Isotye-specific Degradation of Rac Activated by the Cytotoxic Necrotizing Factor 1*

Marius Pop, Klaus Aktories, and Gudula Schmidt‡

From the Institut für Experimentelle und Klinische Pharmakologie und Toxikologie der Albert-Ludwigs-Universität Freiburg, Albert-Strasse 25, D-79104 Freiburg, Germany

Rho GTPases are key regulators of a wide variety of cellular functions, including regulation of actin structures, integrin signaling, phospholipid signaling, transcription, cell cycle progression, and cellular transformation (for review see Ref. 1) (2, 3). Rho GTPases cycle between a GDP-bound inactive and a GTP-bound active form. This GTPase cycle is regulated by different protein families. The GDP-bound form of Rho proteins is complexed with guanosine nucleotide dissociation inhibitors (GDIs). Activation occurs by the exchange of GDP for GTP, which is catalyzed by guanosine triphosphate activating protein (GAP). GAPs stimulate hydrolysis of the bound GTP, a process that is catalyzed by GTPase activating proteins (GAPs) (for review see Ref. 4). A wide variety of bacterial species synthesize protein toxins, which either activate or inactivate Rho GTPases (for review see Ref. 5). One of the Rho-activating toxins is the cytotoxic necrotizing factor 1 from Escherichia coli. CNF1 catalyzes the deamidation of glutamine 63/61 of the Rho family proteins that is critical for GTP hydrolysis (6, 7). Its modification blocks the intrinsic, as well as the GAP-catalyzed, hydrolysis of the bound nucleotide, resulting in a constitutively activated Rho. CNF1-treated cells are characterized by the formation of stress fibers, filopodia, and membrane ruffles, which is due to activation of RhoA, Cdc42, and Rac, respectively (8). DNT from Bordetella pertussis is another toxin that constitutively activates Rho GTPases (9). It modifies the GTPases at the same site as CNFs (i.e., Gin61/63). However, DNT transamidates the Rho GTPases by attaching primary amines, such as putrescine, spermine, and/or lysine onto the Rho proteins.

Recent results have shown that Rac is markedly and preferentially degraded by a proteasome-dependent pathway in CNF1-treated HEK293 cells. Consistent with this is the finding that the c-Jun N-terminal kinase (JNK), which is activated by Rac, is only transiently activated after CNF1 treatment (8). An important functional role of the degradation of Rac in the infection process is proposed, whereby elimination of the Rac GTPase increases the mobility of CNF-affected target cells (10) and facilitates crossing of the epithelial barrier by the CNF-producing E. coli.

In this study, we report that constitutive activation, the presence of a putative destruction box, and effector binding of Rac are necessary for degradation. We show that CNF1-induced Rac degradation is even isotype-specific, and we identify the amino acids in Rac which define this specificity.

MATERIALS AND METHODS

Cell Culture—HEK293 and HeLa cells were grown in Dulbecco’s modified Eagle’s medium (12 mM-glutamine) supplemented with 10% fetal calf serum, penicillin (40 units/ml), and streptomycin (40 μg/ml) in a humidified atmosphere of 5% CO2 at 37 °C. For intoxication, the cells were treated with 500 ng/ml purified GST-CNF1 or partially purified DNT, respectively.

Mutagenesis—Rac1 mutants were constructed by PCR in the presence of two primers (sense and corresponding antisense) carrying a mutation in Rac1. The PCR products were transformed into Epicurean XL-2 Blue ultracompetent cells (Stratagene). Mutations were checked by sequencing.

Transfection—HEK293 cells were transfected with 5 μg of DNA per 6-cm dish using Metafectene (Biontech, Germany) according to the manufacturer’s protocol. For Rac1 (wild-type and mutants) identical transfection rates were obtained by dividing the cells into two or three parts 24 h after transfection. To block protein synthesis cycloheximide (Sigma, Steinheim, Germany, 1 μg/ml) was added 48 h post-transfection.
tion and immediately before addition of the toxins, as indicated. For Rac2 and Rac3 isoforms (wild-type and mutants), respectively, 5 h post-transfection actinomycin D (Sigma, 0.5 μg/ml) was added to the culture medium. CNF1 and cycloheximide were added 24 h post-transfection. Where indicated the proteasome inhibitor MG132 (Sigma, 30 μM final concentration) was added together with CNF1.

**Protein Preparation**—For toxin purification, a BL21 E. coli strain, carrying pGEX-CNF1, was grown in minimal medium (40 mM NaHPO4, 30 mM KH2PO4, 8 mM NaCl, 1 mM MgSO4, 100 mM CaCl2, 18 mM NH4Cl, 3 μM thiamine, 50 mM glucose, 3 mM ZnSO4, 2 mM MnCl2, 50 mM H2BO3, 1 mM NaCl, 7 mM CdCl2, 0.5 mM CuCl2, 13 mM EDTA, and 7 mM FeSO4). At A600 0.2 μM isopropyl-β-D-thiogalactopyranoside was added, and the culture was grown for an additional 4 h. The cells were harvested and the glutathione S-transferase (GST) fusion proteins were purified by means of glutathione-Sepharose (Amersham Biosciences).

**Partial Purification of DNT**—The Bordetella bronchiseptica strain G58BB11 obtained from Dr. Roy Gross (Wuerzburg, Germany) was grown in Steiner-Scholle medium at 37 °C. Cells were collected after growing overnight by centrifugation and lysed by sonication and French press in lysis buffer (20 mM Tris/HCl, pH 7.4, 10 mM NaCl, 5 mM MgCl2). DNT was partially purified from smaller proteins by centrifugation of the cell lysate through a 100-kDa membrane (Microcon, Amicon) and washing with lysis buffer.

**Western Blot Analysis**—HEK293 cells growing on Petri dishes (3 cm) were treated with 500 ng/ml full-length GST-CNFX and 30 μM MG132 (Sigma) as indicated in the figure legends, washed twice with phosphate-buffered saline, and lysed in 30 μl of SDS buffer (20 mM Tris-HCl, 200 mM glycine, and 0.1% SDS). The samples were subjected to SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. Rac was detected with a specific antibody (anti-Rac, BD Biosciences). His-Rac, which was detected with a monoclonal antibody against the His tag (Upstate Biotechnology Inc., Lake Placid, NY). Binding of the second horseradish peroxidase-coupled antibody was detected using enhanced chemiluminescent detection reagent (100 mM Tris-HCl, pH 8.0, 1 mM Luminol (Fluka, St. Gallen, Switzerland), 0.2 mM p-coumaric acid, 3 mM H2O2). Stained bands were quantified by using ImageQuaNT 5.0 software.

**JNK Assay**—HEK293 cells growing on Petri dishes (10 cm) were treated with or without CNF1 (500 ng/ml) or partially purified DNT for different time intervals, as indicated. After treatment with GST-CNFX or DNT, cells were lysed on ice for 15 min in 0.5 ml of lysis buffer (20 mM Tris-HCl, pH 7.4, 137 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 50 mM sodium β-glycerophosphate, 20 mM sodium pyrophosphate, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 5 mM benzamidine, and 1 mM sodium orthovanadate). For determination of kinase activity, the c-Jun N-terminal kinase (JNK) was precipitated from the cytosol with a rabbit anti-JNK polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) bound to protein A-Sepharose (Amersham Biosciences). The immunoprecipitates were washed two times in lysis buffer with 500 mM NaCl and once in kinase buffer (25 mM Hepes, pH 7.5, 10 mM MgCl2, 25 mM sodium β-glycerophosphate, 5 mM benzamidine, 1 mM sodium orthovanadate, and 0.5 mM dithiothreitol). Recombinant GST-c-Jun (2 μg), 100 μM ATP, and 0.5 μCi of [γ-32P]ATP were added, and the reaction mixture was incubated for 30 min at 30 °C. The reaction was stopped with Laemmli sample buffer. The samples were subjected to SDS-PAGE, and the proteins were transferred to nitrocellulose. The 32P-phosphorylated c-Jun was visualized by autoradiography, and subsequently, the amount of precipitated JNK was determined by Western blotting.

**RESULTS**

**Constitutive Activation of Rac Is Necessary**—Recently, we and others have shown that CNF1-activated Rac is degraded in HEK293 and HeLa cells by a proteasome-dependent pathway (10, 11). In the present study, we attempted to characterize the structural and functional requirements for degradation of activated Rac. First, we tested whether constitutive activation is needed. To this end, constitutively active Rac(G12V) and Rac(Q61E) mutants were transfected into POSH-RBD-expressing cells (Fig. 1, lane 5). The data suggest that permanent activation is required for proteasomal degradation of Rac.

**Ephector Binding Is Required**—Doyle et al. reported recently that Rac(T35S), which is not able to bind to any of the known Rac effector molecules (12, 13), is protected from degradation (10), suggesting that an effector molecule may be involved in targeting activated Rac to the proteasome. To get more insights into the structural requirements for specific effectors involved in degradation of Rac, we exchanged tyrosine in position 40 with cysteine. It is well known that Rac(Y40C) does not interact with CRIB (Cdc42/Rac interactive binding)-containing effectors and some effectors without a CRIB domain (e.g. POSH), but it still binds to others (14). Therefore, we examined whether also Rac(Y40C) is degraded after activation with CNF1. We expressed His-activated Rac(Y40C) or His-tagged wild-type Rac (wt) in HEK293 cells. After replating and adding cycloheximide, degradation of the expressed and endogenous Rac proteins was studied with or without CNF1-activation. As a control, degradation was also studied in the presence of the proteasome inhibitor MG132. As shown in Fig. 2A, MG132 blocked degradation of CNF1-activated endogenous and recombinant Rac (lanes 3 and 6). In contrast, although activation of expressed wild-type Rac led to degradation (lane 2), activation of the recombinant effector mutant (Y40C) by CNF1 had no effect on the stability of the GTPase (lane 5). The data suggest that a CRIB-containing effector or a protein like POSH may be involved in the degradation of Rac constitutively activated by CNF1.

Thus, our studies indicated that constitutive activation and effector interaction are necessary for Rac degradation. If such an effector is involved in the degradation process, overexpression of a specific GTPase-binding domain of an effector should prevent Rac degradation. Therefore, we cloned and expressed the GTPase-binding domain of the Rac-specific effector POSH (POSH-RBD) together with His-tagged Rac1 in HEK293 cells. Cotransfected cells were treated with CNF1 in the presence of cycloheximide, and the time-dependent degradation of endogenous and the tagged GTPase were studied by Western blotting. As shown in Fig. 2B, degradation of Rac started between 90 min and 3 h after CNF1 treatment, which is in line with previous results (11). Degradation of CNF1-activated His-Rac was clearly inhibited in POSH-RBD-expressing cells (Fig. 2B, left versus right, upper band), whereas the difference was not so
Isotype-specific Degradation of Rac Activated by CNF1

Effects of DNT-induced Activation of Rac—In addition to deamidation catalyzed by CNF1, transamidation induced by the dermonecrotic toxin (DNT) from Bordetella species also leads to constitutively active Rho proteins (15). The transamidation by DNT with polyamines such as putrescine or spermine (9, 16) occurs at the same amino acid (Gln-63 of RhoA) as deamidation by CNF1. To get further insights into the requirements for Rac degradation concerning effector binding, we studied the activation and degradation of DNT-transamidated Rac by analyzing the fate of DNT-modified cellular Rac with time. As shown by the PAK pull-down assay in Fig. 3A, Rac was activated in cells treated with DNT (upper lane, Rac GTP), as well as in cells treated with CNF (lower lane, Rac GTP) significantly at 90 min. Surprisingly, although the amount of CNF-activated Rac decreased with time, the amount of DNT-activated Rac remained stable for at least 8 h (Fig. 3A, upper lane, Rac1). Recently, we reported that Jun kinase activity, which is regulated in a Rac-dependent manner, is increased after CNF treatment of cells. However, the activation is only transient because CNF-activated Rac is rapidly degraded (11). In line with the activation of Rac by DNT, we also observed that Jun kinase was activated in DNT-treated cells after 2–4 h (Fig. 3B, upper lane). However, in contrast to the transient CNF1-induced Jun kinase activation (Fig. 3B, lower lane (8)), Jun kinase activity in DNT-treated cells was sustained (Fig. 3B, upper lane), indicating that transamidated Rac was not degraded and could still interact with its effectors to maintain Jun kinase activation. Because DNT induces transamidation at Gln61 of Rac, which is located in the switch II region, it is possible that modification of this functionally important region by a bulky polypeptide group inhibits the interaction of Rac with an effector that directs Rac to degradation. This hypothesis is in line with the observation that the two toxins induce different types of changes in cell morphology (Fig. 3C). In contrast to CNF, DNT did not induce membrane ruffling in HeLa cells, whereas CNF led to the formation of lamellipodia after 2–4 h of toxin treatment. We speculate that activation of Rac by transamidation catalyzed by DNT does not allow interactions with effectors involved in membrane ruffling and in control of degradation of Rac.

Involvement of Other Rac-interacting Proteins—Not only effectors but also different proteins interact with activated Rac proteins, including guanine nucleotide dissociation inhibitors (GDIs) and GTPase-activating proteins (GAPs). To test which of these protein families are involved in Rac degradation, we mutated amino acid residues in Rac1, that are known to be essential for the interaction with each of the respective protein family members, we studied binding properties and degradation susceptibility following activation with CNF1.

The interaction between Rac1 and GDIs occurs through hydrogen bonds, involving the amino acids Tyr-64, Arg-66, His-103, and Arg-186 of Rac1 (17, 18). To study degradation of activated Rac, without the involvement of GDI, we expressed Rac1 and the mutants Rac(66E), Rac(H103E), and Rac(Y64H) as His-tagged proteins in HEK293 cells. With H103E Rac1 as one representative of these proteins, we confirmed that this mutant GTPase is not able to interact with GDI in a GDI-pull-down experiment in vitro (not shown). Full activation of this mutant with CNF1 was verified by pull-down experiments with PAK (not shown). Consequently, we used this mutant to study its degradation in CNF1-treated HEK293 cells. As shown in Fig. 4A, endogenous Rac (lower band) and the mutant Rac protein (upper band) were both degraded after activation with CNF1, suggesting that the interaction of Rac1 with GDI is not crucial for the degradation of the CNF1-activated GTPase.

Next, we tested whether the interaction of Rac1 with GAP is necessary for degradation. Asn-92 and Tyr-64 in Rac are involved in extensive hydrogen bonding (as shown for the RhoA-
with GST-fused c-Jun in the presence of endogenous JNK. The beads carrying the precipitated JNK were incubated with a JNK-specific antibody and protein A beads to precipitate the endogenous JNK. The rest of the clear lysates were incubated with beads carrying GST-PAK (CRIB domain). The beads were washed, and bound proteins were separated by SDS-PAGE and transferred onto nitrocellulose for Western blot analysis using a specific antibody against Rac. Shown are typical results of more than three different experiments.

Fig. 3. DNT polyamidation of Rac leads to sustained activation of Rac signaling. A, detection of endogenous Rac1 (Rac1) and activated Rac1 (Rac1 GTP) by pull-down with PAK and Western blotting; HEK 293 cells were treated with CNF1 (lower panel) or partially purified DNT (upper panel) for up to 8 h, as indicated. The cells were lysed and 1/10 of the clear lysates were subjected to Western blotting. The rest of the clear lysates were incubated with beads carrying GST-PAK (CRIB domain). The beads were washed, and bound proteins were separated by SDS-PAGE and transferred onto nitrocellulose for Western blot analysis using a specific antibody against Rac. Shown are typical results of more than three different experiments. B, HEK 293 cells were treated with CNF1 (lower panel) or partially purified DNT (upper panel) for 2–10 h, as indicated. The lysates were incubated with a JNK-specific antibody and protein A beads to precipitate the endogenous JNK. The beads carrying the precipitated JNK were incubated with GST-c-Jun in the presence of [γ-32P]ATP. The samples were separated by SDS-PAGE, proteins were transferred onto nitrocellulose, and the radiolabeling of GST-c-Jun was determined using a PhosphorImager. The amount of precipitated JNK was determined by Western blot using the JNK antibody. Shown are typical results of at least three different experiments. C, morphological changes of HeLa cells induced by CNF1 or DNT. HeLa cells were treated with purified GST-CNFI or partially purified DNT, respectively. Photographs were taken after the indicated time periods. Arrows indicate membrane ruffling.

Fig. 4. A, GAPs and GDIs are not involved in degradation. HEK 293 cells were transfected with DNA encoding for His-tagged wild-type Rac1 (lanes 1 and 2), two Rac1 mutants deficient in GAP interaction Rac1(Y64H) (lanes 3 and 4) or Rac1(N92D) (lanes 5 and 6), respectively, or the mutant Rac1(H103E), which does not interact with GDIs (lanes 7 and 8). After addition of cycloheximide, cells were then harvested in the presence and absence of CNF1 as indicated for further 6 h. Endogenous and expressed Rac of cell lysates was detected by Western blotting. Shown is a typical result of at least three different experiments. B, involvement of a putative destruction box. HEK 293 cells were transfected with DNA encoding for two His-tagged Rac1 proteins mutated in the putative destruction box, Rac1(R174E) and Rac1(R174A,L177A), respectively. After addition of cycloheximide, CNF1 was added for further 6 h as indicated. Lysates of the cells were subjected to SDS-PAGE, and endogenous and expressed Rac was detected by Western blotting. Shown is a typical result of three different experiments.

GAP complex for Tyr-66 and Asn-94, respectively (19). Therefore, we expressed Rac(N92D) and Rac(Y64H) mutants, respectively. We observed complete degradation of these mutants after CNF1 activation within the cell (Fig. 4A), suggesting that interaction of the activated mutant with GAP is not involved in CNF-induced degradation.

A Sequence Similar to the Mitotic Destruction Box in Rac—To further characterize requirements needed for degradation of GTPases, we compared Rac with other degraded proteins. For cyclins a sequence termed the “mitotic destruction box” has been identified, which recruits the mitotic proteins to proteasomal degradation. This sequence carries three conserved amino acids (RXXLXXXXN) in otherwise poorly conserved regions (20, 21). We identified a similar spaced pair of arginine (Arg-174) and leucine (Leu-177) residues (but no asparagine) at the C terminus of all Rac isoforms and determined whether exchange of these residues in Rac1 had any effect on degradation. Inhibition by CNF1 of Rac1(N92D) and Rac1(Y64H) mutants, respectively, activation of each of the isoforms after CNF1 treatment, and involvement of a putative destruction box. HEK 293 cells were transfected with DNA encoding for two His-tagged Rac1 proteins mutated in the putative destruction box, Rac1(R174E) and Rac1(R174A,L177A), respectively. After addition of cycloheximide, CNF1 was added for further 6 h as indicated. Lysates of the cells were subjected to SDS-PAGE, and endogenous and expressed Rac was detected by Western blotting. Shown is a typical result of three different experiments.

Degradation of Activated Rac Is Isotype-specific—We transfected HEK-293 cells with vectors encoding different isoforms of Rac as His-tagged proteins. Because recombinant Rac2 and Rac3 proteins (wild-type and mutants) are expressed in a much higher amount than Rac1 proteins in HEK293 cells, the transfection protocol had to be changed to detect degradation. To block transcription, actinomycin D was added 5 h post-transfection to the culture medium. CNF1 and cycloheximide were added 24 h post-transfection. We found that Rac1 was degraded in the cells exclusively (Fig. 5A). Activation of each of the isoforms after CNF1
or farnesylated (24). To study whether this kind of isoprenylation of the CAAX-box of Rac3 (CTVF) suggests that it can be geranylgeranylated or farnesylated (24). To study whether this kind of isoprenylation is required for Rac degradation, we constructed Rac1 with the CAAX-box of Ras, to allow farnesylation. We expressed the His-tagged Rac isoforms and found that they were not degraded (Fig. 5A), indicating that isoprenylation is required, but it is not sufficient to induce degradation of constitutively activated Rac2 and Rac3, that specify which determinants of these isoforms are required for degradation, we performed site-directed mutagenesis.

C-terminal Determinants of Rac Degradation—In addition to the existence of direct binding interactions between proteins, subcellular localization is important for interaction of GTPases with their effectors in the living cell (22). Because the isoprenylation of the CAAX-Box at the C terminus of Rho GTPases is a crucial determinant of membrane localization, we studied CNF-induced degradation with CAAX-box-deficient Rac1 in HEK293 cells. As shown in Fig. 5C (lanes 1 and 2), CAAX-box-deficient, non-isoprenylated Rac1 was not degraded in CNF-treated cells, whereas the endogenous isoprenylated Rac was degraded. This indicates that the isoprene moiety is important for degradation and might be involved in recognition by the proteasome machinery and/or for binding of a specific effector.

Rac1 and Rac2 are geranylgeranylated, whereas the CAAX-box of Rac3 (CTVF) suggests that it can be geranylgeranylated or farnesylated (24). To study whether this kind of isoprenylation is crucial for degradation, we constructed Rac1 with the CAAX-box of Ras, to allow farnesylation. We expressed the His-tagged Rac isoforms and found that they were not degraded, because there were no substrates of CNF1 (not shown). As a further control we expressed dominant active forms of the Rac isoforms and found that they were not degraded (Fig. 5B). Rac1, Rac2, and Rac3 encompass identical amino acids in the switch-I and switch-II regions but interact with different effectors (22). Moreover, it was reported that the various Rac proteins have different subcellular localizations (23).

The Polybasic Region—First, we analyzed differences in the polybasic region. The polybasic region (amino acids 183–188) of Rac1 consists of 6 Arg/Lys residues, whereas Rac2 and Rac3 have only 3 and 4 Arg/Lys residues, respectively. We exchanged the polybasic region of Rac1 toward either of the two other isoforms blocked degradation of the activated GTPase, whereas endogenous Rac1 is degraded. This indicates that amino acids 183–188 of Rac1 are crucial for degradation of the GTPase. To study, whether the polybasic region is not only crucial but also sufficient to induce degradation of constitutively activated Rac2 and Rac3, we exchanged the polybasic domain of Rac2 and Rac3, respectively, for the corresponding amino acids of Rac1. We expressed the proteins in HEK 293 cells and studied their degradation following activation by CNF1. Farnesylated Rac1 was degraded in the same amount as geranylgeranylated Rac1 (Fig. 5C, lanes 3 and 4), indicating that isoprenylation is required, but it is not important which isoprene moiety is attached. To gain more insights into the differences between Rac1, Rac2, and Rac3, we expressed the proteins following CNF1 exposure was studied by Western blotting. Shown are typical results of three independent experiments.
mutants, respectively making Rac1 similar to Rac2: Rac1(T135A) (lanes 3 and 4) and Rac1(128RLRD131) (Rac1Insert3, lanes 5 and 6). Cells were cultivated with or without CNF1 as indicated for a further 6 h in the presence of cycloheximide. Endogenous and expressed Rac from cell lysates was detected by Western blotting. Shown is a typical result of three different experiments.

We exchanged this residue in Rac1 to the corresponding amino acid of Rac2 or Rac3 and expressed the mutant in HEK293 cells. As shown in Fig. 7, Rac1(T135A) was degraded following activation by CNF1. Moreover, residues 128–131 are different in Rac3 compared with Rac1 and Rac2. Mutation of 128KLEE131 of Rac1 to 128RLRD131 of Rac3 (Rac1Insert3) did not block degradation of Rac1. The data indicate that differences in the insert region are not responsible for isotype-specific Rac degradation.

Further Residues Involved—In addition to differences in the polybasic domain and the insert region, 11 residues of Rac1 are different in either Rac2 or Rac3. We changed every single amino acid in Rac1 and expressed the mutants in HEK293 cells to study their involvement in degradation after activation by CNF1. As shown in Fig. 8, exchange of 6 of these residues to the corresponding amino acids of Rac2 had no influence on Rac degradation, whereas the CNF1-activated mutants Rac1(N107S) and Rac1(A151S) were degraded much less efficiently. Moreover, degradation of the CNF1-activated mutants Rac1(F90Y) and Rac1(K147R) was completely blocked. Taken together, the polybasic domain as well as amino acids 90, 107, 147, and 151 seem to play a role in defining isotype-specific degradation of Rac.

By introducing all these residues into Rac3 one after the other, we studied whether we can induce degradation of Rac3. As mentioned above, exchange of the polybasic domain of Rac3 toward the corresponding Rac1 amino acids was not sufficient to induce degradation of CNF1-activated Rac3. Phe-90 is already present in Rac3. As shown in Fig. 9, additional mutation of His-107 toward Asn did not lead to degradation of Rac3. Mutation S151A led to slight degradation when inserted in Rac3(H107N) containing the polybasic domain of Rac1. Normal levels of degradation were only found after the additional mutation of R147K, indicating that complete reversion of all identified crucial amino acids in Rac3 toward Rac1 is needed for degradation. In this Rac3 mutant only the insert region and amino acid 111, which does not play a role in isotype-specific degradation, are still different compared with Rac1, again indicating that the insert region does not play a role in isotype-specific degradation of Rac.

**DISCUSSION**

Recently, we and others showed that CNF1-catalyzed deamidation of Rac, which causes constitutive activation of the GTPase, induces rapid degradation by a proteasome-dependent pathway (10, 11). Here, we studied the structural and functional requirements for this proteasomal degradation in more detail. First we tested whether constitutive activation was required for Rac degradation. Therefore, we expressed constitutively active Rac(G12V) and Rac(Q61E), as well as the fast cycling mutant Rac(F28L), in HEK293 cells. After blocking further protein synthesis with cycloheximide, the degradation of the recombinant and endogenous Rac was studied by Western blotting. Whereas the constitutively active mutants were degraded, the fast cycling Rac was not. Thus, it could be concluded that permanent activation is a prerequisite for the proteasomal degradation of Rac, which is in line with the two recently reported observations. First, the strength of activation of Rac is linked to the amount of ubiquitination, which was shown by exchanging Q61 of Rac to different amino acids (10). Second, expression of an exchange factor for Rac is not sufficient to induce the same amount of ubiquitination of the activated GTPase as that which occurs for permanently activated Rac (10). Surprisingly, we observed that DNT-induced activation of Rac did not cause degradation of the GTPase. In agreement with this, we found that stimulation of Jun kinase activity via Rac/Cdc42 was transient with CNF, but long lasting after DNT treatment of cells. This indicates that one or more other factors in addition to constitutive activation are necessary for induction of Rac degradation. This further suggests that precise structural requirements define the fate of activated Rac. Because DNT induces transamidation at Gln61 of Rac, which is located in the switch II region, it is possible that modification of this functionally important region by a bulky polyamine group inhibits the interaction of Rac with an effector, that direct Rac to degradation. The hypothesis that DNT-induced activation of Rac favors interaction with different effectors as compared with CNF is supported by our observation that the two toxins induce distinct types of changes in cell morphology. For example, we observed that, in contrast to CNF, DNT did not induce membrane ruffling in HeLa cells. Thus, activation of Rac by transamidation appears to inhibit interactions with effectors involved in membrane ruffling and/or in control of degradation.

Our results indicate that binding to a specific Rac-interacting protein is necessary to target activated Rac to the proteasome. Therefore, we studied different Rac mutants deficient in
and Rac3 similar to Rac1. First the polybasic region was changed (PBD3/1, His-tagged Rac3 single and manifold mutants, respectively, making transfected with DNA encoding for His-tagged Rac3 (lane 1) identified crucial residues of Rac1 into Rac3.

HEK 293 cells were necessary to establish a possible role of POSH in Rac degradation activity (28). However, further studies are and that contains a putative zinc ring finger domain with E3 ubiquitin ligase activity (28). Nevertheless, it is interesting that the putative destruction sequence identified in all Rac isoforms as RAVL is also present in RhoA as RAAL, but not in Cdc42. This may explain why Cdc42 is constitutively activated by CNF but is not subsequently degraded. Activated RhoA is degraded to a much lesser extent than that found for Rac (11).

Activation and effector interactions of small GTPases within the cell depend on the correct subcellular localization. Deletion of the CAAX-box of Rac1 inhibited degradation, indicating that isoprenylation and membrane attachment is important for recognition of the proteasome machinery and/or correct localization, for binding to a specific effector. Our studies with different Rac isoforms provided important additional information about the structural and local requirements for CNF1-induced degradation. The Rac isoforms Rac1, Rac2, and Rac3 share identical amino acids in the switch-I and switch-II regions but differ at the C terminus. Accordingly, they are characterized by different subcellular localization (23). In addition, it has been recently reported that the various Rac isoforms interact with different effectors in mammalian cells (22).

Pull-down experiments clearly showed that Rac2 and Rac3 were substrates for CNF-induced deamidation and activation, whereas we observed that Rac1, but not Rac2 or Rac3, was degraded in the cells. To gain more insights into the differences between the Rac isoforms that specify which determinants are required for degradation, we performed site-directed mutagenesis. Exchange of the single amino acids 90, 107, 147, and 151, respectively, in Rac1 to the corresponding residues of Rac2 or Rac3 blocked degradation of the activated Rac1 mutant, indicating that these amino acids are needed for the interaction with a specific protein or are involved in a special protein fold. It is worth noting that in the three-dimensional structure, all crucial residues are located on one side of the molecule (compare Fig. 10C). Together with the polybasic domain they may constitute an interaction surface for an effector or adaptor molecule. An exchange of these amino acids and the polybasic region of Rac3 into the corresponding amino acids of Rac1 can induce degradation following activation by CNF1.

Recently, amino acids of Rac1 involved in binding of protein kinase C-related kinase (PRK1) have been identified by NMR studies (29). Results of that study clearly show that more than the effector region is involved in protein binding. None of the crucial amino acids 90, 107, 147, and 151 involved in isotype-specific degradation of Rac have been identified. Nevertheless, the polybasic region is crucial for the interaction of Rac1 with PRK. Deletion of the last 7 amino acids of Rac1 or mutation of any of the basic amino acids of the polybasic region showed a dramatic decrease in affinity toward PRK1, whereas they showed no effect on PAK binding (29).

Our findings indicate that localization and/or interaction with a specific protein, which binds to the C terminus, is critical for Rac degradation. Moreover, amino acids 90, 107, 147, and 151 play a role in isotype-specific degradation of Rac. So far, the nature of the interacting proteins is not known; however, some candidates can be offered. Rac1 interacts with Crk and phosphatidylinositol 5 phosphate kinase via its C-
terminal polybasic residues, whereas Rac2 does not interact with these proteins (22). This finding makes Crk and phosphatidylinositol 5 phosphate kinase two possible candidates, which could be responsible for leading to degradation of activated Rac. A further candidate could be POSH, which does not contain a CRIB domain and, similar to CRIB domain proteins, does not interact with the Y40C mutant of Rac1 (27). However, isotype-specific degradation can not be defined only by POSH binding, because pull-down experiments with the Rac binding domain of POSH showed interaction with all activated Rac isoforms (not shown).

All together, our studies give important new information on the requirements for degradation of Rac. First, Rac degradation depends on an effector or an adaptor protein. Interaction of this protein with Rac depends on the integrity of the effector domain and is blocked by Y40 exchange. Second, Rac transamidated by DNT is activated, but not degraded. Third, the C terminus of Rac defines the susceptibility for degradation. CAAX-box-deficient Rac1 and the isoforms Rac2 and Rac3, which differ from Rac1 in their C terminus, are not degraded after activation. This suggests that the localization of the GTPase and/or specific protein interactions at the C terminus are essential. The sequence RXXL must be present, because mutations within this sequence prevent Rac from degradation. The polybasic region and amino acids 90, 107, 147, and 151 define isotype-specific degradation of Rac. Although these res-

Fig. 10. Conclusion: identified overall (A) and isotype-specific (B and C) determinants required for Rac degradation. As shown in this report, constitutive activation, effector binding, the presence of a putative destruction box, and isoprenylation of Rac are necessary for degradation. The polybasic domain as well as amino acids 90, 107, 147, and 151 play a role in defining isotype-specific degradation of Rac. As deduced from the Rac1 structure, amino acids 90, 107, 147, and 151 are located on the same side of the molecule (C). The figure was produced using the RasMol program. Note, that the Rac1 C terminus is missing.

Isotype-specific Degradation of Rac Activated by CNF1
idues have not been identified as crucial for any effector interaction, they are located on the same side of the Rac molecule and may physiologically be involved in binding of a yet not identified effector or adaptor protein or proteins, which specify Rac1 function.

Acknowledgments—We gratefully thank Alan Hall, London for the POSH construct and Reza Ahmadian, MPI-Dortmund for Rac2 and Rac3. We thank Iris Misicka for excellent technical assistance.

REFERENCES
1. Aspenstrom, P. (1999) Curr. Opin. Cell Biol. 11, 95–102
2. Ridley, A. J. (1996) Curr. Biol. 6, 1256–1264
3. Sidéropoulou, A. R., Smith, J. L., Ross, A. H., Qiu, R.-G., Symons, M., and Exton, J. H. (1995) J. Biol. Chem. 270, 8466–8473
4. Schmidt, G., Sehr, P., Wilm, M., Selzer, J., Mann, M., and Aktories, K. (1997) Nature 387, 729–732
5. Lerm, M., Pop, M., Hoffmeyer, A., Rapp, U. R., Aktories, K., and Schmidt, G. (1999) Infect. Immun. 67, 496–503
6. Masuda, M., Betancourt, L., Matsuzawa, T., Kashimoto, T., Takae, T., Shimomizu, Y., and Horiguchi, Y. (2000) EMBO J. 19, 521–530
7. Doye, A., Mettouchi, A., Bossis, G., Clément, R., Buissen-Touati, C., Flatau, G., Gagnoux, L., Piechaczyk, M., Boquet, P., and Lemarche, N. (2002) Cell 111, 553–564
8. Westwick, J. K., Lambert, Q. T., Clark, G. J., Symons, M., Van Aelst, L., Pestell, R. G., and Der, C. J. (1997) Mol. Cell. Biol. 17, 1324–1335
9. Le Marec, N., Tapon, N., Stowers, L., Burbelo, P. D., Aspenström, P., Bridges, T., Chant, J., and Hall, A. (1996) Cell 87, 519–529
10. Owen, D., Mott, H. R., Laue, E. D., and Lowe, P. N. (2000) Biochemistry 39, 1243–1250
11. Schmidt, G., Goebringer, U.-M., Schirmer, J., Lerm, M., and Aktories, K. (1999) Biochemistry 38, 31875–31881
12. Schmidt, G., Goebringer, U.-M., Schirmer, J., Uttenweiler-Joseph, S., Wilm, M., Lohmann, M., Giese, A., Schmalzing, G., and Aktories, K. (2001) Infect. Immun. 69, 7663–7670
13. Di-Pui, N., Faure, J., Grizot, S., Molnar, G., Pick, E., and Dagher, M.-C. (2001) Biochemistry 40, 10014–10022
14. Grizot, S., Faure, J., Fiesschi, F., Vignais, P. V., Dagher, M. C., and Pevay-Peyroula, E. (2001) Biochemistry 40, 10007–10013
15. Rittinger, K., Walker, P. A., Eccleston, J. F., Smerdon, S. J., and Gamblin, S. J. (1997) Nature 389, 758–762
16. Jacobs, H. W., Kudel, E., and Lehner, C. F. (2001) EMBO J. 20, 2376–2386
17. King, R. W., Glotzer, M., and Kirschner, M. W. (1996) Mol. Biol. Cell 7, 1343–1357
18. van Hennik, P. B., ten Klooster, J. P., Halstead, J. R., Voermans, C., Anthony, E. C., Dvicha, N., and Hordijk, P. L. (2003) J. Biol. Chem. 278, 39166–39175
19. Michaelson, D., Silletti, J., Murphy, G., D’Eustachio, P., Rush, M., and Phillips, M. R. (2001) J. Cell Biol. 152, 111–126
20. Moores, S. L., Schaber, M. D., Mosser, S. D., Rands, E., O’Hara, M. B., Garsky, V. M., Marshall, M. S., Pumphianu, D. L., and Gibbs, J. B. (1991) J. Biol. Chem. 266, 14603–14610
21. Li, R., Debrevecz, B., Jia, T. P., Gao, Y., Tigyi, G., and Zheng, Y. (1999) J. Biol. Chem. 274, 29648–29654
22. Karnoub, A. E., Der, C. J., and Campbell, S. L. (2001) Mol. Cell. Biol. 21, 2847–2857
23. Tapon, N., Nagata, K., Lamarche, N., and Hall, A. (1998) EMBO J. 17, 1395–1404
24. Xu, Z., Kukekov, N. V., and Greene, L. A. (2003) EMBO J. 22, 252–261
25. Owen, D., Lowe, P. N., Nieltsch, D., Ewen, C. F., Chirgadze, D. Y., Parker, P. J., Blundell, T. L., and Mott, H. R. (2003) J. Biol. Chem. 278, 50578–50587