Acta Biochimica Polonica

Vol. 48 No. 4/2001

851–865

QUARTERLY

GTP-binding properties of the membrane-bound form of porcine liver annexin VI★✪

Aneta Kirilenko, Marcin Golczak, Sławomir Pikuła and Joanna Bandorowicz-Pikuła✉

Department of Cellular Biochemistry, M. Nencki Institute of Experimental Biology, L. Pasteura 3, 02-093 Warszawa, Poland

Received: 6 November, 2001; accepted: 12 November, 2001

Key words: annexin VI, GTP binding, TNP-GTP, circular dichroism, GTP-binding domain

Annexin VI (AnxVI) of molecular mass 68–70 kDa belongs to a multigenic family of ubiquitous Ca^{2+}- and phospholipid-binding proteins. In this report, we describe the GTP-binding properties of porcine liver AnxVI, determined with a fluorescent GTP analogue, 2′-(or 3′)-*O*-(2,4,6-trinitrophenyl)guanosine 5′-triphosphate (TNP-GTP). The optimal binding of TNP-GTP to AnxVI was observed in the presence of Ca^{2+} and asolectin liposomes, as evidenced by a 5.5-fold increase of TNP-GTP fluorescence and a concomitant blue shift (by 17 nm) of its maximal emission wavelength. Titration of AnxVI with TNP-GTP resulted in the determination of the dissociation constant (K_d) and binding stoichiometry that amounted to 1.3 μM and 1:1 TNP-GTP/AnxVI, mole/mole, respectively. In addition, the intrinsic fluorescence of the membrane-bound form of AnxVI was quenched by TNP-GTP and this was accompanied by fluorescence resonance energy transfer (FRET) from AnxVI Trp residues to TNP-GTP. This indicates that the GTP-binding site within the AnxVI molecule is probably located in the vicinity of a Trp-containing domain of the protein. By controlled proteolysis of human recombinant AnxVI, followed by purification of the proteolytic fragments by affinity chromatography on GTP-agarose, we isolated a 35 kDa fragment corresponding to the N-terminal half of AnxVI containing Trp^{192}. On the basis of these results, we suggest that AnxVI is a GTP-binding protein and the binding of the nucleotide may have a regulatory impact on the interaction of annexin with membranes, e.g. formation of ion channels by the protein.

★Presented at the XXXVIIth Convention of the Polish Biochemical Society, Toruń, Poland, September, 10–14, 2001.

✪This work was supported by grant No. 6 P04A 039 21 from the State Committee for Scientific Research (KBN, Poland).

✉Tel.: (48 22) 659 8571 (ext. 347); fax: (48 22) 822 5342; e-mail: bandor@nencki.gov.pl

Abbreviations: Anx, annexin; CD, circular dichroism; EGF, epidermal growth factor; FRET, fluorescence resonance energy transfer; IPTG, isopropyl-β-D-thiogalactopyranoside; PVDF, polyvinylidenedifluoride; SV40, simian virus 40; TNP-GTP, 2′-(or 3′)-*O*-(2,4,6-trinitrophenyl)guanosine 5′-triphosphate.

Annexins form a family of Ca^{2+}- and phospholipid-binding proteins, represented in higher *Eucaryota* by over 200 species- and tissue-specific isoforms (Morgan *et al.,* 1999; Bandorowicz-Pikuła *et al.,* 2001). The eight-repeat domain AnxVI is the largest member of the family. As a result of alternative splicing, it is expressed in mammalian tissues in two isoforms (Moss & Crumpton, 1990; Kaetzel *et al.,* 1994) that differ by six amino-acid residues (Fig. 1). The crystal structure of the

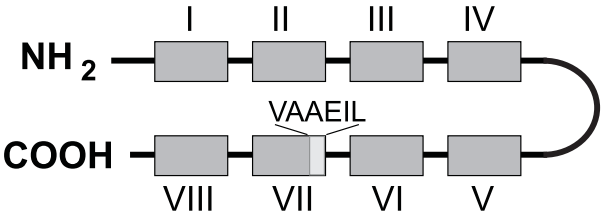

Figure 1. The domain structure of two isforms of AnxVI expressed in mammalian tissues.

The following regions can be recognized in the AnxVI molecule (on the basis of the crystal structure of bovine AnxVI): the N-terminal tail (residues 1–21), repeat domain I (residues 22–91), repeat domain II (residues 92–163), coil II–III (residues 164–172), repeat domain III (residues 173–250 with Trp^{192}), repeat domain IV (residues 251–325), linker region (residues 326–354 with Trp^{343}), coil preceding lobe B (residues 355–365), repeat domain V (residues 366–434), repeat domain VI (residues 435–506), coil VI–VII (residues 507–515), repeat domain VII (residues 516–599), and repeat domain VIII (residues 600–673). As a result of alternative splicing of AnxVI mRNA, the sequence VAAEIL is eliminated, giving rise to a shorter AnxVI isoform.

larger AnxVI isoform of human and bovine origin has been solved (Benz *et al.,* 1996; Avila-Sakar *et al.* 1998; 2000), however, the functional significance of this isoform remains unclear. It was reported that, in A431 cells expressing the larger isoform of AnxVI, EGF-mediated membrane hyperpolarization was attenuated and Ca^{2+} entry abolished (Fleet *et al.,* 1999). Moreover, some evidence has been provided that the lack of the six amino acid residue-long fragment in the shorter AnxVI isoform may have some implications for the folding of the C-terminal half of AnxVI, related to a different function of this isoform (Smith *et al.,* 1994).

From *in vitro* studies, AnxVI was implicated in Ca^{2+}-homeostasis (Hawkins *et al.,* 2000), in vesicular trafficking (Bandorowicz-Pikuła & Pikuła, 1998a), and in signal transduction pathways (Russo-Marie, 1999; Bandorowicz-Pikuła *et al.*, 2001). In all these processes, purine nucleotides are engaged. Membrane trafficking is a GTP-dependent process, and AnxVI has been found to interact with GTP-binding proteins, e.g., with dynamin (Turpin *et al.,* 1998), and with GTPase activating proteins, e.g., $p120^{GAP}$ (Davis *et al.,* 1996; Chow *et al.,* 2000). In addition, it has also been reported that AnxVI interacts with ATP-binding proteins, e.g., with F-actin (Jones *et al.,* 1992), and *in vitro* itself binds ATP (Bandorowicz-Pikuła & Awasthi, 1997; Bandorowicz-Pikuła *et al.,* 1997a; 1997b; 1999a; 1999b) or its analogues (Danieluk *et al.,* 1999a; 1999b). What could be the functional significance of AnxVI–nucleotide interactions?

Recently, two types of interaction of AnxVI with lipid membranes *in vitro* were observed. The first type was induced by acidification of the assay medium (Golczak *et al.,* 2001a), while the second one by the presence of purine triphosphonucleosides, ATP (Bandorowicz-Pikuła & Awasthi, 1997; Bandorowicz-Pikuła & Pikuła, 1998b; 1998c) or GTP (Kirilenko *et al.,* 2002). Both low pH and the presence of GTP in the millimolar concentration range induced voltage-dependent ion channels formed by AnxVI in lipid membranes that can potentially play a role in cellular ion homeostasis (Hawkins *et al.,* 2000; Kourie & Wood, 2000), especially under pathological conditions (Rand, 2000). However, the mechanisms of interaction of AnxVI with membrane lipid bilayers at low pH and in the presence of GTP are probably different. At low pH, AnxVI behaved like an integral membrane protein (Golczak *et al.,* 2001b; 2001c), and at pH 7.4, in the presence of the nucleotide, AnxVI

bound to the membrane as a peripheral protein (Kirilenko *et al.,* 2002). While the existence of membranous AnxVI in various cell types has been reported by other investigators (Giambanco *et al.,* 1993; Ishitsuka *et al.,* 1998; Turpin *et al.,* 1998; Babiychuk *et al.,* 1999; Babiychuk & Draeger, 2000; Ayala-Sanmartin, 2001; Orito *et al.,* 2001), GTP-induced interactions of AnxVI with membranes, leading to the activity of the protein as an ion channel, are still unclear. AnxVI was found secreted into the bile, although no molecular mechanism explaining how the soluble protein can cross the membrane was proposed (Thorin *et al.,* 1995). AnxVI was also reported to regulate the function of the ryanodine receptor in muscle cells. The results were suggestive of AnxVI being present in the lumen of the sarcoplasmic reticulum (Diaz-Munoz *et al.,* 1990). These experiments were, however, performed *in vitro* using the patch-clamp technique and a reconstituted system into which AnxVI was added from the side corresponding to the luminal surface of the sarcoplasmic reticulum membrane. No proof was provided that AnxVI is indeed present inside the sarcoplasmic reticulum compartment, however, it is a ubiquitous protein in skeletal and cardiac muscles (Kaetzel *et al.,* 1994; Luckcuck *et al.,* 1998; Benevolensky *et al.,* 2000). So far, AnxVI has been detected in the lumen of the endocytotic compartment, suggesting that AnxVI is able to cross the membrane of endocytes (Cuervo *et al.,* 2000). In addition, a KFERQ-like sequence was identified within the AnxVI molecule (Cuervo *et al.,* 2000). This sequence is defined as a targeting sequence for the chaperone-mediated lysosomal pathway of protein degradation that is activated in confluent cells in response to the removal of serum growth factors (Dice *et al.,* 1990). This suggests the existence of a mechanism allowing transport of the hydrophilic, at neutral pH, AnxVI through the hydrophobic region of the membrane bilayer.

Various cellular proteins are already known to transit between two major states: soluble in the cytosol and integral membrane protein. Among various examples are bacterial toxins (Prevost *et al.,* 2001) as well as prion proteins (Abdulla *et al.,* 2001; Prusiner, 2001). Protein transformation from the soluble to insoluble form is accompanied, in those cases, by a significant change of protein secondary structure, namely from an α-helix to a β-structure rich conformation (Lesieur *et al.,* 1999). For human AnxV and hydra AnxXII, a mechanism involving changes in protein hydrophobicity (Köhler *et al.,* 1997; Isas *et al.,* 2000; Sopkova-De Oliveira Santos *et al.,* 2000) and protein hexamerisation in response to acidification of the surrounding milieu was reported (Cartailler *et al.,* 2000; Isas *et al.,* 2000). In the case of AnxVI, we have already shown that the formation of low pH-induced channels by AnxVI is accompanied by changes in secondary and tertiary structure of the protein (Golczak *et al.,* 2001a, 2001b), as well as an increase of protein hydrophobicity (Golczak *et al.,* 2001c). In contrast, the GTP-induced ion channel activity of human recombinant AnxVI was not associated with penetration of a hydrophobic region of the membrane lipid bilayer by the protein (Kirilenko *et al.,* 2002).

The GTP-induced interaction of AnxVI with membranes and formation of GTP-dependent channels by the protein molecule in planar lipid membranes suggests that AnxVI may bind the nucleotide. Therefore, in this report we determined the GTP-binding properties of AnxVI (porcine liver isoform) using a fluorescent analog of GTP, TNP-GTP. In addition, we followed conformational changes of AnxVI induced by GTP by circular dichroism (CD) spectroscopy.

MATERIALS AND METHODS

Chemicals. TNP-GTP was purchased from Molecular Probes Inc. (Eugene, OR, U.S.A.). Purine nucleotides, asolectin (soybean lipids), GTP-agarose (3.0 μmoles GTP/ml packed gel) and V8 protease from *Staphylococcus aureus*

were obtained from Sigma-Aldrich (Poznań, Poland). Isopropyl-D-thiogalactopyranoside (IPTG) was provided by BIO 101, Inc. (Vista, CA, U.S.A.). All other chemicals were of the highest purity commercially available.

***Purification of AnxVI from porcine liver and expression of human recombinant AnxVI in* Escherichia coli.** AnxVI was purified to homogeneity from the fraction of calcium-precipitable proteins of porcine liver homogenate (containing in addition AnxIV, AnxII and other proteins as minor impurities), by means of EGTA extraction and ion exchange chromatography, essentially as described in Danieluk *et al.* (2001). The yield of purification was 0.9–1.1 mg protein/100 g tissue (wet mass), as observed earlier (Bandorowicz *et al.,* 1992). The protein was lyophilized and stored at –22°C until use. Its purity was ascertained by SDS/PAGE (Laemmli, 1970) and silver staining (Morrisey, 1981). The proteins were then electrotransferred onto nitrocellulose membranes (Towbin *et al.,* 1979) and identified using monoclonal antibodies against human AnxVI (Transduction Laboratories, Lexington, KY, U.S.A.) also recognizing porcine, rat and mouse isoforms. Human recombinant AnxVI was expressed in *Escherichia coli* strain B121(DE3) after induction with IPTG, and purified as previously described in detail (Golczak *et al.,* 2001c).

Steady-state fluorescence. Fluorescence measurements were performed on a Fluorolog 3 spectrophotometer (Jobin Yvon Spex, Edison, NJ, U.S.A.) with 3 nm slits for both excitation and emission at 25°C. The assay medium consisted of 50 mM Tris/HCl, pH 7.4, 50 mM NaCl, 0.5 mM EGTA or 2 mM Ca^{2+}, 0.25 mg/ml asolectin liposomes. Fluorescence titrations with TNP-GTP were monitored at λ_{ex} = 415 nm and λ_{em} = 545 nm and 550 nm. Fluorescence emission spectra were recorded in the wavelength range of 500–600 nm (λ_{ex} = 415 nm) for TNP-GTP or wavelength range of 305–580 nm (λ_{ex} = 295 nm) for the intrinsic porcine liver AnxVI fluorescence. All titration points were corrected for dilution (not exceeding 10%) and inner filter effects (Lakovicz, 1983). The specific fluorescence change (ΔF) of TNP-GTP in the presence of AnxVI was obtained by subtracting the nonspecific fluorescence measured in the presence of excess GTP (5 mM) from the total fluorescence, as described in Huang *et al.* (1998). To calculate K_ds, the ΔFs were plotted *versus* TNP-GTP concentrations, and the results were analysed using Eqn. 1: $\Delta F = \Delta F_{max} [U_0+D_0+K_d((U_0+D_0+K_d)^2-4U_0D_0)^{1/2}]/(2U_0)$, where U_0 is the concentration of AnxVI and D_0 is the concentration of TNP-GTP (Huang & Klingenberg, 1995). The binding stoichiometry TNP-GTP : AnxVI (n) was obtained from a mass action plot (Eqn. 2): $r/[L]_{free} = n/K_d - r/K_d$, where r is the concentration ratio of the bound ligand to the protein, and $[L]_{free}$ is the concentration of free ligand (Huang *et al.,* 1998).

Binding of proteolytic fragments of human recombinant AnxVI to GTP-agarose. The procedure in general followed that described for binding of AnxVI to ATP-agarose (Bandorowicz-Pikuła, 1998). Briefly, a GTP-agarose column (3 ml) was washed with 50 mM Tris/HCl, pH 7.4, 2 mM Ca^{2+}. In the meantime, 400 μg of AnxVI was treated with 0.4 μg of V8 protease form *S. aureus* at room temperature for 12 min in 50 mM Tris/HCl, pH 7.4, 2 mM Ca^{2+}. The reaction was stopped with the additions of protease inhibitors, phenylmethylsulfonyl fluoride and aprotinin, to final concentrations of 4 mM and 1000 K.I.U./ml, respectively. Annexin fragments were applied to the column by gravity, the column was washed once with 50 mM Tris/HCl, pH 7.4, 2 mM Ca^{2+} to remove non-specifically bound fragments. The annexin fragments that bound to the column were eluted with a 0–300 mM linear gradient of NaCl. The protein fragment that specifically bound to GTP-agarose eluted at 100–150 mM NaCl and was then subjected to SDS/PAGE, electroblotted onto PVDF membrane and sequenced. N-terminal amino-acid sequence analysis was

performed on a gas-phase sequencer (Model 491, Perkin Elmer-Applied Biosystems, Foster City, CA, U.S.A.) at the BioCenter, Jagiellonian University (Kraków, Poland). The phenylthiohydantoin derivatives were analysed by on-line gradient high performance liquid chromatography on a Microgradient Delivery System Model 140C equipped with a Programmable Absorbance Detector Model 785A (Perkin Elmer-Applied Biosystems, Foster City, CA, U.S.A.).

CD Spectra of porcine liver AnxVI. Far-UV CD spectra of AnxVI with and without GTP in the presence of Ca^{2+} and asolectin liposomes were measured at 25°C on an AVIV CD spectrophotometer (AVIV Associates Inc.) with a light source of 450 watts under an N_2 atmosphere. GTP was added to 100 μM. Protein concentration was 3 μM. The assay medium contained 25 mM Tris/HCl, pH 7.4, 0.25 mg/ml asolectin liposomes, 1 mM Ca^{2+} or 0.25 mM EGTA. The spectra were recorded in 0.2-cm quartz cuvettes. The bandwith was 1 nm and the integration time 1 s. Protein secondary structure was calculated using the Contin software, 1999 edition (Provencher & Glockner, 1981).

Other procedures. Protein concentrations were determined by the Bradford method (Bradford, 1976) with bovine serum albumin as a standard. Free calcium concentrations were calculated using the Chelator program (Schoenmakers *et al.,* 1992). SDS/PAGE under reducing conditions was performed on 5% stacking and 12% resolving gels, according to Laemmli (1970).

RESULTS AND DISCUSSION

GTP-binding properties of AnxVI purified from porcine liver

In our previous report we tested the effect of GTP on the human recombinant AnxVI/membrane interaction using planar lipid membranes made of asolectin. We found that milimolar GTP induced voltage-dependent ion channels formed by AnxVI molecules (at low nanomolar concentration) with a single ion channel conductance of 30–35 pS under symmetric conditions (i.e. 200 mM KCl in both chambers) (Kirilenko *et al.,* 2002). Such a value corresponds to the diameter of the hydrophilic pore within the protein molecule of approximately 8 Å, assuming the existence of a single pore. This is larger than predicted from an analysis of the structure of recombinant human AnxVI in a membrane bound form, described by Benz *et al.* (1996). Moreover, these authors suggested the presence of two hydrophilic pores within the AnxVI molecule. This points out to the possibility of various mechanisms of ion channel formation by AnxVI, depending on the conditions. Indeed, at low pH we observed a different interaction of AnxVI with membranes that resembled intrinsic membrane proteins rather than, electrostatic in nature, the surface interaction at pH 7.4 (Golczak *et al.,* 2001a). It has to be stressed that, under our experimental pH 7.4 conditions, the ion channels were induced independently of the fluctuations of Ca^{2+} concentration and that the presence of GTP was a prerequisite. Other nucleotides, such as GDP or cGMP, did not induce channel formation by AnxVI (Kirilenko *et al.,* 2002), suggesting specific interactions.

On the basis of these observations, we decided to test the hypothesis that AnxVI itself binds GTP. To verify this hypothesis we purified native AnxVI from porcine liver and used TNP-GTP as a fluorescent probe to characterize the GTP-binding site of AnxVI. Trinitrophenyl derivatives of nucleotides have succesfully been used to investigate the GTP/ATP-binding properties of various native and recombinant proteins including, for example, Na^+,K^+-ATPase (Moczydlowski & Fortes, 1981), cystic fibrosis transmembrane conductance regulator (Thomas *et al.,* 1991; Randak *et al.,* 1996), nucleotide-binding domain of P-glycoprotein (Baubichon-Cortay *et al.,* 1994), EGF receptor protein tyrosine

kinase (Cheng & Koland, 1996), SV40 T antigen (Huang & Klingenberg, 1995; Huang *et al.,* 1998), and many more. By using TNP-ATP we observed that AnxVI binds the nucleotide, and that the binding is enhanced in the presence of calcium (Bandorowicz-Pikuła & Awasthi, 1997; Bandorowicz-Pikuła *et al.,* 1997a). However, no physiological significance of the binding was proposed.

Considering the effect of calcium and the lipid-binding properties of AnxVI, we decided to test the TNP-GTP-binding properties of AnxVI under conditions promoting the binding of AnxVI to membranes (e.g., Ca^{2+} and asolectin liposomes) or retaining AnxVI in solution (EGTA, no liposomes). We used asolectin instead of phosphatidylserine-enriched liposomes, proven to specifically interact with AnxVI in a Ca^{2+} concentration-dependent manner (Bandorowicz *et al.,* 1992). Firstly, this matches the experimental conditions for previous ion conductance measurements (Golczak *et al.,* 2001a; 2001c; Kirilenko *et al.,* 2002) and secondly, this reduces the electrostatic interactions between AnxVI and liposomes in the presence of Ca^{2+}.

In the assay medium devoid of protein, TNP-GTP, in the micromolar concentration range, displayed little extrinsic fluorescence and the emission maximum was centered at 552 nm. All the constituents of the medium such as calcium ions in combination with asolectin liposomes did not significantly affect either the quantum field of the fluorescent dye or evoke a shift of the fluorescence emission maximum (not shown). The situation was different in the presence of AnxVI in the micromolar concentration range. Even in the absence of calcium there was an enhancement of TNP-GTP fluorescence. The effect was optimal in the presence of Ca^{2+} and asolectin liposomes (Fig. 2A) and this effect was taken as a measure of binding of TNP-GTP to AnxVI. Some binding was also seen in the absence of liposomes (Fig. 2B), but in the presence of Ca^{2+} and asolectin liposomes, a several fold enhancement of TNP-GTP fluorescence was accompanied by a 17-nm shift of the emission maximum (Table 1). Under other tested conditions the shift of the fluorescence maximum was much smaller, not exceeding 5 nm.

Figure 2C shows the results of fluorescence titration experiments in the presence of AnxVI concentration ranging from 1.0 to 2.75 μM, where the TNP-GTP emission fluorescence at λ_{em} = 545 nm (λ_{ex} = 415 nm) was measured. Such experiments allowed us to determine the maximal fluorescence enhancement (ΔF_{max}) and the dissociation constants (K_d) by fitting the experimental data using Eqn. 1 given in the Materials and Methods section. The specificity of the interaction between TNP-GTP and AnxVI were examined in an experiment designed to check the ability of GTP to displace TNP-GTP from its binding sites on the AnxVI molecule. It was found that GTP in the milimolar concentration range diminished the enhancement of TNP-GTP fluorescence in the presence of AnxVI in the medium containing Ca^{2+} and asolectin liposomes (Fig. 2D). This result can be interpreted as the competition of GTP with TNP-GTP for the same binding site in the AnxVI molecule. A similar behavior was observed for the inhibition of the interaction of methylanthryniloyl derivatives of ATP and GTP (Mant-nucleotides) with rat elongation factor eEF-2 by large excess of unmodified respective nucleotides (Gonzalo *et al.,* 2000). The binding of TNP-ATP and TNP-ADP to the C-terminal nucleotide-binding domain of P-glycoprotein was also effectively inhibited by ATP and ADP present in a 1000-times higher concentration than the TNP-nucleotides (Baubichon-Cortay *et al.,* 1994). In both above examples, the interpretation was the same as ours – competition between unmodified nucleotides and their derivatives for the same binding site.

Since we checked the specificity of the interaction, we were able to determine the binding parameters of TNP-GTP to AnxVI. In Fig. 3A, the ΔF_{max} and K_d values obtained as shown in Fig. 2C were plotted against AnxVI concentra-

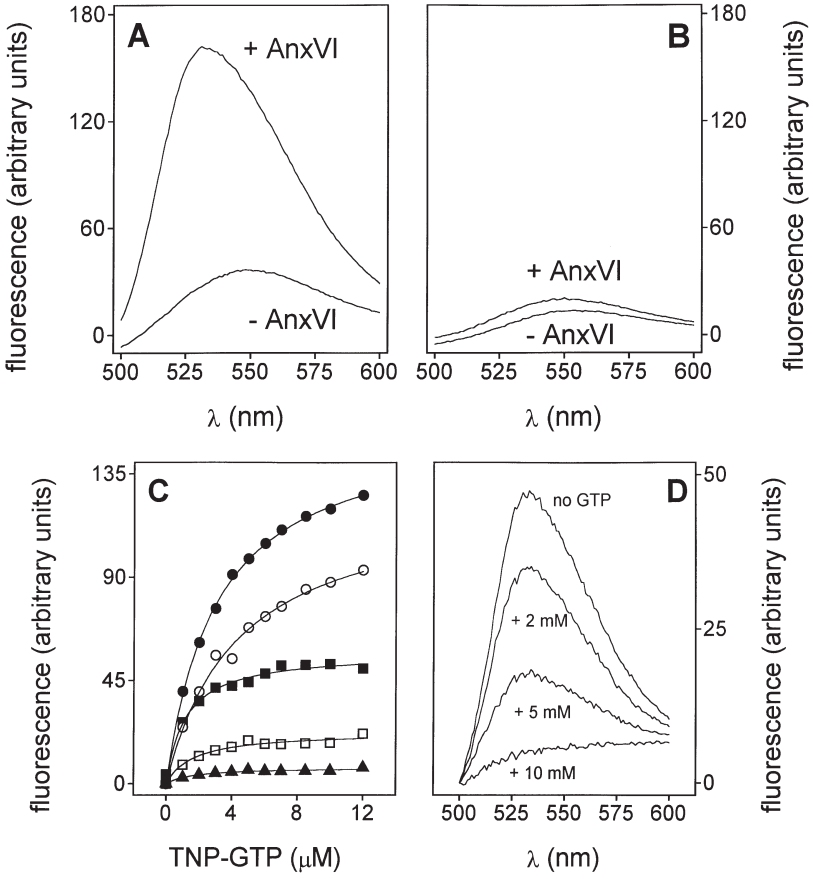

Figure 2. Binding of TNP-GTP to AnxVI as monitored by the enhancement of extrinsic fluorescence of the fluorescent dye in the presence of the protein.

(A) The fluorescence emission spectra of 5 μM TNP-GTP (λ_{ex} = 415 nm) measured in the absence (lower curve) or presence (upper curve) of 2 μM AnxVI. The assay medium was supplemented with 0.25 mg/ml of asolectin liposomes and 2 mM Ca^{2+}. Representative spectra are shown from at least 3 different experiments. **(B)** Same as panel A, except the measurements were performed in the presence of 2 mM Ca^{2+}, without asolectin liposomes. **(C)** Titration of TNP-GTP extrinsic fluorescence with various concentrations of AnxVI. The assay medium contained 25 mM Tris/HCl, pH 7.4, 50 mM NaCl, 2 mM Ca^{2+}, 0.25 mg/ml asolectin liposomes, TNP-GTP (0–12 μM) and AnxVI: 1.0 μM (▲) 1.5 μM (❐), 2.0 μM (■) 2.5 μM (○), and 2.75 μM (●). The specific fluorescence enhancement (ΔF) was calculated as the difference between the fluorescence emission intensity of TNP-GTP + AnxVI, measured in the absence and presence of 5 mM GTP. Mean values of three experiments are shown. They varied by 3–5%. **(D)** The effect of GTP on the binding of TNP-GTP to AnxVI. The fluorescence emission spectra of 5 μM TNP-GTP were recorded in the presence of 2 μM AnxVI, asolectin liposomes (0.25 mg/ml), 2 mM Ca^{2+} and, as indicated in the figure, concentrations of GTP ranging from 0 to 10 mM. Representative spectra from two experiments are displayed.

tions; all determinations were performed under optimal conditions, e.g., in the presence of Ca^{2+} and asolectin liposomes. ΔF_{max} values increased linearly with AnxVI concentration, while K_d values appeared to be independent of the AnxVI concentration. Therefore, by following the procedure described for the analysis of the binding of TNP-ATP and TNP-ADP with SV40 T antigen (Huang *et al.,* 1998), we were able to calculate the binding stoichiometry for AnxVI and TNP-GTP interactions. The stoichiometry was obtained by reploting the titration data from Fig. 2A in the form of a mass action plot (Fig. 3B), according to Eqn. 2 given in the Materials and Methods section. The data fit to a straight line (R = 0.99). On the basis of the plot depicted in Fig. 3B, we calculated the intercept value at the X-axis (Y = 0) that amounted to 1.05, indicating a binding stoichiometry (n) of 1 mole of TNP-GTP per 1

mole of AnxVI. The Hill coefficient of the binding amounted to 1, suggesting no cooperativity in TNP-GTP binding to AnxVI, as also revealed for TNP-ATP and porcine liver AnxVI (Bandorowicz-Pikula *et al.,* 1999a), and TNP-GTP and human AnxVI (Kirilenko *et al.,* 2002).

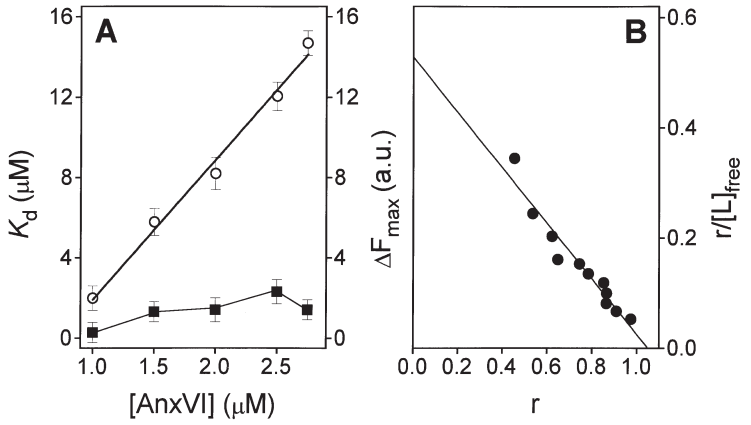

Figure 3. The binding parameters of TNP-GTP to AnxVI.

(**A**) Maximum fluorescence enhancement (ΔF_{max}) (○) and dissociation constant (K_d) (■) dependence on AnxVI concentration. Fluorescence titrations were performed in the presence of 1.0–2.75 μM AnxVI (as indicated on the abscissa), at pH 7.4 in the presence of 0.25 mg/ml of asolectin liposomes and 2 mM Ca^{2+}. Titration curves were fitted according to hyperbolic regression while the straight line is the least-square fit with R = 0.99. Mean values of three experiments, varying by 3–5%, are shown. (**B**) Mass action plot for experiments performed under conditions described in panel A, e.g. ΔF_{max} dependence was determined in the presence of 1.0–2.75 μM AnxVI. The straight line is the least-squares fit with K_d 1.9 μM (slope) and n 1.05 (intercept with the X-axis at Y = 0). Fluorescence was monitored at λ_{em} = 545 nm (λ_{ex} was 415 nm).

Localization of the GTP-binding domain in the AnxVI molecule

Using TNP-GTP as a fluorescent probe to determine the nucleotide-binding properties of AnxVI, we observed that the binding of TNP-GTP is accompanied by the quenching of the intrinsic fluorescence of the protein by the nucleotide, as also observed for the TNP-ATP and AnxVI interaction (Bandorowicz-Pikuła *et al.,* 1997a). Figure 4 shows the fluorescence emission spectra of AnxVI Trp residues (λ_{ex} = 295 nm) titrated with 0–15 μM TNP-GTP. In the absence of the nucleotide, the AnxVI spectrum exhibited an emission maximum at 339 nm. In the presence of raised concentrations of TNP-GTP, Trp fluorescence was significantly quenched (by more than 36%) due to FRET from Trp residues to bound TNP-GTP, as visible by a peak of fluorescence at 535 nm characteristic for TNP-GTP bound to the protein. This result supported the assumption that the GTP-binding site, observed in the presence of Ca^{2+} and asolectin liposomes, is localized in the vicinity of a Trp residue.

As revealed by the crystal structure of the AnxVI molecule, of the two Trp residues present in AnxVI, Trp^{192} is located in the repeat domain III of the protein, and Trp^{343} in the linker between two symmetric lobes (Benz *et al.,* 1996). With this in mind, we performed a test to locate the GTP-binding site by using controlled proteolysis of AnxVI with protease V8 and affinity chromatography of the obtained fragments on a GTP-agarose column. Since the complete primary sequence of porcine liver AnxVI is not known (Bandorowicz-Pikuła *et al.,* 1999a), we used for this purpose human recombinant AnxVI expressed in *E. coli*. We already knew that this recombinant protein is indstinguishable from the native protein with respect to nucleotide binding properties (Danieluk *et al.,* 2001; Kirilenko *et al.,* 2002) and ion channel activity at low pH

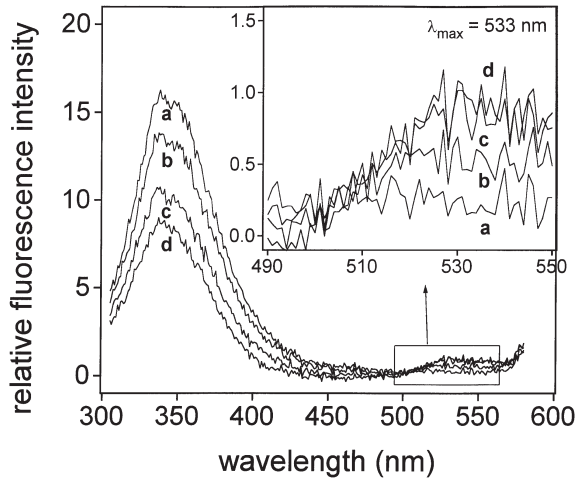

Figure 4. Quenching of intrinsic fluorescence of AnxVI with TNP-GTP.

The assay medium contained 25 mM Tris/HCl, pH 7.4, 50 mM NaCl, 2 mM Ca^{2+}, 0.25 mg/ml asolectin liposomes, 2.0 μM AnxVI and 0 (spectrum a), 5 μM (spectrum b), 10 μM (spectrum c) or 15 μM (spectrum d) TNP-GTP. Samples were excited at λ_{ex} = 295 nm. Representative differential spectra of typical experiments (after subtraction of buffer signal) are shown. Inset represents an enlargement of the main spectra showing significant fluorescence resonance energy transfer as visible from the increase in the intrinsic fluorescence intensity at 533 nm, characteristic for TNP-GTP in the form bound to AnxVI, with the isosbestic point at 500 nm.

(Golczak *et al.,* 2001c). By performing similar experiments to those described for the identification of porcine liver AnxVI proteolytic fragments that are able to bind to ATP-agarose and can be labelled with 8-azido[γ-^{32}P]ATP (Bandorowicz-Pikuła, 1998), we purified the N-terminal fragment of recombinant human AnxVI with an apparent molecular mass of 35 kDa, as identified by partial sequencing. Since this fragment contains Trp^{192}, we suggest that this residue is located in the vicinity of the nucleotide-binding domain of AnxVI. On the other hand, by using a different experimental approach, we were able to purify two different proteolytic fragments of AnxVI on an ATP-agarose column, of molecular masses 33.2 and 18.8 kDa. Both fragments were derived from the C-terminal part of the AnxVI molecule, as determined by partial sequencing and comparison with human isoform of the protein (Bandorowicz-Pikuła *et al.,* 1999a; 2001a). On the basis of these two experiments and the AnxVI/GTP binding stoichiometry, we suggest that the residues forming the GTP-binding domain of AnxVI are evenly distributed within both symmetrical halves of the protein. One has to remember that no sequence similarity to any known nucleotide-binding motif identified to date have been reported for AnxVI and other members of the annexin family of proteins (Bandorowicz-Pikuła *et al.,* 2001). In agreement with the observations presented in this report, other investigators have already observed that purine nucleotides affect the interaction of annexins with membranes, as for example the ion channel activity of AnxI was modulated by cAMP and ATP (Cohen *et al.,* 1995), ATP enhanced binding of AnxVI to hepatocyte plasma membranes (Tagoe *et al.,* 1994), and AnxVII, in the presence of Ca^{2+}/liposomes, exhibited a hydrolytic acitivity towards GTP (Caohuy *et al.,* 1996; Pollard *et al.,* 1998). The latter observation is consistent with the stimulatory effects of GTP on the ion channel activity of AnxVI (Kirilenko *et al.,* 2002). One may, therefore, speculate that GTP-AnxVI interactions occur in the protein domain that is also involved in AnxVI-membrane binding at neutral pH.

The effect of GTP binding on AnxVI secondary structure

Finally, we addressed the question of possible conformational changes occuring during nucleotide-binding to AnxVI. We already knew that the binding of one of the most important ligands for annexins, calcium ions, is not accompanied by a profound conformational transition, as revealed by the crystal structure of AnxVI (Benz *et al.,* 1996). On the other hand, we found that the low pH-induced ion channel formation by AnxVI paralleled changes in protein hydrophobicity in a spe-

Table 1. Characteristics of TNP-GTP binding to porcine liver AnxVI

Conditions of binding	Binding monitored by TNP-GTP fluorescence[a]			Binding monitored by Trp fluorescence quenching[b]
	λ_{em} (nm)[c]	increase in quantum yield[d]	K_d (μM)	maximal quenching (%)
– liposomes – Ca	550 ± 2	× 1.3	5.0 ± 1.2	2.0
– liposomes + Ca	548 ± 2	× 1.6	8.6 ± 2.8	5.0
+ liposomes – Ca	552 ± 2	× 1.5	2.0 ± 0.2	9.3
+ liposomes + Ca	533 ± 1	× 5.5	1.3 ± 0.4	36.6

[a]Fluorescence measurements were performed at the wavelength of maximum emission after excitation at 415 nm. [b]Fluorescence measurements were performed at 337 nm after excitation at 295 nm. [c]The maximum-emission wavelength of free TNP-GTP is 553 ± 1 nm. [d]Compared to the quantum yield of free TNP-GTP.

cific domain (Golczak *et al.,* 2001c), as well as large changes of secondary structure, characterized by a significant increase of α-helix content at the expense of β-structures (Golczak *et al.,* 2001a; 2001b). Conformational changes observed upon GTP binding to porcine liver AnxVI were measured using CD spectroscopy, and are summarized in Table 2. They revealed in the protein and a concomitant increase in the amount of turn-like structures. This may suggest that the initial binding of GTP to AnxVI, that is stimulated by Ca^{2+}, already occurs in solution, and then it may further modulate the interaction of AnxVI with membranes and perhaps the formation of GTP-induced ion channels by annexin in membranes.

Table 2. The effect of GTP on the secondary structure of porcine liver AnxVI

Additions	Regular α-helix	Distorted α-helix	Regular β-strands	Distorted β-strands	Turn-like structures	Unordered structures
Ca	59.6 ± 2.8	32.3 ± 2.5	0.4 ± 0.1	0.3 ± 0.1	7.3 ± 2.5	0
Ca + GTP	52.9 ± 2.1	29.3 ± 2.6	0.4 ± 0.1	0.4 ± 0.1	17.1 ± 1.2	0
Ca + liposomes	48.6 ± 2.1	25.8 ± 2.8	3.4 ± 1.5	0	20.1 ± 2.4	2.0 ± 0.9
Ca/liposomes/GTP	58.5 ± 2.3	22.4 ± 1.9	0	0	5.6 ± 0.9	14.6 ± 1.7

Calculations are based on far-UV CD spectra measured at 25°C. The assay medium contained 0.2 mg/ml of porcine liver AnxVI in 25 mM Tris/HCl, pH 7.4, 1 mM $CaCl_2$ and other additions as indicated in the Table, i.e. 0.1 mM GTP or 0.25 mg/ml of asolectin liposomes. The mean values ± S.D. from three determinations are shown and were calculated with the aid of the Contin software, 1999 version (Provencher & Glockner, 1981). The distorted and regular α- and β-structures are distinguished according to the definition given in the software description.

that the binding of AnxVI to asolectin liposomes in the presence of Ca^{2+} was accompanied by a moderate drop of α-helix content of the protein at the expense of turn-like structures. Surprisingly, addition of GTP under these conditions shifted the AnxVI secondary structure back to the form observed for the soluble protein in the presence of Ca^{2+} with no liposomes. On the other hand, addition of GTP in the absence of liposomes produced a smaller but significant drop of α-helix content Furthermore, by using Fourier-transform infrared spectroscopy and caged-ATP, we observed that, after UV photolysis of the caged compound to release the nucleotide, the binding of ATP to AnxVI in solution affected less than 10 amino-acid residues of the 673 residues of annexin (Bandorowicz-Pikuła *et al.,* 1999a; 2001) and the binding was not accompanied by profound changes in the secondary structure of the protein (Bandorowicz-Pikuła *et al.,* 1997a; 1999a). These results point to the

existence of at least three independent mechanisms of interaction of AnxVI with membranes. At neutral pH, the binding of AnxVI to phosphatidylserine-enriched membranes is stimulated by Ca^{2+} and is accomplished mostly by electrostatic interactions of AnxVI with membrane lipids, without major conformational alterations within the protein molecule (Benz *et al.,* 1996; Avila-Sakar *et al.,* 2000). By replacing phosphatidylserine with asolectin, the binding of AnxVI to membranes is stimulated with GTP and Ca^{2+}, and may result in the formation of ion channels by the annexin molecules (Kirilenko *et al.,* 2002). This type of interaction is accompanied by moderate changes in protein secondary structure (Table 2). The third type of interaction, Ca^{2+}-independent and induced at low pH, is accompanied by a profound change in protein secondary structure and a significant increase of protein hydrophobicity that accounts for the appearance of membrane forms of AnxVI that behave as true integral membrane proteins (Golczak *et al.,* 2001a; 2001c). Such isoforms of annexins have already been observed in nature by other investigators (Bianchi *et al.,* 1992; Turpin *et al.,* 1998; Babiychuk *et al.,* 1999; Lavialle *et al.,* 2000).

CONCLUDING REMARKS

In this report we provide experimental evidence that GTP binds to AnxVI with optimal efficiency in the presence of Ca^{2+} and asolectin liposomes. This is consistent with the formation of ion channels by AnxVI molecules that are induced at neutral pH in the presence of GTP (Kirilenko *et al.,* 2002). The interaction of GTP with AnxVI is already initiated in solution in the presence of Ca^{2+} and is associated with a moderate drop of α-helix content at the expense of turn-like structures. In this form, AnxVI may interact with membranes. Work is in progress in our laboratory to identify the nucleotide-binding domain within the AnxVI molecule, using recombinant peptides, point mutations and crystallographic approaches.

We thank Professor R. Huber from the Max-Planck-Institut für Biochemie (Martinsried, Germany) for providing cDNA for human AnxVI and Professor R. Donato from Universita di Perugia (Italy) for sending *E. coli* cells transformed with AnxVI cDNA. We would like to express our gratitude to Dr. Patrick Groves from the Nencki Institute of Experimental Biology (Warsaw, Poland) for fruitful discussion.

REFERENCES

Abdulla, Y.H. (2001) A plausible function of the prion protein: Conjectures and a hypothesis. *Bioessays* **23**, 456–462.

Avila-Sakar, A.J., Creutz, C.E. & Kretsinger, R.H. (1998) Crystal structure of bovine annexin VI in a calcium-bound state 1. *Biochim. Biophys. Acta* **1387**, 103–116.

Avila-Sakar, A.J., Kretsinger, R.H. & Creutz, C.E. (2000) Membrane-bound 3D structures reveal the intrinsic flexibility of annexin VI. *J. Struct. Biol.* **30**, 54–62.

Ayala-Sanmartin, J. (2001) Cholesterol enhances phospholipid binding and aggregation of annexins by their core domain. *Biochem. Biophys. Res. Commun.* **283**, 72–79.

Babiychuk, E.B. & Draeger, A. (2000) Annexins in cell membrane dynamics. Ca^{2+}-regulated association of lipid microdomains. *J. Cell Biol.* **150**, 1113–1124.

Babiychuk, E.B., Palstra, R.J., Schaller, J., Kampfer, U. & Draeger, A. (1999) Annexin VI participates in the formation of a reversible, membrane-cytoskeleton complex in smooth muscle cells. *J. Biol. Chem.* **274**, 35191–35195.

Bandorowicz, J., Pikuła, S. & Sobota, A. (1992) Annexins IV (p32) and VI (p68) interact with erythrocyte membranes in a calcium-dependent manner. *Biochim. Biophys. Acta* **1105**, 201–206.

Bandorowicz-Pikuła, J. (1998) A nucleotide-binding domain of porcine liver annexin VI. Proteolysis of annexin VI labeled with 8-azido-ATP, purification of proteolytic fragments by affinity chromatography on ATP-agarose and fluorescence studies. *Mol. Cell. Biochem*. **181**, 11–20.

Bandorowicz-Pikuła, J. & Awasthi, Y.C. (1997) Interaction of annexins IV and VI with ATP. An alternative mechanism by which a cellular function of these calcium- and membrane-binding proteins is regulated. *FEBS Lett*. **409**, 300–306.

Bandorowicz-Pikuła, J., Wrzosek, A., Pikuła, S. & Awasthi, Y.C. (1997a) Fluorescence spectroscopic studies on interactions between porcine liver annexin VI and nucleotides — a possible role for a tryptophan residue. *Eur. J. Biochem.* **248**, 238–244.

Bandorowicz-Pikuła, J., Wrzosek, A., Makowski, P. & Pikuła, S. (1997b) The relationship between the binding of ATP and calcium to annexin IV. Effect of nucleotide on the calcium-dependent interaction of annexin with phosphatidylserine. *Mol. Membr. Biol.* **14**, 179–186.

Bandorowicz-Pikuła, J. & Pikuła, S. (1998a) Annexins and ATP in membrane traffic: A comparison with membrane fusion machinery. *Acta Biochim. Polon*. **45**, 721–733.

Bandorowicz-Pikuła, J. & Pikuła, S. (1998b) Modulation of annexin VI-driven liposome aggregation by ATP. *Biochimie (Paris)* **80**, 613–620.

Bandorowicz-Pikuła, J. & Pikuła, S. (1998c) Adenosine 5′-triphosphate — a new regulator of annexin VI function. *Acta Biochim. Polon*. **45**, 735–744.

Bandorowicz-Pikuła, J., Wrzosek, A., Danieluk, M., Pikuła, S. & Buchet, R. (1999a) Changes in secondary structure of annexin VI upon ATP binding, as investigated by photochemical release of nucleotide, infrared difference, circular dichroism and fluorescence spectroscopies. *Biochem. Biophys. Res. Commun.* **263**, 775–779.

Bandorowicz-Pikuła, J., Danieluk, M., Wrzosek, A., Buś, R., Buchet, R. & Pikuła, S. (1999b) Annexin VI: An intracellular target for ATP. *Acta Biochim. Polon.* **46**, 801–812.

Bandorowicz-Pikuła, J., Buchet, R. & Pikuła, S. (2001) Annexins as nucleotide-binding proteins: Facts and speculations. *BioEssays* **23**, 170–178.

Baubichon-Cortay, H., Baggetto, L.G., Dayan, G. & Di Pietro, A. (1994) Overexpression and purification of the carboxyl-terminal nucleotide-binding domain from mouse P-glycoprotein. Strategic location of a tryptophan residue. *J. Biol. Chem.* **269**, 22983–22989.

Benevolensky, D., Belikova, Y., Mohammadzadeh, R., Trouve, P., Marotte, F., Russo-Marie, F., Samuel, J.L. & Charlemagne, D. (2000) Expression and localization of the annexins II, V, and VI in myocardium from patients with end-stage heart failure. *Lab. Invest.* **80**, 123–133.

Benz, J., Bergner, A., Hofmann, A., Demange, P., Gottig, P., Liemann, S., Huber, R. & Voges, D. (1996) The structure of recombinant human annexin VI in crystals and membrane-bound. *J. Mol. Biol.* **260,** 638–643.

Bianchi, R., Giambanco, I., Ceccarelli, P., Pula, G. & Donato, R. (1992) Membrane-bound annexin V isoforms (CaBP33 and CaBP37) and annexin VI in bovine tissues behave like integral membrane proteins. *FEBS Lett.* **296,** 158–162.

Bradford, M.M. (1976) Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.

Caohuy, H., Srivastava, M. & Pollard, H.B. (1996) Membrane fusion protein synexin (annexin VII) as a Ca^{2+}/GTP sensor in exocytotic secretion. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 10797–10802.

Cartailler, J.-P., Haigler, H.T. & Luecke, H. (2000) Annexin XII E105K crystal structure: Identification of pH-dependent switch for mutant hexamerization. *Biochemistry* **39**, 2475–2483.

Cheng, K. & Koland, J.G. (1996) Nucleotide-binding by the epidermal growth factor receptor protein-tyrosine kinase. Trinitro-

phenyl-ATP as a spectroscopic probe. *J. Biol. Chem.* **271**, 311–318.

Chow, A., Davis, A.J. & Gawler, D.J. (2000) Identification of a novel protein complex containing annexin VI, Fyn, Pyk2, and the $p120^{GAP}$ C2 domain. *FEBS Lett.* **469**, 88–92.

Cohen, B.E., Lee, G., Arispe, N. & Pollard, H.B. (1995) Cyclic 3′-5′-adenosine monophosphate binds to annexin I and regulates calcium-dependent membrane aggregation and ion channel activity. *FEBS Lett*. **377,** 444–450.

Cuervo, A.M., Gomes, A.V., Barnes, J.A. & Dice, J.F. (2000) Selective degradation of annexins by chaperone-mediated autophagy. *J. Biol. Chem.* **275**, 33329–33335.

Danieluk, M., Pikuła, S. & Bandorowicz-Pikuła, J. (1999a) Annexin VI interacts with adenine nucleotides and their analogs. *Biochimie (Paris)* **81**, 717–726.

Danieluk, M., Buś, R., Pikuła, S. & Bandorowicz-Pikuła, J. (1999b) Affinity labeling of annexin VI with a triazine dye, Cibacron blue 3GA. Probable interaction of the dye with C-terminal nucleotide-binding site within the annexin molecule. *Acta Biochim. Polon.* **46**, 419–429.

Danieluk, M., Golczak, M., Pikuła, S. & Bandorowicz-Pikuła, J. (2001) UDP hydrolase activity associated with the porcine liver annexin fraction. *Biochim. Biophys. Acta* **1526**, 70–76.

Davis, A.J., Butt, J.T., Walker, J.H., Moss, S.E. & Gawler, D.J. (1996) The Ca^{2+}-dependent lipid binding domain of $P120^{GAP}$ mediates protein-protein interactions with Ca^{2+}-dependent membrane-binding proteins. Evidence for a direct interaction between annexin VI and $P120^{GAP}$. *J. Biol. Chem.* **271,** 24333–24336.

Diaz-Munoz, M., Hamilton, S.L., Kaetzel, M.A., Hazarika, P. & Dedman, J.R. (1990) Modulation of Ca^{2+} release channel activity from sarcoplasmic reticulum by annexin VI (67-kDa calcimedin). *J. Biol. Chem.* **265**, 15894–15899.

Dice, J.F. (1990) Peptide sequences that target cytosolic proteins for lysosomal proteolysis. *Trends Biochem. Sci.* **15**, 305–309.

Fleet, A., Ashworth, R., Kubista, H., Edwards, H., Bolsover, S., Mobbs, P. & Moss, S.E. (1999) Inhibition of EGF-dependent calcium influx by annexin VI is splice form-specific. *Biochem. Biophys. Res. Commun.* **260**, 540–546.

Giambanco, I., Verzini, M. & Donato, R. (1993) Annexins V and VI in rat tissues during post-natal development: Immunochemical measurements. *Biochem. Biophys. Res. Commun.* **196**, 1221–1226.

Golczak, M., Kicińska, A., Bandorowicz-Pikuła, J., Buchet, R., Szewczyk, A. & Pikuła, S. (2001a) Acidic pH-induced folding of annexin VI is a prerequisite for its insertion into lipid bilayers and formation of ion channels by the protein molecules. *FASEB J.* **15**, 1083–1085.

Golczak, M., Kirilenko, A., Bandorowicz-Pikuła, J. & Pikuła, S. (2001b) Conformational states of annexin VI in solution induced by acidic pH. *FEBS Lett.* **496**, 49–54.

Golczak, M., Kirilenko, A., Bandorowicz-Pikuła, J. & Pikuła, S. (2001c) N- and C-terminal halves of human annexin VI differ in ability to form low pH-induced ion channels. *Biochem. Biophys. Res. Commun.* **284**, 785–791.

Gonzalo, P., Sontag, B., Lavergne, J.-P., Jault, J.-M. & Reboud, J.-P. (2000) Evidence for a second nucleotide binding site in rat elongation factor eEF-2 specific for adenylic nucleotides. *Biochemistry* **39**, 13558–13564.

Hawkins, T.E., Merrifield, C.J. & Moss, S.E. (2000) Calcium signaling and annexins. *Cell Biochem. Biophys.* **33**, 275–296.

Huang, S.G. & Klingenberg, M. (1995) Fluorescent nucleotide derivatives as specific probes for the uncoupling protein: Thermodynamics and kinetics of binding and the control by pH. *Biochemistry* **34,** 349–360.

Huang, S.G., Weisshart, K. & Fanning, E. (1998) Characterization of the nucleotide binding properties of SV40 T antigen using fluorescent 3′(2′)-*O*-(2,4,6-trinitrophenyl)adenine nucleotide analogues. *Biochemistry* **37**, 15336–15344.

Isas, J.M., Cartailler, J.P., Sokolov, Y., Patel, D.R., Langen, R., Luecke, H., Hall, J.E. & Haigler, H.T. (2000) Annexins V and XII insert into bilayers at mildly acidic pH and form ion channels. *Biochemistry* **39**, 3015–3022.

Ishitsuka, R., Kojima, K., Utsumi, H., Ogawa, H. & Matsumoto, I. (1998) Glycosaminoglycan binding properties of annexin IV, V, and VI. *J. Biol. Chem.* **273**, 9935–9941.

Jones, P.G., Moore, G.J. & Waisman, D.M. (1992) A nonapeptide to the putative F-actin binding site of annexin-II tetramer inhibits its calcium-dependent activation of actin filament bundling. *J. Biol. Chem.* **267**, 13993–13997.

Kaetzel, M.A., Pula, G., Campos, B., Uhrin, P., Horseman, N. & Dedman, J.R. (1994) Annexin VI isoforms are differentially expressed in mammalian tissues. *Biochim. Biophys. Acta* **1223**, 368–374.

Kirilenko, A., Golczak, M., Pikuła, S., Buchet, R. & Bandorowicz-Pikuła, J. (2002) GTP-induced membrane binding and ion channel activity of annexin VI. Is annexin VI a GTP biosensor? *Biophys. J.*, in press.

Kourie, J.I. & Wood, H.B. (2000) Biophysical and molecular properties of annexin-formed channels. *Prog. Biophys. Mol. Biol.* **73**, 91–134.

Köhler, G., Hering, U., Zschörnig, O. & Arnold, K. (1997) Annexin V interaction with phosphatidylserine-containing vesicles at low and neutral pH. *Biochemistry* **36**, 8189–8194.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* **227**, 680–685.

Lakovicz, J.R. (1983) *Principles of Fluorescence Spectroscopy.* Plenum, New York.

Lavialle, F., Rainteau, D., Massey-Harroche, D. & Metz, F. (2000) Establishment of plasma membrane polarity in mammary epithelial cells correlates with changes in prolactin trafficking and in annexin VI recruitment to membranes *Biochim. Biophys. Acta* **1464,** 83–94.

Lesieur, C., Frutiger, S., Hughes, G., Kellner, R., Pattus, F. & van der Goot, F.G. (1999) Increased stability upon heptamerization of the pore-forming toxin aerolysin. *J. Biol. Chem.* **274**, 36722–36728.

Luckcuck, T., Trotter, P.J. & Walker, J.H. (1998) Localization of annexin VI in the adult and neonatal heart. *Cell Biol. Int.* **22**, 199–205.

Moczydlowski, E.G. & Fortes, P.A.G. (1981) Characterization of 2′,3′-*O*-(2,4,6-trinitrocyclohexadienylidine)adenosine 5′-triphosphate as a fluorescent probe of the ATP side of sodium and potassium transport adenosine triphosphatase. Determination of nucleotide binding stoichiometry and ion-induced changes in affinity for ATP. *J. Biol. Chem.* **256**, 2346–2356.

Morgan, R.O., Jenkins, N.A., Gilbert, D.J., Copeland, N.G., Balsara, B.R., Testa, J.R. & Fernandez, M.P. (1999) Novel human and mouse annexin A10 are linked to the genome duplications during early chordate evolution. *Genomics* **60**, 40–49.

Morrisey, J.H. (1981) Silver staining for protein in polyacrylamide gels: A modified procedure with enhanced uniform sensitivity. *Anal. Biochem.* **117,** 307–310.

Moss, S.E. & Crumpton, M.J. (1990) Alternative splicing gives rise to two forms of the p68 Ca^{2+}-binding protein. *FEBS Lett.* **261**, 299–302.

Orito, A., Kumanogoh, H., Yasaka, K., Sokawa, J., Hidaka, H., Sokawa, Y. & Maekawa, S. (2001) Calcium-dependent association of annexin VI, protein kinase C , and neurocalcin on the raft fraction derived from the synaptic plasma membrane of rat brain. *J. Neurosci. Res.* **64**, 235–241.

Pollard, H.B., Caohuy, H., Minton, A.P. & Srivastava, M. (1998) Synexin (annexin VII) hypothesis for Ca^{2+}/GTP-regulated exocytosis. *Adv. Pharmacol.* **42,** 81–87.

Prevost, G., Mourey, L., Colin, D.A. & Menestrina, G. (2001) Staphylococcal pore-forming toxins. *Curr. Top. Microbiol. Immunol.* **257**, 53–83.

Provencher, S.W. & Glockner, J. (1981) Estimation of globular protein secondary structure from circular dichroism. *Biochemistry* **20**, 33–37.

Prusiner, S.B. (2001) Shattuck lecture – neurodegenerative diseases and prions. *N. Eng. J. Med.* **344**, 1516–1526.

Randak, C., Neth, P., Auerswald, E.A., Assfalg-Machleidt, I., Rosher, A.A., Hadorn, H.-B. & Machleidt, W. (1996) A recombinant polypeptide model of the second predicted nucleotide-binding fold of the cystic fibrosis transmembrane conductance regulator is a GTP-binding protein. *FEBS Lett.* **398,** 97–100.

Russo-Marie, F. (1999) Annexin V and phospholipid metabolism. *Clin. Chem. Lab. Med.* **37**, 287–291.

Rand, J.H. (2000) The annexinopathies: A new category of diseases. *Biochim. Biophys. Acta* **1498**, 169–173.

Schoenmakers, T.J.M., Visser, G.J., Flik, G. & Theuvenet, A.P.R. (1992) CHELATOR: An improved method for computing metal ion concentrations in physiological solutions. *BioTechniques* **12**, 870–874.

Smith, P.D., Davies, A.A., Crumpton, M.J. & Moss, S.E. (1994) Structure of the human annexin VI gene. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 2713–2717.

Sopkova-De Oliveira Santos, J., Fischer, S., Guilbert, C., Lewit-Bentley, A. & Smith, J.C. (2000) Pathway for large-scale conformational change in annexin V. *Biochemistry* **39**, 14065–14074.

Tagoe, C.E., Boustead, C.M., Higgins, S.J. & Walker, J.H. (1994) Characterization and immunolocalization of rat liver annexin VI. *Biochim. Biophys. Acta* **1192,** 272–280.

Thomas, P.J., Shenbagamurthi, P., Ysern, X. & Pedersen, P.L. (1991) Cystic fibrosis transmembrane conductance regulator: Nucleotide binding to a synthetic peptide. *Science* **251**, 555–557.

Thorin, B., Gache, G., Dubois, T., Grataroli, R., Domingo, N., Russo-Marie, F. & Lafont, H. (1995) Annexin VI is secreted in human bile. *Biochem. Biophys. Res. Commun.* **209**, 1039–1045.

Towbin, H., Staehelin, T. & Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354.

Turpin, E., Russo-Marie, F., Dubois, T., de Paillerets, C., Alfsen, A. & Bomsel, M. (1998) In adrenocortical tissue, annexins II and VI are attached to clathrin coated vesicles in a calcium-independent manner. *Biochim. Biophys. Acta* **1402**, 115–130.