

Review Article

Cytoskeleton-Associated Protein 4: Functions Beyond the Endoplasmic Reticulum in Physiology and Disease

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Cytoskeleton-associated protein 4 (CKAP4; also known as p63, CLIMP-63, or ERGIC-63) is a 63 kDa, reversibly palmitoylated and phosphorylated, type II transmembrane (TM) protein, originally identified as a resident of the endoplasmic reticulum (ER)/Golgi intermediate compartment (ERGIC). When localized to the ER, a major function of CKAP4 is to anchor rough ER to microtubules, organizing the overall structure of ER with respect to the microtubule network. There is also steadily accumulating evidence for diverse roles for CKAP4 localized outside the ER, including data demonstrating functionality of cell surface forms of CKAP4 in various cell types and of CKAP4 in the nucleus. We will review the recent studies that provide evidence for the existence of CKAP4 in multiple cellular compartments (i.e., ER, plasma membrane, and the nucleus) and discuss CKAP4's role in the regulation of various physiological and pathological processes, such as interstitial cystitis, drug-induced cytotoxicity, pericellular proteolytic activity, and lung lipid homeostasis.

1. Introduction

Cytoskeleton-associated protein 4 (CKAP4), also known as p63, CLIMP-63, and ERGIC-63, is a 63-kDa nonglycosylated, type II transmembrane protein that is reversibly palmitoylated during interference of intracellular protein transport and mitosis [1–5]. Hans-Peter Hauri and colleagues first identified CKAP4 in 1993 while studying morphogenesis of the endoplasmic reticulum (ER). They found CKAP4 to be a resident protein of the stable ER-Golgi intermediate compartment (ERGIC) and to show signs of being highly enriched in isolated ERGIC [2]. However, early studies following this initial discovery, used rabbit polyclonal antibodies and immunoelectron microscopy to demonstrate that CKAP4 was actually located in the rough ER where it functions as a microtubule-binding protein, anchoring the ER membranes to the microtubular cytoskeleton of epithelial cells *in vivo* and *in vitro* and maintaining the structure of the ER [2, 3, 6–8]. This ability made CKAP4 the first integral membrane protein capable of linking an organelle to microtubules and earned it the name cytoskeletal-linking membrane protein or CLIMP-63 [9]. Further investigation

by mutagenesis demonstrated that the luminal segment of CKAP4 oligomerizes into α -helical complexes. These coiled-coil complexes prevent CKAP4 from being located at the nuclear envelope and mediate its specific localization in the reticular domain of the ER [9].

Currently, CKAP4 is known to also be present in the plasma membrane of vascular smooth muscle cells [10], bladder epithelial cells [11], and type II alveolar pneumocytes [12], where it functions as a binding site for a several different ligands. CKAP4's localization on the surface of vascular smooth muscle cells was first confirmed by the use of a monoclonal antibody and analysis by Western blotting and immunofluorescence microscopy [10]. Additionally, Razzaq et al. (2003) established CKAP4 as a binding site for tissue plasminogen activator (tPA) by demonstrating that the addition of an anti-p63 monoclonal antibody inhibited the binding of tPA on the surface of vascular smooth muscle cells (VSMC). CKAP4 was also shown to be localized to the cell membrane and perinuclear areas of bladder epithelial cells by immunofluorescence microscopy, where it functions as a receptor for antiproliferative factor (APF) [11]. Similarly, Gupta et al. (2006) verified the presence

of both intracellular and membrane CKAP4 on type II pneumocytes by immunofluorescence and immunoblotting, and established its role as a receptor for surfactant protein A (SP-A) [12]. All three of these reports provided preliminary evidence of CKAP4's localization on the outer cell surface of a variety of cell types, suggesting that it might have diverse functions beyond the ER.

In this paper, we will examine recent studies that provide accumulating evidence for diverse roles for CKAP4 localized outside the ER. For example, CKAP4 has been shown to be required for APF-mediated signaling [11, 13–16], for gentamycin-induced cytotoxicity [17], for regulation of tPA function [10], and for SP-A-induced surfactant uptake by pneumocytes [18]. This evidence for CKAP4 mediating a wide array of cellular responses important in physiological and pathological processes, such as interstitial cystitis, drug-induced cytotoxicity, pericellular proteolytic activity, and lung lipid homeostasis, provides new insights into this classically ER-resident protein.

2. CKAP4 Structure

Full-length, human CKAP4 consists of 602 amino acids and is classified as a type II transmembrane protein with a 474 amino acid carboxyl-terminal region (C-terminus) located in the lumen of the ER, a 106 amino acid amino-terminal region (N-terminus) located in the cytoplasm, and a single 24 amino acid transmembrane domain [2] (Figure 1). The N-terminal cytoplasmic stretch contains identically repeating strings of five prolines, seven glycines, six serines, and eight alanines, which explains the guanine-cytosine content richness in the region [4]. Schweizer et al. (1994) demonstrated that the N-terminal amino acids 2–21 function as the ER-anchoring domain, whereas amino acids 24–101 act as the microtubule binding domain [3]. Furthermore, Schweizer et al. (1994) confirmed that all three domains of CKAP4—luminal, cytoplasmic, and transmembrane (luminal and cytoplasmic domains are most important)—were needed for complete intracellular retention. However, it was also discovered through mutation analysis that only the N-terminal 23 amino acids in the cytoplasmic tail are necessary for retention [3].

Between amino acids 36–59, CKAP4 has a proline-rich domain (PRD) which likely functions in protein-protein interactions [20]. Just beyond the microtubule-binding domain CKAP4 contains a potential β -COP binding site—a di-arginine motif located between amino acids 102–103. The N-terminus is also home to a number of polyalanines, polyserines, and polyglycines located between the transmembrane domain and the PRD (amino acids 72–89) which are absent in rodent forms of CKAP4. Earlier studies have also established that CKAP4 is nonglycosylated by comparing biochemical data with the predicted amino acid sequence for the N-terminus which revealed no site for N-linked glycosylation [2, 4].

The C-terminus comprises a large extracellular domain that is predicted to be a coiled-coil, containing five heptad repeats (residues 468–503) which are thought to be a leucine

zipper (<http://www.predictprotein.org/>). This domain was determined to be necessary for the oligomerization of α -helical complexes and the subdomain specific localization of CKAP4, as deletion of this region resulted in the loss of CKAP4 oligomers and the increased mobility of the protein [9]. The sequence of amino acids (503–602) following the leucine zipper are also predicted to form amphipathic helical complexes and possess many positively charged basic amino acid residues, suggesting its possible involvement in intermolecular interactions (<http://www.ebi.ac.uk/Tools/pfa/ipscan/>). Additionally, the arrangement of the leucine zipper followed by the amphipathic helix is a key characteristic of the bZIP DNA-binding transcription factor proteins [19].

CKAP4 is also highly posttranslationally modified via phosphorylation at multiple sites and palmitoylation of a cysteine residue at position 100. There are four sites (serine 3, 17, 19, and 101) for phosphorylation on the cytosolic tail of CKAP4 [7]. Serine 3, 19, and 101 are predicted protein kinase C sites, whereas serine 17 is a predicted casein kinase II site. Preventing the phosphorylation of serine 3, 17, and 19 in CKAP4 has been shown to inhibit binding of CKAP4 to microtubules *in vitro*. Further, the extent of CKAP4 phosphorylation also increases during mitosis [7]. More recently, it has been shown that phosphorylation of these three serine residues is required for CKAP4 nuclear translocation in response to APF binding [16].

Aside from phosphorylation, CKAP4 is also regulated by palmitoylation—the addition of a 16-carbon fatty acid palmitate to a specific cysteine residue(s) by a labile thioester linkage. CKAP4 was found to be reversibly palmitoylated during interference of intracellular protein transport and mitosis by the drug brefeldin A [4]. Schweizer et al. (1995) later discovered two cysteine residues (Cys¹⁰⁰ and Cys¹²⁶) located in/near the transmembrane domain of CKAP4 [5]. Since cysteine residues within close proximity to a membrane bilayer have frequently been found to be palmitoylated by thioester linkages [21], Cys¹⁰⁰ and Cys¹²⁶ were immediately investigated as potential palmitoylation sites in CKAP4. Deletion and point mutation studies soon established Cys¹⁰⁰ as the palmitoylation site on CKAP4 [5]. Further examination of Cys¹⁰⁰ revealed that neither the transmembrane domain nor the neighboring amino acids have been found to be necessary for palmitoylation. However, the distance between Cys¹⁰⁰ and the transmembrane domain is required for palmitoylation, and alterations of this segment in either direction severely weaken acylation [5].

Similar to the results seen for phosphorylation, the blocking of palmitoylation decreases CKAP4 binding to microtubules [22]. A group of at least 23 enzymes called palmitoyl acyltransferases (PATs), which are characterized by an Asp-His-His-Cys (DHHHC) motif, have been determined to mediate palmitoylation [23]. Zhang et al. (2008) identified the DHHHC2 enzyme, an alleged tumor suppressor [24], as the PAT responsible for the palmitoylation of CKAP4 by the use of a procedure called palmitoyl cysteine isolation capture and analysis (PICA) [22]. Palmitoylation by DHHHC2 has been shown to be necessary for CKAP4's nuclear localization and its trafficking from the ER to the plasma membrane

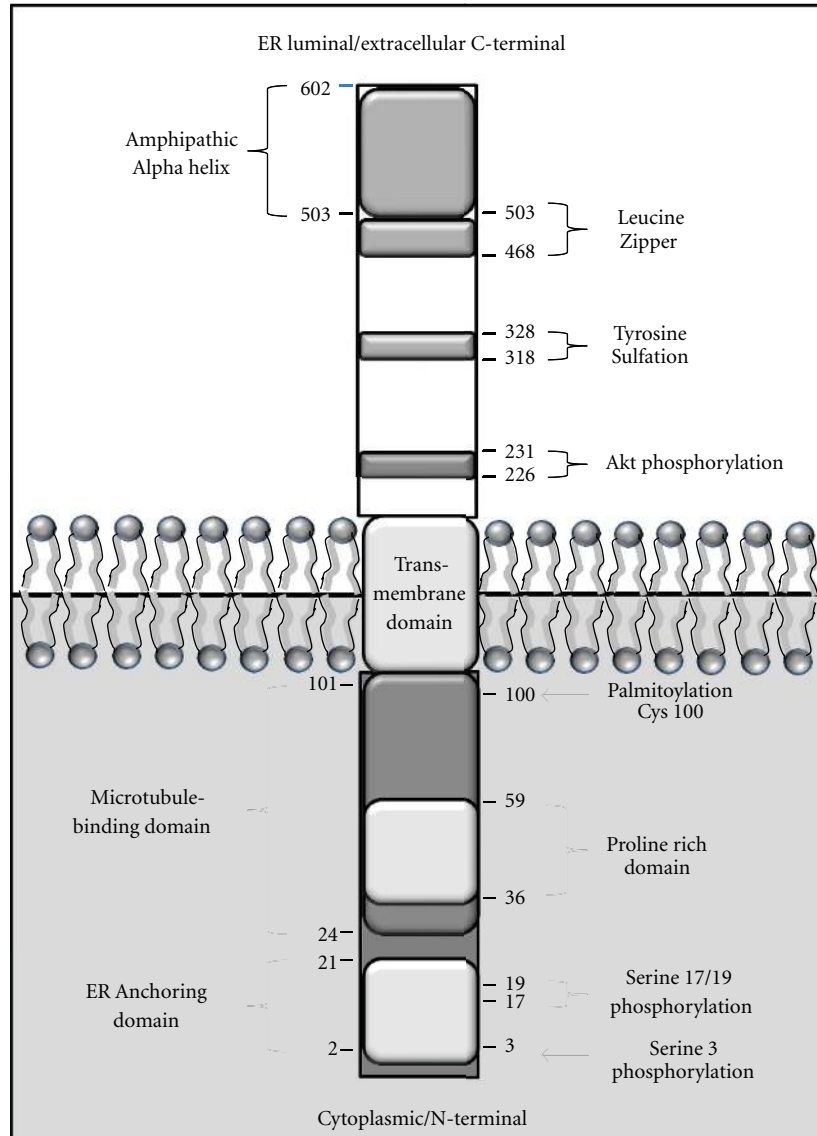


FIGURE 1: CKAP4 structural domains. CKAP4 is a 63-kDa nonglycosylated type II transmembrane protein that is phosphorylated and palmitoylated. The C-terminus is 447 amino acids long and is located in either the lumen of the ER or extracellular space, depending on the localization of the protein. It contains an alpha helical region (503–602) and a leucine zipper (468–503), which are important for oligomerization and are characteristic of the bZIP DNA-binding motif [9, 19]. Additionally, the C-terminus also contains the sites for tyrosine sulfation and Akt phosphorylation. The N-terminus is 106 amino acids long and is located in the cytoplasm. It is comprised of two major domains the microtubule binding domain and the ER anchoring domain. The microtubule-binding domain contains the site of palmitoylation (Cys¹⁰⁰) which is important for the trafficking of the protein to the plasma membrane and a proline rich sequence (36–59), whereas the ER anchoring domain has three serine residues (3, 7, and 19) which are required for phosphorylation-dependent binding to microtubules and nuclear translocation in response to APF.

[14] (Figure 2). It has also been shown that knockdown of DHHC2 by siRNA inhibited plasma membrane localization and the ability of APF to inhibit proliferation [14].

CKAP4 has also been predicted to undergo post-translational modification by tyrosine sulfation, the addition of a negatively charged sulfate group to a tyrosine residue, at tyrosine 323 [25, 26]. The trans-Golgi is home to the enzymes responsible for this sulfation which leads to a tyrosyl O-sulfate that improves protein-protein interaction by the formation of hydrogen bonds and/or salt bridges [25, 26].

The ability of this region to form these complexes with positively charged areas makes it a potential ligand-receptor binding location [27].

3. CKAP4 in the Endoplasmic Reticulum

The ER is part of the endomembrane system and is composed of a large network of membranous tubules and sacs. It encompasses more than half of the total membrane in eukaryotic cells and has an extensive number of functions

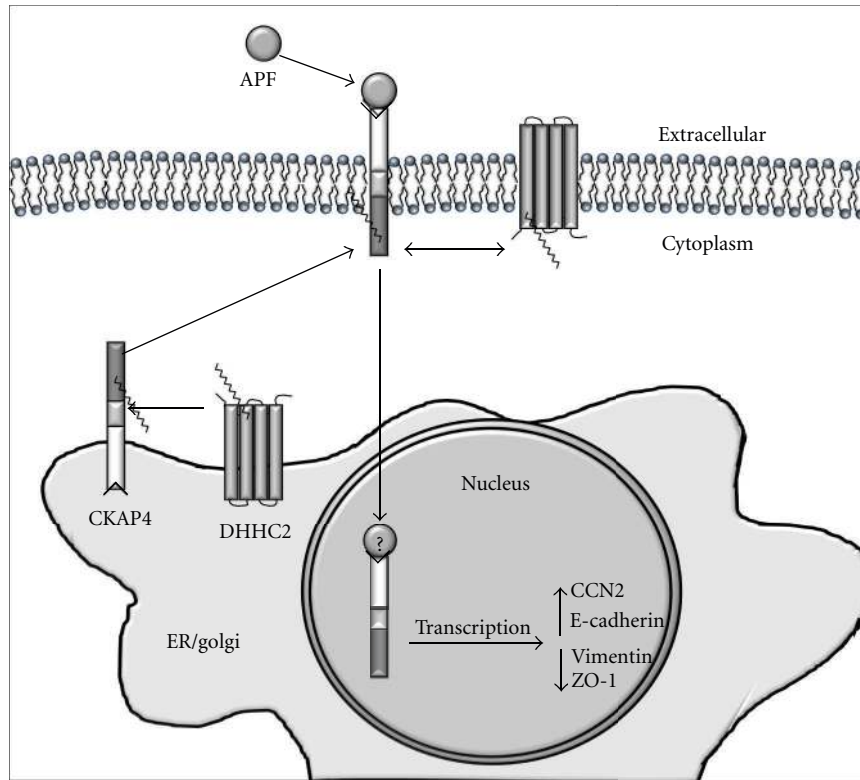


FIGURE 2: DHHC2 palmitoylation of CKAP4 is necessary for APF-mediated signaling. APF is a sialoglycopeptide that inhibits cellular proliferation of bladder epithelial cells (normal and carcinoma) and is capable of binding to the transmembrane protein CKAP4 [11, 28]. The APF/CKAP4 interaction has been shown to be required for APF's inhibition of cellular proliferation and for the alteration of specific gene expression, including E-cadherin, ZO-1, vimentin, p53, and CCN2 [11, 29]. Further, these APF-mediated changes in cellular proliferation and gene expression are regulated by DHHC2-mediated palmitoylation of CKAP4, as CKAP4 must be palmitoylated to traffic to the cell surface [22]. There is a marked increase in the nuclear abundance of CKAP4 in response to APF; however, it is not known whether APF is also present in the nucleus.

such as folding, processing assembling, sorting, synthesizing, degrading, translocating, and glycosylating secretory and membrane proteins; synthesizing and metabolizing lipids; regulating intracellular calcium. The ER is subdivided into the smooth ER and the rough ER (including the reticular domain and the nuclear envelope), which contain bound ribosomes. Although, most proteins in the rough ER can be located in either the reticular domain or the nuclear envelope, there are several exceptions. CKAP4 is one such exception and is restricted to the reticular domain [6]. However, before the detection of CKAP4 in the rough ER, it was originally described as a resident protein of the stable ER-Golgi intermediate compartment (ERGIC), a membrane network located in between the ER and the Golgi apparatus (primarily at the *cis* side of the Golgi) [4]. This notion of CKAP4 being located in the ERGIC was based on the common distribution of CKAP4 and ERGIC-53 (a 53-kD type I transmembrane marker protein of the ERGIC) [2–4].

Schweizer and colleagues first identified the requirement that all three domains (luminal, cytoplasmic, and transmembrane) of CKAP4 are needed for retention [3]. Additionally, they predicted a self-association mechanism by which CKAP4 is retained between the ER and Golgi.

This mechanism suggested that the luminal domain of one CKAP4 molecule forms a dimer with the transmembrane domain of another CKAP4. Schweizer et al. (1994), continued to propose that these dimer complexes act as the foundation for of large Triton X-100-insoluble oligomer structures, which anchor CKAP4 in the ERGIC and do not allow it to travel to the plasma membrane [3].

However, in 1995 this postulated localization of CKAP4 in the ERGIC was reevaluated when a novel polyclonal antibody against human CKAP4 was produced and used to establish the location of CKAP4 in the rough ER [5]. A considerable amount of colabeled CKAP4 and ERGIC-53 overlap was also found throughout the rough ER, which raised questions pertaining to previous reports [5]. ERGIC-53's suspected ability of recycling via the ER explained its localization in rough ER and helped clarify that the overlap with CKAP4 actually occurred in the rough ER rather than in the ERGIC, as previously proposed [5, 30, 31].

Following the discovery of CKAP4's localization to the rough ER, researchers began investigating potential capabilities that the integral membrane protein may possess. Klopfenstein et al. (1998) observed that the overexpression of CKAP4 led to the rearrangement of the ER and microtubule

bundles along the now altered ER [6]. These findings provided the initial evidence to show that CKAP4 is capable of directly binding the ER to microtubules [6] (Figure 3(a)). Further exploration revealed that the first 23 amino acids in CKAP4's cytoplasmic tail contained the information for ER rearrangement, whereas amino acids 24–101 were deemed necessary for linking the ER to microtubules. CKAP4's capability of connecting the ER to microtubules is mediated by the phosphorylation of three serine residues (3, 17, and 19) in the N-terminal domain [7]. Due to its direct anchoring ability, CKAP4 has been suspected of stabilizing the ER and/or stabilizing microtubules [6]. Several observations steered Klopfenstein et al. (1998) to this possible conclusion; for example, overexpression of a CKAP4 construct containing a mutation in amino acids 24–101 caused a rearrangement of the ER from an exterior to a more central area in the cytoplasm and the specific localization of CKAP4 in the reticular domain of the rough ER [6].

Klopfenstein et al. (2001) further investigated the reticular domain specific localization of CKAP4 by performing mutagenesis experiments, which identified the luminal segment as the reason for the protein's reticular domain localization in the rough ER [9]. The lumen is capable of such a restriction by oligomerizing into α -helical, 91 nm long rod shaped complexes that prevent CKAP4 from traveling to the nuclear envelope. These large coiled coil structures are projected to form higher orders of oligomerization stabilized by electrostatic intermolecular forces [9]. Since CKAP4 can be restricted to the reticular domain by transmembrane domain aggregation, the large luminal segment is thought to possess functional ability beyond microtubule binding. Alternatively, other nonmutually exclusive possibilities for the luminal segment are the formation of large support structures that play a role in the ER's morphology and the binding of opposing cisternal membranes to maintain the ER cisternae in a flat configuration [9].

Although there are several microtubule binding proteins, none of them seem to have a microtubule binding domain similar to that of CKAP4, which was found to only be affected by high salt concentrations (>200 mM NaCl) and contain no Src homology 2 domain [6]. Furthermore, the microtubule-binding domain of CKAP4 has identically repeating strings of five prolines, seven glycines, six serines, and eight alanines and a repeated tetrapeptide motif [4, 6]. CKAP4 has also been found to have no sequence similarities to CLIPs, which are soluble proteins that are associated with organelle-microtubules interactions [32].

Evidence of the recombinant cytoplasmic domain causing a concentration dependent effect on tubulin polymerization has led to the determination of at least one microtubule binding site [6]. This single binding domain has also been postulated to bundle microtubules based on turbidity assay experiments which revealed relatively high optical densities (ODs) for the 20 μ M CKAP4 tail recombinant peptide when incubated with a tubulin preparation. The high OD found in these experiments signifies an increased level of microtubule bundling. Yet, another conceivable situation is that CKAP4 may contain two microtubule-binding sites that are capable of combining tubulin subunits together.

4. Precedence for CKAP4 Functioning Outside the ER

4.1. CKAP4 on the Cell Surface. CKAP4 was thought to be a resident protein of the ER since its initial discovery by Hauri and his colleagues in 1993. However, nearly a decade later Razzaq et al. (2003) first reported the likelihood that CKAP4 also functioned as a plasma membrane receptor [10]. Based on previous studies [33, 34], Razzaq et al. had already distinguished that tPA was capable of binding to human VSMC, but did not yet identify the receptor protein responsible for this interaction. Therefore, by labeling VSMC surface proteins with 125 I and isolating proteins with an affinity chromatography procedure based on tPA-binding characteristics, they were able to isolate a 63 kDa protein. Mass spectrometric and western blot analysis identified the protein as being CKAP4. Subsequent investigation by immunofluorescence microscopy revealed CKAP4 to be located in both the rough ER and on the surface of human VSMC. Further evidence supporting CKAP4's functionality as the tPA binding protein was established when a monoclonal antibody against CKAP4 blocked tPA from binding to VSMC [10]. Additionally, COS cells transfected with a CKAP4 mutant (Δ 2-101AA), which lacks its N-terminal and is known to travel to the plasma membrane, expressed an increase level in plasminogen activity by >7 fold and subsequently can be blocked by the use of an antibody against CKAP4 [3, 10].

Although it is not known how CKAP4 interacts with tPA, it is feasible that the protein's coiled-coil region may play an important role. This notion is supported by the evidence that fibrin, myosin, and keratin 8 all possess binding sites with tPA that contain coiled-coil structures and that oligomer formation has exhibited enhancement of tPA binding to both amyloid β and endostatin [35–37]. These results imply that CKAP4 may play an important role in mediating pericellular proteolytic activity in vessel walls.

Shortly after the discovery of CKAP4's ability to function as a receptor for tPA, Gupta et al. (2006) found additional evidence signifying the protein's role as a plasma membrane receptor using two chemical cross linkers and identification by mass spectrometry and blotting. The interaction between CKAP4 and SP-A was confirmed by coimmunoprecipitation experiments and the use of an antibody against CKAP4 which obstructed SP-A's ability to block surfactant secretion [12] (Figure 3(b)). A subsequent study employing electron microscopy revealed that CKAP4 was not only located in the ER and plasma membrane of type II cells, but also in the microvilli where it is in close proximity to SP-A [38]. SP-A characteristically binds to type II cells via calcium-dependent (specific) binding and calcium-independent (nonspecific) binding. The introduction of an antibody against CKAP4 blocked SP-A's specific binding, but did not alter the nonspecific interactions. Similar results were obtained when the antibody was used in experiments that contained cAMP, which increased SP-A binding. Additionally, siRNA knock-down of CKAP4 resulted in an inhibition of SP-A specific binding and further demonstrated CKAP4's ability to bind to SP-A [38].

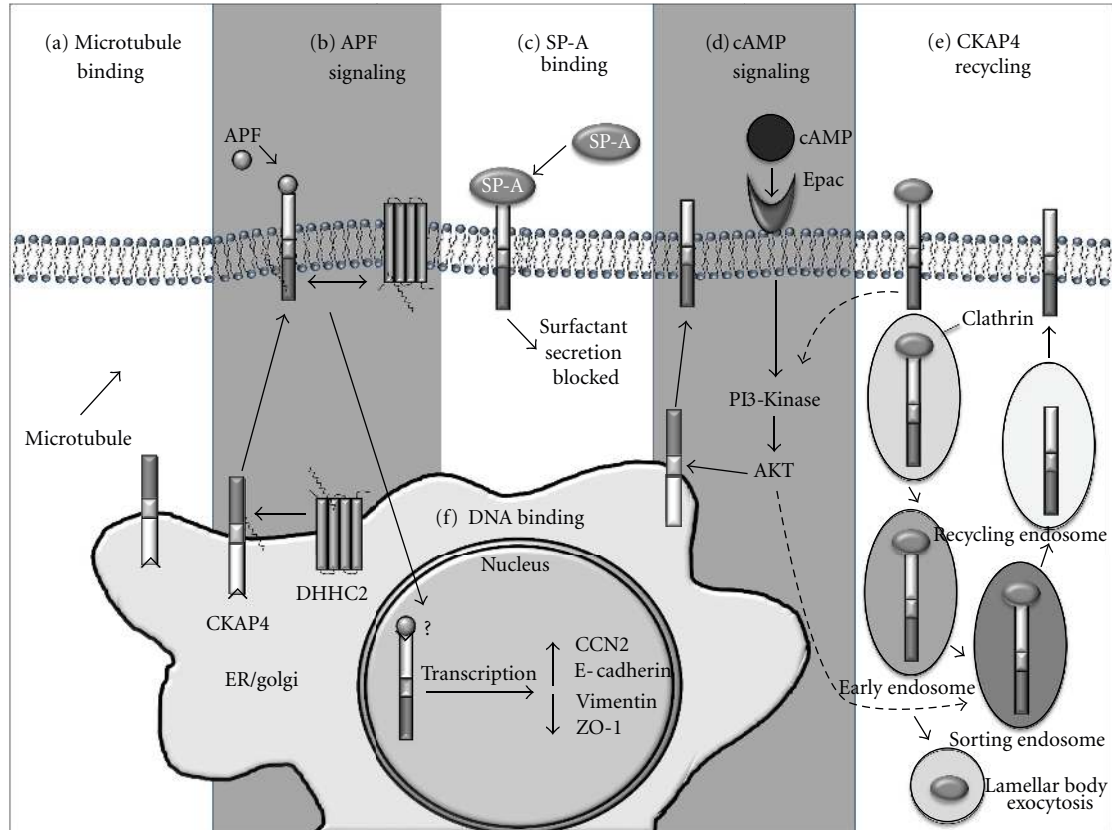


FIGURE 3: Model of CKAP4's functions at the plasma membrane, ER, and nucleus. (a) CKAP4 has been determined to be able to bind the ER to microtubules and is suspected of stabilizing the ER and/or stabilizing microtubules [6]. (b) The palmitoylation of CKAP4 by DHHC2 has been shown to be required for APF mediated signaling [14, 22]. (c) The binding of SP-A to CKAP4 inhibits the secretion of surfactant [12]. (d) An interaction between cAMP and Epac causes a signal cascade stimulating PI3-Kinase which in turn activates Akt and increases the transport of CKAP4 from the ER to the plasma membrane [18]. (e) The SP-A/CKAP4 interaction is recycled via a clathrin pathway and is stimulated by the cAMP signaling cascade [18]. (f) CKAP4 binds to the CCN2 promoter via its C-terminal domain (residues 126–501 which includes the bZIP-like DNA-binding domain) in an APF-dependent fashion [13]. It is not known whether APF binds CKAP4 in the nucleus.

Within the same year that CKAP4 was identified as being a receptor for SP-A, Conrads et al. (2006) was attempting to identify the cellular receptor for APF, a small sialoglycopeptide secreted by bladder cells in individuals with interstitial cystitis that potently inhibits the growth of bladder epithelial cells (normal and carcinoma) *in vitro* [28, 39]. Its potency to inhibit proliferation (IC_{50} in the high picomolar range), ability to alter gene expression, and the requirement for a hexosamine-galactose disaccharide linked in a specific α configuration to the backbone peptide for activity all suggested that APF's cellular effects were mediated by binding to a transmembrane receptor [11]. Using a pull-down strategy, solubilized microsomal preparations from APF-sensitive bladder epithelial cells were passed over an avidin column to which biotinylated, synthetic APF was attached. Following a series of stringent washes, two proteins were observed in the eluate and identified by mass spectrometry as CKAP4 and vimentin. Evidence confirming CKAP4's ability to function as a receptor for APF was exhibited by the use of an antibody against CKAP4 which blocked APF activity and by siRNA knockdown of CKAP4 which reduced cell sensitivity to APF. Subsequent studies confirmed the cell surface

expression of CKAP4 in HeLa cells by immunolocalization of nonpermeabilized cells [14] and by cell surface biotinylation [16] and also demonstrated that CKAP4 expression was required for APF-induced cellular effects, including (1) inhibition of normal bladder epithelial cell and cervical and bladder carcinoma cell proliferation [11, 13–15], (2) altered gene expression (i.e., vimentin, zonular occludens-1 [ZO-1], E-cadherin, p53, MMP2, and CCN2 [13–15]), and (3) Akt/GSK3 β / β -catenin phosphorylation [15].

4.2. CKAP4 in the Nucleus. Interestingly, there is new evidence demonstrating CKAP4 localization in the nucleus of both HeLa [14, 16] and T24 bladder [13] carcinoma cells. Planey et al. (2009) noted a marked increase in nuclear abundance of CKAP4 in HeLa cells following treatment with APF, suggesting that APF binding to CKAP4 on the cell surface induced its translocation into the nucleus. Importantly, this APF-induced change in CKAP4 localization was shown to be dependent on palmitoylation of CKAP4 by DHHC2 [14] (Figure 2). To further test the idea that CKAP4 translocates to the nucleus in response to APF, Zacharias et

al. (2012) used three different techniques—surface labeling with Sulfo-NHS-biotin, cellular fractionation, and immunocytochemistry followed by confocal microscopy—to monitor CKAP4 localization in response to APF binding [16]. Their results showed that biotinylated CKAP4 protein was detected in the nuclear fraction of APF-treated cells but not in the nuclear protein fraction from untreated cells; moreover, they determined by cellular fractionation and Western Blot analysis that exposure of HeLa cells to APF resulted in a ~4-fold increase in the relative abundance of CKAP4 in the nucleus.

To examine the effect of serine phosphorylation and/or palmitoylation on the nuclear localization of CKAP4 in response to APF, Zacharias and colleagues generated a series of mutants that mimic combined states of palmitoylation and phosphorylation [16]. They determined that APF promotes serine phosphorylation of CKAP4; moreover, that phosphorylation of serines 3, 17, and 19 is required for APF-induced nuclear translocation of CKAP4, as mutation of these residues to alanine (ΔA) inhibited this process. Additionally, mutation of these serine residues to glutamic acid to create a phosphomimicking form of CKAP4 (CKAP4S ΔE) promoted the nuclear translocation of CKAP4 in response to APF. Interestingly, a phosphomimicking form of CKAP4 that was also constitutively depalmitoylated localized in the nucleus without APF stimulation. These data suggest that when CKAP4 is bound by APF on the PM, it becomes depalmitoylated and phosphorylated and translocates into the nucleus. In direct support of the idea that CKAP4 must be phosphorylated to enter the nucleus, Zacharias et al. also showed by metabolic labeling with $\gamma^{32}P$ -ATP that nuclear CKAP4 is phosphorylated [16].

Matika et al. (2012) reported similar nuclear localization of CKAP4 in response to APF in T24 bladder carcinoma cells using confocal microscopy and cellular fractionation followed by Western Blot [13]. Of significant importance in this study was the detection of a second, smaller CKAP4-specific band in the nuclear fraction of APF-treated cells, suggesting that CKAP4 may be proteolyzed prior to entering the nucleus; moreover, the detection of a relatively small amount of CKAP4 in the nucleus of untreated cells in this study and in other unpublished experiments suggests that CKAP4 may have some inherent nuclear function not related to APF-induced signal transduction or that may be specific to cancer cells, as nuclear localization of CKAP4 after binding of tPA or SPA was not apparent in previous studies [10, 12].

To explore the potential role of nuclear localized CKAP4 in APF-induced CCN2 expression, Matika and colleagues investigated whether CKAP4 could bind to the CCN2 proximal promoter using two different approaches. By electrophoretic mobility shift assay (EMSA), they showed that recombinant CKAP4 (comprised of C-terminal residues 126–501, which includes the bZIP-like DNA binding domain) bound specifically to the CCN2 proximal promoter; furthermore, they demonstrated that endogenous CKAP4 bound the CCN2 proximal promoter in an APF-dependent manner using a streptavidin-bead pull down approach [40]. These data provided strong evidence that CKAP4 is an essential downstream mediator of APF-induced CCN2 expression

(and potentially other APF-responsive genes) and expanded our knowledge of CKAP4 cellular function to include a potential role in regulating transcription, either directly as a DNA-binding protein or indirectly through recruitment of other DNA-binding proteins [13] (Figure 3(f)).

CKAP4 is 63 kDa, homo-oligomeric, and is embedded in membranes; these are not typical, physical properties of proteins that enter the nucleus by diffusion. Generally, proteins that diffuse into the nucleus are cytosolic and have a molecular weight less than 40 kDa. Proteins too large to diffuse through the nuclear pore translocate with the assistance of a shuttle protein (or karyopherin) that is physically bound to a nuclear localization signal (NLS) sequence of the imported protein. CKAP4 contains a glycines-rich domain that may act as an NLS. Similar domains found in several families of proteins are known to mediate nuclear import [41, 42]. Ongoing work indicates that this domain of CKAP4 is sufficient to drive nuclear localization of a nonrelated protein (Planey and Zacharias, unpublished data).

Because of its size and TM domain, it would seem unlikely that CKAP4 would have a nuclear function. However, there is precedent for large, TM-domain proteins entering the nucleus. The epidermal growth factor (EGF) receptor family is comprised of glycosylated, single-pass TM domain proteins of ~140 kDa that also translocate from the plasma membrane into the nucleus after ligand binding [43]. Within the nucleus, they regulate transcription and participate, enzymatically, in signal transduction pathways. While initially, the idea that these proteins translocated to and had functional activity in the nucleus beyond their tyrosine kinase activity at the plasma membrane was controversial, recent mechanistic details have shed further light on how they make their way into the nucleus and function therein (reviewed in [43]). There is also precedent for several cytoskeletal proteins such as vimentin, actin, BPAG1, α II spectrin, actinin-4, and β II-tubulin being localized to the nucleus where they participate in nuclear related functions such as transcription, DNA repair, mRNA transport, nuclear architecture, and gene reprogramming (reviewed in [44]).

5. CKAP4 in Physiological and Pathological Processes

5.1. CKAP4 and APF-Mediated Signaling in Interstitial Cystitis. APF is a 9-residue, sialoglycopeptide (Neu5 Aca2-3Gal β 1-3GalNAca-O-TVPAVVVA) produced specifically in the bladders of interstitial cystitis (IC) patients [28]. APF has been shown to cause abnormalities in normal bladder epithelial cells and bladder cancer cell lines that mimic changes seen in explanted IC/PBS cells, including profoundly inhibited cell proliferation [15, 39, 45, 46], increased p53 and p21 expression [13, 15, 29], altered epithelial growth factor production [13, 39, 46], and a specifically altered gene expression pattern including increased E-cadherin with decreased ZO-1, occludin, and vimentin [47, 48]. These APF-induced abnormalities have also been observed in an *in vivo* mouse model for IC, suggesting that APF may indeed play

a role in the pathogenesis of IC/PBS [49]. Several lines of evidence suggest that APF's potent cellular effects on bladder cell proliferation and altered gene expression manifest through binding to CKAP4. First, exposure of live bladder epithelial cells to anti-CKAP4 monoclonal antibodies prior to APF exposure abolishes APF activity [11]. Second, reducing CKAP4 expression with siRNA inhibits APF effects on cell proliferation and gene expression, including downregulation of vimentin, ZO-1, and MMP2 and upregulation of E-cadherin, p53, and CCN2, [11, 13–15] as well as APF's effects on Akt/GSK3 β / β -catenin phosphorylation [15]. Third, inhibition of CKAP4 palmitoylation impairs its trafficking to the plasma membrane, thereby blocking the cellular effects of APF [14]. Collectively, these data suggest that APF signals primarily, if not exclusively through CKAP4, and that regardless of whether low affinity receptors also exist, the IC-related effects of APF are mediated through CKAP4.

While the requirement for CKAP4 to promote APF activity is clear, its function in this regard remains undefined and an area of active investigation. New evidence for a direct effect of nuclear CKAP4 on APF-induced upregulation of CCN2 suggests that CKAP4 has both transcriptional and post-translational effects on CCN2 expression [13]. The potential for CKAP4 to bind DNA was hypothesized by Planey and Zacharias following their observation that APF increased the nuclear abundance of CKAP4 in HeLa cells [14] and subsequently, that it specifically promoted CKAP4's nuclear translocation from the cell surface [16]. Interestingly, the luminal/extracellular domain (C-terminus) of CKAP4 contains a leucine zipper domain with five heptad repeats (the "L"s in larger font below) that mediate CKAP4 oligomerization [9] and an adjacent amphipathic helix (underlined) rich in basic residues (basic residues in large, italicized font—(468)-LASTVRSLG $\underline{\text{ETQLVLYGDVEELKRSVGE}}\underline{\text{LPSTVESLQKVQEQVHTLLSQDQAQAARLPPQDFLDRSSLDNLKASVSQVEADLKMRLRTAVDSLVAYSVKIETNENNLESAKGLLDLRLNDLDRLFVKVEKIH}}\underline{\text{EKV}}\text{-(602)}$ —an arrangement that is the hallmark of the bZIP DNA-binding transcription factor proteins [19] (Figure 1). Using genomic DNA affinity chromatography (GDAC) Zacharias et al. (2012) demonstrated that CKAP4 isolated from APF-treated cells bound genomic DNA (gDNA) in a phosphorylation-dependent manner. Further, they showed that recombinant CKAP4 protein consisting of only the C-terminal residues 126–501 (which includes the bZIP-like DNA-binding domain) bound specifically to gDNA [16]. Likewise, this region of CKAP4 was shown to bind specifically to the proximal promoter of an APF-responsive gene, CCN2, by EMSA as described above (Figure 3(f)). While these data shed some light on a potential role for CKAP4 in mediating APF-induced changes in gene expression, the mechanism by which CKAP4 gains entry to the nucleus is not obvious and requires further investigation.

5.2. CKAP4 and Lung Lipid Homeostasis. SP-A is a calcium-dependent lectin composed of 18 protein subunits that is found in the bronchoalveolar fluid and plays a significant role

in the maintenance of lung lipid homeostasis through regulation of surfactant turnover [38, 50]. Type II pneumocytes in the alveoli of the lung produce surfactant, a phospholipid-rich lipid-protein mixture, to lower the surface tension in lung and maintain normal function. Through receptor-mediated specific binding of SP-A to alveolar type II cells, SP-A enhances the uptake of surfactant-like liposomes and profoundly inhibits the secretion of surfactant lipids from the pneumocytes [51, 52]. In 2006, Gupta et al. identified CKAP4 as a SP-A binding protein on type II pneumocytes by chemical cross-linking techniques [12]. Later, it was shown that reduced CKAP4 expression by siRNA blocked CKAP4 specific SP-A binding and reversed the ability of SP-A to prevent surfactant secretion from A549 pneumocyte cells, substantiating the role of CKAP4 as an SP-A receptor protein [38].

Further evidence supporting the interaction between CKAP4 and SP-A, and consistent with a physiological function of CKAP4 in surfactant turnover, comes from studies performed by Kazi et al. [18]. They hypothesized that the phosphatidylinositol-3-kinase (PI3-kinase) signaling pathway was a possible mechanism for the transport of CKAP4 from the ER to the plasma membrane. By blocking the pathway with LY294002, a potent PI3K inhibitor, they were able to prevent restoration of CKAP4 to the plasma membrane, resulting in an interference of SP-A function. Thus, analogous to the findings of Planey et al. (2009) which demonstrated that palmitoylation of CKAP4 by DHHC2 was required for its plasma membrane localization and mediation of APF activity [14], surface localization of CKAP4 is also required for SP-A function (Figure 3(c)).

Based on evidence from prior studies [53, 54], Kazi and colleagues also established that AKR-transforming enzyme (Akt) played a role in the transport of CKAP4 by using Akt inhibitor VIII, which impeded SP-A activities. Therefore, SP-A mediated lipid turnover can be compromised by blocking either the PI3-kinase or Akt signaling pathways (Figure 3(d)). Notably, work by Shahjee et al. (2010) implicated the Akt pathway in APF's effects on proliferation and gene expression, demonstrating specifically that APF decreased Akt phosphorylation in a CKAP4-dependent manner. Collectively, these findings support the idea that CKAP4 is an SP-A receptor and support a role for the PI3-kinase/Akt system in the transport process that retains or increases the concentration of CKAP4 on the plasma membrane.

5.3. CKAP4 and Gentamicin-Induced Cytotoxicity. Aminoglycosides, such as gentamicin, are common antibiotics that are used to treat a variety of bacterial infections. However, they are also capable of inducing cytotoxicity in the proximal tubules of the kidneys and in the inner ear. While attempting to understand how aminoglycosides cause such severe side effects, Karasawa et al. (2010) discovered that gentamicin binds to CKAP4 in mouse kidney proximal tubule (KPT11) cells via pull down experiments with gentamicin agarose conjugates and mass spectrometry analysis. Although other proteins were found to bind to gentamicin, only CKAP4 was localized in the ER and significantly co-localized with

gentamicin, suggesting that CKAP4 plays a role in inducing cytotoxicity [17].

Focusing on CKAP4 led Karasawa and colleagues to observe a possible difference in CKAP4's oligomerization states in proximal and distal tubule cells, based on western blots which showed dithiothreitol (DTT) resistant dimers only in the proximal tubule cells. They determined that gentamicin binds to the ER luminal domain of CKAP4 by gentamicin agarose pull down assays and demonstrated that gentamicin affects CKAP4 by inducing DTT resistant CKAP4 dimerization. These results suggest that CKAP4 plays a role in inducing the toxicity of gentamicin. Although the specific role is uncertain, it is hypothesized that the cytoplasmic domain of CKAP4 is structurally altered. Evidence supporting this postulation was seen when mutations of the Cys¹⁰⁰ palmitoylation site blocked dimerization induced by gentamicin, indicating that CKAP4 dimerization is dependent on palmitoylation. Additionally, using siRNA to reduce CKAP4 expression in kidney proximal tubule (KPT11) cells, Karasawa noted increased cellular resistance to gentamicin-induced cytotoxicity, establishing that CKAP4 dimerization plays an important role in aminoglycoside-dependent cytotoxicity [17].

To explore potential mechanisms by which CKAP4 may induce gentamicin cytotoxicity, Karasawa and colleagues searched for proteins that interact with CKAP4 after gentamicin treatment using immunoprecipitation. They identified 14-3-3 proteins as CKAP4-binding proteins, and observed that 14-3-3 θ directly binds to CKAP4, whereas 14-3-3 β only interacts with 14-3-3 θ . Additional siRNA experiments targeting 14-3-3 β and 14-3-3 θ , demonstrated that only 14-3-3 β significantly contributes to gentamicin-induced cytotoxicity, particularly with increased caspase-3 activity that leads to apoptosis. However, it is likely that 14-3-3 θ is required since it directly links CKAP4 to 14-3-3 β [17]. While the physiological functions of 14-3-3 proteins in regulating cell cycle timing and arrest is well characterized (reviewed in [55]), the role of CKAP4 in cell cycle control is less obvious. CKAP4 has been shown to function in cellular proliferation, as CKAP4 expression is upregulated by a cyclin-dependent kinase inhibitor olomoucine [56]; further, palmitoylation of CKAP4 by DHHC2 is required for cell cycle inhibition by APF. Thus, it is plausible that gentamicin induces cell death by interfering with cellular proliferation control through CKAP4 and 14-3-3 proteins [17].

6. Conclusion

In this paper, we provide evidence for CKAP4 in the regulation of cellular processes from multiple cellular compartments, including the ER, plasma membrane, and nucleus. Given the fact that CKAP4 appears to function as a biologically relevant receptor for three seemingly unrelated proteins in diverse cell types—tPA in vascular smooth muscle cells, SP-A in rat type II pneumocytes, and APF in normal bladder and carcinoma cells—several important questions arise, including (1) what structural and physical characteristics of CKAP4 allow it to function as a receptor for these

various ligands? (2) Does CKAP4 mediate transmembrane signaling or does it merely function as a chaperone protein for cytoplasmic or nuclear translocation of its ligands? (3) What cellular mechanisms affect compartmental transit of CKAP4 from the ER to the cell surface or to the nucleus? (4) What other functions and vital system processes involve a role for CKAP4? These questions underscore the need for a more detailed understanding of CKAP4 and its functionally diverse biology.

Indeed, the existence of an ER resident protein like CKAP4 on the cell surface was initially a difficult concept to accept. However, multiple lines of evidence using biochemical, immunohistochemical, and functional assays eventually provided credible evidence for the existence and, importantly, the functionality of cell surface and nuclear forms of CKAP4. With the more recent discoveries of non-ER functions of CKAP4 playing a significant role in many physiological and pathological processes such as interstitial cystitis, drug-induced cytotoxicity, pericellular proteolytic activity, and lung lipid homeostasis, new insights into downstream intracellular pathways, crosstalk, signaling coreceptors, and mechanisms for the transport of CKAP4 to the cell surface and nucleus are certain to unfold for this ER protein.

Acknowledgments

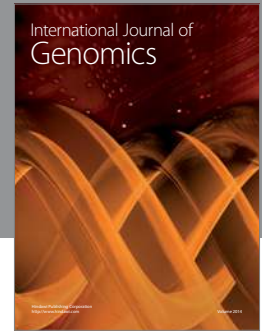
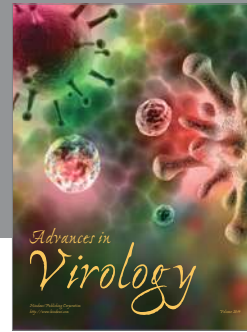
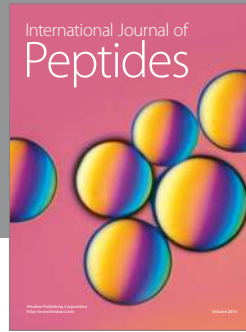
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