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Saccharomyces cerevisiae elongation factor 2 is phosphorylated by an endogenous kinase

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Mammalian cells contain a Ca^{2+}/calmodulin-dependent protein kinase that specifically phosphorylates and inactivates elongation factor 2 (EF-2) in response to hormones and other agents which increase intracellular Ca^{2+} concentrations Therefore, it has been proposed that the rate of translation in mammals is regulated by EF-2 phosphorylation In the present study, EF-2 purified from the yeast *Saccharomyces cerevisiae* is shown to be a substrate for the mammalian EF-2 kinase Furthermore, evidence was obtained using two-dimensional gel electrophoresis and peptide mapping which suggests that yeast EF-2 is a substrate for an endogenous kinase which phosphorylates the same site as the mammalian EF-2 kinase Based on these findings, we propose that in yeast as in higher eukaryotes, the protein synthesis elongation cycle is regulated by phosphorylation of EF-2

Elongation factor 2, Protein phosphorylation, Protein synthesis, Protein kinase, *Saccharomyces cerevisiae*

1 INTRODUCTION

The eukaryotic elongation factor-2 (EF-2) is one of two soluble protein factors required for the elongation phase of protein synthesis Recently, a Ca^{2+}/calmodulin dependent protein kinase was identified in mammalian cells which specifically phosphorylates EF-2 [1] None of the other known protein kinases phosphorylate EF-2 to a significant extent, and EF-2 kinase phosphorylates none of the typical substrates for other protein kinases [2] Mammalian EF-2 is phosphorylated in vitro with a stoichiometry of approximately 1 mol/mol on threonine residues located within amino acids 51–61, a region of the protein which may form part of the ribosome binding site [3] Furthermore, the extent of EF-2 phosphorylation in vivo has been found to increase after treatment of cells with drugs that raise the intracellular level of Ca^{2+} [4] Stimulation of labeled fibroblasts with bradykinin, vasopressin or epidermal growth factor resulted in a rapid 2–10-fold increase in phosphorylation of EF-2 [5] Phosphorylation of EF-2 in vitro inactivates the protein, and the degree of phosphorylation in vivo is inversely correlated with the rate of protein synthesis [6] Indeed, Celis et al [7] observed increased phosphorylation of EF-2 in human amnion cells during mitosis and proposed that this increase may explain the decline in the translation rate during cell division Thus, it is hypothesized that phosphorylation of EF-2 plays a role in the regulation of gene expression in eukaryotes by controlling the rate of nascent polypeptide chain elongation

Saccharomyces cerevisiae, or baker's yeast, is readily amenable to molecular genetic analysis and yet it displays most of the features of higher eukaryotes. Indeed, there is increasing evidence that many cellular processes are mechanistically conserved among different eukaryotic species In this report, we show that yeast EF-2 can be phosphorylated by the mammalian EF-2 kinase. We also show that yeast contains an endogenous kinase which phosphorylates EF-2 The data suggest, therefore, that *S cerevisiae* utilizes EF-2 phosphorylation as a means of regulating the rate of protein synthesis

2 MATERIALS AND METHODS

EF-2 was purified from commercial bakers yeast by a modification of the method of Skogerson [8] Specifically, the DEAE-Sephacel (Pharmacia) and CM-Sephadex (Pharmacia) steps were performed using a batch method in which pre-equilibrated resin was mixed with a yeast lysate for 30 min at 4°C The resins were washed and eluted on a Buchner funnel employing the buffers described by Skogerson EF-2 was further purified using an HPLC system equipped with a MonoQ column (Pharmacia) Protein was applied to the column in buffer A (20 mM Tris-HCl, pH 7 6, 1 mM DTT, 10% glycerol, 0 1 mM phenylmethylsulfonyl fluoride) adjusted to 100 mM KCl and eluted with a linear 100–600 mM KCl gradient in buffer A Purified EF-2 was dialyzed against buffer A and stored at −70°C EF-2 was assayed by diphtheria toxin-catalyzed ADP-ribosylation with [^{32}P]NAD$^+$ (30 Ci/mmol, New England Nuclear) [9] Each 50 μl reaction contained 10 μl sample and 40 μl assay solution (20 mM Tris-HCl, pH 7 6, 10 mM DTT, 0 20 μg diphtheria toxin and 10^6 cpm [^{32}P]NAD$^+$) The reaction mixture was incubated for 15 min at 37°C The radioactivity incorporated was measured after trichloracetic acid precipitation and washing of protein Alternatively, the ADP-ribosylated sample was electrophoresed on a 7 5% SDS-polyacrylamide gel [10] and visualized by autoradiography

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In experiments using yeast cell extracts, *S cerevisiae* YPH 3995 was grown at 30°C in YM-1 medium [11] to a density of 1 OD_{600}/ml Cells were collected by centrifugation (5 min, 5000 rpm, 4°C) and resuspended in lysis buffer (20 mM HEPES-KOH, pH 7 5, 100 mM potassium acetate, 2 mM magnesium acetate, 2 mM DTT, 0 5 mM phenylmethylsulfonyl fluoride) Cells were lysed by vortexing with 4 volumes of chilled, acid-washed 0 45 mm glass beads The lysate was centrifuged for 30 min at 100 000× *g* (4°C), and the supernatant was passed over a Sephadex G-50 column (Pharmacia) equilibrated in buffer B (50 mM HEPES-KOH, pH 7 6, 10 mM magnesium acetate,5 mM DTT, 10% glycerol, 0 1 mM $CaCl_2$)

A ribosome-free extract from rabbit reticulocytes was prepared as described previously [12] To obtain partially purified EF-2 kinase, the reticulocyte lysate was loaded onto QAE-Sephadex A-50 (Pharmacia) equilibrated in buffer C (10 mM Tris-HCl, pH 7 6, 1 mM $MgCl_2$, 50 mM KCl, 7 mM β-mercaptoethanol and 10% glycerol) by stirring for 30 min at 4°C The resin was washed with buffer C adjusted to 200 mM KCl The resin was eluted with a linear KCl gradient (200–600 mM) in buffer C Fractions were collected and assayed for EF-2 kinase activity with and without added yeast EF-2 (2 μg per reaction) The phosphorylation reaction contained in a final volume of 50 μl 50 mM HEPES-KOH, pH 7 6, 10 mM magnesium acetate, 5 mM DTT, 40 ng/ml okadaic acid, 2 μg bovine brain calmodulin, 0 15 mM $CaCl_2$, 50 μM [γ^{32}P]ATP (2000 cpm/pmol) and the kinase sample After 10 min at 37°C, the reaction was stopped by adding 40 μl of the mixture to 10 μl boiling 5× sample buffer (62 5 mM Tris-HCl, pH 6 8, 15 mM DTT, 10% glycerol and 1% SDS) The extent of phosphorylation was analyzed by SDS-polyacrylamide gel electrophoresis (discontinuous, 7 5% slab gel) followed by autoradiography Fractions containing EF-2 kinase were pooled, de-salted by gel filtration through a column of Sephadex G-50 equilibrated in buffer B and stored at −70°C

Two-dimensional gel electrophoresis was performed in accordance with the procedure described by O'Farrell [13] An equal volume of first dimension buffer (9 5 M urea, 2% Triton X-100 5% β-mercaptoethanol, 1 6% pH 5/8 ampholyte (Pharmacia), 0 4% pH 3/9 ampholyte (Pharmacia) was added to the samples Following a 15 min incubation at room temperature, the samples were loaded onto 1 5×12 mm tube gels The second dimension consisted of SDS-PAGE using 7 5% slab gels Radioactive spots were visualized by autoradiography

Cleveland gel analysis [14] was performed using a kit obtained from Promega which contained the proteases and buffers Phosphorylated or ADP-ribosylated samples were electrophoresed on a 7 5% slab polyacrylamide gel The gel was stained with Coomassie stain for 10 min, destained for 45 min, and then rinsed with distilled water Target bands were excised with a razor blade and equilibrated in 1×SDS-PAGE sample buffer The fingerprinting gel was prepared with a 4 5% acrylamide stacking gel and 15% acrylamide resolving gel Stacking was carried out at 150 V for 45 min followed by a 30 min voltage interruption to allow further protease digestion Electrophoresis was resumed at 250 V until the dye front reached the bottom of the gel The labeled bands were visualized by autoradiography

3. RESULTS

3 1. *Yeast elongation factor-2 is a substrate for mammalian EF-2 kinase*

The present study was undertaken to determine whether a functional homolog of the mammalian EF-2 kinase exists in the simple eukaryote *S. cerevisiae*. As a first step, purified yeast EF-2 was tested as a substrate for the EF-2 kinase from rabbit reticulocytes. The mammalian enzyme was purified such that it was free of contaminating EF-2 (Fig 1, lane 1) Thus, in the absence of added EF-2, a phosphorylated species of appropriate size was not detected by autoradiography. However, addition of yeast EF-2 (Fig 1, lane 2) to the

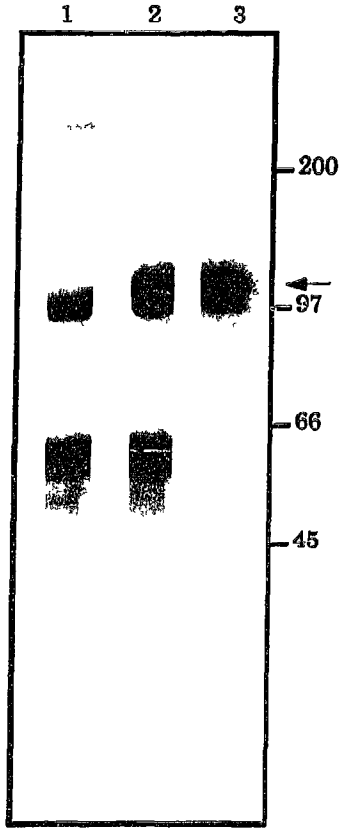

Fig 1 Phosphorylation of yeast EF-2 by the mammalian EF-2 kinase analyzed by SDS-PAGE and autoradiography (Lane 1) Phosphorylation reaction without yeast EF-2, (lane 2) phosphorylation with 2 μg yeast EF-2, (lane 3) diphtheria toxin catalyzed ADP-ribosylation of yeast EF-2 The reactions were conducted as described in section 2 The arrow indicates the location of EF-2

reaction resulted in the appearance of a single phosphorylated species that co-migrated with ADP-ribosylated yeast EF-2 (Fig 1, lane 3) The results of this experiment suggest, therefore, that yeast EF-2 can be phosphorylated by the mammalian EF-2 kinase

3.2 *Yeast EF-2 is phosphorylated by an endogenous protein kinase*

In the experiments illustrated in Fig. 2, two-dimensional equilibrium isoelectric focusing SDS-PAGE was used to determine whether a protein kinase exists in yeast which phosphorylates EF-2 The design of the experiment made use of the fact that EF-2 in a yeast cell lysate can be specifically labeled by diphtheria toxin and [^{32}P]NAD^+ When analyzed by two-dimensional gel electrophoresis, ADP-ribosylated yeast EF-2 present in a cell lysate migrated as a single major spot (Fig 2A) However, when the yeast cell lysate was first incubated in the presence of unlabeled ATP and the phosphatase inhibitor okadaic acid [15] and then incubated together with [^{32}P]NAD^+ and diphtheria toxin, the labeled EF-2 migrated as two spots (Fig. 2B). By comparison with the results depicted in Fig 2A, the first spot corresponds to ADP-ribosylated EF-2. The distance between the first

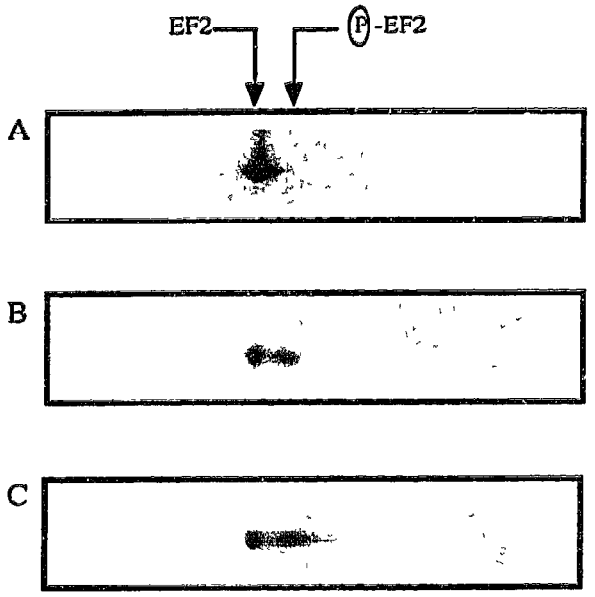

Fig 2 Phosphorylation of yeast EF-2 by an endogenous kinase analyzed by two-dimensional equilibrium isoelectric focusing SDS-PAGE (A) Yeast extract without ATP, (B) yeast extract incubated with 7 μM ATP, (C) yeast extract incubated with 1 μg rabbit reticulocyte EF-2 kinase and 7 μM ATP EF-2 was labeled by diphtheria toxin-catalyzed ADP-ribosylation in the presence of [^{32}P]NAD$^+$ as described in section 2

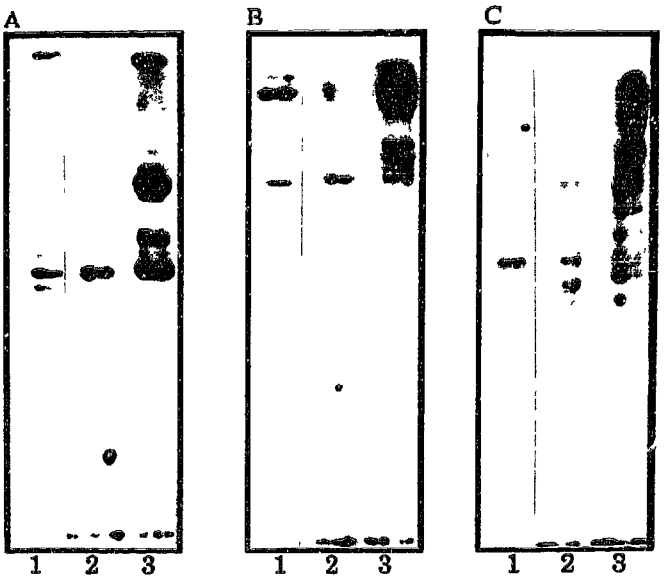

Fig 3 Cleveland gel analysis of yeast EF-2 [^{32}P]phosphopeptides (Lane 1) Yeast EF-2 phosphorylated by rabbit reticulocyte EF-2 kinase, (lane 2) yeast EF-2 phosphorylated in a yeast cell extract, (lane 3) yeast EF-2 ADP-ribosylated by diphtheria toxin (A) 2 5% (w/w) Endo Glu-C, (B) 2 5% (w/w) Endo Lys-C, (C) 0 20% (w/w) alkaline protease

spot and the second more acidic spot suggests the addition of a single phosphate group and its two negative charges. The results of this experiment indicate, therefore, that yeast EF-2 is phosphorylated by an endogenous kinase. When rabbit reticulocyte EF-2 kinase was added to the yeast cell lysate with unlabeled ATP and okadaic acid, the same two spots were observed following ADP-ribosylation and two dimensional equilibrium isoelectric focusing SDS-PAGE (Fig 2C). Thus, no additional species was generated as a result of phosphorylation of yeast EF-2 by the mammalian kinase

3 3. *Yeast EF-2 is phosphorylated at a single site*

Peptide mapping by limited proteolysis was used to determine whether the endogenous yeast kinase phosphorylates the same EF-2 peptide as the mammalian EF-2 kinase. In the method originally described by Cleveland [14], partial proteolytic cleavage of target proteins generates a pattern of peptide fragments that is characteristic of the target protein and the proteases used for cleavage. In the experiment shown in Fig 3, three different proteases were used to generate a range of peptide fragments Endoproteinase Glu-C cleaves polypeptides at the carboxylic side of glutamic acids, and endoproteinase Lys-C cleaves at the carboxylic side of lysine residues Alkaline protease has a broader specificity, cleaving preferentially at aromatic residues, then at large hydrophobic amino acids and finally at alternate sites when used at very high concentrations. Yeast EF-2 phosphorylated by the partially purified mammalian EF-2 kinase in the presence of [γ-^{32}P]ATP (lanes 1) was compared to EF-2 phosphorylated by the endogenous kinase present in a yeast cell lysate (lanes 2) EF-2 labeled by diphtheria toxin with [^{32}P]ADP ribose was included as a control (lanes 3) The digestion patterns observed in lanes 1 were identical to those in lanes 2 suggesting that the yeast and the mammalian kinases phosphorylate the same residue in EF-2

4. DISCUSSION

The present work demonstrates that yeast EF-2 can be phosphorylated by mammalian EF-2 kinase This result is not unexpected considering the high degree of sequence similarity between yeast and mammalian EF 2. The amino acid sequence of yeast EF-2 has been deduced from the nucleotide sequence of the cloned genes [16] Comparison of this sequence with that of mammalian EF-2 reveals complete amino acid identity in the proposed phosphorylation domain [3,16] with the exception that threonine-54 in the mammalian sequence is an alanine in the yeast protein Phosphorylation of EF-2 in intact human fibroblasts was found to occur on a single threonine residue, probably threonine-57 [5]. Similarly, a recent in vitro analysis by Price et al [17] suggests that threonine-57 is the major phosphorylated residue in rabbit reticulocyte EF-2.

In this work we have also presented evidence that a functional homolog of mammalian EF-2 kinase exists in yeast. Incubation of yeast extracts with ATP followed by two-dimensional gel electrophoresis resulted in the appearance of a single major phosphorylated form of EF-2 Similar results were obtained when yeast

extracts were incubated with rabbit reticulocyte EF-2 kinase. It is possible that prolonged exposure to ATP could result in the appearance of a second phosphorylated species Price et al [17] found that rabbit reticulocyte EF-2 kinase phosphorylates threonine-57 ten to twenty times faster than threonine-59 Thus, reaction times longer than those employed here are necessary to observe bisphosphorylated EF-2

An additional, satellite spot was sometimes detected when EF-2 in a yeast cell extract was ADP-ribosylated by diphtheria toxin (Fig 2A) This species may correspond to EF-2 with incompletely synthesized diphthamide [18,19] as was suggested by Celis et al. [7] and Levenson et al [20] on the basis of studies with mammalian EF-2. These authors proposed that this form of EF-2 represents a transient post-translational processing intermediate because it was most visible in cells pulsed briefly with [^{35}S]methionine

The results of Cleveland gel analysis support the existence of a yeast homolog to mammalian EF-2 kinase With each of the three proteases used in the analysis, a peptide pattern was generated for EF-2 phosphorylated by the yeast kinase that was identical to EF-2 phosphorylated by the mammalian kinase The relative simplicity of the digestion patterns observed for phosphorylated EF-2 presumably results from the extreme N-terminal location of the phosphorylated threonine, and the presence of a protease-sensitive region on the C-terminal side of the phosphorylation site [21]. Thus, the yeast and mammalian kinases appear to exhibit identical specificities.

Based on the results presented here, we propose that in yeast as in higher eukaryotes the protein synthesis elongation cycle is regulated by phosphorylation Preliminary experiments suggest that Ca^{2+} chelators inhibit the activity of the yeast EF-2 kinase Although little is known about the Ca^{2+} signalling system in yeast, several Ca^{2+}/calmodulin-dependent kinases have been identified in *S cerevisiae* For example, Londesborough and Nuutinen [22] reported the purification of a Ca^{2+}/calmodulin-dependent protein kinase from yeast that phosphorylates histones and casein. We hypothesize, therefore, that yeast EF-2 kinase is part of a family of enzymes that enable yeast to integrate cellular functions in response to changes in intracellular free Ca^{2+} concentrations

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