Intracellular Hydrogen Peroxide Production Is an Upstream Event in Apoptosis Induced by Down-Regulation of Casein Kinase 2 in Prostate Cancer Cells

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Abstract

We have shown previously that down-regulation of CK2 activity (protein kinase CK2, formerly casein kinase 2) by employing its inhibitors apigenin or 4,5,6,7-tetrabromobenzotriazole promotes apoptosis in prostatic carcinoma cells. In an effort to define the downstream mediators of this action, we show that cell apoptosis observed on down-regulation of CK2 is preceded by intracellular generation of hydrogen hydroxide (H₂O₂) in various normal and cancer cells. In this regard, both androgen-dependent ALVA-41 and androgen-independent PC-3 cells treated with 80 μmol/L apigenin or 4,5,6,7-tetrabromobenzotriazole or with antisense CK2α oligonucleotide or small interfering RNA respond similarly to down-regulation of CK2.

Interestingly, whereas chemical inhibitors of CK2 elicited H₂O₂ production in both cancer and noncancer cells, the antisense CK2α-mediated down-regulation of CK2 showed significant H₂O₂ production in cancer cells but had minimal effect in noncancer cells. The basis of this key difference is unclear at present, but this observation may have implications for the therapeutic potential of antisense CK2 oligonucleotide in cancer therapy. The H₂O₂ production induced by antisense CK2α was associated with robust caspase-3 activity, nuclear factor-κB nuclear translocation, cytochrome c release, and subsequent DNA fragmentation in prostate cancer cells (ALVA-41 and PC-3). These findings describe, for the first time, a relationship between CK2 and reactive oxygen species, such that CK2 inhibition leads to production of intracellular H₂O₂, which may serve as a downstream mediator of apoptosis in cancer cells. (Mol Cancer Res 2006;4(5):331–8)

Introduction

Protein kinase CK2 (formerly casein kinase 2 or II) is a ubiquitous protein serine/threonine kinase consisting of three subunits α, α’, and β (40, 38, and 28 kDa, respectively) present as α2β2, αα’β2, or α2β2 expressed differently in various cells. CK2 is a multifunctional protein kinase involved in several cellular functions, including cell growth and proliferation (for reviews, see, e.g., refs. 1-6). Cellular level of CK2 in different cell types remains fairly constant, except that it is elevated in normal cells during proliferation. However, CK2 has been found to be consistently dysregulated (elevated) in cancer cells, which has prompted much interest in studying its function in cancer pathobiology (see e.g., ref. 5). CK2 is diffusely localized in both the cytoplasm and nucleus in normal cells; however, it is more concentrated in the nucleus in cancer cells (7). In addition, the elevation of CK2 in cancer cells is not simply a reflection of higher proliferative activity but rather an indicator of the pathologic status of the tumor (7).

Our previous work suggested that a mode of CK2 regulation in the cell in response to diverse stimuli was to undergo dynamic shuttling in and out of the nucleus. Our results showed that growth stimuli promoted the shuttling of CK2 to the nucleus where nuclear matrix and chromatin served as its preferential targets. Removal of growth or survival stimuli resulted in dramatic loss of the CK2 from nuclear matrix and chromatin and was associated with induction of apoptosis (8-10). Further, down-regulation of CK2 by inhibitors of CK2, such as 4,5,6,7-tetrabromobenzotriazole (TBB), or molecular agents, such as antisense CK2α oligonucleotide, results in induction of apoptosis (11, 12). We also provided evidence that chemical-mediated apoptosis (such as that induced by etoposide or diethylstilbestrol) was suppressed by prior forced overexpression of CK2 (13). Together, these observations provided the evidence that CK2, besides its role in promotion of cell growth, may have a broad function in regulation of apoptosis. This conclusion is further supported by our recent work showing that overexpression of CK2 can suppress death receptor-mediated apoptosis, whereas inhibition of CK2 augments cell death mediated by agents, such as tumor necrosis factor–related apoptosis-inducing ligand (11, 14). Because cancer cells show a dysregulation not only of proliferation but also of apoptosis (15), the ability of CK2 to act as a suppressor of apoptosis suggests a potential...
mode of its involvement in the cancer cell phenotype. These various considerations provide a strong impetus for investigation of the downstream apoptosis related loci that may be affected by CK2.

Several recent reports suggest a role of reactive oxygen species (ROS) in drug-induced apoptosis, including the engagement of downstream proteins involved in the execution of apoptosis (see, e.g., refs. 16-19). Intracellular generation of hydrogen peroxide (H$_2$O$_2$), the most stable of ROS, has been considered an important mediator of apoptosis, and even exogenous addition of H$_2$O$_2$ is a potent activator of the apoptosis machinery (17-19). Thus, we decided to determine cellular dynamics of H$_2$O$_2$ in response to apoptosis induced by down-regulation of CK2 in cancer cells. For these studies, we have employed prostate cancer cells as our experimental model. Prostate cancer is a major cause of death among males. Initially, the cancer cells are sensitive to androgens; however, therapeutic androgen ablation eventually leads to emergence of the androgen-insensitive phenotype of prostate cancer, which is resistant to therapy (see, e.g., ref. 20). Accordingly, for our studies, we have employed both androgen-sensitive and androgen-insensitive prostate cancer cells. In this report, we show for the first time an involvement of H$_2$O$_2$ in induction of apoptosis as a result of CK2 down-regulation in cancer and noncancer cells. We show that down-regulation of CK2 results in significant generation of intracellular H$_2$O$_2$ and that the known downstream targets of H$_2$O$_2$ signaling, such as nuclear factor-κB (NF-κB), cytochrome c, and caspase-3, are activated with consequent DNA fragmentation. Importantly, cancer and noncancer cells show a differential response to production of H$_2$O$_2$ when chemical inhibitors versus antisense CK2$\alpha$ oligonucleotide are employed for down-regulation of CK2.

Results

Effect of Inhibition of CK2 on H$_2$O$_2$ Production in Prostate Cancer Cells

To investigate the effects of CK2 down-regulation on prostate cancer cells, we employed androgen-sensitive ALVA-41 and androgen-independent PC-3 cells. We used the relatively specific chemical inhibitors of CK2, such as apigenin and TBB. Of these, TBB is a more specific inhibitor. We also employed antisense CK2$\alpha$ oligonucleotide or small interfering RNA (siRNA)-CK2$\alpha$ to achieve molecular down-regulation of CK2 (11, 12, 14). In one series of experiments, ALVA-41 cells were treated with 80 μmol/L TBB or 80 μmol/L apigenin, and H$_2$O$_2$ production was determined. Under these conditions, apigenin and TBB cause significant inhibition of CK2 activity and are known to induce apoptosis in these cancer cells (11, 12). Both apigenin and TBB induced intracellular H$_2$O$_2$ production as early as 6 hours after treatment as indicated by the rightward shift in log fluorescence on flow cytometry (Fig. 1A and B) and subsequent induction of apoptosis (data not shown). As shown in Fig. 2A, molecular down-regulation of CK2 with the apoptosis-inducing concentration of antisense CK2$\alpha$ oligonucleotide (2 μg/ml; ref. 9) had a similar effect on H$_2$O$_2$ production in prostate cancer cells. No significant shift in log fluorescence was detected for samples treated with nonsense CK2$\alpha$ oligonucleotide or N-[1-(2,3-diololesterol)propyl]-N,N,N-trimethylammoniummethyl sulfate (DOTAP) controls (Fig. 2B and C). Similar observations were made in the androgen-insensitive PC-3 cells (data not shown). Together, these data show that chemical or molecular down-regulation of CK2 results in intracellular generation of H$_2$O$_2$.

Comparison of Intracellular H$_2$O$_2$ Production on Molecular Down-Regulation of CK2 in Cancer versus Noncancer Cells

We determined whether the cancer and noncancer cells responded similarly to down-regulation of CK2 with respect to
production of H$_2$O$_2$. Prostatic cancer ALVA-41 and PC-3 cells, nontumorigenic prostate BPH-1 cells, and noncancerous cell line Chinese hamster ovary (CHO) were equally responsive to treatment with apoptosis-inducing concentrations of apigenin or TBB as indicated by the increase in intracellular H$_2$O$_2$ production. However, treatment of these cells with antisense CK2α oligonucleotide showed a distinctly differential response in cancer versus noncancer cells, such that although the H$_2$O$_2$ production was comparable in cancer (ALVA-41 and PC-3) and noncancer (BPH-1 and CHO) cells in response to apigenin or TBB it was minimal in noncancer cells treated with antisense CK2α oligonucleotide (Fig. 3A). Use of the pSilencer-siRNA-CK2α as another method to down-regulate CK2 in the above-mentioned tumorigenic and nontumorigenic cell lines gave results analogous to those observed for antisense CK2α. Both prostate cancer cells (ALVA-41 and PC-3) showed an increase in H$_2$O$_2$ production, although the production in ALVA-41 cells was greater and somewhat delayed in the p53-deficient PC-3 cells. However, the noncancer BPH-1 and CHO cells failed to produce any H$_2$O$_2$ in response to treatment of cells with the siRNA-CK2α as was observed for antisense CK2α oligonucleotide (Fig. 3B). It may be noted that the production of H$_2$O$_2$ by antisense CK2α oligonucleotide treatment was somewhat greater than that by siRNA-CK2α treatment in cancer cells; however, BPH-1 and CHO cells responded similarly to both type of treatments.

Molecular Inhibition of CK2 in Prostate Cancer Cells Induces Apoptosis Downstream of H$_2$O$_2$ Production

Having established that inhibition of CK2 resulted in intracellular H$_2$O$_2$ production, we addressed whether the H$_2$O$_2$ production preceded apoptosis and whether engagement of targets, such as NF-κB and cytochrome c, was upstream or downstream of H$_2$O$_2$ production. Spectrofluorimetric analysis showed that treatment of cells with 2 μg/mL antisense CK2α oligonucleotide induced a robust caspase-3 activation associated with H$_2$O$_2$ production in ALVA-41 cells (Fig. 4A). Western blot analysis for NF-κB and IκB revealed that p65 subunit of NF-κB translocated to the nucleus, whereas IκB degradation was noted in cytosolic fractions at 24 hours in response to antisense treatment, suggesting that these events were downstream of H$_2$O$_2$ production. The release of cytochrome c coincided with the NF-κB translocation and seemed to be downstream of H$_2$O$_2$, suggesting that mitochondria were engaged in induction of apoptosis in response to CK2 inhibition (Fig. 4B).

A cell survival assay using WST-1 showed that ≥50% of the ALVA-41 and PC-3 cells succumbed to death (Fig. 5A and C, respectively). To confirm that this cell death was due to apoptosis, we stained the cells with propidium iodide and analyzed them for DNA fragmentation by flow cytometry. At 24 hours of treatment with the antisense CK2α, significant apoptosis was indicated by the increase in sub-G$_1$ fraction in ALVA-41 (Fig. 5B) and PC-3 (Fig. 5D) cells. There was minimal change in percentage of cells in G$_1$, S, and G$_2$-M phases of cell cycle, suggesting that the cells had undergone complete apoptosis in response to the antisense. Induction of apoptosis by antisense CK2α in BPH-1 and CHO was minimal (data not shown), which accorded with our previous findings (9, 21). Although both androgen-dependent and androgen-independent cell lines showed sensitivity to antisense CK2α,
ALVA-41 cells were relatively more sensitive than PC-3 cells. This may be explained by PC-3 cells being deficient in p53, which is known to transcriptionally regulate proapoptotic molecules (22).

Discussion

In this report, we have examined the dynamics of H$_2$O$_2$ production in response to down-regulation of CK2. Because down-regulation of CK2 results in induction of apoptosis (8, 9, 11, 12), the downstream production of intracellular H$_2$O$_2$ suggests a possible downstream pathway by which cancer cells may undergo apoptosis in response to down-regulation of CK2. Much evidence has implicated H$_2$O$_2$ as a mediator in drug-induced apoptosis (17-19, 23, 24). No previous information exists on the relation of protein kinase CK2 and the intracellular redox function in cells. Based on various observations employing CK2 inhibitors (apigenin and TBB) and antisense CK2α oligonucleotide at apoptosis-inducing concentrations in cancer cells, our data suggest that production of H$_2$O$_2$ precedes the initiation of apoptosis upstream of mitochondria. These observations suggest that H$_2$O$_2$ production under these conditions is not simply owing to the accumulation of ROS over time in cells as a consequence of their death.

It has been hypothesized that ROS signaling is targeted at certain cellular signal transduction components and is not random (25). Among the known downstream targets of H$_2$O$_2$ signaling is NF-κB (26, 27). In the present report, we have documented that NF-κB activation is indeed a target for antisense CK2α oligonucleotide after H$_2$O$_2$ production. In addition, our data suggest that the H$_2$O$_2$ production was upstream of mitochondria, such that cytochrome c release was an event after the production of H$_2$O$_2$. These observations suggest that H$_2$O$_2$ produced on down-regulation of CK2 may lead to activation of mitochondrial pathway to apoptosis.

One of the major challenges in antitumor therapy is to develop strategies that promote death of cancer cells while sparing normal cells. Based on the emerging knowledge of apoptosis signaling, novel treatments have been considered for induction of tumor-specific cell death (28). Understanding
the physiologic differences between normal and cancer cells with respect to generation of ROS may offer an important approach to preferentially induce cancer cell death by using ROS. It has been suggested that because cancer cells are more active than normal cells the intrinsic oxidative stress in these cells could be exploited for generating ROS (such as H₂O₂) for inducing apoptosis (29). However, overwhelming the cells with generation of ROS could also lead to nonapoptotic death or necrosis that induces an inflammatory response in normal cells (30, 31). Thus, it would be important to devise conditions that would generate ROS to induce apoptosis in cancer cells as a primary mode of cell death.

An important outcome of the present study may be that antitumor strategies employing a combination approach for CK2 down-regulation and ROS production may prove to be an even more effective strategy than a single modality for killing tumor cells. Recently, androgen-sensitive LNCaP prostate cancer cells have been shown to be redox regulated by genes, such as Nox (NADPH oxidases; ref. 32). Further, these authors emphasized that CK2 is the p47(phox) kinase and that phosphorylation of p47(phox) by CK2 regulates the deactivation of NADPH oxidase (32). These observations are particularly germane to studies of redox regulation by CK2 as suggested by the present work. It may be noted that our findings on the generation of H₂O₂ on down-regulation of CK2 pertain to both androgen-sensitive and androgen-insensitive prostate cancer cells. This is important because the latter phenotype of prostate cancer is particularly resistant to therapy. We have documented previously that CK2 signal is equally functional in both types of prostate cancer (33) and as shown here equally amenable to generation of H₂O₂ with potential therapeutic implications. Further, it may be noted that PC-3 cell line is p53 deficient; however, the response of the two cell lines (ALVA-41 and PC-3) to induction of apoptosis on down-regulation of CK2 and production of H₂O₂ is analogous, suggesting that the effects mediated by CK2 down-regulation may be independent of the p53 status in these cells. Together, our findings hint at the involvement of CK2 in modulation of ROS and ROS-regulated genes in various cells studied here. In addition, we are investigating the effects of down-regulation of CK2 on H₂O₂ production in several other cancer cell lines, which suggest similar results as obtained for prostate cancer cells. Studies are in progress in our laboratory to define the mechanism(s) that govern signal transduction activated via H₂O₂ on down-regulation of CK2.

In previous work, we documented that whereas chemical inhibitors of CK2 induced apoptosis in both normal and cancer cells the use of antisense CK2α oligonucleotide was relatively more effective toward the cancer cells than normal or benign cells (9, 11, 12, 22). The present work also shows that antisense CK2α oligonucleotide induces H₂O₂ production preferentially in cancer cells compared with that in normal cells, whereas such a differential response is not apparent when chemical inhibitors of CK2 (apigenin and TBB) are employed to down-regulate CK2 activity. Although the basis of this difference is unclear at present, an important implication of these observations is that antisense CK2α oligonucleotide may be useful in targeting CK2 in vivo for achieving cancer therapy

### Figure 4

Antisense CK2α induces caspase-3 activation, NF-κB translocation, and cytochrome c release in cancer cells. ALVA-41 cells (0.25 × 10⁶) were plated overnight in six-well plates followed by treatment with 2 μg/mL nonsense CK2 oligonucleotide and 2 μg/mL antisense CK2α oligonucleotide using a DNA/DOTAP ratio as described in Fig. 2. A, Caspase-3 activity was determined at 4 to 24 hours as described in Materials and Methods. B, Western blot analysis of NF-κB translocation, IκB degradation, and cytochrome c release was done at 18 hours of treatment as described in Materials and Methods. Lane a, control; lane b, antisense CK2α oligonucleotide treated. β-Actin served as the loading control.

1 In preparation.
without significant effect on the normal cells. Although our observations suggest an apparent pharmacologic window, it is necessary to consider specific strategies to deliver antisense CK2α preferentially to the tumor cells in vivo because CK2 is present ubiquitously and is essential for cell survival. We have already initiated such studies by employing specifically designed nanocapsules based on tenfibgen as the capsule material to serve as a vehicle for carrying antisense CK2α to tumors (12, 22, 34, 35).

In summary, this is the first report to link generation of intracellular H2O2 to induction of apoptosis mediated by down-regulation of CK2 in both androgen-sensitive and androgen-insensitive prostate cancer cells. Production of H2O2 may trigger apoptosis associated with action on target genes, such as NF-κB and cytochrome c. It is of particular interest that whereas chemical inhibition of CK2 evokes production of H2O2 in both cancer and noncancer cells to a similar extent antisense CK2α oligonucleotide-mediated down-regulation of CK2 exerts a preferential effect on generation of H2O2 in cancer cells with a minimal effect on noncancer cells. These observations may have additional implications concerning the usefulness of CK2 as a target for cancer therapy (22, 34, 35).

Materials and Methods

Cell Lines and Reagents

Prostate cancer monolayer cell line, PC-3, was maintained in RPMI 1640 (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum and 2 mmol/L L-glutamine and grown in standard T-75 flasks. Similarly, ALVA-41 cells were grown in RPMI 1640 supplemented with 6% fetal bovine serum and 2 mmol/L L-glutamine. CHO cell line was purchased from Clontech (Palo Alto, CA) and maintained in Ham’s F-12 supplemented with 2 mmol/L L-glutamine.
l-glutamine and 6% fetal bovine serum. BPH-1 cell line (immortalized benign prostate epithelial cells) was obtained from Dr. Simon Hayward (Vanderbilt University, Nashville, TN). These cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum and 2 mMol/L l-glutamine. Redox-sensitive dye 5-(and-6)-chloromethyl-2’,7’-dichlorofluorescein diacetate (CM-H2DCFDA) was purchased from Molecular Probes (Eugene, OR).

**Cell Transfection and Treatment**

The sequence for the antisense CK2α oligonucleotide employed in the experiments was 5'-CCTGCTTTGG-CACGGGTCCCGACAT-3'. The corresponding nonsense sequence used as a control was 5'-CTCGAGGTCGAC-GGTATCGATCCG-3'. ALVA-41, PC-3, BPH-1, and CHO cells (0.25 × 10⁶ in each case) were plated overnight in six-well format followed by treatment with apigenin (80 μmol/L), TBB (80 μmol/L), antisense CK2α (2 μg/mL oligonucleotide), or nonsense CK2α (2 μg/mL oligonucleotide). Transfection with the oligonucleotide employed a mixture of 1 μg DNA/5 μL DOTAP. Cells were treated with pSilencer-siRNA-CK2α (2 μg/mL) as described previously (12).

**Cell Viability and Proliferation Assay**

The cell proliferation assay reagent WST-1, a tetrazolium salt that is cleaved by mitochondrial dehydrogenases in viable cells (Roche, Indianapolis, IN), was employed to determine cell viability and proliferation in cells treated with various agents as stated above. An aliquot of 200 μL containing a suspension of treated or untreated cells (2 × 10⁵-5 × 10⁵) was placed in each well of a 96-well plate. Cells were allowed to reattach over a period of 24 hours. Following the treatment of cells as indicated above, medium in each well was replaced with 100 μL fresh medium containing 100 μL/mL WST-1 and incubated at 37°C for an additional 60 minutes. An automated plate reader was employed to measure A₄50. The results were confirmed in at least three independent experiments.

**Determination of DNA Fragmentation**

Propidium iodide staining was done for analyzing DNA fragmentation. ALVA-41 and PC-3 cells (0.25 × 10⁶ in each case) were plated in six-well format. Cells were treated with nonsense CK2α oligonucleotide or antisense CK2α oligonucleotide (DOTAP controls were included) and then collected in Falcon tubes and washed twice with 1× PBS at 100 × g for 5 minutes. Supernatant was removed and 70% ethanol (2.5 mL) was added to the pellet and suspended in 1× PBS by vigorous mixing. Samples were incubated for 1 hour at 4°C and then washed twice with 1× PBS (1.0 mL). The pellet was resuspended in a solution consisting of propidium iodide (2.5 μg/mL) + 12.5 μL of 10 mg/mL RNase A + 500 μL of 38 mMol/L sodium citrate buffer. Samples were incubated at 37°C for 45 to 60 minutes. Stained cells were analyzed by flow cytometry (FACSVantage SE, Becton and Dickinson, San Jose, CA) with the excitation wavelength at 488 nm and emission wavelength at 610 nm. At least 10,000 events were analyzed by CellQuest software.

**Determination of Caspase-3 Activity**

PC-3 and ALVA-41 cells (0.5 × 10⁶ in each case) were plated in six-well format. After the desired treatment, cells were collected and washed twice with 1× PBS. The pellets were suspended in chilled 100 μL of 1× radioimmunoprecipitation assay lysis buffer and incubated on ice for 15 minutes. Caspase-3 activity was determined using the fluorescent caspase substrate (Biomol, Plymouth, PA). A 50 μL aliquot of 2× reaction buffer containing 10 mMol/L DTT and the conjugate caspase-3 assay substrate (10 μmol/L, DEVD-AFC) was added to 50 μL cell lysate in a black 96-well plate. Samples were incubated at 37°C for 1 hour in the spectrofluorometer (SpectraMax Gemini, Basel, Switzerland). Caspase activity was measured every 15 minutes at the relative fluorescence intensity at 505 nm following excitation at 400 nm.

**Measurement of Intracellular H₂O₂ Concentration**

Intracellular concentration of H₂O₂ was determined by staining the cells with the redox-sensitive dye CM-H₂DCFDA. Initially, CM-H₂DCFDA enters the cell where its acetate moiety is cleaved by intracellular esterases to C-H₂DCF. C-H₂DCF is then oxidized by H₂O₂ using the intracellular peroxidases to yield the fluorescent C-DCF. ALVA-41, PC-3, BPH-1, or CHO (0.25 × 10⁶) cells were treated with apigenin or TBB (80 μmol/L) for 4 to 24 hours, 2 μg/mL antisense CK2α oligonucleotide or nonsense CK2α oligonucleotide for 4 to 24 hours, or siRNA-CK2α for 2 to 24 hours. Following treatment, cells were washed once with 1× PBS, exposed to 5 μmol/L CM-H₂DCFDA, and incubated at 37°C for 30 minutes. Subsequently, cells were washed once in plain medium and resuspended in 500 μL of the appropriate growth medium. Flow cytometric analysis was done using the excitation at 488 nm and emission at 525 nm. At least 10,000 events were analyzed using the CellQuest software.

**Preparation of Nuclear and Cytosolic Extracts**

For detection of NF-κB translocation and cytochrome c release, ALVA-41 cells (5 × 10⁶) were subjected to antisense CK2α oligonucleotide and then washed twice with PBS by centrifugation at 100 × g for 5 minutes. The pellet was gently homogenized in the extraction buffer and subjected to differential centrifugation at 600 × g for 5 minutes, and the supernatant fraction was removed. The pellet representing the nuclear fraction was suspended in a mild buffer as described previously (9) and was subjected to gel electrophoresis to determine NF-κB translocation. The supernatant fraction was centrifuged at 14,000 × g for 15 minutes to remove mitochondria, and the resulting supernatant fraction was subjected to gel electrophoresis to determine IκB and cytochrome c expression.

**Western Blot Analysis for NF-κB, IκB, and Cytochrome c Expression**

Details of the Western blotting procedure were as described previously (36). Membranes were subjected to mouse anti–cytochrome c (1:1,000; R&D Systems, Minneapolis, MN),
mouse anti-NF-κB (1:500), and mouse anti-IκBα (1:500; Cell Signaling, Inc., Beverly, MA) primary antibodies. After three washes with TBS-Tween 20, the membranes were reprobed with secondary goat anti-mouse IgG (1:30,000). Membranes were washed again with TBS-Tween 20 and analyzed for chemiluminescence (Western Pico). As a loading control, membranes were stripped using Restore Stripping buffer (Pierce, Inc., Rockford, IL) and reprobed with rabbit anti-actin (1:200; Sigma-Aldrich, Inc., St. Louis, MO).

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References
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