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Characterization and cDNA Cloning of Phospholipase C-γ, a Major Substrate for Heparin-Binding Growth Factor 1 (Acidic Fibroblast Growth Factor)-Activated Tyrosine Kinase

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Heparin-binding growth factors (HBGFs) bind to high-affinity cell surface receptors which possess intrinsic tyrosine kinase activity. A M_r 150,000 protein phosphorylated on tyrosine in response to class 1 HBGF (HBGF-1) was purified and partially sequenced. On the basis of this sequence, cDNA clones were isolated from a human endothelial cell library and identified as encoding phospholipase C-γ. Phosphorylation of phospholipase C-γ in intact cells treated with HBGF-1 was directly demonstrated by using antiphospholipase C-γ antibodies. Thus, HBGF-1 joins epidermal growth factor and platelet-derived growth factor, whose receptor activation leads to tyrosine phosphorylation and probable activation of phospholipase C-γ.

The heparin-binding growth factors (HBGFs) are a group of structurally related polypeptides that are mitogenic for a variety of cell types in vitro and in vivo (reviewed in reference 2). Class 1 HBGF (HBGF-1) is an acidic polypeptide mitogen for endothelial, fibroblast, smooth muscle, and other cell types, and is known commonly as acidic fibroblast growth factor (aFGF), a truncated form of the polypeptide originally cloned (11) and sequenced (3) as β-endothelial cell growth factor.

HBGF-1 binds to specific high-affinity cell surface receptors and is reported to stimulate tyrosine kinase activities (4, 8). Friesel et al. (7) reported that HBGF-1 induces tyrosine phosphorylation of three distinct polypeptides of M_r 150,000, 130,000, and 90,000 in the murine fibroblast cell line NIH 3T3 (clone 2.2). It is presently unknown whether any of these polypeptides correspond to the HBGF-1 receptor, although indirect evidence indicates that the M_r 150,000 and 130,000 polypeptides may contain HBGF-1-binding activity. Recently, we showed that antibodies directed against the human flg protein immunoprecipitate HBGF-1-activated protein tyrosine kinases of approximate M_r 120,000 and 135,000 (30). The flg protein is homologous to the cloned chicken class 2 HBGF (HBGF-2) receptor (17) and is related to but distinct from a second protein tyrosine kinase HBGF receptor, termed bek (15).

Relatively little is known about the mechanism involved in HBGF-1-induced signal transduction. The results of several groups are consistent with the hypothesis that activation of tyrosine kinase activity is an early event in cellular mitogenic response to HBGF-1 (4, 7, 8). In contrast, there is not a consensus regarding a role for phosphoinositide hydrolysis, protein kinase-C activation, or calcium mobilization in the mechanisms of action of HBGF-1. For example, it was reported that neither the aFGF nor the basic FGF (i.e., HBGF-1 or HBGF-2) receptor signalling pathway was coupled to the activation of phospholipase C (PLC) in hamster fibroblasts and that the early mitogenic events induced by HBGFs could be initiated independently of inositol lipid hydrolysis and protein kinase C activation (18). In contrast, other investigators (12, 37) have presented evidence that the addition of basic FGF (HBGF-2) to quiescent cultures of Swiss 3T3 cells rapidly induces diacylglycerol formation, protein kinase C activation, and Ca^{2+} mobilization. In addition, the protein kinase C-activating phorbol ester, 12-O-tetradecanoyl-phorbol-13-acetate inhibits induction of DNA synthesis by basic FGF only in certain lines of NIH 3T3 cells (40).

These studies were undertaken to determine the identities of the proteins phosphorylated on tyrosine residues in response to HBGF-1. The M_r 150,000 protein (7) was chosen initially because it was a likely candidate for the HBGF-1 receptor. We report here the cloning of the cDNA for human PLC-using synthetic oligonucleotides based on the amino acid sequences of trypsin-derived fragments obtained from the M_r 150,000 protein that is phosphorylated on tyrosine in response to HBGF-1. Furthermore, using phosphotyrosine and PLC-γ-specific antibodies, we demonstrate the HBGF-1 stimulates phosphorylation of PLC-γ on tyrosine residues in intact cells.

MATERIALS AND METHODS

Preparation and microsequence analysis of phosphotyrosine-containing proteins. NIH 3T3 (strain 2.2) cells were maintained in roller bottles (850 cm^2, Corning Glass Works) in Dulbecco modified Eagle medium containing 10% calf serum, glutamine, and antibiotics. Subconfluent cultures were made quiescent by incubating them for 16 h in complete medium containing 1% calf serum. Cells were incubated for 3 h in 20 μM Na_3VO_4 and then stimulated at 37°C for 10 min by addition of 10 ng of recombinant human HBGF-1 per ml (10). The cells were washed once with phosphate-buffered saline and then incubated for 5 min with 10 ml of lysis buffer (10 mM Tris, 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 30 mM sodium pyrophosphate, 100 μM sodium orthovanadate, 1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride [pH 7.4]) per roller bottle. The lysates were scraped from the roller bottles, pooled, and clarified by centrifugation for 40 min at 150,000 × *g*. The clarified lysate (1,000 ml) was applied to an 8-ml column of antiphosphotyrosine monoclo-

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nal antibody 1G2 coupled to CNBr-activated Sepharose 4B (Oncogene Sciences, Inc.). The column was washed with 10 volumes of lysis buffer, and phosphotyrosine-containing proteins were eluted with 50 ml of a solution containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 0.1% Triton X-100, 40 mM phenylphosphate, and 0.1 mM phenylmethylsulfonyl fluoride (pH 7.2). The eluate was clarified by centrifugation. The eluate was concentrated by acetone precipitation (acetone-lysate, 4:1 [vol/vol]) at −20°C for 16 h. The precipitate was suspended in Laemmli sample buffer (16) and subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) for Western blot (immunoblot) analysis or electroelution. The preparative gels were stained with Coomassie blue (Bio-Rad Laboratories) for 5 min and destained until the protein bands were easily visualized. The M_r 150,000 band was excised from the gel, and the protein was electroeluted by the procedure of Hunkapiller et al. (9). SDS and Coomassie blue were removed from the protein sample by ion-pair extraction (14). The relative mobility of the eluted protein was identical to that of an apparent M_r 150,000 protein identified by Western blot (immunoblot) analysis of the 1G2 eluate by using antiphosphotyrosine antibodies as described previously (7). Following ion-pair extraction, the protein sample (20 pmol) was suspended in 100 mM ammonium bicarbonate and incubated with trypsin (Worthington Diagnostics) at an enzyme-to-substrate ratio of 1:25 for 18 h at 37°C. The trypsin digest was fractionated by using an RP-300 cartridge (2.1 by 30 mm; Applied Biosystems, Inc.) and a model 130 microbore high-pressure liquid chromatograph (Applied Biosystems, Inc.). Selected fractions were subjected to automated Edman degradations by using an Applied Biosystems model 477A protein sequencer with an on-line model 120 phenylthiohydantoin analyzer.

Isolation of human phospholipase C-γ cDNA clones. (i) Isolation of mouse genomic clones. A 32-base-pair (bp) antisense oligonucleotide, 5' AGCTGGGCAAACTGGCCATA GGTGATGTCCCC 3', was generated from the amino acid sequence of a 12-residue tryptic peptide and end labeled with [γ-^{32}P]ATP with T4 polynucleotide kinase. One million bacteriophage of a mouse genomic library (Clontech no. ML 1009) were plated on a lawn of *Escherichia coli* NM538 (13) and transferred to nitrocellulose as described previously (11). Filters were hybridized overnight with approximately 10^6 cpm of ^{32}P-32-mer per ml at 50°C in 5× SSPE (1× SSPE is 0.18 M NaCl, 0.01 M $NaPO_4$ [pH 7.2], 0.01 M EDTA)–0.25% nonfat dry milk. Filters were washed at 55°C for 20 min, once with 2× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate [pH 7.4])– 0.1% SDS, and then twice with 0.5× SSC–0.1% SDS. After overnight autoradiography, eight clones were identified and purified to homogeneity by additional rounds of screening of the original hybridizing plaques at higher dilution. One clone, G23-5, was grown on a lawn of *E. coli* NM538 and purified from the lysate with LambdaSorb (Promega Biotec). DNA was obtained by phenol extraction, digested with *Hae*III, and ligated into *Sma*I-digested pGEM2. Following transformation of *E. coli* TG1, ampicillin-resistant colonies were transferred to nitrocellulose, hybridized overnight with the ^{32}P-32-mer, and identified by autoradiography as described above. Nucleotide sequence analysis (33) of plasmid DNA from positive colonies containing a 179-bp *Hae*III fragment confirmed its identity to the original mouse tryptic peptide sequence.

(ii) Isolation of human genomic clones. The nucleotide sequence of the 179-bp mouse genomic *Hae*III fragment was used to generate two 30-mer oligonucleotides which were then used to prime polymerase chain reactions (PCR) in human genomic DNA (32). The PCR product was cloned into *Sma*I-digested pGEM2 and sequenced. The nucleotide sequence of the human genomic PCR product was identical to the corresponding mouse sequence.

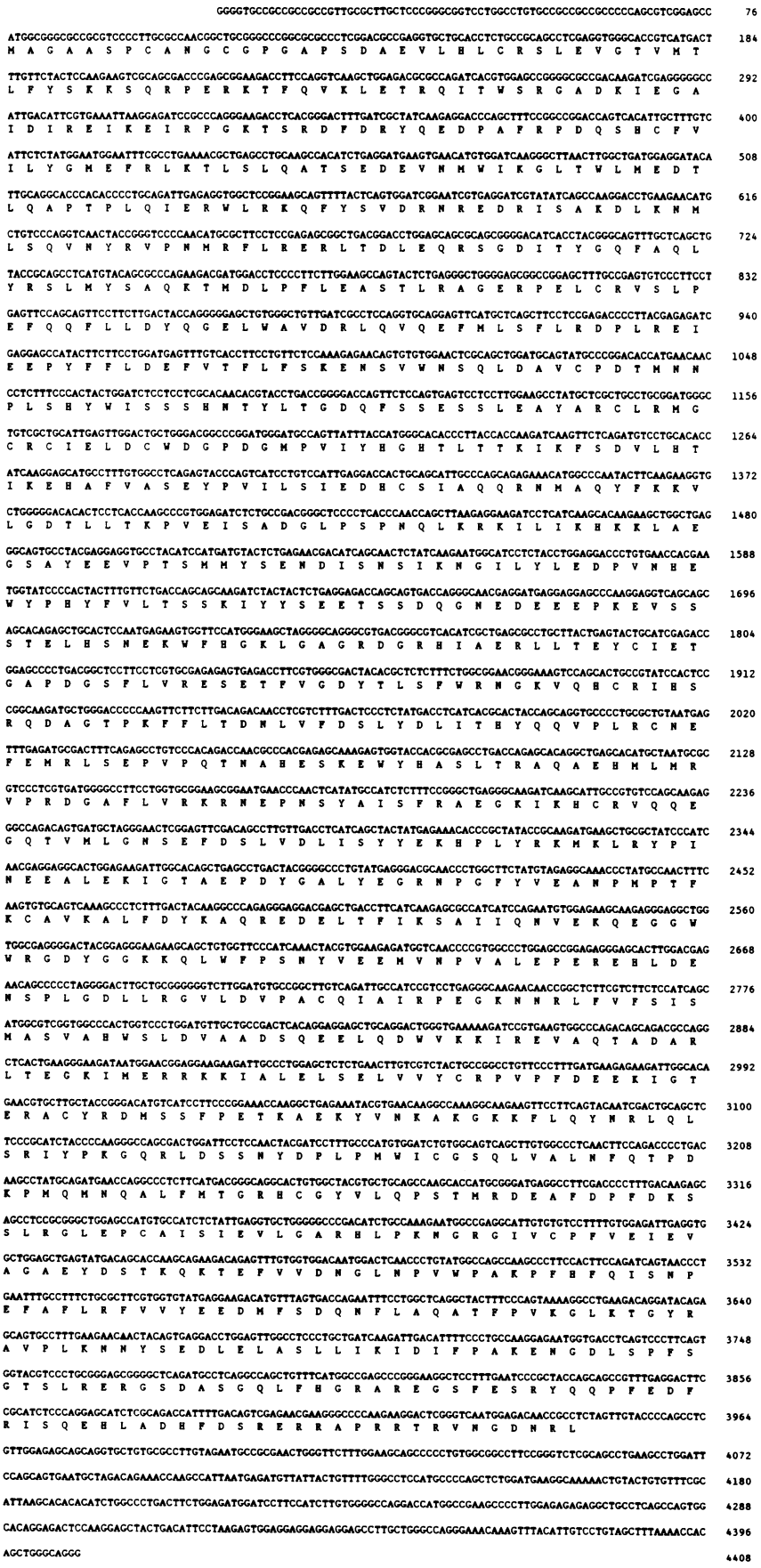
(iii) Isolation and nucleotide sequence analysis of human cDNA clones. A 35-base antisense oligonucleotide (5' ACCG TCTTCTGGGCGCTGTACATGAGGCTGCGGTA 3') derived from the human genomic sequence was used to screen 2.4×10^6 phage of a human umbilical vein endothelial cell cDNA library in λgt11 essentially as described previously (11). Prehybridization and overnight hybridization were performed at 52°C in 5× SSPE–0.25% nonfat dry milk. Filters were washed at 57°C for 20 minutes each wash, once with 1× SSC–0.1% SDS and twice with 0.5× SSC–0.1% SDS. Following overnight autoradiography, six clones were identified and purified, and phage DNA was prepared by using LambdaSorb. Phage DNA was digested with *Eco*RI, and cDNA inserts were subcloned into *Eco*RI-digested M13mp18 and pGEM2. Restriction mapping and preliminary nucleotide sequence analysis established that the 6 clones represented four unique overlapping cDNA fragments whose combined sequence was 9 bp short of the translational initiation ATG. A seventh 508-bp clone which contained 76 bp of 5' untranslated sequence was obtained from a human brain stem library (ATCC 37432) by screening with a radiolabeled double-stranded DNA probe corresponding to nucleotides 131 to 409 in Fig. 1A. The nucleotide sequence shown in Fig. 1A was determined on both strands by chain termination sequencing (33) of all clones.

(iv) Immunoprecipitation and Western blot analysis. Rabbit anti-phospholipase C-γ was prepared and prebound to protein A-Sepharose as described previously (20). Cell lysates from two confluent 100-mm (diameter) dishes were incubated with the antibody-protein A complex for 90 min at 4°C. The beads were washed with HNTG (20 mM HEPES, 0.1% Triton X-100, 150 mM NaCl, 10% glycerol [pH 7.5]). The immunoprecipitated proteins were eluted with 2× Laemmli sample buffer and subjected to polyacrylamide gel electrophoresis in the presence of SDS. Proteins were transferred to nitrocellulose for 2 h at 200 mA with a semidry blot apparatus. The blots were then probed for either PLC-γ or phosphotyrosine immunoreactivity by using the indicated primary antibody followed by ^{125}I-protein A. The dose response of tyrosine phosphorylation of PLC-γ induced by HBGF-1 and its time course were determined as indicated in the Fig. 1 legend.

RESULTS

Large-scale preparations of NIH 3T3 cells (clone 2.2) were extracted, and phosphotyrosine-containing proteins were isolated by immunoaffinity chromatography as described in Materials and Methods. Analysis of samples of the eluates by SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting using anti-phosphotyrosine antibodies revealed a pattern of phosphotyrosine-containing proteins (Fig. 2A) which was similar to one observed previously (7). Three of the phosphotyrosine-containing proteins (apparent molecular weights, 150,000, 130,000, and 90,000) were dependent on HBGF-1 for maximal phosphorylation. The apparent molecular weight, i.e., the relative mobility of the M_w 150,000 species was identical to that of a major Coomassie blue-stained band observed when the remainder of the eluates were pooled and analyzed by SDS-PAGE (Fig. 2B). This protein was chosen for protein sequence analysis.

A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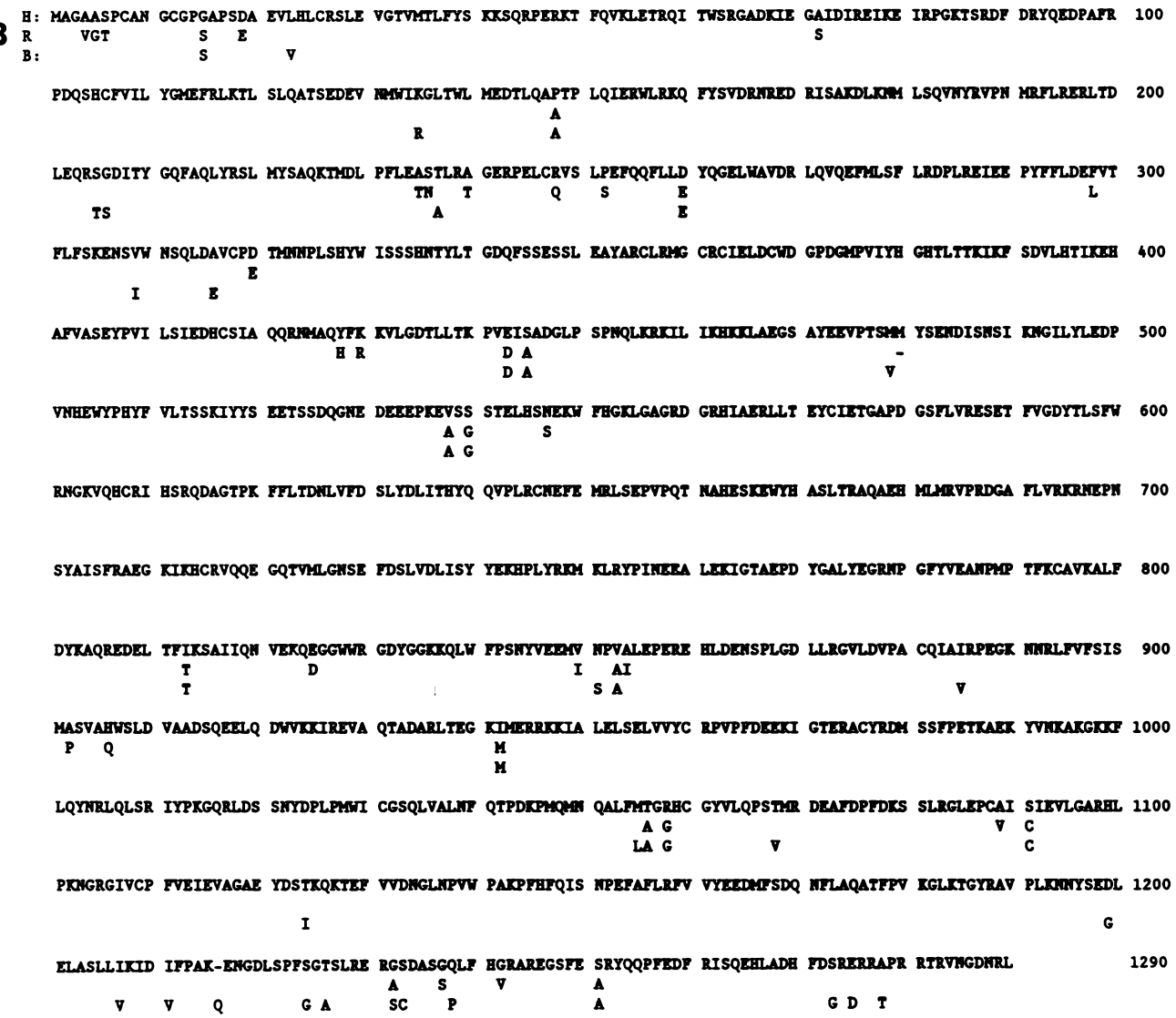

FIG. 1. (A) Nucleotide sequence and deduced amino acid sequence of human phospholipase C-γ. The entire cDNA sequence of human PLC-γ was determined by analysis of overlapping cDNA clones obtained from HUVEC and brain stem cDNA libraries as described in Materials and Methods. The GenBank accession number for this sequence is M34667. (B) Comparison of deduced amino acid sequences of human (H), rat (R), and bovine (B) PLC-γ. Only differences in the rat and bovine sequences compared with the human sequences are shown.

The two Coomassie-stained protein bands at 150,000 shown in Fig. 2B were eluted from the gel and processed for trypsin digestion and protein sequence analysis as described in Materials and Methods. The sequence of one of the peptides obtained (X-G-D-I-T-T-G-Q-F-A-Q-L-Y) was used to design a unique 32-base oligonucleotide probe. When the probe was used to screen a mouse genomic library, eight clones were identified, and the clone which consistently hybridized most strongly under the hybridization and washing conditions utilized was pursued further. In order to rapidly determine the authenticity of this clone, phage DNA was digested with *Hae*III, and the entire mixture was cloned into pGEM2. Recombinant plasmids containing a 179-bp *Hae*III fragment were identified by colony hybridization with the 32-mer, and nucleotide sequence analysis revealed an exact match between the 12-amino-acid tryptic peptide sequence and the deduced amino acid sequence of the 179-bp *Hae*III fragment (data not shown). In order to obtain a human sequence for screening of human libraries, two 30-base oligonucleotides within the 179-bp sequence were synthesized and used to prime PCR in human genomic DNA. Sequencing of the PCR product revealed identity between mouse and human sequence in the 35-bp region between the PCR primers (data not shown). A 35-base oligonucleotide based on this sequence was then used to screen a human umbilical vein endothelial cell cDNA library, which resulted in the isolation of four independent overlapping clones. Nucleotide sequence analysis revealed high homology to the published sequences of both bovine (35) and rat (36) PLC-γ and indicated that the combined sequence encompassed the entire coding region except for the 5′-terminal 9 bp.

A clone containing the 5′-terminal human PLC-γ sequence was obtained by screening a human brain stem cDNA library with a radiolabeled probe corresponding to the 5′ end of the HUVEC cDNA sequence. A single 508-bp clone was isolated which exactly overlapped the previously obtained sequence by 423 bp and extended it 85 bp in the 5′ direction. The amino acid sequence deduced from this composite

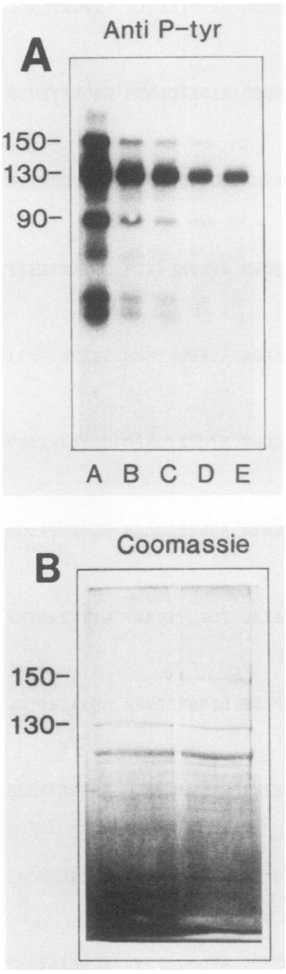

FIG. 2. (A) Analysis of phosphotyrosine-containing proteins eluted from 1G2 columns. Proteins were eluted from the 1G2 monoclonal antibody column with phenylphosphate in five consecutive fractions. Portions of each fraction were subjected to SDS-PAGE, and the proteins were transferred to nitrocellulose and immunoblotted with antiphosphotyrosine antibodies. The blots were incubated with ^{125}I-protein A and phosphotyrosine-containing proteins were visualized by overnight autoradiography. Lanes A through E, Portions of consecutive fractions from the 1G2 column. The position of an apparent M_r 150,000 phosphotyrosine-containing protein is indicated. (B) Coomassie blue-stained SDS-polyacrylamide gel of a pool of fractions eluted from the 1G2 column with phenylphosphate. Fractions shown to contain the apparent M_r 150,000 phosphotyrosine-containing protein by Western blot analysis were pooled and subjected to PAGE in the presence of SDS with a 7% acrylamide gel. The two lanes, each containing 50% of the preparation, are shown. The proteins were visualized with Coomassie blue, and the apparent M_r 150,000 protein was excised for electroelution.

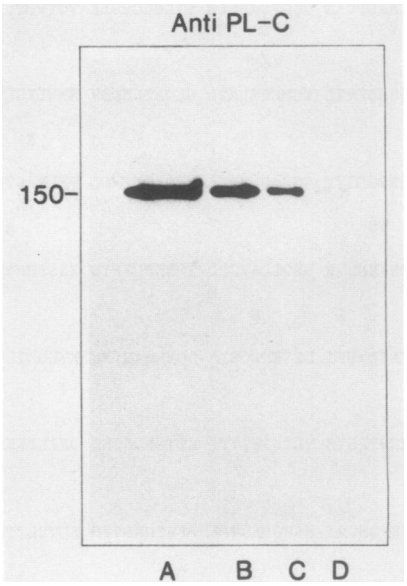

FIG. 3. Western blot analysis of proteins eluted from the 1G2 column by using anti-PLC-γ antibodies. Portions of the pooled fractions shown in Fig. 1 were taken to determine the amount of PLC-γ immunoreactivity in the post-1G2 fractions. Lanes A through D, 30 μl each of 1G2 eluate and 1:3, 1:10, and 1:30 dilutions of the eluate, respectively. The samples were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with anti-PLC-γ antibodies. The immunoreactive proteins were visualized by overnight autoradiography following incubation of the blots with ^{125}I-labeled protein A.

sequence shows 97% identity to both the bovine and rat phospholipase C-γ sequences (Fig. 1B).

Once the amino acid sequences of the trypsin-derived fragment were identified as a portion of human PLC-γ, we analyzed a fraction of the 1G2 eluate for PLC-γ immunoreactivity. Immunoblot analysis (using anti-PLC-γ antibodies) of various concentrations of the 1G2 eluates demonstrated that PLC-γ is a major component of the 150,000 apparent molecular weight region of the eluate (Fig. 3).

We analyzed the HBGF-1 induction of PLC-γ tyrosine phosphorylation in intact cells. Treatment of cells with 10 ng of recombinant HBGF-1 per ml for 10 min induced a high level of tyrosine phosphorylation in PLC-γ while having no effect on the total amount of PLC-γ protein (Fig. 4A). A dose-response curve indicates that HBGF-1-induced tyrosine phosphorylation is concentration dependent, with maximum phosphorylation obtained at 3 to 10 ng/ml (Fig. 4B). Higher concentrations resulted in lower levels of tyrosine phosphorylation under the conditions employed in this experiment. A time course of PLC-γ tyrosine phosphorylation induced with 10 ng of HBGF-1 per ml reveals that phosphorylation is time dependent, with maximal phosphorylation observed 10 min after HBGF-1 addition (Fig. 4C).

DISCUSSION

A major step towards understanding the mechanism of action of the heparin-binding (or fibroblast) family of growth factors is the identification of the protein substrates of HBGF-induced tyrosine kinase activity. We focused our attention on a M_r 150,000 protein phosphorylated on tyrosine in response to HBGF-1 because previous results had indicated that its phosphorylation was one of the earliest events that occurred after binding HBGF-1 to cells. Phosphorylation occurred at 4°C and showed a dependence on HBGF-1 concentration similar to that observed for HBGF-1 stimulation of DNA synthesis (7).

In this report, the identification of the M_r 150,000 protein that is phosphorylated on tyrosine in response to HBGF-1 as PLC-γ has been established, first by cDNA cloning and sequencing and subsequently by immunological criteria. Human PLC-γ consists of 1,290 amino acids and is 97% homologous to both the rat and bovine proteins. The motifs

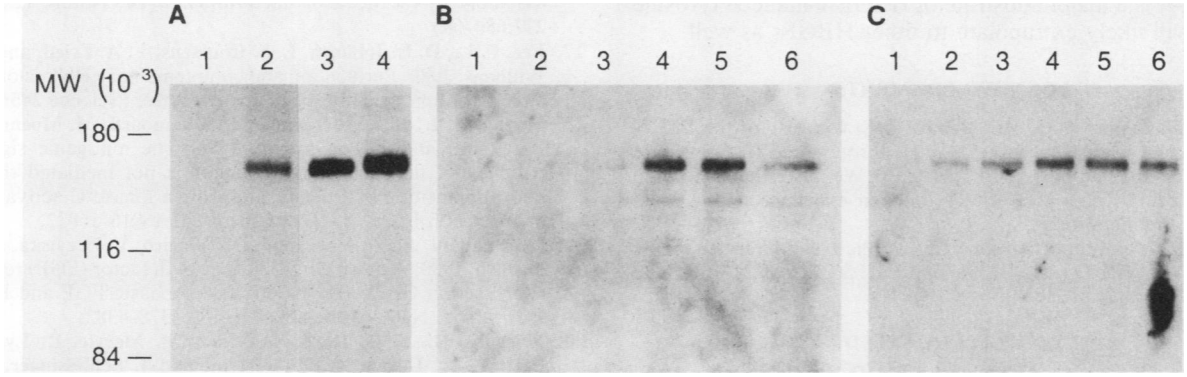

FIG. 4. Stimulation of tyrosine phosphorylation of PLC-γ by HBGF-1 in NIH 3T3 cells. (A) NIH 3T3 (strain 2.2) cells were serum starved overnight, as described in Materials and Methods. The cells were then treated for 10 min with either 10 ng of recombinant HBGF-1 per ml (10) (lanes 2 and 4) or 0.1% bovine serum albumin (lanes 1 and 3). The cells were then lysed, immunoprecipitated with anti-PLC-γ, and subjected to SDS-PAGE, and immunoblot analysis was performed by using either antiphosphotyrosine (lanes 1 and 2) or anti-PLC-γ (lanes 3 and 4) antibodies. Proteins were visualized by using ^{125}I-protein A and overnight autoradiography. (B) Cells were treated as in panel A, and the blots were probed with antiphosphotyrosine antibodies following treatment with 0, 0.3, 1, 3, 10, and 30 ng of recombinant HBGF-1 per ml (lanes 1 through 6, respectively). (C) Cells were treated as in panel A, and the blots were probed with antiphosphotyrosine antibodies following treatment with 10 ng of recombinant HBGF-1 per ml for 0, .3, 1, 3, 10, or 20 min. (lanes 1 through 6, respectively).

and domains found previously in the bovine and rat proteins (reviewed in reference 29) are conserved in the human protein.

Clearly, HBGF-1 induces tyrosine phosphorylation of PLC-γ in living cells with a potency similar to that shown for platelet-derived growth factor (PDGF) and epidermal growth factor (20, 22, 39). Although it has not been directly shown that PLC-γ is activated upon tyrosine phosphorylation, there is a good correlation between the ability of growth factors to stimulate tyrosine phosphorylation of PLC and their ability to stimulate phosphatidylinositol turnover. For example, it was shown that the protein tyrosine kinase activity of the epidermal growth factor receptor is essential for stimulation of phosphatidylinositol bisphosphate hydrolysis and Ca^{2+} release (24). Wahl et al. (38) showed that antiphosphotyrosine antibodies could be used to recover increased PLC activity from A431 cells treated with epidermal growth factor and that the kinetics of PLC-γ phosphorylation are consistent with the increase in cellular phosphatidylinositol turnover. Recently, Margolis et al. (21) showed that PDGF treatment of 3T3 cells overexpressing recombinant PLC-γ results in increased production of inositol 1,4,5-triphosphate, a product of PLC-γ activity. On the basis of the results presented here, it is likely that in addition to epidermal growth factor and PDGF, the HBGFs (or fibroblast growth factors) also affect phosphatidylinositol turnover via enhanced phosphorylation of PLC-γ.

The breakdown of phosphatidylinositol bisphosphate by PLC-γ leads to the generation of inositol 1,4,5-triphosphate, which releases calcium from intracellular stores, and diacylglycerol, which activates protein kinase C. While their findings are still somewhat controversial, several investigators have provided evidence of PLC involvement in signal transduction by HBGFs. Addition of HBGF-2 to quiescent cultures of Swiss 3T3 cells resulted in increased cellular diacylglycerol, cytoplasmic Ca^{2+}, and activation of protein kinase C (12, 37). Brown et al. (1) demonstrated a dose-dependent increase in accumulation of inositol phosphates in response to preparations of recombinant HBGF-1 and HBGF-2. They also found that increasing the concentrations of HBGF-1 or HBGF-2 beyond that necessary for maximum response led to a pronounced dose-dependent decline in inositol phosphate accumulation. These results correlate with the phosphorylation of PLC-γ on tyrosine in response to increasing concentrations of HBGF-1 (Fig. 4B). Although this reduced response is not understood, it is frequently observed in DNA synthesis assays.

In contrast to these results, other workers have found no evidence that the mitogenic signalling pathway of HBGF-1 or HBGF-2 is mediated through PLC in Chinese hamster CCL39 fibroblasts (18), bovine lens epithelial cells (23), or bovine capillary endothelial cells (5). However, these conclusions are weakened by the observation that thrombin and HBGF-2 can synergistically activate PLC in CCL39 cells (25). It has also been shown that HBGF-2 stimulates protein kinase C in fetal bovine aortic endothelial cells, presumably by diacylglycerol produced via activation of PLC (27). Thus, the current consensus on the role of PLC in the HBGF signalling pathway is that the experimental data obtained depend largely on the cell type, the basal cellular PLC activity, and the assay employed. Our results demonstrate that tyrosine phosphorylation and probable stimulation of PLC-γ are early events in the transduction of the signal induced by HBGF-1 in NIH 3T3 cells.

Recently, several groups have isolated cDNA clones for two distinct HBGF receptors (15, 17, 19, 26, 28, 31; C. A. Dionne, G. Crumley, F. Bellot, J. M. Kaplow, G. Searfoss, M. Ruta, W. H. Burgess, M. Jaye, and J. Schlessinger, EMBO J., in press), the analysis of which places them in the family of receptors which includes the PDGF and colony-stimulating factor 1 receptors (34, 41). The most distinctive features of this family of receptors include extracellular domains consisting of 3 (HBGF) or 5 (PDGF, colony-stimulating factor 1) immunoglobulin-like domains and intracellular domains whose tyrosine kinase domains contain insertions. Despite these similarities, it is interesting to note that PLC-γ is phosphorylated in cells only in response to PDGF or HBGF-1, and not in response to colony-stimulating factor 1 (6). The two cloned human HBGF receptors, termed *bek* and *flg*, cross-react with high affinity to both HBGF-1 and HBGF-2 (Dionne et al., in press) as well as to Kaposi fibroblast growth factor (19; F. Bellot et al., unpublished data). Therefore, the results obtained herein, demonstrating

that PLC-γ is a major substrate of HBGF-1-induced tyrosine kinase, will likely extrapolate to other HBGFs as well.

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