Selective Role of Intracellular Chloride in the Regulation of the Intrinsic but Not Extrinsic Pathway of Apoptosis in Jurkat T-cells

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Apoptosis is a genetic program for the removal of unwanted cells from an organism, which is distinct from necrosis by its characteristic volume loss or apoptotic volume decrease. This cell shrinkage is the result of ion redistribution that is crucial for both the activation and execution of apoptosis. Here we report that UV-C but not Fas ligand treatment results in a significant decrease in intracellular chloride that can be abolished by modulation of chloride flux using either the chloride channel inhibitor SITS or medium with a reduced chloride concentration. Accordingly, downstream events are diminished during UV-C-induced apoptosis following chloride flux modulation, whereas Fas ligand-induced apoptotic characteristics are not affected. Moreover, the activation of the mitogen-activated protein kinase signal transduction pathway early in the apoptotic signaling cascade was affected by chloride flux in Jurkat T-cells. Thus, an alteration of intracellular chloride plays an important role in the activation of signaling molecules upstream of the mitochondria, specifically impairing the intrinsic but not extrinsic apoptotic pathway.

Apoptosis or programmed cell death plays an important role in the removal of unwanted cells in multicellular organisms. An impaired or increased rate of apoptosis can lead to diseases such as cancer, neurodegeneration, AIDS, and autoimmune diseases. A typical morphological feature observed during apoptosis is the reduction of cellular volume (apoptotic volume decrease), a feature that distinguishes this form of cell death from necrosis (1).

Healthy cells maintain a constant cell size by keeping a delicate balance of their intracellular ions and osmolytes. Small variations in intracellular ionic composition in response to external osmolarity changes or cell cycle alterations are compensated via inherent volume-regulatory mechanisms (2–4). In order for cells to shrink during apoptosis, a significant loss of intracellular cations has been reported to occur (5–9). Furthermore, the physiological concentration of potassium in non-apoptotic cells has been shown to constrain the activation of caspases and to inhibit the activity of apoptotic nucleases (6, 7). Although the role of potassium and sodium during volume regulation and apoptotic volume decrease has been extensively studied (6, 9), the function of the anion chloride is less well understood. Clearly, a flux of anions should occur in parallel with the loss of sodium or potassium to ensure electroneutrality. Along these lines, the involvement of a volume-sensitive outwardly rectifying chloride channel following several apoptotic stimuli including Fas receptor stimulation in Jurkat cells has been suggested (10, 11). In addition in a rat liver cell line (HTC) an increase of outwardly rectifying potassium current was coincident with a chloride current following tumor necrosis factor α stimulation (8). Chloride channel inhibitors have also been reported to block both staurosporine and tumor necrosis factor α plus cycloheximide induced apoptosis in human epithelial HeLa, lymphoid U937, and endothelial ECV304 cells as well as rat hepatocytes (12–14). However, none of these reports directly evaluate the actual intracellular chloride concentration change; nor do they link chloride to changes in other intracellular ions, such as sodium and potassium, which are necessary requisites for apoptotic volume decrease and the activation of downstream events such as cytochrome c release and caspase activation. Furthermore, there is little understanding of how changes in ions such as chloride affect the signal transduction pathways during apoptosis.

In the current paper, we seek to determine the role of chloride ions early in the process of apoptosis under conditions where the intrinsic (UV-C) and extrinsic (FasL)2 pathway of apoptosis are not overlapping (15). Here we report studies using a medium with reduced chloride to deplete the intracellular chloride content as well as SITS, a chloride channel inhibitor, to block chloride fluxes. We show for the first time that UV-C but not FasL treatment results in intracellular chloride decrease during apoptosis in Jurkat T-cells. Furthermore, we show that chloride flux modulation is linked to intracellular sodium and potassium changes and also that chloride ion flux specifically regulates the UV-C induced activation of the kinase signaling cascade upstream of the mitochondria.

EXPERIMENTAL PROCEDURES

Antibodies and Inhibitors—Mouse antibody anti-caspase-3, rabbit anti-stress-activated protein kinase/JNK, rabbit anti-phospho-stress-activated protein kinase/JNK, rabbit anti-SEK1/MKK4, and rabbit anti-phospho-SEK1/MKK4 were purchased from Cell Signaling Technology (MA). Mouse antibody anti-PARP and mouse antibody against α-tubulin were obtained from BD Transduction Laboratories and Sigma, respectively. Horseradish peroxidase-conjugated secondary antibody to mouse was obtained from Amersham Biosciences. Disodium 4-acetamido-4′-isothiocyanato-stilbene-2,2′-disulfonate (SITS) was dissolved at a final concentration of 100 mM in Me2SO and stored at 4 °C. If not otherwise stated, all other chemicals were purchased from Sigma and were of the highest grade available.

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2 The abbreviations used are: FasL, Fas ligand; SITS, disodium 4-acetamido-4′-isothiocyanato-stilbene-2,2′-disulfonate; AVD, apoptotic volume decrease; DIDS, 4,4′-disothiocyanostilbene; JNK, c-Jun N-terminal kinase; MAP kinase, mitogen-activated protein kinase; MEQ, 6-methoxy-N-ethylquinolinium iodide; SBFI, sodium binding benzofuran isophthalate; AM, acetoxymethyl ester; TNF-α, tumor necrosis factor α; PARP, poly(ADP-ribose) polymerase; PFBf, potassium binding benzofuran isophthalate; PBS, phosphate-buffered saline; PI, propidium iodide; DIC, differential interference contrast; diH-MEQ, 6-methoxy-N-ethyl-1,2-dihydroquinoline; DiBAC4(3), bis(1,3-dibutylbarbituric acid)trimethine oxonol.
**Cell Culture and Treatments**—Jurkat T-cells (clone E6.1) were cultured at 37 °C, 7% CO₂ atmosphere in RPMI 1640 medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum, 4 mM glutamine, 50 µg/ml streptomycin, and 50 units/ml penicillin. For chloride-reduced medium, RPMI 1640 was initially made lacking KCl and NaCl, which account for 99.5% of the total chloride content. To substitute the chloride anion, 5.3 mM potassium gluconate and 103.5 mM sodium gluconate were added to the medium, followed by adjustment to pH 7.5. The medium was examined for isotonicity using a 5500 vapor pressure osmometer (Wescor, Inc.). For modulation of chloride flux, cultured cells were either treated with the chloride channel inhibitor SITS or resuspended in chloride-reduced RPMI 1640 medium followed by incubation for 30–60 min at 37 °C, 7% CO₂ atmosphere. Apoptosis in Jurkat T-cells was induced by either application of 25 ng/ml recombinant FasL (Kamiya Biomedical Co.) or treatment with 30 mJ/cm² UV-C light in a medium to maintain isotonicity. A plot of the ratio of suspension volume per dish area was kept constant to ensure an equal energy dose in each case.

**Determination of Intracellular Chloride by Flow Cytometry**—To determine intracellular chloride changes, the dye MEQ (Molecular Probes, Inc., Eugene, OR) was used. Prior to each experiment, the iodide salt of the dye was reduced to the cell-permeable form diH-MEQ with sodium borohydride as recommended by the manufacturer (16–18). The resulting yellow oil was dissolved in 320 µl of Me₂SO under nitrogen, resulting in a 50 mM stock solution. 5 µl of the 50 mM diH-MEQ stock solution were added to 1 ml of cells (5 × 10⁶ cells/ml), yielding a final concentration of 250 µM. The membrane-permeable diH-MEQ was loaded by diffusion into the cells, where it was oxidized for 30 min at 37 °C, 7% CO₂ atmosphere prior to examination. Changes in the MEQ fluorescence were determined by flow cytometry on a LSRII (BD Biosciences) equipped with FACSDiva software. For each sample, 10,000 cells were examined on a forward scatter versus MEQ fluorescence dot blot. A gate was used to obtain the mean MEQ fluorescence of stained cells with normal cell size. For calibration (Stern-Volmer analysis), cells were equilibrated for 15–20 min at room temperature in media ranging from 0 to 100 mM chloride containing 7.4 µM tributyltin and 10 µM nigericin. Sodium gluconate was used as a substitute for chloride in the media to maintain isotonicity. A plot of the ratio F₀/F versus [Cl⁻] provides a straight line (see Fig. 1A). Changes in intracellular chloride concentration can be estimated with respect to changes in the mean MEQ fluorescence using the resulting equation representing the linear regression of the Stern-Volmer plot. For the determination of intracellular chloride, Jurkat T-cells were incubated for 15–20 min at room temperature in standard RPMI, RPMI containing 500 µM SITS, or chloride-reduced RPMI medium followed by flow cytometric analysis. Chloride measurements were made 4 h after the induction of apoptosis. Immediately prior to analysis by flow cytometry, propidium iodide (PI) was added to a final concentration of 10 µg/ml. The difference in the mean MEQ fluorescence of apoptotic samples and the corresponding negative controls was used to calculate the decrease in intracellular chloride, applying the calibration equation from the Stern-Volmer analysis.

**Intracellular Sodium and Potassium Analysis by Flow Cytometry**—For intracellular sodium and potassium measurements, 2 µl of 2.5 mM SBFI-AM (Na⁺) or PBFI-AM (K⁺) (Molecular Probes) stock (in Me₂SO) were added to 1 ml of cells (5–10 × 10⁶ cells/ml), yielding a final concentration of 5 µM. The dye was loaded into the cells for 1 h at 37 °C, 7% CO₂ atmosphere prior to the time of examination. Immediately prior to analysis by flow cytometry, PI was added to a final concentration of 10 µg/ml. 10,000 cells were analyzed by sequential excitation of the cells containing either SBFI-AM or PBFI-AM and PI at 340–350 and 488 nm, respectively, using an LSR flow cytometer (BD Biosciences) and CellQuest software.

**Measurement of Plasma Membrane Potential Changes**—For the analysis of changes in plasma membrane potential by flow cytometry, DiBAC₄(3) (Molecular Probes) was used. Prior to the measurements, 7.5 µl of a 20 µM DiBAC₄(3) stock (in Me₂SO) were individually added to 1 ml of cells (5–10 × 10⁶ cells/ml), yielding a final concentration of 150 nM. The dye was loaded into the cells for 30 min at 37 °C, 7% CO₂ atmosphere. Immediately prior to analysis by flow cytometry, PI was added to a final concentration of 10 µg/ml. 10,000 cells were examined using a LSRII flow cytometer and FACSDiva software (BD Biosciences) with an excitation at 488 nm and emission at 530 and 585 nm for DiBAC₄(3) and PI, respectively.

**Analysis of Cytochrome c Release**—Jurkat T-cells (2–4 × 10⁶) were collected by centrifugation at times indicated after apoptosis induction and washed with ice-cold PBS. Cytosolic extracts were prepared by using a mitochondria/cytosol fractionation kit (BioVision). The protein content of each sample was analyzed using a protein assay (Bio-Rad). Extracts were stored at −70 °C until further use. The amount of released cytochrome c in the cytosolic extracts was analyzed with a Quantikine human cytochrome c enzyme-linked immunosorbent assay kit (R&D Systems).

**Cell Lysate Preparation**—Jurkat T-cells (5–7 × 10⁶) were collected by centrifugation 5 h after apoptosis induction and washed with ice-cold PBS. Cells were lysed in 100 µl of lysis buffer (25 mM HEPES-NaOH (pH 7.4), 3 mM MgCl₂, 5 mM EGTA, 20 mM sodium molybdate, 10% glycerol, 0.5% Nonidet P-40, and 1 tablet/10 ml of protease inhibitor mixture (Roche Applied Science)) using a Dounce homogenizer and centrifuged at 20,800 × g for 15 min at 4 °C. Supernatants were removed, the protein content was analyzed using a protein assay, and the lysates were stored at −70 °C until further use.

**Cell Lysate Preparation for Kinase Phosphorylation Analysis**—Jurkat T-cells (2.25 × 10⁶) were collected by centrifugation 1 h after apoptosis induction and washed with ice-cold PBS. Cells were lysed in 100 µl of lysis buffer (20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 150 mM NaCl, 0.5% Triton X-100, 10 mM NaF, 10 mM sodium orthovanadate, and 1 tablet/10 ml of protease inhibitor mixture (Roche Applied Science)) by sonication for 10 × 1 s on ice. The protein content was analyzed using a protein assay, and the lysates were stored at −70 °C until further use.

**Protein Detection by Western Blotting**—30 or 50 µg of protein/sample were diluted in Laemmli loading buffer supplemented with β-mercaptoethanol and denatured at 100 °C for 5 min followed by SDS-PAGE on 4–20% precast Tris/glycine gels (Bio-Rad) at 150 V. The separated proteins were electrophoretically transferred onto nitrocellulose membranes (Protran 0.2 µm; Schleicher and Schuell) at 250 mA for 80 min, following standard protocols. After blocking with bovine serum albumin, membranes were incubated with the various primary antibodies, followed by horseradish peroxidase-conjugated secondary antibodies and ECL-based detection (Amersham Biosciences).

**Hoechst Staining**—Jurkat T-cells were loaded for 30 min at 37 °C, 7% CO₂ atmosphere with 5 µg/ml Hoechst 33342 prior to apoptotic stimulation. Cells were washed, resuspended in normal or chloride reduced medium, and induced to undergo apoptosis. After 5 h, a laser-scanning confocal microscope (LSM 510 NLO mounted on an Axiovert 100M microscope; Zeiss), operating in 2-photon excitation mode, was used to obtain the fluorescence and differential interference contrast (DIC) images. The images were obtained simultaneously using the 750 line from the included titanium sapphire laser as the light source and the Zeiss Plan-Apo ×63 oil numerical aperture = 1.4 as the objective lens.
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For fluorescence, a 390–465-nm band pass filter was used for the emission. The software used for acquisition was Zeiss LSM510 version 3.2 for Windows 2000.

**DNA Degradation Analysis by Flow Cytometry**—From various samples, 1–2 ml of cells (5–10 × 10^5 cells/ml) were pelleted 6 h after induction of apoptosis. The cells were fixed by the slow addition of 5 ml of ice-cold 70% ethanol and stored overnight at −20 °C. For flow analysis, the fixed cells were pelleted, washed in ice-cold PBS, and stained in 1 ml of 20 μg/ml PI, 10 units/ml RNase One (Promega) in PBS for 20 min at room temperature in the dark. 7,500 cells were analyzed by flow cytometry using a BD Biosciences FACSort by gating on a PI area versus width dot plot. The percentage of degraded DNA was determined by the number of cells with subdiploid DNA divided by the total number of examined cells.

**DNA Degradation Analysis by Agarose Gel**—For the analysis of DNA degradation, 2–5 × 10^6 cells were collected by centrifugation 6 h after induction of apoptosis and resuspended in ice-cold PBS. The total DNA was extracted using the DNeasy Tissue Kit (Qiagen, CA) and stored at −70 °C until further use. DNA content was determined at 260 nm, and 2.0 μg of DNA/sample were analyzed on a 1.8% agarose gel stained with ethidium bromide.

**Statistical Analysis**—The data are expressed as average ± S.E. Averages were compared by one-way analysis of variance, followed by *post hoc* testing using Dunnett’s analysis for single comparison with a sample of interest. A *p* value of *p* < 0.05 was considered statistically significant.

**RESULTS**

**UV-C- but Not FasL-induced Cell Death Results in an Intracellular Chloride Decrease**—During programmed cell death, an iso-osmotic loss of cellular volume or apoptotic volume decrease (AVD) is routinely observed. This process is driven by the loss of intracellular ions, particularly sodium and potassium, but the role of anions and in particular chloride ions during AVD is far less understood (6, 9). Therefore, we evaluated changes in intracellular chloride concentration during FasL- or UV-C-induced apoptosis in Jurkat T-cells. Cells were loaded with the reduced, cell-permeable dye diH-MEQ to analyze intracellular chloride changes. This dye has previously been used to monitor intracellular chloride changes in various cell types (16–20), but not in apoptotic cells. Inside the cell, diH-MEQ is oxidized to the polar MEQ molecule, which is trapped in the cell, resulting in a uniform staining without significant dye compartmentation (16). The MEQ fluorescence is quenched by increasing halide concentrations. We measured by flow cytometry the dye compartmentation (16). The resulting values from the linear regression analysis were used to calculate the intracellular chloride concentration from mean MEQ fluorescence values of Jurkat T-cells in standard RPMI 1640, medium treated with 500 μM SITS, or RMPI 1640 with reduced chloride content. The cells were then induced to undergo apoptosis by either the addition of 25 ng/ml FasL or exposure to 30 mJ/cm^2 UV-C light (exposure time is about 30 s). After 4 h, the cells were analyzed by flow cytometry as described above. The difference in the mean MEQ fluorescence between the corresponding negative control and an apoptotic sample was used to calculate the intracellular chloride change. In C, the intracellular chloride increase is presented in a bar graph. Shown are averaged values ± S.E. (n = 3). Human Jurkat T-cells were cultured for 30–60 min in RPMI 160 medium, medium treated with 500 μM SITS, or RPMI 1640 with reduced chloride content. The cells were then induced to undergo apoptosis by either the addition of 25 ng/ml FasL or exposure to 30 mJ/cm^2 UV-C light (exposure time is about 30 s). After 4 h, the cells were analyzed by flow cytometry as described above. The difference in the mean MEQ fluorescence between the corresponding negative control and an apoptotic sample was used to calculate the intracellular chloride change. In C, the intracellular chloride decrease is presented in a bar graph. Shown are averaged values ± S.E. (n = 4). The asterisk indicates a statistically significant decrease in the chloride loss as a result of chloride flux modulation compared with the stimulus. UV-C but not FasL induction of apoptosis is accompanied by a decrease in intracellular chloride that is affected by chloride flux modulation.

**FIGURE 1.** UV-C- but not FasL-induced apoptosis is accompanied by an intracellular chloride concentration decrease. Jurkat T-cells were loaded with 250 μM MEQ for 30 min prior to analysis by flow cytometry using a forward-scan (cell size) versus MEQ fluorescence dot plot and a gate drawn around normally sized cells containing MEQ. A shows a representative Stern-Volmer plot for the fluorescence quenching of MEQ by various concentrations of chloride in RPMI 1640 medium. *F₀/F* is the ratio of fluorescence without chloride to fluorescence values in the presence of the various chloride concentrations. The resulting values from the linear regression analysis were used to calculate the intracellular chloride concentration from mean MEQ fluorescence values of Jurkat T-cells in standard RPMI 1640, medium treated with 500 μM SITS, or RPMI 1640 with reduced chloride content. In B, the intracellular chloride content is presented in a bar graph. Shown are averaged values ± S.E. (n = 3). Human Jurkat T-cells were cultured for 30–60 min in RPMI 160 medium, medium treated with 500 μM SITS, or RPMI 1640 with reduced chloride content. The cells were then induced to undergo apoptosis by either the addition of 25 ng/ml FasL or exposure to 30 mJ/cm^2 UV-C light (exposure time is about 30 s). After 4 h, the cells were analyzed by flow cytometry as described above. The difference in the mean MEQ fluorescence between the corresponding negative control and an apoptotic sample was used to calculate the intracellular chloride change. In C, the intracellular chloride decrease is presented in a bar graph. Shown are averaged values ± S.E. (n = 4). The asterisk indicates a statistically significant decrease in the chloride loss as a result of chloride flux modulation compared with the stimulus. UV-C but not FasL induction of apoptosis is accompanied by a decrease in intracellular chloride that is affected by chloride flux modulation.
FIGURE 2. Chloride flux modulation reduces the number of cells showing UV-C- but not FasL-induced AVD mediated by potassium efflux. Human Jurkat T-cells were treated and induced to undergo apoptosis as described in the legend to Fig. 1. 1 h prior to examination, PBFI-AM was added to 1 ml of cells at a final concentration of 5 μM, and incubation was continued. Cell size and potassium content were analyzed after 4 h by flow cytometry using a forward scatter (cell size) versus PBFI fluorescence (potassium content) three-dimensional plot (A) or a dot plot gating for shrunken cells with decreased intracellular potassium (B). The use of the chloride channel inhibitor SITS (500 μM) or intracellular chloride depletion by using RPMI medium with reduced chloride content resulted in decreased number of cells undergoing AVD mediated by potassium efflux following UV-C exposure but not FasL treatment. The presented three-dimensional plots (A) are representative for at least three individual experiments. The percentages presented in the bar graph (B) are averaged values ± S.E. (n = 3). #, samples statistically different from controls after induction of apoptosis. *, a statistically significant decrease in the number of shrunken cells with decreased potassium content as a result of chloride flux modulation compared with the stimulus. ζ, a statistically significant increase in the number of shrunken cells with decreased potassium as a result of SITS treatment.

Chloride Flux Modulation Reduces the Number of Cells Showing UV-C-induced AVD Mediated by Potassium Efflux—AVD is a characteristic hallmark of apoptotic cell death, and it is linked to a loss of intracellular potassium (6). Therefore, it was important to evaluate if chloride flux modulation alters the efflux of intracellular potassium. Jurkat T-cells were cultured and treated with either FasL or UV-C to initiate apoptosis as described above. Cell size and intracellular potassium content were simultaneously analyzed after 4 h by flow cytometry using a forward scatter (cell size) versus PBFI fluorescence (potassium content) three-dimensional plot. Compared with the control cells, treatment with FasL or UV-C in normal medium resulted in an increased population of cells with reduced intracellular potassium (red circle) and a corresponding smaller cell size (Fig. 2A, first row). The treatment with FasL in the presence of SITS enhanced potassium depletion accompanied by cell shrinkage (Fig. 2A, second row). In contrast, UV-C exposure in the presence of SITS reduced cell shrinkage and the corresponding loss of potassium (Fig. 2A, second row). FasL treatment in medium with reduced chloride content did not significantly change the number of cells that shrunk and had low potassium (Fig. 2A, third row). In contrast, UV-C treatment of cells in medium with reduced chloride concentration decreased the number of cells undergoing apoptotic volume decrease and potassium loss (Fig. 2A, third row). These data were also analyzed on a forward scatter versus PBFI fluorescence dot plot, and the percentage of cells gated in a region for cells with decreased intracellular potassium content and decreased cell size is summarized in Fig. 2B. Clearly, FasL and UV-C treatment results in an increased percentage of cells having lost potassium and decreased cell size. In the case of UV-C, either the chloride channel inhibitor or the chloride-reduced medium significantly reduced the number of cells with a smaller cell size and a loss of potassium. In contrast, treatment with FasL in the presence of SITS even enhanced significantly potassium depletion and cell shrinkage. Together, these data suggest that modulation of chloride flux by using either a chloride channel inhibitor or depleting intracellular chloride
affects only the UV-C-induced loss of potassium and thus the number of cells exhibiting AVD in Jurkat T-cells.

Chloride Flux Modulation Inhibits the Rise in Intracellular Sodium during Apoptosis—It has recently been shown that induction of apoptosis is associated with changes in intracellular sodium (6, 26). Thus, we sought to determine how chloride flux modulation affects changes in intracellular sodium in response to stimulation of apoptosis. Following pretreatment with SITS or the change to chloride reduced medium, Jurkat T-cells were examined 4 h after either FasL or UV-C treatment. Shown are histogram plots for the SBFI fluorescence (sodium content). Interestingly, treatment with either FasL or UV-C resulted in a second population of cells with increased intracellular sodium (on the right of the white line) in normal medium (Fig. 3A, first row). In standard RPMI, treatment with either FasL or UV-C resulted in a second population with significantly increased intracellular sodium content. The use of the chloride channel inhibitor SITS (500 μM) or intracellular chloride depletion by using RPMI with reduced chloride content resulted in a slightly broader cell distribution for the intracellular sodium content in the control cells. Interestingly, the rise in intracellular sodium in cells following either FasL or UV-C treatment was completely abolished by chloride flux modulation.

Chloride Flux Modulation Reduces the Number of Cells Showing UV-C-induced Plasma Membrane Depolarization—An association between the movement of monovalent ions, especially sodium, and the plasma membrane depolarization occurring during apoptosis has been suggested (2); therefore, we examined the extent of plasma membrane depolarization following chloride flux modulation. Jurkat T-cells plasma membrane depolarization was examined after 4 h by flow cytometry using DiBAC4(3) following the described pretreatment with SITS or the change to chloride-reduced medium prior to apoptosis induction (Fig. 4). The cells were analyzed on a forward scatter versus DiBAC4(3) fluorescence dot blot gating for nondepolarized cells with normal cell size. In the presence of SITS or intracellular chloride depletion alone, a small but insignificant decrease in the number of normal sized cells with a polarized plasma membrane was observed (Fig. 4). Treatment with either FasL or UV-C both resulted in cellular depolarization (Fig. 4). Neither the chloride channel inhibitor nor chloride depletion altered the response to FasL (Fig. 4). In contrast, both chloride
modulators blocked in part the depolarizing effect of UV-C treatment (Fig. 4). These data suggest a distinct role of chloride in the intrinsic apoptotic pathway. However, it is important to note that pretreatment with SITS and change to chloride-reduced medium per se resulted in an increased number of depolarized cells. Thus, the inhibitory effect of chloride flux onto the UV-C-induced mitochondrial pathway is probably an underestimation. Clearly, ion movement and plasma membrane potential are selectively required by the intrinsic and extrinsic apoptotic pathways.

Chloride Flux Modulation Inhibits UV-C- but Not FasL-induced Cytochrome c Release—UV-C-induced apoptosis primarily proceeds by the mitochondrial signaling pathway (15). Additionally, Jurkat cells are type II cells, which critically require the mitochondria as an amplification signal for extrinsically stimulated apoptosis. Chloride flux modulation selectively affected ion changes, AVD, and plasma membrane potential following UV-C but not FasL treatment, all typical early hallmarks of apoptotic progression. We now sought to determine if and where the signaling cascade is affected. Thus, we investigated whether the release of cytochrome c from the intermembrane space of mitochondria into the cytosol is affected by chloride flux modulation. The release of cytochrome c is a necessary step in the late activation phase of the mitochondrial signaling pathway resulting in the activation of caspases. Jurkat T-cells were either cultured in normal medium or medium with reduced chloride content or treated with SITS prior to induction of apoptosis with either FasL or UV-C. Cytosolic extracts were prepared after 5 h (Fig. 5). The amount of cytochrome c in the cytosol was examined by using a human cytochrome c-specific enzyme-linked immunosorbent assay kit. Pretreatment with 500 μM SITS or use of a medium with reduced chloride content blocked the UV-C-induced release of cytochrome c into the cytosol (B). FasL-induced release of cytochrome c was not affected (A). Shown are averaged values ± S.E. (n = 3). #, samples statistically different from controls after induction of apoptosis. *, a statistically significant increase in the number of nondepolarized cells as a result of chloride flux modulation compared with the stimulus.

only slightly above the control (Fig. 5B). Similarly, suspending the cells in chloride-reduced medium prior to UV-C light exposure also inhibited the release of cytochrome c (Fig. 5B). These data suggest that the modulation of chloride flux affects intrinsically induced signals upstream or at the level of mitochondria prior to the release of cytochrome c.

Chloride Flux Modulation Reduces UV-C-induced Caspase-3 Cleavage—Since UV-C-induced release of cytochrome c, a late activation phase event, was inhibited by modulation of chloride flux, it was of interest to analyze if the downstream activation of executioner caspases was also affected. Apoptosis was induced by either the addition of FasL or exposure to UV-C light, and the cleavage of caspase-3 and one of its targets, PARP, was analyzed by Western blotting. Pretreatment with SITS or chloride depletion both inhibited the cleavage of procaspase-3 compared with UV-C alone but not FasL (Fig. 6). The chloride channel inhibitor also inhibited appearance of the cleaved caspase-3 band following UV-C treatment. Furthermore, the cleavage of PARP following UV-C exposure is significantly reduced in the presence of SITS or in chloride-reduced medium (Fig. 6). All samples from FasL-treated cells showed a similar amount of cleaved PARP irrespective of modulation of chloride flux (Fig. 6). Thus, neither the chloride channel inhibitor SITS nor intracellular chloride depletion altered the effect of FasL on caspase-3 activation or PARP cleavage. In contrast, both SITS and chloride depletion applied prior to UV-C exposure blocked the cleavage of caspase-3 and its intracellular target PARP.
Chloride Flux Modulation Inhibits UV-C-induced Chromatin Condensation—The condensation of chromatin is a classical, late occurring morphological feature of apoptosis. Therefore, we evaluated the influence of chloride flux modulation on chromatin condensation during apoptosis. Hoechst dye-loaded Jurkat T-cells were examined 5 h after induction of apoptosis with either FasL or UV-C by confocal microscopy. Compared with control cells, neither the chloride channel inhibitor SITS alone nor the RPMI medium with reduced chloride content affected the chromatin condensation and cell morphology (Fig. 7, first column, middle and bottom). Treatment with FasL resulted in a condensation of chromatin, cell shrinkage, and apoptotic body formation (Fig. 7, second column, top) in the presence or absence of SITS as well as chloride-reduced medium. In fact, the presence of SITS resulted in an even higher number of shrunken cells (Fig. 7, second column, middle). UV-C treatment in normal medium resulted in the condensation of chromatin, cell shrinkage, and apoptotic body formation (Fig. 7, second column, top). Pretreatment with SITS inhibited chromatin condensation as well as cell shrinkage and apoptotic body formation (Fig. 7, second column, middle). Similarly, chloride-reduced medium also inhibited the effect of UV-C (Fig. 7, second column, bottom). Thus, the morphological changes typical for apoptosis induced by UV-C but not FasL are inhibited by chloride flux modulation.

Chloride Flux Modulation Decreases UV-C-induced DNA Degradation—An important, although late characteristic of apoptosis is the internucleosomal cleavage of the DNA. Therefore, the influence of chloride flux modulation on the pattern and extent of the DNA degradation was analyzed. Six hours after induction of apoptosis with FasL or UV-C the DNA degradation was either visualized by agarose gel electrophoresis (Fig. 8A) or examined by flow cytometry analysis of ethanol-fixed and PI-stained cells (Fig. 8B). Compared with the controls showing only one prominent band of uncleaved genomic DNA, all samples of cells treated with FasL contained a reduced amount of uncleaved DNA and showed a ladder-like pattern, a characteristic of apoptosis (Fig. 8A). UV-C exposure alone shared the same characteristics of reduced genomic DNA and apoptotic laddering (Fig. 8A). In contrast, in samples pretreated with either the chloride channel inhibitor SITS or cultured in chloride-reduced medium UV-C induced DNA degradation was blocked (Fig. 8A). The percentage of cells with degraded DNA was also analyzed by flow cytometry. Treatment with FasL or UV-C significantly increased the number of cells with degraded DNA compared with the controls (Fig. 8B). Pretreatment with the chloride channel inhibitor SITS did not block DNA degradation induced by FasL, whereas SITS treatment prior to UV-C exposure significantly inhibited DNA degradation (Fig. 8B). Chloride-reduced medium enhanced the number of cells showing DNA degradation following FasL treatment. In contrast, UV-C treatment in medium with reduced chloride content significantly inhibited DNA degradation (Fig. 8B). These data show that the DNA degradation following exposure to UV-C but not FasL treatment is reduced by modulation of chloride flux using either a chloride channel inhibitor or intracellular chloride depletion.

Chloride Flux Modulation Affects the UV-C-induced Kinase Signaling Cascade—We were able to show that chloride flux modulation affected UV-C- but not FasL-induced apoptotic characteristics (with the exception of sodium influx). Interestingly, in Jurkat T-cells, which are type II cells requiring the release of cytochrome c as an amplification signal for extrinsic stimulated cell death, the FasL-induced translocation of this apoptogenic factor was not affected by chloride modulation. We now sought to determine where upstream of the mitochondria the UV-C-induced signaling cascade is affected. It is well known that MAP kinases are activated in response to UV irradiation. Therefore, we analyzed the phosphorylation of the MAP kinase family members JNK and JNK kinase (MKK4). Apoptosis was induced by either the addition of FasL or exposure to UV-C light, and the levels of total and phosphorylated JNK and MKK4 were analyzed by Western blotting. Following UV-C but not FasL treatment, JNK and MKK4 are phosphorylated (Fig. 9). The pretreatment with the chloride channel inhibitor SITS and intracellular chloride depletion both significantly reduced the phosphorylation of these two kinases (Fig. 9). In contrast, no effect of chloride modulation occurred in combination with FasL treatment. Thus, chloride flux specifically affects the UV-C-induced kinase signaling cascade upstream of the mitochondria that ultimately results in the release of apoptogenic factors from the mitochondria into the cytosol.

**DISCUSSION**

This report shows for the first time that UV-C- but not FasL-induced apoptosis in Jurkat T-cells is accompanied by a significant decrease in...
intracellular chloride. Furthermore, inhibition of chloride flux by application of the chloride channel inhibitor SITS or intracellular chloride depletion via use of medium with reduced chloride content (21–24) attenuates UV-C- but not FasL-induced apoptotic characteristics in Jurkat T-cells. Moreover, the activation of the MAP kinase family members JNK and MKK4 following UV-C stimulation was abolished by chloride flux modulation, indicating a specific role of chloride flux in the regulation of signaling cascades. Our group recently published that late during apoptosis following high doses of UV-C, the intrinsic pathway cross-activates the extrinsic pathway (27). In the current paper, we focus on early time points and low stimuli doses to ensure conditions were the apoptotic pathways are still independent and not overlapping (15). Together, our data suggest a specific role for an alteration of the intracellular chloride concentration regulating the activation phase of the intrinsically induced apoptotic signaling pathway upstream of the mitochondria. Similar to changes in intracellular potassium, which are thought to be involved in the regulation of caspase and nuclease activation (28), changes in intracellular chloride appear to be crucial for the activation of signaling molecules.

To the best of our knowledge, this is the first report that utilizes the anion-specific dye MEQ for analysis of intracellular chloride changes during apoptosis by flow cytometry. This dye was previously used to monitor intracellular chloride changes in different cell types by confocal microscopy or spectrophotometry (16–20); however, it has not been used to monitor changes during apoptosis. We show that induction of apoptosis with UV-C actually results in a significant decrease in intracellular chloride. It is interesting to note that not the actual chloride decrease but chloride movement seems to be crucial for activation of the apoptotic signaling cascade. This conclusion can be drawn from the fact that either using the chloride channel inhibitor SITS or previous intra-

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**FIGURE 8.** Chloride flux modulation reduces the number of cells undergoing UV-C- but not FasL-induced DNA degradation. Human Jurkat T-cells were treated and induced to undergo apoptosis as described in the legend to Fig. 1. After 6 h, DNA degradation was analyzed either by total DNA isolation followed by conventional agarose gel electrophoresis (A) or by cell fixation in ethanol, PI staining, and flow cytometry (B). Modulation of chloride flux reduced the number of cells with DNA fragmentation after UV-C but not FasL treatment. The gel image presented in A represents multiple similar experiments. The data presented in B are averaged values ± S.E. (n = 3). #, indicates samples statistically different from controls after induction of apoptosis. *, a statistically significant reduction of DNA degradation as a result of chloride flux modulation compared with the stimulus.

**FIGURE 9.** Chloride flux modulation affects UV-C-induced kinase signaling cascades. Human Jurkat T-cells were treated and induced to undergo apoptosis as described in the legend to Fig. 1. 1 h after induction of apoptosis, total cell protein extracts were prepared. 30 μg of total protein/lane were separated by SDS-PAGE and analyzed by Western blotting using antibodies against phosphorylated and total levels of SEK1/MKK4, stress-activated protein kinase/JNK, and α-tubulin. Shown are blots representative for three experiments.
cellular chloride depletion both abolish UV-C-induced apoptosis. It was often suggested that a decrease in intracellular chloride might accompany the loss of potassium that mediates AVD (29, 30). Here we show that the decrease in intracellular chloride during apoptosis of Jurkat cells is not a general feature of apoptosis but depends on the actual apoptotic stimulus applied. Our data using UV-C light as a stimulus for the mitochondrial death pathway is in accordance with other studies suggesting the involvement of chloride ions in the regulation of apoptosis in other cells. One of the first reports showed that the chloride channel inhibitor DIDS inhibited staurosporine-induced apoptosis in endothelial cells and cardiomyocytes (31). Furthermore, various chloride channel inhibitors like DIDS, 5-nitro-2-(3-phenylpropylamino)-benzoic acid, and phloretin, have been reported to prevent cell death, morphological changes, AVD, and cytochrome c release in human epithelial HeLa and human lymphoid U937 cells after staurosporine-induced apoptosis (12). Despite suggesting a role for chloride ions in the mitochondrial signaling cascade at the level of or upstream of the mitochondria, the actual link to ion-mediated AVD has not been reported. In another study, staurosporine-induced apoptosis in human endothelial ECV304 cells was attenuated by the application of the chloride channel inhibitors phloretin and DIDS, although staurosporine only generated a rapid increase in plasma membrane chloride channel activity but did not alter potassium conductance (14). Interestingly, in contrast to potassium or sodium channel blockers, the chloride channel blocker SITS was able to decrease staurosporine-induced cellular and nuclear morphological changes as well as cell death in primary rat cortical cultures (13). Additionally, it is been shown that Fas receptor ligation activates an outwardly rectifying chloride channel in human Jurkat T-cells which is regulated by a Src-like tyrosine kinase (11). In these studies, FasL receptor ligation following intracellular acidification and apoptosis was reduced by the inhibitors glibenclamide and indoleacetic acid, although DIDS was less effective (11). Exposure of a rat liver cell line (HTC) to TNF-α resulted in both increased potassium and chloride currents, which could be blocked by ion substitution or by using the corresponding ion channel blockers (8). However, TNF-α-mediated cell death was only delayed but not blocked by potassium or chloride channel blockage (8). The increase in potassium current was inhibited by chloride removal but not by chloride channel blockers, suggesting that potassium channel activity does not depend on chloride movement but might be affected by chloride binding (8).

Recently, chloride currents showing characteristics of the volume-sensitive outwardly rectifying chloride channel were described within minutes after treatment with staurosporine, anti-Fas antibody, and TNF-α in HeLa cells (10). These currents resembling an actual chloride influx were sensitive to 5-nitro-2-(3-phenylpropylamino)-benzoic acid, DIDS, and cytosolic ATP. A selective role for reactive oxygen species that serves as an upstream signal in volume-sensitive outwardly rectifying chloride channel activation following the intrinsic but not extrinsic acting stimuli was observed (10), suggesting that intrinsic activation of programmed cell death could lead to the activation of outwardly rectifying chloride channels. Unfortunately, in this report, the role of volume-sensitive outwardly rectifying chloride channel-mediated currents in the actual protection against extrinsically induced caspase activation and cell death was not addressed. Also, outwardly rectifying chloride currents resembling chloride influx contrast with our measured decrease in intracellular chloride content.

It is important to note that the modulation of chloride ions not only affects the progression of apoptosis but also affects the intracellular concentration of sodium and potassium. Both ions are known to play an important role during volume regulation, the activation of the apoptotic signaling cascade, and apoptotic volume decrease. Recently, two distinct roles for sodium and potassium were postulated (26). It was shown that whereas the loss of potassium is required for the activation of caspases and nucleases (28), the sodium ions play a crucial role in the regulation of the cellular volume independent of other apoptotic events (26). In the present study, a broader cell distribution for the intracellular sodium content was observed in control and apoptotic samples following modulation of chloride. Furthermore, the major rise in sodium occurring after intrinsic or extrinsic apoptosis induction was not observed when the chloride channel inhibitor was used or intracellular chloride was depleted. In contrast, plasma membrane depolarization was only abolished by chloride flux modulation during UV-C- but not FasL-induced apoptotic progression. Therefore, a rise in intracellular sodium might not account for plasma membrane depolarization under these experimental conditions.

Previous investigations have used various pharmacological inhibitors to block chloride channels and evaluate their role in apoptosis (8, 10–14, 31). We used the stilbene derivative SITS to block chloride channels. It is known that this class of inhibitors besides Cl− channels can also block Cl−/HCO3− exchangers and Na+/HCO3− cotransporters. However, a recent report showed that the protective effect of DIDS against staurosporine-induced apoptosis in rat cardiomyocytes is independent of the presence of HCO3− (32). Therefore, the protective effect of stilbene derivates is most likely mediated by an inhibition of chloride channels. Furthermore, our studies evaluate the effect of a medium with reduced chloride to modulate the intracellular chloride content during programmed cell death. Chloride-reduced media have been widely used to study volume regulatory mechanisms because cells respond to this environment with a depletion of chloride within minutes (21–24). In accordance with these reports, we measured a significant decrease in intracellular chloride for cells cultured in RPMI with reduced chloride content. Furthermore, the observed small reduction in the forward scatter for Jurkat T-cells suspended in chloride-reduced medium (data not shown) is in agreement with previous reports where chloride depletion was accompanied by cell shrinkage (22, 33).

It is well known that MAP kinase family members such as JNK are phosphorylated and thereby activated following intrinsic apoptotic stimuli like UV irradiation, reactive oxygen species, and osmotic shock (34–38). It has been shown that the effect of JNK on cell death does not only depend on the stimulus applied and the cell type used but also appears to be influenced by the length of the actual JNK activation (39). Interestingly, TNF-α-induced apoptosis also depends on JNK family kinase activation (36, 40). In contrast, Fas-induced apoptosis apparently does not involve activation of the ASK1-MKK4-JNK pathway (36, 38, 40–43). Our data showing an activation of the JNK signaling cascade following UV-C but not FasL treatment is in accordance with these reports. Similar to a recent report showing the requirement of K+ channel activity for the activation of JNK (35), we describe for the first time a role of chloride flux in the regulation of the MAP kinase signaling cascade. Moreover, activation of JNK kinases is known to result in the release of apoptogenic factors from the mitochondria, placing these kinases upstream of the mitochondrial checkpoint (38, 40, 44, 45). Thus, the differential role of chloride flux modulation on the release of cytochrome c between FasL and UV-C treatment can be explained with the selective activation of JNK, which is regulated by a chloride concentration change.

In conclusion, this study shows that in Jurkat T-cells UV-C- but not FasL-induced apoptosis results in a significant decrease in intracellular chloride. Furthermore, there is a clear correlation between modulation of chloride ions, potassium and sodium changes, and apoptosis activa-
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REFERENCES

1. Kerr, J. F., Wyllie, A. H., and Currie, A. R. (1972) Br. J. Cancer 26, 239–257
2. Bortner, C. D., Gomez-Angelats, M., and Cidlowski, J. A. (2001) J. Biol. Chem. 276, 4304–4314
3. Lang, F., Ritter, M., Gamper, N., Huber, S., Fillon, S., Tanneur, V., Lepple-Wienhues, A., Szabo, I., and Gulbins, E. (2000) Cell. Physiol. Biochem. 10, 417–428
4. al-Habori, M. (1994) Int. J. Biochem. 26, 319–334
5. Beauvais, F., Michel, L., and Dubertret, L. (1995) J. Leukocyte Biol. 57, 851–855
6. Bortner, C. D., Hughes, F. M., Jr., and Cidlowski, J. A. (1997) J. Biol. Chem. 272, 32436–32442
7. Hughes, F. M., Jr., Bortner, C. D., Purdy, G. D., and Cidlowski, J. A. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 6169–6174
8. Nietsch, H. H., Roe, M. W., Fiekers, J. F., Moore, A. L., and Lidofsky, S. D. (2000) J. Biol. Chem. 275, 20556–20561
9. Barbiero, G., Duranti, F., Bonelli, G., Amenta, J. S., and Baccino, F. M. (1995) Exp. Cell Res. 217, 410–418
10. Shimizu, T., Numata, T., and Okada, Y. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 6770–6773
11. Szabo, I., Lepple-Wienhues, A., Kaba, K. N., Zoratti, M., Gulbins, E., and Lang, F. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 6169–6174
12. Maeno, E., Ishizaki, Y., Kanaseki, T., Hazama, A., and Okada, Y. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 9487–9492
13. Small, D. L., Tauskela, J., and Xia, Z. (2002) Neurosci. Lett. 334, 95–98
14. Porcelli, A. M., Ghelli, A., Zanna, C., Valente, P., Ferroni, S., and Rugolo, M. (2004) Cell Death Differ. 11, 655–662
15. Vu, C. C., Bortner, C. D., and Cidlowski, J. A. (2001) J. Biol. Chem. 276, 37602–37611
16. Riwer, J., and Verkman, A. S. (1991) Biochemistry 30, 7879–7883
17. Inglefield, J. R., and Schwartz-Bloom, R. D. (1999) Methods 18, 197–203
18. Zhou, J. G., Ren, J. L., Qiu, Q. Y., He, H., and Guan, Y. Y. (2005) J. Biol. Chem. 280, 7301–7308
19. Woll, E., Gschwentner, M., Furst, J., Hofer, S., Buernerger, G., Jungwirth, A., Frick, J., Deetjen, P., and Paulmichl, M. (1996) Pfluegers Arch. Eur. J. Physiol. 432, 486–493
20. MacVinish, L. J., Reancharoen, T., and Cuthbert, A. W. (1993) Br. J. Pharmacol. 108, 469–478
21. Butt, A. G., McLaughlin, C. W., Bowler, J. M., Purves, R. D., and Macknight, A. D. (1994) J. Membr. Biol. 142, 1–20
22. Takeuchi, S., Ando, M., and Inama, I. (1997) Hear. Res. 113, 99–109
23. Lambeth, A., and Lowe, A. G. (1978) J. Physiol. (Lond.) 275, 51–63
24. Aull, F. (1979) Biochim. Biophys. Acta. 554, 538–540
25. Pilas, B., and Durack, G. (1997) Cytometry 28, 316–322
26. Bortner, C. D., and Cidlowski, J. A. (2003) J. Biol. Chem. 278, 39176–39184
27. Scocchio, A. B., and Cidlowski, J. A. (2004) Exp. Cell Res. 297, 212–223
28. Hughes, F. M., Jr., and Cidlowski, J. A. (1999) Adv. Enzyme. Regul. 39, 157–171
29. Okada, Y., Maeno, E., Shimizu, T., Deraki, K., Wang, J., and Morishima, S. (2001) J. Physiol. (Lond.) 532, 3–16
30. Gomez-Angelats, M., and Cidlowski, J. A. (2002) Toxicol. Pathol. 30, 541–551
31. Fujita, H., Ishizaki, Y., Yanagisawa, A., Morita, I., Murota, S. I., and Ishikawa, K. (1999) Cell Biol. Int. 23, 241–249
32. Tanabe, S., Wang, X., Takahashi, N., Uramoto, H., and Okada, Y. (2005) FEMS Lett. 579, 517–522
33. Fine, D. M., Lo, C. F., Aguillar, L., Blackmon, D. L., and Montrose, M. H. (1995) J. Membr. Biol. 145, 129–141
34. Galcheva-Gargova, Z., Derijard, B., Wu, I. H., and Davis, R. J. (1994) Science 265, 806–808
35. Gao, J., Wu, D., Guo, T. B., Ruan, Q., Li, T., Lu, Z., Xu, M., Dai, W., and Lu, L. (2004) Exp. Cell Res. 297, 461–471
36. Tobiume, K., Matsuzawa, A., Takahashi, T., Nishihori, H., Morita, K., Takeda, K., Minowa, O., Miyazono, K., Noda, T., and Ichijo, H. (2000) EMBO Rep. 2, 222–228
37. Li, T., Dai, W., and Lu, L. (2001) J. Biol. Chem. 276, 32668–32676
38. Tournoir, C., Hess, P., Yang, D. D., Xu, J., Turner, T. K., Nimmel, A., Bar-Sagi, D., Jones, S. N., Flavell, R. A., and Davis, R. J. (2000) Science 288, 870–874
39. Chen, Y. R., Wang, X., Templeton, D., Davis, R. J., and Tan, T. H. (1996) J. Biol. Chem. 271, 31929–31936
40. Schwabe, R. F., Uchinami, H., Qian, T., Bennett, B. L., Lemasters, J. J., and Brenner, D. A. (2004) FASEB J. 18, 720–722
41. Matsuzawa, K., Matsuzawa, A., Takeda, K., and Ichijo, H. (2004) J. Biochem. (Tokyo) 136, 261–265
42. Villunger, A., Huang, D. C., Holler, N., Tschopp, J., and Strasser, A. (2000) J. Immunol. 165, 1337–1343
43. Lenczowski, J. M., Dominguez, L., Ader, A. M., King, L. B., Zacharchuk, C. M., and Ashwell, J. D. (1997) Mol. Cell Biol. 17, 170–181
44. Lee, K., Nimmul, A., Zong, W. X., Kennedy, N. I., Flavell, R. A., Thompson, C. B., Bar-Sagi, D., and Davis, R. J. (2002) Mol. Cell Biol. 22, 4929–4942
45. Aoki, H., Kang, P. M., Hampe, J., Yoshimura, K., Noma, T., Matsuoka, M., and Izumo, S. (2002) J. Biol. Chem. 277, 10244–10250