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Signal transduction from membrane to cytoplasm: Growth factors and membrane-bound oncogene products increase Raf-1 phosphorylation and associated protein kinase activity

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ABSTRACT **We have examined the phosphorylation and the serine/threonine-specific kinase activity of the protooncogene product Raf-1 (formerly c-raf) in response to oncogenic transformation or growth-factor treatment of mouse 3T3 cells. Expression of the membrane-bound oncogene products encoded by v-*fms*, v-*src*, v-*sis*, polyoma virus middle-sized tumor antigen, and Ha-*ras* increased the apparent molecular weight and phosphorylation of the Raf-1 protein, while expression of the nuclear oncogene and protooncogene products encoded by v-*fos* and c-*myc* did not. Changes in electrophoretic mobility and phosphorylation occurred rapidly in response to treatment of cells with platelet-derived growth factor, acidic fibroblast growth factor, epidermal growth factor, and the protein kinase C activator phorbol 12-myristate 13-acetate, but not insulin. The phosphorylation of the Raf-1 protein occurred primarily on serine and threonine residues. However, a subpopulation of Raf-1 molecules was phosphorylated on tyrosine residues in cells transformed by v-src or stimulated with platelet-derived growth factor. Transformation by v-src, or treatment with platelet-derived growth factor or phorbol 12-myristate 13-acetate, activated the Raf-1-associated serine/kinase activity as measured in immune-complex kinase assays. These findings suggest that proliferative signals generated at the membrane result in the phosphorylation of the Raf-1 protein and the activation of its serine/threonine kinase activity. Raf-1 activation may thus serve to transduce signals from the membrane to the cytoplasm and perhaps on to the nucleus.**

Cytoplasmic serine/threonine protein kinases are candidate signal transducers linking growth stimulatory events at the cell membrane to changes which occur in the cytoplasm and nucleus. Two serine/threonine kinases normally residing in the cytoplasm have been shown to be activated by mitogenic or oncogenic signals, protein kinase C (1), and a ribosomal S6 kinase (2). We have examined the role of a third serine/threonine kinase, the product of the *Raf-1* protooncogene (formerly c-*raf*) (3, 4), in mitogenesis and oncogenesis. *Raf-1* is the cellular homologue of v-*raf*, the transforming gene of the murine sarcoma virus 3611 (5). Transfection of human and rodent tumor DNAs, as well as cytogenetic studies have implicated *Raf-1* in a variety of common human and rodent neoplasms (6). The product of the *Raf-1* gene is a 74-kDa cytoplasmic protein which has an associated serine/threonine-specific protein kinase activity (3, 4, 7, 8). The Raf-1 protein can be divided into three domains: a cysteine-rich domain, a serine/threonine-rich domain, and a carboxyl-terminal kinase domain (4, 6, 9). Oncogenic activation of the Raf-1 protein can occur by an amino-terminal truncation which removes a putative regulatory region (6). In addition, when amino-terminal truncated Raf-1 protein is injected into serum-starved NIH 3T3 cells, DNA synthesis is efficiently induced in those cells (10).

In the following study we examine the phosphorylation and protein kinase activity of the Raf-1 protein in response to oncogenic transformation or mitogen treatment of mouse 3T3 cells. Our results suggest that the Raf-1 protein can be activated by tyrosine and/or serine and threonine phosphorylation as a result of direct or indirect action of membrane-bound oncogene products and growth factor receptors.

MATERIALS AND METHODS

Mitogens. Platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) (receptor grade) were from Collaborative Research (Waltham, MA). Acidic fibroblast growth factor (FGF) (0.4 ng of protein per unit from P. A. D'Amore, Childrens Hospital, Boston) and PDGF were used at a concentration of 10 half-maximal units/ml except where noted (1 unit induces half-maximal stimulation of [^{3}H]thymidine uptake by 3T3 cells). Bovine pancreatic insulin, phorbol 12-myristate 13-acetate (PMA), and dibutyryl cAMP were from Sigma.

Radiolabeling of Cells and Immunoprecipitation. For ^{32}P labeling, cells were incubated at 37°C for 3 hr in phosphate-free medium supplemented with [^{32}P]orthophosphate at 2 mCi/ml (1 Ci = 37 GBq) and 2% (vol/vol) platelet-poor plasma. For growth-factor experiments, mitogens were added directly to the labeling medium. Assays were terminated by the addition of ice-cold isotonic phosphate-buffered saline containing 100 μM sodium orthovanadate. Cell lysates were prepared as described (11), the lysates were normalized for protein content, and labeled proteins were immunoprecipitated for 3 hr at 4°C with anti-phosphotyrosine [Tyr(*P*)] antiserum (12) or with anti-Raf-1 antiserum (anti-SP63) (13). Immune complexes were precipitated and washed as described (11) prior to sodium dodecyl sulfate (SDS)/PAGE analysis.

Phosphatase Analysis. Phosphatase assays were performed as in the figure legend and as described (14).

Peptide Mapping and Phospho Amino Acid Analysis. ^{32}P-labeled proteins were resolved by SDS/PAGE, extracted from the gels, and subjected to trypsin digestion as described (15). Labeled peptides were separated on thin-layer cellulose plates by electrophoresis at pH 1.9 for 20 min at 1000 V followed by ascending chromatography as described (16). Phospho amino acid analysis of phosphorylated proteins was performed as described (15).

Protein Kinase Assays *in Vitro*. Immune complexes were prepared as described above. Immunoprecipitated Raf-1

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Abbreviations: PDGF, platelet-derived growth factor; EGF, epidermal growth factor; FGF, fibroblast growth factor; PMA, phorbol 12-myristate 13-acetate; T, tumor; Tyr(*P*), phosphotyrosine; SDS, sodium dodecyl sulfate.

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protein was then assayed for the ability to phosphorylate histone type V-S *in vitro* as described (8).

RESULTS

PDGF and Membrane-Bound Oncogene Products, but Not Nuclear Oncogene Products, Alter the Electrophoretic Mobility of the Raf-1 Protein. The Raf-1 protein was examined in 3T3 cells transformed by the v-*fms* and v-*src* oncogene products by using immunoblot analysis. As seen in Fig. 1*A*, there is a population of Raf-1 molecules in both v-fms- and v-src-transformed cells which migrates more slowly than the Raf-1 molecules in quiescent 3T3 cells. No change in the mobility of Raf-1 was observed in Rat-1 cells transformed by v-fos or 3T3 cells transformed by overexpression of the c-*myc* gene (Fig. 1*B*).

We next determined whether changes in Raf-1 protein mobility could be observed after addition of the growth factor PDGF to cells (Fig. 1*A*). When quiescent 3T3 cells were treated with PDGF for 5 min, all of the Raf-1 molecules exhibited a slower electrophoretic mobility when compared to the Raf-1 protein from unstimulated cells. This apparent molecular weight increase was transitory. The size increase was maximal 5 min after PDGF treatment; thereafter, multiple faster migrating forms of the Raf-1 protein appeared. By 2 hr after PDGF treatment approximately half of the Raf-1 molecules had returned to a mobility similar to that of the Raf-1 protein isolated from quiescent BALB/3T3 cells.

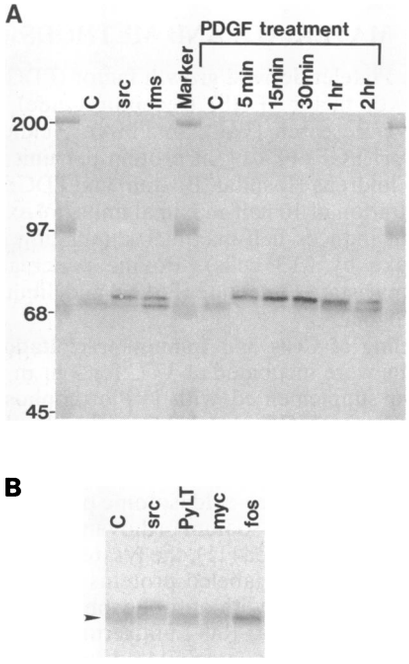

FIG. 1. Migration patterns of Raf-1 protein in cells expressing membrane-bound or nuclear oncogene products or cells treated with PDGF. Experiments were initiated on density-arrested monolayers as described (11). Growth factors were added directly to the medium and incubation continued at 37°C. Transformed cell lines were not fed with fresh medium for at least 48 hr prior to use. Cell lysates were prepared and equalized for protein content before electrophoretic separation on 7.5% SDS/PAGE and transfer to nitrocellulose filters as described (17). Protein blots were probed with anti-Raf-1 antibody (13) and immunological reactions were identified by using an alkaline phosphatase detection system. (*A*) Raf-1 proteins in 3T3 cells were transformed by the v-src (src) (18) or v-fms (fms) (17) oncogenes or in quiescent BALB/3T3 cells (C) treated with PDGF as indicated. Molecular mass markers (in kDa) are shown to the left. (*B*) Raf-1 proteins in cells transformed by v-src (src), by v-fos (fos) [Curran *et al.* (26)] or by c-myc are shown. The c-myc-expressing cells are transformed due to c-myc overexpression from a murine mammary tumor virus-inducible promoter (19). PyLT is an NIH 3T3 cell line expressing polyoma virus large T antigen (D.R.K., unpublished results). Control quiescent BALB/3T3 fibroblasts (C) are also shown. Substrate development time for the blots was 2 min.

The Phosphorylation of the Raf-1 Protein Increases in Response to Oncogenes, Mitogens, and PMA Treatment. To determine whether the changes in the mobility of the Raf-1 protein were accompanied by an increase in phosphorylation, we labeled cells with [^{32}P]orthophosphate and immunoprecipitated the Raf-1 protein (Fig. 2). Alterations in Raf-1 protein phosphorylation were first examined in quiescent BALB/3T3 cells treated for 5 min with the following 3T3 cell mitogens: EGF, FGF, PDGF, and PMA (22, 23). In immunoprecipitates from mitogen-treated cells, the Raf-1 protein exhibited a decreased mobility and increased phosphate content as compared to untreated quiescent cells (Fig. 2*A*). Treatment for 24 hr with the Tyr(*P*) phosphatase inhibitor sodium orthovanadate also increased the phosphorylation and molecular mass of the Raf-1 protein (Fig. 2*A*). However, when quiescent 3T3 cells were treated with insulin for 5 min, or with dibutyryl cAMP, an activator of the cAMP-dependent protein kinase, no size change or increased phosphorylation of the Raf-1 protein was observed (Fig. 2*A*).

Expression of various membrane-bound oncogene products results in changes in Raf-1 protein. As shown in Fig. 2*B*, the Raf-1 protein displayed an altered electrophoretic mobility and enhanced phosphorylation in cells transformed by expression of the v-src, v-sis, v-fms, c-H-ras or polyoma

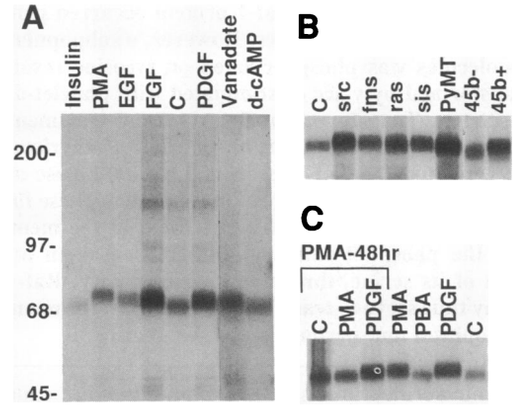

FIG. 2. Changes in Raf-1 phosphorylation in response to membrane-bound oncogene products, mitogens, and PMA treatment. Oncogene-expressing or quiescent 3T3 cells were labeled with [^{32}P]orthophosphate for 3 hr prior to lysis. Anti-Raf-1 immunoprecipitates were analyzed by 7.5% SDS/PAGE. (*A*) Quiescent BALB/3T3 cells were treated for 5 min with insulin (5 μg/ml), PMA (50 ng/ml), EGF (100 ng/ml), acidic FGF (10 units/ml), or PDGF (10 units/ml) prior to lysis. Cells were also treated with 40 μM sodium orthovanadate or 1 mM dibutyryl cAMP (d-cAMP) for 24 hr. Control quiescent BALB/3T3 fibroblasts (C) are also shown. (*B*) 3T3 cells were transformed by v-src, v-fms, c-H-ras (activated for transformation by mutation at codon 61, M. Corbley, and T.M.R., unpublished results), v-sis (C. D. Stiles, personal communication), or polyoma virus middle-sized T antigen (PyMT) (20). 45b is an NIH 3T3 cell line expressing polyoma virus middle-sized T antigen under the control of the dexamethasone-inducible murine mammary tumor virus promoter (21). 45b+, 45b cells treated for 16 hr with 5 μM dexamethasone to induce middle-sized T antigen synthesis; 45b−, untreated 45b cells. (*C*) Quiescent BALB/3T3 cells were chronically treated with PMA at 250 ng/ml for 48 hr to remove PMA binding sites and protein kinase C activity. PMA, chronically treated quiescent cells treated for an additional 5 min with PMA at 50 ng/ml. PDGF, chronically treated quiescent cells treated for 5 min with PDGF (10 units/ml). Also shown are quiescent BALB/3T3 cells (not pretreated with PMA) (C), treated for 5 min with PDGF (10 units/ml), PMA (50 ng/ml), or biologically inactive PMA (phorbol 13-butyrate) (50 ng/ml).

virus middle-sized tumor (T) antigen oncogenes. We also examined Raf-1 protein phosphorylation in cells where expression of the polyoma virus middle-sized T oncogene is under the control of the dexamethasone-regulatable murine mammary tumor virus promoter. Addition of dexamethasone (5 μM for 16 hr) to this cell line, 45b, results in the appearance of anchorage-independent growth concomitant with the induction of middle-sized T antigen synthesis (21). Treatment of quiescent 45b cells with dexamethasone resulted in an increase in phosphorylation and apparent molecular mass of the Raf-1 protein when compared to the Raf-1 protein from untreated 45b cells (Fig. 2*B*). The cells appeared more refractile and the saturation density of the cultures increased after the addition of dexamethasone (data not shown). Dexamethasone treatment of control quiescent BALB/3T3 cells did not result in any alterations of the Raf-1 protein or cellular morphology (data not shown). These results suggest that the changes in Raf-1 protein phosphorylation and mobility observed in transformed cells are due to oncogene expression and correlate with the acquisition of the transformed state.

We next examined the effect of PMA on the migration and phosphorylation of the Raf-1 protein to determine if the changes observed in response to oncogenic transformation or growth-factor stimulation could be correlated with the activation of protein kinase C (Fig. 2*C*). Quiescent BALB/3T3 cells treated for 5 min with biologically inactive PMA (phorbol 13-butyrate) (50 ng/ml) showed no change in the Raf-1 protein, in contrast to the changes observed with biologically active PMA (50 ng/ml). If cells were chronically treated with PMA at 250 ng/ml for 48 hr to remove PMA-binding sites and protein kinase C activity (23), the Raf-1 protein mobility and phosphorylation state returned to that observed in quiescent BALB/3T3 cells (Fig. 2*C*). Subsequent treatment of chronically treated cells with PMA (50 ng/ml for 5 min) still showed no change in the Raf-1 protein (Fig. 2*C*). However, treating these cells for 5 min with PDGF did increase the phosphate content and molecular mass of the Raf-1 molecules (Fig. 2*C*). These results suggest that while PMA activation of protein kinase C can affect the mobility and phosphorylation state of the Raf-1 protein, there are mechanisms independent of protein kinase C through which growth factors can bring about this change.

Electrophoretic Mobility Changes in Raf-1 Protein Are Due to Phosphorylation. To confirm that the changes in Raf-1 protein mobility were due to phosphorylation, the Raf-1 protein immunoprecipitates from PDGF-stimulated and quiescent 3T3 cells were treated with potato acid phosphatase. Fig. 3 shows that phosphatase treatment decreased the apparent molecular mass of Raf-1 molecules in PDGF-stimulated cells from a molecular mass of ≈76 kDa to ≈72 kDa and in unstimulated cells from 74 kDa to a molecular mass identical to that of the phosphatase-treated Raf-1 molecules from PDGF-treated cells. Additional experiments in which the phosphatase was inactivated by treatment with sodium fluoride prior to mixing with the Raf-1 immunoprecipitates confirmed that this size decrease was due to phosphate removal and not to proteolysis (data not shown). Thus the molecular mass increase after PDGF stimulation can be accounted for solely by the enhanced phosphorylation of Raf-1 molecule.

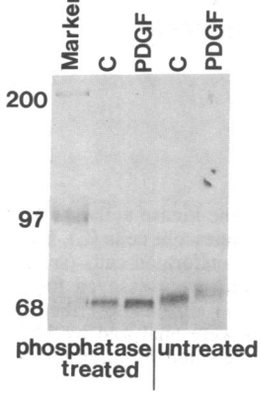

FIG. 3. Phosphatase treatment of Raf-1 protein. Anti-Raf-1 immunoprecipitates from PDGF-stimulated (10 units/ml for 5 min) (PDGF) and quiescent 3T3 cells (C) were treated with potato acid phosphatase (4 μg phosphatase per 40 μl of reaction mixture) in the presence of aprotinin (0.15 unit/ml) and leupeptin (20 μM) for 20 min at 30°C. Treated and untreated immunoprecipitates were electrophoresed by 7.5% SDS/PAGE and transferred to nitrocellulose filters. Raf-1 proteins were detected by probing the blot with anti-Raf-1 antibody.

Raf-1 Protein from PDGF-Treated and v-src-Transformed Cells Is Phosphorylated on Serine and Tyrosine Residues. Since many of the growth factor receptors and membrane oncogene products have associated tyrosine kinase activities, the Raf-1 protein was examined to determine if any of the increased phosphorylation of the protein could be due to phosphorylation on tyrosine residues. First, Tyr(*P*)-containing proteins were immunoprecipitated by using an antibody which specifically recognizes Tyr(*P*) residues from quiescent 3T3 cells and v-src-transformed cells or from quiescent and PDGF-treated 3T3 cells overproducing the Raf-1 protein (10-fold overproduction). Protein blots of the anti-Tyr(*P*) immunoprecipitates were then probed with anti-Raf-1 antibody. Fig. 4*A* shows that the Raf-1 protein is immunoprecipitated by the anti-Tyr(*P*) antisera from v-src-transformed cells and from PDGF-treated Raf-1 overproducer cells (c-raf*) but not from quiescent cells. To continue this examination, quiescent cells stimulated with PDGF and v-src-transformed cells were incubated with [^{32}P]orthophosphate and labeled proteins were immunoprecipitated with either the anti-Raf-1 antibody or the anti-Tyr(*P*) antibody. Only in PDGF-stimulated and v-src-transformed cells did the anti-Tyr(*P*) antibody precipitate a protein whose migration corresponded with at least some of the Raf-1 molecules found in these cells. Two-dimensional tryptic phosphopeptide mapping revealed that the proteins immunoprecipitated by the anti-Tyr(*P*) and anti-Raf-1 antisera from the PDGF-treated cells and by the anti-Raf-1 antibody from unstimulated control cells shared a similar set of ^{32}P-labeled peptides (Fig. 4*C*). The identity of this set of peptides was confirmed by mixing ^{32}P-labeled peptides immunoprecipitated by the anti-Tyr(*P*) and anti-Raf-1 antisera from the PDGF-treated cells (data not shown). Identical results were observed in immunoprecipitates from v-src-transformed cells (data not shown). The tryptic maps also revealed that there are a number of peptides characteristic of the Raf-1 protein observed from control cells as well as from the PDGF-stimulated and v-src-expressing cells (Fig. 4*C*); however, the Raf-1 molecules from the PDGF-stimulated cells contain additional ^{32}P-containing peptides that are not observed in control or v-src-transformed cells (Fig. 4*C*).

Phospho amino acid analysis demonstrated that the population of Raf-1 molecules recognized by the anti-Tyr(*P*) antiserum was phosphorylated on both tyrosine and serine residues (Fig. 4*D*). The amount of ^{32}P-labeled Raf-1 protein in the anti-Tyr(*P*) immunoprecipitates represented only a fraction of the total ^{32}P-labeled cellular Raf-1 molecules observed in the anti-Raf-1 immunoprecipitates. In anti-Raf-1 immunoprecipitates from the PDGF-stimulated cells, we detected threonine phosphorylation and large increases in serine phosphorylation as compared to the quiescent 3T3 cells (Fig. 4*D*). Similar increases in serine and threonine phosphorylation were also observed in anti-Raf-1 immunoprecipitates from v-src cells (data not shown). We were unable to detect Tyr(*P*) residues on the Raf-1 protein precipitated with the anti-Raf-1 antiserum from PDGF-treated BALB/3T3 cells; however, when cells overproducing the Raf-1 protein (c-raf*) were stimulated with PDGF, Tyr(*P*) residues were readily apparent in anti-Raf-1 immunoprecipitates (Fig. 4*D*). We were unable to detect a band comigrating with Raf-1 in anti-Tyr(*P*) immunoprecipitates from PMA-, FGF-, or EGF-treated 3T3 cells or in activated c-H-ras-transformed 3T3 cells (data not shown).

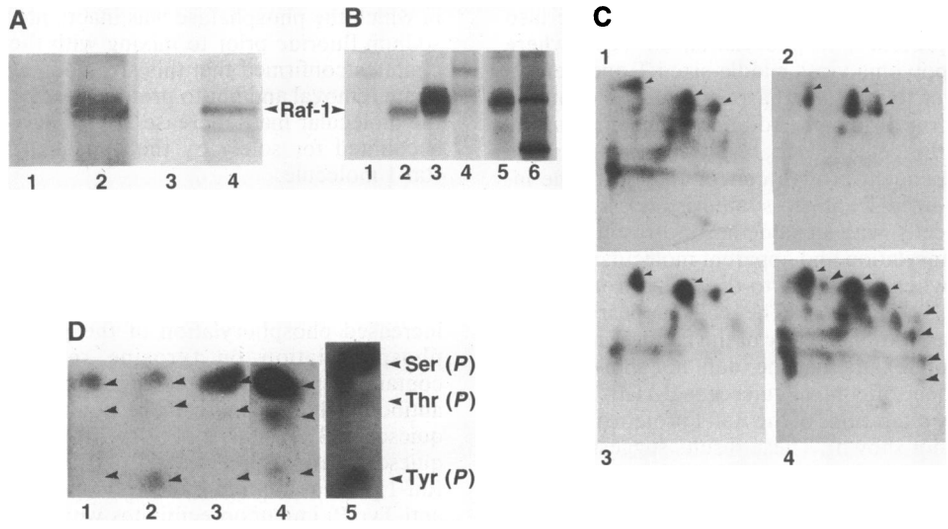

FIG. 4. Enhancement of serine, threonine, and tyrosine phosphorylation in Raf-1 molecules from PDGF-treated and v-src-transformed cells. (*A*) Tyr(*P*)-containing proteins were directly immunoprecipitated by using an anti-Tyr(*P*) antibody from quiescent (control, lane 3) and v-src-transformed (lane 4) cells or from quiescent (control, lane 1) and PDGF-treated (lane 2) c-raf* cells (a NIH 3T3 nontransformed cell line in which the Raf-1 protein is overexpressed 10-fold; U.R., unpublished results). The anti-Tyr(*P*) immunoprecipitates were electrophoresed on SDS/polyacrylamide gels, transferred to nitrocellulose filters, and then probed with anti-Raf-1 antibody. (*B*) Cells were labeled with [^{32}P]orthophosphate for 3 hr. v-src-transformed cells, PDGF-treated cells (10 units/ml for 5 min), and quiescent cells were lysed and immunoprecipitated with either anti-Raf-1 antibody or anti-Tyr(*P*) antibody. Anti-Tyr(*P*) and anti-Raf-1 immunoprecipitates from quiescent cells (control; lanes 1 and 2, respectively), PDGF-treated cells (lanes 3 and 4, respectively), or v-src-transformed (lanes 5 and 6, respectively) cells. (*C*) Two-dimensional tryptic peptide mapping of phosphoproteins immunoprecipitated with anti-Raf-1 antibody from quiescent cells (control, map 2), PDGF-treated cells (map 4), or v-src-transformed (map 1) cells. Map 3 is a map of the phosphoprotein co-migrating with Raf-1 in anti-Tyr(*P*) immunoprecipitates from PDGF-treated cells. Peptides were analyzed on thin-layer cellulose plates by electrophoresis (vertical axis) followed by chromatography (horizontal axis). (*D*) Phospho amino acid analysis of the Raf-1 molecules immunoprecipitated by the anti-Raf-1 and anti-Tyr(*P*) antisera. Ser(*P*), phosphoserine. Thr(*P*), phosphothreonine. The phospho amino acid analysis of the Raf-1 protein immunoprecipitated by anti-Raf-1 antibody was from 3×10^6 cells, while the analysis of Raf-1 protein immunoprecipitated by anti-Tyr(*P*) antibody was from 10^7 cells. The exposure time was 3 days. Lanes: 1, control cells immunoprecipitated with anti-Raf-1; 2, PDGF-treated cells immunoprecipitated with anti-Tyr(*P*); 3, PDGF-treated cells immunoprecipitated with anti-Raf-1; 4, PDGF-treated c-raf* cells immunoprecipitated with anti-Raf-1; 5, src-transformed cells immunoprecipitated with anti-Tyr(*P*).

PDGF and PMA Treatment and v-src Expression Increase Raf-1-Associated Serine/Threonine Kinase Activity. To determine if the physical alterations of the Raf-1 molecules correlate with changes in the serine/threonine kinase activity of the protein, quantitative immune-complex kinase assays were performed *in vitro* with Raf-1 proteins immunoprecipitated from quiescent BALB/3T3 cells, quiescent cells treated for 5 min with either PDGF or PMA, and v-src-transformed cells. To insure that equivalent amounts of Raf-1 protein were present in each sample, cell lysates were normalized for protein content by [^{35}S]methionine-labeling analysis. As shown in Fig. 5, the ^{32}P labeling of histone type V-S increased in the anti-Raf-1 immunoprecipitates from v-src-transformed cells and from cells treated with PMA and PDGF when compared to immunoprecipitates from quiescent control cells. Cerenkov counting to measure the ^{32}P radioactivity in the histone bands revealed that PMA and PDGF treatment of cells resulted in a 3-fold increase in histone phosphorylation above basal levels, while v-src transformation resulted in a 5-fold increase (as averaged from four experiments). Phospho amino acid analysis of the histone molecules phosphorylated *in vitro* revealed that serine was the predominant phosphorylated residue. A slight amount of threonine phosphorylation was detected; however, no Tyr(*P*) residues were observed (data not shown).

DISCUSSION

Cytoplasmic serine/threonine kinases have been postulated to function downstream from membrane events in the mitogenic pathway (24). We have tested this hypothesis by examining the phosphorylation and protein kinase activity of Raf-1 protein isolated from growth-factor-stimulated and oncogene-transformed cells. Membrane-bound oncogene product expression and growth-factor addition increased the phosphate content and apparent molecular weight of Raf-1 protein whereas nuclear oncogene product expression did not. In addition, Raf-1-associated serine/threonine kinase activity was enhanced by growth-factor treatment and membrane-bound oncogene product expression. These results suggest that Raf-1 molecules receive signals from membrane-bound receptors and oncogene products and transmit them to as yet unidentified cellular targets.

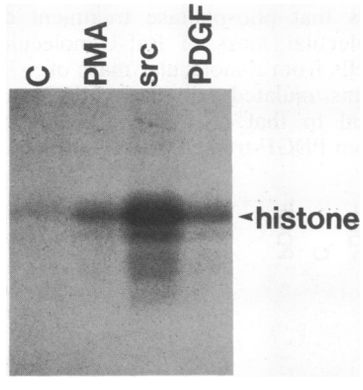

FIG. 5. Raf-1-associated serine/threonine kinase activity. Anti-Raf-1 immunoprecipitates prepared from quiescent cells (C), PMA-treated cells (50 ng/ml for 5 min), v-src-transformed cells (src), or PDGF-treated cells (10 units/ml for 5 min) were assayed for the ability to phosphorylate histone type V-S *in vitro* as described (8). Assay time was 5 min at 25°C. Samples were analyzed by 12.5% SDS/PAGE and phosphoproteins were detected by autoradiography. Protein kinase assays were linear over the time of the assay.

Mechanism of Hyperphosphorylation of Raf-1 Protein in Response to Oncogenic and Mitogenic Signals. Our data suggest that the increased mobility of Raf-1 protein may occur by at least two mechanisms. Phosphatase experiments, phospho amino acid analysis, and phosphopeptide mapping all indicate that the shift in mobility of the Raf-1 protein is due to increased phosphorylation occurring primarily on serine residues. The increase in Raf-1 phosphorylation and apparent molecular mass in response to PMA treatment suggests that these alterations may occur as a result of serine phosphorylation directly mediated by protein kinase C. Consistent with this possibility, chronic treatment of cells with PMA, which reduces membrane protein kinase C activity (25), prevents subsequent induction of Raf-1 protein phosphorylation by PMA. However, PDGF stimulation alters Raf-1 protein phosphorylation and mobility in cells chronically treated with PMA and chronic treatment of v-src-transformed cells does not reverse the Raf-1 protein alterations observed in those cells. These experiments suggest that there are mechanisms, independent of protein kinase C which modulate the Raf-1 protein phosphorylation. An alternative mechanism is suggested by experiments demonstrating that the Raf-1 protein is phosphorylated on tyrosine residues in response to PDGF stimulation and v-src expression. Treatment of cells with Tyr(*P*) phosphatase inhibitor sodium orthovanadate also results in increased serine and tyrosine phosphorylation of Raf-1 molecules. These results imply that increases in cellular tyrosine phosphorylation may affect Raf-1 protein migration and phosphorylation. While tyrosine phosphorylation accounts for little of the total Raf-1 phosphate increase, it could stimulate Raf-1 phosphorylation by at least two mechanisms. (*i*) Raf-1 protein phosphorylation may occur by the action of other serine/threonine kinases whose activity is induced by tyrosine kinases. (*ii*) Tyrosine phosphorylation of the Raf-1 protein by tyrosine kinases may induce Raf-1 protein kinase activity, resulting in enhanced autophosphorylation of the molecule.

Membrane-bound oncogene products and growth-factor receptors might activate Raf-1 activity by the phosphorylation of amino acids residing in the amino-terminal portion of the protein. The amino-terminal domain of the Raf-1 protein is known to have a regulatory role based on experiments showing that Raf-1 can be rendered a transforming protein by removal of this domain and that the v-raf product is truncated in this region (6). Within the amino-terminal domain there is a region rich in serine/threonine residues (9). The experiments presented here suggest that phosphorylation of these residues may activate Raf-1 protein kinase activity thereby propagating proliferative signals to cellular targets mediating mitogenesis. The importance of the serine/threonine kinase activity to Raf-1 function and the nature of the targets of this activity are presently unknown. The precise role of tyrosine, threonine, and serine phosphorylation in modulating Raf-1 activity will be determined by mutational analysis of the Raf-1 protein.

The Role of Raf-1 as a Signal Transducer. Our results indicate that there is a correlation between proliferative events generated at the membrane and alterations in the Raf-1 protein, including activation of the Raf-1 associated serine/threonine kinase activity. Since the expression of nuclear oncogene products did alter Raf-1 protein phosphorylation, we hypothesize that the Raf-1 protein is involved in the mediation of proliferative signals generated at the membrane but not in the mediation of proliferative signals originating in the nucleus. Other experiments suggest that the *raf* gene product functions downstream of membrane-associated oncogene products in the signal-transduction pathway (6, 24). In studies by Smith *et al.* (24), microinjected antibodies directed against Raf-1 were able to inhibit the growth of cells transformed by v-ras and v-src but not those transformed by v-raf. The investigators concluded that the v-raf protein either acts independently of $p21^{c\text{-}ras}$ or that it functions downstream of $p21^{c\text{-}ras}$. The experiments presented here, showing Raf-1 protein phosphorylation in response to activated c-Ha-ras protein expression, suggest the latter possibility. We propose that the activation of Raf-1 by mitogenic stimuli at the membrane provides another route of signal transduction to the nucleus. This route would function in addition to the well established pathways mediated by protein kinases C and A and the calcium-regulated kinases.

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