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BASIC FIBROBLAST GROWTH FACTOR IS A SUBSTRATE FOR PHOSPHORYLATION BY HUMAN NEUTROPHIL ECTO-PROTEIN KINASE ACTIVITY*

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Basic fibroblast growth factor (FGF) has recently been shown to be a phosphoprotein and its receptor affinity is modified by phosphorylation. Since most studies of protein phosphorylation have focused on intracellular protein kinases, a physiologic mechanism of the regulation of basic FGF activity by phosphorylation has been unclear. Evidence for the existence of ecto-protein kinase activity on the surface of several types of cells including human neutrophils has been described. These ecto-protein kinase activities utilize exogenous ATP to phosphorylate exogenous as well as endogenous cell-surface proteins. This report demonstrates that human basic FGF is phosphorylated on serine by human neutrophil ecto-protein kinase activity and this phosphorylation is inhibited by heparin. Regulation of basic FGF activity by phosphorylation may be one of the functions of ecto-protein kinase activity.

Basic fibroblast growth factor (FGF) is highly mitogenic for a variety of cells in tissue culture (1) and induces angiogenesis in vivo (2). Basic FGF has recently been shown to be a phosphoprotein (3) and is a substrate for phosphorylation by both the catalytic subunit of cAMP-dependent protein kinase (PKA) and the calcium- and phospholipid-dependent protein kinase (PKC) (4). PKA

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Abbreviations: FGF, fibroblast growth factor; PKA, cAMP-dependent protein kinase; PKC, calcium-dependent and phospholipid-dependent protein kinase; HBSS, Hanks' balanced salt solution, pH 7.4; NaCl-HEPES, 145 mM NaCl, 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.4; PAGE, polyacrylamide gel electrophoresis; PVDF, Immobilon-P; P-S, phosphoserine; P-T, phosphothreonine; P-Y, phosphotyrosine.

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phosphorylates human basic FGF in the receptor binding domain at Thr^{112} and this phosphorylated form of basic FGF has a higher affinity for its receptor (4). PKC phosphorylates human basic FGF on Ser^{64} (4). Since most studies of protein phosphorylation have focused on intracellular protein kinases (5,6), the physiologic role of the regulation of basic FGF activity by phosphorylation has been unclear. Evidence for the existence of ecto-protein kinase activity on the surface of several types of cells including human neutrophils has been described (7-12). These ecto-protein kinase activities utilize exogenous ATP to phosphorylate exogenous as well as endogenous cell-surface proteins. Few physiologic substrates of ecto-protein kinase activity have been identified. We tested whether basic FGF was a substrate for phosphorylation by human neutrophil ecto-protein kinase activity. This report demonstrates that human basic FGF is phosphorylated on serine by human neutrophil ecto-protein kinase activity and this phosphorylation is inhibited by heparin. Regulation of basic FGF activity by phosphorylation may be one of the functions of ecto-protein kinase activity.

MATERIALS AND METHODS

Cell preparation - Normal peripheral blood neutrophils were prepared by a modification of the method of Boyum as previously described (13,14) and were suspended at the indicated concentrations in Hanks' balanced salt solution, pH 7.4, (HBSS) (GIBCO, Grand Island, NY) or in 145 mM NaCl, 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.4, (NaCl-HEPES) (GIBCO). Differential cell counts on Wright-stained cells routinely revealed greater than 95% neutrophils. Viability as assessed by trypan blue dye exclusion was greater than 98%.

Ecto-protein kinase assay - The ecto-protein kinase assay was performed as previously described with minor modifications (11). Briefly, neutrophils in HBSS, 10^6 cells/50 µl, were allowed to adhere for 30 min to wells of a 96 well microtiter plate (Falcon, Becton Dickinson, Lincoln Park, NJ) in a 37°C incubator containing 5% CO_2. The buffer was then aspirated and the adherent cells were washed twice with 200 µl of NaCl-HEPES at 23°C and 20 µl of NaCl-HEPES with or without 6 µg of sodium heparin (from porcine intestinal mucosa, grade I, 181 U/mg, Sigma Chemical Co., St. Louis, MO), 10 µg of fibronectin, 10 µg of laminin, or 10 µg of type IV collagen (a gift of Dr. A. Skubitz, University of Minnesota, Minneapolis) was added to each well. After 5 min at 23°C, 20 µl of NaCl-HEPES containing 2 µg of basic FGF (carrier free, recombinant human basic FGF, or bovine basic FGF from bovine brain, Research & Diagnostic Systems, Minneapolis, MN) or 2 µg of bovine acidic FGF (Research & Diagnostic Systems) and 20 µl of NaCl-HEPES containing 10 µCi of [γ-^{32}P]ATP (specific activity 4500 Ci/mmol, ICN, Irvine, CA), 300 µM $MnCl_2$, 600 µM Na_3VO_4, and 600 µM Na_2MoO_4 were then added, and the mixture was incubated for 10 min at 37°C. Twenty µl of NaCl-HEPES containing 40 mM diisopropylfluorophosphate (DFP) (Sigma), 400 µM ATP (Sigma), 200 µM Na_3VO_4, and 200 µM Na_2MoO_4 was then added and the samples were incubated for 2 min at 23°C. The supernatant was then removed and added to concentrated Laemmli sample buffer (final concentrations 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.001% bromphenol blue) (15). Sample buffer (80 µl) was then added to each well, and after 1 min, the solubilized cell extract was removed. Samples were immediately incubated for 2 min at 100°C and then analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) in a 15% gel. Control reactions were also performed by incubating basic or acidic FGF in the same reaction mixture without cells. Gel slabs were stained, destained, dried, and subjected to autoradiography by using Dupont Cronex film. In some cases, as indicated, proteins were excised from the dried gels and the radioactivity determined in a liquid scintillation spectrometer using Ecolite (+) (ICN). Molecular weight standards were purchased from Sigma.

Phosphoamino acid analysis - Phosphoamino acid analyses were performed as previously described (16). Briefly, radiolabeled proteins resolved by SDS-PAGE were transblotted onto Immobilon-P (PVDF) paper (Millipore Corp., Bedford, MA), localized by autoradiography and excised. The proteins on the PVDF were then hydrolyzed in 6 M HCl for 2 hr at 110°C. The HCl was removed by evaporation using a Speed Vac (Savant Instruments). The dried, partially hydrolyzed samples were then dissolved in water containing phosphoserine (P-S), phosphothreonine (P-T), and phosphotyrosine (P-Y) (Sigma) each at 1 mg/ml. The samples were then spotted on microcrystalline cellulose TLC plates (Polygram CEL400 by Macherey-Nagel and Co., Brinkman Instruments, Inc.) and the phosphoamino acids were resolved by electrophoresis at pH 3.5 in the first dimension, and then chromatography using isobutyric acid/0.5 M NH_4OH (5/3, v/v) in the second dimension. The radiolabeled phosphoamino acids were detected by autoradiography using X-Omat AR film.

RESULTS

Phosphorylation of FGF by ecto-protein kinase activity. Incubation of recombinant human basic FGF with [γ-^{32}P]ATP in the presence of human neutrophils resulted in the incorporation of ^{32}P into basic FGF in the supernatant (Fig. 1, lane A). In contrast, little ^{32}P was incorporated into basic FGF incubated under identical conditions in the absence of cells (lane E). Preincubation of neutrophils with heparin (final concentration 100 μg/ml), which inhibits casein phosphorylation by neutrophil ecto-protein kinase activity (17), markedly reduced the incorporation of ^{32}P into basic

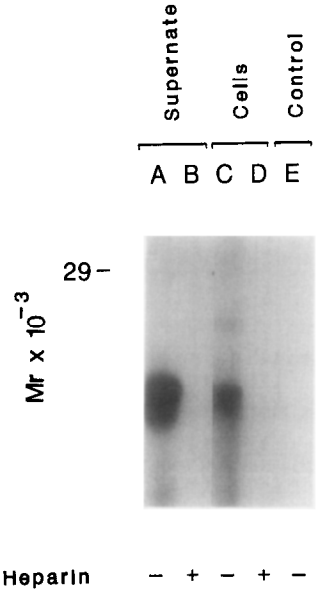

Figure 1. Phosphorylation of basic FGF by human neutrophil ecto-protein kinase activity. Adherent cells were incubated for 5 min at 23°C in 20 μl of NaCl-HEPES without (lanes A and C) or with (lanes B and D) 12 μg of heparin, and then 20 μl of NaCl-HEPES containing 2 μg of recombinant human basic FGF and 20 μl of NaCl-HEPES containing 10 μCi of [γ-^{32}P]ATP, 300 μM $MnCl_2$, 600 μM Na_3VO_4, and 600 μM Na_2MoO_4 were added. After incubating for 10 min at 37°C, 20 μl of NaCl-HEPES containing 40 mM DFP, 400 μM ATP, 200 μM Na_3VO_4, and 200 μM Na_2MoO_4 was added and the mixtures were incubated for 2 min at 23°C. The supernatants (lanes A and B) and adherent cells (lanes C and D) were analyzed separately by SDS-PAGE and autoradiography as described in the text. Control reactions were also performed with basic FGF and [γ-^{32}P]ATP in buffer without cells to determine autophosphorylation as described in the text (control, lane E). Proteins used as molecular weight standards were: ovalbumin, 45,000; and carbonic anhydrase, 29,000.

FGF in the supernatant (lane B). Since basic FGF tends to adhere strongly to surfaces, the amount of basic FGF remaining adherent to the cells and the well were also examined. ^{32}P-labeled basic FGF was readily detected remaining with the adherent cell fraction (lane C) although much more was present in the supernatant (lane A). Preincubation of cells with heparin also markedly reduced the amount of ^{32}P-labeled basic FGF present in the adherent cell fraction (lane D). Similar results were observed using basic FGF purified from bovine brain (data not shown). No release of protein kinase activity was detected when adherent neutrophils were incubated with basic FGF under identical conditions (data not shown). Since some extracellular matrix proteins have been shown to alter the phosphorylation of basic FGF by PKA (4), their effects on basic FGF phosphorylation by neutrophil ecto-protein kinase activity was tested. Fibronectin, laminin, and type IV collagen at 10 μg/well had no effect on basic FGF phosphorylation by the ecto-protein kinase activity. Bovine acidic FGF was not a substrate for ecto-protein kinase activity under the same assay conditions (data not shown).

Phosphoamino acid analysis. To characterize the nature of the ^{32}P-label found on human and bovine basic FGF phosphorylated by human neutrophil ecto-protein kinase activity, the proteins were analyzed for their phosphoamino acid composition by electrophoresis and chromatography following acid hydrolysis as described in the methods. The vast majority of $^{32}PO_4$ incorporated into both human and bovine basic FGF was present in serine (Fig. 2).

DISCUSSION

Basic FGF is a heparin binding growth factor that is highly mitogenic for a variety of cells including capillary endothelial cells, fibroblasts, and smooth muscle cells in vitro (1). While its physiologic role is not clearly defined, it is a potent inducer of angiogenesis in vivo (2) and appears to exert a regulatory role in early hematopoiesis (18). In culture, basic FGF is found in many normal and transformed cells (19,20). Recent studies have suggested that basic FGF is bound to heparan sulfate in the basement membrane and can be released in an active form with the appropriate stimulus (21,22).

Basic FGF is phosphorylated by bovine capillary endothelial cells and human hepatoma cells in culture demonstrating that it exists in vivo as a phosphoprotein (3,4). The physiological significance of this phosphorylation is not clearly defined. PKA phosphorylates basic FGF at

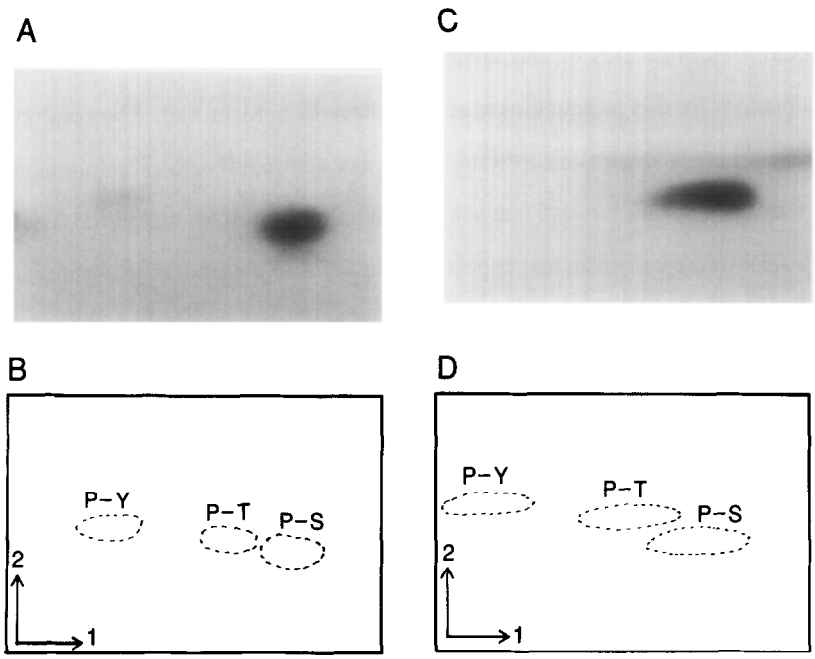

Figure 2. Phosphoamino acid analysis of recombinant human basic FGF and bovine basic FGF phosphorylated by human neutrophil ecto-protein kinase activity. Basic FGF was ^{32}P-labeled by the addition of [γ-^{32}P]ATP in the presence of human neutrophils, resolved by SDS-PAGE as in Figure 1, recovered, and subjected to acid hydrolysis as described in the text. The resultant phosphoamino acids were resolved by electrophoresis at pH 3.5 in the first dimension and chromatography in the second dimension and visualized by autoradiography as described in the text. Panel A, autoradiograph of the resolved phosphoamino acids of recombinant human basic FGF; panel B, schematic representation of the electrophoresis and chromatography dimensions and the positions of migration of authentic phosphoserine (P-S), phosphothreonine (P-T), and phosphotyrosine (P-Y) in panel A; panel C, autoradiograph of the resolved phosphoamino acids of bovine basic FGF; panel D, schematic representation of the positions of migration of P-Y, P-T, and P-S in panel C.

Thr^{112} in its receptor binding domain, and this phosphorylated form of basic FGF has a higher affinity for its receptors on BHK cells than unphosphorylated basic FGF (3). In contrast, PKC phosphorylates basic FGF on Ser^{64}, and this phosphorylated form of basic FGF has the same affinity for its receptor as unphosphorylated basic FGF (3). These studies also demonstrated that heparin can alter the site of phosphorylation of basic FGF by PKA to Ser^{64}, and that the extracellular matrix proteins fibronectin, laminin, and type IV collagen inhibit the phosphorylation by PKA (4). In addition, heparin inhibits the phosphorylation of basic FGF on Ser^{64} by PKC, but fibronectin, laminin, and type IV collagen do not (4).

It is not known what protein kinases are responsible for the phosphorylation of basic FGF in vivo. Since most studies of protein phosphorylation have considered it an intracellular process, the physiologic significance of the regulation of basic FGF phosphorylation by heparin and

extracellular matrix proteins has not been clear. Recent studies have provided evidence for the existence of ecto-protein kinases on the surface of several types of cells (7-12), however few physiologic substrates for this protein kinase activity have been identified. The results described here demonstrate that basic FGF is a substrate for human neutrophil ecto-protein kinase activity. Interestingly, recent studies have found that acidic FGF is released from retinal rod outer segment membranes by ATP by a mechanism that appears to require ATP-dependent phosphorylation (23). However, acidic FGF was not a substrate for the ecto-protein kinase activity.

The neutrophil ecto-protein kinase activity phosphorylated basic FGF on serine, in contrast to PKA. This phosphorylation was potently inhibited by heparin. In contrast, fibronectin, laminin, and type IV collagen had no effect on the phosphorylation of basic FGF by neutrophil ecto-protein kinase activity. While the physiologic significance of the phosphorylation of basic FGF remains unclear, these results demonstrate a mechanism for extracellular phosphorylation of basic FGF and its regulation by heparin, and are consistent with a regulatory function of basic FGF phosphorylation. In addition, this study identifies a physiologically appropriate substrate for neutrophil ecto-protein kinase activity.

Although previous studies have suggested that human neutrophil ecto-protein kinase activity is distinct from casein kinase II, the neutrophil ecto-protein kinase activity does share some characteristics of casein kinase II (17). It is, therefore, of interest that basic FGF is a poor substrate for casein kinase II (3). In addition, acidic FGF is a substrate for PKC (3) but not for human neutrophil-ecto-protein kinase activity. Thus, these studies provide further evidence that casein kinase II is distinct from neutrophil ecto-protein kinase activity.

Although basic FGF interacts with specific cell-surface receptors, the subsequent steps in signal transduction remain to be clearly established. Recent studies suggest that internalized basic FGF is translocated to the nucleolus and has the ability to function as an intracellular messenger, directly regulating gene expression (24,25). Evidence also suggests that nuclear translocation is important for some biological responses to acidic FGF (26). It is possible that phosphorylation of basic FGF could modify these functions as well. It will be of interest to determine whether the phosphorylation of basic FGF by neutrophil ecto-protein kinase activity alters its physiologic effects. Such studies will be complicated by the presence of both ecto-protein kinases and ecto-

protein phosphatases on the responding cell, and will require the purification of ecto-protein kinase activity.

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