M Phase-specific Expression and Phosphorylation-dependent Ubiquitination of the ClC-2 Channel

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Cl⁻ channel activities vary during the cell cycle and are thought to play various roles including regulation of cell volume. We have shown previously that ClC-2 channels are directly phosphorylated and functionally regulated by the M phase-specific cyclin-dependent kinase p34cdc2/cyclin B. We investigate here to determine whether the expression levels of ClC-2 channel protein vary during the cell cycle. Immunoblot and immunocytochemical analyses of cells cycle-synchronized by serum depletion/replenishment reveal that ClC-2 channel protein is expressed predominantly at M phase in cells with two nuclei and a clear constriction ring, whereas RNA blot analysis shows that ClC-2 mRNA expression does not change during the cell cycle. Ubiquitin assays reveal that the ClC-2 channels are ubiquitinated at M phase, whereas the magnitude of ubiquitination is suppressed by incubation with olomoucine, an inhibitor of p34cdc2/cyclin B, and it is almost completely abolished in ClC-2 channels having an S632A mutation, which cannot be phosphorylated by p34cdc2/cyclin B, indicating that ubiquitination of ClC-2 channels requires phosphorylation by M phase-specific p34cdc2/cyclin B. Regulation at the post-transcriptional level, including phosphorylation-dependent ubiquitination, may contribute to M phase-specific expression of ClC-2 channels. Cell cycle-dependent regulation of expression at the protein level in addition to the regulation of function suggests that the ClC-2 channel plays a physiological role in the cell cycle.

Cl⁻ channel activity varies during the cell cycle and is thought to play several roles in the progression of the cell cycle including regulation of membrane potential and cell volume (1, 2). At M phase, activation of inwardly rectifying Cl⁻ currents has been reported in two species, Caenorhabditis elegans oocytes (3) and ascidian embryos (4, 5). The biophysical properties of the Cl⁻ channels activated at M phase in these species (3–6) strongly resemble those of the CIC-2 channel (7–10); both are activated by hyperpolarization, show inward rectification and no inactivation, and are activated by cell swelling. According to the regulation of function suggests that the ClC-2 channel plays a physiological role in the cell cycle.

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MATERIALS AND METHODS

Plasmid Construction—ClC-2 cDNA obtained from rabbit heart (13) was subcloned into pcDNA3.1(+) (Invitrogen) (pcDNA3.1(+)/rbClC-2). The Ser-632 to Ala mutation (S632A) in the consensus sequence of p34cdc2/cyclin B phosphorylation (14) in ClC-2 (rbClC-2/S632A) and the introduction of a hemagglutinin (HA) epitope (YPYDVPDYA) between the third (Ala-3) and fourth (Pro-4) residue of rabbit ClC-2 (rbClC-2-HA) were made by overlap extension using PCR methods (15). The sequences of the plasmid constructs were verified by the dideoxy nucleotide chain termination method using the 377 DNA sequencing system (PerkinElmer Life Sciences).

Cell Culture and Transfection—NIH3T3, HEK293, and NRK-49F cells (ATCC) were maintained and transfected with plasmid DNAs using LipofectAMINE plus reagent (Invitrogen) according to the manufacturer’s instructions. NIH3T3 cells stably expressing rbClC-2-HA (NIH3T3/rbClC-2-HA) were established by selection with Geneticin at a concentration of 1.5 mg/ml.

Cell Cycle Synchronization—Exponentially growing NRK-49F or NIH3T3 cells were introduced into quiescence phase (G0/G1) by serum deprivation for 30–40 h, when the cells were released from G0/G1 phase.

1 The abbreviations used are: HA, hemagglutinin; FCS, fetal calf serum; zLLLal, benzoyloxy carbonyl-leucyl-leucyl-leucinal; Me₂SO, dimethyl sulfoxide.
Fig. 1. Expression levels of CIC-2 protein but not of its mRNA are altered during the cell cycle. A, a representative data of cell-cycle synchronization in NRK-49F cells. G0/G1-arrested NRK-49F cells were released into media with 10% FCS. Samples were processed at the indicated times for fluorescence-activated cell sorter analysis. B, 20 μg of total RNA prepared at the indicated times after serum addition was electrophoresed on a 1% (w/v) agarose gel, stained with ethidium bromide (lower panel), and blotted with 32P-labeled rbCIC-2 (upper panel). C, 60 μg of crude membrane proteins was separated on an SDS, 7% polyacrylamide gel and immunoblotted (IB) with anti-CIC-2 antibody.

Fig. 2. CIC-2 channel proteins are predominantly expressed in dividing cells at M phase. A, G0/G1-arrested NIH3T3(rbClC-2-HA) cells were released with medium containing 15% FCS and immunostained with rat anti-HA antibody followed by fluorescein isothiocyanate-conjugated anti-rat IgG. Cover glasses were mounted on a slide and observed by incubation with 1:500-diluted fluorescein isothiocyanate-conjugated anti-ClC-2 rat antibody (Alomone Laboratories), 20 mM sodium phosphate buffer (pH 6.5), 0.1% SDS, 100 μg/ml sonicated and denatured salmon sperm DNA, 10% (w/v) dextran sulfate, and 3 × 106 cpm/ml 32P-labeled probe (nucleotides 221–2504 of rbClC-2) at 42 °C for 16 h. The nylon membranes were washed in 0.1 × SSC, 0.1% SDS at 50 °C and exposed to x-ray film at −80 °C for 14 days.

Preparation of Crude Membrane Fraction and Immunoblot Analysis—Crude membrane fractions were prepared from untransfected NRK-49F cells or HEK293 cells transiently transfected with pcDNA3.1(+) of rbClC-2-2-HA or pcDNA3.1(+) of rbClC-2(S632A)-HA as described previously (19). Briefly, the cells were washed 3 times with phosphate-buffered saline (80 mM NaH2PO4, 20 mM Na2HPO4, and 100 mM NaCl (pH 7.4)), suspended in buffer A (50 mM Tris (pH 7.5), 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, and 10 μg/ml each aprotinin, leupeptin, and pepstatin), sonicated, and centrifuged at 200 g for 1 h. The pellets were resuspended in 500 μl of buffer A (crude membrane fraction) and stored at −80 °C until immunoblot analysis. The crude membrane proteins (60 μg for NRK-49F cells and 20 μg for HEK293 cells) were boiled in SDS reducing sample buffer, electrophoresed on an SDS, 7% polyacrylamide gel, and transferred to a nitrocellulose membrane by electroblotting overnight at 4 °C at 200 mA. The membrane was incubated with 1:300-diluted anti-CIC-2 rabbit antibody (Alomone Laboratories) for NRK-49F cells or 1:1000-diluted anti-HA rat antibody (Roche Molecular Biochemicals) for NIH3T3 cells followed by incubation with 1:800-diluted horseradish peroxidase-conjugated anti-rat IgG (BioSys), and the proteins were detected using an enhanced chemiluminescence system (Amersham Biosciences). Protein concentrations were determined using the bichinchoninic acid assay (Pierce). The protein expression level was quantified by measuring the immuno-density of the main band at about 100 kDa by NIH Image software.

Ubiquitin Assay—Synchronized NIH3T3(rbClC-2-HA) cells or HEK293 cells transiently transfected with pcDNA3.1(+) empty vector (mock), pcDNA3.1(+) of rbClC-2-HA, or pcDNA3.1(+) of rbClC-2(S632A)-HA were washed twice with wash buffer (10 mM Tris (pH 7.4) and 0.25 M sucrose) and lysed in radioimmunoprecipitation assay buffer (150 mM NaCl, 20 mM Tris (pH 7.4), 2 mM EDTA, 50 mM NaF, 1 mM Na2VO4, 5 mM 2-mercaptoethanol, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each aprotinin, leupeptin, and pepstatin) at 4 °C for 30 min (20). In some
experiments, cells were incubated with 10 μM tripeptide aldehyde benzylxycarbonyl-leucyl-leucyl-leucinal (zLLLal; Wako) and 10 μM olo-
moucine (Peptide Institute Inc.) for 2 h. Cell lysates were cleared by 
centrifugation at 15,000 rpm for 30 min at 4 °C and presorbed by 
incubation with 20 μl of protein G-Sepharose (Amersham Biosciences) 
overnight at 4 °C. The supernatant was incubated with 2 μg/ml of anti-HA high affinity 
antibody (Roche Molecular Biochemicals) and 15 μl of protein G-Sepharose 
(Amersham Biosciences) at 4 °C. Protein-antibody complexes 
were washed five times with the radioimmune precipitation assay 
buffer, eluted with the SDS reducing sample buffer, and subjected to 
immunoblot analysis as described above by incubation with anti-ubiq-
uitin antibody (Medical & Biological Laboratories, Co., Ltd.) at a con-
centration of 5 μg/ml followed by incubation with 1:2000-diluted horse-
radish peroxidase-conjugated anti-mouse IgG (DAKO). The magnitude 
of ubiquitination was quantified by measuring immuno-densities 
greater than 100 kDa by NIH Image software. zLLLal and olomoucine 
were prepared as 10 mM stock solution in dimethyl sulfoxide (Me 2SO) 
and stored below −80 °C until use.

Phosphorylation Assays in Cultured Cells—48 h after transfection of 
HEK293 cells in a 60-mm culture dish with 6 μg of mock, 
pcDNA3.1 (+) rbcIC-2-HA, or pcDNA3.1 (+) rbcIC-2/S632A)-HA, the cul-
ture medium was replaced with 3 ml of phosphate-free Dulbecco’s 
modified Eagle’s medium (Invitrogen) containing 1 mM Ca of
RESULTS

Fig. 1A shows a representative experiment of cell cycle synchronization in NRK-49F cells. The first peak delineates cells in G0/G1 phase, the second peak delineates cells in G2/M phase, and the area between the two peaks delineates cells in S phase. Cells up to 10 h after serum addition are at G0/G1 phase, 12–16 h are at S phase, 18–22 h are at G2/M phase, and 24 h are at G0/G1 phase. To determine whether the expression of CIC-2 mRNA or protein varies during the cell cycle, RNA blot and immunoblot analysis were performed in cells at various time points after serum addition. The expression level of CIC-2 mRNA apparently did not change during the cell cycle (Fig. 1B). In contrast, the expression level of CIC-2 channel protein varied markedly during the cell cycle (Fig. 1C); an immunopositive band was barely detectable between 6 and 20 h after serum addition, but a strong immunopositive band at about 100 kDa, which is approximately the expected molecular size of the CIC-2 channel, was detected at 22 h. At 24 and 26 h, the intensity of the immunopositive band at 100 kDa was markedly diminished compared with that at 22 h.

To further examine cell cycle-specific expression of CIC-2 channel protein, the CIC-2 channel was immunostained at various stages of the cell cycle in NIH3T3 cells stably expressing rbClC-2-HA (NIH3T3(rbClC-2-HA)). Fig. 2A shows representative differential interference contrast images (upper panels) and immunfluorescence images (lower panels) at 18, 22, and 26 h after serum addition. Although the differential interference contrast image shows the presence of many cells at all 3 time points, positive immunofluorescence for the CIC-2 channel was observed only in dividing cells at 22 h and not at 18 or 26 h. Fig. 2B depicts typical immunofluorescence-positive cells observed at 22 h. Positive immunofluorescence was observed mostly in cells close to the exit of M phase, such as those with two nuclei and a constriction ring (panel a), cells just before cell division (panel b), and cells just after division (panel c).

Because the ubiquitination-26 S proteasome pathway is frequently utilized to degrade proteins with short half-lives (less than 2 h) (21, 22), including several cell cycle-related proteins (23), ubiquitination could well be involved in the rapid decrease of CIC-2 channel protein after cell division. To determine whether this is the case, NIH3T3(rbClC-2-HA) cells treated with 26 S proteasome inhibitor zLLLal (10 μM) (24, 25) for 2 h was immunoprecipitated with anti-HA antibody followed by immunoblotting with anti-ubiquitin antibody. A smear pattern was observed at molecular weights greater than about 100 kDa (the third lane in Fig. 3A). In controls, the only weak smear was observed in NIH3T3(rbClC-2-HA) cells without treatment with zLLLal (second lane), and there was no smear in NIH3T3 cells transfected with mock (first lane). These findings indicate that CIC-2 channels are polyubiquitinated. To determine whether ubiquitination of the CIC-2 channels varied during the cell cycle, we performed ubiquitin assay in cells collected at various time points after serum addition. The magnitude of ubiquitination was greater when zLLLal was added between 21–23 h and cells were collected at 23 h rather than at other time points (Fig. 3C).

We found in previous experiments using in vitro and cell-free phosphorylation assays that the CIC-2 channel is phosphoryl-
ated by the M phase-specific cyclin-dependent kinase p34\textsuperscript{cdc2}/cyclin B at Ser-632 in the C terminus (12). Because the tryptophan-tryptophan (WW) domain of Nedd4, an E3 ubiquitin ligase, binds to cdc25 only when it is phosphorylated at the exit of the mitotic phase (26, 27), phosphorylation by p34\textsuperscript{cdc2}/cyclin B might well link the ClC-2 channels with an E3 ubiquitin ligase and ubiquitination. We first determined whether or not ClC-2 channels are phosphorylated in vivo. In vivo phosphorylation assay revealed incorporation of \textsuperscript{32}P at about 100 kDa in HKE293 cells transfected with rbClC-2-HA but not in mock-transfected HKE293 cells (Fig. 4A). \textsuperscript{32}P incorporation was markedly diminished in HKE293 cells transfected with rbClC-2(S632A)-HA, a mutant in which Ser-632 in the consensus sequence of phosphorylation by p34\textsuperscript{cdc2}/cyclin B is replaced with Ala (Fig. 4A). Incubation with olomoucine, a cyclin-dependent kinase inhibitor (28), also diminished \textsuperscript{32}P incorporation (Fig. 4B). To investigate the relationship between ubiquitination of ClC-2 channel and its phosphorylation by p34\textsuperscript{cdc2}/cyclin B, we performed a ubiquitin assay in the presence of olomoucine and in cells transfected with rbClC-2(S632A). The magnitude of ubiquitination was significantly suppressed by olomoucine and was almost completely abolished in cells transfected with rbClC-2(S632A) (Fig. 5).

We examined HKE293 cells transiently transfected with pcDNA3.1(+)/rbClC-2-HA or pcDNA3.1(+)/rbClC-2(S632A)-HA to determine if zLLLal or olomoucine affected the steady-state level of ClC-2 channel expression. Compared with control conditions (first lane in Fig. 6), incubation with zLLLal (10 \mu M) for 6 h (second lane in Fig. 6, A and B) or olomoucine (10 \mu M) for 6 h (third lane in Fig. 6, A and B) significantly increased the expression level of ClC-2 channel protein. In NIH3T3 cells transfected with rbClC-2 containing the S632A mutation, the level of ClC-2 channel expression also was significantly increased (fourth lanes in Fig. 6, A and B) compared with control conditions.

**DISCUSSION**

In the present study, we examined CIC-2 channel expression levels during the cell cycle. We find that CIC-2 channel protein is expressed predominantly in dividing cells at M phase, that CIC-2 channel protein is ubiquitinated strongly at M phase, and that this ubiquitination is dependent on phosphorylation of the channel by p34\textsuperscript{cdc2}/cyclin B, which leads to proteasomal degradation of CIC-2 channel protein.

Immunoblotting and immunocytochemistry show that CIC-2 channel protein is expressed predominantly in dividing cells. Because RNA blot analysis revealed no appreciable alteration in mRNA expression level during the cell cycle, M phase-dominant expression of CIC-2 channel protein is most likely regulated at the post-transcriptional level rather than at the transcriptional level. CIC-2 channel protein expression rapidly decreased immediately after division of the cell, suggesting active degradation of CIC-2 channel protein at this point in the cell cycle. Several proteins containing a PEST sequence of enriched Pro, Glu, Ser, and Thr flanked by positively charged amino acids, particularly in the C terminus, have short intracellular half-lives (less than 2 h) (29, 30). They appear to be important as signals for ubiquitination (30, 31); for example, phosphorylation of the G\textsubscript{1} cyclin in yeast within the PEST region leads to recognition by an E3 ubiquitin ligase (31). Analysis using PEST-FIND software found the five PEST sequences to be clustered in the C terminus of CIC-2 channels, and ubiquitin assays showed CIC-2 channels to be polyubiquitinated. Because ubiquitination of CIC-2 channels is highest around the exit of M phase (21–23 h), it may well be responsible for the rapid disappearance of the CIC-2 channels after cell division. The mechanisms responsible in up-regulation of CIC-2 channel protein expression at M phase, on the other hand, are not known. Although in many cell cycle regulators such as the cyclins, cyclic protein expression occurs due to a burst of transcription that precedes the regulated degradation, cyclic expression of some proteins occurs due to modulation of protein synthesis (32, 33). The latter may be the case in the CIC-2 channel, because there is no noticeable regulation of CIC-2 channel transcription (Fig. 1B). To clarify the mechanism of regulated protein synthesis of the CIC-2 channel requires further investigation and is beyond the scope of the present study. However, it is interesting that cell cycle-dependent phosphorylation of the translational repressor binding protein (4E-BP1) by p34\textsuperscript{cdc2}/cyclin B was recently found to cause its dissociation from the eukaryotic initiation factor 4F (eIF-4F), switching on mRNA translation at M phase (34).

We found previously using in vitro and cell-free phosphorylation assays that CIC-2 channels are phosphorylated by the M phase-specific cyclin-dependent kinase p34\textsuperscript{cdc2}/cyclin B at Ser-632 in the C terminus (12). We show in the present study that CIC-2 channels are phosphorylated in vivo. Application of olomoucine or the presence of the S632A mutation markedly but not completely diminished the magnitude of phosphorylation, suggesting that p34\textsuperscript{cdc2}/cyclin B is at least in part responsible for phosphorylation of the CIC-2 channel at Ser\textsuperscript{632} and also that the CIC-2 channel is phosphorylated by some other kinase at a residue other than Ser-632. This finding is consistent with the previous report that the CIC-2 channel expressed in Xenopus oocytes is functionally regulated by protein kinase A (9). In contrast, CIC-2 channel ubiquitination was almost completely abolished by the replacement of Ser-632 to Ala and was strongly inhibited by olomoucine. These data indicate that ubiquitination of the CIC-2 channel is dependent on phosphorylation of the channel by p34\textsuperscript{cdc2}/cyclin B.

In addition to being a signal of proteasomal degradation, ubiquitination is now recognized to be a signal in a broad spectrum of events, including endocytosis, intranuclear trafficking, viral budding, and endosome trafficking (35). In the present study, inhibition of a 26 S proteasome with zLLLal or of phosphorylation by p34\textsuperscript{cdc2}/cyclin B with olomoucine or insertion of the S632A mutation raised the steady-state level of CIC-2 channel protein, indicating that this ubiquitination participates in proteasomal degradation.

We showed previously that CIC-2 channels are functionally regulated by M phase-specific phosphorylation (12). CIC-2 channel currents expressed in Xenopus oocytes are inhibited by phosphorylation by p34\textsuperscript{cdc2}/cyclin B. Accordingly, p34\textsuperscript{cdc2}/cyclin B may well be important both in regulation of CIC-2 channel function and in expression of channel protein in a cell cycle-dependent manner. Further study is required to clarify the physiological role of the CIC-2 channel in the cell cycle.

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