Comparative Analysis of Phosphorylation of Translational Initiation and Elongation Factors by Seven Protein Kinases*

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Four initiation factors (eIF-2, -3, -4B, and -4F), previously shown to be phosphorylated in vivo, are each phosphorylated to a significant extent in vitro (>0.3 mol of phosphate/mol of factor) by at least three different protein kinases. An S6 kinase from liver, an active form of protease-activated kinase II which modifies the same sites on S6 as those phosphorylated in vivo in response to mitogens, phosphorylates the β subunit of eIF-2, eIF-3 (p120–p130), eIF-4B, and eIF-4F (p220). The Ca²⁺, phospholipid-dependent protein kinase phosphorylates eIF-2β, eIF-3 (p170, p120–p130), eIF-4B, and eIF-4F (p220, p25). The cAMP-dependent protein kinase significantly modifies eIF-4B and, to a lesser extent, eIF-3 (p130). Casein kinase I incorporates phosphate only into eIF-4B, but to a limited extent. Casein kinase II phosphorylates eIF-2α, eIF-3 (p170, p120), and eIF-4B, while protease-activated kinase I modifies eIF-3 (p170, p120–p130), eIF-4B, and eIF-4F (p220). The mitogen-stimulated S6 kinase from 3T3-L1 cells, activated in response to insulin, does not phosphorylate any of the initiation factors. There is no significant incorporation of phosphate into eIF-2α or eIF-4B, or eIF-4C, eIF-4D, EF-1, or EF-2 by any of the protein kinases examined. Phosphopeptide mapping of tryptic digests of the phosphorylated subunits shows that the individual protein kinases modify different sites. The sites phosphorylated in vitro reflect those modified in vivo as shown with eIF-4F in concomitant studies with reticulocytes treated with tumor-promoting phorbol ester (Morley, S. J., and Traugh, J. A., J. Biol. Chem., in press). Thus, we have identified multipotential protein kinases which modify four initiation factors phosphorylated in vivo and have shown that phosphorylation of these translational components can be coordinately regulated.

Four factors required for initiation of protein synthesis are phosphorylated in reticulocytes (1–3) and in cultured cells (3, 5, 6). These include the α and β subunits of initiation factor 2, eIF-3β (p130–p110, p67), eIF-4B, and eIF-4F (p220, p25). Initial evidence for regulation of phosphorylation of initiation factors comes from studies on the inhibition of protein synthesis in reticulocytes by hemin deprivation via phosphorylation of eIF-2α (as reviewed in Refs. 7 and 8). Recently, multiple isolectric forms of eIF-2α and -β, eIF-4B, and eIF-4F (p220, p25) have been identified by two-dimensional isoelectric focusing/gel electrophoresis. Alterations in eIF-2α and eIF-4B are associated with starvation/refeeding (5). Dephosphorylation of eIF-4E (the equivalent of eIF-4F, p25) is observed upon heat shock (6) and during mitosis (9), while phosphorylation of eIF-4F (p220, p25) is stimulated in response to insulin and TPA. Elongation factor 1 is phosphorylated in Artemia, as determined by amino acid sequencing (10), and EF-2 is specifically phosphorylated by Ca²⁺, a calmodulin-dependent protein kinase III in response to agents which elevate Ca²⁺ levels (11, 12). Thus, six of the factors required for protein synthesis have been shown to be phosphorylated in vivo in higher eukaryotes.

Alterations in phosphorylation of other translational components including ribosomal protein S6 (13, 14) and aminoacyl-tRNA synthetases (14, 15) in response to hormones, mitogens, and cAMP have been reported. Extensive analyses of the protein kinases responsible for site-specific phosphorylation of S6 have been carried out (13, 14); less is known of the protein kinases involved in phosphorylation of other translational components. To analyze the function of phosphorylation in the regulation of protein synthesis, it is essential to identify the protein kinases modifying initiation and elongation factors. Thus, a systematic examination of the phosphorylation of seven highly purified initiation factors (2, 3, 4A, 4B, 4C, 4D, 4F) and two elongation factors from rabbit reticulocytes has been completed with seven different protein kinases. The latter include the cAMP-dependent protein kinase, S6 kinase from bovine liver (an active form of protease activated kinase II), mitogen-stimulated S6 kinase from insulin-stimulated 3T3-L1 cells, Ca²⁺, phospholipid-dependent protein kinase, protease-activated kinase I, casein kinase I, and casein kinase II. These studies have been carried out with levels of purified protein kinase sufficient to prevent indiscriminate, nonselective phosphorylation, yet which allow significant incorporation of phosphate into sites modified in vivo by the same enzyme. This technique has been used successfully in our laboratory with a number of different substrates, and sites phosphorylated in vitro are identical with those modified in vivo (16–19). In this study, the four initiation factors phosphorylated in vivo, eIF-2, eIF-3, eIF-4B, and eIF-4F, are specifically phosphorylated in vitro on the same subunits by three or more of the multipotential protein kinases.

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‡The abbreviations used are: eIF, eukaryotic initiation factor; EF, elongation factor, protein kinase C, Ca²⁺, phospholipid-dependent protein kinase; TPA, 12-O-tetradecanoylphorbol-13-acetate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylenediaminetetraacetic acid.

§R. C. Venema and J. A. Traugh, unpublished observations.
EXPERIMENTAL PROCEDURES

RESULTS AND DISCUSSION

The phosphorylation of highly purified initiation and elongation factors from rabbit reticulocytes by catalytic amounts of seven different protein kinases has been systematically examined. With constant amounts of protein kinase and factor, differences in the phosphorylation of each factor and the specificity of the protein kinases were determined. Phosphorylation was carried out by incubation of each factor with \( \gamma^{32P} \)ATP in the presence or absence of each protein kinase. The protein kinases were also incubated alone and with an artificial substrate, to quantify protein kinase activity and to ensure the enzymes were fully active. Reaction mixtures were analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate, and the phosphorylated subunits were identified by autoradiography (Fig. 1). Phosphorylation was specific, and only the subunits modified in vivo were modified in vitro. Care was taken to ensure that the conditions and concentration of enzymes were sufficient to prevent nonspecific phosphorylation and to allow significant amounts of phosphate to be incorporated, as described previously with other substrates (16–19).

Four initiation factors (2, 3, 4B, and 4F) previously shown to be phosphorylated in vivo (1, 5, 6), were significantly phosphorylated in vitro (≥0.3 mol of phosphate/mol of factor) by three or more multipotential protein kinases (Table I). Protein kinase C and the S6 kinase from liver, an active form of protease-activated kinase II, phosphorylated all four factors. Casein kinase II and protease-activated kinase I significantly modified three of the four factors. The cAMP-dependent protein kinase significantly phosphorylated eIF-4B and, to a lesser extent, eIF-3. Casein kinase I modified only eIF-4B, while the mitogen-stimulated S6 kinase, isolated from 3T3-L1 cells stimulated with insulin, was specific for ribosomal protein S6 and did not modify any of the factors. No significant incorporation of phosphate into eIF-2a or -\( \gamma \), eIF-4A, eIF-4C, eIF-4D, or the two elongation factors was observed.

Tryptic phosphopeptide maps of the initiation factors showed that each of the protein kinases phosphorylated distinct residues in the individual proteins or subunits. Although there was some similarity between protein kinase C and the S6 kinase from liver with some of the factors, the patterns were distinct with others. Phosphopeptide maps of eIF-4F (p25 and p220) phosphorylated in reticulocytes in response to TPA show the same sites are modified in vitro by protein kinase C.1 Similar specificity of phosphorylation has been observed with the protein kinases which modulate ribosomal protein S6 (14). Thus, it would be expected that other sites phosphorylated on eIF-4F and other initiation factors would also be representative of physiological conditions with the correct stimuli.

It is possible that interactions between initiation factors and other components of the initiation complex affect the rate and/or extent of phosphorylation of the factors or ribosomal proteins. These interactions may differ in response to different physiological signals that can alter the activities of specific protein kinases and consequently the sites and extent of phosphorylation. Rychlik et al. (3) have identified the single site in eIF-4E (p25 of eIF-4F) phosphorylated in HeLa cells.

1 Portions of this paper (including "Experimental Procedures," part of "Results," and Figs. 2–6) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
Phosphorylation of Translational Initiation Factors

Table I

<table>
<thead>
<tr>
<th>Initiation factor</th>
<th>Subunit M₀ x 10⁻¹⁸</th>
<th>Protein kinase C</th>
<th>S6 kinase</th>
<th>Protease-activated kinase I</th>
<th>cAMP-dependent protein kinase</th>
<th>Casein kinase II</th>
<th>Casein kinase I</th>
<th>Mitogen-stimulated S6 kinase</th>
</tr>
</thead>
<tbody>
<tr>
<td>eIF-2</td>
<td>52</td>
<td>2.2</td>
<td>1.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>eIF-3</td>
<td>170</td>
<td>0.3</td>
<td>-</td>
<td>0.4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>eIF-4B</td>
<td>120-130</td>
<td>0.6</td>
<td>0.4</td>
<td>-</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>eIF-4F</td>
<td>80</td>
<td>0.4</td>
<td>0.7</td>
<td>1.0</td>
<td>0.6</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>3.2</td>
<td>0.8</td>
<td>1.9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*<, < 0.3 mol/mol incorporated. No significant phosphorylation of eIF-4A, eIF-4C, eIF-4D, EF-1, or EF-2 was observed.

Factors is less clear, it is not possible to know what type of control might be exerted by modifying the activity of eIF-3 or eIF-4B, but based upon their general utilization in the 80 S initiation complex pathway, one might expect eIF-3 to be involved in quantitative regulation while eIF-4B might be involved in qualitative (mRNA-specific) regulation. It should also be noted that the multipotent protein kinases used in this study did not modify eIF-2α or γ, eIF-4A, eIF-4C, eIF-4D, EF-1, or EF-2. As eIF-2α, eIF-1, and EF-2 have been observed to be phosphorylated in vivo, additional special circumstances (and other protein kinases) are also operative in effecting control of translation.

Evidence has accumulated implicating initiation as an important site in regulation of translation by phosphorylation. Under conditions of heme deprivation, phosphorylation of the α subunit of eIF-2 culminates in inhibition of hemoglobin synthesis (7, 8). Early responses to growth promotion may also be controlled, at least in part, through initiation (14). Serum stimulation of quiescent cells enhances migration of mRNA into polysomes at early times (44, 45). Coincident with stimulation of polysome formation by growth-promoting compounds, insulin and serum, is the enhanced phosphorylation of ribosomal protein S6 (46, 47). Insulin, serum, and TPA also stimulate the multiple phosphorylation of eIF-4B (5). Addition of serum (48, 49) or epidermal growth factor (49) to quiescent Swiss 3T3 cells stimulates the synthesis of a few specific proteins, and increased synthesis is independent of the level of mRNA at early times.

From the data reported here, it would be expected that the differences in translation observed with differentially phosphorylated 40 S ribosomal subunits (49, 50) would be enhanced by the concomitant site-specific phosphorylation of initiation factors. Stimulation of S6 kinase activity is an early event in mitogen action, and protein kinase C is activated by mitogenic hormones and tumor-promoting phorbol esters. Both protein kinases significantly and differentially phosphorylate in vitro all four of the initiation factors modified in vivo. Thus, following growth stimulation, phosphorylation of S6 would be coincident with changes in phosphorylation of initiation factors. We predict that these phosphorylation events act in concert to promote stimulation of translation of specific mRNAs.

The purpose of these experiments is to identify most, if not all, of the protein kinases modifying the individual factors and to determine the extent of coordinate phosphorylation as a preliminary to examining the effects on protein synthesis. Except for the specific phosphorylation of eIF-2α by the heme-controlled repressor and the double-stranded RNA-activated inhibitor (which results in inhibition of protein synthesis through formation of an inactive eIF-2(αP)-GDP-

References

Phosphorylation of Translational Initiation Factors


SUPPLEMENTARY MATERIAL

COMPARATIVE ANALYSIS OF PHOSPHORYLATION OF TRANSLATIONAL INITIATION AND ELONGATION FACTORS BY SEVEN PROTEIN KINASES

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Figure 1. Two-dimensional tryptic phosphopeptide maps of eIF-4B phosphorylated by six different protein kinases.

Figure 2. Two-dimensional tryptic phosphopeptide maps of eIF-4B phosphorylated by six different protein kinases.

Figure 3. Rate of dephosphorylation of eIF-4B.

Figure 4. Two-dimensional tryptic phosphopeptide maps of eIF-3 phosphorylated by six different protein kinases.

Figure 5. Two-dimensional phosphopeptide maps of eIF-4B phosphorylated by six different protein kinases.