THE JOURNAL OF IMMUNOLOGY

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J Immunol 2006; 177:7889-7897; ;
doi: 10.4049/jimmunol.177.11.7889
http://www.jimmunol.org/content/177/11/7889

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Nucleocytoplasmic Shuttling of HMGB1 Is Regulated by Phosphorylation That Redirects It toward Secretion[1]

Ju Ho Youn* and Jeon-Soo Shin[2]*†‡

The high mobility group box 1 (HMGB1) protein can be secreted by activated monocytes and macrophages and functions as a late mediator of sepsis. HMGB1 contains two nuclear localization signals (NLSs) for controlled nuclear transport, and acetylation of both NLSs of HMGB1 is involved in nuclear transport toward secretion. However, phosphorylation of HMGB1 and its relation to nuclear transport have not been shown. We show here that HMGB1 is phosphorylated and dynamically shuttled between cytoplasmic and nuclear compartments according to its phosphorylation state. Phosphorylation of HMGB1 was detected by metabolic labeling and Western blot analysis after treatments with TNF-α and okadaic acid, a phosphatase inhibitor. Hyperphosphorylated HMGB1 in RAW 264.7 and human monocytes was relocated to the cytoplasm. In a nuclear import assay, phosphorylated HMGB1 in the cytoplasm did not enter the nucleus. We mutated serine residues of either or both NLSs of HMGB1 to glutamic acid to simulate a phosphorylated state and examined the binding of HMGB1 to karyopherin-α1, which was identified as the nuclear import protein for HMGB1 in this study. Substitution to glutamic acid in either NLSs decreased the binding with karyopherin-α1 by $\sim$ 50%; however, substitution of both NLSs showed no binding, and HMGB1 was relocated to the cytoplasm and subsequently secreted. These data support the hypothesis that HMGB1 could be phosphorylated and that the direction of transport is regulated by phosphorylation of both NLS regions. ***The Journal of Immunology,*** **2006, 177: 7889–7897.**

High mobility group box 1 (HMGB1)[3] protein, a highly conserved ubiquitous protein, was first purified almost 30 years ago as a nuclear protein (1). HMGB1 is involved in nucleosome stabilization and gene transcription (2) and can also localize to the cell membrane of neurites for outgrowth (3) and to the cell membranes of tumor cells for metastasis (4). HMGB1 is passively released by necrotic cells, though not by apoptotic cells, and triggers inflammation (5). HMGB1 also functions as a late mediator of endotoxemia, sepsis, and hemorrhagic shock in animals and human patients (6–8). Specific inhibition of endogenous HMGB1 with HMGB1 antagonists could reverse the lethality of established sepsis (9). HMGB1 is released from activated monocytes and macrophages (6, 10) and NK cells (11) and behaves as a proinflammatory cytokine. Exposure to HMGB1 leads to various cellular responses, including the chemotactic cell movement of smooth muscle cells and monocytes (12, 13) and the release of proinflammatory cytokines such as TNF-α, IL-1, IL-6, and IL-8 (14). Furthermore, when NK cells are in close physical contact with immature dendritic cells (DCs), the immature DCs produce IL-18 that causes NK cells to produce HMGB1. HMGB1, in turn, causes DC maturation and Th1 polarization, events that initiate the adaptive immune responses (11, 15).

HMGB1 contains two homologous DNA-binding motifs (HMG boxes A and B) and an acidic tail (16). It also contains two nuclear localization signals (NLSs) and two putative nuclear export signals (10), demonstrating that HMGB1 shuttles between the nucleus and the cytoplasm through a tightly controlled mechanism. In activated monocytes HMGB1 is hyperacetylated and relocated from the nucleus to the cytoplasm for exocytosis, and this is mediated by the nuclear exportin, chromosome region maintenance 1 (CRM1) (10, 17). No evidence of phosphorylation, methylation, or glycosylation has previously been found in HMGB1 from the calf thymus, mouse thymus, or activated human monocytes (10). Phosphorylation of several plant HMG family proteins has been seen and reported to modulate the stability and DNA binding of these proteins (18, 19). Moreover, phosphorylation of NLSs in many proteins influences their binding to nuclear import proteins and consequently increases (20) or decreases (21) their nuclear accumulation, suggesting that phosphorylation of HMGB1 also may affect its nuclear transport. Considering that positively charged residues are abundant in NLSs and are necessary for binding to nuclear importin proteins such as the karyopherins (KAPs) (22), the change in the charge of HMGB1 NLSs that results from phosphorylation may disrupt the interaction of HMGB1 with the nuclear importin. However, the effect of phosphorylation of HMGB1 on its nuclear import has not been previously shown.

The present study shows that HMGB1 was phosphorylated in activated RAW 264.7 cells by TNF-α, a proinflammatory stimulus, and okadaic acid (OA), a phosphatase inhibitor. In addition, phosphorylation of HMGB1 at both NLSs was found to influence its nuclear import in a nuclear import assay and its immunoprecipitation with a nuclear cargo carrier protein, KAP-α1, which was found to bind HMGB1 in this study. Finally, HMGB1 showed reduced binding to KAP-α1 in a phosphorylation-dependent manner.

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Received for publication July 17, 2006. Accepted for publication September 26, 2006.

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[1] This work was supported by the Korea Science and Engineering Foundation through the National Core Research Center for Nanomedical Technology Grant R15-2004-024-00000-0, National Research and Development Program for Cancer Control Grant 0320250-2, and Korean Health 21 Research and Development Program Project 01-PJ10-PG6-01GM03-0002 of the Ministry of Health and Welfare, Yonsei University Biomolecular Secretion Research Center, and Brain Korea 21 Project for Medical Sciences, Republic of Korea.

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[3] Abbreviations used in this paper: HMGB1, high mobility group box 1 protein; CHX, cycloheximide; CRM1, chromosome region maintenance 1; DC, dendritic cell; EGFP, enhanced GFP; KAP, karyopherin; NLS, nuclear localization signal sequence; OA, okadaic acid; PBMo, peripheral blood monocyte; TB, transport buffer; TSA, trichostatin A; WCL, whole cell lysate.

0022-1767/06/$02.00

Materials and Methods

Cell culture

Murine macrophage RAW 264.7 cells (American Type Culture Collection) and HeLa cells were cultured at 37°C under 5% CO_2 in DMEM supplemented with 10% FBS (Invitrogen Life Technologies), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Human peripheral blood monocytes (PBMo) were harvested from the adhesive cells on the culture flask by Ficoll-Hypaque gradient centrifugation after yielding PBMC. Human recombinant TNF-α (R&D Systems), OA (Calbiochem), trichostatin A (TSA; Sigma-Aldrich), and cycloheximide (CHX; Sigma-Aldrich) were purchased.

Western blot analysis

To analyze the secretion of HMGB1 in the supernatants, culture media were replaced with serum-free OPTI-MEM (Invitrogen Life Technologies) medium and concentrated with Amicon Centricon filtration (Millipore) after removing cell debris, and Western blot analysis was performed. The cytoplasmic and nuclear fractions from 5×10^6 cells were separated using a digitonin-based method (23) to observe the levels of HMGB1 in each fraction. The cells were lysed using 1% Nonidet P-40 buffer containing a protease inhibitor mixture (Sigma-Aldrich), and the protein concentrations were measured by Bradford assay (Bio-Rad) for the analysis of whole cell lysates (WCLs). The protein samples underwent 12% SDS-PAGE and were transferred to a nitrocellulose membrane. Western blot analysis was performed using rabbit anti-HMGB1 (BD Pharmingen) and HRP-labeled goat anti-rabbit Ig as primary and secondary Abs, respectively. The signals were revealed with ECL (Pierce).

Immunoprecipitation

To identify the phosphorylated residues of the HMGB1 protein, TNF-α-treated RAW 264.7 cells were lysed with a protease inhibitor mixture. Cell homogenates were centrifuged at 20,000 × *g* for 15 min and precleared by incubation with protein G-Sepharose (Amersham Biosciences) at 4°C for 30 min. The precleared extracts (500 μg) were incubated with rabbit polyclonal anti-phosphoserine, anti-phosphotyrosine, and anti-phosphothreonine (all from Chemicon) and then protein G-Sepharose was added and incubated for 3 h at 4°C. Immune complexes were collected by centrifugation and washed with lysis buffer. Collected complexes were fractionated by SDS-PAGE, transferred to membranes, and blotted with anti-HMGB1 for detection. Anti-pAKT (Cell Signaling Technology) was used as a negative control. To investigate the time-dependent phosphorylation of HMGB1, the WCLs of RAW 264.7 cells treated with TNF-α for the indicated time were immunoprecipitated with anti-HMGB1 and subjected to Western blot analysis using anti-phosphoserine.

Immunofluorescence and GFP imaging

Cells were cultured in LabTek II chambers (Nalgene) and fixed in 3.7% paraformaldehyde in PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, and 4 mM $MgSO_4$ (pH 7.0)) for 10 min at room temperature. After fixation, the cells were washed with PBS and incubated for 3 min at 4°C with HEPES-based permeabilization buffer containing 300 mM sucrose and 0.2% Triton X-100. The cells were blocked with 0.2% BSA in PBS for 15 min and incubated with rabbit anti-HMGB1 for 1 h at room temperature. After three washes with blocking solution, secondary Ab FITC-conjugated goat anti-rabbit Ig (BD Pharmingen) was added. Cells expressing various HMGB1-enhanced GFP (EGFP) proteins were stained with 4′,6-diamidino-3-phenylindole and observed with a BX51 fluorescent microscope (Olympus). Cells expressing HMGB1-EGFP and its derivatives were fixed as described above.

Nuclear import assay in digitonin-permeabilized cells

Nuclear import assays were performed with minor modification as previously described (24). Briefly, the HeLa cell cytosol was first prepared. For this, HeLa cells at a density of 5×10^5 cells/ml were harvested and washed twice in ice-cold PBS and once in washing buffer (10 mM HEPES (pH 7.3), 110 mM KOAc, 2 mM $Mg(OAc)_2$, and 2 mM DTT). They were then homogenized with hypotonic lysis buffer (5 mM HEPES (pH 7.3), 10 mM KOAc, 2 mM $Mg(OAc)_2$, 2 mM DTT, 20 μM cytochalasin B, 1 mM PMSF, and 1 μg/ml leupeptin, pepstatin, and aprotinin, each). The supernatants were sequentially centrifuged at 1,500 × *g* for 15 min, 15,000 × *g* for 20 min, and 100,000 × *g* for 1 h, dialyzed against transport buffer (TB; 20 mM HEPES (pH 7.3), 110 mM KOAc, 2 mM $Mg(OAc)_2$, 5 mM NaOAc, 1 mM EGTA, 2 mM DTT, and 1 μg/ml leupeptin, pepstatin, and aprotinin, each), and frozen in aliquots in liquid nitrogen before storage at −80°C.

For the assays, HeLa cells were washed in TB and permeabilized for 5 min on ice in TB containing 40 μg/ml digitonin. The cells were rinsed for 5–10 min with several changes of TB, and the excess buffer was removed. The cells were incubated with transport mixture for 1 h at 22°C. The transport mixture contained HeLa cell cytosol at a final concentration of 2 mg/ml, which was preincubated for 30 min at room temperature with an ATP-regenerating system (1 mM ATP, 5 mM creatine phosphate, 20 U/ml creatine phosphokinase, and 0.5 mM GTP) either with or without 10 μM OA, and with 30 μg/ml each substrate. The cells were fixed with 3.7% formaldehyde for 10 min and immediately examined by fluorescent microscope.

DNA constructs and mutagenesis

The gene encoding human HMGB1 was cloned upstream of EGFP in pEGFP-N1 (BD Clontech), and the construct was named pHMGB1-EGFP-N1. For the recombinant HMGB1-EGFP protein, a *Sac*I/*Not*I fragment from pHMGB1-EGFP-N1 was subcloned into pET-28a (Novagen). His_6-tagged HMGB1-EGFP, GST-EGFP, and EGFP proteins were produced in *Escherichia coli* BL21(DE3) pLysE (Novagen). The cells transformed with each construct were grown in Luria-Bertani medium containing kanamycin (15 μg/ml) and chloramphenicol (34 μg/ml) to an OD_{600} of 0.4–0.5 at 37°C, cooled to 25°C, induced with 0.1 mM isopropyl-β-D–thiogalactopyranoside, and grown overnight at 25°C. The cells were lysed by sonication, and the clear lysate was loaded onto a Ni^{2+}-NTA column. The bound protein was washed with 50 mM NaH_2PO_4, 300 mM NaCl, and 20 mM imidazole (pH 8) and was eluted in the same buffer supplemented with 200 mM imidazole. All proteins were dialyzed into TB and stored at −80°C.

Site-directed mutations of HMGB1 were generated from pHMGB1-EGFP-N1 as a template using the QuikChange site-directed mutagenesis kit (Stratagene). Six serines were partially or completely mutated into alanine or glutamic acid. Each HMGB1-EGFP mutation was depicted in Fig. 5*A*.

Human KAPs were cloned into *Bam*HI/*Xho*I (KAP-α1, -α2, -α4, and -α6), *Eco*RI/*Xho*I (KAP-α3 and -α5), or *Bam*HI/*Not*I (KAP-β1) sites of pGEX-4T-1 (Pharmacia) to produce GST fusion proteins. KAP-α1 (GenPeptide Protein Database accession no. AAC60648), KAP-α2 (GenPeptide Protein Database accession no. AAA65700), KAP-α3 (GenPeptide Protein Database accession no. AAH17355), KAP-α4 (GenPeptide Protein Database accession no. AAC25605), KAP-α5 (GenPeptide Protein Database accession no. AAH47409), KAP-α6 (GenPeptide Protein Database accession no. AAC15233), and KAP-β1 (GenPeptide Protein Database accession no. AAH03572) were prepared from PCR amplifications of oligo(dT)-selected, HeLa cell-derived cDNA. The primers were as follows: KAP-α1, 5′-CGCGGATCCATGACCACCCCAGGAAAAGAGAAC-3′ (forward) and 5′-CCGCTCGAGAAGCTGGAAACCTTCCATAGGAGC-3′ (reverse); KAP-α2, 5′-CGCGGATCCATGTCCACCAACGAGAATGCTAATAC-3′ (forward) and 5′-CCGCTCGAGAAAGTTAAAGGTCCCAGGAGCCCCAT-3′ (reverse); KAP-α3, 5′-CCGGAATTCATGGCCGAGAACCCCAGCTTGGAG-3′ (forward) and 5′-CCGCTCGAGAAAATTAAATTCTTTTGTTTGAAGGTTGGC-3′ (reverse); KAP-α4, 5′-CGCGGATCCATGGCGGACAACGAGAAACTGGAC-3′ (forward) and 5′-CCGCTCGAGAAACTGGAACCCTTCTGTTGTACA-3′ (reverse); KAP-α5, 5′-CCGGAATTCATGGATGCCATGGCTAGTCCAGGG-3′ (forward) and 5′-CCGCTCGAGAAGTTGAAATCCATCCATTGGTGCTTC-3′ (reverse); KAP-α6, 5′-CGCGGATCCATGGAGACCATGGCGAGCCCAGGG-3′ (forward) and 5′-CCGCTCGAGTAGCTGGAAGCCCTCCATGGGGGCC-3′ (reverse); and KAP-β1, 5′-CGCGGATCCATGGAGCTGATCACCATTCTCGAGAAGACC-3′ (forward) and 5′-ATAAGAATGCGGCCGCAGCTTGGTTGTTGACTTTGGTCAGTTCTTTTG-3′ (reverse). The nucleotide sequences of restriction enzyme sites are underlined. The GST-KAP fusion proteins were produced in *E. coli* BL21. Cells were harvested and disrupted by sonication in lysis buffer with 1% Triton X-100, 10% glycerol, 1 mM EDTA, 1 mM DTT, and a protease inhibitor mix (1 μg/ml leupeptin, pepstatin, and aprotinin and 1 mM PMSF) (Sigma-Aldrich) in PBS. After centrifugation, the supernatants were incubated with glutathione-Sepharose at 4°C. Bound proteins were eluted by incubation at room temperature for 30 min with 10 mM reduced glutathione. SDS-PAGE analysis of each eluted GST-KAP protein revealed a major protein band with the predicted molecular size. For the transfection study, Flag-tagged KAP-α1 was cloned into pCMV-Tag2 (Stratagene). All constructs were confirmed by DNA sequencing (Applied Biosystems). FuGENE 6 (Roche) was used for the transfection study.

Interaction between KAP protein and HMGB1 mutant proteins

Two micrograms of each GST-KAP protein was coupled to glutathione-Sepharose 4B beads and incubated with 500 μg of WCL from RAW 264.7 cells as a HMGB1 source at 4°C overnight. WCLs were obtained after incubating cells in lysis buffer (50 mM HEPES (pH 7.4), 150 mM NaCl, 1.5 mM $MgCl_2$, 10% glycerol, 1% Nonidet P-40, 1 mM EDTA, 50 mM

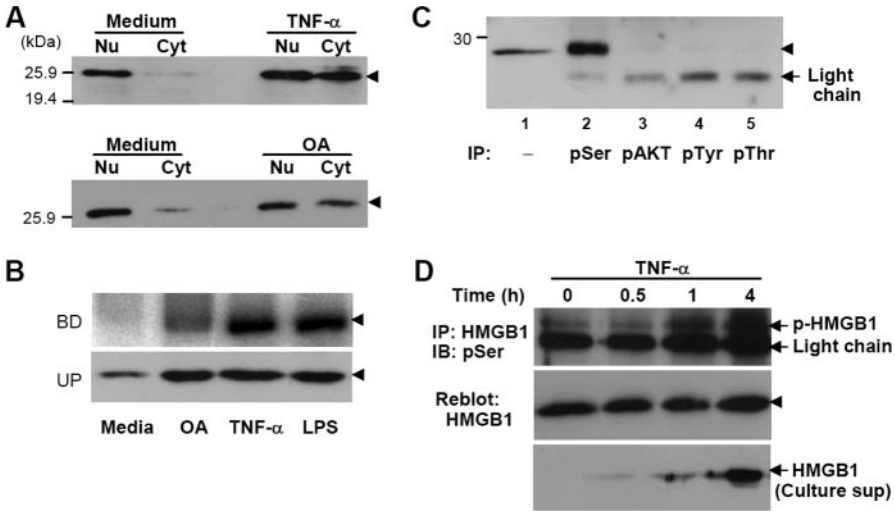

FIGURE 1. HMGB1 is phosphorylated by TNF-α or OA treatment in RAW 264.7 cells. *A*, RAW 264.7 cells were treated with TNF-α (20 ng/ml for 16 h) or OA (100 nM for 8 h). The nuclear (Nu) and cytoplasmic (Cyt) proteins were separated and blotted with anti-HMGB1. *B*, Metabolic ^{32}P-labeling of HMGB1 in RAW 264.7 cells. RAW 264.7 cells were metabolically labeled with [^{32}P]orthophosphate for 4 h and stimulated with OA (100 nM) for 2 h and with TNF-α (20 ng/ml) and LPS (100 ng/ml) for 8 h. WCLs were immunoprecipitated with rabbit anti-HMGB1 from two different vendors, BD PharMingen (BD) and Upstate Biotechnology (UP). The proteins were resolved and visualized by autoradiography. *C*, RAW 264.7 cells were treated with TNF-α, and WCLs were immunoprecipitated (IP) with anti-phosphoserine (pSer), anti-phosphotyrosine (pTyr), and anti-phosphothreonine (pThr) and blotted with anti-HMGB1. WCL was loaded as an HMGB1 control (*lane 1*). Anti-phosphorylated AKT (pAKT) was used for a control Ab (*lane 3*). *D*, RAW 264.7 cells were treated with TNF-α for the indicated length of time. WCLs were immunoprecipitated with anti-HMGB1, immunoblotted (IB) with anti-phosphoserine, and reblotted with anti-HMGB1. The same culture supernatants were concentrated, separated, and blotted with anti-HMGB1.

NaF, 1 mM sodium orthovanadate, 1 mM DTT, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin) for 30 min on ice. Extracts were clarified by centrifugation at 20,000 × *g* for 15 min at 4°C. GST complexes were washed and separated by 12% SDS-PAGE. The blots were probed with anti-HMGB1 and the signals were revealed by ECL detection as described above.

To observe the binding of KAP protein to each mutant HMGB1 in the cells, Flag-tagged KAP-α1 and each mutant HMGB1-GFP plasmid were cotransfected into RAW 264.7 cells. Cell homogenates of transfected RAW 264.7 cells were harvested and incubated with M2 mouse anti-Flag (Sigma-Aldrich) and mouse anti-GFP (Santa Cruz Biotechnology) at 4°C overnight. Immune complexes were collected and the membranes were blotted with anti-Flag and anti-GFP, respectively. The reciprocal experiment was also performed. GST was used as a negative control, and to test the direct binding of HMGB1 to KAP-α1, a GST pull-down assay was performed. For this study, His_6-tagged wild-type HMGB1 and boxes A (aa 1–79) and B (aa 88–162) of HMGB1 were cloned into pRSETB (Invitrogen Life Technologies) and purified proteins were included in this test. The recombinant protein of HMGB1 was incubated with GST-KAP-α1 (10 μg), which was coupled to glutathione-Sepharose 4B beads. After separating on the gel, the membrane was probed with anti-His and reprobed with anti-GST.

Metabolic labeling

RAW 264.7 cells were cultured in phosphate-free DMEM containing 10% dialyzed FBS (Invitrogen Life Technologies) for 4 h and further incubated for 4 h by adding 600 μCi of [^{32}P]orthophosphate (Amersham Biosciences) per milliliter to each dish. After 4 h the cells were stimulated with 100 nM OA for 2 h and 100 ng/ml LPS and 20 ng/ml TNF-α for 8 h. The labeling was terminated by removing the culture medium followed by two immediate washes of the cells with ice-cold PBS. The cells were harvested by scrapping in 0.8 ml of lysis buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM NaF, 10 mM sodium pyrophosphate, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM DTT, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 5 μg/ml pepstatin, and 0.5 mM PMSF) and centrifuged at 21,000 × *g* for 20 min at 4°C. The concentration of total soluble proteins in the supernatant was quantified. Precleared lysates were incubated with 2 μg/ml rabbit anti-HMGB1 from two different companies (BD Pharmingen and Upstate Biotechnology) for 2 h at 4°C. Following the addition of protein G-Sepharose, the tubes were rocked for an additional 1 h and beads were washed 10 times with lysis buffer without SDS. Proteins were eluted in Laemmli sample buffer and separated. The gels were dried and the radioactivity was analyzed by autoradiography.

To observe the phosphorylation of the HMGB1 mutant plasmid, RAW 264.7 cells were transfected with 10 μg each of the wild-type HMGB1-EGFP and HMGB1 NLS1/2A-GFP plasmids (Fig. 5*A*) and incubated for 24 h. The cells were added with [^{32}P]orthophosphate and stimulated with 100 ng/ml LPS for another 8 h. WCLs were immunoprecipitated with anti-GFP and resolved for autoradiography.

Results

HMGB1 serine residues are phosphorylated by TNF-α and OA treatments

To investigate whether HMGB1 is phosphorylated and how its phosphorylation influences its nuclear transport, RAW 264.7 cells were treated with OA, a type 1/2A protein phosphatase inhibitor (25), to induce phosphorylation of HMGB1. OA was used at a low concentration of 100 nM for 8 h or less to minimize the nuclear leakage of HMGB1 and to block entry into the cell cycle (26). Treatment with TNF-α as a positive control cytokine resulted in the translocation of nuclear HMGB1 to the cytoplasm (Fig. 1*A*, *upper panel*), confirming previous reports (17, 27). Treatment of cells with OA also resulted in increased levels of HMGB1 in the cytoplasm (Fig. 1*A*, *lower panel*) similar to that seen with TNF-α-treated cells, suggesting that phosphorylation of HMGB1 is possibly related to its relocation.

Next, to demonstrate the direct evidence of HMGB1 phosphorylation, RAW 264.7 cells were labeled with [^{32}P]orthophosphate and stimulated with 100 nM OA, 20 ng/ml TNF-α, and 100 ng/ml LPS, and the WCLs were immunoprecipitated with anti-HMGB1 for autoradiography. We used rabbit anti-HMGB1 from two different vendors for confirmation. HMGB1 was phosphorylated by OA, TNF-α, and LPS treatments (Fig. 1*B*), and we confirmed the phosphorylation of HMGB1 in LPS-treated RAW 264.7 cells, which were transfected with a HMGB1-GFP plasmid and metabolically labeled (Fig. 5*B*).

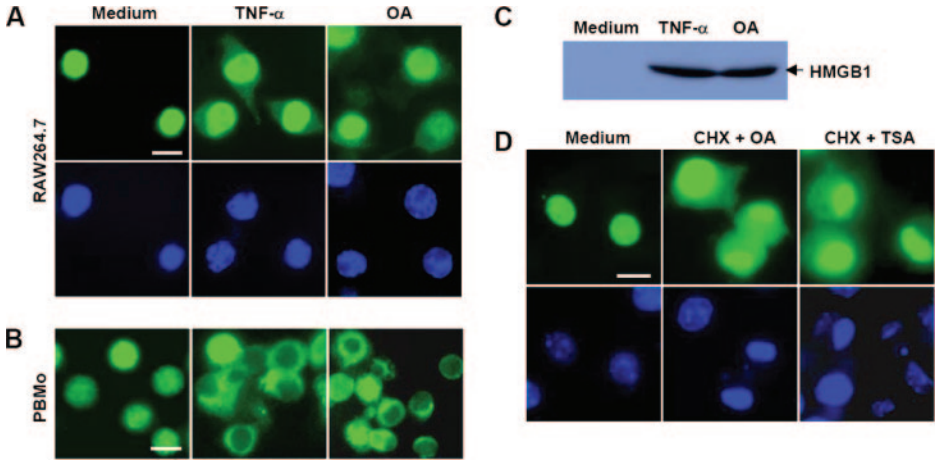

FIGURE 2. Effect of HMGB1 phosphorylation on its location in RAW 264.7 cells and human PBMo cells. *A* and *B*, RAW 264.7 cells (*A*) and human PBMo cells (*B*) were treated with OA (100 nM for 8 h), and immunofluorescent staining was performed to observe the HMGB1. TNF-α (20 ng/ml for 16 h) was used as a positive control cytokine. HMGB1 was exclusively observed in the nuclei of the unstimulated (Medium) RAW 264.7 and PBMo cells but moved to the cytoplasm after OA treatment. *C*, Western blot analysis of the HMGB1 protein in the culture supernatants of PBMo cells, which were from *B*. *D*, HMGB1 in the nucleus is transported to the cytoplasm by phosphorylation. RAW 264.7 cells were transfected with a wild-type HMGB1-GFP plasmid and cultured for 24 h, and then the cells were treated with 2 μg/ml CHX for 1 h followed by OA treatment for 4 h or by TSA treatment for 2 h and green fluorescent images were observed. Bar, 10 μm.

To determine which amino acid residue of HMGB1 is phosphorylated, RAW 264.7 cells were treated with 20 ng/ml TNF-α for 16 h. WCLs from treated cells were immunoprecipitated with anti-phosphoserine, anti-phosphotyrosine, and anti-phosphothreonine Abs, separated, and immunoblotted with anti-HMGB1. Only serine residues of HMGB1 were phosphorylated by TNF-α treatment (Fig. 1*C*).

Next, to observe HMGB1 secretion, RAW 264.7 cells were treated with TNF-α for the indicated length of time and the culture supernatants were harvested and concentrated. WCLs were immunoprecipitated with anti-HMGB1 and then immunoblotted with anti-phosphoserine. The levels of HMGB1 were nearly constant within whole cells but increased in the culture supernatants (Fig. 1*D*, *middle* and *lower panels*), confirming previous reports of time-dependent secretion of HMGB1 (6, 27, 28). The level of phosphorylated HMGB1 also increased after TNF-α treatment in a time-dependent manner (Fig. 1*D*, *upper panel*).

Phosphorylated HMGB1 is relocated toward secretion

To further examine the effect of phosphorylation on the relocation of HMGB1, RAW 264.7 cells were treated with OA and indirect immunofluorescent staining was performed. HMGB1 was mostly observed in the nuclei of unstimulated RAW 264.7 cells (Fig. 2*A*). When the cells were treated with OA for 8 h, HMGB1 was observed in both the nucleus and the cytoplasm, which was similar to that seen in TNF-α-treated cells (Fig. 2*A*). Relocation of HMGB1 after OA treatment was also clearly observed in freshly isolated human PBMo cells (Fig. 2*B*). In addition, HMGB1 was detected in the culture supernatants of PBMo cells after OA treatment (Fig. 2*C*), suggesting the relation of HMGB1 secretion to its phosphorylation.

To exclude the possibility of HMGB1 presence in the cytoplasm due to new protein synthesis, HMGB1 relocation was directly observed using a HMGB-GFP plasmid after treatment with CHX, an inhibitor of new protein synthesis. RAW 264.7 cells were transfected with wild-type HMGB1-GFP plasmid, incubated for 24 h, and then treated with 2 μg/ml CHX. One hour after CHX treatment, OA was added for 4 h in the presence of CHX. We also treated the cells with TSA, a histone deacetylase inhibitor, for 2 h as a positive control because hyperacetylated HMGB1 has been shown to relocate from the nucleus to the cytoplasm (10). As shown in Fig. 2*D*, the HMGB1-GFP protein, which was mostly observed in the nuclei of the cells 24 h after transfection, was relocated to the cytoplasm after OA treatment in the presence of CHX. This result suggests that the HMGB1 observed in the cytoplasm after phosphorylation is not due to new protein synthesis but is caused by the relocation of existing proteins inside the nucleus.

Phosphorylated HMGB1 in the cytoplasm does not enter the nucleus

HMGB1 can traverse the nuclear membrane in both directions. However, HMGB1 molecules are predominantly in the nucleus in an unstimulated state, indicating that import is much more effective than export (10). To further demonstrate whether phosphorylation influences nuclear import of HMGB1, a nuclear import assay was performed using a digitonin-permeabilized HeLa cell-free transport system (24). Digitonin-permeabilized cells have perforated plasma membranes, which release cytosolic components from cells while the nuclear envelope and other major organelle membranes remain intact. As a source of exogenous HMGB1 protein, recombinant HMGB1-GFP protein was purified from *E. coli* (Fig. 3*A*). GST-GFP was prepared as a control protein.

HMGB1-GFP was observed in the nuclei of digitonin-treated HeLa cells when the cells were incubated for 1 h with a transport mixture that contained HMGB1-GFP but not OA, suggesting that HMGB1-GFP entered the nucleus by a default pathway (Fig. 3*B*, *upper left panel*). When the cells were incubated with a HMGB1-GFP-containing transport mixture that included OA, HMGB1-GFP remained in the cytoplasm (Fig. 3*B*, *upper right panel*). Meanwhile, the GST-GFP protein did not enter the nucleus regardless of whether the transport mixture was treated with OA or not (Fig. 3*B*, *middle panels*). Unfused GFP was distributed throughout the cells (Fig. 3*B*, *lower panels*), which is a well-known observation. These results show that HMGB1 once exported to the cytoplasm after phosphorylation or the phosphorylation of HMGB1 occurring in the cytoplasm prevented its nuclear import and plays a critical role in localizing HMGB1 to the cytoplasm. Although GST has no NLS and thus remains in the cytoplasm regardless of its phosphorylation state, HMGB1 has two

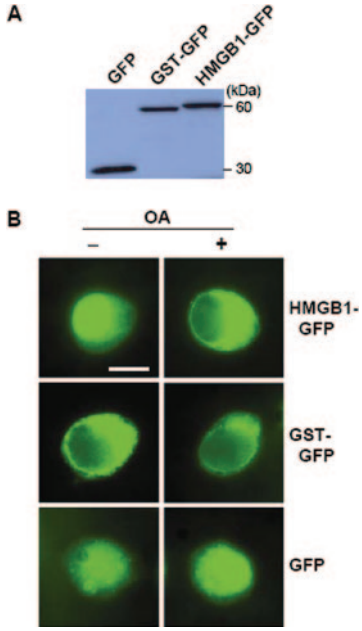

FIGURE 3. Nuclear import assay of HMGB1. *A*, Western blot analysis of His-tagged HMGB1-GFP, GST-GFP, and GFP proteins. His_6-tagged HMGB1-GFP protein was expressed in *E. coli* BL21 (DE3) pLysE for a nuclear import assay. These proteins were purified using a Ni^{2+}-NTA column and blotted with anti-GFP. Each protein was observed at the predicted size. *B*, Nuclear import assay of HMGB1. HeLa cells were permeabilized with digitonin and incubated for 1 h at 22°C with the complete transport mixture. The transport mixture contained recombinant import protein and HeLa cell-derived cytosol, which was preincubated with an ATP-regenerating system in the presence or absence of OA. The cells were fixed and immediately observed by fluorescent microscopy. Bar, 10 μm.

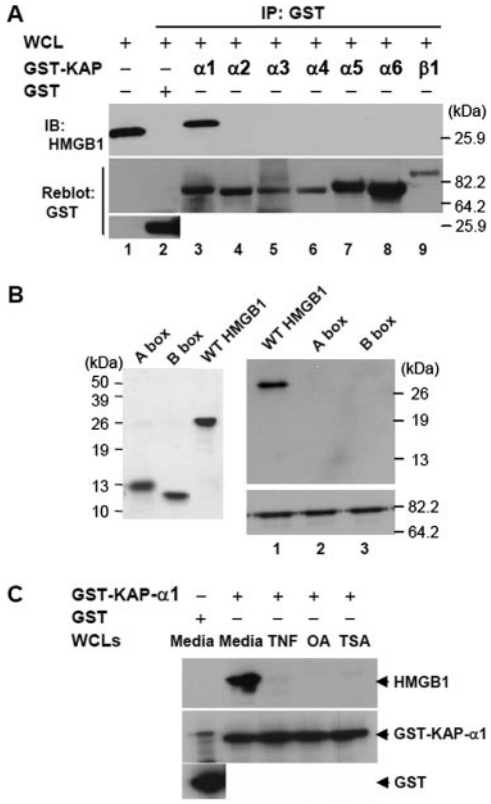

FIGURE 4. The binding of HMGB1 to nuclear import proteins. *A*, GST-KAP-α1, -α2, -α3, -α4, -α5, -α6, and -β1 fusion proteins immobilized on glutathione-Sepharose 4B beads were incubated with WCLs of RAW 264.7 cells overnight at 4°C. Sepharose-bound proteins were separated and the membrane was immunoblotted (IB) with anti-HMGB1 and reblotted (Reblot) with anti-GST. WCL was loaded as an HMGB1 control (*lane 1*), and GST protein was used negative control (*lane 2*). *B*, His_6-tagged wild-type (WT) HMGB1 and boxes A (aa 1–79) and B (aa 88–162) HMGB1 proteins were purified from *E. coli* BL21 and identified at the expected size by Coomassie blue staining. HMGB1 and GST-KAP-α1 was incubated and the precipitate was blotted with anti-His for HMGB1 and reblotted with anti-GST for KAP-α1. *C*, GST-KAP-α1, immobilized on glutathione-Sepharose beads, was incubated with WCLs of RAW 264.7 cells that were treated with OA, TSA, or TNF-α. The precipitates were blotted with anti-HMGB1 and reblotted with anti-GST.

NLSs for nuclear import (10). Therefore, the phosphorylation of HMGB1 at regions close to both NLSs may possibly play an important role in the controlled shuttling mechanism of HMGB1.

HMGB1 binds to KAP-α1, and phosphorylation of HMGB1 decreases its binding to KAP-α1

To investigate whether phosphorylation prevents HMGB1 from interacting with a nuclear import protein, we first determined which KAP protein is involved in binding with HMGB1 as its cargo protein and then observed the interaction of phosphorylated HMGB1 with the KAP protein. KAP family proteins act as shuttling receptors and specifically bind NLS motifs of cargo proteins to facilitate their nuclear import (29). For this study, the GST-KAP fusion proteins α1, α2, α3, α4, α5, α6, and β1 were produced in *E. coli*. GST-KAP-β1 was included because some proteins directly bind KAP-β1 for their nuclear transport (30). For an in vitro protein-protein interaction study, WCLs of unstimulated RAW 264.7 cells, a source of unphosphorylated HMGB1, were incubated with each GST-KAP fusion protein that was bound to glutathione-Sepharose beads. KAP-α1 was identified as the carrier protein for HMGB1 (Fig. 4*A*).

We then tested the direct binding of recombinant HMGB1 protein to KAP-α1 to exclude the possibility that other HMGB1-interacting proteins present in cell lysates could have a role in binding. A purified His_6-tagged wild-type HMGB1 protein and box A (aa 1–79) and box B (aa 88–162) proteins, which were identified at expected sizes by Coomassie blue staining (Fig. 4B, *left panel*), were purified from *E. coli* BL21. The same molar amounts of the wild and truncated forms of HMGB1 were added to GST-KAP-α1, and a GST pull-down assay was performed. Only wild-type HMGB1 was found to bind GST-KAP-α1 (Fig. 4*B*, *right panel*), showing the direct binding of HMGB1 to KAP-α1 without any other interacting proteins. Box A and box B proteins, which include NLS1 (aa 28–44) and do not include a NLS, respectively, showed no binding.

We next tested the binding of phosphorylated HMGB1 to the KAP-α1 protein. When the binding of an OA-treated RAW 264.7 cell lysate to KAP-α1 was tested the interaction was not observed, whereas the binding of a medium-treated RAW 264.7 cell lysate to KAP-α1 was clearly seen (Fig. 4*C*). This result demonstrates that phosphorylation of HMGB1 is one of important modifications that decrease its nuclear import by reducing the binding to KAP-α1. Acetylated HMGB1 from a TSA-treated RAW 264.7 cell lysate also showed no binding to KAP-α1, implying that the re-entry of acetylated HMGB1 to the nucleus is blocked because of an interruption in the binding to KAP-α1.

The effect of phosphorylation of both NLS regions of HMGB1 on the binding to KAP-α1

Because HMGB1 was phosphorylated in serine residues (Fig. 1*C*), we hypothesized that serine phosphorylation close to either or both

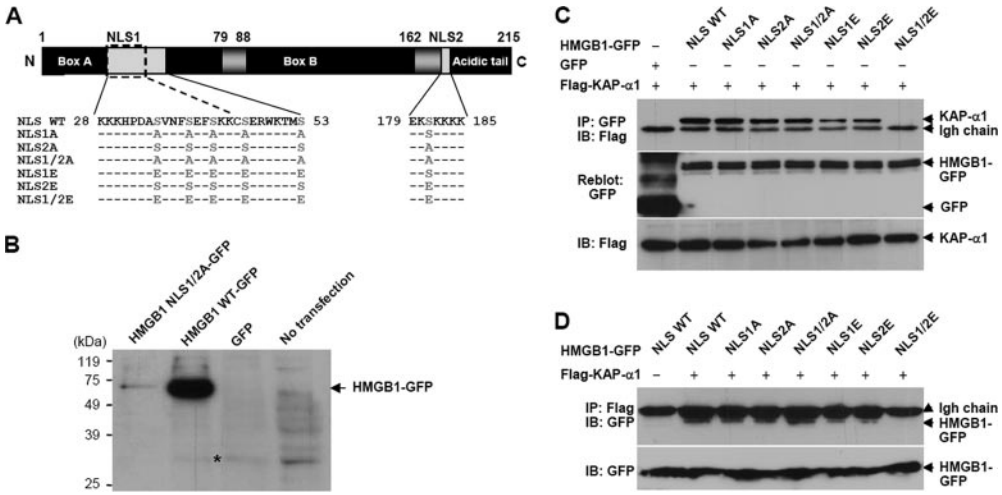

FIGURE 5. The effect of phosphorylation of HMGB1 on binding to KAP-α1. *A*, Schematic presentation of mutated HMGB1-GFPs. Six serines were point-mutated into alanine (A) or glutamic acid (E). The first gray box (aa 28–53) contains NLS1 (dot box) and the adjacent serine-containing region, and another gray box (aa 179–185) is NLS2. Boxes A and B, the acidic tail, and the amino acid numbers are marked. WT, Wild type. *B*, RAW 264.7 cells were transfected with wild-type HMGB1-GFP and HMGB1 NLS1/2A plasmid and metabolically labeled with [^{32}P]orthophosphate as described in *Materials and Methods*. The cells were stimulated with 100 ng/ml LPS for 8 h and immunoprecipitated with anti-GFP. The proteins were resolved and visualized by autoradiography. A GFP plasmid was used as a negative control. GFP is not phosphorylated and nonspecifically observed at the possible position of the asterisk. *C*, RAW 264.7 cells were cotransfected with Flag-tagged KAP-α1 and each HMGB1-GFP mutant plasmid. After 24 h, WCLs were prepared, immunoprecipitated (IP) with anti-GFP, and subjected to Western blotting. The membranes were immunoblotted (IB) with anti-Flag and reblotted with anti-GFP. Flag-KAP-α1 levels were observed to determine whether equal amounts of WCLs were loaded. *D*, The reciprocal experiments were also performed. The m.w. of HMGB1-GFP is similar to that of the Ig H (Igh) chain, and the bands are located just below the IgH chain bands.

NLSs is crucial for its relocation. There are 11 serines throughout HMGB1. Among them, four serines are at 35, 39, 42, and 46 within NLS1, and one is at position 181 within NLS2. The NetPhos 2.0 program (⟨www.cbs.dtu.dk/services/NetPhos/⟩) predicts six serines as the possible phosphorylation sites: the above-mentioned five serines within NLS1 and NLS2 and one more serine at position 53 close to NLS1. To observe whether these six serines are mainly involved in phosphorylation, RAW 264.7 cells were transfected with a wild-type HMGB1-GFP plasmid and a HMGB1 NLS1/2A-GFP plasmid (Fig. 5*A*) for metabolic labeling. Wild-type HMGB1-GFP was strongly phosphorylated; however, HMGB1 NLS1/2A showed a near background level of phosphorylation, which was similar to that of GFP when transfected with a GFP plasmid (Fig. 5*B*). This result suggests that the above-mentioned six serines are the major phosphorylation sites of HMGB1.

To observe the effect of phosphorylation in both NLS regions of HMGB1 on the binding to KAP-α1 and on the subcellular localization of HMGB1 in transfected cells, a number of site-directed mutations of six serines of NLS1 and NLS2 were generated using a HMGB1-GFP fusion construct plasmid (Fig. 5*A*). Serines 35, 39, 42, 46, and 53 within or close to NLS1 and serine 181 within NLS2 were partially or completely mutated into alanine or glutamic acid. Substitution with alanine and glutamic acid simulated an unphosphorylated and a phosphorylated state, respectively (31). RAW 264.7 cells were cotransfected with a Flag-tagged KAP-α1 plasmid and with each mutant HMGB1-GFP plasmid, and immunoprecipitates using anti-GFP for HMGB1 or anti-Flag for KAP-α1 were analyzed. As shown in Fig. 5, *C* and *D*, the interactions of HMGB1 NLS1A, NLS2A, and NLS1/2A with KAP-α1 were similar or slightly decreased as compared with wild-type HMGB1 NLS. Those of HMGB1 NLS1E and NLS2E, which mimicked phosphorylation in either NLS region, were significantly decreased to ~50% of wild type. HMGB1 was predominantly observed in the nucleus after transfection (Fig. 6*A*), possibly suggesting a slow entrance to the nucleus. However, HMGB1 NLS1/2E showed no binding to KAP-α1. These results suggest that phosphorylation at either or both NLSs of HMGB1 differentially reduces the binding to KAP-α1 and has a significant impact on the nuclear import of HMGB1.

Phosphorylation of both NLS regions of HMGB1 is required for its relocation to the cytoplasm

We also investigated the subcellular localization of HMGB1 depending on the state of HMGB1 phosphorylation at either or both NLSs. RAW 264.7 cells were transfected with each mutant HMGB1-GFP plasmid and cultured for 24 h without any stimulation. The mutant fusion proteins from the HMGB1 NLS1A, NLS2A, NLS1/2A, NLS1E, and NLS2E constructs, which showed at least ~50% of interaction with KAP-α1 as compared with wild-type HMGB1, were localized to the nuclei 24 h after transfection (Fig. 6*A*). HMGB1 NLS1/2E, mimicking phosphorylation at both NLSs, however, was located in the cytoplasm. When the same culture supernatants were harvested to observe secreted HMGB1-GFP, HMGB1-GFP was detected only in HMGB1 NLS1/2E-transfected cells (Fig. 6*E*). These data strongly suggest that the concomitant change to the phosphorylated state at both NLSs is important for the cytoplasmic localization and subsequent secretion of HMGB1. When the transfection study was conducted using a nonmyeloid HeLa cell line, similar results were also obtained (data not shown).

Next, RAW 264.7 cells were treated with OA 24 h after each transfection to further investigate the effect of change in the phosphorylation state upon HMGB1 relocation. Wild-type HMGB1 NLS, NLS1E, and NLS2E were relocalized to the cytoplasm after OA treatment, whereas HMGB1 NLS1A, NLS2A, and NLS1/2A were unaffected and remained in the nucleus (Fig. 6*B*). HMGB1 NLS1A, NLS2A, and NLS1/2A remained in unphosphorylated states at either or both NLS regions even after OA treatment. Hence, HMGB1 relocation to the cytoplasm is induced by phosphorylation of both NLS regions. When RAW 264.7 cells were treated with TSA, which induces forced acetylation of HMGB1 regardless of serine phosphorylation, the wild-type and all mutant

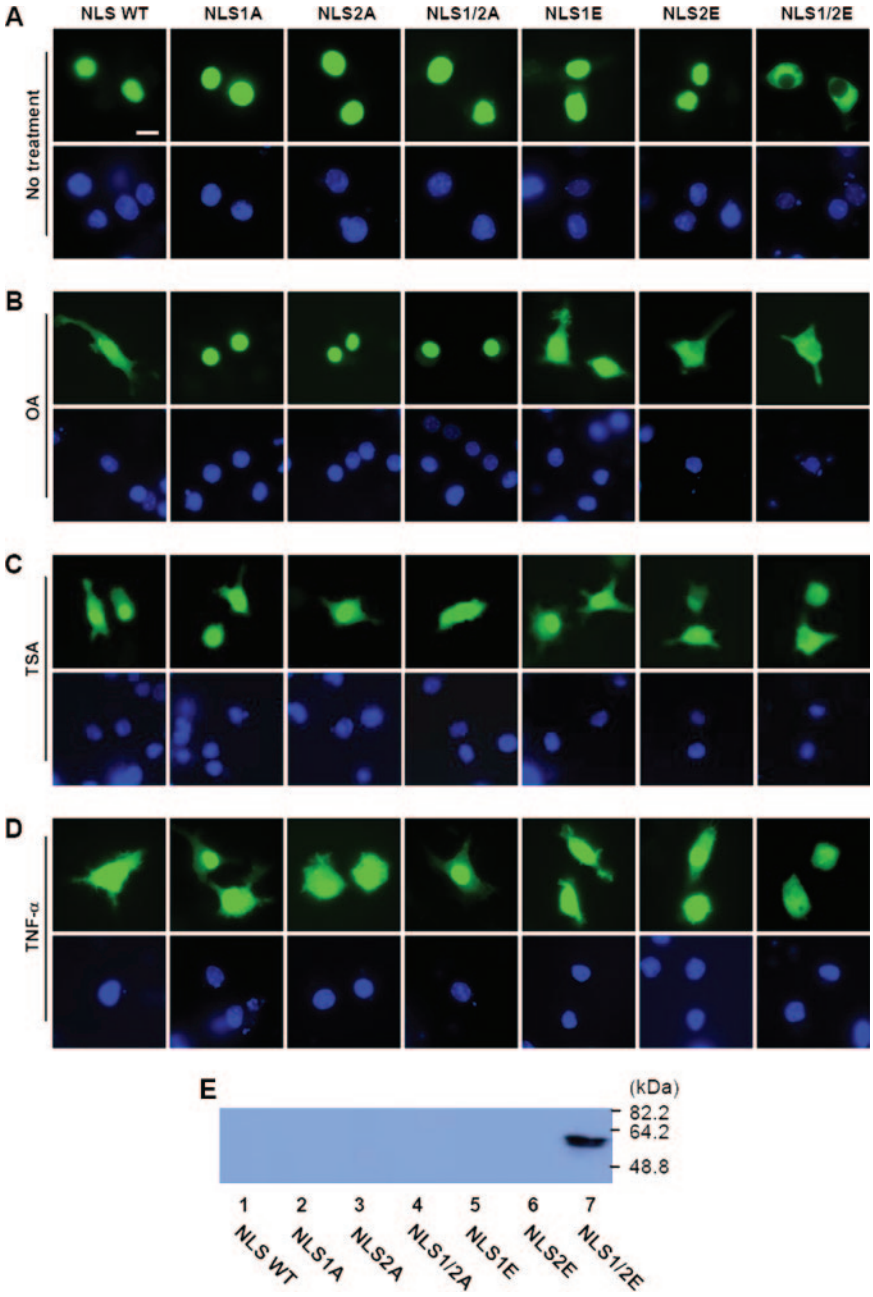

FIGURE 6. Mutation of HMGB1 NLS sites alters subcellular distribution of HMGB1. *A*, RAW 264.7 cells were transfected with wild-type (WT) and each mutant HMGB1-GFP plasmid and immunofluorescent assays were performed 24 h later without any treatment. *B*, *C*, and *D*, OA (100 nM for 4 h) (*B*), TSA (10 ng/ml for 2 h) (*C*), and TNF-α (20 ng/ml for 16 h) (*D*) were applied 24 h after transfection to observe the effect on HMGB1 nuclear export by phosphorylation, acetylation, or both. Some cells showed no GFP, implying no transfection. *E*, Western blot analysis of HMGB1-GFP protein in the culture supernatants, which were from *A*. The membrane was blotted with anti-GFP. Bar, 10 μm.

HMGB1-GFPs showed cytoplasmic relocation (Fig. 6*C*) as expected (10). The same results were observed in TNF-α-treated cells (Fig. 6*D*). Proinflammatory signaling pathways via TNF-α have an impact on enzymes responsible for acetylation/deacetylation (10) and also on those responsible for phosphorylation, according to our data. These results show that, in addition to acetylation, the phosphorylation of both NLS regions of HMGB1 is involved in the cytoplasmic relocation and its eventual secretion.

Discussion

HMGB1 is released from necrotic cells and acts as one of the endogenous danger signals (15). Activated monocytes and macrophages can also secrete HMGB1 in the absence of cell death. Macrophages and monocytes play a central role in coordinating inflammation. TNF-α or IL-1 can stimulate the production of HMGB1 (6), and HMGB1 also induces the production of TNF-α and IL-1 (14). HMGB1 is an important mediator of DC maturation and Th1 polarization (11, 15, 32). Thus, it is important to identify the regulation of HMGB1 secretion, because HMGB1 is involved in the initiation of immune responses in microenvironmental circumstances and the amplification of downstream proinflammatory responses and shock.

In this study we have found that phosphorylation of HMGB1 occurs in vivo after TNF-α and OA treatments and results in the transport of HMGB1 to the cytoplasm for eventual secretion. HMGB1 phosphorylation has not been clearly identified, although phosphorylation of several plant HMG family proteins (18, 19) and of human and insect (*Chironomus*) HMG-Is (33, 34) has been reported. The phosphorylation of the HMG box delays its translocation to the nucleus (35). Our finding that HMGB1 is phosphorylated is in contrast to a previous report showing no evidence of phosphorylation in HMGB1 from the calf thymus and activated human monocytes (10). We currently have no information about this discrepancy. It might be due to a lower extent of phosphorylated HMGB1 in their samples. We

found that HMGB1 phosphorylation occurs at six serines mainly around NLS1 and NLS2, although we did not show which serine was phosphorylated in this study. Early reports also indicated that lamb HMGs1 and 2 are phosphorylated in serines only (36). Human HMG-I of HMG family is phosphorylated at serine and threonine residues (33). These results suggest that the site of phosphorylation of HMG family proteins may vary.

Furthermore, our study discovered that HMGB1 was translocated to the cytoplasm after OA treatment in RAW 264.7 and freshly isolated human PBMo cells. In addition, transfected nuclear HMGB-GFP was relocated to the cytoplasm after OA treatment in the presence of CHX, suggesting that HMGB1 is phosphorylated in the nucleus upon stimulation and is moved toward a secretory direction. Because OA did not induce acetylation (data not shown), it is possible that phosphorylation alone can increase nuclear export. It has been established that nuclear export of HMGB1 is mediated through a CRM1-dependent pathway (10, 17). Hence, phosphorylation may influence the interaction with CRM1 to facilitate nuclear export. Further studies regarding the molecular interactions of phosphorylated HMGB1 with CRM1 will provide a better understanding of the nuclear export of HMGB1.

Fluorescent analysis after the transfection of each HMGB1 NLS mutant plasmid suggests that phosphorylation is important in the accumulation of HMGB1 in cytoplasm and the possible subsequent secretion. In an unstimulated state, HMGB1 protein was imported to the nucleus by KAP-α1 as a nuclear cargo carrier protein after translation and eventually accumulated in the nucleus. However, a significant fraction of HMGB1 cannot re-enter the nucleus if it has been exported from the nucleus due to phosphorylation. One novel feature of the present study is the semiquantitative analysis of mutant HMGB1 protein binding to the KAP-α1 protein. Phosphorylation at both NLS sites is important in blocking the re-entry to the nucleus and in the accumulation in the cytoplasm. Previously, only acetylation has been known to regulate HMGB1 relocation (10, 17). This study, however, showed that phosphorylation is also important in the regulated secretion of HMGB1, although it is unknown which modification is dominant under physiological conditions. Thus, the enzyme that is involved in the phosphorylation of HMGB1 would be a good candidate to target for treating HMGB1-mediated shock.

It would be interesting to know why the concomitant phosphorylation of both NLSs separated by >130 amino acids is necessary for the cytoplasmic localization. In an unstimulated state, the acidic tail region of HMGB1 interacts with basic stretches in the A and B boxes, mainly the A box (37). This observation implies that NLS1 and NLS2 may come close together in three dimensions to bind KAP-α1 because HMGB1 NLS1 and NLS2 are located in the A box and just in front of the acidic tail, respectively. Thus, it is hypothesized that the close proximity of both NLSs in a resting state is disturbed by concomitant phosphorylation at the regions close to the NLSs when stimulated. In the study of HMGB2 (previously HMG2) protein, which has high sequence similarities with HMGB1, the acidic carboxyl terminus influences its retention in the nucleus (38, 39). Thus, we are now studying the role of the HMGB1 acidic tail in the nuclear transport of the protein because HMGB1 and HMGB2 are not structurally and functionally the same (40, 41).

In conclusion, in addition to previously established acetylation, we showed in this study that the subcellular localization of HMGB1 is also finely tuned by phosphorylation at both NLS regions.

Acknowledgments

We thank Won Gun Lee for technical assistance and Sun Mi Ahn for critical reading of manuscript.

Disclosures

The authors have no financial conflict of interest.

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