Evidence for the intracellular location of chloride channel (ClC)-type proteins: co-localization of ClC-6a and ClC-6c with the sarco/endoplasmic-reticulum Ca^{2+} pump SERCA2b

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Chloride channel protein (ClC)-6a and ClC-6c, a kidney-specific splice variant with a truncated C-terminus, are proteins that belong structurally to the family of voltage-dependent chloride channels. Attempts to characterize functionally ClC-6a or ClC-6c in *Xenopus* oocytes have so far been negative. Similarly, expression of both ClC-6 isoforms in mammalian cells failed to provide functional information. One possible explanation of these negative results is that ClC-6 is an intracellular chloride channel rather than being located in the plasma membrane. We therefore studied the subcellular location of ClC-6 isoforms by transiently transfecting COS and CHO cells with epitope-tagged versions of ClC-6a and ClC-6c. Confocal imaging of transfected

cells revealed for both ClC-6 isoforms an intracellular distribution pattern that clearly differed from the peripheral location of CD2, a plasma-membrane glycoprotein. Furthermore, dual-labelling experiments of COS cells co-transfected with ClC-6a or -6c and the sarco/endoplasmic-reticulum Ca^{2+} pump (SERCA2b) indicated that the ClC-6 isoforms co-localized with the SERCA2b Ca^{2+} pump. Thus ClC-6a and ClC-6c are intracellular membrane proteins, most likely residing in the endoplasmic reticulum. In view of their structural similarity to proven chloride channels, ClC-6 isoforms are molecular candidates for intracellular chloride channels.

INTRODUCTION

The ClC chloride channel family forms a group of structurally-related membrane proteins members of which have been identified in bacteria, plants, yeast, fish and mammals [1,2]. Mammals possess at least nine different ClC genes (ClC-1 to -7 and ClC-K1 and -K2) and the number of ClC isoforms is further increased by alternative splicing [3–5]. All ClC proteins, with the exception of some of the splice variants, have a common structure with an even number (10 or 12) of transmembrane domains and with intracellular N- and C-termini [2,6,7]. There is conclusive experimental evidence that some ClC members form plasma-membrane located chloride channels [1,2]. For example, expression of ClC-0 in *Xenopus* oocytes or incorporation of purified ClC-0 into lipid bilayers generates a voltage-dependent chloride current with double-barrelled gating kinetics [8,9]. The functional channel complex is composed of a homodimer with two distinct pores, each of which is independently operated by a voltage-dependent fast gate [10,11]. Furthermore, the permeating anion (and not an intrinsic structural element) serves as the voltage-sensor of the fast gate [12,13]. Thus ClC-0 represents a new paradigm for voltage-dependent ion channels that fundamentally differs from the voltage-dependent cation channels. Similarly, expression of ClC-1 in *Xenopus* oocytes, insect cells or mammalian cells has clearly established that ClC-1 is a plasma-membrane chloride channel that stabilizes the membrane potential in skeletal muscle [14–16]. The detailed biophysical and functional characterization of ClC-0 and ClC-1 contrasts sharply with the lack of knowledge for ClC-6. A major obstacle in this regard is that injection of *Xenopus* oocytes with ClC-6a RNA had either no effect [17] or resulted in the activation of an endogenous chloride conductance [18]. Therefore although ClC-6a structurally belongs to a family of plasma-membrane

chloride channels, it has not yet been possible to ascertain its proper function.

We have recently shown that ClC-6 can be alternatively spliced resulting in four distinct isoforms, ClC-6a to ClC-6d [4]. The ClC-6a isoform is identical to the ClC-6 protein originally described [17]. It has a canonical ClC structure with 10–12 predicted transmembrane domains. In contrast, ClC-6b, -6c and -6d are truncated at their C-terminus and they contain maximally 4 (ClC-6b and ClC-6d) or 7 (ClC-6c) transmembrane domains. ClC-6a and ClC-6c are the most abundant isoforms with ClC-6a being ubiquitously expressed and ClC-6c being limited to the kidney [4,17]. We now demonstrate that ClC-6a and ClC-6c escape functional characterization in *Xenopus* oocytes and in mammalian cells because of their intracellular location. Indeed, ClC-6a and -6c co-localize with the sarco/endoplasmic-reticulum Ca^{2+} pump (SERCA2b) when transiently transfected in COS cells.

EXPERIMENTAL

Vector construction

A c-Myc epitope (amino acids EQKLISEEDL; see [19]) was inserted at the N-terminus of a human ClC-6c cDNA clone [4] via PCR mutagenesis. A *Bam*HI-*Sal*I restriction fragment containing the cMycClC6c open reading frame was subcloned into the mammalian expression vector pCIneo (Promega) resulting in the pCIneo/cMycClC6c vector. c-Myc epitope tagging of human ClC-6a was performed by replacing the ClC-6c-specific *Xba*I-*Hind*III fragment in pCIneo/cMycClC6c with the ClC-6a-specific *Xba*I-*Hind*III fragment generating the pCIneo/cMycClC6a vector. The c-Myc epitope tag was inserted at the C-terminus of human ClC-6a and -6c via PCR mutagenesis. Subcloning into

Abbreviations used: ClC, chloride channel protein; I_{Clin} , nucleotide-sensitive chloride current; SERCA2b, sarco/endoplasmic-reticulum Ca^{2+} pump; CD2, T-lymphocyte cell-surface antigen; CHO, Chinese hamster ovary; TRITC, tetramethylrhodamine isothiocyanate.

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pCIneo of *Bam*HI–*Hind*III restriction fragments containing the C-terminal-tagged open reading frames resulted in the pCIneo/CIC6acMyc and the pCIneo/CIC6ccMyc vectors. The pSV57/SERCA2b and pKG5/CD2 vectors were kindly given by F. Wuytack (Katholieke Universiteit, Leuven, Belgium) and N. Barclay (University of Oxford, Oxford, U.K.) respectively.

cDNA fragments encoding either CIC-6a or CIC-6c were inserted in the bicistronic GFP-expression vector pCIneo/IRES-GFP [20] by blunt-end ligation. To this end, the pCIneo/IRES-GFP vector was digested with *Eco*RI and blunt ended with T4 DNA polymerase. The CIC-6a and CIC-6c fragments were blunt-ended *Bam*HI/*Hind*III restriction fragments of pBS/CIC-6a and pBS/CIC-6c [4] respectively. The c-Myc epitope was inserted at the N-terminus of a rat CIC-1 cDNA clone (a gift from T. J. Jentsch, Hamburg University, Germany) via PCR-mutagenesis. A blunt-ended restriction fragment containing the cMycCIC1 open reading frame was cloned in the *Eco*RI-digested and blunt-ended pCIneo/IRES-GFP vector resulting in the pCIneo/IRES-GFP/cMycCIC1.

Transient transfection of mammalian cells

COS cells and Chinese hamster ovary (CHO) cells were transiently transfected using the polycationic liposome Lipofect AMINE[®] (Life Technologies) following the manufacturer's instructions. In short, exponentially-growing cells were seeded the day before transfection at a density of 15000 cells/cm² in tissue-culture dishes (35-mm diam.). At 50–80% confluency, cells were incubated for 6 h with a transfection cocktail containing 2 µg DNA and 4 µl LipofectAMINE[®] reagent per 35-mm dish. For co-transfection, we used 1.5 µg DNA of each vector (1:1 ratio) and 6 µl LipofectAMINE[®] per 35-mm dish. Cells were transferred after 24 h to gelatin-coated 2-well coverslip culture chambers (Lab Tek, Nunc Inc.) and grown for another 24 h before the start of the immunostaining procedure. For patch-clamp analysis, cells were transfected in 35-mm dishes, transferred to coverslips after 24 h and electrophysiological measurements were started 48 h after transfection. Alternatively, cells were transfected by the Superfect Transfection protocol (Qiagen), using 2.5 µg DNA and 12 µl Superfect reagent per 35-mm dish.

Current measurements in COS cells

For current recordings, fluorescent cells were visualized in a patch-clamp set up (EPC7) containing a Zeiss Axiovert 100 microscope, a Xenon light source and epifluorescence optics (Zeiss XBO 75 and Zeiss-EPI unit fluorescence condenser). A band-pass filter (Zeiss filter set 9,487909; BP 450–490) was used for excitation. Light was transmitted to the cells via a dichroic mirror (Zeiss FT 510). Emitted light passed through a 520-nm long-pass filter (Zeiss, LP 520) and was detected visually. Whole-cell membrane currents were monitored with an EPC7 patch clamp amplifier using the following voltage protocol: from a holding potential of –30 mV cells were pre-pulsed to +80 mV for 100 ms, after which voltage steps of 400 ms were applied from –140 to +80 mV in +20-mV increments. This was followed by a 50 ms after pulse to –100 mV. Currents were sampled at 1 ms intervals. Sampling and stimulation were controlled via the pClamp 5.7.1. software (Axon Instruments, Foster City, CA, U.S.A.). All experiments were performed at room temperature. The extracellular solution was a modified Krebs solution containing 150 mM NaCl, 6 mM KCl, 1.5 mM CaCl₂, 10 mM Hepes and 10 mM glucose, adjusted to pH 7.4 with NaOH. In some experiments, CdCl₂ was added to the bath solution at a concentration of 1 mM. The osmolality of the extracellular solutions, measured with a vapour osmometer

(Wescor 5500, Schlag, Gladbach, Germany), was 320 ± 5 mosmol/l. Pipette solutions contained 40 mM KCl, 100 mM K-aspartate, 4 mM Na₂ATP, 1 mM MgCl₂, 0.1 mM EGTA, 10 mM Hepes, adjusted to pH 7.2 with KOH.

Confocal immunofluorescence study of CIC-6 localization in transiently transfected cells

Cells were fixed in PBS containing 3% (w/v) paraformaldehyde for 15 min, permeabilized with 0.5% (v/v) Triton X-100 for 10 min and blocked with 20% (v/v) goat serum in PBS for 1 h. They were then incubated for 16–72 h at 4 °C with primary antibodies: c-Myc 9E10 (Santa Cruz) for c-Myc epitope-tagged versions of CIC-6a and CIC-6c; IID8 for SERCA2b; and Ox34 for CD2. Thereafter, the cells were treated with FITC-conjugated goat anti-mouse IgG (Sigma ImmunoChemicals) at room temperature for 1 h. Control reactions were performed by incubating transfected cells with the secondary antibodies only and by processing mock-transfected cells with primary and secondary antibodies. Cells were imaged using a Bio-Rad MRC 1000 UV confocal laser-scanning microscope with a 40× oil-immersion objective and a Kalman filter averaging 2 to 5 frames. Both confocal (iris confocal aperture 0.7–3.5 mm) and non-confocal images (768 × 512 lines) were stored.

Co-localization of CIC-6a or -6c and SERCA2b was studied in co-transfected COS cells (see above). SERCA2b expression was detected with polyclonal affinity-purified primary antibodies and tetramethylrhodamine isothiocyanate (TRITC)-labelled anti-rabbit IgG (Sigma ImmunoChemicals) as secondary antibodies. cMyc/CIC-6a or -6c was detected with the 9E10 monoclonal antibody and FITC-labelled secondary antibodies. Two separate epi-detectors filtering FITC and TRITC emission signals allowed simultaneous dual imaging of cMyc/CIC-6 and SERCA2b.

RESULTS

Functional characterization of COS cells transiently transfected with CIC-6a or CIC-6c

Brandt and Jentsch [17] reported that attempts to express human CIC-6a functionally in *Xenopus* oocytes were without success. In our hands, expression of CIC-6a or CIC-6c in *Xenopus* oocytes activated an endogenous nucleotide-sensitive chloride current (*I*_{Cl_{in}) provided that the oocytes were incubated at a higher temperature (24 °C for 2 h or 37 °C for 30 min) before the electrophysiological analysis at room temperature (results not shown and [18]). The *I*_{Cl_{in} is identical with the chloride current observed after expression of mammalian p*I*_{Cl_{in}} in *Xenopus* oocytes [18,21,22]. It has thus been concluded that CIC-6a and CIC-6c can induce expression of an endogenous chloride channel in oocytes.}}

As an alternative to expression in *Xenopus* oocytes we tried transient transfection of CIC-6 isoforms in a mammalian cell line (COS7) (Figure 1). CIC-6a and CIC-6c were subcloned in a bicistronic GFP-expression vector (pCIneo/IRES-GFP) which allows identification of channel-expressing cells using green fluorescence [20]. Electrophysiological analysis of all green cells transfected with either CIC-6a (*n* = 11) or CIC-6c (*n* = 9) was negative and the recorded currents did not differ significantly from the control currents obtained in non-fluorescent cells (*n* = 11) (Figures 1A and 1B). This contrasted sharply with a previous study in which RCK1, a Kv1.1 K⁺ channel, was transiently expressed in COS cells using the pCIneo/IRES-GFP vector. After transfection with RCK1 every green cell tested expressed the typical K⁺ current [20]. As an additional positive control rat CIC-1 (containing an N-terminal c-Myc epitope) was transfected

Figure 1 Functional characterization of COS cells transfected with CIC-6 isoforms

COS cells were transiently transfected with CIC-6a, CIC-6c or cMyc-CIC-1 using the pCIneo/IRES-GFP vector. Whole-cell membrane currents were measured in green fluorescent cells and in non-fluorescent cells (negative control) with the voltage protocol shown above (A). (A) Cells expressing CIC-6a (a) or CIC-6c (b) displayed time-independent currents, which did not differ from the currents measured in non-fluorescent control cells (c). (B) Current (mean current $\pm$ S.E.M. at the beginning of the 400 ms pulse)—voltage relationship for green fluorescent cells expressing CIC-6a (■, $n = 11$) or CIC-6c (▲, $n = 9$) and for non-fluorescent control cells (●, $n = 11$). There were no significant differences between transfected and non-transfected cells. The extracellular solution was a modified Krebs solution. (C) Typical current trace of a green fluorescent COS cell transfected with rat CIC-1. As discussed in the text, currents were recorded in the presence of extracellular 1 mM CdCl_2 . This trace is from a representative example of six CIC-1 expressing cells. (D) Current—voltage relationship for the cell shown in (C). The plotted currents were measured at the beginning (■) and at the end (●) of the 400 ms step.

into COS cells using the pCIneo/IRES-GFP vector. The membrane resistance of all green cells was low (15.2 ± 2.2 MOhm, $n = 7$) most likely because of the overexpression of CIC-1 channels. With an access resistance of 7.6 ± 0.5 MOhm ($n = 21$) it was thus impossible to clamp the green fluorescent cells. We, therefore, partially reduced the overexpressed CIC-1 chloride current by supplementing the extracellular solution with 1 mM Cd^{2+} , which induces approx. 50 % block of CIC-1 currents [23,24]. Under these conditions we were able to measure a voltage-dependent current in green cells which reversed at -20 mV and which rapidly deactivated upon hyperpolarisation, similar to that described for CIC-1 [16] (Figures 1C and 1D). These currents were never observed in dark cells or in non-transfected cells. Thus although transient transfection of CIC-1 yielded typical membrane currents, cells transfected with CIC-6a or CIC-6c remained negative.

Morphological evidence for an intracellular location of CIC-6 proteins

To further investigate why heterologous expression of CIC-6a and CIC-6c in COS cells failed to provide functional information, the expression of CIC-6 isoforms was checked in transiently transfected COS cells by confocal immunofluorescence micro-

scopy (Figures 2 A–D). To facilitate detection of transfected proteins, CIC-6a and CIC-6c proteins were tagged with an N-terminal c-Myc epitope. Confocal images of cells transfected with CIC-6a and CIC-6c showed that the strongest fluorescence was concentrated around the nucleus and that the intensity of the signal decreased at the periphery of the cell (Figures 2A and 2B). No fluorescent signals were observed in transfected cells when the primary anti-c-Myc antibody was omitted during the staining procedure or when mock-transfected cells were processed with primary and secondary antibodies (results not shown). The distribution pattern of the CIC-6 isoforms was then compared with that of SERCA2b, the endoplasmic reticulum Ca^{2+} pump of non-muscle cells [25] and of CD2, a T-lymphocyte plasma-membrane glycoprotein [26]. Interestingly, the SERCA2b distribution pattern (Figure 2C) was virtually identical to that of CIC-6a and CIC-6c. In contrast, the CD2 pattern clearly differed in that staining for CD2 resulted in fluorescent signals that were most intense at the cell periphery (Figure 2D).

To exclude the possibility that the intracellular location of CIC-6 isoforms was an artefactual observation, various control experiments were performed. First, we tested whether the modified N-terminus of the c-Myc tagged CIC-6 isoforms might interfere with the delivery of newly synthesized CIC-6 proteins to the plasma membrane. To this end, the c-Myc epitope was inserted at the C-terminus of the CIC-6 isoforms and their

distribution after transient transfection in COS cells was examined (Figures 2E and 2F). This revealed, for both isoforms, a distribution pattern that was completely identical with that of CIC-6 proteins containing the N-terminal epitope tag. Thus the modified N-terminus cannot account for the intracellular location of the CIC-6 isoforms. Secondly, we checked whether the intracellular distribution might be an artefact of the expression system. COS cells express the SV40 large T antigen, which permits episomal replication of expression vectors such as pCIneo that contain the SV40 ori sequence [27]. To avoid plasmid amplification, we transfected the N-terminal-tagged CIC-6 isoforms in CHO cells, which do not express the SV40 large T antigen. Furthermore, CHO cells have been used successfully to express ion channels at the plasma membrane [28–31]. When expressed in CHO cells, both CIC-6a and CIC-6c displayed an intracellular distribution pattern identical with that in COS cells (Figures 2G and 2H). The strong intracellular perinuclear staining was virtually identical to that of SERCA2b (Figure 2I) and clearly differed from the peripheral pattern of CD2 (Figure 2J). Thus the overexpression system in COS cells is not responsible for the intracellular location. Finally, we tested whether the c-Myc epitope prevented plasma-membrane proteins from reaching their normal location in COS cells. Control experiments were performed by transiently transfecting COS cells with rat CIC-1 containing an N-terminal c-Myc epitope. As shown in Figure 1 (C and D), COS cells transfected with epitope-tagged CIC-1 acquired a voltage-dependent plasma-membrane current indicating that the c-Myc-tagged CIC-1 had reached the plasma membrane. In view of these control experiments we concluded that the intracellular location of CIC-6 isoforms cannot be attributed to artefacts arising from modifications to the N-terminus, the expression system or the c-Myc epitope.

Co-localization of CIC-6a/6c and SERCA2b

Finally, we compared the distribution pattern of CIC-6 isoforms and SERCA2b by double-labelling of co-transfected COS cells. CIC-6a and CIC-6c were visualized using FITC-labelled secondary antibodies, whereas the SERCA2b signal was generated via TRITC-labelled secondary antibodies. Cells that expressed both CIC-6 and SERCA2b were subjected to confocal analysis (Figure 3). Within the same cell there was basically no difference between the CIC-6 and the SERCA2b distribution pattern indicating that both proteins co-localize. The specificity of CIC-6 and SERCA2b detection was checked by omitting either the anti-c-Myc antibodies or the anti-SERCA2b antibodies. In the former case, no FITC signals were detected and no TRITC signals were present in the latter situation (results not shown). Thus these data indicate that CIC-6a and CIC-6c are intracellular membrane proteins that most likely reside in the endoplasmic reticulum.

DISCUSSION

The observation that CIC-6a and CIC-6c are intracellular proteins that most likely reside in the endoplasmic reticulum provides an explanation as to why their expression in *Xenopus* oocytes and in mammalian cells has failed to elucidate their function. Indeed, using the voltage-clamp technique one can readily access ion channels that are present in the plasma membrane, but not intracellularly located channels.

The structural similarity of CIC-6 isoforms to well-established chloride channels such as CIC-0 and CIC-1 is compatible with their being anion channels. It is therefore possible, but not yet proven, that CIC-6a and possibly CIC-6c represent a new class of intracellular chloride (or anion) channels. Intracellular anion channels, other than the mitochondrial porins [32], have been reported (for a review, see [33]). For example, the sarcoplasmic reticulum in skeletal muscle and cardiac muscle contains anion-selective pathways which have been studied by fusing sarcoplasmic-reticulum vesicles with lipid bilayers [34–36]. Similar techniques have permitted the identification of intracellular anion channels in non-excitable cells [37]. A possible function for the sarco/endoplasmic-reticulum-located chloride channels is to dissipate the electrical gradient that is created by the unidirectional movement of Ca^{2+} (release or uptake) across the sarco/endoplasmic-reticulum membrane. Furthermore, it has been postulated that intracellular chloride channels play a permissive role in the acidification of Golgi vesicles, endosomes and lysosomes by dissipating the membrane potential created by the unidirectional transport of H^+ into the organellar lumen [33].

The molecular identification of intracellular anion channels, except for the porins, is still incomplete. Because of its presence in endosomes it is possible that the cystic fibrosis transmembrane conductance regulator functions not only as a cAMP-regulated plasma-membrane chloride channel but also as an intracellular chloride channel involved in organellar acidification [38,39]. Furthermore, there is evidence that two structurally related proteins, p64, a 64 kDa microsomal protein, and NCC27, a 27 kDa protein present in the nuclear envelope, form intracellular chloride channels or that they are regulators thereof [40–42]. Finally, there is growing evidence for CIC proteins as intracellular anion channels. Indeed, our results are consistent with an endoplasmic location for CIC-6 isoforms. Also, immunofluorescence imaging of pancreatic acinar cells showed CIC-2 protein in secretory vesicles close to the apical pole [43]. Furthermore, plant CIC isoforms, when expressed in yeast as well as the endogenous yeast CIC protein, are targeted to intracellular compartments [44]. However, caution must be exercised as functional proof of chloride-channel activity is still lacking for the CIC-6 isoforms as well as for the plant and yeast CIC proteins. Therefore further functions are required before these CIC proteins can be unambiguously classified as intracellular chloride channels.

Figure 2 Intracellular location of CIC-6 isoforms in transiently transfected COS and CHO cells

Confocal-immunofluorescence detection of CIC-6a, CIC-6c, SERCA2b and CD2 in transiently transfected COS (A–F) and CHO (G–J) cells. Tagging of CIC-6 isoforms with a c-Myc epitope allowed detection of CIC-6 isoforms with the monoclonal c-Myc 9E10 antibody. SERCA2b and CD2 were detected with monoclonal antibodies IID8 and OX34 respectively. The specificity of the signals was verified by omitting the primary antibodies and incubating the cells with only the secondary FITC-conjugated antibodies and by processing mock-transfected cells with primary and secondary antibodies (results not shown). In COS cells, N-terminal epitope-tagged CIC-6a (A) and CIC-6c (B) were located intracellularly with a distribution pattern virtually identical to that of SERCA2b (C), an endoplasmic-reticulum membrane protein. This pattern is in sharp contrast with that of CD2 (D), a plasma-membrane protein. Both CIC-6 isoforms were tagged also with a C-terminal c-Myc epitope. As shown in (E) (F) CIC-6a and CIC-6c, displayed an identical intracellular distribution pattern to the N-terminal tagged versions of CIC-6. To exclude the possibility that the intracellular location of CIC-6 isoforms was an artefact of overexpression in COS cells, we studied their subcellular distribution pattern when expressed in CHO cells (G–J). The intracellular staining pattern of N-terminal-tagged CIC-6a (G) and CIC-6c (H) is virtually identical to that of SERCA2b (I) and clearly different from that of CD2 (J). The colour scale (see vertical bar in B and H) ranges from blue (background signal) to white (saturating signal). Scale bars are shown in each panel.

Figure 3 Co-localization of CIC-6 isoforms with SERCA2b in transfected COS cells

Localization of cMyc/CIC-6 isoforms and the SERCA2b Ca^{2+} pump was analysed in co-transfected COS cells by simultaneous dual confocal immunofluorescence. CIC-6 isoforms were visualized using FITC-labelled secondary antibodies, whereas the SERCA2b signal was generated via TRITC-labelled secondary antibodies. The specificity of the signals was first checked by omitting either the anti-c-Myc antibodies or the anti-SERCA2b antibodies (results not shown). (A) Shows the location of SERCA2b (left panel) and of CIC-6c (right panel) in two co-expressing cells. (B) Shows the location of SERCA2b (left panel) and of CIC-6a (right panel) in a co-expressing cell. The colour scale (see vertical bar in B) is identical to that shown in Figure 2. Scale bars represent 20 μm .

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