Changes in gene expression after transfection of pancreatic 6A3 cells with the GTP-binding protein Goα: Verification of microarray data by real-time polymerase chain reaction (RT-PCR)

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RÉSUMÉ

The polyoma-BK-virus-transformed rat pancreatic islet cell line (6A3) expresses viral T-antigen, has highly malignant characteristics and has lost insulin-secreting capacity. In 6A3 cells, the phosphoinositide hydrolysis is constitutively activated, with a substantial loss of regulatory control. The changes in the trans-signal system appear to be located at the level of phospholipase C and its GTP-binding protein. Studies on GTP-binding protein distribution have shown that 6A3 cells lack the expression of the pertussis toxin (PTX)-sensitive GTP-binding protein Goα. To investigate the possibility that the regulation of PLC activity in 6A3 cells is linked to the PTX-sensitive G protein Goα, a transfection vector harbouring Goα was stably introduced into 6A3 cells. The Goα expression vector, denominated pGoαgfp, contains in addition a sequence for the green fluorescence protein (gfp) for monitoring the expression of the protein by fluorescence microscopy. In addition, a control cell line was established expressing gfp alone by introducing a vector denominated pgfp into 6A3 cells. Experiments showed morphological changes for cells expressing Goαgfp. Non-transfected cells and cells transfected with gfp alone did not show any changes in their morphology. The observed changes are most likely linked to differential expression of cytoskeletal proteins. Earlier reports demonstrated that many biological functions of Goα are associated with cytoskeletal system, such microtubule assembly and disassembly, regulation of various intracellular pathways and involvement coupling to calcium signalling and cytoskeleton rearrangements, with the mitotic spindle apparatus and participation in cell division and differentiation. Recent microarray studies have shown that many proteins associated with the cytoskeletal apparatus are upregulated in Goαgfp expressing cells, i.e. actin α1, annexin A2, tropomyosin 4, tropomyosin 3γ, connective tissue growth factor, aurora kinase B and actinin α1. The aim of this study was to verify the changes in cytoskeletal gene expression obtained by microarray experiments with two-step real-time PCR experiments using cDNA as a template. For this purpose, the commercially available ProbeLibrary design software from Roche Applied Biosystems was applied. The relative expression levels were estimated by comparing transcripts from Goαgfp expressing cells with cells expressing gfp alone. Results obtained by microarray analysis could be confirmed by real-time PCR experiments.

INTRODUCTION

The human polyomavirus BK (BKV) was originally isolated from a kidney – transplanted patient in UK [1]. The virus is ubiquitous and infects human populations all over the world [2]. Most primary infections takes place in early childhood, and at an early adult age about 70-80 % of all individuals have anti-BKV serum antibodies as a maker of previous infection [3]. BKV does not, however, became eradicated form the human organism after primary infection. The virus may establish itself in a latent state, from which it may be reactivated during immunosuppressive disease or treatment [4].

BKV has a proven potential for malignant transformation of numerous cell cultures from various species, including humans. Inoculation of BKV into some rodent species results in tumor formation. BKV DNA, mRNA and polypeptides have been identified in human tumors and in cell lines established form tumor tissues. Adenocarcinomas of the endocrine pancreas are among the tumor forms commonly seen following rodent inoculation, and strains of BKV with rearrangement in their non-coding control regions have been identified in human pancreatic insulinomas [5,6]. Recently, a BK-virus transformed cell line (6A3) was established from rat pancreatic islets that expresses viral T-antigen, is highly malignant and has lost its insulin-secreting capacity [7]. A loss of the regulatory control of phosphoinositide hydrolysis with evidence for constitute activation of phopholipase C is most possibly located at the level of its GTP-binding protein [8]

G-proteins, short for guanine nucleotide-binding proteins or GTP-binding proteins, are a family of proteins involved in second-messenger cascades. Their signalling mechanism uses guanosine diphosphate (GDP) for guanosine triphosphate (GTP) as a molecular switch to allow or inhibit biochemical reactions inside the cell. Alfred Gilman and Martin Rodbell were awarded the Nobel Prize in Physiology or Medicine in 1994 for their discovery and research on G – proteins. G-proteins consist of alpha (α), beta (β) and gamma (γ) subunits. Diseases such as diabetes and certain forms of pituitary cancer, amount others, are thought to have some root in the malfunction of G-proteins. A fundamental understanding of their function, signalling pathways, and protein interaction may lead to eventual treatment and possibly the creation of various preventive approaches.

Gα subunits consist of two domains, the GTPase domain, and the alpha-helical domain. There exist at least 20 different alpha units, which are separated into several main families. Most

α subunits were first identified in G proteins that are sensitive to ADP-ribosylation catalyzed either cholera toxin (G_s), pertussis toxin (G_i and G_o proteins), or both toxins. The α subunit of G_o protein, originally isolated from bovine brain [9,10,11,12], exist as monomeric (active) and hetrotrimeric (inactive) forms. It has been reportet that α subunit is associated with mitotic spindle. The report concludes that Goα but not Giα or Gsα is associated with the mitotic spindle; furthermore, the association involves the heterotrimeric Go protein than a single subunit of Goα. There are reports that associates $G_{o\alpha}$ with microtubles and may play some role in regulating the assembly and disassembly of microtubules[13]. Recent results from several laboratories suggest that G protein also interact with microtubules and participate in cell division and differentiation, and localization of Gα and Gβγ with the mitcrotubule cytoskeleton has been reported both in interphase and mitotic cells [14,15,16,17]. The role of G_o proteins has been generally believed to be confined to the brain and heart, several reports indicate that Go proteins may serve to regulate various intracellular pathways in non-neural cell lines[14]. It has also been concluded that G_o proteins play a role in thrombin signalling distinct form $G_{i1/2}$ proteins, which are mediated through their Gβγ subunits and involve coupling to calcium signalling and cytoskeletal rearrangements[18].

It is well established that G-Proteins and other signal proteins are associated with different endocrine functions of the β-cells in islets of Langerhans. This includes the GTP-binding protein Goα. However, BK-virus transformed pancreatic β-cells (6A3) do not express Goα. Therefore, we introduced Goα into 6A3 cells to investigate the possible implications on the biological functions of this protein in these cells. The transformed cells show morphological and biological changes. Microarray experiments revealed that genes encoding for cytoskeletal proteins and some G proteins were overrepresented in cells expressing Goα when compared to control cells. To verify the expression data otained by microarray analysis, the genes of interest were further validated by real-time polymerase chain reaction (RT- PCR).

METHODS USED IN THIS STUDY

Materials

RPMI 1640 medium, fetal calf serum (FCS), lipofectamine PLUS and gentamycin G418 were purchased from Invitrogen, trypsin was from Sigma, TriZol©Reagent from GE Healthcare, Rat Universal Probe Library, FastStart TaqMan Probe Master (ROX) and cDNA synthesis kit was from Roche Applied Sciences. All other chemicals used in this study were of standard laboratory grade.

Cell lines and culture

6A3 cells [7] were cultured in RPMI-1640 (11 mmol glucose) medium supplemented with 2 % FBS (fetal bovine serum), 75 μg/ml penicillin and 50 μg/ml streptomycin and incubated in a humidified atmosphere with 95% air and 5% CO_2. 6A3 cell lines expressing Goαgfp (6A3Goαgfp) and gfp (6A3gfp) were maintained in the same medium containing 200 μg/ml gentamycin (G418) as a selective marker. The cells were trypsinized and sub-cloned once or twice weekly. The medium was changed twice weekly and 24 hours prior to an experiment. The cells were always harvested at a logarithmic phase of growth.

Transfections

6A3 cells were transfected with pGoαgfp and pgfp using Lipofectamine Plus transfection kit (Invitrogen) and according to manufacturer's protocol. One 6 well plate with approximately 60 % confluent cells was prepared. 24 hours after transfection growth medium was replaced with medium containing G418 (400 μg/ml) for selection of pGoαgfp and pgfp expressing cells. Single clonal strains were obtained by dilution series and by monitoring of fluorescence labeled cells.

RNA preparations

Total RNA was isolated directly from cell samples with TriZOL™ and according to the manufacturer's protocol. After removal of the cell culture medium, 750 μL TriZOL™ reagent was added to each cell container to achieve lysis of the cells. The cells were detached from the container by a cell scraper and homogenized before they were placed in a sterile test tube. The

sample suspension was incubated in room temperature for 5 min to permit complete dissociation of the nucleoprotein. To separate the aqueous phase containing RNA, 200 µl chloroform was added and the mixture was incubated for 10 min at room temperature and than centrifuged 15 min at 12,000 x *g* at 2 to 8°C. The aqueous phase was than transferred to a new sterile test tube and 500 µl of isopropanol was added to precipitate RNA. After 10 min incubation at room temperature and centrifugation for 10 min at 12,000 x *g* at 2 - 8°C, the supernatant was removed. The RNA pellet was washed once with 1.0 ml 75 % ethanol. After centrifugation at 7,500 x *g*, the RNA was re-dissolved in 50 µl of RNase-free water. The sample was then incubated 10 min at 59°C in a water-bath to dissolve the RNA completely. Quantity and quality of total RNA was judged by spectrophotometry at a wavelength of 260 and 280 nm, respectively.

Microarray analysis

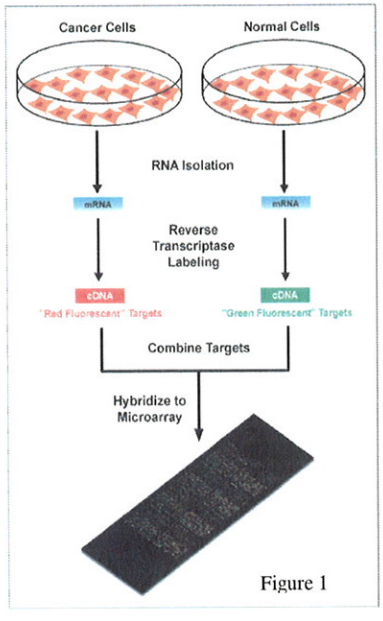

Figure 1

DNA microarray is a collection of microscopic DNA spots attached to a solid surface, such glass, plastic or silicon chip forming an array. The affixed DNA segments are known as probes, thousands of which can be used in a single DNA microarray. Microarray technology evolved from Southern Blotting, where fragments of DNA is attached to substrate and then probed with known gene fragment. Microarray is used to measure the expression levels of large number numbers of genes simultaneously. The most common use of microarrays is to quantify mRNAs transcribed from different protein-encoding genes. RNA is extracted from a cell, and then converted to cDNA. Fluorescent tags (Cy3 and Cy5) are enzymatically incorporated into the newly synthesized cDNA or can be chemically attached to the new strands of DNA. A cDNA molecule that contains a sequence complementary to one of the single stranded probe sequence of the array will hybridize, via baseparing, to the spot at which the complementary reporters are affixed. The spot will then fluoresce when examined using a microarray scanner. The fluorescence intensity of each spot then evaluated in term of the number of copies of a particular mRNA, which ideally indicates the level of expression of a particular gene [19,20,21]. The principle of a micrarray analysis is outlined in **figure 1.** 5.7 K rat oligo microarrays were obtained form the Norwegian Microarray Consortium

(http://www.mikromatrise.no/). 5,7k rat oligonucleotide microarray printed from OPERON rat oligo collection v1.1. Included in the array are 10 different cDNAs from Arabidopsis thaliana (Stratagene Spotreporter). The Arabidopsis probes allow the user to add Arabidopsis mRNA to their samples prior to labeling and hybridization and to use these as controls of the performance of the analysis with respect to labeling and hybridization efficiency as well as normalization procedures.

Total RNA was reverse transcribed and labelled with Cy3- and Cy5- attached dendrimer, respectively, using the Genisphere 3DNA 350HS kit (Genisphere, Montvale, NJ) as described in the manufacturer's protocol. Hybridizations of transcribed probes were carried out in a TECAN HS4800 instrument using the formamide-based hybridization buffer from Genisphere containing 5% dextrane sulfate and 5.5 ng/µl COT1 DNA (GIBCO BRL Life technologies) at 37° for 23 hours. 3DNA dendrimer hybridizations were carried out in formamide-based hybridization buffer alone. Post-hybridization washes were carried out at room temperature with 2xSSC for 1 min, 0.2% SDS /2xSSC for 1 min. and finally with 0.2xSSC for 30 sec. The arrays were scanned with the GenePix 4000B scanner (Axon Instruments Inc). Microarray experiments, including data analysis and statistical analysis was performed by MRCT (www.umn.no/labforum).

Data analysis and statistics

The features were extracted from the arrays using Genepix Pro 6.0 [Axon instruments Inc (2004) Gene Pix 6.0 Pro, http://www.axon.com, documentation]. Spots that displayed a signal-to-noise ratio of less than 2, or that were significantly saturated (more than 20 % saturation among foreground pixels) were filtered out. The median was used as the averaging measure of the foreground pixels. After quality control, genes that were present in less than 50 % of the arrays were filtered out. The arrays were normalized using dye-swap normalization. Statistical significance was assigned to the genes using the SAM methodology [22]. The FDR threshold was set to 15 %. The resulting gene list contained downregulated and upregulated genes (data not shown).

cDNA Synthesis

For cDNA synthesis the *Transcriptor First Strand cDNA Synthesis Kit* (Roche Applied Sciences) was used according to the manufacturer's instructions. All necessary components were

placed on ice and briefly centrifuged before starting the experiment. The following components were combined in a sample tube before incubation at 65°C for 10 min: 0.5 µg total RNA , 2 µl random hexamer primer (600 pmol/µl) and molecular grade water to a final volume of 13 µl. The mixture was transferred on ice prior to addition of 4 µl transcriptor RT reaction buffer (5x), 0,5 µl protector RNase inhibitor, 2 µl deoxynucleotide mix (10 mM each), 0,1µl transcriptor reverse transcriptase for total volume 20 µl. The samle was mixed by pipetting and centrifuged briefly, prior to incubations as follows: 25°C for 10 min, 55°C for 30 min, and finally heating to 85 °C for 5 min to inactivate the reverse transcriptase. The samples were stored at -20°C until further use.

Universal ProbeLibrary

The *Universal ProbeLibrary* is a combination of 90 probes and unique assay design software that delivers real-time PCR assays for virtually any transcript in an organism. So far, Universal ProbeLibrary kits have been developed for Human, Mouse, Rat, *Arabidopsis*, *Drosophila, C.elegans*, and Primates. All primers were designed with the *Assay Design Center* (available at www.universalprobelibrary.com). It contains the free web-based ProbeFinder™ software that optimally designs intron-spanning assays for the target gene. The gene sequences were entered and ProbeFinder designed gene-specific assays using probes from the kit in combination with primers. The sequences, primer names and correlating probe numbers used in this study are listed in table 1.

Real-time polymerase chain reaction (RT-PCR)

Real-time polymerase chain reaction (PCR) uses fluorescent reporter molecules to monitor the production of amplification products during each cycle of the PCR reaction. This combines the DNA amplification and detection steps into one homogeneous assay and obviates the need for gel electrophoresis to detect amplification products. Rather than having to look at the amount of DNA or mRNA target accumulated after a fixed number of cycles, real-time assays determine the point in time during cycling when amplification of a PCR product first is detected. This is determined by identifying the cycle number at which the reporter dye emission intensity raises above background noise. That cycle number is referred to as the threshold cycle (C_t).

The C_t is determined at the exponential to the copy number of the target. Therefore the higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed, and the lower C_t[23,24].

First a dilution was made of ROX (1 mM) 1:10 in 0,1 M Tris, pH 8. Added 9 µl 0,1 M Tris in 1 µl ROX (1 mM), before adding 8,5 µl (1:10) 0,1 mM ROX solution in 1,25 ml FastStart TaqMan Probe Master (Rox). The primers concentrations were made to 200µM. Then dilutions were made for both primers to 1:20 by adding 19µl nuclease free water to 1µl primer. Following samples were mixed and pipetted into the thermal cycler plate wells: Master Mix (ROX) 10 µl, Probe 0,2 µl, reverse primer (1:20) 0,8 µl, forward primer (1:20) 0,8µl, RNase free water 7,2 µl for total 19 µl. 1µl of cDNA (250 ng) was pipetted into well in the end for total 20µl sample volume. Before run in the RealTime PCR machine (Applied Biosystems HT7900), an Optical Cover was put over Thermal Cycler Plate, and centrifuged for 4 sec in a plate centrifuge. The program was set with following parameters: Absolute quantitation, 96 well, blank template. Stage program was set to: Fast 95°C for 10min, 95°C for 1 sec, and 60 ° C for 20 sec. The sample volume to 20µl, and 40 cycles.

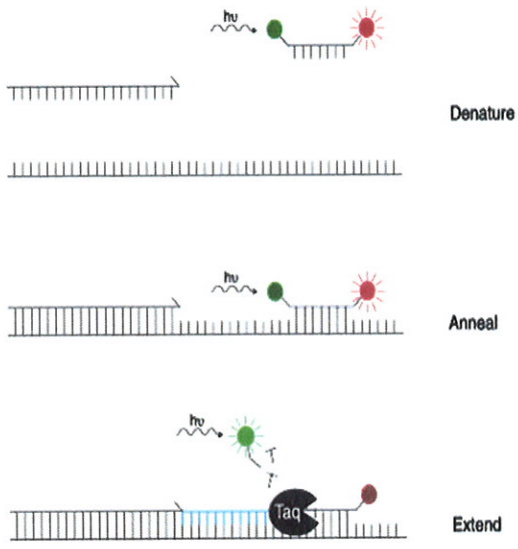

Figure 2: Outlined principle and three step view of the TaqMan probe technology: Before the probe is met with the Taq polymerase, energy is transferred from a short-wavelength fluorophore (green) to a long-wavelength fluorophore (red). When the polymerase adds nucleotides to the template strand, it releases the short-wavelength fluorophore, making it detectable and the long-wavelength undetectable.

RESULTS

Establishment of Goαgfp and gfp expressing 6A3 cell lines

Goα full-length [25] containing the coding region and all of the 3' non-translated sequence of Goα was PCR amplified from RNA prepared from the pancreatic insulin-producing β-cell line Rin5F (Acc.no.ATCC no.: CRL-2058) by introducing the restriction sites for Hind III and Kpn I for unidirectional cloning into pEGFP (Gen Bank Accession #: U55763). Expression of Goαgfp resulted in functional protein with green fluorescence proteine (gfp) located at the N-terminus of Goα protein, as earlier confirmed by Western blot analysis (unpublished data not shown). This protein construction maintains biological functions of Goα in respect to receptor coupling intact. Non-modified, gfp- expressing vector was used as control in transfection experiments. The vectors were denominated pGoαgfp and pgfp and transfected with the lipofectamine method into 6A3 cells. Single cell clones were established by using the selective marker G418 and by dilution experiments. This resulted in four cell lines denominated 6A3Goαgfp-C1-C4 and one 6A3gfp (control cell line). All four 6A3Goαgfp cell lines showed the same morphological characteristics as shown for one single cell clone in figure 3.

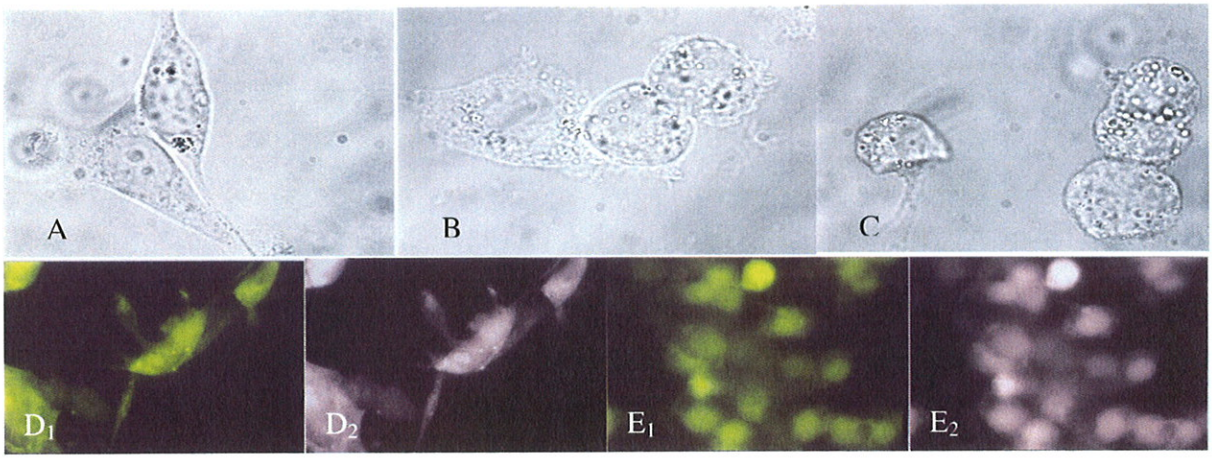

Figure 3: Phase contrast and fluorescence microscopy of 6A3Goαgfp and 6A3gfp cell lines.
A: 6A3Goagfp cells; B: 6A3gfp (control) cells; C: 6A3 cells (non-transfected); $D_{1\text{-}2}$; 6A3Goαgfp cells. D_1: with fluorescence; D_2: without fluorescence; $E_{1\text{-}2}$; 6A3gfp cells. E_1: with fluorescence; E_2: without fluorescence

RNA perparations of cells expressing Goagfp and gfp alone

Total RNA was prepared as described in the Methods section. Results are shown in table 2. Four Goαgfp and two Gfp for control. The quantity and quality of the RNA preparations were judged by spectrophotometry at a wavelength of 260 nm and 280 nm with a Nanodrop instrument. The samples quantity is given in ng/µl. The quality of the RNA is listed up in third column. The value should be between 1,8-2,0. The fourth column shows the grade of contamination, and the value should be >2,0. Four Goαgfp samples and two gfp samples were prepared with 0.88 µg/µl, 0.90 µg/µl, 1.48 µg/µl, 1.39 µg/µl, 1.86 µg/µl and 1.35 µg/µl, respectively. All preparations were of good quality, with virtually no contaminations.

cDNA synthesis of RNA preparations

In order to prepare the RNA samples for a two-step real time polymerase chain reaction (RT-PCR), cDNA was made from the RNA samples as described in the Methods section. RNA obtained from four Goαgfp cell lines and one gfp cell line was used for cDNA synthesis as outlined in table 3. cDNA was prepared form 0.5 µg of each RNA preparation. Finally, the quantity and quality of the cDNA was judged by spectrophotometry with measurements at the wavelengths of 260 nm and 280 nm with a Nanodrop instrument. The concentrations of the synthesised cDNAs were 2.28 µg/µl, 2.38 µg/µl, 2.29 µg/µl, 2.34 µg/µl and 2.35 µl, respectively. The result shows that the synthesis of cDNA of the different RNA preparations is consistent. 0.25 µg of each cDNA preparation was used for RT-PCR as described in the Methods section.

Real- Time PCR of cDNA samples from Goαgfp and gfp expressing cells

cDNA samples prepared from RNA of Goαgfp and gfp expressing cells were used for real-time polymerarse chain reaction (RT_PCR) with the primers designed with ProbeLibrary Software (see also table 1). The Ct values were registered as outlined in table 4. All samples have been measured in duplicate. Eleven primer pairs were able to amplify specific gene products. The housekeeping gene cyclophilin (*Cyc*) was used to standardize the RT-PCR procedure by measuring the efficiency of the reaction (data not shown). Primers encoding for actinin 4 (*Actn4*) and cathepsin S (*Ctss*) did not give any results and were therefore excluded from the table.

Relative quantification using the comparative Ct method

Genes that were significantly up-regulated in microarray experiments were validated real-time PCR. Ten genes out of thirteen were validated by this method (list genes by name). To be able to compare the obtained results, we introduced comparative Ct method, which will give us relative fold-change Goαgfp compared to gfp (control).

The two most commonly used methods to analyze data form real-time, quantitative PCR experiments are absolute quantification and relative quantification. Absolute quantification determines the input copy number, by relating the PCR signal to a standard curve. Relative quantification relates the PCR signal of the target transcript in the Goα transformed cell lines (6A3Goαgfp) to the other not Goα transformed cell line (6A3gfp). The $2^{-\Delta\Delta CT}$ method is a convenient way to analyze the relative changes in gene expression from real-time quantitative PCR experiments and is described in detail by many authors [26,27].

Shortly, ΔCt values were determined by subtracting the average housekeeping gene Ct value from the average Goαgfp and gfp Ct values. It is highly recommended that the internal control gene is properly validated for each RT experiment to determine that the gene expression is unaffected by the experimental treatment. For this purpose, the efficiency of the housekeeping gene cyclophilin A was determined and found to be acceptable (data not shown). Then, ΔΔCt was calculated by subtracting average ΔCt gfp from average ΔCt average respective target gene. Numbers given for Goαgfp relative to gfp were determined by evaluating the expression. The $2^{-\Delta\Delta CT}$ values depict fold-change in expression of Goαgfp relative to gfp. The obtained real time curves were transferred to Microsoft Excel, and the values were calculated as outlined in table 5. Results show a high fold-change of the different GTP-binding proteins tested, with *Gnao* 60 times, *Gnb1* almost 4 times and *Gnai2* for 3,76 times, respectively. Further, connective tissue growth factor *Ctgf* shows also a large fold-change of 58 compared to only 3.2-fold change in the microarray experiment.. The tropomyosins, *Tpm3* and *Tpm4* showed a two-fold change and actin three-fold change, shows us that the factors involved in the cytoskeleton is up regulated and correlates with the values observed by the microarray experiment.

Discussion

The polyoma-BK-virus-transformed rat pancreatic islet cell line (6A3) expresses viral T-antigen, has highly malignant characteristics and has lost insulin-secreting capacity. In 6A3 cells, the phosphoinositide hydrolysis is constitutively activated, with a substantial loss of regulatory control. The changes in the trans-signal system appear to be located at the level of phospholipase C and its GTP-binding protein [8] and earlier studies on GTP-binding protein distribution have shown that 6A3 cells lack the expression of the pertussis toxin (PTX)-sensitive GTP-binding protein Goα (unpublished data). To investigate the possibility that the regulation of PLC activity in 6A3 cells is linked to the PTX- sensitive G protein Goα, a transfection vector harbouring Goα was stably introduced into 6A3 cells. The Goα expression vector, denominated pGoαgfp, contains in addition a sequence for the green fluorescence protein (gfp) for monitoring the expression of the protein by fluorescence microscopy. In addition, a control cell line was established expressing gfp alone by introducing a vector denominated pgfp into 6A3 cells. Experiments showed morphological changes for cells expressing Goαgfp. Non-transfected cells and cells transfected with gfp alone did not show any changes in their morphology. Goα expressing cells grew strictly in monolayers and had a stretched and spheric shape, whereas nontransfected cells were round-shaped with visible rapid growth and formation of foci. The observed changes are most likely linked to differential expression of cytoskeletal proteins as recent microarray experiments have shown that some cytoskeletal and related proteins are significantly up-regulated in Goα expressing cells (unpublished data). Candidate genes were tropomyosin 4 (*Tpm4*), tropomyosin 3 (*Tpm3*), actin α1 (*Acta 1*), actinin α4 (*Atcn4*), aurora kinase B (*Aurkb*), connective tissue growth factor (*Ctgf*) and annexin A2 (*Anxa2*). In addition, some GTP-binding proteins were differentially expressed, such as the GTP-binding protein α-subunit Gi2α (*Gnai2*) and β1-subunit (*Gbn1*). As expected, the GTP-binding protein Goα was over-expressed in the transfected cells. As known from other published reports, Goα is associated with mitotic spindle formation, microtubules assembly and disassembly, regulation of various intracellular and involvement coupling to calcium signalling and cytoskeleton rearrangements [14, 15, 16]. It is well known that actin is a globular structural protein that polymerizes in a helical fashion to form an actin filament. These form the cytoskeleton, provide mechanical support for the cell, determine the cell shape, enable cell movements and participate in

contraction of the cell during cytokinesis. Our results may confirm the connections between Goα and cytoskeletal rearrangements. However, it would be interesting to test if the morphological changes are directly coupled to Goα. One possibility to test this, is to inhibit Goα expression by si-RNA technologies to see if the morphological changes can be circumvented.

The obtained results by microarray analysis could be confirmed by RT-PCR experiments, although for some genes to a different extend. It is interesting to note, that connective tissue growth factor (*Ctgf*) expression was considerably higher when confirmed by RT-PCR as with microarry analysis. This is most possible to the limits of detection in microarray experiments. Ideally, efficiencies of all target primers should be tested for optimal results. This would probably explain some of the differences between expression levels obtained from microarrays and RT-PCR. In addtition, the Goα gene was not represented on the microarrays. Verification of expression could be done by Northern blot analysis, by using Goα cDNA- or oligo probe for detection. With this approach, a semi-quantitative validation of Goα expression would be possible. Primers encoding for actinin 4 (*Actn4*) and cathepsin S (*Ctss*) were not able to amplify the expected products. This may be due to the nature of the primer or inhibitory factors in the reaction mixture but it is also possible that these signals are solely artefacts.

Taken all observations together, it was possible to verify gene expression data by real time polymerase chain reaction (RT-PCR). However, one should keep in mind that labelling-procedures/products for microarray analysis are improving and results form this type of analysis becoming more accurate, making RT-PCR verifications unnecessary in the future.

Table

Table 1: Universal ProbeLibrary for Rat. Roche Applied Science (Probefinder)				
Name	***Abbreviation***	***Left Primer (FW)***	***Right Primer (REV)***	***Probe***
Rattus norvegicus actinin alpha 4	*Actn4*	cgggatggcttaaacttcat	cgctctggcttaggcaac	#67
Rattus norvegicus actin, alpha 1	*Acta1*	tgaagcctcacttcctaccc	cacacatggtgtctagtttctgc	#81
Rattus norvegicus guanine nucleotide binding protein, alpha inhibiting 2	*Gnai2*	tcaatgactcagccgcttac	gggatatagtcactctgtgctatgc	#85
Rattus norvegicus guanine nucleotide binding protein, alpha o	*Gnao*	caccettgaccatctgcttt	cagctgcgtcttcataggtg	#116
Rattus norvegicus PDZ and L IM domain 1	*Pdlim1*	gctgcgtagacaacatgacg	ctcggtcactagaggagacca	#85
Rattus norvegicus annexin A2	*Anxa2*	ccttcgcctaccagaggag	cagacaaggccgacttcatc	#121
Rattus norvegicus connective tissue growth factor	*Ctgf*	gctgacctagaggaaaacattaaga	ccggtaggtcttcacactgg	#129
Rattus norvegicus aurora kinase B	*Aurkb*	ctttggctggtctgtgcat	ctgggggcagatagtccag	#69
Rattus norvegicus cathepsin S	*Ctss*	ccagagagacctaccctgga	acgtcgtacgtcttcttcattct	#118
Rattus norvegicus tropomyosin 3, gamma	*Tpm3*	ctgatgatgcggaggagag	ggttcaaggaagccacctc	#41
Rattus norvegicus tropomyosin 4	*Tpm4*	ggcggaggtgtctgaactaa	tcagattgttagttacgttcttgagc	#85
Rattus norvegicus guanine nucleotide binding protein, beta 1	*Gnb1*	cacatctgttcccttctccaa	gactcggttgtcatgtccag	#63
Rattus norvegicus Cyclophilin A	*Cyc*	tctgcactgccaagactgag	catgccttctttcaccttcc	#42

Table 2: RNA extractions			
Sample ID	ng / µl	260 / 280	260 / 230
6A3 Goαgfp-C1	882,44	1,93	2,20
6A3 Goαgfp-C2	901,58	1,93	2,20
6A3 Goαgfp-C3	1482	1,93	2,04
6A3 Goαgfp-C4	1395	1,96	1,86
6A3 gfp	1856	1,96	2,02
6A3 gfp	1346	2,00	2,18

Table 3: cDNA preparations			
Sample ID	ng / µl	260 / 280	260 / 230
6A3 Goα-C1	2284,8	1,74	2,23
6A3 Goα-C2	2379,6	1,74	2,22
6A3 Goα-C3	2291,1	1,74	2,22
6A3 Goα-C4	2336,4	1,74	2,22
6A3 Gfp-C4	2345,2	1,74	2,22

Table 4: Results of RT-PCR reactions (Ct-values) from Goαgfp and gfp-transformed 6A3 cells

Sample name	Detector name	Ct	Sample name	Detector name	Ct
6A3-Goαgfp	*Tpm3*	25.650553	6A3-gfp	*Gnb1*	29.60527
6A3-Goαgfp	*Tpm3*	25.380262	6A3-gfp	*Gnb1*	30.368662
6A3-gfp	*Tpm3*	26.06897	6A3-Goαgfp	*Ctgf*	31.045052
6A3-gfp	*Tpm3*	27.455338	6A3-Goαgfp	*Ctgf*	32.398582
6A3-Goαgfp	*Tpm4*	25.31026	6A3-gfp	*Ctgf*	25.347662
6A3-Goαgfp	*Tpm4*	25.35945	6A3-gfp	*Ctgf*	26.38434
6A3-gfp	*Tpm4*	26.095186	6A3-Goαgfp	*Anxa2*	20.327587
6A3-gfp	*Tpm4*	26.609653	6A3-Goαgfp	*Anxa2*	20.819614
6A3-Goαgfp	*Pdlim1*	24.259266	6A3-gfp	*Anxa2*	21.88714
6A3-Goαgfp	*Pdlim1*	24.211826	6A3-gfp	*Anxa2*	23.173279
6A3-gfp	*Pdlim1*	23.068249	6A3-Goαgfp	*Gnao*	20.988958
6A3-gfp	*Pdlim1*	23.542744	6A3-Goαgfp	*Gnao*	21.146921
6A3-Goαgfp	*Aurkb*	24.973883	6A3-gfp	*Gnao*	29.981707
6A3-Goαgfp	*Aurkb*	24.96179	6A3-gfp	*Gnao*	30.984217
6A3-gfp	*Aurkb*	24.284195	6A3-Goαgfp	*Cyc*	22.469341
6A3-gfp	*Aurkb*	24.513506	6A3-Goαgfp	*Cyc*	22.339424
6A3-Goαgfp	*Gnai2*	23.195808	6A3-gfp	*Cyc*	22.595495
6A3-Goαgfp	*Gnai2*	23.355505	6A3-gfp	*Cyc*	22.270254
6A3-gfp	*Gnai2*	25.087595	6A3-Goαgfp	*Acta1*	36.096767
6A3-gfp	*Gnai2*	27.108869	6A3-Goαgfp	*Acta1*	36.921024
6A3-Goαgfp	*Gnb1*	28.87475	6A3-gfp	*Acta1*	38.19964
6A3-Goαgfp	*Gnb1*	28.435616	6A3-gfp	*Acta1*	37.76223

Tabel 5: Validation of miroarray results by RT-PCR			
Gene name	Acc.no.	Fold-change RT-PCR	Fold-change microarray
Gnao	NM_017327	60	Not on array
Gbn1	NM_030987	3.97	7.05
Ctgf	NM_02266	58.1	3.20
Pdlim1	NM_017365	2.17	0.97
Gnai2	NM_031035	3.76	2.02
Anxa2	NM_019905	3.36	1.22
Tpm4	NM_012678	2.01	1.24
Tpm3	NM_057208	2.51	1.16
Acta1	NM_019212	3.09	1.04
Aurkb	NM_053749	1.49	1.28

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