Chapter 2
The Cell Biology of the SARS Coronavirus Receptor, Angiotensin-Converting Enzyme 2

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Abstract The identification of angiotensin-converting enzyme 2 (ACE2) as a cellular receptor for the SARS coronavirus (SARS-CoV) rejuvenated research into what was regarded by some as a minor player in the renin–angiotensin system. The discovery of its double life led to breathtaking advances in the understanding of virtually all aspects of its biology, including its structure, physiological and pathophysiological roles and cell biology. ACE2, like its well-known homologue, ACE, is a metallopeptidase which resides on the cell surface of the epithelial, and sometimes endothelial, cells of the heart, kidney, testes, lung and gastrointestinal tract. It is a type I transmembrane protein with a large catalytic extracellular domain which acts as both a peptidase and a viral receptor. This extracellular domain can be cleaved from the cell surface by other peptidases, modulating its activity. The levels of the enzyme on the cell surface are also thought to be regulated by internalisation on S-protein binding and by clustering in membrane microdomains known as lipid rafts. This chapter summarises the current understanding of how the cell biology of ACE2 is regulated and may influence and determine its function, and concludes by discussing the future challenges and opportunities for studies of this increasingly important enzyme.

2.1 Introduction

Angiotensin-converting enzyme 2 (ACE2) was first identified in 2000 simultaneously by two groups using distinct methodologies (Donoghue et al. 2000; Tipnis et al. 2000). Its close mammalian homologue, angiotensin-converting enzyme (ACE), is a well-characterised angiotensinase and prominent therapeutic target in

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hypertension, leading to a concentration of early studies of ACE2 on its substrate specificities and role in the renin–angiotensin system (RAS). Like ACE, ACE2 is a zinc metallopeptidase which is able to hydrolyse a wide variety of substrates. Of these, the best studied in the context of ACE2 are angiotensin I (Ang I) and angiotensin II (Ang II), peptides involved in regulating blood pressure and tissue fibrosis. Although able to cleave both peptides, it has become clear that the mitogenic and hypertensive peptide Ang II is the predominant physiological substrate of ACE2, being cleaved to the vasodilatory peptide angiotensin-(1–7). This suggested that ACE2 is therefore likely to have a beneficial role in cardiovascular disease, a finding which slowed research efforts due to its unsuitability as a target for conventional pharmacological intervention. The discovery of a role for ACE2 as a receptor for the SARS coronavirus (SARS-CoV) (Li et al. 2005), however, led to a reinvigoration and diversification of research effort toward understanding the tissue distribution and cell biology of ACE2.

2.2 Clues from Homologous Proteins

ACE2 is an 805-amino-acid glycoprotein bearing significant sequence homology in its N-terminal domain to somatic ACE and in its cytoplasmic, C-terminal domain to collectrin, also known as Tmem27 (Fig. 2.1). Analysis of the amino acid sequence of ACE2 reveals a putative signal peptide and transmembrane domain, indicating that it, like its homologue ACE, is expressed as a type I (N-terminal domain extracellular) transmembrane protein. The extracellular domain shares significant sequence identity with the equivalent region of ACE, but unlike somatic ACE, contains only a single HEMGH zinc-binding catalytic motif, as is the case with the germinal isoform of ACE (Fig. 2.1). The intracellular, carboxy-terminal region of ACE2, however, shares no homology with ACE but instead closely resembles that

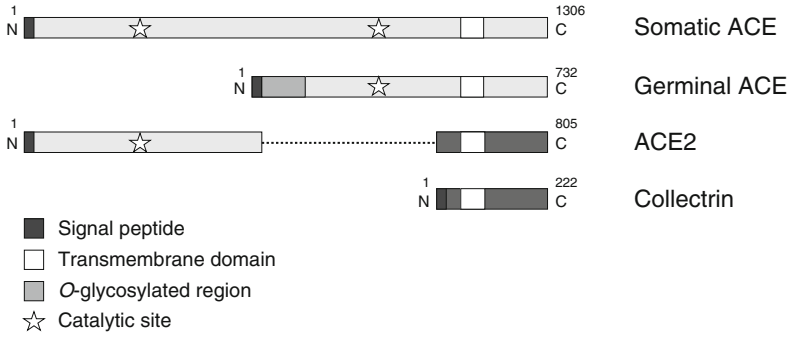

Fig. 2.1 Alignment of ACE2 sequence with homologous proteins. Regions of homology are indicated with *shading*. All four proteins contain signal peptides and transmembrane regions, but collectrin contains no catalytic residues. ACE2 is homologous with both the N terminus of somatic ACE and the C terminus of collectrin

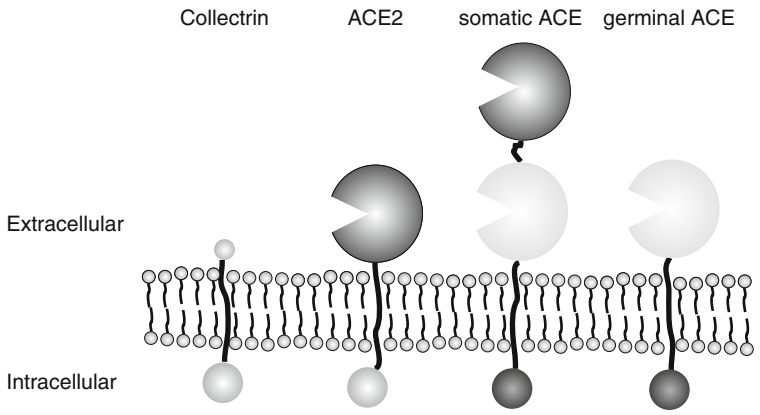

Fig. 2.2 Orientation of ACE2 and its homologues in the plasma membrane. ACE2 and its homologues are type I membrane proteins, with an extracellular amino-terminal domain and an intracellular carboxy-terminal domain. ACE2, somatic ACE and germinal ACE contain catalytic sites (represented as "*Pacman*" *shapes*) in the extracellular domain; collectrin does not

of collectrin, a non-catalytic protein with a small extracellular domain expressed in the kidney (Zhang et al. 2001) and pancreas (Fukui et al. 2005). This structure suggests the possibility that ACE2 may represent a gene fusion product between ACE and collectrin. The regions of homology between the four proteins are further illustrated in Fig. 2.2, which illustrates the orientation of the proteins in the plasma membrane.

The membrane localisation of ACE2 and ACE is in keeping with their roles in the RAS, allowing their extracellular catalytic sites to cleave circulating angiotensin (and other) peptides. In polarised epithelial cells in culture, ACE2 is trafficked predominantly to the apical membrane, with little detectable in the basolateral compartment (Warner et al. 2005). Interestingly, ACE displays a different localisation, being equally distributed between apical and basolateral membrane compartments. While the mechanisms responsible for this difference have yet to be identified, it is likely that distinct targeting motifs may reside in the disparate cytoplasmic domains of the two proteins. This suggestion is reinforced by the primarily apical expression of collectrin (Zhang et al. 2001), which shares homology in its cytoplasmic domain with ACE2 but not ACE, in collecting duct epithelial cells in the kidney.

In vivo, ACE2 is expressed predominantly in the heart, kidneys and testes (Tipnis et al. 2000), and to a lesser extent the lung and gastrointestinal tract, with low levels detectable in most tissues (Hamming et al. 2004). In the heart, ACE2 is expressed predominantly in cardiac myofibroblasts (Guy et al. 2008), cardiac myocytes and endothelial cells (Burrell et al. 2005), although this distribution is reported to vary between species. In the kidney, ACE2 is expressed in proximal and distal tubular epithelial cells, with low levels detectable in the glomeruli. Immunohistochemical analysis demonstrates a predominantly membranous expression pattern for ACE2 in these cells, with immunoreactivity strongest in the apical brush

border (Brosnihan et al. 2003). These findings are in keeping with the observed localisation in polarised kidney epithelial cells in culture (Warner et al. 2005). In the lung ACE2 is primarily confined to the epithelium, with cell surface expression detected in Clara and type II cells, but is also found in smooth muscle and endothelial cells (Wiener et al. 2007). In lung epithelial cells grown in culture, ACE2 is expressed predominantly in the apical membrane compartment (Ren et al. 2006), in keeping with its role as a receptor for SARS-CoV.

2.3 Regulation of ACE2 Expression on the Cell Surface

The levels and function of cell-surface proteins may be controlled in a number of ways, including modulation of gene expression, shedding of the protein from the cell surface, internalisation and clustering in lipid microdomains within the plasma membrane. This chapter will concentrate on the mechanisms regulating the levels of the mature ACE2 protein on the cell surface.

2.3.1 Proteolytic Cleavage Secretion

Many membrane proteins, particularly type I transmembrane proteins, undergo a proteolytic cleavage secretion event, more commonly referred to as "shedding," in which the ectodomain of the protein is cleaved by a proteinase, often a member of the matrix metalloproteinase (MMP) or a disintegrin and metalloproteinase (ADAM) families, and released into the extracellular milieu (illustrated in Fig. 2.3) (Huovila et al. 2005). This process may serve to release a ligand, allowing it to bind to its receptor (e.g., cytokines such as TNF-α), or simply to downregulate

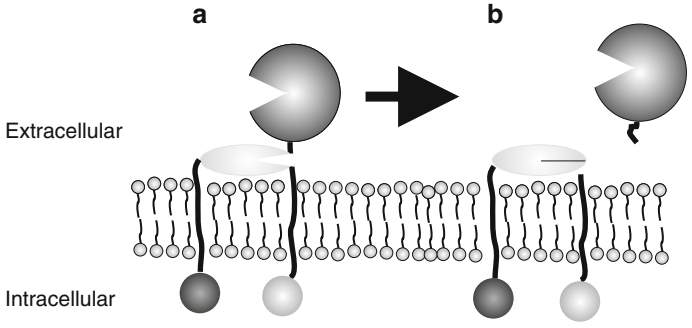

Fig. 2.3 Ectodomain shedding. Many transmembrane proteins, particularly those with an extracellular amino-terminal domain, are subject to a "shedding" event in which an intramembrane proteinase cleaves the juxtamembrane region of the target protein (**a**), releasing its ectodomain into the extracellular milieu (**b**)

the levels or activity of a protein on the cell surface. ACE2 (along with its homologue ACE) is subject to such an ectodomain shedding event, releasing a catalytically active ectodomain, a process regulated by protein kinase C activation and involving a member of the ADAM family, TACE (TNF-α converting enzyme) (Lambert et al. 2005, 2008). While the physiological significance of this shedding event is not clear, increased levels of circulating ACE2 have been detected in cardiovascular disease (Shaltout et al. 2008), and the ability of cleaved (soluble) ACE2 to reduce SARS-CoV infectivity is well established (Li et al. 2003). Intriguingly, however, siRNA-mediated TACE downregulation reduces the ability of SARS to infect Huh7 cells (Haga et al. 2008), suggesting the role of ACE2 shedding in SARS infection is more complex than is readily apparent.

Commonly, the transmembrane regions of shed proteins are subsequently subject to further intramembrane cleavage, generating a short carboxy-terminal fragment, a process termed regulated intramembrane proteolysis (RIP) (Medina and Dotti 2003). It has been demonstrated for a number of proteins, most notably notch and Alzheimer's precursor protein (APP) but also the ACE2 homologue, ACE (Fleming 2006), that this carboxy-terminal fragment is able to trigger signalling events leading to changes in the expression of target genes. Whether such a signalling mechanism occurs following ectodomain shedding of ACE2 remains to be established. The cytoplasmic domain of ACE2 is known, however, to have a regulatory role, both in terms of ectodomain shedding (Lambert et al. 2008) and SARS infectivity (Haga et al. 2008). Association of the cytoplasmic tail with a ubiquitous calcium-binding protein, calmodulin, reduces the release of its ectodomain suggesting a role for calmodulin in regulating ACE2 expression on the cell surface. The role of the cytoplasmic domain on SARS infection is controversial; Haga et al. (2008) recently reported that entry of SARS-CoV is dependent on the presence of the cytoplasmic domain of ACE2, a finding in direct contrast to those of Pohlmann et al. (2006) and Inoue et al. (2007) who suggest that entry is not dependent on the presence of this domain. These differences remain to be resolved but are likely due to the different experimental systems used.

2.3.2 The Role of Membrane Microdomains

It is thought that within the plane of plasma membranes, clusters of lipids such as sphingolipids and cholesterol form microdomains often termed lipid rafts. Although still somewhat controversial, a large body of evidence indicates that lipid rafts influence signalling and protein–protein interactions by partitioning and clustering proteins. Much of this evidence comes from studies in which cellular cholesterol is depleted using agents such as methyl-β-cyclodextrin. Cholesterol depletion alters the ability of a number of viruses to infect mammalian cells, including SARS-CoV. Studies by Glende et al. (2008) have revealed cholesterol dependence for SARS-CoV entry into cells on the presence of lipid rafts, possibly due to clustering of ACE2 into these microdomains. Furthermore, it has been

demonstrated that virus entry is mediated by internalisation of ACE2 upon S-protein binding into endosomes by a clathrin- and caveolin-independent mechanism involving lipid rafts (Wang et al. 2008). A degree of controversy remains about the role of membrane microdomains in regulating SARS-CoV entry, however, as others have failed to detect ACE2 in lipid raft preparations (Warner et al. 2005). The reasons for these discrepancies remain unclear, but are likely to be due to the use of heterologously- or endogenously-expressed ACE2 and/or differences in lipid raft preparation methodologies.

2.4 Conclusions and Future Perspectives

The serendipitous discovery of ACE2 as the cellular receptor for SARS-CoV rejuvenated studies analysing the cell biology of a protein previously thought by some only to be a minor player in the RAS. This reinvigoration of research not only led to important discoveries regarding the mechanisms regulating the expression of ACE2 at the cell surface, impacting on its function as the SARS-CoV receptor, but also helped stimulate studies which revealed an unexpectedly significant role for ACE2 in the RAS. Further work is required to fully elucidate the mechanisms regulating the cell surface function of ACE2; it is likely to interact with as-yet-unidentified proteins and may turn out to have intracellular signalling functions which influence its function and the function of other proteins. At present, most of the cell biological studies of ACE2 have been directed at analysing post-transcriptional events regulating its function. Changes in the levels of ACE2, however, have been identified in a wide variety of pathologies, suggesting that transcriptional and post-transcriptional regulatory mechanisms may also have an important role. Indeed, recent studies have indicated a number of pathways which may regulate ACE2 at the molecular level. Whatever the focus of future studies turns out to be, however, it seems unlikely that ACE2 has given up all its secrets yet.

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