Ras Binding Triggers Ubiquitination of the Ras Exchange Factor Ras-GRF2

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Ras is a small GTPase that is activated by upstream guanine nucleotide exchange factors, one of which is Ras-GRF2. GRF2 is a widely expressed protein with several recognizable sequence motifs, including a Ras exchange motif (REM), a PEST region containing a destruction box (DB), and a Cdc25 domain. The Cdc25 domain possesses guanine nucleotide exchange factor activity and interacts with Ras. Herein we examine if the DB motif in GRF2 results in proteolysis via the ubiquitin pathway. Based on the solved structure of the REM and Cdc25 regions of the Son-of-sevenless (Sos) protein, the REM may stabilize the Cdc25 domain during Ras binding. The DB motif of GRF2 is situated between the REM and the Cdc25 domains, tempting speculation that it may be exposed to ubiquitination machinery upon Ras binding. GRF2 protein levels decrease dramatically upon activation of GRF2, and dominant-negative Ras induces degradation of GRF2, demonstrating that signaling downstream of Ras is not required for the destruction of GRF2 and that binding to Ras is important for degradation. GRF2 is ubiquitinated in vivo, and this can be detected using mass spectrometry. In the presence of proteasome inhibitors, Ras-GRF2 accumulates as a high-molecular-weight conjugate, suggesting that GRF2 is destroyed by the 26S proteasome. Deleting the DB reduces the ubiquitination of GRF2. GRF2 lacking the Cdc25 domain is not ubiquitinated, suggesting that a protein that cannot bind Ras cannot be properly targeted for destruction. Point mutations within the Cdc25 domain that eliminate Ras binding also eliminate ubiquitination, demonstrating that binding to Ras is necessary for ubiquitination of GRF2. We conclude that conformational changes induced by GTPase binding expose the DB and thereby target GRF2 for destruction.

The Ras proto-oncogenes encode low-molecular-weight, membrane-bound GTPases that play a central role in ensuring an appropriate cellular response to growth and differentiation factors by transducing and integrating extracellular signals (4, 27). Despite this pivotal role, little is known about how Ras is regulated. Ras acts as a critical intermediate in the transduction of signals from membrane receptors by acting as a molecular switch, transmitting signals to downstream components only when in an active GTP-bound form. Cycling of Ras between the inactive GDP-bound form and the active GTP-bound conformation is regulated by the opposing actions of guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs).

Ras-GRF2 (GRF2) is a widely expressed GEF which catalyzes nucleotide exchange on Ras through its Cdc25 domain (7, 14). GRF2 is a bifunctional GEF; in addition to having activity on Ras, GRF2 is capable of binding to another small G protein, Rac1, through its DbI homology (DH) domain. Through its interaction with Ras and Rac, GRF2 is capable of activating both the extracellular signal-regulated kinase (ERK) and the stress-activated protein kinase-mitogen-activated protein kinase (MAPK) cascades (14, 15). GRF2 is a modular protein containing a number of protein motifs in addition to the Cdc25 and DH domains. It contains, in amino-to-carboxy-terminal order, a pleckstrin homology (PH) domain, coiled-coil motif, illimaquinone motif, DH domain, a second PH domain, a Ras exchange motif (REM), a PEST-like region (rich in proline, glutamic acid, serine, and threonine) that contains a candidate destruction box (DB), and, finally, the Cdc25 domain (14). PH domains in other proteins are involved in protein-protein or protein-lipid interactions; the illimaquinone motif in GRF2 appears to be important for allowing activated Ras to couple to the MAPK pathway (11); the REM in a related exchange factor, Son-of-sevenless (Sos), has been implicated in stabilizing the structure of the Cdc25 domain (5). Between the REM and the Cdc25 domains of GRF2 is a motif similar to the DB of B-type cyclins, as well as a stretch of amino acids C-terminal to the DB that is rich in proline, glutamate, serine, and threonine (PEST sequences). Both motifs have been implicated in targeting proteins for ubiquitination and subsequent degradation via the 26S proteasome.

The ubiquitin system is a highly conserved method of protein degradation which involves the posttranslational modification of proteins by the small protein ubiquitin and delivery of these modified proteins to the 26S proteasome for degradation (reviewed in reference 24). The attachment of ubiquitin to a protein occurs via a biochemical “bucket-brigade” of enzyme activity. First, free ubiquitin is activated by an E1 enzyme and is then transferred to an E2 enzyme which, in cooperation with an E3 ubiquitin ligase protein (or protein complex), covalently links ubiquitin to a lysine residue on the target protein. The process can be repeated to add an additional ubiquitin to the previous one, commonly on Lys48 of ubiquitin. Ubiquitin conjugation continues, resulting in a high-molecular-weight complex containing a polyubiquitin chain that is essential for rec-
ognition and degradation by the 26S proteasome with concomitant recycling of ubiquitin. Recently, a fourth component, called E4, that is required for ubiquitin chain elongation was cloned (23).

Various signals can target proteins for ubiquitination. The DB, first found in mitotic cyclins, is a 9-amino-acid motif that targets proteins for ubiquitination usually in a cell cycle-specific manner, through the anaphase-promoting complex (APC), an E3 ligase (8). Another signal, the KEN box, targets a subset of proteins different from those targeted by the APC (36). A third putative signal is a PEST sequence: G, cycles are an example of proteins that contain this signal (47). The E3 ligase involved in degrading these substrates is the SCF protein complex, which consists of the following proteins: a cullin family member, Skp1; Rbx1 (also called Roc1); and an F-box protein which binds the targeted substrate (9). A requirement for phosphorylation of the substrate prior to recognition by the SCF complex appears to be common to all SCF-substrate interactions.

Given the presence of the putative ubiquitination signals in GRF2, we have chosen to study the targeting of GRF2 by the ubiquitination system. The location of the REM in the crystal structure of Sos bound to Ras suggests that the two domains of Sos, REM and Cdc25, interact with each other during binding to Ras (5). In Sos, the REM and Cdc25 domain are situated in close proximity to one another, whereas in GRF2 there is a large block of intervening sequence. Interestingly, it is this stretch of amino acids in GRF2 that contains the PEST region and the DB. It is possible, therefore, that, upon the binding of GRF2 to Ras, the DB is exposed to the ubiquitination machinery, resulting in ubiquitination of GRF2. Here, we show that GRF2 contains a targeting signal for ubiquitination and that GRF2 is ubiquitinated following binding to Ras.

MATERIALS AND METHODS

Construction of plasmids. The cloning of the ΔDH and the ΔCdc25 GRF2 deletions has been previously described (15).

The mutant GRF2s were generated by PCR. The ΔREM construct, in which GRF2 codons 638 to 686 were deleted, was obtained by doing two rounds of PCR. The first two reactions used the following primers: (i) a 5′ primer flanking the unique BamHI site in GRF2 and a 3′ primer that deletes the above-named codons and (ii) a 5′ primer from which the above codons were deleted and a 3′ primer that flanks the unique XhoI site in GRF2. These two PCR products were mixed and used as a template for a second round of PCR using the BamHI- and XhoI-flanking primers, resulting in a PCR product with codons 638 to 686 deleted. The PCR product was digested with BamHI and XhoI and subcloned into BamHI and XhoI-digested pcDNA3-Flag-GRF2.

The ΔDB construct, with codons 742 to 751 deleted, was obtained using the same method. Reaction 1 used a 5′ primer starting at codon 541 and a 3′ primer with the above-named codons deleted. Reaction 2 used a 5′ primer with the above-named codons deleted and a 3′ primer that flanks the SacI site in the pcDNA multiple cloning site. These two PCR products were mixed and used as a template for a second round of PCR using the outside flanking primers, resulting in a PCR product with codons 742 to 751 deleted. This PCR product was digested with EcoRI and the 1,763-bp fragment was used to replace the 1,783-bp fragment from pcDNA3-Flag-GRF2.

The point mutations R1022E and R1092A were constructed using a Transgen RNA extraction. Northern analysis was performed as described previously (22). Northern analysis was performed as follows: 20 μg of total RNA was separated on a agarose-formaldehyde gel and transferred to a nylon filter. The filter was prehybridized in FSB (100 mM NaH2PO4, 50 mM sodium pyrophosphate, 7% SDS, 1 ml EDTA, 100 μg of denatured salmon sperm DNA per milliliter) for 5 h at 68°C and then hybridized with a random-prime synthesized probe overnight at 68°C. The probe used was DNA corresponding to codons 666 to 935 of GRF2. The filter was washed twice for 45 min each in FSB with SDS lowered to 1% and then exposed to X-ray film. The blot was stripped and reprobed with a random-prime synthesized probe corresponding to an internal fragment of a housekeeping gene, β-actin. The Northern blot data were quantified using a Bio-Rad GS-250 phosphorimagier and Molecular Analyst software.
ERK 1 assay. 293T cells were transiently cotransfected with 3 μg of p3JM-ERK1 (expressing Myc epitope-tagged ERK 1) and 5 μg of either the pcDNA3 vector or the pcDNA3-Flag-GRF2 constructs as indicated in the figures. After 48 h, the cells were serum starved for 18 h and then stimulated with 4 μM ionomycin for 5 min at 37°C, washed in PBS, and then lysed in NP-40 lysis buffer. A Bradford assay was performed using clarified lysates. Ten micrograms of lysate was separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-Flag, anti-Myc (9E10), anti-MAPK (New England Biolabs, Inc.), and anti-phospho-MAPK (New England Biolabs, Inc.) antibodies.

Ubiquitination assays. 293T cells were transiently transfected with 4 μg of the pcDNA-Flag-GRF2 constructs as indicated in the figures, with or without 1 μg of pCMV-HA-Ubiquitin (44). After 48 h, cells were rinsed in PBS and lysed in NP-40 lysis buffer and the lysates were clarified. Lysates were precleared with a search engine (PeptideScan). The search engine was used to identify the sequence was manually determined, generating a sequence tag, which was fed into a database search engine (PeptideScan). The search engine was used to identify the provenance of the peptide with protein-DNA database searches. Every peptide identified was confirmed manually.

RESULTS

GRF2 contains a candidate DB. Sequence analysis revealed that GRF2 contains a sequence with some similarity to one found in mitotic cyclins and other unstable proteins (Fig. 1). The region in mitotic cyclins required for ubiquitin-mediated proteolysis contains an amino acid motif called the DB (18, 48). The consensus for this motif is RXLGXNXX; the R and L residues are conserved in all the DBs of A- and B-type cyclins, while the N residue is conserved only in B-type cyclins (18). GRF2's PEST-rich region contains a motif very similar to the DB of A-type cyclins. The Ras exchange factor in Saccharomyces cerevisiae, Cdc25p, has been shown to contain a functional DB with the sequence RSSLNLGSLN (21). It is interesting that GRF2's DB, KLSLTSSLN, resembles the yeast exchange factor's DB more closely than the DBs of mitotic cyclins.

GRF2 is an unstable protein in stimulated cells. If GRF2 is a protein that is targeted for destruction upon Ras binding, then one predicts that activating GRF2 and therefore activating Ras would result in a decrease in GRF2 protein levels. To test this, a 293 cell line stably transfected with GRF2 protein and expressing low levels of the protein was grown to 90% confluence. Cells were then stimulated with ionomycin, a calcium ionophore that raises intracellular calcium levels, leading to the activation of GRF2 (11, 14, 15). Cells were harvested at various time points, and equal amounts of total protein were resolved by SDS-PAGE. GRF2 was detected by Western blotting with M2 anti-Flag antibody, and the amount of GRF2 present under each condition was quantified. The region of total protein were resolved by SDS-PAGE. GRF2 was de-

FIG. 1. Schematic representation of the domain structure of murine GRF2 with a sequence alignment of the DB motif of GRF2 (codons 743 to 751); the Ras exchange factor in yeast, Cdc25p (codons 148 to 156); and the consensus sequence of mitotic A-type cyclins. The boxed residues are those that are conserved in known DBs; the R and L residues are conserved in A- and B-type cyclins, whereas the N residue is conserved only in B-type cyclins. CC, coiled-coil motif; IQ, illimaquinone motif.
suggests that the disappearance of GRF2 is a signal-triggered event.

**GRF2 destruction depends upon its DB motif.** In order to determine if the DB motif is responsible for the instability of GRF2 in lysates, a construct of GRF2 in which the DB was deleted was used. 293 cells were transiently transfected with GRF2 or the ΔDB construct, with or without N17 Ras (Fig. 3). N17 Ras displays a dominant-negative behavior as a consequence of its inability to coordinate magnesium properly; it prevents activation of endogenous Ras by sequestering exchange factors into dead-end complexes (16). We have shown previously that this Ras mutation prevents GRF2 from signaling to the MAPK pathway (15). By sequestering exchange factors in this method, N17 Ras results in a prolonged interaction between GRF2 and Ras rather than the usual, presumably transient, interaction. As a control, the cells were also cotransfected with a construct encoding β-galactosidase to ensure that any effects seen were specific to GRF2 and not to other transfected proteins. After equal amounts of protein were loaded on a gel and immunoblotting for Flag-GRF2 was performed, the level of GRF2 protein was found to be reduced approximately 90% in the presence of N17 Ras while the levels of the ΔDB construct were reduced only slightly (Fig. 3A). The levels of β-galactosidase did not change. The nitrocellulose membrane was exposed to a phosphorimager, and the image and quantitation obtained from the scanned phosphorimager screen are shown in Fig. 3B.

Northern blot analysis was performed to determine if this observed effect was at the protein level or if it was a result of much lower transcript levels in the WT samples. A histogram of the results of the Northern blots (Fig. 3C) confirms that the protective effect of deleting the DB was at the protein level. The Northern blotting showed that the introduction of N17 Ras resulted in a decrease in transcript levels; however, the

![FIG. 2. Ras-GRF2 protein levels decrease in response to stimulation by Ca2+-ionomycin. Serum-starved 293 cells stably expressing Flag-tagged GRF2 were stimulated for 5 min at 37°C with 4 μM ionomycin and harvested at the indicated periods of time. GRF2 protein levels in 60 μg of lysate were assessed by Western blotting with the M2 monoclonal anti-Flag antibody to detect GRF2; actin levels were assessed by blotting with an anti-actin monoclonal antibody. The data shown are representative of four experiments.](http://mcb.asm.org/)

![FIG. 3. Deleting the DB protects GRF2 from N17 Ras-induced degradation. (A) The assays were carried out 2 days after transfection of 293 cells with the indicated GRF2 construct. Thirty micrograms of lysate was separated by SDS-PAGE and transferred to nitrocellulose membranes. In the upper gel, the samples were Western blotted for GRF2 using M2 anti-Flag monoclonal antibody. In the middle gel, the presence of N17 Ras was verified by Western blotting of lysates with the LAO45 anti-Ras monoclonal antibody. In the lower gel, the lysate was Western blotted for β-galactosidase (β-gal) using an anti-β-galactosidase monoclonal antibody. The data shown are representative of four experiments. (B) The bar graph shows the quantitated data as relative protein levels. These data were generated from the phosphorimager image shown below the histogram, and this image was generated from the same membrane used for the upper gel of panel A. The data shown are representative of four experiments. (C) N17 Ras expression does not affect ΔDB transcript levels differently than it affects WT transcript levels. Northern analysis was performed as described in Materials and Methods. A random-primed probe of DNA corresponding to codons 686 to 933 of GRF2 was used to probe RNA extracted from cells transiently transfected with the indicated construct. The data shown are representative of two experiments. (D) ERK 1 activity in 293T cells. The assay was carried out 3 days after transfection of 293T cells with the indicated GRF2 construct and myc-ERK 1. Cells were serum starved for 20 h and then either left untreated (−) or treated with 4 μM ionomycin for 5 min at 37°C (+). In the upper gel, GRF2 protein expression was verified by Western blotting with a polyclonal phospho-ERK 1 and 2 antibody. Equal levels of expression of myc-ERK 1 were confirmed by Western blotting of the lysate with 9E10 myc monoclonal antibody (middle gel). The data shown are representative of three experiments.)
decrease was the same for the WT and the ΔDB construct. This was not surprising, as N17 Ras has been shown previously to globally reduce transcription (1).

To address the possibility that the ΔDB protein was more stable as a result of its being misfolded or improperly localized, GRF2 or the DB was cotransfected with myc-ERK and levels of MAPK activity were assessed by using a phospho-specific antibody to MAPK (Fig. 3D). Both WT GRF2 and the ΔDB protein signaled efficiently to the MAPK cascade, suggesting that the differences seen in levels of destruction are not because the deletion of the DB resulted in an unfolded protein that is not targeted properly. We have found that the expression level of GRF2 influences the amount of degradation that can be observed in these experiments in that a significant amount of overexpression of GRF2 can result in the ubiquitination machinery responsible for its targeting becoming saturated (data not shown), an effect that can also be seen in other systems (20). The high level of expression in 293T cells in this experiment resulted in the ubiquitination pathway becoming saturated so that no difference was seen when cells were stimulated with ionomycin. These findings imply that the destruction of GRF2 is dependent upon the presence of the DB and that the observed difference in protein levels is not a result of lower transcript levels. These data also suggest that activation of Ras and subsequent signaling are not required for the destruction of GRF2, as downstream signaling is blocked in the presence of N17 Ras.

**GRF2 is ubiquitinated in vivo.** Because DBs in other proteins target those proteins for destruction via the ubiquitin degradation pathway, we wanted to determine if GRF2’s DB motif targets it for ubiquitination. To do this, GRF2 was transiently cotransfected with an HA-tagged ubiquitin construct into 293T cells. GRF2 was immunoprecipitated from cell lysates prepared from these cells and blotted with 12CA5 antibody cross-reacting with the large amount of GRF2 present in the gel; there are ubiquitin conjugates of the appropriate size (Fig. 4D, lane 5). This ladder was not seen in vector-transfected control cells or in cells not expressing the ubiquitin construct (lane 4 and lanes 1 to 3, respectively). A ΔDH protein (lanes 2 and 6) was used as a control in this experiment to show that an irrelevant deletion does not cause a significant decrease in GRF2’s ubiquitination state. The ΔDH construct was ubiquitinated to a level similar to that of WT GRF2, while ubiquitination of the ΔDB construct was compromised.

The possibility exists that merely overexpressing a protein can result in its ubiquitination due to large amounts of misfolded polypeptides. However, this possibility does not appear to be a factor in this assay, as other proteins such as p120GAP and RACK1 were not ubiquitinated when they were overexpressed under these conditions (Fig. 4B and C, respectively). These data provide further evidence that the DB is important for targeting GRF2 for ubiquitination.

The ubiquitin moiety is covalently attached to the GRF2 protein, and the ubiquitin conjugates seen in the GRF2 IPs are not the result of an unknown ubiquitinated protein coimmunoprecipitating with GRF2. This possibility was tested by boiling the immune complex in a buffer containing 1% SDS, thereby disrupting all protein-protein interactions. TX-100 buffer was added to dilute the SDS to allow a second immunoprecipitation to be performed. GRF2 was immunoprecipitated again, and this sample was tested for the presence of ubiquitin conjugates of the appropriate size (Fig. 4D, lane 5). The bands seen in lanes 1 and 4 are the result of the 12CA5 antibody cross-reacting with the large amount of GRF2 present in the gel; there are no HA-tagged proteins present in these lanes. The data shown are representative of three experiments.

**FIG. 4.** GRF2 ubiquitination in vivo. The assays were carried out 2 days after transfection of 293T cells with the indicated construct. (A) Flag-GRF2 or the mutant proteins were isolated by anti-Flag antibody immunoprecipitation. The washed immune complexes were Western blotted with M2 Flag antibody for GRF2 (lower gel) and with 12CA5 antibody for ubiquitin (Ub) (upper gel). The data shown are representative of five experiments. (B) p120 Ras-GAP (GAP) was isolated by anti-KT3 antibody immunoprecipitation. The washed immune complexes were Western blotted with anti-KT3 ascsites fluid for GAP (lower gel) and with 12CA5 antibody for ubiquitin (upper gel). The data shown are representative of two experiments. (C) Flag-RACK1 was isolated by anti-Flag immunoprecipitation. The washed immune complexes were Western blotted with M2 Flag antibody for RACK1 (right gel) and with 12CA5 antibody for ubiquitin (left gel). The data shown are representative of two experiments. (D) GRF2 was isolated by anti-Flag antibody immunoprecipitation. Two samples were then boiled in SDS buffer for 5 min, allowed to cool, and then diluted in TX-100 buffer. GRF2 was again isolated from these samples by anti-Flag antibody immunoprecipitation. All of the washed immune complexes were Western blotted with M2 Flag antibody for GRF2 (upper gel) and with 12CA5 antibody for ubiquitin (lower gel). The bands seen in lanes 1 and 4 are the result of the 12CA5 antibody cross-reacting with the large amount of GRF2 present in the gel; there are no HA-tagged proteins present in these lanes. The data shown are representative of three experiments.
tion of GRF2 at 135 kDa (apparent molecular mass), two larger bands were identified at 175 and 200 kDa. All peptides located in these bands were identified as originating from GRF2, with the exception of a doubly charged peptide ion located at an m/z of 894.3. An MS-MS spectrum of this ion revealed it to be the tryptic peptide TITLEVEPSDTIENVK found only in ubiquitin (Fig. 5A, lower panel). An MS-MS spectrum of a diagnostic peptide derived from GRF2 is shown in Fig. 5A, upper panel. The abundance of the ubiquitin ion increased with the increasing molecular weight of the GRF2-containing bands (Fig. 5B), and this effect was more pronounced when its signal was normalized to one of the GRF2 peptide signals in the same band. This evidence suggests that the increasing apparent molecular weight of the GRF2 protein is caused by an increasing load of conjugated ubiquitin.

**GRF2 accumulates as a ubiquitinated complex in the presence of proteasome inhibitors.** In order to determine if the proteasome plays a role in the destruction of GRF2, we looked at the ubiquitination state of GRF2 in the presence of two proteasome inhibitors, LLnL and MG-132 (10, 17, 25). A 293 cell line stably expressing Flag-GRF2 and HA-ubiquitin was grown to 85% confluence and then treated for various times with a carrier (DMSO), 50 μM LLnL, or 50 μM MG-132. Cells were then lysed, GRF2 was immunoprecipitated, and its ubiquitination state was assessed by immunoblotting for HA-tagged ubiquitin (Fig. 6). As the length of treatment with the inhibitors increased, high-molecular-weight complexes of GRF2-ubiquitin began to accumulate (lanes 7 to 9 and 12 to 14). These complexes had barely exited the stacking gel and were therefore very large (>200 kDa). Similar results were seen with lactacystin, another proteasome inhibitor (data not shown). We have no explanation as to why treatment with MG-132 increased the levels of GRF2 whereas treatment with another proteasome inhibitor, LLnL, did not. This was, however, a repeatable result. As the 26S proteasome is known to degrade ubiquitinated proteins, this finding indicates that GRF2 is likely destroyed by the 26S proteasome.

**Cdc25 mutant proteins that cannot bind Ras are not ubiquitinated.** Deletion of the Cdc25 domain severely reduced the susceptibility of GRF2 to ubiquitination (Fig. 7C, lane 3). We have shown that the Cdc25 domain is required for interaction with Ras (15), so these data suggest that the exchange factor’s interaction with Ras is important for targeting GRF2 for ubiquitination. However, as deleting the Cdc25 domain removes 18 lysines from the protein, we could not exclude the possibility that the reduced ubiquitination of the ΔCdc25 protein was a result of the smaller number of lysines available for attachment of ubiquitin moieties. To address this, point mutant proteins were generated at conserved arginine residues in the Cdc25 domain at positions 1022 and 1092 (R1022E and R1092A);
The data shown are representative of four experiments.

FIG. 7. Ability of proteins with the Cdc25 domain point mutations R1022E and R1092A to bind Ras and become ubiquitinated. (A) In vitro association of GRF2, R1022E, and R1092A with GST-Ras. GST-Ras complexed with agarose beads was incubated with lysate from transiently transfected 293T cells expressing Flag-GRF2 or the indicated point mutant protein. Bound GRF2 proteins were detected by Western blotting with the M2 monoclonal Flag antibody. GRF2 and mutant protein expression in lysate was detected by Western blotting with the M2 monoclonal Flag antibody (lanes 1 to 3). The GST fusion protein had been prepared in either its nucleotide-free (NF), GDP-bound (GDP), or GTPγS-bound (GTPγS) form. The -- and + refer to the absence or presence of cell lysate during the incubation. In lane 5, lysate from cells expressing WT GRF2 was added. The data shown are representative of four experiments. (B) ERK 1 activity in 293T cells. The assay was carried out 2 days after transfection of 293T cells with the indicated GRF2 construct. In the upper gel, GRF2 protein expression was verified by Western blotting of the lysates. In the lower gel, activated ERK was detected by Western blotting with a polyclonal phospho-MAPK antibody. Equal levels of expression of ERK 1 and 2 were confirmed by Western blotting of the lysate with a polyclonal MAPK antibody (middle gel). The data shown are representative of three experiments. (C) Ubiquitination of mutant proteins. The assays were carried out 2 days after transfection of 293T cells with the indicated constructs. Flag-GRF2 or the mutant proteins were isolated by anti-Flag antibody immunoprecipitation. The washed immune complexes were Western blotted with M2 Flag antibody for GRF2 (lower gel) and with 12CA5 antibody for ubiquitin (Ub) (upper gel). The asterisk indicates a degradation product of the R1092A protein in lane 5. The data shown are representative of four experiments.

these mutations have been shown in other Ras GEF proteins to abrogate binding to Ras (6, 32, 35). These mutant GRF2 proteins should, therefore, be unable to be ubiquitinated as a consequence of this diminished binding.

The mutant proteins were tested for their ability to bind Ras in a pulldown assay (Fig. 7A). Bacterially produced, purified GST-Ras, either nucleotide-free or bound to GDP or to GTPγS, was incubated with lysate containing the indicated GRF2 protein, and the amount of GRF2 bound to the fusion protein was assessed by Western blotting. A common property of Cdc25 domains is their relatively high affinity for the nucleotide-free form of Ras, likely reflecting their catalytic mechanism of stabilizing an otherwise unfavorable nucleotide-free intermediate state. WT GRF2 (lanes 7 to 9) was able to bind specifically to nucleotide-free Ras, as previously shown (15). The binding of the R1022E protein was barely detectable (lanes 10 to 12); the binding of the R1092A protein was severely reduced, to approximately 20% that of WT GRF2 (lanes 13 to 15).

To test whether this impaired binding translates into an inability to signal to the MAPK cascade, phospho-MAPK immunoblotting was performed (Fig. 7B). Lysates from cells expressing the indicated GRF2 protein were immunoblotted with anti-Flag antibody to detect GRF2, anti-MAPK antibody to detect ERK 1 and 2, and anti-phospho-MAPK antibody to detect phosphorylated, activated ERK 1 and 2. GRF2 (lane 2) significantly activated ERK compared to the level of activation in the vector-alone control (lane 1). The R1022E protein activated ERK to a level similar to that of the vector-alone control, and the R1092A protein activated ERK slightly; these results correlate with the proteins’ limited ability to bind Ras.

In addition, the effect of reduced Ras binding and signaling on GRF2 ubiquitination was tested (Fig. 7C). Again, ability of the ΔCdc25 protein to be ubiquitinated was remarkably decreased and the R1022E and R1092A proteins were also severely impaired (lanes 4 to 5). This finding strongly supports the notion that GRF2 must bind Ras in order to be targeted for ubiquitination.

The ΔREM protein does not bind Ras and is not ubiquitinated. The REM appears to be involved in stabilizing the structure of the Cdc25 domain; it may orientate or stabilize a helical hairpin of the Cdc25 domain so that, upon binding, the hairpin can alter the structure of Ras to allow for nucleotide release (5). After deletion of the REM, GRF2 lost its ability to bind to Ras as shown in Fig. 8A (lanes 9 to 11). Concomitant with this, the ΔREM protein was severely impaired in ERK activation (Fig. 8B). Along with this inability to bind Ras, the ΔREM protein was also no longer ubiquitinated (Fig. 8C), again implying that binding to Ras is a necessary event in targeting GRF2 for ubiquitination.

Overexpressing Ras increases ubiquitination of GRF2. If binding to Ras is important for ubiquitination, then one can predict that overexpression of Ras would have an effect on the ubiquitination of GRF2. In Fig. 9, H-Ras was overexpressed in 293 cells and the ubiquitination of GRF2 was assessed. Increasing cellular Ras levels increased the ubiquitination of WT GRF2 (Fig. 9, lanes 2 and 3), as predicted. Interestingly, it also increased the amount of ubiquitination of the R1022E and R1092A proteins (Fig. 9, lanes 4 and 5 and 6 and 7). Without overexpressing Ras, the R1022E and R1092A proteins have barely detectable levels of ubiquitination; increasing Ras levels presumably shifts the equilibrium to increase binding between Ras and the mutant proteins, thereby increasing ubiquitination.

GRF2 is phosphorylated near the DB. GRF2 was analyzed for the presence of phosphorylated residues by deconvolution of the mass spectrum of a tryptic digest of the protein and searching for peptides differing by a mass of 80 Da (neutral mass of HPO₃) (Fig. 10A). Two phosphate-containing peptides were located, and their sequences were confirmed by MS-MS. Both peptides are located close to the DB and are within the median size of phosphorylation sites.
PEST region of GRF2. The peptide KFSSPPPLAVSR (residues 723 to 734 of GRF2), located on the N-terminal side of the DB, was found to contain a single phosphorylation site (Fig. 10B). The peptide IGALDLTNSSSSSSPTTTTHSPAA SPPPHTAVLESAPADK (residues 754 to 793), located on the C-terminal side of the DB, was found to have four phosphorylated residues (data not shown). For both peptides, the mass spectra did not provide enough information to determine the exact amino acid(s) that is phosphorylated.

**DISCUSSION**

Here we have tested the role of the DB motif in the ubiquitination of GRF2 and whether conformational changes induced by GTPase binding expose the DB and thereby target GRF2 for destruction. The deletion of the DB did not appear to result in a mislocalized or misfolded protein, as the ΔDB protein was still fully functional in terms of its ability to activate the MAPK pathway. N17 Ras induces degradation of GRF2, demonstrating that signaling downstream of Ras is not required for the destruction of exchange factor; however, there does not appear to be any cell cycle regulation of this destruction and direct involvement of ubiquitin was not demonstrated (21). Kaplon and Jacquet (21) suggest that these factors indicate that regulation of Ras in yeast may be directly modulated by the cellular content of the exchange factor rather than variations in cellular localization or activity. In vitro-translated mouse Sos2 has been shown to be ubiquitinated, but the ubiquitination of Sos in vivo has not been explored (34). The data in this paper provide the first demonstration of the in vivo ubiquitination of an activator of Ras, as well as a model to explain how it is targeted for destruction.
FIG. 10. Mass spectra of peptides generated from a trypsin digest of GRF2. (A) GRF2 was immunoprecipitated from a stably expressing 293 cell line and separated by SDS-PAGE, and the GRF2-specific band was analyzed by MS as described in Materials and Methods. Shown are two peptides that differ in size by the molecular weight of a phosphate group. This indicates that this peptide is phosphorylated. (B) Both peptides from panel A were isolated and sequenced by MS-MS. The peptide is located at amino acids 723 to 734 of GRF2. The upper graph shows the spectrum derived from the 643.3-Da peptide; the lower graph shows the spectrum derived from the 683.3-Da peptide. The arrow depicts the loss of the phosphate group from the larger ion. amu, atomic mass units; TOF, time of flight.

GRF2. This result strongly indicates that binding to Ras is necessary for degradation.

GRF2 is ubiquitinated in vivo, but it is not possible to see the GRF2-ubiquitin conjugates by Western blotting for GRF2, suggesting that only a small portion of GRF2 is ubiquitinated. The experiments assaying ubiquitination were done with exponentially growing cell cultures, so if ubiquitination of GRF2 is linked to cell cycle events, then only a small portion of the total cell culture would be in the correct phase. This may explain why we did not find a larger population of GRF2 becoming ubiquitinated. An equally plausible explanation may just be that the sensitivities of the antibodies used are not sufficient to detect the portion of GRF2 protein that is modified with ubiquitin. However, in a GRF2 IP from unsynchronized cells, ubiquitin peptides can be detected using MS techniques. Ubiquitin sequences can be found associated with GRF2 sequences in the absence of other proteins at apparent molecular weights much higher than that of GRF2 or ubiquitin alone. This circumstance is highly suggestive of a covalent interaction between the two proteins. Furthermore, if the mass spectrometer detector response of the identified ubiquitin peptide is normalized to any peptide from GRF2, a stoichiometric estimate can be made. From this, it is apparent that on an SDS-polyacrylamide gel the slower-migrating GRF2 is more highly ubiquitinated.

The ΔCdc25 protein is not ubiquitinated, suggesting that a protein that cannot bind Ras cannot be properly targeted for degradation. To test this further, and to ensure that this effect was not due to the removal of a large number of lysines, point mutant proteins that are severely impaired in their ability to bind Ras were made. The point mutations within the Cdc25 domain also eliminated ubiquitination, demonstrating that binding to Ras is required for ubiquitination of GRF2.

While the ubiquitination of the ΔDB protein is impaired, it is not eliminated. It is possible that the DB is not the sole determinant for ubiquitination of GRF2. As mentioned above, GRF2 also contains PEST sequences that are thought to be signals for ubiquitination, and perhaps these PEST sequences cooperate with the DB in targeting GRF2 for destruction. Preliminary observations suggest that this may be the case. The DB, first found in mitotic cyclins, is a 9-amino-acid motif that targets proteins for ubiquitination through the E3 ligase called the APC, usually in a cell cycle-specific manner (48). Substrate recognition by the APC is thought to require one of several adapter proteins containing WD40 motifs. The known adapter proteins responsible for degrading various proteins such as the mitotic cyclins are Cdh1 and Cdc20, each of which appears to be responsible for specific substrates (45). All known Cdc20 substrates contain a DB, while Cdh1 substrates are recognized by the presence of a DB or a KEN box (36). It is unknown at this point if GRF2 is ubiquitinated by the APC pathway with a specific adapter protein that links it to the APC.

The APC is active from the end of mitosis and throughout G1 phase, according to literature that considers the proteolysis of G2 cyclins (2). Preliminary results suggest that GRF2 protein begins to be degraded at the end of G1 phase (C. L. de Hoog, unpublished observations). This finding suggests that either an unknown adapter protein for the APC regulates its destruction or GRF2’s ubiquitination is actually regulated by the SCF complex. To date, all known SCF substrates are recognized in a strictly phosphorylation-dependent manner (9). Interestingly, GRF2 is phosphorylated on multiple residues within its PEST region, as shown by MS. We do not know the role these phosphorylation events play, but it is interesting that they fall within the PEST region of GRF2 as it is the PEST region in G1 cyclins that must be phosphorylated in order for them to be targeted for ubiquitination (9). It is also possible that the phosphorylation events are important in the activation of GRF2, as is the case for GRF1 (29–31).

The precedent has been set for proteins in the Ras pathway being destroyed by ubiquitin-mediated proteolysis, as evidenced by the destruction of tramtrack (Ttk), a transcriptional repressor in the Drosophila Ras signaling pathway that is required to specify R7 cell fate in the Drosophila eye (26, 42). The destruction of Ttk is dependent upon the presence of phyllopod (Phyl), which is induced by the Ras pathway downstream of the sevenless receptor tyrosine kinase. Phyl binds to a nuclear protein, seven in absentia (Sina), and this complex then binds Ttk, stimulating its ubiquitination and destruction. The Sina protein also binds to a ubiquitin-conjugating enzyme,
Ubc9, which presumably contributes to the ubiquitination of Ttk (42).

There are other examples in the literature of a binding-triggered signal for ubiquitination. Human papillomavirus protein E6 binds the cellular factor E6-AP, and this pair associates with p53, whereupon p53 is targeted for destruction via the ubiquitin-mediated proteolytic pathway (38). An example of activation-triggered ubiquitination is found in a report regarding protein kinase C (PKC) (28). Treatment of cells with phorbol esters activates and then depletes some PKC isoforms. This depletion is a result of ubiquitination that is stimulated upon activation of PKC; blocking activation blocks ubiquitination (28). Lu et al. (28) speculate that activation of the ubiquitin-conjugating system is likely stimulated by a conformational change in PKC that occurs upon ATP binding or hydrolysis, resulting in a suicide model for PKC regulation.

We propose that in an unstimulated cell, GRF2 is in an inactive complex or conformation, perhaps involving intramolecular interactions or an interaction with an unknown negative regulator. Upon stimulation of the cell with an agent that causes an increase in intracellular calcium levels, GRF2 is activated such that it is capable of binding to Ras. If the REM of GRF2 is involved in the stabilization of the Cdc25 domain as appears to be the case with Sos (5), this binding may “loop” out the stretch of amino acids containing the DB. As a consequence of the interaction with Ras, the intervening DB is displaced in an active state or conformation, causing the protein to be targeted for destruction (Fig. 11). Another Ras exchange factor, CNrasGEF, contains a PDZ domain between its REM and Cdc25 domains (37), and it is tempting to speculate that the activity of the PDZ domain is regulated by Ras binding in a manner similar to that of the DB of GRF2.

One possible explanation for the existence of multiple Ras-dependent signaling systems is that different signals are required at specific stages of the cell cycle. In addition to being able to transform cells, Ras has been established as an important cell cycle regulator. Microinjection of activated Ras into quiescent fibroblasts drives entry into S phase. Moreover, in some cell types, injection of neutralizing antibodies to endogenous Ras results in the cell cycle arrest of cells growing in serum and the inability to progress through to S phase (12, 13, 33). More recently, use of a novel method for detecting Ras-GTP, which involves affinity precipitation of activated Ras using its binding partner Raf, allowed the activation state of Ras to be monitored throughout the cell cycle (43). Using this method, Taylor and Shalloway demonstrated that in quiescent HeLa cells treated with serum, activation of Ras is achieved immediately after serum addition. Four to 5 h later in mid G1 phase, there is a second, much stronger activation of Ras, which does not appear to involve tyrosine phosphorylation (and therefore Grb2-Sos complexes). The pattern of Ras activation is the same when cells are grown in the presence or absence of serum or in suspension or attached to a substrate. These results point to a mechanism of Ras activation that is integral to the cell cycle machinery and is not solely linked to receptor-tyrosine kinase activation. It is possible that this mid-G1-phase activation of Ras is stimulated by GRF2, making this event calcium dependent.

As other DB-containing proteins are regulated in a cell cycle-dependent manner, perhaps Ras-GRF2 is also regulated in this way. Protein destruction is an excellent way to drive a pathway in one direction, as evidenced by the numerous cell cycle regulatory proteins whose levels or activities are controlled in this manner. It is also a mechanism that can be utilized to prevent an event from occurring at an inopportune time, and it may be for this reason that GRF2 is destroyed: in order to prevent activation of Ras at an inappropriate time in the cell cycle, perhaps in G2/M phase, when calcium oscillations are observed (3). An equally plausible explanation is that this is a method of turning off the signal following Ras activation. This downregulation is presumably part of a complex series of signaling events occurring following stimulation of a cell in order to elicit the desired response, be it progression through the cell cycle or differentiation. In either case, the regulated destruction of an exchange factor is a unique method of regulation in the Ras pathway in mammalian cells.

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