GOLGI-ASSOCIATED ANION EXCHANGER, AE2

Identification, cell type specific targeting and
structural role in the Golgi complex

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Abstract

Anion exchanger 2 (AE2) is a member of the anion exchanger gene family, which includes three additional members, AE1, AE3, and AE4. They are also known as Na^+-independent Cl^-/HCO_3^- exchangers, and their major function is to regulate intracellular pH and chloride concentration. All four isoforms have several N-terminally truncated variants that are often expressed cell type specifically. Red blood cells express the full-length AE1 isoform that interacts with ankyrin, an adapter protein linking plasma membrane to the spectrin-based membrane skeleton. This membrane skeleton association is essential for maintaining the membrane integrity of red blood cells. AE3 variants are mainly found in the brain and heart, whereas AE4 is localized in the kidney.

Anion exchanger 2 is expressed in every cell line and tissue studied thus far, and it has been mainly localized to the plasma membrane. However, we found two types of localization/targeting of the AE2 protein in several of the cell lines studied. The protein was localized to either the plasma membrane or the Golgi complex, depending on the cell type. The AE2 variant expressed in these cells was identified as the full-length AE2 protein.

The determinants of differential intracellular targeting were assessed. We hypothesized that Golgi-AE2 is anchored to the Golgi membranes via its association with the Golgi membrane skeleton. We were able to show that the Golgi localization of AE2 correlated with the cell type specific expression of Ank_{195}, a Golgi membrane skeletal protein. In cells where AE2 was targeted to the plasma membrane, Ank_{195} was not expressed. In addition, the detergent insolubility and co-redistribution properties of AE2 and Ank_{195} strongly suggested that these proteins interact with each other.

The Golgi membrane skeleton has been shown to be necessary for maintaining the Golgi structure. Our studies were consistent with these findings, showing that in cells in which AE2 expression was reduced by using AE2-specific antisense oligonucleotides, the Golgi complex was dispersed. The spectrin-based membrane skeleton was probably partially detached from the Golgi membranes leading to breakdown of the Golgi structure and disorganization of the microtubules associated with it.

The present findings suggest that the targeting of AE2 is cell type specific, and that Golgi-localized AE2 serves as a membrane association site for the spectrin-based Golgi membrane skeleton, thereby participating in the maintenance of the Golgi structure.

Keywords: AE2 protein, ankyrins, Golgi complex, Golgi membrane skeleton, membrane proteins

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Abbreviations

aa	amino acid
ADP	adenosine diphosphate
AE	anion exchanger
AE1e	erythrocyte AE1 isoform
AE1k	kidney AE1 isoform
ARF	ADP ribosylation factor
ATP	adenosine triphosphate
CA	carbonic anhdrase
cDNA	complementary DNA
CGN	cis-Golgi network
CHO-K1	Chinese hamster ovary cells
COP	coatomer protein
COS-7	African green monkey kidney cells
DMEM	Dulbecco's modified essential medium
DNA	deoxyribonucleic acid
DTT	dithiotreitol
EM	electron microscopy
ER	endoplasmic reticulum
ERGIC	ER-Golgi intermediate compartment
kDa	kilodalton
F89	human skin fibroblast cells
HeLa	human adenocarcinoma cells, cervix
MDBK	Madine Darby bovine kidney cells
MDCK	Madine Darby canine kidney cells
MEM	minimal essential medium
NRK	Normal rat kidney cells
MAD	membrane association domain (of spectrin)
MPR	mannose-6-phosphate receptor
mRNA	messenger RNA
MTOC	microtubule organizing center
NSF	N-ethylmaleimide sensitive factor

PAGE	polyacrylamide gel electrophoresis
PGC	post-Golgi carrier
RNA	ribonucleic acid
ROS	rat osteosarcoma cells
RT	room temperature (about 25°C)
SDS	sodium dodecyl sulfate
SNAP	soluble NSF attachment protein
SNARE	SNAP receptor
tER	transitional ER
TGN	trans-Golgi network
VTC	vesicular tubular membrane clusters

List of original articles

I   Holappa K, Mustonen M, Parvinen M, Vihko P, Rajaniemi H and Kellokumpu S (1999) Primary structure of a sperm cell anion exchanger and its messenger ribonucleic acid expression during spermatogenesis. Biol Repr 61: 981-986.

II   Holappa K, Suokas M, Soininen P and Kellokumpu S (2001) Identification of the full-length AE2 (AE2a) isoform as the Golgi-associated anion exchanger in fibroblasts. J Histochem Cytochem 49(2): 259-269.

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IV   Holappa K, Muñoz MT, Egea G. and Kellokumpu S (2004) The AE2 anion exchanger is necessary for the structural integrity of the Golgi apparatus in mammalian cells. FEBS letters 564: 97-103.

Contents

1 Introduction

Anion exchangers are transmembrane proteins that contribute to electroneutral exchange of bicarbonate (HCO_3^-) anions for chloride (Cl^-). Thus, they regulate the intracellular pH, cell volume and chloride concentration of cells. In red blood cells, anion exchange is coupled to CO_2 transport via the activity of carbonic anhydrase enzyme, which catalyzes the reversible conversion of bicarbonate to CO_2 (Vince & Reithmeier 1998).

Anion exchangers are usually targeted to the plasma membrane, and they traverse the bilayer 12 or 14 times. The large amino-terminus and the short carboxy-terminus are both on the cytosolic side. The anion exchanger gene family includes four members; AE1, AE2, AE3, and AE4. All these isoforms have alternatively spliced or promoted variants, which show both cell type and tissue specificity (Alper *et al.* 2002). The most thoroughly studied anion exchanger protein is the AE1 isoform, formerly known as the band 3 protein of the red blood cell membrane. It is the most abundant protein in the red blood cells, and it serves as an attachment site for the plasma membrane skeleton. The membrane skeleton is a spectrin-based network that lies just beneath the plasma membrane, and its anchorage to the plasma membrane is essential for maintaining the biconcave shape of red blood cells. In cells where AE1 has been inactivated by gene targeting (Southgate *et al.* 1996), the plasma membrane skeleton appears normal, but membrane integrity and the shape of red blood cells are markedly altered.

Previous studies have shown that anion exchangers also accumulate in intracellular compartments. This has been mainly interpreted as an artifact or as a sign of recycling into the Golgi complex of proteins otherwise localized in the plasma membrane. This work was undertaken to study the cell type specific, intracellular targeting of the anion exchanger isoform AE2. The potential determinants of the plasma membrane and/or Golgi targeting of the AE2 protein, identified as a full-length AE2a variant, were assessed. Special interest was focused on the Golgi-localized AE2a. The structural role of AE2a in the Golgi complex was studied by using the AE2a-specific antisense oligonucleotides or the overexpression of GFP-tagged AE2a.

2 Review of the literature

2.1 Introduction to transmembrane ion transport

Three classes of transmembrane proteins, i.e., pumps, carriers and channels, control the ion transport across cell membranes. Pumps are enzymes that utilize energy from adenosine triphosphate (ATP) or light to pass ions and other solutes across membranes. Carriers move ions down their concentration gradients, but the energy derived from this may be used to pass another ion up along its concentration gradient. Channels are membrane pores that open transiently and let ions pass quickly across the membrane (Pollard & Earnshaw 2002).

ATP-driven pumps include the F_0F_1 family of membrane pumps, the P-Type ATPase family of membrane pumps, and ABC Transporters. The F_0F_1 family is further divided in F-type and V-type ATPases. F-type ATPases are found in mitochondrial and chloroplast membranes, whereas V-type ATPases are localized to other intracellular membranes. The substrate for ATP-driven pumps is H^+. Proton flux drives the ATP synthesis in F-type ATPases, whereas V-type ATPases use ATP energy to acidify the intracellular compartments, such as endosomes, lysosomes, the Golgi complex, and some vesicles. P-type ATPases include e.g., Na^+K^+ ATPases that use ATP energy to produce the primary gradients of Na^+ and K^+. P-type ATPases are localized to the plasma membrane. P-type ATPases as well as F-type and V-type ATPases generate electrical and chemical gradients across memranes. Some carriers use these primary ion gradients to drive the movement of other ions and solutes up their concentration gradients. Therefore, carriers are sometimes called secondary transporters. The ABC transporter family is a large protein family, which includes proteins localized to the plasma membrane, endoplasmic reticulum, and other organelles. They differ from the other two families of ATP-driven pumps in that they do not generate ion gradients. The substrates for ABC transporters are various (Pollard & Earnshaw 2002).

Membrane carriers use the ion gradients produced by membrane pumps to drive the movement of ions across membranes. The movement of one ion down its concentration gradient is often concurrent with the rise of other ions. Membrane carriers are integral membrane proteins that usually traverse the membrane 12 times. Carriers are further

divided into three categories according to the type of substrate transport. Uniporters transport a single substrate down its concentration gradient, whereas symporters transport several substrates into the same direction. Symporters are also known as cotransporters. The most well-known uniporters are GLUT carriers, which are responsible for glucose uptake in red blood cells, fat, and muscle. One example of a symporter is the SGLT1 protein of the gut that transports Na^+ and glucose, resulting in glucose uptake. Antiporters are exchangers that move substrates in an opposite direction. Antiporters include Na^+/H^+ exchangers and HCO_3^-/Cl^- exchangers. The latter are also known as anion exchanger proteins, and they mediate the electroneutral exhange of bicarbonate for chloride. Overall, antiporters usually exchange like substrates, i.e., anions for anions and cations for cations (Pollard & Earnshaw 2002).

Membrane channels do not create ion gradients, but instead allow specific ions to move down their concentration gradients across membranes. As a result, the electrical charges separate and produce a membrane potential. The electrical signal spreads over the surface of the cell through the opening and closing of channel pores. This is especially important in the excitable cells of nerve and muscle, where high-speed communication is needed to generate action potentials. Membrane channels respond to membrane potential, intracellular or extracellular ligands, or mechanical forces. Voltage-gated cation channels respond to membrane potential, and they are found in excitable cells. They include voltage-gated Na^+, K^+, Ca^{2+}, and Cl^- channels. Epithelial cells express Na^+, Cl^-, K^+, and water channels (Pollard & Earnshaw 2002).

The co-operation of membrane pumps, carriers and channels provides means for such processes as cellular volume regulation, salt and water reabsorption in kidney, glucose uptake, synaptic transmission at neuromuscular junctions, cardiac contraction, and ATP production by oxidative phosphorylation in mitochondria (Pollard & Earnshaw 2002).

2.2 Anion exchangers

2.2.1 AE gene family

Two gene families encode the proteins responsible for bicarbonate transport, the SLC4 and the SLC26 gene families. The SLC4 gene family consists of ten genes encoding two electrogenic Na^+/HCO_3^- cotransporters (NBCe1, NBCe2) and three electroneutral Na^+-coupled HCO_3^- transporters (NBCn1, NDCBE, NCBE) (Romero *et al.* 2004). The SLC4 gene family also includes three Na^+-independent Cl^-/HCO_3^- exchanger genes (SLC4A1, SLC4A2, and SLC4A3) (Kopito *et al.* 1987, Schofield *et al.* 1994, Yannoukakos *et al.* 1994, Medina *et al.* 1997, Alper *et al.* 2002), encoding proteins known as AE anion exchangers (AE1, AE2, and AE3). The anion exchangers AE1-AE3 all have several alternatively spliced or promoted variants. A fourth member of the family of anion exchanger proteins, AE4, has also been recently found and characterized (Parker *et al.* 2001, Tsuganezawa *et al.* 2001). However, AE4 is more similar to the Na^+/HCO_3^- cotransporters than to the members of the AE family (Romero *et al.* 2004). Another member of the SLC4 gene family, BTR1, is a bicarbonate transporter-related protein of

unknown function (Parker *et al.* 2001). The SLC26 gene family includes several sulfate transporters and anion transporters (Everett & Green 1999, Lohi *et al.* 2000, Lohi *et al.* 2002).

The human AE genes have been localized to the chromosomes 17q21 (AE1: Showe *et al.* 1987), 7q35-q36 (AE2: Palumbo *et al.* 1986), 2q36 (AE3: Su *et al.* 1994, Yannoukakos *et al.* 1994), and 5q23-q31 (AE4: Parker *et al.* 2001). The human AE1 gene spans 18 kb and has 20 exons (Schofield *et al.* 1994). The mouse anion exchanger 1 gene spans approximately 17 kb, consisting of 20 exons (Kopito *et al.* 1987). The anion exchanger 2 gene spans 17 kb and contains 23 exons both in humans (Medina *et al.* 1997) and in mice (Lecanda *et al.* 2000). The AE3 gene from rat spans approximately 12.5 kb and contains 24 exons (Linn *et al.* 1992). The anion exchanger AE4 gene has 22 exons in humans (Parker *et al.* 2001).

2.2.2 *Protein structure*

The anion exchangers AE1-AE3 share a common basic structure. They consist of a large N-terminal cytosolic domain (~400-700 aa in full-length AEs), a large C-terminal transmembrane domain (~500 aa) and a short cytoplasmic C-terminal tail (Figure 1). The anion exchangers share considerable homology in their C-terminal transmembrane domains, but the cytosolic N-terminal domain has been shown to be highly variable (Brosius III *et al.* 1989, Kudrycki & Shull 1989, Linn *et al.* 1992, Kollert-Jöns *et al.* 1993, Cox & Cox 1995, Cox *et al.* 1996). The anion exchangers, AE1-AE3, and their N-terminally truncated variants are often species-specific and also show notable tissue or cell type specificity (**AE1**: Kopito & Lodish 1985, Kopito *et al.* 1987, Cox & Lazarides 1988, Tanner *et al.* 1988, Brosius III *et al.* 1989, Kim *et al.* 1989, Kudrycki & Shull 1989, Lux *et al.* 1989, Kudrycki & Shull 1993, Cox *et al.* 1995, Adair-Kirk *et al.* 1999, Richards *et al.* 1999, **AE2**: Demuth *et al.* 1986, Alper *et al.* 1988, Chow *et al.* 1992, Gehrig *et al.* 1992, Wang *et al.* 1996, Medina *et al.* 1997, Stuart-Tilley *et al.* 1998, Lecanda *et al.* 2000, Medina *et al.* 2000, **AE3**: Kopito *et al.* 1989, Kudrycki *et al.* 1990, Morgans & Kopito 1993b, Yannoukakos *et al.* 1994).

The N-terminal domain attaches with various proteins, depending on the variant. Some variants are bound to ankyrin, for example, while others are incapable of binding to this protein, which links the membrane skeleton to the plasma membrane. This membrane skeleton association is crucial for maintaining the biconcave shape of red blood cells and thus for membrane integrity (Southgate *et al.* 1996). There are 12 or 14 (Passow *et al.* 1992, Reithmeier 1993, Tanner 1993) transmembrane domains in the large C-terminal domain that contributes to anion exchange. The C-terminal tail has a binding site for carbonic anhydrase II – an enzyme that catalyzes the conversion of carbon dioxide to bicarbonate: $CO_2 + H_2O \rightarrow HCO_3^- + H^+$ (Vince & Reithmeier 1998).
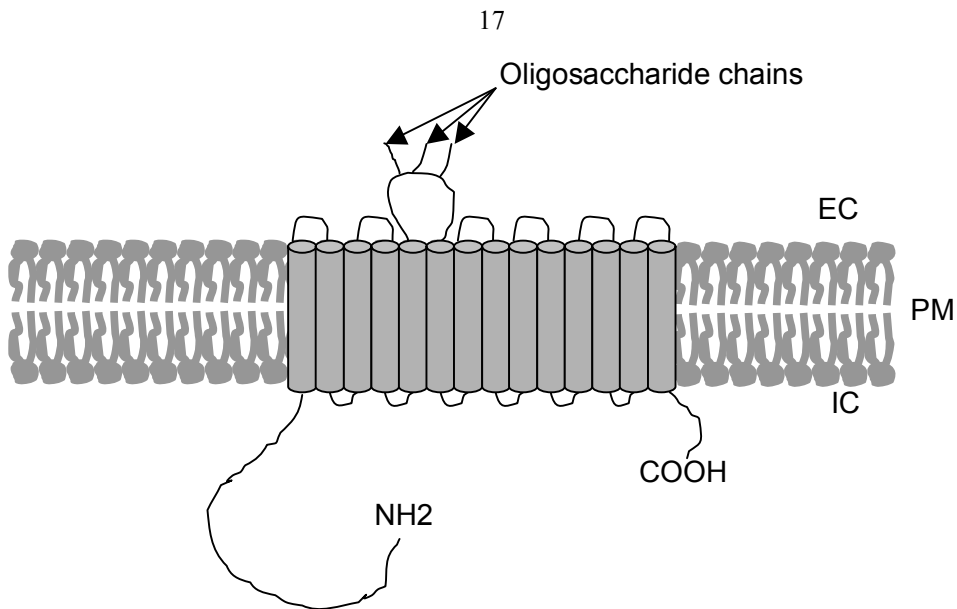
Fig. 1. The putative topography of anion exchanger proteins. Anion exchangers have 12 or 14 transmembrane spans. Both the N-terminus and the C-terminus are cytosolic (IC=intracellular space and EC=extracellular space, PM=plasma membrane). The number of N-linked oligosaccharide chains depends on the isoform. Anion exchanger 2 (in figure) has three potential N-glycosylation sites in its third extracellular loop.

2.2.2.1 Oligomerization

Many plasma membrane glycoproteins form oligomeric complexes in the endoplasmic reticulum prior to their transport out of the organelle (Hurtley & Helenius 1989). Generally, there are two types of oligomerization, and homo-oligomerization occurs between identical and hetero-oligomerization between different polypeptide chains. Anion exchangers form homo-oligomers, but conflicting data exist about the state of association. In addition to monomers, anion exchangers are known to exist as dimers or tetramers (Tomida *et al.* 1986, Casey & Reithmeier 1991, Pinder *et al.* 1995, Blackman *et al.* 1998, Hanspal *et al.* 1998, Van Dort *et al.* 1998, Zoloratev *et al.* 1999, Pushkin *et al.* 2000). According to most studies, ankyrin preferentially binds to the tetrameric form of the anion exchanger. Tetramerization is thought to occur between the N-terminal portions whereas dimerization derives from interactions between the C-terminal domains (Casey & Reithmeier 1991, Cölfen *et al.* 1998, Taylor *et al.* 2001). Tetramers were completely absent from the red blood cells of ankyrin-deficient mice, indicating that ankyrin binding is absolutely required for tetramer formation (Yi *et al.* 1997). Ankyrin has been shown to bind to AE1 already in the endoplasmic reticulum, and ankyrin seems to be needed for the delivery of this complex from the ER to the Golgi complex (Gomez & Morgans 1993).

2.2.2.2 Glycosylation

Another feature of anion exchangers is that they are glycoproteins and thus contain oligosaccharide chains. Anion exchanger 1 has one N-glycosylation site at asparagine 642 in its fourth extracellular loop (Drickamer 1978). The N-glycosylation site is known to be occupied, and the glycan structure is either an elongated polylactosaminyl or complex type (Tsuji *et al.* 1981, Fukuda *et al.* 1984, Landolt-Marticorena *et al.* 1998). The oligosaccharide residue of AE1, however, is not required for oligomerization or anion transport since the protein deglycosylated with N-glycosidase F was able to dimerize and retained its activity (Casey *et al.* 1992). In addition, mutation of the acceptor site to aspartate (N642D) did not prevent the transport of AE1 to the plasma membrane, showing that N-glycosylation is not needed for cell surface expression (Li *et al.* 2000). Similarly, N-glycosylation is not needed for the transport activity of AE2 or AE3 (Fujinaga *et al.* 2003). In this study, N-glycosylation was inhibited with tunicamycin. AE2 has three consensus N-glycosylation sites, and AE3 has one such site in the third extracellular loop (Fujinaga *et al.* 2003).

2.2.2.3 Phosphorylation

Anion exchanger 1 is known to be phosphorylated on serine/threonine and tyrosine residues (Dekowski *et al.* 1983, Low *et al.* 1987, Minetti *et al.* 1998, Brunati *et al.* 2000). Brunati and co-workers demonstrated the sequential action of Syk and Lyn tyrosine kinases on the Tyr-8/Tyr-21 and Tyr-359/Tyr-904 residues of anion exchanger 1, respectively (Brunati *et al.* 2000). The importance of phosphorylation is largely unknown, but it has been shown to take place in response to hypertonic shrinkage (Minetti *et al.* 1996, Minetti *et al.* 1998). The tyrosine 8 of AE1 (Dekowski *et al.* 1983, Yannoukakos *et al.* 1991) is in the region where glycolytic enzymes bind. The phosphorylation of Tyr-8 inhibits enzyme binding, indicating that AE1 may be involved in the regulation of glycolysis, because glyceraldehyde-3-phosphate dehydrogenase and other glycolytic enzymes are inactive when bound to AE1 (Tsai *et al.* 1982, Low *et al.* 1987, Low *et al.* 1993). Chemical compounds that stimulated the tyrosine phosphorylation of AE1 were also shown to increase glycolysis (Harrison *et al.* 1991). Anion exchanger AE2 transport activity may be regulated by serine/threonine phosphorylation. Anion exchanger AE2a has one highly conserved protein kinase C consensus phosphorylation site. The phorbol ester that activates protein kinase C was shown to stimulate the Cl^-/HCO_3^--exchange of AE2a in rabbit mucous cells (Rossmann *et al.* 2001).

2.2.3 Function of anion exchangers

Anion exchanger proteins are responsible for the electroneutral exchange of chloride and bicarbonate anions (Alper *et al.* 2002), which activity they already acquire in the endoplasmic reticulum (Ruetz *et al.* 1993). Thus, they regulate the intracellular pH and chloride concentration of cells (Alper *et al.* 2002). They are also involved in the

regulation of cellular volume together with other membrane carriers and channels (Humphreys *et al.* 1995, Fievet *et al.* 1998). In red blood cells, the activity of the Cl^-/HCO_3^- exchanger (AE1) increases the capacity of blood to carry carbon dioxide from tissues into the lungs for excretion (Romero *et al.* 2004). Carbon dioxide together with H_2O is converted to H^+ and HCO_3^- by carbonic anhydrases. Bicarbonate is then transported out of the cells by anion exchangers that permit the uptake of additional CO_2 (Romero *et al.* 2004).

In kidney collecting ducts, Cl^-/HCO_3^- exchangers together with H^+-ATPases partipicate both in acid secretion by type A intercalated cells (AE1k; Drenckhahn *et al.* 1985, Alper *et al.* 1989) and in base secretion by type B intercalated cells (AE4; Parker *et al.* 2001, Tsuganezawa *et al.* 2001). In type A intercalated cells, the anion exchanger protein is present in the basolateral membrane and plays a key role in the reabsorption of bicarbonate from the lumen to blood. The H^+-ATPase in the apical membrane of type A intercalated cells secretes protons (Alper *et al.* 1989). In type B intercalated cells, the anion exchanger protein is present in the apical membrane transporting bicarbonate to the lumen (Alper *et al.* 1989). H^+-ATPase is probably present in both apical and basolateral membranes in these cells (Alper *et al.* 1989). In addition, AE2 is expressed in the basolateral membranes of many epithelial cells of renal tubules (Alper *et al.* 1997) and involved in the transport of acid from blood into lumenal fluid (Castillo *et al.* 1999). The parietal cells of gastric glands secrete protons that acidify the stomach fluid. To prevent these cells from alkalinization, anion exchanger 2 exports bicarbonate in exchange for Cl^- into the basolateral membrane of parietal cells (Jöns *et al.* 1994, Stuart-Tilley *et al.* 1994, Jöns & Drenckhahn 1998). Co-operation of the basolateral Cl^-/HCO_3^- exchanger with the apical Na^+/H^+ exchanger in villus enterocytes results in net absorption of NaCl (Knickelbein *et al.* 1985).

The activity of anion exchange is dependent on pH in such a way that the peak activity occurs at extracellular pH~7 for AE1 and at pH~9 for AE2 (Zhang *et al.* 1996). AE2 activity is fully suppressed at extracellular pH 5.0, but AE1 maintains 63% of its activity at extracellular pH 5.0 (Humphreys *et al.* 1994, Zhang *et al.* 1996, Stewart *et al.* 2001). In these experiments, intracellular pH remained nearly at its steady state values. AE2 activity was shown to be slightly less pH-dependent in experiments where both intracellular and extracellular pH were changed together (Sterling & Casey 1999), or where intracellular pH was changed at constant extracellular pH (Jiang *et al.* 1994, Stewart *et al.* 2001). Anion exchanger AE3 is insensitive to changes in intracellular pH (Sterling & Casey 1999).

The steep pH dependence (both intra- and extracellular) of AE2 activity is thought to reflect its function as a cellular defense against alkaline loads and in the maintenance of a resting intracellular pH and chloride concentration (Humphreys *et al.* 1994, Jiang *et al.* 1994). Anion exchanger AE2 activity, but not AE1 activity, also increases in hypertonic conditions (Humphreys *et al.* 1995). Both the cytoplasmic and transmembrane domains of AE2 contribute to the regulation of anion exchange by intracellular pH (Zhang *et al.* 1996). The cytoplasmic, N-terminal pH modifier region has been recently localized between aa 311 and aa 347 of AE2 (Stewart *et al.* 2001, Stewart *et al.* 2002). Instead, the membrane domain alone is sufficient to carry out the anion transport function of AE1 (Lepke *et al.* 1992). Erythroid AE1 maintains most of its activity despite the changes in

pH, because of its role in transporting CO_2 out of tissues for excretion by the lungs (Zhang *et al.* 1996).

2.2.4 Cellular and subcellular localization

Anion exchangers and their variants show remarkable tissue and cell type specificity (Table I). The full-length AE1e (911 aa in humans; e for erythrocyte) is mainly expressed in red blood cells, where it comprises about 25% of the total protein content (Fairbanks *et al.* 1971). Instead, the N-terminally truncated forms (AE1k; k for kidney) of human (Wagner *et al.* 1987, Kollert-Jöns *et al.* 1993), mouse (Brosius III *et al.* 1989), and rat AE1 (Drenckhahn *et al.* 1985, Kudrycki & Shull 1989) are expressed in kidney, localizing in the basolateral membrane of acid-secreting type A intercalated cells of cortical and medullary collecting ducts (Drenckhahn *et al.* 1985, Alper *et al.* 1989). The rodent AE1k isoform lacks the first 79 amino acids (Brosius III *et al.* 1989, Kudrycki & Shull 1989), and the human AE1k lacks the first 65 amino acids of full-length AE1e (Kollert-Jöns *et al.* 1993, Figure 2).

Table 1. Cellular and subcellular localization of anion exchanger proteins.

Isoform	Variant	Species	Tissue distribution	Intracellular localization
AE1	AE1e	human, rodents	red blood cells	plasma membrane
	AE1k	human, rodents	kidney	basolateral membrane
	AE1-1	chicken	erythrocyte	plasma membrane
	AE1-2	chicken	erythrocyte	plasma membrane
	AE1-3	chicken	kidney, erythroid cells	apical membrane
	AE1-4	chicken	kidney	basolateral membrane
	AE1-5	chicken	kidney, erythroid cells	basolateral membrane
AE2	AE2a	human, rodents	ubiquitous	plasma membrane
	AE2a	rabbit	stomach, parietal cells	basolateral membrane
	AE2b(1)	human	liver, kidney	basolateral membrane
	AE2b(2)	human	liver, kidney	basolateral membrane
	AE2b(1)	rodents	mainly stomach, liver	basolateral membrane
	AE2b(2)	rodents	mainly stomach, liver	basolateral membrane
	AE2c1	rodents	stomach	basolateral membrane
	AE2c2	rodents	mainly stomach	basolateral membrane
	AE2-1	chicken	stomach	basolateral membrane
	AE2-2	chicken	stomach	basolateral membrane
	AE2b	rabbit	stomach, mucous cells	basolateral membrane
AE3	AE3b	human, rodents	brain	plasma membrane
	AE3c	human, rodents	heart	plasma membrane
AE4	AE4	human	kidney	apical membrane

The alternative NH_2- termini of the chicken kidney variants AE1-3, AE1-4, and AE1-5 (Cox & Cox 1995) have been suggested to play a role in polarized sorting and recycling

between PM and the Golgi complex (Adair-Kirk *et al.* 1999). Basolateral accumulation of the AE1-4 variant and its recycling to the Golgi are dependent on two tyrosine residues (Tyr 44 and Tyr 47). The tyrosine 47 resides in the sequence YVEL (Adair-Kirk *et al.* 1999), which resembles the internalization signal found in other proteins recycling to the Golgi (YXXZ, Y=tyrosine, X=any amino acid, Z=hydrophobic residue) (•Kirchhausen *et al.* 1997, Gu *et al.* 2001). During the recycling to the Golgi, chicken AE1 anion exchangers associate with the cytoskeleton and receive complex N-linked sugars (Adair-Kirk *et al.* 1999, Ghosh *et al.* 1999), the reason of which is currently unknown. The YVEL sequence is also present in the other chicken AE1 variants except AE1-3. The AE1-3 variant accumulates in the apical membrane of chicken kidney epithelial cells, and it lacks the first 63 amino acids of the AE1-4 variant including the two tyrosine residues (Adair-Kirk *et al.* 1999). Consequently, the AE1-3 variant does not recycle to the Golgi (Adair-Kirk *et al.* 1999, Adair-Kirk *et al.* 2002). The AE1-1 and AE1-2 variants are expressed in chicken erythrocytes (Kim *et al.* 1989).

The full-length anion exchanger 2, AE2a (1241 aa in humans, 1237 aa in rat and mouse), is widely expressed in nearly every tissue examined thus far, and it is therefore considered to be a "housekeeping" protein. The highest levels of expression have been found in gastric epithelial cells (Jöns *et al.* 1994, Stuart-Tilley *et al.* 1994, Cox *et al.* 1996), choroid plexus (Lindsey *et al.* 1990, Alper *et al.* 1994), intestine (Chow *et al.* 1992, Alper *et al.* 1999), and kidney (Brosius III *et al.* 1995, Alper *et al.* 1997, Castillo *et al.* 1999). In polarized epithelial cells, it is mainly localized to the basolateral membrane (Alper *et al.* 1994), with the exception of the villus and crypt enterocytes in ileum, where AE2a may be apically located (Chow *et al.* 1992, Alper *et al.* 1999). In addition, a Golgi-localized AE2 has been detected in the rat (Alper *et al.* 1997) and mouse (Stuart-Tilley *et al.* 1998) kidney medulla as well as in a number of tissue culture cells (Kellokumpu *et al.* 1988).

Alternative splicing and alternative promoters produce additional AE2 variants (Figure 2). In rat and mouse, these include AE2b and AE2c, the former having an alternative promoter in intron 2. AE2c has two alternatively spliced isoforms, AE2c1 and AE2c2, the latter with an alternative promoter in intron 5 resulting in a 1035-aa protein (Wang *et al.* 1996). AE2a and AE2c2 are ubiquitously expressed in rat and mouse tissues, whereas AE2c1 is only present in stomach (Stuart-Tilley *et al.* 1998, Lecanda *et al.* 2000). The mouse AE2b(1) and AE2b(2) variants are mainly found in stomach, although they are also detected in other tissues (Lecanda *et al.* 2000). Two variant AE2 anion exchangers, AE2-1 and AE2-2, with an alternative N-terminus are expressed in chicken stomach (Cox *et al.* 1996). In human, in addition to the full-length AE2a, two variants, AE2b(1) and AE2b(2), closely related to the rodent AE2b variants have been identified (Medina *et al.* 2000). These variants are expressed in liver and kidney. The AE2c isoforms have not been detected in humans (Medina *et al.* 2000). In addition, studies on the expression of AE2 in rabbit stomach have shown that the AE2b isoform is the predominant form in the basolateral membrane of parietal cells and AE2a in mucous cells (Rossmann *et al.* 2001).

Anion exchanger 3 has two isoforms, one of which is expressed mainly in heart (AE3c) and the other in brain (AE3b) (Kopito *et al.* 1989, Kudrycki *et al.* 1990, Yannoukakos *et al.* 1994, Richards *et al.* 1999, Figure 2). Both isoforms have also been detected in rat retina, where they are expressed cell type specifically either in neurons ("heart" isoform) or in glial cells ("brain" isoform) (Kobayashi *et al.* 1994). AE3c is also

expressed in smooth muscle cells (Brosius III *et al.* 1997). The mouse cardiac isoform is a 1030 aa (1034 in human) protein, whereas the brain isoform consists of 1227 amino acids (1232 aa in human) (Linn *et al.* 1992, Yannoukakos *et al.* 1994). They differ in their N-terminus in such a way that the first 270 amino acids of brain AE3 are replaced by a peptide sequence of 73 amino acids in cardiac AE3. These different translation products are a consequence of alternative promoter and exon usage (Linn *et al.* 1992). Mouse AE3 also has two additional isoforms in brain (AE3b-14nt and AE3b-311nt) that lack the transmembrane domain and are functionally inactive as anion exchangers (Morgans & Kopito 1993b). Anion exchanger 4 (AE4) has been localized to the apical membrane of base-secreting type B intercalated kidney cells in rabbit and human (Parker *et al.* 2001, Tsuganezawa *et al.* 2001, Figure 2). The localization of AE4 may be cell type specific since, in rat and mouse, AE4 has been localized to the basolateral membrane of type A intercalated kidney cells (Ko *et al.* 2002).
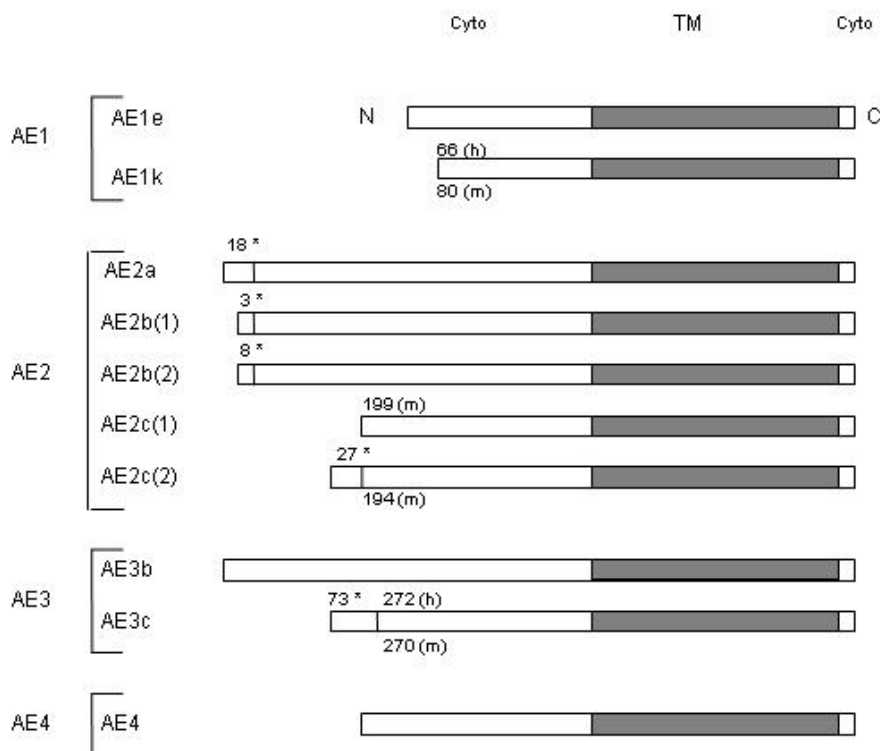
Fig. 2. Schematic representation of the human and mouse anion exchanger isoforms and variants (figure modified from Alper et al. 2002). Numbers refer to the length of the peptide sequence that is different (asterisk) or the position of the first amino acid (h=human, m=mouse) in N-terminally truncated variants compared to the full-length protein in human and mouse anion exchanger isoforms (Cyto=cytoplasmic portion, TM=transmembrane portion).

2.2.5 Interacting proteins

Anion exchanger 1, the earliest known member of anion exchangers, interacts with a number of other proteins in the plasma membrane (Low 1986). The most widely studied of these is interactions is that with erythrocyte ankyrin (Bennett & Stenbuck 1980, Drenckhahn *et al.* 1985, Michaely & Bennett 1995), which constitutes a link with the spectrin network (Bennett & Stenbuck 1979, Davis *et al.* 1991) and, thus, with the membrane cytoskeleton. The rat kidney isoform, AE1k lacks the first 79 amino acids (or 65 aa in humans; Kollert-Jöns *et al.* 1993) of full-length AE1 and does not bind ankyrin (Ding *et al.* 1994, Wang *et al.* 1995). The brain anion exchanger AE3 is capable of binding to the N-terminal domain of erythrocyte ankyrin *in vivo* (Morgans & Kopito 1993a). In these co-immunoprecipitation assays, AE2 was unable to bind to erythrocyte ankyrin. In more recent studies, however, anion exchanger 2 has been shown to bind erythrocyte ankyrin *in vitro* (Jöns & Drenckhahn 1998). In this study, anion exchanger 2 was suggested to be immobilized in the basolateral membrane through ankyrin-mediated cytoskeletal linkage.

   In addition to ankyrin, the N-terminal domain of the erythrocyte anion exchanger AE1e interacts with several other proteins. These include membrane skeletal proteins, such as protein 4.1 (Pasternack *et al.* 1985) and protein 4.2 (Korsgren & Cohen 1988), and the glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase (Kliman & Steck 1980, Rogalski *et al.* 1989), phosphofructokinase (Jenkins *et al.* 1985), and aldolase (Murthy *et al.* 1981). In addition, AE1e binds to the oxygen-carrying protein hemoglobin or preferentially to its deoxygenated form (Walder *et al.* 1984) and to hemichromes, which are early intermediates in the denaturation of hemoglobin (Waugh & Low 1985). Deoxyhemoglobin and glycolytic enzymes compete for the same binding site in AE1. Thus, the activity of glycolytic enzymes may be regulated by the oxygenation state of hemoglobin since glycolytic enzymes become active when released from AE1 (Walder *et al.* 1984, Low *et al.* 1993). AE1e also binds to the protein tyrosine kinase (p72[syk]) responsible for tyrosine phosphorylation of AE1 (Harrison *et al.* 1994). Glycophorin A binding is essential for the cell surface trafficking and anion transport activity of AE1 (Nigg *et al.* 1980, Groves & Tanner 1992, Young & Tanner 2003).

   The alternatively spliced kidney isoform of AE1 also has binding sites for and co-localizes with glyceraldehyde-3-phosphate dehydrogenase (Ercolani *et al.* 1992). The human kidney isoform of AE1k (Kollert-Jöns *et al.* 1993), however, does not interact with protein 4.1 or aldolase (Wang *et al.* 1995), but it has been shown to bind kanadaptin (Chen *et al.* 1998), a protein that may be involved in polarized expression of AE1 in the intercalated cells of kidney (Drenckhahn *et al.* 1985, Alper *et al.* 1989). The full-length erythroid isoform of AE1 does not bind kanadaptin (Chen *et al.* 1998).

   Most recently, the binding of carbonic anhydrase II (CAII; Vince & Reithmeier 1998, Vince & Reithmeier 2000) to the C-terminal, cytosolic portion of anion exchangers has been demonstrated. Carbonic anhydrase is an enzyme that hydrates carbon dioxide to bicarbonate, which is then transported out of the cells by the AE1 protein. These reactions are coupled to the oxygen-carrying function of red blood cells. Both proteins need one another for activation, i.e., CAII binding is required for the anion transport activity of AE1 (Sterling *et al.* 2001), and AE1 binding activates the formation of bicarbonate by

CAII (Scozzafava & Supuran 2002). These proteins are thus suggested to form a metabolon, i.e., a weakly associated complex of proteins that allows rapid channeling of products from one active site to another (Srere 1985, Vince & Reithmeier 2000, Sterling *et al.* 2001). Also, the anion exchangers AE2 and AE3 bind to CAII and CAIV (Sterling *et al.* 2001, Sterling *et al.* 2002). CAIV forms the extracellular component of a transport metabolon, also facilitating the anion exchange activity of AE proteins (Sterling *et al.* 2002).

2.3 Plasma membrane skeleton

Anion exchanger 1 serves as an attachment site for the underlying spectrin-based membrane skeleton (Figure 3). This attachment is mediated by the adapter protein ankyrin, which also interacts with the spectrin network. Spectrin oligomers are cross-linked by short actin filaments to form a two-dimensional network. About 30-50 % of AE1 is tightly bound to ankyrin. The red blood cells lacking AE1 are fragile and have decreased membrane stability (Peters *et al.* 1996, Southgate *et al.* 1996). The membrane skeleton architecture was, however, shown to be nearly normal, indicating that AE1 is not a crucial component in the formation of the plasma membrane skeleton. AE1-null red blood cells possessed normal levels of spectrin, protein 4.1, and actin in a ratio identical to that in normal membrane skeletons. Also, ankyrin-deficient red blood cells contained normal plasma membrane skeletons, indicating that ankyrin is not required for membrane skeleton assembly (Yi *et al.* 1997). The spectrin content, however, was reduced to about 50% of normal, and AE1 tetramers were absent in ankyrin-deficient red blood cells.

The stabilization of membranes may be achieved by direct AE1-lipid or spectrin-lipid interactions (Peters *et al.* 1996, Southgate *et al.* 1996). In good accordance, asymmetric distribution of membrane lipids between the inner and outer leaflets of the plasma membrane both increases the association of the spectrin-actin skeleton with membrane lipids and improves the mechanical stability of the red blood cell membrane (Manno *et al.* 2002). Direct interactions of spectrin with membrane lipids have been previously characterized (Cohen *et al.* 1986, Maksymiw *et al.* 1987). Another major attachment site of the spectrin network to the plasma membrane is formed by protein 4.1 (Figure 3), which is bound to anion exchanger 1 and glycophorin C in the plasma membrane. Overall, the intact plasma membrane skeleton of red blood cells has a crucial role in controlling membrane shape, stability, and organization.
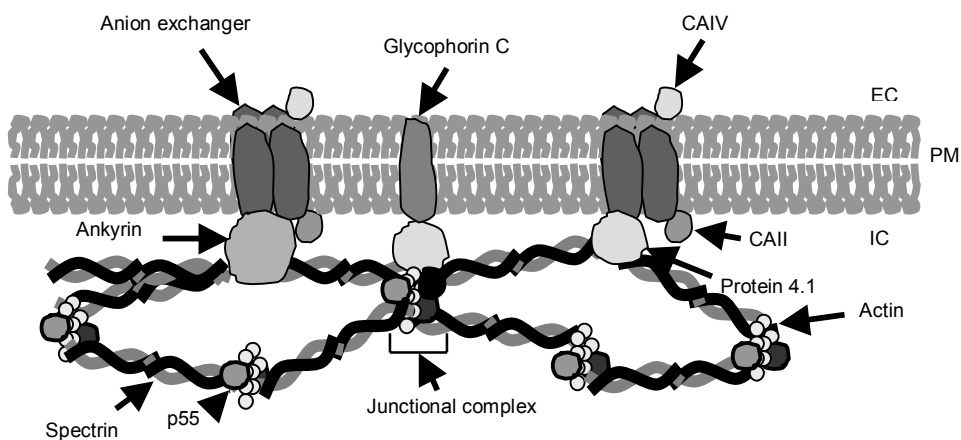
Fig. 3. Schematic presentation of the plasma membrane skeleton. Anion exchanger, AE1, is bound to ankyrin and protein 4.1 via its N-terminus. Protein 4.1 is also bound to glycophorin C, but not simultaneously with the anion exchanger protein. Actin cross-links spectrin tetramers at junctional complexes (see text for details). In addition, anion exchanger binds several other proteins, e.g. carbonic anhydrase II (CAII) and IV (CAIV), that catalyze the reversible conversion of CO_2 to HCO_3^-. (EC=extracellular space, IC=intracellular space, PM=plasma membrane)

2.3.1 Ankyrin

Three distinct ankyrin genes, ANK1, ANK2, and ANK3, encode the polypeptides designated as Ank1 ("erythrocyte" ankyrin R), Ank2 ("brain" ankyrin B), and Ank 3 ("general" ankyrin G) (Bennett 1992, De Matteis & Morrow 1998, Rubtsov & Lopina 2000). These isoforms contain three domains: the N-terminal membrane-binding, central spectrin/fodrin-binding, and C-terminal regulatory domains (Bennett 1992, Rubtsov & Lopina 2000). The membrane-binding domain (89 kDa) contains 24 tandemly organized repeat motifs, called ankyrin repeats, that consist of 33 amino acids each (Lux *et al.* 1990, Michaely & Bennett 1992). This domain and especially the ankyrin repeats 7-12 and 13-24 have been shown to be responsible for high-affinity binding to anion exchanger 1 (Davis & Bennett 1990a, Davis *et al.* 1991, Michaely & Bennett 1995). The N-terminal membrane binding domain also has distinct binding sites for tubulin (Davis & Bennett 1984, Davis *et al.* 1991) and Na$^+$/K$^+$-ATPase (Davis & Bennett 1990b). Na$^+$/K$^+$-ATPase has binding sites also in the spectrin binding domain of ankyrin (Davis & Bennett 1990b). Ankyrin binds to one β-subunit of the spectrin filament (Kennedy *et al.* 1991) with its central spectrin-binding domain (Platt *et al.* 1993). The spectrin-binding domain (62 kDa) consists of N-terminal, central, and C-terminal subdomains. The N-terminal subdomain (Davis & Bennett 1990a) contains proline and acidic amino acids, and it is variable between the ankyrins B and R. The central sub-domain and the C-terminal sub-domain of the spectrin-binding domain are highly conserved between the ankyrins B and R (Bennett

1992). The C-terminal regulatory domain (55 kDa) modulates the affinities of the other two ankyrin domains (Davis & Bennett 1990a, Bennett 1992).

Ankyrin R has several isoforms that result from alternative splicing or proteolysis (Rubtsov & Lopina 2000). The full-length ankyrin R (206 kDa) is the major isoform in erythrocytes, and it interacts with the plasma membrane localized anion exchanger 1 (Davis & Bennett 1990a). Ankyrin B has two isoforms, a 220 kDa isoform and a 440-kDa isoform that is also expressed in nervous tissue and has a 220 kDa rod-shaped domain inserted between its C-terminal and spectrin-binding domains (Rubtsov & Lopina 2000). Ankyrin G has several isoforms that are expressed in epithelial cells, myocytes, hepatocytes, megakaryocytes, and neurons (Rubtsov & Lopina 2000).

The anion exchanger interacts with ankyrin via its N-terminal portion. The first 79 amino acids and the "hinge" region between 155 and 195 seem to be involved in ankyrin binding (Davis *et al.* 1989, Ding *et al.* 1994, Ding *et al.* 1996, Chang & Low 2003). Also, the redox state of the cysteines 201 and 317 has been suggested to be critical for ankyrin binding (Thevenin *et al.* 1989, Willardson *et al.* 1989). Disulfide bond formation between Cys-201 and Cys-317 may block the ankyrin binding by conformational change or steric hindrance (Thevenin *et al.* 1989).

2.3.2 Spectrin

Spectrin is a major component of the membrane skeleton, and its ability to bind several integral membrane proteins, cytosolic proteins, phospholipids, and all major filaments makes it a central organizing protein in the cell. The spectrin molecule consists of α- and β-subunits twisted around each other (Yu *et al.* 1973). These heterodimers can form higher oligomers - mostly tetramers in red blood cells, by head-to-head interactions (Ungewickell & Gratzer 1978). The predominant subunits in red blood cells are αI and βI, and the general plasma membrane isoforms are αII and βII spectrins (De Matteis & Morrow 2000). βIII is the Golgi isoform (Beck *et al.* 1994, Stankewich *et al.* 1998), but no corresponding α-subunit has been found, and III may thus form homo-oligomeric spectrin filaments. Also, two other β-isoforms are known, βIV and βV (De Matteis & Morrow 2000). The main role of the spectrin-based membrane skeleton seems to be to control the lateral distribution of integral membrane proteins and the formation of discrete membrane domains (Hammerton 1991, Bennett & Gilligan 1993, Beck & Nelson 1996).

The plasma membrane-localized spectrin also associates directly with membrane lipids via its membrane association domains (MAD), although with low affinity (Cohen *et al.* 1986, O'Toole *et al.* 1999). The spectrin-lipid interactions are short-termed (O'Toole *et al.* 1999) and inhibited by ankyrin binding to spectrin (Bialkowska *et al.* 1994, Diakowski *et al.* 1999). The exact role of spectrin-lipid binding is not known, but it has been suggested to facilitate the formation of the membrane skeleton (O'Toole *et al.* 1999). It may also provide an alternative binding site for spectrin if ankyrin is not present (Bialkowska *et al.* 1994, Diakowski *et al.* 1999) or if the ankyrin-spectrin binding has been down-regulated by ankyrin phosphorylation (Lu *et al.* 1985, Cianci *et al.* 1988). The ankyrin/spectrin cytoskeleton seems to be continuously remodeled by ankyrin

phosphorylation (Ghosh & Cox 2001). Spectrin becomes phosphorylated and redistributed to the cytosol during mitosis (Fowler & Adam 1992).

2.3.3 Protein 4.1 and protein 4.2

Another major site of interaction between the plasma membrane and the membrane skeleton occurs between spectrin and protein 4.1 (Correas *et al.* 1986). Protein 4.1 serves as an adapter protein between the spectrin network and glycophorin C (Reid *et al.* 1990, Marfatia *et al.* 1994) or AE1 (Pasternack *et al.* 1985). However, protein 4.1 is not able to bind simultaneously to these integral membrane proteins (Pasternack *et al.* 1985). Instead, the ternary complex that includes protein 4.1, glycophorin C, and p55 forms an independent attachment site for the spectrin network to the plasma membrane (Reid *et al.* 1990, Alloisio *et al.* 1993, Marfatia *et al.* 1994). The association of protein 4.1 with glycophorin C is regulated by polyphosphoinositides PtdIns(4,5)P$_2$ (Anderson & Marchesi 1985). Protein 4.1 is also bound to actin, which cross-links spectrin tetramers in the plasma membrane skeleton (Correas *et al.* 1986). The spectrin-actin junctional complexes also include tropomyosin, tropomodulin, adducing, and protein 4.9 (Gilligan & Bennett 1993). Each junctional complex binds six spectrin filaments, resulting in a hexagonal array of the plasma membrane skeleton (Liu *et al.* 1987, Gilligan & Bennett 1993).

About 50% of the membrane-bound protein 4.1 is linked to AE1 (An *et al.* 1996). The binding interface between AE1 and protein 4.1 has been mapped to the cytoplasmic sequence IRRRY/LRRRY of AE1 and to the membrane-binding domain sequence LEEDY of protein 4.1 (Jöns & Drenckhahn 1992). Another protein 4.1 binding site may be present in the N-terminus of AE1 (Lombardo *et al.* 1992), since the kidney AE1k that contains the IRRRY sequence but lacks the first 65 aa of full-length AE1 (AE1e) is unable to bind to protein 4.1 (Wang *et al.* 1995). Protein 4.1 binding to AE1 has been suggested to modulate the interaction between AE1 and ankyrin (An *et al.* 1996). The synthetic peptide IRRRY dissociated AE1 from protein 4.1, which in turn resulted in an increase of interaction between AE1 and ankyrin. This increased association decreased membrane deformability and increased the mechanical stability of the membrane (An *et al.* 1996). This study has been challenged by gene knockout experiments that generated complete deficiency of protein 4.1 in mice (Shi *et al.* 1999). In protein 4.1-deficient mice, the erythrocyte membrane exhibited lower stability and increased fragmentation of red blood cells with concomitant reduction in the expression of spectrin, ankyrin, glycophorin C, and p55. Protein 4.2 (Korsgren & Cohen 1986), in turn, is potentially involved in strengthening the linkage of AE1 to the cytoskeleton (Bennett & Stenbuck 1979, Rybicki *et al.* 1996). Anion exchanger 1 seems to provide the sole attachment site for protein 4.2 since it was totally absent in AE1-deficient red blood cells (Peters *et al.* 1996, Southgate *et al.* 1996). The RNA levels of protein 4.2 were normal, indicating that the protein is unstable in AE1-deficient cells (Peters *et al.* 1996).

2.4 Golgi complex

2.4.1 Golgi structure

The Golgi complex was first described by Camillo Golgi in 1898 (Golgi 1898). The first electron microscopic examinations in the 1950's showed the Golgi complex to consist of several flattened cisternae or saccules with dilated rims (reviewed by Farquhar & Palade 1981). These cisternae are often slightly curved, with the concave (trans) side facing the nucleus or the plasmalemma and the convex (cis) side facing the rough endoplasmic reticulum. Today, the Golgi complex is thought to be composed of several interconnected stacks that form a continuous ribbon-like structure. Each stack is formed of five functionally different compartments: CGN (*cis*-Golgi network), *cis*-Golgi, medial-Golgi, *trans*-Golgi, and TGN (*trans*-Golgi network). In addition, or maybe equivalent to CGN, an ER-Golgi intermediate compartment (ERGIC) has been described (Saraste & Kuismanen 1984, Schweizer *et al.* 1990, Balch *et al.* 1994, Bannykh *et al.* 1996, Bannykh & Balch 1997, Bannykh *et al.* 1998). The ERGIC components are composed of vesicular tubular membrane clusters (VTCs) that are transported from the ER towards the cis-Golgi along microtubules (Figure 4) (Saraste & Svensson 1991, Mizuno & Singer 1994, Presley *et al.* 1997).

CGN and TGN are structurally somewhat divergent structure from the *cis*-, medial-, and *trans*-Golgi. They consist of interconnected, tubular membrane structures with vesicles budding off or fusing to the membrane. The CGN side is typically associated with small vesicles, while the TGN side has larger secretory granules or vacuoles on its side (Farquhar & Palade 1981). Although the Golgi compartments are functionally differentiated to some extent, they may be physically connected with each other. Tubular connections have been shown to exist between individual Golgi stacks (Cooper *et al.* 1990, Rambourg & Clermont 1990, Tanaka & Fukudome 1991, Mironov *et al.* 1997, Ladinsky *et al.* 1999). Transient tubular connections have also been suggested to exist between the neighboring cisternae, and they may be involved in protein trafficking between the Golgi compartments (Weidman 1995).

The dynamic nature of the Golgi complex with protein-carrying vesicles fusing to and budding from its membranes requires continuous remodeling of its structure. The cis-Golgi is thought to be formed by the fusion of ERGIC elements, which originate from vesicles or tubules produced at ER exit sites (Figure 4) (Bannykh *et al.* 1998). The cisterna formation is initiated by tethering reactions. The vesicle-localized giantin (Linstedt & Hauri 1993) binds to a complex formed by GM130 (Nakamura *et al.* 1995), GRASP65 (Barr *et al.* 1997), and p115 (Waters *et al.* 1992) in the target cisternae (Sönnichsen *et al.* 1998, Linstedt 1999, Dirac-Svejstrup *et al.* 2000). Vesicle targeting is mediated by soluble NSF attachment protein receptors (SNARE). The SNARE pairing of GOS28 on the vesicle (v-SNARE or vesicle SNARE) and syntaxin 5 on the target membrane (t-SNARE or target SNARE) completes the fusion process (Söllner *et al.* 1993, Subramaniam *et al.* 1997). p115 may have a catalytic role in SNARE pairing (Shorter *et al.* 2002). After membrane fusion, the SNARE complexes are dissociated by the activity of α-SNAP/NSF (Subramaniam *et al.* 1997) or the p47/p97 ATPase complex

(Rabouille *et al.* 1995b, Kondo *et al.* 1997). The former has been implicated in the control of heterotypic fusion (v-t-SNARE pairing) and the latter in homotypic fusion (t-t-SNARE pairing) (Rabouille *et al.* 1998). They may also act sequentially so that p97 produces larger structures from the cisternae first formed by NSF-mediated vesicle fusion (Acharya *et al.* 1995).

The stacking of cisternae is mediated by giantin-p115-GM130-GRASP65 tethering factors (Barr *et al.* 1997, Barr *et al.* 1998, Shorter & Warren 1999) and a tether-independent medial-Golgi protein, GRASP55 (Shorter *et al.* 1999). These Triton X-100-insoluble Golgi matrix proteins have been shown to be sufficient to generate the ribbon-like Golgi structure (Seemann *et al.* 2000a). GRASP55 interacts with golgin-45, their interaction being regulated by GTPase rab2 (Short *et al.* 2001). Recently, the prevalent role of GM130 in maintaining the Golgi structure has been questioned, since the Golgi structure and function were not significantly altered in cells deficient of GM130 (Vasile *et al.* 2003). This indicates that other proteins may also contribute to Golgi stacking. One such protein may be another member of the golgin family (Barr & Short 2003, Gillingham & Munro 2003), the cis-Golgi-localized golgin-84 (Bascom *et al.* 1999). The exact role of golgin-84, however, has not yet been established, but it is known to be important for the Golgi structure (Satoh *et al.* 2003, Short & Barr 2003).

In mitosis, the Golgi complex loses its ribbon-like structure due to the separation of individual stacks, which are further fragmented into clusters of vesicles and tubules that are distributed throughout the cytoplasm (Colman *et al.* 1985, Warren *et al.* 1995). This fragmentation is accomplished by the continuous budding of COPI-coated vesicles unable to fuse with the target membrane and by the generation of heterogenous tubules and vesicles via the COPI-independent pathway (Misteli & Warren 1994, Warren *et al.* 1995). The phosphorylation of GM130 coincides with the initiation of Golgi fragmentation in the prophase, and the protein becomes dephosphorylated in the telophase as the Golgi fragments start to reassemble (Lowe *et al.* 2000). GM130 is a substrate for the cyclin-dependent kinase CDK1-cyclin B, which is responsible for mitotic Golgi fragmentation (Misteli & Warren 1994, Lowe *et al.* 1998). The phosphorylation of the serine 25 of GM130 inhibits its binding to p115 and may provide the explanation for the COPI-dependent Golgi fragmentation pathway (Lowe *et al.* 2000). The Golgi reassembly from mitotic fragments requires phosphorylation of the p115 protein at serine 941 (Dirac-Svejstrup *et al.* 2000).

2.4.2 Golgi function

2.4.2.1 Glycosylation

The hydroxyl groups of the serine and threonine residues of some secretory and membrane proteins become O-glycosylated in the Golgi. The first sugar residue is usually N-acetylgalactosamine, which can give rise to more complex structures bearing additional monosaccharide residues (Hanisch 2001). The asparagine-linked N-glycosylation of proteins and lipids begins in the endoplasmic reticulum, where they

acquire a core oligosaccharide consisting of glucose, mannose, and N-acetylglucosamine (Roth 2002). The consensus acceptor site for N-glycosylation is in the sequence Asn-X-Ser/Thr, where X is any amino acid other than proline. During their subsequent passage from CGN to TGN, glycoproteins acquire a number of sequential modifications in their sugar residues (Roth 2002). This is accomplished by glycosyltransferases that have distinct but overlapping sites of activity within the Golgi complex (Dunphy *et al.* 1981, Roth & Berger 1982, Dunphy *et al.* 1985, Dunphy & Rothman 1985, Nilsson *et al.* 1993a, van den Eijnden & Joziasse 1993, Rabouille *et al.* 1995a, Prescott *et al.* 1997) and glycosidases that are involved in the trimming of the sugar chains (Hersovics 1999). Glycosyltransferases add sugar residues to the growing oligosaccharide chain attached to a protein or lipid, and glycosidases remove them. The sugar-nucleotide donors are made in the cytosol and transported to the Golgi by specific transporters (Abeijon *et al.* 1997). Most glycosylation reactions take place on the lumenal side of the Golgi, where the active site of transmembrane glycosyltransferases lies. The glycosylation reactions are numerous, and there are more than two hundred different glycosyltransferases and glycosidases involved. These are typically type II integral membrane proteins (N-terminus in the cytosol), and many of them carry out similar reactions. They have different pH optima, and the lumenal pH, which is known to decrease from ER to the trans-Golgi network, thus affects their activity (Demaurex 2002).

2.4.2.2 Sorting of proteins

The second major function of the Golgi complex is the sorting of the numerous proteins that arrive in the Golgi from their site of synthesis, the endoplasmic reticulum. This results in a continuous flow of proteins through the Golgi complex to TGN, where the sorting mainly takes place. According to the 'bulk flow' model (Pfeffer & Rothman 1987, Wieland *et al.* 1987), proteins are continuously delivered to the cell surface or secreted, unless they contain a specific retention signal (e.g., Brands *et al.* 1985, Munro & Pelham 1987, Wieland *et al.* 1987, Nilsson *et al.* 1989, Jackson *et al.* 1990, Pelham 1990, Munro 1991, Shin *et al.* 1991). This view has been challenged by more recent studies, which have shown that many proteins are concentrated and selectively sorted in the endoplasmic reticulum prior to exit (reviewed by Barlowe 2003). Some soluble proteins seem to be transported on a 'bulk flow' basis, but receptor-mediated transport may contribute to the more selective export of soluble proteins from ER (Barlowe 2003). Similarly, at TGN, proteins are selectively packaged into vesicles targeted to lysosomes, secretory vesicles, or the plasma membrane (reviewed by Gu *et al.* 2001).

2.4.3 Golgi pH

The organelles of the secretory pathway show a pH gradient that decreases toward the plasma membrane (pH~7 in endoplasmic reticulum -> pH~6 in TGN -> pH~5.5 in secretory granules) (Demaurex 2002). Also, the lumen of the organelles of the endocytic pathway represents a decreasing pH gradient (pH~6.5 in early endosomes -> pH<6 in late

endosomes -> pH<5.5 in lysosomes) (Demaurex 2002). In endosomes, the dissociation of receptors from their ligands requires acidic pH (Gu *et al.* 2001). Thus, the pH of the lumen of the organelles is generally more acidic than that of the surrounding cytosol (pH7.0-7.5). This acidification is attained mainly by the activity of vacuolar-type H^+-ATPases, which are present in nearly all intracellular organelles (Demaurex 2002). The pH of the organelle lumen may also be regulated by passive proton leaks (Demaurex 2002). In addition, the Na^+/K^+-ATPase involved in pH regulation, may be present in endosomes (Cain *et al.* 1989, Fuchs *et al.* 1989). The ER membrane seems to be more permeable to ions and other small molecules than other organellar membranes, and its pH is thus close to that of the cytosol (Le Gall *et al.* 2004). However, the regulation of organelle acidity is still poorly understood.

The localization/activity of Golgi glycosyltransferases (Axelsson *et al.* 2001), and the sorting of proteins at TGN (Caplan *et al.* 1987, Carnell & Moore 1994, Henkel *et al.* 2000) are pH-dependent processes and require an acidic environment. As in other organelles, acidification in the Golgi is achieved primarily by vacuolar type H^+-ATPases (Moriyama & Nelson 1989, Demaurex 2002). In addition, the Na^+/H^+ exchanger has been shown to be present in Golgi membranes, and it may be involved in the cation homeostasis of TGN (Numata & Orlowski 2001). From electrophysiological recordings, the Golgi-associated chloride channel, GOLAC, has been suggested to provide counterions into the TGN lumen, to prevent charge accumulation resulting from the activity of H^+-ATPase (Nordeen *et al.* 2000).

2.4.4 Golgi membrane skeleton

Similarly to the plasma membrane skeleton, Golgi-localized counterparts have been identified that form the Golgi membrane skeleton (Beck & Nelson 1998). These proteins include Golgi-specific βIII spectrin (Beck *et al.* 1994, Stankewich *et al.* 1998) and two ankyrins, AnkG119 (Devarajan *et al.* 1996) and Ank_{195} (Beck *et al.* 1997) (the numbers refer to the molecular mass of each protein). AnkG119 is an ANK3 gene product and Ank_{195} is immunologically related to ankyrin R (De Matteis & Morrow 1998). Ankyrin G119 is a truncated and alternatively spliced isoform of AnkG of the nervous system. The ankyrin repeat domains of this protein consist of 13 rather than 24 repeats, and the regulatory domain is shorter, 5 kDa, compared to the 55 kDa of the erythrocyte ankyrin (Devarajan *et al.* 1996). The lack of regions previously shown to contribute to membrane binding indicates that AnkG119 is unable to bind to AE1, which renders the protein more soluble (Devarajan *et al.* 1996). The domain structure of Ank_{195} is not known because the protein has not been cloned. The molecular size of the protein, however, indicates that it includes most of the regions found in erythrocyte ankyrin. Both Golgi ankyrins bind to the spectrin network surrounding the Golgi, but they differ in their solubility properties. The trans-Golgi protein, ankyrin 195, resists Triton X-100 extraction (Beck *et al.* 1997), whereas the ankyrin G119 localizing to the cis-Golgi is Triton X-100-soluble (Devarajan *et al.* 1996). At the present, no Golgi-specific isoforms for α-spectrin, protein 4.1, or protein 4.2 are known. The Golgi spectrin may also be homo-oligomeric, bearing only -subunits.

In red blood cells, the spectrin oligomers are cross-linked by short actin filaments to generate the two-dimensional lattice of the membrane skeleton. In the Golgi, the actin-related protein centractin (Arp1) may have a similar role. Centractin is a component of the dynactin complex, and it has been shown to interact with the Golgi spectrin (Holleran *et al.* 1996, Holleran *et al.* 2001). Dynactin, in turn, is associated with the minus end directed microtubule motor protein dynein (Karki & Holzbaur 1995), indicating that a link exists between the spectrin membrane skeleton and microtubules. The positioning of the Golgi complex and the Golgi morphology are known to be dependent on microtubules and associated motor proteins (Rogalski & Singer 1984, Thyberg & Moskalewski 1985, Burkhardt 1998). Thus, the Golgi membrane skeleton and microtubules may co-operate to maintain the Golgi structure.

The plasma membrane skeleton is crucial for membrane integrity (Southgate *et al.* 1996) and the lateral distribution of integral membrane proteins (Beck & Nelson 1996). Similarly, the Golgi membrane skeleton has been suggested to be required for the integrity of Golgi membranes and the formation of discrete membrane domains within the Golgi (Beck *et al.* 1994, Beck & Nelson 1998). Accumulating evidence exists to suggest that the Golgi spectrin is involved in vesicle trafficking by forming a vesicle coat together with the ankyrin G119. Na^+/K^+-ATPase protein transport from the ER to the Golgi has been shown to require the Golgi spectrin-ankyrin G119 skeleton (Devarajan *et al.* 1997). Also, the ER to Golgi transport of vesicular stomatitis virus (VSV)-G protein requires spectrin (Godi *et al.* 1998).

2.4.5 Localization mechanisms of Golgi proteins

The targeting signals can act in two different ways – either a protein is retained in the right organelle via a retention signal, or there is a retrieval signal that returns the protein, if it has escaped, to the right organelle. Depending on whether anterograde cargo moves by cisternal maturation or by vesicles within the Golgi, the Golgi resident proteins must either enter the retrograde vesicles/tubules or have a retention signal, respectively. The Golgi localization mechanisms have mainly been studied by using glycosyltransferases localized within the Golgi cisternae according to the oligosaccharides they modify in the glycosylation sequence. N-acetylglucosaminyltransferase I (GlcNAc-TI) is localized mainly in the medial-Golgi (Dunphy *et al.* 1985, Nilsson *et al.* 1993a), 1,4-galactosyltransferase (β1,4GalT) in the trans-Golgi/TGN (Roth & Berger 1982, Suganuma *et al.* 1991, Nilsson *et al.* 1993a), and 2,6-sialyltransferase (α2,6ST) in TGN (Roth *et al.* 1985, Taatjes *et al.* 1988). In addition, mannosidase II has been localized to the medial-Golgi (Velasco *et al.* 1993, Rabouille *et al.* 1995a). Although glycosyltransferases occupy distinct Golgi cisternae, their distributions overlap considerably (Nilsson *et al.* 1993a, Rabouille *et al.* 1995a).

The Golgi glycosidases and glycosyltransferases share a common domain structure (short N-terminal cytoplasmic tail, single transmembrane domain, large C-terminal luminal domain and a stem region between the luminal and transmembrane portions), but they lack sequence homology, which means that the Golgi retention mechanism cannot be deduced from their protein sequences. Thus, these Golgi enzymes lack the clear-cut

retention/retrieval signals found in, for example, ER-localized proteins (Munro & Pelham 1987, Paabo *et al.* 1987, Nilsson *et al.* 1989, Jackson *et al.* 1990, Pelham 1990). However, the membrane-spanning domain of glycosyltransferases has been shown to partly determine the Golgi localization of these proteins (Munro 1991, Nilsson *et al.* 1991, Aoki *et al.* 1992, Burke *et al.* 1992, Russo *et al.* 1992, Tang *et al.* 1992, Teasdale *et al.* 1992, Wong *et al.* 1992, Burke *et al.* 1994). The cytoplasmic tail and the luminal domain may also have a role in Golgi retention (Colley *et al.* 1992, Dahdal & Colley 1993, Burke *et al.* 1994, Tang *et al.* 1995, Nilsson *et al.* 1996). The lack of specific targeting signals in Golgi transmembrane proteins has led to two hypotheses of Golgi retention, i.e., the kin recognition and membrane thickness models.

2.4.5.1 Kin recognition

The oligomerization or kin recognition model first presented by Machamer (1991) suggests that the Golgi glycosyltransferases form oligomeric structures that are too bulky to enter the transport vesicles and thus to leave the Golgi. Two medial-Golgi enzymes, N-acetylglucosaminyltransferase I and mannosidase II, have been shown to form hetero-oligomers with each other, but not with the trans-Golgi enzyme β1,4-galactosyltransferase (Nilsson *et al.* 1994). Thus, glycosyltransferases form hetero-oligomers with their kin enzymes, i.e., enzymes localized in the same Golgi compartment, but not with the enzymes of different Golgi compartments (Nilsson *et al.* 1993b). β1,4-galactosyltransferase (Teasdale *et al.* 1994, Yamaguchi & Fukuda 1995), α-2,6-sialyltransferase (Chen *et al.* 2000), and β1,6-N-acetylglucosaminyltransferase V (Sasai *et al.* 2001) are all able to oligomerize. Several other proteins are retained in the Golgi by a similar mechanism, including the avian coronavirus M protein (Weisz *et al.* 1993), the intermediate compartment protein p63 (Schweizer *et al.* 1994), and the mouse hepatitis virus M protein (Krijnse Locker *et al.* 1995). In support of the kin recognition model, the ability of 2,6-sialyltransferase to form oligomers has been shown to correlate with its localization in the Golgi (Chen *et al.* 2000).

2.4.5.2 Membrane thickness

The second model of Golgi retention is based on the relatively thin lipid bilayer of the Golgi membranes as compared to the plasma membrane. The plasma membrane contains sphingolipids and sterols (Orci *et al.* 1981), which make the bilayer thicker and more ordered than the bilayer in the ER and Golgi (Nezil & Bloom 1992). In addition, the cholesterol concentration has been shown to increase within the Golgi in the cis to trans direction (Orci *et al.* 1981). According to the membrane thickness model, which is also known as the lipid-sorting model proteins that cross the bilayer and are retained in the Golgi have shorter hydrophobic domains than the proteins destined to the cell surface (Munro 1998). The transmembrane domains of Golgi enzymes have been shown to contain an average of five residues less than the plasma membrane proteins (Bretscher &

Munro 1993, Masibay *et al.* 1993, Munro 1998). The Golgi enzymes have more bulky phenylalanine residues than the PM proteins.

By adding six leucine residues to the transmembrane domain of α-2,6-sialyltransferase, the protein was targeted to the plasma membrane instead of the Golgi (Munro 1991). Similar results have been obtained later with β1,4-galactosyltransferase (Masibay *et al.* 1993). In addition, the influenza virus neuraminidase, which is a plasma membrane protein, accumulated in the Golgi and rough endoplasmic reticulum when the length of the hydrophobic transmembrane domain was reduced (Sivasubramanian & Nayak 1987). Overall, this model proposes that the shorter hydrophobic domains of Golgi proteins prevent them from entering the cholesterol-rich transport vesicles that are to be transported to the plasma membrane. In favor of this model, the difference in cholesterol concentration has been shown to be sufficient for the regulation of membrane insertion of transmembrane domains (Ren *et al.* 1997).

2.4.5.3 Targeting sequences

Although no specific targeting signals have been identified for transmembrane Golgi resident proteins, this is not the case with Golgi recycling proteins. In fact, recycling may be a common feature of Golgi-localized proteins since glycosyltransferases, which were previously considered to be permanent residents of Golgi, have also been suggested to recycle through ER (Storrie *et al.* 1998). A number of TGN-localized membrane proteins have a retrieval signal (YQRL) in their cytoplasmic tails that serves to return the proteins from the cell surface to TGN (Kirchhausen *et al.* 1997, Gu *et al.* 2001). This tyrosine-based motif, YXXZ (Y=tyrosine, X=any amino acid, Z=hydrophobic residue), interacts directly with μ-subunits of the adaptor complexes, thus directing them to clathrin vesicles (Ohno *et al.* 1996). The tyrosine-based internalization signal is found in, for instance, TGN38, furin, and gpI (varicella zoster virus glycoprotein I) (Kirchhausen *et al.* 1997, Gu *et al.* 2001). Some proteins, e.g., furin and gpI, also require a dileucine motif together with a cluster of acidic amino acids for endocytosis and TGN recycling (Molloy *et al.* 1999, Gu *et al.* 2001, Alconada *et al.* 1996). The acidic cluster contains casein kinase II phosphorylation sites (Jones *et al.* 1995), and the intracellular trafficking of furin and gpI is regulated by phosphorylation of these sites (Jones *et al.* 1995, Alconada *et al.* 1996). The connector protein PACS-1 links the phosphorylated acidic cluster sorting motifs to AP-1 adaptor complexes (Crump *et al.* 2001).

There are a number of peripheral proteins that are attached to the cytosolic side of the Golgi membranes. These are mainly proteins capable of forming coiled-coil structures, including the Golgi matrix proteins giantin, p115, and GM130 (Waters *et al.* 1992, Linstedt & Hauri 1993, Nakamura *et al.* 1995), cytoskeleton-associated proteins (e.g., ankyrin and spectrin) (Beck *et al.* 1994, Devarajan *et al.* 1996), and several vesicular transport proteins (Gleeson 1998, Munro 1998). Until recently, the mechanisms of Golgi localization and retention of these proteins were largely unknown. The Golgi matrix protein, GM130, interacts with GRASP65, and both proteins are involved in the stacking of the Golgi cisternae. The interaction site of GM130 has been mapped to its extreme carboxy-terminus (Barr *et al.* 1998), which seems to form a unique Golgi-targeting signal

(as discussed in Munro & Nichols 1999). The coiled-coil protein Golgin-45 binds to the medial-Golgi protein GRASP55, and they are involved in the stacking of the Golgi cisternae (Short *et al.* 2001). The 'GRIP' domain of ~42 amino acids with one conserved tyrosine found in several coiled-coil proteins has been shown to be sufficient for Golgi targeting (Munro &Nichols 1998, Barr 1999, Kjer-Nielsen *et al.* 1999, Gillingham & Munro 2003). The GRIP domain lies in the carboxy-terminal non-coiled domain of several peripheral Golgi proteins.

2.5 Protein and membrane trafficking in the Golgi region

Proteins are synthesized in the endoplasmic reticulum where they also acquire post-translational modifications and become properly folded. Proteins are transported from ER via VTCs to the cis-Golgi in COP II-coated vesicles (Barlowe *et al.* 1994, Barlowe 2003, Figure 4) that bud from transitional ER (ER exit site), a specialized region of the endoplasmic reticulum (Palade 1975, Bannykh *et al.* 1996, Bannykh & Balch 1997). This forward trafficking i.e., from ER to TGN is also called anterograde transport. The components of vesicle formation and some ER recycling proteins are returned from the cis-Golgi or VTCs to ER. This retrograde traffic is generally thought to be mediated by COPI-coated vesicles (Letourneur *et al.* 1994, Orci *et al.* 1997, Bannykh *et al.* 1998, Martinez-Menárguez *et al.* 1999, Figure 4). COPI- and COPII-coated vesicle formation, transport, and fusion with the membrane are regulated by GTPases. Tethering factors and SNARE proteins are involved in the vesicle fusion events. Some of the protein components involved in COP-coated vesicle fusion also play a role in Golgi cisterna formation and stacking (section 2.4.1.). Proteins move through the following Golgi compartments in the cis-trans direction, acquire some additional modifications, and arrive in TGN, where they are sorted to their correct destinations.
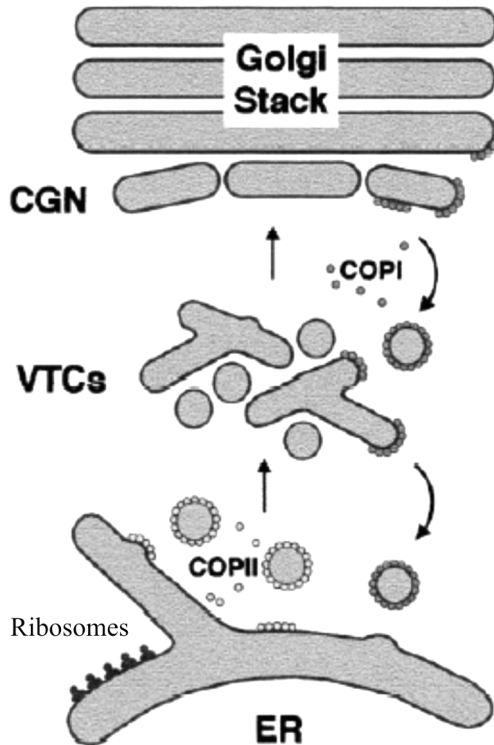
Fig. 4. Coat protein complexes of the early secretory pathway. COPII-coated vesicles bud from the transitional zones of ER (tER) and then fuse with or form vesicle tubular clusters (VTCs or ERGIC). VTCs fuse with or form the *cis*-Golgi network, and COPI-coated vesicles mediate the retrograde trafficking of recycling proteins (Barlowe 2000).

2.5.1 ER-Golgi interface

2.5.1.1 Anterograde transport

COPII-coated vesicles are thought to deliver their cargo from ER to vesicular tubular membrane clusters (VTCs) (Bannykh *et al.* 1996, Figure 4). These pleiomorphic structures are then translocated along the microtubules to the cis-Golgi by using dynein as a motor protein (Saraste & Svensson 1991, Mizuno & Singer 1994, Presley *et al.* 1997). Alternatively or additionally, the COPI-coated vesicles may be responsible for anterograde trafficking from VTCs (Aridor *et al.* 1995, Scales *et al.* 1997). VTCs (or ERGIC) have also been suggested to constitute a specialized domain of ER instead of being a distinct, individual compartment. In this case, transport of cargo from ER to Golgi would need only one vesicular transport step (Krijnse-Locker *et al.* 1994).

The ER-Golgi trafficking has been mainly studied in yeast, but mammalian homologues of the proteins involved have been found, and the molecular mechanisms of membrane trafficking seem to be highly conserved. Secretory proteins have been shown to be selectively sorted at ER exit sites by the activity of Sar1p-GTPase and COPII (Nakano & Muramatsu 1989, Aridor *et al.* 1998, Aridor *et al.* 2001). Heterodimers of Sec23/24 (Paccaud *et al.* 1996, Pagano *et al.* 1999) and Sec13/31 (Salama *et al.* 1997, Shugrue *et al.* 1999) assemble into COPII-coated vesicles that bud from the ER membrane (Salama *et al.* 1993, Barlowe *et al.* 1994). The GTPase activity of Sec23 is responsible for uncoating the vesicles required for fusion to occur, and Sec24 is involved in the selection of the cargo molecules (Miller *et al.* 2002, Barlowe 2003). Short cytoplasmic peptide sequences (tyrosine and the diacidic or di-hydrophobic motif) of cargo proteins bind to the Sec23-Sec24 complex and thus serve as a sorting motif directing the cargo to the correct vesicles (Bannykh *et al.* 1998, Barlowe 2003 and refs therein). Transmembrane proteins of the p24 family bind to coat proteins, and they may be involved in vesicle formation and cargo selection (Barlowe 2003). In addition, lectin ERGIC-53 serves as a transport receptor for some glycoproteins in the ER to Golgi trafficking (Hauri *et al.* 2000, Barlowe 2003).

2.5.1.2 *Retrograde transport*

The main purpose of retrograde transport is to recycle the proteins and lipids needed for vesicle formation in ER and to retrieve escaped ER-resident proteins. The dilysine motif of transmembrane proteins and the KDEL sequence of soluble proteins serve as cargo selection signals directing the proteins to the correct vesicles (Pelham 1990, Cosson & Letourneur 1994, Letourneur *et al.* 1994, Teasdale & Jackson 1996, Andersson *et al.* 1999). The recruitment of COPI coat is regulated by a small GTP-binding protein, ADP-ribosylation factor (ARF) (Serafini *et al.* 1991, Donaldson *et al.* 1992a, Palmer *et al.* 1993, Lanoix *et al.* 1999, Malsam *et al.* 1999, Pepperkok *et al.* 2000). The movement of COPI-coated vesicles may be mediated by myosin motors along actin filaments, shown to facilitate retrograde trafficking (Valderrama *et al.* 2001, Durán *et al.* 2003). In favor of this, β- and γ-actin are present in COPI-coated and non-coated vesicles (Valderrama *et al.* 2000).

The involvement of COPI-coated vesicles in retrograde trafficking is not, however, generally accepted, and alternative explanations have been put forward. Membrane tubules that are translocated along microtubules by kinesin motor proteins may be involved in retrograde trafficking (Lippincott-Schwartz *et al.* 1990, Orci *et al.* 1991, Lippincott-Schwartz *et al.* 1995, Sciaky *et al.* 1997, Allan *et al.* 2002). In favor of this, a COPI-independent transport route has been shown to contribute to part of the recycling (Girod *et al.* 1999, Storrie *et al.* 2000). The COPI-independent pathway is regulated by Rab6, which binds the motor protein Rabkinesin-6 (Echard *et al.* 1998).

2.5.1.3 Molecular mechanisms of vesicle fusion

Vesicle fusion with the target membrane is thought to include two consecutive events, tethering and SNARE (soluble NSF attachment protein receptor) pairing (Chen & Scheller 2001). In the initial tethering of vesicles to the cis-Golgi membrane, the COPI-coated vesicles that contain giantin (Linstedt & Hauri 1993) are bridged to GM130 (Nakamura *et al.* 1995) in the cis-Golgi via p115 protein (Sönnichsen *et al.* 1998, Seemann *et al.* 2000b). GM130 is anchored to the Golgi by interaction with GRASP65 (Barr *et al.* 1998). The p115-independent tethering involving GM130 and GRASP65 is responsible for the inititation of COPII-coated vesicle fusion with the cis-Golgi membrane (Moyer *et al.* 2001). The tethering leads to SNARE pairing, i.e., the v-SNAREs in vesicles (GOS-28; Nagahama *et al.* 1996) and the t-SNAREs in the target membrane (Syntaxin 5; Dascher *et al.* 1994) interact, bringing the membranes sufficiently close together for membrane fusion to occur (Söllner *et al.* 1993, Hay & Scheller 1997, Weber *et al.* 1998). The N-ethylmaleimide sensitive factor (NSF) complexed with α-SNAP (Block *et al.* 1988, Beckers *et al.* 1989, Clary *et al.* 1990) then catalyzes the disassembly of SNARE complexes, enabling the SNARE proteins to be used for another cycle of docking and fusion (Söllner *et al.* 1993). Vesicle transport and fusion are regulated by GTPases. Rab1 GTPase regulates ER-Golgi trafficking by interacting with GM130 and p115 (Plutner *et al.* 1991, Tisdale *et al.* 1992, Allan *et al.* 2000, Moyer *et al.* 2001), whereas rab2 interacts with golgin-45 (Tisdale *et al.* 1992, Tisdale & Balch 1996, Short *et al.* 2001).

2.5.1.4 Role of Golgi membrane skeleton

The Golgi membrane skeleton may play an important role in protein and membrane trafficking in the ER-Golgi interspace and at TGN (Devarajan *et al.* 1997, Godi *et al.* 1998, De Matteis & Morrow 1998, Lippincott-Schwartz 1998, De Matteis & Morrow 2000). The presence of a spectrin-based matrix in pre-Golgi intermediates (Beck *et al.* 1994, Devarajan *et al.* 1996), the ability of III spectrin to bind the Arp1 of the dynein-dynactin complex (Waterman-Storer *et al.* 1995, Holleran *et al.* 2001), and dynein's ability to power the translocation of pre-Golgi intermediates (Saraste & Svensson 1991, Mizuno & Singer 1994, Presley *et al.* 1997) indicate that spectrin plays an important role in protein and membrane trafficking in the Golgi region (Lippincott-Schwartz 1998) and also in maintaining the Golgi structure (Siddhanta *et al.* 2000). Spectrin associates directly with Golgi membranes via its membrane association domains MAD1 and MAD2 (Godi *et al.* 1998, De Matteis & Morrow 2000). The binding of spectrin via the MAD2 domain to the pre-Golgi and Golgi membranes is regulated by a small GTP-binding protein ADP ribosylation factor, ARF (Godi *et al.* 1998), which increases Golgi phosphatidylinositol 4,5 bisphosphate (PtdInsP$_2$) concentrations (Godi *et al.* 1998, Godi *et al.* 1999, Siddhanta *et al.* 2000). Local rearrangement or disassembly of the spectrin-based membrane skeleton could result in membrane deformation, thus allowing the formation of secretory vesicles (Lorra & Huttner 1999). The dissociation of spectrin III from the Golgi membranes seems to be regulated by phosphorylation that has been shown

to correlate with Golgi fragmentation (Siddhanta *et al.* 2003). In addition, both Golgi ankyrin Ank_{195} and spectrin III dissociate from Golgi membranes during the mitotic breakdown of the Golgi complex (Beck *et al.* 1994, Beck *et al.* 1997).

2.5.2 Intra-Golgi transport

For protein transport within the Golgi, three models have been presented. According to the first, proteins are transported from one compartment into another by transport vesicles (COPI-coated), which bud from the dilated rims of one compartment and then fuse with the next (Palade 1975, Balch *et al.* 1984, Duden *et al.* 1991, Ostermann *et al.* 1993, Orci *et al.* 1997). In this model, Golgi enzymes are thought to be permanent residents of each corresponding compartment. The wealth of vesicles at the rims of the Golgi cisternae (Palade 1975, Ladinsky *et al.* 1999) supports this mechanism. In the cisternal progression model, the cisternae are thought to mature from the cis- to trans-Golgi through recycling of Golgi enzymes (Franke *et al.* 1971, Allan & Balch 1999, Martínez-Menárguez *et al.* 2001, Mironov *et al.* 2001). In this model, the new cisternae are proposed to be continuously formed from vesicular-tubular membrane clusters of ERGIC, and TGN is consumed during the formation of transport carriers. This model has been supported by the fact that many proteins, e.g., procollagen, are simply too large to be packaged into transport vesicles (Clermont *et al.* 1993, Becker *et al.* 1995, Bonfanti *et al.* 1998). In the third model, transient membrane tubules have been suggested to be important for protein transport through the Golgi (Weidman 1995). These three mechanisms may, however, co-exist or be cell type dependent (Mironov *et al.* 1997, Glick & Malhotra 1998).

2.5.3 Post-Golgi transport

The trans-Golgi network is the sorting station for proteins targeted to various destinations, such as lysosomes, secretory granules, or cell surface (Keller & Simons 1997, Gu *et al.* 2001). Clathrin-coated vesicles are formed at TGN and then delivered to lysosomes or endosomes (Ladinsky *et al.* 1999, Gu *et al.* 2001). Adaptor protein AP1 binds to the cytoplasmic tails of some membrane proteins and to clathrin. The transport of lysosomal enzymes from TGN to lysosomes is the best understood instance of this mechanism. These enzymes are tagged with terminal mannose-6-phosphates in the Golgi. The mannose-6-phosphates associate with the mannose-6-phosphate receptors (MPRs) bound to AP1 and ARF-recruited GGA proteins at TGN (Doray *et al.* 2002), thus directing the complex to the appropriate transport vesicles (Pearse & Robinson 1990, Ludwig *et al.* 1995). Lysosomal enzymes dissociate from mannose-6-phosphate receptors in the acidic environment of endosomes, after which MPRs are recycled to TGN and lysosomal enzymes are delivered to lysosomes (Gu *et al.* 2001).

Some plasma membrane localized proteins associate with the lipid rafts that contribute to cell surface delivery in non-polarized cells and to apical membrane delivery in polarized epithelial cells (Simons & Ikonen 1997). Lipid rafts are membrane microdomains enriched in sphingolipids and cholesterol on the outer leaflet and

phospholipids and cholesterol on the inner leaflet of the lipid bilayer. Lipid rafts can bind GPI-anchored proteins, some transmembrane glycoproteins, and acylated tyrosine kinases of the Src family (Simons & Ikonen 1997).

N-glycans and O-glycans as well as glycosylphosphatidylinositol (GPI) anchors may function as sorting signals for protein delivery to the apical membrane of polarized epithelial cells (Rodriguez-Boulan & Gonzalez 1999, Huet *et al.* 2003). Lectins that bind to sugar residues on proteins may play a role as cargo receptors (Rodriguez-Boulan & Gonzalez 1999). The well-known glycan signal is the mannose-6-phosphate signal mentioned above targeting proteins to lysosomes in clathrin-coated vesicles (Ludwig *et al.* 1991). The mannose-6-phosphate receptor, in turn, contains an acidic-cluster/dileucine signal that binds to GGAs (Puertollano *et al.* 2001). Tyrosine or Leu-Leu motifs are found in proteins destined to the basolateral membrane of polarized epithelial cells. Basolateral sorting signals seem to be dominant over apical signals (Casanova *et al.* 1991).

The secretory vesicles or larger pleiomorphic, tubular structures (post-Golgi carriers, PGCs) bud from TGN and deliver proteins to the plasma membrane along microtubules (Hirschberg *et al.* 1998, Toomre *et al.* 1999, Polishchuk *et al.* 2000). Recently, similar pleiomorphic structures that contain clathrin and AP1 were described in long-range transport of cargo (Puertollano *et al.* 2003). The exact destination of these carriers was not found. They were, however, shown to be distinct from PGCs. Dynein and kinesin power the microtubulus-directed transport between TGN and the plasma membrane (for a review, see Allan *et al.* 2002). Also, actin filaments may be involved in the Golgi to plasma membrane vesicle trafficking, since the actin-based motor protein, myosin II, is needed for membrane budding at TGN (Narula & Stow 1995, Ikonen *et al.* 1997). Another actin-based myosin I motor protein has been shown to direct vesicle transport from the Golgi to the apical membrane of polarized epithelial cells (Fath & Burgess 1993, Jacob *et al.* 2003).

3 Aims of the present work

Anion exchangers are generally thought to reside in the plasma membrane. However, accumulating evidence exists to suggest that anion exchanger proteins are also present in the membranes of intracellular organelles. Our group has previously shown that an anion exchanger protein is present in Golgi membranes. The main purpose of this work was to identify the Golgi anion exchanger isoform, to study its subcellular localization mechanism, and to clarify its role in the Golgi. The specific aims were:

1. To identify a Golgi anion exchanger isoform and to confim that it is a permanent resident of the Golgi.

2. To study the cell-type specific targeting of AE2a.

3. To study the localization and expression of Golgi AE2a with respect to Golgi membrane skeletal proteins.

4. To study the role of AE2a in maintaining the Golgi structure.

4 Materials and methods

4.1 cDNA cloning

A commercial human testis gt-11 cDNA library (5'-STRETCH) was obtained from Clontech (Palo Alto, CA). The library was screened with a ^{32}P-labeled PstI-fragment (0.4 kb) probe derived from rat AE2 cDNA. Prehybridization and hybridization were performed at 42°C in 50% formamide, 5-strength SET (125 mM Tris, 2 mM EDTA, 0.75 M NaCl, pH 8.0), 5-strength Denhardt's, 0.1% SDS, and 50 µg/ml tRNA. Positive clones were selected for plaque purification and sequencing. The missing 5' end that included a translation initiation codon was amplified by a reverse transcription polymerase chain reaction. Primers at the 5'-end were synthesized according to the human kidney AE2 sequence (Gehrig *et al.* 1992).

4.2 Preparation of GFP-AE2 construct and transfection

All three cDNA clones (T2, T3, 5'-1000) were digested with appropriate restriction enzymes and ligated to form full-length AE2 cDNA, which was then subcloned to the EcoRI site of the pGEM-4z plasmid vector. For expression in eukaryotic cells, the full-length AE2 cDNA was inserted into the pE-GFP-C1 (Clontech, Palo Alto, CA) vector by using HindIII and EcoRI restriction enzymes and DNA ligase (Boehringer Mannheim). A 5'-HindIII site was created by using the Quick change mutagenesis kit (Stratagene; La Jolla, CA). The cDNA (1-2 µg) coding for the GFP-AE2 fusion protein was transfected to cells plated one day earlier by using the FUGENE-6 transfection reagent (Boehringer Mannheim). Transfected cells were examined 20-24 hrs after transfection.

4.3 Antibodies

A polyclonal antibody against the AE2 C-terminal peptide (amino acids 1229-1241 of AE2) was used that had been prepared in rabbits and affinity-purified as described earlier (Parkkila *et al.* 1993). The antibody was used to detect endogenous AE2 in several cell lines (COS7, CHO-K1, HeLa, MDBK, MDCK, and NRK). The antibodies against KDEL receptor, GM130, and giantin were used as Golgi markers. The anti-KDEL receptor was from Stressgen (Victoria, Canada), and anti-GM130 was from Transduction Laboratories (Lexington, KY, USA). The monoclonal antibody against giantin was a kind gift from Dr. Hans-Peter Hauri (Basel, Scwitzerland). Alexa fluor-conjugated secondary antibodies (wavelengths 488 nm or 594 nm) were purchased from Molecular Probes Inc. (Eugene, Oregon). The monoclonal antibody against GFP was used in immunoblotting studies, and it was obtained from Clontech. The monoclonal anti-α-tubulin antibody was used for the staining of microtubules, and it was obtained from Sigma (St. Louis, MO, USA). The anti-Ank_{195} antibody was from Dr. Kenneth Beck (University of California, Davis, CA). The peroxidase-conjugated secondary anti-mouse and anti-rabbit antibodies (Fab fragments) used in immunoblottings and immunoelectron microscopy were from P.A.R.I.S. (Compiegne, France).

4.4 Cell culture

Each cell line (COS7, CHO-K1, HeLa, MDBK, MDCK, and NRK) was cultured in the media and the supplements recommended by ATCC (Rockville, MD). The media, fetal calf serum, 1M Hepes, penicillin-streptomycin, and non-essential amino acids were obtained from Gibco (Grand Island, NY). Human embryonic skin fibroblasts were grown in MEM with glutamax supplemented with 10% newborn calf serum, penicillin-streptomycin (Gibco), and 50 µg/ml ascorbic acid. Cells were grown on Petri dishes or on glass coverslips in 5% CO_2.

4.5 Drug treatments

The cells were treated with cycloheximide (15 µg/ml) for 0, 3, or 5 hours. For brefeldin A treatment, the cells were incubated in the presence of the drug (5 µg/ml) for 0, 15, or 30 min. Nocodazole (20 µg/ml) was added directly to the culture medium 4 hours before fixation. Latrunculin B was added to the culture medium (0.2 µg/ml) 15 minutes before fixation. Tunicamycin was added directly to the culture medium (1 µg/ml) and the cells were cultured for an additional 16 hours in the presence of the drug.

4.6 Indirect immunofluorescence

Cells grown on glass coverslips were washed 2-3 times with PBS and fixed with 3.7% formaldehyde in PBS for 20 minutes at room temperature. After rinsing 2-3 times with PBS, the cells were permeabilized with 0.1% saponin/0.1% BSA in PBS for 1 hour at RT. The incubations with primary and secondary antibodies were carried out in PBS with 0.1% BSA and 0.1% saponin for 1 hour at RT. The sample slides were mounted with Immu-Mount (Shandon) and examined under an epifluorescence microscope (Olympus BX-60).

4.7 Triton X-100 extraction

COS-7 and HeLa cells were subjected to detergent extraction as follows. Cells cultured on glass coverslips were washed 2-3 times with PBS and cooled on ice for 5 minutes. The cells were then incubated with 1% Triton X-100 in TKM buffer (20 mM Tris, 10 mM KCl, 1m M $MgCl_2$, pH6.5 or pH7.4) for 30 minutes on ice. In GFP-AE2 transfected cells, the pH of the TKM buffer was adjusted to pH6.5, because it reduced the background of diffuse GFP fluorescence that appeared after the Triton X-100 extraction. In immunoblottings and in quantitative experiments, pH7.4 TKM buffer was used. After detergent extraction, the cells were rinsed with PBS and fixed. In some experiments, the cells were stained with primary and secondary antibodies prior to microscopy. In control experiments, the cells were fixed before the detergent extraction.

4.8 Immunoblotting

Immunoblotting of endogenous AE2 protein was performed using native PAGE gels, because the epitope recognized by the AE2 antibody is SDS-sensitive (Alper *et al.* 1997). The cells were washed 2-3 times with PBS and lysed directly into ice-cold lysis buffer (50 mM Tris, 150 mM NaCl, 1% Nonidet P-40, pH8.0) supplemented with protease inhibitors (Mini protease inhibitor tablets, Roche Diagnostics) (Papageorgiou *et al.* 2001). After removal of insoluble material by centrifugation, 2 x PAGE sample buffer was added, and the samples were heated for 3 minutes at 70°C before separation by polyacrylamide gel electrophoresis. Size-separated samples were transferred onto a nitrocellulose filter and immunoblotted with anti-AE2 antibody.

Immunoblotting of Ank_{195} was performed as above, but in the presence of SDS and 100 mM DTT. In addition, 1% Triton X-100 was used instead of 1% Nonidet P-40. The cells were treated with 1% Triton X-100 for 30 minutes, the extract was transferred into an Eppendorf tube, and 2 x SDS-PAGE sample buffer was added. The Triton X-100 extracted cells on the dish were then lysed in 2 x SDS-PAGE sample buffer. Both the Triton X-100 extracts and the cell lysates were subjected to SDS-PAGE and immunoblotting with anti-Ank_{195} antibody.

For immunoblotting of the GFP-AE2 fusion protein, the cells were washed 2-3 times with PBS and lysed directly with 2 x SDS-PAGE sample buffer supplemented with protease inhibitors and 100 mM DTT. The samples were agitated in an Eppendorf shaker for two hours to reduce viscosity and heated for three minutes at 94°C before subjecting to SDS-PAGE. Size-separated samples were transferred onto a nitrocellulose filter and immunoblotted with anti-GFP antibody.

4.9 Antisense oligonucleotide treatment

Phosphorothioate antisense oligonucleotides corresponding to the translational initiation site of AE2 mRNA (5'-gcGCTGCTCATGGCCGAATCTtag-3') were obtained from Cybergene (Huddinge, Sweden). The COS-7 cells were plated one day before the antisense oligonucleotide treatment. The culture medium was then replaced with a serum-free medium, and antisense or sense oligonucleotides were added directly to the cells at a final concentration of 25 µg/ml. After 4-6 hours of incubation, serum was added and the cells were cultured for an additional 2-3 days.

4.10 Electron microscopy

The cells grown on Petri dishes were washed twice with 0.1 M Na-phosphate buffer (pH7.4) (Na-P) at RT. The cells were then fixed on plates with 2.5% glutaraldehyde for 2 hours at room temperature. After rinsing, the cells were post-fixed on the dishes with 1% osmium tetroxide (in H_2O) before dehydration in ethanol and embeddening in ethanol-Epon LX112 and finally with Epon only. Thin sections were cut and post-stained with uranyl acetate and lead citrate. The specimens were examined using a Philips CM100 transmission electron microscope and photographed with a CCD camera.

Peroxidase staining was performed as described earlier (Kellokumpu *et al.* 1997). Briefly, cells grown on Petri dishes were rinsed with 0.1 M Na-P and fixed on plates with 4% paraformaldehyde (in 0.1 M Na-P) for 60 min at 20°C. After blocking (1% BSA in Na-P), the cells were stained with the primary antibodies (anti-GFP) followed by peroxidase-conjugated secondary antibodies. All antibody incubations were done in the presence of 0.1% saponin and 0.1% BSA. After post-fixing with 2.5% glutaraldehyde (in Na-P buffer), diaminobenzidine (0.5 mg/ml) and H_2O_2 were added and the peroxidase reaction was allowed to proceed for 15–30 min on ice. Epon embedding on plates and thin sectioning were performed using conventional procedures.

In the labeling of cells with protein A-coated gold particles (5 and 10 nm), the cells were fixed with 4% paraformaldehyde in PBS, scraped from the culture plates, embedded in 4% gelatin, and snap-frozen in liquid nitrogen in the presence of 2.3 M saccharose. Thin sections (80-100 nm) were cut and immunostained with appropriate primary antibodies, secondary rabbit anti-mouse antibodies, and finally with colloidal gold coated with protein A.

5 Results

5.1 Cloning and sequencing of AE2 (I)

The human testis cDNA library screening produced three independent AE2 cDNA clones (T1, T2, T3). Two overlapping clones (T2, T3) were chosen for further analysis and sequencing. These clones were shown to be almost identical to the previously published AE2 mRNA sequence (Gehrig *et al.* 1992, Medina *et al.* 1997), but a sequence of about 600 bp at the 5'-end, including the translation initiation codon, was missing. The missing 5'-end and a 379 bp region overlapping T2 were amplified by using RT-PCR. The amplification product (954 bp) was ligated together with T2 and T3 cDNA clones to form the full-length AE2 cDNA sequence (I, Figure 1), which was then subcloned to the EcoRI-site of the pGEM-4z vector and thoroughly sequenced. For eukaryotic cell expression and visualization, the AE2 cDNA sequence was inserted into the p-EGFP-C1 vector. The N-terminal GFP tag has been recently shown not to alter the targeting or chloride transport properties of AE1 (Beckmann *et al.* 2002). When compared to the AE2 sequence obtained from the genomic clones, six nucleotide disparities were found that did not cause amino acid changes (A instead of C in position 285 and so forth: A^{285}- C^{285}; T^{486}- C^{486}; C^{936}- T^{936}; T^{1071}- C^{1071}; T^{1464}- C^{1464}; G^{2202}- A^{2202}), and four nucleotide disparities (G instead of A in position 470 and so forth: G^{470} - A^{470}; C^{1090} - T^{1090}; C^{1455} - G^{1455}; G^{1456} - C^{1456}) that caused amino acid changes (Q instead of R in position R and so forth: Q^{157} - R^{157}; Y^{364} - H^{364}; E^{485} - D^{485}; L^{486} - V^{486}, respectively). Three of the amino acid changes were found to be identical in other mammalian species (Q^{157}, D^{485}, and L^{486}). Site-directed mutagenesis of tyrosine Y^{364} to histidine H^{364} did not alter the intracellular localization of GFP-tagged AE2 (unpublished observation).

5.2 Cell type dependent targeting of AE2 (II, III, IV)

The anti-AE2 C-terminal antibody and the antibodies against the Golgi markers GM130 (Nakamura *et al.* 1995), KDEL receptor (Kelly 1990), and giantin (Linstedt & Hauri

1993) were used to study the subcellular localization of AE2 in different cell lines. In HeLa and MDCK cells, the anti-AE2 antibody stained the lateral borders and the whole cell surface indicating that AE2 is targeted to the cell surface in these cells (III, Figures 1 and 8). The anion exchanger AE2 has been generally known as a plasma membrane protein and it is directed to the basolateral membrane of polarized epithelial cells (Alper *et al.* 1994). The cell density of HeLa cells had no profound effect on the cell surface localization of AE2.

Immunological evidence for Golgi localization of AE2 also exists (Kellokumpu *et al.* 1988, Alper *et al.* 1997, Stuart-Tilley *et al.* 1998). In our studies, the anion exchanger AE2 was shown to be localized to the Golgi in several cell lines. These included F89, ROS, NRK, CHO-K1, COS-7, and MDBK cells (III, Figures 1 and 8). Additional support for Golgi localization came from drug treatment studies. Brefeldin A blocks the ER to Golgi trafficking and results in rapid tubulation and redistribution of the Golgi enzymes into ER (Klausner *et al.* 1992). However, the trans-Golgi network and its resident proteins have been shown to be separated from the Golgi stacks and to merge with the components of the endocytic pathway upon brefeldin A treatment (Nebenführ *et al.* 2002). At the molecular level, brefeldin A prevents COPI vesicle formation by inhibiting nucleotide exchange of the ADP-ribosylation factor (ARF, Helms & Rothman 1992) that recruits the coat proteins (Donaldson *et al.* 1992b). This may lead to inability of ER-Golgi transport vesicles (COPII coated) to fuse with the altered Golgi cisternae (Nebenführ *et al.* 2002) thus preventing ER-Golgi trafficking. Alternatively, or perhaps additionally, extensive tubulation of Golgi membranes results in increased retrograde trafficking (Lippincott-Schwartz *et al.* 1990). In brefeldin A-treated cells, the AE2 protein was shown to be redistributed to the endoplasmic reticulum (II, Figure 3). Instead, the peripheral Golgi protein Ank_{195} has been shown to dissociate from Golgi membranes in brefeldin-A treated cells. Ank_{195} was distributed throughout the cytoplasm with a loss of co-localization with the medial-Golgi marker mannosidase II (Beck *et al.* 1997). Furthermore, cycloheximide treatment was used to study whether AE2 is in transit toward the cell surface. Cycloheximide inhibits protein synthesis but it does not prevent transport to the cell surface. The cells were incubated in the presence of cycloheximide and stained with the anti-AE2 antibody. The distribution of AE2 was not affected by the treatment, i.e., AE2 remained in the Golgi, and no plasma membrane staining was evident after 5 hours of incubation. The result indicates that AE2 is a permanent resident of this organelle (II, Figure 4).

5.2.1 Identification of Golgi-associated anion exchanger as AE2a

Anion exchangers share significant homology in the C-terminal transmembrane domain, but the N-terminus is highly variable (Brosius III *et al.* 1989, Kudrycki & Shull 1989, Linn *et al.* 1992, Kollert-Jöns *et al.* 1993, Cox & Cox 1995, Cox *et al.* 1996). The anti-AE2 C-terminal antibody also recognizes, at least to some extent, the AE1 isoform of the anion exchanger gene family. Therefore, the immunofluorescence staining by the C-terminal anti-AE2 antibody could not be directly interpreted as AE2 staining, and we therefore used the anti-AE2 N-terminal antibody to confirm that the isoform detected was

AE2. Similar Golgi-like staining was observed by using the N-terminal antibody (II, Figure 2). Important evidence for the specificity of anti-AE2 antibody staining was obtained by using AE2-specific, 5'-end antisense oligonucleotides to reduce AE2 expression in COS-7 cells. The antisense oligonucleotides used were designed so that they hybridize only with the mRNA of the full-length AE2 protein (AE2a variant). The expression of AE2 was shown to be decreased by 77%, indicating that the isoform expressed in these cells is AE2a (IV, Figure 1). The AE2 expression levels were measured based on fluorescence intensities in immunofluorescence stainings.

The mRNA analysis was done using northern blotting and RT-PCR. The rat osteosarcoma cells (ROS) and human skin fibroblasts (F89) that showed prominent Golgi staining by C-terminal anti-AE2 antibody were shown to express the AE2 mRNA of about 4.4 kb that corresponds to the full-length isoform of AE2 (II, Figure 5). The probe used in Northern blotting experiments corresponds to a portion in the C-terminal transmembrane domain and should thus detect other AE2 variants if present. In RT-PCR, four overlapping AE2 cDNA fragments were amplified that were found to be identical in size regardless of whether they originated from F89, human testis tissue, or the AE2a plasmid construct (II, Figure 5).

The plasmid construct encoding the N-terminally GFP-tagged AE2a protein was transfected into cells by using the FUGENE-6 transfection reagent. Similarly to endogenous AE2, the GFP-AE2a fusion protein was localized to the Golgi in COS-7 (II, Figure 6 and III, Figure 2) and CHO-K1 cells (III, Figure 8), whereas HeLa (III, Figure 2) and MDCK cells (III, Figure 8) showed prominent plasma membrane localization of the GFP-AE2a fusion protein. Thus, the targeting of the AE2 protein was not dependent on the variant expressed in these cell lines. Instead, the full-length AE2a variant was localized in a cell type-dependent manner. The Golgi localization of GFP-AE2 was confirmed by double staining with the Golgi markers giantin and -COP (II, Figure 6). The GFP-AE2 co-localized with marker proteins within the Golgi region, but not with the vesicle coat protein -COP. The nocodazole treatment was also indicative of Golgi localization of GFP-AE2. Nocodazole severes microtubule polymerization leading to dispersal of the Golgi to peripheral sites (Cole *et al.* 1996, Yang & Storrie 1998). In nocodazole-treated cells, the GFP-AE2 fusion protein was found in small punctate structures scattered throughout the cytosol (II, Figure 6).

5.2.2 Post-translational modification of AE2a

The two cell lines, COS-7 and HeLa, that showed differential localization of AE2 protein were chosen for further analysis. The molecular weights of the endogenous AE2 proteins in these cells were compared. The Golgi epitope recognized by the anti-AE2 antibody is SDS-sensitive (Alper *et al.* 1997, Stuart-Tilley *et al.* 1998). Therefore, we used native PAGE gels in immunoblotting experiments. In both cell lines, one major band was detected, and the protein was shown to be of identical molecular weight, indicating that AE2 is similarly processed in the secretory pathway of COS-7 and HeLa cells (III, Figure 3).

Also, the GFP-AE2 fusion proteins expressed in COS-7 and HeLa cells were of identical molecular size in the two cell lines as assessed by SDS-PAGE and Western blotting (III, Figure 3). They also showed a similar glycosylation pattern as evidenced by site-directed mutagenesis. The three potential N-glycosylation sites of AE2 were sequentially mutated (N859Q, N868Q, N882Q) to glutamines. A single mutation (N859Q) did not alter the molecular size or the localization of the GFP-tagged AE2 protein. The double mutation (N859+868Q) reduced the molecular size of the GFP-tagged AE2 identically in COS-7 and HeLa cells. The triple mutant protein (N859+868+882Q) migrated as a sharp band in SDS-PAGE gel, and it was of the same molecular size in both COS-7 and HeLa cells. Both the double and triple mutant proteins were localized to the endoplasmic reticulum. We concluded that the last two N-glycosylation sites are occupied, and that N-glycosylation seems to be needed for proper folding of the protein and for protein transport out of the ER. The two potential tyrosine phosphorylation sites were similarly eliminated, and this did not affect the localization of the fusion protein in either of the two cell lines studied. In addition, the GFP-AE2 fusion protein seemed to form oligomers of identical size in both cell lines, as evidenced by immunoblotting, indicating that oligomerization state is not a determining factor in differential targeting of the fusion protein. From these studies, we could conclude that there are no major differences in post-translational modification between COS-7 and HeLa cells that might explain the differential targeting of AE2a.

5.3 Golgi AE2 and Golgi membrane skeleton (II, III)

5.3.1 Detergent extraction

Anion exchanger 1 in the plasma membrane of red blood cells resists detergent extraction in cold. This is known to be due to its association with the plasma membrane skeleton (Yi *et al.* 1997). The plasma membrane ankyrin is a 220-kDa adapter protein that links the spectrin-based cytoskeletal network to integral plasma membrane proteins, including AE1 (Bennett & Stenbuck 1980, Drenckhahn *et al.* 1985, Michaely & Bennett 1995). Our detergent extraction studies of the Golgi-associated, overexpressed, and GFP-tagged AE2 protein showed that it is also mainly insoluble in 1% Triton X-100 (II, Figure 8 and III, Figure 4). Instead, the plasma membrane-localized GFP-AE2 of HeLa cells was completely solubilized with the detergent (III, Figure 4). The detergent insolubility and Golgi localization of AE2a led us to hypothesize that the protein might associate with the Golgi membrane skeleton, possibly via another detergent-insoluble Golgi-associated protein, ankyrin Ank_{195} (Beck *et al.* 1997). Ank_{195} is a 195 kDa protein, suggesting that it may include the ankyrin repeat motif required for anion exchanger binding (Beck *et al.* 1997). Instead, another Golgi ankyrin, AnkG119, lacks this binding site and is therefore Triton X-100-soluble (Devarajan *et al.* 1996).

5.3.2 Co-localization and correlation of the expression of AE2 and Ank₁₉₅

Cells transfected with the plasmid construct coding for GFP-tagged AE2 were treated with 1% Triton X-100 to remove the detergent-soluble ankyrin isoforms also recognized by the anti-Ank$_{195}$ antibody (Beck *et al.* 1997). The transfected and detergent-extracted cells were immunostained with anti-Ank$_{195}$ antibody. Both GFP-AE2 and Ank$_{195}$ resisted detergent extraction and remained in the Golgi region of COS-7 cells (III, Figure 9). The localization of these proteins was shown to be highly overlapping. In addition, the Ank$_{195}$ protein showed more concentrated and compact localization in the cells that expressed the GFP-AE2 fusion protein. However, in the cells that expressed the fusion protein at high levels as assessed by fluorescence intensity, the Golgi was fragmented. Consequently, Ank$_{195}$ was more widely distributed, again co-localizing with the GFP-AE2 fusion protein. This indicated a strong correlation between GFP-AE2 and Ank$_{195}$ localization. In COS-7 cells, localization of the GFP-AE2 fusion protein at the plasma membrane was sometimes seen, suggesting that the presumed ankyrin-dependent association sites on the Golgi can be saturated. Plasma membrane localization has also been detected previously with other TGN proteins upon overexpression (Humphrey *et al.* 1993).

We found a correlation between Ank$_{195}$ and AE2 or GFP-AE2 expression in various cell lines in which AE2 was localized to the Golgi (COS-7, NRK, CHO-K1, and MDBK), but not in cells where AE2 was localized to the cell surface (HeLa, MDCK). Accordingly, in immunoblots of total cell lysates derived from these cell lines, the anti-Ank$_{195}$ antibody detected a 193 kDa, Triton-insoluble protein band in COS-7, NRK, CHO-K1, and MDBK cells, but this band was absent in HeLa or MDCK cells (III, Figures 6 and 7).

5.4 Anion exchanger AE2 has a structural role in the Golgi (IV)

5.4.1 Antisense treatments

The structural role of AE2 in the Golgi was studied using antisense oligonucleotides. AE2 mRNA-specific antisense oligonucleotides were used to deplete or reduce the expression of AE2 in COS-7 cells. The average reduction of the anion exchanger 2 expression levels was found to be 77% of that in control cells measured by fluorescence intensity (IV, Figure 1). In AE2-depleted cells, the Golgi complex was dispersed into small punctate structures that were scattered around the nucleus as assessed by anti-GM130 and anti-KDEL receptor antibody staining (IV, Figure 1). Electron microscopy showed the Golgi stacks to be less organized and generally smaller than in control cells. They also appeared to reside further apart from each other. The cisternae were swollen and often fenestrated (IV, Figure 2). Thus, the AE2 protein seems to be a component essential for the structural integrity of the Golgi complex. The reduction in AE2 expression was, however, not evenly distributed, and there were cells that had normal levels of expression according to

the fluorescence intensity of antibody staining. The Golgi in these cells appeared similar to that in the control cells. Statistical analyses revealed an inverse correlation (r=-0.60) between the level of AE2 expression and Golgi fragmentation. The fluorescence intensities and the maximal distance between the Golgi fragments were measured using the SIS AnalySIS software (Olympus).

Ank$_{195}$ remained associated with the Golgi membranes in antisense-treated cells as assessed by anti-Ank$_{195}$ antibody staining (IV, Figure 3). This is most probably due to its association with the Golgi spectrin, which has other association sites within the Golgi membranes. For example, it binds to Golgi membrane lipids via its membrane association domains (MAD).

5.4.2 GFP-AE2 overexpression

Dispersion of the Golgi complex was evident in cells that expressed large amounts of GFP-AE2 fusion protein (estimated by fluorescence intensity). Anti-GM130 antibody staining showed numerous, vesicular-like structures in the perinuclear cytoplasm of highly overexpressing cells (IV, Figure 5). Electron microscopy analysis showed accumulation of large, interconnected, tightly packed, lamellar or tubular membrane structures in transfected cells (IV, Figure 5). That these membraneous aggregates represented, at least partly, Golgi membranes was shown by double immunogold labeling with antibodies to the KDEL receptor or GFP (IV, Figure 5). In cells that expressed the GFP-AE2 fusion protein at a low level, the Golgi remained unaltered. Similarly, the GFP-tagged, N-terminally truncated version of the AE2 protein that lacks the ankyrin-binding site did not alter Golgi structure (IV, Figure 5).

5.4.3 Role of microtubules

Microtubules are needed for correct Golgi organization and positioning. Nocodazole is a drug that severs microtubule polymerization, and thus, in cells treated with nocodazole, the Golgi complex is scattered into "mini-stacks" throughout the cytosol (Cole *et al.* 1996, Yang & Storrie 1998). Spectrin has been suggested to provide a link between the Golgi membranes and the microtubule network (Waterman-Storer *et al.* 1995, Holleran *et al.* 2001). In addition, erythrocyte ankyrin has been shown to directly associate with tubulin (Davis & Bennett 1984, Davis *et al.* 1991). Therefore, we studied the microtubule organization in AE2 antisense-treated cells. The antisense-treated cells were stained with antibodies to -tubulin and AE2. Distinct disorganization of microtubules and a lack of discrete microtubule organization center (MTOC) was seen in the antisense-treated cells (IV, Figure 4). Microtubules seemed to emanate from multiple points along the nuclear envelope. Similar redistribution of microtubules was also found in AE2-overexpressing cells stained with antibodies to -tubulin (IV, Figure 6).

6 Discussion

6.1 Golgi-associated AE2a

Anion exchangers have generally been regarded as plasma membrane localized proteins. However, some evidence for intracellular localization also exists (Kellokumpu *et al.* 1988, Alper *et al.* 1997, Stuart-Tilley *et al.* 1998). Golgi-like staining of epithelial cells of the inner medullary collecting duct and medullary thick ascending limb cells of the kidney has been detected by using a polyclonal anti-AE2 antibody (Alper *et al.* 1997, Stuart-Tilley *et al.* 1998). Furthermore, the plasma membrane localized chicken AE1 variants have been shown to recycle to the Golgi (Adair-Kirk *et al.* 1999). During the recycling, the AE1 variants acquire complex N-linked sugars, the reason for which is not known (Adair-Kirk *et al.* 1999). In addition, an AE1-related polypeptide (45 kDa) has been detected in mitochondrial membranes (Ostedgaard *et al.* 1991). The present study was based upon initial work by Kellokumpu and co-authors (1988), showing that a polypeptide immunologically related to the erythrocyte AE1 (band 3) is present in Golgi membranes both *in vivo* and *in vitro*. In this study, we gathered several lines of evidence to show that the isoform localized to the Golgi is the AE2a variant. Our data thus provides further evidence for the intracellular localization of anion exchangers. We cannot, however, exclude the possibility that AE2 cycles between the plasma membrane and the Golgi, although we rarely detected AE2 in the plasma membrane in COS-7 cells. This was only evident in GFP-AE2-overexpressing cells, which is in accordance with the previous findings showing that overexpression of TGN-localized protein may lead to plasma membrane localization (Humphrey *et al.* 1993).

6.2 Cell-type specific targeting of AE2a

Anion exchangers show substantial tissue and cell-type specificity. Generally, this specificity has been thought to be related to structurally different anion exchanger variants. For example, the full-length AE1e variant is mainly expressed in red blood cells,

whereas N-terminally truncated AE1k is expressed in the kidneys. Thus, our study is the first to show that cell-type specificity is not due to the specific structure of the anion exchanger protein, since the same full-length AE2 variant, i.e., AE2a, was differentially targeted in a number of cell lines. The Golgi targeting of the AE2a variant was found in COS-7, NRK, CHO-K1, MDBK, F89, and ROS cells, whereas in HeLa and MDCK cells, the protein was localized mainly to the plasma membrane. Conclusions were based on immunostainings (all cell lines), GFP-AE2 expressions (COS-7, HeLa, CHO-K1, MDBK, and MDCK), mRNA analysis (F89, ROS), RT-PCR analysis (F89), and immunoblottings of endogenous AE2 (COS-7, HeLa, NRK) or GFP-AE2 fusion protein (COS-7, HeLa, CHO-K1, MDBK, MDCK). The full-length AE2 variant has been considered a 'house-keeping' protein expressed in every tissue and cell types. Our results further confirmed this assumption, showing AE2 to be ubiquitously expressed in every cell line studied, although its cellular localization was variable.

Although the AE2a variant is most likely to be the predominant form, we cannot exclude the possibility that other AE2 variants or AE isoforms are expressed in at least some of the cell lines studied. The human AE2b(1) and AE2b(2) variants are known to be expressed in liver and kidney (Medina *et al.* 2000). The corresponding rodent variants are found mainly in stomach, but also in kidney and other tissues (Wang *et al.* 1996, Lecanda *et al.* 2000). The rodent AE2c2 variant is ubiquitously expressed whereas AE2c1 is present only in stomach (Stuart-Tilley *et al.* 1998, Lecanda *et al.* 2000). The expression of these variants has not been studied in tissues from which CHO-K1 (ovary), HeLa (cervix), F89 (skin), and ROS (bone) cells have been isolated. Instead, the COS-7 (monkey), NRK (rat), MDBK (bovine), and MDCK (canine) cells are all kidney cells and may express the known kidney AE2b and AE2c variants or the kidney AE1k protein. From our results, however, we can conclude that, in COS-7 and NRK cells, most of the AE2 protein present is a full-length variant since the Golgi staining was significantly reduced or eliminated by using AE2a-specific antisense oligonucleotides. In mRNA analysis of F89 and ROS cells, a single band (4.4 kb) corresponding to AE2 mRNA was detected, indicating that other variants (of about 4.2 kb and 3.8 kb) are either absent or only present in minute amounts.

6.3 AE2 and Golgi membrane skeleton

We were able to exclude the possibility that major post-translational modifications determine the cell-type specific targeting of AE2. These included such modifications as N-glycosylation and tyrosine phosphorylation, which are required for the targeting of some proteins to the cell surface (Rodriguez-Boulan & Gonzalez 1999, Huet *et al.* 2003). Our results indicated that N-glycosylation of the AE2 protein may be needed for its correct folding and exit from the endoplasmic reticulum. In addition, unglycosylated AE2 seemed to be more easily degraded. Similarly, oligomerization, which is a potential Golgi retention mechanism (Machamer 1991), could be ruled out as a main sorting mechanism of AE2. We therefore suggest that AE2 might be anchored to Golgi membranes via its association with the Golgi membrane skeleton. Two known candidates for mediating this association are the cis-Golgi ankyrin AnkG119 (Devarajan *et al.* 1996) and the trans-

Golgi/TGN-localized Ank$_{195}$ (Beck *et al.* 1997). Both of these have been shown to associate with Golgi III spectrin, but only Ank$_{195}$ is known to be Triton X-100 insoluble. The Triton X-100 insolubility of a protein is often due to its oligomerization or cytoskeletal association (Sheetz & Sawyer 1978). In addition, ankyrin G119 lacks a large part of the 33-residue ankyrin repeat structure (Devarajan *et al.* 1996) found in other ankyrin G isoforms, which is needed for binding to the anion exchanger protein (Davis *et al.* 1991, Michaely & Bennett 1995). It has been suggested that AnkG119 together with III spectrin form a vesicle coat that contributes to vesicle formation and transport at the ER-Golgi interface (Devarajan *et al.* 1997, Godi *et al.* 1998). Accordingly, AnkG119 and III spectrin have been localized to the pre-Golgi intermediate compartment and the cis-Golgi (Beck *et al.* 1994, Devarajan *et al.* 1996). Thus, the Triton X-100 insolubility and the highly overlapping co-localization, the correlation of expression and the co-redistribution properties of AE2 and Ank$_{195}$ suggested that they are mutually associated. Unfortunately, we were unable to directly assess the association between Ank$_{195}$ and AE2, since both the anti-AE2 antibodies and the anti-Ank$_{195}$ antiserum proved to be quite inefficient in immunoprecipitation. The possibility to address this potential interaction was further complicated by the fact that GFP-AE2 and Ank$_{195}$ have the same molecular size, and that the Ank$_{195}$ protein sequence is not known.

Our data is in good accordance with the Golgi mesh hypothesis and the studies made by Beck and co-authors (1994) showing that III spectrin is important for the Golgi structure. Likewise, in AE2 antisense-treated cells, the Golgi structure was severely altered, showing that AE2 is needed for the maintenance of the Golgi structure. It is tempting to speculate that the AE2/Ank$_{195}$ complex could represent a major attachment site for III spectrin, and that the lack of this site might lead to partial dissociation of the spectrin meshwork from the Golgi membranes. The local dissociation of the Golgi membrane skeleton, in turn, could result in dispersion of the Golgi complex (Beck & Nelson 1998). The Golgi spectrin is also known to associate directly with membrane lipids (Godi *et al.* 1998, De Matteis & Morrow 2000), and this might explain why Ank$_{195}$ (likely in association with the spectrin network) remained in the close proximity of the Golgi membranes in AE2 antisense-treated cells. One possibility is that the spectrin-actin network remains associated with the Golgi membranes via some additional interactions, such as the protein 4.1 found in PM of red blood cells (Alloisio *et al.* 1993, Marfatia *et al.* 1994), although its presence has not been demonstrated in the Golgi.

6.4 Functional significance of AE2 in the Golgi

Anion exchanger 2 may not merely be a structural protein of the Golgi, but it may also be involved in the regulation of Golgi pH. Acidic Golgi pH is known to be attained by the activity of vacuolar H^+-ATPase (Moriyama & Nelson 1989, Demaurex 2002). The Cl^-/HCO_3^- exchanger might provide counterions to maintain the electroneutrality of the Golgi lumen. In fact, Cl^- depletion has been reported to dissipate the acidic pH of the Golgi (Llopis *et al.* 1998). Recently, however, the rate of proton pumping has been shown to be independent of counterion permeability (Schapiro & Grinstein 2000, Demaurex 2002). The role of AE2 in the regulation of pH and pH-dependent processes, such as

sorting (Caplan *et al.* 1987, Carnell & Moore 1994, Henkel *et al.* 2000) and protein glycosylation (Varki 1998, Axelsson *et al.* 2001), are interesting issues to be studied in the future. Recently, it has been shown that drugs that dissipate the pH gradient in the Golgi induce similar morphological changes as those detected in AE2-depleted cells (Kellokumpu *et al.* 2002).

7 Conclusions

We have identified a Golgi-associated anion exchanger as a full-length variant of AE2 and shown it to be a permanent resident of the Golgi. The intracellular targeting of AE2 was shown to be cell type dependent, since the same AE2 variant was expressed mainly in the plasma membrane in some cell lines. Anion exchangers have been previously considered to be proteins localized in the plasma membrane. Our study, however, shows that anion exchangers are also localized in intracellular organelles. The Golgi anion exchanger might be involved in the regulation of lumenal pH together with the vacuolar type H^+-ATPases that acidify the lumen of organelles. Acidic environment is crucial for protein trafficking in the secretory pathway and for the modification of proteins in the Golgi.

The AE2 protein may play a role in the assembly or maintenance of the Golgi membrane skeleton. The assembled Golgi membrane skeleton and the associated microtubules are essential for Golgi positioning and structure. The lack of AE2 in the Golgi may cause the partial dissociation of the Golgi membrane skeleton from the Golgi membranes, leading to Golgi dispersal. Alternatively or additionally, AE2 may affect the Golgi organization indirectly by controlling the pH of the Golgi lumen, and altered pH could cause the impairment of the pH-dependent vesicular trafficking processes, eventually leading to Golgi dispersal.

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