Covalent Linkage between Nucleotides and Platelet-derived Endothelial Cell Growth Factor*

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Platelet-derived endothelial cell growth factor (PD-ECGF) is a 45-kDa peptide mitogen which is present in platelets and placlenta and produced by certain cultured cell lines. Immunoprecipitation of A431 cells metabolically labeled with $^{32}$Porthophosphate revealed the incorporation of $^{32}$P radioactivity into PD-ECGF. Phosphoamino acid analysis showed that serine residues of PD-ECGF were phosphorylated in vivo. Forskolin, 12-O-tetradecanoylphorbol-13-acetate, and epidermal growth factor had no effect on the in vivo phosphorylation of PD-ECGF. Moreover, incubation of pure PD-ECGF with $\gamma$-$^{32}$P]ATP led to labeling of PD-ECGF. Optimal labeling was achieved by incubation at 95°C for 5 min in the presence of sodium dodecyl sulfate, dithiothreitol, and Mg2+ or Mn2+. PD-ECGF was also labeled with [2,8-$^3$H]ATP, [2,5',8-$^3$H]ATP, or [2-$^3$P]ATP. ATP and GTP were the preferred nucleotide substrates by comparison with other nucleotides and related components. Partial amino acid hydrolysis liberated a significant amount of O-$^{32}$Pphosphoserine from PD-ECGF labeled in vitro with $\gamma$-$^{32}$P]ATP. Furthermore, $^{32}$P-radiolabeled nucleotides were released after snake venom phosphodiesterase or piperidine treatment from PD-ECGF labeled in vitro with [2-$^3$P]ATP or [2,7-$^3$P]ATP, as well as from PD-ECGF labeled in vivo with [2-$^3$P]orthophosphate. These data indicate that serine residues of PD-ECGF can be covalently linked to phosphate groups of nucleotides, resulting in a nucleotidylated protein. The functional significance of this post-translational modification remains to be determined.

Platelet-derived endothelial cell growth factor (PD-ECGF) stimulates the growth and chemotaxis of endothelial cells in vitro and angiogenesis in vivo (1). Its DNA sequence predicts a 482-amino acid protein without signal sequence which has no striking homology to other known proteins (1). PD-ECGF was initially purified from human platelets as a 45-kDa single chain polypeptide (2) and more recently from human placenta (3); no striking homology to other known proteins (1). PD-ECGF was also labeled with [2,8-$^3$H]ATP, [2,5',8-$^3$H]ATP, or [2-$^3$P]ATP. ATP and GTP were the preferred nucleotide substrates by comparison with other nucleotides and related components. Partial amino acid hydrolysis liberated a significant amount of O-$^{32}$Pphosphoserine from PD-ECGF labeled in vitro with $\gamma$-$^{32}$P]ATP. Furthermore, $^{32}$P-radiolabeled nucleotides were released after snake venom phosphodiesterase or piperidine treatment from PD-ECGF labeled in vitro with [2-$^3$P]ATP or [2,7-$^3$P]ATP, as well as from PD-ECGF labeled in vivo with [2-$^3$P]orthophosphate. These data indicate that serine residues of PD-ECGF can be covalently linked to phosphate groups of nucleotides, resulting in a nucleotidylated protein. The functional significance of this post-translational modification remains to be determined.

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1 The abbreviations used are: PD-ECGF, platelet-derived endothelial cell growth factor; DTT, dithiothreitol; EGF, epidermal growth factor; EGTA, [ethylenbis(oxyethylenenitrito)]tetracetic acid; FGF, fibroblast growth factor; PEI, polyethyleneimine; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulfate; TPA, 12-O-tetradecanoylphorbol-13-acetate.
Covalent Linkage between Nucleotides and PD-ECGF

RESULTS

Immunoprecipitation of \( ^{32}P \)Orthophosphate-labeled Cells with a PD-ECGF Antiserum—The human epidermoid carcinoma cell line A431, which produces PD-ECGF (4), was metabolically labeled with \( ^{32}P \)orthophosphate. After rapid cooling, cells were lysed in a buffer containing detergents and phosphatase inhibitors. The cell lysates were then incubated with PD-ECGF antiserum (18), in the presence or absence of unlabeled pure PD-ECGF, and the precipitates were analyzed by SDS-gel electrophoresis and autoradiography. Labeling of a 45-kDa component was noticed in the precipitate with the immune serum, but not with nonimmune serum (Fig. 1). This component was not seen when an excess amount of PD-ECGF was present during immunoprecipitation, indicating that \( ^{32}P \) radioactivity is incorporated in vivo into PD-ECGF.

Characterization of PD-ECGF \( ^{32}P \)-Labeled in Vivo—In order to characterize which type of linkage occurred between phosphate and PD-ECGF, aliquots of immunoprecipitates of cells labeled metabolically with \( ^{32}P \)orthophosphate were incubated at acidic or basic pH, or in hydroxylamine. Treatment with 0.1 M HCl for 1 h at 37 °C did not result in any significant loss of label as analyzed by SDS-gel electrophoresis and autoradiography (Fig. 2, lanes b and d). No label was lost after incubation with 0.2 M hydroxylamine at pH 7.5 (Fig. 2, lane h) or 3.86 M hydroxylamine at pH 4.75 for 30 min at 37 °C (data not shown). In contrast, most of the label was lost after incubation with 0.1 M NaOH for 1 h at 37 °C (Fig. 2, lane f).

Using 0.75 M KH₂PO₄, pH 4.3, as solvent (17). The chromatogram was then subjected to autoradiography. As standards were included \( ^{32}P \)orthophosphoric acid, as well as unlabeled ATP, ADP, and AMP, the latter visualized under UV light.

FIG. 1. Immunoprecipitation of a lysate of A431 cells labeled metabolically with \( ^{32}P \)Orthophosphate. A431 cells were metabolically labeled with \( ^{32}P \)orthophosphate, lysed, and then precipitated either with nonimmune serum (lane a) or with anti-PD-ECGF serum in the absence (lane b) or presence (lane c) of an excess amount of PD-ECGF. Samples were analyzed by SDS-gel electrophoresis and autoradiography, as described under “Experimental Procedures.”

As described by Blobel and Dobberstein (12) using 10–18% gradient polyacrylamide gels. The gels were run under reducing conditions at constant current overnight at room temperature. After completion of electrophoresis, the gels were fixed (except for when proteins later were to be electrotransferred from gels) in 10% trichloroacetic acid and then stained with Coomassie Brilliant Blue R-250, followed by destaining. Alternatively, for the experiment of the susceptibility of radiolabeled PD-ECGF, the gels were stained in 0.1% Coomassie Brilliant Blue R-250, in 25% isopropyl alcohol, adjusted to pH 7.4 by the addition of NaHCO₃, and then destained in 10% isopropyl alcohol, in order to fix and stain the gels at neutral pH. The gels were dried under vacuum and heat. Occasionally, the gels were treated with alkali (1 M KOH, 2 h, 55 °C) in order to hydrolyze most of the phosphoserine (14). Autoradiography and fluorography were performed with pre-exposed Fuji RX films using Du Pont Lightning Plus intensifying screens. Exposure was at −70 °C for 1–5 days. Alkalitreated gels were exposed 3 times as long as their untreated counterparts.

Phosphoamino Acid Analysis—Cells labeled with \( ^{32}P \)orthophosphoric acid were immunoprecipitated with PD-ECGF antiserum, as described above. Electrottransfer from gels to PVDF membranes was performed essentially as described (15). The 45-kDa PD-ECGF band was localized by autoradiography and cut out from the PVDF membrane. Partial amino acid hydrolysis and two-dimensional electrophoresis were performed as described (14). Briefly, the PVDF strip was exposed for 1 h at 110 °C to 6 M HCl, 0.1% phenol. After evaporation of HCl, two-dimensional separation of phosphoamino acids was performed on cellulose TLC plates by high voltage electrophoresis at pH 1.9 in the first dimension and electrophoresis at pH 3.5 in the second dimension. Unlabeled phosphoamino acids were included as internal markers and were visualized by ninhydrin staining. The electrophoretograms were analyzed by autoradiography after drying. Phosphoamino acid analysis was also performed on pure PD-ECGF labeled with [α-\(^{32}P\)]ATP or [γ-\(^{32}P\)]ATP, as described above.

Nucleotide Analysis—Radiolabeled PD-ECGF immobilized on PVDF membranes (see above) was cut out and incubated with snake venom phosphodiesterase or piperidine as described (16). After lyophilization, the samples were loaded onto PEI-TLC plates. Nucleotides were separated by one-dimensional ascending chromatography using 0.75 M KH₂PO₄, pH 4.3, as solvent (17). The chromatogram was then subjected to autoradiography. As standards were included \( ^{32}P \)orthophosphoric acid, as well as unlabeled ATP, ADP, and AMP, the latter visualized under UV light.

FIG. 2. Susceptibility of PD-ECGF labeled metabolically with \( ^{32}P \)Orthophosphate. A431 cells were metabolically labeled with \( ^{32}P \)orthophosphate, lysed, and then precipitated with nonimmune serum (lanes a, c, e, and g) or with PD-ECGF antiserum (lanes b, d, f, and h). The immunoprecipitates were then exposed to 0.1 M HCl (lanes c and d), 0.1 M NaOH (lanes e and f), or 0.2 M NH₄OH (lanes g and h) and analyzed by SDS-gel electrophoresis and autoradiography. For details, see “Experimental Procedures.”
Covalent Linkage between Nucleotides and PD-ECGF

The loss of the labeling of PD-ECGF also occurred when a gel of immunoprecipitates of cells metabolically labeled with $[^32P]_{\text{orthophosphate}}$ was soaked in $1 \text{ M KOH}$ for 2 h at 55 °C (data not shown). These data suggest that phosphate is O-linked to a serine residue in PD-ECGF and are inconsistent with the presence of O-phosphoester bonds to threonine or tyrosine residues, phosphoamidates, acyl phosphates, thio- phosphates (19), or ADP-ribosylation (20).

**Phosphoamino Acid Analysis of PD-ECGF $^{32P}$-Labeled in Vivo**—In order to verify more directly that PD-ECGF in intact cells contains serine phosphate, A431 cells were labeled with $[^32P]_{\text{orthophosphate}}$. A lysate from the cells was then treated with anti-PD-ECGF serum, and the precipitate was subjected to SDS-gel electrophoresis followed by electrotransfer to a PVDF membrane. The 45-kDa protein was localized by autoradiography, cut out from the PVDF membrane, and subjected to partial acid hydrolysis. Phosphoamino acids were then separated by electrophoresis at pH 1.9 in the first dimension, followed by electrophoresis at pH 3.5 in the second dimension. Analysis by autoradiography revealed that only O-phosphoserine was present (see below, Fig. 7 c).

**The Effect of Kinase Stimulants on the Phosphorylation of PD-ECGF in Vivo**—In order to investigate which kinases phosphorylate PD-ECGF, we examined the effect of various kinase stimulants, including TPA, which stimulates protein kinase C, forskolin, which stimulates AMP-dependent kinase, and EGF, which stimulates the tyrosine kinase activity of the EGF receptor overexpressed in A431 cells. Cells were metabolically labeled with $[^32P]_{\text{orthophosphate}}$ for 4 h. At the end of the labeling period, cells were incubated for 10 min in the presence of 200 ng/ml EGF, 16 μM TPA, or 400 μM forskolin. The lysates were immunoprecipitated with PD-ECGF-specific antisera, and the precipitates were analyzed by SDS-gel electrophoresis and autoradiography. However, no significant increase in labeling of the 45-kDa PD-ECGF component was seen after any of the treatments (data not shown).

In Vitro Phosphorylation of PD-ECGF—Since we were unable to modulate the phosphorylation of PD-ECGF in A431 cells, we attempted to establish an in vitro PD-ECGF phosphorylation assay in order to identify the kinases involved. In the presence of Mg$^{2+}$ and DTT, pure PD-ECGF was incubated with $[^\gamma-\text{P}]_{\text{ATP}}$ in the presence or absence of A431 cell lysate at 37 °C for 15 min. Samples were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Surprisingly, the control sample which contained no kinase source showed a labeling of the 45-kDa PD-ECGF (data not shown). In order to exclude the possibility that the PD-ECGF preparation contained a kinase as a contamination, pure recombinant PD-ECGF was first denatured with SDS in the presence or absence of DTT at 37 °C for 15 min or at 95 °C for 5 min. The denatured samples were then incubated with Mg$^{2+}$, DTT, and $[^\gamma-\text{P}]_{\text{ATP}}$ at 37 °C for 15 min. After addition of SDS-sample buffer, samples were analyzed by $[^\gamma-\text{P}]_{\text{ATP}}$ and with the following additions: 5 mM EGTA (lane a), 5 mM DTT (lane b), 5 mM DTT and 15 mM Mg$^{2+}$ (lane c), 5 mM DTT and 15 mM Mn$^{2+}$ (lane d), 5 mM DTT and 15 mM Ca$^{2+}$ (lane e), or 5 mM DTT and 15 mM Zn$^{2+}$ (lane f). Incubations were terminated by the addition of 100 mM EDTA; samples were analyzed by SDS-gel electrophoresis and autoradiography. C, pure PD-ECGF was incubated with $[^\gamma-\text{P}]_{\text{ATP}}$ in the presence of 5 mM DTT and 15 mM Mg$^{2+}$ during the following conditions: at 37 °C for 0 min (lane a) or for 30 min with (lane c) or without (lane b) 2% SDS. At 37 °C for 30 min without SDS followed by the incubation with SDS at 37 °C for 30 min with SDS (lane d), or at 95 °C for 5 min with SDS (lane e). Incubations were terminated by the addition of 100 mM EDTA; samples were then analyzed by SDS-gel electrophoresis and autoradiography. For details, see “Experimental Procedures.”

**Fig. 3.** Characterization of optimal conditions for the labeling of PD-ECGF. A, pure PD-ECGF was pretreated with 2% SDS at 37 °C for 15 min (lane a) or at 95 °C for 5 min (lane b) and then incubated further with $[^\gamma-\text{P}]_{\text{ATP}}$ at 37 °C for 15 min and analyzed by SDS-gel electrophoresis and autoradiography. B, pure PD-ECGF was incubated at 95 °C for 5 min in the presence of 2% SDS and $[^\gamma-\text{P}]_{\text{ATP}}$ and with the following additions: 5 mM EGTA (lane a), 5 mM DTT (lane b), 5 mM DTT and 15 mM Mg$^{2+}$ (lane c), 5 mM DTT and 15 mM Mn$^{2+}$ (lane d), 5 mM DTT and 15 mM Ca$^{2+}$ (lane e), or 5 mM DTT and 15 mM Zn$^{2+}$ (lane f). Incubations were terminated by the addition of 100 mM EDTA; samples were analyzed by SDS-gel electrophoresis and autoradiography. C, pure PD-ECGF was incubated with $[^\gamma-\text{P}]_{\text{ATP}}$ in the presence of 5 mM DTT and 15 mM Mg$^{2+}$ during the following conditions: at 37 °C for 0 min (lane a) or for 30 min with (lane c) or without (lane b) 2% SDS. At 37 °C for 30 min without SDS followed by the incubation with SDS at 37 °C for 30 min with SDS (lane d), or at 95 °C for 5 min with SDS (lane e). Incubations were terminated by the addition of 100 mM EDTA; samples were then analyzed by SDS-gel electrophoresis and autoradiography.
Characterization of the in Vitro Phosphorylation of PD-ECGF—The prerequisites for in vitro phosphorylation of PD-ECGF were investigated as follows. PD-ECGF was incubated with [γ-32P]ATP in the presence of SDS and other additions and then analyzed by SDS-gel electrophoresis and autoradiography. Incubation with EGTA, DTT, or Mg2+ at 24 °C for 15 min did not result in labeling of 45-kDa PD-ECGF (data not shown). In contrast, a significant amount of label was detected after incubation of PD-ECGF with DTT for 5 min at 95 °C (Fig. 3B, lane b). No labeling occurred without DTT (Fig. 3B, lane a), and the addition of Mg2+ or Mn2+ considerably increased the labeling (Fig. 3B, lanes c and d, respectively); Ca2+ or Zn2+ (Fig. 3B, lanes e and f, respectively) could not replace Mg2+ and Mn2+.

To further investigate the optimal phosphorylation conditions, we performed the following series of experiments. PD-ECGF was incubated with [γ-32P]ATP under reducing conditions in the presence of Mg2+, the incubation was then terminated by the addition of EDTA, and the samples were analyzed by SDS-gel electrophoresis and autoradiography. No labeling of 45-kDa PD-ECGF was detected in the absence of SDS, with or without the incubation at 37 °C for 30 min (Fig. 3C, lanes a and b). By addition of SDS during the incubation at 37 °C for 50 min, the labeling of PD-ECGF was seen (Fig. 3C, lane c). The labeling was increased further by incubation at 95 °C for 5 min (Fig. 3C, lane d). Preincubation without SDS at 37 °C did not result in an increase of the labeling (Fig. 3C, lane d). Addition of guanidine HCl instead of SDS did not result in the labeling of PD-ECGF (data not shown). Thus, the phosphorylation of PD-ECGF is promoted by denaturation of SDS at high temperature and is dependent on Mg2+ or Mn2+ and reducing conditions.

In Vitro Labeling of PD-ECGF with Different ATP Radioisotopes—Since PD-ECGF denatured by SDS at 95 °C was phosphorylated in vitro after incubation with [γ-32P]ATP, it is unlikely that PD-ECGF itself has kinase activity. Notably, however, the amino acid sequence of PD-ECGF contains two repeats of nucleotide binding motif (Gly-X-Gly) in amino acid sequence of PD-ECGF. Therefore, we examined if an intact ATP molecule can bind covalently to PD-ECGF. PD-ECGF was incubated at 95 °C for 5 min under reduced conditions in the presence of Mg2+ and SDS, either with [2,8-3H]ATP, [2,5',8-3H]ATP, [α-32P]ATP, or [γ-32P]ATP, and then analyzed by SDS-gel electrophoresis and fluorography. Radioactivity was found to be bound to PD-ECGF, regardless of in which part the ATP molecule was labeled (Fig. 5), indicating that ATP becomes covalently labeled in vitro with [γ-32P]ATP in the presence of a 1000-fold molar excess of various unlabeled nucleotides and related compounds as competitive inhibitors. Unlabeled ATP and GTP (Fig. 6A, lanes b and j) competed efficiently with [γ-32P]ATP, whereas ADP, UTP, and CTP partially inhibited the labeling (Fig. 6A, lanes c, k, and l). In contrast, AMP, cAMP, adenosine, adenine, pyrophosphate, and phosphate had only limited effects (Fig. 6A, lanes d–i). Deoxyribonucleotides and ribonucleotides were almost equally efficient competitors, since the radioactive labeling of PD-ECGF was inhibited to the same extent by dATP and dGTP (Fig. 6B, lanes b and d) as by ATP and GTP (Fig. 6B, lanes c and e). However, dCTP competed less efficiently than CTP (Fig. 6B, lanes f and g), and dTTP less efficiently than UTP (Fig. 6B, lanes i and h).

Phosphoamino Acid Analysis of PD-ECGF—Since a serine residue of PD-ECGF is phosphorylated in vivo as described above, it is possible that the linkage between the nucleotide and PD-ECGF formed in vitro is through a phosphodiester bond between a serine residue and a phosphate group of the nucleotide. To test this possibility, pure PD-ECGF was labeled with [α-32P]ATP or [γ-32P]ATP in the presence of Mg2+, SDS, and DTT. After SDS-gel electrophoresis, followed by electrotransfer to a PVDF membrane, the 45-kDa PD-ECGF was localized by autoradiography and cut out from the PVDF membrane. Partial amino acid hydrolysis liberated a significant amount of 32P-labeled phosphoserine from PD-ECGF labeled in vitro with [γ-32P]ATP (Fig. 7a), as well as from PD-ECGF labeled in vivo with [32P]orthophosphate as described above (Fig. 7c). In contrast, only trace amounts of O-[32P]phosphoserine were released from PD-ECGF labeled in vitro with [α-32P]ATP (Fig. 7b). The presence of small amounts of O-[32P]phosphothreonine in PD-ECGF labeled in vitro with [α-32P]ATP or [γ-32P]ATP was also detected. These data suggested that nucleotides can form a covalent linkage.
Covalent Linkage between Nucleotides and PD-ECGF

FIG. 6. Competition of in vitro [γ-32P]ATP labeling of PD-ECGF with various nucleotides and related compounds. Pure PD-ECGF was incubated with [γ-32P]ATP in the absence (lanes a in A and in B) or presence of a 1000-fold molar excess of unlabeled nucleotides and related components: in A, ATP (lane b), ADP (lane c), AMP (lane d), cAMP (lane e), adenine (lane f), adenosine (lane g), pyrophosphate (lane h), phosphate (lane i), GTP (lane j), and UTP (lane l); in B, ATP (lane b), dATP (lane c), GTP (lane d), dGTP (lane e), CTP (lane f), dCTP (lane g), UTP (lane h), and dTTP (lane i). Samples were analyzed by SDS-gel electrophoresis and autoradiography, as described under “Experimental Procedures.”

with serine residues via their phosphate groups at the γ-position. Other possibilities have, however, not been excluded, e.g. that PD-ECGF is phosphorylated on serine residues and in addition contains an ADP nucleotide covalently bound to a separate residue.

Nucleotide Analysis of in Vivo and in Vitro 32P-Labeled PD-ECGF—In order to further explore the possibility that nucleotides form covalently bound complexes with PD-ECGF, the following experiments were performed. PD-ECGF was labeled in vitro with [α-32P]ATP or [γ-32P]ATP. After SDS-gel electrophoresis and electrotransfer to a PVDF membrane, the 45-kDa PD-ECGF was localized by autoradiography and cut out from the PVDF membrane; the strips were then exposed to snake venom phosphodiesterase (data not shown) or to 0.5 M piperidine, both of which cleave phosphate bonds, and then analyzed by PEI-TLC. 32P radioactivity was found in free phosphate, ADP, and a significant amount in AMP from PD-ECGF labeled in vitro with [α-32P]ATP (Fig. 8, lane b), and

FIG. 7. Phosphoamino acid analysis of in vitro- and in vivo-labeled PD-ECGF. A431 cells were metabolically labeled with [32P]orthophosphate, solubilized, and subjected to immunoprecipitation with the anti-PD-ECGF-specific serum (c). In parallel, pure PD-ECGF was incubated with [α-32P]ATP or [γ-32P]ATP (b and a, respectively). Samples were size-fractionated by SDS-gel electrophoresis and electrotransferred to a PVDF membrane. The 45-kDa PD-ECGF was localized by autoradiography, cut out from the PVDF membrane, and subjected to partial amino acid hydrolysis. Two-dimensional separation of phosphoamino acid was performed by electrophoresis at pH 1.9 on cellulose TLC in the horizontal direction followed by electrophoresis at pH 3.5 in the vertical direction. Unlabeled phosphoamino acids were included as internal controls. The positions where samples were applied are marked (+), as well as orthophosphate (Pi), phosphoserine (PS), phosphothreonine (PT), and phosphotyrosine (PY). For details, see “Experimental Procedures.”

FIG. 8. Nucleotide analysis of in vitro- or in vivo-labeled PD-ECGF. A431 cells were metabolically labeled with [32P]orthophosphate, solubilized, and subjected to immunoprecipitation. In parallel, pure PD-ECGF was incubated with [α-32P]ATP or [γ-32P] ATP. Samples were analyzed by electrophoresis and electrotransferred to a PVDF membrane. The 45-kDa PD-ECGF was localized by autoradiography, cut out from the PVDF membrane, and exposed to 0.5 M piperidine. Samples were then analyzed by PEI-TLC using 0.75 M KH2PO4, pH 4.3, as a developer. [32P]Orthophosphate and unlabeled nucleotides were used as controls. For details, see “Experimental Procedures.”
in free phosphate, AMP, and ATP from PD-EGF labeled in vitro with \([\gamma^32P]ATP\) (Fig. 8, lane c). Radioactivity was noticed also in the position between ATP and the origin. To explore whether the same type of complex is formed also in vivo, lysates of cells labeled metabolically with \([32P]orthophosphate\) were immunoprecipitated and subjected to SDS-gel electrophoresis. After electrophoresis to a PVDF membrane, the 45-kDa PD-EGF was localized and cut out. Pimentdine treatment liberated \(32P\) radioactivity as free phosphate and nucleotide monophosphate (Fig. 8, lane d), as did treatment with phosphodiesterase (data not shown). In conclusion, PD-EGF in vivo contains covalently bound nucleotide, and pure recombinant PD-EGF can be covalently bound in vitro with nucleotide.

**DISCUSSION**

In this study, we report that PD-EGF synthesized in A431 cells contains nucleotide covalently bound to a serine residue. Furthermore, we show that ATP can become covalently bound to PD-EGF in vitro in a nonenzymatic reaction. PD-EGF could be labeled in vitro with different ATP isotopes which were radiolabeled in adenine, in ribose, in phosphate at the \(\alpha\)-position, or in phosphate at the \(\gamma\)-position. Pimentdine treatment, which cleaves phosphoester bonds at random, liberated nucleotide monophosphate and free orthophosphate from in vivo radiolabeled PD-EGF, and ATP, ADP, AMP, and free orthophosphate from in vitro radiolabeled PD-EGF. Partial acid hydrolysis released significant amounts of \([32P]\)serine from \([\gamma^32P]\)ATP-labeled PD-EGF; but only a trace amount of \([32P]\)serine from \([\alpha^32P]\)ATP-labeled PD-EGF. These observations suggest that the \(\gamma\)-phosphate of ATP can form a covalent linkage with a serine residue of PD-EGF (Fig. 9). ATP and GTP were the preferred nucleotides for nucleotidylation of PD-EGF; our data suggest that PD-EGF in vivo is also covalently linked to a nucleotide triphosphate, but the exact type of nucleotide involved remains to be elucidated.

PD-EGF was phosphorylated in vitro at \(95^\circ C\) under reducing conditions in the presence of ATP and SDS. Nonenzymatic \([\gamma^32P]\)ATP labeling at high temperature of poly(\(E_{\alpha}N_{\omega}\)) co-polymer, angiotensin-II peptides, and rabbit muscle enolase at serine and tyrosine residues has been demonstrated (21). The phosphorylation occurred in the presence of Mn\(^{++}\), but not in the presence of Mg\(^{++}\). It is not clear whether these labelings represented nucleotidylation similar to those discussed in the present work or a true phosphorylation. Similar to the observations by Schieve and Martin (21), phosphoamino acid analysis of in vitro-labeled PD-EGF revealed a small amount of phosphotyrosine; tyrosine phosphorylation was, however, not observed, when in vivo-labeled PD-EGF was analyzed. However, in contrast to the observations by Schieve and Martin (21), the in vitro phosphorylation of PD-EGF occurred also in the presence of Mg\(^{++}\). Serial experiments of various \([\gamma^32P]\)ATP concentrations in reactions with a fixed amount of PD-EGF revealed that the efficiency of nucleotidylation of PD-EGF in vitro was low (0.1 mmol of ATP per mol of PD-EGF; data not shown).

Given the low stoichiometry of nucleotidylation of PD-EGF in vitro and the harsh conditions needed to promote the reaction, it is likely that the nucleotidylation of PD-EGF in vivo is catalyzed by an enzyme, the identity of which, however, remains to be determined. We do not know whether the same serine residue in PD-EGF is involved in binding to nucleotides in vitro and in vivo, or whether any of the nucleotide binding motifs in PD-EGF are involved. Direct attempts to determine nucleotide binding of PD-EGF in vitro by the methods used for GTP-binding proteins (22, 23) were negative (data not shown).

Several growth factors have been found to be phosphorylated, including FGF, transforming growth factor-\(\beta\), and interleukin-1. Phosphorylation occurs on mannose residues in the case of the transforming growth factor-\(\beta\) precursor (24, 25) and pre-interleukin-1\(\alpha\) (26, 27). Furthermore, the transforming growth factor-\(\beta\) precursor has been shown to bind to the receptor for mannose 6-phosphate (25). In contrast, acidic and basic FGF, which, similar to PD-EGF, lack hydrophobic signal sequences (5), have been found to be phosphorylated on serine residues in cultured cells (6). The functional significance of the phosphorylation of FGFs remains to be determined, but it may regulate receptor binding affinity (6). In addition, it was reported that kinase C-dependent phosphorylation releases acidic FGF from rod outer membrane, to which acidic FGF is tightly associated (28, 29).

Several examples of nucleotidylated proteins that are covalently bound to nucleotides of RNA or DNA have been described. UMP at the 5'-terminal end of poliovirus genome RNA has been found to be bound covalently to a tyrosine residue of an unknown protein (30, 31), and the 5'-terminal dAMP of Bacillus subtilis phase \(\phi 29\) DNA has been found to be bound covalently to a serine residue of the terminal protein p3 (32, 33).

Furthermore, a serine residue of the oncogene product SV40 large T has been demonstrated to be linked to cellular RNA via the phosphate group of AMP of RNA (11, 34, 35), and a serine residue of the tumor suppressor gene product p53 has recently been found to be linked to RNA covalently (36). In addition, AMP or UMP has been shown to be covalently bound to glutamine synthetase in Excherichia coli at tyrosine residues and to regulate its activity (37, 38). In contrast to these examples, however, PD-EGF appears to be covalently bound to a nucleotide triphosphate.

The biological function of the nucleotidylation of PD-EGF remains to be elucidated. PD-EGF lacks hydrophobic signal sequence (1) and is released from cells very slowly (4). One possibility is that PD-EGF is covalently bound to DNA or RNA and that PD-EGF inside the cells has another function than as a growth factor. Alternatively, nucleotidylation of PD-EGF could provide a high energy bond or an address label which might be of importance in the secretion of PD-EGF from cells in a signal sequence-independent manner. Further studies are required to prove or disprove these possibilities.

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