# Annexin II is associated with mRNAs which may constitute a distinct subpopulation

Anni VEDELER[1] and Hanne HOLLÅS

Department of Biochemistry and Molecular Biology, University of Bergen, Årstadveien 19, N-5009 Bergen, Norway

Protein–mRNA interactions affect mRNA transport, anchorage, stability and translatability in the cytoplasm. During the purification of three subpopulations of polysomes, it was observed that a 36-kDa protein, identified as annexin II, is associated with only one specific population of polysomes, namely cytoskeleton-associated polysomes. This association appears to be calcium-dependent since it was sensitive to EGTA and could be reconstituted *in vitro*. UV irradiation resulted in partial, EGTA-resistant cross-linking of annexin II to the polysomes. Binding of $^{32}$P-labelled total RNA to proteins isolated from the cytoskeleton-bound polysomes on a NorthWestern blot resulted in a radioactive band having the same mobility as annexin II and, most importantly, purified native annexin II immobilized on nitrocellulose specifically binds mRNA. The mRNA population isolated from cytoskeleton-bound polysomes binds to annexin II with the highest affinity as compared with those isolated from free or membrane-bound polysomes. Interestingly, the annexin II complex, isolated from porcine small intestinal microvilli was a far better substrate for mRNA binding than the complex derived from transformed Krebs II ascites cells. When cytoskeleton-associated polysomes were split into 60 S and 40 S ribosomal subunits, and a peak containing mRNA complexes, annexin II fractionated with the mRNAs. Finally, using affinity purification of mRNA on poly(A)$^+$-coupled magnetic beads, annexin II was only detected in association with messenger ribonucleoproteins (mRNPs) present in the cytoskeletal fraction (non-polysomal mRNPs). These results, derived from both *in vitro* experiments and cell fractionation, suggest that annexin II binds directly to the RNA moiety of mRNP complexes containing a specific population of mRNAs.

Key words: actin, cytoskeleton, mRNP, RNA-binding proteins.

## INTRODUCTION

Cytoskeletal elements are important for the transport and localization of specific mRNAs in oocytes and early embryonic cells as well as in somatic cells (reviewed in [1–5]). Recently, unconventional myosins and other proteins have been identified as putative adapter proteins for the association of specific mRNAs with the cytoskeleton [6,7]. The localization signal for sorting appears almost exclusively in the 3′ untranslated region of the mRNAs [4].

We have previously developed a combined detergent/salt extraction procedure for the isolation of three fractions containing different polysome populations from Krebs II ascites cells, i.e. 'free' (or loosely cytoskeleton-associated) polysomes (fraction A), cytoskeleton-bound polysomes (fraction B) and membrane-bound polysomes (fraction C) [8]. These three fractions differ in their content of cytoskeletal proteins, phospholipids, as well as specific mRNAs [8,9] and complement of poly(A)$^+$-binding proteins [10]. For example, c-*myc* mRNA is found enriched in cytoskeleton-bound polysomes, whereas actin mRNA is found in both free and cytoskeleton-bound polysomes, and $\beta_2$-microglobulin mRNA is highly enriched in membrane-bound polysomes [9,11]. Analysis of the protein composition of the different fractions revealed that a protein of about 36 kDa in apparent molecular mass is specifically enriched, and represents one of the most abundant proteins in the B fraction containing cytoskeleton-bound polysomes [9]. Here we identify this protein as annexin II.

Annexin II (p36) is a member of a family of calcium- and lipid-binding proteins characterized by repeated, conserved consensus sequences (reviewed in [12–14]). In the cytoplasm, it exists as a soluble monomer or as a membrane-associated heterotetrameric complex ($p36_2p11_2$) together with p11, a protein belonging to the S-100 multigene family. Binding to p11 involves the N-terminal domain of annexin II, which also contains phosphorylation sites for pp60$^{v\text{-}src}$ and protein kinase C [15–17]. Despite the detailed biochemical and structural characterization of annexin II, its physiological role is still not known. However, annexin II appears to be a multifunctional protein involved in endocytosis (reviewed in [14]), exocytosis (reviewed in [18]), DNA binding [19,20] and even extracellular processes (reviewed in [21]). In addition, annexin II is able to bind and bundle actin filaments *in vitro* [22,23]. Earlier studies by two different groups suggested that annexin II is associated with cytoplasmic RNA [24,25]. Annexin II was found to co-precipitate with a cytoplasmic high-molecular-mass factor suggested to be nucleic acid [24]. Also, annexin II sedimented together with polysomes and small messenger ribonucleoprotein (mRNP) complexes in sucrose gradients [25].

The present results, involving both cross-linking, *in vitro* binding of RNA to purified annexin II and immunological detection of annexin II in affinity-purified mRNP complexes, strongly suggest that annexin II binds directly to the mRNA moiety of inactive mRNPs and cytoskeleton-bound polysomes, indicating that a specific population of mRNAs is associated with annexin II.

## MATERIALS AND METHODS

### Chemicals and media

Except where indicated, all the chemicals were purchased from Sigma (St. Louis, MO, U.S.A.). All media were purchased from Gibco (Inchinnin, Scotland, U.K.).

Abbreviations used: TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; mRNP, messenger ribonucleoprotein.

[1] To whom correspondence should be addressed (e-mail Anni.Vedeler@pki.uib.no).

### Cell culture

Krebs II ascites cells (a mouse plasmacytoma cell line) and MPC-11 (a mouse lymphosarcoma cell line) were grown as described previously [8,26]. L-929 (a mouse cell line derived from aveolar/adipose tissue) cells were grown as roller suspension cultures to a density of about $10^6$ cells/ml in combined (equal volumes) RPMI 1640 and Dulbecco's modified Eagle's medium, containing 15 % heat-inactivated horse serum and 20 $\mu$g/ml gentamicin. Human lung fibroblasts were cultivated as monolayers in minimum essential medium supplemented with 10 % foetal calf serum. All cells were grown in an incubator with a 5 % $CO_2$ atmosphere.

### Purification of the annexin II–p11 complex from pig intestinal epithelium and Krebs II ascites cells

The heterotetrameric annexin II–p11 complex was purified from porcine intestinal epithelial cells derived from pig intestines as described by Gerke and Weber [22]. The B fraction enriched in annexin II was obtained from Krebs II ascites cells using the sequential detergent/salt fractionation protocol [8], including 10 mM EGTA in the buffer. The fraction was centrifuged for 60 min at 100000 ***g*** at 4 °C and, subsequently, the salt concentration was reduced to 65 mM KCl before application of the material on to a DE 52 column (Whatman International, Maidstone, Kent, U.K.). The rest of the purification protocol was identical to the procedure described by Gerke and Weber [22].

### Cell fractionation and isolation of polysomes

The procedure was originally developed for Krebs II ascites cells and has been presented in detail before [8]. Polysomes present in the individual fractions were isolated by centrifugation through a 20 % sucrose cushion for 2 h at 226000 $\boldsymbol{g}_{max}$ in a 50 Ti rotor (Beckman) or, for small volumes, for 25 min at 436000 $\boldsymbol{g}_{max}$ in a TLA 100.2 rotor (Beckman) and finally resuspended in the 130 mM KCl buffer. Polysomes were stabilized by the addition of cycloheximide to the culture medium at a final concentration of 20 $\mu$g/ml 5 min before harvesting of cells.

### Polysome profiles

To obtain polysome profiles, 10 absorbance units of fraction A, measured at 260 nm, and equivalent volumes of the two other fractions (B and C) were layered on 15–40 % sucrose gradients. Polysome profiles were obtained as described previously [8].

### Isolation of polysomal mRNAs and inactive mRNP complexes

Polysomes were split into 40 S, 60 S and mRNA-containing peaks by incubating 10 $A_{260}$ units of polysomes for 1 h with 30 $\mu$g/ml *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) [27] in a high-salt buffer (1 M KCl) before centrifugation for 4 h at 285000 $\boldsymbol{g}_{max}$ in a SW 41 rotor (Beckman) in a linear (12.5–25 %) sucrose gradient prepared in the same buffer. The collection of the peaks was monitored at 260 nm. Collected material from six gradients was concentrated by centrifugation for 20 h at 226000 $\boldsymbol{g}_{max}$ in a 50 Ti rotor (Beckman), followed by resuspension of the pellets in 130 mM KCl buffer. Inactive mRNAs (mRNP complexes) were isolated by incubating the post-polysomal supernatants derived from the different fractions with magnetic oligo($dT_{25}$) beads (Dynal A. S., Oslo, Norway), followed by extensive washing, according to the manufacturer's instructions, except that the binding of poly(A)$^+$ mRNA to oligo(dT) was performed for at least 2 h.

### Preparation of EGTA extracts containing $Ca^{2+}$-binding proteins

Cells were first lysed in the 25 mM KCl buffer (see above), containing 2 mM $CaCl_2$, centrifuged for 10 min at 1100 ***g***, and washed three times in the same buffer lacking detergent. Subsequently, calcium-binding proteins were extracted from the resulting pellet by a 20-min incubation with 10 mM EGTA in the 130 mM KCl buffer, followed by centrifugation for 10 min at 1100 ***g***. During the preparation of the extracts 2 mM benzamidine and 1 × CLAP (1 $\mu$g/ml each of chymostatin, leupeptin, antipain and pepstatin) were included as protease inhibitors in all buffers in addition to 1 mM PMSF. The EGTA extract was dialysed overnight with three changes of buffer (130 mM KCl buffer) to remove EGTA.

### Protein determination

Protein concentrations were measured by the Coomassie Brilliant Blue dye-binding assay of Bradford [28] using BSA as standard.

### Electrophoresis and immunoblotting

The proteins in the different fractions (40 $\mu$g of total protein per lane, except in Figure 4, lanes 5–7, see below) were separated in 10 % SDS/polyacrylamide gels [29] and transferred on to 0.2-$\mu$m nitrocellulose filters (Schleicher and Schüell, Dassel, Germany) [30]. Annexin II was detected using an affinity-purified polyclonal antibody [22] at a concentration of 1 $\mu$g/ml and actin by using a monoclonal IgM antibody (N350; Amersham, Little Chalfont, Bucks., U.K.), diluted according to the supplier's instructions. Monoclonal annexin II antibodies (clone 5, Transduction Laboratories, Lexington, KY, U.S.A.) were used at a concentration of 0.25 $\mu$g/ml in Figure 4, lanes 5–7. To detect the different proteins of the annexin family, a polyclonal antibody raised against a conserved peptide motif located in repeat 2 of all annexins [31] was used at a concentration of 1 $\mu$g/ml. Dr Volker Gerke (University of Münster, Münster, Germany) generously provided the different annexin and p11 antibodies. Affinity-purified polyclonal rab4 protein antibodies and the corresponding antiserum were generous gifts from Dr Bruno Goud (Curie Institute, Paris, France). For optimal detection of the rab4 protein, 150 $\mu$g of total protein per lane was loaded for SDS/PAGE. The binding of the primary antibodies was detected using alkaline phosphatase-conjugated anti-mouse IgM, goat anti-rabbit or anti-mouse IgG (Promega, Madison, WI, U.S.A.) and a dye reaction with the substrates 5-bromo-4-chloro-3-indolyl phosphate/Nitro Blue Tetrazolium (BCIP/NBT). $^{125}$I-Protein A (Amersham) was used in experiments requiring quantification of annexin II.

### NorthWestern blotting

Krebs II ascites cells (a total of $10^9$ cells) were incubated *in vivo* for 1 h in serum-containing medium supplemented with 2 mCi [$^{32}$P]inorganic phosphate (Amersham). After preparation of A, B and C fractions and further purification of polysomes, total RNA was isolated from each of the three polysome populations according to the single-step method described by Chomczynski and Sacchi [32]. Nitrocellulose membranes, containing SDS/PAGE separated proteins, were pre-incubated for 1 h in RNA-binding buffer (10 mM Tris, pH 7.0, 50 mM KCl, 2 mM $CaCl_2$ and 0.02 % of each of polyvinylpyrollidone, Ficoll and BSA) to block unspecific binding. Subsequently, 5 $\mu$g of $^{32}$P-labelled RNA in the RNA-binding buffer was added and incubated for 1 h. The blots were autoradiographed after extensive washing in the above buffer.

### UV cross-linking

RNA–protein cross-linking of the B fraction was carried out using UV irradiation (254 nm) during a 10-min incubation at 0 °C in a 1-ml cuvette at a protein concentration of 3–4 mg/ml.

### RNA-binding assays

Aliquots (3 $\mu$l) containing 0.75, 1.5 and 3.0 $\mu$g of annexin II complex isolated from Krebs II ascites cells or pig microvillar epithelium were spotted on to nitrocellulose membranes and allowed to air-dry. Protein concentration in each spot was adjusted to 3.0 $\mu$g with BSA. The membranes were blocked overnight at 4 °C in binding buffer (10 mM triethanolamine, pH 7.4, 50 mM KCl, 1 mM dithiothreitol, 2 mM $MgSO_4$, 1 mM $CaCl_2$ and 500 $\mu$g/ml yeast tRNA). A sample of $^{32}$P-labelled RNA corresponding to 50000 c.p.m. was added and the membranes were incubated for 30 min at room temperature in binding buffer containing 20 units/ml RNasin (Promega). Radiolabelled total RNA from each polysome population was isolated as described above and mRNA and rRNA were further purified using magnetic oligo($dT_{25}$) beads (Dynal A. S.) as described by the supplier. After incubation with RNA, the membranes were washed for $4 \times 15$ min at room temperature in binding buffer without yeast tRNA. The binding of RNA was quantified and visualized using a Canberra Packard Instant Imager™.

## RESULTS

### The presence of annexin II in cytoskeleton-bound polysomes

We have demonstrated previously that polysomes from different cell types can be fractionated into three different populations, corresponding to 'free' (or loosely cytoskeleton-associated), cytoskeleton-bound and membrane-bound polysomes [8]. These fractions are referred to as A, B and C, respectively, and the polysomes purified from each fraction similarly A, B and C polysomes. These polysomes are enriched in different mRNA species [8–11,26], and also differ in their complement of poly(A)$^+$-binding proteins [10]. Short-term stimulation of cells with insulin increases the amount of cytoskeleton-bound B polysomes in Krebs II ascites cells [33].

Analysis of the protein composition of the different fractions, obtained from Krebs II ascites cells, revealed an approx. 36-kDa protein as one of the most abundant proteins in the B fraction [9], suggesting a structural protein. Since the B fraction is also enriched in actin [8] and the 36-kDa protein could be released by EGTA, it was of interest to see if it might be a member of the annexin family of $Ca^{2+}$-binding proteins. The three fractions, as well as the isolated polysomes, were subjected to immunoblotting using a peptide antibody raised against a conserved motif present in repeat 2 of the annexins [31]. As shown in Figure 1, the antibody recognized a protein of about 36 kDa that was enriched in the B fraction and, most interestingly, was found associated with the purified B polysomes. The immunoreactive 36-kDa protein was also recovered in the C fraction, but it was absent in C polysomes. In addition, the antibody detected weakly two additional proteins in the 50–55-kDa range in the B and C polysomes (Figure 1).

To show that the enrichment of the 36-kDa annexin in the B fraction was not unique to the Krebs II ascites cells, similar fractions were prepared from two other cell lines, MPC-11 and L-929 [2,34]. These are transformed mouse cell lines that grow both in suspension and as monolayers, but display different organization of the cytoskeleton since the MPC-11 cells are known to lack vimentin filaments [35]. The different fractions (A, B and C) were again analysed by immunoblotting, in this case employing an affinity-purified antibody against the 36-kDa annexin II since it has the expected size and is known to bind actin [22,23]. The results showed that the 36-kDa protein that was also found enriched in the B fractions derived from MPC-11 and L-929 cells appears to be annexin II (Figure 2). Furthermore, the relative amounts of annexin II recovered in the B and C fractions of L-929 cells appeared to depend on culture conditions, as more annexin II, like actin (results not shown), was present in the C fraction isolated from monolayer L-929 cells. In the corresponding suspension cells, both annexin II (Figure 2) and actin (results not shown) were more concentrated in the B fraction containing the cytoskeleton-bound polysomes. In gen-

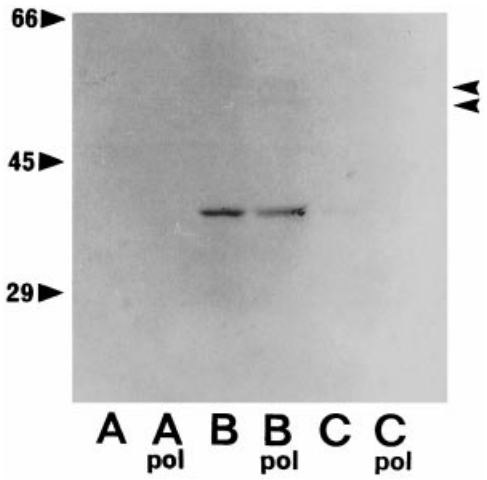


**Figure 1 Identification of annexins in the fractions (A, B and C) isolated from Krebs II ascites cells and the respective polysomal subfractions**

The three fractions were prepared and the corresponding polysomes (pol) were isolated as described in Materials and methods section. Proteins (40 $\mu$g/lane) were separated by SDS/PAGE (10% gels), transferred to nitrocellulose filters, and probed with antibodies against the consensus sequence 2 present in all annexins (CP-2). The positions of the prestained molecular-mass markers (29, 45 and 66 kDa) are indicated. Note the enrichment of the 36-kDa annexin in the B fraction and B polysomes. The arrows indicate two additional annexins in the 50–55-kDa range.

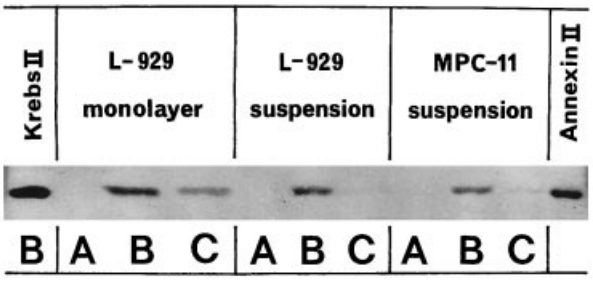


**Figure 2 Distribution of the 36-kDa annexin II in the A, B and C fractions isolated from MPC-11 cells and from L-929 cells, grown either as monolayers or in suspension**

Proteins (40 $\mu$g/lane) were separated by SDS/PAGE (10% gels), transferred to nitrocellulose filters, and immunostained with affinity-purified antibodies against annexin II. The lanes to the left and right contained the B fraction from Krebs II ascites cells and purified annexin II, respectively. Note the enrichment of annexin II in the B fractions and the increased recovery of the protein in the C fraction from monolayer L-929 cells, as compared with the suspension cells.

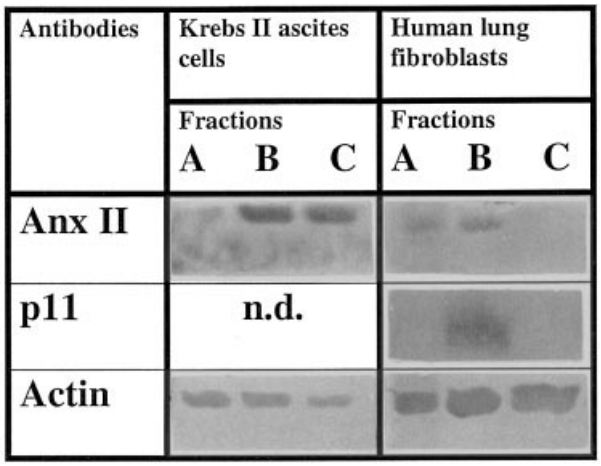


**Figure 3 Distributions of annexin II (Anx II), p11 and actin in A, B and C fractions isolated from Krebs II ascites cells and human lung fibroblasts**

Proteins (40 $\mu$g/lane) from the different fractions (A, B or C) were separated by SDS/PAGE (10% gels), transferred to nitrocellulose filters, and analysed by immunoblotting using antibodies against annexin II, p11 or actin antibodies as described in the Materials and methods section. n.d., not determined.

eral, we observed that the relative amounts of annexin II in the B and C fractions varied to some extent, possibly due to heterogeneity of the cells based on the cell cycle. Despite these variations, annexin II reproducibly was only found associated with one population of polysomes, namely the B polysomes.

The distributions of actin, annexin II and p11, the intracellular protein ligand of annexin II [12], in the A, B and C fractions derived from Krebs II ascites and human lung fibroblasts are shown in Figure 3. The human fibroblasts were chosen to provide an untransformed control cell type and to facilitate the detection of p11, since the available antibody only recognizes the human or bovine protein. A marked difference in the distribution of annexin II was observed in lung fibroblasts, as compared with Krebs II ascites cells. Namely, in addition to the B fraction, a considerable pool of annexin II was also found in the 'soluble' A fraction, whereas no annexin II was detectable in the C fraction. In contrast, p11 was only detected in the B fraction, suggesting that the A fraction contains the soluble monomeric annexin II, while the B fraction contains the annexin II–p11 complex, i.e. the form that is thought to be associated with membranes and/or the cortical cytoskeleton [36–38]. All fractions isolated from human lung fibroblasts contained a substantial amount of actin and the apparent ratio of annexin II to actin was much lower in these cells as compared with the transformed Krebs II ascites cells.

### Binding *in vitro* of cytoskeleton-bound B polysomes to annexin II after freezing

In the course of these experiments we observed that freezing in liquid nitrogen and thawing of the B fraction before isolation of B polysomes resulted in the loss of annexin II associated with those polysomes (Figure 4, lanes 1 and 2). This made it possible to carry out binding experiments *in vitro* in which the annexin II-depleted polysomes were incubated in the presence of calcium and an annexin II-enriched extract prepared from Krebs II ascites cells (Figure 4, lane 4). Subsequently, the polysomes were re-purified by centrifugation through a sucrose cushion. As shown in Figure 4 (lane 3), the binding of annexin II to B polysomes could be reconstituted *in vitro*. The contribution of annexin II in polysomes present in the annexin II-enriched extract was negligible, as shown in Figure 4, lanes 5–7.

More detailed binding experiments were performed either by incubating a constant amount of B annexin II-depleted polysomes in the presence of increasing amounts of the annexin II-enriched extract, or vice versa, followed by isolation of the polysomes as above and quantification of bound annexin II by immunoblotting. The use of both the annexin II antibody and $^{125}$I-Protein A in excess ensured linearity of annexin II detection. The binding curves (Figures 5I and 5II) show that in both cases the association between annexin II and B polysomes increased linearly. Also, at higher concentrations of the variable component, saturation of binding was observed. The binding curve in Figure 5(I) shows a decrease in annexin II binding when the incubation was carried out in the presence of high concentrations of the annexin II-enriched extract. This is probably caused by the formation of annexin II-containing aggregates that do not pellet together with the polysomes.

### Evidence for direct association of annexin II with mRNA

To further analyse the nature of the association between annexin II and B polysomes, the B fraction isolated from the Krebs II ascites cells was used in UV-cross-linking experiments. During the UV treatment the amount of aggregation that can occur during or shortly after cross-linking was followed and the duration of the UV irradiation was restricted to avoid the run-off of ribosomes. Polysome profiles were recorded from both control and UV-cross-linked B fractions to make sure that UV-induced aggregation, which would have caused the polysomes to behave abnormally in the sucrose gradients, had not occurred (Figure 6I).

Krebs II ascites cells contain a high level of endogenous RNAses which, during the long procedure used for isolation of polysome subfractions, can cleave the covalently cross-linked RNA to fragments consisting of only a few nucleotides. Interestingly, UV cross-linking caused a slight decrease in the mobility of annexin II (Figure 6II, see particularly the second lane from left), which could be due to binding of the protein to such short RNA fragments. To further assess whether covalent binding of annexin II to RNA had indeed occurred as a result of UV cross-linking, control and UV-treated B fractions were treated with EGTA, which releases the protein from cytoskeletal and/or membrane fractions [22]. Actin was included in the analysis since it associates with annexin II *in vitro* [22,23] and co-purifies with the cytoskeleton-bound polysomes [8] but is not known to bind directly to RNA. Figure 6(II) shows that EGTA efficiently released annexin II from the control B polysomes, whereas in the UV-treated B polysomes the protein became partly resistant to the treatment. In contrast, EGTA treatment did not seem to significantly affect the amount of actin in either the UV-irradiated or control polysomes as judged by Western blotting which, however, provides only a semi-quantitative measure.

As another approach to study the possible interaction between annexin II and RNA, we examined the binding of $^{32}$P-labelled total RNA isolated from Krebs II ascites cells to B polysomal proteins (Figure 7I, lane 1), or a mixture of $Ca^{2+}$-binding proteins, enriched in annexin II, that could be released by EGTA (Figure 7I, lane 3), on a NorthWestern blot. Both preparations were observed to contain an approx. 36-kDa protein that bound the RNA probe, and displayed the same mobility as immunologically detected annexin II from the same blot (Figure 7II), suggesting direct binding of RNA to annexin II.

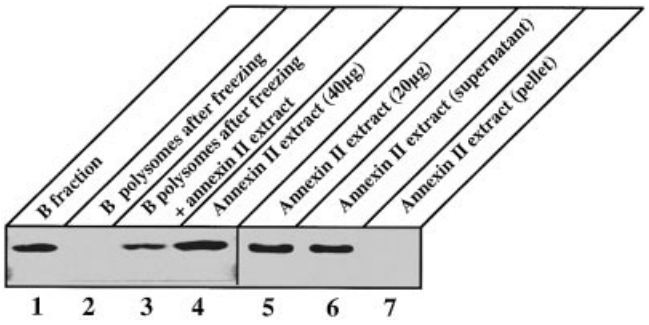


**Figure 4 Annexin II reassociates with annexin II-depleted B polysomes *in vitro***

The B fraction (lane 1) was prepared from Krebs II ascites cells and, following one freeze-and-thaw cycle, the B polysomes were isolated. These annexin II-depleted B polysomes (lane 2) were then incubated with a protein extract (a dialysed EGTA extract) enriched in annexin II (lane 4) in the presence of 2 mM calcium and, following incubation, polysomes were re-isolated by centrifugation through at sucrose cushion (lane 3). The annexin II-enriched extract (lane 5) was subjected to high-speed centrifugation used to purify polysomes (see the Materials and methods section), resulting in a supernatant (lane 6) and a pellet (lane 7). Subsequently, 40 $\mu$g of protein/lane (lanes 1–4) and 20 $\mu$g/lane of annexin II-enriched extract (lane 5) and equivalent volumes of the supernatant (lane 6) and the pellet (lane 7) were loaded on the gel. Proteins were separated by SDS/PAGE (10% gels), transferred to nitrocellulose, and immunostained with affinity-purified polyclonal antibodies (lanes 1–4) or monoclonal antibodies (lanes 5–7) against annexin II.

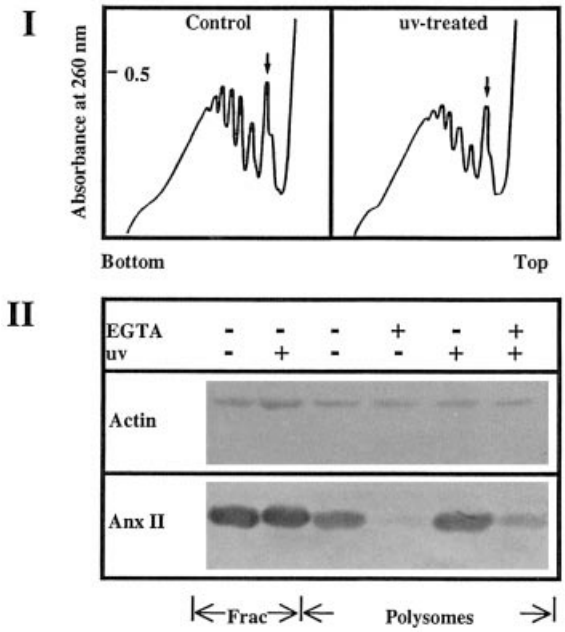


**Figure 6 The effect of UV cross-linking and EGTA treatment on the association of annexin II with the B polysomes of Krebs II ascites cells**

(**I**) Sedimentation profiles of polysomes from control and UV-treated B fractions. Arrows indicate the 80 S monosomal peak. (**II**) The detection of actin and annexin II in control and UV-irradiated B fractions (Frac) and the corresponding polysomes that were further incubated in the presence or absence of EGTA. Proteins (40 $\mu$g/lane) were separated by SDS/PAGE (10% gels), transferred to nitrocellulose filters, and analysed by immunoblotting using antibodies against actin and annexin II as indicated in the Figure. Note that in response to UV cross-linking part of annexin II in the polysomes becomes resistant to release by EGTA.

However, the detection of binding of radiolabelled RNA to the 36-kDa protein on NorthWestern blots (Figure 7) required very long exposure times, suggesting that this putative annexin II in its denatured form may be a rather poor substrate for binding

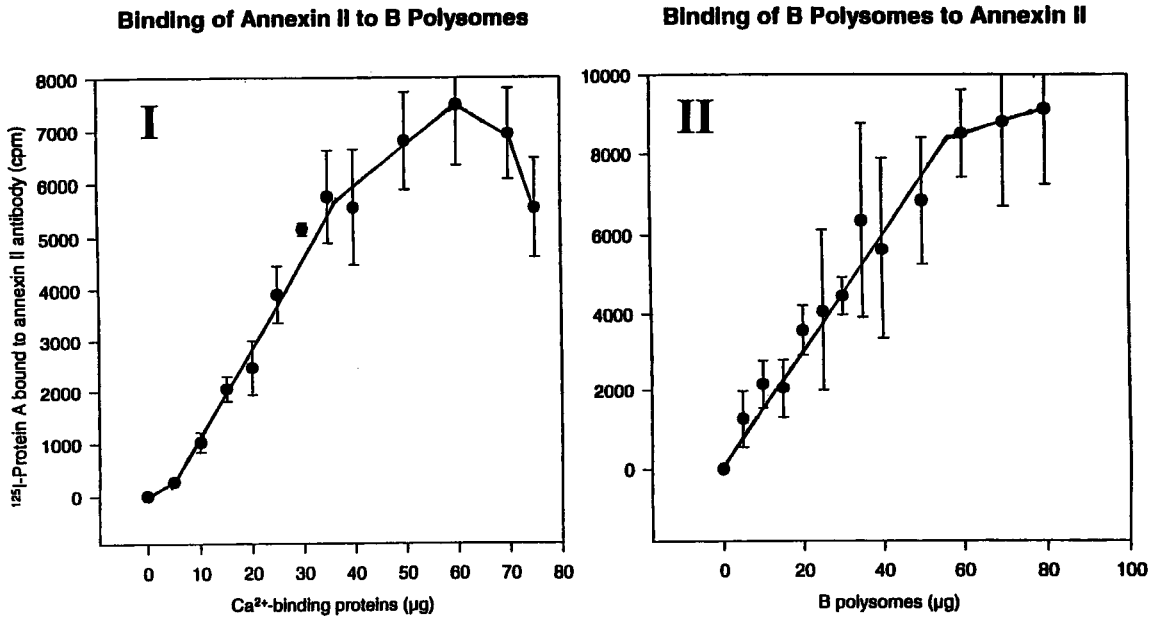


**Figure 5 Assays *in vitro* show linear and saturable binding between annexin II and B polysomes**

B polysomes and the dialysed EGTA-released protein extract, enriched in annexin II, were prepared from Krebs II ascites cells. In the binding assays, the concentration of either B polysomes (**I**), or the protein extract (**II**), was kept constant (40 $\mu$g/assay). Incubations were carried out in a total volume of 100 $\mu$l in 130 mM KCl buffer supplemented with 2 mM calcium. Subsequently the polysomes were re-isolated, and solubilized in SDS sample buffer. The proteins were separated by SDS/PAGE (10% gels), transferred to nitrocellulose, and immunostained with affinity-purified antibodies against annexin II, followed by detection of bound annexin II antibodies using $^{125}$I-Protein A. Quantification was performed using a Canberra Packard Instant Imager™. The data points show the average of three experiments, and the standard deviations are indicated.

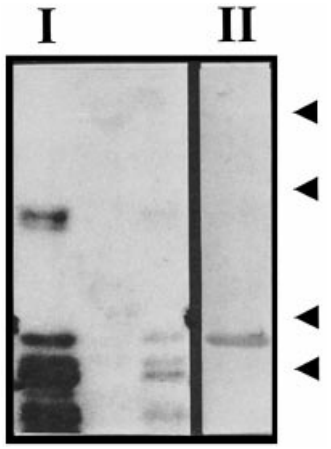


**Figure 7 The binding of $^{32}$P-labelled total RNA to a protein identified as annexin II by immunodetection**

(**I**) B polysomes (lane 1), a post-polysomal supernatant (the proteins were precipitated by chloroform/methanol; lane 2), and an extract of $Ca^{2+}$-binding proteins (lane 3) were prepared from Krebs II ascites cells. Proteins (40 $\mu$g/ml) were separated by SDS/PAGE (10% gels), transferred to nitrocellulose filters, and probed with total $^{32}$P-labelled RNA (isolated from Krebs II ascites cells) on a NorthWestern blot. (**II**) Immunostaining of B polysomal proteins with an affinity-purified antibody against annexin II (lane 4). Lane 4 was excised from and, following immunostaining, realigned with the same filter that was used for the NorthWestern blot. The arrowheads on the right indicate the positions of molecular-mass markers (34, 37, 50 and 67 kDa).

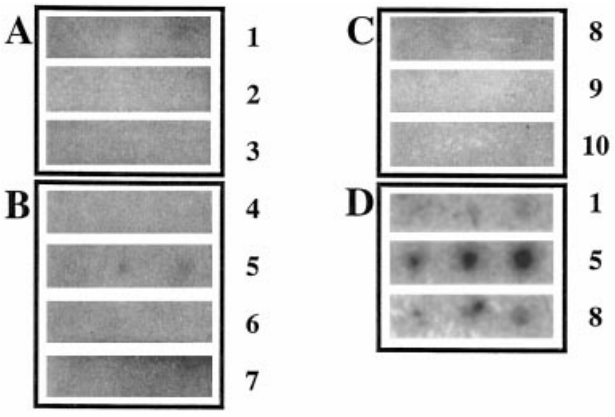


**Figure 8 The binding of $^{32}$P-labelled total, ribosomal and messenger RNAs, isolated from the A, B and C polysomes of Krebs II ascites cells, to purified annexin II**

The annexin $II_2p11_2$ complex was isolated from Krebs II ascites cells (**A**, **B** and **C**) or porcine microvillar epithelium (**D**). Then, 0.75, 1.5 or 3.0 $\mu$g of the purified protein was spotted on to nitrocellulose membranes and the total protein concentration in each spot was adjusted to 3.0 $\mu$g with BSA. Binding assays involving total RNA (rows 3, 7 and 10), mRNA (rows 1, 4, 5 and 8) or rRNA (rows 2, 6 and 9), isolated from A polysomes (**A**), B polysomes (**B**) and C polysomes (**C**), were performed as described in the Materials and methods section. mRNA was isolated from A (**D**, row 1), B (**D**, row 5) or C polysomes (**D**, row 8). Binding of mRNA isolated from the B fraction was also performed in the presence of EGTA (**B**, row 4). The bound radiolabelled RNA was visualized using an Canberra Packard Instant Imager™.

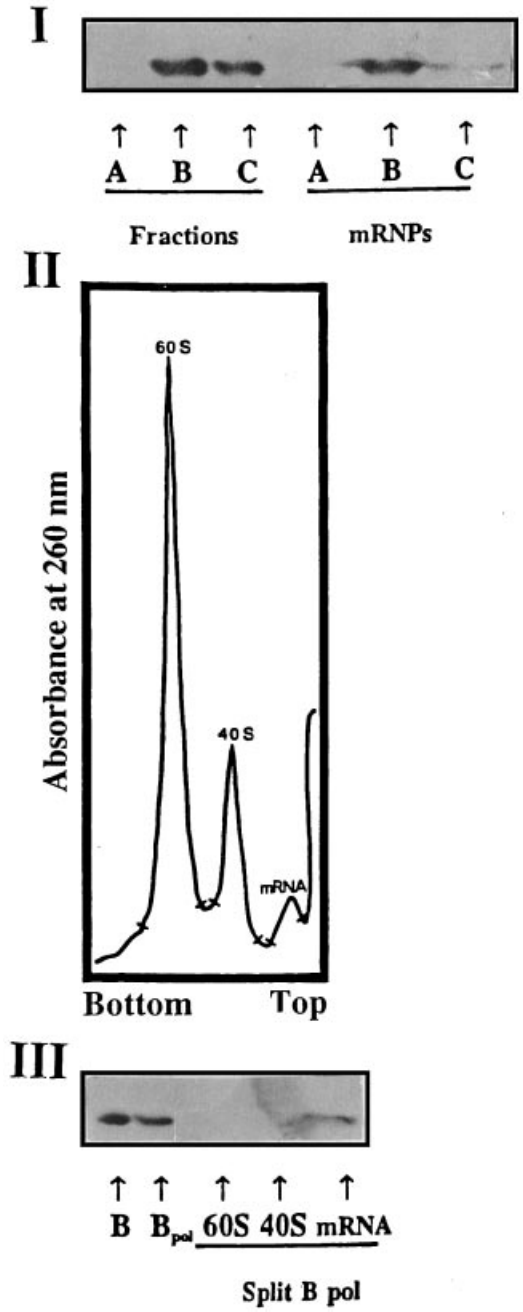


**Figure 9 Annexin II in the B fraction associates with both mRNAs and inactive mRNPs**

(**I**) The distribution of annexin II in the A, B and C fractions of Krebs II ascites cells and inactive mRNP complexes, isolated from the respective post-polysomal supernatants using poly(A)$^+$ magnetic beads. (**II**) Sedimentation profile after splitting of the cytoskeleton-bound B polysomes, isolated from a UV-treated B fraction, by TPCK into ribosomal subunits and mRNAs. (**III**) Detection by immunoblotting, of annexin II in the B fraction (B), B polysomes ($B_{pol}$), and the 60 S and 40 S ribosomal subunits, as well as the mRNA-containing peak. The contents of the collected peaks (indicated in **II**) were pelleted by centrifugation. Proteins (40 $\mu$g/lane) were separated by SDS/PAGE (10% gels), transferred to nitrocellulose filters, and immunostained with affinity-purified antibodies against annexin II.

of RNA. Therefore, to demonstrate directly the binding of RNA to the native protein, the annexin II–p11 complex was purified from both Krebs II ascites and porcine intestinal epithelial cells, immobilized on nitrocellulose membranes, and incubated with $^{32}$P-labelled RNA. The presence of a high molar excess of yeast tRNA had no effect on RNA binding. Thus immobilized annexin II was tested for its possible binding specificity to rRNA (Figure 8, rows 2, 6 and 9) or mRNA (Figures 8A–8C, rows 1, 4, 5 and

8; in row 4 the binding was performed in the presence of EGTA). Interestingly, weak, but specific binding of mRNA isolated from B polysomes to the annexin II complex could be detected (Figure 8B, row 5), whereas mRNAs isolated from A (Figure 8A, row 1) or C polysomes (Figure 8B, row 8), as well as rRNA (Figure 8, rows 2, 6 and 9) showed negligible binding. Also, the association of B mRNAs with the protein, observed in this assay, was calcium-dependent as EGTA abolished the binding (Figure 8B, row 4). Binding of total RNA isolated from the respective polysomes to immobilized annexin II (Figure 8, rows 3, 7 and 10) was also included as a further test of specificity. Since rRNA constitutes about 98–99 % of total RNA [39], no binding of total RNA to the annexin II–p11 complex was observed in this case.

Finally, we also examined the binding of mRNAs obtained from the three polysome populations of Krebs II ascites cells to the annexin II–p11 complex, purified from porcine intestine microvillar epithelium using a well-established procedure [22], and observed that this annexin II complex bound selectively B mRNA, and with a much higher affinity than the protein isolated from the transformed Krebs II ascites cells (Figure 8D, row 5).

### Annexin II is associated with both active and inactive mRNAs

The results obtained from the above *in vitro* studies suggest that annexin II binds to mRNA. To obtain further evidence for this conclusion, polysomes were sub-fractionated to separate the mRNAs from ribosomal subunits. In addition, inactive mRNAs (non-polysomal) present as mRNP complexes were isolated from the post-polysomal A, B and C supernatants using oligo(dT) magnetic beads. Routine procedures for splitting the polysomes/ribosomes with puromycin or TPCK, and their separation on sucrose gradients into 60 S, 40 S and mRNA-containing peaks, require both high salt and the presence of 10 mM EDTA in the buffer [40]. Since both conditions release annexin II, UV cross-linking of the polysomes was performed before their splitting with TPCK and sub-fractionation on sucrose gradients (Figure 9II). Subsequently, the 60 S, 40 S and the top mRNA peaks were pooled and analysed for their content of annexin II. As shown in Figures 9(I) and 9(III), respectively, annexin II was observed to associate with the B mRNP complexes and, in addition, was only found in the distinct peak, obtained after the splitting of the B polysomes, which contained the mRNAs (Figure 9II).

## DISCUSSION

Earlier studies involving sucrose-gradient sedimentation analysis and immunoprecipitation have shown the association of annexin II with small RNA particles (RNPs), isolated both from transformed and non-transformed chick embryo fibroblasts [25]. Here we show that annexin II is enriched in a fraction that can be prepared from a number of cell types and contains cytoskeleton-bound polysomes. Further purification of the polysomes from this B fraction resulted in the presence of annexin II in these polysomes, whereas the protein was not found associated with polysomes purified from the two other fractions studied (A and C). Our results demonstrate further that two additional proteins, which can be classified as annexins by immunological criteria, are present in the different polysomal fractions. The characteristics of the polysome-associated annexin II are not known, but the observation that it could be co-purified with B polysomes even in the absence of externally added calcium (results not shown) would indicate that the calcium requirement for binding is in the lower concentration range, but is required for association of annexin II with mRNA (Figure 8).

The presence of annexin II in the B fraction is presumably caused by its binding to actin, an interaction that has been well established *in vitro* [22,23]. An increased annexin II-to-actin ratio was observed in all the transformed cell lines examined (Krebs II, L-929, MPC-11), as compared with the non-transformed human lung fibroblasts (Figure 3). The results obtained with L-929 cells, grown either in suspension or as monolayers, showing the partial shift of both annexin II and actin to the C fraction in the monolayer cells also argues in favour of their association. Further, the release of annexin II to the B fraction by increasing the salt concentration from 25 to 130 mM KCl is indeed reminiscent of the binding of F-actin to annexin VI, which is optimal at 10–30 mM KCl [41]. Krebs II ascites cells, an aggressive transformed cell line, contain elevated levels of annexin II as compared with the L-929 and MPC-11 cells and, in particular, the human lung fibroblasts. Malignant transformation of different cell lines has previously been shown to involve an increased expression of annexin II, both at the mRNA and protein levels, as well as an increase in its phosphorylation [42].

Not only was annexin II present in the purified cytoskeleton-bound polysomes, but it could also reassociate with annexin II-depleted cytoskeleton-bound polysomes *in vitro*, and remained associated to these polysomes after a new purification of these polysomes by centrifugation through a sucrose cushion, indicating that the association of the protein with these polysomes is specific. Also, the isolation of polysomes as such did not cause trapping of annexin II since no annexin II was found in the polysomes purified from the fractions after freezing and thawing (Figure 4, lane 2), even though the corresponding fraction contained substantial amounts of the protein (Figure 4, lane 1).

A number of additional experiments, including UV cross-linking in combination with fractionation of ribosomes, North-Western blotting and binding of $^{32}$P-labelled RNA to annexin II immobilized on nitrocellulose, further indicate that annexin II binds directly to the mRNA moiety of the cytoskeleton-bound polysomes. In particular, immobilization of the native annexin II complex on nitrocellulose followed by incubation with $^{32}$P-labelled RNA resulted in a weak but specific binding of mRNAs isolated from B polysomes when compared with rRNA and the other two populations of mRNAs isolated from the A or C polysomes (Figure 8). Using an identical binding assay, but with the annexin II complex isolated from porcine intestinal microvillar epithelium, the preference of annexin II to bind mRNAs isolated from B polysomes was even more evident and the binding was of much higher affinity. The reason for this is not clear but it could be caused by different post-translational modifications of the two preparations. rRNA did not bind to the annexin II complex isolated from porcine intestinal microvillar epithelium (results not shown). Since a high molar excess of tRNA was present during binding, we conclude that a specific population of mRNAs appears to bind selectively to annexin II. Our data are also in accordance with earlier studies suggesting that annexin II associates with RNA [24,25] in the form of small RNPs [25]. However, in the latter case the apparent binding of annexin II to less complex RNA species could have been caused by mRNA degradation.

Further, UV irradiation of the B fraction resulted in covalent binding of annexin II to RNA, as demonstrated by the finding that UV-generated RNA–protein cross-links rendered part of the protein resistant to EGTA-mediated release. In contrast, if the treatment was carried out in the presence of EGTA, no cross-linking of annexin II to B polysomes was observed (A. Vedeler and H. Hollås, unpublished work). L5 is a ribosomal protein of the 60 S subunit [43] and UV cross-linking did not result in an increased recovery of this protein, as measured by immuno-

detection, in the polysomes (results not shown). Since this protein is an intrinsic member of the 60 S ribosomal subunit, this suggests that UV-induced cross-linking of annexin II to mRNA is not an artefact.

Using affinity purification of mRNA on poly(A)$^+$ magnetic beads, annexin II was only found in association with the mRNA moiety of cytoskeleton-bound polysomes and mRNPs present in the cytoskeletal fraction. Whereas the association of polysomes with actin filaments and microtubules appears to represent a rather non-specific and more general type of attachment [9], our data indicate that the mRNAs that bind annexin II could constitute a distinct subpopulation.

There was no enrichment of annexin II in the B polysomes relative to the fraction itself, suggesting that the annexin II that associates with polysomes could represent a distinct post-translational modification or isoform of the protein (see below) not involved in endocytosis or exocytosis. It is noteworthy that several annexin II variants or isoforms have been described [44]. The data shown in Figure 9 also show that annexin II binds to the mRNA moiety of mRNPs and polysomes and thus an enrichment of annexin II in polysomes would not be expected as the mRNA-binding proteins, not taking into account initiation, elongation and termination factors, constitute only a minor part of the proteins present in polysomes.

It is possible that the annexin II, which associates with cytoskeleton-bound polysomes, represents a distinct variant/isoform that is different from the one(s) that bind to membranes. This is currently being investigated to confirm this suggestion. Earlier findings showing that only about 3 % of total cellular phospholipids are recovered in the B fraction [9] together with no immunological detection of rab4 protein in this fraction (results not shown), support the conclusion that the B fraction is not significantly contaminated by early endosomal membranes.

By calculating the amount of annexin II in B polysomes by Western blotting using the ECL detection method and an annexin II standard curve, one arrives at an estimate of $3 \pm 1$ annexin II molecules per ribosome. This calculation is based on the assumption that the protein content of polysomes is about 50 % of the total ribosomal mass, not taking into account the mRNAs. Although this is a rough estimate, it is in the same order of magnitude as obtained for Y-box proteins (reviewed in [45]). These phosphoproteins function both as DNA- (role in transcription) as well as RNA-binding proteins and have been proposed to be involved in mRNA packaging and to bundle actin.

In conclusion, the present biochemical analysis shows that annexin II may be associated with a specific population of mRNAs and strongly suggests that the binding involves its direct interaction with mRNA. Thus annexin II seems to be involved in association with both vesicles and mRNA. In this regard it is very interesting that one of the protein ligands of testis and brain RNA-binding protein is the transitional endoplasmic reticulum ATPase [46] involved in the ATP-dependent fusion of vesicles in transport from endoplasmic reticulum to Golgi.

Our data also indicate that annexin II binds to both translationally active and inactive mRNAs and that this binding may be regulated by calcium. Studies to identify specific mRNAs belonging to the subpopulation of polysomes with which annexin II interacts, as well as the specific sequences that are involved in this association, are in progress.

We would like to express special gratitude to Dr Volker Gerke for his generous gift of antibodies, for numerous discussions and constructive suggestions during the various phases of this work as well as for critical comments on the manuscript. In addition, we are grateful to Dr Jaakko Saraste and Dr Robert Kretsinger for critically reading the manuscript and giving valuable comments. Dr Bruno Goud and Dr Joachim Stahl are appreciated for their generous gifts of rab4p and L5 antibodies, respectively. Finally, Dr Torgeir Flatmark is thanked for discussions concerning the binding experiments. This work was supported in part by the Norwegian Cancer Society.

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Received 5 November 1999/8 March 2000; accepted 30 March 2000