Raf-1 is a potential substrate for mitogen-activated protein kinase *in vivo*

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MAP (mitogen-activated protein) kinase is shown to phosphorylate baculovirally expressed Raf-1 *in vitro*, generating one major tryptic phosphopeptide which co-migrated with a peptide from Raf-1 ³²P-labelled *in situ*. This peptide also undergoes an insulin-dependent increase in labelling. Thus the serine/threonine kinase Raf-1 may be a substrate for MAP kinase *in vivo*.

INTRODUCTION

Many growth factors and hormones initiate transmembrane signalling by interacting with specific cell surface receptors which possess an intrinsic tyrosine kinase activity [1]. Activated receptors trigger an array of intracellular events, including ion fluxes, generation of second messengers and multiple protein phosphorylations. Most of these protein phosphorylations occur on serine or threonine residues, and a key question is to understand how an initial tyrosine phosphorylation signal is converted to one which targets the serine and threonine phosphorylation sites of relevant proteins. A potential mechanism is the phosphorylation and activation of key serine/threonine kinases by tyrosine phosphorylation. Evidence for this came from the discovery that the growth-factor-stimulated serine/threonine kinase mitogen-activated protein (MAP) kinase is phosphorylated on tyrosine residues *in situ* [2].

MAP kinase was originally isolated from insulin-treated 3T3-L1 adipocytes [3], but is now known to be activated by a diverse array of hormones and growth factors in many cell types (reviewed in [4]). Both tyrosyl and threonyl phosphorylations of MAP kinase are required for activity [5], suggesting that the enzyme integrates signals from more than one phosphorylation pathway. MAP kinase is known to phosphorylate and partially reactivate member(s) of the 90 kDa family of S6 kinases *in vitro* [6]. Identification of other physiologically relevant MAP kinase substrates will be crucial to a full understanding of the role played by MAP kinase in growth factor signal transduction.

Many other serine/threonine kinases are known to be activated upon stimulation of cells with mitogenic agents (reviewed in [4]), including Raf-1, a 74 kDa serine/threonine kinase encoded by the cellular homologue of *v-raf*, the transforming gene of mouse sarcoma virus 3611 [7]. Exposure of mouse 3T3 cells to various mitogenic agents increased the kinase activity of Raf-1 and was accompanied by serine/threonine phosphorylation as well as tyrosine phosphorylation of the protein [8]. The subsequent finding [9] of a direct physical association of Raf-1 with the platelet-derived growth factor (PDGF) receptor in intact cells suggested that Raf-1 kinase might be activated by direct phosphorylation on tyrosine residues by the PDGF receptor kinase. However, in several recent reports, activation of Raf-1

kinase by various agonists was shown to occur without concomitant tyrosyl phosphorylation [10–13]. Thus physiological activation of Raf-1 may be mediated through its phosphorylation by other serine/threonine protein kinases.

Since the protein kinases which phosphorylate Raf-1 may themselves be regulated, we considered MAP kinase to be among potential protein kinases acting on Raf-1. In this report, Raf-1 is demonstrated to be a substrate for MAP kinase *in vitro*. Furthermore, the site phosphorylated on Raf-1 by MAP kinase appears to undergo increased phosphorylation following stimulation of intact HIRC-B cells with insulin.

MATERIALS AND METHODS

Expression of Raf-1 proteins using baculovirus and cell lysis

Spodoptera frugiperda (Sf9) cells were obtained from American Type Culture Collection. The cDNA of a kinase-inactive Raf-1 mutant, Raf-1a, in which Trp replaces Lys-374, was provided by Dr. Ulf Rapp (NCI, Frederick, U.S.A.). Raf-1 and Raf-1a baculovirus were constructed as described [9]. To generate baculoviral Raf-1 and Raf-1a proteins, Sf9 cells were seeded at 2×10^7 cells/150 mm plate, followed by infection with baculovirus at a multiplicity of infection of 10. At 48 h after infection, the cells were washed once with ice-cold phosphate-buffered saline and lysed as described [9].

Culture, radiolabelling and lysis of HIRC-B cells

HIRC-B cells, a Rat-1 fibroblast derivative that overexpresses the normal human insulin receptor [14], were grown to confluence in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and methotrexate (500 nM). Prior to use, the cells were serum-starved for 18 h in DMEM, followed by metabolic labelling for 4 h with [³²P]P_i (1 mCi/ml). After hormone treatment (100 nM-insulin, 37 °C, 10 min), the cells (from five 100 mm dishes) were washed once with ice-cold 0.15 M-NaCl and scraped into 1 ml of lysis buffer [20 mM-Tris, pH 8.0 at 4 °C, 137 mM-NaCl, 2 mM-EDTA, 2 mM-sodium pyrophosphate, 1 mM-sodium orthovanadate, 1 mM-phenylmethanesulphonyl fluoride (PMSF), 10 µg of leupeptin/ml, 10 µg of aprotinin/ml, 1% (v/v) Triton X-100, 10% (v/v) glycerol] at 4 °C. Lysis was

Abbreviations used: (b)MAP kinase, (baculovirus) mitogen-activated protein kinase; DMEM, Dulbecco's modified Eagle's medium; PDGF, platelet-derived growth factor; PMSF, phenylmethanesulphonyl fluoride; PKC, protein kinase C; MBP, myelin basic protein.

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allowed to proceed for 20 min at 4 °C followed by centrifugation (12000 *g*, 10 min), and the resulting supernatant was used for immunoprecipitation with anti-Raf-1 antiserum.

Immunoprecipitation

Anti-Raf-1 antiserum, raised against the C-terminal peptide sequence CTLTSPRLPVF, was generously provided by Ken Wood (Dana-Farber Cancer Institute). Lysates from Sf9 cells or ³²P-labelled HIRC-B cells were immunoprecipitated for 3 h at 4 °C with antibody bound to Protein A-Sepharose followed by washing and processing for SDS/PAGE as described [9].

Phosphopeptide mapping

³²P-labelled Raf-1 protein was resolved by SDS/PAGE (8% gels), extracted from the gels and subjected to trypsin digestion as described [15]. The labelled peptides were then separated by t.l.c. on cellulose plates by electrophoresis at pH 1.9 for 30 min at 1000 V, followed by ascending chromatography for 5 h as described [16].

Kinase reactions *in vitro*

Sf9 cells were lysed and immunoprecipitated as described above. The immunoprecipitates were washed finally in kinase buffer (25 mM-Hepes, pH 7.5, 25 mM-β-glycerophosphate, 1 mM-dithiothreitol, 5 mM-MnCl₂, 15 mM-MgCl₂) and then incubated for 10 min at 30 °C in kinase buffer (total volume 50 μl) containing an appropriate amount of MAP kinase (transferring 2–4 pmol/min in the standard myelin basic protein (MBP) kinase assay [5]) and 50 μM-ATP (5000 c.p.m./pmol).

Preparation of MAP kinase

MAP kinase was purified extensively from EL4 thymoma cells, essentially as described [17], by sequential chromatography using DE52 cellulose, phenyl-Superose and Superose 12.

Materials

[γ-³²P]ATP and [³²P]P_i were obtained from New England Nuclear-Du Pont. Methatrexate and all other chemicals were purchased from Sigma.

RESULTS AND DISCUSSION

Activation of Raf-1 protein kinase by various growth factors in fibroblasts [8], H35 cells [10], HeLa cells [11], T-lymphocytes [12] and macrophages [13] is associated with increased serine/threonine phosphorylation of the protein. To test whether MAP kinase could be responsible for all or part of this phosphorylation, we first investigated the ability of MAP kinase to phosphorylate baculoviral Raf-1 (bRaf-1) *in vitro*.

Incubation of purified MAP kinase with anti-Raf-1 immunoprecipitates from Sf9 cells infected with recombinant Raf-1 baculovirus (see the Materials and methods section) resulted in the phosphorylation of a protein of molecular mass approx. 74 kDa (Fig. 1, lane 2). No such phosphorylation was observed when MAP kinase was incubated with immune complexes prepared from cells infected with wild-type baculovirus (Fig. 1, lane 6), confirming that the 74 kDa protein undergoing increased phosphorylation was bRaf-1. Prior inactivation of MAP kinase by incubation with protein phosphatase 2A [6] completely blocked its ability to elicit phosphorylation of bRaf-1 (results not shown).

To determine whether MAP kinase was causing increased autophosphorylation of bRaf-1, MAP kinase was incubated with immune complexes containing a Raf-1 mutant (bRaf-1a) which lacks autophosphorylating kinase activity (see the Materials and methods section). This also resulted in phosphorylation of a

74 kDa protein (Fig. 1, lane 4). We have no reliable estimate of the stoichiometry of bRaf-1 phosphorylation by MAP kinase *in vitro* due to the difficulty in measuring the amount of bRaf-1 protein associated with the immune complex. However, immunoblotting with anti-Raf-1 antibodies of immune complexes containing bRaf-1 (Fig. 1, lanes 1 and 2) or bRaf-1a (Fig. 1, lanes 3 and 4) indicated that approximately equal amounts of bRaf-1 protein were present in each case (results not shown). Therefore, since bRaf-1a exhibited increased phosphorylation following incubation with MAP kinase, at least part of the observed increase in bRaf-1 phosphorylation following incubation with MAP kinase cannot be attributed simply to increased autophosphorylation.

Some structural features of Raf-1 may be relevant to recognition by MAP kinase of Raf-1 as a protein substrate. MAP kinase phosphorylates MBP with high efficiency at a unique site with the amino acid sequence Thr-Pro-Arg-Thr*-Pro-Pro-Pro (* represents the phosphorylation site) [17]. More recently, Rosner and co-workers demonstrated that MAP kinase phosphorylates the epidermal growth factor receptor at Thr-669, in the sequence Pro-Leu-Thr-Pro [18]. Thus, although the exact amino acid sequence requirements for recognition by MAP kinase are not yet known, an adjacent proline residue at the C-terminal side of the acceptor amino acid is probably important. Inspection of the amino acid sequence of Raf-1 [19] reveals 11 serine or threonine residues that are followed immediately on the C-terminal side by at least one proline residue. One such sequence occurs in a region which is highly conserved among all Raf-1 proteins [19]. Another sequence, Pro-Lys-Thr-Pro-Val-Pro (residues 307–312), closely resembles that phosphorylated by MAP kinase in MBP. Determination of the exact site of phosphorylation of Raf-1 by MAP kinase will further our understanding of the substrate recognition requirements for MAP kinase.

Both MAP kinase and Raf-1 are activated within approx. 5 min of stimulation of mouse 3T3 cells with growth factors [3,8], compatible with the idea that one kinase may be involved in the

Fig. 1. Phosphorylation of recombinant Raf-1 by MAP kinase

Sf9 cells infected with Raf-1 baculovirus (lanes 1 and 2), Raf-1a baculovirus (lanes 3 and 4) or wild-type baculovirus (lanes 5 and 6) were immunoprecipitated with anti-Raf antibody and incubated without (lanes 1, 3 and 5) or with (lanes 2, 4 and 6) MAP kinase. Samples were analysed by SDS/PAGE (8% gels) and phosphoproteins were detected by autoradiography as described in the Materials and methods section. Raf-1 and molecular mass markers are indicated by arrows to the left of the Figure, and the top of the gel and dye front are indicated by arrowheads to the right of the Figure.

Fig. 2. Phosphopeptide mapping of Raf-1 immunoprecipitated from resting or insulin-stimulated HIRC-B cells

Raf-1 was immunoprecipitated from five 100 mm dishes of ^{32}P -labelled HIRC-B cells and subjected to tryptic phosphopeptide analysis as described in the Materials and methods section. Phosphopeptides were resolved on cellulose t.l.c. plates by electrophoresis at pH 1.9 (horizontal axis) followed by chromatography (vertical axis). Equal quantities of radioactivity (410 c.p.m.) were applied to each plate at the origin, marked by an arrowhead. (a) Unstimulated cells, (b) insulin-treated (50 nM, 10 min) cells. The major peptides exhibiting increased phosphorylation are indicated by arrows.

regulation of the other *in vivo*. Thus we evaluated the potential phosphorylation of Raf-1 by MAP kinase *in vivo* using peptide mapping criteria. A recent study of Blackshear *et al.* [10] demonstrated increased phosphorylation of Raf-1, on serine and threonine residues only, in HIRC-B cells treated with insulin. HIRC-B cells are a Rat-1 fibroblast line induced to overexpress the human insulin receptor [14]. They also appear to express both Raf-1 and MAP kinase at high levels [10,20]. Therefore we decided to use this system to investigate the phosphorylation of Raf-1 *in situ*.

Two-dimensional tryptic phosphopeptide maps of Raf-1 immunoprecipitated from ^{32}P -labelled HIRC-B cells are shown in Fig. 2. Treatment of cells with insulin caused an increase in the phosphorylation of three major tryptic peptides (labelled 1, 2 and 4 in Fig. 2). In addition, several new phosphopeptides were generated, albeit of relatively low intensity. The changes in peptide phosphorylation seen in Fig. 2 underestimate the actual changes in Raf-1 phosphorylation due to insulin treatment, because equal amounts of radioactivity were applied to each t.l.c. plate. In the experiment shown, insulin induced a 75% increase in Raf-1 phosphorylation (measured by Čerenkov counting of the excised protein band). Qualitatively similar phosphopeptide maps have been reported for Raf-1 immunoprecipitated from PDGF-treated fibroblasts [8]. A more recent report showed the appearance of a new tryptic phosphopeptide in Raf-1 immunoprecipitated from T-lymphocytes stimulated with either phorbol esters or antibodies [12].

Fig. 3. Comparison of phosphopeptide maps of Raf-1 phosphorylated *in vitro* and *in situ*

(a) bRaf-1a immunoprecipitated from Sf9 cells and incubated with MAP kinase; (c) Raf-1 immunoprecipitated from insulin-stimulated HIRC-B cells; (b) mixture of (a) and (c). Phosphopeptides were resolved as described in the legend to Fig. 2. Equal quantities of radioactivity (310 c.p.m.) were loaded on to each plate. The major phosphopeptides resolved from tryptic digestion of Raf-1 are indicated by arrows. Peptides 1, 2 and 3 (c) are equivalent to those in Fig. 2.

In separate experiments, comparisons were made between the tryptic phosphopeptide map of Raf-1 phosphorylated in intact cells and that from bRaf-1a phosphorylated by MAP kinase *in vitro*. Tryptic digestion of bRaf-1a phosphorylated by MAP kinase *in vitro* generated a single major phosphopeptide (labelled α in Fig. 3a). Tryptic digestion of bRaf-1 following phosphorylation by MAP kinase also generated phosphopeptide α as well as several other peptides resulting from Raf-1 autophosphorylation, but phosphopeptide α was absent from the tryptic peptide map of bRaf-1 autophosphorylated alone (results not shown). In mixing experiments, peptide α from bRaf-1a co-migrated with peptide 1 from Raf-1 phosphorylated *in situ* (Fig. 3b), suggesting that MAP kinase phosphorylates a site(s) on Raf-1 which also undergoes increased phosphorylation on treatment of HIRC-B cells with insulin.

The complex phosphopeptide pattern of Raf-1 observed after insulin stimulation suggests that, in addition to MAP kinase, other protein kinases are involved in the phosphorylation of Raf-1 *in vivo*. In light of this, it was perhaps not surprising that we were unable to demonstrate any reproducible effects on Raf-1 kinase activity following phosphorylation by MAP kinase; these experiments were performed with bRaf-1 immunoprecipitated from Sf9 cells and with Raf-1 immunoprecipitated from resting or stimulated HIRC-B cells. Although it is also possible that the above experiments failed for technical reasons, our results suggest

that Raf-1 may require phosphorylation by more than one protein kinase in order to be activated. Interestingly, MAP kinase itself exhibits an absolute requirement for tyrosine and threonine phosphorylation in order to be activated [5], and a similar phenomenon exists for p34^{cdc2}, which requires both tyrosine and threonine dephosphorylation for activation [21]. A requirement for phosphorylation/dephosphorylation on multiple sites may prove to be a common mechanism for regulating protein kinases of critical importance in the control of cell division.

Regarding other protein kinases potentially involved in the regulation of Raf-1, several reports suggest a role for the Ca²⁺- and phospholipid-sensitive serine/threonine kinase protein kinase C (PKC). Thus phorbol esters, potent activators of PKC, caused the activation and phosphorylation of Raf-1 in 3T3 fibroblasts [8] and in T-lymphocytes [12]. Indeed, PKC may be required for activation of Raf-1 in T-lymphocytes, since down-regulation of cellular PKC by prolonged treatment with phorbol esters prevented activation of Raf-1 via the T-cell receptor [12]. In contrast, activation of Raf-1 by PDGF in 3T3 fibroblasts [8] or by insulin in H35 cells [10] was unaffected by PKC down-regulation. Thus activation of Raf-1 may occur via PKC-dependent and PKC-independent routes. Interestingly, similar studies suggest that activation of MAP kinase can also be mediated via PKC-dependent and -independent pathways [4,17,22].

Although phosphorylation of Raf-1 appears to be important in its regulation, other factors may also be involved. For example, Raf-1 possesses a hydrophobic 'zinc-finger'-like domain, a structure postulated to be responsible for the binding of PKC to diacylglycerol. However, there have been as yet no reports on the existence of a factor which might interact allosterically with this or any other putative regulatory domain in Raf-1.

In summary, these results demonstrate that Raf-1 can be phosphorylated by MAP kinase *in vitro*. Our data also suggest that this phosphorylation may occur *in vivo*. Since both Raf-1 and MAP kinase are activated within minutes of the addition of growth factors to intact cells, they may together constitute part of a common mitogenic signalling pathway. Further studies are now necessary to establish the role of phosphorylation by MAP kinase, and perhaps other protein kinases, in regulating the activity of Raf-1. Such information will in turn help to delineate the critical pathways which mediate intracellular signalling by growth factors.

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