Annexin A2 recognises a specific region in the 3′-UTR of its cognate messenger RNA

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Abstract

Annexin A2 is a multifunctional Ca2+- and lipid-binding protein. We previously showed that a distinct pool of cellular Annexin A2 associates with mRNP complexes or polysomes associated with the cytoskeleton. Here we report in vitro and in vivo experiments showing that Annexin A2 present in this subset of mRNP complexes interacts with its cognate mRNA and c-myc mRNA, but not with β2-microglobulin mRNA translated on membrane-bound polysomes. The protein recognises sequence elements within the untranslated regions, but not within the coding region, of its cognate mRNA. Alignment of the Annexin A2-binding 3′-untranslated regions of annexin A2 mRNA from several species reveals a five nucleotide consensus sequence 5′-AA(C/G)(A/U)G. The Annexin A2-interacting region of the 3′-untranslated region can be mapped to a sequence of about 100 nucleotides containing two repeats of the consensus sequence. The binding elements appear to involve both single and double stranded regions, indicating that a specific higher order mRNA structure is required for binding to Annexin A2. We suggest that this type of interaction is representative for a group of mRNAs translated on cytoskeleton-bound polysomes.

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Keywords: Annexin A2; mRNA; 3′-UTR; mRNA-binding; consensus sequence; c-myc

1. Introduction

In eukaryotic cells mRNA-binding proteins modulate gene expression by regulating the activity, abundance and stability of both the coding and non-coding mRNAs present in mRNP complexes. These proteins contribute to the organisation of mRNAs into structurally and functionally related subsets of such complexes, thus coordinating mRNA localisation and the synthesis of proteins involved in complex cellular processes [1–4]. Most of the localised mRNAs require components of the cytoskeleton for their transport from the nucleus to specific target sites in the cell. Both actin filaments and microtubules, and their associated motor molecules, play a role in mRNA targeting. In general, the targeting signal resides in the 3′-UTRs of the mRNAs. In the case of Vg1 and actin mRNAs, these elements (also referred to as Zip codes) are small single stranded RNA (ssRNA) sequences [5,6]. However, the precise targeting sequences are often difficult to define since they are most likely related to a higher order structure of the mRNAs. Accordingly, secondary structures within cis-acting elements, in association with trans-acting proteins, have been implicated in the targeting of most mRNAs [7]. Different steps in the localisation of mRNAs are often mediated by separate cis-acting elements. For example, a sequence of 11 nucleotides (nt) is sufficient to direct the transport of myelin basic protein mRNA from the cell body into the oligodendrocyte processes, whereas another region of approximately 340 nt, containing a stable secondary structure, mediates its localisation to the myelin compartment [8]. Repression of translation during the transport process ensures that protein expression occurs selectively at the target site [1].

Abbreviations: AnxA2, Annexin A2 protein; anxA2, annexin A2 mRNA or cDNA; AnxA2t, Annexin A2 heterotetramer (AnxA2,p112); BSA, bovine serum albumin; CBP, cytoskeleton-bound polysomes; CDR, coding region; ds, double stranded; FL, full-length; FP, free polysomes; HC, heavy chain; MBP, membrane-bound polysomes; mRNP, messenger ribonucleotide particle; nt, nucleotides; PCR, polymerase chain reaction; RAM, rabbit anti-mouse IgG; RT-PCR, reverse transcriptase PCR; ss, single stranded; TEV, Tobacco Etch Virus; UTR, untranslated region

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example, it has been shown that only localised nos mRNA, which codes for the Nanos protein in Drosophila, is translated [9].

A 3′-UTR-binding protein, in complex with other proteins, is involved in the targeting of β-actin mRNA to actin filaments in fibroblasts [3,10]. A homologue of this protein targets Vgl mRNA to microtubules in Xenopus oocytes [11]. Hence, alternative filament-associated proteins appear to determine the specificity of the association of distinct mRNAs with the cytoskeleton. Annexin A2 (AnxA2) may function as such a protein since it has been identified both as a F-actin-binding [12–14] and a mRNA-binding protein [15–17], associated with a specific subpopulation of mRNA complexes [15]. Interestingly, regulation of mRNA-cytoskeleton interactions would only be one of the cellular roles of this multifunctional protein, which has also been implicated in signal transduction, membrane trafficking and regulation of membrane/cytoskeleton contacts [18–22].

We have previously developed a method for the isolation of three specific subpopulations of polysomes from cultured cells, i.e., “free” (or loosely cytoskeleton-bound; FP), cytoskeleton-bound (CBP) and membrane-bound polysomes (MBP) [23]. This well-characterised fractionation protocol [15,24–29] made it possible to analyse the compartmentalisation of specific mRNAs and study their interactions with the cytoskeleton. As a result of such analysis, AnxA2 was identified as a mRNA-binding protein in the CBP fractions [15]. Since c-myc mRNA is translated on CBP in Krebs II cells [24] and fibroblasts [25], the identity of the components mediating the interaction of c-myc and other specific mRNAs with the CBP was further studied. Here we show that AnxA2 in mRNP complexes is associated with c-myc and anxA2 mRNAs translated on CBPs in vivo. Moreover, we provide evidence supporting the conclusion that AnxA2 interacts with a ~100 nt region in the 3′-UTR of its cognate mRNA.

2. Materials and methods

2.1. Cell fractionation and isolation of polysomes

Three subpopulations of polysomes were isolated from Krebs II cells essentially as described [23].

2.2. Northern blot analysis

Total RNA was isolated from each of the three individual polysomal subpopulations purified from Krebs II cells by the single-step guanidinium–isothiocyanate–phenol–chloroform extraction method [30]. The conditions for Northern blotting were essentially as described by Hesketh et al. [25]. The probes were generated from bovine anxA2 (corresponding to a sequence in the N-terminal coding region with 86% homology to the same region in mouse anxA2), mouse c-myc or β2-microglobulin cDNAs using Multiprime kits and R[α-32P]dCTP (3000 Ci/mmole), both obtained from Amersham Pharmacia Biotech.

2.3. Immunoprecipitation and reverse transcriptase polymerase chain reaction (RT-PCR)

Immunoprecipitation of AnxA2–mRNP complexes was performed essentially as published by Chu et al. [31]. In brief, Krebs II cells were fractionated as described [23] and 1.5 mg of the cytoskeletal fraction was immunoprecipitated using monoclonal AnxA2 antibodies (kindly provided by Volker Gerke, Münster) coupled to protein A-Sepharose via rabbit anti-mouse IgG (RAM) after pre-clearance by RAM. The immunoprecipitation was performed in NET-buffer (50 mM Tris–HCl, pH 7.4, 150 mM KCl and 0.05% Triton) containing 40 U/ml of RNasin (Promega). The immunoprecipitates were phenol/chloroform extracted twice. The first water-phase was incubated with RNase-free DNase (Promega) before the second extraction. Nucleic acids were precipitated with carrier RNA and resuspended in RNAase-free water. The interphase from the first phenol/chloroform extraction was resuspended in NET-buffer and acetone precipitated. AnxA2 was identified by Western blot analysis using monoclonal AnxA2 antibodies (Transduction Laboratories). Next, immunoprecipitated RNA was used as template for RT-PCR in the Access RT-PCR System (Promega) using the conditions recommended by the manufacturer. 5 μM of primers (Eurogentec) were used for the amplification of the 3′-UTRs of mouse c-myc, anxA2 and β2-microglobulin cDNAs (Table 2A).

2.4. Purification of the AnxA2 heterotetramer (AnxA2t)

The AnxA2t was purified from epithelial cells derived from pig intestines as described [12].

2.5. Construction of recombinant AnxA2

The bovine anxA2 cDNA, originally from the pcD vector (ac. no. M14056, ATCC) [32], was cloned into the pGEM-Zf(+) vector and used as a template for the construction of recombinant AnxA2 by PCR. The PCR product was synthesised using specific forward (5′-ATCCGGAGAACCTCATGTTG-TGTTG-3′) and reverse (5′-ATCCGG-GGAAGACTGTACCTCAGTCATCCACCACACAGG-3′) primers obtained from Eurogentec and TIB MOLBIOL. The PCR fragment was ligated into the TOPO vector (Invitrogen) using TA cloning for amplification before digestion by restriction enzymes (NcoI and Acc65I) and ligation of the PCR product into the pETM-41 expression vector (kindly provided by Gunter Stier, EMBL, Heidelberg). The vector was derived from the pET24d expression vector (Novagen) and contains a 6His-Maltose Binding Protein tag followed by a Tobacco Etch Virus (TEV)-protease sensitive linker (ENLYFQG). DNA sequencing verified the identity of the final construct.

2.6. Protein expression and purification

The AnxA2 fusion protein (see above) was expressed overnight at 15 °C in the BL-21 Escherichia coli strain. Cells were harvested by centrifugation and resuspended in breakage buffer (50 mM Na2HPO4 (pH 8.0), 0.5 M NaCl, 10 mM imidazole, 5% (w/v) glycerol, 0.5 μg/ml DNase I, 0.25 μg/ml RNase A, protease inhibitor cocktail (1 tablet/50 ml) (Complete, EDTA-free, Roche) and 1 mM DTT). Cells were disrupted by French press (type FA-073, SLM Instruments). The homogenate was centrifuged at 100000 g, for 1 h at 4 °C, and the supernatant was loaded on His-Select Nickel Affinity Gel (Sigma) equilibrated with buffer A (50 mM Na2HPO4 (pH 8.0), 0.3 M NaCl and 10 mM imidazole) and incubated for 1 h at 4 °C on a rotating wheel. The resin was washed for 5 min with buffer A containing protease inhibitor cocktail (1 tablet/100 ml), then 5 min with buffer B (50 mM Na2HPO4 (pH 8.0), 1 M NaCl, 10 mM imidazole and protease inhibitor cocktail (1 tablet/500 ml)) and finally with buffer A containing protease inhibitor cocktail (1 tablet/500 ml) until the A280 had reached approximately zero. The His-tagged protein was eluted from the resin with buffer A containing 250 mM imidazole and protease inhibitor cocktail (1 tablet/500 ml). 2 mM EGTA was added to remove any traces of Ni2+ bound to the protein, and subsequently the eluted protein was immediately loaded on a PD-10 column (Sephadex G-25, Pharmacia) equilibrated with 20 mM Tris, pH 8. The protein was digested with TEV protease at 4 °C (molar ratio 1:50) during centrifugation at 4800×g (overnight). Digested AnxA2 was separated from the undigested form and from His-tagged Maltose Binding Protein by loading it to Q-Sepharose (Amersham Biosciences) that had been equilibrated overnight using 100 mM Tris (pH 8) and 500 mM NaCl. The resin was washed twice in 100 mM Tris (pH 8) to remove the salt, before loading the digestion mixture to the Q-Sepharose. Due to the difference in charge between Maltose Binding Protein and AnxA2, the former binds to the Q-Sepharose while AnxA2 remains unbound. The purity of recombinant AnxA2 was determined by SDS-PAGE [33] followed by Coomassie Brilliant Blue staining and the concentration was measured spectrophotometrically by using the theoretical extinction coefficient at A280 of 30250 M−1 cm−1 as determined from the amino acid content.
The respective *anxA2* transcripts, the restriction enzyme sites used for cloning and linearisation of the templates, and the RNA polymerases used for in vitro transcription are indicated. The numbers of the *anxA2* transcripts in parenthesis refer to the numbers of the transcripts shown in Fig. 3. The length and the specific regions of the different *anxA2* transcripts are shown in Fig. 3.

### 2.7. Preparation of cDNA templates for in vitro transcription

The bovine *anxA2* cDNA [32], cloned into the pGEM-3Zf(+) vector, was used as a template for the construction of all the *anxA2* transcripts. The bovine *anxA2* transcript Restriction sites

<table>
<thead>
<tr>
<th>Restriction sites for cloning</th>
<th>Linearised by</th>
<th>Promoter</th>
</tr>
</thead>
<tbody>
<tr>
<td>BamHI</td>
<td>EcoRI</td>
<td>SP6</td>
</tr>
<tr>
<td>BglII</td>
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<td>SP6</td>
</tr>
<tr>
<td>HindIII</td>
<td>T7</td>
<td></td>
</tr>
</tbody>
</table>

The respective *anxA2* transcripts, the restriction enzyme sites used for cloning and linearisation of the templates, and the RNA polymerases used for in vitro transcription, are indicated. The numbers of the *anxA2* transcripts in parenthesis refer to the numbers of the transcripts shown in Fig. 3. The length and the specific regions of the different *anxA2* transcripts are shown in Fig. 3.

### Table 1

| DNA templates used for the in vitro generation of different *anxA2* transcripts |
|---------------------------------|------------------|----------|
| *anxA2* transcript             | Restriction sites | Linearised Promoter |
| 1–131nt *anxA2* (1)            | BamHI            | EcoRI    | SP6      |
| Δ1–964nt *anxA2* (2)           | BamHI–BglII     | BglII    | SP6      |
| Δ55–1074nt *anxA2* (3)         | BamHI–HindIII   | HindIII  | T7       |
| Δ473–1381nt *anxA2* (4)        | PstI–EcoRI      | EcoRI    | SP6      |
| Δ473–964nt *anxA2* (5)         | PstI–BglII     | BglII    | SP6      |
| Δ1075–1381nt *anxA2* (6)       | PstI–EcoRI      | EcoRI    | SP6      |

The length and specific regions of the different *anxA2* transcripts are shown in Fig. 3. The T7 promoter site is underlined.

The length and specific regions of the different *anxA2* transcripts are shown in Fig. 3. The T7 promoter site is underlined.

### Table 2

Sequences of primers used in RT-PCR of AnxA2-immunoprecipitated RNA in Fig. 2(A) and PCR of different *anxA2* transcripts in Fig. 3(B)

#### A

<table>
<thead>
<tr>
<th>Amplified fragment</th>
<th>Primer sequence</th>
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<tr>
<td>Δ1095–1373nt <em>anxA2</em></td>
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<tr>
<td></td>
<td>5'-CTCAGAATTCAGGCTACGACA-3' (rev)</td>
</tr>
<tr>
<td>Δ1900–2265nt c-myc</td>
<td>5'-TACTGCGAGCTAGCTAATCAGGAGGAG-3' (forw)</td>
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<tr>
<td></td>
<td>5'-CGGGAATTCTATGTCGATCTAAATC-3' (rev)</td>
</tr>
<tr>
<td>Δ414–862nt β2-microglobulin</td>
<td>5'-CCTGCGAGCTTTCATGCT-3' (forw)</td>
</tr>
<tr>
<td></td>
<td>5'-CGTGAAGCTTTGTTTTATTTT-3' (rev)</td>
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</tbody>
</table>

#### B

<table>
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<tr>
<th><em>anxA2</em> transcript</th>
<th>Primer sequence</th>
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</thead>
<tbody>
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<td>5'-ATGTCTACGCGATGAGAAAATCC-3' (forw)</td>
</tr>
<tr>
<td></td>
<td>5'-GTCGTAAGCTTTCATGCT-3' (rev)</td>
</tr>
<tr>
<td>3'UTR <em>anxA2</em> transcripts</td>
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<td>Δ1075–1381nt <em>anxA2</em> (6)</td>
<td>5'-TACTGCGAGCTTCAGGCTACGACA-3' (forw)</td>
</tr>
<tr>
<td></td>
<td>5'-CCTGCGAGCTTTCATGCT-3' (rev)</td>
</tr>
<tr>
<td>Δ1075–1224nt <em>anxA2</em> (7)</td>
<td>5'-TATACGCTTACATGAGAGCTTTCACG-3' (forw)</td>
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<tr>
<td></td>
<td>5'-CAAACGATATATATT-3' (rev)</td>
</tr>
<tr>
<td>Δ1225–1381nt <em>anxA2</em> (8)</td>
<td>5'-TATACGCTTACATGAGAGCTTTCACG-3' (forw)</td>
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<td></td>
<td>5'-CAAACGATATATATT-3' (rev)</td>
</tr>
<tr>
<td>Δ1123–1131nt <em>anxA2</em> (9)</td>
<td>5'-TATACGCTTACATGAGAGCTTTCACG-3' (forw)</td>
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<td>5'-CAAACGATATATATT-3' (rev)</td>
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<tr>
<td>Δ1124–1224nt <em>anxA2</em> (10)</td>
<td>5'-TATACGCTTACATGAGAGCTTTCACG-3' (forw)</td>
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<td>5'-CAAACGATATATATT-3' (rev)</td>
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<tr>
<td>Δ1075–1149nt <em>anxA2</em> (11)</td>
<td>5'-TATACGCTTACATGAGAGCTTTCACG-3' (forw)</td>
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<td>5'-CAAACGATATATATT-3' (rev)</td>
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<td>5'-CAAACGATATATATT-3' (rev)</td>
</tr>
<tr>
<td>Δ1150–1224nt <em>anxA2</em> (13)</td>
<td>5'-TATACGCTTACATGAGAGCTTTCACG-3' (forw)</td>
</tr>
<tr>
<td></td>
<td>5'-CAAACGATATATATT-3' (rev)</td>
</tr>
</tbody>
</table>

The length and specific regions of the different *anxA2* transcripts are shown in Fig. 3. The T7 promoter site is underlined.
synthesised by PCR, using proof-reading RNA polymerase with forward primers (Genosys) containing the T7 promoter site (underlined in Table 2B). These transcripts were used directly for in vitro transcription.

2.8. In vitro transcription

RNA probes were uniformly [α-32P]UTP-labelled (3000 Ci/mmol; Amersham Pharmacia Biotech) in vitro by transcription of linearised templates (Table 1) or PCR products (Table 2) for 90 min, by standard procedures as described in the Promega protocol. A specific activity of 1.0–2.5 × 10^8 cpm/μg RNA was obtained. Labelled RNA probes were analysed for integrity and purity by agarose gel electrophoresis.

2.9. AnxA2–RNA binding assays

AnxA2–RNA binding assays were performed essentially as described by Kwon and Hecht [34]. RNA was incubated with AnxA2 or recombinant AnxA2 in RNA binding solution (10 mM triethanolamine, pH 7.4, 50 mM KCl, 1 mM DTT, 2 mM MgSO4, 1 mM CaCl2 and 1 μg/μl of yeast tRNA), supplemented with 20 μM RNAin (Promega) containing 2.5% (w/v) Ficoll for 20 min in a final volume of 20 μl. After incubation, the RNA probes were covalently crosslinked to the proteins by a 10 min exposure to UV light at 5 cm distance using a Hoefer UVC 500. RNases T1 and A were then added for 30 min at 37 °C to a final concentration of 0.01 U/μl and 0.1 μg/μl, respectively, to digest unprotected RNA. Nucleotide–protein complexes were separated from degraded RNA probe by 10% (w/v) SDS-PAGE [33] and visualised using a Canberra Packard Instant Imager. Coomassie Brilliant Blue staining was carried out to verify the amount of protein loaded in each lane. The binding assays of the competition experiments consisted of both radiolabelled and unlabelled competitor RNA.

3. Results

3.1. AnxA2 and c-myc mRNAs associate with CBP and AnxA2 in vivo

To identify the subpopulation of polysomes involved in translation of anxA2 mRNA, polysomal sub-fractionations were prepared from Krebs II cells [23,24] and Northern blot analysis was performed. The results showed that anxA2 mRNA is highly enriched in the CBP (Fig. 1). β2-microglobulin mRNA, translated on MBP, and c-myc mRNA, translated on CBP of these cells, were used as negative and positive controls, respectively [23–25]. Further, to verify that AnxA2 interacts with c-myc and anxA2 mRNAs in intact cells, AnxA2 was immunoprecipitated from the cytoskeletal fraction of Krebs II cells, and RT-PCR of the associated RNA was performed using specific primers for the 3′-UTR regions of these mRNAs. Whereas no amplification of β2-microglobulin cDNA was observed (Fig. 2A, lanes 14–17), this cDNA was efficiently amplified when total cellular RNA was used (Fig. 2A, lane 20), indicating that the primers and RT-PCR conditions were specific. By contrast, cDNA fragments corresponding to the 3′-UTRs of anxA2 and c-myc mRNAs were amplified (Fig. 2A, lanes 6 and 12, respectively) and their identities were confirmed by sequencing (data not shown). The specificity of immunoprecipitation

Fig. 2. AnxA2 and c-myc mRNAs, but not β2-microglobulin mRNA, associate with cytoskeleton-bound mRNPs in Krebs II cells. (A) Amplified RT-PCR/PCR products resolved on 1.8% (w/v) agarose gel stained with ethidium bromide. The AnxA2-bound mRNP complexes in the cytoskeleton fraction were immunoprecipitated using monoclonal AnxA2 antibodies (α-AnxA2) coupled to Protein A-Sepharose via RAM (lanes 3, 4, 6, 9, 10, 12, 14–17). The anxA2, c-myc and β2-microglobulin fragments in the isolated immunoprecipitates were then RT-PCR amplified (lanes 4, 6, 10, 12, 14–17) or, as a negative control, PCR amplified (lanes 3, 9 and 19) using anxA2-, c-myc- and β2-microglobulin-specific primers, respectively. As negative controls, the cytoskeleton fraction was also subjected to immunoprecipitation with RAM-coated Protein A-Sepharose alone and RT-PCRs were performed using the specific primers as indicated (lanes 5 and 11). As further negative controls, the immunoprecipitated samples were either treated with RNase prior to RT-PCR (lanes 4 and 10) or the RT-PCRs were performed with no template present (lanes 2, 8 and 18). No β2-microglobulin fragment appeared after Mg2+ titration of the RT-PCR reaction (lanes 14–17). Therefore, the specificity of the β2-microglobulin primers and the RT-PCR reaction conditions in the presence of 2 mM Mg2+ were verified using total RNA purified from Krebs II cells (lane 20). The 500 bp DNA size marker (lanes 1, 7 and 13) is indicated to the left. (B) Western blot analysis of the immunoprecipitates obtained from the cytoskeleton fraction in the absence (lane 21) or presence (lane 22) of AnxA2 monoclonal antibodies. AnxA2 was detected using monoclonal primary AnxA2 antibodies followed by HRP-coupled goat anti-mouse IgG. This secondary antibody also reacts with the heavy chain (HC) of mouse IgG present in the immunoprecipitates.
was confirmed by various RT-PCR controls (Fig. 2A) and by Western blot analysis (Fig. 2B). The latter showed that AnxA2 is present in the specific immunoprecipitates (lane 22), but not in control precipitates containing rabbit anti-mouse IgG (RAM) alone (lane 21). Similar results were obtained using total lysates of Krebs II cells (data not shown), but as expected, less mRNA was recovered in these immunoprecipitates, since only a fraction of the total cellular AnxA2 is associated with mRNPs.

3.2. Sequence elements of anxA2 mRNA involved in its binding to AnxA2

The above experiments established that AnxA2 interacts with anxA2 mRNA in vivo. Moreover, we have recently shown that it interacts directly with the localisation signal in the 3′-UTR of c-myc mRNA [17]. To establish a direct mRNA-binding and identify the region of anxA2 mRNA involved in AnxA2 binding, an UV cross-linking assay was used to monitor the

![Fig. 3. Mapping of the sequence elements of anxA2 mRNA involved in its binding to AnxA2. (A) Schematic presentation of the different anxA2 transcripts generated in vitro from the full-length mRNA. The numbers to the left refer to the transcripts used in the AnxA2–RNA binding assays shown in panel B. The AnxA2-binding transcripts are shown in red. (B) The binding of 2 fmol (∼100000 cpm) of the radiolabelled transcripts indicated in (A) to 2 μg of AnxA2 or BSA (lane to the right). (C) Schematic presentation of the different anxA2 3′-UTR transcripts generated in vitro. The numbers to the left refer to the transcripts used in the AnxA2–RNA binding assays shown in panel D. The AnxA2-binding transcripts are shown in red. (D) The binding of 150 fmol (∼100000 cpm) of the radiolabelled transcripts indicated in (C) to 2 μg of purified recombinant AnxA2. (E) Prediction of metastable secondary structures (Mfold programme) of the Δ1075–1149 nt transcript revealed a putative stem–loop structure containing the AACAG sequence (boxed region). The AnxA2–RNA binding reactions were performed as described in Materials and methods.]
binding of different in vitro radiolabelled \textit{anx}A2 transcripts (Fig. 3A and C). As shown in Fig. 3B, full-length mRNA (transcript 1), as well as the Δ1–964 nt (transcript 2) and the Δ473–1381 nt (transcript 4) transcripts, overlapping in the coding region, were all found to bind to AnxA2 purified from porcine intestinal epithelial cells. By contrast, transcript 3, corresponding to the coding region of the \textit{anx}A2 mRNA (Δ55–1074 nt) (Fig. 3B) and the Δ473–964 nt transcript (Fig. 3B, transcript 5), corresponding to the coding region of transcript 4, do not interact with AnxA2. Thus, comparison of the binding activities of the different \textit{anx}A2 transcripts indicated that a binding motif resides in the 3′-UTR (Fig. 3B, compare transcripts 3 and 5 with transcript 4). This conclusion was further supported by the binding of transcript 6 (Δ1075–1381 nt) to AnxA2 (Fig. 3D).

Comparison of the coding region transcript 3 (Δ55–1074 nt) with the partly overlapping Δ1–964 nt transcript (Fig. 3B, compare transcripts 2 and 3), suggested that an additional binding region may reside in the 55 nt 5′-UTR of \textit{anx}A2 mRNA. However, we were unable to confirm this by binding of the radiolabelled \textit{anx}A2 Δ1–55 nt transcript to its cognate protein, presumably because of loss of a particular secondary structure present in the longer RNA transcripts (data not shown). No \textit{anx}A2 mRNA binding was observed when BSA was used in the UV cross-linking assays (Fig. 3B, lane to the right).

Since our previous results showed that AnxA2 interacts with the 3′-UTR of \textit{c}-myc mRNA [17] and the present study shows that it also interacts with that of its cognate mRNA (Fig. 3B), it was of interest to map the binding region in the 3′-UTR of \textit{anx}A2 mRNA in more detail. Different transcripts of the 3′-UTR of the \textit{anx}A2 mRNA were generated by PCR using specific primers and subsequent in vitro transcription of the PCR fragments (Fig. 3C). The Δ1075–1381 nt (the entire 3′-UTR) and the Δ1075–1224 nt transcripts, but not the Δ1225–1381 nt transcript, were found to bind to AnxA2, indicating the presence of a binding site in the 5′ end of the \textit{anx}A2 3′-UTR (Fig. 3D, compare transcripts 6, 7 and 8). The difference between the non-binding Δ1075–1123 nt (Fig. 3D, transcript 9) and the AnxA2-binding Δ1075–1149 nt 3′-UTR region (Fig. 3D, transcript 11) is a short sequence of 26 nt, which may form a stem–loop structure (Fig. 3E, boxed region), as determined using the algorithms of the Mfold programme [35,36]. Therefore, this short nucleotide sequence could be involved in

![Fig. 4](image-url). Competition experiments provide evidence that AnxA2 binds to two separate regions of the \textit{anx}A2 mRNA (A), to both ssRNA and dsRNA (B) and interacts with the Δ1124–1224 nt region of the \textit{anx}A2 3′-UTR (C). (A) Increasing amounts (10-, 50- or 100-fold molar excess) of unlabelled in vitro transcribed full-length \textit{anx}A2 mRNA (lanes 1–4) or \textit{anx}A2 3′-UTR (lanes 5–8) as indicated above the figure were added to 2 μg AnxA2t to compete with 1 fmol of uniformly radiolabelled full-length \textit{anx}A2 transcript as indicated below the figure. (B) 2 μg of AnxA2t was incubated with 2 fmol of radiolabelled in vitro transcribed \textit{anx}A2 mRNA in the absence (control, lane 9) or presence of 1 μg of either poly(rA), poly(rU), poly(rC) or poly(rI-rC) (lanes 10–13). Note that poly(rG) was not included as it may form complex secondary structures. (C) Increasing amounts (10-, 100- or 500-fold molar excess) of the unlabelled Δ1124–1224 nt transcript (lanes 1–4), Δ1075–1149 nt transcript (lanes 5–8), or Δ1150–1224 nt transcript (lanes 9–13) were added to 2 μg of recombinant AnxA2 to compete with 150 fmol of the corresponding uniformly radiolabelled transcripts. (D) The unlabelled Δ1124–1224 nt transcript was either excluded or added in 500-fold excess to 2 μg of recombinant AnxA2 to compete with 50 fmol of the uniformly radiolabelled Δ1075–1149 nt (lanes 13 and 14) and Δ1150–1224 nt (lanes 15 and 16) transcripts. 1 μg/ml yeast tRNA was present to inhibit non-specific RNA binding in all reactions. Binding was performed as described in Materials and methods.
the interaction of mRNA with AnxA2. This suggestion is corroborated by the finding that the Δ1150–1224 nt transcript (Fig. 3D, transcript 13) interacted with AnxA2 more weakly than the Δ1124–1224 nt transcript (Fig. 3D, transcript 10). However, a transcript corresponding to this short 26 nt sequence was not able to bind to AnxA2 (Fig. 3D, transcript 12).

To further examine whether anxA2 mRNA contains two separate AnxA2 binding sites, competition experiments were conducted in which unlabelled full-length anxA2 mRNA and its 3′-UTR competed with the radiolabelled full-length anxA2 mRNA transcript for binding to AnxA2 (Fig. 4A). Effective competition was observed at 100-fold molar excess of unlabelled anxA2 mRNA (Fig. 4A, lane 4). By contrast, the unlabelled 3′-UTR transcript did not significantly compete with the full-length anxA2 mRNA (Fig. 4A, lane 5–8), suggesting that the mRNA contains two independent AnxA2 binding regions. Further competition experiments, using radiolabelled anxA2 mRNA, were performed with ssRNA or dsRNA of random size (Fig. 4B, lanes 9–13). The results suggested that ssRNA homopolymers are more efficient competitors than the poly(r1-c) dsRNA. Poly(rG) was not tested as G-rich ssRNA homopolymers are more efficient competitors than the poly(rI-rC) homopolymers, respectively. These data support the conclusion that the AnxA2-binding region of the anxA2 mRNA has both ss and ds character, with a possible weak preference for U-rich sequences. However, complete competition was not observed with any of the homopolymers, indicating that specific higher order mRNA structures are involved in the AnxA2–mRNA complex formation. The presence of uridine in the AnxA2-binding region was also supported by the UV cross-linking experiments in which the signal observed on instant imaging, is due to the UV cross-linked [$^{32}$P]-labelled uridine nucleotides bound to AnxA2. Since the relative electrophoretic mobility of nucleotide-bound AnxA2 did not significantly change, it appears that only a few nucleotides could be UV cross-linked to AnxA2 (data not shown).

To obtain further information on the identity of the specific AnxA2-binding 3′-UTR region of the anxA2 mRNA, additional competition experiments were performed. Thus, in these experiments three different unlabelled transcripts of the 1075–1224 nt 3′-UTR region of anxA2 mRNA (transcripts 10, 11 and 13) were used at 10-, 100- and 500-fold molar excess, to compete with the corresponding radiolabelled transcripts (Fig. 4C). It is evident that only the Δ1124–1224 nt transcript (transcript 10) competed effectively (Fig. 4C, lanes 1–4), suggesting that most of this region is required for specific interaction with AnxA2. The finding that the unlabelled Δ1124–1224 nt transcript (transcript 10) was also able to compete for the binding of the radiolabelled Δ1075–1149 nt (transcript 11) and Δ1150–1224 nt (transcript 13) transcripts (Fig. 4D, lanes 13–14 and 15–16, respectively), further support this conclusion.

High concentrations of KCl completely abolished the binding of anxA2 mRNA to the AnxA2t (Fig. 5), indicating that electrostatic interactions are crucial for the interaction. These forces are most likely contributed by basic amino acids and the phosphate backbone of RNA [38]. The interaction was preserved at physiological KCl concentrations.

To compare the 3′-UTRs of anxA2 mRNA in different mammalian species, an alignment of the nucleotide sequences was performed (Fig. 6). In agreement with the binding data, showing that the 86 nt localisation signal of mouse c-myc mRNA [17] and the 1124–1224 nt region of anxA2 mRNA (present results) are involved in interaction with AnxA2, a five nucleotide consensus sequence, 5′-AA(C/G)/(A/U)G, could be identified in these regions (Fig. 6). This sequence is repeated

![Fig. 5. The effect of increasing KCl concentration on the binding of anxA2 mRNA to its cognate protein. 2 fmol of radiolabelled in vitro transcribed anxA2 mRNA was incubated with 2 μg AnxA2 in the presence of 50 (lane 1), 100 (lane 2), 200 (lane 3) or 500 (lane 4) mM KCl. Binding was performed as described in Materials and methods.](image)

![Fig. 6. Alignment of partial 3′-UTR regions of anxA2 mRNAs from different mammalian species and mouse c-myc mRNA. The two repeats of the consensus sequence are indicated in grey. The numbers above refer to the nucleotide sequence of bovine anxA2 mRNA.](image)
once in the bovine, human and porcine 3′-UTRs of anxA2 mRNA, whereas the repeat in the mouse and rat sequences appears to be degenerated. Moreover, the 3′-UTRs of anxA2 mRNAs contain a common sequence, CCAG, which is not present in the localisation signal of c-myc mRNA (Fig. 6, in bold). It appears to be unrelated to the interaction with AnxA2 since it is located in the non-binding Δ1075–1123 nt region. However, it could represent a cis-acting sequence for another trans-acting factor.

4. Discussion

4.1. The interaction of AnxA2 with its cognate mRNAs involves the 3′-UTR

We have previously shown that AnxA2 is a mRNA-binding protein associated with CBP of Krebs II cells [15]. This finding indicated that specific AnxA2-associated mRNAs are bound to cytoskeleton elements and translated on CBPs. One of these specific mRNAs is c-myc [24], which binds to AnxA2 via the localisation signal in its 3′-UTR [17]. The presence of c-myc mRNA in AnxA2-containing mRNP complexes has been confirmed by an independent study [16].

In the present study we show that AnxA2 interacts with specific mRNAs in the CBP fraction and identify two such mRNAs as anxA2 and c-myc mRNAs (Figs. 1 and 2). Immunoprecipitation of AnxA2 from cell fractions, followed by RT-PCR of the AnxA2-associated RNAs using specific primers, revealed that these mRNAs, but not β2-microglobulin mRNA, are present in the AnxA2-associated mRNP complexes (Fig. 2), indicating that this interaction is specific for a subset of cellular mRNAs. These results are in accordance with recent data showing that structurally and/or functionally related mRNAs are transported together in large mRNP complexes [39]. The localisation signal in the AnxA2-binding 3′-UTR of c-myc mRNA [17], forms a stem–loop structure that targets the mRNA to CBP in the perinuclear region for translation [40,41]. Also anxA2 mRNA is translated on CBP (Figs. 1 and 2), suggesting that it contains a similar stem–loop structure in its 3′-UTR.

To identify the sequence elements in anxA2 mRNA involved in its binding to AnxA2, several truncated forms of anxA2 mRNA were generated. Collectively, the in vitro binding studies (Figs. 3B and 4A) suggest the presence of two independent binding regions in full-length anxA2 mRNA. One such region resides in the 3′-UTR and another possibly in the 5′-UTR, enabling AnxA2 to interact with both regulatory ends of the mRNA. UTRs at both termini of mRNA are known to interact via associated proteins such as poly(A)-binding protein and initiation factor 4G [42,43]. Furthermore, by binding to trans-acting proteins, the 5′-UTR is mainly involved in modulating initiation of translation, while the 3′-UTR is primarily involved in mRNA transport and stability [44]. AnxA2 does not seem to play a role in mRNA stability since binding of AnxA2 to c-myc mRNA or its cognate mRNA in cell lysates has no effect on mRNA degradation in vitro (unpublished results). Translation is repressed during mRNA transport to ensure specific targeting of proteins. Thus, it is possible that AnxA2 functions in both localisation and repression of translation during transport of specific mRNAs.

Further binding studies, using different short truncated transcripts of the 3′-UTR, indicated that the 1124–1224 nt region of anxA2 mRNA is involved in the binding to its cognate protein (Fig. 3C and D, compare transcripts 9–13). Competition studies corroborate this proposal since only the entire 1124–1224 nt transcript (transcript 10) was able to compete for the other AnxA2-binding transcripts (transcripts 11 and 13) of the 3′-UTR of anxA2 mRNA (Fig. 4D). The interaction with AnxA2 appeared to essentially involve the 1124–1149 nt region, which may form a putative stem–loop structure, although a short transcript corresponding to this region was not able to bind to AnxA2 (Fig. 3D, transcript 12), most likely due to its inability to form a particular higher order structure or due to the formation of an equilibrium of metastable RNA structures of low energy. Interestingly, this short region contains a putative binding motif of five nucleotides, 5′-AAC(G/C)(A/U)G, that appears twice within this 100 nt sequence (Δ1124–1224 nt transcript) (Fig. 6) and most likely is part of a specific higher order RNA structure. The Δ1150–1224 nt transcript (transcript 13) that interacts only weakly with AnxA2 contains only the second repeat, suggesting that the first one in the Δ1124–1149 nt region represents the principal AnxA2-binding region. However, the second repeat may also contribute to the interaction since the Δ1124–1224 nt transcript (transcript 10) is able to compete for the Δ1075–1149 nt transcript (transcript 11) (Fig. 4D). Repeated binding motifs have been implicated in the targeting of other mRNAs such as Fgl or VegT [45]. In general, most perinuclear mRNA localisation signals have been delimited to 100–200 nt [28,40,46] and the signal in the 3′-UTR of anxA2 mRNA appears to be of the same size. Notably, the five-nucleotide consensus sequence (AACAG) is also present in the 86 nt localisation signal of mouse c-myc mRNA, which was previously found to interact with AnxA2 [17]. Furthermore, the perinuclear localisation signal in the 3′-UTR of vimentin mRNA [46] that is translated on CBP (unpublished results), contains two repeats of this consensus sequence. However, the perinuclear localisation signals of the mouse slow/cardiac troponin C and chicken α-cardiac actin mRNAs [47] lack this sequence, suggesting that it may target mRNAs to CBP, outside the perinuclear region.

Several lines of evidence support the conclusion that the interaction of AnxA2 with anxA2 and c-myc mRNAs is specific. Firstly, β2-microglobulin mRNA was not associated with immunoprecipitated AnxA2 (Fig. 2A). Secondly, AnxA2 does not bind to the coding region of its cognate mRNA nor to the Δ1075–1123 nt, Δ1122–1143 nt or Δ1225–1381 nt transcripts (transcripts 9, 12 and 8, respectively) of the 3′-UTR. Thirdly, the binding is not caused by non-specific mRNA–AnxA2 interactions since BSA (Fig. 3), recombinant p11, α-actin and denatured AnxA2 did not show any binding (data not shown). Finally, a high concentration of tRNA (1 μg/ml) was present in the binding assays to prevent non-specific RNA–protein interactions and we have previously reported that AnxA2 does not bind rRNA [15].
4.2. The binding of mRNAs to AnxA2 involves higher order RNA structures

Using the MFold programme we were not able to predict an identical RNA secondary structure either for the aligned sequences shown in Fig. 6 or the entire 3′-UTR of the same mRNAs. Also, previous prediction of secondary structures in c-myc mRNA from several species failed to identify identical stem–loop structures, regardless of a rather long consensus sequence [40]. However, both rat and mouse anxa2 mRNAs interact with AnxA2 (unpublished results), indicating that a specific secondary structure of the 3′-UTR is required for binding. In favour of this suggestion, mutating the GUAAUUUA sequence in the localisation signal of c-myc mRNA, leading to disruption of a secondary stem–loop structure, also partially abolished its interaction with AnxA2 [17]. Moreover, the presence of 30% (v/v) formamide in the binding assay, which destroys secondary structures in mRNA, greatly diminished their binding to AnxA2, while Mg2+, which promotes the formation and stabilisation of RNA secondary structures, enhanced the binding (data not shown).

Previous well-known RNA–protein interactions have been demonstrated to rely mainly upon higher order structures [48]. For example, mutations disrupting the formation of the stem–loop structure in the localisation signal in the 3′-UTR of K10 mRNA all block its localisation to the anterior pole of the Drosophila oocyte. By contrast, mutations that alter only the nucleotide sequence have little or no effect on the localisation of this mRNA [49]. In the present study, competition with both ssRNA or dsRNA polymers partially inhibited the binding of both full-length anxa2 (Fig. 4B) and c-myc mRNAs (unpublished results) to AnxA2, with the ssRNA homopolymers being the most effective competitors. Thus, the binding of these mRNAs to AnxA2 appears to involve their higher order structure including both ssRNA and dsRNA regions.

Based on these results, we conclude that cells contain a distinct pool of AnxA2 that is an integral component of mRNP complexes associated with the cytoskeleton, and suggest that it functions in the transport and targeting of specific mRNAs. This proposal is further supported by the demonstration that AnxA2 also acts as a nuclear shuttle protein [50,51] and that it binds to the localisation signal of c-myc mRNA [17], which is translated on CBP [15]. Importantly, the present results indicate that a region of about 100 nucleotides in the 3′-UTR of anxa2 mRNA is involved in the interaction with its cognate protein. This region contains a specific higher order RNA structure and two repeats of a five nucleotide consensus sequence, 5′-AA (C/G)(A/U)G. The interaction of AnxA2 with its cognate mRNA may imply a post-transcriptional feed-back mechanism that ultimately regulates the synthesis of the protein.

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