Both Cleavage Products of the mCLCA3 Protein Are Secreted Soluble Proteins*

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Members of the chloride channels, calcium-activated (CLCA) family of proteins and in particular the murine mCLCA3 (alias gob-5) and its human ortholog hCLCA1 have been identified as clinically relevant molecules in diseases with secretory dysfunctions including asthma and cystic fibrosis. Initial studies have indicated that these proteins evoke a calcium-activated chloride conductance when transfected into human embryonic kidney cells 293 cells. However, it is not yet clear whether the CLCA proteins form chloride channels per se or function as mediators of other, yet unknown chloride channels. Here, we present a systematic biochemical analysis of the posttranslational processing and intracellular trafficking of the mCLCA3 protein. Pulse-chase experiments after metabolic protein labeling of mCLCA3-transfected COS-1 or human embryonic kidney 293 cells revealed cleavage of a primary 110-kDa mCLCA3 translation product in the endoplasmic reticulum into a 75-kDa amino-terminal and a 35-kDa carboxyl-terminal protein that were glycosylated and remained physically associated with each other. Confocal fluorescent analyses identified both cleavage products in vesicles of the secretory pathway. Neither cleavage product was associated with the cell membrane at any time. Instead, both subunits were fully secreted into the extracellular environment as a soluble complex of two glycoproteins. These results suggest that the two mCLCA3 cleavage products cannot form an anion channel on their own but may instead act as extracellular signaling molecules. Furthermore, our results point toward significant structural differences between mCLCA3 and its human ortholog, hCLCA1, which is thought to be a single, non-integral membrane protein.

Several members of the CLCA2 family of proteins have been shown to mediate an anion current that is activated by intracellular calcium. Electrophysiological data derived from whole-cell or single-channel patch clamp analyses of HEK 293 or COS-1 cells heterologously transfected with different CLCA homologues consistently identified a transmembrane current that was activated by calcium ionophores including ionomycin. These currents were blocked by several chloride channel blockers including 4,4’-disothiocyanostilbene-2,2’-disulfonic acid (1, 2–4, 6, 8, 12–16, 18–21). Hence, the gene products have been tentatively designated as chloride channels, calcium-activated (1).

At present, 15 CLCA members are known in 6 mammalian species. These include four human homologues (hCLCA1 (2), hCLCA2 (4, 5), hCLCA3 (6), and hCLCA4 alias hCaCC2 (3)), six murine homologues (mCLCA1 (6, 7), mCLCA2 (8), mCLCA3 alias gob-5 (9–11), mCLCA4 (13), mCLCA5, and mCLCA6 (14)), two bovine members (bCLCA1 alias CaCC (15, 16) and bCLCA2 alias Lu-ECAM-1 (17, 18)), one porcine member (pCLCA1 (19)), one equine member (eCLCA1 (20)), and one rat homologue (21).

Initial protein analyses have identified for the majority of CLCA members a consensus protein model of a 100-kDa primary translation product that contains an amino-terminal signal peptide. This primary protein is thought to become glycosylated to form an ~130-kDa glycoprotein that is then cleaved into an ~90-kDa and an ~40-kDa subunit. The two subunits were hypothesized to form a transmembrane protein with four or five membrane spanning domains, possibly shaping a pore through the membrane, consistent with the proposed function as a channel (2, 4). This hypothetical protein conformation was established based on several protein prediction programs. In addition to an unequivocal cleavable signal peptide at their amino terminus, computer-aided hydrophobicity plots based on the Kyte-Doolittle analysis predicted four putative transmembrane domains for bCLCA1 (15), bCLCA2 (18), hCLCA1 (2), and mCLCA1 (6) and five potential transmembrane domains for hCLCA2 (4). However, this consensus model has remained questionable so far. For example, the first murine homologue, mCLCA1, was further analyzed based on other transmembrane prediction programs. Although the TMPRED software predicted only two potential transmembrane domains, the SOSUI and PSORT II algorithms predicted only one possible transmembrane domain at the very carboxyl terminus of the polypeptide (7). An alternative model with only a single transmembrane domain was established for hCLCA1 using the SMART program (22, 23). Sequence homologies with the extracellular α2-integrin collagen receptor domain in the

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§ The abbreviations used are: CLCA, chloride channels, calcium-activated; mCLCA3, murine CLCA3; hCLCA1, human CLCA1; endo H, endo-β-acetylglucosaminidase H; PBS, phosphate-buffered saline; GT, galactosyltransferase; YFP, yellow fluorescent protein; ER, endoplasmic reticulum; HEK, human embryonic kidney; PNGase, peptide N-glycosidase F.
central region of the hCLCA1 protein suggested a single trans-
membrane domain only close to the carboxyl terminus. In
sharp contrast to all these models are recent studies which have
proposed that the larger amino-terminal cleavage products of
hCLCA1 and mCLCA3 may be membrane-associated proteins
without any transmembrane domain (24).

Other CLCA family members that comprise only the amino-
terminal third of the CLCA consensus sequence were shown to
encode soluble, fully secreted proteins, namely a variant (clone
4) of the bovine CLCA2 (18) and the human hCLCA3 (5). Inter-
estingly, a secreted nature was also observed for mCLCA3 in
immunolocalization studies using native murine tissues; immuno-
electron microscopy demonstrated the protein in mucin granules of intestinal goblet cells and in the extracellular
mucous layer but no association with the cell membrane
(11). In summary, previous results led to contradictory
hypotheses on the transmembrane topology of CLCA pro-
teins, even for the same protein.

Here, we present a systematic biochemical analysis of the
posttranslational processing and intracellular trafficking of the
mCLCA3 protein. The results clearly show that the primary
mannose-rich glycosylated 110-kDa mCLCA3 translation
product is cleaved into a 75-kDa amino-terminal and a 35-kDa
carboxyl-terminal protein in the endoplasmic reticulum. No
association with the plasma membrane was observed using bio-
chemical and immunofluorescent assays. Instead, both sub-
units were fully secreted as a complex glycosylated protein
complex into the extracellular environment. The subunits
remained associated with each other inside and outside the cell.
Our results are at variance with a previously reported model for
its human ortholog, hCLCA1, as a single, non-integral mem-
brane protein (24) but support the notion that mCLCA3 cannot
form an anion channel on its own but may instead act as a
soluble signaling molecule.

EXPERIMENTAL PROCEDURES

Computer-aided Analyses—The mCLCA3 amino acid
sequence (GenBank™ accession number NP_059502) was
screened for a signal sequence using the SignalP 3.0 software (25–
27). Hydrophobicity analyses were carried out using the Kyte-
Doolittle (28), SOSUI (29), HMMtop (30), TMPRED (31), DAS
(32), and PSORT II (33) software to identify putative transmem-
brane regions of the mCLCA3 protein. The software NetNGly was
used to identify potential N-linked glycosylation sites.

Materials—Reagents used for PCR were purchased from
Peqlab, Erlangen, Germany, and nucleotides were obtained
from Invitrogen. Tissue culture dishes were obtained from
Greiner, Hamburg, Germany. Streptomycin, penicillin, Dul-
becco’s modified Eagle’s medium (DMEM), methionine-free
DMEM, and trypsin were purchased from PAA, Pasching, Aus-
tria. Fetal calf serum, peptatin, leupeptin, aprotinin, trypsin
inhibitor, and molecular mass standards for SDS-PAGE were
purchased from Sigma. Phenylmethylsulfonyl fluoride and anti-
pain were obtained from Roche Diagnostics. Lipofectamine
was purchased from Invitrogen. L-[35S]Methionine (>1000
Ci/mmol) and protein A-Sepharose were obtained from Amer-
sham Biosciences. Acrylamide and tetramethylethylenediam-
ine were purchased from Carl Roth GmbH, Karlsruhe,
Germany. SDS, ammonium persulfate, dithiothreitol and Tri-
ton X-100 (TX-100) were obtained from Merck. Endo-β-
acetylglucosaminidase H (endo H) and PNGase F were pur-
bought from New England Biolabs, Frankfurt, Germany. The
pEYFP-N1, galactosyltransferase (GT)-DsRed2, and
dsRed2-ER vectors were purchased from Clontech Laborato-
ries Inc., Heidelberg, Germany. Restriction enzymes and
prestained SDS marker were obtained from MBI Fermentas, St.
Leon-Rot, Germany. For immunoprecipitation and Western blot
analyses of YFP-tagged mCLCA3 protein, the BD living colors™
full-length Aequorea victoria polyclonal antibody or mono-
clonal antibody (IL-8) (anti-YFP; BD Biosciences) was used.
For Western blot analyses of YFP-tagged mCLCA3 protein and
for immunofluorescence microscopy, the rabbit polyclonal anti-
p3b was used. Western blot analyses of YFP-tagged mCLCA3 protein were carried out
using the rabbit polyclonal antibodies anti-p3a and p3bs used
which were generated against synthetic oligopeptides corre-
spending to amino acids 83 to 97 (p3a) or 253 to 267 (p3b) of
the mCLCA3 protein (11). Anti-rabbit horseradish peroxidase
(HRP)-linked IgG antibody (anti-rabbit HRP; Cell Signaling,
Beverly, MA) or anti-mouse IgG (H+L) (Biologo, Kronshagen,
Germany) were used as secondary antibody for Western blot
analyses. Enhanced chemiluminescence was used to visualize
the protein labeling in the Western blot analyses (ECL; Amer-
sham Biosciences). Goat anti-rabbit IgG-AlexaFluor 568 or
IgG-AlexaFluor 433 (anti-rabbit Fluor; Molecular Probes) were
used as secondary antibody for immunofluorescence analyses.

Construction of a YFP-tagged mCLCA3 Protein—The previously
cloned mCLCA3 open reading frame (11) was transferred
without the stop codon into the pEYFP-N1 vector. For inser-
tion into the vector, oligonucleotide primers flanking the
mCLCA3 open reading frame and containing appropriate link-
ers were designed for PCR (upstream primer, 5’-ATCTCGAG-
CTATGGAATCTTTCAGAGTCGCTGCTG-3’; with the Xhol
restriction site underlined; downstream primer 5’-ATAGCT-
TTGTCAAACCTAGTGTCACCTGC-3’ with the HindIII
restriction site underlined). PCR was performed using Pwo
DNA polymerase and the following conditions: 35 cycles of
95 °C for 2 min, 65 °C for 40 s, and 72 °C for 2 min with a time
increment of 8 s per cycle for each extension step (72 °C) fol-
lowed by a final extension at 72 °C for 10 min. The amplified
product was cut with XhoI and HindIII and subsequently
cloned into the respective XhoI/HindIII sites of the pEYFP-N1
vector upstream of the YFP open reading frame. Complete
sequencing excluded PCR and reading frame errors (Gen-
Bank™ accession number AB 016592) and verified correct car-
boxyl-terminal linkage to the YFP open reading frame.

Transient Transfection of COS-1 Cells and HEK 293 Cells—
Cells were kept in 10-cm culture dishes with 10 ml of Dulbecco’s
modified Eagle’s medium containing 10% fetal calf serum and
1% penicillin/streptomycin in culture medium at 37 °C in the
presence of 5% CO2. Between medium changes cells were
washed with prewarmed phosphate-buffered saline (PBS)
unless mentioned otherwise. For confocal fluorescence and all
biochemical analyses, COS-1 cells were transiently transfectioned with the mCLCA3-YFP vector or the mCLCA3 vector (11)
using DEAE-dextran as described previously (34). Briefly, 5 μg
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of plasmid-DNA were incubated with 10 μl of DEAE-dextran (50 mg/ml) in 1.5 ml of Dulbecco’s modified Eagle’s medium at room temperature for 30 min. Subsequently, cells at 40% confluency were incubated for 90 min with the DNA-DEAE complex. The complex was then replaced for 3 h with culture medium containing chloroquine (5 mg/ml) followed by incubation in unsupplemented culture medium. HEK 293 cells were transiently transfected with the same vectors using Lipofectamine according to the manufacturer’s protocol to confirm and reproduce the data in a second cell line. Mock transfection was performed with the vector alone.

Biosynthetic Labeling and Immunoprecipitation—48 h after transfection COS-1 cells were biosynthetically labeled. After precubination with 5 ml of methionine-free minimum Eagle’s medium for 2 h, the medium was replaced by a similar medium containing 100 μCi of [35S]methionine. Labeling was performed for 6 h. In pulse-chase experiments the cells were pulsed with 100 μCi of [35S]methionine for 20 min followed by a chase with nonradioactive methionine for different time intervals. Cells and medium were analyzed separately. The medium was removed and centrifuged for 5 min at 1000 × g at 4 °C, and the supernatant was kept on ice until immunoprecipitation. Cells were washed twice with cold PBS and solubilized with 1 ml of lysis buffer for 30 min on ice. The lysis buffer contained 25 mM Tris-Cl (pH 8.0), 50 mM NaCl, 0.5% sodium deoxycholate, and 0.5% Triton X-100 and was supplemented with 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 μg/ml antipain, and 50 μg/ml trypsin. The cell lysate was centrifuged at 4 °C at 10,000 × g to remove cell debris, and the supernatant was subjected to immunoprecipitation. After a preclearing step of medium and cell lysates with protein A-Sepharose beads, immunoprecipitation was performed using anti-YFP or anti-p3b antibodies. In a fashion similar to Jacob et al. (35), medium and cell lysates were incubated separately for 2 h at 4 °C with the respective antibody first (1:5000) and then with protein A-Sepharose beads (100 or 20 μl, respectively). The immunoprecipitates were subjected to SDS-PAGE on 10% slab gels, and the radioactive proteins were visualized using a Phospho-Imager (Bio-Rad).

Western Blot Analysis—48 h after transfection, COS-1 and HEK 293 cells and medium were processed as described above. Immunoprecipitates were boiled in 3-fold SDS-PAGE Laemml buffer, split in two, and analyzed by 10% SDS-PAGE. Native protein lysates from murine colon mucosa (20 μg/lane (11)) served as positive controls. After SDS-PAGE, proteins were electroblotted onto nitrocellulose membranes. Membranes were blocked for 90 min at room temperature with Tris-buff- ered saline containing 0.2% Tween 20 and 5% (w/v) nonfat milk and subsequently probed for 1 h at room temperature with anti-YFP (1:1,000) or anti-p3b (1:750) or anti-p3a (1:100) diluted in blocking buffer. Membranes were then incubated for 1 h at room temperature with secondary horseradish peroxi- dase-conjugated anti-rabbit (1:2,000) or anti-mouse IgG (H+L) (1:200) diluted in Tris-buffered saline-Tween. Protein labeling was developed using enhanced chemiluminescence.

Endoglycosidase Treatment—Digestion of the immunopre- cipitates of mCLCA3 protein or mCLCA3-YFP tagged with endo H or PNGase F before SDS-PAGE analysis and Western blotting was performed as described previously (36).

Confocal Fluorescence Microscopy—Confocal images of living cells were generated 48 h after transfection using a Leica TCS SP2 microscope equipped with a ×65 water planapo- chromat lens and an argon laser (Leica Microsystems, Solms, Germany). Dual color YFP and dsRed images were obtained by sequential scans with the 514-nm excitation line of an argon laser or the 543-nm excitation line of a He/Ne laser, respectively.

Immunofluorescence Microscopy—COS-1 cells transfected with mCLCA3 and YFP tagged mCLCA3 were grown for 48 h on coverslips. The attached cells were fixed with 4% paraformaldehyde for 4 h, washed twice with PBS, and quenched twice for 10 min with 50 mM NH4Cl. After blocking in PBS containing 1% BSA and 0.5% saponin (blocking buffer) for 30 min, the samples were incubated with anti-p3b (1:250) in blocking buffer for 45 min at room temperature. After 4 washes in blocking buffer, cells were incubated with secondary fluorochrome-labeled anti-rabbit antibody (1:1000; AlexaFluor 568 or 433) in blocking buffer for 45 min. After repeated washes in blocking buffer and in PBS at room temperature, the coverslips were fixed on a glass slide with moviol. Dual color images were generated by excitation of the YFP protein and the AlexaFluor dye at 514 or 568 nm, respectively. The AlexaFluor antibody 433 was excited at 433 nm.

RESULTS

Computer-aided Hydrophobicity Plots and Glycosylation Site Prediction of the mCLCA3 Primary Polypeptide

To date, prediction of CLCA transmembrane domains has mainly been based on Kyte-Doolittle analyses, leading consistently to CLCA protein conformation models with a single hydrophobic, amino-terminal, cleavable signal sequence, and four or five potential transmembrane domains (2, 4, 6, 7, 16, 18). In the present study analyses of the mCLCA3 protein using the transmembrane prediction programs Kyte-Doolittle (Fig. 1A), SOSUI, HMMtop, TMPRED, DAS, and PSORT II all confirmed the existence of a hydrophobic amino-terminal sequence consistent with a cleavable signal sequence. However, analyses of the mCLCA3 amino acid sequence using Kyte-Doolittle, SOSUI, HMMtop, DAS, and PSORT II did not predict any transmembrane domain. In contrast, TMPRED software suggested two potential transmembrane domains at amino acids 375–396 and 464–484 (Fig. 1B) but no carboxyl-terminal transmembrane domain as was predicted for mCLCA1 using this software (7). In summary, computer-aided prediction of potential transmembrane models for mCLCA3 resulted in ambiguous predictions depending on the software used, predicting either no or two membrane spanning regions.

To identify potential sites of N-linked glycosylation within the mCLCA3 protein, the amino acid sequence was analyzed using the NetNGly software program. Complete analysis revealed six potential sites at amino acids 504, 770, 804, 810, 836, and 887. Considering the conserved posttranslational cleavage of all CLCA primary translation products studied to date into an ~80–90-kDa amino-terminal and an ~30–40-
compartment. After 20 min of pulsing with [35S]methionine (0
p3b antibody directed against the mCLCA3 amino terminus, pulse-chase experiments. After immunoprecipitation using the
transiently transfected COS-1 cells and their supernatant in
ecessing of the mCLCA3 protein was investigated in detail using
microscopic analyses of the subcellular mCLCA3 protein dis-
tribution pattern in intestinal goblet cells suggested an at least
terminal cleavage product (1). Because several attempts to gen-
erate antibodies against the carboxy terminus of different
CLCA proteins including mCLCA3 in our and other laborato-
ries have been unsuccessful to date (not shown), we generated a
mCLCA3-YFP fusion construct to study the cellular processing
and transport of the carboxy-terminal mCLCA3 cleavage
product. This fusion protein was analyzed in transiently trans-
sected COS-1 cells, and the YFP-containing carboxy-terminal
mCLCA3 protein species was recovered by immunoprecipita-
tion using anti-YFP antibodies. Pulse-chase experiments
revealed several [35S]methionine-labeled protein species both
in the cell lysate and the medium (Fig. 3). At 0 h of chase, three
labeled bands were detected in the cell lysate at ~105, 130, and
140 kDa, whereas the medium did not contain any labeled
mCLCA3-YFP protein (Fig. 3, left panels). Three additional
weaker bands at ~60, 75, and 115 kDa were also present in the
mock-transfected cells and, thus, were considered unspecific.
After 0.5 h of chase, two additional strongly labeled protein
bands appeared in the cell lysate at ~65 and 75 kDa. Only after
1 h of chase, two labeled bands were detected in the medium at
~77 and 68 kDa. Up to 6 h of chase, labeled bands in the cell
lysate gradually diminished, whereas the intensities of the two
labeled proteins in the medium slowly increased (Fig. 3). Longer
chases of up to 23 h resulted in a total loss of the labeled
mCLCA3-YFP protein species in the cell lysate, whereas the
protein bands in the medium were preserved. In conclusion, the
results of this experiment identified three distinct early intra-
cellular mCLCA3 protein species with sizes similar to the ami-
no-terminal-labeled protein species plus ~30 kDa of the added
tag. In addition, two proteins of 65 and 75 kDa were detected
in the cells at 0.5 h of chase and two further proteins of 68 and 77
kDa in the medium at 1 h of chase and thereafter. Only the latter
two proteins persisted in the medium, whereas all intracellular
labeled proteins disappeared after ~6 h of chase.

Analysis of Cellular Protein Processing and Secretion by Pulse-
chase Experiments

mCLCA3 Protein Species—Our previous immunoelectron
microscopic analyses of the subcellular mCLCA3 protein dist-
ribution pattern in intestinal goblet cells suggested an at least
in part secreted protein (11). In the present study cellular pro-
cessing of the mCLCA3 protein was investigated in detail using
transiently transfected COS-1 cells and their supernatant in
pulse-chase experiments. After immunoprecipitation using the
p3b antibody directed against the mCLCA3 amino terminus,
cell lysates and medium were analyzed side by side to detect
mCLCA3-derived amino-terminal protein fragments in either
compartment. After 20 min of pulsing with [35S]methionine (0
h chase), 3 [35S]methionine-labeled protein bands were
detected in the cell lysate at ~72, 100, and 110 kDa, whereas the
medium did not contain any labeled mCLCA3 protein (Fig. 2,
left panels). After 0.5 h of chase, an additional band was
detected in the cell lysate at ~75 kDa, whereas after 1 h of chase
an ~77-kDa band appeared in the medium (Fig. 2, asterisk).
Occasionally, two faint bands at 100 and 110 kDa were detected
in the medium. Possibly, these bands represent secreted unprocessed mCLCA3-proteins.
Up to 12 h of chase, labeled bands in the cell lysates gradually
disappeared, whereas the intensity of the 77-kDa band in the
medium increased (Fig. 2, right panels). These data clearly iden-
tified three primary intracellular protein species, one intracel-
lular 75-kDa processing product and a single secreted 77-kDa
protein, all containing the epitope of the p3b antibody close to
the amino terminus of the primary translation product.

mCLCA3-YFP Fusion Protein Species—CLCA precursor pro-
teins have consistently been shown to be posttranslationally
processed into a large amino-terminal and a smaller carboxyl-
terminal cleavage product (1). Because several attempts to gen-
erate antibodies against the carboxy terminus of different
CLCA proteins including mCLCA3 in our and other laborato-
ries have been unsuccessful to date (not shown), we generated a
mCLCA3-YFP fusion construct to study the cellular processing
and transport of the carboxy-terminal mCLCA3 cleavage
product. This fusion protein was analyzed in transiently trans-
sected COS-1 cells, and the YFP-containing carboxy-terminal
mCLCA3 protein species was recovered by immunoprecipita-
tion using anti-YFP antibodies. Pulse-chase experiments
revealed several [35S]methionine-labeled protein species both
in the cell lysate and the medium (Fig. 3). At 0 h of chase, three
labeled bands were detected in the cell lysate at ~105, 130, and
140 kDa, whereas the medium did not contain any labeled
mCLCA3-YFP protein (Fig. 3, left panels). Three additional
weaker bands at ~60, 75, and 115 kDa were also present in the
mock-transfected cells and, thus, were considered unspecific.
After 0.5 h of chase, two additional strongly labeled protein
bands appeared in the cell lysate at ~65 and 75 kDa. Only after
1 h of chase, two labeled bands were detected in the medium at
~77 and 68 kDa. Up to 6 h of chase, labeled bands in the cell
lysate gradually diminished, whereas the intensities of the two
labeled proteins in the medium slowly increased (Fig. 3). Longer
chases of up to 23 h resulted in a total loss of the labeled
mCLCA3-YFP protein species in the cell lysate, whereas the
protein bands in the medium were preserved. In conclusion, the
results of this experiment identified three distinct early intra-
cellular mCLCA3 protein species with sizes similar to the ami-
no-terminal-labeled protein species plus ~30 kDa of the added
tag. In addition, two proteins of 65 and 75 kDa were detected
in the cells at 0.5 h of chase and two further proteins of 68 and 77
kDa in the medium at 1 h of chase and thereafter. Only the latter
two proteins persisted in the medium, whereas all intracellular
labeled proteins disappeared after ~6 h of chase.

Protein Processing and Cellular Distribution of mCLCA3-YFP
Fusion Protein Species

Western blot analyses were used to immunologically iden-
tify the [35S]methionine-labeled protein species detected in the
pulse-chase experiments. For simultaneous detection of
mCLCA3 amino-terminal and mCLCA3-YFP carboxy-terminal
protein species derived from transiently transfected COS-1
and HEK 293 cells, immunoprecipitation was performed on cell
lysates and medium using anti-YFP antibodies followed by
Western blot analyses with anti-YFP or anti-mCLCA3 (p3b)
antibodies, respectively.

![Figure 1: Software-based predictions of mCLCA3 structural features. A, no peaks were present in the Kyte-Doolittle algorithm with scores greater than 1.8 kcal/mol (horizontal line, default threshold in the algorithm used), suggesting no transmembrane regions in addition to the signal sequence. B, in contrast, TMPRED software with a window size between 17 and 33 amino acids predicted two potential transmembrane domains (TM1 and TM2) at amino acids 375 to 396 and 464 to 484 (black segments). No other prediction software (SOSUI, HMMtop, DAS, and PSORT II) identified potential transmembrane domains. C, NetNGlyc predicted six potential asparagine-linked glyco-
sylation sites (tree like structures), three of which (tree like structures, *) were actually predicted to get glycosylated.](https://example.com/figure1.png)

![Figure 2: Biosynthesis, processing, and secretion of the mCLCA3 protein in transfected COS-1 cells. The mCLCA3 protein was immunoprecipitated with anti-mCLCA3 antibody p3b from the cell lysate (L) or medium (M) of mCLCA3-transfected and [35S]methionine-labeled COS-1 cells. The immunoprecipitated samples were analyzed by SDS-PAGE and autoradiography. After 1 h of chase and at all later times a 77-kDa protein (asterisk) was detected in the medium. Mock, vector-alone-transfected cells.](https://example.com/figure2.png)
Both Cleavage Products of mCLCA3 Are Secreted

FIGURE 3. Biosynthesis, processing, and secretion of the carboxyl-terminal-labeled mCLCA3 proteins and their processing products. COS-1 cells were transiently transfected with mCLCA3 labeled with YFP at the carboxyl terminus and pulsed with [35S]methionine followed by different chase times. The mCLCA3-YFP protein was immunoprecipitated from the cell lysate (L) or medium (M) with anti-YFP antibody, and the immunoprecipitated samples were analyzed by SDS-PAGE and autoradiography. After 1 h of chase two protein bands (68 and 77 kDa) were detected in the medium. Mock, vector-alone-transfected cells.

COS-1 and HEK 293 cells were used in parallel to identify potential intrinsic effects of the respective heterologous cell systems used are shown. The arrow indicates the putative cleavage site at amino acid 83–97 (37). B, the mCLCA3-YFP protein was immunoprecipitated from the lysate (L) or medium (M) of pEYF-N1-transfected COS-1 cells using the anti-YFP antibody, and the immunoprecipitated samples were separated by SDS-PAGE followed by Western blot analysis using anti-mCLCA3 antibody p3b or anti-YFP antibody, respectively. Protein extracted from mouse colon lysates (colon) was loaded for comparison with the in vivo protein, C, same experiments as in B, with HEK 293 cells transfected instead of COS-1 cells. The 72-kDa protein in the cell lysate and the 77-kDa protein in the medium were identified as the amino-terminal cleavage product, whereas the 65-kDa protein in the lysate and the 68-kDa in the medium were identified as the carboxyl-terminal subunit in both cell systems (sizes including the YFP tag of -30 kDa).

COS-1 and HEK 293 cells were used in parallel to identify potential intrinsic effects of the respective heterologous cell system on the processing of the mCLCA3-YFP fusion protein. Virtual identical data were obtained from both cell systems (Fig. 4, B and C), suggesting that the mCLCA3-YFP protein was processed identically in the two cell lines. Both antibodies, anti-mCLCA3 (p3b) directed against amino-terminal mCLCA3 protein fragments and anti-YFP directed against carboxyl-terminal mCLCA3-YFP fusion protein fragments (Fig. 4A), labeled the cell lysate bands at -130 and 140 kDa previously found in the pulse-chase experiments using mCLCA3-YFP-transfected cells (compare with Figs. 3 and 4, B and C). These findings in combination with the molecular masses corresponding to those predicted for the fusion protein indicated that the two bands represented precursor mCLCA3-YFP polypeptides before posttranslational cleavage (Fig. 4, B and C). Interestingly, the previously detected protein band of -105 kDa in the cell lysates (Fig. 3) was also present in the Western blot analyses with either antibody (Fig. 4, B and C). In contrast, this band was not detected by anti-mCLCA3 antibody p3a, which recognizes the epitope corresponding to amino acids 83–97 of the mCLCA3 polypeptide (11) (data not shown), suggesting that the 105-kDa protein fragment is generated by a cleavage event between the p3a and p3b epitopes. The antibody p3b identified the -75-kDa band in the cell lysate and the 77-kDa band in the medium (Fig. 3) as the amino-terminal cleavage products of the processed mCLCA3-YFP fusion protein (Fig. 4, B and C). The size of these protein species perfectly corresponded to the molecular weight of the native mCLCA3 protein as derived from murine colonic mucosal protein lysates (Fig. 4B, colon). Anti-YFP antibodies identified the -65-kDa band in the cell lysate and the -68-kDa protein in the medium (Fig. 3) as the carboxyl-terminal cleavage products of the processed mCLCA3-YFP fusion protein (Fig. 4, B and C). In summary, as previously shown for its human ortholog hCLCA1 (2), both cleavage products remained associated within the cell and in the medium at any time and were co-precipitated.

Subcellular Localization of the mCLCA3-YFP Fusion Protein in Living Cells

Confocal immunofluorescence detection of the untagged mCLCA3 protein in transfected COS-1 cells revealed an intracellular, vesicular distribution. The plasma membrane itself did not contain any mCLCA3 protein (Fig. 5A).

Similarly, confocal fluorescence imaging of mCLCA3 fused to YFP in transfected COS-1 cells revealed an intracellular, vesicular location of the mCLCA3-YFP fusion protein with some vesicles migrating toward the plasma membrane (Fig. 5B). The plasma membrane, however, did not contain any mCLCA3-YFP. To confirm association of the amino-terminal and carboxyl-terminal cleavage products as revealed by immunoprecipitation analyses (Fig. 4), p3b antibodies directed against the amino terminus of the mCLCA3 protein were used for immunofluorescence microscopy of the same cells. AlexaFluor 568-labeled secondary antibodies co-localized the mCLCA3 amino terminus in intracellular vesicles that also contained the mCLCA3-YFP carboxyl terminus (data not shown).
To confirm the intracellular localization, COS-1 cells were co-transfected with the mCLCA3-YFP construct (Fig. 5C1) and the pDsRed-ER (Fig. 5C2) containing the signal sequence of calreticulin, a specific marker for the ER, and a carboxyl-terminal KDEL ER retention sequence. The co-localization demonstrated the mCLCA3-protein reaching the ER. Moreover, COS-1 cells were co-transfected with the mCLCA3-YFP construct (Fig. 5D1) and the GT-DsRed vector as a Golgi marker (Fig. 5D2). The co-localization with the galactosyltransferase protein (Fig. 5D3) demonstrated the mCLCA3 protein passing through the Golgi.

**Glycosylation Pattern of the mCLCA3 Protein**

As shown in the pulse-chase experiments in COS-1 cells, the cell extracts and the media contained molecular forms of mCLCA3 that differed only slightly in their apparent molecular weights (i.e. 110 versus 100 kDa versus 77 versus 75 and 72 kDa, respectively; Fig. 2). To test whether each group consisted of different glycosylation forms of the same protein, immunoprecipitates were specifically deglycosylated with endo H or PNGase F treatments for specific N-deglycosylation of the proteins. Indeed, the 110-kDa protein species in the cell lysate was reduced to 100 kDa by endo H and PNGase F, whereas the 100-kDa protein was resistant to both enzymes (Fig. 6, left panels). Similarly, both enzymes reduced the 75-kDa protein species in the cell lysate to a 73-kDa protein band, whereas the 72-kDa protein in the cell lysate was resistant to both endo H and PNGase F treatments. Thus, the 110- and 75-kDa proteins were identified to be mannose-rich glycosylation forms processed from the endoplasmic reticulum, whereas the 100- and 72-kDa proteins represented the unglycosylated precursors. On the other hand, the 77-kDa species detected in the medium was endo H-resistant but shifted to 73 kDa upon treatment with PNGase F, indicating that it corresponded to a complex glycosylated protein form processed in the Golgi apparatus (Fig. 6, right panels, asterisk).

**Biosynthesis and glycosylation pattern of the mCLCA3 protein.** Immunoprecipitates of cell lysate (L) or medium (M) of mCLCA3 transfected and biosynthetically [35S]methionine-labeled COS-1 cells were divided into three aliquots and treated with endo H (H), PNGase F (F), or not treated (–). Autoradiography identified the 110-kDa protein and the 75-kDa protein in the cell lysate as mannose-rich glycosylated forms, reduced in size by both endo H and PNGase F. In contrast, the 77-kDa protein form in the medium was only susceptible to PNGase F treatment (asterisk), indicating a protein that was glycosylated in the Golgi apparatus.

**Glycosylation Pattern of the mCLCA3-YFP Fusion Protein**

We also analyzed the glycosylation patterns of the protein products identified in the pulse-chase experiments with the mCLCA3-YFP fusion protein (Fig. 3). The 140-kDa precursor protein in the lysates was reduced to a 130-kDa precursor protein with both endo H and PNGase F (Fig. 7, A and B, left panels) and, thus, identified a mannose-rich glycosylated form, whereas no size reduction of the 130-kDa protein was observed upon treatment with both enzymes, indicating that was the unglycosylated precursor (primary translation product).

Similarly, the 105-kDa protein species in the cell lysate was also resistant to both deglycosylating enzymes and most likely represents an unglycosylated protein. The 75-kDa amino-terminal cleavage product in the cell lysate (Fig. 7A, left panels) was reduced to a 73-kDa species by both endo H and PNGase F, identifying a mannose-rich type glycosylation of the endoplasmic reticulum. In contrast, the amino-terminal 77-kDa protein secreted into the medium was sensitive to PNGase F only and shifted to a 73-kDa protein band, indicating its complex type of glycosylation (Fig. 7A, right panels). Endoglycosidase treatments of the carboxyl-terminal cleavage products revealed similar results; the 65-kDa carboxyl terminus in the cell lysate was sensitive to both endo H and PNGase F treatments (Fig. 7B, left panels), and the 68-kDa carboxyl terminus released into the medium was sensitive to PNGase F only (Fig. 7B, right panel).
Both Cleavage Products of mCLCA3 Are Secreted

A mCLCA3 protein with c-terminal YFP tag

| kDa | L | L | L | M | M | M |
|-----|---|---|---|---|---|---|
| 140 | - | H | F | - | H | F |
| 130 |   |   |   |   |   |   |
| 105 |   |   |   |   |   |   |
| 75  |   |   |   |   |   |   |

B anti-YFP

| kDa | L | L | L | M | M | M |
|-----|---|---|---|---|---|---|
| 65  | - | H | F | - | H | F |

FIGURE 7. Glycosylation pattern of the mCLCA3-YFP fusion protein and its processing products. A, immunoprecipitates of the cell lysate (L) or medium (M) of pEYFP-N1 mCLCA3-transfected COS-1-cells were divided into aliquots and treated with endo H (H), PNGase F (F), or not treated (–). Western blot analysis using anti-mCLCA3 antibody p3b identified the 140-kDa protein and the 75-kDa proteins in the cell lysate as mannose-rich glycosylated forms, whereas the 77-kDa protein in the medium was only susceptible to PNGase F treatment (right panels), indicating a protein form that has passed the Golgi. B, Western blot analysis using anti-YFP antibody (JL-8) identified the 65-kDa carboxyl-terminal cleavage product in the cell lysate as a mannose-rich form. It shifted to ~55 kDa. In contrast, the 68-kDa band in the medium (right panels) was only susceptible to PNGase F, suggesting complex glycosylation of a protein that has passed the Golgi.

DISCUSSION

The molecular identity of calcium-activated anion channels is still elusive. The CLCA proteins including mCLCA3 have been proposed to be molecular candidates for calcium-activated chloride channels based on the appearance of a novel calcium-dependent chloride conductivity in cultured cells heterologously transfected with CLCA family members including mCLCA3 (12). This hypothesis gave rise to the designation of the gene family as channels (1). To form ion channels, however, the CLCA proteins should possess transmembrane domains forming a conductive ion pore. Computer-aided hydrophobicity plots helped predict potential transmembrane domains within a given polypeptide sequence. However, Kyte-Doolittle, SOSUI, HMMTOP, DAS, and PSORT II software failed to predict any transmembrane domain within the mCLCA3 amino acid sequence, whereas the TMPRED analysis suggested a transmembrane topology for mCLCA3 with two transmembrane spanning domains. This difference may be due to an entirely different approach used by the TMPRED algorithm, which is based on a comparison of the sequence in question with a data base of known transmembrane proteins (TMBase (31)). In contrast, the other algorithms used largely employ computational calculation of the hydrophobicity and other properties of a chosen sequence of amino acids. Thus, the results of the computational transmembrane analyses vary depending on the type of algorithm used, and it is difficult to establish a transmembrane model on the predicted data alone. This may be a problem specific to putative channel proteins that may possess strongly charged amino acid residues in membrane-spanning domains that obscure the otherwise typically hydrophobic properties of such regions. Therefore, the current study was designed to establish potential transmembrane domains and the cellular processing of mCLCA3. In particular, we wanted to test an alternative hypothesis according to which mCLCA3 is a fully secreted protein. This hypothesis was first raised in a previous immune electron microscopic study in which the mCLCA3 protein was observed to be at least in part secreted from murine intestinal goblet cells in vivo (11).

In full accordance with a previously established model of posttranslational cleavage of CLCA precursor proteins (1, 18), our present study revealed that the 110-kDa mannosé-rich glycosylated untagged mCLCA3 primary translation product is cleaved to yield a 75-kDa amino-terminal subunit. This subunit was complex glycosylated in the Golgi and fully secreted into the medium. Due to the lack of available antibodies that specifically recognize the carboxyl terminus of the mCLCA3 protein, a YFP fusion construct was generated that allowed specific detection of the carboxyl terminus and carboxyl-terminal cleavage products. The primary translation product of this YFP fusion protein was also cleaved into a 75-kDa amino-terminal and a 65-kDa YFP-tagged carboxyl-terminal subunit including the 33-kDa tag, showing that the tag did not interfere with intracellular protein processing. These data are consistent with previous observations for other CLCA proteins with regard to the cleavage event and the sizes of cleavage products (2, 4, 6, 13, 14, 18). In a recent study the cleavage site of the mCLCA3 protein has been identified at amino acid 686 (37). Based on this location, the calculated molecular mass of the amino-terminal subunit of 75.3 kDa or 73.3 kDa after cleavage of the amino-terminal signal sequence is close to that of the non-glycosylated amino-terminal fragment of the mCLCA3 protein detected in this study. Moreover, complex glycosylation yielded an ~75-kDa glycoprotein that was consistent with immunoblot detection of the natural amino-terminal cleavage product of mCLCA3 extracted from mouse colon mucosa.

The observation that the cleaved products appeared at early chase time points as the mannosé-rich glycosylated forms indicates that cleavage of the mCLCA3 precursor occurs in the early secretory pathway, most likely in the endoplasmic reticulum. Although the cleavage is well documented for several CLCA proteins, the identity of the cleaving protease is still obscure. In view of the facts that the cleavage occurs in vivo (11) and in different heterologous cell systems, the protease could be a ubiquitously expressed molecule. The cleavage signal, on the other hand, is poorly conserved throughout the CLCA family (1), suggesting the implication of a conformational motif rather than a primary amino acid sequence. A recent study suggested that the cleavage may be an autoproteolytic event (38).

Immunoprecipitation of the mCLCA3-YFP fusion protein in this study demonstrated that the amino-terminal mCLCA3 product is co-precipitated with the carboxyl-terminal subunit intracellularly as well as extracellularly. This close association of both cleaved mCLCA3 products is similar to the results...
observed for its human ortholog hCLCA1 in an earlier study. In that study the Myc-tagged hCLCA1 amino-terminal cleavage product had been shown to co-precipitate with the untagged carboxyl-terminal subunit (2). The four CLCA proteins hCLCA1 (human), mCLCA3 (mouse), pCLCA1 (pig), and eCLCA1 (horse) seemingly represent orthologous species-specific variants of the same protein and represent a single phylogenetic cluster (20). It, thus, seems plausible to assume that the strong association of the two subunits may be a characteristic feature of these family members. The nature of this association and its structural and functional relevance, however, are unclear so far.

On the other hand, our present study also disclosed significant differences between mCLCA3 and other orthologs of this cluster, in particular the first human CLCA protein, hCLCA1. The carboxyl-terminal cleavage product of hCLCA1 was not detected in the cell lysate and only at minute amounts in the medium in a recent study. It was, thus, concluded that this smaller cleavage product may be unstable once cleavage has taken place (24). By contrast, the corresponding carboxyl-terminal cleavage product of mCLCA3 was detected at significant amounts in this study both in the lysate and in the medium virtually over the entire observation period at amounts that were similar to the amino-terminal cleavage product, probably indicating equimolarity of the two cleavage products. Importantly, the cell system used for heterologous transfection was the same in both studies, namely HEK 293 cells. A second important difference was the binding of the amino-terminal cleavage product of hCLCA1 to the cell surface of transfected HEK 293 cells, clearly visualized by immunofluorescence (24). This binding was abolished by acid treatment, and it was, thus, concluded that the surface association was due to binding of this larger cleavage product to an as yet unidentified surface protein on HEK 293 cells. By contrast, no such surface association was observed for any mCLCA3 protein in this study using the same heterologous cell system. These inconsistencies may point toward structural differences between the human hCLCA1 and the murine mCLCA3, which are otherwise considered direct orthologs in the two species (39). Also, recent data from our laboratories have shown that the second human CLCA protein, hCLCA2, is clearly anchored in the cell membrane by a carboxyl-terminal true transmembrane domain.3 Such differences would be consistent with the well-established notion of an intra-species evolution of CLCA gene family members, resulting in substantial differences between different CLCA proteins, even between direct orthologs of different species (1, 39).

Our data strongly suggest that the mCLCA3 protein does not belong to the group of CLCA proteins that contain four to five transmembrane segments (1). In fact, neither the amino-terminal nor the carboxyl-terminal fragments contain a transmembrane domain but, instead, are secreted into the extracellular medium. Furthermore, we failed to detect any mCLCA3 cleavage product at the cell surface.

Thus, the results of this study clearly argue that the mCLCA3 protein cannot form an anion channel by itself. The appearance of a novel calcium-activated chloride conductance in mCLCA3-transfected HEK 293 cells (12), which were also used in this study, therefore points toward an indirect activation of the chloride secretory pathway, possibly via a signaling function of mCLCA3. Besides these biochemical data there is growing electrophysiological evidence suggesting that CLCA proteins do not act as channels themselves. The porcine pCLCA1, a member of the phylogenetic cluster also containing hCLCA1, mCLCA3, and eCLCA1, induces a calcium-activated chloride conductance as well as a cAMP-dependent chloride conductance (19, 40, 41). When expressed in Caco-2 cells, the calcium-dependent chloride conductance disappears through differentiation of these cells, whereas the pCLCA1 mRNA and protein expressions were retained. Furthermore, the cAMP-dependent chloride conductance modulated by pCLCA1 was observed throughout differentiation of Caco-2 cells (42). These electrophysiological data pointed toward a regulatory effect of the pCLCA1 protein on other chloride channels rather than to an inherent chloride channel function. It, therefore, appears reasonable to propose to rename mCLCA3 and pCLCA1 and possibly other CLCA proteins once their true function and significance have been identified.

Further functional analyses are required to explain how CLCA proteins can evoke calcium-activated chloride conductivity. The CLCA proteins possess structural elements including a von Willebrand factor domain, which is known to mediate protein-protein interactions (22, 24). In addition, endothelial CLCA proteins share a common β3 binding motif in their subunits (43, 44). Binding of mCLCA1 to the β3 integrin activates a focal adhesion kinase and downstream extracellular signal-regulated kinase (43, 44). Moreover, the mCLCA1 protein directly interacts with the large conductance potassium channel β subunit (45). Thus, the CLCA proteins are able to interact with other proteins and could have the potential to act as signaling molecules. The way of action on the chloride secretory pathway and possible interacting partners have yet to be identified.

The discovery that the mCLCA3 open reading frame encodes two secreted soluble proteins stands in sharp contrast to the previous transmembrane ion channel model (9, 11, 12). This change of paradigm necessitates a correction of the putative function and biomedical significance of this molecule that has been shown to play a significant role in diseases with secretory disorders including asthma and cystic fibrosis (46–50). The mCLCA3 mRNA is exclusively expressed in goblet cells and other mucin producing cells (9, 11), which are highly specialized components of the epithelial surfaces of the intestinal, respiratory, and reproductive tracts and the conjunctiva. The main function of these goblet cells is to secrete a mucus gel layer that serves as a protective coating against harmful factors such as digestive fluids, pathogenic microorganisms, toxins, physical damage, and drying out. The main structural components of goblet cell-derived mucus are MUC5AC, MUC5B, and MUC2 mucins (51, 52). In addition, a number of secreted proteins with specific functions are solubilized in the mucus layer of different organs, including lysozyme, lactoferrine, and trefoil factor family proteins. Although the significance of many of these soluble proteins is well understood, others still await identification of

3 Eble, R. C., Walia, V., Cheng, H.-c., Connolly, C. J., Mundhenk, L., Gruber, A. D., and Pauli, B. U. (2006) J. Biol. Chem. 281, 29448–29454.
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their functions in normal and diseased epithelium. For example, members of the trefoil factor family proteins are thought to play a role in wound healing and epithelial restitution by activating cell migration, reducing cell-cell adhesion, exerting anti-apoptotic properties, and influencing rheological properties (53). Interestingly, certain trefoil factor family proteins also elicit a luminal chloride secretory response when added to epithelial monolayers (54). However, neither the way of action nor the binding partners of trefoil factor family proteins have been identified. The results of the present study suggest that the murine mCLCA3 and possibly other CLCA proteins may have to be added to the list of soluble proteins of mucinous layers with as yet unknown functions and interacting partners. Future studies will have to address such interesting partners and the way these secreted proteins modulate the chloride secretory pathway and goblet cell metaplasia.

REFERENCES

1. Gruber, A. D., Elble, R. C., and Pauli, B. U. (2002) in Current Topics in Membranes (Fuller, C. M., ed) Vol. 53, pp. 368–388, Academic Press, San Diego, CA
2. Gruber, A. D., Elble, R. C., Ji, H. L., Schreur, K. D., Fuller, C. M., and Pauli, B. U. (1998) Genomics 54, 200–214
3. Agnel, M., Vermat, T., and Culouscou, J. M. (1999) FEBS Lett. 455, 295–301
4. Gruber, A. D., Schreur, K. D., Ji, H. L., Fuller, C. M., and Pauli, B. U. (1999) J. Biol. Chem. 274, 32096–32101
5. Romio, L., Musante, L., Cinti, R., Seri, M., Moran, O., Zegarra-Moran, O., and Galletta, L. I. (1999) Gene (Amst.) 228, 181–188
6. Lee, D., Ha, S., Cho, K., Baik, M., and Choi, Y. (1999) Biochem. Biophys. Res. Commun. 264, 933–937
7. Komiyama, T., Tanigawa, Y., and Hirohashi, S. (1999) Biochem. Biophys. Res. Commun. 255, 347–351
8. Wessendorf, I., and Gruber, A. D. (2000) Cytogenet. Cell Genet. 88, 208–209
9. Wessendorf, I., and Gruber, A. D. (2002) J. Histochem. Cytochem. 50, 829–838
10. Gruber, A. D., Elble, R. C., Ji, G. L., Schreur, K. D., Fuller, C. M., and Pauli, B. U. (1999) J. Biol. Chem. 274, 18586–18591
11. Evans, S. R., Thoreson, W. B., and Beck, C. L. (2004) J. Biol. Chem. 279, 41792–41800
12. Cingh, S. A., Aways, M. S., Bubien, J. K., Iasmalow, I. I., Arata, M. P., Berdev, B. K., Benos, D. I., and Fuller, C. M. (1995) J. Biol. Chem. 270, 31016–31026
13. Ran, S., Fuller, C. M., Arata, M. P., Latorre, R., and Benos, D. J. (1992) J. Biol. Chem. 267, 20650–20657
14. Zhu, D., and Pauli, B. U. (1993) Int. J. Cancer 53, 628–653
15. Elble, R. C., Widom, J., Gruber, A. D., Abdel-Ghany, M., Levine, R., Goodwin, A., Cheng, H. C., and Pauli, B. U. (1997) J. Biol. Chem. 272, 27853–27861
16. Gaspar, K. J., Racette, K. J., Gordon, J. R., Loewen, M. E., and Forsyth, G. W. (2000) Physiol. Genomics 9, 101–111
17. Anton, F., Leverkoehe, I., Munchen, L., Thoreson, W. B., and Gruber, A. D. (2005) J. Histochem. Cytochem. 53, 1011–1012
18. Jeong, S. M., Park, H. K., Yoon, I. S., Lee, J. H., Kim, J. H., Jang, C. G., Lee, C. J., and Nah, S. Y. (2005) Biochem. Biophys. Res. Commun. 334, 569–576