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Isolation and characterisation of the human rab18 gene after stimulation of endothelial cells with histamine

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Abstract Here we report the isolation of a cDNA encoding the complete human rab18 protein from histamine-stimulated endothelial cells using differential display. The amino acid sequence showed 98% homology with the previously isolated mouse rab18 protein, which is implicated in apical/basolateral endocytosis. Northern blot analysis revealed two transcripts (2.5 kb and 1 kb) ubiquitously expressed in all examined organs, as well as in human umbilical vein endothelial and aortic cells. In blood cells rab18 transcripts were hardly detectable. The histamine-induced time-dependent increase of rab18 mRNA expression in human umbilical vein endothelial cells and peripheral blood mononuclear cells indicates for the first time a link between receptor-mediated signal transduction and the regulation of rab gene expression. This finding might also imply a role for rab proteins in inflammation.

Key words: GTP-binding protein; Histamine; Lymphocyte; Human umbilical vein endothelial cell; Regulation of gene expression

1. Introduction

Rab proteins, a family of ras-related small GTPases, have been known to be key regulators of membrane trafficking for more than a decade (for a recent review see [1]). More than 30 different rab proteins have been identified and localised on different organelles and transport vesicles in mammals [2].

We report here the isolation of the complete human rab18 cDNA from human umbilical vein endothelial cells (HUVECs) stimulated by the inflammatory mediator histamine. Isolation and characterisation of the rab18 gene from mouse pituitary AtT-20 cells and adult mouse kidney has shed some light on the possible function of the gene product. The mouse rab18 mRNA seems to be equally expressed in polarised and non-polarised cells [3,4]. However, intracellular localisation in organ cryosections demonstrated the rab18 protein to be expressed in a tissue- and cell type-dependent manner in mouse kidney and intestines [4]. The rab18 protein was detected in apical endocytic structures in polarised epithelial cells of kidney proximal tubules. However, the rab18 protein does not seem to be restricted to the apical transport machinery of all polarised cells, since the protein was also localised on both the apical and basolateral domains in epithelial cells of the intestines. A role of rab18 in the transport between the plasma membrane and early endosomes was discussed in this context.

The genetic mapping of rab18 on the mouse chromosome 18 and alignment with the consensus map of mouse chromosome 18 showed rab18 to be coincident with two neighbouring mutations, *Tw* (twirler) and *ax* (ataxia) which result in neurological abnormalities. These findings and the detection of rab18 transcript in the brain [4] led to the suggestion that rab18 might be a potential candidate gene for these mutations [5]. Furthermore, rab proteins have been shown in previous studies to participate in the function of brain synaptic vesicles [6,7].

The recognition of rab proteins as key elements in vesicle transport has led to further investigations into the physiological regulation of rab-mediated membrane trafficking. Thus, agonist-regulated post-transcriptional processing, such as phosphorylation or geranylgeranylation, has been described for rab3, rab3B, rab4, rab6, and rab8 proteins. The activation of platelets by thrombin, a potent inducer of secretion, resulted in the phosphorylation of rab3B, rab6, and rab8 proteins [8]. The active form of rab8 has also been shown to interact with a stress-activated protein kinase in transfected 293 T cells, indicating that rab-regulated protein phosphorylation may serve to modulate secretion in response to stress stimuli [9]. In both 3T3 L1 fibroblasts and adipocytes the amount of geranylgeranylated rab3 and rab4 proteins was increased by insulin, indicating insulin as an activator of the membrane trafficking machinery in the above mentioned cell systems [10].

Although agonist- or stress-induced post-translational processing of rab proteins has been described to some extent, reports on the regulation of gene expression of rab proteins are scarce. Differential expression of rab genes has been shown in differentiating phagocytes [11] and during developmental regulation in *Drosophila*, *Dictyostelium* and maize [12–14].

It was our aim to identify and characterise genes which are differentially expressed in human endothelial cells during early inflammation. The chosen approach, that is stimulation of HUVECs with histamine and subsequent analysis of gene expression by differential display, was based on the very well documented inflammatory characteristics of histamine. Histamine has been proven to be one of the mediators released early during inflammatory events, such as anaphylactic reactions, shock and sepsis [15–17]. Focusing on the histamine-induced inflammatory effects, we were able to show that histamine rapidly stimulates endothelial cell responses, such as adhesion of neutrophils to endothelial cells [18] and mediator release (unpublished observations).

This report demonstrates that expression of the human rab18 gene isolated from activated HUVECs is also rapidly induced by histamine. To our knowledge regulation of rab

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gene expression as a response to physiological stimuli has only been shown in plants. This is the first report demonstrating effector-regulated mRNA expression of a human rab gene.

2. Materials and methods

2.1. Cell culture

Cryopreserved HUVECs (Cell Systems) were cultured in EBM medium (Clonetics) supplemented with EGM SingleQuots (0.01 μg/ml hEGF, 1 μg/ml hydrocortisone, 50 μg/ml gentamicin sulphate, 50 μg/ml amphotericin B, 12 μg/ml bovine brain extract) and 10% foetal calf serum (Life Technologies) at 37°C, 5% CO_2, 95% air. Endothelial cells were cultured at a concentration of 7×10^5 cells/5 ml medium in 25 cm^2 Falcon flasks (Becton Dickinson). Medium was exchanged every 48 h. Endothelial cell cultures were used from the second through the seventh passage as recommended by Clonetics.

Culture medium was removed from tightly confluent cells and substituted by medium containing histamine (1 mM). The histamine concentration was determined previously [18]. The cells were incubated for various times in an atmosphere of 5% CO_2, 95% air at 37°C.

2.2. Isolation of human blood cells

Human blood cells were isolated from venous blood of healthy donors by a two-step Percoll density gradient centrifugation. 7 ml of heparinised (100 IU/ml) venous blood was carefully layered over a discontinuous gradient of isotonic Percoll (solution 1 = 1.1026 g/ml, solution 2 = 1.0776 g/ml) and centrifuged for 25 min at $350\times g$ at room temperature. After centrifugation the granulocytes were collected from the first interphase. Mononuclear cells were isolated from the second interphase. Both cell types were washed twice with PBS.

In a second step monocytes were isolated from mononuclear cells by adhesion to cell culture flasks. Mononuclear cells were suspended in RPMI medium and cultured for 2 h in Falcon flasks at 37°C. After washing twice with phosphate-buffered saline (PBS) RNA was isolated from collected non-adherent mononuclear cells and adherent monocytes as described below.

2.3. Preparation and stimulation of peripheral blood mononuclear cells (PBMNCs)

PBMNCs were isolated from heparinised venous blood of healthy donors of both genders and various ages, using a Ficoll-Paque® gradient (Pharmacia, Uppsala, Sweden). 7.5 ml of RPMI containing 0.1% bovine serum albumin and penicillin (100 U/ml)/streptomycin (100 μg/ml) was added to 7.5 ml of heparinised venous blood, then carefully layered on 15 ml of Ficoll-Paque gradient. After centrifugation at 2000 rpm for 20 min, the interface was isolated and cells were washed twice and then resuspended in RPMI medium. Cells were counted in a Neubauer chamber. 2×10^6 cells were incubated in a total volume of 10 ml of RPMI medium in the presence or absence of histamine (10^{-5} M) at 37°C, 5% CO_2, 95% humidified air. At this concentration viability always exceeded 98% as judged by trypan blue exclusion. Incubation of PBMNCs with higher histamine concentrations resulted in toxic effects. Stimulation times were 5, 15, 120 or 360 min. Stimulation was terminated by centrifugation at 2000 rpm for 10 min, then cells were washed once and diluted in 1 ml RPMI medium.

2.4. RNA isolation

Culture medium was decanted, cells were washed in PBS (NaCl 120 mM, KH_2PO_4 2.7 mM, Na_2HPO_4 10 mM) and lysed by incubation with Trizol reagent (Life Technologies). After phenol/chloroform extraction and ethanol precipitation, samples were redissolved in diethylpyrocarbonate-treated sterile water.

2.5. Differential display

Differential display was performed according to the protocol published by Liang and Pardee [19]. 2 μg total RNA was treated with RNase-free DNase (Boehringer Mannheim) in the presence of 40 U recombinant RNase inhibitor (Life Technologies) to remove contaminating genomic DNA. After phenol/chloroform extraction and ethanol precipitation, samples were redissolved in diethylpyrocarbonate-treated sterile water at a concentration of 250 ng/μl. 500 ng RNA from each sample was reverse transcribed with Thermoscript (Life Technologies) at 60°C in the presence of 1 μM 5′-AAAGACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTTTV-3′ and 0.5 mM dNTPs for 60 min. After reverse transcription, 2 μl of the reaction mixture was taken for each of the following PCR reactions using 5′-GACCACGCGTATCGATGTCGAC-3′ as 3′-primer and 5′-TGTGAGGAATGTGGGAAA-3′ as 5′-primer. PCR reaction was performed in the presence of 0.4 μM 3′-primer, 0.4 μM 5′-primer, 0.2 mM dNTPs, and 2.5 U Taq polymerase (Life Technologies). Cycling parameters (25 cycles) were 94°C for 20 s, 58°C for 30 s, 68°C for 4 min and a final 10 min extension step at 68°C after initial incubation at 94°C for 3 min. PCR products were separated on a denaturing 5% polyacrylamide sequencing gel and visualised by silver staining (Silver Stain Plus Kit, Bio-Rad) according to the manufacturer's protocol. Fragments of interest were isolated by excising the band from the wet gel. Gel slices were dissolved in 0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA pH 8, and 0.1% SDS. Following ethanol precipitation, the fragments were reamplified using the PCR conditions described above. PCR fragments were cloned into the pCR′ II Vector (Invitrogen) according to the manufacturer's protocol.

2.6. RACE protocol

The RACE protocol was performed using the 5′/3′RACE kit (Boehringer Mannheim) according to the manufacturer's protocol. For the amplification of the 3′-end 5′-GGGAAAGTGAGAACCAGAATAAAGGAG-3′ was used as a forward primer for first round PCR and 5′-AAGGAGGAGGAGCCTGTGGTGG-3′ was used as a nested forward primer for second round PCR. For the amplification of the 5′-end 5′-GACTGCAAACTTGTGCATGCTTACC-3′ was used for reverse transcription. For first round PCR 5′-CTGCAAGGTCCCTAAATAGCAGTT-3′ was used as a nested reverse primer and 5′-GCAAGAGATGGAATTTCCCAGAG-3′ was used as a second nested reverse primer for second round PCR. PCR fragments were cloned into the pCR′ II Vector (Invitrogen) according to the manufacturer's protocol.

2.7. Sequence analysis

Sequence analysis was performed using the ABI Prism 277 Automated Sequencer (Applied Biosystems, USA). The sequence was determined on both strands.

2.8. Preparation of probes for Northern blot hybridisation and Northern blot analysis

The pCR″ II Vector (Invitrogen) was linearised with *Bam*HI (Promega) at the multiple cloning site. Linearised vector DNA was isolated from a 1% agarose gel according to the manufacturer's protocol (QIAquick Gel Extraction Kit, Qiagen). 1 μg linearised vector DNA was in vitro transcribed in a total volume of 20 μl using the DIG RNA labeling kit (Boehringer Mannheim) according to the manufacturer's protocol with T7 RNA polymerase. Labelling efficiency was estimated in a spot test with a DIG-labelled control (Boehringer Mannheim) as recommended by Boehringer.

For Northern blot analysis, 5 μg total RNA was loaded per lane. 40 ng DIG-labelled RNA molecular weight marker I (Boehringer Mannheim) was loaded in one lane as a marker. Probes were run for 16 h at 23 V in formaldehyde-containing agarose gels (1%). Gels were hydrolysed in 0.05 M NaOH for 20 min, washed three times in DEPC-treated sterile water, and soaked in 20×SSC for 2×15 min. Gels were blotted on a positively charged Nylon membrane (Boehringer Mannheim) with 20×SSC for 6 h at room temperature. Filters were crosslinked using Stratagene's crosslinker. After prehybridisation, filters were hybridised at 68°C for 16 h with 25 ng DIG-labelled RNA probe per ml hybridisation solution (Dig easy hyb, Boehringer Mannheim). Stringency washes were carried out twice with 0.4×SSC/0.1% SDS at 68°C after washing twice with 2×SSC/0.1% SDS at room temperature. Filters were blocked for 30 min in 1×block solution (Boehringer Mannheim), incubated with anti-digoxigenin-AP Fab fragments (Boehringer Mannheim) 1:30 000 in 1×block solution for 30 min, washed in 1×wash solution (Boehringer Mannheim) and incubated with CDP-star (Boehringer Mannheim) for 5 min. Finally filters were exposed to Biomax light-1 films (Kodak). All experiments were repeated at least twice.

2.9. Reverse transcription and polymerase chain reaction

In order to rule out genomic DNA contamination as an amplification source the RNA solution was incubated with DNase I (Boehringer, Mannheim) for 15 min at 37°C. Reverse transcription and

PCR was performed using the Titan™ One Tube RT-PCR system (Boehringer Mannheim). Total RNA (250 ng) was added to all components of the system in a total volume of 50 μl: 0.2 mM each of dATP, dCTP, dTTP and dGTP, 0.4 μM sense and antisense primer, 40 U RNase inhibitor (Life Technologies) in a buffer of 100 mM Tris-acetate (pH 6.5), 10 μl 5×RT-PCR buffer (7.5 mM $MgCl_2$), 2.5 μl DTT solution (100 mM) and 1 μl enzyme mixture consisting of Taq DNA polymerase, Pwo DNA polymerase and AMV reverse transcriptase. The RT-PCR reaction was carried out using specifically designed primer sets for GAPDH: sense 5′-CCATGGAGAAGGCTGGGG-3′, antisense 5′-CAAAGTTGTCATGGATGACC-3′ [20], the GAPDH primers were located in two different exons; human rab18: 5′- GCCGTCATCAACGACCTACATGG-3′ as a forward primer and 5′-CCCATAGGAGTTGAAGCAGCGC-3′ reverse primer. The temperature cycles for amplifying GAPDH and rab18 cDNA consisted of: 30 min reverse transcriptase reaction at 55°C, 3 min of denaturing at 94°C (1 cycle), 30 s of denaturing at 94°C, 30 s primer annealing at 58°C, 90 s of primer extension at 72°C (35 cycles), 7 min primer extension at 72°C (1 cycle) (Thermocycler PC-9606/Corbett). 15 μl aliquots of the PCR product were fractionated by horizontal gel electrophoresis on a 1.5% agarose ethidium bromide-stained gel.

2.10. Tissue distribution

Tissue distribution was evaluated using PCR amplification with the human rab18 gene-specific primers described above from normalised cDNA (Clontech) according to the manufacturer's protocol. In brief 2 μl from each tissue-specific cDNA was used as template. PCR reaction was performed in the presence of 0.3 μM of each primer, 0.2 mM dNTPs, and 2.5 U Taq polymerase (Life technologies). Cycling parameters (35 cycles) were 94°C for 20 s, 64°C for 30 s, 72°C for 60 s and a final 5 min extension step at 72°C after initial incubation at 94°C for 3 min. PCR products were separated on a 1.5% agarose gel and photographed. Experiments were repeated three times.

2.11. High performance liquid chromatography

PCR reaction products were quantified by size fractionation through anionic exchange high performance liquid chromatography (HPLC) in a Waters LCM1 plus system, which includes two pumps 444, an automated gradient controller, an autosampler 712, a UV detector 486 and a data module 746 for data analysis. The column was recalibrated for 10–12 min with a buffer ratio of 46/54 (A/B). The total flow rate was 1 ml/min. 10 μl of the PCR reaction probe was loaded on a prepacked DEAE-bonded non-porous resin particle column (diameter: 2.5 μm). PCR products were separated by the following gradient program: 0.01 min, 46/54 buffer A (25 mmol/l Tris–HCl, pH 9.0, 1 mol/l NaCl)/buffer B (25 mmol/l Tris–HCl, pH 9.0); 0.50 min, 54/46 (A/B); 8 min, 64/36 (A/B). The elution profile was assessed at 260 nm. Peak areas corresponding to the PCR products, detected at 260 nm by HPLC, were integrated and when essential normalised to levels of GAPDH message (Millennium software, Waters Corporation).

2.12. Statistical analysis

Normalised PCR products were integrated and displayed as percentage of control value. PCR product levels amplified from untreated endothelial cell mRNA served as control values. Differences in time course between control values and the different time points were tested using the paired Student's *t*-test. Due to multiple comparisons, only P-values ≤ 0.02 were considered significant. The results are displayed as means (±S.E.M.).

3. Results

3.1. Identification of the human rab18 protein sequence

Stimulation of HUVECs with histamine and subsequent analysis of gene expression by differential display led to the identification of a differentially expressed 612 bp cDNA fragment. Cloning and sequence analysis revealed the cDNA fragment to encode the 3′ region of the human rab18 protein. The complete rab18 cDNA clone was isolated using a RACE protocol.

The nucleotide and amino acid sequence of the human rab18 are shown in Fig. 1a,b, respectively. Alignment of the human rab18 protein sequence and the rab18 protein sequences previously isolated from other species, such as mouse, snail and roundworm, revealed a high degree of homology across the phylum (98%, 92% and 85%, respectively) (Fig. 1b).

Analysis of the amino acid sequences revealed a markedly high interspecies homology at the structural features contributing towards GTP binding and functional specificity (Fig. 1b). The 100% interspecies homology observed in the conserved nucleotide binding motifs PM1 (phosphate/Mg^{2+} binding motif), PM3, as well as G1 (guanine base binding) and G3 extends into the PM1 adjacent β-strand 1 and the PM3 adjacent switch II region, which includes loop 4/helix 2/loop 5 [21,22]. On the other hand, the highest degrees of variability between the rab18 protein sequences of the analysed species were observed in conserved regions defining organelle targeting, such as helix 3/loop 7 and the C-terminal region [23,24]. These observations indicate a functional interspecies specificity of the rab18 protein which might not coincide with the species-specific localisation of rab18 on distinct membrane compartments.

3.2. rab18 mRNA expression in different cell types and organs

Northern blot analysis using the 612 bp cDNA fragment as a hybridisation probe resulted in two hybridisation signals with a size of about 2.5 kb and 1 kb (Fig. 2). The size of the larger transcript was consistent with the mouse rab18 mRNA size [4]. The expression of the smaller transcript coincided with the expression pattern of the 2.5 kb transcript.

Both signals were detected in venous and aortic endothelial cells. The expression of both transcripts were hardly detectable in smooth muscle cells, PBMNCs, monocytes and neutrophils.

Tissue distribution of rab18 transcript was assessed by PCR amplification. Using gene-specific primers rab18 cDNAs were amplified from tissue cDNA preparations (MTC™), which were normalised using different housekeeping genes. Agarose gel electrophoresis of the PCR products demonstrated cDNA fragments corresponding to the expected rab18 cDNA fragment size (592 bp) (Fig. 3). To confirm the origin of the cDNA fragment, the PCR product was cloned and sequenced. The homology between rab18 and the PCR product sequence was 100%. Although rab18 mRNA was expressed ubiquitously in all organs examined, the intensity of expression varied between organs. Quantification of rab18 cDNA fragments by HPLC revealed the rab18 transcript to be strongly expressed in heart, kidney, pancreas, lung and liver (Fig. 3, lower panel). Comparatively weak expression of rab18 mRNA was observed in brain, placenta and skeletal muscle.

3.3. Histamine-induced upregulation of rab18 mRNA in endothelial cells and PBMNCs

HUVECs and PBMNCs were stimulated with histamine (1 mM and 10 μM, respectively) for increasing periods of time in order to analyse the effect of histamine on rab18 mRNA expression. The method of RT-PCR was employed on total RNA extracted from stimulated and unstimulated cells to detect changes in rab18 expression. GAPDH mRNA was reverse transcribed and amplified from the same RNA preparations as a control. In order to utilise the RT-PCR products for semi-quantitation, the optimum number of am-

a.

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atggacgagg acgtgctaac caccctgaag atcctcatca tcggcgagag tggggtgggc aagtccagcc tgctcttgag gttcacagat gatacgtttg
atccagaact tgcagcaaca ataggtgttg actttaaggt gaaaacaatt tcagtggatg gaaataaggc taaacttgca atatgggata ctgctggtca
agagaggttt agaacattaa ctcccagcta ttatagaggt gcacagggtg catgctagtt ggaaataaaa tcgataagga aaatcgtgaa gtcgatagaa
aattggttaa atgaattgga aacatactgt acaagaaatg acatagtaaa ttatattagt ttatgatgtc acaagaagag atacatttgt taaactggat
atgaaggcct gaaatttgca cgaaagcatt ccatgttatt tatagaggca agtgcaaaaa cctgtgatgg tgtacaatgt gcctttgaag aacttgttga
aaagatcatt cagacccctg gactgtggga aagtgagaac cagaataaag gagtcaaact gtcacacagg gaagaaggcc aaggaggagg agcctgtggt
ggttattgct ctgtgttata aactctggga aattccatct cttgcatatt tgatcagata gtgacatctt tctgtatata aactctttaa ctgctatttt
agggaccttg cagtttgcac ataattgttt tatatcatag cagtaaatat ttgcaagaaa tcccactcat cgaccccggg taaaatgtta tggtaagcat
gcacagtttg cagtctacag ttttttttatg tagcacaaaa taggtgtacc tttataagta cattcaattt tatgatttac atttatcatg taatttttaa
aaaaatccat ctatctagga tatgttgata caaagtctgc ttttgctatt ctttttgctt aaatactcct atcattttct gaattacttg gtatttagaa
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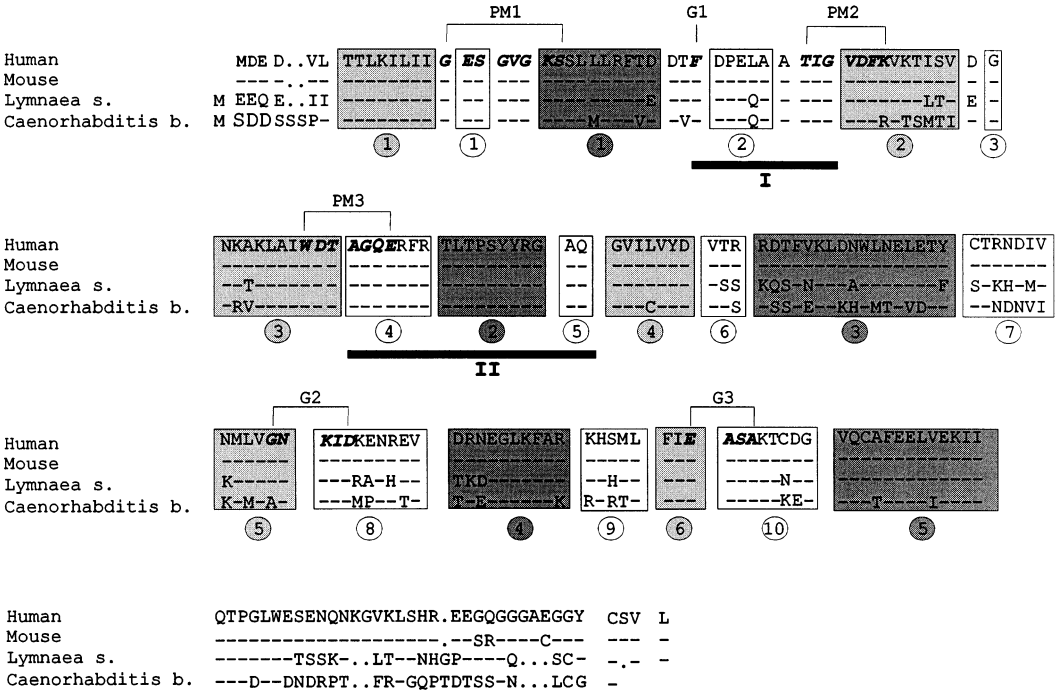
b.

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Human                QTPGLWESENQNKGVKLSHR.EEGQGGGAEGGY  CSV  L
Mouse                --------------------.--SR----C---  ---  -
Lymnaea s.           -------TSSK-..LT--NHGP----Q...SC-  -.-  -
Caenorhabditis b.    ---D--DNDRPT..FR-GQPTDTSS-N...LCG  -
```

Fig. 1. Nucleotide and amino acid sequence of the human rab18 gene. a: The nucleotide sequence of the human rab18 cDNA coding region. b: Amino acid alignment of human rab18 protein with rab18 proteins of other species. White boxes: loops; grey boxes: β-strands; dark grey boxes: α-helices; encircled figures: consecutive numbering of motifs. Black bars: switch region I and II. PM1–3 represent the phosphate/Mg^{2+} binding motifs and G1–3 the guanine base binding motifs. The highly conserved amino acid residues are indicated in black letters.

plification cycles that were still in the linear range was determined for the two sets of gene-specific primers.

The amplification products proved to be in the linear amplification phase at 32 cycles as determined by absorbance at 260 nm by HPLC (data not shown).

During 24 h of continuous histamine stimulation (1 mM) the rab18 mRNA levels in endothelial cells increased by $92.2 \pm 14\%$ ($P \leq 0.001$) as compared to control levels (unstimulated endothelial cells) (Fig. 4). The histamine-stimulated changes in rab18 level were concentration-dependent. After 24 h endothelial cell stimulation with 100 μM histamine the rab18 message was increased by $80.7 \pm 4.7\%$ ($P \leq 0.001$). The incubation of endothelial cells with 10 μM histamine did not result in changes of rab18 mRNA levels (results not shown).

Initially a fast increase of rab18 transcript was observed within 4 h of histamine stimulation ($52.3 \pm 9.1\%$; $P \leq 0.001$), with a significant upregulation of rab18 transcript after 30 min ($12 \pm 5.2\%$ ($P \leq 0.001$). These observations were in accordance with results initially obtained by Northern blot analysis showing an increase of the 2.4 kb and 1 kb transcripts in endothelial cells after 2 h of histamine stimulation (Fig. 4a, inset). Although a histamine-induced upregulation of rab18 transcript was also observed in PBMNCs, the time-dependent pattern of rab18 mRNA upregulation was not comparable (Fig. 4a). A significant histamine-stimulated increase of the rab18-encoding mRNA as compared to cells which were in-

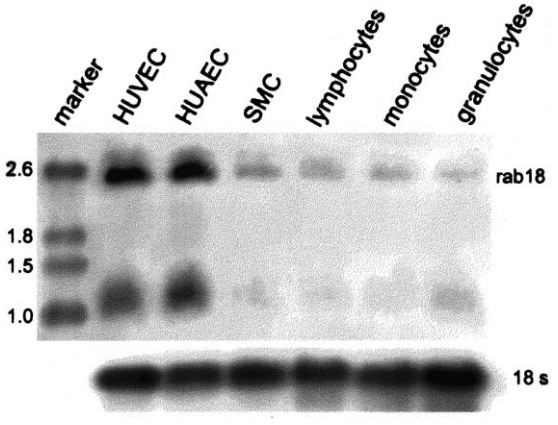

Fig. 2. Northern blot analysis of the expression pattern of human rab18 mRNA in different cell systems.

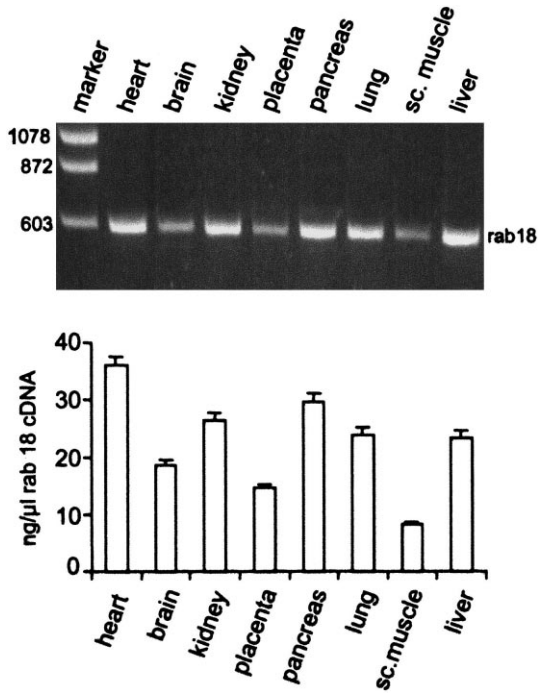

Fig. 3. Human rab18 expression pattern in a variety of organs examined by RT-PCR of normalised tissue cDNA panels ($n = 3$).

cubated for the same time period without histamine was only observed after 2 h of stimulation ($78 \pm 10.6\%$; $P \leq 0.001$). Eight hours of histamine stimulation led to a further increase of rab18 expression ($107.1 \pm 10.5\%$; $P \leq 0.001$).

It has to be taken into consideration that rab18 expression decreases in untreated PBMNCs incubated for the same time period as histamine-stimulated cells. After 2 h of incubation the rab18 transcript level in untreated cells decreased by 33.1% as compared to freshly isolated cells (Fig. 4b). At this time point the decrease of rab18 mRNA level in untreated cells is statistically significant ($P \leq 0.01$). However, a histamine-dependent increase of rab18 transcript can also be observed when compared to freshly isolated PBMNCs (8 h: $71 \pm 28.6\%$). Although this observation is not statistically significant, the statistically significant enhancement of rab18 expression in comparison to the relevant control groups in endothelial cells and peripheral mononuclear cells indicates histamine as a potent inducer of rab18 transcription in these cell systems.

4. Discussion

In the present work we have identified a histamine-regulated small GTPase belonging to the rab family using differential display. The human rab cDNA was isolated, cloned and sequenced. Sequence analysis of the rab cDNA revealed 100% homology with the partial sequence of the human rab18 mRNA previously isolated from skeletal muscles [25]. The complete human rab18 amino acid sequence showed high amino acid identity (98%) to the corresponding mammalian rab18 sequence isolated from mouse [3,4]. The highest degree of conservation in the rab18 amino acid sequence of different species was observed in the effector/switch I and switch II region. Small GTP binding proteins are thought to be molecular switches, because local conformational differences between GDP- and GTP-bound forms of the protein are the cause for the interaction of rab proteins with specific effector and GTPase activating proteins. The functional specificity of the rab protein is thus mainly defined by the amino acid sequence of the switch I and switch II domains. The evolutionarily conserved high degree of homology in the effector/

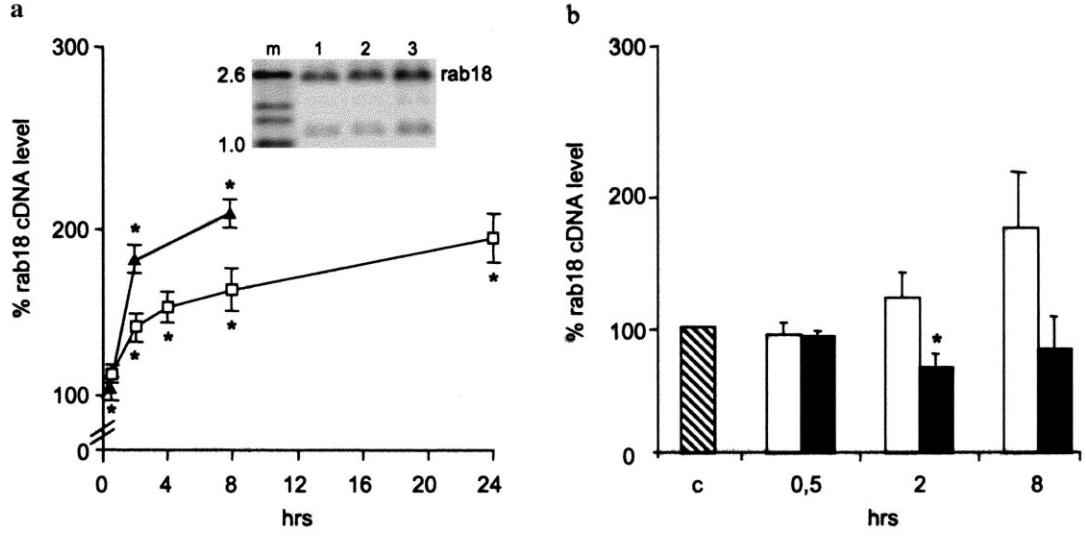

Fig. 4. Histamine-induced time-dependent regulation of rab18 expression in HUVECs and PBMNCs. HUVECs (open squares) and PBMNCs (closed triangles) were incubated with histamine (1 mM and 10 mM, respectively) for various time periods as indicated. Gene products were analysed by RT-PCR, normalised to levels of GAPDH message and quantified by HPLC. a: Graphic representation of histamine-induced changes of rab18 cDNA levels displayed as percentage of control values. (Control values for PBMNC rab18 expression were obtained from unstimulated cells incubated for the same time periods as histamine-stimulated PBMNCs); inset: Northern blot analysis of histamine-induced rab18 expression in HUVECS. b: Graphic representation of the time-dependent increase of rab18 cDNA fragment in histamine-stimulated PBMNCs displayed as a percentage of freshly isolated PBMNCs (control). Open bar: histamine-stimulated PBMNCs; black bars: unstimulated PBMNCs incubated for indicated time periods. The results displayed are means ($\pm$S.E.M.) of three separate stimulations. The RT-PCRs were performed in duplicate.

switch I and switch II region of the rab18 amino acid sequences indicates that rab18 interacts with the same or closely related effector protein(s) and regulatory proteins within different species.

The C-terminal region of rab proteins is thought to contain information for the correct targeting of the proteins to their specific locations within the cell [1]. Consequently the targeting domain is the least conserved region of the members of the rab subfamily. The rab18 amino acid sequence downstream of the effector/switch I and switch II region is also hypervariable in length and sequence composition in different species. The species-dependent hypervariability includes the cysteine-containing motif, CXC, which is a prerequisite for the membrane association of rab proteins. The rab-associated CXC motif subject to isoprenyl (geranylgeranyl) modification is conserved in *Lymnea stagnalis* and *Caenorhabditis briggsae*. On the other hand, the two mammalian rab18 proteins have the C-terminal sequence CSVL. It has been discussed that the mammalian rab18 protein is modified by the CAAX GGL transferase [3].

These observations suggest that the distinct localisation of rab18 proteins to specific compartments might be species-dependent.

Cell type-specific expression of rab18 mRNA was investigated by Northern blot analysis. Northern blot analysis revealed two mRNA species with lengths of 2.5 and 1.0 kb. Strong signals of both transcripts were detected in polarised cells such as venous and aortic endothelial cells. A rather weak expression pattern was exhibited in smooth muscle cells and non-polarised cells such as PBMNCs, monocytes and granulocytes. In order to investigate whether the two transcripts are splicing variants of the same gene, as described for rab proteins isolated from rat [26,27], primers directed against the start codons and the 3′-untranslated region were constructed. RT-PCR reactions with these primers resulted in a single band corresponding to an unspliced rab18 product (results not shown). A difference in length of the 3′-untranslated region, due to different polyadenylation signals as described for the rab11a mRNA, cannot be excluded by these observations [28]. However, the size of the large transcript is in accordance with the size of the rab18 transcript isolated from mouse [4]. The origin of the small transcript awaits further analysis.

The tissue distribution of the rab18 mRNA was assessed by PCR amplification using normalised tissue cDNA preparations. A ubiquitous but uneven expression of the rab18 mRNA was observed in human organs. The expression pattern of the human rab18 mRNA varies slightly from the expression pattern reported in mouse [3,4]. The strongest expression of human rab18 was detected in heart, liver, kidney, lung and pancreas. A strong mouse rab18 signal was also observed in brain. The human rab18 mRNA signal is comparatively weak in brain. The ubiquitous expression of the rab18 transcript is in accordance with the Bourne hypothesis, which points out the large number of intracellular membrane trafficking events common to all mammalian cells [29]. Furthermore, the ubiquitous expression of rab RNA does not necessarily indicate an equal level of expression in the various cell types [1]. Detailed analysis of rab18 and rab20 protein expression in mouse revealed distinct cell type-specific expression patterns in sections of mouse kidney and intestines [4].

This aspect should also be taken into consideration in regard to our further findings. We were able to show that the regulation of rab18 expression can be modified by external factors. The stimulation of polarised (HUVECs) and non-polarised cells (PBMNCs) with histamine resulted in a statistically significant time- and concentration-dependent increase of rab18 transcript in both cell types. The histamine-induced increase of rab18 message in endothelial cells lasted for a period of 24 h. Histamine-induced long-term changes in gene expression have also been shown for the histamine receptor subtype H_2 [30] and a zinc finger transcription factor (unpublished observations). In particular the continuous downregulation of one of the histamine receptor subtypes suggests that long-term stimulation of endothelial cells with histamine results in a new steady-state level of the cells. Although the differential receptor-mediated modulation by growth factors and hormones has been well established for Ras proteins, a differential regulation of mammalian rab proteins has only been shown for developmental and cell cycle events at the post-translational level. Histamine is one of the mediators secreted by mast cells and basophils during early inflammatory events [15,31]. The release of the endogenous amine results in inflammatory reactions induced in a variety of cell types [18,32,33]. The histamine-induced upregulation of rab18 mRNA indicates for the first time a link between receptor-mediated signal transduction and regulation of rab gene expression. It also suggests a possible role of rab proteins in inflammation. This is in accordance with data demonstrating a significant enhancement of the basal GTPase activity in neutrophils of severely burned patients [34]. A role of rab proteins in immunological reactions such as antigen presentation has also been discussed previously [35]. However, the results obtained in this study invariably lead to a number of questions which have to be studied in more detail in order to assess the precise effect of external factors on the regulation and function of rab proteins and the role of rab proteins in inflammatory/immunological reactions. For a start, the effect of histamine on rab18 protein levels in these cells has to be elucidated. Furthermore, in view of the results obtained by Lütcke et al. [4], it might also be of great interest to study the subcellular distribution of the rab18 protein in histamine-stimulated and unstimulated cells. In order to determine the physiological function of the rab18 protein it is necessary to extend from cell culture experiments to a study of rab protein function in humans. An inflammatory role of rab18 for example could be established by examining rab18 protein concentration and distribution in PBMNCs of septic patients.

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