Protease-activated kinase II as the mediator of epidermal growth factor-stimulated phosphorylation of ribosomal protein S6

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Epidermal growth factor stimulates phosphorylation of ribosomal protein S6 in serum-starved Swiss 3T3 cells, leading to the formation of highly phosphorylated derivatives containing 4–5 phosphates. Two-dimensional analysis of tryptic phosphopeptides of S6 shows an identical pattern to the ones obtained previously in other cells in response to insulin and the tumor promoting phorbol ester, 12-O-tetradecanoyl phorbol-13-acetate. This suggests a common intracellular mediator of S6 phosphorylation by different growth promoting agents. It is proposed that the potential mediator of this phosphorylation is the Ca^{2+}-independent, cAMP-independent protein kinase, protease activated kinase II, as shown by the extent of phosphorylation and the tryptic phosphopeptide maps of S6 with highly purified enzyme [(1983) J. Biol. Chem. 258, 13998–14002].

Protein kinase *Epidermal growth factor* *Phosphorylation* *Ribosome* *Protease-activated kinase* *Swiss 3T3 cell*

1. INTRODUCTION

Ribosomal protein S6 is phosphorylated in a variety of cells during growth factor regulated cell growth and division [1–7], upon stimulation of cells by the phorbol ester tumor promoter, TPA [8–10], as well as in malignant cell growth in virally transformed cells deprived of serum [10–12]. The pathways by which all of these diverse agents lead to increased phosphorylation of S6 are under intense study. Elucidation requires isolation and characterization of the serine protein kinase(s) responsible for S6 phosphorylation under the various growth conditions. Although the cAMP-dependent protein kinase has been shown to phosphorylate S6 both in vivo and in vitro [13–16], it does not appear to be responsible for S6 phosphorylation in growth-stimulated cells [5,17–20].

Several groups have reported increased protein kinase activity for S6 in the soluble fraction of cell extracts from cells treated with growth factors [5,7,20]. The active form has been partially purified from insulin-treated 3T3-L1 preadipocytes by Perisic and Traugh [5]. Another approach in elucidating regulation of the protein kinase is by isolation of the inactive, proenzyme form and investigation of possible modes of activation in vitro. Such an enzyme, denoted as protease activated kinase II (PAK II), has been isolated from rabbit reticulocytes [21] and 3T3-L1 preadipocytes [5]. The inactive proenzyme form of PAK II is activated in vitro by limited proteolysis and phosphorylates the same sites on S6 that are phosphorylated in cultured cells stimulated with insulin [5,9] or with TPA [9]. A similar pro-

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Abbreviations: EGF, epidermal growth factor; TPA, 12-O-tetradecanoyl phorbol-13-acetate; PAK II, protease activated kinase II

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teolytically activated enzyme, called H4 protein kinase, has been isolated from lymphoid cells in inactive form [22]. Here, we report that the EGF-stimulated phosphorylation of S6 is mediated by PAK II as shown by the extent of phosphorylation and phosphopeptide mapping.

2. MATERIALS AND METHODS

2.1. *Materials*

Carrier-free [^{32}P]orthophosphate was obtained from ICN, mouse EGF (culture grade) from Collaborative Research and Swiss 3T3 mouse embryo fibroblasts from American Type Culture Collection. Trypsin (DPCC treated) was from Sigma and thin-layer cellulose sheets from Eastman-Kodak.

2.2. *Phosphorylation and analysis of ribosomal protein S6 in Swiss 3T3 cells*

Swiss 3T3 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, in six 175 cm^2 tissue culture flasks. At approx. 85% confluency, cells were rinsed twice with serum-free, phosphate-free medium at 37°C, and incubated in the same medium for 1 h. Cells were then radioactively labeled in the medium supplemented with [^{32}P]orthophosphate (0.1 mCi/ml) for 2 h. During the last hour of labeling, to one-half of the culture flasks, EGF (10^{-8} M) was added. Following incubation, cells from control and EGF-treated cultures were collected, lysed and the ribosomes prepared as described previously [5]. Ribosomes from Swiss 3T3 cells (0.045 mg) were mixed with nonlabeled carrier ribosomes from rabbit reticulocytes (0.20 mg) and total ribosomal protein was extracted and analyzed by 2-dimensional polyacrylamide gel electrophoresis as described [18].

2.3. *Phosphopeptide mapping of ribosomal protein S6*

^{32}P-labeled S6 was excised from the gel and digested extensively with trypsin as described [5]. Two-dimensional phosphopeptide mapping, with electrophoresis in the first dimension followed by ascending chromatography, was carried out according to Traugh et al. [14] and the phosphopeptides were detected by autoradiography.

3. RESULTS

Phosphorylation of ribosomal protein S6 in Swiss 3T3 cells, serum-starved or stimulated by EGF, was analyzed by 2-dimensional polyacrylamide gel electrophoresis and 2-dimensional phosphopeptide mapping. Swiss 3T3 cells were serum-starved for 1 h then labeled with ^{32}P for 2 h. Little

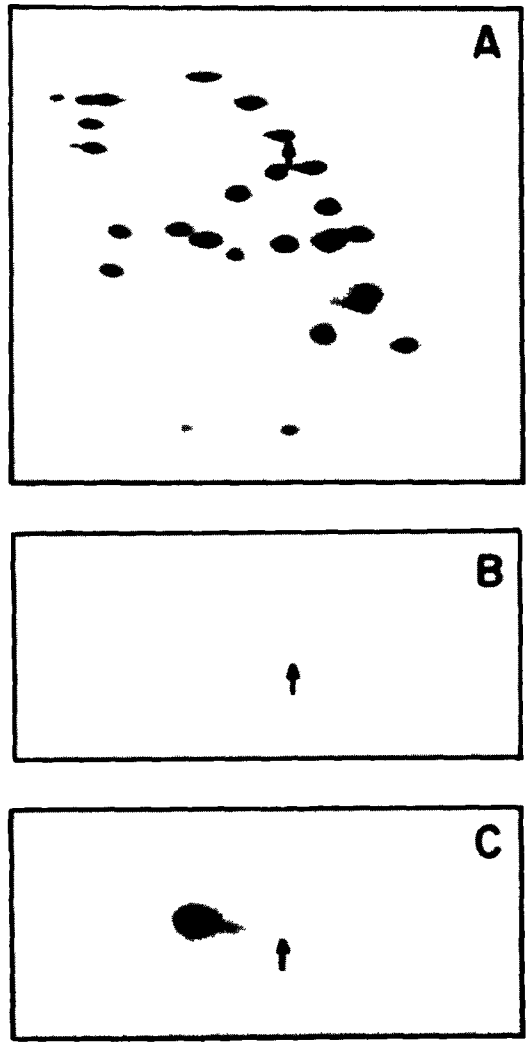

Fig.1. Two-dimensional gel electrophoresis of ribosomal proteins from serum-starved and EGF-treated Swiss 3T3 cells. Swiss 3T3 cells deprived of serum were labeled with [^{32}P]orthophosphate in the absence (B) or presence of 10^{-8} M EGF (C). A represents the stained protein pattern and B and C are autoradiograms. Arrows indicate ribosomal protein S6; numbers indicate the state of phosphorylation.

incorporation of radioactive phosphate into ribosomal protein S6 was observed under these conditions (fig.1), although other cellular proteins were highly phosphorylated. If, during the last hour of incubation, EGF (10^{-8} M) was added to the medium, a selective enhancement of slowly migrating derivatives of S6 containing 4–5 phosphates was observed. Thus, EGF stimulated incorporation of total phosphate into S6 with an accumulation of highly phosphorylated derivatives of the protein.

The site specificity of S6 phosphorylation induced by EGF was analyzed by 2-dimensional phosphopeptide mapping on thin-layer cellulose sheets. Fig.2 shows the tryptic phosphopeptide pattern of S6 labeled with [^{32}P]orthophosphate in Swiss 3T3 cells stimulated with EGF. The complex pattern of tryptic phosphopeptides is identical to that obtained previously with S6 phosphorylated in 3T3-L1 cells stimulated with insulin [5] and in Reuber H35 cells stimulated with insulin and/or TPA [9], with the exception that a small amount of phosphate is observed in peptide B. The pattern observed in Swiss 3T3 cells following stimulation with EGF contains the same phosphopeptides as those from 40 S ribosomal subunits phosphorylated with purified PAK II from rabbit reticulocytes and insulin-stimulated 3T3-L1 cells [5,22]. Thus, it appears that PAK II is activated in response to EGF with the concomitant stimulation of phosphorylation of S6 as shown by phosphopeptide mapping.

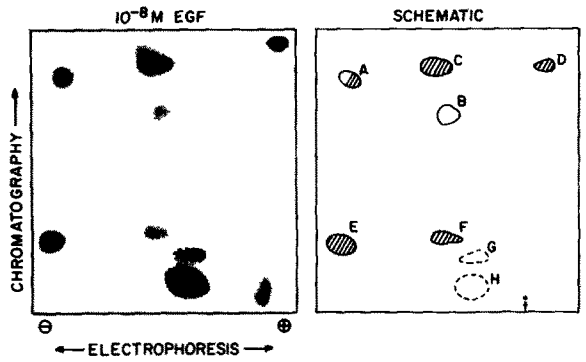

Fig.2. Comparison of phosphopeptide patterns of S6 phosphorylated in Swiss 3T3 cells stimulated by EGF with previously determined maps. An autoradiogram of the tryptic phosphopeptide map of S6 from serum-starved Swiss 3T3 cells incubated for 1 h with EGF is shown on the left. The schematic on the right identifies the tryptic phosphopeptides: crosshatching, obtained with 40 S ribosomal subunits and PAK II in vitro [5,18]; open, obtained with the cAMP-dependent protein kinase [13,14]; solid lines, observed both in cultured cells and in vitro; dotted lines, peptides detected in 3T3-L1 cells stimulated with insulin [5] and Reuber H35 cells stimulated with insulin and/or TPA [9].

4. DISCUSSION

In this study, we have characterized the extent and site specificity of the phosphorylation of ribosomal protein S6 in Swiss 3T3 cells stimulated by EGF. We compared these data with those obtained with other growth-promoting stimuli, insulin in 3T3-L1 cells [5] and TPA and/or insulin in Reuber H35 hepatoma cells [9]. All of these agents induce the same, maximal degree of S6 phosphorylation and lead to phosphorylation of the same sites on S6, as shown by 2-dimensional analysis of tryptic digests of ^{32}P-labeled S6. The same basic phosphopeptide pattern is also observed in vitro when 40 S ribosomal subunits are phosphorylated with PAK II [18]. These data suggest that PAK II is responsible for the EGF-stimulated phosphorylation of S6 in Swiss 3T3 cells and is activated in response to the hormone.

A small amount of phosphate is also observed in peptide B. This phosphopeptide is not detected in S6 from other cell types stimulated with insulin or TPA [5,9] or in vitro with purified PAK II [18]. Peptide B has previously been shown to contain sites phosphorylated by the cAMP-dependent protein kinase [13,14]. Martin-Perez et al. [19] have also identified a small amount of phosphorylation of S6 in response to EGF which is attributable to the cAMP-dependent protein kinase. This suggests that Swiss 3T3 cells may contain higher levels of cAMP and/or cAMP-dependent protein kinase or that the cAMP-dependent protein kinase may be involved in partially mediating EGF action.

PAK II is isolated from the postribosomal supernatant fraction of rabbit reticulocytes in an inactive form, and is activated in vitro by limited proteolysis [21], or by diacylglycerol and phosphatidylserine in a Ca^{2+}-independent manner [23]. We have shown previously that the enzyme is present as an inactive proenzyme in serum-starved 3T3-L1 cells and is rapidly activated upon addition of insulin to the cells. The activated form of the enzyme is separable from the inactive form by ion-

exchange chromatography, cannot be activated further by limited proteolysis, and the activity is expressed in the absence of Ca^{2+} or phospholipids [5].

Recent reports indicate that the same sites on S6 are phosphorylated not only during the normal growth processes regulated by different growth factors [7,9], but also in serum-deprived cells transformed by retroviruses [10,12]. All of these data suggest a unique serine protein kinase that acts as a common intracellular mediator of growth-promoting agents. From our studies, this protein kinase has been identified as PAK II. Mechanism(s) which regulate the activity of PAK II in vivo are unknown. A common feature in the action of both growth factors and transforming RNA viruses is the activation of tyrosine-specific protein kinases [24–30]. These protein kinases could regulate the activity of S6 kinase through phosphorylation. This idea is supported by the finding that the presence of phosphatase inhibitors during cell extraction is crucial in obtaining the fully active form of the S6 kinase in serum or EGF-induced Swiss 3T3 cells [20]. Our recent investigations show phosphorylation of a protein in the PAK II preparation on tyrosine with the concomitant activation of PAK II by three different tyrosine-specific protein kinases, the EGF receptor, the insulin receptor and Abelson kinase (in preparation). Nevertheless, this and other alternative modes of activation need further investigation before all possible physiological modes of activation for the enzyme are determined.

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