Serine 209, Not Serine 53, Is the Major Site of Phosphorylation in Initiation Factor eIF-4E in Serum-treated Chinese Hamster Ovary Cells*

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Ser-53 has previously been considered the major phosphorylation site in eukaryotic initiation factor (eIF)-4E, and this appeared to be supported by studies using a S53A mutant. Recently, however, several lines of evidence have indicated that Ser-53 might not be the true phosphorylation site. This prompted us to re-examine the phosphorylation site in eIF-4E using factor purified from 32P-labeled, serum-treated Chinese hamster ovary cells. Isoelectric focusing and phosphoamino acid analysis indicated the existence of a single phosphorylated serine. Edman degradation of the major radiolabeled tryptic product from 32P-labeled eIF-4E showed that the phosphorylated site was positioned three residues from the N terminus of this peptide. There are three serines in the sequence of eIF-4E that are three residues away from a tryptic cleavage site (i.e. lysine or arginine). 32P-Labeled eIF-4E was digested with trypsin, Lys-C, or trypsin followed by Glu-C and subjected to two-dimensional mapping; the data obtained eliminated two of these potential sites, leaving Ser-209. Comigration of the synthetic peptide SGS(P)209TTK with the radiolabeled tryptic product on (i) reverse-phase chromatography and (ii) two-dimensional mapping at different pH values confirmed that Ser-209 is the major phosphorylation site in eIF-4E in serum-stimulated Chinese hamster ovary cells.

Phosphorylation of eukaryotic initiation factor (eIF)-4E (also known as eIF-4F) plays a critical role in regulating cellular translation and particularly that of certain mRNAs. Although it remains unclear how phosphorylation of eIF-4E affects its activity, it has been reported to lead to increased association of eIF-4E with high molecular mass complexes, perhaps including the other subunits of eIF-4F. Recent work also suggests that phosphorylation of eIF-4E may increase its affinity for the cap (8), although phosphorylation is certainly not a prerequisite for it to bind.

Several years ago, Rychlik et al. (9) presented evidence that the major site of phosphorylation of eIF-4E in rabbit reticulocytes was Ser-53. This apparent identification of Ser-53 as the site of phosphorylation was backed up by several studies employing a site-directed mutant of eIF-4E in which Ser-53 was altered to alanine. Such a mutant showed a number of differences from the wild-type protein when overexpressed in intact cells or when studied in vitro in that it did not induce cell transformation or promote abnormal morphology (10, 11), and it was not incorporated into 48S initiation complexes (12).

Evidence has, however, been presented that other phosphorylation sites exist in eIF-4E based on the fact that the S53A mutant still underwent phosphorylation (11–14). In addition, Kaufman et al. (13) found no discernible differences in the ability of wild-type or S53A mutant eIF-4E to support translation of selected mRNAs or to become incorporated into the eIF-4F complex. Furthermore, we recently observed that the major insulin-stimulated eIF-4E kinase in Chinese hamster ovary (CHO) cells was able to phosphorylate recombinant eIF-4E (S53A) to a similar extent compared with the wild-type recombinant protein. Taken together, these findings prompted us to reinvestigate the site of phosphorylation in eIF-4E. Our data show that the major site of phosphorylation in eIF-4E in serum-stimulated CHO cells is Ser-209, close to the C terminus of the protein.

**EXPERIMENTAL PROCEDURES**

Materials—m7GTP-Sepharose was from Pharmacia Biotech Inc. Carrier-free [32P]orthophosphate was purchased from Amersham Corp. Chinese hamster ovary (CHO.K1) cells were kindly provided by Dr. L. Ellis (Houston, TX). Materials for tissue culture were obtained from Life Technologies, Inc. Modified trypsin and sequencing-grade Lys-C and Glu-C were from Promega. Microcystin-LR was obtained from Calbiochem. Unless otherwise stated, all other reagents were from Sigma.

Cell Culture, Treatment, and Labeling—CHO.K1 cells were grown and maintained in culture as described by Dickens et al. (15). Cells were seeded at an initial density of 5 × 10^4/60-mm dish and grown to near confluence (3–4 days) in Ham's F-12 medium containing 10% (v/v) fetal calf serum before being "stepped down" to 0.1% serum for 16 h. For 32P labeling, the cells were washed (2 × 5 ml) with phosphate-free Dulbecco's modified Eagle's medium and then incubated for 3 h at 37 °C in 2 ml of the same medium containing 1–1.5 mCi/ml 32P-P, fetal calf serum was then added to a final concentration of 30% (v/v), and the cells were incubated for a further 10 min. Cell extracts were prepared as described in a previous study.

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The abbreviations used are: eIF, eukaryotic initiation factor; CHO, Chinese hamster ovary; m7GTP, 7-methylguanosine triphosphate; HPLC, high pressure liquid chromatography.

2 A. Flynn and C. G. Proud, unpublished data.

3 Early in this study, we learned that Prof. R. E. Rhoads (Shreveport, LA) had also obtained data indicating that Ser-53 was not the major phosphorylation site in eIF-4E.
Phosphorylation Site in eIF-4E

Results and Discussion

As we have previously reported, 4 isoelectric focusing analysis reveals the existence of only two distinct species of eIF-4E in CHO cells as detected by immunoblotting, and treatment of isolated eIF-4E with alkaline phosphatase causes conversion of all the factor to the more basic (i.e. unphosphorylated) form. Fig. 1A shows an immunoblot of eIF-4E from control and serum-treated CHO.K1 cells: in the former case, only 20% of the eIF-4E is phosphorylated, while in serum-treated cells, almost all the factor (~80%) is in the more acidic (phosphorylated) form. These findings are consistent with the data from a range of other cell types indicating that eIF-4E contains one major phosphorylation site (20).

To determine which type of amino acid was phosphorylated in eIF-4E in CHO.K1 cells, serum-starved cells were incubated with radioactive inorganic phosphate and briefly treated with serum, and eIF-4E was then isolated and subjected to phosphoamino acid analysis. Only phosphoserine was observed (Fig. 1B), which is consistent with several other reports using a range of different cell types (see Ref. 20, for a review), although phosphothreonine has been reported in eIF-4E under some conditions (see, for example, Ref. 7).

It thus appeared likely that eIF-4E was phosphorylated at a single serine residue in serum-treated CHO.K1 cells. To investigate further the location of the phosphosericine residue in eIF-4E, radiolabeled factor was isolated from serum-treated cells and subjected to tryptic digestion followed by separation of the resulting peptides by reverse-phase chromatography (Fig. 2). Two separate experiments were performed, and in both cases, one major peak of radioactive material was observed. This peak eluted at ~8% acetonitrile, indicating that the material was very hydrophilic. Some additional radioactive material eluted later, at 20–30% acetonitrile, and may have represented products of incomplete tryptic digestion of radiolabelled eIF-4E. In both experiments, the first peak contained ~75% of the total radioactive material, and this was contained within one fraction, whereas the later material emerged as a broad smear.

Two-dimensional phosphopeptide mapping was performed to characterize radiolabeled material contained in the main peak from reverse-phase chromatography. Fig. 3A (panel i) shows that one positively charged phosphopeptide was obtained when electrophoresis was performed at pH 1.9; this phosphopeptide migrated slightly faster than cyano (which has a net charge of +1 at this pH). The same result was obtained at pH 3.5, except that the peptide and cyano did not migrate as quickly toward the cathode (data not shown). Consistent with the findings of other groups (14, 19, 21), the radiolabeled tryptic product from eIF-4E did not move from the origin on chromatography, again testifying to its extremely hydrophilic nature.

To determine the position of the phosphosericine residue in the tryptic product, manual Edman analysis was performed, with samples being retained after every cycle for further analysis by manual Edman degradation, performed exactly as described earlier (19). An aliquot of starting material and samples from each round of degradation were loaded onto a thin-layer cellulose plate and electrophoresed in one dimension (pH 3.5) for 25 min at 1 kV. 2P was loaded as a standard, and the dried plate was subjected to autoradiography.

Two-dimensional Peptide Mapping—Samples were analyzed by two-dimensional peptide mapping, as described previously (19), at pH 3.5 and 1.9. The thin-layer cellulose plates were dried and either subjected to autoradiography (for 32P-labeled samples) or sprayed with ninhydrin (to detect the unlabeled synthetic phosphopeptide). A mixture of 2,4-dinitrophenyllysine/cyanol (~1 µg of each) was loaded as a marker.

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one-dimensional mapping. The initial peptide and the products obtained after one or two cycles had a net positive charge (Fig. 3B). However, after the third cycle, 75% of the radioactivity no longer migrated with positively charged material, but instead moved in the opposite direction and migrated with inorganic phosphate. Furthermore, the fourth round of degradation yielded [32P]orthophosphate only, with no counts remaining in the position of the original phosphopeptide (data not shown). These data indicate that the third residue in the peptide bears the radiolabel, and given the conclusions drawn from the phosphoamino acid analysis (Fig. 1B), the tryptic peptide therefore contains a phosphoserine at position 3.

There are no sequence data available for eIF-4E from the Chinese hamster. However, there is a very high degree of identity between rabbit, mouse, and human eIF-4E (22–24); the only significant differences are in the extreme N terminus of the factor (residues 12, 16, and 17), while the remainder of the eIF-4E sequence is 99.5% identical. We therefore examined the sequences of these proteins to identify possible candidates for the tryptic peptide obtained in our studies. In each case, there are three serines that lie three residues C-terminal to a tryptic cleavage site (Lys or Arg). Attempts to purify sufficient amounts of the tryptic phosphopeptide from eIF-4E in CHO.K1 cells were unsuccessful. Therefore, to distinguish between these three possible phosphorylation sites, further proteolytic digests were performed. In the case of Ser-64 in the tryptic peptide LIS64K, digestion of the intact32P-labeled factor with Lys-C would yield a much larger and more hydrophobic phosphopeptide (TWQANLRLISK) than that obtained by tryptic digestion, which would migrate quite differently on two-dimensional peptide mapping. However, as shown in Fig. 3A (panel ii), the migration of the phosphopeptide produced with Lys-C was identical to that of the tryptic product at pH 1.9 (and also at pH 3.5; data not shown), ruling out Ser-64 as the phosphorylation site.

In the case of Ser-24 in the tryptic peptide TES24-NQEVANPEHYIK, treatment of the tryptic phosphopeptide with Glu-C would be expected to generate a smaller species with altered mobility on electrophoresis and chromatography. However, Fig. 3A (panel iii) shows that after Glu-C treatment, the migration of the phosphopeptide produced with Lys-C was identical to that of the tryptic product at pH 1.9 (and also at pH 3.5; data not shown), indicating that Ser-24 is not the radiolabeled residue. By a process of elimination, then the phosphorylation site in eIF-4E in CHO cells appears to be Ser-209 in the tryptic pep-

**Fig. 1.** *Analysis of eIF-4E in CHO cells.* A, isoelectric focusing was carried out using eIF-4E isolated from serum-starved CHO.K1 cells that had no further addition (lane 1) or that were re-challenged with serum (10%, v/v) for 10 min (lane 2). Shown is a Western blot; the position of phosphorylated eIF-4E (P) is indicated. B, phosphoamino acid analysis of [32P]-labeled eIF-4E from serum-treated CHO.K1 cells was performed as described under "Experimental Procedures." The positions of migration of phosphoserine (S) and phosphothreonine (T) markers (−1 μg each) are indicated. P, [32P]orthophosphate; o, origin.

**Fig. 2.** *HPLC analysis of the tryptic phosphopeptides from 32P-labeled eIF-4E.* Reverse-phase chromatography on a C18 column was performed for the products of trypsinolysis of [32P]-labeled eIF-4E from serum-treated CHO.K1 cells (A) or for a nonradiolabeled synthetic phosphopeptide, SGS(P)TTK (B), as described under "Experimental Procedures." The flow rate was 0.1 ml/min; 100-μl fractions were collected.

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As a final confirmation of the identity of the phosphorylation site in eIF-4E, a synthetic phosphopeptide (SGS(P)TTK) that corresponds to residues 207–212 of eIF-4E was prepared. This peptide showed identical behavior to that of the tryptic product from \( ^{32}\)P-labeled eIF-4E on (i) reverse-phase chromatography (Fig. 2), (ii) electrophoresis at pH 1.9 (Fig. 3A (panel iv)) or (iii) at pH 3.5 (data not shown), and (iv) thin-layer chromatography (Fig. 3A (panel iv)). This strongly supports the identification of the labeled phosphopeptide and confirms that the phosphorylated residue is indeed Ser-209.

In conclusion, all these data indicate that the major (and probably the only) phosphorylation site in CHO cells is Ser-209. Reappraisal of the role of phosphorylation of eIF-4E is therefore required using mutants based on Ser-209. This work also highlights the need for caution when using Ser to Ala mutants of proteins to investigate the role of phosphorylation, as this approach has given phenotypes not related to phosphorylation in two translation initiation factors, i.e., eIF-4E (discussed above) and eIF-2\(\alpha\) (25, 26).

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