Spatiotemporal activation of Rac1 for engulfment of apoptotic cells

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The engulfment of apoptotic cells requires phagocytes to coordinately activate Rho family GTPases that regulate actin dynamics. Here, we used a FRET biosensor to visualize the spatiotemporal activation of Rac1 during engulfment of apoptotic cells. We report that apoptotic cells were usually engulfed by the phagocytes' lamellipodia, where Rac1 was activated. Often, apoptotic cells were engulfed successively at the same lamellipodial site, suggesting the presence of portals for apoptotic cells. At this location, the activated Rac1 was recruited to form phagocytic cups that were comprised of actin patches. When the phagocytic cup was closed, Rac1 was down-regulated, and the actin patches were abruptly broken down. The constitutively active Rac1 remained at phagocytic cup for a longer period than the wild-type Rac1, and the closure of the phagocytic cup was significantly delayed in cells expressing a constitutive active form of Rac1, resulting in inefficient engulfment. These results indicate that activated Rac1 is necessary to assemble F-actin, but closing the phagocytic cup requires Rac1 to be deactivated.

apoptosis | FRET | small GTPase

During animal development, many harmful or useless cells are generated and undergo apoptosis (1). In adults, cells infected by virus or bacteria, senescent cells, and neutrophils and lymphocytes that are involved in inflammation are killed by apoptosis (2, 3). To prevent the release of noxious materials from dying cells, apoptotic cells are swiftly engulfed by phagocytes, professional phagocytes such as macrophages and immature dendritic cells, and nonprofessional phagocytes such as fibroblasts and epithelial cells (4, 5). If dead cells were not smoothly cleared, these cells would undergo secondary necrosis and release noxious materials that could cause strong inflammation and autoimmune diseases (6–8).

Phagocytes engulf apoptotic cells but not living healthy cells, indicating that apoptotic cells present an "eat me" signal to the phagocytes (9). Among many molecules proposed as an "eat me" signal, one of the most likely candidates is phosphatidylserine that is exposed to the surface of apoptotic cells in a caspase-dependent manner (10). We previously showed that a factor, Milk Fat Globule EGF Factor 8 (MFG-E8), secreted from the thioglycolate-elicited peritoneal macrophages, works as a bridge between apoptotic cells and phagocytes by recognizing apoptotic cells via phosphatidylserine and phagocytes via integrin $\alpha_v\beta_3$ (11).

The actin cytoskeleton must be reorganized in phagocytes for engulfment of apoptotic cells (12, 13). The signal transduction leading to the actin reorganization for engulfment of apoptotic cells has been genetically studied in *C. elegans* (14–17). These studies revealed two pathways that converge at Ced-10/Rac-1; one pathway comprised of Ced-2/CrkII, Ced-5/DOCK180, and Ced-10/Rac1, and another comprised of Ced-1/CD91 or LPR, Ced-6/Gulp, Ced-7/ABCA1, and Ced10/Rac1. Subsequent studies of the engulfment of apoptotic cells with mammalian system confirmed the involvement of the Rho family GTPases and mammalian homologues of the CED proteins (14, 18, 19). However, how and when the Rac1 pathway is activated and how it regulates the engulfment of apoptotic cells has been elusive (20).

Using the above-mentioned MFG-E8-integrin system for the engulfment, we previously showed that Rac1 and RhoG positively regulate the engulfment of apoptotic cells, whereas RhoA inhibits the process (21), suggesting the spatio and temporal activation of Rho family GTPases in the engulfment of apoptotic cells. In this report, we used the FRET probe to monitor the activation of Rac1 in this process and found that apoptotic cells are often engulfed from the defined area of the phagocytes. The active Rac1 is recruited to the area where actin patch is formed for phagocytic cup. The subsequent closure of phagocytic cup is well correlated with the down-regulation and detachment of Rac1 from the phagocytic cup. These results indicate that two different signaling pathways, recruitment of active Rac 1 and down-regulation of Rac 1, are involved in formation and closure of phagocytic cup.

Results

Engulfment of Apoptotic Cells at Lamellipodia. We previously established a probe, Raichu (Ras and interacting chimeric unit)-Rac1/PM and Raichu-Rac1, which consist of YFP, the Cdc42-, Rac-interacting binding motif (CRIB) for Pak, Rac1, CFP, and C terminus of K-Ras (Raichu-Rac1/PM) or C terminus of Rac1 (Raichu-Rac1) (Fig. 1*A*) (22). These probes detect activated Rac1. That is, when Rac is activated, the intramolecular binding of Rac1 to CRIB enhances FRET between YFP and CFP. In many cases, Raichu-Rac1/PM and Raichu-Rac1 gave essentially the same results (23, 24). However, because the signal obtained with Raichu-Rac1/PM probe is usually stronger than that with Raichu-Rac1, we used the Raichu-Rac1/PM probe in most experiments of this study. Swiss 3T3 or NIH 3T3 cells were stably transformed to express $\alpha_v\beta_3$ integrin and transfected with the pRaichu-Rac1/PM expression vector.

Mouse thymocytes were induced to undergo apoptosis by dexamethasone treatment, and the phagocytosis of the apoptotic cells was carried out in the presence of Milk Fat Globule EGF Factor 8 (MFG-E8) (11). The cells were imaged for YFP and CFP, and the YFP/CFP intensity ratio, reflecting the FRET efficiency, was transformed into an intensity-modulated display. As shown in Fig. 1*B* and supporting information (SI) Movie S1, a high FRET efficiency was observed in phagocyte processes where the plasma membranes ruffled vigorously, confirming

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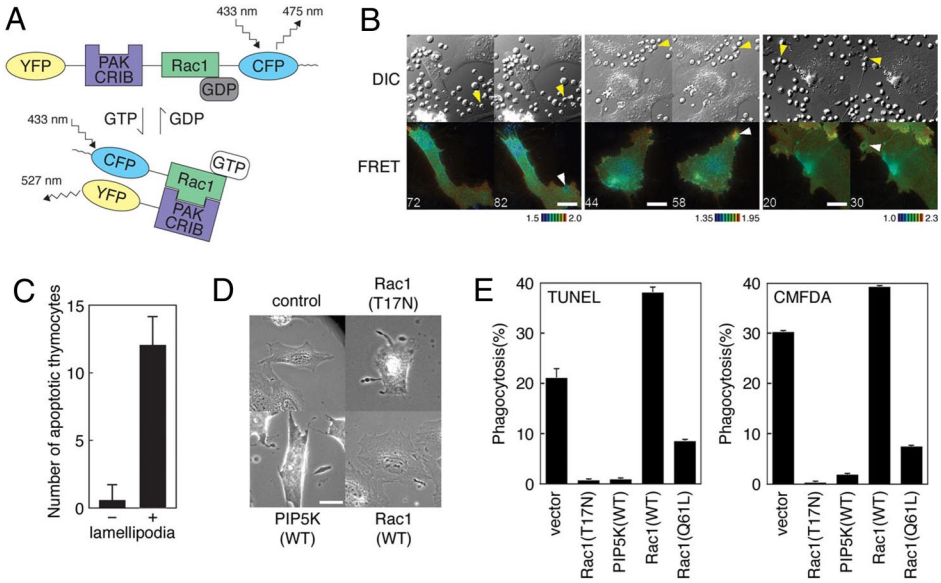

Fig. 1. Engulfment of apoptotic cells at lamellipodia. (*A*) The FRET probe for Rac1 (Raichu-Rac1/PM and Raichu-Rac1) is shown schematically. When GDP is bound to Rac1, the excitation of CFP at 433 nm produces fluorescence at 475 nm. When GTP is bound to Rac1, the intramolecular binding of Pak1 to Rac1 brings YFP into close proximity with CFP, causing FRET and fluorescence at 527 nm from YFP. Raichu-Rac1/PM carries the C-terminal region of Ki-Ras4B at the C terminus, whereas Raichu-Rac1 carries the C-terminal region of Rac1. (*B*) NIH 3T3/integrin cells (*Left*) and Swiss 3T3/integrin cells (*Center* and *Right*) were transfected with pRaichu-Rac1/PM. After the addition of apoptotic thymocytes, CFP, YFP, and DIC images were obtained every 2 min. The FRET and DIC images taken before and upon engulfment are shown for three representative cases. Numbers indicate elapsed time (minutes) from the addition of apoptotic cells. The engulfed cells are indicated by arrowheads, and the eight colors used for FRET efficiency are shown below each image. (Scale bars: 20 μm.) (*C*) The number of apoptotic thymocytes engulfed by 5 Swiss 3T3/integrin cells in 180 min at regions with (+) or without (−) lamellipodia was scored. The experiments were done three times, and the average numbers are shown with SD. (*D*) Phase-contrast images of Swiss 3T3/integrin cells expressing the dominant-negative form (T17N) of Rac1, wild-type (WT) PIP5Kα, or wild-type (WT) Rac1. (Scale bar: 20 μm.) (*E*) The cells infected with the empty vector or the virus carrying the cDNA for Rac1(T17N), PIP5K(WT), Rac1(WT), or Rac1(Q61L) were subjected to the engulfment assay with $CAD^{-/-}$ thymocytes (*Left*) or CMFDA-labeled wild-type thymocytes (*Right*) as preys. Percentages of the phagocytes carrying the engulfed thymocytes were determined by FACS as described in *Materials and Methods*. The experiments were done at least three times, and the average numbers are shown with SD.

that Rac1 was activated at the lamellipodia (22, 25). The Rac1 activation and formation of lamellipodia were observed when NIH 3T3/integrin cells were plated on fibronectin- but not laminin-coated plates, suggesting that Rac1 was activated through cell-matrix adhesion.

In most cases (40 of 44), the engulfment of apoptotic cells occurred at the lamellipodia (Fig. 1 *B* and *C*). When the wild-type Rac1 was overexpressed, the cells extensively formed lamellipodia (Fig. 1*D*). In contrast, the overexpression of the dominant-negative form of Rac1 or wild-type PIP5Kα inhibited the formation of lamellipodia (Fig. 1*D*) (26, 27). Using these cells that do or do not form lamellipodia, we assayed the engulfment of apoptotic cells. In one assay, we followed the DNA degradation (TUNEL staining) in phagocytes of caspase-activated DNase $(CAD)^{-/-}$ apoptotic thymocytes, whereas in another assay, the engulfment was assayed with CMFDA-labeled apoptotic thymocytes as preys. In both assays, the wild-type Rac1 strongly enhanced the engulfment, whereas the dominant-negative Rac1 as well as PIP5Kα severely inhibited the process (Fig. 1*E*). These results indicate that the formation of lamellipodia is important for engulfment of apoptotic cells.

Formation of Phagocytic Cups, and Engulfment of Apoptotic Cells at Restricted Sites of Phagocytes. Ingestion of apoptotic thymocytes began after they bound to the phagocytes, and the initiation times for engulfment varied for individual apoptotic cells (Fig. 1*B* and Movie S1). However, once ingestion started, it progressed quickly, and engulfment was detected as a change in the brightness of the apoptotic cells, as viewed by differential interference contrast. To monitor the engulfment of apoptotic cells with more detail, Swiss 3T3/integrin cells were transfected with red fluorescent protein (RFP)-actin, and the actin dynamics were followed during phagocytosis. As reported for the Fc-mediated engulfment of IgG-coated erythrocytes (28), when the apoptotic cells were added, a ring of F-actin in the lamellipodia of the phagocytes surrounded the dead cells and formed the phagocytic cup (Fig. 2*A* and Movie S2). The phagocytic cup lasted a few minute and was then abruptly broken down. Phagocytosed dead cells were seen thereafter as dark shadows in the RFP images.

There are a few systems to monitor the closure of phagocytic cups (28–30). In our system, monitoring the closure by the phase-bright to phase-dark transition in the phase-contrast images gave a better result than observing the sealing of the phagosomes in GFP-GPI images (Fig. S1 and Movie S3). The phase-dark thymocytes were never stained with Annexin V, supporting that the phase-dark thymocytes were engulfed (Fig. S1). By plotting the intensities of the phase-contrast and RFP-actin, we found that the engulfment of apoptotic cells was always accompanied by the gradual accumulation and then breakdown of F-actin around the dead cells (Fig. 2*A*). Monitoring of integrin using the integrin β_3-EGFP fusion protein indicated that integrin β_3 was recruited to the site of the engulfment with the same kinetics as the actin patch formation (Fig. 2*B* and Movie S4), suggesting that the binding of apoptotic cells to integrin via MFG-E8 induced the clustering of integrins, leading to actin polymerization.

In the course of the experiments to monitor the phagocytic cup formation for engulfment of apoptotic cells, we noticed that the engulfment took place at restricted locations. As shown in Fig. 3*A* and Movie S5, at least 10 apoptotic cells were engulfed within 50 min at 2 neighboring positions in one case. In another case, F-actin-enriched protrusions were generated on the plasma membrane and served as gates for apoptotic cells; in these cases,

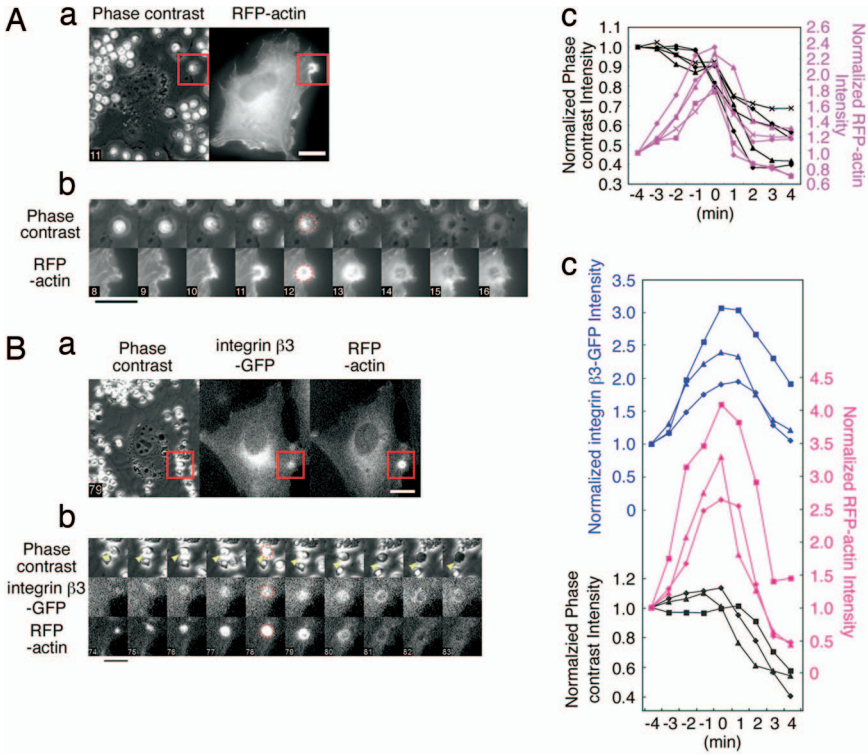

Fig. 2. Formation of phagocytic cups for engulfment of apoptotic cells. Swiss 3T3/integrin cells (*A*) or Swiss 3T3 cells expressing integrin β_3-EGFP (*B*) were transfected with RFP-actin. Forty-eight hours later, apoptotic cells were added, and RFP, GFP, and phase-contrast images were obtained every 1 min. Shown are representative phase contrast, RFP, and GFP images at the indicated time (minutes) after the addition of the apoptotic cells (*b*). Yellow arrowheads point to the apoptotic cells being engulfed. (Scale bars: 20 μm.) The boxed area in *a* is magnified in *b*. The intensities of the images in phase-contrast, GFP, and RFP in the circled regions (diameter, 7.3 μm) (*b*) were monitored, and the results of five (*A*) or three (*B*) representative experiments are shown in *c*. Time 0 was taken when the intensity of RFP-actin was at maximum. The intensities of RFP (magenta), GFP (blue), and phase-contrast (black) were normalized by defining the value at a time of −4 min as 1.0.

three to four apoptotic cells successively entered the phagocytes in a few minutes without breakdown of the phagocytic cup (Fig. 3*B* and Movie S6). In 35 phagocytes that engulfed at least 4 apoptotic cells for 180 min, more than two apoptotic cells entered from a single portal in 86% cases.

Down-Regulation of Rac1 at the Closure of Phagocytic Cup. The swift polymerization and de-polymerization of actin suggested Rho GTPase might be involved. The CFP intensity from Raichu-Rac1 imaging indicated that Rac1was recruited to the phagosomes (Fig. 4 and Movies S7 and S8). Rac1 was found first at the edge of the phagocytic cup, then broadly at the dead cells. Within a few minutes, the concentration of Rac1 around the phagosome

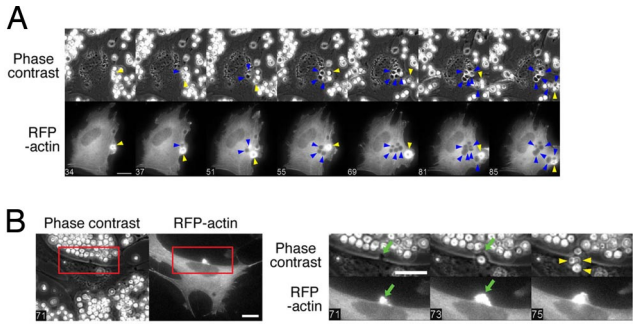

Fig. 3. Engulfment of apoptotic cells at specific regions of phagocytes. Swiss 3T3/integrin cells were transfected with RFP-actin. Forty-eight hours after the transfection, apoptotic cells were added, and RFP and phase-contrast images were obtained every 1 min (*A*) or 2 min (*B*). Representative phase-contrast and RFP images at the indicated time (minutes) after the addition of the apoptotic cells. Yellow and blue arrowheads point to the apoptotic cells being engulfed and the apoptotic cells that were already engulfed, respectively. (Scale bars: 20 μm.) In *B*, the boxed area in *Left* is enlarged in *Right*. The phagocytic protrusion formed for engulfment is indicated by green arrows.

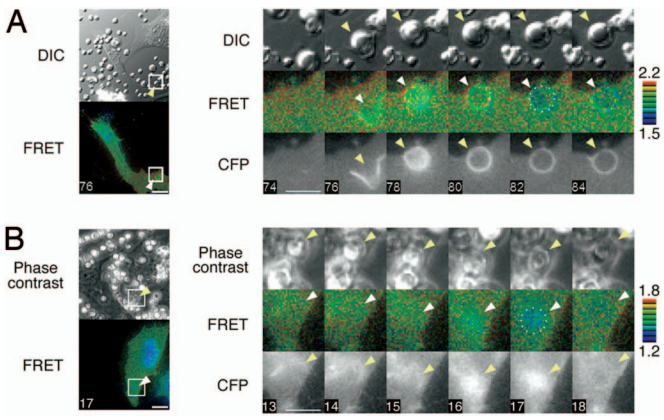

Fig. 4. Down-regulation of Rac1 at the closure of the phagocytic cup. NIH 3T3/integrin cells were transfected with pRaichu-Rac1/PM (*A*) or pRaichu-Rac1 (*B*), apoptotic thymocytes were added to the cells, and CFP fluorescence, YFP fluorescence, and DIC or phase-contrast images were obtained every 2 min (*A*) or 1 min (*B*). Images were taken at the indicated time points (minutes). The eight colors used for FRET efficiency are shown to the right. Arrowheads indicate an engulfed cell. (*Left*) Wide-angle images of the cells at 76 min (*A*) and 17 min (*B*). The boxed areas are magnified in *Right*. Experiments were done at least five times, and representative results are shown. (Scale bars: 10 μm.)

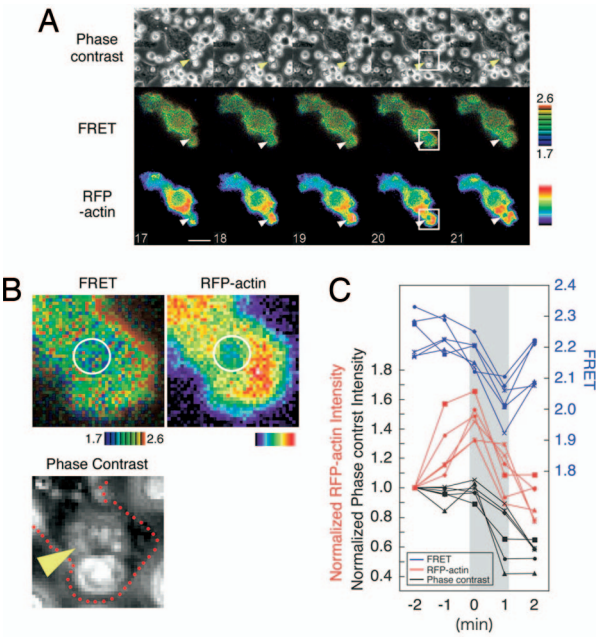

Fig. 5. Regulation of Rac1 during engulfment of apoptotic cells in a macrophage cell line. BAM3 cells were cotransfected with pRaichu-Rac1/PM and mRFP-actin. Forty-eight hours later, apoptotic cells were added to the BAM3 cells, images for CFP, YFP, RFP, and phase-contrast were obtained every 1 min, and the FRET efficiency was determined. The experiments were carried out at least 10 times, and a representative result is shown. RFP images were converted into pseudocolor images by using the MetaMorph software. The pseudocolor representing the intensity of RFP fluorescence is shown in bars, in which blue to red colors denote a low to high level of RFP-actin. Numbers indicate the time elapsed from the addition of apoptotic cells. Arrowheads indicate an engulfed cell. (Scale bar: 20 μm.) (*B*) Phase-contrast, FRET, and RFP images for the boxed area (*A*) when the FRET efficiency was minimum (20 min), shown at a higher magnification. The BAM3 cell is depicted by a red dotted line in the phase-contrast image, and an engulfed apoptotic cell is indicated by an arrowhead. (*C*) Intensities of the FRET (blue line), RFP (red line), and phase-contrast (black line) images in the circled area (*B*) plotted for five cases. Time 0 was set at the time when the intensity for RFP-actin was at the maximum. The intensities of RFP and phase contrast were normalized by defining the value at a time of −2 min as 1.0.

decreased, which was accompanied by closure of the phagocytic cup by a membranous structure covering the apoptotic cells. During this process, in particular, once the phagocytic cup was sealed, the FRET efficiency of Raichu-Rac1/PM decreased (Fig. 4*A*), indicating that Rac1 was inactivated. Similar results were obtained with Raichu-Rac1 that retains the authentic C terminus of Rac1 (Fig. 4*B* and Movie S8). The down-regulation of Rac1 was specific and not due to intermolecular interaction, because little changes in the FRET efficiency were observed with Raichu-Rac1G12V/PM carrying the GTPase-negative Rac1 (Fig. S2) and Raichu-RhoA/PM (Fig. S3).

Macrophages are professional phagocytes for apoptotic cells. To examine whether our finding, the down-regulation of Rac1 during the engulfment of apoptotic cells, can be generalized or not, the pRaichu-Rac1/PM probe and pRFP-actin were expressed in a mouse macrophage cell line BAM3 that efficiently engulfs apoptotic cells in an MFG-E8-independent manner (31). As shown in Fig. 5*A* and Movie S9, the FRET efficiency of Raichu-Rac1/PM was high throughout BAM3 cells. Accordingly, the cell membranes of BAM3 cells intensely ruffled, and the cells moved aggressively. When apoptotic thymocytes were added to BAM3 cells, actin patches formed to engulf them. Each patch was disassembled when the phagocytic cup was sealed (Fig. 5 *A* and *B*). Coincident with the disassembly of the actin patch, the Rac1 activity, monitored by FRET was transiently suppressed at sites where a dead cell was engulfed. The results from FRET, RFP-actin, and phase-contrast data indicated that the assembly of the actin patch took ≈2 min, but the disassembly of the actin, closure of the phagocytic cup, and down-regulation of Rac1 occurred concomitantly and within 1 min (Fig. 5*C*).

Requirement of Down-Regulation of Rac1 for Efficient Engulfment. Rac1 is known to regulate actin dynamics (32, 33). The above results suggested that it also regulates the closure of the phagocytic cup for the engulfment of apoptotic cells. To confirm that the down-regulation of Rac1 is necessary for the efficient engulfment of apoptotic cells, the constitutive active form (Q61L) of Rac1 was expressed in NIH 3T3/integrin cells. These cells showed the enhanced membrane ruffling like the cells expressing the wild-type Rac1 (data not shown), but their ability to engulf apoptotic cells was strongly reduced (Fig. 1*E*). Swiss 3T3/integrin cells were then cotransfected with the expression vector for Rac1Q61L and EYFP-actin, and the actin-patch formation was followed. As shown in Fig. 6*B* and Movie S10, actin patches and phagocytic cups formed to engulf individual apoptotic cells in the presence of the constitutive active form of Rac1. However, the disassembly of the actin patch and the closure of phagocytic cups were significantly delayed. In fact, although F-actin began to disassemble at the site of phagocytosis, the molecule began to reassemble concurrently. This cycle of assembly and disassembly was often repeated several times until the phagocytic cups were completely closed. Such a delay in the closure of phagocytic cup was not observed in the cells expressing the wild-type Rac1 (data not shown). Accordingly, the wild-type Rac1, monitored with RFP-Rac1, only transiently bound to phagocytic cups during the engulfment of apoptotic cells. On the other hand, the constitutive-active Rac1 indecisively remained on the phagocytic cups for a longer period, in average 4 times longer (Fig. 6*C* and Movie S11). These results indicated that Rac1 must be inactivated for the actin patch to be disassembled in the final stage of engulfment.

Discussion

We showed here that Rac1 has two different roles in the engulfment of apoptotic cells. The first is to mobilize plasma membranes and establish lamellipodia, and the second is to regulate the actin dynamics during engulfment. Apoptotic cells were engulfed in the lamellipodia of phagocytes, where Rac1 was activated. Using biochemical approaches, Tosello-Trampotent *et al.* (34) previously showed that the engulfment of carboxylate-modified latex beads that may mimic apoptotic cells activates Rac1. When we followed the engulfment of apoptotic cells by the time-lapsed video, we noticed that the engulfment of apoptotic cells, at least with our system (the engulfment of apoptotic thymocytes by mouse macrophages or NIH 3T3 cells), is not a synchronous process. One phagocyte engulfs apoptotic cells one by one, 3–10 apoptotic cells in one hour, and we could not observe by the biochemical method the clear activation of Rac1 by apoptotic cells (data not shown). On the other hand, monitoring the Rac1 activation by FRET probe indicated that Rac1 is preactivated before addition of apoptotic cells. Growing cells receive signals from various growth factors that can activate Rac1 (32). The cell-matrix adhesion through integrins and cadherins also activates Rac1 (35). It is likely that the binding of apoptotic cells to the phagocyte induces the clustering of integrins, leading to recruitment of the activated Rac1 to phagosomes.

Monitoring the phagocytic cup formation, we noticed that several restricted locations in the lamellipodia seemed to be responsible for the engulfment of apoptotic cells. Recently, a hot spot called "eisosome" was identified in yeast as an endocytic portal (36). This immobile structure is composed of cytoplasmic

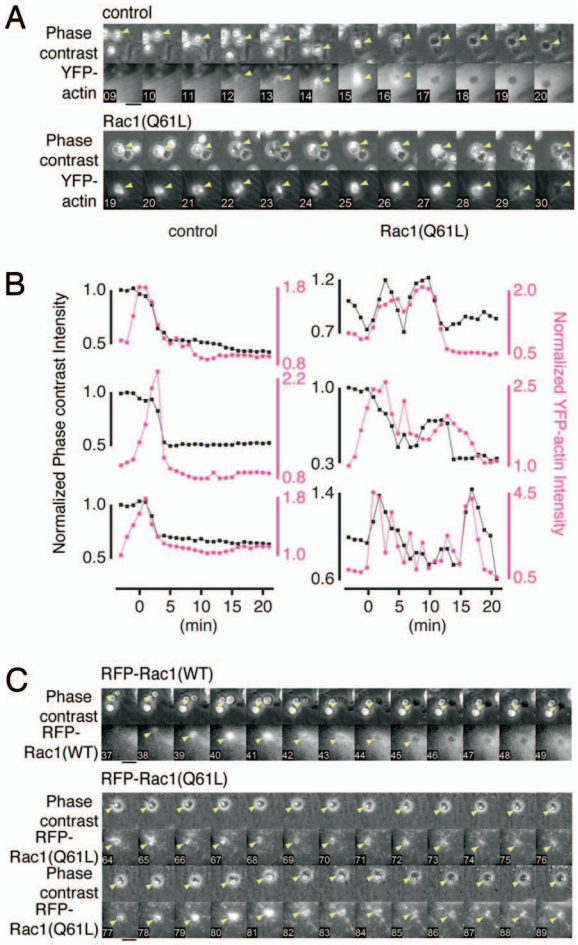

Fig. 6. Effect of the constitutive-active Rac1 on the engulfment of apoptotic cells. Swiss 3T3/integrin cells were cotransfected with EYFP-actin together with pRedNES-Rac1Q61L or control vector. Forty-eight hours later, apoptotic thymocytes were added, and YFP, dsRed-Express, and phase-contrast images were obtained every 1 min. (*A*) Images of phase-contrast and YFP at the indicated time points (minutes) from the addition of apoptotic cells. Engulfed apoptotic cells are indicated by arrowheads. (*B*) Intensities of the phase-contrast (black) and YFP (magenta) images in the region containing an engulfed apoptotic cell were followed with the control or Rac1(Q61L)-expressing cells. The data with three different cells are shown. The experiments were repeated at least three times. (*C*) Swiss 3T3/integrin cells were transfected with pmRFP-Rac1 or pmRFP-Rac1Q61L. Forty-eight hours later, apoptotic thymocytes were added, and RFP and phase-contrast images were taken every 1 min. Numbers on the images represent the elapsed time (minutes) after the addition of apoptotic cells. The engulfed apoptotic cells are indicated by arrowheads.

and transmembrane proteins and provides the basement for the actin patch that is formed for the endocytosis of membrane receptors, soluble nutrients and lipids. The engulfment of apoptotic cells at limited locations suggests that some eisosome-like structure is responsible for the engulfment of apoptotic cells as well. It will be interesting to study whether the same or different portals in macrophages are used for engulfment of apoptotic cells, necrotic cells, bacteria, nutrients, and so on.

In many processes where Rac1 is involved, the Rac1 activity is regulated positively and negatively, by guanine exchange factors (GEF) and GTPase-activating proteins (GAP), respectively (33). After the formation of actin patches and the phagocytic cup, Rac1 was abruptly down-regulated, suggesting that GAP is recruited to phagocytic cups to down-regulate Rac1. Many GEF and GAP have been identified for Rac1 (37). To understand the molecular mechanism of the engulfment of apoptotic cells, it will be necessary to determine which GEF and GAP are responsible to regulate Rac1 and how they are activated. For activation of T cells, many molecules in the antigen-presenting cells interact with the molecules in T cell, forming "immunological synapse" (38). Similarly, many ligand-receptor pairs seem to be involved in the engulfment of apoptotic cells, and a term "engulfment synapses" has been proposed (5, 39). Recruitment of integrin to the apoptotic cells to form phagocytic cup suggests that integrin is one of the components of the "engulfment synapse," and plays a role in recruitment of the active Rac1. Whether any of the known components of the "engulfment of synapse" is involved in down-regulation of Rac1 remains to be determined.

Other Rho family GTPases such as RhoG and RhoA positively and negatively regulate the engulfment of apoptotic cells, while Rab5 seems to be involved in transport of apoptotic cells to lysosomes (21, 40). We recently observed that Rab5 was activated at phagosomes immediately after breakdown of the actin patch, and Gapex-5 GEF seems to work in the upstream of Rab5 (41). Another GEF ALS2 was recently shown to link Rac1 to Rab5 in transferrin-mediated endocytosis (42). How Gapex-5 and/or ALS2 function between Rac1 and Rab5 in the engulfment of apoptotic cells remains to be studied. In addition to the MFG-E8/integrin-mediated engulfment system, macrophages use other systems, in particular phosphatidylserine receptors such as Tim-4, stabilin-2 and BAI1 for engulfment of apoptotic cells (43–45). It will be interesting to study the spatiotemporal activation of Rac1 and Rab5 using FRET analyses in these engulfment systems.

Materials and Methods

FRET Probes and Plasmids. FRET probes for Rac1 (pRaichu-Rac1/PM and pRaichu-Rac1) are described in refs. 22 and 23. pRaichu-Rac1/PM carries the C-terminal region of the Ki-Ras4B at the C terminus to localize the probe at plasma membrane, whereas pRaichu-Rac1 carries the C-terminal region of Rac1. pmRFP-actin was produced from pEYFP-actin (BD Biosciences) by replacing EYFP with RFP. The cDNAs of constitutive-active (Rac1Q61L) and dominant-negative (Rac1T17N) Rac1 mutants were constructed by PCR-mediated mutagenesis. The coding sequence of mouse PIP5Kα was prepared by RT-PCR from mouse BAM3 cells. pRedNES-Rac1Q61L is a polycistronic expression vector that carries the cDNA for Flag-tagged Rac1Q61L and cDNA for dsRed-Express (46) placed downstream of an internal ribosome entry site. To express the integrin-EGFP fusion protein, the coding region of mouse integrin β_3 (11) was fused to EGFP at its C terminus and introduced into pMX retrovirus vector (47). To monitor the localization of Rac1, the wild-type and constitutive active Rac1 (Q61L) were fused to mRFP at the N terminus and expressed with pCXN2 vector (48).

Mice, Cell Lines, and Recombinant Proteins. C57BL/6 mice were purchased from Japan SLC. *CAD*$^{-/-}$ mice are described in ref. 49. Mouse fibroblast cell lines NIH 3T3 and Swiss 3T3, and macrophage cell line BAM3 (50) were maintained in DMEM containing 10% FCS. Transformation of these cell lines to express the integrin $\alpha_v\beta_3$, PIP5Kα, Rac1 mutants, and integrin β_3-EGFP was carried out by the retrovirus-mediated transfection (11). In brief, Plat-E cells (47) were transfected with the pMX vectors, and the supernatant was used to infect NIH 3T3 or Swiss 3T3 cells in the presence of 10 μg/ml polybrene (Sigma). The FLAG-tagged recombinant mouse MFG-E8 was produced in 293T cells and purified by using the anti-Flag M2 affinity gel as described in ref. 11.

Microscopy, Image Acquisition, and Image Processing. FRET imaging was performed essentially as described in refs. 22 and 23. Briefly, NIH 3T3 cells expressing integrin $\alpha_v\beta_3$ (NIH 3T3/integrin), Swiss 3T3 cells expressing integrin $\alpha_v\beta_3$ (Swiss 3T3/integrin), or BAM3 cells were plated on fibronectin-coated 35-mm glass-base dishes (Asahi Techno Glass). The pRaichu vectors, pmRFP-actin, pERedNES-Rac1, and pRFP-Rac1 were introduced into Swiss 3T3/integrin and NIH 3T3/integrin cells with Polyfect (Qiagen) and into the BAM3 cells with FuGENE 6 (Roche Diagnostics). Twenty-four hours after transfection, the cells were imaged in phenol red-free DMEM/F12 (Invitrogen) containing 10% FBS, in a heated closed chamber. Images were obtained every 1 or 2 min by using an Olympus IX81 inverted microscope, a cooled charge-coupled device camera

(CoolSNAP HQ; Roper Scientific), and MetaMorph software (Universal Imaging).

For emission ratio imaging, the following filter sets (excitation and emission filters; Omega Optical) were used: 440AF21 and 480AF30 for CFP, 440AF21 and 535AF25 for YFP, and 580AF20 and 636DF55 for RFP. A 455DRLP dichroic mirror (Omega Optical) was used for CFP and YFP, and an 86006bs dichroic mirror (Chroma Technology) was used when RFP fluorescence was monitored with FRET probes. Cells were illuminated with a 75-W Xenon lamp through a 6% neutral density filter (Omega Optical) and a $\times 60$ oil-immersion objective lens. At each time point, three to four images were recorded with the following exposure times: CFP, 300 ms; YFP, 300 ms; RFP, 400 ms; differential interference contrast (DIC), 50 ms; and phase-contrast, 10 ms at binning 4×4. The ratio images of YFP/CFP, reflecting the FRET efficiency, were created with the MetaMorph software and displayed in eight-color mode (red to blue) in the intensity-modulated display (IMD) mode.

***In Vitro* Phagocytosis of Apoptotic Cells.** For engulfment of apoptotic cells, thymocytes from C57BL/6 mice were treated for 5 h with 10 μM dexamethasone as described in ref. 11 and added to NIH 3T3/integrin, Swiss 3T3/integrin, or BAM3 cells in the presence (for the NIH 3T3 and Swiss 3T3 cells) or absence (BAM3 cells) of 0.1 μg/ml MFG-E8.

To study the effect of PIP5Kα and Rac1 on the engulfment, the cDNAs for PIP5Kα, Rac1, and its derivatives were inserted into the pMXs-puro vector and expressed in NIH 3T3/integrin cells. The engulfment of apoptotic cells was assayed with *CAD*$^{-/-}$ thymocytes as preys, as described in ref. 11. After a 120-min incubation at 37°C, the cells were subjected to TUNEL staining with FITC-labeled dUTP (Roche Diagnostics) and analyzed by flow cytometry with a FACSCalibur flow cytometer (BD Biosciences). The engulfment was also assayed with CMFDA-labeled wild-type thymocytes. In brief, thymocytes were labeled with 1 μM CMFDA (Molecular Probes) and induced to undergo apoptosis as above. NIH 3T3/integrin cells were incubated with CMFDA-labeled thymocytes, washed thoroughly with PBS, gently treated with 0.25% trypsin containing 1 mM EDTA to remove unengulfed thymocytes, and analyzed by flow cytometry.

Other materials and methods used in this study ar described in *SI Materials and Methods*.

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