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Two tales of Annexin A2 knock-down: One of compensatory effects by antisense RNA and another of a highly active hairpin ribozyme

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ABSTRACT

Besides altering its own expression during cell transformation, Annexin A2 is upregulated during the progression of many cancer types and also plays key roles during viral infection and multiplication. Consequently, there has been great interest in Annexin A2 as a potential drug target. The successful design of efficient in vivo delivery systems constitutes an obstacle in full exploitation of antisense and RNA-cleaving technologies for the knock-down of specific targets. Efficiency is dependent on the method of delivery and accessibility of the target. Here, hairpin ribozymes and an antisense RNA against rat *annexin* A2 mRNA were tested for their efficiencies in a T7-driven coupled transcription/translation system. The most efficient ribozyme and antisense RNA were subsequently inserted into a retroviral vector under the control of a tRNA promoter, in a cassette inserted between retroviral Long Terminal Repeats for stable insertion into host DNA. The Phoenix package system based on defective retroviruses was used for virus-mediated gene transfer into PC12 cells. Cells infected with the ribozyme-containing particles died shortly after infection. However, the same ribozyme showed a very high catalytic effect in vitro in cell lysates, explained by its loose hinge helix 2 region. This principle can be transferred to other ribozymes, such as those designed to cleave the guide RNA in the CRISPR/Cas9 technology, as well as to target specific viral RNAs. Interestingly, efficient down-regulation of the expression of Annexin A2 by the antisense RNA resulted in up-regulation of Annexin A7 as a compensatory effect after several cell passages. Indeed, compensatory effects have previously been observed during gene knock-out, but not during knock-down of protein expression. This highlights the problems in interpreting the phenotypic effects of knocking down the expression of a protein. In addition, these data are highly relevant when considering the effects of the CRISPR/Cas9 approach.

1. Introduction

Annexin A2 (AnxA2) is a multifunctional protein [1–4] participating in many cellular processes, such as exocytosis [5,6], endocytosis [7–10], exophagy [11], phagocytosis [12], actin dynamics [13,14], specific mRNA transport and translation [15–17], DNA repair [18], replication [19] and transcription [20], as well as cell-cell contact, cell adhesion [21,22] and migration [23]. The discrimination between these cellular processes is mediated by its post-translational modifications, subcellular localisation and specific ligand interactions [24–26]. AnxA2 is a key protein in tumour proliferation and progression, related to its roles in tumour cell adhesion, proliferation and migration, which facilitate metastasis and neo-angiogenesis [27]. It is therefore not surprising that the expression of AnxA2 is modulated in several cancer types, being upregulated in e.g. hepatocellular [28], colorectal [29], and gastric carcinoma [30], pancreatic [31], lung [32] and breast [33], as well as glioma [34] cancers. Upregulation of AnxA2 correlates with enhanced cancer progression and poor prognosis [30,35], due to increased tumour recurrence and resistance to therapy [31,36–39]. To influence cancer progression, it would therefore be beneficial to reduce

Abbreviations: AnxA1/A2/A7/A11, Annexin A1/A2/A7/A11 protein; *anx*A1/A2/A7/A11, *annexin* A1/A2/A7/A11 cDNA or mRNA; bp, base pair; cpm, counts per minute; eGFP, enhanced green fluorescent protein; env, envelope gene from Moloney murine leukemia virus; FACS, Fluorescence-activated cell sorting; gag, capsid-group antigen gene from Moloney murine leukemia virus; HRP, horseradish peroxidase; LTR, long terminal repeat; NGF, nerve growth factor; nt, nucleotide; PC12, rat pheochromoctyoma cell line; pol, polymerase gene from Moloney murine leukemia virus; pol III, RNA polymerase III; Rib, ribozyme

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the expression of AnxA2 back to normal physiological levels. Furthermore, since AnxA2 is ubiquitously expressed, it would be highly advantageous to selectively regulate its expression in cancer cells to avoid severe side effects. Viruses have the ability to target specific cells by recognising their cell surface proteins. Thus, viruses loaded with catalytic or antisense RNAs could be used to knock-down the expression of the protein of interest – in this case, AnxA2 – in cancer cells.

Ribozymes are small catalytic RNA molecules that possess sequence-specific RNA cleavage activity and can inactivate the expression of specific gene products. Ribozymes have been successfully used to target and destroy viral and cellular RNAs both in cell culture and animal model systems [40]. Successful use of ribozymes to knock-down target gene expression is dependent on a number of factors, including target site selection/availability, ribozyme gene delivery, expression, stability and intracellular localisation. Notably, similar considerations are important for the use of the CRISPR/Cas9 system. A variety of natural ribozyme motifs have been identified. Naturally occurring ribozymes are the self-splicing group I and II introns, Ribonuclease P, hepatitis D virus, as well as the hairpin and hammerhead ribozymes [41–43]. Ribozymes can also be artificially created to act on specific target sequences in *cis* (intramolecular RNA catalysed reactions) or *trans* (intermolecular cleaving reactions, where other RNA molecules act as substrate) [44].

The two types of ribozymes that have been used most extensively are the hairpin- and hammerhead-ribozymes. This study employed hairpin ribozymes. The hairpin ribozyme consists of two independently folding domains, A and B, which must interact through tertiary contacts to form a functional catalytic complex (Fig. 1). After having cleaved the substrate RNA in the region that forms part of loop A (Fig. 1), the ribozyme dissociates from its substrate. The ribozyme can thereafter hybridise to and cleave new substrate RNA molecules. This reaction does not require protein cofactors [45]. However, the nucleotides within the internal loops are critically important for generating the required structure that forms an active site for the reaction [46].

A carefully designed antisense RNA selectively targets the mRNA encoded by an individual gene, thereby inhibiting the expression of the corresponding protein. By binding to its substrate mRNA, an antisense RNA can translationally inactivate this mRNA, leading to knock-down of the corresponding protein. It has been hypothesised that the association of an mRNA with a complementary sequence could specifically block the synthesis of the encoded protein in a dose-dependent manner, as the formation of a double-stranded hybrid would prevent the message from being translated by ribosomes. The prevention of ribosomal assembly or ribosomal sliding along the mRNA molecule also appears as possible important mechanisms of antisense action [47].

AnxA2 has been suggested by several studies to be a potential therapeutic target for the treatment of cancer in general, as well as for targeting metastasis and cancer-associated drug resistance [48,49]. Inhibition of angiogenesis has been targeted by inactivation of the vascular endothelial growth factor (VEGF). However, this type of therapy caused severe side effects due to the formation of more invasive and metastatic tumours [50]. As AnxA2 is involved in both angiogenesis and metastasis [51–53], therapeutic targeting of AnxA2 in cancer cells/cancer associated tissues could dramatically reduce most or all of these side effects. The highly potent ribozyme designed against rat *anx*A2 mRNA can easily be adapted to human *anx*A2 mRNA and further developed for delivery by a viral infection system targeting cancer cells, or using an RNA nanotechnology approach [54].

2. Materials and methods

2.1. Construction of ribozymes

The purified N-*ras*-pMJT plasmid, originally harbouring a cDNA coding for a hairpin ribozyme against HIV-1 [55], was used as a template for PCR amplification of the catalytic sites of the ribozymes against *anx*A2 mRNA. The use of five different specific 5′ primers (the specific 5′end primer for the very active RatRib120 is 5′-acaggatcc-tagggtttagaacataaccagagaaacacacgtt with the *Bam*HI site underlined; the other forward primers are: RatRib116, 5′-acaggatccgtttgactagaacataaccagagaaaca cacgtt; BovRib128, 5′-acaggatcctttgactagaacgtaaccagagaaacacacgtt; BovRib132, 5′-acaggatcctacgctttagaagaccaccagagaaacacacgtt; BovRatRib389/401, 5′-acaggatcctcttcagtagaactttaccagagaaacacacgtt) resulted in the amplification of five different *anx*A2 ribozymes. However, the same 3′ end primer was used in all PCR amplifications, namely 5′-aaaacgcgtaccaggtaa with the *Mlu*I site underlined. Thus, the specificity of the hairpin ribozyme cDNA in the expression cassette between the tRNA promoter and the tRNA termination sequences in the pMJT vector [56] was changed. All primers were from GenoSys, Sigma-Aldrich (USA). The ribozyme constructs were also inserted in the TOPO vector under the control of the T7-promoter for in vitro assays in the rabbit reticulocyte lysate system. Furthermore, the entire expression cassettes containing the two different constructs (*anx*A2 RatRib120 or antisense RNA) were excised from the pMJT

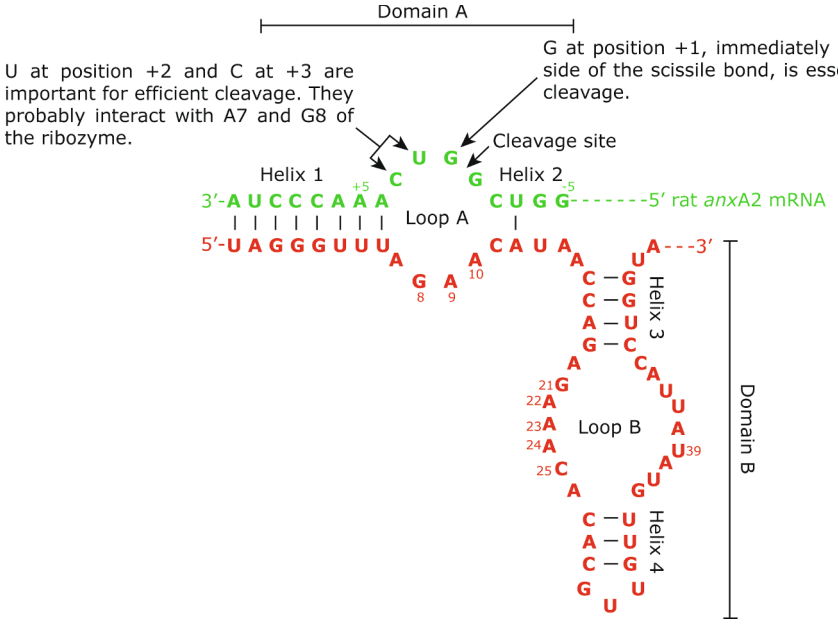

Fig. 1. The structural framework of the *trans*-acting *anx*A2 RatRib120 hairpin ribozyme and its substrate. Helices 1 and 2 are responsible for substrate recognition and loop A, which has certain base requirements (see text for further details), contains the cleavage site. Loop A is formed by both the ribozyme and target mRNA. Two helices, helix 3 and helix 4, separate a large asymmetrical internal loop, the loop B, and are entirely of ribozymal origin.

vector and inserted into the *Hind*III site of the pJIM vector, which contains the cDNA encoding the enhanced green fluorescence protein (eGFP) under the control of a CMV promoter. All constructs were verified by sequencing.

2.2. Cloning of cDNAs coding for rat anxA2 mRNA and antisense RNA

There are two variants of rat *anx*A2 mRNA; a 1356 nucleotide (nt) form and a 1362 nt form, which have probably arisen through alternative splicing of the gene [57]. A consecutive sequence of 6 nt in the 5′end of the coding region of the *anx*A2 mRNA constitutes the only difference between the two forms, which otherwise show a homology of 100%. The introduction of 6 extra nt results in 2 extra amino acids, including a Ser in the N-terminal domain of rat AnxA2 [57]. The 1356 nt form of rat *anx*A2 was obtained by RT-PCR using total RNA isolated from PC12 cells. It should be noted that the more abundant isoforms of mRNAs are preferred by this method [58]. PC12 cells were used as the source of good quality total RNA isolated by the use of TRIzol reagent (Thermo Fisher Scientific, USA), followed by RNA precipitation from the aqueous phase. First strand cDNA synthesis was performed using the SuperScript II RNase H-Reverse Transcriptase kit (Thermo Fisher Scientific, USA) and mRNA was degraded by RNase H (Thermo Fisher Scientific, USA). Second strand synthesis was performed with the GC-RICH PCR System kit (Sigma-Aldrich; USA). The specific primers were *ratBamH1*-forward (5′-cgtggatccggaggctctctgcaataggt) and *ratHindIII*reverse (5′-cgaagctttttttttttttttttaaag). The full-length rat *anx*A2 cDNA (including the UTRs) was inserted into the *Bam*HI and *Hind*III sites in the transcription pGEM3Z(f) + vector (Promega, USA) under the control of the T7 promoter. For the construction of the cDNA coding for the 141 nt rat antisense *anx*A2 RNA, full-length rat *anx*A2 cDNA was used as a template with the primers *ratMlu*1forward (5′-taacgcgtggaggctctctgcaataggt) and *ratanx*143*BamHI*reverse: (5′-taggatccgaagttggtgtagggtttga). The rat *anx*A2 antisense cDNA was also inserted in the expression cassette in the *Mlu*I and *Bam*HI sites of the pMJT vector, as well as in the TOPO vector similar to the ribozymes. The expression cassette was subsequently inserted into the *Bam*HI site of the pJIM vector. Restriction enzymes were obtained from Thermo Fisher Scientific (USA). All cDNA constructs were verified by sequencing.

2.3. Culture and infection of PC12 cells

The rat adrenal pheochromocytoma (PC12) cells, representing a readily adherent sub-clone derived from the original PC12 cell line [59] (a generous gift from professor Jaakko Saraste), were grown in Gibco RPMI 1640 medium (Thermo Fisher Scientific, USA) supplemented with 10% (v/v) heat-inactivated horse-serum, 5% (v/v) foetal bovine serum, 2 mM L-glutamine, 100 units penicillin/ml and 100 μg streptomycin/ml. All supplements were from Sigma-Aldrich (USA). As described previously [60], the cells were routinely cultured at 37 °C in a humidified atmosphere of 21% O_2 supplemented with 5% CO_2. For transfer of the cDNAs encoding the ribozyme and the antisense RNA into the genome of the PC12 cells, a retroviral packaging system using the ecotropic Phoenix cell line was used [61]. The genome of murine retroviruses (Moloney murine leukaemia virus) consists of three genes: The capsid-group antigen (*gag*), polymerase (*pol*) and envelope gene (*env*) flanked on both sides by LTRs. Phoenix is an amphotropic 293T-based split packaging cell line and has the *gag-pol* and *env* genes each expressed from a different non-Moloney murine leukaemia virus-based promoter. Phoenix packaging cells provide the structural and regulatory proteins necessary for assembling an infectious viral particle. The retroviral vector (the recombinant pJIM vector containing the inserted ribozyme or antisense RNA sequence in the expression cassette), was mixed with calcium chloride (Sigma-Aldrich, USA) and HEPES-buffered saline to form a precipitate. The packaging cell line took up the precipitate and the plasmid was transcribed. The retroviral vector also contains a packaging signal, which causes the Phoenix cells to assemble virus particles “loaded” with the recombinant pJIM. These particles bud from the host cells and can be collected and used to infect the target cells – in this case, the PC12 cells. PC12 cells express the murine multi-spanning membrane protein that is the retroviral receptor recognised by the envelope protein of Moloney murine leukaemia virus and required for the infection of virions harbouring this envelope protein. Infection involves the binding of the retroviral *env* to this specific cell surface receptor in the PC12 cells, resulting in the internalisation of the complex. Once inside the cell, the viral nucleoprotein complex gains access to intracellular DNA nucleotide triphosphate pools, whereupon the retroviral reverse transcriptase initiates the synthesis of a double-stranded DNA copy of the viral genome, in preparation for its integration into the host cell chromosome. For this purpose, the linear double-stranded cDNA made in the cytoplasm is first transported to the nucleus. It is subsequently inserted into the host genome due to the presence of the LTRs in the cDNA and the viral enzyme Integrase. Infection of a host cell with a retrovirus results in the establishment of a lifelong carrier state because the virus remains integrated as provirus within the chromosome of the infected cell.

2.4. Selection of the infected cells using FACS

Infected PC12 cells were separated from uninfected PC12 cells by two-way flow-cytometry using Cytomation’s MoFlo modular flow cytometer with a 70 μm nozzle. The PC12 cells infected (5–25% efficiency) with the RatRib120 ribozyme, the antisense *anx*A2 construct, or the empty pJIM vector were grown in culture to reach approximately 70% confluency. The total amount of cells was estimated to 1.5×10^6. The cells were mechanically detached from the flasks, transferred to centrifuge tubes, and centrifuged at 500*g* for 5 min. The supernatant was discarded, and the pellet resuspended in 1 ml of Gibco RPMI 1640 medium (Thermo Fisher Scientific, USA) containing 2 mM L-glutamine, 10% (v/v) heat-inactivated horse serum, 5% (v/v) foetal bovine calf serum, 100 u/ml penicillin and 100 μg/ml streptomycin. All supplements were from Sigma-Aldrich (USA). The cell suspension was placed in the MoFlo Cytomation apparatus and sorted according to the manual provided by the manufacturer. Subsequently, the cells were seeded in 96-well plates at low density to obtain uniform cultures of infected cells.

2.5. Preparation of cell lysates from PC12 cells

Whole PC12 cell lysates were obtained by incubation of cells for 15 min in RIPA buffer (Thermo Fisher Scientific, USA) with 2 mM EGTA (Sigma-Aldrich, USA) and protease inhibitor cocktail (Roche, Mannheim, Germany; EDTA-free), followed by centrifugation for 20 min at 12,000*g*, as detailed in Aukrust et al. [62]. Protein determination was performed using the infrared spectroscopy method and Direct Detect® Infrared Spectrometer according to the manufacturer (Millipore, USA).

2.6. SDS-PAGE and western blot analysis

SDS-PAGE was performed using 10% (w/v) gels (Mini-Protean TGX; Bio-Rad Laboratories, USA) and the proteins were transferred onto nitrocellulose membranes (0.2 μm pore size) (Amersham, GE Healthcare Life Science, Germany) by blotting performed at 50 V for 2 h. AnxA2 (610069; BD Biosciences (USA); dilution 1:1000), AnxA7 (610668; BD Biosciences (USA); dilution 1:1000) and tubulin (86298; Cell Signaling Technology (USA); dilution 1:5000) were detected by mouse monoclonal antibodies, while AnxA1 was detected by rabbit polyclonal antibodies (71-3400; Invitrogen (USA); diluted 1:500). AnxA11 was detected by goat polyclonal antibodies (sc-9322; Santa Cruz Biotechnology (USA); dilution 1:500). Subsequently, horseradish peroxidase (HRP)-conjugated anti-mouse, anti-rabbit or anti-goat

secondary antibodies (Bio-Rad Laboratories (USA) or Abcam (UK)) were used. The reactive protein bands were visualised using the WesternBright ECL HRP substrate (Advansta, USA).

2.7. Coupled transcription/translation reactions in the rabbit reticulocyte lysate (TNT)

The TNT reticulocyte lysate system (Promega, USA) was used to perform a coupled transcription/translation reaction to determine the effect of the different ribozymes and the antisense RNA on the expression of the rat AnxA2, measured as [^{35}S]-Met (10 mCi/ml) (Perkin Elmer, USA) incorporated into the protein. Circular plasmid DNA (1 μg of each) was used as templates. The plasmids with cDNAs coding for the ribozymes or the antisense RNA were pre-incubated for 10 min to initiate their transcription before the plasmid containing the rat *anx*A2 cDNA (or control vector with the *luciferase* cDNA) was added to the reaction. The samples were incubated for a further 90 min at 30 °C. 0.2 mg/ml RNase A (Thermo Fisher Scientific, USA) was added to the samples, which were then incubated for another 5 min at 30 °C. The newly synthesised proteins were resolved by 10% SDS-PAGE and visualised using a Canberra Packard Instant Imager (Austria).

3. Results

3.1. Design of ribozymes and an antisense RNA against rat annexin A2 mRNA

We designed ribozymes against both rat and bovine *anx*A2 mRNA. However, the latter were not used in infection studies due to the lack of a suitable bovine cell line. Instead, the ribozymes directed against bovine *anx*A2 mRNA served as controls of the specificity of the ribozymes against rat *anx*A2 mRNA. The first part of the name of the ribozyme indicates the species of the substrate mRNA, while the following number indicates the position of the nt immediately next to the 5′ end of the point of cleavage in the substrate mRNA, based on the rat and/or bovine *anx*A2 cDNA sequences (NM_019905.1 and NM_174716.1, respectively) (Fig. 1). Altogether 5 ribozymes (Rib) were designed: RatRib116, RatRib120, BovRib128, BovRib132 and RatBovRib389/401 (Fig. 2). In order to determine the specificity of the three rat *anx*A2 ribozymes, different features were adopted in their design. Thus, it should be noted that RatRib116 has full base-pairing in helices 1 (7 bp) and 2 (4 bp) and a loop A of 10 nt in total, while RatRib120 has full base-pairing in helix 1 (8 bp), a loop of 8 nt in total, but only one base-pairing in helix 2. Furthermore, RatBov389/401 has only 6 bp in helix 1 and full base-pairing in helix 2 (4 bp) and a large loop A (12 nt in total). However, loop A in RatBov389/401 does not contain the G that is supposed to be important for cleavage of the mRNA. A hairpin ribozyme is supposed to have a substrate sequence requirement of 5′-BN * GUC-3′ in the part of loop A formed by the substrate mRNA, where the * indicates the site of cleavage, the nucleotide B is G, U or C, and N is any base [63]. The BovRib128 has no match to the rat *anx*A2 mRNA in the region forming helix 2 and BovRib132 has no significant match to rat *anx*A2 mRNA (Fig. 2).

A target region in the rat *anx*A2 mRNA comprising the first 141 nt starting from its very 5′ end was selected for designing the rat *anx*A2 antisense RNA sequence. Specific primers were designed to amplify the cDNA sequence corresponding to this region in the antisense direction. It has been considered preferable that the target region resides in the 5′ end of the mRNA, comprising the AUG start codon [64], in order to inhibit or hamper initiation of translation.

3.2. In vitro effects of the anxA2 ribozymes and antisense RNA on rat anxA2 mRNA stability/translatability

The different ribozyme and antisense RNA constructs were inserted into the TOPO vector in the correct orientation relative to the T7 promoter. Subsequently, their effect on the rat *anx*A2 mRNA stability/translatability was analysed in the rabbit reticulocyte lysate (TNT system) by monitoring the incorporation of [^{35}S]-Met into AnxA2 protein following T7-driven transcription of the rat *anx*A2 mRNA from its cDNA in the pGEM3Zf(+) vector, and its subsequent translation (Fig. 3A, D). The incorporation of [^{35}S]-Met after coupled transcription/translation of the rat *anx*A2 cDNA alone (lane 1) was used as control (100%), while the co-transcription of the ribozymes resulted in either no significant effect (BovRib128; lane 2), (BovRib132; lane 3), (RatRib116; lane 4) or reduction in the incorporation of [^{35}S]-Met into AnxA2 by approximately 90% (RatRib120; lane 5) or 70% (RatBovRib389/401; lane 6), respectively, as compared to the transcription/translation of *anx*A2 mRNA alone (100%) (Fig. 3A, C). The effect of the different ribozyme transcripts on the synthesis of a 61 kDa luciferase protein was used as negative controls (Fig. 3B).

The rat *anx*A2 antisense RNA sequence led to a ~70% decrease of AnxA2 expression in the coupled transcription/translation system (Fig. 3D and F, compare lanes 7 and 8). The expression of the luciferase protein was again used as a control (Fig. 3E). Despite the appearance of

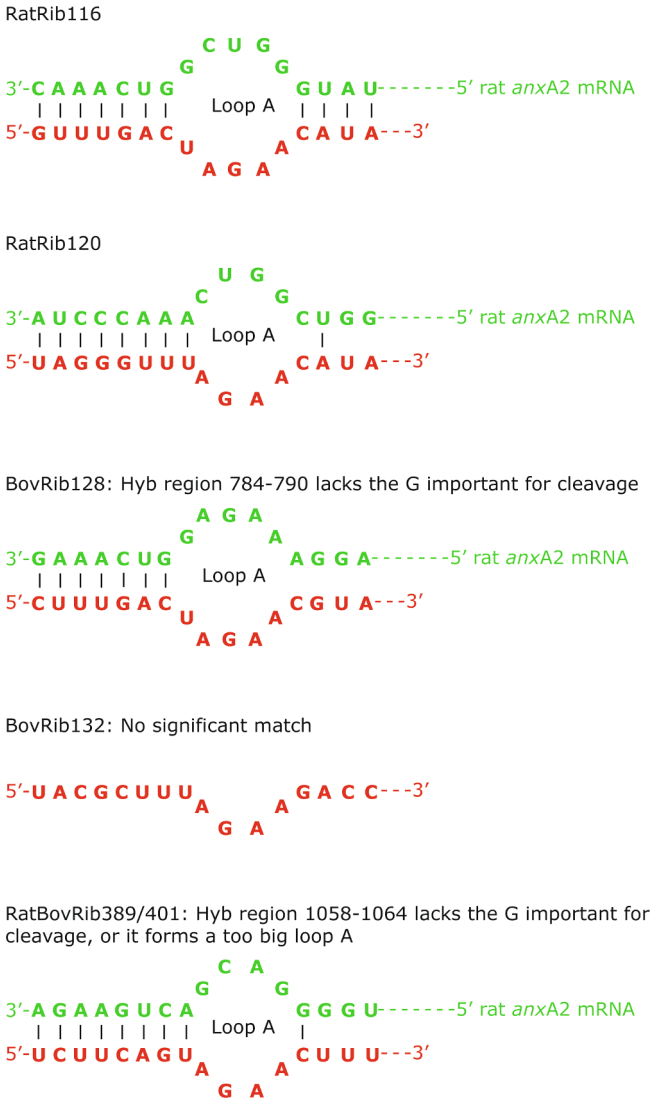

Fig. 2. The 5′ end of the different ribozymes directed against rat and/or bovine anxA2 mRNA. Only the nucleotides of the ribozymes complementary to the rat *anx*A2 substrate mRNA and the ribozymal nucleotides involved in forming loop A are shown. The names of the ribozymes indicate whether the ribozyme is designed to cleave rat or bovine *anx*A2 mRNA, and also provides the position of cleavage in the substrate mRNA.

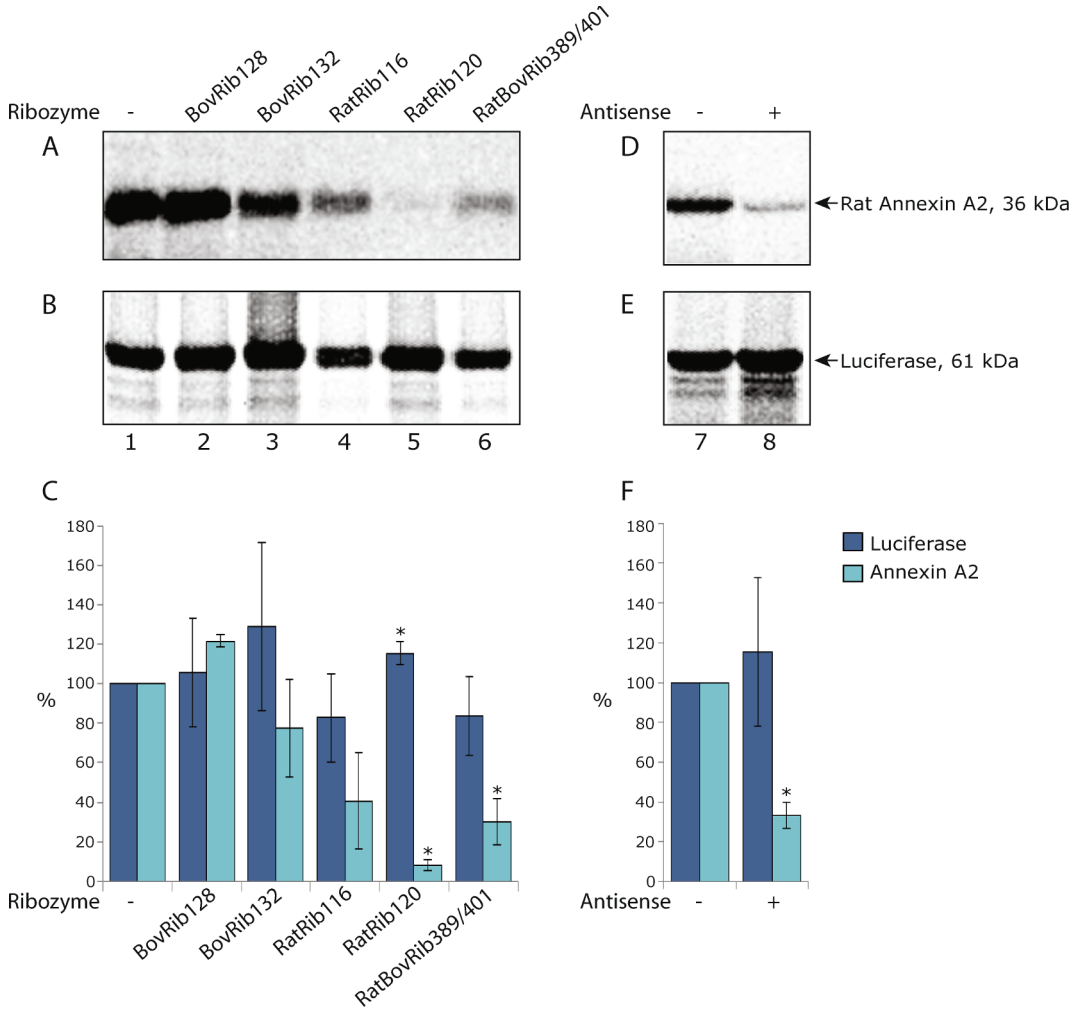

Fig. 3. The effect of *anx*A2 hairpin ribozymes (A) or antisense RNA (D) on the translation of rat *anx*A2 mRNA (A and D) in a coupled transcription-translation in vitro system (TNT). *anx*A2 mRNA was transcribed and translated from 1 μg of pGEM3Zf(+) vector containing the rat *anx*A2 cDNA alone (lanes 1 and 7), or in combination with the transcripts BovRib128 (lane 2), BovRib132 (lane 3), RatRib116 (lane 4), RatRib120 (lane 5), RatBovRib391/401 (lane 6), or of rat *anx*A2 antisense RNA transcribed from 1 μg of recombinant TOPO vector. Similar experiments were performed using 1 μg of Control Vector containing the *luciferase* cDNA as a negative control, as indicated in the Figure (B and E). [^{35}S]-Met labelled AnxA2 was resolved by 10% SDS-PAGE, and visualised using a Canberra Packard Instant Imager. 10 μl reaction mix was applied in each well. The 36 kDa AnxA2 and the 61 kDa luciferase are indicated by arrows to the left. Results (C and F) are the mean ± SD of 3 independent experiments each. Statistical significance was determined by the unpaired Student's *t*-test ($^{*}p < 0.05$).

some degradation products below the main band of ~61 kDa, the intensity of the luciferase bands did not decrease in the presence of the rat *anx*A2 antisense RNA (lane 8).

When using the coupled in vitro transcription/translation rabbit reticulocyte system it was important to establish and quantify the presence of AnxA2 in the lysate. Although only newly synthesised AnxA2 will contain [^{35}S]-Met, the presence of endogenous AnxA2 in the lysate might interfere with the results obtained using this in vitro system, since AnxA2 binds to its cognate mRNA [65]. It is evident from Fig. 4, lanes 1–3 that the rabbit reticulocyte lysate does not contain endogenous AnxA2. This corroborates the findings of others showing that AnxA2 is not present in erythrocytes [66].

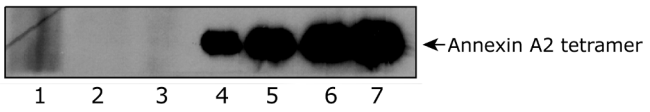

Fig. 4. The absence of endogenous AnxA2 in rabbit reticulocyte lysate. Proteins in 50 μg (lane 1), 100 μg (lane 2), or 150 μg (lane 3) rabbit reticulocyte lysate or increasing amounts of purified AnxA2 tetramer; 0.5 μg (lane 4), 1.0 μg (lane 5), 2.0 μg (lane 6) and 3.0 μg (lane 7), to serve as a standard curve for AnxA2, were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. Subsequently, AnxA2 was immunodetected by affinity purified monoclonal AnxA2 antibodies.

3.3. Transfer of anxA2 antisense and RatRib120 constructs into a retroviral vector expressing GFP allows visualisation of infection and sorting of infected PC12 cells

The rat pheochromocytoma PC12 cell line is useful for morphological studies since they are rather flat and become polarised during differentiation, being particularly well suited for confocal microscopy. Finally, contributing to the usefulness of transfections and/or infections is their relative stability, homogeneity, high efficiency of differentiation in response to nerve growth factor (NGF), potential for genetic manipulation and the large number of studies characterising their properties.

As the RatRib120 ribozyme proved to be the most effective in the in vitro experiments employing the TNT coupled transcription/translation system, it was chosen for further testing in PC12 cells. In order to perform infection experiments with the two cDNAs, encoding either the RatRib120 ribozyme or the antisense RNA under the control of a tRNA

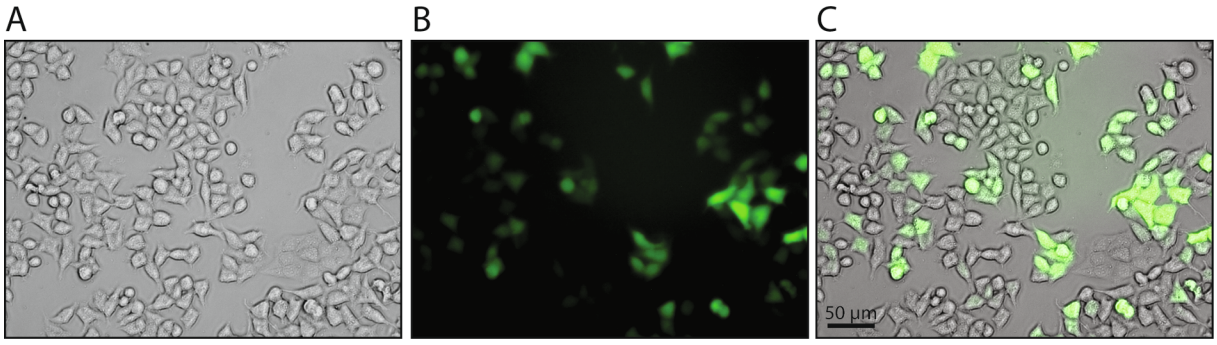

Fig. 5. Cultured PC12 cells infected with the rat *anx*A2 antisense cDNA together with the eGFP marker. PC12 cells after infection as seen with normal light (A), or with a blue light (B). The two images are merged in C. The positive infected PC12 cells can be seen as green fluorescence specs, due to the expression of the eGFP marker, whereas the uninfected cells do not express eGFP.

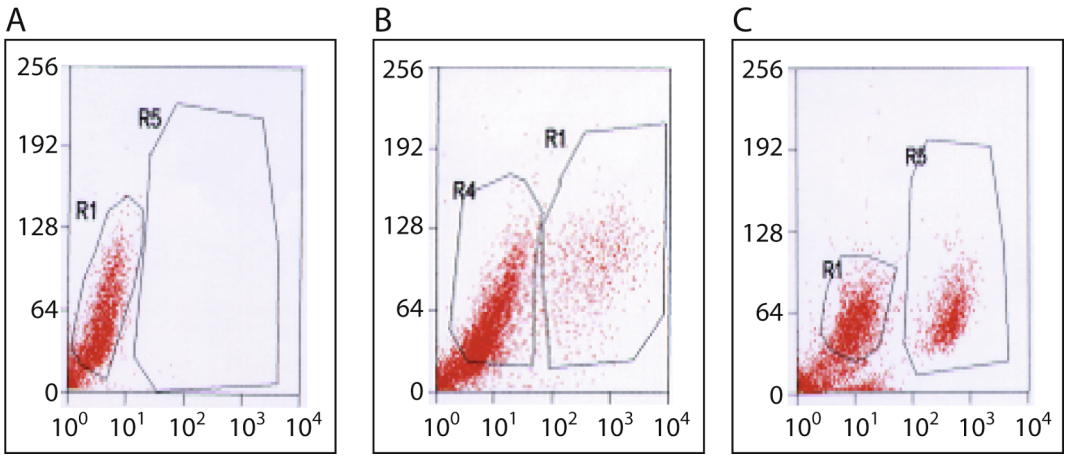

Fig. 6. Graphic recordings of the PC12 cells during the sorting process, indicating the level of fluorescence (FL1) and granulation (SSC) in uninfected PC12 cells (graph 1), PC12 cells infected with the eGFP marker alone (graph 2), or a combination of the eGFP marker and the rat antisense *anx*A2 (graph 3). Graphs 1–3 were recorded using cycle mode as the cells pass through the flow-cytometer, and display the distribution of fluorescent cells versus non-fluorescent cells at any given time. Two areas (R1 and R5) were marked in each of the graphs, and the cells within these two areas were sorted using two-way sorting as the fluorescent cells (R5) were to be sorted from the non-fluorescent cells (R1). The R5 and R1 population of cells were collected in separate tubes, and the R5 population containing PC12 cells infected with both the eGFP marker and the rat antisense *anx*A2 RNA was used for further applications.

promoter, they were inserted into the retroviral pMJT vector between the tRNA promoter site for polymerase III and the tRNA termination sequence using *Mlu*I and *Bam*HI restriction enzymes [55]. Subsequently, the resulting expression cassettes containing the two different constructs were excised from this vector and inserted into the pJIM vector, which also contains the cDNA encoding eGFP, and therefore could be used as a reporter gene for the identification of infected PC12 cells. pJIM also contains a CMV promoter and is a CRU5-GFP variant described in [56].

The PC12 cells infected with the RatRib120 cDNA together with the eGFP marker did not survive the infection despite several attempts. However, the in vitro assays showed that we had designed a very active ribozyme by making the helix region 2 formed by the ribozyme together with the mRNA very flexible in just having one base-pairing.

The PC12 cells infected with the cDNA coding for the 141 nt rat *anx*A2 antisense RNA sequence together with the eGFP marker survived. Approximately 25% of the PC12 cells in the infected cultures displayed green fluorescence (Fig. 5), indicating positive infection with the *anx*A2 antisense construct. Both PC12 cells infected with the eGFP marker only, and cells infected with eGFP together with the *anx*A2 antisense RNA, were sorted by FACS (Fig. 6) in order to study the effect of the *anx*A2 antisense RNA on the expression of AnxA2 in rat PC12 cells. The cells from each culture were sorted separately using two-way sorting based on eGFP expression of the infected cells.

3.4. Specificity of the anxA2 antisense RNA

Immediately following the infection with the *anx*A2 antisense RNA, the infected PC12 cells were not able to respond to NGF and initiate the outgrowth of long neurite-like extensions. This was expected since AnxA2 has been reported to play a role in the outgrowth of PC12 cell extensions [67] and is also present in their growth cones [68]. However, after some passages, many of the cells that were infected with the antisense RNA formed long extensions, also in the absence of NGF, which was completely unexpected. The analysis of cell lysates prepared from wild-type PC12 cells, cells infected with an empty vector (pJIM), or *anx*A2 antisense RNA in the same vector, showed that AnxA2 is down-regulated in cells infected with the antisense construct (Fig. 7A, lane 3), in particular when compared with the empty vector control (Fig. 7A, lane 2). It should be noted that infection with the empty vector alone evidently causes up-regulation of AnxA2 expression, perhaps due to the presence of the strong CMV promoter in the vector. As AnxA1 is the closest relative of AnxA2 [1], we hypothesised that there could be a redundancy between AnxA1 and AnxA2 functions in the infected cells. Thus, we also investigated the expression level of AnxA1 and two more distantly related Anxs; namely AnxA7 and AnxA11. It is evident that neither AnxA1 (Fig. 7A-B, lane 3) nor AnxA11 (Fig. 7A-B, lane 3) is upregulated following AnxA2 knock-down, while the expression of AnxA7 is about 6-fold upregulated (Fig. 7A-B, lane 3).

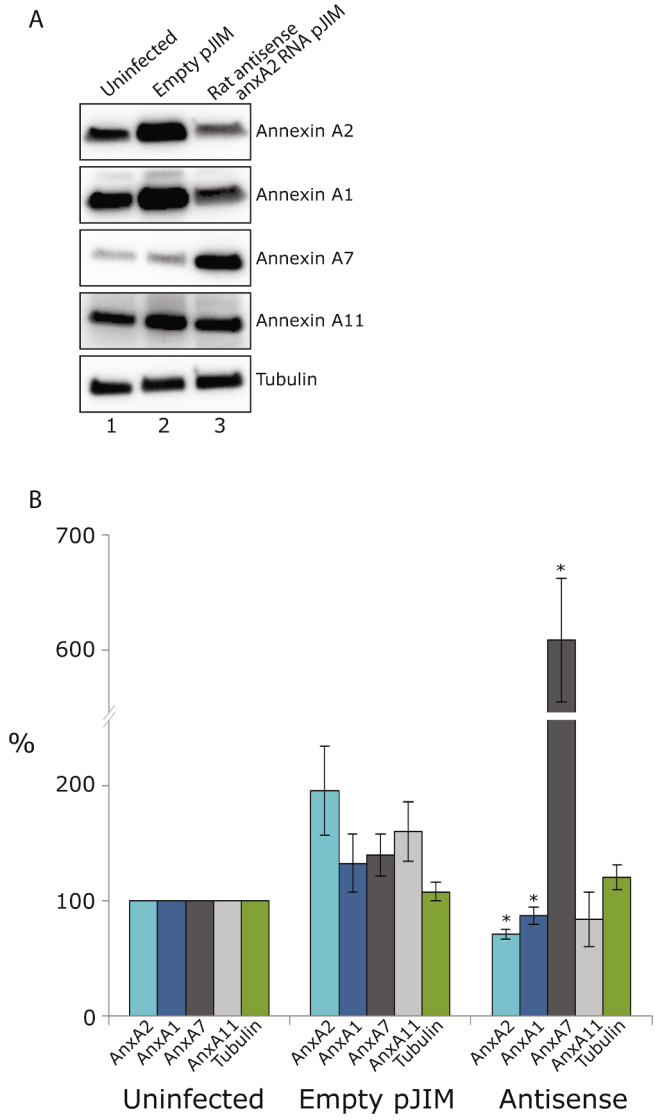

Fig. 7. Expression levels of Anxs A1, A2, A7 and A11 in PC12 cells after infection with rat *anx*A2 antisense RNA. 20 µg of total cell lysates from control cells (lane 1), cells infected with empty pJIM vector (lane 2) or with rat *anx*A2 antisense RNA in pJIM (lane 3) were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes and probed against AnxA1, AnxA2, AnxA7 and AnxA11 as indicated to the right. Probing against tubulin was used as a loading control. Results (B) are the mean ± SD of 3 independent experiments each. Statistical significance was determined by the unpaired Student's *t*-test ($^{*}p < 0.05$).

4. Discussion

4.1. Design of ribozymes and their efficiency in vitro in the coupled transcription/translation system

Due to their catalytic effects in cleaving specific mRNAs, ribozymes have been regarded as promising tools both in basic research and disease therapy. Several ribozymes have already been used in the treatment of infectious diseases, cancer and genetic disorders. Recently, ribozymes have also been used in transgenic animal research, as well as gene target validation and pathway elucidation. One of the advantages of using ribozymes is that their effect is equivalent to a gene knock-out without the need for homologous recombination and inactivation of both alleles [69].

Several investigators have stated the need for the nucleotide G in position 11 of the ribozyme sequence, which forms the first base pair in helix 2, next to loop A [63,70–72]. However, of the ribozymes generated in this project, only BovRib132 has the nucleotide G in position 11. By contrast, all the remaining ribozymes have C in this position, including RatRib120 with the highest cleavage activity and thus the strongest knock-down effect on rat *anx*A2 mRNA (Figs. 1 and 2). Only ribozyme RatRib116, contains full complementarity to the rat *anx*A2 mRNA within the 4 nt helix 2 segment, whereas the highly effective RatRib120 ribozyme is only capable of Watson-Crick base-pairing to one nt in the target mRNA within this region (Figs. 1 and 2). The high efficiency of the RatRib120 ribozyme (Fig. 2) could be caused by greater freedom of the hinge region, as there is no "constraining" complementarity in the helix 2 region. This could allow a more precise interaction between the loop A and loop B domains of the ribozyme (see Fig. 1), which is required for its catalytic activity. However, it is generally considered that helix 2 should be fixed at 4 bp for optimal activity, whereas the length of helix 1 giving optimal catalytic efficiency is dependent on its sequence and must be optimised for each target sequence [73]. Since flanking sequences of 5–9 bp typically yield optimal activity, all five *anx*A2 mRNA specific ribozymes contain 7–8 nt, which hybridise to the target mRNA to form helix 1.

The coupled in vitro transcription/translation system based on the rabbit reticulocyte lysates closely mimics in vivo conditions, containing all the necessary components for efficient synthesis of proteins. This system has been used with success to analyse the effect of ribozymes and antisense RNA on target mRNAs [74]. Thus, it is also suitable for testing the effect of *anx*A2 specific ribozymes and antisense RNA on the translation of rat *anx*A2 mRNA. In this coupled transcription/translation system, the ribozyme constructs directed against rat *anx*A2 mRNA (RatRib116 and RatRib120) were more specific for their substrates than the ribozyme constructs directed against bovine *anx*A2 mRNA (BovRib128 and BovRib132) (Fig. 3). The RatRib120 and BovRib132 ribozymes resulted in higher knock-down efficiency of rat *anx*A2 mRNA as compared to the RatRib116 and BovRib128 ribozymes, respectively. Both pairs of ribozymes were directed against two homologous sites in the respective mRNAs. Accordingly, the different catalytic activities of these pairs might be caused by better accessibility of the RatRib120 and BovRib132 ribozymes to their substrate, due to the secondary structure of the *anx*A2 mRNA. The ribozyme directed against a region common to both rat and bovine *anx*A2 mRNAs, BovRatRib389/401, was also quite effective in inhibiting the translation of rat *anx*A2 mRNA. The *anx*A2 specific ribozymes did not affect the synthesis of luciferase serving as a negative control, indicating a certain degree of specificity. In conclusion, all the three ribozymes targeting specifically the rat *anx*A2 mRNA were very efficient in the knock-down of rat AnxA2 expression in vitro.

4.2. Efficiency of the rat anxA2 antisense RNA and ribozymes in the in vitro coupled transcription/translation system and upon retroviral infection of PC12 cells

The rat *anx*A2 antisense RNA leads to about 70% reduction in AnxA2 expression in the reticulocyte lysate-based coupled transcription/translation system (Fig. 3D, compare lanes 7 and 8). Thus, the antisense was selected for retroviral infection of PC12 cells. All the PC12 cells used in this project are derived from the same sub-clone, which is an important requirement since different sub-clones of PC12 cells vary in their ability to form extensions upon stimulation with NGF.

In order to transcribe the ribozymes and the antisense RNA sequence under the control of a strong promoter, the cDNA constructs were ligated into an expression cassette containing a tRNA promoter and a tRNA termination sequence. The promoter present in the expression cassette is an RNA polymerase III promoter shown to yield very high intracellular levels of ribozymes, in addition to providing a high transcription rate and stability of the mature ribozyme transcript [75]. Promoters naturally involved in the synthesis of small RNAs, like tRNAs and snRNAs, have well-defined transcription termination signals and thus can transcribe short artificial sequences with well-defined

flanking sequences. A retroviral vector (pMJT) containing a strong tRNA promoter was chosen for this study. Furthermore, in the course of the project, a retroviral vector containing the eGFP cDNA (pJIM) became available. Since eGFP would also serve as a good transfection/infection marker, the ribozyme expression cassette with the tRNA promoter was cloned into this vector. The retroviral pJIM vector contains a multiple cloning site just upstream of the eGFP gene within a region of the plasmid flanked by LTRs and the viral packaging signal. This vector was used to transfect the Phoenix cell lines which would package the region flanked by the LTRs into replication-defective viral particles.

The intracellular transcription of a ribozyme or an antisense coding gene is termed endogenous ribozyme/antisense delivery. Alternatively, ribozymes or antisense RNAs can be introduced either by direct injection or by transfection. Endogenous delivery of ribozymes and the antisense RNA, as carried out in this project, requires a vector, preferably a retroviral one, containing the gene for the ribozyme or antisense controlled by a suitable promoter. Transcription of the ribozyme or antisense can then be achieved either in stably infected or transiently transfected cell lines. It is very important that the ribozyme or antisense gene is placed downstream of a strong promoter, resulting in its efficient expression, as the high cellular concentration of ribozyme or antisense ensures that substrate binding is not a limiting factor. This also introduces a higher risk for off-target effects. The major advantage of the endogenous application of ribozymes and antisense lies in their continuous expression. Both are transcribed in the nucleus and transported into the cytoplasm [76]. The *in situ* production of the regulatory agent, and hybridisation between the ribozyme/antisense RNA and messenger RNAs, may take place within the nucleus or in the cytoplasm. Therefore, effects on gene expression might occur after transcription, but prior to translation [77]. In the infection experiments carried out in this project, the DNA sequence, which was inserted into the host DNA, consists of the 5′LTR, followed by the packaging signal (Ψ), the tRNA promoter, the cDNA of interest (encoding either the RatRib120 ribozyme or the rat *anx*A2 antisense RNA), the tRNA termination sequence, the eGFP encoding gene and the 3′LTR. The ribozyme is under direct control of the tRNA promoter.

4.3. Retroviral infection with the RatRib120 ribozyme

In vitro experiments assessing the effect of the RatRib120 ribozyme on the translation of rat *anx*A2 mRNA showed approximately 90% reduction when using the translation of rat *anx*A2 alone as a reference. The RatRib120 ribozyme was expected to be more effective in vivo than in vitro because cells contain a number of unwinding activities that may give the ribozyme better access to the target mRNA. The transfection of the Phoenix cell line with the retroviral vectors was successful, resulting in efficient production of virus particles containing the RatRib120 ribozyme cDNA. Nevertheless, the three separate infections of PC12 cells with the RatRib120 ribozyme resulted in only a few weakly eGFP-positive cells, which died within 30–40 h after infection. The AnxA2 protein has a lifespan of approximately 15 h [78]. Thus, one possible explanation for the observed cell death is that the AnxA2 protein is vital to PC12 cells. Since the AnxA2 knock-out mice are viable, developmentally normal and have a normal lifespan [79], PC12 cell death due to the ribozyme infection was unexpected. Infection with the empty pJIM vector (which only contains the cDNA for eGFP between the LTRs) did not kill the cells; neither did the infection with *anx*A2 antisense RNA, suggesting that cell death was related to the ribozyme. It is possible that the ribozyme construct was inserted via the LTRs into a site in the host DNA, which activated certain strong promoters. As a consequence, increased expression of proteins compromising cell viability could be activated. Since it is not likely that the *anx*A2 ribozyme cDNA was inserted into the same site in all cells (although insertion is not a completely random event), it is more likely that the ribozyme also targets other mRNAs and in this manner causes cell death. A Blast (megablast) of the target substrate sequence of RatRib120, namely 5′-ggucggucaaacccua, did not give a full match to any other sequences than to *anx*A2 cDNA or pseudogene. Since it is possible that the ribozyme binds partially to sequences, thus creating off-target effects, such effects can also be expected for small RNAs including the guide RNA employed in the CRISPR/Cas9 technology. Namely, there is no requirement for a 100% hybridisation between the mRNA and the ribozyme/small RNA. However, there is still another possibility which is not related to off target effects; namely, in the AnxA2 knock-out mouse the expression of genes can be subject to adaptation during development. Thus, the knock-out mouse can over time gain the ability to survive due to compensatory effects, while knock-down cells loaded with a highly active ribozyme are left with no choice or enough time for similar adaption. The findings showing that in the AnxA1 knock-out mouse, several other Anxs are upregulated (depending on the tissue), giving rise to redundancy/compensatory effects, corroborate this possibility [80]. Our primary goal was to knock-down AnxA2 in viable cells. Preliminary in vitro experiments in which the effect of RatRib120 was tested on radiolabelled *anx*A1, *anx*A7 or *anx*A11 mRNAs showed no effect on these mRNAs, indicating a certain degree of specificity (results not shown). This does not rule out that other off-target mRNAs could be involved. However, this is a problem, which should be considered in all knock-out and knock-down experiments.

4.4. Retroviral infection with the anxA2 antisense RNA

The infection studies also demonstrated the survival of the PC12 cells infected with the rat *anx*A2 antisense RNA, although cell proliferation was decreased as compared to uninfected PC12 cells or PC12 cells infected with the eGFP marker only. For further investigation, the infected cells were sorted using FACS to obtain a homogenous population of *anx*A2 antisense-infected PC12 cells, as well as of control PC12 cells infected with the eGFP marker only (Figs. 5 and 6).

Generally, the infected cells appeared larger than the uninfected cells. Furthermore, the increase in size was more apparent in cells infected with the rat *anx*A2 antisense RNA. A possible explanation might lie in the fact that AnxA2 anchors the cortical actin cytoskeleton to the plasma membrane [80]. Thus, due to down-regulation, this anchoring function could be compromised in the PC12 cells expressing the rat *anx*A2 antisense RNA. Consequently, the interaction between the cortical actin network and the plasma membrane would become less tight, resulting in an apparent increase in cell size. In accordance with this idea, it has been shown that many cells contain a large AnxA2 pool just underneath the plasma membrane [1,81].

There was a marked decrease in the expression of AnxA2 in the PC12 cells infected with the eGFP marker together with the rat *anx*A2 antisense RNA as compared to PC12 cells infected with eGFP alone. Thus, the antisense RNA indeed inhibited the translation of the rat *anx*A2 mRNA in these cells, resulting in decreased endogenous AnxA2 levels that could affect the ability of the cells to grow at a normal rate. However, the uninfected PC12 cells expressed AnxA2 at a level that was lower than that observed in cells infected with the eGFP marker alone (Fig. 7). A likely explanation could be that the insertion of the eGFP marker is quasi-random. It is possible that its insertion enhances the transcription of AnxA2 directly or via other transcription factors.

4.5. Ribozymes and antisense RNAs as tools to knock-down target mRNAs

As ribozymes can act catalytically by destroying a particular mRNA, they might be more efficient tools to down-regulate the expression of a particular gene than an antisense RNA, which cannot recycle. An antisense RNA does not dissociate from the RNA substrate, thereby blocking its translation. A 1:1 ratio between an antisense RNA-molecule and its mRNA substrate is therefore theoretically required to achieve a complete inhibition of a specific gene. However, antisense RNAs can also act via an enzymatic mechanism by inducing the activity of RNase

H [82]. Thus, although these antisense RNAs are not inherently catalytically active, they could induce such an activity. Our studies showed that the rat *anx*A2 antisense RNA inhibited the expression of rat AnxA2 by 70% in a coupled in vitro transcription/translation assay. This inhibition was higher in vivo, indicating that more factors are operational in the PC12 cells than in the rabbit reticulocyte lysate system used for the in vitro assays. In addition, all ribozyme constructs contain two regions forming helices 1 and 2 together with the target mRNA by hybridisation and could thus evoke an antisense RNA effect.

When uninfected PC12 cells were treated with NGF, they formed the long extensions typical for neuron-like differentiation. However, when the cells were first infected with rat *anx*A2 antisense RNA, they were initially unable to form such long extensions. However, after several passages, the infected cells formed extensions spontaneously. This could be due to the upregulation of AnxA7, in line with results showing its presence in dendrites [83]. Like AnxA2, AnxA7 is involved in the organisation of the membrane cytoskeleton [84] and is present in lipid rafts [85]. These overlapping functions of AnxA2 and AnxA7 could explain the apparent compensatory effects of the latter.

AnxA2 knock-out mice have been produced [79,86]. The mice are viable, but their cells show defects in membrane organisation and transport along the secretory and endocytic pathways. In recent years, there has been a concern regarding genetic compensation induced by deleterious mutations, which has been suggested to be absent in gene knock-downs [87]. The knock-down of AnxA2 resulting in the upregulation of AnxA7 may indicate that compensation could take place also as a knock-down effect.

4.6. Conclusions and future perspectives

In conclusion, the success of RatRib120 in knocking-down the expression of rat AnxA2 is due to the high catalytic effect of this ribozyme, the accessibility of the target site in *anx*A2 mRNA, and its stable integration into host DNA. Its high catalytic effect is due to the very flexible hinge region, helix 1, which is made by base-pairing with a region of the mRNA. Several other studies have employed knock-down of the expression of AnxA2 [88–92], but RatRib120 appears to be the most efficient. The ribozyme can easily be adapted to target the human *anx*A2 mRNA. The second most active *anx*A2 ribozyme, RatRib116, targets a site only 4 nt away from that of RatRib120. Therefore, it may be possible to generate a highly active and more specific ribozyme against *anx*A2 mRNA by increasing the length of the 5′ end of the ribozyme, thereby possibly avoiding off-target effects. Since ribozymes have already been used in clinical trials with great success [93], this ribozyme could provide a good candidate for optimisation and future use as a therapeutic cancer drug. The *anx*A2 antisense RNA was less efficient in knocking down the expression of AnxA2. Moreover, with time, an upregulation of AnxA7 as a compensatory effect was observed, showing how important it is to distinguish between short and long term effects. Off-target and compensatory effects also apply in case of the CRISPR/Cas9 technique [94], a novel method that still needs refinement. Our preliminary experiments indicated that extracellular vesicles isolated from PC12 cells infected with the *anx*A2 antisense RNA reduced the AnxA2 expression in PC12 cells infected with the empty vector. As this finding opens the possibility that extracellular vesicles from cell cultures infected with these RNAs can be harvested and administrated to patients, this approach should be further explored and refined in relation to virus infections, using an antisense RNA or a ribozyme against viral RNA.

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Author contributions

E.Aa. and A.V. wrote the paper with contributions from A.K.G. and J.R.L. Furthermore, E.Aa., A.K.G., M.G., H.H. and A.V. performed the experiments and analysed the data. J.R.L. contributed with the infection and cell sorting experiments.

Conflicts of interest

The authors declare no conflict of interest.

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