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Annexin I Is an Endogenous Ligand that Mediates Apoptotic Cell Engulfment

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Summary

Engulfment of apoptotic cells requires presentation of new cell surface ligands by the dying cells. Using a differential proteomics technology, we identify that annexin I is a caspase-dependent engulfment ligand; it is recruited from the cytosol and exported to the outer plasma membrane leaflet, colocalizes with phosphatidylserine, and is required for efficient clearance of apoptotic cells. Furthermore, phosphatidylserine receptor (PSR) clustering around apoptotic cells indicates a requirement for annexin I. In the nematode *Caenorhabditis elegans*, downregulation of the annexin homolog prevents efficient engulfment of pharyngeal cell corpses. These results provide novel mechanistic insights into how apoptotic cells are removed and may explain a pathogenic mechanism of chronic inflammatory diseases where annexin I autoantibodies have been described.

Introduction

Apoptosis is a physiological cell death mechanism that is essential for development and tissue homeostasis in multicellular organisms. Deregulation of apoptosis is the cause for multiple pathological conditions where excessive proliferation, degeneration, or inflammation has been documented (Fadok et al., 2001; Henson et al., 2001). Apoptotic cells are rapidly and selectively recognized and engulfed by the phagocytes or neighboring cells, even before overt morphological features are exhibited (Kerr et al., 1972; Sulston and Horvitz, 1977). This recognition process is theoretically the sum of repulsive and adhesive forces and thought to be initiated by the cell surface presentation of specific engulfment ligands, also termed "eat me" signals, by the dying cells (Fadok et al., 2001; Henson et al., 2001; Brown et al., 2002; Caron and Hall, 1998). Phagocytes recognize apoptotic cells by a repertoire of cell surface receptors that directly bind to engulfment ligands exposed on the surface of dying cells (Savill and Fadok, 2000; Henson et al., 2001). Although molecular identities of engulfment ligands are largely unknown, the most well-studied marker of apoptosis is the exposure of phosphatidylserine (PS) to the outer leaflet of the plasma membrane (Fadok et al., 1992; Bratton et al., 1997). Several classes of receptors from engulfing cells have been implicated in the recognition of apoptotic cells, possibly binding directly to the exposed PS or indirectly via bridging molecules. These receptors include the phosphatidylserine receptor (PSR), *C. elegans* CED-1, the tyrosine kinase receptor MER, *Drosophila* Croquemort, several classes of scavenger receptors, integrins, complement receptors, and CD14 (reviewed by Platt et al., 1998; Adachi et al., 1997; Franc et al., 1999; Fadok et al., 2000; Zhou et al., 2001). Blocking each of these receptors alone caused partial inhibition of apoptotic cell engulfment, suggesting redundancy in the engulfment pathways (Platt et al., 1998). Although the diversity of these receptors suggests the complexity of ligands, identities of endogenous engulfment ligands that are necessary for apoptotic cell clearance are not known. Specifically, a major challenge in the field is the lack of knowledge on endogenous protein ligands that possess three essential features: exposure mediated by the direct consequence of intracellular caspase activation, specific interaction with the engulfment receptors, and a general requirement in engulfment of apoptotic cells across phylogeny. This lack of knowledge is due, in part, to the difficulty in profiling cell surface-bound membrane proteins. To identify and functionally characterize engulfment protein ligands, we have developed and utilized a recently described proteomics technology (Gygi et al., 1999; Han et al., 2001). This technology, termed *3D* chromatography-*i*sotope-*c*oded *a*ffinity *t*ags (3D-ICAT), and mass spectrometry, allows identification and simultaneous quantification of complex membrane proteins (Han et al., 2001). Thus, we triggered Jurkat T lymphocytes with anti-Fas IgM and differentially profiled membrane proteins that were upregulated in apoptotic cells.

Results

Proteomics Profiling of Apoptotic Membrane Proteins

Membrane proteins from naive and apoptotic (3 hr anti-Fas IgM-treated) Jurkat T lymphocytes were labeled with light and heavy ICAT reagents (Figure 1A), and protein identities and quantitative changes were determined (Figure 1B). Among the differentially regulated peptide pairs, we have focused on a doubly charged peptide pair, with *m/z*s of 872.0 and 876.0, which showed high levels of upregulation by anti-Fas IgM treatment (7.9-fold) (Figure 1C). MS/MS analysis of the 876.0 peak and database analysis unequivocally identified residues 269–280 of human annexin I, a known calcium-dependent PS binding protein (Figure 1D). Two additional peptides from annexin I were also identified and quantified with similar upregulation in the anti-Fas IgM-triggered membrane fractions. The rapid upregulation of annexin I in the early phases of apoptosis as well as

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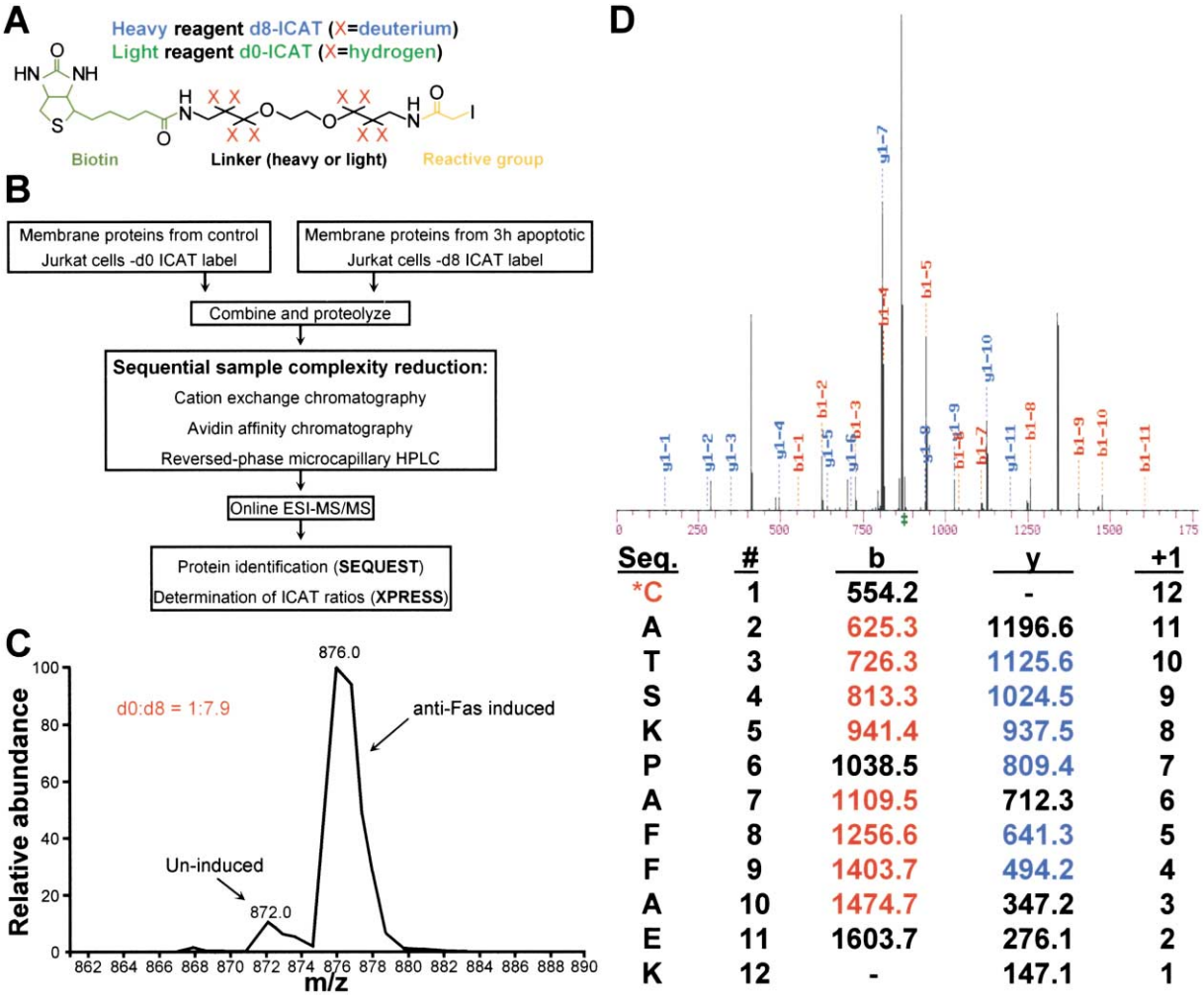

Figure 1. Identification of Annexin I as a Candidate Engulfment Ligand

(A) Structure of the *i*sotope-coded *a*ffinity *t*ags (ICAT).

(B) Strategy for identification and simultaneous quantification of protein ligands that are upregulated in apoptotic cells. The software tools utilized to identify (SEQUEST) and quantify (XPRESS) during the analysis are indicated.

(C) Detection of a doubly charged, ICAT-labeled peptide pair showing upregulation during apoptosis.

(D) MS/MS analysis of the 876.0 isotope peak and peptide identification. The tandem mass spectrum derived by collision-induced dissociation and identified amino acid sequences from the human annexin I protein is shown. Theoretically predicted (black) and experimentally identified (red or blue) N- and C-terminal fragment ions are shown (b and y ions, respectively). An asterisk on the cysteine residue represents the heavy-isotope modification.

its known biochemical activity in membrane aggregation and PS binding prompted us to further characterize the recruitment of this protein.

Recruitment and Export of Annexin I to the Apoptotic Cell Surface

We next examined the localization and export of annexin I. Immunofluorescence analysis of unpermeablized naive cells revealed that annexin I was not detectable on the cell surface (Figure 2A). However, in unpermeablized apoptotic Jurkat cells, the gradual increase in exposure of annexin I as discrete patches on the apoptotic cell surface was seen (Figures 2B–2D). Furthermore, in permeablized Jurkat cells, anti-Fas IgM treatment revealed the sequestration of cytosolic annexin I to the PS-rich, inner leaflet of the plasma membrane by 3 hr (Figures 2E–2H). Similarly, annexin I externalization into discrete membrane patches was also observed in human primary vascular smooth muscle cells after anti-Fas IgM-mediated apoptosis, indicating that this is a general mechanism for apoptotic cells (Figures 2I–2K). The appearance of annexin I patches, especially in human vascular smooth muscle cells as distinct ring-like structures that were distributed as concentrated foci throughout the cell surface, was seen by 3 hr and similarly increased by 6 hr after the death trigger (Figures 2I–2K). Consistent with these results, cell fractionation and Western analyses of Jurkat T cells revealed that annexin I was recruited from the cytosol to the membrane fractions by anti-Fas IgM treatment (Figure 2L). Moreover, the recruitment was specific among the expressed annexins, as only the significant upregulation of annexin I was seen in the

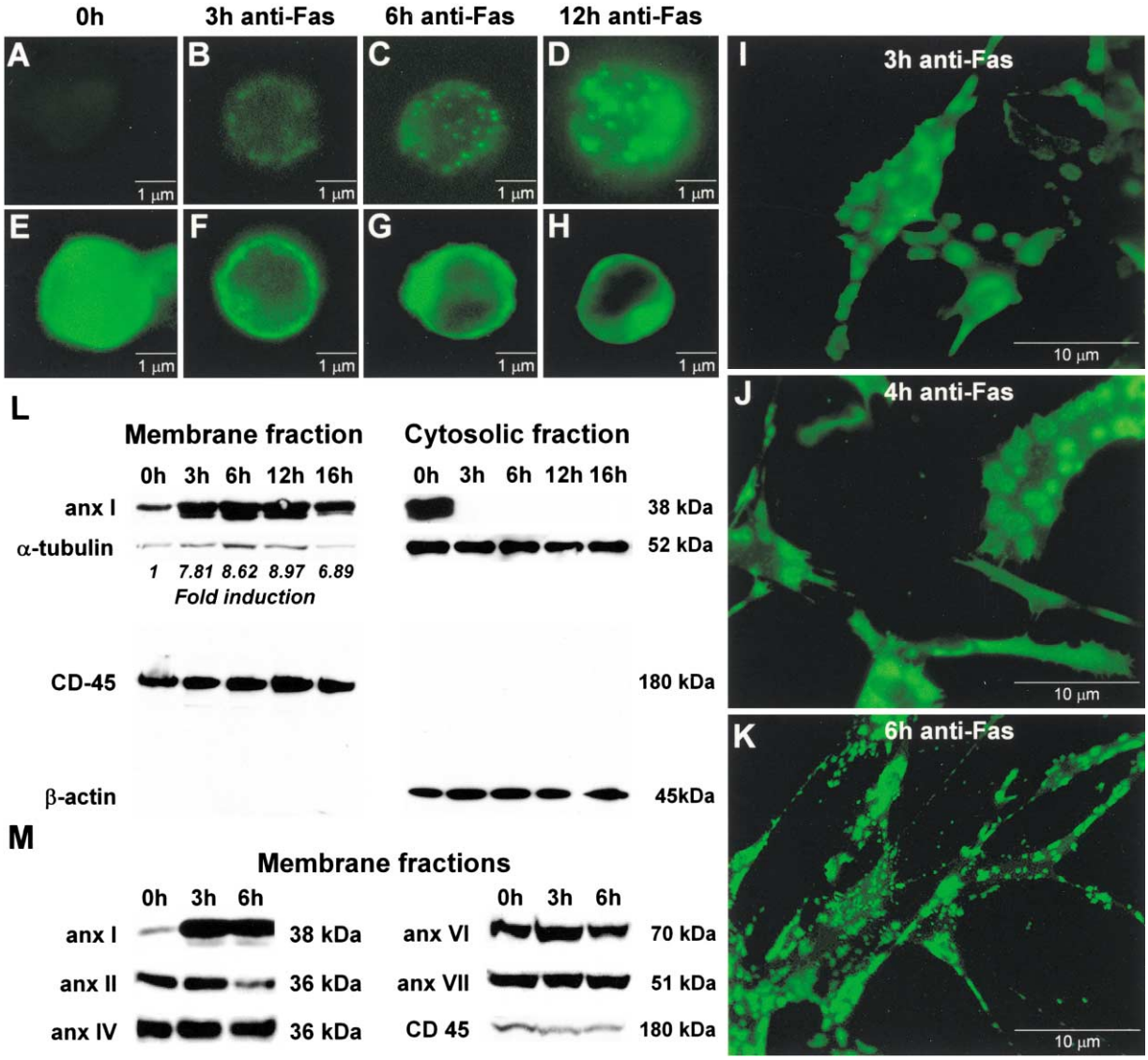

Figure 2. Annexin I is Specifically Recruited to the Cell Surface during Apoptosis

(A–D) Immunofluorescence detection of annexin I in unpermeablized Jurkat cells after the anti-Fas IgM treatment. Tangential images were taken with identical excitation and detection conditions to demonstrate relative levels of annexin I on the cell surface.

(E–H) Time-dependent recruitment of annexin I in permeablized Jurkat T cells by immunofluorescence detection of equatorial sections. Induction of apoptosis by anti-Fas IgM results in the recruitment of annexin I from the cytosol to the plasma membrane.

(I–K) Cell surface exposure of annexin I in unpermeablized human vascular smooth muscle cells after the anti-Fas IgM-mediated induction of apoptosis.

(L) Recruitment of annexin I from the cytosol to the membrane fractions by cell fractionation of human Jurkat T cells during apoptosis and detection by Western blotting are shown. CD45 and β-actin Western analyses demonstrate the efficiency of cell fractionation.

(M) Western analyses of annexin family of proteins in the membrane fractions during apoptosis are shown.

membrane fractions after the death trigger (Figure 2M). These results confirmed that annexin I is specifically recruited from the cytosol and exported to the cell surface as discrete patches during anti-Fas IgM-mediated apoptosis and further suggest that specific export of annexin I may be a signal for the engulfing cells.

The Requirement for Caspase Activity and Calcium Mobilization for the Recruitment of Annexin I to the PS Patches on the Cell Surface

The mechanism of annexin I recruitment was further investigated. We tested whether recruitment of annexin I during apoptosis required intracellular caspase activity and mobilization of calcium (reviewed by Hawkins et al., 2000). As shown in Supplemental Figures S1A and S1B at http://www.developmentalcell.com/cgi/content/full/4/4/587/DC1, anti-Fas IgM-induced apoptosis and PS externalization in Jurkat T cells were effectively blocked by preincubating Jurkat cells with a broad-spectrum caspase inhibitor, Z-VAD-FMK. We next tested the release of intracellular calcium by culturing Jurkat T cells in the calcium-free media with or without the addition of anti-Fas IgM and measured the release of calcium by Fluro-3 fluorescence (Scoltock et al., 2000). As shown in

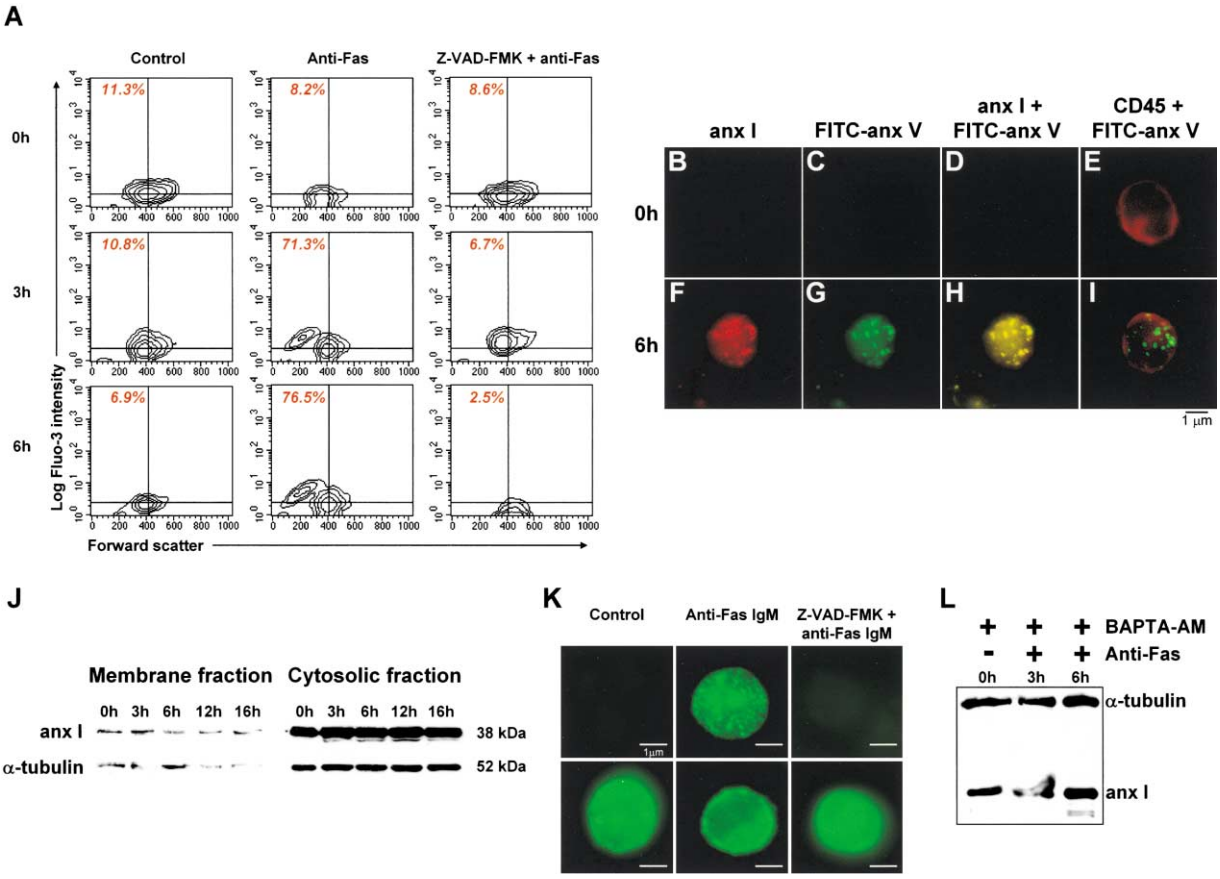

Figure 3. Requirement for Caspase Activity and Calcium for the Recruitment and Export of Annexin I

(A) Inhibition of intracellular calcium release during anti-Fas-mediated apoptosis by Z-VAD-FMK. Jurkat cells were treated with anti-Fas antibody for 3 hr and 6 hr in calcium-free medium with or without the preincubation with Z-VAD-FMK. The contour plots show the calcium levels along the *y* axis (Fluo-3 fluorescence intensities) and cell size (forward scatter) along the *x* axis.

(B–I) Specific colocalization of annexin I (red, monoclonal anti-annexin I staining) with PS (green, FITC-annexin V staining) in unpermeablized, apoptotic Jurkat cells. Immunofluorescence colocalization of PS with annexin I (F, G, and H), but not with CD45 receptor (E and I), at 6 hr after anti-Fas IgM treatment is shown.

(J and K) Inhibition of annexin I recruitment and export by pretreatment with Z-VAD-FMK.

(J) Jurkat cells were treated with anti-Fas IgM for the indicated time points, membrane and cytosolic fractions were isolated, and the relative distribution of annexin I was analyzed by Western blotting.

(K) Immunofluorescence analysis in nonpermeablized (top panel) and permeablized (bottom panel) cells is shown.

(L) Buffering the intracellular calcium with BAPTA-AM effectively blocks translocation of annexin 1 from the cytosol. Western analysis of cytosolic fractions from control cells or anti-Fas IgM-treated cells in the presence of BAPTA-AM is shown. Note the failure of annexin I depletion from the cytosolic fractions. A control antibody against α-tubulin was used to normalize for equal protein loading.

Figure 3A, anti-Fas IgM-mediated release of intracellular calcium was observed by 3 hr, and this release was also blocked by pretreatment of the Jurkat T cells with Z-VAD-FMK. Since the gradual increase in the PS exposure coincided with the annexin I exposure on the apoptotic cell surface, (Figure 2A; see Supplemental Figure S1A), colocalization of externalized PS and annexin I during apoptosis was analyzed in Jurkat cells and in human vascular smooth muscle cells with FITC-annexin V (Fadok et al., 1992). Immunofluorescence analysis demonstrated specific colocalization of PS and annexin I as discrete patches on the cell surface of unpermeablized, apoptotic Jurkat cells and primary human vascular smooth muscle cells (Figures 3B–3I; see Supplemental Figure S2 at http://www.developmentalcell.com/cgi/content/full/4/4/587/DC1). The requirement for caspase activity for the recruitment of annexin I was further analyzed. As shown in Figures 3J and 3K, pretreatment of Jurkat cells with Z-VAD-FMK prevented the recruitment of annexin I from the cytosol to the membrane fractions and subsequent export to the cell surface. Furthermore, buffering the intracellular calcium with BAPTA-AM alone effectively blocked the translocation of annexin I from the cytosol to the plasma membrane (Figure 3L). These results demonstrate that caspase activity and intracellular calcium release were essential for the recruitment and export of cytosolic annexin I to the PS-rich cell surface.

Cell Surface-Exposed Annexin I Is Necessary for Efficient Engulfment of Apoptotic Cells

We next tested whether annexin I is functionally required for the engulfment of apoptotic cells. Apoptotic cell engulfment is a well coordinated process that involves at least five defined events: (1) tethering of apoptotic cells

to engulfing cells, (2) engulfing cell pseudopodia formation, (3) membrane zippering between apoptotic cells and engulfing cells, (4) pseudopodia tip fusion and internalization of apoptotic cells, and (5) digestion of apoptotic cells (Fadok et al., 2001; Henson et al., 2001). To distinguish defined events using an in vitro engulfment system, we carefully selected and improved a previously described in vitro engulfment system where apoptotic cells were shown to be efficiently engulfed by endothelial cells (Oka et al., 1998; see Supplemental Figure S3 at http://www.developmentalcell.com/cgi/content/full/4/4/587/DC1; Experimental Procedures). First, using control cells, we performed series of single or double fluorescence-labeling experiments (engulfing cells, red fluorescence; apoptotic Jurkat T cells, green fluorescence) to establish the time required for maximal tethering and internalization (see Supplemental Figure S3; Experimental Procedures). Second, to test the functional role of annexin I, we established two Jurkat cell lines where annexin I protein is downregulated by targeting its mRNA with a small interfering RNA (siRNA) (Elbashir et al., 2001; Harborth et al., 2001). As shown in Figures 4A–4D, *annexin I* siRNAs, but not one-base mutant siRNA, resulted in the effective downregulation of annexin I protein, while apoptosis and PS exposure in annexin I-downregulated cells remained unchanged (see Supplemental Figure S4 at http://www.developmentalcell.com/cgi/content/full/4/4/587/DC1). We next tested the possible tethering defects in annexin I-downregulated Jurkat T cells. As shown in Figure 4E, dose-dependent reduction of tethering was observed in annexin I-downregulated cells. Similarly, dose-dependent disruption of tethering was also observed with two different antibodies against human annexin I (Figures 4F and 4G), while no significant effect was seen with antibodies against other expressed annexins (Figure 4H). We tested the morphologically distinguishable defects during the engulfment of annexin I-silenced apoptotic cells. Quantitative Nomarski microscopy experiments revealed that, even after a prolonged attachment to engulfing cells, among the fewer cells that are tethered, internalization was quantitatively reduced in annexin I-silenced apoptotic cells (Figures 4I and 4J; see Supplemental Table S1 at http://www.developmentalcell.com/cgi/content/full/4/4/587/DC1).

To examine the absolute requirement for annexin I in apoptotic cell engulfment, we purified and characterized endogenous annexin I from apoptotic Jurkat cell membranes (Figures 5A and 5B) and tested whether the reconstituted annexin I could effectively rescue the tethering defects seen in two cell lines lacking annexin I. In this experiment, purified annexin I bound to PS-rich membranes from the wild-type apoptotic Jurkat cells were first eluted and reconstituted in the media during the tethering assay. We presumed that annexin I would efficiently bind to cell surface-exposed PS of annexin I-silenced apoptotic cells and, thus, likely restore the engulfment defects exhibited in these cells. As shown in Figure 5C, soluble annexin I significantly enhanced tethering of annexin I-silenced apoptotic cells to ~90% of the level seen for the wild-type apoptotic cells. These results provide compelling evidence that the presence of annexin I on the outer plasma membrane leaflet is required for efficient tethering and internalization of apoptotic cells.

Involvement of PSR in the Annexin I-Mediated Engulfment Pathway

The colocalization of annexin I and PS on the outer plasma membrane leaflet of apoptotic cells suggests that PS recognition receptors may recognize annexin I and mediate engulfment of apoptotic cells. One of these receptors, termed the phosphatidylserine receptor (PSR), has been implicated in recognition of externalized PS present in apoptotic cells (Fadok et al., 2000). It is known that functional recognition of apoptotic cells by an engulfment receptor typically involves clustering of engulfment receptors around apoptotic cell corpses, elegantly demonstrated in *C. elegans* with an engulfment receptor, CED-1 (Zhou et al., 2001). Thus, we examined possible clustering of PSR around apoptotic Jurkat T cells. We first expressed PSR in 293T cells, cocultured with untreated or apoptotic Jurkat T cells, and examined the PSR clustering by immunofluorescence microscopy. As demonstrated by the images and quantification shown in Figure 6A, PSR clustering as complete rings around apoptotic Jurkat T cells was seen (top two panels). Interestingly, PSR clustering was diminished or not detectable around annexin I-silenced apoptotic cells or around viable Jurkat cells, even though they are attached to the PSR-expressing 293T cells (Figure 6A). Time course analysis revealed reduced clustering of PSR around annexin I-silenced apoptotic cells (Figure 6A; see Supplemental Table S2 at http://www.developmentalcell.com/cgi/content/full/4/4/587/DC1). These results suggest the functional involvement of PSR in annexin I-mediated apoptotic cell recognition and engulfment.

We next examined whether annexin I and PSR participate in the same engulfment pathway. Toward this goal, we tested the extent of tethering defects mediated by single and double silencing of annexin I and PSR. First, we utilized two different siRNAs targeting the PSR mRNA and derived two endothelial cell strains where PSR protein is efficiently downregulated (Figure 6B). The wild-type endothelial cells or PSR downregulated endothelial cells were then incubated with wild-type or annexin I-silenced apoptotic Jurkat cells, and the extent of tethering was analyzed. As shown in Figure 6C, comparable reduction of tethering was seen in single silencing experiments where PSR in engulfing cells or annexin I in apoptotic Jurkat were tested. Interestingly, double silencing did not cause further reduction in tethering, suggesting that PSR and annexin I may function in the same engulfment pathway (Figure 6C). To confirm the above observations, we tested the effects of neutralizing antibodies against annexin I and PSR. Consistent with the siRNA-silencing effects, the use of a single antibody against either annexin I or PSR resulted in a similar reduction in tethering, and no further reduction was seen by the addition of double antibodies (Figure 6D). These results, together with observed interaction of annexin I with PS, implicate the functional role of PSR and annexin I in the same engulfment pathway.

Downregulation of *C. elegans* Annexin nex-1 Prevents Efficient Engulfment of Cell Corpses

We extended our study in the nematode *C. elegans*, where timing and position of engulfed cell corpses, some of the genes required for apoptotic cell en-

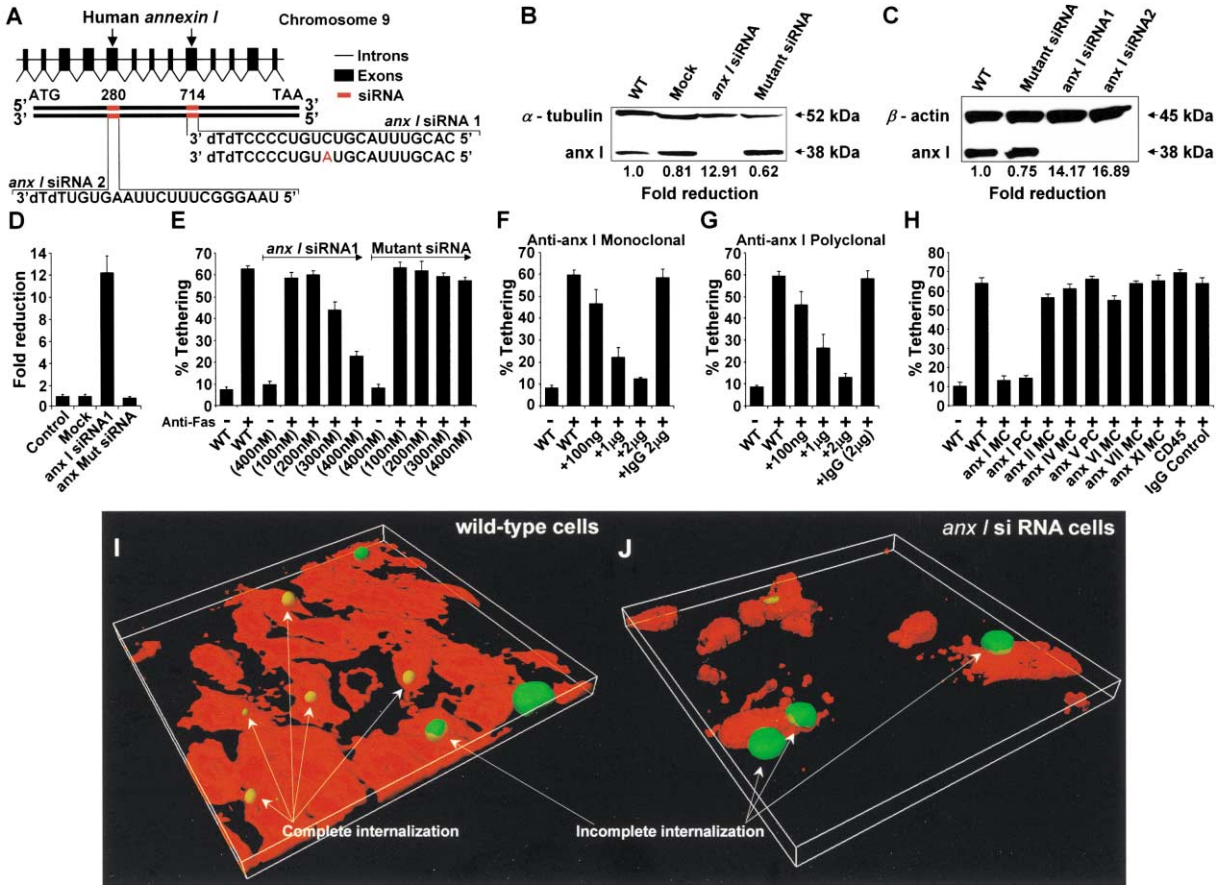

Figure 4. Annexin I Is Required for Efficient Engulfment of Apoptotic Cells

(A) Genomic structure of the human annexin I, two antisense siRNA sequences targeting the wild-type annexin I, and the one-base mutant (C to A) are shown. The siRNA-targeted exons are indicated by arrows.

(B–C) Specific downregulation of annexin I by siRNA. Western analysis of total cell lysates from Jurkat cells with the indicated treatment is shown. Effective downregulation of annexin I protein is observed by the use of two specific siRNAs (400 nM). α-tubulin and β-actin levels were used to normalize the fold downregulation.

(D) Quantitative analysis of annexin I protein downregulation by *anx-1* siRNA 1. Fold reduction was calculated from four independent experiments with a single concentration of siRNA (400 nM).

(E) Dose-dependent tethering defects in annexin I-downregulated, apoptotic cells. Results shown are mean percentages ±SD; n = 3. Tethering was determined as described in the Experimental Procedures.

(F–H) The specific inhibition of tethering by an anti-annexin I monoclonal antibody (F) and a polyclonal antibody (G), but not by antibodies against other expressed annexins (H), is shown.

(I and J) Defective engulfment of annexin I-silenced cells by endothelial cells. Wild-type and annexin I-silenced (*anx-1* siRNA) cells treated with anti-Fas IgM and labeled with a green fluorescence dye (CMFDA). Engulfing endothelial cells were labeled with a red fluorescence dye (SP-DiI-C-18), and a phagocytosis assay was performed as described in the Experimental Procedures. Three-dimensional isosurface reconstructions of serial optical sections of engulfed cells are shown. Almost complete internalization of a large majority of wild-type apoptotic cells was observed, while incomplete internalization of most of the annexin I downregulated, apoptotic cells is apparent.

gulfment, and mutant genetic phenotypes have been well characterized (reviewed by Gumienny and Hengartner, 2001). Among the four predicted *annexin* genes, although RNA expression can be detected for three of the genes, protein expression of a single annexin family member, *nex-1*, has been described in this model system (Creutz et al., 1996; Daigle and Creutz, 1999). To downregulate the nex-1 protein, we microinjected *nex-1* siRNA or full-length double-stranded RNA (*nex-1* dsRNA) into the ovaries of wild-type N2 (Bristol) worms (Fire et al., 1998). As shown in Figure 7A, Western analysis with a polyclonal antibody against nex-1 protein demonstrated specific silencing in the progeny of the injected worms. Defects in engulfment of apoptotic cell corpses were examined in the nex-1-downregulated L1 larvae. As previously described by Ellis and colleagues, no detectable cell corpses were seen in the wild-type or in the control siRNA-injected larvae (Figure 7B) (Ellis et al., 1991). In contrast, significant numbers of unengulfed cell corpses were apparent in the pharynx and head of the nex-1-silenced worms (Figure 7C). Scoring the unengulfed pharyngeal corpses from the pooled nex-1-silenced larvae revealed an average of 0.9 per animal (56 corpses in 62 animals; Table 1, first experiment). Since the silencing effect of siRNA is dependent on the microinjection efficiency and the genetics of siRNA sensitivity (Sijen et al., 2001), we repeated the microinjection experiment in two *C. elegans* strains, isolated each of the injected worms, and examined the engulfment defects in nex-1-silenced progeny. An average

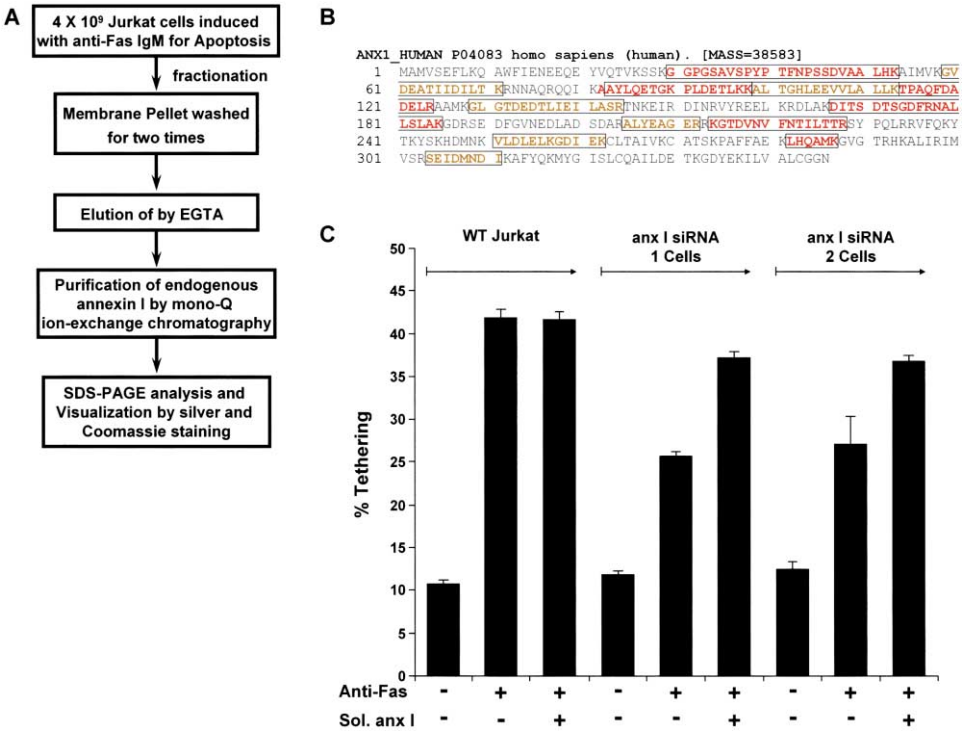

Figure 5. Purified Annexin I from Apoptotic Cell Membranes Rescues Engulfment Defects in Annexin I-Silenced Apoptotic Cells

(A) Schematic flowchart describing the purification strategy for endogenous annexin I. The EGTA-eluted sample contained over eighty proteins, whereas the mono-Q flow-through fraction contained only annexin I.

(B) In-gel trypsin digestion and microcapillary LC-MS/MS analysis of a 38 kDa band from the mono-Q flow-through fraction identifies multiple peptides corresponding to the endogenous human annexin I. Each of the peptides independently identified by the micro-LC-MS/MS procedure is boxed. Adjacent tryptic peptides were colored with red or brown.

(C) Soluble annexin I protein rescues tethering defects observed in annexin I-silenced apoptotic cells. Representative results from four separate experiments, analyzed in triplicate, are shown. The rescue effect is observed in two different cell lines where distinct siRNAs were used to silence endogenous annexin I.

of 0.3–1.0 unengulfed pharyngeal cell corpses were observed in N2 larvae, while a less variable effect was observed in the RNAi-sensitive mutant strain (PD8489 *rrf-3* [*pk1246*]) (Table 1, second experiment). These results indicate that nex-1 protein, similar to the human annexin I, is required for efficient engulfment of apoptotic cells and suggest that engulfment ligands involved in removing apoptotic cells are evolutionarily conserved.

Since the homology of the *C. elegans nex-2* gene is slightly closer to annexin I than is *nex-1,* we also microinjected siRNAs and dsRNA targeting the *nex-2* gene and scored for the presence of unengulfed pharyngeal corpses (Table 1, third experiment). Among the progeny from seven mothers that were injected, no unengulfed pharyngeal cell corpses were observed. Similarly, we injected PSR dsRNA into the ovaries of N2 worms and scored for unengulfed pharyngeal cell corpses, but only 1 corpse in 52 larvae was detected (Table 1, fourth experiment).

Discussion

Quantitative Proteomics Analysis of Apoptotic Cell Membrane Proteins

We have utilized a newly developed proteomics methodology, termed 3D-ICAT, coupled with mass spectrometry, to identify membrane proteins that are upregulated during the anti-Fas IgM-mediated apoptosis. Analysis of approximately thirty fractions separated and purified by three chromatography steps resulted in unambiguous identification and simultaneous quantification of over 300 proteins (Han et al., unpublished data). We have further characterized annexin I because of the rapid upregulation in the membrane fractions as well as the known properties of PS binding (Bitto and Cho, 1998; Montaville et al., 2002; Hawkins et al., 2000). Our analysis supports the fact that annexin I is a novel endogenous engulfment ligand identified by the quantitative proteomics strategy. Four lines of evidence support the fact that annexin I is an endogenous engulfment ligand: (1) caspase-dependent recruitment, export, and colocalization with PS on the cell surface during apoptosis, (2) annexin I-dependent clustering of PSR, (3) the requirement of annexin I in engulfment of apoptotic human cells, and (4) the requirement for an annexin homolog in *C. elegans* for engulfment of pharyngeal cell corpses. Thus, our results suggest that the function of annexin I as an endogenous engulfment ligand is preserved across phylogeny and further imply that annexin I-mediated apoptotic cell recognition and subsequent engulfment is a general mechanism. However, it is possible that other annexin homologs or additional unidentified engulfment ligands participate in cells and tissues that do not express annexin I. It is conceivable that tissue- and cell-specific engulfment mechanisms may also play a

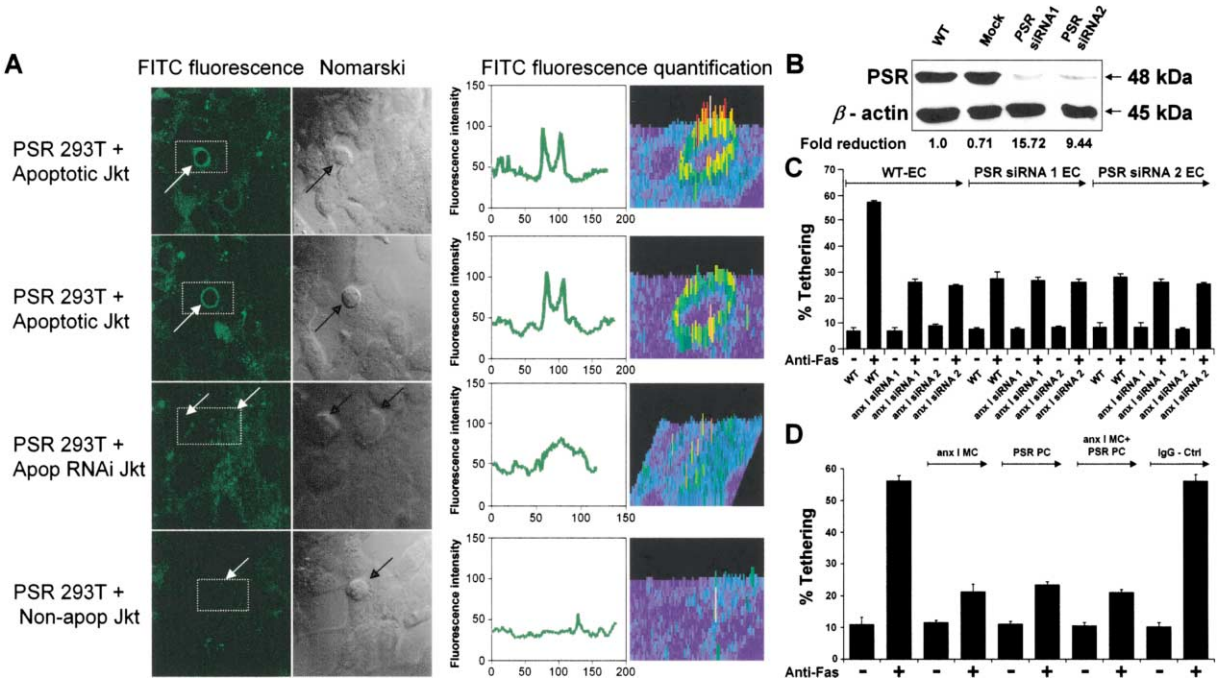

Figure 6. Involvement of PSR and Annexin I in Engulfment of Apoptotic Cells

(A) Clustering of PSR around apoptotic Jurkat cells requires the presence of annexin I. 293T cells transiently transfected with human PSR cDNA and apoptotic Jurkat cells (top two panels), *anx-1* siRNA silenced apoptotic cells (third panel), or naive Jurkat cells (bottom panel) were added to the dish. Cells were fixed with 1% paraformaldehyde, and PSR clustering was visualized by immunofluorescence detection with a rabbit polyclonal antibody against human PSR (Santa Cruz). Fluorescence and Nomarski images are shown. FITC fluorescence quantification of boxed areas are represented either by a graphical plot, which quantifies total fluorescence along the *x*-axis of the image, or a computer-generated fluorescence plot, which displays fluorescence intensities from each pixel in a color scale from blue (lowest) to red (highest). Note the defective clustering of PSR in *anx-1* siRNA-treated apoptotic cells, even though the expression of PSR is high (third panel). Uneven PSR fluorescence is most likely due to the variation in transfected plasmid uptake by the cells.

(B) Downregulation of endogenous PSR protein expression by siRNA-mediated gene silencing. Endothelial cells treated with indicated siRANs were examined for PSR expression by Western analysis with a polyclonal antibody against human PSR.

(C) Functional involvement of PSR and annexin I in apoptotic cell engulfment. Similar defective tethering of wild-type apoptotic Jurkat cells or annexin I-silenced cells to PSR-downregulated endothelial cells is shown. No significant additive effect was seen when signal-silenced cells in the assay (either PSR-silenced EC alone or annexin I-silenced Jurkat alone) were compared against double-silenced cells (PSR in EC and annexin I in Jurkat cells).

(D) Defective tethering exhibited by anti-annexin I antibody or anti-PSR antibody. No further reduction in tethering was observed when double antibodies were used.

role in recognition and uptake of apoptotic cells. Further characterization of apoptotic cells and engulfing cells from different tissues, as well as the comprehensive characterization of ligand-receptor pairs and signaling mechanisms, is required to fully understand the well-coordinated events that control apoptotic cell recognition and uptake.

A number of proteins have been proposed to be receptors for apoptotic cells or bridging molecules between apoptotic cells and engulfing cells (Savill et al., 1992; Hanayama et al., 2002; Nakano et al., 1997; Platt et al., 1998; Savill and Fadok., 2000; Fadok et al., 2000; Ogden et al., 2001). However, their true nature of interaction to the apoptotic cells, as well as the mechanism of engulfment mediated by these proteins, is not comprehensively understood. A more comprehensive characterization of membrane proteins, such as our approach, may not only help identify adhesive ligands, but it may also help identify the proteins that mediate the repulsive signals.

Mechanism Mediated by Exposed Annexin I to Promote Apoptotic Cell Engulfment

We have demonstrated that annexin I is recruited to the PS-rich domains on the cell surface and that this recruitment requires the caspase activity and the release of intracellular calcium, since BAPTA-AM and Z-VAD-FMK effectively blocked the translocation of annexin I. These results suggest that the presentation of annexin I and possibly other engulfment ligands may require coordinate regulation of intracellular caspases and calcium. In addition, cell surface exposure of annexin I promotes clustering of PSR on engulfing cells, and the exposed annexin I is necessary for efficient engulfment of apoptotic cells. Silencing annexin I protein by siRNA resulted in defective tethering and internalization of apoptotic cells (Figure 4 and Supplemental Table S1). Similarly, in the nematode *C. elegans*, downregulating the nex-1 annexin prevents efficient engulfment of cell corpses (Figure 7 and Table 1). The exact signal transduction mechanism mediated by annexin I to promote

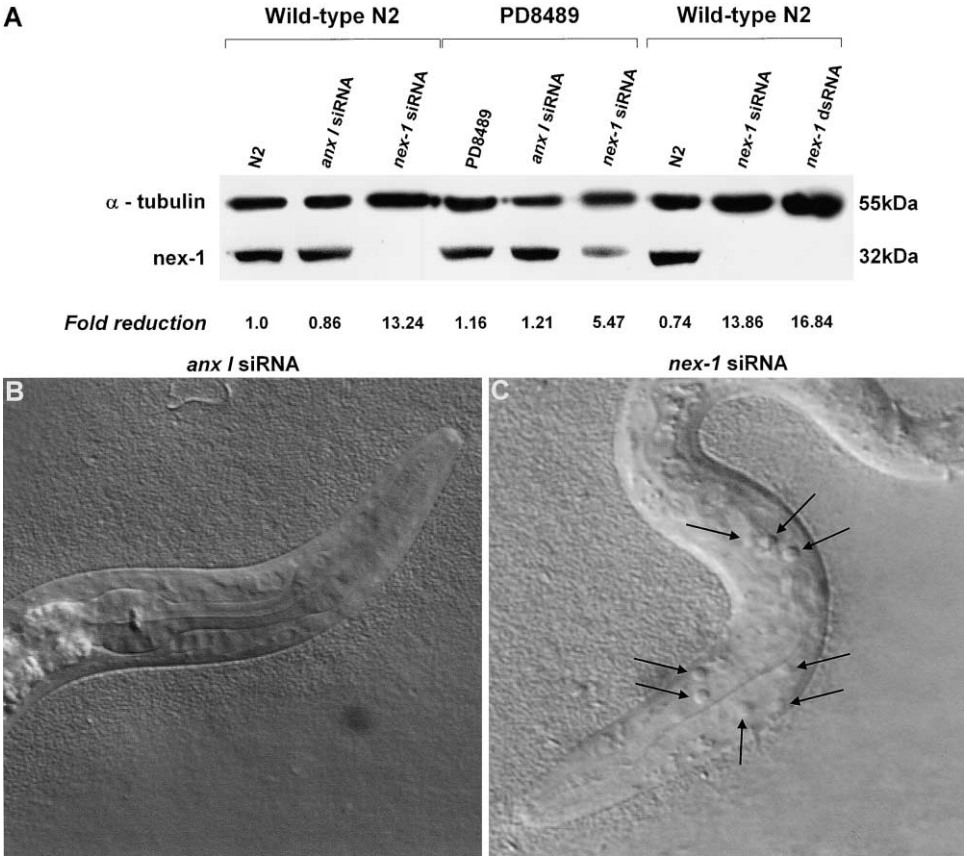

Figure 7. Downregulating the nex-1 Annexin in *C. elegans* Prevents Efficient Cell Corpse Engulfment

(A) Specific silencing of the nex-1 protein by microinjection of *nex-1* siRNAs and dsRNA is shown. Equal amounts of total protein lysates (30 μg) from the wild-type N2, *anx-1* siRNA-, *nex-1* siRNA-, or *nex-1* dsRNA-injected worms were analyzed by Western analysis with a specific polyclonal antibody (Creutz et al., 1996). The fold reduction was quantified by normalizing with corresponding α-tubulin signals. The range of silencing nex-1 protein varies from 5- to 16-fold in four separate experiments.

(B–C) Nomarski images of a representative larva injected with *anx-1* siRNA (B) or *nex-1* siRNA (C) are shown. Arrows indicate the button-like apoptotic cells.

engulfment requires further studies. We postulate that annexin I-mediated engulfment signals are transmitted by receptors such as the PSR from engulfing cells. Although PSR was first identified as a candidate PS-recognizing receptor, the exact binding sites present on the apoptotic cells for PSR are not known (Fadok et al., 2000). We have identified that PSR clustering is enhanced in the presence of annexin I on the apoptotic cell surface. Furthermore, our results suggest that PSR and annexin I function in the same engulfment pathway (Figures 6C and 6D). It has been demonstrated that apoptotic cells signal to the engulfing cells, possibly via exposed cell surface ligands such as annexin I. For example, clustering of the *C. elegans* CED-1 receptor around cell corpses is mediated by the signal(s) from dying cells that require a functional *ced-7* gene (Zhou et al., 2001). Similarly, the induction of Croquemort cell surface receptor in *Drosophila* has been shown to be mediated by the unknown signals from the dying cells (Franc et al., 1999). Also, in the mammalian culture system, apoptotic cells have been shown to induce secretion of TGFβ in macrophages (Fadok et al., 2000). Thus, signals presented from apoptotic cells may induce a variety of responses in engulfing cells, and this process is likely mediated by a number of cell surface ligands to coordinate the precise sequence of events associated with engulfment (Henson et al., 2001). We have demonstrated that annexin I is a signal that mediates tethering and internalization of apoptotic cells. Downregulating annexin I does not completely inhibit tethering or internalization, suggesting that other uncharacterized engulfment ligands may also contribute toward the apoptotic cell engulfment. However, the phenotype of annexin I-silenced apoptotic cells, which show drastic inhibition of tethering and defects in internalization, further suggests that signaling initiated by annexin I may influence PSR clustering and cytoskeletal rearrangement associated with apoptotic cell engulfment. Although we could demonstrate the functional participation of annexin I and PSR in the same engulfment pathway in mammalian cells, silencing *C. elegans* PSR did not show a significant level of unengulfed pharyngeal corpses. This could be due to inefficient silencing of PSR protein or functional divergence of worm PSR with human PSR. Further studies are required to elucidate the signal transduction pathways mediated by the PSR.

Table 1. nex-1 Protein Is Required for Efficient Engulfment of Apoptotic Cell Corpses

	Number of Unengulfed Pharyngeal Cell Corpses in L1 Larvae		
Parental Genotype	dsRNA Trigger	Cell Corpses/Animals	
First Experiment			
Control injections			
Wild-type N2	*anx-1* siRNA	0/50	(n = 50)
Wild-type N2	*anx-1* dsRNA	0/60	(n = 60)
Test injections			
Wild-type N2	*nex-1* siRNAs	56/62	(n = 62)
Wild-type N2	*nex-1* dsRNA	74/81	(n = 81)
Second Experiment			
Control injections			
Wild-type N2	*anx-1* siRNA	0/8	(n = 8)
Wild-type N2	*anx-1* siRNA	0/5	(n = 5)
Wild-type N2	*anx-1* dsRNA	0/21	(n = 21)
Wild-type N2	*anx-1* dsRNA	0/12	(n = 12)
Test injections			
Wild-type N2	*nex-1* siRNAs	5/15	(n = 15)
Wild-type N2	*nex-1* siRNAs	16/19	(n = 19)
Wild-type N2	*nex-1* siRNAs	17/18	(n = 18)
Wild-type N2	*nex-1* siRNAs	10/14	(n = 14)
PD8489 (*rrf-3* [*pk1246*])	*nex-1* siRNAs	11/11	(n = 11)
PD8489 (*rrf-3* [*pk1246*])	*nex-1* siRNAs	11/12	(n = 12)
Wild-type N2	*nex-1* dsRNA	20/21	(n = 21)
Wild-type N2	*nex-1* dsRNA	7/14	(n = 14)
Wild-type N2	*nex-1* dsRNA	14/15	(n = 15)
Wild-type N2	*nex-1* dsRNA	14/16	(n = 16)
Third Experiment			
Wild-type N2	*nex-2* siRNA	0/8	(n = 8)
Wild-type N2	*nex-2* siRNA	0/7	(n = 7)
Wild-type N2	*nex-2* siRNA	0/8	(n = 8)
Wild-type N2	*nex-2* dsRNA	0/12	(n = 12)
Wild-type N2	*nex-2* dsRNA	0/7	(n = 7)
Wild-type N2	*nex-2* dsRNA	0/5	(n = 15)
Wild-type N2	*nex-2* dsRNA	0/12	(n = 12)
Fourth Experiment			
Wild-type N2	PSR dsRNA	0/7	(n = 7)
Wild-type N2	PSR dsRNA	1/13	(n = 13)
Wild-type N2	PSR dsRNA	0/11	(n = 11)
Wild-type N2	PSR dsRNA	0/9	(n = 9)
Wild-type N2	PSR dsRNA	0/12	(n = 12)
Control Quantification of L1Pharyngeal Cell Corpses			
Mutant Genotype	Allele	Cell Corpses/Animals	
ced-1	*n1995*	9/20	(n = 20)
ced-5	*n1812*	59/20	(n = 20)
ced-7	*n1892*	42/20	(n = 20)

The injected double-stranded RNA trigger is indicated. For the first experiment, six adult hermaphrodites were injected with the indicated RNA trigger and transferred to a single agar plate 24 hr after the injection, and hatched L1 larvae were scored together for unengulfed pharyngeal cell corpses. For the second experiment, wild-type N2 worms (ten) or *rrf3*, (*pk1246*) worms (two) were injected with the indicated RNA trigger and transferred to individual agar plates 24 hr after the injection, and hatched larvae were scored for unengulfed pharyngeal cell corpses. For the third experiment, wild-type N2 worms were injected with two siRNAs or dsRNA targeted against the *nex-2* mRNA. The scoring of the indicated *ced* mutant strains were performed to demonstrate the accuracy of scoring unengulfed pharyngeal cell corpses. n, number of larvae scored.

Anti-Inflammatory Role of Annexin I

Annexin I was first discovered over 20 years ago as a protein that mediates the anti-inflammatory action of glucocorticoids (Hawkins et al., 2000; Hirata and Axelrod, 1980). It is well documented that glucocorticoids induce the expression and secretion of annexin I in cell

culture models and that exogenous administration of annexin I confers anti-inflammatory activity in animal models (Perretti, 1997, 1998; Goulding et al., 1998). Moreover, administration of annexin I antibodies in animal models of inflammation mediates the increased inflammation and abrogates the anti-inflammatory activities of glucocorticoids (Duncan et al., 1993; Mancuso et al., 1995; Yang et al., 1997, 1999). Also, the recent observation that annexin I N-terminal peptide could bind to the formyl peptide receptor (FPR) on neutrophils and prevents *trans*-endothelial extravasation supports the anti-inflammatory role of annexin I (Walther et al., 2000). However, neutrophil extravasation alone does not encompass a wide spectrum of inflammatory responses; in chronic inflammatory conditions, lymphocytes and macrophages make up the major inflammatory cells. Most importantly, anti-annexin I IgM and IgG antibodies have been detected in patients with systemic lupus erythematosus (SLE), a chronic autoimmune disorder where episodic inflammation is seen throughout the body (Hirata et al., 1981; Goulding et al., 1989; Pruzanski et al., 1994). SLE patients invariably develop autoantibodies against a number of self-antigens, such as anti-DNA antibodies and anti-nucleoprotein antibodies, and mounting evidence suggests that SLE autoantigens are derived from apoptotic cells (Casciola-Rosen et al., 1994; Cohen et al., 2002; Rosen and Casciola-Rosen, 1999). Supporting this hypothesis is the experimental observation of defective apoptosis demonstrated in SLE patients (Hermann et al., 1998; Pickering et al., 2000). Although a number of candidate proteins have been proposed, a cause and effect relationship between autoantibodies and engulfment defects has not been established, and the exact sequence of events that culminate in the generation of autoantibodies is not well understood. However, in light of our findings, antibodies against annexin I in SLE patient sera, similar to annexin I monoclonal and polyclonal antibodies, may block the cell surface-exposed annexin I presented on apoptotic cell surfaces and, thus, likely prevent efficient tethering and complete removal of apoptotic cells. This, in turn, may cause the release of cellular constituents into the blood stream and tissues and accelerate the further generation of autoantibodies. Comprehensive identification of engulfment ligands will likely provide a better understanding of pathogenic mechanisms associated with chronic inflammatory diseases where defects in engulfment of apoptosis have been implicated.

Experimental Procedures

Cell lines, reagents, antibodies, methods for induction of apoptosis, cell fractionation and 3D-ICAT analysis, fluorescence colocalization experiments of annexin I with PS, Western analysis, siRNA-targeting experiments, phagocytosis and tethering assays, purification and characterization of annexin I, measurement of intracellular calcium concentrations, methods for preparation of dsRNA, and silencing of worm genes by microinjection are described in the Supplemental Experimental Procedures at http://www.developmentalcell.com/cgi/content/full/4/4/587/DC1.

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