DelGEF, a homologue of the Ran guanine nucleotide exchange factor RanGEF, binds to the exocyst component Sec5 and modulates secretion

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Abstract In order to identify the function of deafness locus putative guanine nucleotide exchange factor (DelGEF), a protein homologous to the nucleotide exchange factor for the small GTPase Ran, a cDNA library was screened for interacting proteins using a yeast two-hybrid system. The human homologue of Sec5, a protein involved in vesicle transport and secretion, was identified as a binding partner. The interaction between DelGEF and Sec5 was found to be dependent on Mg^{2+} and stimulated by guanosine triphosphate (GTP) or deoxycytidine triphosphate (dCTP). Downregulation of endogenous DelGEF in HeLa cells induced increased extracellular secretion of proteoglycans indicating a possible role for DelGEF in the secretion process.
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Key words: Deafness locus putative guanine nucleotide exchange factor; Sec5; Exocyst; Yeast two-hybrid; Deoxycytidine triphosphate; Guanosine triphosphate

1. Introduction

The DelGEF (deafness locus-associated putative guanine nucleotide exchange factor) protein [1] is a homologue of RanGEF, the guanine nucleotide exchange factor for the small GTPase Ran [2]. DelGEF is a ubiquitously expressed and soluble protein with a size of 49 kDa. A structural motif consisting of seven internal repeats is found in RanGEF and defines the three-dimensional structure of RanGEF which resembles a seven-bladed propeller [3,4]. A similar repeat structure, in which structurally important residues are conserved, is found in DelGEF suggesting that DelGEF may adopt a similar tertiary structure as RanGEF. The gene encoding DelGEF is located at a 225 kb genomic sequence of chromosome 11p14, critical for *DFNB18*, a locus for non-syndromic sensorineural deafness [5].

In the present study we aimed at identifying proteins interacting with DelGEF, using the yeast two-hybrid system. DelGEF was found to bind to the human homologue of Sec5, a subunit of the sec6/8 multiprotein complex. Binding was detected in the presence of Mg^{2+} and could be significantly stimulated by addition of guanosine triphosphate (GTP) or deoxycytidine triphosphate (dCTP). Furthermore, downregulation of endogenous DelGEF in HeLa cells using antisense oligonucleotides induced extracellular secretion of proteoglycans, indicating a possible role for DelGEF in the secretion process.

2. Materials and methods

2.1. Yeast two-hybrid selection

The complete open reading frame of human DelGEF1 was cloned into the vector pGBT9 (Clontech) in frame with a Gal4 binding domain. *Saccharomyces cerevisiae* strains Y187 (MATα, ura3–52, his3–200, ade2–101, trp1–901, leu2–3112, gal4Δ, met⁻, gal80Δ, URA3::GAL1$_{UAS}$-GAL1$_{TATA}$-lacZ) and PJ69-4A (MATa, trp1–901, leu2–3112, ura3–52, his3–200, gal4Δ, gal80Δ, GAL2-ADE2, LYS2::GAL1-HIS3, met2::GAL7-lacZ) were transformed with the bait plasmid and with a human T-cell lymphocyte cDNA library fused to the Gal4 activating domain in the vector pACT2 (Clontech), respectively. The transformed strains were mated on nitrocellulose filters. Transformants were grown on selection plates lacking leucine, tryptophan, histidine and adenine. Plasmids were recovered from growing colonies and amplified through *Escherichia coli* transformation. Recovered plasmids encoding potential interactors were cotransformed with bait plasmid into yeast strain Y190 (MATa, ura3–52, his3–200, lys2–801, ade2–101, trp1–901, leu2–3112, gal80Δ, cyhʳ, LYS::GAL1$_{UAS}$-HIS3$_{tata}$-HIS3, URA3::GAL1$_{UAS}$-GAL1$_{TATA}$-lacZ) to confirm the Try⁺ His⁺ LacZ⁺ phenotype. Cotransformation with pGBT9 (without insert) was done as negative control to verify specific interaction.

2.2. Rapid amplification of complementary deoxyribonucleic acid ends-polymerase chain reaction (RACE-PCR)

RACE-PCR primers were designed based on the human sec5 sequence obtained in the yeast two-hybrid screening as follows (5′-GCTGCCCTCACTCTTCTTGTTAGCC-3′, 5′-CCTTCATTTGGA-GAGATGCCGGTCA-3′). RACE-PCR was performed according to manufacturers instructions using the Marathon cDNA amplification kit (Clontech). A human brain RACE-ready cDNA library (Clontech) was used as template.

2.3. Expression of DelGEF-glutathione S-transferase (GST) fusion protein

Full-length DelGEF cDNA was cloned into the vector pGEX-4T-2 (Amersham Pharmacia Biotech) allowing the expression of a fusion protein with an N-terminal GST domain and a C-terminal DelGEF domain. Bacterial expression of DelGEF-GST was done as described previously [1] and the fusion protein was purified by glutathione Sepharose 4B affinity chromatography (Amersham Pharmacia Biotech) according to manufacturers instructions.

2.4. In vitro binding assay

A cDNA containing the full-length coding sequence of human Sec5 with an additional T7 promotor sequence was amplified by PCR (upper primer: 5′-TAATACGACTCACTA TAGGGAGACCAC-ATGTCTCGA-3′, lower primer: 5′-TCAATATGTGCCACGCCAT

TCC-3′) from a human brain cDNA library (Clontech). A cDNA corresponding to the C-terminal DelGEF binding domain (amino acid residues 447–924) of human Sec5 with an additional T7 promotor sequence was amplified by PCR (upper primer: 5′-AAAATTG TAA-TACGACTCACTATAGGGCGACCACCA AACCCAAAAAAAG-AG-3′, lower primer: 5′-ACTTGCGGGGTTTTTCAGTATCTA-CGAT-3′) from a pACT2 plasmid containing the DelGEF cDNA. These cDNAs were used as templates for coupled in vitro transcription and translation using the TnT Coupled Reticulocyte Lysate System (Promega). Sec5 was radiolabelled by incorporation of [35S]methionine (Amersham Pharmacia Biotech) during the translation reaction. 0.2 mg of DelGEF-GST or GST alone was immobilised on 10 μl glutathione Sepharose 4B beads (Amersham Pharmacia Biotech). 5 μl of the Sec5 transcription/translation reaction was added to the beads and incubated for 1 h at 25°C in 500 μl binding buffer (20 mM Tris–HCl, pH 8.0, 100 mM NaCl and 0.1% Triton X-100) supplemented with ethylenediamine tetraacetic acid (EDTA), $MgCl_2$, $CaCl_2$, $MnSO_4$ or nucleotide as indicated in the text. The beads were extensively washed in binding buffer. Bound proteins were eluted in electrophoresis sample buffer by heating at 95°C for 5 min and separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). The gel was incubated with 1 M sodium salicylate (pH 6.0) for 15 min, dried and exposed to Biomax MR film for 12 h. Computerised images of fluorographs were used for determination of band intensities using the NIH Image software.

2.5. Northern blot analysis

A cDNA corresponding to nucleotides 1370–2815 of human sec5 was amplified by PCR and used as template in an oligo-labelling reaction using the NonaPrimer kit (Appligene). The 32P-labelled probe was hybridised to human multiple tissue Northern blots (Clontech) according to manufacturers instructions. Exposure was for 72 h using X-OMAT AR film (Kodak).

2.6. Antisense oligonucleotide treatment

HeLa (human cervix carcinoma epithelial) cells (ATCC#CCL-2) were maintained under standard conditions. For antisense treatment, cells were plated at 50 000/ml and incubated for 4 h to allow attachment. To a solution containing 100 μM antisense or scrambled oligonucleotide was added 5 vol. Dulbecco's modified Eagle's medium (DMEM) and 0.25 vol. Superfect transfection reagent (Qiagen). The solution was incubated at 25°C for 10 min and added to the cells at a final concentration of 500 nM oligonucleotide. After 24 h cells were used for analysis of DelGEF expression or proteoglycan release. The following phosphorothioate-modified oligonucleotides (MWG Biotech) were used: DG1 (5′-GCTCGCGCTCCATGC-3′), DG2 (5′-TATTCCCTACTCTCTGAC-3′), DG3 (5′-GTTCTCTTCACCCAC-CATTA-3′). Corresponding scrambled control oligonucleotides were synthesised as follows: DG1ctrl (5′-CGTACCTCGCGCTCG-3′), DG2ctrl (5′-CAGTCTCTCATCCCTTAT-3′), DG3ctrl (5′-ATTAC-CACCCACTTCTCTTG-3′).

2.7. RNA isolation and quantitative reverse transcriptase (RT)-PCR

Total RNA was isolated using the RNeasy mini kit (Qiagen) and quantitated by spectrophotometric measurements. 0.5 μg of RNA was used for first-strand cDNA synthesis using superscript II reverse transcriptase (Life Technologies). The cDNA was used as template for PCR using DelGEF-specific primers (upstream: 5′-TGGGATTT-TACGATTATGCTCA-3′, downstream: 5′-GCACAACCGTCGTC-CAC-3′). As RNA quality control, PCR was performed with primers specific for 18S ribosomal RNA using the QuantumRNA Classic 18S primer set (Ambion). PCR products from reactions in linear phase (22–24 cycles) were quantitated on an Agilent 2100 Bioanalyzer using the DNA 7500 LabChip kit (Agilent Technologies).

2.8. Labelling and detection of secreted proteoglycans

Cells were incubated with 0.1 mCi/ml of $^{35}SO_4$ (Amersham Pharmacia Biotech) in labelling buffer (20 mM HEPES, 125 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1.8 mM $MgCl_2$, 5.6 mM glucose, pH 7.2) for 30 min. The labelling buffer was removed and cells were incubated in complete medium for 1–3 h. The medium was removed and briefly centrifuged to remove detached cells. Four vol. of acetone were added to the medium and protein precipitation was allowed to proceed overnight at −20°C. Proteins were pelleted by centrifugation (10 000×g, 20 min), resuspended in SDS loading buffer and resolved by SDS–

PAGE. The gel was incubated with 1 M salicylate (pH 6.0) for 30 min, dried and exposed to Biomax MR film (Kodak). Computerised images of the fluorographs were obtained by scanning, allowing for determination of band intensities using the NIH Image software.

3. Results

3.1. Identification of Sec5 as a protein interacting with DelGEF

In order to identify proteins interacting with DelGEF, a human cDNA library was screened using a yeast two-hybrid system. Of an estimated 4×10^6 transformants screened, 60 clones expressing reporter activity (β-galactosidase) could be identified and prey constructs were recovered from these colonies. The isolated plasmids were sequenced and the cDNAs from these clones were compared with nucleotide sequences of known proteins using the BLASTN algorithm. Two human cDNA sequences encoding potential DelGEF binding partners were obtained, one of these was found to be a human homologue of Sec5. The specific interaction between the pGBT9-DelGEF bait plasmid and the isolated pACT2 plasmid carrying the Sec5 cDNA was verified by cotransformation. Only upon cotransformation with pGBT9-DelGEF and not with pGBT9 (without insert) did the yeast colonies display reporter activity (not shown). Transformation with pGBT9-DelGEF alone did not induce reporter activity (not shown). The human Sec5 sequence obtained in the yeast two-hybrid screening was 95% identical to the C-terminal region (amino acid residues 447–924) of rat Sec5 [6] (GenBank acc. no. AF032666) indicating that DelGEF interacts with the C-terminal region of human Sec5. By using RACE-PCR, additional sequence information was obtained (not shown) yielding the complete coding sequence of human sec5 (deposited in the EMBL database, acc. no. AJ414403). The open reading frame consists of 2772 nucleotides encoding a protein of 924 amino acid residues (Fig. 1). The complete human Sec5 sequence was found to be 94% identical with rat Sec5 at the amino acid level. A nucleotide binding motif (A/G,X,X,X,X,G,K,S/T) [7] was identified in close proximity to

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  1  MSRSRQPPLVTGISPNEGIPWTKVTIRGENLGTGPTDLIGLTICGHNCLL
 51  TAEWMSASKIVCRVGQAKNDKGDIIVTTKSGGRGTSTVSFKLLKPEKIGI
101  LDQSAVWVDEMNYYDMRTDRNKGIPPLSLRPANPLGIEIEKSKFSQKDLE
151  MLFHGMSADFTSENFSAAWYLIENHSNTSFEQLKMAVTNLKRQANKKSEG
201  SLAYVKGGLSTFFEAQDALSAIHQKLEADGTEKVEGSMTQKLENVLNRAS
251  NTADTLFQEVLGRKDKADSTRNALNVLQRFKFLFNLPLNIERNIQKGDYD
301  VVINDYEK AKSLFGKT EVQVFKKYYAEVETRIEALRELLLDKLLETPSTL
351  HDQKRYIRYLSDLHASGDPAWQCIGAQHKWILQLMHSCKEGYVKDLKGNP
401  GLHSPMLDLDNDTRPSVLGHLSQTASLKRGSSFQSGRDDTWRYKTPHRVA
451  FVEKLTKLVLSQLPNFWKLWISYVNGSLFSETAEKSGQIERSKNVRQRQN
501  DFKKMIQEVMHSLVKLTRGALLPLSIRDGEAKQYGGWEVKCELSGQWLAH
551  AIQTVRLTHESLTALEIPNDLLQTIQDLILDLRVRCVMATLQHTAEEIKR
601  LAEKEDWIVDNEGLTSLPCQFEQCIVCSLQSLKGVLECKPGEASVFQQPK
651  TQEEVCQLSININIMQVFIYCLEQLSTKPDADIDTTHLSVDVSSPDLFGSIH
701  EDFSLTSEQRLLIVLSNCCYLERHTFLNIAEHFEKHNFQGIEKITQVSMA
751  SLKELDQRLFENYIELKADPIVGSLEPGIYAGYFDWKDCLPPTGVRNYLK
801  EALVNIIAVHAEVFTISKELVPRVLSKVIEAVSEELSRLMQCVSSFSKNG
851  ALQARLEICALRDTVAVYLTPESKSSFKQALEALPQLSSGADKKLLEELL
901  NKFKSSMHLQLTCFQAASSTMMKT
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Fig. 1. Amino acid sequence of a human homologue of Sec5. The complete sequence of human Sec5 was obtained by yeast two-hybrid interaction cloning and subsequent RACE-PCR. The sequence has been deposited in the EMBL database (acc. no. AJ414403). The C-terminal region of human Sec5 interacting with DelGEF is underlined. A putative nucleotide binding site is boxed.
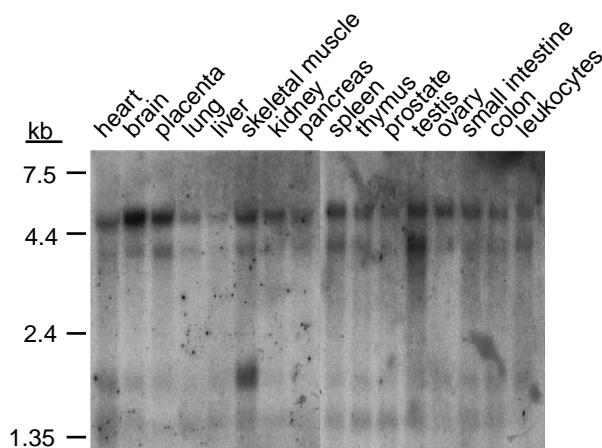
Fig. 2. Expression of sec5 mRNA in human tissues. A ^{32}P-labelled cDNA probe specific for human sec5 was hybridised to human multiple tissue RNA blots. RNA size markers are indicated.

the domain interacting with DelGEF in the yeast two-hybrid system (Fig. 1).

The second cDNA sequence obtained in the yeast two-hybrid screening encodes a protein which displays no significant homology to other proteins and has not been described in the literature. It is currently being characterised in our laboratory.

3.2. Sec5 mRNA tissue distribution

The tissue distribution of human Sec5 was investigated by Northern blot analysis (Fig. 2). A radiolabelled probe was prepared and hybridised to a human multiple tissue RNA blot. The predominantly expressed mRNA was found to have a size of around 4.5 kb. This transcript was detectable

in all tissues and was highly expressed in brain and placenta. In addition, shorter transcripts could be observed. A transcript of approximately 1.5 kb was strongly expressed in skeletal muscle.

3.3. Effects of metal ions and nucleotides on the DelGEF/Sec5 interaction

In order to verify the interaction between DelGEF and Sec5, in vitro binding experiments were performed. Recombinant GST-tagged DelGEF was bound to glutathione Sepharose beads. ^{35}S-labelled Sec5, obtained by coupled in vitro transcription/translation, was incubated with the beads. Finally, the material binding to the beads was analysed by gel electrophoresis. It was found that radiolabelled Sec5 could bind to DelGEF-GST coated beads only in the presence of 1 mM Mg^{2+} or 1 mM Mn^{2+} (Fig. 3). Binding could not be detected in the presence of 1 mM Ca^{2+} or 1 mM EDTA. Beads coated with GST alone did not retain Sec5, independent of buffer conditions.
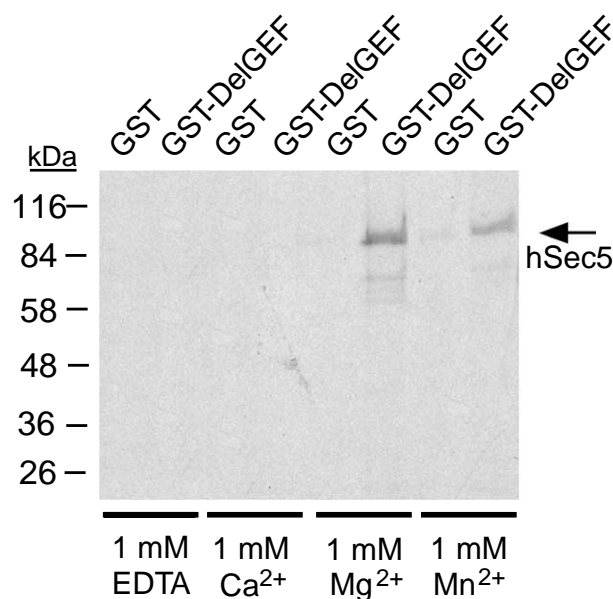

Fig. 3. In vitro binding between DelGEF and Sec5. GST alone or recombinant DelGEF-GST was immobilised on glutathione Sepharose beads and incubated with ^{35}S-labelled Sec5 produced by coupled in vitro transcription and translation. The binding buffer (20 mM Tris–HCl, pH 8.0, 100 mM NaCl and 0.1% Triton X-100) was supplemented with 1 mM EDTA, 1 mM Mg^{2+}, 1 mM Mn^{2+} or 1 mM Ca^{2+}. Radiolabelled material binding to the beads was detected by SDS–PAGE and subsequent fluorography.
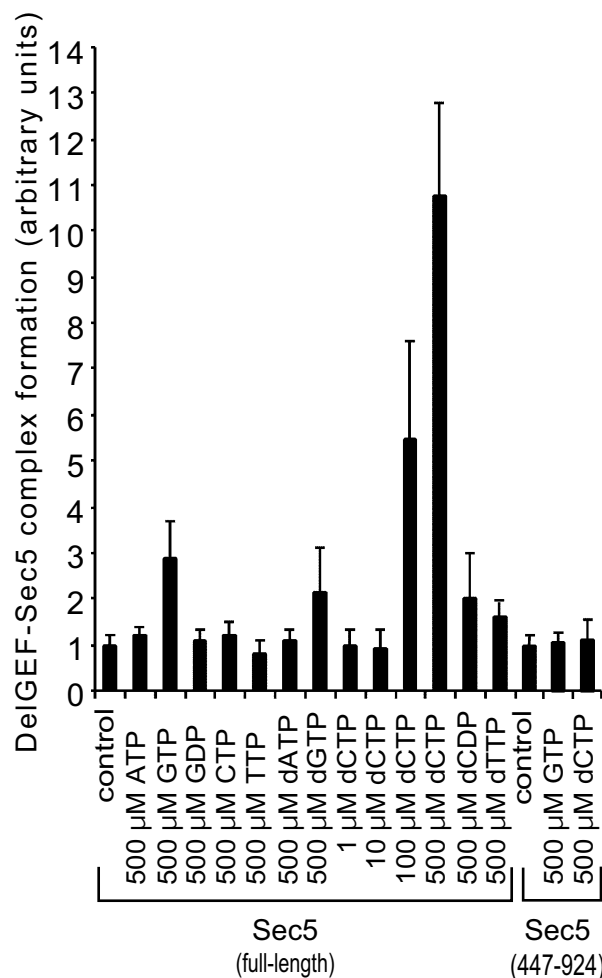

Fig. 4. In vitro binding between DelGEF and Sec5 in the presence of nucleotides. Recombinant DelGEF-GST was immobilised on glutathione Sepharose beads and incubated with ^{35}S-labelled full-length or truncated Sec5 produced by coupled in vitro transcription and translation. The binding buffer (20 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM Mg^{2+} and 0.1% Triton X-100) was supplemented with nucleotide as indicated. Radiolabelled material binding to the beads was detected by SDS–PAGE and subsequent fluorography. Binding was estimated by measuring band intensities using the NIH Image software. Values indicate means ± S.D. of four experiments.

Since human Sec5 was found to contain a nucleotide binding motif and since DelGEF is a homologue of RanGEF [2], we investigated if the addition of nucleotides to the in vitro binding reaction could affect the binding between DelGEF and Sec5 (Fig. 4). All binding reactions were performed in the presence of 1 mM Mg^{2+}. Addition of dCTP (500 μM) or GTP (500 μM) stimulated the interaction leading to an approximately 10- or three-fold increase of Sec5 binding to DelGEF, respectively. Other nucleotide triphosphates (ATP, CTP, TTP, dATP, dGTP and dTTP) and the nucleotide diphosphates (GDP and dCDP) did not significantly alter the amount of Sec5 binding to immobilised DelGEF at 500 μM nucleotide concentration. At 100 μM dCTP there was a five-fold stimulation of complex formation whereas 1 and 10 μM dCTP were without effect. When using a truncated Sec5 protein (residues 447–924) containing only the DelGEF binding domain indicated in Fig. 1, and not the putative nucleotide binding domain, there was no stimulation of complex formation by 500 μM dCTP or 500 μM GTP (Fig. 4).
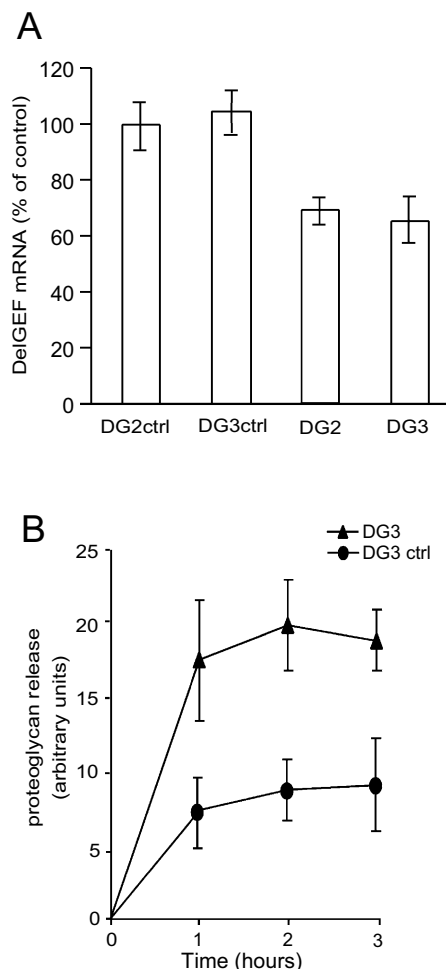

Fig. 5. Extracellular release of proteoglycans following DelGEF downregulation. HeLa cells were plated at 50 000/ml and transfected with control or DelGEF antisense oligonucleotides (500 nM). A: After 24 h, total RNA was isolated and used as template in quantitative RT-PCR using DelGEF-specific primers. B: After 24 h, the extracellular secretion of $^{35}SO_4$-labelled proteoglycans was measured at different timepoints in cells transfected with control oligonucleotide DG3ctrl or DelGEF antisense oligonucleotide DG3. Values indicate means ± S.D. of four experiments.

3.4. Effect of endogenous DelGEF downregulation on proteoglycan secretion

The interaction between DelGEF and Sec5 prompted us to investigate the role of DelGEF in secretion. We attempted to downregulate the expression of endogenous DelGEF in cultured human cells by antisense oligonucleotide treatment. A set of phosphorothioate-modified oligonucleotides were tested for their ability to lower the levels of DelGEF mRNA in HeLa cells. Two antisense oligonucleotides, DG2 and DG3, were found to induce downregulation of DelGEF mRNA (Fig. 5A). Double-stranded RNA oligonucleotides were recently shown to mediate gene silencing [8] but we could not detect DelGEF mRNA downregulation when transfecting with DelGEF-specific RNA duplexes (not shown).

The secretion of sulphated proteoglycans is a commonly used fluid phase marker of constitutive secretion [9–12]. In HeLa cells, chondroitin sulphate proteoglycans are the predominant proteoglycan species released upon sulphate labelling [13]. The expression of DelGEF in HeLa cells was downregulated by treatment with antisense oligonucleotide DG3. Thereafter the extracellular release of $^{35}SO_4$-labelled sulphated proteoglycans was determined (Fig. 5B). The release of sulphated proteoglycans was complete after 2 h in both control and antisense-treated cells. Sulfated proteoglycan release was found to increase two-fold in cells subjected to DelGEF antisense treatment. In contrast, there was no effect of the antisense treatment on cell growth and cell viability as judged by proliferation assays and trypan blue exclusion (not shown). The total (intracellular+extracellular) levels of labelled proteoglycans were not significantly changed upon antisense treatment indicating that proteoglycan synthesis was not altered by DelGEF downregulation (not shown).

4. Discussion

Using the yeast two-hybrid system, a human cDNA library was screened for proteins capable of interacting with DelGEF. We found that DelGEF interacts with a human homologue of Sec5, a protein involved in secretion. Sec5 is a component of the Sec6/8 multiprotein complex [14,15], the mammalian counterpart of the yeast exocyst complex [16–18]. The Sec6/8 complex is required for secretion and provides a mechanism for targeting secretory vesicles to sites of plasma membrane fusion [19,20]. We have determined the complete coding sequence of human Sec5 (Fig. 1) and found it to be 94% identical to rat Sec5 [6] at the amino acid level. Multiple tissue Northern blot analyses (Fig. 2) indicated that human Sec5 is ubiquitously expressed.

The interaction between DelGEF and Sec5 was confirmed by in vitro binding experiments and was found to be dependent on addition of Mg^{2+} or Mn^{2+} (Fig. 3). In addition, the binding could be stimulated by the addition of GTP or dCTP, whereas other ribonucleotides and deoxyribonucleotides were ineffective (Fig. 4). The physiological concentrations of GTP and dCTP have been estimated to be around 500 and 50 μM, respectively [21]. We observed significant stimulation of complex formation at 500 μM GTP and 100 μM dCTP suggesting that GTP and possibly also dCTP can regulate the DelGEF–Sec5 interaction in vivo. The results suggest the presence of nucleotide binding sites within the DelGEF–Sec5 complex. In accordance, a putative nucleotide binding motif was observed in close proximity to the DelGEF-interacting domain of hu-

man Sec5. When using a truncated form of Sec5 without the putative nucleotide binding site, there was no stimulation of in vitro binding between DelGEF and Sec5 by GTP or dCTP. GTP binding proteins are known to regulate many different functions in the cell whereas dCTP functions mainly as a substrate for enzymes involved in nucleotide metabolism, DNA synthesis and DNA repair. The exact mechanisms whereby GTP and dCTP stimulate formation of the DelGEF–Sec5 complex will require further investigation. For example, dCTP may stabilise the DelGEF–Sec5 complex by replacing another nucleotide normally promoting a hydrolysis-associated interaction of transient character. In the case of the enzyme aspartate transcarbamoylase, dCTP functions as a competitive inhibitor of ATP-dependent activation [22].

Since DelGEF interacts with Sec5, we investigated whether DelGEF plays a functional role in secretion. Using antisense oligonucleotides, the expression of endogenous DelGEF could be selectively downregulated in HeLa cells (Fig. 5). Subsequently, the extracellular secretion of chondroitin sulphate proteoglycans was measured. The extracellular release of proteoglycans is a commonly used fluid phase marker of constitutive secretion [10,11]. Upon inhibition of DelGEF expression, secretion of chondroitin sulphate proteoglycans was significantly upregulated, indicating a regulatory function for endogenously expressed DelGEF in the secretion process. In contrast, total synthesis of chondroitin sulphate proteoglycans was not significantly affected by DelGEF antisense treatment, indicating that proteoglycan synthesis was not affected by downregulation of endogenous DelGEF.

Ongoing work in our laboratory is aimed at studying the mechanism whereby GTP and dCTP regulate formation of the DelGEF–Sec5 complex and to define the cellular role of Del-GEF in the secretion process.

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