Phosphorylation *in vivo* of non-ribosomal proteins from native 40S ribosomal particles of Krebs II mouse ascites-tumour cells

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Four non-ribosomal proteins from native 40S ribosomal subunits with mol.wts. of 110 000, 84 000, 68 000 and 26 000 were phosphorylated *in vivo* when ascites cells were incubated in the presence of [^{32}P]P_i. The 110 000-, 84 000- and 26 000-dalton proteins are identical with phosphorylated products from native 40S subunits after phosphorylation *in vitro* by a cyclic nucleotide-independent protein kinase. Phosphoserine was the major phosphorylated amino acid of the proteins phosphorylated *in vivo* and *in vitro*.

Initiation factors from rabbit reticulocytes are phosphorylated *in vitro* (Issinger *et al.*, 1976; Traugh *et al.*, 1976; Farrell *et al.*, 1977) and *in vivo* (Benne *et al.*, 1978). However, so far little information exists on the exact location of these phosphorylated protein factors within the cell. For practical reasons, initiation factors are usually isolated from the total postmitochondrial supernatant. This isolation procedure, though very economic, precludes any specific information about the origin of the factors isolated. Since the non-ribosomal protein moiety of native 40S particles has been shown to contain nearly all known initiation factors (Sundkvist & Staehelin, 1975; Freienstein & Blobel, 1975; Thompson *et al.*, 1977), we wanted to find out whether any of these proteins are phosphorylated *in vivo*.

Native 40S particles are mostly devoid of mRNA (Sundkvist & Staehelin, 1975; Safer *et al.*, 1978), but do contain eIF-3, whose binding is considered as one of the first steps in initiation-complex formation. Thus it is speculated that phosphorylation might eventually represent an early event in the initiation of protein synthesis in eukaryotes.

We report here the phosphorylation *in vivo* of four non-ribosomal proteins from native 40S subunits, two of which are identical in size with subunits 2 and 4 from eIF-3.

Experimental

Krebs II mouse ascites-tumour cells were pas-

Abbreviations used: eIF-3, initiation factor 3; eIF-2, initiation factor 2.

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saged in Balb/c-strain mice. The labelling conditions with [^{32}P]P_i were virtually the same as those described by Leader & Coia (1977); 500 ml of ascites cells (10^7 cells/ml) were incubated in the presence of [^{32}P]P_i (0.1 mCi/ml) for 3–12 h at 37°C. The incubation time for the individual experiments was as specified in the legends to Figures.

Native 40S particles were isolated by a modification of the method of Sundkvist & Staehelin (1975). Proteins were separated in one-dimensional polyacrylamide gels (11% or 15% acrylamide) in the presence of sodium dodecyl sulphate (Laemmli, 1970). Gels were run in parallel with proteins of known molecular weights (*Escherichia coli* RNA polymerase β -subunit 165 000, β' -subunit 155 000, β -galactosidase 116 000, phosphorylase *a* 94 000, bovine serum albumin 68 000, fumarase 49 000, carbonic anhydrase 28 000 and cytochrome *c* 12 300).

Phosphorylation of native 40S particles was essentially as described by Issinger & Reichert (1979); 0.3 A_{260} unit of native 40S particles was suspended in the presence of [γ - ^{32}P]ATP and incubated for 15 min at 37°C. The reaction was stopped by the addition of sodium dodecyl sulphate sample buffer [80 mM-Tris/HCl, pH 6.8, 20 mM-dithioerythritol, 4% (v/v) sodium dodecyl sulphate, 20% (v/v) glycerol].

For the determination of phosphorylated amino acids of the proteins phosphorylated *in vivo* and *in vitro*, the non-ribosomal protein moiety was isolated by high-salt treatment. Usually about 26 A_{260} units of native 40S particles were incubated in the presence of 500 mM-KCl at 0°C for about 60 min. This mixture was applied to a linear glycerol gradient

(15–30%, v/v) in 20 mM-Tris/HCl (pH 7.3)/3 mM-MgCl₂/500 mM-KCl. Centrifugation was for 7 h at 40000 rev./min, 4°C, in a Beckman SW 40 rotor. Material sedimenting slower than 18S was pooled, dialysed against 20 mM-NH₄HCO₃ and concentrated by freeze-drying.

The protein fraction obtained (0.5 M-KCl wash from native 40S particles) was hydrolysed in 2 M-HCl at 110°C for 6 h in a sealed glass tube. The hydrolysed sample was then analysed for [³²P]phosphoserine and [³²P]phosphothreonine by ion-exchange chromatography (Dowex 50W-X8) (Olson *et al.*, 1974).

Results and discussion

The phosphorylation experiments *in vivo* were carried out under various conditions with different incubation times. In all cases the overall phosphorylation pattern remained the same; however, quantitative changes were observed (results not

Fig. 1. Radioautographs of the total native-40S-particle proteins phosphorylated *in vivo* (a) and *in vitro* (b). Ascites cells were incubated in the presence of [³²P]P_i and incubated for 3 h on a rotary shaker. Native 40S subunits were isolated and analysed on 11%-polyacrylamide gels in the presence of sodium dodecyl sulphate (a). Phosphorylation conditions *in vitro* were as described in the Experimental section (b). The numbers to the right refer to the mol.wt. ($\times 10^{-3}$) of the phosphorylated proteins.

shown). More detailed studies are necessary to find out what the basis for the observed quantitative changes in the phosphorylation pattern *in vivo* really is.

Fig. 1 shows radioautographs of polyacrylamide gels of the total proteins of native 40S particles from ascites cells. The proteins that are phosphorylated *in vivo* have mol.wts. of 110000, 84000, 68000 and 26000 (Fig. 1a). All four proteins are non-ribosomal in nature, as shown by Issinger & Reichert (1979). When native 40S particles were incubated in the presence of [γ -³²P]ATP or [γ -³²P]GTP, the same proteins that were shown to be phosphorylated *in vivo* also became phosphorylated *in vitro*, with the exception of the 68000-daltons protein (Fig. 1b). The reason for this lack of phosphorylation is unknown. Issinger & Reichert (1979) had shown that the phosphorylation *in vitro* is due to a protein kinase associated with native 40S particles. The protein kinase can use either [γ -³²P]ATP or [γ -³²P]GTP as a phosphate donor. Analysis of phosphorylated amino acids showed serine to be the major phosphate acceptor. Analysis of the phosphoproteins *in vivo* also gave evidence that serine is the major phosphate acceptor (Fig. 2).

It seems likely that the phosphorylated products *in vivo* and *in vitro* from native 40S particles might be phosphorylated by the same enzyme. However, it

Fig. 2. Analysis of phosphorylated amino acids in the non-ribosomal protein fraction from phosphorylated proteins

The high-salt-wash fraction from native 40S particles phosphorylated *in vivo* was hydrolysed in 2 M-HCl and then analysed on a Dowex 50 column (the sample was mixed with 1 mg of O-phospho-DL-serine and O-phospho-DL-threonine in 0.1 M-HCl). Fractions of volume about 0.2 ml were collected. Portions (50 μ l) were removed from each fraction and directly pipetted on to filter paper. The positions of phosphoserine and phosphothreonine were identified by staining the filters with ninhydrin reagent. Finally all filters were counted for radioactivity in a toluene-based scintillation fluid.

cannot be excluded that there are two different protein kinases that phosphorylated the same substrate. Observations supporting such a notion have been made by Ernst *et al.* (1980) for the haem-regulated and double-stranded-RNA-activated eIF-2 kinase. Both kinases phosphorylate the same site of the α -subunit of eIF-2, yet they are distinguishable by immunological means, suggesting that they are indeed two distinct enzymes.

The importance of the phosphorylation of the 26000- and the 84000-daltons proteins cannot be judged, since these proteins have not been assigned any function (Figs. 1a and 1b).

The two other phosphoproteins from native 40S particles phosphorylated *in vivo*, with mol.wts. 110000 and 68000, are of the same size as the subunits 2 and 4 of purified eIF-3. Further evidence that these proteins are identical comes from earlier observations by Issinger *et al.* (1976) and Traugh *et al.* (1976), who had shown that the 68000- and 110000-dalton subunits of eIF-3 can be phosphorylated by a cyclic nucleotide-independent protein kinase. Binding of eIF-3 to 40S particles is supposed to be the first step in the initiation-complex formation (Safer & Anderson, 1978, and references therein). This step is followed by the binding of the ternary complex (eIF-2 • Met-tRNA^{fMet} • GTP) and the subsequent binding of mRNA. Since the proportion of mRNA-containing native 40S complexes is usually below 5% of the total native 40S subunits (Sundkvist & Staehelin, 1975; Safer *et al.*, 1978), phosphorylation of eIF-3 subunits is likely to occur before the binding of mRNA. So far no quantitative determinations of the actual amount of phosphorus associated with eIF-3 bound to native 40S particles have been carried out.

The possible role of this phosphorylation event, however, has to await the elucidation of the specific function of eIF-3 during translation.

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