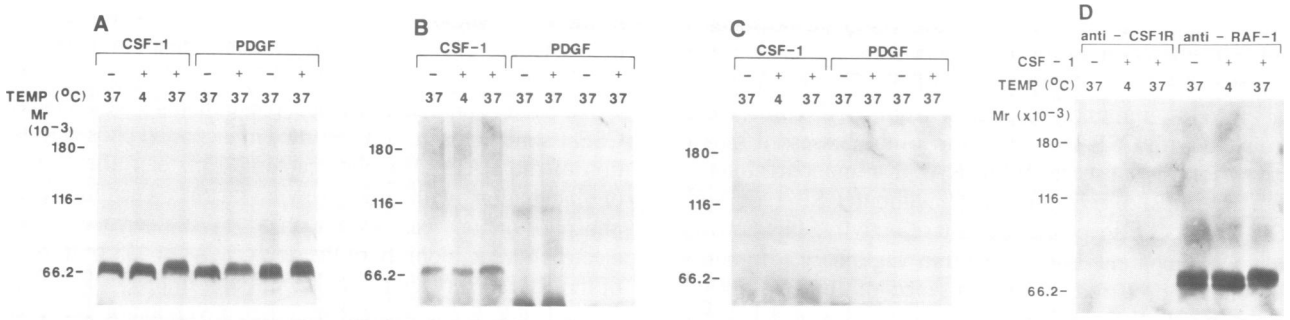


Fig. 6. RAF-1 is not detected in CSF-1R or PDGFR immunoprecipitates. **(A)** Anti-RAF-1 (SP63) immunoblot of 7.5% SDS—PAGE of: CSF-1; RAF-1 immunoprecipitates from 10^7 control cells (−, 37°C), or from 10^7 cells incubated with CSF-1 (13.2 nM) at 4°C for 60 min (+, 4°C) or at 37°C for 5 min (+, 37°C). PDGF; RAF-1 immunoprecipitates from control cells and from cells treated with PDGF (50 ng/ml) at 37°C for 5 min. RAF-1 immunoprecipitates from 3×10^6 c-raf* cells (two left lanes) and from 10^7 NIH3T3 cells expressing the human PDGFR (two right lanes). **(B)** Anti-RAF-1 (SP63) immunoblot of 7.5% SDS−PAGE of: CSF-1; CSF-1R immunoprecipitates from 10^7 control cells (−, 37°C) or from 10^7 cells incubated with CSF-1 (13.2 nM) at 4°C for 60 min (+, 4°C) or at 37°C for 5 min (+, 37°C). PDGF; PDGFR immunoprecipitates from control cells and from cells treated with PDGF (50 ng/ml) at 37°C for 5 min. Murine PDGFR immunoprecipitates from 3×10^6 c-raf* cells (two left lanes) and human PDGFR immunoprecipitates from 10^7 NIH3T3 cells (two right lanes). **(C)** Duplicate of blot shown in B probed with anti-RAF-1 antiserum (SP63) in the presence of 10 μg/ml SP63 peptide. **(D)** Anti-v-*raf* 30 kd immunoblot of 7.5% SDS−PAGE of CSF-1R (anti-CSF-1R) or anti-v-*raf* 30 kd (anti-RAF-1) immunoprecipitates from 10^7 control cells and from 10^7 cells treated with CSF-1 (13.2 nM) at 4°C for 60 min or at 37°C for 5 min.

expressing the human β-type PDGFR using the PR7212 monoclonal antibody (Hart *et al.*, 1987), that recognizes the extracellular domain of the human β-type PDGFR (lanes 6 and 7). A band of ~72 kd was recognized by the anti-RAF-1 antiserum in CSF-1R immunoprecipitates from both control and CSF-1 stimulated cells. This band possessed a similar mobility to RAF-1, but its apparent molecular weight was not altered as a consequence of CSF-1 stimulation. PDGFR immunoprecipitates isolated with either antibody did not contain any protein reacting with the anti-RAF-1 (SP63) antiserum. The trivial possibility of poor human PDGFR expression in the 3T3 cells was excluded by immunoblotting with anti-PDGFR antibodies (data not shown). A duplicate blot, shown in Figure 6C, was probed with anti-RAF-1 antiserum (SP63) in the presence of 10 μg/ml SP63 peptide. The appearance of the 72 kd band observed in Figure 6B was specifically blocked by this treatment. Although these data seemed consistent with a CSF-1 independent association of the CSF-1R with the RAF-1 protein, we were intrigued by the fact that the same amount of protein was present in CSF-1R immunoprecipitates from factor stimulated or resting cells and by the fact that the mobility of the 72 kd band did not change after CSF-1 stimulation, as would be expected if this band represented RAF-1. We therefore ran CSF-1R and RAF-1 immunoprecipitates on the same blot and probed the blot with a second anti-RAF-1 antiserum (anti-v-*raf* 30 kd), also directed against the carboxy-terminal portion of the RAF-1 molecule. Figure 6D shows that the anti-v-*raf* 30 kd, although able to recognize RAF-1 in the RAF-1 immunoprecipitates, did not recognize any protein in the CSF-1R immunoprecipitates from either control or CSF-1 stimulated cells. We therefore conclude that the 72 kd band present in the CSF-1R immunoprecipitates, although specifically recognized by one of the two anti-RAF-1 antisera used, was not RAF-1. These data fail to provide any evidence for an association of RAF-1 with either the CSF-1R or the PDGFR.

CSF-1 dependent stimulation of RAF-1 kinase activity

To determine whether the CSF-1 stimulated phosphorylation of RAF-1 in serine/threonine *in vivo* was associated with an increase in its serine/threonine kinase activity, RAF-1

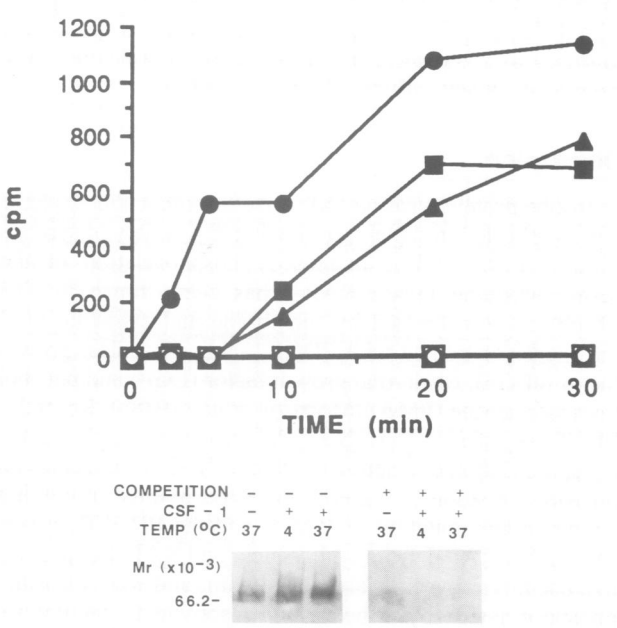


Fig. 7. Kinase assay of RAF-1 immunoprecipitates from control and CSF-1 treated cells. **A**, anti-RAF-1 (SP63) immunoprecipitates from 2.5×10^6 control (■) or CSF-1 treated [4°C, 60 min (▲); 37°C 5 min (●)] cells were incubated with [γ^{32}P]ATP and a RAF-1 amino-terminal peptide substrate for different time periods at 20°C. Results are expressed as means of duplicate assay that differed by ≤10%. Background radioactivity, present in aliquots taken immediately after [γ-^{32}P]ATP addition, has been subtracted from all experimental values. Open symbols, results of assays of anti-RAF-1 (SP63) antiserum precipitates prepared in the presence of competing SP63 peptide (2.5 μg/μl antiserum). **B**, RAF-1 immunoblot of aliquots of the immunoprecipitates used in the kinase assays shown in A, that have been incubated for 30 min at 20°C in kinase assay buffer. The position of the 66.2 kd marker protein is indicated.

immunoprecipitates from 2.5×10^6 control and CSF-1 treated cells were tested for kinase activity by incubation with a RAF-1 peptide substrate (amino acids 31−50, IVQQFGFQRRASNNGKLTN, in which Y37 has been substituted for by F) and [γ-^{32}P]ATP. The choice of this peptide as a RAF-1 kinase substrate was based on the

speculation that this region of the RAF-1 molecule might occupy the substrate site of the quiescent enzyme in a manner similar to the 'pseudosubstrate' peptide at the N-terminus of protein kinase C (Rapp *et al.*, 1988). Figure 7A shows that RAF-1 immunoprecipitates from BAC1.2F5 cells incubated with CSF-1 at 37°C for 5 min possessed kinase activity for this substrate. While RAF-1 immunoprecipitates from BAC1.2F5 cells incubated without CSF-1 or with CSF-1 at 4°C also possessed kinase activity, this activity was only apparent after a 10 min lag phase. Immunoprecipitates prepared in the presence of the appropriate competing RAF-1 peptide antigen were without detectable kinase activity. A RAF-1 immunoblot of aliquots of the immunoprecipitates used in the kinase assay presented in Figure 7A, that were incubated for 30 min in the kinase assay buffer, are shown in Figure 7B to indicate that similar amounts of RAF-1 were immunoprecipitated in each case and that the RAF-1 peptide prevented RAF-1 immunoprecipitation completely. Indistinguishable results were obtained in the kinase assay when RAF-1 immunoprecipitates were prepared using the anti-v-*raf* 30 kd antibody (data not shown). RAF-1 immunoprecipitates from cells stimulated with CSF-1 at 37°C that had been treated with CIAP, followed by removal of the phosphatase by washing, were devoid of kinase activity (data not shown).

Discussion

Cytosolic protein tyrosine and serine/threonine kinases have been implicated in signal transduction in a variety of systems (Smith *et al.*, 1986). Increased phosphorylation of the serine/threonine kinase RAF-1 has been shown in 3T3 fibroblasts transformed by membrane and cytosolic viral oncogenes or treated with PDGF, acidic fibroblast growth factor (aFGF), epidermal growth factor (EGF) and phorbol myristate acetate (PMA) (Morrison *et al.*, 1988b). Recently, PDGF stimulation has been described to result in the phosphorylation of a subpopulation of RAF-1 molecules on tyrosine residues. Based on experiments involving coimmunoprecipitation of RAF-1 and the PDGFR, it was shown that RAF-1 from cell lysates, and RAF-1 expressed in a baculoviral vector, associated with, and were tyrosine-phosphorylated by, the PDGF activated baculoviral expressed PDGFR *in vitro* (Morrison *et al.*, 1989). On the basis of these studies, RAF-1 was claimed to be a candidate substrate for the PDGFR tyrosine kinase.

The CSF-1R is very similar, in its structural organization, to the PDGFR. Both molecules have a distinct pattern of cysteine spacing within their extracellular domains that is characteristic of the members of the immunoglobulin gene superfamily (Williams and Barclay, 1988). Moreover, their kinase domains are interrupted by stretches of hydrophilic amino acids (74 and 104, respectively) that are unique to each receptor and could therefore represent the site of interaction with specific substrates (Yarden *et al.*, 1987). The genes for the PDGFR and the CSF-1R are physically linked in a head-to-tail array and separated by <500 bp (Roberts *et al.*, 1988). They display a very similar genomic organization, suggesting that the two genes arose through duplication. Because of this striking similarity, it was important to establish whether the serine/threonine kinase RAF-1 was involved in CSF-1 signal transduction and whether it was possible to identify it as a candidate substrate of the CSF-1R kinase.

The data reported in this paper show that RAF-1 is present in the unstimulated macrophage cell line used throughout the study as a 72/74 kd protein doublet, most of the protein (60%) migrating on SDS−PAGE as a 72 kd band. The 74 kd band contained more phosphate than the 72 kd band. CSF-1 stimulation at 37°C resulted in increased phosphorylation of the RAF-1 doublet reaching a plateau after 5 min of incubation with the growth factor. The increased phosphorylation was accompanied by a decrease in the electrophoretic mobility of the doublet. After 1.5 min, 63% of the RAF-1 molecules could be found in the 74 kd band. These results are consistent with the conversion of the 72 kd band into the 74 kd band being due to a phosphorylation event. Dephosphorylation with CIAP as well as PAP was able to abrogate the CSF-1 effect on RAF-1 migration pattern, indicating that phosphorylation is responsible for the formation of the 74 kd species from the 72 kd species.

A particularly important aspect of this study was to determine whether RAF-1 was one of the proteins rapidly phosphorylated on tyrosine in response to CSF-1 in intact cells and thereby a candidate substrate for the CSF-1R kinase. Several pieces of evidence indicate that this is not the case. First, we were unable to demonstrate phosphorylation of RAF-1 on tyrosine by phosphoamino acid analysis despite its increased phosphorylation in serine/threonine. Second, RAF-1 could not be precipitated from the anti-PY reactive protein fraction of CSF-1 stimulated cells but was completely recovered from the anti-PY unreactive fractions. A 73 kd protein band with the molecular weight of RAF-1 was present among the phosphotyrosyl proteins but was unable to react with two different anti-RAF-1 antipeptide antisera. Furthermore, RAF-1 was consistently not detected in the anti-PY eluate by immunoblotting. Third, stimulation of cells at 4°C, under conditions that lead to tyrosine phosphorylation of the full complement of proteins phosphorylated at 37°C (Sengupta *et al.*, 1988), failed to stimulate phosphorylation or activation of RAF-1. Even at 37°C, the phosphorylation of RAF-1 occurs later than the CSF-1 induced protein tyrosine phosphorylation (Sengupta *et al.*, 1988). These data clearly indicate that RAF-1 is not phosphorylated on tyrosine in response to CSF-1 under conditions in which other proteins are, and that the phosphorylation of RAF-1 can be resolved kinetically from such tyrosine phosphorylation events. Thus, under physiological conditions, RAF-1 is not a substrate of the CSF-1R tyrosine kinase.

Our inability to demonstrate tyrosine phosphorylation after growth factor stimulation was not a peculiarity of the CSF-1 system. Similar results were obtained using RAF-1 overexpressing NIH3T3 fibroblasts (c-raf* cells) incubated with PDGF, indicating that the absence of tyrosine phosphorylation of RAF-1 in response to growth factors is a more general phenomenon. These results are at variance with previously reported work (Morrison *et al.*, 1988b), which suggested that RAF-1 is phosphorylated on tyrosine and associates with the receptor in response to PDGF. In this work, the evidence of RAF-1 tyrosine phosphorylation in response to PDGF relied on similarities between the peptide maps of RAF-1 and a 74 kd protein band found in the anti-PY eluate of PDGF treated cells; in fact, while these maps share common peptides, this does not necessarily establish identity (Morrison *et al.*, 1988b). A second piece of evidence was derived from immunoblots of RAF-1 immunoprecipitates probed with an anti-PY antibody and anti-PY

eluates probed with anti-RAF-1 antiserum. However, specificity controls for these experiments were not mentioned, and since RAF-1 is heavily phosphorylated on serine residues and can be detected non-specifically at least by our own αPY antiserum (data not shown), these data should be interpreted with caution, especially as comparatively large amounts of protein were being loaded and blotted. Finally, it should be noted that RAF-1 was not amongst the proteins that were immunoprecipitated by the same αPY antibody used in the studies by Morrison *et al.* (1988, 1989).

In addition, we could not demonstrate coprecipitation of RAF-1 with either the CSF-1R or the PDGFR from unstimulated as well as growth factor stimulated cells. While these results, in the case of the PDGFR, are again at odds with the report of Morrison *et al.* (1989), it should be noted that the antibodies used to immunoprecipitate the PDGFR in the two studies were not the same, although one of our antibodies (PR7212) recognizes an extracellular epitope on the PDGFR and was therefore not expected to interfere with the coprecipitation of proteins associated with the intracellular domain of the receptor. It is still possible, therefore, that the detection of the association between RAF-1 and the PDGFR is strictly dependent on the antibody used for PDGFR immunoprecipitation.

To establish whether the CSF-1 induced changes had any effect on RAF-1 kinase activity, we performed kinase assays on RAF-1 immunoprecipitates from control and CSF-1 stimulated cells using an amino-terminal RAF-1 peptide substrate. Activation of RAF-1 kinase activity was detected in immunoprecipitates from cells incubated with the growth factor at 37°C. Interestingly, neither CSF-1 induced phosphorylation of the RAF-1 molecule nor the accompanying shift in the apparent molecular weight or activation of its *in vitro* kinase activity could be achieved by stimulating BAC1.2F5 cells with CSF-1 at 4°C, even for a very prolonged time period (4 h). Thus, in contrast, to CSF-1 induced protein tyrosine phosphorylation, which is not blocked at 4°C (Sengupta *et al.*, 1988), CSF-1 mediated phosphorylation of RAF-1 appeared to be a strictly temperature dependent phenomenon. The nature of the temperature dependence remains to be investigated. It is conceivable that activation might be dependent upon phenomenon related to the internalization of the CSF-1−CSF-1R complex, which is completely blocked at 4°C. Alternatively, it might require recompartmentalization of RAF-1 itself or of serine/threonine kinase(s) that may mediate the CSF-1 induced phosphorylation of RAF-1. It is also possible that the phenomenon is simply temperature dependent and independent of any recompartmentalization. The function performed by RAF-1 in CSF-1 signal transduction and the relationship between the activation of this serine threonine kinase, achieved without its tyrosine phosphorylation, and the rapid phosphorylation on tyrosine of an array of other proteins with as yet unknown function are particularly interesting questions raised by the present study. RAF-1 is ubiquitously present in most tissues and cell lines, suggesting that this protein performs a basic regulatory function required by most cell types (Rapp *et al.*, 1988). Although its role in cell physiology is still obscure, it is known that truncation of its amino-terminal domain activates kinase activity and that RAF-1 proteins deregulated by amino-terminal truncation induce proliferation in serum-starved 3T3 cells, indicating a role of this kinase in mediating cell proliferation (Rapp *et al.*, 1987, 1988). A number of observations in different growth factor systems (PDGF, Morrison *et al.*, 1988b; EGF, Harald App and Ulf R.Rapp, unpublished results; and CSF-1, this study) have indicated that RAF-1 phosphorylation and activation plays a role in signal transduction pathways initiated by receptor tyrosine kinases. In addition, recent experiments have shown that the *Drosophila* homolog of c-*raf-1*, D-*raf* encodes a potential serine/threonine kinase that acts downstream of the protein tyrosine kinase encoded by *torso*, *torso* is one of the maternally expressed terminal class of genes regulating the correct formation of the most anterior and posterior segments of the larva and seems to be a transmembrane protein with a receptor-like extracellular domain. These results are consistent with the induction of terminal development being mediated through a signal transduction pathway involving the interaction of D-*raf* with a receptor-like tyrosine kinase (Ambrosio *et al.*, 1989).

In the CSF-1 response, activation of RAF-1 could be achieved simply by binding of a regulatory molecule to the amino-terminal region of RAF-1, followed by RAF-1 autophosphorylation; alternatively, a conformational change could be caused by phosphorylation of a specific site by a CSF-1 inducible serine/threonine kinase activity. The activation of RAF-1 could thus represent a step in a phosphorylation cascade initiated by the CSF-1R at the cell surface and ultimately leading to cell growth. Further studies will investigate the possibility of an interaction between RAF-1 and the proteins rapidly phosphorylated on tyrosine after CSF-1 stimulation as well as the nature of the temperature dependent event that permits phosphorylation and activation of the RAF-1 kinase.

Materials and methods

Cell culture and labeling

BAC1.2F5 cells (Morgan *et al.*, 1987) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% newborn calf serum (NCS) and 1.32 nM purified human recombinant CSF-1 (a gift from Cetus Corp., Emeryville, CA). Confluent cultures (10^7 cells per 100 mm tissue culture dish) were cultured in medium without CSF-1 for 16 h to upregulate receptors and then incubated for 90 min in phosphate-free DMEM supplemented with 25 mM HEPES buffer (GIBCO, Grand Island, NY) and 10% heat inactivated, dialyzed NCS as previously described. Cells were then either labeled by incubation for 3 h in phosphate-free medium containing 1 mCi/ml carrier-free *o*-[^{32}P]phosphate (9000 Ci/mmol, Amersham, New England; ^{32}P-labeling, Sengupta *et al.*, 1988); or by incubation for 3 h in leucine-deficient, phosphate-free DMEM containing 1 mCi/ml of *o*[^{32}P]phosphate and 0.5 mCi/ml [^{3}H]leucine (140 Ci/mmol, Amersham, UK; dual labeling). All DMEM labeling media contained 1/10 normal $NaHCO_3$, 25 mM HEPES buffer and 10% dialyzed NCS. NIH3T3 cells overproducing the RAF-1 protein (c-raf* cells Morrison *et al.*, 1988b) and NIH3T3 cells expressing the human β-type PDGF receptor, gift of Dr Andrius Kazlauskas (Fred Hutchison Cancer Research Center, Seattle) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS). Density arrested fibroblasts were labeled with *o*-[^{32}P]phosphate as described above, except that they were kept serum-free from the time of addition of phosphate-free medium.

Growth factor stimulation

Cells were equilibrated at the desired temperature and incubated for different periods of time with either 13.2 nM human recombinant CSF-1 (Cetus, Emeryville; BAC1.2F5 cells) or 50 ng/ml human recombinant PDGF (R & D Systems, Minneapolis, MN; NIH3T3 cells). Incubations were terminated by aspirating the medium and washing five times with ice-cold PBS.

Cell lysis and immunoprecipitation

Cells were lysed by addition of 1 ml of ice cold lysis buffer (20 mm Tris−HCl, 137 mM NaCl, 1% Triton X-100, 10% glycerol, 2 mM EDTA,

1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM, sodium vanadate, pH 8.0). Insoluble material was removed by centrifugation (16 000 *g*, 30 min, 4°C) and lysates were normalized on the basis of protein determinations carried out using the method of Bradford (1976). Normalized volumes of lysates were precleared by incubation (60 min at 4°C) with BSA–Sepharose beads prior to incubation for 6–12 h at 4°C with different antibodies. The goat polyclonal anti-CSF-1R antiserum was raised against the purified, native CSF-1R molecule (A.W.Ferrante, Y.G.Yeung and E.R.Stanley, unpublished). The IgG fraction of rabbit polyclonal antisera raised against the murine PDGFR β-type sequence 739–757 (YMAPYDNYVPSAPGRTYRA, Kumjian *et al.*, 1989) was kindly provided by Dr Tom Daniel, Vanderbilt University, Nashville. The monoclonal antibody PR7212, which recognizes an extracellular epitope of the β-subunit of the human PDGFR (Hart *et al.*, 1987) was a gift of Dr Dan Bowen-Pope, University of Washington, Seattle. The mouse monoclonal anti-PY antibody generated using phosphotyramine as an immunogen (B.Druker, D.K,Morrison, J. Burkhart and T.M.Roberts) was provided by Dr D.K.Morrison, University of California, San Francisco. Rabbit polyclonal antisera raised against a carboxy-terminal peptide of v-*raf* (SP63, CTLTTSPRLPVF, Schultz *et al.*, 1985) or against a 30 kd C-terminal fragment of v-*raf* produced in *Escherichia coli* (Kolch *et al.*, 1988) were used to immunoprecipitate RAF-1 molecules. Immune complexes were collected following incubation (1–2 h at 4°C) with Protein A–Sepharose beads. Immunoprecipitates were washed once with lysis buffer, twice with 0.5 M LiCl–0.1 M Tris–HCl pH 7.4 and once with 10 mM Tris–HCl pH 7.4. The beads were eluted by boiling in Laemmli sample buffer (Laemmli, 1970) and the eluates were analyzed by 7.5% SDS–PAGE. The gels were stained, dried and subjected to autoradiography at −70°C using preflashed Kodak XAR 5 films. Alternatively, proteins were electrophoretically transferred onto Immobilon PVDF paper (Millipore Co., Bedford, MA) in 50 mM Tris, 384 mM glycine, 20% methanol, 0.01% SDS at 30 V for 2 h, followed by 70 V for 16 h and finally 100 V for 4 h at 4°C. Residual binding sites were blocked by incubation with 3% gelatin (Biorad) in 25 mM Tris–HCl, 150 mM NaCl, pH 8.0 (TBS) for 1 h at room temperature. Blots were subsequently incubated overnight with anti-RAF-1 (anti-SP63, 1:1000; anti v-*raf* 30 kd, 1:500) antisera in TBS containing 1% gelatin and 0.05% Tween-20 (antibody buffer). After washing, blots were incubated for 2 h at room temperature with goat anti-rabbit affinity purified alkaline phosphatase conjugated antibodies (Biorad, 1:3000) in antibody buffer. Blots were washed and antigen–antibody complexes visualized by incubation in 100 mM Tris–HCl, pH 9.5, containing 100 mM NaCl, 5 mM $MgCl_2$, 330 μg/ml of 5-bromo-4-chloro-3-indolyl-phosphate and 150 μg/ml nitroblue tetraziolium for 5–30 min.

Isolation of anti-phosphotyrosine reactive proteins

Cells were lysed in 2 ml extraction buffer (10 mM Tris–HCl, 50 mM NaCl, 0.5% NP-40, 30 mM sodium pyrophosphate, 50 mM NaF, 100 μM sodium vanadate, 5 μM $ZnCl_2$, 1 mM PMSF, 0.1% bovine serum albumin, pH 7.05) for 20 min at 4°C. Insoluble materials were removed by centrifugation as described above and lysates were incubated with 30 μl of packed Sepharaose 4B to which the monoclonal anti-phosphotyrosine antibody 1G2 had been coupled (Huhn *et al.*, 1987). Phosphotyrosine containing proteins were competitively eluted in 40 μl of extraction buffer containing 1 mM phenyl-phosphate (15 min at 4°C). The eluates were either analyzed by 5–15% gradient SDS–PAGE or diluted to 1 ml in extraction buffer and immunoprecipitated with anti-RAF-1 antipeptide antisera.

Phosphoamino acid analysis

^{32}P-labeled proteins were resolved by SDS–PAGE, extracted from the gels and subjected to tryptic digestion. The digests were hydrolyzed in 6 N HCl at 110°C for 1 h and the hydrolysates were separated by two-dimensional electrophoresis on cellulose-coated TLC plates as described (Cooper *et al.*, 1983). Phosphoamino acids were visualized by autoradiography at −70°C on preflashed Kodak XAR 5 films.

Phosphatase assays

RAF-1 immunoprecipitates were treated with either calf intestine alkaline phosphatase (Boehringer-Mannheim, 24 U/μl, 0.24 U/ml) in a reaction buffer consisting of 50 mM Tris–HCl, 5 mM $MgCl_2$, 1 mM PMSF, pH 8.3, for 45 min at 34°C. Controls in buffer containing a mixture of phosphatase inhibitors (30 mM sodium pyrophosphate, 50 mM NaF, 1 mM sodium vanadate) were included in the assays. Assays were terminated by boiling in Laemmli sample buffer.

Immune complex kinase assay

Immunoprecipitates were prepared as described above. Specificity controls for the immunoprecipitation reaction were performed by preincubating the anti-RAF-1 (SP63) antiserum with an excess of the peptide antigen SP63 (2.5 μg peptide/μl antiserum for 1 h at room temperature) and subsequenty using the mixture in place of the antiserum alone in the immunoprecipitation. In either case, the immunoprecipitates were washed twice in kinase buffer (40 mM Tris–HCl, 120 mM NaCl, 0.8 mM DTT, 10 mM $MnCl_2$, 0.8% Tween-20, 10 μg/ml leupeptin, 0.2 U/ml aprotinin, 1000 U/ml soybean trypsin inhibitor, pH 7.4) prior to assay. The kinase activity of immune complexes was assessed by including immunoprecipitates from 2.5 × 10^6 cells with an amino-terminal RAF-1 peptide substrate (amino acids 31–50 IVQQFGFQRRASNNGKLTN, in which Y37 has been substituted for by F) (1 mg/ml) in 100 μl of kinase buffer containing 20 μCi [γ-^{32}P]ATP (Amersham, 3000 Ci/mmol) for different times at 20°C. Incubations were terminated by spotting duplicate aliquots of the reaction mixtures on Whatman P81 phosphocellulose filters. The filters were washed four times each for 30 min with 0.85% phosphoric acid, dried (30 min, 37°C) and the incorporated radioactivity was determined by Cerenkov counting.

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