RBEL1 Is a Novel Gene That Encodes a Nucleocytoplasmic Ras Superfamily GTP-binding Protein and Is Overexpressed in Breast Cancer*5

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Rab family proteins are generally known as regulators of protein transport and trafficking. A number of Rab proteins have been implicated in cancer development and/or progression. Here we report the identification of a novel Rab-like protein, which we have named RBEL1 (Rab-like protein 1) for its higher similarity to the Rab subfamily members. We have characterized two isoforms of RBEL1 including the predominant RBEL1A and the less abundant RBEL1B that results from alternative splicing. Both isoforms harbor conserved N-terminal guanine nucleotide phosphate (GTP) binding domains and, accordingly, are capable of binding to GTP. Both isoforms contain variable C termini and exhibit differential subcellular localization patterns. Unlike known Rabs that are mostly cytosolic, RBEL1B predominantly resides in the nucleus, whereas RBEL1A is localized primarily to the cytosol. Interestingly, a point mutation affecting RBEL1B GTP binding also alters the ability of mutant protein to accumulate in the nucleus, suggesting GTP binding potential to be important for RBEL1B nuclear localization. Our results also indicate that RBEL1A is overexpressed in about 67% of primary breast tumors. Thus, RBEL1A and RBEL1B are novel Rab-like proteins that localize in the nucleus and cytosol and may play an important role in breast tumorigenesis.

The Ras superfamily consists of five structurally distinct subfamilies including Ras, Rho, Rab, Sar1/Arf, and Ran with the Rab group of proteins constituting the largest subfamily (1). Some Rabs are expressed ubiquitously, although others exhibit tissue specificity. In general, the key function of many of the known Rab proteins is to regulate protein transport/trafficking pathways and to determine the specificity of membrane transport steps within the cell (1, 2). In a manner similar to other GTP-binding proteins, many Rab proteins cycle between a GTP-bound active state and a GDP-boundinactive state. The GTP-bound Rabs interact with their effectors and thereby regulate important steps in the vesicular transport affecting the compartmentalization of their effector proteins. The GDP-bound Rabs, on the other hand, disassociate from their effectors and return to the donor compartment for the next delivery cycle (2). Different Rab members have been shown to regulate different steps in vesicle trafficking including cargo selection and budding, movement, docking, and fusion (2, 3). For example, Rab1, Rab2, Rab5, and Rab9 are implicated in the first stage (selection and budding) of secretory transport; Sec4, Rab5, Rab6, and Rab7 are involved in the second stage (vesicle movement), and Ypt1, Ypt7, Sec4, Rab1, and Rab5 are implicated in the third stage (vesicle tethering/docking). Some Rabs, such as Rab5, are believed to regulate several stages (i.e. stages 1, 2, and 3) of protein transport (3). Because Rabs are involved in different steps in the exocytic and endocytic pathways, their subcellular localization also varies. For example, Rab1 and Rab6 are found in the endoplasmic reticulum and Golgi respectively, whereas Rab5 and Rab7 reside in the early and late endosomes, respectively (2, 4).

Recent evidence suggests that some Rab proteins are involved in cancer development and/or progression (5). For example, overexpression of Rab1B, Rab4B, Rab10, Rab22A, Rab24, and Rab25 genes has been reported in liver cancers (6, 7). Increased expression of Rab25 has also been linked to prostate cancer progression (8). Elevated levels of Rab25, due to gene amplification, have been found in ovarian and breast cancers and linked to increased aggressiveness and poor prognosis. Enforced expression of Rab25 is reported to also enhance anchorage-independent growth and cell proliferation and inhibit apoptosis induced by several genotoxic and nongenotoxic stresses involving UV irradiation, nutrition starvation, and taxol treatment (9).

Despite recent progress made in delineating the function of Rabs in protein transport/trafficking, our understanding of the role of this class of proteins in human cancer development and/or progression is still very limited, and thus, further studies are needed. In this manuscript we report the identification and characterization of a novel Rab-like protein that we have named RB1L1 (Rab-Like protein 1). We have characterized two splice variants, RBEL1A and RBEL1B, and demonstrated that they are novel GTP-binding proteins. RBEL1A and RBEL1B reside in the cytosol as well as the nucleus, although to different degrees. Interestingly, the nuclear localization of RBEL1B is associated with its GTP binding ability, as the mutant defective in GTP binding does not accumulate in the nucleus. Our results further...
indicate that RBEL1A is overexpressed in the majority of primary breast tumors. Thus, RBEL1A and -B, to the best of our knowledge are the first Rab-like proteins that localize to the nucleus and may play an important role in modulating cell survival and tumorigenesis.

**EXPERIMENTAL PROCEDURES**

**Reagents and Expression Constructs**—GFP

antibody was from Roche Applied Science. HA-tag monoclonal antibody (HA.11) was from Covance (Berkeley, CA). β-Actin monoclonal antibody was from Sigma. All RBEL1 point mutations were generated by the QuikChange site-directed mutagenesis kit (Stratagene). Anti-human RBEL1 antibodies were generated through a commercial source (Pocono Rabbit Farm and Laboratory, Canadensis, PA) by immunizing rabbits with a peptide containing 18 amino acids (ALKKLVGSDQAPGRDKNC) specific for both A and B variants of the RBEL1 protein and does not show homology to any known protein including other Rab and Ras family members.

To generate the GFP-tagged wild type and mutant RBEL1A and RBEL1B constructs, full-length RBEL1A and RBEL1B cDNAs were subcloned into pEGFPc1 expression construct (BD Bioscience Clontech). HA-tagged RBEL1A and RBEL1B were generated by inserting the open reading frame of RBEL1A and -B into the pSRRHA mammalian expression vector. All vectors were sequenced to confirm the correct sequences. HA-tagged RBEL1AT57N and RBEL1BT57N point mutations were generated using a site-directed mutagenesis kit (Stratagene) as per the manufacturer’s protocol.

**RT-PCR**—RT-PCR was performed as we have previously described (10). Primers for the PCR reactions included sense primer, 5'-AATGCGGCCCCACCATCCTGTG-3', and antisense primer, 5'-GACTTGCTTCCCTTCCTTCTGC-3'. RBEL1A and RBEL1B were simultaneously amplified in the same PCR reaction, and these primers amplified the 1200- and 360-bp products representing RBEL1A and RBEL1B, respectively.

**RNA Blot Hybridizations**—Northern blotting and hybridization for Cancer Profiling Arrays were performed according to standard procedures as we have previously described (11, 12). To detect RBEL1 expression, human RBEL1 cDNA fragments that recognize both RBEL1A and RBEL1B isoforms or are specific to RBEL1A isoform were used as probes. Human multiple tissue Northern blot membrane containing poly-A+ RNA from various human tissues was purchased from Panomics (Fremont, CA). Human cancer profiling arrays carrying the cDNAs of transcripts isolated from matching normal and tumor tissues representing 241 individual patients were purchased from BD Bioscience/Clontech.

**Western Blotting, Immunoprecipitation, and Immunostaining**—Western blotting, immunoprecipitations, and immunostaining were performed by standard procedures as we previously described (13).

**β-N-Acetylhexosaminidase (NAHase) Digestion**—NAHase digestion was performed according to the manufacturer’s protocol (New England Biolabs). Briefly, 50 μg of total protein from lysates of 293T- or RKO cell-transfected RBEL1A and RBEL1B expression vectors were incubated in reaction buffer (provided by the vendor) plus NAHase (25 units) as per the manufacturer’s protocol. Twenty-four hours after digestion, all samples were analyzed by Western blotting using RBEL1-specific antibodies.

**RBEL1A Suppression by RNA Interference**—To suppress RBEL1A expression, cells were transiently transfected with negative control medium GC content Stealth RNAi-mediated interference (RNAi) duplex or a combination of three Stealth RNAi duplexes specific for RBEL1 (Invitrogen) according to the manufacturer’s protocol. The small interfering RNAs used are 5’-UGACGAAGGACACGAUUUGUCAGC-3’, 5’-UCGU-UCUCCAUCUUAAAGCGCUGCA-3’, and 5’-UCCCCGGA-GAUGUAAUUUGAGGUC-3’.

**Nuclear and Cytosolic Fractionations**—Nuclear and cytosolic fractions were prepared as previously described (14).

**In Vitro GTPase Assay**—RBEL1 binding to GTP was performed by the procedures as we have previously described (15). Briefly, 293T cells were transiently transfected with either GFP or pSRα-HA empty vectors (negative control), HA-T21WT (positive control), GFP-tagged RBEL1A and RBEL1B, and HA-tagged RBEL1A or -B (wild type and T57N mutant) and labeled with [32P]orthophosphoric acid. GFP, GFP-tagged RBEL1 proteins or HA only, HA-tagged RBEL1A, RBEL1B, or wild type-T21 were immunoprecipitated with anti-tag antibodies. Bead-bound immunoprecipitants were then eluted, and equal amounts of each immunoprecipitate were spotted and run on a cellulose polyester foil-backed layers (PEI-F) plate and exposed to phosphorimaging.

**RESULTS**

**Identification of RBEL1, a Novel Member of the Ras Superfamily**—Using a computer-based approach involving alignment of redundant expressed sequence tags (ESTs) to delineate novel open reading frames, we have recently identified and cloned several novel stress-regulated genes that exhibit altered expression in human tumors (11, 12). Using a similar approach, we also identified a novel gene encoding a Rab-like protein, which we have named RBEL1 for its unique protein sequence. We have characterized two isoforms of RBEL1 designated as RBEL1A and RBEL1B (Fig. 1, A and B; supplemental Fig. 1, A and B). The RBEL1 gene maps to chromosome 9q34.3, and according to the genomic organization, RBEL1A harbors 15 exons, whereas the splice variant corresponding to RBEL1B is devoid of exons 10–14 and is also missing parts of the exons 9 and 15 (Fig. 1, A and B). Thus, RBEL1A and -B proteins contain identical N-terminal sequences but harbor divergent C termini, and the data base searches revealed the existence of only hypothetical proteins indicating RBEL1 to be a novel protein. At the N termini, both RBEL1A and -B contain the hallmark sequence of all the GTP-binding proteins harboring five highly conserved domains involved in nucleotide binding and hydrolysis (shown in bold and underlined in supplemental Figs. 1, A and B). It is known that the members of the Ras superfamily

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2 The abbreviations used are: GFP, green fluorescent protein; HA, hemagglutinin; RT, reverse transcription; NAHase, β-N-acetylhexosaminidase.
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We performed a pairwise alignment to compare RBEL1 with the canonical Ras GTPase, H-Ras, and found that RBEL1, within its GTP binding domain, was 33% homologous to H-Ras GTPase. A similar degree of homology between H-Ras and several other members of Ras superfamily has been reported, including, for example, Rnd2 (34%), RHOT1 (38%), Sar1 (35%), and Arf (33%). RBEL1, however, did not show similar homology to non-Ras family GTPases such as eEF1A, GBP1, eIF5B, and Dynamin, suggesting that it belongs to the Ras superfamily. Next, we compared the RBEL1 sequence with that of other members in various Ras subfamilies including Ras, Ran, Rab, Rho/Rac, and Arf/Sar subfamilies and found RBEL1 to show a higher degree of homology to the Rab (31–34%) and Ras (30–33%) subfamily members than with the Rac/Rho (26–28%) and Arf/Sar (27–29%) subfamily proteins. Overall, because clearly more Rab proteins align with RBEL1, we have named it so (Rab-like protein 1); however, RBEL1 also harbors several unique features not present in members of these subfamilies. For example, both isoforms of RBEL1 deviate from the Rab canonical GTP binding consensus sequences in the third (DXYG(K/Q)→DXDK), fourth (NKXD→NYXD), and fifth (EXSAX→EXSMX) GDP/GTP binding domains (supplemental Fig. 1, A and B, and supplemental Table 1). Comparison of the GTP binding domains of RBEL1 with those of other Ras superfamily proteins is shown in supplemental Table 1. Thus, RBEL1 is a novel protein that belongs to a unique and perhaps new subfamily within the Ras superfamily.

We also noted that both isoforms of RBEL1 contain proline-rich domains within their central regions (RBEL1A, amino acids 293–397, and RBEL1B, amino acids 293–363). Proline-rich regions have been implicated in protein-protein interactions, subcellular localization, and are often the recognition site for binding SH3 (Src homology 3) and WW domain-containing proteins (18, 19). In addition, the A isoform also harbors a number of putative nuclear localization signals at its C terminus (shown in bold and shaded in supplemental Fig. 1A) that are absent in RBEL1B.

To determine RBEL1 expression and tissue distribution, we performed Northern blot analyses, and as shown in Fig. 1C, the RBEL1 cDNA-specific probe (pan RBEL1 probe), which was expected to detect both A and B isoforms, recognized a predominant transcript of ~3.6 kilobases in all the tissues examined with stronger signals seen in lung, skeletal muscle, heart, stomach, and testis. The fact that the pan RBEL1 probe predominantly detected ~3.6-kilobase RBEL1-specific transcripts that corresponded to the RBEL1A isoform, it was likely that RBEL1A was the more abundant variant. To further confirm that the ~3.6-kilobase transcripts detected by the pan probe...
were indeed RBEL1A, we next utilized a RBEL1A-specific probe to do Northern blotting. A cDNA probe designated as Probe 2 that corresponds to the region specific for RBEL1A but is absent in RBEL1B (Fig. 1A) was prepared, and Northern analysis was performed on a number of cell lines including MCF-7, T47D, MDA231, MDA468, and Hs578T breast cancer cells and MCF10A normal breast cells as well as RKO colon cancer cells. Fig. 1D, upper panel, shows that the RBEL1A-specific probe detected the ~3.6-kilobase transcripts in all cell lines, with MCF10A normal breast cells exhibiting a low level of expression. The same blot was also probed (after stripping the first probe and then waiting for the signals of first probe to decay) with the pan RBEL1 probe, and as shown in Fig. 1D, lower panel, the pan RBEL1 probe also predominantly detected the same-size RBEL1A signal. These results, therefore, indicate that RBEL1A is the predominant isoform, whereas the RBEL1B transcripts, not detected by Northern blotting, exists in low abundance. Next, we performed the more sensitive RT-PCR to compare the expression of the A and B isoforms, and as shown in Fig. 1E, the RBEL1B mRNA was indeed expressed at a low level compared with RBEL1A mRNA. Taken together, these results suggest that the RBEL1A transcripts exist in high abundance and that the A isoform is the predominant variant.

**RBEL1A Is Overexpressed in Human Breast Cancer**—Because we noted that RBEL1A expression was higher in the breast cancer cell lines (MCF-7, T47D, MDA231, MDA468, and Hs578T) than in normal breast epithelial cells MCF10A (Fig. 1D), next we examined RBEL1A expression in primary breast tissues using cancer profiling arrays representing tumor and matching normal tissue mRNAs of 13 different human tissue types including breast, uterus, colon, stomach, ovary, lung, kidney, rectum, and thyroid. We found that RBEL1A was predominately overexpressed in primary breast tumors. Fig. 2A shows the results of RBEL1A expression in cancer profiling arrays of representative breast samples, and the overall results for various malignancies are summarized in Fig. 2B. As is shown (Fig. 2, A and B), 32 of 48 (67%) breast tumors exhibited increased expression of RBEL1A mRNA when compared with their matching normal tissues from the same individuals. A relatively fewer number of samples representing colon, gastric, ovarian, and uterine cancers also displayed increased RBEL1A mRNA expression (Fig. 2B). However, we did not find any considerable changes in RBEL1A mRNA expression in primary tumors of the thyroid, prostate, rectum, and lung compared with their corresponding matched normal tissues (data not shown), indicating that RBEL1A was specifically overexpressed in human breast cancers. Ubiquitin expression signals (provided by the vendor) were used as loading controls to ensure comparable loading for the cancer profiling arrays. We also performed Northern blot analysis using four pairs of matched breast normal and tumor samples (three adenocarcinomas and one benign fibroadenoma), and the results revealed that RBEL1A mRNA expression was elevated in all three breast carcinomas (Fig. 2C, lanes 1, 3, and 4) but with no obvious changes in the benign fibroadenoma (Fig. 2C, lane 2). Furthermore, examination of the Serial Analysis of Gene Expression (SAGE) data base to explore the expression profile of RBEL1A-specific tags revealed that in general, breast cancer tissues expressed much higher levels of RBEL1A mRNA than the normal breast tissues. Together these results indicate that RBEL1A overexpression occurs at a high frequency in human breast cancer and that altered RBEL1A expression and consequently its function could be a common feature of breast cancer.

**RBEL1A Protein Expression and O-Linked Glycosylation**—To study the expression of RBEL1 at protein level, anti-RBEL1 antibodies were generated. Western blot analysis revealed that the endogenous RBEL1 detected by the anti-RBEL1 antibodies (Fig. 3A, lanes 1–7) migrated as four major species of ~125, ~110, ~100, and ~80 kDa (Fig. 3A) with the 125-kDa species being the predominant one. To further confirm the specificity of the anti-RBEL1 antibody, the expression of HA-tagged RBEL1A was analyzed either by anti-HA Western blotting of the total cell lysates (Fig. 3B) or by anti-HA immunoprecipitation of the cell lysates from HA-RBEL1A-expressing cells followed by anti-RBEL1 Western blotting (Fig. 3C). As shown in Fig. 3, the band pattern of exogenous HA-RBEL1A (Fig. 3, B and C) is largely consistent with that of the endogenous RBEL1 protein detected by anti-RBEL1 antibody (Fig. 3A), thus, confirming the specificity of the anti-RBEL1 antibody. We also used the RBEL1A mRNA-mediated interference knockdown approach to further assess the specificity of anti-RBEL1 antibody. Results shown in Fig. 3D indicate that, although control scramble small interfering RNA had no effect on the expression of RBEL1A protein detected by the anti-RBEL1 antibody, RBEL1-specific
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However, it is possible that post-translational modifications other than glycosylation may also contribute to the differential molecular mass of RBEL1A. We also sought to investigate the glycosylation status of RBEL1B that contains 520 amino acids and has a predicted mass of 57 kDa. Given that endogenous RBEL1B isoform exists in low abundance and is not detectable by Western blotting, we therefore used vector expressing HA-tagged RBEL1B. The HA-tagged RBEL1B expressed from this construct has a predicted molecular mass of 62 kDa, and we noted that exogenous RBEL1B also displayed multiple bands ranging from ~62 to ~75 kDa, with a major band migrating at ~75 kDa. However, unlike RBEL1A, treatment of RBEL1B with NAHase did not alter the HA-RBEL1B protein band pattern (Fig. 3E, right panel, compare lane 1 and lane 2). These results, therefore, suggest that RBEL1B may not be modified by O-linked glycans.

The Nucleocytoplasmic Localization of RBEL1—To determine the subcellular localization of RBEL1, we tagged both isoforms with GFP knockdown of endogenous RBEL1 in RKO cells remarkably suppressed the endogenous RBEL1A protein expression. These results, thus, also confirm the specificity of anti-RBEL1 antibody. RBEL1A has 729 amino acids with predicted molecular masses of ~80 kDa, which is less than the molecular mass of the ~125-, ~110-, and ~100-kDa species detected by Western blotting. Computer-based glycosylation prediction revealed that the RBEL1A protein harbors a number of potential O-linked glycosylation sites but no N-linked glycosylation sites. Next, we sought to investigate whether the differential molecular mass of the RBEL1A was due to post-translational events involving O-linked N-acetylgalactosamine glycosylation. NAHase is an enzyme that is known to hydrolyze O-linked glycan chains (20). We, therefore, used NAHase to digest the RBEL1A protein to determine whether RBEL1A was indeed a glycosylated protein. Fig. 3E, left panel, shows that the molecular mass of the ~125-kDa species was significantly reduced in the cell lysates digested with NAHase (lane 2) when compared with the undigested sample (lane 1), and there was a concomitant increase in the abundance of molecular species in the ~110-kDa to less than 100-kDa range (Fig. 3E, compare lanes 1 and 2). Interestingly, we did not find any noticeable increase in the abundance of ~80-kDa species in the NAHase-digested sample (lane 2). These results, therefore, suggest that the differential molecular mass of RBEL1A noted on Western blotting appear to occur at least in part due to O-linked glycosylation.

FIGURE 3. A, Western blots (WB) showing expression of endogenous RBEL1A protein in human breast cancer (lanes 2–6), colon cancer (RKO, lane 7), and normal (lane 1) breast cells. Western analyses were performed using anti-RBEL1 antibodies as described under “Experimental Procedures.” The same blots were probed with β-actin as a loading control. MW, molecular weight. B, Western blot showing expression of exogenous HA-tagged RBEL1A in MCF-7 breast cancer cells detected by anti-HA antibodies. C, confirmation of anti-RBEL1A specificity. RKO cells were transiently transfected with empty vector or HA-RBEL1A expression vector. Immunoprecipitation (IP) was performed using anti-HA antibody followed by Western blotting analysis using anti-RBEL1 antibody. Note that the protein band pattern of HA-RBEL1A is similar to that of the endogenous RBEL1 (A). D, confirmation of anti-RBEL1A specificity by RNA-mediated interference (RNAi) approach. Western blot analysis was performed using anti-RBEL1 antibody on the lysates of RKO cells harboring scrambled small interfering RNA (lane 1) or RBEL1-specific siRNA (lane 2). Cont, control. E, RBEL1A is an O-linked glycosylated protein. Western blot analysis showing exogenous HA-RBEL1A detected by anti-RBEL1 antibodies (left panel) or exogenous HA-RBEL1B detected by anti-HA antibody (right panel) in HEK 293T cells before (lane 1) or after (lane 2) treatment with β-N-acetylgalactosaminidase (NAHase).
FIGURE 4. Subcellular distribution of exogenous and endogenous RBEL1A and RBEL1B. A, B, and C, representative photomicrographs show subcellular localization of exogenous GFP-only, GFP- and HA-tagged RBEL1A and RBEL1B isoforms in MCF-7 breast cancer cells, MCF10A normal breast cells, colon cancer RKO cells, and NIH3T3 mouse fibroblasts. Cells growing on lab-tekII chamber slides were transiently transfected with the indicated vectors. Approximately 24 h after transfection, cells were fixed with 4% paraformaldehyde, and nuclei were counterstaining with 4′,6-diamidino-2-phenylindole (DAPI) nuclear dye. Photomicrographs were taken under an Olympus fluorescent microscope using appropriate filters. D, subcellular distribution of endogenous RBEL1. RKO colon cancer cells seeded on lab-tekII slides were fixed and processed for immunostaining anti-RBEL1 specific antibodies or preimmune serum and rhodamine-conjugated secondary antibodies. Nuclei were stained with 4′,6-diamidino-2-phenylindole nuclear dye. Arrows point to cells with nucleocytoplasmic distribution of RBEL1.

E and F, nuclear and cytoplasmic fractionations showing the subcellular distribution of endogenous RBEL1A. The cytosolic (CYT) and nuclear (NUC) fractions were separated as described (14). Endogenous RBEL1A was detected in both nuclear and cytosolic fractions. Lamin B and β-actin serve as protein markers for nuclear and cytosolic portions, respectively. 2XNUC, double amount of the nuclear fraction was loaded; TCL, total cell lysate; MW, molecular weight. The β-actin blot in F was overexposed to rule out the possibility of cytosolic contamination.
RBEL1 proteins was not influenced by the size of GFP, we also generated the HA-tagged RBEL1A expression construct and analyzed its expression in MCF-7 and RKO cells. Anti-HA immunostaining performed on the HA-RBEL1A-expressing cells revealed that the distribution pattern of HA-RBEL1A was largely similar to that seen with the GFP-tagged RBEL1A (Fig. 4B, a” to c”). The subcellular localization of GFP- and HA-tagged RBEL1B, on other hand, was predominantly nuclear but excluded from the nucleoli (Fig. 4B, g” to h”), and C, a” to d”), although a low level of distribution was noted in the cytoplasm in a punctate pattern (Fig. 4C, a and d).

Using anti-RBEL1 antibodies, we also performed immunostaining to determine the distribution of endogenous RBEL1. As shown in Fig. 4D, the expression of endogenous RBEL1 in RKO cells was predominantly noted in the cytoplasm (Fig. 4D) with some cells also displaying a nucleocytoplasmic distribution (Fig. 4D, arrows). Thus, the subcellular distribution pattern of endogenous RBEL1 was similar to that noted for GFP-RBEL1A and HA-RBEL1A detected in the same cell line. The anti-RBEL1 antibody is expected to detect both RBEL1A and -B isoforms, but given that RBEL1A is the predominant isofrom, whereas RBEL1B is expressed at very low levels, it is, therefore, likely that the signals detected by the anti-RBEL1 antibody mainly represent endogenous RBEL1A.

We also performed cell fractionation assays to evaluate the nucleocytoplasmic localization pattern of endogenous RBEL1A. As shown in Fig. 4E and F, higher levels of endogenous RBEL1A were detected in the cytosolic fraction, whereas modest levels of RBEL1A were seen in the nuclear fraction in MCF-7 and RKO cells. The same blot was probed for β-actin (cytoplasmic) and lamin B (nuclear) to evaluate the purity of the cytosolic and nuclear fractions (Fig. 4E and F, lower panels). Together these results indicate that RBEL1A and RBEL1B exhibit rather unique nucleocytoplasmic expression patterns. To date, only a few GTPases are known to localize in the nucleus including Ran, Rac1, Rap1, and PIKE-A and MxB (17, 21–24).

RBEL1A and RBEL1B Are GTP-binding Proteins—Amino acid sequence comparison revealed that both isoforms of RBEL1A share a significant homology to other proteins in the Ras/Rab families, particularly at their N terminus. Specifically, both RBEL1A and RBEL1B harbor all five GTP binding domains found in other GTPases of the Ras/Rab family. Next, we performed GTPase assays to determine the GTP binding potential of RBEL1. After cell labeling with [32P]orthophosphate, GFP, GFP-tagged RBEL1 proteins, and HA-tagged TC21 were immunoprecipitated with respective anti-tag antibodies. HA-tagged wild type TC21 (also known as R-Ras2, which belongs to R-Ras subfamily of the Ras superfamily) was used as a positive control in these GTPase assays to evaluate the GTP/GDP binding. We have previously reported that the wild type TC21/R-Ras2 binds to both GTP and GDP with a higher GTP:GDP-bound ratio (15). As shown in Fig. 5A, top panels, TC21/R-Ras2 binds to both GTP and GDP with
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higher ratio in the GTP-bound state, a finding that is consistent with our previous results (15). RBEL1A and RBEL1B are also GTP-binding proteins, but unlike TC21/R-Ras2, both of these isoforms predominantly exist in the GTP-bound configuration (Fig. 5A, top panels). To exclude the possibility of the size of GFP tag affecting RBEL1A and -B to hydrolyze GTP, we also performed the in vitro GTPase assays using the HA-tagged RBEL1A and -B and obtained similar results (Fig. 5A, bottom left panel, lane 2, and bottom right panel, lanes 2 and 3). The presented results therefore, indicate that (i) both RBEL1A and RBEL1B are novel GTP-binding proteins that are predominantly GTP-bound in the cell and (ii) that both RBEL1 isoforms possess a low intrinsic GTPase activity for hydrolyzing GTP to GDP.

The RBEL1B Mutant Deficient in GTP Binding Loses Its Ability to Accumulate in the Nucleus—Next, we sought to investigate whether the GTP binding is important for RBEL1 to localize to nucleus, as is the case for Ran (25). For this purpose, we studied the RBEL1B isoform because this isoform predominantly localizes in the nucleus. We performed site-directed mutagenesis to generate two RBEL1 mutants. One is a gain-of-function mutant that involved the replacement of arginine with valine at position 52 (RBEL1B-R52V), and the other is a loss-of-function mutant involving the replacement of threonine with asparagine at position 57. The RBEL1B-R52V gain-of-function mutant corresponds to H-Ras-G12V, whereas the RBEL1B-T57N loss-of-function mutant matches the H-Ras-S17N. Previous studies of H-Ras and a number of Ras-like GTPases have demonstrated that the gain-of-function mutations occurring at the relevant residue of the first GTP binding domain diminish the intrinsic GTPase activity and lock the small GTPases in the GTP-bound state; the loss-of-function mutation, on the other hand, impairs their GTP binding potential (25, 26). We expressed the GFP-tagged wild-type and mutant-RBEL1B in NIH3T3 cells and examined the subcellular localization of these proteins. As shown in Fig. 5B, the subcellular localization of GTP-tagged wild type RBEL1B and GFP-tagged RBEL1B-R52V was similar and exhibited nuclear and punctate cytosolic subcellular distributions. By contrast, the GFP-RBEL1B-T57N mutant was predominantly localized in the cytosol with less accumulation in the nucleus.

To ensure the subcellular distribution of RBEL1B-T57N was not affected by the tag protein, we also performed immunostaining (anti-HA tag) to detect the expression of the HA-tagged wild type and mutant (T57N)-RBEL1B, and a similar cellular distribution was observed as that seen with the GFP-tagged counterparts (Fig. 5C). In vitro GTPase assays also demonstrated that whereas the wild type counterpart was mainly GTP-bound, the RBEL1B-T57N mutant was not able to bind to GTP or GDP (Fig. 5A, bottom right panel, lane 4). Interestingly, we found that RBEL1A harboring the same mutation (T57N) also lost its ability to bind to GTP or GDP (Fig. 5A, bottom left panel, lane 3).

In addition, we also examined the expression and subcellular distribution of the wild type- and mutant-RBEL1B (T57N) proteins by cell fractionation. Interestingly, we found that the expression levels of the mutant RBEL1B (T57N) were consistently lower than those of the wild type protein (Fig. 5D, left panel, lanes 1 and 2) even though the same amounts of each plasmid were used for transfections. The molecular basis for this finding remains unclear at the present time, and future studies will provide some insight as to whether or not the GTP binding deficiency alters the expression and/or stability of the mutant protein. We also found that wild type RBEL1B predominantly localized to the nuclear fraction (Fig. 5D, right panel, compare lanes 1 and 3), whereas RBEL1B-T57N was mainly cytosolic (Fig. 5D, right panel, compare lanes 2 and 4). These results are consistent with our immunofluorescence studies (Fig. 5, B and C). Thus, our results indicate that the lack of GTP binding ability also affects the subcellular localization of RBEL1B. These results, therefore, suggest that GTP binding may be important for RBEL1B to localize in the nucleus and could prove critical for its nucleocytoplasmic distribution and its overall function.

DISCUSSION

In this study we have identified and characterized a novel gene that encodes a Rab-like GTP-binding protein, RBEL1. We have characterized two isoforms of RBEL1 including RBEL1A and RBEL1B, and both isoforms exhibit significant amino acid similarity with other Ras superfamily proteins at their N termini and specifically share a higher homology with members of the Rab subfamily. Just like Rab proteins and other members of Ras superfamily, RBEL1A and -B harbor all five GTP binding domains and exhibit GTP binding capability. In addition to these commonalities, however, both RBEL1A and RBEL1B also have several features that are not typical for most Rab-like proteins. For example, both RBEL1A and RBEL1B exhibit variable subcellular distributions, and although RBEL1A predominantly exhibits cytoplasmic distribution, RBEL1B is mostly nuclear. Interestingly, although RBEL1A contains several putative nuclear localization signals, only a small portion of cells exhibits a nucleocytoplasmic distribution, whereas RBEL1B, which does not contain a classical nuclear localization signal, predominantly resides in the nucleus. Although further studies are needed to study the molecular mechanisms that regulate the subcellular distribution of RBEL1, the nucleocytoplasmic subcellular distribution of RBEL1 could be potentially important for its function. Previous studies have established that Rab and Rab-like proteins are important for facilitating protein transport from one cellular compartment to another (2–4). Although Rab and Rab-like proteins generally do not reside in the nucleus, Ran GTPase, which belongs to Ran subfamily but is also considered a Rab-like protein (27), exhibits a nucleocytoplasmic distribution pattern (28–30). Ran is a key regulator for protein transport across the nuclear pore complex (28–30). Our results suggest that both isoforms of RBEL1 and Ran share several similar features. First, RBEL1 proteins and Ran share high degree of sequence homology at their N-terminal GTP binding domain (supplemental Figs. 2 and 3). Second, Ran and RBEL1A and -B lack cysteine residues at their C termini and, therefore, are unlikely to be prenylated. Third, both Ran and RBEL1A contain stretches of acidic amino acids at the C termini (DEDDDL for Ran and DYEEL for RBEL1A). The C-terminal sequence of Ran is...
important in modulating the interaction of Ran with regulatory factors and is essential for its function in regulation of cell cycle progression (31). Fourth, Ran and RBEL1 proteins exhibit similar nucleocytoplasmic distribution patterns. The nucleocytoplasmic movement of Ran is essential for its role in modulating the transport of macromolecules across the nuclear envelope (29). Whether RBEL1A and -B also have Ran-like function in regulation of protein transport across the nuclear membrane is an important issue that will be investigated in our future studies.

Compared with most of the Rab and Ras family GTPases, both RBEL1A and -B harbor changes in three of five GDP/GTP binding domains (supplemental Table 1). RBEL1A and RBEL1B deviate from the canonical consensus sequences in the third (DXXG/K/Q)→DXXDK), fourth (NKX→NXYD), and fifth (EXSAX→EXSMX) domains. These changes are found in the human RBEL1 cDNAs from multiple EST libraries prepared from different tissues and are conserved among RBEL1A homologs in mouse and Caenorhabditis elegans (supplementary Fig. 4). Thus, these changes are unlikely to occur due to sequencing artifacts. Previous studies have demonstrated that each consensus sequence within these GTP binding motifs has particular functions in nucleotide binding and hydrolysis, and mutations/alterations within these sequences either enhance or inhibit nucleotide binding and hydrolysis (32, 33). Our results in this study indicate that both RBEL1A and RBEL1B predominantly bind to GTP (Fig. 5). These results would suggest that RBEL1A and RBEL1B are either unable to hydrolyze GTP or hydrolyze GTP at a very slow intrinsic rate. Previous studies have shown that another Rab protein named Rab24 is also locked in GTP-bound state presumably due to two inherent changes in the highly conserved GTP binding motifs (NKX→TKXD and DXXG/K/Q)→DXXGS) (34). Interestingly, Rab24 and RBEL1A/RBEL1B harbor similar inherent changes in their GTP binding motifs, suggesting that the deviations from the consensus sequence may account for their ability to predominantly exhibit the GTP-bound state. Our results also indicate that the GTP binding potential is important for nuclear localization of RBEL1. For example, the GTP binding-deficient RBEL1B (T57N) mutant is excluded from the nucleus and predominantly resides in the cytoplasm (Fig. 5), a finding that suggests that the GTP binding may play an important role in the function of RBEL1B.

Members of the Ras family including H-, K-, and N-Ras are highly conserved GTP binding motifs (NKX→TKXD and DXXG/K/Q)→DXXGS) (34). Interestingly, Rab24 and RBEL1A/RBEL1B harbor similar inherent changes in their GTP binding motifs, suggesting that the deviations from the consensus sequence may account for their ability to predominantly exhibit the GTP-bound state. Our results also indicate that the GTP binding potential is important for nuclear localization of RBEL1. For example, the GTP binding-deficient RBEL1B (T57N) mutant is excluded from the nucleus and predominantly resides in the cytoplasm (Fig. 5), a finding that suggests that the GTP binding may play an important role in the function of RBEL1B.

Members of the Ras family including H-, K-, and N-Ras are frequently mutated in human malignancies, and although the mutations generally activate these proteins, overexpression is an infrequent mechanism of activation in human tumors (16). Rab and Rab-like genes, by contrast, are rarely mutated in human cancers, but alterations in the expression of several Rab genes including Rab1B and Rab4B have been reported. It is known that Rab and Rab-like family proteins are generally involved in regulating protein trafficking and transport pathways, and they mediate such effects via interactions with other binding partners (1, 2). Because RBEL1A and RBEL1B reside both in the cytoplasm and in nucleus, identification of their interacting partner(s) may help elucidate their exact function. While this manuscript was in preparation, Tompkins et al. (35) reported the identification of several p14ARF-binding proteins by yeast two-hybrid screen and found that one of the many Arf/INK4a-interacting proteins showed sequence similarity to a hypothetical protein FLJ10101 (GenBank accession number DQ141240). Interestingly, the sequence of FLJ10101 is same as RBEL1A. Arf is a tumor suppressor, which stabilizes p53 tumor suppressor by binding and sequestering Mdm2, a p53 E3 ubiquitin ligase (36, 37). We have found that RBEL1A is overexpressed in a number of human malignancies. Future studies will, therefore, define the molecular basis of RBEL1A interactions with Arf and other as yet unidentified proteins particularly in context to its function in normal and malignant cells.

In summary, our results provide valuable information that both isoforms of RBEL1 represent a new group of Rab-like proteins, which may play an important role in human tumorigenesis.

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**Novel Nucleocytoplasmic Ras Superfamily Proteins**