Repression of classical nuclear export by S-nitrosylation of CRM1

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Summary

The karyopherin chromosomal region maintenance 1 (CRM1) is the major receptor for classical nuclear protein export. However, little is known about the regulation of CRM1 itself. Here, we report that cellular CRM1 became S-nitrosylated after extensive exposure to endogenous or exogenous nitric oxide (NO). This abrogated the interaction of CRM1 with nuclear export signals (NESs) and repressed classical protein export. Analysis by mass spectrometry and involving the use of S-nitrosylation mimetic mutations indicated that modification at either of two specific cysteines of CRM1 was sufficient to abolish the CRM1-NES association. Moreover, ectopic overexpression of the corresponding S-nitrosylation-resistant CRM1 mutants rescued NO-induced repression of nuclear export. We also found that inactivation of CRM1 by NO facilitated the nuclear accumulation of the antioxidant response transcription factor Nrf2 and transcriptional activation of Nrf2-controlled genes. Together, these data demonstrate that CRM1 is negatively regulated by S-nitrosylation under nitrosative stress. We speculate that this is important for promoting a cytoprotective transcriptional response to nitrosative stress.

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Key words: CRM1, Nrf2, Nitric oxide, Nitrosation, Nitrosylation, Nuclear export

Introduction

Regulation of the nucleocytoplasmic localization of signaling components, especially transcription factors, is often an important aspect of signal transduction. Proteins are transported into and out of the nucleus through the nuclear pore complex (NPC). Most nucleocytoplasmic protein transport is carried out by shuttling receptors of the karyopherin family, which recognize specific nuclear localization sequences (NLSs) or nuclear export sequences (NESs) on cargoes to mediate their translocation through the NPC (Bednenko et al., 2003; Terry et al., 2007). Chromosomal region maintenance 1 (CRM1; also known as Exportin-1) is an abundant evolutionarily conserved export receptor for the 'classical' leucine-rich NESs (Fornerod et al., 1997). The current model is that CRM1, NES-containing protein and the GTP-bound form of Ran assemble into a trimeric complex in the nucleus that undergoes translocation through the NPC. Once in cytoplasm, the complex could undergo a disassembly reaction triggered by GTP hydrolysis, an event stimulated by the GTPase-activating protein RanGAP1 and the co-activator RanBP1 (Pemberton and Paschal, 2005; Hutten and Kehlenbach, 2007). Although CRM1 has been considered to be a crucial gate-keeper in controlling nucleocytosolic movement of numerous signaling components and transcriptional regulators, it remains unknown whether the activity of CRM1 can be regulated by signaling.

Nitric oxide (NO) exerts a ubiquitous influence on cellular signaling, in large part by means of S-nitrosylation of protein cysteine residues (Foster et al., 2003; Hess et al., 2005; Hara et al., 2005). It is also increasingly apparent that dysregulated S-nitrosylation might play a causal role in a spectrum of human diseases (Foster et al., 2003; Chung et al., 2004; Uehara et al., 2006; Lim et al., 2008; Cho et al., 2009). Intracellular NO, possibly in its S-nitrosothiol (SNO) form [e.g. *S*-nitrosoglutathione (GSNO)], can interact with a susceptible cysteine residue, resulting in S-nitrosylation that then modulates cell signaling, transcription and enzyme activity (Hess et al., 2005). Recently, NO has been implicated in regulating the nucleocytoplasmic trafficking of a number of proteins (Schneiderhan et al., 2003; Buckley et al., 2003; Hara et al., 2005; Qu et al., 2007a), but it is not known whether it can modulate the nuclear transport machinery.

The notions that the nuclear export receptor CRM1 is inactivated by the sulfhydryl-alkylating agent *N*-ethylmaleimide (Holaska and Paschal, 1998) and that CRM1 contains multiple evolutionally conserved cysteines suggest the possibility that the function of CRM1 could be redox-sensitive and regulated by S-nitrosylation. In this study, we found that CRM1 was extensively S-nitrosylated under nitrosative stress, and that S-nitrosylation of CRM1 at C528 and C585 compromised its ability to recognize classical NESs. Because this facilitates transcription by the antioxidant response transcription factor Nrf2, we speculate that S-nitrosylation-mediated deactivation of CRM1 counteracts nitrosative stress-induced cell death.

Results

Nitric oxide blocks CRM1-dependent protein nuclear export

Our initial goal was to test the possibility that the classical CRM1-mediated nuclear export could be affected by oxidative or reductive cellular environments. We employed a live-cell-based nuclear export system by evaluating the nuclear export capability of Rev,

a nucleocytoplasmic shuttling HIV protein with both a basic amino-acid-rich NLS recognized by multiple importins (Arnold et al., 2006) and a classical NES recognized by CRM1 (Askjaer et al., 1998). We used expression vectors derived from Rev-GFP in which the endogenous Rev NES was mutationally inactivated (Henderson and Eleftheriou, 2000). One of these vectors (Rev1.4) contained no additional NES and was competent only for nuclear import, whereas a second vector (Rev1.4+NES) contained an ectopically added wild-type Rev NES, and was competent for nucleocytoplasmic shuttling. We performed the analysis in HEK293 cells, where the steady-state localization of wild-type Rev is cytoplasmic (Fig. 1A). With this system, we evaluated the effects of a series of redox-related proteins and small molecules on nuclear export by ectopic overexpression of these proteins in cells, or incubation of cells with these reagents (supplementary material Table S1). The proteins or small molecules demonstrating an effect on localization of Rev1.4+NES but no effect on Rev1.4, were chosen as the potential regulators for classical nuclear export (positive hits). Among the proteins/molecules we examined, the only repeatable hit showing obviously inhibitory effect on nuclear export was GSNO, an endogenous SNO form and widely used nitrosating reagent (Hess et al., 2005; Hara et al., 2005; Qu et al., 2007a). As shown in supplementary material Fig. S1A, Rev1.4-GFP was concentrated in nucleoli in HEK293 cells, and this localization was unaffected by GSNO, However, Rev1.4+NES, which was localized to the cytoplasm at steady state in HEK293 cells, relocalized to nucleoli in the presence of GSNO or the CRM1 inhibitor leptomycin B (LMB) (Fig. 1A). In addition, GSNO was found to repress actinomycin-D-induced nuclear export of wild-type Rev in HeLa cells (supplementary material Fig. S1B). The GSNO-exerted effect cannot be attributed to the nonspecific oxidative stress, because it could not be mimicked by treatment with the strong oxidant H_2O_2 (Fig. 1A; supplementary material Fig. S1B). Using a Rev1.4-based assay, we also observed that GSNO similarly inactivated nuclear export mediated by the CRM1-dependent NES from the cellular caspase inhibitor CrmA (Rodriguez et al., 2003) (supplementary material Fig. S1A), suggesting that the effect of NO on nuclear export is independent of the NES context.

Next, we sought to determine whether NO selectively represses the classical CRM1 protein export pathway. The transcriptional co-activator MokA contains at least one classical CRM1-dependent NES (NES2) and one non-classical NES (NES3) (Smaldone and Ramirez, 2006). We analyzed these NESs expressed as GFP fusions. By itself, GFP is small enough to cross the NPC by passive diffusion and becomes modestly concentrated in the nucleus at steady state (Chatterjee and Stochaj, 1998). Although the addition of both NES2 and NES3 caused GFP to be excluded from the nucleus (Fig. 1B), only NES2-GFP was localized to the nucleus in the presence of LMB, as shown previously (Smaldone and Ramirez, 2006). Similarly, only NES2-GFP accumulated in the nucleus in response to GSNO treatment (Fig. 1B). This observation further demonstrates that exposure to NO represses classical CRM1-dependent export, but also indicates that it does not affect export by at least one non-classical NES.

To determine whether endogenous production of NO exerts an inhibitory effect on nuclear export, the RAW264.7 macrophage line expressing Rev1.4+NES-GFP was treated with lipopolysaccharide (LPS), a stimulus that induces high levels of inducible nitric oxide synthase (iNOS) expression and NO generation in macrophages (Hara et al., 2005; Qu et al., 2007b) (Fig. 1D). Using this model, we observed that LPS treatment, similarly to GSNO treatment,

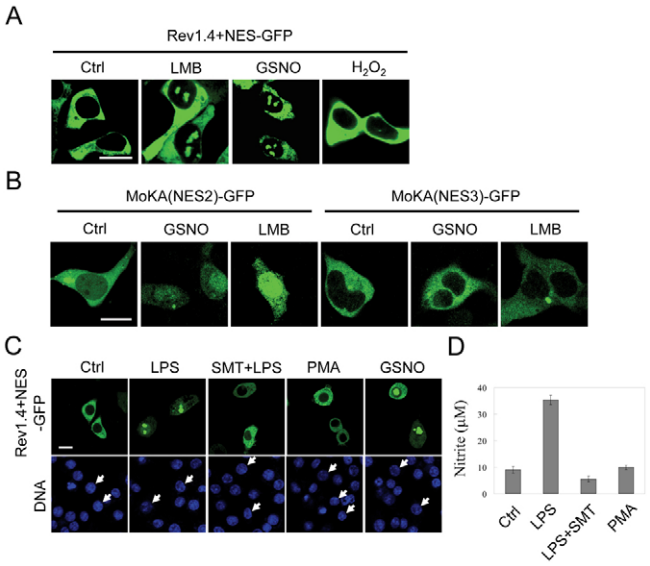


Fig. 1. NO represses CRM1-dependent classical nuclear export. (A) HEK293 cells expressing Rev1.4+NES-GFP were untreated (Ctrl) or treated with LMB (5 ng/ml) for 20 minutes or with GSNO (1 mM) or H_2O_2 (1 mM) for 4 hours. Images of the GFP fluorescence are shown. (B) HEK293 cells expressing MoKA(NES2)-GFP (left panels) or MoKA(NES3)-GFP (right panels) were treated with GSNO or LMB for 4 hours. The GFP fluorescence was then examined. (C) RAW264.7 cells transiently expressing Rev1.4+NES-GFP were treated with LPS (100 ng/ml), LPS+SMT (100 μM) or PMA (0.5 μM) for 24 hours, or with GSNO (1 mM) for 4 hours, and the GFP fluorescence examined. Arrows denote the nuclei of transfected cells. (D) Nitrite concentration in the medium of samples in panel C; error bars denote s.d. Scale bars: 10 μm.

resulted in strong nuclear accumulation of the NES reporter within 24 hours, indicating a block in CRM-mediated export of the reporter. Co-treatment with *S*-methylisothiourea (SMT), a NOS inhibitor (Qu et al., 2007b), prevented NO production and correspondingly abrogated the ability of LPS to inhibit nuclear export (Fig. 1C; supplementary material Fig. S1C). As control, phorbol 12-myristate 13-acetate (PMA), an inducer of reactive oxygen species through activation of NADPH oxidase (Johann et al., 2006) exerted no such effect (Fig. 1C). A similar repression of nuclear export by endogenously produced NO was observed with another CRM1-dependent reporter, MokA(NES2)-GFP (supplementary material Fig. S1D). Together, these data support a role of nitrosative stress (mediated by NO), but not of oxidative stress (mediated by H_2O_2), in repression of classical nuclear export.

Nitric oxide represses CRM1-NES interaction

Subsequently, we investigated the molecular basis for repression of CRM1-dependent classical nuclear export. Immunoblot analysis (supplementary material Fig. S2A) showed that the protein levels of CRM1, Ran, RanGAP1, Nup214, and Nup88 did not diminish after GSNO treatment. Moreover, GSNO treatment did not change the nuclear envelope localization of Nup88, RanGAP1 and CRM1, and did not change nuclear morphology (supplementary material Fig. S2B-D). To determine whether NO interferes with interaction of CRM1 with a classical NES, a glutathione-*S*-transferase (GST)-pulldown assay was employed. This involved in vitro incubation of recombinant GST-NES with CRM1 and RanGTP, in the absence or presence of GSNO. Whereas a specific interaction was observed

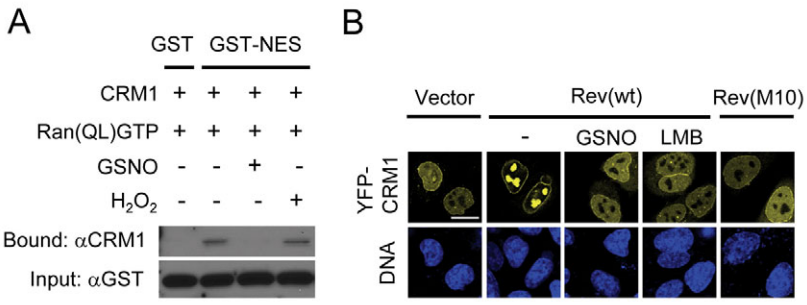


Fig. 2. NO represses the interaction of CRM1 with a classical NES. (A) Glutathione Sepharose-bound GST-NES or GST was incubated for 1 hour with purified CRM1 and RanQ69L-GTP. The beads then were washed and incubated with 100 μM GSNO or 100 μM H_2O_2 for 1 hour. Afterwards, the bound proteins were analyzed by immunoblotting with CRM1 antibody. The input GST-NES was detected with an anti-GST antibody. (B) HeLa cells were transfected with an expression plasmid for YFP-CRM1 together with either an empty vector or with a vector encoding Rev or Rev(M10). Transfected cells were treated with 1 mM GSNO or 5 ng/ml LMB for 4 hours, and then the YFP fluorescence was detected. Scale bar: 10 μm.

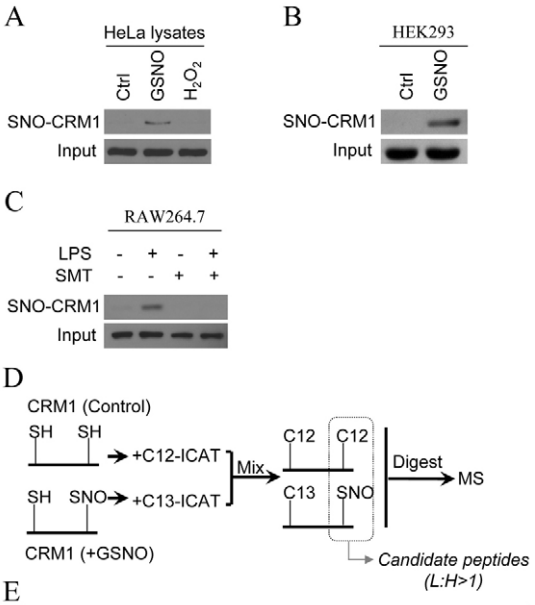
between CRM1 and GST-NES (Fig. 2A), this interaction was abolished by adding 100 μM GSNO to the binding assay. By contrast, addition of 100 μM H_2O_2 showed no inhibition.

We also evaluated the effect of NO on the CRM1-NES association in vivo. In HeLa cells, yellow fluorescent protein (YFP)-CRM1 was localized at the nuclear rim as well as within the nucleus. When Rev was co-expressed, a significant fraction of CRM1 was found in the Rev-containing nucleoli (Fig. 2B), as observed previously (Daelemans et al., 2002). This relocalization was dependent on the interaction between the Rev-NES and CRM1, because expression of the NES-deficient Rev M10 mutant or incubation with LMB completely abolished the Rev-mediated concentration of CRM1 in the nucleolus. After GSNO treatment, the Rev-dependent CRM1 nucleolar localization was also abolished (Fig. 2B). Furthermore, in vivo co-immunoprecipitation experiments confirmed that GSNO was able to inhibit the interaction between NES-containing protein and endogenous CRM1 (supplementary material Fig. S3). Taken together, both in vitro and in vivo data indicated that NO was able to abrogate the classical NES-CRM1 association. This effect could explain the NO-induced repression of classical nuclear export.

S-nitrosylation of CRM1 regulates the CRM1-NES interaction

NO has been reported to regulate protein-protein interactions via S-nitrosylation of targets (Hara et al., 2005; Qu et al., 2007b; Palmer et al., 2008). The finding that NO impaired the CRM1-NES interaction suggested that CRM1 could be a direct target for post-translational modification by NO. To investigate whether CRM1 can be S-nitrosylated, HeLa lysates were treated with 100 μM GSNO or 100 μM H_2O_2, and then subjected to the biotin-switch analysis, which exchanges the relatively unstable cysteine-linked NO with a stable biotin adduct (Jaffrey and Snyder, 2001; Hess et al., 2005). As shown in Fig. 3A, CRM1 in cell lysates was S-nitrosylated when exposed to GSNO. Furthermore, CRM1 was S-nitrosylated when HEK293 cells were treated with GSNO in vivo (Fig. 3B). To know whether intracellular CRM1 could be S-nitrosylated by endogenously produced NO, the endotoxin-stimulated RAW264.7 cells were employed. As shown in Fig. 3C, endogenous CRM1 became S-nitrosylated after LPS treatment, which was prevented by co-treatment with SMT. Interestingly, CRM1 appeared to be more readily modified by S-nitrosylation than did glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (supplementary material Fig. S4A). Together, these results established CRM1 as a direct target for S-nitrosylation by NO, suggesting that it could be a novel sensor for NO stress.

Because CRM1 has multiple cysteines, we implemented a novel mass spectrometry approach for identifying its S-nitrosylated sites. As shown in Fig. 3D, separate samples of recombinant CRM1 protein that were either untreated or treated with GSNO were labeled with [^{12}C]- or [^{13}C]cysteine-reactive isotope-coded affinity tag (ICAT) reagents, respectively, and then the mixture of the two samples was subjected to analysis using mass spectrometry. For the GSNO-treated CRM1, free cysteines that are S-nitrosylated cannot be labeled by heavy [^{13}C] ICAT reagent, whereas the same residues in the untreated sample are labeled with the light [^{12}C] ICAT reagent. This would lead to an increased signal ratio of light:heavy label for cysteines that are partially modified by GSNO. Using this approach, we identified seven S-nitrosylation sites on CRM1 (Fig. 3E). Four



E

Peptides	Cys	L:H
R.FLVTVIKDLLGL**C**EQK.R	528*	5.14
R.TSESL**C**QNNMVILK.L	164*	4.05
K.LFEFMHETHDGVQDMA**C**DTFIK.I	585*	3.3
K.LDINLLDNVVN**C**LYHGEGAQQR.M	34	2.94
F.QL**C**QFVM*ENSQNAPLVHATLETLLR.F	209*	2.67
R.LAYSNGKDDEQNFIQNLSLFL**C**TFLK.E	327	2.4
K.**C**LSENISAAIQANGEMVTK.Q	723	2.39

Fig. 3. S-nitrosylation of CRM1 in vivo and in vitro. (A) Samples of HeLa cell lysate were incubated with either 100 μM GSNO or H_2O_2 for 1 hour. Then, the S-nitrosylated CRM1 in the lysates was detected by the biotin-switch assay followed by immunoblotting with CRM1 antibody. HEK293 (B) or RAW264.7 cells (C) were treated with 1 mM GSNO for 4 hours or 100 ng/ml LPS (and/or 100 μM SMT) for 24 hours, respectively. Then the cell lysates were subjected to the biotin switch assay. (D) Identification of S-nitrosylation sites in CRM1 by ICAT-MS. Candidate peptides are those with light (L):heavy (H) ratios >1. (E) Identified sites of S-nitrosylation. Asterisks denote conserved cysteines.

of these cysteines are highly conserved during evolution: C164, C209, C528 and C585.

Mutation of cysteine to tryptophan has recently been shown to mimic the S-nitrosylation modification (Palmer et al., 2008). To investigate a potential function of S-nitrosylation at each of the four evolutionarily conserved cysteines of CRM1, we generated single-site cysteine-to-tryptophan mutants at each site (Fig. 4A). The S-nitrosylation mimetics at C528 and C585, but not at C164 and C209, completely abrogated the CRM1-NES interaction, as evidenced by co-immunoprecipitation experiments and by the lack of nucleolar localization of CRM1 in Rev-expressing HeLa cells (Fig. 4B,C; supplementary material Fig. S5A). Examination of the primary sequence of CRM1 revealed that both C528 and C585 are followed by a conserved acidic amino acid (aspartic acid or glutamic acid) (supplementary material Fig. S4B,C). The 3D structure shows that both C528 and C585 are surrounded by acid/base S-nitrosylation motifs (Fig. 5) (Hess et al., 2005). Importantly, C528 and C585 are the only two cysteine residues localized in the NES-binding region of CRM1 (Ossareh-Nazari and Dargemont, 1999; Dong et al., 2009) (Fig. 4A), and C528 is located in the NES binding groove of CRM1 (Dong et al., 2009) (Fig. 5). Thus, covalent addition of an NO group to the sulfydryl of C528 is predicted to block access to the NES binding site as part of the NO group occupies the groove. In fact, LMB covalently modifies C528 to stably inactivate CRM1 (Kudo et al., 1999). It should be noted that C585 is a newly identified cysteine that is sensitive to NO, but not to LMB (supplementary material Fig. S5B). We speculate that S-nitrosylation of CRM1 at C585 might alter the local conformation of the NES-binding region or provide a steric constraint, and therefore inhibit the CRM1-NES interaction. Together, these observations indicate that S-nitrosylation at C528 or C585 is critical for inhibiting the interaction between CRM1 and the classical NES.

To explore whether S-nitrosylation of CRM1 at C528 and C585 is sufficient for repression of nuclear export by GSNO, we generated the C528S/C585S double mutant of CRM1 (here abbreviated as 2CS). Cysteine-serine substitutions are commonly used to create non-S-nitrosylatable mutants (Palmer et al., 2008). We observed that overexpression of CRM1(2CS) completely rescued the GSNO-induced repression of nuclear export, using Rev1.4+NES-GFP as a reporter (Fig. 4D). By contrast, the NES-binding-deficient CRM1(C585W) or CRM1 (C528W) (Fig. 4B,C) were unable to rescue nuclear export of the reporter (Fig. 4D). Interestingly, despite having a weaker effect than CRM1(2CS), wild-type CRM1 that was overexpressed also showed partial rescue activity (data not shown). This is consistent with the notion that a fraction of CRM1 molecules remain active (non-S-nitrosylated at the crucial cysteines) when cells are exposed to GSNO. When CRM1 is present at a sufficiently high concentration in cells (i.e., with overexpression), the non-modified protein is sufficient to complement the inactivated endogenous CRM1 to yield a steady-state cytoplasmic localization. Therefore, we only used CRM1(2CS), but not CRM1(wild-type), for the following rescue experiments. Collectively, these experiments suggested that S-nitrosylation of CRM1 at C528 or C585 is sufficient for repression of nuclear export by CRM1.

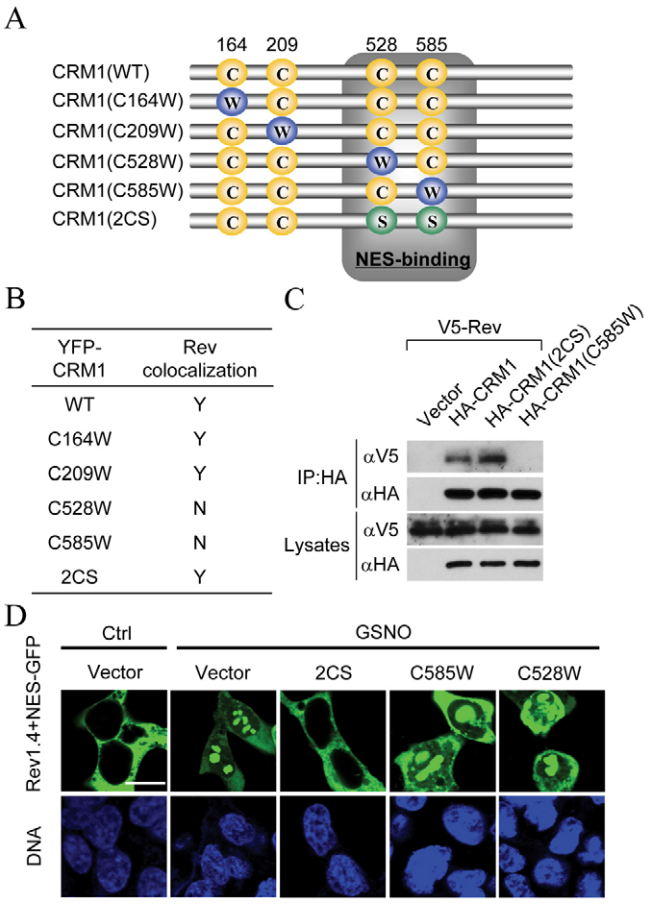


Fig. 4. S-nitrosylation of CRM1 at C528 or C585 represses its activity. (A) Schematic representation of CRM1 and CRM1 derivatives containing cysteine-to-tryptophan or cysteine-to-serine mutations. (B) HeLa cells were transfected with Rev together with the indicated YFP-fused CRM1 mutants, and then the YFP fluorescence was observed as Fig. 2B. (C) HeLa cells were transfected as indicated and 48 hours later the cell lysates were subjected to immunoprecipitation with HA antibody, followed by immunoblotting with V5 or HA antibodies. (D) HEK293 cells were transfected with plasmids expressing Rev1.4+NES-GFP and either CRM1(C528S/C585S), CRM1(C585W) or an empty vector, and then were incubated with or without GSNO for 4 hours. The GFP fluorescence was observed. Scale bar: 10 μm.

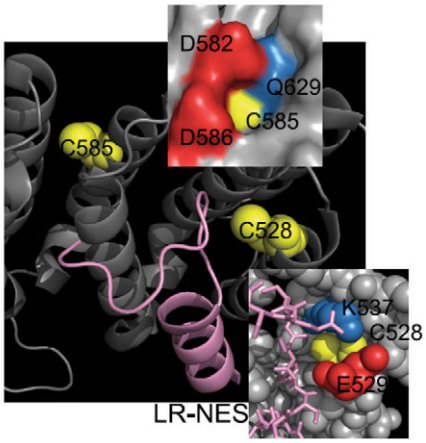


Fig. 5. Structure of CRM1-NES complex showing putative acid/base S-nitrosylation motifs around C528 and C585. Grey, CRM1; pink, leucine-rich nuclear export signal (LR-NES); yellow, cysteines; blue, basic amino acids; red, acidic amino acids. Images were created and rendered by PyMol.

S-nitrosylation of CRM1 facilitates Nrf2 signaling

We next explored whether S-nitrosylation of CRM1 arising from exposure of cells to nitrosative stress functions as part of an antioxidant protective response. We focused our analysis on NF-E2-related factor-2 (Nrf2), a key transcription factor that activates the expression of cytoprotectant genes by binding to an antioxidant response element (ARE) in their promoter regions (Dhakshinamoorthy and Porter, 2004; Motohashi and Yamamoto, 2004; Li et al., 2007; Liu et al., 2008). Previous work revealed that Nrf2 is a nucleocytoplasmic shuttling protein, and that Nrf2 localization can be regulated by the activity of CRM1 (Li et al., 2005). For our analysis, HEK293 cells expressing HA-Nrf2 were treated with LMB or GSNO, and the subcellular distribution of Nrf2 was determined by immunofluorescence. Consistent with the ability of these treatments to inhibit classical nuclear export, both LMB and GSNO resulted in relocalization of HA-Nrf2 from the cytoplasm to the nucleus (Fig. 6A,C). Moreover, overexpression of CRM1(2CS), but not CRM1(C585W), completely reversed GSNO-induced nuclear accumulation of Nrf2 (Fig. 6B,C).

To extend these observations, we analyzed the localization of endogenous Nrf2 in response to GSNO treatment, using SH-SY5Y neuroblastoma cells that were stably transfected with either CRM1(2CS), CRM1(C585W) or an empty vector. As shown in Fig. 6D, GSNO treatment significantly increased the nuclear level of Nrf2, in agreement with a study involving the use of two other NO donors (Dhakshinamoorthy and Porter, 2004). As predicted, the nuclear accumulation of Nrf2 was inhibited in cells overexpressing CRM1(2CS). This was accompanied by diminished activation of ARE-driven reporter (Fig. 6E), as well as diminished expression of Nrf2-target genes, including heme oxygenase-1 (HO-1) (Fig. 6D,F) and NAD(P)H:quinone oxidoreductase (NQO1) (Fig. 6F). By contrast, overexpression of CRM1(C585W) had no significant effect on the activation of Nrf2 targets (Fig. 6D,E,F). Because S-nitrosylation of CRM1 increased the expression of Nrf2-regulated genes implicated in combating nitrosative stress (Dhakshinamoorthy and Porter, 2004), we examined whether this effect could diminish NO-induced neural cell death. As shown in Fig. 6G, cells overexpressing CRM1(2CS), but not CRM1(C585W), showed decreased cell viability in the presence of increasing concentrations of GSNO. Although it is not clear why overexpression of CRM1(2CS) did not show statistically significant effect on cell survival when cells were treated with 1 mM GSNO, it is possible that other nuclear export pathways [e.g. nuclear export of p53 (Kanai et al., 2007) or GAPDH (Brown et al., 2004)] facilitated by overexpressed CRM1(2CS) also contribute to the regulation of cell survival under relatively mild NO stress (see below for more discussion on the characteristics of CRM1 regulation). Together, the results of our study indicate that inactivation of CRM1 via S-nitrosylation facilitates the Nrf2-ARE pathway and thereby helps to counteract nitrosative-stress-induced neural cell death.

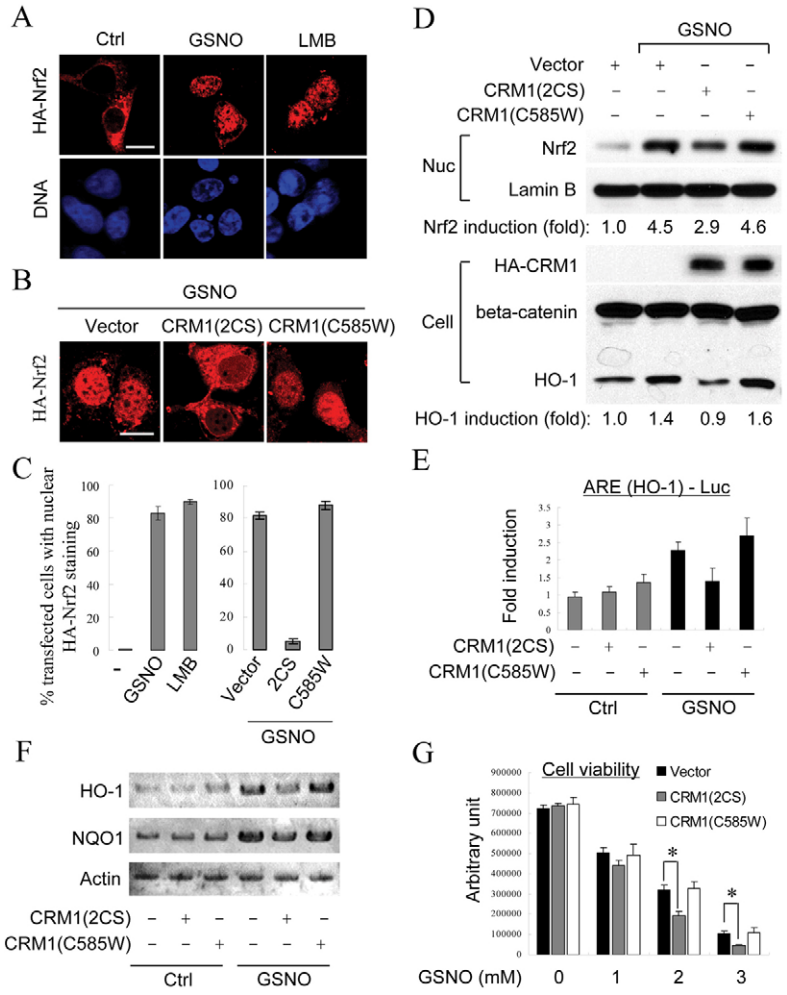


Fig. 6. Regulation of Nrf2-ARE signaling by S-nitrosylation of CRM1. (A,B) HEK293 cells were transfected with expression plasmids for HA-Nrf2 and CRM1(2CS), CRM1(C585W) or an empty vector as indicated, and then incubated with medium containing GSNO, LMB or no addition for 4 hours. The subcellular localization of HA-Nrf2 was determined by immunofluorescence staining with HA antibody. Scale bars: 10 μm. (C) The percentages of transfected cells with nuclear HA-Nrf2 staining in A and B. At least 100 cells were counted. Error bars denote s.d. (D) SH-SY5Y cells that were stably transfected with vectors encoding HA-CRM1(2CS), HA-CRM1(C585W), or with empty vector were treated with 1.5 mM GSNO for 7 hours. Then, the Nrf2 and lamin B1 in the nuclear fraction (nuc) were detected by immunublotting. In parallel samples, the expression of HO-1, β-catenin, and HA-CRM1 in unfractionated cells were detected by immunoblotting 10 hours after GSNO treatment. Fold induction of nuclear Nrf2 and cellular HO-1 normalized with loading control was quantified. (E) SH-SY5Y cells described in D were transfected with ARE(HO-1)-Luc and β-galactosidase. 24 hours after transfection, cells were treated with 500 μM GSNO for 8 hours, and the β-galactosidase-normalized luciferase activity was determined. Error bars denote s.d. (F) Stably transfected SH-SY5Y cells were treated with 1 mM GSNO for 5 hours. Total RNA was extracted from the cells after GSNO treatment. RT-PCR was performed using 2 μg of total RNA for the HO-1 and NQO1 genes. β-actin is shown as a control. (G) SH-SY5Y cells described in D were treated with the indicated concentrations of GSNO for 24 hours and the cell viability was determined. Error bars denote s.d.

Discussion

Here, we provide evidence that the S-nitrosylation of CRM1 negatively regulates classical nuclear export under nitrosative stress. First, exogenous and endogenous NO represses CRM1-dependent nuclear export of various NES-containing reporters and known CRM1 cargos, such as IκB (our unpublished data) and Nrf2. Second, NO stress abrogates CRM1-NES interaction both in vivo and in vitro. Third, CRM1 undergoes S-nitrosylation under nitrosative stress, and S-nitrosylation of CRM1 at C528 or C585 (located at the NES-binding domain) abolishes NES-CRM1 interactions. Lastly, S-nitrosylation-resistant CRM1 mutant is able to rescue nuclear export under nitrosative stress.

The NO regulation of classical nuclear export bears the following characteristics: First, consistent with an S-nitrosylation-dependent mechanism, the CRM1-dependent nuclear export is selectively responsive to NO stress but not to oxidative stress. The latter and other stresses such as heat shock and UV radiation have been known to influence classical nuclear import in a nonspecific manner (Miyamoto et al., 2004; Kodiha et al., 2004). Second, we observed a substantial nuclear export repression during prolonged exposure of cells to millimolar GSNO, or in 24-hour-endotoxin-treated macrophages, when endogenous NO was continuously and strongly induced. Furthermore, we also noticed that the NO-caused repression of nuclear export was reversible (the nuclear export was recovered after GSNO removal; data not shown), although the rate of CRM1 denitrosylation remains to be further determined as this event would equally influence CRM1 activity. Thus, these observations suggest that a sustained local concentration of NO or SNO around CRM1 might be required to repress CRM1-dependent nuclear export. Because biological regulatory systems are typically tolerant of very small changes in protein concentrations, we speculate that only S-nitrosylation of a specific fraction of the CRM1 pool would lead to a discernible repression of nuclear export. The recent reports that iNOS is localized in perinuclear aggresome-like structures as well as in the nucleus (Giordano et al., 2002; Kolodziejska et al., 2005; Saini et al., 2006; Jones et al., 2007) imply a possibility that CRM1 could be efficiently S-nitrosylated, at least when iNOS is induced.

Third, the NO regulation of classical nuclear export is likely to be a complicated and multilayered regulation for the following reasons: (1) The fact that only a fraction of CRM1 is S-nitrosylated might be important in preventing the pleiotropic effects of inactivating the entire general export pathway, although we cannot exclude the possibility that the fraction of S-nitrosylated CRM1 could exert a dominant negative effect in vivo, as sumoylation usually does. (2) Nucleocytoplasmic localization of endogenous proteins is balanced by several factors (such as nuclear import-export and nuclear-cytoplasmic retention). In most cases, modification of CRM1 by NO might only alter localization of a subset of its cargoes. (3) The relative number and strength of CRM1-recognized NES varies from cargo to cargo, which might result in differential responsiveness of cargos to NO stress. (4) NO could exert an additional, direct effect on some CRM1 cargos or their binding partners (Qu et al., 2007a). Thus, although CRM1 is an essential factor that exports hundreds of different cargoes out of the nucleus, only a few are expected to be 'selectively' involved in a response to NO stress.

Accumulation of SNO can produce a stress on the mammalian organism that influences survival; in particular, the nitrosative stress that is identified with GSNO is implicated in disease (Foster et al., 2003; Liu et al., 2004; Hess et al., 2005). Our study links cell survival against increased reactive nitrogen species (RNS) to their ability to activate defence mechanisms that aim to increase antioxidant potential. We demonstrate that S-nitrosylation of CRM1 facilitates nuclear accumulation of Nrf2, and subsequent activation of Nrf2-target genes under NO stresses.

During preparation of this manuscript, an elegant experiment was performed by Ashino and co-workers to show that NO induction is required for endotoxin-induced activation of Nrf2-target genes in macropages. Their results showed that the activation was abrogated in macrophages isolated from *iNOS*$^{-/-}$ animals (Ashino et al., 2008). In this regard, our observations on S-nitrosylation of CRM1 and the repression of classical nuclear export in LPS-challenged RAW264.7 macrophages could, at least in part, explain the induction of the Nrf2-ARE pathway under endotoxic shock. We would like to point out that, in addition to blockage of CRM1-mediated nuclear export of Nrf2, NO might also target other Nrf2-regulatory protein(s)/or pathway(s). For example, the Nrf2 inhibitory protein Keap1 serves as a cytosolic redox sensor for various electrophile agents (Itoh et al., 2004), and it needs to be determined whether Keap1 (or Nrf2 itself) could be modified and functionally regulated by S-nitrosylation. In addition, activation of the MAPK pathway has recently been involved in NO-induced Nrf2 activation in the vascular endothelium (Buckley et al., 2003). Thus, activation of the Nrf2-mediated antioxidant defensive response under NO stress is likely to be a complicated and multilayered response; however, it forms an important cell defence mechanism against increased RNS.

Interestingly, organisms do not appear to employ this mechanism against 'pure' oxidative stress, as evidenced by both our result that the classical nuclear export remains functional under oxidative stress, and a by recent report that the classical NES of Nrf2 is insensitive to oxidative stress (Li et al., 2005). It should be pointed out that S-nitrosylation is also involved in other NO-related cell survival mechanisms. For instance, caspases employ S-nitrosylation to mediate cell survival (Mannick et al., 1999).

In summary, this study complements previous knowledge on the biological effects of NO, as well as on the function of S-nitrosylation in human health and disease. Whereas this study was analyzed with conditions that emulate nitrosative stress, it is plausible that more subtle and transient spatiotemporal regulation of CRM1 by NO occurs in other cellular contexts [e.g. in NMDA-treated or β-amyloid-treated primary neurons (Hara et al., 2005; Uehara et al., 2006; Takahashi et al., 2007; Tian et al., 2008; Cho et al., 2009; Sen et al., 2009)]. Addressing the spatiotemporal regulation of CRM1 by NO, identifying other substrates of CRM1 that might be involved in NO regulation, and revealing the functional implication of CRM1 S-nitrosylation in pathophysiologically relevant animal and/or primary cell models provide important challenges for future research. Because NO is connected to multiple pathological processes such as endotoxic shock and neurodegenerative diseases, the present findings will improve our understanding of how nitrosative stress is relevant to these processes at the nuclear transport level, and have the potential to yield therapeutic strategies to fight such neurodegenerative diseases.

Materials and Methods

Reagents, plasmids and antibodies

Leptomycin B (LMB), lipopolysaccharides (LPS; from *E. coli* serotype 0111:B4), and *S*-methylisothiourea (SMT) were purchased from Sigma. Methyl methanethionsulfonate (MMTS) and *N*-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio) propionamide (Biotin-HPDP) were purchased from Pierce. Hoechst 33342 was purchased from Molecular Probes. Other reagents have previously been described (Qu et al., 2007a). The following plasmids were described in previous investigations:

Rev(wt) and Rev(M10) (Edgcomb et al., 2008); Rev1.4+NES(Rev)-GFP (Henderson and Eleftheriou, 2000); MoKA(NES2)-GFP and MoKA(NES3)-GFP (Smaldone and Ramirez, 2006); HA-CRM1 (Knauer et al., 2005); HA-Nrf2 and ARE(HO-1)-Luc (Liu et al., 2008). V5-Rev was cloned with pcDNA Directional TOPO Expression Kit (Invitrogen). Various CRM1 mutants were generated using the QuikChange site-directed mutagenesis kit (Stratagene) and confirmed by sequencing. Antibodies to the following antigens used in this study include: Rev (sc-69729), Actin (sc-1616), GFP (sc-9996, sc-8334), Ran (sc-1156), RanGAP1(sc-28322), HA (sc-7392), GAPDH (sc-32233) and Nrf2 (sc-13032) (Santa Cruz Biotechnology); CRM1 (E11620-150) and NUP88 (611896) (BD Pharmingin); V5 (Invitrogen); V5 (AB3792) (Millipore); NUP214 (A300-717A-1) (Bethyl Laboratories, Montgomery, TX); HO-1 (SPA-895) (StressGen, Ann Arbor, MI); anti-HA agarose (Sigma); anti-GFP agarose (MBL International, Woburn, MA).

Cell transfection and immunoblotting

HeLa, HEK293, RAW264.7 and SH-SY5Y cells (ATCC) were transiently transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The SH-SY5Y cell populations stably expressing CRM1 mutants or empty vector (pcDNA) were enriched by selection by 500 μg/ml G418 for 3 weeks. Cell fractionation, and immunoblotting were performed as previously described (Liu et al., 2008).

GST pull-down assay

Glutathione Sepharose-bound GST-NES or GST was incubated for 1 hout with 1 μmol CRM1 and 1.25 μmol RanQ69L-GTP. After washing twice with buffer B (20 mM HEPES pH 7.3, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 70 mM NaCl, 1 mM EGTA, and 0.5% NP40), the beads were treated with GSNO or H_2O_2 for 1 hour at 4°C in the dark. The bound proteins were eluted by boiling with SDS loading buffer, and then subjected to immunoblot analysis.

Co-immunoprecipitation

The CRM1-cargo association was determined by co-immunoprecipitation as previously described (Tajima et al., 2003; Brown et al., 2004; Du et al., 2008), with minor modification. In brief, agarose-conjugated antibodies were incubated with cell lysates in ice-cold lysis buffer (50 mM Tris-HCl pH 7.5, 250 mM NaCl, 0.5% Triton X-100, 10% glycerol, and protease inhibitor cocktail) overnight at 4°C with constant rotation. Because we observed that the CRM1-cargo association was not discernibly improved when RanQ69L-GTP was included in the lysis buffer (to stabilize the CRM1 trimeric complex), we only used the lysis buffer without RanQ69L-GTP. The beads were extensively washed with lysis buffer, and the immunoprecipitates analyzed by immunoblotting with the relevant antibodies. The immunoprecipitations were performed strictly in the dark to prevent the loss of S-nitrosylation.

Biotin-switch and Griess assay

For biotin-switch assay, cell lysates were prepared in lysis buffers (50 mM Tris pH 7.5, 150 mM NaCl, 0.5% deoxycholic acid, 0.1% SDS, 1% NP-40, 0.1 mM neocuproine, and protease cocktail). The supernatant of cell lysis was assayed for protein concentration by the bicinchoninic acid (BCA) method, and then adjusted to the appropriate concentration. Blocking buffer (2.5% SDS, 20 mM MMTS) was mixed with the samples and incubated for 30 minutes at 50°C to block free thiol groups. After removing excess MMTS by acetone precipitation, nitrosothiols were then reduced to thiols and biotinylated using reducing buffer (1% SDS, 10 mM ascorbate, 4 mM biotin-HPDP). Then, the biotinylated proteins were pulled-down by streptavidin-agarose beads, eluted with SDS sample buffer, and subjected to immunoblot analysis. The Griess assay to measure nitrite in the medium was performed as previously described (Hu et al., 1996).

Mass spectrometry determination of GSNO-modified cysteines

Purified CRM1 (100 μg) was treated with or without 1 mM GNSO for 30 minutes in the dark at room temperature. The excess GSNO was removed by centrifugation in an Amicon Ultra-15. Then, twice the sample volume of 8 M urea was added. The control and GSNO-treated samples were respectively labeled by light [^{12}C] and heavy [^{13}C] ICAT reagents (Applied Biosystems) at 37°C for 2 hours. After removing excess labeling reagent, the combined samples were subjected to enzymatic digestion. The digested peptides were purified according to the ICAT kit instructions and analyzed by nano-LC ESI MS/MS on a thermo LTQ linear trap instrument (Thermo Finnigan). MS/MS spectra were extracted from the raw files by using SEQUEST with the following parameters: molecular weight range, 600-3500; threshold, 1000; precursor mass tolerance, 3; group scan tolerance, 1; minimum group count, 1; minimum number of ions, 25; precursor charge state, auto; MSn level, 2. SEQUEST output files were created by using the following parameters: peptide mass tolerance, 3; fragment ion tolerance, 1. MS/MS spectra were searched against a database downloaded from the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/; human) using SEQUEST in Bioworks 3.2 (Thermo Finnigan). The SEQUEST database search criteria included a static modification of cysteine residues of 227 Da and a variable modification of 9 Da for cysteines. Peptides were permitted to have up to two missed cleavages. XC>2 for doubly charged and >2.5 for triply charged ions; DCn>0.1; RS*P*<5; and preliminary score (Sp)>500. Bioworks 3.2 was used to identify and quantify the modified cysteines. The signal ratios (L:H) of light [^{12}C]/heavy [^{13}C] is proportional to GSNO-mediated modification on cysteines (S-nitrosylation or S-nitrosylation-derived higher oxidation forms). Seven cysteines with a signal ratio over 2.0 were regarded as highly reliable sites for S-nitrosylation. The detailed MS data with all identified peptides will be sent by request.

Immunofluorescence

Indirect immunofluorescence analysis was performed with various primary antibodies and with Alexa Fluor488 or Alexa Fluor594 anti-IgG antibody (Invitrogen), as previously described (Qu et al., 2007a). The cell nuclei were stained with Hoechst 33342. Pictures of cells were taken with a laser-scanning confocal microscope (Olympus FV500, Tokyo, Japan).

Luciferase assay

The β-galactosidase-normalized luciferase activities were assayed as previously described (Liu et al., 2006). Each transfection was performed with 300 ng of ARE-Luc construct and 50 ng of β-galactosidase plasmid.

Semi-quantitative RT-PCR

Total RNA was isolated from SH-SY5Y cells with Trizol reagent (Invitrogen). Semi-quantitative RT-PCR analysis of HO-1 and NQO1 expression was performed as previously described (Dhakshinamoorthy and Porter, 2004). The PCR products were analyzed on a 1% agarose gel with ethidium bromide.

Cell viability analysis

SH-SY5Y cells cultured at 80% confluence were treated with GSNO. The number of viable cells was determined by CellTiter-Glo Luminescent Cell Viability Assay (Promega) according to the manufacturer's protocol.

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