Evidence for a Conserved Role for CrkII and Rac in Engulfment of Apoptotic Cells*

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Apoptosis or programmed cell death occurs in multicellular organisms throughout life. The removal of apoptotic cells by phagocytes prevents secondary necrosis and inflammation and also plays a key role in tissue remodeling and regulating immune responses. The molecular mechanisms that regulate the engulfment of apoptotic cells are just beginning to be elucidated. Recent genetic studies in the nematode Caenorhabditis elegans have implicated at least six genes in the removal of apoptotic cell corpses. The gene products of ced-2, ced-5, and ced-10 are thought to be part of a pathway that regulates the reorganization of the cytoskeleton during engulfment. The adapter proteins CrkII and Dock180 and the small GTPase Rac represent the mammalian orthologues of the ced-2, ced-5 and ced-10 gene products, respectively. It is not known whether CrkII, Dock180, or Rac proteins have any role during engulfment in mammalian cells. Here we show, using stable cell lines and transient transfections, that overexpression of wild-type CrkII or an activated form of Rac1 enhances engulfment. Mutants of CrkII failed to mediate this increased engulfment. The higher CrkII-mediated uptake was inhibited by coexpression of a dominant negative form of Rac1 but not by a dominant negative Rho protein; this suggested that Rac functions downstream of CrkII in this process, which is consistent with genetic studies in the worm that place ced-2, ced-5, and ced-10 in another (15–19). Elegant genetic studies in the nematode Caenorhabditis elegans have implicated at least six genes segregated into two partially redundant complementation groups as important in the engulfment of apoptotic cells (with ced-1, ced-6, and ced-7 in one group and ced-2, ced-5, and ced-10 in another) (15–19).

Programmed cell death is a process that occurs in various tissues of the body throughout ontogeny (1). The apoptosis of specific cells is closely linked to the removal of apoptotic bodies/cell corpses (2–5). The removal of the dying cells before the integrity of the cell membrane is lost prevents the release of potentially harmful contents, thus protecting the neighboring cells. Engulfment also plays a key role in tissue remodeling and in regulating immune responses (2–5). In mammals, macrophages and immature dendritic cells are thought to carry out the majority of the apoptotic cell removal, although clearly other cell types can perform this function in different tissues (1, 4, 6).

The dying cells expose several “eat-me” signals or apoptotic markers that can be recognized by the phagocytic cells through one or more cell surface receptors (2). The subsequent intracellular signaling events in the phagocyte lead to cytoskeletal rearrangements that facilitate the engulfment of the dying cells. The signaling during engulfment also regulates the cytokine secretion by the phagocyte (1, 2). One of the eat-me signals is the exposure on the cell surface of phosphatidylserine (PS), a lipid normally found only on the inner leaflet of the plasma membrane and that can be detected by annexin V staining (7). PS recognition by the phagocytes can occur via CD36 (8, 9) and CD14 (10) as well as the recently cloned unique PS receptor (11). PS exposure on the phagocyte has also been seen under certain conditions (12). Modifications to glycosylation patterns on cell surface proteins and a change in the surface charge of the dying cells can also serve as apoptotic markers, although the mechanisms for their recognition have been less well defined (13, 14). Although progress has been made in identifying some of the phagocytic receptors, relatively little is known about coordination of the specific intracellular signaling events downstream of these receptors and how they regulate engulfment of apoptotic cells.

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1 The abbreviations used are: PS, phosphatidylserine; SH2/SH3, Src homology 2/3; wt, wild type; FACS, fluorescence-activated cell sorter; GFP, green fluorescent protein; EGFP, enhanced green fluorescent protein.
creased uptake is dependent on downstream signaling through CrkII and the GTPase Rac1 in mammalian engulfment. We have indicated a role for the CED-2 orthologue CrkII in the removal of apoptotic bodies (19). To test the role of Crk proteins in engulfment in mammalian cells, we overexpressed Myc-tagged CrkII in the phagocytic LR73 cell line. Stable LR73 clones expressing various levels of CrkII were isolated (see "Experimental Procedures"). The fraction of LR73 cells that became fluorescent due to engulfment of thymocytes was analyzed by flow cytometry. The backgrounds after incubation with live thymocytes have been subtracted. The engulfment by control LR73 was set at 100%, and the engulfment from four independent experiments (each performed on duplicate samples) is shown (p < 0.01, n = 4). b, control LR73 cells and Crk-LR73 (clone 32) were analyzed for engulfment of 2 μm carboxy-ylate beads or E. coli bacteria (50 particles/cell) by flow cytometry.

In this report, we examined the role of the adapter protein CrkII and the GTPase Rac1 in mammalian engulfment. We observe that overexpression of CrkII or Rac in the LR73 cell line enhances phagocytosis and that the CrkII-mediated increased uptake is dependent on downstream signaling through Rac. These data suggest that CED-2/CrkII and CED-10/Rac are part of an evolutionarily conserved pathway in engulfment of apoptotic cells.

EXPERIMENTAL PROCEDURES

Cells—The phagocytic LR73 Chinese hamster ovary cell line was cultured in Alpha’s modified Eagle’s medium supplemented with 10% fetal calf serum and penicillin/streptomycin/pen-glutamine as described previously (31).

Plasmids and Antibodies—The plasmids encoding Myc-tagged wt or mutant CrkII were kindly provided by Michiyuki Matsuda (Tokyo, Japan). The plasmids encoding Rac1L61, Rac1N17, and RhoN19 were provided by Dr. J. T. Parsons (University of Virginia), and the pEGRFEGFP3 plasmid was provided by Dr. Ian Macara (University of Virginia). pEBG-Shc, from our laboratory, has been described previously (32). The anti-Myc and anti-CrkII antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Transfections—For stable transfections, the LR73 cells were transfected using the calcium phosphate method with 20 μg of linearized plasmids encoding Myc-tagged rat CrkII wt or mutants (28) along with 0.5 μg of a puromycin plasmid for selection. Stable single colonies were picked and analyzed for CrkII expression by immunoblotting using anti-c-Myc or anti-CrkII. Immunoprecipitation and immunoblotting were performed as described previously (31). For transient transfections, LipofectAMINE-2000 reagent (Life Technologies, Inc.) was used as recommended by the manufacturer. At least two wells were independently transfected for each condition/plasmid combination, the duplicate samples were processed separately in the rest of the experiment, and the mean and standard deviation were calculated. All transient transfection experiments were performed multiple times with the data presented being representative of at least three independent experiments. Briefly, 150,000 LR73 cells/well were plated in 24-well plates. The cells were transfected the next day by incubation with plain medium containing 2 μl LipofectAMINE-2000 and 0.8 μg of each plasmid for 6 h, washed, and incubated with fresh medium (10% serum and no antibiotic) for 24 h before the phagocytosis assays were performed as described below.

Engulfment Assays—20,000 LR73 cells/well were plated in 24-well plates and used 2–3 days later. Apoptotic thymocytes used as targets were labeled in one of two ways. Thymocytes from 6-week-old mice were harvested and labeled with the lipophilic red fluorescent dye PKH-26 (6 μM for 5 min). The excess dye was quenched and the viable cells were purified using Lympholite M gradient (Cedarlane). Alternatively, thymocytes were washed and resuspended in Hanks’ balanced salt solution (Life Technologies, Inc.) at 5 million cells in 100 μl, and mixed v/v with Hanks’ balanced salt solution containing 240 μM of the cytoplastmic cell tracker dye CM-orange (5-(and-6)-4-chloromethyl-benzoylamino tetra-methylrhodamine) (Molecular Probes, Eugene, OR). After 2 min of incubation at room temperature, the cells were incubated at 37 °C for 25 min followed by a further incubation with fetal calf serum (v/v) for 25 min at 37 °C. Thymocytes labeled with either dye were induced to undergo apoptosis by dexamethasone (2 μg/ml) for 24 h. Under these conditions, about 40–50% of the thymocytes were Annexin V positive and less than 10% of Annexin V positive cells were propidium iodide positive (i.e. necrotic). The thymocytes were washed once and overlayed (at 500,000 cells/well in 300 μl of growth medium) on LR73 cells. Routinely, the assays were carried out in duplicates or triplicates for each condition with a 50-min incubation for thymocytes and a 2-h incubation for beads. The wells were then aspirated and washed twice with cold phosphate-buffered saline. The cells on the plate were trypsinized, resuspended in cold medium (with 0.2% sodium azide), and analyzed by flow cytometry. Unengulfed thymocytes were gated out by their forward and side scatter. Routinely, 10,000–20,000 events were collected and the data analyzed using CellQuest software. The controls included the use of live thymocytes (not treated with dexamethasone) and apoptotic thymocytes incubated with LR73 cells at 4 °C. Although we trypsinized the cells prior to analysis, the FACs assay cannot distinguish between fluorescence derived from bound versus engulfed thymocytes. However, as determined by confocal microscopy, the majority of the fluorescent phagocytes scored in the FACs assay represents cells that have engulfed the thymocytes or are in the process of engulfment (data not shown). For engulfment assays with 0.1 μm carbonate-modified beads (Sigma), indicative of phagocytosis and pinocytosis, respectively, the beads were incubated with cells for 2 h, washed extensively, and analyzed as described above. Forward scatter was used to gate out the unbound beads. To test the involvement of Rho family GTPases, Clostridium difficile toxin B (provided by Dr. Chang Hahn, University of Virginia) was preincubated with the phagocytes at 10 ng/ml for 15 min and was present throughout the engulfment assay.

RESULTS AND DISCUSSION

Overexpression of CrkII Enhances Phagocytosis—Genetic studies in C. elegans have indicated a role for the CED-2 orthologue CrkII in the removal of apoptotic bodies (19). To test the role of Crk proteins in engulfment in mammalian cells, we overexpressed Myc-tagged CrkII in the phagocytic LR73 cell line. Stable LR73 clones expressing various levels of CrkII proteins (Crk-LR73) were established. Compared with parental LR73 cells, Crk-LR73 cells had an increased uptake of apoptotic cells when the ratio of apoptotic versus live cell uptake was assessed (an increase of 1.5–2-fold in four independent experiments, p < 0.01) (Fig. 1a). CrkII-overexpressing cells also showed an enhanced uptake of 2 μm carbonate-modified latex beads, which are thought to mimic the negative charge on dying cells and have been used previously as a surrogate for apoptotic cells (33, 34). The increased phagocytosis was specific, as there was no detectable difference between parental and Crk-LR73 cells in the uptake of Escherichia coli (Fig. 1b) or...
0.1 μm beads (indicative of pinocytosis) (data not shown). An analysis of multiple clones expressing different levels of wt CrkII showed a roughly dose-dependent increase in engulfment of 2 μm carboxylate-modified beads (2–5-fold in 10 independent experiments) (Fig. 2a). The primary data obtained by flow cytometry to derive the data presented in the bar graphs is also shown (bottom panel). In the histograms, in the gate setting to determine the percentage of cells containing engulfed particles, we excluded the sharp first peak as it was seen even in the presence of azide, suggesting that this may reflect binding rather than internalization (see data in Fig. 2b below).

b, to distinguish whether the enhanced engulfment seen was due to greater binding or internalization, parental LR73 or Crk-LR73 cells were incubated with 2 μm carboxylate beads in the presence or absence of 1% sodium azide at 4 °C or 37 °C. Compared with cells incubated at 4 °C, the greater percentage of fluorescent cells at 37 °C in the presence of azide is considered to reflect cells with bound fluorescent beads, whereas incubation at 37 °C without azide reflects both binding and internalization. The primary flow cytometric data is presented below the bar graphs.

CrkII is composed of one Src homology 2 (SH2) and two Src homology 3 (SH3) domains (Fig. 3). We compared the engulfment by LR73 cells stably expressing either wt CrkII or CrkII carrying mutations in either the SH2 domain (R38V) or the first SH3 domain (W169L), the latter being implicated in Dock180 binding (Fig. 3). Although LR73 clones expressing the lowest levels of Myc-tagged wt CrkII still enhanced engulfment, this was not seen with LR73 expressing the highest
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levels of the mutants. There was no difference between LR73 cells expressing wt and mutant CrkII when assessed for uptake of 0.1 μm beads (data not shown). It is possible that both the SH2 and the first SH3 domain of CrkII are critical for Crk recruitment/function in this system and that mutation of either domain may render the protein functionally inactive and may explain the lack of enhancement or inhibition with either mutant.

Rac Functions Downstream of CrkII in Mammalian Engulfment—Genetic studies using Drosophila Myoblast city (a homologue of CED-5/Dock180) and overexpression studies in 293T cells have indicated that CrkII binds to Dock180 via its SH3 domain and that overexpression of Crk and Dock180 can enhance the formation of GTP-bound Rac, leading to cytoskeletal reorganization (26–28). In wt Crk-LR73 cells, as expected, the immunoprecipitation of Myc-tagged CrkII co-precipitated Dock180 (data not shown). When we attempted to generate Dock180-overexpressing cell lines to test the role of Dock180 in engulfment, we were unable to obtain clones expressing good levels of Dock180 protein. To determine whether the enhanced phagocytosis due to CrkII overexpression is a result of Crk signaling via Rac1, we examined the effect of C. difficile toxin B, which has been shown to inactivate Rho family GTPases including Rac1 (35). Toxin B treatment abolished the engulfment by parental as well as Crk-LR73 cells (Fig. 4a). To directly test the role of Rac, we transiently transfected LR73 cells with plasmids coding for CrkII, the constitutively active Rac1L61, or the dominant negative Rac1N17. In all cases, we cotransfected a plasmid coding for the green fluorescent protein (GFP) as a marker for transfected cells. We then examined the engulfment of “red”-labeled 2 μm carboxylate beads by the GFP positive “green” cells and scored the percentage of “double positive” cells within the transfected population by flow cytometry. Compared with GFP only transfected cells, transient expression of CrkII enhanced the engulfment of carboxylate-modified beads by about 4-fold, whereas Rac L61 increased the uptake by 3–4-fold (Fig. 4b). Under the same conditions, the expression of Rac1N17 inhibited the uptake to about 50% of the GFP control transfection (representative of three independent experiments). Similarly, when LR73 cells transiently transfected with Rac1N17 and GFP marker were tested for their ability to take up CM-orange-labeled apoptotic thymocytes, there was a significant decrease in the uptake of dying cells compared with control plasmid transfected cells (Fig. 4c). Under the same conditions, as seen in stable transfectants (Fig. 1a), Crk overexpression had an increased uptake of dead cells (170% compared with Shc; data not shown). These data suggested that functional endogenous Rac proteins are important for basal engulfment of apoptotic cells.

We then tested whether the increased uptake due to Crk overexpression would also be sensitive to inhibition by Rac1N17 coexpression. The N17Rac1 inhibited the Crk-mediated enhancement, suggesting that Rac1 functions downstream of CrkII in this process (Fig. 4d). In contrast, expression of the dominant negative form of Rho (RhoN19) along with CrkII did not inhibit Crk-mediated enhanced uptake. Moreover, under these conditions, expression of another adapter protein, Shc, did not increase the uptake or affect the RacL61- or CrkII-mediated enhanced engulfment (Fig. 4d).

The data presented here indicate that the adapter protein CrkII, through a pathway that involves the downstream signaling via Rac, regulates engulfment of apoptotic cells. Together with the genetic studies in the worm, the data presented here suggest that CrkII and Rac proteins play an evolutionarily conserved role in the clearance of apoptotic cells. The enhancement of engulfment due to wt CrkII overexpression suggests that this signaling pathway may be one of the rate-limiting steps. It was surprising to us that the CrkII proteins with a mutation in the SH2 domain or the first SH3 domain failed to function as a dominant negative in engulfment. One possibility is that both the SH2 and the first SH3 domain of CrkII may be critical for Crk recruitment/function and that mutation of either domain may render the protein “null.” This likelihood may explain the lack of enhancement or failure of inhibition of the basal engulfment due to expression of either mutant. At present, the receptor(s) that function upstream of CrkII during phagocytosis is not precisely known. Genetic studies in C. elegans have not identified a receptor in the second complementation group of genes (which encode the Crk, Dock180, and Rac orthologues). Because some members of the integrin family of surface receptors have been shown to signal via Crk (25, 36), the potential role of an integrin receptor functioning upstream of CrkII during engulfment seems plausible. During the review of this manuscript, Albert et al. (37) reported that αβi integrin may play a role in signaling upstream of Crk during engulfment of apoptotic cells. Moreover, using a model system of HEK 293T cells as phagocytes, these authors observed that overexpression of CrkII caused a decreased uptake of apoptotic cells. Whether the discrepancy between our results and those of Albert et al. reflects the different cell types being used as phagocytes, or perhaps the much greater overexpression of transfected plasmids achievable in T antigen-expressing 293T cells compared with LR73 cells, remains to be determined. Nevertheless, both sets of data implicate a function for CrkII-mediated signaling during engulfment.

Figure 3. CrkII-mediated enhanced engulfment requires both the SH2 and SH3 domains of Crk. Different stable LR73 clones expressing either the wt, W169L (mutant in the SH3 domain), or R38V (mutant in the SH2 domain) were analyzed for engulfment with 2 μm carboxylate beads. The wt clones are the same ones shown in Fig. 2a above. The relative expression levels of the transfected proteins are shown in the middle panel. The bottom panel depicts the schematic diagram of wt CrkII and the mutants.

Figure 4. Rac Functions Downstream of CrkII in Mammalian Engulfment. A. The data presented here indicate that the adapter protein CrkII, through a pathway that involves the downstream signaling via Rac, regulates engulfment of apoptotic cells. Together with the genetic studies in the worm, the data presented here suggest that CrkII and Rac proteins play an evolutionarily conserved role in the clearance of apoptotic cells. The enhancement of engulfment due to wt CrkII overexpression suggests that this signaling pathway may be one of the rate-limiting steps. It was surprising to us that the CrkII proteins with a mutation in the SH2 domain or the first SH3 domain failed to function as a dominant negative in engulfment. One possibility is that both the SH2 and the first SH3 domain of CrkII may be critical for Crk recruitment/function and that mutation of either domain may render the protein “null.” This likelihood may explain the lack of enhancement or failure of inhibition of the basal engulfment due to expression of either mutant. At present, the receptor(s) that function upstream of CrkII during phagocytosis is not precisely known. Genetic studies in C. elegans have not identified a receptor in the second complementation group of genes (which encode the Crk, Dock180, and Rac orthologues). Because some members of the integrin family of surface receptors have been shown to signal via Crk (25, 36), the potential role of an integrin receptor functioning upstream of CrkII during engulfment seems plausible. During the review of this manuscript, Albert et al. (37) reported that αβi integrin may play a role in signaling upstream of Crk during engulfment of apoptotic cells. Moreover, using a model system of HEK 293T cells as phagocytes, these authors observed that overexpression of CrkII caused a decreased uptake of apoptotic cells. Whether the discrepancy between our results and those of Albert et al. reflects the different cell types being used as phagocytes, or perhaps the much greater overexpression of transfected plasmids achievable in T antigen-expressing 293T cells compared with LR73 cells, remains to be determined. Nevertheless, both sets of data implicate a function for CrkII-mediated signaling during engulfment.
Our observations that Rac overexpression alone can enhance engulfment and that CrkII-mediated enhanced uptake is inhibited by dominant negative RacN17 expression are consistent with the genetic studies in the worm, where the deficiency of cell corpse removal in ced-2 (crk)-deficient animals was rescued by ced-10 (rac) overexpression. Interestingly, we also observe that CrkII, Dock180, and Rac proteins can localize to the glycosphingolipid-enriched membrane microdomains referred to as lipid rafts (data not shown). Moreover, the disruption of lipid rafts inhibited the engulfment of apoptotic cells. Because actin polymerization has been linked to the lipid rafts, and occurs downstream of the CrkII/Dock180/Rac proteins, our working hypothesis is that engagement of the engulfment receptor(s) prelocalized or recruited to these microdomains may lead to the initiation of signaling events via the Crk/Dock180/Rac signaling pathway. In turn, through already known or yet to be defined Rac effectors, this could lead to a reorganization of the actin cytoskeleton and facilitate engulfment. Testing this model and defining the specific localization of molecules during engulfment of apoptotic cells might lead to a better understanding of the molecular details of this fundamentally important biological process.

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