

## Immunocytochemical detection of extracellular annexin II in cultured human skin keratinocytes and isolation of annexin II isoforms enriched in the extracellular pool

Alice Shau Ping Ma<sup>1,\*</sup>, Daniel J. Bell<sup>1</sup>, Anshu A. Mittal<sup>1</sup> and Harold H. Harrison<sup>2</sup>

Section of Dermatology, <sup>1</sup>Department of Medicine, and <sup>2</sup>Department of Pathology, The University of Chicago, Chicago, Illinois 60637, USA

\*Author for correspondence

### SUMMARY

Monoclonal antibodies were raised against trypsinized human skin epidermal cells and selected for their staining of the epidermal cells in a cell periphery pattern. One antibody, CP-1, immunoprecipitated a 36 kDa protein that was identified as annexin II heavy chain by microsequencing of a CNBr-generated peptide fragment from the antigen and by cross-identification with another anti-annexin II antibody. In addition to staining a broad cell periphery band in keratinocytes, CP-1 also detected annexin II outside and in between the top layer cells before cell permeabilization. Double-labeling of annexin II and F-actin revealed a distinct topographical relationship between the two, with intercellular annexin II flanked by the submembranously located actin of the juxtapositioned cells. Annexin II was isolated from cultured keratinocytes via immunoaffinity column chromatography in one step, using the same monoclonal antibody CP-1 and was found to be resolved into multiple isoforms when analyzed by

two-dimensional gel electrophoresis. The predominant components of annexin II were basic, with pI of 6.5-8.5, and some of them formed disulfide-linked monomeric multimers under non-reducing conditions. Acidic annexin II isoforms with pI 5.4-5.8 were barely detectable among the total annexin II isolated but were selectively enriched in an extracellular pool created by 0.05% ethylenediaminetetraacetic acid (EDTA) dispersion of the cultured cells into single cell suspensions. Furthermore, they can be separated from the rest of annexin II by using a different elution condition. A 46 kDa protein, the identity of which is unclear, co-eluted with the acidic isoforms in the EDTA washes. These acidic isoforms, which co-eluted with the 46 kDa protein, are suspected of corresponding to the extracellular annexin II detected immunocytochemically.

Key words: annexin II, isoforms, extracellular, immunolocalization, isolation

### INTRODUCTION

In order to gain a better understanding of the interactions among epidermal cells during tissue organization, we raised monoclonal antibodies to detect soluble cell periphery proteins, which may occupy either side of the plasma membrane of these cells. Monoclonal antibodies were raised against intact single-cell suspensions obtained by trypsinization of normal human skin epidermis, and screened by immunolocalization studies on cryostat skin sections and on cultured keratinocytes. One antibody, CP-1, which was not epidermal cell-specific was found to immunoprecipitate a 36 kDa protein, which was coincidentally identified to be annexin II heavy chain. However, in addition to detection in the submembranous region of cultured human keratinocytes on methanol-permeated cells, the antigen was also detected intercellularly on some top layer cells before cell permeabilization. Annexin II's submembranous location but not its extracellular presence has been reported by various investigators (Cheng and Chen, 1981; Cooper and Hunter,

1983; Greenberg and Edelman, 1983; Lehto et al., 1983; Nigg et al., 1983; Radke et al., 1983).

Data on annexin II presented by various groups of investigators, who originally named the protein lipocortin II (Huang et al., 1986; Flower, 1988), calpactin I (Glenney, 1986), p36 (Gerke and Weber, 1984) or chromobindin 8 (Creutz et al., 1983; Ali et al., 1989), have been increasing substantially. The only accepted function of this protein, so far, is its role as chromobindin in aggregating adrenal chromaffin granules to the submembranous region during exocytosis (Creutz, 1992). The annexin family of proteins now consists of more than 22 members (Burgoyne and Geisow, 1989; Barton et al., 1991). These proteins are defined as calcium-dependent phospholipid-binding proteins with four characteristic repeats, each composed of about 70 amino acids and a variable amino terminus (Geisow, 1986; Weber and Johnsson, 1986; Saris et al., 1986; Huang et al., 1986). Because of the abundance of these proteins in multiple mammalian cell types, in addition to the specialized adrenal chromaffin cells, and their presence in other species including

*Drosophila* (Johnston and Sudhof, 1990) and slime molds (Greenwood and Tsang, 1991), it has been speculated that they may participate in some very basic cellular processes involving membranes. Their function in bringing about membrane fusion has been suggested (Creutz, 1992). Annexin II, like many other annexins, is not cell type-specific and has been found to be especially abundant in skin (Huang et al., 1986; Pepinsky et al., 1988). Epidermal keratinocytes have been shown to synthesize lipids that contribute to the barrier function of skin as an organ (Elias and Feingold, 1988). These cells have also been demonstrated to be a source of a number of secretory products (Milstone and Edelson, 1988; Barra et al., 1994), although the modes of secretion remain unclear. The annexins, as a whole, have neither a leading signal sequence nor an extended hydrophobic domain to span the plasma membrane. However, another annexin, annexin V, originally isolated from chondrocyte membrane, has been reported to be found extracellularly (Pfäffle et al., 1988). X-ray crystallography studies of annexin V have found its four characteristic repeats to be arranged roughly in a plane forming a central hydrophilic channel, not unlike that of transmembrane ion channels (Huber et al., 1990, 1992). Furthermore, both annexins V and VII were reported to form voltage-dependent ion channels across synthetic lipid bilayers (Pollard and Rojas, 1988; Karshikov et al., 1992). Because of the sequence homology of the four repeats among the annexins, the three-dimensional structure of annexin V is likely to be similar to that of the other annexins.

Partly because of these intriguing data from previous reports on the extracellular presence of another annexin and partly because of our original goal to investigate the functions of this abundant protein in keratinocytes, we decided to verify further our immunocytochemical detection of extracellular annexin II in cultured keratinocytes. We isolated annexin II from the cultured keratinocytes and examined the isolates from the intact cultures in comparison with those isolated from the artificially created intracellular and extracellular compartments or pools. The cultured cells were first separated into single-cell suspensions with a solution of 0.05% EDTA in PBS. The cation chelator EDTA was chosen to disperse cultured keratinocytes mainly because it had been traditionally used, in addition to trypsin, for the preparation of single-cell suspensions from skin tissue for primary cultures and in the subsequent propagation of the cultured epidermal cells. Trypsin was eliminated because of its potential for degrading proteins in the intercellular region. Previous investigators had reported that the extraction of annexin II from vesicles of intestinal brush border was facilitated by another cation chelator, ethylene glycol-*O,O'*-bis( $\alpha$ -aminoethyl)-*N,N,N',N'*-tetraacetic acid (EGTA) (Gerke and Weber, 1984; Glenney and Glenney, 1985; Glenney, 1986). The significance of magnesium ions in relation to annexin II has not been well documented although the calcium-dependent phospholipid-binding property is a characteristic feature of the annexin family of proteins. Our objective was to find qualitative differences between the annexin II isolated from the intra- and extracellular compartments.

## MATERIALS AND METHODS

### Human tissues and immunological reagents

Foreskin specimens from neonates were obtained from the nursery at

The University of Chicago Hospitals. Specimens of other normal human tissues were obtained from autopsies performed within 24 hours after death. An alternative anti-annexin II heavy chain monoclonal antibody as well as anti-annexins I, IV and VI, and anti-annexin II light chain monoclonal antibodies, were purchased from ICN Bio-medical Inc. Goat anti-mouse antisera and mouse peroxidase anti-peroxidase antibody were purchased from Sternberger-Meyer, and ECL chemiluminescence substrate was a product of Amersham. Rhodamine-phalloidin (Molecular Probe) and avidin-biotin staining kit, avidin-fluorescein and avidin-DCS (Vector) were used for immunofluorescent staining. Pre-immune mice sera were used as negative controls.

### Monoclonal antibody production

Whole-cell preparations of human skin epidermal cells trypsinized from newborn foreskin, were used as antigens in monoclonal antibody production (Ma and Sun, 1986). Briefly, female Balb/c mice were inoculated subcutaneously with  $5 \times 10^4$  cells in PBS, and the mice sera were screened by immunofluorescence staining on frozen skin sections. Mice whose sera yielded staining of skin epidermal cells in skin sections in a cell periphery pattern at a titer higher than 1:500 were sacrificed, and their spleen cells were fused with X63.Ag8.653 mouse myeloma cells in polyethylene glycol (PEG) 1500 and selected in hypoxanthine/aminopterin/thymidine (HAT) medium (Sigma) according to the method described by Kohler and Milstein (1975).

### Cell culture

Human epidermal cells were grown from newborn foreskin, with mitomycin-C-treated 3T3 fibroblasts used as feeder layers according to the method described by Rheinwald and Green (1975). The culture medium contained hydrocortisone (Sigma) at 0.4  $\mu$ g/ml and cholera toxin (Schwarz-Mann) at  $10^{-12}$  M/ml in addition to penicillin and streptomycin (Gibco). Epidermal growth factor (Collaborative Research) at 4 ng/ml was added 3-4 days after plating of the epidermal cells. Cultured cells from passages 2 through 4 were used for biochemical analysis and immunocytochemical staining unless otherwise stated. For immunocytochemical staining, cells were cultured on acid-washed glass coverslips or in multichambered slides (Lab-Tek). MDCK cells (ATCC, ccl 22), grown on coverslips in minimal essential medium with Earle's basal salt solution, and early cultures of human keratinocytes, plated in Keratinocyte Serum-Free medium (Gibco) were also tested in preliminary immunolocalization experiments. Normal human fibroblasts were a gift from Dr Alan Horwitz, University of Illinois.

### Radiolabeling of cultured cells

Radiolabeling experiments were carried out on 90% confluent cultures in 60 mm or 100 mm dishes. The best yield was obtained by addition of [ $^{35}$ S]methionine (Amersham) at 125  $\mu$ Ci/ml in a methionine-deficient medium for 4 hours, followed by a 15 hour overnight chase in normal culture medium, prior to cell lysis.

### Cell lysis and protein extraction, and gel electrophoresis

Cell lysate preparations of cultures, either labeled or unlabeled, were obtained in 50 mM Tris-HCl, pH 7.4, 0.5% NP40, 0.5% sodium deoxycholate, 50 mM NaCl, in the presence of 1 mM EGTA, 1 mM EDTA, 5  $\mu$ g/ml antipain, 5  $\mu$ g/ml pepstatin and 1 mM phenylmethylsulfonyl fluoride on ice, clarified by centrifugation at 100,000 *g* for 1 hour at 4°C, and stored frozen. These lysates were analyzed by SDS-gel electrophoresis (Laemmli, 1970) on 12% gels and stained with Coomassie Blue or silver nitrate. Alternatively, the SDS-gel was electroblotted onto nitrocellulose or Immobilon paper (Millipore), and stained with Fast Green or Coomassie Blue, respectively. The radiolabeled extracts analyzed by SDS-gel electrophoresis were autoradiographed on X-OMAT AR films (Eastman Kodak).

### Immunoprecipitation

Protein A attached to Sepharose CL-4B beads (Sigma), linked to purified CP-1 ascites fluid via rabbit anti-mouse serum (Cappel), was incubated overnight at 4°C with [<sup>35</sup>S]methionine-labeled cell lysate containing 10<sup>6</sup> cpm for each 10 µl of beads. The beads were rinsed extensively with Buffer A consisting of essentially the same solution as that used for the cell lysate preparation but without EDTA, EGTA or anti-proteases. The final rinse was carried out in Buffer B, which was the same as Buffer A except for an increase in NaCl to 1.5 M and the addition of 0.1% SDS. For negative controls, normal mouse serum-linked immunobead preparations were used.

### Immunoaffinity column chromatography

CP-1 antibody in ascites fluid after purification by ammonium sulfate precipitation was coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia) and packed into 1 ml mini-columns. Cell lysates, of unlabeled or radiolabeled cultures of human epidermal cells or EDTA washes, as described below, were passed through the CP-1 antibody-coupled column by means of a peristaltic pump and collected with a fraction collector. The protein content in the fractions was monitored at 280 nm. The column was then extensively rinsed with coupling buffer consisting of 0.1 M NaHCO<sub>3</sub>, pH 8.3, containing 0.5 M NaCl and extensively with Buffers A and B described in the immunoprecipitation experiment until the baseline was reached. The column was then eluted with phosphate-citrate buffer, pH 2.8, for removal of the antigen bound to the antibody attached column. The column was regenerated stepwise with a 0.1 M sodium acetate buffer, pH 4, containing 0.5 M NaCl, followed by 0.1 M Tris buffer, pH 8, with 0.5 M NaCl. The eluted proteins were analyzed by SDS-gel electrophoresis, using silver nitrate stain for assessment of purity. For quantitative purification of antigen, 15 ml Bio-Rad columns containing 5 g of CNBr-activated Sepharose 4B beads bound to 30-45 mg of antibody were used. The purified antigen was extensively dialyzed in double-distilled water and then lyophilized for further characterization.

### Cyanogen bromide cleavage and amino acid sequencing

When the purified 36 kDa protein was subjected to analysis with a Beckman microsequence analyzer, no amino acid residues were obtained, suggesting a block at the amino terminus. The purified protein was then cleaved with cyanogen bromide, and the resulting peptides were separated by SDS-gel electrophoresis and transblotted onto Immobilon paper (Millipore). The peptide bands were cut out and subjected to an Applied Biosystems-478 Protein Sequencer equipped with an on-line Applied Biosystems 128 PTH analyzer. The PTH derivatives were subjected to reverse-phase chromatography on a Brownlee C-18 column. Protein microsequencing was performed in the laboratory of Dr James Tam, Vanderbilt University.

### Subcellular immunolocalization

Methods and fixatives used for subcellular localization were chosen by prior testing on cryostat skin sections by comparing staining on unfixed sections with those fixed with methanol and/or acetone at 4°C or combinations of different concentrations of freshly prepared glutaraldehyde and paraformaldehyde. Methods that yielded the strongest staining were employed. For submembranous or cytoplasmic staining, a brief treatment of the cultured cells gave the best contrast between positive and negative signals although staining was found to diminish in direct relationship to duration of treatment. CP-1 antibody was found to be non-reactive with glutaraldehyde- or paraformaldehyde-treated specimens and, therefore, for detection of cell surface staining a pre-fixative labeling method was adopted. Briefly, human skin keratinocytes cultured on coverslips or chamber slides were rinsed with DMEM. The cells were incubated with undiluted supernatant CP-1 antibody, or purified ascites (10-20 mg/ml) at 1:20 dilution, for 30 minutes at 37°C in a humidified chamber before rinsing. Phosphate buffered saline (PBS) used in rinsing steps and in fixatives contained

2 mM MgCl<sub>2</sub> and 0.1 mM CaCl<sub>2</sub>. The cells were then fixed in 0.5% freshly prepared glutaraldehyde and 1% paraformaldehyde in PBS at room temperature for 20 minutes, followed by 50 mM NH<sub>4</sub>Cl in PBS for 5 minutes before rinsing in PBS. Biotinylated anti-mouse antibody (Vector) was used as secondary antibody and it was followed by avidin-fluorescein with rinsing steps in between. Pre-immune mouse serum at 1:20 dilution or supernatant from the myeloma parent cell line were used as negative controls. Double-labeling experiments with rhodamine-phalloidin staining, for F-actin (Molecular Probe), were carried out by additional treatment with cold acetone to permeate the cultured cells, which had been labeled with CP-1 and fixed with glutaraldehyde and paraformaldehyde as described. To match up with the strong rhodamine staining of actin in this double-labeling experiment, avidin DCS (Vector) was used instead of avidin-fluorescein. For comparison, MDCK cells and keratinocytes grown in Keratinocyte Serum-free medium (Gibco), both monolayer cells were immunostained. Stained slides were mounted on Gelvatol and viewed with a Zeiss photomicroscope III equipped with appropriate barrier filters.

### Cell dispersion by EDTA

Confluent cultures were first rinsed with DMEM and then treated briefly with a solution of 0.05% EDTA in PBS. Most suprabasal keratinocyte cultures could be dispersed into single cells after 15 to 30 minutes. The rest of the basal epithelial cells were removed by pipetting. The collected cells were washed once in DMEM for 5 minutes, and cell viability, assessed with Trypan Blue, was maintained at 95% or above. After low-speed centrifugation (600 g), the pellets obtained were lysed. The EDTA solution and the DMEM used for pellet rinsing were collected and centrifuged sequentially at low speed (600 g) and high speed (100,000 g) for removal of cells or cell debris. The EDTA solutions were neutralized by addition of equal molar concentrations of CaCl<sub>2</sub> (Sigma) and stored frozen for further analysis and for immunoaffinity column chromatography. For comparison, undispersed cell cultures, scraped with a rubber policeman, were also used.

The lysates obtained from the pellets of dispersed cells were used to represent the intracellular compartment. The suspending EDTA washes represented an arbitrarily defined extracellular compartment that includes intercellular proteins released during this artificial dispersion process. For comparison, the lysates of the undispersed cultures, in theory, would represent the total of the intracellular and the extracellular compartments. Annexin II from each compartment was then isolated by immunoaffinity column chromatography and compared by one- and two-dimensional gel electrophoresis and by western immunoblotting. This crude fractionation of cultured cells and the separation of annexin II into intracellular, extracellular and total pools are represented diagrammatically in Table 1.

### Non-equilibrium pH gradient (NEpHG) gel electrophoresis

Two-dimensional, non-equilibrium pH gradient gel electrophoresis was performed as described previously (O'Farrell et al., 1977), using combinations of Servalyte, pH 5-7 and pH 3-10 (Serva). The separated proteins were stained with silver nitrate or transblotted onto nitrocellulose paper (Millipore) and stained with Fast Green.

### High-resolution isoelectric focusing (IEF) gel electrophoresis

High-resolution two-dimensional isoelectric focusing gel electrophoresis was performed, with a combination of ampholytes containing Servalyte, pH 3-0 (Serva), Pharmalyte, pH 3-10 (Pharmacia), and Ampholine, pH 5-7 (LKB), in an ISO-DALT two-dimensional electrophoresis system (Large Scale Biology, Inc.), running for 14,000 V/hour at an average of 700-900 V. The system was employed, since it allowed highly reproducible two-dimensional analysis of protein samples as well as isoelectric focusing. The second-dimension gel was run in SDS-Tris-glycine buffer, pH 8.6 (Harrison et al., 1992).



**Annexin II was also detected extracellularly in immunolocalization**

Using cold methanol-treated fibroblasts grown on coverslips, annexin II was detected submembranously essentially as reported previously (Greenberg and Edelman, 1983; Lehto et al., 1983; Nigg et al., 1983; Radke et al., 1983). En face staining of the methanol-treated cultured keratinocytes showed a broad band pattern at the cell periphery (data not shown). This broad marginal pattern suggests that annexin II is submembranously located. However, intercellular annexin could not be excluded, because no consistent non-staining of intercellular space as found in the immunostaining of cytoplasmic proteins such as keratins was detected (Ma and Lorincz, 1988). Another drawback of using methanol as a fixative was the observation that staining diminished in direct relationship to the duration of treatment.

All small growing cells exhibited much stronger staining than their mature counterparts, as shown both by cold methanol fixation or by the pre-fixation labeling method. Using the pre-fixation labeling method, a much weaker but striking intercellular staining was detected among top layer keratinocytes in passage 3 and 4 cultures. Although the positive signals required higher magnification to be discernible, the clear-cut intercellular staining pattern and some of its cell surface staining features were not detected in negative controls stained with pre-immune mouse serum. Nor was this extracellular presence detected in monolayer keratinocytes grown in Keratinocyte Serum-Free medium (Gibco). MDCK cultures also tested negative.

Double-labeling experiments with pre-fixation labeling for annexin II and acetone treatment for F-actin were then carried out, in an effort to decipher the different patterns of extracellular annexin II staining. Annexin II staining was found occupying the midline between the double-lined actin staining of the juxtapositioned cells (Fig. 3a and b). Some cell surface staining was also observed on some of these top cells.

**Annexin II isoforms**

Annexin II was positively identified in the cell-free supernatant in keratinocyte cultures pulse-chase labeled with [<sup>35</sup>S]methionine (data not shown). It was noted that the quantity of this extracellular annexin II was too small for further analysis. When annexin II isolated from the intra- and extracellular compartments of dispersed keratinocytes were compared by non-

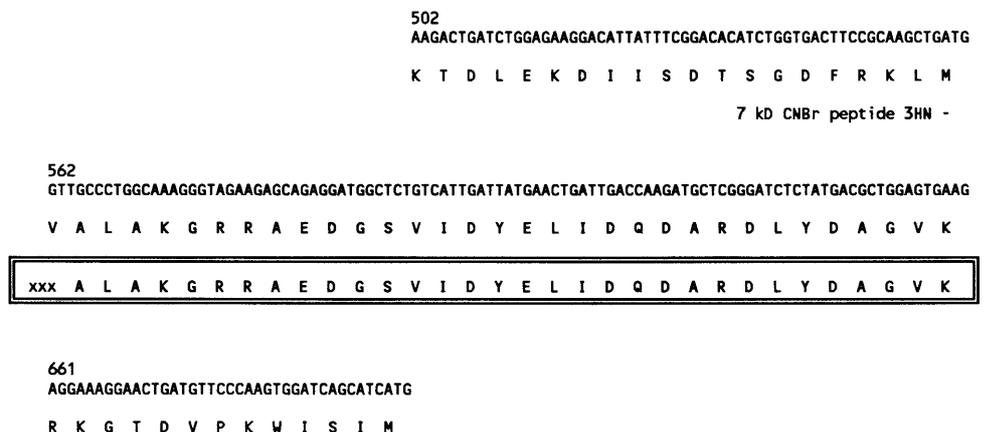
equilibrium pH gradient (NEpHG) gel electrophoresis, their pI values were noted to be consistently different (Fig. 4, I). The Fast Green-stained annexin II from the intracellular compartment occupied a wider range of pI values (Fig. 4, Ib), with the basic isoforms being the major components whereas annexin II from the extracellular compartment occupied only a narrower acidic range (Fig. 4, Ic). When the two fractions were combined in a reversed proportion (Fig. 4, Ia), not only were the pI range and the configuration restored, but the change in the ratio between the basic isoforms to the acidic ones was also evident. This showed that the difference in the pI of the annexin II isolated was not an artifact resulting from smearing due to protein overloading in this gel system. The extracellular annexin II, therefore, differed from the intracellular counterpart by lacking the major basic isoforms. When the isolate from the intracellular compartment was underloaded and the NEpHG gel stained with silver nitrate, the prominent basic spot was further resolved into at least four different isoforms (insert in Fig. 4, Ib).

In order to double check these results and to compare the annexin II isolated with that in the pre-column cell lysate, the highly reproducible system (Harrison et al., 1992) of isoelectric focusing was employed. Annexin II isolated from the intracellular compartment was found to exist in isoforms with the pI of major components at 6.5, 7.5 and 8.5, respectively (Fig. 4, II, lower panel). Minor components with pI between 5 and 6 were barely discernible (Fig. 4, II, lower panel, unmarked). When pre-column cell lysate was analyzed in parallel, the putative annexin II was found to occupy exactly the same spots (Fig. 2, II, upper panel) with a similar configuration. This suggests that the isoform nature of annexin II was not an artifact created by immunoaffinity column chromatography.

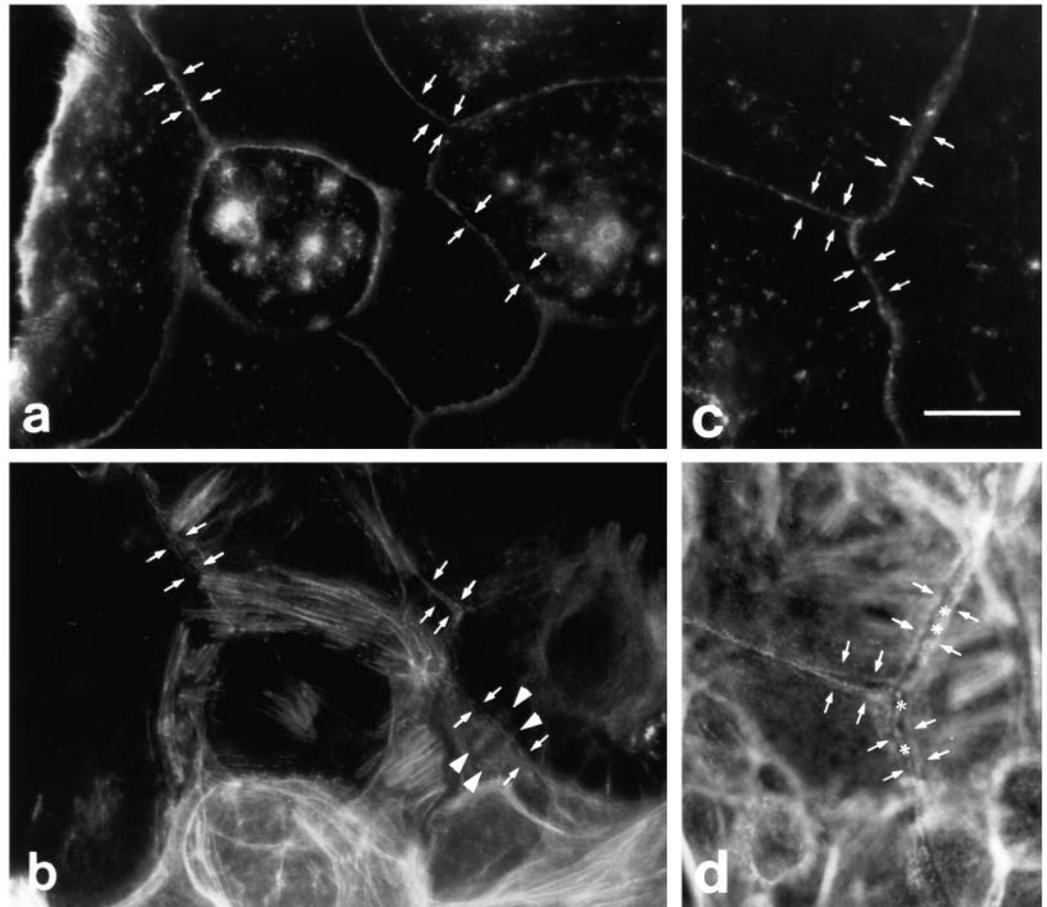
**Further fractionation of annexin II isoforms**

In the aforementioned experiments, all the elution fractions, as shown on chromatographic tracings, were collected and combined for analysis to represent annexin II of each compartment. However, the chromatography tracings were noted to show reproducible intrinsic ratio differences between the individual peaks found in the intra- and extracellular compartments (Fig. 5A and B). While the phosphate-citrate elution constituted a major protein peak, marked a, from the intracellular compartment, the major protein peak from the extracellular compartment was at peak c, when 0.1 M Tris-HCl, pH 8,

**Fig. 2.** Mapping of a CNBr-cleaved 7 kDa peptide fragment from the immunoaffinity-purified 36 kDa protein with human lipocortin II (Huang et al., 1986). N-terminal sequences of the peptide show complete identity with the predicted protein residues 173 to 204 derived from human lipocortin II cDNA (accession no. A23942). The 33-amino-acid segment is denoted by a box, and the first residue not recognized during sequencing is marked as xxx.



**Fig. 3.** Double-labeling with CP-1 by the pre-fixation method (a and c), followed by rhodamine-phalloidin staining for F-actin after cold acetone treatment (in b and d) on near-confluent keratinocyte cultures. In a and c, opposing sets of small arrows point to single fluorescence staining, indicating intercellular annexin II. These single lines of annexin II staining are flanked by double lines of staining by actin, marked by sets of opposing small arrows in b and d. In d, where the intercellular spaces are wider, mid-lines of actin, denoted by asterisks, are also present. Actin staining, as stress fibers (denoted by arrowheads), which appear to be in line and perpendicular to the cell surface joining the adjacent cells (b). Bar, 20  $\mu$ m.



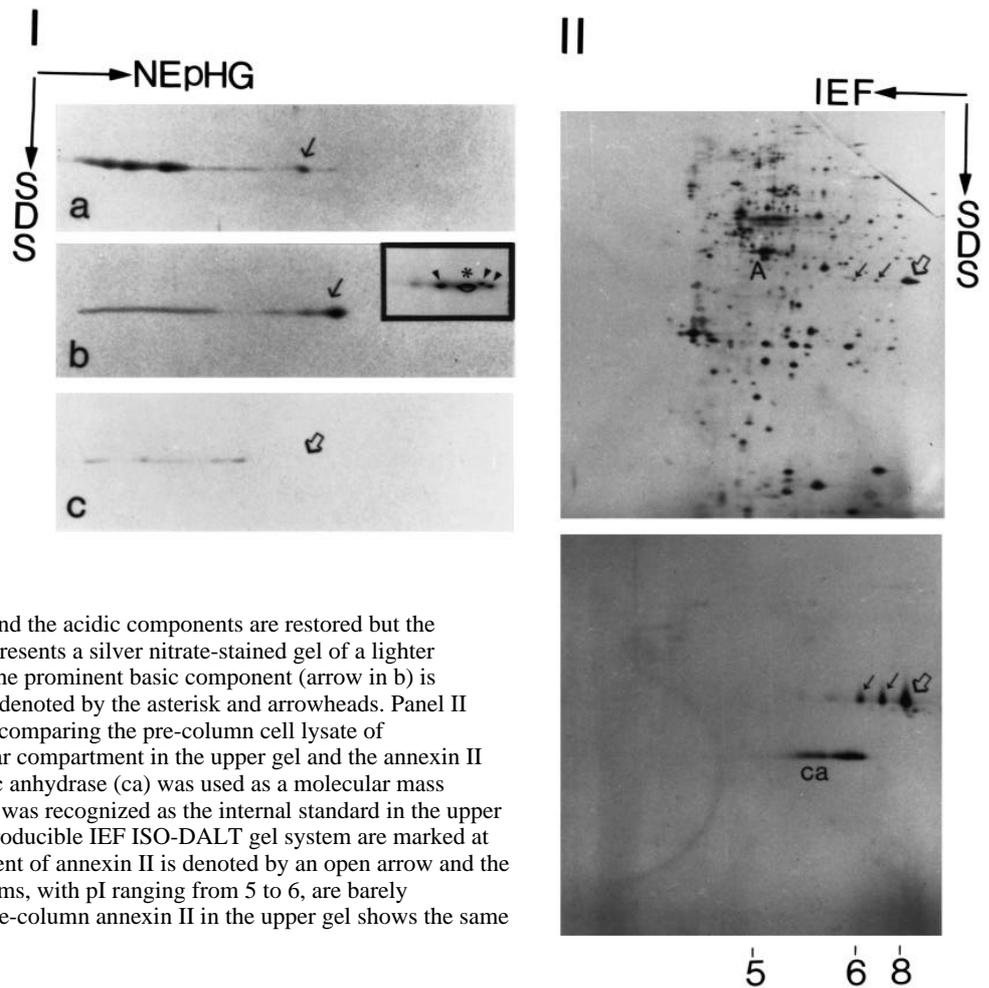
containing 0.5 M NaCl was the elution buffer. Both tracings showed small middle peaks, marked b. Peaks b and c were still not eluted if the use of phosphate-citrate buffer, pH 2.8, was prolonged through several column volumes, indicating that peaks b and c were not a result of incomplete elution.

To distinguish between the components obtained by these different non-specific buffers, the proteins were collected separately from the peaks and further analyzed by one- and two-dimensional electrophoresis. A combination of internal standards consisting of bovine serum albumin (molecular mass 66 kDa, pI 5.4, 5.6) and carbonic anhydrase (molecular mass 29 kDa, pI 5.9, 6.0) were used (Fig. 5C). Due to the twenty-fold difference in the quantities of intracellular isolates as compared to the extracellular pool, the loading of the gels was not proportional to the total quantity eluted. About 1/5 of fraction a of the intracellular compartment was loaded compared to each of the fractions b, c, a', b' and c'. Peak a and peak a' (Fig. 5C) showed essentially the same pI whereas proteins from peaks b and b' (Fig. 5C b and b') showed a minor shift of pI to 8.25 and a blurred configuration when compared with peaks a and a' (Fig. 5C). Heavier loading of peak b from the extracellular compartment showed no change in configuration. The protein from peak c of the extracellular component (Fig. 3c') showed a distinct shift of pI to 5.4-5.8. In addition, a 46 kDa, pI 5.7, protein was conspicuously present. This confirmed our earlier observation that annexin II exists in isoforms (Fig. 4) and ensured the separation of these different annexin IIs by individual collection of the elution peaks. The

acidic isoforms were enriched roughly ten times with the combination of crude cell fractionation followed by elution from an immunoaffinity column.

#### Verification of annexin II isoforms

To reaffirm that the 36 kDa protein collected in each fraction was indeed annexin II heavy chain, an alternate monoclonal antibody to annexin II heavy chain (ICN Biomedical, Inc.) was used in western immunoblotting using chemiluminescence ECL (Amersham) as substrate (Fig. 6). This antibody was found to be not only specific for annexin II in the cell lysate but also sensitive enough to detect small amounts of annexin II present in the supernatant (Fig. 6, III). It was noted that the reactivities to the 36 kDa protein eluted from the three peaks were positive but of disproportional intensity. Further immunoblotting of NEpHG gel analysis of the individual fractions indicated that this anti-annexin II antibody immunoreacted strongly with the major basic component (data not shown). The 46 kDa band detected in peak c of the extracellular compartment was not reactive with anti-annexin II antibody (Fig. 6, II, lane 6'). The panels of elutions also tested negative against anti-annexin II light chain (p10), anti-annexins I (35 kDa), IV (32.5 kDa) and VI (68 kDa) monoclonal antibodies (data not shown). These results indicate that the isoforms isolated were not similar sized annexins cross-reactive to our antibody CP-1. Instead, reactivity to a 56 kDa band protein, proven to be the heavy chain of CP-1 immunoglobulin (Fig. 6, II, lanes a-d) was detected by this



**Fig. 4.** Two-dimensional gel electrophoresis comparison of annexin II isolated from intra- and extracellular compartments of keratinocytes. Panel I shows NEpHG gel electrophoresis analysis of annexin II transblotted onto nitrocellulose paper and stained with Fast Green (except insert in b). Intracellular annexin II is shown in b while extracellular annexin II is shown in c. Note the lack of the prominent basic spot in c (pointed to by open arrow). When an increased quantity of extracellular isolate was combined with the intracellular isolate, as shown in a, the pH range, the prominent basic component (arrow) and the acidic components are restored but the proportion is reversed. The insert in b represents a silver nitrate-stained gel of a lighter loading of intracellular annexin II when the prominent basic component (arrow in b) is resolved into at least four components as denoted by the asterisk and arrowheads. Panel II consists of silver nitrate-stained IEF gels comparing the pre-column cell lysate of keratinocytes representing the intracellular compartment in the upper gel and the annexin II isolated from it in the lower gel. Carbonic anhydrase (ca) was used as a molecular mass standard in the lower gel, while actin (A) was recognized as the internal standard in the upper gel. Estimates of the pI in this highly reproducible IEF ISO-DALT gel system are marked at the bottom. The most prominent component of annexin II is denoted by an open arrow and the other components by arrows. Faint isoforms, with pI ranging from 5 to 6, are barely discernible (unmarked). The presumed pre-column annexin II in the upper gel shows the same configuration as that in the lower gel.

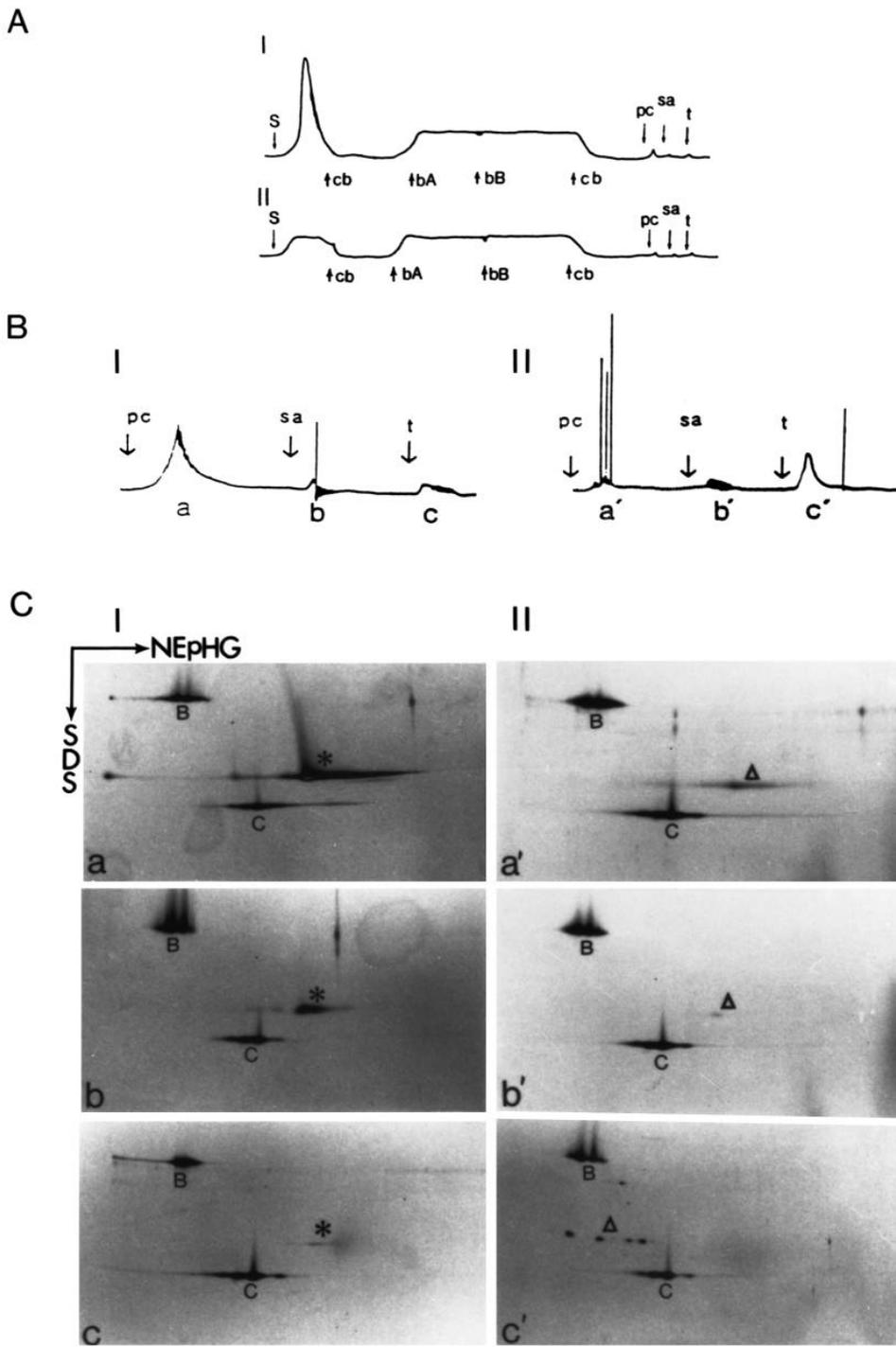
sensitive technique (Fig. 6, II, lanes 5' and 6'), although the immunoglobulin was not detectable by Fast Green (Fig. 6, I, lanes 5 and 6). It was unlikely that the 46 kDa protein was eluted, due to 0.5 M NaCl in the Tris buffer, pH 8, since it did not elute with buffer A and buffer B, which consist of low salt (50 mM NaCl) and high salt (1.5 M NaCl) washes, respectively.

To investigate whether the 46 kDa protein was non-specifically bound to the immune matrix, peak c from the extracellular compartment was collected and passed a second time through the column, and was again subjected to the same buffers in the same sequence. The peak c elution, consisting of the 36 kDa annexin and the 46 kDa protein identified by gel electrophoresis, were again eluted together with Tris buffer, pH 8, passed in the same sequence, while peaks a and b remained void. The three elution fractions collected from lysates of the undispersed cultures again showed the presence of a 46 kDa protein with the 36 kDa annexin II in the third fraction (Fig. 6, I, lanes 7,8 and 9). Immunoblotting of these three fractions showed essentially similar reactivity to anti-annexin II antibody with a strong reaction only to peak a elution (data not shown). It is of interest to note that the ratios of the 46 kDa to the 36 kDa annexin II proteins in the EDTA washes and the undispersed cells (Fig. 6, I, lanes 6 and 9) were different. Western immunoblotting with anti-annexin II antibody was also carried out on the three elution fractions analyzed in SDS-

PAGE, under non-reducing conditions (Fig. 6, IV). Reactive bands were detected not only in peak a but also in peak c elutions, unlike those analyzed under reducing conditions (Fig. 6, I and II). These two fractions reacted to a distinctly different set of higher molecular mass bands (Fig. 6, IV) except for the 160 kDa disulfide-linked immunoglobulin in peaks b and c (lanes 13' and 14' in Fig. 6, IV). The relative lack of a disulfide-linked polypeptide in peak b was also noted, distinguishing it from the other two peaks. The results of the analysis of annexin II isoforms isolated by this crude cell fractionation followed by immunoaffinity column chromatography have been tabulated (Table 2).

## DISCUSSION

In a search for small soluble cell periphery proteins with which to study the organization of epidermal cells, annexin II was coincidentally found. That the 36 kDa antigen identified in the present study is annexin II is indicated by a shared identical 33-residue stretch of amino acid sequence (Wallner et al., 1986; Saris et al., 1986; Huang et al., 1986) from a cyanogen bromide-cleaved peptide of this protein and by cross-identification using another anti-annexin II monoclonal antibody. At variance with previous reports (Cheng and Chen, 1981; Cooper and Hunter, 1983; Greenberg and Edelman,

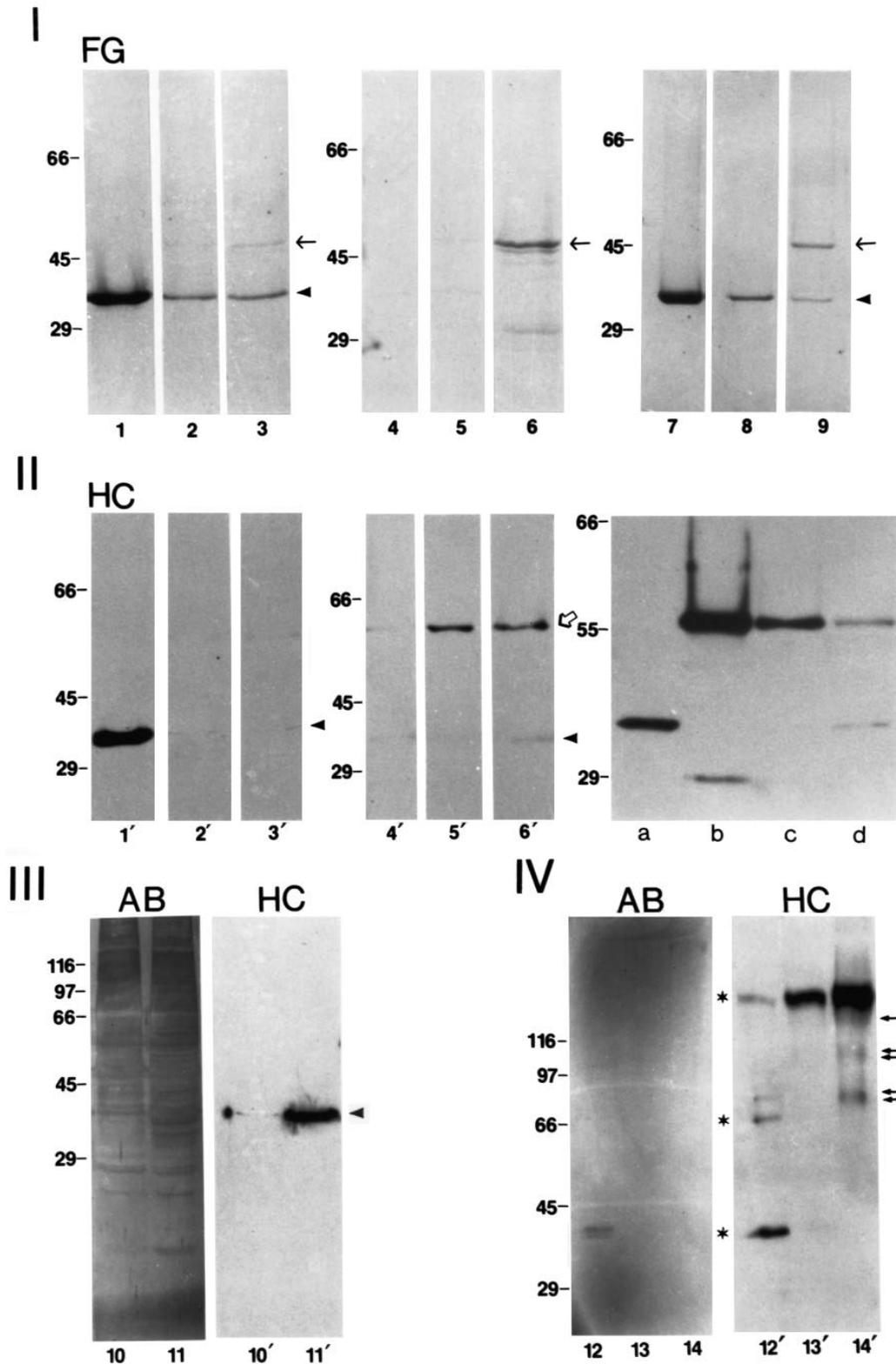


**Fig. 5.** NEpHG gel electrophoresis showing further fractionation of annexin II isolated from the intracellular compartment (marked as panel I) and the extracellular compartment (marked as panel II) of cultured keratinocytes. Chromatography tracings of I and II are shown in A and their respective elution tracings starting with the addition of phosphate-citrate buffer, pH 2.8 (PC), are magnified in B. Arrows in A and B denote addition of cell lysate (S) or the EDTA washes: cb for coupling buffer; bA for buffer A; bB for buffer B; pc for phosphate citrate buffer, pH 2.8; sa for sodium acetate, pH 4; and t for 50 mM Tris buffer, pH 8.2. The three elution peaks collected separately, denoted a, b, c in A and a', b', c' in B, were analyzed by NEpHG gel electrophoresis, stained with silver nitrate, and shown in C, named accordingly. A combination of molecular mass standards added to each fraction are shown as bovine serum albumin, marked B (66 kDa, pI 5.4, 5.6) and carbonic anhydrase, marked C (29 kDa, pI 5.9, 6.0). The 36 kDa protein isolated from the intracellular elution fractions are marked by asterisks and the extracellular ones are marked by triangles. While peak a constitutes the major eluent of the intracellular compartment, as reflected by the protein marked with an asterisk in gel a, peak c' is the major eluent of the extracellular compartment and is represented by the protein marked by a triangle in c'. Note the shift of the 36 kDa protein in c' to the acidic side. A 46 kDa protein is also shown in c'. Both peaks b and b' eluent show a minor shift to the acidic side and a blurred configuration.

1983; Lehto et al., 1983; Nigg et al., 1983; Radke et al., 1983), that annexin II is an exclusively intracellular protein, is the present finding, that it is also detectable intercellularly in stratified keratinocytes.

The lack of extracellular annexin II in previous immunolocalization experiments can be explained in part by the very soluble nature of this amphipathic protein. Extracellular annexin II was found in pre-permeabilized cells only in two subset populations. It was found to be present in all small dividing and newly formed cells. Apart from the brightness of

staining of these small cells, no definite pattern of staining was found. The striking but much weaker intercellular annexin II, when detected, was found among all top layer keratinocytes in passage 3 and 4 cultures where the top polygonal cells assumed larger lateral dimensions. It is unclear how the extracellular annexin II in the small dividing cells is related to the intercellular annexin II in the more differentiated keratinocytes. Double-labeling experiments of annexin II with actin, however, revealed the distribution pattern of extracellular annexin II among the more differentiated cells. Extracellular



**Fig. 6.** Western blotting of the three separate elutions collected from cultured keratinocytes, transblotted onto nitrocellulose paper, stained with Fast Green (FG) or Amido Black (AB) and immunoblotted with anti-annexin II heavy-chain antibody (HC) (ICN Biomedical Inc.) using chemiluminescence (Amersham) as substrate. The three consecutive fractions denoted as peaks a, b and c on the chromatographic tracings shown in Fig. 5 are represented in lanes 1-3, respectively, for the intracellular compartment. The collections from the extracellular compartment shown in tracings a', b' and c' are represented in lanes 4-6. The same three collections obtained from the cell lysates of undispersed cultures are analyzed and represented in lanes 7-9. The 36 kDa annexin II is denoted by an arrowhead and the 46 kDa protein is pointed to by an arrow. Panel II shows the result of a western immunoblot with an alternative anti-annexin II monoclonal antibody (ICN Biomedical, Inc.) on lanes 1-6 shown in panel I. Positive reactions to the 36 kDa band are denoted by arrowheads. The inconsistency in intensity of reaction is noted between lanes 1' and 3'. Lanes a-d represent a western immunoblot to peak a collection from the intracellular compartment (lane a), 10 µg (lanes b) and 5 µg of purified CP-1 ascites fluid (lane c), and peak c collection from the extracellular compartment (lane d). This indicates that the 56 kDa reactive bands in lanes 5' and 6' collections are

due to the presence of monoclonal antibody in the elutions in fractions b' and c'. Panel III shows the positive control of this ICN anti-annexin II antibody on pre-column EDTA washes (lane 10) and cell pellet lysate (lane 11). Only one reactive band on these lanes was detected, showing the specificity and sensitivity of this antibody. Panel IV shows the three collected fractions from undispersed cultures (lanes 12, 13 and 14), electrophoresed under non-reducing conditions and immunoblotted with the same antibody (lanes 12'-14'). The numbers of positive reactive bands in lane 12', denoted by asterisks, are distinctly different from those in lane 14', denoted by arrows.

**Table 2. Analysis of annexin II isoforms isolated by immunoaffinity column**

CP-I immunoaffinity chromatography elution peaks	~pI					Suggested topographical location	Western blots with ICN anti-annexin II	
	5.4	5.8	6.5	7.5	8.5		Reducing conditions	Non-reducing conditions
a. Phosphate-citrate buffer, pH 2.8			●	●	○ ○ ●	Intracellular	Reactive	Reactive to (36-S-S-36) <sub>x</sub>
b. 0.1 M sodium acetate with 0.5 M NaCl, pH 4					○ ○	? Both compartments	Weakly reactive	?
c. 0.1 M Tris with 0.5 M NaCl, pH 8		○				Extracellular	Non-reactive to 46 kDa	Reactive directly to (46-S-S-46) <sub>x</sub> , or indirectly to (46-S-S-36) <sub>x</sub>
	○ ○	○ ○					Weakly reactive to 36 kDa	

annexin II in these cells was found on the side of the cell surface opposite to where submembranous actin was present. This subcellular finding may impart additional significance to reports (Gerke and Weber, 1984; Glenney and Glenney, 1985) on the *in vitro* bundling effect on actin by annexin II and our own finding of annexin II's *in vitro* effect on actin and keratins (Ma et al., 1994). Annexins I and II are the only two annexins reported to bind actin, non-erythroid spectrin (Gerke and Weber, 1984) and fodrin (Cheney and Willard, 1989). Immunohistochemical staining of annexin I, a 35 kDa protein, in skin tissues *in vivo* also suggests its association with cytoskeletal components (Fava et al., 1993). The cellular processes whereby annexin II became translocated to an extracellular position on the opposite side of the plasma membrane from where the submembranous actin is located have not been defined. Such findings would be useful in delineating the functions of this abundant cellular protein situated at the periphery of these skin cells.

We were able to purify annexin II from cultured keratinocytes with CP-1-attached immunoaffinity column chromatography in one step. The annexin II isolated was found to exist in isoforms with their pI ranging from 5.4 to 8.5. The basic isoforms with pI of 6.5-8.5 were the major components of annexin II in cultured epidermal keratinocytes. The acidic isoforms with pI of 5.4-5.8 constituted the main components present in the extracellular pool of annexin II. These acidic isoforms were detectable also in the undispersed cells. This suggests that the acidic annexin II was preferentially released during cell dispersion by EDTA treatment. Isoforms of annexin II have been reported (Cruetz et al., 1987), although the pI of annexin II is recognized to be in the range of 7-9, corresponding to the basic isoforms designated in this study. Modifications of annexin II such as myristylation (Soric and Gordon, 1985) and phosphorylation (Erikson and Erikson, 1980; Cooper and Hunter, 1983; Radke et al., 1983) have been reported, although all these experiments were performed on chicken embryonic fibroblasts transformed by Rous sarcoma virus. Glycosylation of annexin II has been demonstrated by biosynthetic labeling experiments in a human squamous carcinoma cell line, SCC/Y1 (Goulet et al., 1992). The acidic annexin II isoforms isolated from the extracellular compartment in our study differ from the basic isoforms also by their co-elution with a prominent 46 kDa protein.

Under reducing conditions, this 46 kDa protein was non-reactive both to monoclonal antibodies against annexin II (CP-1 and the ICN anti-annexin II antibody), and to those against annexins I, IV and VI in western immunoblots. Neither was this 46 kDa protein significantly detected in the third elution fraction when the cell lysate of the dispersed cells, which consisted of more abundant cellular proteins, was passed through the column. Interaction of this 46 kDa protein non-specifically with the Sepharose beads, for that reason, appeared less likely. When the fraction containing the acidic annexin II isoforms and the 46 kDa protein was passed a second time through the immunoaffinity column, the collected eluate again yielded the two proteins together in the same fraction instead of being separated. This suggests that the 46 kDa protein might be associated with the extracellular acidic annexin II isoforms. Similarly, the results of western blotting of the acidic annexin II co-eluted with 46 kDa analyzed under non-reducing conditions suggest that the two proteins are related to each other. These findings indicate that the extracellular immunocytochemical staining by CP-1 was not due to cross-reactivity to another protein, i.e. the 46 kDa protein, since the latter is related to the acidic annexin II isoforms.

The stepwise elution of various annexin II isoforms from the immunoaffinity column by non-specific elution buffers could be explained by the different degrees of immunobinding of annexin II in its various nascent forms to the immunobeads. Isolation of the three elution fractions and NEpHG gel electrophoresis analysis of each individual fraction showed consistent isoforms of annexin II in each, according to the sequence of elution, irrespective of which compartment was loaded onto the column. If the major protein eluted from each compartment was taken to represent the main annexin II component in the respective compartment and the minor proteins eluted were taken to represent contamination by the other compartment, we would be able to distinguish the extracellular annexin II from the rest. However, the crude cell fractionation method cannot possibly eliminate contamination of one compartment by the contents from the other. Also, the disparity between the quantities of annexin II in the intracellular pool and the extracellular one do not allow one to conclude successfully that the minor components are contaminants. In theory, finding a specific antibody that can distin-

guish between the acidic annexin II and the rest of annexin II would be an ideal way to provide direct evidence.

In conclusion, our studies have revealed that the monoclonal antibody CP-1 identifies annexin II heavy chain. The same antibody immunolocalized annexin II in-between and on the outside of the top layer keratinocytes in culture, before cell permeabilization. The annexin II isolated from these keratinocytes was found to exist in multiple isoforms. The acidic isoforms with pI 5.4-5.8, which co-eluted with a 46 kDa protein, were found to be enriched in the extracellular pool created by using the chelator EDTA. These co-eluted proteins could be purified by using different elution conditions with immunoaffinity column chromatography.

The authors thank Dr Nancy B. Schwartz for her invaluable suggestions and support, Dr James Tam for kindly microsequencing the peptides, Brett Ayers and Gracita Ramos for their technical assistance, Dr Michelle M. LeBeau for allowing use of her fluorescence microscope and Loretta Armstead for typing the manuscript. This work was supported by United States Public Health Service Grant AR-01714, a Dermatology Foundation research grant and the Beedie Scleroderma Foundation.

## REFERENCES

- Ali, S. M., Geisow, M. J. and Burgoyne, R. D. (1989). A role for calpactin in calcium-dependent exocytosis in adrenal chromaffin cells. *Nature* **340**, 313-315.
- Barra, R. M., Fenjves, E. S. and Taichman, L. B. (1994). Secretion of apolipoprotein E by basal cells in cultures of epidermal keratinocytes. *J. Invest. Derm.* **102**, 61-66.
- Barton, G. J., Newman, R. H., Freemon, P. S. and Crumpton, M. J. (1991). Amino acid sequence analysis of the annexin super-gene family of proteins. *Eur. J. Biochem.* **198**, 749-760.
- Burgoyne, R. D. and Geisow, M. J. (1989). The annexin family of calcium-binding proteins. *Cell Calcium* **10**, 1-10.
- Cheney, R. E. and Willard, M. B. (1989). Characterization of the interaction between calpactin I and fodrin (non-erythroid spectrin). *J. Biol. Chem.* **264**, 18068-18075.
- Cheng, Y.-S. E. and Chen, L. B. (1981). Detection of phosphotyrosine-containing 34,000 dalton protein on the framework of cells transformed with Rous sarcoma virus. *Proc. Nat. Acad. Sci. USA* **78**, 2388-2392.
- Clark, D. M., Moss, S. E., Wright, N. A. and Crumpton, M. J. (1991). Expression of annexin VI (p68, 67 kDa-calclectrin) in normal human tissues: evidence for developmental regulation in B- and T-lymphocytes. *Histochemistry* **96**, 405-412.
- Cooper, J. A. and Hunter, T. (1983). Regulation of cell growth and transformation by tyrosine-specific protein kinases: the search for important cellular substrate proteins. *Curr. Top. Microbiol. Immunol.* **107**, 125-161.
- Creutz, C. E., Dowling, L. G., Sando, J. J., Villar Palasi, C., Whipple, J. H. and Zaks, W. J. (1983). Characterization of the chromobindins. *J. Biol. Chem.* **258**, 14664-14674.
- Creutz, C. E., Zaks, W. J., Hamman, H. C., Crane, S., Martin, W. H., Gould, K. L., Oddie, K. M. and Parsons, S. J. (1987). Identification of chromaffin granule-binding proteins. *J. Biol. Chem.* **262**, 1860-1868.
- Creutz, C. E. (1992). The annexins and exocytosis. *Science* **258**, 924-931.
- Elias, P. M. and Feingold, K. R. (1988). Lipid-related barriers and gradients in the epidermis. *Ann. NY Acad. Sci.* **548**, 4-14.
- Erikson, R. L. and Erikson, E. (1980). Identification of a cellular protein substrate phosphorylated by the avian sarcoma virus-transforming gene product. *Cell* **21**, 829-836.
- Fava, R. A., Nanney, L. B., Wilson, D. and King, L. E. Jr (1993). Annexin-I localization in human skin: possible association with cytoskeletal elements in keratinocytes of the stratum spinosum. *J. Invest. Derm.* **101**, 732-737.
- Flower, R. J. (1988). Lipocortin and the mechanism of action of the glucocorticoids. *Br. J. Pharmacol.* **94**, 987-1015.
- Geisow, M. J. (1986). Common domain structure of Ca<sup>2+</sup> and lipid-binding proteins. *FEBS Lett.* **293**, 99-103.
- Gerke, V. and Weber, K. (1984). Identity of p36K phosphorylated upon Rous sarcoma virus transformation with a protein purified from brush borders; calcium-dependent binding to non-erythroid spectrin and F-actin. *EMBO J.* **3**, 227-233.
- Glenney, J. R. Jr and Glenney, P. (1985). Comparison of Ca<sup>++</sup>-regulated events in the intestinal border. *J. Cell Biol.* **00**, 754-763.
- Glenney, J. R. Jr and Tack, B. F. (1985). Amino-terminal sequence of p36 and associated p10: identification of the site of tyrosine phosphorylation and homology with S-100. *Proc. Nat. Acad. Sci. USA* **82**, 7884-7888.
- Glenney, J. R. Jr (1986). Phospholipid-dependent Ca<sup>2+</sup> binding by the 36 kDa tyrosine kinase substrate (calpactin) and its 33 kDa clone. *J. Biol. Chem.* **261**, 7247-7252.
- Glenney, J. R. Jr, Boudreau, M., Galyean, R., Hunter, T. and Tack, B. (1986). Association of the S-100-related calpactin/light chain with the NH<sub>2</sub>-terminal tail of the 36 kDa heavy chain. *J. Biol. Chem.* **261**, 10485-10488.
- Goulet, F., Moore, K. G. and Sartorelli, A. C. (1992). Glycosylation of annexin I and annexin II. *Biochem. Biophys. Res. Commun.* **188**, 554-558.
- Greenberg, M. E. and Edelman, G. M. (1983). The 34 kDa pp60<sup>src</sup> substrate is located at the inner face of the plasma membrane. *Cell* **33**, 767-779.
- Greenwood, M. and Tsang, A. (1991). Sequence and expression of annexin VII of Dictyostelium discoideum. *Biochim. Biophys. Acta* **1088**, 429-432.
- Harrison, H. H., Miller, K. L., Dickinson, C. and Danfeldt, J.A. (1992). Quality assurance and reproducibility of high-resolution two-dimensional electrophoresis and silver staining in polyacrylamide gels. *Amer. J. Clin. Path.* **97**, 97-104.
- Huang, K. S., Wallner, B. P., Mattliano, R. J., Tizard, R., Burne, C., Frey, A., Hession, C., McGray, P., Sinclair, L. K., Chow, E. P. C., Browning, J. L., Ramachandran, K. L., Tang, J., Smart, J. E. and Pepinsky, R. B. (1986). Two human 35 kD inhibitors of phospholipase A<sub>2</sub> are related to substrates of pp60<sup>src</sup> and of the epidermal growth factor receptor/kinase. *Cell* **46**, 191-199.
- Huber, R., Schneider, M., Mayr, I., Römisch, J. and Paques, E. P. (1990). The calcium bindings sites in human annexin V by crystal structure analysis at 2.0 Å resolution. Implications for membrane binding and calcium channel activity. *FEBS Lett.* **275**, 15-21.
- Huber, R., Berendes, R., Burger, A., Schneider, M., Karshikov, A. and Luecke, H. (1992). Crystal and molecular structure of human annexin V after refinement. Implications for structure, membrane binding and ion channel formation of the annexin family of proteins. *J. Mol. Biol.* **223**, 683-701.
- Johnston, P. A. and Sudhof, T. C. (1990). The lipocortin/ calpactin family of calcium-binding proteins. *Biochem. Soc. Trans.* **18**, 1097-1098.
- Karshikov, A., Berendes, R., Burger, A., Cavalié, Lux, H.D. and Huber, R. (1992). Annexin V membrane interaction: an electrostatic potential study. *Eur. Biophys. J.* **20**, 337-344.
- Kristensen, T., Saris, C. J. M., Hunter, T., Hicks, L. J., Noonan, D. J., Glenney, J. R. Jr and Tack, B. F. (1986). Primary structure of bovine calpactin I heavy chain (p36), a major cellular substrate for retroviral protein-tyrosine kinases: homology with the human phospholipase A<sub>2</sub> inhibitor lipocortin. *Biochemistry* **25**, 4497-4503.
- Kohler, G. T. and Milstein, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **256**, 495-497.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.
- Lehto, V. P., Virtanen, L., Paasivuo, R., Ralston, R. and Litalo, K. (1983). The p36 substrate of tyrosine-specific protein kinases co-localizes with non-erythrocyte  $\alpha$ -spectrin antigen, p230, in surface lamina of cultured fibroblasts. *EMBO J.* **2**, 1701-1705.
- Ma, A. S. P. and Sun, T. T. (1986). Differentiation-dependent changes in the solubility of a 195-kD protein in human epidermal keratinocytes. *J. Cell Biol.* **103**, 41-48.
- Ma, A. S. P. and Lorincz, A. L. (1988). Immunofluorescence localization of peripheral proteins in cultured human keratinocytes. *J. Invest. Dermatol.* **90**, 331-335.
- Ma, A. S. P., Bystol, M. E. and Tranvan, A. (1994). The in vitro modulation of filament bundling in F-actin and keratins by annexin II and calcium. *In Vitro* (in press).
- Milstone, L. M. and Edelson, R. L. (1988). Endocrine, metabolic and immunologic functions of keratinocytes. *Ann. NY Acad. Sci.* **548**.
- Nigg, E. A., Cooper, J. A. and Hunter, T. (1983). Immunofluorescent localization of a 39,000-dalton substrate of tyrosine protein kinases to the cytoplasmic surface of the plasma membrane. *J. Cell Biol.* **96**, 1601-1609.
- O'Farrell, P. A., Goodman, H. M. and O'Farrell, P. H. (1977). High

- resolution two-dimensional electrophoresis of basic as well as acidic proteins. *Cell* **12**, 1133-1142.
- Pepinsky, R. B., Tizard, R. T., Mattaliano, R. J., Sinclair, L. K., Miller, G. T., Browning, J. L., Chow, P., Burn, C., Huang, K. S., Pratt, D., Wachter, L., Hession, C., Frey, A. Z. and Wallner, B.** (1988). Five distinct calcium and phospholipid binding proteins share homology with lipocortin I. *J. Biol. Chem* **263**, 10799-10811.
- Pfäffle, M., Ruggiero, F., Hofmann, H., Fernández, M. P., Selmin, O., Yamada, Y., Garrone, R. and von der Mark, K.** (1988). Biosynthesis, a secretion and extracellular localization of anchorin CII, a collagen-binding protein of the calpactin family. *EMBO J.* **7**, 2335-2342.
- Pollard, H. B. and Rojas, E.** (1988).  $Ca^{2+}$ -activated synexin forms highly selective, voltage-gated  $Ca^{2+}$  channels in phosphatidylserine bilayer membranes. *Proc. Nat. Acad. Sci. USA* **85**, 2974-2978.
- Radke, K., Carter, C., Moss, P., Dehazya, P., Schliwa, M. and Martin, S.** (1983). Membrane association of a 36,000-dalton substrate for tyrosine phosphorylation in chicken embryo fibroblasts transformed by avian sarcoma viruses. *J. Cell Biol.* **97**, 1601-1611.
- Rheinwald, J. G. and Green, H.** (1975). Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell* **6**, 331-343.
- Saris, C. J. M., Tack, B. F., Kristensen, T., Glenney, J. R. Jr and Hunter, T.** (1986). The cDNA sequence for the protein-tyrosine kinase substrate p36 (calpactin I heavy chain) reveals a multidomain protein with internal repeats. *Cell* **46**, 201-212.
- Soric, J. and Gordon, J. A.** (1985). The 36-kilodalton substrate of pp60<sup>V-SRC</sup> is myristylated in a transformation-sensitive manner. *Science* **230**, 563-566.
- Walker, J. H., Obrocki, J. and Südhof, T. C.** (1983). Calelectrin, a calcium-dependent membrane-binding protein associated with secretory granules in Torpedo cholinergic electromotor nerve endings and rat adrenal medulla. *J. Neurochem.* **41**, 139-145.
- Wallner, B. P., Mattaliano, R. J., Hession, C., Cate, R. L., Tizard, R., Sinclair, L. K., Foeller, C., Chow, E. P., Browning, J. L., Ramachandran, K. L. and Pepinsky, R. B.** (1986). Cloning and expression of human lipocortin, a phospholipase A<sub>2</sub> anti-inflammatory activity. *Nature* **320**, 77-81.
- Weber, K. and Johnsson, N.** (1986). Repeating sequence homologies in the p36 target protein of retroviral kinases and lipocortin, the p37 inhibitor of phospholipase A<sub>2</sub>. *FEBS Lett.* **203**, 95-98.

(Received 28 September 1993 - Accepted, in revised form, 28 March 1994)