Phosphorylation of HMGB1 and its effect on the nucleocytoplasmic shuttling and secretion

Ju Ho Youn

Department of Medical Science

The Graduate School, Yonsei University

Phosphorylation of HMGB1 and its effect on the nucleocytoplasmic shuttling and secretion

Ju Ho Youn

Department of Medical Science

The Graduate School, Yonsei University

Phosphorylation of HMGB1 and its effect on the nucleocytoplasmic shuttling and secretion

Directed by Professor Jeon-Soo Shin

The Doctoral Dissertation submitted to the Department of Medical Science, the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Ju Ho Youn

June 2008

This certifies that the Doctoral Dissertation of Ju Ho Youn is approved.

Thesis Supervisor : Jeon-Soo Shin

Thesis Committee Member #1: In-Hong Choi

Thesis Committee Member #2: Sang-Nae Cho

Thesis Committee Member #3: Young Soo Ahn

Thesis Committee Member #4: Tai-Soon Yong

The Graduate School
Yonsei University

June 2008

Acknowledgements

박사학위를 무사히 마칠 수 있어 기쁩니다. 어둡고도 긴 터널을 빠져 나온 듯 홀가분한 마음이지만 돌이켜 보면 고난의 시간들을 연구에 대한 열정과 인내로 잘 견딘 것 같습니다. 많은 도움을 주신 고마운 분들이 없었다면 결코 혼자 걸어 올 수 없었을 것 같습니다.

연세대학교 의과대학 미생물학교실 모든 교수님이하 교실원들에게 감사 드립니다. 특히 지도교수님이신 신전수 교수님에게 모든 영광을 돌립니다. 정말이지 힘든 때가 있었습니다. 그 때마다 항상 교수님께서 힘을 주셨습니다. 또한 진리 탐구의 진실성과 의미에 있어 진정한 연구자의 자세를 가르쳐주셨습니다. 그리고 항상 따듯한 격려와 가르침 주신 최인홍 교수님에게 감사 드립니다. 늘 바쁘시지만 관심 가져주시고 지도해주신 조상래 교수님께도 감사 드립니다. 오랜 시간 동안 한 가족처럼 지내며 도움주신 김은숙, 연수인 선생님에게 감사의 마음의 전하고 싶습니다. 그리고 부족한 제 논문을 꼼꼼히 지도해주신 안영수 교수님과 용태순 교수님께도 감사인사 드립니다. 끝으로 인고의 시간들을 잘 견딜 수 있도록 힘과 용기주신 부모님과 동생에게 이 논문을 바치고 싶습니다.

Table of contents

Abstract 1

I. Introduction 3

II. Materials ans Methods 7
1. Cell culture 7
2. Western blotting analysis 7
3. Immunoprecipitation 8
4. Immunofluorescence and GFP imaging 9
5. Nuclear import assay in digitonin-permeabilized cells 10
6. DNA constructs and mutagenesis 12
7. Interaction between KAP protein and HMGB1 mutant proteins 16
8. Metabolic labeling 18

III. Results 20
1. HMGB1 serine residues are phosphorylated by TNF-α and OA treatments 20
2. Phosphorylated HMGB1 is relocated towards secretion 23
3. Phosphorylated HMGB1 in the cytoplasm does not enter the nucleus 26
4. HMGB1 binds to KAP-α1 and phosphorylation of HMGB1 decreases its binding to KAP-α1 29
5. The effect of phosphorylation of both NLS regions of HMGB1 on the binding to KAP-α1 32
6. Phosphorylation of both NLS regions of HMGB1 is required for its relocation to the cytoplasm 35

IV. Discussion 38

V. Conclusion 42

References 44

Abstract (in Korean) 51

Publication List 53

LIST OF FIGURES

Figure 1. HMGB1 is phosphorylated by TNF-α or OA treatment in RAW 264.7 cells ……...……..22

Figure 2. Effect of HMGB1 phosphorylation on its location in RAW 264.7 cells and human PBMo cells……. ……..25

Figure 3. Nuclear import assay of HMGB……28

Figure 4. The binding of HMGB1 to nuclear import protein……31

Figure 5. The effect of phosphorylation of HMGB1 on binding to KAP-α……...34

Figure 6. Mutation of HMGB1 NLS sites alters subcellular distribution of HMGB1……..……...37

LIST OF TABLES

Table 1. Primers used for site-directed mutagenesis……..……..13

Table 2. Primers used for karyopherin cloning……..…….15

LIST OF ABBREVIATIONS

HMGB1	high mobility group box 1 protein
RAGE	receptor for advanced glycation end products
TLR	toll-like receptor
CHX	cycloheximide
CRM1	chromosome region maintenance 1
NK cells	natural killer cells
Th1	T helper lymphocyte, type 1
DC	dendritic cell
TNF	tumour necrosis factor
IL	interleukin
EGFP	enhanced GFP
KAP	karyopherin
NLS	nuclear localization signal
NES	nuclear export signal
OA	okadaic acid
PBMo	peripheral blood monocytes
TB	transport buffer
TSA	trichostatin A
WCL	whole cell lysate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

Abstract

Phosphorylation of HMGB1 and its effect on the nucleocytoplasmic shuttling and secretion

Ju Ho Youn

Department of Medical Science

The Graduate School, Yonsei University

(Directed by Professor Jeon-Soo Shin)

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 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. This study demonstrates 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. Serine residues of either or both NLSs of HMGB1 were mutated to glutamic acid to simulate a phosphorylated state and the binding of HMGB1 to karyopherin-α1, which was identified as the nuclear import protein for HMGB1 in this study, was examined. Substitution to glutamic acid in either NLSs decreased the binding with karyopherin-α1 by about 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.

Key words: HMGB1, NLSs, phosphorylation, karyopherin-α1, nuclear transport

Phosphorylation of HMGB1 and its effect on the nucleocytoplasmic shuttling and secretion

Ju Ho Youn

Department of Medical Science

The Graduate School, Yonsei University

(Directed by Professor Jeon-Soo Shin)

I. INTRODUCTION

The high mobility group box 1 (HMGB1) protein, a highly conserved, ubiquitous protein, was first purified almost 30 years ago as a nuclear protein[1]. HMGB1 is 80% amino acid sequence identity among higher eukaryotic species, and over 98% identity between rodents and man[2, 3]. The 215 amino acid residues of human HMGB1 are organized into three functional domains. The N-terminus is rich in positively charged lysine residues, while the highly conserved C-terminus (the 'acidic tail') is rich

in negatively charged aspartic and glutamic acid residues. HMGB1 has a structural motif known as the 'HMG box', an ~80-residue, L-shaped domain that mediates DNA binding[4]. HMGB1 bends DNA and facilitates binding of various transcription factors to their cognate sequences, including the steroid nuclear hormones progesterone and estrogen, HOX proteins, p53, and transcription factor II B. These observations suggest a possible role for HMGB1 in DNA recombination, repair, replication and gene transcription[3]. HMGB1-deficient mice are viable only for a few hours after birth, although parenterally administered glucose can prolong survival for a few days[5].

The lack of chromosomal HMGB1 protein does not disrupt cell growth, but may affect the transcriptional regulation of certain genes, such as those activated by the glucocorticoid receptor. HMGB1 can also localize to the cell membrane of neurites for outgrowth[12] and to the cell membranes of tumor cells for metastasis[13]. HMGB1 is passively released from necrotic cells, though not by apoptotic cells, and triggers inflammation[14]. HMGB1 also functions as a late mediator of endotoxemia, sepsis, and hemorrhagic shock in animals and human patients[15-17]. Specific inhibition of endogenous HMGB1 could reverse the lethality of established sepsis with HMGB1 antagonists[18]. HMGB1 is released from activated monocytes and macrophages[15, 19] and natural killer (NK) cells[20]and behaves as a proinflammatory cytokine.

Once present in the extracellular space, HMGB1 can signal tissue injury and initiate inflammatory response through binding to a range of receptors, including the receptor for advanced glycosylation end products (RAGE), toll-like receptor (TLR)2 or TLR4[6, 7]. HMGB1 is a terminal mediator of sepsis and can induce hepatic injury during ischemia/reperfusion in a TLR4-dependent manner[8]. Exposure to HMGB1 leads to various cellular responses, including the chemotactic cell movement of smooth muscle cells, monocytes[21, 22] and the release of proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, and IL-8[23]. NK cells are in close, physical contact with immature dendritic cells (DCs). IL-18, produced by immature DCs, causes NK cells to produce HMGB1. HMGB1, in turn, causes DC maturation and Th1 polarization that initiate the adaptive immune responses[20, 24].

HMGB1 contains two homologous DNA-binding motifs (HMG boxes A and B) and an acidic tail[25]. It also contains two nuclear localization signals (NLSs) and two putative nuclear export signals (NESs)[19], demonstrating that HMGB1 shuttles between the nucleus and 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)[19, 26]. No evidence of phosphorylation, methylation, or

glycosylation has previously been found in HMGB1 from calf thymus, mouse thymus, and activated human monocytes[19]. Phosphorylation of several plant HMG-family proteins has been seen and reported to modulate the stability and DNA binding of these proteins[27, 28]. Also, phosphorylation of NLSs in many proteins influence their binding to nuclear import proteins and consequently increases[29] or decreases[30] their nuclear accumulation, suggesting that phosphorylation of HMGB1 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)[31], the difference in charge of HMGB1 NLSs that results from phosphorylation may disrupt the interaction of HMGB1 with the nuclear importin. However, the phosphorylation of HMGB1 to the nuclear import has not been previously shown.

The present study shows that HMGB1 could be 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 immunoprecipitation to 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.

II. MATERIALS AND METHODS

1. Cell culture

Murine macrophage RAW 264.7 cells (American Type Culture Collection, Manassas, VA, USA) and HeLa cells were cultured at 37^{o}C under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (Invitrogen, Carlsbad, CA, USA), 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, after yielding peripheral blood mononuclear cells, by Ficoll-Hypaque gradient centrifugation. Human recombinant TNF-α (R&D Systems, Minneapolis, MN, USA), okadaic acid (OA, Calbiochem, San Diego, CA, USA), trichostatin A (TSA, Sigma-Aldrich, St. Louis, MO, USA), and cycloheximide (Sigma-Aldrich) were purchased.

2. Western blotting analysis

To analyze the secretion of HMGB1 in the supernatants, culture media were

replaced with serum-free OPTI-MEM (Gibco-BRL, Bethesda, MD, USA) medium and concentrated with Amicon Centricon filtration (Millipore, Bedford, MA, USA) after removing cell debris, and Western blot analysis was performed. The cytoplasmic and nuclear fractions from $5x10^6$ cells were separated using a digitonin-based method[32] to observe the levels of HMGB1 in each fraction. The cells were lysed using 1% Nonidet P-40 buffer containing a protease inhibitor cocktail (Sigma-Aldrich), and the protein concentrations were measured by Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA) for the analysis of whole cell lysates. 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, San Diego, CA, USA) and HRP-labeled goat anti-rabbit Ig as primary and secondary antibodies, respectively. The signals were revealed with enhanced chemiluminescence (ECL, Pierce, Rockford, IL, USA).

3. Immunoprecipitation

To identify the phosphorylated residues of the HMGB1 protein, TNF-α-treated RAW 264.7 cells were lysed with a protease inhibitor cocktail.

Cell homogenates were centrifuged at 20,000 × *g* for 15 min and precleared by incubation with protein G-Sepharose (Amersham Biosciences, Piscataway NJ, USA) at 4°C for 30 min. The precleared extracts (500 μg) were incubated with rabbit polyclonal anti-phosphoserine (pSer), anti-phosphotyrosine (pTyr) and anti-phosphothreonine (pThr) (all from Chemicon, Temecula, CA, USA) 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 Technologies, Beverly, MA, USA) 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-pSer.

4. Immunofluorescence and GFP imaging

Cells were cultured in LabTek II chambers (Nalgene, Rochester, NY, USA) and were fixed in 3.7% paraformaldehyde in PHEM buffer (60 mM PIPES, 25 mM

HEPES, 10 mM EGTA, 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^{o}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 were incubated with rabbit anti-HMGB1 for 1 h at RT. After 3 washes with blocking solution, secondary antibody FITC-conjugated goat anti-rabbit Ig (BD Pharmingen) was added. Cells expressing various HMGB1-EGFP proteins were stained with DAPI and the cells were observed with a BX51 fluorescent microscope (Olympus, Tokyo, Japan). Cells expressing HMGB1-GFP and its derivatives were fixed as described above.

5. Nuclear import assay in digitonin-permeabilized cells

Nuclear import assays were performed with minor modification as previously described[33]. Briefly, HeLa cell cytosol was first prepared. For this, HeLa cells at a density of $5x10^5$ 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 $Mg(OAc)_2$, 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, 1 μg/mL each of leupeptin, pepstatin, and aprotinin). 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 each of leupeptin, pepstatin, and aprotinin), 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, 0.5 mM GTP) either with or without 10 μM OA, and 30 μg/mL of each substrate. The cells were fixed with 3.7% formaldehyde for 10 min and immediately examined by fluorescent microscope.

6. DNA constructs and mutagenesis

The gene encoding human HMGB1 was cloned upstream of GFP in pEGFP-N1 (BD Biosciences Clontech, Palo Alto, CA, USA), and the construct was named pHMGB1-EGFP-N1. For the recombinant HMGB1-GFP protein, a *Sac*I/*Not*I fragment from pHMGB1-EGFP-N1 was subcloned into pET-28a (Novagen, Madison, WI, USA). Six-His-tagged HMGB1-EGFP, GST-EGFP, and EGFP proteins were produced in *E. coli* BL21(DE3) pLysE (Novagen). The cells transformed with each construct were grown in LB 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 (IPTG), 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 QuickChange™ Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). Site-directed mutagenesis was performed with QuickChangeTM

Site-Directed Mutagenesis Kit (Stratagene). The primers used for mutagenesis are listed in Table 1. The underlined and bold letters indicate the mutant sequences.

Table 1. Primers used for site-directed mutagenesis.

Mutation	sense primer sequence (5'-3') / anti-sense primer sequence (5'-3')
S35A, S39A	GAAGAAGCACCCAGATGCT**GCA**GTCAACTTC**GCA**GAGTTTTCTAAGAAGTGC / GCACTTCTTAGAAAACTCT**GCG**AAGTTGACT**GCA**GCATCTGGGTGCTTCTTC
S35E, S39E	GAAGAAGCACCCAGATGCT**GAA**GTCAACTTC**GAA**GAGTTTTCTAAGAAGTGC / GCACTTCTTAGAAAACTC**TTC**GAAGTTGAC**TTC**AGCATCTGGGTGCTTCTTC
S42A, S46A	GTCAACTTCGCAGAGTTT**GCT**AAGAAGTGC**GCA**GAGAGGTGGAAGACCATG / CATGGTCTTCCACCTCTC**TGC**GCACTTCTT**AGC**AAACTCTGCGAAGTTGAC
S42E, S46E	GTCAACTTCGAAGAGTTT**GAG**AAGA AGTGC**GAA**GAGAGGTGGAAGACCATG / CATGGTCTTCCACCTCTC**TTC**GCACTTCTT**CTC**AAACTCTTCGAAGTTGAC
S53A	GAGGTGGAAGACCATGGCT**GCT**AAAGAGAAAGG / CCTTTCTCTTTAGC**AGC**CATGGTCTTCCACCTC
S53E	GTGCGAAGAGAGGTGGAAGACCATG**GAG**GCTAAAGAGAAAGGAAAATTTGAAG / CTTCAAATTTTCCTTTCTCTTTAGC**CTC**CATGGTCTTCCACCTCTCTTCGCAC
S181A	GGAGTTGTCAAGGCTGAAAAA**GCC**AAGAAAAAGAAGGAAGAGG / CCTCTTCCTTCTTTTTCTT**GGC**TTTTTCAGCCTTGACAACTCC
S181E	GGAGTTGTCAAGGCTGAAAAA**GAG**AAGAAAAAGAAGGAAGAGG / CCTCTTCCTTCTTTTTCTT**CTC**TTTTTCAGCCTTGACAACTCC

Briefly, sense and anti-sense mutagenic oligonucleotides were annealed to the corresponding strands of the heat-denatured (at 95°C for 30 sec) pHMGB1-EGFP-N1 plasmid. New strands containing the mutations were amplified with *Pfu Turbo* DNA polymerase in the 16 thermal cycles, consisting of 95°C for 30 sec, 55°C for 1 min, and 68°C for 12 min. The amplified products were treated with *Dpn* I to digest the methylated parental strands. The mutated plasmids were recovered by transformation of the XL1-Blue competent cells, and sequenced to ensure that the mutations were actually introduced. Human KAPs were cloned into *Bam*HI/*Xho*I (KAPs-α1, α2, α4, α6), *Eco*RI/*Xho*I (KAPs-α3, α5) or *Bam*HI/*Not* I (KAP-β1) sites of pGEX-4T-1 (Amersham-Pharmacia) to produce GST-fusion proteins. KAP-α1 (GenPeptide Protein Database accession: AAC60648), α2 (AAA65700), α3 (AAH17355), α4 (AAC25605), α5 (AAH47409), α6 (AAC15233), and β1 (AAH03572) were prepared from PCR amplifications of oligo(dT)-selected HeLa cell-derived cDNA. The primers used for mutagenesis are listed in Table 2. The nucleotide sequences of restriction enzyme sites are underlined.

Table 2. Primers used for karyopherin cloning.

Name	Sequence (5'-3')
KAP-α1 forward	CGCGGATCCATGACCACCCCAGGAAAAGAGAAC
KAP-α1 reverse	CCGCTCGAGAAGCTGGAAACCTTCCATAGGAGC
KAP-α2 forward	CGCGGATCCATGTCCACCAACGAGAATGCTAATAC
KAP-α2 reverse	CCGCTCGAGAAAGTTAAAGGTCCCAGGAGCCCCAT
KAP-α3 forward	CCGGAATTCATGGCCGAGAACCCCAGCTTGGAG
KAP-α3 reverse	CCGCTCGAGAAAATTAAATTCTTTTGTTTGAAGGTTGGC
KAP-α4 forward	CGCGGATCCATGGCGGACAACGAGAAACTGGAC
KAP-α4 reverse	CCGCTCGAGAAACTGGAACCCTTCTGTTGGTACA
KAP-α5 forward	CCGGAATTCATGGATGCCATGGCTAGTCCAGGG
KAP-α5 reverse	CCGCTCGAGTAGCTGGAAGCCCTCCATGGGGGCC
KAP-α6 forward	CGCGGATCCATGGAGACCATGGCGAGCCCAGGG
KAP-α6 reverse	CCGCTCGAGTAGCTGGAAGCCCTCCATGGGGGCC
KAP-β1 forward	CGCGGATCCATGGAGCTGATCACCATTCTCGAGAAGACC
KAP-β1 reverse	ATAAGAATGCGGCCGCAGCTTGGTTGTTGACTTTGGTCAGTTCTTTTG

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 of 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, Foster City, CA, USA). Fugene6 (Roche, Indianapolis, IN, USA) was used for the transfection study.

7. 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% NP40, 1 mM EDTA, 50 mM NaF, 1 mM sodium-orthovanadate, 1 mM DTT, 1 mM PMSF, 10 µg/mL aprotinin, 10 µg/mL leupeptin, 10 µgmL pepstatin) for 30 minutes 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 co-transfected into RAW 264.7 cells. Cell homogenates of transfected RAW 264.7 cells were harvested and incubated with mouse anti-Flag (M2, Sigma-Aldrich) and mouse anti-GFP (Santa Cruz Biotechnology, Santa Cruz, CA, USA) 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, GST pull-down assay was performed as described above. Recombinant protein of HMGB1 was incubated with GST-KAP-α1 (10 μg) which was coupled to glutathione sepharose 4B beads. For this study, Six-His-tagged wild-type HMGB1 and boxes A (aa 1-79) and B (aa 88-162) of HMGB1 were cloned into pRSETB (Invitrogen) and purified proteins were included in this test. After separating on the gel, the membrane was probed with anti-His and reprobed with anti-GST.

8. Metabolic labeling

RAW 264.7 cells were cultured in phosphate starved condition of phosphate-free DMEM containing 10% dialyzed FBS (Gibco-BRL) for 4 h and further incubated for 4 h by adding 600 μCi of [^{32}P]orthophosphate (Amersham-Pharmacia) per milliliter to each dish. After 4 h, the cells were stimulated with 100 nM OA for 2 h, 100 ng/mL of LPS and 20 ng/mL of TNF-α for 2 and 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% NP-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 using the Bradford reagent (Bio-Rad Laboratories). Pre-cleared lysates were incubated with 2 μg/mL of rabbit anti-HMGB1 from two different companies of 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 ten 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.

III. RESULTS

1. 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[34], 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 block entry into the cell cycle[35]. Treatment with TNF-α as a positive control cytokine resulted in the translocation of nuclear HMGB1 to the cytoplasm (Fig. 1A, upper panel), confirming previous reports[26, 36]. Treatment of cells with OA also resulted in increased levels of HMGB1 in the cytoplasm (Fig. 1A) 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 labelled with [^{32}P]orthophosphate and stimulated with 100 nM OA, 20 ng/mL TNF-α and 100 ng/mL LPS for the indicated length of time, and the cell lysates were immunoprecipitated with anti-HMGB1 for autoradiography.

The rabbit anti-HMGB1 antibodies from two different vendors were uesd for confirmation. HMGB1 was phosphorylated by OA, and by TNF-α and LPS treatments. Phosphorylation was increased by the increment of treatment time from 2 h to 8 h (Fig. 1B). 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. 1C).

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 to observe HMGB1 secretion. WCLs were immunoprecipitated with anti-HMGB1 and then immunoblotted with anti-phosphoserine. The levels of HMGB1 were nearly unchanged within whole cells but were increased in the culture supernatants (Fig. 1D), confirming previous reports of time-dependent secretion of HMGB1[15, 36, 37]. The level of phosphorylated HMGB1 was also increased by TNF-α in a time-dependent manner (Fig. 1D).

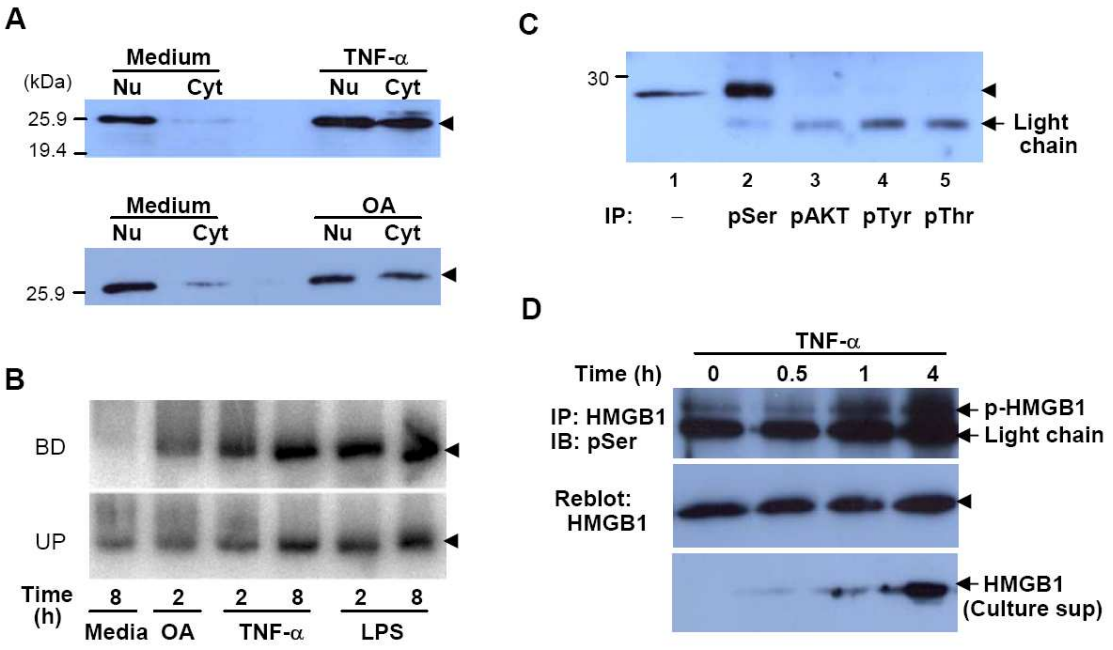

Figure 1. HMGB1 is phosphorylated by TNF-α or OA treatment in RAW 264.7 cells. (A) RAW 264.7 cells were treated with TNF-α (20 ng/mL for 16 h) or OA (100 nM for 8 h). The nuclear (Nu) and cytoplasmic (Cyt) proteins were separated and blotted with anti-HMGB1. (B) Metabolic [^{32}P] labeling of HMGB1 in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

2. Phosphorylated HMGB1 is relocated towards 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. 2A). 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. 2A). Relocation of HMGB1 after OA treatment was also clearly observed in freshly isolated human PBMo cells (Fig. 2B), confirming the phosphorylation effect of HMGB1. The same culture supernatants of human PBMo cells were harvested and assayed for HMGB1 secretion. HMGB1 was detected in the culture supernatants of PBMo cells after OA treatment (Fig. 2C), 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 any 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. As shown in Fig. 2D, 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 HMGB1 observed in the cytoplasm after phosphorylation is not due to new protein synthesis but due to relocation of existing proteins inside the nucleus.

Treatment of cells with the histone deacetylase inhibitor trichostatin A (TSA) as a positive control because the hyperacetylated HMGB1 is relocated from the nucleus to the cytoplasm[19]. As shown in Fig. 2D, the HMGB1-GFP protein, which was mostly observed in the nuclei of the cells 24 hour 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.

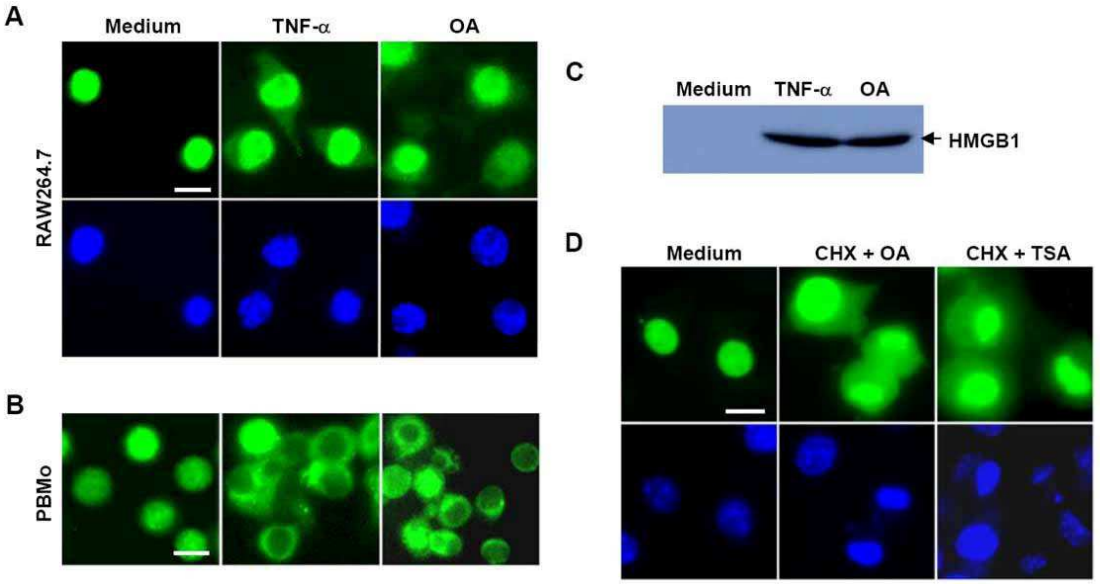

Figure 2. Effect of HMGB1 phosphorylation on its location in RAW 264.7 cells and human PBMo cells. RAW 264.7 cells (A) and human PBMo cells (B) were treated with OA (100 nM for 8 h), and immunofluorescent staining was performed to observe the HMGB1. TNF-α (20 ng/mL for 16 h) was used as a positive control 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 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.

3. Phosphorylated HMGB1 in the cytoplasm does not enter the nucleus

HMGB1 can transverse 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[19]. 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 [33]. 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. 3A). Glutathione-*S*-transferase (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 the transport mixture that contained HMGB1-GFP but not OA, suggesting that HMGB1-GFP entered the nucleus by default way (Fig. 3B). When the cells were incubated with the HMGB1-GFP-containing transport mixture that included OA, HMGB1-GFP remained in the cytoplasm (Fig. 3B). Meanwhile, the GST-GFP protein did not enter the nucleus,

regardless of whether the transport mixture was treated with OA or not (Fig. 3B, middle). GST has no NLS and thus was located in the cytoplasm regardless of its phosphorylation in the presence of an ATP-regenerating system. Finally, unfused GFP was distributed throughout the cells (Fig. 3B), which is a well-known observation. These results show that the phosphorylation of HMGB1 occurred in the cytoplasm prevented its nuclear import and plays a critical role in localizing HMGB1 to the cytoplasm.

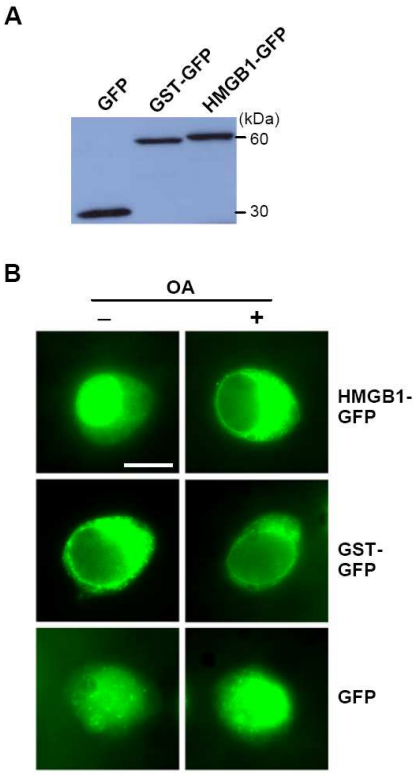

Figure 3. Nuclear import assay of HMGB1. (A) Western blot analysis of His-tagged HMGB1-GFP, GST-GFP and GFP proteins. Six-His-tagged HMGB1-GFP protein was expressed in *E. coli* BL21 (DE3) pLysE for a nuclear import assay. These proteins were purified using a Ni^{2+}-NTA column and blotted with anti-GFP. 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.

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

To investigate whether phosphorylation prevents HMGB1 from interacting with the nuclear import protein, it was determined which KAP protein is involved in binding with HMGB1 as its cargo protein and then observed the interaction of p-HMGB1 with the KAP protein. The KAP family proteins act as shuttling receptors and specifically bind the NLS motifs of cargo proteins to facilitate their nuclear import [38]. For this study, GST-KAP fusion proteins of α1, α2, α3, α4, α5, α6, β1 were produced in *E. coli*. GST-KAP-β1 was included because some proteins directly bind to KAP-β1 for their nuclear transport [39]. 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, which was bound to glutathione-Sepharose beads. KAP-α1 was identified as the carrier protein for HMGB1 (Fig. 4A).

To exclude the possibility that other proteins in cell lysates require for interaction between HMGB1 and KAP-α1, GST pull-down assay was carried out using recombinant HMGB1. Purified six-His-tagged wild-type HMGB1 protein and boxes A (aa 1-79) and B (aa 88-162) proteins, which were identified at expected size by

Coomassie blue staining (Fig. 4B, left), were purified from *E. coli* BL21. Same molar amounts of all wild and truncated forms of HMGB1 were added to GST-KAP-α1, and GST pull-down assay was performed. Only wild type HMGB1 was found to bind GST-KAP-α1 (Fig. 4B, right), showing the direct binding of HMGB1 to KAP-α1 without any other interacting proteins. Box A and B proteins, which include NLS1 (aa 28-44) and no NLS respectively, showed no binding.

The binding of phosphorylated HMGB1 to KAP-α1 protein was next tested. When the binding of KAP-α1 to OA-treated RAW 264.7 cell lysate was tested, the interaction was not observed while the binding to medium-treated RAW 264.7 cell lysate was clearly seen (Fig. 4C). 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 TSA-treated RAW 264.7 cell lysate showed no binding to KAP-α1, implying that re-entry of acetylated HMGB1 to the nucleus is blocked because of no binding to KAP-α1.

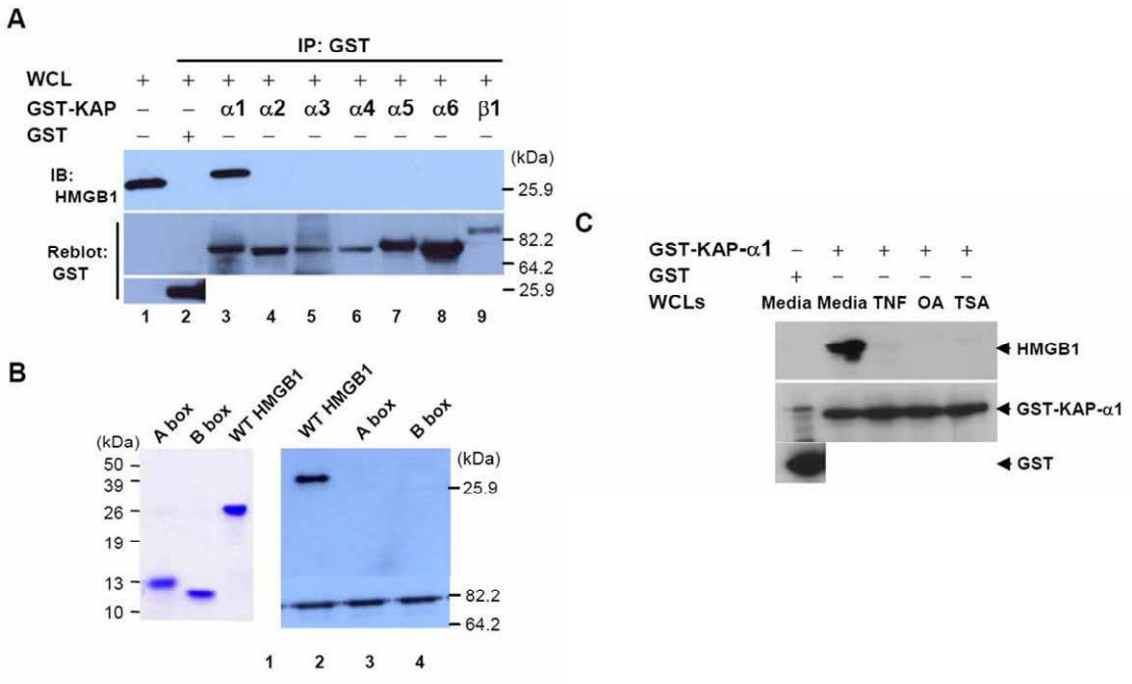

Figure 4. The binding of HMGB1 to nuclear import proteins. (A) GST-KAP-α1, -2, -3, -4, -5, -6, and -β1 fusion proteins immobilized on glutathione-Sepharose 4B beads were incubated with WCLs of RAW 264.7 cells overnight at 4°C. Sepharose-bound proteins were separated and the membrane was blotted with anti-HMGB1 and reblotted with anti-GST. WCL was loaded as an HMGB1 control (lane 1), and GST protein was used negative control (lane 2). (B) Six-His-tagged wild type HMGB1 and boxes A (aa 1-79) and B (aa 88-162) HMGB1 proteins were purified from *E. coli* BL21 and identified at expected size by Coomassie blue staining. HMGB1 and GST-KAP-α1 was incubated for, 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 which were treated with OA, TSA, or TNF-α. The precipitates were blotted with anti-HMGB1 and reblotted with anti-GST.

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

Because HMGB1 was phosphorylated in serine residues (Fig. 1C), thus it is proposed that serine phosphorylation close to either or both NLSs is crucial for its relocation. There are 11 serines throughout the 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. They are five serines within NLS1 and NLS2 described above and one more serine at 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.5A) 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. 5B). 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. 5A). 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 [40].

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. 5C and 5D, 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 about 50% of wild type. HMGB1 was predominantly observed in the nucleus after transfection (Fig. 6A), 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.

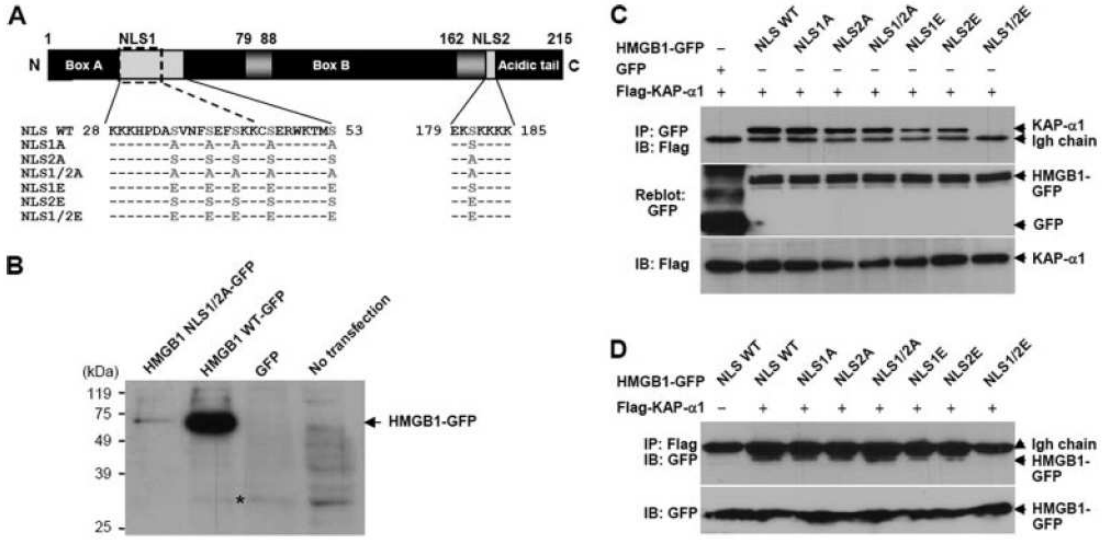

Figure 5. The effect of phosphorylation of HMGB1 on binding to KAP-α1.

(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 molecular weight of HMGB1-GFP is similar to that of the Ig H (Igh) chain, and the bands are located just below the IgH chain bands.

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

The subcellular localization of HMGB1 depending on the state of HMGB1 phosphorylation at either or both NLSs was also investigated. 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 about 50% of interaction with KAP-α1 as compared with wildtype HMGB1, were localized to the nuclei 24 h after transfection (Fig. 6A). 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. 6E). 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.

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. 6B). 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 HMGB1-GFPs showed cytoplasmic relocation (Fig. 6C) as expected[19]. The same results were observed in TNF-α-treated cells (Fig. 6D).

Proinflammatory signaling pathways via TNF-α have an impact on enzymes responsible for acetylation/deacetylation[19] and also on those responsible for phosphorylation, according to present 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.

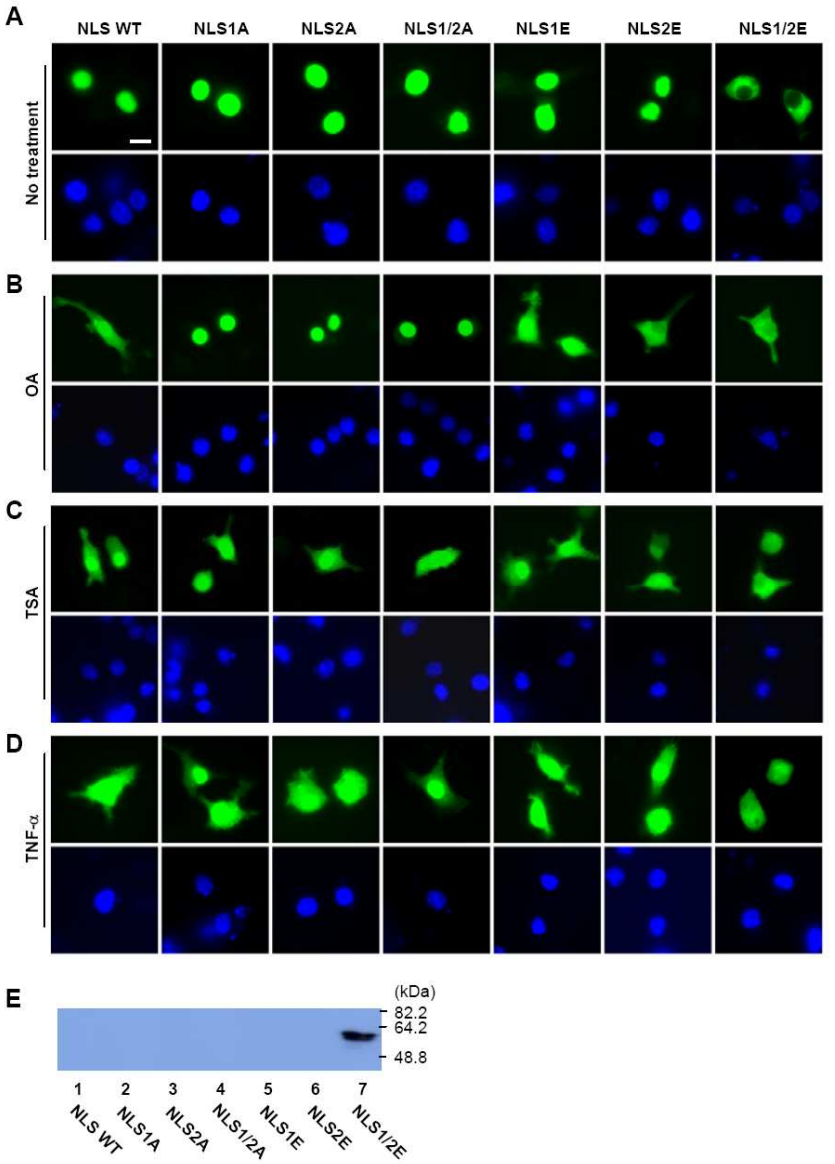

Figure 6. Mutation of HMGB1 NLS sites alters subcellular distribution of HMGB1. (A) RAW 264.7 cells were transfected with wild-type (WT) and each mutant HMGB1-GFP plasmid and immunofluorescent assays were performed 24 h later without any treatment. (B, C, and D) OA (100 nM for 4 h), TSA (10 ng/mL for 2 h), and TNF-α (20 ng/mL for 16 h) 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) Secretion of wild-type and each mutant HMGB1-GFP was tested 24 h after transfection by Western blotting. The culture supernatants were concentrated and blotted with anti-GFP. Bar: 10 μm.

IV. DISCUSSION

HMGB1 is released from necrotic cells and acts as one of the endogenous danger signals[24]. 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[15], and HMGB1 also induces the production of TNF-α and IL-1[23]. HMGB1 is an important mediator of DC maturation and Th1 polarization[20, 24, 41]. So 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.

Tthe present study demonstrates 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[27, 28] and of human and insect (Chironomus) HMG-Is[42, 43] has been reported. The phosphorylation of this HMG box delays its translocation to the nucleus[44]. The study results of HMGB1 phosphorylation is different from a previous report, that showed no evidence of

phosphorylation in HMGB1 from calf thymus and activated human monocytes[19].

There is currently no information about this discrepancy. It may be due to a lower extent of phosphorylated HMGB1 in their samples. The results obtained in this study revealed that HMGB1 phosphorylation at serine residues although did not show which serine is phosphorylated. Early reports indicated that lamb HMGs1 and 2 are phosphorylated in only serines[45]. Human HMG-I of HMG family is phosphorylated at serine and threonine residues[42]. These results suggest that phosphorylation of HMGB family proteins are different from each HMG family protein.

Furthermore, the present study discovered that HMGB1 was translocated to the cytoplasm in RAW 264.7 and freshly isolated human PBMo cells, which were treated with OA. In addition, 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 towards secretory direction. Since OA did not induce acetylation, it is possible that the phosphorylation alone can increase the nuclear export. It has been established that the nuclear export of HMGB1 are mediated through CRM1-dependent pathway[19, 26]. So phosphorylation may influence the interaction with CRM1 to facilitate the nuclear export. Further studies regarding the

molecular interactions of p-HMGB1 with CRM1 will provide a better understanding of the nuclear export of HMGB1.

Fluorescent analysis using HMGB1 NLS mutant plasmids suggests that phosphorylation is important in the accumulation of HMGB1 in the cytoplasm and possible subsequent secretion. In an unstimulated state, HMGB1 protein was imported by KAP-α1 as a nuclear cargo carrier protein to the nucleus after translation and accumulated in the nucleus. However, a significant fraction of HMGB1 can not re-enter the nucleus if it has been exported from the nucleus by 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 to block the re-entry to the nucleus and accumulate in the cytoplasm. Previously, acetylation only has been known to regulate HMGB1 relocation[19, 26]. 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. So the enzyme that is involved in the phosphorylation of HMGB1 would be a good candidate to target in order to treat HMGB1-mediated shock.

It would be interesting to know why the concomitant phosphorylation of both NLSs separated by more than 130 amino acids is necessary for the cytoplasmic

localization. In an unstimulated state, HMGB1's acidic tail region interacts with basic stretches in A and B boxes, mainly with A box[46], implying that NLS1 and NLS2 probably come close together in 3-dimension to bind KAP-α1 since HMGB1 NLS1 and NLS2 are located in the A box and just in front of the acidic tail, respectively. Thus, it is proposed that the close proximity of both NLSs is disturbed by phosphorylation at the regions close to two NLSs concomitantly when stimulated. In the study of HMGB2 (previously HMG2) protein, which has high sequence similarities with HMGB1, acidic carboxyl terminus influences its retention in the nucleus[47, 48].

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

V. CONCLUSION

High Mobility Group Box chromosomal protein 1 (HMGB1) is a nuclear DNA-binding protein acting as a proinflammatory cytokine when secreted into the extracellular space by activated macrophages and dendritic cells. Recently HMGB1 has been shown to be a proinflammatory cytokine with a role in the immunopathogenesis of sepsis. Thus, it is important to identify the regulation of HMGB1 secretion. However, the endogenous factors involved in the control of HMGB1 secretion are poorly known. The purpose of the present study is to demonstrate whether HMGB1 protein is phosphorylated and its phosphorylation influences the secretion of HMGB1.

1. HMGB1 serine residues are phosphorylated by TNF-α or okadaic acid treatment and phosphorylated HMGB1 in the cytoplasm does not enter the nuclei of RAW264.7 and PBMo cells.

2. Karyopherin-α1 is the nuclear import protein of HMGB1 and phosphorylation of HMGB1 decreases its binding to KAP-α1.

3. Mutation of six serine residues to aspartic acid to simulate phosphorylation state in both NLS1 and NLS2 of HMGB1 decreased its binding to karyopherin-α1 and HMGB1 was relocated to the cytoplasm and subsequently secreted into extracellular space.

4. Serine phosphorylation in or adjacent NLS1 and NLS2 is important to nucleocytoplasmic transport of HMGB1 and phosphorylation of HMGB1 is a potential mechanism of regulating the secretion of HMGB1.

In this study, it is shown 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.

REFERENCES

1. Goodwin, GH, Sanders C, Johns EW. A new group of chromatin-associated proteins with a high content of acidic and basic amino acids. Eur J Biochem 1973;38:14-9.
2. Bustin M, Reeves R. High-mobility-group chromosomal proteins: architectural components that facilitate chromatin function. Prog Nucleic Acid Res Mol Biol. 1996;54:35–100.
3. Czura CJ, Wang H, Tracey KJ. Dual roles for HMGB1: DNA binding and cytokine. J Endotoxin Res 2001;7:315–21.
4. Czura CJ, Tracey KJ. Targeting high mobility group box 1 as a late-acting mediator of inflammation. Crit Care Med 2003;31:S46–50.
5. Calogero S, Grassi F, Aguzzi A, Voigtlander T, Ferrier P, Ferrari S, et al. The lack of chromosomal protein Hmg1 does not disrupt cell growth but causes lethal hypoglycaemia in newborn mice. Nat Genet 1999;22:276–80.
6. Park JS, Gamboni-Robertson F, He Q, Svetkauskaite D, Kim JY, Strassheim D. High mobility group box 1 protein interacts with multiple Toll-like receptors. Am J Physiol Cell Physiol 2006;290:C917–24.
7. Rovere-Querini P, Capobianco A, Scaffidi P, Valentinis B, Catalanotti F, Giazzon M. HMGB1 is an endogenous immune adjuvant released by necrotic cells. EMBO Rep 2004;5:825–30.
8. Tsung A, Sahai R, Tanaka H, Nakao A, Fink MP, Lotze MT. The nuclear factor HMGB1 mediates hepatic injury after murine liver ischemia–reperfusion. J Exp Med 2005**;**201:1135–43.

9. Park JS, Arcaroli J, Yum HK, Yang H, Wang H, Yang KY, et al. Activation of gene expression in human neutrophils by high mobility group box 1 protein. Am J Physiol Cell Physiol 2003;284:C870-9.
10. Park JS, Svetauskaite D, He Q, Kim JY, Strassheim D, Ishizaka A, et al. Involvement of Toll-like receptors 2 and 4 in cellular activation by high mobility group box 1 protein. J Biol Chem 2004;279:7370-7.
11. Lotze MT, KJ Tracey. High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. Nat Rev Immunol 2005;5:331-42.
12. Merenmies J, Pihlaskari R, Laitinen J, Wartiovaara J, Rauvala H. 30-kDa heparin-binding protein of brain (amphoterin) involved in neurite outgrowth. Amino acid sequence and localization in the filopodia of the advancing plasma membrane. J Biol Chem 1991;266:16722-29.
13. Taguchi A, Blood DC, del Toro G, Canet A, Lee DC, Qu W, et al. Blockade of RAGE-amphoterin signalling suppresses tumour growth and metastases. Nature 2000;405:354-60.
14. Scaffidi P, Misteli T, Bianchi ME. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature 2002;418:191-5.
15. Wang H, Bloom O, Zhang M, Vishnubhakat JM, Ombrellino M, Che J, et al. HMG-1 as a late mediator of endotoxin lethality in mice. Science 1999;285:248-51.
16. Sunden-Cullberg J, Norrby-Teglund A, Rouhiainen A, Rauvala H, Herman G, Tracey KJ, et al. Persistent elevation of high mobility group box-1 protein (HMGB1) in patients with severe sepsis and septic shock. Crit Care Med 2005;33:564-73.

17. Ombrellino M, Wang H, Ajemian MS, Talhouk A, Scher LA, Friedman SG, et al. Increased serum concentrations of high-mobility-group protein 1 in haemorrhagic shock. Lancet 1999;354:1446-7.
18. Yang H, Ochani M, Li J, Qiang X, Tanovic M, Harris HE, et al. Reversing established sepsis with antagonists of endogenous high-mobility group box 1. Proc Natl Acad Sci USA 2004;101:296-301.
19. Bonaldi T, Talamo F, Scaffidi P, Ferrera D, Porto A, Bachi A, et al. Monocytic cells hyperacetylate chromatin protein HMGB1 to redirect it towards secretion. EMBO J 2003;22:5551-60.
20. Semino C, Angelini G, Poggi A, Rubartelli A. NK/iDC interaction results in IL-18 secretion by DCs at the synaptic cleft followed by NK cell a ctivation and release of the DC maturation factor HMGB1. Blood 2005;106:609-16.
21. Degryse B, Bonaldi T, Scaffidi P, Müller S, Resnati M, Sanvito F, et al. The high mobility group (HMG) boxes of the nuclear protein HMG1 induce chemotaxis and cytoskeleton reorganization in rat smooth muscle cells. J Cell Biol 2001;152:1197-1206.
22. Rouhiainen A, Kuja-Panula J, Wilkman E, Pakkanen J, Stenfors J, Tuominen RK, et al. Regulation of monocyte migration by amphoterin (HMGB1). Blood 2004;104:1174-82.
23. Andersson U, Wang H, Palmblad K, Aveberger AC, Bloom O, Erlandsson-Harris H, et al. High mobility group 1 protein (HMG-1) stimulates proinflammatory cytokine synthesis in human monocytes. J Exp Med 2000;192:565-70.

24. Messmer D, Yang H, Telusma G, Knoll F, Li J, Messmer B, Tracey KJ, et al. High mobility group box protein 1: an endogenous signal for dendritic cell maturation and Th1 polarization. J Immunol 2004;173:307-13.
25. Bustin M, Lehn DA, Landsman D. Structural features of the HMG chromosomal proteins and their genes. Biochim Biophys Acta 1990;1049:231-43.
26. Gardella S, Andrei C, Ferrera D, Lotti LV, Torrisi MR, Bianchi ME, et al. The nuclear protein HMGB1 is secreted by monocytes via a non-classical, vesicle-mediated secretory pathway. EMBO Rep 2002;3:995-1001.
27. Stemmer C, Leeming DJ, Franssen L, Grimm R, Grasser KD. Phosphorylation of maize and Arabidopsis HMGB proteins by protein kinase CK2alpha. Biochemistry 2003;42:3503-8.
28. Stemmer C, Schwander A, Bauw G, Fojan P, Grasser KD. Protein kinase CK2 differentially phosphorylates maize chromosomal high mobility group B (HMGB) proteins modulating their stability and DNA interactions. J Biol Chem 2002;277:1092-8.
29. Briggs LJ, Stein D, Goltz J, Corrigan VC, Efthymiadis A, Hübner S, et al. The cAMP-dependent protein kinase site (Ser312) enhances dorsal nuclear import through facilitating nuclear localization sequence/importin interaction. J Biol Chem 1998;273:22745-52.
30. Zhang F, White RL, Neufeld KL. Phosphorylation near nuclear localization signal regulates nuclear import of adenomatous polyposis coli protein. Proc Natl Acad Sci USA 2000;97:12577-82.

31. Conti E, Uy M, Leighton L, Blobel G, Kuriyan J. Crystallographic analysis of the recognition of a nuclear localization signal by the nuclear import factor karyopherin alpha. Cell 1998;94:193-204.
32. Bird CH, Blink EJ, Hirst CE, Buzza MS, Steele PM, Sun J, et al. Nucleocytoplasmic distribution of the ovalbumin serpin PI-9 requires a nonconventional nuclear import pathway and the export factor Crm1. Mol Cell Biol 2001;21:5396-407.
33. Adam SA, Marr RS, Gerace L. Nuclear protein import in permeabilized mammalian cells requires soluble cytoplasmic factors. J Cell Biol 1990;111:807-16.
34. Bialojan C, Takai A. Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases. Specificity and kinetics. Biochem J 1988;256:283-90.
35. Cobb J, Cargile B, Handel MA. Acquisition of competence to condense metaphase I chromosomes during spermatogenesis. Dev Biol 1999;205:49-64.
36. Taniguchi N, Kawahara K, Yone K, Hashiguchi T, Yamakuchi M, Goto M, et al. High mobility group box chromosomal protein 1 plays a role in the pathogenesis of rheumatoid arthritis as a novel cytokine. Arthritis Rheum 2003;48:971-81.
37. Rendon-Mitchell B, Ochani M, Li J, Han J, Wang H, Yang H, et al. IFN-gamma induces high mobility group box 1 protein release partly through a TNF-dependent mechanism. J Immunol 2003;170:3890-7.
38. Macara IG. Transport into and out of the nucleus. Microbiol Mol Biol Rev 2001;65:570-94.

39. Kurisaki A, Kose S, Yoneda Y, Heldin CH, Moustakas A. Transforming growth factor-beta induces nuclear import of Smad3 in an importin-beta1 and Ran-dependent manner. Mol Biol Cell 2001;12:1079-91.
40. Liu X, Sun Y, Constantinescu SN, Karam E, Weinberg RA, Lodish HF. Transforming growth factor beta-induced phosphorylation of Smad3 is required for growth inhibition and transcriptional induction in epithelial cells. Proc Natl Acad Sci USA 1997;94:10669-74.
41. Dumitriu IE, Baruah P, Valentinis B, Voll RE, Herrmann M, Nawroth PP, et al. Release of high mobility group box 1 by dendritic cells controls T cell activation via the receptor for advanced glycation end products. J Immunol 2005;174:7506-15.
42. Xiao D-M, Pak JH, Wang X, Sato T, Huang FL, Chen H-C, et al. Phosphorylation of HMG-I by protein kinase C attenuates its binding affinity to the promoter regions of protein kinase C gamma and neurogranin/RC3 genes. J Neurochem 2000;74:392-9.
43. Schwanbeck R, Wiśniewski JR. Cdc2 and mitogen-activated protein kinases modulate DNA binding properties of the putative transcriptional regulator Chironomus high mobility group protein I. J Biol Chem 1997;272:27476-83.
44. Wiśniewski JR, Schulze E, Sapetto B. DNA binding and nuclear translocation of insect high-mobility-group- protein-1 (HMG1) proteins are inhibited by phosphorylation. Eur J Biochem 1994;225:687-93.
45. Ramachandran C, Yau P, Bradbury EM, Shyamala G, Yasuda H, Walsh DA. Phosphorylation of high-mobility-group proteins by the calcium-phospholipid-

dependent protein kinase and the cyclic AMP-dependent protein kinase. J Biol Chem 1984;259:13495-503.

46. Knapp S, Müller S, Digilio G, Bonaldi T, Bianchi ME, Musco G. The long acidic tail of high mobility group box 1 (HMGB1) protein forms an extended and flexible structure that interacts with specific residues within and between the HMG boxes. Biochemistry 2004;43:11992-7.
47. Pallier C, Scaffidi P, Chopineau-Proust S, Agresti A, Nordmann P, Bianchi ME, et al. Association of chromatin proteins high mobility group box (HMGB) 1 and HMGB2 with mitotic chromosomes. Mol Biol Cell 2003;14:3414-26.
48. Shirakawa H, Tanigawa T, Sugiyama S, Kobayashi M, Terashima T, Yoshida K, et al. Nuclear accumulation of HMG2 protein is mediated by basic regions interspaced with a long DNA-binding sequence, and retention within the nucleus requires the acidic carboxyl terminus. Biochemistry 1997;36:5992-9.
49. Ronfani L, Ferraguti M, Croci L, Ovitt CE, Schöler HR, Consalez GG, et al. Reduced fertility and spermatogenesis defects in mice lacking chromosomal protein Hmgb2. Development 2001;128:1265-73.

Abstract (in Korean)

HMGB1의 인산화가 분비조절에 미치는 영향

<지도교수 신 전 수>

연세대학교 대학원 의과학과

윤 주 호

High mobility group box 1 (HMGB1) 단백질은 내독소 혈증, 균혈증, 출혈성 쇼크 발생시 활성화된 단구(monocyte), 대식세포(macrophage), 자연살(NK) 세포에서 분비되어 전염증성(proinflammatory) 사이토카인과 같은 기능을 수행함으로써 세균 감염 및 염증성 질환의 병인기전에 관여한다. HMGB1 은 2 개의 DNA 결합 모티브 (HMG box A 및 B)와 산성을 띠는 꼬리 (acidic tail)를 갖는 구조이고 2 개의 핵 국재화 시그널(nuclear localization signal, NLS)과 2 개로 예상되는 핵 방출 시그널(nuclear export signal, NES)이 있다. 즉, 핵과 세포질 사이의 이동이 엄격하게 조절될 것으로 예상할 수 있으나 HMGB1 의 세포 밖 분비기전은 아직까지 분명치 않다.

본 연구에서는 전염증성 사이토카인인 TNF-α, 내독소 그리고 포스파타제(phosphatase) 억제제인 okadaic acid(OA)를 RAW 264.7세포와 사람의 말초혈액 단구 (peripheral blood monocytes, PBMo)에 처치하였을 경우 HMGB1의 serine 잔기가 인산화됨을 관찰하였고, 인산화된 HMGB1은 세포질에서 핵으로의 이동이 저해됨을 확인하였다.

HMGB1의 NLS 부위의 serine이 주요 인산화 부위이며 인산화가 일어나면 HMGB1의 핵 내 이동을 담당하는 karyopherin-α1 핵 유입 단백질과의 결합이 억제되어 핵 내 이동이 방해됨을 알 수 있었다. 그리고 NLS 부위의 serine 잔기들을 음전하를 띤 잔기들로 치환하였을 경우 인산화 상태와 유사하게 HMGB1의 핵 내 유입이 억제되는 현상을 관찰하였다. 이는 인산화에 의해 야기되는 HMGB1의 NLS부위에서의 전하의 변화가 핵 유입 단백질과의 결합을 저해할 수 있음을 확인할 수 있었다. 이러한 결과는 HMGB1의 인산화 상태가 세포 밖 방출을 야기하는 핵에서 세포질로의 이동을 촉진하고, 이러한 HMGB1의 인산화 조절이 HMGB1 분비 함께 HMGB1의 활성을 제어함에 있어 중요하다고 생각된다.

핵심되는 말: HMGB1, 인산화, NLS, karyopherin-α1

Publication List

1. Youn JH, Oh YJ, Kim ES, Choi JE, Shin JS. High Mobility Group Box 1 Protein Binding to Lipopolysaccharide Facilitates Transfer of Lipopolysaccharide to CD14 and Enhances Lipopolysaccharide-Mediated TNF-{alpha} Production in Human Monocytes. J Immunol 2008;180: 5067-74.

2. Youn JH, Shin JS. Nucleocytoplasmic shuttling of HMGB1 is regulated by phosphorylation that redirects it toward secretion. J Immunol 2006;177:7889-97.

3. Park IH, Youn JH, Choi IH, Nahm MH, Kim SJ, Shin JS. Anti-idiotypic antibody as a potential candidate vaccine for *Neisseria meningitidis* serogroup B. Infect Immun 2005;73:6399-406.

4. Park IH, Yeon SI, Youn JH, Choi JE, Sasaki N, Choi IH, Shin JS. Expression of a novel secreted splice variant of the receptor for advanced glycation end products (RAGE) in human brain astrocytes and peripheral blood mononuclear cells. Mol Immunol 2004;40:1203-11.

5. Youn JH, Myung HJ, Liav A, Chatterjee D, Brennan PJ, Choi IH, Cho SN, Shin JS. Production and characterization of peptide mimotopes of phenolic glycolipid-I of Mycobacterium leprae. FEMS Immunol Med Microbiol 2004;41:51-7.