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Heparin-Binding Growth Factor 1 Stimulates Tyrosine Phosphorylation in NIH 3T3 Cells

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Tyrosine phosphorylation of cellular proteins induced by heparin-binding growth factor 1 (HBGF-1) was studied by using the murine fibroblast cell line NIH 3T3 (clone 2.2). HBGF-1 specifically induced the rapid tyrosine phosphorylation of polypeptides of M_r 150,000, 130,000, and 90,000 that were detected with polyclonal and monoclonal antiphosphotyrosine (anti-P-Tyr) antibodies. The concentration of HBGF-1 required for half-maximal induction of tyrosine phosphorylation of the M_r-150,000 M_r-130,000, and M_r-90,000 proteins was approximately 0.2 to 0.5 ng/ml, which was consistent with the half-maximal concentration required for stimulation of DNA synthesis in NIH 3T3 cells. HBGF-1-induced tyrosine phosphorylation of the M_r-150,000 and M_r-130,000 proteins was detected within 30 s, whereas phosphorylation of the M_r-90,000 protein was not detected until 3 min after HBGF-1 stimulation. All three proteins were phosphorylated maximally after 15 to 30 min. Phosphoamino acid analysis of the M_r-150,000 and M_r-90,000 proteins confirmed the phosphorylation of these proteins on tyrosine residues. Phosphorylation of the M_r-150,000 and M_r-90,000 proteins occurred when cells were exposed to HBGF-1 at 37°C but not at 4°C. Exposure of cells to sodium orthovanadate, a potent P-Tyr phosphatase inhibitor, before stimulation with HBGF-1 resulted in enhanced detection of the M_r-150,000, M_r-130,000, and M_r-90,000 proteins by anti-P-Tyr antibodies. Anti-P-Tyr affinity-based chromatography was used to adsorb the HBGF-1 receptor affinity labeled with ^{125}I-HBGF-1. The cross-linked HBGF-1 receptor-ligand complex was eluted with phenyl phosphate as two components: M_r 170,000 and 150,000. P-Tyr, but not phosphoserine or phosphothreonine, inhibited adsorption of the ^{125}I-HBGF-1–receptor complex to the anti-P-Tyr antibody matrix. Treatment of cells with sodium orthovanadate also enhanced recognition of the cross-linked ^{125}I-HBGF-1–receptor complex by the anti-P-Tyr matrix. These data suggest that (i) the ^{125}I-HBGF-1–receptor complex is phosphorylated on tyrosine residues and (ii) HBGF-1-induced signal transduction involves, in part, the tyrosine phosphorylation of at least three polypeptides.

Class 1 heparin-binding growth factor (HBGF-1) is an acidic polypeptide mitogen for endothelial cells and fibroblasts (24, 31) and is commonly known as acidic fibroblast growth factor (aFGF), a truncated derivative of its precursor, β-endothelial cell growth factor (4). aFGF is a member of a family of polypeptides that presently includes basic FGF (bFGF) (1) and the oncogenes *hst*/KS (2, 34), *int-2* (9), and FGF-5 (35). Although these polypeptides are related structurally to one another (1, 2, 9, 21, 34), the highest degree of structural similarity exists between aFGF and bFGF (1, 21).

HBGF-1 binds to specific high-affinity cell surface receptors on a variety of cell types (17, 27, 29, 33). The interaction of HBGF-1 with its receptor has been reported to stimulate tyrosine kinase activities (8, 18). Several growth factor receptors have been shown to have intrinsic tyrosine kinase activity; these include the receptors for epidermal growth factor (EGF) (6, 7), platelet-derived growth factor (PDGF) (11, 14), insulin (22), insulinlike growth factor I (20), and colony-stimulating factor I (30). Several of these receptors have been shown to be the cellular homologs of viral oncogenes (19). These results indicate a role for tyrosine phosphorylation in the control of normal and malignant cell proliferation (19).

Clarification of the mechanism of HBGF-1-induced signal transduction via its receptor is essential to understanding the relationship of the HBGF-1 receptor to other growth factor receptors. Huang and Huang (18) reported that brain-derived growth factor stimulates the tyrosine phosphorylation of an M_r-135,000 protein in Swiss 3T3 membranes. In addition, Coughlin et al. (8) reported that stimulation of Swiss 3T3 cells with aFGF and bFGF results in the tyrosine phosphorylation of an M_r-90,000 protein. Here we report that HBGF-1 treatment of intact cells results in the sequential tyrosine phosphorylation of three polypeptides of M_r 150,000, 130,000, and 90,000 and that this tyrosine phosphorylation is time and temperature dependent. Furthermore, the M_r-150,000 and M_r-130,000 polypeptides contain both an HBGF-1 ligand-binding domain and phosphotyrosyl residues.

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MATERIALS AND METHODS

Materials. HBGF-1 was purified (3) and radioiodinated (15) as described previously. The specific activity of ^{125}I-HBGF-1 was 60 to 100 μCi/μg. Tissue culture media were purchased from GIBCO Laboratories (Grand Island, N.Y.). $^{32}P_i$ was from Amersham Corp. (Arlington Heights, Ill.), and ^{125}I-protein A was prepared essentially as described elsewhere (10). Protein A-Sepharose was purchased from Pharmacia, Inc. (Piscataway, N.J.). Phosphoamino acids and phenylmethylsulfonyl fluoride were from Sigma Chemical Co. (St. Louis, Mo.). The immobilized antiphosphotyrosine (anti-P-Tyr) monoclonal antibody 1G2 was purchased from

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Oncogene Science, Inc. Thin-layer cellulose plates were purchased from Analtech. High-molecular-weight protein electrophoresis standards were from Bio-Rad Laboratories (Richmond, Calif.). All other reagents were analytical grade.

Cell culture. The murine fibroblast cell line NIH 3T3 (clone 2.2) was a generous gift of C. Fryling (Rorer Biotechnology Inc.). Cells were maintained in Dulbecco modified Eagle medium (DME) supplemented with 10% calf serum (Hyclone). For experiments, cells were plated in either 100- or 35-mm-diameter dishes and grown to near confluence. The medium was removed and replaced with DME supplemented with 0.5% calf serum for 24 to 48 h. For some experiments, cells were exposed to various concentrations of sodium orthovanadate (Sigma) for 18 h before stimulation of the cells with HBGF-1. Cells were prepared for anti-P-Tyr Western blot (immunoblot) analysis by incubation with HBGF-1 for the times and temperatures specified in the figure legends. DNA synthesis by cells was measured by the incorporation of [^{3}H]thymidine (6.7 mCi/mmol; Dupont, NEN Research Products, Boston, Mass.) as described previously (3).

Preparation and characterization of anti-P-Tyr sera. Anti-P-Tyr antisera were prepared essentially as described by Ek and Heldin (12). Briefly, 2 mg of P-Tyr was coupled to 10 mg of bovine immunoglobulin by incubation for 24 h at 22°C with constant agitation in the presence of 20 mg of *N*-ethyl-*N*-(3-dimethylamino-propyl)carbodiimide in a final volume of 1 ml of phosphate-buffered saline (PBS). After coupling, the conjugate was dialyzed exhaustively at 22°C against PBS. Female New Zealand White rabbits were given 200 μg of the P-Tyr–immunoglobulin conjugate in 0.5 ml of PBS mixed with an equal volume of Freund complete adjuvant by multiple intradermal injections. The rabbits were boosted every 3 weeks with the same amount of antigen suspended in an equal volume of Freund incomplete adjuvant. Rabbits were bled after a total of three injections and tested for production of anti-P-Tyr antiserum.

To test the reactivity of immune anti-P-Tyr serum, the capacity of the serum to precipitate proteins known to contain phosphorylated tyrosine residues was evaluated. Specificity was evaluated further by determining whether different phosphoamino acids could inhibit this precipitation. A431 cells were grown to confluence in 100-mm-diameter dishes containing DME with 10% (vol/vol) fetal bovine serum. The cells were then permeabilized with digitonin, and cellular proteins were rapidly labeled with exogenously added [γ-^{32}P]ATP as described previously (16). Briefly, monolayers of A431 cells were starved overnight in DME containing 0.5% fetal bovine serum and then permeabilized and labeled simultaneously by incubation in 2 ml of permeabilization buffer (145 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 2 mM $MnCl_2$, 2 mM $ZnCl_2$, 20 μM sodium orthovanadate, 75 μg of digitonin per ml 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.4) containing 20 μCi of [γ-^{32}P]ATP (5,000 Ci/mmol; Amersham) for 5 to 10 min at 22°C. Incubation was carried out in the presence of 100 ng of EGF per ml. The reaction was terminated by aspirating the labeling solution and washing the preparation twice with permeabilization buffer without digitonin; labeled proteins were then extracted in 0.5 ml of HNTG-1 buffer (150 mM NaCl, 10% glycerol, 1.5 mM $MgCl_2$, 1.0 mM EGTA, 0.01 mg of aprotinin per ml, 1% Triton X-100, 20 mM HEPES, pH 7.4). Cell lysates were scraped from the dish and spun at 10,000 × *g* for 10 min to remove insoluble material. Samples were immunoprecipitated by addition of 10 μl of antiserum per 100 μl of labeled cell extract and incubated at 4°C for 2 h with constant agitation. For some experiments, antisera were added in the presence or absence of 1 mM P-Tyr, phosphoserine, phosphothreonine, or phenyl phosphate. Then 50 μl of a 50% suspension of protein A-Sepharose was added, and incubation was continued for 1 h.

Samples were centrifuged at 10,000 × *g* for 30 s, the supernatant was removed, and the protein A-Sepharose beads were washed three times in HNTG-1 and once in HNTG-2 (15 mM NaCl, 10% glycerol, 0.1% Triton X-100, 20 mM HEPES, pH 7.4). A 100-μl amount of sodium dodecyl sulfate (SDS) sample buffer was added, and samples were heated to 95°C for 4 min. Samples were cooled and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) on 7.5% polyacrylamide slab gels. The gels were stained, destained, dried, and subjected to autoradiography at −80°C with X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.) with Cronex Lightning-Plus intensifying screens (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.).

Rabbit antisera that were positive for reactivity to P-Tyr-containing proteins were affinity purified by phosphotyramine-Sepharose essentially as described elsewhere (28).

Immunoblot analysis of cells exposed to HBGF-1 by using anti-P-Tyr antibodies. NIH 3T3 cells were grown to near confluence in either 35- or 100-mm-diameter dishes as described above. Cells were exposed to HBGF-1 at the concentrations, times, and temperatures indicated. The cells were rinsed once in cold PBS containing 100 μM sodium orthovanadate. The cells were then lysed in the dish in a buffer containing 10 mM Tris, 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 30 mM sodium pyrophosphate, 100 μM sodium orthovanadate, 1.0% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride pH 7.4 (lysis buffer), immediately scraped from the plates with a rubber policeman, vortexed, and centrifuged at 10,000 × *g* for 10 min at 4°C. Total protein content of the lysate was determined by the BCA protein assay (Pierce Chemical Co., Rockford, Ill.).

Lysates (300 μg per lane) were mixed with an equal volume of SDS sample buffer, and proteins were separated by SDS-PAGE on 7.5% polyacrylamide slab gels. Alternatively, cell lysates were adsorbed to monoclonal anti-P-Tyr antibodies immobilized on a solid support, and P-Tyr-containing proteins were purified from the lysates as described below for in vivo labeling experiments. The P-Tyr-containing proteins eluted from the anti-P-Tyr matrix were then separated on 7.5% SDS-polyacrylamide gels. Proteins were transferred electrophoretically from the gels to nitrocellulose filters (0.45-μm pore size; Bio-Rad) in transfer buffer (25 mM Tris, 192 mM glycine, 0.02% SDS, 20% methanol) for 2 h at 4°C (32). Filters were then blocked by incubation in 50 mM Tris hydrochloride–150 mM NaCl, pH 7.4 (TBS), containing 5% bovine serum albumin (BSA) for 2 h at 45°C. Blots were washed once in TBS and then incubated for 3 h at 22°C with anti-P-Tyr antibody at a concentration of 20 μg/ml in TBS containing 5% BSA and 0.05% sodium azide (NaN_3). The anti-P-Tyr-containing solution was removed; blots were washed four times in TBS containing 0.05% Triton X-100 and then incubated with 5 × 10^5 cpm of ^{125}I-protein A per ml for 30 min at 22°C. Blots were washed four times in TBS containing 0.05% Triton X-100 and air dried. Binding of ^{125}I-protein A was visualized by autoradiography with Kodak XAR film and Cronex Lightning-Plus intensifying screens at −80°C for 12 to 36 h.

In vivo labeling and immunoprecipitation of P-Tyr-containing proteins. NIH 3T3 cells were grown to near confluence as described above and starved for 24 h in DME

containing 0.5% calf serum. Cells were washed once with DME without phosphate and containing 0.1% BSA. Cultures were incubated at 37°C in 3 ml of this medium per 100-mm-diameter plate with 1.0 mCi of $^{32}P_i$ per ml. The labeling time was 2.5 h, and HBGF-1 was added at the indicated concentrations for various times at the end of the labeling period. Labeling was terminated by washing the cells twice with PBS containing 100 μM sodium orthovanadate and then adding 0.5 ml of lysis buffer to each plate. Lysates were scraped quickly from the plates with a rubber policeman, vortexed intermittently for 20 min, and centrifuged at 10,000 × *g* for 10 min at 4°C. The supernatants were mixed with 20 μl of a 50% suspension of immobilized anti-P-Tyr monoclonal antibody for 1 h at 4°C with constant agitation. The beads were washed with 1.0 ml of lysis buffer four times, and P-Tyr-containing proteins were eluted with 10 mM phenyl phosphate–10 mM Tris hydrochloride (pH 7.4)–50 mM NaCl–0.1% Triton X-100, with 0.01% ovalbumin as the carrier protein. Eluted phosphoproteins were analyzed by SDS-PAGE on 7.5% polyacrylamide slab gels. The gels were prepared for autoradiography as described above.

Phosphoamino acid analysis of ^{32}P-labeled proteins. ^{32}P-labeled proteins were separated by electrophoresis as described above and visualized by autoradiography, and the bands of interest were cut out of the dried gel. The gel pieces were rehydrated in 0.5 ml of water for 10 min. Each gel slice was homogenized in 0.5 ml of gel band buffer (0.1% SDS, 50 mM ammonium bicarbonate, 1 mM EDTA, 10 μg of BSA per ml) (13), and 40 μl of β-mercaptoethanol was added. The mixture was boiled for 2 min and then incubated at 37°C for 18 h. The gel was pelleted at 10,000 × *g* for 5 min, and the supernatant was removed. The remaining gel was reextracted with 0.5 ml of gel band buffer for 2 h at 37°C. The eluate was dried in a Speed Vac concentrator, dissolved in water containing 20 μg of BSA per ml, and precipitated with 20% (vol/vol) trichloroacetic acid. The precipitates were pelleted at 10,000 × *g* for 5 min, washed once with 0.5 ml of ethanol, and air dried. Acid hydrolysis was performed for 1 h at 110°C in 6 N constantly boiling HCl (Pierce). Hydrolysates were dried under vacuum in a Speed Vac concentrator. The resulting residue was washed three times with 0.5 ml of water and finally suspended in 2.0 μl of pyridine-acetic acid-water (5:45:945, pH 3.5; TLE buffer) containing 2 mg each of phosphoserine, phosphothreonine, and P-Tyr per ml as standards. Samples were spotted onto thin-layer cellulose plates (Analtech) and separated in a single dimension by electrophoresis in TLE buffer, pH 3.5, at 4°C. Plates were air dried and then sprayed with 3% (wt/vol) ninhydrin in butanol (Pierce) to visualize phosphoamino acid standards. Plates were then subjected to autoradiography with Kodak X-Omat AR film with Cronex Lightning-Plus intensifying screens at −80°C for 5 to 7 days to visualize radiolabeled phosphoamino acids.

Cross-linking of ^{125}I-HBGF-1 to NIH 3T3 cells and immunoprecipitation with anti-P-Tyr antibodies. NIH 3T3 cells in 100-mm-diameter dishes were incubated for 20 min at 22°C with 4 ml of binding buffer containing 10 ng of ^{125}I-HBGF-1 per ml in the presence or absence of 1 μg of unlabeled HBGF-1 per ml. Cells were transferred to 37°C for 5 min and then rapidly washed in warm (37°C) PBS and disuccinimidyl suberate added to a final concentration of 0.3 mM. Dishes were incubated for an additional 15 min at 37°C and then rapidly washed; cell lysates were prepared for immunoprecipitation with anti-P-Tyr as described above. Samples were either prepared directly for electrophoresis or immunoprecipitated with immobilized monoclonal anti-P-Tyr antibody. Before the addition of immobilized anti-P-Tyr, soluble cell lysates either received control diluent or were made 0.5 mM with P-Tyr, phosphoserine, or phosphothreonine. After incubation with the anti-P-Tyr beads for 1 h with constant agitation, the beads were washed as described above, and bound proteins were eluted with anti-P-Tyr elution buffer. Eluted proteins were then subjected to SDS-PAGE on 7.5% polyacrylamide slab gels and prepared for autoradiography as described above.

RESULTS

Stimulation by HBGF-1 of tyrosine phosphorylation in intact NIH 3T3 cells. Anti-P-Tyr antibodies have been shown to be sensitive reagents for identification of proteins phosphorylated on tyrosine residues (19). Several growth factor receptors (PDGF, EGF, insulin, insulinlike growth factor I, and colony-stimulating factor I) have been shown to possess intrinsic protein tyrosine kinase activity that is thought to play a role in ligand-induced signal transduction (19). P-Tyr-specific antisera were used to detect HBGF-1-induced tyrosine phosphorylation of proteins in extracts of NIH 3T3 (clone 2.2) cells. Equal numbers of serum-starved NIH 3T3 cells were stimulated with various concentrations of HBGF-1; then cell lysates were prepared and subjected to electrophoresis and immunoblotting with affinity-purified anti-P-Tyr antibodies. HBGF-1 induced the specific tyrosine phosphorylation of polypeptides of M_r 150,000 and 90,000 (Fig. 1A). In addition, there was an increase in tyrosine phosphorylation of proteins of M_r 130,000, 78,000, and 60,000 that were constitutively phosphorylated in unstimulated cells. HBGF-1 stimulation of tyrosine phosphorylation was concentration dependent, with as little as 0.2 ng of HBGF-1 per ml stimulating phosphorylation of the M_r-150,000, M_r-130,000, and M_r-90,000 polypeptides (Fig. 1A). This concentration was consistent with the amount of HBGF-1 required for stimulation of DNA synthesis in these same cells as measured by [^3H]thymidine incorporation (Fig. 1B).

As an alternative method for assaying HBGF-1-stimulated tyrosine phosphorylation in vivo, equal numbers of NIH 3T3 cells were incubated for 2.5 h with $^{32}P_i$. Cells were then extracted with Triton X-100-containing buffer, and the ^{32}P-labeled proteins were adsorbed onto a monoclonal anti-P-Tyr matrix. The bound proteins were eluted with 10 mM phenyl phosphate and separated by SDS-PAGE. ^{32}P-labeled proteins of M_r 150,000 and 90,000 were immunoprecipitated specifically from extracts of labeled cells stimulated with 10 ng of HBGF-1 per ml (Fig. 2A, lane 2) but not from extracts of unstimulated cells (Fig. 2A, lane 1). In addition, ^{32}P-labeled bands of M_r 130,000 and 78,000 that were present in the unstimulated control were increased in intensity in the eluates from the immunoprecipitates of HBGF-1-stimulated cells.

The ^{32}P-labeled M_r-150,000 and M_r-90,000 bands were excised from the stained and dried gel and subjected to acid hydrolysis, and the radioactive phosphoamino acid content was determined as described in Materials and Methods. The M_r-150,000 protein was phosphorylated on both tyrosine and serine residues but not on threonine residues (Fig. 2B, lane 1). Phosphoamino acid analysis of the M_r-90,000 protein showed that ^{32}P was incorporated into tyrosine in addition to serine and threonine. The M_r-130,000 protein was also phosphorylated on tyrosine residues, as determined by phosphoamino acid analysis (data not shown). These data confirmed the results obtained by immunoblotting experiments;

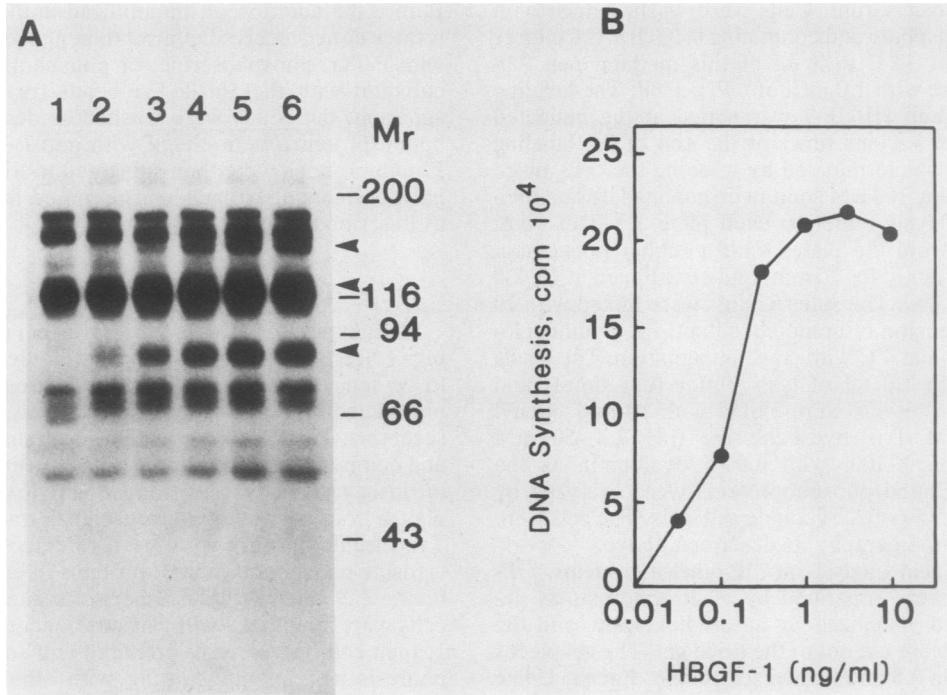

FIG. 1. Stimulation of tyrosine phosphorylation in intact NIH 3T3 cells by HBGF-1. (A) NIH 3T3 cells were incubated at 37°C for 10 min in the presence of diluent (lane 1) or HBGF-1 at a concentration of 0.1 (lane 2), 0.2 (lane 3), 0.5 (lane 4), 1 (lane 5), or 10 (lane 6) ng/ml and then processed for immunoblotting as described in the text. Migration of the molecular weight standards is indicated on the right. Arrowheads indicate positions of the M_r-150,000, M_r-130,000, and M_r-90,000 polypeptides that were phosphorylated on tyrosine in response of HBGF-1. (B) NIH 3T3 (clone 2.2) cells were grown to near confluence and then starved as described in Materials and Methods. Cells received various concentrations of HBGF-1 after 24 h, were incubated for an additional 18 h, and were then pulsed with 0.5 μCi of [^{3}H]thymidine per ml for 4 h. The cells were then harvested, and incorporation of radioactivity was determined as described elsewhere (3).

i.e., the M_r-150,000, M_r-130,000, and M_r-90,000 proteins were phosphorylated on tyrosine residues, and the polyclonal antibodies used in the immunoblotting experiments recognized the same set of P-Tyr-containing proteins as did the anti-P-Tyr monoclonal antibody.

Kinetic analysis of HBGF-1-induced tyrosine phosphorylation. To examine the kinetics of HBGF-1-induced tyrosine phosphorylation, equal numbers of NIH 3T3 cells were stimulated with 10 ng of HBGF-1 per ml for various lengths of time after labeling for 2.5 h with $^{32}P_i$. Tyrosine phosphorylation of the M_r-150,000 protein occurred within 30 s of HBGF-1 addition and became maximal at 15 to 30 min after HBGF-1 addition (Fig. 3). In contrast, tyrosine phosphorylation of the M_r-90,000 protein was not detectable until 3 min after HBGF-1 addition, and it also became maximal after 15 to 30 min (Fig. 3). In addition, the increased tyrosine phosphorylation of the M_r-130,000 protein observed in immunoblotting experiments occurred within 30 s of HBGF-1 addition and peaked after 15 to 30 min. Small increases in phosphorylation of polypeptides of M_r >200,000, 78,000, and 40,000 were also observed (Fig. 3).

Effects of temperature on HBGF-1 induced tyrosine phosphorylation. To examine the effects of temperature on HBGF-1-induced tyrosine phosphorylation, NIH 3T3 cells were incubated at 37°C for 10 min or at 4°C for various lengths of time in the presence or absence of 10 ng of HBGF-1 per ml. Cell lysates were prepared and adsorbed onto an anti-P-Tyr matrix, and the eluates were processed as described for immunoblotting with anti-P-Tyr antibodies. The M_r-150,000 and M_r-90,000 polypeptides were phosphorylated on tyrosine at 37°C in the presence (Fig. 4, lane 8) but not the absence (lane 7) of HBGF-1. When NIH 3T3 cells were exposed to HBGF-1 at 4°C for 10 (lane 2) and 45 (lane 4) min, no phosphorylation of the M_r-150,000 or M_r-90,000 proteins on tyrosine residues was detected. Incubations with HBGF-1 at 4°C for up to 4 h did not result in detectable tyrosine phosphorylation of the M_r-150,000 and M_r-90,000 polypeptides (data not shown). To rule out the possibility that exposure to cold had an irreversible effect on the HBGF-1-induced tyrosine kinase activity, cells were exposed to HBGF-1 at 4°C for 45 min and then placed at 37°C for 10 min. These cells were still able to phosphorylate the M_r-150,000 and M_r-90,000 polypeptides in response to HBGF-1 stimulation when placed at 37°C (Fig. 4, lane 6). The M_r-130,000 polypeptide that was constitutively phosphorylated in untreated cells was also sensitive to the effects of temperature. Its level of phosphorylation increased when cells exposed to HBGF-1 were shifted from 4 to 37°C (Fig. 4, lane 6) but did not increase in unstimulated cells.

Effects of sodium orthovanadate on HBGF-1-induced tyrosine phosphorylation. Sodium orthovanadate has been shown to be a potent inhibitor of P-Tyr phosphatases (16). To determine the effect of sodium orthovanadate on HBGF-1-induced tyrosine phosphorylation, NIH 3T3 cells were incubated with various concentrations of sodium orthovanadate for 18 h. The cells were then exposed to 10 ng of HBGF-1 per ml at 37°C for 10 min. With concentrations as low as 10 μM sodium orthovanadate, HBGF-1-induced tyrosine phosphorylation of the M_r-150,000, M_r-130,000, and M_r-90,000 proteins was greater than in untreated controls

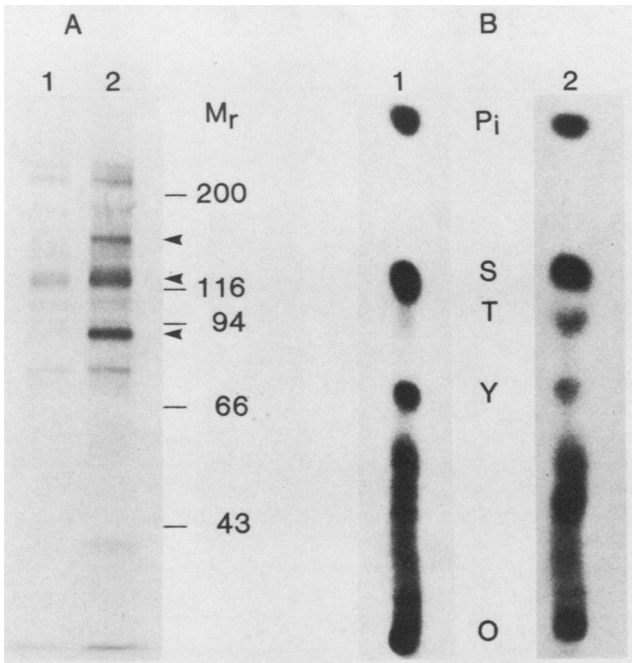

FIG. 2. HBGF-1 stimulation of tyrosine phosphorylation in NIH 3T3 cells labeled in vivo with $^{32}P_i$ (A) NIH 3T3 cells were metabolically labeled with $^{32}P_i$ for 2.5 h at 37°C and then exposed to diluent (lane 1) or 20 ng of HBGF-1 per ml (lane 2) for 10 min at 37°C. Cell lysates were prepared and immunoprecipitated with immobilized monoclonal anti-P-Tyr antibodies as described in Materials and Methods. Bound proteins were eluted with 10 mM phenyl phosphate and subjected to SDS-PAGE and autoradiography. Shown is an autoradiogram of the stained, dried gel. Arrowheads indicate positions of the M_r-150,000, M_r-130,000, and M_r-90,000 polypeptides. Shown on the right is migration of the molecular weight standards. (B) ^{32}P-labeled M_r-150,000 (lane 1) and M_r-90,000 (lane 2) protein bands were excised from the gel and processed for phosphoamino acid analysis as described in Materials and Methods. The ^{32}P-labeled phosphoamino acids were visualized by autoradiography, and migration was compared with that of phosphoamino acid standards (P-Tyr, phosphoserine, and phosphothreonine), visualized by ninhydrin as indicated in the center margin.

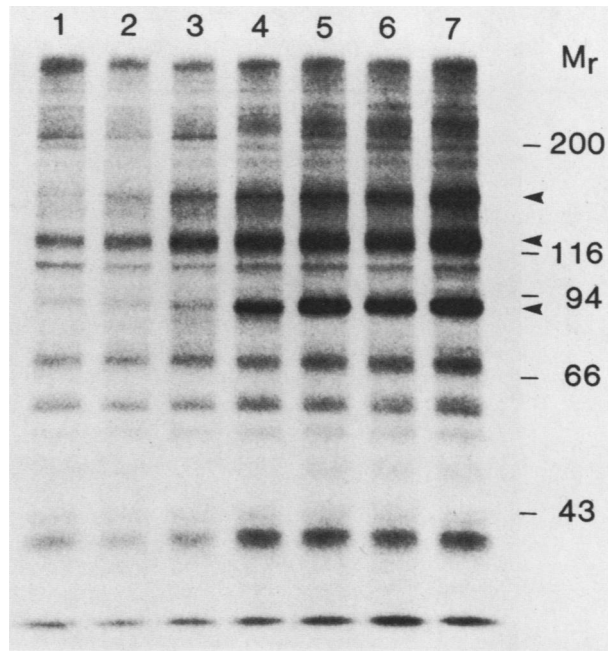

FIG. 3. Time course of HBGF-1-induced tyrosine phosphorylation. NIH 3T3 cells were metabolically labeled for 2.5 h at 37°C and then exposed to 10 ng of HBGF-1 per ml for 0 (lane 1) 0.5 (lane 2), 1 (lane 3), 3 (lane 4), 5 (lane 5), 15 (lane 6), or 30 (lane 7) min. Cell lysates were prepared and immunoprecipitated with anti-P-Tyr as described in Materials and Methods. Bound proteins were eluted with 10 mM phenyl phosphate and subjected to SDS-PAGE and autoradiography. Arrowheads indicate positions of the M_r-150,000, M_r-130,000, M_r-90,000 polypeptides phosphorylated on tyrosine in response to HBGF-1.

(Fig. 5). Sodium orthovanadate concentrations of 50 μM or more resulted in enhanced tyrosine phosphorylation of many other proteins even without HBGF-1 stimulation (Fig. 5, lanes 5 and 6).

Recognition of cross-linked ^{125}I-HBGF-1–receptor complexes by anti-P-Tyr antibodies. To determine the relationship between the M_r-150,000 and M_r-130,000 P-Tyr-containing proteins and the HBGF-1 receptor, we examined the ability of anti-P-Tyr antibodies to recognize specifically the HBGF-1 receptor covalently cross-linked to ^{125}I-HBGF-1. The HBGF-1 receptor-ligand complex was adsorbed specifically to immobilized anti-P-Tyr and eluted with phenyl phosphate as cross-linked receptor-ligand complexes of M_r 170,000 and 150,000 (Fig. 6, lane 3). P-Tyr (lane 4) but not phosphoserine (lane 5) or phosphothreonine (lane 6) prevented the adsorption of the cross-linked ^{125}I-HBGF-1–receptor complexes to the anti-P-Tyr affinity matrix. These results demonstrated that the cross-linked ^{125}I-HBGF-1–receptor complexes contained at least one P-Tyr residue.

Since pretreatment of NIH 3T3 cells with sodium orthovanadate resulted in enhanced detection of HBGF-1-induced tyrosine phosphorylation by immunoblotting, we examined the effect of sodium orthovanadate pretreatment on recognition of the cross-linked ^{125}I-HBGF-1–receptor complexes by anti-P-Tyr antibodies. NIH 3T3 cells were incubated overnight in the presence or absence of 25 μM sodium orthovanadate. Cells were then incubated at 22°C in the presence of 10 ng of ^{125}I-HBGF-1 per ml with or without 1 μg of unlabeled HBGF-1 per ml and then cross-linked with disuccinimidyl suberate at 37°C for 15 min. The demonstration that anti-P-Tyr antibodies recognized the ^{125}I-HBGF-1–receptor complex (Fig. 7, lane 1) and that this recognition was enhanced by sodium orthovanadate treatment (lane 3) strongly suggested that the ^{125}I-HBGF-1–receptor complex was phosphorylated on tyrosine residues. The increase in cross-linked ^{125}I-HBGF-1–receptor complexes immunoprecipitated from cells pretreated with sodium orthovanadate was not due to increased binding of the labeled ligand to cells, since the same amount of ^{125}I-HBGF-1 bound to both treated and untreated cells (data not shown).

DISCUSSION

We report here that HBGF-1 specifically induces rapid tyrosine phosphorylation of proteins of M_r 150,000, 130,000, and 90,000 in intact NIH 3T3 (clone 2.2) cells, as detected by polyclonal and monoclonal anti-P-Tyr antibodies. Consequently, the HBGF-1 receptor may be similar to other growth factor receptors, since HBGF-1 mediates the tyrosine phosphorylation of cellular proteins in a manner similar to that of EGF and PDGF. Neither EGF nor PDGF was able to induce tyrosine phosphorylation of the M_r-150,000, M_r-130,000, or M_r-90,000 protein (data not shown).

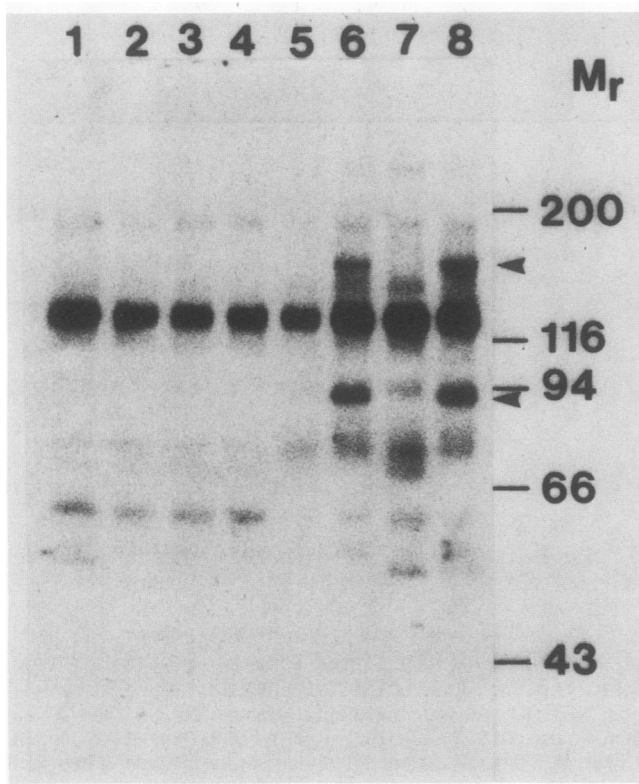

FIG. 4. Effect of temperature on HBGF-1-induced tyrosine phosphorylation. NIH 3T3 cells were incubated in either the presence (lanes 2, 4, 6, and 8) or the absence (lanes 1, 3, 5, and 7) of 10 ng of HBGF-1 per ml at 4°C for 10 (lanes 1 and 2) or 45 (lanes 3 and 4) min, at 4°C for 45 min and then at 37°C for 10 min (lanes 5 and 6), or at 37°C for 10 min (lanes 7 and 8). Cell lysates were prepared and tyrosine-phosphorylated proteins adsorbed onto an anti-P-Tyr matrix. The phenyl phosphate-eluted proteins were subjected to SDS-PAGE and immunoblotted with anti-P-Tyr as described in Materials and Methods. Arrowheads indicate positions of the M_r-150,000 and M_r-90,000 P-Tyr-containing proteins. Migration of SDS-PAGE molecular weight standards is indicated on the right.

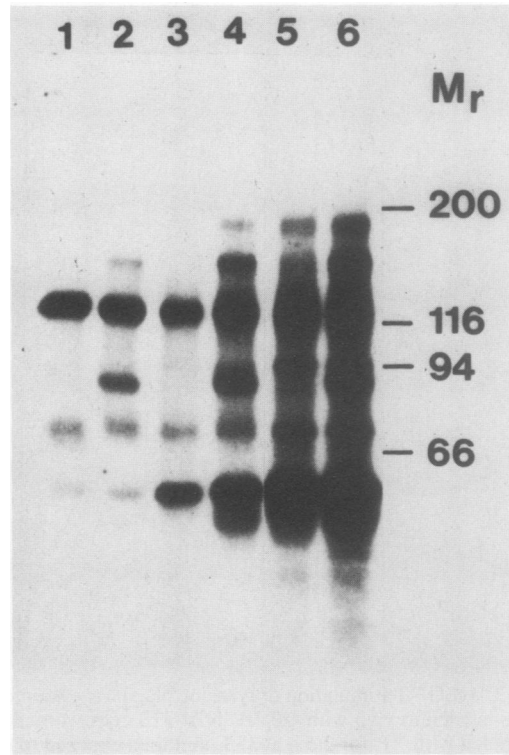

FIG. 5. Effect of sodium orthovanadate on HBGF-1-induced tyrosine phosphorylation. Equal numbers of NIH 3T3 cells were incubated for 18 h in the presence of diluent (lanes 1 and 2) or of 10 (lanes 3 and 4) or 50 (lanes 5 and 6) μM sodium orthovanadate. After 18 h, cells were incubated in the presence (lanes 2, 4, and 6) or absence (lanes 1, 3, and 5) of 10 ng of HBGF-1 per ml for 10 min at 37°C. Cell lysates were prepared and adsorbed to an anti-P-Tyr matrix. Phenyl phosphate-eluted P-Tyr-containing proteins were subjected to SDS-PAGE and anti-P-Tyr immunoblotting. Migration of the SDS-PAGE molecular weight standards is shown on the right.

The recent report that the HBGF-1 signal transduction mechanism seems not to involve inositol lipid breakdown or protein kinase C activation (25) supports the notion that protein tyrosine phosphorylation may be a major element of the HBGF-1-induced signal transduction pathway.

In vivo labeling of NIH 3T3 cells with $^{32}P_i$, immunoprecipitation of the ^{32}P-labeled proteins with monoclonal anti-P-Tyr antibodies, and phosphoamino acid analysis of the M_r-150,000, M_r-130,000, and M_r-90,000 proteins confirmed that these proteins are phosphorylated on tyrosine residues in addition to serine and threonine residues. The receptors for EGF (16) and PDGF (12, 14), which have been shown to autophosphorylate on tyrosine residues, have also been shown to contain phosphoserine and phosphothreonine residues after stimulation with ligand in intact cells. Therefore, phosphorylation of the M_r-150,000 and M_r-130,000 proteins on tyrosine and serine residues and of the M_r-90,000 protein on tyrosine, serine, and threonine residues in response to HBGF-1 stimulation of intact cells is similar to the responses of other receptor tyrosine kinases when analyzed in vivo.

The kinetics of tyrosine phosphorylation of the M_r-150,000, M_r-130,000, and M_r-90,000 proteins were found to be different. Phosphorylation of the M_r-150,000 and M_r-130,000 proteins occurred within 30 s of exposure to HBGF-1 and reached a maximum after 15 to 30 min of stimulation. In contrast, tyrosine phosphorylation of the M_r-90,000 protein was not detectable until 3 min after exposure to HBGF-1 but also became maximal after 15 to 30 min of stimulation. Therefore, we conclude that tyrosine phosphorylation of the M_r-150,000 and M_r-130,000 proteins is one of the earliest events associated with HBGF-1 stimulation and that the M_r-90,000 polypeptide may be a substrate for an HBGF-1-induced tyrosine kinase.

Tyrosine phosphorylation of the EGF (16) and PDGF (12, 14) receptors has been shown to occur in vivo at both 37 and 4°C in response to ligand stimulation. In contrast, the HBGF-1-induced tyrosine phosphorylation of the M_r-150,000, M_r-130,000, and M_r-90,000 proteins was not detectable at 4°C but only at 37°C. In this regard, Coughlin et al. (8) observed HBGF-induced tyrosine phosphorylation of a 90-kilodalton polypeptide in Swiss 3T3 cells at 37°C but not at 4°C. It is possible that HBGF-1-induced tyrosine phosphorylation requires noncovalent association between membrane components for tyrosine kinase activity, as has been demonstrated recently for the bombesin receptor complex (5). Alternatively, tyrosine phosphorylation may occur in HBGF-1-stimulated cells at 4°C but at levels below the detection limit of our system.

Sodium orthovanadate has been shown to be a potent inhibitor of P-Tyr phosphatases (16). Treatment of NIH 3T3

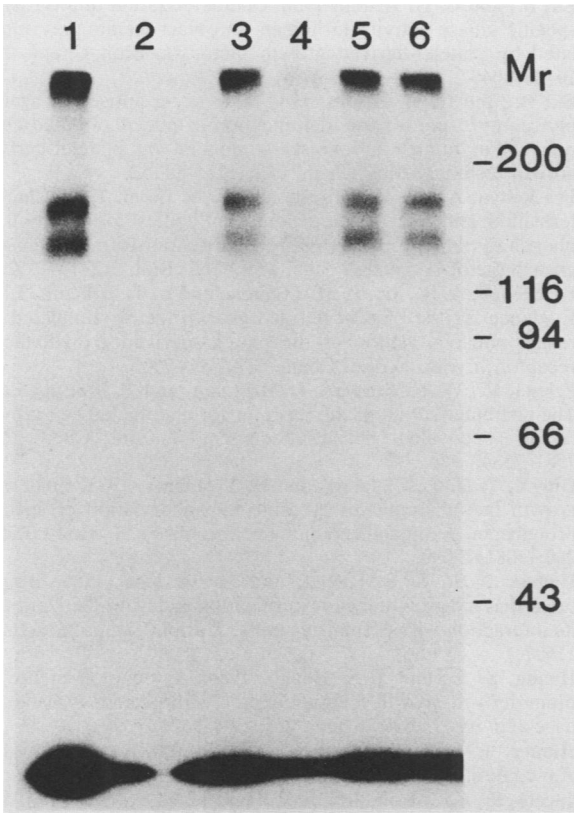

FIG. 6. Binding of cross-linked ^{125}I-HBGF-1–receptor complexes to anti-P-Tyr antibodies. NIH 3T3 cell lysates were prepared for immunoprecipitation as described in Materials and Methods. Solubilized cellular extracts representing approximately 10% of cell-bound counts in either the absence (lane 1) or the presence (lane 2) of 1 μg of unlabeled HBGF-1 per ml were prepared directly for electrophoresis. Before the addition of immobilized anti-P-Tyr, soluble cell lysates were treated with control diluent (lane 3) or made 0.5 mM with P-Tyr (lane 4), phosphoserine (lane 5), and phosphothreonine (lane 6). The lysates were then immunoprecipitated with monoclonal anti-P-Tyr and washed extensively. The bound proteins were eluted with 10 mM phenyl phosphate, separated by electrophoresis on a 7.5% SDS-polyacrylamide gel, and visualized by autoradiography. Migration of the molecular weight standards is shown on the right.

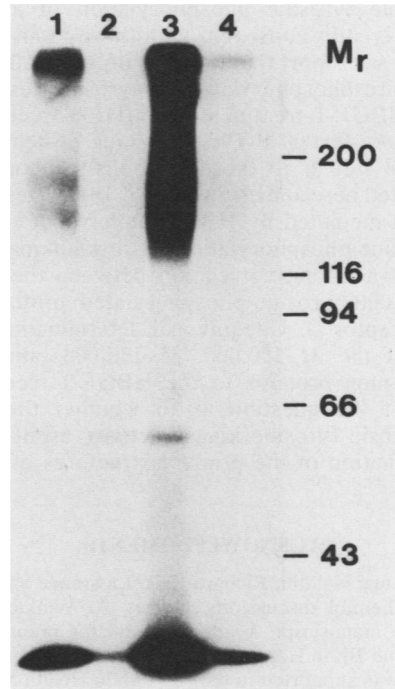

FIG. 7. Effect of sodium orthovanadate on the recognition of cross-linked ^{125}I-HBGF-1–receptor complexes by anti-P-Tyr. NIH 3T3 cells were incubated for 18 h in the absence (lanes 1 and 2) or presence of 25 μM sodium orthovanadate and incubated for 20 min at 22°C with 10 ng of ^{125}I-HBGF-1 per ml in 4 ml of binding buffer in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of 1 μg of unlabeled HBGF-1 per ml. Cells were then transferred to 37°C for 5 min, cross-linked, and prepared for immunoprecipitation with anti-P-Tyr antibodies as described in Materials and Methods. The immunoprecipitates were washed, and bound P-Tyr-containing proteins were eluted with 10 mM phenyl phosphate, separated by SDS-PAGE, and visualized by autoradiography.

cells with sodium orthovanadate before stimulation with HBGF-1 results in enhanced detection of proteins phosphorylated on tyrosine residues in response to HBGF-1 treatment. This increase in sensitivity is presumably due to the inhibition of phosphatases before stimulation of HBGF-1-induced tyrosine phosphorylation. Several other P-Tyr-containing proteins can be detected in orthovanadate-treated cells in the presence or absence of HBGF-1 stimulation. Sodium orthovanadate at concentrations of between 1 and 50 μM potentiates the mitogenic response of NIH 3T3 cells to HBGF-1 while inducing only a weak increase in [^{3}H]thymidine incorporation in the absence of HBGF-1 (data not shown). These data serve to emphasize the potential for tyrosine phosphorylation as part of HBGF-1-induced signal transduction.

To determine the relationship between the M_r-150,000 and M_r-130,000 polypeptides and the HBGF-1 receptor, ^{125}I-HBGF-1 was cross-linked to NIH 3T3 cells under conditions that were optimal for the induction of tyrosine phosphorylation in these cells. Two bands, of M_r 170,000 and 150,000, were specifically immunoprecipitated with anti-P-Tyr antibodies from the ^{125}I-HBGF-1 affinity-labeled cell extracts. These M_r-170,000 and M_r-150,000 bands are consistent with the cross-linked HBGF-1–receptor complexes previously reported (8, 15, 18, 26, 27, 33). The apparent molecular weights of the two ^{125}I-HBGF-1–receptor complexes are in good agreement with the apparent molecular weights of the M_r-150,000 and M_r-130,000 HBGF-1-induced P-Tyr-containing proteins when the corresponding shift in molecular weight due to the covalent attachment of the ligand is taken into consideration. The enhanced recognition of the ^{125}I-HBGF-1–receptor complexes by pretreatment of cells with sodium orthovanadate is also consistent with the enhanced recognition of tyrosine phosphorylation of the M_r-150,000 and M_r-130,000 polypeptides by anti-P-Tyr antibodies in response to HBGF-1 stimulation in vanadate-treated cells.

It was recently reported that HBGF-1 induces tyrosine kinase activity in Swiss 3T3 cells (8, 18). Huang and Huang (18) reported that brain-derived growth factor, an HBGF-1-like polypeptide, stimulates tyrosine phosphorylation of an M_r-135,000 protein in plasma membranes isolated from Swiss 3T3 cells. Although tyrosine phosphorylation of this protein was clearly demonstrated, no additional tyrosine-phosphorylated substrates were observed (18). In addition, Coughlin et al. (8) reported that aFGF and bFGF stimulate the tyrosine phosphorylation of an M_r-90,000 protein in intact Swiss 3T3 cells. However, they did not observe

FGF-inducible tyrosine phosphorylation of any proteins with characteristics consistent with those of an FGF receptor (8). Here we report that proteins of M_r 150,000, 130,000, and 90,000 are phosphorylated on tyrosine residues in response to HBGF-1 treatment of NIH 3T3 cells. The M_r-130,000 and M_r-90,000 P-Tyr-containing proteins described here may be similar to those previously reported (8, 18). Data presented here and elsewhere (8, 18) suggest that signal transduction mediated by HBGFs involves a complex pattern of tyrosine phosphorylations. Cross-linking and kinetic data suggest a strong correlation between the M_r-150,000 and M_r-130,000 tyrosine-phosphorylated proteins and the HBGF-1 receptor(s). Unequivocal determination of the relationship of the M_r-150,000, M_r-130,000, and M_r-90,000 P-Tyr-containing proteins to the HBGF-1 receptor(s) and resolution of the question as to whether these proteins contain intrinsic tyrosine kinase activity await purification and determination of the primary structures of these polypeptides.

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