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Review

Sjögren's syndrome and the epithelial target: A comprehensive review

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ABSTRACT

The most difficult component in our understanding of human autoimmunity remains a rigorous dissection of etiological events. Indeed, the vast literature on autoimmune diseases focuses on the inflammatory response, with the hope of developing drugs that reduce inflammation. However, there is increasing recognition that understanding the immunobiology of target tissues will also have direct relevance to disease natural history, including breach of tolerance. Sjögren's syndrome is essentially an epitheliitis and there are major changes to normal architectural salivary organization. We propose that loss of homeostasis is the initial event that precipitates inflammation and that such inflammatory response includes not only the adaptive response, but also an intense innate immune/bystander response. To understand these events this review focuses on the architecture, phenotype, function and epithelial cell organization. We further submit that there are several critical issues that must be defined to fully understand epithelial cell immunobiology in Sjögren's syndrome, including defining epithelial cell polarity, cell–cell and cell to extracellular matrix interactions and a variety of chemical and mechanical signals. We also argue that disruption of tight junctions induces disorganization of the apical pole of salivary acinar cells in Sjögren's syndrome. In addition, there will be a critical role of inflammatory cytokines in the apico-basal relocation of tight junction proteins. Further, the altered disorganization and relocation of proteins that participate in secretory granule formation are also dysregulated in Sjögren's syndrome and will contribute to abnormalities of mucins within the extracellular matrix. Our ability to understand Sjögren's syndrome and develop viable therapeutic options will depend on defining these events of epithelial cell biology.

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1. Introduction

The most difficult component in our understanding of autoimmune diseases remains defining the etiological events that lead to clinical pathology. Sjögren's syndrome (SS) illustrates this enigma. Indeed, there is an enormous data base [1–42] on the clinical aspects, diagnostic criteria, treatment protocols and natural history, but the origin of the disease remains elusive. In fact it is interesting that the majority of studies have focused more on the inflammatory response than on the target or the victim of the disease. In the case of Sjögren's syndrome dissection of the inflammatory response may eventually prove useful in developing new therapies, but thus far, in contrast to rheumatoid arthritis, this has been relatively nonproductive. One feature of human autoimmunity that seems to be constant is the finding that there is a pre-autoimmune phase in which patients have many of the serologic abnormalities of autoimmune disease before they develop clinical pathology. Such appears to be the case in some murine models of autoimmune disease as well. In this respect an understanding of the immunobiology of the epithelial cells within the salivary gland would appear to be a critical area to understand immunological homeostasis and ultimately breach of tolerance. In fact, changes in expression of proteins involved in maintaining cell–cell and cell–extracellular matrix relationships that are essential for epithelial glandular

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architecture organization have been described in the pre-autoimmune phase [43].

In this review we propose that study of the epithelial architecture of Sjögren's syndrome must be considered to be the primary insult that leads to epitheliitis, or the inflammation of the salivary and lacrimal glands. We note in fact that Sjögren's syndrome is not the only autoimmune epitheliitis and many of our comments can well be applicable to a similar and, in fact, in some respects, an overlapping disease, primary biliary cirrhosis. Our thesis, however, will focus only on Sjögren's syndrome and indeed we emphasize that the pre-autoimmune phase of the salivary epithelial cells do not depend on the proximity or the amount of inflammatory cells present in the gland [44,45].

Cellular architecture is fundamental for the maintenance of morphological and functional homeostasis of mono-stratified epithelia. Several studies show that one of the relevant elements involved in the development of autoimmunity is loss of the protective function of mucosal barriers. A review of the main aspects related to the organization of epithelia is relevant to understanding the pathophysiological alterations observed in autoimmune diseases, such as Sjögren's syndrome, where the protective function of epithelia is lost. A considerable body of evidences suggests that the loss of homeostasis in epithelial cells of the salivary and lachrymal glands plays a major role in the initiation and progression of Sjögren's syndrome. Bearing this in mind, the disease has been referred to in the literature as autoimmune epitheliitis, a term that was coined early on by Moutsopoulos and his colleagues [24,46,47]. To expand upon our understanding of epithelial cells, we will first review the architecture and function of epithelial cells as exemplified by salivary acinar cells. In these cells, the maintenance and regulation of cell polarity is essential. Emphasis is placed on the discussion of master regulators of cell polarity, such as tight junctions, hemidesmosomes and polarity complexes and the consequences of alterations that affect these structures. For example, the loss of asymmetry of the specific plasma membrane domains leads to relocation of the molecular machinery (Rab GTPases and SNARE proteins) inducing an ectopic exocytosis of secretory granules to the extracellular matrix. Such alterations in destination of proteins can trigger an inflammatory response. The role of pro-inflammatory cytokines in the loss of barrier function of the epithelium and their relevance to autoimmunity is also analyzed. In brief, several examples are provided to demonstrate the importance architectural organization for appropriate epithelia function and the consequential changes may have for developing autoimmune responses.

2. Architecture and function of salivary acinar cells

Salivary glands are composed of both acini and ducts. In the latter case intercalated, striated and excretory ducts are distinguished, which all participate in conducting secreted products (saliva) towards the mucosal surfaces. Acinar cells, on the other hand, are responsible for the synthesis of saliva components, a process that involves a number of discrete steps that take place in different subcellular compartments. Saliva components are first synthesized in the rough endoplasmic reticulum (RER), then processed in RER and the Golgi complex, concentrated and targeted in the Golgi complex and stored in secretory granules [48,49]. Sorting and movement of the secretory granules to the apical plasma membrane involves primarily microtubules [50]. Exocytosis of proteins, glycoproteins and mucins occurs upon fusion of secretory granules with the plasma membrane. The sum of these processes constitutes what is referred to as "*regulated secretion*" that can be triggered both by external biochemical molecules (agonists) and/or mechanical signals [51,52]. Examples of the former group include alpha- and beta-adrenergic agonists, cholinergic agonists, peptidenergics and vasoactive intestinal peptide, among others [52]. These ligands bind to and activate specific receptors on the basal plasma membrane of the acinar cell, which couple via transducer proteins to different pathways that generate the second messengers responsible for both inducing exocytosis and the *de novo* synthesis of secretion products [53]. The turnover of plasma membrane components and certain extracellular matrix (ECM) components follow the same route described above. However, since no external signals are required for activation, this process is referred to as "*constitutive secretion*". The membrane components, in this instance, are stored in secretory vesicles that traffic either to the apical or basolateral plasma membrane. In addition, other vesicles containing ECM products fuse with the basal plasma membrane and release their contents toward the ECM [53].

Saliva contains electrolytes and water [49]. The water flows from the blood stream via channels (aquaporin) in the basal membrane of acinar cells. Likewise, electrolytes are mobilized by transporters and/or ion channels present within the same membrane. Subsequently, the release of water and electrolytes to the acinus lumen occurs through specific aquaporins and different channels or electrolyte transporters, respectively, located in the apical membrane of acinar cells. The bulk flow of water and electrolytes is transcellular, although paracellular flow mediated by ion channels of the claudin family, a component of tight junctions (TJ) located in the apical pole of acinar cells also occurs to a lesser extent [54,55].

Acinar cell are shaped as truncated pyramid with the subcellular organelles asymmetrically distributed in the cytoplasm. The nucleus localizes in the basal region, the RER in the basolateral region around the nucleus, the Golgi complex in close proximity of the nucleus facing the apical membrane, and secretory granules in the apical region. The localization of the organelles depends on the cytoskeleton, particularly microtubules [54]. The plasma membrane is also highly asymmetric whereby two membrane domains are distinguished, referred to as apical and basolateral. Each of these membranes contains specific proteins and lipids, which do not intermix, since TJ prevent diffusion of membrane constituents from the apical to the basolateral domain and *vice versa* [56].

Given that acinar cells are highly polarized, the delivery of secretory products is a vectorial process [57]. Cell polarity is regulated at least by two types of interactions, cell–cell (TJ) and cell–ECM (hemidesmosomes). In this review we will focus on discussing the organization of the apical pole of acinar cells. This domain is particularly important because some of the proteins involved in the fusion of secretory granules with the plasma membrane (exocytic machinery) are present there.

3. Structural organization of the apical pole of the acinar cell under physiological conditions

An important structural element of apical pole is the actin cytoskeleton, which is part of the terminal web and the microvilli, which emerge from the apical surface of the acinar cells [58]. Actin microfilaments (F-actin) at the apical pole interact with proteins, such as α-actinin, profilin, villin, type II non-muscle myosin, fodrin, ezrin, radixin, moesin and indirectly with scaffolding and plasma membrane proteins. In addition, F-actin organizes the cortical cytoplasm of the basolateral domain [59,60].

Ezrin is a cytoplasmic protein that together with radixin and moesin constitutes the ERM protein family. Members of this family contain an N-terminal domain (N-ERMAD), an α-helix central region and a C-terminal region (C-ERMAD). The C-ERMAD binds to the N-ERMAD region of all family members and the last 30 aminoacids of C-ERMAD contain a binding site for F-actin [60]. In its inactive state, the C-ERMAD domain and N-ERMAD are associated,

thus masking the F-actin binding site [60]. The interaction of PtdIns(4,5)P2 (PIP2) with lysine residues of the N-ERMAD liberates restraints imposed by the interaction of these domains. Moreover, phosphorylation of thr-567 destabilizes intramolecular association of inactive ezrin, promoting the interaction with F-actin [60,61]. Phosphorylated ezrin is found exclusively in specialized structures of the plasma membrane [60]. The mechanism by which ezrin is inactivated remains unidentified, although ezrin is sensitive to calpain [62], a protease regulated by intracellular Ca^{+2} levels [60]. In gastric cells and endothelial cells, an increase in Ca^{+2} levels results in degradation of ezrin, as evidenced by the generation of a 55 kDa protein fragment [60]. In support of this mechanism, transfection of calpastatin, a cellular inhibitor of calpain, causes a significant accumulation of ezrin [60]. Another mechanism proposed for ezrin inactivation is the dephosphorylation of the active protein. However, the identity of the phosphatase involved in this process remains to be determined.

The dynamics of cortical organization of F-actin are important to organize the cell surface structures, but also for the correct functioning of cell membrane proteins. In this context, several pieces of evidence indicate that ezrin expression is necessary to maintaining epithelium integrity [63]. For example, suppression of ezrin expression using antisense oligonucleotides in cultures of epithelial cells prevents formation of microvilli, cell–cell adhesions and cell–substrate interactions. As a consequence, plasma membrane ruffles are generated and cell motility increases [64]. 2D cell cultures transfected with a mutant thr-567 that mimics phosphorylated ezrin (active form) show ruffle-like structures on the cell surface, presumably due to an increase in the number of bridges between F-actin and the plasma membrane [60]. Conversely, the same construct, transfected to 3D epithelial cell cultures, induces shape changes and decreases the ability to organize tubules. Under these conditions, enhanced ezrin activation alters cell–cell adhesion, as well as induces changes in E-cadherin distribution and Rac-GTPase activation [60]. Ezrin−/− mice show defects in the terminal web and the brush border of intestinal microvilli that are irregular in length and shape [63,65]. These and other data indicate that ezrin represents a regulated connection between cortical F-actin and plasma membrane proteins. This feature is important in many cellular processes, such as determination of cell shape, organization of microvilli, formation of ruffles, cell adhesion, motility, signal transduction and secretion.

4. Characteristics of apical intercellular junctional complexes in epithelial cells

The barrier function of epithelial cells is regulated by the apical intercellular junctional complex. The major constituents of this complex are TJs and adjacent adherens junctions [66]. TJs are a multiprotein complex composed of transmembrane and cytosolic proteins (Fig. 1). The most important functions of TJs include: 1) to restrict the lateral diffusion of plasma membrane proteins, thus forming asymmetric apical and basolateral domains with different functions that containing distinct molecules and structures; and 2) to form the major barrier that prevents the paracellular transport of solutes [56,66]. TJ plasma membrane proteins include claudins, occludins, junction adhesion molecule (JAM) and Coxsackievirus and adenovirus adhesion receptor (CAR). Moreover, membrane associated guanylate kinases (MAGUKs) represent another group of relevant TJ proteins located in cytoplasm [67].

Claudins are a family of 24 proteins with molecular masses ranging 20–27 kDa that form homo- or heteromeric complexes to generate ion selective pores. Within their tertiary structure, four transmembrane segments generate 2 extracellular loops and one intracellular loop with both the N-terminal and C-terminal regions

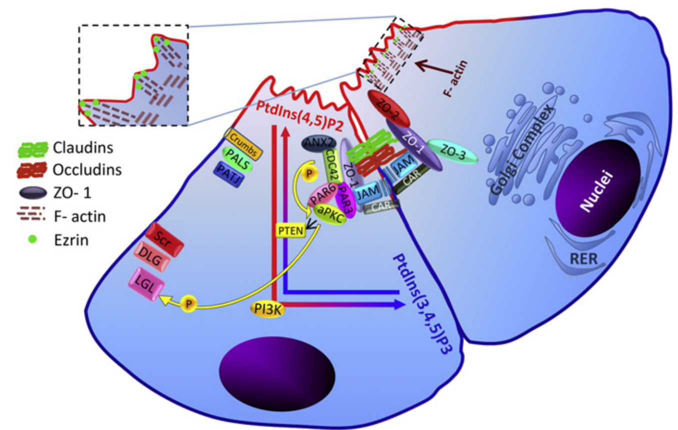

Fig. 1. Tight junctions (TJ) and protein complexes modulate cell polarity. TJ are multiprotein complexes formed by claudins, occludin, JAM (junction adhesion molecule), CAR (Coxsackievirus and adenovirus adhesion receptor) and MAGUK (membrane associated guanylate kinases) proteins. The protein complexes that modulate apical polarity are the sub-apical Par, the apical Crumbs and the basolateral Scribble.

oriented towards the cytoplasm. Claudin cytoplasmic domains associate with proteins containing PDZ domains (i.e. PSD-95 = post synaptic protein-95, DLG = discs large, ZO-1 = zonula occludens) through highly conserved aminoacids in the C-terminal region [68]. Mutations in this region prevent binding of claudin 1 and 5 to ZO-1, which in turn precludes ZO-1 association with TJs [69].

Occludin is an integral membrane protein of 65 kDa with four transmembrane domains that generate two extracellular loops, whereby both N- and C-terminal sequences face the cytoplasm. The extended C-terminal domain (150 aa) contains binding sites for protein scaffolds as ZO-1, ZO-2 and ZO-3 [68,70,71]. The functional significance of occludin in regulation of the paracellular barrier function is controversial. Cells overexpressing occludin form TJs strands of normal appearance (Reviewed in Ref. [68]). Studies using knockout mice for occludin in the intestinal epithelium revealed that other proteins, including claudin 3, did not alter their expression levels and those TJs appeared normal morphologically and in terms of their barrier function [72]. Taken together, this evidence indicates that the TJ strands can be formed in the absence of occludin. However, Saitou et al. studied the *in vivo* function of occludin, disrupting the endogenous mouse occludin locus. Both heterozygous and homozygous mutant mice were viable, but the homozygous mutant mice showed very complex abnormalities in various organs [72].

The *JAM* is a glycosylated protein, with a molecular mass of 43 kDa. JAM possesses three distinct structural domains: 1) an extracellular region of 215 aminoacid that contains two immunoglobulin variable domains, 2) a single transmembrane segment and 3) a short intracellular branch (45 aa) with a classic PDZ binding motif type II (Treo-X-aa hydrophobic) [70,73]. Indeed, JAM interacts with the PDZ domains of proteins such as, AF6, ASIP/Par-3 and ZO-1, via the C-terminal region [70]. JAM is also suggested to restrict the free flow of proteins in the intermembrane space. In inflammatory processes, the passage of leukocytes from the blood to the ECM requires TJ disassembly. In this situation, the immunoglobulin-like domains of JAM proteins are thought to bind to and thereby preclude the paracellular transport of proteins that is facilitated by leukocyte-induced TJ damage [74].

The *CAR protein* is an integral membrane protein of 46 kDa, with only one transmembrane spanning domain, an extended cytoplasmic stalk and an extracellular region consisting of two immunoglobulin-type domains [70]. In epithelial cells, CAR co-immunoprecipitates with ZO-1 and also co-localizes with TJ. The

C-terminal region contains PDZ type I (Ser-X-aa hydrophobic) binding motifs, which may explain the interaction observed between CAR and ZO-1. CAR binds serum IgG and IgM, and is overexpressed at sites of inflammation [75]. Therefore, it has been speculated that CAR together with JAM facilitate to the trans-epithelial migration of immune cells [76].

MAGUKs are cytoplasmic proteins that contain three PDZ domains, an SH3 domain and a guanylate kinase-like domain (GUK). PDZ domains bind to the C-terminal of several proteins, including occludin and claudins. MAGUK proteins correspond to a family of 3 members, ZO-1, -2 and -3, with molecular masses of 220 kDa, 160 kDa and 130 kDa, respectively. These proteins localize exclusively to the cytoplasmic surface of the TJ, in the immediate vicinity of the plasma membrane [67,71]. ZO-1 or -2 directly bind to F-actin via their C-terminal region, suggesting that these molecules crosslink TJs and F-actin [71,77,78]. ZO-2 associates with ZO-1 through a PDZ II/PDZ II interaction. ZO-3 can also associate with ZO-1, but not ZO-2. Finally, the PDZ I domain of the three ZOs binds to the Tyrosine/Valine sequence present in the C-terminal region of the claudins [71].

5. Regulation of polarity of apical pole in epithelial cells

Three protein complexes modulate apical polarity: the sub-apical Par complex, the apical Crumbs complex and the basolateral Scribbs complex (Fig. 1) [79]. The Par complex contains among other proteins Par6 bound to aPKCs through its N-terminal PB1 domain [80], which additionally binds to Par3 through its PDZ domain [81]. Par3 also interacts with aPKCs and Cdc42 through its C-terminal tail and the Par3–aPKC complex recruits the lipid phosphatase, PTEN. In the apical Crumbs complex, the cytoplasmic tail of the transmembrane protein Crumbs interacts directly with the PDZ domain of Pals1 [81]. The interactions between Pals1 and PATJ involve binding of the L27 domain of each protein [79]. Finally, in the basolateral Scribble complex, Scrib associates indirectly with Dlg via a GUK holder protein [79,81,82]. The interaction between Lgl and the rest of the complex components remains to be defined [79]. To establish and maintain cell polarity, polarity complexes are thought to interact with one another [82].

The relative levels of PtdIns(3,4,5)P3 and PtdIns(4,5)P2 are crucial factor in determining cell polarity [83,84]. Phosphatidylinositol-phosphates (PtdInsPs) are phospholipids that are singly or multiply phosphorylated at different positions of the inositol head group. In this context of particular interest are events altering phosphorylation at the positions 3, 4 and/or 5 [56]. PtdIns(3,4,5)P3 is generated from PtdIns(4,5)P2 via phosphorylation by PtdIns3-kinases (PI3K). Alternatively, PtdIns(3,4,5)P3 can be dephosphorylated again to yield PtdIns(4,5)P2 by the phosphatase PTEN. Asymmetric PtdIns(4,5)P2:PtdIns(3,4,5)P3 distribution is observed in various cell types, including migrating neutrophils and polarized kidney epithelia [85,86]. In polarized MDCK cells PtdIns(3,4,5)P3 localizes exclusively to the basolateral membrane, whereas PtdIns(4,5)P2 is apically enriched [56,84].

Different signaling pathways and proteins are involved in TJ assembly including protein kinase A (PKA), heterotrimeric G-proteins, and various PKC isoforms and RhoGTPases. The effects of PKA on the assembly and disassembly of TJ remain controversial. In thyroid cell cultures, deprived of extracellular calcium, the addition of thyroid stimulating hormone (TSH) elevates cAMP levels and prevents TJ dissociation. Thus, PKA activation has been suggested to prevent TJ disassembly [87]. However, in MDCK cells incubated with PKA inhibitor, TJ dissociation was also reduced [88]. Several PKC isoforms localize to TJs [89,90], but the best described molecular mechanism is that of aPKCs. These kinases form complexes with Par3 (Par3 = ASIP in mammals) and Par6 [91]. Furthermore, recruitment of additional TJ proteins is mediated by binding of Par3 to JAM [91]. Par6 binds Cdc42, a RhoGTPase essential for cell polarity implicated in the activation of aPKCs [91,92]. aPKCs activity is negatively regulated by protein phosphatase2A (PP2A), which interacts directly with dephosphorylated aPKC [93]. Overexpression of the catalytic subunit of PP2A in MDCK cells correlates with dephosphorylation of TJ proteins and an increase in the paracellular permeability. Both observations tend to indicate that PP2A is a negative regulator of TJs [94]. These data suggest that the aPKC-Par3-Par6 complex and PP2A have opposite functions in regulating the assembly and disassembly of TJs [94].

Rho-GTPases are also involved in regulating TJ assembly, as well as paracellular permeability [95]. The role of GTPases in TJ assembly was inferred from experiments where GTPγS was assayed in permeabilized T84 cells inhibiting this process [96]. Also, the microinjection of C3 transferase in MDCK cells (a toxin that inactivates Rho) inhibited TJ assembly [97,98]. Moreover, Rho-GTPase has been suggested to stimulate phosphorylation of the C-terminal domain of occludin and enhance cytoplasmic distribution of this protein. Importantly, phosphorylation of occludin prevents its interaction with F-actin adjacent to the apical plasma membrane (Reviewed in Ref. [99]).

6. Altered organization of the apical pole of salivary acinar cells from Sjögren's syndrome patients

In initial studies observing salivary acini from SS-patients at low resolution by light microscopy, we determined that some acinar cells had lost their anchorage to the basal lamina. These cells were released from the acinus and accumulated in the lumen of the ducts. Somewhat surprisingly, the nuclei of such cells did not display overt signs of apoptosis [100]. Results from our laboratory and others [101] demonstrated that acinar cells synthesize both matrix metalloproteinases (MMPs), which degrade the basal lamina components, as well as their tissue inhibitors (TIMPs) [101–103]. An MMP/TIMP expression ratio greater than 1 was taken to indicate that degradative activity was increased. For laminin and collagen IV changes in immuno staining intensity were apparent in the basal lamina and ducts of acini, while no such changes were detected in capillaries situated in close proximity of these structures [104,105]. These observations indicated that salivary glands from SS-patients produced enzymes that destroyed their own basal lamina, which did not diffuse through the ECM. Interestingly, mRNA and protein levels of some laminin subunits were only increased in glands from SS-patients with a low degree of fibrosis [104]. This finding suggests that glands of SS-patients activate a mechanism to remodel the basal lamina, probably as part of a survival strategy that seeks to avoid cell loss. Another relevant observation in this context was that these changes were independent of the proximity of inflammatory cells. Concomitantly, protein levels of α6β4 integrin, an adhesion receptor known to interact with the basal lamina, were decreased in SS-patients with disrupted basal lamina [106]. This integrin is crucial to the organization of adhesion complexes called hemidesmosomes, which are responsible for cell anchorage to the basal lamina. Changes in α6β4 integrin and other components of this basal adhesion complex detected in SS-patients have been reviewed extensively elsewhere [105,107].

Additionally, we observed luminal expansion in the SS-patient acini by transmission electron microscopy (TEM) [100]. Moreover, residual microvilli on the apical surface of acinar cells were decreased in number and the characteristic finger-shaped morphology was lost [100]. As previously mentioned, microvilli structure is determined by the high density of F-actin filaments

linked to the plasma membrane via ezrin. Subsequent cDNA microarray studies using an epithelial cell-enriched fraction from SS-patients salivary glands revealed that ezrin expression was increased [108]. Then, we determined that ezrin protein levels were increased in SS-patient and that localization of the protein changed from preferentially present in the apical plasma membrane in controls, to the accumulation in the basal cytoplasm in SS-patients [44]. Studies using immuno-gold immunohistochemistry combined with TEM confirmed this location [44]. Ezrin activation by thr-567 phosphorylation (P-ezrin) promotes F-actin binding. In controls, P-ezrin is detected exclusively at the apical pole of acinar cells, whereas in SS-patients the protein localizes preferentially to the basal cytoplasm. In controls, the localization of P-ezrin coincides with high levels of F-actin at the apical pole of acinar cells, while in SS-patients colocalization was only observed in the basal cytoplasm [44]. Since P-ezrin represents the active form of the protein, decreases in P-ezrin levels at the apical pole of the acinar cells from SS-patients may explain the reported microvilli disorganization.

Microvilli are connected through F-actin to the terminal web, which consists of an array of F-actin bundles anchored to the cytoplasmic proteins of TJs. Therefore; TJ disruption is likely to trigger the observed loss of microvilli architecture. To corroborate this notion, the expression and localization of key proteins that organize this junctional multiprotein complex, such as occludin, ZO-1, claudins 1, 3 and 4 was compared in SS-patients and control individuals [45]. In SS-patients, occludin and ZO-1 protein levels were down-regulated; claudin 1 and claudin 4 were upregulated, while claudin 3 was similar to controls (Table 1). Surprisingly, for claudin 3, immuno staining was redistributed from apical to basolateral plasma membrane of acinar cells in SS-patients and this change was not associated with the proximity of inflammatory cells (Fig. 2) [45]. TEM revealed the presence of electron dense material extending from apical to basolateral surface in a manner similar to what was observed for claudin 3. Interestingly, for acini with these alterations, cytoplasmic endocytic vesicles were detected with accumulation of electron dense material in membranes as described above. In addition, a large number of exosome-like microvesicles, ranging 30–300 nm in size were detected in the lumen of such acini [45]. Endocytosis may lead to the formation of exosomes, among other vesicles participating in the formation of multivesicular bodies. Exosomes have been described in autoimmune diseases, such as SS, where they have been ascribed at least two roles: 1) Antigen presentation and 2) miRNA carriers [109–111].

Reportedly, cytokines induce TJ disorganization [45,96,112]. In our laboratory, acini isolated from salivary glands of control individuals were incubated with TNF-α and/or IFN-γ and then claudin 3 or occludin were detected using specific antibodies. Both proteins redistributed following cytokine exposure from the apical to the basolateral acinar plasma membrane [45]. The importance of this experiment resides in the fact that for the first time well-documented observations *in vivo* could be reproduced in vitro. Others reported that disruption of the epithelial barrier by pro-inflammatory cytokines, such as IFN-γ, represents a major pathophysiological consequence of intestinal inflammation [96]. These authors previously showed that IFN-γ increases paracellular permeability in a T84 epithelial cell model by inducing endocytosis of TJ proteins, such as occludin, JAM, and claudin 1. TJ proteins internalize into large actin-coated vacuoles derived from the apical plasma membrane. These structures resemble the vacuolar apical compartment (VAC), which had been described previously in epithelial cells that lost cell polarity. These changes were dependent on ATPase activity of a myosin II motor. Pharmacological analysis revealed that formation of VACs and endocytosis of TJ proteins was mediated by Rho-associated kinase (ROCK) rather than myosin light chain kinase. These studies also showed that IFN-γ treatment resulted in Rho activation and up-regulation of ROCK. Taken together, these results suggest that IFN-γ induces endocytosis of epithelial TJ proteins via RhoA/ROCK-mediated and myosin II-dependent formation of VACs [96]. Moreover, TNF-α is a pro-inflammatory cytokine that alters epithelial cell function by inducing changes in the actin cytoskeleton [112], promoting microtubule disassembly and reducing barrier function [113]. The mechanisms underlying these changes include the formation of reactive oxygen species [114] and RhoA GTPase [115,116], as well as MAPK activation [113,117], increased transcription of myosin light chain kinase [115,118] and heat shock proteins [119] and endocytosis of TJ proteins [120]. Presumably as a consequence of these changes, loss of barrier function, redistribution of apical and basolateral components of the acinar plasma membrane and cellular polarity are observed. These alterations undoubtedly affect severely the functionality of the secretory machinery of salivary acinar cells from SS-patients.

Table 1
Relative protein levels of tight junction proteins of labial salivary gland from SS-patients.

Protein	Relative protein levels (Mean ± SD)			
	Controls	Patients	*P*	Ratio P/C
Claudin 1	0.2 ± 0.2	0.4 ± 0.2	0.02	2.0
Claudin 3	1.5 ± 0.4	1.2 ± 0.5	0.66	0.8
Claudin 4	0.13 ± 0.1	0.34 ± 0.4	0.04	2.6
Occludin	1.1 ± 1.2	0.13 ± 0.15	0.004	0.12
ZO-1	0.5 ± 0.6	0.1 ± 0.1	0.002	0.2

In SS-patients, Claudin 1 and 4 were up-regulated, while Occludin and ZO1 were down-regulated. However, Claudin 3 showed no change. Modified of Ewert et al. [45].

7. Organization of the secretory machinery

In eukaryotic cells, trafficking of secretory vesicles to the plasma membrane is essential for normal cell function. This process is also required for intercellular communication via the release of relevant molecules in multicellular organisms. The fusion of secretory vesicles with the plasma membrane occurs essentially by constitutive exocytosis in all cells. Alternatively, both constitutive and regulated exocytosis is observed in exocrine and endocrine cells.

The molecular machinery involved in membrane recognition and fusion of vesicles within the secretory pathway in eukaryotic cells is mediated by Rab GTPases and SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptors). Additionally, these processes are modulated by other factors, including Sec1/Munc18-like proteins and synaptotagmins [121,122].

7.1. Overview of Rab GTPases

Rab, Ras, Rho, Ran and Sar1/Arf proteins, among others, form the superfamily of small GTP-binding proteins. These proteins have molecular masses ranging 18–40 kDa [123]. To date, 11 types of Rab proteins have been identified in yeast and over 60 in mammals, all with a high degree of homology to one another [124].

Rab proteins, like other members of the superfamily of small GTP-binding proteins, can be found in the active GTP-bound or the inactive GDP-bound state [123,125]. The activation/inactivation cycle is regulated by interaction with accessory proteins such as GDIs (GDP dissociation inhibitors), GDFs (GDI displacement factors), GEFs (guanine nucleotide exchange factors) and GAPs (GTPase-activating proteins). GDIs bind to inactive Rab proteins, masking their C-terminal regions. These complexes localize to the

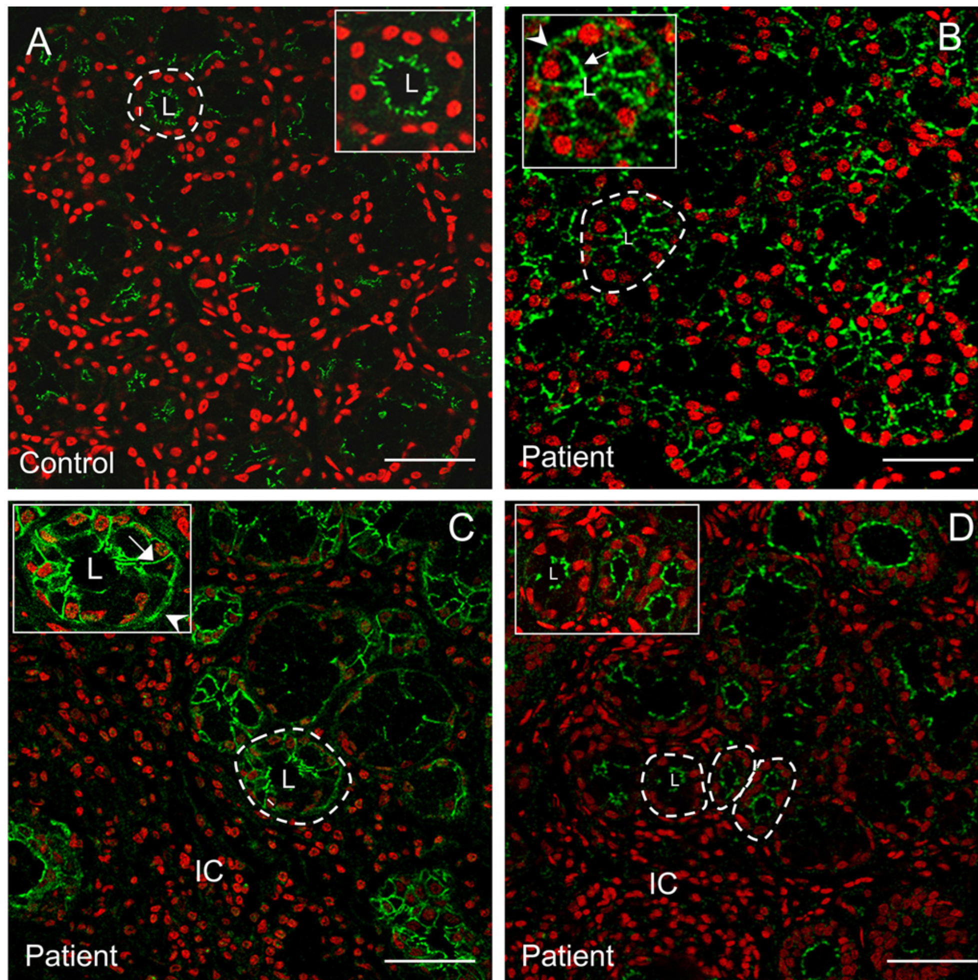

Fig. 2. Claudin 3 redistribution in acini of SS-patients is not associated to inflammatory cells. Microphotographs of a control subject (A) and a representative SS-patient (B, C, D) are shown. Specific claudin-3 staining is green, while nuclei are stained red. Claudin-3 redistribution to the basolateral plasma membrane of acini localized either distant from or close to inflammatory cell foci in acini of SS-patients is shown (B and C, respectively). Normal claudin-3 distribution in the apical plasma membrane of acini and ducts in close proximity of inflammatory cell foci is shown in acini of SS-patients (D). Dotted white lines in A, B, C, and D indicate acini that are highlighted in the respective insets. White arrows in the insets (panels B and C) indicate claudin-3 redistribution to the acinar lateral surface and white arrowheads redistribution to the acinar basal surface. L: lumen. IC: inflammatory cells. Bars correspond to 40 μm. Images reproduced under permission of Arthritis and Rheumatism [45].

cytosol, since GDIs mask elements responsible for the insertion of Rab proteins into target membranes. GDFs displace GDIs from the GDI-Rab complexes, permitting Rab translocation to membranes where they adopt a different conformation, which is recognized by GEF protein that induces the exchange of GDP by GTP. Once activated, Rab-proteins recruit specific effectors to the respective membranes. Finally, GAP proteins augment the intrinsic GTP-ase activity of Rabs, thereby favoring GTP hydrolysis and the return of Rabs to their inactive state (Fig. 3) [125].

Rab proteins regulate intracellular trafficking by controlling vesicular transport, recognition, tethering and fusion to the acceptor compartment [126]. As indicated above, they represent molecular switches, which in the active GTP-bound state develop conformational changes that permit interaction with specific effectors. Examples of interactions between specific effectors and the respective Rabs include: EEA1 and Rab5, involved in endocytic vesicle tethering and fusion; Rabphilin and Rab3A that regulate exocytosis of synaptic vesicles; TIP47 and Rab9, which participate in recycling of mannose 6-phosphate receptors; Melanophilin and Rab27A that participate in tethering and retention of melanosomes in the cell periphery for transfer to neighboring keratinocytes [126].

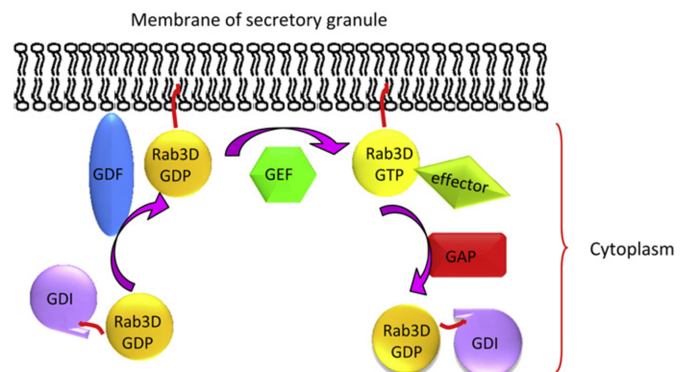

Fig. 3. The activation/inactivation cycle of Rab3D in cell secretion. Cycling is regulated by interaction with accessory proteins such as GDI (GDP dissociation inhibitor), that is bound to inactive Rab protein, masking its C-terminal region; GDF (GDI displacement factor), GEF (guanine nucleotide exchange factor) protein inducing exchange of GDP by GTP and GAP (GTPase-activating protein), that catalyzes GTP hydrolysis returning Rab to its inactive form.

Rab proteins are specifically located in different compartments along the exocytic and endocytic route, including the RER, the Golgi complex, secretory granules, secretory vesicles, and endosomes, among others. Also, they are expressed differentially according to cell types. For detailed description of Rab protein distribution between subcellular compartments and their respective roles in intracellular trafficking processes the interested reader is referred elsewhere [126,127].

Consistent with a role for Rab3 (A-D) proteins in exocytosis, they are specifically localized in secretory cells. For example, Ohnishi et al. reported that Rab3D localizes to the sub-apical region of rat pancreatic acini [128]. Alternatively, Raffaniello et al. observed Rab3D in the membrane of secretory granules from rat parotid glands [129]. Schlüter et al. confirmed these results comparing the localization and function of Rab3 isoforms in various rat tissues. In brain and cerebellum Rab3A, B and C isoforms represented the prevalent isoforms, while in exocrine tissues, such as salivary, lachrymal and pituitary glands Rab3D was detected [130]. Subsequent studies reported on Rab3D localizing to the membrane of mature secretory granules [131,132].

7.2. Alterations of Rab-GTPases in SS-patients

In particular salivary acinar cell from SS-patients have severe alterations of localization and expression of cell polarity markers that may influence intracellular trafficking, which could be related to Rab proteins. These GTPases are implicated directly or indirectly in altering the intracellular trafficking of vesicles in a variety of diseases including diabetes type 2, Bardet-Biedl syndrome and Griscelli syndrome, among others [133]. As Rab3D is a marker of mature secretory granules and is the predominant isoform in exocrine tissue, alterations in Rab3D distribution will affect secretory function. Rab3D localizes in close proximity of the apical membrane in lacrimal glands from control BALB/c mice [131]. While in lacrimal gland of NOD mice, a murine model of SS [134]. Rab3D was observed in areas close to the basolateral plasma membrane [131]. Also, in these mice the secretory granules are highly heterogenous in size and shape when observed by TEM, this finding agrees with the altered distribution pattern observed for Rab3D by immunofluorescence analysis [131].

In control acinar cells from labial salivary glands, Rab3D localized primarily to granular structures of the apical region. However, in SS-patients, Rab3D distribution throughout the cytoplasm was observed for approximately 60% of serous acini. In this study, Rab8A, used as a control, was found to localize to the basolateral region where it participates in constitutive exocytosis. No changes in Rab8A localization were detected in SS-patient acini (Fig. 4) [132]. Interestingly, Rab3D redistribution was accompanied by mislocalization of ezrin from the apical to basolateral membrane, loss of nuclear polarity, increased volume of the Golgi complex, MUC7 accumulation in the cytosol [132] and STX4 localization to the basolateral plasma membrane of serous acini [1]. These results are indicative of drastic alterations in the normal destination of the secretory granules to the apical plasma membrane. These events may be linked to cytoskeletal changes since alterations in polarity are apparent in acinar cells of SS-patients. In addition, such alterations could explain the observed accumulation of secretory granules in the cytosol that cannot be exocytosed to the acinar lumen. Possibly even complexes with SNARE proteins (STX4) localized in basal compartment are formed that result in misdirected exocytosis to the ECM. In the salivary glands of SS-patients also a significant decrease in the Rab3D protein levels were detected. These changes did not, correlate with the unstimulated salivary flow ($r = 0.2185$), indicating that Rab3D function is not related to the process of water and electrolyte release. Alternatively, an inverse correlation with changes in scintigraphy ($r = -0.5123$, $p = 0.0249$) was detected, which therefore provides a morphofunctional test for salivary gland performance [132].

7.3. Membrane fusion receptor: SNAREs

SNARE proteins play a key role in all membrane fusion events in eukaryotes [135]. SNAREs constitute a subcellular protein family specific for every compartment, consisting of 36 members in *Homo sapiens*. SNARE proteins are 100–300 aminoacids in size and most

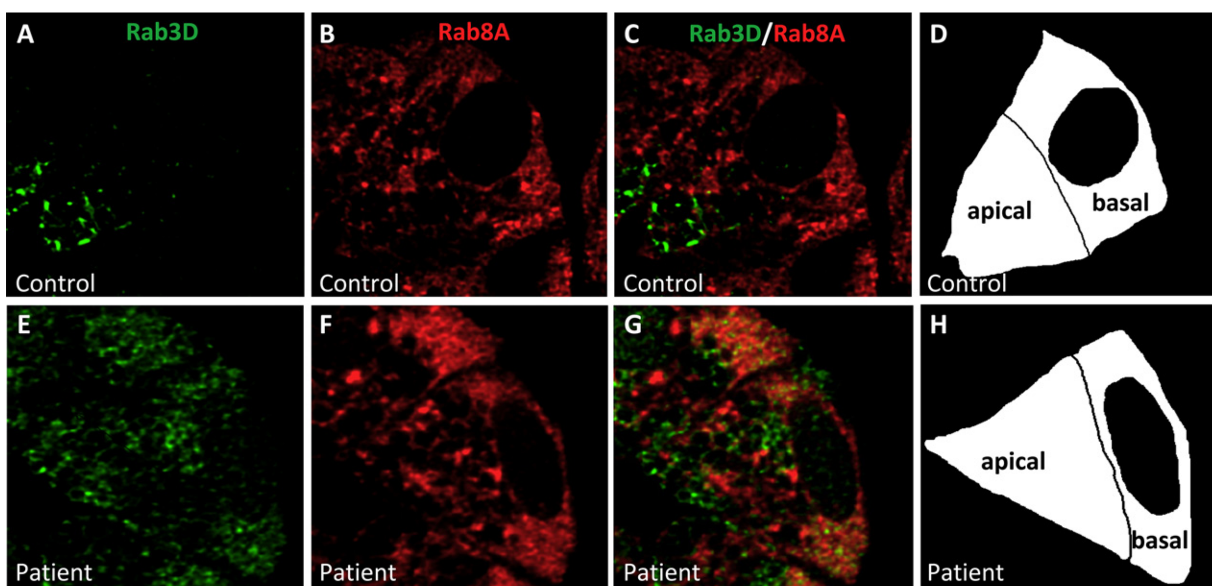

Fig. 4. Altered localization of Rab3D in acinar cells from SS-patients indicates loss of cell polarity. In control acinar cells Rab3D is localized in mature secretory granules. In SS-patients acinar cells Rab3D distributed throughout the cytoplasm. This change resulted in loss of cell polarity, implying that these secretory granules may ectopically merge with basolateral plasma membrane. Rab8A, a control for expression and localization, was only found in the basolateral region both in controls and SS-patients. These observations demonstrate a differential behavior of Rab GTPases.

of them are small type II membrane proteins [136]. They are the core constituents of the protein machinery that facilitates membrane fusion. SNARE proteins are membrane receptors that bind SNAPs (soluble NSF attachment proteins) and NSF (N-ethylmaleimide Sensitive Factor) [122,137]. Cell membrane fusion requires SNARE proteins localized both to a donor (v-SNAREs) and an acceptor membrane (t-SNAREs) and specific binding between the different SNAREs is thought to mediate membrane fusion [135,137,138].

To date, 3 families of highly conserved SNARE proteins have been described: Vamps (Vesicle associated membrane proteins), Syntaxins (STXs) and SNAP-class SNAREs (SNAP-25 and their homologs) [136,139]. The SNARE family members vary in structure and size, although all retain a conserved 60-70 aminoacid sequence known as the SNARE motif, which contains 8 repeats of a heptameric sequence, usually flanked by a C-terminal transmembrane domain [138]. However, SNAP-25 (25 kDa synaptosome-associated protein; not related to SNAPs by NSF) and its homologs (SNAP-23, SNAP-29 and SNAP-47, among others) lack a transmembrane domain, but instead are anchored to the membrane by post-translational modifications, such as palmitoylation [122,137]. Furthermore, Vamps and STXs are inserted into the phospholipid bilayer of the plasma membrane via their C-terminal hydrophobic regions, resulting in exposure of their coiled-coil motifs towards the cytoplasm [122]. STXs and Vamps have one SNARE motif, whereas SNAP-class SNAREs have two motifs. Four classes of SNARE motifs are structurally distinguished (referred to as R-, Qa-, Qb, and Qc-SNARE motifs) [122,138].

Membrane fusion occurs through formation of a heterotrimeric complex between SNARE proteins. This complex involves four different alpha-helix SNARE motifs (R/Q rule) of which frequently three t-SNARE motifs are located in the acceptor membrane and one v-SNARE is located in the donor membrane. The ternary complex formed is highly stable and can be dissociated only by the action of NSF (a soluble ATPase), which acts in concert with cofactors called SNAPs [122,137]. This fusion complex provides the energy required to break the hydrophilic barrier that separates both membranes. Initially, a "trans" SNARE complex forms followed by a "cis" complex in which the four motifs remain localized to the acceptor membrane [136–138]. During assembly of the complex, SNARE motifs interact sequentially from the N-terminus towards the C-terminus, forming a zipper structure and generating a driving force, which causes the opposing membranes to gradually connect. Finally, membranes fuse when the SNARE motifs interaction sequence is completed [122].

SNARE proteins were first described in neurons. The neural SNAREs VAMP2/synaptobrevin, (localized in synaptic vesicle membrane) is forming fusion complexes with STX1A and SNAP-25 (localized in the pre-synaptic plasma membrane) modulating the releasing of neurotransmitters [121,136,137]. In pancreatic acinar cells and submandibular gland cells, SNARE proteins such as VAMP2, VAMP8, STX2, STX3, STX4 and SNAP-23 have important functions in both constitutive and regulated secretion [140–144]. VAMP2 and STX2 are primarily associated to constitutive secretion, while STX4 and VAMP8 are involved in regulated secretion [140,141]. On the other hand, STX3 participates in homotypic granule–granule fusion [145], whereas SNAP-23 is involved in all three processes described [140,145]. STX4 and STX2 localize to the plasma membrane [140,144], VAMP2 and VAMP8 in secretory granules [140], and SNAP-23 and STX3 in secretory granules as well as the plasma membrane [140]. Interactions between SNARE proteins to form complexes involved in the fusion of secretory granules with the apical plasma membrane (STX4/VAMP8/SNAP-23 or STX2/VAMP2/SNAP-23) or granule–granule homotypic fusion (STX3/VAMP8/SNAP-23) have been described [140–143,145].

VAMP8 is highly relevant to the process of regulated secretion [141,142,146]. VAMP8 knockout mice present a reduced stimulated secretion towards the apical plasma membrane in several different exocrine tissues (i.e. salivary gland, lachrymal, breast, sweat glands and prostate) [141]. Moreover, antibodies against VAMP8 prevent enzyme exocytosis triggered by calcium in pancreatic acinar cells [140]. These data indicate that VAMP8 participates in exocytosis at the apical membrane and represents the putative SNARE present in secretory granules. Changes in the location of SNARE proteins have been associated with severe clinical manifestations, such as acute pancreatitis [145].

7.4. Altered expression and localization of SNARE proteins in salivary glands of SS-patients

The correct expression and localization of SNARE proteins is crucial to preserve vectoriality of the secretion process and an efficient fusion of secretory granules from the apical plasma membrane. Studies of SNARE proteins involved in regulated secretion in salivary glands of SS-patients identified a significant decrease in relative protein levels of VAMP8 and STX4. On the other hand, for STX3, an SNARE protein involved in granule–granule fusion, a significant increase in protein levels was detected in SS-patients. Also, STX3 presence increased in the cytoplasm and the basolateral plasma membrane [1], presumably, favoring granule–granule fusion, as was observed in previous studies [100]. Additionally, also apico-basal redistribution of STX4, SNAP-23 and VAMP8 is observed in SS-patient acinar cells [1]. These redistributions were similar those observed for claudin 3, ezrin and Rab3D [44,45,132].

Interestingly, in the absence of secretory exogenous stimulation, atypical STX4-RAB3D colocalization was observed in the basal and

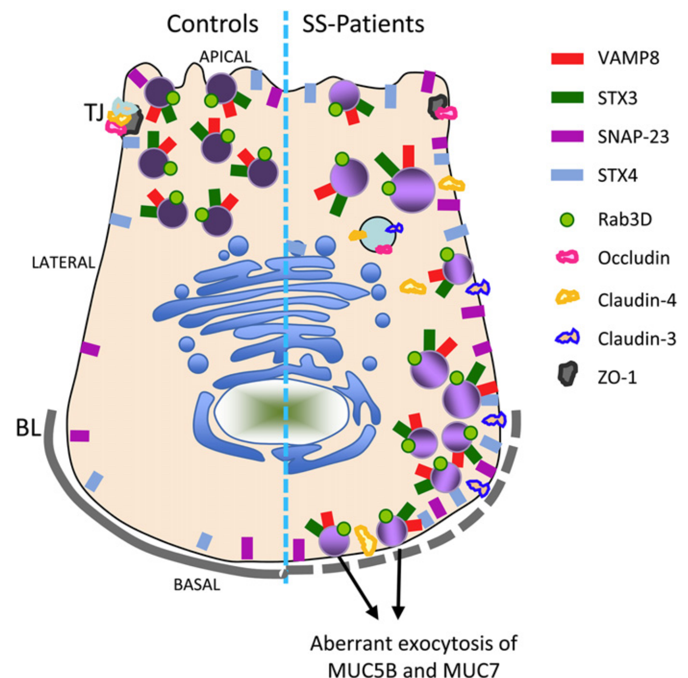

Fig. 5. Disruption of tight junctions induces relocalization of secretory machinery proteins. Altered localization of tight junction (TJ) proteins in salivary glands of SS-patients promotes redistribution of both apico-basal Rab3D and SNARE proteins. The presence of high levels of functional SNARE complexes in the basolateral plasma membrane under unstimulated conditions, may explain the aberrant presence of MUC5B and MUC7 in the ECM. As these mucins are normally exocyted by the apical pole of acinar cells, its altered localization may be an inflammation inducer. BL: basal lamina.

lateral plasma membrane domains for SS-patients [1]. Additionally, we observed an increase in the formation of SNARE complexes for VAMP8, STX4 and SNAP-23 under basal conditions. The VAMP8-STX4 complexes aberrantly localized to the basal acinar cell pole in SS-patients. Increased functional SNARE complexes with anomalous localization likely represent the cause of ectopic exocytotic processes, such as aberrant secretion of mucins from acinar cells towards the ECM, as was observed for MUC7 and MUC5B (Fig. 5) [1]. In this context, it is intriguing to speculate that such aberrantly secreted mucins may promote inflammation and disease progression.

8. Concluding remarks

Cell polarity is important for the organization and function of all epithelia, including secretory epithelium. The interaction of many proteins is required to establish and maintain cell polarity. Some, considered the most relevant, were discussed here (TJ, hemidesmosomes, polarity complexes, cytoskeleton). Alterations in the expression and localization of proteins that are part of TJ lead to significant changes in salivary gland cell polarity in SS-patients. Pro-inflammatory cytokines, such as TNF-α and IFN-γ, which are also synthesized by salivary acinar cells of SS-patients, induce redistribution of TJ proteins in normal salivary acini, indicating their importance in the regulation of cell polarity. In these patients, the changes observed in cell organization affect the localization and, as a consequence, the functionality of the secretory machinery. In this context, apico-basal redistribution of proteins was found to be important for determining the destination of mature secretory granules (Rab3D) and in membrane fusion (SNARE). Moreover, formation of functional SNARE complexes in the basolateral domain of acinar cells was associated with misdirected exocytosis towards the ECM (i.e. mucins) (Fig. 5). These changes are important; due to alterations in mucin distribution/secretion may possibly contribute to the development of inflammatory processes.

In general, disruption of the epithelial barrier (i.e. TJ) is linked to the development of chronic immune diseases. If epithelial barrier function is a major contributing factor in the predisposition to disease remains unclear. However, clinical and experimental evidence supports a role for epithelial barrier dysfunction in a wide variety of diseases, including cystic fibrosis, cancer, type 1 diabetes, celiac disease, Crohn's disease and SS. Recent studies have identified a role for a number of exogenous factors, including pathogens and components of the innate and adaptive immune system, in processes leading to the loss of epithelial barrier function. Understanding the relevant interactions between innate and adaptive immunity and epithelial barrier function will provide important insights to the pathogenesis of inflammatory and autoimmune diseases. Furthermore, a better understanding of the molecular pathways involved in the regulation of epithelial barrier function can be expected to have important implications both for the treatment and prevention of chronic inflammatory diseases, since such insight should permit the development of therapeutic agents that modulate epithelial barrier function in a more specific manner.

Disclosure statement

The authors have declared no conflicts of interest.

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