A novel approach for expression cloning of small GTPases: identification, tissue distribution and chromosome mapping of the human homolog of *rheb*

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Abstract **We report a novel approach for identifying monomeric GTP-binding proteins that is based on probing cDNA expression libraries with [α-^{32}P]GTP. In short, a nitrocellulose replica from a plated cDNA expression library is treated with 2% SDS to block the GTP-binding activity of various G proteins expressed by *E. coli*, thus allowing the direct identification of positive clones. Using this procedure we have cloned several small GTP-binding proteins from human keratinocytes including the human homolog of *rheb*, a novel member of the *ras*-related GTP-binding proteins. Human *rheb* cDNA shares 90% identity with the rat counterpart and it is highly upregulated in transformed human cells of various origin. Northern analysis showed that human *rheb* is ubiquitously expressed, with the highest levels observed in skeletal and cardiac muscle, and not in brain, as it is the case for rat *rheb*. The human *RHEB* gene was mapped to chromosome 10q11.**

Key words: ras-related GTPase; GTP-binding blot overlay assay; Northern blot hybridization

1. Introduction

Small *ras*-related GTP-binding proteins constitute a surprisingly large superfamily of monomeric regulatory proteins that play key roles in cell proliferation and differentiation [1–3], cytoskeletal organization [4,5] and protein transport [6,7]. Most of these proteins have been identified by low stringency hybridization of cDNA libraries with probes derived from previously cloned *ras*-like proteins, mainly ras p21. To date, over 40 different *ras*-related GTP-binding proteins from mammalian cells have been cloned and sequenced [8], and more are likely to be discovered. Given the high sequence similarity among members of the *ras* superfamily, however, efforts to reveal novel GTPases by screening cDNA libraries with *ras*-derived probes may not be so effective, particularly, if novel members are distant from those already known.

An alternative approach for detecting low molecular weight GTP-binding proteins in eukaryotic cells is provided by the nitrocellulose blot overlay assay with [α-^{32}P]GTP as a ligand [9–11]. By using this assay we have detected more than 40 low molecular weight GTP-binding proteins in normal human keratinocytes, determined their relative abundance in normal proliferating and SV40 transformed keratinocytes (K14), and identified several unknown GTPases that are strongly down-regulated in the transformed cells and that may be required for the maintenance of the normal phenotype [10]. Based on the same approach, a 2D gel database of many cloned *ras*-like GTPases has been established by Huber et al. [11]. The number of small GTPases that can be revealed by this technique is limited however by the low abundancy of these proteins.

The main obstacle hampering the application of the GTP-binding blot overlay assay to the screening of expression libraries has been the presence in *E. coli* of various proteins, such as for example, elongation factors Tu and G, initiation factors as well as other proteins that in native form bind GTP in the blot overlay assay, thus giving a very high background on the nitrocellulose replica from a plated cDNA library [12]. To overcome this problem, we thought to take advantage of the fact that small GTPases comprising the *ras* superfamily are able to bind GTP following denaturation with sodium dodecyl sulfate (SDS) and transfer to nitrocellulose membranes [9,11,13]. Based on the above premises we have developed a novel and simple approach for the identification and cloning of low molecular weight GTP-binding proteins. Using this procedure we have cloned several GTP-binding proteins including the human homolog of rheb, a low abundance protein that may play a role in long-term activity dependent neuronal responses in rat brain [14].

2. Materials and methods

2.1. Materials

Hybond N- and C membranes, [α-^{32}P]GTP (3000 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were purchased from Amersham. The cloning kit was obtained from Stratagene. The multiple tissue northern blot was purchased from Clontech.

2.2. Expression of small GTP-binding proteins in E. coli

Recombinant small GTP binding proteins were expressed by using standart procedure as described [15]. The culture was grown at 37°C to an A_{600} of about 0.4. Thereafter, isopropyl thio-β-D-galactoside (IPTG) was added to a final concentration of 1 mM and the culture was continued to grow for 2 h at 37°C. Bacteria were lysed by sonication and the extracts were analysed by dot-blot and by the [α-^{32}P]GTP blot overlay assay.

2.3. cDNA expression library

An oligo-dT primed expression cDNA library was constructed in

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Abbreviations: GTP, guanosine 5′-triphosphate; DTT, dithiothreitol; IPTG, isopropyl thio-β-D-galactoside.

The sequence of human *rheb* was submitted to the EMBL/GeneBank databases with accession number Z29677.

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phage λ ZAPII (Stratagene) using poly(A)$^+$ RNA from whole human epidermis [16]. RNA was prepared using the guanidin isothiocyanate/CsCl method [15], and was purified using 'pushcolumns' (Stratagene). cDNA was prepared according to Gubler [17] and prior to ligation it was size fractionated on a low melting agarose gel: 200–600 bp, 600–2000 bp and above 2000 bp. The primary cDNA library was amplified according to the manufacturer's description (Stratagene).

2.4. Screening of the cDNA expression library with [α-^{32}P]GTP

Amplified phages (600–2000 bp) were plated on 500 cm^2 dishes at a density of approximately 125,000 pfu per plate, using the bacterial strain LE 392 (Stratagene). When plaques were visible, they were overlaid with nitrocellulose membranes (Amersham) for 12 h. Prior to overlay, the membranes were soaked in 10 mM IPTG and dried. Thereafter, the nitrocellulose replica was washed two times in a solution containing 50 mM Tris-HCl, 0.3% Tween 20 or NP40, pH 7.5 to remove the attached bacterial lawn and to reduce the high viscosity resulting from bacterial lysis. The filters were then incubated in 2% SDS/100 mM DTT for 15 min at 50°C. The replica was then washed three times with 25 mM Tris/190 mM glycine buffer, pH 8.3 containing 20% methanol (25 min per wash) to remove SDS, and subjected for [α-^{32}P]GTP blot overlay assay as described below [10,18]. Following removal of the nitrocellulose filters from the plates, replicas were taken using nylon membranes which were then used for hybridization with previously identified cDNAs. Several random labeled cDNAs were hybridized at the same time. The corresponding DNA hybridization autoradiographs were then superimposed with the ^{32}P X-ray film containing all clones revealed by [α-^{32}P]GTP-binding assay. After picking the first round of clones, the plates were placed at −70°C for subsequent isolation of additional clones.

2.5. [α-^{32}P]GTP-binding assay

[α-^{32}P]GTP blot overlay assay was performed as described [10,18]. Briefly, the nitrocellulose filters were rinsed twice with a solution containing 50 mM Tris-HCl, pH 7.4, 0.3% Tween 20 and 10 μM $MgCl_2$, and then incubated for 60 min in the same buffer containing 100 mM DTT, 100 μM ATP and 1.0 μCi of [α-^{32}P]GTP/ml. The nitrocellulose filters were then washed several times in the same buffer lacking [α-^{32}P]GTP, DTT and ATP for 30 min, dried and exposed for autoradiography for 3 to 72 h at −70°C with an intensifying screen. A fraction (up to 20) of the positive clones was subcloned and full length cDNAs were isolated and sequenced to assess their identity.

2.6. DNA sequencing

Cloned cDNAs were rescued as a Bluescript plasmids using the automatic excission process of λ ZAP. The *Xho*I/*Not*I fragments were cloned into M13mp20 and sequenced in both directions by generating of *Bal*31 deletion fragments which were subfractionated on a low melting agarose gel and subcloned into M13mp20 or 21 vectors.

2.7. Northern blot analysis

RNAs from various human tissues and transformed cell lines were prepared as described [19]. Total RNA was denatured, fractionated on a 1.2% agarose-formaldehyde gel and transferred onto a nylon membrane as described [20]. ^{32}P-labeled cDNA was generated by random priming and used for hybridization. mRNA tissue distribution was analysed using a multiple tissue Northern blot obtained from Clontech.

2.8. Chromosome mapping of the human rheb gene (RHEB)

The full length *rheb* cDNA clone was used directly for fluorescence in situ hybridization (FISH) as previously described [21].

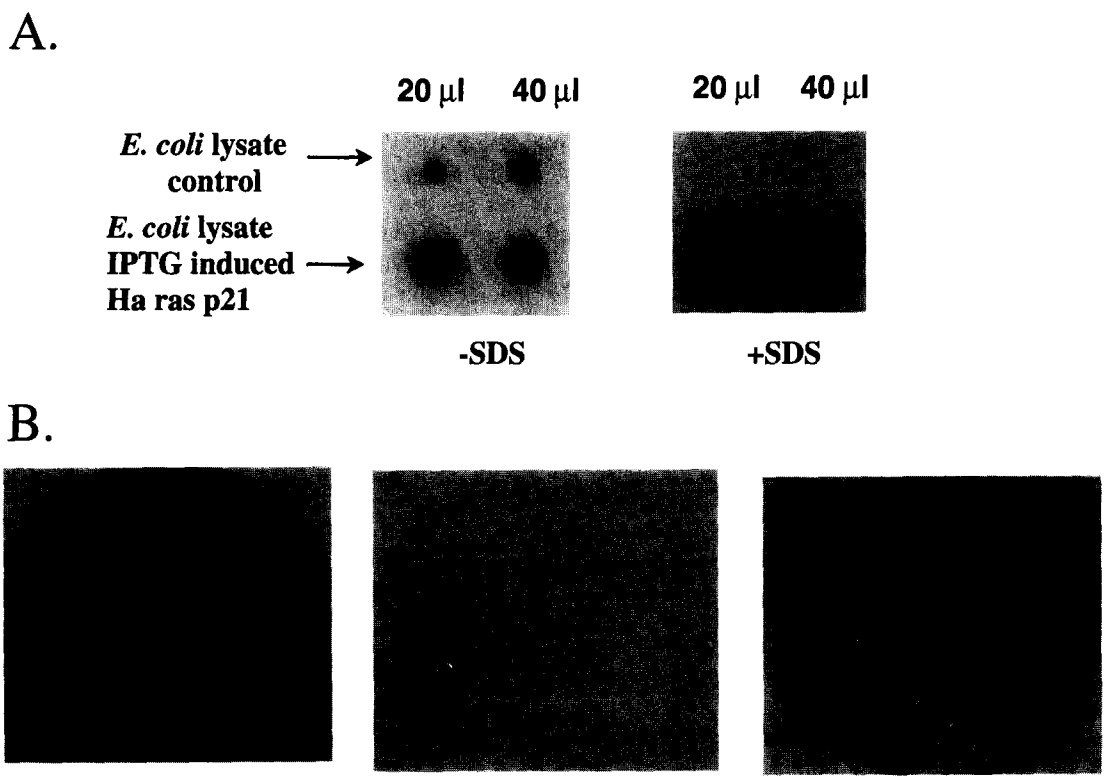

Fig. 1. (A) [α-^{32}P]GTP-binding activity of *E. coli* extracts blotted on nitrocellulose filters. Equal amounts (20 and 40 μl) of control (upper dots) and IPTG-induced Ha-ras p21 transformed bacterial lysates (bottom dots) were dot blotted to nitrocellulose membranes. Right panel: The blot was treated with 2% SDS, washed, renatured with 0.3% Tween 20 and subjected to [α-^{32}P]GTP blot overlay assay. Left panel: untreated, control blot. (B) Treatment of nitrocellulose replicas from plated λ ZAPII library with 2% SDS/100 mM DTT drastically decreases the background and allows the identification of clones overexpressing small *ras*-like GTPases using the [α-^{32}P]GTP ligand blotting procedure. Only a fraction of the library was plated in a few Petri dishes. (1) Nitrocellulose membrane laid on the plated library and incubated with [α-^{32}P]GTP in the absence of SDS/DDT treatment. This particular plate had no positive plaques as shown in (2). The exposure time for autoradiography was 3 h. (2) Replica from the plate shown in (A) but incubated with 2% SDS and washed with 20% methanol prior to incubation with [α-^{32}P]GTP. The X-ray film was exposed for 48 h. (3) Replica from a plate containing plaques with GTP-binding activity (Ha-ras p21) treated as in (2) and exposed to an X-ray film for 16 h.

3. Results

3.1 The [α-^{32}P]GTP blot overlay assay for probing of expression cDNA libraries

To examine the effect of SDS denaturation on the ability of various bacterial G proteins to bind GTP, *E. coli* cells were lysed by sonication and the extracts were dot blotted on nitrocellulose membrane. Following SDS treatment and renaturation with nonionic detergent (NP40 or Tween 20) the dot blots were probed with [α-^{32}P]GTP. As shown in Fig. 1A, treatment of the blots with 2% SDS almost completely abolished the GTP-binding activity of bacterial G proteins, but had no major effect on the GTP-binding activity of recombinant Ha-ras p21 overexpressed in *E. coli*.

To test if the GTP overlay assay modified as described above could be used to screen expression cDNA libraries we treated the nitrocellulose replica of a plated λ ZAPII library of human psoriatic keratinocytes with 2% SDS/100mM DTT at 60°C for 10 min to denature *E. coli* GTP-binding proteins. Blots were then washed with 20% methanol prior to [α-^{32}P]GTP ligand blotting (see section 2). As shown in Fig. 1B, the detergent treatment resulted in a drastic reduction of the extensive binding of *E. coli* nucleotide binding proteins, and allowed the detection of IPTG induced GTP-binding activity of the clones overexpressing various small *ras*-like GTPases. Moreover, by using high concentrations of ATP (up to 100 μM) as an effective competitor for nonspecific GTP-binding to the nitrocellulose membrane it has been possible to further reduce the background and thus improve the signal-to-noise ratio [10,18].

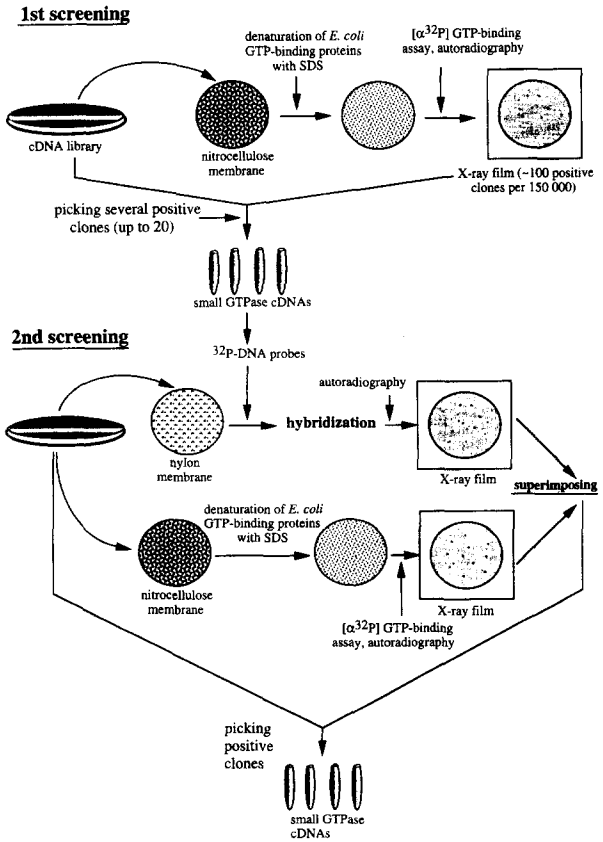

Fig. 2. Schematic diagram of the strategy for screening expression libraries.

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1                                   M  P  Q  S  K  S  R  K  I  A  I  L
1   CCGGGGCTGAGGAGGCCGCCAAGATGCCGCAGTCCAAGTCCCGGAAGATCGCGATCCTG

13  G  Y  R  S  V  G  K  S  S  L  T  I  Q  F  V  E  G  Q  P  V
60  GGCTACCGGTCTGTGGGGAAATCCTCATTGACGATTCAATTTGTTGAAGGCCAATTTGTG

33  D  S  Y  D  P  T  I  E  N  T  F  T  K  L  I  T  V  N  G  Q
120 GACTCCTACGATCCAACCATAGAAAACACTTTTACAAAGTTGATCACAGTAAATGGACAA

53  E  Y  H  L  Q  L  V  D  T  A  G  Q  D  E  Y  S  I  F  P  Q
180 GAATATCATCTTCAACTTGTAGACACAGCCGGGCAAGATGAATATTCTATCTTTCCTCAG

73  T  Y  S  I  D  I  N  G  Y  I  L  V  Y  S  V  T  S  I  K  S
240 ACATACTCCATAGATATTAATGGCTATATTCTTGTGTATTCTGTTACATCAATCAAAAGT

93  F  E  V  I  K  V  I  H  G  K  L  L  D  M  V  G  K  V  Q  I
300 TTTGAAGTGATTAAAGTTATCCATGGCAAATTGTTGGATATGGTGGGGAAAGTACAAATA

113 P  I  M  L  V  G  N  K  K  D  L  H  M  E  R  V  I  S  Y  E
360 CCTATTATGTTGGTTGGGAATAAGAAAGACCTGCATATGGAAAGGGTGATCAGTTATGAA

133 E  G  K  A  L  A  E  S  W  N  A  A  F  L  E  S  S  A  K  E
420 GAAGGGAAAGCTTTGGCAGAATCTTGGAATGCAGCTTTTTTGGAATCTTCTGCTAAAGAA

153 N  Q  T  A  V  D  V  F  R  R  I  I  L  E  A  E  K  M  D  G
480 AATCAGACTGCTGTGGATGTTTTTCGAAGGATAATTTTGGAGGCAGAAAAAATGGACGGG

173 A  A  S  Q  G  K  S  S  C  S  V  M  End
540 GCAGCTTCACAAGGCAAGTCTTCATGCTCGGTGATGTGATTCTGCTGCAAAGCCTGAGGA
                                    ***
600 CACTGGGAATATATTCTACCTGAAGAAGCAAACTGCCCGTTCTCCTTGAAGATAAACTAT
660 GCTTCTTTTTTCTTCTGTTAACCTGAAAGATATCATTTGGGTCAGAGCTCCCCTCCCTTC
720 AGATTATGTTAACTCTGAGTCTGTCCAAATGAGTTCACTTCCATTTTCAAATTTTAAGCA
780 ATCATATTTTCAATTTATATATTGTATTTCTTAATATTATGACCAAGAATTTTATCGGCA
840 TTAATTTTTCAGTGTAGTTTGTTGTTTAAAATAATGTAATCATCAAAATGATGCATATTG
900 TTACACTACTATTAACTAGGCTTCAGTATATCAGTGTTTATTTCATTGTGTTAAATGTAT
960 ACTTGTAAATAAAATAGCTGCAAACCTC polyA 987
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Fig. 3. Nucleotide sequence of human *rheb*. The cDNA consists of 987 bases with a polyA tail. The deduced amino acid sequence is shown above the encoding region from the intiation codon at positions 24–26 (bold typed) to the termination codon at positions 577–579 (asterisks). The polyadenylation signal AATAAA is underlined.

Since the expression cloning procedure is based on the functional property of small GTPases to bind GTP, the only limitations lie on the degeneracy and representation of the cDNA library. To partly circumvent these problems and to avoid the repetitive picking of the same clones we proceeded as follows (Fig. 2). After plating the library, only a fraction (up to 20) of the positive clones was subcloned and full length cDNAs were isolated and sequenced to assess their identity. Next, the cDNA expression library was replated and, following [α-^{32}P]GTP probing, the plaques were transfered to a nylon filter and hybridized – under high stringency – with the labeled cDNA probes identified in the previous step. By superimposing the two X-ray films it was possible to deduct the clones identified at each step by [α-^{32}P]GTP binding. Clones having high homology, or identical to those detected at previous steps, were not considered in further screenings. The screening of the plated expression library with both independent probes, homologous DNAs and [α-^{32}P]GTP, enhanced considerably the effectiveness of the screening.

3.2. Identification of the human homolog of rheb

Using the strategy described above we carried out a systematic screening of a λ ZAPII expression library from human keratinocytes which yielded up to 100 positive clones per 125,000 colonies. DNA sequence analysis of some of the isolated clones has so far identified several known low molecular weight GTP-binding proteins including Ha-ras p21, human analogs of rac1, rab11, ypt1 and rab5b – which belong to different subgroups of the *ras*-superfamily – and the human ADP ribosylation factor(s) (results not shown). The validity of the approach was also confirmed by the identification of the human homolog of *rheb* [14], a novel low abandance small GTP-bind-

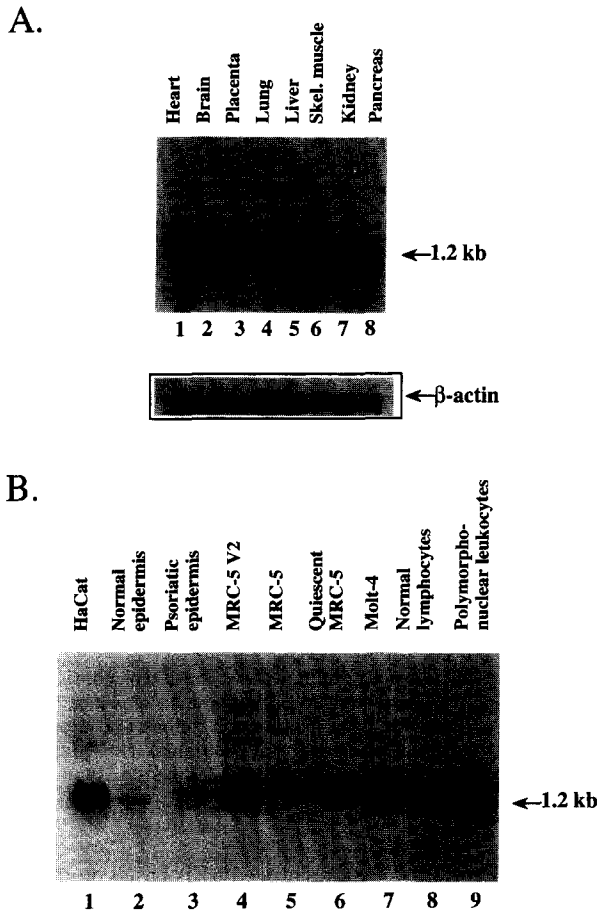

Fig. 4. (A) Transcripts of *rheb* gene in adult human tissues. Multiple tissue northern blot (Clontech) containing 2μg of pure poly A⁺RNA per line isolated from human tissues was consecutively hybridized with ^{32}P-labeled *rheb* cDNA (panel 1) and β-actin control probe (panel 2) under high stringency. The samples were: heart (1), brain (2), placenta (3), lung (4), liver (5), skeletal muscle (6), kidney (7), pancreas (8). (B) Northern analysis of rheb gene expressed in several transformed human cells. Total RNAs were isolated from different human transformed cell lines and their normal counterparts: HaCat (1); normal epidermis (2); psoriatic epidermis (3); SV40 transformed embryonic lung fibroblasts (MRC-5 V2) (4); normal proliferating embryonic lung fibroblasts (MRC-5) (5); quiescent MRC-5 (6); Molt-4 (7); normal lymphocytes (8); polymorphonuclear leukocytes (9). The RNAs (10 μg per line) were denatured, electrophoresed through an agarose-formaldehyde gel, transferred to a nylon membrane and hybridized with the ^{32}P-labeled *rheb* cDNA.

ing protein, which may belong to a hitherto unknown branch of the *ras* superfamily.

The full length copy of the human *rheb* clone (987 bp) was sequenced in both direction and found to share 90% identity with a cDNA coding for the rat counterpart [14]. The deduced protein sequence of 184 amino acid residues is almost identical in human and rat with the exception that Met170 in human is replaced by Ile170 in rat (Fig. 3). As in the case of rat *rheb*, the human homolog exhibited Arg-Ser at positions 15–16 (corresponding to positions 12 and 13 in ras), two amino acid replacement that are only found in oncogenic Ha-ras p21 mutants [22]. The possibility that these amino acid substitutions are due to the fact that we screened a cDNA library from psoriatic keratinocytes (a hyperproliferative disease) has been excluded as a cDNA clone isolated from normal human keratinocytes by probing with *rheb* exhibited an identical sequence in the coding region, with the exception of one base substitution (codon 164 (Ile): ATT→ATC) (result not shown).

3.3. *Differential expression of rheb gene in normal and transformed human cells*

Northern analysis of poly A⁺ RNA isolated from various human tissues was performed using a commercial blot from Clontech containing equal amounts (2 μg per line) of RNA from several organs: heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas. The levels of RNA applied to each line were confirmed using β-actin as a probe. Fig. 4A shows that the *rheb* gene is expressed in all adult human tissues as one transcript of about 1.2 kb (high stringency) in agreement with data obtained for rat *rheb* [14]. The highest levels of *rheb* mRNA were found in skeletal and cardiac muscle and the lowest in lung and liver very much resembling the expression pattern of Ha-ras p21 in adult mouse tissues [23]. We have also shown that the *rheb* gene is transcribed in human epidermis, lymphocytes and polymorfonuclear leukocytes (Fig. 4B), suggesting that it is ubiquitiously expressed in human cells.

The levels of *rheb* transcripts in several normal and transformed human cells were also determined by northern hybridyzation. Total RNAs were prepared from the transformed cells and their normal counterparts: these included the keratinocyte cell line HaCat (control, normal and psoriatic epidermis), SV40 transformed embryonic lung fibroblasts MRC-5 V2 (control, normal proliferating and quiescent MRC-5) and Molt-4 (control, normal lymphocytes). Equal amounts of RNA were fractionated on a 1.2% agarose-formaldehyde gel, blotted onto nitrocellulose and hybridized under high stringency conditions (Fig. 4B). Taken together, the results indicated that the

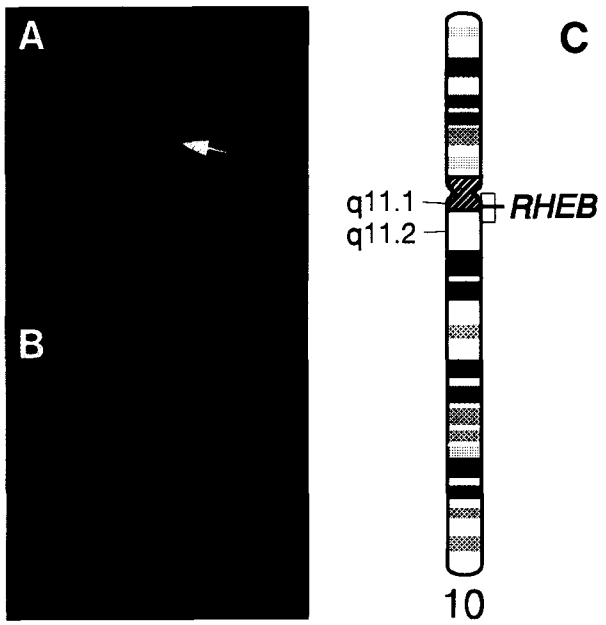

Fig. 5. FISH mapping of RHEB to 10q11.1–q11.2, with (A) a partial metaphase with specific signals on the chromosome 10 (arrow), (B) the corresponding DAPI-stained chromosomes and (C) a digitized chromosome diagram with the location of RHEB according to mean Flpter value. The horizontal box indicates the variation on individual chromosomes.

rheb transcript is several folds upregulated in the transformed cells as compared to their normal counterparts.

3.4. Chromosome mapping of human RHEB

Human genomic DNA digested with several restriction enzymes showed only one fragment hybridizing under high stringency with *rheb* both in *Bgl*II and *Bam*HI digests and two bands in the case of *Hin*dIII. Since the *rheb* exon contains one *Hin*dIII site, we concluded that there is only a single-copy RHEB gene in the human genome, a fact that was further confirmed by quantitative comparison with Ha-*ras* p21 (data not shown). In addition, only one consistent signal was observed by FISH analysis, corresponding to the very proximal region of 10q, with 24 of 50 analysed metaphases (48%) expressing at least one specific signal, and with 76 of the chromatids (38%) being labelled. The Flpter value based on measurments of 12 signal-bearing chromosomes was 0.37 ± 0.03, corresponding to a location within 10q11.1–q11.2 (Fig. 5).

4. Discussion

Using a simple expression cloning/screening procedure we have identified the human homolog of *rheb*, a novel member of the *ras*-superfamily that is present in less than 20,000 molecules per cell in normal human keratinocytes ([24], unpublished observation), and that exhibits interesting amino acid residues at positions 15 (Arg) and 16 (Ser) that correspond to residues 12 and 13 in ras. Considering that the Gly12→Arg12 mutation has been implicated in *ras*-associated carcinogenesis [22,25,26], the occurrence of Arg at this position in *rheb* is intriguing. The oncogenic potential of the Gly12→Arg12 substitution is thought to be due to the guanidinium group of Arg12 taking the position of Wat175, thus physically blocking the access to the γ-phosphate and inhibiting GTP hydrolysis [27]. In vitro studies have shown that the replacement of the normal Gly at position 12 with a wide range of amino acids has the potential to reduce the intrinsic GTPase activity [28] and sensitivity to effector molecules (GAP and neurofibromin) of *ras* p21 [29, 30]. So far, this particular amino acid replacement has not been observed in *rho, ypt/rab* and *ran/tc4;* in these subgroups variations in this motif (codons 12 and 13) are relatively limited: Gly-Gly in *ras* and *ran/tc4*; Gly-Ala in *rho* and Ser(Thr, Ala or Gln)-Gly(Ser or Ala) in *ypt/rab*. Accordingly, *rheb* may provide with an interesting model for further studies concerning (i) the role of the glycine-12 residue (also, glycine-13 as well) on the structure of the GTP-binding site and (ii) the mechanisms underlying GTP hydrolysis, which may differ in details between various small GTPases.

Presently, we have no information as to the function of *rheb*, although the fact that it exhibits interesting sequence features and that its mRNA is strongly upregulated in transformed human cell lines underlie an important role in cell proliferation and/or transformation. This is in line with published data showing that the rat homolog behaves as an immediate-early gene in fibroblasts [14]. In addition, rat *rheb* has been shown to be enriched in brain and to be regulated by synaptic activity suggesting a putative role in neuronal responses [14]. Our studies concerning the destribution of *rheb* mRNA in human tissues indicate that its levels are much higher in skeletal and cardiac muscle as compared to brain implying that the human homolog may have other yet unknown functions.

Finally, we would like to emphasize that the expression cloning/screening procedure we have developed may serve as a powerful means for identifying novel small GTPases that may escape detection by cDNA hybridization with homologous probes or by the direct analysis of the proteins using [α-^{32}P]GTP blot overlay assay. Identification of the full complement of GTPases in a given cell type will provide new insights into the diverse spectrum of cellular processes orchestrated by these proteins as well as to the 'crosstalk' between various members of the *ras* superfamily.

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