Annexin A2 Is a Novel RNA-binding Protein

Annexin A2 (ANXA2) is a Ca\(^{2+}\)-binding protein that is up-regulated in virally transformed cell lines and in human tumors. Here, we show that ANXA2 binds directly to both ribonucleotide homopolymers and human c-myc RNA. ANXA2 was shown to bind specifically to poly(G) with high affinity \((K_d = 60 \text{ nM})\) and not to poly(A), poly(C), or poly(U). The binding of ANXA2 to poly(G) required Ca\(^{2+}\) \((A_{50} = 10 \mu\text{M})\). The presence of RNA in the immunoprecipitates of ANXA2 isolated from HeLa cells established that ANXA2 formed a ribonucleoprotein complex \textit{in vivo}. Sucrose gradient analysis showed that ANXA2 associates with ribonucleoprotein complexes and not with polyribosomes. Reverse transcriptase-PCR identified c-myc mRNA as a component of the ribonucleoprotein complex formed by ANXA2 \textit{in vivo}, and binding studies confirmed a direct interaction between ANXA2 and c-myc mRNA. Transfection of LNCaP cells with the ANXA2 gene resulted in the up-regulation of c-Myc protein. These findings identify ANXA2 as a Ca\(^{2+}\)-dependent RNA-binding protein that interacts with the mRNA of the nuclear oncogene, c-myc.

The annexins are a family of more than 160 unique annexin proteins that are present in more than 65 different species ranging from fungi and protists to plants and higher vertebrates (1). ANXA2\(^1\) consists of an amino-terminal domain (ATD), which comprises the first 30 amino acid residues of the protein and the carboxyl core domain (CCD) composed of the remaining residues. The CCD of ANXA2 contains sites for binding Ca\(^{2+}\), phospholipid, F-actin and heparin (2, 3). The ATD contains regulatory phosphorylation sites for both protein kinase C (Ser-25) and Src (Tyr-23). In addition to the phosphorylation sites, the ATD also contains the site for interaction with the Src (Tyr-23) and Ser-25. In addition to the phosphorylation sites, the ATD also contains the site for interaction with the S100A10 (also referred to as p11), a member of the S100 family of Ca\(^{2+}\)-binding proteins (7–10). The crystal structure of an amino-terminally truncated form of ANXA2 has been reported (12). The protein is planar and curved with opposing convex and concave sides. The convex side faces the biological membrane and contains the Ca\(^{2+}\) and phospholipid-binding sites. The concave side faces the cytosol and contains both the amino and carboxyl termini.

A multitude of intracellular functions have been suggested for ANXA2, including roles as a mediator of Ca\(^{2+}\)-regulated exocytosis (13–16) or endocytosis (17–19) as well as a role in modulating sarcolemmal phospholipid raft organization during smooth muscle cell contraction (20, 21) and regulation of ion channels (22). Since an ANXA2 knockout mouse has not been developed, it is not clear whether these reported in vitro functions represent actual physiological functions of the protein. Nevertheless, knowledge of these putative functions of ANXA2 has not provided clues as to the role that ANXA2 may play \textit{in vivo}.

The expression of ANXA2 is induced in various transformed cells, including v-src, v-H-ras, v-mos, or SV40-transformed cells (23). Furthermore, the ANXA2 gene is growth-regulated, and its expression is stimulated by growth factors such as insulin, fibroblast growth factor, and epidermal growth factor (24). Up-regulated ANXA2 has also been reported in human hepatocellular carcinoma (25), pancreatic adenocarcinoma (26), high grade glioma (27), gastric carcinoma (28), and acute myelocytic leukemia (29). Since overexpression of the ANXA2 gene is commonly observed in both virally transformed cell lines and human tumors, it has been suspected that this up-regulated level of ANXA2 might link ANXA2 to a key step in cellular transformation. However, without a detailed knowledge of its intracellular role, it is difficult to envision the role that up-regulation of ANXA2 expression would have on cellular transformation.

Typically, ANXA2 has been reported to display two distinct intracellular distributions, with the majority of the protein...
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localized to the cytoplasmic face of the plasma membrane and a secondary diffuse cytoplasmic distribution (30). The first indication that ANXA2 might interact with RNA was a report that utilized subcellular fractionation to show that a significant portion of ANXA2 was associated with ribonucleoprotein particles in cytoplasmic extracts of both normal and transformed cells. It was also shown that ANXA2 immunoprecipitated from UV-irradiated cultured cells associated with RNA and formed a RNA-ANXA2 cross-linked ribonucleoprotein complex. These authors also showed by biochemical fractionation experiments that about 10–15% of the total cellular ANXA2 was associated with the nucleus (31). Ensuing studies showed that ANXA2 could bind to deoxyribonucleic acid structures such as Z-DNA (32) or Alu subpopulations (33). Other studies have identified nuclear ANXA2 in immunoblots of nuclei as and part of a primer recognition complex that stimulates DNA polymerase α activity (6, 34). ANXA2 was also shown to localize with cytoskeleton-associated mRNA subpopulations (35). Most recently, it was shown that ANXA2 possessed a nuclear export sequence, and it was proposed that ANXA2 readily enters the nucleus but is rapidly exported (36).

In the present report, we have examined HeLa cell extracts for the presence of ANXA2-binding proteins. Surprisingly, we found that several RNA-associated proteins bound to an ANXA2 affinity column, and this association was blocked by pretreatment with RNase A. We also show that in the presence of Ca2+, ANXA2 binds to ribonucleic homopolymers with a high affinity for poly(G) and in a salt-resistant manner. Subsequently, we show that ANXA2 is an RNA-binding protein that forms a messenger ribonucleoprotein (mRNP) particle. We identify c-myc RNA as a component of the ANXA2-ribonucleo- protein complex and show that ANXA2 binds directly to c-myc mRNA. Last, the expression of ANXA2 in a cell line normally devoid of this protein results in an increase in both ANXA2 and c-Myc protein. Overall, these studies identify ANXA2 as a novel RNA-binding protein that may regulate the translation of c-myc RNA.

MATERIALS AND METHODS

Cell Lines, DNA Vectors, and Cell Lysis—HeLa, LNCaP, and 293 HEK cells were obtained from the American Type Culture Collection (Manassas, VA) and were grown at 37°C in 5% CO2 in Dulbecco’s modified Eagle’s medium containing 10% (v/v) fetal bovine serum (Invitrogen). The cDNA for ANXA2 and S100A10 were PCR-amplified and ligated into pcDNA3.1-neomycin (pcDNA-S100A10) or pcDNA 3.1/Hyg/pDNAA2X (Invitrogen). The c-myc vector (pBluescript) was a generous gift from Dr. Robert Orlowski (Chapel Hill, NC).

LipofectAMINE 2000 (Invitrogen) was used as outlined in the manufacturer’s instructions to transfect 10-cm2 dishes of the human prostate carcinoma cell line, LNCaP, with the pcDNA 3.1 vectors, pcDNA-S100A10 and pcDNA-ANXA2. Stably transfected cells were selected with 7.5 μg/ml neomycin and 5 μg/ml hygromycin, respectively. Clonal cell lines were selected by ANXA2 and S100A10 protein expression.

For detergent based lysis, cells were lysed with Nonidet P-40 buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40) supplemented with protease inhibitors and clarified by centrifugation at 12,000 g for 10 min at 4°C. Where indicated, cells were hypotonically lysed by resuspending cells (from one 10-cm2 dish) in 1 ml of hypotonic lysis buffer (20 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 25 mM NaCl plus protease inhibitors) and drawing the solution through a 27-gauge needle five times, followed by 25 strokes in a Dounce homogenizer. After incubation on ice for 10 min, a postnuclear supernatant was obtained by clarifying the cell lysate for 10 min at 8,000 g. Soluble protein fractions were quantitated by BCA assay (Pierce).

Immunoprecipitation and Western Blot Analysis—HeLa cell lysate (50 μg in 0.5 ml of 0.2% Nonidet P-40 buffer) was precleared with 1 μg of either nonimmune mouse or rabbit IgG and 20 μl of protein G-PLUS beads (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1 h at 4°C. ANXA2 (a kind gift from Tony Hunter, La Jolla, CA) or ANXA5 (FL-319; Santa Cruz Biotechnology) antibody (1 μg) were then added to the precleared lysate, and the reactions were rocked for 1 h at 4°C, followed by the addition of Protein G-PLUS beads (20 μl/reaction) for an additional 1 h. The immune complexes were washed three times and either boiled in SDS-PAGE sample buffer for Western blot analysis or extracted with phenol/chloroform/isooamyl alcohol (PCI 25:24:1) for RNA isolation.

For Western blot analysis, proteins in sample buffer were resolved on SDS-PAGE, transferred to 0.2-μm nitrocellulose membranes, immuno- stained, and visualized using SuperSignal chemiluminescent substrate (Pierce). Primary antibodies (1:1000 dilution) were obtained from the following sources: Becton Dickinson/Transduction Laboratories (annexin A2 and S100A10), Santa Cruz Biotechnology (annexin A5 (FL-319), and Cell Signaling Technology (86 ribosomal protein).

Sucrose Gradient Complex RNA Extraction, RNA Binding, and RT-PCR—The ANXA2 or ANXA5 immune complexes were washed three times with RNase-free Nonidet P-40 buffer and diluted to a final volume of 200 μl with diethylpyrocarbostyril-treated water. The beads were extracted with one volume of PCI and treated with 2 units of DNase I for 30 min at 37°C. The DNAase-treated RNA was then extracted with one volume of PCI, followed by ethanol precipitation using linear polyacrylamide (Sigma) as a nuclear acid carrier. The precipitated RNA was diluted to 20 μl with diethylpyrocarbostyril-treated water and stored at 80°C.

The bound RNA was labeled using RNA ligase and cytidine 3′-β-(phosphate) (pCP; 5′ 3′-labeled, PerkinElmer Life Sciences) as previously described (37). Briefly, 8 μl of the extraneous RNA was mixed with 40 units of RNAsin, 2 μl of Me2SO, 50 μM of pCP, and 2 μl of RNA ligase in a final volume of 20 μl and incubated for 2 h at 37°C, followed by ethanol and protein extraction as described above. Incorporation of the pCP label was assessed quantitatively by scintillation counting of trichloroacetic acid precipitates. Qualitative analysis of incorporation was assessed by electrophoresis of 3 μl of labeled RNA on a 1% (w/v) agarose gel. The gel was dried under vacuum, and the labeled RNA was visualized by autoradiography.

RT-PCR analysis of the bound RNA was carried out using the OneStep RT-PCR kit (Qiagen). To detect the 275-nucleotide segment at the 3′-end of the c-myc mRNA as described previously (38), the following primers were used: forward 5′-GGCCGAACACACAAGCCTCTTGAGG3′; reverse, 5′-GCCTCAGGACATTTCTGTTAGAAG3′.

Sucrose Gradient Analysis of Cell Lysates—Linear sucrose gradient was performed essentially as described previously (39). Postnuclear supernatants from hypotonically lysed cells were sedimented in a 15–50% (w/v) sucrose gradient (sucreose solutions made in hypotonic lysis buffer adjusted to 100 mM NaCl) by centrifugation for 2 h at 37,000 rpm in a Beckman SW41 rotor. After centrifugation, samples were fractionated into 1-ml fractions by top displacement using a gradient fractionator (Buchler). For Western blot analysis, 20 μl of each gradient fraction was boiled for 5 min with SDS-PAGE sample buffer and analyzed as described previously.

Homoribopolymer Binding Assay—Binding of cell lysates to homoribopolymer beads was carried out essentially as described previously with a few modifications (40). Cell lysate (100 μg in 0.5 ml of Nonidet P-40 buffer) was incubated with a 25-μl packed volume of homoribopolymer beads (Sigma) and rotated for 30 min at 4°C. The beads were washed three times with Nonidet P-40 buffer, boiled in 25 μl of SDS-PAGE sample buffer, and probed for ANXA2 by Western blot as described above. For purified ANXA2 binding to homoribopolymer beads, 1 μg of ANXA2 was added to 25 μl of packed homoribopolymer beads in 0.5 ml of TBS. Protein binding analysis was carried out as described above either by Western blot or by staining with Coomassie Blue where indicated.

Immunohistological Analysis—Immunohistological analysis was carried out on sections of paraffin-embedded tissue from primary and metastatic tumors from patients with prostate cancer. The primary antibodies used were anti-ANXA2 monoclonal antibodies (1:1000 dilution). Positive immunostaining was considered present if the protein was detected in the cytoplasm of prostate cells. Negative controls included omission of the antibody or the use of isotype-matched control antibody. The intensity of staining was scored from 0 to 3+, with 3+ indicating strong positive staining. Statistical analysis was performed using the one-way ANOVA test.
preincubated at 37°C with either 200 units/ml RNasin (Promega), 50 units/ml DNase I (Ambion), or 500 μg/ml RNase A (Qiagen) for 30 min. After the incubation, the cell lysates were rotated in the presence of 1 mM CaCl₂ with the Tris-blocked matrix followed by incubation with the ANXA2 affinity column. Bound proteins were analyzed as described above.

**Protein Identification by In-gel Tryptic Digestion and Mass Spectrometry**—Stained bands were excised, and an automated in-gel tryptic digestion was performed on a Mass Prep Station (Micromass, UK). The gel pieces were destained, reduced (dithiothreitol), alkylated (iodoacetamide), and digested with trypsin (Promega sequencing grade modified), and the resulting peptides were extracted from the gel and analyzed via liquid chromatography/mass spectrometry. Liquid chromatography/mass spectrometry was performed on a CapLC (Waters) high-pressure liquid chromatograph and a Q-ToF-2 (Micromass) Mass Spectrometer, using a Picofrit C18 reversed-phase capillary column (New Objective). Proteins were identified from the mass spectrometry/mass spectrometry data using MASCOT (Matrix Science, UK) and searching the NCBI database.

**Expression and Purification of Recombinant ANXA2**—The galactose-inducible *Saccharomyces cerevisiae* expression vector (pYeDP0) as well as the vector containing the cDNA for ANXA2 (pYeDP60-ANXA2) were kindly supplied by Jesus Ayala-Sanmartin (INSERM, Paris, France) and have been described previously (41). The cDNA of the S100A10 protein was PCR-amplified and inserted into the pYeDP60 vector, followed by the transformation of the vectors into the protease-deficient *S. cerevisiae* strain FKY282 (kindly supplied by Francois K Relief, Genopole, Envy, France). The growth and induction of the yeast cultures was done as described previously (41), with only slight modifications. Prior to purification, 1-liter cultures of each ANXA2 and ANXA2 heterotetramer using the purification detailed previously. The isolated proteins were purified further using gel permeation chromatography equilibrated in 40 mM Tris-HCl, 140 mM NaCl, 0.1 mM EGTA, and 0.1 mM dithiothreitol. Proteins were aliquoted and stored at –80°C.

**Ultraviolet Cross-linking Assay**—Full-length (1.8 kb) c-myc message was transcribed and labeled in vitro with T7 polymerase (Stratagene) with [32P]UTP. [32P]-Labeled RNA probes were synthesized by in vitro transcription with the Riboprobe® system (Promega). [32P]-Labeled c-myc mRNA (1.77 × 10⁷ cpm/μg) was incubated with 1.5 μg of purified recombinant ALII with or without unlabeled c-myc RNA, positive control template RNA, and homoribopolymers (poly(G) and poly(C)). The RNA-protein mixture binding reaction was carried out in a 20-μl reaction mixture containing 10 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM MgCl₂, 1 mM CaCl₂, 5% glycerol, and 2 μg of yeast tRNA. The mixtures were incubated at 30°C for 30 min, after which they were irradiated with a UV Stratalinker (Stratagene). The RNA samples were digested with 1 μl of RNase A (10 mg/ml) at 37°C for 15 min and analyzed by 12% SDS-PAGE.

**Surface Plasmon Resonance**—ANXA2 heterotetramer was coupled to a CM5 sensor chip in a BIAcore 3000 instrument (BIAcore, Uppsala, Sweden) using the manufacturer’s amine-coupling kit. Homoribopolymer-binding assays were conducted in 10 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM CaCl₂, at 25°C and a flow rate of 30 μl/min. A nonderivitized flow cell was used as a control for the contribution of the bulk refractive index to the surface plasmon resonance signal. After each injection, the surface was regenerated with an injection of 2 mM EGTA, 10 mM HEPES, pH 7.4, 0.15 M NaCl. An approximate equilibrium dissociation constant (Kd) was obtained by measuring the equilibrium resonant units (Rₑ) at several poly(G) concentrations (10 nM to 1 μM) at equilibrium. Binding data were analyzed by Scatchard analysis using the BIAevaluation software according to the following relationship: Rₑ/C = KₑRₑₘₐₓ – KₑRₑₛ where Rₑₘₐₓ is the resonance signal at saturation, C is the concentration of free analyte, and Kₑ is the equilibrium association constant.

**RESULTS**

**ANXA2 Forms a Ribonucleoprotein Complex**—As a first step in elucidation of the possible physiological function(s) of ANXA2, we attempted to isolate intracellular proteins that interacted with ANXA2. In order to isolate these binding partners, we utilized CNBr-activated Sepharose matrices conjugated with ANXA2 or a blocked, unconjugated resin (resin control). Annexin A5 (ANXA5), which has considerable sequence and structural similarity to ANXA2, was also conjugated to the matrix as a specificity control.

Cell lysates prepared from either HeLa or 293 HEK cells were first precleared with unconjugated Sepharose matrix, followed by application to either the ANXA2 or ANXA5 matrices. These cell types were chosen because of their differing levels of endogenous ANXA2. HeLa cells have an abundance of endogenous ANXA2, whereas 293 HEK cells have substantially less ANXA2 by comparison (data not shown). Since the annexins are known to require Ca²⁺ to bind to cellular targets, the cell lysates were incubated with the ANXA2 or ANXA5 matrices. Upon completion of the binding reactions, the proteins bound to the matrices were removed by boiling in SDS-PAGE sample buffer, followed by separation on 8% polyacrylamide gels. We observed that several cellular proteins associated with the ANXA2 matrix (Fig. 1A, lane 3). These cellular proteins did not associate with the control matrix or the ANXA5 affinity matrix, and the association of these proteins with the ANXA2 matrix required Ca²⁺. It is interesting to note that nearly identical results were obtained using 293 HEK cell lysate as starting material (data not shown). This suggests that the bound material does not require high levels of ANXA2 expression; nor does it appear to bind to ANXA2 stoichiometrically.

Having established that ANXA2 binds to a number of distinct cellular proteins in a specific and Ca²⁺-dependent manner, the bands were excised and digested, and the fragments were analyzed by liquid chromatography-tandem mass spectrometry. A portion of the mass spectrometry results is shown in Table I. Surprisingly, we noticed that many of the proteins identified as specific ANXA2-binding proteins were either ribosomal proteins or proteins that are known to interact with cellular RNA. For example, the major proteins that bound to the ANXA2 affinity column included poly(A)-binding protein-1 (42), ribosomal protein L4 (43), ribosomal protein P0 (44), ribosomal protein P1 (45), ribosomal protein P2 (46), and ribosomal protein P3 (47).
tRNA as a competitor (Fig. 2 bound to poly(G) in the presence of as high as 1 mg/ml yeast in the HeLa cell extracts (Fig. 3). This finding suggested that either RNA or RNA-binding proteins were interacting with ANXA2.

To differentiate between these two possibilities, the ANXA2 affinity matrix binding experiments were repeated with cell lysates pretreated with RNase A, RNasin (an RNase inhibitor), or DNase I. As shown in Fig. 1B, the association between ANXA2 and the cellular proteins was abolished by pretreatment with RNase A. In contrast, the pretreatment of the cellular extracts with either DNase I or RNasin did not affect the profile of cellular proteins bound to the ANXA2 affinity matrix. These results therefore indicate that ANXA2 assembles with cellular proteins to form a ribonucleoprotein (RNP) complex and that intact RNA is required for the interaction of ANXA2 with its cellular binding partners.

ANXA2 Binds RNA in Vitro—The possible interaction of ANXA2 with RNA was tested by incubating HeLa cell lysates with agarase-conjugated RNA homopolymers. This assay system has been typically used to characterize the RNA binding properties of many other RNA-binding proteins (40, 47, 48). HeLa cell lysates were incubated with the RNA homopolymers, and bound proteins were eluted and analyzed by Western blot using an ANXA2 monoclonal antibody. We observed that endogenous ANXA2 bound selectively to poly(G)-agarose (Fig. 2A) and that this interaction was dependent on Ca$^{2+}$ at ~10 μM (Fig. 2B). Furthermore, when the bound proteins were analyzed with a monoclonal antibody to S100A10, the Western blot confirmed the presence of the S100A10 binding partner (data not shown), suggesting that some of the ANXA2 bound to poly(G) was complexed to S100A10 as the heterotetramer. Thus, the interaction of ANXA2 with its S100A10 binding partner did not block its ability to interact with poly(G).

We subsequently used a battery of binding conditions to characterize the interaction of HeLa cell lysate ANXA2 with poly(G). The binding of the endogenous ANXA2 to poly(G) was stable in 1 mg/ml heparin and in NaCl concentrations up to 0.25 M (Fig. 2, C and D, respectively). Furthermore, ANXA2 bound to poly(G) in the presence of as high as 1 mg/ml yeast tRNA as a competitor (Fig. 2E).

In order to visualize the endogenous proteins that bound to the poly(G)-agarose, the poly(G)-binding protein fraction obtained from HeLa cell lysates was also analyzed by SDS-PAGE and Coomassie Blue staining. We found that a 35-kDa protein was the predominant poly(G)-binding protein in these lysates. Mass spectrometry identified this protein band as ANXA2, confirming that ANXA2 was the major poly(G)-binding protein in the HeLa cell extracts (Fig. 3).

The formation of ANXA2-containing RNP complexes suggested that either ANXA2 directly interacts with RNA or interacted via other RNA-bound protein(s). To distinguish between these possibilities, purified ANXA2 was assayed for intrinsic RNA binding activity using the agarose-immobilized RNA homopolymers. We used the heterotetrameric form of ANXA2 for these analyses, since it is the prevalent form of the protein in most cell types. As shown in Fig. 4A, recombinant heterotetrameric ANXA2 showed specific binding to poly(G) with very little binding to poly(A), poly(C), or poly(U). Similar results were observed with the monomeric form of ANXA2 (data not shown). We also assessed the specificity of the poly(G)-agarose binding by performing competition assays with unconjugated homoribopolymers. ANXA2 was incubated with the poly(G) beads in the presence of a molar excess of free competitor homoribopolymers. We observed that the binding of ANXA2 to the poly(G) beads was blocked by the free poly(G) but not by free poly(A), poly(C), or poly(U) (Fig. 4B). Similarly to the interaction of endogenous HeLa cell ANXA2 with poly(G) (Fig. 2), the binding of recombinant ANXA2 to poly(G) was not blocked by yeast tRNA (Fig. 4C) or heparin and was stable in the presence of physiological NaCl concentrations (data not shown).

In addition to the competition experiment described above, the interaction of unconjugated poly(G) homopolymers with the ANXA2 heterotetramer was further characterized using two distinct methods. First, ANXA2 was conjugated to a biosensor chip, and the binding to the RNA homopolymers was examined by surface plasmon resonance. Of the four homoribopolymers examined, ANXA2 bound only to poly(G). These experiments further established that ANXA2 bound selectively and with high affinity (K_D of 60 nM) to poly(G) (Fig. 4D). Additionally, only the binding of poly(G) resulted in a substantial conformational change in ANXA2 as assessed by circular dichroism (data not shown), providing further evidence of the specificity of poly(G) binding.

Monomeric ANXA2 Harbors the RNA-binding Site within the ANXA2 Heterotetramer—The previous data established that poly(G) and ANXA2 form a specific and tight complex. It was unclear whether both subunits of ANXA2 heterotetramer contribute to poly(G) binding. Shown in Fig. 5A, ANXA2 binds to poly(G) whether it is complexed as the heterotetramer or as a monomer. In contrast, the purified S100A10 subunit does not bind the homoribopolymers.

ANXA2 monomer consists of an ATD, which comprises the first 30 amino acid residues of the protein, and a CCD composed of the remaining residues (49). The ATD is released from the molecule by proteolysis. Having established that the ANXA2 monomer contains the RNA-binding site, the RNA binding activity of the CCD fragment was compared with the intact ANXA2 monomer to crudely map the region of ANXA2 responsible for binding to poly(G). As is shown in Fig. 5B, both the intact monomer and the CCD fragment were precipitated by poly(G) beads, demonstrating that the poly(G) binding site is found within the CCD of ANXA2. This was unexpected, since it suggested that the CCD, which is highly conserved among the annexins, contained a key RNA-binding motif that was unique to ANXA2. Incubation of a tissue annexin fraction containing annexins A1–A7 (50) with poly(G)-agarose resulted in the selective binding of only a single annexin, which was confirmed by Western blot to be ANXA2 (Fig. 5C). Additionally, a double-stranded DNA agarose resin did not interact with any of the annexins in the tissue fraction (Fig. 5C). These data established that the RNA binding activity of ANXA2 was probably not a property shared by other members of the annexin family of proteins; nor was general nucleic acid binding activity a common function of the annexin family.

### Table I

Proteins bound to Annexin A2 Affinity Matrix

| Protein                                | Binding Activity |
|----------------------------------------|------------------|
| Small (40 S) ribosomal subunit proteins |                  |
| S2, S3a, S4, S5, S9, S13, S14, S18, S19, S25, S32 |                  |
| Large (60 S) Ribosomal Subunit Proteins |                  |
| L4, L7, L10, L14, L15, L17, L19, L22, L27a, L29, L31, L34, L35 |                  |
| Y-box-binding protein                  |                  |
| β-Actin                               |                  |
| Myb-binding Protein                   |                  |
| Nucleolin                             |                  |
| hnRNP K                               |                  |
| Scar protein                          |                  |
| Poly (A)-binding protein              |                  |
| α-Tubulin                             |                  |
| β-Tubulin                             |                  |
| Ribonucleoprotein U                    |                  |
| Elongation factor-1a                  |                  |
ANXA2 Forms an RNP in Vivo—To determine whether ANXA2 interacted with RNA in vivo, we immunoprecipitated ANXA2 from HeLa cell lysates and analyzed the content of the immunoprecipitate. As a control, immunoprecipitates of ANXA5 were also examined. The specificity of the antibodies was confirmed by Western blot (Fig. 6A). The RNA was then isolated from the immunoprecipitates and labeled by the pCP method (37). As shown in Fig. 6B, the ANXA2 immunoprecipitate isolated from HeLa cell lysate was labeled with pCP and therefore contained RNA, whereas the ANXA5 immunoprecipitate was not labeled. As an additional control, immunoprecipitates were prepared using nonimmune mouse IgG. We found that these immunoprecipitates did not contain significant amounts of RNA, suggesting that neither the antibodies alone nor the agarose beads used for the precipitation were responsible for the coprecipitation of RNA with ANXA2.

To further investigate the possibility that ANXA2 formed RNP complexes in vivo, we fractionated HeLa cell lysates on linear sucrose gradients and analyzed the ANXA2 distribution by Western blot. As a control for these experiments, ribosomal protein S6 was used as a marker for free ribosomes and polyribosomes. The location of the free (cytosolic) protein fraction was monitored by assaying for tyrosine phosphatase activity using p-nitrophenyl phosphate as a substrate (data not shown). As shown in Fig. 7A, ANXA2 co-sedimented with the RNPs in the upper fractions of the gradient. In contrast, pretreatment of the HeLa lysate with RNase A resulted in the appearance of ANXA2 in the free protein fraction. This result showed that ANXA2 formed an RNP complex and that RNA was critical for the formation of this complex.

Identification of c-myc mRNA as a Component of the mRNP—The ANXA2-RNP complex resolved on the sucrose gradient was pooled and immunoprecipitated with the ANXA2 antibody (Fig. 7B). The RNA was isolated from the immunoprecipitate and analyzed by Northern blot for the presence of c-myc mRNA. As shown in Fig. 7C, c-myc mRNA was specifically coprecipitated with the ANXA2-RNP complex, suggesting that this mRNA was a component of the complex.

Fig. 2. RNA binding properties of ANXA2. For A–E, Nonidet P-40-soluble lysate from HeLa cells (100 μg/lane) were incubated with 25 μl of the indicated homoribopolymer beads for 30 min (for D and E), the Ca2+ concentration used was 100 μM. The beads were washed three times with Nonidet P-40 buffer, boiled in sample buffer, and analyzed by Western blot analysis for ANXA2 binding. F, the eluted proteins were analyzed by Coomassie Blue staining of the polyacrylamide gels. A, ANXA2 binds specifically to poly(G) in a Ca2+–dependent manner. HeLa cell lysates were left either untreated or incubated with 100 μM Ca2+ or 5 mM EGTA prior to the addition of the indicated homoribopolymers. B, the Ca2+ requirement for poly(G) binding to ANXA2 was analyzed by incubating HeLa lysates with the indicated concentrations of Ca2+ prior to the addition of the poly(G) beads. C, heparin does not inhibit the interaction between ANXA2 and poly(G). Cell lysates were treated as in A, except heparin (1 mg/ml) was added either during the wash steps (“wash only”) or during both the binding reaction and the wash steps (“incubation and wash”). D, ANXA2 binds to poly(G) in a salt-resistant manner. The concentration of salt in the HeLa cell lysate was adjusted as indicated prior to the addition to the poly(G) beads. After the 30-min incubation period, the beads were washed with Nonidet P-40 buffer containing the indicated concentration of salt. E, ANXA2 binding to poly(G) was assessed in the presence of the indicated amounts of yeast tRNA. The tRNA was present during both the binding reaction in addition to the wash steps.
tate and subjected to RT-PCR. Since c-Myc expression is necessary for Src transformation (51) and ANXA2 is elevated in Src-transformed cells, we explored the possibility that ANXA2 might play a role in the regulation of c-myc mRNA. As shown in Fig. 7C, we detected the presence of c-myc mRNA in the RNA isolated from the ANXA2 immunoprecipitates. In contrast, we did not detect glyceraldehyde-3-phosphate dehydrogenase mRNA in the immunoprecipitates (data not shown). Our inability to detect glyceraldehyde-3-phosphate dehydrogenase mRNA indicated that ANXA2 is not a general, nonspecific RNA-binding protein but binds to a distinct subset of cellular mRNAs. These results establish that ANXA2 is part of an mRNA complex in vivo that contains c-myc mRNA and possibly other RNA transcripts.

ANXA2 Binds to c-myc mRNA—The presence of c-myc RNA in the ANXA2-ribonucleoprotein complex could be due to the direct interaction of ANXA2 with c-myc mRNA or to the indirect interaction of ANXA2 with a c-myc-binding component of this complex. To distinguish between these possibilities, we performed a UV-cross-linking assay with purified ANXA2 and ²⁵P-labeled full-length c-myc mRNA. As shown in Fig. 8A, ANXA2 binds directly to c-myc mRNA. Furthermore, the interaction between ANXA2 and c-myc mRNA was blocked by either poly(G) homoribopolymer or unlabeled c-myc mRNA but not by poly(C) homoribopolymer. In addition, the interaction between ANXA2 and c-myc mRNA was Ca²⁺/H₁₁₀₀₁-dependent (Fig. 9B). To further examine the specificity of the interaction of c-myc mRNA with ANXA2, we transcribed an irrelevant RNA (pGEM
Express positive control template) and observed that this RNA did not compete with the c-myc transcript for binding to ANXA2 (Fig. 9B, lane 3). Therefore, these data establish that the interaction between ANXA2 and c-myc mRNA is direct, specific, and Ca\(^{2+}\)-dependent.

**Expression of ANXA2 Increases c-Myc Protein Levels**—The predominant form of ANXA2 in cultured cells is complexed to its S100A10 binding partner as a heterotetramer. Previous studies have established that although ANXA2 is a prominent protein in human prostate cell lines such as DU-145 and PC-3, ANXA2 and its S100A10 binding partner are not present in the human prostate, LNCaP cell line (52). We examined the c-Myc protein levels in these three prostate cell lines. As shown in Fig. 9A, the human prostatic cell line, LNCaP, is devoid of ANXA2 and also has low expression of c-Myc protein compared with the DU-145 and PC-3 cell lines (Fig. 9B). To further explore the relationship between ANXA2 and c-Myc, we transfected the LNCaP cells with the gene for both ANXA2 and its S100A10 binding partner. Stable transfectants were cloned, and cell lines expressing both ANXA2 and S100A10 were selected. Two permanent cell lines, expressing both proteins, were then further characterized. As shown in Fig. 9C, LNCaP clonal cell lines expressing ANXA2 (Fig. 9C) have significantly up-regulated levels of c-Myc protein compared with the control cells (Fig. 9D).

**Fig. 6.** ANXA2 is part of a ribonucleoprotein complex in vivo. A, ANXA2 and ANXA5 can be specifically immunoprecipitated from HeLa cell lysate (500 µg) by incubation with 1 µg of antibody followed by the addition of protein G-PLUS-agarose for 1 h. Immunocomplexes were resolved by SDS-PAGE, followed by immunostaining using antibodies specific for ANXA2 and ANXA5. B, ANXA2 immunoprecipitates with RNA in HeLa cells. Immunoprecipitation was performed as in A in addition to a nonimmune mouse IgG control (mIgG). Immune complexes were washed, and the bound RNA was extracted and 3' labeled with [\(^{32}\)P]pCp. Results were quantitated by scintillation counting or by agarose gel electrophoresis (inset).

**Fig. 7.** Characterization of the interaction of ANXA2 with RNA. A, ANXA2 binds to RNA in vivo. Hypotonically lysed HeLa cells were fractionated through a linear sucrose gradient either in the presence of an RNase inhibitor (top panels) or RNase A (bottom panels). Each gradient was collected in 10 fractions, and an aliquot (20 µl) of each fraction was analyzed by Western blot for ANXA2 and, as a control, ribosomal protein S6 (rS6). B, ANXA2 can be immunoprecipitated from sucrose gradient fractions. A 100-µl aliquot (pooled from the second and third fractions from the RNase-inhibited gradient in A) was diluted to 1 ml with TBS and immunoprecipitated as in Fig. 6A for ANXA2. Nonimmune mouse IgG was used as an antibody control. C, ANXA2 co-immunoprecipitates with c-myc mRNA. Bound RNA was extracted from the immunoprecipitations performed in B, followed by RT-PCR analysis for c-myc mRNA. Marker was 100 bp (New England Biolabs).

**DISCUSSION**

In the current report, we establish that ANXA2 is a unique RNA-binding protein. The absence of the well-established RNA-binding domains from the sequence of ANXA2 as well as the selectivity of the protein for poly(G) homoribopolymers implies that ANXA2 possesses a unique RNA-binding domain. We also demonstrate that the binding of poly(G) homoribopolymer to ANXA2 is totally dependent on Ca\(^{2+}\). Thus, our report is the first demonstration of a Ca\(^{2+}\)-dependent RNA-binding protein. This observation further establishes that ANXA2 is a unique RNA-binding protein.

Approximately six distinct RNA-binding motifs have been identified (reviewed in Ref. 53). These include the RNP motif, the arginine-rich motif, the RGG box, the KH motif, the double-stranded RNA-binding motif, and the zinc finger-knuckle motif. The absence of these structures from ANXA2 implies that the RNA-binding domain of this protein is unique among the RNA-binding proteins. Since of the seven annexins tested for RNA binding activity, only ANXA2 bound to RNA (Fig. 5C), it is likely that the presence of an RNA-binding domain in ANXA2 is unique to the annexin family of proteins. Direct binding studies have shown that ANXA2 is a low affinity Ca\(^{2+}\)-binding protein that binds Ca\(^{2+}\) with a Kd of about 0.5 mM. However, as shown in Fig. 2B, the interaction of the protein with RNA occurred with a Kd of about 10 µM. This
suggests that the interaction of ANXA2 with RNA induces a conformational change resulting in a change in the architecture of the Ca\(^{2+}\)-binding sites from low affinity to higher affinity Ca\(^{2+}\)-binding sites. Our observation of a Ca\(^{2+}\)-dependent conformational change in ANXA2 upon RNA binding, as measured by circular dichroism (data not shown), is consistent with this suggestion. Although we were unable to determine the exact RNA-binding site of ANXA2, we did determine that the RNA-binding domain of ANXA2 is located in the carboxyl domain of the protein.

c-Myc is a multifunctional nuclear phosphoprotein that can promote cell cycle progression, apoptosis, and cellular transformation. c-Myc regulates these activities at the molecular level by functioning as a regulator of gene transcription, activating or repressing specific target genes. The half-life of c-myc mRNA is regulated when cells change their growth rates or differentiate. Two regions within c-myc mRNA determine its short half-life; one is in the 3'-untranslated region, and the other is in the coding region. A cytoplasmic RNA-binding protein, the coding region determinant-binding protein, binds to the c-myc coding region in vitro and shields it from endonuclease digestion and thereby prolongs the mRNA half-life (54). The 5'-untranslated region may also play a role in the translation regulation of c-myc, since the interaction of the cap binding protein, eIF4E, with this region relieves the translation repression imposed on the c-myc mRNA by its structured 5'-untranslated region (55). In this work, we have used an immunoprecipitation-RT-PCR technique to show that ANXA2 forms a RNP complex in HeLa cells, and we identify one species of mRNA in this complex as c-myc mRNA (Fig. 7C). Furthermore, we show that ANXA2 binds directly to c-myc mRNA (Fig. 8).

Although the exact binding site on c-myc mRNA was not identified in our report, it was observed that the interaction of ANXA2 with c-myc mRNA results in the up-regulation of c-Myc protein (Fig. 9), suggesting that the binding of ANXA2 to c-myc mRNA may have an important physiological role in the regulation of c-myc mRNA.

In general, the RNA-binding proteins serve a number of functions including regulating mRNA stability and the rate and efficiency of mRNA translation. In addition, the RNA-binding proteins can participate in the specific targeting of mRNA in the cytoplasm (reviewed in Ref. 56). Considering that...
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