Dissecting the Cellular Functions of Annexin XI Using Recombinant Human Annexin XI-specific Autoantibodies Cloned by Phage Display*

Received for publication, October 23, 2002, and in revised form, June 9, 2003
Published, JBC Papers in Press, June 11, 2003, DOI 10.1074/jbc.M210852200

Lauge Farnaes and Henrik J. Ditzel‡

From the Department of Immunology, The Scripps Research Institute, La Jolla, California 92037

Functional studies of cellular proteins are often complicated by the lack of well-defined monoclonal antibodies, the production of which is hampered by the highly conserved nature of these cellular proteins across species. Annexin XI, a member of the Ca\(^{2+}\)-dependent, phospholipid-binding protein family, is an example of such a protein and was used in studies to devise a strategy using human autoimmune phage display libraries to generate reagents for biological studies of conserved cellular proteins. An IgG phage display library was generated from bone marrow of an autoimmune patient with high serum antibody titer against annexin XI, which was identified recently as an autoantigen targeted by autoantibodies in several systemic autoimmune diseases. From this phage library, a panel of human monoclonal annexin XI-specific Fab s were isolated and applied to studies of the cellular functions of annexin XI. Confocal microscopy showed a cell cycle-specific redistribution of annexin XI from the cytoplasm to the mitotic spindle. In metaphase, annexin XI was up-regulated and co-stained with α-tubulin. The subcellular distribution of annexin XI in COS-7 cells was shown to be Ca\(^{2+}\)-dependent, and exhibited a predominantly nuclear pattern at low concentrations and a cytoplasmic pattern at high Ca\(^{2+}\) concentrations. Calcyclin, found previously to bind annexin XI in vitro, in vivo coated the nuclear membrane of cultured cell lines and did not colocalize with annexin XI. Ultrastructural analysis by immunoelectron microscopy revealed that annexin XI associated with specific granules in both neutrophils and eosinophils, suggesting a role in the exocytotic pathway. Our results illuminate the multifunctional nature of annexin XI, provide the first evidence that annexin XI associates with the mitotic spindles and might play a role in cell division, and clearly illustrate the potential of phage display-derived human autoantibodies in broader analyses of the function of highly conserved cellular proteins.

Annexin XI is a member of the annexin superfamily, which is characterized by Ca\(^{2+}\)-dependent binding to negatively charged phospholipids, e.g. as found in biomembranes (1–4). All annexin proteins consist of a conserved C-terminal annexin homology domain containing either 4 or 8 repeating units of 70 amino acids and an individually unique N-terminal regulatory domain. The N-terminal of annexin XI is hydrophobic, rich in glycine, tyrosine, and proline residues, and is larger than that of the other annexin members. Annexin XI is ubiquitously expressed in a variety of tissues and cell types of eukaryotes, but its subcellular distribution varies considerably. Some growth and differentiation conditions favor the presence of annexin XI in the nucleus, whereas others favor either a cytoplasmic distribution or both. Annexin XI has been found localized predominantly in the nucleus of rapidly developing tissue, such as undifferentiated mesenchymal cells of 14-day-old rat embryos (5). However, once this tissue differentiated into connective tissue, as in the 18-day-old embryo, annexin XI was found equally distributed in the nucleus and the cytoplasm. The N-terminal domain of annexin XI has been proposed to contain a nuclear localization signal, because deletion of the N-terminal domain shifted the distribution of annexin XI from nuclear to cytoplasmic within COS-7 cells, although no traditional nuclear localization signal has been identified (6). Interestingly, the N-terminal domain also contains a binding site for calcyclin, a Ca\(^{2+}\)-binding and growth cycle-regulated EF hand protein (7).

Annexin XI also seems to play a role in the secretion or degradation of neutrophil granules, suggested by its binding to granules in a Ca\(^{2+}\)-dependent manner (8, 9). Supporting this concept, annexin XI has been shown to be secreted by activated neutrophils in response to an internal calcium signal (10). Annexin XI has also been found in insulin-containing granules and suggested to play a role in insulin release (11). Other studies have suggested that annexin XI is involved in vesicle formation, because transfection of insect Sf9 cells with recombinant annexin XI-A caused the formation of large "annexin XI-associated vesicles" in the cytoplasm of the cells that were clearly visible with a light microscope (12).

Autoantibodies to many nuclear and cytoplasmic proteins are found in the sera of patients with diverse autoimmune conditions (13, 14). Antibodies to a particular autoantigen are often associated with a specific autoimmune disease or a disease process that is common to a group of autoimmune diseases. Recently, annexin XI was identified as an autoantigen in ~10% of patients with systemic autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus, and Sjögren’s syndrome (15, 16). The anti-annexin XI response is predominantly of the IgG subclass and targeted against the unique N-terminal domain of the protein, demonstrated by lack of cross-reactivity with other annexins and with recombinant C-terminal-truncated annexin XI in immunoprecipitation studies (15). It has been suggested that anti-annexin XI antibodies might have relevance for thrombosis because of some degree of co-occurrence (17). To explain the mechanism behind the asso-
cation with thrombosis, it has been hypothesized that if annexin XI, similar to annexin V, has anticoagulant properties, anti-annexin XI autoantibodies may inhibit this regulatory function (17).

Although more data on annexin XI are becoming available, its cellular function remains to be clarified. One limiting factor is the lack of annexin XI-specific monoclonal antibodies, which are needed to generate transgenic mice because of their conserved structure in different species. Because autoantibodies against annexin XI are found in patients with different autoimmune diseases, we decided to clone a panel of annexin XI-specific antibodies by phage display technology and use them as probes to examine the subcellular localization of annexin XI in various human cells using confocal microscopy, immunohistochemistry, and electron microscopy. Our results show for the first time that annexin XI is up-regulated in mitotic cells and stains mitotic spindles. Ca\(^{2+}\) was found to influence both the association of annexin XI with tubulin and the nuclear or cytoplasmic subcellular localization of annexin XI. Phage display cloned human autoantibodies provide valuable tools for the biological study of conserved cellular proteins.

**MATERIALS AND METHODS**

**Library Construction and Phage Selection**—Preparation of RNA from bone marrow lymphocytes and subsequent construction of an IgG Fab library using the pComb3 M13 surface display system have been described (18, 19). For antibody selection, the phage library generated from a rheumatoid arthritis patient was panned separately against freshly isolated neutrophils in suspension and recombinant annexin XI coated on enzyme-linked immunosorbent assay (ELISA)\(^{1}\) wells. Neutrophils were prepared from fresh heparinized whole blood by a combination of dextran sedimentation and centrifugation through discontinuous plasma-Percol gradients as described (20). This technique was designed to minimize exposure to bacterial endotoxin and preserve the neutrophils in a minimally activated state. Subsequently, the neutrophil pellet was washed twice with 0.9% saline and resuspended to \(-5 \times 10^8\) cells/ml. Fab displaying phage resuspended in PBS containing 1% bovine serum albumin (BSA) were added to the microtube containing neutrophils and to recombinant annexin XI coated onto ELISA wells and incubated for 1 h at 4 °C or 37 °C, respectively. Unbound phage were removed by washing 10 times with PBS containing 1% BSA. Bound phage, enriched for those bearing antigen-binding surface Fabs, were removed by washing 10 times with PBS containing 1% BSA. Bound phage were amplified by infection of Escherichia coli XL1-Blue cells to produce clones secreting soluble Fab fragments. Fabs were purified from bacterial supernatants by affinity chromatography, as described previously, with minor modifications (19, 21).

**Cloning of Annexin XI—RNA was isolated from HeLa cells and reverse-transcribed into cDNA, and the gene encoding annexin XI was amplified using specific primers. The PCR product was cloned into the pCEX-4T-1 vector (Amer sham Pharmacia) using the BanHI and XhoI restriction sites. The ligated vector was heat pulse-transformed into BL21 E. coli (Stratagene, La Jolla, CA). A single clone, containing the correct insert as determined by sequencing, was grown in Luria-Bertani media, and protein expression was induced with isopropyl-1-thio-\(\beta\)-d-galactopyranoside for 4 h.

**ELISA Analysis**—To assess specificity, Fabs were screened by ELISA against recombinant annexin XI and a panel of other antigens including annexin V, ovalbumin, BSA, the Fc part of IgG (all from Sigma), tubulin (Cytoskeleton, Inc., Denver, CO), HIV-1 gp120 (Intracel, Issaquah, WA), whole recombinant human annexin VII (kindly provided by Dr. H. B. Pollard, Uniformed Services University School of Medicine, Bethesda, MD), and the N-terminal region of annexin VII and the N-terminal region of annexin XI expressed as glutathione S-transferase fusion proteins (the plasmids pGST-Anx7N and pGST-Anx11N were kindly provided by Dr. M. Maki, Nagoya University, Japan (20)). As negative controls, a human anti-HIV-1 gp120 Fab,Fab\(_2\) and an anti-\(\alpha\)-tubulin antibody (Sigma) were included. Human Fabs were incubated with the test antigen for 1.5 h at 37 °C, followed by washing with PBS-0.05% Tween. Detection of bound human Fabs was carried out with alkaline phosphatase-labeled goat anti-human IgG Fab\(_2\) antibody (Pierce) and visualized with nitrophenol substrate (NPP substrate) (Sigma) by reading absorbance at 405 nm.

**SDS-PAGE and Western Blot Analysis**—Electrophoresis of HeLa nuclear and cytoplasmic extract (Computer Cell Culture Center, Mons, Belgium), resolved under denaturing and reducing (100 mM dithiothreitol) conditions, was performed in a discontinuous buffer system on 10% polyacrylamide gels. Separated proteins were electroblotted onto polyvinylidene difluoride membranes. Immunoblotting (Immobilon-M, Millipore, Bedford, MA), blocked for 2 h in 5% skimmed milk in 10 mM Tris, 350 mM NaCl, pH 7.6, washed three times with Western blot buffer (50 mM Tris, 350 mM NaCl, 15 mM Na\(_2\)SO\(_4\) and 0.1% Tween 20), and incubated with patient sera diluted in 1:50, 1:100, and 1:200 in incubation buffer (50 mM Tris, 150 mM NaCl, 0.5% skimmed milk, and 0.1% Tween 20, pH 7.6) or control antibodies for 2 h at room temperature. The membrane was washed in Western blot buffer and incubated with alkaline phosphatase-conjugated rabbit anti-human IgG antibody (diluted 1:500, Dako, Glostrup, Denmark) for 1 h at room temperature. After three washes in PBS, the membrane was fixed with 0.2% glutaraldehyde in PBS for 15 min at room temperature and finally washed in PBS. Bound alkaline phosphatase-conjugated antibody was visualized by 5-bromo-4-chloro-3-indoly phosphate (Bio-Rad). A low range protein marker (Bio-Rad) was used to indicate the molecular weight of the fragments.

**Immunocytochemical Analysis by Immunofluorescence and Confocal Laser Scanning Microscopy**—Mammalian cell lines HeP-2, HeLa (human squamous carcinoma), and A549 (human lung cancer) were grown in RPMI 1640-supplemented 10% fetal calf serum (Tissue Culture Biologists), 100 units/ml penicillin, and 100 units/ml streptomycin, whereas COS-7 (Green Monkey kidney), MCF-7, MB157, BrCaM201 (human breast carcinoma), RD (human embryonal rhabdomyosarcoma), and U97 (human glioblastoma) were grown in DMEM supplemented with 10% fetal calf serum, 1% nonessential amino acids, 5 mM \(\mu\)-glutamine, 1% sodium pyruvate, 100 units/ml penicillin, and 100 units/ml streptomycin. The cells were allowed to adhere to coverslips (Nunc, Kamstrup, Denmark) for 48 h at 37 °C, 5% CO\(_2\), to form monolayers. In some experiments, the cells were washed in either 2 mM CaCl\(_2\) or 5 mM EGTA (Sigma) and incubated with 0.005% Triton X-100 in either 2 mM CaCl\(_2\) or 5 mM EGTA for 2 s before fixation in ice-cold ethanol. In other experiments, HEp-2 cells were treated with either 10 \(\mu\)M nucodazole for 2–10 h, 10 \(\mu\)M colchicine for 2 h, or 10 \(\mu\)M cytochalasin D for 2 h at 37 °C, 5% CO\(_2\). Neutrophils were purified as described above and stained in suspension. Glass slides with HEp-2 cells attached (ANA-screen, Immuno Concepts, Sacramento, CA) were also used. Cells were fixed with ice-cold ethanol for 5 min, followed by four washes with PBS and blocking with 5% normal goat serum (BioSource International, Camarillo, CA) for 30 min. Cells were incubated with the following antibodies: purified human anti-annexin Fabs (20–100 \(\mu\)g/ml), donor serum (1:840), rabbit anti-human calcyclin antibody (1:400, Sigma), and mouse anti-human \(\alpha\)-tubulin antibody (1:100, Sigma) for 1.5 h at room temperature. The cells were washed five times for 3 min each time with PBS and incubated with FITC-labeled (Fab)\(_2\) goat anti-human IgG (Fab)\(_2\) antibody, Texas Red-labeled goat anti-mouse IgG antibody, or CY-5-labeled goat anti-mouse IgG antibody (all from Jackson). When appropriate, the cells were also incubated with propidium iodide (5 \(\mu\)g/ml, Sigma) in the final step at 5 \(\mu\)g/ml for 10 min. The slides were mounted with SlowFade (Molecular Probes, Eugene, OR) before analysis using a ZEISS Axiosvert S100 TV confocal microscope. As controls, all experiments were carried out omitting the primary antibody.

**Labeling of Fabs with Fluorescein Isothiocyanate and Nanogold—**Conjugation with fluorescein isothiocyanate and Nanogold was performed using kits from Rockland Immunochemicals Corp., Gilberts, and Nanoprobe (Stony Brook, NY), respectively, according to the manufacturer’s guidelines. In brief, human Fab (100 \(\mu\)g) was mixed with 9.5 \(\mu\)l of Flous solution (2 mg/ml in MeSO) and allowed to incubate for 2 h at room temperature on a Lab-quake shaker. The mixture was then added to a G-25 Sephadex gel filtration column (Pharmacia) to separate the Fab from the unreacted reagents. The Fab was eluted in PBS, and fractions and concentrated. Fluorescein isothiocyanate-labeled antibody (300 \(\mu\)l) was mixed with 220 \(\mu\)l of Nanogold reagent (first dissolved in 20 \(\mu\)l of MeSO), then 200 \(\mu\)l of deionized H\(_2\)O. The reagents were incubated for 1 h at room temperature on a Lab-quake shaker. Again,
RESULTS

Cloning of Human Anti-Annexin XI Antibodies—The need to understand the cellular function of the Ca\(^{2+}\)-binding protein annexin XI led us to clone human IgG anti-annexin XI antibodies using phage display. Initially, a panel of sera from patients with systemic autoimmune disease were examined for anti-annexin XI reactivity by Western blotting. Serum from one patient with rheumatoid arthritis contained IgG antibodies that bound to a band with a molecular mass of 56 kDa in the cytoplasmic fraction of HeLa cells, indicative of annexin XI. The IgG anti-annexin XI reactivity of the serum was confirmed by its binding to recombinant annexin XI in ELISA. This patient’s serum also exhibited an uncharacteristic staining pattern of the squamous cell line HeLa, a cell line commonly used for anti-nuclear antibody identification in the clinic. The serum also stained neutrophils, but not lymphocytes, using immunofluorescence microscopy. To clone a panel of human anti-annexin XI autoantibodies, an IgG/λ phage display library of ~6 × 10^8 members was constructed from the bone marrow RNA of the rheumatoid arthritis patient. The antibody library was selected against freshly isolated neutrophils in suspension and against recombinant annexin XI coated on ELISA wells. After four rounds of biopanning, a 20- and 80-fold amplification in eluted phage was observed in the two selection protocols, respectively, indicating enrichment for antigen-binding Fab-phages. Individual clones, expressed as soluble Fabs by excision of the gene III from the pcomb3 phagemid DNA from the last round of selection, were tested for binding to recombinant annexin XI that was sensitive to the SDS treatment. The Fabs did not bind to whole human annexin VII or the N-terminal region of annexin VII, despite annexin V and XI having highly homologous C-terminal cores. The Fabs did not bind to whole human annexin VII or the N-terminal region of annexin VII, despite exhibiting the closest homology with annexin XI among all annexins (largest and most hydrophobic N-terminal tails), and also did not bind to the other control antigens, thus demonstrating that they were specifically directed against the N-terminal tail of annexin XI. An anti-HIV-1 gp120 Fab, b12 used as a control, did not bind to the recombinant annexin XI, but bound strongly to HIV-1 gp120. Immunofluorescence analysis and laser scanning confocal microscopy of HeP-2 cells demonstrated that all four anti-annexin Fabs examined as bacterial supernatants exhibited a cytoplasmic staining pattern similar to the serum of the patient from whom the Fabs were cloned. The anti-annexin Fabs also bound to dot blots of HeP-2 cell lysates but not to Western blots of the same HeP-2 cell lysates, indicating that the Fabs recognized a conformational epitope on annexin XI that was sensitive to the SDS treatment.

Probing the Subcellular Distribution of Annexin XI Using Recombinant Fabs—To detail the subcellular localization of annexin XI, a panel of human cell lines were examined with laser scanning confocal microscopy using one of the anti-annexin Fabs, AN5-17, as a probe. HEp-2, as a probe. HEp-2 cells showed intense Fab AN5-17 staining in the cytoplasm and weak, speckled staining in the nucleus (Fig. 3). A similar cytoplasmic staining and weak speckle or no staining in the nucleus were observed in the other cells tested, including breast cancer cell lines MCF-7 and BrCaMZ01, lung cancer cell line A549, rhabdomyosarcoma cell line RD, squamous carcinoma cell lines HeP-2 and HeLa, glioblastoma cell line U87, and Green Monkey kid-
Localization of annexin XI in HEp-2 cells was examined by laser scanning confocal microscopy using Fab AN5-17 (H9251). Partial colocalization of annexin XI and mitotic spindles in dividing cells, where it colocalized with mitotic cells; annexin XI is up-regulated and displayed on mitotic and multiple spindles (Fig. 3). These results confirm the implication that annexin XI was up-regulated during cell division. This up-regulation in mitotic Hep-2 cells after release from nocodazole block was confirmed using Fab AN5-17 in ELISA (data not shown). In nondividing cells, a relatively constant amount of annexin XI from cell to cell was observed in most cell types, with the exception of rhabdomyosarcoma RD cells, wherein staining with Fab AN5-17 showed cells that were either intensely or weakly stained for annexin XI (data not shown).

Calcium influences the subcellular distribution of Annexin XI—In contrast to our finding by confocal microscopy, a previous report on annexin XI in COS-7 cells using conventional immunofluorescence microscopy described annexin XI as being located predominantly in the nucleus (6). When we used the same detection technique, annexin XI also appeared to exhibit a nuclear stain, but the ability to take optical sections through the cells showed that although there was weak nuclear staining, cytoplasmic staining predominated.

To evaluate whether the discrepancy of annexin XI cellular distribution between our finding and those of others may be attributable to growth conditions, we examined whether Ca\(^{2+}\) influenced the subcellular annexin XI localization. The cell surface membrane of COS-7 cells was briefly permeabilized in the presence of 2 mM CaCl\(_2\) or 5 mM EGTA before fixation. Staining with the anti-annexin XI Fab and analysis using confocal microscopy revealed a significantly different cell distribution of annexin XI in the cells treated with Ca\(^{2+}\) and those treated with the Ca\(^{2+}\) chelator EGTA. In EGTA-treated COS-7 cells, annexin XI was predominantly nuclear and colocalized with propidium iodine by Ca\(^{2+}\) and fluorescein isothiocyanate-labeled Fab AN5-17 (B and D). Annexin XI in EGTA-treated cells was predominantly localized in the nucleus (B) and colocalized with propidium iodine (A). In addition, weak diffuse annexin XI staining was also found in the cytoplasm (B). In Ca\(^{2+}\)-treated cells, annexin XI staining was not found in the nucleus but was found in the cytoplasm associated with the tubulin microtubules radiating in all directions from the centrosome (D).

Interaction between Annexin XI and Calcyclin—Because previous immunoprecipitation studies have shown that annexin XI binds specifically to calcyclin (23, 24), it was of interest to study the colocalization of these two proteins in cultured cells. Calcyclin is a product of a growth-regulated gene (249) and was found to be an EF-hand protein, localizing at the nuclear envelope and at plasma membrane only in the presence of Ca\(^{2+}\).

FIG. 3. Analysis of annexin XI distribution in dividing and mitotic cells; annexin XI is up-regulated and displayed on mitotic spindles in dividing cells, where it colocalized with α-tubulin. Localization of annexin XI in HEp-2 cells was examined by laser scanning confocal microscopy using Fab AN5-17 (A, D, and C). The cells were costained with a mouse monoclonal anti-α-tubulin antibody (B, E, and H) displaying the α-tubulin-rich kinetochore microtubules in a regular mitotic cell (E), an irregular three-polar spindle mitotic cell (H), and the α-tubulin cytoskeleton network in nondividing cells (E). Similar to α-tubulin staining (E and H), spindle-like structures were stained by Fab ANA5-17 (D and G), and superimposition of the images (F and I, annexin XI, green; α-tubulin, red) showed partial colocalization between the annexin XI and the α-tubulin at the kinetochore microtubules. Partial colocalization of annexin XI and α-tubulin in nondividing cells was also observed, although less distinct (A–C).

FIG. 4. The subcellular localization of annexin XI is influenced by Ca\(^{2+}\). COS-7 cells in the presence of 5 mM EGTA (A and B) or 2 mM CaCl\(_2\) (C and D) were permeabilized, fixed, and stained with propidium iodine (A and C) and fluorescein isothiocyanate-labeled Fab AN5-17 (B and D). Annexin XI in EGTA-treated cells was predominantly localized in the nucleus (B) and colocalized with propidium iodine (A). In addition, weak diffuse annexin XI staining was also found in the cytoplasm (B). In Ca\(^{2+}\)-treated cells, annexin XI staining was not found in the nucleus but was found in the cytoplasm associated with the tubulin microtubules radiating in all directions from the centrosome (D).
analyzed by human Fab AN5-17 and a mouse anti-calcyclin antibody as probes. Hep-2 (A–C) and COS-7 (D–F) cells were fixed, permeabilized, and stained with human Fab AN5-17 (A and D) and mouse anti-calcyclin (B and E). The images in column 3 are superimpositions of columns 1 and 2 showing Fab AN5-17 staining in green and anti-calcyclin antibody in blue. Calcyclin associated with the nuclear membrane in nondividing cells, whereas annexin XI was largely cytoplasmic and no cosntaining was observed. In mitotic cells (arrows), annexin XI staining was enhanced compared with resting cells and localized associated with mitotic microtubules as well as in the cytoplasm, whereas calcyclin staining gradually disappeared as the nuclear envelope disintegrated.

Thus, the previously reported subcellular distribution of calcyclin differs from that of annexin XI. We performed double-staining for annexin XI and calcyclin in various cell types using Fab AN5-17 and a mouse monoclonal anti-calcyclin antibody and showed that whereas annexin XI was cytoplasmic in the HEp-2, HeLa, MB157, and BrCaMZ01 cells, calcyclin was localized at the nuclear membrane. During mitosis, when the nuclear envelope disassembles into membrane vesicles, calcyclin staining also gradually changes from the ring-like structure of the intact nuclear envelope to single spots corresponding to the membrane vesicles; finally, in metaphase, calcyclin completely disappears. No colocalization between annexin XI and calcyclin was observed either in COS-7, A549, or RD cells (Fig. 5). Interestingly, calcyclin staining varied considerably from cell to cell, some showing strong and others very weak stain.

**Immunoelectron Microscopy Analysis of Annexin XI**—To evaluate the subcellular distribution of annexin XI in more detail, Fab AN5-17 was labeled with fluoro-Nanogold and used to stain fixed and permeabilized human granulocytes and cultured cells for electron microscopy analysis. A fluoro-Nanogold-labeled anti-HIV-1 human Fab, b12, was used as control. Initially, the human granulocytes were examined with confocal microscopy, demonstrating staining of the Fab AN5-17; however, the small size of these cells made subcellular localization of annexin XI impossible using this technique. Instead, electron microscopic analysis of neutrophils showed accumulation of silver-enhanced gold particles in certain granules, while other granules and the surrounding cytoplasm were unlabeled (Fig. 6B). Interestingly, in eosinophils, the Fab AN5-17 was concentrated corresponding to the large, ovoid, specific granules surrounding the crystalloids, whereas no staining was found elsewhere in the cytoplasm or nucleus (Fig. 6A). Nondividing HEp-2 cells were also examined, and Fab AN5-17 showed diffuse distribution of the silver-enhanced gold particles in the cytoplasm without clear association with any cell organelles, whereas no staining was observed in the nucleus (Fig. 6C).

**The Cloned Human Anti-Annexin XI Antibodies Appear to be Derived from an Antigen-driven, Affinity-matured Response**—Why annexin XI is recognized as an autoantigen in patients with several types of systemic autoimmune diseases remains unclear. To evaluate whether the cloned anti-annexin IgG Fab fragments were derived from plasma B cells involved in an active antigen-driven immune response, the variable heavy and light chain genes were sequenced and compared with germ-line sequences in the GenBank database (Fig. 1, Table I). The variable heavy chain genes of the four Fabs were significant somatically mutated, characteristic of an affinity-matured antibody, exhibiting 76–94% nucleotide and 65–90% amino acid identity to four different heavy chain germ lines as the closest germ-line gene. Three of the four variable heavy chains belonged to the dominant VH3 subgroup, whereas the last belonged to the VH1 subgroup. The variable light chain genes were also mutated, but to lesser degree than the heavy chain genes, exhibiting 90–98% amino acid identity to the closest germ-line gene. Somatic mutations in the framework regions (FRs) and complementarity-determining regions (CDRs) were analyzed by measuring the replacement (R) to silent (S) mutation ratio (R/S ratio) for the CDRs (CDR1 and CDR2) and FRs (FR1, FR2, and FR3), which were 1.0–3.0 and 0.6–4.3 for the heavy chain and >1.0–>6.0 and <1.0–2.0 for the light chain, respectively.

**DISCUSSION**

We investigated the subcellular distribution of annexin XI using human autoantibodies cloned by phage display and showed that annexin XI associates with the tubulin cytoskeleton in a Ca$^{2+}$-dependent manner. Annexin XI and tubulin colocalized in mitotic cells to the mitotic spindles and in interphase cells corresponding to the cytoplasmic cytoskeletal network. The association of annexin XI with the cytoplasmic tubulin-based cytoskeletal network was particularly distinct in the presence of high Ca$^{2+}$ concentrations, whereas it was disrupted in cells treated with EGTA. The mitotic spindles, built of microtubules, function as the framework that allows the chromosomes to be segregated during mitosis (reviewed in Refs. 26 and 27). This process is highly dynamic and involves the activity of a variety of microtubule-based motor proteins, many of which have been identified. Staining of mitotic cells with antibodies specific for these proteins often yields distinct staining patterns that suggest their function. Interestingly, annexin XI staining was observed corresponding to the full length of the kinetochore microtubules rather than only on one end, as seen with some associating proteins. In addition, annexin XI was found overexpressed in dividing cultured cells, as determined by confocal microscopy and ELISA. Although the precise role of annexin XI in mitosis remains unclear; annexin XI might have a stabilizing/destabilizing effect on the kinetochore microtubules, thus influencing the catastrophe rate (27).

Annexin XI has been shown to be nuclear in some cell types and cytoplasmic in others. The nuclear localization of annexin XI has been shown to be cell type-specific and developmentally dependent (5). Transient expression of N-terminally-deleted annexin XI in COS-7 cells showed cytoplasmic localization, in contrast to intact and C-terminally-deleted annexin XI, which exhibited a nuclear localization in this cell type, demonstrating...
that the N-terminal domain of annexin XI is required for nuclear localization (6). In this report, we show that the nuclear localization of intact annexin XI is Ca\(^{2+}\)-dependent. In COS-7 cells, annexin XI in the presence of high concentrations of Ca\(^{2+}\) was localized in the cytoplasm corresponding to the tubulin network, whereas in EGTA-treated COS-7 cells, annexin XI was predominantly nuclear. Our results suggest that the observed cell type-specific and developmentally dependent nuclear localization of annexin XI may relate to the subcellular Ca\(^{2+}\) concentration of these cells.

A series of studies have shown that annexin XI binds calcyclin in a Ca\(^{2+}\)-dependent manner, and the calcyclin-binding site has been localized to the N-terminal part of rabbit annexin XI, corresponding to amino acids 49–66 (24). The annexin XI/calcyclin binding was examined in vitro using assays such as liposome coprecipitation and Western blots of cells transfected with annexin XI and calcyclin. The lack of appropriate reagents, however, has prevented in vivo analysis of annexin XI/calcyclin interactions in different cell lines (23, 28). We found calcyclin associated with the nuclear envelope in nondividing cells. During mitosis, where the nuclear envelope disassembles, calcyclin also gradually disappeared and was absent in metaphase cells. Interestingly, it has been found that calcyclin regulation is also cell cycle-dependent and is up-regulated in various cutaneous tumors (29). In contrast, annexin XI was found to be either predominantly cytoplasmic or nuclear. Although the two proteins were consistently present in a large panel of cultured cell lines tested, no colocalization was observed, even when the Ca\(^{2+}\) concentration was varied. This does not rule out the possibility that annexin XI/calcyclin interactions may play an important biological role in vivo, but it shows that the interaction is a rare event, probably related to a distinct biological function. As discussed above, the N-terminal of annexin XI is necessary for its nuclear localization. Because there are no traditional nuclear localization signals within the N-terminal of the annexin XI, it could be that Ca\(^{2+}\)-dependent interaction with calcyclin is required for annexin XI to enter the nucleus.

Immunoprecipitation studies of fractionated neutrophil vesicles indicate that annexin XI translocates from the cytosol to the specific granules in Ca\(^{2+}\)-dependent manner, as seen during activation (8, 9). We examined the subcellular distribution of annexin XI in fixed and permeabilized neutrophils using fluoro-Nanogold-labeled Fab AN5-17 and electron microscopy. Interestingly, annexin XI was localized within distinct granules, while other granules and the cytosol were unstained. From their size and morphology, these granules seem to correspond to specific granules, although we were unable to confirm this. Neutrophils are very sensitive cells, and it seems likely that they were activated during cell purification, in agreement with earlier observations. Our ultrastructural analysis also showed strong staining in eosinophils corresponding to the specific granules surrounding the crystalloids. Eosinophils, they appear less microbicidal. The specific granules con-

![Image](59x556_to_563x737)

**Fig. 6.** Immunoelectron micrograph of eosinophils (A), neutrophils (B), and HEp-2 cells (C) stained with fluoro-Nanogold-labeled anti-annexin XI Fab AN5-17. A, in eosinophils, the silver-enhanced gold particles were concentrated in the specific granules consisting of crystalloids (arrows), whereas no staining was found elsewhere in the cytoplasm or nucleus. B, in neutrophils, the silver-enhanced gold particles were evenly distributed in the cytoplasm (arrowheads), whereas other vesicles were unlabeled. C, in contrast, silver-enhanced gold particles were found to be either predominantly cytoplasmic or nuclear. Although the two proteins were consistently present in a large panel of cultured cell lines tested, no colocalization was observed, even when the Ca\(^{2+}\) concentration was varied. This does not rule out the possibility that annexin XI/calcyclin interactions may play an important biological role in vivo, but it shows that the interaction is a rare event, probably related to a distinct biological function. As discussed above, the N-terminal of annexin XI is necessary for its nuclear localization. Because there are no traditional nuclear localization signals within the N-terminal of the annexin XI, it could be that Ca\(^{2+}\)-dependent interaction with calcyclin is required for annexin XI to enter the nucleus.

**Table 1**

Comparison of the nucleotide and deduced amino acid sequences of the heavy chain variable (V\(_H\)) and light chain variable (V\(_L\)) domains of the cloned annexin XI-specific Fabs with the closest germline sequences demonstrates the frequency of silent (S) and replacement (R) mutations.

| Clone   | Closest germline | Amino acid homology (%) | Nucleotide homology (%) | R/S ratio FRs | R/S ratio CDRs | JH or JL usage |
|---------|------------------|-------------------------|-------------------------|---------------|---------------|----------------|
| AN5-14 VH | VH3-9            | 90                      | 94                      | 7/4: 1.8      | 2/2: 1.0      | JH5            |
| AN5-17 VH | VH3-48           | 88                      | 91                      | 4/7/0.6       | 7/4: 1.8      | JH4            |
| AN5-20 VH | IGHV-1L1         | 65                      | 76                      | 23/16: 1.4    | 16/8: >2.0    | JH1            |
| AN4-16 VH | VH3-23           | 74                      | 86                      | 17/4: 4.3     | 12/4: 3.0     | JH4            |
| AN5-14 VL | A27              | 98                      | 98                      | 0/1: <1.0     | 2/0: >2.0     | JK5            |
| AN5-17 VL | A27              | 94                      | 96                      | 3/2: 1.5      | 2/1: 2.0      | JK1            |
| AN5-20 VL | L1               | 90                      | 95                      | 4/2: 2.0      | 6/0: >6.0     | JK3            |
| AN4-16 VL | V2-1             | 98                      | 98                      | 1/1: 1.0      | 1/0: >1.0     | JL2            |

\(^\text{a}\) In addition, the percent homologies, in terms of nucleotide and amino acid sequence as compared to the closest germline sequence, are shown. The anti-annexin XI IgG Fabs were highly somatically mutated and exhibited high R/S ratios.
tain a variety of hydrolytic enzymes, including histaminase. The precise function of annexin XI associated with these specific granules remains uncertain, but it has been suggested to play a role in degranulation (8, 9). In support of this, annexin XI has been shown to be secreted by activated neutrophils in response to an internal calcium signal (10). In addition, annexin XI has been shown to be present inside insulin-containing granules and has been suggested to play a role in insulin release (11). Importantly, both neutrophil and eosinophil plasma membranes contain Fc and complement receptors, which may cause binding of human immunoglobulins independently of the antibody specificity. The use of directly labeled Fab fragments circumvented this problem. These data suggest that whereas annexin XI is localized in the cytoplasm of nonsecretory cells, annexin XI is found inside granules in secretory cells.

The generation of monoclonal antibodies recognizing conformational epitopes to study the biological function of conserved cellular proteins is often difficult, as exemplified by annexin XI, the least changed of all the annexins over the last 500 million years (30). Therefore, because human autoimmune sera recognize such conserved molecules, they have been used for analysis of cellular proteins (31, 32). However, because most autoimmune sera contain antibodies against multiple autoantigens, it is often impossible to determine what component of the autoreactive response results in which cell-staining patterns. Cloning the individual autoantibodies from such autoimmune responses may be valuable, but this approach has been limited by inefficient techniques for generating human monoclonal antibodies. Phage display technology seems to overcome this.

In conclusion, we examined some of the biological features of annexin XI using recombinant human autoantibodies as probes. Our localization data suggest that annexin XI plays a role in cell cycle regulation and vesicle exocytosis. Our results also demonstrate that Ca\(^{2+}\), through its influence on the interaction between annexin XI and multiple molecules, significantly influences the subcellular distribution of annexin XI. The use of the generated recombinant Fabs should permit further exploration of these interactions.

Acknowledgments—We thank Drs. D. R. Burton and R. Fox for fruitful discussions and patient samples and Elizabeth Kompfner and Vivian Benoit for technical assistance.

REFERENCES

1. Creutz, C. E., Pazoles, C. J., and Pollard, H. B. (1978) J. Biol. Chem. 253, 3798–3802
2. Blackwood, R. A., and Ernst, J. D. (1990) Biochem. J. 266, 195–200
3. Francis, J. W., Balazovich, K. J., Smolen, J. E., Margolis, D. I., and Boxer, L. A. (1992) J. Clin. Invest. 90, 537–544
4. Emans, N., Gerver, J. P., Walter, C., Gerke, V., Kellner, R., Griffiths, G., and Grunenberg, J. (1993) J. Cell Biol. 120, 1357–1369
5. Mamiya, N., Iino, S., Mizutani, A., Kobayashi, S., and Hidaka, H. (1994) Biochem. Biophys. Res. Commun. 202, 403–409
6. Mizutani, A., Watanabe, N., Kitao, T., Tekumitsu, H., and Hidaka, H. (1995) Arch. Biochem. Biophys. 318, 157–165
7. Sudo, T., and Hidaka, H. (1999) FEBS Lett. 444, 11–14
8. Sjölin, C., and Dahlgren, C. (1996) Blood 87, 4817–4823
9. Sjölin, C., Movitz, C., Lundqvist, H., and Dahlgren, C. (1997) Biochim. Biophys. Acta 1326, 149–156
10. Boussac, M., and Garin, J. (2000) Electrophoresis 21, 665–672
11. Iino, S., Sudo, T., Niwa, T., Fukasawa, T., Hidaka, H., and Niki, I. (2000) FEBS Lett. 479, 46–50
12. Sudo, T., Mamiya, N., Goto, M., Watanabe, Y., and Hidaka, H. (1996) Biochem. Biophys. Res. Commun. 225, 706–711
13. Tan, E. M., Chan, E. K., Sullivan, K. F., and Rubin, R. L. (1988) Clin. Immunol. Immunopathol. 47, 121–147
14. Nepartstek, Y., and Plotz, P. H. (1985) Annu. Rev. Immunol. 11, 79–104
15. Misaki, Y., Pruijn, G. J. M., van der Kemp, A. W. C. M., and van Venrooij, W. J. (1994) J. Biol. Chem. 269, 4240–4246
16. Misaki, Y., Van Venrooij, W. J., and Pruijn, G. J. M. (1995) J. Rheumatol. 22, 97–102
17. Jorgensen, C. S., Levantino, G., Houen, G., Jacobsen, S., Halberg, P., Ullman, S., Khamashita, M. A., Asmussen, K., Oehlum, P., Jorgensen, M. K., van Venrooij, W. J., and Wik, A. (2000) Lupus 9, 515–520
18. Barbas, C. F., III, Kang, A. S., Lerner, R. A., and Benkovic, S. J. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 7978–7982
19. Burton, D. R., Barbas, C. F., III, Persson, M. A. A., Koenig, S., Chanock, R. M., and Lerner, R. A. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 10138–10137
20. Haslett, C., Guthrie, L. A., Kopanikar, M. M., Johnston, R. B., and Henson, P. M. (1985) Am. J. Pathol. 119, 101–110
21. Dittrich, H. J., Biele, J. M., Moore, J. P., Sodroski, J., Sullivan, N., Sawyer, L. S. W., Hendry, R. M., Yang, W. P., Barbas, C. F., III, and Burton, D. R. (1995) J. Immunol. 154, 893–906
22. Satoh, H., Nakano, Y., Shihata, H., and Maki, M. (2002) Biochim. Biophys. Acta 1590, 661–677
23. Minami, H., Tekumitsu, H., Mizutani, A., Watanabe, Y., and Hidaka, H. (1992) FEBS Lett. 305, 217–219
24. Sudo, T., and Hidaka, H. (1998) J. Biol. Chem. 273, 6351–6357
25. Stradal, T. B., and Gimona, M. (1999) J. Biol. Chem. 274, 31593–31596
26. Karsten, E., and Vernes, I. (2001) Science 294, 543–547
27. Wittmann, T., Hyman, A., and Desai, A. (2001) Nat. Cell Biol. 3, E28–E34
28. Tekumitsu, H., Mizutani, A., Minami, H., Kobayashi, R., and Hidaka, H. (1992) J. Biol. Chem. 267, 8919–8924
29. Brink, U., Osihi, H. J., Zeng, F.-Y., Gerke, V., Lazareu, D., Zografski, C., Tsambaos, D., and Berger, H. (1996) J. Dermatol. Sci. 10, 181–190
30. Morgan, R. O., Bell, D. W., Testa, J. R., and Fernandez, M. P. (1998) Genomics 48, 100–110
31. Morø, Y., Peesely, C., Fritzler, M. J., Steigerwald, J., and Tan, E. M. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 1627–1631
32. Whitehead, C. M., Winkfein, R. J., Fritzler, M. J., and Rattner, J. B. (1996) Arthritis Rheum. 39, 1635–1642