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Artículo original

Heme responsiveness *in vitro* is a common feature shared by the eukaryotic initiation factor 2α kinase family

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ABSTRACT

Four distinct eukaryotic initiation factor 2α (eIF2α) kinases phosphorylate eIF2α at Ser-51 and regulate protein synthesis in response to cellular stress conditions. This kinase family includes the hemin-regulated inhibitor (HRI); the double-stranded RNA-dependent kinase (PKR); the GCN2 protein kinase; and the endoplasmic reticulum-resident kinase (PERK). HRI mediates protein synthesis inhibition in heme-deficient reticulocyte lysates. Although HRI contains two putative heme regulatory motifs (HRMs) that are not present in other eIF2α kinases, the significance of these motifs in heme regulation is not clear. In fact, it had been characterized two novel eIF2α kinases from *Schizosaccharomyces pombe* that lacked any of the HRMs, but were sensitive to heme regulation *in vitro*. To investigate the importance of different regions in the regulation of HRI by heme, specific HRI mutants were generated, and kinase activities and heme responsiveness were analyzed *in vitro*. Mutational analysis indicated that the heme regulatory motifs

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Abbreviations: HRM, heme regulatory motif; SEK1 & SEK2, *S. pombe* eIF2α kinases.

were spread around some regions in the HRI catalytic domain, outside of the HRMs. In accordance with these results, both the autokinase and the eIF2α kinase activities of three distinct eIF2α kinases, including the human PKR, the mouse GCN2 and the *Drosophila* PERK were inhibited *in vitro* by hemin. Although the known regulatory mechanisms of these eIF2α kinases are very different, the data reported here indicate that all known eIF2α kinases are regulated *in vitro* by hemin. This finding provides evidence that hemin represents a regulatory mechanism unique to eIF2α kinases and underscores the role of hemin in the translational regulation of eukaryotic cells.

Key words: eIF2α kinases.—Translational control.—Heme-regulated kinase.—Heme regulatory domain.

RESUMEN

La respuesta a hemina *in vitro* es una característica compartida por todos los miembros de la familia de las eIF2α quinasas

Las cuatro eIF2α quinasas eucarióticas fosforilan el residuo Ser-51 de la subunidad alfa del factor de iniciación 2 y regulan la síntesis de proteínas en respuesta a situaciones de estrés celular. Esta familia de proteínas quinasas está formada por el inhibidor regulado por hemina (HRI); la quinasa dependiente de RNA de doble cadena (PKR); la proteína quinasa GCN2 y la quinasa residente en el retículo endoplásmico (PERK). El HRI inhibe la síntesis de proteínas en lisados de reticulocitos de conejo deficientes de hemina. Aunque el HRI contiene dos supuestos motivos reguladores de hemina (HRMs), que no están presentes en las otras eIF2α quinasas, no está claro aún el papel de estos motivos en la regulación por hemina. De hecho, se han caracterizado dos nuevas eIF2α quinasas de *Schizosaccharomyces pombe* que carecen de dichos HRMs, pero son sensibles a la regulación por hemina *in vitro*. Un análisis mutacional indicó que los motivos reguladores de hemina estaban dispersos a lo largo del dominio catalítico, fuera de los HRMs. De acuerdo con estos resultados, las actividades autoquinasa y eIF2α quinasa de tres eIF2α quinasas distintas, la PKR humana, la GCN2 de ratón y la PERK de *Drosophila*, se inhibían por hemina *in vitro*. Aunque los mecanismos de regulación de todas estas eIF2α quinasas son muy diferentes, nuestros resultados indican que todas las eIF2α quinasas se regulan por hemina *in vitro*. Este descubrimiento soporta la evidencia de que la hemina representa un mecanismo de regulación específico de las eIF2α quinasas, y subraya su papel en la regulación de la traducción de células eucarióticas.

Palabras clave: eIF2α quinasas.—Control de la Traducción.—Quinasa regulada por hemina.—Dominio regulador de hemina.

INTRODUCTION

In eukaryotic cells, protein synthesis is mainly regulated at the level of initiation of mRNA translation. The reversible phosphorylation of the α-subunit of eukaryotic translation initiation factor 2 (eIF2α) is a well-characterized mechanism of translational control in response to a wide variety of cellular stresses, including nutrient starvation, iron deficiency, heat shock, UV irradiation and viral infection (1, 2). Four different eIF2α kinases have been identified that specifically phosphorylate eIF2α on Ser-51. All known eIF2α kinases share a conserved kinase domain linked to unique regulatory regions. Thus, HRI is activated both by heme deficiency and under conditions of heat shock and oxidative stress (3). PKR is induced by interferon (IFN) and activated by double-stranded RNA (dsRNA) during viral infection (4). GCN2 (general control non-derepressible-2) is an eIF2α kinase that is activated by amino acid or serum deprivation and UV irradiation (5-7). The fourth eIF2α kinase, PERK, is activated by unfolded proteins in the endoplasmic reticulum (ER) (8, 9). It has recently cloned and characterized two novel eIF2α kinases from the fission yeast *S. pombe* (named SEK1/Hri1p and SEK2/Hri2p) both of which show higher homology with mammalian HRI than with other eIF2α kinases (10).

HRI, found most abundantly in erythroid cells, serves to limit globin protein synthesis when the levels of available heme are low. However, the presence of HRI mRNA and HRI activity in non-erythroid tissues and in NIH 3T3 cells raises the possibility of additional regulatory roles for HRI (11). The eIF2α kinase GCN2 was originally characterized in *Saccharomyces cerevisiae* and GCN2 homologs have been identified in *Drosophila melanogaster* (12) and mammals (5), suggesting that GCN2 might be the founding and also the best conserved member of the eIF2α kinase family (2). PERK was originally identified in rat pancreatic islet cells (8) and, recently, PERK homologs have been found in mouse, human, and *Drosophila melanogaster* (9, 13). Interestingly, when activated by their cognate upstream stress signals, the mammalian PERK and GCN2 repress translation of most mRNAs but selectively increase translation of ATF4 mRNA, a member of the activating transcription family (14).

All distinct eIF2α kinases share extensive homology in the kinase catalytic domain. Apart from the 12 conserved subdomains found in most protein kinases, they have additional characteristic features, including an insert region between subdomains IV and V and a conserved sequence in subdomain V that distinguishes them from other serine/threonine kinases (15). Especially notable is the presence of this conserved motif, essentially LY/HIQME/QY/LC, located N-terminal to subdomain V in all of the known members (up to 20) of this kinase family, suggesting that this motif might contain a putative substrate-specific recognition domain (16).

HRI is regulated by heme, and it seems that heme binds directly to HRI and blocks kinase activity (17). In fact, it has been shown that HRI is a hemoprotein with two distinct heme-binding sites (17, 18). Both of them, the N-terminus and the kinase insertion, which are unique to HRI, appear to be involved in the heme regulation of HRI (18). Furthermore, HRI is among six hemoproteins that have a putative heme regulatory motif (HRM) (19); HRI contains two of these motifs that are not present in other eIF2α kinases, although the role of the HRMs in the heme regulation of full-length HRI remains uncertain. Thus, the heme-binding site responsible for the reversible heme regulation of HRI must be determined in order to understand the mechanism of the regulation of HRI by heme.

In this study we have examined the effects of several deletions and point mutations in specific regions of HRI on its activity by *in vitro* assays. We have also tested these mutants of HRI for responsiveness to heme. Our findings indicate that none of three unique regions in HRI, the N-terminus, the kinase insertion and the C-terminus are required for the regulation of HRI by heme. Furthermore, we have found that both the autokinase and the eIF2α kinase activities of the other members of the eIF2α kinase family, such as PKR, GCN2 and PERK, were also sensitive to hemin *in vitro*. We conclude that the heme-binding site responsible for the reversible heme regulation is located within the eIF2α kinase domain and this conserved motif(s) is characteristic of all known members of the eIF2α kinase family.

MATERIAL AND METHODS

HRI Mutagenesis and Plasmid Construction

All HRI expression constructs were generated from pRSETB-MHRI plasmid, which harbors wild type (wt) mHRI (11). Single point mutations C409A and C550A were made by using the QuickChange™ site-directed mutagenesis kit (Strategene) as described by the manufacturer, using the mutagenic primers shown in Table I. HRI Δ8 and HRI Δ8/C550A were generated from pRSETB-MHRI and pRSETB-MHRI/C550A plasmids, respectively, by substitution of the cDNA fragment encoding aa 1 to 413 with a PCR generated fragment encoding aa 1 to 405. The resultant plasmids, pRSETB-MHRI Δ8 and pRSETB-MHRI Δ8/C550A, encode HRI proteins lacking the 406 to 413 aa residues alone, or together with the C550A point mutation. To generate the HRI Δ137/C409A/C550A mutant, the pRSETB-MHRI C409A/C550A plasmid was cut with *Bam*HI, yielding three fragments. The fragment corresponding to the HRI central region (aa 138-501) was subcloned to produce a plasmid encoding an HRI product that lacks the 1 to 137 aa residues and contains the C409A and C550A mutations. HRI 138-580/C409A/C550A was generated by substituting the Gln-581 CAA codon for a stop codon (UAA) in the pRSETB-MHRI Δ137/C409A/C550A plasmid.

HRI Q4 chimera was obtained as follows: the whole pRSETB-MHRI plasmid without the region corresponding to the HRI kinase insert domain (residues 235-389) (11) was amplified by PCR, introducing restriction sites at both ends. The kinase insert domain (residues 656-807) of GCN2 (5) was also amplified by PCR introducing the same restriction sites. Both generated DNA fragments were ligated to produce a pRSETB-MHRI Q4 vector encoding an HRI protein with the MGCN2 insert sequence.

Prokaryotic expression of HRI proteins

The pRSETB-MHRI-wt and the pRSETB-MHRI-mutant plasmids were used to transform a competent BL21 (DE3) pLys S strain of *E. coli*. Protein expression and bacterial lysis were done as previously

described (11). Bacterial lysates were diluted (1/100) in 20 mM Tris-HCl (pH 7.4) and assayed for their ability to phosphorylate eIF2α as described below.

TABLE I. *Sequences of mutagenic HRI oligonucleotides*

Construct	Mutagenic primer*
HRI C409A	5'-GTGGACGAAGCTGCT**GC**TCCCTATGTTATGGC (sense)
	5'-GCCATAACATAGGGA**GC**AGCAGCTTCGTCCAC (antisense)
HRI C550A	5'-CCCTCAGTAAAAGG**GC**TCCGGTGCAAGCC (sense)
	5'-GGCTTGCACCGGA**GC**CCTTTTACTGAGGG (antisense)
HRI 138-580	5'-CAGAGTGAGCTTTTT**TAA**ACAACTGGAAATG (sense)
	5'-CATTTCCAGTTGTTT**A**AAAAAGCTCACTCTG (antisense)

* Underlined codons correspond to the indicated amino acid mutations and the generated stop codon. The changed nucleotides are marked in bold.

Cell Culture and Transfection

HEK 293T cells were grown and transfected as previously described (20). The plasmids used were the pcDNA3.1/Myc-His vectors (Invitrogen) containing the coding region of mouse GCN2 (pcMGCN2-WT) (5), mouse HRI (pcMHRI-WT) (11), human PKR (provided by Dr. J. Gil), *Drosophila melanogaster* PERK (13), or rat ζPKC (21) in frame with a C-terminal tag encoding the *myc* epitope and a polyhistidine metal-binding peptide.

Affinity Purification and *in vitro* Phosphorylation Assays

Affinity purification of proteins was performed as previously described (20). The eluted proteins from the metal affinity resin as well as the *E. coli* lysates containing the HRI wild type and mutant proteins were assayed for their ability to phosphorylate eIF2α as reported previously (12, 22), with modifications as described. In a total volume of 20 μl, 5 μl of kinase fractions were incubated with various concentrations of hemin (0-4 μM) for 30 min at 30º C in the kinase buffer (20 mM Tris-HCl (pH 7.6), 2.5 mM $MgCl_2$, 2.5 mM

$Mg(OAc)_2$, 0.25 mg/ml BSA, 50 μM ATP) containing purified rabbit reticulocyte eIF2 (0.5 μg) and 3 μCi of [γ-^{32}P] ATP (3000 Ci/mmol). When PKR or GCN2 was assayed the reaction mixtures also included 1 μg/ml poly(I)·poly(C) dsRNA or 2.5 μg/ml (0.65 nM) Sindbis virus RNA, respectively. Phosphorylation of casein (Sigma) by highly purified casein kinase II (CKII) from rat liver was carried out as described (22). Briefly, purified CKII (0.01 U/ml) was incubated for 10 min at 30° C with 66 μg/ml casein as substrate and 3 μCi of [γ-^{32}P] ATP (3000 Ci/mmol) in a final volume of 30 μl containing 20 mM Tris-HCl (pH 7.2), 8 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM EDTA, 1 mM dithiothreitol and 50 μM ATP. ζPKC kinase assay was as for eIF2α kinases, except for the substitution of $Mg(OAc)_2$ by $MgCl_2$ and eIF2α by 3 μg of myelin basic protein (MBP) as substrate. Incubations were terminated by the addition of SDS sample buffer and phosphoproteins were analyzed by both SDS-PAGE (23) on a 10 or 12.5% polyacrylamide gel (28.5:1 (w/w) acrylamide/bisacrylamide) and autoradiography using Agfa Curix RP2 film and an Amersham-Pharmacia intensifying screen. The areas corresponding to the phosphorylated α-subunit of eIF2 were scanned at 633 nm in a computing 300 A densitometer (Molecular Dynamics, Inc.).

RESULTS

All known HRIs contain two putative heme regulatory motifs (HRM): ACPYVM and RCPVQA, which are located within the catalytic domain [(11) and Fig. 1B] and are not present in other eIF2α kinases. To date, the significance of these motifs in the heme regulation of HRI is unknown.

To understand the regulation mechanism of HRI by heme, we designed and constructed a series of mutants (Fig. 1A), including point mutations of the two invariant cysteine residues in the HRMs (HRI C409A/C550A) alone or together with either a large N-terminal deletion (HRI Δ137/C409A/C550A) or deletions that remove both the N-terminal and the C-terminal domains (HRI 138-580/C409A/C55A). The HRI Δ8 mutant contains a deletion of amino acids 406-413 that removes the entire HRM1. The HRI Δ8/C550A mutant also contains a single point mutation of conserved cysteine in HRM2. In addition,

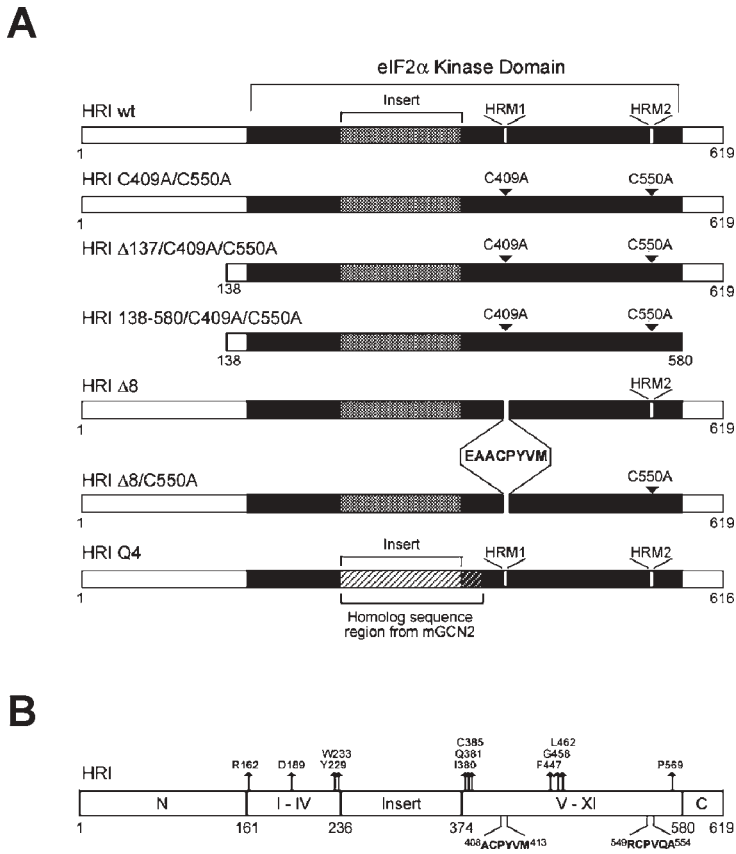

FIGURE 1. ***A,*** *the 619 amino acid wild type HRI sequence is illustrated by a large box and shown at the top. The black boxes represent the conserved two lobes of the eIF2α kinase domain, separated by a large insert region (stippled boxes) between subdomains IV and V. Position of the two heme regulatory motifs (HRM1 and 2) within the catalytic domain is shown. Six generated mutants are shown: HRI C409A/C550A (in which the conserved cysteines in both HRM1 and HRM2 are mutated to alanine); HRI Δ137/C409A/C550A (previous mutant in which the first 137 amino acids were deleted); HRI 138-580/C409A/C550A (previous mutant with the indicated deletion at the C-terminus); HRI Δ8 (an eight-amino acid deletion in HRM1); HRI Δ8/C550A (mutant containing HRM1 deletion and C550A substitution); HRI Q4 (in which the insert region of HRI kinase was replaced by the corresponding insert region from mouse GCN2). The numbers refer to the amino acid residues.* ***B,*** *the five domains of mouse HRI included the N-terminus (N); the conserved two lobes of the eIF2α kinase domain separated by a large insert (amino acids 236-373); and the C-terminus (C) are indicated. The 11 invariant residues and the two HRMs, within the eIF2a kinase domain, that are absent in other Ser/Thr protein kinases are also indicated.*

we constructed the HRI Q4 mutant, in which the entire kinase insert together with the N-terminal of HRI kinase subdomain V (11) was replaced by the corresponding amino acid sequence of mouse GCN2 (5). All the HRI constructs in pRSETB vector (11) were efficiently expressed in *E. coli* and the amounts of recombinant proteins, tested by Western blot, were similar (data not shown).

To assess the kinase activity of the HRI mutants, an *in vitro* kinase assay was performed in which bacterial extracts containing these mutants were tested for phosphorylation of exogenously added eIF2. Under these conditions, the extent of inhibition of eIF2α kinase activity of the wild type and HRI mutants by heme was dependent upon the amount of HRI used.

To determine the role of the HRMs in the heme regulation of HRI we studied the effect of hemin on the eIF2α kinase activity of three different HRI mutants, C409A/C550A, Δ8, and Δ8/C550A. For all of the mutants, eIF2α kinase activity was similar to that of the wild type HRI. Furthermore, the eIF2α kinase activity of HRI mutants was also hemin-sensitive (Fig. 2A). Interestingly, the extent of inhibition of HRI mutants by heme was similar to that of HRI wt at all hemin concentrations tested (Fig. 2B). The concentration of hemin required for 50% inhibition of eIF2α kinase activity was about 0.3-0.5 μM for wild type HRI, in good agreement with earlier studies (3, 18), and 0.3 μM, 0.4 μM and 0.7 μM for C409A/C550A, Δ8 and Δ8/C550A mutants, respectively. These results strongly suggest that the entire HRM1 and the cysteine residue in HRM2 have no apparent effect on the heme-responsiveness of HRI *in vitro*.

Similarly, to determine the role of both amino and carboxyl ends in the heme regulation of HRI, the inhibition by heme of the eIF2α kinase activity of the two indicated HRI deletion mutants, also containing point mutations into the HRMs (Δ137/C409A/C550A and 138-580/C409A/C550A), were compared. In this case, the eIF2α kinase activity of mutants was also inhibited by the same concentration range of hemin (Fig. 2A), although we obtained slightly different values in the hemin concentration required for their half-maximal inhibition (Fig. 2B). These results suggest that neither N- nor C-terminal domains are essential in the heme regulation of HRI. Finally, the mutant HRI Q4, in which the HRI kinase insert had been

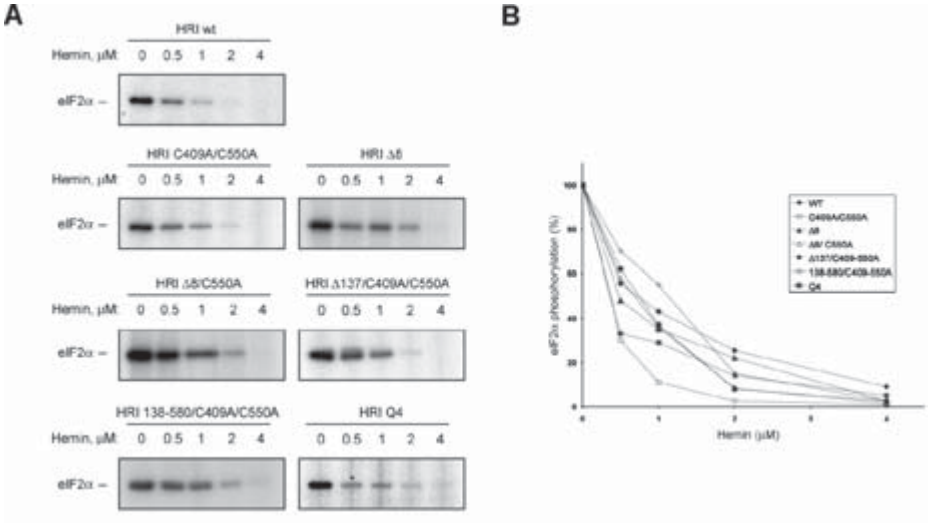

FIGURE 2. ***A,*** *samples of recombinant HRI wt, as a control; HRI C409A/C550A; HRI Δ8; HRI Δ8/C550A; HRI Δ137/C409A/C550A; HRI 138-580-C409A/C550A; and HRI Q4 proteins were analyzed for their ability to phosphorylate purified rabbit reticulocyte eIF2, in the presence of increasing concentrations of hemin as indicated. Position of phosphorylated eIF2α is indicated.* ***B,*** *the phosphorylation of eIF2α was estimated by quantifying the corresponding band density of the autoradiograms. The intensity of the eIF2α band at zero concentration of hemin was defined as 100%. The figure shows a representative experiment of at least three independent experiments with very similar results.*

replaced by the mouse GCN2 corresponding region, showed lower eIF2α kinase activity (Fig. 2A). Probably due to its low specific activity, HRI Q4 was more sensitive to inhibition by heme (0.4 μM for 50% inhibition) than that of wild type HRI (Fig. 2B). This result suggests that the kinase insert region, which does not show any significant homology with those of the other eIF2α kinases, may not be the important region for achieving the heme responsiveness of HRI. Altogether, our mutational analysis indicates that the heme regulatory region(s) are spread around the 12 conserved subdomains characteristic of all eukaryotic Ser/Thr protein kinases.

Because the kinase catalytic region of HRI was responsible for heme regulation, we tested whether the other eIF2α kinases, besides mammalian HRI, could be inhibited by hemin *in vitro*. To this end, we used purified eIF2α kinases from transfected HEK 293T cells:

mouse HRI (as positive control), mouse GCN2, human PKR, and *Drosophila* PERK. In order to achieve full activity of GCN2 and PKR, they were assayed in the presence of Sindbis virus RNA (20) and poly(I)·poly(C), respectively (Fig. 3A). Of great interest was the finding that both the autokinase and eIF2α kinase activities of the affinity-purified recombinant eIF2α kinases were inhibited by low

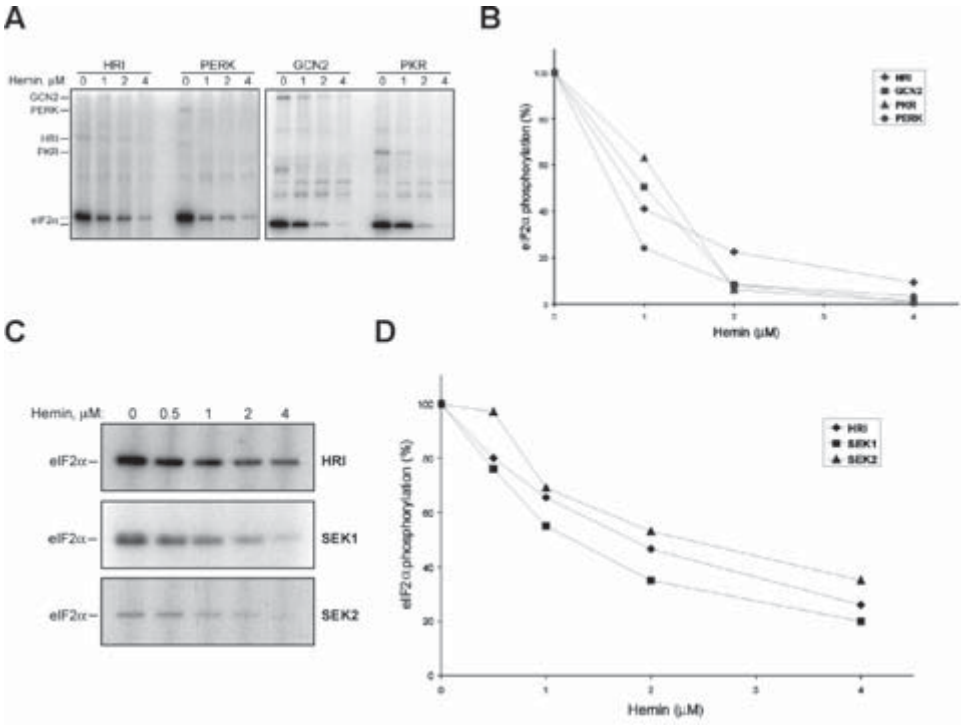

FIGURE 3. ***A,*** *autokinase and eIF2α kinase activities of distinct eIF2α kinases are inhibited by hemin. All four eIF2α kinases (mouse HRI and GCN2, human PKR and Drosophila PERK) were expressed in HEK 293T cells as Myc-His fusion proteins and purified by affinity chromatography. In vitro phosphorylation assays were performed in the absence or presence of the indicated increasing concentrations of hemin, as described under Material and Methods. The positions of phosphorylated HRI, GCN2, PKR, PERK and eIF2α are indicated.* ***B,*** *eIF2α phosphorylation was estimated by denstitometric analysis as described for Fig. 2.* ***C,*** *eIF2α kinase activity of both S. pombe eIF2α kinases, SEK1 and SEK2, is inhibited by hemin. All three eIF2α kinases (mouse HRI and S. pombe SEK1 and SEK2) were expressed in E. coli, as previously reported. In vitro phosphorylation assays were performed in the absence or presence of the indicated concentrations of hemin.* ***D,*** *eIF2α phosphorylation was estimated and represented as in B.*

concentrations of hemin (Fig. 3A). Moreover, the extent of inhibition of all of them by heme was similar to that of mouse HRI at all hemin concentrations tested (Fig. 3B). We therefore conclude that the kinase activities of different eIF2α kinases are also regulated *in vitro* by hemin.

Moreover, the two novel eIF2α kinases from *S. pombe* (SEK1/Hri1p and SEK2/Hri2p) were also heme-responsive *in vitro* (Fig. 3C). Again, the extent of the inhibition of SEK1 and SEK2 by heme was similar to that of mouse HRI at all concentrations tested (Fig. 3D). Altogether, our results indicate that the responsiveness to hemin *in vitro* is a common property of all the other eIF2α kinases.

We were interested in ascertaining the possible specificity for the hemin effect reported here. In order to see if other protein kinases, in addition to eIF2α kinases, were heme responsive, we tested the effect of hemin on the protein kinase activity of two other Ser/Thr protein kinases, CKII and ζPKC. The data presented in Fig. 4 demonstrated that the *in vitro* phosphorylation of either casein, by CKII, or MBP, by ζPKC, was not inhibited by the presence of hemin. We therefore conclude that the eIF2α kinases, but not other serine-

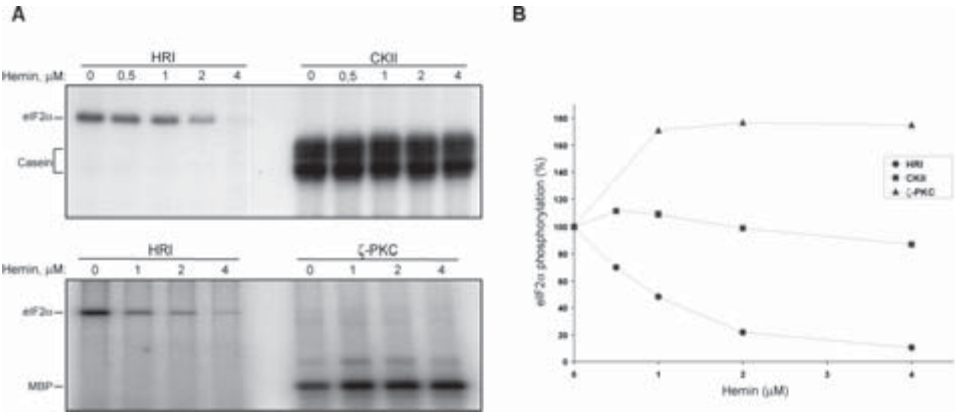

FIGURE 4. ***A,*** *shown are the results from the in vitro phosphorylation of eIF2α by recombinant mouse HRI, as a control, of casein by purified casein kinase II, and of MBP by purified His-tagged ζPKC, in the absence or presence of the indicated concentrations of hemin. The positions of phosphorylated eIF2α, casein and MBP are indicated.* ***B,*** *the phosphorylation of eIF2α, casein and MBP was estimated as described in Figure 2.*

threonine protein kinases, are regulated *in vitro* by hemin, suggesting that the putative heme-regulatory motifs are shared by all members of the eIF2α kinase family.

DISCUSSION

The molecular mechanism(s) by which hemin regulates mammalian HRI has yet to be determined. Furthermore, the precise heme-binding domain responsible for rapid regulation of HRI by heme is not characterized. Some previous reports have suggested that purified HRI is a homodimer and a hemoprotein with two distinct heme-binding sites. Binding of heme to an N-terminal domain (amino acids 1-138) of HRI is stable and autonomous, whereas binding to a kinase-insertion domain (amino acids 241-406) is dynamic and may be responsible for the reversible heme regulation of HRI (18). It should be noted that this reported kinase-insertion domain contains not only the entire mouse kinase insert sequence unique to HRI but also includes the kinase subdomain V and the adjacent amino acids (LHIQMQLC), a highly conserved motif among eIF2α kinases. Additionally, HRI is among six hemoproteins that have a putative heme regulatory motif (HRM) where Cys at position 2 seems to be essential (19). HRI contains two of these HRMs, which are not present in the other eIF2α kinases. The data reported here indicate that the HRMs do not play an essential role in the heme-responsiveness of HRI. Thus, Cys-409 and Cys-550 were mutated individually to alanine and the entire HRM1 was deleted with no apparent effect on either the eIF2α kinase activity or the heme responsiveness of the HRI mutants. Furthermore, two novel eIF2α kinases from *S. pombe,* highly similar to HRI, show heme responsiveness *in vitro* although amino acid sequence analyses revealed that they lacked any of this HRM.

Comparison of the deduced amino acid sequences of mammalian HRI with the other eIF2α kinases reveals the existence in HRI of three unique regions, very different in sequence and size, in addition to the two kinases lobes containing the 12 conserved catalytic subdomains characteristic of all eukaryotic Ser/Thr protein kinases. Thus, mouse HRI comprises these five domains (Fig. 1B): the amino

terminus, the first kinase lobe (catalytic subdomains I to IV), the kinase insert (amino acids 236-373), the second kinase lobe (catalytic subdomains V to XI) and the carboxyl terminus (11). The data reported here strongly suggest that the three unique regions in HRI (the N- and C-terminus and the kinase insert) are not involved in the regulation of HRI activity by heme. Furthermore, we report here that the kinase insert domain of mouse HRI is required for kinase activity but does not play a significant role in the heme regulation of HRI. Thus, we also observed that the HRI Q4 mutant, that contains the kinase insert domain of mouse GCN2 instead of its own domain, is still heme-regulated. These findings demonstrate that the binding domain responsible for the heme regulation of HRI is located within its two kinase lobes and, therefore, raise the possibility that some other eIF2α kinases may also be sensitive to inhibition by heme.

To test this possibility, we attempted to determine whether members of the other known eIF2α kinase subfamilies had the ability to be regulated by hemin *in vitro*. Here, we have shown that all recombinant mouse GCN2, *Drosophila* PERK and human PKR exhibit both the autokinase and the eIF2α kinase activities *in vitro* and, strikingly, these two kinase activities are inhibited by low concentrations of hemin. Our results indicate that the heme binding to a still unknown but well conserved region, may block the binding of ATP and thus inhibit both the autokinase and the eIF2α kinase activities of all known eIF2α kinases *in vitro*.

By comparing the amino acid sequence of mouse HRI with all other known eIF2α kinases (up to 20 members) we have found 11 invariant positions within the kinase domain that are absent in the majority of other Ser/Thr protein kinases. These conserved residues are as follows: R162, D189, Y229, W233, I380, Q381, C385, F447, G458, L462 and P569 (Fig. 1B). It is of particular interest that three of these residues (I380, Q381 and C385), uniquely conserved among eIF2α kinases, are located in the kinase lobe that binds ATP. Thus, according to sequence alignment with the catalytic subunit of cAMP-dependent protein kinase (PKA-Cα) (15), and assuming that all Ser/Thr protein kinases fold into topologically similar three-dimensional core structures, it was predicted that the conserved sequence LHIQMQLCE will form a very hydrophobic β-strand (b5) in the small kinase lobe and will connect the two lobes of the catalytic domain.

In addition, some residues of this motif help to anchor ATP by forming hydrogen bonds with either the adenine or the ribose ring (15). Moreover, previous results indicate that the cysteine residue in the HRM of six different hemoproteins is absolutely essential for binding to heme (19). It has been suggested that such cysteine serves the critical function of donating electrons to the iron atom of heme (19). Therefore, it is possible that these three amino acids (I380, Q381 and C385), the Cys-385 in particular, may be directly involved in hemin binding, although the exact heme regulatory sites within the eIF2α kinases remain to be determined.

Collectively, our results indicate that, although the known regulatory mechanisms of eIF2α kinases *in vivo* are very different, all known eIF2α kinases are regulated *in vitro* by hemin. Thus, hemin may represent a regulatory mechanism unique to eIF2α kinases. On the other hand, heme has been shown to function as an effector molecule that can regulate many biological processes, including transcription, translation, protein translocation and erythroid differentiation. Also, heme plays key roles in oxygen sensing and utilization in all living organisms ranging from bacteria to humans (24). Our studies provide a new example of how heme may control the activity of a family of translational inhibitors and how a regulatory system may be conserved from yeast to mammals to control protein synthesis. Thus, the eIF2α kinases may provide a feedback mechanism to coordinate the synthesis of some hemoproteins that are essential for the function of all aerobic cells, according to heme concentration in eukaryotic cells. When cells grow in a limiting supply of oxygen, the low concentration of heme synthesized would allow the activation of the eIF2α kinases, and a low level of protein synthesis would result. In a more aerobic environment, heme would repress the eIF2α kinases, eliciting full induction of protein synthesis.

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