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Phosphorylation of Basic Fibroblast Growth Factor by Purified Protein Kinase C and the Identification of a Cryptic Site of Phosphorylation

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We have further characterized the protein kinase C (PK-C) dependent phosphorylation of basic fibroblast growth factor (FGF). Intact recombinant basic FGF and a series of ten peptide fragments of basic FGF were phosphorylated by PK-C and the products were analyzed by SDS-PAGE and autoradiography. As expected, peptide fragments containing the known site of phosphorylation (Ser^{64}) are substrates for phosphorylation. Surprisingly however, peptides containing the receptor binding domain of the mitogen [basic FGF(106-115)] are also phosphorylated. An examination of this sequence reveals the presence of a consensus sequence (Ser^{108}-Ala^{109}-Lys^{110}) that mediates the reaction. Accordingly, all peptides that contain the core amino acids basic FGF(106-111) are substrates for phosphorylation. Peptide mapping of basic FGF confirms that Ser^{64} is the primary site of phosphorylation, suggesting that Ser^{108} is a cryptic consensus sequence. Because basic FGF is metabolized to sequence specific fragments after its binding and internalization into target cells, this cryptic site may in fact be phosphorylated *in vivo*.

Basic fibroblast growth factor (FGF) is a pluripotent mitogen that is characterized by the large number of its biological activities *in vitro* and *in vivo*, by its capacity to bind immobilized heparin, and by the fact that it appears to be regulated by its sequestration in the extracellular matrix (1-4). Because it is a potent angiogenic factor that has been associated with normal embryonic growth and development, solid tumor growth, the complications of diabetes and angiofibromas, there has been considerable interest in identifying the mechanisms that regulate its activity. We recently identified one potential mechanism when we reported that basic FGF is synthesized as a phosphoprotein (5,6).

The phosphorylation and de-phosphorylation of proteins has long been recognized as a potential mechanism to regulate biological activity (7). It has only been recently recognized, however, that growth factors themselves are substrates for this process (5). Both acidic and basic FGFs are phosphorylated by the phospholipid dependent protein kinase C (PK-C) although only basic FGF is phosphorylated by the cAMP-dependent kinase. Epidermal growth factor-related mitogens, transforming growth factor-β and the insulin-like growth factors are also substrates for phosphorylation by the purified kinases. Of these however, only acidic and basic FGF have been shown to be phosphorylated by intact cells *in vivo* .

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Recent studies by several investigators have suggested that basic FGF may also exist as a cytoplasmic protein that is translocated to the nucleus (8-10). Furthermore, Bouché *et al.* (11,12) have found that, through some unknown mechanism, basic FGF is transported to the nucleolus. In the present study, we further characterize the phosphorylation of basic FGF by PK-C. We establish the existence of a cryptic site of phosphorylation in a sequence adjacent to the receptor binding domain of basic FGF. Because this phosphorylation reaction is only detectable in peptide fragments of basic FGF, the results are discussed in light of the observation that basic FGF is metabolized to sequence specific and long lived peptide fragments after its internalization into mesenchymal (13) and neuronal (14) cells; these fragments may thus be intracellular substrates for PK-C *in vivo*.

Material and Methods

Materials: PK-C (purified from bovine brain) was a generous gift of Dr. G. Walton. [γ^{32}P]-ATP was purchased from ICN Biochemicals (Irvine, CA) and recombinant basic FGF was a gift from Dr. Paolo Sarmientos, Farmitalia-Carlo Erba (Milan, Italy). Trypsin-TPCK was purchased from Worthington (Freehold, NJ) and all other biochemicals purchased from Sigma (St Louis MO). The solid phase synthesis of the peptides was performed as described (15,16). Peptides were purified to homogeneity, verified by reverse phase HPLC and their identity confirmed by amino acid analyses. The amino acid numbering system used is based on the sequence reported by Esch *et al.* (17).

Phosphorylation assays: Basic FGF and the indicated peptide fragments were phosphorylated by purified PK-C as described (5). Briefly, assays were performed in 20 ul of 15 mM Tris-HCl, pH 7.5 containing 84 ng of enzyme, 1 μCi of labelled ATP, 10mM $MgCl_2$ and 0.6 mM $CaCl_2$. Sonicated phospholipids (final concentration of 40 ug/ml phosphatidyl serine and 0.8 ug/ml diacylglycerol) were added as indicated. The assays were performed for 15 minutes at 37°C and the reaction was terminated by the addition of Laemmli's sample buffer (18). Phosphorylated proteins were visualized by autoradiography after SDS-PAGE.

Phosphoamino acid analyses: Phosphorylated peptides and proteins were prepared for phosphoamino acid analyses as described by Cooper *et al.* (19). After extraction from the dried gel and TCA precipitation, the phosphoproteins were hydrolyzed in 6N HCl for 90 min. at 110°C. The phosphoamino acids were separated by high voltage electrophoresis on cellulose plates at pH 1.9 and pH 3.5 and the separation visualized by autoradiography. Unless indicated otherwise, 500 cpm (Cerenkov) were applied to each plate.

Tryptic mapping: Peptides and proteins were prepared for tryptic mapping as described by Hunter and Sefton (20). After extraction from the dried gel and TCA precipitation, the phosphoprotein was solubilized and hydrolyzed with trypsin-TPCK. The tryptic fragments were then separated on cellulose thin layer plates by electrophoresis at 1.1 kV for 25 min. (pH 1.9) followed by ascending chromatography in the second dimension. The peptide fragments were visualized by autoradiography.

RESULTS

Basic FGF is phosphorylated in vitro by PK-C on a serine residue: Human recombinant basic FGF is phosphorylated in a phospholipid-dependent manner by purified preparations of PK-C (Fig. 1A). When the phosphorylated basic FGF is extracted and acid hydrolyzed, a two-dimensional separation of the phosphoamino acids reveals that the radioactive phosphate is incorporated exclusively into serine (Fig. 1B). This residue has been identified# in a previous report (6) as Ser ^{64}.

#The amino acid numbering system used is based on the sequence reported by Esch *et al.* (17).

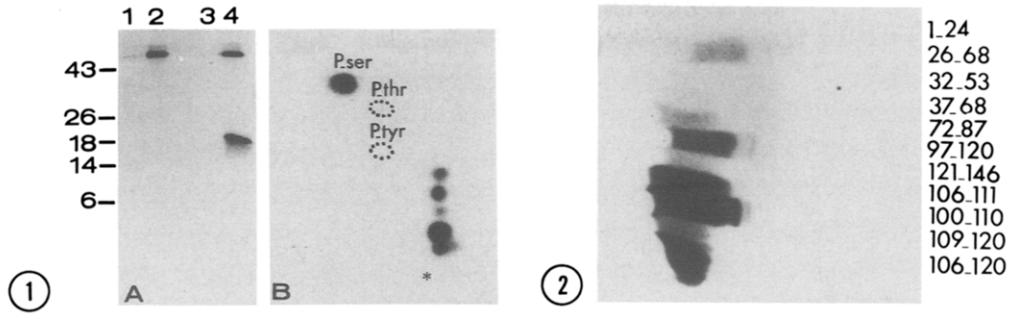

Figure 1. Phosphorylation of basic FGF by PK-C. **PANEL A**: The phosphorylation of human recombinant basic FGF was performed under the conditions described in Material and Methods. Lane 1: PK-C, no phospholipids, no basic FGF; Lane 2: PK-C, phospholipids, no basic FGF; Lane 3: PK-C, no phospholipid, basic FGF; Lane 4: PK-C, phospholipids, basic FGF. The autoradiogram of the phosphorylated proteins was obtained after separation by 15% SDS-PAGE. The left lane shows the position of the molecular weight standards (kDa). **PANEL B:** Phosphoamino acid analysis of basic FGF phosphorylated by PK-C. The radioactive band corresponding to basic FGF (Fig. 1A, lane 4) was cut out of the gel, extracted and acid hydrolyzed. The phosphoamino acids were separated as described in Material and Methods and the ^{32}P-labelled amino acids were visualized by autoradiography. The circles indicate the position of the standard unlabeled phosphoamino acids: phosphoserine (P-ser), phosphothreonine (P-thr) and phosphotyrosine (P-tyr).

Figure 2. Phosphorylation of basic FGF fragments by PK-C. 5 μg of the indicated peptides were phosphorylated as described in Materials and Methods and analyzed by 25% SDS-PAGE. The picture represents the autoradiogram of the corresponding gel.

Phosphorylation of basic FGF-related peptide fragments by PK-C: We identified three potential target serines at positions Ser^{64}, Ser^{108}, and Ser^{143} that meet the criteria (21) to be considerd consensus sequences for PK-C phosphorylation (Ser/Thr-Xaa-Lys/Arg, where Xaa is usually an uncharged amino acid). Additional basic residues, amino or carboxyl to the target amino acid enhance the Vmax and Km of the phosphorylation reaction (21), suggesting that Ser^{108} (which is in the sequence **Arg-Ser**-Ala-**Lys**) is a better candidate than Ser^{64} (Val-**Ser**-Ile-**Lys**) or Ser^{143} (Met-**Ser**-Ala-**Lys**). These predictions were confirmed by experiments with synthetic peptide fragments of basic FGF. Peptides containing Ser^{108} such as basic FGF(97-120), basic FGF(106-111) and basic FGF(106-120) are the most extensively phosphorylated among 11 different basic FGF fragments tested in the phosphorylation assay (Fig. 2).

The same series of peptides was also tested as competitive inhibitors for the phosphorylation of basic FGF by PK-C. As shown in Fig. 3, basic FGF(97-120) and basic FGF(106-111) are the most effective competitors for basic FGF phosphorylation and are also inhibitors of PK-C autophosphorylation. The fragments basic FGF(22-68) and basic FGF(37-68) both contain the known target residue Ser^{64} but are much less potent as competitors for basic FGF phosphorylation.

Comparisons with the site phosphorylated in the intact basic FGF molecule and in the peptide fragments: To confirm the site phosphorylated by PK-C in intact basic FGF, we performed a comparative tryptic mapping analysis of basic FGF and peptides (97-120) and (106-111) after their phosphorylation by PK-C (Fig. 4). Trypsin digestion of the basic FGF fragments generated a common [^{32}P]-labeled peptide which

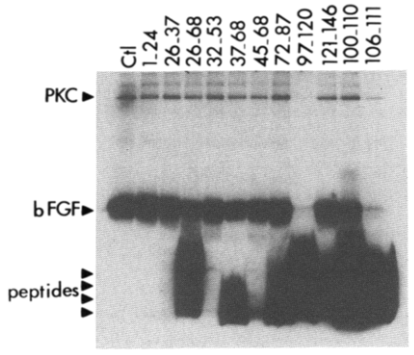

Figure 3. Phosphorylation of basic FGF by PK-C in the presence of basic FGF fragments. 0.5 μg basic FGF was phosphorylated by purified PKC in the absence (Ctl) or in the presence of 10 μg of the indicated basic FGF peptide fragments. The reaction was performed as described in Materials and Methods and the phosphoproteins were then analyzed by 15% SDS -PAGE and autoradiography. The arrows indicate the position of PK-C, basic FGF and the peptides.

migrated very low in the second dimension (ascending chromatography). In the case of peptide basic FGF(106-111), an additional peptide was observed which migrated less in the first dimension (high voltage electrophoresis at pH 1.9), but had the same Rf in the second dimension. On the basis of trypsin specificity, digestion of the peptide basic FGF(106-111) (Tyr-Arg-**(Phospho)Ser**-Arg-Lys-Tyr) generates two related phosphopeptides: **(Phospho)Ser**-Arg and **(Phospho)-Ser**-Arg-Lys. In contrast, digestion of basic FGF(97-120) generates only the most basic peptide **(Phospho)Ser**-Arg-Lys.

When intact basic FGF is phosphorylated by PK-C and analyzed under identical conditions, two phosphopeptides are detected (Fig. 4C). None of these peptides co-migrate with the peptides generated from phosphorylated basic FGF(106-111) or basic FGF(97-120). Instead, they migrate higher in the second dimension with the increased hydrophobicity predicted for the sequence surrounding Ser^{64}. Thus, although Ser^{108} is an excellent potential site of phosphorylation by PK-C, intact basic FGF is phosphorylated on a different residue by

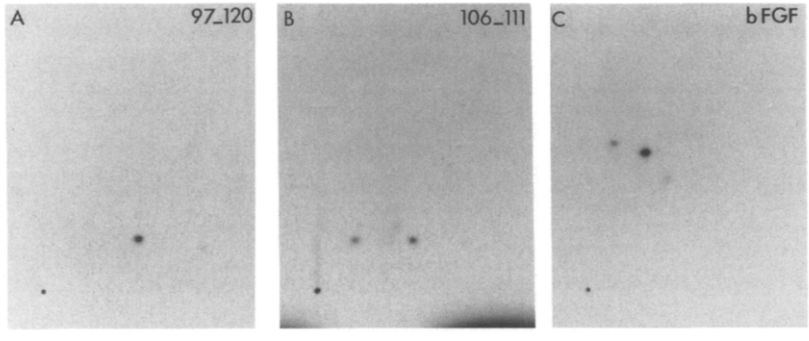

Figure 4. Comparative tryptic mapping of basic FGF(97-120), basic FGF(106-111) and intact basic FGF after phosphorylation by PK-C. Basic FGF and basic FGF(97-120) and basic FGF(106-111) were phosphorylated *in vitro* by PK-C. After analysis by 15% SDS-PAGE, the phosphopeptides were extracted from the gels and digested with trypsin. Tryptic peptides (500 Cerenkov cpm of each digest) were separated by two-dimensional analysis as described in Materials and Methods and visualized by autoradiography.

this kinase. In view of these differences, these results support the notion that, due to constraints in the tertiary structure of basic FGF, Ser^{108} is inaccessible to the kinase.

DISCUSSION

PK-C is able to phosphorylate protein substrates on serine and/or threonine residues. We recently demonstrated that several growth factors, including acidic and basic FGF are substrates for PK-C *in vitro* (5). We show here that phosphorylation of basic FGF occurs exclusively on a serine residue. Among the nine serine residues present in basic FGF, three of them present the appropriate environment to be considered potential targets of PK-C: Ser^{64} Ser^{108} and Ser^{143}. The additional arginine residues at both the C- and N-terminal sites of Ser^{108} increase the probability of this residue being a preferential site of phosphorylation in the intact growth factor. This, however, was not found to be the case. While peptides containing Ser^{108} are the best substrates for PK-C *in vitro* and the best inhibitors of basic FGF phosphorylation, intact basic FGF molecule is phosphorylated at a different site (Ser^{64}).

These observations indicate that the use of peptide fragments for the identification of sites of phosphorylation is misleading if there exist cryptic consensus sequences. Specifically, Ser^{64} which is weakly phosphorylated in basic FGF fragments, is the site of phosphorylation in intact basic FGF. Under the conditions of the experiments described here, basic FGF is thus presumably folded in a structural conformation that precludes phosphorylation of Ser^{108}. This is not to say that these findings preclude a role for the phosphorylation of basic FGF at this site. Indeed, there may exist experimental and/or physiological conditions where this site may be unmasked and thus becomes a target for PK-C dependent phosphorylation. As an example, the amino acid substrate of the cAMP-dependent phosphorylation of basic FGF can be directed by the interaction between basic FGF and heparin (6). Accordingly, in instances where metabolic fragments of basic FGF are generated, the smaller peptides may well be phosphorylated on Ser^{108}.

It is particularly interesting to note that after the binding and internalization of basic FGF to its high affinity receptor, it is internalized and metabolized to three distinct peptide fragments that are long lived *in vitro* (13,14) and *in vivo* (22,23). One of these peptides contains the basic FGF(106-120) epitope suggesting that it is a potential substrate for protein phosphorylation at Ser^{108}. Further studies will be required to establish if there exists a functional effect of this phosphorylation in the receptor binding domain of basic FGF and whether these peptide fragments are phosphorylated *in vivo*.

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