Nucleotides bind to the C-terminus of CIC-5

Leigh WELLHAUSER*†, Hsin-Hen KUO*†, Fiona L. L. STRATFORD*, Mohabir RAMJEESINGH*, Ling-Jun HUAN*, Winnie LUONG*†, Canhui LI*†, Charles M. DEBER*† and Christine E. BEAR*†‡¹

*Programme in Structural Biology and Biochemistry, Research Institute, Hospital for Sick Children, 555 University Avenue, Toronto, ON, Canada M5G 1X8,

†Department of Biochemistry, University of Toronto, Toronto, ON, Canada M5S 1A8, and ‡Department of Physiology, University of Toronto, Toronto, ON, Canada M5S 1A8

Mutations in CIC-5 (chloride channel 5), a member of the CIC family of chloride ion channels and antiporters, have been linked to Dent's disease, a renal disease associated with proteinuria. Several of the disease-causing mutations are premature stop mutations which lead to truncation of the C-terminus, pointing to the functional significance of this region. The C-terminus of CIC-5, like that of other eukaryotic CIC proteins, is cytoplasmic and contains a pair of CBS (cystathionine β -synthase) domains connected by an intervening sequence. The presence of CBS domains implies a regulatory role for nucleotide interaction based on studies of other unrelated proteins bearing these domains [Ignoul and Eggermont (2005) *Am. J. Physiol. Cell Physiol.* **289**, C1369–C1378; Scott, Hawley, Green, Anis, Stewart, Scullion, Norman and Hardie (2004) *J. Clin. Invest.* **113**, 274–284]. However, to date, there has been no direct biochemical or biophysical evidence to support nucleotide interaction with CIC-5.

In the present study, we have expressed and purified milligram quantities of the isolated C-terminus of CIC-5 (CIC-5 Ct). CD studies show that the protein is compact, with predominantly α -helical structure. We determined, using radiolabelled ATP, that this nucleotide binds the folded protein with low affinity, in the millimolar range, and that this interaction can be competed with 1 μ M AMP. CD studies show that binding of these nucleotides causes no significant change in secondary structure, consistent with a model wherein these nucleotides bind to a preformed site. However, both nucleotides induce an increase in thermal stability of CIC-5 Ct, supporting the suggestion that both nucleotides interact with and modify the biophysical properties of this protein.

Key words: CD, chloride channel 5 (CIC-5), chloride ion channel, cystathionine β -synthase, nucleotide, protein purification.

INTRODUCTION

Mutations in CIC-5 (chloride channel 5), a member of the CIC family of chloride ion channels and transporters, have been linked to Dent's disease, a renal disease associated with proteinuria [1–3]. Protein wasting by the kidney is thought to reflect impaired protein reabsorption across the apical membrane of the proximal tubule via the multiligand megalin/cubulin receptor [4,5]. Studies in mammalian cell lines and in CIC-5 knockout mice have implicated a primary defect in endosomal acidification in CIC-5-depleted cells and tissues [6–8], an endosomal function thought to be critical in modifying ligand–receptor interactions [9]. Our understanding of the molecular mechanisms underlying CIC-5 function is limited, however, and significant development in this area is required before we can define the molecular basis for disease.

CIC-5 belongs to a large family of CIC proteins, some of which function as Cl^-/H^+ antiporters (CIC-3, CIC-4 and CIC-5) and some as chloride channels (CIC-0, CIC-1 and CIC-2) [10]. The molecular basis for function of CIC-5 as a Cl^-/H^+ antiporter remains unclear. However, X-ray crystal structures of prokaryotic CIC family members have already proven invaluable in predicting several important structural features of the mammalian proteins. These membrane proteins are dimeric, with the ion translocation pathway(s) located within each monomer [11]. However, unlike the prokaryotic proteins described above, eukaryotic CIC proteins possess extensive N- and C-termini, both of which have been implicated in the regulation of CIC protein function [12–15].

Sequence analysis of the C-terminus of all mammalian CIC proteins, including CIC-5, reveals the existence of a pair of CBS (cystathionine β -synthase) domains separated by a 'linker segment' [10,16]. Crystal structures of bacterial enzymes bearing two CBS domains show how the two domains associate to form a pair. The fold of each domain, comprising a $\beta 1$ – $\alpha 1$ – $\beta 2$ – $\beta 3$ – $\alpha 2$ pattern, associates with the other domain in an antiparallel arrangement and a cleft is formed between these two domains [16]. Recently, the first crystal structure of the C-terminus of a CIC protein, the *Torpedo marmorata* CIC channel (CIC-0), was determined, revealing that the overall fold of the CBS domain pair is conserved among different families of proteins [17]. However, the structure of the 'linker segment' was not resolved in this structure, possibly reflecting its inherent disorder and the extreme C-terminus was absent due to its proteolysis. Therefore uncertainty remains regarding the structure of these regions, both of which have been directly implicated in CIC protein regulation [13,15].

There is increased support for the idea that CIC proteins bind and are regulated by nucleotides [16,18]. For example, voltage-dependent activation of CIC-1 chloride channel activity is inhibited by either AMP or ATP binding [19]. CIC-2, another channel-forming member of this family, is regulated differently, with replacement of ATP with AMP causing an increase in the rate of activation [20]. These results suggest that CIC-2 is normally inhibited by ATP and this inhibition is relieved by AMP binding. The functional consequences of nucleotide binding to CIC-5 are yet to be determined. However, it was shown by Vanoye and George [21] that chloride transport function by CIC-4 (a very close relative of CIC-5, with 78 % sequence identity) is dependent

Abbreviations used: CBS domain, cystathionine β -synthase domain; CFTR, cystic fibrosis transmembrane conductance regulator; CIC-5, chloride channel 5; CIC-5 Ct, C-terminus of CIC-5; hCIC-5, human CIC-5; IPTG, isopropyl β -D-thiogalactoside.

¹ To whom correspondence should be addressed, at Research Institute, Hospital for Sick Children, 555 University Avenue, Toronto, ON, Canada M5G 1X8 (email bear@sickkids.ca).

on ATP and activity is inhibited upon replacement of ATP with the non-hydrolysable analogue, p[NH]ppA (adenosine 5'-[β , γ -imido]triphosphate), implicating a possible role for hydrolysis in the ATP-dependent regulation of CIC-4.

Scott et al. [18] recently showed that several proteins containing CBS domain pairs [i.e. AMPK (AMP-activated protein kinase) and IMPDH (inosine-5'-monophosphate dehydrogenase)] bind nucleotides. These authors also showed that the C-terminus of CIC-2 is capable of binding ATP as a glutathione S-transferase-fusion protein [18]. However, as the available crystal structures, including the recent crystal structure of a C-terminal construct of CIC-0 [17], failed to show nucleotide bound to the CBS domain pair, the site to which nucleotides bind remains unclear. Bennetts et al. [19] used molecular modelling to predict that ATP binds within the cleft formed by the pair of CBS domains in the C-terminus of CIC-1. Mutagenesis of the residues predicted to co-ordinate nucleotides in the CBS domains of the C-terminus altered nucleotide-dependent changes in CIC-1 channel gating, supporting the role for these domains in nucleotide binding. However, as yet there were no direct biochemical studies to confirm nucleotide binding to CIC-1. Interestingly, a disease-causing mutation in CIC-2, located in the linker between the two CBS domains, caused a decrease in ATP binding in the context of the C-terminal fusion protein described above. At present, it is unclear whether the CBS domains alone or together with the intervening sequence linking these regions comprise the nucleotide-binding pocket. Together, these findings point to the potential physiological significance of the C-terminus in CIC protein function as well as the putative regulatory role of nucleotide binding and/or hydrolysis. However, the above reports highlight the remaining uncertainties in the field, several of which can be resolved in direct biochemical assays of nucleotide binding and hydrolysis by the C-terminus of different CIC proteins.

In the present study, we describe the procedure for expressing and purifying a protein corresponding to the entire C-terminus of CIC-5, containing both CBS domains plus the intervening region. The high yield of protein permitted the first direct assessments of nucleotide interaction using both biochemical and biophysical methods.

METHODS

Construct of CIC-5 C-terminus

Recombinant hCIC-5 (human CIC-5) was a gift from T. Jentsch [ZMNH (Zentrum für Molekulare Neurobiologie Hamburg), University of Hamburg, Hamburg, Germany]. The pcDNA plasmid containing hCIC-5, as described previously, was used as a template for PCR [8]. Primers were designed to amplify the DNA encoding the predicted residues of the C-terminus of the full-length hCIC-5 (575–746 amino acids) and to add a C-terminal hexahistidine tag. Primers were flanked by 5'-BamHI and 3'-NotI ends, and the PCR products were cloned into pET28c using these restriction sites.

Expression and purification of the C-terminus of CIC-5

The *Escherichia coli* BL21 (DE3) strain was used for the expression of the pET28c-hCIC-5 Ct (where CIC-5 Ct is C-terminus of CIC-5) plasmid. The culture containing pET28c-hCIC-5 Ct was grown at 37 °C in Luria–Bertani medium (0.5 litre) supplemented with 30 μ g/ml kanamycin until the absorbance at 600 nm reached 0.6, upon which the culture was induced overnight at room temperature (25 °C) with 5 mM IPTG (isopropyl β -D-thiogalactoside; Sigma, U.S.A.). The cells were collected by centrifugation

(6370 g, 15 min and 4 °C) and lysed by a French press (Spectronic Instruments, New York, NY, U.S.A.) into 100 mM Tris/HCl (pH 8.0) containing complete protease inhibitors (Roche Diagnostics, Mannheim, Germany). A final centrifugation (81 620 g, 2 h and 4 °C) removed the insoluble fraction, and the supernatant was filtered through a 0.22 μ m pore filter prior to being applied at 1 ml/min to a 1 ml HisTrap HP column (Amersham Biosciences, Baie d'Urfé, QC, Canada). Using an AKTA FPLC system (Amersham Biosciences), an imidazole gradient was applied to the column by titrating buffer 1 (20 mM Tris/HCl, 20 mM NaCl and 20 mM imidazole, pH 8.0) and buffer 2 (20 mM Tris/HCl, 20 mM NaCl and 300 mM imidazole, pH 8.0), and the gradient was held at 90 and 160 mM imidazole to elute impurities and peptide respectively. Fractions containing purified hCIC-5 Ct were identified by SDS/PAGE, silver staining and Western blotting, and were pooled and concentrated to a final volume of 1–2 ml. The protein was concentrated using an Amicon Ultra-15 centrifugal filter unit, molecular-mass cut-off 10 kDa (1770 g and 4 °C).

Assays of ATP binding and hydrolysis

Purified CIC-5 Ct (10 μ g) diluted in ATP binding buffer (20 mM Tris/HCl, 40 mM NaCl and 4 mM MgCl₂, pH 7.5) was incubated with indicated concentrations of ATP and 1 μ Ci of [α -³²P]ATP for indicated time periods at 33 °C. Unbound [α -³²P]ATP was separated from [α -³²P]ATP bound to the CIC-5 Ct by applying the sample to a filter column (Fischer Scientific, Fairlawn, NJ, U.S.A.) packed with Sephadex G-50 fine resin (Amersham Biosciences) and centrifuging for 5 min at 110 g. Radioactivity eluted from the column was quantified through scintillation counting. Non-specific binding was determined by control assays with urea (6 M)-denatured CIC-5 Ct.

ATPase activity was measured as the production of [α -³²P]ADP from [α -³²P]ATP by the CIC-5 Ct as described previously [22]. The assay was carried out in a reaction mixture (15 μ l) containing 1 μ g of CIC-5 Ct, 10% (v/v) glycerol, 50 mM Tris/HCl, 50 mM NaCl, 2 mM MgCl₂, 0.1 μ M dodecyl maltoside and 8 μ Ci of [α -³²P]ATP (3000 Ci/mmol) and 1.0 mM unlabelled ATP (pH 7.5).

CD studies

CD studies were performed with the Jasco J-810 spectropolarimeter (Jasco, Easton, MD, U.S.A.) using a quartz cuvette with a 1 mm path length and 13 μ M CIC-5 Ct. The CD spectrum of thermal denaturation of CIC-5 Ct was obtained from 195–250 nm at 1° increments between 25 and 70 °C. Thermal denaturation of CIC-5 Ct in the presence and absence of nucleotides was monitored by CD at 222 nm. The melting temperature, T_m , was determined by curve fitting of the transition region of the melting profile using Spectra Manager software (Jasco). The temperature was controlled by a circulating bath and increased with a programmable Jasco PFP-425S temperature controller at a scan rate of 1 °C/min. All CD spectra are generated from duplicate scans and are representative of four experiments (different aliquots) obtained from three different protein preparations. Each point in the thermal denaturation profile is representative of four separate experiments, employing two independent preparations of CIC-5 Ct protein.

Analyses

The time dependence for [³²P]ATP binding was fitted using a logarithmic function, and the ATP dose–response curve was fitted using the single-site binding algorithm using the curve-fitting program Prism (GraphPad, San Diego, CA, U.S.A.). The statistical significance of differences between experimental groups was evaluated using the unpaired *t* test (Prism; GraphPad).

Figure 1 Expression of the C-terminus of CIC-5

(A) A fragment of CIC-5 corresponding to residues 575–746 of hCIC-5 was expressed in bacteria using the pET28c expression vector. The N-terminus of this fragment corresponds to a glutamic residue (Glu⁵⁷⁵) four positions from the putative boundary of the membrane domain of CIC-5 (Ala⁵⁷¹) as predicted in the structural alignment of CIC-5 with the bacterial membrane proteins ecCIC (CIC from *E. coli*) and stCIC (CIC from *Salmonella Typhimurium*) [11]. A hexahistidine tag was inserted on to the C-terminus of this protein for subsequent purification. (B) Western blot (anti-His antibody) of 20 µg of total protein in the supernatant (S) and pellet (P) shows that a protein of the predicted mass, approx. 23 kDa, was specifically expressed following an overnight induction with 5 mM IPTG at room temperature. Lysis in 100 mM Tris/HCl (pH 8.0) buffer led to optimal solubilization of this protein with 162% enrichment in the supernatant relative to the pellet fraction assessed through band densitometry using Image J (NIH Imaging).

Figure 2 Purification of the C-terminus of CIC-5

(A) The C-terminus of CIC-5 (CIC-5 Ct-His) was purified to near homogeneity by nickel affinity. A prominent band running as a 23 kDa protein was detected by Coomassie Blue staining and the protein was sequenced by MS following gel extraction to confirm the identity of the protein and to determine that the N-terminus was intact. Molecular masses are given in kDa (kD). (B) The stability of the purified protein was assessed following a 48 h incubation in the presence of varying imidazole concentrations, various salts and additives. Stability was determined as the abundance of soluble protein in a dot-blot assay, assessed using densitometry. As imidazole concentration should be minimized for spectroscopic methods, we chose a buffer containing 10 mM imidazole in the presence of 0.5 M NaCl to optimize protein stability. TCEP, tris-(2-carboxyethyl)-phosphine.

RESULTS

Expression and purification of the C-terminus of CIC-5

A protein corresponding to the C-terminus of CIC-5, extending from residues 575 to 746 and engineered to possess a polyhistidine tag, was expressed in *E. coli*. This fragment encompasses the entire cytoplasmic C-terminus, including both CBS domains (Figure 1A). The protein, detected by SDS/PAGE analysis and immunoblotting using an anti-polyhistidine antibody, is expressed exclusively in induced bacterial cultures and migrates as a 23 kDa protein as expected (Figure 1B). Previous purification studies of bacterially expressed C-terminal fragments of CIC proteins reported that such proteins exhibit limited solubility [23]. Therefore we initially subjected the bacterial lysates to a solubility screen in order to optimize the yield of soluble protein. Buffering agents as well as salt composition were varied in this screen, and the effect of the relatively mild detergent DDM (n-dodecyl β-D-maltoside) was also determined. Regardless of the salt employed (except for CaCl₂), we found that solutions buffered using 100 mM Tris/HCl at pH 8.0 provided the best yield of soluble protein. The presence of a low concentration of detergent (50 µM) did not appreciably

increase the yield in the presence of this buffer. Therefore large-scale bacterial cultures (0.5–1.0 litre) were lysed in the presence of buffers containing 100 mM Tris/HCl (pH 8.0). As shown in Figure 1(B), the ratio of CIC-5 Ct protein was enriched in the supernatant relative to the cell pellet under these conditions. Accordingly, we purified this protein from the supernatant rather than attempting to extract protein from the pellet and inclusion bodies.

CIC-5 Ct protein was purified to near homogeneity by virtue of the affinity of its polyhistidine tag for a nickel column. The purified protein was stained with Coomassie Blue and migrated as expected (23 kDa) (Figure 2A). The identity of the protein and the integrity of the N-terminus were confirmed following band extraction and analysis by MS. We performed an additional screen in order to determine the optimal conditions for maintaining protein stability as the protein exhibited a tendency to precipitate with time, consistent with previous reports of the C-terminal fragments isolated from the related protein, CIC-0 [22]. Imidazole concentrations were varied in the 'stability' screen, as were salt concentrations and the presence of various additives [i.e. tris-(2-carboxyethyl)-phosphine and glycerol; Figure 2B]. The presence of imidazole tended to increase stability (Figure 2B).

Figure 3 CD spectra of purified CIC-5 Ct

CD spectra of purified CIC-5 Ct obtained at various temperatures ranging from 25 to 55 °C, labelled a–g, in 5 °C increments. Purified CIC-5 Ct was exchanged into 5 mM Tris/HCl and 500 mM NaCl (pH 8.0) and used at a concentration of 13 μ M. At 25–40 °C, the spectra exhibited double minima (208 and 222 nm) characteristic of α -helical structure. The amplitude of both bands decreased at temperatures > 40 °C, consistent with temperature-sensitive loss of helical structure.

However, even in the presence of low imidazole concentrations (10 mM), the protein remained relatively stable in the presence of 500 mM NaCl. Therefore we chose this solution to preserve stable protein for subsequent spectroscopic studies.

Analysis of the secondary structure of the CIC-5 Ct peptide

The CD spectrum of purified CIC-5 Ct in 5 mM Tris/HCl and 500 mM NaCl (pH 8.0) buffer exhibited the double minima at 208 and 222 nm characteristic of α -helical structure at room temperature (Figure 3). The molar ellipticity values of the bands observed in the spectrum (near -10000°), along with the skewing of the classical helical pattern towards 200 nm, suggested that the folded protein also contains a proportion of unstructured ('random') regions in addition to its helical content. The protein was then subjected to increasing temperatures (in 1 °C increments) in order to determine its thermal stability. Spectra were monitored as a function of the decrease in ellipticity (θ) measured at 222 nm. As shown in Figure 3, the peptide retained its characteristic α -helical spectrum until the temperature was increased to approx. 45 °C; at that temperature and above, there was a dramatic monotonic decrease in helical structure. We found that this temperature-dependent change in CD was not reversible (results not shown), suggesting that the unfolded protein is likely prone to aggregation at these higher temperatures.

The C-terminus of CIC-5 binds but does not hydrolyse ATP

It has been suggested that pairs of CBS domains confer a nucleotide-binding pocket in related proteins containing such pairs [19]. Also, the function of the CIC proteins CIC-1, CIC-2 and CIC-4 has been shown to be modified by nucleotide binding; for example, it has been reported that the function of CIC-4, the closest relative to CIC-5, is regulated by ATP but not by non-hydrolysable analogues, suggesting that CIC-4 may bind and hydrolyse ATP [21]. These reports prompted our analysis of the direct binding of [32 P]ATP to the purified C-terminus of CIC-5 as well as our study of its ATPase activity.

In the binding studies, [32 P]ATP was added to CIC-5 Ct in varying concentrations in order to measure the concentration dependence of binding. Unbound [32 P]ATP was removed from [32 P]ATP bound to the C-terminal peptide by gel filtration. Figure 4(A) presents the time course for [32 P]ATP binding to CIC-5 Ct, showing that binding is complete within 2 h. Urea (6 M)-

Figure 4 ATP binds to CIC-5 Ct

(A) The time course for [32 P]ATP binding to purified CIC-5 Ct ($\square$, $n = 4$) and denatured CIC-5 Ct (treatment with 6 M urea, ∇ , $n = 4$) was determined in the presence of 1 mM unlabelled ATP. In each trial, 10 μ g of protein was assayed. Means $\pm$ S.E.M. are shown. Data for native CIC-5 Ct were fitted using logarithmic function ($r = 0.8$). (B) ATP concentration dependence of [32 P]ATP binding was fitted using the single-site binding algorithm ($r = 0.85$) to yield the binding affinity constant, $K_d = 1.25$ mM ATP. The means and S.E.M. for three trials are shown. Non-specific binding was corrected by subtracting mean binding to urea-denatured protein, 25 ± 2 c.p.m. (mean $\pm$ S.E.M.). (C) This bar graph shows the effect of pretreatment with 1 μ M MgAMP on [32 P]ATP binding (1 mM) to CIC-5 Ct as determined in three comparative studies; non-specific binding was corrected as described above. The difference between the two data sets is significant ($P = 0.0035$). Means and S.E.M. are shown.

denatured CIC-5 Ct failed to exhibit increased [32 P]ATP binding over time, supporting the specificity of binding to the native protein. Figure 4(B) shows the concentration dependence for MgATP $^{2-}$ binding to CIC-5 Ct, following subtraction of non-specific binding by denatured protein (25 ± 2 c.p.m.; mean $\pm$ S.E.M.) and these data were fitted using the single-site binding algorithm to yield a K_d value of approx. 1.25 mM MgATP $^{2-}$. Pretreatment of the CIC-5 Ct peptide with the non-hydrolysable nucleotide MgAMP at 1 μ M effectively competed binding of [32 P]ATP in the presence of 1 mM MgATP $^{2-}$ (Figure 4C). In further experiments, we examined the ATPase activity of the CIC-5 Ct using an assay system commonly used to assess the ATPase activity of the full-length CFTR (cystic fibrosis transmembrane conductance regulator) protein (Figure 5) [24,25]. Relative to the CFTR protein, we found that the CIC-5 Ct protein exhibited negligible levels of ATPase activity, comparable with that exhibited by the denatured CIC-5 Ct protein (Figure 5).

Binding of nucleotides to CIC-5 Ct protein increases its thermal stability

As the studies shown in Figure 4 revealed that the CIC-5 Ct is capable of binding MgAMP and MgATP $^{2-}$, we next asked whether nucleotide binding affects its secondary structural and/or propensity for unfolding and aggregation. To address this question, we used CD spectroscopy to compare the secondary structure and temperature-sensitivity of the CIC-5 Ct protein in the presence or absence of the nucleotides, MgAMP and MgATP $^{2-}$. We first noted that regardless of the presence or absence of nucleotide, the CIC-5 Ct protein maintains a similar content of helical structure at room temperature, i.e. spectra at 25 °C were unaltered by

Figure 5 CIC-5 Ct does not exhibit ATPase activity

ATPase activity was measured as the production of [α - 32 P]ADP from [α - 32 P]ATP as previously described [22,24,25]. The ATPase activity by the CIC-5 Ct protein was similar before and after heat denaturation (open and hatched bars respectively; results shown are means $\pm$ S.E.M.). The activity exhibited by CIC-5 Ct ($n = 4$) was negligible compared with that previously determined for purified, reconstituted CFTR (solid bar, $n = 3$, $*P = 0.006$, [25]).

addition of 500 μ M AMP or ATP (Figure 6A). Although 1 μ M MgAMP is physiologically relevant [18] and competes MgATP $^{2-}$ binding to CIC-5 Ct, as shown in Figure 4, this concentration is low relative to the molar concentration of CIC-5 Ct required for these CD analyses (13 μ M) and is expected to bind less than 10% of the protein in the assay. We thus evaluated the relative effects of 500 μ M MgAMP and 500 μ M MgATP $^{2-}$ on the protein's unfolding transition. Thermal stability was monitored by measuring the melting temperature (T_m), the temperature at which 50% of the original secondary structure was lost, at 222 nm. We found that the addition of 500 μ M MgAMP caused a significant increase in the melting temperature of the CIC-5 Ct, from $44.2 \pm 0.8^\circ\text{C}$ ($n = 4$) to $47.1 \pm 0.4^\circ\text{C}$ ($n = 4$, $P = 0.02$) (Figure 6B). Addition of 500 μ M MgATP $^{2-}$ induced an increase in T_m to $49.0 \pm 0.2^\circ\text{C}$ ($n = 4$, $P = 0.008$), a value significantly greater than that induced by 500 μ M MgAMP. These overall findings suggest that while nucleotide interaction does not affect the secondary structure of the CIC-5 Ct protein itself, the presence of bound AMP or ATP is signalled by the increased resistance to CIC-5 Ct denaturation.

DISCUSSION

The present paper is the first to describe the biochemical features of the C-terminus of human CIC-5. The boundaries of this protein extend from the residues predicted to exit from the membrane domain of CIC-5 to the extreme C-terminus. This protein includes a pair of CBS domains, a feature common to all mammalian CIC proteins and predicted to be vital for the regulated function of all of these family members [10,13]. In the present study, we have provided direct biochemical evidence that this protein fragment binds the nucleotides AMP and ATP, supporting predictions from studies of related proteins [18,19]. We also determined that this isolated fragment cannot mediate the hydrolysis of ATP, resolving a question relevant to our understanding of the mechanism of action of this clinically relevant protein [21]. Importantly, analysis of the protein by CD spectroscopy suggests that this protein has a well-folded structure and, hence, is potentially amenable for future high-resolution structural studies.

To date, the molecular basis for nucleotide binding to the C-terminus of CIC proteins is unclear. There are no Walker consensus motifs in the primary sequence of this family of proteins, and none of the available crystal structures of CBS domains contains

Figure 6 Nucleotide binding induces an increase in thermal stability of CIC-5 Ct

(A) CD spectra of purified CIC-5 Ct (13 μ M) exchanged into 5 mM Tris/HCl and 500 mM NaCl (pH 8.0) in the absence of nucleotide (solid line), plus 500 μ M MgAMP (dashed line) or 500 μ M MgATP $^{2-}$ (dotted lines) obtained at 25 $^\circ\text{C}$. These spectra are representative of four samples, obtained from two different protein purifications. (B) Thermal stability of the secondary structure was determined as the change in CD at 222 nm caused by 1 $^\circ\text{C}$ increments in temperature starting at 25 $^\circ\text{C}$ and normalized relative to the maximum change observed at 55 $^\circ\text{C}$. A representative thermal stability experiment for CIC-5 Ct alone ($\blacktriangle$), CIC-5 Ct plus 500 μ M Mg-AMP ($\blacktriangledown$) or 500 μ M MgATP $^{2-}$ -bound CIC-5 Ct proteins ($\diamond$) is shown. The curves for each experiment were generated using Spectra Manager software (Jasco) and the temperature at which 50% of the signal at 222 nm remains (0.5) was determined to obtain T_m ($^\circ\text{C}$). Four studies of CIC-5 Ct yielded a mean T_m of $44.2 \pm 0.8^\circ\text{C}$ (mean $\pm$ S.E.M.), four studies of CIC-5 Ct + 500 μ M MgAMP yielded a mean T_m of $47.1 \pm 0.4^\circ\text{C}$ (mean $\pm$ S.E.M.) and four studies of CIC-5 Ct + 500 μ M MgATP $^{2-}$ yielded a mean T_m of $49.0 \pm 0.2^\circ\text{C}$ (mean $\pm$ S.E.M.).

nucleotides. Bennetts et al. [19] have presented a model wherein ATP could be docked *in silico* within a cleft formed between the two CBS domains of CIC-1. According to these authors, the adenine base binds deeply within this cleft with the ribose ring and phosphate groups located closer to the top of the pocket. In support of this model, these authors found that mutation of residues within this putative binding pocket affected the nucleotide-regulated gating of CIC-1 channel activity [19]. On the other hand, in the related CIC protein CIC-2, a disease-causing residue located in the intervening sequence between the CBS domains perturbed ATP binding to a CIC-2 Ct fusion protein [18], implicating this intervening region in binding. Interestingly, the recent biochemical and crystallographic studies of a C-terminal fragment of CIC-0 (including both CBS domains and the intervening segment) did not detect nucleotide binding, and the authors suggested that a region distinct from the C-terminus of CIC-0 may instead be required for stable nucleotide binding [17]. These later findings may reflect the variation in the experimental approaches employed and divergence in the primary sequence between these family members, particularly within the intervening sequence between the CBS domains (< 11% sequence identity).

Both AMP and ATP nucleotides tended to increase the melting temperature of the native CIC-5 Ct conformation, with ATP

displaying a significantly greater effect (Figure 6B). The latter result likely arises from the fact that the three negatively charged phosphate groups available in ATP (versus one in AMP) provide an opportunity for electrostatic cross-linking interactions involving proximal protein polar side chains: this added binding energy must first be overcome before the protein secondary structure begins to denature. As suggested by Tratschin and co-workers [26], protein thermal stability can be achieved by changes in the fractional polar surface of proteins with the concomitant decrease in external hydrophobicity. High-resolution structural studies of the C-terminus of CIC-5 will be required to determine whether such nucleotide-dependent changes in thermal stability may reflect subtle changes in local electrostatic interactions or changes in tertiary or quaternary structure.

It has been postulated that CIC-5 normally functions as a Cl^-/H^+ antiporter to facilitate endosomal acidification [27,28], a function that is important in efficient dissociation of ligand-receptor interactions and receptor presentation on the cell surface [9,29]. It is well known that endosomal acidification is an ATP-dependent process as it is mediated by the V-type ATPase [30]. The present studies showing that CIC-5 directly binds nucleotide, together with previous functional data showing that the closest family relative CIC-4 is activated by ATP [21], support the idea that there may be co-ordinated regulation of the V-type ATPase and CIC-5. Such a parallel regulation is consistent with the putative facilitative role for CIC-5 in endosomal acidification.

Finally, the biochemical and biophysical studies described in the present paper provide the groundwork not only for high-resolution structural studies of the CIC-5 Ct, but also provide the rationale for future investigations of the regulation of CIC-5 by nucleotides. These studies show that ATP and AMP bind the isolated CIC-5 C-terminus, and future studies will focus on understanding the molecular basis for and the functional consequences of this interaction in the isolated fragment as well as the full-length protein. Such studies will also provide a template with which to understand the molecular consequences of disease-causing mutations described in the gene coding for CIC-5.

This research was supported primarily through a grant to C. E. B. from the Canadian Institutes of Health Research and in part through a grant to C. M. D. from the Canadian Cystic Fibrosis Foundation. L. W. and W. L. were supported by scholarships awarded by Natural Sciences and Engineering Research Council. H.-H.K. holds a Research Training Award from the Research Institute, Hospital for Sick Children (Toronto, ON, Canada). We also acknowledge contributions by Dr P. Lynne Howell (Head of the Programme in Structural Biology and Biochemistry, Hospital for Sick Children) in designing the solubility and stability screens for this protein, tools that were essential in optimizing the yield of protein for biophysical studies.

REFERENCES

- Thakker, R. V. (1999) Chloride channels in renal disease. *Adv. Nephrol. Necker Hosp.* **29**, 289–298.
- George, Jr, A. L. (1998) Chloride channels and endocytosis: CIC-5 makes a dent. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 7843–7845.
- Jentsch, T. J. (2005) Chloride transport in the kidney: lessons from human disease and knockout mice. *J. Am. Soc. Nephrol.* **16**, 1549–1561.
- Christensen, E. I., Devuyst, O., Dom, G., Nielsen, R., Van der Smissen, P., Verroust, P., Leruth, M., Guggino, W. B. and Courtois, P. J. (2003) Loss of chloride channel CIC-5 impairs endocytosis by defective trafficking of megalin and cubilin in kidney proximal tubules. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 8472–8477.
- Piwon, N., Gunther, W., Schwake, M., Bosl, M. R. and Jentsch, T. J. (2000) CIC-5 Cl^- -channel disruption impairs endocytosis in a mouse model for Dent's disease. *Nature (London)* **408**, 369–373.
- Hara-Chikuma, M., Wang, Y., Guggino, S. E., Guggino, W. B. and Verkman, A. S. (2005) Impaired acidification in early endosomes of CIC-5 deficient proximal tubule. *Biochem. Biophys. Res. Commun.* **329**, 941–946.
- Gunther, W., Piwon, N. and Jentsch, T. J. (2003) The CIC-5 chloride channel knock-out mouse – an animal model for Dent's disease. *Pflügers Arch.* **445**, 456–462.
- Mohammad-Panah, R., Harrison, R., Dhani, S., Ackerley, C., Huan, L. J., Wang, Y. and Bear, C. E. (2003) The chloride channel CIC-4 contributes to endosomal acidification and trafficking. *J. Biol. Chem.* **278**, 29267–29277.
- van Weert, A. W., Dunn, K. W., Guez, H. J., Maxfield, F. R. and Stoorvogel, W. (1995) Transport from late endosomes to lysosomes, but not sorting of integral membrane proteins in endosomes, depends on the vacuolar proton pump. *J. Cell Biol.* **130**, 821–834.
- Jentsch, T. J., Neagoe, I. and Scheel, O. (2005) CLC chloride channels and transporters. *Curr. Opin. Neurobiol.* **15**, 319–325.
- Dutzler, R., Campbell, E. B., Cadene, M., Chait, B. T. and MacKinnon, R. (2002) X-ray structure of a CIC chloride channel at 3.0 Å reveals the molecular basis of anion selectivity. *Nature (London)* **415**, 287–294.
- Jordt, S. E. and Jentsch, T. J. (1997) Molecular dissection of gating in the CIC-2 chloride channel. *EMBO J.* **16**, 1582–1592.
- Estevez, R., Pusch, M., Ferrer-Costa, C., Orozco, M. and Jentsch, T. J. (2004) Functional and structural conservation of CBS domains from CLC chloride channels. *J. Physiol.* **557**, 363–378.
- Hebeisen, S. and Fahlke, C. (2005) Carboxy-terminal truncations modify the outer pore vestibule of muscle chloride channels. *Biophys. J.* **89**, 1710–1720.
- Denton, J., Nehrkke, K., Yin, X., Beld, A. and Strange, K. (2005) Altered gating and regulation of a carboxy-terminus CIC channel mutant expressed in the *C. elegans* oocyte. *Am. J. Physiol. Cell Physiol.* **290**, C1109–C1118.
- Ignoul, S. and Eggermont, J. (2005) CBS domains: structure, function, and pathology in human proteins. *Am. J. Physiol. Cell Physiol.* **289**, C1369–C1378.
- Meyer, S. and Dutzler, R. (2006) Crystal structure of the cytoplasmic domain of the chloride channel CIC-0. *Structure* **14**, 299–307.
- Scott, J. W., Hawley, S. A., Green, K. A., Anis, M., Stewart, G., Scullion, G. A., Norman, D. G. and Hardie, D. G. (2004) CBS domains form energy-sensing modules whose binding of adenosine ligands is disrupted by disease mutations. *J. Clin. Invest.* **113**, 274–284.
- Bennett, B., Rychkov, G. Y., Ng, H. L., Morton, C. J., Stapleton, D., Parker, M. W. and Cromer, B. A. (2005) Cytoplasmic ATP-sensing domains regulate gating of skeletal muscle CIC-1 chloride channels. *J. Biol. Chem.* **280**, 32452–32458.
- Niemeyer, M. I., Yusef, Y. R., Cornejo, I., Flores, C. A., Sepulveda, F. V. and Cid, L. P. (2004) Functional evaluation of human CIC-2 chloride channel mutations associated with idiopathic generalized epilepsies. *Physiol. Genomics* **19**, 74–83.
- Vanoye, C. G. and George, Jr, A. L. (2002) Functional characterization of recombinant human CIC-4 chloride channels in cultured mammalian cells. *J. Physiol.* **539**, 373–383.
- Kogan, I., Ramjeesingh, M., Li, C. and Bear, C. E. (2002) Studies of the molecular basis for cystic fibrosis using purified reconstituted CFTR protein. *Methods Mol. Med.* **70**, 143–157.
- Maduke, M., Williams, C. and Miller, C. (1998) Formation of CLC-0 chloride channels from separated transmembrane and cytoplasmic domains. *Biochemistry* **37**, 1315–1321.
- Li, C., Ramjeesingh, M., Wang, W., Garami, E., Hewryk, M., Lee, D., Rommens, J. M., Galley, K. and Bear, C. E. (1996) ATPase activity of the cystic fibrosis transmembrane conductance regulator. *J. Biol. Chem.* **271**, 28463–28468.
- Ramjeesingh, M., Li, C., Garami, E., Huan, L. J., Galley, K., Wang, Y. and Bear, C. E. (1999) Walker mutations reveal loose relationship between catalytic and channel-gating activities of purified CFTR (cystic fibrosis transmembrane conductance regulator). *Biochemistry* **38**, 1463–1468.
- Argos, P., Rossmann, M. G., Grau, U. M., Zuber, H., Frank, G. and Tratschin, J. D. (1979) Thermal stability and protein structure. *Biochemistry* **18**, 5698–5703.
- Piccolo, A. and Pusch, M. (2005) Chloride/proton antiporter activity of mammalian CLC proteins CIC-4 and CIC-5. *Nature (London)* **436**, 420–423.
- Scheel, O., Zdebik, A. A., Lourdel, S. and Jentsch, T. J. (2005) Voltage-dependent electrogenic chloride/proton exchange by endosomal CLC proteins. *Nature (London)* **436**, 424–427.
- van Weert, A. W., Geuze, H. J., Groothuis, B. and Stoorvogel, W. (2000) Primaquine interferes with membrane recycling from endosomes to the plasma membrane through a direct interaction with endosomes which does not involve neutralisation of endosomal pH nor osmotic swelling of endosomes. *Eur. J. Cell Biol.* **79**, 394–399.
- Wagner, C. A., Finberg, K. E., Breton, S., Marshansky, V., Brown, D. and Geibel, J. P. (2004) Renal vacuolar H^+ -ATPase. *Physiol. Rev.* **84**, 1263–1314.