Regulation of Calcyclin (S100A6) Binding by Alternative Splicing in the N-terminal Regulatory Domain of Annexin XI Isoforms*

(Received for publication, July 3, 1997, and in revised form, December 3, 1997)

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Annexin XI is a Ca\(^{2+}\)/phospholipid-binding protein that interacts with a member of S100 protein family, calcyclin (S100A6), in a Ca\(^{2+}\)-dependent manner. There are two isoforms of annexin XI, annexin XI-A and -B, generated by alternative splicing in the N-terminal regulatory domain. To determine the role of the alternative splicing region in the calcyclin-binding, we identified and characterized its calcyclin binding site. Experiments with glutathione S-transferase fusion proteins with N-terminal sites of annexin XI-A showed the calcyclin binding site to be in residues Gln\(^{19}\)-Thr\(^{62}\) of rabbit annexin XI-A, which contains part of the splicing region. A synthesized peptide corresponding to Tyr\(^{43}\)-Thr\(^{62}\) of annexin XI-A inhibited the interaction of annexin XI with calcyclin in liposome co-pelleting assay. The calcyclin binding site possesses a hydrophobic residue cluster conserved among S100 binding sites of annexin I and II. Recombinant annexin XI isoforms were expressed in Sf9 cells using a baculovirus expression system. In contrast to annexin XI-A, it was found that annexin XI-B protein could not bind to calcyclin by the liposome co-pelleting assay. In Sf9 cells coexpressing calcyclin with annexin XI isoforms, the calcyclin binding was observed only for annexin XI-A isofrom. These results indicate that the calcyclin binding ability of annexin XI is an annexin XI-A isoform-specific character, suggesting that annexin XI isoforms might play distinct roles in cells through each alternative splicing regions.

Annexins are Ca\(^{2+}\)/phospholipid-binding proteins that are present in a wide variety of cells and tissues. They form a structurally related family, all members consisting of a C-terminal core domain with either four or eight repeating units of about 70 amino acids and an individually unique N-terminal tail domain. Due to the Ca\(^{2+}\)/phospholipid binding ability through the core domain, it has been shown that annexins can bind to biomembranes in a Ca\(^{2+}\)-dependent manner and aggregate and fuse them (1–4). Therefore, it has been suggested that annexins participate in a number of cellular functions including exocytosis, endocytosis, blood coagulation, regulation of phospholipase A2 activity, cell proliferation, and inflammatory responses (5). However, the exact functions of individual annexin forms remain to be determined.

To elucidate the physiological functions of annexins, their binding proteins have been investigated. It has been established, for example, that synapsin I and calsectpin bind to annexin VI, whereas plasminogen and tissue plasminogen activator interact with annexin II (6). Among the various binding proteins, three members of the S100 protein family, calgizzarin (S100C), p11 (S100A10), and calcyclin (S100A6) specifically interact with annexin I, II, and XI, respectively (7–9). The S100 protein family are Ca\(^{2+}\)-binding proteins with 2 EF-hand Ca\(^{2+}\) binding structures. Since p11 forms heterotetramers with annexin II and affects its subcellular localization and affinity for Ca\(^{2+}\) (10–12), annexin-S100 protein interactions have been suggested to play important roles in intracellular functions of annexin family members. However, compared with annexin II, there is less accumulation of biochemical and biophysical information for the interaction of S100 proteins with annexin I and XI.

We discovered annexin XI as a calcyclin-associated protein (CAP-50) in rabbit lung (9, 13) and subsequently demonstrated that it is widely distributed in various tissues including the lung, heart, aorta, testis, and liver (14). Annexin XI is localized mainly in the nucleus and membranes of SY1 and COS-7 cells, whereas it is predominantly found in the cytoplasm of primary cultured hepatocytes (14–16). The nuclear localization of annexin XI is cell type-specific and developmental-dependent (17). Since its binding protein calcyclin is overexpressed in tumor cells with the mRNA levels specifically increased in the G\(_1\) phase of the cell cycle when stimulated by serum, platelet-derived growth factor, or epidermal growth factor (18, 19), it is possible that the annexin XI-calcyclin complex may play a role in cell proliferation and division.

The N-terminal domains of the annexin family are generally considered as regulatory sites, since they contain sequences responsible for phosphorylation, proteolysis, and interaction with other proteins including S100 proteins described above. Annexin XI has the longest N-terminal domain, composed of 197 amino acids, of all known annexins (20). There are two isoforms, annexin XI-A and annexin XI-B, generated by alternative splicing in the N-terminal regulatory domain of identically primary transcripts (21). The primary isoform of annexin XI has been named annexin XI-A, and the new isoform is termed annexin XI-B (20–23). We previously reported that the region encompassing residues Tyr\(^{37}\)-Leu\(^{44}\) of the N-terminal regulatory domain of rabbit annexin XI-A is critical for its calcyclin interaction based on a truncated mutant study (24). Although the exact binding site was not defined, the critical region is located in the alternative splicing region of annexin XI isoforms. In addition, the N-terminal domain of annexin XI is essential for its nuclear localization (16). Therefore, it is important to determine the exact nature of the calcyclin binding site.

In the present study, the calcyclin binding site of annexin XI-A was identified and shown to possess a hydrophobic residue cluster conserved among S100 binding sites of annexins.

* This work was supported in part by Grants-in-aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan 06044105, 06040109, and 06507001. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ”advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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This paper is available on line at http://www.jbc.org
We also demonstrated that the calcyclin binding of annexin XI was an annexin XI-A isoform-specific feature, and annexin XI-B had no binding ability. These findings suggest that the alternative splicing-derived annexin XI isoforms play distinct roles in cell function.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases and DNA-modifying enzymes were from Takara Shuzo, Boehringer Mannheim Biochemistry, New England Biolabs, and Fermentas MBi. Spodoptera frugiperda SF9 cells, baculovirus transfer vector pVL1392, pBlueBacIII, baculovirus NPV DNA, and Grace’s insect cell medium were cultured from Invitrogen. Baculovirus transfer vector pFastBac1 and DH10Bac competent cells were obtained from Life Technologies, Inc. All other materials and reagents were of the highest quality available from commercial suppliers.

Construction of Annexin XI and Calcyclin Recombinant Baculovirus—A rabbit annexin XI-A cDNA sequence, present in pBlueBacIII was amplified by the polymerase chain reaction (PCR) with Pfu DNA polymerase and purified after cutting with BamHI and PstI. The PCR product was verified by DNA sequencing. The employed oligonucleotides corresponding to bases 20–39 (with the initiation site) and the antisense to bases 1641–1661 (with sequence 3’-AAGGATTCTTGCGGCCTTTTCCTGT-5’), respectively. The sequences of the oligonucleotides used for PCR were as follows: 5’-AAGGATCCCATGAGCTACCCTGGCTAT-3’ and 5’-AATC-GCAGATTCTGGCGGCCATTTGGTG-3’. The purified PCR product was introduced into the BamHI/PstI-digested pBlueBacIII plasmid. Recombinant baculovirus of rabbit annexin XI-A was prepared with the pBlueBacIII rabbit annexin XI-A as described previously (25). For cloning of rabbit calcyclin cDNA into the pFastBac donor plasmid, the coding region of the rabbit calcyclin in the pGEX-3X vector (26) was subcloned into the BamHI/EcoRI sites of the pFastBac donor plasmid. pFastBac rabbit calcyclin was transformed into DH10Bac competent cells for transfection into the bacmid. Bacmid DNA of calcyclin was isolated from the transformed cells was transferred into SF9 insect cells. High-titer recombinant SF9-A rabbit annexin XI-A vector was used as the template, was cloned into the pGEX-3X vector, the coding region of rabbit annexin XI-A cDNA in pME188 (16) was amplified by the polymerase chain reaction (PCR) with Pfu DNA polymerase and purified after cutting with BamHI and PstI. The PCR product was verified by DNA sequencing. The employed oligonucleotides corresponding to bases 20–39 (with the initiation site) and the antisense to bases 1641–1661 (with sequence 3’-AAGGATTCTTGCGGCCTTTTCCTGT-5’), respectively. The sequences of the oligonucleotides used for PCR were as follows: 5’-AAGGATCCCATGAGCTACCCTGGCTAT-3’ and 5’-AATCGCAGATTCTGGCGGCCATTTGGTG-3’. The purified PCR product was introduced into the BamHI/PstI-digested pBlueBacIII plasmid. Recombinant baculovirus of rabbit annexin XI-A was prepared with the pBlueBacIII rabbit annexin XI-A as described previously (25). For cloning of rabbit calcyclin cDNA into the pFastBac donor plasmid, the coding region of the rabbit calcyclin in the pGEX-3X vector (26) was subcloned into the BamHI/EcoRI sites of the pFastBac donor plasmid. pFastBac rabbit calcyclin was transformed into DH10Bac competent cells for transfection into the bacmid. Bacmid DNA of calcyclin was isolated from the transformed cells was transferred into SF9 insect cells. High-titer recombinant SF9-A rabbit annexin XI-A was prepared with the coding region of rabbit calcyclin in the pGEX-3X vector (26) was subcloned into the BamHI/EcoRI sites of the pFastBac donor plasmid. pFastBac rabbit calcyclin was transformed into DH10Bac competent cells for transfection into the bacmid. Bacmid DNA of calcyclin was isolated from the transformed cells was transfected into SF9 insect cells. High-titer recombinant SF9-A rabbit calcyclin was a generous gift from Dr. W. J. Chazin (The Scripps Research Institute, San Diego, CA). This vector was transformed into Escherichia coli BL21 cells to express recombinant proteins. Overnight cultures were grown, and the recombinant proteins were induced with isopropyl-1-thiogalactopyranoside. The cell culture was harvested, resuspended in extraction buffer, and prepared as described above. Fractons containing calcyclin were collected and dialyzed against H2O.

Expression and Purification of Recombinant Annexin XI—Expression and Purification of recombinant rat annexin XI-A and bovine annexin XI-B as fusion proteins with GST in E. coli cells, cDNAs encoding various rabbits of domain XI-A and residues Val52-Pro60 of bovine annexin XI-B, generated by PCR with pM188-rab annexin XI-A (16) or pVL1392-bov annexin XI-B as the template, were cloned into a pGEX-3X vector. The oligonucleotides employed for forward and reverse primers were synthesized with BamHI and EcoRI sites on their 5’ ends, respectively. Their sequences were as follows: GAST-A31–57, 5’-TTGATCCCCGGCGATCAGGCCCCAT-3’ and 5’-TAGAATTCATGGGAGGCAGGGTG-3’; GST-A31–42, 5’-TTGAGTTGACCAGGGGGTCGAGGGTG-3’ and 5’-TTGAGATTGATCGGAGGGTG-3’; GST-A31–55, 5’-TTGAGATGCTGGCGGGCCGAGGGTG-3’ and 5’-TTGAGATTGATCGGAGGGTG-3’; GST-A31–65, 5’-TTGAGATGCTGGCGGGCCGAGGGTG-3’ and 5’-TTGAGATTGATCGGAGGGTG-3’; GST-A31–77, 5’-TTGAGATGCTGGCGGGCCGAGGGTG-3’ and 5’-TTGAGATTGATCGGAGGGTG-3’; GST-A31–91, 5’-TTGAGATGCTGGCGGGCCGAGGGTG-3’ and 5’-TTGAGATTGATCGGAGGGTG-3’; GST-A31–103, 5’-TTGAGATGCTGGCGGGCCGAGGGTG-3’ and 5’-TTGAGATTGATCGGAGGGTG-3’. A schematic diagram of the fusion proteins was shown in Fig. 1A. PCR products were verified by DNA sequencing. The construct of GST N-terminal domain of annexin XI fusion protein was transformed into E. coli XL1-Blue cells.

1 The abbreviations used are: AXI, annexin XI; PCR, polymerase chain reaction; DTT, dithiothreitol; MES, 4-morpholinethanesulfonic acid; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis.
were applied to glutathione-Sepharose columns. After extensive washing of the columns with a high salt resuspension buffer (1 M NaCl) and subsequently resuspension buffer, elution was carried out with resuspension buffer containing 10 mM glutathione. The fractions containing purified protein were dialyzed against resuspension buffer and stored at –80 °C.

Calcyclin Affinity Sepharose Chromatography of Annexin XI Isoforms and GST Fusion Proteins—Binding capacities of annexin XI isoforms and GST fusion proteins for calcyclin were determined by calcyclin affinity Sepharose chromatography. Purified annexin I, XI (18 μg), or GST fusion proteins (20 μg) were incubated in 40 mM Tris-HCl, pH 7.5, 0.2 M NaCl, 0.2 mM CaCl2, and 300 μL of a 50% slurry of calcyclin-Sepharose. After rotation for 4 h at 4 °C, calcyclin-Sepharoses were washed three times in buffer containing 40 mM Tris-HCl, pH 7.5, 1 mM NaCl, 0.2 mM CaCl2, and 300 μL of GST fusion proteins were specifically eluted from the Sepharose beads with buffer containing 40 mM Tris-HCl, pH 7.5, 0.2 M NaCl, 5 mM EGTA and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) followed by Coomassie staining.

Subcellular Fractionation—Three days post-inoculation with recombinant baculovirus of annexin XI isoforms, SF9 cells were harvested and washed three times in phosphate-buffered saline and resuspended in 40 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, 5 μg/ml leupeptin. The cell suspensions were lysed by sonicating them for 30 s and centrifuged at 100,000 × g for 30 min, yielding the soluble cytosol and membrane pellets. Aliquots of 30 μL of a 50% slurry of calcyclin-Sepharose were incubated in 40 mM Tris-HCl, pH 7.5, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, 5 μg/ml leupeptin. Soluble cytosol and membrane pellet fractions were analyzed by SDS-PAGE followed by immunoblotting.

Coexpression of Annexin XI Isoforms and Calcyclin in SF9 Cells—Rabbit calcyclin baculovirus was infected into SF9 cells in 25-cm2 T flasks with and without baculovirus of bovine annexin XI isoforms. Cell monolayers were harvested by scraping from T flasks 48 h after infection. Cell pellets were obtained by centrifugation at 1000 × g for 5 min, washed with phosphate-buffered saline and resuspended in 40 mM Tris-HCl, pH 7.5, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, 5 μg/ml leupeptin. Subcellular fractionation was performed as described above except for gentle sonication to avoid the proteolysis of calcyclin and annexin XI proteins.

Other Procedures—Liposome co-pelleting assays were performed as described previously (27), and in peptide competition study, 0.3 (μM) 10 nM (100 μM) of the peptide was incubated with 0.06 nmol (3 μg) of annexin XI protein and 0.2 nmol of calcyclin protein. Annexin I protein was purified from bovine lung as described by Glenney et al. (28). Immunoblotting was performed with an antibody that can detect both annexin XI isoforms and calcyclin to annexin XI in a dose-dependent manner. This result supports that the residues Gln49-Thr62 of rabbit annexin XI-A functions as the calcyclin binding site.

Identification of Calcyclin Binding Site of Annexin XI—A previous truncated mutant study indicated that the Tyr57-Leu58 residues of rabbit annexin XI-A are critical for its calcyclin binding (24). To identify the calcyclin binding site of annexin XI, GST fusion proteins with N-terminal sites of rabbit annexin XI-A were constructed and expressed in E. coli. On the basis of our previous study with truncated mutants, we chose 5 sites containing 29 particular amino acid sequences in the region between Pro29 and Tyr81 of rabbit annexin XI-A (Fig. 1A). These fusion proteins were examined for calcyclin binding with calcyclin affinity Sepharose. These proteins were incubated with calcyclin-Sepharose in the presence of CaCl2. After washing three times with buffer containing 1 M NaCl to exclude nonspecific binding, the protein bound to calcyclin were eluted with buffer containing EGTA (Fig. 1B). Full-length rabbit annexin XI-A protein but not control GST protein bound to calcyclin-Sepharose in a Ca2+-dependent manner. Three GST fusion proteins, GST-AXI 34–62, GST-AXI 40–68, GST-AXI 49–77, also bound to calcyclin-Sepharose, whereas the two other, GST-AXI 29–57 and GST-AXI 53–81, did not bind. The common sequence for these demonstrating binding is Gln49-Thr62 of annexin XI-A. This result shows that the calcyclin binding site is residues Gln49-Thr62 of rabbit annexin XI-A.

Effect of Synthesized Peptide Corresponding to Tyr43-Thr62 of Annexin XI on the Annexin XI-Calcyclin Interaction—To confirm the calcyclin binding site of annexin XI obtained with GST fusion proteins with N-terminal sites of annexin XI, the synthesized peptide corresponding to residues Tyr43-Thr62 of annexin XI (AXI 43–62 peptide) was examined to see if it inhibited the annexin XI-calcyclin interaction. Calcyclin was incubated with annexin XI and Ca2+/phosphatidylserine liposome in the presence or absence of the AXI 43–62 peptide. Almost all calcyclin protein bound to annexin XI and was coprecipitated with annexin XI-phosphatidylserine liposome complex in a Ca2+-dependent manner in the absence of the peptide. AXI 43–62 peptide inhibited the binding of calcyclin to annexin XI in a dose-dependent manner. This result supports that the residues Gln49-Thr62 of rabbit annexin XI-A functions as the calcyclin binding site.

Expression and Purification of Annexin XI Isoforms in Bacu-
We expressed and purified recombinant annexin XI isoforms in Sf9 cells with recombinant baculoviruses. Recombinant bovine annexin XI-A was highly expressed as a 54-kDa protein and a 60-kDa form due to a post-translational modification as previously reported (Fig. 3A) (25). Recombinant annexin XI-A proteins were solubilized from Sf9 cells with EGTA-containing buffer and purified by 30% ammonium sulfate precipitation and Q- and S-Sepharose column chromatography. After purification, a major 54-kDa protein and a minor 48-kDa protein appeared on SDS-PAGE. The 54-kDa protein could bind to calcyclin affinity Sepharose in a Ca$^{2+}$-dependent manner, but 48-kDa protein could not. Both proteins were detected on Western blots with anti-annexin XI antibody (data not shown). Therefore, the 54-kDa protein was concluded to be the full-length form of annexin XI and the 48-kDa protein is a not shown). Therefore, the 54-kDa protein was concluded to be the full-length form of annexin XI and the 48-kDa protein is a result of proteolysis during purification, possibly around amino acid position 60 in the N-terminal domain. Recombinant annexin XI-B protein was extracted in 8 M urea-containing buffer and purified by 30% ammonium sulfate precipitation and Q- and S-Sepharose column chromatography. After purification, a major 54-kDa protein and a minor 48-kDa protein appeared on SDS-PAGE. The 54-kDa protein could bind to calcyclin affinity Sepharose in a Ca$^{2+}$-dependent manner, but 48-kDa protein could not. Both proteins were detected on Western blots with anti-annexin XI antibody (data not shown). Therefore, the 54-kDa protein was concluded to be the full-length form of annexin XI and the 48-kDa protein is a result of proteolysis during purification, possibly around amino acid position 60 in the N-terminal domain. Recombinant annexin XI-B protein was extracted in 8 M urea-containing buffer and purified by 30% ammonium sulfate precipitation and Q- and S-Sepharose column chromatography. After purification, a major 54-kDa protein and a minor 48-kDa protein appeared on SDS-PAGE. The 54-kDa protein could bind to calcyclin affinity Sepharose in a Ca$^{2+}$-dependent manner, but 48-kDa protein could not. Both proteins were detected on Western blots with anti-annexin XI antibody (data not shown). Therefore, the 54-kDa protein was concluded to be the full-length form of annexin XI and the 48-kDa protein is a result of proteolysis during purification, possibly around amino acid position 60 in the N-terminal domain.

Competitive inhibition of annexin XI binding to calcyclin by synthesized AXI 43–62 peptide. Rabbit annexin XI-A and calcyclin proteins were mixed with phosphatidyserine liposome in the presence or absence of the AXI 43–62 peptide. After centrifugation at 100,000 × g, supernatant (S) and pellet (P) fractions were subjected to SDS-PAGE (15% gel) followed by Coomassie staining. Molecular masses are indicated on the left (kDa). cont. control.

Fig. 2. Competitive inhibition of annexin XI binding to calcyclin by synthesized AXI 43–62 peptide. Rabbit annexin XI-A and calcyclin proteins were mixed with phosphatidyserine liposome in the presence or absence of the AXI 43–62 peptide. After centrifugation at 100,000 × g, supernatant (S) and pellet (P) fractions were subjected to SDS-PAGE (15% gel) followed by Coomassie staining. Molecular masses are indicated on the left (kDa). cont. control.

Coexpression of Annexin XI Isoforms with Calcyclin in Sf9 Cells—To determine whether annexin XI isoforms form complexes with calcyclin in cells, lysates from normal Sf9 cells, cells expressing calcyclin, and cells coexpressing calcyclin and each annexin XI isoform were subjected to coprecipitation assays with membrane components of Sf9 cells followed by SDS-PAGE to detect calcyclin or immunoblotting to detect annexin XI. In Sf9 cells expressing only recombinant calcyclin, about 50% of the calcyclin existed in the cytosolic fraction under the experimental conditions applied (Fig. 5). When calcyclin was coexpressed with annexin XI-A, almost all was translocated to the precipitated membrane fraction by binding to annexin XI-A in a Ca$^{2+}$-dependent manner. However, when coexpressed with annexin XI-B, calcyclin was not translocated, remaining in the cytosolic fraction in both the presence and absence of Ca$^{2+}$. This result confirms that the annexin XI-B isoform is not a calcyclin-binding protein. In this subcellular fractionation study, a difference for subcellular distributions of annexin XI isoforms was observed. Annexin XI-A translocated from the cytosolic fraction to the membrane fraction in a Ca$^{2+}$-dependent manner (Fig. 5, lower panel). In contrast, annexin XI-B was localized completely in the membrane fraction both in the absence and presence of Ca$^{2+}$. This localization was also observed in Sf9 cells expressing only recombinant annexin XI-B (data not shown).
In the present study, use of multiple GST fusion proteins with N-terminal sites of rabbit annexin XI-A identified its calcyclin binding site, located within residues Gln49-Thr62 of the N-terminal regulatory domain. Previously, we reported that a truncated form lacking the N-terminal 26 amino acids of annexin XI-A retained calcyclin binding ability, whereas loss of the N-terminal 52 amino acids abolished the interaction (24), suggesting that the region of Tyr27-Leu52 is critical for the calcyclin binding or might be the calcyclin binding site. Between the residues Tyr27-Leu52 of rabbit annexin XI-A and the residues Gln49-Leu52 of the calcyclin binding site presented in this study, the common sequence is Gln49-Leu52. As shown in Fig. 1B, GST-AXI 49–77, but not GST-AXI 53–81, could bind to calcyclin. Therefore, it is strongly suggested that the region between Gln49-Leu52 of annexin XI-A is essential for the binding, and the region of Tyr27-Ans148 is not necessarily essential. In addition, it is also suggested that the region of Asn58-Thr62 of annexin XI-A is essential for the binding, since GST-AXI 34–62, but not GST-AXI 29–57, could bind to calcyclin. Considering that the binding of annexin XI with calcyclin is mediated by a hydrophobic interaction (26), it is probable that only hydrophobic residues, Leu52 and Met59, within Gln49-Leu52 and Asn58-Thr62, respectively, may play an important role.

In the members of the annexin family, annexin I and II have been shown to bind to other S100 proteins, S100C (calgizzarin) and p11 (S100A10), by interaction of the 13 and 12 N-terminal amino acids, respectively (29–32). These are again mediated by hydrophobic-hydrophobic bonding (30, 32–34). Comparison of the primary structures of the S100 binding sites reveals a conserved amino acid arrangement consisting of four hydrophobic amino acids, residues Leu52, Met55 (Val in bovine),...
Aβ 51-59 of annexin XI-A, residues Val9, Phe7, Leu8, Ala11 of annexin XI, and residues Val7, Ile7, Leu8, Leu11 of annexin II (Fig. 6A). CD spectroscopy indicates that the p11 binding site of annexin II forms an amphipathic α-helix (29), and Chou-Fasman secondary analysis has predicted that both the S100C binding site of annexin I and the calcyclin binding site of annexin XI present here form similar amphipathic α-helices. In these α-helices, the hydrophobic residues described above are aligned on one side, whereas hydrophilic residues are aligned on the opposite side (Fig. 6B). Thus the secondary structures of S100 binding sites appear to be conserved among annexin I, II, and XI. Despite this similarity, annexin XI protein only binds to calcyclin among the S100 protein family including S100C and p11 (27). In addition, calcyclin does not interact with other annexin family proteins (annexin II, V, VI, VII) as described previously (9). In this study, annexin I protein could not bind to calcyclin (Fig. 4B). These suggest that differences of amino acids other than hydrophobic residues in sequences of S100 binding sites or three-dimensional structures of each annexins and S100 proteins might be involved in determining the binding specificity.

Among protein-protein interactions through hydrophobic faces within amphipathic helices, perhaps the best characterized are those of calmodulin with calmodulin-dependent enzymes such as myosin light chain kinase (35, 36). Calmodulin, a Ca2+-binding protein with four EF-hand structures similar to the two EF-hands of S100 proteins, binds to specific peptide regions of many target proteins to regulate their activity in a Ca2+-dependent manner. Formation of amphipathic α-helices has been shown by CD spectroscopy and NMR analysis (36). By analogy it would be expected that the functions of annexins might be affected by binding with S100 proteins. Indeed, the formation of heterotetramers of annexin II and p11 alters the subcellular localization and the affinity for Ca2+ in phospholipid binding and chromaffin granule aggregation of annexin II, which is suggested to play an essential role in catecholamine secretion in chromaffin cells (2, 10–12, 37). Both annexin I and S100C have been identified as components of the cornified envelope in human epidermal keratinocytes (38). Sites of cross-linkage have been identified at residues within the S100C binding site of annexin I and the Lys8 and C-terminal residues near the annexin I binding site of S100C. Thus the annexin I-S100C interaction might be required for anchorage to the envelope. Annexin XI was discovered as calcyclin-associated protein (CAP-50) in our laboratory (9, 13) and has been shown to localize in the nucleus of continuous cell lines such as 3Y1 and COS-7 cells (16). It has been suggested that calcyclin is involved in cell proliferation and division, since it is overexpressed in tumor cells, and transcription of its mRNA can be induced by serum and growth factors (18, 19). Although a nuclear localization signal of annexin XI has still been unidentified, it has been indicated that the nuclear localization of annexin XI is mediated through its N-terminal regulatory domain (16). Since the calcyclin binding site of rabbit annexin XI-A is located at residues Glu29-Thr52 in this domain, it is possible that the nuclear localization of annexin XI might be regulated by its binding of calcyclin.

Annexin XI isoforms are generated by alternative splicing of identical primary transcripts (21). Annexin XI-A has a 37-amino acid insert, whereas annexin XI-B has a totally different 39-amino acid insert in the splicing region. As it was speculated that their calcyclin binding ability would differ in view of the results of our previous truncated mutant study (24), it was found that annexin XI-B could not bind to calcyclin by a liposome co-pelleting assay (Fig. 4B). By contrast between the amino acid sequences of annexin XI isoforms, two arrange-
ments consisting of four hydrophobic amino acids shown in the calcyclin binding sites Pro$_{30}$-Asp$_{43}$ and Asp$_{43}$-Arg$_{56}$ are also found in the alternative splicing region of annexin XI-B (Fig. 4A). However, since annexin XI-B has no calcyclin binding ability, these sites do not function as calcyclin binding sites.

In Val$_{52}$-Thr$_{65}$ of annexin XI-B, corresponding to the calcyclin binding site of annexin XI-A, the four hydrophobic amino acid arrangement is not conserved (Fig. 4A). Indeed, this region of annexin XI-B could not function as a calcyclin binding site (Fig. 4C, GST-AXIB 52–80).

It was observed that the subcellular distributions of recombinant annexin XI isoforms were distinct in SF9 cells (Fig. 5). Annexin XI-B existed in the membrane fraction, both in the absence and presence of Ca$^{2+}$, in contrast to the Ca$^{2+}$-dependent membrane association of annexin XI-A. In hydrophobicity plot analysis with the method by Kyte and Doolittle (39), it was revealed that annexin XI-B possesses a long hydrophobic amino acid region between Leu$_{28}$ and Met$_{62}$ containing part of the splicing region (Fig. 7). The hydrophobic region consisting of 30 residues is of sufficient length to span the membrane lipid bilayer and to possess a high probability of $\alpha$-helix formation. Such a long hydrophobic site was not found in the splicing region of annexin XI-A. It is possible that annexin XI-B might be an integral membrane protein generated through a hydrophobic interaction and that the alternative splicing region of annexin XI-B might function as the Ca$^{2+}$-dependent membrane-associating domain, not as a calcyclin binding site.

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