Small GTP-binding proteins and regulated secretion of von Willebrand factor by endothelial cells

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CHAPTER 2

SMALL GTP-BINDING PROTEINS
IN ENDOTHELIAL CELLS

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ABSTRACT

Small GTP-binding proteins of the Ras superfamily control an extensive number of intracellular events by alternating between GDP- and GTP-bound conformation. The presence of members of this protein family was examined in human umbilical vein endothelial cells employing RT-PCR. Sequence analysis of 215 cDNA clones revealed the presence of a total of 28 different partial cDNAs encoding small GTP-binding proteins. Two sequences corresponded to novel isoforms of Rab2 and Rab9. In addition, human analogues of Rab4, Rab7, Rab9, Rab14, and Rab15 were identified. Besides Rab proteins, members of other subfamilies were detected as well. As a first step towards elucidation of the function of the different small GTP-binding proteins identified we have isolated full length cDNA corresponding to Rab30 from a human endothelial cell cDNA library. In order to assess the subcellular localization of Rab30, we expressed epitope tagged Rab30 cDNA in monkey kidney COS-1 cells. Immunoelectronmicroscopy of transfected COS-1 cells indicated that Rab30 is associated with Golgi stacks.

INTRODUCTION

Endothelial cells constitute a continuous monolayer which has a crucial function in haemostasis, thrombosis and atherosclerosis. Under quiescent conditions the endothelium provides a non procoagulant surface which serves to maintain the integrity of the vascular system. Perturbation of endothelial cells by inflammatory reagents has been shown to alter its adhesive properties dramatically. Several studies have suggested a role for small GTP-binding proteins in the regulation of endothelial cell function. Injection of H-Ras into bovine aortic endothelial cells greatly alters motility of the cells suggesting the presence of a H-Ras sensitive pathway (Fox et al., 1994). Pronounced effects were observed on endothelial cell morphology and migration by clostridium botulinum C3 transferase, a reagent specific for the Rho-members of the superfamily of small GTPases (Yano et al., 1996). A number of small GTP-binding proteins of the Rab subfamily which mediate vesicular transport along the secretory pathway have been found in endothelial cells. An early report described the molecular cloning of an isoform of Rab5 from endothelial cells (Wilson and Wilson, 1992). Subsequent studies utilized specific antibodies directed against the highly variable carboxy terminal part of Rab proteins to establish the localization of Rab1, Rab3b, Rab4, Rab6 and Rab8 in endothelial cells (Karniguan et al., 1993).

To obtain additional information on the repertoire of small GTP-binding proteins in human endothelial cells, we have used RT-PCR with primers directed to conserved regions of the Rab subfamily essentially as described previously (Chavrier et al., 1992). As a first step in the characterization of the identified small GTP-binding proteins we have cloned full length cDNA of Rab30 from a human endothelial cell cDNA library and studied the subcellular localization of this protein by heterologous expression in COS-1 cells.
MATERIALS AND METHODS

Materials

Oligonucleotide primers and the vector pSVL were obtained from Pharmacia-LKB (Roosendaal, The Netherlands). Radioactive chemicals were obtained from The Radiochemical Centre (Amersham, UK). Hybridoma producing monoclonal antibody 9E10, directed against the first 11 amino acids of c-myc was purchased from American Type Culture Collection (Rockville, USA). All other reagents and DNA modifying enzymes used were of analytical grade.

Isolation of cDNA sequences homologous to small GTP-binding proteins

Human umbilical vein endothelial cells (HUVEC) were grown as described previously (Brinkman et al., 1994). RNA was isolated using RNAzol™B and reverse transcribed in cDNA (Wak-chemie, Bad Homburg, Germany). CDNA was used as template in the polymerase chain reaction using oligonucleotide primers corresponding to the conserved GTP-binding regions PM3 and G2 of the family of Rab proteins (WDTF (5'-TGGGA(T/C)ACNGCNGGNCA(G/A)GA-3') and NKXD (5'-NA(G/A)(G/A)TCNNN(T/C)TT(G/A)TTNCC-3' respectively. PCR fragments of approximately 200 bp were obtained that were cloned into the vector pGEM-T (Promega, Madison, USA), yielding pCLB-GTP-19701 to pCLB-GTP-20099. Plasmid DNA of 215 clones was isolated and the nucleotide sequence of the insert was determined using a 373 DNA sequencer (Applied Biosystems, Gouda, The Netherlands). All DNA sequences found were compared and aligned with sequences of mammalian small GTP-binding proteins available from protein and nucleotide databases, using PCGENE software (Intelligenetics, Oxford, UK).

Cloning of full length Rab30 cDNA from an endothelial cell cDNA library

A human umbilical vein endothelial cell cDNA library in the vector pcDNA-I consisting of approximately 8 x 10^4 independent clones was screened to obtain full length Rab30 cDNA. Clone pCLB-GTP-19902, which contained a partial cDNA that corresponds to Rab30, was digested with Ncol and Notl and a 192 bp insert was radioactive labeled and used as probe. The nucleotide sequence of the isolated clone (pcDNA-I-Rab30) with an insert of 1.6 kb was determined as described above.

Construction of epitope tagged Rab30 cDNA and expression in COS-1 cells

We have used pcDNA-I-Rab30 and pBluescript-c-myc (kindly provided by Dr. F. Michiels, Netherlands Cancer Institute) as templates to construct epitope tagged Rab30 in the eukaryotic expression vector pSVL termed pSVL-Myc-Rab30. In the resulting construct the coding sequence of Rab30 is preceded by 11 amino acids that constitute the epitope of monoclonal antibody 9E10 (Evan et al., 1985). Monkey kidney COS-1 cells were transfected and immunoelectronmicroscopy was performed as described previously (Voorberg et al.,
For quantification we have used the pictures of 5 different cells expressing epitope tagged Rab30. The surface area of the Golgi stacks and of the rest of the cytoplasm was measured and the number of gold particles in each area was counted.

RESULTS

Identification of small GTP-binding proteins in human endothelial cells

We have addressed the presence of mRNA encoding small GTP-binding proteins in endothelial cells employing RT-PCR. Oligonucleotide primers corresponding to conserved regions of small GTP-binding proteins of the Rab family were used to amplify partial cDNA fragments. Partial cDNA sequences were cloned and sequence analysis revealed the presence of a large number of small GTP-binding proteins (Table I). The majority of the partial cDNA sequences corresponded to Rab proteins that have not been identified in endothelial cells previously (Table IA). The amino acid sequence encoded by clone pCLB-GTP-19833 was 84% homologous to the corresponding part of Rab9 (Figure 1a) as reported by other authors (Chavrier et al., 1990). This suggests that clone pCLB-GTP-19833 represent a novel isoform of Rab9 and we propose to term this protein Rab9b in accordance with current nomenclature rules. Similarly, clone pCLB-GTP-19940 showed 94% homology to human Rab2 (Tachibana et al., 1988) and most likely represent a novel isoform of Rab2 (Figure 1b). A number of other Ras-related small GTP-binding proteins were detected as well (Table IB).

Expression of epitope tagged Rab30 in eukaryotic cells

In the previous paragraph we have provided an overview of the repertoire of small GTP-binding proteins in endothelial cells. As a first step towards functional characterization of the small GTP-binding proteins encountered in endothelial cells, we have isolated full length cDNA corresponding to human Rab30. The sequence was compared with the published sequence of Rab30 (Chen et al., 1996) and one difference was observed at nucleotide position
where A is instead of G is found, predicting a substitution of glycine for aspartic acid at amino acid position 45. We have constructed a fusion protein in which the first 11 amino acids of the proto-oncogene c-myc are fused to the amino terminus of Rab30. The localization of Rab30 in transfected COS-1 cells was studied using immuno-electronmicroscopy (Figure 2). Weak diffuse labelling was found all over the cytoplasm but a high labelling was always observed on all cisternae of the Golgi apparatus (Figure 2a). In some COS-1 cells transfected with pSVL-Myc Rab30 a large amount of label associated with an altered Golgi apparatus where the Golgi stacks are highly circularized and are partly disassembled into vesicular structures (Figure 2b). Figure 2c shows an untransfected COS-1 cell in the same section in which some background labelling is present. However, no reactivity was observed on the

elaborate Golgi apparatus in the untransfected cell. Quantitative analysis revealed that 97.5 gold particles/μm² were associated with the Golgi apparatus in transfected cells. In non-transfected cells 5.4 gold particles/μm² were found to be associated with the Golgi apparatus. The amount of gold particles present in the cytoplasm did not significantly differ among transfected and non-transfected cells (respectively 8.2 and 5.5 gold particles/μm²). It should be noted that overexpression of Rab30 in COS-1 cells had no effect on the morphology of other organelles present in the transfected cell and this points at a specific interaction of Rab30 with the Golgi apparatus.

**DISCUSSION**

Endothelial cells constitute a highly regulatory barrier between the bloodstream and tissue that controls processes like inflammation and atherosclerosis. In this study we have provided
an inventory of small GTP-binding proteins in human endothelial cells derived from umbilical vein. Previous studies have established that endothelial cells may exhibit heterogeneity (Risau, 1995). Consequently, care should be taken in extrapolation of our data to endothelial cells of different vascular origin. Using RT-PCR we have found cDNA fragments corresponding to 28 different small GTP-binding proteins. The use of oligonucleotide primers derived of the nucleotide sequence of the Rab family may have hampered the detection of additional members of the family of small GTPases in endothelial cells. The majority of GTP-binding proteins identified in this study has not been detected in human endothelial cells.

**Figure 2.** Immunoelectron microscopy of COS-1 cells transfected with Myc-Rab30 cDNA. COS-1 cells transfected with epitope tagged Rab30 cDNA were incubated with monoclonal antibody 9E10 and 10 nm gold conjugate. (a), a cell expressing Myc-Rab30 shows labeling on all cisternae from the Golgi apparatus (G). The outline of the two Golgi stacks are marked by arrows. (b) a cell overexpressing Myc-Rab30. Labeling is high on the altered Golgi apparatus. The Golgi stacks (arrows) are disassembling in vesicular structures (v). Nucleus (n). (c) a cell from the same section that does not express Myc-Rab30. The two Golgi stacks (G) do not show labeling above the background. Bars = 200 nm.
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previously (Table 1). One clone which we have termed Rab9b was only 84% homologous to Rab9 indicating the presence of a novel isoform of Rab9. In addition, we have found a novel sequence, termed Rab2b, that displayed 94% homology with the amino acid sequence of human Rab2 (Tachibana et al., 1988).

The inventory of small GTP-binding proteins presented in this study provides an attractive starting point to address the function of these proteins in endothelial cells. Here, we show that overexpression of myc-tagged Rab30 in COS-1 cells results in association of Rab30 with the Golgi-apparatus. We cannot exclude that association of Rab30 with Golgi stacks is due to the high level of Rab30 in transfected COS-1 cells. Definite assignment of the subcellular localization of Rab30 in endothelial cells awaits the development of antibodies specific for Rab30. Similarly, specific antibodies directed towards other small GTP-binding proteins identified in this study should greatly facilitate future studies which aim at dissection of the secretory pathway in endothelial cells.

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LITERATURE


