Evidence for differential localization of annexin VI during mammary secretory differentiation

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Introduction

Mammary glands contain a group of Ca$^{2+}$-dependent proteins [1-3] that appear to be developmentally regulated [4]. The proteins are included in a novel group of Ca$^{2+}$-binding phospholipid-sensitive molecules that share considerable genetic relatedness for which the name annexin has been proposed (for reviews see [5-7]).

In our studies, we observed a protein species of approximately 70 kDa size; at low concentration, the protein often describes proteins of similar molecular size variously is equivalent to liver 67 kDa calelectrin originally described by Sudhof [8]. The two proteins are now recognized to be members of the annexin VI group [7]. Other workers have described proteins of similar molecular size variously referred to as 73 kDa intestine protein, 68 kDa lymphocyte membrane-binding protein and 68 kDa calcimedin [5, 6].

The function of 67 kDa liver calelectrin and the mammary annexin VI is currently unknown. Close relatives of these proteins have been implicated in several physiological processes (for a review see [10]).

In an effort to establish the cellular function of mammary annexin VI, we examined the tissue localization of the protein during mammary gland secretory differentiation. In this report we present evidence that the mammary annexin VI exists as an intracellular protein in luminal ductal epithelia of virgin mammary glands. In lactating glands the protein appears to be located on the external basal surfaces of alveoli. Since extracellular matrix molecules are known to influence developmental events, it is possible that the protein may bind matrix components and perhaps play some role in signalling and/or maintaining mammary secretory differentiation. Consistent with this argument, we have reported annexin VI binding to type I collagen in vitro [11].

Results

Immunohistochemical localization of annexin VI in developing mammary glands. The localization of annexin VI was investigated in virgin mammary glands of 8-week-old mature virgin mice, of 12–14-day-pregnant mice and of mice that had progressed 1 week into lactation. In mature virgin glands there is no secretory differentiation; only ductal structures resulting from penetration of the fatty stroma by highly mitotic end buds is observed in juvenile animals. The end bud structure is not observed in mature glands. In lactating glands, some but not all of the ductal epithelia differentiate into secretory epithelia as they develop into lobular alveolar structures. The lumen of these alveoli are connected to ductal elements that all converge into larger collecting ducts that eventually lead to the gland nipple [12].

Antiser to the liver annexin VI reacted in immunofluorescence microscopy with tissue from virgin and lactating mammary glands (Fig. 1). Ductal cells of mature virgin mammary glands showed intense staining (Fig. 1A). While ductal epithelia showed prominent staining, some connective tissue cells also demonstrated limited staining. Little staining of adipocytes was evident. The 70 kDa antigen is clearly enriched in the ductal epithelia of virgin mammary glands.

In lactating glands, immunofluorescence reactivity was restricted to the basolateral surfaces of the alveolar epithelia in a manner suggesting that the annexin VI is on the external cell surface, or completely extracellular, perhaps in the basal lamina (Fig. 1E). Alveolar cell junctions and alveoli basal regions showed distinct staining.

Mammary glands from mice sacrifice through pregnancy were also examined for annexin VI staining (Fig. 1C). Glands midway through pregnancy showed staining intermediate between that of virgin and lactating gland tissue. Ductal elements showed cells that stained intensely, and other cells that showed no staining. Furthermore, the epithelia of newly emerging alveolar structures showed little or no staining; however, some alveolar basal staining was evident.

Identical results as obtained in Figs. 1A, 1C and 1E were obtained using a monoclonal antibody raised against the 73 kDa annexin VI from bovine intestine (data not shown).

None of the tissue section used in these experiments showed staining when treated with antisera that was adsorbed with annexin VI before use in immunohistochemistry. In addition, normal controls such as non-immune serum, and preimmune serum also showed no reactivity in histology.

Mammary annexin VI localization and matrix digestion. Mammary gland tissue sections (mounted on glass slides) were treated for various time periods with collagenase–hyaluronidase to determine whether annexin VI immunohistochemical staining is retained following conditions that remove the extracellular matrix from the preparation. We reasoned that the apparently external annexin VI in secretory alveoli might be released from its presumed anchored position following digestion of the basal lamina–extracellular matrix. Thus released it would not be available for histochemical staining. Digestion of tissue sections from lactating glands resulted in loss of the alveolar basal staining material observed in sections not treated (cf. Figs. 1E and 1F). Identical treatment of virgin gland sections resulted in no loss in annexin VI staining intensity (cf. Figs. 1A and 1B); the expected result if the reactive antigen is an intracellular protein in ductal epithelia. Furthermore, treatment of tissue sections from glands midway through pregnancy resulted in loss of staining associated with emerging alveolar structure, but maintained ductal staining of epithelia that presumably have not yet been committed to alveolar differentiation (cf. Figs. 1C and 1D).

Mammary annexin VI in collagenase–hyaluronidase-harvested ductal and alveolar epithelia. Mammary glands were obtained from virgin and lactating animals. Epithelial cells were recovered from these tissues by collagenase–hyaluronidase digestion which removes the
Fig. 1. Tissue immunofluorescence localization of annexin VI in virgin and lactating mammary glands

(A) Mammary gland from 8-week-old mature virgin mouse. Ductal epithelia show intense staining (solid arrow). (C) Mid-pregnancy mammary gland showing some limited staining of newly developing alveoli (open arrow) and more intense staining of ductal epithelia (solid arrow). (E) Mammary gland from 7-day lactating mouse. Staining is sharply defined and confined to lateral alveolar cell junctions and basal regions of alveoli (solid arrows). Collagenase-hyaluronidase digestion of microscopy tissue section from mammary glands. Ductal structure from virgin mammary gland. (A) Tissue before digestion (as above). (B) Tissue following 15 min of digestion. Note retention of annexin VI staining (solid arrow). Mammary glands midway through pregnancy showing developing alveoli and ductal structures. (C) Tissue prior to digestion (as above). (D) Tissue after 15 min of digestion. Ductal staining is retained (solid arrow), but alveolar staining is lost (open arrow). Alveoli of lactating mammary gland. (E) Tissue before diges- (as above). (F) Tissue following 15 min of digestion. External alveoli staining is completely lost following digestion (solid arrow). All panels are 300× (original magnification).

basal lamina/basement membrane structure. The resulting epithelia were then monitored for annexin VI. The separated proteins were visualized with gold staining and identified with immunoblotting (Fig. 2). Examination of identical amounts of protein from cell lysates of virgin and lactating gland preparation clearly showed that alveolar epithelia from lactating glands have no detectable annexin VI compared with significant levels of the protein in ductal epithelia from virgin glands. Apparently, removal of the basal lamina–basement membrane of secretory alveolar cells results in removal of the annexin VI while the same treatment of virgin glands yielded epithelia that required cell rupture to release the protein.

Mammary annexin VI isolation from hyaluronidase–collagenase lactating gland digest. Mammary glands from 7-day-lactating animals were digested for 1 h using collagenase–hyaluronidase and epithelia were harvested from the digest. After sedimentation of the particulate material, the digest was assayed for mammary annexins. The only mammary annexin recovered in the digest was the annexin VI. The annexin VI recovered was cleaved into smaller immunoreactive species of approximately 32–34 kDa molecular size in SDS/PAGE (Fig. 3B). Recovery of a degraded version of the annexin VI is to be expected given the presence of minor contaminating proteinases in the digesting solution. Collagenase–hyaluronidase digestion of
Epithelial cells were recovered from virgin and lactating glands by collagenase–hyaluronidase digestion which removes the basal lamina–basement membrane structure. The resulting epithelial cell preparations were then monitored for annexin VI. Identical amounts of protein from cell lysates of virgin and lactating glands were examined. Annexin VI from virgin gland ductal epithelia, protein visualized with gold staining. (B) As in (A); protein visualized with annexin VI antiserum. (C) Annexin VI from lactating gland alveolar epithelia, protein visualized with gold staining. (D) As in (C); protein visualized with annexin VI antiserum. Arrowhead notes the position of the annexin VI. Note the absence of the annexin VI from alveolar epithelia.

![Image](image_url)

**Fig. 2. Direct assay of annexin VI in collagenase–hyaluronidase-harvested epithelia**

**Discussion**

The immunohistochemical localization presented confirms our previous report that annexin VI is an intracellular protein of mammary luminal ductal epithelia [8]. In these cells, staining was prominent at the inner cell surface. In contrast, the location of staining in lactating glands is restricted to the external basolateral cell surface of secretory epithelia of alveoli; the protein appears not to be an abundant intracellular component of these cells. The protein may be a membrane species with exposure on the external alveolar epithelial cell surface. It is also possible that the protein resides in the basal lamina; however, it appears unlikely that it is a component of the extracellular matrix per se. Similar immunofluorescence data were obtained with both polyclonal and monoclonal antisera.

That the annexin VI is localized in the external basolateral space of alveoli in lactating mammary glands is supported by the observation that tissue sections from these glands treated with collagenase–hyaluronidase do not retain staining in immunohistochemistry. Virgin gland sections treated in an identical manner retain their annexin VI-staining character. The data with virgin gland sections clearly establish that the loss of annexin VI staining in the collagenase–hyaluronidase-digested tissue sections from lactating glands is not the consequence of antigen destruction of minor contaminating proteinases which would have abolished antigen staining in both virgin and lactating tissue. Low levels of proteinases may account for some cleavage of the annexin VI, but not to the extent that immunohistochemical reactivity is destroyed (see below).

We suggest that the externally located annexin VI is somehow anchored to a component of the alveolar basal lamina–extracellular matrix complex (e.g. type I collagen). The loss of protein staining in the collagenase–hyaluronidase tissue section digestion experiments results from dissolution of the basal lamina–extracellular matrix. After dislodgement, the protein is lost during washing of the sections before immunohistochemical staining.

External alveolar basolateral localization of the annexin VI would explain why collagenase–hyaluronidase-harvested alveolar epithelia from lactating glands demonstrated none of the protein. In contrast, epithelia similarly harvested from virgin mammary glands revealed abundant levels of the protein.
tein following rupture of the cells; precisely the result expected for an intracellular protein.

Finally, as would be expected for an external protein, the annexin VI can be recovered from the digest of collagenase-hyaluronidase-harvested secretory cells. We recovered a cleaved version of the protein that remained immunoreactive to annexin VI antisera. Cleavage of the annexin VI would be expected given that low levels of proteinases are known to be present in collagenase preparations which could degrade the 70 kDa protein in a manner similar to that observed in controlled proteolysis experiments [8].

Thus, it seems clear that during mammary gland secretory differentiation a change in the localization of annexin VI occurs. Initially, the protein is intracellular in luminal ductal epithelia of virgin glands. In secretory alveolar epithelia of lactating glands the protein assumes an external position on the basal-lateral surface of alveoli. The mechanism and reason for such a change in the localization of the protein is not apparent at this time. Glenney [13] has reported that the 73 kDa intestinal Ca"-binding protein is located in the lamina propria of intestinal extracellular matrix. In addition, anchoran CII has recently been reported to be related to the calpain family [14]; furthermore, these workers report the secretion of this protein which they argue binds to collagen in the extracellular matrix of chondrocytes [15].

A working hypothesis we have adopted for the externally located basolateral position of annexin VI in lactating mammary glands is that it serves to influence and somehow impact the secretory phenotype of mammary alveolar epithelia. The annexin VI would play such a role through interactions with components of the basal lamina-basement membrane. We find this model attractive, since changes in the extracellular matrix-basal lamina chemical composition are known to influence ductal and secretory development in the mammary gland [16]. In support of this model, we have reported binding in vitro of annexin VI to type II collagen [11]. Wirl & Pfaffle [17] have also reported collagen binding by a group of rat mammary Ca"- and lipid-sensitive proteins that are probably members of the annexin group. We are currently attempting to answer several questions regarding the translocation and function of annexin VI in mammary gland development and physiology.

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Hormonal regulation of an avian annexin I gene

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We have had an abiding interest in the mechanisms by which the pituitary hormone prolactin (PRL) and its relatives [growth hormone (GH), placental lactogens, etc.] affect the phenotype of their target cells by altering cell-specific gene expression. Differentiation-specific gene regulation by polypeptide hormones is generally a poorly understood process. Many polypeptide hormones and growth factors are known to affect cell growth through shared pathways, especially proto-oncogene expression. However, the mechanisms by which these hormones affect the differentiation state of specific target cells through altering nuclear gene expression are largely unknown. In contrast, the small lipophic hormones (steroids, thyroid, isoprenoids, etc.) are well known to affect specific gene expression and the differentiation state of target cells by interacting with ligand-dependent transcription factors [i.e. their receptors [1]]. As a member of a wide class of "helix-bundle peptides" [2], which includes not only PRL and GH, but also many cytokines (interleukins, erythropoetin, granulocyte-macrophage colony-stimulating factor, etc.), PRL is archetypal as a polypeptide which not only stimulates cell growth but also alters phenotypic differentiation. One of the differentiation-state specific genes which is induced by PRL in a classic target tissue model is an annexin I-like gene (cp35) in the pigeon crop sac [3,4]. In this paper, I will summarize the state of our understanding of this gene and its regulation by PRL, and also speculate on its evolution and the function of cp35 and related gene products.

The cp35 gene was discovered by a differential hybridization strategy [3] as a consequence of the dramatic induction of its mRNA in crop sac tissue stimulated by PRL injection. In the pigeons and doves (Class Aves, family Columbidae), PRL secretion during egg incubation and brooding leads to the production of crop milk [5]. This cheese-like substance is a lipid- and protein-laden nourishment which is the only

Abbreviations used: PRL, prolactin; GH, growth hormone.

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