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SPECIFIC PHOSPHORYLATION OF THE β SUBUNIT OF eIF-2 FACTOR FROM BRAIN BY THREE DIFFERENT PROTEIN KINASES

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The eukaryotic initiation factor 2 (eIF-2) from calf brain has been purified to homogeneity and free of endogenous kinase activity. Phosphorylation of eIF-2 factor has been examined with four different protein kinases. Casein kinase II, calcium/ phospholipid-dependent protein kinase and cyclic AMP-dependent protein kinase from brain, phosphorylate the β subunit of eIF-2, whilst hemin-controlled inhibitor phosphorylate the α subunit of the factor. According to the peptide maps obtained, the phosphorylation sites of the factor by the three β kinases are specific and distinct. These data suggest a different regulation for the β subunit through this modification.

Eukaryotic initiation factor 2 (eIF-2) is involved in the initial steps of peptide chain-initiation, which appears to be a general control point in the short-term control of translation in response to stimuli such as hormone, nutritional conditions, mitogens and serum growth factor, interferon and/or viral infection, fertilization of oocytes and environmental conditions (1).

Initiation factor eIF-2 mediates the binding of the initiator Met-tRNA$_i$ to the small ribosomal subunit 40S via formation of a ternary complex containing GTP. The factor is isolated as a trimeric specie containing non-identical subunits of apparent molecular weights: α(35,000-38,000 dalton), τ(48,000-

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Abbreviations: eIF-2, eukaryotic initiation factor 2; CK II, casein kinase II; PKA, cyclic AMP-dependent protein kinase; PKC, calcium/phospholipid-dependent protein kinase; HCI, hemin-controlled inhibitor; TFA, trifluoracetic acid.

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52,000 dalton) and β(52,000-56,000 dalton) (2). eIF-2 is phosphorylated in its α subunit by two protein kinases: the hemin-controlled inhibitor (HCI) and a double-stranded RNA-activated inhibitor (DAI); this phosphorylation affects the activity of the factor and leads to the inhibition of initiation of protein synthesis (3).

eIF-2 from reticulocytes is also phosphorylated in the β subunit and eIF-2β kinases have also been described: casein kinase II (CK II) (4,5), protease-activated kinase II (6), and calcium/ phospholipid-dependent protein kinase or protein kinase C (PKC) (7). The function of the β subunit and the physiological significance of its phosphorylation has been questioned for many years and still remains unclear.

Previous studies from our laboratory with crude extracts, suggested that eIF-2 from brain tissue might be phosphorylated by CK II, PKC, and cyclic AMP-dependent protein kinase (PKA) (8). In the present work we demonstrate that purified eIF-2 from brain is phosphorylated only in its β subunit by the three above mentioned protein kinases.

MATERIAL AND METHODS

eIF-2 factor from calf brain was purified to homogeneity by introducing several modifications to our previous purification scheme (9), as follows: cIF fraction from 2.6 Kg of calf brain (from a slaughter house) (2.9 g of protein), was fractionated with a 30-70% ammonium sulphate precipitation and dialyzed for 12 h against buffer A (20 mM Tris-HCl, pH 7,6, 1 mM DTT; 0.2 mM EDTA; 1mM phenylmethylsulphonyl fluoride; 10% glycerol) containing 0.2 mM KCl. The second step consists of a heparin-Sepharose chromatography and elution with a KCl (0.2-0.5 M) linear gradient in buffer A. eIF-2 activity eluted in this chromatography represents 55% of the original activity and shows a 35-fold purification. Pooled, active fractions from heparin-Sepharose were adjusted to 0.35 M KCl, applied to a phosphocellulose column and eluted with a KCl (0.35-1.0 M) linear gradient. The recovery and the purification in this step were 24% and 146-fold respectively. Pooled active fractions were concentrated by ultrafiltration and adjusted to 0.35 M KCl before being loaded into a GDP-agarose column and eluted with a KCl (0.35-3.0 M) linear gradient. Although the recovery of activity in this step was very low (1.1%), the eIF-2 factor is purified 308-fold and is completely free of endogenous phosphorylation.

CK II from calf brain was purified by a relative new and simple purification scheme from our laboratory (unpublished results) based on a previously reported scheme used for the enzyme from other sources (10). PKA (catalytic subunit) and PKC from brain were purified by previously described procedures (11,12). Purified CK II, PKC and PKA from brain were completely free of other protein kinase activities. HCI was a gift from Dr. C. de Haro.

eIF-2 phosphorylation was performed in a final volume of 50 µl containing: 25 mM Tris-HCl, pH 7.5; 5 mM $MgCl_2$; 5 mM NaF; 1.5 and 4.5 µg of purified eIF-2 (for electrophoresis and tryptic digestion respectively), 0.5 µg of the correspondent protein kinase and its modulators (0.3 mg/ml phospholipids plus 0.5 mM calcium). After 2 min preincubation at 30°C, the reaction was started by addition of [γ-^{32}P]ATP (100 µM, 5 or 10 µCi) and maintained for 2 min. For SDS-PAGE the reaction mixture was stopped and analyzed directly as previously described (8), and both the silver stained gels and the autoradiographies were scanned at 450 or 800 nm respectively. For tryptic digestion the phosphorylated factor was separated from the reaction mixture by precipitation with 10% trichloroacetic acid (13).

Tryptic digestion was performed in 100 µl of 0.2 M N-ethyl-morpholine buffer, pH.8.2, with a protein:trypsin ratio of 20:1 for 12 h at 37ºC, after which the sample was made 0.1% in trifluoroacetic acid (TFA) and loaded directly onto the HPLC column.

Peptide fractionations were carried out by HPLC chromatography on reversed-phase column. A C-18 Novopack column (5µm, 3.9 x 150 mm) was employed for the separation of tryptic peptides using 0.1% TFA as solvent A and 0.1% TFA in acetonitrile as solvent B. All analyses were carried out at room temperature at a constant flow rate of 0.5 ml/min, and fractions of 1 ml were collected to measure their radioactivity by Cerenkov counting.

RESULTS AND DISCUSSION

eIF-2 from brain tissue isolated and characterized for the first time in our laboratory (9), contained protein kinase activities responsible for its phosphorylation (14). The modified procedure used for purification of eIF-2 from brain in this paper, yields an almost homogeneous protein (99% pure) as determined by SDS-PAGE, completely free of endogenous phosphorylation activity. The purified factor consists of three subunits α, β, and γ with molecular weights of above 38,000, 52,000 and 48,000 respectively (Fig. 1, panel eIF-2).

The densitometric analysis of phosphorylated eIF-2 by the three kinases CK II, PKA and PKC also from brain, shows that all are able to phosphorylate purified eIF-2 specifically only in its β subunit. However the degree of phosphorylation is quite different depending of the kinase used (Fig.1, panels CK II, PKC and PKA). CK II phosphorylation of the β subunit of the factor is much higher than that produced by PKC or PKA with a relative ratio of 17.5:3.7:1.0. The phosphorylation of the factor by PKA, which is hardly visible when compared with CK II or PKC, is confirmed by longer exposure of the autoradiogram (Fig.1, panel PKA "s"). Phosphorylation of the β subunit in the factor from reticulocytes by CK II, PKC and protease-activated

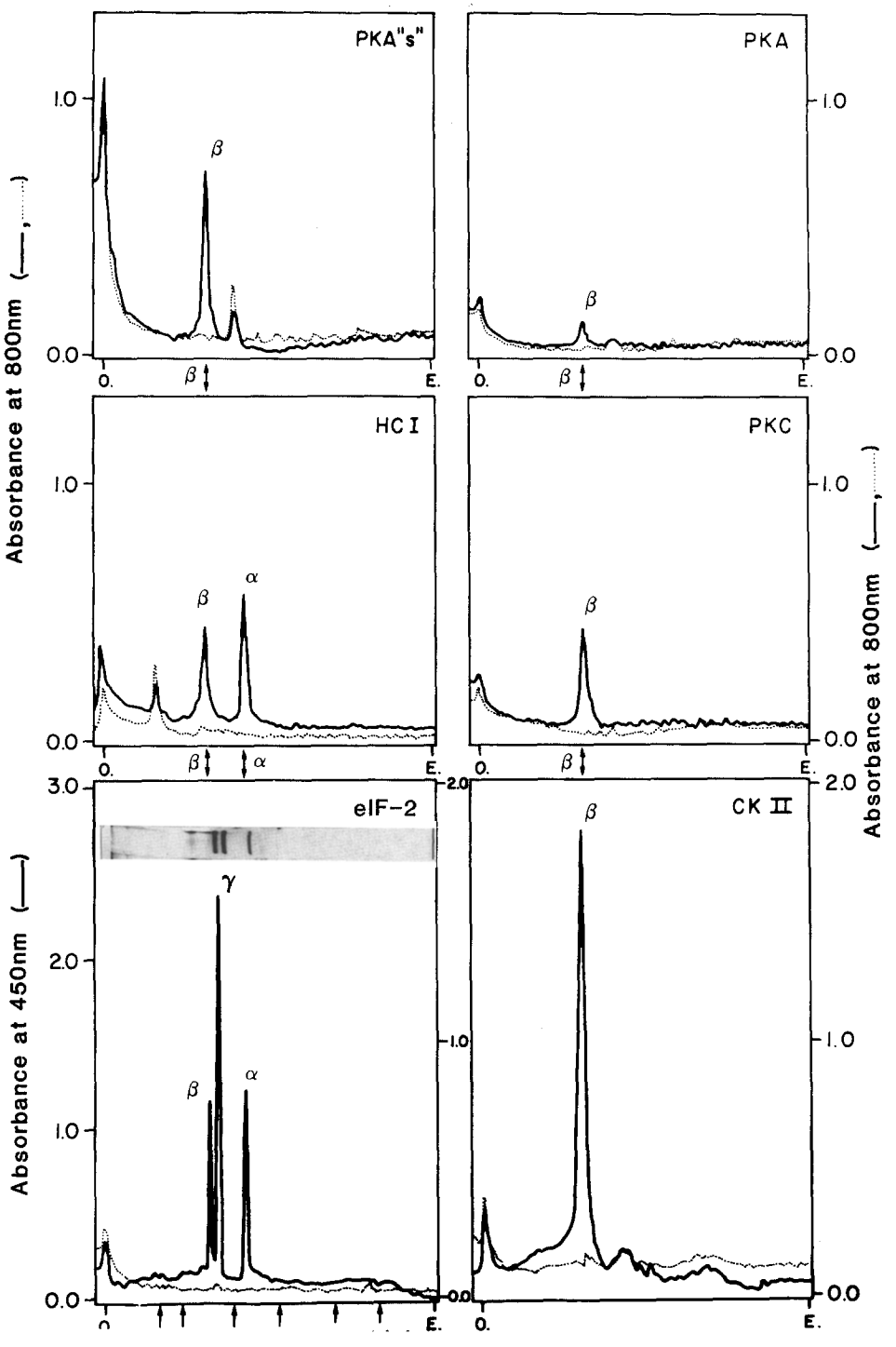

Figure 1. Densitometric scanning from SDS-PAGE of eIF-2 phosphorylated with different protein kinases. eIF-2 panel: (1.5 ug), silver stain (——), autoradiogram (···); the corresponding silver staining is inserted. Panels CK II, PKC, PKA and HCI: autoradiograms in the presence (——) or absence (···) of eIF-2. Panel PKA"s": overexposed panel PKA. Phosphorylated α and β subunits, mobilities of standard molecular weights proteins, O (origin), and E (end), of the gel, are indicated.

kinase II has already been described by other authors (4,7), who discard the possible phosphorylation of the factor by PKA. Our results show a much higher phosphorylation by CK II when compared with PKC and confirm the phosphorylation of the factor from brain tissue by PKA. The phoshorylation of brain factor by PKA is highly interesting and deserves more complete and detailed studies.

Under the same experimental conditions, HCI is able to phosphorylate the α subunit of the factor 2 (Fig.1, panel HCI). This fact indicates, (i) eIF-2 purified from brain is susceptible to be phosphorylated by HCI from rabbit reticulocytes, (ii) CK II, PKC and PKA are specific eIF-2(β) kinases. Phosphorylation of β subunit by HCI (Fig. 1, panel HCI) is due to contamination of the kinase with CK II (Dr. C. de Haro, personal communication).

Isolated eIF-2 from other eukaryotic cells, are also phosphorylated in its α subunit by HCI (3). This result reflects the high degree of similarity in this subunit between factors from different sources and strengthen the possibility that polypeptide chain initiation in nonerythroid cells could be controlled by the state of phosphorylation of the α subunit of eIF-2, as occurs in erythroid cells.

To determine whether phosphorylation by the three eIF-2(β) kinases (CK II, PKC and PKA) was at the same or different sites, the radiolabeled factor from brain was analyzed by HPLC phosphopeptide mapping. As shown on Fig.2, each protein kinase gave a distinctive phosphopeptide pattern. The map of eIF-2 phosphorylated by CK II contained 2 major and 4 minor phophopeptides (h, j and b, e, g, i, respectively), while that obtained by PKC contained one major (h) and four minor (b, c, d, and g) phosphopeptides and the one by PKA gave only four minor phosphopeptides (a, c, d and f). These results demonstrate that the factor from brain is phosphorylated by the three eIF-2(β) kinases in at least one or more different and specific site. Comparing the degree of phosphorylation obtained by the three kinases (Table 1), we observe that the total amount of radioactivity incorporated in the presence of CK II is much higher than that incorporated with PKC or PKA, with a ratio of 20.3: 2.1: 1.0 respectively (Table 1). This ratio is quite similar to that estimated from the scanned densitograms.

Evidence of different phosphopeptides produced from phosphorylated β subunit of eIF-2 from reticulocytes by CK II and

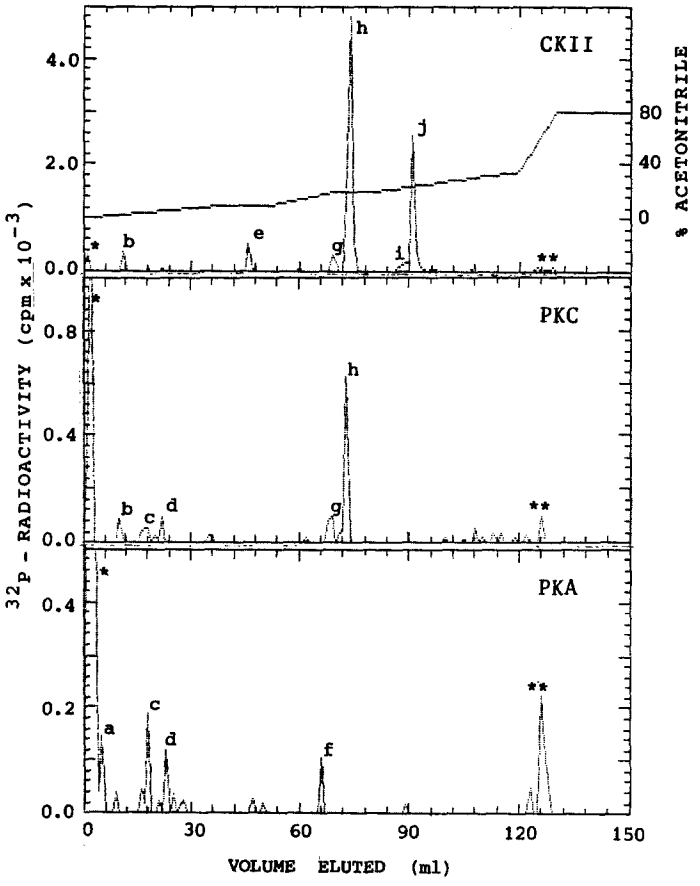

Figure 2. Separation of tryptic digest of phosphorylated eIF-2 by CK II, PKC and PKA kinases. The digested material (4.5 µg of eIF-2 and 0.5 µg of the corresponding kinase) was applied on a C18-Novapack column and the elution of peptides was carried out with a gradient of acetonitrile in 0.1% TFA as indicated in the figure. The radioactivity incorporated in the corresponding kinase in the absence of eIF-2, was considered as a blank, and substrate to its chromatogram. Peaks were designated a to j according to the order of elution from the column. Peaks below 100 cpm, and those indicated with asterisks, which correspond to the non-absorbed (*), and the washed (**) material, were not considered.

protease-activated kinase II has been reported by other authors (4). Furthemore it has been also sugested that CK II and PKC phosphorylated the β subunit of eIF-2 from reticulocytes at different sites (7).

The function of phosphorylation of the β subunit is not yet clear. It has been suggested that modification of β subunit may affect the rate of phosphorylation or dephosphorylation of α subunit (4) or produce a small stimulation of eIF-2-dependent binding of Met-tRNA$_i$ to 40S ribosomal subunit (5). Recent data

Table 1. Distribution of ^{32}P radioactivity in tryptic peptides of brain eIF-2 phosphorylated by three different protein kinases

Peak	a	b	c	d	e	f	g	h	i	j	Ratio[1]
CK II		344[2]	564				521	6647	413	3182	20.3
PKC		111	109	110			175	720			2.1
PKA	143		207	121		104					1.0

[1] Ratio was calculated by normalyzing total counts obtained from PKA peptides to unity.
[2] Numbers represent the cpm incorporated on each tryptic peptide

have suggested that β subunit function is to allow optimal guanine nucleotide exchange factor interaction with eIF-2 (15).

Our results prove that eIF-2 factor from brain is phosphorylated by three different eIF-2(β) kinases: CK II, a cyclic nucleotide and calcium independent kinase; PKC a calcium-dependent kinase; and PKA a cyclic nucleotide-dependent kinase. The differences in the modification produced by the β kinases are not only in the degree of phosphorylation, but also in its specificity.

Modification of the β subunit by phosphorylation seems to be produced in eIF-2 factors from the brain and reticulocytes by the same and also by a different protein kinase. This fact may suggest a distinct regulation or even function for the β subunit, depending on the type of cell studied.

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