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Rap1 up-regulation and activation on plasma membrane regulates T cell adhesion

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Rap1 and Ras are closely related GTPases that share some effectors but have distinct functions. We studied the subcellular localization of Rap1 and its sites of activation in living cells. Both GFP-tagged Rap1 and endogenous Rap1 were localized to the plasma membrane (PM) and endosomes. The PM association of GFP-Rap1 was dependent on GTP binding, and GFP-Rap1 was rapidly up-regulated on this compartment in response to mitogens, a process blocked by inhibitors of endosome recycling. A novel fluorescent probe for GTP-bound Rap1 revealed that this GTPase was transiently activated only on the PM of both fibroblasts and T cells. Activation on the PM was blocked by inhibitors of endosome recycling. Moreover, inhibition of endosome recycling blocked the ability of Rap1 to promote integrin-mediated adhesion of T cells. Thus, unlike Ras, the membrane localizations of Rap1 are dynamically regulated, and the PM is the principle platform from which Rap1 signaling emanates. These observations may explain some of the biological differences between these GTPases.

Introduction

The Ras superfamily of monomeric GTPases controls a wide range of cellular processes. The prototypical member of this class of regulatory molecules, Ras, plays a role in >30% of human cancers. Rap1 is a member of the subset of monomeric GTPases that are most closely related to Ras itself. Whereas the biological functions of Ras, particularly its roles in cellular growth and differentiation, are well established, the functions of Rap1 are poorly understood. Originally described as a suppressor of Ras-mediated oncogenic transformation (Kitayama et al., 1989), one model held that Rap1 functions by competing for Ras effectors, a view that was supported by the ability of Rap1 to bind to Raf-1 but to not activate the MAPK cascade (Bos, 1998). This view lost some credence when it was reported that Rap1 can stimulate MAPK through B-Raf (Vossler et al., 1997) and that overexpressed Rap1 was capable of inducing oncogenic transformation in Swiss 3T3 fibroblasts (Altschuler and Ribeiro-Neto, 1998). Other Ras effectors that promote cellular growth (e.g., RalGDS) are also activated by interaction with Rap1 (Kishida et al., 1997).

Growth control is but one of a variety of processes in which Rap1 has been implicated. The Rap1 guanine nucleotide exchange factor (GEF) Epac2 has been linked to cAMP-regulated exocytosis, implicating Rap in the control of vesicular trafficking (Ozaki et al., 2000). Overexpression of Rap1 stimulated integrin-dependent adhesion of human T cells, and adhesion of T cells was blocked by expression of dominant-negative Rap1 (Reedquist et al., 2000). Particularly illustrative of the distinct functions of Ras and Rap is the recent finding that these GTPases have opposing effects on AMPA receptor trafficking (Zhu et al., 2002).

The search for functions of Rap1 has included analyses in lower eukaryotes. Bud1, a *Saccharomyces cerevisiae* orthologue of Rap1, is critical for the establishment of yeast polarity through the assembly of the actin cytoskeleton during bud formation (Park et al., 1999). In *Dictyostelium discoideum*, membrane ruffling and lamellipodia formation, both actin-based morphological changes, were regulated by Rap1 (Rebstein et al., 1997). In *Drosophila melanogaster*, the distribution of adherens junctions in epithelium is controlled by an orthologue of Rap1 (Knox and Brown, 2002).

The distinct functions of Ras and Rap1 suggest that despite 70% sequence identity within the effector binding region, including complete identity among residues shown to make contact with the Ras binding domain (RBD) of effectors (Bos et al., 2001), these GTPases are differentially regulated. Indeed, although some GEFs are shared between Ras and Rap1 (e.g., Ras-GRP2), others are Rap1 specific (e.g., C3G; Bos et al., 2001). Once loaded with GTP, modulation of the nucleotide binding state differs between Ras and Rap because, unlike Ras and most Ras-related GTPases that have a glutamine residue at position 61, Rap1 has a threonine and therefore very low intrinsic GTPase activity. In addition to this intrinsic difference, Rap1-specific GTPase activating proteins (GAPs) have been described previously (Polakis et al., 1991). Despite the similarities of the effector domains of Ras and Rap1, particularly in the switch 1 domain, the relative affinities for effectors differs considerably, perhaps due to differences in the switch 2 domain. For example, whereas the RBD of Raf-1 binds to Ras with a 50-fold higher affinity than to Rap1, the opposite is true for the RBD of RalGDS (Herrmann et al., 1996).

Ras and Rap1 also differ in their COOH-terminal hypervariable regions that direct posttranslational modification and membrane targeting. Whereas Ras proteins are modified with a farnesyl isoprenoid, Rap1 is modified with a geranylgeranyl lipid. Consistent with their distinct membrane-targeting motifs, the subcellular localizations of Ras and Rap1 have been reported to differ, a feature that could explain, in part, differential function. In primary myeloid cells, Rap1, but not Ras, is associated with specialized vesicular compartments that serve as pools of membrane that can be rapidly mobilized to the cell surface during degranulation (Maridonneau-Parini and de Gunzburg, 1992; Mollinedo et al., 1993; Berger et al., 1994). Indirect immunofluorescence analysis of cultured fibroblasts and epithelial cells has revealed Rap1 in the Golgi region (Beranger et al., 1991) and on endosomes (Pizon et al., 1994) but not on the plasma membrane (PM). In contrast, in the same cells, Ras proteins are expressed at steady state on both the PM and the Golgi apparatus (Choy et al., 1999). Rap1, like Ras, has been shown to undergo GTP/GDP exchange in response to various growth factors (Zwartkruis et al., 1998). Using a fluorescent probe, we have recently determined that both the PM and Golgi pool of Ras is activated as a consequence of growth factor signaling (Chiu et al., 2002). Recent works have examined the subcellular location of Rap signaling by using a chimeric FRET-based GTPase sensor, Raichu-Rap1 (Mochizuki et al., 2001; Ohba et al., 2003). Despite its targeting to the PM with the hypervariable region of K-Ras4B, this probe reported activation of Rap1 on endomembranes in living cells stimulated with EGF.

By analyzing GFP-tagged Rap1 proteins in living cells and by localizing endogenous Rap1 using subcellular fractionation, we found that the steady-state localization of Rap1 includes endosomes and the PM but not the Golgi apparatus, that PM localization was dependent on GTP binding, and that growth factors stimulated a rapid increase in Rap1 expression on the PM that was dependent on exocytosis. By fusing GFP to the RBD of RalGDS (GFP-RBD$_{RalGDS}$), we have developed a Rap1-specific probe that has allowed us to determine where and when Rap1 is activated in living cells. We found that, in contrast to Ras (Chiu et al., 2002), only the pool of Rap1 associated with the PM became activated in response to growth factors. Similar results were obtained in T cells in which Rap1-mediated adhesion was blocked by inhibition of exocytosis.

Results

PM localization of GFP-Rap1 in living cells is nucleotide dependent

To determine the steady-state subcellular distribution of Rap1 in living cells, we expressed the GTPase tagged at the NH$_2$ terminus with GFP in a variety of cell lines and observed localization of the fusion protein by laser scanning confocal microscopy. In both COS-1 and MDCK cells, GFP-Rap1 was observed on the PM as well as on the nuclear envelope and on cytoplasmic vesicles that were most abundant in the paranuclear region (Fig. 1, A and B). In COS-1 cells, PM expression was most prominent on membrane ruffles. To determine if the guanine nucleotide binding state of Rap1 influences its subcellular localization, we tagged GTP-bound (Rap1V12) and nucleotide-free (Rap1N17) mutants with GFP. Whereas the GTP-bound form displayed a steady-state localization indistinguishable from wild type, the nucleotide-free form was observed on cytoplasmic vesicles but not on the PM (Fig. 1, A and B). Thus, unlike GFP-H-Ras (Chiu et al., 2002), the steady-state distribution of Rap1 depends on its guanine nucleotide binding state.

Because Rap1 has been reported previously on endosomes (Pizon et al., 1994), we tested whether the intracellular vesicles that expressed GFP-Rap1 were derived from this compartment. Both the peripheral and paranuclear vesicles marked by GFP-Rap1 incorporated Texas-red–conjugated transferrin and were therefore endosomal (Fig. 1 C). The intracellular vesicles marked by GFP-Rap1V12 and GFP-Rap1N17 also accumulated transferrin (Fig. 1 C). Thus, unlike localization at the PM, the endosomal targeting of Rap1 does not depend on guanine nucleotide binding state.

In addition to endosomes, intracellular Rap1 has been reported on the Golgi apparatus (Beranger et al., 1991). To determine if Golgi membranes were a component of the paranuclear compartment marked by GFP-Rap1, we co-transfected cells with GFP-Rap1 and a Golgi marker, CFP-tagged galactosyl transferase (CFP-GalT), and resolved the two proteins by laser scanning confocal microscopy coupled with spectral deconvolution. Although both proteins were highly enriched in the paranuclear region where the Golgi apparatus, the endosome recycling compartment, and the microtubule organizing center are known to interdigitate, GFP-Rap1 was not enriched on the structure clearly delineated by CFP-GalT (Fig. 1 D). In contrast, GFP-N-Ras, previously shown to reside on the Golgi apparatus and PM (Choy et al., 1999), decorated the identical structures marked by CFP-GalT (Fig. 1 E). Thus, unlike N-Ras, Rap1 is not enriched on the Golgi apparatus.

Endogenous Rap1 is expressed on the PM

To compare the localization of GFP-Rap1 with that of endogenous Rap1, we analyzed a variety of cell lines for Rap1
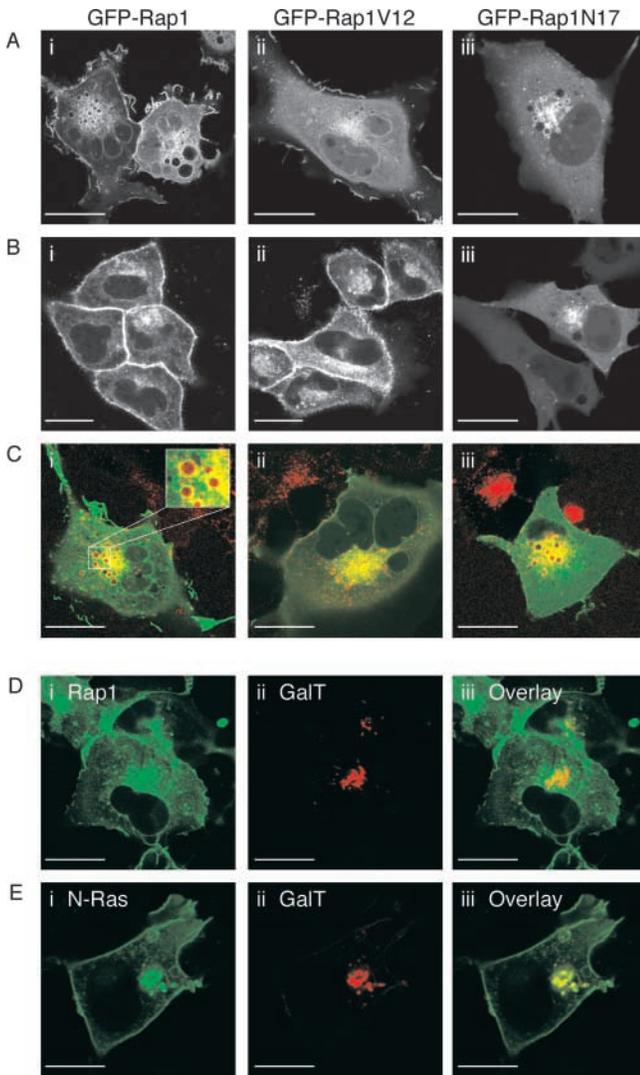
Figure 1. **Localization of GFP-Rap1 in living cells.** COS-1 (A) or MDCK (B) cells were transfected with GFP-Rap1 (i), GFP-Rap1V12 (ii), or GFP-Rap1N17 (iii) and imaged 24 h after transfection with a laser scanning confocal microscope. Results are representative of three independent experiments (>30 cells examined per condition per experiment). (C) COS-1 cells were transfected as in A and incubated at 37°C for 30 min with Texas red–conjugated transferrin. Dual color confocal images were acquired showing colocalization in yellow. Enlarged inset in panel i shows transferrin in the lumen of endosomes decorated with GFP-Rap1. (D and E) COS-1 cells were cotransfected with CFP-GalT and either GFP-Rap1 (D) or GFP-N-Ras (E) and imaged with a laser scanning confocal microscope equipped with the Meta™ system of spectral deconvolution capable of separating CFP from GFP. Bars, 10 μM.

Figure 2. **Localization of endogenous Rap1.** Subcellular fraction-ation of MDCK cells using sucrose followed by Optiprep gradients or biotinylation cell surface expression assay. Immunoblot analysis for EEA1, Na/K ATPase, and Rap1 was performed on the total membrane fraction (TM) and on membrane fractions highly enriched for the Golgi complex (Golgi), plasma membranes (PM), or endosomes (Endo) obtained by Optiprep, or on PM obtained by biotin affinity purification (Biotin PM). Results are representative of two independent experiments.

stained for Rap1, revealed staining only on intracellular membranes (unpublished data). This result suggests that either indirect immunofluorescent staining with currently available anti-Rap1 antibodies is not sensitive enough to detect Rap1 at the PM or that fixation and permeabilization leads to selective loss of the Rap1 protein or its relevant antigenic epitope at the PM. Accordingly, we analyzed the PM for endogenous Rap1 using an independent method, subcellular fractionation. Using a discontinuous sucrose density gradient followed by a continuous Optiprep gradient, we obtained fractions from homogenates of MDCK cells highly enriched in membranes derived from the Golgi apparatus, endosomes, and PM. Golgi, endosomal, and PM fractions were highly enriched for galactosyl transferase, EEA-1, and Na/K ATPase, respectively. Immunoreactive Rap1 was present in both endosomal and PM fractions (Fig. 2). Importantly, neither the Golgi complex nor PM fractions were contaminated by EEA1-positive membranes. The trace amount of immunoreactive Rap1 detected in Golgi fractions was similar to levels of immunoreactive Na/K ATPase in those fractions, supporting the conclusion that the small amount of Rap1 detected in Golgi fractions could derive from the inevitable contamination of Golgi membranes with PM-derived vesicles. To further establish that endogenous Rap1 was expressed on the PM, we used a well-characterized method for the affinity purification of PM-derived vesicles. COS-1 cells were biotinylated at 4°C to allow modification only of surface membrane; after Dounce homogenization, biotinylated vesicles were affinity purified using immobilized streptavidin (Mammen et al., 1997). Rap1 was found in the biotinylated fraction, which also contained Na/K ATPase but not EEA-1 (Fig. 2, Biotin PM lane). Thus, the results from subcellular fractionation were concordant with those obtained by localizing GFP-Rap1 in living cells. From these data, we conclude that the localization of overexpressed GFP-Rap1 on both PM and endosomes reflects the localization of the endogenous GTPase.

Mitogens induce rapid, exocytosis-dependent up-regulation of Rap1 on the PM

The localization of Rap1 on intracellular vesicles including endosomes, as well as on the PM, raised the possibility that PM-associated Rap1 might be regulated by exocytosis. Indeed, Rap1 associated with intracellular granules of neutrophils (Maridonneau-Parini and de Gunzburg, 1992) and

using indirect immunofluorescence. We found that, unlike the reproducible results obtained by imaging GFP-Rap1 in living cells, the pattern of endogenous Rap1 revealed by indirect immunofluorescence was sensitive to fixation and permeabilization methods. In both COS-1 and MDCK cells, the most reproducible pattern observed was one of widely distributed cytoplasmic vesicles consistent with endosomes, in agreement with Pizon et al. (1994). PM staining was not observed. However, cells transfected with GFP-Rap1 that—when imaged alive, revealed unambiguous PM localization of the fusion protein—when fixed, permeabilized, and
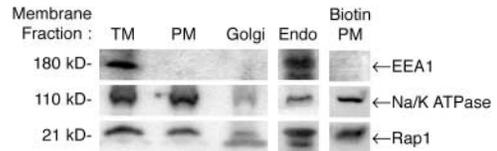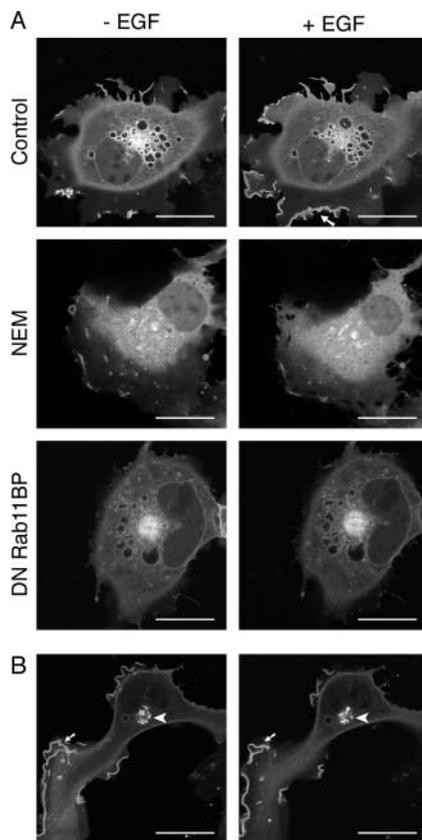
Figure 3. **Growth factor stimulates exocytosis-dependent up-regulation of Rap1 on the PM.** (A) COS-1 cells were transfected with GFP-Rap1 wild type without (top and middle) or with cotransfection of dominant-negative Rab11BP (bottom). Cells were serum starved 24 h after transfection, stimulated with EGF in the absence (top and bottom) or presence (middle) of NEM, and imaged before and 5 min after stimulation. Arrow indicates areas of GFP-Rap1 up-regulation on PM ruffles. PM up-regulation of Rap1 was observed in all control cells and validated by measurement of relative fluorescence intensity as described in Materials and methods. In contrast, only $16 \pm 9\%$ and $8 \pm 8\%$ (mean $\pm$ SEM) of NEM-treated and dominant-negative Rab11BP (DN Rab11BP) transfected cells, respectively, showed up-regulation on PM ($n = 4$; $P < 0.0001$ for each condition compared with control). (B) COS-1 cells expressing GFP-H-Ras that were serum starved and stimulated as in A showed no change in Golgi apparatus (arrowhead) or PM (arrow) expression. Images shown are representative of seven Z slices acquired to compensate for minimal focal drift. Bars, 10 μM.

platelets (Nagata and Nozawa, 1995) serves as a pool that can be rapidly mobilized to the PM upon degranulation. Therefore, we used GFP-Rap1 to determine if PM expression was rapidly modulated. In serum-starved COS-1 cells, EGF stimulated up-regulation of PM-associated GFP-Rap1 (Fig. 3 A). The rapidity of this response (evident by 5 min) was inconsistent with new synthesis of GFP-Rap1 and suggested translocation from another compartment, in this case, intracellular vesicles. To confirm that EGF-stimulated up-regulation of PM-associated GFP-Rap1 was mediated by exocytosis, we used *N*-ethylmaleimide (NEM), a reagent known to block a wide range of vesicular fusion events including those associated with endocytic recycling (Galli et al., 1994). Pretreatment of cells with NEM blocked EGF-stimulated up-regulation of Rap1 on the PM (Fig. 3 A).

Among the various subclasses of endosomes, it is the recycling endosomes that traffic to the cell surface and fuse with the PM in an NEM-sensitive process (Galli et al., 1994). Endosome recycling has been shown to be controlled by Rab11 and negatively regulated by a dominant-negative Rab11 binding protein (Rab11BP; Zeng et al., 1999). We overexpressed dominant-negative Rab11BP with GFP-Rap1 and observed markedly decreased GFP-Rap1 at the PM at baseline and inhibition of EGF-stimulated up-regulation of GFP-Rap1 at the PM, confirming regulation of PM-associated Rap1 expression by endosomal recycling (Fig. 3 A). In contrast to GFP-Rap1, GFP-H-Ras was expressed in serum-starved cells on the PM and the Golgi apparatus, and the distribution was not influenced by stimulation with EGF (Fig. 3 B). Thus, as in hematopoetic cells, Rap1 is expressed on the PM of fibroblasts and the extent of PM expression can be rapidly up-regulated by exocytosis from a Rab11BP-sensitive compartment.

Recruitment of GFP-RBD$_{RalGDS}$ from the cytosol to membranes reports localization of GTP-bound Rap1

We have shown that the RBD of Raf-1 tagged with GFP is a fluorescent probe that can report where and when Ras is activated in living cells without significant interaction with GTP-bound Rap1 (Chiu et al., 2002). To develop an analogous probe specific for Rap1, we used the RBD of RalGDS, an effector for Ras and Rap1 that in contrast to Raf-1 has a higher affinity for Rap1 (Herrmann et al., 1996). When expressed alone in serum-starved cells, GFP-RBD$_{RalGDS}$ had a homogeneous distribution in the cytosol and nucleoplasm revealing negatively imaged organelles and accumulating on no membrane compartment (Fig. 4 A, i). This pattern was indistinguishable from that of GFP-RBD$_{Raf-1}$ (Fig. 4 B, vi) or GFP expressed alone in the same cells. However, when coexpressed with wild-type Rap1, GFP-RBD$_{RalGDS}$ accumulated on PM in peripheral ruffles (Fig. 4 A, ii). When coexpressed with Rap1V12, the reporter accumulated on prominent PM ruffles as well as on paranuclear vesicles (Fig. 4 A, iii). No redistribution was observed when GFP-RBD$_{RalGDS}$ was coexpressed with nucleotide-free, dominant-negative Rap1N17 (Fig. 4 A, iv). Thus, membrane recruitment of GFP-RBD$_{RalGDS}$ was dependent on the GTP-bound state of Rap1.

Because in addition to Rap1, H-Ras, M-Ras, and R-Ras may interact with the RBD of RalGDS (Ehrhardt et al., 2002), we determined the specificity of membrane recruitment of GFP-RBD$_{RalGDS}$ for reporting GTP-bound Rap1. We coexpressed the probe with GTP-bound H-Ras61L, M-Ras71L, or R-Ras87L and observed no membrane recruitment (Fig. 4 B, iii–v) in serum-starved COS-1 cells. Conversely, GFP–RBD$_{Raf–1}$ was a sensitive probe for GTP-bound H-Ras61L, M-Ras71L, or R-Ras87L (Fig.4B, viii–x) but not GTP-bound Rap1 (Fig. 4 B, vii). Moreover, dominant-negative Rap1N17 blocked wild-type Rap1-mediated recruitment of GFP-RBD$_{RalGDS}$ to membrane ruffles (Fig. 4 C, i and ii). Thus, GFP-RBD$_{RalGDS}$ is an in vivo probe specific for activated Rap1.

To validate GFP-RBD$_{RalGDS}$ recruitment to membranes as a readout of Rap1 activation and to confirm the PM localization of activated Rap1, we stimulated Rap1 via an alternative, growth factor–independent pathway. Activated
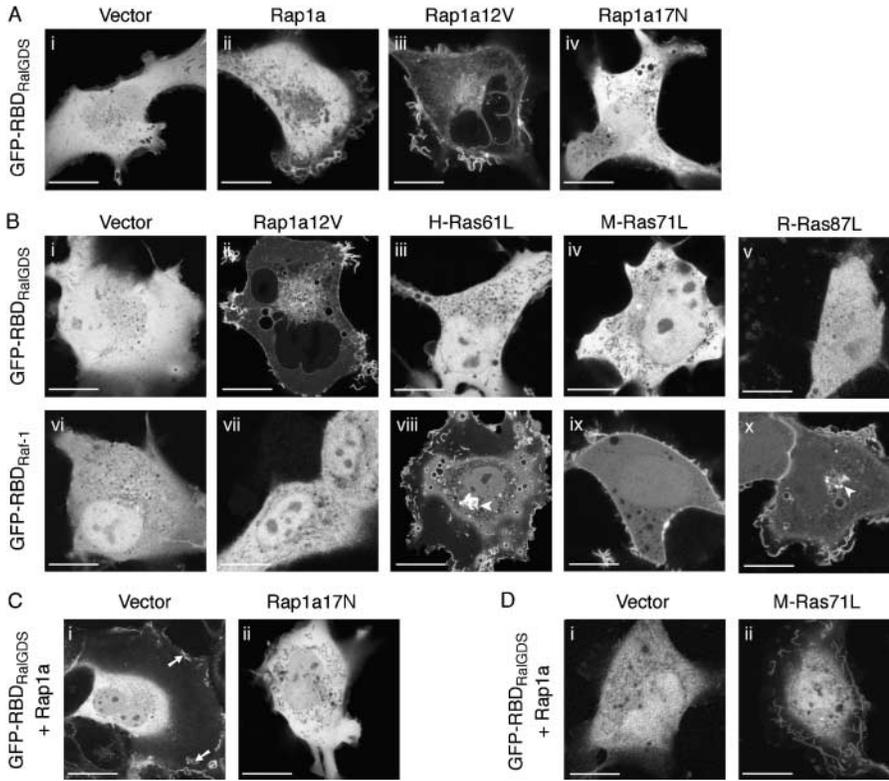
Figure 4. **Rap1 activation in living COS-1 cells.** (A) COS-1 cells were transfected with GFP-RBD$_{RalGDS}$ and vector (i), untagged Rap1 wild type (ii), Rap1V12 (iii), or Rap1N17 (iv), and cells were imaged alive 24 h after transfection under conditions of growth in serum. (B) Cos-1 cells were cotransfected with GFP-RBD$_{RalGDS}$ (i–v), GFP–RBD$_{Raf-1}$ (vi–x), and either vector (i and vi), untagged Rap1V12 (ii and vii), untagged H-Ras61L (iii and viii), untagged M-Ras71L (iv and ix), or untagged R-Ras87L (v and x); serum starved; and imaged as in A. Arrowheads indicate the Golgi apparatus. (C) COS-1 cells were cotransfected with GFP–RBD$_{RalGDS}$, untagged Rap1 wild type, and either vector (i) or untagged Rap1N17 (ii); grown in serum; and imaged as in A. (D) COS-1 cells were cotransfected with GFP–RBD$_{RalGDS}$, untagged Rap1 wild type, and either vector (i) or M-Ras71L (ii); serum starved; and imaged as in A. Arrows indicate PM. Bars, 10 μM. Results are representative of three independent experiments (>30 cells examined per condition per experiment).

M-Ras71L has been shown to activate Rap1 via the Rap-specific GEF, RA GEF 2 (Gao et al., 2001). Whereas expression of M-Ras71L in COS-1 cells expressing GFP-RBD$_{RalGDS}$ failed to induce any redistribution of the reporter (Fig. 4 B, iv), coexpression of wild-type Rap1 induced a marked redistribution of the probe to the PM but not intracellular vesicles (Fig. 4 D), demonstrating that, in the presence of activated M-Ras, Rap1 is activated specifically on PM. In addition to M-Ras, cAMP has been shown to regulate Rap1 in some cells independently of growth factors via Epac1, a Rap1-specific GEF (Kawasaki et al., 1998). Recently, a novel cAMP analogue that does not activate PKA, 8CPT-2Me-cAMP, was shown to specifically activate Rap1 (Enserink et al., 2002). In contrast to growth factor stimulation, we were unable to detect activation of endogenous or ectopically expressed Rap1 in COS-1, NIH 3T3, or 293 cells treated with 8CPT-2Me-cAMP using either membrane recruitment of GFP-RBD$_{RalGDS}$ in living cells or GST-RalGDS RBD pull-down in cell lysates (unpublished data), suggesting that Epac1 was not expressed in these cells.

Mitogen-stimulated activation of Rap1 occurs only at the PM and is dependent on exocytosis

The differential membrane localization of GFP-Rap1 (PM and endosomes) versus GFP-RBD$_{RalGDS}$ in cells coexpressing Rap1 (PM only) was striking and suggested that under conditions of growth in serum, the GTP-bound pool of Rap1 is limited to the PM. Therefore, we sought to use GFP-RBD$_{RalGDS}$ to study dynamic changes in Rap1 activation after cell activation.

We have shown that GFP-RBD$_{Raf-1}$ can report in living cells dynamic spatio-temporal activation of Ras in response to mitogenic stimulation (Chiu et al., 2002). To determine if GFP-RBD$_{RalGDS}$ could report similar activation for Rap1, we ex-

pressed this probe in COS-1 cells, serum starved the cells, and then stimulated them with EGF. GFP-RBD$_{RalGDS}$ was distributed homogenously throughout the cytosol and nucleoplasm of >90% of transfected, serum-starved cells without accumulation on any membrane (Fig. 5 A). Within 5 min of exposure to EGF, the GFP-RBD$_{RalGDS}$ reporter was recruited to membrane ruffles by endogenous Rap1 (Fig. 5 A). This recruitment was transient, reversing by 20 min (Fig. 5 A). When wild-type Rap1 was overexpressed along with GFP-RBD$_{RalGDS}$, a similar, but more robust, recruitment to membrane ruffles was observed that reversed with the same kinetics (Fig. 5 B) as that observed with endogenous Rap1 (Fig. 5 A). Similar observations were made in NIH 3T3 fibroblasts (Fig. 5 C).

To verify that upon stimulation with growth factor, the RBD of RalGDS can discriminate between activated Rap1 versus Ras, we stimulated COS-1 cells with EGF and immunoprecipitated whole cell lysates with either GST-RBD$_{RalGDS}$ or GST-RBD$_{Raf-1}$. The level of each activated GTPase was detected by immunoblot using antibodies specific for either Ras or Rap1. Whereas, GST-RBD$_{RalGDS}$ detected activated Rap1 but not Ras, the converse was true for GST-RBD$_{Raf-1}$ (Fig. 5 D). Thus, the RBD of RalGDS is specific for growth factor–mediated Rap1 activation. We conclude that although Rap1 is expressed on both the PM and internal membranes, only the pool associated with the PM becomes GTP bound upon stimulation with EGF. Thus, intracellular pools of Rap1 differ from those of H-Ras that can be activated in situ in response to mitogens (Chiu et al., 2002).

To determine if activation of Rap1 on PM requires exocytosis, we analyzed EGF-stimulated recruitment of GFP-RBD$_{RalGDS}$ in the presence of NEM or dominant-negative Rab11BP and found that it was blocked completely by either condition (Fig. 6 A). In contrast, inhibition of clathrin-
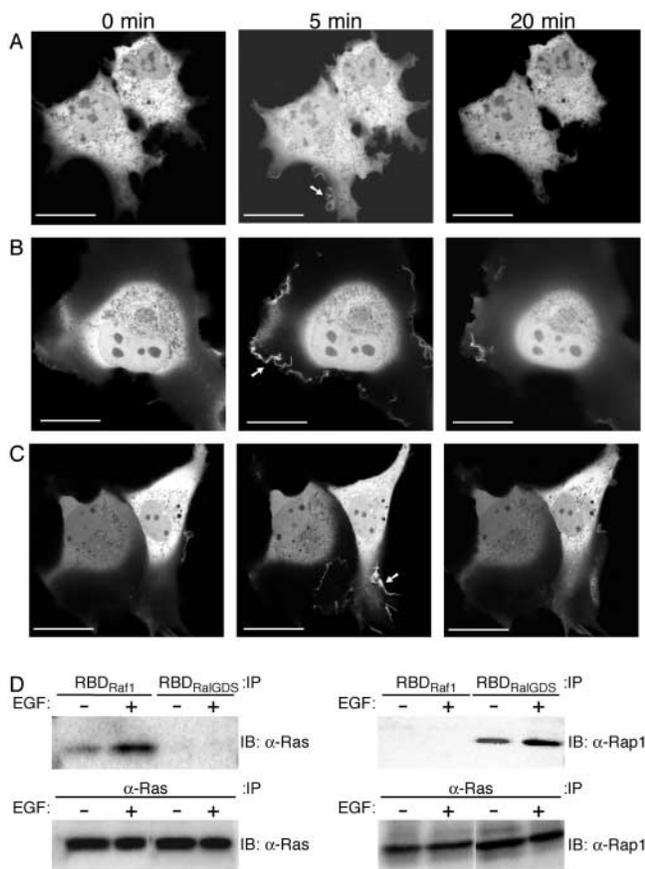
Figure 5.   **Growth factor–stimulated activation of Rap1 in living cells.** (A) COS-1 cells were transfected with GFP-RBD$_{RalGDS}$, serum starved 24 h after transfection, and imaged alive at the indicated times after stimulation with EGF at 37°C. Endomembrane recruitment of the probe was not observed in any COS-1 cell. PM recruitment of the probe was observed in 60 ± 5% (mean ± SEM) of transfected cells ($n = 4$). (B) COS-1 or (C) NIH 3T3 cells were cotransfected with GFP-RBD$_{RalGDS}$ and untagged Rap1 wild type, serum starved 24 h after transfection, and imaged as in A. Arrows indicate GFP-RBD$_{RalGDS}$ recruitment to PM. Bars, 10 μM. Endomembrane recruitment of the probe was observed in no COS-1 or NIH-3T3 cell. PM recruitment of the probe was observed in 75 ± 8% and 71 ± 10% (mean ± SEM) of COS-1 and NIH-3T3 cells, respectively ($n = 4$). (D) COS-1 cells were serum starved, and then stimulated with vehicle or 100 ng/ml EGF for 5 min. Clarified cell lysates were immunoprecipitated with either Sepharose-conjugated GST-RBD$_{Raf-1}$ and GST-RBD$_{RalGDS}$ (top) or anti-Ras and anti-Rap1 polyclonal antisera (bottom). Immunoprecipitates were immunoblotted with anti-Ras (left) or anti-Rap1 (right) mAbs. Results shown are representative of two independent experiments.

mediated endocytosis with dominant-negative epsin had no effect (Fig. 6 B). Thus, both up-regulation and GTP/GDP exchange of Rap1 on PM require exocytosis, suggesting that the two processes are linked.

Rap1 is activated at the PM of Jurkat T cells and regulates integrin-dependent adhesion in an exocytosis-dependent fashion

Among the best characterized functions of Rap1 is the regulation of integrin-mediated adhesion of Jurkat T cells (Reedquist et al., 2000; Katagiri et al., 2002). Accordingly, to analyze the role of endosome recycling in Rap1 func-
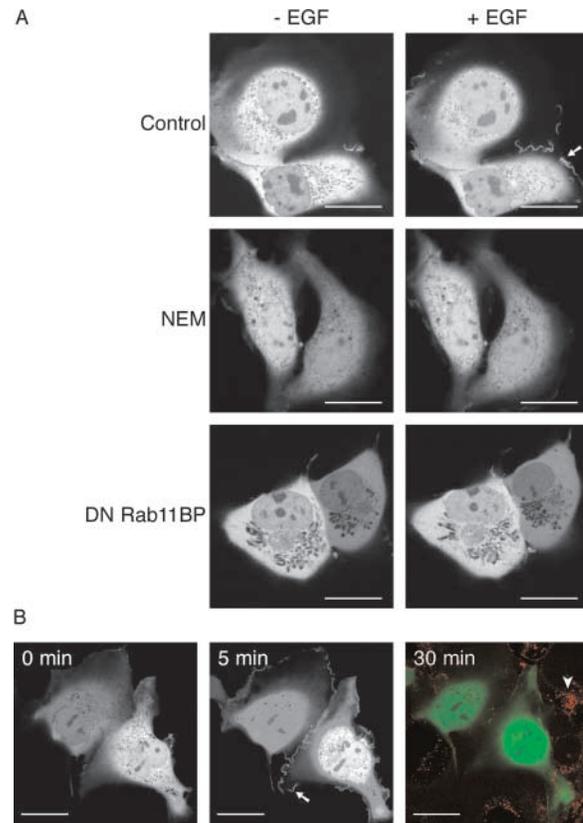

Figure 6.   **Activation of Rap1 at the PM is dependent on exocytosis but not endocytosis.** (A) COS-1 cells were transfected with GFP-RBD$_{RalGDS}$ alone (top and middle) or with dominant-negative Rab11BP (DN Rab11BP; bottom), serum starved 24 h after transfection, and stimulated at 37°C with EGF in the absence (top and bottom) or presence (middle) of NEM. Endomembrane recruitment of the probe was observed in no cell. PM recruitment was observed in 63 ± 14%, 25 ± 8%, and 29 ± 10% of control, NEM-treated, and DN Rab11BP transfected cells (mean ± SEM), respectively ($n = 4$; $P < 0.02$ for each condition compared with control). (B) COS-1 cells were cotransfected with GFP-RBD$_{RalGDS}$, untagged Rap1 wild type, and dominant-negative epsin; serum starved; stimulated with EGF in the presence of Texas red–conjugated transferrin; and imaged after 5 min as in A. The dual color image shown in the far right panel was acquired 30 min after stimulation following removal of excess transferrin. Only the untransfected cell to the right accumulated transferrin in endosomes (arrowhead). PM recruitment of the probe was observed in 71 ± 17% (mean ± SEM) of epsin-transfected, EGF-stimulated cells ($n = 4$). Arrows indicate GFP-RBD$_{RalGDS}$ recruitment to PM. Bars, 10 μM.

tion, we used this system. GFP-Rap1 was localized in Jurkat cells (Fig. 7 A) in a pattern similar to that of COS-1 and MDCK cells: the wild-type and GTP-bound (Rap1V12) forms were observed on PM as well as on cytoplasmic vesicles and the nuclear envelope, whereas the nucleotide-free (Rap1N17) form was expressed predominantly on intracellular membranes. Unstimulated Jurkat cells were round and exhibited a mixed population of two distinct patterns of GFP-Rap1 expression where all of the cells expressed GFP-Rap1 on endomembrane, but the minority of cells clearly expressed the protein on the PM. After stimulation of the TCR by cross-linking CD3, a condition previously shown to activate Rap1 (Reedquist and Bos, 1998), the majority of cells were asymmetrical and exhibited a distribution of GFP-Rap1 on both endomem-
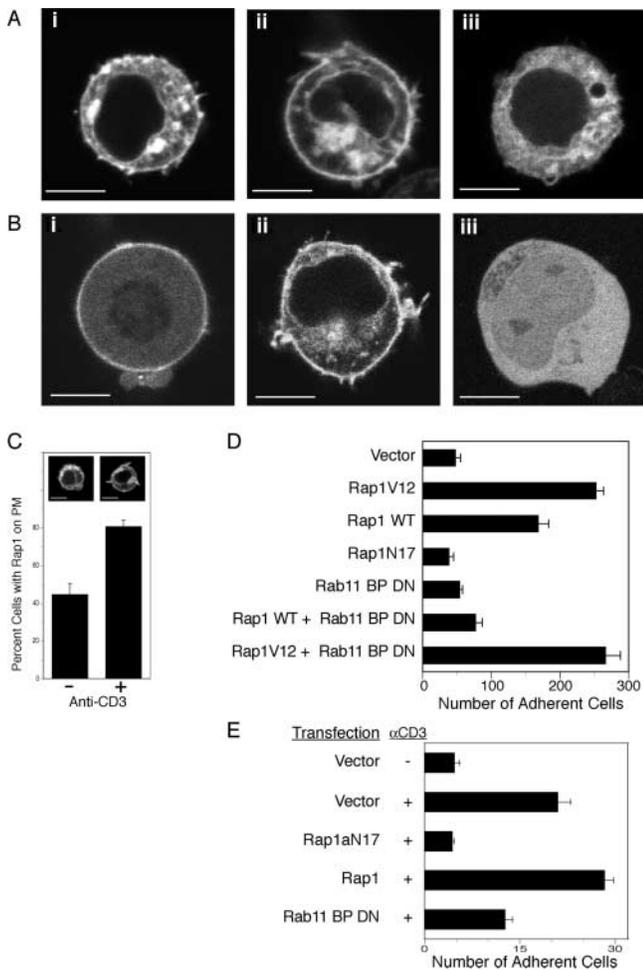
Figure 7. **Up-regulation and activation of Rap1 on PM controls T cell adhesion.** (A) Jurkat T cells were transfected with either GFP-Rap1 wild type (i), GFP-Rap1V12 (ii), or GFP-Rap1N17 (iii) and imaged alive 48 h after transfection and growth in serum. (B) Jurkat cells were transfected with GFP-RBD$_{RalGDS}$ and untagged Rap1 wild type (i), Rap1V12 (ii), or Rap1N17 (iii) and imaged as in A. Bars, 2 μM. Results are representative of three independent experiments (>30 cells examined per condition per experiment). (C) Jurkat cells were transfected with GFP-Rap1, serum starved, and treated with or without anti-CD3 antibodies. Cells were scored for unambiguous PM expression and the percentage of such cells before and after cross-linking TCRs was plotted (mean ± SEM, $n = 4$). Representative cells before (left) and after (right) anti-CD3 treatment are shown as insets. Bars, 2 μM. (D) Jurkat cells were transfected as indicated and plated on fibronectin-coated multi-well plates 48 h later. (E) Jurkat cells were transfected as indicated and plated on ICAM-1–coated multi-well plates 48 h later with or without treatment with anti-CD3 antibodies. Cells per well (in duplicate) bound to fibronectin (D) or ICAM-1 (E) after 1 h were counted and presented as the mean ± SEM ($n = 4$).

We confirmed that overexpression of wild-type Rap1 augmented the ability of Jurkat cells to adhere to fibronectin (Fig. 7 D). Expression of GTP-bound Rap1V12 had an even greater effect than that of wild-type Rap1. Whereas coexpression of dominant-negative Rab11BP had no effect on the ability of constitutively GTP-bound Rap1V12 to promote adhesion of Jurkat cells, coexpression of this protein markedly inhibited wild-type Rap1-stimulated adhesion. To assess the role of Rap1 and endosomal recycling in a system where the TCR stimulates inside-out signaling to a specific integrin, we studied adhesion to ICAM-1–coated surfaces that is dependent on the activation of LFA-1 (Katagiri et al., 2002; Fig. 7 E). Although fewer cells adhered to ICAM-1–coated surfaces than fibronectin-coated surfaces, the LFA–1/ICAM-1–mediated adhesion could be markedly stimulated by cross-linking CD3. Whereas overexpression of wild-type Rap1 slightly augmented adhesion, expression of dominant-negative Rap1N17 inhibited TCR-stimulated adhesion. Importantly, dominant-negative Rab11BP also inhibited this process. The results with both fibronectin and ICAM-1 suggest that endosomal recycling is required for regulation by Rap1 of integrin-mediated adhesion.

Discussion

Membrane targeting of GTPases that, like Rap1 and Ras, contain a CAAX motif is determined by posttranslational processing of the CAAX sequence (Casey et al., 1989), secondary membrane-targeting sequences adjacent to the CAAX motif (Hancock et al., 1991), and the capacity to interact after processing with cytosolic chaperones such as RhoGDI (Michaelson et al., 2001). Unlike the CAA(S/M) motif of Ras proteins, Rap1 ends in a CAAL motif that becomes geranylgeranylated, a modification that is more hydrophobic than that of farnesylated Ras proteins (Silvius and l'Heureux, 1994). The Rap1 CAAL motif is flanked by a relatively strong polybasic region (net charge +5) similar to that of K-Ras4B. In this regard, Rap1 is most similar to Rac1, a geranylgeranylated Rho family GTPase that is targeted to the PM (Michaelson et al., 2001). However, unlike processed Rac1 that is sequestered in the cytosol by its interaction with RhoGDI, Rap1 has no known cytosolic binding protein and is therefore predicted to have a strong affinity for the PM.

Despite its Rac1-like membrane-targeting sequence, Rap1 has not been previously localized to the PM of cultured cells. Originally localized to the Golgi complex (Beranger et al., 1991), both endogenous and overexpressed Rap1 were later found to be associated exclusively with late endosomes/lysosomes (Pizon et al., 1994). Thus, whereas our localization of intracellular GFP-tagged Rap1 is consistent with earlier reports, our observation of GFP-Rap1 on the PM of cultured cells is new but not unexpected. That GFP-Rap1N17 did not localize, like GFP-Rap1, to the PM argues against a simple overexpression artifact for the PM localization of the wild-type protein. Our confirmation by subcellular fractionation, including affinity purification of biotinylated surface membrane, that endogenous Rap1 is expressed on PM suggests that the inability to visualize PM-associated Rap1 by indirect immunofluorescence of fixed and permeabilized cultured

brane and PM (Fig. 7 C). Despite robust expression of wild-type GFP-Rap1 on intracellular membranes, GFP-RBD$_{RalGDS}$ was recruited in Jurkat cells coexpressing untagged wild-type Rap1 exclusively to the PM (Fig. 7 B, i), indicating that, like in COS-1 cells, the GTP-bound pool of wild-type Rap1 is limited to the PM. In contrast, constitutively GTP-bound Rap1V12 recruited GFP-RBD$_{RalGDS}$ to both PM and intracellular membranes (Fig. 7 B, ii). As in COS-1 cells, nucleotide-free Rap1N17 failed to recruit GFP-RBD$_{RalGDS}$ to any membrane (Fig. 7 B, iii).

cells is a result of low sensitivity of the assay, and that the localization of overexpressed GFP-Rap1 on the PM reflects the true subcellular distribution of the GTPase. This view is supported by the observation that in primary myeloid cells, Rap1 has been localized to the PM (Quinn et al., 1992). In lymphoid cells, a recently identified effector of Rap1, RapL, has been shown to associate with the surface adhesion molecule LFA-1 and mediate in a Rap1-dependent fashion its relocalization to the leading edge of the cell (Katagiri et al., 2003), confirming a functional role for Rap1 on the PM.

More intriguing than the PM localization of Rap1 is the rapid up-regulation of the GTPase on this compartment that we observed after growth factor stimulation. The rapidity of the increase in GFP-Rap1 surface expression rules out new protein synthesis as a source. Cytosolic pools of processed GFP-Rap1 were not observed, which is consistent with the absence of a GDI-like binding partner. Thus, the additional GFP-Rap1 that appeared on the PM is most likely derived from an intracellular membrane compartment. Indeed, the source of Rap1 that rapidly appears on the PM of terminally differentiated myeloid cells in response to inflammatory agonists is a pool associated with two classes of specialized vesicles known as secondary (or specific) and tertiary granules that serve as intracellular reservoirs of PM (Maridonneau-Parini and de Gunzburg, 1992; Mollinedo et al., 1993). Although the cultured epithelial cells and fibroblasts used in this work do not contain specialized secretory granules, the large pool of intracellular Rap1 present on endosomes is a potential source of rapidly mobilizable protein. The sensitivity of Rap1 surface up-regulation to NEM suggests that membrane fusion events are required, and the inhibition by dominant-negative Rab11BP implicates recycling endosomes as the source of additional PM Rap1. Thus, specialized secretory organelles are not required for the regulation of Rap1 surface expression by exocytosis.

Given the large pool of intracellular Rap1 and the recent observation that intracellular Ras is activated in situ by growth factor signaling (Chiu et al., 2002), it was somewhat surprising to observe Rap1 activation only at the PM. This suggests that the GEFs that activate Rap1 after EGFR ligation are localized at the PM. EGFR stimulation causes recruitment to the PM of the GEF SOS, which activates several Ras-related GTPases including M-Ras (Quilliam et al., 1999). We observed that GTP-bound M-Ras71L stimulated activation of Rap1 at the PM. Because activation of M-Ras recruits to the PM RA GEF 2, a Rap-specific GEF (Gao et al., 2001), we hypothesize that M-Ras links EGFR stimulation with Rap1 activation at the PM.

The reversibility of Rap1 activation at the PM implicates GAP activity on this compartment. Indeed, Rap1GAP was localized to the PM (Polakis et al., 1991). Recently, Rap1GAP has been shown to be dynamically recruited to the PM by G_z in NGF-stimulated PC12 cells (Meng and Casey, 2002). Because inactive Rap1 traffics through the endosomal recycling compartment, the balance of GEFs and GAPs in this compartment might favor the latter, and endocytosis may serve as a mechanism of down-regulating Rap1.

The coincidence of both up-regulated GFP-Rap1 and activated Rap1 on membrane ruffles lends further support to the view that the two processes are linked. Exocytosis to the leading edge of the cell where extending lamellipodia require rapid expansion of the surface membrane is a well established paradigm in cell biology and one that may explain the appearance of GFP-Rap1 in ruffles. Moreover, the preferential up-regulation of Rap1 in membrane ruffles suggests that the GTPase may play a role in actin-based processes such as cell motility and adhesion. Indeed, Rap1 has been implicated in the regulation of integrin-mediated adhesion in lymphoid cells downstream of the T cell receptor and of CD31 (Reedquist et al., 2000). We confirmed the role of Rap1 in integrin-mediated T cell adhesion and showed that the Rap1-mediated regulation was sensitive to agents that block endosome recycling. This observation provides functional evidence for regulation by Rap1 of adhesive events at the PM and for modulation of that function by exocytosis. Rap1-regulated LFA-1–dependent adhesion at the ruffling leading edge of the cell and detachment at the uropod have recently been shown to depend on a residue in the LFA-1 β chain that is required for receptor internalization and recycling (Tohyama et al., 2003). It is tempting to speculate that the endosomal compartment storing the intracellular pool of LFA-1 is the same as the one that contains a mobilizable pool of Rap1.

Our results differ substantially from those of recent works that observed Rap1 activation only on internal membranes (Mochizuki et al., 2001; Ohba et al., 2003). The basis for this discrepancy is not entirely clear but is undoubtedly related to the distinct methods applied. Those authors did not directly measure Rap1 activation but rather used an overexpressed chimeric FRET probe to sample, in a spatio-temporal fashion, the relative balance of GEFs and GAPs active against the chimera. Unfortunately, the Raichu-Rap1 FRET probe was not unbiased in its subcellular distribution but rather incorporated the membrane-targeting sequence of K-Ras4B, well established to target proteins exclusively to the PM (Hancock et al., 1990; Choy et al., 1999). Moreover, in these studies, the spatial resolution of the FRET readout was relatively low, such that specific subcellular compartments could not be distinguished and the conclusion that Rap1 was activated on endomembranes was based on a diffuse perinuclear signal. The ability of PM-targeted Raichu-Rap1 to report Rap1 activation on endomembranes has not been explained, and a Raichu-Rap1 probe with a native membrane-targeting sequence has not been reported. Interestingly, Raichu-Rap1V12, a K-Ras4B–targeted GAP-resistant probe with a constitutively high degree of GTP binding, reported activity only at the PM that was surprisingly EGF sensitive (Ohba et al., 2003), which is consistent with our results using GFP-RBD$_{RalGDS}$. However, in our work, each Rap isoform analyzed was targeted to membranes with its native hypervariable region, and our fluorescent reporter was untargeted and thus had unbiased access to the cytosolic leaflet of all membrane compartments. Most importantly, GFP-RBD$_{RalGDS}$ proved capable of reporting the spatio-temporal activation of endogenous Rap1.

In summary, our in vivo imaging of Rap1 localization and activation has provided insight into the dynamic regulation of Rap1 in response to growth factor stimulation and has highlighted important differences between Rap1 and Ras. Whereas the subcellular distribution of Ras is unaffected by

growth factor stimulation and pools of Ras on intracellular compartments are activated in situ (Chiu et al., 2002), Rap1 is up-regulated at the PM in conjunction with its activation principally on that compartment. Moreover, when up-regulation was blocked, so was Rap1 function. We propose that localization of Rap1 and Ras to different membrane compartments contributes to their distinct cellular functions.

Materials and methods

Plasmids

The full or partial coding sequences of the relevant human cDNAs (Ras isoforms or mutants thereof, Rap1 or mutants thereof, Raf-1, and Ral GDS) were amplified by PCR and cloned in frame into the mammalian expression vectors pEGFP-N1 or pEGFP-C3 (CLONTECH Laboratories, Inc.) or into pcDNA3.1(+)/Neo (Invitrogen) as indicated. Production of GFP-Raf-1 RBD is performed as described previously (Chiu et al., 2002). The Ral GDS RBD was generated by PCR amplification of a cDNA encoding amino acids 786–883 of human RalGDS and was inserted in frame with GFP into the pEGFP-N1 vector. All plasmid constructs were verified by bidirectional sequencing. Mammalian expression vectors encoding M-Ras71L and R-Ras87L were obtained from A. Cox (University of North Carolina School of Medicine, Chapel Hill, NC). Mammalian expression vectors encoding dominant-negative epsin (DPW domain) and dominant-negative Rab11BP were provided by P. DeCamilli (Yale University School of Medicine, New Haven, CN) and D. Sabatini (New York University [NYU] School of Medicine, New York, NY), respectively.

Cell culture and transfection

COS-1, MDCK, and NIH 3T3 cells were maintained in 5% CO_2 at 37°C in DME containing 10% FBS (COS-1 and MDCK) or FCS (NIH 3T3; Colorado Serum Co.). Jurkat cells were maintained in 5% CO_2 at 37°C in RPMI containing 10% FBS. Cells to be examined by fluorescence microscopy were plated at 2×10^5 per plate into 35-mm dishes containing a glass coverslip-covered 15-mm cutout (MatTek) and transfected the next day. Transfection of COS-1, MDCK, and NIH 3T3 cells was performed with SuperFect® (QIAGEN) according to the manufacturer's instructions, and cells were examined the following day. Transfection of Jurkat cells was performed with DMRIE-C (QIAGEN), and cells were examined 48 h later. Fluorescent loading of endosomes was accomplished by incubating cells with 5 μg/ml of Texas red–conjugated transferrin (Molecular Probes) for 30 min at 37°C followed by removal of the unincorporated probe. Jurkat cells were stimulated with 5 μg/ml of mouse anti–human CD3 and anti–human CD28 (Ancell).

Subcellular fractionation

MDCK cells were grown to 90–95% confluence and washed in ice-cold PBS before harvesting. Cells were suspended in homogenization buffer (0.3 M sucrose, 10 mM Tris-HCl, pH 7.5, 10 mM KCl, 1 mM DTT, and protease inhibitors) and disrupted by ball-bearing homogenizer (12-μm clearance). To remove unbroken cells and nuclei, the homogenate was centrifuged at 600 g for 5 min. Membrane and soluble fractions of the 600-g supernatant were separated by centrifugation at 160,000 g for 120 min. The total membrane pellet was resuspended in 1.35 M sucrose and separated by flotation through a discontinuous sucrose density gradient (0.25, 0.90, and 1.35 M) by centrifugation (350,000 g for 120 min). Purified Golgi membranes were isolated at the interface between the 0.25- and 0.9-M sucrose layers. Endosomes were harvested from the 1.35-M sucrose layer. A mixed fraction of PM and smooth ER was isolated from the interface between the 0.9- and 1.35-M sucrose layers. PM was further purified by loading this fraction on top of a linear gradient of 5–20% Optiprep (Nycomed) and centrifuging for 18 h at 95,000 g. After centrifugation, PM was harvested from fractions near the top of the gradient. 50 μg of each fraction (determined by BCA assay [Pierce Chemical Co.]) was analyzed by immunoblot for Na^+/K^+ ATPase (rabbit polyclonal 1:500; gift of T. Morimoto), EEA-1 (mouse monoclonal 1:2,500; Transduction Laboratories), and Rap-1 (mouse monoclonal 1:1,000; Transduction Laboratories). Immunoreactive proteins were detected using rabbit anti–mouse Ig antisera and ^{125}I-protein A and visualized by phosphorimager (Molecular Dynamics).

Biotinylation surface expression assay

Affinity purification of PMs was performed essentially as described previously (Mammen et al., 1997). In brief, COS-1 cells were grown to confluency in 10-cm dishes, rinsed, and incubated for 20 min on ice with 10 μg/

ml Sulfo-NHS-biotin in biotinylation buffer (10 mM sodium borate, pH 8.8, and 150 mM NaCl). 10 mM NH_4Cl was added to terminate the reaction and cells were scraped into 1 ml of cytosolic lysis buffer (10 mM Hepes, 10 mM NaCl, 1 mM KCl, 5 mM $NaHCO_3$, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1 mM PMSF, 100 U/ml aprotinin, and 5 mM EDTA) and incubated for 5 min on ice. Membranes were disrupted on ice using a Dounce homogenizer, and nuclei and unbroken cells were pelleted at 1,000 g. Crude membranes were incubated with immobilized streptavidin for 1 h at RT, and affinity-purified membranes were pelleted. Purified membranes and membranes in the unbound fraction were solublized in Laemmli buffer, boiled, and subjected to SDS-PAGE followed by immunoblot analysis. 50 μg of each fraction (determined by BCA assay) was analyzed by immunoblot for Na^+/K^+ ATPase (rabbit polyclonal 1:500; gift of T. Morimoto), EEA-1 (mouse monoclonal 1:1,000; Transduction Laboratories), and Rap-1 (rabbit polyclonal 1:1,000, gift of J. de Gunzburg, Institut Curie, Paris, France). Immunoreactive proteins were detected using rabbit anti–mouse Ig antisera and ^{125}I-protein A and visualized by phosphorimager.

Ras/Rap in vitro activation assays

Detection of activated Ras or Rap1 was performed as described previously (de Rooij and Bos, 1997). Immunoblot analyses were performed using either an anti-panRas mAb (Ras10; Upstate Biotechnology) or an anti-Rap1 mAb (Transduction Laboratories). Proteins were detected using ^{125}I-protein A by phosphorimager analysis.

Imaging and stimulation

Cells were examined alive with an inverted laser scanning confocal microscope (model Zeiss 510 LSM; Carl Zeiss MicroImaging, Inc.). TIFF images were processed with Adobe Photoshop 6.0. For stimulation with mitogens, the cells in 35-mm MatTek plates were maintained at 37°C using a micro-incubator (model PDMI-2; Harvard Apparatus). Stimulations were performed by adding 100 ng/ml EGF (COS-1 cells) or 5 μg/ml (Jurkat cells) of mouse anti–human CD3 antibodies (Ancell) to the media while continuously observing selected cells. Stimulations in the presence of NEM were performed by preincubating cells with 1 mM NEM for 30 min at 37°C before EGF addition. Jurkat T cells expressing GFP-Rap1 were readily divided into two populations: one manifesting clear PM fluorescence and one with only endomembrane fluorescence. Up-regulation of Rap1 on Jurkat cell PM was measured as the percentage of cells manifesting the former phenotype before and after cross-linking CD3. Statistical analysis was performed on results obtained from a minimum of six cells monitored in each of at least three independent experiments, and p-values were calculated using a one-tailed t test.

Cell attachment assay

Adhesion assays using either fibronectin or ICAM-1–coated multi-well plates were performed as described previously (Katagiri et al., 2002). Recombinant ICAM-1 was provided by M. Dustin (NYU School of Medicine). Adhesion of Jurkat cells was quantified as the number of cells remaining bound to either fibronectin or ICAM-1 after removal of nonadherent cells with PBS.

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