Production and Characterization of a Highly Specific and Potent Antibody Capable of Detecting Endogenous RhebL1 Protein in Mammalian Tissues and Cell Lines

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Rheb (Ras homolog enriched in brain) is a small G-protein and a critical component of the insulin/TSC/mTOR pathway involved in the regulation of cell growth. Although a single gene for Rheb exists in most species, in mammals, two genes have been identified- Rheb1 and RhebL1 (Rheb2). Most studies to date have explored the expression and localization patterns of Rheb protein in mammalian cell lines using exogenously expressed tagged versions of Rheb1 for lack of a suitable antibody capable of detecting endogenous Rheb. The commercial antibodies available are either not able to detect the endogenous protein or are not specific enough to distinguish between the two Rheb proteins. We have developed and characterized a highly specific and potent affinity-purified antibody toward the mouse RhebL1 protein. This antibody does not cross react with Rheb1 and is highly sensitive as it can detect endogenous RhebL1 protein. Using this antibody, we have shown that RhebL1 protein is not ubiquitously expressed in mouse tissues and that the brain exhibits the highest level of expression. In addition, RhebL1 is expressed in two cell lines-3T3-L1 and L1C2, and its expression is induced in 3T3-L1 cells as they undergo differentiation from pre-adipocytes to adipocytes. This antibody now serves as a useful tool to study RhebL1 protein exclusively without any non-specific cross-reactivity with the Rheb1 protein.

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Abbreviations: Rheb (Ras homolog enriched in brain), GAP (GTPase Activating Protein), EGF (Epidermal Growth Factor), FGF (Fibroblast Growth Factor), MBP (Maltose Binding Protein), RT-PCR (Reverse Transcriptase Polymerase Chain Reaction), DMEM (Dulbecco's Modified Eagle Media), mTOR (mammalian Target of Rapamycin), kD (kilodalton), MIX (methylisobutylxanthine), DEX (dexamethasone), FBS (Fetal Bovine serum)

Introduction

Rheb is a GTP-binding homolog of Ras which was first identified as a gene rapidly induced in rat brain neurons by seizure activity and long-term potentiation paradigm [1]. Rheb has been shown to be a critical component of the insulin/mTOR/S6K pathway [2, 3] and has been shown to directly function in the control of cell cycle and cell growth. Genetic epistases as well as biochemical studies indicate that Rheb is negatively regulated by the TSC1/TSC2 tuberous sclerosis complex proteins which function as a GAP (GTPase Activating Protein) for Rheb [3, 4]. Rheb has been shown to regulate S6K and 4E-BP1 by phosphorylation in an mTOR dependent manner suggesting that Rheb lies upstream of mTOR [3, 5].

In most lower organisms (yeast, *C. elegans*, *Drosophila*), Rheb is encoded by a single gene while in mammals, two distinct genes exist for

Rheb classified as Rheb1 and Rheb2 - also known as RhebL1 [6]. The mouse Rheb1 and RhebL1 have 78 % homology at the nucleotide level while at the amino acid level, they share a 51 % identity. Both human Rheb1 and RhebL1 are known to activate S6K when over-expressed transiently in mammalian cells [7] in a rapamycin dependent manner suggesting that they may have similar roles in the insulin/mTOR pathway. However, not much work has been done to elucidate the functional significance of RhebL1 in detail. In addition, most of the studies have been done with recombinant RhebL1 over-expressed in cell lines and not with the endogenous protein itself.

The expression profile of both the genes has shown that while human Rheb1 mRNA is expressed ubiquitously [8], RhebL1 is primarily expressed in the brain [9]. It was also shown that Rheb1 is rapidly induced in 3T3 fibroblasts upon serum stimulation as well as in PC 12 cells in response to growth factors such as EGF and FGF [1]. Additionally, Rheb1 and RhebL1 mRNA levels were also found to be elevated in human tumor cell lines [9, 10] as well as in UV-irradiated fibroblasts causing sensitization to UV killing [11]. Thus, Rheb expression is likely to be regulated by a number of different factors. It will be interesting to determine whether RhebL1 is regulated differently than Rheb1 considering its tissue specificity.

Studies of Rheb1 and RhebL1 *in vitro* using mammalian cell lines have primarily been performed using over-expressed tagged proteins which strictly do not reflect the physiological conditions of the endogenous proteins themselves. Although a number of antibodies exist for Rheb1 and RhebL1, they are either non-reactive or cross-react with both Rheb proteins. In this study, we have focused on detecting the expression of endogenous mouse RhebL1 using various mouse cell lines and tissues. We have succeeded in generating an antibody which is highly specific for mouse RhebL1 and does not cross react with Rheb1, which has enabled us to monitor RhebL1 exclusively. In addition, we have also shown that RhebL1 is induced in 3T3-L1 cells in response to adipogenic signals.

Materials and Methods

Generation of mouse RhebL1 Antibody

Polyclonal antisera toward mouse RhebL1 protein was generated by Washington Biotechnology, Inc. (Maryland). A 14-residue long C-terminal peptide of mouse RhebL1 (IARVENSYGRQDRR) was synthesized and conjugated to KLH. Two New Zealand rabbits were immunized and titers determined by ELISAs. Crude antiserum was collected and affinity purified using a peptide column. Affinity purified material was stored at 4^{o}C in the presence of 0.1% thimerosol. The concentration of this purified anti-RhebL1 was 5.4mg/ml.

Purification of MBP-mRheb1 and MBP-mRhebL1 proteins

Mouse Rheb1 and RhebL1 EST clones (IMAGE clones 3710454 and 4527910 respectively) were obtained from ATCC and the coding sequences were amplified by PCR and inserted into the pMal-c2 vector (New England Biolabs). These constructs were transformed into *E. coli* BL21 (DE3) strain and expression induced by the addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at 37^{o}C for 3 h. Proteins were expressed as MBP (maltose binding protein) fusion proteins. Cells were harvested by centrifugation and suspended in lysis buffer comprising of 20 mM Tris–HCl, pH 7.4, containing 200 mM NaCl, 1 mM EDTA, 10 mM

β-mercaptoethanol and protease inhibitor cocktail (Roche Applied Science). Cells were lysed by sonication (3 pulses of 30 s each) at 4°C. The suspension was clarified by centrifugation and the supernatant loaded onto a column containing amylose resin (New England Biolabs) equilibrated with the lysis buffer. The column was washed with five volumes of lysis buffer and the fusion proteins were eluted with lysis buffer containing 10 mM maltose. The eluted fractions were analyzed by SDS-polyacrylamide gel electrophoresis followed by staining with Coomassie Brilliant Blue which revealed a single band of ~62kD. Purified proteins were stored in 50% glycerol at -20°C. Protein concentrations were determined by Bradford protein assay.

Mammalian Cell Lines, Culture Conditions and Transfection

Eph4 and NMuMg (mouse mammary epithelial cells) cell lines were kindly provided by T. Lane (University of California, Los Angeles- UCLA); JS1 (rat schwann) and RT4 (rat schwannoma) cells by D. Scoles (Cedars-Sinai, Los Angeles); 3T3-L1 (murine pre-adipocytes) by P. Tontonoz (UCLA); L1C2 (murine lung carcinoma) by S. Sharma (VA medical Center, Los Angeles); C6 (rat glioma) by S. Kumar (UCLA) and B16 (mouse melanoma) by M. Kolodney (UCLA).

Eph4, NMuMg, JS1 and RT4 cells were maintained in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), GlutaMax supplement (Invitrogen) and penicillin-streptomycin. 3T3-L1 pre-adipocytes were maintained in Dulbecco's modified eagle's medium supplemented with 10% heat-inactivated bovine serum, GlutaMax and penicillin-streptomycin. Differentiation was induced by replacing the medium with differentiation media- DMEM supplemented with 10% fetal bovine serum containing 0.5 mM methylisobutylxanthine (MIX), 1 μM dexamethasone, and 5 μg/ml insulin when the cells reached confluency [12, 13]. After 48 h in differentiation media, medium was changed to DMEM containing 10% FBS and 5 μg/ml insulin and was subsequently replaced with fresh medium containing 10% FBS (without insulin) every two days. L1C2 and B16 melanoma cells were maintained in RPMI 1640 containing 10% FBS, GlutaMax and penicillin-streptomycin. C6 glioma cells were maintained in DMEM-F12 medium containing 10% FBS, GlutaMax and penicillin-streptomycin. All cell lines were maintained at 5% CO_2.

L1C2 and HEK293 cells were seeded as monolayers in a 12-well plate and transfected with myc-mRhebL1 plasmid (1 μg) using the Polyfect transfection reagent (Qiagen). Transfection guidelines were followed as indicated in the Polyfect handbook. Cells were harvested 48 hours post transfection for preparation of protein extracts.

Preparation of Lysates and Protein Estimation

Total cell lysates were prepared by suspending the harvested cells in lysis buffer (10 mM Tris, pH 7.5, 130 mM NaCl, 1% Triton, 10 mM NaF, 10 mM NaPi, 10 mM NaPPi and protease inhibitor cocktail (Roche Applied Science) on ice for 30min. Lysates were then centrifuged at 20,000 x g for 15 min. at 4°C and the supernatants removed and frozen at -20°C until used. These supernatants were analyzed via immunoblotting. Protein estimation was done using the Bradford's assay using bovine serum albumin (BSA) as the standard.

Mouse Tissues

Mouse tissues were kindly provided by S. Young (UCLA). Brain, heart, liver, kidney, muscle and

testis were placed in ice-cold buffer (5 ml/g wet weight) containing 250 mM sucrose, 10 mM Tris-HCl, 1 mM disodium EDTA, pH 7.4, and protease inhibitor cocktail.. The tissues were disrupted in a glass homogenization tube (200 strokes) on ice. The homogenates were placed in 1.5-ml tubes and centrifuged at 20,000 × *g* for 10 min. The resulting supernatant fractions were kept frozen until used.

Immunoblot analysis

Equal amounts of protein were loaded on a 12% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. Membranes were immunoblotted with anti-human Rheb1 (Cell Signaling), anti- mouse RhebL1 (Washington Biotech) and anti-myc (Cell Signaling) primary antibodies and anti-Rabbit IgG HRP-linked (Cell Signaling) was used as the secondary antibody. All dilutions of the antibodies were done in phosphate-buffered saline containing 0.1% Tween and 5% milk powder. Antibodies were used at 1000-fold dilution unless otherwise stated. Blots were developed using the ECL Western Blotting Substrate (Pierce) via chemiluminescence.

Peptide competition assays

Anti-RhebL1 (5.4mg/ml) was mixed with Rheb L1 peptide (IARVENSYGRQDRR , 1mg/ml) at various ratios (1:0, 1:5, 1:20 and 1:50 v/v corresponding to 1:0, 1:1, 1:4 and 1:10 w/w) and incubated on a rotator at 4^{o}C for 2h and then at 37^{o}C for 2h. The mixture was used for immunoblotting in competition assays. Anti-RhebL1 (5.4 mg/ml) was mixed with lamin-B peptide (HDNKSQLYTVKYKDGTE, 1 mg/ml, Abcam Inc.) at a 1:5 w/w ratio in a similar fashion.

Microscopy

Images of differentiating 3T3-L1 cells were analyzed using a Nikon Eclipse TE300 Diaphot microscope with Epifluorescence attachment and a CCD camera system (Photometrics, CoolSNAPfx) supported by the MetaMorph software. Specifically fat droplets in undifferentiated as well as differentiated cells were observed eight days post induction.

Results

Generation of Mouse RhebL1 Antibody and its Characterization

Rheb is a highly conserved protein, present in most species from yeast to human. Although it is a popular view that only mammals have two Rheb genes- Rheb1 and RhebL1 (Rheb2) [6] while all other organisms have only Rheb1, here we show that RhebL1 is also present in *Xenopus*, a non-mammalian organism. RhebL1 protein sequence is present in both *Xenopus tropicalis* and *Xenopus laevis* (African clawed frog) and is distinct from the Rheb1 sequence, bearing a 54% identity to human RhebL1 (Figure 1). In addition, a hypothetical protein also exists in *Danio rerio* (zebrafish) which closely resembles human RhebL1 (55% identity). This suggests that RhebL1 is not exclusive to mammals and may have functional significance in lower organisms as well.

A RhebL1 Antibody

Although the expression profile of human RhebL1 mRNA has been reported by several groups [9, 10, 14], results have been conflicting. While Yuan *et al*., [14] report that RhebL1 is expressed in all human tissues using RT-PCR, Saito *et al*., [9] report that RhebL1 is predominantly present in the brain, using northern blotting. Basso *et al.*, [10] reported that RhebL1 mRNA level is elevated in tumor cell lines relative to normal cells. In all cases, the expression profile was determined based on

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Home sapiens            MPLVRYRKVVILGYRCVGKTSLAHQFVEGEFSEGYDPTVENTYSKIVTLG
Mus musculus            MPLVRYRKVAILGYRSVGKTSLAHQFVEGEFLEGYDPTVENTYSKTVTLG
Danio rerio             MPQPKYRKIAVLGYRSVGKSSLTIQFVEGQFVDSYDPTIENTFNKMVSVN
Xenopus tropicalis      MAPVKHRKIVMLGYPSVGKSSLALQFIKGDFPKDYEPTIENTWSKTFVMG
Xenopus laevis          MAPVKHRKIVMLGYPSVGKSSLALQFFKGDFPKDYEPTIENTWSKTFVMG
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Home sapiens            KDEFHLHLVDTAGQDEYSILPYSFIIGVHGYVLVYSVTSLHSFQVIESLY
Mus musculus            KDEFHLHLVDTAGQDEYSILPYSLIIGVHGYVLVYSVNSLRSFQIVKNLY
Danio rerio             GQDFNLQLVDTAGQDEYSIFPQSHSMDIHGYVLVYSVTSMKSFEVVQVLH
Xenopus tropicalis      SDEFELDVVDTAGQDEYSLLPQSFIFGIHGYIVVYSVACSRSFQIARAIH
Xenopus laevis          SDEFELDVVDTAGQDEYSLLPQSFIFGIHGYIVVYSVACSRSFQIARAIH
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Home sapiens            QKLHEGHGKTRVPVVLVGNKADLS-PEREVQAVEGKKLAESWGATFMESS
Mus musculus            QKLHEGHGKTRLSVLLVGNKADLS-PEREVQAVEGKKLAESWGAMFMESS
Danio rerio             DKLLDMVGKIQVPTVLVGNKKDLH-MERVIKPEEGKKLADSWGAAFMESS
Xenopus tropicalis      SILVDRRGKCLMPIVLVGNKNDLPTHCREVKPEDGKKLAESWGAAFLEVS
Xenopus laevis          SILVDKRGKCLMPIVLVGNKNDLPTHCREVKPEDGKKLAESWGAAFLEVS
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Home sapiens            ARENQLTQGIFTKVIQEIARVENSYG-QERRCHLM
Mus musculus            ARDNQLTQDVFIKVIQEIARVENSYGRQDRRCYLM
Danio rerio             AKENQTAVEVFKRIILEMEKVDGNAPSEEKKCAVM
Xenopus tropicalis      AKDPERSKLIFTKMIEEIDRVERSFG-EEKKCCVM
Xenopus laevis          AKDPERSKLIFTKMIEEIDRVERSFD-EEKKCCVM
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Figure 1. Sequence alignment of human, mouse, zebrafish and Xenopus RhebL1 proteins
Alignment was performed using ClustalW program (http://align.genome.jp/sit-bin/clustalw). Accession numbers: Human RhebL1 (NP_653194.1), mouse RhebL1 (NP_081243.1), zebrafish (*Danio rerio*) RhebL1 (NP_001070216.1), *Xenopus tropicalis* RhebL1(NP_988937.1), *Xenopus laevis* RhebL1(NP_001088227.1). Identical residues are marked with an asterisk (*) and conserved residues are marked with (:)

the mRNA and not on the protein itself. To date no study has been conducted to analyze endogenous RhebL1 protein levels in mammalian cell lines or tissues; all studies have been done using plasmid encoded over-expressed RhebL1. It is critical that the expression pattern of the endogenous protein be analyzed to determine whether RhebL1 is in fact tissue specific or not as the limited expression pattern of RhebL1 may highlight its possible role in specific tissues under different conditions. Although a number of antibodies are available against RhebL1 commercially, in our hands most of the antibodies have not been able to detect endogenous RhebL1 and/or have cross-reacted with overexpressed Rheb1 (data not shown). As a result, we sought to develop an antibody against mouse RhebL1 with an intention to detect the endogenous protein and utilize it specifically for RhebL1 with minimal cross-reactivity toward Rheb1.

Mouse Rheb1 and RhebL1 proteins are highly conserved with a 51% identity at the amino acid level (Figure 2a). In order to raise an antibody which is specific for RhebL1 exclusively, the protein sequences were examined to delineate regions of highest variability. The region with least homology was found to be at the C-terminus of the protein. A 14-residue C-terminal peptide spanning from amino acid 167 to 180 was selected as the epitope to generate the antibody. Within this peptide stretch, there is no amino acid identity between the two proteins and hence was deemed most suitable to serve as the antigen (Figure 2a). The antibody was synthesized by Washington Biotechnology Inc. (Maryland) in two New Zealand rabbits. Crude polyclonal serum was tested against purified recombinant Rheb1 and RhebL1

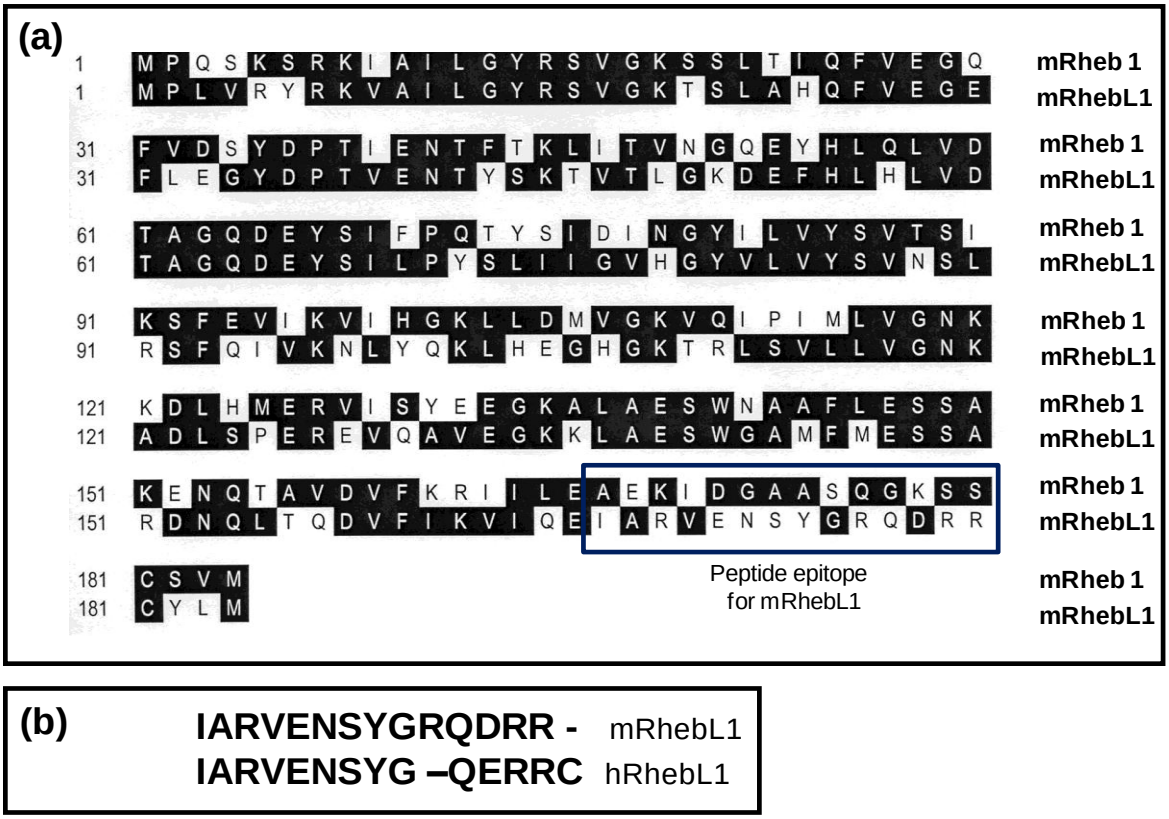

Figure 2. Comparison of mouse and human Rheb sequences

(a) Alignment of mouse Rheb1 and RhebL1 protein sequences. Sequence alignment was done using Clustal program. Accession numbers: mRheb1 (NP_444305.2), mRhebL1 (NP_081243.1). Boxed region shows the mRhebL1 14-residue peptide that was chosen as the epitope for raising the antibody. (b) C-terminal peptide sequences of mouse and human RhebL1 proteins.

proteins as well as other unrelated recombinant proteins and was found to cross-react with most of them in a non-specific manner (data not shown). As a result, the antibody was subject to affinity purification using a peptide column (performed by Washington Biotechnology) and the affinity purified antibody was characterized for its specificity and sensitivity.

Specificity and Selectivity of RhebL1 Antibody

In order to determine its specificity, the affinity purified antibody was tested against purified recombinant mouse MBP-Rheb1 and MBP-RhebL1 proteins. In addition, purified recombinant human, *Drosophila* and *S. pombe* proteins were also tested as potential antigens. As observed in Figure 3a, the antibody did not cross-react with Rheb1. In addition, a variety of purified G-proteins were also analyzed to test whether the antibody cross reacts with other G proteins. Several purified recombinant tagged proteins were used such as Ras, Rho, and Rab which share a 30-35% identity with RhebL1. As shown in Figure 3a, the RhebL1 antibody was found to be highly specific toward the mouse RhebL1 protein exclusively. Surprisingly, the antibody was found not to cross-react with human His-RhebL1 as well. Comparison of the amino acid sequence within the antigenic peptide between the human and mouse RhebL1 proteins revealed an 85% identity (Figure 2b) although gaps were found between the regions of homology. The antibody also did not exhibit

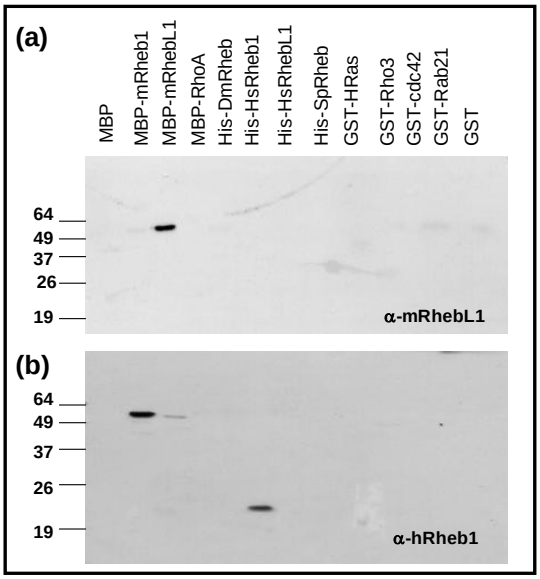

Figure 3. Reactivity of anti-mRhebL1 and anti-hRheb1
Immunoblot of purified fusion or tagged Rheb proteins from various species as well as other recombinant G-proteins fractionated on a 12 % SDS polyacrylamide gel. 1-2 μg of each protein was loaded except MBP-mRheb1 and MBP-mRhebL1 which were 0.5 μg each. Anti-mRhebL1 (5.4 mg/ml) (panel a) and anti-hRheb1 (panel b) antibodies were used at a 1000-fold dilution.

any activity toward non-mammalian Rheb proteins from *Drosophila* (DmRheb) and *S. pombe* (SpRheb). It needs to be stressed, however, that the *Drosophila* and yeast Rheb proteins, which are encoded by single genes, are homologues of mammalian Rheb1. A similar study using an antibody toward the human Rheb1 protein showed that this antibody reacted with both mouse and human Rheb1 purified recombinant proteins and had some cross-reactivity with mouse RhebL1 as well (Figur3b). These results indicate that the antibody generated against mouse RhebL1 is highly specific and selective for mouse RhebL1 alone with no cross-reactivity against Rheb1.

Affinity of RhebL1 Antibody

We were able to show that the mRhebL1 antibody is highly specific and selective toward mouse RhebL1. Next, we examined the affinity of the antibody for RhebL1. In the initial experiments, the antibody was used at a dilution of 1000 fold for detecting 0.5 μg of purified protein. Higher dilutions were subsequently used and its ability to detect the protein (1 μg of purified protein) was analyzed. As shown in Figure 4a, the antibody was found to have an unusually high affinity for its target mRhebL1 protein. The antibody was tested at dilutions ranging from 100,000 to 5000,000 fold and even at the highest dilution, it was found to react with RhebL1. This finding suggests that the titer as well as the affinity of the antibody for RhebL1 is high and that it may serve as a useful tool for detecting endogenous RhebL1, even if it is expressed at low levels, in cell lines/tissues with minimum background reactivity with Rheb1.

Sensitivity of RhebL1 Antibody

After establishing that the antibody has high affinity and specificity for RhebL1, we sought to determine its sensitivity limits. Earlier we had demonstrated that anti-human Rheb1 cross-reacts with mouse RhebL1 protein when the latter is used in the amount of 0.5 μg. To compare the sensitivities of both these antibodies for their target proteins in parallel, varying amounts of MBP-mRheb1 and MBP-mRhebL1 proteins (0.1 to 1 μg) were probed with anti-human Rheb1 and anti-mRhebL1. As observed in Figure 4b, the anti-mRhebL1 was capable of detecting 100 ng of RhebL1 protein while the lowest amount that anti-hRheb1 could detect was 500 ng of Rheb1. Although anti-human Rheb1 cross reacted with mRhebL1, this reactivity was dependent on the amount of mRhebL1. The reactivity was found to decrease as the concentration of the protein was lowered from 1 μg to 0.1 μg.

To define the sensitivity thresholds of anti-RhebL1 further, the concentration of the

antigen protein was lowered to picogram levels and the antibody was tested at various dilutions. As shown in Figure 4c, the antibody could detect as low as 10 ng of mRhebL1 when used at a dilution of a 1000 fold. Thus, its detection limits are governed by a combination of the concentration of the antigen as well as the dilution at which the antibody is used. Detection limits of 10 ng of purified target protein suggest that this antibody may be efficient in reacting with low levels of endogenous RhebL1 protein.

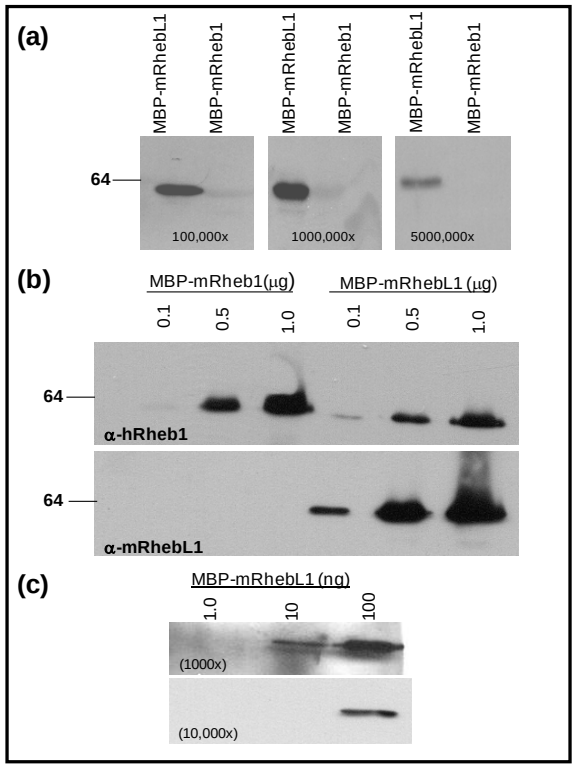

Figure 4. Affinity and sensitivity of anti-mRhebL1.

Immunoblot of purified fusion mouse Rheb1 and RhebL1 recombinant proteins fractionated on a 12 % SDS polyacrylamide gel.

(a) 1 μg of each protein was loaded and anti-mRhebL1 (5.4 mg/ml) was used at a 100,000-fold, 1000, 000-fold and 5000, 000-fold dilutions.

(b) Varying quantities of the proteins were loaded ranging from 0.1 to 1.0 μg. Anti-hRheb1 and anti-mRhebL1 antibodies were used at a 1000-fold dilution.

(c) Varying quantity of the protein was loaded ranging from 1.0 ng to 100 ng. Anti-mRhebL1 was used at 1000-fold and 10, 000-fold dilutions.

The characterization of the specificity, selectivity and sensitivity of the mouse RhebL1 antibody clearly indicates that it may be utilized for detecting endogenous RhebL1 expression without any contaminating cross-reactivity with Rheb1. This finding is of great significance as RhebL1 can be studied exclusively using this antibody.

Detection of Endogenous RhebL1 using anti-mRhebL1

Expression of RhebL1 in Mouse Cell Lines and Tissues

Earlier reports demonstrated that RhebL1 mRNA is expressed primarily in brain tissues [9] and is elevated in tumor cell lines [10]. No studies have been conducted to monitor the protein itself. We analyzed various mouse cell lines with the antibody we raised against RhebL1. Our previous attempts to detect human RhebL1 in human cell lines routinely used in the lab (HEK293, HeLa, etc.) were unsuccessful (data not shown) due to the high specificity of the antibody toward mouse RhebL1 exclusively as shown in the previous observations. Hence, different normal and tumor mouse cell lines as well mouse tissue extracts were acquired and used for detection purposes. 20-25 μg of protein extract was loaded and the antibody was used at a dilution of 5000 fold. As shown in Figure 5a, endogenous RhebL1 could be detected in two cells lines- 3T3-L1 adipocytes (differentiated) and L1C2 lung carcinoma cells. This is the first study reporting the detection of endogenous mouse RhebL1 protein in cell lines and suggests that the antibody is, in fact, capable of reacting with cellular RhebL1. This finding has significant implications as it indicates that RhebL1 has a limited expression pattern and may have specific functions to perform in different cell

lines. 3T3-L1 adipocytes represent a model for adipogenesis [15] and it will be critical to examine whether RhebL1 is expressed in this cell line under all conditions or is an inducible gene in response to adipogenic stimuli. To explore this possibility, we studied this cell line further (as explained in the next section).

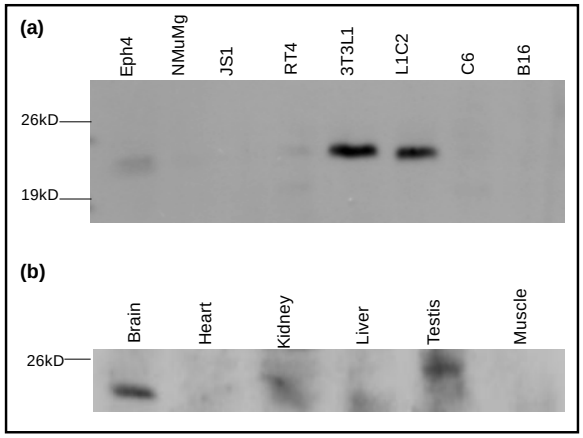

Figure 5. Detection of endogenous RhebL1 in mouse cell lines and tissues.

Immunoblot of total cell lysates prepared from several mouse cell lines and tissues (as described in Materials and Methods) fractionated on a 12 % SDS polyacrylamide gel.

(a) 20 µg of each cell line lysate was analyzed. Anti-mRhebL1 was used at 5000-fold dilution.

(b) 25 µg of each tissue lysate was analyzed. Anti-mRhebL1 was used at 5000-fold dilution.

The detection of RhebL1 in mouse cell lines prompted us to examine mouse tissues in a similar manner. Crude protein extracts from brain, heart, kidney, liver, testis and muscle were probed with anti-mRhebL1. As shown in Figure 5b, a strong reactivity was observed in brain extract with minor reactivity also observed in kidney, liver and testis (a higher molecular weight signal) extracts. Based on the findings of Saito *et al.* [9] who found that RhebL1 mRNA is expressed mostly in the brain we conclude that RhebL1 protein is also predominantly expressed in the brain and the cross-reactivity with other tissues observed in our study is likely to be a background reaction. Thus, this preliminary observation indicates that RhebL1 protein expression is indeed, brain specific.

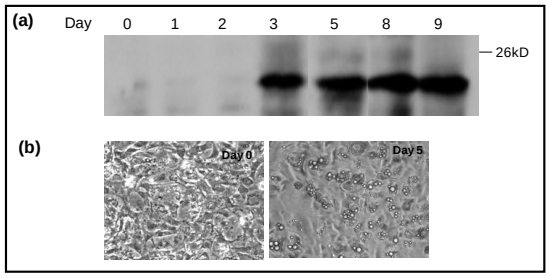

Figure 6. Expression of RhebL1 in differentiating 3T3-L1 cells

(a) 3T3-L1 pre-adipocytes were grown to confluence on a 12-well dish and differentiated into adipocytes as described in Materials and Methods. Cells were harvested and total lysates prepared at various time points. 25 µg of protein was analyzed. Anti-mRhebL1 was used at a 2000-fold dilution. The time points indicate the number of days after treatment of the cells with the differentiation media.

(b) Accumulation of fat droplets in differentiating 3T3-L1 cells.

Induction of RhebL1 in 3T3-L1 Cells

Adipogenesis involves the differentiation of pre-adipocytes into adipocytes in response to differentiation cues. The most well studied model system for this process is the 3T3-L1 fibroblast cell line. These cells are immortalized murine pre-adipocytes which differentiate into mature adipocytes upon appropriate hormonal stimulation and experimental manipulation [15]. These cells are known to express genes which are also expressed in adipocytes *in vivo*. We wanted to determine whether RhebL1 is expressed in both pre-adipocytes as well as differentiated adipocytes or whether it follows a specific expression pattern. 3T3-L1 cells were grown in DMEM containing 10% calf bovine serum to full confluence (48 h post seeding) and then differentiated as described in materials and methods. Briefly, media was changed to DMEM containing 10% fetal bovine serum (providing a richer source of growth factors) and the cells treated with a differentiation cocktail containing 0.5 mM methylisobutylxan-

thine (MIX), 0.5 μM dexamethasone (DEX) and 5 μg/ml insulin. 48h later, media was changed to DMEM containing 10% FBS and insulin without DEX and MIX and fresh media was replaced every 48 h until day 9 post differentiation. The day when the media was changed to differentiation media is denoted as day 0. As shown in Figure 6a, RhebL1 expression was first detected three days after the cells were treated with differentiation media and was continuously observed nine days post differentiation. Differentiation of pre-adipocytes into adipocytes involves the accumulation of triglyceride lipid droplets as the differentiation progresses (Figure 6b). Although RhebL1 was found to be expressed in adipocytes and not in pre-adipocytes, we observed that the level of protein remained the same as differentiation progressed further. In other words, RhebL1 levels were not found to increase with increased fat accumulation in this initial study. We are presently addressing this issue in greater detail to monitor whether RhebL1 expression does in fact, correlate with differentiation in a time dependent manner. Our study is the first to show that RhebL1 is inducible by adipogenic factors and may be involved in adipogenesis. However, it is too early to ascertain whether RhebL1 is a gene which has a causative effect in the process of adipogenesis itself or whether it is a gene which is responsive to the signaling cascade which ensues during adipogenesis such as transactivation from PPARγ peroxisome proliferator activated receptor gamma [16]. Nevertheless, it will be interesting to determine the exact role of RhebL1 in these cells.

Size of Endogenous RhebL1

The theoretical molecular size of the mouse RhebL1 protein based on its amino acid sequence is 20.4kD, hence we expected to observe a signal close to the 19kD protein marker. However, close examination of the endogenous RhebL1 in 3T3-L1 and L1C2 cells as well as in brain extracts revealed that the signal is approximately 23kD (Figures 5a, 5b and 6a). In order to determine whether this signal is in fact corresponding to RhebL1 and not a non-specific cross reactivity, we performed competition assays using RhebL1 specific peptide. Our aim was to observe whether this signal in L1C2 cells disappears after the antibody has been pre-absorbed by the RhebL1 specific peptide. As a control, we also transfected myc-mRhebL1 into HEK293 cells and used the lysates for comparing the size of the myc-tagged exogenously expressed protein with the native endogenous version. In addition, we also used the purified recombinant MBP-mRhebL1 protein. For the peptide competition assays, the antibody was pre-incubated with the RhebL1 peptide at 4^{o}C for 2h followed by 2h at 37^{o}C and then utilized for probing the blots at 1000-fold dilution. The antibody was mixed with the peptide at various concentrations (1:0, 1:5, 1:20 and 1:50 v/v corresponding to 1:0, 1:1, 1:4 and 1:10 w/w respectively, antibody: peptide ratios). As observed in Figure 7a, the endogenous RhebL1 in L1C2 is larger in size (~23kD) compared to the myc-mRhebL1 expressed in HEK293 cells (~19kD). The signal at 23kD disappears when the antibody is competed away by the peptide in increasing concentrations suggesting that this protein does correspond to RhebL1 and is not a non-specific cross-reaction. The positive controls- myc-mRhebL1 and MBP-mRhebL1 are also competed away by the peptide. In addition, the antibody is only competed away by the RhebL1 peptide and not an unrelated lamin-B peptide (Figure 7b). These results indicate that endogenous RhebL1 may likely be modified by a post-translational modification which increases its

molecular size by approximately 2-3kD. To further confirm our findings, we over-expressed myc-mRhebL1 in L1C2 cells to determine whether we can observe signals at two different molecular weights - one at ~23kD corresponding to endogenous RhebL1 and an additional one corresponding to over-expressed myc-mRhebL1. Earlier, expression of myc-mRhebL1 (Figure 7) in HEK293 cells yielded only the 19kD signal because the antibody is highly specific for the mouse protein and does not react with the endogenous human protein in

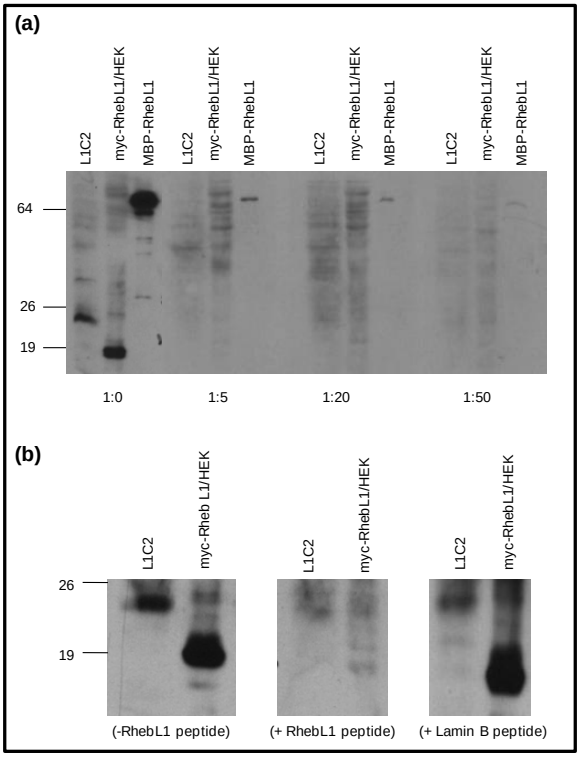

Figure 7. Peptide competition assays

(a) Extracts of HEK293 cells transfected with myc-RhebL1 and L1C2 cells were probed with anti-mRhebL1 in the absence and presence of competing RhebL1 peptide. Anti-RhebL1 (5.4 mg/ml) was pre-incubated with RhebL1 peptide (IARVENSYGRQDRR, 1 mg/ml) at various dilutions (1:0, 1:5, 1:20 and 1:50 v/v) and was used at 1000-fold dilution.

(b) Extracts of HEK293 cells transfected with myc-mRhebL1 and L1C2 cells were probed with anti-mRhebL1 which had been pre-incubated either with a competing RhebL1 peptide (1 mg/ml, IARVENSYGRQDRR) or a non-competing Lamin B peptide (1 mg/ml, HDNKSQLYTVKYKDGTE) peptide at a ratio of 1:20, v/v (Antibody: Peptide).

HEK293 cells. As evident in Figure 8a, there are in fact two signals observed in L1C2 cells upon transfection with myc-mRhebL1- one at ~19kD corresponding to the over-expressed myc-RhebL1 and the other at ~23kD corresponding to the endogenous version of RhebL1. The signal at ~19kD corresponds to the exogenously supplied RhebL1 as evidenced by its reactivity with the myc antibody (Figure 8b). The signal at ~23kD is observed in both untransfected and transfected

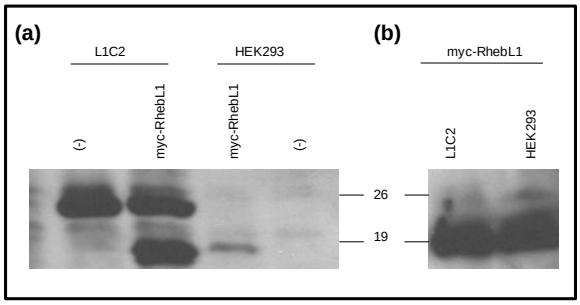

Figure 8. Assessing the molecular size of endogenous mouse RhebL1

Immunoblot of total cell lysates prepared from L1C2 cells and HEK293 cells untransfected or transfected with myc-mRhebL1 and fractionated on a 12 % SDS polyacrylamide gel. (20 µg of protein was loaded). Antibodies were used at 1000-fold dilution. (a) Extracts probed with anti-mRhebL1. (b) Extracts probed with anti-myc.

L1C2 cells but not in HEK293 cells as the antibody is capable of reacting only with the mouse protein and not the human counterpart. These observations suggest that the size of the endogenous mouse RhebL1 protein, at least in L1C2 cells, is larger than that deduced from its coding sequence and that RhebL1 may be subject to post-translational modification in these cells. Assuming that such a modification does occur, an intriguing question for us was then to determine why the over-expressed myc-RhebL1 was itself not getting modified. Our hypothesis is that since the myc-tag was placed at the N-terminus of RhebL1, it is likely that the N-terminus of RhebL1 may be getting modified

and the modification site may be getting masked by the myc tag. These observations obviously warrant a closer examination of the RhebL1 protein in mammalian systems and may imply a unique regulation of RhebL1 via a post-translational modification.

Discussion

The mammalian insulin/mTOR pathway has recently garnered tremendous interest as the key player of this cascade- mTOR has been shown to be responsible for controlling a wide variety of processes including cell growth, translation and nutrient sensing [17]. The Ras homolog - Rheb has been reported to activate mTOR and thus, play a key regulatory role. In mammals, two genes exist for Rheb which we classify as Rheb1 and RhebL1. RhebL1 has also been shown to play a similar role in the insulin pathway as evidenced by its ability to activate S6K [7], although it has not yet been shown to be a direct target of the TSC1/TSC2 complex as has been demonstrated for Rheb1 [18]. Most studies in mammalian cells have been conducted using over-expressed Rheb1 and RhebL1 proteins which do not represent the endogenous protein behavior in the true sense. No study has yet been conducted which explores the differences between the two proteins encoded by these genes primarily due to the lack of suitable antibodies specific for each protein. We were interested in studying the endogenous RhebL1 protein and determine whether it differs significantly from Rheb1. We chose to synthesize an antibody toward the mouse RhebL1 protein due to the easier availability of mouse tissues and ease of *in vivo* studies. In this report, we show the successful generation of a mouse RhebL1 antibody and its characterization. The antibody has been determined to be highly specific for RhebL1 with no cross-reactivity with Rheb1, which is extremely useful to monitor the individual differences between the two proteins in terms of their expression pattern and regulation.

The antibody was characterized extensively using purified recombinant proteins in terms of its specificity, selectivity and sensitivity toward the target protein. The antibody could react with mouse RhebL1 but not human RhebL1 which suggests that a very small change in the peptide epitope (as observed between the mouse and human RhebL1 sequences) can confer unusual affinity for species specific target. The antibody was subsequently utilized for the detection of endogenous protein using several mouse cell lines. RhebL1 was detected in a limited number of cell lines suggesting that its expression may be subjected to regulation depending on the cell type. Two particular cell lines, namely the adipocyte 3T3-L1 fibroblasts and lung carcinoma L1C2 cell line showed expression of RhebL1. We were also able to show that the protein was found to be predominantly expressed in mouse brain tissues.

The regulation of Rheb1 and RhebL1 may be quite different despite having a similar function in the insulin/mTOR pathway, as evidenced by their differential pattern of expression. It is not known whether RhebL1 has additional functions distinct from those of Rheb1 in different tissues. We were able to show that RhebL1 expression is regulated in 3T3-L1 adipocyte cells. RhebL1 was detectable upon the differentiation of the pre-adipocytes to adipocytes suggesting that its expression may be regulated directly by adipogenic factors or indirectly by transactivation mediated by transcription factors such as the nuclear hormone receptor-PPARγ, whose role in the process of adipogenesis has been very well studied. We

are presently studying the RhebL1 expression in both 3T3-L1 and L1C2 cells under various conditions in a time-dependent manner.

This study thus, provides evidence of the generation of a highly specific antibody for mouse RhebL1 and its ability to detect endogenous RhebL1 without cross reacting with Rheb1. We envisage that this antibody could serve as a suitable reagent to study RhebL1 in terms of its localization and regulation and provide valuable insights into its function.

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