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Generation of a Mutant Form of Protein Synthesis Initiation Factor eIF-2 Lacking the Site of Phosphorylation by eIF-2 Kinases

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The phosphorylation of the α-subunit of initiation factor eIF-2 leads to an inhibition of protein synthesis in mammalian cells. We have performed site-directed mutagenesis on a cDNA encoding the α-subunit of human eIF-2 and have replaced the candidate sites of phosphorylation, Ser-48 and Ser-51, with alanines. The cDNAs were expressed in vitro by SP6 polymerase transcription and rabbit reticulocyte lysate translation, and the radiolabeled protein products were analyzed by high-resolution two-dimensional gel electrophoresis. The wild-type and Ser-48 mutant proteins became extensively phosphorylated by eIF-2 kinases present in the reticulocyte lysate, and when additional heme-controlled repressor or double-stranded RNA-activated kinase was present, phosphorylation of the proteins was enhanced. The Ser-51 mutant showed little covalent modification by the endogenous enzymes and showed no increase in the acidic variant with additional eIF-2 kinases, thereby suggesting that Ser-51 is the site of phosphorylation leading to repression of protein synthesis.

Protein synthesis initiation factor eIF-2 promotes the binding of the initiator tRNA (Met-tRNA$_i$) to 40S ribosomal subunits during the initiation phase of protein synthesis (11). The factor comprises three nonidentical subunits: α (36 kilodaltons [kDa]), β (38 kDa), and γ (52 kDa). Phosphorylation of the α-subunit is correlated with the inhibition of protein synthesis in many systems, including hemin deprivation in rabbit reticulocyte lysates, interferon treatment followed by viral infection, serum deprivation, and other environmental insults (for reviews, see references 13 and 14). The phosphorylation of eIF-2 interferes with the eIF-2B-catalyzed guanine nucleotide-exchange reaction required for efficient recycling of eIF-2 after each round of initiation. Two highly specific protein kinases, the hemin-controlled repressor (HCR) and the double-stranded RNA-activated inhibitor (DAI), apparently phosphorylate the same site in the α-subunit of eIF-2 (eIF-2α), as determined by phosphopeptide analyses (5, 6, 15). The target amino acid is serine (18), which appears to be located near the N or C terminus of the protein, since mild tryptic hydrolysis generates 34- and 4-kDa peptide fragments, with only the latter containing phosphoserine (20).

We have recently cloned and sequenced human and rat cDNAs encoding the eIF-2α subunit (4). The deduced protein sequence of 315 amino acids lacks serine residues in the C-terminal 63 amino acids, thereby suggesting that the 4-kDa phosphopeptide is generated from the N terminus. There are six serine residues within the first 60 amino acids of the N-terminal sequence that could serve as the target site for the kinases. To define the site of phosphorylation, we initiated a project to mutate each of the serine codons to those coding for alanine and then test the resulting protein products as substrates for HCR and DAI. In the course of this work, Wettenhall et al. (19) reported Edman degradation analyses of eIF-2α phosphorylated in vitro with HCR and concluded that Ser-48 is a phosphorylation site. Since these workers (9) subsequently showed that a synthetic peptide corresponding to eIF-2α (positions 41 to 54) is phosphorylated only at Ser-51 and since the work of Colthurst and co-workers (1) indicated that Ser-51 may be the sole site of phosphorylation, we focused on both Ser-48 and Ser-51.

The human cDNA insert in pHh2a-1 (4) was excised and cloned into the *Eco*RI and *Hin*cII site of pSP65 (10) to generate pSP65-2a. The gapped heteroduplex method described by Strauss et al. (17) was used to generate plasmids containing mutations at either Ser-48 or Ser-51 (Fig. 1). Both mutations resulted in the substitution of an alanine for a serine residue. Mutations were selected by screening with oligonucleotides and by restriction enzyme digestions. The Ser-48 mutation was designed to destroy a *Dde*I restriction site, whereas the Ser-51 mutation generated a *Hae*III site. The structures of the Ser-48 and Ser-51 mutant cDNAs were confirmed by sequencing the relevant portions of the DNAs (data not shown).

To evaluate whether or not the mutant forms of eIF-2α can serve as substrates for the eIF-2α protein kinases, the wild-type and mutant cDNAs were transcribed in vitro with SP6 polymerase (10) and the resulting uncapped mRNAs were translated in mRNA-dependent, hemin-supplemented rabbit reticulocyte lysates (7) in the presence of [^{35}S]methionine. The de novo-synthesized eIF-2α protein products were analyzed by high-resolution two-dimensional gel electrophoresis (3), which resolves each of them into two forms: a basic form corresponding to the nonphosphorylated species and an acidic form which comigrates with and corresponds to the phosphorylated form, as demonstrated previously (4). Each of the SP6 transcripts was translated in the reticulocyte lysate either without exogenous kinases (control rows) or with addition of exogenous kinases during translation (+ DAI or + HCR rows in Fig. 2). In the first set (upper two rows), translation was carried out for 35 min, with DAI and reovirus double-stranded RNA added at 20 min (+ DAI panels). In the second set (lower two rows), translation was carried out for 60 min, with HCR added at 45 min (+ HCR panels). A substantial amount (ca. 50%) of the Ser-48 mutant protein was present in the more acidic spot in control reactions, and addition of either DAI or HCR led to a further increase in the acidic versus basic variant. The Ser-51 mutant, on the other hand, exhibited only a minor amount (ca. 10 to 25%) of the acidic variant, and addition of DAI or

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SER-48 MUTATION: ATT CTT CTT GCt GAA TTA TCC AGA CGA CGT ATC
Ile Leu Leu ALA Glu Leu Ser Arg Arg Arg Ile
SER-51 MUTATION: ATT CTT CTT AGT GAA TTG GCC AGA CGA CGT ATC
Ile Leu Leu Ser Glu Leu ALA Arg Arg Arg Ile
48 51

FIG. 1. Site-directed mutagenesis of eIF-2α cDNA. The gapped heteroduplexing method of Strauss et al. (17) was used to generate two mutants in pSP65-2a, a pSP65 derivative (10) containing eIF-2α cDNA (4). The mutant cDNAs were cloned into MP19 (12), and the relevant portions of the DNA were sequenced for verification of the nucleotide changes (16). A portion of the DNA sequence of the Ser-48 and Ser-51 mutants and the nucleotide changes is shown. The base changes in the Ser-48 mutant (AG to GC) resulted in the destruction of a *Dde*I site and a change in eIF-2α of Ser-48 to Ala. Similarly, nucleotide changes in the Ser-51 mutant (AT to GG) resulted in the creation of a *Hae*III restriction site and a change in eIF-2α of Ser-51 to Ala.

HCR did not cause an increase in apparent phosphorylation. The wild-type eIF-2α protein behaved like the Ser-48 mutant, showing enhanced phosphorylation when DAI was added. In the control experiment in the HCR series, the extent of wild-type eIF-2α phosphorylation was so great that further phosphorylation with HCR was not seen. However, when the time of translation was reduced to 30 min, with HCR added at 15 min, increased phosphorylation of the wild-type form also was observed (data not shown). The failure of the Ser-51 mutant form to be further phosphorylated is most readily explained by the hypothesis that Ser-51 is the sole site of phosphorylation for these kinases. The high-level phosphorylation of the Ser-48 mutant form and its increased phosphorylation with DAI or HCR indicates that if Ser-48 is indeed a target site, it is not the only target site for these enzymes.

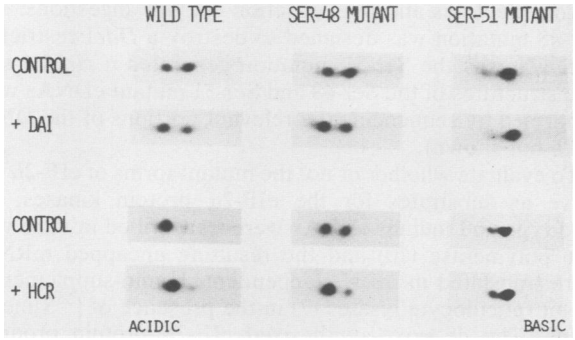

FIG. 2. Analysis of mutant forms of eIF-2α. The cDNAs for wild-type, Ser-48 mutant, and Ser-51 mutant forms were each transcribed by SP6 polymerase and then translated in an mRNA-dependent reticulocyte lysate for 35 (top two rows) or 60 (lower two rows) min. The de novo-synthesized protein products labeled with [^{35}S]methionine were analyzed by isoelectric focusing/sodium dodecyl sulfate-polyacrylamide gel electrophoresis as previously described (3), except that the isoelectric focusing runs were done for 20 h, as the longer runs were shown to give more reproducible focusing of the phosphorylated form of eIF-2α. A segment of the resulting autoradiogram is shown in each row. In the control rows, only eIF-2 kinases endogenous to the reticulocyte lysate were present. In the + DAI rows, DAI and 100 ng of reovirus double-stranded RNA per ml were included in the translation mixes during the last 15 min of incubation. In the + HCR rows, HCR was included in the last 15 min of incubation. All reactions were terminated by the addition of 5 to 10 volumes of the following solution: 1.2 g of urea, 680 μl of double-distilled H_2O, 400 μl of 10% Nonidet P-40, and 100 μl of ampholytes (pH 3 to 10). In all cases, the spots on the left correspond to the phosphorylated form; the spots on the right are the nonphosphorylated form.

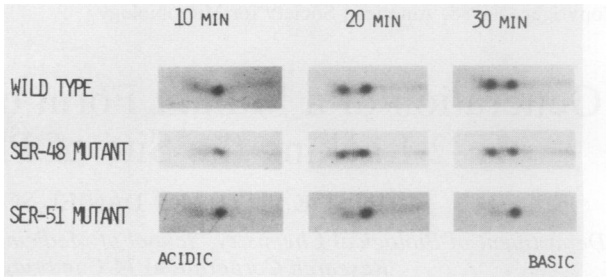

FIG. 3. Kinetics of phosphorylation of mutant forms of eIF-2α. The time course of phosphorylation of de novo-synthesized products labeled with [^{35}S]methionine by eIF-2α kinases present in the reticulocyte lysate was determined. Synthesis of the wild type (top rows), the Ser-48 mutant (middle rows), and the Ser-51 mutant (bottom rows) was stopped at 10, 20, or 30 min as described in the legend to Fig. 1; the ^{35}S-labeled products were then analyzed by isoelectric focusing/sodium dodecyl sulfate-polyacrylamide gel electrophoresis; in all cases, the leftward spots correspond to the phosphorylated form, and the rightward spots correspond to the nonphosphorylated form.

It appears that the Ser-51 mutant form is phosphorylated at low efficiency by a protein kinase at a site other than Ser-51. To investigate the nature of the low-level covalent modification of the Ser-51 mutant, we analyzed the time course of phosphorylation of the wild-type, Ser-48 mutant, and Ser-51 mutant forms (Fig. 3). The phosphorylated form of the wild type (top rows) and the Ser-48 mutant (middle rows) became detectable between 10 and 20 min of incubation. The acidic variant of the Ser-51 mutant became detectable at 10 min of incubation, and the extent of modification did not increase with further incubation. The result suggests that the low-level covalent modification of Ser-51 may not be related to the activation of endogenous HCR or DAI.

To further determine whether the Ser-51 mutant protein was phosphorylated by HCR or DAI, we investigated the effect of 2-aminopurine, an inhibitor of eIF-2 kinases (2), on the phosphorylation of the wild-type, Ser-48 mutant, and Ser-51 mutant forms (Fig. 4). In each case, translation was carried out for 30 min, either without (control rows) or with (+ 2-AP rows) the addition of 10 mM 2-aminopurine. The wild-type and the Ser-48 mutant proteins become exten-

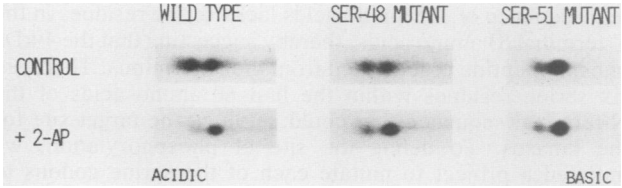

FIG. 4. Effect of 2-aminopurine on the phosphorylation of mutant forms of eIF-2α. mRNAs encoding the wild-type, Ser-48 mutant, or Ser-51 mutant forms of eIF-2α were translated in rabbit reticulocyte lysates for 30 min in the absence (control rows) or presence (+ 2-AP rows) of 10 mM 2-aminopurine. The translations were stopped as described in the legend to Fig. 1; the de novo-synthesized products labeled with [^{35}S]methionine were analyzed by isoelectric focusing/sodium dodecyl sulfate-polyacrylamide gel electrophoresis; the leftward spots correspond to the phosphorylated form, and the rightward spots correspond to the nonphosphorylated form.

sively phosphorylated, and the level of phosphorylation was substantially reduced in the presence of 2-aminopurine. The results are consistent with the view that the acidic variants of the wild-type and Ser-48 mutant proteins are generated by the phosphorylating activity of HCR and DAI. In contrast, only a minor fraction of the Ser-51 mutant form was modified to generate the acidic variant, and the presence of 2-aminopurine did not have any apparent effect on this covalent modification. The lack of reduction in the acidic variant of Ser-51 by 2-aminopurine indicates that the modification of the Ser-51 mutant protein is not dependent on activation of eIF-2 kinases.

The view that Ser-51 is the site of phosphorylation for HCR and DAI is consistent with the results of Colthurst and co-workers (1) obtained by protein chemical analyses. The discrepancy with the results of Wettenhall et al. (19) is difficult to explain but may be due to in vitro phosphorylation conditions. These workers recently reported that in the presence of α- or β-spectrin, HCR preferentially phosphorylates the Ser-51 residue (8). The significance of the acidic modification of Ser-51 mutant is not known, but the kinetics of this covalent modification suggest that the nascent polypeptide is covalently modified at low efficiency by one or more enzymes at some site other than Ser-51. In the complete protein, this site is resistant to further modification, and only the Ser-51 site is used by HCR and DAI.

The Ser-51 mutant DNA may be useful in determining whether or not eIF-2α phosphorylation is necessary for the inhibition of protein synthesis in cells subjected to a variety of physiological conditions. We have placed the wild-type and Ser-51 mutant cDNAs in expression vectors suitable for transfections leading to transient or long-term expression. It is possible that the expressed mutant eIF-2α will inhibit the eIF-2α kinases, thereby sparing the phosphorylation of endogenous eIF-2 and preventing the repression of initiation in such cells. Experiments are in progress to determine the feasibility of studying translational control by such methods.

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