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REVIEW

Proteases, cystic fibrosis and the epithelial sodium channel (ENaC)

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Abstract Proteases perform a diverse array of biological functions. From simple peptide digestion for nutrient absorption to complex signaling cascades, proteases are found in organisms from prokaryotes to humans. In the human airway, proteases are associated with the regulation of the airway surface liquid layer, tissue remodeling, host defense and pathogenic infection and inflammation. A number of proteases are released in the airways under both physiological and pathophysiological states by both the host and invading pathogens. In airway diseases such as cystic fibrosis, proteases have been shown to be associated with increased morbidity and airway disease progression. In this review, we focus on the regulation of proteases and discuss specifically those proteases found in human airways. Attention then shifts to the epithelial sodium channel (ENaC), which is regulated by proteolytic cleavage and that is considered to be an important component of cystic fibrosis disease. Finally, we discuss bacterial proteases, in particular, those of the most prevalent bacterial pathogen found in cystic fibrosis, *Pseudomonas aeruginosa*.

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Proteases

Proteases (peptidases, proteinases) are broadly classified as hydrolases that act on the peptide bond (Levene 1905). These enzymes are ubiquitous and control a range of biological functions across all kingdoms of life (Lopez-Otin and Bond 2008). This includes polypeptide cleavage in digestion and nutrient absorption to complex cell signaling, as in the caspase and blood-clotting cascades. Cleavage of the peptide bond results in the liberation of amino acid or peptide sequences from the original polypeptide polymer. These hydrolysis events can be used post-translationally to regulate substrate activity, localization and expression levels. A diversity of proteases are expressed from prokaryotes to humans and have been broadly classified into six family groups based on their active site chemistry, namely, serine proteases, threonine proteases, cysteine proteases, aspartate proteases and metalloproteases (MMPs) (Rawlings et al. 2010; Studholme et al. 2003). In addition, a small number of glutamic proteases have been isolated, although their identification has been limited to filamentous fungi, to date (Oda 2012).

Over 550 proteases have been identified in the human genome (Puente et al. 2003; Quesada et al. 2009). Of these, ~90 are thought to be homologs that are catalytically inactive, potentially contributing to physiological regulation via competitive interactions with their respective substrates. The location of cleavage is specific to each protease (or family) and can be broadly categorized as being either endoprotease (internal cleavage site) or exoprotease (cleavage of terminal residues) in nature (Overall et al. 2004). The substrate sequence and cleavage site is also specific to each protease or protease family. Specificities can be extremely permissive

when cleavage sites are defined by a small number of amino acids, as with Arg-C, Asp-N, Lys-C, or trypsin (Quesada et al. 2009; Rawlings et al. 2012). Protease specificity can be greatly increased when the recognition sequences are more extended peptide sequences, as with thrombin and multiple caspases (Di Cera and Cantwell 2001; Talanian et al. 1997). Additional specificity is seen in proteases that recognize protein tertiary structures, as with the de-sumoylating and de-ubiquitylating proteases (Mossessova and Lima 2000).

The specific reaction mechanisms vary by active site composition and, in some cases, are still disputed. However, the general mechanism of hydrolysis proceeds via a nucleophilic attack on the backbone carbonyl of the protein/peptide substrate (Erez et al. 2009). Protease residues can accomplish this attack directly or through activated water and/or the coordinate metal ion in the active site. In addition to the catalytic domains, many proteases contain additional domains that regulate activity and localization. These domains serve to regulate protease function by changes in post-translational modification, ligand binding and cleavage. In addition, accessory domains serve to regulate membrane and cellular localization and facilitate protein-protein interactions in proteolytic cascades.

In this review, the regulation of proteases will be briefly discussed and then our focus will shift to proteases in the human airway and their role in the disease progression of cystic fibrosis (CF). Particular attention will be paid to proteases linked to pathogenesis and CF disease and to the impact of proteases on the regulation of the epithelial sodium channel (ENaC).

Protease regulation

Protease activity is tightly regulated at a minimum of four levels, which include transcriptional regulation, post-translational modification, physical compartmentalization and functional inhibition (Chow et al. 1995; Gorogh et al. 2006; Lopez-Otin and Matrisian 2007; Mirghomizadeh et al. 2009; Muzio et al. 1997). The multiple levels of protease regulation provide for tight tissue, temporal and environmentally responsive control of activity. Dysregulation of these regulatory events putatively contributes to multiple disease pathophysiologies. Transcriptional and epigenetic regulation of protease expression has been shown to be a major determinant across multiple families of proteases. A variety of cancer models suggest that the overexpression of proteases responsible for tissue remodeling is correlated with tumor proliferation and disease progression (Lopez-Otin and Matrisian 2007).

In addition to phosphorylation and glycosylation, a common means of protease regulation is through multiple post-translational modifications. Whereas a variety of mechanisms have been described for specific proteases, one of the most common is the proteolytic activation of pro-proteases (Salvesen and Riedl 2008). A variety of protease families are initially expressed, trafficked and/or secreted in an inactive state. Often, this inactive state is associated with a pro-peptide sequence that inhibits enzymatic activity. Removal of the pro-peptide, by protease cascades or by autoproteolytic cleavage, results in a disinhibition of protease activity and an active enzyme (Egnell and Flock 1992). This type of proteolytic regulation is often associated with secreted proteases and the pro-peptide cleavage is accomplished after secretion from the cell or with specific environmental triggers. Intracellular proteases can be regulated in a similar manner with the most well-studied cascade being that of the caspase apoptotic pathway (Salvesen and Riedl 2008). Such cascades often rely on an initial receptor or another physiological sensor that mediates the activation of an upstream protease. The cascade is triggered when cleavage of the downstream pro-peptides occurs as a result of upstream protease activation.

In addition to pro-peptide cleavage, ligand-induced activation is associated with a variety of proteases (Baumann 1994; Ravaud et al. 2003). Recent work on serralysin proteases has demonstrated that Ca^{2+} serves as a critical co-factor for protease folding and subsequent activation (Zhang et al. 2012). In the absence of Ca^{2+}, the proteases remain in an unfolded conformation and are inactive. Calcium binding induces the folding of a chaperone domain, which subsequently serves to facilitate the folding and activation of the proteolytic domain.

Compartmentalization also serves as a major mechanism to regulate protease activities. Membrane and organelle targeting are key to regulating the functions of the extracellular MMPs and organelle-specific proteases (Fritz et al. 1987; Kametaka et al. 2003). In many cases, pro-peptide cleavage and/or additional post-translational modifications are associated with proper secretion or trafficking. This compartmentalization serves to regulate the activities of the activated protease spatially, with classic examples being lysosomal proteases and furin, a protease found in the trans-Golgi network. In addition, a unique mechanism to regulate protease activity intracellularly is the auto-compartmentalization seen with the proteasome (Song et al. 2003; Tomisugi et al. 2000; Unno et al. 2002). Structurally, the proteasome is a barrel formed of 28 core polypeptides. Within the core of the barrel, multiple protein subunits form active sites that provide for proteolysis with a range of substrate specificities. Regulatory complexes found at either or both ends of the proteasome regulate access to the central cavity and active sites. As a complex, access to the active sites within the barrel provide for the regulation of this important enzyme. Coupled with the ubiquitin modification system, the proteasome is recognized as one of the most critical components regulating the proteome in cells, degrading a majority of folded and misfolded substrates and regulating a wide range of physiological processes (Ciechanover 1998; Hershko and Ciechanover 1998).

Finally, inhibitors (or anti-proteases) provide an additional layer of protease regulation. Emerging evidence suggests that the protease-inhibitor balance is critical to a variety of normal and disease states in humans (Gorogh et al. 2006; Guyot et al. 2008; Myerburg et al. 2006; Quesada et al. 2009). On their initial discovery, protease inhibitors were thought to protect the host from unwanted hydrolytic activities, both spatially and temporally. However, recent work suggests that the protease-anti-protease balance is a mechanism to fine-tune protease activities. As an example, work on the airway surface fluid in patients with compromised pulmonary systems (to be discussed later in this review) suggests fluid volume is sensed via changes in this protease/anti-protease balance (Kleyman et al. 2009; Mall et al. 2004; Myerburg et al. 2006; Tan et al. 2011; Tarran et al. 2006). These changes in the protease/anti-protease balance serve as a regulatory loop that alters water secretion across the epithelia. Thus, the changes in the protease/anti-protease balance both act as a signal and regulate water secretion across the airway epithelium.

Proteases, inhibitors and disease

Altered protease function and regulation have been associated with a large number of pathophysiological conditions (Quesada et al. 2009). Both sporadic and hereditary diseases are associated with endogenous protease dysregulation or dysfunction (Table 1). A large body of work has evolved looking specifically at the role of MMPs in cancer development and progression and in cardiac disease. The roles of these proteases were originally thought to be restricted to modification of the extracellular matrix. However, recent work suggests broader roles for the protease in other physiological and pathophysiological states.

Table 1 Human proteases, inhibitors and disease (adapted from Quesada et al. 2009)

Protease	Gene	Disease
Angiotensin-converting enzyme	ACE	Renal tubular dysgenesis
ADAM9	ADAM9	Cone-rod dystrophy
ADAMTS-13	ADAMTS-13	Thrombotic thrombocytopenic purpura
Afg3-like protein 2	AFG3L2	Ataxia(s)
Complement factor B	BF	Atypical hemolytic uremic syndrome
Calpain-3	CAPN3	Limb-girdle muscular dystrophy type 2A
Caspase-2	CASP2	Autosomal recessive intellectual disability
Caspase-8	CASP8	Autoimmune lymphoproliferative syndrome (I)
Carboxypeptidase E	CPE	Hyperproinsulinemia and diabetes
DJ-1	DJ1	Parkinson's disease type VII
Neutrophil elastase	ELA2	Cyclic neutropenia
Thrombin	F2	Hyperprothrombinemia/hypoprothrombinemia
Mitochondrial inner membrane protease2	IMMP2L	Tourette syndrome
Neurotrypsin	PRSS12	Nonsyndromic mental retardation
Presenilin 1	PSEN1	Alzheimer type III
Presenilin 2	PSEN2	Alzheimer type IV
Renin	REN	Renal tubular dysgenesis
Paraplegin	SPG7	Spastic paraplegia
Transmembrane protease, serine 3	TMPRSS3	Deafness
Ubiquitin C-terminal hydrolase I	UCHL1	Parkinson's disease type V
Ubiquitin-specific protease 26	USP26	Sertoli-cell-only syndrome
Ubiquitin-specific protease 9Y	USP9Y	Azoospermia and hypospermatogenesis
FACE1/ZMPSTE24	FACE1	Progeria, mandibuloacral dysplasia
Chymotrypsin C	CTRC	Hereditary pancreatitis
Procollagen C-proteinase	BMP1	Osteogenesis imperfect
Ataxin 3	MJD1	Machado-Joseph disease
Proprotein convertase 1	PCSK1	Obesity
Lysosomal carboxypeptidase A	PPGB	Galactosialidosis
Proteasome catalytic subunit 3i	PSMB8	Nakajo-Nishimura sundrome

Table 2 Protease inhibitors and human disease (adapted from Quesada et al. 2009)

Inhibitor	Disease
α1 Antitrypsin	Thrombosis, emphysema
A1-Antichymotrypsin	Vascular disease
Kallistatin	Pancreatitis
Angiotensinogen	Hypertension, hypotension
Protein Z-dependent protease inhibitor	Venous thrombosis
Vaspin	Diabetes
Maspin	Cancer progression
Megsin	IgA nephropathy
Antithrombin II	Venous thrombosis
Heparin cofactor II	Venous thrombosis
Plasminogen activator inhibitor 1	Bleeding disorders, myocardial infarction
Pigment epithelium-derived factor	Age-related macular disease

In addition, a growing number of hereditary diseases are associated with alterations in protease inhibitor expression and function (Table 2) (Quesada et al. 2009). Whereas the exact mechanisms associated with the pathophysiological states vary by disease, evidence for both the direct and indirect involvement of proteases and protease inhibitors suggests that balanced protease activities are critical for regulating physiological processes.

Cystic fibrosis

CF is a disease of altered salt and water movement across epithelial tissues. Mutations within the CF transmembrane conductance regulator (CFTR) that result in a loss of CFTR function at the plasma membrane underlie the pathophysiologies of CF and CF-related diseases (Dean et al. 2001; Drumm et al. 1990; Mall et al. 1999; Riordan et al. 1989). The primary complications associated with CF are found in the digestive and pulmonary systems. Pancreatitis and associated nutritional deficiencies are associated with abnormal buffering of the pancreatic duct leading to premature zymogen activation (Choi et al. 2001; Ko et al. 2002; Marino et al. 1991; Stuhrmann et al. 1990). Similarly, lung function is chronically degraded as a result of decreased mucocilliary clearance and the persistence of airway pathogens (Rich et al. 1990). The ensuing inflammatory and immune responses result in chronic injury to the airway epithelium and a decrease in pulmonary function.

CFTR functions as a protein kinase A (PKA-) and ATP-regulated Cl^- channel, facilitating the secretion of Cl^- across the apical membranes of epithelial cells (Cheng et al. 1991; Gregory et al. 1990). In the airway, Cl^- secretion is thought to impact the regulation of Na^+ absorption through ENaC, either by an alteration in the electrogenic driving force for Na^+ or by the direct regulation of ENaC by CFTR itself (Briel et al. 1998; Hopf et al. 1999; Ji et al. 2000; Kunzelmann et al. 1997; Reddy et al. 1999; Schreiber et al. 1999; Stutts et al. 1997). The luminal surfaces of the conducting airways are lined with a thin layer of fluid known as the airway surface liquid (ASL), which facilitates mucus clearance from the lung. The height of the ASL is determined by the net osmotic gradient established by Na^+ absorption and Cl^- secretion through these apically located ion channels. ENaC, in conjunction with the basolateral Na^+ / K^+ ATPase, is believed to be the predominant means for Na^+ absorption across the airway epithelium. The loss of Cl^- channel activity (as in CF) or an increase in Na^+ absorption putatively results in increased water absorption and dehydration of the ASL. This dehydration, in turn, increases mucus viscosity, decreases the efficacy of the mucocilliary clearance and facilitates pathogen adherence and colonization in the lung (Tarran et al. 2006; Voynow et al. 2008).

The role of ENaC in contributing to human CF lung pathophysiology is still under investigation. Studies of the recently generated CF pig suggest that Na^+ hyperabsorption is not associated with the CF phenotype (Abu-El-Haija et al. 2011; Chen et al. 2010; Itani et al. 2011). In *ex vivo* tissue and cell studies, significant changes in Cl^- conductance but not in Na^+ conductance have been observed. Similarly, *ex vivo* studies of human CF tracheal and bronchial epithelial cells indicate a primary role for altered Cl^- conductance, associated with the loss of CFTR, in the CF tissues. However, they fail to show widespread changes in Na^+ absorption (Itani et al. 2011; Reddy and Quinton 2003). These data stand in contrast to several in vivo and in vitro experiments that have implicated altered Na^+ absorption in CF pathophysiology (Boucher 2004; Jiang et al. 2000; Myerburg et al. 2006). Thus, the exact nature of ENaC involvement has still not been fully elucidated from a functional and physiological perspective.

Although CFTR is thought to play the dominant role in CF pathophysiology, emerging evidence suggests a role for ENaC in the airway (Huber et al. 2010). Consistent with a role for ENaC in the CF phenotype, genetic studies of atypical CF patients have identified mutations in ENaC that putatively underlie their CF pathophysiology (Azad et al. 2009; Rauh et al. 2010). Further evidence for a role of ENaC in regulating lung function comes from transgenic mice overexpressing β-ENaC (Zhou et al. 2011). Disruption of the CFTR locus does not induce full CF-like lung pathophysiology in mice as a result of the divergent lung physiology (Kent et al. 1997). However, transgenic overexpression of ENaC recapitulates the CF-like lung physiology, consistent with a role for ENaC-associated Na^+ absorption in the CF lung (Mall et al. 2004). Together, these data suggest that ENaC may partially regulate or contribute to the CF lung phenotype under specific spatial or physiological conditions.

Epithelial sodium channel

ENaC is expressed in the epithelial cells of several tissues, including the kidneys, airways, salivary ducts, sweat ducts, colon and taste cells (Butterworth 2010; Butterworth et al. 2009; Eaton et al. 2009; Hamm et al. 2010; Kleyman et al. 2009; Lang et al. 2010; Rossier and Stutts 2009). ENaC is a sodium-selective ion channel comprising three homologous subunits, namely, α-, β-, γ-, each composed of two membrane-spanning domains, a large folded extracellular domain and intracellular amino- and carboxy-termini (see Fig. 1). ENaC is the limiting step in the reabsorption of sodium across epithelia. Because of its role in the regulation of sodium homeostasis, ENaC has been associated with clinical defects of salt and water transport and implicated in a number of disease conditions including defects in airway surface hydration in CF (Capasso et al. 2005; Eaton et al. 2009; Ecelbarger and Tiwari 2006; Edelheit et al. 2005; Freundlich and Ludwig 2005; Hummler 1999; Li and Wang 2007; Matthay et al. 2005; Sun et al. 2011; Wagner et al. 2008).

ENaC is regulated by a number of intrinsic and external factors, the details of which have been reviewed previously (Bens et al. 2006; Bhalla and Hallows 2008; Butterworth 2010; Butterworth et al. 2009; Eaton et al. 2009; Ecelbarger and Tiwari 2006; Kleyman et al. 2009; Pochynyuk et al. 2006; Rossier and Stutts 2009). In every example of ENaC regulation, a limited number of options alter Na^+ transport. ENaC activity can be changed either by altering the amount of time that the channel spends open (open probability or P_O) or by modulating the surface membrane density (channel number or n) of the channel (Rossier 2002). Once fully active, the predominant mechanism to reduce sodium transport is to remove ENaC from the membrane surface by endocytosis. More recently, a new mode of regulation was appreciated that involved the activation of the channels by proteolytic cleavage (Adebamiro et al. 2007; Bruns et al. 2007; Ergonul et al. 2006; Hughey et al. 2003, 2004a, 2004b; Kleyman et al. 2009; Liu et al. 2002; Passero et al. 2008; Planes and Caughey, 2007; Planes et al. 2005; Tan et al. 2011; Vallet et al. 1997; Vuagniaux et al. 2002; Vuagniaux et al. 2000). In this instance, the P_O is dramatically increased, in some cases altering ENaC from an electrically silent state to a fully active channel (Caldwell et al. 2004, 2005). Once ENaC is activated by proteases, however, the same mechanisms of retrieval and

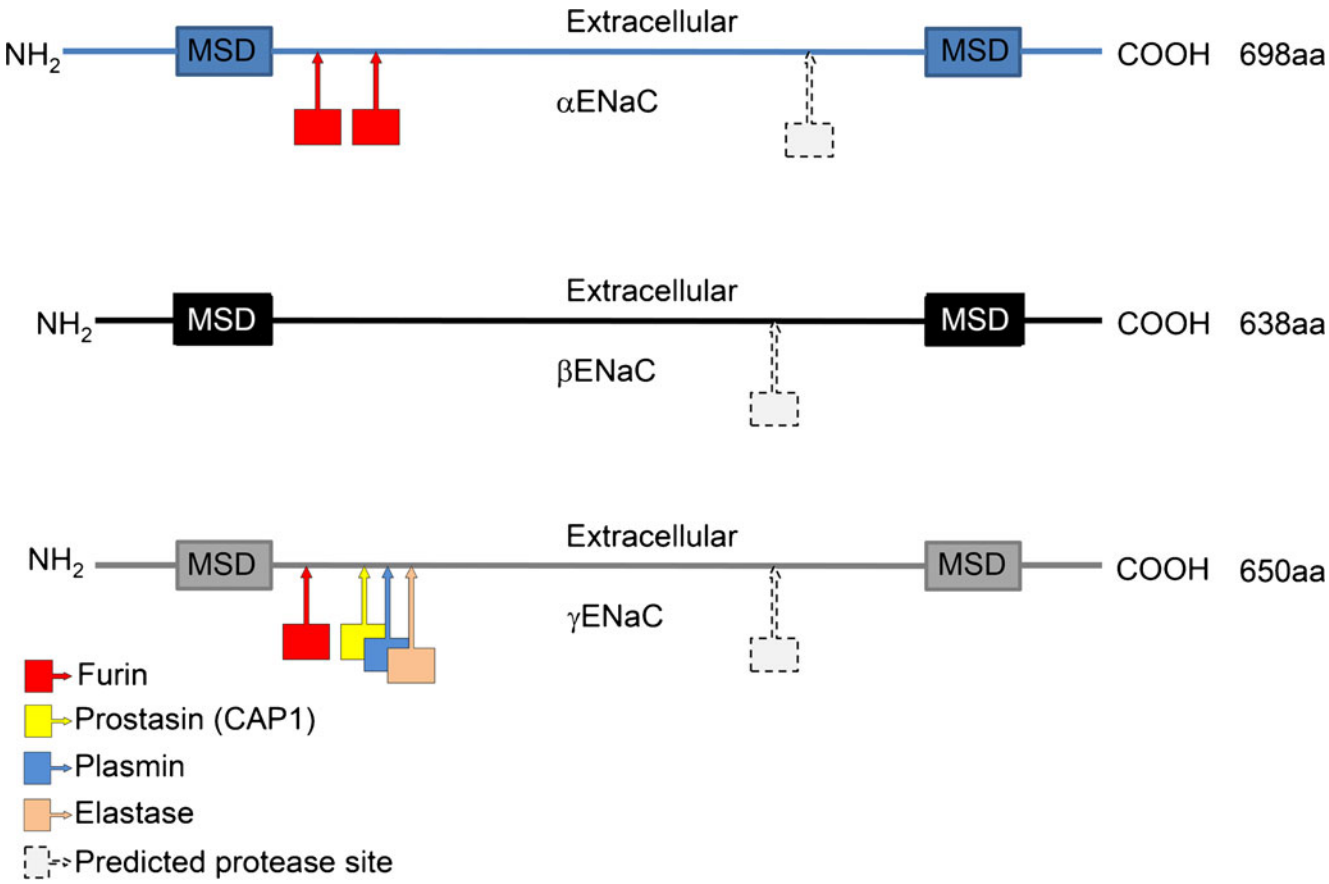

Fig. 1 Representation of α-, β- and γ-ENaC structures with the approximate locations of confirmed and predicted protease cleavage sites (*aa* amino acids, *MSD* membrane spanning domain). Based on figures presented in Kleyman et al. (2009), Rossier and Stutts (2009) and Hamm et al. (2010)

degradation need to be employed to reduce the net Na^+ reabsorption.

ENaC activation by proteases

The potential for proteases and protease inhibitors to alter ENaC activity and Na^+ transport was described in the early 1980s, before the molecular identity of the channel was known. The ability of protease inhibitors to block sodium flux was described in a number of model tissues without an underlying knowledge of the mechanisms behind these observations (Orce et al. 1980, 1981). The description of a serine protease that specifically activated ENaC, called a channel-activating protease (CAP1), opened the door to a new field of investigation in the regulation of ENaC (Liu et al. 2002; Vallet et al. 1997; Vuagniaux et al. 2000). CAP1 was eventually identified as prostasin or TMPRSS8 and these initial observations were quickly followed up with the descriptions of two additional channel-activating proteases, CAP2 (TMPRSS4) and CAP3 (matriptase; Planes and Caughey 2007; Vuagniaux et al. 2002). To date, the major class of proteases involved in ENaC cleavage belongs to the broad family of serine proteases, which all have a conserved serine in their active sites. The action of all these proteases is to cleave sites on the extracellular loops of the ENaC subunits at specific recognition sequences. Confirmed cleavage sites have been found clustered predominantly toward the n-terminal ends of α− and γ−ENaC, an area that is presumably exposed and accessible to protease action (Fig. 1; Adebamiro et al. 2007; Bruns et al. 2007; Caldwell et al. 2004; Gormley et al. 2003; Hamm et al. 2010; Hughey et al. 2004a, 2004b; Kleyman et al. 2009; Passero et al. 2008; Planes and Caughey 2007; Rossier 2004). However, a number of putative protease recognition sites have been mapped on all three subunits at both the N- and C-terminal sides of the extracellular loops of each subunit (Rossier and Stutts 2009). Protease action at these predicted sites has yet to be demonstrated experimentally. ENaC is acted on by proteases at intracellular locations or at the surface membrane, either with membrane-bound/anchored proteases or extracellular/free proteases.

Intracellular proteases

To be fully active, with a high P_O, ENaC needs to be cleaved more than once on the large extracellular domains of the α− and γ−subunits. One of the first steps in ENaC cleavage appears to occur by the action of pro-protein convertases, most likely located intracellularly (Fig. 2a; Schafer et al. 1995). The convertase furin is the most well-studied and best candidate for this intracellular protease function (Bosshart et al. 1994; Bruns et al. 2007; Hughey et al. 2004a, 2004b; Schafer et al. 1995). Furin is responsible for cleaving both α− and γ−subunits through a consensus sequence R/S-XX-R (where R is an arginine, S a serine and X any amino acid; Bruns et al. 2007; Hughey et al. 2004a, 2004b; Sheng et al. 2006). In the case of the α-subunit, furin is able to cleave at two distinct sites in the extracellular domain of the channel (Fig. 1). Cleavage at both of these locations releases a small inhibitory peptide. This cleavage putatively relieves structural constraints and allows the channel to become partially activated. Likewise, a furin site has been found on the extracellular domain of the γ-subunit (Bruns et al. 2007). A second cleavage event by an additional protease is required to achieve full activation of ENaC. The location of the distal γ-ENaC cleavage site varies with the specific protease investigated; however, it is within approximately 60 amino acids from the first furin cleavage site (Fig. 1). This second cleavage event also releases an inhibitory fragment, similar to α-ENaC. To date, a number of other serine proteases have been implicated in the cleavage of ENaC subunits and include members of the elastase family, plasmin, prostasin, chymotrypsin and trypsin (Adebamiro et al. 2007; Caldwell et al. 2005; Carattino et al. 2008; Kleyman et al. 2009; Passero et al. 2008; Rossier and Stutts 2009; Vuagniaux et al. 2002). In most cases, a double-cleavage event appears to release a small portion of the extracellular domain of ENaC, thereby activating the channel.

Functional and biochemical studies investigating ENaC cleavage do not always make it clear precisely where in the cell and when in the life of the channel the cleavage events occur. However, the need for the double-cleavage seems to be a requirement for full activation of ENaC. Therefore, ENaC might be found in a range of processed states depending on the longevity of the channel and the opportunity for interaction with both intracellular and extracellular proteases. A range of channel-open probabilities are therefore likely until full activation has been achieved; this observation has been made in the biophysical description of ENaC (Caldwell et al. 2004, 2005).

Membrane-bound extracellular proteases

Following biosynthesis and trafficking to the Golgi, ENaC is likely to encounter the intracellular protease furin, as this convertase has been shown to recycle between the Golgi and plasma membrane (Fig. 2a). Once ENaC is inserted into the plasma membrane, the opportunity exists for full proteolytic activation of the channel by extracellular proteases (Fig. 2b). The three CAPs described above are all reported to be membrane bound. CAP1 is anchored by glycosylphosphatidylinositol (GPI) and can be shed from the membrane surface. The CAP2 and CAP3 proteases are membrane-bound

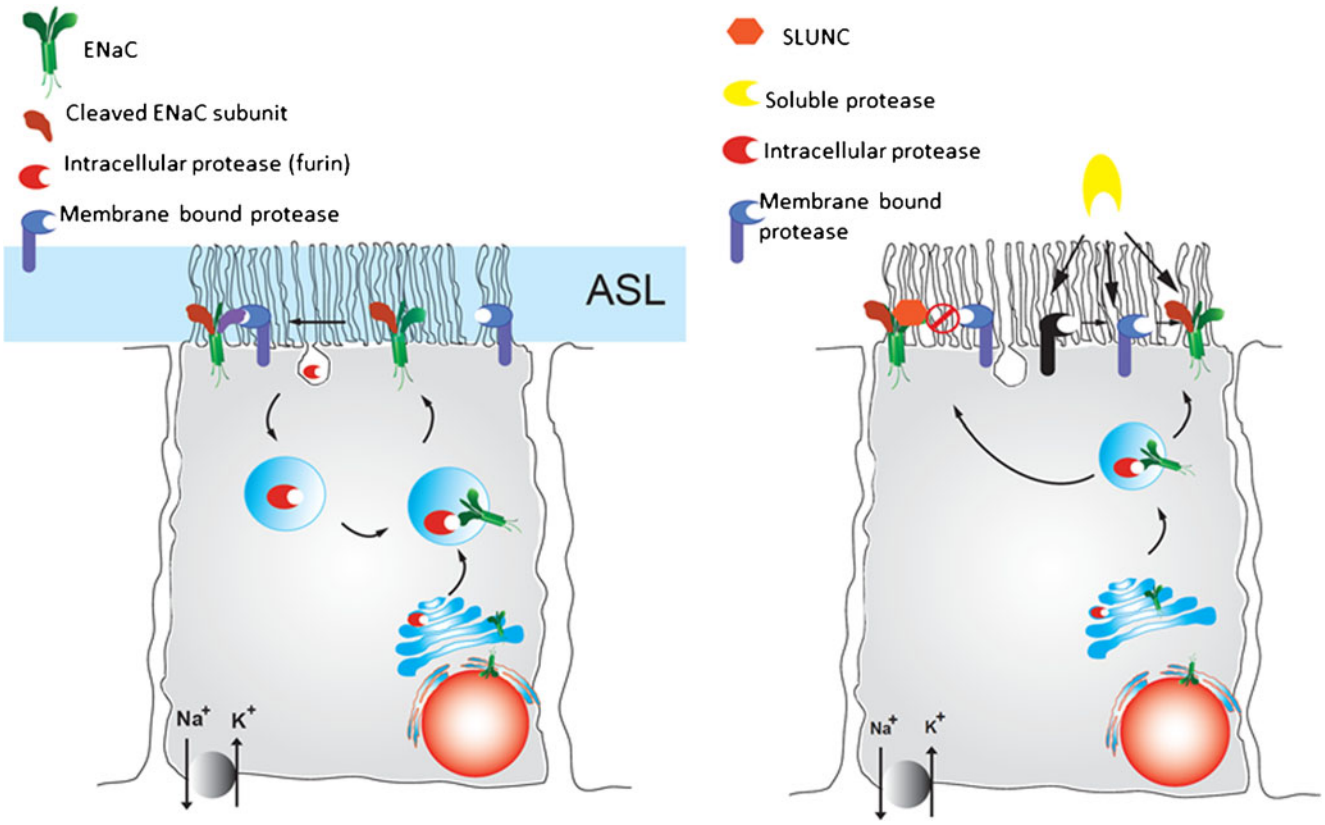

Fig. 2 Representation of protease activation of ENaC in airway epithelial cells. **a** A double-cleavage event is required for full ENaC activation (*ASL* airway surface liquid). The first step probably occurs by intracellular proteases (furin), followed by a second cleavage event at the surface (membrane protease). **b** Multiple cleavage events or a cascade of proteolysis might be involved in achieving full ENaC activation. In this scenario, ENaC is cleaved by intracellular proteases and reaches the membrane surface in either an uncleaved or partially cleaved state. It can then be further activated by membrane-bound proteases or by soluble proteases. In addition, soluble protease might initiate a cascade of proteolytic events eventually resulting in full ENaC activation. Soluble protease inhibitors and proteins that prevent ENaC cleavage (SPLUNC) might modulate the extent of ENaC activation. Images based on previous reviews (Rossier and Stutts 2009; Gaillard et al. 2010)

serine proteases with portions of these proteins being located intracellularly. Therefore, a direct association between ENaC and these proteases would probably be required to achieve proteolytic cleavage (Gormley et al. 2003; Rossier 2004; Vuagniaux et al. 2002).

Some uncertainty exists about the absolute requirement for the catalytic activity of CAP1 to achieve ENaC activation. Purported CAP1 catalytic mutants were still capable of activating ENaC; however, mutation of the proposed prostasin recognition sequence on γENaC (RKRK) eliminates the ability of prostasin to activate ENaC (Harris et al. 2008; Kleyman et al. 2009; Rossier 2004; Rossier and Stutts 2009). Several authors have suggested the possibility of an indirect mechanism for CAP1 activation of ENaC or the role of another protease in a cascade of proteolytic activity (Gaillard et al. 2010; Rossier and Stutts 2009; Tarran et al. 2006). Both of these possibilities remain plausible. In line with these suggestions and observations, we consider that a multi-step process might be required for full ENaC activation (see below; unpublished observations; Fig. 2b). Unlike the discrepancies for CAP1, greater consensus has been achieved with regard to the requirement for catalytic activities of CAP2 and CAP3 to activate ENaC fully and these cleavage sites have been mapped by using model expression systems (Planes and Caughey 2007; Planes et al. 2005; Vuagniaux et al. 2002).

One of the factors that might contribute to the seemingly contradictory studies for CAP1 activation of ENaC is that protease activity can itself be modulated by protease inhibitors, as described above. The interplay between protease activity and the action of protease inhibitors has been proposed as an underlying factor in excessive airway surface liquid reabsorption in the CF airway (Donaldson et al. 2002; Gaillard et al. 2010; Hughey et al. 2007; Myerburg et al. 2010; Rossier and Stutts 2009; Tan et al. 2011). Several protease inhibitors have been identified that result in the direct inhibition of the target proteases. These include the Kunitz-like inhibitors (aprotinin) and the serpins (protease nexin 1; Adebamiro et al. 2002, 2005, 2007; Myerburg et al. 2010; Orce et al. 1980, 1981; Planes et al. 2005; Tarran et al. 2006;

Tong et al. 2004; Vallet et al. 1997). Alternatively, inhibitors might bind to ENaC directly and prevent protease access and subsequent activation of the channel (Fig. 2b). The protein SPLUNC1 appears to bind to ENaC and prevent access of proteases to cleavage sequences, thereby inhibiting proteolytic activation of ENaC (Gaillard et al. 2010; Rollins et al. 2010).

Added to this interplay between proteases and their inhibitors is the possibility that a series of proteolytic steps is required for full ENaC activation. In this scenario, the activity of a protease might activate a protease pathway or a cascade of proteases that cleave ENaC. This could involve the inactivation or degradation of a protease inhibitor or conversion of a protease from an inactive to active form (Fig. 2b). These possibilities have been recently suggested in the literature but evidence for this activation and the nature of these proposed cascades have yet to be demonstrated experimentally for ENaC (Chambers et al. 2007; Gaillard et al. 2010; Tarran et al. 2006).

ENaC and proteases in the airway

Previous work from our group and others indicates that a balance between the protease activity of membrane-tethered channel-activating proteases (CAPs) and soluble protease inhibitors in the ASL modulates ENaC activity and therefore Na^+ absorption across human bronchial epithelial (HBE) cells (Gaillard et al. 2010; Kleyman et al. 2006; Mall 2008; Myerburg et al. 2006, 2010; Rossier and Stutts 2009). In this scheme, when the ASL volume is low, soluble protease inhibitors would achieve a sufficiently high local concentration in the ASL to minimize constitutive activation of ENaC by CAPs. This would lead to reduced Na^+ and water reabsorption. Conversely, when the ASL volume is expanded, the soluble protease inhibitors are diluted, relieving the inhibition of CAPs. ENaC activation would presumably occur from membrane-bound proteases that cannot be removed from the airway surface.

A number of proteases have been identified in the airway, not all of which are associated with ENaC activation (Cottrell et al. 2004; Elizur et al. 2008). The most common and abundant proteases found in the extracellular milieu in human airway are the serine and metallo-proteases (Conese et al. 2003; Elizur et al. 2008; Voynow et al. 2008). In the airways of CF patients, extracellular proteases are derived predominantly from two sources: external and intrinsic. Proteases are released by invading bacterial pathogens as virulence factors that facilitate infection and colonization. As a consequence of immune, inflammatory and cell damage/repair responses, the host also secretes proteases (Bainbridge and Fick 1989; Conese et al. 2003; Elizur et al. 2008; Terheggen-Lagro et al. 2005; Voynow et al. 2008). These proteases, combined with a number of endogenous and epithelial-cell-derived proteases, make up the large number of active proteases in the surface fluid overlaying the airway epithelium and have a range of impacts in the airway. Serine proteases have been shown to increase mucus expression, which decreases ciliary beating and results in ciliary injury (Griese et al. 2008). In addition, serine proteases induce goblet cell hyperplasia and degrade extracellular matrix (Coraux et al. 2008; Elizur et al. 2008; Voynow et al. 2008; Wynn 2008). The proteases also induce increased interleukin expression (IL-8), causing downstream pro-inflammatory signaling. Finally, the serine proteases degrade innate and adaptive immune molecules, altering immune signaling in the airway (Downey et al. 2007a, 2007b, 2009).

On balance these proteases are detrimental to the host, while aiding the pathogenesis of bacterial infection. In response to invasion, the host innate immune response might exacerbate the inflammation and protease damage, particularly in patients with CF and progressive lung diseases. One of the downstream consequences of infection is the accumulation of neutrophils at the site of infection (Downey et al. 2009). A large number of proteases are released by neutrophils themselves. These include neutrophil serine proteases, neutrophil elastase (which is known to activate ENaC), proteinase 3 and cathepsin G. Metalloproteases, collagenase and gelatinase have also been detected in the ASL under inflammatory conditions (Downey et al. 2009; Elizur et al. 2008; Voynow et al. 2008). In addition, bacterial pathogens are known to secrete a variety of proteases that are capable of host remodeling (see below). These external bacterial proteases might also serve to activate ENaC (Fig. 2b).

Bacterial infection in CF

The major opportunistic pathogen associated with CF lung disease is *Pseudomonas aeruginosa*, a gram-negative rod-shaped bacterium (Burke et al. 1991; Jagger et al. 1982; Parmely et al. 1987). *Pseudomonas* infection and colonization presents early in the patient's life and persists in the CF lung as a result of the ability of the bacterium to evade and neutralize the host's defenses (Lazdunski et al. 1990; Lyczak et al. 2000; Suter 1994). *Pseudomonas* colonization is aided by the compromised mucociliary clearance associated with CF. The persistence of *Pseudomonas* induces lung injury by both direct and indirect mechanisms (Hobden 2002; Kharazmi et al. 1984a, 1984b; Lyczak et al. 2002; Parmely et al. 1990; Sarkisova et al. 2005a; Suter 1994). The secretion of multiple virulence factors results in direct injury to the epithelial tissue (Kudoh et al. 1994; Wiener-Kronish et al. 1993). Additionally, the chronic involvement of immune and inflammatory responses, resulting from *Pseudomonas* colonization, leads to indirect injury (Granstrom et al. 1984; Jagger et al. 1982; Kharazmi et al. 1984a; Parmely and

Horvat 1986; Parmely et al. 1984; Reeves and McElvaney 2012; Saadane et al. 2006; Sagel et al. 2002). Specifically, the long-term recruitment and activation of neutrophils is associated with epithelial damage as a result of sustained secretion of bacterial killing proteins (Conese et al. 2003; Kercsmar and Davis 1993).

Pseudomonas has evolved multiple mechanisms to facilitate adherence and colonization. Among these, the secretion of virulence factors and the formation of biofilms are thought to contribute to *Pseudomonas* resistance to host and antibiotic insults (Kudoh et al. 1994; Lazdunski et al. 1990). Biofilm formation provides a physical barrier between the bacterial cells and the extracellular environment, decreases the efficacy of host and pharmacological agents and facilitates adherence (Byrd et al. 2011; Kobayashi 2005; Lee et al. 2011; Sarkisova et al. 2005b). Composed of DNA, polysaccharides and proteins, the biofilms form a matrix that encapsulates the bacterial cells (Byrd et al. 2011; Lee et al. 2011). The development of this matrix is a compounding factor in the treatment of *Pseudomonas* infection. Virulence factors also play key roles in modulating the virulence of many pathogens and include proteins that modulate innate and adaptive immune signaling and inflammatory signaling and that break down epithelial barriers (Kobayashi 2005; Sarkisova et al. 2005b; Tomlin et al. 2001).

Multiple proteases have been implicated in the virulence of *Pseudomonas*, including elastase A and B (LasA and LasB), protease IV (PIV) and alkaline protease (AP) (Jagger et al. 1982, 1983). Bronchiolar lavage and sputum from CF patients have demonstrated the presence of multiple bacterial proteases in the *Pseudomonas*-infected lung. Similarly, the expression and secretion of these proteases have been reported in other modes of *Pseudomonas* infection, including burn and ocular injury (Hobden 2002; Kharazmi et al. 1984a). Multiple proteolytic targets are associated with the expression of these proteases and might contribute to changes in the physiology of infected tissue.

The two elastase proteases are metalloproteases and are both capable of degrading human elastin; LasB protease is also capable of degrading collagen (Goldberg and Ohman 1987; Johnson et al. 1967; Kessler and Safrin 1988; Kessler et al. 1993; Rust et al. 1996; Saulnier et al. 1989; Toder et al. 1994; Uss et al. 1969; Voynow et al. 2008). Both elastin and collagen are significant biopolymers and contribute to tissue plasticity. Changes in elastin and collagen content in the lung are associated with pulmonary fibrosis (Voynow et al. 2008). In addition, both proteases have also been shown to be involved in the invasive phenotype of multiple *Pseudomonas* strains (Azghani et al. 2002; Fleiszig et al. 1997). LasB has also been shown to degrade IgA and IgG and surfactant proteins A and D (SP-A and D), both being associated with the identification and elimination of pathogens (Bainbridge and Fick 1989; Heck et al. 1990; Mariencheck et al. 2003).

PIV is a serine protease that has been implicated in corneal virulence of *Pseudomonas* (Engel et al. 1997; O'Callaghan et al. 1996). As with the elastase proteases, PIV can degrade surfactant and iron-binding proteins isolated from bronchoalveolar lavage (Britigan et al. 1993; Malloy et al. 2005). In addition, PIV can bind plasminogen and cleave fibrinogen, which are both involved in regulating blood clotting (Caballero et al. 2001; Engel et al. 1998). Specifically, the degradation of fibrinogen is associated with hemorrhage, which is often associated with *Pseudomonas* infection.

AP is a Ca^{2+}-regulated Zn^{2+} metalloprotease (Baumann 1994; Baumann et al. 1993; Duong et al. 1992; Guzzo et al. 1990; Zhang et al. 2012). The active protease is capable of degrading γ-interferon and putatively inactivates multiple protease inhibitors (Guyot et al. 2010; Horvat et al. 1989; Leidal et al. 2003; Parmely et al. 1990). In addition, AP is thought to alter the function of neutrophils and leukocytes, facilitating evasion of host defenses (Kharazmi et al. 1984a, 1984b; Parmely et al. 1984). Whereas its substrate specificity is currently unknown, the protease is thought to be capable of cleaving a broad range of physiological targets (Louis et al. 1998). Previous studies suggest that AP expression is regulatory, both spatially and temporally (Lazdunski et al. 1990). AP expression is modulated temporally during the infection and colonization of the host tissue and is highly enriched in biofilms (Manos et al. 2008, 2009; Salunkhe et al. 2005).

Bacterial proteases, ENaC and CF

Although long-term and widespread changes in ENaC activation might not directly contribute to the CF phenotype *per se*, they might contribute significantly to local and acute changes within the lung. Recent work suggests that, in addition to the host proteases that are present in the lung, secreted pathogen proteases might contribute to colonization and infection, as detailed above. In such models, the secretion of bacterial proteases would lead to the activation of ENaC, either directly through its cleavage or indirectly through the activation of a host protease. Evidence for the possibility of an indirect cascade activation is suggested by the ability of a number of bacterial proteases to cleave host proteases and protease inhibitors in vitro (Guyot et al. 2008, 2010; Johnson et al. 1967; Kessler and Safrin 1994; Kessler et al. 1998; Leidal et al. 2003). The nature and molecular identities of these putative cascades have not been characterized at this point in vivo.

The roles for the bacterial proteases in colonization, infection and exacerbation are also not well established. Secreted bacterial proteins and peptides are often immunogenic and, in many cases, become neutralized after recognition by the adaptive immune system (Granstrom et al. 1984; Jagger et al. 1982). However, both the initial response and generation of neutralizing antibodies occurs on timescales

that would not be predicted to alter acute expression and secretion during early steps in infection and colonization. Evidence for the acute expression of *Pseudomonas* proteases is found in analysis of bronchoalveolar lavage, wherein protease secretion and activation occurs extremely early in infection and colonization (Burke et al. 1991; Lyczak et al. 2000; Suter 1994). The increase in local protease activity might directly influence host defenses. In addition, the increase in protease activity might result in local tissue remodeling that facilitates adhesion and colonization. Moreover, the secreted proteases, including elastase and alkaline protease, might contribute to local remodeling by ENaC cleavage and alterations in its activity. The resulting changes in ASL would putatively facilitate adhesion by decreasing ASL and mucociliary clearance. This, coupled with changes in epithelial permeability, might lead to an increase in pathogen invasion and infection.

Concluding remarks

The role of proteases in activating Na^+ conductance through ENaC is now a well-established mechanism in ENaC regulation. The appreciation of this mode of channel modulation has prompted studies into underlying misregulation of proteases in pathophysiological disease states such as CF. From the brief summary provided above, we can clearly see that the airway is awash with a large number of proteases and protease-interacting proteins that are derived from a variety of sources, both host and invading pathogen. Comprehension of the pathways that link the potential protease interactions and cascades remains a challenging undertaking for a full understanding of the physiology of the airway.

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