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Protein kinase CK2 phosphorylates and interacts with deoxyhypusine synthase in HeLa cells

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Abbreviations: DHS, deoxyhypusine synthase; eIF5A, eukaryotic initiation factor 5A

Abstract

Deoxyhypusine is a modified lysine and formed posttranslationally to be the eukaryotic initiation factor eIF5A by deoxyhypusine synthase, employing spermidine as butylamine donor. Subsequent hydroxylation of this deoxyhypusine-containing intermediate completes the maturation of eIF5A. The previous report showed that deoxyhypusine synthase was phosphorylated by PKC *in vivo* and the association of deoxyhypusine synthase with PKC in CHO cells was PMA-, and Ca^{2+}/phospholipid-dependent. We have extended study on the phosphorylation of deoxyhypusine synthase by protein kinase CK2 in order to define its role on the regulation of eIF5A in the cell. The results showed that deoxyhypusine synthase was phosphorylated by CK2 *in vivo* as well as *in vitro*. Endogenous CK2 in HeLa cells and the cell lysate was able to phosphorylate deoxyhypusine synthase and this modification is enhanced or decreased by the addition of CK2 effectors such as polylysine, heparin, and poly(Glu, Tyr) 4:1. Phosphoamino acid analysis of this enzyme revealed that deoxyhypusine synthase is mainly phosphorylated on threonine residue and less intensely on serine. These results suggest that phosphorylation of deoxyhypusine synthase is CK2-dependent cellular event as well as PKC-mediated effect. However, there were no observable changes in enzyme activity between the phosphorylated and unphosphorylated forms of deoxyhypusine synthase. Taken together, besides its established function in hypusine modification involving eIF5A substrate, deoxyhypusine synthase and its phosphorylation modification may have other independent cellular functions because of versatile roles of deoxyhypusine synthase.

Keywords: eukaryotic initiation factor 5A; phosphoamino acids; phosphorylation; post-translational modification; protein kinases; protein processing

Introduction

Hypusine biosynthesis occurs exclusively in one cellular protein, the precursor of eukaryotic translation initiation factor eIF5A, through a unique two-step posttranslational modification. In the first step, deoxyhypusine synthase facilitates the transfer of the 4-aminobutyl moiety from spermidine to the eIF5A precursor protein containing lysine to form a deoxyhypusine-containing protein (Park *et al.*, 1982; Park and Wolff, 1988; Wolff *et al.*, 1990). Subsequent hydroxylation of this intermediate by deoxyhypusine hydroxylase completes hypusine synthesis and eIF5A maturation (Abbruzzese *et al.*, 1988).

Disruption of any step involved in hypusine synthesis leads to arrested cell proliferation. In the yeast *Saccharomyces cerevisiae*, inactivation of two eIF5A genes (Schnier *et al.*, 1991; Wöhl *et al.*, 1993) or the deoxyhypusine synthase gene (Sasaki *et al.*, 1996; Park *et al.*, 1998) causes suppression of growth and eventual loss of cell viability. The growth inhibition of mammalian cells depleted of the polyamine spermidine was attributed to deprivation of mature eIF5A containing hypusine (Byers *et al.*, 1994). Inhibitors of deoxyhypusine synthase and/or deoxyhypusine hydroxylase exert strong antiproliferative effects in various mammalian cells, including many human cancer cell lines (Hanauske-Abel *et al.*, 1994; Park *et al.*, 1994; Lee *et al.*, 1995). As the first enzyme in the hypusine synthetic pathway, deoxyhypusine synthase occupies a critical position, and could provide a potential target for intervention in cell proliferation.

As is the case for eIF5A, the amino acid sequence of deoxyhypusine synthase is highly conserved among various eukaryotic species (Joe *et al.*, 1995; Kang *et*

al., 1995; Tao and Chen, 1995a; Wolff *et al.*, 1995; Chen and Liu, 1997; Wolff and Park, 1999); both eIF5A and deoxyhypusine synthase also appear to be functionally conserved throughout eukaryotic evolution. Deoxyhypusine synthases from several species share similar physical and catalytic properties. Experimental evidence from gel filtration and/or ultracentrifugation studies suggests that the enzymes from rat, human, yeast (*Saccharomyces cerevisiae*) and *Neurospora crassa* exist as a homotetramer of identical subunits (Wolff *et al.*, 1990; Joe *et al.*, 1995; Kang *et al.*, 1995; Tao and Chen, 1995a; 1995b; Wolff *et al.*, 1995). They exhibit narrow specificity toward spermidine and NAD^+.

The eIF5A undergoes two types of posttranslational modifications, hypusination and phosphorylation, where the activity of the factor is dependent on the first but is not influenced by the latter *in vitro* (Kang *et al.*, 1993). For the probable regulation of eIF5A activity, a reversible modification may be one of the possibilities. Deoxyhypusine synthase, the first enzyme involved in hypusination, could thus be an eligible target.

The reversible phosphorylation of proteins is a major mechanism for the regulation of a broad spectrum of fundamental cellular processes (Krebs, 1994; Hunter, 2000). Given the importance of this covalent modification, it may not be surprising that the human genome encodes several hundred distinct protein kinases (Lander *et al.*, 2001; Venter *et al.*, 2001), that a third of all cellular proteins appear to be phosphorylated (Ahn and Resing, 2001). Accordingly, on average, each protein kinase may phosphorylate a few dozen proteins within a cell. Protein kinase CK2 (formerly known as CKII, casein kinase II) exhibits a much broader specificity and are likely to phosphorylate over 160 distinct proteins within cells (Pinna, 1990; Allende and Allende, 1995).

On all its substrates, CK2 phosphorylates Ser/Thr residues specified by clusters of acidic residues, mostly located on the C-terminal side of the target amino acid, with the residue at position n+3 being responsible for the minimum consensus, S/T-X-X-Neg, where Neg is the negatively charged side chain of either a carboxylic residue (Glu or Asp) or a previously phosphorylated residue (Ser-P or Tyr-P) (Meggio *et al.*, 1994a).

Recent discovery by Park *et al.* (2003) exhibited the efficient reversal of deoxyhypusine synthesis when eIF5A ([^{3}H]deoxyhypusine) was incubated with human deoxyhypusine synthase, NAD, and 1,3-diaminopropane, [^{3}H] spermidine was formed. Purified human deoxyhypusine synthase also exhibited homospermidine synthase activity when incubated with spermidine, NAD, and putrescine. Thus, it was found that putrescine can replace eIF5A precursor protein as an acceptor of the 4-aminobutyl moiety of spermidine to form homospermidine (Park *et al.*, 2003).

Due to their sequence homology, there is no doubt that the homospermidine synthase originated from deoxyhypusine synthase by gene duplication and recruitment for pyrrolizidine alkaloid biosynthesis (Ober and Hartmann, 1999a; 2000). The two enzymes share the same reaction mechanism, except for the loss of the ability of homospermidine synthase to bind the eIF5A precursor protein to its surface and deoxyhypusine synthase, in addition to its intrinsic activity, already catalyzes the aminobutylation of putrescine yielding homospermidine. Thus, homospermidine synthase has to be considered a deoxyhypusine synthase which lost its intrinsic activity. It provides new insights into the intricate specificity and versatility of the deoxyhypusine synthase reaction.

Lately, we demonstrated that yeast recombinant deoxyhypusine synthase was phosphorylated by PKC *in vitro* (Kang and Chung, 1999). Furtheremore, deoxyhypusine synthase was found to be phosphorylated by endogenous kinases in various cell lines and shown to have close association with PKC in intact CHO cells (Kang *et al.*, 2002). Deoxyhypusine synthase contains putative phosphorylation sequence motifs for CK2 as well as for PKC. In this report, we presented phosphorylation modification of deoxyhypusine synthase by CK2, another Ser/Thr kinase, *in vivo* and *in vitro*, and evaluated the relationship between phosphorylation of deoxyhypusine synthase and the activity of the enzyme.

Materials and Methods

Materials

[1,8-^{3}H]Spermidine·HCl (15 Ci/mmol), [γ-^{32}P]ATP (6,000 Ci/mmol), and [γ-^{32}P]GTP (6,000 Ci/mmol) were obtained from NEN Life Science Products. DMEM, antibiotic-antimycotic mixture, and trypsin-EDTA were purchased from Gibco/BRL Life Technologies. FBS was from Hyclone. CK2 was from Upstate Biotechnology, protein size marker from MBI Fermentas (Lithuania), nitrocellulose and PVDF membranes from Millipore. Mouse anti-phosphothreonine monoclonal antibody, and mouse anti-CK2α monoclonal antibody were obtained from Santa Cruz Biotechnology, mouse anti-phosphoserine monoclonal antibody from Abcam (UK). The luminol-enhanced chemiluminiscence (ECL) Western blotting detection kit was purchased from Amersham Pharmcia Biotech. Protein A agarose, polylysine, heparin, and poly (Glu, Tyr) 4:1 were obtained from Sigma. Cellulose thin layer chromatography (TLC) plate (without fluorescent indicator) was from Merck, and other chemicals were purchased as described in the text. Human deoxyhypusine synthase and eIF5A precursor proteins expressed in *E. coli*

were purified from *E. coli* lysates after overexpression of human deoxyhypusine synthase and eIF5A cDNAs, respectively, are described (Joe *et al.*, 1995). Deoxyhypusine synthase protein from rat testis and its specific antibody were prepared as described (Wolff *et al.*, 1995).

Cell culture

HeLa cells, Chinese hamster ovary (CHO) cells, NIH3T3 cells, and HEK 293 cells were grown in DMEM, supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, and 10% (v/v) FBS in a humidified 5% CO_2 incubator at 37°C. Serum deprivation was carried out by lowering the concentration of FBS from 10% to 0.5% for the period of 24 h prior to specific treatment.

Protein kinase assays

Purified His-tagged deoxyhypusine synthase was incubated at 37°C for 30 min in kinase buffer (20 mM Tris/HCl, pH 7.5, 10 mM magnesium acetate, 0.4 mM $CaCl_2$, and 1 mM DTT) by CK2 with various effectors such as polylysine, heparin, and poly(Glu, Tyr) 4:1 in the presence of [γ-^{32}P]ATP. The reaction was terminated by mixing with SDS sample buffer, and separated on a 10% SDS-polyacrylamide gel (Laemmli, 1970). Radiolabeled proteins were visualized by autoradiography.

In vitro phosphorylation of deoxyhypusine synthase with HeLa cell lysate was performed by adding total cell extract with 1 μg of human recombinant deoxyhypusine synthase protein. To obtain total cellular protein, cells were washed with cold PBS and harvested with a rubber policeman, resuspended in whole cell suspension buffer (10 mM Tris/HCl, pH 7.5, and 2 mM $MgCl_2$) and centrifuged at 2,700 *g* at 4°C for 5 min. Then they were freeze-thawed, suspended in 100 μl of suspension buffer containing 1% Triton X-100, 5 mM DTT, and 1 mM PMSF, vortexed, and then stood for 10 min on ice. Cell lysates were cleared by centrifugation at 15,000 *g* at 4°C for 10 min. The amount of cellular protein present in the clarified supernatant was calculated by using the Bio-Rad protein assay kit. Kinase assays using HeLa cell lysates were incubated with kinase buffer with various CK2 effectors in the presence of [γ-^{32}P]ATP at 37°C for 30 min. The phosphorylated reaction mixtures were processed for immunoprecipitation.

For *in vivo* phosphorylation of deoxyhypusine synthase, growth-arrested HeLa cells plated onto 150-mm dishes were treated with mock or polylysine, heparin, and poly(Glu, Tyr) 4:1 for 30 min at 37°C. After treatments, cell lysates were generated using repeated freeze-thawed cycle. The resulting cell lysates containing equal amounts of protein were used for immunoprecipitation and Western blotting.

Phosphoamino acid analysis

Affinity purified human deoxyhypusine synthase was radiolabeled by CK2, electrophoretically separated on a 10% SDS-polyacrylamide gel and transferred to Immobilon (Millipore) (Kamps and Sefton, 1989; Duclos *et al.*, 1991). The subsequent ^{32}P-labeled deoxyhypusine synthase band was excised, transferred to screwcap microfuge tube, and hydrolyzed at 110°C for 2 h in 6 M HCl under nitrogen gas. Hydrolysates were dried in speedvac concentrator, dissolved in distilled water, and applied to cellulose TLC plate. Phosphoamino acids were separated by chromatography in isobutyric acid/0.5 M ammonium hydroxide (5:3, v/v). Phosphoamino acid standards were visualized by ninhydrin staining while radiolabeled phosphoamino acids were detected by autoradiography.

Immunoprecipitation and Western blot analysis

For the immunoprecipitation experiments, 2 mg of cellular extract was incubated with polyclonal rabbit anti-deoxyhypusine synthase antibody for 2 h at 4°C. Protein A agarose was then added to precipitate the immune complex and incubated for further 30 min at 4°C with constant rocking. The beads were washed and the final pellets were separated by SDS-PAGE followed by Western blot analysis with appropriate indicated antibody.

For Western blotting, precipitated immunocomplexes were resolved by SDS-PAGE and transferred to nitrocellulose membrane by standard procedures (Towbin *et al.*, 1992). Immunoblotting was done with specific antibodies and visualized by using the ECL Western blotting detection kit (Amersham Pharmacia Biotech) with manufacturer's instructions.

Assay of deoxyhypusine synthase

The enzyme activity was measured as described previously (Wolff *et al.*, 1990; Kang *et al.*, 1995). A typical reaction mixture contained, in total volume of 20 μl, 0.2 M glycine/NaOH buffer, pH 9.5, containing 1 mM DTT, 25 μg of bovine serum albumin, 1 mM NAD^+, 7-9 mM (2-5 μCi) [1,8-^{3}H]spermidine, 10 μM human eIF5A precursor protein, and enzyme. Incubations were at 37°C for 1 h. The radioactivity of [^{3}H]-deoxyhypusine was measured after its separation from other components of the acid hydrolyzed protein fraction by ion exchange chromatography as described previously (Wolff *et al.*, 1990). One unit of activity is defined as the amount of enzyme catalyzing the formation of 1 pmol of deoxyhypusine h^{-1}.

Results

Deoxyhypusine synthase was phosphorylated by purified recombinant PKC [PKC (α+β+γ) mixture, and PKC δ] *in vitro* (Kang and Chung, 1999), and also phosphorylated by CHO cell lysate. Furthermore, we demonstrated this enzyme was phosphorylated and associated with PKC *in vivo* (Kang *et al.*, 2002). Human deoxyhypusine synthase contains several potential phosphorylation sequence motifs for CK2, in addition to PKC. Study was extended to confirm the phosphorylation of deoxyhypusine synthase by CK2 and define its role on the regulation of eIF5A in the cell. Protein kinase CK2 is a pleiotropic Ser/Thr kinase which has been shown to phosphorylate numerous substrates. CK2 is ubiquitous in eukaryotes and its amino acid sequences are highly conserved, and the enzyme has been shown to be elevated in rapidly growing cells (Pinna, 1990).

In order to examine whether deoxyhypusine synthase could be a substrate for CK2, we analyzed the *in vitro* phosphorylation of human recombinant deoxyhypusine synthase by protein kinase CK2 in the presence of [γ-^{32}P]ATP as phosphate donor. As shown in Figure 1A, the phosphorylated deoxyhypusine synthase was clearly detectable (lower panel, lane 1). Furthermore, to confirm CK2-mediated phosphorylation in deoxyhypusine synthase, this enzyme was incubated in the presence of various CK2 effectors such as polylysine (100 nM), heparin (10 μg/ml), and poly(Glu, Tyr) 4:1 (1 mg/ml). Polylysine, CK2 activator, stimulates the phosphorylation of human deoxyhypusine synthase by CK2 *in vitro*. Figure 1A shows the amount of ^{32}P-incorporation into deoxyhypusine synthase at 100 nM polylysine resulted in 1.4-fold increase compared with that of control (lower panel, lane 2). Whereas, treatment of typical polyanionic CK2 inhibitors such as heparin and poly(Glu, Tyr) 4:1 showed 54%, and 60% radioactivity in the degree of phosphorylation, respectively, compared with mock-treated control served as 100 percent (lower panel, lane 3 and 4). This CK2-mediated phosphorylation was blocked by heparin (up to 50 μg/ml) treatment and poly(Glu, Tyr) 4:1 (up to 5 mg/ml) treatment in a dose-dependent manner (data not shown).

To verify the phosphorylation of deoxyhypusine synthase by endogenous kinases, HeLa cell lysate was allowed to incubated in kinase reaction buffer containing 1 μg of purified rat deoxyhypusine synthase and various CK2 effectors for 30 min at 37°C in the presence of [γ-^{32}P]ATP, and then immunoprecipitated with rabbit polyclonal anti-deoxyhypusine synthase antibody. The immunocomplexes bound to protein A agarose were detected by SDS-PAGE followed by autoradiography. Figure 2A shows that phosphorylated deoxyhypusine synthase appears in autoradiogram,

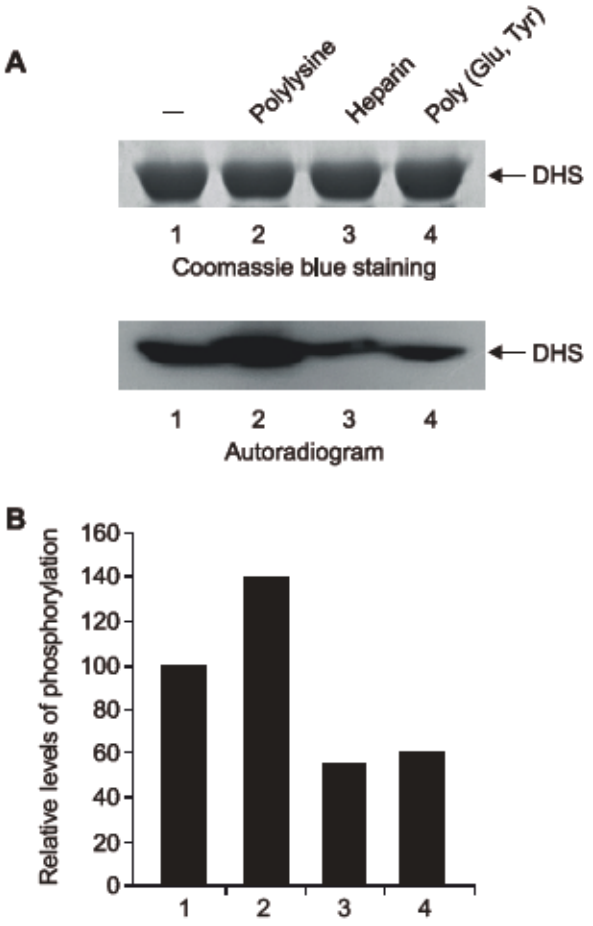

Figure 1. *In vitro* phosphorylation of human recombinant deoxyhypusine synthase by CK2. Human recombinant deoxyhypusine synthase (DHS) was incubated at 37°C for 30 min with CK2 and various effectors such as polylysine (100 nM), heparin (10 μg/ml), and poly-(Glu, Tyr) 4:1 (1 mg/ml) in the presence of [γ-^{32}P]ATP. Phosphorylated deoxyhypusine synthase with a molecular mass of 41 kDa was visualized by SDS-PAGE followed by autoradiography (A). (B) Histogram depiction of the relative levels of phosphorylation of deoxyhypusine synthase in the absence of effector served as 100 percent control.

and the levels of phosphorylation were definitely elevated with polylysine treatment (lane 2) and diminished with the treatment of heparin or poly(Glu, Tyr) 4:1 (lane 3 and 4). In order to confirm the phosphorylation of deoxyhypusine synthase by CK2, purified rat deoxyhypusine synthase was incubated with HeLa cell lysate in the presence of [γ-^{32}P]GTP under assay conditions as described in Figure 2A, because it has been shown that, unlike other protein kinases, CK2 can utilize GTP instead of ATP as the phosphate donor (Hathaway and Traugh, 1982). As shown in Figure 2B, endogenous cellular kinase(s) employing GTP in HeLa cell lysate clearly phosphorylated deoxyhypusine synthase. Phosphoamino acid analysis of human deoxyhypusine synthase phosphorylated by CK2 demonstrated that this protein was phosphorylated

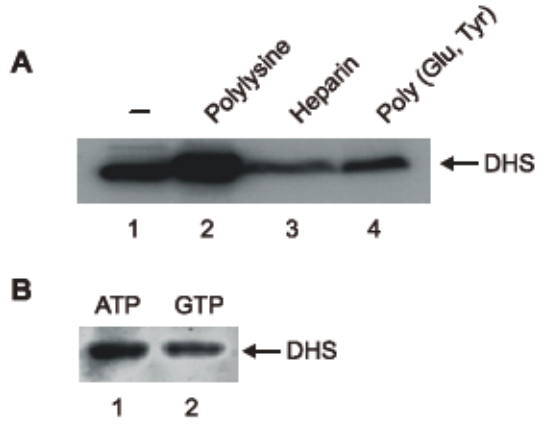

Figure 2. Phosphorylation of deoxyhypusine synthase with HeLa cell lysate *in vitro*. (A) HeLa cell lysate (2 mg) was precleared with 15 μl of protein A agarose, and incubated in kinase reaction buffer containing 1 μg of purified rat deoxyhypusine synthase and various CK2 effectors (lane 1, control; lane 2, 100 nM polylysine; lane 3, 10 μg/ml heparin; lane 4, 1 mg/ml poly(Glu,Tyr) 4:1) at 37°C for 30 min in the presence of [γ-^{32}P]ATP, and then further incubated with anti-deoxyhypusine synthase (DHS) antibody at 4°C for 2 h. The immune complexes were bound to protein A agarose, washed, and separated on a SDS-polyacrylamide gel. Phosphorylated deoxyhypusine synthase was detected by autoradiography. Arrow in the right margin indicates the phosphorylated deoxyhypusine synthase. (B) HeLa cell lysate was phosphorylated in the presence of [γ-^{32}P]ATP (lane 1) or [γ-^{32}P]GTP (lane 2) under standard assay conditions as described in A.

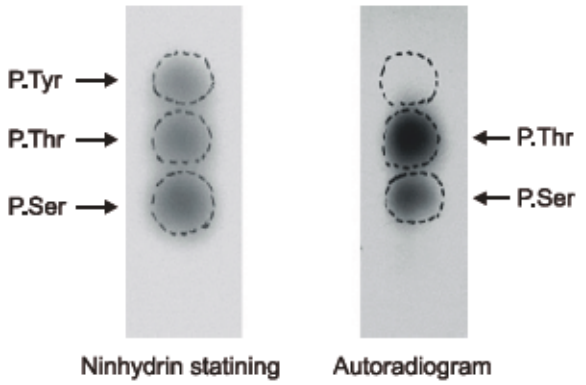

Figure 3. Phosphoamino acid analysis of human deoxyhypusine synthase phosphorylated by CK2. ^{32}P-labeled deoxyhypusine synthase by CK2 was separated by SDS-PAGE, and transferred to Immobilon membrane. The membrane piece corresponding to deoxyhypusine synthase was hydrolyzed under vacuum in 6 M HCl at 110°C for 2 h and dried in speedvac concentrator. Dried sample was subjected to thin layer chromatography in isobutyric acid/0.5 M ammonium hydroxide (5:3). ^{32}P-labeled phosphoamino acids were detected by autoradiography. Standard phosphoamino acids were visualized by ninhydrin staining.

Table 1. Relationship between phosphorylation of deoxyhypusine synthase (DHS) and the activity of the enzyme.

Phosphorylation state of DHS	DHS enzyme activity (cpm)
1. Unphosphorylated	21,610
2. Phosphorylated	
by PKC	22,005
by CK2	22,405
by HeLa cell lysate	19,750

mainly on threonine, and much less amount of ^{32}P-incorporation into serine was apparent. But there was no detectable spot on tyrosine residue on TLC plate (Figure 3).

To determine whether deoxyhypusine synthase is phosphorylated *in vivo*, HeLa cells treated with various stimuli were harvested, immunoprecipitated with anti-deoxyhypusine synthase antibody, and then the immunocomplexes were resolved in SDS-gel and immunoblotted with anti-phosphothreonine or anti-phosphoserine antibody. Figure 4 demonstrated phosphorylated deoxyhypusine synthase was distinctively visible (lane 1 of A and B) and markedly increased after the addition of polylysine (lane 2). Heparin or poly (Glu, Tyr) treatment in intact HeLa cells resulted in considerable reduction in the degree of phosphorylated deoxyhypusine synthase (lane 3 and 4). The immunoreactivity of anti-phosphothreonine antibody to deoxyhypusine synthase is notably strong compared with that of anti-phosphoserine antibody to the protein in equal titer. This is a good agreement with the result of previous phosphoamino acid analysis (Figure 3).

To strengthen that CK2 present in cell lysates is directly involved in the phosphorylation of deoxyhypusine synthase, we searched for an association between CK2α and deoxyhypusine synthase *in vitro*. As shown in Figure 5A, there is a definite interaction between deoxyhypusine synthase and CK2α, and the highest level of CK2α subunit was observed in HeLa and HEK 293 cells, followed by CHO and NIH3T3 cells. To further confirm that CK2 is directly implicated in deoxyhypusine synthase phosphorylation, we examined for an *in vivo* association. Figure 5B shows deoxyhypusine synthase could be closely correlated with CK2α in various eukaryotic cells, and this interaction was nearly equivalent in all cell lines examined.

To evaluate intracellular role of deoxyhypusine synthase phosphorylation, the enzyme was tested for the possible alteration of deoxyhypusine synthase activity during the phosphorylation processes by CK2, PKC, and HeLa cell lysate. Table 1 suggests that there was no observable changes in enzyme activity between the phosphorylated and unphosphorylated forms of deoxyhypusine synthase.

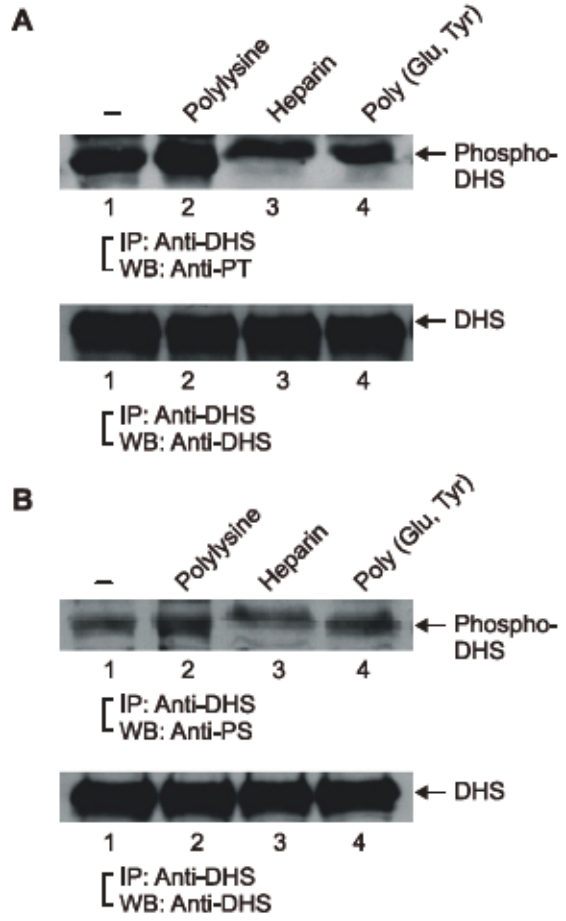

Figure 4. *In vivo* phosphorylation of deoxyhypusine synthase in HeLa cells. HeLa cells were starved of serum and stimulated with polylysine (1 μM), heparin (50 μg/ml) or poly (Glu, Tyr) 4:1 (10 mg/ml) for 30 min at 37°C. The cells were harvested, precleared with protein A agarose, and deoxyhypusine synthase was immunoprecipitated with anti- deoxyhypusine synthase (DHS) antibody. The immunocomplexes were subjected to SDS-PAGE and then immunoblotted with anti-phosphothreonine (PT) antibody (A) or anti-phosphoserine (PS) antibody (B). Lane 1 in A and B shows mock-treated control. Phospho-DHS denotes phosphorylated deoxyhypusine synthase.

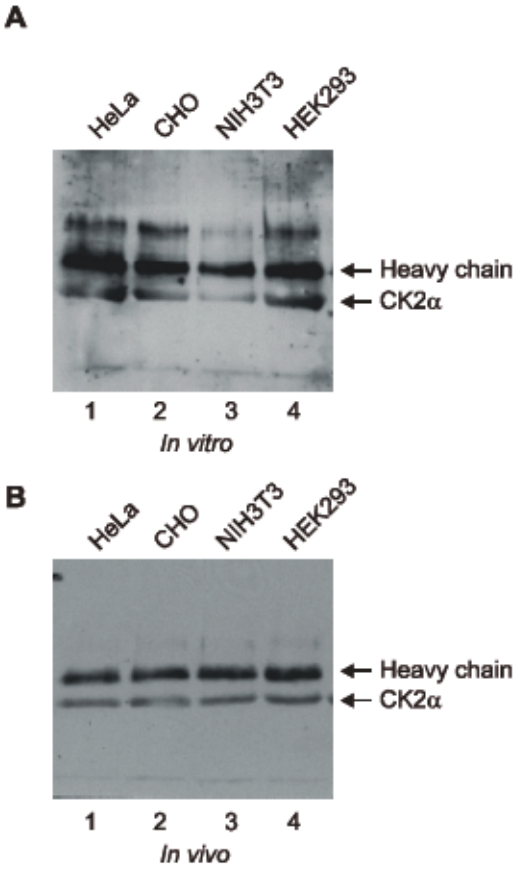

Figure 5. Association of deoxyhypusine synthase with CK2α *in vitro* and *in vivo*. Lysates (2 mg each) obtained from HeLa (lane 1), CHO (lane 2), NIH3T3 (lane 3), and HEK 293 cells (lane 4) were phosphorylated in the presence of 1 μg of purified rat deoxyhypusine synthase and 250 μM ATP *in vitro*, and then immunoprecipitated with polyclonal anti-deoxyhypusine synthase antibody and subjected to SDS-PAGE followd by immunoblotting with anti-CK2α antibody (A). Same amounts of cell lysates listed in A were incubated with anti-deoxyhypusine synthase antibody and the immune complexes were separated by SDS-PAGE and subsequent immunoblot analysis with anti-CK2 antibody (B).

Discussion

Deoxyhypusine is a modified lysine. It is formed post-translationally in eukaryotic initiation factor eIF5A by deoxyhypusine synthase, employing spermidine as butylamine donor. Subsequent hydroxylation of this deoxyhypusine-containing intermediate completes the maturation of eIF5A (Park *et al.*, 1993a; 1993b). Hypusine-modified eIF5A, the active form of the protein, is the only cellular protein known to contain hypusine, and this modification is essential for eIF5A activity (Park *et al.*, 1993a). Despite of eIF5A being a constitutive component, for maintaining a homeostasis of cell, there should be some point of regulating its production due to this protein's function as one of the translation initiation factors involved in the synthesis of other cellular proteins. The most predictable target for reversible regulation of eIF5A is deoxyhypusine synthase, the first enzyme involved in its hypusination. Yeast recombinant deoxyhypusine synthase was phosphorylated with CK2 as well as PKC *in vitro* (Kang and Chung, 1999). In previous report, we observed deoxyhypusine synthase was phosphorylated by PKC *in vitro* and the association of deoxyhypusine synthase with PKC in CHO cells was PMA-, and Ca^{2+}/phospholipid-dependent (Kang *et al.*, 2002). The study was extended on the phosphorylation of deoxyhypusine synthase by CK2 to characterize its cellular role on the regulation of eIF5A.

Protein kinase CK2 is an ubiquitously expressed

pleiotropic and probably constitutively active serine and/or threonine kinase that can use ATP as well as GTP as cosubstrate. In many organisms, protein kinase CK2 is a heterotetramer composed of two catalytic α- or α'-subunits and two regulatory β-subunits (Pinna, 1990; Allende and Allende, 1995). CK2β seems to play at least a trifunctional role: it confers stability to the holoenzyme (Meggio *et al*., 1992), it increases enzyme activity and its activity is stimulated by polycations (Grankowski *et al*., 1991), and it determines substrate specificity (Bidwai *et al*., 1992; Meggio *et al*., 1992). The catalytic α-subunit is regulated not only by the β-subunit but also by polyanions; very little is known about the α'-subunit.

Treatment with polylysine specifically increased (1.4-fold) the relative phosphorylation of deoxyhypusine synthase in human, supporting CK2 was implicated in the control of this system. The basal activity of the CK2 holoenzyme is even high in this phosphorylation reaction concerning human recombinant deoxyhypusine synthase (Figure 1A). There are a few notable exceptions, however, exemplified by calmodulin, where the β subunit has been shown to display a predominantly negative function. It has been suggested that the phosphorylation of these substrates either is catalyzed by intracellular pools of free catalytic subunits or is triggered by basic polypeptides, as it is known that polylysine counteracts the inhibitory effect of the β subunit (Meggio *et al*., 1994b). Polyanionic heparin and poly(Glu, Tyr) 4:1, the CK2 specific inhibitors, reduce the phosphorylation of deoxyhypusine synthase by about 50-60%, indicating that CK2 is involved in deoxyhypusine synthase signaling mechanism (Figure 1A and B).

The *in vitro* phosphorylation of deoxyhypusine synthase by recombinant CK2 elicited us to investigate the phosphorylation of deoxyhypusine synthase with endogenous kinases. As shown in Figure 2A, HeLa cell lysates induced the phosphorylation of deoxyhypusine synthase and the degree of phosphorylation by the cell lysate was influenced by the treatment of polylysine, heparin, or poly(Glu, Tyr) in the same manner of Figure 1. In addition, the phosphorylation of deoxyhypusine synthase by cell lysate was also detected in the presence of GTP as the phosphate donor, suggesting involvement of CK2 in this pathway. Phosphoamino acid analysis of human recombinant deoxyhypusine synthase phosphorylated by CK2 revealed that this enzyme was phosphorylated on threonine by about 70% of the total phosphorylation and the remainder of the phosphorylation on serine. Deoxyhypusine synthase is indeed Ser/Thr phosphorylated in intact HeLa cells. Such a phosphorylation is especially increased if HeLa cells are incubated with polylysine (Figure 4). It is also detectable, however, in untreated cells. Such an *in vivo* phosphorylation is markedly decreased by specific inhibitor of CK2, heparin and poly(Glu, Tyr). On the basis of these results, it could be possible to suggest that CK2 might be the *in vivo* kinase in the phosphorylation of deoxyhypusine synthase.

To confirm our *in vitro* data, the association of CK2 with deoxyhypusine synthase was examined in various cells, that is, HeLa, CHO, NIH3T3, and HEK 293 cells. The fact that deoxyhypusine synthase and CK2α subunit can be co-immunoprecipitated from different cells, clearly support the specificity of association as well as possible cellular signal link. Based on the results showing different intensities in the association of CK2α with deoxyhypusine synthase between the *in vitro* (Figure 5A) and *in vivo* (Figure 5B), one could speculate that a signaling mechanism turning off the stimulation of deoxyhypusine synthase by CK2 other than the direct molecular interaction between the two proteins presents in intact cells.

Deoxyhypusine synthase is definitively phosphorylated by CK2 *in vivo*. However, intracellular role of phosphorylation of this enzyme is still unclear. Unexpectedly, there was no clear differences of enzymatic activity in hypusination between phosphorylated and unphosphorylated forms of deoxyhypusine synthase (Table 1). Deoxyhypusine synthase, as well as eIF5A, is ubiquitous in all eukaryotes, and both are essential for eukaryotic cell proliferation and survival. The vital role of this enzyme and the hypusine modification has been demonstrated from gene disruption studies in yeast (Park *et al*., 1998). It was not known whether deoxyhypusine synthase can catalyze other reaction in polyamine metabolism or perform other cellular functions. Indeed, deoxyhypusine synthase is involved in the posttranslational activation of the eIF5A and, as a side-reaction, catalyzes the formation of homospermidine if its substrate, the eIF5A precursor protein, is replaced by putrescine in tobacco (Ober and Hartmann, 1999b) and *senecio vernalis* (Ober and Hartmann, 1999a; Ober *et al*., 2003). Another plant enzyme, homospermidine synthase, which can also catalyze the synthesis of homospermidine, was identified (Ober and Hartmann, 1999a) in the roots of certain plants that produce pyrrolizidine alkaloids. Plant homospermidine synthase is assumed to be phylogenetically derived from deoxyhypusine synthase; it represents a deoxyhypusine synthase having lost its intrinsic activity. The synthesis of homospermidine as an enzymatic by-product of an essential enzyme is considered in respect to the evolutionary origin of homospermidine synthase and the biosynthetic pathway of pyrrolizidine alkaloids. Besides its established function in hypusine modification, and a proposed role in the production of homospermidine and unusual polyamines in a few mammalian tissues, deoxyhypusine synthase may have other indenpendent cellular func-

tions.

Although the phosphorylation of deoxyhypusine synthase does not influence on the activity of the enzyme in hypusination reaction involving eIF5A substrate, it may implicate other cellular functions because of diverse and versatile roles of deoxyhypusine synthase. Lately, the deoxyhypusine synthase gene has been reported as one of the eight upregulated gene signature associated with metastasis in human cancer (Ramaswamy *et al.*, 2003). Cellular functions of deoxyhypusine synthase and the significance of phosphorylation of the enzyme in cells have to be studied in detail.

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