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Caspase 3-mediated Proteolysis of the N-terminal Cytoplasmic Domain of the Human Erythroid Anion Exchanger 1 (Band 3)*

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The N-terminal cytoplasmic domain of the anion exchanger 1 (AE1 or band 3) of the human erythrocyte associates with peripheral membrane proteins to regulate membrane-cytoskeleton interactions, with glycolytic enzymes such as glyceraldehyde-3-phosphate dehydrogenase and aldolase, with the protein-tyrosine kinase p72^{syk}, with hemoglobin and with hemichromes. We have demonstrated that the N-terminal cytoplasmic domain of band 3 (CDB3) is a substrate of the apoptosis executioner caspase 3 (1). CDB3 has two non-conventional caspase 3 cleavage sites, TATD^{45} and EQGD^{205} (2). *In vitro* treatment of recombinant CDB3 with caspase 3 generated two fragments, which could be blocked by pretreatment with the caspase 3 inhibitor Z-DEVD-fmk (3). Recombinant CDB3 in which the caspase 3 cleavage sites Asp^{45} and Asp^{205} were mutated, was resistant to proteolysis (4). Proteolytically derived fragments cross-reactive with polyclonal anti-band 3 antibody appeared with simultaneous cleavage of poly (ADP-ribose) polymerase and procaspase 3 in staurosporine (STS)-treated HEK293 cells transiently transfected with CDB3 (5). *In vivo* cleavage of CDB3 could be blocked by pretreatment of cells with Z-DEVD-fmk or in cells transfected with mutant CDB3 (D45A, D205A) (6). Co-transfection experiments showed that STS-mediated cleavage of CDB3 diminished its interaction with the N-terminal domain of protein 4.2, confirming that such cleavage interferes with the interaction of CDB3 with cytoskeletal proteins (7). Active caspase 3 was observed in aged red cells but not in young cells. This red cell caspase 3 could cleave band 3 present in inside-out vesicles prepared from young erythrocytes arguing in favor of a physiological role of caspase 3 in aged erythrocytes.

Apoptosis is required to maintain the balance between cell proliferation and cell death. Nuclear collapse and DNA fragmentation are universal features of apoptosis in nucleated cells. However, the nucleus itself is not required for apoptosis, since apoptotic stimuli induce apoptotic morphological features in anucleate cells (1–3). The signaling events that culminate in some of the key plasma membrane changes associated with cell death *in vivo*, such as externalization of PS,[1] operate independently of the nucleus (3). While the process of apoptosis has been studied in-depth in nucleated cells, the role of apoptosis regulatory molecules in anucleate cells is poorly understood. The anucleate mature circulating human erythrocyte is cleared by macrophages after its 120-day life span. The senescent erythrocyte shares at least one feature in common with nucleated cells undergoing apoptosis. The externalization of PS on the outer leaflet of the red cells results in recognition by macrophages probably through a receptor that engages PS (4). We hypothesized that the circulating human erythrocyte potentially shares part of the apoptotic machinery of nucleated cells, and that this machinery may be triggered as a consequence of red cell senescence, pathology such as in diseases like sickle cell anemia, thalassemia, and oxidative insult to red cells.

Proteases play a critical role in the expression of mammalian apoptosis. The caspases are a specialized family of cysteine-dependent aspartate-directed proteases (5–7). The caspases recognize a tetrapeptide sequence on their substrates with an aspartate residue at the fourth position. Caspase activation is a critical event in the onset of apoptosis (5). Human caspase 3 is perhaps the most universal apoptosis mediator. It is present in most mammalian cells (8). Its deletion by gene knockout blocks neuronal death during brain development with consequent lethality (9). Studies in our laboratory have demonstrated that caspase 3 is present in mature human erythrocytes and activated by oxidative stress (10). Berg *et al.* (11) have also demonstrated that caspases 3 and 8 are present in mature human erythrocytes.

The cytoskeleton of an apoptotic cell undergoes profound changes. Underlying these changes is the cleavage of many cytoskeletal proteins by caspases. These include lamins (12, 13), gelsolin (14), actin (15), and fodrin (16). We speculate that loss of anchorage of the lipid bilayer to the cytoskeleton could also contribute to the loss of membrane integrity that is a feature of erythrocyte senescence.

The two most abundant integral membrane proteins in the human erythrocyte membrane are the sialoglycoprotein, glycophorin and the anion exchanger, band 3. Both play a crucial structural role in linking the bilayer with the spectrin-based skeletal network. There are 1.2×10^6 copies of band 3 per cell of which 40–60% is associated with the spectrin-based skeleton. Erythrocyte band 3 is a multifunctional protein with its N- and C-terminal domains on the cytoplasmic face of the lipid bilayer and ~14 membrane spans (17–19). While the C-termi-

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[1] The abbreviations used are: PS, phosphatidylserine; CDB3, cytoplasmic domain of band 3; PARP, poly (ADP-ribose) polymerase; STS, staurosporine; Z-DEVD-fmk, benzyloxycarbonyl-Asp(OCH_3)-Glu(OCH_3)-Val-Asp(OCH_3)-fluoromethylketone; PIPES, 1,4-piperazinediethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethyl ammonio]-1-propanesulfonic acid; DTT, dithiothreitol; NTA, nitrilotriacetic acid; IOV, inside-out vesicles; HEK, human embryonic kidney.

nal 52 kDa membrane domain is responsible for anion transport across the membrane, the 43 kDa N-terminal cytoplasmic domain of band 3 (CDB3) plays a crucial structural role in linking the bilayer with the spectrin-based skeletal network. It functions as an anchoring site for the membrane-associated cytoskeletal proteins ankyrin (20), protein 4.2 (21) and protein 4.1 (22); for the glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (23), phosphofructokinase (24), and aldolase (25); for hemoglobin (26, 27), for hemichromes (28) and for the protein-tyrosine kinase $p72^{syk}$ (29). These interactions regulate key elements of red cell structure and function including cell flexibility (30), regulation of glucose metabolism, and lifespan of the cell.

The non-erythroid counterpart of the major erythrocyte membrane cytoskeletal protein spectrin is a substrate of caspase 3. The caspase 3 cleavage sites on both α- and β-non-erythroid spectrin (16, 17) have been mapped. Since little is known about the molecular consequences of caspase activation in red cells, we have chosen to address this question by attempting to identify the substrates of caspase 3 in the mature human erythrocyte. In the present study we have tested the possibility that the cytoplasmic domain of band 3 may contain caspase 3 cleavage sites. Proteolysis at these sites could potentially alter each of the characteristics of red cell structure and function regulated by the N-terminal CDB3. We demonstrate for the first time that CDB3 is indeed a substrate for caspase 3. We have identified two specific but non-conventional cleavage sites of caspase 3 on CDB3. We also demonstrate that CDB3 expressed in HEK293 cells undergoes staurosporine-induced degradation, which can be blocked by caspase 3 inhibitor; and that this impairs the interaction of CDB3 with the cytoskeletal protein 4.2. Defining CDB3 as a caspase substrate suggests a mechanism by which caspase 3 activation in human erythrocytes under conditions such as oxidative stress, might lead to important consequences such as uncoupling of the bilayer from the underlying spectrin-based skeleton, and modulation of glucose metabolism. That caspase 3 is likely to have a physiological role in erythrocytes, is best supported by our observations that active caspase 3 is present in aged red cells but not in young cells, and that this red cell-derived caspase 3 is capable of cleaving band 3 present in inside-out vesicles prepared from young red cells. Our demonstration, for the first time, that active caspase 3 is present in density-separated aged erythrocytes as opposed to young cells, is the best evidence that caspase 3 is likely to play a physiological role in the mature human erythrocyte.

EXPERIMENTAL PROCEDURES

Materials—The plasmids containing human caspase 3 and caspase 8 in pET 15b were gifts from Kevin Wang, University of Michigan, Ann Arbor, MI. Band 3 cDNA was a gift from Joseph Casey, University of Alberta, Edmonton. The caspase 3 inhibitor Z-DEVD-fmk was a product of CN Biosciences, San Diego, CA, staurosporine (STS) was from Roche Applied Science. Protein A/G-agarose, anti-caspase 3 antibody, and goat anti-rabbit IgG linked to alkaline phosphatase were from Santa Cruz Biotechnology, Santa Cruz, CA. Antibody against cleaved poly (ADP-ribose) polymerase (PARP) was from Cell Signaling Technology, Beverley, MA; anti-FLAG antibody was from Sigma Chemical Co., and anti-His antibody was from Roche Applied Science.

Purification of Recombinant Caspase 3—Caspase 3 was expressed in *Escherichia coli* BL21 (DE3) by induction with 0.5 mM isopropylthiogalactopyranoside (IPTG) at 25 °C and purified from the cytosol of the expressed cells by affinity chromatography on Ni^{2+}-NTA-agarose (Qiagen).

Cloning and Expression of the N-terminal Cytoplasmic Domain of Band 3 (CDB3)—The gene encoding amino acids 1–390 of CDB3 was amplified from band 3 cDNA by PCR using the primers 5′-CGC GGA TCC ATG GAG GAG CTG CAG GAT-3′ (sense) and 5′-GTA GCG GCG CCG GAT ATC-3′ (antisense), digested with BamHI (underlined) and cloned in the vector pBAD-HisB (Invitrogen) between the BglII and PvuII sites to generate the vector pBD101 encoding residues 1–390 of band 3 with an N-terminal His tag. *E. coli* Top10/pBD101 was grown to an OD_{600} of 0.6. Arabinose was added to a final concentration of 0.002%, and growth was continued at 37 °C with shaking for 4 h. Cells were harvested, reconstituted in 10 mM sodium phosphate, pH 7.4, 150 mM NaCl (PBS), and kept at −70 °C overnight. Frozen cells were thawed and treated with 1 mg/ml lysozyme, 5 mM DTT, 1% (v/v) Triton X-100 containing 1 μg/ml DNase, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 0.1 mM pefabloc for 30 min at 4 °C. The lysed cells were centrifuged at 20,000 × *g* for 30 min at 4 °C, and the supernatant was loaded on a Ni^{2+}-NTA- agarose column equilibrated in 50 mM sodium phosphate, pH 7.4, containing 0.5 M NaCl, 1% (v/v) Triton X-100 (buffer A). After washing the column with 100 mM imidazole in buffer A, N-terminally His-tagged CDB3 was eluted with 200 mM imidazole in buffer A.

Site-directed Mutagenesis—Site-directed mutagenesis was performed by the principle of overlap extension PCR (31) using primers detailed below. The first rounds of PCR were performed with primer pairs a and b for one reaction and primer pairs c and d for the second reaction. The second round of PCR was performed using the products of the first round as templates and the primers a and d. Sequencing was done on both strands to confirm incorporation of the mutations. Primers b and c were respectively 5′-ACAGCCACAGCCTACCACACC-3′ and 5′-GGTGTGGTAGGCTGTGGCTGT-3′ for the mutation D45A; 5′-ACAGCCACAGAATACCACACC-3′ and 5′-GGTGTGGTATTCTGTGGCTGT-3′ for the mutation D45E; 5′-GAGCAGGGAGCTGGGGGCACA-3′ and 5′-TGTGCCCCCAGCTCCCTGCTC-3′ for the mutation D205A; 5′-GAGCAGGGAGAAGGGGGCACA-3′ and 5′-TGTGCCCCCTTCTCCCTGCTC-3′ for the mutation D205E. Primers a and d were 5′-CGCGGATCCATGGAGGAGCTGCAGGAT-3′ and 5′-GTAGCGGCGCCGGATATCACGCACCAG-3′, respectively.

Cloning in pcDNA 3.1(−) and pFLAG-CMV-6b—The genes encoding wild-type and mutant CDB3 were amplified by PCR with the primer pair 5′-CCA CCA TGG AGG AGC TGC AGG ATG ATT AT-3′ (sense) and 5′-CCC AAG CTT CTA CAC AGG CAT GGC CAC TTC-3′ (antisense) and cloned into the vector pcDNA3.1(−) (Invitrogen) between the EcoRV and HindIII (underlined) sites. An N-terminal Kozak sequence (-CCACC-) was introduced just upstream of the intiation codon and a stop codon was incorporated in the antisense primer. All the clones were sequenced. The protein 4.2 gene was amplified from the protein 4.2 cDNA in pGEM-3z using the primer pair 5′-AAGGATCCATATGGGACAGGCACTGGGT-3′ (sense) and 5′-AGCTGATAGTTCAGGGGCTACCACGGT-3′ (antisense), digested with BamHI (underlined) and cloned between the BglII and EcoRV sites of pFLAG-CMV-6b (Sigma Chemical Co.). The N-terminal 690 bp of the 4.2 gene (encoding the N-terminal 230 amino acids) was amplified using the same sense primer and the antisense primer 5′-GGTGGGCAGGACCCTCTGCTCCTTGAG-3′ and cloned as described above.

Preparation of Ghosts and Purification of CDB3—Human blood was obtained from normal healthy volunteers. Ghosts were prepared by washing cells thrice with 15 volumes of PBS followed by lysis in lysis buffer (6.8 mM Na_2HPO_4, 2.25 mM NaH_2PO_4, pH 8.0 containing 10 μg/ml pefabloc, 1 μg/ml pepstatin, 10 μg/ml aprotinin, and 1 μg/ml leupeptin). CDB3 was prepared from ghosts according to Bennett (32) after chymotryptic cleavage from spectrin-depleted, acid-stripped vesicles. Purified red cell CDB3 was used to raise polyclonal antibody in rabbits. This antibody recognized the 43 kDa N-terminal cytoplasmic domain of band 3 and all subfragments of this domain.

Preparation of Inside-Out Vesicles (IOV)—IOV were prepared by treating ghosts with 30 volumes of 0.1 mM EGTA, pH 8.5 for 30 min at 37 °C, followed by centrifugation at 30,000 × *g* for 20 min at 4 °C as described by Korsgren and Cohen (33). Vesicles were washed once with the same buffer.

Separation of Young and Aged Red Blood Cells on Percoll-Bovine Serum Albumin Gradient—Density-dependent separation of red cells was performed as described by Corsi *et al.* (34) 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)-bovine serum albumin gradient (34) followed by centrifugation at 1,500 × *g* for 20 min at 20 °C. Young and aged erythrocytes were collected separately from the 68 and 84% Percoll layers respectively, and washed in PBSG buffer.

Western Blotting—Proteins were electrotransferred to polyvinylidene difluoride (PVDF) membranes (Immobilon P, Millipore, Bedford, MA). After blocking for 1 h with PBS-0.05% Tween 20 (PBS-T) containing 5% nonfat dry milk (NFDM), the blots were washed in PBS-T and probed with anti-CDB3 antibody (1:5,000) by incubation overnight at 4 °C.

After three washes in PBS-T containing 0.5% NFDM, the blots were incubated for 1 h with peroxidase-conjugated anti-rabbit IgG (1:2,000). The blots were then washed three times with PBS-T containing 0.5% NFDM and developed by chemiluminescence using the Lumiglo reagent (Cell Signaling Technology, Beverley, MA). When performing Western blotting for detection of caspases, 6×10^5 cells were pelleted and freeze-thawed three times in 20 μl of cell extraction buffer (50 mM PIPES/NaOH (pH 6.5), 2 mM EDTA, 0.1% (w/v) CHAPS, 5 mM DTT, 20 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin, 1 mM pefabloc). The lysates were centrifuged at 10,000 $\times$ *g* for 5 min at 4 °C, and the supernatants were collected for the detection of caspases.

In Vitro Proteolysis of CDB3 and Recombinant CDB3 by Caspase 3—Purified red cell CDB3 or recombinant CDB3 (1 μg) was incubated with purified recombinant caspase 3 (0.2 μg) or caspase 8 (0.2 μg) in 100 mM HEPES (pH 7.2) containing 10 mM DTT and 10% (v/v) glycerol at 25 °C for 120 min. The reaction was stopped by the addition of an equal volume of SDS-PAGE sample buffer. Triplicate samples were subjected to electrophoresis. One gel was stained with Coomassie Blue, whereas two other samples were electroblotted onto polyvinylidene difluoride membranes. One blot was subjected to Western blotting using anti-CDB3 antibody. The other blot was used for N-terminal sequencing of bands predicted to be derived from band 3 by development with anti-band 3 antibody.

N-terminal Sequencing—After SDS-PAGE and electrotransfer, the desired bands were cut out and subjected to N-terminal sequencing in an Applied Biosystems protein sequencer at the protein sequencing facility of Eurosequence, Groningen, The Netherlands.

Cell Culture and Transfection—The human embryonic kidney cell line HEK293 was obtained from the National Centre for Cell Science, Pune, India. Cells were grown in modified Eagle's medium (MEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C with 5% CO_2 in a humidified atmosphere. Cells were transfected using the Polyfect reagent (Qiagen) according to the manufacturer's protocol. Briefly, cells (6×10^5/well) were plated on 35-mm dishes and grown to 50–70% confluence. Cells were incubated at 37 °C with Polyfect complex (formed by incubating 2 μg of DNA and 20 μl of Polyfect reagent in 100 μl of serum-free MEM for 15 min) in 2 ml of complete MEM. After 5 h of incubation, medium was replaced with fresh MEM, and growth was permitted for another 36 h. For co-transfection experiments, 1 μg of each DNA was used.

Immunoprecipitation Experiments—HEK293 cells after treatment were lysed in lysis buffer containing 20 mM Tris-HCl, pH 8, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 20 mM NaF, 10 mM Na-β -glycerophosphate, 1 mM sodium orthovanadate, 1 mM pefabloc, and 10 μg/ml aprotinin. The lysate was centrifuged at 15,000 $\times$ *g* for 15 min at 4 °C. The supernatant, where required, was incubated overnight with 1:100 dilution of polyclonal anti-CDB3 antibody at 4 °C followed by addition of 5 μl of protein A/G-agarose. After incubation for 3 h at 4 °C, the beads were pelleted, washed once in lysis buffer, boiled in SDS gel denaturing buffer, and separated on SDS-polyacrylamide gels prior to Western blotting.

Red cells were lysed with 5 volumes of 7.5 mM sodium phosphate buffer (pH 7.4) containing 1 mM EDTA and 10 mM DTT. Immunoprecipitates were prepared by incubation overnight with 1:100 dilution of anti-caspase 3 antibody (C-277, Santa Cruz Biotechnology) followed by addition of protein A/G-agarose as described above.

Caspase 3 Activity Assay—DEVD-dependent protease activity was determined using Ac-DEVD-pNA as substrate and the immunosorbent caspase 3 activity assay kit from Roche Applied Science. Briefly, caspase 3 was captured from lysates from 100-μl erythrocytes (5% hematocrit) in microtiter plates coated with anti-caspase 3 monoclonal antibody. Following washing the plates, Ac-DEVD-*p*NA was added and the released *p*NA was determined spectrophotometrically at 405 nm. A *p*NA calibration curve was plotted from a *p*NA stock solution and the caspase 3 activity was measured relative to this curve.

Apoptosis Induction—HEK293 cells were grown in 6-well plates and transfected as described above. Apoptosis was induced by adding 10 μM STS followed by incubation for 24 h. Where indicated, cells were incubated with Z-DEVD-fmk 1 h prior to STS treatment. After incubation, cells were resuspended in SDS gel denaturing buffer, and proteins were denatured at 100 °C for 3 min.

Cleavage of IOV with Caspase 3 Immunoprecipitates from Young and Aged Erythrocytes—IOV were incubated at 37 °C with caspase 3 immunoprecipitates obtained from lysates of young and aged erythrocytes (described above) in 20 mM HEPES, pH 7.5 containing 10 mM NaCl, 10 mM DTT, and 0.5% Nonidet P40. The assay was carried out in the presence or absence of the caspase 3 inhibitor Z-DEVD-fmk (10 μM). Samples were separated on SDS-12% polyacrylamide gels, electrotransferred onto polyvinylidene difluoride membranes, and probed with polyclonal anti-CDB3 antibody as described earlier.

RESULTS

In Vitro Degradation of CDB3 by Recombinant Caspase 3—We have previously shown that mature human erythrocytes contain caspase 3, and that caspase 3 can be activated under oxidative stress (10). Cytoskeletal proteins such as spectrin have previously been demonstrated to be substrates of caspase 3 (16, 17). Since the major erythrocyte membrane integral membrane protein band 3 plays a pivotal role in regulating structure and function of the mature human erythrocyte, this study was undertaken to test the possibility that cytosolic domains of band 3 could be substrates of caspase(s). The N-terminal cytosolic domain of band 3 (CDB3) has been chosen as the starting point of these investigations. CDB3 was purified from red cells and tested as a substrate of caspase 3 by incubating with recombinant purified caspase 3. Cleavage of CDB3 was observed. Two bands migrating with M_r 18 and 21 were reproducibly obtained (Fig. 1*A*, *lane b*). The appearance of these bands was blocked when CDB3 was pretreated with the caspase 3 inhibitor Z-DEVD-fmk (Fig. 1*A*, *lane c*). Controls in which no caspase 3 had been added did not show significant degradation of CDB3 (Fig. 1*A*, *lane a*). The two cleaved fragments cross-reacted with anti-CDB3 antibody when subjected to Western blotting (data not shown). This confirmed that they were derived from CDB3. These bands were subjected to N-terminal sequencing. The N-terminal sequences mapped to the sequences ^{46}YHTTS and ^{206}GGTEG for the bands migrating with M_r 18 and 21, respectively. From these sequences we inferred that caspase 3 cleavage had occurred on the C-terminal side of the sequences TATD^{45} and EQGD^{205}, respectively. On the other hand, incubation of red cell CDB3 with recombinant caspase 8 did not result in any cleavage of CDB3 (data not shown), suggesting that cleavage was specific for caspase 3. In order to facilitate mapping of cleavage sites by mutational analyses, further experiments of *in vitro* cleavage of CDB3 by caspase 3 were performed using recombinant His-tagged CDB3. To reconfirm that caspase 3 cleavage sites mapped to TATD^{45} and EQGD^{205}, His-tagged CDB3 was incubated with caspase 3. Recombinant His-tagged CDB3 migrated with an apparent molecular mass of 60 kDa on SDS gels (Fig. 1, *panel B*, *band a*). The migration of cleaved fragments was also slower and reflected anomaly. The reason for this is unclear. However, the migration pattern was reproducible. The anomaly may possibly be attributed to the fact that CDB3 exhibits a highly elongated morphology (35) combined with the addition of the N-terminal hexahistidine tag. Nevertheless, incubation of purified His-tagged recombinant CDB3 with caspase 3 gave two cleaved fragments of apparent molecular masses 25 and 22 kDa reproducibly (Fig. 1*B*, *bands d* and *e*). These bands were confirmed to be derived from CDB3 by Western blotting with anti-CDB3 antibody (data not shown). Cleavage at the two sites TATD^{45} and EQGD^{205} were predicted to generate fragments of size 4.5, 17.3, and 21.1 kDa corresponding to residues 1–45, 46–205, and 206–390 respectively (Fig. 1, *panel E* and Table I). N-terminal sequencing of the *bands e* and *d* observed on SDS-PAGE showed that they were indeed derived from cleavage at TATD$^{45}\downarrow$ YYHTTS and EQGD$^{205}\downarrow$ GGTEG, respectively. As expected, neither of these bands gave a positive reaction with anti-His antibody (Fig. 1, *panel C*). Any proteolytic fragment corresponding to residues 1–45 along with the hexahistidine tag with a predicted molecular mass of 4.5 kDa was, as expected, not visible on 10 or 12% polyacrylamide SDS gels. The bands derived from red cell CDB3 migrated differently from those derived from recombinant CDB3 despite the absence of the His tag in both cases. This may be due to the C terminus of

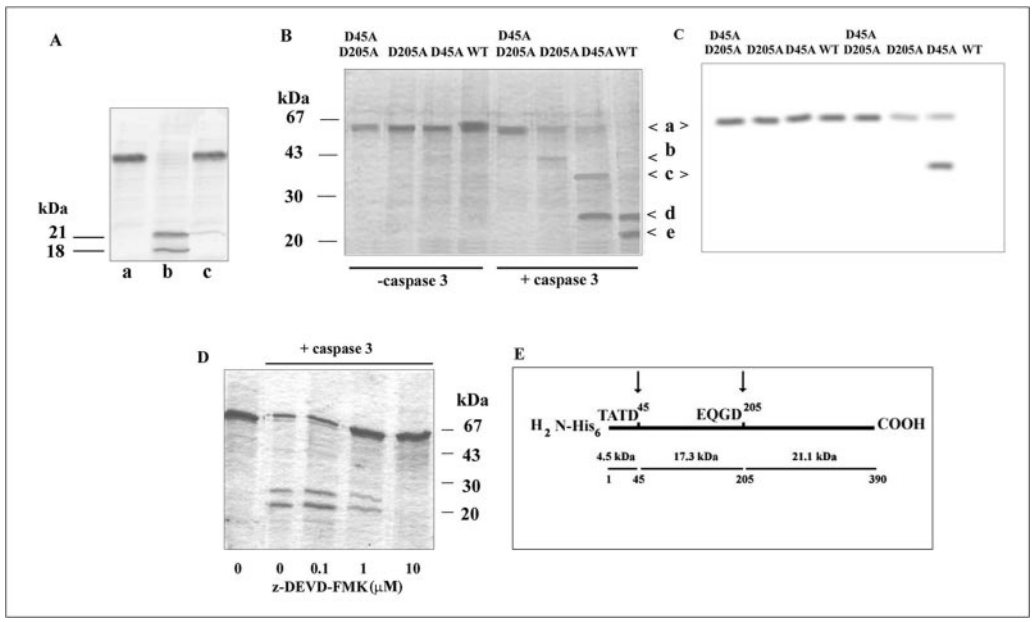

FIG. 1. **Cleavage of CDB3 *in vitro* by caspase 3.** *Panel A*, purified red cell CDB3 was incubated without (*lane a*) or with (*lanes b* and *c*) recombinant caspase 3 in the absence (*lane b*) or in the presence (*lane c*) of Z-DEVD-fmk (10 μM) at 37 °C for 4 h. Recombinant (N-terminal hexahistidine-tagged) wild type (*WT*) (*panels B*, *C*, and *D*) or mutant (*panels B* and *C*) CDB3 expressed *in E. coli* were either left untreated or treated with caspase 3 for 2 h either in the absence (*panels B* and *C*) or in the presence (*panel D*) of caspase 3 inhibitor Z-DEVD-fmk at different concentrations as indicated. The samples were then denatured by SDS gel denaturing buffer, separated on SDS-polyacrylamide gels, followed by staining with Coomassie Blue (*panels A*, *B*, and *D*) or probing with anti-His antibody after electroblotting (*panel C*). *Panel E* is a diagrammatic representation of the caspase 3 cleavage sites (indicated by *arrows*) identified in CDB3 by N-terminal sequencing.

TABLE I
Predicted and deduced caspase 3 cleavage sites in the cytoplasmic domain of band 3

Recombinant	N-terminal sequence obtained	Assumed caspase cleavage site	Predicted mol. wt.	Mol. wt. on SDS-PAGE
			kDa	*kDa*
Wild type	YHTTS and GGTEG	$TATD^{45}$YHTTS and $EQGD^{205}$GGTEG	4.5, 17.3, and 21.1	Two bands of 22 and 25 (e and d, respectively)[a]
Mutant D45A	GGTEG	$EQGD^{205}$GGTEG	21.8 and 21.1	35 and 25 (c and d, respectively)
Mutant D205A	YHTTS	$TATD^{45}$YHTTS	4.5 and 38.4	One band of 40 (b)
Mutant D45A, D205A	N.D.[b]	None	42.9	One band of 60 (a)

[a] a, b, c, d, and e refer to the bands depicted in Fig. 1.
[b] N.D., not determined.

red cell-derived CDB3 (obtained by chymotryptic cleavage) being different from that of the recombinant CDB3 (*i.e.* residue 390). In summary, red cell-derived CDB3 and recombinant CDB3 behaved similarly with respect to their recognition by caspase 3 as substrate. Caspase 8, on the other hand, was unable to cleave CDB3 (data not shown).

Proteolysis of CDB3 by Caspase 3 in the Presence of the Inhibitor Z-DEVD-fmk—The cleavage sites on CDB3 mapped by us did not match the preferred caspase 3 consensus sequence D*XX*D (36, 37). In order to confirm the specificity of caspase 3-mediated cleavage, incubations of CDB3 with caspase 3 were carried out in the presence of different concentrations of the caspase 3-specific inhibitor Z-DEVD-fmk. A dose-dependent effect of the inhibitor in blocking caspase 3-mediated proteolysis was observed (Fig. 1*D*). Complete inhibition of caspase 3-mediated cleavage was observed at an inhibitor concentration of 10 μM. This confirmed the specificity of cleavage by caspase 3.

Proteolysis of Site-specific Mutants of CDB3 by Caspase 3—Site-specific mutants were generated in which the site $TATD^{45}$ was mutated to TATA and the site $EQGD^{205}$ was mutated to EQGA individually and in combination. Caspase 3 specifically cleaves at the C-terminal end of aspartate residues and it was surmised that neither of the mutants would be cleaved at the sites of mutation by caspase 3. This was indeed found to be the case. Cleavage by caspase 3 was blocked at each of the mutated sites and products of altered size were obtained (Fig. 1*B*). CDB3 harboring the D45A mutation (CDB3 [D45A])gave the bands d and c. Predictably, from the N-terminal sequence GGTEG of band d, we could deduce that cleavage had occurred on the C-terminal side of Asp^{205}. Band e, generated in the wild type from cleavage on the C-terminal side of Asp^{45} encompassing residues 46–205 was expectedly absent in caspase-cleaved CDB3[D45A]. Another fragment (residues 1–205) of calculated M_r 21.8 carrying the N-terminal His tag could be predicted (Table I). We observed the appearance of band c migrating with M_r 35 and carrying the His tag (as probed by Western blotting with anti-His antibody (Fig. 1*C*)), suggesting that this probably represented the fragment of CDB3 encompassed by residues 1–205 along with the N-terminal hexahistidine tag. Band c was, as expected, also positive on Western blots probed with anti-CDB3 antibody (data not shown). No other band was positive when probed with anti-His antibody (Fig. 1*C*) excluding the possibility that the extreme N terminus was present in any of the other bands. Anomaly in migration was therefore assumed to be the reason for obtaining a band of M_r 35. This anomaly was most pronounced on SDS gels for bands a and c, both of which retain the extreme N-terminal 45 residues. This suggested that residues 1–45 of CDB3 probably encompass the critical determinants of an elon-

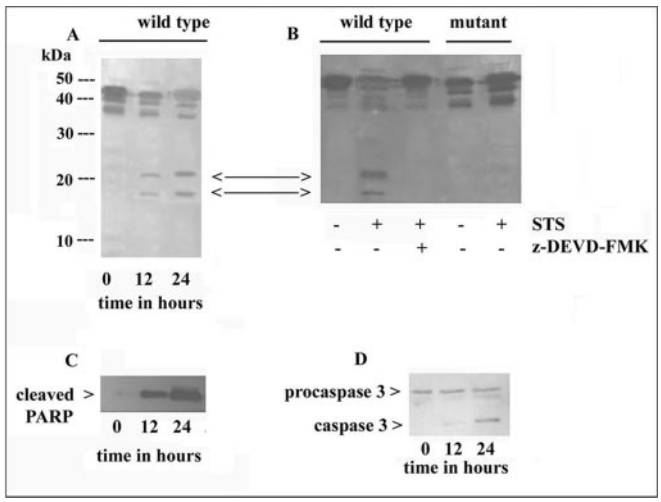

FIG. 2. **CDB3 is cleaved during apoptosis in a time-dependent manner.** HEK293 cells expressing wild-type CDB3 (*panels A–D*) or CDB3[D45A/D205A] (*panel B*) were treated with staurosporine (10 μM) for the indicated periods of time (*panels A*, *C*, and *D*) or for 24 h (*panel B*, where indicated). Equivalent amounts of total cell lysates were separated on SDS-polyacrylamide gels and CDB3 or its cleaved products (indicated by *arrows*) were detected in Western blots using a rabbit polyclonal antibody raised against CDB3. Cleaved PARP (89 kDa) was detected by Western blotting using antibody against cleaved PARP (*panel C*), procaspase 3, and caspase 3 (32 and 17 kDa, respectively) were detected by antibody against caspase 3 (*panel D*).

gated conformational state of CDB3 when expressed in *E. coli* leading to its anomalous migration on SDS gels. When CDB3[D205A] was cleaved by caspase 3 only one proteolytically derived band b (M_r 40) was visible on SDS gels. The predicted size of the fragment encompassing residues 46–390 was 38.4 kDa (Table I). The N-terminal sequence of band b mapped to the sequence YHTTS enabling us to infer that cleavage had occurred on the C-terminal side of Asp^{45}. Unlike the wild type CDB3, a proportion of uncleaved CDB3 was always present in both the mutants even upon prolonged incubation with caspase 3. The reason for this is unclear at present. CDB3[D45A/D205A] was resistant to cleavage by caspase 3, confirming that Asp^{45} and Asp^{205} represent the two caspase 3 cleavage sites on CDB3. Mutants D45E and D205E behaved identically with D45A and D205A, respectively (data not shown).

Cleavage of CDB3 in Cells Undergoing Apoptosis—In order to examine whether CDB3 undergoes caspase 3-mediated cleavage *in vivo*, we transfected HEK293 cells with CDB3. CDB3 rather than intact band 3 was chosen for transfection in order to facilitate the confirmation of the caspase 3 cleavage sites by mutational analysis. Lysates from transiently transfected cells were probed with anti-CDB3 antibody. Western analysis demonstrated that CDB3 was expressed in transfected cells (Fig. 2*A*). Immunoblot analysis showed an apparent heterogeneity in migration of CDB3 on SDS gels (M_r ranging from 40 to 43). Similar heterogeneity in migration of CDB3 has also been reported by Bennett (32). Apoptosis was induced by treatment with STS (10 μM) for different periods of time. Cells after STS treatment for 24 h did not lose their membrane integrity since they did not stain with the dye trypan blue. This indicated that the cells were undergoing apoptosis rather than necrosis. Lysates from untreated cells or cells treated with STS were analyzed by SDS-PAGE followed by Western blotting using polyclonal anti-CDB3 antibody. STS-treated cells showed the time-dependent appearance of two bands which were cross-reactive with anti-band 3 antibody (Fig. 2*A*) and absent in untreated cells. STS-treated untransfected cells did not show these bands (data not shown). To examine whether caspase 3 was involved in the generation of these bands, cells were treated with the cell-permeable caspase 3 inhibitor Z-DEVD-fmk for 1 h prior to STS treatment. The appearance of both these bands was blocked in cells that had been treated with Z-DEVD-fmk (Fig. 2*B*). This suggested that these bands were derived from caspase 3 activation. To verify whether the two aspartate residues 45 and 205 (which were identified *in vitro*), represent the apoptosis-dependent caspase 3 cleavage sites of CDB3 *in vivo*, we performed experiments in STS-treated cells expressing CDB3[D45A/D205A]. Mutant CDB3 did not show any degradation on exposure to STS (Fig. 2*B*), suggesting that Asp^{45} and Asp^{205} represent STS-induced caspase 3 cleavage sites. Caspase 3 activation, a hallmark of apoptosis, was followed by Western blotting to detect the activated form of caspase 3 (17 kDa). (Fig. 2*D*). Caspase 3 activation was also supported by the observation that there was a time-dependent increase in the appearance of cleaved PARP, a caspase 3 substrate (Fig. 2*C*).

Interaction of Protein 4.2 with CDB3 in HEK293 Cells Undergoing Apoptosis—Protein 4.2 was chosen as one of the natural binding partners of CDB3. Attempts were made to study the interaction of full-length protein 4.2 with CDB3. However, the level of expression of protein 4.2 was very low as observed by Western blotting with anti-FLAG antibody. Moreover, the expressed protein 4.2 was readily degraded, possibly by proteases, during preparation of cell lysates. Since our previous work has demonstrated that an N-terminal 23-kDa polypeptide derived from protein 4.2 interacts with CDB3 (38), we chose to study the binding of the N-terminal domain of protein 4.2 encompassing the first 230 amino acid residues of protein 4.2 carrying a FLAG epitope (designated as C1), in STS-treated HEK293 cells in order to analyze whether the ability of CDB3 to interact with its natural binding partners is compromised in HEK293 cells undergoing apoptosis. Lysates were prepared from cells transfected with FLAG-C1 (24 kDa) or CDB3 alone or co-transfected with FLAG-C1 and CDB3. The cell-free supernatant was incubated with polyclonal anti-CDB3 antibody, followed by pull-down with protein A/G-agarose. Western blotting of the immunoprecipitates with anti-FLAG antibody demonstrated that FLAG-tagged C1 could be pulled down by anti-CDB3 antibody in the co-transfected cells only (Fig. 3*A*, *lane 4*). Immunoprecipitates obtained from cell lysates transfected with CDB3 (Fig. 3*A*, *lane 1*) or with C1 (Fig. 3*A*, *lane 2*) alone, did not develop any band with anti-FLAG antibody. This clearly demonstrated the interaction of C1 with CDB3 in HEK293 cells after cotransfection. Lysates were also prepared from cells transfected with C1 alone and pulled down with protein A/G-agarose in order to rule out the possibility that C1 could be pulled down nonspecifically by protein A-agarose (Fig. 3*A*, *lane 1*). When HEK293 cells co-transfected with C1 and CDB3, were treated with STS, immunoprecipitates from cell lysates showed a significant reduction in the intensity of the band corresponding to C1 when developed with anti-FLAG antibody (Fig. 3*A*, *lane 5*), suggesting that there was a reduction of C1-CDB3 interaction in HEK293 cells undergoing STS-induced caspase 3-mediated apoptosis. The interaction was restored in cells pretreated with the cell-permeable caspase 3 inhibitor Z-DEVD-fmk prior to STS treatment (Fig. 3*B*) indicating a role of caspase 3. This was further supported by the observation that C1-CDB3 interaction was not affected by STS treatment (Fig. 3*A*, *lanes 6* and *7*) when the mutant CDB3[D45A/D205A] was used in co-transfection experiments. Finally, Western blotting of lysates obtained from C1-transfected STS-treated cells with anti-FLAG antibody showed that C1 itself is resistant to caspase 3-mediated cleavage (Fig. 3*C*). No degradation of C1

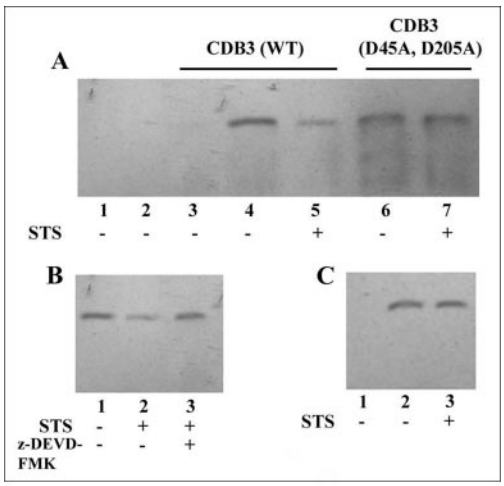

FIG. 3. **Interaction of CDB3 with a protein 4.2-derived domain in HEK293 cells.** *Panel A*, HEK293 cells were transfected with C1 alone (*lanes 1* and *2*), or CDB3 alone (*lane 3*) or with C1 and CDB3 (*lanes 4* and *5*) or with C1 and CDB3[D45A/D205A] (*lanes 6* and *7*). Cells were either left untreated (*lanes 1–4* and *6*) or treated with staurosporine (10 μM) for 24 h (*lanes 5* and *7*). Cells were lysed, lysates were incubated without (*lane 1*) or with (*lanes 2–7*) anti-CDB3 antibody, followed by immunoprecipitation with protein A-agarose. Immunoprecipitates were subjected to SDS-PAGE followed by Western blotting with anti-FLAG antibody. *Panel B*, cells co-transfected with C1 and CDB3 were left untreated (*lanes 1* and *2*) or pretreated with caspase 3 inhibitor (Z-DEVD-fmk) (*lane 3*), followed by staurosporine treatment (*lanes 2* and *3*). Immunoprecipitation and Western blotting with anti-FLAG antibody were carried out as described under *panel A*. *Panel C*, cells, not transfected (*lane 1*) or transfected with C1 alone (*lanes 2* and *3*), were left untreated (*lanes 1* and *2*) or treated with staurosporine (*lane 3*) for 24 h, followed by lysis and Western blotting using anti-FLAG antibody.

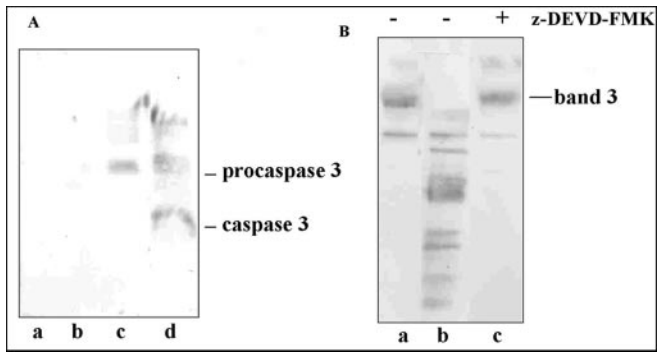

FIG. 4. **Detection of active caspase 3 in red cells and cleavage of band 3 by red cell caspase 3.** *Panel A*, cytosol obtained from young (*lanes a* and *c*) and old erythrocytes (*lanes b* and *d*) were incubated overnight without (*lanes a* and *b*) or with (*lanes c* and *d*) caspase 3 antibody, followed by pull-down with protein A/G-agarose. The immunoprecipitates were separated on SDS-polyacrylamide gels prior to Western blotting with anti-caspase 3 antibody. The blot is representative of similar results obtained with three samples from three different volunteers. *Panel B*, inside-out vesicles from young erythrocytes were incubated with caspase 3 immunoprecipitates (obtained as described under *panel A*) from young (*lane a*) or aged erythrocyte cytosol (*lanes b* and *c*) in the absence (*lanes a* and *b*) or in the presence (*lane c*) of the caspase 3 inhibitor Z-DEVD-fmk for 4 h at 37 °C. Samples were separated on SDS-polyacrylamide (12%) gels, followed by Western blotting using polyclonal anti-CDB3 antibody. The blot is representative of similar results obtained in three different experiments.

was detected on immunoblots even when probed with polyclonal anti-4.2 antibody (data not shown).

Role of Endogenous Red Cell Caspase 3 in the Cleavage of Band 3—Based on our previous observations that oxidative stress could activate caspase 3 in mature human erythrocytes (10) we argued that caspase 3 could possibly be activated during erythrocyte senescence and that this activation could play a role in red cell senescence by cleavage of band 3. During the course of its 120-day life span, the erythrocyte undergoes a time-dependent increase in cell density. This has been the basis of the widely used method of density-dependent separation of young and old erythrocytes (34, 39, 40). In order to test our hypothesis that caspase 3 activation could play a role in red cell senescence, young and old human red blood cells were separated on a Percoll gradient. Cells were lysed and the cytosol were used for the analysis of soluble intracellular caspase 3. Erythrocyte cytosol from old red blood cells were found to contain detectable levels of activated, cleaved caspase 3 as assessed by Western blotting (Fig. 4*A*). On the other hand, cleaved caspase 3 could not be detected in lysates from young red blood cells, although procaspase 3 was visible on Western blots. This was further supported by assaying the caspase activity using the substrate Ac-DEVD-pNA. Caspase 3 captured from lysate prepared from 100 μl aged erythrocytes (of 5% hematocrit) released 650 $\pm$ 40 pmol of pNA/h. This was 10-fold more than the activity that could be detected in young erythrocyte lysates. In order to test whether endogenous red cell caspase 3 could cleave red cell band 3, IOV were prepared from young erythrocytes. Caspase 3 was immunoprecipitated from the lysates of old erythrocytes with anti-caspase 3 antibody and the immunoprecipitate was incubated with IOV from young erythrocytes. Considerable fragmentation of band 3 was observed (Fig. 4*B*, *lane b*) in IOV incubated in the presence of red cell caspase 3 with the appearance of bands ranging in size 18–70 kDa cross-reactive with anti-band 3 antibody. This was blocked when IOV were pretreated with the caspase 3 inhibitor Z-DEVD-fmk (Fig. 4*B*, *lane c*). Incubation of IOV with the immunoprecipitate derived from young red cells, did not show significant fragmentation of band 3 (Fig. 4*B*, *lane a*).

DISCUSSION

Proteolysis by caspases plays a central role in the execution of apoptosis in different cell types. Based on the premise that red cells though anucleated, undergo senescence, recognition by macrophages and removal from the circulation over the course of a 120-day life span, we sought to investigate the potential role of caspases on membrane remodeling in the mature human red cell. In our previous studies we have demonstrated that red cells contain caspase 3 (10), the central player in most apoptotic signaling pathways, and that caspase 3 is activated in response to oxidative stress. These findings prompted us to look for potentially crucial caspase 3 substrates. Through interactions with a diverse array of proteins, CDB3 regulates among others, cell shape, glucose metabolism, ion transport, and cell clearance from the circulation. Considering its central role, we asked the question whether CDB3 contains caspase 3 cleavage sites. CDB3 was purified from mature human erythrocytes and used as a substrate for recombinant caspase 3. It was cleaved by caspase 3 to give two major fragments which were sequenced from the N terminus. This enabled us to deduce that cleavage occurs on the C-terminal side of the two sites: TATD^{45} and EQGD^{205}. In order to conduct further detailed studies, recombinant CDB3 was expressed in *E. coli* and used as a substrate for recombinant caspase 3. The same sites of cleavage could be demonstrated with recombinant CDB3 as substrate. The deduced two sites fit the preferred P4 but not P1 consensus for caspase 3 (41). These sites represent a growing list of cleavage sites of caspase 3, which do not fit the D*XX*D consensus motif (42–44). This was further confirmed by the following experiments: (1) when caspase 3 inhibitor was added, the cleavage of CDB3 by caspase 3 could be blocked; and (2) site-directed mutagenesis of Asp^{45} and Asp^{205} (to either Ala or Glu) could prevent the caspase 3-mediated cleavage of

CDB3. The crystal structure of CDB3 suggests that residues 1–54 and 202–211 represent regions in CDB3 with segmental flexibility (45). These regions would therefore be likely to be accessible for caspase-mediated proteolysis. The glycolytic enzymes (GAPDH, aldolase, and phosphofructokinase) (23–25) bind to the extreme N terminus of CDB3. Caspase-mediated cleavage of CDB3 at position 45 would be sufficient to disrupt each of these interactions. The binding site of protein 4.2 on CDB3 may involve, among other residues, Glu-40, since mutation at Glu-40 leads protein to 4.2 deficiency and hereditary spherocytosis (46). Cleavage at Asp-45 and Asp-205 could therefore be sufficient to abrogate protein 4.2 binding. In order to analyze whether CDB3 could be cleaved by caspase 3 *in vivo*, HEK293 cells were transiently transfected with CDB3, since red cells are not amenable to transfection. To investigate the apoptosis-induced fragmentation of CDB3, we treated transiently transfected HEK293 cells with the protein kinase inhibitor STS, which is known to induce apoptosis in many cell types (47, 48). We observed that these cells were undergoing apoptosis rather than necrosis. Immunoblot analysis of STS-treated cell lysates showed the partial degradation of CDB3 as well as formation of two anti-band 3 antibody cross-reactive bands in a time-dependent manner. The kinetics of formation of these two fragments from CDB3 was parallel to that of the fragmentation of PARP, a widely recognized caspase 3 substrate. There was a concomitant increase in the formation of cleaved caspase 3 also. The specificity of caspase 3-mediated proteolysis was confirmed by the observation that pretreatment with Z-DEVD-fmk prior to STS treatment could block the cleavage of CDB3. This was further confirmed by the fact that HEK 293 cells expressing mutant CDB3[D45A/D205A] did not show any cleavage of CDB3 upon STS treatment.

Protein 4.2, a 72 kDa peripheral membrane protein binds band 3 (21) and stabilizes the linkage of the cytoskeleton to the lipid bilayer. Protein 4.2 associates with band 3 and controls the lateral and rotational mobility of band 3 oligomers (49). From *in vitro* analysis we have previously demonstrated that the N-terminal domain of protein 4.2 is one of the domains critical for the protein 4.2-CDB3 interaction (38). It was therefore tempting to speculate that the induction of caspase 3-mediated apoptosis may lead to the disruption of band 3-protein 4.2 interaction. In order to study the interaction of protein 4.2 with CDB3 *in vivo*, we co-transfected HEK293 cells with FLAG-tagged C1 (encompassing the N-terminal 230 amino acid residues of protein 4.2) and CDB3. Immunoprecipitates obtained using anti-CDB3 antibody from lysates of cells expressing both CDB3 and C1, showed the presence of Flag-tagged C1 by Western analysis. This demonstrated the *in vivo* interaction between C1 and CDB3 in HEK293 cells. STS treatment after co-transfection significantly diminished such interaction between C1 and CDB3. The interaction was not abolished since a proportion of intact band 3 was invariably present in STS-treated cells. Interaction could be restored when the caspase 3 inhibitor Z-DEVD-fmk was used prior to STS treatment. This was evidence of the STS-induced caspase 3-mediated cleavage of CDB3 compromising its function of serving as the binding site for cytoskeletal proteins.

Finally, it was of utmost importance to demonstrate that active caspase 3 is present in human erythrocytes and that it indeed cleaves red cell band 3. Considering that aged erythrocytes show reduced glutathione levels, and our earlier observations that oxidative stress activates caspase 3 in mature human erythrocytes, we asked the question whether caspase 3 activation was a feature associated with erythrocyte senescence. Caspase 3 was indeed observed to be activated in aged red cells both by Western analysis, which showed the presence of cleaved caspase 3 and by spectrophotometric activity assay. Lysates from aged or young red cells were used as source of caspase 3 and tested in terms of their ability to degrade red cell band 3. Band 3 present in IOV from young erythrocytes was found to be cleaved by anti-caspase 3 immunoprecipitates of lysates derived from aged red cells only, further supporting the argument of a possible physiological role of active caspase 3 in erythrocyte aging. Band 3 degradation mediated by caspase 3 could serve as a senescence signal for the removal of aged cells from the circulation. The cleavage pattern of band 3 showed the presence of several bands of different sizes. This was not surprising, since, in addition to the sites present in CDB3, other cytosolic domains of band 3 could contain caspase 3 cleavage sites, which are yet to be identified.

In conclusion, we have demonstrated for the first time the cleavage of any of the isoforms of the anion exchanger band 3 by caspase 3. Red cell CDB3 is cleaved by caspase 3 *in vivo* at two non-conventional sites $TATD^{45}$ and $EQGD^{205}$, and this weakens CDB3-protein 4.2 interactions. The cleavage sites identified in this study are specific for the erythroid anion exchanger AE1 and are not conserved in AE2 and AE3. The mature human erythrocyte is devoid of a nucleus and mitochondria. Nevertheless, we and other workers (10) have shown that at least some players of the apoptotic program known in nucleated cells are present in the mature human erythrocyte. We have also shown that caspase 3 can be activated under oxidative stress. It is therefore interesting to speculate that caspases when activated in the mature human erythrocyte could participate in the degradation of crucial erythrocyte proteins involved in the maintenance of shape and function. Exemplary of this is our demonstration for the first time that caspase 3-mediated cleavage of CDB3 could disrupt its interaction with the peripheral membrane protein 4.2, a finding, which could have potentially serious consequences under conditions of stress or pathology, and possibly during senescence. Of foremost importance is our observation that active caspase 3 from aged erythrocytes can cleave red cell band 3, strongly suggesting that caspase-mediated cleavage of substrates such as band 3 is likely to play a crucial role in erythrocyte senescence. These findings open avenues of future investigation on the consequences of caspase 3-directed cleavage on the many functions associated with the cytoplasmic domains of band 3.

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