Atypical properties displayed by annexin A9, a novel member of the annexin family of Ca^{2+} and lipid binding proteins

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Received 14 April 2003; revised 27 May 2003; accepted 28 May 2003

First published online 11 June 2003

Edited by Guido Tettamanti

Abstract **Annexin A9 is a novel member of the annexin family of Ca^{2+} and phospholipid binding proteins which has so far only been identified in EST data bases and whose deduced protein sequence shows mutations in residues considered crucial for Ca^{2+} coordination in other annexins. To elucidate whether the annexin A9 protein is expressed as such and to characterize its biochemical properties we probed cell extracts with specific anti-annexin A9 antibodies and developed a recombinant expression system. We show that the protein is found in HepG2 hepatoma cell lysates and that a green fluorescent protein-tagged form is abundantly expressed in the cytosol of HeLa cells. Recombinant expression in bacteria yields a soluble protein that can be enriched by conventional chromatographic procedures. The protein is capable of binding phosphatidylserine containing liposomes albeit only at Ca^{2+} concentrations exceeding 2 mM. Moreover and in contrast to other annexins this binding appears to be irreversible as the liposome-bound annexin A9 cannot be released by Ca^{2+} chelation. These results indicate that annexin A9 is a unique member of the annexin family whose intracellular activity is not subject to Ca2+ regulation.**

Key words: Acidic phospholipid; Actin binding; Calcium signaling; Membrane binding

1. Introduction

Annexins are a family of evolutionarily conserved proteins characterized by their ability to interact with membrane phospholipids in a Ca^{2+}-dependent manner. This property most likely underlies annexin functions in membrane organization and traffic and has been proposed to depend on so-called type II Ca^{2+} binding sites found in annexin proteins (for recent reviews on annexins see [1,2]). Type II sites are located in the protein core domains which are structurally conserved within the annexin family and are built of four or eight homologous segments known as annexin repeats. The repeats form a compact and slightly curved disc with the type II Ca^{2+} sites protruding at the convex side of the disc (for reviews on annexin structure see [3–5]). Typically, these type II sites are formed by an interhelical loop and an acidic amino acid, called cap residue, with the latter residing approximately 40 residues C-terminal to the loop and providing carboxyl oxygens for Ca^{2+} coordination in the folded molecule [6]. In membrane-bound annexins Ca^{2+} bound in the type II sites serves a bridging function to the phospholipid headgroups by coordinating not only to carbonyl and carboxyl oxygens of the protein but also to the phosphoryl moiety of membrane phospholipids [7]. Amino acids participating in the formation of type II binding sites are all found in an individual annexin repeat and a typical four repeat annexin core should thus contain four type II sites. While this has been proven correct for annexin A5, albeit only at higher Ca^{2+} concentrations [8], two annexins, A1 and A2, have suffered amino acid substitutions of the cap residue in the first annexin repeat. In both cases the elimination of the acidic residue at the cap position most likely renders the respective site incapable of binding Ca^{2+} as revealed by structural analyses of Ca^{2+}-loaded annexin crystals [9,10].

All annexins structurally or biochemically characterized to date contain at least three of the type II Ca^{2+} sites and it is Ca^{2+} binding to these sites that enables the proteins to dock peripherally onto the cytoplasmic leaflets of cellular membranes [11,12]. As this property has been considered a hallmark of the annexin family it was highly unexpected that an annexin sequence lacking the acidic cap residue in all four annexin repeats was identified by Morgan and Fernandez in a data base search of human expressed sequence tags (ESTs) [13]. This annexin, initially termed annexin 31 and with the new annexin terminology in place now known as annexin A9, has its closest relative in annexin A2, a protein thought to function in the organization and regulation of membrane/cytoskeleton linkages [14]. While this classification is based on sequences of the annexin core region similar sequence features are also found in the N-terminal domains of annexins A2 and A9. Typically, the N-terminal domains of different annexins are highly divergent and are believed to confer specificity to individual members of the family (for review see [2]). Although showing limited sequence identity, the N-terminal regions of annexins A2 and A9 share the potential of forming an amphipathic α-helix. In annexin A2 this helix formation has been experimentally verified with the hydrophobic side of the helix representing the binding surface for a specific intracellular protein ligand, S100A10 [15,16].

Given the unique sequence features displayed by annexin A9 we sought to determine whether the protein is expressed in cultured cells and whether it shows unique biochemical characteristics. By employing annexin A9-specific antibodies we detect low-level protein expression in HepG2 but not HeLa

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0014-5793/03/$22.00
doi:10.1016/S0014-5793(03)00634-3

cells. Moreover we show that the annexin A9 protein expressed in bacteria is capable of interacting with phospholipid liposomes in a Ca^{2+}-dependent manner. However, the interaction requires Ca^{2+} concentrations in the millimolar range and occurs in an irreversible manner. This argues against a participation of Ca^{2+} in regulating annexin A9 function within cells and supports the view that the protein fulfills highly specific functions not displayed by other members of the family.

2. Materials and methods

2.1. Cell culture and preparation of cell lysates

HeLa cells were maintained in Dulbecco's modified Eagle's medium (BioWhittaker Europe, Verviers, Belgium) with 10% fetal calf serum, glutamine and antibiotics in a 7% CO_2 incubator at 37°C. HepG2 cells were maintained in RPMI 1640 medium (BioWhittaker Europe) supplemented with 10% fetal calf serum, glutamine and antibiotics in a 5% CO_2 incubator at 37°C.

Cells grown to 70–80% confluence were harvested, resuspended in lysis buffer (20 mM HEPES, pH 7.4, 0.1% sodium dodecyl sulfate (SDS), 0.5% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF)) and incubated for 30 min at 4°C. Cell lysates were then centrifuged at $10\,000\times g$ for 10 min at 4°C to pellet cellular debris. Supernatants were resolved on a 12% SDS–polyacrylamide gel electrophoresis (PAGE) gel and transferred to a polyvinylidene difluoride(PVDF) membrane. Blots were blocked with 5% milk powder in TBS-T (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.2% Tween 20) prior to probing with immunoaffinity-purified anti-annexin A9 antibodies.

2.2. Cloning of green fluorescent protein (GFP) expression constructs

Annexin A1–GFP and annexin A2–GFP constructs have been described previously [11]. To obtain annexin A9–GFP, with enhanced GFP fused to the carboxy-terminus of protein, full-length annexin A9 cDNA was amplified by polymerase chain reaction (PCR) (forward primer with 5′-flanking *Eco*RI site 5′-accatgtctgtgactggcgggaag-3′, reverse primer with a *Sal*I site replacing the stop codon 5′-catgtcttcagccctgcacaaggc-3′) using the bacterial expression vector pET-23a(+)$_{mod.}$/*anx*A9 (see below) as template. The PCR fragment was inserted into the linearized mammalian expression vector pEGFP-N3 (Clontech, Heidelberg, Germany) and correctness verified by sequence analysis.

2.3. Transfections and fluorescence microscopy

Transient transfections employed HeLa cells grown on coverslips which were transfected using Effectene (Qiagen, Hilden, Germany). Twenty-four hours after transfection, cells were fixed with 4% formaldehyde in phosphate-buffered saline for 10 min at room temperature. Localization of the respective annexin was visualized by GFP fluorescence using a DM RXA fluorescence microscope (Leica, Wetzlar, Germany).

2.4. Expression of annexin A9 in a coupled transcription–translation reticulocyte lysate system

Full-length annexin A9 was expressed in a coupled in vitro transcription–translation system using a vector containing the complete cDNA of annexin A9 under T7 promoter control and the TNT–T7 reticulocyte lysate system in the presence of methionine essentially as described by the supplier (Promega, Madison, WI, USA). 1 mM methionine and 1 μg plasmid DNA were used in one 50 μl assay. As a negative control an assay was performed with the empty vector. Reactions were incubated at 30°C for 90 min.

2.5. Reverse transcription and PCR (RT-PCR)

Total RNA was isolated using the Qiagen RNeasy kit according to the manufacturer's protocol with the Qiashredder columns for cell lysis. Total RNA (5 μg) was subjected to reverse transcription using the SuperScript II RNase H^- Reverse Transcriptase (Invitrogen Life Technologies, Carlsbad, CA, USA) and reaction conditions specified by the manufacturer. Reactions were primed with 100 pM random hexamers. The PCR reaction was heated for 2 min at 94°C and started in a 25 μl volume using 5 μl of the cDNA mix as a template (primers see below), followed by 35 cycles of denaturing at 94°C for 45 s, annealing at 56°C for 1 min and extension at 72°C for 1 min. In the final cycle, extension time was increased to 7 min.

2.6. Generation and immunoaffinity purification of anti-annexin A9 antibodies

Rabbit polyclonal antiserum to a synthetic peptide corresponding to amino acids 8–29 of human annexin A9 was obtained by standard immunization procedures (Eurogentec, Seraing, Belgium). For affinity purification, column fractions enriched in bacterially expressed annexin A9 were separated by SDS–PAGE and transferred onto PVDF membrane by Western blotting. Immobilized proteins were visualized by Ponceau staining and the membrane strip carrying the immobilized annexin A9 was cut out. Serum samples were diluted with TBS-T containing 5% non-fat milk powder and incubated for 2 h with the membrane strip, then washed three times with TBS-T. Adsorbed anti-annexin A9 antibodies were eluted by placing the membrane strip in 100 mM glycine pH 2.7. Eluted antibodies were immediately neutralized with 1/10 volume 1 M Tris and 10×TBS-T. Concentration of the affinity-purified antibodies employed Microcon YM-30 centrifugation filter devices (30 MWCO, Millipore, Bedford, MA, USA).

2.7. Expression and purification of human annexin A9

The complete cDNA encoding human annexin A9 was amplified from a human fetal liver MATCHMAKER cDNA library (BD Clontech, Heidelberg, Germany) by PCR using the following primers, each with 5′-flanking *Eco*RI sites: 5′-atgtctgtgactggcgggaag-3′ (forward) and 5′-tcacatgtcttcagccctg-3′ (reverse). The purified PCR product was digested with *Eco*RI and cloned into the bacterial expression vector pET-23a(+) (Novagen, Heidelberg, Germany) modified to allow expression of full-length annexin A9 without a T7 tag [17]. Correct insertion of the cDNA was confirmed by sequencing.

Escherichia coli expression strain BL21(DE3)pLysS (Stratagene, Amsterdam, The Netherlands) was transformed with the pET-23a(+)$_{mod.}$/*anx*A9 expression vector. Transformed bacteria were grown at 37°C in LB medium containing 150 μg/ml ampicillin until the OD_{600} reached 0.6. Expression of annexin A9 was induced with 1 mM isopropyl-β-D-thiogalactose (IPTG) for 3 h at 37°C and the bacteria were then harvested by centrifugation. The cell pellet was resuspended in 1/10 culture volume of buffer L (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM dithiothreitol (DTT), 1 mM EGTA, 0.5 mM PMSF) and lysed by three freeze/thawing cycles and sonication (3×30 s). Insoluble material was removed by centrifugation at $30\,000\times g$ for 30 min at 4°C followed by an additional centrifugation step at $100\,000\times g$ for 40 min at 4°C. The annexin A9-containing supernatant was dialyzed against buffer D (20 mM imidazole–HCl, pH 7.5, 10 mM NaCl, 1 mM DTT, 1 mM EGTA, 0.5 mM PMSF). Annexin A9 was enriched by ion exchange chromatography on DEAE Sephacel (Amersham Pharmacia Biotech AB, Uppsala, Sweden) using buffer D as column buffer. Under these conditions annexin A9 bound to the column and was eluted using 100 mM NaCl in buffer D.

2.8. Phospholipid binding assay

For the phospholipid binding assays, fractions enriched in annexin A9 were dialyzed against buffer D plus 100 mM NaCl but without EGTA. Binding properties of annexin A9 were assayed in a liposome pelleting assay [18]. Briefly, liposomes were generated by sonicating brain extract (Folch fraction I from bovine brain, Sigma B-1502, Steinheim, Germany) in water. Binding experiments were performed in EGTA-free buffer D (plus 70 mM NaCl and 2 μM E64) containing different $CaCl_2$ concentrations (0.5, 1 and 2.5 mM Ca^{2+}) or 1 mM EGTA. Protein fractions in the respective binding buffer were first centrifuged at $100\,000\times g$ for 15 min at 4°C to pellet any remaining insoluble material. Liposomes were then added to a final concentration of 2 mg/ml and the reaction mixtures were incubated for 1 h at room temperature. Liposomes were pelleted by centrifugation at $100\,000\times g$ for 1 h at 4°C, resuspended in buffer D containing 100 mM EGTA or 100 mM EGTA plus 0.2% Triton X-100, to release Ca^{2+}-dependently bound annexin A9, and again pelleted by centrifugation. Equivalent amounts of supernatants and pelleted liposomes were analyzed by SDS–PAGE and subsequent immunoblotting.

2.9. Actin co-sedimentation assay

The ability of annexin A9 to bind or bundle actin was investigated

as described [19] by co-sedimentation with F-actin in bundling buffer (2 mM Tris–HCl, pH 7.5, 50 mM KCl, 1 mM $MgCl_2$, 0.33 mM ATP, 0.5 mM DTT). Briefly, 1/50 volume of actin polymerization solution (2.5 mM KCl, 100 mM $MgCl_2$, 50 mM ATP) was added to 1 mg/ml rabbit skeletal muscle actin (Cytoskeleton, Denver, CO, USA) in G-actin buffer (5 mM Tris–HCl, pH 8.0, 0.2 mM $CaCl_2$, 0.2 mM ATP) and the solution was then incubated for 30 min at room temperature. Polymerized F-actin was stored at 4°C prior to use. In a final volume of 200 μl, 25 μg F-actin and the fraction enriched in annexin A9 were incubated for 20 min at room temperature in the presence (4 mM Ca^{2+}) or absence (1 mM EGTA) of Ca^{2+}. The reaction mixture was centrifuged at low speed (15 600 × *g*, 15 min, 20°C) to pellet F-actin bundles, followed by a high speed centrifugation (350 000 × *g*, 15 min, 20°C) to pellet F-actin. Proteins left in the supernatants were precipitated with 10% trichloroacetic acid (TCA) and pelleted at 20 000 × *g* for 15 min at 4°C. The different samples were analyzed by SDS–PAGE and subsequent Western blotting using the polyclonal anti-annexin A9 antibodies. F-actin was visualized by Coomassie staining.

2.10. S100A10 overlay assay

S100A10 overlay assays were carried out using protein extracts from *E. coli* BL21(DE3)pLysS expressing human annexin A9, a sample of human annexin A9 enriched by DEAE chromatography (see above) and heterotetrameric annexin A2–S100A10 complex isolated from porcine mucosa as positive control [20]. The protein samples were separated by SDS–PAGE and then transferred onto PVDF membrane. The membrane was blocked with 2% (w/v) bovine serum albumin (BSA) in TBS and then washed three times for 10 min with TBS. Subsequently the membrane was incubated for 1 h at 37°C with 10 μg purified recombinant human S100A10 [21] in 2 ml TBS containing 2% (w/v) BSA and 2 mM DTT. After extensive washing with TBS, bound S100A10 was detected using a monoclonal anti-S100A10 antibody [22]. To visualize annexin A2 and annexin A9 on the same blot, the membrane was stripped for 30 min at 50°C in stripping buffer (67 mM Tris–HCl, pH 6.8, 2% (w/v) SDS and 96 mM β-mercaptoethanol) and probed using a monoclonal anti-annexin A2 antibody [12] or the polyclonal anti-annexin A9 antibody.

3. Results and discussion

3.1. Expression of the annexin A9 protein in cultured cells

Annexin A9 is a very unusual annexin in that all type II high affinity Ca^{2+} binding sites characteristic for the annexin protein family are mutated and therefore predicted to be dysfunctional [13]. Positive selection of an annexin with ablated type II sites might point towards Ca^{2+}-independent functions.

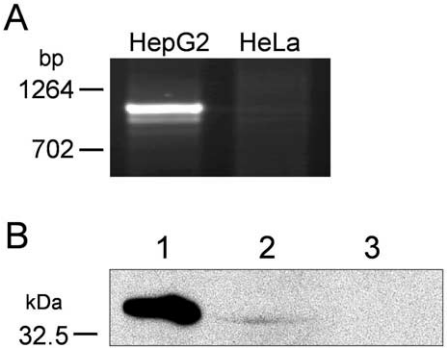

Fig. 1. Expression of endogenous annexin A9 in HepG2 cells. A: RT-PCR performed with total RNA isolated from HepG2 and HeLa cells. The size of the PCR product as deduced from the cDNA sequence is estimated to be 1038 bp. B: Western blotting with affinity-purified anti-annexin A9 antibodies detects an immunoreactive band at the same apparent molecular mass as the bacterially expressed annexin A9 (lane 1) in total cell lysates from HepG2 (lane 2) but not HeLa cells (lane 3).

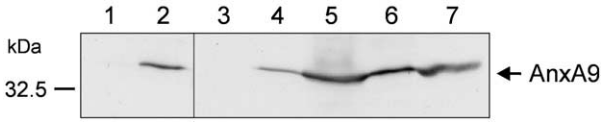

Fig. 2. Expression of human annexin A9 in *E. coli*. For comparison, annexin A9 expressed in an eukaryotic in vitro translation reaction is shown in lane 2. As a negative control the result of a TNT reaction using an empty vector is depicted in lane 1. *E. coli* BL21(DE3)-pLysS transformed with pET-23a(+)$_{mod.}$/*anx*A9 was grown in LB medium containing ampicillin and recombinant protein expression was induced with 1 mM IPTG. Annexin A9 was then enriched as described in Section 2 and protein samples of the different purification steps were analyzed by Western blotting using anti-annexin A9 antibodies. Lane 3, proteins from non-transformed bacteria; lanes 4 and 5, bacterial proteins prior to and following IPTG induction for 3 h, respectively; lane 6, soluble protein pool from the induced bacteria; lane 7, annexin A9 eluted from the DEAE column at 100 mM NaCl.

Although annexin A9 transcripts have been found in several human EST libraries [13], the existence of the protein as such in human cells has not been reported. Therefore, we performed RT-PCR on total RNA from different cultured cells. While a number of cells including HeLa did not yield any specific signal, we could generate a single PCR product of the expected size in HepG2 cells (Fig. 1A), confirming previous reports of a matching EST clone originating from this cell line [13]. To monitor the expression of annexin A9 protein in cultured cells we prepared polyclonal antibodies directed against a synthetic peptide resembling a part of the N-terminal domain of the protein. The sequence was chosen because it showed a high degree of variation to N-terminal sequences of other annexins and thus was likely to generate annexin A9-specific antibodies. The antibodies obtained recognized the cognate, recombinantly expressed annexin A9 and not the most closely related family members, annexins A1 and A2 (not shown). The immunoaffinity-purified anti-annexin A9 antibodies were then employed to stain Western blots of whole cell lysates. As shown in Fig. 1B, a band corresponding in size to that of recombinantly expressed annexin A9 was stained in lysates from HepG2 hepatoma but not HeLa cells. This signal was not observed with the pre-immune serum indicating that it is due to specific recognition of the endogenous HepG2 annexin A9 protein (not shown). A limited expression pattern of the protein is in line with previous observations on the limited abundance of annexin A9 sequences in EST libraries from different tissues or cells [13] and with the limited presence of annexin A9 mRNAs in different murine organs [23]. The expression profiling and an immunocytochemical analysis of mouse embryos employing the antibody described here [23] reveals that the protein appears to be restricted to certain types of tissues, in particular spleen and liver. Such a specific expression pattern contrasts with that of most other annexins (for review see [2]) and argues for a rather specialized function of annexin A9.

3.2. Expression of the annexin A9 protein in E. coli

To characterize the protein biochemically, we chose bacterial expression of annexin A9 as full-length authentic protein. Recombinant annexin proteins generated by bacterial expression systems in previous analyses have been shown to retain their typical properties such as Ca^{2+}-regulated phospholipid

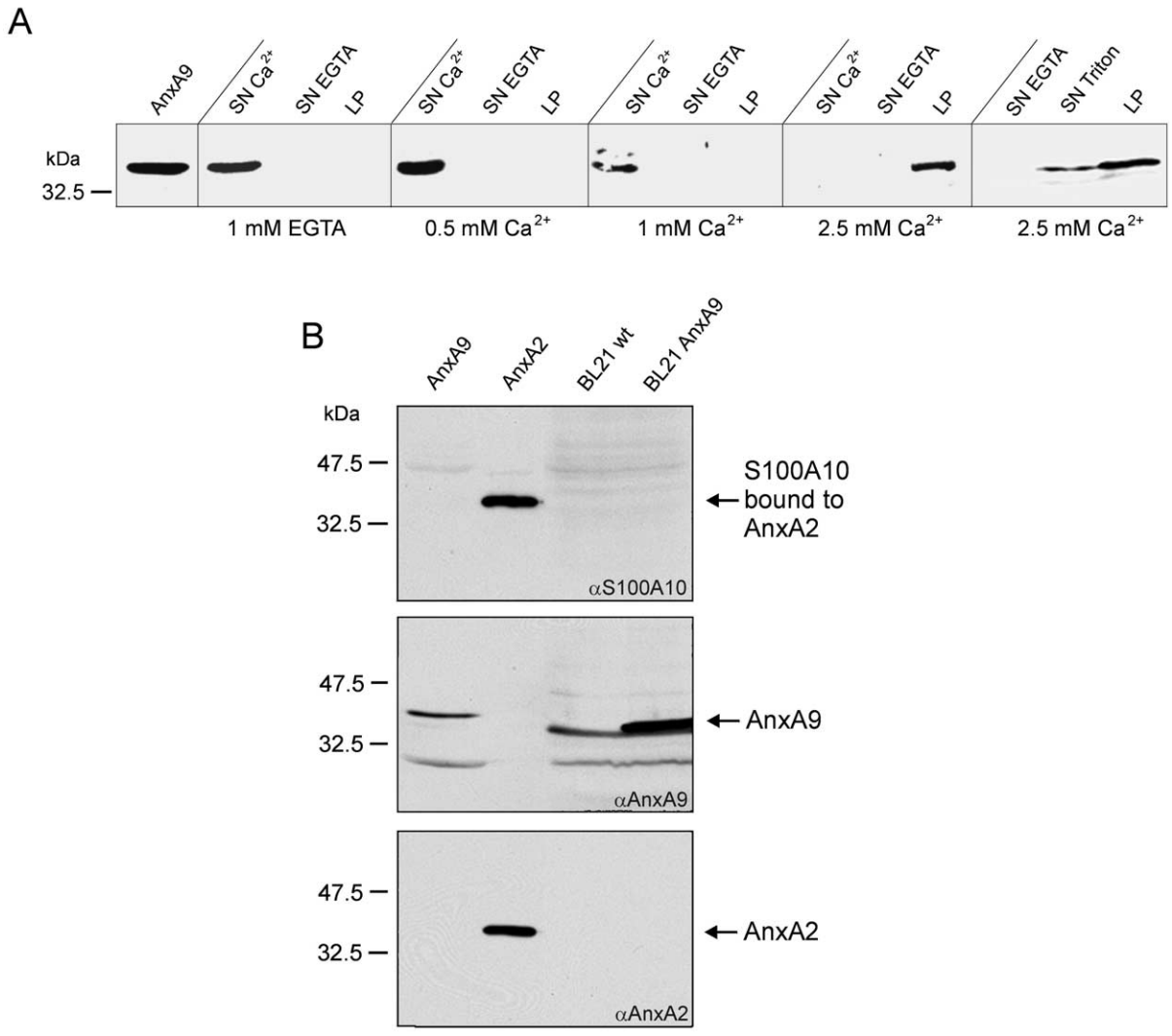

Fig. 3. Ca^{2+}-dependent phospholipid binding of human annexin A9 (A) and S100A10 ligand binding blot (B). A: Annexin A9 present in the DEAE eluate was mixed with phospholipid liposomes in the presence of either EGTA or increasing Ca^{2+} concentrations. Liposomes were pelleted, re-extracted with 100 mM EGTA and pelleted again. Supernatants of the first (SN Ca^{2+}) and second (SN EGTA) centrifugation and the liposome pellets (LP) were analyzed by Western blotting using the anti-annexin A9 antibodies. In the case of annexin A9 bound in the presence of 2.5 mM Ca^{2+} the liposome-bound material was first extracted in the buffer containing 100 mM EGTA (SN EGTA) and then subjected to extraction with 100 mM EGTA and 0.2% Triton X-100. Following an additional centrifugation the detergent-extracted annexin A9 (SN Triton) as well as the protein still bound to liposomes (LP) were visualized by Western blotting. Note the Ca^{2+}-dependent binding of annexin A9 which cannot be reversed by EGTA treatment. Only the inclusion of detergent leads to an extraction of some annexin A9 from the liposome pellet. B: Annexin A9 present in the DEAE eluate (AnxA9), annexin A2 present in the annexin A2–S100A10 complex purified from intestinal epithelial cells (AnxA2), a protein extract from non-transformed *E. coli* BL21 (BL21 wt) and an extract from transformed BL21 bacteria expressing annexin A9 (BL21 AnxA9) were subjected to SDS–PAGE, transferred to PVDF membrane and then incubated with purified S100A10 following a ligand blot protocol developed for visualizing the S100A10–annexin A2 interaction [31]. Bound S100A10 was detected with a specific monoclonal antibody. Subsequently the membrane was stripped and probed with anti-annexin A9 and anti-annexin A2 antibodies, respectively. Note the binding of S100A10 to the immobilized annexin A2 which is not observed in the case of annexin A9.

binding (see, for example, [24,25]). As shown in Fig. 2, IPTG induced the transformed bacteria to produce annexin A9. To verify the correct synthesis as full-length protein in *E. coli*, we additionally used a system for eukaryotic translation in vitro. Immunoreactive bands obtained by either bacterial expression or eukaryotic in vitro translation migrated at the same apparent molecular mass. Following cell lysis, the bacterially expressed protein was found in the soluble protein pool. Interestingly, expression of annexin A9 was rather limited compared to the expression of other annexins, e.g. annexin A1, using the same expression system and mode of induction (not shown).

3.3. *Biochemical characteristics of recombinantly expressed annexin A9 and expression of GFP-tagged annexin A9 in HeLa cells*

Annexin A9 present in the soluble bacterial lysate was enriched by ion exchange chromatography on DEAE cellulose. Under the conditions chosen (pH 7.4) the protein eluted from the column at approximately 100 mM NaCl (Fig. 2). This correlates with the calculated isoelectric point of 5.29. Inspection of the eluted fraction revealed a number of additional (bacterial) polypeptides with annexin A9 representing approximately 10% of the total protein (not shown).

Annexin A9 present in the DEAE eluate was subjected to

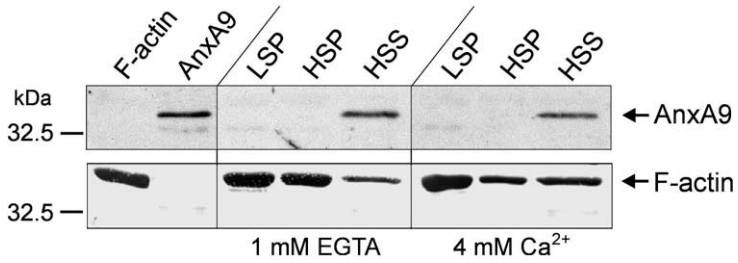

Fig. 4. Annexin A9 does not bind to F-actin. The ability of annexin A9 to bind or bundle F-actin was investigated in a co-sedimentation assay. Annexin A9 present in the DEAE eluate was incubated with F-actin in the presence or absence of Ca^{2+}. F-actin bundling was detected by low speed centrifugation (LSP) and F-actin binding by high speed centrifugation (HSP). Proteins left in the high speed centrifugation supernatant (HSS) were precipitated with TCA. The different samples were analyzed by SDS–PAGE and then transferred to a PVDF membrane. After immunodetection with the polyclonal anti-annexin A9 antibody (upper panel), the membrane was stained with Coomassie to visualize F-actin (lower panel).

biochemical analyses. First, we performed liposome pelleting experiments using brain extract liposomes containing approximately 50% phosphatidylserine and increasing free Ca^{2+} concentrations. At Ca^{2+} concentrations of up to 1 mM, binding of annexin A9 to the negatively charged liposomes was negligible (Fig. 3A). This is in sharp contrast to the behavior of all other annexins analyzed so far which show complete or almost complete binding to acidic liposomes at such Ca^{2+} levels (see, for example, [26]), indicating that the amino acid substitutions in the cap residues of the type II Ca^{2+} binding sites indeed have rendered these sites inactive in annexin A9. However, when Ca^{2+} was increased to 2.5 mM annexin A9 was co-pelleted together with the liposomes. Most likely this low affinity binding is mediated through type III Ca^{2+} sites which have also been found in all annexins crystallized in their Ca^{2+}-bound form so far and are possibly unaltered in annexin A9. Type III sites differ considerably from type II sites, both in architecture and in affinity for Ca^{2+}. They typically provide carboxyl oxygens of an acidic residue and carbonyl oxygens of a nearby site in the backbone for Ca^{2+} coordination and have a Ca^{2+} affinity which is at least an order of magnitude lower than that of canonical type II sites [10,24,25]. Type III sites have thus been considered irrelevant for intracellular activities of annexins although they might participate in regulating extracellular functions reported for some members of the annexin family (for review see [2,27]). Interestingly and in contrast to other type II site annexin mutants engineered recombinantly [24,25], annexin A9 bound to liposomes at the elevated Ca^{2+} levels could not be released by chelating Ca^{2+} with EGTA. Only the inclusion of Triton X-100 in the EGTA elution buffer resulted in a significant liberation of annexin A9 from the liposomes (Fig. 3A). These binding characteristics suggest some conformational protein alteration in the annexin A9–Ca^{2+}–liposome complex which probably slows down the off rate for Ca^{2+} or leads to an exposure of Ca^{2+}-insensitive hydrophobic surfaces in the annexin A9 protein. Future experiments have to address this question, e.g. by probing the hydrophobic nature of the protein under different conditions and elucidating the thermodynamics of ligand binding.

The liposome binding data indicate that annexin A9 is not regulated by intracellular Ca^{2+} thus constituting a distinct and so far non-described subgroup of the annexin family. Whether or not the protein is capable of interacting with certain types of cellular membranes in a manner not dependent on Ca^{2+} remains to be determined. Such considerations are noteworthy since the closest annexin A9 relative, annexin A2, can bind to cholesterol-rich subdomains of cellular membranes in a Ca^{2+}-independent manner [28]. Most likely, this interaction is mediated through the unique N-terminal sequence which shows some features reminiscent of the N-terminal domain of annexin A9 [29,30].

Another property uniquely displayed by the N-terminal domain of annexin A2 is its capability of interacting with the S100 protein S100A10 [15]. This interaction, which can be demonstrated by ligand overlay blotting [31], is mediated through the N-terminal 12 residues of annexin A2 forming an amphipathic α-helix. The corresponding sequence at the N-terminal end of annexin A9 also shows a potential to form an amphipathic α-helix and some residues participating in annexin A2–S100A10 complex formation are conserved in the N-terminal annexin A9 sequence. Therefore, we performed S100A10 ligand overlays employing annexin A9 and annexin A2 immobilized on PVDF membrane to elucidate whether annexin A9 can also interact with S100A10. Fig. 3B reveals that in contrast to annexin A2 the recombinantly expressed annexin A9 protein is not able to bind to S100A10. While this argues against any annexin A9–S100A10 complex formation it remains to be determined whether other protein

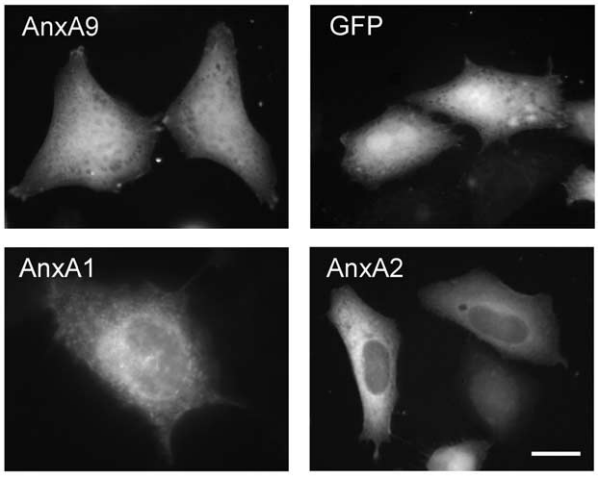

Fig. 5. Intracellular distribution of annexin A9 in transfected HeLa cells. HeLa cells were transfected with an expression construct encoding an annexin A9–GFP fusion protein (AnxA9) and the intracellular distribution was visualized by recording the GFP signal. Expression constructs encoding GFP alone (GFP), annexin A1–GFP (AnxA1) or annexin A2–GFP (AnxA2) were used as controls. Scale bar: 10 μm.

ligands can interact with the unique N-terminal domain of annexin A9.

Since annexin A2 is also an F-actin binding protein [20] and since a sequence constituting a potential actin binding site is conserved between annexins A2 and A9 [19], the recombinantly expressed annexin A9 was also used in F-actin co-sedimentation experiments (Fig. 4). However, in contrast to annexin A2, annexin A9 was unable to bind to actin filaments even in the presence of high (4 mM) Ca^{2+} concentrations. Thus the F-actin binding sequence mapped in annexin A2 is per se not sufficient to mediate F-actin binding in an annexin background. Additional parameters, most likely including intact type II Ca^{2+} binding sites, are required.

The inability of annexin A9 to interact with F-actin is also supported by our analysis of the intracellular distribution of a C-terminally GFP-tagged annexin A9 in HeLa cells. Fig. 5 reveals that the fusion protein shows no indication of a colocalization with actin stress fibers or cortical actin filaments. It is present throughout the cytoplasm as well as nucleoplasm. Annexins A1 and A2 used as controls in these experiments are found on endosomal vesicles and in the cytoplasm as well as at the plasma membrane [11]. A nuclear export sequence found in the N-terminal domain of annexin A2 has been shown to confer nuclear exclusion for this annexin [32]. Interestingly, a similar sequence involving regularly spaced leucine residues is also present in annexin A9 but seems to be ineffective in this case.

In conclusion, our data confirm that annexin A9 is an atypical member of the annexin family, since it is not capable of binding acidic phospholipids in the presence of submillimolar Ca^{2+} concentrations. This suggests that the protein fulfills a rather special function which due to its low abundance and biochemical properties does not involve the providing of a membrane scaffold proposed for other annexins (for review see [2]). High resolution structural studies have to reveal whether annexin A9 shares a common structural fold with other annexins with the core constituting an entity designed to interact with negatively charged macromolecular surfaces. Specific functional insights are likely to be obtained by gene disruption or silencing experiments as functional redundancy is not expected in the case of annexin A9.

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