Inflammation-induced transcriptional regulation of Golgi transporters required for the synthesis of sulfo sLex glycan epitopes

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Received on May 27, 2004; revised on July 12, 2004; accepted on July 14, 2004

The de novo synthesis and expression of sulfo sLex glycan on vascular endothelial glycoproteins has a central role in the initiation of inflammatory reactions, serving as a putative ZIP code for organ-specific trafficking of leukocytes into sites of inflammation. The synthesis of sulfo sLex requires energy carrying donors, CMP-sialic acid (CMP-SA), GDP-fucose (GDP-Fuc), and adenosine 3′-phosphate 5′-phosphosulphate (PAPS) for donation of SA, Fuc, and sulfate, respectively. These donors are synthesized in the nucleus or cytosol and translocated into Golgi by specific transporters where corresponding transferase and proteins as well as enzymatic activities increase on inflammatory stimuli. Here we analyze the transcriptional coregulation of CMP-SA, GDP-Fuc, and PAPS transporters with in situ hybridization and real-time PCR in acute inflammation using kidney and heart allografts as model systems. Our results indicate that these three transporters display transcriptional regulation exists for Golgi membrane transporters required for the synthesis of the inflammation-inducible ZIP code sulfo sLex glycans.

Key words: Transporter/Golgi/fucosylation/sialylation/sulfation

Introduction

The traffic of leukocytes from blood circulation into tissues is termed extravasation. Infiltration of leukocytes from blood circulation into various target tissues is the hallmark of all inflammatory reactions. L-selectin on leukocytes and its endothelial sulfo sLex-decorated ligands have been shown to be crucial mediators of the tethering and rolling phases of the leukocyte extravasation cascade (Hemmerich and Rosen, 2000; Lowe, 2003; Rosen, 1999; Vestweber, 2003; von Andrian, 2003). Although sulfo sLex glycan epitopes are constitutively expressed in lymph node high endothelium, they are induced de novo to vascular endothelium at sites of inflammation in both rodents and humans (Fukuda, 2002; Lowe and Marth, 2003; Paavonen, and Renkonen, 1992; J. Renkonen et al., 2002; Toppila et al., 2000; Turunen et al., 1995; van Zante and Rosen 2003; Vestweber, 2002). These epitopes have been suggested to vary slightly based on their differential reactivity detected with a panel of sLex and extended core 1 sulfated polyacactosines (J. Renkonen et al., 2002).

Sulfo sLex decorations are added to the proteins as post-translational modifications in the Golgi as a result of consecutive enzymatic reactions. Specific transferases use the high-energy nucleotide derivatives, CMP-sialic acid (CMP-SA), GDP-fucose (GDP-Fuc), and adenosine 3′-phosphate 5′-phosphosulphate (PAPS) as donors to yield the sulfo sLex epitope. The synthesis of sulfo sLex glycans starts with α2,3 sialylation of the galactose followed by β fucosylation of the N-acetylgalcosamine (Bistrup et al., 1999; Ellies et al., 1998; Hiraoa et al., 1999; Homeister et al., 2001; Maly et al., 1996; Mitoma et al., 2003; Yeh et al., 2001). The CMP-SA is synthesized in the nucleus via the CMP-SA synthase (CMAS, LocLink 5590) (Eckhardt et al., 1996; Krapp et al., 2003). Two different cytosolic pathways lead to the formation of GDP-L-fucose. The GDP-Fuc de novo pathway involves conversion of GDP-α-L-mannose to GDP-β-L-fucose by two enzymes, the GDP-α-mannose-4,6-dehydratase (GMDS, LocLink 2762) and GDP-α-keto-6-deoxy-α-mannose-3,5-epimerase-4-reductase (TSTA3 or FX, LocLink 7264). In the alternative, that is, salvage biosynthetic pathway, l-fucokinase (FUK, LocLink 197258) synthesizes L-fucose-1-phosphate from l-fucose and ATP. GDP-L-fucose pyrophosphorylase (FUK, LocLink 8790) catalyzes the formation of GDP-L-fucose from l-fucose-1-P and GTP (Becker and Lowe, 2003; Niittymaki et al., 2004). PAPS serves as the universal sulfonate donor compound for all sulfotransferase reactions. PAPS is synthesized in two sequential steps and in humans the bifunctional PAPS synthase (isoforms PAPSS1 and PAPSS2, LocLinks 9060 and 9061, respectively) catalyzes...
the biosynthesis. Inorganic sulfate first combines with ATP to form adenosine 5'-phosphosulfate (APS) and pyrophosphate catalyzed by ATP sulfurylase domain. In the second step, APS combines with another molecule of ATP to form PAPS and ADP catalyzed by APS kinase domain (Xu et al., 2000, 2003).

After synthesis, all these donors are transported to the Golgi via specific transporters to serve as donors in the transferase reactions leading to the decoration of glycoproteins (see Figure 1). The genes coding for the CMP-SA transporter (SLC35A1, LocLink 10559) (Aoki et al., 2001; Eckhardt et al., 1996, 1998, 1999; Ishida et al., 1998; Oelmann et al., 2001), the GDP-Fuc transporter (FUCT1, LocLink 55343) (Hidalgo et al., 2003; Lubke et al., 2001; Luhn et al., 2001; Puglielli and Hirschberg, 1999; Roos et al., 2002) and for the PAPS transporter (SLC35B2, LocLink 347734) (Kamiyama et al., 2003) have been recently characterized. They all are members of the multispan membrane proteins.

The integrity of these three different pathways, which all participate to yield sulfo sLex glycan structure, has been shown to be crucial for the viability and homeostasis of an organism. Mice bearing a deletion of the UDP-N-acetylgalactosamine-2-epimerase/N-acetyllmannosamine enzyme (GNE, LocLink 10020) responsible for the CMP-SA synthesis display embryonic lethal phenotype (Schwarzkopf et al., 2002). Likewise, mice lacking the fx gene are embryonic lethal if the dietary fucose is depleted (Smith et al., 2002), and human patients with dysfunctional GDP-Fuc transporter show a severe form of leukocyte adhesion deficiency (LADII) and also suffer from mental retardation (Lubke et al., 2001; Luhn et al., 2001). Finally a recent study in fruit flies also indicated that PAPS transporter (slalom in Drosophila) is crucial for cell signaling during development and essential for differentiation and viability (Luders et al., 2003).

Immunohistochemical analyses have suggested that a rapid de novo induction of endothelial sulfo sLex epitopes is the key event guiding leukocyte infiltration into tissues at the initiation of an inflammatory process (Toppila et al., 1994; Turunen et al., 1995). Thus much interest has been focused on understanding how this process is regulated. Both the enzymatic activity as well as the protein levels of relevant glycosyltransferases, such as the FucTVII enzyme (FUT7, LocLink 2529), crucial for the synthesis of the α1,3 fucosylation into sialylated acceptors, are induced within the Golgi during the inflammatory processes (Majuri et al., 2003; Toppila et al., 1999). In addition, the expression levels of various glycosyltransferase transcripts, such as sialyl- and fucosyltransferases, have been shown to be up-regulated in various settings related to inflammatory episodes or lymphocyte development (Gillespie et al., 1993; Kannagi, 2002; Smithson et al., 2001).

Here we analyze the expression of CMP-SA-, GDP-Fuc-, and PAPS transporters by in situ hybridization and real-time polymerase chain reaction (PCR) during rat organ allograft rejection as a model for sulfo sLex-dependent inflammatory events. Moreover, the expression profiles of CMP-SA and GDP-fucose transporters were analyzed in silico using data from 230 Affymetrix U133A human transcriptome gene chips.

Results and discussion

The CMP-SA transporter was the first sugar nucleotide transporter cloned from a mutant Chinese hamster ovary cell line (Eckhardt et al., 1996). Recently a rare LADII (CDGIIc) disease with essentially no fucosylated glycans was shown to be caused by a dysfunctional GDP-fucose transporter (Lubke et al., 2001; Luhn et al., 2001). The first hint of the molecular basis of the PAPS transport was obtained nearly a decade ago (Mandon et al., 1994), when the rat PAPS transporter was purified to apparent homogeneity from the liver Golgi membranes. It was found to be a 75-kDa homodimeric protein, and the Drosophila and mammalian homologs of the PAPS transporter gene sequence and the corresponding putative proteins were identified and verified by biochemical activity assays (Kamiyama et al., 2003; Luders et al., 2003).

Kidney and heart allograft rejection after transplantation between major histocompatibility complex–incompatible inbred rat strains was chosen as a model of acute inflammation. Here de novo expressed sulfo sLex epitopes on the graft endothelium have been shown to play a crucial role in the recruitment of leukocytes into the site on inflammation (Kirveskari et al., 2000; Toppila et al., 1999; Turunen, 1995), and thus we analyzed whether the transcription of these three transporters, crucial for the synthesis of sulfo sLex glycans, would be coregulated.

In situ labeling of the sugar nucleotide transporters

In control kidneys, the level of GDP-Fuc transporter mRNA was below the detection limit with the in situ hybridization. Three days after transplantation, a clear up-regulation of transcription was seen in the kidney cortex and outer medulla, the site of leukocyte infiltration during the early phases of acute rejection. The inner medullary area was not infiltrated by leukocytes during allograft rejection and remained negative throughout the experiment. The signal was strong at the corticomедullary junction and in the transitional epithelium of renal pelvis. During the fourth postoperative day, the signal for GDP-Fuc transcripts was very strong and evenly distributed in the cortex, outer medulla, and transitional epithelium, after
which it decreased and displayed a patchy expression pattern (Figure 2A).

On higher magnification, the glomeruli of the cortex were devoid of signal (Figure 2B), whereas it was detected in peritubular capillaries both in cortex and medulla (Figure 2C, D). Peritubular capillaries have previously been shown to de novo express sulfo sLex, acquire high endothelial morphology, and display lymphocyte-specific adhesion during acute rejection episodes (Kirveskari et al., 2000; Renkonen et al., 1990; Turunen et al., 1994). The transitional epithelium also expressing sLex glycan showed a clear GDP-Fuc signal, and in the kidney tubules no signal was observed (data not shown).

To show that the inductions observed were not solely linked to kidney inflammation, we also performed rat heart allograft transplantations. Our previous studies have shown that the induction of inflammatory leukocyte extravasation in rat heart allografts is due to the induction of endothelial sulfo sLex glycans (Toppila et al., 1999). Although no signal for GDP-Fuc transporter was evident in control hearts, a specific signal was recorded under the epicardium 3 days after transplantation that coincides with the appearance of the first signs of acute allograft rejection, that is, inflammation. After 4 days, a very strong signal was evident, especially in the walls of the right ventricle and under the pericardium at sites where the leukocyte

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**Fig. 2.** GDP-Fuc transporter. In the control kidney (cont) no signal is evident. At 3 days after transplantation, a clear signal could be seen in the cortex (co) and the outer medulla (me), whereas levels in the inner medulla (papilla = pa) were low. A strong signal was seen in the corticomedullary junction (arrowheads) and the transitional epithelium of renal pelvis (arrows). At 4 days after transplantation a strong signal was present in the cortex and outer medulla. The papillary region showed a lower signal, and at 5 days the expression was diminished (A). In dipped section (4 days) the glomerulus (arrowheads) and adjacent arteriole (ar) were devoid of labeling (B). The tubule of the nephron (arrowheads) and arteriole (ar) were negative in the kidney cortex (C). In the medulla, tubules (arrowheads) were nonlabeled and surrounding tissue exhibited signal (D). Control hearts (cont) showed no signal. A low signal was present at 3 days under the epicardium (arrowheads), and a strong signal could be seen especially in the walls of the right ventricle (arrowheads) at 4 days. The signal decreased at 5 days (E). In dipped section the signal was present in the cells (arrows) of the lymphatic infiltrate (li) and intramuscular capillaries (arrowheads) and the cardiac muscle was devoid of labeling (F). An artery was negative (ar) and the surrounding cells showed a signal (G). A clear signal was seen in the endothelium of a capillary (arrowheads) (H).
infiltration caused acute rejection. Low diffuse labeling was detected at 5 days after transplantation (Figure 2E). The GDP-Fuc was present in the lymphocytes and capillary endothelium, whereas cardiac muscle cells were nonlabeled (Figure 2F, H). The arteries did not exhibit any signal.

No signal for the PAPS transporter was seen in control kidneys, but after 3 days a clear signal was present in cortex and outer medulla, that is, in the same area where the rejection occurred as well as the GDP-Fuc signal was induced (Figure 3A). The strongest signal was seen in the corticomedullary junction and in the transitional epithelium of renal pelvis. At 4 days both the cortex and inner medulla showed a strong signal for PAPS transporter, which then declined at day 5 (Figure 3A). In high-power micrographs, the glomeruli were not labeled (not shown), whereas peritubular capillaries in the cortex and medulla and the transitional epithelium displayed a clear signal (Figure 3B, C, D). The renal tubules and the inner medulla showed no labeling for the PAPS transporter.
In control heart, no labeling could be seen. Concomitant with the early signs of acute allograft rejection at 3 days, low labeling of PAPS transporter was seen under the pericardium. At day 4 a moderate signal was seen in the walls of the right ventricle, which then diminished a day later (Figure 3E). On higher magnification, a signal could be seen in lymphocytes and capillary endothelium, but cardiac muscle cells remained nonlabeled (Figure 3F, G, H).

Our results with oligoprobes against the rat CMP-SA transporter sequence gave no signals in the normal nor the transplanted heart or kidney allografts undergoing fulminant rejection at 3–5 days after surgery (data not shown).

**Real-time PCR analysis of the transporters**

To have a more quantitative evaluation (even without spatial information) of the expression levels of the transporter transcripts during kidney and heart allograft rejection, we performed quantitative real-time PCR. Both the GDP-Fuc and PAPS transporters were clearly induced in a time-dependent manner during the rejection episodes in both organs, and these data are very much in concordance with the data obtained with the *in situ* hybridizations (Figure 4). Usually the kidney allograft rejection affected larger areas of tissue and thus could at least partly explain the difference between the levels of expression of the transporter mRNAs in kidneys and hearts.

Real-time PCR analyses indicated that also the CMP-SA transporter was induced during the inflammatory reaction both in the kidney and in the heart (Fig. 4). The most probable explanation why we failed to see CMP-SA transporter signal with *in situ* hybridization is the higher sensitivity of the PCR assay over the hybridizations, because the levels of the mRNA was at least on the same level as for the other transporters analyzed both by the quantitative real-time PCR as well as the predicted from the Affymetrix analysis.

**In silico analysis of the expression of CMP-SA and GDP-Fuc transporters**

Our wet lab results suggested that the two genes, CMP-SA and GDP-Fuc transporters, are coregulated on an inflammatory stimuli caused by the allograft rejection-induced inflammation. Next we wanted to know whether these two genes would be coregulated over a large number of experiments from diverse cell types and a broad range of physiological and experimental conditions.

The current rapidly increasing, publically available transcriptome data from gene chip analysis allows one to rapidly perform experiments *in silico*, which used to a require extensive wet lab experimentation. After extracting data from 230 Affymetrix human U133A gene chip experiments containing the probes for both CMP-SA and GDP-Fuc transporters (Affymetrix codes 203306_s_at and 218485_s_at, respectively), we were able to show that they are not substantially coregulated, because their correlation coefficient for these two transporters was only 0.53 (Figure 5). Thus there seems to be many other conditions in which the cell prefers to synthesize either sialylated or fucosylated but perhaps not dually decorated glycans. Unfortunately the PAPS transporter and the endothelial-specific sulfotransferases were not present in the first edition of the Affymetrix U133A/B, and thus their expression levels could not be analyzed in this *in silico* assay.

The mean expression levels were calculated for all 22,215 genes over the 230 experiments. The genes were then divided into quartiles based on these values; the mean expression levels within each quartile were 1396.1, 282.9, 115.3, and 35.9, respectively. Furthermore, the genes were divided into four categories based on ranges of mean expression values; 0–50, 51–100, 101–1000, and 1001-max (Table I). When comparing the mean expression values for CMP-SA and GDP-Fuc transporters, we could show that they were expressed relatively abundantly in most of the experiments (mean expression 505.8 and 259.6, respectively; see Table II), although their expression levels varied extensively depending on experimental conditions or tissue/cell sources.

After demonstrating that the two transporters are essentially very poorly coregulated, the next questions was if any other genes would be coregulated with either of them over the 230 Affymetrix U133A gene chip data collection. From these analyses we picked the 10 best coregulated genes (proteins) for both transporters (Table III). The best coregulated genes for CMP-SA transporter included receptor-type protein tyrosine phosphatase 2, interferon-inducible protein and myc-binding protein linked to signal transduction and regulation of transcription, MUC5AC, a mucin-type membrane glycoprotein very rich in glycan...
decorations, and GALNT12, a glycosyltransferase gene involved in O-linked glycan synthesis. Likewise, the genes coregulated best with the GDP-Fuc transporter included genes involved in the regulation of transcription, such as PAX6 and KLF5 transcription factors. Also aquaporin 5, prostaglandin E synthase, linked to inflammatory process, and GMDS, an enzyme participating in the synthesis of GDP-Fuc, were present on the list of coregulated genes. Considered together, previous studies have shown that inflammation-related stimuli can up-regulate the expression levels of several glycosyltransferase transcripts, as well as the corresponding enzymatic activities and protein levels (Gillespie et al., 1993; Kannagi, 2002; Majuri et al., 1994; Smithson et al., 2001; Toppila et al., 1999). Our data suggest that there is an endothelial temporospatial induction of GDP-Fuc and PAPS transporters and at least a time-dependent induction of CMP-SA transporter at the time the sulfo sLex-dependent leukocyte extravasation begins into sites of tissue inflammation. Furthermore these same genes can also be up-regulated in the infiltrating leukocytes participating the rejection.

Materials and methods

Rats and transplantations

Major histocompatibility–incompatible inbred WF (RT1v) and DA (RT1a) rats obtained from the Laboratory Animal Center, University of Helsinki, Finland, were used for the transplantations. The rats were 12–14 weeks old with a mean weight of 330 g. A modified microvascular technique as described previously was applied and permission to perform the experiments was received from the provincial office of Uusimaa (R. Renkonen et al., 2002; Turunen et al., 1995). DA hearts transplanted into WF recipients were allografts, WF grafts to WF and DA grafts to DA served as syngeneic controls, and normal nontransplanted hearts were controls. The study plan was approved by the Institutional Ethical Review Board. All animals received humane care in compliance with the Principles of Laboratory Animal Care and Guide for the Care and Use of Laboratory Animals prepared and formulated by the National Institutes of Health (NIH Publication no. 86-23, revised 1985).

In situ hybridization

After decapitation of the animals, the kidneys and hearts were excised and frozen on dry ice. Serial 14-μm-thick sections were cut with a Microm HM-500 cryostat (Microm, Heidelberg, Germany), the sections were thawed on polystyrene, and stored at −20°C until used. For each gene three oligonucleotide probes were designed and used as a mixture in in situ hybridization experiments.

Oligonucleotide probes for PAPS transporter (interim name: solute carrier family 35, member B2; gene symbol: SLC35B2; LocusLink ID: 316241) were designed based on GeneBank/EMBL/DDBJ entry AC096454 as follows: 5’-CGCCAGCACCTGGGTTAGGAAGCTGA-3’ (covering

Table I. The demographic values of the expression levels of all the 22,215 genes over 230 Affymetrix U133A chip array experiments

<table>
<thead>
<tr>
<th>Range</th>
<th>Mean intensity</th>
<th>No. of genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–50</td>
<td>28.1</td>
<td>4162</td>
</tr>
<tr>
<td>50–100</td>
<td>73.6</td>
<td>3494</td>
</tr>
<tr>
<td>100–1000</td>
<td>339.9</td>
<td>12358</td>
</tr>
<tr>
<td>1000–max</td>
<td>2539.2</td>
<td>2201</td>
</tr>
</tbody>
</table>

Table II. The demographic values of the expression levels of the GDP-Fuc transporter (218485_s_at) and CMP-SA transporter (203306_s_at) over 230 Affymetrix U133A chip array experiments

<table>
<thead>
<tr>
<th></th>
<th>GDP-Fuc transporter</th>
<th>CMP-SA transporter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Min</td>
<td>7.3</td>
<td>121.9</td>
</tr>
<tr>
<td>Max</td>
<td>854.3</td>
<td>1496.1</td>
</tr>
<tr>
<td>Range</td>
<td>847</td>
<td>1374.2</td>
</tr>
<tr>
<td>Mean</td>
<td>259.6</td>
<td>505.8</td>
</tr>
<tr>
<td>SD</td>
<td>130.5</td>
<td>240.2</td>
</tr>
</tbody>
</table>
nucleotides 137370 to 137403), 5'-CATGATGATGGGAAA-GCTTGTTCTCGGCGCAGC-3' (covering nucleotides 137346 to 137313), and 5'-TGTCACTGTGGTGGGAGGA-CTGGGAGTAGCTGTG-3' (covering nucleotides 136848 to 136815).

Oligonucleotide probes for rat protein similar to GDP-Fuc transporter 1 (gene model name: LOC311204; LocusLink ID: 311204) were designed based on GeneBank/EMBL/DDBJ entry BF557232 as follows: 5'-AAGATGGGGGTATCCAGCTGCAGGGAGGGCT-3' (covering nucleotides 9 to 42), 5'-ACCATGCCAGGGGCAGGAGCT-3' (covering nucleotides 90 to 123), and 5'-CATGCGTGAATAAGAC-GAGCGGAGCGGAGCACA-3' (covering nucleotides 166 to 199).

Oligonucleotide probes for rat protein similar to CMP-SA transporter (gene model name: LOC313139; LocusLink ID: 313139) were designed based on GeneBank/EMBL/DDBJ entry AI072449 as follows: 5'-GGGCTGGTTTC-CCACTGTACAAGTGTGACCCCACC-3' (covering nucleotides 343 to 376), 5'-GGTCACCTGGTACACT-GCCGCATCCAGGTTACTG-3' (covering nucleotides 473 to 506), and 5'-CCACCACTGACGTGTAGAGGCCTCCCACACTAGC-3' (covering nucleotides 43 to 76).

Table III. 10 best correlating transcripts (proteins) for either GDP-Fuc transporter (218485_s_at) and CMP-SA transporter (203306_s_at) over 230 Affymetrix U133A chip array experiments

<table>
<thead>
<tr>
<th>Name</th>
<th>Gene symbol, entry name</th>
<th>Correlation</th>
<th>SwissProt</th>
<th>Affymetrix</th>
<th>GO:Process and Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMP-SA tr</td>
<td>PTPRN2, Receptor-type protein-tyrosine phosphatase</td>
<td>0.827</td>
<td>Q92932</td>
<td>203029_s_at</td>
<td>protein amino acid dephosphorylation</td>
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<tr>
<td></td>
<td>KIAA0471 protein</td>
<td>0.824</td>
<td>O75059</td>
<td>203020_at</td>
<td>na</td>
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<tr>
<td></td>
<td>IFI16, Gamma-interferon-inducible protein IIf-16</td>
<td>0.813</td>
<td>Q16666</td>
<td>208966_x_at</td>
<td>regulation of transcription, cell proliferation; hemopoiesis</td>
</tr>
<tr>
<td></td>
<td>ibid</td>
<td>0.805</td>
<td>Q16666</td>
<td>206332_s_at</td>
<td>regulation of transcription, cell proliferation; hemopoiesis</td>
</tr>
<tr>
<td>MYCBP, C-Myc binding protein</td>
<td>0.804</td>
<td>Q96KG0</td>
<td>203359_s_at</td>
<td>na</td>
<td></td>
</tr>
<tr>
<td>MUC5AC, Mucin 5AC</td>
<td>0.799</td>
<td>O60460</td>
<td>214303_x_at</td>
<td>cell adhesion</td>
<td></td>
</tr>
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<td>ibid</td>
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<td>O60460</td>
<td>214385_s_at</td>
<td>cell adhesion</td>
<td></td>
</tr>
<tr>
<td>IL20RA, Class II cytokine receptor ZCYTOR7</td>
<td>0.799</td>
<td>Q9UHF4</td>
<td>219115_s_at</td>
<td>blood coagulation, cytokine receptor activity</td>
<td></td>
</tr>
<tr>
<td>RAB11A, Ras-related protein, endosomal traffic</td>
<td>0.798</td>
<td>P24410</td>
<td>200864_s_at</td>
<td>RAB small monomeric GTPase activity</td>
<td></td>
</tr>
<tr>
<td>GALNT12, UDP-GalNAc-transferase 12</td>
<td>0.797</td>
<td>Q8IXK2</td>
<td>218885_s_at</td>
<td>transferase activity</td>
<td></td>
</tr>
<tr>
<td>SSH3BP1, Spectrin SH3 domain binding protein 1</td>
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<td>O76049</td>
<td>209028_s_at</td>
<td>na</td>
<td></td>
</tr>
<tr>
<td>TFF1, Trefoil factor 1</td>
<td>0.794</td>
<td>P04155</td>
<td>205009_at</td>
<td>carbohydrate metabolism; cell growth/maintenance</td>
<td></td>
</tr>
<tr>
<td>GDP-fuc tr</td>
<td>AQP5, Aquaporin 5, Pax6, transcription factor</td>
<td>0.816</td>
<td>P55064</td>
<td>213611_at</td>
<td>excretion; water transport</td>
</tr>
<tr>
<td></td>
<td>KLF5, Krueppel-like factor 5</td>
<td>0.809</td>
<td>P26367</td>
<td>205646_s_at</td>
<td>organogenesis; vision; CNS development</td>
</tr>
<tr>
<td></td>
<td>PTGES, Prostaglandin E synthase</td>
<td>0.808</td>
<td>P35287</td>
<td>209212_s_at</td>
<td>transcription from Pol II promoter</td>
</tr>
<tr>
<td></td>
<td>MSLN, Mesothelin</td>
<td>0.801</td>
<td>O14684</td>
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<td>prostaglandin metabolism; signal transduction</td>
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<td></td>
<td>RARRES1</td>
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<td></td>
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<td>0.791</td>
<td>P49788</td>
<td>221872_at</td>
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<tr>
<td>LXN, Latexin protein</td>
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<td>P49788</td>
<td>206392_s_at</td>
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<tr>
<td>Q8TEF8, Hypothetical protein</td>
<td>0.787</td>
<td>Q9BS40</td>
<td>218729_at</td>
<td>na</td>
<td></td>
</tr>
<tr>
<td>GMDS, GDP-mannose 4,6 dehydratase</td>
<td>0.787</td>
<td>Q8TEF8</td>
<td>210367_s_at</td>
<td>carbohydrate metabolism; GDP-L-fucose biosynth.</td>
<td></td>
</tr>
<tr>
<td>TMPRSS4, Transmembrane protease</td>
<td>0.757</td>
<td>Q9NRS4</td>
<td>218960_at</td>
<td>proteolysis and peptidolysis</td>
<td></td>
</tr>
</tbody>
</table>
The oligonucleotide probes were labeled with \([\alpha^32P]dATP\) (NEN, Boston, MA) using terminal deoxynucleotidyltransferase (Amersham, Bucks, UK) to a specific activity of \(6 \times 10^7\) cpn \(\mu\)g\(^{-1}\). The \textit{in situ} hybridization was carried out as described (Schultz \textit{et al.}, 2003).

The sections were briefly air-dried and hybridized at 42°C for 18 h with 5 ng/ml of the probes in the hybridization cocktail. After hybridization, the sections were rinsed four times at 55°C in 1× saline sodium citrate buffer (SSC) for 15 min each and subsequently left to cool down for 1 h at room temperature. The sections were dipped in distilled water, dehydrated with 60% and 90% ethanol, and air-dried. Thereafter the sections were covered with Kodak MR autoradiography film (Kodak, Rochester, NY) or dipped in Kodak NTB2 emulsion. The autoradiography films were developed using Kodak LX24 developer and AL4 fixative. The dipped sections were developed with D19 (Kodak) developer, fixed with G333 (Agfa Gevaert, Cologne, Germany) fixative, counterstained with cresyl violet, and coverslipped.

**Reverse transcription and quantitative real-time PCR**

Frozen kidney and heart allograft specimens were homogenized, and total RNA was isolated using Qiagen RNeasy Midi kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. The samples were analyzed for RNA quality and quantity using Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). cDNA synthesis from total RNA was performed using the Invitrogen SuperScript cDNA synthesis kit (Invitrogen, Carlsbad, CA). Prior to cDNA synthesis, RNA was treated with amplification-grade DNase I (1 U/\(\mu\)g RNA, Invitrogen). DNase-treated RNA (1 \(\mu\)g) was reverse transcribed with random hexamers according to the manufacturer’s instructions. Parallel reactions were run in the absence of SuperScript II (-RT) controls to assess the degree of contaminating genomic DNA.

The resulting cDNA samples were subjected to real-time quantitative PCR assay (Heid \textit{et al.}, 1996) to detect the expression levels of CMP-SA, GDP-Fuc, and PAPS transporter mRNAs. Primers and probes were designed using the Primer Express (version 1, PE Applied Biosystems, Foster City, CA), a software provided with the ABI 7000 Sequence Detection System (PE Applied Biosystems). Forward and reverse primers were positioned as close as possible to each other without overlapping with the probe. Probes were synthesized with the fluorescent reporter FAM (6-carboxy-fluorescein) in the 5’ end and the quencher TAMRA (6-carboxy-tetramethyl-rhodamine) in the 3’ end. The primers and probes used were as follows: GDP-Fuc transporter forward: 5’ATCTTCGTCCACTCTTACCAATGC3’, reverse 5’GCAGCAGGGTGCCAGAGT3’, probe 5’CTCACTGCTGTGAAA GCCGCTCAG3’, CMP-SA transporter forward 5’GGATTTTTCTATGGCTACAC- GTATTATG3’, reverse 5’TTCACACCACCTGAGCT- GTAGAG3’, probe 5’CCCTCCCACACTAGCAAGGAA- GATAACCAACAG3’, and PAPS transporter forward 5’TCTTGGCCAGG TCCTGAAAGC3’, reverse 5’TGCAGTACACCCCAAGTCAGAT3’, probe 5’CTTCTGTGCGC- CGCGGGACTGCCAG3’.

cDNA was amplified in a total volume of 25 \(\mu\)l containing 1× Universal Master Mix (PE Applied Biosystems) on an ABI Prism 7000 Sequence Detection System (PE Applied Biosystems) using 1 \(\mu\)l cDNA as a template. Assays for each transcript were carried out as duplicates, and PCR amplification was repeated twice. Any inefficiencies in RNA input or reverse transcription were corrected by normalization to a housekeeping gene (18S rRNA Control Reagents; PE Applied Biosystems). Primer concentrations for target amplicons were optimized to yield maximal amplification with minimal primer concentration. Primer concentrations used were 300 nM/300 nM (F/R) for GDP-Fuc and CMP-SA transporters and 300 nM/900 nM (F/R) for the PAPS transporter, respectively. Concentration of the FAM-TAMRA-labeled probe was 200 nM. Relative amounts of GDP-Fuc, CMP-SA, and PAPS transporter mRNAs were calculated based on standard curves (Applied Biosystems User Bulletin 2) prepared by a serial dilution of control cDNA.

**Microarray data set**

We used our own 37 experiments with human conjunctival epithelial cells taken from healthy and allergic patients. These data are under analysis process and will be published in another article. The study was approved by the ethical committee of the Helsinki University Central Hospital.

One hundred forty-three experiments were downloaded from Gene Expression Omnibus (Edgar \textit{et al.}, 2002) (GEO; www.ncbi.nlm.nih.gov/geo). The following experiments were downloaded from Microarray Center of Childrens National Medical Center (Washington, DC) (http://microarray.cnmresearch.org). Twenty-nine experiments came from PGA human CD4+ lymphocytes. Twenty-four experiments came from PGA + human + muscle + obese. Six experiments from: PGA human obstructive pulmonary.

**Gene expression profiling data analysis**

CEL files were analyzed using MicroArray Suite 5.0 software (Affymetrix). Average intensities for each array were scaled to a target intensity of 100. Signal intensities were normalized across all the experiments using quantile normalization method (Bolstad \textit{et al.}, 2003). Pearson’s correlation coefficients were calculated as a similarity measure of any two different expression profiles. For each gene the variance, mean and median were calculated over the entire data set.

**Acknowledgments**

The work was supported in part by Academy of Finland, Technology Development Center, Helsinki, Sigrid Juselius Foundation, the Helsinki University Central Hospital Research Fund, and the Medical Research Fund of Tampere University Hospital. The authors thank Dr. Loubomir Petrov for performing the rat transplantations. Kiikka Kauranen for the PCR, Ulla Jukarainen for performing the \textit{in situ} hybridizations, Leena Saraste for help with the manuscript, and Meri Renkonen for artwork.
Abbreviations
APS, adenosine 5′-phosphosulfate; LAD, leukocyte adhesion deficiency; PAPS, adenosine 3′-phosphate 5′-phosphosulfate; PCR, polymerase chain reaction; SA, sialic acid.

References


