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Purification and Characterization of the Functional Catalytic Domain of PKR-Like Endoplasmic Reticulum Kinase Expressed in *Escherichia coli*

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Abstract PKR-like endoplasmic reticulum (ER) kinase (PERK) is a type I transmembrane ER-resident protein containing a cytoplasmic catalytic domain with a Ser/Thr kinase activity, which is most closely related to the eukaryotic translation initiation factor-2α (eIF2α) kinase PKR involved in the antiviral defense pathway by interferon. We cloned and expressed the PERK C-terminal kinase domain (cPERK) in *Escherichia coli*. Like PERK activation in cells under ER stress, wild-type cPERK underwent autophosphorylation when overexpressed in *E. coli*, whereas the cPERK(K621M) with a methionine substitution for the lysine at amino acid 621 lost the autophosphorylation activity. The activated form cPERK which was purified to near homogeneity, formed an oligomer and was able to *trans*-phosphorylate specifically its cellular substrate eIF2α. Two-dimensional phosphoamino acids analysis revealed that phosphorylation of cPERK occurs at the Ser and Thr residues. The functionally active recombinant cPERK, and its inactive mutant should be useful for the analysis of biochemical functions of PERK and for the determination of their three-dimensional structures.

Key words: Autophosphorylation, ER stress, PERK, PKR, Ser/Thr kinase

Protein biosynthesis in eukaryotes is promptly adjusted in response to a variety of different environmental stimuli, such as nutrient starvation, heat shock, and viral infection. A key step in the inhibition of protein synthesis under these conditions occurs at the level of initiation through phosphorylation of the α-subunit of eukaryotic translation initiation factor eIF2 (eIF2α) [7, 18, 23]. To date, four specific eIF2α kinases have been identified. These include (i) the heme-regulated translational inhibitor in erythroid cells, which adjusts globin synthesis to heme availability [2]; (ii) the GCN2 kinase that is activated upon amino acid deprivation to paradoxically increase translation of the GCN4 mRNA encoding a transcription factor for the genes involved in amino acid biosynthesis [6]; (iii) the interferon-inducible, double-stranded RNA-dependent kinase PKR that plays an important role in cellular antiviral defense mechanisms [17]; and (iv) the PKR-like endoplasmic reticulum (ER) kinase (PERK) that is implicated in transducing ER stress to eIF2α phosphorylation [5].

Human PERK is a Ser/Thr kinase consisting of two functionally distinct domains: an N-terminal regulatory domain and a C-terminal catalytic domain. The N-terminal lumenal domain is similar to the ER-stress-sensing domain of the ER-resident kinase Ire1. The C-terminal domain is most closely related to that of the eIF2α kinases GCN2, HRI, and PKR [5, 19, 20]. Various cellular stresses, including overexpression of recombinant proteins in eukaryotic cells and virus infection, contribute to the accumulation of misfolded proteins in the ER, resulting in the triggering of an unfolded protein response (UPR) [4, 21]. The UPR is associated with an increased transcription of ER chaperone genes, GRP78/Bip and GRP94, to facilitate protein maturation in the ER lumen. The GRP78 binds to the unfolded proteins in the lumen, leaving PERK free to dimerize or oligomerize, which results in autophosphorylation of its C-terminal cytoplasmic kinase domain, rendering the PERK active [12]. Activated PERK phosphorylates the Ser51 of eIF2α, which stabilizes the eIF2/GDP/eIF2B complex and prevents the GDP-GTP exchange reaction. Therefore, phosphorylation of eIF2α limits translation initiation events on cellular mRNA [5].

Even though PERK is known to be hyperphosphorylated by ER stress in cells, very little is known about where the multiple phosphorylations occur at its kinase domain and how its function is controlled by other cellular proteins. In order to characterize the biochemical properties of the C-

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terminal kinase domain of PERK(cPERK), we cloned and expressed cPERK in *Escherichia coli* as a fusion protein with a $(His)_6$-tag. The wild-type cPERK overexpressed in *E. coli* underwent autophosphorylation mainly at the Ser and Thr residues and was functionally active. The activated cPERK forming an oligomer was able to phosphorylate its physiological substrate, eIF2α.

MATERIALS AND METHODS

Construction of Plasmids

For the cloning of cPERK, total RNA was extracted from HepG2 human hepatoma cells (ATCC, U.S.A.), which were cultivated, as described previously [15], using TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.). Total RNA (2 μg) was subjected to reverse transcription (RT) using Superscript II reverse transcriptase (Gibco-BRL, Gaithersburg, MD, U.S.A.) with a PERK-specific primer, 5'-TTCCGCG-GCCGCTATGGCCGACGTCGACCTAATTCTTGGCA AAGGGC-3'. The resulting cDNA was then used for PCR amplification using Vent DNA polymerase (New England BioLabs, Beverly, MA, U.S.A.) with oligonucleotides 5'-CTAGCTAGCCGCAGGCTTTTCCATCCTCA-3' and 5'-GGGGTACCCTAATTGCTTGGCAAAGGGC-3'. The PCR product was digested with NheI and XhoI and ligated into the pET28a(+) expression vector (Novagen, Madison, WI, U.S.A.) to obtain pET-cPERK. The gene encoding a mutant PERK kinase domain cPERK(K621M), in which Lys621 was replaced with Met, was generated by a bridged PCR method using pET-cPERK as a template, as described previously [13], and cloned into the pET28a(+) vector to obtain pET-cPERK(K621M).

The cDNA for eIF2α was synthesized from the total RNA of HepG2 cells with Superscript II reverse transcriptase and an eIF2α-specific oligonucleotide, 5'-CCGCTCGAGT-TAATCTTCAGCTTTGGCTT-3'. After PCR-amplification of the cDNA with oligonucleotides 5'-GGAATTCATGC-CGGGTCTAAGTTGTAG-3' and 5'-CCGCTCGAGTTA-ATCTTCAGCTTTGGCTT-3', the resulting PCR product was digested with EcoRI and XhoI and ligated into the expression vector pGEX-4T-1 (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) to obtain pGEX-eIF2α. The gene encoding a mutant eIF2α(S51A), in which Ser51 was replaced with Ala, was generated by a bridged PCR using pGEX-eIF2α as a template and cloned into the pGEX-4T-1 to obtain pGEX-eIF2α(S51A). The integrity of the reading frames and coding sequences, as well as the presence of mutations in the obtained clones, were verified by DNA sequencing.

Expression and Purification of Recombinant PERK Kinase Domain and eIF2α

E. coli strain BL21(DE3) cells (Novagen) transformed with pET-cPERK or pET-cPERK(K621M) were grown at 37°C in LB medium containing ampicillin (100 μg/ml). At a 600 nm optical density (OD_{600}) of 0.6, protein expression was induced at 25°C for 8 h by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). N-Terminal $(His)_6$-tagged cPERK and PERK(K621M) were purified with Ni-nitrilotriacetic acid (NTA)-Sepharose resin (Qiagen, Hilden, Germany), as described previously [14]. PERK kinase domain protein peaks were combined, dialyzed against a buffer containing 50 mM Tris-HCl (pH 8.0), 1 mM DTT, 50 mM NaCl, 5 mM $MgCl_2$, and 10% glycerol, and subjected to gel-filtration chromatography (GFC) using a Sephacryl S-200HR column (Amersham Pharmacia Biotech). Proteins were eluted from the column with a GFC buffer (50 mM Tris-HCl, pH 7.8, 1 mM DTT, 150 mM NaCl, 0.5 mM EDTA) at a flow rate of 0.5 ml/min [16]. The eluates were monitored by a UV detector at a wavelength of 280 nm. The column was calibrated with known molecular weight standards to construct a calibration curve from which the molecular weights of the eluted proteins were calculated.

E. coli DH5α cells transformed with pGEX-eIF2α or pGEX-eIF2α(S51A) were grown at 37°C in LB medium containing ampicillin to an OD_{600} of 0.8, and protein expression was induced at 30°C for 15 h by the addition of 1 mM IPTG. Cell pellets obtained from 1-liter culture were washed once with PBS and resuspended in 40 ml of sonication buffer (50 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 5 mM DTT, 20% glycerol). After sonication, Triton X-100 was added to a final concentration of 1% (v/v), and unbroken cells and cell debris were removed by centrifugation. The supernatant was mixed with Glutathione Sepharose 4B beads (Amersham Pharmacia Biotech) for 1 h at 4°C. Bound proteins were eluted with elution buffer (50 mM Tris-HCl, pH 7.5, 50 mM KCl, 5 mM DTT, 20% glycerol, 10 mM reduced glutathione), and further purified by anion-exchange chromatography using the Q-Sepharose FF (Amersham Pharmacia Biotech) column preequilibrated with a buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM β-mercaptoethanol, 100 mM KCl, and 20% glycerol. After the column was washed with the same buffer, bound proteins were eluted with 100 to 500 mM KCl. The fractions containing GST-eIF2α and GST-eIF2α(S51A) were pooled and dialyzed against a storage buffer (50 mM Tris-HCl, pH 7.5, 50 mM KCl, 5 mM DTT, 20% glycerol), and aliquots were kept at −80°C. Total protein concentrations were measured by the Bradford assay (Bio-Rad, Hercules, CA, U.S.A.).

SDS-PAGE and Western Blot Analysis

Proteins were resolved by sodium dodecyl sulfate (SDS)-8% polyacrylamide gel electrophoresis (PAGE) and Western blot analysis was performed, using an anti-penta-His monoclonal antibody (Qiagen), as described previously

[8, 11]. Membrane-bound antibodies were detected with the enhanced ChemiLuminescence kit (ECL, Amersham Pharmacia Biotech).

In Vitro Dephosphorylation of cPERK

cPERK and cPERK (K621M) were incubated with or without 400 units of λ-phosphatase (PPase) (New England BioLabs) in 30 μl of λ-PPase reaction buffer (50 mM Tris-HCl, pH 7.5, 2 mM $MnCl_2$, 0.1 mM EDTA, 5 mM DTT, 0.01% Brij 35) including a protease inhibitor cocktail (Roche, Mannheim, Germany) at 30°C for 30 min. Then, the reaction mixtures were analyzed by electrophoresis on an SDS-8% polyacrylamide gel, followed by Western blot analysis.

In Vitro Autophosphorylation and Protein Kinase Assays

Autophosphorylation activity of the PERK kinase domain was assayed in 20 μl of kinase buffer (20 mM HEPES, pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM DTT) containing 1 μg of either purified cPERK or cPERK (K621M), 100 μM ATP, and 10 μCi of [γ-^{32}P]ATP (Amersham Biosciences) for 30 min at 30°C. For kinase activity assay of cPERK, 0.4 μg of GST-eIF2α or GST-eIF2α(S51A) was added to the autophosphorylation activity assay mixtures. The reaction mixtures were then analyzed by SDS-8% PAGE. The gels were then dried and subjected to autoradiography.

Two-Dimensional Phosphoamino Acids Analysis

For analysis of phosphorylated amino acids in cPERK, phosphoamino acid assay was performed as described previously [1]. After the *in vitro* kinase assay, the autophosphorylated, labeled cPERK was resolved by SDS-PAGE and transferred to a Hybond-P poly(vinylidene difluoride) (PVDF) membrane (Amersham Biosciences). The membrane was exposed to X-ray film, and the labeled cPERK band was visualized by autoradiography. The strip of PVDF membrane containing the radiolabeled cPERK was hydrolyzed in 6 M HCl at 110°C for 1 h. Radiolabeled phosphoamino acids were analyzed by two-dimensional thin-layer electrophoresis using an HTLE-7002 apparatus (CBS Scientific, Del Mar, CA, U.S.A.). Positions of the phosphoserine, phosphothreonine, and phosphotyrosine (Sigma-Aldrich, St. Louis, MO, U.S.A.) used as markers were visualized by ninhydrin (Sigma-Aldrich) staining. Radiolabeled phosphoamino acids were visualized by autoradiography.

RESULTS

Autophosphorylation of Recombinant cPERK Overexpressed in *E. coli*

The gene encoding PERK has 4,325 nucleotides containing a 72-base pair 5'-untranslated sequence, 3,345-base pair open reading frame, and 908-base pair 3'-untranslated sequence [19]. The single open reading frame predicts a 1,115-residue polypeptide with a deduced molecular weight of 125 kDa. We cloned the cDNA for the C-terminal kinase domain of PERK (amino acids 535-1115; cPERK) by RT-PCR (Fig. 1A). The cPERK was expressed in *E. coli* BL21(DE3) as a fusion protein with an N-terminal $(His)_6$-tag. Its mutant cPERK(K621M) with Met substitution at Lys621 conserved among all the eIF2α kinases was also expressed in parallel. The Lys621 residue, which corresponds to the Lys618 in mouse PERK, close to the catalytic center of PERK has been shown to be important for PERK autokinase activity in cells [5, 9]. After protein expression had been induced by the addition of IPTG, the cell extract was prepared and analyzed by SDS-PAGE (Fig. 2B). Although the protein gel stained with Coomassie blue did not clearly show protein bands for cPERK and cPERK(K621M), these proteins could be detected by immunoblotting with an anti-penta-His antibody (Fig. 1C). The wild-type cPERK, which has a predicted molecular weight of 68 kDa, slowly migrated and was detected as a protein band with an apparent molecular weight of approximately 97 kDa, whereas its mutant

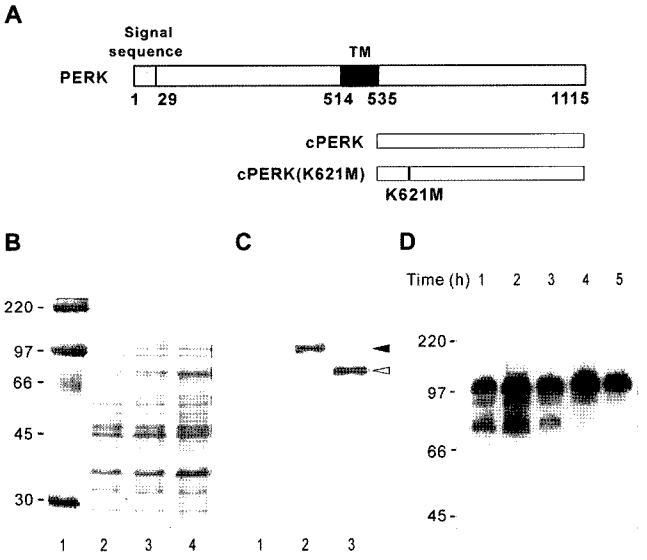

Fig. 1. Autophosphorylation of cPERK expressed in *E. coli*.
A. Schematic representation of PERK and location of the kinase domain (amino acids 535-1115). The signal sequence element, transmembrane domain (TM), and the location of mutation introduced in cPERK(K621M) are indicated. **B–C**. Coomassie blue staining (**B**) and Western blot analysis (**C**) of the total proteins from *E. coli* cells transformed with the empty vector (lane 1), the cPERK-expressing vector (lane 2), or the cPERK (K621M)-expressing vector. Western blot analysis was performed with an anti-penta-His antibody. Closed and open arrowheads indicate the positions of the recombinant cPERK and cPERK(K621M), respectively. **D**. Kinetics of autophosphorylation of cPERK. After induction of protein expression by the addition of IPTG for the indicated time shown above each lane, equal amounts of total cell extracts were resolved by SDS-8% PAGE, and Western blot analysis was performed as in (**C**). Protein size markers are shown at the left of each gel, in kDa.

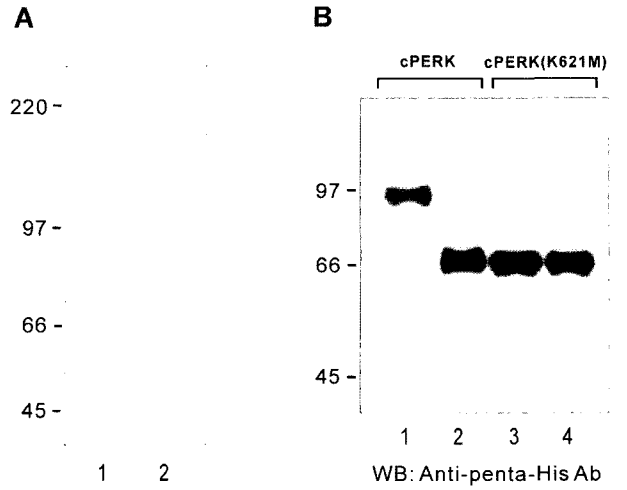

Fig. 2. Purification of recombinant cPERK and cPERK(K621M) and *in vitro* dephosphorylation.
A. Recombinant cPERK and cPERK(K621M) were expressed and purified from *E. coli.* **B.** The purified cPERK (lanes 1 and 2) and cPERK (K621M) (lanes 3 and 4) were incubated without (lanes 1 and 3) or with (lanes 2 and 4) 400 units of λ-PPase for 30 min. Proteins were separated by SDS-8% PAGE and analyzed by Western blotting (WB) with the indicated antibody. Protein size markers are shown at the left of each gel, in kDa.

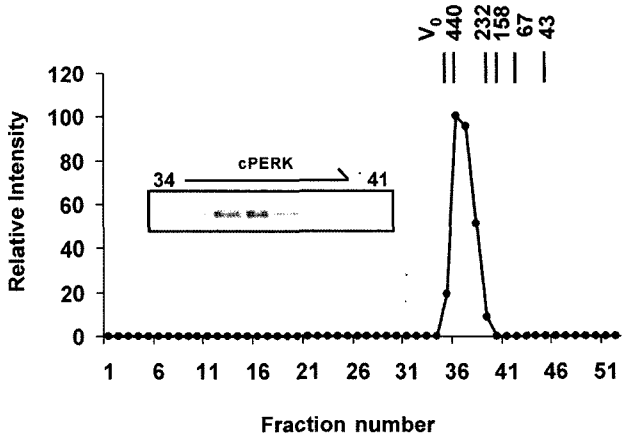

Fig. 3. Gel-filtration chromatography of cPERK: oligomerization of cPERK.
GFC elution profile. The cPERK eluates were resolved by SDS-PAGE and stained with Coomassie blue (inset). Fraction numbers are indicated at the top of the protein gel. The migration of protein standards is indicated along the elution profile.

cPERK(K621M) had the predicted molecular weight. This result suggested that the cPERK expressed without the N-terminal PERK domain sensing the unfolded protein might undergo autophosphorylation at multiple sites, resulting in a significant decrease in electrophoretic mobility, as seen in the cells under ER stress [5]. The high molecular weight protein band started to appear at the early time point of protein expression with multiple protein bands of lower molecular weights (Fig. 1D, lanes 1–3), which later disappeared, probably because of saturation of phosphorylation on the cPERK.

To prove that cPERK is indeed hyperphosphorylated when expressed in *E. coli*, we treated both cPERK and its mutant cPERK(K621M) purified by GFC (Fig. 2A) with λ phosphatase, and compared their electrophoretic mobility changes by SDS-PAGE. As expected, cPERK migrated to the same position as that of the catalytically inactive cPERK (K621M) upon treatment with λ phosphatase, which dephosphorylates phosphoserine, phosphothreonine, and phosphotyrosine in protein [24] (Fig. 2, compare lanes 1 and 2). In contrast, the inactive mutant cPERK(K621M) did not show any electrophoretic mobility change, demonstrating that the mobility shift was due to phosphorylation of cPERK. Moreover, a phosphospecific antibody for PERK recognized the cPERK, but failed to detect the cPERK(K621M) by Western blot analysis (data not shown), again confirming that cPERK is indeed in a highly phosphorylated form.

Oligomerization of cPERK

It has been reported that activated PERK forms an oligomer in cells under ER stress by releasing the Grp78/BiP protein from the N-terminal region of PERK residing in the ER lumen [12]. We investigated whether recombinant cPERK also forms an oligomer *in vitro*. cPERK proteins eluted from the Ni-NTA column were further purified by GFC using a Sephacryl S-200HR column. The hyperphosphorylated form of cPERK was eluted from the column in an early peak (Fig. 3). Its apparent molecular weight of ~400 kDa, as calculated from the elution volume, was remarkably larger than the molecular weight of the cPERK on the denaturing protein gel shown in Fig. 1C. This result is consistent with a previous report showing that PERK oligomerizes *in vivo* to become an active form phosphorylating its physiological substrate eIF2α [12].

Autophosphorylation and Substrate Phosphorylation Activities of Recombinant cPERK

Various kinases, but not all, require autophosphorylation in their kinase domain for activation. In the case of PKR, intermolecular *trans*-autophosphorylation is shown to be mediated by its dimerization [22]. We investigated whether hyperphosphorylated cPERK has an autophosphorylation activity, and a protein kinase activity on its physiological substrate eIF2α. *In vitro* kinase assay results, shown in Fig. 4, indicated that cPERK is able to phosphorylate itself (Fig. 4A, lane 1) probably through intermolecular interaction. It also phosphorylated *in trans* GST-eIF2α substrate (lane 3), but not GST-eIF2α(S51A) with an Ala substitution for the phosphorylation site Ser51 (Fig. 4B, lane 3). In contrast, the cPERK(K621M) mutant showed no autophosphorylation and *trans* activation activities (data not shown), indicating that cPERK(K621M) is completely defective for the autophosphorylation that is essential for activation of the kinase function of PERK. These results also indicate a

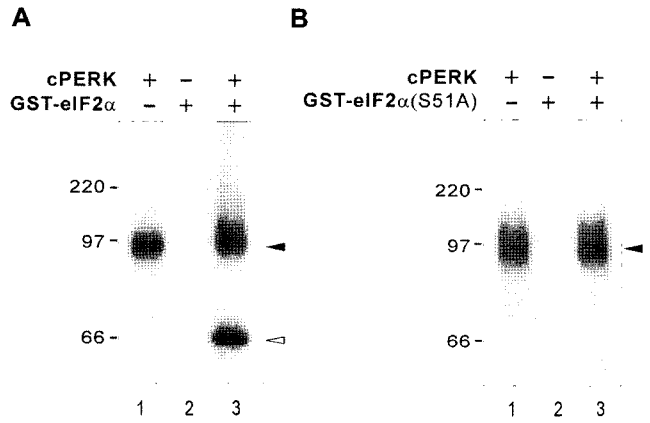

Fig. 4. Autophosphorylation and *trans*-phosphorylation activities of PERK.

A. *In vitro* autophosphorylation activity of cPERK and its *trans*-phosphorylation activity on GST-eIF2α (lane 3). Purified cPERK and GST-eIF2α were used for *in vitro* kinase assays. **B.** A similar experiment was performed with GST-eIF2α(S51A). Closed and open arrowheads indicate the autophosphorylated cPERK and phosphorylated GST-eIF2α, respectively. Protein size markers are shown at the left of each gel, in kDa.

specific phosphorylation activity of cPERK on its physiological substrate eIF2α *in vitro*.

cPERK is Phosphorylated at Ser and Thr Residues

Based on the sequence similarity between PERK and PKR, PERK has been considered to be a Ser/Thr kinase [3]. However, little is known about what amino acids are phosphorylated in the autophosphorylated form of PERK. In addition, it is still controversial whether PKR phosphorylates only Ser and Thr residues. A previous study has indeed shown that PKR is also able to phosphorylate a Tyr residue that was substituted for the normal target Ser on eIF2α. It was therefore necessary to determine what amino acids are phosphorylated in the recombinant cPERK. Purified cPERK was autophosphorylated *in vitro* in the presence of [γ-^{32}P]ATP and the labeled cPERK was subjected to two-dimensional phosphoamino acids analysis. As shown in Fig. 5C, cPERK autophosphorylated *in vitro* both the Ser and Thr residues at approximately a 3:1 ratio.

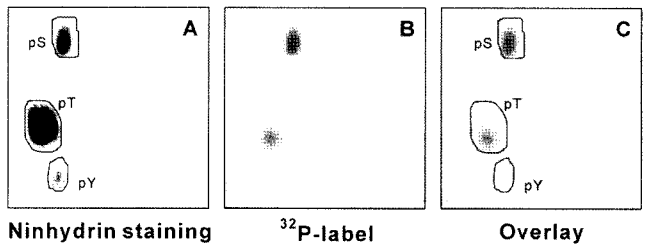

Fig. 5. cPERK is phosphorylated at Ser and Thr residues.

In vitro labeled cPERK was resolved by SDS-10% PAGE and then transferred to a PVDF membrane. The cPERK band was excised and then subjected to acid hydrolysis followed by two-dimensional phosphoamino acid analysis. Labeled phosphoamino acids were detected by autoradiography. Circles indicate the ninhydrin staining pattern of phosphoamino acid standards resolved with the acid hydrolysates (**A**). Both Ser and Thr were detectably labeled (**B** and **C**). pS, phosphoserine; pT, phosphothreonine; pY, phosphotyrosine.

DISCUSSION

The mammalian ER-stress response consists of an early phase in which protein synthesis is inhibited by eIF2α phosphorylation, and a later phase in which genes that promote increased ER capacity are induced. The early, PERK-dependent phase plays an important role in acutely reducing the load of client proteins that the ER must handle and is strongly protective against ER stress [23]. The PERK kinase domain plays an essential role in eIF2α phosphorylation that results in the inhibition of protein translation. In this study, we have expressed cPERK in *E. coli*, and purified the activated form cPERK to near homogeneity. We demonstrated that the recombinant cPERK is functionally active *in vitro* and has both autophosphorylation and *trans*-phosophorylation activities. The level of the hyperphosphorylated form cPERK increased during the time course of its expression in *E. coli* (Fig. 1D), which is likely due to enhanced oligomerization of cPERK. This result is consistent with an activation mechanism for PERK in which release of the ER chaperone GRP78/Bip from PERK occurs when misfolded proteins accumulate in the ER, leading to activation of its kinase activity through dimerization or oligomerization of the kinase domain [12]. Lack of an N-terminal domain in the cPERK might explain why its overexpression in *E. coli* can lead to its autophosphorylation through oligomerization. It seems likely that overexpression of cPERK in bacteria cells allow for cPERK oligomerization without ER stress by bringing the cytoplasmic kinase domain into close proximity. Such oligomerization-mediated activation of eIF2α kinases was also observed when PRK was expressed in *E. coli*. The recombinant PRK was fully active and did not respond further to an RNA regulator *in vitro* [10]. Even though oligomerization of PERK appears to be a prerequisite for its activation *in vivo*, it has remained unclear how many molecules of PERK are involved in the formation of the PERK oligomer upon ER stress. A previous study suggested that the complex might consist of 2–6 molecules of PERK [12]. From the gel-filtration experiment, we could conclude that cPERK forms a tetramer *in vitro* with an apparent molecular weight of ~400 kDa (Fig. 3).

The functionally active cPERK should be useful for the isolation and characterization of other cellular substrates or viral/cellular proteins that might modulate the kinase activity of PERK. Furthermore, both inactive and active cPERK recombinant proteins can be used to determine and compare their three-dimensional structures to

elucidate what structural changes are accompanied by the hyperphosphorylation of PERK.

Acknowledgments

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