The Rac1 C-terminal Polybasic Region Regulates the Nuclear Localization and Protein Degradation of Rac1*

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We observed evolutionary conservation of canonical nuclear localization signal sequences (K(K/R)(X)(K/R)) in the C-terminal polybasic regions (PBRs) of some Rac and Rho isoforms. Canonical D-box sequences (RXL), which target proteins for proteasome-mediated degradation, are also evolutionarily conserved near the PBRs of these small GTPases. We show that the Rac1 PBR (PVVKRRKSK) promotes Rac1 nuclear accumulation, whereas the RhoA PBR (RRGKKKRG) keeps RhoA in the cytoplasm. A mutant Rac1 protein named Rac1 (pbrRhoA), in which the RhoA PBR replaces the Rac1 PBR, has greater cytoplasmic localization, enhanced resistance to proteasome-mediated degradation, and higher protein levels than Rac1. Mutating the D-box by substituting alanines at amino acids 174 and 177 significantly increases the protein levels of Rac1 but not Rac1(pbrRhoA). These results suggest that Rac1 (pbrRhoA) is more resistant than Rac1 to proteasome-mediated degradation pathways involving the D-box. The cytoplasmic localization of Rac1(pbrRhoA) provides the most obvious reason for its resistance to proteasome-mediated degradation, because we show that Rac1(pbrRhoA) does not greatly differ from Rac1 in its ability to stimulate membrane ruffling or to interact with SmgGDS and IQGAP1-calmodulin complexes. These findings support the model that nuclear localization signal sequences in the PBR direct Rac1 to the nucleus, where Rac1 participates in signaling pathways that ultimately target it for degradation.

Members of the Ras superfamily of small GTPases, which includes the Ras and Rho families, participate in a wide variety of physiological and pathophysiological processes (reviewed in Refs. 1 and 2). The signaling pathways utilized by different members of the Ras and Rho families may act synergistically, antagonistically, or independently to elicit many cellular responses, including changes in gene transcription, proliferation, motility, adhesion, and contraction (reviewed in Refs. 1–4). Similar structural features in these GTPases may promote their interactions with the same activator or effector, resulting in the convergence of their signaling pathways. Characterizing the similar structural features that allow Ras and Rho family members to interact with shared activators or effectors will help determine how the signaling pathways of these small GTPases overlap.

The C-terminal polybasic region (PBR)1 is a structural feature that is present in many small GTPases in the Ras and Rho families (reviewed in Ref. 2). The PBR is comprised of a series of lysines and arginines and often immediately precedes the C-terminal CAAX sequence (Table I). Several investigators have proposed that a major function of the PBR is to promote the interactions of small GTPases with the nucleocytoplasmic shuttling protein SmgGDS (2, 5–8). SmgGDS interacts with small GTPases that have a PBR, including RhoA, Rac1, Rac2, Cdc42, K-Ras4B, Rap1A, Rap1B, and RalA (reviewed in Ref. 2). Thus, SmgGDS is a strong candidate to regulate overlapping signaling pathways utilized by different members of the Ras and Rho families.

We observed that canonical nuclear localization signal (NLS) sequences (K(K/R)(X)(K/R)) (9) are present in the PBRs of many small GTPases (2). We previously demonstrated that the nuclear accumulation of Rac1 requires the PBR (8), consistent with the presence of canonical NLS sequences in the Rac1 PBR (Table I). In contrast, RhoA exhibits minimal nuclear accumulation (8), consistent with the absence of NLS sequences in the RhoA PBR (Table I). We noticed that Rac and Rho proteins exist as different isoforms that are distinguished by the presence or absence of canonical NLS sequences in their PBRs (Table I). This expression of isoforms differing by the presence or absence of a canonical NLS sequence in the PBR is evolutionarily conserved (Table I). Isoforms of small GTPases that differ by the presence or absence of a functional NLS will have different nucleocytoplasmic shuttling capabilities, allowing them to operate in different subcellular compartments.

The evolutionary conservation of canonical NLS sequences in the PBRs of Rac and Rho isoforms (Table I), as well as other small GTPases (2), indicates that nucleocytoplasmic shuttling may be an important characteristic of these GTPases. However, the purpose of this nucleocytoplasmic shuttling is currently unknown. Because small GTPases are ~21 kDa, they should be small enough to passively diffuse through nuclear pores and enter the nucleus without the aid of an NLS. However, the NLS may be required for the nuclear entry of small GTPases when they are associated with other proteins (such as SmgGDS) in protein complexes that are too large to passively diffuse through nuclear pores. NLS-containing small GTPases may participate in signaling pathways inside the nucleus.

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The abbreviations used are: PBR, polybasic region; aa, amino acids; CA, constitutively active; CHO, Chinese hamster ovary; DN, dominant negative; GFP, green fluorescent protein; HA, hemagglutinin; NLS, nuclear localization signal; E3, ubiquitin-protein isopeptide ligase; FACS, fluorescence-activated cell sorting; OD, optical density.

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possibility is consistent with reports that signaling proteins that physically interact with these small GTPases are present in the nucleus (reviewed in Ref. 2).

Nucleocytoplasmic shuttling can direct proteins to different nuclear and cytoplasmic pathways of ubiquitination and proteasomal degradation (reviewed in Ref. 10). Many proteins that are ubiquitinated and degraded by the 26S proteasome have a functional NLS. These proteins can be degraded in the cytoplasm or nucleus. For example, the tumor suppressor protein p53 is believed to be initially monoubiquitinated when it enters the nucleus but only fully ubiquitinated and degraded after it leaves the nucleus and returns to the cytoplasm. In contrast, the Pyr1 protein is ubiquitinated and degraded in the nucleoplasm (reviewed in Ref. 10). Both nuclear and cytoplasmic pathways degrade some proteins, such as the MyoD transcription factor (11).

Although Rac1 is ubiquitinated and subjected to proteasomal degradation (12–14), it is not known where these events occur in the cell. Most studies indicate that it is the activated, GTP-bound forms of Rac that are ubiquitinated and degraded by the 26S proteasome (reviewed in Ref. 10). The activated, GTP-bound forms of Rac are ubiquitinated and degraded by the 26S proteasome (12–14). The activated, GTP-bound forms of Rac, but not the GDP-bound forms, associate with proteins that are members of several different E3 ubiquitin ligase complexes, including the anaphase-promoting complex (also known as APC/C) (12). Proteins that are ubiquitinated by APC/C often have a consensus sequence known as the “destruction box” or “D-box,” which consists of the sequence RXLR (reviewed in Ref. 15). Most interestingly, we observed evolutionary conservation of canonical D-box sequences at the C termini of Rac proteins, as well as Rho proteins (Table I).

Based on the observation that Rac1 activation enhances both its nuclear entry (8) and its proteasome-mediated degradation (13, 14), we hypothesized that the nuclear entry of Rac1 promotes its proteasome-mediated degradation. If Rac1 is targeted for degradation in the nucleus, then blocking the nuclear entry of Rac1 should prevent its degradation. A similar line of reasoning was used to demonstrate the nuclear degradation of Far1 (16). To test this prediction, we designed a mutant Rac1 protein in which the Rac1 PBR is replaced with the RhoA PBR, which lacks a functional NLS (8). We show that this Rac1 mutant, which is named Rac1(pbrRhoA), has greater cytoplasmic localization and significantly elevated protein levels compared with Rac1. Inhibition of proteasome activity increases the protein level of Rac1 to the level of Rac1(pbrRhoA), indicating that Rac1 is more susceptible than Rac1(pbrRhoA) to proteasome-mediated degradation. The cytoplasmic localization of Rac1(pbrRhoA) provides the most obvious reason for its resistance to proteasome-mediated degradation, because Rac1(pbrRhoA) does not greatly differ from Rac1 in its ability to stimulate membrane ruffling or to interact with SmgGDS and IQGAP1-calmodulin complexes. Furthermore, we show that mutation of the D-box from RAVL to AAVA (aa 174–177) significantly elevates the protein levels of Rac1 but not Rac1(pbrRhoA). Taken together, these results support the model that nucleocytoplasmic shuttling directs Rac1, and potentially other NLS-containing GTPases, to proteasome-mediated degradative pathways located in the nucleus.

### Experimental Procedures

cDNA Constructs—cDNA inserts were placed in the HA-pcDNA3.1 vector or Myc-pcDNA3.1 vector (8) or pEGFP-C1 vector (Clontech) to express proteins with an N-terminal tag consisting of the hemagglutinin (HA) epitope, Myc epitope, or green fluorescent protein (GFP), respectively (9). Site-directed mutagenesis of the inserts was performed using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The Myc-SmgGDS construct encoding SmgGDS (isoform 2; reviewed in Ref. 10) was obtained from the Guthrie cDNA Resource Center (www.cDNA.org). To generate the Rac1(pbrRhoA), CA-Rac1(pbrRhoA), and DN-Rac1(pbrRhoA) constructs, the PVKKRKRK sequence at aa 181–188 of Rac1 was replaced with PVKKRKRK, which is the sequence corresponding to the Rac1 PBR (aa 181–188 in human).
We previously described the RhoA(pbrQ), CA-RhoA(pbrQ), and DN-RhoA(pbrQ) constructs, in which glutamines replace the lysines and arginines in the RhoA PBR (aa 182–187), as depicted in Fig. 1B (8).

**Culture and Transfection of Cells**—CHO-m3 cells are Chinese hamster ovary (CHO) cells stably transfected with the M₃ muscarinic acetylcholine receptor. These cells, which do not exhibit differences from parental CHO-K1 cells in the absence of muscarinic agonist, were cultured as previously described (8). The A7r5 line of rat aortic smooth muscle cells, and the COS-7 line of monkey kidney cells, were cultured in Dulbecco’s modified Eagle’s medium containing fetal bovine serum (10%), HEPES (1%), nonessential amino acids (1%), glutamine (0.3 µg/ml), penicillin (20 units/ml), and streptomycin sulfate (20 µg/ml). The cells were transfected with cDNAs by electroporation in Ham’s
In each experiment investigators ranked the nuclear localization of software. To quantify nuclear localization of the GFP-tagged proteins, in each experiment investigators ranked the nuclear localization of proteins in at least 20 different cells transfected with the same cDNA. Equal numbers of cells were lysed in buffer containing 0.5% Nonidet P-40 with protease and phosphatase inhibitors. The lysates were centrifuged (13,000 g, 10 min, 4 °C), and the resulting supernatants were immunoprecipitated using the 9E10 Myc antibody (Santa Cruz Biotechnology) or HA antibody (Covance). The immunoprecipitates were subjected to SDS-PAGE followed by autoradiography.

Immunoprecipitation of Transiently Expressed Proteins—These assays were performed as described by Lanning et al. (8). The cells were electroporated with cDNAs, cultured in complete medium for 3 h, and then cultured for 16 h in labeling medium containing [35S]methionine. Equal numbers of cells were lysed in buffer containing 0.5% Nonidet P-40 with protease and phosphatase inhibitors. The lysates were centrifuged (13,000 × g, 10 min, 4 °C), and the resulting supernatants were immunoprecipitated using the 9E10 Myc antibody (Santa Cruz Biotechnology) or HA antibody (Covance). The immunoprecipitates were subjected to SDS-PAGE followed by autoradiography.

Enhanced Chemiluminescence Western Blotting—The cells were electroporated with cDNAs and cultured for 3 h in the absence of drugs. These electroporated cells, or nonelectroporated cells, were then cultured in the absence or presence of 10 μM MG-132 or 20 μM lactacystin for 20 h. Equal numbers of cells were suspended in Nonidet P-40 buffer containing 0.5% Nonidet P-40 with protease and phosphatase inhibitors (8) and incubated for 20 min on ice with periodic vortexing. To generate whole cell lysates, the cells suspended in Nonidet P-40 buffer were mixed directly with an equal volume of 2× Laemmli sample buffer. To generate soluble cell extracts, the cells suspended in Nonidet P-40 buffer were centrifuged (13,000 × g, 10 min, 4 °C) to generate a supernatant and pellet. The supernatant was collected as the soluble cell extract and mixed with an equal volume of 2× Laemmli sample buffer. After SDS-PAGE, the proteins were transferred to polyvinylidene difluoride membranes and immunoblotted using doubling dilutions of antibody to HA (Covance), Rac1 (Transduction Labs), RhoA (Santa Cruz Biotechnology), or actin (Sigma). Bound antibodies were visualized by ECL (8). The nuclear proteins are solubilized by these cell lysis techniques, as indicated by our ability to detect the nuclear protein RCC1 in ECL-Western blots of both the soluble cell extracts and whole cell lysates (data not shown).

FACS Analysis—CHO-m3 cells were electroporated with pEGFP-C1 (as a control) or with constructs encoding GFP-tagged GTPases and cultured for 24 h. The live cells were suspended in phosphate-buffered saline containing 5 mM EDTA and analyzed for GFP fluorescence using a fluorescence-activated cell sorter (FACSCaliber, Becton-Dickinson).

RESULTS

Switching the PBRs between Rac1 and RhoA Alters Their Nuclear Accumulation.—We previously reported that mutant Rac1(pbrQ) proteins, in which neutral glutamines replace the positively charged lysines and arginines in the Rac1 PBR (Fig. 1A), have less nuclear accumulation than Rac1 proteins (8). Because this glutamine substitution destroys both the positive charge and the specific order of aa within the PBR, it was not determined whether it is the charge or the order of aa in the PBR that promotes Rac1 nuclear accumulation. To address this question, we generated cDNA constructs encoding Rac1 (pbrRhoA) proteins, in which the Rac1 PBR is replaced with the RhoA PBR, as depicted in Fig. 1A. This replacement results in a mutant Rac1(pbrRhoA) protein that still has a positively charged PBR but no longer has the canonical NLS sequences (KKRK and KRKR) found in the wild type Rac1 PBR. To examine the effects of the activation states of the GTPases, constructs encoding constitutively active and dominant negative forms of the GTPases were also generated (Fig. 1A). Transient expression of the GFP-tagged Rac1 and Rac1(pbrRhoA) proteins in CHO-m3 cells indicated that there is significantly less nuclear accumulation of the Rac1(pbrRhoA) proteins than their Rac1 counterparts possessing a normal PBR (Fig. 1, C and D). Thus, a specific PBR sequence, rather than the positive charge of the PBR, is required for Rac1 nuclear accumulation. The PBR sequence that is required for Rac1 nuclear accumulation is most likely KKKRR and KRKR, because it contains the canonical NLS sequences KKKR and KRKR.

Although GFP-tagged CA-Rac1(pbrRhoA) exhibits reduced nuclear accumulation, it still induces the formation of membrane ruffles and accumulates in these structures (Fig. 1C, panel 6), similar to the responses induced by GFP-tagged CA-Rac1 (Fig. 1C, panel 5). Thus, replacement of the Rac1 PBR with the RhoA PBR does not prevent Rac1(pbrRhoA) from participating in signaling pathways leading to membrane ruffling.

We also examined the intracellular localization of GFP-tagged RhoA(pbrRac1) proteins in which the RhoA PBR is replaced with the Rac1 PBR, as depicted in Fig. 1B. This replacement results in a mutant RhoA(pbrRac1) protein that possesses the canonical NLS sequences (KKRR and KRRR) found in the wild type Rac1 PBR. The GFP-tagged RhoA(pbr-Rac1) proteins exhibit significantly greater nuclear accumulation than their GFP-tagged RhoA counterparts possessing a normal PBR (Fig. 1, C and E). Thus, the Rac1 PBR acts as a potent NLS for RhoA(pbrRac1) proteins.

We also examined the distributions of the GTPases in living COS-7 kidney cells (Fig. 2, A and B) and A7r5 smooth muscle cells (Fig. 2, C and D). The distributions of the GFP-tagged Rac1(pbrRhoA) and RhoA(pbrRac1) proteins in these cells (Fig. 2) are similar to their distributions in CHO-m3 cells. The Rac1(pbrQ) proteins, which have neutral PBRs because of glutamine substitutions (Fig. 1A), exhibit significantly reduced nuclear accumulation in COS-7 cells (Fig. 2, A and B) and A7r5 cells (Fig. 2, C and D), similar to their reduced nuclear accumulation in CHO-m3 cells (8). The CA-Rac1(pbrQ) protein does not induce membrane ruffling in COS-7 cells (Fig. 2A, panel 6) and A7r5 cells (Fig. 2C, panel 6). In contrast, membrane ruffling is induced by both CA-Rac1 and CA-Rac1(pbrRhoA) in COS-7 cells (Fig. 2A, panels 4 and 5) and A7r5 cells (Fig. 2C, panels 4 and 5).

Switching the PBRs between Rac1 and RhoA Alters Their Interactions with SmgGDS.—We compared the protein interactions of HA-Rac1 and HA-Rac1(pbrRhoA) in 35S-labeled CHO-m3 cells co-expressing Myc-tagged SmgGDS. Immunoprecipitation of the HA-tagged GTPases (Fig. 3A) or Myc-SmgGDS (Fig. 3, B and C) indicated that Myc-SmgGDS co-precipitates strongly with both HA-DN-Rac1 and HA-DN-Rac1(pbrRhoA) (lanes 4 and 5). Shorter exposures of the autoradiographs revealed that Myc-SmgGDS forms only slightly less stable complexes with HA-DN-Rac1(pbrRhoA) than with HA-DN-Rac1 (Fig. 3C, lanes 4 and 5). These results indicate that replacing the Rac1 PBR with the RhoA PBR only moderately diminishes the stability of the complexes formed between Rac1 and SmgGDS.

IQGAP1-calmodulin complexes associate strongly with both HA-CA-Rac1 and HA-CA-Rac1(pbrRhoA) (Fig. 3A, lanes 6 and 7,
in agreement with our previous report that IQGAP1-calmodulin complexes preferentially associate with activated Rac1 (17). Thus, the PBR switch does not inhibit the ability of Rac1 (pbrRhoA) to interact with IQGAP1-calmodulin complexes.

We also examined the interactions of the RhoA (pbrRac1) proteins with SmgGDS (Fig. 3, D–F). Significantly greater amounts of Myc-SmgGDS associate with HA-RhoA than with HA-RhoA (pbrRac1) (Fig. 3, D–F, lanes 2 and 3). Shorter exposures of the autoradiographs revealed that Myc-SmgGDS also forms more stable complexes with HA-DN-RhoA than with HA-DN-RhoA (pbrRac1) (Fig. 3F, lanes 4 and 5). These results indicate that replacing the RhoA PBR with the Rac1 PBR significantly diminishes the ability of RhoA (pbrRac1) to associate with SmgGDS.

When the HA-tagged GTPases and Myc-SmgGDS are allowed to interact in vitro, Myc-SmgGDS forms only slightly more stable complexes with HA-DN-Rac1 than with HA-DN-Rac1 (pbrRhoA) (Fig. 4A, lanes 6 and 8). In contrast, Myc-SmgGDS associates much more strongly with HA-DN-RhoA than with HA-DN-RhoA (pbrRac1) (Fig. 4B, lanes 6 and 8). Thus, the interactions of SmgGDS with the wild type and mutant GTPases in vitro (Fig. 4) are similar to their interactions in cells (Fig. 3).

Previous reports indicate that SmgGDS extracts small GTPases from membranes, increasing the levels of the GTPases in the soluble fraction of cells (18). We observed that the co-expression of Myc-SmgGDS significantly increases the solubility of HA-Rac1 but not HA-Rac1 (pbrRhoA) in the cells (Fig. 5, A and B). We also examined the effects of Myc-SmgGDS on the total protein levels of the GTPases in the cells and found that the protein levels of the GTPases are not affected by Myc-SmgGDS co-expression (Fig. 5, C and D).

The PBR Regulates Rac1 Protein Degradation—We observed a significantly greater protein level of HA-Rac1 (pbrRhoA) than HA-Rac1 in the cells, regardless of the presence or absence of co-transfected Myc-SmgGDS (Fig. 5, C and D). The higher level of HA-Rac1 (pbrRhoA) than HA-Rac1 is probably not due to differences in the transfection efficiency or transcription rates of the constructs, because all of the HA-tagged GTPases are expressed from the same promoter in the HA-pcDNA3.1 vector. To further quantify differences in expression, we compared the fluorescence levels of the expressed GFP-tagged GTPases in live CHO-m3 cells using FACS analysis. FACS analysis similarly indicated significantly greater levels of the GFP-tagged Rac1 (pbrRhoA) proteins than the GFP-tagged Rac1 proteins in the cells, regardless of the activation state of the GTPases (Fig. 6).

We speculated that the different protein levels of Rac1 and Rac1 (pbrRhoA) result from their different subcellular distributions. If Rac1 is similar to proteins such as Far1, which is degraded in the nucleus (16), then Rac1 may be degraded more readily than Rac1 (pbrRhoA) because Rac1 enters the nucleus more readily than Rac1 (pbrRhoA). This model predicts that the difference between the protein levels of Rac1 and Rac1 (pbrRhoA) will be lessened by inhibiting proteasome-mediated degradation. To test this prediction, CHO-m3 cells expressing the HA-tagged GTPases were cultured for 20 h in the absence or presence of the proteasome inhibitor MG-132 and subjected to ECL-Western blotting using HA antibody or actin antibody, as a control (Fig. 7). There are significantly lower levels of HA-Rac1 than HA-Rac1 (pbrRhoA) in untreated cells (Fig. 7, A and D, lanes 1–5, and M) but not in MG-132-treated cells (Fig. 7, A and D, lanes 6–10, and M). Similarly, there are significantly lower levels of HA-CA-Rac1 than HA-
CA-Rac1(pbrRhoA) in untreated cells (Fig. 7, B and E, lanes 1–5, and M) but not in MG-132-treated cells (Fig. 7, B and E, lanes 6–10, and M). Similar increases in the levels of HA-Rac1 and HA-CA-Rac1 occurred when the cells were incubated with the proteasome inhibitor lactacystin (20 μM, 20 h) instead of MG-132 (data not shown). Treatment with MG-132 induces the appearance of a 38-kDa protein in several of the immunoblots, most noticeably in the immunoblots of HA-CA-Rac1 and HA-CA-Rac1(pbrRhoA) (Fig. 7, B and E, lanes 6 and 7). This 38-kDa protein may be monoubiquitinated forms of HA-CA-Rac1 and HA-CA-Rac1(pbrRhoA) that did not become fully ubiquitinated and degraded because of proteasome inhibition by MG-132. Previous reports similarly show a build up of ubiquitinated, activated Rac1 in cells treated with MG-132 (13).

We found that the effects of MG-132 on the protein levels of the HA-tagged wild type and mutant RhoA proteins depend on the activation states of the GTPases. Incubation with MG-132 increases the protein levels of HA-DN-RhoA and HA-DN-RhoA(pbrRac1) (Fig. 7, I, L, and N) but does not significantly alter the protein levels of HA-CA-RhoA and HA-CA-RhoA(pbr-Rac1) (Fig. 7, H, K, and N). Interestingly, MG-132 diminishes the protein level of HA-RhoA (Fig. 7, G and N) but does not

Fig. 3. The PBR regulates the protein interactions of Rac1 and RhoA in cells. CHO-m3 cells were co-transfected with Myc-SmgGDS plus one of the indicated HA-tagged wild type or mutant Rac1 (A–C) or RhoA (D–F) proteins. In control samples, the Myc-pcDNA3.1 vector replaced Myc-SmgGDS, and the HA-pcDNA3.1 vector replaced the HA-tagged GTPases (lane 8). Equal numbers of 35S-labeled cells were lysed, and the HA-tagged GTPases were immunoprecipitated from the lysates using HA antibody (A and D). Alternatively, Myc-SmgGDS was immunoprecipitated from the lysates using Myc antibody (B, C, E, and F). A representative autoradiograph from six independent experiments is shown. The names of the identified proteins in the immunoprecipitates are indicated to the left of the figures. The identity of the protein that migrates with a relative molecular mass of 24 kDa (B and E) has not yet been established.
significantly alter the protein level of HA-RhoA(pbrRac1) (Fig. 7, J and N).

To determine whether the N-terminal HA tag alters the proteasome-mediated degradation of the GTPases, we measured the effects of MG-132 on the degradation of untagged Rac1 and RhoA proteins transiently expressed in CHO-m3 cells. MG-132 increases the protein levels of the untagged Rac1 proteins (Fig. 8, A–D), similar to its effects on the HA-tagged Rac1 proteins. Somewhat surprisingly, MG-132 also increases the protein levels of RhoA and CA-RhoA (Fig. 8, E, F, and H), in contrast to its inability to increase the protein levels of HA-RhoA and HA-CA-RhoA (Fig. 7, G, H, and N). These findings indicate that the HA tag does not apparently alter the proteasome-mediated degradation of Rac1 proteins, but it may interfere with the proteasome-mediated degradation of RhoA proteins. The HA tag may also affect post-translational processing of the HA-DN-RhoA protein, because DN-RhoA migrates as two immunoreactive proteins in immunoblots (Fig. 8G),
whereas HA-DN-RhoA migrates as a single immunoreactive protein (Fig. 7I).

We were concerned that the transiently transfected Rac1 and RhoA proteins may be subjected to proteasomal degradation simply because they are overexpressed in the cells. If overexpression is the only reason why the Rac1 and RhoA proteins are degraded, then endogenous Rac1 and RhoA proteins, which are not overexpressed, should be resistant to proteasome-mediated degradation. However, we observed that MG-132 increases the protein levels of endogenous Rac1 and RhoA (Fig. 9), similar to the effects of MG-132 on transiently transfected Rac1 and RhoA (Fig. 8). This finding indicates that the degradation of the transiently expressed Rac1 and RhoA proteins reflects a normal physiological process and is not simply a response to overexpression or mutation of the transiently expressed proteins.

The Canonical D-box Sequence Regulates Rac1 Protein Degradation—To test the function of the canonical D-box sequence at aa 174–177 in Rac1 (Table I), we generated a cDNA encoding a mutant Rac1(R174A,L177A) protein in which alanines replace the arginine at aa 174 and the leucine at aa 177. We also generated a cDNA encoding Rac1(pbrRhoA)(R174A,L177A), which also has a disrupted D-box because of alanine substitutions at aa 174 and 177. These cDNAs were inserted in the HA-pcDNA3.1 vector and transiently expressed in CHO-m3 cells. We observed significantly higher levels of HA-Rac1 and HA-RhoA compared to the levels of HA-Rac1(R174A,L177A) and HA-RhoA(pbrRhoA)(R174A,L177A) (Fig. 10, A and D, lanes 1–5 and E). This indicates that the D-box is essential for the degradation of Rac1 and RhoA proteins.

DISCUSSION

The results of this study indicate that the PBRs of Rac1 and RhoA contribute significantly to the functions of these GTPases. The PBR provides at least two types of signals to regulate the GTPases. The first signal emanates from the positive charge of the basic lysines and arginines within the PBR that attracts the negative charges of the acidic residues in the guanine nucleotide-binding domain.
which may enhance the association of the GTPase with membranes and other proteins through electrostatic interactions (reviewed in Ref. 2). The second signal emanates from the specific order of lysines and arginines in the PBR, which produces a functional NLS in the Rac1 PBR but not in the RhoA PBR. These signals from the PBR direct the small GTPases to specific subcellular locations, where they participate in different signaling pathways.

Our results indicate that the PBR directs Rac1 to the nucleus, where it may participate in nuclear signaling pathways. Several lines of reasoning support the idea that Rac1 has specific nuclear functions. First, the evolutionary conservation of canonical NLS sequences in the PBRs of Rac proteins and other small GTPases (Table I and Ref. 2) indicates that the nuclear entry of these GTPases contributes to their cellular functions. Second, the nuclear accumulation of Rac1 in CHO cells, rat smooth muscle A7r5 cells, and monkey kidney COS-7 cells (Figs. 1 and 2) indicates that Rac1 has nuclear functions in a variety of different cell types. Finally, the growing number of guanine nucleotide exchange factors, scaffolding proteins, and effectors for small GTPases that are found in the nucleus (reviewed in Ref. 2) supports the proposed participation of Rac1 and other NLS-containing small GTPases in nuclear signaling pathways.

In addition to regulating nuclear signaling, the nucleocytoplasmic shuttling of Rac1 may also regulate Rac1 signaling pathways in the cytoplasm. The nuclear accumulation of Rac1 may diminish its interactions with signaling proteins in the cytoplasm, resulting in diminished cytoplasmic signaling. If this suggestion is correct, then the nuclear accumulation of Rac1 should reduce Rac1-mediated membrane ruffling. Although we did not perform quantitative morphological assays to examine the relationship between Rac1 nuclear localization and membrane ruffling, a recent study found that enhanced nuclear accumulation of Rac1 in COS-7 cells correlates with diminished Rac1-mediated membrane ruffling (19). It was also previously reported that actin dynamics in the cytoplasm are altered by the nuclear accumulation of LIM-kinase 1, which is a Rac1 effector (20).

Several reports have indicated that mutating the PBR alters the interactions of Rac1 with several protein partners (reviewed in Ref. 2). It has not been determined whether these PBR-dependent protein interactions of Rac1 are the cause or the result of Rac1 nucleocytoplasmic shuttling. The PBR-dependent association of Rac1 with certain proteins may promote Rac1 nucleocytoplasmic shuttling. Conversely, the PBR-dependent nucleocytoplasmic shuttling of Rac1 may allow it to associate with specific proteins located in distinct subcellular compartments. Establishing the causal relationships between the PBR-dependent protein interactions of Rac1 and its nucleocytoplasmic shuttling will help define the function and regulation of Rac1 in the nucleus.

Our results indicate that nucleocytoplasmic shuttling regulates Rac1 degradation. It is intriguing to speculate that Rac1 participates in signaling pathways inside the nucleus and is targeted for degradation only after these nuclear functions are completed. This model is similar to the degradation of transcription factors that have completed their nuclear duties (reviewed in Ref. 10). Although our results indicate that the nuclear entry of Rac1 enhances its degradation, it has not yet been established where Rac1 degradation actually occurs. Rac1 may be degraded in the nucleus, similar to Far1 (reviewed in Ref. 10). Alternatively, Rac1 may be degraded only after it exits the nucleus and returns to the cytoplasm, similar to the p53 protein (reviewed in Ref. 10).

Overexpression and mutation of proteins can lead to their degradation. However, our observation that endogenous Rac1 is subjected to proteasomal degradation indicates that Rac1 degradation is a physiological process that may serve to regulate Rac1-dependent signaling. If mutations in the transiently expressed proteins promote their degradation, we would expect Rac1(pbrRhoA) to be more susceptible to proteasomal degradation than Rac1. However, we observed that Rac1(pbrRhoA) is less susceptible to proteasomal degradation than Rac1 (Fig. 7). The main characteristics that distinguish Rac1(pbrRhoA) from Rac1 are the enhanced cytoplasmic localization and elevated protein levels of Rac1(pbrRhoA). These characteristics suggest that the nuclear exclusion of Rac1(pbrRhoA) protects it from degradation.

![Graph of peptide fluorescence](image.png)

**Fig. 6.** The PBR regulates Rac1 protein levels. Live CHO-m3 cells expressing GFP or the indicated GFP-tagged GTPases were examined by FACS analysis for GFP fluorescence. The results shown are the means ± S.E. from three to six independent experiments. The brackets above the columns indicate a statistical comparison between the two bracketed samples. *, p < 0.05; NS, not significant.
FIG. 7. The PBR regulates Rac1 degradation. A–L, CHO-m3 cells expressing an HA-tagged GTPase were incubated in the absence (lanes 1–5) or presence (lanes 6–10) of 10 μM MG-132 for 20 h. Whole cell lysates from equal numbers of cells were subjected to ECL-Western blotting using serial doubling dilutions of HA antibody, starting at a 1:800 dilution (lanes 1 and 6). As a control, the same immunoblots were reacted with serial doubling dilutions of actin antibody, starting at a 1:400 dilution (lanes 1 and 6). Representative blots from three independent experiments are shown. M and N, densitometry was used to determine the OD of the HA-tagged GTPases detected by a 1:1600 dilution of HA antibody in the ECL-Western blots. The results shown are the means ± S.E. from three independent experiments. The brackets above the columns indicate a statistical comparison between the two bracketed samples. The symbols within a column indicate a statistical comparison between the OD of the indicated HA-tagged GTPase isolated from untreated versus MG-132-treated cells. *, p < 0.05; NS, not significant.

C-terminal Polybasic Regions of Rac1 and RhoA
proteasomal degradation, supporting the model that the nuclear entry of Rac1 ultimately targets it for degradation.

Both DN-Rac1 and DN-Rac1(pbrRhoA) apparently are targeted for proteasomal degradation in the cytoplasm, because the MG-132-mediated inhibition of the proteasome elevates the protein levels of these dominant negative GTPases, which are sequestered in the cytoplasm. Both DN-Rac1 and DN-Rac1(pbrRhoA) exhibit abnormal protein interactions, as indicated by their stable association with SmgGDS and their inability to interact with IQGAP1-calmodulin complexes. The cytoplasmic mislocalization of these dominant negative GTPases and their abnormal protein interactions may cause DN-Rac1 and DN-Rac1(pbrRhoA) to be degraded by cytoplasmic pathways that do not normally degrade Rac1 proteins.

The excessive nuclear accumulation of RhoA(pbrRac1) indicates that RhoA will readily enter the nucleus if it has a PBR with a canonical NLS sequence. Based on this finding, spontaneous mutations that produce a functional NLS in the RhoA PBR will cause significant mislocalization of the mutant RhoA proteins. This mislocalization probably generates a strong selection pressure against mutations that produce canonical NLS sequences in the RhoA PBR. This probability is consistent with our observation that the absence of canonical NLS sequences in the PBR of RhoA is evolutionarily conserved (Table I).

A recent report indicates that RhoA undergoes proteasomal degradation at the plasma membrane (21). Thus, a slightly higher protein level of RhoA(pbrRac1) compared with RhoA (Fig. 7N) is consistent with the nuclear accumulation of RhoA(pbrRac1) protecting it from degradative pathways at the plasma membrane. However, conclusions about RhoA degradation should not be drawn from our data, because the N-terminal tag apparently interferes with RhoA degradation, as indicated by our finding that MG-132 diminishes the level of HA-RhoA but enhances the levels of transiently expressed RhoA.
and endogenous RhoA. Previous studies similarly indicate that an N-terminal tag occasionally interferes with the proteasomal degradation of some proteins (11).

The evolutionary conservation of canonical D-box sequences in both Rac1 and RhoA (Table I) suggests that these GTPases are subjected to proteasomal degradation following their ubiquitination by APC/C, which is the ubiquitin ligase complex that is generally believed to recognize the D-box (reviewed in Ref. 10). Proteins that make up APC/C have been reported to be in interactions with small GTPases in the nucleus and cytoplasm, then different regions in RhoA and Rac1 regulate the association of the different small GTPases with SmgGDS. For example, if the association of Rac1 with SmgGDS depends on different regions in RhoA and Rac1, then replacing the Rac1 PBR with RhoA PBR may have relatively minor effects on the association of Rac1 with SmgGDS. However, this property of the Rac1 PBR is apparently valid only in the context of RhoA, because SmgGDS interacts almost equally well with DN-Rac1(pbrRhoA) and DN-Rac1(pbrQ) (8).

Several lines of evidence indicate that the positive charge of the PBR promotes the interaction of SmgGDS with RhoA and Rac1. Neutralizing the PBR diminishes interactions between SmgGDS and these GTPases, as indicated by the diminished association of SmgGDS with DN-RhoA(pbrQ) and DN-Rac1(pbrQ) (8). The ability of SmgGDS to interact with different GTPases that have PBRs with similar positive charges but different aa sequences (reviewed in Ref. 2) also indicates that a positively charged PBR promotes the interaction of a GTPase with SmgGDS.

Interestingly, we found that SmgGDS associates more with DN-RhoA than with DN-RhoA(pbrRac1), indicating that the RhoA PBR is better than the Rac1 PBR in promoting the association of RhoA with SmgGDS. However, this property of the RhoA PBR is apparently valid only in the context of RhoA, because SmgGDS interacts almost equally well with DN-Rac1 and DN-Rac1(pbrRhoA). These results may occur because different regions in RhoA and Rac1 regulate the association of the small GTPases with SmgGDS. For example, if the association of Rac1 with SmgGDS depends on multiple regions in Rac1 in addition to the PBR, then replacing the Rac1 PBR with the RhoA PBR may have relatively minor effects on the association of Rac1 with SmgGDS. In contrast, if the association of RhoA with SmgGDS depends mainly on the PBR, then replacing the RhoA PBR with the Rac1 PBR may have relatively major effects on the association of RhoA with SmgGDS. This line of reasoning is supported by previous studies indicating that Rac1 and RhoA interact with SmgGDS through different mechanisms (22).

Our observation that the PBR switch results in greater mislocalization of DN-RhoA(pbrRac1) than DN-Rac1(pbrRhoA) (Fig. 1, D and E) is consistent with the PBR promoting the interaction of SmgGDS with RhoA and Rac1. This probability is supported by the co-localization of CA-Rac1 with IQGAP1 at membrane ruffles of CHO-m3 cells (17).

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inner surface of the nuclear envelope (reviewed in Ref. 23). The C terminus of amphibian lamin B3b, which consists of TKRRKKKCCSVS, acts both as an NLS and as a membrane-targeting signal for the isoprenylated lamin B3b protein (24). We previously reported that GFP fused to a peptide corresponding to the Rac1 C terminus (PPPVKKRKRKCLLL) exhibits greater nuclear accumulation than GFP (8). These and other findings indicate that the PBR of Rac1 acts as a functional NLS even in the presence of a functional CAAX region (reviewed in Ref. 2).

The nuclear targeting activity of the Rac1 PBR may vary depending on the cell type in which Rac1 is expressed. The accumulation of Rac1 at cell junctions in epithelial cells (reviewed in Ref. 17) may diminish its nuclear accumulation in these cells. Thus, Rac1 nuclear accumulation should be most apparent in cells that lack mature adherens junctions. This prediction is supported by published images showing the nuclear accumulation of Rac1 in COS-7 cells (19), NIH3T3 cells (25), and HeLa cells (26). The participation of Rac1 in both junctional and nuclear functions is consistent with the participation of other proteins, such as β-catenin, in both junctional and nuclear signaling pathways. Interestingly, nucleocytoplasmic shuttling regulates the degradation of β-catenin (reviewed in Ref. 27), just as nucleocytoplasmic shuttling may regulate the degradation of Rac1.

While this report was in review, Pop et al. (28) reported that cytotoxic necrotizing factor 1 induces the proteasome-mediated degradation of Rac1, but not Rac2 or Rac3. These investigators showed that the degradation of Rac1 is completely blocked when the Rac1 PBR is replaced with the Rac2 PBR or the Rac3 PBR (28). These findings are consistent with our model that nucleocytoplasmic shuttling regulates Rac1 degradation. According to our model, Rac1 is more susceptible than Rac2 and Rac3 to proteasome-mediated degradation because Rac1 has functional NLS sequences in its PBR, whereas Rac2 and Rac3 lack NLS sequences in their PBRs (Table I). Replacing the Rac1 PBR with either the Rac2 PBR or Rac3 PBR should block the nuclear entry and subsequent degradation of Rac1.

Our findings indicate that the C-terminal PBR regulates the intracellular localization and protein interactions of Rac1 and RhoA. The evolutionary conservation of Rac and Rho isoforms that differ by the absence or presence of a canonical NLS sequence in their PBR (Table I) suggests that the PBR keeps some isoforms in the cytoplasm, whereas the PBR allows other isoforms to enter the nucleus. Our findings support the model that Rac1 is directed by its PBR to participate in nuclear

**Fig. 10. The D-box regulates Rac1 degradation.** A–D, CHO-m3 cells expressing an HA-tagged GTPase were incubated in the absence (lanes 1–5) or presence (lanes 6–10) of 10 μM MG-132 for 20 h. Whole cell lysates from equal numbers of cells were subjected to ECL-Western blotting using serial doubling dilutions of HA antibody, starting at a 1:800 dilution (lanes 1 and 6), or actin antibody, starting at a 1:400 dilution (lanes 1 and 6). Representative blots from three independent experiments are shown. E, densitometry was used to determine the OD of the HA-tagged GTPases detected by a 1:1600 dilution of HA antibody in the ECL-Western blots. The results shown are the means ± S.E. from three independent experiments. The brackets above the columns indicate a statistical comparison between the two bracketed samples. The symbols within a column indicate a statistical comparison between the OD of the indicated HA-tagged GTPase isolated from untreated versus MG-132-treated cells. *, p < 0.05; NS, not significant.
pathways that ultimately target it for degradation. The purpose for the nuclear entry of Rac1 is probably not just its degradation but rather the participation of Rac1 in currently uncharacterized nuclear signaling pathways. These nuclear functions may distinguish Rac1 from Rac2 and Rac3, which lack canonical NLS sequences in their PBRs (Table I). Our findings indicate that RhoA is excluded from the nucleus because it lacks an NLS in its PBR. This nuclear exclusion of RhoA may distinguish it from RhoC, which possesses a canonical NLS sequence in its PBR (Table I). The signaling pathways of all of these GTPases may converge when their PBRs direct them to interact with the nucleocytoplasmic shuttling protein SmgGDS. These diverse roles of the PBR demonstrate the importance of this multifunctional region in signaling by small GTPases.

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