J. Biochem. **135**, 479–485 (2004)
DOI: 10.1093/jb/mvh055

In Vitro Phosphorylation of Initiation Factor 2α (aIF2α) from Hyperthermophilic Archaeon *Pyrococcus horikoshii* OT3

Maino Tahara[1], Akiko Ohsawa[1], Sakura Saito[2] and Makoto Kimura[*,1]

[1]Laboratory of Biochemistry, Department of Bioscience and Biotechnology, Faculty of Agriculture, Kyushu University, Hakozaki 6-10-1, Higashi-ku, Fukuoka 812-8581; and [2]Department of virus 3, Laboratory-5, National Institute of Infectious Diseases, Tokyo 208-0011

Received January 15, 2004; accepted February 6, 2004

Eukaryotic initiation factor 2 (eIF2) is a heterotrimeric protein composed of α, β, and γ subunits, of which the α subunit (eIF2α) plays a crucial role in regulation of protein synthesis through phosphorylation at Ser51. All three subunit genes are conserved in Archaea. To examine the properties of archaeal initiation factor 2α (aIF2α), three genes encoding α, β, and γ subunits of aIF2 from the hyperthermophilic archaeon *Pyrococcus horikoshii* OT3 were expressed in *Escherichia coli* cells, and the resulting proteins, aIF2α, aIF2β, and aIF2γ, were characterized with reference to the properties of eIF2. aIF2α preferentially interacts with aIF2γ, but does not interact with aIF2β, which is consistent with data obtained with eIF2, of which eIF2γ serves as a core subunit, interacting with eIF2α and eIF2β. It was found that aIF2α was, albeit to a lower degree, phosphorylated by double-stranded RNA-dependent protein kinase (hPKR) from human, and a primary target site was suggested to be Ser48 within aIF2α. This finding led us to the search for a putative aIF2 specific kinase gene (PH0512) in the *P. horikoshii* genome. The gene product Ph0512p unambiguously phosphorylated aIF2α, and Ser48, as in the phosphorylation by hPKR, was suggested to be a target amino acid residue for the PKR homologue Ph0152p in *P. horikoshii*. These findings suggest that aIF2α, like eIF2α in eukaryotes, plays a role in regulation of the protein synthesis in Archaea through phosphorylation and dephosphorylation.

Key words: archaea, double-stranded RNA-dependent protein kinase, initiation factor 2α, phosphorylation, *Pyrococcus horikoshii* OT3.

Abbreviations: aIF2, archaeal initiation factor 2; aIF2α, α subunit of aIF2; aIF2β, β subunit of aIF2; aIF2γ, γ subunit of aIF2; eIF2, eukaryotic initiation factor 2; eIF2α, α subunit of eIF2; eIF2β, β subunit of eIF2; eIF2γ, γ subunit of eIF2; h-eIF2α, recombinant eIF2α from human; hPKR, double-stranded RNA-dependent protein kinase from human; IPTG, isopropyl-β-D-thiogalactopyranoside; MALDI-TOF MS, matrix assisted laser desorption ionization time of flight mass spectrometry; Met-tRNA$_i$, methionyl-initiator tRNA; OB-fold, oligosaccharide/oligonucleotide binding-fold; RSW, ribosomal salt wash fraction; Trx, thioredoxin.

Phosphorylation of the α subunit of eukaryotic translation initiation factor 2 (eIF2α) is a well characterized mechanism of translational control (*1*, *2*). Eukaryotic initiation factor 2 (eIF2) is a multifunctional protein in the translation initiation process in eukaryotes (for a review see ref. *3*). It is a heterotrimeric GTP-binding protein composed of α, β, and γ subunits (*4–6*) and forms a ternary complex with GTP and Met-tRNA$_i$ to deliver the Met-tRNA$_i$ into the 40S ribosomal subunit. Upon formation of an 80S initiation complex, the GTP is hydrolyzed and eIF2 is released as an eIF2·GDP binary complex (*7*). The GDP bound to eIF2 is then exchanged for GTP, a reaction catalyzed by a guanine nucleotide exchange protein, termed eIF2B (*8*, *9*). In delivery of Met-tRNA$_i$, eIF2β is involved in the binding of Met-tRNA$_i$, mRNA and guanine nucleotides (*10*), and eIF2γ is responsible for the binding of guanine nucleotides as well as Met-tRNAi (*11*). eIF2α is, however, dispensable for the binding of Met-tRNA$_i$ to the small ribosomal subunit: rather, it plays a key role in translational regulation. Thus, phosphorylation of eIF2α converts eIF2 from a substrate to a competitive inhibitor of eIF2B, impairing formation of the ternary complex and thereby inhibiting translation initiation (*12*, *13*). eIF2B contains five subunits and is composed of catalytic (ε and γ) and regulatory subcomplexes (α, β, and δ). It was suggested that tight binding of phosphorylated eIF2α to the eIF2B regulatory subcomplex is crucial for the inhibition of eIF2B (*13*).

Several protein kinases that phosphorylate eIF2α in response to conditions of stress have been identified: these include PKR (response to double-stranded RNA in viral-infected cells) (*14*), HCR (heme regulated protein kinase) (*15*), GCN2 (response to amino acid starvation) (*16*, *17*), and PERK (response to unfolded proteins in the endoplasmic reticulum) (*18*). While activated by distinct stress stimuli, each eIF2α protein kinase phosphorylates eIF2α at the identical residue, Ser51, suggesting a common mode of substrate recognition. The interaction mechanism of eIF2α and its specific kinases has been studied by structural and mutational analyses of the K3L protein, a structural mimic of eIF2α (*19*). The result identified two distinct regions of eIF2α as important for PKR recognition: the region from Ile45 to Arg56 (sequence ILLSELSRRRIR), where the target Ser51 is located; and

*To whom correspondence should be addressed. Tel/Fax: +81-92-642-2853, E-mail: mkimura@agr.kyushu-u.ac.jp

 © 2004 The Japanese Biochemical Society.

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on September 29, 2012

the region from Lys79 to Asp83 (sequence KGYID), which is referred to as the PKR recognition motif. Recently, the crystal structures of the N-terminal two-thirds of eIF2α from human (*20*) and yeast (*21*) were determined at 1.9 Å and 2.5 Å resolutions, respectively. The structures showed that eIF2α is composed of two major domains: the N-terminal domain has a five-stranded β-sheet and is folded into an OB-fold, while the C-terminal domain has α helical structure. The phosphorylation site Ser51 is located at the exposed loop connecting strands β3 and β4 of the N-terminal OB-fold domain.

It is generally accepted that various components of the translation apparatus in Archaea, a third phylogenetic kingdom (*22*), share many sequence and functional features with their eukaryotic counterparts (*23*, *24*). Indeed, Archaea possess all three subunit homologues of eIF2. Three subunits in the hyperthermophilic archaeon *Pyrococcus abyssi* were overproduced in *Escherichia coli* cells, and their reconstitution to form a trimeric protein was reported (*25*). In addition, the crystal structure of the γ subunit of the *P. abyssi* IF2 was established: it displays significant similarities to elongation factors, such as EF-Tu (*25*). Furthermore, the β subunit from *Methanococcus jannashii* was produced in *E. coli* cells, and the solution structure was reported (*26*). The β subunit was composed of two distinct domains: the N-terminal α/β-fold domain contains a four-stranded β-sheet with two α helices, and the C-terminal domain has a zinc-binding motif of three anti-parallel β strands. Although homologues of eIF2α, eIF2β, and eIF2γ were identified in Archaea, and the structural information about aIF2β and aIF2γ provided valuable insights into the mechanism and regulation of eukaryotic translation initiation, the presence of a translational regulation *via* phosphorylation of the α subunit in Archaea remained unclear.

In our own study, the hyperthermophilic archaeon *Pyrococcus horikoshi* OT3 was chosen as a target archaeal organism, which was isolated from the hot waters of the Okinawa Trench. *P. horikoshii* can grow at high temperatures, favoring a temperature of 98°C. The entire length of the genome is 1.74 million base pairs, comprising 2,061 open reading frames (*27*). The functions of about 27% of the gene products can be assigned using sequence-based methods. Three protein genes (PH0961, PH0605, and PH1706) encoding α, β, and γ subunits of aIF2 were identified in the *P. horikoshii* genome. To gain more insights into the structure-function relationships of aIF2 as well as eIF2, we attempted to overproduce aIF2α, aIF2β, and aIF2γ in *E. coli* cells. In this study, we first characterized aIF2α in terms of its interaction with aIF2β and aIF2γ. Next, we examined whether aIF2α could be a substrate for a double-stranded RNA dependent protein kinase (hPKR) from human. Furthermore, we overproduced a putative aIF2-specific kinase from *P. horikoshii* in *E. coli* cells and showed that it could specifically phosphorylate Ser48 within the aIF2α molecule.

MATERIALS AND METHODS

Materials—*P. horikoshii* OT3 strain and its genomic DNA were kindly given by Prof. I. Tanaka (Hokkaido University). Oligonucleotides used in this study were purchased from Hokkaido System Science. *ExTaq* DNA polymerase and DNA ligation kit were purchased from Takara Shuzo and used as recommended by the supplier. Restriction endonucleases and DNA modifying enzymes were purchased from MBI Fermentas. The plasmid vectors used in this work were pGEM™-T EASY vector from Promega, and pET-22b and pET-32a expression vectors from Novagen. *E. coli* strain JM109 was used as a host strain for cloning, and *E. coli* strain BL21-Codon Plus™ (DE3)-RIL (Stratagene) was used as a host strain for expression of recombinant proteins. All other chemicals were of analytical grade for biochemical use.

Overproduction—The genes PH0961, PH0605, and PH1706 encoding aIF2α, aIF2β and aIF2γ, respectively, were amplified by PCR from the *P. horikoshii* OT3 genomic DNA. The DNA fragments for aIF2α and aIF2γ were cloned into pET-22b, and that for aIF2β was cloned into pET-32a. For expression, the resulting plasmids were introduced into *E. coli* BL21-Codon Plus™ (DE3)-RIL cells, and expression was induced by adding 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when OD_{600} reached at 0.6 (*28*). After induction, the culture was further incubated for 4 h at 37°C.

Purification of Proteins—aIF2α: *E. coli* cells cultured in 1 liter of broth were disrupted by sonication in 20 ml of the buffer (50 mM HEPES, pH 7.5, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF). After centrifugation, the supernatant was loaded onto a SP-Sepharose column (1.5 × 10 cm) equilibrated in the same buffer. The proteins were eluted with a linear gradient of increasing NaCl concentration from 0 to 1.0 M in 200 ml of the buffer. The fractions containing aIF2α were further purified on a butyl Toyopearl 650M column (1.5 × 10 cm), from which the proteins were eluted with a linear gradient of decreasing $(NH_4)_2SO_4$ concentration from 1.5 to 0 M in 200 ml of the buffer. In this hydrophobic column chromatography, aIF2α was eluted at around 0.45 M $(NH_4)_2SO_4$ concentration.

aIF2β: *E. coli* cells cultured in 500 ml of broth were disrupted by sonication in 20 ml of the buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9). After centrifugation, the supernatant was heated for 10 min at 70°C. The thermostable proteins were purified by affinity chromatography using His·Bind Resin column (Amersham), as described by the manufacturer. The fusion protein (26 mg) of aIF2β with thioredoxin was digested with thrombin (26 U) in 20 mM Tris-HCl, pH 7.4, containing 50 mM NaCl and 2 mM $CaCl_2$ for 3 h at room temperature. The digests were again purified by His·Bind Resin column chromatography.

aIF2γ: *E. coli* cells cultured in 1 liter of broth were disrupted by sonication in 20 ml of the buffer (50 mM Tris-HCl, pH 6.5, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF). After centrifugation, the supernatant was loaded onto a SP-Sepharose column (1.5 × 10 cm) equilibrated in the same buffer. The proteins were eluted by a linear gradient of increasing NaCl concentration from 0 to 1.0 M in 200 ml of the buffer. aIF2γ was eluted at 0.2 M NaCl concentration.

Analysis using His·Bind Resin Column—Three subunits of aIF2 were purified as described above. aIF2α and/or aIF2γ were incubated with His-tagged aIF2β in 50 μl of binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl pH7.9) at room temperature. The mixture was loaded onto the nickel-charged His·Bind Resin column

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on September 29, 2012

(50 μl), previously equilibrated with 150 μl of binding buffer. After washing with 6 column volumes of the wash buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl pH7.9), His-tagged aIF2β and bound proteins were eluted with the elution buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl pH7.9).

Preparation of the aIF2α Mutants—Site-directed mutagenesis was done by the unique site elimination method (*31*) using a Chameleon double-stranded site-directed mutagenesis kit (Stratagene). The resulting genes were cloned into pET-22b previously digested with the same enzymes, and the proteins were overproduced in BL21-Codon Plus™(DE3)-RIL cells by the procedures described for aIF2α.

Purification of the Gene Product Ph0512p—The fusion construct containing the thioredoxin gene and PH0512 (Trx-PH0512) was generated by the PCR amplification with specific primer sets containing the *Nco*I and *Xho*I restriction sites. The Trx-PH0512 fusion constructs were transfected into the *E. coli* BL21-Codon Plus™ (DE3)-RIL for protein expression. The transformed bacteria were grown in an LB medium with 0.1 mg/ml ampicillin at 37°C to an A_{600} of 0.8, then cultured for a further 4 h after being induced with 0.5 mM IPTG. The cells were harvested by centrifugation at 10,000 rpm for 10 min, resuspended in binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9), then disrupted by ultrasonication. After removing the cell debris, the supernatants were loaded onto a His·Bind Resin column equilibrated with a binding buffer. After washing with the wash buffer (60 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl pH 7.9), the protein was eluted with 250 mM imidazole in the same buffer. The fusion protein of thioredoxin and Ph0512p (Trx-Ph0512p) was further purified on a ResourceQ column (6 ml). Ph0512p was prepared by thrombin digestion of the fusion protein Trx-Ph0512p.

In Vitro Phosphorylation—Ribosomal salt wash fraction (RSW) was prepared from ribosomal fraction of IFN-β–treated human amnion FL cells (6×10^7) by extraction using buffer B (20 mM HEPES, pH7.5, 4 mM $Mg(OAc)_2$, 1 mM DTT, 10% glycerin) containing 500 mM KCl, as previously described (*32*). For *in vitro* phosphorylation assay, we used a more purified DEAE-cellulose flowthrough fraction obtained essentially by the method described by Samuel (*33*). RSW fraction dialyzed against buffer B containing 50 mM KCl was passed through a 1.0 × 6 cm column of DEAE cellulose (Seikagaku Kogyo, Tokyo) equilibrated in buffer B containing 50 mM KCl and the flowthrough fraction was obtained as semippurified PKR (hPKR fraction). The *in vitro* phosphorylation by hPKR fraction was carried out as described in (*32*). Briefly, aIF2α (20 μg) or eIF2α (2 μg) was incubated in 50 μl of reaction mixture of buffer B containing 100 mM KCl, 0.1% NP40, 100 μM ATP, 0.25 μCi of [γ-^{33}P]ATP (Amersham), 1 μg/ml of poly(I) : poly(C), and 10 μl of hPKR. After incubation at 30°C for 20 min, the phosphorylated proteins were analyzed by SDS-PAGE. The phosphorylated proteins were detected by autoradiography using Fuji Film Bas 2500. As a reference, 2.5 μg of His-human eIF2α (h-eIF2α), which was expressed in *E. coli* and purified by use of an Ni-column, was used.

The phosphorylation assays of aIF2α and its mutants (20 μg) using the *P. horikoshii* protein Ph0512p were carried out in 30 μl mixtures containing 20 mM HEPES (pH 7.5), 10 mM KCl, 5 mM $Mg(OAc)_2$, 1 mM DTT, 1 μM PMSF, 1× protease inhibitor cocktail 10% (v/v)glycerol, and 200 μCi/ml [γ-^{33}P]ATP. The mixtures were incubated for 60 min at 42°C, and the reaction was terminated by the addition of 10 μl of 2× SDS gel loading buffer. The samples were boiled for 2 min, then subjected to 15% polyacrylamide SDS gel electrophoresis followed by phosphorimaging.

Miscellaneous—SDS-PAGE using 15% polyacrylamide gel concentration was done, as described in (*33*). The N-terminal amino acid sequence was analyzed by use of a gas phase protein sequencer PSQ-1 (Shimadzu). The molecular mass was measured on a Voyager MALDI-TOF MS (Applied Biosystems). α-Cyano-4-hydroxycinnamic acid in acetonitrile–water–0.1% trifluoroacetic acid (50:40:10) was used as a matrix.

RESULTS AND DISCUSSION

Preparation of aIF2 Subunits α, β, and γ—The genes PH0961, PH0605, and PH1706 were predicted to encode aIF2α (32 kDa), aIF2β (16 kDa), and aIF2γ (45 kDa), respectively. The amino acid sequences of three subunits can be aligned to those of corresponding proteins from both human and yeast, having 30%–47% identical residues. The γ subunit is the most conserved between Eukarya and Archaea (47% identity), while the α subunit shows 23% identity between Eukarya and Archaea. The β subunit of Archaea consists of only the C-terminal two-thirds of the eukaryal sequence. The region that is common to eIF2β and aIF2β has 27% identity.

To characterize properties of the *P. horikoshii* aIF2α, all three subunits (aIF2α, aIF2β and aIF2γ) were overproduced in *E. coli* cells and the resulting proteins were purified by a set of column chromatographies, as described under MATERIALS AND METHODS. The homogeneities of the three purified proteins were analyzed by SDS-PAGE, as shown in Fig. 1. Proteins aIF2α and aIF2γ migrated as single bands, while aIF2β gave two bands of slightly different mobility on SDS-PAGE

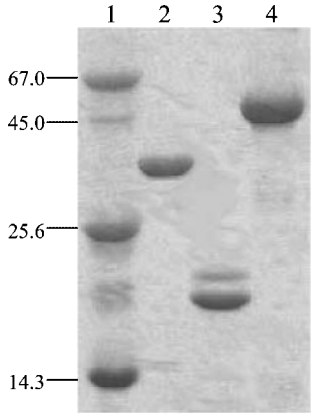

Fig. 1. **SDS–PAGE analysis of aIF2 subunits α, β and γ.** Lane 1, marker proteins; lane 2, aIF2α (32 kDa); lane 3, aIF2β (16 kDa); lane 4, aIF2γ (45 kDa). Proteins were analyzed by SDS-PAGE using 15% polyacrylamide gel and stained with CBB. Marker proteins include bovine serum albumin (67.0 kDa), ovalbumin (45.0 kDa), α-chymotrypsin (25.6 kDa), and lysozyme (14.3 kDa).

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on September 29, 2012

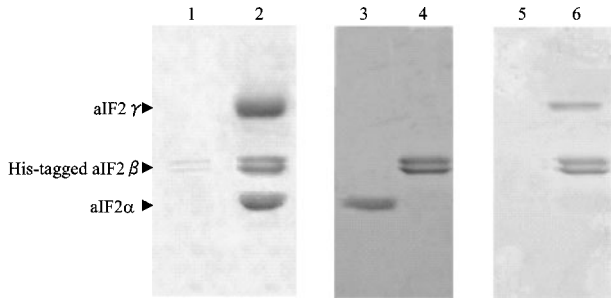

Fig. 2. ***In vitro* assembly of the three purified subunits.** aIF2α and aIF2γ were loaded onto the His·Bind Resin in the presence of His-tagged aIF2β. After washing (lane 1), His-tagged aIF2β and bound proteins ware eluted with the elution buffer containing 1 M imidazole (lane 2). In addition, either aIF2α (lanes 3 and 4) and aIF2γ (lanes 5 and 6) were each loaded onto the His·Bind Resin in the presence of His-tagged aIF2β. After washing (lanes 3 and 5), His- tagged aIF2β and bound proteins were eluted, as described above (lanes 4 and 6). The arrowheads indicate aIF2α, His-tagged aIF2β, and aIF2γ.

(Fig. 1, lane 3). The N-terminal sequencings and MALDI-TOF MS analyses demonstrated their homogeneities. aIF2β gave two bands on SDS-PAGE, even though it was purified to apparent homogeneity. Since aIF2β contains a zinc-binding cysteine cluster at the C-terminus (*24*) and the C-terminal deletion mutant (residues 1–102) of aIF2β showed a single band on SDS-PAGE (Tahara *et al.* unpublished results), the heterogeneity of aIF2β observed on SDS-PAGE is probably caused by two distinct conformations within the C-terminal 30 residues. The yields of aIF2α, aIF2β, and aIF2γ from 1 liter of culture broth were 8 mg, 12 mg, and 3 mg, respectively.

Previously, the two subunit forms of eIF2 (eIF2αγ) were purified from mammals (*34* and references therein) and α subunit-deficient eIF2 (eIF2βγ) was purified from rabbit reticulocyte lysate (*35*). These results indicated that eIF2γ serves as a core subunit to form a trimeric form of eIF2. To evaluate a protein structure of the aIF2 subunits thus produced, we examined the interaction of aIF2α with aIF2β and aIF2γ by immobilizing aIF2β to His·Bind Resin column, as described under "MATERIALS AND METHODS." As shown in Fig. 2, aIF2α was coeluted with aIF2β and aIF2γ (lane 2), although it could not bind to the column in the absence of aIF2γ (lane 3). Furthermore, aIF2γ exhibited binding activity to the His·Bind Resin column in the presence of aIF2β (lane 5) and was coeluted with aIF2β (lane 6). These results indicated that aIF2γ bridges aIF2α and aIF2β, which is consistent with data obtained from eIF2 subunits (*34*, *35*) and the *P. abyssi* IF2 (*25*). The results also suggested that the three subunits are correctly folded in to an active conformation.

In Vitro Phosphorylation by hPKR—The phosphorylation of eIF2α is known to be an important translational control mechanism in eukaryotes, including plants (*36*), and the regulation of eIF2α activity is directly governed by the specific phosphorylation of Ser51. We examined whether aIF2α could be recognized by human double-stranded RNA dependent protein kinase (hPKR). The sequence comparison showed that amino acids flanking Ser51 in eIF2α that comprise the phosphorylation motif (ILLSELSRRRIR) are not conserved in aIF2α (Fig. 3). Instead, aIF2α has two Ser residues, Ser44 and Ser48, around the region corresponding to the phosphorylation motif in eIF2α. Furthermore, the recognition motif (KGYID) in eIF2α required for the specific phosphorylation is slightly changed. That is, the conserved Tyr in eIF2α is replaced by His in aIF2α. Hence, the mutant H77Y, in which His77 in aIF2α was replaced by the corresponding residue Tyr in the possible recognition motif in eIF2α, was generated by site-directed mutagenesis, and the wild-type aIF2α and its mutant H77Y were subjected to the *in vitro* phosphorylation assay by hPKR.

hPKR was a semipurified fraction obtained from human FL cells induced by interferon, as described in "MATERIALS AND METHODS." Many phosphorylated proteins, including autophosphorylated hPKR and endogenous eIF2α appeared to be generated by the usual phosphorylation reaction, even when h-eIF2α was added exogenously as a substrate for hPKR (Fig. 4A, lanes 1–3). In order to show clearer phosphorylated bands of exogenously added a-IF2α, we tried to decrease these endogenous bands by preincubation in the reaction mixture containing cold ATP and poly (I): poly(C) before *in vitro* phosphorylation reactions using [γ-^{33}P]–ATP (Fig. 4A, lanes 4–9). The preliminary phosphorylation of archaeal proteins by hPKR provided no signal in autoradiography (data not shown). Hence, 20 μg of archaeal proteins, which were concentrated by use of Ultrafree (Millipore) to about 10-fold the concentration of h-eIF2α, were included in the reaction mixtures, as shown in Fig. 4B.

```
                          44  48                          77
                          |   |                           |
P.h. aIF2α      KEAFMHISEVASTWVRNIRDYLKEGQKVVAKVIRVDPRKGHIDLSLRRVTQQQ
M.j. aIF2α      KEGMIHISEVTSGWVKNIRDHVKVGQRVVAKVLRVDERKGHIDLSLKRVTEQQ
M.t. aIF2α      KEAFIHISEVSSGWVKNIRDFVRENQKIVARVLRVNPRKGHVDVSMKRIREDQ
A.f. aIF2α      REGMVHISEVASGWIKDIREHVKKGQKVICKVLDVNPKRGHIDLSIKDVNERQ
H.s. eIF2α      IEGMILLSELSRRRIRSINKLIRIGRNECVVVIRVDKEKGYIDLSKRRVSPEE
D.m. eIF2α      IEGMILLSELSRRRIRSINKLIRVGKTEPVVVIRVDKEKGYIDLSKRRVSPED
C.e. eIF2α      KEGMILLSELSRRRIRSVNKLIRVGRSESVVVIRVDKDKGYIDLSKRRVYQKD
S.c. eIF2α      IEGMILLSELSRRRIRSIQKLIRVGKNDVAVVLRVDKEKGYIDLSKRRVSSED
                ==========                  ==========
                          *
```

Fig. 3. **Alignment of amino acid sequences of archaeal and eukaryotic IF2α.** The relevant region of aIF2α where mutations were introduced is shown as an alignment of α subunit sequences derived from several organisms. The first four sequences are archaeal proteins, P.h., *P. horikoshii*, M.j., *M. jannaschii*, M.t., *Methanobacterium thermoautotrophicum*, A.f., *Archaeglobus fulgidus*, and the last four are eukaryotic proteins, H.s., *Homo sapiens*, D.m., *Drosophila melanogaster*, C.e., *Caenorhabditis elegans*, and S.c., *S. cerevisiae*. The phosphorylation motif (ILLSELSRRRIR) and recognition motif (KGYID) in eIF2α are indicated by single and double underlines, respectively. * denotes the phosphorylation sites in eIF2α.

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on September 29, 2012

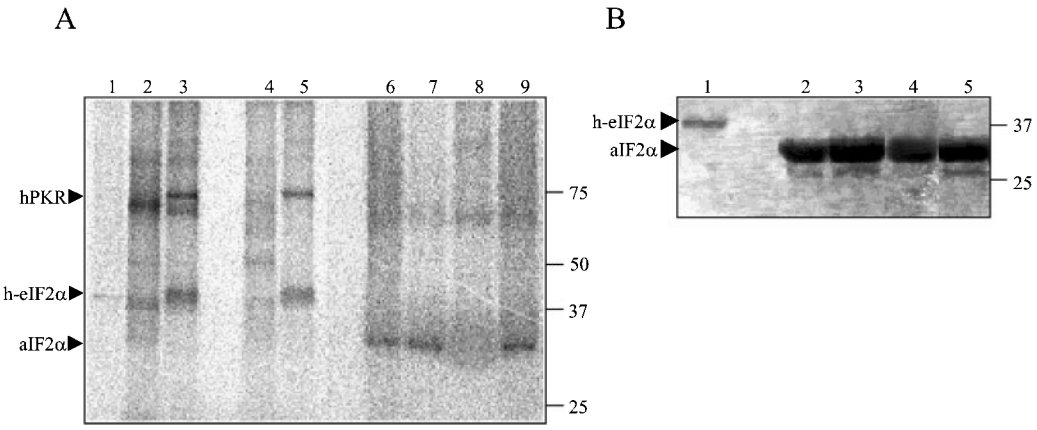

Fig. 4. ***In vitro* phosphorylation of aIF2α and its mutants by hPKR.** A, Phosphorylation of h-eIF2α, aIF2α, and its mutants by hPKR. Fractions containing hPKR were subjected to *in vitro* phosphorylation, in the absence or presence of 1 mM poly(I):poly(C) (lanes 1 and 2, respectively), and in the presence of recombinant human eIF2α (h-eIF2α) (lane 3), without preincubation in the presence of cold ATP. After preincubation with poly(I): poly(C) and 0.5 mM cold ATP, neither h-eIF2α nor aIF2α (lane 4), h-eIF2α (lane 5), aIF2α (lane 6), S44A (lane 7), S48A (lane 8) and H77Y (lane 9) were subjected to phosphorylation. The proteins were then separated by SDS-PAGE and visualized by autoradiography. The migrations of h-eIF2α, aIF2α and hPKR are indicated on the left. Molecular markers (kDa) are shown on the right. B, The proteins used for phosphorylation reactions were shown by gel staining with CBB after SDS-PAGE. Lane 1, h-eIF2α; lane 2, aIF2α; lane 3, S44A; lane 4, S48A; lane 5, H77Y.

The *in vitro* phosphorylation assay after preincubation showed that h-eIF2α was efficiently phosphorylated, together with secondary phosphorylation of hPKR, when h-eIF2α was added exogenously (Fig. 4A, lane 5). However, without exogenous h-eIF2α, the phosphorylations of endogenous eIF2α and hPKR were almost undetectable (Fig. 4A, lane 4). This result indicated that hPKR had a potential activity for phosphorylation of exogenously added h-eIF2α under these conditions. As for phosphorylation of aIF2α, only a single band estimated to be of 32 kDa could be detected in Fig. 4A, lane 6, suggesting that the aIF2α was phosphorylated by hPKR. In addition, the mutant H77Y appeared to be phosphorylated by hPKR to the same extent as the wild-type aIF2α (Fig. 4A, lane 9). Since the conserved Tyr in the recognition motif (KGYID) in eIF2α was found to be dispensable for phosphorylation of aIF2α by hPKR and the mutation of the Tyr residue resulted in the most potent disrupter of PKR binding (*19*), the present result suggested that aIF2α may be recognized by hPKR in a manner different from eIF2α.

To corroborate the phosphorylation of aIF2α and also to localize phosphorylation site(s), mutants S44A and S48A, in which Ser44 and Ser48 in aIF2α were respectively replaced by Ala, were generated by site-directed mutagenesis and analyzed again by the *in vitro* phosphorylation. The phosphorylation of S44A yielded 32-kDa bands identical to that of aIF2α (Fig. 4A, lane 7), while that of S48A did not produce a band of 32-kDa (Fig. 4A, lane 8). The results suggest that aIF2α could be a specific substrate for hPKR, and that Ser48 is the primary phosphorylation site in aIF2α.

In Vitro Phosphorylation by Ph0512p—The phosphorylation of aIF2α by hPKR described above led us to expect that there would be genes in the *P. horikoshii* genome which encode PKR homologue(s). The homology search detected two genes PH0445 and PH0512 which encode possible aIF2α specific kinase proteins. An attempt was then made to express the two genes in *E. coli* cells using the expression vector pET-22b as an expression plasmid, but both protein products were produced as inclusion bodies. Next, the proteins were produced in *E. coli* cell as fusion proteins with thioredoxin, as described under MATERIALS AND METHODS. This attempt resulted in a production of a soluble fusion protein of thioredoxin and Ph0512p (Trx-Ph0512p), although no stable gene product for PH0445 has yet been obtained. Trx-Ph0512p was subjected to the *in vitro* phosphorylation assay for aIF2α and its mutants. Figure 5 shows that the Trx-Ph0512p unambiguously phosphorylated aIF2α as well as mutants S44A and H77Y, whereas the mutant S48A was little phosphorylated by Trx-Ph0512p, which is consistent with the result obtained by the *in vitro* phosphorylation with hPKR. The results indicated that aIF2α is phosphorylated by the PKR homologue Ph0512p; the primary target site was

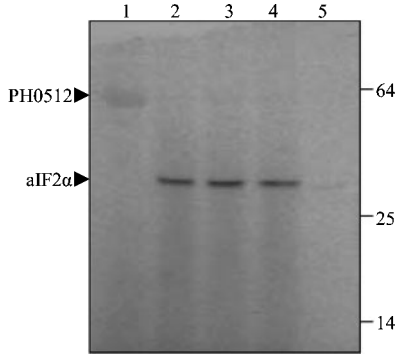

Fig. 5. ***In vitro* phosphorylation of aIF2α and its mutants by Trx-Ph0512p.** Trx-Ph0512p readily phosphorylated aIF2α. *In vitro* kinase assays using [γ-^{33}P]ATP were performed using the purified Trx-Ph0512p. Trx-Ph0512p incubated for 1 h at 42°C with *P. horikoshii* aIF2α (lane 2), H77Y (lane 3), S44A (lane 4), S48A (lane 5). Reaction products were analyzed by SDS-PAGE followed by autoradiography. The migrations of Trx-Ph0512p and aIF2α are indicated on the left. Molecular markers (kDa) are shown on the right.

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on September 29, 2012

found to be Ser48, and His77 was found to be dispensable for the recognition by Trx-Ph0512p.

To rule out the possibility that the phosphorylation of aIF2α and its mutants observed was an artifact of the fusion protein with thioredoxin, the same experiment was done using Ph0512p obtained by digestion of Trx-Ph0512p with thrombin, and the results were essentially identical (data not shown).

The present results demonstrated that aIF2α could be phosphorylated by hPKR and the PKR homologue Ph0512p from *P. horikoshii*, and the primary target site was suggested to be Ser48 in the aIF2α molecule. It was reported that phosphorylation sites are often preceded or followed by several arginine residues (*37*, *38*); arginine side-chains have the potential to form multiple hydrogen bonds with the oxygen atoms of the phosphate group. In the case of eIF2α, the targeted Ser51 is followed immediately by three conserved arginine residues, and two other positively charged side-chains Arg56 and Lys60 are nearby in the three-dimensional structure. However, the three conserved arginine residues in eIF2α are completely lacking in aIF2α, and only two positively charged residues, Arg52 and Arg55, which are highly conserved in Archaea, follow the target Ser48 (Fig. 3). It is likely that the inefficient phosphorylation of aIF2α compared with that of eIF2α is due to the lack of the consecutive Arg residues around the target site in aIF2α. Alternatively, the targeted Ser48 in aIF2α may be recognized by hPKR and the PKR homologue Ph0512p in a manner different from Ser51 in eIF2α. A definite answer as to how aIF2α is recognized by its specific kinase must await a detailed analysis of the interaction of aIF2α and Ph0512p.

In conclusion, the present study strongly suggested the presence of an aIF2α phosphorylation pathway in Archaea. We recently identified and overproduced the *P. horikoshii* homologues of the regulatory subcomplex of eIF2B. A further study on the interaction of aIF2α with the archaeal regulatory subcomplex of aIF2B will provide valuable insights into the inhibitory mechanism of eukaryotic translation initiation *via* phosphorylation of eIF2α.

We thank Prof. I. Tanaka and Dr. M. Yao of Hokkaido University for valuable comments and suggestions throughout this study, and Dr. A. Kato of National Institute of Infectious Diseases for providing h-eIF2α. This work was supported in part by a grant from the National Project on Protein Structural and Functional Analyses, and by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

REFERENCES

1. Hinnebusch, A.G. (1993) Gene-specific translational control of the yeast GCN4 gene by phosphorylation of eukaryotic initiation factor 2. *Mol. Microbiol.* **10**, 215–223
2. Rhoads, R.E. (1993) Regulation of eukaryotic protein synthesis by initiation factors. *J. Biol. Chem.* **268**, 3017–3020
3. Sonenberg, N. and Dever, T.E. (2003) Eukaryotic translation initiation factors and regulators. *Curr. Opin. Struc. Biol.* **13**, 56–63
4. Ernst, H., Duncan, R.F., and Hershey, J.W.B. (1987) Cloning and sequencing of complementary DNAs encoding the α-subunit of translational initiation factor eIF2. *J. Biol. Chem.* **262**, 1206–1212
5. Pathak, V.K., Nielsen, P.J., Trachsel, H., and Hershey, J.W.B. (1988) Structure of the β subunit of translational initiation factor eIF-2. *Cell* **54**, 633–639
6. Gaspar, N.J., Kinzy, T.G., Scherer, B.J., Humbelin, M., Hershey, J.W.B., and Merrick, W.C. (1994) Translation initiation factor eIF-2. Cloning and expression of human cDNA encoding the γ-subunit. *J. Biol. Chem.* **269**, 3415–3422
7. Hershey, J.W.B. (1991) Translational control in mammalian cells. *Annu. Rev. Biochem.* **60**, 717–755
8. Cigan, A.M., Bushman, J.L., Boal, T.R., and Hinnebusch, A.G. (1993) A protein complex of translational regulators of GCN4 mRNA is the guanine nucleotide-exchange factor for translation initiation factor 2 in yeast. *Proc. Natl Acad. Sci. USA* **90**, 5350–5354
9. Price, N. and Proud, C. (1998) The guanine nucleotide-exchange factor, eIF-2B. *Biochimie* **76**, 748–760
10. Merrick, W.C. (1992) Mechanism and regulation of eukaryotic protein synthesis. *Microbiol. Rev.* **56**, 291–315
11. Dorris, D.R., Erickson, F.L., and Hannig, E.M. (1995) Mutations in GCD11, the structural gene for eIF2γ in yeast, alter translational regulation of GCN4 and the selection of the start site for protein synthesis. *EMBO J.* **14**, 2239–2249
12. Kaufman, R. (2000) Double-stranded RNA-activated protein kinase PKR in *Translational Control of Gene Expression* (Sonnenberg, N., Hershey, J.W.B., and Mattews, M.B., eds.) pp. 503–527, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
13. Chen, J.J. (2000) Heme-regulated eIF2α kinase in *Translational Control of Gene Expression* (Sonnenberg, N., Hershey, J.W.B., and Mattews, M.B., eds.) pp, 529–546, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
14. Dever, T.E., Feng, L., Wek, R.C., Cigan, A.M., Donahue, T., and Hinnebusch, A.G. (1992) Phosphorylation of initiation factor 2α by protein kinase GCN mediates gene-specific translational control of GCN4 in yeast. *Cell* **68**, 585–596
15. Hinnebusch, A.G. and Natarajan, K. (2002) Gcn4p, a master regulator of gene expression, is controlled at multiple levels by diverse signals of starvation and stress. *Eukaryot. Cell* **1**, 22–32
16. Ron, D. and Harding, H.P. (2000) PERK and translational control by stress in the endoplasmic reticulum in *Translational Control of Gene Expression* (Sonnenberg, N., Hershey, J.W.B. and Mattews, M.B., eds.) pp. 547–560, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
17. Dar, A.C. and Sicheri, F. (2002) X-ray crystal structure and functional analysis of vaccinia virus K3L reveals molecular determinants for PKR subversion and substrate recognition. *Mol. Cell* **10**, 295–305
18. Norato, M.C., Widom, J., and Clardy, J. (2002) Crystal structure of the N-terminal segment of human eukaryotic translation initiation factor 2α. *J. Biol. Chem.* **277**, 17057–17061
19. Dhaliwal, S. and Hoffman, D.W. (2003) The crystal structure of the N-terminal region of the alpha subunit of translation initiation factor (eIF2α) from *Saccharomyces cerevisiae* provides a view of the loop containing serine 51, the target of the eIF2α-specific kinases. *J. Mol. Biol.* **334**, 187–195
20. Woese, C.R. and Fox, G.E. (1977) Phylogenetic structure of the prokaryotic domain: the primary kingdoms. *Proc. Natl Acad. Sci. USA* **74**, 5088–5090
21. Kyrpides, N.C. and Woese, C.R. (1998) Universally conserved translation initiation factors. *Proc. Natl Acad. Sci. USA* **95**, 224–228
22. Kyrpides, N.C. and Woese, C.R. (1998) Archaeal translation initiation revisited: the initiation factor 2 and eukaryotic initiation factor 2B α-β-δ subunit families. *Proc. Natl Acad. Sci. USA* **95**, 3726–3730
23. Schmitt, E., Blanquet, S., and Mechulam, Y. (2002) The large subunit of initiation factor aIF2 is a close structural homologue of elongation factors. *EMBO J.* **21**, 1821–1832
24. Cho, S. and Hoffman, D.W. (2002) Structure of the β subunit of translation initiation factor 2 from the archaeon *Methanococ-*

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on September 29, 2012

cus jannashii: a representative of the eIF2 β/eIF5 family of proteins. *Biochemistry* **41**, 5730–5742
25. Kawarabayashi, Y., Sawada, M., Horikawa, H., Haikawa, Y., Hino, Y., Yamamoto, S., Sekine, M., Baba, S., Kosugi, H., Hosoyama, A., Nagai, Y., Sakai, M., Ogura, K., Otsuka, R., Nakazawa, H., Takamiya, M., Ohfuku, Y., Funahashi, T., Tanaka, T., Kudoh, Y., Yamazaki, J., Kushida, N., Oguchi, A., Aoki, K., and Kikuchi, H. (1998) Complete sequence and gene organization of the genome of a hyper-thermophilic archaebacterium, *Pyrococcus horikoshii* OT3. *DNA Res.* **5**, 55–76
26. Studier, F.W., Rosenberg, A.H.R., Dunn, J.J., and Dubendorff, J.W. (1990) Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.* **185**, 60–89
27. Deng, W.P. and Nickoloff, J.A. (1992) Site-directed mutagenesis of virtually any plasmid by eliminating a single site. *Anal. Biochem.* **200**, 81–88
28. Saito, S. (1989) Possible involvement of virus-induced oritein kinase in the antiviral state induced with interferon-γ against sindbis virus. *J. Interfern Res.* **9**, 23–34
29. Karlsson, R., Michaelsson, A., and Mattsson, L. (1991) Kinetic analysis of monoclonal antibody-antigen interaction with a new biosensor based analytical system. *J. Immunol. Methods* **145**, 229–240
30. Iwanaga, S., Yamasaki, N., and Kimura, M. (1998) Chymotrypsin inhibitor from *Erythrina variegate* seeds: Involvement of amino acid residues within the primary binding loop in potent inhibitory activity toward chymotrypsin. *J. Biochem.* **124**, 663–669
31. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of head of bacteriophage T4. *Nature* **227**, 680–685
32. Saito, S. (1989) Possible involvement of virus-induced oritein kinase in the antiviral state induced with interferon-γ against sindbis virus. *J. Interfern Res.* **9**, 23–34
33. Samuel, C.E. (1981) Procedures for measurement of phosphorylation of ribosome-associated proteins in interferon-treated cells. *Methods Enzymol.* **79**, 168–178
34. Kimball, S.R., Everson, W.V., Myers, L.M., and Jefferson, L.S. (1987) Purification and characterization of eukaryotic initiation factors and a guanine nucleotide exchange factor from rat liver. *J. Biol. Chem.* **262**, 2220–2227
35. Mouat, M.F. and Manchester, K. (1998) An α subunit-deficient form of eukaryotic protein synthesis initiation factor eIF2 from rabbit reticulocyte lysate and its activity in ternary complex formation. *Mol. Cell. Biochem.* **183**, 69–78
36. Krishnamoorthy, T., Pavitt, G.D., Zhang, F., Dever, T.E., and Hinnebusch, A.G. (2001) Tight binding of the phosphorylated α subunit of initiation factor 2 (eIF2α) to the regulatory subunits of guanine nucleotide exchange factor eIF2B is required for inhibitor of translation initiation. *Mol. Cell. Biol.* **21**, 5018–5030
37. Johnson, L.N. and Barford, D. (1993) The effects of phosphorylation on the structure and function of proteins. *Annu. Rev. Biophys Biomol. Struct.* **22**, 199–232
38. Pearson, R.B. and Kemp, B.E. (1991) Protein kinase phosphorylation site sequences and consensus specificity motifs: tabulations. *Methods Enzymol.* **200**, 62–81

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on September 29, 2012