Epidermal Growth Factor or Okadaic Acid Stimulates Phosphorylation of Eukaryotic Initiation Factor 4F*

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Eukaryotic initiation factor 4F, a multi-protein mRNA cap binding complex, was isolated by m'GTP-Sepharose affinity chromatography from human mammary epithelial cells (184A1N4) incubated with [32P]orthophosphate. Treatment of cells with epidermal growth factor resulted in enhanced phosphorylation of both p28 (eIF-4E) and p220 subunits. The identities of the p28 and p220 subunits were confirmed by immunoprecipitation. The phosphorylation was both rapid and sustained in duration; p28 attained maximal levels (2–3-fold) within 30 min of treatment and remained elevated for at least 2 h, while p220 reached one-half maximal levels by 30 min, and maximal levels (3–4-fold) by 2 h of treatment. Two phosphorylated isoforms of p28 and multiple phosphorylated forms of p220 were detected by two-dimensional isoelectic focusing/sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Phosphoamino acid analysis of 6 N HCl hydrolyzates of p28 and p220 isolated from epidermal growth factor-treated and control cells indicated that serine is the predominant phosphorylated amino acid in both instances. In no case was phosphothreonine observed. Pretreatment of cells with 1 μM okadaic acid resulted in the hyperphosphorylation of both p28 and p220 subunits. These results suggest that mitogenic growth factors and cellular serine/threonine phosphatases (pp1 and/or pp2A) serve essential roles in regulating phosphorylation levels of eukaryotic initiation factor 4F and support the concept that translational control is a component of the signal transduction mechanisms involved in growth regulation.

Protein phosphorylation is an essential regulatory mechanism involved in modulating cellular responses to hormones and mitogenic growth factors. Treatment of cells with epidermal growth factor (EGF)* results in the activation and rapid autophosphorylation of its receptor, followed by the direct tyrosine phosphorylation of numerous substrates (1, 2). Shortly thereafter, the activities of several cytosolic serine/threonine kinases rise dramatically (3). One major substrate for the mitogen-induced serine kinase activity(ies) is the ribosomal protein S6 (4). Although the role of ribosomal S6 phosphorylation is unknown, treatment of cells with EGF has been shown to result in the rapid conversion of monosomes to polysomes and enhanced protein synthesis (5–7). It has been suggested that one likely area for control of protein synthesis would be in the initiation process through enhanced mRNA selection and binding to the ribosomal complex. At least four polypeptides cooperate to select the capped 5′ terminus of eukaryotic mRNA, denature 5′-terminal structure, and transfer the mRNA to the 43 S ribosomal initiation complex (reviewed in Refs. 8 and 9). eIF-4F exists as a multi-protein complex comprised of eIF-4E, the actual cap-binding peptide (termed either p25 or p28), eIF-4A, the mRNA helicase peptide (p46), and p220, a peptide whose function is unclear, but has been suggested to align eIF-4A with eIF-4E and the mRNA cap (10–13). eIF-4F (an 80-kDa peptide) has been shown to associate with the eIF-4F cap-binding complex under certain conditions and has been suggested to aid in the recycling eIF-4F (14).

eIF-4E (p28) is reported to be the least abundant initiation factor (15, 16) and evidence has been presented that mRNA binding is the rate-limiting step in translation (17). Of the mRNA cap-binding initiation factors, subunits p220, eIF-4E, and eIF-4B have been shown to be phosphorylated in vivo following treatment of cells with phorbol 12-myristate 13-acetate or insulin (18, 19). Diphosphorylation of these subunits has been noted upon heat shock (16), during mitosis (20), or upon serum starvation (21). Initial studies indicated that phosphorylation of eIF-4E did not alter recognition or binding to the mRNA cap structure (16, 20, 22). However, more recently, Sonenberg and coworkers (23) have shown that overexpression of wild type eIF-4E, but not that of serine 53-mutated eIF-4E resulted in malignant transformation of NIH 3T3 cells. The authors suggested that eIF-4E is an intracellular transducer of extracellular signals and an intermediary in growth control pathways.

We examined the effect of EGF on the extent of phosphorylation of eIF-4F in the human mammary epithelial cell line 184A1N4. These cells contain 300,000 EGF receptors/cell and require EGF for growth (24). Treatment of cells with EGF resulted in enhanced phosphorylation of both p28 (eIF-4E) and p220 subunits on serine residues. We further found that pretreatment of cells with 1 μM okadaic acid resulted in the hyperphosphorylation of both p28 and p220 subunits.

EXPERIMENTAL PROCEDURES

Materials—[32P]Orthophosphate was obtained from ICN. Insulin, hydrocortisone, m'GTP-Sepharose, m'GTP, GTP, ATP, phosphoser-

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Results

Stimulation of Phosphorylation of eIF-4F in Quiescent 184A1N4 Cells in Response to EGF—To examine whether the phosphorylation state of eIF-4F in quiescent 184A1N4 cells was changed in response to EGF, cells were incubated with $[^{32}P]$orthophosphate in the absence of serum for 3 h, and then incubated in the presence or absence of 100 ng/ml EGF for an additional 1 h. eIF-4F was isolated by m'GTP-Sepharose adsorption in the presence of protease and phosphatase inhibitors and the adsorbed proteins examined by polyacrylamide gel electrophoresis and autoradiography as described under “Experimental Procedures.” Some incorporation of $[^{32}P]$ into eIF-4F was observed in the absence of EGF (Fig. 1, lane 1). The presence of EGF resulted in a large increase in phosphorylation of both p28 and p220 subunits of eIF-4F (lane 2). EGF stimulated increases in the phosphorylation states of p28 and p220 had no apparent effect on the binding of eIF-4F to m'GTP-Sepharose. There were no control experiments were carried out by using either $[^{35}S]$methionine metabolically labeled cells, or by staining the m'GTP eluted $[^{32}P]$labeled proteins with Coomassie Blue or silver (data not shown). These results are in agreement with studies using either reticulocyte lysates (22), or site-directed mutants of eIF-4F, containing alanine substituted for serine (33). The identities of p28 and p220 phosphoproteins as subunits of eIF-4F were confirmed by several methods. Adsorption to m'GTP-Sepharose was blocked by addition of 100 μM m'GTP to the binding buffer (Fig. 1, lanes 3 and 4), while GTP or ATP had no effect on binding or recovery (data not shown). eIF-4F subunit identities were further established by immunoprecipitation of m'GTP-Sepharose eluates with either anti-eIF-4E (Fig. 1, lanes 5 and 6) or anti-p220 (Fig. 1, lanes 7 and 8). With either antibody, polyacrylamide gel electrophoresis of immunoprecipitates contained both p28 and p220 subunits, indicating that the subunits remained associated after elution from m'GTP-Sepharose.

We observed an additional phosphorylated protein in m'GTP elutes, Mr ~ 58,000, whose degree of phosphorylation appears to diminish somewhat upon EGF treatment. It is conceivable that this phosphoprotein is the eIF-4A component of eIF-4F. Alternatively this protein might be capable of phosphorylation at Thr~200.

Fig. 1. Increased phosphorylation of eIF-4F in human mammary epithelial cells in response to EGF. Human mammary epithelial cells (184A1N4) were incubated with $[^{32}P]$orthophosphate for 4 h. One h prior to cell lysis, 100 ng/ml EGF was added to the indicated dishes. Cell lysates from individual dishes were adsorbed to m'GTP-Sepharose and eluted with 200 μM m'GTP as described under “Experimental Procedures.” The total eluates from individual dishes were resolved by SDS-PAGE and autoradiography or immunoprecipitated by the indicated antisera prior to SDS-PAGE analysis. Lanes 1, 2, 5–8, lysates adsorbed in the absence of 100 μM m'GTP. Lanes 3 and 4, lysates adsorbed in the presence of 100 μM m'GTP. Lanes 5 and 6, eluates immunoprecipitated by anti-p28. Lanes 7 and 8, eluates immunoprecipitated by anti-p220.
independently binding to the mGTP moiety. The absence of this phosphoprotein from immunoprecipitates of mGTP elutes and the different molecular mass of eIF-4A (M, \( \approx 46,000 \)) suggests that this protein may not be eIF-4A.

We then examined the time course of EGF-stimulated phosphorylation of eIF-4F. Human mammary epithelial cells (184A1N4) were labeled with \([^{32}P]orthophosphate for 4 h, and treated with 100 ng/ml EGF for 0, 10, 30, 60, or 120 min during the labeling period, prior to cell lysis. Phosphorylation of p28 and p220 subunits was both rapid and sustained in duration (Fig. 2). p28 attained maximal levels of phosphorylation (2-3-fold increase) within 30 min of EGF treatment and remained elevated for at least 2 h. p220 showed different kinetics, reaching one-half maximal levels by 30 min, and maximal levels (3-4-fold) by 2 h of treatment.

In separate experiments a 3-fold increase in phosphorylation of ribosomal protein S6 due to addition of EGF was observed. Phosphorylation of ribosomal S6 showed maximal phosphorylation by 2 h of treatment.

Two-dimensional Polyacrylamide Gel Electrophoresis and Phosphoamino Acid Analysis of eIF-4F—Two-dimensional isoelectric focusing/polyacrylamide gel electrophoresis of eIF-4F, labeled \( \text{in vivo} \) in the presence or absence of EGF, showed the presence of two phosphorylated forms of p28 (Fig. 3). EGF treatment appeared to stimulate the phosphorylation of both variants. Although the recovery of p220 relative to p28 on two-dimensional gels was poor, the data suggested that multiple isoforms of p220 were present. Multiple phosphorylated forms of p220 have been reported following exposure of cells to PMA or insulin (18, 19).

The identities of the amino acid residues phosphorylated in p28 and p220 in the presence or absence of EGF was determined by limited acid hydrolysis of the isolated proteins and high voltage electrophoresis as described under "Experimental Procedures." As seen in Fig. 4A, p28 was phosphorylated only on serine residues. Similarly, p220 was phosphorylated predominantly on serine residues (Fig. 4B). Longer exposures revealed trace amounts of threonine phosphate on p220. In no case, was phosphotyrosine observed.

**Fig. 2. Time course of eIF-4F phosphorylation in response to EGF.** Cells were labeled with \([^{32}P]orthophosphate as described under "Experimental Procedures." Each dish was treated with 100 ng/ml EGF for 0, 10, 30, 60, or 120 min at 37 °C prior to lysis. The lysates were adsorbed to mGTP-Sepharose. The total mGTP eluate from each lysed dish was analyzed by SDS-PAGE and autoradiography. Each time point represents the average counts/min (Cherenkov) of the actual gel slice from duplicate determinations.

**Fig. 3. Analysis of EGF-stimulated phosphorylation of eIF-4F by two-dimensional isoelectric focusing/sodium dodecyl sulfate-polyacrylamide gel electrophoresis.** 184N1A4 cells were incubated with \([^{32}P]orthophosphate for 3 h. The cells were then incubated for an additional 60 min in the absence (panel A) or presence (panel B) of 100 ng/ml EGF prior to cell lysis. The lysates were then adsorbed to mGTP-Sepharose. The total mGTP eluate from each lysed dish was analyzed by two-dimensional isoelectric focusing/SDS-PAGE (7.5% acrylamide) and autoradiography as described under "Experimental Procedures."
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phosphorylation. These results indicate that phosphatase 1 and/or 2A are involved in regulating the phosphorylation state of eIF-4F.

**DISCUSSION**

We have demonstrated that two subunits of eIF-4F (p28 and p220) are phosphorylated in human mammary epithelial cells (184A1N4) and that, in response to EGF, the extent of phosphorylation of these subunits is elevated. Typically, the phosphorylation of p28 is raised 2–3-fold while that of p220 is raised 3–4-fold. Phosphorylation of p28 peaked at 30 min after the addition of EGF and remained elevated for at least 2 h; phosphorylation of p220 attained half-maximal values by 30 min and maximal phosphorylation was noted at the end of the 2-h interval examined.

Phosphoamino acid analysis indicated that serine is the predominant phosphorylation site on both p28 and p220; phosphotyrosine was not detected. Two-dimensional isoelectric focusing/SDS-PAGE analysis revealed the presence of two phosphorylated isoforms of p28; EGF appeared to stimulate the phosphorylation of both variants to similar extents. Although tryptic cleavage of phosphorylated p28 has been reported to yield a single radioactive peptide containing only 1 serine residue, Ser-53 (35), other reports (22, 36) suggest that more than one site of phosphorylation exists.

Enhanced phosphorylation of eIF-4E (p28) appears to be a common cellular response to mitogenic stimulation and has been observed following exposure to phorbol 13-myristate 13-acetate (18), insulin (19), or tumor necrosis factor (37). In vitro phosphorylation of p28 has been detected with protein kinase C (38, 39) and a nonabundant eIF-4E-specific kinase (40); phosphorylation of p220 has been detected with protein kinase C (38, 39) protease-activated kinases I and II (38) and cAMP-dependent kinase (39). In view of the many reports that a variety of serine/threonine kinases are activated following mitogenic stimulation (see Ref. 41, and reviewed in Refs. 3 and 42), it would seem probable that the activation of one or more of these is involved in the EGF-induced increased phosphorylation of p28 and p220. Our observations that treatment of 184A1N4 cells with okadaic acid, an inhibitor of phosphoprotein phosphatases 1 and 2A, also results in an elevated phosphorylation of p28 and p220 suggest that the regulation of the activity of specific phosphatases may also be involved. In this regard, dephosphorylation of eIF-4E has been observed during heat shock (16) and mitosis (20).

Initial studies indicated that the phosphorylation state of eIF-4E does not alter recognition of, or binding to the mRNA cap structure (16, 20, 22). However, it has recently been reported that overexpression of wild type eIF-4E<sup>Ser<sup>53</sup></sup>, but not mutated eIF-4E<sup>D<sub>18</sub></sup><sup>Ser<sup>53</sup></sup>, results in malignant transformation of NIH 3T3 cells (23). This observation suggests a growth controlling function for eIF-4F independent of m<sup>GTP</sup> binding and indicates that phosphorylation of Ser-53 is required for this eIF-4F function.

It also has been suggested that phosphorylation of ribosomal protein S6 could influence rates of global protein synthesis (5). Our early observation on the effect of EGF on protein synthesis and polysome formation (6) and our present observations on the effects of EGF and okadaic acid on the phosphorylation state of p28, p220, and ribosomal S6 in human mammary cells support the concept that translational control may be an important signalling mechanism in growth regulation.

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