Re-expression of Detachment-inducible Chloride Channel mCLCA5 Suppresses Growth of Metastatic Breast Cancer Cells*

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The calcium-activated chloride channel hCLCA2 has been identified as a candidate tumor suppressor in human breast cancer. It is greatly down-regulated in breast cancer, and its re-expression suppresses tumorigenesis by an unknown mechanism. To establish a mouse model, we identified the mouse ortholog of hCLCA2, termed mCLCA5, and investigated its behavior in mammary epithelial cell lines and tissues. Expression in the immortalized cell line HC11 correlated with slow or arrested growth. Although rapidly dividing, sparsely plated cells had low levels of expression, mCLCA5 was induced by 10-fold when cells became confluent and 30-fold when cells were deprived of growth factors or anchorage. The apoptosis effector Bax was induced in parallel. Like hCLCA2, mCLCA5 was down-regulated in metastatic mammary tumor cell lines such as 4T1 and CSML-100. Ectopic re-expression in 4T1 cells caused a 20-fold reduction in colony survival relative to vector control. High mCLCA5 expression in stable clones inhibited proliferation and enhanced sensitivity to detachment. Moreover, mCLCA5 was induced in lactating and involuting mammary gland, correlating with differentiation and onset of apoptosis. Together, these results establish mCLCA5 as the mouse ortholog of hCLCA2, demonstrate that mCLCA5 is a detachment-sensitive growth inhibitor, and suggest a mechanism whereby these channels may antagonize mammary tumor progression.

Metastatic tumor cells commonly activate growth and survival pathways and inactivate tumor suppressive growth arrest and apoptosis mechanisms (1). Epithelial cells depend for their survival upon continuous signaling from ligated growth factor receptors and integrins bound to extracellular matrix proteins (2–6). Loss of such signaling triggers a default pathway of cell death called anoikis, detachment-induced apoptosis (7). Metastatic tumor cells are usually nearly impervious to anoikis due to mutations that inactivate apoptotic signaling, enhance survival signaling, or both (1). Much effort has been invested in identifying new tumor suppression mechanisms that are lost in the evolution of the metastatic cell.

Epithelial cells, from which most human cancers are derived, exist at a boundary between the organism and its external environment and so must maintain a vast array of plasma membrane ion channels, sensitive to a range of internal or external cues, to maintain homeostasis in the face of sudden environmental changes. A diverse suite of chloride channels in the plasma membrane help to regulate cell volume, membrane potential, and intracellular pH (pHi)

1. All of these properties vary with the proliferative state of the cell (10–12) and exert global effects on gene expression, the cytoskeleton, cell division, and apoptosis (11, 13–18).

Gruber and Pauli (19, 20) recently described a calcium-activated chloride channel, hCLCA2, that is down-regulated in breast tumors and tumor cell lines. Ectopic expression of hCLCA2 in cancer cell lines greatly reduced tumorigenicity in nude mice, suggesting that hCLCA2 is a tumor suppressor in breast cancer. hCLCA2 belongs to a recently discovered family of proteins, currently comprising about a dozen members, that are widely expressed in mammals but apparently not beyond (19–28). Various family members are found in epithelia, endothelia, and smooth muscle of multiple organs (21, 24, 25, 27, 28). CLCAs do not resemble any other known Cl− channel (i.e. CFTR, ligand-gated channels, or the CLC or CLIC families) or any other protein. The family is deeply divergent, some members sharing as little as 36% identity, yet all share certain signatory features. The prototypical CLCA family member is a type I transmembrane protein about 900 amino acids in length with a proteolytic cleavage near amino acid 680 that results in products of 90 kDa and 30–40 kDa that are found in close association on the exterior surface of the plasma membrane (19, 24, 26, 28). Another common feature is a symmetrical multiple-cysteine motif, C12XnC12XnC12XnC12Xn, in the amino-terminal tail. Potential phosphorylation sites for calmodulin kinase II and protein kinase C are consistent with calcium regulation, and ectopic expression of CLCA proteins leads to a novel chloride current that is activated by calcium and blocked by typical chloride channel inhibitors such as DIDS (19, 23, 26, 28). However, it has not been firmly established whether CLCAs conduct chloride directly or are instead channel regulators. In addition to chloride conductance, several members of this family have been shown to interact with integrin β4 via a short, semiconserved motif found in both the 90- and 30–40-kDa subunits (29).

Four CLCA family members have been reported in mouse, all only distantly related (~40% identical) to hCLCA2, an evolutionary outlier of the family (25). To establish an animal model of hCLCA2 in mammary gland development and neoplasia, we

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sought a true ortholog. We report here the identification of this ortholog, termed mCLCA5, and show that, like hCLCA2, it is expressed in normal mammary epithelium but down-regulated in metastatic cell lines. Furthermore, mCLCA5 is a detachment-inducible gene, the ectopic expression of which suppresses the growth and survival of a metastatic tumor cell line while having little effect on the growth of normal mammary epithelial cells. These results demonstrate that mCLCA5 is the murine counterpart of hCLCA2 and provide insight into the likely tumor suppression mechanism.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture—The mouse mammary epithelial cell line, HC11, was obtained from Dr. Nancy Hynes (Friedrich Miescher Institute, Basel, Switzerland). HC11 was maintained in complete medium (CM: Dulbecco’s modified Eagle’s medium, 100 units/ml penicillin, 100 μg/ml streptomycin, 10% fetal bovine serum, and 10 ng/ml recombinant human epidermal growth factor). CM, CM-100, and CM-100 were a gift from Dr. S. Zain (University of Rochester School of Medicine). The cell line series, 67NR, 4T07, and 4T1, was obtained from Dr. Fred Miller (Karmanos Cancer Institute). All tumor cell lines were grown in normal medium (NM: Dulbecco’s modified Eagle’s medium, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum). Seroma-free medium (C3M: Dulbecco’s modified Eagle’s medium, 100 units/ml penicillin, and 100 μg/ml streptomycin) was used in starvation and anchorage deprivation experiments.

Population doubling times were determined by seeding cells at different densities in triplicate in 24-well plates, trypanosinizing, and manually counting at 24-h intervals. Doubling times (D) were calculated using the formula D = log Nt/No/t, where t is time, No is the starting cell number, and Nt is the cell number at time t.

Identification and Cloning of mCLCA5—A BLAST search using the cDNA sequence of hCLCA2 revealed a previously unknown mouse CLCA that had sequence identity to hCLCA2. Two expressed sequence tags were obtained, corresponding to the 5′ and 3′ ends of the ORF (5′-CACAAAGATGGTCACTGTCTG-3′ and 5′-AGTCATCGGGCT/H11032/TCCCCGTGAATAAACCAGAG-3′), respectively, of the hCLCA2 ORF. Primers 5′-AGTCATCGGGCT/H11032/TCCCCGTGAATAAACCAGAG-3′ corresponding to the ends of the ORF (5′-CACAAAGATGGTCACTGTCTG-3′ and 5′-AGTCATCGGGCT/H11032/TCCCCGTGAATAAACCAGAG-3′) were reverse-transcribed and used as template for PCR with 5′-AGTCATCGGGCT/H11032/TCCCCGTGAATAAACCAGAG-3′ and 5′-CACAAAGATGGTCACTGTCTG-3′ as forward and reverse primers, respectively. The resulting colonies were harvested, reverse-transcribed, and subjected to PCR for mCLCA5 and GAPDH as described above. Bax primers were 5′-TCCCCGGACG-GCTTGGGAGC-3′ and 5′-CACAAAGATGGTCACTGTCTG-3′ (94 °C, 20 s; 55 °C, 45 s; 72 °C, 20 s; 35 cycles) (21).

Cell Death Assays—Aliquots of cells were plated after trypsinization (zero time point) or end-over-end rotation (24-h time point) and stained with propidium iodide (4 μg/ml). Cells that took up propidium iodide were counted as dead cells. Random fields of cells were counted totaling 500–1000 cells/time point, and average absolute percentages plus one standard deviation were plotted for comparison.

Survival Assays—4T1 and HC11 cells were electroporated with 5 μg of either pcDNA3.1Zeo vector or pcDNA3.1Zeo-mCLCA5 plus 1 μg of pEGFP-C3 (Clontech) to normalize for transfection efficiency. The cells were replated, allowed to recover for 72 h, and then treated with trypsin, resuspended, and counted with trypsin blue (In Vitrogen). Equal numbers of cells were seeded in 6-well plates and subjected to antibiotic selection for 10 days (NM plus 750 μg/ml zeocin for 4T1 and CM plus 400 μg/ml zeocin in HC11). Vector- and mCLCA5-transfected populations were normalized for transfection efficiency by counting the percentage of cells expressing GFP in five random fields (~150 cells/field). The resulting colonies were stained with crystal violet (5% solution in 20% methanol). After photography, the crystal violet was eluted with 10% acetic acid overnight, and the absorbance at 562 nm was determined to quantify differences in survival between vector- and mCLCA5-transfected cells.

RESULTS

Discovery and Cloning of mCLCA5—A BLAST search of the Ensembl Mouse Genome Server (32) using the hCLCA2 ORF revealed a series of expressed sequence tags clustered on chromosome 3 (H3 region) with sequence identity to hCLCA2. Two expressed sequence tags were identified with similarity to the 3′ and 5′ ends, respectively, of the hCLCA2 ORF.

Primers based on these sequences were used to amplify the entire ORF of the suspected ortholog by RT-PCR of mouse mammary epithelial cell line RNA. The resulting product showed high sequence identity to hCLCA2, 78% at the nucleotide level and 72% at the amino acid level (Fig. 1A), and was named mCLCA5 in accordance with established CLCA nomenclature, which is chronological within species (27). By contrast, other family members retain only 38–45% amino acid identity with hCLCA2 and cluster independently on a phylogenetic tree (Fig. 1B). In addition to the higher primary sequence identity, mCLCA5 shares two other distinct properties of hCLCA2: the spacing of the symmetrical multicysteine motif, C4X6C4X6C4X6C, as opposed to C4X2C4X2C4X2C4X2C in the rest of the family; and a frameshift mutation that results in an ~40-amino-acid carboxyl-terminal extension (20, 24). In addition, the conservation of most of the putative N-linked glycosylation and protein kinase C phosphorylation sites suggests structural and functional conservation.

To determine whether mCLCA5 is processed similarly to other family members, an HA epitope tag was appended to the
Immunoblotting of HEK293T cells transfected with this construct detected bands of ~110 kDa and ~20 kDa, corresponding respectively to the precursor and the carboxy-terminal processing product (Fig. 1).

Tissue Expression Pattern of mCLCA5—To determine whether mCLCA5 occurs in the same tissues as hCLCA2, its expression was assessed in a broad range of tissues by RT-PCR, first using a commercial cDNA array. Positive signals were then confirmed using manually isolated tissues. Although most adult tissues were negative, mCLCA5 was detected in many of the same locations as hCLCA2, including lung, kidney, uterus, and endothelial cells (20, 22) (Fig. 2A). However, mCLCA5 was also detected in heart and spleen and throughout the gastrointestinal tract. Thus, mCLCA5 tissue distribution overlaps, but is broader than, that of hCLCA2.

Most importantly for this study, hCLCA2 was reported in luminal epithelial cells of the mammary gland (19, 20). mCLCA5 was detected in mammary gland as well but only in fully differentiated lactating and involuting gland, correlating with slow or arrested growth and anoikis (Fig. 2B).

Expression of mCLCA5 in Mammary Cell Lines—hCLCA2 is highly expressed in the spontaneously immortalized mammary epithelial cell line MCF10A but down-regulated in tumor cell lines (19). To find whether mCLCA5 behaved similarly, we determined relative mRNA levels by RT-PCR in a series of mouse cell lines ranging from normal mammary epithelial cells to metastatic tumor cells. (The alternative of measuring protein levels was not possible due to the inability to develop an anti-mCLCA5 antibody despite five attempts.) The immortalized cell line HC11 has been used extensively to model the growth and differentiation of mammary epithelial cells in vitro. Like other immortalized mammary epithelial cell lines, HC11 cells undergo apoptosis when deprived of growth factors and anchorage (33–36). Starvation-induced apoptosis is potentiated
Fig. 2. Tissue expression pattern of mCLCA5. A, RNA was extracted from various mouse tissues, reverse-transcribed, and subjected to PCR (38 cycles) using primers spanning bp 27–687 of mCLCA5. GAPDH was amplified for 25 cycles as a control for RNA amount. The array had been normalized to \( \beta \)-actin. 

B, mCLCA5 expression was post-proliferative and correlated with growth arrest and predisposition to apoptosis.

Related sets of tumor cell lines with varying metastatic properties were selected for comparison. CSML-0 and CSML-100 are respectively non-metastatic and metastatic derivatives from the same tumor (37). Similarly, 67NR, 4T07, and 4T1 are respectively non-metastatic, very poorly metastatic, and highly metastatic lines derived from a single tumor and have been employed extensively to model breast cancer metastasis (38, 40). Accordingly, we found that the combination of starvation and detachment accelerated mCLCA5 induction in HC11. Expression was induced already by 1 h after detachment, peaked at 30-fold by 4 h, and abated by 48 h after detachment. Bax, an early apoptosis marker, was induced and declined in parallel (Fig. 5A). On the other hand, in 4T1 cells, mCLCA5 was induced only briefly by detachment (Fig. 5A), and cell death after 24 h was much lower (5% versus 18% for HC11; Fig. 5B). The co-induction of mCLCA5 with Bax preceding apoptosis again suggests that mCLCA5 plays some role in cell cycle arrest and/or apoptosis induction.

Reduced Survival of HC11 and 4T1 upon Ectopic Expression of mCLCA5—To investigate this possibility, we employed a colony formation assay to determine the effect of ectopic expression of mCLCA5 on growth and survival of HC11 and 4T1 cells. This assay has been used extensively to demonstrate the growth inhibitory effects of tumor suppressors such as p21WAF1, p16INK4A, and p19ARF (41, 42). Cells were electroporated with mCLCA5 cDNA cloned into pcDNA3.1-Zeo or
with empty vector, along with pEGFP-C3 as a control for transfection efficiency. The cells were seeded at equal densities, subjected to zeocin selection for 10 days, and then stained with crystal violet. Although mCLCA5 overexpression decreased HC11 colony formation by about 2-fold (Fig. 6A), overexpression in 4T1 caused a 20-fold decrease in colony number, confirming that metastatic tumor cells are especially sensitive to mCLCA5 (Fig. 6B).

To better understand how mCLCA5 impaired tumor cell survival, 4T1 clones were selected that expressed mCLCA5 at different levels (Fig. 7A, inset). Doubling times varied with mCLCA5 expression. Although the vector-transfected clone doubled in 17.5 h, the moderate (15-fold) mCLCA5 expresser doubled in 25.7 h, and the highest (30-fold) expresser doubled in 42.2 h (Fig. 7A). In addition to retarding growth, mCLCA5 overexpression suppressed focus formation by confluent 4T1 cells (Fig. 7B). To determine whether mCLCA5 sensitized 4T1 cells to anoikis, cells were detached, and cell death was assayed by propidium iodide uptake after 24 h in suspension. Although only a slight increase was observed for the moderate expresser, the higher expresser exhibited a 2-fold increase in cell death relative to vector-transfected cells (Fig. 7C). Nuclear fragmentation typical of apoptosis was observed in propidium iodide-stained cells. This association between mCLCA5 expression and growth arrest or apoptosis in vitro may explain its induction late in mammary development in vivo, in fully differentiated lactating or involuting gland (Fig. 2B), and its loss from metastatic tumor cells.

**DISCUSSION**

Here we describe the mouse ortholog of the human CLCA2 breast cancer tumor suppressor, mCLCA5. This orthology is evidenced by primary amino acid sequence, similar tissue distribution, and loss of expression with tumor progression. Several aspects of mCLCA5 behavior in mouse may provide insight into tumor suppression by hCLCA2 in human breast cancer. 1) First, expression in normal cells correlates with slow or arrested growth. 2) Second, induction by cell detachment or starvation occurs in normal cells but inefficiently or not at all in metastatic tumor cells. 3) Third, metastatic cells lose expression, and forced re-expression greatly inhibits proliferation and enhances detachment-sensitivity. 4) Finally, mCLCA5 expression in mammary gland is induced in fully differentiated gland when cell division is reduced or arrested. These results suggest that mCLCA5, and perhaps by extrapolation hCLCA2, somehow inhibits cell division in response to loss of signaling from growth factor- and integrin-dependent pathways. The conditional expression in mammary gland is in contrast with its behavior in other adult organs such as pancreas and gastrointestinal tract. It is possible that mCLCA5 is regulated differently in those organs at a post-transcriptional level or that it is only expressed in tissues or subpopulations of cells that are growth-arrested or apoptotic. These questions await the development of antibodies suitable for higher resolution immunohistochemical studies.

How might a channel protein evoke such effects? A number of recent studies link plasma membrane ion channels to progression of the cell cycle and cancer. Evidence is especially compelling that K+ channels are required for cell cycle progression. For example, pharmacological blockade of K+ channels inhibits
proliferation of multiple cell types, including normal lymphocytes, melanoma, breast and prostate cancer cell lines, and glial cells (43–47). Concordantly, some K\textsubscript{+}/H11001 channels are up-regulated during tumorigenesis and are pro-oncogenic. For example, the HERG channel is constitutively activated in a broad range of cancer cell types, and HERG-mediated K\textsuperscript{+} currents stimulate proliferation of several cell types (48, 49). Similarly, the TASK3 channel is up-regulated in a variety of cancers and has been demonstrated to stimulate tumor cell proliferation and apoptosis resistance; mutation of the K\textsuperscript{+}/H11001 filter abrogated both channel function and proliferative effects (50).

Plasma membrane chloride currents also vary with the cell cycle, but their relation to specific chloride channels and to cell proliferation is less well established. In glioma cells (16), B lymphocytes (51), and nasopharyngeal carcinoma cells (10), Cl\textsuperscript{-} currents were observed to peak in G\textsubscript{1}, ebb in S phase, and peak again in mitosis (10). Blockade of Cl\textsuperscript{-} channels has variable effects depending on the cell type and the blocking agent. For example, cell proliferation was enhanced by chlorotoxin treatment of astrocytoma cells (15) or DIDS treatment of Schwann cells (52) or B cells (53), but channel blockade in other cell types slowed or arrested proliferation (10, 11). Because of the multiplicity of Cl\textsuperscript{-} channel subtypes, the lack of subtype-specific blockers, and the fact that some channels are still known only by their electrophysiological signatures, the genetic identity of the molecules responsible for these currents remains obscure (8). At present, the best genetic evidence comes from studies of CLCA down-regulation in various cancers. In addition to the loss of hCLCA2 in human breast cancer and the data reported herein, we also detected down-regulation of other mouse CLCA family members, mCLCA1 and -2, in mammary tumor cell lines (21) and transformed endothelial cells.\textsuperscript{2} Others have reported the near universal down-regulation of hCLCA1 and -4 in human colon cancer biopsies when compared with autologous normal tissues (54). Whether CLCA-mediated chloride currents vary with the cell cycle has not been established.

The induction of mCLCA5 by growth factor and anchorage deprivation in parallel with Bax suggests that mCLCA5 plays some role in anoikis and may explain why metastatic cells lose expression, whereas non-metastatic cells do not. The enabling role of Cl\textsuperscript{-} channels in apoptosis is increasingly well documented (55, 56), and two mechanisms have been proposed, regulation of either cell volume or pH\textsubscript{i}. For example, Maeno \textit{et al.} (57) showed that chloride efflux is essential for cell shrinkage early in the apoptotic cascade and that channel blockade effectively prevented shrinkage and rescued a variety of cell types from staurosporine-induced apoptosis. On the other hand, Szabo \textit{et al.} (58) observed an outwardly rectified

\textsuperscript{2} R. C. Elble, unpublished data.
chloride current in response to Fas ligation in lymphocytes, and again, blocking the current prevented apoptosis but apparently by inhibiting the reduction of pH, an early and obligate step in the apoptotic cascade for many cell types (59, 60). Likewise, in two model systems, CFTR has been shown to potentiate apoptosis by promoting intracellular acidification. Gottlieb et al. (68) found that transfection of wild-type CFTR into mammary epithelial cells potentiated apoptosis by inducing intracellular acidification, whereas inactive mutant CFTR did not (60). Similarly, Barriere et al. (9) demonstrated that transfection of CFTR sensitized PS120 lung fibroblasts to apoptosis by reducing pH and that preventing the change in pH by co-transfection of the NHE1 Na+/H+ exchanger also prevented apoptosis. Mechanistically, the drop in pH has been shown to activate apoptosis effectors such as caspases and acid endonucleases (61, 62). Thus, it will be important to determine whether mCLCA5 expression in stressed cells reduces pH or changes cell volume. In addition to these effects, another early event in the apoptotic cascade that is mediated by ion channels is the release of calcium from intracellular stores (63). This is noteworthy in view of the findings that Bax expression is induced in parallel to mCLCA5, and both proteins are functionally activated by calcium release from the sarcoplasmic reticulum (25, 64).

The effects of CLCA channels with respect to cell growth and stress-induced apoptosis are reminiscent of those produced by the mostly nuclear CLIC family of intracellular chloride channels (65, 66). The nuclear channel NCC27 is expressed at G2/M, and specific blockade of the channel arrests cells at G2/M (65). On the other hand, CLIC4 is a p53- and stress-inducible channel that potentiates apoptosis by an unknown mechanism (66). Since members of both the CLCA and the CLIC families are expressed in many of the same cell types, it is possible that they perform similar functions for different cellular compartments. mCLCA5 is not the only CLCA gene induced in mammary involution. We previously described a pair of genes, mCLCA1 and mCLCA2, that are nearly identical to each other but reciprocally regulated (21). mCLCA1 is repressed during mammary involution and by detachment in vitro, whereas mCLCA2 is induced by these conditions, implying opposing functions. The behavior of mCLCA5 differs from that of mCLCA2 however in two respects: mCLCA5 is induced more rapidly by stress, and it is not repressed by prolactin (21). It is possible that mCLCA2 and mCLCA5 perform similar functions but that mCLCA5 is an early response gene, whereas mCLCA2 acts later, after the cessation of lactational signaling and intensification of apoptotic signaling. In humans, no orthologs exist for mCLCA1 or mCLCA2, so hCLCA2 must perform all CLCA functions in human mammary development (67). It will be interesting to determine whether hCLCA2 is similarly responsive to starvation and detachment and has growth-retarding and proapoptotic properties in human mammary epithelial cells. These studies set the stage for a more definitive investigation of the role of mCLCA5 in mammary development and neoplasia by gene ablation in mouse.

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3 R. C. Elble and J. R. Beckley, unpublished data.
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