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Expression of Carbohydrate-Binding Protein p33/41 in Human Tumor Cell Lines

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We previously reported a new type of lectin, p33/41 (annexin IV), which was isolated from a bovine tissue extract [Kojima, K. *et al.* (1992) *J. Biol. Chem.* 267, 20536-20539]. When the expression of p33/41 (annexin IV) was surveyed in the lysates of 39 human tumor cell lines by SDS-PAGE, followed by Western blot analysis with polyclonal anti-bovine p33/41 and monoclonal anti-annexin IV (Z016, Zymed) antibodies, 21 cell lines were found to be reactive with the polyclonal antibody, whereas all 39 cell lines were stained with Z016. These results together with those obtained with standard proteins, annexins IV and V, suggested that the monoclonal antibody, Z016, recognizes annexin V, but not p33/41 (annexin IV). Therefore, we performed cDNA cloning of human p33/41 (annexin IV) to prepare a recombinant protein and raised monoclonal antibodies against the protein. Northern blot analysis with the cDNA as a probe showed that a human colon cancer cell line, HT29, contains p33/41 (annexin IV) mRNA of two sizes, 2.0 and 3.0 kb. The two monoclonal antibodies, AS11 and AS17, against the recombinant protein generated were useful for flow cytometric analysis, ELISA, Western blot analysis and immunoprecipitation. Flow cytometric analysis with AS17 showed that p33/41 (annexin IV) is located in the cytoplasm of HT29 cells, but not on the cell surface. However, one of the cell surface proteins first labeled with biotin and then solubilized with a detergent was immunoprecipitated with AS17. The results suggest the existence of a membrane spanning form of p33/41 (annexin IV).

Key words: annexin, lectin, monoclonal antibody, tumor cell line.

A number of researchers have investigated the carbohydrates that play important roles in various biological phenomena on the cell surface such as cell-cell interactions, cell adhesion, and tumor metastasis. To completely elucidate such biological phenomena, however, it is essential to identify the biological molecules that recognize these carbohydrates. The most predominant molecules among them are carbohydrate-binding proteins, lectins. Most animal lectins have been classified into two types, C-type lectins and S-type lectins (also called galectins). Each lectin has a carbohydrate recognition domain with a specific consensus amino acid sequence (*1, 2*). Various C-type lectins with a membrane spanning domain have been found to be responsible for some of the above-mentioned phenomena, whereas the functions and secretion mechanism of S-type lectins containing no signal peptide have not been completely elucidated.

We previously reported that the protein, p33/41, isolated from bovine tissues is a carbohydrate-binding protein specific to sialoglycoproteins and various glycosaminoglycans, including heparan sulfate, and is identical to annexin IV with no consensus sequence of the C-type or S-type lectins (*3, 4*).

The annexins are a family of structurally related proteins that bind to phospholipids in a calcium-dependent manner. Their expression has been demonstrated in many organisms from mammals to molds and plants. In mammals, several different annexin gene products are expressed in various cells other than red blood cells (*5-7*). Their exact biological functions *in vivo* have not yet been completely elucidated, although a number of *in vitro* experiments indicated that some annexins exhibit membrane channel activity, inhibit phospholipase A_2 and blood coagulation, transduce mitogenic signals, and are involved in membrane-cytoskeleton interactions [reviewed by P. Raynal and H.B. Pollard (*8*)]. In addition, the primary structures of annexins have been intensively investigated. Annexins are composed of 4 or 8 repeating domains of around 70 amino acids and an N-terminal domain specific for each type. The fact that annexins have no signal peptide coincides with the observation that they are abundant in the cytoplasm, *i.e.*, they are intracellular proteins (*9-12*).

Our histochemical studies involving a specific polyclonal

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Abbreviations: AMV, avian myeloblastoma virus; CBB, Coomassie Brilliant Blue; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; IPTG, isopropyl-1-thio-β-D-galactoside; ORF, open-reading frame; PVDF, polyvinylidene difluoride; sulfo-NHS-biotin, *N*-hydroxysulfosuccinimide ester of biotin.

antibody against p33/41 (annexin IV) showed that p33/41 (annexin IV) is localized in specific cells within specific tissues, *e.g.*, the apical plasma membrane of renal epithelial cells in the proximal tubules (*13*). Mollenhauer *et al.* reported that annexin V is located on the cell surface and functions as a receptor for collagen of the extracellular matrix (*14*). Massey *et al.* reported that annexin IV is located in the basolateral plasma membrane of intestinal absorbing cells, hepatocytes, and pancreatic acinar cells (*15*). Yeatman *et al.* showed, by flow cytometric analysis with monoclonal anti-annexin antibodies, that annexins are expressed on the tumor cell surface and that these antibodies inhibit tumor cell metastasis (*16*), although one of the monoclonal antibodies used, anti-annexin IV (Zymed), was found not to be specific for annexin IV, as described later in this report. All these results raised the possibilities that annexins are secreted outside of cells, are located on the cell surface, and function as receptors for free glycoconjugates, which reside on the surface of other cells or on extracellular matrices besides collagen. Therefore, it is now important to determine how annexins are expressed in certain cells, transported across the plasma membrane, and localized on the cell surface, and what their actual ligands are. Monoclonal antibodies specific to individual annexins are essential for these purposes. At present, however, only two monoclonal antibodies, Z016 and CPIII16-5, are available on the market as anti-annexin IV monoclonal antibodies.

In the course of our study on the expression of p33/41 (annexin IV) in 39 human tumor cell lines, however, both the monoclonal antibodies, Z016 and CPIII16-5, were found not to recognize p33/41 (annexin IV). Therefore, we performed cDNA cloning of p33/41 (annexin IV) in the human colon adenocarcinoma cell line, HT29, and prepared two monoclonal antibodies, AS11 and AS17, against the recombinant human p33/41 (annexin IV). The two monoclonal antibodies obtained were found to be useful in ELISA, Western blot analysis, immunoprecipitation and flow cytometric analysis. These antibodies provided some important results that suggested for the first time the presence of a membrane spanning form of p33/41 (annexin IV), and are expected to be essential for further studies on the biological functions of p33/41 (annexin IV).

MATERIALS AND METHODS

Cells—We established 5 breast cancer cell lines, HBC-4, HBC-5, HBC-7, BSY-1, and BSY-2, from tumors of patients in the Cancer Institute Hospital, Tokyo; HBC-4 from an adenocarcinoma tubulare medullare of a 72-year-old female, HBC-5 from an adenocarcinoma papillotubulare of a 53-year-old female, HBC-7 from an adenocarcinoma scirrhousm of a 40-year-old female, and BSY-1 and BSY-2 from a pleural exudate of a 56-year-old female with an adenocarcinoma solid tubulare. MDA-MB-231 and SK-BR-3 were purchased from the American Type Culture Collection (Rockville, MD). Stomach cancer cell lines MKN-1, 7, 28, and 45 were described in Ref. *17*. The other cell lines were provided by Dr. Robert Shoemaker, Division of Cancer Treatment, Frederick Cancer Research and Development Center, N.C.I. (Frederick, MD) (*18, 19*).

Antibodies—Polyclonal anti-bovine p33/41 (annexin IV) antibodies were prepared and characterized as previously described (*3*). Horseradish peroxidase (HRP)-conjugated affinity purified goat anti-mouse IgG and anti-rabbit IgG were purchased from Bio-Rad and Kirkegaard & Perry Labs, respectively. The monoclonal anti-annexin IV antibodies (Z016) were from Zymed. For flow cytometric analysis, the fluorescein isothiocyanate (FITC)-conjugated mAb, clone 187.1, rat anti-mouse IgG κ chain was used.

Cell Culture—All the human tumor cell lines were grown as cell monolayers in RPMI 1640 medium supplemented with 5% heat-inactivated fetal calf serum (FCS) without antibiotics. All cells were cultured at 37°C under a humidified atmosphere containing 5% CO_2. At 80% confluency, the tumor cell monolayers were passaged by treatment with 0.125% trypsin, 2 mM EDTA, and phosphate-buffered saline (PBS) at 37°C, and then harvested within 5 min in fresh medium. All cells used in these experiments were free of mycoplasma infection.

Preparation of Cell Lysates—Cell lysates were prepared by the method of Yamori *et al.* (*20*). At 80% confluency, tumor cell monolayers were washed with PBS and then frozen with liquid nitrogen. The monolayers were solubilized with 1 ml of 10 mM Tris-HCl buffer (pH 7.5) containing 50 mM $NaVO_3$, 30 mM NaP_2O_7, 50 mM NaF, 50 mM NaCl, 5 mM EDTA, 100 kat/ml aprotinin (Sigma), 1 mM phenylmethanesulfonyl fluoride (PMSF), and 0.5% NP-40 (PI buffer). The lysates were centrifuged at 35,000 rpm in a Beckman TLA-45 rotor for 20 min at 100,000 $\times g_{av}$ and the supernatants were collected. The protein concentrations of the lysates were determined with a TONEIN-TPII (Otsuka Pharm., Tokyo).

SDS-PAGE and Immunoblot Analysis—SDS-PAGE and immunoblot analysis were performed as previously described (*3, 4*). Briefly, the lysates were dissolved in SDS-sample buffer [0.1 M Tris-HCl buffer (pH 6.8) containing 2% SDS and 40% glycerol] with or without 2% 2-mercaptoethanol and then separated by SDS-PAGE on 10% gels according to the procedure of Laemmli (*21*). The target proteins in the lysates were detected by SDS-PAGE and subsequent Western blot analysis. After electrophoresis, the proteins were electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Millipore). The membrane was first blocked with 0.3% skim milk and then was incubated with the primary antibody at room temperature for 1 h. The blot was washed three times and subsequently incubated with HRP-conjugated anti-mouse or rabbit IgG at a final dilution of 1:1,000 at room temperature for 1 h. The membrane was washed three times and then developed with 4-chloro-1-naphthol. As the primary antibodies, we used monoclonal antibodies at a final concentration of 5 μg/ml and polyclonal antibodies at a final concentration of 20 μg/ml. The intensity of the color development was measured with a densitometer, model CS-930 (Shimadzu, Kyoto).

cDNA Cloning of Human p33/41 (Annexin IV)—Total RNA was prepared from the human colon cancer cell line, HT29, by the standard guanidium thiocyanate extraction procedure (*22*). Poly(A) RNA was selected by oligo(dT)-cellulose Type 7 (Pharmacia) chromatography, and 5 μg was reverse-transcribed into cDNA with avian myeloblastoma virus (AMV) transcriptase (Life Science). Double-stranded cDNA was prepared by the method of Gubler and Hoffman (*23*). After the addition of the *Eco*RI adapter, the cDNAs were ligated into λgt10 and packaged with a GIGA

PACK GOLD (STRATAGENE) *in vitro*. The phages were plated on *Escherichia coli* NM514. About 1.1×10^5 independent recombinant plaques were obtained. The library was screened by the plaque-hybridization method (*24*) with ^{32}P-random prime labeled oligonucleotides of the bovine p33/41 (annexin IV) open-reading frame (ORF) sequence as a probe. The positive phages were plaque-purified and phage DNAs were extracted. The cDNA inserts were subcloned into the pBluescript II SK$^+$ plasmid for sequencing. Sequence analysis was performed with an A.L.F. DNA sequencer (Pharmacia), and the size of the p33/41 (annexin IV) mRNA was determined by Northern analysis.

Expression and Analysis of p33/41 (Annexin IV) in E. coli—The p33/41 (annexin IV) protein was expressed as a glutathione-*S*-transferase (GST)-p33/41 (annexin IV) fusion protein in *E. coli*. The expression vector, pGEX-2T, was obtained from Pharmacia. For expression of p33/41 (annexin IV), pGEX-2T was first digested with *Eco*RI and then ligated to the 5′ end non-coding region deleted p33/41 (annexin IV) cDNA. *E. coli* strain DH5α was transformed with this plasmid and protein expression was induced by adding a final concentration of 0.1 mM isopropyl 1-thio-β-D-galactoside (IPTG). The protein was isolated from *E. coli* extracts by incubation with glutathione (GSH)-Sepharose 4B (Pharmacia). The isolated protein was separated and analyzed by SDS-PAGE under reducing conditions.

Preparation of mAb to p33/41 (Annexin IV)—BALB/c mice were immunized by subcutaneous injection with 10 μg of the purified GST-p33/41 (annexin IV) fusion protein in Freund's complete adjuvant and boosted 30 days later by subcutaneous injection with 5 μg of the purified GST-p33/41 (annexin IV) fusion protein in Freund's incomplete adjuvant. After an additional 20 days, *i.e.*, 3 days before fusion, 2.5 μg of purified GST-p33/41 (annexin IV) in PBS was administered by intravenous injection. Splenocytes from the immunized BALB/c mice were fused with X63Ag8.653 myeloma cells in 50% polyethylene glycol 4000. Hybrid cells were selected in RPMI 1640 medium containing hypoxanthine/aminopterin/thymidine. Hybridomas producing antibodies against p33/41 (annexin IV) were screened by differential ELISA using GST-p33/41 (annexin IV) and GST as antigens, and subsequently tested by Western blot analysis with GST-p33/41 (annexin IV) and GST. Cells from positive wells were subcloned twice by limiting dilution in the presence of BALB/c mouse peritoneal macrophages as feeder cells. Ascitic fluid was produced by intraperitoneally injecting hybridomas into BALB/c mice primed 1-week earlier with pristane. The IgG fractions were purified from the ascites fluid by ammonium sulfate precipitation, followed by affinity chromatography on protein A-Sepharose (Pharmacia).

DNA Transfection and Flow Cytometric Analysis—cDNA was ligated to the *Pst*I-*Kpn*I site of the expression vector, pcDL-SRα296 (*25*). The plasmid DNA (2.5 μg) was purified by CsCl density-gradient centrifugation and transfected by the spheroplast fusion method (*26*) into COS-1 cells that had been seeded at 1×10^5 cells/60 mm tissue culture dish. The cells at 48–72 h post transfection were used in the following studies. One million cells were collected and washed with PBS. For cytoplasmic staining, the cells were fixed with 1% formalin and permeabilized with 0.1% Triton X-100. The cells were incubated with the hybridoma conditioned medium as the primary antibody, and then washed with PBS and treated with the FITC-anti mouse IgG κ chain. The cells were washed with PBS and then resuspended in 100 μl of PBS. Flow cytometric analysis was performed with a flow cytometer, model FACScan (Becton-Dickinson). Controls comprised cells treated with normal mouse IgG.

Cell Surface Biotinylation and Immunoprecipitation—Cell surface biotinylation was performed as described by Groettrup *et al.* (*27*). In brief, cells were washed four times with PBS containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$ [PBS(+)], resuspended in 1.5 ml of a freshly prepared solution of 0.5 mg/ml of *N*-hydroxylsulfosuccinimide ester of biotin (sulfo-NHS-biotin) (Pierce), and then agitated at 4°C for 30 min. The cells were washed four times with 25 mM lysine and their viability was confirmed by trypan blue exclusion to be >97%. The cells were solubilized with 1 ml of PI buffer. The lysates were centrifuged at 35,000 rpm in a Beckman TLA-45 rotor for 20 min at $100{,}000\times g_{av}$, and the supernatants were collected. Antibodies and protein A-Sepharose were added to the supernatants, and the mixtures were agitated at 4°C for 4 h. The Sepharose beads were washed four times with PI buffer. The immune complexes on the beads were dissolved in SDS-sample buffer and then separated by SDS-PAGE. The gels were blotted onto a PVDF membrane. The membrane was blocked and incubated with streptavidin-alkaline phosphatase conjugate (Vector) and washed three times, and then the blots were developed with a NBT/BCIP detection system (Amersham).

RESULTS

Screening of p33/41 (Annexin IV) in Human Tumor Cell Lines—The expression of p33/41 (annexin IV) in tumor cells was surveyed in the lysates of 39 human tumor cell lines by SDS-PAGE and subsequent Western blot analysis involving polyclonal anti-bovine p33/41 (annexin IV) and monoclonal anti-annexin IV (Z016, Zymed) antibodies. Bands stained with polyclonal anti-bovine p33/41 (annexin IV) antibodies and monoclonal antibody Z016 were observed at similar but clearly different positions, corresponding to those of 33–35 kDa proteins (data not shown). The bands observed with polyclonal anti-bovine p33/41 (annexin IV) antibodies migrated further than those observed with monoclonal antibody Z016. The results are shown as the relative intensities of the stained bands in Fig. 1. The results obtained with polyclonal anti-bovine p33/41 (annexin IV) antibodies were not identical to those with monoclonal antibody Z016. Lysates of 21 cell lines were stained with polyclonal anti-bovine p33/41 (annexin IV) antibodies, whereas all 39 cell lines were stained with monoclonal antibody Z016; *i.e.*, the lysates of 21 cell lines were stained with both antibodies. The results suggested that the antibodies recognize different proteins. Although p33/41 (annexin IV) was expressed differently in the various cell lines (as detected with anti-bovine p33/41 (annexin IV) antibodies) (Fig. 1), certain patterns of expression were found in histologically similar cell types, *i.e.*, cell lines of adenocarcinomas such as lung adenocarcinoma A549, colon adenocarcinoma HT29, WiDr, and ovarian adenocarcinoma SK-OV-3.

cDNA Cloning of p33/41 (Annexin IV)—Southern blot analysis of human genomic DNA was performed with

^{32}P-labeled cDNA of bovine p33/41 (annexin IV) prior to cDNA cloning. Only one band, with a size of about 8 kb, was detected with the probe for the *Eco*RI digests of genomic DNA (data not shown). The size of the mRNA coding for human p33/41 (annexin IV) was determined by Northern blot analysis. Total RNAs from human colon cancer cell line HT29 and human erythroleukemia cell line K562 were separated in an agarose gel containing formaldehyde, blotted onto a GENE SCREEN PLUS membrane (Du Pont), and subsequently hybridized to the probe (Fig. 2). Strong staining was detected for total RNA and poly(A) RNA of HT29, while no staining was detected for the total RNA of K562. The probe hybridized to two mRNA species of about 2.0 and 3.0 kb. The two mRNA species were also detected in human normal tissues and other carcinoma cell lines (data not shown). These values were larger than those previously described, 1.4 and 2.5 kb (*28*). After hybridization of the HT29 cDNA library with the probe, 22 positive plaques were found on duplicate filters. Phage DNAs of the positive plaques were isolated and digested with *Eco*RI, and the sizes of the cDNA inserts were determined by agarose gel electrophoresis. Only one *Eco*RI insert could be detected in each phage. After the cDNA inserts had been subcloned into the pBluescript II SK$^+$ plasmid, the complete cDNA sequence of p33/41 (annexin IV) was determined with an A.L.F. DNA sequencer. The size of the 2.0 kb cDNA (1,972 bases) is in close agreement with the estimated length of its mRNA (Fig. 2). The cDNA with the insert size of 2.0 kb had the complete open reading frame sequence for human annexin IV (about 1.0 kb) previously described (*29*), followed by a 3′ non-coding region with a longer poly(A) sequence than that previously described (*29*). A rare, but efficient polyadenylation signal (ATTAAA; Wickens (*30*)) spans nucleotides 1954-1959. The mRNA of p33/41 (annexin IV) stabilized by the efficient poly(A) signal may be the reason why p33/41 (annexin IV) is highly expressed in these cell lines. These sequence data for human p33/41 (annexin IV) are available in the EMBL/GenBank DDBJ under accession number, D78152.

Expression of p33/41 (Annexin IV) in E. coli—The *E. coli* expression vector, pGEX, was used to express human p33/41 (annexin IV) in *E. coli*. The vector expresses a recombinant protein in the form of a GST-fusion protein. The 2.0 kb human p33/41 (annexin IV) cDNA modified so as to delete the stop codon in the 5′ untranslated region was ligated to pGEX-2T, and the vector was transformed in *E. coli* DH5α. Protein expression was induced with IPTG and then the expressed protein was purified by affinity chromatography on a GSH-Sepharose 4B column. The fusion

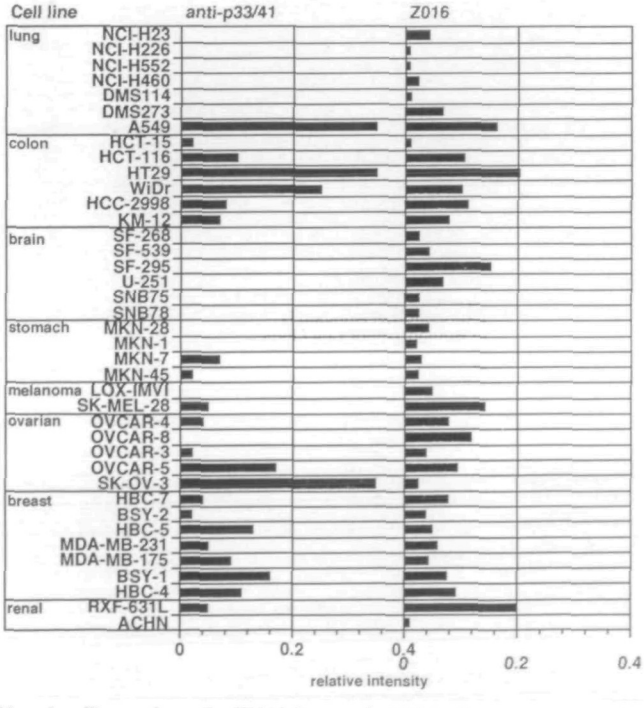

Fig. 1. **Screening of p33/41 (annexin IV) in human tumor cell lines.** Human tumor cells were grown in RPMI 1640 medium supplemented with 5% FCS. The cells were solubilized with the lysis buffer containing 0.5% NP-40 Sample solutions containing 40 μg of protein from a cell lysate per lane were subjected to SDS-PAGE. The separated target proteins were detected by immunoblot analysis with polyclonal anti-bovine p33/41 antibodies and monoclonal antibody Z016 (Zymed). The relative reactivities to the antibodies were expressed as the relative intensities of the color development to that in the control experiment; 1 4 μg of bovine p33/41 (annexin IV) IV= 1 for polyclonal anti-bovine p33/41 (annexin IV) antibodies, and 1.0 μg of bovine annexin V=1 for monoclonal antibody Z016.

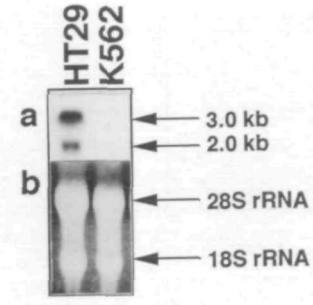

Fig 2 **Northern analysis of p33/41 (annexin IV) mRNA.** Ten micrograms of total RNA from human colon adenocarcinoma cell line HT29 and human erythroleukemia cell line K562, respectively, was probed for the polynucleotide of the ORF sequence of bovine p33/41 (annexin IV) 1 0 kb cDNA The RNAs were stained with ethidium bromide in the gel prior to blotting (b) RNA species of 2.0 and 3 0 kb were detected on hybridization in HT29 but not in K562 cells (a)

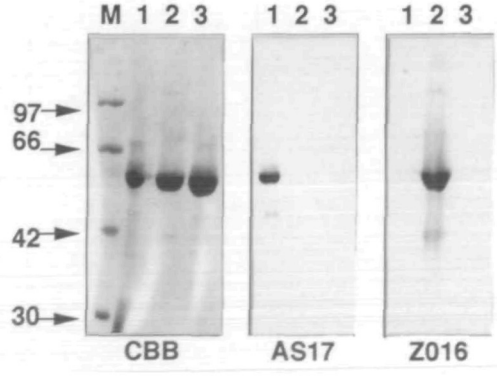

Fig. 3. **Reactivities of antibodies to annexins on immunoblot analysis.** Lane M, molecular mass standards; lane 1, GST-human p33/41 (annexin IV); lane 2, GST-bovine annexin V; lane 3, GST-bovine p33/41 (annexin IV). The fusion proteins were separated by SDS-PAGE under non-reducing conditions, blotted, and then stained with CBB, AS17, or Z016. Each lane was loaded with 5 μg (for CBB staining) or 1 μg (for Western blotting) of protein

protein obtained exhibited a size of 60 kDa on SDS-PAGE. The GST-p33/41 (annexin IV) fusion protein cross-reacted with the polyclonal anti-bovine p33/41 (annexin IV) antibody (data not shown). Computer sequence analysis demonstrated that the amino acid sequence of human p33/41 (annexin IV) was 91.4% identical to that of bovine p33/41 (annexin IV).

Preparation and Properties of Monoclonal Antibodies against Human p33/41 (Annexin IV)—Twenty-two out of three hundred hybridomas cultured in conditioned media were found to react with GST-p33/41 (annexin IV) but not with GST in the ELISA system, and two of them reacted with GST-p33/41 (annexin IV), as found on Western blot analysis. The two monoclonal antibodies thus established (named AS11 and AS17) belong to the mouse IgG_{2b} family.

AS17 reacted, as found on Western blot analysis, with recombinant human p33/41 (annexin IV), but not with recombinant bovine p33/41 (annexin IV) or annexin V (Fig. 3, AS17), or AS11 (data not shown). Monoclonal antibody Z016 reacted with the recombinant annexin V, but did not recognize the recombinant bovine or human p33/41 (annexin IV), as found on Western blot analysis (Fig. 3, Z016).

Flow cytometric analyses were carried out to determine whether these monoclonal antibodies could recognize p33/41 (annexin IV) in COS cells transfected with p33/41 (annexin IV) cDNA; *i.e.*, whether they reacted to expressed proteins in not only *E. coli* but also COS cells, because it was thought that the latter was a more intact protein (Fig. 4). Cell surface staining of the cDNA-transfected COS cells is shown in Fig. 4, (a) and (b), and cytoplasmic staining shown in (c) and (d). The antibodies used were AS17 for (a) and (c), and Z016 for (b) and (d). The lower figures are the results obtained with mock transfectants. The FACS patterns in (a) and (c) showed that AS17 recognizes only the protein located in the cytoplasm of the COS cells transfected with p33/41 (annexin IV) cDNA. The patterns in (b) and (d) showed that Z016 does not recognize the protein

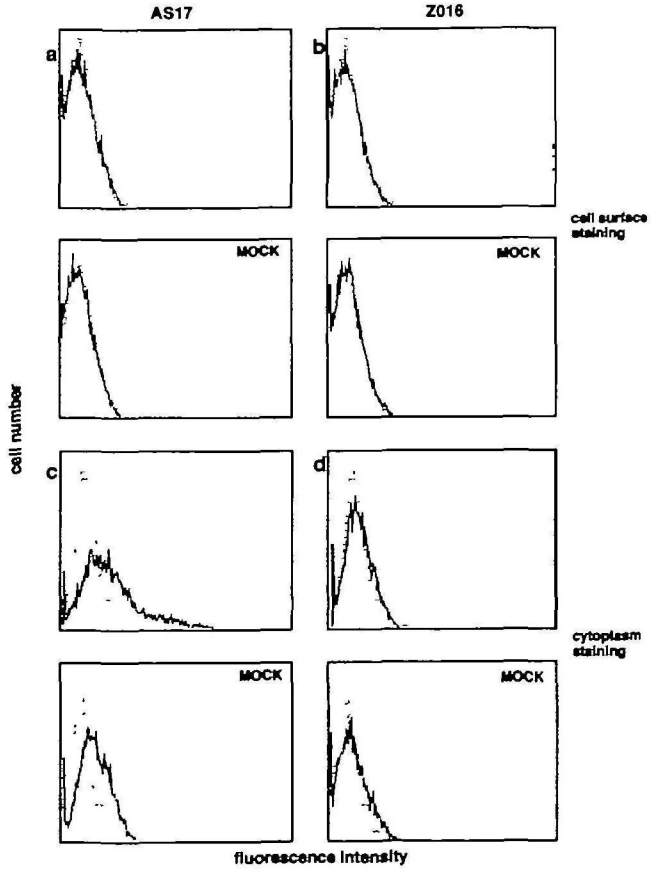

Fig 4. **Flow cytometric analysis of p33/41 (annexin IV) cDNA in transfected-COS cells.** COS cells transfected with cDNA were subjected to cell surface staining, (a) and (b), and cytoplasmic staining, (c) and (d). The p33/41 (annexin IV) cDNA transfectant was analyzed with AS17, (a) and (b), and Z016, (c) and (d). The lower panels are the results of mock transfection. The results are represented by solid lines and those of control experiments involving mouse IgG are represented by dashed lines.

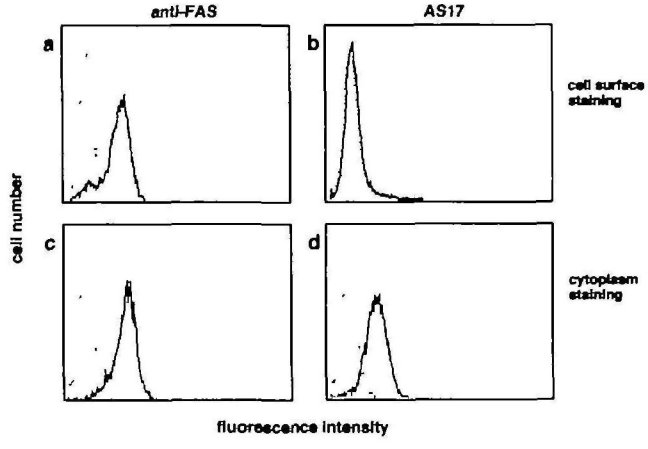

Fig. 5 **Flow cytometric analysis of adenocarcinoma cell line HT29.** Cell surface staining of HT29 is shown in (a) and (b), and cytoplasmic staining in (c) and (d) HT29 was stained with monoclonal antibody AS17, (b) and (d), and the monoclonal anti-Fas antibody as positive controls, (a) and (c) The results are represented by solid lines and those of control experiments involving normal mouse IgG are represented by dashed lines.

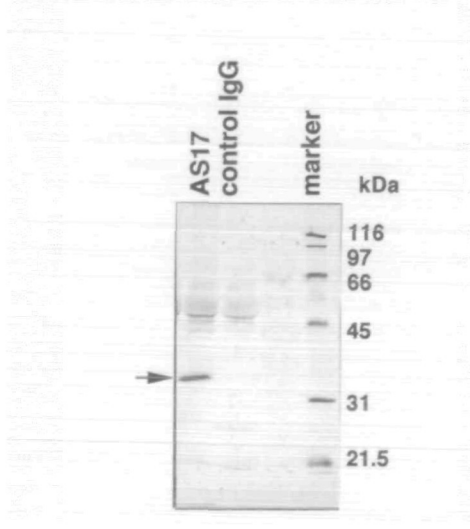

Fig. 6 **Analysis of cell surface proteins of HT29 with monoclonal antibody AS17.** The cell surface proteins of HT29 were biotinylated, lysed, and then immunoprecipitated with monoclonal antibody AS17 or normal mouse IgG. The precipitates obtained were analyzed by SDS-PAGE under reducing conditions. The biotinylated proteins were detected by color development with streptavidin-alkaline phosphatase.

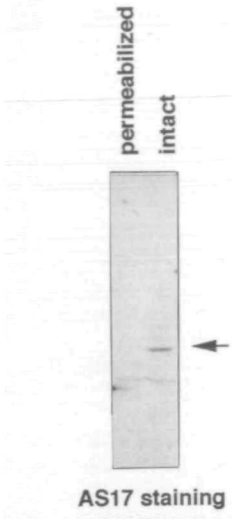

Fig 7. **Immunoblot analysis of p33/41 (annexin IV) in biotinylated HT29 cell lysates.** Intact cells and permeabilized cells were separately biotinylated and then solubilized with the lysis buffer containing 0 5% NP-40 Sample solutions containing 40 μg of protein from a cell lysate per lane were subjected to SDS-PAGE under reducing conditions The separated target protein was detected by immunoblot analysis with AS17.

expressed in the COS cells transfected with p33/41 (annexin IV) cDNA. The same results were obtained with CPIII16-5 (monoclonal anti-annexin IV antibody, BIODESIGN) (data not shown). AS17 was also successfully used for immunoprecipitation (see below), as was AS11 (data not shown).

Cellular Localization of p33/41 (Annexin IV) in Adenocarcinoma Cell Line HT29—Flow cytometric analysis using AS17 was performed on a human colon adenocarcinoma cell line, HT29 (Fig. 5). The antibody against the Fas antigen, a tumor cell surface marker, was used as a positive control. Intact HT29 cells were not stained with AS17; *i.e.*, p33/41 (annexin IV) was not detected on the surface of the intact HT29 cells (Fig. 5b). When the cells were treated with Triton X-100 to make the plasma membrane permeable, the cytoplasm was strongly stained with AS17 (Fig. 5d). The results may simply indicate that p33/41 (annexin IV) is expressed only in the cytoplasm of HT29, *i.e.*, not on the plasma membrane. To confirm this, the cell surface proteins were analyzed after labeling with sulfo-NHS-biotin, which reacts with ϵ-amino groups of lysine residues on cell surface proteins. The biotinylation of HT29 was performed on intact HT29 cells (cell surface protein biotinylation) and on permeabilized HT29 cells treated with Triton X-100 (cytosolic protein biotinylation). After lysis of the cells, p33/41 (annexin IV) was immunoprecipitated with AS17 and then analyzed by SDS-PAGE. The gel was then blotted onto a PVDF membrane and the biotinylated proteins were reacted with streptavidin-alkaline phosphatase for visualization. The 33 kDa biotinylated cell surface protein was detected on intact cells, as shown in Fig. 6, but not on permeabilized cells (data not shown). Furthermore, the lysates obtained from cells biotinylated by means of the two different procedures were also submitted to immunoblot analysis with AS17. As shown in Fig. 7, an AS17-sensitive band was clearly observed for the lysate of the surface biotinylated HT29 cells, but not for that of the HT29 cells biotinylated after permeabilization, indicating that AS17 recognizes neither the membrane p33/41 (annexin IV), whose cytosolic part is biotinylated, nor the biotinylated cytosolic p33/41 (annexin IV). In other words, these results suggest the existence of a membrane spanning type of p33/41 (annexin IV), whose epitope for AS17 is located in the cytoplasmic domain and contains the lysyl residue important for the interaction with AS17.

DISCUSSION

A number of monoclonal antibodies to annexin family proteins are available on the market. One of them, a monoclonal anti-annexin IV antibody (Z016 as well as CPIII16-5), has been most widely used in various studies on tissue distribution and cellular localization (*16, 31-33*). However, in the course of our study on p33/41 (annexin IV) expression in human tumor cells, it was found that the monoclonal anti-annexin IV antibodies available on the market do not recognize p33/41 (annexin IV). It appears from the results in Figs. 1 and 2 that Z016 as well as CPIII16-5 (data not shown) recognized annexin V, and annexin V is ubiquitously expressed in various human tumor cell lines whereas p33/41 (annexin IV) is mainly expressed in adenocarcinoma cell lines. Therefore, we cloned the p33/41 (annexin IV) cDNA of human tumor cells, produced the recombinant protein in *E. coli*, and raised monoclonal antibodies against the protein. Two of the monoclonal antibodies were found to be useful for ELISA and Western blotting of p33/41 (annexin IV), flow cytometric analysis of the COS cells transfected with human p33/41 (annexin IV) cDNA, and immunoprecipitation. The monoclonal antibodies obtained recognized human p33/41 (annexin IV), but not human annexin V, which has an amino acid sequence 58.6% identical to that of human p33/41 (annexin IV).

Annexin family proteins have been called by various common names (*34*). Annexin IV has been called by many different names: endonexin I, protein II, chromobindin 4, 32.5K-calelectrin, placental anticoagulant protein-II, placental protein PP4-X, 35-β-calcimedine, and lipocortin IV. Annexin IV has also been reported to exhibit a number of *in vitro* and biological activities: anticoagulant activity, mediation of exocytosis, composition of cytoskeleton, and regulation of chloride ion channels (*35-38*). We showed that a carbohydrate binding protein, p33/41, is identical to annexin IV; *i.e.*, it binds carbohydrate in a calcium-dependent manner (*4*). The recombinant protein produced in this study by human p33/41 (annexin IV) cDNA exhibits calcium-dependent carbohydrate binding activity, as does bovine p33/41 (annexin IV) (data not shown). Since carbohydrates are mostly located on the outside of cells, to function as a carbohydrate binding protein, p33/41 (annexin IV) must be transported to the plasma membrane and then be finally translocated onto the cell surface or be extracellularly secreted.

The results of flow cytometric analysis of HT29 showed that p33/41 (annexin IV) is located in the cytoplasm, not on the cell surface. However, after biotinylated cells had been solubilized with a detergent, one of the cell surface proteins labeled with biotin was immunoprecipitated with the

monoclonal antibodies, and in a control experiment involving permeabilized cells no such biotin-labeled protein was observed. Furthermore, an AS17-sensitive band was clearly observed for the lysates of cell surface biotinylated HT29 cells, but not for that of the HT29 cells biotinylated after permeabilization (Fig. 7). These results suggested that p33/41 (annexin IV) of HT29 cells is located not only in the cytoplasm as a soluble form but also in the plasma membrane as a membrane spanning form, and the epitope against the antibody is located on the cytoplasmic side of the membrane p33/41 (annexin IV), not on the cell surface side. These results suggest for the first time the existence of a membrane spanning form of p33/41 (annexin IV).

In spite of that annexins as well as galectins (S-type lectins) have no known leader sequence required for their import into the lumen of the endoplasmic reticulum (*39*), some of them have been shown to be secreted or located on the cell surface (*40, 41*). Annexins including annexin IV exist in a soluble form and two membrane protein forms: a peripheral protein form which binds to the membrane in a calcium-dependent manner, and an intrinsic membrane protein form which is resistant to calcium chelators but can be solubilized with detergents (*13, 42*). The crystal structure of annexin IV has not yet been elucidated, while those of annexins I, V, and VII have been reported (*43-45*). Certain annexins other than annexin IV been reported to exhibit calcium channel activity (*46*). For the mechanism underlying the activity, a central hydrophilic pore and surrounding four-helix bundles oriented perpendicular to the membrane have been proposed. However, the lengths of the alpha helices reported for the crystal structures are shorter than those required for known membrane spanning helices. Therefore, the conformations of the intrinsic membrane form of annexin and the mechanisms involved in annexin translocation across the plasma membrane are of great interest.

Further questions to be answered are whether the membrane spanning form is a major stable form among the intrinsic membrane form proteins, and whether it retains the carbohydrate binding activity or has to be further transferred onto the cell surface to exhibit the activity. Our monoclonal antibody should be useful for *in vitro* investigations, including ones on the mechanisms of annexin IV penetration and translocation across the membrane.

REFERENCES

1. Drickamer, K. (1988) Two distinct classes of carbohydrate-recognition domains in animal lectins. *J. Biol. Chem.* **263**, 9557-9560
2. Samuel, H.B., Douglas, N.W.C., Michael, A.G., and Hakon, L. (1994) Galectins. *J. Biol. Chem.* **269**, 20807-20810
3. Kojima, K., Ogawa, H.K., Seno, N., and Matsumoto, I. (1992) Affinity purification and affinity characterization of carbohydrate-binding proteins in bovine kidney. *J. Chromatogr.* **597**, 323-330
4. Kojima, K., Ogawa, H.K., Seno, N., Yamamoto, K., Irimura, T., and Matsumoto, I. (1992) Carbohydrate-binding proteins in bovine kidney have consensus amino acid sequence of annexin family proteins. *J. Biol. Chem.* **267**, 20536-20539
5. Smallwood, M.F., Gurr, S.J., McPherson, M.J., Roberts, K., and Bowles, D.J. (1992) The pattern of plant annexin gene expression. *Biochem. J.* **281**, 501-515
6. Schlaepfer, D.D., Fisher, D.A., Brandt, M.E., Bode, H.R., Jones, J.M., and Haigler, H.T. (1992) Identification of a novel annexin in *Hydra vulgaris*. Characterization, cDNA cloning, and protein kinase C phosphorylation of annexin XII. *J. Biol. Chem.* **267**, 9529-9539
7. Clark, G.B., Dauwalder, M., and Roux, S.J. (1992) Purification and immunolocalization of an annexin-like protein in pea seedlings. *Planta* **187**, 1-9
8. Raynal, P. and 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
9. Raynal, P., Van Bergen en Henegouwen, P.M.P., Hullin, F., Ragabthomas, J., Fauvel, J., Verkleij, A., and Chap, H. (1992) Morphological and biochemical evidence for partial nuclear localization of annexin 1 in endothelial cells. *Biochem. Biophys. Res. Commun.* **186**, 432-439
10. Schlaepfer, D.D., Bode, H.R., and Haigler, H.T. (1990) Expression of annexins as a function of cellular growth state. *J. Cell Biol.* **111**, 229-238
11. Sun, J., Salem, H.H., and Bird, P. (1992) Association of annexin V with mitochondria. *FEBS Lett.* **314**, 425-429
12. Koster, J.J., Boustead, C.M., Middleton, C.A., and Walker, J.H. (1993) The sub-cellular localization of annexin V in cultured chick-embryo fibroblasts. *Biochem. J.* **291**, 595-600
13. Kojima, K., Utsumi, H., Ogawa, H.K., and Matsumoto, I. (1994) Highly polarized expression of carbohydrate-binding protein p33/41 (annexin IV) on the apical plasma membrane of epithelial cells in renal proximal tubules. *FEBS Lett.* **342**, 313-318
14. Mollenhauer, J., Bee, A., Lizarbe, M.A., and Von der Mark, K. (1984) Role of anchorin CII, a 31,000-mol-wt membrane protein, in the interaction of chondrocytes with type II collagen. *J. Cell Biol.* **98**, 1572-1578
15. Massey, D., Traverso, V., Rigal, A., and Maroux, S. (1991) Cellular and subcellular localization of annexin IV in rabbit intestinal epithelium, pancreas and liver. *Biol. Cell* **73**, 151-156
16. Yeatman, T.Y., Updyke, T.V., Kaetzel, M.A., Dedman, J.D., and Nicolson, G.L. (1993) Expression of annexins on the surfaces of non-metastatic and metastatic human and rodent tumor cells. *Clin. Exp. Metastasis* **11**, 37-44
17. Motoyama, T., Hojo, H., Suzuki, T., and Oboshi, S. (1979) Evaluation of the regrowth assay method as an in vitro drug sensitivity test and its application to cultured human gastric cancer cell lines. *Acta Med. Biol.* **27**, 49-63
18. Stinson, S.F., Alley, M.C., Kopp, W.C., Fiebig, H.-H., Mullendore, L.A., Pitman, A.F., Kenney, S., Keller, J., and Boyd, M.R. (1992) Morphological and immunocytochemical characteristics of human tumor cell lines for use in a disease-oriented anticancer drug screen. *Anticancer Res.* **12**, 1035-1054
19. Alley, M.C., Scudiero, D.A., Monks, A., Hursey, M.L., Czerwinski, M.J., Fine, D.L., Abbott, B.J., Mayo, J.G., Shoemaker, R.H., and Boyd, M.R. (1988) Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. *Cancer Res.* **48**, 589-601
20. Yamori, T., Iizuka, Y., Takayama, Y., Nishiya, S., Iwashita, S., Yamazaki, A., Takatori, T., and Tsuruo, T. (1990) Insulin-like growth factor I rapidly induces tyrosine phosphorylation of a M_r 150,000 and a M_r 160,000 protein in highly metastatic mouse colon carcinoma 26 NL-17 cells. *Cancer Res.* **51**, 5859-5865
21. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
22. Chirgwin, J.J., Przbyla, A.E., MacDonald, R.J., and Rutter, W.J. (1987) Isolation of biologically active ribonucleic acid from sources enriched in ribonucleases. *Biochemistry* **18**, 5295-5300
23. Gubler, U. and Hoffman, B.J. (1983) A simple and very efficient method for generating cDNA libraries. *Gene* **25**, 263-269
24. Lathe, R. (1985) Synthetic oligonucleotide probes deduced from amino acid sequence data. *J. Mol. Biol.* **183**, 1-12
25. Takebe, Y., Seiki, M., Fujisawa, J., Hoy, P., Yokota, K., Arai, K., Yoshida, M., and Arai, N. (1988) SRα promoter: An efficient and versatile mammalian cDNA expression system composed of the simian virus 40 early promoter and the R-U5 segment of human T-cell leukemia virus type 1 long terminal repeat. *Mol. Cell. Biol.* **8**, 464-472
26. Seed, B. and Aruffo, A. (1987) Molecular cloning of the CD2

antigen, the T-cell erythrocyte receptor, by a rapid immunoselection procedure. *Proc. Natl. Acad. Sci. USA* **84**, 8573-8577
27. Groettrup, M., Ungewiss, K., Azogui, O., Palacios, R., Owen, M.J., Hayday, A.C., and von Boehmer, H. (1993) A novel disulfide-linked heterodimer on pre-T cells consists of the T cell receptor β chain and a 33 kd glycoprotein. *Cell* **75**, 283-294
28. Grundmann, U., Amann, E., Abel, K.-J., and Küpper, H.A. (1988) Isolation and expression of cDNA coding for a new member of the phospholipase A_2 inhibitor family. *Behring. Inst. Mitt.* **82**, 59-67
29. Tait, J.F., Smith, C., Frankebberry, D.A., Miao, C.H., Adler, D.A., and Disteche, C.M. (1992) Chromosomal mapping of the human annexin IV (ANX4) gene. *Genomics* **12**, 313-318
30. Wickens, M. (1990) How the messenger got its tail: Addition of poly(A) in the nucleus. *TIBS* **15**, 277-281
31. Eberhard, D.A., Brown, M.D., and VandendBerg, S.R. (1994) Alteration of annexin expression in pathological neuronal and glial reactions. *Am. J. Pathol.* **145**, 640-649
32. Sjoelin, C., Stendahl, O., and Dahlgen, C. (1995) Calcium-induced translocation of annexins to subcellular organelles of human neutrophils. *Biochem. J.* **300**, 325-330
33. Blanchard, S., Waker, J.H., and Vaughan, P.F.T. (1993) Expression of annexins in the human neuroblastoma SH-SY5Y. *Biochem. Soc. Trans* **21**, 315S
34. Crumpton, M.J. and Dedman, J.R. (1990) Protein terminology tangle. *Nature* **345**, 212
35. Grundmann, U., Amann, E., Abel, K.-J., Bohn, H., Löbermann, H., Lottspeich, F., and Küpper, H.A. (1988) Characterization of cDNA encoding human placental anticoagulant protein (PP4): Homology with the lipocortin family. *Proc. Natl. Acad. Sci. USA* **85**, 3708-3712
36. Zaks, W.J. and Creutz, C.E. (1991) Ca^{2+}-dependent annexin self-association on membrane surfaces. *Biochemistry* **30**, 9607-9615
37. Gerhard, W. and Reinhard, S.-A. (1990) Collagen-binding proteins of mammary epithelial cells are related to Ca^{2+}- and phospholipid-binding annexins. *J. Cell. Physiol.* **144**, 511-522
38. Kaetzel, M.A., Chan, H.C., Dubinsky, W.P., Dedman, J.R., and Nelson, D.J. (1994) A role of annexin IV in epithelial cell function. *J. Biol. Chem.* **269**, 5294-5320
39. Whitney, P.L., Powell, J.T., and Sanford, G.L. (1986) Oxidation and chemical modification of lung β-galactoside-specific lectin. *Biochem. J.* **238**, 683-689
40. Cooper, D.N.W. and Barondes, S.H. (1990) Evidence for export of a muscle lectin from cytosol to extracellular matrix and for a novel secretory pathway. *J. Cell Biol.* **110**, 1681-1891
41. Rubaratelli, A., Bajetto, A., Allavena, G., Wollman, E., and Sitia, R. (1992) Secretion of thioredoxin by normal and neoplastic cells through a leaderless secretory pathway. *J. Biol. Chem.* **267**, 24161-24164
42. Bianchi, R., Giambanco, I., Ceccarelli, P., Pula, G., and Donato, R. (1992) Membrane-bound annexin V isoforms (CaBP33 and CaBP37) and annexin VI in bovine tissues behave like integral membrane proteins. *FEBS Lett.* **296**, 158-162
43. Weng, X., Luecke, H., Song, I.S., Kang, D.S., Kim, S.-H., and Huber, R. (1993) Crystal structure of human annexin I at 2.5 Å resolution. *Protein Sci.* **2**, 448-458
44. Concha, N.O., Head, J.F., Kaetzel, M.A., Dedman, J.R., and Seaton, B.A. (1993) Rat annexin V crystal structure: Ca^{2+}-induced conformational changes. *Science* **261**, 1321-1324
45. Pollard, H.B., Rojas, E., and Burns, A.L. (1992) Synexin (annexin VII) and membrane fusion during the process of exocytotic secretion. *Prog. Brain Res.* **92**, 247-255
46. Berendes, R., Voges, D., Demange, P., Huber, R., and Burger, A. (1993) Structure-function analysis of the ion channel selectivity filter in human annexin V. *Science* **262**, 427-430