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CD45-Mediated Fodrin Cleavage during Galectin-1 T Cell Death Promotes Phagocytic Clearance of Dying Cells[1]

Mabel Pang,* Jiale He,[2]* Pauline Johnson,‡ and Linda G. Baum[3]*†

Disassembly and phagocytic removal of dying cells is critical to maintain immune homeostasis. The factors that regulate fragmentation and uptake of dying lymphocytes are not well understood. Degradation of fodrin, a cytoskeletal linker molecule that attaches CD45 to the actin cytoskeleton, has been described in apoptotic cells, although no specific initiator of fodrin degradation has been identified. CD45 is a glycoprotein receptor for galectin-1, an endogenous lectin that can trigger lymphocyte apoptosis, although CD45 is not required for phosphatidylserine externalization or DNA degradation during galectin-1 death. In this study, we show that fodrin degradation occurs during galectin-1 T cell death and that CD45 is essential for fodrin degradation to occur. In the absence of CD45, or if fodrin degradation is prevented, galectin-1-induced cell death is not accompanied by membrane blebbing, although phosphatidylserine externalization and DNA degradation proceed, indicating that fodrin degradation occurs via a distinct pathway compared with the pathway that leads to these other hallmarks of cell death. Moreover, there is slower phagocytic uptake by macrophages of T cells in which fodrin degradation is prevented, relative to T cells in which CD45-mediated fodrin degradation occurs. These studies identify a novel role for CD45 in regulating cellular disassembly and promoting phagocytic clearance during galectin-1-induced T cell death. *The Journal of Immunology,* **2009, 182: 7001–7008.**

Apoptosis is a complex process that culminates in the disassembly and removal of dying cells. Clearance of apoptotic cells is critical for proper tissue development and homeostasis. Efficient phagocytic clearance of dying cells has also been proposed to prevent inflammation triggered by release of intracellular contents, as well as avoiding exposure of sequestered self-Ags that could provoke autoimmune disease (1–4).

Several apoptotic triggers for T cells have been described. Galectin-1 is a mammalian lectin expressed by many cell types, including activated T cells, thymic epithelial cells, dendritic cells, and endothelial cells, that induces death of specific subsets of thymocytes and peripheral T cells. Galectin-1 regulates thresholds of positive and negative selection in the thymus, and selectively kills CD4 Th1 vs Th2 cells, due to expression of preferred glycan ligands on the former (5–8). The galectin-1 death pathway is distinct from death pathways initiated by other triggers, such as Fas, in that galectin-1 T cell death requires CD7 as a galectin-1 receptor, appears to be caspase independent, and involves a unique pattern of mitochondrial events, with no cytochrome *c* release but selective release of endonuclease G that moves to the nucleus to degrade cellular DNA (9–11).

In addition to CD7, galectin-1 also binds to CD45 on the T cell surface, although CD45 expression is not absolutely required for galectin-1 death of T cells (9, 12–14). However, CD45 can both positively and negatively regulate susceptibility to galectin-1; if CD45 is expressed, the extracellular domain of CD45 must be appropriately glycosylated for galectin-1 binding and signaling to occur (8, 12). The CD45 cytoplasmic region has two domains, a membrane-proximal tyrosine phosphatase domain and a C-terminal domain that has homology to the phosphatase domain but lacks enzymatic activity. The C-terminal cytoplasmic domain interacts with the cytoskeletal linker protein fodrin (αII-spectrin); CD45 interaction with fodrin is critical for CD45 delivery to the plasma membrane and also regulates enzymatic activity of the tyrosine phosphatase domain (15–17).

Fodrin is the major spectrin family member expressed in nonerythroid cells. Fodrin degradation accompanies apoptosis triggered by many death signals in many cell types, such as T cell death initiated by Fas ligation, B cell death initiated by TGF-β, and neural cell death initiated by staurosporine (18–20), although different proteases, including caspases and calpains, appear to be responsible for fodrin degradation in different apoptotic pathways (19–25). Fodrin degradation is proposed to contribute to apoptotic cell blebbing and other morphologic changes that occur during breakdown of dying cells (18, 21, 22). In the present study, we have found that, during galectin-1 T cell death, CD45-associated fodrin is proteolytically degraded in a parallel but separate process from the intracellular death pathway that results in phosphatidylserine externalization and nuclear DNA cleavage and that CD45 is essential for this to occur. Fodrin degradation was required for the full spectrum of morphologic changes observed during galectin-1 T cell death. Moreover, fodrin degradation enhanced the rate of macrophage engulfment of galectin-1-treated T cells.

Materials and Methods

Cell lines and reagents

Jurkat E6-1 cells (gift from F.-T. Liu, University of California, Davis, CA) and J45.01 cells (American Type Culture Collection) were maintained in RPMI 1640 (Invitrogen) with 10% FBS (HyClone), 10 mM HEPES, 2 mM

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Glutamax, 2 mM sodium pyruvate in 5% CO_2 at 37°C. J45.01 cells transfected with CD45 or vector alone were maintained in the above medium with 0.6 μg/ml G418. RAW 264.7 cells (American Type Culture Collection) were maintained in DMEM (Invitrogen), 10% FBS, and 2 mM Glutamax. Abs were from the indicated sources: monoclonal mouse anti-fodrin (Chemicon International), polyclonal rabbit CD45 (Abcam), monoclonal mouse phospho-tyrosine (Cell Signaling), monoclonal mouse CD45(LCA) (DakoCytomation), monoclonal mouse CD45 Alexa Fluor 488 (BioLegend), goat anti-mouse Alexa Fluor 594, goat anti-rabbit Alexa Fluor 488 (Invitrogen), goat anti-mouse HRP (Bio-Rad). Additional reagents used were: annexin V-Alexa Fluor 594 (Invitrogen), Prolong Gold antifade mounting medium, annexin V-FITC (Molecular Probes), caspase inhibitor I (z-vad-fmk), calpain inhibitor IV (z-lly-fmk) and calpain inhibitor VI (SJA 6017; Calbiochem), APO-Direct kit (BD Biosciences), and ECL (GE Healthcare). Galectin-1 was prepared as previously described (9).

T cell death assays

T cell death assays were done as previously described (9–11). Briefly, 1.25×10^6 Jurkat or J45.01 cells in triplicate were pretreated with or without 20 μM calpain inhibitors for 1 h at 37°C in 200 μl of the relevant media with or without G418 before adding 20 μM galectin-1 or buffer control for 6 h at 37°C/5% CO_2. Cells were dissociated with 0.1 M lactose in PBS. Fifty microliters in duplicate from each sample was removed for detection of cell death with annexin V-FITC and propidium iodide. Data were acquired on a FACScan flow cytometer and analyzed using CellQuest software (BD Biosciences). The remainder of the samples were combined for parallel experiments (immunoblot, immunoprecipitation, immunofluorescence). TUNEL was performed on cells fixed in 1% paraformaldehyde according to the manufacturer's directions (APO-Direct kit). Data were acquired and analyzed as above.

Immunoblotting

For fodrin analysis, cells were treated with galectin-1 or buffer control as described above. Cells were suspended in 0.1 mM pervanadate for 30 min at 37°C before lysing in 50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 5 mM EDTA, 150 mM NaCl, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 mM sodium orthovanadate for 15 min on ice (22). Lysates were microfuged for 15 min at 10,000 rpm. Ten micrograms of lysates was separated on a 3–8% Tris-acetate gel (Invitrogen NuPAGE Electrophoresis System) and electroblotted onto nitrocellulose (Whatman). The membrane was blocked and probed as previously described (9) and proteins were visualized by ECL. For immunoprecipitation, 1×10^7 cells treated with galectin-1 or buffer control were incubated with 3 μg of fodrin Ab or CD45 Ab with protein G beads (Pierce) overnight and washed extensively with lysis buffer before processing as above. Samples were denatured in NuPAGE reducing agent and NuPAGE SDS Sample buffer (Invitrogen) before loading.

Confocal immunofluorescence microscopy

Cells were treated with galectin-1 or buffer control as above, washed with cold PBS, fixed with 1 ml of 4% paraformaldehyde in PBS for 30 min on ice, and quenched with 3 ml of 0.2 M glycine in PBS. Pelleted cells were washed with PBS and blocked overnight with 2% goat serum in PBS at 4°C. These nonpermeabilized cells were incubated with CD45-Alexa Fluor 488 (1/200) and annexin V-Alexa Fluor 594 (1/50) for 1 h at room temperature (RT).[4] After washing with PBS, cells were refixed briefly with 4% paraformaldehyde in PBS and quenched. Cells were mounted on slides with Prolong Anti-fade Gold mounting medium, dried overnight at RT in the dark, and stored at 4°C. Alternatively, cells were permeabilized in 0.1% Triton X-100/1% BSA in PBS for 5 min, washed with PBS, refixed with 4% paraformaldehyde for 1 min, quenched, washed with PBS, and blocked with 1 ml of 10% goat serum in PBS for 1 h at RT. Cells were incubated with mouse anti-human fodrin (1/100) and rabbit anti-CD45 (1/100) in 1% goat serum/PBS overnight at 4°C. Cells were washed with PBS and stained with goat anti-mouse Alexa Fluor 594 (1/100) and goat anti-rabbit Alexa Fluor 488 (1/00) in 1% goat serum/PBS for 1 h at RT in the dark. Cells were washed and mounted as above. Slides were visualized on a Fluoview laser-scanning confocal microscope (Olympus) and images were processing using Fluoview imaging analysis software (version 2.1.39).

Phagocytosis assay

RAW 264.7 macrophages (3×10^5) were cultured on coverslips in 6-well plates in complete DMEM. Jurkat or J45.01 cells were labeled with 2 μM CFSE (Molecular Probes) for 15 min in PBS at 37°C in the dark. Unbound CFSE was neutralized with complete medium before resuspending cells in fresh prewarmed medium and incubating another 30 min. CFSE-labeled T cells were treated with galectin-1 or buffer control as described above. One $\times 10^6$ labeled T cells were added to the macrophage monolayer for the indicated time at 37°C. Nonphagocytosed target cells were removed by three washes with PBS. Digital images of randomly selected fields under phase-contrast microscopy were captured by confocal fluorescence microscopy and the number of engulfed cells in >300 macrophages was counted. The phagocytic index was calculated as follows: phagocytic index = (total number of engulfed T cells/total number of counted macrophages) × (number of macrophages containing engulfed cells/total number of counted macrophages) × 100, exactly as previously described (26, 27); in cases where only T cell fragments were observed, this was counted as one engulfed T cell per macrophage. The fraction of macrophages containing phagocytosed material was also calculated (number of macrophages containing engulfed cells or cell fragments/total number of counted macrophages × 100).

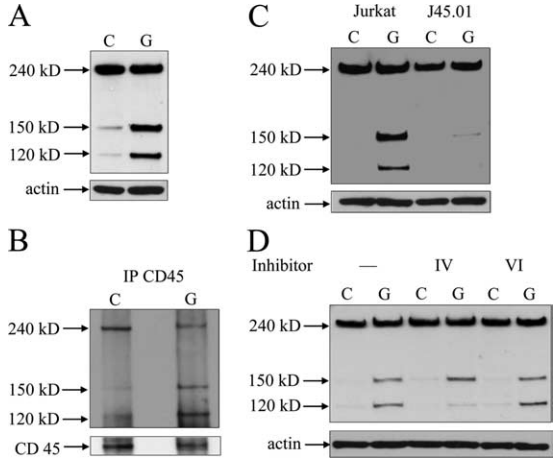

FIGURE 1. Galectin-1 binding to T cells results in fodrin degradation. *A*, Immunoblot of whole cell lysates of Jurkat cells treated with galectin-1 (G) or control buffer (C). Fodrin cleavage products of 150 and 120 kDa are abundant in galectin-1-treated cells compared with control-treated cells. *B*, Fodrin associates with CD45 before and after cleavage. CD45 was immunoprecipitated from control (C)- and galectin-1 (G)-treated Jurkat cells and fodrin was detected by immunoblotting. Full-length fodrin associates with CD45 in control cells, while the 150- and 120-kDa cleavage products associate with CD45 in galectin-1-treated cells. *C*, Lack of fodrin cleavage in cells that do not express CD45. Jurkat and J45.01 cells were treated with buffer control (C) or galectin-1 (G). Immunoblots of whole cell lysates demonstrate that fodrin cleavage products are abundant in galectin-1-treated Jurkat cells, but not in galectin-1-treated J45.01 cells. *D*, Fodrin degradation involves m-calpain. Immunoblots of whole cell lysates of Jurkat cells treated with or without galectin-1 demonstrate that fodrin cleavage was reduced in galectin-1-treated cells in the presence of calpain inhibitor IV that inhibits m-calpain, while calpain inhibitor VI that inhibits μ-calpain had no effect on fodrin cleavage. Data are representative of three or more independent experiments for each panel.

Results

CD45-associated fodrin degradation during galectin-1-induced T cell death

Treatment of Jurkat T cells with galectin-1 resulted in cleavage of fodrin from the 240-kDa intact molecule into 150- and 120-kDa fragments (Fig. 1*A*). These fodrin fragments have been described during apoptosis of several cell types treated with a variety of apoptotic triggers; both the 150- and 120 kDa fragments are derived from the C-terminal portion of fodrin and the 120-kDa fragment is generated by further proteolysis of the 150-kDa fragment (18–25). The 150-kDa fragment, in particular, can be generated by cellular proteases after cell lysis, even in control-treated cells;

[4] Abbreviation used in this paper: RT, room temperature.

therefore, several protease inhibitors are included in lysis buffers (24), although a faint band at 150 kDa can sometimes be detected in control-treated cells. However, robust fodrin cleavage was clearly seen in galectin-1-treated cells, with abundant 150- and 120-kDa fragments generated. Fodrin that was cleaved during galectin-1 death was associated with CD45, as immunoprecipitation of CD45 after galectin-1 treatment demonstrated reduced association of full-length fodrin and the presence of 150- and 120-kDa fodrin fragments (Fig. 1*B*).

CD45 expression appeared to be required for galectin-1- induced fodrin degradation, as J45.01 cells that do not express CD45 demonstrated no fodrin cleavage after galectin-1 treatment (Fig. 1*C*) compared with parental Jurkat cells. The requirement for CD45 for fodrin degradation appears to be specific for galectin-1 cell death, as we observed equivalent fodrin degradation in Jurkat and J45.01 cells after Fas ligation, indicating that CD45 is not required for fodrin cleavage during Fas-mediated T cell death (data not shown).

Several proteases can participate in fodrin degradation in different cell death pathways, including caspases and m- and μ-calpains (21–24). Fodrin degradation during galectin-1 death, especially production of the 120-kDa fragment, was reduced by an m-calpain inhibitor but not by a μ-calpain inhibitor (Fig. 1*D*), while a panel of caspase inhibitors had no effect on fodrin cleavage in galectin-1-treated cells (data not shown). Thus, fodrin cleavage, especially generation of the 120- kDa fragment, occurs at least in part via m-calpain-mediated proteolysis during galectin-1 T cell death, and CD45 appears to be essential for this process.

Fodrin degradation occurs by a separate and parallel pathway from other apoptotic events in galectin-1-treated T cells

As shown in Fig. 1*C*, we observed no fodrin degradation in J45.01 cells. However, although CD45 is a major receptor for galectin-1 on T cells and CD45 can regulate T cell susceptibility to galectin-1, CD45 expression is not absolutely required for galectin-1 T cell death (12, 14). This suggests that fodrin degradation occurs through a separate and parallel pathway from other events in galectin-1 T cell death. This would be in distinct contrast to other cell death pathways in which fodrin degradation is tightly coupled to other events in the pathways (18, 22, 25).

To address whether fodrin degradation occurs via a separate pathway from other events in galectin-1-mediated T cell death, we examined other markers of cell death in Jurkat and J45.01 cells and Jurkat cells treated with calpain inhibitors during galectin-1 cell death. As shown in Fig. 2*A*, phosphatidylserine externalization was virtually identical in Jurkat and J45.01 cells treated with galectin-1, although we observed minimal fodrin cleavage in J45.01 cells (Fig. 1*C*). Similarly, phosphatidylserine externalization was virtually identical in Jurkat cells treated with or without calpain inhibitors (Fig. 2*B*), although m-calpain inhibitor IV reduced fodrin degradation (Fig. 1*D*). Thus, inhibition of fodrin degradation either by absence of CD45 or by m-calpain inhibition did not affect the early event of phosphatidylserine externalization during galectin-1-mediated cell death.

We also examined DNA degradation using the TUNEL assay. We observed no effect of the m-calpain inhibitor on the extent of TUNEL labeling of apoptotic cells (Fig. 2*C*), indicating that DNA degradation proceeded normally despite m-calpain inhibition and reduced fodrin degradation. Thus, fodrin degradation is not required upstream of DNA degradation in galectin-1-mediated T cell death; this is consistent with a prior report that demonstrated equivalent DNA fragmentation during galectin-1-induced cell death in Jurkat and J45.01 cells (14). We also asked whether inhibition of DNA cleavage affected fodrin degradation. Blocking

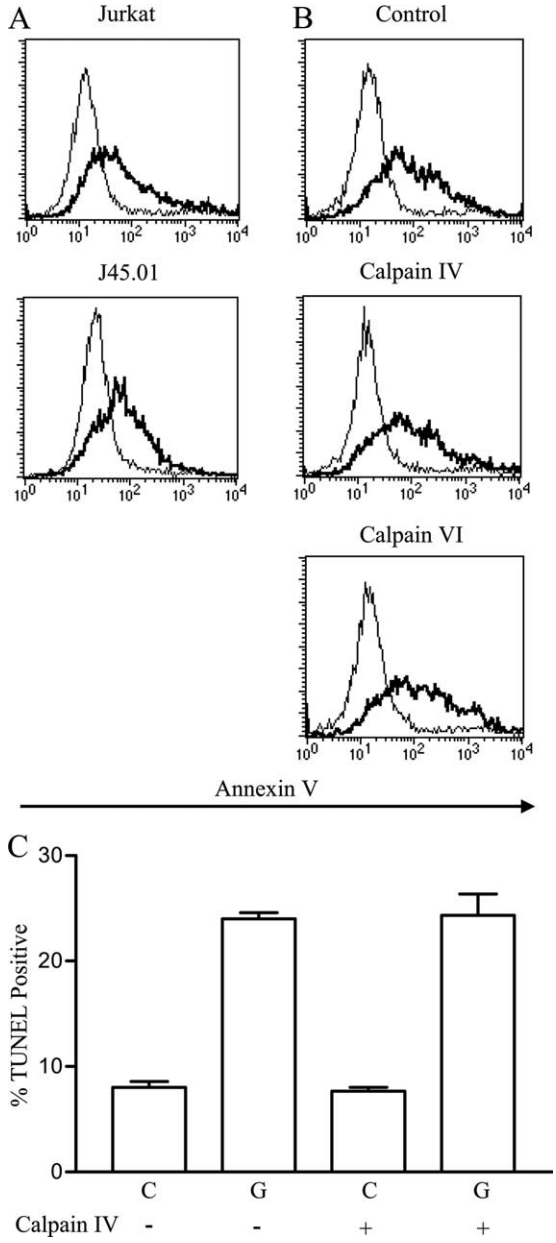

FIGURE 2. Fodrin cleavage is not required for other hallmarks of cell death in galectin-1-treated Jurkat T cells. *A*, Annexin V binding to Jurkat and J45.01 cells treated with buffer control (thin line) or galectin-1 (thick line). *B*, Annexin V binding to Jurkat cells treated with buffer control (thin line) or galectin-1 (thick line) in the absence or presence of the indicated calpain inhibitors. *C*, DNA degradation detected by TUNEL in control- or galectin-1-treated cells in the absence or presence of the indicated calpain inhibitors. Data are representative (*A* and *B*) or mean ± SD of triplicate samples (*C*) from one of three independent experiments.

mitochondrial release of endonuclease G reduced TUNEL labeling, but did not affect fodrin degradation in galectin-1-treated cells (data not shown). Thus, fodrin proteolysis and the events leading to plasma membrane changes and DNA degradation appear to occur via separate pathways during galectin-1 T cell death.

Fodrin degradation is important for CD45 clustering and morphologic changes during galectin-1 T cell death

During galectin-1-induced T cell death, CD45 clusters on membrane blebs and colocalizes with externalized phosphatidylserine on the blebs; this is in contrast to the uniform phosphatidylserine

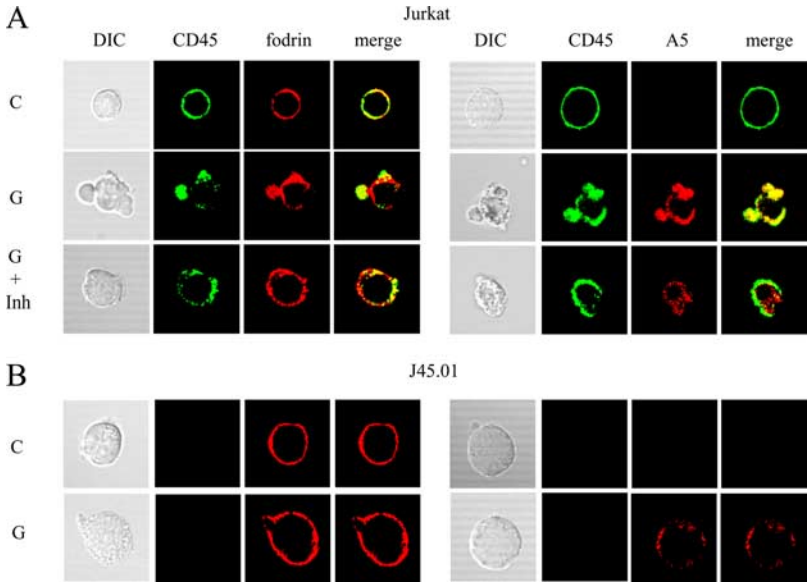

FIGURE 3. Fodrin cleavage promotes CD45 clustering and membrane blebbing during galectin-1 T cell death. *A* (*left*), Jurkat cells were treated with buffer control (C) or galectin-1 (G) for 60 min in the absence or presence of calpain inhibitor IV. CD45 (green) and fodrin (red) were detected by immunofluorescence confocal microscopy. *Right*, CD45 (green) and annexin V (A5, red) binding was detected on cells treated as above. CD45 clustering, fodrin redistribution, and membrane blebbing were reduced in the presence of calpain inhibitor IV compared with cells treated with galectin-1 alone. Annexin V binding was evident on cells treated with calpain inhibitor IV, but clustering on blebs was reduced compared with cells treated with galectin-1 alone. *B*, Jurkat and J45.01 cells were treated with buffer control (C) or galectin-1 (G), and CD45 and fodrin (*left*), or CD45 and annexin V binding (*right*), detected as in *A*. No CD45 staining was detected on J45.01 cells. Fodrin redistribution, membrane blebbing, and annexin V clustering were markedly reduced in J45.01 cells compared with Jurkat cells. Data are representative of three independent experiments.

externalization seen on T cells during Fas-induced cell death (8, 9, 12). To ask whether fodrin degradation is important for membrane blebbing and other morphologic changes that occur during galectin-1 T cell death, we examined cells treated with galectin-1 in the presence or absence of the m-calpain inhibitor IV.

Cells treated with galectin-1 displayed massive membrane blebbing typical of apoptotic cells (Fig. 3*A*). In contrast, cells treated with galectin-1 in the presence of m-calpain inhibitor IV displayed minimal blebbing or other morphologic changes detected by light microscopy. In control- treated cells, CD45 and fodrin were uniformly distributed at the plasma membrane. After galectin-1 treatment, CD45 clustered primarily on apoptotic blebs, as we have previously described (8, 9). Moreover, the continuous ring of fodrin staining seen in control cells was disrupted in galectin-1-treated cells, with pronounced fodrin accumulation near apoptotic blebs. Because the fodrin Ab does not distinguish intact from degraded fodrin by immunofluorescence, it is not clear whether fodrin that associates with CD45 in membrane blebs is intact or degraded; however, the continuous fodrin staining observed in control cells was disrupted after galectin-1 binding. In the presence of m-calpain inhibitor IV, CD45 was present in small patches evenly distributed on the cell surface, in contrast to the large CD45 clusters on membrane blebs on cells treated with galectin-1 alone. Similarly, in the presence of m-calpain inhibitor IV, fodrin colocalized with CD45 on galectin-1-treated cells, but remained relatively evenly distributed around the cell perimeter, in contrast to the pronounced clustering of fodrin at membrane blebs seen in cells treated with galectin-1 alone. Annexin V binding demonstrated that phosphatidylserine externalization occurred on cells in the absence or presence of the m-calpain inhibitor. However, the clustered annexin V staining on apoptotic blebs that was evident on cells treated with galectin-1 alone was not seen when the cells were treated with galectin-1 plus m-calpain inhibitor IV.

Although CD45 is not essential for phosphatidylserine externalization and DNA fragmentation during galectin-1 death (12, 14), we have not previously examined membrane blebbing on $CD45^-$ cells treated with galectin-1. Galectin-1 treatment of J45.01 cells resulted in phosphatidylserine externalization, detected by annexin V binding, but we did not observe the membrane blebs on J45.01 cells that are typically seen on galectin-1-treated $CD45^+$ cells (Fig. 3*B*). Moreover, as seen with m-calpain inhibitor IV (Fig. 3*A*), fodrin remained relatively evenly distributed around the cell perimeter in J45.01 cells after galectin-1 treatment, in contrast to the fodrin clustering seen on Jurkat cells after galectin-1 treatment. Thus, reducing fodrin degradation, either by loss of CD45 expression or by m-calpain inhibition, decreased membrane blebbing during galectin-1 death.

Reconstitution of CD45 expression restores fodrin degradation, CD45 clustering, and membrane blebbing during galectin-1 T cell death

To directly ask whether CD45 is required for fodrin degradation during galectin-1 cell death, we examined fodrin degradation in J45.01 cells reconstituted with full-length CD45 (RABC) or CD45 lacking the D1 tyrosine phosphatase domain ($D1^-$) (28). J45.01 cells transfected with vector alone demonstrated no significant fodrin degradation after galectin-1 treatment, while reconstitution with full-length CD45RABC restored fodrin degradation after galectin-1 treatment (Fig. 4*A*). However, expression of CD45 lacking the D1 domain was not sufficient to restore fodrin cleavage in $D1^-$ cells treated with galectin-1. All four cell lines demonstrated other hallmarks of cell death after galectin-1 treatment, including annexin V binding and increased membrane permeability (data not shown). In addition, fodrin coimmunoprecipitated with both full-length CD45 in the RABC cells and with mutant CD45 in the $D1^-$ cells (data not shown), demonstrating that lack of fodrin degradation in the $D1^-$ cells did not result from loss of fodrin association with CD45.

We next examined morphologic changes in these cells during galectin-1-induced cell death (Fig. 4*B*). J45.01 cells transfected with vector alone did not display the massive blebbing seen with Jurkat cells, nor did fodrin cluster in these cells, after galectin-1 treatment. In contrast, cells reconstituted with full-length CD45RABC demonstrated membrane blebbing and colocalization of clustered CD45 with fodrin after galectin-1 treatment. However, cells transfected with the $D1^-$ CD45 construct had no apparent membrane blebbing after galectin-1 treatment, and cells had patchy but continuous CD45 and fodrin localization around the plasma membrane. These results suggested that the phosphatase domain of CD45 is important for fodrin cleavage.

To ask whether inhibiting tyrosine phosphatase activity affected fodrin degradation, we treated cells with the tyrosine phosphatase inhibitor bpV(phen) and examined fodrin degradation after galectin-1 binding. As previously described (12), bpV(phen) did not

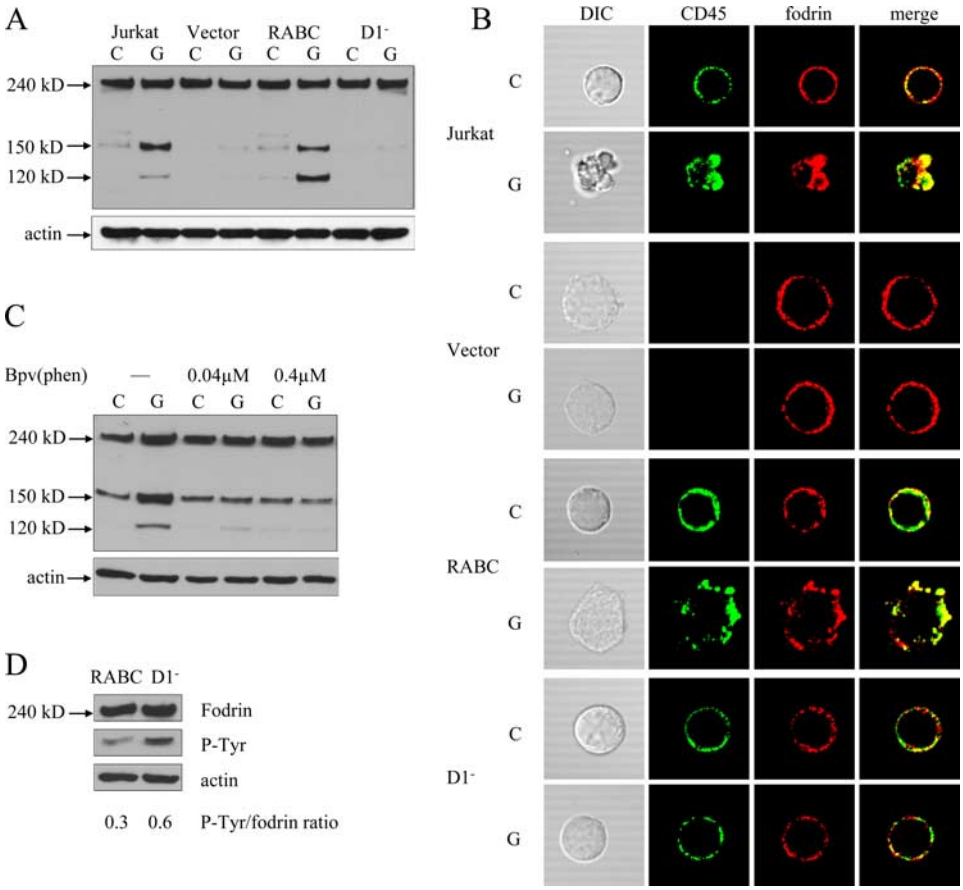

FIGURE 4. Expression of CD45 is sufficient to restore fodrin cleavage after galectin-1 binding. J45.01 T cells were transfected with vector alone, full-length murine CD45 (RABC), or CD45 lacking the intracellular D1 phosphatase domain (D1$^-$). *A*, Immunoblots of whole cell lysates were probed to detect fodrin and fodrin cleavage products. Fodrin cleavage was not detected in J45.01 cells transfected with vector alone. Expression of CD45RABC restored fodrin cleavage after galectin-1 binding, while no fodrin cleavage was detected in D1$^-$ cells. *B*, CD45 (green) and fodrin (red) localization was examined by immunofluorescence confocal microscopy. Cells expressing CD45RABC demonstrated membrane blebbing, CD45 clustering, and fodrin redistribution after galectin-1 treatment, comparable to that observed for Jurkat cells, while these changes were not seen in D1$^-$ cells. *C*, Tyrosine phosphatase inhibition reduced fodrin degradation. Jurkat cells were treated with indicated concentrations of the tyrosine phosphatase inhibitor bpV(phen) before galectin-1 treatment, and immunoblots of whole cell lysates were probed to detect fodrin and fodrin cleavage products. Phosphatase inhibitor treatment markedly reduced fodrin degradation. *D*, Reduced fodrin phosphorylation in cells with intact CD45 phosphatase domain. Fodrin was precipitated from RABC and D1$^-$ cells and immunoblots were probed for phosphotyrosine and fodrin. The ratio of phosphorylated fodrin:total fodrin in D1$^-$ cells was increased compared with RABC cells. Data are representative of three independent experiments.

inhibit galectin-1 death of the cells, as detected by annexin V binding and membrane permeability (data not shown). However, inhibition of tyrosine phosphatase activity markedly reduced fodrin degradation after galectin-1 treatment, indicating that tyrosine dephosphorylation is important for fodrin cleavage (Fig. 4*C*). This is consistent with previous reports documenting that phosphorylation of a specific tyrosine residue in fodrin confers resistance to calpain-mediated cleavage (23, 24).

A specific phosphatase responsible for fodrin dephosphorylation in mammalian cells has not been previously identified; however, fodrin binding to the D2 intracellular domain of CD45 promotes CD45 PTPase activity (15, 28). To ask whether loss of the D1 tyrosine phosphatase domain of CD45 affected fodrin phosphorylation, we determined baseline tyrosine phosphorylation status of fodrin in J45.01 cells expressing either full-length RABC or the D1$^-$ CD45 construct. Tyrosine phosphorylation of fodrin was increased ~2-fold in cells expressing the D1$^-$ CD45 construct compared with cells expressing CD45RABC, as shown by examining the ratio of phosphorylated intact fodrin to total intact fodrin in these cells (Fig. 4*D*). These data imply that the CD45 tyrosine phosphatase domain modulates fodrin phosphorylation status, which may regulate fodrin susceptibility to proteolytic cleavage during galectin-1 cell death.

Enhanced phagocytosis of apoptotic T cells with degraded fodrin

Because fodrin degradation was not essential for other events in galectin-1-induced T cell death, such as phosphatidylserine externalization and DNA degradation, we asked whether fodrin degradation would enhance phagocyte clearance of dying cells. Clearance of apoptotic cells by phagocytes is important for normal tissue homeostasis, for prevention of inflammation resulting from release of cellular contents, and for removal of intracellular Ags that could be recognized as non-self and thus trigger autoimmunity (1–4, 29).

We compared phagocytosis of control or galectin-1-treated Jurkat and J45.01 cells by RAW 264.7 macrophages. T cells were labeled with CFSE to visualize engulfed T cells within macrophages. CFSE-labeled T cells were treated with galectin-1 for the indicated period of time and added to a monolayer of RAW 264.7 cells for 1 h before microscopic examination. After 1 h, there was minimal blebbing and disintegration of either Jurkat or J45.01 cells

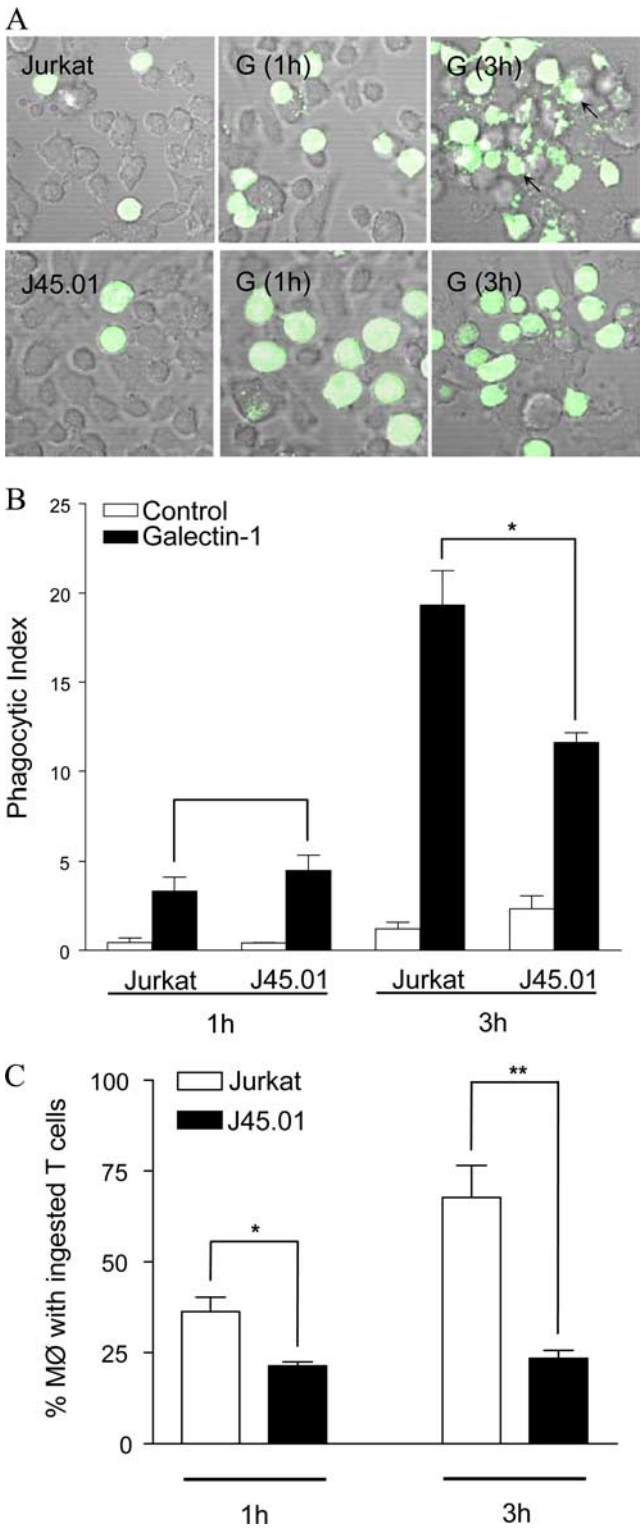

FIGURE 5. Fodrin cleavage promotes phagocytosis of dying cells. *A*, Jurkat and J45.01 cells were labeled with CFSE before addition of buffer control or galectin-1 for 6 h. Galectin-1- or control-treated T cells were added to RAW 264.7 macrophage monolayers for 1 or 3 h. Phagocytosis of labeled T cells was visualized by confocal microscopy. *B*, Phagocytic index of macrophages with Jurkat or J45.01 cells. At 1 h, $p = 0.39$ for Jurkat vs J45.01 cells treated with galectin-1. *, at 3 h, $p < 0.02$ for Jurkat vs J45.01 cells treated with galectin-1. *C*, The fraction of macrophages with ingested cells. *, at 1 h, $p = 0.02$ for Jurkat vs J45.01 cells treated with galectin-1. **, at 3 h, $p < 0.01$ for Jurkat vs J45.01 cells treated with galectin-1. *B* and *C*, Values are mean ± SD of triplicate samples from one of three independent experiments.

and similar numbers of Jurkat and J45.01 cells were observed on the RAW 264.7 monolayer. However, after 3 h, there was pronounced fragmentation of galectin-1-treated Jurkat cells and numerous RAW 264.7 cells displayed internalized T cell fragments. In contrast, J45.01 cells remained generally intact 3 h after galectin-1 treatment, with minimal blebbing and cell fragmentation, as in Figs. 3 and 4. Although T cells engulfed by macrophages were visible, the RAW 264.7 cells contained fewer fragments of J45.01 cells compared with Jurkat cells.

Phagocytosis of control-treated or galectin-1-treated cells was quantified in two ways. We calculated the phagocytic index (defined in *Materials and Methods*) (26, 27) (Fig. 5*B*), as well as the fraction of macrophages containing engulfed T cells or T cell fragments (Fig. 5*C*). When cells treated with galectin-1 for only 1 h were added to RAW 264.7 cells, there was a significant increase in the phagocytic index for both Jurkat and J45.01 cells compared with control -treated cells, but no difference in phagocytosis of the two T cell lines compared with each other. However, after 3 h of galectin-1 treatment, we observed a significant reduction in the phagocytic index for J45.01 cells compared with Jurkat cells (Fig. 5*B*), consistent with the reduced fragmentation of J45.01 cells compared with Jurkat cells by this time point (Fig. 5*A*). In addition, the fraction of macrophages ingesting Jurkat cells treated with galectin-1 for 3 h was greater than that observed for Jurkat cells treated for 1 h. In contrast, there were fewer macrophages containing J45.01 cells after 1 or 3 h of galectin-1 treatment, compared with Jurkat cells, and increased time of galectin-1 treatment did not appreciably increase the fraction of macrophages with ingested J45.01 cells (Fig. 5*C*). Thus, fodrin cleavage enhanced the efficiency of macrophage phagocytosis of $CD45^+$ dying cells compared with $CD45^-$ dying cells in which fodrin proteolysis was prevented.

Discussion

The current study demonstrates that fodrin degradation occurs in a CD45-mediated process during galectin-1 T cell death (Fig. 1). Although fodrin degradation occurs in many cell types in many death pathways, proteolytic degradation of fodrin has previously been described as an integral part of a general cell death pathway. In contrast, during galectin-1-mediated T cell death, fodrin degradation occurs via a separate and parallel pathway from the pathways that lead to phosphatidylserine externalization and DNA degradation (Fig. 2). No specific cell surface receptor required for fodrin degradation during apoptosis has been previously proposed, and no role for CD45 in cytoskeletal disassembly during apoptosis has been previously described. Although fodrin is essential for proper delivery and localization of CD45 on the lymphocyte cell surface (17), a role for CD45 in fodrin degradation has not been demonstrated. CD45 is a glycoprotein receptor for galectin-1 on T cells, but we and others have previously found that CD45 expression is not essential for galectin-1-mediated T cell death (9, 12–14). Thus, this work demonstrates a novel and unique role for CD45 in galectin-1-mediated T cell death.

The present study also demonstrates that macrophages more efficiently phagocytose dying T cells with cleaved fodrin, compared with dying cells in which fodrin proteolysis is prevented (Fig. 5). Although fodrin proteolysis accompanied membrane blebbing in $CD45^+$ galectin-1-treated cells (Figs. 3 and 4), membrane blebbing alone may not be sufficient to promote efficient phagocytosis, as inhibition of membrane blebbing during Jurkat cell apoptosis by blocking the Rho kinase ROCK-I did not alter the rate of macrophage ingestion of the cells (30). This suggests that it is cytoskeletal disassembly, rather than blebbing, that contributes to efficient phagocytic clearance of dying cells. Thus, these experiments

identify a novel function for CD45 during galectin-1 T cell death in promoting fodrin degradation to enhance phagocytic clearance of dying T cells.

Alterations in the intracellular and extracellular domains of CD45 are associated with autoimmune disease in humans and mice (31, 32). The requirement for the CD45 D1 tyrosine phosphatase domain for efficient fodrin cleavage (Fig. 4) suggests that mutations in any CD45 domains that affect fodrin cleavage could affect the rate of phagocytic clearance of cells killed by galectin-1 in vivo and could thus contribute to the increased frequency of autoimmunity associated with specific CD45 mutations.

These data describe a novel role for CD45 in regulating fodrin degradation during galectin-1 death. Conversely, is there a role for fodrin degradation in regulating CD45 function? Clustering of CD45 by galectin-1 or spontaneous oligomerization of CD45 have both been shown to reduce CD45 tyrosine phosphatase activity, although the precise mechanism by which this occurs has not been elucidated (32, 33). Association of the CD45 D2 domain with fodrin enhances tyrosine phosphatase activity of the D1 domain, perhaps by keeping the CD45 cytoplasmic tail in an extended conformation (15, 26). It will be of interest to determine whether fodrin degradation contributes to reduced CD45 tyrosine phosphatase activity after galectin-1 binding.

Galectin-mediated clustering of cell surface glycoproteins is proposed to regulate a variety of events on the cell surface, including segregation of the TCR from CD8 to induce anergy in tumor-infiltrating CD8 T cells, restricting TCR signaling during thymocyte selection and peripheral T cell response to Ag, determination of cytokine receptor residency time on mammary tumor cells and glucose transporter residency time on pancreatic β cells, polarized sorting of glycoproteins and retention of cell surface sodium channels on renal epithelial cells, and glycoprotein association with lipid rafts on intestinal epithelial cells (7, 34–40). It is possible that these events are regulated solely by galectin binding to glycans on the extracellular domains of membrane glycoproteins. However, none of these studies asked whether changes in cytoskeletal tethering via glycoprotein intracellular domains were required for galectin-mediated glycoprotein clustering on the cell surface. Our data demonstrate that the dramatic clustering of CD45 observed after galectin-1 binding involves fodrin degradation and thus release of the CD45 intracellular domain from cytoskeletal tethering.

These studies demonstrate a novel role for CD45 in cytoskeletal disassembly during a specific cell death pathway, describe a mode of cytoskeletal disassembly that is separable from other cellular events in this death pathway, and support a role for CD45-mediated cytoskeletal disassembly in efficient phagocytic clearance of apoptotic cells. Understanding the processes that regulate apoptotic cell clearance may promote new approaches to halting initiation or progression of autoimmune disease, while identification of novel activities for CD45 will further define the role of this abundant and complex glycoprotein during lymphocyte development and function in the periphery.

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Disclosures

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