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Human axillary apocrine glands: proteins involved in the apocrine secretory mechanism

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Summary. The apocrine secretory mechanism is a mode of secretion by which the apical part of the cell cytoplasm is pinched off, which leads to the formation of an aposome. The distinct mechanism of formation and decapitation of the aposome is not well investigated. Only few proteins are known that are involved in this secretory mechanism. We studied the human axillary apocrine gland and looked at proteins associated with cytokinesis, a process that is comparable to the pinching-off mechanism of apocrine glandular cells. By immunohistochemistry, we detected actin, myosin II, cytokeratin 7 and 19, α- and ß-tubulin, anillin, cofilin, syntaxin 2, vamp8/endobrevin and septin 2. In highly active glandular cells, these proteins are located at the base of the apical protrusion when the aposome is in the process of being released or are concentrated in the cap of the apical protrusion. These findings demonstrate new insights on apocrine secretory mechanisms and point to similarites to the terminal step of cytokinesis, which is regulated by a SNARE-mediated membrane fusion event.

Key words: Axillary apocrine gland, Secretion mechanism, Cytoskeleton, Cytokinesis

Introduction

It was the aim of the present study to investigate proteins that are involved in the decapitation mechanism of apocrine glandular cells in the human axilla. In particular, we investigated cytoskeletal proteins and proteins of the SNARE fusion machinery.

Apocrine glands are distributed in numerous areas of the body, the axillae, external female genitalia, inguinal, perianal, periumbilical and periareolar regions, and also the prostate apparently uses apocrine mechanisms to release its secretory products (Requena et al., 1998; Saga, 2002; Wilhelm et al., 2003). Specialized apocrine glands are located at the margin of the eyelids, the glands of Moll (Vaughan and Asbury, 1980; Stoeckelhuber et al., 2003, 2008) and in the skin of the human external auditory canal, the ceruminous gland (Perry and Shelley, 1955; Stoeckelhuber et al., 2006). The common feature of apocrine glands is a secretory mechanism by which apical parts of the cell are demarcated and released into the lumen of the gland. In addition, glandular cells exhibit secretion by exocytosis and the two secretion modes apparently alternate (Saga, 2002). In the case of the apocrine secretory mechanism, the apical protrusion is of homogenous structure and no organelles are found in the protrusions (Schaumburg-Lever and Lever, 1975; Stoeckelhuber et al., 2003). Molecules secreted by the apocrine mechanism are synthesized within the cytoplasm and concentrated at the apical protrusion-forming region of the plasma membrane (Wilhelm et al., 1998). So, these molecules can be secreted although they do not have a signal-peptide targeting them to the plasma-membrane for exocytosis.

The release of secretory material of apocrine glands in the lumen is called decapitation and pinching-off (Gesase and Sato, 2003). The decapitation mechanism in apocrine glandular glands of the human axilla was investigated by Schaumburg-Lever and Lever (1975) by electron microscopy. The first step is the formation of an apical cap, then the formation of a horizontal dividing

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Abbreviations: SNARE, soluble NSF attachment protein receptor.

membrane at the base of the apical cap and finally the appearance of tubules over and parallel to the dividing membrane causing the decapitation of the cap by supplying the plasma membrane for both the undersurface of the apical cap and the luminal plasma membrane. These processes are similar to those during cytokinesis in dividing cells, which is triggered and regulated by many components. Mainly cytoskeletal proteins are involved in cytokinesis. Actin and myosin II dominate the contractile ring (Eggert et al., 2006) and actin has already been detected in the decapitation formation of apocrine scent glands (Metzler et al., 1992). Anillin is localized to the cleavage furrow and binds to myosin II (Field and Alberts, 1995; Hickson and O'Farrell, 2008) and septin (Maddox et al., 2007). Septin 2, in turn, directly binds myosin II and this association is important for fully activating myosin II in interphase of dividing cells (Joo et al., 2007; Cao et al., 2009). Septin filaments were reported to interact in vivo with assemblies of actin (Kinoshita et al., 2002) and tubulin (Nagata et al., 2003). Cofilin, a small actin-binding protein is temporarily concentrated at the contractile ring during cytokinesis (Nagaoka et al., 1995). Cofilin accumulates during late stages of furrowing and is finally enriched at the midbody. Syntaxin 2 and endobrevin/VAMP-8 are required for the terminal step of cytokinesis (Low et al., 2003; Gromley et al., 2005). These two proteins are members of the SNARE membrane fusion machinery.

In the present study, we attempted to compare cytokinesis and apocrine secretory cell mechanisms in order to obtain a closer insight into the mechanism of apocrine secretion.

Materials and methods

Tissue

Tissue of human axillary apocrine glands were obtained from the Institute of Dermatopathology, Buchholz, Germany, director: PD Dr. Dr. Christoph Schubert. We received specimens from 21 patients (10 male and 11 female) aged 17 to 87 years undergoing surgery for different skin and gland diseases. Healthy tissue used for this study was adjacent to pathological tissue. In accordance with the Helsinki Principles, all patients were consulted in respect of our intention to study the axillary apocrine glands. The material was immediately fixed in buffered formalin (4.5%), dehydrated with ethanol and embedded in paraffin.

Immunohistochemical staining

Immunohistochemical staining was performed with antibodies to actin (Sigma-Aldrich, Munich, Germany), myosin II (Sigma-Aldrich, Munich, Germany), cytokeratin 7 (Dako, Hamburg, Germany), cytokeratin 19 (Dako, Hamburg, Germany), α-tubulin (Zytomed, Berlin, Germany), ß-tubulin (Zytomed, Berlin, Germany), anillin (Abcam, Cambridge, UK), cofilin (Abcam, Cambridge, UK), syntaxin 2 (Santa Cruz Biotechnology, Santa Cruz, USA) , vamp8 (Abcam, Cambridge, UK) , septin 2 (Santa Cruz Biotechnology, Santa Cruz, USA). Five μm paraffin sections were dewaxed and pretreated with microwave irradiation in citrate buffer at pH 6.0 for 15 min for all antibodies except septin 2, myosin II and actin, where no pre-treatment was necessary. After blocking the endogenous peroxidase activity with 3% hydrogen peroxide for 15 min, the sections were incubated with 3% normal goat, swine or rabbit serum (Vector Laboratories, Burlingame, USA). The primary antibodies were applied according to the avidin-biotin-horseradish-peroxidase complex method using the Vectastain Kit from Vector Laboratories (Burlingame, USA) and diluted as follows: anti-actin 1:50, anti-myosin II 1:2000, anti-CK 7 1:100, anti-cytokeratin 19 1:50, anti-α-tubulin 1:200, anti-ß-tubulin 1:200, anti-anillin 1:100, anti-cofilin 1:30,000, anti-syntaxin 2 1:100, anti-vamp8 1:100, anti septin 2 1:80. In control sections, the primary antibody was replaced by buffer. Sections were incubated for 1h at room temperature and overnight at 4°C. The secondary antibody (Vector Laboratories, Burlingame, U.S.A.) was diluted 1:200 and applied for 45 min at room temperature. Diaminobenzidine was used as chromogen. Partially, the sections were counterstained with hematoxylin. An overview of the staining procedures for every antibody is listed in table 1.

Sections were studied with a Nikon microscope and images were captured by a digital Nikon camera (Nikon, Duesseldorf, Germany). Labeling results were categorized as weak, medium and strong staining.

Results

General morphology

Axillary apocrine glands were located in the dermis and the subcutis. The cross-sectioned tubules consist of an inner layer of secretory cells and an outer layer of myoepithelial cells. Tall prismatic active glandular cells alternated with flat and cuboidal inactive glandular cells often within the same secretory tubule. The apocrine secretory mechanism implies that a portion of the apical cytoplasm is released from the cell. This is a continuous process which can be morphologically divided into a few steps: at first a broad protrusion is formed, which then increasingly narrows at its base and then finally is pinched off. The zone at the base of the protrusion was investigated particularly in regard to the localization of the proteins mentioned below.

Actin

Actin was expressed in active and inactive glandular cells in the whole cytoplasm with a medium to strong staining. Especially the apical protrusions were strongly stained (Fig. 1). A faint line of a strong actin-staining

was seen at the base of the protrusions in few cells. Myoepithelial cells showed a strong actin expression.

Myosin II

The antibody to myosin II marked the whole glandular cells in the active and inactive secretion status with rather strong signal intensity. Especially the entire apical protrusion showed a very strong expression with the antibody to myosin II (Fig. 2a). A myosin II-positively marked zone at the base of the protrusion was seen only in these cells where the protrusion was about to be released (Fig. 2b). The myoepithelial cells were strongly stained.

Cytokeratin 7 and 19

The staining intensity for cytokeratin 7 and 19 was identical. Active as well as inactive glandular cells express CK 7 and 19 with a medium to strong intensity. In active glandular cells, CK 7 and 19 were also strongly

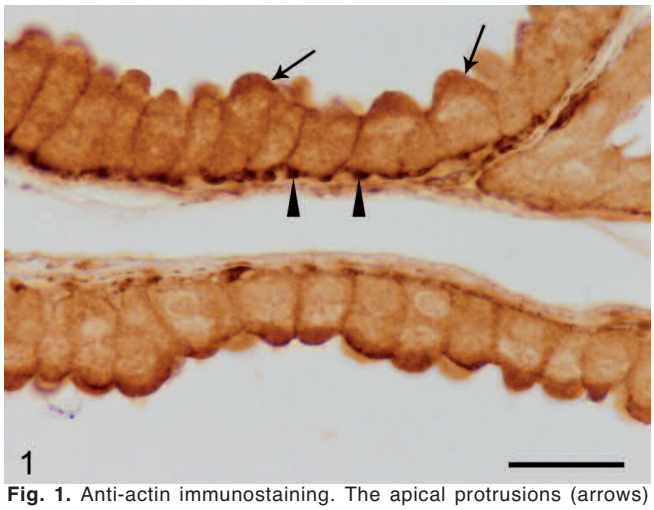


Fig. 1. Anti-actin immunostaining. The apical protrusions (arrows) showed a stronger staining with the antibody to actin. In a few cells, a fine demarcation-line below the protrusion was positive for actin. The arrowheads mark the more strongly stained myoepithelial cells. Scale bar: 25 μm.

Table 1. Primary antibodies.

antibody	host	clone	dilution	blocking serum (3%)
anti-actin	rabbit	polyclonal	1:50	swine
anti-myosin II	rabbit	polyclonal	1:2,000	goat
anti-cytokeratin 7	mouse	OV-TL 12/30	1:100	swine
anti-cytokeratin 19	mouse	BA17	1:50	swine
anti-α- tubulin	mouse	Z022	1:200	swine
anti-ß-tubulin	mouse	Z023	1:200	swine
anti-anillin	goat	polyclonal	1:100	rabbit
anti-cofilin	rabbit	polyclonal	1:30,000	goat
anti-syntaxin 2	goat	polyclonal	1:100	rabbit
anti-vamp 8	rabbit	polyclonal	1:100	goat
anti-septin 2	goat	polyclonal	1:80	rabbit

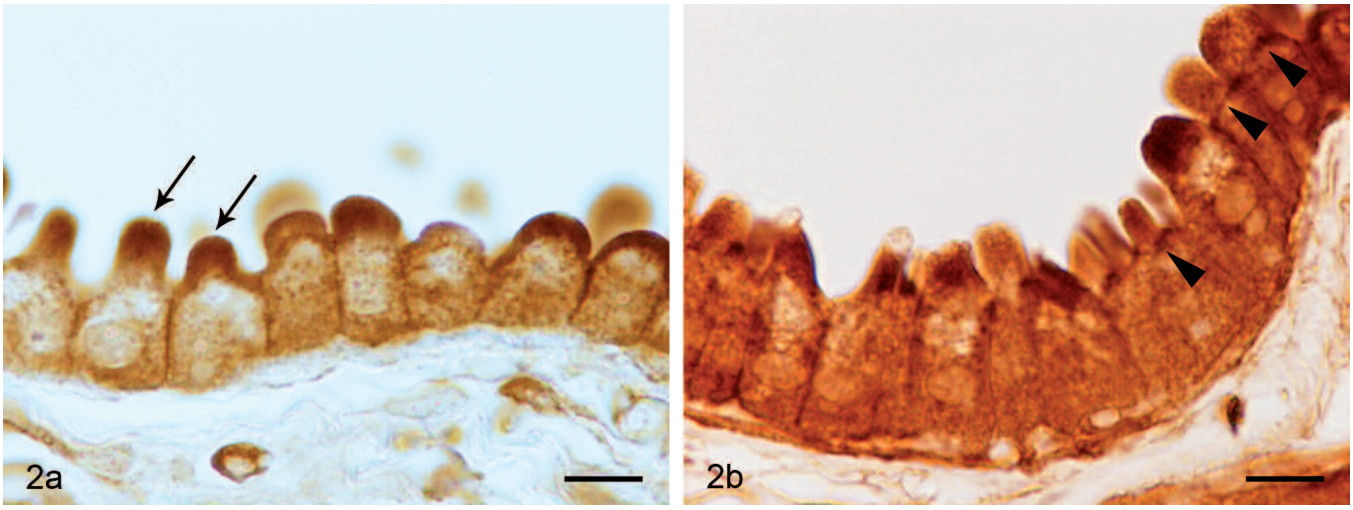


Fig. 2. Anti-myosin II immunostaining of axillar apocrine cells. **a.** A strong anti-myosin II-staining in the protrusions of glandular cells (arrows). **b.** A myosin II-positively marked zone at the base of the protrusion (arrowheads) was seen in cells where the protrusion was about to be released. Scale bars: 10 μm.

expressed in the bulging apex forming a kind of a cap on top of the protrusion (Fig. 3). CK 7 and 19 were concentrated in a region at the base of the apical protrusion when the protrusion was in the process of being released (Fig. 4). In some glandular tubules, the elongated protrusion itself remained unstained, in other tubules the protrusion showed a medium staining.

α- and ß-tubulin

Antibodies to α- and ß-tubulin showed the same staining results. Both active and inactive glandular cells express tubulins with a medium to strong staining in the cellular apex (Fig. 5). In tall apical protrusions, the staining was particularly strong. In most cells, the whole protrusion was stained, only few cells showed no apical staining.

Anillin

A weak to medium staining of the apical protrusion could be detected in active but also in rather inactive glandular cells (Fig. 6). The apical protrusions were more strongly marked when the glandular cells were highly prismatic, and here the stain was concentrated at the base of the protrusion. Immediately before being

Fig. 3. Anti-CK7 immunostaining. Active glandular cells show a strong staining of the apical part of the cell. Scale bar: 25 μm.

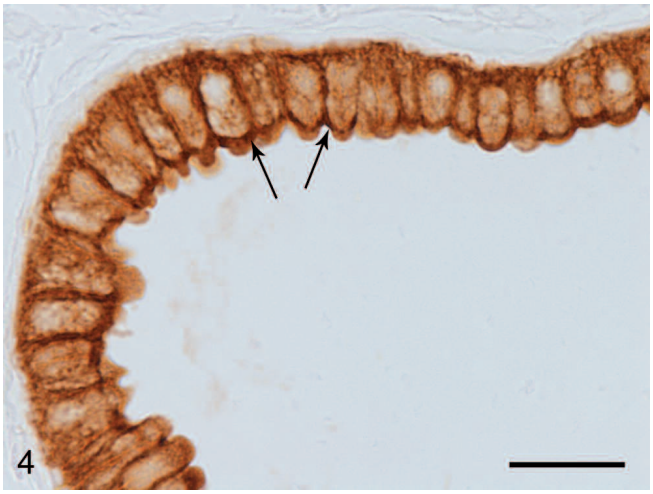


Fig. 4. Anti-CK19 immunostaining. In highly active glandular cells, CK 19 is concentrated in a region directly below the apical protrusion (arrows). Scale bar: 25 μm.

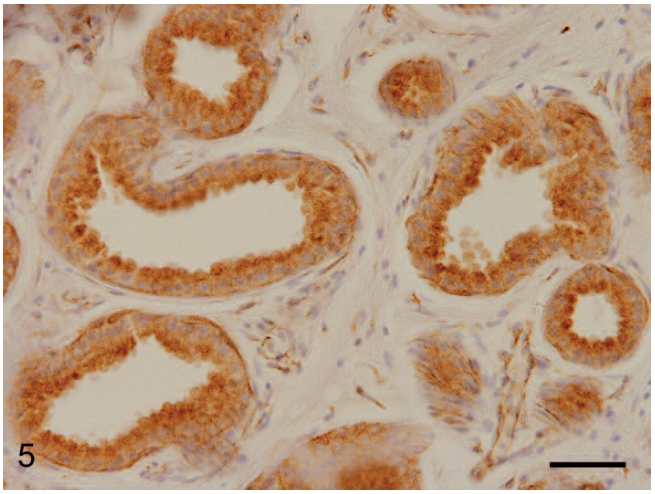


Fig. 5. Anti-ß-tubulin immunostaining. ß-Tubulin was strongly expressed in the cellular apex. Scale bar: 50 μm.

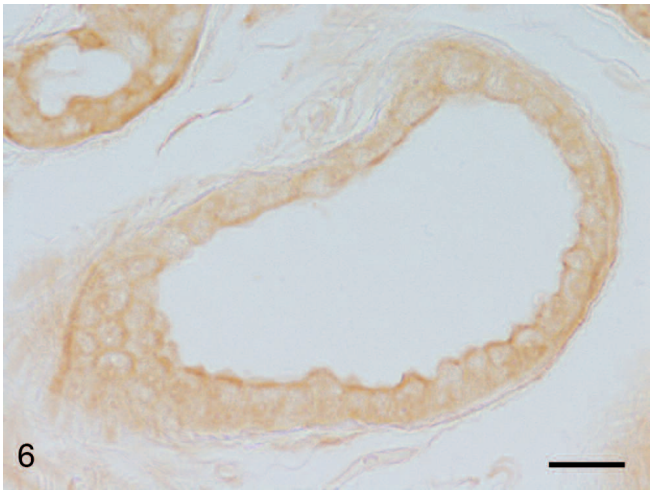


Fig. 6. Anti-anillin immunostaining. Anilin showed a weak to medium expression of the glandular cells with a stronger expression at the apical part of the cell. Scale bar: 25 μm.

pinched off, the protrusions were unstained.

Cofilin

Cofilin staining showed a variable pattern (Fig. 7). The glandular cells showed a medium to strong expression of cofilin. Variations concern also the apical protrusions. In some active glandular cells, a strong staining of the apical part of the cell could be observed. In other cells, the apical protrusion was totally unstained. These differences can occur even within one tubule. A more strongly stained zone at the base of the protrusion followed by a narrow unstained area and an again stained upper part of the protrusion were detected in a few cells.

Syntaxin 2

The staining of the syntaxin-antibody showed a granular staining of weak, medium or strong intensity of active glandular cells (Fig. 8a). Inactive cells were

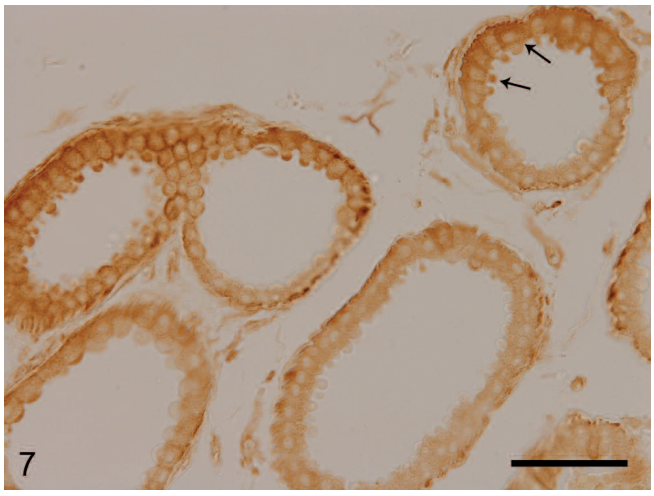
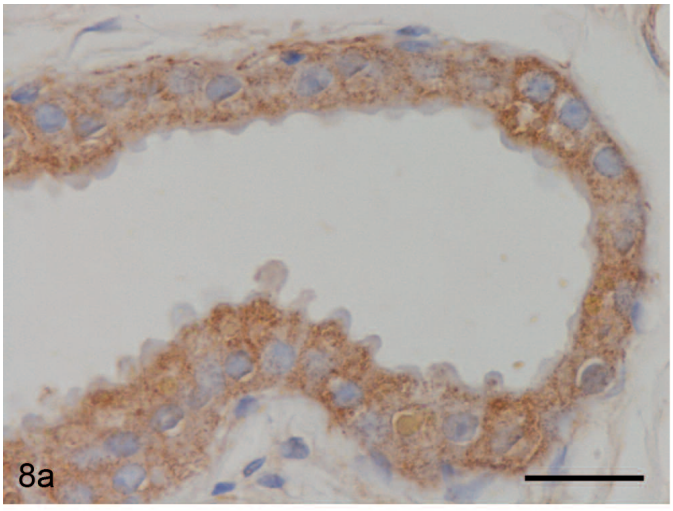
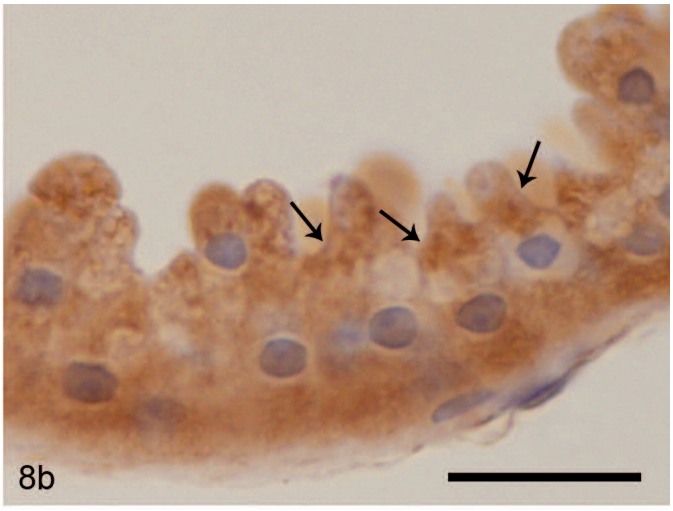


Fig. 7. Anti-cofilin immunostaining, more strongly stained glandular cell protrusions alternate with unstained prostrusions, few cells showed a more strongly stained zone beneath the protrusion (arrows). Scale bar: 50 μm.





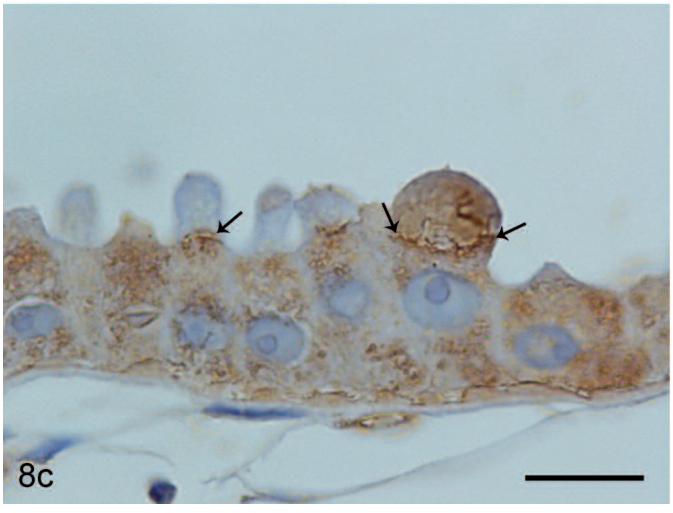


Fig. 8. Anti-syntaxin 2 immunostaining showed a variable staining pattern. **a.** In general, a granular staining pattern of apocrine glandular cells without syntaxin-expression in the protrusion was observed. **b.** In some sections, syntaxin 2 was also expressed in an area below the protrusions (arrows). **c.** Positively stained demarcation line below the protrusion (arrows). Scale bars: a, b, 25 μm; c, 10 μm.

generally more weakly stained. The apical protrusions of active glandular cells were predominantly unstained, but in some rare sections the apical protrusion showed a strong staining, which is weaker before the release (Fig. 8b). In some sections (Fig. 8c), a line demarcating the upper cap of the cell was very clearly visualized by the syntaxin 2 antibody.

Vamp8 (endobrevin)

Vamp8, the vesicle associated membrane protein, was strongly expressed at the base of protrusions in active glandular cells (Fig. 9a). In some cells at higher magnifications, positive-stained vesicles in this region were visible (Fig. 9b). These vesicles have a round or tubular structure. The protrusion itself was unstained. The intensity of the staining of the cytoplasm in active and inactive glandular cells varied from weak to medium.

Septin 2

The staining results with the antibody to septin 2 were variable. There were cross-sectioned tubules which showed a weak cytoplasmic granular staining with a stronger staining in the area below the protrusion. The

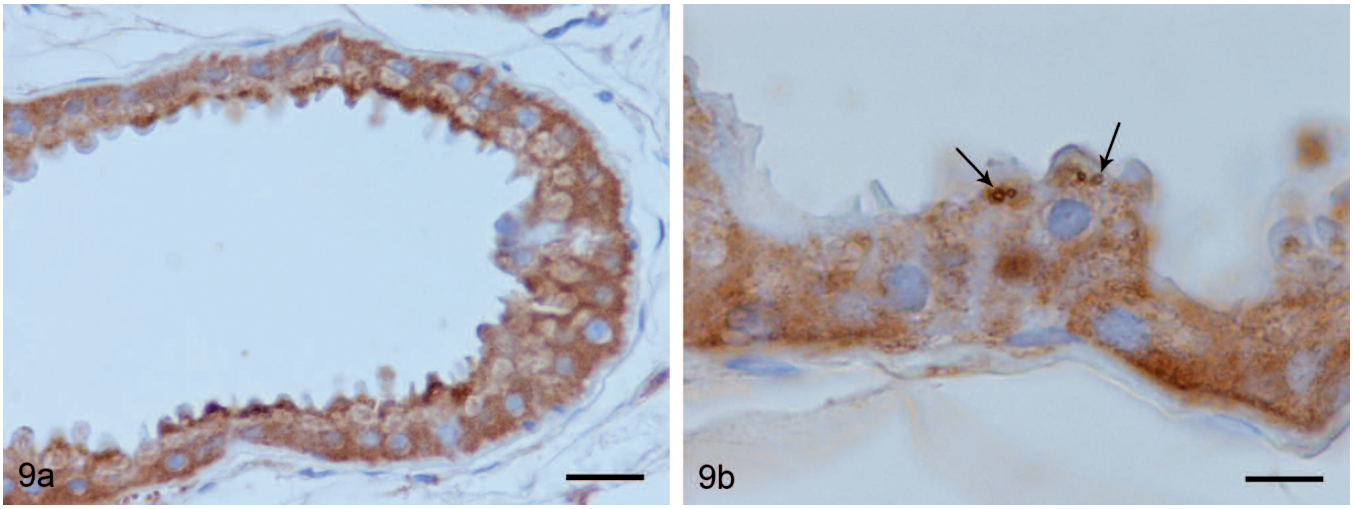


Fig. 9. Anti-Vamp 8 immunostaining. **a.** Vamp 8 was strongly stained at the base of the protrusion. **b.** Positively stained vesicles were visible in the protrusions of glandular cells (arrows). Scale bars: a, 25 μm; b, 10 μm.

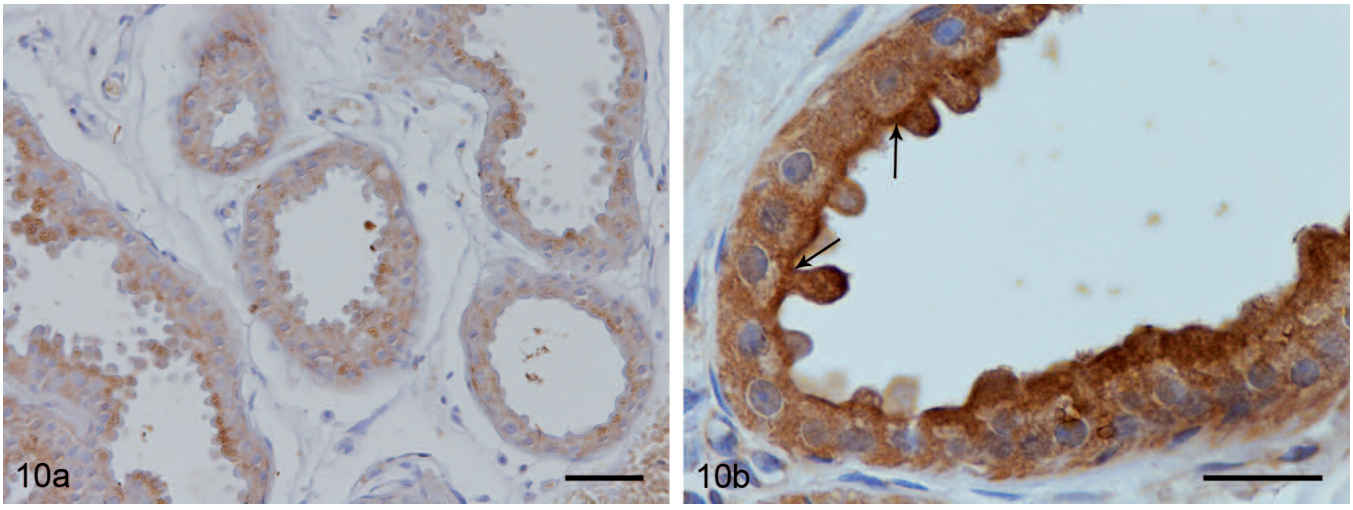


Fig. 10. Anti-septin-2 immunostaining. Granular septin-staining of the glandular cells **(a)** alternates with more strongly stained sections with a clear concentration of the protein below the protrusion **(b)** (arrows). Scale bars: a, 50 μm; b, 25 μm.

apex of the glandular cells was then unstained (Fig. 10a). In other sections septin 2 had a medium to strong expression, the staining was more homogenous and the apical protrusion was strongly stained. Glandular cells which were in the process of detaching the apex showed a stronger staining in the area below the apical protrusion (Fig. 10b). Inactive glandular cells expressed septin 2 with weak to medium strong intensity.

There was no correlation with age or sex of the patients in regard to the staining results of the tested antibodies.

Discussion

The apocrine secretory mechanism in different glands is a process by which the apical part of the cell detaches. The findings of the exact mechanism were obtained mainly by morphological studies with transmission and scanning electron microscopy (Charles, 1959; Hashimoto et al., 1966; Schaumburg-Lever and Lever, 1975; Testa-Riva and Puxeddu, 1980; Agrawal and Vanha-Pertulla, 1988; Stoeckelhuber et al., 2003, 2006). Very few studies have been carried out on cytoskeletal proteins that are part of, and involved in, the apocrine secretion machinery (Metzler et al., 1992; Atoji et al., 1998; Stoeckelhuber et al., 2000, 2003, 2006).

Interactions between myosin II and actin in the ring formed during cytokinesis are believed to generate the force that constricts the cell in two daughters (Murthy and Wadsworth, 2005). We have found actin and myosin II strongly concentrated in the apical protrusions of active glandular cells. In cells that are in an advanced stage of detaching the protrusion we found actin and myosin II strongly expressed at the base of the protrusion, a zone that is described by Schaumburg-Lever and Lever (1975) in an ultramicroscopic study as the dividing membrane.

Anillin serves as a scaffold protein for the contractile ring (Piekny and Glotzer, 2008). Syntaxin 2 is a ubiquitously expressed t-SNARE that has been reported to be targeted to the plasma membrane in several cell types (Benett et al., 1993), it was identified with a factor called epimorphin that promotes branching morphogenesis in lung (Hirai et al., 1992) and mammary epithelial cells (Chen et al., 2009). Epimorphin is identical to syntaxin 2. Inhibition of the function of syntaxin 2 and endobrevin by overexpression causes failure of midbody abscission in cytokinesis while earlier steps are unaffected (Low et al., 2003). These results indicate that the terminal step of cytokinesis is not a passive "ripping-apart" or "pinching-off" mechanism but is regulated by a SNARE-mediated membrane fusion event that is distinct from exocytotic events that are involved in prior ingression of the plasma membrane (Low et al., 2003). In the electronmicroscopic study of Testa-Riva and Puxeddu (1980), the morphology of the apocrine secretory mechanism in the ceruminous glands of the ear is described in detail. These authors found secretory vesicles at the base of the apical protrusion. The membranes of the vesicles fuse and they also fuse with the lateral plasmamembrane. This fusion event marks the base of the projection until the protrusion detaches from the cell. The demarcation membrane located below the vesicle area as described by Schaumburg-Lever and Lever (1975) could not be found regularly by Testa-Riva and Puxeddu (1980). Positively stained vesicles with the antibody to syntaxin 2 and vamp8 were detected in our study at the demarcation line of the protrusion. This finding suggests that these proteins could be involved in the release of the apocrine protrusion as well.

Septin 2 colocalizes in the apocrine protrusion with myosin II, anilin and tubulin as is postulated for the cleavage furrow in dividing cells (Nagata et al., 2003; Maddox et al., 2007; Cao et al., 2009). Cofilin is expressed in the whole cell, a more strongly stained zone beneath the apical protrusion was found only in few cells. The reason for that might be the fact that in dividing cells cofilin was found during late stages of furrowing (Nagaoka et al., 1995). So, only in a narrow time frame of decapitation is cofilin expressed in that zone.

The behaviour of keratin filaments during cell division was examined in a wide range of epithelial cell lines from several species, almost half of them show keratin disruption by forming aggregates and spheroids within the cytoplasm (Knowles and Franks, 1977; Keydar et al., 1979; Lane et al., 1982). In cytokinesis, all major structural elements of the cell are substantially reorganized (Brown et al., 1983). However, in apocrine secreting cells reorganisation takes place only in the apical part. In our studies, we could not really observe keratin disruption, which, however, may not be detectable at the light microscopy level. In glandular cells, however, in which the apical protrusion is on the point of being decapitated, this part of the cell is unstained and without visible aggregates. It could be speculated that in this region the cytokeratin filaments might be disrupted. Evidence for that would be visible only at a higher resolution of electron microscopy. The stronger cytokeratin staining intensity at the base and top of the protrusion may serve as a temporarily stabilizing framework.

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