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Thiol Oxidation Enforces Phosphatidylserine Externalization in Apoptosis-Sensitive and -Resistant Cells Through a $\Delta\psi m$/Cytochrome *c* Release-Dependent Mechanism

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ABSTRACT

Previous studies have shown that unlike most apoptotic cells, Raji cells do not externalize phosphatidylserine (PS) upon apoptosis. Here we show that Raji cells are resistant to intrinsic apoptogenic agents, but sensitive to extrinsically triggered Fas-induced apoptosis. Treatment of intrinsic apoptosis-competent Jurkat cells with vitamin E implicated reactive oxygen species in intrinsic apoptosis because, like Raji cells, they became resistant to actinomycin D- but not Fas-triggered apoptosis. Oxidation of sulfhydryls in both cell types with *N*-ethylmaleimide resulted in rapid disruption of the mitochondrial membrane potential, release of cytochrome *c* from the mitochondria to the cytoplasm, and externalization of PS by a mechanism that was not inhibited by the pan caspase inhibiter zVAD-fmk. These results suggest that although cell death and PS externalization are both cytochrome *c*-dependent, they are distinct and separable processes. *Antioxid. Redox Signal.* 6, 203–208.

INTRODUCTION

REACTIVE OXYGEN SPECIES (ROS) produced as a result of disrupted mitochondrial electron transport are important mediators of apoptosis, especially apoptosis triggered through the intrinsic mitochondria-dependent pathway (1, 2, 4, 12). This pathway involves the rapid release of cytochrome *c* (cyt *c*) from the mitochondria to the cytosol, where it assembles into the apoptosome that regulates the activation of downstream caspases (16). Despite the importance of cyt *c* release to apoptosis, little is known about its mechanism of release and the role ROS play in this process. Clearly, the barrier function of the outer mitochondrial membrane must be disrupted to an extent that facilitates cyt *c* release. This could occur through perturbation of the permeability transition pore complex (PTPC), physical disruption of the membrane, or the formation of a nonspecific protein-permeable pore.

Two of the earliest measurable events in apoptosis are the release of cyt *c* (7) and the reorientation of phosphatidylserine (PS) (11) from the cell's inner to outer plasma membrane leaflet. Detailed kinetic analyses have indicated that cyt *c* release precedes PS exposure and loss of plasma membrane integrity (7). Recent evidence indicates, however, that certain cell types can undergo apoptosis without externalizing PS (5, 8, 15). Importantly, the apparent resistance to PS externalization in these cells is completely reversed by treatment with thiol-reactive reagents (5, 8). As disulfide cross-linkers can promote apoptosis by interacting with Cys 56 of the mitochondrial adenine nucleotide translocater (ANT) (3, 18), we reasoned that resistance to PS exposure might be due to the inability of the cells to oxidize critical sulfhydryls. Here we report that ROS likely play a critical role in sulfhydryl oxidation because ROS-dependent intrinsic apoptosis, cyt *c* release, and PS exposure, but not extrinsic apoptosis, are abolished with vitamin E. Our results suggest that ROS produced through the intrinsic pathway directly result in PS externalization by a cyt *c*-dependent mechanism. This likely occurs through a thiol-dependent pathway that disrupts the mitochondrial membrane

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potential ($\Delta\psi m$) and barrier properties of the mitochondrial membrane, resulting in cyt *c* release and activation of a specific PS externalization pathway.

MATERIALS AND METHODS

Materials and routine procedures

Human Jurkat T cells and EBV-transformed Raji B cells were purchased from the American Type Tissue Collection (Manassas, VA, U.S.A.). Cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 m*M* glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cultures were grown at 37°C in a humidified 5% CO_2 environment. Anti-Fas IgM antibodies (clone CH-11) were obtained from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Actinomycin D, staurosporine, and sulfhydryl compounds were purchased from Sigma (St. Louis, MO, U.S.A.). Fluorescein isothiocyanate (FITC)-labeled annexin V was from Trevegin (Gaithersburg, MD, U.S.A.). Fluorescence was quantified with a PerkinElmer LS-50B spectrofluorometer at room temperature.

Annexin V labeling for PS exposure

PS exposure was measured by the binding of FITC-annexin V according to the protocol supplied by the manufacturer. Labeling was monitored by fluorescence-activated cell sorting (FACS) analysis.

Cyt c *release*

The release of cyt *c* from the mitochondria to the cytoplasm was monitored by immunoblotting cytosolic extracts or by immunostaining fixed and permeabilized cells.

Immunoblotting. Cells were washed in ice-cold Tris-buffered saline (TBS; pH 7.2), and the pellet was resuspended in 0.5 ml of hypotonic extraction buffer (20 m*M* HEPES, 1.5 m*M* MgCl, 10 m*M* KCl, 1 m*M* EDTA, 1 m*M* EGTA, 0.1 m*M* phenylmethylsulfonyl fluoride, 10 µg/l leupeptin, 5 µg/ml pepstatin) for 30 min. The cells were then broken in a glass Dounce. The lysates were centrifuged at 100,000 *g* for 15 min, and the supernatants were collected and run on 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels. The separated proteins were then transferred to nitrocellulose membranes. The membranes were blocked with ovalbumin (1%) for 2 h and then probed with an appropriate dilution of anti-human cyt *c* antibodies (Pharmingen). Antibody binding was detected with secondary horseradish peroxidase-conjugated antibody using enhanced chemiluminescence.

Immunostaining. Cells were washed with TBS, and 100 µl of cells (10^6/ml) was plated onto polylysine-coated glass slides for 5 min at 4°C. The adherent cells were fixed (3.5% paraformaldehyde for 30 min), permeabilized (0.01% Tween 20 for 20 min), and blocked (0.5% bovine serum albumin for 15 min). The cells were then incubated with an appropriate dilution of cyt *c* antibody (Pharmingen) followed by FITC-anti-mouse antibody (Sigma).

Determination of $\Delta\psi m$

$\Delta\psi m$ was determined by monitoring the fluorescence emission profile of cells labeled with JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide; 15 µ*M*) (13) for 10 min at room temperature. After washing, the cells were adjusted to 10^6 cells/ml in TBS, and emission scans were recorded from 500 nm to 600 nm (λ_{ex} = 490 nm). $\Delta\psi m$ of the entire cell population was assessed by the ratio of the green/red emission peaks at 534 and 594 nm, respectively.

RESULTS

Cell type-specific apoptosis and PS externalization

Initiation of apoptosis through the extrinsic pathway with Fas antibody results in apoptosis of both Jurkat and Raji cells. However, in contrast to Jurkat cells, Raji cells did not externalize PS as determined by FACS analysis of annexin V-labeled cells (Table 1). To directly trigger ROS-dependent intrinsic apoptosis, both cell types were treated with actinomycin D or staurosporine. Unlike Jurkat cells that became apoptotic and expressed PS, Raji cells were completely resistant to the effects of the drugs (Table 1 and Fig. 1). In fact, the growth rate of Raji cells incubated with 10-fold higher concentration of actinomycin D than that required to induce apoptosis in Jurkat cells was identical to the growth rate of control cells incubated in the absence of the drug (data not shown). These data raise the possibility that the resistance of Raji cells to drug-induced apoptosis through the intrinsic mitochondrial-dependent pathway could be related to the cells' inability to externalize PS upon Fas-induced apoptosis through the extrinsic pathway. Consistent with previous observations (5, 8), incubation of both cell types with *N*-ethylmaleimide (NEM) resulted in the immediate externalization of PS (Fig. 2) irrespective of their sensitivity or resistance to intrinsic apoptogenic compounds.

To study the mechanism that might be involved in the inability of Raji cells to express PS, we elected to mimic their resistance to drug-induced apoptosis by treating intrinsic apoptosis-competent Jurkat cells with vitamin E to inhibit the

TABLE 1. APOPTOSIS AND PS EXTERNALIZATION IN JURKAT AND RAJI CELLS

	Jurkat cells		*Raji cells*	
*Treatment**	*Apoptosis*†	*PS*‡	*Apoptosis*†	*PS*‡
Control cells	1.3%	2.8%	1.1%	0.42%
Actinomycin D	34.5%	75.1%	7.4%	0.52%
Etoposide	37.5%	66.6%	6.4%	6.4%
Staurosporine	34.4%	76.6%	9.0%	8.4%
Anti-Fas	49.7%	86.4%	30.5%	11.4.%

*Actinomycin D, 1 µg/ml, 15 h; etoposide, 25 µg/ml, 15 h; staurosporine, 5 µ*M*, 15 h; anti-Fas, 250 ng/ml, 5 h.

†Apoptosis was determined by FACS analysis of the fraction of cells in sub G0/G1 after staining with propidium iodide.

‡PS exposure was determined by binding of FITC-annexin V.

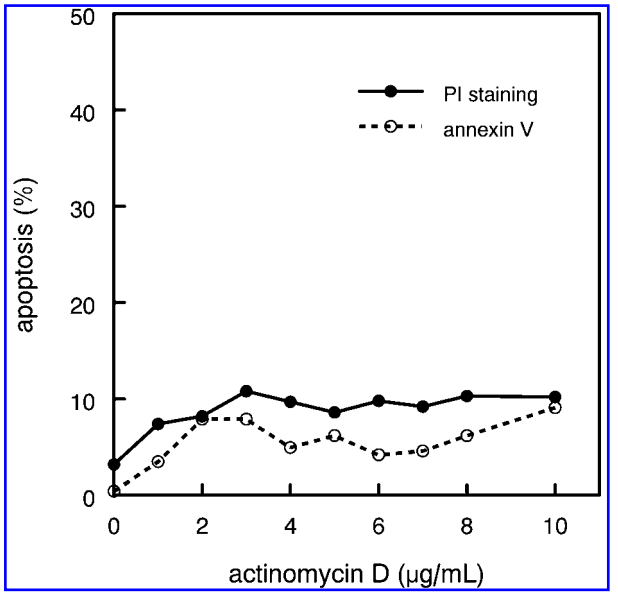

FIG. 1. Resistance of Raji cells to actinomycin D-triggered apoptosis. Raji cells were incubated with increasing concentrations of actinomycin D for 10 h. Apoptosis (sub G0/G1) and PS externalization were determined by staining with propidium iodide (PI) and FITC-annexin V, respectively.

generation of ROS. Figure 3 shows that, similar to the inherent resistance of Raji cells, vitamin E-treated Jurkat cells were completely resistant to actinomycin D-induced apoptosis. As opening of the PTPC pore and concomitant loss of $\Delta\psi m$ are likely required for cyt *c* release, the ability of vitamin E to inhibit collapse of $\Delta\psi m$ was determined. It can be seen that Jurkat cells treated with anti-Fas and actinomycin D underwent apoptosis that was associated with a loss in $\Delta\psi m$ as shown by the shift in the ratio of green/red (534 nm/595 nm) fluorescence in JC-1-labeled cells (Fig. 4). Similar to the data on apoptosis shown in Fig. 3, vitamin E had no effect on $\Delta\psi m$ in anti-Fas-treated cells (Fig. 4A). In contrast, treatment with vitamin E abrogated collapse of $\Delta\psi m$ in actinomycin D-treated cells (Fig. 4B).

Effect of thiol-reactive reagents on $\Delta\psi m$ and PS exposure

Several studies have reported that incubation of cells with thiol-disulfide reagents enforces opening of the PTPC and disruption of $\Delta\psi m$ by cross-linking the mitochondrial ANT through Cys 56. Figure 5 shows the concentration dependence of NEM on $\Delta\psi m$ and PS externalization in Jurkat cells as determined by the ratio of green/red fluorescence and FACS analysis of cells labeled with the membrane potential-sensitive dye JC-1 and annexin V, respectively. The data show a relationship between the concentrations of NEM, disruption of $\Delta\psi m$, cyt *c* release, and PS externalization. However, PS exposure seemed to occur at NEM concentrations below the threshold required for disruption of $\Delta\psi m$.

Even though drug-induced apoptosis is generally associated with decreased $\Delta\psi m$ and PS exposure, uncoupling agents that directly disrupt $\Delta\psi m$ do not commit cells to apoptosis (6). As a key step in initiating apoptosis is opening of the mitochondrial membrane and subsequent release of cyt *c* and apoptosis-inducing factor, we examined the relationship between $\Delta\psi m$ and cyt *c* release to determine whether PS externalization was dependent on decreased $\Delta\psi m$, sulfhydryl oxidation/acylation, or both. Jurkat cells were treated with carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) to disrupt $\Delta\psi m$ without perturbation of sulfhydryls. The results shown in Table 2 indicate that, in the absence of SH-reactive reagents, disruption of $\Delta\psi m$ did not lead to release of cyt *c* or externalization of PS. In contrast, SH reagents, irrespective of whether they acylate (NEM) or cross-link (diamide) target sulfhydryls, induced a concomitant and dramatic disruption in $\Delta\psi m$ that coincided with cyt *c* release and subsequent PS exposure.

DISCUSSION

There is increasing evidence that apoptosis triggered through the intrinsic pathway is dictated by ROS-dependent signaling mechanisms that can alter the redox status of cellular thiols. Several studies have shown that disulfide-generating thiol cross-linkers, but not monovalent thiol acylating reagents, in-

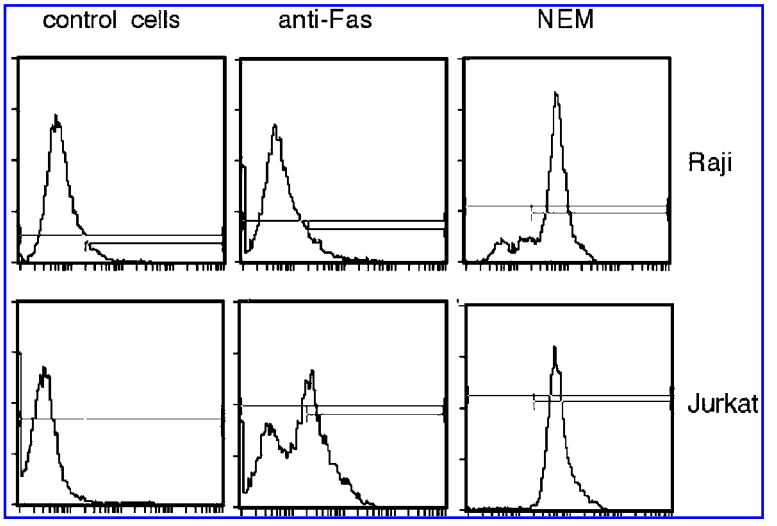

FIG. 2. PS externalization in anti-Fas- and NEM-treated cells. Jurkat and Raji cells were incubated with Fas antibody for 5 h or with 2 m*M* NEM for 30 min. The cells were then washed, and PS externalization was monitored with FITC-annexin V.

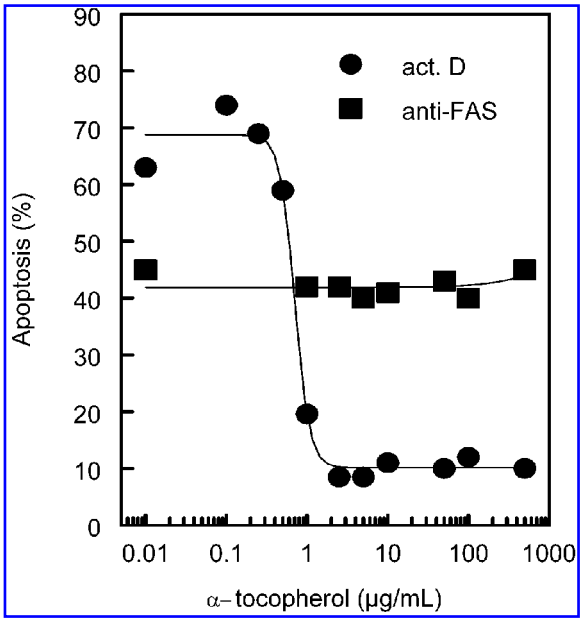

FIG. 3. Inhibition of actinomycin D-triggered apoptosis by vitamin E. Jurkat cells were treated with actinomycin D (1 μg/ml) in the presence or absence of vitamin E for 10 h. Apoptosis was determined by staining with propidium iodide.

duce apoptosis by targeting the mitochondrial matrix (3, 18). Specifically, treatment of cells with cysteine cross-linkers such as diamide or dithiopyridine oxidizes the ANT at a critical cysteine residue (Cys 56) that results in disruption in Δψm, opening of the PTPC with release of cyt *c* and apoptosis-inducing factor, that results in poly(ADP-ribose) polymerase cleavage, PS exposure, and DNA fragmentation by a Bcl-2-independent mechanism. In contrast, incubation of cells with monovalent thiols to produce thiol esters prevented diamide-dependent loss of Δψm (monitored with the potential sensitive dye, DiOC6) (10, 18).

Here we show that incubation of both Jurkat cells and PS externalization-resistant Raji cells with "monovalent" NEM or diamide resulted in reduction in Δψm that was followed by cyt *c* release and PS exposure. Unlike SH reagents, uncoupling reagents like CCCP caused dramatic changes in Δψm without concomitant cyt *c* release or PS exposure.

Previous studies have shown that collapse of Δψm is not required for apoptosis (6) and that the pan caspase inhibitor zVAD-fmk inhibits Δψm collapse and DNA degradation, but not cyt *c* release after incubation of cells with intrinsic apoptogenic reagents (7, 17). Taken together with our data showing that collapse of Δψm alone does not result in cyt *c* release suggests that mitochondrial depolarization is not a prerequisite for its release. Moreover, once released, its presence in the cytosol does not ensure enforcement of the apoptotic program.

Our results suggest that the release of cyt *c* into the cytosol plays an essential role in PS externalization. As extrinsic apoptotic stimuli can initiate apoptosis in the absence of major mitochondrial involvement (9, 15), one would predict that apoptosis could occur in the absence of ROS-dependent cyt *c* release, in contrast to intrinsically induced apoptosis where mitochondrial participation is essential to the apoptotic program. This concept is supported by results that showed that Bcl-2 inhibits both apoptosis (14) and PS externalization in type II cells, but only PS externalization in type I cells (15). This seems to be the case in Raji cells, a property that can be dupli-

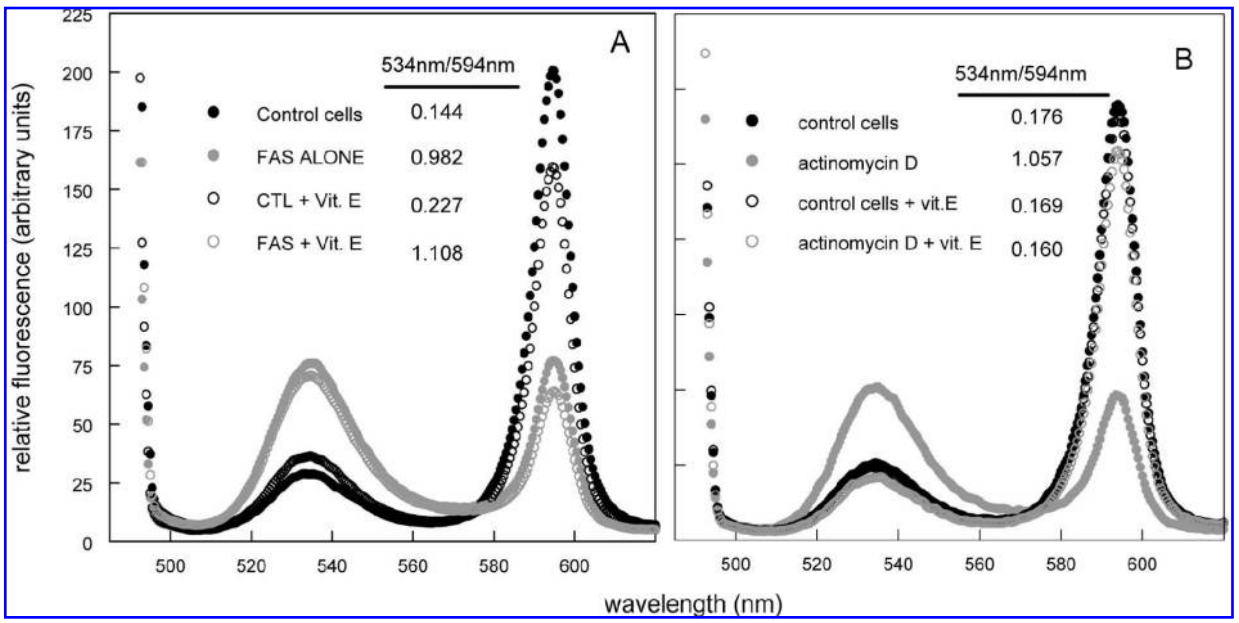

FIG. 4. Loss of Δψm in Fas- and actinomycin D-triggered apoptosis. (**A**) Jurkat cells were treated in the presence or absence of vitamin E (50 μ*M*) overnight and then incubated with Fas antibody for 6 h. (**B**) Cells were incubated with actinomycin D (1 μg/ml) for 10 h in the presence or absence of vitamin E. Δψm was determined by assessing the ratio of green (534 nm)/red (594 nm) fluorescence (λ_{ex} = 485 nm) in JC-1-labeled cells.

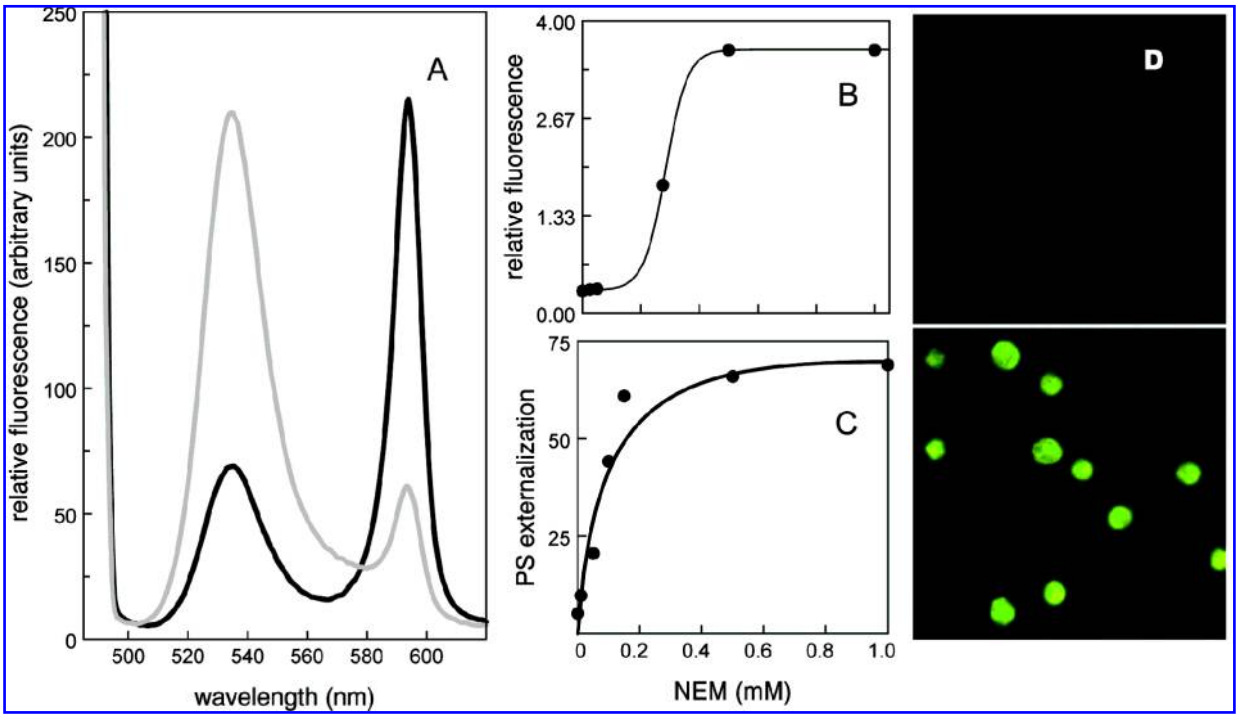

FIG. 5. Effects of NEM on Δψm, PS exposure, and cyt *c* release. Jurkat cells were incubated with increasing concentrations of NEM for 30 min at 37°C. Δψm and PS externalization were determined with JC-1 and annexin V, respectively. Cyt *c* release was determined by immunostaining detergent-permeabilized cells. (**A**) Typical JC-1 scan (black trace, control cells; gray trace, NEM-treated cells). (**B**) Ratio of green (534 nm)/red (594 nm) fluorescence (λ_{ex} = 485 nm). (**C**) FITC-annexin V labeling. (**D**) Immunofluorescence of cyt *c* in (**top**) control cells and (**bottom**) NEM-treated cells.

cated, in part, by vitamin E-treated Jurkat cells. As shown in Results, vitamin E-treated Jurkat cells, like normal Raji cells, are resistant to (ROS-dependent) intrinsic apoptogenic stimuli such as actinomycin D. Observations that Raji cells, in contrast to Jurkat cells, do not externalize PS during Fas-triggered extrinsic apoptosis suggest that Raji are phenotypically type I cells that undergo apoptosis in the absence of ROS-mediated cyt *c* release, a property consistent with their resistance to intrinsic mitochondria/cyt *c* release-dependent stimuli.

In summary, our data support the notion that apoptosis and PS externalization are distinct and separable processes. Thus, although cyt *c* clearly plays an important role in promoting the intrinsic apoptotic cascade, extrinsic apoptosis can proceed in the absence of PS externalization (15). Although conclusive evidence is still lacking, all of the available data suggest that PS externalization depends on cytosolic cyt *c*. This can be induced chemically with sulfhydryl reagents that *a priori* do not trigger apoptosis, or physiologically through cyt *c*-dependent apoptotic pathways.

TABLE 2. RELATIONSHIP BETWEEN ΔΨM, PS EXTERNALIZATION, AND CYT *c* RELEASE*

	Δψm		*PS externalization*		*Cyt c release*	
Treatment†	*Jurkat*	*Raji*	*Jurkat*	*Raji*	*Jurkat*	*Raji*
Control	0.43	0.1	No	No	No	No
Diamide (5 m*M*)	3.71	4.4	Yes	Yes	Yes	Yes
NEM (2 m*M*)	5.2	0.88	Yes	Yes	Yes	Yes
NEM + zVAd-fmk	5.5	ND	Yes	Yes	Yes	Yes
CCCP (10 μ*M*)	6.23	0.68	No	No	No	No

*Δψm was determined by the ratio of fluorescence emission (534 nm/594 nm) in JC-1-labeled cells. PS exposure was determined by FACS analysis of FITC-annexin V-labeled calls. Cyt *c* release was determined by immunoblotting or by immunofluorescence in permeabilized cells.

†Jurkat and Raji cells were incubated with the indicated reagents for 30 min at 37°C.

ABBREVIATIONS

ANT, adenine nucleotide translocator; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; cyt *c*, cytochrome *c*; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide; NEM, *N*-ethylmaleimide; PS, phosphatidylserine; PTPC, permeability transition pore complex; ROS, reactive oxygen species; TBS, Tris-buffered saline; $\Delta\psi m$, mitochondrial membrane potential.

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