Random Mutagenesis and Functional Analysis of the Ran-binding Protein, RanBP1*

(Received for publication, August 27, 1999, and in revised form, November 12, 1999)

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Ran GTPase is required for nucleocytoplasmic transport of many types of cargo. Several proteins that recognize Ran in its GTP-bound state (Ran-GTP) possess a conserved Ran-binding domain (RanBD). Ran-binding protein-1 (RanBP1) has a single RanBD and is required for RanGAP-mediated GTP hydrolysis and release of Ran from nuclear transport receptors (karyopherins). In budding yeast (Saccharomyces cerevisiae), RanBP1 is encoded by the essential YRB1 gene; expression of mouse RanBP1 cDNA rescues the lethality of Yrb1-deficient cells. We generated libraries of mouse RanBP1 mutants and examined 11 mutants in vitro and for their ability to complement a temperature-sensitive yrb1 mutant (yrb1-51ts) in vivo. In 9 of the mutants, the alteration was a change in a residue (or 2 residues) that is conserved in all known RanBDs. However, 4 of these 9 mutants displayed biochemical properties indistinguishable from that of wild-type RanBP1. These mutants bound to Ran-GTP, stimulated RanGAP, inhibited the exchange activity of RCC1, and rescued growth of the yrb1-51ts yeast cells. Two of the 9 mutants altered in residues thought to be essential for interaction with Ran were unable to rescue growth of the yrb1ts mutant and did not bind detectably to Ran in vitro. However, one of these 2 mutants (and 2 others that were crippled in other RanBP1 functions) retained some ability to co-activate RanGAP. A truncated form of RanBP1 (lacking its nuclear export signal) was able to complement the yrb1ts mutation. When driven from the YRB1 promoter, 4 of the 5 mutants most impaired for Ran binding were unable to rescue growth of the yrb1ts cells; remarkably, these mutants could nevertheless form ternary complexes with importin-5 or importin-β and Ran-GTP. The same mutants stimulated only inefficiently RanGAP-mediated GTP hydrolysis of the Ran-GTP-importin-5 complex. Thus, the essential biological activity of RanBP1 in budding yeast correlates not with Ran-GTP binding per se or with the ability to form ternary complexes with karyopherins, but with the capacity to potentiate RanGAP activity toward GTP-bound Ran in these complexes.

In eukaryotic cells, DNA replication and transcription are compartmentalized in the nucleus. Access to the nucleoplasm is provided by thousands of pores that penetrate the double-membrane envelope of the nucleus. These pores are complex structures that, while permitting the diffusion of small molecules, only allow the passage of most proteins and nucleic acids when they are associated with soluble factors called karyopherins, which are specialized for either import (importins) or export (exportins) (for reviews, see Refs. 1–5). The direction of transport and accumulation against a concentration gradient are driven by the Ran GTPase (6, 7), which cycles between GTP- and GDP-bound states, like other G proteins.

Ran itself is predominantly nuclear, and likewise, the guanine nucleotide exchange factor for Ran, RCC1, is associated with chromatin, ensuring that nuclear Ran is largely GTP-bound (8–10). Nuclear Ran-GTP binds to importins and thereby dissociates incoming importin-cargo complexes; conversely, nuclear Ran-GTP cooperatively promotes formation of exportin-cargo complexes (11–18). Importin-Ran-GTP complexes recycle back to the cytosol (19). Thus, as the result of both import and export, Ran-GTP ends up in the cytosol in association with a transport factor and must be released to permit further rounds of transport. Release is driven by GTP hydrolysis. The GTPase-activating protein (RanGAP),1 responsible for catalyzing hydrolysis is present both in the cytosol and is attached to fibrils that extend from the cytoplasmic face of the nuclear pores (20–22). However, RanGAP alone only weakly stimulates hydrolysis of Ran-GTP bound to importins or exportins (11, 23). A cofactor is required to permit efficient RanGAP action and to dissociate transport factor complexes from nuclear pores. In mammalian cells, two proteins, RanBP1 (24) and Nup358 (also called RanBP2) (25, 26), can perform this function. RanBP1 contains one copy and Nup358 contains four copies of a highly conserved, 135-residue domain that can bind Ran-GTP with high affinity, form a ternary complex with Ran-GTP and importins, and co-activate RanGAP (27–31). This domain is referred to as the Ran-binding domain or RanBD.

Nup358 is a giant nucleoporin that is a component of the fibrils that extend into the cytosol from the cytoplasmic face of the nuclear pore and associates with a form of RanGAP that is modified via attachment of a small ubiquitin-like polypeptide, SUMO-1 or Smt3 (32, 33). A related protein in the nematode, Caenorhabditis elegans, Ranup96, contains 2 RanBDs (27).

1 The abbreviations used are: RanGAP, GTPase-activating protein; RanBP1, Ran-binding protein-1; RanBD, Ran-binding domain; Gp-pNHP, 5-quinuclidinylimidophosphatase; PCR, polymerase chain reaction; IPTG, isopropylthio-D-galactoside; PMSF, phenylmethylsulfonfonyl fluoride; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; MOPS, 4-morpholinepropanesulfonic acid; NES, nuclear export signal.

* This work was supported by NCI Postdoctoral Traineeship CA09041 from the National Institutes of Health (to J. T.) and National Institutes of Health Research Grants GM50526 (to I. G. M.) and GM21841 (to J. W. T.). 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.

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This paper is available on line at http://www.jbc.org
crystal structure of the first RanBD (RanBD1) from Nup358, in a complex with Ran bound to a non-hydrolyzable GTP analog (GppNHz), has been solved (34). RanBD1 possesses a β-barrel fold, similar to those of pleckstrin homology, phosphotyrosine binding, and Wiskott-Aldrich syndrome protein homology-1 domains. The N terminus of RanBD1 loops around Ran, and the C-terminal extension of Ran almost completely encircles RanBD1, in a mutual embrace. The Switch I effector loop in Ran makes contact with an invariant sequence, EWWKGR, within the RanBD (residues 66–71 in mouse RanBP1). Other conserved regions in the RanBD, such as an RXXMRRD motif (residues 87–93 in mouse RanBP1), also make direct contact with Ran.

In contrast to Nup358, RanBP1 is a small (25 kDa) cytosolic protein. The budding yeast (Saccharomyces cerevisiae) homolog of RanBP1, Yrb1, is essential for viability (35), and two temperature-sensitive mutations, yrb1-1ts (E146D/F151S) and yrb1-2ts (L55P) (numbering according to equivalent positions in mouse RanBP1), display defects in both nuclear protein import and RNA export (36). Yrb1(E146D/F151S), but not Yrb1(L55P), still binds to activated (GTP-bound) yeast Ran, Gsp1(G21V), at the restrictive temperature (36). Correspondingly, in vivo, the yrb1-1ts mutant shows a less pronounced phenotype than the yrb1-2ts mutant with respect to nuclear protein import; interestingly, however, the potency of these same mutations in preventing RNA export is the reverse (36). Yeast contains no obvious counterpart of Nup358, but other smaller proteins with less well conserved RanBDs are present, including Yrb2 and Nup2. However, unlike a yrb1Δ mutant, yrb2Δ and nup2Δ cells are viable. Thus, Yrb1 is most likely the primary agent available to bind to Ran-GTP in transport receptor complexes in the cytosol and to assist yeast RanGAP (Rna1) to catalyze GTP hydrolysis and dissociation of Ran-GTP from these complexes. There is some evidence, however, that RanBP1 and its homologs may possess other functions, distinct from Ran binding (37, 38).

As one approach to examine the essential physiological function(s) of RanBP1, we first randomly mutagenized the RanBD segment of mouse RanBP1, generating a library of mutants, many of which contain alterations of conserved residues in the RanBD. Second, the RanBP1 mutants were expressed in and analyzed by cleavage with purified thrombin (provided by Paula Tracy, University of Vermont). Importin-5 (RanBP5) was expressed as an N-terminally (His)6-tagged protein from plasmid pQE670 (provided by Dirk Görlich, University of Heidelberg). S-tagged importin-β (provided by S. Adam, Northwestern University) was induced in bacterial strain BL21(DE3) containing a pET-ρ267 construct as described previously (41) and purified by binding to S-agarose. Protein concentrations were determined by the Bradford protein assay or from their calculated extinction coefficients at 280 nm. Protein integrity during preparation was monitored by SDS-PAGE and staining with Coomassie Blue dye.

Ran overlay assays were performed as described previously, using Ran loaded with [γ-32P]GTP (3000 Ci/mmol) (40). Solution binding assays were performed using 20 pmol of GST-RanBP1 (or mutants thereof) attached to glutathione-Sepharose beads (20 µl of a 50:50 buffer/beads slurry). Ran that had been preloaded with [γ-32P]GTP (10 µM, 5 Ci/mmol) was incubated with the beads for 30 min on ice and then rapidly washed, and the amount of radioactivity retained was counted in a liquid scintillation counter.

Inhibition of RCC1-mediated GTP/GDP exchange on Ran was determined in a similar manner to that described previously, using recombinant RCC1 with [γ-32P]GTP-loaded Ran (42). Briefly, 80 pmol of Ran (produced by thrombin cleavage of GST-Ran) was loaded with 20 µCi of [α-32P]GTP (3000 Ci/mmol, NEN Life Science Products) and then diluted 10-fold in buffer containing 50 mM MOPS, pH 7.1, 1 mM diethiothreitol, 10 mM MgCl₂, and 0.1 mg of bovine serum albumin/ml. RCC1 was expressed as a GST fusion protein and cleaved from the GST using thrombin (Amersham Pharmacia Biotech). The [α-32P]GTP-Ran complex was dialyzed into the GTP exchange reaction containing 50 mM MOPS, pH 7.1, 6.25 mM MgCl₂, and 0.63 mM each of GDP, GTP, and NaH₂PO₄ plus GST-RanBP1 (or mutants) at the desired concentration. RCC1 was added at time 0 to a final concentration of 1.5 nM, in a volume of 50 µl, at 30 °C. At 3 min, 20 µl of the sample was subjected to filter binding through nitrocellulose and quantitated by scintillation counting. GTP dissociation rate constants (kₗ) were calculated assuming
ing simple exponential decay of a single species. Curves were fit to the RanBP1 inhibition data assuming a competitive inhibition model, using Kaleidagraph software.

Co-activation of RanGAP by RanBP1 was measured using recombinant GST-mouse RanBP1 (42). Ran was loaded with [γ-32P]GTP, as described above. The Ran-GTP complex was diluted by vigorous vortex mixing with glass beads. Insoluble materials were removed by centrifugation, and the protein extracts were resolved by SDS-PAGE, transferred to nitrocellulose, and analyzed for expression of the (HA1)3-tagged RanBP1 proteins by immunoblotting with an anti-HA monoclonal antibody (12CA5).

RESULTS

Identification of RanBP1 Mutants—Libraries of RanBP1 mutants containing mutations within the RanBD sequence were constructed in two ways (see under “Experimental Procedures”). For the libraries of mutants produced using spiked oligonucleotides, no selection or screen was performed because mutations were introduced at a defined rate and within a known region of the RanBD. Rather, clones were chosen at random and sequenced. Mutant sequences were obtained at a frequency of about 15%.

To identify mutants from the library obtained using template-limited PCR, we developed and applied an overlay method for screening colonies to assess the ability of the RanBP1 mutants to associate with Ran-GTP (Fig. 1). Bacteria containing pGEX alone or mutated pGEX-RanBP1 were plated onto LB agar plus ampicillin and grown overnight. To induce expression of GST or the GST fusion proteins, the colonies were overlaid with a nitrocellulose filter soaked in IPTG and incubated for 3 h. Colonies adherent to the filter were lysed, and the released filter-bound proteins were incubated with [α-32P]GTP:Ran and then washed to remove unbound probe. Colonies expressing GST alone did not retain detectable radioactivity, upon exposure to X-ray film. In contrast, colonies expressing GST-RanBP1 bound [α-32P]GTP-Ran, and the exposed film could be readily aligned with the original colonies. Moreover, it was relatively easy to identify colonies that appeared to lack robust binding of [α-32P]GTP-Ran, and the exposed film could be readily aligned with the original colonies. Moreover, it was relatively easy to identify colonies that appeared to lack robust binding of [α-32P]GTP-Ran, and the exposed film could be readily aligned with the original colonies.

Binding of Ran-GTP by the Mutant RanBP1s—Expression of the 11 mutant RanBP1 fusions was equivalent to that of the GST fusion to normal RanBP1 (Fig. 2A, lower panel). As judged by the overlay assay, three double mutants (W67R/K68M, G71C/K76E, and R91S/K97T) and one single mutant (E37K) showed a severe defect in binding Ran-[γ-32P]GTP, and another double mutant (R92K/D93Y) showed a less pronounced binding defect (Fig. 2A, upper panel). Surprisingly, Glu-37 is not a side chain that contributes any contacts at the Ran-GTP interface, as deduced from the crystal structure of the RanBP2-RanGppNHp complex (34), but it resides in the vicinity of conserved residues that provide hydrophobic interactions with the conserved residues that provide hydrophobic interactions with the heparinogenon (2% each) as the carbon source and incubated either at 20 or 37 °C.

To detect expression of the HA-tagged RanBP1 mutants in yeast, JY604 transformants were grown overnight in 5 ml of SC-Glc-Ura at 23 °C. When the cultures reached an A600nm = 1.0, 3 ml of each was centrifuged, and the cell pellet was resuspended in 5 ml of SCGal-Raf-Ura and incubated at 30 °C for 4 h. The yeast were then harvested by centrifugation, washed with water, resuspended in 0.1 ml of water containing protease inhibitors (4 μM aprotinin, 30 μM leupeptin, and 250 μM PMSF), and disrupted by addition of boiling SDS-PAGE sample buffer containing 2% sodium dodecyl sulfate and 0.5% mercaptoethanol. The cell extracts were centrifuged, and the protein extracts were resolved by SDS-PAGE, transferred to nitrocellulose, and analyzed for expression of the (HA1)3-tagged RanBP1 proteins by immunoblotting with an anti-HA monoclonal antibody (12CA5).

Expression of Yeast yrb1ts Mutant by Mouse RanBP1—Two vectors were used for heterologous expression of RanBP1 in yeast. To obtain relatively high level expression, the pYES2 vector (Invitrogen) was modified by introduction of a triple HA1 tag between the unique NcoI and HindIII sites downstream of the galactose-inducible GAL1 promoter. The tag was designed to possess 5′-terminally (His)6-tagged importin-5 was used. N-terminally (His)6-tagged importin-5 was expressed in bacteria and purified using Ni2+-saturated nitriultraetatic-agarose beads (43). GAP assays were performed essentially as described by Deane et al. (44), using 0.6 ng Ran that had been loaded with [γ-32P]GTP and preincubated for 30 min in the presence or absence of 40 nM GST RanBP1 and/or 40 nM importin-5. Ran-GAP was added to a final concentration of 400 nM, for 5 min at 30 °C, and [γ-32P]GTP remaining bound to the Ran was determined by filter binding, as described above.

Assays of binding to importin-5 were performed in 1.0 ml of Ran binding buffer (20 mM MOPS, pH 7.1, 100 mM sodium acetate, 5 mM magnesium acetate, 5 mM dithiothreitol, 0.05% Tween), using 8 nM importin-5, 20 nM GST-RanBP1, plus wild-type Ran (20 nM) that had been preloaded with GTP (28). After incubation at 4 °C for 90 min, complexes were captured onto glutathione-Sepharose beads (40 μl) and washed three times with binding buffer containing 0.1 mM GTP. Proteins were separated by SDS-PAGE. After transfer to nitrocellulose, bound proteins were detected using anti-Ran (Signal Transduction Technology) and anti-His (Qiagen) antibodies. Binding to importin-β was performed using S-tagged importin-β attached to S-agarose beads (45). Formation of a GST-RanBP1-GDP-importin-β complex was performed in a similar manner, except that α-[32P]GDP-loaded Ran replaced Ran(G19V), and the complex was detected by washing the beads and counting for bound radiolactivity.

Complementation of Yeast yrb1ts Mutant by Mouse RanBP1—Two vectors were used for heterologous expression of RanBP1 in yeast. To obtain relatively high level expression, the pYES2 vector (Invitrogen) was modified by introduction of a triple HA1 tag between the unique EcoRI/HindIII sites downstream of the galactose-inducible GAL1 promoter. The tag was designed to possess RanBP1 coding sequences were transferred from pGEX into both pGEX-2T, yielding yCplacTY. The RanBP1 mutants of interest were then inserted into this vector, and the resulting plasmids were then introduced by DNA-mediated transformation into either yeast strain JY525 (yrb1-51ts fus1Δ) containing the 2-micro kilobase pair fragment (SpeI-ClaI) containing CEN4-A53, which was excised from yCplac33 and had its 5′-overhang filled in by incubation with the Klenow fragment of E. coli DNA polymerase I and four NTPs, yielding pYEChE. Wild-type and mutant RanBP1 coding sequences were transferred from pGEX into both pYESH3 and pYEC3. To express HA-tagged RanBP1 mutants at levels comparable with the endogenous Yrb1, the 570-base pair promoter region 5′ to the YRB1 gene was amplified from yeast genomic DNA by PCR and subcloned (as a PstI-XbaI fragment) into yCplac22, yielding yCplacTY. The RanBP1 mutants of interest were then inserted into this vector, and the resulting plasmids were then introduced by DNA-mediated transformation into either yeast strain JY525 (MATα his3Δ1 leu2Δ2 hist1Δ22 trpl1Δ63 ade2-101) or its otherwise isogenic derivative, JY604 (MATα his3Δ1 leu2Δ2 hist1Δ22 trpl1Δ63 ade2-101). Due to the yrb1-51 mutation strain JY604 cannot grow at a temperature above 30 °C, whereas the parental strain JY525 grows well, even at temperatures as high as 37 °C. Growth of JY604 at restrictive temperature is fully restored by expression of either Y RB1 or mouse RanBP1 cDNA (HTF9a)3. Transformants were selected on synthetic medium containing 2% glucose but lacking uracil (SC-Glc-Ura) at 20 °C. The resulting colonies were grown in liquid SC-Glc-Ura medium under the same conditions and then patched onto agar plates containing 10% DMSO, 2% glucose, and 2% galactose. All four mutants that appeared to bind Ran-GTP normally, two (T72S and K79E) alter residues that are not highly conserved among RanBDs. Remarkably, however, the other four mutants that bind Ran-GTP normally (E69A, D93A, D93N, and A100T) all change residues that are invariant or nearly so in all known RanBDs. To confirm these conclusions, two other assays were used.

First, the ability of the GST-RanBP1 mutants to compete for Ran-GTP binding to RanBP2/Nup358 was assessed (Fig. 2B). Detergent-solubilized extracts of COS cells were separated by

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3 J. Trueheart and J. Thorner, unpublished observations.
Fig. 1. Colony overlay assay for binding of Ran-GTP. [α-32P]GTP-Ran colony overlay. Bacterial colonies transformed with pGEX (left half of each plate) or mutated pGEX RanBP1 (right half of each plate) were plated onto LB + ampicillin plates. Replicas were made (right side), and the originals were overlaid with nitrocellulose soaked in IPTG (1 mM) for 3 h to induce expression from the pGEX plasmids. The bacteria associated with the filters were lysed overnight as described under “Experimental Procedures” and then incubated with [α-32P]GTP-Ran (50 μCi, 3000 Ci/mmol; 1 μg of Ran, total protein, per plate) for 20 min. After washing to remove excess unbound [α-32P]GTP-Ran, the filters were exposed to x-ray film overnight (left side). Arrows indicate candidate RanBP1 mutants defective in Ran-GTP binding.

SDS-PAGE, transferred to nitrocellulose, and then probed using Ran-[γ-32P]GTP that had been preincubated with a molar excess of either wild-type GST-RanBP1 or each of the mutant fusion proteins. Preincubation with wild-type RanBP1 titrated the available supply of Ran-[γ-32P]GTP and thereby successfully blocked binding of this radioactive probe to RanBP2. Reassuringly, all of the mutants that scored as normal for Ran-GTP binding in the overlay assay also behaved like wild-type RanBP1 in this competition assay. Likewise, the mutants that showed impairment in the overlay assay were all unable to compete effectively for Ran-[γ-32P]GTP binding to RanBP2. As judged by this competition method, the RanBP1 mutants most defective in Ran-GTP binding were W76R/K68M and R91S/K97T.

Second, the ability of the GST-RanBP1 mutants to bind Ran-[γ-32P]GTP in solution was measured (Fig. 2C). GST-RanBP1 was bound to glutathione-Sepharose beads and incubated with Ran-[γ-32P]GTP. After 30 min the beads were washed extensively and counted for retained radioactivity. Representative results for four of the RanBP1 mutants are shown in Fig. 2C. The mutants appeared to fall into two distinct classes. All of the mutants that exhibited unimpaired Ran binding in the overlay and competition assays (for example, A100T), displayed saturation curves for Ran-[γ-32P]GTP binding similar to that of wild-type GST-RanBP1. Conversely, all of the mutants defective for Ran binding in the overlay and competition assays (for example, E37K/G71C/K76E and W67R/K68M) also displayed no detectable Ran binding in solution (Fig. 2C).

Given the sequence conservation among all known RanBDs (from yeasts, nematodes, plants, mammals, and other vertebrates), some of the above results are quite unexpected. For example, the WKERG motif (residues 67–71) is invariant and, based on the crystal structure of the RanBD1-RanGppNHP complex, Glu-69 appears to participate in electrostatic interactions with the Switch I effector loop in Ran (Fig. 3); yet we found that the E69A mutation had no detectable effect on binding of RanBP1 to Ran-GTP. Similarly, the CANH motif (residues 108–111) is also invariant; yet we found that an A100T mutation had no effect on the ability of RanBP1 to bind Ran-GTP. Likewise, at the position equivalent to Asp-93 of mouse RanBP1, there is an acidic residue (Asp or Glu) in nearly all other known RanBDs, but, again, neither a D93A nor a D93N mutation was able to abrogate binding of RanBP1 to Ran-GTP. However, the residues in RanBD1 equivalent to Ala-100 and Asp-93 in RanBP1 do not make direct contacts with Ran in the crystal structure (34). Ala-100 is buried within the β-barrel, near the end of β-strand 4. On the other hand, two of the mutants completely defective in Ran-GTP binding, W67R/K68M and G71C/K76E, do alter invariant residues in or near the WKERG motif, and the other mutant most defective in Ran-GTP binding, R91SK97T, changes invariant residues in or near the conserved RXXMRRD motif.

Inhibition of RCC1 by RanBP1 Mutants—RanBP1 can potentially inhibit the release of GTP from Ran in the presence of either EDTA (to chelate Mg2+) or the Ran-specific guanine nucleotide exchange factor, RCC1 (46). RanBD1 does not bind to Ran-GppNHP so as to occlude the nucleotide-binding site (34) but rather appears to preferentially stabilize the effector loops of Ran in their GTP-binding conformation. If so, it might be predicted that only those RanBP1 mutants able to bind Ran-GTP with high affinity should be capable of inhibiting RCC1 activity. The ability of the GST-RanBP1 mutants to block the action of RCC1 were assessed in vitro under conditions in which a fusion of GST to wild-type RanBP1 inhibited the initial rate of [α-32P]GTP release by about 90%. Under these conditions, and in agreement with the above prediction, the six mutants that retained the ability to bind to Ran-GTP were all able to inhibit RCC1-catalyzed GTP release to an extent similar to wild-type RanBP1, whereas the W67R/K68M mutant, which was completely defective for Ran-GTP binding, was unable to inhibit the exchange activity of RCC1 (data not shown). Likewise, three other mutants with substantial impairments in Ran-GTP binding all showed a significant reduction (Ki values at least 10-fold higher than wild-type RanBP1) in their ability to block RCC1 action (Fig. 4A). The R92K/D93Y mutant, which had the mildest defect in Ran-GTP binding, displayed an intermediate degree of reduction in inhibition of RCC1 activity (Ki value only about 2-fold higher than normal RanBP1) (Fig. 4A). Overall, the ability of RanBP1 to block RCC1-mediated GTP release correlated well with the ability of RanBP1 to bind Ran-GTP, as expected, if RanBP1 binding stabilizes Ran in the GTP-bound state.

Activation of RanGAP by RanBP1 Mutants—Wild-type RanBP1 has no intrinsic GAP activity toward Ran, but the presence of RanBP1 increases the rate of RanGAP-stimulated GTP hydrolysis several fold (45). If RanBP1 alone stabilizes the GTP-bound state of Ran, then the conformation of RanBP1 must change significantly in the ternary complex with RanGAP. To determine if our collection of RanBP1 mutants might shed some light on residues required for RanBP1 to promote RanGAP action, the effects of the mutant RanBP1 proteins on RanGAP-stimulated Ran GTPase activity were measured under conditions where wild-type RanBP1 increased the rate of
GTP hydrolysis maximally, with a $K_d$ of 1–2 nM (Fig. 4B). The six mutants that retained Ran-$z$GTP binding ability were all capable of activating RanGAP, whereas the W67R/K68M mutant that was completely defective for Ran-$z$GTP binding was also completely defective in RanGAP activation (data not shown). Unexpectedly, however, the correlation between Ran-$z$GTP binding and ability to stimulate RanGAP activity did not hold for several mutants. For example, two mutants (G71C/K76E and R91S/K97T) severely defective in Ran binding and the mutant (R92K/D93Y) only moderately defective in Ran-$z$GTP binding all displayed a significant and comparable ability to stimulate RanGAP-mediate GTP hydrolysis (Fig. 4B). Most revealingly, a mutant (E37K) that is nearly as defective in Ran-$z$GTP binding as G71C/K76E and R91S/K97T, and is significantly more defective than R92K/D93Y, was essentially unable to stimulate RanGAP action (estimated $K_d > 100$ nM) (Fig. 4B), suggesting that Glu-37 in RanBP1 may directly contact RanGAP and help to promote formation of a functional RanGAP-RanBP1-Ran-$z$GTP ternary complex.

To determine whether RanBP1 can bind directly to RanGAP in the absence of Ran-$z$GTP, a GST-RanGAP fusion protein was immobilized on glutathione-Sepharose beads, and either wild-type RanBP1 or the R91S/K97T mutant (released from the GST fusions by thrombin cleavage) was mixed with the beads. After rapid washing, bound protein was solubilized in SDS-PAGE sample buffer, resolved by SDS-PAGE, transferred to nitrocellulose, and analyzed by immunoblotting with anti-RanBP1 antibody and by the overlay assay with $\gamma^32$P-GTP:Ran. As judged by these detection methods, no significant quantity of RanBP1 was bound to the immobilized GST-RanGAP, suggesting that the interaction between RanBP1 and RanGAP is very weak in the absence of Ran-$z$GTP (data not shown). Thus, it was not possible to test directly the effect of the RanBP1 mutations on the association between RanBP1 and RanGAP.

**Fig. 3. Structure of the RanBD1-Ran-GppNHp complex showing locations of residues that, when mutated, interfere with Ran-GTP binding.** Figure was generated using RAS-MOL using Protein Data Bank coordinates (code 1A2K). RanBD1 is shown in grey; Ran is shown in green. The N terminus of RanBP1 (N) and the C terminus of Ran (C) are indicated.
mutants permitted the binding of the Ran-[γ-32P]GTP probe in the overlay to a band of about 95 kDa (Fig. 5A). This apparent molecular mass corresponds to the size of importin-β. This observation suggested that all of mutants are able to form stable ternary complexes of RanBP1-Ran-GTP-importin-β (or another member of the importin-β family of similar size present in COS cells, such as importin-5). Even the W677R/K687M mutant, which does not bind Ran-GTP detectably and cannot compete with Nup358 for binding to Ran-GTP, was able to form such a complex (Fig. 5A).

To test directly the ability of the RanBP1 mutants to form ternary complexes with Ran-GTP and importin proteins, bacterially expressed (His)_6-tagged importin-5 (42) was incubated with Ran that had been preloaded with GTP, plus either wild-type or mutant GST-RanBP1 fusions. The GST-RanBP1 proteins were then captured on glutathione-Sepharose, washed, and analyzed by SDS-PAGE and immunoblotting to determine if Ran and importin-5 were bound. As expected, wild-type GST-RanBP1 binds importin-5 in the presence of Ran-GTP but not in the absence of Ran-GTP (Fig. 5B). Surprisingly, however, each of four mutants (E37K, G71C/K76E, R91S/K97T, and R92K/D93Y) defective in binding Ran-GTP in three other assays were nonetheless able to bind importin-5 in the presence of Ran-GTP, confirming the results of the overlay assay (Fig. 5A). In other experiments, similar results were obtained using S-tagged importin-β (data not shown).

To determine whether, like normal RanBP1, the RanBP1 mutants could also utilize Ran-GDP to form ternary complexes, wild-type Ran was loaded with [α-32P]GDP and incubated with S-importin-β immobilized on agarose beads along with all (but one) of the mutant RanBP1 proteins. After extensive washing, radioactivity retained by the beads was measured. In this case, only those RanBP1 mutants that were previously shown to be capable of associating with Ran-GTP were able to bind Ran-GDP in an importin-dependent manner (Fig. 5C), suggesting that there is a fundamental difference in the manner in which Ran-GTP and Ran-GDP influence RanBP1-mediated complex formation with importin-β.

The principal function of RanBP1 in nucleocytoplasmic transport is believed to be termination of the transport cycle and regeneration of free transport receptors in the cytosol (18, 31, 47, 48). RanBP1 (and Nup358) performs this function by binding to Ran-GTP importin complexes and permitting access by RanGAP, which then hydrolyzes the GTP, thereby triggering dissociation of the Ran and the release of free importin. Formation of RanBP1-Ran-GTP-importin complexes in the cytosol may also provide a mechanism to promote dissociation of transport receptor-Ran-GTP complexes from binding sites in the nuclear pore. To test whether the RanBP1-Ran-GTP-importin ternary complexes formed by the RanBP1 mutants were substrates for RanGAP action, Ran was preloaded with [γ-32P]GTP, then bound to importin-5 in the presence of either wild-type GST-RanBP1 or the GST-RanBP1 mutants, then incubated in the absence or presence of a high concentration of RanGAP, and the amount of radioactivity remaining bound was quantitated (Fig. 5D). As has been demonstrated by others (46), importin-5 potently inhibits GTP hydrolysis on Ran by RanGAP, and wild-type RanBP1 relieved this inhibition. Despite the fact that the RanBP1 mutants were fully capable of forming ternary complexes under the conditions of the assay, they were either incapable of promoting RanGAP-mediated GTP hydrolysis (E37K) or did so only very inefficiently (G71C/K76E, R91S/K97T, and R92K/D93Y). Therefore, a critical defect in these RanBP1 mutants appears to lie in their ability to support the action of RanGAP on Ran-GTP-importin complexes.

Function of the RanBP1 Mutants Examined in Vivo by
Complementation of a Yeast yrb1ts Strain—A temperature-sensitive allele (yrb1-51) of the yeast RanBP1 homolog was isolated as described in detail elsewhere. Yeast cells (strain JY604) harboring this mutation are unable to grow at 37 °C, whereas otherwise isogenic YRB1 cells (strain JY525) grow well at this temperature (Fig. 6). When JY604 was transformed with pGK-RanBP1, which expresses a cDNA (HTF9a) encoding mouse RanBP1 from the constitutive PGK1 promoter carried on a high copy number (2 \( \mu m \) DNA) vector, ability to grow at 37 °C was restored, whereas the same cells transformed with the empty vector (pGK) were unable to grow at the restrictive temperature (Fig. 6). This result demonstrates that mammalian RanBP1 can substitute for a defect in yeast Yrb1 function. Although wild-type RanBP1 was capable of complementing the ts defect, we noted that the cells did not grow robustly. We next tested the ability of each RanBP1 mutant to complement the yrb1-51 allele using pGK constructs. Of the 11 RanBP1 mutants tested in the same fashion, only two (E69A and T72S) grew, but only poorly, at 37 °C. We noted that, even at permissive temperature (RT or 37 °C for 3 days, as indicated).

**Fig. 5.** Formation of RanBP1-Ran-importin ternary complexes. A, detergent-solubilized extracts of COS cells were resolved by SDS-PAGE and transferred to nitrocellulose, as described in the legend to Fig. 2, probed with a mixture of \( ^{32}P \)GTP plus a 2-fold molar excess of GST-RanBP1 or the indicated mutants, washed thoroughly, and then used to expose x-ray film to detect binding to importin-\( \beta \). B, purified (His)\(_6\)-importin-5 (8 nM) was incubated with 20 nM GST-RanBP1 or the indicated mutants plus 20 nM of Ran-GDP for 90 min at 4 °C in binding buffer. GST-RanBP1 was captured on glutathione-Sepharose beads (40 \( \mu l \)) and washed 3 times with binding buffer. Bound proteins were separated by SDS-PAGE, transferred to nitrocellulose, and detected by immunoblotting with anti-Ran, anti-RanBP1, and anti-(His)\(_6\) tag antibodies. C, to detect ternary complexes between RanBP1, Ran-GDP, and importin-\( \beta \), assembly was performed, as in B, but Ran(G19V) was replaced by \( ^{32}P \)GDP-Ran, which was detected, after extensive washing of the beads, by counting the radioactivity present. D, RanGAP assays were performed on wild-type Ran (0.6 nM) loaded with \( ^{32}P \)GTP and preincubated for 30 min in the presence or absence of GST-RanBP1 (40 nM) and/or importin-5 (40 nM). Incubations with RanGAP (400 nM) were for 5 min at 30 °C. wt, wild type.

**Fig. 6.** Mammalian RanBP1 substitutes in vivo for its yeast counterpart, Yrb. Yeast strain JY604 (yrb1-51\(^{ts} \)) and its otherwise isogenic YRB1\(^{+} \) parent (strain JY525), as indicated, were plated on YPD medium (upper panels), and JY604 was plated on selective medium (SCGlc-Ura) after transformation with either an empty high copy number URA3-containing vector (pGK) or the same vector expressing the cDNA for mouse RanBP1 (bottom panels), and the plates were incubated at either 23 (room temperature, RT) or 37 °C for 3 days, as indicated.
sive temperature, transformants could only be obtained with wild-type RanBP1, and the E69A and T72S mutants, and all grew very slowly. These observations confirm other reports that high level overexpression of RanBP1 is growth-inhibitory (35) and shows that overexpression of many of the mutants was especially toxic to yeast cell growth.

To avoid this problem, we next subcloned each RanBP1 construct into a low copy CEN4-containing vector, pYECH3, that we created by modification of pYES2 (Invitrogen), in which expression was controlled by a galactose-inducible GAL1 promoter. Excision of the RanBP1 coding sequences from the pGEX-2T plasmids and insertion into pYECH3 also yielded in-frame fusions that introduced a triple HA1 epitope tag at the N terminus of each RanBP1 construct. Transformants of strain JY604 were selected on SCGlc-Ura medium at room temperature and were then spotted at various dilutions onto SCGal/Raf-Ura plates at either room temperature or 37 °C. All of the transformants were able to grow at room temperature on either glucose or galactose/raffinose (Fig. 7A). At 37 °C, 9 of the 11 mutants permitted readily detectable growth of the cells, although none supported growth as vigorously as that supported by wild-type RanBP1. Neither W67R/K68M nor R91S/K97T was able to support detectable growth of the yrb1-51° cells. Similar results were obtained when individual colonies selected from each transformation on SCGlc-Ura plates were subsequently patched onto ScGal/Raf-Ura plates and incubated at permissive and non-permissive temperatures; no growth was observed for cells expressing W67R/K68M or R91S/K97T at 37 °C. Thus, the mutations that appeared to cause the most severe defect in the ability of RanBP1 to bind Ran-GTP (see especially Fig. 2B) were unable to substitute functionally for the defective endogenous Yrb1. Of course, it cannot be ruled out from this experiment alone that W67R/K68M or R91S/K97T mutations, in addition to abrogating Ran-GTP binding, also cause the structure or function of RanBP1 to be temperature-sensitive. Likewise, the inability of these two mutants to rescue growth of strain JY604 at the non-permissive temperature could, in principle, be the consequence of a low level of expression or of rapid degradation of these particular mutants if they are unstable proteins at 37 °C. To rule out at least these possibilities, transformants were grown on glucose at 18 °C and then shifted to galactose medium at 37 °C for 3 h. After harvesting and cell lysis, extracts were analyzed by SDS-PAGE and immunoblotting with an anti-HA monoclonal antibody. All of the HA-tagged RanBP1 mutants were expressed at the non-permissive temperature at a level equivalent to wild-type RanBP1. Therefore, the inability of W67R/K68M or R91S/K97T to rescue growth reflects some defect in function rather than in expression or stability.

On the other hand, two other mutants, E37K and G71C/K76E, which are almost as defective in Ran-GTP binding in vitro as W67R/K68M or R91S/K97T (Fig. 2, A–C) and unable to inhibit RCC1 effectively (Fig. 4A), nonetheless complemented the yrb1-51° strain. Thus, neither efficient Ran-GTP binding nor efficient inhibition of RCC1 (Prp20 in yeast) seems to be critical to the essential physiological function of RanBP1. RanBP1 possesses a nuclear export signal (NES) in its C-terminal region, outside of the RanBD, and an HA1-tagged fragment of RanBP1 that contains only its RanBD accumulates within the nucleus of transfected mammalian cells, where it is toxic to cell growth (49). Moreover, micro-injected RanPB1 lacking an NES can also inhibit both nuclear import and export pathways (7). Likewise, yeast Yrb1 contains a potential NES in a short N-terminal extension outside of its RanBD (36), and removal of this sequence causes Yrb1 to accumulate in the yeast cell nucleus. 4 To determine whether the essential function of RanBP1 in yeast requires an aspect of its sequence flanking the RanBD on either side, a fragment containing just the RanBD of RanBP1 was subcloned into pYECH3 and introduced into strain JY604. The isolated RanBD was stably expressed in yeast (Fig. 7B) and, surprisingly, was able to substitute functionally for the defective Yrb1 at the restrictive temperature (Fig. 7A).

Although the pYECH3 vector used for the above complementation tests is a low copy number plasmid, the RanBP1 constructs harbored in pYECH3 are expressed from a strong inducible promoter (GAL1), and consequently, the RanBP1 proteins were all produced at a level significantly higher than that of native Yrb1 produced from its chromosomal locus and endogenous promoter. Hence, one might argue that functional deficiencies in some of the RanBP1 mutants might have been obscured by the fact that overexpression of the mutant proteins (or the isolated RanBD domain) could compensate for defects in Ran-GTP binding or other properties. To test this possibility, constructs were prepared that expressed each RanBP1 mutant from the authentic YRB1 promoter (obtained by PCR from yeast genomic DNA) in a low copy number vector. Under these conditions of reduced expression, wild-type RanBP1 and even its isolated RanBD were still capable of rescuing growth at the non-permissive temperature (Fig. 7C). Likewise, several RanBP1 mutants, including E69A, D93A, and A100T, all altered in highly conserved positions within the RanBD, were still able to complement the yrb1-51°, even at this lower level of expression. However, revealingly, three mutants (E37K, G71C/K76E, and R92K/D93Y), which were able to rescue growth at restrictive temperature when expressed at a higher level, were unable to support growth at a lower level of expression (Fig. 7C). Since two of these mutants (E37K and G71C/K76E) were quite defective in Ran-GTP binding by every test applied, whereas the R92K/D93Y mutant exhibited only a minor defect in Ran binding, these findings confirmed that the ability of the RanBP1 mutants to substitute for Yrb1 function in vivo does not correlate with the ability to bind Ran-GTP per se. Rather, the common property shared by these three RanBP1 mutants is a defect in stimulating RanGAP action on Ran-GTP in solution (Fig. 4B) and on Ran-GTP in complexes with transport receptors (importins) (Fig. 5D).

**DISCUSSION**

We used two independent methods for introducing substitution mutations within the RanBD region of mammalian RanBP1. Some randomly generated mutants were chosen for further study because they contained alterations (one or two) in highly conserved residues. Other mutants were selected for further study on the basis of the fact that they displayed a reduction in binding to Ran-GTP, as judged by an overlay assay. The in vitro biochemical properties and in vivo phenotype of the 11 mutants characterized in this study are summarized in Table I.

Four of the five mutants that were found to be defective in binding Ran-GTP were double mutants that altered residues within sequence segments now known to form the major interface with Ran, based on the three-dimensional structure of the RanBP2 RanBD1-Ran-GppNHp complex (34). However, the altered residue (Glu-37) in the fifth mutant defective for Ran-GTP binding faces solvent in the crystal structure. Given that the alteration characterized is a charge reversal mutation (E37K) in the N-terminal “arm” of RanBP1 that grasps Ran, one possible explanation for its ability to disrupt RanBP1 binding to Ran-GTP might be that placing a positively charged

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4 M. Künzler and J. Thorner, unpublished results.
residue at this position alters the conformation or overall charge of the arm so as to prevent (sterically or electrostatically) close approach of the two proteins. Contrary to intuition, alterations of certain invariant (or nearly invariant) residues in RanBP1, including E69A, D93A, and A100T caused no significant defects in the biochemical properties of RanBP1 in vitro and manifested no detectable deficiency in the ability of RanBP1 to substitute for its yeast counterpart (Yrb1) in vivo. The lack of effect of the E69A mutation is particularly surprising because Glu-69 appears to make a direct electrostatic contact with the Switch I region of Ran in the RanBD1-Ran-GppNHp crystal. Although the residue in RanBD1 (Glu-83) equivalent to Asp-93 does not form any apparent bond with Ran, the lack of effect of the D93A mutation is still rather surprising because Asp-93 sits immediately adjacent to the invariant RXMRK motif that lies at RanBP1-Ran interface and does contact the Switch I region of Ran.

Those mutants unable to bind Ran-GTP also do not inhibit RCC1-mediated GTP release from Ran, as expected. In contrast, the capacity of the different RanBP1 mutants to bind Ran-GTP did not correlate well with their ability to stimulate RanGAP action. Of the five mutants unable to bind Ran-GTP, three of them (G71C/K76E, R91S/K97T, and R92K/D93Y) were able nonetheless to function, at high concentration, as co-activators of RanGAP. In contrast, the other two mutants (E37K and W67R/K68M) were unable to stimulate RanGAP. This anomaly suggests that RanBP1 may participate in promoting the association of RanGAP with Ran-GTP, perhaps by making specific side chain contacts with RanGAP. Indeed, E37K is solvent-exposed and thus well positioned for such an interaction. Moreover, RanGAP is a protein that is more than twice the size of either RanBP1 or Ran and thus could conceivably make contact with both proteins simultaneously or with two different parts of RanBP1 (possibly explaining the fact that both the E37K and W67R/K68M mutations abrogated RanBP1 stimulation of RanGAP function).

Most remarkably, all of the mutants tested were able to form stable ternary complexes with Ran-GTP and importin-β (or importin-5). A simple explanation for this result is that side chains in the importins make direct contacts with sites in RanBP1 distinct from those in the RanBD involved in the association of RanBP1 with Ran-GTP. However, this suggestion is not in accord with a model recently presented by Vetter et al. (50) in which the RanBD of RanBP1 is proposed to make no contact with importin-β in the ternary complex (50). An alternative explanation for our results is that binding of Ran-GTP to importins dramatically increases the on-rate for RanBP1 binding. What might be the mechanistic basis for this effect? We propose that the conformation of the unique C-terminal segment of Ran is critical for this regulation. In the absence of other factors, the C terminus of Ran, even in the GTP-bound state, most likely remains in close association with the surface of Ran due to the interaction of an acidic motif (DEDDDD) in the C terminus with a patch of basic side chains (residues 139–142) on the surface of the body of the protein (51). Revealingly, however, in the RanBD1-Ran-GppNHp crystal structure, the C-terminal portion of Ran is swung out and wrapped tightly around the RanBD. Apparently, association of importin-β with Ran-GTP also causes the C terminus to swing

**Fig. 7. Mammalian RanBP1 substitutes in vivo for its yeast counterpart, Yrb1.** A, yeast strain JY604 (yrb1-51ts) was transformed with an empty vector (pYECH3) or the same vector expressing N-terminally (HA1)-tagged versions of either wild-type (wt) RanBP1 or the RanBP1 mutants indicated or a fragment comprising just the RanBD of RanBP1. Cell suspensions of equal titer from individual transformants were spotted onto selective medium to either repress (SCGlc-Ura) or induce (SCGal/Raf-Ura) expression of the indicated proteins. Plates were incubated at the indicated temperatures for 2 days. B, yeast cells expressing each RanBP1 protein indicated from the vector pYEXH3 (see "Experimental Procedures") were grown in 5 ml of selective medium (SCGlc-Ura) at 23 °C to an A600nm = 1.0, and then a 3-ml sample of each culture was collected by centrifugation and resuspended in 0.1 ml of water containing protease inhibitors (4 μM aprotinin, 30 μM leupeptin, and 250 μM PMSF). After addition of SDS-PAGE sample buffer, the yeast was broken by vigorous vortex mixing with glass beads, then clarified by centrifugation to remove insoluble material, and analyzed for expression of the (HA1)3-tagged RanBP1 by SDS-PAGE, transfer to nitrocellulose, and immunoblotting with the anti-HA1 monoclonal antibody 12CA5. C, the RanBP1 mutants indicated were subcloned behind the authentic YRB1 promoter in the low copy vector, yCPlacTY (see "Experimental Procedures"), and introduced into strain JY604 by DNA-mediated transformation. Individual transformants were suspended in water at equivalent titer, spotted onto selective medium (SCGlc-Ura), and grown at the indicated temperatures for 2 days.
away from the body of the protein, as judged by its enhanced accessibility to various probes (such specific antibodies and proteases) (19, 34). This importin-induced change in the freedom of motion of the C terminus of Ran-GTP places Ran in a state more receptive to RanBP1 binding. Thus, in a Ran-GTP-importin complex, the free energy barrier for binding of RanBP1 should be reduced and, hence, the on-rate for RanBP1 binding should increase. This effect may be sufficient to account for the observed ability of the RanBP1 mutants that have bindable RanBDs of Nup358 or importin- to facilitate a new round of import. This complex is, however, completely resistant to RanGAP. Only in the presence of RanBP1, experimental evidence has shown that Ran has a role in the regulation of aster formation during mitosis that is unrelated to its function of motion of the C terminus of Ran. Consistent with the mechanism we propose above, a simple explanation for these findings would be that, when Ran is in the GDP-bound state, binding of importin cannot release the constraint on the positioning of the C terminus of Ran and, thus, cannot increase the on-rate for RanBP1 binding.

Do our results shed any additional light on what the essential function of RanBP1 in nucleocytoplasmic transport might be? The current consensus view, supported by considerable experimental evidence, is that RanBP1 acts, together with RanGAP, to trigger GTP hydrolysis and the dissociation of transport complexes in the cytosol to complete the cycle of transport. For example, importin-β exits the nucleus in a complex with Ran-GTP, from which it must be dissociated to initiate a new round of import. This complex is, however, completely resistant to RanGAP. Only in the presence of RanBP1, or the RanBDs of Nup358 or importin-α, can RanGAP catalyze GTP hydrolysis and release of importin-α (11, 23). Likewise, RanBP1 is able to promote the release of Yrb4 or importin-5 from Ran-GTP (44, 46). Similarly, RanBP1 can release the exportin, Cse1 (and CAS, its mammalian homolog) from nuclear pores, Ran-GTP and its cargo, importin-α (18, 48, 53). Disruption of the Yrb1 gene in S. cerevisiae is lethal, presumably because nuclear transport ceases rapidly in the absence of some function of RanBP1. Of the mammalian RanBP1 mutants unable to support growth of the Yrb1-deficient cells when expressed at a level presumably equivalent to endogenous Yrb1, each of them (and all of the other RanBP1 mutants described here) can nonetheless form a ternary complex with Ran-GTP and importin-β. However, in contrast to the mutants that can complement the yrb1-51 mutation, the mutants that are non-functional in vivo all share the property that they cannot facilitate RanGAP function, at least on RanBP1-Ran-GTP-importin-5A.

The residues marked (YES) are not invariant but are highly conserved (i.e. D may be an E in some species). ND, not determined. JY604 is the S. cerevisiae strain harboring the temperature-sensitive allele yrb1-51.

### Table I

| Mutant | Invariant residue? | Binds Ran? | Inhibits RCC1? | Activates RanGAP? | Complex with importing/Ran? | Terinary complex with RanGTP/importins? | Rescue JY604 (high copy)? | Rescue JY604 (low copy)? |
|--------|-------------------|------------|---------------|-------------------|-----------------------------|-----------------------------------------|--------------------------|--------------------------|
| E37K   | YES               | --         | --            | --                | ++                          | ++                                     | --                       | --                       |
| W67R/K68M | YES/YES         | --         | --            | --                | ++                          | ++/++                                  | --                       | --                       |
| E69A   | YES               | ++         | ++            | ++                | ++                          | ++/++                                  | --                       | --                       |
| G71C/K76E | YES/YES         | --         | --            | --                | ++                          | ++                                     | --                       | --                       |
| T27S   | NO                | ++         | ++            | ND                | ++                          | ++                                     | --                       | --                       |
| K96E   | NO                | ++         | ++            | ND                | ++                          | ++                                     | --                       | --                       |
| R91S/K97T | YES/YES         | --         | --            | --                | ++                          | ++                                     | --                       | --                       |
| R92D/K93Y | YES/YES         | --         | --            | --                | ++                          | ++/++                                  | --                       | --                       |
| D93A   | (YES)             | ++         | ++            | ND                | ++                          | ++/++                                  | --                       | --                       |
| D93N   | (YES)             | ++         | ++            | ++                | ++                          | ++/++                                  | --                       | --                       |
| A100T  | YES               | ++         | ++            | ++                | ++                          | ++/++                                  | --                       | --                       |

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