CLIC4, an Intracellular Chloride Channel Protein, Is a Novel Molecular Target for Cancer Therapy

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Chloride intracellular channel (CLIC)4 is a p53- and tumor necrosis factor α (TNFα)-regulated chloride channel protein that is localized to the mitochondria and cytoplasm of mouse and human keratinocytes. CLIC4 protein increases in differentiating keratinocytes and in keratinocytes exposed to DNA-damaging agents and metabolic inhibitors. Increasing CLIC4 levels by transduction of recombinant CLIC4 causes apoptosis. CLIC4 translocates to the nucleus under a variety of conditions of cell stress, and nuclear CLIC4 is associated with cell cycle arrest and accelerated apoptosis. Reduction of CLIC4 and several other CLIC family members by expressing a doxycycline-regulated CLIC4 antisense also causes apoptosis in squamous cancer cell lines. Expressing antisense CLIC4 in tumors derived from transplanting these cells into nude mice inhibits tumor growth, increases tumor apoptosis, and reduces tumor cell proliferation. Co-administration of TNFα intraperitoneally enhances the tumor-inhibitory influence of CLIC4 antisense expression. Together, these results suggest that CLIC4 is important for keratinocyte viability and may be a novel target for anti-cancer therapy.

Key words: chloride channel/stress/apoptosis/differentiation/cancer therapy
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Chloride is a major intracellular anion of all living organisms. Intracellular chloride is essential for regulating electrogenic cation transport across intracellular and plasma membranes and maintaining membrane potential in organelles. As a consequence, both organelle volume and pH are maintained, and these are fundamental to organelle function. The importance of intracellular chloride ion regulation is emphasized by the evolution of at least five independent chloride channel families in mammalian cells (Table I). The number of family members in each class of chloride channels is variable, but mutations in genes from several families are associated with neuromuscular, respiratory, osseous, and renal diseases in humans (Thakker, 1999; Dworakowska and Dolowy, 2000; Li and Weinman, 2002).

The most recently discovered and still evolving family of chloride channel proteins is the chloride intracellular channel (CLIC) family. Currently, seven highly homologous proteins are recognized that are encoded by independent genes, except for p64 (CLIC5B) and CLIC5A (Table II). The parent member of this family, p64, was isolated from microsomes of bovine kidney and trachea, and have been shown to possess chloride-selective channel function in lipid bilayers (Redhead *et al*, 1997). All subsequently discovered members of the CLIC family possess the central core domains of p64, including a transmembrane region, but vary in the amino and carboxy termini (Fig 1). Consensus phosphorylation sites for PKA, PKC, CKII, and tyrosine kinases on CLIC family members suggest that post-translational modification contributes to the physiology of CLIC proteins. A structural and functional breakthrough in understanding CLIC took place with the crystal structure studies of CLIC1 (Harrop *et al*, 2001). This analysis confirmed the similarity of CLIC1 to the omega class of glutathione transferase proteins, and provided a structural basis through protein folding for CLIC proteins to partition into soluble or membranous compartments of mammalian cells. Structural studies also indicated that channel activity was likely related to the formation of homodimers or tetramers of CLIC creating a hydrophilic pocket for ion transport.

Among the various CLIC family members, the biological activity of CLIC4 has been studied most extensively (Table III). CLIC4 (also known as mtCLIC, p64H1, RS43) is ubiquitously expressed with particularly high expression in the skin, kidney, liver, and brain. The subcellular localization of CLIC4 varies with cell type and has been localized to golgi, endoplasmic reticulum, large dense core vesicles in the brain, and the inner mitochondrial membrane and cytoplasm in mouse and human keratinocytes. In keratinocytes and other cell types, the expression of CLIC4 transcripts is regulated by p53 and tumor necrosis factor α (TNFα), and CLIC4 is a direct response gene for both c-myc and p53, with functional consensus binding sites identified for both of these transcription factors in the CLIC4 promoter (Fernandez-Salas *et al*, 1999, 2002). Increased or reduced expression of CLIC4 induces apoptosis in several cell types including keratinocytes, and CLIC4 expression increases in keratinocytes undergoing an apoptotic response to an external stimulus. Furthermore, CLIC4 upregulation is a

Abbreviations: CLIC, chloride intracellular channel; TNFα, tumor necrosis factor α

Table I. Intracellular chloride channel families

Types of chloride channels	Physiological function	Basic structure	Activation	Associated human diseases
Ligand-gated channels (acetylcholine receptor family)	Synaptic neurotransmission, neurotransmitters	4 membrane spanning, hetero-oligomeric (pentamer)	GABA and glycine, phosphorylation (PKA)	Hyperekplexia, or startle disease
Cystic fibrosis transmembrane—conductance regulator (CFTR)	Control the movement of water in tissues, maintain the fluid consistency	12 membrane spanning, monomeric	Voltage, ATPase dependent, phosphorylation (PKA)	Cystic fibrosis, congenital bilateral absence of vas deferens
Voltage-gated chloride channels (CLC)	Transmembrane potential stabilization, transepithelial transport, cell volume regulation, acidification of intracellular organelles	12–13 membrane spanning, two pore, symmetric homodimeric	Voltage, volume, pH, and phosphorylation (PKA, CaMKII, PKC)	Hypercalciuric syndromes (Dent's disease), epilepsy, myotonias, Bartter's syndrome
Calcium-activated chloride channels (CLCA)	Modulator of Cl channel, transepithelial ion transport, excitability of neurons and muscle cells, and oocyte fertilization	4–5 membrane spanning, hetero-oligomeric	Calcium, phosphorylation (PKA)	Best's vitelliform macular dystrophy
Chloride intracellular channels (CLIC)	Control of pH gradient generated by H + ATPase in intracellular organelles, kidney, neural, and sperm function	Exist as soluble and membrane forms, 1–2 membrane spanning, possibly homo-tetrameric, structurally similar to GST	Voltage, pH, and phosphorylation (PKA, PKC, CKII, p59fyn)	Not determined yet

GST, glutathione transferase.

Table II. Chloride intracellular channels

Family members	Chloride channel activity	Subcellular location	Activation and features
p64/CLIC5B (49 kDa)	Indanyloxy acetic acid (IAA)-sensitive Cl transporter, voltage-dependent Cl channel activity in microsomal compartment, anti-p64-mediated immunodepletion of Cl-transporting activity	Cytoplasm, plasma membrane, intracellular membranes	Phosphorylation (PKA, p59fyn, Src)
CLIC1 (27 kDa)	Anion channel activity in planar lipid, channel activity blocked by IAA-94, partitions among soluble and membrane compartment, possibly channel modulator	Nuceoplasm, nuclear membrane, cytoplasm, plasma membrane	Activation by reactive oxygen, related to Ω class of GST
CLIC2 (28 kDa)	Not determined	Interact with Sedlin; not extensively verified	Not determined
CLIC3 (24 kDa)	Overexpression stimulates chloride channel activity	Primarily in the nucleus	Interact with ERK7 but not a direct substrate of ERK7
CLIC4 (29 kDa)	Chloride selective channel activity, C-term-specific antibody abolishes Cl-selective activity, possibly a channel modulator	Nucleus, cytoplasm, mitochondria, golgi, ER, intracellular membrane, plasma membrane, cytoskeleton, secretory vesicles	Local pH changes, possibly phosphorylation status or cellular stress
CLIC5A (28 kDa)	Concentration-dependent chloride efflux, sensitive to IAA-94	Primarily cytoplasmic, cytoskeleton associated	Not determined
CLIC6/parchorin (65 kDa)	Potentiates chloride ion efflux in water-secreting cells, interact with dopamine receptors	Primarily cytosolic, plasma membrane	Not determined

GST, glutathione transferase; CLIC, chloride intracellular channel.

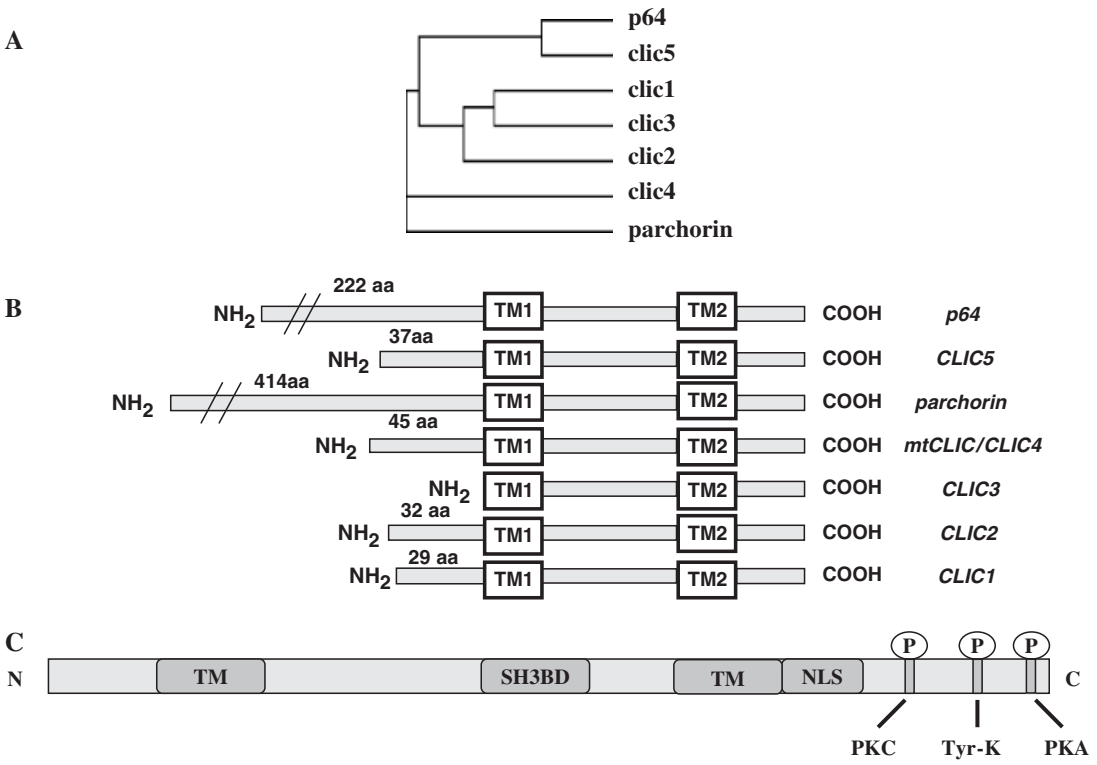

Figure 1
Molecular signatures of CLIC family members are evolutionarily conserved and include two transmembrane domains, nuclear localization signal (NLS), protein–protein interaction motif and phosphorylation sites. (*A*) Hierarchical dendrogram of chloride intracellular channel (CLIC) family members based on protein sequence homology (NCBI). (*B*) Alignment of CLIC proteins based on structure, transmembrane domains (TM), and size. Differences in the number of amino acids in N-termini are designated. (*C*) Locations of various structural motifs of CLIC4 are illustrated as TM (transmembrane region), SH3BD (SH3 binding domain), NLS (nuclear localization signal), and circled "P" as the putative phosphorylation sites of PKC, tyrosine kinase (Tyr-K), and PKA.

requirement for p53-induced apoptosis (Fernandez-Salas *et al*, 2002). The apoptotic response to overexpression of CLIC4 is directed through a mitochondrial pathway, resulting in decreased mitochondrial membrane potential, release of cytochrome C, and activation of caspases (Fernandez-Salas *et al*, 1999). Cytoplasmic CLIC4 translocates to the nucleus in keratinocytes undergoing apoptosis or growth arrest in response to stress including exposure to metabolic inhibitors, DNA-damaging agents, inhibitors of cell cycle progression, and TNFα. Nuclear translocation involves interaction with the Ran/Importin nuclear transport complex (Suh *et al*, 2004). Nuclear targeting of CLIC4 through transduction of a modified CLIC4 construct accelerates apoptosis and induces apoptosis in cells that are defective in the mitochondrial apoptotic pathway through genetic deletion of Apaf1 (Suh *et al*, 2004). Together, these results indicate that CLIC4 is an integral part of the cellular stress–response pathway in keratinocytes and other cell types.

Murine CLIC4 was first isolated in keratinocytes induced to differentiate terminally by calcium (Fernandez-Salas *et al*, 1999). Since this discovery, modifications in CLIC4 have been associated with 3T3-L1 fibroblasts differentiating into adipocytes, and mammary fibroblasts differentiating into myofibroblasts under the influence of transforming growth factor β (Ronnov-Jessen *et al*, 2002). CLIC4 is likely to be involved in other signaling pathways through its interaction in complexes of dynamin, tubulin, actin, and 14-3-3 proteins in rat brain (Suginta *et al*, 2001), and its association with AKAP 350 at the centrosome and midbody of dividing mammalian cells (Berryman and Goldenring, 2003). The recent discovery that the *Caenorhabditis elegans* CLIC homologue is essential for the proper formation of the worm's excretory canal (Berry *et al*, 2003) supports a wider involvement of CLIC proteins in cellular functions beyond or requiring chloride transport. The importance of these functions is emphasized by the discovery that CLIC4 is among a small number of genes whose expression is upregulated in cutaneous stem cells (Morris *et al*, 2004).

The involvement of CLIC4 in the differentiation of several cell types, its importance in maintaining cell viability, its relationship with TNFα, p53, and c-myc, and the essentiality of chloride homeostasis in cell signaling prompted us to test whether CLIC4 could be a target for tumor therapy. To address this question, we created several keratinocyte-derived tumor cell lines constructed to express an antisense CLIC4 under the regulation of a tetracycline-sensitive promoter (Suh *et al*, 2005). Upon withdrawal of doxycycline from culture medium, the antisense construct is induced and CLIC4 levels decrease (Fig 2). Subsequent studies have indicated that expression of CLIC4 antisense also reduces the level of CLIC1 and CLIC5 in these cell lines (Suh *et al*, 2005). When these cells are grafted to a dermal graft bed on the dorsum of athymic nude mice, they grow as aggressive squamous cell carcinomas while the mice are fed a diet containing doxycycline (Fig 3). If doxycycline is withdrawn from the diet to induce CLIC4 antisense either at the time of

Table III. Characterization and biological activities of CLIC4

Induced by p53, TNFα, DNA damage, and differentiation
Localized to the cytoplasm and inner mitochondrial membrane
Contains functional NLS domain, consensus phosphorylation sites, and TM domain
Translocates to the nucleus from the cytoplasm in response to cell stress
Binds to Ran, NTF2, and importin-α
Overexpression or nuclear targeting causes cell cycle arrest or apoptosis
Direct response gene for p53 and c-myc
Upregulated in cutaneous stem cells (Morris *et al*, 2004)
Interacts with dynamin, tubulin, 14-3-3, and actin in rat brain (Suginta *et al*, 2001)
Induced by TGFβ in mammary myofibroblast transdifferentiation (Ronnov-Jessen *et al*, 2002)
Induced in 3T3-L1 cells transdifferentiating to adipocytes (Kitamura *et al*, 2001)

TNFα, tumor necrosis factor α; TGFβ, transforming growth factor β.

grafting or after tumor establishment, however, tumor growth is inhibited. Histological and immunohistochemical analyses of tumors indicate that the expression of the antisense CLIC4 is associated with a 3-fold increase in apoptotic cells (TUNEL assay) and a significant reduction in proliferating cells (BrdU staining). Further reduction in tumor growth is obtained by injecting TNFα intraperitoneally twice weekly, together with antisense expression (Fig 3). Together, these results suggest that CLIC4 reduction could be a novel target for therapy of cutaneous tumors.

The forgoing results indicate a role for CLIC4 in both keratinocyte differentiation and carcinogenesis. Previous studies have suggested that chloride transport participates in epidermal homeostasis (Ashley, 2003), but the discovery of CLIC proteins adds a new dimension to the process. A number of questions arise as a consequence of this discovery. What is the function of CLIC4 induction in differentiating keratinocytes? Does CLIC4, or other CLIC, proteins participate in the growth arrest and loss of viability associated with epidermal maturation? Does the increase in p53-mediated transcription in differentiating keratinocytes (Spandau, 1994; Fernandez-Salas *et al*, 1999) regulate

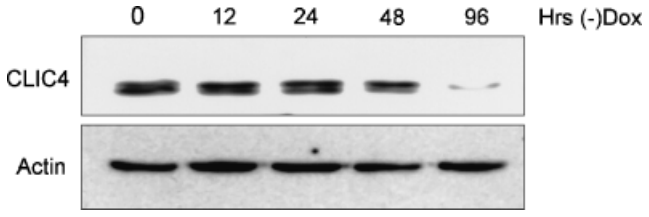

Figure 2
Genetically modified SP1 (chloride intracellular channel (CLIC)4AS SP1) cells were cultured in doxycline-containing media (0 h (−) Dox) and then the doxycycline was withdrawn to induce CLIC4 antisense. Cells were harvested at different times (12–96 h (−) Dox) after the doxycycline withdrawal and CLIC4-antisense-expressing cell lysates were analyzed by CLIC4 and actin immunoblots.

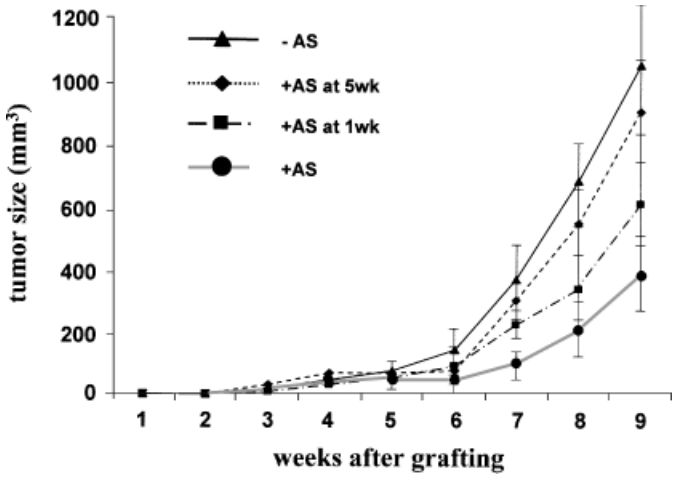

Figure 3
Chloride intracellular channel (CLIC)4AS SP1 cells were grafted on the back of athymic nude mice and the treated mice were divided into four groups (N = 10 for each group; a similar experiment was repeated three times) to analyze the anti-tumor effect of CLIC4 antisense *in vivo*. Mice were fed with mouse chow with (−AS, solid triangle with solid black line) or without (+AS, solid circle with gray line) doxycline (200 μg per kg) during the duration of the experiment or the mouse diet was changed from a doxycline-containing feed to a regular feed either at 1 wk (+AS at 1 wk, solid diamond with a fine dotted line) or 5 wk (+AS at 5 wk, solid square with a semi-dotted line) after grafting.

CLIC4 expression? Does the increase in PKC activation in maturing epidermal cells modify CLIC4 function? How does the subcellular localization of CLIC4 contribute to differentiation or carcinogenesis? Can we develop small molecule inhibitors for the CLIC proteins that would have a therapeutic benefit in the treatment of cutaneous or other malignancies? Will combined treatments with inhibitors of CLIC proteins and standard chemotherapeutic drugs enhance cancer regression? All of these questions are readily approachable in experimental models. The real challenge will be to translate results from these basic studies into clinical advances for the prevention and treatment of cutaneous diseases.

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