Cell, Vol. 95, 81–91, October 2, 1998, Copyright ©1998 by Cell Press

G Protein Signaling Events Are Activated at the Leading Edge of Chemotactic Cells

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Summary

Directional sensing by eukaryotic cells does not require polarization of chemoattractant receptors. The translocation of the PH domain–containing protein CRAC in *D. discoideum* to binding sites on the inner face of the plasma membrane reflects activation of the G protein–linked signaling system. Increments in chemoattractant elicit a uniform response around the cell periphery. Yet when cells are exposed to a gradient, the activation occurs selectively at the stimulated edge, even in immobilized cells. We propose that such localized activation, transmitted by the recruitment of cytosolic proteins, may be a general mechanism for gradient sensing by G protein–linked chemotactic systems including those involving chemotactic cytokines in leukocytes.

Introduction

How do cells in multicellular organisms find and maintain their appropriate locations? Increasing evidence suggests that directed cell movement, or chemotaxis, plays a more significant role than previously thought. This essential physiological process is displayed by many eukaryotic cells including keratinocytes, endothelial cells, neurons, and cells of the immune system (Downey, 1994; Lauffenburger and Horwitz, 1996; Baggiolini, 1998). Phagocytic cells, including leukocytes and free-living amoebae such as *D. discoideum*, although evolutionarily distant, share remarkable similarities in behavior as well as in the molecular components involved in chemotaxis (Devreotes and Zigmond, 1988; Chen et al., 1996). Both cell types exhibit a polarized shape, move by pseudopod extension, and sense gradients through a series of repeated excitation and adaptation events. Most interestingly, their chemotactic responses appear to be mediated via a common set of G protein–linked signaling events. This conservation, coupled with its accessible genetics and biochemistry, make *D. discoideum* an excellent model to study the mechanism of chemotaxis (Parent and Devreotes, 1996a).

Whereas bacterial chemotaxis is relatively well understood, the mechanisms by which eukaryotic cells sense chemical gradients have remained cryptic. In prokaryotes, directed cell movement is mediated by the temporal regulation of a two-component histidine kinase signaling system that controls the direction of flagellar rotation (Alex and Simon, 1994). Cells moving up the gradient experience an increase in receptor occupancy and "swim" longer, while those choosing the wrong direction encounter a decrease in receptor occupancy and "tumble" sooner (Koshland, 1977). Eukaryotic cells also respond temporally to increases in receptor occupancy. Indeed, in leukocytes and amoebae, stimulus increments elicit sharp rises in the proportion of polymerized actin, in IP_3 and Ca^{2+} levels, as well as the phosphorylation of myosins, the production of intracellular cAMP, and the activation of MAPK and STAT (Devreotes and Zigmond, 1988; Caterina and Devreotes, 1991; Thompson et al., 1994; Lai et al., 1996; Maeda et al., 1996; Parent and Devreotes, 1996a; Krump et al., 1997; Araki et al., 1998). All of these responses are transient and reoccur with each increase in receptor occupancy. However, in contrast to bacteria, where there is no communication between the "front" and the "back" of the cell, eukaryotic cells appear to also be capable of spatial sensing. When a stimulus is applied, both leukocytes and amoebae preferentially extend pseudopods up the gradient, suggesting that they have the ability to sense the surrounding chemoattractant concentration prior to moving (Zigmond, 1974; Zigmond et al., 1981; Devreotes and Zigmond, 1988).

In order for cells to sense gradients, some of the signaling proteins must become asymmetrically distributed or activated. In *D. discoideum* the localization of several cytoskeletal and signal transduction components in chemotaxing cells has been studied using GFP technology and immunohistochemical staining. Actin, coronin, talin, cofilin, and CAP (cyclase-associated-protein) have all been shown to transiently accumulate at the leading edge (Gerisch et al., 1995; Kreitmeier et al., 1995; Gottwald et al., 1996; Aizawa et al., 1997; Westphal et al., 1997). These cytoskeletal rearrangements have been proposed to be mediated by an asymmetric distribution of chemoattractant receptors and/or G proteins. However, studies using GFP fusion proteins, have shown that neither chemoattractant receptors nor G protein β–subunits become unevenly distributed during chemotaxis (Xiao et al., 1997; T. Jin et al., unpublished results; G. Servant and H. Bourne, personal communication). Therefore, it must be the *activation* of the G protein–linked signaling pathways that preferentially occurs at the leading edge of chemotaxing cells.

In an effort to better understand how cells sense chemical gradients, we set out to visualize signaling events using a GFP-fusion protein known to be recruited to the plasma membrane following chemoattractant receptor stimulation. CRAC (cytosolic regulator of adenylyl cyclase), a novel protein that contains a PH domain at its N terminal, must be translocated to the plasma membrane during each transient activation of adenylyl cyclase (Insall et al., 1994). Biochemical and genetic analyses have shown that the receptor-mediated redistribution of CRAC does not take place in cells lacking

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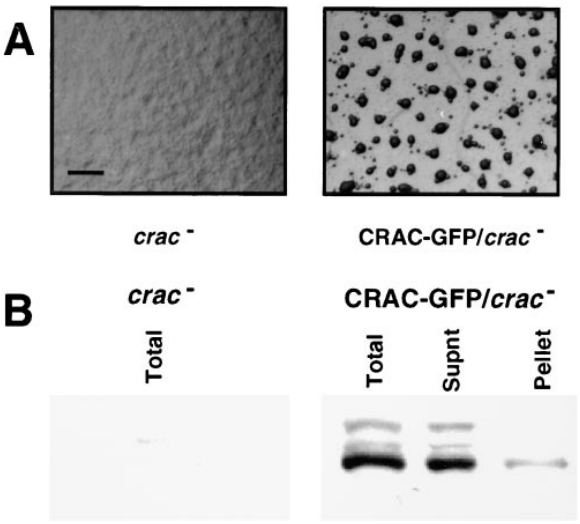

Figure 1. The CRAC-GFP Fusion Protein Retains Wild-Type Activity

(A) Developmental phenotype of *crac*$^-$ and CRAC-GFP/*crac*$^-$ cells on nonnutrient agar. Photographs were taken 24 hr after the cells were plated. Bar represents 8 mm.

(B) Western analysis of whole cells (total), supernatant (supnt), and pellet fractions after lysis and centrifugation. Samples were loaded on 7.5% SDS-PAGE and immunoblotted using an antibody directed against GFP. The multiple bands correspond to the fusion protein (see Experimental Procedures).

the unique G protein β–subunit expressed in these cells, suggesting that the CRAC-binding site is either Gβγ or that the generation of the CRAC binding site requires Gβγ (Lilly and Devreotes, 1995). By using the relocalization of CRAC as a marker to report the spatial and temporal activation of G protein–coupled signaling, we sought to gain insights into the mechanisms of directed cell movement.

Results

The CRAC-GFP Fusion Protein Retains Wild-Type Activity

Cyclic AMP plays a critical role in the early development of *D. discoideum* during which 10^5 cells spontaneously aggregate and differentiate to form multicellular structures (Parent and Devreotes, 1996a). Cells lacking CRAC do not produce cAMP because they cannot mediate G protein–coupled activation of adenylyl cyclase and therefore remain as smooth monolayers of single cells (Insall et al., 1994). Rescue of the aggregation defect in *crac*$^-$ cells thus provides a stringent test for the integrity of the CRAC-GFP fusion protein. The *crac*$^-$ cells were stably transfected with the CRAC-GFP expression plasmid, and a few clones were selected, expanded, and tested for differentiation by depositing washed cells on nonnutrient agar plates. Figure 1A depicts the developmental phenotype of the *crac*$^-$ and CRAC-GFP/*crac*$^-$ cells. Expression of CRAC-GFP in *crac*$^-$ cells suppressed the aggregation-deficient phenotype and led to the formation of differentiated structures. A GFP antibody specifically recognized bands of ~110 kDa only in CRAC-GFP/*crac*$^-$ cells. Crude subcellular fractionation showed that, as is the case with CRAC, CRAC-GFP was almost entirely found in the cytoplasmic fraction of cells (Figure 1B).

The biochemical properties of the CRAC-GFP/*crac*$^-$ cells were characterized by measuring G protein–mediated adenylyl cyclase activity. In lysates of *crac*$^-$ cells the ratio of GTPγS-stimulated to basal adenylyl cyclase activity was 1.9, while in lysates of CRAC-GFP/*crac*$^-$ cells it was greater than 30 (data not shown). This extent of activation is comparable to that displayed by wild-type AX_3 cells. To further characterize the activity of the CRAC-GFP fusion protein, supernatants prepared from wild-type AX_3 cells or CRAC-GFP/*crac*$^-$ cells were used to reconstitute the GTPγS-stimulated activity of lysates derived from *crac*$^-$ cells. Each of these supernatants restored the GTPγS-mediated activation of adenylyl cyclase to wild-type levels (data not shown). Taken together, these results show that the wild-type and GFP-tagged CRAC proteins are essentially interchangeable.

Chemoattractants Cause a Transient Translocation of CRAC-GFP from the Cytosol to the Plasma Membrane

As predicted from the subcellular fractionation data, nonstimulated, differentiated CRAC-GFP/*crac*$^-$ cells showed an even cytoplasmic fluorescent signal when observed by conventional fluorescence microscopy (Figure 1B, Figure 2 upper panel). When the cells were stimulated by the addition of a saturating dose of cAMP (1 μM), a dramatic translocation of CRAC-GFP from the cytosol to the plasma membrane ensued. The relocalization began instantaneously after the addition of the stimulus and peaked within 5–10 s. By 30–40 s the original uniform cytoplasmic distribution was reestablished (Figure 2, upper panel). Similar responses were observed when the cells were stimulated with concentrations of cAMP as low as 1 nM (data not shown). This translocation event occurred specifically following receptor activation. During random movement little or no change in the overall signal was observed even at the tips of extended pseudopods where many cytoskeletal proteins are known to accumulate.

Quantification of the stimulus-induced translocation response showed that the enrichment of CRAC-GFP at the plasma membrane resulted in a depletion in the amount of CRAC-GFP in the cytoplasm. The bottom panel of Figure 2 shows the difference in fluorescence intensities before and after the addition of chemoattractant at points along a line through the central portion of the cell. As depicted by the three basal readings, the distribution of the signal did not change in unstimulated cells. However, a dramatic increase in the fluorescent signal at the periphery of the cell coupled to a reduction in the intracellular signal was observed following receptor stimulation (for clarity only the peak translocation point is presented). The transient kinetics of the translocation event is illustrated in the inset as a measure of the peak fluorescent signals measured at the periphery of the cells. These results show that CRAC-GFP is a highly sensitive marker that can be used to visualize activated G protein–coupled signaling pathways.

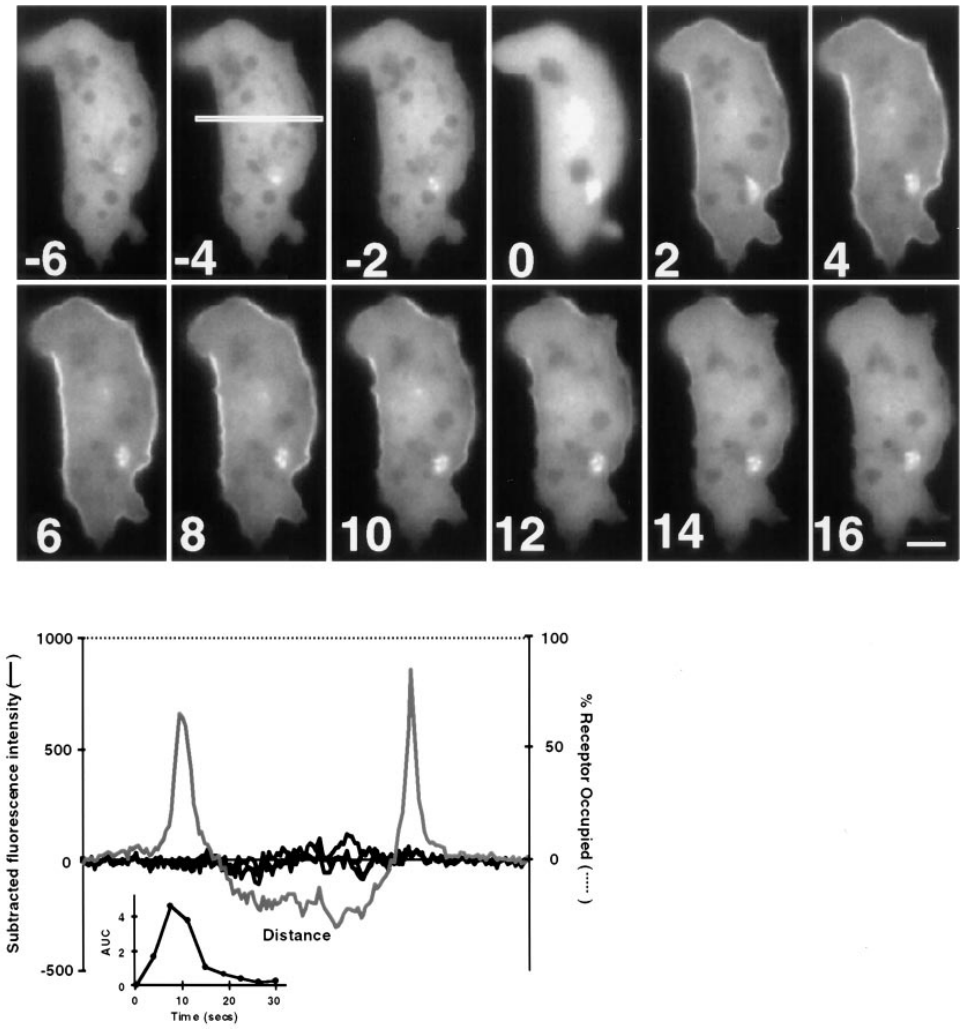

Figure 2. Kinetic Analysis of the Chemoattractant-Mediated Translocation of CRAC-GFP

(Top panel) Montage of images of Redi CRAC-GFP/*crac*$^-$ cells taken before and after the addition of 1 μM cAMP, a saturating dose of chemoattractant. Numbers in the lower left corner are seconds before and after stimulation. Chemoattractant was added at time 0 (note that the cell goes out of focus in this frame). Calibration bar represents 6 μm. An animated version of this experiment can be found in the supplemental data section of the on-line version of the journal (http://www.cell.com/supplemental/95/1/81). Similar results were observed in over 30 independent sessions.

(Bottom panel) Quantitative analysis of the translocation event. Fluorescence intensities were measured along a thin line through the central portion of the cell (see schematic representation on frame taken at −4 s on the top panel). Intensities from the −2 s frame were subtracted from each of the other frames and plotted. For clarity, only the 6 s time point is presented. The % receptor occupancy is illustrated as 100% across the length of the cell. The kinetics of the translocation event is presented as a measure of the area under the curve of the florescent signal at the left periphery of the cell for each time point (inset). Similar results were obtained by analysis of at least five cells recorded on different days.

To determine whether actin polymerization was required for the translocation of CRAC-GFP to the plasma membrane, we pretreated the cells with agents that prevent de novo actin filament polymerization. Latrunculin A or Cytochalasin A were used at final concentrations of 0.5 μM and 1 μM, respectively. A 3–5 min pretreatment with either drug left the cells rounded up and immobile (see below and Figure 6A). When these cells were stimulated with a uniform increase of chemoattractant at a final concentration of 10 μM, a robust translocation of the fluorescent signal to the plasma membrane occurred (data not shown). Thus, the transient relocalization of CRAC-GFP to the plasma membrane does not require reorganization of the actin cytoskeleton.

CRAC-GFP Is Associated with Membrane Fractions following Chemoattractant Receptor Stimulation

The association of CRAC-GFP with the plasma membrane observed in living cells was also apparent in membranes collected from lysed cells. CRAC-GFP/*crac*$^-$ cells were stimulated with cAMP, and at specific times, aliquots of cells were lysed and quickly processed for membrane isolation and immunoblotting. The amount

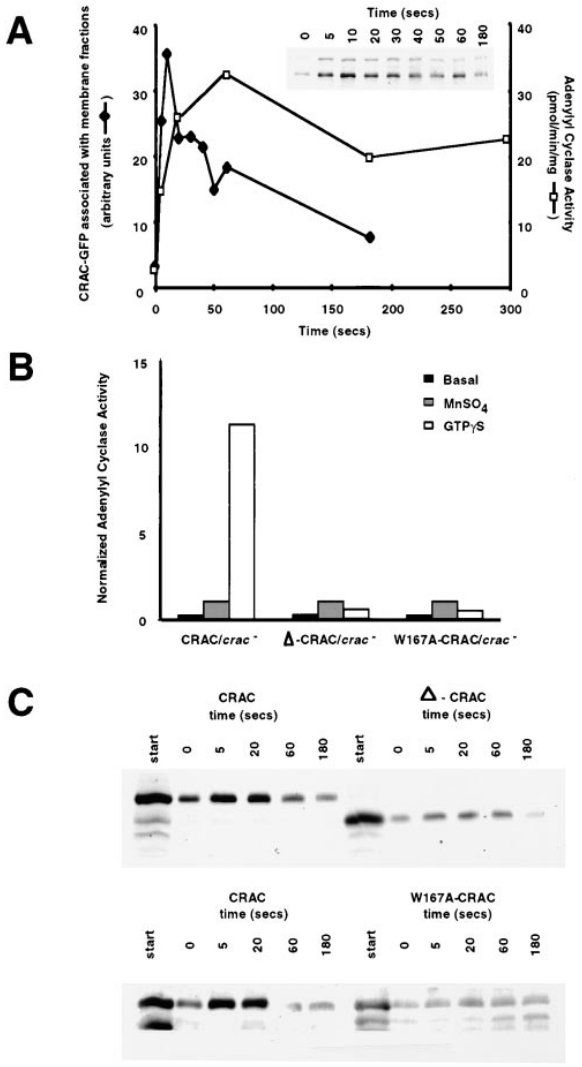

Figure 3. Biochemical and Functional Analysis of CRAC-GFP

(A) Redi CRAC-GFP/*crac*⁻ cells were stimulated with 10 μM cAMP, and at specific times, aliquots of cells were lysed and processed for membrane isolation and immunoblotting or assayed for adenylyl cyclase activity. The closed diamonds represent a densitometric analysis of the translocation of CRAC-GFP to the membrane fractions. The open squares depict the adenylyl cyclase activity.

(B) Basal, unregulated ($MnSO_4$), and G protein–mediated (GTPγS) activation of adenylyl cyclase in CRAC/*crac*⁻, Δ-CRAC/*crac*⁻, and W167A-CRAC/*crac*⁻ (see Experimental Procedures).

(C) Amount of CRAC, Δ-CRAC, or W167A-CRAC that becomes associated with membrane fractions following chemoattractant addition. Cells were stimulated and processed for membrane isolation and immunoblotting as in (A). The results presented here are representative of at least two independent experiments.

of CRAC-GFP associated with membrane fractions transiently increased following chemoattractant addition, with kinetics that paralleled those observed by fluorescence microscopy. Taking aliquots at 10 s intervals revealed that the translocation is rapid and subsides about 40 s after the addition of the stimulus (Figure 3A). Quantification of the signal by densitometry routinely showed a 5- to 10-fold increase in the amount of CRAC-GFP translocated to the plasma membrane. Moreover, we calculated that, at the peak stimulation, ~10% of total CRAC-GFP was associated with membranes.

As is the case for the translocation of CRAC-GFP to the plasma membrane, chemoattractant-mediated activation of adenylyl cyclase is also transient. Previously published data have shown that the kinetics of enzyme activation are slower than the rapid redistribution of CRAC-GFP (Theibert and Devreotes, 1986; Lilly and Devreotes, 1995). To further define this possible lag between CRAC translocation and peak enzyme activity, each response was measured in parallel at short time points following chemoattractant addition. Figure 3A shows that the chemoattractant-mediated activation of adenylyl cyclase indeed peaks about 60 s after receptor stimulation, as was previously observed. Consequently, while CRAC-GFP translocates quickly after receptor stimulation, the maximum activation of adenylyl cyclase occurs slightly later suggesting that there are additional steps between CRAC translocation and the activation of adenylyl cyclase.

The PH Domain Appears to Be Required for Recruitment to the Plasma Membrane

To assess the molecular determinants involved in the translocation of CRAC to the plasma membrane, we studied two CRAC mutants. First we engineered a point mutation in the PH domain of CRAC by substituting the Trp residue conserved in all PH domain sequences to an Ala residue (W167A). This Trp residue is located on the C-terminal α helix, buried within the β sandwich of the structure, and is postulated to contribute to the stability of the entire PH domain (Ferguson et al., 1994, 1995). Second, we used a mutant of CRAC, isolated from a chemical mutagenesis screen as an aggregation-deficient clone, which harbored an in-frame 53 amino acid deletion of residues 467 to 518 near the C terminal (Δ-CRAC) (P. J. Lilly et al., unpublished results). Although CRAC, W167A-CRAC, and Δ-CRAC transformed in *crac*⁻ cells expressed protein, only cells transformed with the wild-type version of CRAC successfully rescued the aggregation-deficient phenotype of *crac*⁻ cells (data not shown). Neither W167A-CRAC nor Δ-CRAC/*crac*⁻ transformants showed significant GTPγS-mediated activation of adenylyl cyclase (Figure 3B). We next assessed the amount of wild-type or mutant CRAC that became associated with membranes following chemoattractant addition. As shown in Figure 3C, Δ-CRAC displayed a transient association with the membrane fraction following cAMP treatment, while the mutant harboring a point mutation in the PH domain failed to do so. Thus, while neither mutant protein was able to activate adenylyl cyclase, only the one bearing a mutation in the PH domain was unable to associate with membranes in response to cAMP. These results suggest that CRAC has at least two distinct functional domains and that translocation is necessary but not sufficient for the activation of adenylyl cyclase.

CRAC-GFP Associates with the Plasma Membrane at the Leading Edge of Chemotaxing Cells

CRAC-GFP appears to be a highly specific marker for the visualization of G protein–coupled activation in living

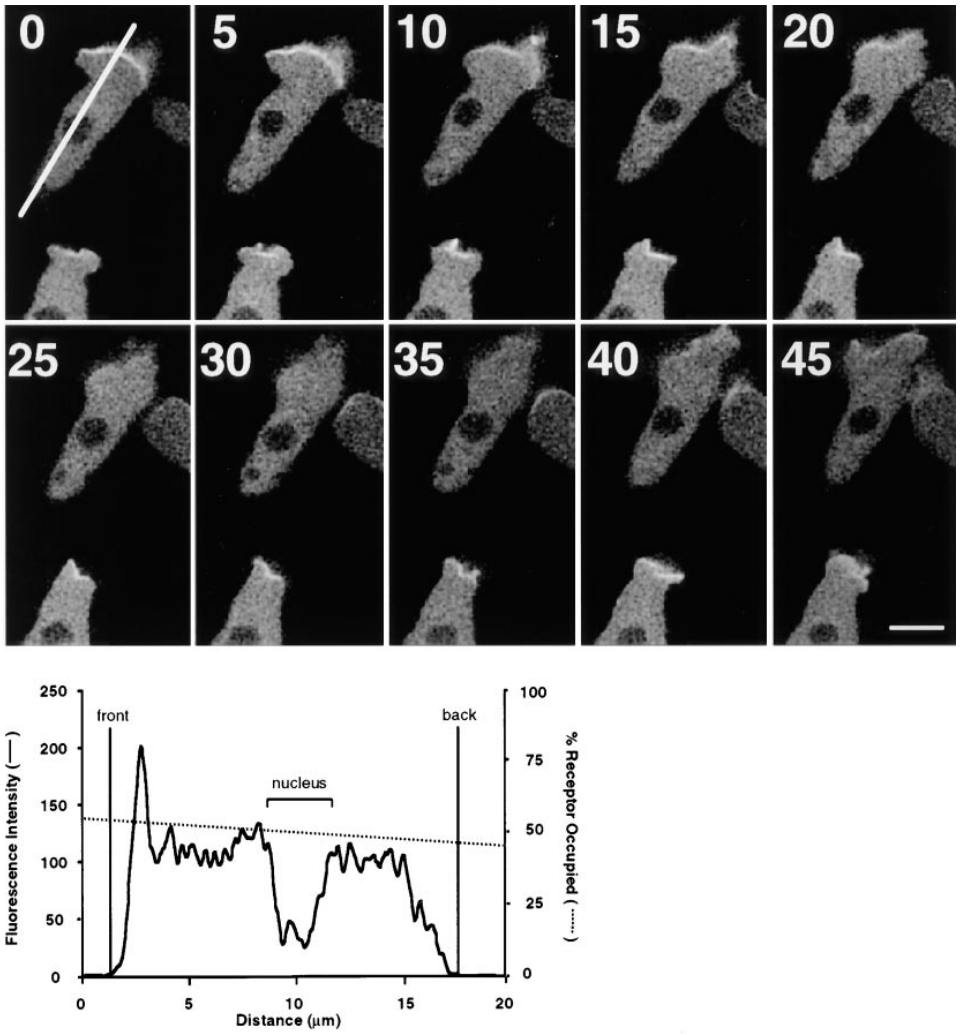

Figure 4. CRAC-GFP Translocates to the Leading Edge of Newly Elicited Pseudopods

(Top panel) Montage of confocal images of Redi CRAC-GFP/*crac*$^-$ cells taken as the cells moved toward a micropipette containing 1 μM cAMP. The pipette is located 25 μm above the upper right corner of the frames. Numbers in the upper left corner are seconds. Calibration bar represents 5.6 μm. This behavior was consistently observed over the course of 15 independent sessions. An animated version of this experiment can be found in the supplemental data section of the on-line version of the journal (http://www.cell.com/supplemental/95/1/81).

(Bottom panel) Quantitative analysis of the translocation event. Fluorescence intensities were measured along a thin line through the central portion of the upper cell (see schematic representation on frame taken at 0 s on the top panel) and plotted as a function of distance. Similar results were obtained by analysis of at least five cells recorded on different days. The dotted line depicts the estimated level of receptor occupancy at different points assuming that the concentration at the midpoint of the cell is near K_d. If it were not near K_d, the changes would be smaller. See Experimental Procedures for details.

cells. To study the spatial localization of signaling events in chemotaxing cells, we placed the CRAC-GFP/*crac*$^-$ cells in a gradient of cAMP and used a micropipette to deliver a constant amount of chemoattractant at a specific location on the cover glass. In this assay, cells rapidly move toward the micropipette tip, which can be repositioned to allow the formation of new chemotactic fronts. As cells carried out chemotaxis, a redistribution of CRAC-GFP from the cytosol to the plasma membrane occurred exclusively on elicited pseudopods. In the sequence of events presented in the top panel of Figure 4, the micropipette containing 1 μM cAMP was placed in the upper right corner and frames were captured every 5 s using confocal microscopy. As the cells were moving toward the chemoattractant, an intense fluorescent signal was observed at the ruffling front of the cells. Note that cells located closest to and furthest from the needle display similar localization of the signal at the leading edge. The association of CRAC-GFP with the plasma membrane in chemotaxing cells was not continuous; the three cells presented in Figure 4 show the appearance and disappearance of fluorescent signal at the plasma membrane within a 20–40 s time frame. Quantification of the translocation event revealed that while receptor occupancy changed only slightly across the cell length (see Experimental Procedures), the CRAC-GFP signal was strongly enriched at the front of the cell. The bottom panel of Figure 4 presents the change in

CRAC-GFP fluorescence intensity along a thin line through the center of a cell. It is evident that a fraction of CRAC is sharply relocalized at the leading edge and that the remaining cytosolic CRAC is uniformly distributed along the length of the cell. Consequently, the highly polarized redistribution of CRAC-GFP at the front of chemotaxing cells greatly amplifies the small differences in receptor occupancy.

When we changed the position of the micropipette, the cells either extended pseudopods from the original migrating front and made a 180° turn toward the newly formed gradient or, alternatively, the cells created a new front thereby reversing the direction of their movement (Devreotes and Zigmond, 1988). Regardless, we always detected a bright fluorescent signal solely on the plasma membrane of the leading edge of the cell. For example, in Figure 5A (top left panel), a cell is moving up a gradient of cAMP toward the micropipette located above and to the right of the frame. The pipette was then moved below and to the left of the frame, and images were captured. As the new cAMP gradient was restored, a chemotactic front began to appear at the back of the cell and we observed small amounts of CRAC-GFP on the plasma membrane. By 200 s, a new leading edge was firmly established and showed a bright fluorescent signal.

We also observed the response of the cells to a falling gradient. The pressure through the needle was first increased to a high level via the micropipette injector and then released to allow reestablishment of the gradient. Immediately upon increasing the pressure, a uniform translocation of CRAC-GFP from the cytosol to the plasma membrane occurred. As the gradient reestablished, the cells showed distinct polarized translocation of CRAC-GFP and, within 70 s, the cells displayed robust chemotaxis. An animated version of this experiment can be found in the supplemental data section of the online version of the journal (http://www.cell.com/supplemental/95/1/81). These results show the rapid and transient nature of the translocation event and underscore the dynamics of the response when the cells are subjected to sudden changes in chemoattractant concentrations.

We next subjected cells to a brief increase in chemoattractant, which swept across the cells like a wave. In these experiments, the micropipette was positioned near a cell and at specific times a pulse of chemoattractant was released by applying a pressure increase on the microinjector for only 5 s. As illustrated in Figure 5B, the side of the cell that encountered the approaching gradient responded by selectively translocating CRAC-GFP along the entire membrane facing the needle. The cell also briefly extended a ruffled edge toward the micropipette. After repeating this stimulation routine three times on the right side of the cell, the pipette was repositioned on the left side and similar directional translocations of CRAC-GFP were elicited (Figure 5B). These observations imply that cells must have mechanisms that allow for the inhibition of the signal at their "back" even when the concentration of chemoattractant is rapidly increasing at both ends of the cell.

To further assess the role of cell movement in chemotaxis, we set out to measure if nonmotile cells retained the capacity to perceive gradients. As noted above, de novo actin polymerization is not required for the translocation event to occur in response to a uniform increase in chemoattractant. We now wanted to assess whether

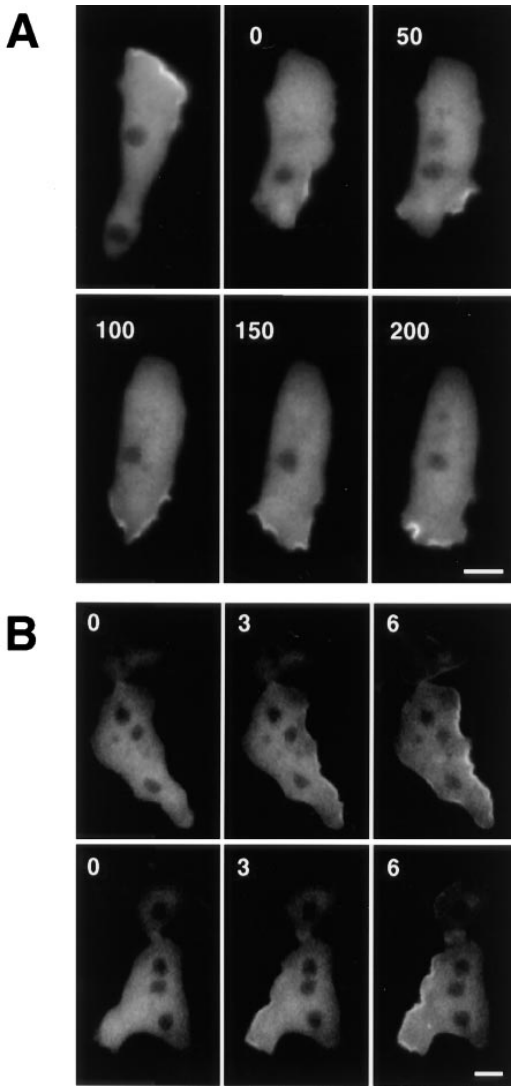

Figure 5. Redistribution of CRAC-GFP after Repositioning of the Micropipette and in Response to Brief Waves of Chemoattractant

(A) The upper left frame was taken as the Redi CRAC-GFP/*crac*$^-$ were moving toward the micropipette, which was located at the upper right corner of the image (the needle contained 1 μM cAMP). The needle was then moved to the lower left corner, and within 1 min, sequential images were captured. Numbers in the upper left corner are seconds after the observation began. Calibration bar represents 6 μm. This behavior was consistently observed over five independent sessions.

(B) A micropipette containing 1 μM cAMP was positioned near Redi CRAC-GFP/*crac*$^-$ cells, and a brief pulse of chemoattractant was released by applying a pressure increase on the microinjector for 5 s. The numbers in the upper left corner are seconds after the chemoattractant was released. The micropipette was placed on the right side of the cell for the top three panels and on the left side of the cell for the bottom three panels. Calibration bar represents 7.6 μm. This behavior was observed in at least four independent sessions. Animated versions of these experiments can be found in the supplemental data section of the on-line version of the journal (http://www.cell.com/supplemental/95/1/81).

actin polymerization was needed to locally activate G protein–coupled signaling in cells exposed to a gradient. In these experiments, cells moving in a gradient of cAMP were treated with 0.5 μM Latrunculin A. After the addition of the drug, the cells gradually stopped extending pseudopods, became progressively rounded, and eventually showed little motility. Surprisingly, even under these conditions, the cells retained the ability to translocate CRAC-GFP specifically at the membrane directed up the chemoattractant gradient (Figure 6A). Figure 6B shows another field of cells examined approximately 20 min after the addition of Latrunculin A. The upper and lower images, taken 90 s apart, illustrate that the cells are essentially immobile. Even so, a strong fluorescent signal was observed at the membrane facing the micropipette (the star depicts where the micropipette was positioned). Although these results strongly suggest that eukaryotic cells are able to perform spatial sensing, we cannot rule out that slight superficial membrane ruffling exhibited by the rounded cells is important for sensing (see animated version of this experiment in the supplemental data section of the on-line version of the journal [http://www.cell.com/supplemental/95/1/81]).

We have observed that CRAC-GFP is specifically redistributed to membranes when cells are stimulated with chemoattractant. We also noted that CRAC is spontaneously associated with "crown-like" structures, thought to be precursors of macropinosomes, in randomly moving vegetative cells. These circular ruffles are regions where F actin accumulates along with the actin-binding protein coronin and myosin IB (Fukui et al., 1989; Jung and Hammer, 1990; Maniak et al., 1995). Figure 7 shows resting vegetative cells displaying an even cytoplasmic fluorescent signal with intense labeling of crowns. The signal associated with these structures took the form of rings and was present as the structures were forming but dissipated as soon as the macropinosomes closed and entered the cell. Developed cells had fewer macropinosomes and, when they were present, they behaved independently of chemoattractant stimulation (see Figure 2, upper panel, bright structure on the lower right corner of the cell). Furthermore, CRAC-GFP was associated with macropinosomes in the $g\beta^-$ cells (data not shown) where we have shown that chemoattractant stimulation does not induce CRAC translocation (Lilly and Devreotes, 1995).

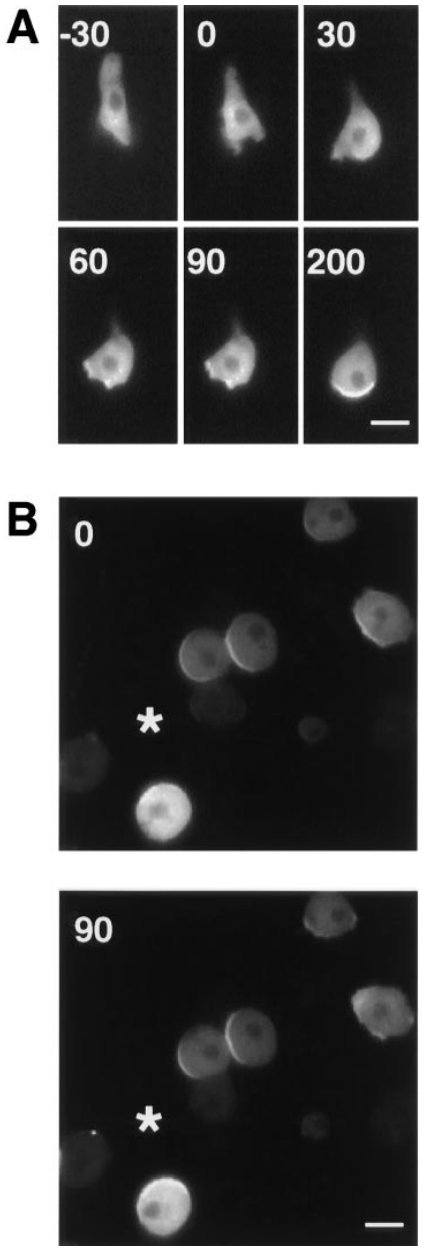

Figure 6. Latrunculin A–Treated Cells Retain the Ability to Selectively Translocate CRAC-GFP at the Membrane Directed up the Chemoattractant

Redi AX_2 cells expressing CRAC-GFP were placed in a chamber.
(A) A micropipette containing 1 μM chemoattractant is located 10 μm below the lower edge of the field. At time 0, Latrunculin A (final concentration 0.5 μM) was added and images were captured. Numbers in the upper left corner are seconds before and after the addition of Latrunculin A. Calibration bar represents 12 μm.
(B) These images, obtained from the same chamber as in (A), were captured approximately 20 min after the addition of Latrunculin A. The position of the micropipette is indicated by the asterisk. Numbers in the upper left corner are seconds. Calibration bar represents 9 μm. Animated versions of these experiments can be found in the supplemental data section of the on-line version of the journal (http://www.cell.com/supplemental/95/1/81).

Discussion

Our results demonstrate that in gradients of chemoattractant the *activation* of the G protein–coupled signaling system, reflected by the transient translocation of CRAC-GFP to the membrane, is spatially localized. Other observations have shown that chemoattractant receptors and G protein subunits are uniformly localized at the cell periphery (Xiao et al., 1997; T. Jin et al., unpublished results; G. Servant and H. Bourne, personal communication). Biochemical analysis has shown that the stimulus-induced translocation of CRAC-GFP is an indication of the appearance of CRAC binding sites on the inner face of the plasma membrane (Lilly and Devreotes, 1995 and see below). These sites appear transiently

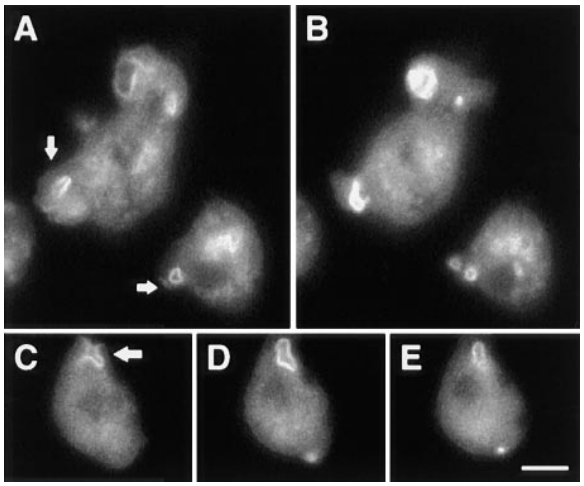

Figure 7. CRAC-GFP Is Associated with Macropinosomes

CRAC-GFP/*crac*$^-$ cells were harvested in their vegetative state, washed once, resuspended in phosphate buffer, and directly observed using conventional microscopy. (A) and (B) were captured at 10 s intervals. The cell shown in (C), (D), and (E) (also captured every 10 s) comes from another field. The arrows point to macropinosomes. Calibration bar represents 8 μm. This behavior was observed over the course of five independent sessions.

in response to uniform stimuli and asymmetrically in response to gradients. Recruitment of a cytosolic protein to localized regions of activation, as we report here, therefore provides a means to transmit already-localized signals. We propose that such localized activation will be a general phenomenon for G protein–linked signaling chemotactic systems including those involving chemotactic cytokines in leukocytes.

Oancea et al. have shown that proteins harboring cysteine-rich domains from PKC become transiently associated with the membrane following G protein– or tyrosine kinase–coupled receptor stimulation (Oancea et al., 1998). In other experiments, the activation of several different G protein–coupled receptors caused the translocation of β-arrestin-GFP to the membrane, and the guanine nucleotide exchange factor, ARNO, became associated with plasma membranes following treatment with insulin (Barak et al., 1997; Venkateswarlu et al., 1998). We report here the redistribution of a signaling protein from the cytosol to the plasma membrane specifically at the leading edge of chemotaxing cells. Although actin and several actin-binding proteins, including coronin, talin, cofilin, and CAP, become associated with newly extended pseudopod extensions in *D. discoideum* cells, this relocalization is observed in randomly moving, as well as chemotaxing, cells, and the redistribution of coronin still occurs in *g*β$^-$ cells, which lack sensitivity to all chemoattractants (Gerisch et al., 1995; Kreitmeier et al., 1995; Gottwald et al., 1996; Aizawa et al., 1997; Westphal et al., 1997; Peracino et al., 1998; T. Jin, N. Zhang, and P. N. Devreotes, unpublished results). Moreover, while the actin-binding proteins tend to fill the entire F-actin rich region of the pseudopod, CRAC-GFP specifically localizes to the plasma membrane at the front edge of the projection. Thus, CRAC-GFP represents a specific marker for the signal transduction events occurring at the leading edge of chemotactically sensitive cells.

Several models for gradient sensing by eukaryotic cells have been proposed (Devreotes and Zigmond, 1988; Caterina and Devreotes, 1991). First, a "purely temporal" model depicts cells as extending pilot sensors in all directions, responding only to those extended up the gradient. Second, a "spatial-temporal" model sees cells as requiring temporal increases in receptor occupancy to respond, but also needs inhibition to occur at the side of the cell receiving a later or lower stimulus. Third, a "purely spatial" model has cells capable of intrinsically detecting differences in receptor occupancy between its two ends, even when receptor occupancy shows no temporal change. Our findings rule out the "purely temporal" model since nonmotile cells are able to polarize their signal transduction responses (Figure 6). Rather, our results favor the "purely spatial" model. For spatial detection, cells must sense small differences in receptor occupancy at their two ends. Hence, the need for chemotaxing cells to express their receptors and G proteins uniformly around the cell perimeter and to possess mechanisms that allow rapid polarization of the activation event.

We have a good understanding of the signal transduction events involved in the activation of adenylyl cyclase. Chemoattractant-mediated activation of adenylyl cyclase requires, in addition to the G protein βγ–subunits, two novel cytosolic proteins: CRAC and Pianissimo (Insall et al., 1994; Chen et al., 1997). Following chemoattractant or GTPγS stimulation, it is thought that CRAC binds to the plasma membrane and somehow, in concert with Pianissimo, activates adenylyl cyclase. The data presented here show that, in living cells, CRAC transiently associates with plasma membrane following stimulation of chemoattractant receptors. This is consistent with previous reconstitution experiments that have shown that the receptor- and GTPγS-mediated activation of adenylyl cyclase in *crac*$^-$ cells can be restored by adding exogenous supernatant preparations derived from unstimulated cells (Lilly and Devreotes, 1995). These biochemical experiments have also established that (1) the GTPγS-induced association of CRAC with membranes does not require chemoattractant receptor, Gα2, or adenylyl cyclase, but depends essentially on Gβ, (2) stimulus-induced modifications of CRAC are not required prior to binding, (3) the appearance of the binding sites is transient in the absence of CRAC, and (4) once created in cells, the CRAC binding sites remain stable in lysates. Since there is a lag of about 50 s between the peak CRAC translocation event and maximal adenylyl cyclase activation, there must be additional steps between the two events. These steps may involve Pianissimo, since CRAC translocation takes place in *pia*$^-$ cells (B. J. B. and P. N. D., unpublished observation).

The identity of the CRAC binding site remains to be established. Our mutant analysis showed that CRAC is composed of at least two distinct functional domains. A region in the C terminal is essential for adenylyl cyclase activation, and based on the Trp to Ala mutation, the integrity of the N-terminal PH domain of CRAC is required for membrane association. Numerous reports have suggested that PH domains may associate with either Gβγ-subunits or phosphatidylinositol lipids (reviewed in Lemmon et al., 1996 and Shaw, 1996). Although chemoattractant-mediated translocation of CRAC

to membranes depends essentially on Gβ, the fact that CRAC can associate with macropinosomes in *g*β⁻ cells suggests that Gβγ itself may not be the direct CRAC binding site. The chemoattractant-mediated generation of the CRAC binding site could depend on a downstream effector of Gβγ. While the Gβγ-sensitive PI3 kinase is an attractive candidate, neither wortmanin nor LY294002 inhibitors of PI3 kinase altered the translocation of CRAC-GFP to the plasma membrane (data not shown). Consequently, at the present time, we do not know the identity of the CRAC binding site. Additional experiments are currently under way to further address this point.

The highly localized translocation of CRAC-GFP in chemotaxing cells indicates that the activation of adenylyl cyclase occurs within a very defined region of the plasma membrane at the leading edge of cells even though the adenylyl cyclase itself is uniformly distributed around the cell periphery (C. A. P. and P. N. D., unpublished results). The exact mechanism of action by which this localized production of intracellular cAMP is involved in chemotaxis remains to be determined. In leukocytes, it has been suggested that cAMP is involved in directed cell movement possibly by blocking adhesion via its effects on PKA and RhoA (Laudanna et al., 1997). We propose that other key components that signal directly to the cytoskeleton might also be recruited to the stimulated edge of the cell by associating with the same sites that recruit CRAC. By using a common binding site at a local region of the inner face of the membrane to initiate multiple signal transduction cascades, the cell can coordinate its behavior in response to the dynamic environments present in multicellular organisms.

Experimental Procedures

CRAC-GFP Fusion Protein Construct and Transformation into *crac*⁻ Cells

A mutant GFP gene with greater brightness and slower photobleaching than the wild-type version was used (Xiao et al., 1997). The full-length cDNA sequence of CRAC was amplified by high fidelity PCR using primers that created an EcoRI site at the 3′-end before the STOP codon. The resulting PCR product was cloned into a unique EcoRI site located upstream of the ATG of the GFP gene. The resulting CRAC-GFP construct was sequenced using chain terminator chemistry (DNA Analysis Facility, Johns Hopkins School of Medicine) and finally cloned into the B18 *D. discoideum* expression plasmid, which gives constitutive levels of expression (Johnson et al., 1991). This CRAC-GFP plasmid was then electroporated into *crac*⁻ cells using a Bio-Rad gene pulser, and stable transformants were selected in 20 μg/ml G418 (Howard et al., 1988; Insall et al., 1994). Individual clones were isolated as plaques on *Klebsiella aerogenes* lawns and grown in the presence of G418 (Sussman, 1987). The phenotypes of these clones were assessed by plating cells on non-nutrient agar at 22°C, as previously described (Devreotes et al., 1987). The cells were designated CRAC-GFP/*crac*⁻. Alternatively, we also transfected the CRAC-GFP construct in wild-type AX_2 cells. Both cell lines behaved similarly and were used in this study.

Δ-CRAC and W167A-CRAC Constructs

The hemaglutinin A tag was fused to the N terminal of the CRAC coding sequence, and the resulting DNA fragment was cloned into the Ddp1-based *D. discoideum* extrachromosomal expression plasmid, p88d1 (Hughes et al., 1994). The W167A mutation was constructed using the MORPH Kit (5 Prime → 3 Prime, Boulder, CO) with a primer encoding the mutated codon. These constructs were then electroporated into *crac*⁻ cells as described above and grown in the presence of 20 μg/ml G418.

Cell Growth and Differentiation

Wild-type AX_3 and *crac*⁻ cells were used as controls (Insall et al., 1994). CRAC-GFP/*crac*⁻ cells were grown in shaking suspension to densities of ~5×10^6 cells/ml in HL5 media containing 20 μg/ml G418, harvested by centrifugation, and allowed to differentiate by resuspending them at 2×10^7 cells/ml in DB (5 mM Na_2HPO_4, 5 mM NaH_2PO_4 [pH 6.2], 2 mM $MgSO_4$, 200 μM $CaCl_2$) and shaking them at 100 rpm for 5 hr with repeated pulses of 50 nM cAMP (Devreotes et al., 1987; Parent and Devreotes, 1996b). After a 30 min treatment with 2 mM caffeine (an inhibitor of adenylyl cyclase, used to bring the cells to a basal state), cells were harvested, washed twice, and finally resuspended at 8×10^7 cells/ml in PM (5 mM Na_2HPO_4, 5 mM NaH_2PO_4 [pH 6.2], 2 mM $MgSO_4$) or SLB (10 mM TrisHCl [pH 7.5], 0.2 mM EGTA, and 200 mM sucrose). The cells, designated Redi (*re*sting and *di*fferentiated), were then processed according to the assay performed.

Subcellular Fractionation and Immunoblotting

Redi cells were resuspended in SLB and lysed by filtration through 5 μm-pore-size Nucleopore filters. The resulting lysates were separated into soluble and pellet fractions by centrifugation at 12,000 × g for 30 min at 4°C (Lilly and Devreotes, 1994). The pellets were resuspended in SLB, and samples were subjected to 7.5% SDS-PAGE (Laemmli, 1970). Immunoblotting was performed on nitrocellulose membranes using a polyclonal antibody directed against GFP (Clontech Lab, Palo Alto, CA) or a polyclonal antibody directed against the last 15 amino acids of CRAC. Detection was performed by chemiluminescence using a donkey anti-rabbit horseradish peroxidase–coupled antibody (Amersham, Arlington Heights, IL). Some of the gels show multiple bands. Evidence suggests that these bands result from degradation that occurs after sample preparation. When a dilute suspension of cells was added directly to sample buffer, nearly all the signal was in the high molecular weight band. Quantification was performed using the NIH Image 1.59 software.

Adenylyl Cyclase Assays

Adenylyl cyclase enzymatic activity was measured in differentiated cells as previously described (Parent and Devreotes, 1995). Briefly, Redi cells were resuspended in PM, filter-lysed in the presence of 2 mM $MgSO_4$, with or without the addition of 40 μM GTPγS and 1 μM cAMP, incubated on ice for 4 min, and assayed for 2 min at room temperature. The unregulated intrinsic activity of the enzyme was measured in the presence of 5 mM $MnSO_4$. For receptor-mediated adenylyl cyclase activation, Redi cells were treated with 10 μM cAMP at room temperature, lysed in the presence of 2 mM $MgSO_4$ at specific time points, and assayed for 30 s. In the reconstitution assays, Redi *crac*⁻ cells were lysed into tubes containing cytosol derived from differentiated wild-type (AX_3) or CRAC-GFP/*crac*⁻ cells as previously described (Parent and Devreotes, 1995).

CRAC Translocation Assay

Redi CRAC-GFP/*crac*⁻ cells were resuspended in PM buffer, stimulated with 10 μM cAMP, and, at specific time points, filter-lysed into 1.5 ml microfuge tubes containing 1 ml ice-cold PM buffer. Samples were centrifuged for 1 min at 15,000 × g and the pellet solubilized in Laemmli buffer (Laemmli, 1970). The samples were finally subjected to 7.5% SDS-PAGE and immunoblotted as described above.

Fluorescence Microscopy Analysis on Live CRAC-GFP/*crac*⁻ Cells

Fluorescence microscopy was performed as previously described (Xiao et al., 1997). Briefly, Redi cells expressing CRAC-GFP were harvested by centrifugation, resuspended in phosphate buffer (5 mM Na_2HPO_4, 5 mM NaH_2PO_4 [pH 6.2]), plated in a small spot on a chambered coverglass (Lab-Tek, Nalge Nunc, Naperville, IL), allowed to adhere, and covered with phosphate buffer to prevent drying. The coverglasses were mounted onto a brass holder and observed using an inverted Zeiss microscope (Axiovert 135 TV, Thornwood, NY) equipped with 40 or 100X oil-immersion plan neofluor objective lenses, an HBO 100 watt mercury lamp, and filter

sets for viewing GFP (Z16, Carl Zeiss; XF100, Omega Optical Co.). The cell movement was recorded with a cooled PXL CCD camera (Photometrics, Tucson, AZ) controlled by IPLab-Spectrum software (Scanalytics, Fairfax, VA) on a Power Macintosh computer. Figures presented were obtained using 50–150 ms exposures.

Generation and Quantitation of Chemoattractant Gradients

Gradients were generated using a microinjector connected to micropipettes containing 1 to 10 μM cAMP (Femtotips, Eppendorf, Germany). To assess the gradients generated, we placed rhodamine (MW = 479) in the micropipette and observed the fluorescence signal after the gradient reached steady state, under a variety of experimental conditions. There was a radially symmetric exponential decrease in the rhodamine concentration centered on the tip (see below for quantitation). At the typical working distances of 10 to 50 μm, we observed 10% to 30% changes in concentration over a distance corresponding to the length of a cell. Near K_d, similar changes in cAMP (MW = 329) concentration would give rise to changes of 2% to 10% in receptor occupancy across the cell, as illustrated in Figure 4. In the course of these experiments we also showed that, following the application of a pressure increase on the microinjector, the cAMP gradients were reestablished within 30 s.

Fluorescence Confocal Microscopy Analysis on Live CRAC-GFP/crac$^-$ Cells

Cells were prepared as described above and analyzed using a confocal laser scanning microscope (Noran OZ; Noran, Middleton, WI) with an inverted microscope (IX-50; Olympus, New Hyde Park, NY) equipped with a U plan-apo 100×/1.35 NA oil immersion lens. An excitation wavelength of 488 nm was used (Krypton-Argon multiline laser), and emissions of 500–550 nm were detected.

Image Processing

In IPLab-Spectrum, each series of images was optimized by histogram stretching and converted to TIFF files. For each series, a script was written in Corel Photo Paint to crop, adjust contrast, and sharpen. All frames of the series were processed identically. Selected frames were built into montages in Corel Draw or PowerPoint. Quantification of fluorescence intensities for both GFP and rhodamine was performed using the IPLab-Spectrum software.

Acknowledgments

We thank Dr. Saskia van Es for helpful discussions and technical assistance, Mr. Rodrigo Bustos for his assistance with the fluorescence microscopy, and Mr. Brian Kang for help with the cloning of CRAC-GFP. We are also grateful to Dr. Denis Wirtz for providing the Latrunculin A and Dr. Pierre A. Coulombe for helpful discussions and excellent suggestions. Videos depicting similar experiments as the ones presented in this paper as well as GFP-tagged chemoattractant receptors and G proteins can be viewed at www.med.jhu.edu/devreotes. This work was supported by The American Cancer Society Grant DB-1c to P. N. D., National Institutes of Health Grant GM57874 to C. A. P. and P. N. D., and National Institutes of Health Grant GM33171 to D. B. M.

Received July 8, 1998; revised August 13, 1998.

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