A Family of Proteins That Stabilize the Ran/TC4 GTPase in Its GTP-bound Conformation*

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Ran/TC4, referred to here as Ran1, is a 25-kilodalton nuclear GTP-binding protein with an acidic C terminus that lacks any consensus prenylation sites. Here, we use a nitrocellulose overlay assay to identify potential effector proteins that bind specifically and with high affinity to the GTP-bound form of Ran1. GTP-Ran1 is shown to bind to a variety of proteins, present in many eukaryotic tissues and cell extracts. A 28-kDa protein is cytosolic, whereas others, consisting of proteins of 86–900 kDa, are primarily localized in the nucleus. Binding is highly specific and is not detected by other small GTPases, such as c-Ha-Ras or Rab3A. Both deletion of the C-terminal -DEDDDEL acidic sequence or alteration of the N terminus of Ran1 inhibits binding. However, these altered forms of Ran1 maintain the capacity to bind guanyl nucleotides and interact with the nucleotide exchange factor. The Ran1-binding proteins potently inhibit release of GTP from Ran1. These proteins can therefore maintain Ran1 in the “on” state and are potential downstream effectors for Ran1-dependent cellular processes.

Ran1 was initially identified as the gene product of TC4, an open reading frame cloned from a human teratocarcinoma cell line by homology to ras (1). Members of the Ras superfamily of small GTPases are regulated by the binding, hydrolysis, and release of guanyl nucleotides (2). Unlike other small GTPases, Ran1 is primarily localized in the nucleus and lacks any consensus sequences for prenylation at the C terminus (3–5). The C terminus of Ran1 consists, instead, of a string of negatively charged amino acid residues. Ran1 is almost identical to a 25-kDa protein isolated as a 1:1 complex with RCC1 (Regulator of Chromosome Condensation) (6). RCC1, a 45-kDa nuclear DNA-binding protein, was identified as the source of a temperature-sensitive mutation in the hamster cell line, tsBN2 (7–10). These cells exhibit a pleiotropic phenotype of premature chromosome condensation or G1 arrest, depending on whether temperature-induced loss of RCC1 occurs during S phase or G1 phase (11, 12). The demonstration that RCC1 acts as a nucleotide exchange factor for Ran1 suggests that the GTP/GDP ratio of Ran1 may be an important parameter in the control of cell cycle progression (13). Similar conclusions have been reached from studies on spil and piml in Schizosaccharomyces pombe, which are fission yeast homologs of Ran1 and RCC1, respectively (14).

RCC1 and Ran1 have also been linked to functions involving transport across the nuclear membrane. The Saccharomyces cerevisiae homolog of RCC1, PRP20/SRM1, is required for mRNA processing and nuclear export (15, 16), and overexpression of the Ran1 homolog, GSP1, or mammalian RCC1 can complement the temperature-sensitive phenotype of prp20/srm1 mutants (17). The abrogation of mRNA export has also been demonstrated in tsBN2 cells upon temperature-induced loss of RCC1 (18). Whether these functions are related to the RCC1-associated cell cycle phenomena is not known; however, the mRNA transport effects occur in minutes versus the cell cycle effects, which occur in hours. In addition, whereas the cell cycle events are dependent upon p34cdc28 kinase activation, the nuclear transport effects are not (11). Additional evidence for a role of Ran1 in nuclear transport stems from the purification of Ran1 as a cytosolic component in Xenopus oocytes necessary to stimulate import of a nuclear localization signal-containing protein (19).

To elucidate components of the pathways in which Ran1 operates, it is necessary to identify specific target proteins with which it interacts. By analogy with Ras, in which the GTP-bound state confers an oncogenic phenotype (20), it is likely that downstream targets of Ran1 will interact preferentially with the GTP-bound state. We have used a nitrocellulose overlay assay to detect putative targets and to determine the mechanism by which they interact with Ran1. While this manuscript was in preparation, Coutavas et al. (21) described the cloning of a 28-kDa cytosolic Ran-binding protein, RanBP1, which is almost identical to a previously identified open reading frame, HTP9A (22). With our assay, we have identified numerous RanBP s, including a 28-kDa cytosolic protein potentially representing RanBP1. Information is presented showing that GTP-Ran1 binds to multiple proteins that exhibit a regulatory function by inhibiting the EDTA-induced release of GTP from Ran1.

MATERIALS AND METHODS

Cell Culturing and Protein Extraction—Chinese hamster ovary (CHO) cells were maintained in F-12 medium (Life Technologies, Inc.) supplemented with 5% fetal bovine serum. Subcellular fractionation was accomplished by harvesting cells into hypertonic lysis buffer containing 10 mM HEPES pH 8.0, 5 mM KCl, and 2 mM MgCl2. Cells were lysed by expression through a 26.5-gauge needle six times, and nuclei were separated by centrifugation at 1,000 × g for 5 min. The supernatant was then centrifuged at 100,000 × g for 30 min to generate S100 and P100 fractions. Soluble nuclear proteins were extracted from the

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1 In light of the possibility that additional proteins exist in the “Ran family” and to prevent future confusion, we refer to the gene product of TC4 as Ran1 based upon its high sequence homology with the polypeptide originally designated Ran (6).

2 The abbreviations used are: RCC1, regulator of chromosome condensation; GDI, guanine nucleotide dissociation inhibitor; CHO, Chinese hamster ovary; MOPS, 3-(N-morpholino)propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.
1,000 x g pellet by incubation with 0.5% Triton X-100, 5 mM MgCl₂, and 1 mg/ml DNase (Sigma) for 30 min on ice followed by treatment with 2 M NaCl. Insoluble nuclear proteins were then separated by centrifugation at 1,200 x g for 5 min. For experiments requiring total cell extract, cells were harvested in the presence of 20 mM HEPES pH 7.4, 0.1 mM EDTA, 100 mM NaCl, and 0.5% Triton X-100. After 10 min of solubilization, lysis was completed by passing the extract 10 times through a 26.5-gauge needle. Insoluble particulate material was removed by centrifugation at 10,000 x g for 5 min.

Ran1 Overlay Assay—Proteins were separated by 8% SDS-PAGE and transferred to nitrocellulose for 3 h at 800 mA. Protein transfers were incubated at 4 °C for 1–2 h in “renaturation buffer” containing 20 mM MOES, pH 7.1, 100 mM sodium acetate, 5 mM magnesium acetate, 0.5% Tween 20, 0.5% bovine serum albumin, and 5 mM dithiothreitol and then preincubated for 30 min at room temperature in “binding buffer” consisting of 20 mM MOES, pH 7.1, 100 mM potassium acetate, 5 mM magnesium acetate, 0.05% Tween 20, 0.5% bovine serum albumin, and 5 mM dithiothreitol in the presence of 100 μM GTP. Blots were rinsed with binding buffer alone and then overlaid with [α-32P]GTP-Ran1 in binding buffer for 30 min at room temperature. Nonspecific binding was removed by five successive rinses in binding buffer, the second containing 50 μM cold GTP. A similar assay was reported by Coutavas et al. (21) while this manuscript was in preparation. Radioactivity associated with the protein bands was quantitated using a GS-250 PhosphorImaging System (Bio-Rad).

The Ran1 used in these assays was produced by isopropyl-1-thio-β-p-galactopyranoside-induced expression from a pET11a vector in BL21(DE3)LysS cells and subsequently purified by Mono Q or DEAE ion-exchange chromatography. [α-32P]GTP-Ran1 was generated by loading 2 μg of recombinant Ran1 with 10 μCi [α-32P]GTP (DuPont NEN) for 20 min on ice in the presence of 10 mM MOES, pH 7.1, 1 mM EDTA, and 1 mg/ml bovine serum albumin in a 15-μl reaction. The complex was subsequently trapped by addition to 15 ml of binding buffer.

Deletion Mutagenesis and Expression of Glutathione S-Transferase Fusion Proteins—The C-terminal deletion mutant of Ran1 (ΔDEDDDL-Ran1) was generated by PCR using pUC19-TC4, a bacterial expression vector, pETlla, and expressed as described above. Strips were then subjected, at room temperature, to the Ran1 overlay assay as described under “Materials and Methods.” The Ranl overlay assay was reported by Coutavas et al. (21) while this manuscript was in preparation. Radioactivity associated with the protein bands was quantitated using a GS-250 PhosphorImaging System (Bio-Rad).

Nuclear Guanine Nucleotide Release Factor Activity—Guanine nucleotide release was measured by the filter binding method of Burstein and Macara (23) with the following modifications. The buffer control consisted of hypotonic lysis buffer containing 10 mM HEPES pH 8.0, 5 mM KCl, and 2 mM MgCl₂. Activity was measured using 2 mg/ml nuclear extract. Residual GDP bound after 30 min can be attributed to nonspecific binding to the nitrocellulose filters by the nuclear extract.

RESULTS AND DISCUSSION

Detection of Ran1-binding Proteins—To observe proteins that interact with Ran1, soluble CHO cell lysate proteins were separated by SDS-PAGE and transferred to nitrocellulose. The nitrocellulose was incubated with a complex of recombinant c-Ha-Ras and Rab3A loaded with [α-32P]GTP. Under these conditions, no bands were detected, even after long exposure (Fig. 1A). These results indicate that the proteins exhibit selective binding to Ran1.
A variety of conditions were tested in an effort to characterize the binding of Ran1 to proteins that had been transferred from SDS gels to nitrocellulose. Binding of Ran1-GTP to cell proteins increased over time to a maximum at 20 min of incubation (Fig. 3B). Use of acetate buffers gave optimal binding; however, washing the nitrocellulose blots in chloride buffers after Ran1 overlay assay was then performed as in Fig. 1.

Together, these data suggest that Ran1 recognizes a contiguous region within the proteins rather than a three-dimensional conformation and that denaturation is not necessary for Ran1 binding to occur.

To establish whether the Ran1-binding proteins are ubiquitous, extracts were tested from a variety of cultured mammalian cells and tissues, as well as Escherichia coli and S. cerevisiae. All extracts, with the exception of bacteria, contain proteins that bind Ran1 (Fig. 2). Proteins corresponding to 28 and 86 kDa were observed in all mammalian cell extracts examined. Similar proteins were also detected in extracts of Xenopus oocyte and SF9 insect cells (not shown). Interestingly, the Ran1-binding proteins above 100 kDa varied in size and intensity among cell type. The intensity of these high molecular weight proteins suggests a strong affinity for Ran1, since these proteins transfer to nitrocellulose with low efficiency. We estimated a minimum dissociation constant of 10^{-10} M based on the amount of Ran1 in the assay.

Ran1-binding Proteins Are Predominantly Localized in the Nucleus—Because Ran1 localizes to the nucleus (16), it was of interest to determine the subcellular distribution of the proteins to which it binds. CHO cells were first fractionated by lysis in a hypotonic buffer and centrifuged to obtain nuclear (P1), cytosol (S100), and membrane (P100) fractions. The nuclei were washed and then extracted with 0.5% Triton X-100 plus DNase, and finally with 2 M NaCl. The remaining insoluble fraction is primarily comprised of the nuclear matrix (24). Equal proportions of each fraction were then analyzed for Ran1-binding proteins as above (Fig. 3A). Interestingly, the majority of Ran1-binding proteins are contained in the soluble nuclear fraction. Greater than 80% of the binding to proteins of 40, 50, and 200-300 kDa is found in this fraction. Proteins corresponding to 28, 65, and 100 kDa were distributed throughout the various fractions, as demonstrated by the presence of lactate dehydrogenase activity in the S100 fraction and retinoblastoma protein exclusively in the soluble nuclear fraction (Fig. 3B). A measure of the specificity of Ran1 binding is demonstrated by the lack of preferential binding by Ran1 to any of the more abundant proteins visualized in a parallel gel stained with Coomassie Blue (Fig. 3C). Some Ran1-binding proteins were also present in the insoluble nuclear matrix fraction, but these proteins are not enriched by the fractionation procedure, and they do not correspond to any major proteins in a preparation of purified rat liver nuclear matrix proteins. The association of Ran1 in the GTP-bound state to components of the nucleus is consistent with the finding that RCC1, the Ran1 exchange factor, is necessary for the localization of Ran1 to the nucleus (25). Although localized to the nucleus, the Ran1-binding proteins were unable to bind to dsDNA cellulose, suggesting...
that, unlike RCC1, they do not represent DNA-binding proteins (8).

To address the possibility that Ran1 recognizes a post-translational modification rather than an amino acid sequence in the binding proteins, a variety of treatments designed to remove covalent modifications (incubations with alkaline phosphatase, potato acid phosphatase, hydroxylamine, or 10% acetic acid) were tested. None of these treatments had any effect upon the capacity of Ran1 to bind to cell proteins. In addition, treatment of 3T3 cells with tunicamycin (to block glycosylation) or lovastatin (to inhibit prenylation) also had no effect on the amount or pattern of Ran1 binding. Furthermore, none of the Ran-binding proteins were immobilized by wheat germ agglutinin, suggesting that they are not glycosylated, as would be the case for nuclear pore proteins (26).

**Altering the C or N Terminus of Ran1 Inhibits Binding**—The presence of an acidic C-terminal sequence in Ran1, absent from other small GTP-binding proteins that do not exhibit specific binding in this overlay assay, suggested that this sequence may be necessary for binding to target proteins. We therefore generated a mutant Ran1 protein with a 7-residue deletion of its acidic C-terminal tail. The mutant protein was less soluble than wild type, and its capacity for binding to GTP was 10-fold less, indicating that the C terminus is important for the proper folding of Ran1, and its deletion may lead to denaturation. To account for the lower GTP binding activity, an equal amount of C-terminal acidic tail in the GTP-specific binding of Ran1 to a specific class of target proteins.

Interaction of Ran1 through the C terminus has implications regarding the recent finding that Ran1 is necessary for nuclear import (19). The C-terminal sequence of Ran1 (PDEDDDL) is almost a mirror image of the SV40 T antigen nuclear localization sequence (PKKKRRK) (26). In fact, antiserum against the peptide, DDDED, has been demonstrated to block subsequent nuclear import of nucleoplasmin (27). Yoneda et al. (27) described a 69-kDa protein recognized by this antiserum. However, they also noted the recognition of other proteins by this antiserum; Ran1 is potentially one of these proteins.

To test the possibility that the acidic C-terminal tail of Ran1 interacts with basic nuclear localization signals, 1 µg of an estrogen receptor hormone-binding domain, expressed as a glutathione S-transferase fusion protein, was run on an SDS-PAGE gel, transferred to nitrocellulose, and blotted with Ran1. This domain of the estrogen receptor contains two nuclear localization sequences. No binding of Ran1 to the fusion protein was observed. These data indicate that more than a polybasic localization signal is necessary for a protein to accommodate specific interaction with Ran1. Additionally, a number of known nuclear proteins, including the retinoblastoma gene product (28), NumA (29), and a recently identified nuclear matrix protein, p250, were examined for their ability to bind to Ran1, but none gave a detectable signal in the overlay assay.

Manipulations of Ran1 at the N terminus were also inhibitory to its ability to bind in the overlay assay. A glutathione S-transferase fusion protein of Ran1 was unable to detect any proteins in cell extracts, although its GTP binding activity was unaffected (not shown). Remarkably, the ability to interact with target proteins was not recovered by thrombin treatment of glutathione S-transferase-Ran1. Thrombin cleaves all but 2 residues (Gly-Ser) from the N terminus of the fusion protein. This result indicates that the interactions with target proteins are exquisite sensitive to small changes at both the N and C termini of Ran1. Interestingly, however, both the C-terminal deletion mutant of Ran1 and the glutathione S-transferase-Ran1 fusion protein were able to interact with guanine-nucleotide exchange factor (Fig. 5). Controls performed using the recombinant Ran1 expressed from pET11a demonstrated that the loss of [α-32P]GDP counts bound to Ran1 was not a consequence of protein degradation. Additionally, accelerated binding of nucleotide to Ran1 was also observed following addition of nuclear extract, confirming that the extract contains bona fide exchange activity. These data suggest that the epitopes used to interact with guanine nucleotide release factors differ
Nitrocellulose-transferred total CHO cell extract was incubated with Ranl. A, release of GTP from Ranl bound to CHO extract proteins. The lane labeled Re-bound represents a CHO sample incubated with cold GTP-Ran for 30 min and GTP release buffer for 30 min. B, GTP release from Ranl 1 versus Ranl bound to CHO cell proteins. Open circles represent release of GTP from Ranl bound to nitrocellulose. Closed circles indicate the capacity of the Ranl 1 to rebind GTP after 30 min of release. Triangles, diamonds, and squares represent release of GTP from Ranl bound to the 86-, 28-, and 100-kDa Ran-binding proteins, respectively, as determined by PhosphorImaging.

from those involved in interactions with putative target proteins. The data further indicate that both the N- and C-terminal regions of the protein (which are predicted to be in close proximity, based on the Ras crystal structure (30)) are essential for high affinity interaction with most of the observed binding proteins.

Ran1-binding Proteins Inhibit GTP Release—To determine whether the Ran1-binding proteins detected by the overlay assay play a functional role in the regulation of nucleotide binding and/or hydrolysis, we measured the rate of release of [α-32P]GTP from Ranl bound to these proteins in the presence of excess EDTA. As with most other Ras-like proteins, GTP release is very slow in the absence of magnesium and is rapid in its absence (31). Remarkably, GTP release was almost completely inhibited by association of Ran1 with the binding proteins (Fig. 6). Additionally, Ran1-binding proteins that were incubated with a complex of Ran1 and cold GTP followed by elution with GTP release buffer were unable to bind [α-32P]GTP-Ran1. As expected, release of GTP from Ranl 1 bound alone to nitrocellulose was rapid in the absence of magnesium. This release might, in principle, however, be a result of denaturation of Ran1, rather than of guanine nucleotide dissociation, and the apparent inhibition of release might, in this case, be ascribed to stabilization of Ran1 by the Ran-binding proteins, rather than to a reduction in koff for nucleotide. To distinguish these possibilities, the ability of Ran on nitrocellulose to rebind [α-32P]GTP was tested. A complex of Ran1 bound to [α-32P]GTP was bound to nitrocellulose and treated with EDTA for 30 min to release nucleotide. A binding mixture containing [α-32P]GTP was then added and trapped with magnesium. As can be seen in Fig. 6B, a rapid and complete rebinding of GTP to Ran1 was observed. This result demonstrates that loss of bound GTP was not caused by rapid denaturation of the Ran1 on the nitrocellulose. Therefore, the inhibition of GTP release by Ran1-binding proteins must be a functional inhibition of the dissociation rate.

This effect is unique among the guanine nucleotide dissociation inhibitors (GDIs) that have been reported to date. The Rab3-GDI, for example, interacts only with the GDP-bound state of Rab3A (32), while the Rho-GDI interacts equally with Rho-GDP and Rho-GTP (33). The physiological role of a family of GDIs that interact only with the GTP-bound state of Ran1 remains to be explored. One possibility is that they are components of a complex involved in nuclear transport and act to maintain Ran1 in the GTP-bound state until interaction with a Ran1-GTPase-activating protein triggers nucleotide hydrolysis and complex disassembly. A cyclical association-dissociation of a complex involving a karyophile, nuclear localization signal receptor, hsc70, and other components has been proposed previously to be necessary for nuclear protein import (34). As these binding proteins are identified, a clearer picture of the role Ran1 plays in nuclear transport and/or mitotic regulation can be established.

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