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The glomerulosclerosis gene *Mpv17* encodes a peroxisomal protein producing reactive oxygen species

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The mutant mouse strain Mpv17 carries a retroviral insert in its genome which inactivates the *Mpv17* gene. At a young age these mice develop glomerulosclerosis and nephrotic syndrome which resembles human disease. We show here that the *Mpv17* gene product is highly conserved and encodes a peroxisomal protein. Loss of the Mpv17 protein does not impair peroxisome biogenesis but instead leads to a reduced ability to produce reactive oxygen species (ROS). In turn, overproduction of the *Mpv17* gene in transfected cells results in dramatically enhanced levels of intracellular ROS indicating a direct involvement of Mpv17 in ROS production. These data reveal a role for the Mpv17 protein in peroxisomal reactive oxygen metabolism and establish a novel link between peroxisomal ROS production and glomerulosclerosis.

Key words: glomerulosclerosis mouse model/peroxisomes/reactive oxygen species

Introduction

Peroxisomes are small membrane bound organelles present in most eukaryotic cells. Their functions include a number of catabolic and anabolic reactions such as H_2O_2 based respiration, β oxidation of very long chain fatty acids, plasmalogen synthesis, and cholesterol synthesis and degradation. The peroxisomal membrane is involved in the transport of metabolites into or out of the peroxisome, as well as in the unique biogenesis of this organelle. Recently, the genes for several peroxisomal membrane proteins have been reported. Mutations of these genes cause human peroxisomal disease. They affect genes involved either in peroxisome biogenesis (Gärtner *et al.*, 1992; Shimozawa *et al.*, 1992) or in specific metabolic pathways taking place within the peroxisome (Lazarow and Moser, 1989; Mosser *et al.*, 1993). About 40 enzymes of the peroxisomal matrix are known, and several causal relationships between peroxisomal enzymes and inherited diseases have been described. However, no animal model exists for these diseases or for any peroxisomal functions.

The mouse strain Mpv17 is a recessive mouse mutant which develops glomerulosclerosis at a young age (Weiher *et al.*, 1990). The phenotype results from a loss of function of a gene coding for a hydrophobic protein of 176 amino acids which is destroyed by a retroviral integration. The human homologue may also have a role in the development of human kidney disease (Weiher, 1993). The *Mpv17* gene is expressed in a variety of cell types despite the kidney-specific pathology of the Mpv17 strain (Weiher *et al.*, 1990). The human homologue was isolated on the basis of cross hybridization and is expressed in liver, kidney and several human cell lines (Karasawa *et al.*, 1993; Weiher 1993). Here we describe the subcellular localization of the Mpv17 protein and address the question of its molecular function in the cell and in the organism. We hereby uncover a novel connection between a peroxisomal defect and kidney disease as well as disclose a new function in the metabolism of reactive oxygen species (ROS).

Results

The human and mouse Mpv17 genes are conserved and the gene products show homology to a peroxisomal membrane protein

The *Mpv17* gene is a single copy gene in humans and mice alike and is expressed in a variety of murine and human cell lines of different origin. Thus, we found single transcripts of 1.7 kb in confluent NIH 3T3 murine fibroblasts and of 1.2 kb in human GM637 fibroblasts (Figure 1a). The 92% interspecies conservation between the two proteins at the predicted amino acid level is displayed in Figure 2. An EMBL database search revealed a significant homology to the peroxisomal membrane protein pmp22 (Kaldi *et al.*, 1993), on the basis of both hydrophilicity profiles and primary sequence comparison. As also shown in Figure 2, the rat pmp22 protein is identical to Mpv17 in >25% of all positions. Based on the protein similarity algorithm in the GCG program (Genetics Computer Group, 1991) the degree of conservation between the two proteins is >50%.

The Mpv17 protein localizes to peroxisomes

In order to characterize the Mpv17 protein, antibodies were raised against the translation product of the the human *Mpv17* open reading frame. Two bacterially expressed fusion proteins were used as antigens. One contained the 27 kDa glutathione-*S*-transferase from *Schistostoma japonicum* (GST 9), while the other contained the 34 kDa Trp E protein of *Escherichia coli* (Ausubel *et al.*, 1994) at the N-terminus. Both had the entire coding region of the human *Mpv17* gene at the C-terminus. Polyclonal rabbit antisera as well as several monoclonal mouse antibodies were derived and used in the following analyses. A Western blot analysis of proteins from mouse 3T3 cells identified a Mpv17-specific protein of 20 kDa in size as illustrated in Figure 1b. This fits very well with the expected size of a protein of 176 amino

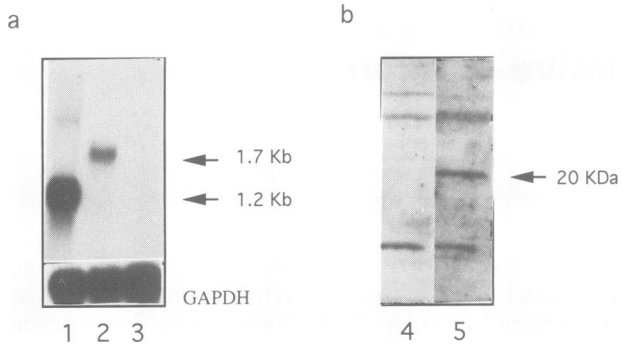

Fig. 1. *Mpv17* expression in human and murine fibroblasts. (**a**) Poly(A)$^+$ RNA was prepared from GM637 (lane 1), NIH3T3 (lane 2) and lung fibroblasts from *Mpv17* homozygous mice (lane 3). 4 μg were loaded per lane. As a probe a human cDNA clone p31710 (Karasawa *et al.*, 1993) was used. (**b**) Protein extracts from NIH3T3 cells were prepared, 100 μg loaded, blotted and probed with pre-immune serum (lane 4) and antiserum (lane 5) from rabbits immunized with GST−Mpv17 fusion protein.

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Mpv17          MALWRAYQRALAA.......HPWKVQVLTAGSLMGVGDM
Hmpv17         MALWRAYQRALAA.......HPWKVQVLTAGSLMGLGDI
pmp22   MAPAASRLRVESELRSLPKRALAQYLLFLKFYPVVTKAVSSGILSALGNL

        ISQQLVERRGLQQHQAGRTLTMVSLGCGFV......GPVVGGWYKVLDHL
        ISQQLVERRGLQEHQRGRTLTMVSLGCGFV......GPVVGGWYKVLDRF
        LA.QMIEKK...QKKDSRSLEVSGLLRYLVYGLFVTGPLSHYLYLFMEYW

        IPGTTKVHALKKMLLDQGGFAPCFLGCFLPLVGILNGMSAQDNWAKLKR
        IPGTTKVDALKKMLLDQGGFAPCFLGCFLPLVGALNGLSAQDNWAKLQR
        VPPEVPWARVKRLLLDRLFFAPTFLLLFFFVMNLLEGKNISVFVAKMRS

        DYPDALITNYYLWPAVQLANFYLVPLHYRLAVVQCVAIVWNSYLSWKAHQF
        DYPDALITNYYLWPAVQLANFYLVPLHYRLAVVQCVAIWNSYLSWKAHRL
        GFWPALQMNWRMWTPLQFININYVPLQFRVLFANMAALFWYAYLASLGK
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Fig. 2. Comparison of the protein sequences of murine and human *Mpv17* genes with the rat pmp 22 (Kaldi *et al.*, 1993) protein. Identities between all three proteins indicated in bold letters constitute 26% of the *Mpv17* sequence. Conservation on the basis of the algorithms of similarity of the GCG program (Genetics Computer Group, 1991) is >52%.

acids, and suggested that the Mpv17 protein may not be processed or modified *in vivo*. This was also consistent with a hypothetical peroxisomal localization suggested by its similarity to pmp22 (see above), since peroxisomal proteins are generally synthesized in the cytoplasm and imported into the organelle without modification (Lazarow and Fujiki, 1985).

To determine the subcellular localization of the Mpv17 protein, immunofluorescence analyses were performed (Figure 3). When murine 3T3 fibroblasts were analysed with the rabbit anti-GST−Mpv17 sera intracytoplasmic spots were observed. A sheep anti-catalase serum was used as a marker for peroxisomal localization (Lazarow and Moser, 1989) in a double label staining (Figure 3a and b). Both staining patterns were superimposable. Similar staining patterns were observed in these cells with a rabbit anti-TrpE−Mpv17 antiserum, as well as with a Mpv17-specific monoclonal antibody (data not shown). Moreover, in HepG2 cells, a human hepatoma cell line which is frequently used in peroxisome studies, a similar subcellular distribution of Mpv17 was seen (not shown). The specificity of the Mpv17 antibodies was verified using primary skin fibroblasts from homozygous Mpv17 mice and from heterozygous control animals, respectively. In a double staining experiment, both anti-catalase and Mpv17 antibodies showed the characteristic peroxisomal staining pattern in heterozygous cells (Figure 3c and d), whereas homozygous cells showed normal catalase staining (Figure 3f) but lacked Mpv17 staining (Figure 3e). Thus, the Mpv17 antibodies specifically recognize the protein missing from homozygous Mpv17 cells. In summary, these immunofluorescence studies using various poly and monoclonal antibodies against Mpv17 demonstrate colocalization with peroxisomal markers and hence peroxisomal localization of the Mpv17 protein. Based on the amino acid sequence it is suggested that the Mpv17 protein may localize to the membrane of the peroxisome but this needs further investigation by immune electron microscopy.

A function of the Mpv17 in ROS metabolism

Several human genetic diseases are known in which the primary defect is within the peroxisome (Lazarow and Moser, 1989). Some of them represent defects in single biochemical pathways, while others such as Zellweger syndrome affect peroxisome biogenesis. Two genes in which a defect can cause Zellweger syndrome have been identified and both of them are known to encode peroxisomal membrane proteins (Gärtner *et al.*, 1992; Shimozawa *et al.*, 1992). We were therefore interested in whether the Mpv17 phenotype represents a biogenesis defect or rather a specific dysfunction of a particular peroxisomal pathway. As shown in Figure 4, mutant liver peroxisomes show no structural abnormality at the level of electron microscopy. As demonstrated by diaminobenzidine staining of the peroxisomes, catalase activity appears to be present as well, indicating that at least for this enzyme the peroxisomes are not deficient or leaky. Moreover, the Mpv17 protein seems not to influence the proliferation of peroxisomes which can be induced in rodents by hypolipidaemic drugs such as bezafibrate (Reddy and Lalwani, 1983; data not shown).

In order to determine the function of the Mpv17 protein and the primary consequences of its absence, we tested mutant and non-mutant mice for several markers of peroxisomal deficiency. No significant difference concerning the C22/C26 ratio, plasmalogen synthesis, catalase activity and bile acid synthesis between the homozygous Mpv17 mice and controls where detected. Considering that peroxisomes are a site of detoxification of O_2^- radicals and peroxides, we investigated mutant and normal cells for intermediates of these pathways. A causative role of overproduction of ROS in the development of glomerulosclerosis has been hypothesized based on clinical data and measurements of H_2O_2 in experimental animal models of glomerulosclerosis (Salahudeen *et al.*, 1991; Wardle, 1993). To study the production of ROS in mutant Mpv17 cells we derived primary skin fibroblasts from mutant and non-mutant mice and tested them for the ability to produce reactive oxygen. Cells were loaded with hydroethidine which reacts with intracellular O_2^- radicals and other ROS to form the fluorescent dye ethidium (Rothe and Valet, 1990). The production of intracellular ROS was then measured by FACS analysis. Surprisingly, the result was in contrast to our expectations. While normal mouse primary fibroblasts showed a single peak of labelled cells, cells from homozygous mutant animals displayed a second peak of fluorescence at low intensity (Figure 5a and b). This second peak is coincident with that obtained with

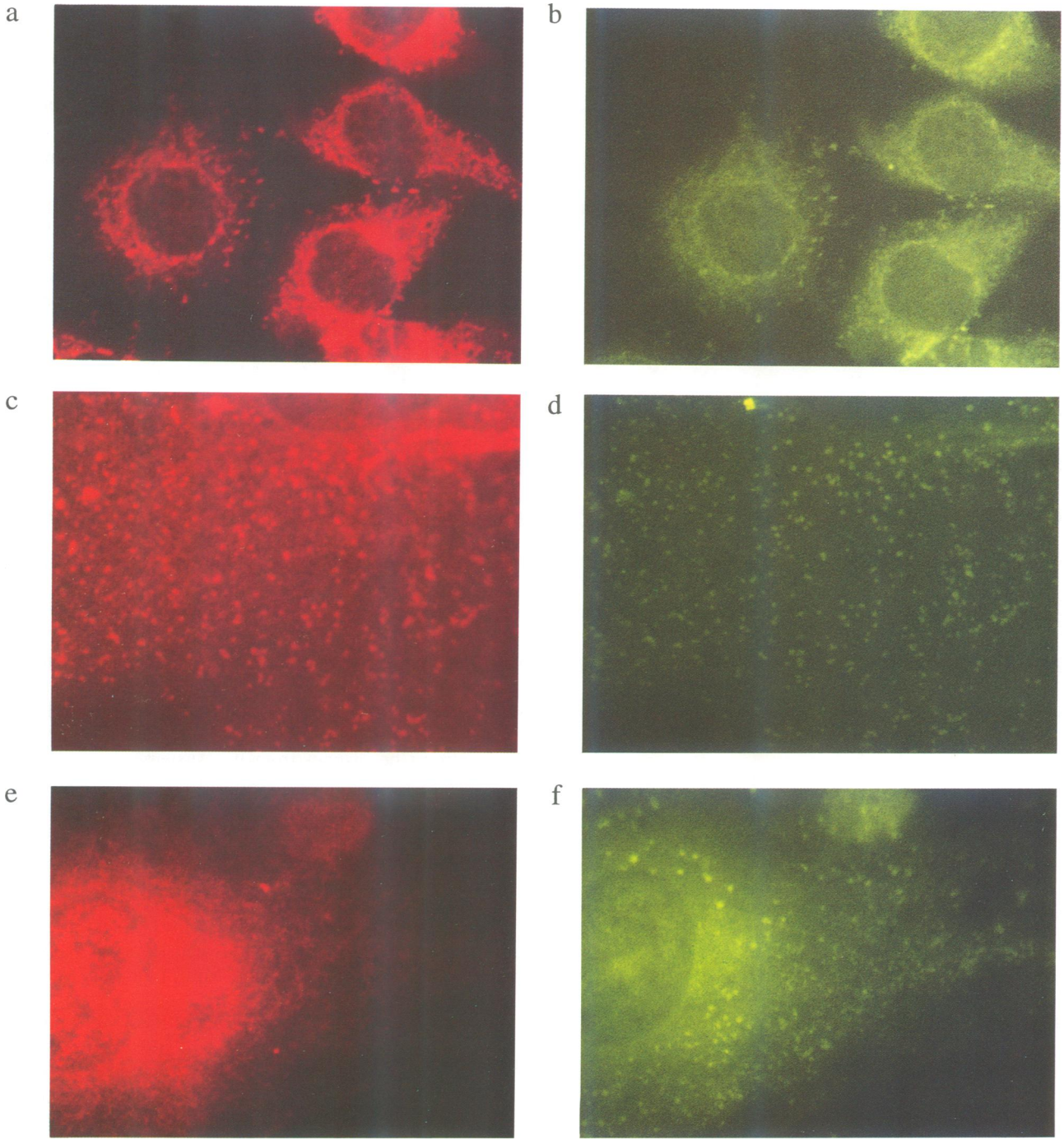

Fig. 3. Colocalization of Mpv17 protein and catalase in peroxisomes. Immunofluorescence studies were performed on murine fibroblasts: 3T3 cells (**a** and **b**), primary fibroblasts from Mpv17 heterozygous (**c** and **d**) and homozygous (**e** and **f**) mice were analysed. Mpv17 protein was visualized by a rabbit anti-GST−Mpv17 antiserum (a) and a monoclonal anti-Mpv17 antibody (c and e) decorated with TRITC labelled secondary antibodies. Catalase was stained by a sheep anti-bovine catalase antibody, which was decorated with a FITC labelled goat anti-sheep IgG antibody (b, d and f).

non-labelled cells assayed at the same wavelength (data not shown). This indicates that, at least in a subpopulation of the primary fibroblasts, lack of *Mpv17* expression leads to a complete failure to produce ROS, while in the other subpopulation different mechanisms of ROS production may be in effect. In order to investigate whether the lack of *Mpv17* expression simply interferes with a pathway involved in ROS production or if the Mpv17 protein by itself has a ROS producing activity, we analysed cells overproducing the Mpv17 protein. Therefore we studied the ROS production of cells overexpressing the *Mpv17* gene product. Thus, 3T3 cells were transfected with a construct constitutively overexpressing high levels of Mpv17 protein. Untransfected 3T3 cells, when grown subconfluently express only very low levels of Mpv17 protein (not shown) and display a distribution divided in a majority of unlabelled and a minority of labelled cells (Figure 5c). In contrast, in several independent RSVMpv17 transfectant clones expressing high levels of Mpv17 protein only one peak representing labelled cells is seen. This

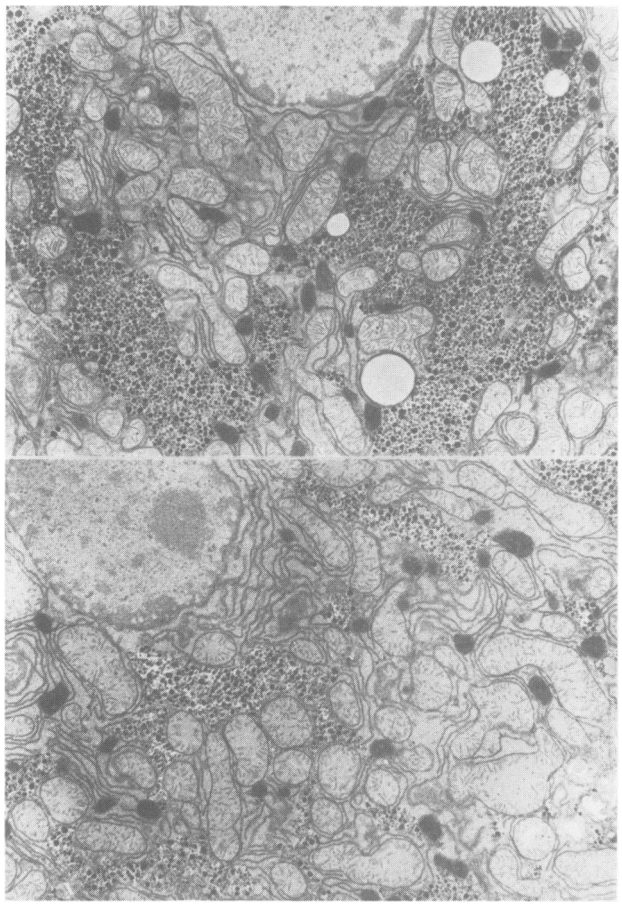

Fig. 4. Electron microscopy of periportal hepatocytes from a 45 day old Mpv17 mouse (bottom) and a control mouse (top). The peroxisomes are stained by diaminobenzidine due to their catalase activity (Gorgas, 1985). Magnification, ×11 000.

is exemplified for one clone shown in Figure 5d. These results indicate that the *Mpv17* gene product itself constitutes a major ROS producing activity in the cell or at least accomplishes a rate limiting step in this process. In summary, our results uncover an new peroxisomal function, namely the production of ROS, the absence of which intriguingly causes glomerulosclerosis in mice.

Discussion

Several transgenic mouse lines have been developed to serve as genetic animal models of glomerulosclerosis, in which overproduction of a transgene causes the phenotype (Kopp *et al.*, 1992, Dressler *et al.*, 1993). In contrast, the Mpv17 strain is a recessive mutant identifying a single endogenous gene whose loss of function induces the development of the disease. Thus, the *Mpv17* gene behaves in mice like a recessive disease gene in humans. The gene is highly conserved and is present as a single copy in mice and humans alike, suggesting that mutations in it may lead to disease in humans too. In earlier work we localized the human homologue on chromosome 2 (Karasawa *et al.*, 1993) and the question of whether Mpv17 plays a role in familial glomerulosclerosis in man is presently under investigation.

Widespread expression of the *Mpv17* gene (Weiher *et al.*, 1990) contrasts with the apparent kidney specificity of the loss-of-function phenotype and raises the question of how the primary defect causes the disease. We have addressed this by investigating the normal function of the Mpv17 protein. First, we determined its intracellular localization by immunofluorescence techniques using antibodies raised against bacterially produced Mpv17 proteins. We found that the Mpv17 protein localizes to the peroxisome. Because none of the known peroxisomal disorders in man include glomerulosclerosis, it is likely that we have identified an exciting functional link between a peroxisomal defect and a glomerular disease. Second, we investigated the function of the *Mpv17* gene product in the peroxisome. While other parameters of peroxisomal metabolism appeared unchanged, mutant cells are largely defective for the production of ROS. This was in contrast to our expectation and seems intriguing evidence for a beneficial effect of ROS production on glomerular function. In addition, we demonstrated that the Mpv17 protein itself has a ROS producing activity when overexpressed in murine fibroblast tissue culture. This activity is distinct from other enzymatic mechanisms which produce ROS in mammals, namely NADPH oxidase and xanthine oxidase (XO) (Cross and Jones, 1991). The NADPH oxidase in neutrophils consists of several membrane bound proteins and is responsible for the respiratory burst crucial for phagocytotic defence mechanisms. Mutations in this TPA inducible system in humans lead to chronic granulomatosis disease (CGD; Smith and Curnutte, 1991; Royer-Pokora *et al.*, 1986). Based on its protein sequence, intracellular localization and lack of TPA inducibility (data not shown), Mpv17 must be distinct from this system. On the other hand, XO is a peroxisomal enzyme producing O_2^- radicals upon oxidation of xanthine to urate. Mpv17 is not related to this enzyme and the phenotype of XO deficiency does not involve the kidney, making a role of Mpv17 in this pathway unlikely. Considering the peroxisome as the site of generation and degradation of H_2O_2 by superoxide dismutase and catalase respectively, Mpv17 may represent an early step in this pathway. Elevated levels of H_2O_2 in transfectants overexpressing the Mpv17 protein support this view (data not shown).

The question of whether the effect of the Mpv17 defect on the glomerulus is a direct one is still open. In this regard it is provocative that high Mpv17 protein expression is found in podocytes (R.Waldherr *et al.*, unpublished), a cell type which plays an important role in producing the glomerular basement membrane (GBM), the primary site of damage in glomerulosclerosis. This damage could be imposed directly by the lack of O_2^- or by the accumulation of a metabolic intermediate, which in the presence of the Mpv17 protein is detoxified in a reaction producing O_2^- as a by-product. Regardless, preliminary evidence indicates that the expression of Mpv17 in cells regulates the expression of genes which are potentially involved in the biosynthesis of the glomerular basement membrane. Such a mechanism could also account for resolution of the apparent contradiction that the loss of a ubiquitously expressed gene function causes the tissue-specific phenotype observed. It will therefore be interesting to investigate if the Mpv17 protein is indeed a general regulator of intracellular reactive oxygen and thereby reactive oxygen dependent gene expression.

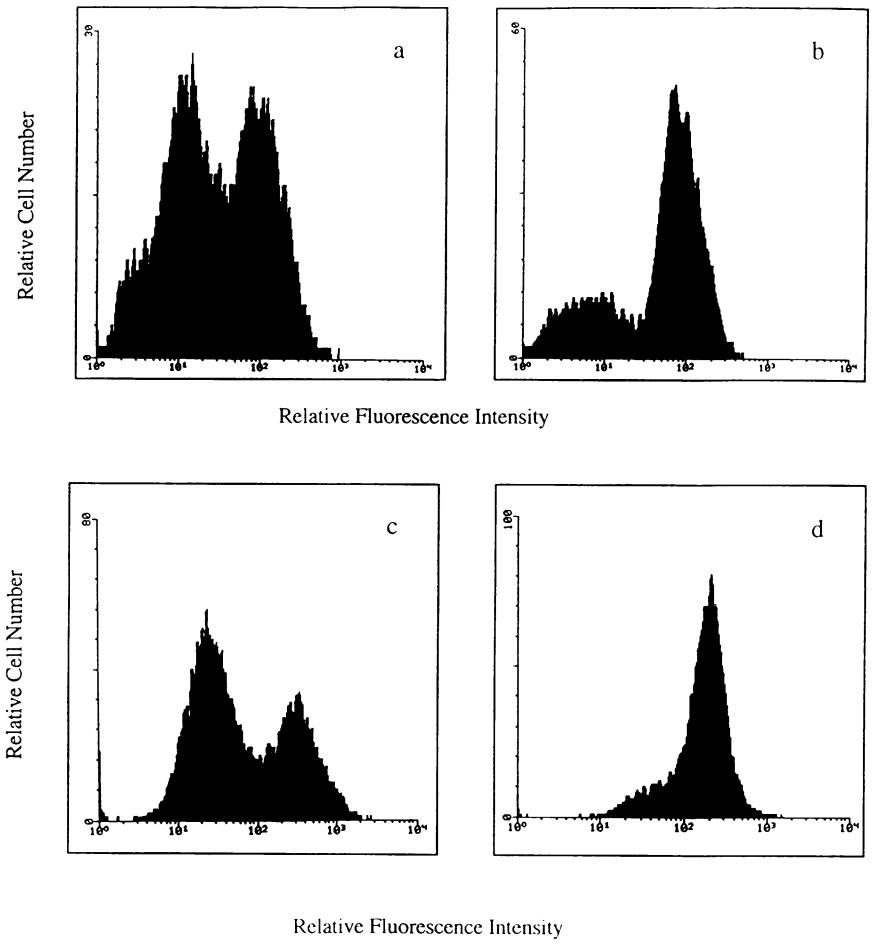

Fig. 5. Reactive oxygen species produced in primary skin fibroblasts from Mpv17 and control mice. Hydroethidine (HE) histograms each representing 10 000 cells after HE staining and analysis by FACS. (**a**) Mpv17 homozygous primary fibroblasts, (**b**) Mpv17 heterozygous primary fibroblasts, (**c**) mouse NIH 3T3 fibroblasts and (**d**) RSVMpv17.5 cells: NIH 3T3 cells transfected with a gene construct expressing the Mpv17 protein of a RSV promoter conferring at least 10-fold overexpression compared with non-transfected cells (A.Reuter and H.Weiher, in preparation).

Recently, a mouse mutant has been described, in which the *bcl2* gene has been inactivated by insertion (Veis *et al.*, 1993). Bcl2 protein, an inhibitor of apoptosis, has a role in protection against H_2O_2 and lipid peroxidation (Hockenbery *et al.*, 1993), in contrast to the Mpv17 protein. Interestingly, a null mutation for *bcl2* in mice also exhibits a phenotype in the kidney, although the pathology of kidney disease in these mice is different from that in the Mpv17 mice. This suggests that the proper function of this organ critically depends on an intricate regulation of radical metabolism. Future experiments will be aimed at defining the particular biochemical reaction and the respective pathway affected by the absence of the Mpv17 protein.

Materials and methods

Cell culture

NIH 3T3, mouse lung fibroblasts, GM 637 cells and primary skin fibroblasts were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

Preparation of primary mouse skin fibroblasts

A piece of skin was taken from newborn mice, 24 h after birth, and placed in Dulbecco's modified Eagle's medium containing 0.05% collagenase A (Boehringer Mannheim). After overnight incubation at 4°C, the epidermal cell layer was carefully removed. The remaining dermis was suspended by pipetting in normal medium containing 10% serum and cultured until cells reached confluency.

Preparation and analysis of poly(A)$^+$ RNA

Poly(A)$^+$ RNA was isolated from five 14 cm plates, which were all grown to 90% confluency. Cells were lysed in 100 mM NaCl, 20 mM Tris–HCl pH 7.4, 10 mM EDTA, 0.1% SDS and proteinase K (300 μg/ml).

The lysate was incubated overnight with oligo(dT)-cellulose type VII (Pharmacia). After several washing steps with 400 mM NaCl, 20 mM Tris pH 7.4, 10 mM EDTA, 0.2% SDS and 100 mM NaCl, 20 mM Tris pH 7.4, 10 mM EDTA, 0.2% SDS, RNA was eluted with diethyl pyrocarbonate (DEPC) treated water and ethanol precipitated. Subsequently the RNA pellet was purified with phenol and chloroform and again precipitated. 4 μg of poly(A)$^+$ RNA were loaded on a 1% agarose gel supplemented with 9% formaldehyde, which was run in MOPS buffer [20 mM morpholino-propyl-sulfonate (MOPS), 5 mM Na acetate, 0.5 mM EDTA, pH 7.0]. The RNA was transferred to a nylon membrane (Hybond N, Amersham), which was prehybridized in 6× SSC, 0.5% SDS, 1% Ficoll, 1% polyvinylpyrrolidone, 1% bovine serum albumin and 250 mg/ml denatured salmon sperm DNA for 2 h at 65°C. The radioactively labelled probe (Feinberg and Vogelstein, 1983) was subsequently added and incubated overnight at 65°C. The filter was washed 15 min with 2×SSC, 1% SDS and twice for 15 min in 0.1×SSC, 0.5% SDS. All washing steps were performed at 65°C in a rocking water bath. The probe was the coding region of human *Mpv17* cDNA.

Generation of antibodies

The human *Mpv17* cDNA was cloned into the pGex vector system (Pharmacia) and the glutathione-*S*-transferase (GST)–Mpv17 fusion protein expressed in *E.coli* according to Smith and Johnson (1988).

The protein was cut out of a large scale SDS–PAGE (Laemmli, 1970) and purified using an electro-elution device (Schleicher & Schuell) in 25 mM Tris, 192 mM glycine, 0.025% SDS. For the production of monoclonal antibodies two Balb/c mice were injected intraperitoneally with 100 μg of the GST–Mpv17 fusion protein. Three further injections with 50 μg of the same antigen in incomplete Freund's adjuvant were given intraperitoneally in 3 week intervals. Test bleeds were used in an ELISA assay and the mouse showing the best immune response received three intravenous injections of 50 μg of purified GST–Mpv17 diluted in PBS. Three days later the spleen was removed from this animal and spleenocytes were fused with Ag 8 myeloma cells according to standard procedures (Harlow and Lane, 1988). From three positve clones, which were identified by ELISA and Western blot analysis, one turned out also to recognize the native form of the Mpv17 protein in immunofluorescence experiments: MAb 3D8. MAb 3D8 producing cells were grown in mass culture and the antibodies were purified using an anti-mouse IgM affinity column (Sigma).

Of the two rabbits immunized according to standard protocols (Harlow and Lane, 1988), one antiserum was positive and was purified via a protein A–Sepharose column (Pharmacia) and then used in Western analyses and immunofluorescence studies in comparison with the also affinity purified pre-immune serum.

Western blot analysis

Total protein extracts were prepared from tissue culture cells grown on 10 cm plates. The culture medium was removed and the adherent cells were washed twice with PBS. Cells were scraped off using a rubber policeman and spun down in 1.5 ml sample tubes. The cell pellet was resuspended in Laemmli loading buffer, heated to 95°C for 5 min and subsequently sonified for 10 s. 100 μg of total protein were electrophoresed on a 12.5% SDS–PAGE and transferred to an Immobilon-P membrane (Millipore) using a semi-dry electroblotting apparatus (Bio-Rad). The filter was blocked in 5% non-fat dried milk in TBS containing 0.3% Tween 20 for 1 h and incubated with the polyclonal rabbit anti-Mpv17 serum and pre-immune serum, respectively. The filter was then treated with horseradish peroxidase conjugated anti-hobbit Ig (Amersham) and signals were detected using the ECL system (Amersham).

Immunofluorescence microscopy

Cells were grown on glass cover slips for 2 days, after which the medium was removed and the cells were washed in PBS before they were fixed with freshly prepared 4% paraformaldehyde. After washing with PBS, cells were permeabilized using 0.2% Triton X-100 followed by rinsing the coverslips with PBS. Primary antibodies were applied in 10% FCS in PBS and were incubated overnight at room temperature.

The *Mpv17* gene product and catalase were visualized using the following antibodies: MAb 3D8, rabbit anti-Mpv17 and sheep anti-bovine catalase (Binding Site). The primary antibodies were decorated with TRITC-conjugated goat anti-mouse (Jackson), TRITC-conjugated goat anti-rabbit and FITC-conjugated donkey anti-sheep secondary antibodies, which were incubated for 30 min at 37°C in PBS containing 10% FCS. After each incubation the cells were washed extensively in PBS. The coverslips were mounted on glass slides using Dako Glycergel and the fluorescent staining pattern was viewed in a fluorescence microscope (model Zeiss Axioskop). Images were recorded on Kodak Ektachrome Panther P1600X film.

Fluorescent measurement of intracellular ROS

Exponentially growing cells (5×10^5) were washed twice with PBS, trypsinized, pelleted and stained with hydroethidine (Polysciences) according to the manufacturer's recommendations. Cells (10 000) were analysed using a Becton Dickinson FACStar with excitation and emission settings described elsewhere (Rothe and Valet, 1990).

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