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Acute hypoxia enhances proteins' *S*-nitrosylation in endothelial cells

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ABSTRACT

Hypoxia-induced responses are frequently encountered during cardiovascular injuries. Hypoxia triggers intracellular reactive oxygen species/nitric oxide (NO) imbalance. Recent studies indicate that NO-mediated *S*-nitrosylation (S-NO) of cysteine residue is a key posttranslational modification of proteins. We demonstrated that acute hypoxia to endothelial cells (ECs) transiently increased the NO levels via endothelial NO synthase (eNOS) activation. A modified biotin-switch method coupled with Western blot on 2-dimentional electrophoresis (2-DE) demonstrated that at least 11 major proteins have significant increase in S-NO after acute hypoxia. Mass analysis by CapLC/Q-TOF identified those as Ras-GTPase-activating protein, protein disulfide-isomerase, human elongation factor-1-delta, tyrosine 3/tryptophan 5-monooxygenase activating protein, and several cytoskeleton proteins. The *S*-nitrosylated cysteine residue on tropomyosin (Cys 170) and β-actin (Cys 285) was further verified with the trypsic peptides analyzed by MASCOT search program. Further understanding of the functional relevance of these *S*-nitrosylated proteins may provide a molecular basis for treating ischemia-induced vascular disorders.

Hypoxia have been demonstrated to play integral roles in functional responses in vascular endothelial cells (ECs), including cell proliferation, angiogenesis, and cell migration [1,2]. Manipulation of these responses is important for biomedical aspects such as ischemia, stroke, and tumorigenesis [3]. Vascular ECs cope with hypoxia to maintain vascular homeostasis by expressing a number of genes, which are mediated by a variety of signaling cascades [4,5]. The transcriptomic and proteomic studies revealed possible roles of novel genes and proteins on hypoxic physiology [6,7]. Hypoxia was found to result in an oxidative stress due to the change in the reactive oxygen species (ROS)/nitric oxide (NO) balance [8], which was thought to exert opposing effects on the stability of hypoxia-inducible factor-1α(HIF-1α) [9].

NO, a labile molecule that constantly produced via the activation of nitric oxide synthase (NOS) [10,11]. Endothelial NOS (eNOS) is essential to maintain vessel integrity. NO/redox disequilibrium results in cellular dysfunction and cardiovascular diseases [12,13]. It is well known that NO-mediated cGMP-dependent effects on cardiovascular system contribute to the anti-thrombosis, vasodilation and antiproliferation [14–16]. However, cGMP-independent effects by NO have been recognized [17]. Recent information indicates that a covalent addition of NO to Cys-sulfur in proteins, or *S*-nitrosylation, plays pervasive roles in the modulation of protein functions [11]. Knowledge of those proteins that possess *S*-nitrosylation is fundamental to understand NO-mediated cellular responses in ECs under stress condition. Previous study demonstrated that ECs exposed to acute hypoxia transiently stimulated NO production via activation of eNOS [18]. An *S*-nitrosothiol-based signal originating from red blood cells (RBCs) mediates hypoxic vasodilation by RBCs, and that vasorelaxation by RBCs dominates NO-based vasoconstriction under hypoxic conditions [19]. However, a comprehensive screen of acute hypoxia-induced *S*-nitrosylation in ECs is lacking. Biotin-switch method has been used to analyze the *S*-nitrosylation of proteins [20]. In this study, we have adopted a modified biotin-switch method coupled with Western-based 2-dimentional electrophoresis (2-DE) to screen *S*-nitrosylated proteins in ECs under acute hypoxia. There were 40 major *S*-nitrosoproteins detected, 11 of which were shown to have an enhanced *S*-nitrosylation under acute hypoxia. The increased *S*-nitrosylated proteins in ECs under acute hypoxia may protect ECs from stress-induced injuries.

Materials and methods

Cell culture and hypoxia apparatus. ECs used were EAhy 926 cell line kindly donated by Cora-Jean S. Edgell, University of North Carolina, Chapel Hill. EAhy 926 cells was cultured in DMEM supplemented with fetal bovine serum (FBS, 10%). After starvation overnight in 0.5% FBS, ECs were exposed to hypoxic conditions (pO_2 = 8 mm Hg) that were achieved by adding medium

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pre-equilibrated with nitrogen gas to cells prior to the incubation in a Plexiglas chamber, which was purged with water-saturated nitrogen gas to a pO_2 = 8 mm Hg by a oxygen controller (PROOX model 110, BioSpherix, Ltd., Redfield, NY). The partial pressure of oxygen (pO_2) of culture medium under hypoxia was monitored using ISO_2 dissolved oxygen meter (World Precision Instruments, Inc., Sarasota, FL). The measurement indicated that a steady-state pO_2 = 8 mm Hg in the culture medium was maintained during experiments [18].

S-nitrosylation analysis by biotin-switch and two-dimensional gel electrophoresis. For the analysis of *S*-nitrosylation, the biotin-switch method was utilized as previously described [20]. In brief, the whole cell lysate was obtained by the ultrasound vibration with lysis buffer [Hepes (250 mM, pH 7.7), EDTA (1 mM), neocuproine (0.1 mM) and CHAPS (0.4%, w/v)]. Cell lysate was blocked with 4 volumes of blocking buffer [Hepes (225 mM, pH 7.7), EDTA (0.9 mM), neocuproine (0.09 mM), SDS (2.5%, w/v) and MMTS (20 mM)] at 50 °C for 20 min with agitation. After precipitation with acetone, the pellet was reduced and biotinylated in HENS buffer [Hepes (250 mM, pH 7.7), EDTA (1 mM), neocuproine (0.1 mM) and SDS (1%, w/v)] that contained 1 mM ascorbate and 1/3 volume of 4 mM *N*-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio) propionamide (biotin-HPDP) (Pierce Biotech., IL, USA). This biotinylated protein lysates were precipitated with acetone and re-suspended in the DTT-free 2-DE sample buffer [urea (9 M), CHAPS (4%, w/v), IPG buffer (2%, v/v, pH 4–7)] (GE Healthcare BioSci., NJ, USA). Proteins were mixed with rehydration solution [Urea (8 M), CHAPS (2%, w/v) and IPG buffer (0.5%, v/v, pH 4–7)] to have final volume of 340 μl and then soaked into DryStrip (pH 4–7, GE Healthcare BioSci.) on Ettan IPGphor system (GE Healthcare BioSci.). After IEF analysis, stripped gels were equilibrated with buffer containing 2% (w/v) SDS, 50 mM Tris–HCl, pH 8.8, 6 M urea and 30% (v/v) glycerol. The equilibrated IEF gels were subjected to a vertical SDS–PAGE system. Reducing agents, DTT and TCEP [tris(2-carboxyethyl)phosphine], were avoided in the processes above.

Gel staining, Western blotting, and image analysis. After DTT-free 2-DE separation, the gels were stained by VisPRO fast stain dye (Visual Protein Biotech., Taipei, Taiwan) for 10 min and scanned by UMAX Astra 1200 S scanner. The spots densities were calculated by ImageMaster software (GE Healthcare BioSci.). The VisPRO dye was then destained and Western- blotted onto nitro-cellulose membrane. The membranes were blocked with skim milk (5%, w/v) and hybridized with streptavidin-HRP (1:4000, PerkinElmer LifeSci., MA, USA). The membranes were developed with SuperSignal West Femto Maximum chemiluminescence regent (Pierce Biotech.) on X-ray film. The densities of each spot that represent the *S*-nitrosylation level were scanned by UMAX Astra 1200S scanner and analyzed by ImageMaster software. Standard mark proteins on the images of VisPRO-stained gels and X-ray films were used to facilitate the protein pecking from the corresponding gel under the same 2-DE condition.

In-gel digestion and mass spectrometric analysis. The *S*-nitrosylated proteins that show significant increases in spot density (>1.5-fold) under acute hypoxia were excised from the corresponding gel stained by VisPRO dye. The gel slices were under in-gel digestion at 37 °C for 4 h according to the manual (In-Gel Tryptic Digestion Kit, Pierce BioSci.). The trypsic peptides were desalting with Proteomics C18 Column (C SUN Ltd., Taipei, Taiwan) and mass analyzed by CapLC/Q-TOF (Micromass, Manchester, UK). MS data were searched against the NCBInr database using MASCOT in-house search program (Matrixscience, London, UK). The biotinylated cysteines sites, i.e., *S*-nitrosylation sites were analyzed by the mass shift of 428.2 Da with ESI-QUAD-TOF. The search parameters were decided by the protein modifications: Acetyl (Protein N-term), Oxi-

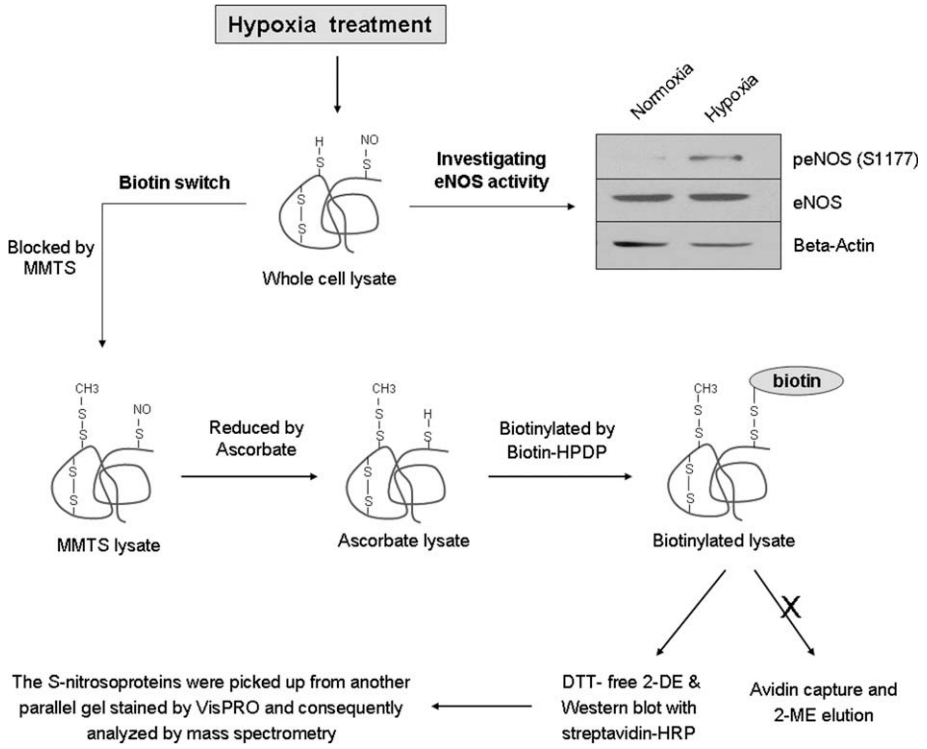

Fig. 1. Hypoxia-induced eNOS activation and approaches to screen *S*-nitrosylated proteins. An aliquot (50 μg) of whole cell lysates from EAhy 926 ECs under normoxia or hypoxia (pO_2 = 8 mm Hg) treatment for 1 h were immunoblotted with antibodies to phospho-eNOS (S1177). eNOS and beta-actin were shown to indicate equal amount of proteins loaded. To screen the *S*-nitrosoproteins, a modified approach on the basis of biotin-switch method was utilized. Whole cell lysate was subjected to biotin-switch processes with three steps: MMTS-blocking, ascorbate-reduction, and biotin-HPDP substitution. The biotinylated proteins, i.e., *S*-nitrosylated proteins, were separated by DTT-free 2-DE and detected by Western blot using streptavidin conjugated with HRP. The *S*-nitrosoproteins were pecked from the parallel gel that stained by VisPRO and analyzed by mass spectrometry.

dation (M) and Biotin-HPDP (C). Mass value was settled as monoisotopic. Peptide Mass Tolerance was fixed at ±0.4 Da. Max Missed Cleavages was set as 1.

Results

Acute hypoxia increased eNOS activity in ECs

To study the eNOS activity in ECs under acute hypoxia, EAhy 926 cells were subjected to hypoxia for 1 h and eNOS was examined for the phosphorylation at ser1179. As shown in Fig. 1, the eNOS phosphorylation was increased after acute hypoxia (pO_2 = 8 mm Hg). This result indicates that application of acute hypoxia to ECs induce a transient increase in eNOS activity.

Analysis of S-nitrosoproteins

In order to validate the specificity of biotin-switch method toward the *S*-nitrosylated proteins, biotin labeling of proteins was examined for its reaction to the reduced thiol (S–H) in proteins collected from each step during the biotin-switch experiment (Fig. 1). As expected, the biotin labeling in whole cell lysate after treating with or without MMTS to block free thiols showed great difference in the biotin labeling proteins (Fig. 2A). Cell lysate with MMTS pretreatment nearly abolished the biotin labeling to proteins, indicating that biotin reacts specifically to free thiols. However, a subsequent ascorbate treatment, an essential step to reduce S-NO bond, recovered some proteins that subsequently could be biotin labeled (Fig. 2A). Those ascorbate-recovered biotin labeling (Cys-*S*-*S*-biotin) proteins were almost diminished after further treatment of these proteins with strong reducing agents, 2-ME or DTT (Fig. 2A). These observations of biotin labeling on ascorbate-reduced proteins are completely in agreement with the nature of labile S-NO bond. Our results validate the specificity of biotin-switch method for the *S*-nitrosylated proteins encountered in ECs after acute hypoxia exposure.

In order to screen the *S*-nitrosylated proteins, biotin labeling proteins from hypoxia or normoxia, instead of pulled-down by streptavidin, were directly subjected to DTT-free 2-DE (Fig. 2B). The gels were fast stained by VisPRO and the location was revealed and density of each protein spot was quantitatively analyzed. The gels were then destained and blotted onto nitro-cellulose membrane followed by the hybridization with the HRP-tagged streptavidin. There were more than 37 *S*-nitrosoproteins to be detected. Among those identified *S*-nitrosylated proteins (Supplementary material), 11 of them showed significant increase (>1.5-folds) in the *S*-nitrosylation after hypoxic treatment (Fig. 2C). These 11 identified *S*-nitrosoproteins were heterogeneous origins, including structural proteins such as vimentin (Spot Nos. 3 and 4), desmin (Spot No. 2), beta-actin (Spot Nos. 6 and 7), tropomyosin 3 (Spot No. 11) and signaling, and stress-related proteins such as Ras-GTPase-activating protein SH3-domain-binding protein (Spot No. 1), human elongation factor-1-delta (Spot No. 9), protein disulfide-isomerase A3 precursor (Disulfide isomerase ER-60) (Spot No. 5) and Tyrosine 3/Tryptophan 5-monooxygenase activation protein (Spot No. 10).

Determination of S-nitrosylated sites

To further validate the occurrence of *S*-nitrosylation of proteins in ECs under acute hypoxia, the biotinylated site, i.e., the *S*-nitrosylated site, was analyzed with nLC-ESI-MS/MS and MASCOT in-house search program (Supplementary material) on two *S*-nitrosylated proteins, beta-actin and tropomyosin. With the peptide mass shift of 428.2 Da, beta-actin and tropomyosin 3 were confirmed to exhibit *S*-nitrosylation at Cys170 and Cys285, respectively (Fig. 3A and B). These data confirm that *S*-nitrosylation did occur in the susceptible Cys residues in endothelial proteins after acute hypoxia exposure.

Discussions

In the present study, we have utilized modified biotin-switch method that coupled with streptavidin-labeled Western analysis on 2-DE. These approaches increased the yield efficiency and the

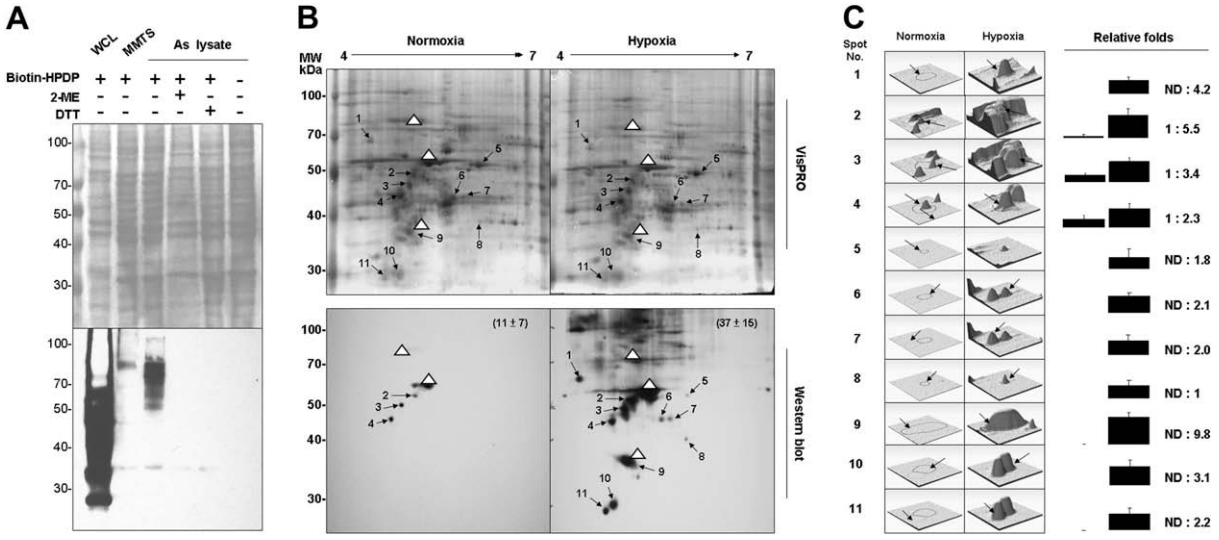

Fig. 2. Acute hypoxia enhanced protein *S*-nitrosylation. The specificity of biotin-HPDP in *S*-nitrosocysteine labeling was evaluated prior to the *S*-nitrosoproteins' screen by 2-DE (A). Fifty micrograms of whole cell lysate (WCL), MMTS lysate (MMTS) and ascorbate-reduced lysate (As) from hypoxia-treated ECs were biotinylated with biotin-HPDP. DTT and 2-mercaptoethanol (2-ME) were added to the As lysate that had been biotinylated. The As lysate without biotinylation was loaded as negative control. The blotted membrane was prestained by Ponceau S (A, upper panel) and immunoblotted by streptavidin-HRP (A, lower panel). Based on the biotin-HPDP that specifically reacted to *S*-nitrosothiol, equal amount of biotinylated lysate (1 mg) from normoxia or hypoxia treatments was subjected to DTT-free 2-DE. The gel was prestained by VisPRO to establish the reference protein markers (solid triangle) (B, upper panel). After de-staining, the gel was Western blotted with streptavidin-HRP to detect the *S*-nitrosoproteins (B, lower panel). The *S*-nitrosylation levels that showed significant increases under acute hypoxia were indicated as arrow head. The increased *S*-nitrosylation after hypoxia was illustrated as relative folds in comparison to the normoxia controls (C). ND: Non detected.

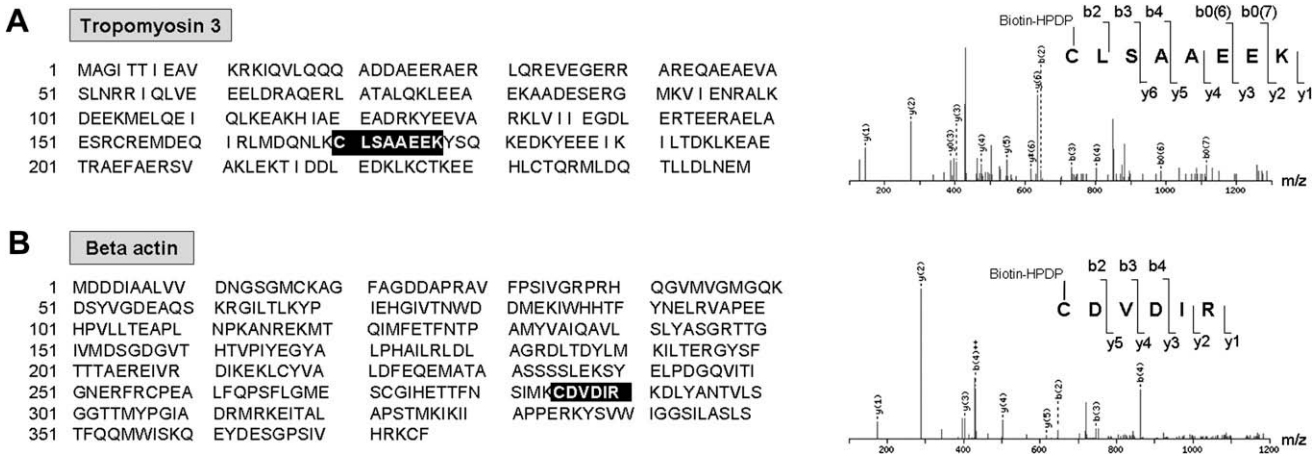

Fig. 3. Determination of *S*-nitrosylation sites on the *S*-nitrosoproteins by nLC-ESI-MS/MS. The MS/MS data of peptide ions was searched against NCBInr database using MASCOT in-house algorithm. Two biotin-HPDP bound peptides sequences of tropomyosin 3 (Cys170) and beta-actin (Cys285) were determined due to a mass shift of 428.2 Da. (A, B) and the peptide lists of each protein were illustrated in the right panel.

sensitivity to detect the endogenous *S*-nitrosylated proteins in ECs under acute hypoxia. With this modified method, we have identified endothelial proteins that can be *S*-nitrosylated under acute hypoxia condition. Further characterization also confirmed the *S*-nitrosylated site in some of those proteins. In this study, 37 proteins in static ECs were detected to have *S*-nitrosylation. However, among those only 11 proteins were shown to have increased *S*-nitrosylation under hypoxia condition. Those *S*-nitrosylated proteins are heterogeneous origin covering from structural-, signaling-, and stress-related proteins. Whether the *S*-nitrosylation of these proteins plays important functional roles in ECs under hypoxia remain unclear. Previous studies reported that ECs exposed to a transient hypoxia induced a burst of ROS [21]. In addition, bovine ECs exposed to acute hypoxia have been shown to increase eNOS protein expression and a sustained increase of eNOS activity [22]. This increased eNOS activity under hypoxia is in agreement with our observation, which showed an increased *S*-nitrosylation in ECs under acute hypoxia. Our previous studies demonstrated the atheroprotective role of NO in ECs [23–25]. Despite unclear mechanism, those NO-mediated protective effects were shown to be cGMP-independent. Accumulating evidence indicates that *S*-nitrosylation is a reversible and selective posttranslational modification that regulates protein activity, stability, and localization. *S*-nitrosylation may act as a sensor when cells are under redox changes [11]. NO-mediated *S*-nitrosylation of dynamin-2 has been shown to promote endothelial survival [26]. Thus, the NO-mediated *S*-nitrosylation of endothelial proteins may exert adaptive or protective effects in ECs under stress condition such as acute hypoxia. Vimentin is a member of the intermediate filament family of proteins associated with cytoskeleton. *S*-nitrosylation of actin was shown to inhibit the neutrophil integrin function [27]. Whether the *S*-nitrosylation of vimentin affects endothelial adhesion under hypoxia remains to be determined. Protein disulfide-isomerase (PDI) is an enzyme that catalyzes the formation and dissociation of disulfide bonds and regulates protein folding under pathophysiological conditions, including hypoxia and ischemia [28]. A recent study showed that *S*-nitrosylation of PDI led to a protein misfolding and resulted in neurodegeneration [29]. PDI was demonstrated to be continuously secreted from NO-producing cells and hence may be involved in the transfer of *S*-nitrosothiol bound-NO to cytosol [30]. *S*-nitrosylation of PDI in ECs may be essential for maintaining endothelial integrity under oxidative stress. Elongation factor-1-δ participates in the elongation step during the translation of mRNA. EF-1δ was shown to co-localize with the protein disulfide-isomerase [31,32]. Recent study showed *S*-nitrosylation of elongation factor 2 and elongation factor-1A-1 in human vascular smooth muscle cells [33]. Whether this *S*-nitrosylation regulates this protein's stability remains to be determined. Ras-GTPase-activating protein SH3-domain-binding protein (G3BP) is essential for Ras signaling [34]. Protein *S*-nitrosylation has been emerged as an important post-transcriptional modification of proteins in cells. The physiological roles of these *S*-nitrosylated proteins may be essential for endothelial protection and adaptation under acute hypoxia. With the modified biotin-switch method coupled with the Western blot-based 2-DE and with the high recovery of trypsic peptide, 11 hypoxia enhanced *S*-nitrosoproteins were identified. The detailed downstream effects of these *S*-nitrosylations remain important questions and warrant further investigation.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.10.144.

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