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Evidence for a second phosphorylation site on eIF-2α from rabbit reticulocytes

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Ser 51 in the NH_2-terminal sequence of the α-subunit of eukaryotic peptide initiation factor 2 (eIF-2) has been identified as a second phosphorylation site for the heme-controlled eIF-2α kinase from rabbit reticulocytes. Increased phosphorylation of this serine relative to the previously described phosphorylation site (Ser 48) is observed when the kinase reaction is carried out in the presence of the α-subunit of spectrin. A synthetic peptide corresponding to eIF-2α(41–54) is phosphorylated only in Ser 51 by the eIF-2α kinase.

Protein synthesis; Peptide initiation; Protein kinase; Phosphorylation site; Amino acid sequence

1. INTRODUCTION

The importance of eIF-2α phosphorylation for translational control in mammalian reticulocytes and other cells has been reviewed (cf. [1]). Recently we determined the NH_2-terminal amino acid sequence of HPLC-purified eIF-2α through Arg 52 to be: Pro-Gly-Leu-Ser-X-Arg-Phe-Tyr-Gln-Ser-Lys-Phe-Pro-Glu-Val-Gln-Asp-Val-Val-Met-Val-Asn-Met-Arg-Ser-Ile-Ala-Glu-Met-Gly-Ala-Tyr-Val-Ser-Leu-Leu-Glu-Tyr-Asn-Asn-Ile-Glu-Gly-(Arg)-Ile-Leu-Leu-Ser_{48}-Glu-Leu-Ser_{51}-Arg [2]. The major phosphorylation sites for the reticulocyte heme-controlled kinase were recovered in the tryptic derivative corresponding to eIF-2α(45–52) [2] containing two possible serine phosphorylation sites. Ser 48 was identified as a phosphorylation site by chymotryptic subdigestion of both whole eIF-2α and a tryptic fragment termed the T-44 peptide. In these experiments Ser 51 could not be unequivocally excluded as a second phosphorylation site. Here we show that Ser 51 of eIF-2α can be phosphorylated by the eIF-2α kinase and that the extent of Ser 51 phosphorylation is greater in the presence of a spectrin subunit peptide. This serine residue was found to be the primary phosphorylation site in a synthetic peptide analog of eIF-2α corresponding to the sequence eIF-2α(41–54). The two additional arginine residues at positions 53 and 54 were deduced from the rat and human cDNA sequences [3].

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Abbreviations: eIF-2α, α-subunit of eukaryotic initiation factor 2; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid

2. MATERIALS AND METHODS

2.1. *Synthesis of a peptide analog of eIF-2α*

The peptide Ile-Glu-Gly-Arg-Ile-Leu-Leu-Ser-Glu-Leu-Ser-Arg-Arg-Arg corresponding to

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eIF-2α(41–54) [2,3] was synthesized as the COOH-terminal amide using the Merrifield procedure [4] on an Applied Biosystems 430A peptide synthesizer. Purification by chromatography on an Amicon C_{18} reversed-phase column (250 Å pore size) was carried out, using the elution conditions in [5].

2.2. *Phosphorylation of eIF-2 and isolation of phosphorylated eIF-2α*

Preparation of eIF-2 from the 0.5 M salt wash fraction of rabbit reticulocytes has been described [6]. The reticulocyte heme-regulated eIF-2α kinase was purified through the preparative gel electrophoresis step [7]. Spectrin was isolated from a washed rabbit reticulocyte membrane fraction by the method of Litman et al. [8] then chromatographed on calmodulin-Sepharose (Sigma, St. Louis, MO) to separate its α- and β-subunits [9]. Phosphorylation of eIF-2 was carried out as described [7]. For the experiment described below, about 0.8 mg eIF-2 was phosphorylated for 20 min at 35°C in a total volume of 0.5 ml with 40 μg eIF-2α kinase, 150 μg α-spectrin and 0.1 mM [γ-^{32}P]ATP (3 Ci/mmol). After incubation an equal volume of 6 M guanidine-HCl, 2 mM EDTA and 1% (v/v) TFA was added and the sample loaded onto a wide-pore 0.46 × 25 cm C_8 HPLC column (Bakerbond, J.T. Baker) fitted with a Brownlee RP 300 C_8 guard column. The column was washed with 0.1% TFA before proteins were eluted with an acetonitrile gradient. The details of the reversed-phase HPLC, including the elution position of phosphorylated and unphosphorylated eIF-2α, have been reported [2].

2.3. *Phosphorylation of the synthetic peptide*

The synthetic eIF-2α(41–54) peptide (71 μg) was phosphorylated with 21 μg eIF-2α kinase and 0.25 mM [γ-^{32}P]ATP (0.7 Ci/mmol) for 3.5 h at 35°C in a total volume of 300 μl. The reaction mixture was adjusted to contain 1% TFA and 1 mM EDTA, then the peptide was reisolated by HPLC as described above for eIF-2α.

For K_m determination reaction mixtures were incubated in a total volume of 30 μl for 30 min at 35°C (the reaction rate was constant for at least 2 h) containing 0.12 μg eIF-2α kinase and substrate concentrations from 16 to 266 μM with 0.25 mM [γ-^{32}P]ATP (about 0.2 Ci/mmol). Reactions were terminated by adding acetic acid to give 30%. Aliquots of 40 μl were pipetted onto Whatman P81 paper squares and their radioactivity determined as described [10].

2.4. *Protease digestion and separation of phosphopeptides*

Conditions for trypsin digestion of eIF-2α have been reported previously [2]. Incubation with trypsin was done for 20 min at 35°C after which time the sample was made 3 M in guanidine-HCl, 1 mM in EDTA and 1% in TFA and loaded and chromatographed on the C_8 Bakerbond column. The isolated tryptic phosphopeptides or the phosphorylated synthetic eIF-2α(41–54) were digested further with *Staphylococcus aureus* V_8 protease (Sigma).

Incubation was carried out in a total volume of 100 μl of 0.1 M NH_4HCO_3, pH 7.8, containing 0.5–1.5 nmol phosphopeptide and 2 μg V_8 protease at 35°C for the times indicated in the figure legends. V_8 incubation mixtures were made 1% in TFA and 1 mM in EDTA and loaded on the C_8 HPLC column equilibrated and then developed as described above. Fractions of 1 ml were collected and their radioactivity determined by Cerenkov counting or from an aliquot by liquid scintillation counting in 10% (v/v) Biosolve as indicated.

3. RESULTS AND DISCUSSION

3.1. *Tryptic derivatives of eIF-2α phosphorylated in the presence of spectrin*

The isolated α- or β-subunit of erythroid spectrin or certain of their large peptide derivatives cause an increase in the extent of eIF-2α phosphorylation by the reticulocyte heme-controlled kinase (Kudlicki et al., submitted). In the experiments described in figs 1 and 2 eIF-2 was extensively phosphorylated by this enzyme in the presence of α-spectrin. The phosphorylated eIF-2α subunit was isolated, subjected to tryptic digestion for 20 min and the tryptic phosphopeptides resolved by HPLC. Two major phosphopeptide peaks were observed, one corresponding to the previously defined T-44 peptide [2]. The second peak which eluted at 39% acetonitrile (termed T-39; fig.1) was relatively diminished when phosphorylation was carried out in the absence of

spectrin (fig.1, inset). The T-39 peptide was determined to have the same NH_2-terminal sequence as the T-44 peptide (residues identified Ser-Ile-Ala-Glu-Met-Gly-Ala-Tyr-Val-Ser-Leu-Leu) which suggests the possibility that T-39 might represent an extended or more extensively phosphorylated form of the T-44 sequence.

3.2. *Identification of Ser 51 of eIF-2α as a phosphorylation site for the reticulocyte heme-controlled kinase*

To investigate further the phosphorylation status of Ser 48 and 51 in the T-44 and T-39 sequences, subdigestion with protease V_8 from *Staphylococcus aureus* was performed. A potential cleavage site for V_8 protease occurs within the T-44 peptide at Glu 49 that would separate Ser 48 and Ser 51. Both T-39 and T-44 were subdigested separately with V_8 protease and the resulting phosphopeptides were analyzed by HPLC. The V_8 digest of the T-39 peptide contained two phosphopeptides that eluted at about 44 and 9% acetonitrile, termed the V-44 and V-9 peptides, respectively, with little radioactivity eluting at the T-39 position (fig.2A). The amino acid sequence of the V-9 peptide was determined to be Leu-Ser-Arg, which corresponds to the eIF-2α(50–52) sequence and includes Ser 51, which thus appears to have been phosphorylated in the original T-39 peptide. V_8 protease digestion of the T-44 peptide generates one prominent phosphopeptide eluting in a position similar to that of V-44 and only a trace of V-9 (fig.2B). This suggests that the T-44 peptide was not phosphorylated appreciably at Ser 51.

The V-44 phosphopeptides were subjected to exhaustive trypsin digestion and reanalyzed by HPLC. A major phosphopeptide eluting at 16% acetonitrile (T-16) was detected together with a trace amount of the later eluting T-26 derivative [2] that would have been generated from any T-44/T-39 peptides undigested by the V_8 protease (not shown). The NH_2-terminal sequence of the T-16 peptide was determined to be Ile-Leu-Leu-Ser... corresponding to the sequences of eIF-2α from Ile 45 to Ser 48 and presumably terminating at Glu 49, thus confirming that Ser 48 is also a phosphorylation site, as reported earlier [2]. Considered together these results suggest that the T-39 peptide differs from T-44 in that it is phosphorylated at both Ser 48 and Ser 51, whereas

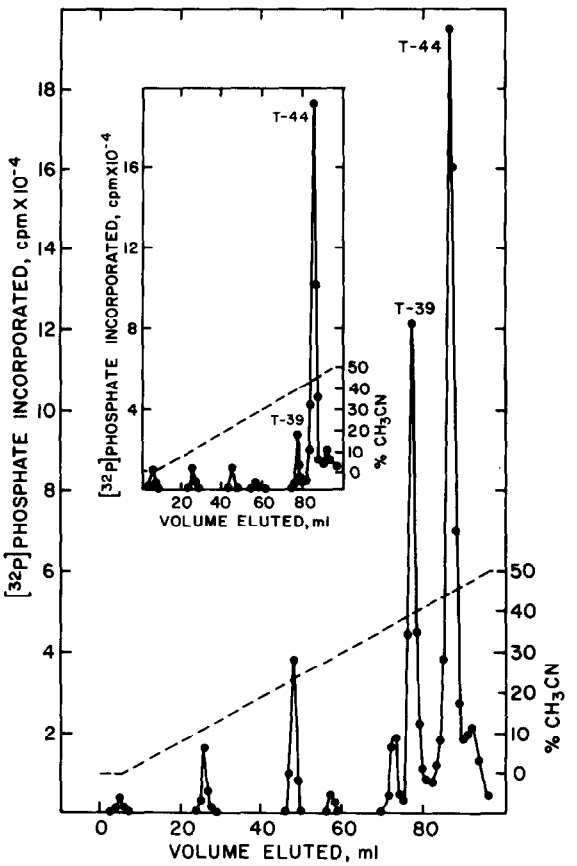

Fig.1. Tryptic phosphopeptides generated from phosphorylated eIF-2α. Phosphorylation of eIF-2 was carried out by the reticulocyte heme-regulated kinase in the presence of α-spectrin using [γ-^{32}P]ATP as described in section 2. The phosphorylated α-subunit of eIF-2 was separated from the β- and γ-subunits and unphosphorylated α-subunit by HPLC as described in [2]. About 30 μg phosphorylated eIF-2α was incubated with 5 μg trypsin for 20 min as in [2], then the resulting peptides were separated by HPLC using a reversed-phase C_8 column. Radioactivity of the resulting fractions was determined by Cerenkov counting. Inset: tryptic phosphopeptides obtained under similar conditions except that the phosphorylation reaction was carried out in the absence of α-spectrin (cf. [2]).

the T-44 peptide appears to be phosphorylated predominantly at Ser 48.

Most of the phosphopeptides evident in fig.1 were tryptic variants containing the Ser 48/Ser 51 sites. However, analysis of a minor phosphopeptide fraction eluting at ~15% acetonitrile, which was more apparent in 12 h digests and was variable

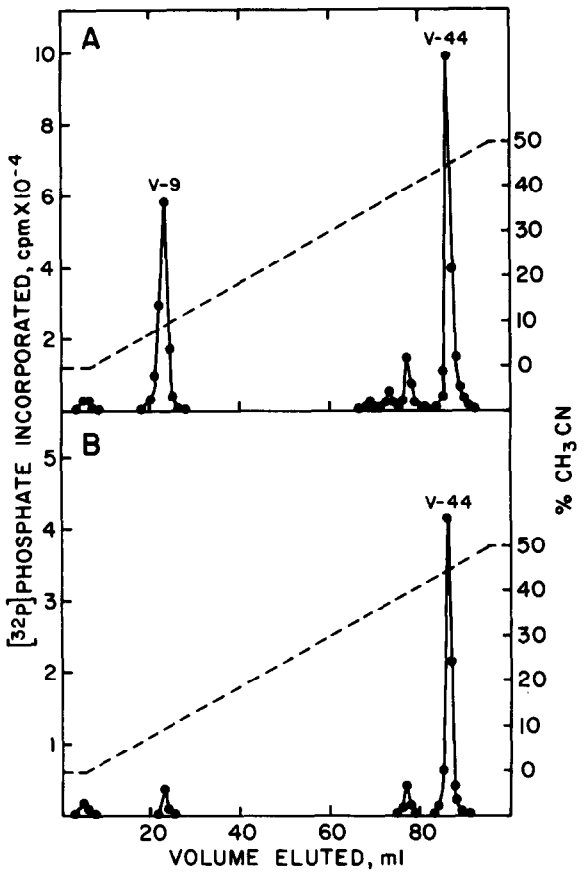

Fig.2. V_8 peptides generated from the T-39 and T-44 tryptic phosphopeptides of eIF-2α. The T-44 and T-39 tryptic peptides (see fig.1) were digested separately with V_8 protease under the conditions given in section 2. The resulting peptides were fractionated by HPLC and radioactivity determined as described for fig.1. (A) V_8 peptides derived from T-39 after 3.5 h incubation. (B) V_8 peptides derived from T-44 after 10 h incubation.

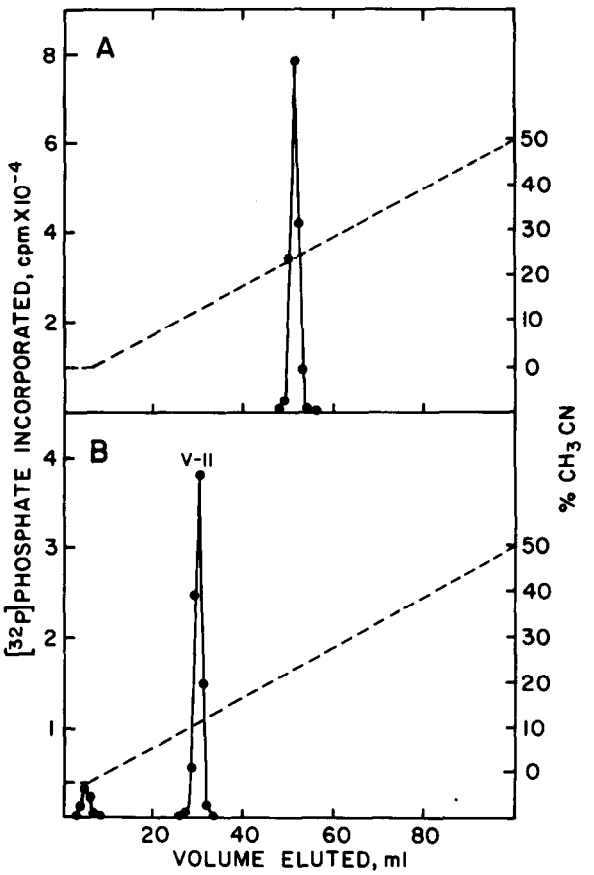

Fig.3. Phosphorylated synthetic eIF-2α(41–54) corresponding to the eIF-2α sequence from Ile 41 to Arg 54. The synthetic peptide was phosphorylated with [γ-^{32}P]ATP by the eIF-2α kinase as described in section 2, then analyzed by HPLC as described in fig.1. (A) HPLC of the phosphorylation reaction mixture (see text). A large radioactive peak of ATP at 0% acetonitrile is not shown. Fractions were collected after the onset of the gradient and their radioactivity determined by liquid scintillation counting from a 50 μl aliquot. (B) HPLC of V_8 digest of the phosphorylated synthetic peptide. Fractions from A containing the phosphopeptide were pooled, taken to dryness, and then the peptide was digested with V_8 protease as described in section 2 and analyzed. Radioactivity was determined as in A with 25-μl aliquots of the resulting fractions.

in amount (not shown), gave the sequence $^{Thr}{}_{Val}{}^{Leu}{}_{Ser}$. A sequence Thr-Val-Tyr-Ser is found as amino acids 106–109 in both rat and human eIF-2α [3], whereas Ser-Val-Leu-Ser occurs in human eIF-2α only at positions 248–251 [3]. It is not clear which amino acids in these sequences are phosphorylated. However, they present the possibility of additional phosphorylation sites in eIF-2α not restricted to the amino-terminal sequence and possibly involving phosphorylation by another contaminating kinase.

3.3. *Phosphorylation of a synthetic peptide containing the eIF-2α phosphorylation sites*

A peptide corresponding to the amino acid sequence of eIF-2α(41–54) which includes the Ser 48 and Ser 51 phosphorylation sites has been syn-

thesized. The peptide was phosphorylated by the eIF-2α kinase with a K_m of 80 μM. The specific activity of the enzyme with this substrate was determined to be approx. 5 nmol phosphate incorporated/min per mg protein. The phosphorylated peptide eluted as a single radioactive peak from a reversed-phase HPLC column at 24% acetonitrile (fig.3A). Digestion of this peptide with V_8 protease followed by HPLC analysis gave a single symmetrical peak of radioactive material that eluted at 11% acetonitrile (fig.3B). The amino acid sequence of this peptide was determined to be Leu-Ser-Arg-Arg-Arg, corresponding to the eIF-2α sequence around Ser 51. This must have been the only site phosphorylated since there is no indication in the elution profile of a phosphopeptide containing Ser 48. Recovery of radioactivity was over 90%. Including α-spectrin in the phosphorylation reaction did not affect the results presented in fig.3. No phosphorylation of Ser 48 was observed under these conditions. Apparently, in the synthetic peptide corresponding to eIF-2α(41–54) Ser 51 is the only phosphorylation site for the heme-controlled eIF-2α kinase. Thus features of eIF-2α besides its primary sequence from amino acids 41 to 54 must convert Ser 48 to a predominant phosphorylation site for the heme-controlled eIF-2α kinase.

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