ARTICLE
Journal of Cellular Biochemistry 108:791–801 (2009)

Journal of Cellular Biochemistry

The Nuclear Architectural Protein HMGA1a Triggers Receptor-Mediated Endocytosis

Wuwei Wu, Wei Wan, and Alexander D.Q. Li*

Department of Chemistry and Center for Materials Research, Washington State University, Pullman, Washington 99164

ABSTRACT

High mobility group proteins A (HMGA), nuclear architectural factors, locate in the cell nuclei and mostly execute gene-regulation function. However, our results reveal that a HMGA member (HMGA1a) has a unique plasma membrane receptor; this receptor specifically binds to HMGA-decorated species, effectively mediates endocytosis, and internalizes extracellular HMGA-functionalized cargoes. Indeed, dyes or nanoparticles labeled with HMGA1a protein readily enter Hela cells. Using a stratagem chemical cross-linker, we covalently bonded the HMGA receptor to the HMGA1a-GFP fusion protein, thus capturing the plasma membrane receptor. Subsequent Western blots and SDS–PAGE gel revealed that the HMGA receptor is a 26-kDa protein. Confocal live-cell microscopic imaging was used to monitor the whole endocytic process, in which the internalized HMGA1a-decorated species are transported by motor proteins on microtubules and eventually arrive at the late endosomes/lysosomes. Cell viability assays also suggested that extracellular HMGA1a protein directly influences the survival ability of Hela cells in a dose-dependent manner, implying versatility of HMGA1a protein and its potent role to suppress cancer cell survivability and to regulate growth. J. Cell. Biochem. 108: 791–801, 2009. © 2009 Wiley-Liss, Inc.

KEY WORDS: HMGA1a; RECEPTOR; ENDOCYTOSIS

A superfamily of nonhistone nuclear architectural proteins, the high mobility group (HMG) proteins consists of three families: HMGA, HMGB, and HMGN [Hock et al., 2007], and each family has a few members sharing common characteristic motifs. Two genes encode four HMGA family members. Alternative spliced mRNA forms and subsequent translation of the *Hmga1* gene produce HMGA1a, HMGA1b, and HMGA1c, and yet another gene encodes HMGA2. The common motifs of HMGA proteins—the so-called AT-hooks bind the AT-rich DNA minor groove efficiently. Totally flexible and structureless, HMGA proteins exhibit interesting and unique interactions with their partners; often they act as core elements organizing multiple proteins into a complex, the so-called enhanceosome [Liu et al., 2001]. Consequently, HMGA proteins behave as architectural transcriptional factors interacting with transcriptional factors including NF-κB and AP1, tumor suppressors p53, mRNA processing proteins, DNA processing/remodeling proteins and structural proteins [Sgarra et al., 2005]. As a result, HMGA proteins bind to AT-rich DNA regions, change DNA conformation, and frequently participate in the regulation of specific genes transcription, either positively or negatively together with other transcription factors.

As nuclear architectural factors, HMGA proteins as well as the broader HMG families execute their function mostly inside cell nuclei, where HMGA proteins and histone H1 compete dynamically for the chromatin-binding site and constantly remodel the structure of the chromatin fiber, thus influencing the gene transcription [Reeves, 2003]. HMGA proteins also inhibit nuclear excision repair, suggesting HMGA protein overexpression might accumulate mutated DNA and cause genomic instabilities associated with many types of human cancers [Adair et al., 2005]. In normal adult tissue, HMGA1 and HMGA2 are hardly detectable, but their levels intensify in malignant epithelia tumors, leukemia, and neoplastic transformation. Interacting with their nuclear partners, HMGA proteins may affect several distinct nuclear processes such as mRNA splicing, DNA replication and chromatin assembly [Pierantoni et al., 2007]. Thus far, literature evidences suggest that HMGA proteins function as ''tumor biomarkers'' and their high level expression has been observed in every carefully and specifically investigated tumor [Giancotti et al., 1989; Ferranti et al., 1992; Rogalla et al., 1998].

However, some HMG family members also assume responsibility outside cell nuclei. For example, HMGB1 protein functions as a matrix-bound or soluble molecule in the extracellular medium of

Grant sponsor: National Institute of General Medicine Sciences; Grant number: GM065306.
*Correspondence to: Prof. Alexander D.Q. Li, Department of Chemistry, PO Box 644630, Pullman, WA 99164-4630. E-mail: dequan@wsu.edu
Received 17 February 2009; Accepted 15 June 2009 • DOI 10.1002/jcb.22281 • © 2009 Wiley-Liss, Inc.
Published online 8 September 2009 in Wiley InterScience (www.interscience.wiley.com).

different cell types. When added to the cell culture, HMGB1 behaves as a potent signaling molecule, stimulating specific responses such as differentiation and cytoskeletal reorganization [Raucci et al., 2007]. In murine NIH3T3 cells, a dynamic, cell cycle-dependent HMGA1 translocation from the nuclei into the cytoplasm and mitochondria has been observed, such observation implies that HMGA1 protein may also play a role in regulating mitochondria function [Dement et al., 2007].

In this study, we report a specific interaction between HMGA1a and its plasma membrane receptor that enables the cell to internalize extracellular HMGA1a protein, HMGA1a conjugates, and HMGA1a functionalized nanoparticles (NP). The receptor effectively mediates endocytosis of HMGA1a-decorated species. Consequently, HMGA1a molecules labeled with Oregon Green 488 or polymer fluorescent nanoparticles readily enter Hela cells. Using a stratagem chemical cross-linker, we photo-activated the cross-linking reaction to covalently bond the HMGA receptor to the HMGA1a-GFP fusion protein and thus captured the plasma membrane receptor. Subsequent Western blots and SDS–PAGE gel revealed that the HMGA receptor is a 26-kDa protein. This receptor protein subsequently orchestrated the whole endocytosis process, in which the internalized HMGA1a-decorated species were transported by motor proteins on microtubules and eventually arrived at the late endosomes/lysosomes. Live-cell imaging was carried out using the confocal microscope, which monitored the endocytic processes. Cell viability assays suggested that extracellular HMGA1a directly influenced the survival ability of the cultured cells in a dose-dependent manner, implying versatility of HMGA1a protein and its potent role in suppressing cancer cells survivability and regulating growth.

MATERIALS AND METHODS

LIVE-CELL FLUORESCENCE IMAGING

Hela cell monolayers were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in CO_2 incubator. Live cells were imaged using Zeiss Axiovert 200 inverted microscope equipped with a liquid nitrogen cooled CCD detector (Princeton Instruments, Roper Scientific). Red-fluorescence cell imaging was acquired using 365-nm excitation and a 590-nm long pass red filter to clean up the stray light. Green fluorescence cell imaging was obtained using 488-nm excitation and a 510 ± 40 nm green bandpass filter to suppress noises.

HMGA1a FUNCTIONALIZED NANOPARTICLES

Following our previous published results, we further modified the optically switchable dual color nanoparticle (dcNP) [Zhu et al., 2007] or photoswitchable single red color nanoparticle (rcNP) [Zhu et al., 2006] surface carboxyl groups into activated esters. Typically, 200 µl dcNP or rcNP in 0.1 M $pH \approx 6.2$ NaH_2PO_4 buffer ($\sim 1 \times 10^{-10}$ mol) was activated by adding 20-µl freshly made 50 mg/ml EDC (*N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride), followed by 5 mg/ml NHS (*N*-hydroxysulfosuccinimide sodium salt) at room temperature for 20 min. Next the solution buffer was exchanged to MES (2-(*N*-morpholino) ethanesulfonic acid hydrate) buffer (0.05 M, $pH \approx 5.0$) using Millipore 30-kDa cutoff centrifugal filters. In 500-µl MES reaction buffer, 50-µl 2.3-mM HMGA1a proteins were added to the activated dcNP or rcNP and the coupling reaction was allowed to proceed at room temperature for 2 h. The reaction buffer was then removed using Millipore 30-kDa cutoff centrifugal filters and the remaining HMGA1a-nanoparticles were washed with PBS buffer (pH 7.4) and finally dispersed in 500 µl PBS to yield a final HMGA-dcNP or rcNP concentration of 0.2 µM and stored at 4°C.

OREGON GREEN 488 LABELED HMGA1a PROTEIN

To a 0.1 nmol HMGA1a protein dissolved in 200 µl 0.1 M sodium bicarbonate buffer ($pH \sim 8.3$), added 1.5 nmol Oregon Green 488 (Invitrogene, CA) and the coupling reaction was allowed to proceed for 1 h at room temperature in dark. The labeled HMGA1a protein was dialyzed against PBS ($pH \sim 7.2$) buffer to remove the unreacted dyes. Optical absorption revealed that each HMGA1a protein was labeled by approximately four Oregon Green 488 dyes.

TRACKING HOW CELLS INTERNALIZE HMGA1a-dcNP

Six 35-mm MatTek's glass bottom culture dishes (MatTek Corp.) were used to grow Hela cells at 37°C in CO_2 incubator. When the cell density reached 5×10^5 per dish, HMGA1a functionalized dcNP was added such that the final dcNP concentration was 5 nM. After 15 min incubation, the old medium was removed and cell was washed with PBS (pH 7.2) for three times, and fresh DMEM medium with 10%FBS was added. The cells were then continuously cultured up to 90 min. Every 15 min, one plate of cells was took out from the incubator and fixed by adding formaldehyde to the medium to reach a final concentration 4%. The fixed cells were imaged using 488-nm illumination and a green band-pass filter.

TREATING HELA CELLS WITH CARBONYL CYANIDE CHLOROPHENYLHYDRAZONE (CCCP)

CCCP inhibits ATP synthesis in cells and ATP depletion will stall the ATP-dependent microtubule transportation. About 5×10^5 Hela cells, grown on a 35-mm MatTek's glass bottom culture dish, were incubated with 5 nM of HMGA1a-dcNP for 1 h, at which time the HMGA1a-dcNP have been endocytosed and arrived at the endosomes. Next, the cells were washed twice with PBS buffer (pH 7.2), nourished under fresh DMEM medium, and treated with various amounts of CCCP drug for 30 min. The final concentrations of the CCCP drug in the DMEM medium were: 0, 50, 100, 200, and 400 µM, respectively. Cells were then imaged continuously using 488-nm laser for 5 min at the speed of 2 s per frame to determine whether the CCCP drug had any effects on motor protein transportation of endosomes. The live-cell images were processed with software ImageJ and the movement of endosomes containing HMGA-dcNP was statistically analyzed by counting the bright moving spots (>600 counts per sample). The endosome transportation speeds were classified into five groups: group 1 has a speed of 0 µm/s; group 2, 0–0.1 µm/s; group 3, 0.1–0.2 µm/s; group 4, 0.2–0.3 µm/s, and group 5 >0.3 µm/s. The relative occurrence was normalized using the occurrence number of the non-moving spots (speed = 0 µm/s) as 100% in the same sample.

To inhibit the ATP-dependent receptor-mediated endocytosis, CCCP was used to treat about 5×10^5 Hela cells, grown on 35 mm

MatTek's glass bottom culture dishes. After the cells were incubated with different amounts of CCCP (final concentration: 0, 50, and 200 μM, respectively) for 1 h, HMGA-dcNP were added (5 nM) to the extracellular medium and cell culture continued at 37°C in CO_2 incubator up to 4 h. The cells were then imaged using 488 nm illumination and a green band-pass filter to determine whether the CCCP drug disabled the endocytosis.

MONITORING HOW LIVE CELLS INTRACELLULLARLY TRANSPORT THE INTERNALIZED HMGA1a PROTEIN CARGOES

Colocalization imaging experiments were performed to determine whether HMGA1a protein cargoes associated with the recycling or early endosome protein Rab11. Using Lipofectamine™ 2000 (Invitrogene), we transfected Hela cells (5×10^5) with plasmid pEGFP-Rab11 (4 μg) that encoded an GFP labeled early endosome protein Rab11 [Chen et al., 1998]. After 24 h, cells were examined using 488-nm illumination to ensure that >80% cells had expressed the desired EGFP-Rab11 protein. The transfected cells were further incubated with 5 nM HMGA-rcNP for 4 h. Finally, live cells were washed with PBS buffer (pH ≈ 7.2) and imaged using 488-nm excitation to acquire the green fluorescence images or 365-nm illumination to obtain the red-fluorescence images. The images were analyzed and processed with software ImageJ to determine whether the HMGA1a cargoes colocalized with early endosome protein Rab11.

To determine whether the HMGA1a cargoes colocalized with late endosomes/lysosomes, we used HMGA1a protein labeled with Oregon Green 488 and LysoTracker Red DND-99 (Invitrogen), a dye that emitted red-fluorescence in lysosomes. Accordingly, Hela Cells (5×10^5) were incubated with 1 μM HMGA-Oregon Green 488 for 4 h, and stained with LysoTracker Red DND-99 (for 10 min). Next, live cells were then rinsed with PBS (pH ≈ 7.2) and imaged using a Zeiss LSM 510 Laser Scanning Confocal Microscope.

EXPRESSING AND PURIFYING HMGA1a–GFP FUSION PROTEIN

HMGA1a gene and enhanced green fluorescence protein (EGFP) gene were fused together using overlapping polymerase chain reactions (PCR). The fused gene was cloned into pET24b vector, transfected into *Escherichia coli* strain DH5α to amplify the plasmid, and sequenced to verify its correct construct. The correct plasmid containing GFP-HMGA1a fusion gene was transfected into *E. coli* strain BL21. Inducing the transformed *E. coli* strain BL21 using 1 mM IPTG for 3 h produced GFP-HMGA1a fusion protein, which was purified using His-Select Nickel Affinity Gel (Sigma, Saint Louis, MI). The fusion-protein concentration was determined by measuring solution optical density at 280-nm wavelength OD280 using a Cary 100 UV-visible spectrophotometer. The extinction coefficient of the fusion protein is 1.82 mg/ml/cm at 280 nm. The purity of the protein was determined to be greater than 80% using SDS–PAGE.

USING CROSS-LINKING STRATEGY TO CAPTURE THE HMGA1a RECEPTOR

To identify the potential HMGA1a plasma membrane receptor, we used a photo-activated cross-linking strategy to covalently connect HMGA1a protein to its receptor. The cross-linker *N*-succinimidyl-6-[4′-azido-2′-nitrophenylamino]hexanoate or SANPAH (Pierce, IL) had two reactive groups: a activated ester group and another photo-reactive azido group. We first attached the SANPAH linker to the GFP-HMGA1a-6his fusion protein by reacting the activated ester with an amino group in the fusion protein. Briefly, 0.5 mg GFP-HMGA1a-6his proteins were dissolved in 0.3 ml 20 mM sodium phosphate, 0.15 M NaCl buffer (pH ~ 8.0) and 30 μl 12 mM SANPAH dissolved in DMSO were added. The reaction was allowed to proceed in the dark at 37°C for 30 min before it was stopped by adding 20 μl 0.1 M Tris buffer (pH ~ 8.0). The SANPAH labeled HMGA1a proteins were purified from non-reacted cross-linker by passing through a 10-kDa cutoff Millipore centrifugal filter and dissolved in a PBS (pH ≈ 7.2) buffer to yield a final concentration of 0.5 mg/ml.

Approximately 1×10^7 Hela cells were incubated with 0.5 mg GFP-HMGA1a-SANPAH for 15 min. Then the cells were washed twice with PBS (pH ~ 7.2) buffer to remove unbound SANPAH cross-linking molecules. At this time, GFP-HMGA1a-SANPAH had docked on the HMGA1a plasma membrane receptor and UV illumination (5 min 365 nm) photo-activated the azido group and subsequent photochemical reaction covalently linked the GFP-HMGA1a-6his to the HMGA1a receptor. After the cross-linking reaction, the cells were collected and lysed using 0.5-ml lysis buffer containing 20 mM Tris pH 7.5, 100 mM NaCl, 0.5% NP-40, 0.5 mM EDTA, 0.5 mM PMSF, and 0.5% protease inhibitor cocktail (Sigma, MO), and the lysate was stored at −80°C for further usage.

Western blots revealed the covalently bonded GFP-HMGA1a-SANPAH-receptor, which was detected by an anti-GFP antibody (Clontech). Briefly, 20 μl cell lysate was mixed with SDS-loading buffer and loaded on a 12% SDS–PAGE gel without boiling. After running the gel, we transferred the proteins bands to a PVDF membrane, where anti-GFP antibody revealed those bands containing GFP with SuperSignal West Pico Mouse IgG Detection Kit (Pierce).

To identify the HMGA1a receptor, we further purified the GFP-HMGA1a-SANPAH-receptor from cell lysate using Ni-NTA beads. Briefly, 0.5-ml cell lysate was diluted to 10-ml using a buffer containing 1% CHAPS, 1 mM $CaCl_2$ and 8 M urea. Both GFP-HMGA1a-SANPAH-receptor and GFP-HMGA1a-SANPAH contained His-tag and bound to the Ni-NTA resin (Sigma, MO); the diluted cell lysate was shaking with the Ni-NTA resin at 0°C for at least 12 h in 10 mM imidazole to promote binding. The resin was then isolated and washed three times with a buffer containing 0.1% dodecyl-maltoside, 0.9% CHAPS, 1 mM $CaCl_2$ and 10 mM imidazole in PBS, once with another buffer containing 1% Triton X-100 and 1% NP-40 in PBS, and finally, the cross-linked GFP-HMGA1a-SANPAH-receptor complex was eluted using PBS buffer containing 250 mM imidazole, 1% Triton X-100 and 1% NP-40. The eluted materials were mixed with SDS-loading buffer and loaded on a 12% SDS–PAGE. Since the concentration of GFP-HMGA1a-SANPAH-receptor was very low, a sensitive silver staining (http://www.biochem.uiowa.edu/donelson/Database items/Rapid Silver Stain.doc) was performed to reveal the protein bands on the gel.

MTT ASSAY AND CELL GROWTH CURVE SUGGEST THAT HMGA1a PROTEIN INFLUENCES THE SURVIVABILITY OF HELA CELLS

Different amounts of the HMGA1a protein were added to the DMEM medium and cultured with Hela cells, in 96-well plates (6,000 cells per well) for 72 h; the HMGA1a protein concentration in the medium was 0, 1.15, 5.75, 11.5, 23, 34.5, and 46 μM, respectively. Standard MTT tests were performed using Vybrant MTT Cell Proliferation Assay Kit (Invitrogen) to test the cancer cell proliferation rate. The MTT test measured the absorbance at 570 nm, which was obtained with a SpectraMax 190 spectrophotometer (Molecular Devices, CA).

To directly measure whether HMGA1a protein influenced cancer cell growth, we incubated Hela cells in 1-ml DMEM medium with or without 10 μM HMGA1a on a 12-well plate for 3 days ($\sim 1.9 \times 10^5$ cells per well initially). The medium either with or without 10 μM HMGA1a was freshly replaced daily, and the viable cell and total cell population were counted using Beckman Coulter every 24 h.

FLOW CYTOMETRY STUDIES OF HMGA1a-INDUCED APOPTOSIS

Hela cells were grown in a 6-well plate to 80% confluence. HMGA1a protein was added until the total concentration reached 10 μM. Following treatment for 12 h, cells were harvested and pelleted by centrifugation at 1,000*g* for 5 min at room temperature. Next, cells were washed twice with cold PBS and resuspended in 1X binding buffer (10 mM HEPES pH 7.5, 140 mM NaCl, 0.25 mM $CaCl_2$) at a concentration of 1×10^6 cells/ml. To stain apoptotic cells, we used Annexin V-FITC Apoptosis Detection Kit, APOAF (Sigma–Aldrich) following the manufacturer's instructions. Briefly, to a 12 mm × 75 mm test tube, Annexin V-FITC (5 μl) and PI (10 l) were added to 500 μl of cells (5×10^5 cells), which were vortexed gently and incubated in the dark for 10 min at room temperature. The samples were analyzed by FACScalibur (BD biosciences) and data were processed with FlowJo software.

Negative control used the same protocol except HMGA1a protein was not added. Positive control used UV to induce Hela cells undergoing apoptosis; HMGA1a protein was not added and the rest procedure was same as above.

RESULTS

HELA CELLS READILY INTERNALIZE HMGA1a PROTEIN AND ITS CONJUGATES INCLUDING HMGA1a FUNCTIONALIZED NANOPARTICLES

Previously, we have synthesized a new class of polymeric nanoparticles, suitable for living cell imaging and whose fluorescence can be photoswitched between two distinct colors and hereafter named as the dual-color nanoparticles (dcNP) [Zhu et al., 2007]. The dcNP have surface carboxyl groups, which can be activated to link the primary amino group of proteins. In this work, we covalently attached the HMGA1a protein to the dual-color nanoparticles, and incubated the HMGA1a-dcNP with Hela cells. After 2 h incubation, the free HMGA1a-dcNP was washed away and the treated Hela cells were illuminated using either 488- or 365-nm light to acquire green- and red-fluorescence images, respectively. Comparing the HMGA1a-dcNP images in both the green and red fluorescence channels, we could clearly observe that the HMGA1a-dcNP were located inside the Hela cells (Fig. 1A), whereas live cells did not internalize the same nanoparticles without HMGA1a modification. Thus HMGA1a found a pathway into living cells. When Hela cells and the Oregon Green 488 labeled HMGA1a protein were incubated for 2 h, live cell imaging using a 488-nm laser revealed that fluorescence lighted up the cytoplasma (Fig. 1B). This result further confirms that extracellular HMGA1 proteins have the ability to penetrate intact cellular membranes, thus entering live Hela cells.

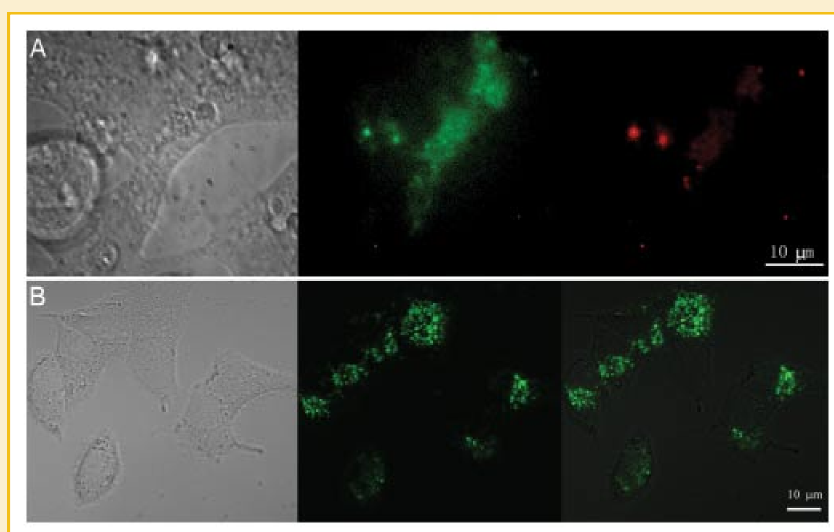

Fig. 1. Hela cells internalize HMGA proteins. A: HMGA-labeled dual color nanoparticles (HMGA-dcNP) were added to the DMEM medium where Hela cells were cultured for two additional hours. Images were taken under white light (left), 488-nm (middle) or 365-nm illumination (right). B: Same as A except that Oregon Green 488 labeled HMGA proteins were used to replace HMGA-dcNP. Images were taken under white light (left) and 488-nm laser (middle). The merge image is showed on right. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

HMGA1a PROTEIN, ITS CONJUGATES, AND HMGA1a FUNCTIONALIZED NANOPARTICLES UTILIZE ENDOCYTIC PATHWAY TO ENTER LIVE CELLS

To investigate the pathway through which HMGA1a protein entered Hela cells, we labeled the HMGA1a protein with dual-color nanoparticles, and monitored the complete internalization process of HMGA1a-dcNP (Fig. 2). The HMGA-dcNPs quickly attached to Hela cell membranes and within 30 min some HMGA-dcNP effectively crossed the cell membranes and after 75 min, the HMGA-NPs had already reached the perinuclear area, where most late endosomes/lysosomes were located. Such fast internalization and transportation hint cell machinery-mediated endocytosis.

Endocytosis is a mechanism that allows cells to internalize external molecules into cell organelles. Receptor-mediated endocytosis, the most common and best-known endocytic pathway, starts with ligand binding to its receptor, and subsequently triggers clathrin-coated pit formation, internalizes ligand-receptor complexes; the uncoated pits emerge as cell organelles—the early endosomes, which are transported along microtubules and delivered to late endosomes/lysosomes. In our experiments, continuous live cell imaging using HMGA-dcNPs offers not only the initial HMGA-dcNP docking positions on the membrane but also the final destinations inside the Hela, a process involving actively transporting HMGA-dcNP on the microtubules.

Figure 3 exemplifies that the motor proteins such as kinesin and dynein use microtubules to transport early endosomes containing HMGA-dcNP. The two green spots in Figure 3A represent the early endosomes containing HMGA-dcNP cargoes because the HMGA-dcNP emit green fluorescence when excited by 488-nm laser illumination. The brighter spot remains still during our investigation, while the dimmer spot (white arrow) moves about 2.3 μm in 30 s, a velocity of 77 nm/s at ambient temperature, which represents a typical microtubule-facilitated transportation [Reck-Peterson et al., 2006]. This observation also supports the idea that

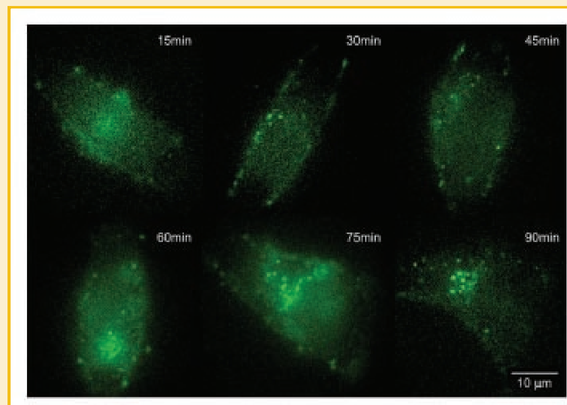

Fig. 2. HMGA1a-dcNP endocytic time trajectories. HMGA-functionalized nanoparticles and Hela cells were incubated together in DMEM medium (10% FBS) for 15 min. Subsequently, the used medium was removed; cells were washed with PBS (pH ≈ 7.2) for three times and nourished using fresh DMEM medium with 10% FBS. The cells were continuously cultured up to 90 min and every 15 min, a plate of cells was fixed by adding formaldehyde to the medium to a final concentration of 4%. The fixed cells were imaged using a 488-nm laser and representative images revealed HMGA-dcNP inside the cell endosomes (green spots). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

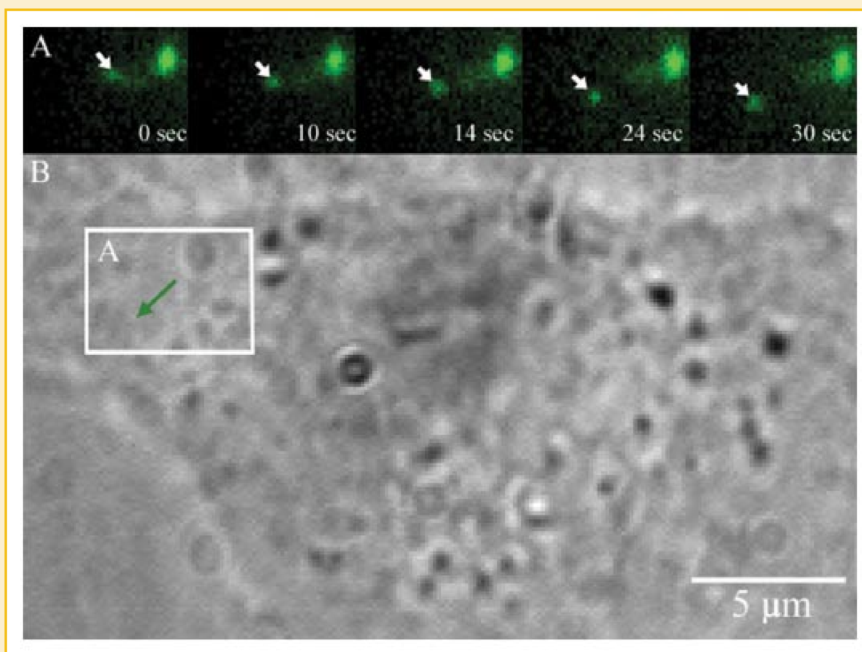

Fig. 3. Motor proteins transport HMGA-dcNP on microtubules in Hela cells. A: Motor proteins (kinesin or dynein) use microtubules to transport early endosomes (A: white arrows) containing HMGA-nanoparticles at high speeds (77 nm/s) in a live cell at room temperature. B: White light image of the whole cell reveals the position (white rectangle) of microtubule-facilitated early endosome transportation relative to the whole cell. The green arrow represents the direction and starting and ending points of microtubule-facilitated transportation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

HMGA-dcNP use receptor-mediated endocytic pathway to enter Hela cells, because microtubule-dependent transportation is one of the hallmarks of early endosomes.

ATP-DEPENDENT MOTOR PROTEIN TRANSPORTATION

The microtubule-facilitated and motor-protein driven transportation of early endosomes requires ATP. To validate our hypothesis that those intracellular fast-moving bright spots are early endosomes transported by motor proteins using ATP, we treated the cell with a protonophore drug carbonyl cyanide chlorophenylhydrazone (CCCP) for 30 min after HMGA-dcNP internalization. The CCCP drug collapsed the mitochondrial proton electrochemical gradient, partially or completely disabled ATP production from mitochondria [Terada, 1981], and therefore controlled all ATP-dependent processes. Continuous monitoring revealed that occurrence of the fast-moving bright spots depended on the drug concentration. Thus, the transportation was ATP-dependent (Fig. 4). Without the CCCP treatment, fast-moving bright spots dominated the whole cellular image. With the CCCP treatment, however, the occurrence of the fast-moving bright spots within live cells abated as the drug concentration increased, concomitant with increasingly non-moving bright spots. When the CCCP concentration reached 200 μM, all intracellular motions came to a halt, indicating that ATP was completely depleted and the drug blocked its production in "live" cells.

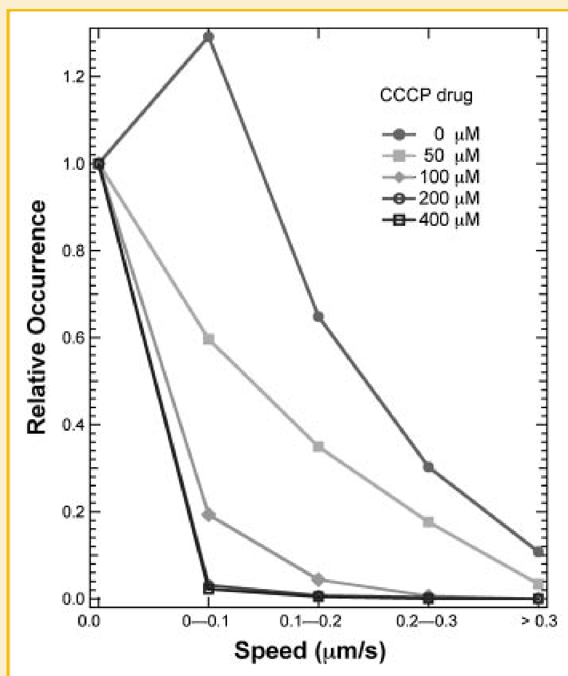

Fig. 4. The CCCP drug inhibits motor protein transporting HMGA cargoes inside Hela cells. Hela cells and HMGA-dcNP were incubated for 1 h before the cells received a 30-min treatment of CCCP drug at various concentrations. Live cells were then imaged continuously using a 488-nm laser for 5 min at 2-s per frame speed. The images were processed using software ImageJ, the moving spots manifested the endosome movement containing HMGA-dcNP cargoes, and their traveling velocities at ambient temperature were statistically counted (>600 counts per sample). The traveling velocities were classified into 5 groups according to their speeds: 0, 0–0.1, 0.1–0.2, 0.2–0.3, and >0.3 μm/s and positioned on the horizontal axis evenly. Their relative occurrences were normalized to the non-moving spots (0 μm/s) in the same sample and plotted against the five speed groups.

Receptor-mediated endocytosis also requires ATP as an energy source. To prove ATP-dependent HMGA endocytosis, we treated the cells with CCCP before exposing them to HMGA-dcNP, and found that HMGA-dcNP were stuck on the cell membrane and could not be endocytosed. Indeed, at or above 200-μM CCCP concentrations, ATP production was disabled and ATP reserves were sufficiently depleted and Hela cells could not internalize any HMGA-dcNP even after 4 h, although the control Hela cells had endocytosed many HMGA-dcNP at this time (Fig. 5).

HMGA1a CARGOES COLOCALIZE WITH MARKERS OF ENDOSOMES OR LYSOSOMES

Two colocalization experiments revealed the final destination of the endocytosed HMGA. The first experiment used optically switchable red color nanoparticles (rcNP) and early endosome marker protein Rab11. We added HMGA-rcNP to the cultured Hela cells transfected with pGFP-Rab11 plasmids to study the colocalization of HMGA and GFP. The results after 4 h incubation displayed that HMGA-rcNP partially colocalized with early endosome marker, GFP-Rab11 protein (Fig. 6), indicating that most HMGA-rcNP had already left the early endosome at this time. The second experiment used LysoTracker Red DND-99 and HMGA-Oregon Green 488 to probe their colocalization in late endosomes/lysosomes. The confocal imaging results suggested that HMGA-Oregon Green 488 and LysoTracker Red DND-99 signals were colocalized in their final destination, the late endosomes/lysosomes (Fig. 7).

HMGA1a HAS A SPECIFIC RECEPTOR ON THE CELL CYTOPLASMIC MEMBRANE

The observation that HMGA1a-dcNP undergoes receptor-mediate endocytosis suggests that there must be an HMGA1a receptor, possibly very specific to HMGA1a protein on the cell membrane. We decide to use a chemical cross-linker stratagem (Fig. 8A) to capture this specific receptor. The cross-linker, *N*-succinimidyl-6-[4′-azido-2′-nitrophenylamino]hexanoate (SANPAH), has dual functional groups: an activated ester and a photochemically reactive azido group. The succinimidyl activated esters reacted with a lysine residue on the HMGA1a-GFP fusion protein first and the resulting product, HMGA1a-GFP-SANPAH, was incubated with Hela cells for 15 min to bind the cell membrane receptor. UV illumination induced the azido group to undergo a "click" photochemistry reaction that snatched another lysine residue on the receptor protein on the live Hela cell membrane. After the cross-linking reaction, the cells were washed to remove unbound proteins and then lysed. An anti-GFP antibody was used to detect the covalently cross-linked product, HMGA1a-GFP-SANPAH-receptor, which Western blots determined that it had a size between 55 and 72 kDa (Fig. 8B).

SDS–PAGE results provided further proof that HMGA1a had a cell membrane receptor. Using a histidine tag, we further purified the cross-linked product, HMGA1a-GFP-SANPAH-receptor, from the cell lysate using an Ni-NTA column. Silver staining revealed that

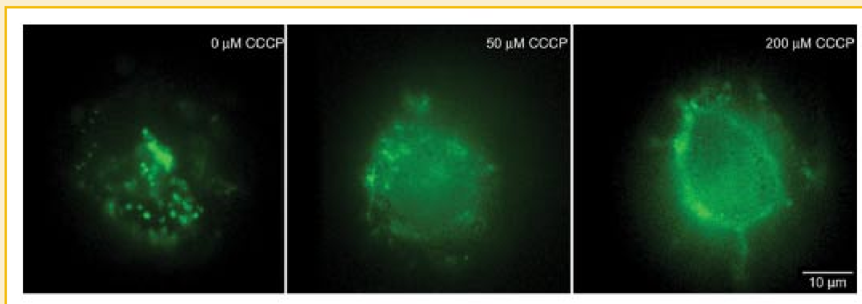

Fig. 5. The CCCP drug prevents Hela cells to internalize HMGA-dcNP. Hela cells were treated with the CCCP drug for 1 h at various concentrations, then incubated with the same amount HMGA-dcNP for 4 h, and finally imaged using a 488-nm laser. Without CCCP drug, Hela cells efficiently internalized many HMGA-dcNP within 4 h as revealed by the intracellular bright spots; however, 200 μM CCCP completely disabled ATP production and blocked the nanoparticles at the cell membrane even after 4 h, although HMGA-dcNP still effectively bound to the cell membrane, lighting up the cell contour. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the cross-linked product lane divulged a unique 66-kDa band on the SDS–PAGE gel, whereas the cell control and the fusion protein controls had no band at this region (Fig. 8C). This concludes that the HMGA1a receptor has a molecular weight of ~26 kDa after subtracting HMGA1a-GFP fusion protein molecular weight.

EXTRACELLULAR HMGA1a PROTEIN SUPPRESSES CELL VIABILITY

Because the HMGA1a protein has a specific plasma membrane receptor, one hypothesis is that extracellular HMGA1a protein may affect intracellular activity through this signaling receptor. To validate this hypothesis, we used MTT assay to measure the cell viability under different extracellular HMGA1a concentrations. The MTT Cell Proliferation Assay measures the cell proliferation rate and the cell viability; both reduce when the cells are under metabolic pressures such as apoptosis or necrosis. Experimental results revealed that when extracellular HMGA1a concentration increased, the cell viability decreased (Fig. 9A), implying the extracellular HMGA1a did influence the cell proliferation rate. This effect had a pattern of dose saturable characteristics, which also supported the receptor-mediated endocytosis hypothesis.

Measuring the cell growth curves demonstrated that HMGA1a protein unambiguously influences the growth of Hela cell. As shown in Figure 9B, the DMEM medium containing 10 μM HMGA1a significantly diminishes the growth of Hela cells. These results further support that extracellular HMGA1a protein suppresses Hela cell survivability.

However, does HMGA1a protein suppress cellular growth through apoptotic or necrotic mechanism? Apoptotic cells expose characteristic phosphatidylserine outside of their plasma membranes, whereas normal cells restrict their phosphatidylserine to cytosolic compartments. Cellular protein Annexin V binds phosphatidylserine specifically. Figure 9C reveals that Annexin V labeled with FITC binds to HMGA1a treated cells considerably more than untreated cells. UV-induced apoptosis also enhances Annexin V binding, and effectively serves as a positive control. Thus HMGA1a effectively induces apoptosis in Hela cells. Figure 9D exemplifies propidium iodide, which only penetrates necrotic or late apoptotic cellular membranes, also effectively stains the HMGA1a treated cells, but not untreated cells. Therefore propidium iodide membrane penetration also confirms that HMGA1a induces apoptosis in Hela cells.

DISCUSSION

HMGA proteins, the traditional nuclear architectural factors, typically execute their function inside the cell nuclei, mostly associating with DNA binding and gene regulation. HMGA1 proteins in murine NIH3T3 cells, however, have been recently discovered to translocate from the nuclei into the cytoplasm and mitochondria dynamically and periodically in accord with the cell cycle, implying a new role for HMGA1 protein to regulate mitochondria function [Dement et al., 2005, 2007].

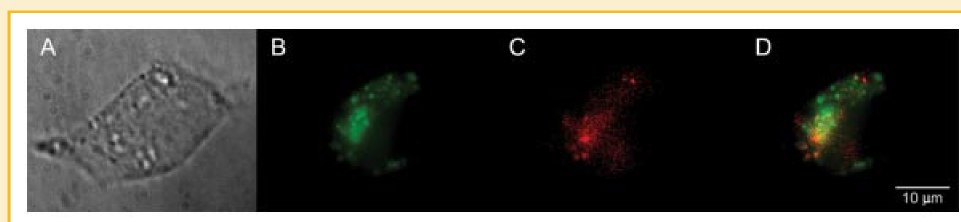

Fig. 6. HMGA-rcNP and GFP-rab11 protein partially colocalize. Hela cells (A) carrying pGFP-Rab11 plasmids expressed GFP-labeled early endosome marker protein Rab11, fluorescing green under 488-nm excitation (B); endocytosed HMGA-rcNPs emitted red fluorescence when excited by 365-nm illumination (C). Partial colocalization (overlaying B and C produces D) suggests that HMGA-rcNPs, after entering the cell for 4 h, have been departing early endosomes. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

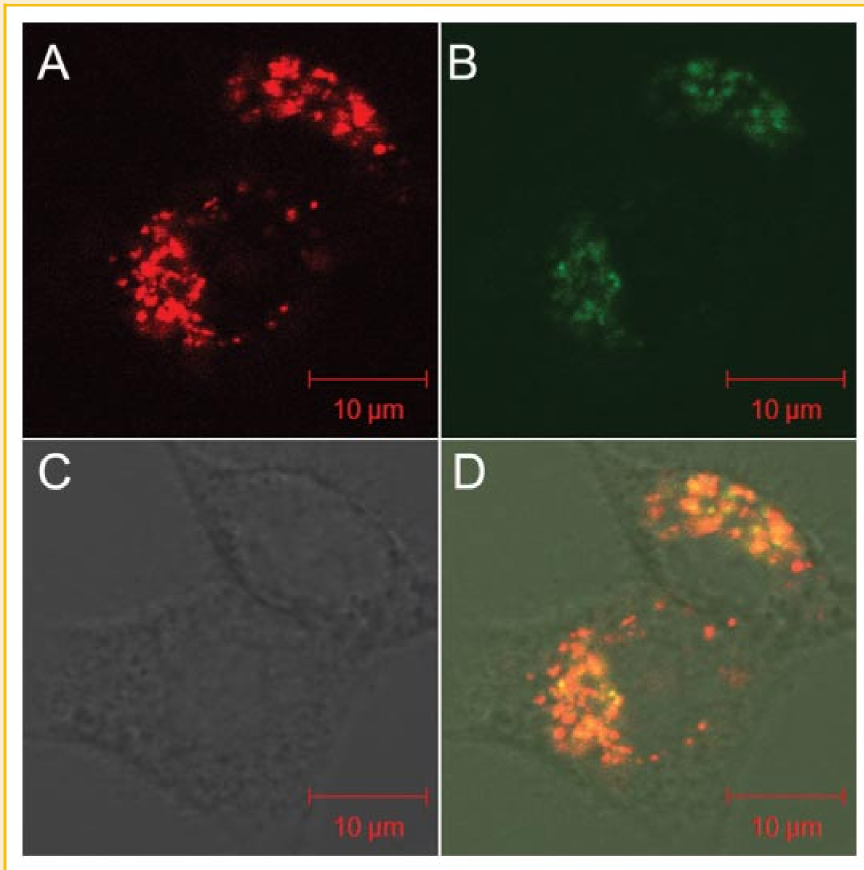

Fig. 7. HMGA-Oregon Green 488 and LysoTracker Red DND-99 colocalize in cells. Hela Cells and HMGA-Oregon Green 488 were incubated for 4 h before staining the cells with LysoTracker Red DND-99 for 10 min. Cells were then washed and imaged using a confocal microscope (Zeiss LSM510 META). LysoTracker red DND-99 was used to stain the lysosomes (A) of Hela cells (C), resulting red-fluorescing lysosomes. HMGA protein labeled by Oregon Green 488 dye had endocytosed into Hela cells for 4 h, thus fluorescing green (B). The good colocalization (overlaying A and B yields D) suggested that the departing HMGA proteins in Figure 6 had mostly arrived at the lysosomes. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

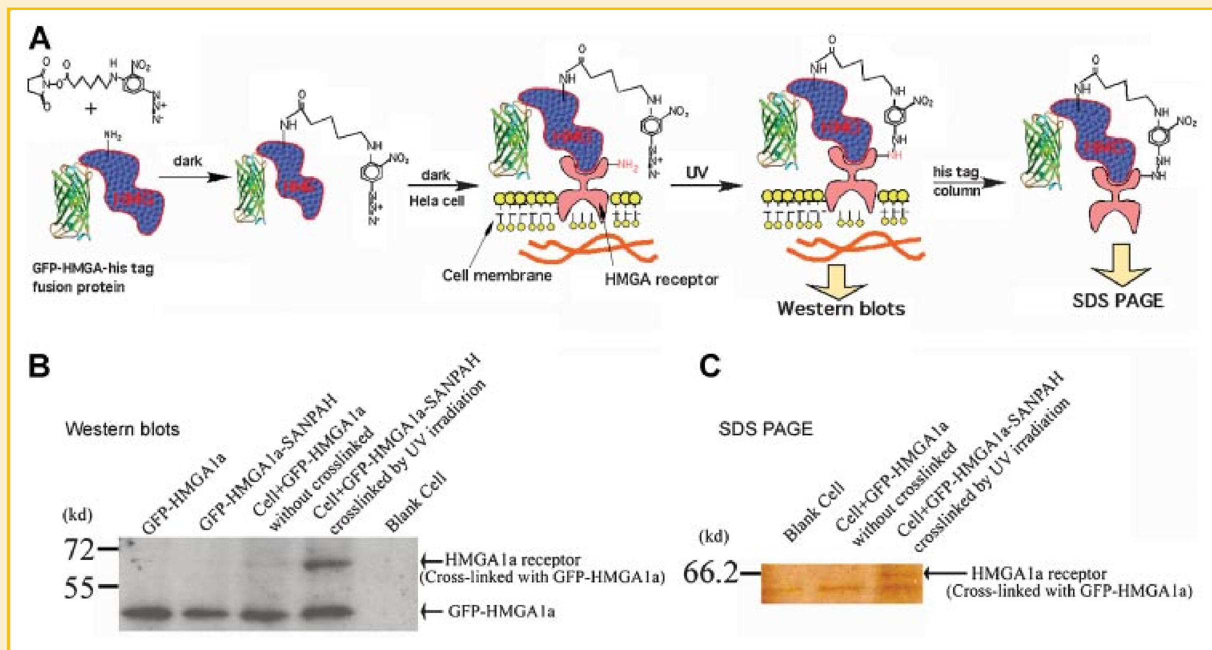

Fig. 8. Capture the HMGA1a specific plasma membrane receptor. GFP and HMGA1a fusion protein reacts with an activated ester bearing a photo-activated cross-linker in dark and the resulting product seeks and binds to the receptor on the Hela cell membrane (A). Photo-activation covalently bonds the GFP-HMGA1a to the receptor (A) and the captured receptor together with GFP-HMGA1a reveals a new 66-kDa band in Western Blots (B) and SDS-PAGE (C). All control lanes are negative in this region. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

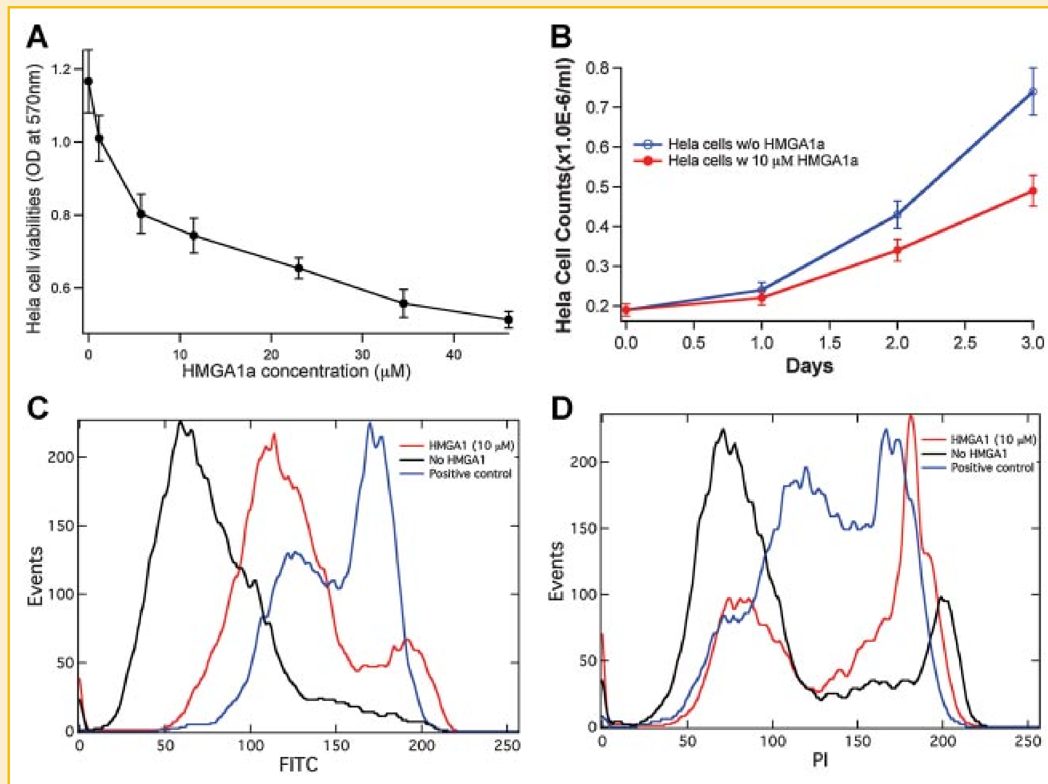

Fig. 9. Extracellular HMGA1a protein affects cell viability. A: When HMGA1a protein was added extracellularly, MTT assay demonstrated that the proliferation rate of Hela cells or their viability abated monotonously as the HMGA1a concentration increased, suggesting that extracellular HMGA1a protein inhibited the growth of Hela cells. Four to six independent experiments were performed, and the average results with standard deviation bars were plotted here. B: The growth rate of Hela cells cultured with 10 μM HMGA1a (red line) was considerably slower than the Hela cells cultured without HMGA1a (blue line). C: Annexin V-FITC was used to bind to Hela cells (black line), HMGA1a treated Hela cells (red line), and UV-irradiated Hela cells (blue line). Flow cytometry reveals that HMGA1a treated cells are characteristically brighter than normal cells because HMGA1a-binding induces apoptosis and thus increases phosphatidylserine or Annexin V-FITC-binding sites at the outer membranes. D: Same as C except propidium iodide, which stains the nuclear DNA of dead cells, was used to replace Annexin V-FITC. HMGA1a treated cells are divided into two categories, probably corresponding to early (left peak) and late (right peak) apoptotic cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Those findings expand the utilities of these small nuclear proteins, and promote us to investigate other functions HMGA1 proteins may have when they leave cell nuclei. HMGA1a protein belongs to the HMG superfamily, and its superfamily member HMGB1 protein behaves like a cytokine extracellularly to many cell types, sending signals to stimulate growth [Passalacqua et al., 1997; Lotze and Tracey, 2005]. Logic reasoning leads the hypothesis that HMGA proteins may have similar extracellularly activities.

Our results reveal that extracellular HMGA1a protein significantly suppresses Hela cell's viability. Inside cell nuclei, HMGA1a cooperates with other transcriptional factors, such as NF-κB, AP1, and stimulates the gene transcription that keeps cell proliferating and prevents the cell from dying [Perrella et al., 1999; Bouallaga et al., 2003]. When interacting with the p53 protein, HMGA1a protein inhibits its tumor suppressor function and prevents cell from apoptosis [Pierantoni et al., 2006]. Generally, nuclear HMGA proteins stimulate gene expression and promote cell proliferation; thus HMGA proteins are named as cancer biomarker. Contrarily, our findings suggest that extracellular HMGA1a protein suppresses Hela cell's viability, a role opposite of its nuclear function. HMGA1 protein does not have any secretary motif, and thus cannot be transported to the extracellular environment through secretion pathways. One possible mechanism is that HMGA1-expressing cells release its nucleus content including HMGA1 into the extracellular environment after undergoing apoptosis or necrosis. This might be a regulation mechanism after cellular damage for HMGA1-expressing cells. Further evidences revealed that extracellular HMGA1a protein could not enter the cell nucleus, hinting that HMGA extracellular role might be signaling. Ironically, although both HMGB1 protein and HMGA1 proteins function as signaling molecules, HMGB1 stimulates cell growth, whereas HMGA1 proteins suppress cell growth and proliferation.

Live cell imaging experiments demonstrated that HMGA1a conjugates or HMGA1a functionalized nanoparticles could not enter the cell nucleus. Although HMGA1a protein labeled Oregon Green 488 dyes or HMGA1a-dcNP in extracellular medium were readily endocytosed into live cells, their final destination located at the late endosomes/lysosomes where their nuclear functions were prohibited. However, extracellular HMGA1a proteins do reduce the Hela cell survivability and such a mechanism must have involved signal transduction pathways.

Signal transduction usually involves a signaling molecule and its membrane receptor. When an extracellular signal molecule binds to its membrane receptor, signals will be transmitted from outside to inside of the cell through mechanisms such as changing the receptor conformation or protein self-assembly formation. One typical example is the epidermal growth factor (EGF) and its receptor (EGFR). The binding of EGF to EGFR activates downstream signaling pathways such as the MAPK and PI3K/Akt pathways, thus regulating the cell development and tumorigenesis [Jorissen et al., 2003]. Once bound, the EGF-EGFR complex will be endocytosed very quickly and the endocytosis in-turn rapidly depletes the receptor density on the cell membrane [Wiley et al., 1991]. Live-cell imaging reveals that the HMGA1a endocytosis shares striking similar characteristics. When added extracellularly, the labeled HMGA1a protein attached the cell quickly, and underwent a typical endocytosis process. The HMGA1a endocytosis typically began within the first 15 min of binding and microtubule-dependent transportation of the HGMA1a protein was observed continuously up to 4 h. After 4 h, most HMGA1a cargoes arrived at their final destinations—late endosomes/lysosomes and mostly ceased moving. A non-specific protein like bovine serum albumin does not inhibit the uptake of HMGA1 protein labeled with Oregon-Green, suggesting that the endocytic mechanism is not fluid-phase, non-specific endocytosis. Competition between labeled and unlabeled HMGA1 proteins reveals that the presence of 10-times unlabeled HMGA1 (100 μM) produced a reproducible and measurable difference in the uptake amount of labeled HMGA1 (10 μM). These observations strongly support that the receptor-mediated endocytosis on the Hela cell membrane coordinates, facilitates, and finally internalizes the HMGA1a cargoes, implying that HMGA1a protein has a new signaling role.

HMGA1a behaves differently than small cell penetrating peptides. Some small basic peptides such as TAT, penetratin and VP22 penetrate live cell membranes and enter into the cytoplasma, and therefore named as cell penetrating peptide (CPP). Actively being debated, the cell penetrating mechanisms are unfortunately unclear. For instance, Wadia et al. [2004] reported that TAT-fusion proteins were internalized by lipid raft-dependent macropinocytosis, Fittipaldi et al. [2003] demonstrated that TAT fusion proteins were internalized through a temperature-dependent endocytic pathway that originated from cell membrane lipid rafts following caveolar endocytosis, and Richard et al. [2005] explained that TAT peptide underwent heparan sulfate receptors mediated clathrin-dependent endocytosis. Although HMGA1a is also a small basic protein, its endocytosis mechanism shares little resemblance to the abovementioned peptides. First, most CPPs can escape endosomes and find their way into the cell nucleus, whereas most HMGA1a conjugates only arrives at late endosomes/lysosomes. Second, no report claims that CPPs have any effects on cell viability, or suggest that CPPs act as signaling molecules, but extracellular HMGA1a can inhibit the proliferation of cancer cells such as the Hela cells.

Hariton-Gazal et al. [2003] reported that extracellular histone also had the ability to penetrate the cell membrane. Histone proteins are the major proteins in cell nuclei, where histone H1 and HMGA protein compete for the same DNA binding site of chromatin [Reeves and Beckerbauer, 2001]. However, cellular uptake of the histone proteins differs significantly from the mechanism of HMGA1a protein uptake. HMGA1a protein undergoes endocytosis, whereas histones directly translocate through the cell plasma membrane, involving no endocytic pathway. Because both are basic protein, the dramatically different mechanisms that they utilize to enter cells suggest that HMGA1a cellular uptake are not merely due to its positive charges generated by basic amino acid residues, but rather it participates specific binding to and signaling via its receptor. Thus far, histone proteins have not been reported to have signaling effects, but HMGA1a does.

As a signal molecule, the HMGB1 protein uses its membrane receptor RAGE (receptor for advanced glycation end products) to stimulate cell growth [Bidwell et al., 2007]. As a signal molecule, HMGA1a protein suppresses cell growth and must have its own receptor as well. To search such possible HMGA1a receptor, we use a photoactivated protein cross-linker, attempting to capture such a membrane signaling receptor. Sparatore et al. [2002] used similar strategies to demonstrate a new HMGB1 receptor co-existed with RAGE. In our experiments, we use the GFP-HMGA1a fusion protein instead of HMGA1a protein to cross-link the elusive receptor. One reason to use GFP-HMGA1a fusion protein is that live cells internalize the fusion protein in a similar way as they internalize HMGA1a protein alone, indicating both interact with the receptor. Another reason is that when identified the cross-linked proteins by Western blots, anti-GFP antibody is extremely specific and has little background, whereas anti-HMGA1a antibody may cross-react with other proteins such as histone proteins. Using this method, we identified a 26-kDa HMGA1a receptor; this discovery hints that the old nuclear protein may have new biological roles, where HMGA1a protein functions beyond the cell nuclei.

What is the signaling role of HMGA1a? Flow cytometry results suggest that HMGA1a functions as a death ligand. The presence of extracellular HMGA1a effectively causes cancerous Hela cells to undergo apoptotic pathways. These results agree with aforementioned observation that extracellular HMGA1a suppresses Hela cell growth and the mechanism of such suppression of HMGA1a appears to send apoptotic signals rather than inhibit cell division.

In conclusion, we report a new phenomenon that Hela cells readily internalize HMGA1a protein using a receptor-mediated endocytic pathway and extracellular HMGA1a protein induces Hela cells to undergo apoptosis. These results imply a new role for HMGA1a in regulating cell proliferation and expand our knowledge regarding this traditional nuclear architectural factor. Further work will identify the HMGA1a receptor and the HMGA1a signaling pathway and its mechanism.

ACKNOWLEDGMENTS

The authors acknowledge the support of National Institute of General Medicine Sciences (Grant GM065306). We thank Dr. R. Reeves for providing *E. coli* strain BL21 carrying pET7C-hHMG-I, which expresses human HMGA1 proteins and Dr. A. Wandinger-Ness for offering pEGFP-Rab11 plasmid expressing GFP labeled Rab11 protein. Additionally, we thank Dr. J.K. Hurst for many helpful discussions.

REFERENCES

Adair JE, Kwon Y, Dement GA, Smerdon MJ, Reeves R. 2005. Inhibition of nucleotide excision repair by high mobility group protein HMGA1. J Biol Chem 280:32184–32192.

Bidwell JP, Yang J, Robling AG. 2007. Is HMGB1 an osteocyte alarmin? J Cell Biochem 103:1671–1680.

Bouallaga I, Teissier S, Yaniv M, Thierry F. 2003. HMG-I(Y) and the CBP/p300 coactivator are essential for human papillomavirus type 18 enhanceosome transcriptional activity. Mol Cell Biol 23:2329–2340.

Chen W, Feng Y, Chen D, Wandinger-Ness A. 1998. Rab11 is required for trans-golgi network-to-plasma membrane transport and a preferential target for GDP dissociation inhibitor. Mol Biol Cell 9:3241–3257.

Dement GA, Treff NR, Magnuson NS, Franceschi V, Reeves R. 2005. Dynamic mitochondrial localization of nuclear transcription factor HMGA1. Exp Cell Res 307:388–401.

Dement GA, Maloney SC, Reeves R. 2007. Nuclear HMGA1 nonhistone chromatin proteins directly influence mitochondrial transcription, maintenance, and function. Exp Cell Res 313:77–87.

Ferranti P, Malorni A, Marino G, Pucci P, Goodwin GH, Manfioletti G, Giancotti V. 1992. Mass spectrometric analysis of the HMGY protein from Lewis lung carcinoma. Identification of phosphorylation sites. J Biol Chem 267:22486–22489.

Fittipaldi A, Ferrari A, Zoppe M, Arcangeli C, Pellegrini V, Beltram F, Giacca M. 2003. Cell membrane lipid rafts mediate caveolar endocytosis of HIV-1 Tat fusion proteins. J Biol Chem 278:34141–34149.

Giancotti V, Buratti E, Perissin L, Zorzet S, Balmain A, Portella G, Fusco A, Goodwin GH. 1989. Analysis of the HMGI nuclear proteins in mouse neoplastic cells induced by different procedures. Exp Cell Res 184:538–545.

Hariton-Gazal E, Rosenbluh J, Graessmann A, Gilon C, Loyter A. 2003. Direct translocation of histone molecules across cell membranes. J Cell Sci 116: 4577–4586.

Hock R, Furusawa T, Ueda T, Bustin M. 2007. HMG chromosomal proteins in development and disease. Trends Cell Biol 17:72–79.

Jorissen RN, Walker F, Pouliot N, Garrett TP, Ward CW, Burgess AW. 2003. Epidermal growth factor receptor: Mechanisms of activation and signalling. Exp Cell Res 284:31–53.

Liu F, Chau KY, Arlotta P, Ono SJ. 2001. The HMG I proteins: Dynamic roles in gene activation, development, and tumorigenesis. Immunol Res 24:13–29.

Lotze MT, Tracey KJ. 2005. High-mobility group box 1 protein (HMGB1): Nuclear weapon in the immune arsenal. Nat Rev Immunol 5:331–342.

Passalacqua M, Zicca A, Sparatore B, Patrone M, Melloni E, Pontremoli S. 1997. Secretion and binding of HMG1 protein to the external surface of the membrane are required for murine erythroleukemia cell differentiation. FEBS Lett 400:275–279.

Perrella MA, Pellacani A, Wiesel P, Chin MT, Foster LC, Ibanez M, Hsieh CM, Reeves R, Yet SF, Lee ME. 1999. High mobility group-I(Y) protein facilitates nuclear factor-kappaB binding and transactivation of the inducible nitric-oxide synthase promoter/enhancer. J Biol Chem 274:9045–9052.

Pierantoni GM, Rinaldo C, Esposito F, Mottolese M, Soddu S, Fusco A. 2006. High Mobility Group A1 (HMGA1) proteins interact with p53 and inhibit its apoptotic activity. Cell Death Differ 13:1554–1563.

Pierantoni GM, Esposito F, Giraud S, Bienvenut WV, Diaz JJ, Fusco A. 2007. Identification of new high mobility group A1 associated proteins. Proteomics 7:3735–3742.

Raucci A, Palumbo R, Bianchi ME. 2007. HMGB1: A signal of necrosis. Autoimmunity 40:285–289.

Reck-Peterson SL, Yildiz A, Carter AP, Gennerich A, Zhang N, Vale RD. 2006. Single-molecule analysis of dynein processivity and stepping behavior. Cell 126:335–348.

Reeves R. 2003. HMGA proteins: Flexibility finds a nuclear niche? Biochem Cell Biol 81:185–195.

Reeves R, Beckerbauer L. 2001. HMGI/Y proteins: Flexible regulators of transcription and chromatin structure. Biochim Biophys Acta 1519:13–29.

Richard JP, Melikov K, Brooks H, Prevot P, Lebleu B, Chernomordik LV. 2005. Cellular uptake of unconjugated TAT peptide involves clathrin-dependent endocytosis and heparan sulfate receptors. J Biol Chem 280:15300–15306.

Rogalla P, Drechsler K, Schroder-Babo W, Eberhardt K, Bullerdiek J. 1998. HMGIC expression patterns in non-small lung cancer and surrounding tissue. Anticancer Res 18:3327–3330.

Sgarra R, Tessari MA, Di Bernardo J, Rustighi A, Zago P, Liberatori S, Armini A, Bini L, Giancotti V, Manfioletti G. 2005. Discovering high mobility group A molecular partners in tumour cells. Proteomics 5:1494–1506.

Sparatore B, Pedrazzi M, Passalacqua M, Gaggero D, Patrone M, Pontremoli S, Melloni E. 2002. Stimulation of erythroleukaemia cell differentiation by extracellular high-mobility group-box protein 1 is independent of the receptor for advanced glycation end-products. Biochem J 363:529–535.

Terada H. 1981. The interaction of highly active uncouplers with mitochondria. Biochim Biophys Acta 639:225–242.

Wadia JS, Stan RV, Dowdy SF. 2004. Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. Nat Med 10:310–315.

Wiley HS, Herbst JJ, Walsh BJ, Lauffenburger DA, Rosenfeld MG, Gill GN. 1991. The role of tyrosine kinase activity in endocytosis, compartmentation, and down-regulation of the epidermal growth factor receptor. J Biol Chem 266:11083–11094.

Zhu MQ, Zhu L, Han JJ, Wu W, Hurst JK, Li AD. 2006. Spiropyran-based photochromic polymer nanoparticles with optically switchable luminescence. J Am Chem Soc 128:4303–4309.

Zhu L, Wu W, Zhu MQ, Han JJ, Hurst JK, Li AD. 2007. Reversibly photoswitchable dual-color fluorescent nanoparticles as new tools for live-cell imaging. J Am Chem Soc 129:3524–3526.