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Fas-, Caspase 8-, and Caspase 3-dependent Signaling Regulates the Activity of the Aminophospholipid Translocase and Phosphatidylserine Externalization in Human Erythrocytes*

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Apoptosis and erythrocyte senescence share the common feature of exposure of phosphatidylserine (PS) in the outer leaflet of the cells. Western analysis showed that mature red cells contain Fas, FasL, Fas-associated death domain (FADD), caspase 8, and caspase 3. Circulating, aged cells showed colocalization of Fas with the raft marker proteins $G\alpha_s$ and CD59; the existence of Fas-associated FasL, FADD and caspase 8; and caspase 8 and caspase 3 activity. Aged red cells had significantly lower aminophospholipid translocase activity and higher levels of PS externalization in comparison with young cells. In support of our contention that caspases play a functional role in the mature red cell, the oxidatively stressed red cell recapitulated apoptotic events, including translocation of Fas into rafts, formation of a Fas-associated complex, and activation of caspases 8 and 3. These events were independent of calpain but dependent on reactive oxygen species (ROS) as evident from the effects of the ROS scavenger *N*-acetylcysteine. Caspase activation was associated with loss of aminophospholipid translocase activity and with PS externalization. ROS was not generated by treatment of cells with *t*-butyl hydroperoxide at 10 °C, and Fas did not translocate into rafts. Concomitantly, neither formation of a Fas-associated signaling complex nor caspase activation could be observed, supporting the view that translocation of Fas into rafts was the trigger for the chain of events leading to caspase 3 activation. Our data demonstrate for the first time the novel involvement of Fas/caspase 8/caspase 3-dependent signaling in an enucleated cell leading to PS externalization, a central feature of erythrophagocytosis and erythrocyte biology.

Programmed cell death or apoptosis is a physiological process that contributes to the homeostasis of multicellular organisms and maintains the balance between cell proliferation and cell death. The survival of nucleated mammalian cells depends on the constant repression of their self-destruction program by signals provided by other cells (1, 2). Mature erythrocytes, like nucleated cells, share the capacity to self-destruct at the end of a 120-day life span or in response to environmental signals. It is therefore likely that erythrocyte survival, lifespan, and death are tightly regulated as in other cells. Whereas the process of apoptosis has been studied in depth in nucleated cells, the role of apoptotic regulatory molecules in enucleated cells such as platelets and erythrocytes is poorly understood. Although the presence of aspartate proteases has been documented for both cell types (3, 4), the function of these proteases, if any, in red cell physiology is just beginning to be appreciated.

The terminal steps of apoptosis in nucleated cells involve the proteolytic activation of a family of aspartate-directed cysteine proteinases, the best studied being the caspases. Activation of the effector caspases may occur by the engagement of death receptors (5). In addition, most apoptotic stimuli turn on a mitochondrion-dependent pathway (6–8). Mature erythrocytes are terminally differentiated and lack mitochondria as well as nucleus and other organelles. However, erythrocyte senescence shares features common to apoptosis in nucleated cells, such as the externalization of phosphatidylserine (PS)[2] on the outer leaflet of the cells. Erythrocytes were until recently considered to lack pathways that have been traditionally linked to activation of the apoptotic machinery in eukaryotic, nucleated cells. However, recent studies have pointed in a direction indicating that part of the machinery that is associated with execution of apoptosis in nucleated cells not only exists in the mature human erythrocyte but is likely to be of physiological significance. We have demonstrated that oxidative stress (9) and erythrocyte aging (10) lead to stimulation of caspase 3 and caspase 3-mediated degradation of band 3.

Apoptotic signaling pathways that lead to caspase 3 activation in various cell types can be subdivided into two major categories: extrinsic and intrinsic. In the extrinsic pathway, apoptotic signals are initiated by cell surface death receptors exemplified by Fas, also known as CD95 or APO-1, leading to the caspase 8-dependent activation of effector caspases, such as caspase 3. Fas is widely expressed. Upon binding to FasL, Fas recruits a cytosolic adapter protein, Fas-associated death domain (FADD), which, in turn, recruits procaspase 8 and forms the death-inducing signaling complex (DISC) (11, 12). Recruitment of procaspase 8 to the DISC results in activation and release of active caspase 8, which directly activates caspase 3 and initiates cell death (13).

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[2] The abbreviations used are: PS, phosphatidylserine; FADD, Fas-associated death domain; DISC, death-inducing signaling complex; APLT, aminophospholipid translocase; *t*-BHP, *t*-butyl hydroperoxide; NEM, *N*-ethylmaleimide; Ph-hyz, phenylhydrazine; MβCD, methyl-β-cyclodextrin; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; *p*NA, *p*-nitroanilide; AFC, 7-amino-4-trifluoromethyl coumarin; Ac-IETD-AFC, Ac-Ile-Glu(OMe)Thr-Asp(OCH_3)-AFC; FMK, fluoromethyl ketone; Z-DEVD-FMK, benzyloxycarbonyl-Asp(OCH_3)-Glu-Val-Asp(OCH_3)-FMK; Z-IETD-FMK, benzyloxycarbonyl-Ile-Glu(OCH_3)-Thr-Asp(OCH_3)-FMK; Z-LEHD-FMK, Z-Leu-Glu(OCH_3)-His-Asp(OCH_3)-FMK; NAC, *N*-acetylcysteine; NBD-PS, 1-palmitoyl-2-[6-[7(nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-caproyl]-*sn*-glycerol-3-phosphoserine; DTSSP, 3,3′-dithiobis(sulfosuccinimidyl propionate); ROS, reactive oxygen species; DCF, 2′,7′-dichlorofluorescein; TBS, Tris-buffered saline; BSA, bovine serum albumin; PIPES, 1,4-piperazinediethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethyl ammonio]1-propanesulfonic acid; PBS, phosphate-buffered saline; DRM, detergent-resistant membrane; PS, phosphatidylserine.

Considering that erythrocytes lack mitochondria, yet undergo activation of aspartate-proteases such as caspases, we reasoned that a machinery involving caspase 8 activation via death receptors prior to activation of caspase 3 might exist in the mature erythrocyte. Our present studies show that Fas, FasL, FADD, and caspase 8 localize to detergent-resistant membrane microdomains of aged and oxidatively stressed red cells. Concomitant with formation of this Fas-associated complex, aged or oxidatively stressed red cells showed caspase 8 and caspase 3 activity, as well as reduced aminophospholipid translocase (APLT) activity in comparison with young red cells. Our studies demonstrate unequivocally that signaling pathways, culminating in activation of the prototypical executioner caspase 3, play an essential role in PS externalization, a central event linked to erythrophagocytosis.

EXPERIMENTAL PROCEDURES

Reagents—Anti-Fas and anti-caspase 8 rabbit polyclonal antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse monoclonal antibody against Fas was from Upstate Biotechnology, Inc. (Charlottesville, VA). Mouse monoclonal anti-FADD and anti-FasL antibodies were from BD Biosciences. Rabbit polyclonal anti-FADD and anti-caspase 3 antibodies were from Cell Signaling Technology (Beverley, MA). Goat anti-rabbit Alexa 488 and goat anti-mouse Alexa 546 antibodies were from Molecular Probes, Inc. (Eugene, OR). Annexin-V-fluos was a product of Roche Applied Science. Anti-Gα_s antibody, *t*-butyl hydroperoxide (*t*-BHP), diamide, *N*-ethylmaleimide (NEM), phenyl hydrazine (Ph-hyz)-HCl, horseradish peroxidase-conjugated goat anti-rabbit antibody, methyl-β-cyclodextrin (MβCD), and the fluorescent probe dichlorofluorescein diacetate (DCFH-DA) were from Sigma. Caspase 3 substrate Ac-DEVD-*p*NA, caspase 8 substrate, Ac-IETD-AFC, caspase 3 inhibitor, Z-DEVD-FMK, caspase 8 inhibitor Z-IETD-FMK, caspase 9 inhibitor Z-LEHD-FMK, calpain inhibitor calpeptin, and *N*-acetylcysteine (NAC) were from EMD Biosciences (La Jolla, CA). Anti-CD59 antibody was from R & D Systems (Minneapolis, MN), 1-palmitoyl-2-[6-[7(nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-caproyl]-*sn*-glycerol-3-phosphoserine (NBD-PS) was from Avanti Polar Lipids (Birmingham, AL), and 3,3′-dithiobis(sulfosuccinimidyl propionate) (DTSSP) was from Pierce. All other reagents were of analytical grade.

Separation and Treatment of Red Blood Cells—Fresh blood was collected from normal healthy volunteers with informed consent, and packed erythrocytes were collected after centrifugation over a Ficoll gradient. The erythrocytes were then washed three times in 25 mM HEPES, pH 7.5, 150 mM NaCl (buffer A) and diluted to 2% hematocrit in the same buffer. Erythrocytes were treated for 15 min at 37 °C with diamide, Ph-hyz, *t*-BHP, or NEM at the indicated concentrations.

Separation of Red Blood Cells on Percoll-Bovine Serum Albumin (BSA) Gradients—Density-dependent separation of red cells was performed as described by Corsi *et al.* (14) with slight modifications. Briefly, the plasma was removed, and erythrocytes were washed three times with 10 mM sodium phosphate (pH 7.4), 150 mM NaCl, 5 mM glucose (PBSG buffer) and layered at 25% hematocrit on a discontinuous Percoll (55–85%, v/v)-BSA gradient followed by centrifugation at 1,500 × *g* for 20 min at 20 °C. Young and aged erythrocytes were collected separately and washed in PBSG buffer.

Measurement of Reactive Oxygen Species (ROS)—ROS produced within erythrocytes were detected with the membrane-permeable probe DCFH-DA. The probe freely enters the cell and is incorporated into hydrophobic regions. The acetate moiety is cleaved off by cellular esterases, leaving a nonfluorescent and impermeable form of DCFH (15). ROS produced by cells oxidizes DCFH to fluorescent dichlorofluorescein (DCF). Erythrocytes were incubated with 100 μM DCFH-DA in PBS for 30 min at 37 °C, prior to application of stress. Fluorescence was monitored using an excitation wavelength of 485 nm and an emission wavelength of 538 nm (16).

Western Blotting—Erythrocytes (2×10^8 cells) were usually lysed in 20 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10% glycerol, and protease inhibitors (20 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin, and 1 mM pefabloc) (buffer B). Lysates were clarified, and proteins were separated on SDS-polyacrylamide gels and then transferred electrophoretically to polyvinylidene difluoride membranes. The blots were blocked with 5% nonfat dry milk and subsequently incubated overnight at 4 °C with primary antibodies in Tris-buffered saline (TBS; 25 mM Tris, 150 mM NaCl, pH 7.4) containing Tween 20 (1%, v/v) (TBST) and 5% (w/v) BSA. After washing, the blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Cell Signaling Technology) (or appropriate secondary antibody) in blocking buffer for 1 h at room temperature, followed by development with BM chemiluminescence reagent (Roche Applied Science) and exposed to x-ray film (Eastman Kodak Co. XAR5). When performing Western blotting for detection of caspases, 6×10^5 cells were pelleted, freeze-thawed in 20 μl of extraction buffer (50 mM PIPES/NaOH, pH 6.5, 2 mM EDTA, 0.1% (w/v) CHAPS, 5 mM dithiothreitol, and protease inhibitors). Rabbit polyclonal anti-FADD, anti-FasL, anti-caspase 3, and anti-caspase 8 antibodies were used at a dilution of 1:1,000.

Immunoprecipitation—Erythrocytes (2×10^8 cells) were lysed in buffer B for 15 min at 4 °C. Lysates were centrifuged at 25,000 × *g* for 15 min, and supernatants were immunoprecipitated overnight with rabbit anti-Fas antibody (1:200) or anti-caspase 8 antibody (1:100) at 4 °C. The supernatant was further incubated with 10 μl of protein A/G-agarose slurry for 3 h at 4 °C. Beads were pelleted, washed three time in lysis buffer, and finally boiled in SDS gel denaturing buffer. Proteins were separated by SDS-PAGE prior to Western blotting.

Fixation of Cells, Staining, and Confocal Microscopy—Treated and untreated erythrocytes were fixed in 95% methanol, 5% PBS for 5 min on glass slides. Cells were permeabilized further with 0.1% Triton X-100 in the same fixative solution for 1 min. Blocking was done in PBS containing 3% BSA (PBSB) for 30 min at room temperature. Staining with antibodies and washing of cells was done in PBSB. Mouse monoclonal anti-Fas, rabbit polyclonal anti-caspase 8, and anti-Gα_s were used at a dilution of 1:1,500 to stain the cells for 1 h at room temperature. Alexa 546-conjugated anti-mouse- and Alexa 488-conjugated anti-rabbit-specific secondary antibodies were used at dilutions of 1:1,500 for 30 min at room temperature. After each staining, cells were washed thoroughly, and samples were mounted with glycerol/PBS (4:1) and observed under a Zeiss LSM 510 confocal microscope.

Determination of Aminophospholipid Translocase Activity—Flippase activity was assessed from the extent of fluorescent phospholipid retained in the plasma membrane after back-extraction with BSA, which extracts only exogenously added fluorescent phospholipids and not endogenous phospholipids, from the outer monolayer (17). Briefly, NBD-PS was reconstituted in 5 mM HEPES, pH 7.5, 14 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 10 mM glucose (buffer C). The lipid was added to a final concentration of 1 μM to erythrocytes and incubated on ice for 5 min in buffer C, followed by incubation at 37 °C for 1 h. 100-μl aliquots were removed at different time intervals and added to 1 ml of cold buffer B containing 1% fatty acid-free BSA in order to extract NBD-PS present in the outer leaflet. Fluorescence associated with the supernatant was measured in a spectrofluorimeter at an excitation wavelength of 472 nm and an emission wavelength of 534 nm.

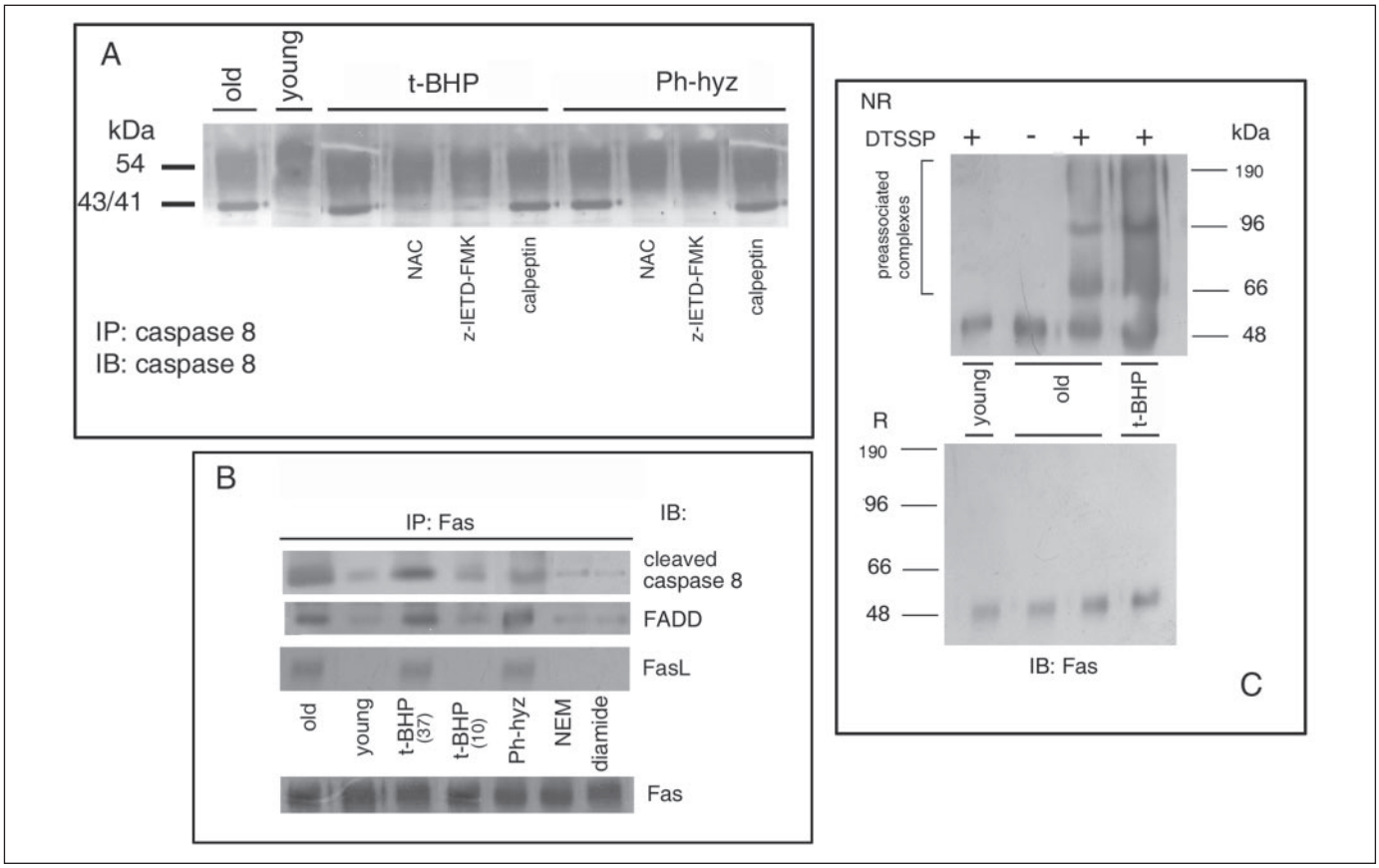

FIGURE 1. **Aging and oxidative stress activate caspase 8 and are associated with formation of a Fas-associated signaling complex in mature human erythrocytes.** Untreated old (*A* and *B*) or young erythrocytes (*A* and *B*) or cells treated for 15 min with *t*-BHP (2 mM) at 37 °C (*A* and *B*) or at 10 °C (*B*) or with Ph-hyz (2 mM) (*A* and *B*), diamide (2 mM) (*B*), or NEM (2 mM) (*B*) were lysed and immunoprecipitated with anti-caspase 8 (*A*) or anti-Fas antibody (*B*). In separate experiments, cells were pretreated with NAC (10 mM) or Z-IETD-FMK (100 μM) or calpeptin (20 μM) for 30 min prior to treatment with t-BHP or Ph-hyz (*A*). The immunoprecipitates were separated by SDS-PAGE and blotted with anti-caspase 8 (*A* and *B*), anti-FADD (*B*), or anti-FasL (*B*) antibody. Blots were reprobed with anti-Fas antibody to ensure equal loading of the gels (*B*). The bands of pro-caspase 8 (54 kDa) and cleaved caspase 8 (43/41 kDa) have been indicated in *A*. *C*, young, old, and *t*-BHP-treated cells were either left untreated (−) or treated (+) with the thiol-cleavable cross-linker DTSSP as described under "Experimental Procedures." Cell lysates were separated by SDS-PAGE under reducing (*R*) and nonreducing (*NR*) conditions and immunoblotted with anti-Fas antibody. High molecular weight species of preassociated Fas induced by DTSSP were visible under NR conditions (*upper blot*) in the case of old cells (*third lane*) as well as *t*-BHP-treated cells (*fourth lane*) but not in the case of cells that had not been treated with *t*-BHP (*first lane*). Blots are representative of the results obtained in three separate experiments.

Determination of Annexin Binding to Red Cells—Labeling with annexin-fluos was performed using the annexin-fluos kit from Roche Applied Science according to the manufacturer's instructions. Data acquisition was performed on a BD Biosiences FACScaliber flow cytometer, and analysis was done with Cell Quest software. 10,000 events/sample were acquired to ensure adequate mean fluorescence levels. The percentage of annexin V-positive erythrocytes was determined from the fluorescence signal in excess of that obtained with a negative (unlabeled) control that was run in each case.

Isolation of Lipid Rafts from Erythrocyte Ghosts—Erythrocyte ghosts were prepared from young, aged, or oxidatively stressed cells separated on Percoll-BSA gradients as described above. Detergent-resistant membrane (DRM) fractions were isolated from ghosts according to the method of Samuel *et al.* (18) with some modifications. Briefly, ghosts from 2×10^9 cells were extracted at 4 °C in 1.5 ml of TBS containing 1% Triton X-100, protease inhibitors, and 1 mM EDTA and subjected to a sucrose flotation gradient. After 30 min at 4 °C, the extract was mixed with an equal volume of 80% (w/v) sucrose, overlaid with 30% (w/v) sucrose and 5% sucrose, and subjected to ultracentrifugation in an AH-629 rotor (Sorvall) at 27,000 rpm for 3 h at 4 °C. The top 4 ml was collected as the first fraction. Proceeding down the gradient, fractions 2–6 were collected as 3-ml aliquots. Samples were precipitated with chilled acetone, and 40 μg of each protein sample was separated by SDS-PAGE, followed by Western blotting with different antibodies.

Cholesterol Depletion of Erythrocytes—"Lipid rafts" are now widely accepted as membrane microdomains enriched in sphingolipids and cholesterol (19, 20). Cyclodextrins selectively extract cholesterol and disrupt membrane rafts (21). Cholesterol depletion was accomplished by treating erythrocytes (at 5% hematocrit) in PBS containing 3 mM KCl with 4 mM MβCD for 2 h at 37 °C (23). The erythrocytes were washed with PBS to remove MβCD and associated cholesterol. Approximately 90% of the total cholesterol was removed by this process, since erythrocyte cholesterol is almost entirely associated with the plasma membrane.

Chemical Cross-linking by DTSSP—DTSSP is a water-soluble, impermeable, thiol-cleavable cross-linker. For cross-linking, 2×10^8 erythrocytes/ml were incubated with 1 mM DTSSP in PBS at 25 °C (24) for 30 min. The reaction mix was further incubated with 10 mM Tris-HCl for 15 min at 25 °C to terminate the cross-linking reaction. Cells were then lysed in 1% Triton X-100, 5 mM iodoacetate, protease inhibitors, 150 mM NaCl, 15 mM EDTA, and 10 mM Tris-HCl. Lysates were immunoprecipitated with anti-Fas antibody as described earlier. After immunoprecipitation, samples were divided in two portions and boiled separately in SDS gel denaturing sample buffer (reducing condition) and β-mercaptoethanol-free sample buffer (nonreducing condition). Samples were separated by 7.5% SDS-PAGE and probed with anti-Fas antibody.

Caspase Activity Assays—For caspase 3 or caspase 8 activity assays, each well of microtiter plates was coated overnight with 2 μg of rabbit

polyclonal caspase 3 or caspase 8 antibody in sodium carbonate/bicarbonate buffer, pH 9.6. After washing with TBST, wells were incubated with TBST containing 3% BSA for 30 min for blocking. Cell-free lysates (from 2×10^8 cells) were loaded in appropriate wells, incubated for 4 h at room temperature, and washed, and DEVDase- or IETDase-dependent protease activity was determined using the colorimetric substrate Ac-DEVD-*p*NA or the fluorimetric substrate Ac-IETD-AFC, respectively, at a concentration of 100 μM in 50 mM HEPES, pH 7.4, containing 1% sucrose, 0.1% CHAPS, and 10 mM DTT. *p*NA release was determined spectrophotometrically at 405 nm. AFC release was measured in a spectrofluorimeter using excitation and emission wavelengths of 360 and 530 nm, respectively.

Statistical Analysis—Data are represented as the means ± S.D. of separate experiments. Student's *t* test was performed to test statistical significance. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Activation of Caspase 8 in Old Red Cells—In normal density-dependent methods of separation, the youngest red cells are the lightest, whereas the aged cells are in the dense fractions (25). Despite some controversy over the years, the dense fraction of circulating red cells is widely accepted to be enriched in aged erythrocytes (26). Therefore, for the purpose of this study, the dense fraction of red cells (density higher than 1.1 g/ml) will be referred to as aged (or old) red cells, and the fraction with a density lower than 1.082 g/ml will be referred to as young red cells. Our previous observations had demonstrated that active caspase 3 is present in old red cells, whereas it could not be detected in young cells (10). Since erythrocytes are devoid of mitochondria, a mitochondria-independent pathway of caspase 3 activation appeared possible. We therefore considered the possibility that caspase 3 activation could be preceded by formation of a DISC-like assembly involving a death receptor, followed by caspase 8 activation. This was further explored as a likelihood, since caspase 8 and the death receptor Fas have been shown to play an important role in erythroid development (27). The cleaved 43/41-kDa caspase 8 band could be detected by Western blotting in old but not in young red cells (Fig. 1*A*). An activity assay using the substrate Ac-IETD-AFC showed a 3–4-fold increase in proteolytic activity in old cells compared with young cells over three independent sets of experiments.

Association of Fas with FADD and Caspase 8—In order to analyze whether caspase 8 activation is associated with formation of complexes involving death receptors and death-linked adapter proteins, coimmunoprecipitation experiments were performed. Red cell lysates were immunoprecipitated with anti-Fas antibody, followed by Western blotting either with anti-caspase 8 or with anti-FADD antibody. Both caspase 8 and FADD were found in the immunoprecipitates from old cells (Fig. 1*B*) but not from the young cells. In addition, FasL was also found to associate with Fas in old cells (Fig. 1*B*). The association of caspase 8 with Fas was further confirmed by confocal microscopy. Fas was present in the plasma membrane, whereas caspase 8 displayed punctate staining in the cytosol of young cells (Fig. 2*A*). However, caspase 8 translocated to the membrane in old cells. Merging of the images suggested that caspase 8 colocalizes with Fas in patches observed in old cells (Fig. 2*B*).

Clustering of Fas in Membrane Microdomains in Old Red Cells—Redistribution of Fas into plasma membrane rafts is one possible mechanism for regulating the efficiency of Fas-mediated death signaling (28, 29). The death-inducing signaling complex is recruited into rafts during Fas-induced apoptosis (24). DRM rafts have been demonstrated in the mature human erythrocyte (18, 30) and shown to regulate erythrocyte β_2-adrenergic receptor and guanine nucleotide-binding protein ($G\alpha_s$)-dependent signaling (31). Considering that translocation of Fas into lipid rafts generates death-inducing signaling platforms (32) and that clusters of Fas were observable in old cells by confocal microscopy, we asked the question whether Fas translocates into membrane rafts in the aged red cell. The consistent localization of $G\alpha_s$ in erythrocyte rafts is documented (18, 30). Confocal microscopy showed that the Fas clusters observed in old cells colocalized with $G\alpha_s$ (Fig. 3*A*). However, no colocalization of Fas with $G\alpha_s$ was observed in young cells (Fig. 3*B*). Further, DRM lipid rafts were isolated by discontinuous sucrose density gradient centrifugation (33). Fractions from the gradient were analyzed by SDS-PAGE and Western blotting. The position of the raft-associated proteins in the gradient was determined by the presence of $G\alpha_s$ and the glycosylphosphatidylinositol-anchored protein CD59, which were enriched in the upper part of the sucrose gradient (fraction 2) with a secondary localization at the bottom of the gradient (fraction 6) (Fig. 4), in accordance with the observations of Samuel *et al.* (18) and Murphy *et al.* (30), indicating a separation of lipid rafts from the Triton X-100-soluble membranes (30). Using a specific anti-Fas antibody, we found that Fas was localized in the bottom fraction of the gradient in untreated, young red cells (Fig. 4*B*), whereas it was recruited into the top (lightest) fractions in aged cells, where it cofractionated with the raft markers $G\alpha_s$ and CD59 (Fig. 4*A*). We also found that FasL, FADD, and

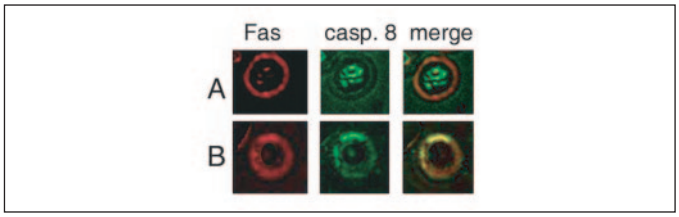

FIGURE 2. **Association of Fas with caspase 8 as demonstrated by confocal microscopy.** Young (*A*) and old (*B*) cells were incubated with anti-Fas and anti-caspase 8 (*casp. 8*) antibody. Cells were washed and labeled with Alexa 488-conjugated rabbit IgG (*green* for caspase 8) and Alexa 546-conjugated mouse IgG (*red* for Fas) as described under "Experimental Procedures." Colocalization of Fas with caspase 8 upon merging (*yellow patches*) was observed in the case of old (*B*) cells only. The results shown are representative of three different experiments.

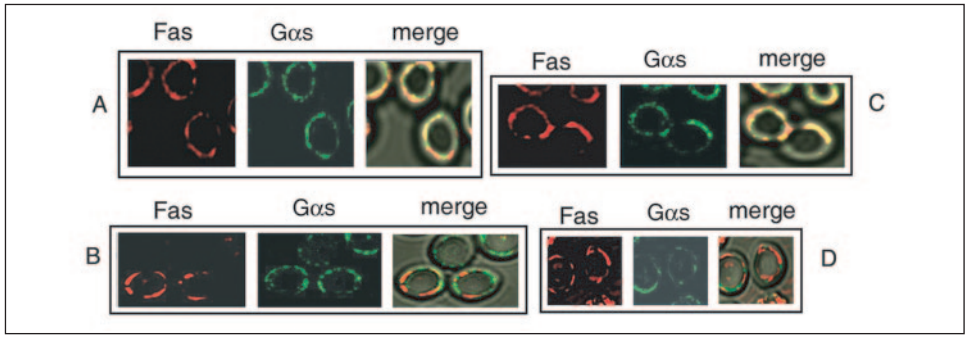

FIGURE 3. **Partitioning of Fas in membrane rafts as demonstrated by confocal microscopy.** Old cells (*A*), young cells (*B*), and cells treated with *t*-BHP at 37 °C (*C*) or at 10 °C (*D*) were incubated with antibodies against Fas and $G\alpha_s$. Cells were washed and labeled by incubating with Alexa 546-conjugated mouse IgG (*red*, Fas) or Alexa-conjugated 488-conjugated rabbit IgG (*green*, $G\alpha_s$). Areas of colocalization between Fas and $G\alpha_s$ (a raft marker) in the *merge panels* (*yellow*) are observable in *A* and *C* but not in *B* and *D*. The results shown are representative of three different experiments. In all the experiments, >70% of the total cell population showed the same labeling pattern, indicating similar localization of markers.

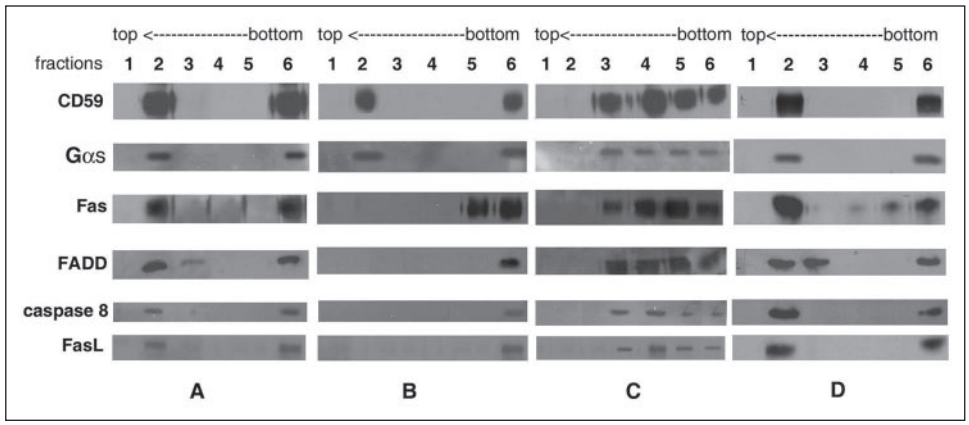

FIGURE 4. **Association of lipid rafts with DISC formation.** Old cells (*A*), young cells (*B*), cholesterol-depleted old cells obtained after treatment with MβCD (*C*) as described under "Experimental Procedures," or *t*-BHP-treated cells (*D*) were washed with PBS and lysed, and the cell-free supernatants were subjected to sucrose density gradient fractionation. Fractions 1–6 (*top* to *bottom*) were collected as described. Proteins in each fraction were resolved by SDS-PAGE and blotted with antibodies against CD59, $G\alpha_s$, Fas, FADD, caspase 8, and FasL, as indicated.

procaspase 8 were recruited in the same light fractions (as the raft markers) in aged cells (Fig. 4*A*). However, in our experimental system, it was not possible to address the question whether Fas is recruited first into the rafts, before recruitment of other death-inducing molecules, as is the case for anti-CD3-stimulated T cells (24). MβCD-mediated cholesterol depletion is known to disrupt lipid rafts. Incubation of aged erythrocytes with MβCD resulted in a randomization of the distribution of Fas, FADD, caspase 8, and FasL and a shift to the heavier fractions (Fig. 4*C*). This finding favored our notion that Fas, FADD, caspase 8, and FasL localize to rafts in aged erythrocytes. Our results demonstrate that events in the lifespan of the circulating erythrocyte trigger the movement of these molecules into distinct microdomains in aged, red cells.

ROS in Aged Red Cells—Since ROS has been extensively documented to be linked to death signaling in a variety of cell types (24), we investigated whether ROS could be detected in aged red cells. The oxidation of 2′,7′-DCFH-DA has been used in many laboratories to detect cellular radical generation. We used this approach to measure ROS in aged cells by measuring DCF in aged cells by fluorimetry. A >3-fold increase in ROS was observed in aged cells compared with young cells (Fig. 5*A*).

The Oxidatively Stressed Red Cell as a Model—Since aged red cells represent the terminal state of the mature erythrocyte in its lifespan and are not amenable to manipulation, we needed a model to validate some of our hypotheses. Considering that it appeared likely that ROS could be playing a role, we chose the oxidatively stressed red cell as our model. Treatment of cells with *t*-BHP (2 mM) or Ph-hyz (2 mM) led to an increase in ROS production over untreated, young cells comparable with that observed in aged cells (Fig. 5*A*). Our previous studies have documented that *t*-BHP-treated red cells showed caspase 3 activation (9). The caspase 3 activity observed in aged cells was also comparable with that observed in *t*-BHP-treated cells (Fig. 5*C*). We therefore chose *t*-BHP-treated red cells as our model for further studies aimed at determining the causal involvement of caspase 8 in caspase 3 activation in red cells.

Caspase 8 Activation Is Upstream of Caspase 3 Activation in Oxidatively Stressed Red Cells—Western analyses showed the appearance of a 43/41-kDa band (Fig. 1*A*) in stressed but not in control cells, suggesting that oxidative stress is associated with caspase 8 activation. IETDase activity measured by AFC release was also consistently more than 5-fold higher in oxidatively stressed cells compared with untreated cells (data not shown). Pretreatment of cells with NAC or Z-IETD-FMK resulted in abrogation of oxidative stress-induced caspase 8 (Fig. 1*A*). Since calpeptin had no effect on caspase 8 activation (Fig. 1*A*), the involvement of calpain in oxidative stress-induced caspase 8 activation was ruled out. In order to investigate whether caspase 8 activates caspase 3 in oxidatively stressed cells, cells were pretreated with cell-permeable caspase inhibitors prior to *t*-BHP treatment and Western blotting to assess caspase 3 activation. Caspase 3 activation was inhibited specifically by the caspase 3- and caspase 8-specific inhibitors Z-DEVD-FMK and Z-IETD-FMK, respectively, but not by the caspase 9 inhibitor Z-LEHD-FMK (Fig. 5*B*), suggesting that caspase 8 was probably the upstream protease involved in caspase 3 cleavage. Caspase 3 activity was also assayed using the substrate Ac-DEVD-*p*NA. Inhibition of activity by Z-DEVD-FMK and Z-IETD-FMK but not by Z-LEHD-FMK (Fig. 5*D*) corroborated the observation that caspase 3 is activated in a caspase 8-dependent manner in oxidatively stressed cells. Considering that Ca^{2+} influx has been implicated in erythrocyte apoptosis (34), young cells were loaded with $CaCl_2$ and the ionophore A23187 prior to assessing caspase 3 activation. No increase in caspase 3 activity was observed by Western blotting (Fig. 5*B*) or the DEVDase activity assay (Fig. 5*C*), suggesting that Ca^{2+} does not play a role in caspase activation. Calpeptin pretreatment did not influence caspase 3 activation (Fig. 5, *B* and *D*), ruling out the involvement of calpain in the signaling pathway leading to the activation of caspase 3.

FADD, FasL, and Caspase 8 Associate with Fas in Oxidatively Stressed Cells—In order to analyze whether caspase 8 activation is linked to formation of a death receptor-associated complex, cells after treatment were lysed, and lysates were immunoprecipitated with anti-Fas antibody. The immunoprecipitates were immunoblotted with anti-FADD or anti-caspase 8 antibody. Both FADD and caspase 8 could be detected in the immunoprecipitates of cells treated with *t*-BHP or Ph-hyz at 37 °C (Fig. 1*B*), suggesting that a Fas-associated complex is formed following exposure of red cells to agents generating ROS. This complex could not, however, be detected in cells treated with the thiol-reactive reagents diamide and NEM, suggesting that ROS generation was specifically required for formation of the Fas-associated complex. FasL was also recruited in this complex, as evidenced by the fact that it could be coimmunoprecipitated with Fas. In harmony with these observations, Fas, FasL, FADD, and procaspase 8 were recruited in the detergent-resistant raft fractions, mirroring their distribution in aged cells (Fig. 4*D*).

Oligomerization of Fas in Aged and in t-BHP-treated Cells—The presence of proteins in rafts favors their self-association (35, 36). The formation of higher order oligomers of Fas has been reported to be associated with death signaling. We analyzed the oligomerization of Fas by incubating red cells with the thiol-cleavable cross-linker DTSSP for 30 min. After terminating the cross-linking reaction, samples were immunoprecipitated with anti-Fas antibody, separated by 7.5% SDS-PAGE under reducing and nonreducing conditions, electrotransferred onto polyvinylidene difluoride membranes, and immunoblotted with anti-Fas antibody. High molecular mass bands (70–200 kDa) appeared

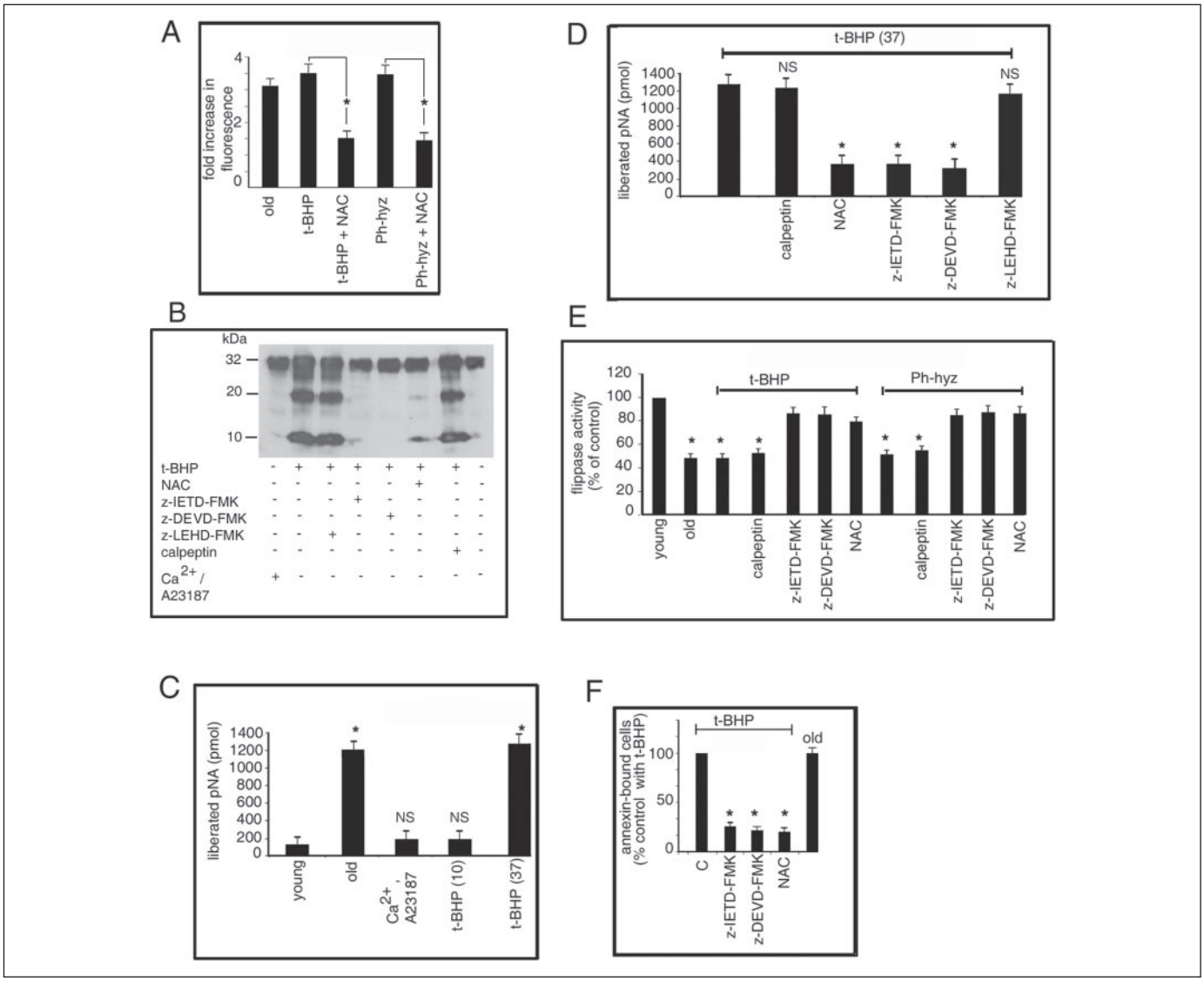

FIGURE 5. **Reactive oxygen species and caspase activation are associated with impairment of aminophospholipid translocase and PS externalization in human erythrocytes.** *A*, cells were left untreated or treated with NAC (10 mM) followed by incubation without or with *t*-BHP or Ph-hyz as described in the legend to Fig. 1*A*. The ROS present in these treated cells as well as in untreated old or young cells was measured in a spectrofluorimeter using the probe DCFH-DA as described under "Experimental Procedures" using excitation and emission wavelengths of 485 and 538 nm, respectively. The results are expressed as -fold increase in comparison with the data obtained in the case of untreated young cells only. Data shown are means ± S.D. ($n = 5$). *, $p < 0.001$. *B*, erythrocytes were either left untreated or treated with NAC (10 mM) or Z-IETD-FMK (100 μM) or Z-DEVD-FMK (100 μM) or Z-LEHD-FMK (100 μM) or calpeptin (20 μM) for 30 min followed by incubation with *t*-BHP. In separate experiments, erythrocytes were treated with $CaCl_2$ (1 mM) and the ionophore A23187 (2 μM) at 37 °C. Lysates were immunoprecipitated with anti-caspase 3 antibody and analyzed for caspase 3 by Western blotting with the same antibody. The bands attributable to the pro (32 kDa) and cleaved (20 and 10 kDa) forms of caspase 3 are indicated. This blot is representative of three independent experiments. *C*, erythrocytes were treated with $CaCl_2$ (1 mM) and the ionophore A23187 (2 μM) or with *t*-BHP at 37 or 10 °C. *D*, erythrocytes were treated with calpeptin, NAC, Z-IETD-FMK, Z-DEVD-FMK, or Z-LEHD-FMK as described in *B*, followed by incubation with *t*-BHP at 37 °C. For *C* and *D*, caspase 3 present in the lysates of treated cells or in the lysates of untreated old and young cells (*C*) was captured on microtiter plates coated with anti-caspase 3 antibody. Ac-DEVD-*p*NA (100 μM) was added to each well, and the release of *p*NA was measured spectrophotometrically at 405 nm. Results represent the mean of five sets of determinations ± S.D. *, $p < 0.001$; *NS*, nonsignificant ($p > 0.5$). In *C*, comparisons have been made with respect to young, untreated cells. In *D*, comparisons have been made with respect to *t*-BHP-treated cells that had not been subjected to any prior treatment. *E*, cells were treated with calpeptin or Z-IETD-FMK or Z-DEVD-FMK or NAC, followed by incubation with *t*-BHP or Ph-hyz. In separate experiments, cells were treated with *t*-BHP or with Ph-hyz alone. Untreated young and old cells as well as the treated cells were used to measure flippase activity. The amount of BSA-extractable PS in the outer monolayer was followed spectrofluorimetrically as described under "Experimental Procedures." The percentage of flippase activity is expressed relative to flippase activity in untreated young cells (*young*, *100*). Results represent the means ± S.D. of five sets of determinations. *, $p < 0.001$ in comparison with untreated, young cells. *F*, cells were treated with the indicated reagents as described in *E*, followed by treatment with *t*-BHP. PS externalization was assessed by flow cytometry using annexin-fluos as described under "Experimental Procedures." The percentage of annexin-bound cells is expressed relative to that obtained after *t*-BHP treatment of cells (*C*, 100%). Untreated, young cells did not show any detectable annexin-fluos binding. Results represent the means ± S.D. of five sets of determinations. *, $p < 0.001$ in comparison with *t*-BHP-treated cells.

in nonreducing gels in both aged and *t*-BHP-treated cells (Fig. 1*C*) but not in untreated, young erythrocytes.

Functional Relevance of the Translocation of Fas into Rafts—To test whether *t*-BHP-mediated ROS generation and Fas clustering is relevant to the downstream activation of caspases, the effects of *t*-BHP were examined at 10 °C. Fluorescence attributable to DCF production following *t*-BHP treatment at 10 °C was comparable with that of normal cells (data not shown). Taking into consideration that ROS generation at 10 °C was not significantly different from that of untreated cells and that 10 °C is below the transition temperature of the membrane (37), we reasoned that cells exposed to t-BHP at 10 °C would not be able to undergo translocation of Fas into rafts, thereby leading to loss of death-inducing function. Colocalization of Fas with $G\alpha_s$ was observed in cells treated with *t*-BHP at 37 °C (Fig. 3*C*). However, at 10 °C, Fas did not

colocalize with the raft marker $G\alpha_s$ (Fig. 3*D*), consistent with the view that the translocation of Fas into rafts was possibly a prerequisite for formation of the Fas-associated complex, since FADD, caspase 8, and FasL could not be coimmunoprecipitated with Fas in cells treated with *t*-BHP at 10 °C (Fig. 1*B*). Caspase 3 activity was also not detectable in these cells (Fig. 5*C*).

ROS and Active Caspases Regulate Aminophospholipid Translocase Activity in Erythrocytes in t-BHP-treated Cells—Different phospholipid classes are asymmetrically distributed over the two halves of the lipid bilayer in erythrocytes (38). The choline phospholipids phosphatidylcholine and sphingomyelin are concentrated in the outer leaflet, whereas the aminophospholipids, phosphatidylethanolamine, and phosphatidylserine (PS) are enriched in the outer leaflet (39–42). Oxidant-induced damage has been proposed to be the underlying mechanism for loss of membrane PS asymmetry in erythrocytes observed in diseases, such as sickle cell disease, thalassemia, and diabetes, as well as in senescent erythrocytes (43–46). The ATP-dependent aminophospholipid translocase transports PS and, to a lesser extent, phosphatidylethanolamine from the outer to the inner leaflet of the plasma membrane against the concentration gradient, thereby compensating for any escape of PS or phosphatidylethanolamine to the outer leaflet (47–49). In contrast to the translocase, an ATP-dependent nonspecific outward movement ("flop") of phospholipids (50) and calcium-dependent nonspecific, bidirectional movement of phospholipids across the membrane, termed scrambling (51, 52), also occur in the red cell. Impaired APLT activity and/or increased scrambling of phospholipids may contribute to loss of phospholipid asymmetry. Our own studies have demonstrated that oxidant-induced damage in erythrocytes activates caspase 3, which inhibits APLT, leading to PS externalization and subsequent erythrophagocytosis (9). Increased ROS generation has also been linked to impairment of APLT activity in sickle cell disease (53). Taking these findings into consideration, we investigated whether oxidant-induced ROS generation and caspase 8 activation could be directly linked to APLT activity. APLT activity was significantly lower in cells treated with *t*-BHP or Ph-hyz (Fig. 5*E*), compared with young, untreated cells. The activity in aged cells was comparable with that of *t*-BHP-treated cells (Fig. 5*E*). The inhibition of APLT activity of red cells by *t*-BHP could be blocked in cells pretreated with NAC, the caspase 8 inhibitor Z-IETD-FMK, or the caspase 3 inhibitor Z-DEVD-FMK (Fig. 5*E*). Calpeptin could not block *t*-BHP-induced loss of APLT activity, ruling out the involvement of calpain in the caspase-dependent inhibition of APLT. Concomitant with the loss of APLT activity, *t*-BHP-treated cells and aged cells displayed comparable PS externalization (Fig. 5*F*) as assessed by annexin binding. As expected, the proportion of PS-exposing cells diminished when cells were pretreated with NAC, Z-DEVD-FMK, or Z-IETD-FMK prior to exposure to *t*-BHP (Fig. 5*F*).

DISCUSSION

Until recently, the mature human erythrocyte was not considered to share any of the stimulus-triggered death signaling pathways that are associated with apoptosis in nucleated cells. However, although erythrocytes lack nuclei and mitochondria, they undergo some of the changes associated with apoptosis, such as membrane blebbing and exposure of PS. In recent years, several studies have demonstrated that triggers such as oxidative stress and osmotic shock lead to "apoptotic" features in the mature red cell. Some studies have focused on Ca^{2+}-dependent processes in erythrocyte death (54) and PS externalization (55). Our own studies have demonstrated that oxidative stress is associated with caspase 3-dependent impairment of APLT activity, leading to PS externalization and erythrophagocytosis (9), and that caspase 3 is activated in aged erythrocytes (10). PS normally localizes to the inner leaflet of cell membranes. It is widely recognized that exposure of PS on the outer leaflet serves as a signal for removal of aged red cells from the circulation. It is a feature that the aged, enucleated mature human erythrocyte shares with nucleated cells undergoing apoptosis. PS-exposing red cell populations are associated with diseases such as sickle cell anemia (56), thalassemia (57–59), and chronic renal failure (60). PS-exposing red cells found among the very high and very low density fractions of sickle cells have been reported to share the feature of loss of APLT activity (59). This was interpreted as evidence that mechanisms of PS exposure that are dependent on loss of APLT activity can be activated both at early stages of red cell maturation and during its life in the circulation. We have therefore investigated the events probably contributing to PS exposure in circulating, human red cells, extending our previous observations that caspase 3 was activated under oxidative stress in red cells, that this was directly linked to impaired APLT activity (9), and that denser human red cells had detectable caspase 3 activity (10), unlike the lighter cells.

Although death receptors have been extensively studied in other cells, their potential functions in erythroid cells have been investigated only recently. Fas is expressed at most stages throughout the maturation of the human erythroid cell, whereas FasL is expressed only by mature erythroblasts (27). Under conditions of limiting erythropoietin, apoptosis of erythroid precursors is induced by activation of Fas mediated by FasL. de Maria *et al.* (27) have shown that maturation of erythroid cells is blocked by activation of the Fas/FasL system through caspase-dependent cleavage of GATA-1. However, the role of death receptors and caspases in the mature human erythrocyte remains an unexplored area. Based on our observations of caspase 3 activation in the mature red cells, we pursued these investigations to explore whether this could occur in a death receptor-dependent manner. The denser (aged) fractions of red cells were found to harbor the cleaved form of caspase 8, strengthening the notion that the death receptor Fas could be a possible upstream player in this pathway. These views were supported by our observations that both the adapter protein FADD and caspase 8 were coimmunoprecipitated with Fas in aged but not in young cells. Finally, in aged cells, Fas, FasL, FADD, and caspase 8 were observed to localize in detergent-resistant membrane microdomains. Disruption of rafts resulted in a randomization of Fas, FasL, FADD, and caspase 8 distribution in old cells. Membrane FasL and Fas have been reported to form supramolecular clusters preceding recruitment and activation of downstream signaling components (61, 62). Whether the translocation of FasL and its interaction with Fas is a prerequisite for formation of a signaling complex and downstream events leading to caspase activation is open to further investigation.

Oxidatively stressed red cells mirrored the aged cells in terms of association of Fas with FasL, FADD, and caspase 8 and their localization in lipid rafts. These associations, as well as activation of caspases 8 and 3, could be blocked by the ROS scavenger NAC, making it appear likely that the ROS-induced association of Fas with rafts is a prerequisite for formation of a signaling complex at the red cell membrane, leading to a sequence of events culminating in caspase 3 activation.

While acknowledging that the Ca^{2+}-dependent phospholipid scramblase and the ATP-dependent floppase are among the effectors of scrambled phospholipid distribution in general, we focused here on the APLT activity, which we observed to be caspase-sensitive. It was significantly lower in old or oxidatively stressed cells compared with young cells. Hermann and Devaux (63) have demonstrated that ATP enrichment of the dense, aged red cell population could not cancel the differences in APLT activity between young and old cells. Connor *et al.* (25)

have demonstrated that differences in ATP levels to the extent observed between young and aged red cells separated by the density-dependent method adopted by us do not contribute to alterations in APLT activity (63). Taking these reports into consideration, it appeared unlikely that the diminished APLT activity observed by us could be attributed to diminished ATP levels. Intracellular Ca^{2+} levels in young and in aged cells separated by density gradient centrifugation have been reported to be 8.4 and 31.2 nM, respectively (64). We also observed a 4-fold increase in Ca^{2+} levels in aged red cells compared with young cells (data not shown). Bitbol *et al.* (65) have demonstrated that the inhibitory effect of Ca^{2+} on the APLT activity starts at a Ca^{2+} concentration of 200 nM. It therefore appeared unlikely that an increase in Ca^{2+} to the extent observed by us in aged cells could account for the difference in APLT activity between young and old cells.

On the other hand, pretreatment of red cells with the caspase 8- or caspase 3-specific inhibitors or with NAC prior to application of oxidative stress blocked the stress-induced inhibition of APLT activity, leading to the conclusion that caspase 8-dependent caspase 3 activation could negatively regulate APLT activity either through direct proteolytic cleavage of the APLT or through an indirect modulatory role involving intermediate regulators of the flippase.

We have provided several lines of evidence suggesting that Fas-dependent signaling processes play a role in regulating PS externalization, one of the signals for red cell clearance from the circulation, by down-regulating APLT in a caspase-dependent manner. These experiments provide the first evidence of a role of death receptor-dependent signaling in the mature enucleated erythrocyte. It can perhaps now be claimed with some confidence that death signaling components that have an established role in erythropoiesis are not merely bystanders that have been transferred to the mature human erythrocyte during development. They play a distinct role, at least under certain conditions, in regulating red cell survival.

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