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Cell Biology

Basic fibroblast growth factor is a substrate for protein phosphorylation and is phosphorylated by capillary endothelial cells in culture

(growth factors/cAMP-dependent protein kinase/protein kinase C)

JEAN-JACQUES FEIGE AND ANDREW BAIRD

Laboratories for Neuroendocrinology, The Salk Institute, La Jolla, CA 92037

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ABSTRACT **A phosphorylated basic fibroblast growth factor (FGF) can be detected in extracts of bovine capillary endothelial cells and human hepatoma cells. Accordingly, human basic FGF contains consensus sequences that account for its phosphorylation on Thr-112 by the catalytic subunit of the cAMP-dependent protein kinase A (PK-A) and on Ser-64 by the calcium- and phospholipid-dependent protein kinase C (PK-C). A kinetic analysis of both of these reactions revealed that basic FGF is among the better substrates for these enzymes. Although the kinase responsible for the phosphorylation *in vivo* has not yet been identified, we examined the effects of phosphorylation on the biological activity, heparin-binding capacity, and receptor-binding capacity of phosphorylated basic FGF. No effects of phosphorylation were observed when the mitogen was phosphorylated by PK-C. In contrast, when basic FGF was phosphorylated in the receptor-binding domain with PK-A, the growth factor was 3–8 times better at displacing radiolabeled basic FGF in the radioreceptor assay. No effects were seen on the binding of this FGF to immobilized heparin or cell-associated glycosaminoglycans, suggesting that this phosphorylation modifies the affinity of basic FGF for its receptor. Biological assays for basic FGF failed to identify differences between the phosphorylated and unphosphorylated forms of recombinant basic FGFs presumably because of the presence of ectophosphatases and the experimental conditions of proliferation and mitogenic assays (37°C, 24–96 hr). Because the relative affinity of basic FGF for its receptor and cell-associated glycosaminoglycans may regulate its activity, the identification of a modified form of basic FGF may be of particular importance in understanding the mechanisms that regulate its biological activity, bioavailability, and processing to and from the extracellular matrix.**

Although the acidic and basic fibroblast growth factors (FGFs) have been clearly established to participate in the regulation of the proliferation of many cell types, the structural characterization of these molecules (1, 2) has raised many questions regarding their possible mechanism of action. As an example, both mitogens lack a classical signal sequence (3, 4) that would allow their secretion and access to their putative receptor located on the extracellular surface of the plasma membrane (5, 6). Because both acidic and basic FGFs have been found in the extracellular matrix, it has been suggested that they utilize an alternative secretion pathway to the outside of the cell and that their activity is tightly regulated by limiting their bioavailability (7–10). In view of the fact that both FGFs are also characterized by their high affinity for immobilized heparin and that they remain cell-associated even when expressed with consensus signal peptide sequences (11, 12), we have been investigating the possibilities that these proteins are cytoplasmic and that posttranslational changes might modulate their processing to and from the extracellular matrix.

Protein phosphorylation is a posttranslational modification that has been implicated in the regulation of almost all steps of cell division (13–16). Recently, Plouët *et al.* (17) reported that the release of acidic FGF from rod outer segment membranes requires ATP-dependent protein phosphorylation. Because basic and acidic FGFs contain consensus sequences for phosphorylation by the phospholipid- and calcium-dependent protein kinase C (PK-C) and basic FGF contains a consensus sequence for the cAMP-dependent protein kinase A (PK-A), we tested the hypothesis that growth factors, and in particular FGFs, are potential substrates for protein phosphorylation.

MATERIALS AND METHODS

Materials. PK-C was a gift from G. Walton (University of California–San Diego, La Jolla) and I. Vilgrain and J. M. Pelarin (Unité 244, Institut National de la Santé et de la Recherche Médicale, Grenoble, France). The catalytic subunit of PK-A was provided by S. Taylor (University of California–San Diego, La Jolla). Casein kinases I and II were purified from bovine lung as previously described and were provided to us by C. Cochet (Unité 244, Institut National de la Santé et de la Recherche Médicale, Grenoble) (18, 19). Recombinant human basic and acidic FGFs (20) were obtained from P. Barr and L. Cousens (Chiron). Synthetic transforming growth factor α (TGF-α) was supplied by Nicholas Ling (Salk Institute), and platelet-derived growth factor (PDGF) was a gift of R. Ross (University of Washington, Seattle). Recombinant insulin-like growth factor I (IGF-I) was obtained from Fujisawa Pharmaceuticals (Osawa, Japan); nerve growth factor (NGF), from D. Schubert (Salk Institute); and tumor necrosis factor α (TNF-α), from Suntory Institute for Biomedical Research (Osaka, Japan). [γ-^{32}P]ATP (4000 Ci/mmol; 1 Ci = 37 GBq) and [γ-^{32}P]orthophosphate were purchased from ICN. Antiserum 773 is an antiserum against basic FGF-(1–24) conjugated to bovine serum albumin and was raised in rabbits. Protein A-Sepharose was obtained from Pharmacia. Reagents for sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) were obtained from Bio-Rad, and all other materials were from Sigma.

Phosphorylation of Growth Factors by PK-C and PK-A. Different growth factors (≈0.5 μg) were incubated for 15 min at 30°C in the presence of purified PK-C from bovine brain

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Abbreviations: FGF, fibroblast growth factor; PK-A, cAMP-dependent protein kinase; PK-C, calcium- and phospholipid-dependent protein kinase; IGF, insulin-like growth factor; TGF, transforming growth factor; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; NGF, nerve growth factor; TNF, tumor necrosis factor; ACE cells, adrenocortical capillary endothelial cells.

(21), the catalytic subunit of PK-A purified from porcine skeletal muscle (22), or bovine lung casein kinases I and II (18, 19). PK-C assays were performed in 20 μl of 10 mM Tris·HCl (pH 7.5) containing 10 μM [γ-^{32}P]ATP (1500 cpm/pmol), 10 mM $MgCl_2$, 0.6 mM $CaCl_2$, 40 μg of phosphatidylserine per ml, 0.8 μg of dioctanoylglycerol per ml; and 1 μg of PK-C per ml. PK-A assays were performed in 20 μl of 10 mM Tris·HCl (pH 7.5) containing 5 μM [γ-^{32}P]ATP (3000 cpm/pmol), 10 mM $MgCl_2$, and 3 μg of the catalytic subunit of PK-A per ml. Casein kinase assays were performed in 20 μl of 10 mM Tris·HCl (pH 7.5) containing 10 μM [γ-^{32}P]ATP (3000 cpm/pmol), 50 mM $MgCl_2$, and either 10 μg of CK-I or 4 μg of CK-II per ml. The reactions were stopped with Laemmli sample buffer, and the phosphorylated proteins were separated by 15% or 20% SDS/PAGE (22) on a mini-slab-gel apparatus (Idea Scientific, Corvallis, OR) and visualized by overnight autoradiography.

Phosphoamino Acid Analysis and Tryptic Mapping. Radiolabeled peptides were extracted from the polyacrylamide gel in 0.05 M ammonium bicarbonate (pH 7.3–7.6) supplemented with 0.1% SDS and 1% 2-mercaptoethanol. After precipitation with 50% trichloroacetic acid, the pellet was dissolved in 6 M HCl, and the protein was hydrolyzed for 60 min at 110°C. Phosphoamino acids were separated by two-dimensional high-voltage electrophoresis as described (23). The plates were run for 20 min at 1.5 kV in pH 1.9 buffer in the first dimension and then for 16 min at 1.3 kV in pH 3.5 buffer in the second dimension.

Phosphorylation of Basic FGF by Cells in Culture. Three 10-cm dishes of SK-HEP-1 hepatoma cells and adrenocortical capillary endothelial cells (ACE cells) (21 × 10^6 cells) were labeled overnight at 37°C in phosphate-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% dialyzed calf serum, 0.5% (SK-HEP-1) or 5% (ACE) normal DMEM, and 0.25 μCi (SK-HEP-1) or 0.40 μCi (ACE) of [^{32}P]orthophosphate per ml. At the end of the labeling period, cells were washed twice with phosphate-buffered saline and lysed in radioimmunoprecipitation assay (RIPA) buffer (24) for 30 min at 4°C. After centrifugation in an Eppendorf Microfuge, the supernatants were immunoprecipitated by using the polyclonal antibody 773 (1:200) in the presence or absence of 5 μg of basic FGF. Twenty-five microliters of protein A-Sepharose suspension (50% in RIPA buffer) was added to the tube and mixed for 30 min. At the end of this incubation, the solution was centrifuged, and the pellet was washed three times with RIPA buffer, resuspended in Laemmli sample buffer and analyzed by 15% SDS/PAGE (22).

Biological Assays of FGF Activity. Basic FGF was phosphorylated with PK-C or the catalytic subunit of PK-A in the presence of unlabeled ATP under the conditions described above. Controls consisted of basic FGF treated with an identical incubation, but in the absence of enzyme. The phosphorylated and nonphosphorylated mitogens were tested for their capacity to bind the FGF receptor by using the BHK cell assay described by Moscatelli (25). Their effects on the proliferation of capillary endothelial cells was performed as described (26), and cell numbers were determined with a Coulter Counter.

RESULTS

Acidic and Basic FGFs Are Substrates for Protein Phosphorylation. Human basic FGF was phosphorylated by both PK-C and the catalytic subunit of PK-A (Fig. 1). In the absence of substrate, there was clear self-phosphorylation of PK-C, PK-A, and casein kinase II (lanes 1, 2, and 4), and as expected (19, 21) casein kinase I showed little, if any, self-phosphorylation (lane 3). Basic FGF was a good substrate for PK-C (lane 5) as shown by the appearance of an intense radiolabeled band corresponding to 18 kDa. This

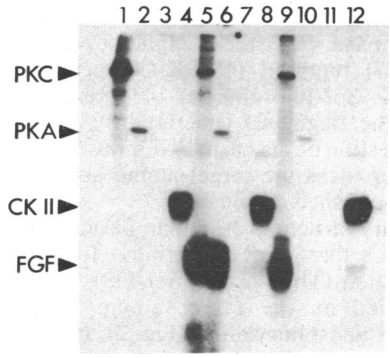

FIG. 1. Phosphorylation of recombinant acidic FGF and basic FGF by various purified protein kinases: Enzymes alone (lanes 1–4) or with 0.5 μg of basic (lanes 5–8) or acidic (lanes 9–12) FGF were tested for their protein kinase activity. Purified PK-C (lanes 1, 5, and 9), PK-A (lanes 2, 6, and 10), casein kinase I (lanes 3, 7, and 11) or casein kinase II (CK II) (lanes 4, 8, and 12) were incubated under the standard phosphorylation conditions described in *Materials and Methods*, and the radiolabeled proteins were separated by SDS/15% PAGE and visualized by autoradiography. Arrowheads show the position of the autophosphorylated enzymes and of the phosphorylated growth factor.

phosphorylation was also shown to be strongly phospholipid dependent (not shown). Similar results shown in lane 6 of Fig. 1 establish that human basic FGF is a good substrate for PK-A as well. There was no evidence that basic FGF is a substrate for casein kinase I (lane 7), and only trace amounts of the mitogen were phosphorylated by casein kinase II (lane 8). An analysis of the results with acidic FGF are presented in lanes 9–12. While human acidic FGF was a substrate for PK-C-dependent phosphorylation (lane 9), PK-A and casein kinase I were ineffective (lanes 10 and 11), and casein kinase II had a small but negligible effect (lane 12).

Identification of the Site of Phosphorylation. Phosphoamino acid analysis of radiolabeled human basic FGF revealed the presence of phosphoserine after incubation with PK-C and phosphothreonine after incubation with PK-A (Fig. 2). Thus, the amino acid target sites phosphorylated by PK-C and

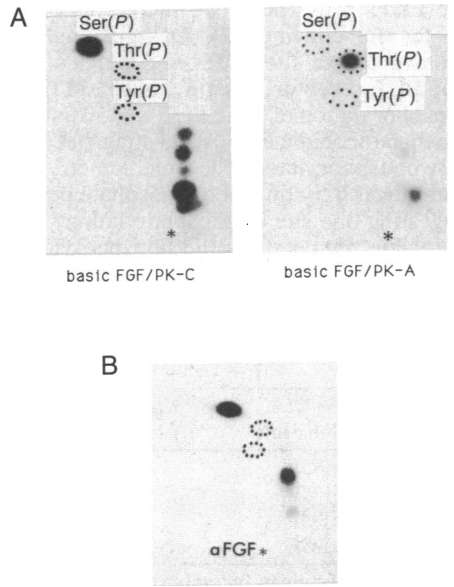

FIG. 2. Identification of the amino acids targeted by PK-C and PK-A. Recombinant human basic FGF was phosphorylated by PK-C or PK-A, and recombinant acidic FGF was phosphorylated by PK-C. Radiolabeled amino acids were identified by two-dimensional electrophoresis after partial hydrolysis in 6 M HCl. PK-A has no effect on acidic FGF and was not analyzed.

PK-A in human basic FGF are distinct. Three sites in the sequence of basic FGF are compatible with the consensus sequence (27) required for PK-C-dependent phosphorylation: Ser-64, Ser-108, and Ser-143. Reverse-phase HPLC analysis of the fragments generated by *Staphylococcus* V_8 protease digestion of the basic FGF phosphorylated by PK-C identified Ser-64 as the target amino acid for this enzyme (J.-J.F., unpublished data).

The primary structure of human basic FGF contains two potential sites that meet the criteria for PK-A-dependent phosphorylation (Thr-112 and Ser-113). Because threonine was identified as the target amino acid for the PK-A-dependent phosphorylation (Fig. 2), it is possible to propose Thr-112 as the targeted amino acid for PK-A. This is further supported by the observation that bovine basic FGF, in which Thr-112 has replaced Ser-112, was phosphorylated by PK-A on a target serine rather than threonine (result not shown). It is particularly interesting to note that this PK-A site (Thr-112) is located in the receptor-binding domain of basic FGF (28) and that all forms of basic FGF (human, bovine, ovine, rat, and frog) contain this potential site of phosphorylation. This is in contrast to the other members of the FGF family including acidic FGF (2) and the proteins encoded by the oncogenes *int-2* (29), *FGF-5* (30) and *hst/ks* (31, 32).

Specificity of FGF Phosphorylation. Several protein kinases were tested for their ability to phosphorylate acidic and basic FGFs in an effort to establish the possible specificity of PK-A and PK-C. Casein kinases I and II, enzymes that usually prefer acidic substrates (18, 21), did not significantly catalyze the phosphorylation of either mitogen (Fig. 1). The tyrosine kinase of the epidermal growth factor (EGF) receptor was also unable to phosphorylate basic FGF (C. Cochet, personal communication), although gag-fps, on oncogenic fusion protein with broad protein tyrosine kinase activity (provided to us by K. Gould, Salk Institute), was able to phosphorylate basic FGF and, to a larger extent, acidic FGF (not shown).

Kinetics of Phosphorylation. The K_m and V_{max} values were deduced from Lineweaver–Burke analyses of the reaction, and are shown in Table 1. The K_m values (1.5 and 3.4 μM) measured for the PK-C-dependent phosphorylation of basic and acidic FGFs rank these two factors among the better substrates for this enzyme (27). As an example, EGFR1, a peptide analog of the site of PK-C-dependent phosphorylation in the EGF receptor, has a K_m of 15 μM (21). The V_{max} for the phosphorylation of basic FGF was 50 times greater for basic FGF than for acidic FGF, suggesting that it can be more extensively phosphorylated. The stoichiometry of phosphate incorporation was 0.5–0.6 mol of phosphate per mol of basic FGF, suggesting one site of phosphorylation.

The K_m value obtained for the phosphorylation of basic FGF by PK-A (17 μM) ranks basic FGF among the better substrates for this kinase (33). Although the rate (V_{max}, 0.06 nmol/min per mg) of this reaction is relatively low, 0.8–1.2 mol of phosphate are incorporated per mol of basic FGF. Acidic FGF is not a substrate for this enzyme.

Table 1. Kinetic parameters of the phosphorylation of FGFs by PK-C and PK-A

Protein kinase	Substrate	K_m, μM	V_{max}, nmol/min per mg
PK-C	bFGF	1.5	18
	aFGF	3.4	0.37
PK-A	bFGF	17.0	0.06
	aFGF	NS	NS

Various amounts of human recombinant acidic FGF (aFGF) and basic FGF (bFGF) were phosphorylated for 15 min at 30°C in the presence of [γ-^{32}P]ATP (10 μM; 1500 cpm/pmol) and PK-C or PK-A under the conditions described in Fig. 1. Radioactivity incorporated into the trichloroacetic acid-precipitable proteins was determined and corrected for the autophosphorylation of the kinase. The kinetic parameters were calculated from Lineweaver–Burke double-reciprocal plots of the results. NS, not a substrate.

Basic FGF Exists as a Phosphorylated Protein in Cells Grown in Culture. The hepatoma cell line SK-HEP-1 and bovine ACE cells were selected for analyses because they synthesize basic FGF (7, 8, 34). Detergent extracts of the phosphate-labeled cells were immunoprecipitated with a specific anti-basic FGF antibody (35, 36), and two major radiolabeled bands were detected by autoradiography after electrophoresis (Fig. 3). In each instance, one of the bands could be identified as being basic FGF by virtue of antibody specificity, the estimated molecular weights of the radiolabeled bands (16–18 kDa), and their displacement by the addition of unlabeled FGF during the immunoprecipitation. The phosphorylated basic FGF synthesized by ACE cells was further characterized by showing that it could be extracted with 2 M NaCl and eluted with a characteristic 1.4–1.6 M NaCl from the heparin-Sepharose column (not shown). No radiolabeled basic FGF was detected in conditioned media.

Effects of Phosphorylation on the Biological Activities of Basic FGF. No differences were found when PK-C-phosphorylated basic FGF was tested on capillary (Fig. 4*A*) or vascular (not shown) endothelial cell proliferation assays. There also were no effects on thymidine incorporation into 3T3 cells (not shown). Because each of these assays are long-term assays consisting of incubations of 24–96 hr, this basic FGF was also tested in a radioreceptor assay (Fig. 4*B*). There were no differences between this form of phosphorylated basic FGF and the recombinant material as determined by their capacity to displace labeled basic FGF bound to either high- or low-affinity sites on BHK cells. There were also no effects on the binding of basic FGF to immobilized heparin (not shown).

It was of particular interest to determine the effects of PK-A-phosphorylated FGF because its target amino acid (Thr-112) is located in the receptor-binding domain of basic FGF (28). As expected, the time course and experimental conditions of the proliferation (Fig. 4*C*) and mitogenesis (not shown) assays precluded any detection of difference between the unphosphorylated and PK-A-phosphorylated basic FGF. In contrast, the phosphorylated FGF was 3–8 times more potent at displacing ^{125}I-labeled FGF binding to its receptor than the unphosphorylated recombinant basic FGF. The effect of PK-A-phosphorylated FGF was shown to be specific for the high-affinity receptor because the displacement of FGF bound to cell-associated glycosaminoglycans was not different between the phosphorylated and unphosphorylated

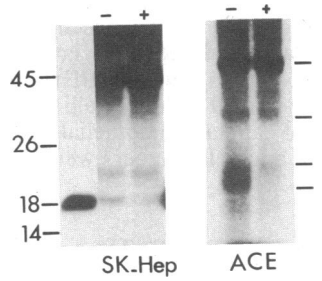

FIG. 3. Phosphorylation of basic FGF in cultured bovine ACE cells and human hepatoma SK-HEP-1 cells. Immunoprecipitates from ^{32}P-labeled cells were prepared as discussed, analyzed by SDS/15% PAGE, and visualized by autoradiography. Immunoprecipitations were performed in the absence (−) or in the presence (+) of an excess of nonradioactive basic FGF. The position of the molecular weight standards ($\times 10^{-3}$) is shown on both sides of the figure, and radioiodinated recombinant FGF is shown in the far-left lane.

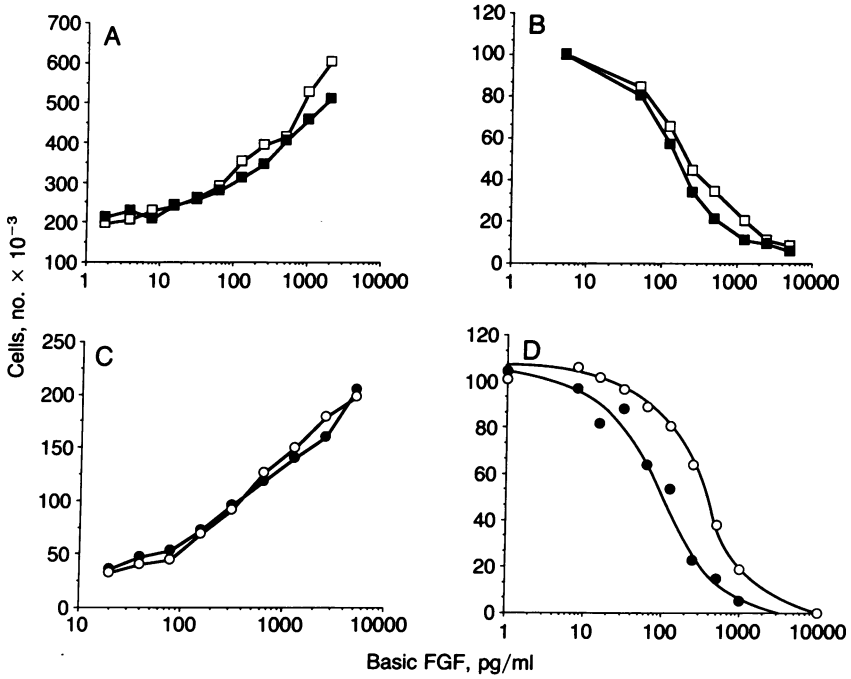

FIG. 4. Biological activities of phosphorylated basic FGFs. Recombinant human basic FGF was phosphorylated (■) by PK-C and tested for its capacity to stimulate the proliferation of ACE cells (*A*) and for its ability to displace ^{125}I-labeled FGF binding to its receptor in BHK cells (*B*). Basic FGF was phosphorylated by PK-A (●) and tested for its capacity to stimulate the proliferation of ACE cells (*C*) and to displace ^{125}I-labeled FGF binding to the FGF receptor on BHK cells (*C*). In each instance, control samples (□, ○) were treated under identical conditions, but in the absence of enzyme.

basic FGFs. Accordingly, the affinity of the phosphorylated FGF for immobilized heparin was also found to be unchanged by phosphorylation (not shown). We also exploited the fact that the site of phosphorylation of basic FGF by PK-A can be modified by heparin so that the enzyme phosphorylates Ser-64 rather than Thr-112 (37). Under these conditions, there were no differences in any of the assays between the phosphorylated and unphosphorylated forms of basic FGF (data not shown).

Growth Factors Are Substrates for Phosphorylation. TGF-α, TGF-β, and IGF-I were substrates for PK-C-mediated phosphorylation (Fig. 5*A*). Not all growth factors were substrates; however, EGF, PDGF, and NGF were either poorly or not-at-all phosphorylated by this kinase. In the case of PK-A, TGF-α and TNF were substrates for phosphorylation, but EGF, PDGF, and NGF were not. The spectrum of growth factors phosphorylated by different kinases varied presumably by virtue of the presence of consensus phosphorylation sequences. Structurally related factors exhibited very different substrate specificities. Basic FGF and TGF-α were phosphorylated by both PK-C and PK-A, yet the structural homolog acidic FGF, which has 55% structural identity with basic FGF, was not phosphorylated by PK-A, and EGF, which is 33% homologous to TGF-α, was not phosphorylated by either kinase.

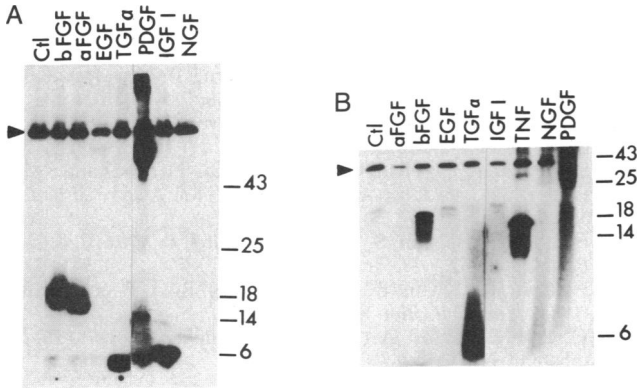

FIG. 5. Phosphorylation of growth factors by purified PK-C (*A*) and PK-A (*B*). The growth factors indicated (0.5 μg per lane) were phosphorylated as described in the text. The reactions were stopped by the addition of Laemmli sample buffer, and the phosphorylated proteins were visualized by autoradiography after separation by SDS/15% PAGE (*A*) or SDS/20% PAGE (*B*). Positions of the molecular weight markers (shown × 10^{-3}) are indicated in the far-right lane. Arrowheads indicate the position of the autophosphorylated protein kinases. Because PDGF was stored in the presence of bovine serum albumin, only 0.1 μg of this factor was used, and the phosphorylation of bovine serum albumin can be detected as a 67-kDa band. Ctl, control; bFGF, basic FGF; aFGF, acidic FGF.

DISCUSSION

It is not known at what step in its synthesis basic FGF is phosphorylated or in fact what enzymes are responsible for the phosphorylation of basic FGF in intact cells. Attempts to identify the amino acid phosphorylated *in vivo* have so far failed because of the small amounts of growth factor made by the cells (7–10) and the low recoveries from immunoprecipitation, peptide hydrolysis, and two-dimensional electrophoresis. The availability of transfected cells with a high expression of basic FGF (11, 12, 38) should alleviate this problem.

Little, if anything, is known about the mechanisms that regulate synthesis, secretion, and bioavailability of growth factors, and the results presented here suggest that it will be important to consider the possible roles of phosphorylation. In the case of basic FGF, one such role includes targeting to specific subcellular organelles. The metabolism of basic FGF after binding to its receptor is different from many other growth factors (39). It is specifically metabolized to three long-lived fragments that continue to retain their capacity to bind heparin. Bouché *et al.* (40) have suggested that it is targeted to and accumulates in the nucleolus after binding to its receptor on the plasma membrane. Whether this transport is kinase dependent is not known. With the observation that FGFs have no obvious signal sequence, remain cell-associated, and may not be secreted in a classical sense (1, 2, 7), the targeting of these mitogens to specific locations for processing in the cell may be of particular importance.

The observation that the PK-A-phosphorylated basic FGF has a greater capacity to displace FGF from its receptor supports the notion that it possesses a higher affinity for the FGF receptor. If this is the case, then this posttranslational change may well contribute to the regulation of FGF activity.

Increased binding of FGF to its receptor has recently been proposed as one of the mechanisms that might enable cells to recruit FGF from the extracellular matrix. Thus, it is interesting to speculate that protein kinases mediate the release of FGFs from the basement membranes. This notion is supported by the work of Plouët *et al.* (17), who recently have demonstrated that the release of acidic FGF from retinal rod outer segment membranes requires ATP-dependent phosphorylation. Any involvement of protein kinases would invoke the existence of these enzymes either in the extracellular milieu or associated with the outer plasma membrane.

The results presented here may have several important implications for our understanding of the mechanisms regulating growth factor activity. Because basic FGF, and possibly other growth factors, are phosphorylated in intact cells, their biological activities, bioavailability, and/or specificities may well be different from the unphosphorylated forms currently being examined in experimental models. The failure to establish that the phosphorylation of basic FGF results in modified activities in the proliferation and mitogenic assays most likely reflects the lability of this posttranslational modification. This supports a potential role for ectokinases and ectophosphatases in regulating the biological activities of basic FGF. The balance between kinases and phosphatases would effectively force a local action for phosphorylated basic FGF. Because the site of phosphorylation of basic FGF by PK-A is modified by heparin (37) and this site of phosphorylation is required for increased receptor binding, protein kinase action is ineffective at modifying FGF activity when it is bound to glycosaminoglycans in the extracellular matrix. The generation of basic FGF free of any heparin or related glycosaminoglycans will be prerequisite to observing any effect of PK-A on basic FGF *in vivo* and, thus, supports the potential role of heparinases in regulating FGF activity (8).

It is important to note that the experimental parameters examined here represent only three (binding, mitogenesis, and proliferation) effects of basic FGF. It will be of importance to establish whether the phosphorylation of FGF modulates cell differentiation (41) and cell adhesion (42). The phosphorylation of potentially cytoplasmic and certainly cell-associated growth factors like the FGFs may well provide a critical regulatory step on signal transduction and any one of their many diverse biological end points.

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