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HUMAN COLONY STIMULATING FACTOR-1 RECEPTOR ACTIVATES THE C-*raf*-1 PROTO-ONCOGENE KINASE

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The proto-oncogene c-*raf*-1 encodes a 74 kD serine/threonine kinase. Recently, it has been shown that Raf kinase activity is stimulated by platelet derived growth factor (PDGF) treatment of receptor bearing cells, and that p74 is a direct substrate for PDGF receptor. CSF-1 treatment of BeWo cells, a human choriocarcinoma cell line, and mouse NIH 3T3 cells expressing a transfected human CSF-1 receptor cDNA, was associated with a 3-4 fold increase in phosphorylation of a 74 kD protein immunoprecipitated with affinity purified Raf-1 antibody. The kinase activity of p74 was increased 2-3 fold against two exogenous substrates following CSF-1 treatment of the transfected cells. These observations suggest that Raf-1 protein is a downstream second messenger molecule in CSF-1 mediated signal transduction.

Mononuclear phagocytes and their precursors require the hemopoietin colony stimulating factor-1 (CSF-1) for their growth, differentiation, and survival (1). CSF-1 binds specifically to a 150 kD transmembrane glycoprotein, and stimulates tyrosine kinase activity in the cytoplasmic domain of the receptor (2). The activated receptor tyrosine phosphorylates cellular substrates that are believed to be important for transducing and amplifying the CSF-1 induced signal (3). Candidate signal transducing molecules engaged by tyrosine kinase receptors include phosphatidylinositol 3-kinase (PI 3-kinase), phospholipase Cγ, c-Raf-1 protein, and the GTPase activating protein (GAP) (4-9). Epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor beta (PDGFRβ) phosphorylate PLCγ on tyrosine residues (6,7), whereas CSF-1R does not (10). PDGFR and CSF-1R also have been shown to activate PI 3-kinase, which catalyzes the formation of the novel phospholipid phosphatidylinositol 3-phosphate (5,11,12). PI 3-kinase has been shown

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o form a complex with polyoma virus middle T antigen and the *src* proto-oncogene rotein, and with a number of retroviral tyrosine kinase oncogene products (13-14).

Microinjection of antibodies specific for the *ras* protein can inhibit cell ransformation by several tyrosine kinase oncogenes. However, anti-*ras* antibody loes not inhibit v-*raf* induced transformation (15). This suggests that c-*raf*-1 unctions downstream of *ras* during cellular transformation. As c-*raf*-1 encodes a erine/threonine kinase, it has been suggested that a cascade of activated kinases ight be involved in growth regulation (16). We tested whether the serine/threonine inase proto-oncogene product c-Raf-1 might become activated upon treatment with SF-1 of mouse NIH3T3 cells expressing a transfected human CSF-1R cDNA. A 74 kD rotein specifically immunoprecipitated with anti-Raf-1 antibody displays increased hosphorylation after treatment of CSF-1R bearing cells with recombinant CSF-1. SF-1 treatment was correlated with an increase in kinase activity toward two xogenous substrates in c-Raf-1 immunoprecipitates.

MATERIALS AND METHODS

Materials. CSF-1R monoclonal antibody, goat anti-rat IgG and Protein A-Agarose eads were obtained from Oncogene Science. Recombinant human CSF-1 (lot no. 38322A, pecific activity 100 U/μl) was from Cellular Products, Buffalo, N.Y. PY20 anti-hosphotyrosine monoclonal antibody was from ICN. Rabbit skeletal muscle actin was rom Sigma. Chicken histone H5 was a gift from Dr. Mike Bustin. γ^{32}P-ATP, ^{32}P-rthophosphate and ^{125}I-Protein A were purchased from New England Nuclear. All other eagents used were analytical grade.

Cell lines. The human placental trophoblast line BeWo was routinely grown in 12K* medium in presence of 15% fetal bovine serum. The mouse NIH 3T3 cells ransfected with a retroviral construct containing CSF-1R cDNA (clone 2-9) were rown in Dulbecco's modified Eagle medium containing 10% fetal bovine serum and 0.8 g/ml G418 (12). The cells were made quiescent by incubating confluent monolayers n medium containing 0.5% serum for 20 hours and stimulated with 1000 U/ml ecombinant human CSF-1 for 15 minutes.

Labelling and immunoprecipitation. Confluent cell monolayers were labeled with mCi of ^{32}P-orthophosphate for 4 hours in phosphate-free medium and lysed in the ish with RIPA buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 5mM EDTA, 1% NP-40, 1 mM Na_3VO_4, 1mM PMSF, 0.25% aprotinin) at 4° for 20 minutes. The cell debris was iscarded after centrifugation at 4°C for 20 minutes. The supernatant was incubated vith 15 μl of CSF-1R monoclonal antibody on ice for 30 minutes. Anti-rat IgG oupled to agarose beads was added and the mixture was placed on a rocking platform t 4°C for 3 hours. The beads were washed once with RIPA buffer, twice with 0.5 M iCl, 0.1 M Tris-HCl pH 7.5, once more with RIPA buffer and finally twice with 50 mM Tris-HCl pH 7.4, 1 mM Na_3VO_4. The beads were resuspended in SDS sample buffer and nalyzed on SDS polyacrylamide gels. Following electrophoresis the gel was treated hour with 1 M KOH (17), dried and autoradiographed with an intensifying screen at -80°C.

Immunokinase assay. The cell monolayer was lysed in RIPA buffer containing 1 mM DTT and immunoprecipitated with affinity purified antipeptide antibody prepared gainst a peptide from the c-Raf-1 carboxyterminus (18). The immunobeads were ashed three times with RIPA buffer containing 1 mM DTT and twice with 50 mM Tris-Cl pH 7.5, 1 mM DTT. The beads were resuspended in Raf-1 kinase buffer (50 mM ris-HCl pH 7.5, 50 mM NaCl, 1 mM DTT, 10 mM $MnCl_2$). Either 1 μg of chicken histone 5 or 5 μg of rabbit skeletal muscle actin was added. 20 μC_i of γ^{32}P ATP was added nd the whole reaction mixture was incubated at 37°C for 10 minutes. The reaction as stopped with concentrated SDS gel sample buffer and the labeled proteins were nalyzed by SDS polyacrylamide gel electrophoresis. The gel was fixed, dried and utoradiographed on Kodak X-OMat AR film with an intensifying screen at -80°C.

RESULTS

Detection of a 74 kD protein in BeWo cells. A variety of proto-oncogene products have been detected in placental tissue. The proto-oncogene c-*fms*/CSF-1R protein is expressed in placental trophoblasts where it might play a role in placental development (19). We used the human trophoblastic cell line BeWo to detect association of proteins with the CSF-1R protein. ^{32}P-orthophosphate labeled cells were stimulated with recombinant human CSF-1 and the cell lysate was immunoprecipitated with CSF-1R monoclonal antibody. The results are shown in Figure 1 (lanes 1 and 2). Along with the small amount of phosphorylated receptor protein (indicated by small arrowheads) a 74 kD protein is detected in receptor immunoprecipitates prepared from CSF-1 treated cells only (large arrow-head).

Recently, it has been shown that the insulin receptor phosphorylates the c-*raf*-1 proto-oncogene product p74 only upon stimulation of receptor bearing cells with insulin (20). This observation suggested that the 74 kD protein detected in BeWo cells might include the c-Raf-1 protein kinase. To test this idea, ^{32}P-orthophosphate labeled BeWo cells were stimulated with CSF-1 and immunoprecipitated with an affinity purified Raf-1 specific antibody (18). The results (Fig. 1, lanes 3 and 4) show a 3-fold stimulation of phosphorylation of c-Raf-1 protein in CSF1 treated BeWo cells as determined by scanning densitometry of the autoradiogram.

Raf-1 phosphorylation in NIH3T3 cells. Mouse NIH3T3 cells do not express endogenous CSF-1R, thus providing a good system for studying some of the functions of this receptor in transfected cells. Recently we have expressed biologically active human CSF-1R in NIH3T3 cells (clone 2-9) (12). To study Raf-1 phosphorylation we labeled these cells with ^{32}P-orthophosphate and stimulated with recombinant human CSF-1. The cell lysate was immunoprecipitated with c-Raf-1 antibody and the proteins were separated by SDS gel electrophoresis (Fig. 2). The data show that CSF-1 treatment of 2-9 cells significantly increases the phosphorylation of the c-Raf-1 protein. Scanning densitometry revealed a 4-fold increase in c-Raf-1 phosphorylation in CSF-1 treated cells. In addition, CSF-1 treatment induced a shift in mobility of the c-Raf-1 protein presumably as a result of the change in phosphorylation state. This shift has also been observed in previous reports of c-Raf-1 phosphorylation (8,20).

Activation of c-*raf*-1 kinase activity. The oncogene v-*raf*-1 of the murine sarcoma virus 3611 is an activated serine/threonine kinase (21), and the transforming potential of the proto-oncogene product c-Raf-1 can be activated by truncation of its amino terminus (22). To determine if increased phosphorylation of c-Raf-1 protein observed in CSF-1 treated cells leads to activation, we tested the kinase activity of the protein *in vitro*. The c-Raf-1 protein was immunoprecipitated from CSF-1 stimulated and nonstimulated 2-9 cells. The immunobeads were subsequently used in an *in vitro* immunokinase assay with the exogenous substrates actin or chicken histone H5 (Fig. 3). The results indicate that after treatment of receptor bearing cells with CSF-1, the c-Raf-1 kinase

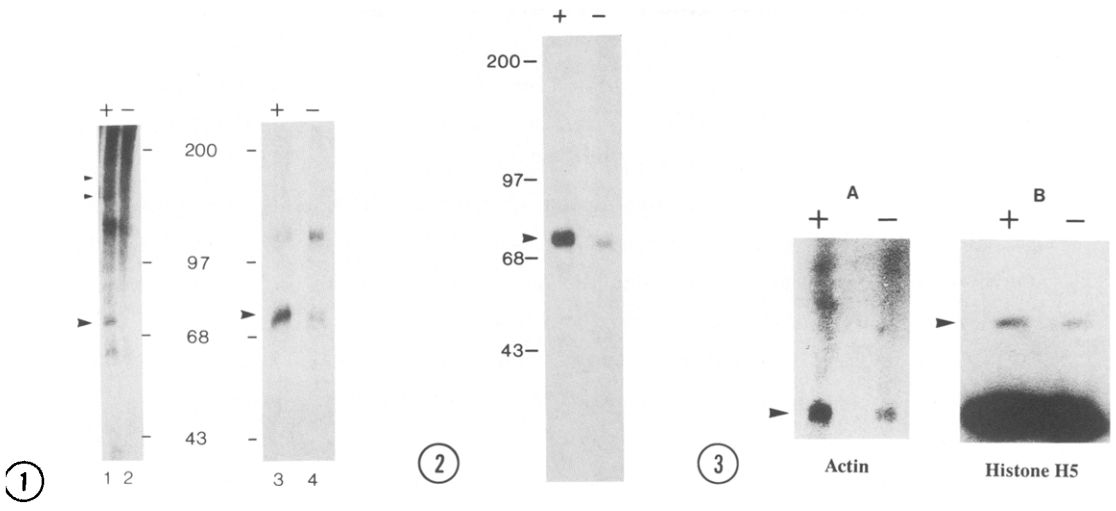

Fig. 1. Phosphorylation of a 74 kD protein in BeWo cells: Confluent cells were made quiescent and labeled with ^{32}P-orthrophosphate as described in Materials and Methods. The cells were stimulated with 1000 U/ml CSF-1 for 15 minutes. The cell lysate was immunoprecipitated either with CSF-1 receptor monoclonal antibody or Raf-1 antibody. The immunobeads were analyzed on a 7.5% SDS polyacrylamide gel. The gel was treated with 1 M KOH (17), dried and autoradiographed at -80°C with an intensifying screen. Lanes 1 and 2: immunoprecipitation with CSF-1 receptor antibody; lanes 3 and 4: Raf-1 antibody. + and - indicate the presence or absence of CSF-1, respectively. Small arrowheads indicate the receptor bands and the large arrowhead shows the 74 kD protein. The molecular weight markers are given in kilodaltons.

Fig. 2. Detection of the 74 kD Raf-1 phosphoprotein in CSF-1 receptor bearing NIH 3T3 cells: ^{32}P-orthophosphate labeled quiescent 2-9 cells were stimulated with 1000 U/ml CSF-1 for 15 minutes. The cell lysates were immunoprecipitated with anti-Raf-1 antibody and the precipitates were analyzed on a 7.5% SDS gel. The gel was treated with alkali (17). + and - indicate the presence or absence of CSF-1 and the arrowhead indicates the p74 Raf-1 protein.

Fig. 3. Activation of Raf-1 kinase activity: Quiescent 2-9 cells were stimulated with 1000 U/ml of CSF-1 for 15 minutes and immunoprecipitated with anti-Raf-1 antibody. The immunobeads were used in an immunokinase assay either with 5 μg of rabbit skeletal muscle actin (Panel A) or with 1 μg of chicken histone H5 (Panel B) as substrates as described in Materials and Methods. + and - indicate the presence or absence of CSF-1. Arrowheads indicate the actin (Panel A) and histone H5 (panel B) bands.

activity is stimulated three-fold with actin (Panel A) and two-fold with chicken histone H5 (Panel B) as substrates (as determined by densitometric scanning of the autoradiograms).

DISCUSSION

In response to growth factor treatment of cells, a number of proteins become phosphorylated, some on tyrosine residues. The role and function of these proteins have not been demonstrated in the growth factor mediated early and late events involved in mitogenesis. EGF and PDGF receptors phosphorylate PLCγ on tyrosine residues (6,7) which might stimulate its enzymatic activity to produce the second messengers diacylglycerol and inositol 1,4,5-trisphosphate from phosphatidyl-inositol-4,5-bisphosphate. In contrast, CSF-1R does not induce phosphatidylinositol

turnover or tyrosine phosphorylation of PLCγ (10, Ghosh Choudhury unpublished). Belonging to the same receptor family, CSF-1 and PDGF receptors do share some common properties. Recently, our laboratory and others have demonstrated that as with PDGFR, CSF-1R can associate with and activate a novel phosphatidylinositol 3-kinase which produces PI 3-phosphate from PI (11,12).

It has been shown that other substrates of PDGFR are the proto-oncogene product c-Raf-1 and GTPase activating protein (GAP) (8,9). C-Raf-1 is expressed in almost all hematopoietic cells and in various differentiated tissues (16). Activation of c-Raf-1 serine/threonine kinase activity has been implicated during cellular differentiation and transformation (16,22). Here we have shown that stimulation of CSF-1R leads to increased phosphorylation of the 74 kD c-Raf-1 protein (Fig. 1 and Fig. 2), and was correlated with increased kinase activity in Raf-1 immunoprecipitates (Fig. 3). Although the phosphorylation observed in p74 was alkali resistant (17), it is not clear if tyrosine was the target. Use of the anti-phosphotyrosine monoclonal antibody PY20 in immunoblot analyses of CSF-1 stimulated 2-9 cell extracts did not reveal tyrosine phosphorylation of a 74 kD protein (unpublished observation). However, it is tempting to suggest that stimulation of CSF-1R at the plasma membrane can transduce proliferative signals through phosphorylation of several substrate proteins, or by indirectly stimulating other substrates. c-Raf-1, activated in response to CSF-1 treatment of receptor bearing cells, could phosphorylate additional downstream proteins involved in CSF-1 mediated proliferation and differentiation. For example, CSF-1 stimulates rapid induction of c-*fos* in receptor-bearing cells (23). Activated Raf-1 also stimulates c-*fos* transcription through a serum responsive element (24), thus implicating c-Raf-1 as an important intermediate in CSF-1 mediated signal transduction.

Recently the *Drosophila* homolog of the c-Raf-1 gene product, 1 (1) polehole (*1(1)ph*), has been shown to be necessary as a substrate for a transmembrane tyrosine kinase protein (*tor*) to express a gain-of-function *tor* mutant phenotype (25). This indicates that c-Raf-1 phosphorylation and activation may have a developmental role in growth factor mediated pathways. Since CSF-1R and c-Raf-1 proteins are expressed at high levels in placental cell lines, they may also play important roles in embryonic development (19).

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