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Dimerization and Release of Molecular Chaperone Inhibition Facilitate Activation of Eukaryotic Initiation Factor-2 Kinase in Response to Endoplasmic Reticulum Stress*

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Phosphorylation of eukaryotic initiation factor-2 (eIF2) by pancreatic eIF2 kinase (PEK), induces a program of translational expression in response to accumulation of malfolded protein in the endoplasmic reticulum (ER). This study addresses the mechanisms activating PEK, also designated PERK or EIF2AK3. We describe the characterization of two regions in the ER luminal portion of the transmembrane PEK that carry out distinct functions in the regulation of this eIF2 kinase. The first region mediates oligomerization between PEK polypeptides, and deletion of this portion of PEK blocked induction of eIF2 kinase activity. The second characterized region of PEK facilitates interaction with ER chaperones. In the absence of stress, PEK associates with ER chaperones GRP78 (BiP) and GRP94, and this binding is released in response to ER stress. ER luminal sequences flanking the transmembrane domain are required for GRP78 interaction, and deletion of this portion of PEK led to its activation even in the absence of ER stress. These results suggest that this ER chaperone serves as a repressor of PEK activity, and release of ER chaperones from PEK when misfolded proteins accumulate in the ER induces gene expression required to enhance the protein folding capacity of the ER.

A well characterized mechanism regulating translation initiation in response to different cellular stresses involves phosphorylation of the α subunit of eukaryotic initiation factor-2 (eIF2)[1] (1, 2). In mammalian cells, four eIF2 kinases have been identified, and each directly senses distinct stress signals and modulates downstream response pathways by translational control. These eIF2 kinases include PKR, important for an antiviral defense pathway mediated by interferon (3–5); HRI, which couples protein synthesis to the availability of heme in erythroid cells (6, 7); GCN2, which is activated by nutritional stresses; and the subject of this report (1, 8–11), pancreatic eIF2 kinase, PEK (also known as Perk, encoded by the *EIF2AK3* gene), important for remedying protein misfolding in the endoplasmic reticulum (11–15).

In mammalian cells, a block in glycosylation or disulfide linkages in the ER or release of calcium from this organelle leads to impaired assembly of proteins slated for the secretory pathway and induced phosphorylation of eIF2 by PEK (16–18). The eIF2, combined with initiator methionyl-tRNA and GTP, associates with the 40 S ribosomal subunit and participates in the recognition of the start codon during initiation of translation (19). During the joining of the small and large ribosomal subunit, GTP complexed with eIF2 is hydrolyzed to GDP. Phosphorylation of eIF2 by PEK reduces the exchange of eIF2-GDP to the GTP-bound form that is catalyzed by the guanine nucleotide exchange factor, eIF2B. The resulting reduction in eIF2-GTP levels impedes translation initiation in the cell, allowing the cell sufficient time to correct the folding problem incurred by the ER stress prior to synthesizing additional proteins.

Accompanying this reduction in translation during ER stress is the unfolded protein response (UPR) (17, 18, 20). The UPR involves the expression of a large number of secretory pathway genes, including those involved in protein folding, such as ER chaperones GRP78/BiP and GRP94, disulfide bond formation, protein glycosylation, retrograde protein degradation, translocation, and vesicle trafficking (21). Another ER transmembrane protein kinase, IRE1, induces the transcription of UPR genes (20, 22–25). Phosphorylation of eIF2 by PEK is proposed to work in concert with IRE1 to enhance the coordinate expression of proteins linked with the UPR by a mechanism involving preferential translation of certain of mRNAs (18, 26). In addition to sharing a common topological arrangement in the ER membrane, a portion of the ER luminal sequences of PEK shares homology with IRE1, suggesting that there is a common mechanism activating the cytoplasmic protein kinase activities in response to the ER stress (13, 14, 27). Loss of PEK (Perk) function in mouse embryonic stem cells exposed to ER stress leads to inappropriately elevated protein synthesis that further exacerbates protein misfolding in this organelle, triggering apoptosis (15). Loss of PEK (*EIF2AK3* gene) in humans leads to a rare autosomal recessive disorder, Wolcott-Rallison syndrome, that is characterized by neonatal insulin-dependent diabetes accompanied by a characteristic destruction of the pancreatic beta cells (28). However, Wolcott-Rallison syndrome patients do not display autoantibodies diagnostic of type I diabetes. At later ages, there is an occurrence of epiphyseal dysplasia, osteoporosis, and growth retardation. Frequently, afflicted patients also suffer from multisystemic pathologies including hepatic and renal complications, cardiovascular disease, and mental retardation (29, 30). PEK−/− mice display similar pancreatic defects and succumb to complications related to severe hyperglycemia within several weeks of birth (11).

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[1] The abbreviations used are: eIF2, eukaryotic initiation factor-2; UPR, unfolded protein response; dsRNA, double-stranded RNA; dsRBD, dsRNA binding domain; HEK, human embryonic kidney; ER, endoplasmic reticulum; PEK, pancreatic eIF2 kinase.

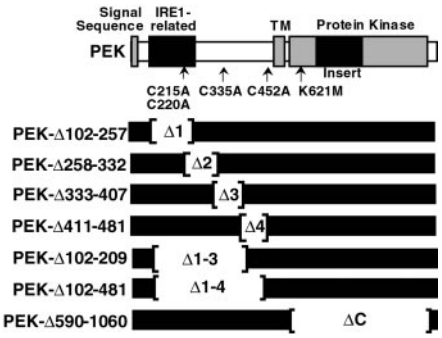

FIG. 1. **Diagram of PEK proteins used to delineate mechanisms important for induced eIF2 phosphorylation in response to ER stress.** Human PEK, 1115 residues in length, is represented by a *box*. PEK contains an amino-terminal signal sequence proposed to facilitate translocation into the ER, sequences homologous to IRE1 (13, 14, 27), and an ER transmembrane region (*TM*). The carboxyl-terminal portion of PEK, located in the cytoplasm, contains the protein kinase domain. In this catalytic region, there is a 223-residue insert region dividing subdomains IV and V, a feature conserved among eIF2 kinases. *Below* the PEK box are mutant versions of PEK used in this study. Residue substitutions in PEK are indicated by the wild-type amino acid, position of residue, and the mutant residue. Portions of PEK that were deleted in-frame are indicated by *brackets* with *numbers 1*, *2*, *3*, or *4* or *C* for carboxyl terminus, and the amino acid residues removed are listed to the *left* of the mutant diagrams.

PEK and each of the other eIF2 kinases are proposed to share common features in their mechanisms of activation in response to stress. Direct binding with ligands whose concentrations are impacted by stress or with proteins whose levels or properties are altered by stress can trigger an activated eIF2 kinase conformation. For example, dsRNA produced during different viral infections binds cooperatively with two dsRNA binding domains (dsRBDs) in PKR, leading to enhanced eIF2 kinase activity (3–5). In the case of HRI, association with chaperones HSP90 and HSC70 is thought to be important for modulating HRI activity in response to heme deprivation or heat shock (6, 31, 32). Binding with ER chaperone GRP78 or its yeast homologue KAR2 is proposed to regulate the ER transmembrane protein kinase IRE1 and possibly PEK (33, 34). With the accumulation of misfolded protein during ER stress, the chaperone may be titrated from the regulatory sequences shared between IRE1 and PEK, facilitating autophosphorylation and eIF2 kinase activity. Accompanying this activated conformation is autophosphorylation involving threonine residues in the so-called activation loop of the kinase domain. PEK was reported to be autophosphorylated *in vitro* at 10 different sites, including threonine 980 located in the activation loop, and hyperphosphorylation as judged by retarded migration of PEK in SDS-PAGE is thought to participate in kinase activation in response to cellular ER stress (13, 14, 35).

In this report, we find that PEK dimerization is required for hyperphosphorylation and induced eIF2 kinase activity during ER stress, and this oligomerization is mediated by ER luminal sequences extending beyond the IRE1 homology domain. PEK association with ER chaperones GRP78 and GRP94 is released upon ER stress, and deletion of sequences in PEK that facilitate GRP78 binding leads to activation as judged by hyperphosphorylation, independent of ER stress. Together, these results support the model whereby different ER chaperones bind and repress PEK, and release of this interaction in response to protein misfolding in the ER facilitates PEK dimerization, autophosphorylation, and induced eIF2 phosphorylation.

MATERIALS AND METHODS

PEK Mutants and Plasmid Constructions—cDNAs encoding wild-type or mutant versions of PEK illustrated in Fig. 1 were inserted downstream of the cytomegalovirus promoter in plasmid pcDNA3 (Invitrogen). These plasmids include pKM10 (wild-type PEK), pKM24 (PEK-K621M), pKM68 (PEK C-A), pKM39 (PEK-Δ1), pKM42 (PEK-Δ2), pKM43 (PEK-Δ3), pKM47 (PEK-Δ4), pKM66 (PEK-Δ4-K621M), pKM56 (PEK-Δ1–3), pKM67 (PEK-Δ1–3-K621M), pKM57 (PEK-Δ1–4), pKM29 (PEK-ΔC), and pKM65 (PEK-ΔC C-A). PEK-K621M, which contains Lys for Met-621 and PEK C-A containing Ala substitutions for Cys at residues 215, 220, 335, and 452), were made by using the QuikChange mutagenesis kit (Stratagene). Deletions in PEK were constructed by standard PCR methods (36). To facilitate immunoprecipitation, the c-Myc epitope was inserted at the carboxyl terminus of full-length PEK, PEK-Δ1–3, PEK-Δ1–4, and PEK-ΔC.

Cell Culture, Transfection, and ER Stress Treatment—Human embryonic kidney (HEK) 293T cells that express SV40 T antigen were cultured in Dulbecco's modified Eagle's medium (BioWhittaker), supplemented with 2 mM glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, 10% fetal bovine serum (Hyclone) in humidified air with 5% CO_2 at 37 °C. Cells grown in 100-mm dishes were transfected with plasmid DNA encoding wild-type or mutant versions of PEK or vector alone by using LipofectAMINE (Invitrogen). After culturing the transfected cells for 48 h, cells were treated with 1 μM thapsigargin for 1 h or 5 mM homocysteine for 3 h. Cells then were washed twice with 10 ml of ice-cold phosphate-buffered solution. Cell lysates were prepared in 500 μl of lysis buffer (1% Triton X-100 in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 100 mM NaF, 17.5 mM β-glycerolphosphate, 10% glycerol) supplemented with protease inhibitors (100 μM phenylmethylsulfonyl fluoride, 0.15 μM aprotinin, 1 μM leupeptin, and 1 μM pepstatin) and clarified by centrifugation.

Immunoblots, Immunoprecipitations, and Kinase Assays—Rabbit polyclonal antibody was raised against recombinant protein containing the carboxyl terminus of human PEK, and the antibody was further prepared by affinity purification. Polyclonal antibody against c-Myc, GRP78, GRP94, and HSP70 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Equal amounts of lysates prepared from HEK 293T cells transfected with the pcDNA3 plasmid expressing PEK were separated by electrophoresis in an SDS-polyacrylamide gel and transferred onto nitrocellulose filters. Filters were blocked in a TBS-T solution containing 20 mM Tris-HCl (pH 7.9), 150 mM NaCl, and 0.2% Tween 20 supplemented with 4% nonfat milk. Filters were then incubated in TBS-T-containing PEK-specific antibody and 4% nonfat milk, washed three times in TBS-T, and incubated with TBS-T-containing goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (Bio-Rad). Filters were washed three times in TBS-T solution, and the PEK-antibody complex was detected by enhanced chemiluminescence. Immunoblots measuring eIF2α phosphorylation were carried out with antibody that specifically recognizes phosphorylated eIF2 at Ser-51 (Research Genetics) (37). Total eIF2α in lysates was detected by immunoblot using monoclonal antibody generously provided by Dr. Scot Kimball (Pennsylvania State University, College of Medicine, Hershey, PA) that recognizes either phosphorylated or nonphosphorylated forms of this initiation factor. The eIF2α-antibody complex was visualized using horseradish peroxidase-labeled anti-rabbit or anti-mouse secondary antibody and chemiluminescent substrate. To establish linearity in the assay, proteins were serially diluted in the SDS-PAGE, and multiple autoradiographic exposures were performed. Immunoblot analyses measuring phosphorylated PEK were performed using rabbit polyclonal antiserum that specifically recognizes PEK phosphorylated at Thr-980.

In experiments incorporating immunoprecipitations, equal amounts of protein lysates prepared from 293T-derived cells were precleared with protein G-agarose (Roche Molecular Biochemicals) for 3 h at 4 °C with gentle rocking. Agarose was collected by centrifugation for 20 s at 12,000 × *g*, and the supernatants were incubated with goat polyclonal antibody against GRP78 or with mouse monoclonal antibody against the c-Myc tag for 1 h at 4 °C with gentle rocking. Antibody complexes were collected by using protein G-agarose incubated for 3 h at 4 °C, followed by centrifugation for 20 s at 12,000 × *g*. Agarose pellets were washed twice with lysis buffer, twice with high salt buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% Triton X-100), and twice with low salt buffer (50 mM Tris-HCl, pH 7.5, 0.1% Triton X-100). Immunoprecipitated proteins were heated at 95 °C for 3 min in the presence of SDS sample buffer and clarified by centrifugation. Proteins were separated by SDS-PAGE, and PEK, GRP78, GRP94, or HSP70 was visualized by immunoblot analysis using specific antibodies. Proteins associated with antibodies were visualized using horseradish peroxidase-labeled secondary antibody and chemiluminescent substrate. Linearity of the immunoblot analyses was confirmed by using serial dilutions of protein sample and by performing multiple autoradiographic exposures of different length times. PEK immunoprecipitation kinase assays were car-

ried out as described using recombinant eIF2α substrate and [γ-^{32}P]ATP in a final ATP concentration of 50 μM (38, 39).

In the study measuring PEK binding with GRP78, we used antibody specific to this ER chaperone in the immunoprecipitation, followed by PEK or GRP78 immunoblot analysis of proteins in the immune complex. GRP78 binding was calculated as the level of PEK in the complex normalized for the level of immunoprecipitated GRP78. Values were presented relative to wild-type PEK co-precipitated with GRP78.

In the dimerization study that defined the dimerization region of PEK, the relative density was determined for each PEK band in the immunoblot analysis. Oligomerization was calculated as the ratio of the nontagged PEK protein to the c-Myc-tagged version of PEK-ΔC in each immunoprecipitation sample. Values were normalized for wild-type PEK co-immunoprecipitated with PEK-ΔC. It is noted that the PEK antibody was prepared against recombinant protein containing residues 588–1115. Although it can recognize PEK-ΔC, which includes residues 1061–1115, immunoblots using this PEK antibody presumably underrepresent PEK-ΔC levels compared with PEK containing the entire carboxyl terminus. Additionally, we carried out immunoblots using antibody specific to the c-Myc tag of PEK-ΔC and found that the relative levels of the truncated kinase between the preparations were identical to that measured with the PEK antibody (data not shown). It is noted that HEK 293T cells expressing PEK-Δ4 consistently expressed less of the co-transfected c-Myc-PEK-ΔC. Similar results were obtained with three independent experiments.

Glycerol Gradient Centrifugation—Proteins in cell lysates were separated on a 12-ml exponential 20–40% glycerol gradient in cell lysis buffer described above. The gradient was subjected to ultracentrifugation using a Beckman SW41 rotor, at 39,000 rpm for 42 h at 4 °C. One-ml fractions were collected from the gradients. Equal volumes from each fraction were separated by SDS-PAGE, and PEK levels were measured by immunoblot analysis using PEK-specific antibody and densitometry. Size standards used in the glycerol gradients included albumin, aldolase, catalase, ferritin, and thyroglobulin (Amersham Biosciences).

RESULTS

Different ER Stress Conditions Induce PEK Oligomerization—To address the multimeric state of PEK during nonstressed and ER-stressed conditions, lysates from HEK 293T cells were characterized by glycerol gradient centrifugation, and fractions were assayed for the presence of PEK by immunoblot. ER stress was induced by adding thapsigargin to the cells, resulting in the release of calcium from this organelle. In nonstressed conditions, PEK sedimented with a molecular weight of appropriately 210,000, which increased to 320,000 in response to ER stress (Fig. 2). The higher molecular weight form observed in response to ER stress was confirmed using HEK 293T cells that overexpressed PEK by transfection of a pcDNA3 derivative encoding PEK downstream of the cytomegalovirus promoter. Migration of PEK in either condition was significantly higher than its monomeric size of 125,000, indicating that PEK oligomerized or interacted with other proteins. Furthermore, immunoblot analysis of PEK in the higher molecular weight form indicated that it sedimented more slowly in the SDS-PAGE as compared with nonstressed conditions (Fig. 2, *B* and *C*). The slower mobility in the denaturing gel electrophoresis is the result of hyperphosphorylation of PEK in response to ER stress, and this migration difference is absent following phosphatase treatment or when kinase-defective mutants of PEK are similarly analyzed (13, 14).

It has been suggested that oligomerization involving the luminal portion of the ER transmembrane protein kinase IRE1, and possibly PEK, is important for their activation in response to ER stress (27, 33, 40, 41). The higher molecular weight measured for PEK during this cellular stress would be consistent with dimerization between PEK molecules. To address this premise, we expressed a c-Myc-tagged version of PEK that was substantially deleted for its cytoplasmic portion (Fig. 1). If PEK oligomerization is required for PEK phosphorylation in *trans* and enhanced eIF2 kinase activity, we reasoned that the PEK-ΔC would function as a dominant-negative. By binding

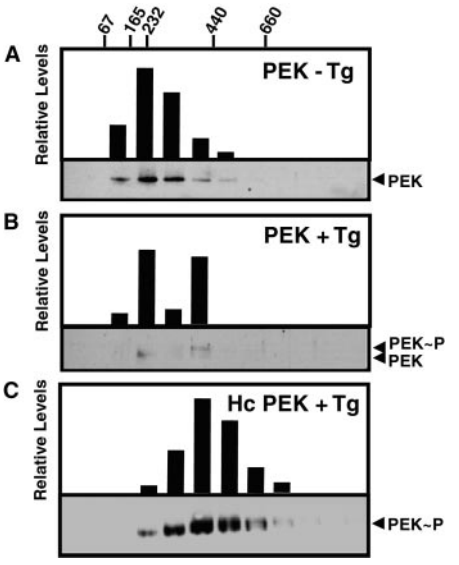

FIG. 2. **PEK oligomerizes in response to ER stress.** HEK 293T cells incubated in the presence or absence of the ER stressing agent, thapsigargin, for 1 h, and cellular lysates were separated using a 20–40% glycerol gradient. PEK levels were measured in gradient fractions by immunoblot using antibody specific to this eIF2 kinase. Autoradiograms generated by the immunoblot analyses are illustrated *below histograms* that present the relative levels of PEK in each gradient fraction as judged by densitometry. *A*, HEK 293T cells were prepared in the absence of ER stress; *B* and *C*, cells were subjected to thapsigargin treatment. *C*, experiment carried out using HEK 293T cells that were transfected with plasmid pcDNA3 encoding wild-type PEK under the transcriptional expression of the cytomegalovirus promoter as described under "Materials and Methods." Marker molecular weights are ×1,000.

with the luminal sequences of the endogenous full-length PEK, PEK-ΔC would block appropriate autophosphorylation required to facilitate an activated conformation of the full-length kinase. Indeed, expression of the truncated PEK-ΔC in HEK 293T cells blocked hyperphosphorylation of PEK in response to thapsigargin treatment as judged by the retarded migration of PEK in the SDS-PAGE (Fig. 3*A*). Moreover, expression of PEK-ΔC significantly diminished the induction of eIF2α phosphorylation in response to thapsigargin exposure. Levels of total eIF2 were unchanged between cell lysates.

To determine whether PEK-ΔC functioned as a dominant-negative in response to other ER stress agents, we carried out an analogous experiment using homocysteine, a known inducer of the UPR (42, 43). Consistent with our earlier observation, PEK hyperphosphorylation was induced in response to homocysteine treatment of the HEK 293T cells, and expression of PEK-ΔC blocked both autophosphorylation and eIF2α phosphorylation in response to this ER stress agent (Fig. 3*B*). We conclude that PEK-ΔC can function as a dominant-negative in response to different ER stress conditions. To ascertain whether PEK-ΔC directly interacts with full-length PEK, the two forms of the eIF2 kinase were co-expressed in HEK 293T cells, and the c-Myc-tagged PEK-ΔC was immunoprecipitated using c-Myc-specific antibody. Proteins in the immune complexes were separated by SDS-PAGE, and PEK was visualized by immunoblot. Wild-type PEK co-immunoprecipitated with PEK-ΔC, whereas no full-length kinase immunoprecipitated with the c-Myc antibody in the absence of PEK-ΔC expression (Fig. 3*C*). Together, our results are consistent with the idea that in response to ER stress PEK dimerizes through ER luminal sequences.

ER Luminal Sequences Extending beyond the IRE1 Homology Region Mediate PEK Dimerization and Activation—The observation that PEK-ΔC complexes with the full-length ver-

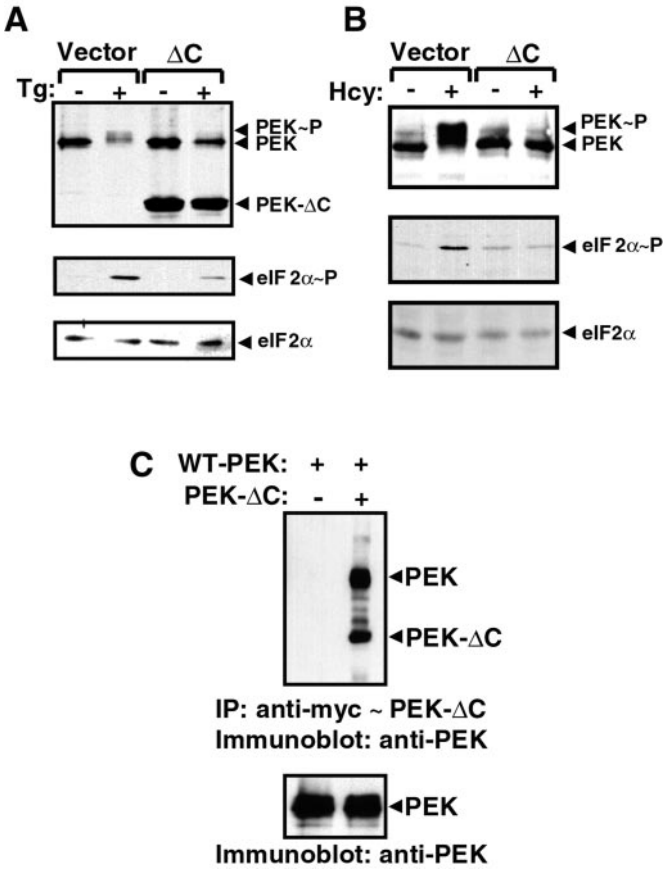

FIG. 3. **Dominant-negative mutant PEK-ΔC complexes with full-length PEK and inhibits phosphorylation of eIF2.** Plasmids expressing PEK-ΔC or vector alone were introduced into HEK 293T cells. Following transfection, the cultured cells were incubated in the presence or absence of thapsigargin (*Tg*, *panel A*) or homocysteine (*Hcy*, *panel B*) as indicated, collected, and analyzed by immunoblot using antibodies specific to PEK, phosphorylated eIF2α, or total eIF2α. *Panel C*, HEK 293T cells were transfected with plasmids expressing both wild-type (*WT*) PEK and c-Myc-tagged PEK-ΔC or with wild type PEK alone as indicated. PEK-ΔC was immunoprecipitated from cell lysates using c-Myc antibody. Proteins in the immune complexes were subjected to SDS-PAGE, followed by immunoblot analysis using antibodies that specifically recognize PEK. In the *lower portion* of *panel C*, wild-type PEK in the cell lysates was measured by immunoblot, showing similar levels of the eIF2 kinase in the transfected HEK 293T cell preparations.

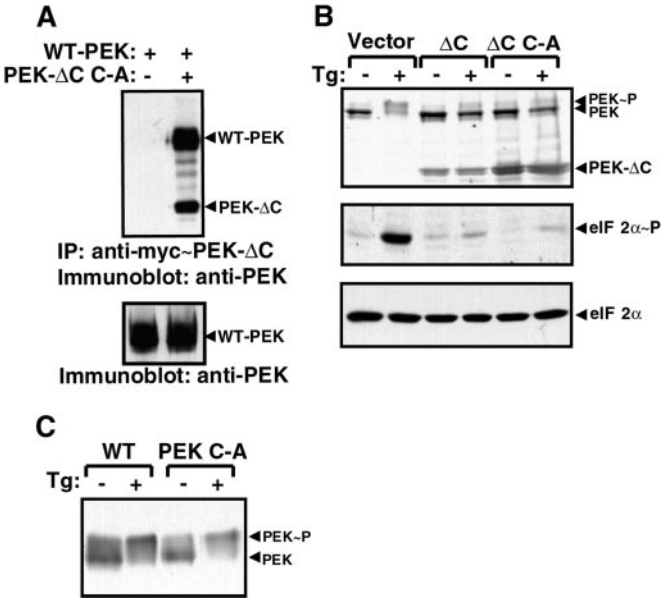

FIG. 4. **Cysteine residues in the ER luminal region are dispensable for dimerization and activation of PEK.** *A*, wild-type PEK was co-expressed in HEK 293T cells with a c-Myc-tagged version of PEK-ΔC containing alanine substituted for each of four cysteine residues in the ER luminal portion of the eIF2 kinase (PEK-ΔC C-A) or with vector alone. Following preparation of cellular lysates, PEK-ΔC C-A was immunoprecipitated using c-Myc antibody. Wild type (*WT*) or the indicated truncated PEK in the immune complexes was visualized by SDS-PAGE, followed by immunoblot analysis using PEK-specific antibodies. In the *lower portion* of *A*, wild-type PEK in the cell lysates was measured by immunoblot, showing similar levels of the kinase in HEK 293T preparations. *B*, expression plasmids for PEK-ΔC, PEK-ΔC C-A, or vector alone were transfected into HEK 293T cells and incubated in the presence or absence of thapsigargin as indicated. PEK, phosphorylated eIF2α, or total eIF2α was visualized by immunoblot using antibodies that specifically recognize these proteins. *C*, wild-type (*WT*) PEK or PEK C-A with alanine substituted at each of the four cysteine residues was expressed into HEK 293T cells. Following incubation in the presence or absence of thapsigargin, cell lysates were prepared and subjected to SDS-PAGE, followed by immunoblot analysis. PEK was visualized using antibodies specific to this eIF2 kinase, and hyperphosphorylation was assessed by the molecular weight shift.

sion of the eIF2 kinase provided a tool to address the portions of the kinase that mediate oligomerization. At least four cysteine residues are present in the luminal portion of PEK from humans, mice, *Drosophila melanogaster*, and *Caenorhabditis elegans* (14). Notably, two cysteine residues at positions 215 and 220 in the human eIF2 kinase are invariant between these PEK homologues and IRE1. We substituted alanine for each of the four cysteine residues in the luminal portion of PEK-ΔC and analyzed the ability of this form of the truncated PEK (PEK-ΔC C-A) to complex with wild-type PEK. Following the experimental strategy described earlier, full-length eIF2 kinase was found to co-immunoprecipitate with the c-Myc-tagged version of PEK-ΔC C-A (Fig. 4*A*). No wild-type PEK was observed to immunoprecipitate using lysates devoid of the truncated kinase. We conclude that cysteine residues in the ER luminal portion of PEK and potential disulfide linkages mediated by these residues are not required for dimerization between PEK molecules. Consistent with the ability of the PEK-ΔC C-A to oligomerize, expression of this mutant in HEK 293T cells blocked both the hyperphosphorylation of PEK in response to ER stress and the subsequent induction of eIF2α phosphorylation (Fig. 4*B*). Furthermore, the full-length PEK containing the four cysteine substitutions was activated, as judged by hyperphosphorylation, in response to thapsigargin treatment (Fig. 4*C*). We conclude that the ER luminal cysteine residues and possible inter- or intramolecular disulfide bonds are dispensable for PEK dimerization and the mechanism of activation of this eIF2 kinase.

To demarcate the portions of PEK required for dimerization, we next deleted four different regions in the ER luminal portion of PEK and analyzed the ability of these mutant proteins to complex with PEK-ΔC (Fig. 1). As described earlier for the co-immunoprecipitation experiments using the full-length version of PEK, the different deleted forms of PEK were distinguishable based on their molecular weight differences as viewed in the SDS-PAGE, followed by immunoblot analysis using PEK-specific antibody (Fig. 5). Deletion of either regions 1, 2, or 3 reduced co-immunoprecipitation with c-Myc-tagged PEK-ΔC to between 10 and 22% of that measured for full-length PEK. By comparison, PEK deleted for region 4 retained the ability to dimerize (Fig. 5). By combining deletions 1, 2, and 3 into a single c-Myc-tagged PEK mutant, we found a near abolishment of dimerization, as measured by co-immunoprecipitation with full-length PEK (Fig. 5*B*). A similar inability to dimerize was obtained when PEK deleted for all four regions was analyzed for co-immunoprecipitation with wild-type PEK. We conclude that sequences extending beyond the IRE1 homol-

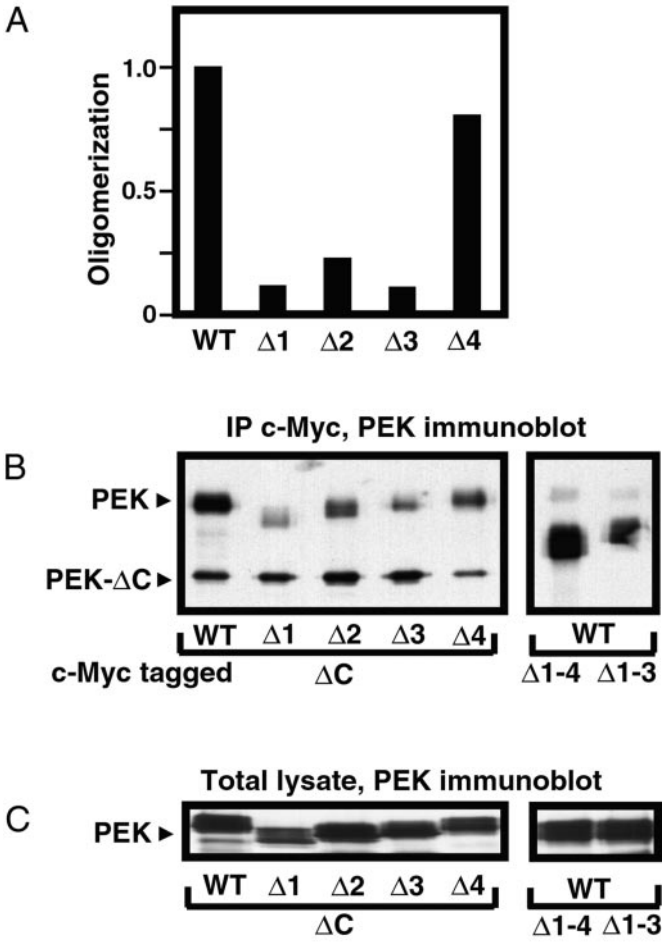

FIG. 5. **Sequences extending beyond the IRE1 homology region facilitate PEK dimerization.** PEK mutants deleted for different portions of the ER luminal domain were co-expressed in HEK 293T cells with either PEK-ΔC or wild-type (*WT*) PEK as indicated. PEK-ΔC, PEK-Δ1–4, or PEK-Δ1–3 tagged with c-Myc as highlighted was immunoprecipitated using equal amounts of cell lysates and antibody specific to the c-Myc epitope. Wild-type or truncated PEK in the immune complexes were separated by SDS-PAGE and visualized by immunoblot analysis using PEK-specific antibodies (*B*). *A*, a *histogram* representing the oligomerization between the Myc-tagged PEK-ΔC and the co-expressed versions of PEK that were not tagged. *Histograms* represent the ratio of nontagged PEK to c-Myc-tagged PEK-ΔC in each immune complex (see "Materials and Methods"). Ratios were normalized for wild-type PEK. It is noted that HEK 293T cells expressing PEK-Δ4 consistently expressed less of the co-transfected c-Myc-PEK-ΔC. *C*, nontagged PEK in the HEK 293T lysates was measured by immunoblot using PEK-specific antibody, showing similar levels of these nontagged kinase versions in the cell preparations.

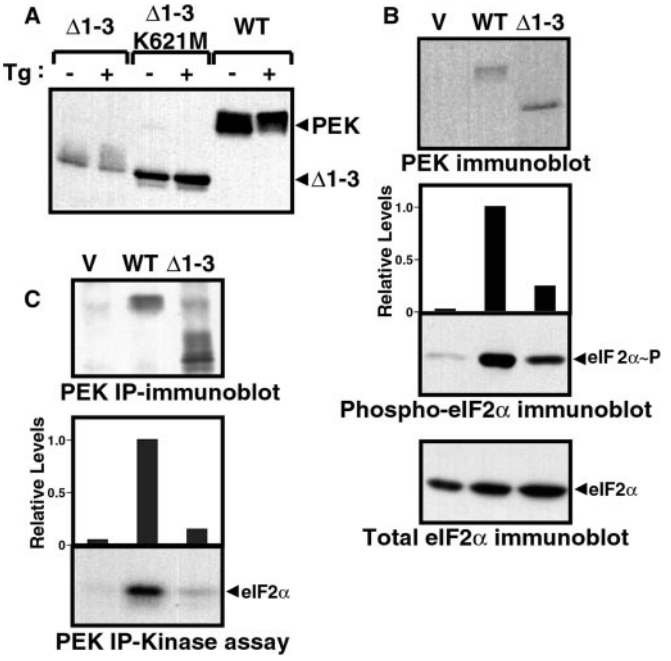

FIG. 6. **Deletion of PEK dimerization sequences reduces eIF2 kinase activity.** HEK 293T cells were transfected with pcDNA3-derived plasmids encoding wild-type PEK, PEK-Δ1–3, the kinase-inactivated PEK-Δ1−3-K621M, or vector alone and subjected to ER stress using thapsigargin. *A*, cellular lysates were prepared from the HEK 293T-derived cells in the presence or absence of ER stress, and PEK was visualized by immunoblot. *B*, phosphorylated eIF2α and total eIF2α in the transfected HEK 293T cells subjected to ER stress were visualized by immunoblot analysis using antibody specific to the phosphorylated form of the protein or using antibody that recognizes both phosphorylated and nonphosphorylated eIF2α. Relative levels of phosphorylated eIF2α are represented in the *histogram*. PEK levels in the samples were analyzed by immunoblot. *C*, PEK in the HEK 293T lysates was immunoprecipitated using PEK-specific antibody and immune complexes containing equal amounts of transfected wild-type or PEK-Δ1–3 were incubated in the presence of [γ-^{32}P]ATP and recombinant eIF2α. Following separation of proteins in the kinase reaction mixture by SDS-PAGE, radiolabeled eIF2α was visualized by autoradiography. Relative levels of *in vitro* phosphorylated eIF2α as determined by liquid scintillation counting are represented as a *histogram*. PEK levels in the immune complexes were measured by immunoblot analysis.

ogy region in the ER luminal portion of PEK are important for dimerization.

Dimerization is proposed to be an important step leading to hyperphosphorylation of PEK and induced eIF2 phosphorylation in response to ER stress. We characterized the levels of autophosphorylation of PEK-Δ1–3 as judged by the molecular weight shift following SDS-PAGE and immunoblot (Fig. 6). While wild-type PEK was hyperphosphorylated in response to ER stress, minimal phosphorylation of PEK-Δ1–3 was detected in the thapsigargin-exposed cells, with a molecular weight identical to that observed for the repressed conditions, or in PEK-Δ1–3 protein containing a kinase-inactivating Met substitution for Lys-621 (Fig. 6*A*). We next examined the impact of Δ1–3 on the eIF2 kinase activity of PEK. The levels of phosphorylation of the α subunit of eIF2 in ER-stressed HEK 293T cells overexpressing wild-type PEK were significantly elevated compared with those transfected with a PEK-Δ1–3 cDNA or vector alone (Fig. 6*B*). Levels of overexpressed wild-type PEK and PEK-Δ1–3 were similar as judged by immunoblot of whole cell lysates. To further compare eIF2 kinase activities between wild-type PEK and PEK-Δ1–3, the protein kinases were immunoprecipitated using antibody specific to PEK and assayed for phosphorylation of recombinant eIF2α substrate. Consistent with the earlier *in vivo* analysis, eIF2 kinase activity of the immunoprecipitated PEK-Δ1–3 was significantly reduced compared with wild-type PEK (Fig. 6*C*). We conclude that ER luminal sequences in PEK that mediate dimerization are required for full induction of eIF2 kinase activity.

Regulation of PEK Involves Association of Chaperones with ER Luminal Portion of the eIF2 Kinase—ER chaperone GRP78 is proposed to control the kinase activity of IRE1, and possibly PEK, through direct protein-protein interaction (33, 34). To address whether PEK associates in a regulated fashion with chaperones, we immunoprecipitated the ER chaperones GRP78 and GRP94 or the cytoplasmic chaperone HSP70 from lysates prepared from HEK 293T cells cultured in the presence or absence of ER stress. Proteins in the immune complexes were separated by SDS-PAGE, followed by immunoblot analysis using antibodies specific for each chaperone or for PEK (Fig. 7). PEK co-immunoprecipitated with GRP78 and GRP94, and this

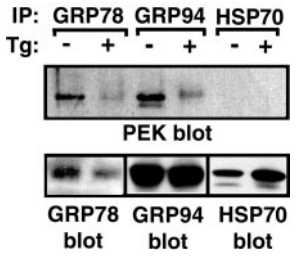

FIG. 7. **PEK complexes with ER-resident chaperones GRP78 and GRP94 but not cytoplasmic chaperone HSP70.** HEK 293T cells were grown in the presence or absence of thapsigargin, and following lysate preparations, antibodies specific to GRP78, GRP94, or HSP70 were used to immunoprecipitate the indicated chaperone. In the *bottom panel*, proteins from each immune complex were separated by SDS-PAGE, and antibodies against GRP78, GRP94, or HSP70 that were used in the immunoprecipitation were used to visualize this chaperone by immunoblot. *Top panel*, in parallel, the presence of PEK was measured in each of the immune complexes using PEK-specific antibody and immunoblot analysis.

association was significantly reduced in response to ER stress. By comparison, no association was detected between PEK and the cytoplasmic chaperone HSP70. These results combined with our earlier glycerol gradient analysis are consistent with the idea that PEK associates with ER chaperones such as GRP78 or GRP94 during nonstressed conditions.

The association of PEK with ER chaperones indicates that sequences in the luminal portion of PEK facilitate this protein-protein interaction. To address which region of PEK is required for interaction with the chaperones, we expressed the PEK mutants deleted for different ER luminal segments in HEK 293T cells and characterized their association with GRP78 as judged by co-immunoprecipitation using antibody specific to GRP78. We found that deletion of regions 1–3, required for dimerization between PEK molecules, were dispensable for interaction with GRP78 (Fig. 8). By contrast, PEK deleted for region 4, which had no impact on PEK dimerization, significantly reduced GRP78 to levels found for the PEK mutant devoid of regions 1–4. As a control, we found similar amounts of GRP78 in the immune complexes as judged by immunoblot (Fig. 8*B*). Furthermore, similar amounts of wild-type and mutant versions of PEK were expressed in the HEK 293T cells in whole cell lysates (Fig. 8*C*), confirming that although PEK-Δ4 and PEK-Δ1–4 were available for interaction with GRP78, there was no complex formed between this ER chaperone and the eIF2 kinase. We conclude that the major domain for PEK interaction with GRP78 chaperone resides in the ER luminal sequences immediately flanking the transmembrane region of PEK. This GRP78 binding region is distinct from the portion of the ER luminal sequences that mediates PEK dimerization.

PEK Mutant That Is Blocked for GRP78 Association Is Constitutively Hyperphosphorylated—GRP78 is proposed to function as a repressor of PEK, binding with the eIF2 kinase and maintaining it in an inactive conformation. With the accumulation of misfolded proteins in the lumen of the ER during stress, GRP78 would be titrated from PEK, allowing PEK to hyperphosphorylate and assume an activated kinase state. Deletion of region 4 significantly reduced binding between GRP78 and PEK. In the immunoblot in Fig. 8*C*, PEK-Δ4 migrated more slowly than the wild-type PEK, suggesting a higher degree of autophosphorylation. To further explore the model, we measured PEK hyperphosphorylation by migration in SDS-PAGE using cells containing the different versions of PEK in response to the presence or absence of ER stress. In response to thapsigargin treatment, expressed wild-type PEK shifted to a higher molecular weight form (Fig. 9, *bottom panel*). To independently confirm that wild-type PEK is phosphorylated in

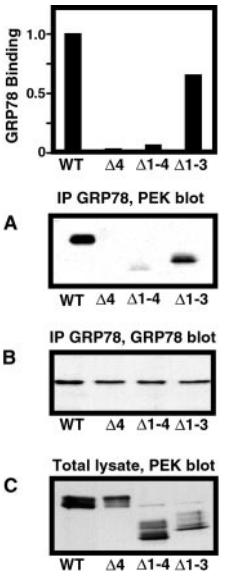

FIG. 8. **Identification of the luminal portion of PEK that associates with GRP78.** GRP78 was immunoprecipitated in lysates prepared from HEK 293T cells expressing wild type PEK, PEK-Δ4, PEK-Δ1–3, or PEK-Δ1–4. Proteins in the immune complexes were separated by SDS-PAGE, and the presence of PEK (*A*) or GRP78 (*B*) was visualized by immunoblot analysis using antibodies specific to these proteins. As a control, PEK levels were measured in whole cell lysates (*C*). Levels of PEK bound to GRP78 are illustrated in the *histogram* in the *top panel*. GRP78 binding was calculated as the level of PEK in the complex normalized for the level of immunoprecipitated GRP78. Values were presented relative to wild-type (*WT*) PEK co-precipitated with GRP78.

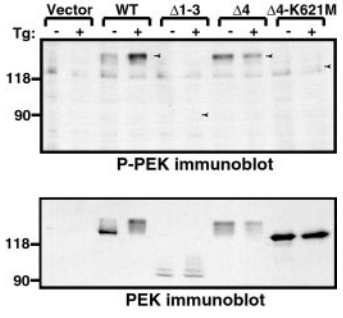

FIG. 9. **Deletions in PEK that block GRP78 interaction lead to constitutive hyperphosphorylation of PEK.** HEK 293T cells expressing wild-type PEK, PEK-Δ1–3, PEK-Δ4, kinase-inactive PEK-Δ4–K621M, or vector alone, were treated in the presence of absence of thapsigargin. Cellular lysates were prepared from the HEK 293T-derived cells and phosphorylated PEK (*top panel*) or total PEK (*bottom panel*) were visualized by immunoblot.

response to the ER stress, we carried out a second immunoblot analysis using antibodies specific to PEK phosphorylated at Thr-980. Phosphorylated wild-type PEK was readily visible in the thapsigargin-exposed cells, whereas minimal phosphorylation was detected in the absence of this ER stress (Fig. 9, *top panel*). By comparison, PEK-Δ1–3 that is severely impaired for dimerization and eIF2 kinase activity showed little change in migration as judged by the immunoblot analysis in response to ER stress and exhibited no phosphorylation as measured using the antibody specific to phosphorylated PEK. These results are consistent with the model that dimerization is a prerequisite for hyperphosphorylation of PEK in response to ER stress. In the example of PEK-Δ4, protein migration in the immunoblot analysis suggests that this PEK mutant is constitutively hyperphosphorylated independently of ER stress (Fig. 8*C* and 9). Supporting this premise is the observation that expression of a version of PEK-Δ4 containing the kinase-defective K621M substitution migrated faster compared with the kinase-active

counterpart (Fig. 9, *bottom panel*). Finally, immunoblot measurements using antibody specific to phosphorylated PEK demonstrated that PEK-Δ4 is phosphorylated independently of ER stress (Fig. 9, *top panel*). As expected, no PEK phosphorylation is detected in the PEK-Δ4 version containing the K621M substitution. Together, these results are consistent with the idea that mutations contributing to the release of GRP78 binding to PEK lead to constitutive hyperphosphorylation and activation of PEK.

DISCUSSION

We describe the characterization of two regions in the ER luminal portion of PEK that carry out distinct functions in the regulation of this eIF2 kinase in response to ER stress. The first described region mediates PEK oligomerization. Using c-Myc-tagged versions of PEK in immunoprecipitation assays, it was found that sequences including, but not limited to, the IRE1 homology region are required for interaction between PEK molecules (Fig. 5). Deletion of this oligomerization region blocked induction of eIF2 kinase activity in response to ER stress, emphasizing the importance of this protein-protein interaction in the mechanism of PEK activation (Fig. 6).

The second characterized region of PEK facilitates interaction with ER chaperones. During nonstressed conditions, PEK was observed to associate with ER chaperones GRP78 and GRP94 (Fig. 7). Minimum chaperone interaction was detected during ER stress conditions. Because ER stress and the accompanying phosphorylation of eIF2 significantly reduce translation, it could be suggested that diminished PEK association with ER chaperones occurs simply because there is less synthesis of the eIF2 kinase and therefore reduced chaperone-mediated folding of PEK. Arguing against this idea is our observation that the observed chaperone interaction with PEK is restricted to ER-based chaperones, with no detectable association with cytoplasmic HSP70 (Fig. 7). More definitively, we observed that ER luminal sequences flanking the transmembrane domain are required for GRP78 interaction, and deletion of this portion in PEK-Δ4 abolished binding with this ER chaperone and led to phosphorylation of this eIF2 kinase even in the absence of ER stress (Figs. 8 and 9). These results suggest that GRP78 serves as a repressor of PEK activity, and its release in response to ER stress facilitates phosphorylation of eIF2 kinase accompanying its activation. PEK autophosphorylation also requires the ability to homo-oligomerize, suggesting that this phosphorylation takes place between PEK molecules during ER stress conditions (Fig. 9). Analysis of PEK by glycerol gradient centrifugation supports the model that there is a dynamic change in the molecular weight of the PEK complex when the ER organelle is subjected to stress (Fig. 2).

Mechanisms Regulating Protein Kinases Involved in the ER Stress Response—In mammalian cells, PEK and IRE1 function in concert to recognize ER stress and implement a stress response. It is curious that while the UPR is also conserved in yeast, only IRE1 is present in its stress pathway. As predicted by the sequence homology in their ER luminal regions, recognition of ER stress by IRE1 and PEK appears to share certain common features. Shamu and Walter (40) reported that overexpression of truncated IRE1, removed for both the carboxyl-terminal kinase domain and the endoribonuclease region important for processing of *HAC1* mRNA, in yeast cells also expressing wild-type IRE1 blocked the UPR. Similar to the PEK oligomerization described in Fig. 5, hetero-oligomerization was found between the truncated and full-length IRE1 proteins, although visualization of the yeast IRE1 protein complex required cross-linking treatment (40). Liu *et al.* (27) reported that a chimeric yeast IRE1 substituted for its ER luminal region with sequences from *C. elegans* PEK could function to induce the UPR in yeast cells, suggesting common features in the control of IRE1 and PEK.

Oligomerization also appears to be important for IRE1 function in mammalian cells. IRE1 was found to shift to a higher molecular weight form, interpreted to be a homodimer, upon treatment of rat pancreatic cells AR42J with the reducing agent, dithiothreitol (33). Furthermore, PEK also shifted to a complex of greater than 600,000 molecular weight, suggestive of upwards of six PEK polypeptides, in response to dithiothreitol exposure. In a similar analysis using HEK 293T cells treated with thapsigargin, we observed a shift in PEK size to ~320,000, suggestive of a complex between two PEK molecules (Fig. 2). This may represent a functional difference between these two cell types. In both studies, the higher molecular weight form of PEK appeared to be phosphorylated as judged by the slower mobility in SDS-PAGE.

Regulation of IRE1 activity also appears to involve interaction between this protein kinase and GRP78. Overexpression of GRP78 (BiP) in Chinese hamster ovary cells prevented the UPR as measured by endogenous GRP78 expression and facilitated continued translation of cellular mRNAs in response to calcium ionophore (A23187) or tunicamycin treatment (44). It was uncertain whether such GRP78 expression was the result of increased GRP78 repression of PEK or IRE1 or rather the result of facilitated protein folding accompanying increased amounts of this ER chaperone. Our characterization of the GRP78 binding region in PEK would support the repressor model. Direct GRP78 interaction with mouse IRE1 and PEK was also reported and was suggested to be involved in inhibition of the kinase activities during the nonstressed state (33). The observation that PEK-Δ4, reduced for interaction with GRP78, is phosphorylated independently of ER stress suggests that ER chaperone binding with PEK is the predominant mechanism of repressing PEK in nonstressed conditions in the HEK 293T cells. We observed that in addition to GRP78, GRP94 can complex with PEK, and this association is significantly reduced in response to thapsigargin treatment. Thus, different ER chaperones may participate in the regulation of PEK.

Molecular Chaperones in the Regulation of eIF2 Kinases—Molecular chaperones are also important for regulation of members of the eIF2 kinase family. Matts and colleagues (6, 31, 32, 45) have championed the role of cytoplasmic chaperones in the stress recognition and activation of HRI. HSP90 and HSC70 associate with HRI and are thought to function as molecular chaperones. Upon converting HRI to a conformation capable of binding to heme, maintaining the eIF2 kinase in an inactive state, HSP90 is released from the so-called transformed HRI. However, HSC70 retains its association with transformed HRI, maintaining the eIF2 kinase in an inactive state (32, 45). In this role, HSC70 would function analogously to GRP78 as a repressor of eIF2 kinase activity. In addition to activation by heme deficiency, the activity of HRI is induced by heat shock or oxidative stress in hemin-supplemented lysates (6, 46). Misfolded protein that accumulates in the cytoplasm of cells subjected to heat or oxidative stress would sequester HSC70 from HRI, facilitating kinase activation and eIF2 phosphorylation. The structural basis for HSC70 inhibition of HRI activity is not currently understood. In the example of PEK, accumulation of unfolded protein in the ER lumen in response to an excessive secretory pathway load or upon exposure of ER stressing agents would titrate ER chaperones from PEK, allowing for activation of the eIF2 kinase. The cytoplasmic chaperone HSP90 is also thought to facilitate maturation and serve as an inhibitor of PKR and yeast GCN2 (47, 48).

Role of Oligomerization and Autophosphorylation in Activation of PEK and eIF2 Kinases—Oligomerization and autophos-

phorylation are two conserved features in the mechanisms activating eIF2 kinases. In the well studied example of PKR, two dsRBDs are required for dimerization between PKR polypeptides (4, 5, 49–51). Such oligomerization is facilitated by the cooperative binding of dsRNA between the dsRBDs, although protein-protein contacts may also participate. Additionally, we have proposed that association of PKR to ribosomes enhances the localized concentration of PKR required to facilitate dimerization (37, 52). Binding of dsRNA to the dsRBDs also contributes to an activated conformational change in PKR involving the release of a proposed inhibitory interaction between a region flanking dsRBD-2 and the kinase catalytic domain (39, 53). In the case of PEK, the inactive state is maintained by GRP78 association with region 4. Such GRP78 binding is suggested to preclude oligomerization between PEK polypeptides involving regions 1–3. GRP78 binding with region 4 may sterically hinder association between PEK polypeptides or maintain regions 1–3 in a conformation not conducive to homodimerization. Release of ER chaperones from PEK in response to ER stress would allow for PEK oligomerization involving the ER luminal sequences. PEK association with the ER membrane would ensure that this eIF2 kinase is present in a localized fashion in the cell, analogous to the ribosome association of PKR. However, the division of the ER transmembrane PEK into cytoplasmic and ER luminal portions would preclude an amino-terminal regulatory domain interaction with the kinase domain as proposed for PKR. Instead, oligomerization between PEK luminal sequences would bring cytoplasmic domains in close proximity.

Following oligomerization, autophosphorylation in *trans* is important for promoting activation of eIF2 kinases. In the example of PKR, phosphorylation occurs between polypeptides at multiple serine and threonine residues. Notably, Thr-446 and Thr-451 in the activation loop of the catalytic domain of PKR are a prerequisite for induced eIF2 kinase activity (37, 54). Mass spectrometric analysis of PEK phosphorylated *in vitro* has identified 10 serine, threonine, and tyrosine residues in the kinase domain (35), including the activation loop residue Thr-980 that was specifically recognized by our phospho-PEK antibody (Fig. 9). Given the importance of the analogous residue in PKR (Thr-446), Thr-980 and the adjacent Thr-985, a residue not yet identified as being phosphorylated, are important candidates for regulation of PEK activation. PEK phosphorylation of eIF2 would contribute to an increased UPR and a dampened general translation, enhancing the protein folding capacity of the ER as required to remedy the cellular stress condition.

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