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ORIGINAL PAPER

The CD44 standard/ezrin complex regulates Fas-mediated apoptosis in Jurkat cells

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Abstract The transmembrane receptor CD44 conveys important signals from the extracellular microenvironment to the cytoplasm, a phenomena known as "outside-in" signaling. CD44 exists as several isoforms that result from alternative splicing, which differ only in the extracellular domain but yet exhibit different activities. CD44 is a binding partner for the membrane-cytoskeleton cross-linker protein ezrin. In this study, we demonstrate that only CD44 standard (CD44s) colocalizes and interacts with the actin cross-linkers ezrin and moesin using well-characterized cell lines engineered to express different CD44 isoforms. Importantly, we also show that the association CD44s-ezrin-actin is an important modulator of Fas-mediated apoptosis. The results highlight a mechanism by which signals from the extracellular milieu regulate intracellular signaling activities involved in programmed cell death.

Keywords CD44 · Ezrin · Apoptosis · Actin cytoskeleton · Interaction

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Introduction

CD44 is a ubiquitously expressed cell adhesion transmembrane receptor which exists as different isoforms. The large number of isoforms results from alternative splicing of the extracellular domain, while all isoforms retain a common transmembrane and intra-cytoplasmic domain. Post-translational modifications (such as glycosylation) of the extracellular domain and alternative splicing are known to influence CD44 function, but nonetheless do not completely explain the multifunctional activity of CD44.

Many studies have reported the ability of the CD44 cytoplasmic domain as critical in coordinating signaling events. Indeed, signaling via CD44 can induce the activation of focal adhesion kinase and mediate resistance to apoptosis [1]. CD44 may also associate with protein kinases, enhance T cell receptor signaling and thereby support proliferation, or cell death [2]. CD44 expression and ligation can promote actin cytoskeleton rearrangement during T cell activation [3]. The carboxy terminal cytoplasmic domain of CD44 supports the binding of proteins with crucial functions in cytoskeletal organization and signaling (reviewed in [4]). These proteins include ankyrin [5], annexin II [6] and members of the ERM (Ezrin, Radixin, Moesin, Merlin) family [7–10]. ERM proteins act as cross-linkers between actin cytoskeleton and membrane

receptors [11]. ERM proteins can become activated either by phospholipid binding by their N-terminal domain or by phosphorylation of C-terminal threonine residue [9, 10]. Once in their "open and active" conformation, the ERM C-terminal domain can bind actin cytoskeleton, while the N-terminal domain is able to associate with a number of transmembrane receptors, including CD44 [11, 12]. These linker proteins have been shown to localize to membrane ruffles, microvilli, filopodia and cleavage furrows [11]. In addition to their structural function, they have been implicated in several cellular functions that involve actin cytoskeleton rearrangement, including cell polarization, migration, morphology, survival and apoptosis [13–18].

The prototype member of the ERM protein family is ezrin. Ezrin and CD44 are ubiquitously expressed and associate via the binding of ezrin to the cytoplasmic domain of CD44, proximal to the transmembrane domain [9]. This association may function to regulate its subsequent interactions with downstream signaling pathways (reviewed by [7]). Phosphorylation of serine 291 by protein kinase C (PKC), and dephosphorylation of serine 325 in the cytoplasmic tail of CD44 result in dissociation of ezrin, and promote cell migration [18]. In myeloid cells, the binding of phosphorylated ERM proteins to CD44 regulates hyaluronan binding to CD44 [19]. Supporting this idea, reports in other cell types suggest that phosphorylated ERM and CD44 play a critical role in microvilli formation, cell adhesion and cell migration [16, 17]. Confocal microscopy studies on endothelial cells show colocalization of CD44, ERM proteins, actin filaments and PKC during wound healing [20]. Recruitment of ezrin and the actin cytoskeleton by CD44 has also been shown to be required for c-met signaling and activation of Ras [21]. In contrast, a recent study reveals that merlin, a member of the ERM family suppresses tumor growth by inhibiting CD44-hyaluronic acid interaction [22].

Actin stabilization and destabilization have both been shown to be implicated in regulation of apoptosis [23–25]. Many reports also suggest that ERM proteins regulate the initial phase of apoptosis through their effects on the actin cytoskeleton rearrangement. Indeed, it has been demonstrated that formation of blebs and disruption of nuclear integrity during apoptosis requires an intact actin cytoskeleton, and that actin accumulates at the basis of the apoptotic bodies [25]. All these studies together suggest that membrane receptors, actin cross-linker proteins and cytoskeleton remodeling are tightly regulated and implicated in the control of the cell's fate. Nonetheless, the impact of CD44 on the actin cytoskeleton, via its effector protein ezrin, has not been elucidated in the context of apoptosis. Recently, we showed that CD44 variant (CD44v) isoforms block Fas mediated apoptosis [26] whereas the shorter standard isoform (CD44s) does not. This mechanism explains the different behavior of CD44s and CD44v observed during apoptosis induction. Here, we extend these studies, investigating the interaction between ERM proteins and different CD44 isoforms, in Jurkat cells in the context of cell death. Our results implicate CD44-ERM interaction as an important regulator of Fas-mediated killing.

Material and methods

Cell lines and generation of CD44 transfectants

Jurkat cells (human T cell leukemia line) obtained from the American Type Culture Collection (ATCC) were co-transfected by electroporation as described in [26] with a neomycin resistance plasmid and CD44s, CD44v or CD44s lacking the cytoplasmic domain (CD44sΔcyt) encoding DNA. The constructs contain EGFP (enhanced green fluorescence protein) fused at the CD44 carboxy terminal end (Fig. 1A).

Expression of CD44 isoforms was confirmed by flow cytometry with fluorescently labeled antibodies for panCD44 and CD44v isoforms. Cells with similar green fluorescence intensity were sorted on a MoFlo high-speed cell sorter (DakoCytomation, Zug, Switzerland).

Cytochalasin D treatment

Cells were exposed to 0.5 μg/ml of cytochalasin D (CD, Sigma) for 90 min before apoptosis induction for 5 h with FasL. The short-term treatment used in this study inhibits actin polymerization without general cytotoxicity, as assessed by analytical cytology analyses [27]. Apoptosis was evaluated by flow cytometry by measuring the sub G1 population.

Induction and evaluation of apoptosis

FasL and anti-human Fas treatment

As previously described [26], Neuro-2a cells producing recombinant mouse FasL were kindly provided by A. Fontana (University Hospital, Zürich, Switzerland). Jurkat transfectants (5×10^5 cells/ml) were incubated with serial dilutions of FasL or with a Fas cross-linking antibody (100 ng/ml) (clone 7C11, Immunotech, Marseille, France) for 1, 3 or 6 h at 37°C.

Untreated and Fas triggered cells were collected, and evaluated for apoptosis with different methods.

1. *AnnexinV/PI.* Cells were stained with annexinV labeled with allophycocyanine (APC) (Alexis, Basel,

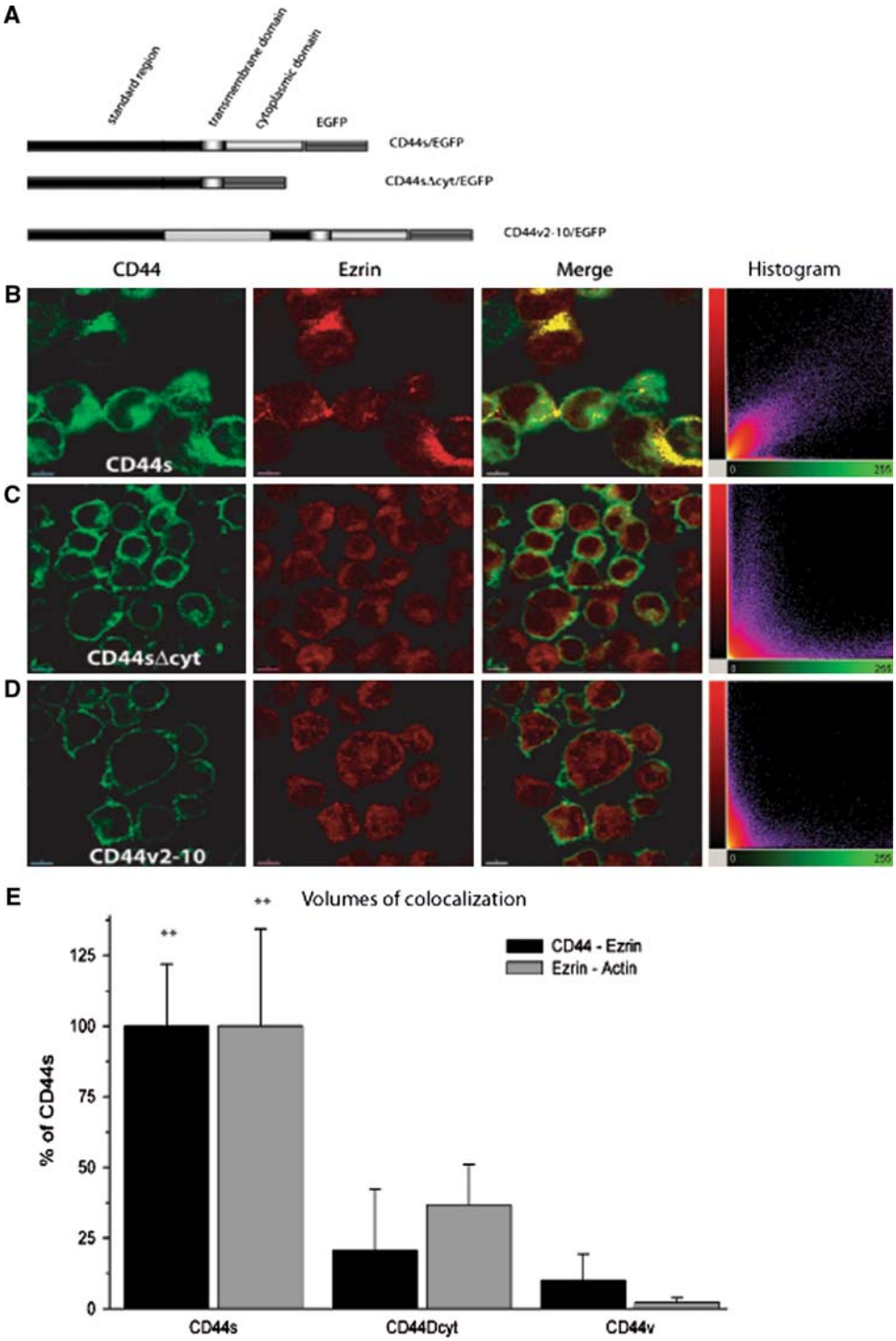

Fig. 1 Expression of CD44 isoforms in Jurkat transfectants and CD44s/ezrin colocalization. (**A**) Scheme of the different CD44-EGFP constructs used for transfection of Jurkat cells. Jurkat cells transfected with CD44s (**B**), CD44sΔcyt (**C**) and CD44v2-10 (**D**). Cells were stained with anti-panCD44 antibody (Hermes-3), followed by Cy2 labeled goat anti-mouse antibody (green fluorescence) and with anti-ezrin antibody followed by TxR labeled goat anti-rabbit antibody. The merge in yellow represents colocalization between CD44s and ezrin. The 2D histograms display the voxel pattern of colocalization between CD44 and ezrin. (**E**) Histogram representing the percentage of colocalization between CD44 and ezrin (black bars) and ezrin and actin (gray bars). Data are means of three to four data sets and were analyzed with the student *t*-test, ($*P < 0.05$; $**P < 0.01$)

Switzerland) and 5 μg/ml propidium iodide (PI) (Sigma, Buchs, Switzerland) and percentage of apoptotic cells was evaluated as described in [26].

2. *Hypodiploid DNA*. Cells were incubated overnight, at 4°C, in the dark in a hypotonic DNA binding buffer (see description in [28]). Apoptotic rates of untreated and FasL treated cells were compared by flow cytometry. Apoptosis was evaluated by measuring loss of PI staining (corresponding to the subdiploid DNA population also called subG1 population which represents the apoptotic population) [28].
3. *PARP p85*. Cells were treated as described in [26] and percentage of apoptotic cells was evaluated by flow cytometry by measuring the PE positive cells.

The data obtained from these three methods were analyzed with the CellQuest program (Becton Dickinson).

Confocal microscopy

For confocal analysis Jurkat CD44s, Jurkat CD44sΔcyt and Jurkat CD44v cells were washed in PBS, fixed 20 min at room temperature with 2% PFA, washed twice with PBS and permeabilized 10 min on ice with 1% triton. Staining was performed with mouse monoclonal anti-human pan-CD44 (Hermes 3) followed by secondary goat anti-mouse labeled with Cy2 (Amersham). Cells were washed and also stained with rabbit anti-human ezrin antibody (Cell signaling, Allschwil, Switzerland) followed by secondary goat anti-rabbit labeled with TxR fluorochrome (Southern Biotechnology, Reinach, Switzerland). Incubations with the antibodies were performed on ice, in the dark, during 1 h and the cells were extensively washed with PBS between the different steps. All antibodies were diluted in PBS with 1% BSA. After staining, the cells were spun onto slides (Cytospin) and mounted in Mowiol. For quantitative colocalization, cells were grown on fibronectin-coated coverslips and immunoreacted as described above. Confocal image stacks were recorded on a Nikon confocal microscope operating in the simultaneous acquisition mode. Images were recorded without clipping of the dynamic range, with detector settings calibrated to the brightest signal (CD44s) and analyzed for colocalization using the Imaris software package (Bitplane AG, Zürich). After application of a $3 \times 3 \times 3$ median filter preventing the formation of noise induced, single voxel signal in the colocalization maps [29], a region of interest corresponding to the CD44 signal was defined by masking the datasets with a threshold at 5% of maximum green intensity. Thresholds for colocalization were picked automatically by an algorithm excluding intensity pairs that exhibit no correlation (Pearson's coefficient < 0) [30] and corrected manually in a few cases with thresholds below the noise level ($\sim$8% of maximum intensity). Quantification included determination of colocalized volume and Pearson's correlation coefficient, which has the advantage of being independent of background.

SDS-PAGE and western blotting

As described in [26], Jurkat cells were collected, lysed in RIPA buffer and extracts were separated on 10% SDS polyacrylamide gels. Proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Schleicher and Schuell, Dassel, Germany) and blocked for 1 h at room temperature in PBS with 3% Top Block (Juro, Luzern, Switzerland). Expression of CD44, ezrin, and tubulin were detected with mouse monoclonal anti-human CD44 (clone Hermes 3, kindly provided by E. Butcher, Stanford, CA), mouse monoclonal anti-human ezrin (clone 18, Transduction Laboratories, Basel, Switzerland), mouse monoclonal anti-tubulin (clone 236-10501, Molecular Probes, Leiden, The Netherlands), followed by goat anti mouse-HRP (Pierce). The signal was visualized by chemoluminescence with Super Signal Substrate (Pierce).

Nitrogen cavitation bomb

A total of 10^8 cells were collected, washed with PBS, centrifuged 5 min at 1,300 rpm and incubated for 30 min at 4°C on a rotating wheal with magnetic beads coated with a secondary goat anti-mouse IgG antibody (Dynal, Hamburg, Germany). The beads were consecutively coated with anti-panCD44 antibody (clone Hermes 3) according to the manufacturer's protocol. The cells coated with beads were then treated with FasL for 10 min at 37°C or left untreated, washed with ice cold H-Buffer (250 mM sucrose, 10 mM Na-Hepes pH 7.2, 2 mM $MgCl_2$, 10 mM NaF, 1 mM sodium vanadate) and retrieved with a magnetic device (Dynal) several times. The cells coated with the beads were then surface cross-linked for 2 h at 4°C in a rotating wheel with 1 mg/ml DTSSP [3,3'-Dithiobis-(sulfosuccinimidylpropionate)] (Pierce). Cross-linking was stopped by incubating the cells with 20 mM Tris-HCl pH 7.5 for 15 min on ice. Cells were intensively washed in H-Buffer, resuspended in H-Buffer+ (H-Buffer with 6.6 μM Pervanadate and 1 mini tablet of a protease inhibitor cocktail (Roche, Penzberg, Germany) for 10 ml of solution. The number of cells coated with beads was counted under the microscope and approximately 40×10^5 cells per sample were disrupted in a nitrogen cavitation bomb at −196°C and 600 psi (pounds per square inch) [31]. After disruption of the membranes, the samples were washed and retrieved four times and finally resuspended in 2× SDS sample buffer. For further western blotting analysis, the extracts were processed as described above and immunoblotted with mouse monoclonal anti-ezrin antibody (Transduction Laboratories) followed by goat anti mouse-HRP (Pierce). The signal was visualized by chemoluminescence (Pierce).

Results

CD44s and ezrin colocalize and interact together

To determine if ezrin interacts with any isoform of CD44 during apoptosis, we used the Jurkat cell model previously established which selectively express different CD44 isoforms. Jurkat cells normally express Fas, but not CD44, constitutively and can easily be induced to undergo apoptosis with FasL or a Fas cross-linking antibody (clone

7C11). Our Jurkat cell lines stably expressed cDNA constructs for CD44s (Ju CD44s), CD44v2-10 (Ju CD44v2-10), CD44v3 (Ju CD44v3), CD44v6 (Ju CD44v6), CD44v9 (Ju CD44v9) and CD44sΔcyt (Ju CD44sΔcyt) (Fig. 1A). As a control we used Jurkat cells transfected with the neomycin resistance plasmid alone (Ju neo). Transfected cells were analyzed by flow cytometry for CD44, Fas and ezrin expression levels (data not shown). Only cell lines with comparable CD44, Fas and ezrin levels were used for these studies.

Confocal data sets of the cells were analyzed for co-localization. CD44s showed considerable overlap between CD44 and ezrin which was found predominantly at the plasma membrane (yellow merge) (Fig. 1B) and which contained 32% (vol/vol) of the green signal. Accordingly, the 2D histogram displayed a distinct distribution of voxels arranged in a diagonal pattern, thus indicating colocalization. Ezrin interacts with the cytosolic domain of CD44, accordingly, colocalization between CD44sΔcyt and ezrin was strongly decreased (Fig. 1C). Thus, the cytosolic domain is critical for recruitment of ezrin to CD44.

Surprisingly, only 10% of the CD44v2-10 isoform, which shares the cytoplasmic domain with CD44s, showed overlap with ezrin (Fig. 1D). Both constructs, CD44sΔcyt and CD44v, displayed histograms with voxels arranged along the two axes and lacking the diagonal distribution (Fig. 1C and D). In addition, correlation coefficients, which provide a measure for the linear intensity relationship of two channels (1 = absolute linear relation, 0 = no relation), are quite high in CD44s (0.864) and show significant decreases in CD44sΔcyt and CD44v that paralleled the results using volumetric data, as shown in Fig. 1E. We observed a strong colocalization between CD44s, ezrin or actin but this colocalization did no longer exist when we substituted the standard form of CD44 (CD44s) by the variant isoform (CD44v2-10) or the CD44sΔcyt isoforms (Fig. 1E). Moreover, CD44sΔcyt, as well as CD44v, displayed colocalization parameters in the range of those observed in Jurkat neo cells used as negative control (data not shown). Our results clearly support a molecular interaction between ezrin, actin and the cytoplasmic domain of CD44s, but not CD44v. Therefore, while the cytosolic domain of CD44 may be necessary for interaction with ezrin, it is insufficient to mediate this interaction. Jurkat cells also express moesin, and the active phosphorylated forms of ezrin and/or moesin (Unpublished observations) are detected in these cells. When Jurkat transfectants were stained with either anti-moesin or anti-phospho-ERM (pERM) specific antibodies, we observed a strong colocalization between CD44s and moesin or pERM. No colocalization was observed with CD44v or CD44sΔcyt (Unpublished observations). Together, these results support the notion that active forms of ezrin, and likely moesin, interact with actin and the cytosolic domain of CD44s.

To extend and confirm these results, we performed co-immunoprecipitation experiments using the nitrogen cavitation bomb method. This alternative procedure to "standard" co-immunoprecipitations offers the advantage that it is detergent-free, and therefore provides an excellent method to examine protein interactions among membrane proteins found in lipid rafts [31]. To study the interaction between CD44 and ezrin, we targeted CD44 in Jurkat neo, Jurkat CD44s, Jurkat CD44v2-10, v3, v6, v9 and Jurkat CD44sΔcyt cells by cavitation bomb extractions, followed by immunoblotting with anti-ezrin antibody (Fig. 2A). An interaction was readily observed with the CD44s isoform,

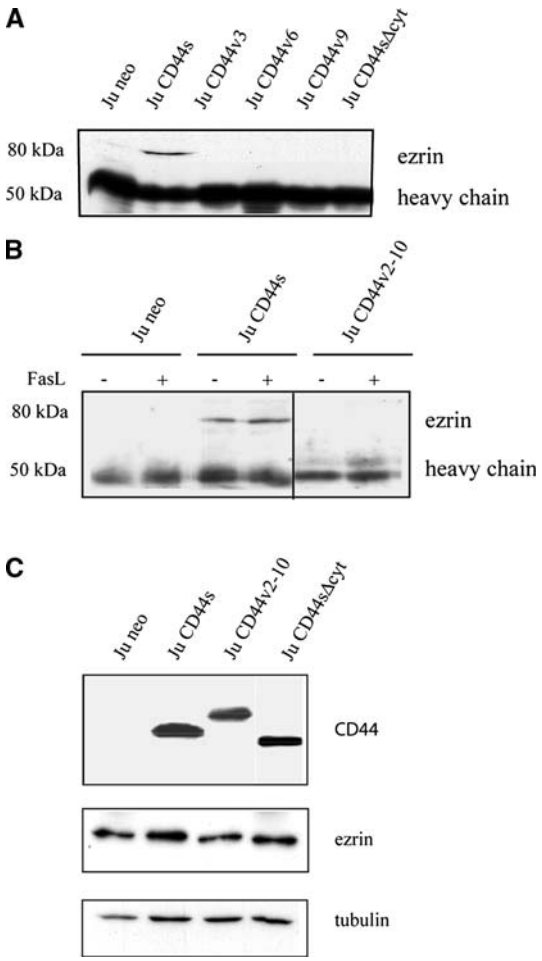

Fig. 2 Interaction between CD44s and ezrin. (**A**) Western blotting for ezrin on CD44 cavitation bomb extractions from Jurkat neo, Jurkat CD44s, Jurkat CD44v3, Jurkat CD44v6, Jurkat CD44v9, and Jurkat CD44sΔcyt. Cells were incubated with magnetic beads coated with mouse anti-human CD44 antibody. (**B**) Western blotting for ezrin on CD44 cavitation bomb extractions from Jurkat neo, Jurkat CD44s, and Jurkat CD44v2-10, untreated or treated for 15 min with FasL, before nitrogen cavitation bomb extraction. (**C**) Western blotting showing the expression of panCD44, ezrin and tubulin as a loading control in the different transfectants

yet not with other isoforms of CD44. Furthermore, anti-CD44 cavitation bomb extractions from Jurkat neo, Jurkat CD44sΔcyt and Jurkat CD44v2-10 did not yield any evidence of interaction with ezrin.

These studies were reproduced under two different conditions: cells untreated and cells treated with FasL for 15 min (Fig. 2B). An 80 kDa band corresponding to ezrin was evident in the extracts from Ju CD44s cells treated (and untreated) with FasL. A band at 50 kDa corresponding to the heavy chain of the antibody coupled to the magnetic beads was observed in all the samples, suggesting that equal quantities of immunprecipitants were analyzed in each case. As an additional control, we confirmed that equal expression of CD44 was maintained in all transfectants via immunoblotting (Fig. 2C). These data confirm that ezrin interacts selectively with the intracellular domain of CD44s and that this interaction is present when the cells are induced to undergo apoptosis with FasL. Interestingly, all CD44 isoforms share an identical cytoplasmic domain, thus it is particularly intriguing that interaction between the CD44v isoforms and ezrin was not observed.

Cells expressing CD44sΔcyt are more resistant to Fas mediated apoptosis

To understand the role of the CD44 cytoplasmic domain in apoptosis induction we conducted in vitro functional assays using Jurkat neo, Jurkat CD44s, Jurkat CD44sΔcyt and Jurkat CD44v2-10 transfected cells. We analyzed Jurkat cells expressing CD44s, which interacts with ezrin together with Jurkat CD44v2-10 as a characterized negative control for cell apoptosis in our model [26], and Jurkat CD44sΔcyt as a negative control for ezrin interaction. The different transfectants were treated with a Fas cross-linking antibody for 1, 3, or 6 h, and apoptotic cells were detected by AnnexinV/PI staining (Fig. 3A). The control cell Jurkat CD44v2-10 was highly protected from Fas-mediated apoptosis as previously described [26]. Interestingly, Ju CD44sΔcyt cells were also significantly more resistant to apoptosis than Ju CD44s cells. In fact, after 3 and 6 h treatment with Fas cross-linking antibody, Ju CD44sΔcyt showed only 7 and 15% of apoptotic cells, respectively. Ju CD44s and Ju neo cells exhibited higher rates of cell death, about 20% at 3 h induction and 35% at 6 h induction (Fig. 3A). Similar results were obtained by measuring hypodiploid DNA after treating the cells for 3 and 6 h with FasL (Fig. 4B). Indeed, after 6 h treatment we observed 38% of apoptotic cells in the Ju CD44s but only 15% in the Ju CD44sΔcyt cells. We obtained similar results when the cells were treated with FasL (Fig. 3C) or a Fas cross-linking antibody for 3 or 6 h (Fig. 3D) and the amount of apoptotic cells was detected by quantifying PARP cleavage. JuCD44s exhibited 41.6% of apoptotic cells, whereas JuCD44sΔcyt showed only 19.2% (Fig. 3D). These results suggest that the deletion of the cytoplasmic domain of CD44s plays a critical role in governing the susceptibility of cells to undergo apoptosis.

Cells expressing CD44s show reduced apoptosis after treatment with cytochalasin D

Others have described that the cytoplasmic domain of CD44 can interact with the actin cytoskeleton via linker proteins such as the ERM proteins [7–9]. To examine the relevance of the actin cytoskeleton during apoptosis, we examined the impact of depleting F-actin prior to treating the different transfectants with Fas. Jurkat cells expressing CD44s, CD44sΔcyt and CD44v2-10 were either treated for 5 h only with FasL or briefly pre-treated with the inhibitor of actin polymerization Cytochalasin D (CD), then exposed for 5 h to FasL to induce apoptosis. Similar amounts of apoptotic cells were observed in the Ju neo, Ju CD44v2-10, and Ju CD44sΔcyt before and after treatment with CD (Fig. 4A). However, although all Jurkat transfectants express similar levels of Fas, only Ju CD44s showed a significant decrease in apoptosis from 35 to 18%, when they were pre-treated with CD (Fig. 4A). Interestingly, we could decrease apoptosis either by deleting the cytoplasmic domain of CD44s or by disrupting the actin cytoskeleton (Fig. 4A, gray bars, CD44s vs. CD44sΔcyt). The data implicate that the actin-CD44s interaction is critical in inducing apoptosis, and that disruption of the actin cytoskeleton affects the pro-apoptotic effect of CD44s. When the cytoskeleton was disrupted with CD prior to apoptosis induction, Ju CD44s cells showed a much lower increase in apoptosis, only 16.5% of apoptotic cells (Fig. 4B, second row, third panel) compared to 34.7% observed in the absence of CD (Fig. 4B, second row, middle panel). CD treatment had no influence on the apoptosis susceptibility of Ju neo, Ju CD44sΔcyt, and Ju CD44v2-10 (Fig. 4B, first, third, and fourth row). In the presence or absence of CD, all three transfectants exhibit similar rates of apoptosis. Together, these results suggest that the cytoplasmic domain of CD44s but not the one of CD44v is required for actin organization and consequently apoptosis induction.

Discussion

CD44s–actin interaction is required for Fas-mediated apoptosis

Many of the direct interactions between ERM proteins and transmembrane proteins involve adhesion receptors. ERM proteins were first found to interact with the cell adhesion

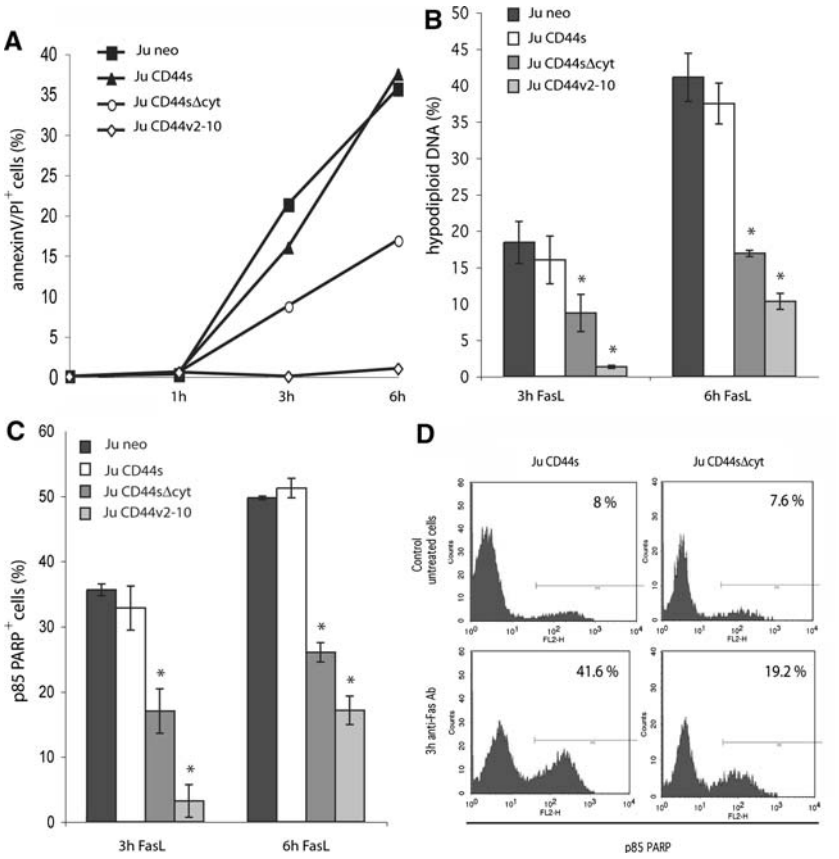

Fig. 3 Jurkat CD44sΔcyt cells are more protected from Fas mediated apoptosis than Jurkat CD44s. (**A**) Jurkat neo, Jurkat CD44s, Jurkat CD44sΔcyt and Jurkat CD44v2-10 were treated with Fas cross-linking antibody for 1, 3 or 6 h. Apoptotic cells were determined with annexinV/PI staining. Values are given for one experiment representative of three. (**B**) Jurkat transfectants were treated with recombinant FasL for 3 or 6 h. The amount of apoptotic cells was determined by measuring hypodiploid DNA (hypodiploid DNA = subG1 apoptotic population). Data are representative of three independent experiments and were analyzed with the Wilcoxon signed rank test ($*P < 0.05$). (**C**) Jurkat neo, Jurkat CD44s, Jurkat CD44sΔcyt and Jurkat CD44v2-10 were treated with recombinant FasL for 3 or 6 h. The percentage of apoptotic cells was determined by flow cytometry with an antibody recognizing the cleaved form of PARP followed by a secondary antibody labeled with PE. Apoptosis was calculated by subtracting the control values (between 2 and 4%) from the values obtained with FasL treatment. Data are representative of three independent experiments and were analyzed with the Wilcoxon signed rank test ($*P < 0.05$). (**D**) FACS analysis of Jurkat CD44s and Jurkat CD44sΔcyt untreated and treated for 3 h with Fas cross-linking antibody. Percentage of p85 PARP positive cells was evaluated by flow cytometry with an anti-p85 PARP antibody followed by a secondary antibody labeled with PE. The bar is limiting the living cells on the left according to the control. Numbers in the upper right corner represent the percentage of apoptotic cells

receptor CD44 [7, 8] through a positively charged juxtamembrane region in the CD44 cytoplasmic domain [9]. The interaction between ezrin and CD44 is tightly regulated by Rho GTPases [6] and PKC [18]. The ability of ezrin to interact with CD44 in a highly regulated fashion strongly indicates that ezrin might have a role in regulating cytoskeleton rearrangement. In this report, we demonstrate that ezrin differentially binds to CD44 isoforms. All isoforms share a common cytoplasmic domain and a different extracellular domain. Here we show that ezrin only colocalizes with the CD44s, but not with CD44 lacking the cytosolic domain or CD44v.

A similar behavior has been observed in other cell adhesion molecules such as integrins. Integrins are also subjected to alternative splicing and exist in various isoforms differing intra- and extra-cellularly. Interestingly, ligation of integrins with substrate ligands leads to actin polymerization and the formation of stable adhesion complexes, and can promote resistance to Fas-mediated apoptosis, while antagonism of integrins instead promotes apoptosis (reviewed in [32]). It is possible that CD44 and integrins work in an integrated fashion to modulate cellular predisposition to apoptosis based on the local microenvironment. Future experiments will address the role of CD44 and integrins in the coordination of susceptibility to programmed cell death.

Interestingly, differences in the extracellular domains of integrins, or in the composition of integrin heterodimers,

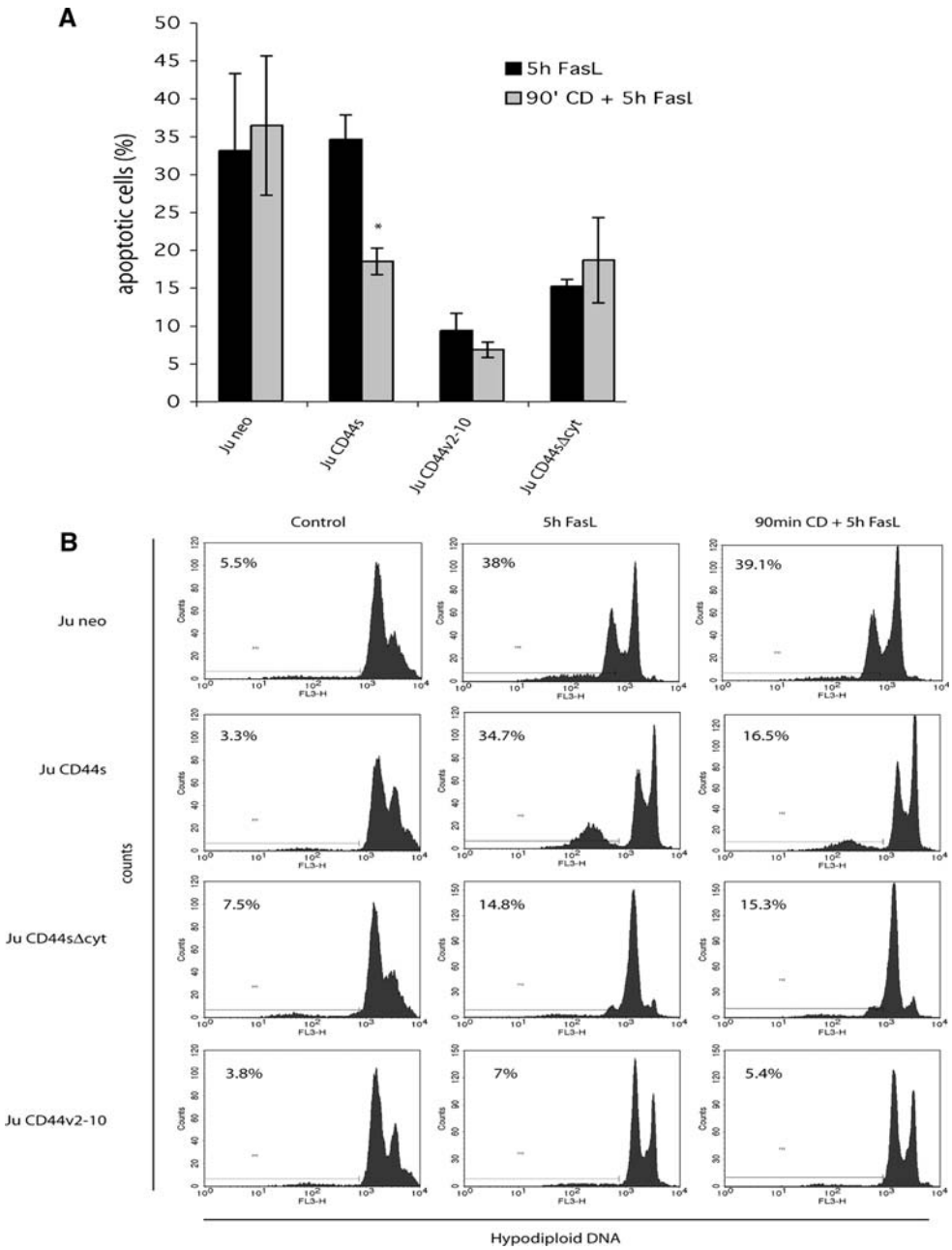

Fig. 4 Disruption of the actin cytoskeleton protects Jurkat CD44s cells from Fas mediated apoptosis. (**A**) Jurkat transfectants were either treated for 5 h only with recombinant FasL or pre-treated for 90 min with Cytochalasin D (CD) followed by 5 h treatment with FasL. Apoptotic cells were evaluated by measuring hypodiploid DNA. Data are representative of three independent experiments and were analyzed with the Wilcoxon signed rank test ($*P < 0.05$). (**B**) FACS analysis of Jurkat neo, Jurkat CD44s, Jurkat CD44sΔcyt and Jurkat CD44v2-10 untreated cells, treated with FasL for 5 h or pre-treated with Cytochalasin D (CD) for 90 min followed by incubation for 5 h with FasL. The numbers in the quadrants represent the percentage of apoptotic cells obtained in one experiment representative of three. The percentage of apoptotic cells was evaluated by measuring hypodiploid DNA (hypodiploid DNA = subG1 apoptotic population). The bar is limiting the living cells on the right according to the control

leads to differences in candidate ligands. Interactions with extracellular ligands can trigger diverse intracellular signals and modulate cellular behavior via a process known as outside-in signaling [33, 34]. Our data provide evidence that isoforms of CD44 differing only extracellularly can similarly regulate intracellular signaling events in a distinct manner and that interaction between CD44s, ezrin, and actin directly impacts the efficiency of the proapoptotic signal.

Ezrin can interact with various membrane proteins such as Fas, E-cadherin, CD43, and CD44. These interactions lead to ezrin phosphorylation, activation and promote subsequent actin polymerization required for diverse biological processes such as cell death, adherens junctions formation, immunological recognition, and/or microbial pathogenesis [35–38]. Interestingly, the interaction of the death receptor Fas with ezrin has been suggested to be required for apoptosis induction (reviewed in [39]). Our results show that the interaction of ezrin to CD44s leads to similar events observed when ezrin interacts with Fas. The linkage of CD44 to the actin cytoskeleton mediated by ezrin, is required for the polarization of the cell preceding apoptosis signaling (Fig. 5). Our study conditions did not permit us to duplicate the interaction between Fas and ezrin

reported by others, (data not shown), however this could be due to differences in interacting partner affinities or differences in cell type. In our system, both CD44 and Fas are present and ezrin/moesin would almost certainly engage in a higher affinity interaction with CD44 than Fas. Regardless of which membrane receptor ezrin or moesin interact with (Fas or CD44), the consequences are identical. In both cases the interaction of ezrin or moesin with CD44 or Fas leads to actin organization and is crucial for maintaining susceptibility to Fas-mediated apoptosis.

In general, the connection between cell membrane proteins and actin, via the ERM proteins, appears to be a key regulator of cell homeostasis. From a metabolic perspective, it would seem to be more efficient and probably easier, for a cell to exploit the capacity to concentrate proteins in a specific site rather than synthesizing additional protein *de novo*.

CD44 regulates apoptosis

One way to explain these observations could be that different ligand binding or extracellular partner(s) may change the conformation of CD44 or its phosphorylation state, thus affecting the recruitment of signaling molecules and the anchorage of the cytoskeleton. In a previous report we have demonstrated that CD44v isoforms but not CD44s, interact with the death receptor Fas and, inhibit the death-signaling cascade [26]. Hence, it is very likely that CD44v isoforms have a higher affinity for Fas. Tight interactions with Fas might reasonably be expected to prevent the interaction with ezrin. This may provide one explanation for the controversial literature surrounding CD44, which has been described as both a pro-apoptotic molecule [40–42], and an anti-apoptotic and pro-metastatic protein [43–47]. The present study together with our previous data [26] provide one explanation for this controversy. We suggest, on the one hand that CD44v isoforms, which are upregulated early in thymic development [48] and later during conditions such as chronic inflammations and cancer, exhibit an anti-apoptotic effect by sequestering Fas [26]. On the other hand, we show that the standard form of CD44 selectively interacts with ezrin/moesin and the actin cytoskeleton forming a multimeric complex, that maintains or enhances susceptibility to apoptosis (Fig. 5). We also demonstrate that the CD44sΔcyt construct, which is similar to the naturally spliced isoform expressing a short cytoplasmic tail of only three amino acids, exerts an anti-apoptotic effect.

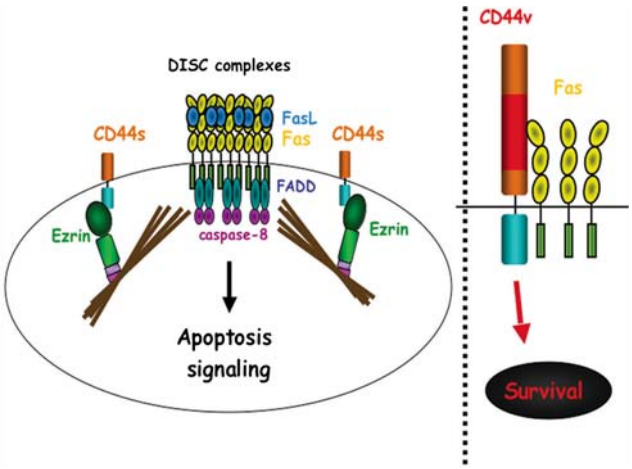

Fig. 5 Model of regulation of cell death by CD44s Molecular mechanism suggesting how CD44s regulates apoptosis. We postulate that the interaction between the cytoplasmic domain of CD44s and ezrin and the consequent organization of the actin cytoskeleton are essential for proning the extrinsic apoptotic pathway. The organized actin filaments resulting from CD44s/ezrin interaction might function as a scaffold, which stabilizes the DISC (Death inducing signaling complexes) and consequently facilitates apoptosis signaling

In contrast to CD44v, the CD44s isoform lacking the cytosolic domain does not interact with Fas (data not shown). Here we document a different anti-apoptotic mechanism; the CD44s truncated form exerts a protective effect towards cell death due to its inability to organize the actin cytoskeleton. Hence, the isoforms of CD44 can exhibit different and even opposite functions concerning regulation of cell death and can regulate apoptosis by diverse mechanisms.

These findings provide an enhanced understanding of the mechanisms by which CD44 regulates apoptosis, and provide new information concerning the function of the CD44s "truncated" isoform. The data demonstrate that potential therapeutic strategies for cancer or autoimmune diseases that target CD44 should be very specific. As shown here, the standard isoform contributes to actin organization, which is crucial for the efficient induction of Fas-mediated apoptosis. Therefore, nonspecific therapies, which target all CD44 isoforms, may be counter-productive.

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