Nedd4 Regulates Ubiquitination and Stability of the Guanine-Nucleotide Exchange Factor CNrasGEF*

Nam Pham‡ and Daniela Rotin§

From the Program in Cell Biology, Hospital for Sick Children, and Department of Biochemistry, University of Toronto, Toronto, Ontario MSG 1X8, Canada

Cyclic nucleotide ras GEF (CNrasGEF) is a guanine-nucleotide exchange factor previously isolated in a screen for Nedd4-WW domain interacting proteins (Pham, N., Cheglakov, I., Koch, C. A., de Hoog, C. L., Moran, M. F., and Rotin, D. (2000) Curr. Biol. 10, 555–558). It activates Ras in a cAMP-dependent manner and Rap-1 independent of cAMP. Here we show that CNrasGEF is a likely substrate of the ubiquitin protein ligase Nedd4. CNrasGEF possesses two PY motifs at its C terminus that are responsible for binding to Nedd4 in vitro. Moreover, Nedd4 and CNrasGEF co-immunoprecipitate from 293T cells expressing ectopic CNrasGEF and endogenous Nedd4, and this co-immunoprecipitation is abrogated in PY motif-mutated CNrasGEF (CNrasGEFΔ2PY). CNrasGEF is ubiquitinated in cells, and this ubiquitination is augmented upon overexpression of wt-CNrasGEF but is inhibited in cells overexpressing a catalytically inactive Nedd4 (Nedd4(CS)) or in cells expressing CNrasGEFΔ2PY, which cannot bind Nedd4. Moreover, pulse-chase experiments have demonstrated that the half-life of CNrasGEF is reduced 5-fold (from ~10 to ~2 h) in cells co-expressing Nedd4 with CNrasGEF but not with CNrasGEFΔ2PY (~2h). CNrasGEF is also stabilized in cells co-expressing Nedd4(CS) or following treatment with lactacystin, indicating proteasomal degradation of this protein. Deletion/mutation of the CDC25 domain to abrogate Ras (or Rap-1) binding leads to impaired ubiquitination of CNrasGEF, suggesting that such binding is critical for ubiquitination. Treatment of cells with the cAMP analogue 8-bromo-cAMP does not affect the ability of CNrasGEF to bind Nedd4 nor its level of ubiquitination, suggesting that Ras binding per se and not its activation is the critical step in triggering ubiquitination of CNrasGEF. These results suggest that CNrasGEF is a substrate for Nedd4, which regulates its ubiquitination and stability in cells.

Nedd4 is an E3 ubiquitin protein ligase comprised of a C2 domain, three or four tandem WW domains, and a ubiquitin ligase Hect domain (1). Our earlier work has demonstrated that Nedd4 binds to the epithelial Na⁺ channel (ENaC) and regulates channel stability at the cell surface (2, 3). The association between these two proteins is mediated by binding of the Nedd4-WW domains to conserved PY motifs (PPPXY) found in ENaC (2, 4). However, our own work and that of others (e.g. Ref. 5) has suggested that Nedd4 likely has other cellular targets aside from ENaC. In our search for such putative targets, we had performed an expression screen of 16-day-old mouse embryo library with the second Nedd4-WW domain as a probe. This screen resulted in the isolation of a C-terminal fragment of the cyclic nucleotide Ras GEF (CNrasGEF) (5); also known as KIAA0313, nRap-GEF1, PDZ-GEF1, and RA-GEF (7–9)).

CNrasGEF belongs to a family of guanine-nucleotide exchange factors of the small GTPase Ras superfamily (10, 11). It has several domains and motifs, including a cyclic nucleotide (cAMP/cGMP)-binding domain (cNMP-BD), Ras exchange motif, PDZ domain, Ras association domain, a CDC25 domain similar to that of Sos1/2, GRF1/2, GRP, as well as Rap GEFs such as Epac, two PY motifs (PPGy and PPDYy, see Fig. 1A) and a C-terminal SAV sequence conforming to a PDZ binding motif (6). Ras proteins play a key role in controlling many cellular processes, including proliferation, differentiation, and apoptosis (10–12). Rap proteins were originally identified by their ability to antagonize Ras signaling but have been shown recently to play overlapping roles with Ras and/or have their own signaling pathways (13). Our recent work has demonstrated that CNrasGEF can activate Ras in vitro and is able to bind cAMP via its cNMP-BD and to activate Ras in cells following treatment with the cAMP analogue 8-Br-cAMP or following elevation of intracellular cAMP with forskolin plus IBMX. This activation is not dependent on protein kinase A (6). CNrasGEF also activates Rap-1, but this activation is independent of cAMP (6–9). The PDZ domain of CNrasGEF is likely involved in targeting or retaining the protein at the plasma membrane (6).

Because we identified CNrasGEF as a Nedd4-interacting protein, the present work was aimed at investigating the possibility that CNrasGEF is a substrate for Nedd4. Here we report that CNrasGEF binds via its PY motifs to Nedd4, that it is ubiquitinated in a Nedd4-dependent manner, that the stability of CNrasGEF in cells is regulated by Nedd4, and that ubiquitination is reduced in mutant CNrasGEF which can not

* This work was supported in part by the Canadian Medical Research Council (MRC)/Canadian Institute of Health Research (CIHR). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ A recipient of an MRC/CIHR Studentship.

§ Supported by an MRC/CIHR Scientist award. To whom correspondence should be addressed: Program in Cell Biology, The Hospital for Sick Children, 555 University Ave., Toronto, Ontario M5G 1X8, Canada. Tel.: 416-813-5098; Fax: 416-813-5771; E-mail: drotin@sickkids.ca.

The abbreviations used are: ENaC, epithelial Na⁺ channel; CNrasGEF, cyclic nucleotide Nedd4, neuronal precursor cell expressed developmentally downregulated; ras guanine-nucleotide exchange factor; GST-CNrasGEF.Cterm, C-terminal region of human CNrasGEF; cNMP-BD, cyclic nucleotide (cAMP/cGMP)-binding domain; PDZ, postsynaptic density protein-95, disc large, zonula occludens-1; IBMX, isobutylmethylxanthine; GST, glutathione S-transferase; PMSF, phenylmethylsulfonyl fluoride; HA, hemagglutinin; Ub, ubiquitin; 8-Br-cAMP, 8-bromo-cAMP; RBD, Ras binding domain; DB, destruction box.

The abbreviations used are: ENaC, epithelial Na⁺ channel; CNrasGEF, cyclic nucleotide Nedd4, neuronal precursor cell expressed developmentally downregulated; ras guanine-nucleotide exchange factor; GST-CNrasGEF.Cterm, C-terminal region of human CNrasGEF; cNMP-BD, cyclic nucleotide (cAMP/cGMP)-binding domain; PDZ, postsynaptic density protein-95, disc large, zonula occludens-1; IBMX, isobutylmethylxanthine; GST, glutathione S-transferase; PMSF, phenylmethylsulfonyl fluoride; HA, hemagglutinin; Ub, ubiquitin; 8-Br-cAMP, 8-bromo-cAMP; RBD, Ras binding domain; DB, destruction box.

N. Pham and D. Rotin, unpublished.
bind Ras. This work, therefore, suggests that CNrasGEF is a novel substrate for Nedd4 and that Ras binding to CNrasGEF is likely necessary for ubiquitination of this exchange factor.

EXPERIMENTAL PROCEDURES

Construction of Plasmids—Expression cloning of mouse CNrasGEF was previously detailed (6). The corresponding full-length cDNA of human CNrasGEF (KIAA0313) was kindly provided by T. Nagase (Kazusa Institute, Japan) and was used in all subsequent studies. The FLAG-tagged CNrasGEF and GST-CNrasGEF-Cterm (amino acids 1348–1499) were constructed as previously described (6). The FLAG-tagged CNrasGEF was used as a template to generate FLAG-CNrasGEF(Y50H9004PY) with a Y^3A mutation at the first PY motif (PPGY^3PPGA) using the QuikChange kit (Stratagene). FLAG-CNrasGEF(Y50H9004PY) was then used as template to construct FLAG-CNrasGEF(Y50H90042PY) with a Y^3A mutation in the second PY motif (PPDY^3PPDA) (Fig. 1A). CNrasGEF lacking its CDC25 domain (ΔCDC25) was constructed as previously described (6). CNrasGEF bearing a mutation in the CDC25 domain, which inhibits Ras binding (R898D), was constructed using the QuikChange kit.

Cell Culture and Transfections—HEK 293T cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 100 units of penicillin plus 100 μg of streptomycin per ml. Cells were transfected using the calcium phosphate precipitation method as described, with a total of 20 μg of DNA per 10-cm dish (equal amounts of DNA per construct). Carrier DNA was added to make up to 20 μg of DNA when single transfections were used.

In Vitro Binding Assays—HEK 293T cells were transfected as above. Where indicated, transfected cells were serum-starved overnight and treated with 0.5 mM 8-Br-cAMP (Sigma) for 15 min. Cells were then lysed in lysis buffer as above. Protein concentrations of the cell lysates were determined using the BCA method (Pierce). Lysates (20 μg) were immunoblotted with either anti-Nedd4 antibodies to detect endogenous Nedd4, or with non-immune serum (control). The bottom panel depicts 5 μg of the GST or GST-CNrasGEF-Cterm proteins used in the pull-down experiment, stained with Coomassie Blue.

Fig. 1. Binding of Nedd4 to the C terminus of CNrasGEF. A, alignment of the C-terminal region of CNrasGEF (amino acids 1348–1499). The partial coding sequence of murine CNrasGEF (mCNrasGEF) isolated from the screen was aligned with its human orthologue (hCNrasGEF) using ClustalW (1.7). The PY motifs are shown in boldface. B, the C terminus of CNrasGEF containing two PY motifs interacts with full-length Nedd4 in a pull-down assay. Equal amount (~50 μg) of GST fusion protein containing the C terminus of CNrasGEF (GST-CNrasGEF-Cterm) or GST alone was incubated with 1 μg of HEK 293T cell lysates. Bound proteins were resolved on SDS-PAGE and immunoblotted with anti-Nedd4 antibodies. Lysates (~20 μg) of HEK 293T cells were also immunoblotted with either anti-Nedd4 antibodies to detect endogenous Nedd4, or with non-immune serum (control). The bottom panel in B depicts 5 μg of the GST or GST-CNrasGEF-Cterm proteins used in the pull-down experiment, stained with Coomassie Blue.
different lysates were determined using the Bradford Bio-Rad protein assay (Sigma). Equal amounts of different lysates (~2 mg) were used in immunoprecipitations. FLAG-tagged CNrasGEF was immunoprecipitated using anti-FLAG-M2 agarose (Sigma) for 1.5 h. Immunocomplexes were washed with lysis buffer (2×) and HNTG (3×) and immunoblotted with anti-HA antibodies to detect HA-ubiquitin conjugation, followed by anti-mouse horseradish peroxidase secondary antibodies and ECL detection. To ensure ubiquitination of CNrasGEF and not of associated proteins, in certain experiments the cell lysates were treated with 1% SDS and boiled for 5 min. These boiled lysates were then diluted 11 times with lysis buffer to dilute the SDS prior to their use in the immunoprecipitation step described above.

**Pulse-chase Experiments**—HEK 293T cells that had been transfected with the same plasmids were pooled and re-seeded in 10-cm dishes to normalize the variation in transfection efficiency. Cells were washed (3×) with Met/Cys-deficient medium (Life Technologies, Inc.) within 30 min and then incubated in that medium containing 0.1 mCi/ml [35S]Met/Cys (Promix, Amersham Pharmacia Biotech) for 2 h. Cells were washed (3×) with chasing medium (Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 100 units of penicillin plus 100 μg of streptomycin per ml, and 10 μM unlabeled Met and Cys) and then chased for the indicated times. They were then washed (2×) with phosphate-buffered saline and lysed in lysis buffer. Protein concentrations of the different lysates were determined using the Bradford Bio-Rad protein assay and equal amounts of total protein (~ 1 mg) were incubated with anti-FLAG-M2-agarose to immunoprecipitate CNrasGEF. Precipitated proteins were washed as described above and resolved on SDS-PAGE. Gels were then dried and exposed to an x-ray film. The amount of labeled CNrasGEF was determined by cutting the corresponding areas on the gel and counting radioactivity by liquid scintillation counting. Where indicated, 10 μM lactacystin (Sigma) for 30 min. Cells were then lysed in lysis buffer supplemented with 50 μL LLLN (N-acetyl-Leu-Leu-norleucinal, Sigma). Equal amounts of different lysates (~ 2 mg) were used for the immunoprecipitations. FLAG-tagged CNrasGEF was immunoprecipitated using anti-FLAG-M2 agarose for 1.5 h. Immunocomplexes were washed with lysis buffer (2×) and HNTG (3×) and immunoblotted with anti-HA antibodies to detect HA-ubiquitin conjugation, followed by anti-mouse horseradish peroxidase secondary antibodies and ECL detection. To ensure ubiquitination of CNrasGEF and not of associated proteins, in certain experiments the cell lysates were treated with 1% SDS and boiled for 5 min. These boiled lysates were then diluted 11 times with lysis buffer to dilute the SDS prior to their use in the immunoprecipitation step described above.

**RESULTS**

**Interaction of CNrasGEF with Nedd4 in Vitro and in Cells**—In search of proteins that bind to CNrasGEF, we performed an expression screen of a 16-day mouse embryo library using the second WW domain of rat Nedd4 as a probe. The screen identified a murine cdna fragment encoding a 149-amino acid polypeptide that has 75% sequence identity (95% sequence similarity) to the C-terminal region of the human GenBankTM entry KIAA0313. We renamed KIAA0313 to CNrasGEF (cyclic nucleotide ras guanine-nucleotide exchange factor) and showed that CNrasGEF is a novel exchange factor that activates Ras in response to elevation of intracellular cAMP or cGMP (6) and Rap-1 independent of cAMP (7, 8). Co-immunoprecipitation of Nedd4 with CNrasGEF requires the PY motifs of CNrasGEF. A, FLAG-tagged CNrasGEF was transfected (or not) into HEK 293T cells and immunoprecipitated with anti-FLAG antibodies. The immune complex was washed and resolved on SDS-PAGE and immunoblotted with anti-HA antibodies to detect co-immunoprecipitated Nedd4 (left panel). B, similar co-immunoprecipitation experiments as in A, only using either wild-type (Wt) FLAG-tagged CNrasGEF or FLAG-CNrasGEF bearing a Y → A mutation in both PY motifs (Δ2PY). The last lane (upper panel) depicts endogenous expression of Nedd4 in untransfected 293T cell lysate.

**Fig. 2. Co-immunoprecipitation of Nedd4 with CNrasGEF requires the PY motifs of CNrasGEF.** A, FLAG-tagged CNrasGEF was transfected (or not) into HEK 293T cells and immunoprecipitated with anti-FLAG antibodies. The immune complex was washed and resolved on SDS-PAGE and immunoblotted with anti-HA antibodies to detect co-immunoprecipitated Nedd4 (left panel). B, similar co-immunoprecipitation experiments as in A, only using either wild-type (Wt) FLAG-tagged CNrasGEF or FLAG-CNrasGEF bearing a Y → A mutation in both PY motifs (Δ2PY). The last lane (upper panel) depicts endogenous expression of Nedd4 in untransfected 293T cell lysate.
with Nedd4 is mediated via the PY motifs of CNrasGEF. Collectively, these and our previous results (6) show that Nedd4 can bind CNrasGEF in vitro and in cells, and that this interaction is mediated by binding of one or more Nedd4 WW domains to the PY motifs of CNrasGEF.

**Nedd4 Mediates Ubiquitination of CNrasGEF in Cells**—To test whether the interaction of Nedd4 with CNrasGEF promotes ubiquitination of this exchange factor, we studied Nedd4-mediated ubiquitination of CNrasGEF. We first tested if CNrasGEF becomes ubiquitinated in HEK 293T cells. FLAG-tagged CNrasGEF was co-transfected with HA-tagged ubiquitin (HA-Ub) (14) into HEK 293T cells. CNrasGEF was then immunoprecipitated with anti-FLAG antibodies to precipitate CNrasGEF and immunoblotted with anti-HA antibodies to detect ubiquitinated CNrasGEF (CNrasGEF-Ub) (top panel). Corresponding lysates were immunoblotted with either anti-T7 antibodies to verify expression of Nedd4 (middle panel) or anti-FLAG antibodies to verify expression of CNrasGEF (bottom panel). Ubiquitinated CNrasGEF in the upper panel appears as a smear of high molecular weight bands. The lower molecular weight smear likely corresponds to partially degraded ubiquitinated CNrasGEF. As in A, only HEK 293T cells were transfected with either wild-type CNrasGEF (Wt) or CNrasGEF bearing a Y → A mutation in the first PY motif (ΔPY) or in both PY motifs (Δ2PY). Immunoprecipitated CNrasGEF (Wt or mutant) was then analyzed for the extent of its ubiquitination using anti-HA antibodies (top panel), as in A. Corresponding lysates were immunoblotted with either anti-T7 antibodies (middle panel) or anti-FLAG antibodies (bottom panel), as in A. C, the pattern of CNrasGEF ubiquitination is retained following sample boiling in SDS: Cells were transfected with FLAG-CNrasGEF (Wt) and HA-Ub (as in A and B), and ubiquitination of CNrasGEF was analyzed without (lane 3) or following (lane 4) boiling of sample in SDS (Boil). Bottom panel: corresponding lysate of CNrasGEF.

**Fig. 3. Nedd4-mediated ubiquitination of CNrasGEF.** A, HEK 293T cells were transfected (tfxn) with the indicated plasmids (FLAG-CNrasGEF, HA-Ub, T7-tagged Nedd4, either wild-type (Wt) or catalytically inactive (CS) mutant). Lysate from the transfected cells were immunoprecipitated with anti-FLAG antibodies to precipitate CNrasGEF and immunoblotted with anti-HA antibodies to detect ubiquitinated CNrasGEF (CNrasGEF-Ub) (top panel). Corresponding lysates were immunoblotted with either anti-T7 antibodies to verify expression of Nedd4 (middle panel) or anti-FLAG antibodies to verify expression of CNrasGEF (bottom panel). Ubiquitinated CNrasGEF in the upper panel appears as a smear of high molecular weight bands. The lower molecular weight smear likely corresponds to partially degraded ubiquitinated CNrasGEF. B, as in A, only HEK 293T cells were transfected with either wild-type CNrasGEF (Wt) or CNrasGEF bearing a Y → A mutation in the first PY motif (ΔPY) or in both PY motifs (Δ2PY). Immunoprecipitated CNrasGEF (Wt or mutant) was then analyzed for the extent of its ubiquitination using anti-HA antibodies (top panel), as in A. Corresponding lysates were immunoblotted with either anti-T7 antibodies (middle panel) or anti-FLAG antibodies (bottom panel), as in A. C, the pattern of CNrasGEF ubiquitination is retained following sample boiling in SDS: Cells were transfected with FLAG-CNrasGEF (Wt) and HA-Ub (as in A and B), and ubiquitination of CNrasGEF was analyzed without (lane 3) or following (lane 4) boiling of sample in SDS (Boil). Bottom panel: corresponding lysate of CNrasGEF.
immunoprecipitated from transfected cells using anti-FLAG antibodies and subsequently immunoblotted with anti-HA antibodies to detect conjugation of HA-Ub. As shown in Fig. 3A (top panel), a high molecular weight smear representing ubiquitinated CNrasGEF (CNrasGEF-Ub) is clearly apparent in cells expressing CNrasGEF and HA-Ub (lane 3). The smear below the 172-kDa marker in lane 3 probably represents degradation products of ubiquitinated CNrasGEF, because the protein tends to become partially degraded in these cells (Fig. 3A, bottom panel), particularly upon interaction with active Nedd4. Indeed, boiling of the samples in SDS prior to precipitation of CNrasGEF (to ensure these bands do not represent associated proteins of CNrasGEF) revealed the same pattern of ubiquitination as that of untreated samples (Fig. 3C), suggesting that the lower molecular weight ubiquitinated bands are indeed fragments of CNrasGEF that are covalently attached to ubiquitin. To test whether Nedd4 is the E3 involved or responsible for the ubiquitination of CNrasGEF, we overexpressed T7-tagged Nedd4, either wild-type (Nedd4(wt)) or catalytically inactive (Nedd4(CS)), bearing a Cys to Ser mutation at the Hect domain, with CNrasGEF and HA-Ub and analyzed the extent of CNrasGEF ubiquitination in these cells. As seen in Fig. 3A (top), overexpression of Nedd4(wt) (lane 5) led to a marked increase in CNrasGEF ubiquitination. In contrast, overexpression of Nedd4(CS) almost completely abolished ubiquitination of CNrasGEF (lane 4). These results suggest that Nedd4 is the ubiquitin ligase responsible for ubiquitination of CNrasGEF and that the Nedd4(CS) mutant is acting in a dominant negative fashion to block ubiquitination of CNrasGEF by endogenous Nedd4.

To further confirm the involvement of Nedd4 in CNrasGEF ubiquitination, we repeated the ubiquitination experiments, only using CNrasGEF in which one (ΔPY) or both (Δ2PY) tyrosines in the PY motifs were mutated to alanines; the latter double-mutant is unable to bind Nedd4 (Fig. 2). As evident from Fig. 3B (top panel), mutation of the first PY motif (lane 4) did not significantly block ubiquitination of CNrasGEF, but mutating both PY motifs led to an almost complete blockade of CNrasGEF ubiquitination (lane 5), similar to the effect of Nedd4(CS) (Fig. 3A). This suggests that either the second or both PY motifs is/are required for Nedd4-mediated ubiquitination. Taken together, these results demonstrate that ubiquitination of CNrasGEF requires binding to Nedd4, further confirming our conclusion that Nedd4 is the E3 responsible for the ubiquitination of CNrasGEF in cells.

The Stability of CNrasGEF Is Regulated by Nedd4—Because our results above demonstrate that Nedd4 is responsible for CNrasGEF ubiquitination, and because ubiquitination of proteins usually results in their subsequent degradation, we proceeded to test whether Nedd4 regulates the stability of CNrasGEF using pulse-chase experiments. To this end, transfected CNrasGEF was pulsed with [35S]Met/Cys for 2 h and chased for 0–18 h (Fig. 4, A and B). Our results show that CNrasGEF expressed in HEK 293T cells has a half-life of ~10 h. This half-life was drastically reduced to ~2 h upon co-expression of wt-Nedd4 together with CNrasGEF. In contrast, co-expression of Nedd4 with CNrasGEF with mutated PY motifs (CNrasGEFΔ2PY) led to stabilization of the mutant protein, with a half-life of ~14 h (Fig. 4B). In accord, CNrasGEF stabilization was also observed in cells co-expressing Nedd4(CS) relative to those co-expressing wt-Nedd4 (Fig. 4A, bottom). Thus, Nedd4 appears to regulate protein stability of CNrasGEF.

CNrasGEF Is Sensitive to Proteasomal Degradation—Several Nedd4 substrates appear to be transmembrane proteins that are targeted for endocytosis and lysosomal degradation following ubiquitination (e.g. ENaC, numerous yeast amino acid permeases (17)), whereas other, cytoplasmic proteins, appear to be targeted to the proteasome (e.g. Ref. 18). We thus tested whether ubiquitination of CNrasGEF, which is not a transmembrane protein, is sensitive to proteasome inhibitors. Fig. 5 shows that treatment of cells with lactacystin for 30 min enhanced the accumulation of ubiquitinated CNrasGEF (Fig. 5A) and stabilized the protein due to inhibition of the proteasome (Fig. 5B), suggesting that ubiquitinated CNrasGEF is targeted for proteasomal degradation.

Reduced Ubiquitination in CNrasGEF Mutants That Can Not Bind Ras—Wild-type CNrasGEF is able to co-immunoprecipitate Ras and Nedd4 (Fig. 6C), suggesting that the three proteins may form a complex in cells. Interestingly, CNrasGEF...
lacking its CDC25 (ΔCDC25) domain is unable to bind Ras (Fig. 6B). To test whether Ras binding is involved in CNrasGEF ubiquitination, we analyzed ubiquitination of the ΔCDC25 mutant of CNrasGEF in cells. As seen in Fig. 6A, ubiquitination of the ΔCDC25 mutant was severely impaired. Because the CDC25 domain possesses 26 lysine residues, which could serve as potential ubiquitin acceptor sites (hence their loss could theoretically lead to reduced ubiquitination irrespective of Ras binding), we generated a point mutation (R898D) in the CDC25 domain that cannot bind Ras (Fig. 6B). Similar to the ΔCDC25 mutant, the R898D mutant showed impaired ubiquitination (Fig. 6A). These results suggest that Ras binding is critical for the ubiquitination of CNrasGEF. To test whether Ras binding to CNrasGEF affects the ability of Nedd4 to associate with this exchange factor, we tested the ability of Nedd4 to co-immunoprecipitate with the R898D mutant, which is incapable of binding Ras. Fig. 6D shows that Nedd4 can still bind the R898D mutant, suggesting that Ras binding to the CDC25 domain is not affecting Nedd4 binding to the PY motifs of CNrasGEF. Thus, Ras binding is not required for Nedd4 binding to CNrasGEF, but rather, for its subsequent ubiquitination.

**DISCUSSION**

We had identified CNrasGEF as an interacting protein of Nedd4 in an expression library screen (6) and have now characterized the interactions between these two proteins. In this report, we show that (i) Nedd4 binds in vitro and in cells to CNrasGEF; this association is mediated by one or more WW domains of Nedd4 (6) and the PY motifs of CNrasGEF; (ii) CNrasGEF is ubiquitinated in cells in a Nedd4-dependent manner, because it requires both active Hect domain of Nedd4 and intact PY motifs of CNrasGEF; (iii) Nedd4 regulates the stability of CNrasGEF in cells; (iv) removal of the Ras (or Rap-1) binding region on CNrasGEF leads to impaired ubiquitination of CNrasGEF; and (v) Binding of Nedd4 to CNrasGEF and the regulation of its ubiquitination are not dependent on cAMP-mediated activation of Ras by CNrasGEF.

---

**FIG. 5.** Accumulation of ubiquitinated CNrasGEF and its increased stability in cells treated with the proteasome inhibitor lactacystin. 293T cells expressing FLAG-CNrasGEF with or without HA-Ub were treated (or not) with 10 μM lactacystin for 30 min. They were then lysed, and the lysate was immunoprecipitated with anti-FLAG (CNrasGEF) antibodies and precipitated CNrasGEF immunoblotted with anti-HA antibodies to detect accumulation of ubiquitinated protein (CNrasGEF-Ub) (top panel). The bottom panel depicts the amount of transfected CNrasGEF in the corresponding lysates. B, pulse-chase experiments using 2-h pulse with [35S]Met/Cys followed by 0- or 3-h chase in the absence or presence of 10 μM lactacystin. CNrasGEF stability increased ~1.7 ± 0.15-fold (mean ± S.E., n = 3) by the lactacystin treatment. The experiment was performed as in Fig. 4, and one representative of three independent experiments is shown. The numbers at the bottom of panel B depict the average amount of remaining CNrasGEF protein (n = 3).
Only a few studies have investigated regulation of Ras exchange factors by the ubiquitin system. In an earlier report, the yeast Cdc25p was shown to possess a destruction box (DB) similar to that found in mitotic cyclins, which controls Cdc25p stability in vivo. The effect of this DB on Cdc25p ubiquitination was not investigated, however. Moreover, Cdc25p degradation appeared constitutive and not cell cycle-dependent (19). Another report has demonstrated in vitro ubiquitination and shortening of half-life of mSos2, which contains two sequences resembling the DB but not mSos1 (where the equivalent sequences are somewhat different) (20). More recently, GRF2 was shown to become ubiquitinated and destabilized in cells upon elevation of cellular calcium, effects dependent on a DB sequence present between the Ras exchange motif and CDC25 domains of this protein. Moreover, Ras binding to the CDC25 domain of GRF2 seems to be critical for its subsequent ubiquitination (21). In all three cases, a destruction box was implicated in the ubiquitination process, but the E3 involved was not identified.

The work presented here demonstrates ubiquitination-mediated regulation of the stability of the Ras/Rap-1 exchange factor CNrasGEF in cells, which is mediated by a different recognition signal: PY motifs that are recognized by WW domains of the ubiquitin ligase Nedd4. This study thus identifies the E3 involved in the regulation of stability of CNrasGEF.

Our recent work has demonstrated that CNrasGEF activates Ras in cells in response to cAMP (6) and Rap-1 independent of cAMP (6–9). The present results showing impaired ubiquitination of CNrasGEF by mutants that are unable to bind Ras/Rap-1 (ΔCDC25, R898D) suggest that Ras or Rap-1 binding is important for the ensuing ubiquitination of CNrasGEF by Nedd4. This is in agreement with the recent demonstration of requirement for Ras binding to GRF2 for its ubiquitination (21) and implicates Ras/Rap-1 binding as an important signal for destruction of these exchange factors. Interestingly, our work shows that stimulation of Ras activation by cAMP is not required for Nedd4 binding to, or Nedd4-mediated ubiquitination of, CNrasGEF. These results suggest that Ras (or Rap-1) binding per se, not the ensuing activation of this protein by CNrasGEF, provides the trigger for ubiquitin-mediated destruction. How the binding of Ras to CNrasGEF leads to recognition by Nedd4 is currently unknown. Clearly, Ras binding to the CDC25 domain is not affecting the ability of Nedd4 to bind the PY motifs of CNrasGEF. Thus, a possible explanation, albeit speculative, may be that Ras binding mediates conformational changes of CNrasGEF that may expose key lysine residues, thus allowing the Hect domain of Nedd4 to attach ubiquitins onto them. A similar scenario may exist for GRF2, whereby Ras binding may allow ubiquitination of exposed lysine residues by an as yet unknown E3 ligase (21).

Nedd4 and its yeast orthologue Rap5p have been shown to

FIG. 6. Reduced ubiquitination of CNrasGEF(ΔCDC25) or CNrasGEF(R898D), which cannot bind Ras. A, 293T cells were transfected with WT or mutant CNrasGEF that either lack its CDC25 domain (ΔCDC25) or bear a point mutation in the CDC25 domain, which renders it incapable of binding Ras (R898D) (see B). As a control, WT CNrasGEF was also transfected with catalytically inactive Nedd4 (Nedd4ΔCS). Following cell lysis, CNrasGEF was immunoprecipitated with anti-FLAG antibodies, and the immune complex was immunoblotted with anti-HA antibodies to detect ubiquitinated CNrasGEF (CNrasGEF-Ub). The lower panel depicts expression of CNrasGEF or mutated CNrasGEF in lysates corresponding to those in the upper panel. B, 293T cells expressing either WT CNrasGEF, ΔCDC25, or R898D mutants were lysed, and the lysate was incubated with GST-Ras to co-precipitate CNrasGEF or its mutant (upper panel). The lower
regulate ubiquitination and cell surface stability of several transmembrane proteins (17). Nedd4 is best characterized by its interactions with the epithelial Na⁺ channel (ENaC) (2). ENaC is composed of three subunits (α, β, γ), each containing one PY motif (22) that can bind to the Nedd4-WW domains (2, 4). Mutations or deletion of the PY motif of β or γ ENaC cause Liddle syndrome (reviewed in Ref. 23), a genetic form of hypertension caused by increased retention and activity of the channel at the cell surface (22, 24). Nedd4 was demonstrated to be a suppressor of ENaC, which regulates channels numbers at the plasma membrane (3, 25), and indeed cell surface stability of ENaC is regulated by ubiquitination (26). This regulation by Nedd4 is impaired in Liddle syndrome due to impaired binding of Nedd4 to ENaC (3) and impaired endocytosis of ENaC (27). Thus, ENaC is a well-documented substrate for Nedd4. Interestingly, recent studies have reported regulation of ENaC by K-Ras-2A, which increases channel activity while decreasing its numbers at the cell surface (28). Because cAMP is a known regulator of ENaC in native tissues (29), it is possible, although speculative, that the association of Nedd4 with CNrasGEF may be somehow involved in connecting ENaC to the Ras pathway.

Although numerous studies have demonstrated regulation of endocytosis and cell surface stability of transmembrane proteins by Nedd4 and Nedd4 family members (e.g. ENaC, the chloride channel ClC-5, the voltage-gated sodium channel H1, LMP2A, and several yeast amino acid permeases and receptors (30, 31; reviewed in Refs. 17, 32)), several studies have shown that Nedd4 and its family members regulate stability of intracellular proteins as well. For example, Smurf1 and -2 regulate ubiquitination and stability of Smads proteins involved in transforming growth factor-β signaling (33, 34), and Rsp5p binds to and regulates stability of RNA polymerase II (18). Thus, although CNrasGEF is localized to the plasma membrane in a PDZ-domain-dependent manner (6), it is nevertheless an intracellular protein. Accordingly, our results here suggest that ubiquitination of CNrasGEF targets it for proteasomal degradation. Thus, Nedd4 can ubiquitinate and target its substrates for either endocytosis/lysosomal degradation or for proteasomal degradation.

In summary, we have shown here that Nedd4 regulates
ubiquitination and stability of CNrasGEF and that such ubiquitination is likely linked to Ras binding to this exchange factor. The mode of ubiquitination of Ras-bound CNrasGEF by Nedd4 remains to be characterized.

REFERENCES

1. Kumar, S., Tomooka, Y., and Noda, M. (1992) Biochem. Biophys. Res. Commun. 185, 1155–1161
2. Staub, O., Dho, S., Henry, P., Correa, J., Ishikawa, T., McGlade, J., and Rotin, D. (1996) EMBO J. 15, 2371–2380
3. Abriel, H., Leffing, J., Rehun, J. F., Pratt, J. H., Schild, L., Horisberger, J. D., Rotin, D., and Staub, O. (1999) J. Clin. Invest. 103, 667–673
4. Kanelis, V., Rotin, D., and Forman-Kay, J. D. (2001) J. Biol. Chem. 276, 38125–38130
5. Treier, M., Staszewski, L. M., and Bohmann, D. (1994) Cell 78, 787–798
6. Taylor, S. J., and Shalloway, D. (1996) Curr. Biol. 6, 1621–1627
7. de Rooij, J., and Bos, J. L. (1997) Oncogene 14, 623–625
8. Huibregtse, J. M., Yang, J. C., and Beaudenon, S. L. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 3656–3661
9. Kaplon, T., and Jacquet, M. (1998) J. Biol. Chem. 273, 20742–20747
10. Nielsen, K. H., Papageorge, A. G., Vass, W. C., Willumsen, B. M., and Lowy, D. R. (1997) Mol. Cell. Biol. 17, 7132–7138
11. de Hoog, C. L., Koehler, J. A., Geldstein, M. D., Taylor, P., Figeys, D., and Moran, M. P. (2001) Mol. Cell. Biol. 21, 2107–2117
12. Schild, L., Lu, Y., Gautschi, I., Schneeberger, E., Lifton, R. P., and Rossier, B. C. (1996) EMBO J. 15, 2381–2387
13. Bonny, O., and Hummler, E. (2000) Kidney Int. 57, 1313–1318
14. Firsov, D., Schild, L., Gautschi, I., Merillat, A. M., Schneeberger, E., and Rossier, B. C. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 15370–15375
15. Goulet, C. C., Volk, K. A., Adams, C. M., Prince, L. S., Stokes, J. B., and Snyder, P. M. (1998) J. Biol. Chem. 273, 30012–30017
16. Staub, O., Gautschi, I., Ishikawa, T., Breitschopf, K., Ciechanover, A., Schild, L., and Rotin, D. (1997) EMBO J. 16, 6325–6336
17. Shimkets, R. A., Lifton, R. P., and Canessa, C. M. (1997) J. Biol. Chem. 272, 25357–25361
18. Bar-Sagi, D., and Hall, A. (2000) Cell 103, 227–238
19. Aylton, V., and Rebollo, A. (2000) Mol. Membr. Biol. 17, 65–73
20. Bos, J. L. (1998) EMBO J. 17, 6766–6782
21. Treier, M., Staszewski, L. M., and Bohmann, D. (1994) Cell 78, 787–798
22. Taylor, S. J., and Shalloway, D. (1996) Curr. Biol. 6, 1621–1627
23. Bar-Sagi, D., and Hall, A. (2000) Curr. Opin. Cell Biol. 12, 157–165
24. Ayllon, V., and Rebollo, A. (2000) Mol. Membr. Biol. 17, 65–73
25. Schwake, M., Friedrich, T., and Jentsch, T. J. (2001) J. Biol. Chem. 276, 12049–12054
26. Hicke, L. (1997) FASEB J. 11, 1215–1226
27. Zhu, H., Kavsak, P., Abdollah, S., Wrana, J. L., and Thomsen, G. H. (1999) Nature 400, 687–693
28. Kavask, P., Rasmussen, R. K., Causing, C. G., Bonni, S., Zhu, H., Thomsen, G. H., and Wrana, J. L. (2000) Mol. Cell 6, 1365–1375
