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QUARTERLY

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*°

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Annexin VI (AnxVI) from porcine liver, a member of the annexin family of Ca^{2+}- and membrane-binding proteins, has been shown to bind ATP *in vitro* with a K_d in the low micromolar concentration range. However, this protein does not contain within its primary structure any ATP-binding consensus motifs found in other nucleotide-binding proteins. In addition, binding of ATP to AnxVI resulted in modulation of AnxVI function, which was accompanied by changes in AnxVI affinity to Ca^{2+} in the presence of ATP. Using limited proteolytic digestion, purification of protein fragments by affinity chromatography on ATP-agarose, and direct sequencing, the ATP-binding site of AnxVI was located in a C-terminal half of the AnxVI molecule. To further study AnxVI-nucleotide interaction we have employed a functional nucleotide analog, Cibacron blue 3GA (CB3GA), a triazine dye which is commonly used to purify multiple ATP-binding proteins and has been described to modulate their activities. We have observed that AnxVI binds to CB3GA immobilized on agarose in a Ca^{2+}-dependent manner. Binding is reversed by EGTA and by ATP and, to a lower extent, by other adenine nucleotides. CB3GA binds to AnxVI also in solution, evoking reversible aggregation of protein molecules, which resembles self-association of AnxVI molecules either in solution or on a membrane surface. Our observations support earlier findings that AnxVI is an ATP-binding protein.

Annexin VI (Anx VI) with apparent molecular mass of 68 kDa is expressed at high levels in mammalian organs such as liver [1] and pancreas [2] where it exhibits specific polar-

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Abbreviations: Anx, annexin; B_{max}, maximal binding; CB3GA, Cibacron blue 3GA; IC_{50}, concentration of an effector for half-maximal inhibition of an effect; $K_{1/2}$, free ligand concentration for half-maximal response; SDS/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis.

ized subcellular localization. In hepatocytes, AnxVI is concentrated on some apical vesicles around the bile canaliculi [2]. Such localization suggests that expression of AnxVI is finely regulated (for review see [3, 4]), and that AnxVI may play a physiological role in vesicular transport in epithelial cells [2, 5]. These findings are in agreement with the suggested role of annexins in exo- and endocytosis (for review see [6]). For example, AnxVI is required for the budding of clathrin-coated pits from human fibroblast plasma membrane. It is thought that AnxVI activates a cysteine protease, disconnecting the clathrin lattice from the spectrin membrane cytoskeleton during the final stages of budding [7]. Moreover, AnxXIIIb was found to associate with lipid microdomains to function in apical transport from the trans-Golgi network in epithelial kidney cells [8] and the AnxII/p11 complex was postulated to participate in regulation of exocytosis in the bovine pulmonary artery epithelial cells [9].

Membrane traffic processes, with their complex machinery including multiple cytosolic and membrane proteins, are also regulated by ATP, GTP, and various nucleotide-binding proteins [6]. We have recently provided evidence that AnxVI of porcine liver binds ATP *in vitro*. This could be demonstrated using different experimental approaches, including photolabeling of the protein with 8-azido-[γ-^{32}P]ATP [10], binding of a fluorescent ATP analog [10–12], or affinity chromatography on ATP-agarose [10, 13]. The specific binding of nucleotide to AnxVI was accompanied by changes in functional characteristics of the protein, including binding to liposomes and biological membranes [10], interaction with cytoskeletal elements [10], and AnxVI-driven aggregation of liposomes [14]. The observed alterations in functional properties of AnxVI were accompanied by changes in the conformation of the protein [11], resulting in lower affinity of the protein for Ca^{2+}. These observations prompted some investigators to speculate about the possible implications of the annexin-nucleotide interaction *in vivo* [15, 16]. It should be noted, however, that the primary structure of AnxVI has no sequence homology with that of other ATP-binding proteins [13, 17]. This phenomenon was also reported for other annexins revealing nucleotide-binding properties, AnxI [18, 19], AnxVII [15, 20], and AnxIV [12]. Except perhaps in the case of AnxVII [15], the observations about annexin-nucleotide interactions have not been substantiated *in vivo*, although the *in vitro* binding suggests that ATP is a functional ligand for some members of the annexin family.

In the present study we have identified an ATP-binding site of AnxVI within the C-terminal half of the AnxVI molecule. Therefore, we have applied Cibacron blue 3GA (CB3GA), a functional, but not structural nucleotide analog (Fig. 1), which has been successfully used for precipitation and purification by affinity chromatography of various nucleotide-binding proteins [21, 22]. The CB3GA-protein interaction was found to be promoted by metal ions [23]. We have found that AnxVI interacts in a calcium-dependent manner with Cibacron blue 3GA immobilized on agarose, and this interaction is reversed by EGTA, ATP and, to a lower extent, by ADP. In solution, CB3GA promoted aggregation of AnxVI molecules. We conclude that the interaction of AnxVI with CB3GA in the presence of Ca^{2+} may involve a mechanism similar to self-association of annexin molecules reported to occur either on membrane surfaces [24–26] or in solution [27, 28]. Moreover, our results provide further evidence that AnxVI does indeed belong to a superfamily of intracellular ATP-binding proteins.

MATERIALS AND METHODS

Chemicals. V8 protease from *Staphylococcus aureus*, ATP-agarose (3.0 μmoles ATP/ml packed gel), adenine nucleotides and glutaraldehyde were purchased from Sigma (U.S.A.).

Cibacron blue 3GA (other commercial names: reactive blue 2, Procion blue H-B) was purchased from Fluka Chemie AG (Switzerland), and Cibacron blue 3GA immobilized on 4% beaded agarose (at 2.6 mg/ml packed resin), from Sigma (U.S.A.). All other chemicals were of the highest purity commercially available.

Purification of AnxVI from porcine liver. AnxVI was purified to apparent homogeneity from porcine liver homogenate, as described earlier [1], with the yield of about 1 mg protein/100 g tissue (wet mass). Dialyzed protein preparations were stored at -80°C at a protein concentration of 0.3-0.7 mg/ml in 20 mM Tris/HCl, pH 7.4, 100 mM NaCl, and 5 mM EGTA. The protein was thawed and concentrated to 1.2-1.8 mg/ml by centrifugation in Centriprep 10 (Millipore, Austria).

Preparation of proteolytic fragments of AnxVI. AnxVI (98 μg) was digested with V8 protease (14 μg) for 5 min at room temperature in 70 μl of 50 mM Tris/HCl, pH 7.4, 0.5 mM EGTA. Then the reaction mixture was supplemented with 2.0 mM $CaCl_2$ and 2 mM phenylmethylsulfonyl fluoride, and was incubated for 60 min with 30 μl of packed ATP-agarose resin. Fragments of AnxVI which did not bind to the resin were washed out with 50 mM Tris/HCl, pH 7.4, 2 mM $CaCl_2$, while proteolytic fragments attached to the resin were eluted with 70 μl of 50 mM Tris/HCl, pH 7.4, 10 mM EGTA, then separated by SDS/PAGE, and electroblotted to Immobilon-P (PVDF) transfer membrane (0.45 μm pore size) (Millipore, U.S.A.). Direct sequencing was performed in the Protein Chemistry Laboratory, University of Texas Medical Branch at Galveston (TX, U.S.A.).

Binding of AnxVI to CB3GA-agarose. AnxVI at various protein concentrations was incubated with 5 μl of packed CB3GA-agarose (corresponding to 15 nmoles of CB3GA) for 60 min at room temperature in 50 μl of 50 mM Tris/HCl, pH 7.4, 100 mM NaCl (Tris/NaCl buffer). Ca^{2+} at various concentrations and other additions were made as indicated in legends to figures. Before use, the resin was washed twice with 1 ml of Tris/NaCl buffer without Ca^{2+}. After incubation, the resin with the attached protein was centrifuged for 2 min at 2000 × *g* and the pellet was washed twice in the presence of Ca^{2+} to remove non-specifically bound AnxVI. To dissociate AnxVI from CB3GA-agarose, the resin was treated for 30 min with 25 μl of 10 mM EGTA, and/or adenine nucleotides and centrifuged again. Finally, the resin was boiled for 10 min in the presence of 25 μl SDS/PAGE sample buffer containing 10 mM EGTA. The protein pattern of supernatants and pellet was examined by SDS/PAGE on a 12% running gel and 5% stacking gel, according to Laemmli [29]. Gels were stained with Coomassie brilliant blue and converted to computer images with the aid of a Molecular Dynamics densitometer (Japan). The obtained images were analyzed using the Image Quant program, version 3.3. The samples incubated without the resin or with agarose without covalently linked CB3GA served as controls.

Aggregation of AnxVI in the presence of CB3GA in solution. In addition to CB3GA, the assay medium contained Tris/NaCl buffer, Ca/EGTA, and the protein at various concentrations. Other additions are indicated in the text. Aliquots were incubated for 30 min at room temperature and centrifuged for 10 min at 6500 × *g*. Both the supernatant and pellet protein fractions were examined by SDS/PAGE and the resulting gels were analyzed by densitometry.

Determination of visible difference spectra of CB3GA in the presence of AnxVI. The visible difference spectra at increasing concentrations of CB3GA (119, 178 and 238 μM) in the absence or presence of 1.2 μM AnxVI were recorded in a buffer containing 50 mM Tris/HCl, pH 7.4, 100 mM NaCl and 20 mM $CaCl_2$, using a UV-160A UV-visible recording spectrophotometer (Shimadzu, Japan). Measurements were performed in 1-ml quartz cuvettes containing the same concentrations of CB3GA in 50 mM Tris/HCl, pH

7.4, 100 mM NaCl, 20 mM $CaCl_2$, and no protein. The appearance of a maximum at 686 nm and a minimum at 616 nm in the visible difference spectra at increasing concentrations of CB3GA recorded in the presence of a fixed concentration of AnxVI were taken as a measure of formation of the dye–protein complexes, as it has been described for yeast hexokinase [30].

Other procedures. The protein concentration was determined according to Bradford [31], with bovine serum albumin as a standard. Ca^{2+} concentrations were calculated using the CHELATOR program [32].

RESULTS

Localization of a nucleotide-binding site within the AnxVI molecule

The ability of AnxVI to bind adenine nucleotides *in vitro* led us to the conclusion that a nucleotide-binding domain does exist and within the protein molecule is probably located in its C-terminal half [13]. This assumption is based on the differences in the nucleotide binding capacity between homologous annexins from porcine liver, AnxVI and AnxIV [10, 12]. Indirect evidence in favor of this localization was also provided by experiments in which a fluorescence energy transfer from tryptophan residue located in the linker region within porcine AnxVI molecule and trinitrophenyl-ATP bound to AnxVI, has been observed [11]. This tryptophan residue corresponds to a Trp-343 residue in human AnxVI [33, 34]. To confirm this localization we subjected AnxVI to a limited proteolytic digestion with V8 protease in the presence of EGTA. Then, the proteolytic fragments which retained the ability to bind to ATP-agarose in the presence of $CaCl_2$ (see the Materials and Methods section), were purified by affinity chromatography. The N-terminus of the major fragment with molecular mass of about 35 kDa revealed the amino-acid sequence: LSAVARVELK..., which was found to be identical with the amino-acid sequence of the C-terminal half of human AnxVI starting from Leu-345 in the linker region [33, 34] between the Ca^{2+}- and phospholipid-binding domains IV and V (Fig. 2). This localization is based on the crystal structure of recombinant human [35] and bovine [36] AnxVI. Previously, we have found that the 35-kDa proteolytic fragment retains covalently attached 8-azido-[γ-^{32}P]ATP (see Fig. 2A in Ref. 13). The described localization of a nucleotide-binding domain of AnxVI suggests the possibility of a fluorescence energy transfer from Trp-343 to trinitrophenyl-ATP bound to AnxVI [11], and indicates that the two symmetrical lobes of AnxVI (lobes A and B) [35] have different binding properties for nucleotides.

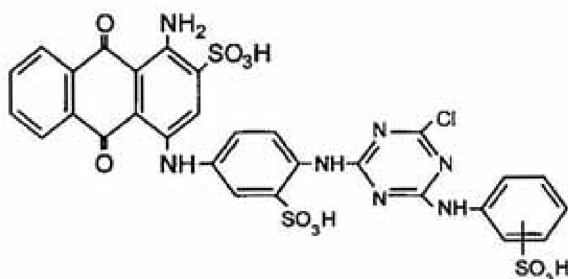

Figure 1. Chemical formula of a functional analog of ATP, Cibacron blue 3GA.

Binding of AnxVI to CB3GA immobilized on beaded agarose

To further examine nucleotide-binding properties of AnxVI we have applied a functional analog of nucleotides, CB3GA. This compound, cross-linked to agarose, has proven useful in the purification of multiple ATP-binding proteins. To examine whether AnxVI binds specifically to CB3GA-agarose we employed the experimental assay system used by us previously to study the binding of AnxVI to ATP-agarose [10, 13]. We found that AnxVI bound to CB3GA-agarose in a Ca^{2+}-dependent manner with a $K_{1/2}$ for Ca^{2+} of 81 μM. The

binding was non-covalent, as it was reversible by addition of EGTA (Fig. 3A inset). It is worth noting, however, that even extraction with EGTA did not result in complete dissociation of AnxVI from the resin, suggesting that the mechanism of interaction of AnxVI with CB3GA requires Ca^{2+} initially, but then becomes relatively Ca^{2+}-independent. Therefore, the ionic detergent SDS was used to completely solubilize the protein. Moreover, under conditions described in Fig. 3, AnxVI did not bind to agarose which had no covalently attached ligand. The binding of AnxVI to CB3GA was also protein-concentration dependent and occurred with half-maximal efficiency at 1.9 μM AnxVI, at fixed Ca^{2+} and CB3GA concentrations (Fig. 3A). Analysis of the data presented in Fig. 3A using the Hill equation gave a Hill coefficient (η_H) value below 1.0, maximal binding (B_{max}) of 12.9 pmoles of AnxVI per nmol of CB3GA at 2 mM Ca^{2+}, indicative of negative cooperativity.

To examine the specificity of the binding we have used various adenine nucleotides to detach AnxVI from the resin. The most efficient was ATP (IC_{50} 0.55 mM), then ADP with a 3-fold higher IC_{50} value, and AMP which was more than 10 times less effective than ATP in solubilizing AnxVI from the resin. The addition of Mg^{2+} did not intensify the inhibitory

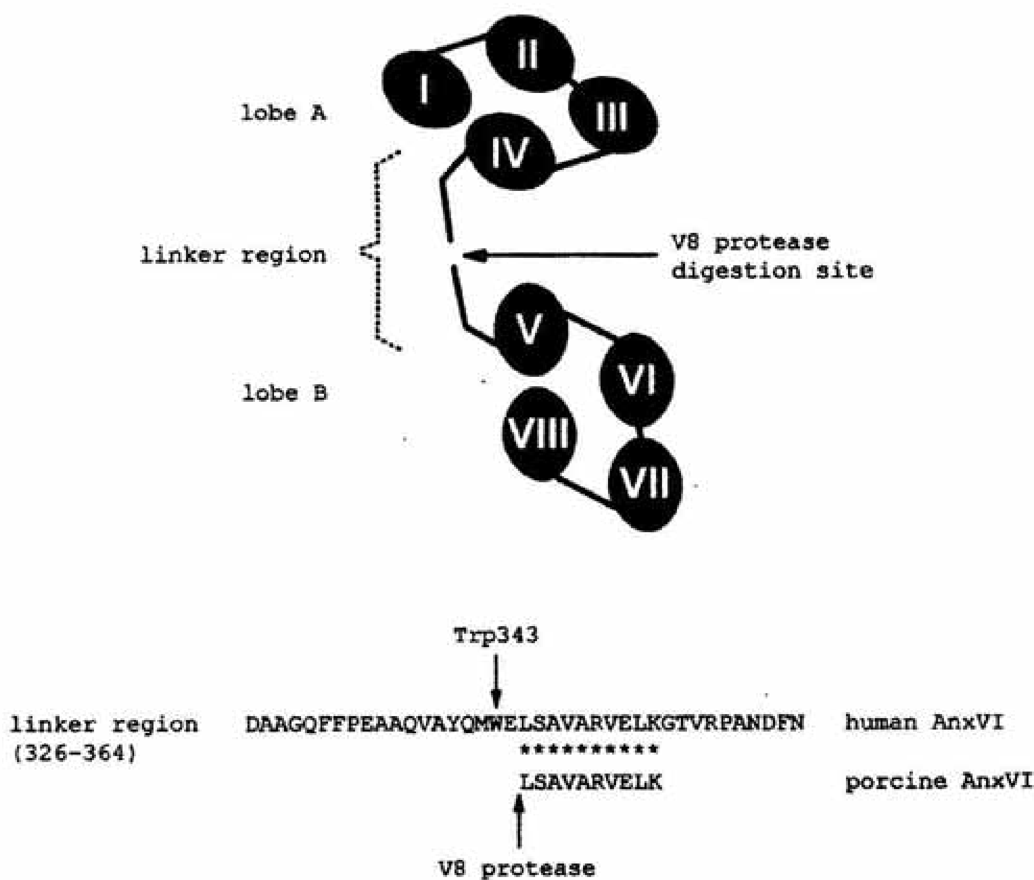

Figure 2. The localization of a nucleotide-binding domain within the AnxVI molecule, digested with V8 protease.

The amino-acid sequence of the porcine AnxVI fragment is shown under the amino-acid sequence of the linker region of human AnxVI [33, 34]. Asterisks denote identical amino-acid residues. Roman numbers stand for the AnxVI Ca^{2+}- and phospholipid-binding domains. The position of Trp343 residue is depicted on the basis of crystal structure of human [35] and bovine [36] AnxVI.

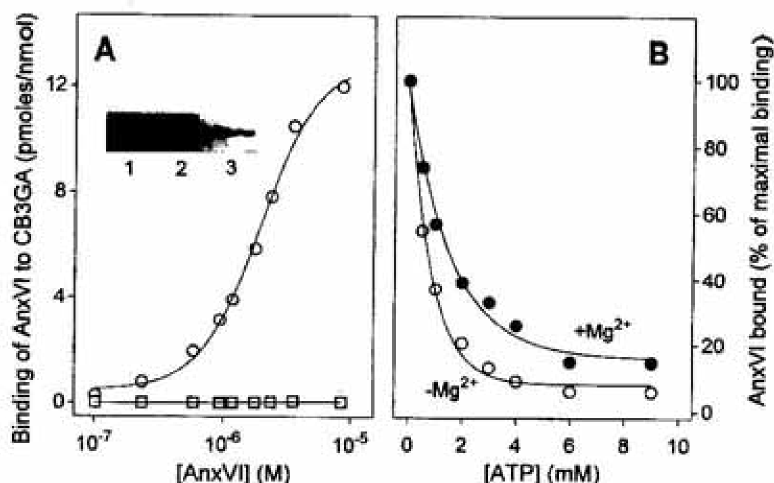

Figure 3. Binding of AnxVI to CB3GA-agarose.

(A) AnxVI at concentration range from 10^{-7} to 10^{-5} M was preincubated with 15 nmoles of CB3GA-agarose in the presence of 2 mM Ca^{2+} (○) or 1 mM EGTA (□) in 50 μl of Tris/NaCl buffer. Inset, AnxVI (180 pmoles) was incubated with CB3GA-agarose (15 nmoles of CB3GA) in the presence of 2 mM Ca^{2+}. The sample was centrifuged and the protein pattern of the supernatant (lane 1), and pellet after extraction with 10 mM EGTA (lane 2), was examined by SDS/PAGE. In addition, CB3GA-agarose was boiled in the presence of SDS/PAGE sample buffer (lane 3). (B) AnxVI (180 pmoles) was incubated with 15 nmoles of CB3GA-agarose in the presence of 2 mM Ca^{2+}. The protein bound to the resin was extracted from agarose with rising concentrations of ATP, in the absence (○) or presence (●) of 10 mM $MgCl_2$. The experiments shown in panels A and B were repeated three times each and mean values are presented. Binding varied by less than 10%.

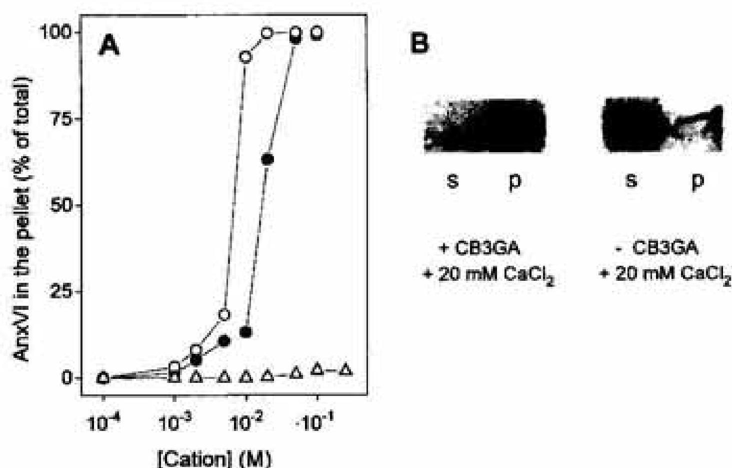

Figure 4. Interaction of AnxVI with CB3GA in solution.

(A) The effect of divalent and monovalent cations. AnxVI (0.25 mg protein/ml) was preincubated with CB3GA (0.2 mg/ml) in the presence of rising concentrations of K^{+} (Δ), Ca^{2+} (○) or Mg^{2+} (●). Mean values, which varied by 6–8%, are shown. Protein was precipitated by centrifugation at 6500 × *g* for 10 min, subjected to SDS/PAGE and quantified by densitometry. AnxVI incubated with CB3GA in the presence of 10 mM EGTA revealed no aggregation. The amount of AnxVI in the pellet after incubation of the protein with both CB3GA and 20 mM $CaCl_2$ was taken as 100%. (B) AnxVI aggregation in the presence of 20 mM $CaCl_2$ requires the presence of CB3GA. AnxVI (180 pmoles) was preincubated in 50 μl of Tris/NaCl buffer and 20 mM $CaCl_2$, with or without CB3GA (15 nmoles). Lane "s" represents the protein pattern of the supernatant and lane "p" of the pellet. The experiments shown in panels A and B were performed in duplicate.

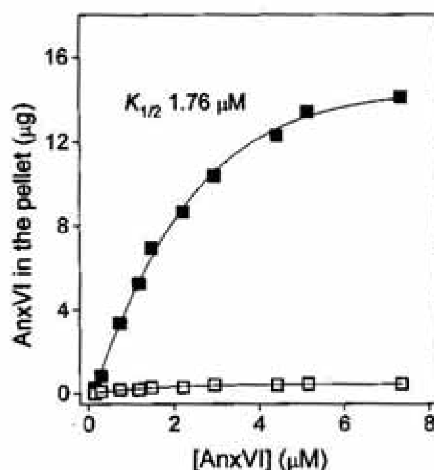

Figure 5. The effect of CB3GA on self-association of AnxVI molecules in the presence of Ca^{2+}.

AnxVI in the concentration range indicated on the abscissa was incubated with 0.3 mM CB3GA (■) or without the triazine dye (□) in 50 μl of Tris/NaCl buffer and 20 mM $CaCl_2$. Samples were centrifuged for 10 min at 6500 × *g* and the protein pattern of pellets was examined by SDS/PAGE and densitometry. Experiments were performed in duplicate. Mean values, which varied by 6–8%, are shown.

effect of ATP on binding of AnxVI to CB3GA-agarose (Fig. 3B). The effect of ATP on detachment of AnxVI from CB3GA-agarose could be, at least partially, ascribed to the ability of the nucleotide to chelate Ca^{2+}.

Interaction of AnxVI molecules with CB3GA in solution in the presence of Ca^{2+}

The effects described in the preceding paragraph prompted us to examine the binding of CB3GA to AnxVI also in solution, with the reasoning on the assumption that binding of multiple triazine dye molecules per one AnxVI molecule might change the solubility of the protein. Indeed, we have observed that CB3GA precipitates AnxVI from solution in a Ca^{2+}-dependent manner (Fig. 4A), with a $K_{1/2}$ for Ca^{2+} of 6.0 mM. Other divalent cations could partially substitute for Ca^{2+}, as did Mg^{2+} ($K_{1/2}$ of 22.3 mM), but monovalent cations, Na^+ or K^+, could not (Fig. 4A). The precipitates observed after low speed centrifugation (see the Materials and Methods section) were formed exclusively in the presence of CB3GA and relatively high Ca^{2+} concentrations. Omitting either Ca^{2+} or the triazine dye from the assay medium resulted in no precipitation of AnxVI (Fig. 4B). The precipitation was reversed by EGTA and by ATP (IC_{50} 0.5 mM) and we suggest that this process reflects a direct interaction of CB3GA with AnxVI saturated with Ca^{2+}. This was confirmed by determining the visible spectra of CB3GA in the presence of AnxVI, which revealed formation of the dye-AnxVI complexes (see the Materials and Methods section). High Ca^{2+} concentrations are known to evoke significant conformational changes in Ca^{2+}-binding loops of AnxVI, as revealed by analysis of the crystal structure of the protein [35, 36], and as was also observed for AnxV [37]. Therefore, it is possible that the conformational rearrangement of AnxVI at high Ca^{2+} concentration and upon CB3GA binding may be responsible for aggregation of the protein, as was shown for AnxXIII which assembles to trimers upon Ca^{2+} binding and may form heterotrimers with other members of the annexin family [38].

The precipitation of AnxVI by CB3GA was dye-concentration dependent ($K_{1/2}$ of 16 μM CB3GA at 3.7 μM AnxVI in the presence of 20 mM $CaCl_2$), as well as protein concentration-dependent ($K_{1/2}$ of 1.8 μM AnxVI in the presence of 0.3 mM CB3GA and 20 mM $CaCl_2$; Fig. 5). The formation of AnxVI precipitates in the presence of CB3GA and at high Ca^{2+} concentrations reflected, in our opinion, formation of AnxVI multimers, since annexins are able to interact with each other, undergoing self-association when bound to membrane phospholipids [24–26] or in solution [27, 28]. By using glutaraldehyde as a cross-linker to study formation of AnxVI multimers we have found on SDS/PAGE that cross-linking of AnxVI molecules (as measured by disappearance of AnxVI monomer from the solution) is enhanced by Ca^{2+} with a $K_{1/2}$ for the cation of

2.9 mM. Supplementation of the medium with CB3GA at a dye/AnxVI molar ratio of 100:1 decreased the $K_{1/2}$ for Ca^{2+} to 0.6 mM. On the basis of these observations it can be concluded that CB3GA does indeed enhance the formation of AnxVI multimers in solution. This is consistent with the view that the nucleotide analog stimulates self-association of AnxVI molecules.

DISCUSSION

In the present report we took advantage of the ability of CB3GA to precipitate nucleotide-binding proteins in a cation-dependent manner [23]. This binding is accompanied by inhibition of their activity, as it has been shown, for example, for mitochondrial adenine nucleotide translocase [39] and yeast hexokinase [30]. For the first time we have shown that AnxVI of porcine liver of 68 kDa binds to CB3GA immobilized on beaded agarose in a Ca^{2+}-dependent manner. The specificity of binding is determined by Ca^{2+} since other cations are less effective in promoting binding, and it is also evident from the fact that homologous AnxIV of 32 kDa does not bind to CB3GA under the same conditions as described for AnxVI (not shown). The different behavior of the two related proteins may be explained by the difference in affinity of AnxVI [10, 11] and AnxIV [12] for ATP and its analogs. In fact, the affinity of AnxIV for ATP is one order of magnitude lower than that of AnxVI [12]. The difference between these two homologous proteins is also confirmed by the localization of the nucleotide-binding domain within the C-terminal half of AnxVI (Fig. 2).

The calcium dependence of binding of AnxVI to CB3GA-agarose may reflect the existence of a relationship between Ca^{2+}-binding sites and the nucleotide-binding domain within the AnxVI molecule [13], as also postulated for AnxI [19] and AnxVII [15]. Indeed, we have found that binding of various ATP analogs, such as 8-azido-ATP or trinitrophenyl-ATP, is dependent on Ca^{2+} concentrations. Binding of hydrophilic 8-azido-ATP is optimal in the absence of Ca^{2+} while the binding of more hydrophobic trinitrophenyl-ATP is stimulated by submillimolar concentrations of Ca^{2+} [10]. Moreover, by using various functional assays we have found that ATP, when bound to AnxVI, modulates the affinity of the protein for Ca^{2+} [10, 14].

The affinity of AnxVI for CB3GA-agarose ($K_{1/2}$ of 81 μM) compared to ATP-agarose ($K_{1/2}$ of 0.6 μM [10]) is by two orders of magnitude lower. This may suggest a somehow different mechanism of interaction. Indeed, CB3GA, when bound to AnxVI in solution, precipitates the protein saturated with Ca^{2+}. This was not observed with ATP as a ligand. Precipitation of AnxVI by CB3GA was due to the stimulation of formation of AnxVI multimers by the triazine dye, which seems analogous to the self-association of annexin molecules in solution also promoted by millimolar concentrations of Ca^{2+} [27, 28]. One important point of our investigation is the question whether ATP prevents the binding of CB3GA to AnxVI. In both types of binding experiments, with CB3GA-agarose and with the soluble form of the triazine dye, binding of AnxVI was prevented by ATP at similar concentrations, i.e. with IC_{50} of 0.5–0.6 mM. This value was, however, by two orders of magnitude higher than that found in competition experiments for 8-azido-ATP labeling or trinitrophenyl-ATP binding to AnxVI [10].

In conclusion, the observations described in the present report are consistent with the mechanism of CB3GA action relying on stimulation of the specific interaction of AnxVI molecules with each other in the presence of Ca^{2+}. This effect seems to be of particular importance in taking advantage of CB3GA as an affinity chromatography ligand for purification of annexins, as well as for studying self-association of annexin molecules, the process postulated to play a role in the interaction of annexin with biological

membranes [3, 4, 40]. We are also planning to examine the effect of CB3GA on annexin activities, since CB3GA was found to be a potent effector acting on numerous biological activities, e.g. being an antagonist of purinergic P2 receptors [41] or counteracting the ATP-mediated increase of Ca^{2+} current in rabbit renal proximal tubules [42].

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REFERENCES

1. Bandorowicz, J., Pikuła, S. & Sobota, A. (1992) Annexins IV (p32) and VI (p68) interact with erythrocyte membrane in a calcium-dependent manner. *Biochim. Biophys. Acta* **1105**, 101–106.
2. Massey-Harroche, D., Mayran, N. & Maroux, S. (1998) Polarized localizations of annexins I, II, VI and XIII in epithelial cells of intestinal, hepatic, and pancreatic tissues. *J. Cell. Sci.* **111**, 3007–3015.
3. Raynal, P. & Pollard, H.B. (1994) Annexins: The problem of assessing the biological role for a gene family of multifunctional calcium- and phospholipid-binding proteins. *Biochim. Biophys. Acta* **1197**, 63–93.
4. Gerke, V. & Moss, S.E. (1997) Annexins and membrane dynamics. *Biochim. Biophys. Acta* **1357**, 129–154.
5. Pikuła, S. & Bandorowicz-Pikuła, J. (1996) Bile flow, vesicular trafficking, and interactions of cytoskeleton with the canalicular domain of hepatocyte plasma membrane. *Cell. Mol. Biol. Lett.* **1**, 119–128.
6. Bandorowicz-Pikuła, J. & Pikuła, S. (1998) Annexins and ATP in membrane traffic: A comparison with membrane fusion machinery. *Acta Biochim. Polon.* **45**, 721–733.
7. Kamal, A., Ying, Y.S. & Anderson, R.G.W. (1998) Annexin VI-mediated loss of spectrin during coated pit budding is coupled to delivery of LDL to lysosomes. *J. Cell Biol.* **142**, 937–947.
8. Lafont, F., Lecat, S., Verkade, P. & Simons, K. (1998) Annexin XIIIb associates with lipid microdomains to function in apical delivery. *J. Cell Biol.* **142**, 1413–1427.
9. Konig, J., Prenen, J., Nilius, B. & Gerke, V. (1998) The annexin II-p11 complex is involved in regulated exocytosis in bovine pulmonary endothelial cells. *J. Biol. Chem.* **273**, 19679–19684.
10. 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.
11. Bandorowicz-Pikuła, J., Wrzosek, A., Pikuła, S. & Awasthi, Y.C. (1997) 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.
12. Bandorowicz-Pikuła, J., Wrzosek, A., Makowski, P. & Pikuła, S. (1997) 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.

13. 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.

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

15. 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.

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

17. Szewczyk, A. & Pikuła, S. (1998) ATP – an intracellular metabolic messenger. *Biochim. Biophys. Acta* **1365**, 333-353.

18. 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.

19. Han, H.-Y., Lee, Y.-H., Oh, J.-Y., Na, D.-S. & Lee B.-J. (1998) NMR analyses of the interactions of human annexin I with ATP, Ca^{2+}, and Mg^{2+}. *FEBS Lett.* **425**, 523-527.

20. 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.

21. Gupta, M.N., Kaul, R., Guoqiang, D., Dissing, U. & Mattiasson, B. (1996) Affinity precipitation of proteins. *J. Mol. Recognit.* **9**, 356-359.

22. Worall, D.M. (1996) Dye-ligand affinity chromatography. *Methods Mol. Biol.* **59**, 169-176.

23. Guoqiang, D., Benhura, M.A., Kaul, R. & Mattiasson, B. (1995) Affinity thermoprecipitation of yeast alcohol dehydrogenase through metal ion-promoted binding with Eudragit-bound Cibacron blue 3GA. *Biotechnol. Prog.* **11**, 187-193.

24. Zaks, W.J. & Creutz, C.E. (1991) Ca(2+)-dependent annexin self-association on membrane surfaces. *Biochemistry* **30**, 9607-9615.

25. Concha, N.O., Head, J.F., Kaetzel, M.A., Dedman, J.R. & Seaton, B.A. (1992) Annexin V forms calcium-dependent trimeric units on phospholipid vesicles. *FEBS Lett.* **314**, 159-162.

26. Evans, T.C., Jr. & Nelsestuen, G.L. (1994) Calcium and membrane binding properties of monomeric and multimeric annexin II. *Biochemistry* **33**, 13231-13238.

27. Alvarez-Martinez, M.T., Porte, F., Liautard, J.P. & Sri Widada, J. (1997) Effects of profilin-annexin I association on some properties of both profilin and annexin I: Modification of the inhibitory activity of profilin on actin polymerization and inhibition of the self-association of annexin and its interaction with liposomes. *Biochim. Biophys. Acta* **1339**, 331-334.

28. Mailliard, W.S., Luecke, H. & Haigler, H.T. (1997) Annexin XII forms calcium-dependent multimers in solution and on phospholipid bilayers: A chemical cross-linking study. *Biochemistry* **36**, 9045-9050.

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

30. Puri, R.N. & Roskoski, R., Jr. (1994) Inactivation of yeast hexokinase by Cibacron blue 3GA: Spectral, kinetic and structural investigations. *Biochem. J.* **300**, 91-97.

31. Bradford, M.M. (1976) Rapid and sensitive method for quantition of microgram quantities of protein utilizing the principle of

protein-dye binding. *Anal. Biochem.* **72,** 248-254.

32. 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.

33. Crompton, M.R., Owens, R.J., Totty, N.F., Moss, S.E., Waterfield, M.D. & Crumpton, M.J. (1988) Primary structure of the human, membrane-associated Ca^{2+}-binding protein p68: A novel member of a protein family. *EMBO J.* **7,** 21-27.

34. Südhof, T.C., Slaughter, C.A., Leznicki, I., Barjon, P. & Reynolds, G.A. (1988) Human 67-kDa calelectrin contains a duplication of four repeats found in 35-kDa lipocortins. *Proc. Natl. Acad. Sci. U.S.A.* **85,** 664-668.

35. 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.

36. 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.

37. Sopkova, J., Vincent, M., Takahashi, M., Lewit-Bentley, A. & Gallay, J. (1998) Conformational flexibility of domain II of annexin V studied by fluorescence of tryptophan 187 and circular dichroism: The effect of pH. *Biochemistry* **37,** 11962-11970.

38. Langen, R., Isas, J.M., Luecke, H., Haigler, H.T. & Hubbell, W.L. (1998) Membrane-mediated assembly of annexins studied by site-directed spin labeling. *J. Biol. Chem.* **273,** 22453-22457.

39. Powers, M.F., Smith, L.L. & Beavis, A.D. (1994) On the relationship between the mitochondrial inner membrane anion channel and the adenine nucleotide translocase. *J. Biol. Chem.* **269,** 10614-10620.

40. Kaetzel, M.A. & Dedman, J.R. (1995) Annexins: Novel Ca^{2+}-dependent regulators of membrane function. *News Physiol. Sci.* **10,** 171-176.

41. Simon, J., Webb, T.E., King, B.F., Burnstock, G. & Barnard, E.A. (1998) Characterisation of a recombinant P2Y purinoreceptor. *Eur. J. Pharmacol.* **291,** 281-289.

42. Yamada, H., Seki, G., Taniguchi, S., Uwatoko, S., Suzuki, K. & Kurokawa, K. (1996) Mechanism of $[Ca^{2+}]_i$ increase by extracellular ATP in isolated rabbit renal proximal tubules. *Am. J. Physiol.* **270,** C1096-C1104.