The AE2 anion exchanger is necessary for the structural integrity of the Golgi apparatus in mammalian cells

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Abstract The structural integrity of the Golgi apparatus is known to be dependent on multiple factors, including the organizational status of microtubules, actin and the ankyrin/spectrin-based Golgi membrane skeleton, as well as vesicular trafficking and pH homeostasis. In this respect, our recently identified Golgi-associated anion exchanger, AE2, may also be of importance, since it potentially acts as a Golgi pH regulator and as a novel membrane anchor for the spectrin-based Golgi membrane skeleton. Here, we show that inhibition (>75%) of AE2 expression by antisense oligonucleotides in COS-7 cells results in the fragmentation of the juxtanuclear Golgi apparatus and in structural disorganization of the Golgi stacks, the cisternae becoming generally shorter, distorted, vesiculated and/or swollen. These structural changes occurred without apparent dissociation of the Golgi membrane skeletal protein Ankyrin$_{195}$, but were accompanied by the disappearance of the well-focused microtubule-organizing center (MTOC), suggesting the involvement of microtubule reorganization. Similar changes in Golgi structure and assembly of the MTOC were also observed upon transient overexpression of the EGFP-AE2 fusion protein. These data implicate a clear structural role for the AE2 protein in the Golgi and in its cytological positioning around the MTOC.

Key words: Golgi apparatus; Anion exchange; pH; Cytoskeleton; Spectrin; Microtubule

1. Introduction

The Golgi apparatus is a highly organized, dynamic organelle that is specialized in the processing of newly synthesized glycoproteins and -lipids, and in their delivery to the correct intracellular or extracellular destinations. Over the last decade major progress has been made in understanding the molecular mechanisms that regulate Golgi architecture and cisternal stacking, including vesicle tethering proteins (giantin, p115, GM130, rab1, rab2), docking and fusion proteins (GOS28, syntaxin5, NSF and SNAPS, p47, p97) and the stacking proteins GRASP65 and GRASP55 [1–4]. The assembly/disassembly cycles of the cisternae mediated by these proteins during mitotic Golgi breakdown are regulated by phosphorylation [5,6]. Other proteins/cytoskeletal elements that associate directly, or indirectly, with the above Golgi matrix (or exoskeletal) proteins include medial Golgi enzymes [7], members of the p24 cargo receptor family [8], microtubules, associated molecular motors, and the dynactin complex [9–13], as well as actin [14,15], myosins [16,17], and the ankyrin/spectrin-based Golgi membrane skeleton [18–22].

The Golgi membrane-associated membrane skeleton is known to consist of at least two distinct ankyrin isoforms, ANKG$_{119}$ and ANK$_{195}$, and one spectrin isoform, βIII-spectrin [23–26]. The exact role(s) of the Golgi membrane skeleton is unclear, but it is thought either to provide a structural scaffold, or to form a novel vesicle coat complex [18–22]. Accordingly, spectrin is released from the Golgi membranes either during mitotic Golgi breakdown, or by allowing brefeldin A-mediated fusion of the Golgi membranes with the endoplasmic reticulum (ER). Overexpression of a homologous βI-spectrin peptide has also been shown to impair ER-to-Golgi transport of some membrane proteins, such as vesicular stomatitis virus (VSV)-G protein and Na/K-adenosine triphosphatase (ATPase) [27].

Previously, it has been reported that Golgi spectrin binds directly to Golgi membrane lipids via its membrane association domains (MADs). This binding is regulated by adenosine diphosphate (ADP) ribosylation factor (ARF), independently of its ability to stimulate COPI–coat formation [28]. Golgi spectrin also interacts directly with both ANKG$_{119}$ and ANK$_{195}$ [24,25], and with the Arp1 subunit of the dynactin complex [29], suggesting additional interactions between spectrin and Golgi membranes (via ankyrin) or microtubules (via the dynactin/dynein complex). One potential ankyrin-interacting Golgi membrane protein is the anion exchanger 2 (AE2), which we have previously identified in a number of cell types [30,31]. In erythrocytes an analogous ankyrin/spectrin meshwork is known to associate with the plasma membrane AE1 (also termed Band 3), a founder member of the anion exchanger (AE) gene family. Studies with knockout mice [32] have also demonstrated that this interaction is essential for the maintenance of erythrocyte cell shape and mechanical stability. The AE2 protein may thus serve a similar function in Golgi membranes by providing a membrane anchorage site for the underlying ankyrin/spectrin-based Golgi membrane skeleton. Therefore, AE2 probably has an important role in the maintenance of normal Golgi architecture in mammalian cells, although no evidence supporting this possibility currently exists. Here we show that the AE2 protein is indeed

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Abbreviations: ER, endoplasmic reticulum; AE2, anion exchanger 2; MTOC, microtubule-organizing center

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required for the structural organization of the Golgi apparatus in COS-7 cells.

2. Materials and methods

2.1. Antibodies and plasmid constructs

A polyclonal antibody against the AE2 C-terminal peptide (amino acids 1229–1241 of AE2) was affinity purified as described earlier [31]. Monoclonal antibodies against GM130 and the KDEL receptor were purchased from Transduction Laboratories (Lexington, KY, USA) and Stressgen (Victoria, Canada) respectively. A monoclonal antibody against α-tubulin was obtained from Sigma (St. Louis, MO, USA). The anti-giantin monoclonal antibody was a kind gift from Dr. Hans-Peter Hauri (Basel, Switzerland). Rabbit antisera against Ank_{195} and $AnkG_{119}$ were kindly donated to us by Dr. Kenneth Beck (University of California, Davis, CA, USA) and Dr. Jon Morrow (Yale University, CT, USA), respectively. A monoclonal anti-green fluorescent protein (GFP) antibody was purchased from Clontech (Palo Alto, CA, USA). Alexa fluor-conjugated secondary antibodies were purchased from Molecular Probes Inc. (Eugene, OR, USA) and peroxi-

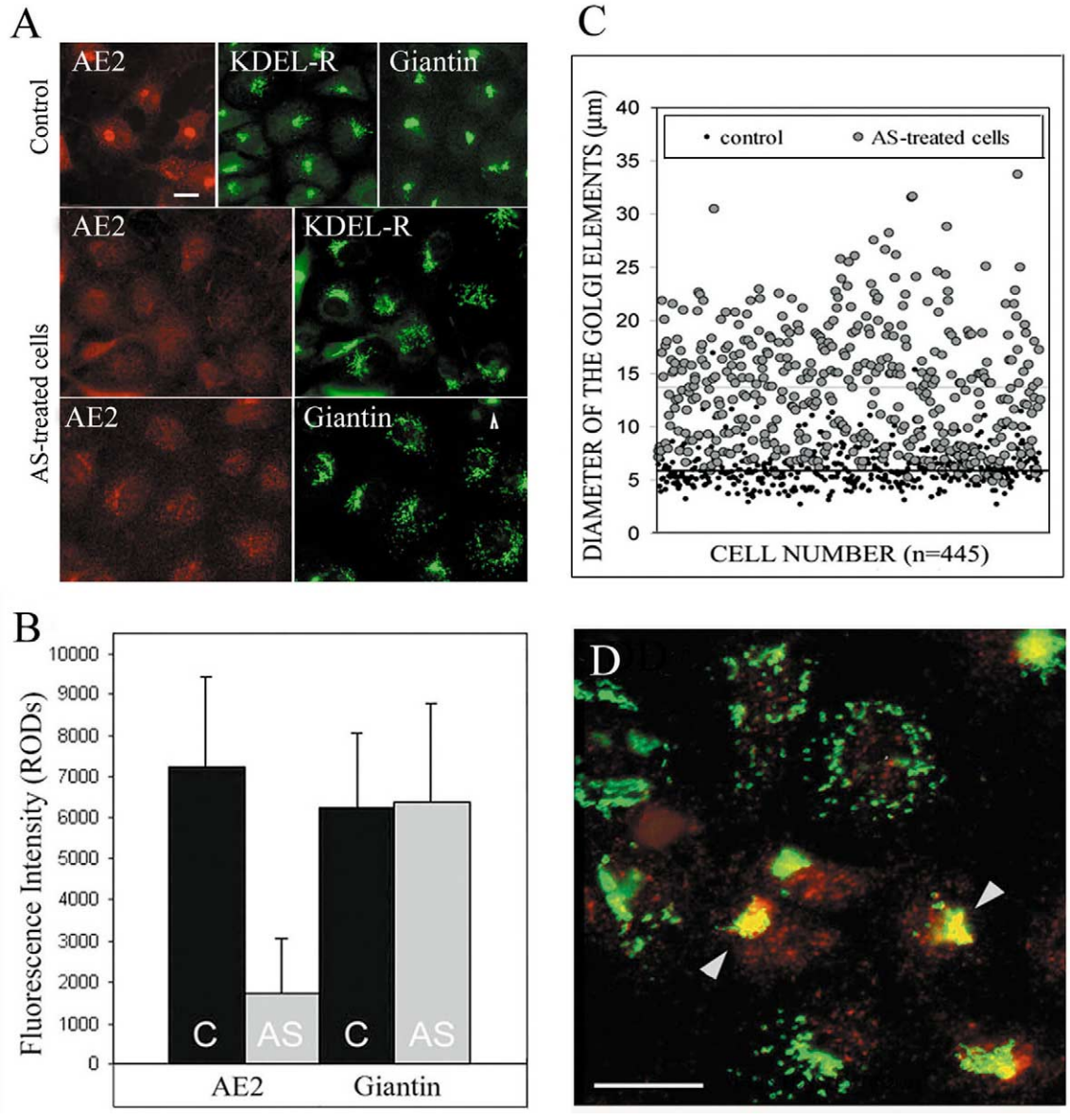


Fig. 1. Inhibition of the expression of AE2 protein in the Golgi by antisense oligonucleotides in COS-7 cells. A: After antisense oligonucleotide treatments, cells were double-stained with antibodies against AE2, giantin and the KDEL receptor (bars = 10 μm). B: The staining intensities of AE2 and Golgi markers were quantified using AnalySIS software (Soft Imaging System, USA). The intensity values (mean ± S.D./400 μm^2; $n = 60$ cells/group) were obtained by subtracting the background values from the intensity values measured in selected Golgi regions. Note that the expression of AE2 protein in the Golgi is inhibited by 77% in antisense oligonucleotide (AS)-treated cells, relative to control cells. C, control cells; AS, antisense oligonucleotide-treated cells. C: Golgi fragmentation in AE2 depleted COS-7 cells. Maximal diameters of the Golgi elements (giantin) were measured in control cells (black circles, $n = 445$) and in antisense oligonucleotide-treated cells (gray circles, $n = 445$) using the AnalySIS software. D: Correlation between AE2 expression and Golgi morphology. Cells were treated and stained as above. In the merged figure, notice the fragmented Golgi elements (green) and the absence of AE2 protein (red). The yellow color denotes to cells that still have a compact Golgi apparatus and express the AE2 protein (red, arrowheads). The staining intensities of AE2 protein and the maximal diameters of the Golgi elements (giantin) were quantitated from 60 double-stained control or antisense oligonucleotide-treated cells and used for regression analyses. Bars, 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

dase-conjugated secondary antibodies from PARIS (Compiegne, France). Plasmid constructs (encoding EGFP- and DsRed-AE2 fusion proteins) and cell transfections with the FUGENE 6 transfection reagent have been described previously [31].

2.2. Cell cultures and antisense oligonucleotide treatments

Phosphorothioate antisense oligonucleotides corresponding to the translational initiation site of AE2 mRNA (5′-gcGCTGCTCATGGCCGAATCTtag-3′) were obtained from Cybergene (Huddinge, Sweden). Several different thiol-modified oligonucleotide batches were tested, of which the one possessing two modified bases at the 5′ end and three bases at the 3′ end (small letters) was most effective when applied directly onto cells.

COS-7 cells (ATCC, Rockville, MD, USA) were grown on Petri dishes, or on glass coverslips, in Dulbecco's modified Eagle's medium (DMEM)-high-glucose medium supplemented with Glutamax (Gibco, Grand Island, NY, USA), 10% fetal calf serum (FCS) and penicillin–streptomycin. Cells were plated 1 day before the addition of the antisense oligonucleotides (25 μg/ml serum-free medium). After 4–6 h, the serum was added and the cells were cultivated for additional 2–3 days without changing the medium.

2.3. Indirect immunofluorescence

Cells were processed for indirect immunofluorescence, as described elsewhere [30]. Briefly, cells were washed with phosphate-buffered saline (PBS), fixed with 3.7% formaldehyde in PBS and stained sequentially with appropriate primary and secondary antibodies in the presence of 0.05% saponin. Stained specimens were examined using an epifluorescence microscope equipped with a CCD camera. AnalySIS software (Soft Imaging System Corp., Lakewood, CO, USA) was used for quantification.

2.4. Electron microscopy

Cells grown on plastic Petri dishes were rinsed twice with 0.1 M Na-phosphate buffer (pH 7.4), fixed on plates with 2.5% glutaraldehyde for 2 h at room temperature and post-fixed on plates in 1% osmium tetroxide before dehydration and embedding in ethanol-epon LX112, and finally with epon only. Thin sections were cut and post-stained with uranyl acetate and lead citrate before examination. Peroxidase staining, when applicable, was performed on *p*-formaldehyde (*p*-FA, 4%) fixed cells before embedding. Saponin was used as permeabilizing agent. Labeling of cells with protein A-coated gold particles (5 and 10 nm) has been described elsewhere [33]. Briefly, cells were fixed with 4% *p*-FA 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 before cutting thin sections (80–100 nm) and immunostaining of the sections with appropriate primary antibodies, secondary rabbit anti-mouse antibodies and finally with colloidal gold coated with protein A.

3. Results

3.1. Inhibition of AE2 expression by antisense oligonucleotides

To assess the structural role of AE2 protein in the Golgi we utilized AE2a isoform-specific antisense oligonucleotides and COS-7 cells, as these cells possess a highly compact and juxtanuclear Golgi apparatus, respond well to antisense oligonucleotides and exhibit a strong labeling for AE2 protein [31]. We found that AE2-specific antisense oligonucleotides significantly reduced the expression of AE2 protein in the Golgi (Fig. 1A). Based on the measured relative fluorescence intensities (Fig. 1B), the expression level of AE2 protein was reduced by 77% from that found in control cells. This inhibition was specific to AE2 protein, as the expression levels of the Golgi marker proteins, giantin and the KDEL receptor (Fig. 1A,B), remained practically unchanged. Furthermore, nonsense oligonucleotides, or differentially thiol-modified AE2 antisense oligonucleotides, did not markedly inhibit the expression of the AE2 protein under identical conditions (data not shown).

3.2. Golgi morphology is altered in AE2 depleted cells

In contrast to control cells, the Golgi morphology in AE2 cells was markedly altered, as assessed by staining the cells with the two Golgi marker antibodies. Specifically, the Golgi was fragmented and scattered into the perinuclear region in

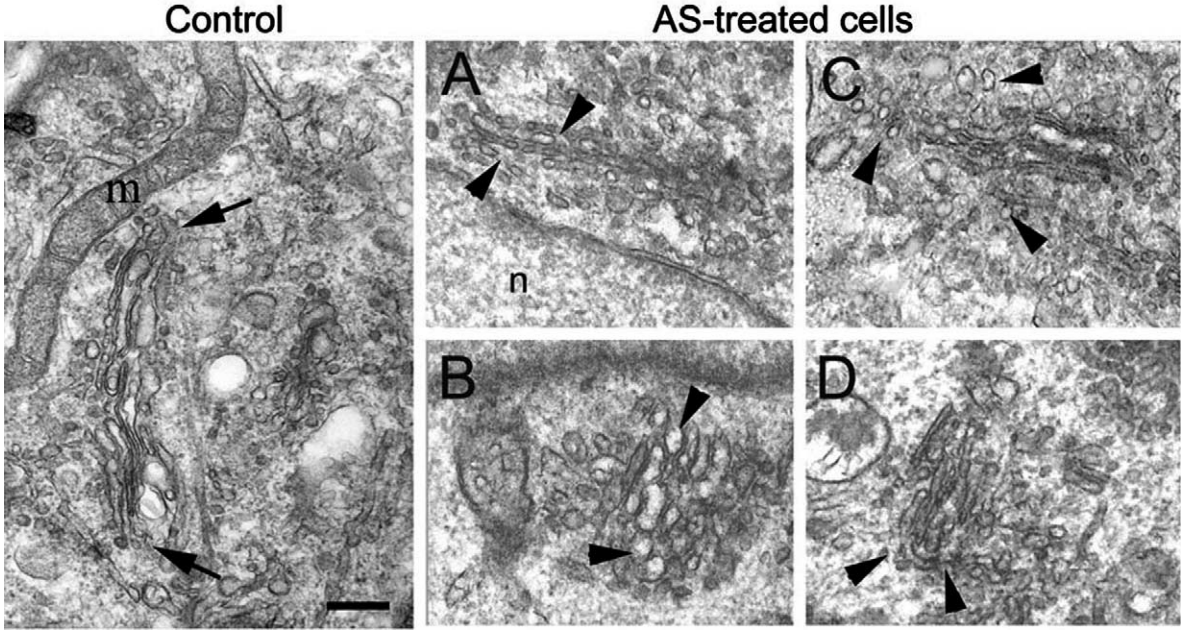


Fig. 2. Electron microscopy of Golgi stacks in AE2 depleted cells. Cells were grown on plastic culture plates and treated, or not, with antisense oligonucleotides. After fixation cells were processed for conventional transmission electron microscopy. Note that in control cells (left) the Golgi stacks (arrows) are well organized and consist of tightly packed and flattened cisternae. In antisense oligonucleotide-treated cells, the Golgi stacks of cisternae show pleiomorphic structural changes (A–D; arrowheads), including fenestrated (A), swollen (B), vesiculated (C) or distorted (D) cisternae. Bar 200 nm (m, mitochondria; n, nucleus).

AE2 depleted cells (Fig. 1A). To allow quantitative estimates we measured the maximal distance between the scattered Golgi elements in each cell ($n = 445$ cells/group). We found that the average maximal diameter (Fig. 1C) of the Golgi elements in control cells was 5.9 μm (± 1.9 μm, S.D.), whereas it was 13.8 μm (± 5.5 μm, S.D.) in AE2 depleted cells (Fig. 1C; gray circles). Thus, the maximal distance between the fragmented Golgi elements was more than doubled, relative to control cells.

Double-staining experiments with anti-AE2 and anti-giantin antibodies showed that AE2 depleted cells, particularly those that possessed a compact Golgi apparatus, still expressed AE2 protein at a considerable level (Fig. 1D), suggesting that these two parameters may correlate with each other. Regression analyses revealed that AE2 expression and fragmented Golgi structure do correlate inversely to some extent (with a correlation coefficient of −0.60). Taken together the results implicate an important role for AE2 protein in the maintenance of a compact and juxtanuclear Golgi apparatus in COS-7 cells.

Electron microscopy was utilized to assess whether AE2 depletion perturbed the morphology of the Golgi stacks. In control cells over 90% of the Golgi stacks were well organized and typically consisted of few flattened and stacked cisternae with adjacent transport vesicles (Fig. 2, left). However, in AE2 depleted cells (Fig. 2A–D), pleiomorphic structural changes in the Golgi stacks of cisternae were observed. The stacks themselves were more dispersed and smaller in size than those found in control cells and the individual cisternae often showed fenestrations (Fig. 2A), were swollen (Fig. 2B) or distorted (Fig. 2C,D) with or without adjacent cytoplasmic vesicles. None of the AE2 depleted cells examined (out of 50 sections) was found to contain structurally normal Golgi stacks. Thus, AE2 protein seems to be required also for the structural organization of the Golgi stacks of cisternae.

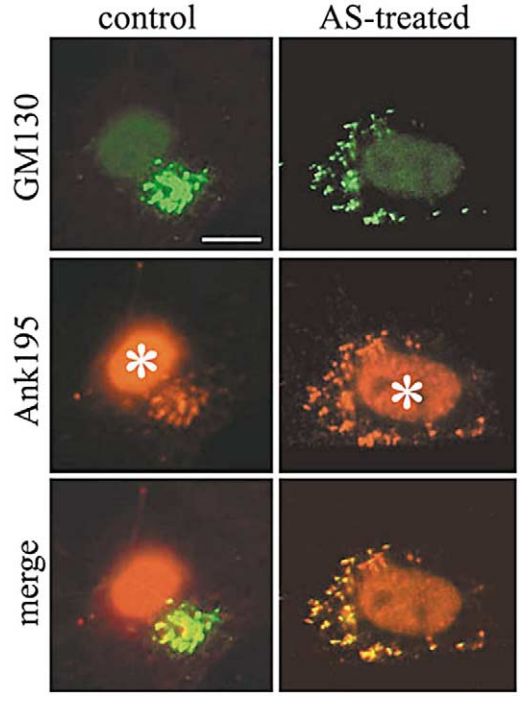


Fig. 3. Association of Ank_{195} with the Golgi membranes. Control and antisense oligonucleotide-treated cells were first detergent extracted, washed, fixed and double-stained with anti-GM130 (green) and anti-ankyrin$_{195}$ (red) antibodies. Note the presence and apparent colocalization of the ANK_{195} (red) with the Golgi marker (green) in both untreated and treated cells. Note also that the nuclei (stars) are stained with the anti-Ank_{195} antibody in detergent-extracted cells. This is probably due either to non-specific staining or the existence of as yet unknown nucleus/ER-associated ankyrin isoform(s). Bar 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

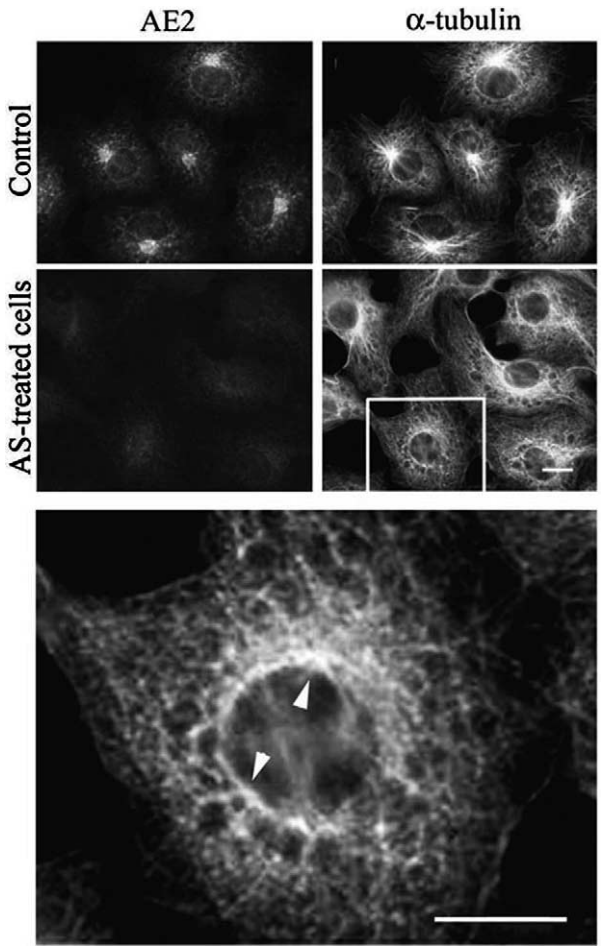
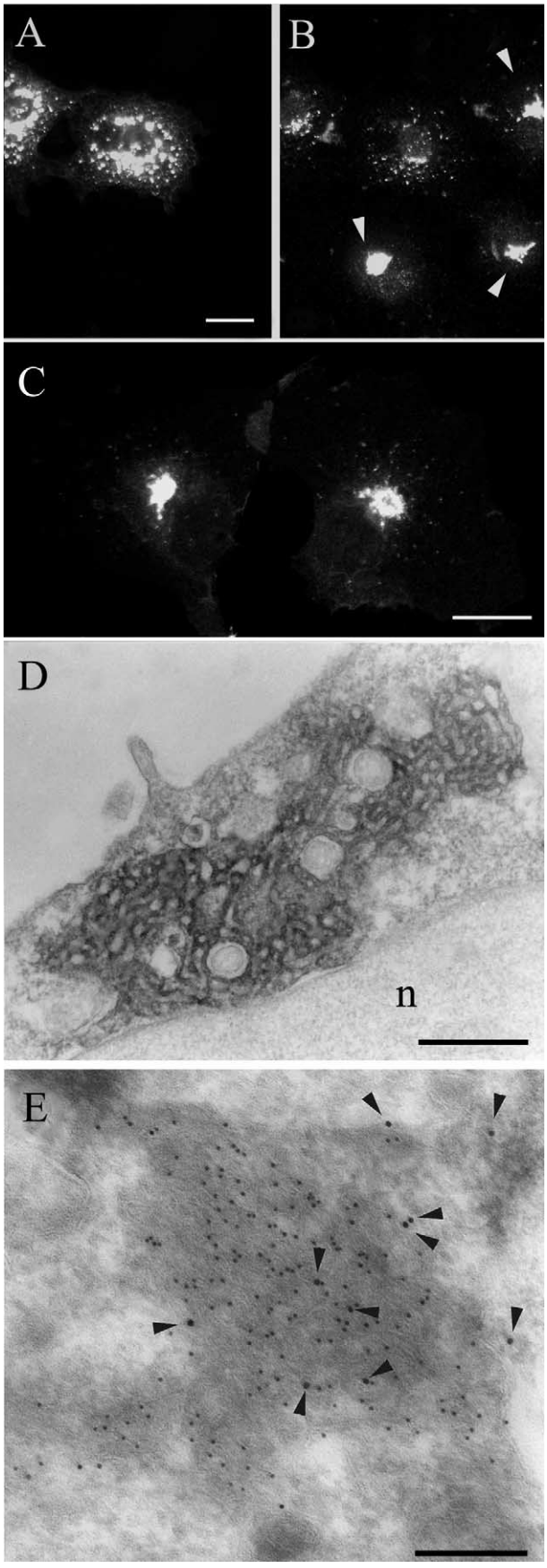


Fig. 4. Disorganization of the MTOC in antisense oligonucleotide-treated cells. COS-7 cells treated, or not, with the antisense oligonucleotides were fixed and stained with anti-AE2 or anti α-tubulin antibodies. In AE2 depleted cells, the MTOC is virtually absent and microtubules appear to nucleate from multiple points around the nuclear envelope (arrowheads). The insert in the middle is shown as a higher magnification at the bottom. Bars, 10 μm.

3.3. *Ank_{195} remains associated with the Golgi membranes in AE2 depleted cells*

Previous studies have shown that both Ank_{195} [25] and AE2 ([30,34], our unpublished data), localize predominantly to the trans-side of the Golgi stack, are resistant to Triton X-100 extraction and also co-redistribute upon overexpression of a EGFP-AE2 fusion protein in COS-7 cells. This suggests that the two proteins may interact directly with each other, thereby linking Golgi membranes to the underlying spectrin-based Golgi membrane skeleton. To evaluate whether the observed Golgi fragmentation in the absence of AE2 protein could result from the dissociation of Ank_{195} from the Golgi mem-



branes, we stained control and AE2 depleted cells with antibodies against ANK_{195}. Prior to staining, cells were extracted with Triton X-100 to remove detergent-soluble ankyrin isoforms that are known to react with this anti-Ank_{195} polyclonal antibody [25]. We found equal levels of Ank_{195} in both control and AE2 depleted cells (Fig. 3) and that Ank_{195} remained in close association with the Golgi membranes despite the apparent absence of AE2 protein. Other Golgi membrane skeletal proteins ($AnkG_{119}$, spectrin) are more widely distributed in COS-7 cells and only partially colocalize with the Golgi markers (data not shown), and therefore are not useful for these studies.

3.4. *The microtubule network is disorganized in AE2 depleted cells*

Because Golgi morphology is known to depend on intact microtubules [9,10], we next examined whether microtubule organization was disrupted in AE2 depleted cells. We stained both control and AE2 depleted cells with an anti-α-tubulin antibody (Fig. 4) and found that in contrast to a well-organized microtubule network and a focused microtubule-organizing center (MTOC) detected in control cells, the microtubule network was disorganized in AE2 depleted cells (Fig. 4). The cells also lacked a well-focused MTOC and microtubules appeared to emanate from multiple points along the nuclear envelope (Fig. 4, bottom). Thus, Golgi fragmentation induced by AE2 depletion is accompanied by changes in the assembly of the microtubule network and the MTOC.

3.5. *Overexpression of EGFP-AE2 fusion protein also interferes with Golgi structure*

To provide additional evidence for a structural role of AE2 protein in Golgi membranes, we transiently overexpressed an EGFP-tagged version of the AE2 protein in COS-7 cells. Similarly to AE2 depletion, we found that overexpression of AE2 fusion protein (Fig. 5A) caused a marked fragmentation/vesiculation of the Golgi, as assessed by staining the cells with the anti-GM130 Golgi marker antibody (Fig. 5B). In neighboring non-transfected cells, or in cells that expressed the fusion protein at a low but detectable level (data not shown), the Golgi apparatus remained practically unaltered and juxtanuclear. Similarly, Golgi structure was also preserved when the cells were transfected with a truncated version of the AE2 protein (Fig. 5C) that lacked the first 563 N-terminal amino acids,

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Fig. 5. Overexpression of the EGFP-tagged AE2 fusion protein induces accumulation of membranous aggregates in COS-7 cells. Cells were transfected with EGFP-AE2 (A–B) or an N-terminally truncated AE2 (C) encoding plasmid before fixation for indirect immunofluorescence (A–C), or for electron microscopy (D,E). Double-staining of the transfected cells (A) with the anti-GM130 antibody (B) shows that the Golgi apparatus is fragmented in cells that express the fusion protein at high levels. In neighboring non-expressing cells (arrowheads) or in cells expressing the N-terminally truncated AE2 variant (C), the Golgi apparatus remains compact and juxtanuclear. Similar results were obtained with DsRed-AE2 protein (data not shown). D: Electron micrograph of the peroxidase-stained EGFP-AE2 protein in transfected cells. Note the accumulation of large tubular or lamellar membrane aggregates near the nuclei (n). E: Double immunogold labeling of the membrane aggregates with anti-GFP (5 nm gold) and anti-KDEL receptor antibodies (10 nm gold, black arrowheads). Note the coexistence of the two proteins within the membranous aggregates. Bars (A,B) 10 μm; (C,D) 200 nm.

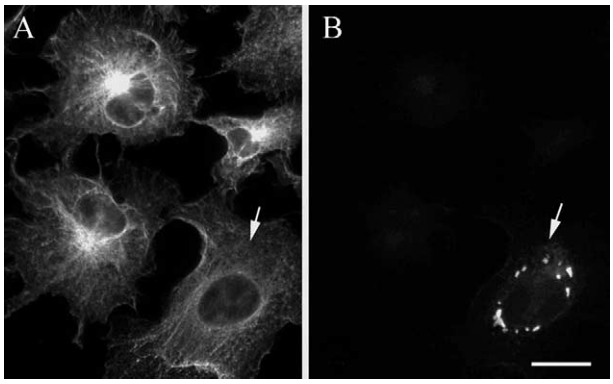

Fig. 6. Disassembly of the MTOC in AE2 overexpressing cells. Cells were transfected with a plasmid encoding EGFP-AE2 before fixation and staining with the anti-α-tubulin antibody. Note the distinct MTOCs in control (non-expressing) cells (A) and the apparent lack of an MTOC in an AE2 overexpressing cell (B, white arrows). Bar 10 μm.

including the potential ankyrin binding domain of the AE2 protein. Ankyrin interaction(s) thus appears to be crucial for the ability of overexpressed AE2 protein to cause structural disorganization of the Golgi.

Electron microscopy with an anti-GFP antibody demonstrated further that overexpression of the full length AE2 fusion protein induced the accumulation of large, interconnected and tightly packed lamellar or tubular membranes in the transfected cells (Fig. 5D). Accumulation of these 'membranous aggregates' was also accompanied by the virtual lack of any morphologically identifiable Golgi stacks. That these membranous aggregates represent at least partly Golgi membranes was demonstrated by double-staining with antibodies against the KDEL receptor and giantin, both of which decorated these EGFP-rich membranous structures (Fig. 5E). In addition, protein disulfide isomerase (PDI), a luminal ER protein, was not present in these structures (data not shown).

Overexpression of the EGFP-AE2 fusion protein, similarly to AE2 depletion, was also accompanied by changes in the organization of the microtubule network and the MTOC. Although equivalent amounts of polymerized microtubules were detected both in non-transfected and transfected cells (Fig. 6), it was found that the well-focused MTOC seen in control cells was virtually absent in cells that expressed the fusion protein. Microtubules also appeared to radiate, similarly to AE2 depleted cells, from multiple points along the nuclear envelope, often forming randomly oriented arrays in the cell periphery. Similar results were also obtained with a DsRed-AE2 fusion protein (data not shown). Thus, similarly to AE2 depletion, overexpression of the EGFP-tagged AE2 protein interferes with the normal organization of the Golgi and the MTOC.

4. Discussion

In this work we have exploited both antisense oligonucleotide-mediated depletion and overexpression to assess the structural role of AE2 protein in the Golgi apparatus in COS-7 cells. Both approaches were found to induce marked alterations in the overall Golgi structure, organization of the polarized Golgi stacks and the MTOC. We also showed that these alterations required the presence of putative ankyrin interaction domain(s), as a truncated version of the AE2 protein lacking these (but containing the ion transport domain) did not markedly affect Golgi morphology. This finding makes it highly unlikely that the disorganization of the Golgi results simply from non-specific interactions due to overexpression of any polytopic membrane protein. The possibility that these structural changes reflect a mere accumulation of mitotic or apoptotic cells with fragmented Golgi elements was also excluded on the basis that the cells contain polymerized microtubules and intact nuclei. Thus, our data implicate a role for the AE2 protein in the maintenance of normal Golgi structure and in the assembly of a well-focused MTOC.

Our data are consistent with the suggested membrane-stabilizing and/or transport-facilitating roles of the ankyrin/spectrin-based Golgi membrane skeleton [21,22], and with AE2 protein being an important part of this multiprotein scaffold. Although we have not been able to formally proof this yet (due to lack of immunoprecipitating antibodies and Ank_{195} cDNA), there are several lines of indirect evidence to support this view. For example, both the AE2 protein and Ank_{195} are detergent resistant ([25,30], see also Fig. 3), colocalize in the trans-Golgi and co-redistribute upon overexpression of the anion exchanger [34]. Moreover, by analogy to severe endovesiculation of erythrocytes despite the apparently unaltered ankyrin–spectrin membrane skeleton in AE1 knockout mice [32], we observed that the Golgi stacks had a more vesiculated appearance in AE2 depleted cells than those found in control cells (Fig. 2), and that in our case, vesiculation also did not require dissociation of Ank_{195} (and perhaps also of other Golgi membrane skeletal components) into the cytoplasm (Fig. 3). One possible explanation for this is that even in the absence of AE2 protein, Ank_{195} remains in a detergent-resistant pool together with other Golgi membrane skeletal and Golgi matrix proteins, or is held near Golgi membranes via its binding to spectrin and the known interactions of spectrin with Golgi membrane lipids. These observations, together with the accumulation of large membranous 'aggregates' in AE2 overexpressing cells, suggest that one role for AE2 protein is to provide additional stability to Golgi membranes by linking the underlying membrane skeleton more firmly to cellular membranes. Consistent with this, Siddhanta et al. [35] have shown that reduced spectrin binding to Golgi membranes in the absence of phosphatidylinositol-4,5-bisphosphate synthesis also results in fragmentation of the Golgi.

Although the exact molecular details remain to be clarified, an impaired interplay between these proteins and Golgi membranes in AE2 depleted cells could also interfere normal vesicular trafficking or routing along microtubules, with the result that the Golgi and the MTOC become disorganized. Consistent with this, spectrin has been shown to associate with the Arp1 subunit of the dynactin complex, which also includes $p150^{Glued}$, dynamitin (p50) and p62 [29]. The spectrin–dynactin complex in turn associates with the cytoplasmic motor protein dynein, a microtubule minus end directed motor protein. Interestingly, overexpression of a spectrin peptide encompassing the actin binding and the MADs (lipid binding domains) has been reported to impair ER-to-Golgi transport of certain membrane proteins [27,28], and up to half of the cells that overexpress the dynamitin p50 subunit exhibit a fragmented Golgi and a less focused MTOC due to the disruption of the dynactin complex [12]. In addition, Ma et al. [36] have reported that overexpressed and mutated dynein

intermediate chain also induces Golgi fragmentation and disorganization of interphase microtubules by perturbing the dynactin–dynein interaction. Collectively, these findings suggest that interfering with any of the above protein–protein interactions that appear to be involved in the binding of Golgi membranes to microtubules, is likely to cause organizational changes in the Golgi and the microtubule network. Based on the above scenario, it is likely that in AE2 depleted cells the structural disorganization of the Golgi and MTOC also results from an impaired association of Golgi membranes with Golgi membrane skeletal proteins.

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