

## The Binding of Human CLIC1 with SEDL and Its Characterization *in vitro*

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Full-length chloride intracellular channel protein 1 (CLIC1) is a member of the family of proteins related to bovine chloride intracellular channel p64. Mutations in the SEDL gene cause spondyloepiphyseal dysplasia tarda (SEDT), a rare X-linked chondrodysplasia. The link between the intracellular chloride channels and SEDL is an important step toward understanding their functional interplay. In the present study, CLIC1 protein was subcloned into the pGEX-KG vector and overexpressed in XL-1 blue cells. We developed a large-scale expression system composed of glutathione S-transferase (GST) fused with a 240-amino-acid CLIC1 protein in *Escherichia coli*. The soluble CLIC1 protein was successfully purified to homogeneity, and its purity, identity, activity and conformation were determined using SDS-PAGE, MALDI-MS, biophotometer and circular dichroism spectroscopic studies. The binding of both CLIC1 and SEDL proteins *in vitro* was detected by BIAcore biosensor and fluorescence measurements.

**Key Words** : Subcloning, Overexpression, Purification, Characterization, Chloride intracellular channel (CLIC1)

### Introduction

Chloride channels in plasma membranes play important roles in defense of cell volume, in transepithelial transport, setting the membrane potential, bone resorption, and the response to certain neurotransmitters.<sup>1,2</sup> Chloride channels are also present in intracellular membranes where they are known to play important roles in acidification of intracellular compartments in exocytosis.<sup>3,4</sup> Malfunction in these channels can lead to severe disease states.<sup>5</sup>

There are seven members of the chloride intracellular channel protein (CLIC) family: CLIC1, CLIC2, CLIC3, CLIC4, CLIC5, p64, and panchorin.<sup>6-10</sup> All of these exist as soluble globular proteins that can form ion channels in organellar and plasma membranes.<sup>7,11-13</sup> Five CLICs of wide distribution have been identified in the membranes of mitochondria, the endoplasmic reticulum (ER), and nuclei, among others.<sup>14,15</sup> CLIC1, the first member of the family have been identified in humans, is a transmembrane protein sufficient to form a functional ion channel as a tetrameric assembly of subunits.<sup>11,16</sup>

All of the CLIC proteins are expressed in a wide variety of tissues and appear to have diverse physiological functions. The renal chloride channel p64 is associated with kidney function, whereas CLIC1 and CLIC4 appear to have a broad tissue distribution.<sup>7,18</sup> P64 co-purifies with channel activity solubilized from bovine kidney microsomes. CLIC1 is expressed to some extent in most tissues and cell types that have been studied and is particularly highly expressed in muscle.<sup>17</sup> It is expressed in a punctate intracellular vesicular pattern in a variety of cultured cells, in the apical domain of renal proximal tubule cells, in an intracellular distribution in

placenta, and in the nuclei of Chinese hamster ovary cells.<sup>8,17</sup> Expression of a third family member, CLIC3, leads to increased whole-cell chloride conductance.<sup>19</sup>

SEDL mRNA is widely distributed in a variety of tissues, including heart, liver, and placenta. SEDL is highly conserved from yeast to humans.<sup>20</sup> Its ortholog in budding yeast, Trs20p, is a subunit of the transport protein particle (TRAPP) complex that plays key roles in vesicle docking/fusion processes during endoplasmic reticulum (ER)-to-Golgi transport.<sup>21-23</sup> Recent studies indicate that the TRAPP complex is a nucleotide exchanger for Ypt1 and Ypt31/32, which are GTPases essential to membrane trafficking.<sup>24,25</sup>

The unusual surface property of SEDL appears to be well suited for multiple protein-protein interactions involving different surface areas. Establishing the link between SEDL and the chloride intracellular channel is necessary in order to understand their functional interplay. Since both CLIC and SEDL proteins are membrane-associated proteins, their interactions might be important either to functions of the TRAPP complex or to the proper targeting and functions of the chloride intracellular channels.

In order to identify the interaction of the CLIC1-SEDL proteins, in the present study we developed methods for efficient overexpression and purification that generate pure and homogeneous CLIC1 protein, large quantities of such protein being required in order to pursue structural studies such as X-ray crystallography. Mouse SEDL was expressed in *E. coli* BL21(DE3)RIL strain and purified as described previously.<sup>26</sup>

The purity, identity, activity and conformation of the CLIC1 protein were determined using SDS-PAGE, MALDI-MS, biophotometer and CD spectroscopic studies. Furthermore, the binding of both CLIC1 and SEDL proteins *in vitro* was

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detected by BIAcore biosensor and fluorescence measurements.

### Materials and Methods

**Overexpression and extraction.** To generate glutathione S-transferase (GST)-fusion protein, CLIC1 was subcloned into the N-terminal GST-tagged fusion protein vector pGEX-KG. The construct was transformed into the expression host *Escherichia coli* XL-blue (Stratagene). A single colony was inoculated with 5 mL of LB (Luria-Bertani) medium containing 50  $\mu\text{g}/\text{mL}$  ampicillin for XL-blue. Bacteria were grown overnight at 37 °C, and were used to inoculate two flasks, each containing 1 L of LB with antibiotics. The cultures were grown at 37 °C until the OD<sub>600</sub> reached 0.5, and protein expression was induced with 1 mM of isopropyl- $\beta$ -D-galactopyranoside (IPTG). The bacterial cells were induced for 4–6 h at 37 °C, then harvested by centrifugation at 4,600 rpm for 30 min. The cell pellets were either immediately used or stored frozen at –70 °C. The cells were resuspended in 1 x PBS buffer (4.3 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.47 mM KH<sub>2</sub>PO<sub>4</sub>, 137 mM NaCl, and 2.7 mM KCl, pH 7.3). The cells were disrupted by sonication on ice, and the cell debris was discarded by centrifugation at 14,000 rpm for 45 min. The insoluble fractions were directly resuspended in a 2 x SDS loading buffer and incubated at 95 °C for 5 min. The whole cells, both the soluble and the insoluble fractions, were fractionated on 12% SDS-PAGE gels and visualized by Coomassie blue staining.

**Purification.** The clear supernatant was loaded onto a glutathione-Sepharose 4 fast flow (Amersham-Pharmacia Biotech; binding capacity 10 mg GST per ml of resin) at a flow rate of 2.5 mL/min and washed extensively with 20 mL of 1 x PBS buffer. The GST-CLIC1 fusion protein was eluted with 10 mL of 5 mM reduced glutathione dissolved in 50 mM of Tris-HCl (pH 8.0). The eluted fraction was dialyzed against elution buffer and reacted with thrombin for 12 h at 4 °C. The reaction mixture was loaded at 2.5 mL/min onto a Q-Sepharose fast-flow (Amersham-Pharmacia Biotech) anion exchange chromatography column pre-equilibrated with buffer A (50 mM Tris-HCl and 200 mM NaCl, pH 8.0). Elution was performed with the salt gradient of the NaCl solution. The protein was passed through the Q-Sepharose column to remove cellular DNA and negatively charged acidic protein. The CLIC1 protein was concentrated to a final volume of 20 mL by centrifuging it at 2,500 rpm using ultrafiltration devices. Then, gel filtration was performed using a Superdex 75 column on an HPLC. The protein was loaded onto a column equilibrated with buffer A and separated at a flow rate of 1.5 mL/min. The protein elution was monitored at 280 nm, and the resulting fraction was analyzed by electrophoresis on 12% SDS-PAGE gel.

**Protein concentration and activity analyses.** The protein concentration was determined by biophotometer using a UVette of 10 mm optical path length, and measured using the Bradford method with the Bio-Rad protein assay kit, with bovine serum albumin utilized as a standard.<sup>27</sup> The

effects of temperature and pH on the protein activity were examined. The assaying of protein activity was carried out by biophotometer in a reaction mixture (100  $\mu\text{L}$ ) consisting of 20 mM of Tris-HCl buffer (pH 7.5), 150 mM of NaCl, and 20  $\mu\text{L}$  of protein.

**Circular dichroism measurement.** A circular dichroism measurement was carried out with a spectropolarimeter (JASCO J-715) using a 0.1 cm cell at 0.2 nm intervals at 25 °C. The CD spectrum of the purified CLIC1 protein (the average of five scans) was recorded in the 200–260 nm range, and a far-UV CD spectrum at the protein concentration of 0.24 mg/mL was required. The CD spectrum was obtained in milli-degrees and converted to molar ellipticity prior to secondary structure analysis. Calculation of the content of the protein's secondary structure elements was performed using the CDNN program.<sup>28</sup>

**MALDI-MS analysis.** For in-gel digestion, 10  $\mu\text{L}$  of trypsin solution (2 ng/L in 25 mM of ammonium bicarbonate, pH 8.0) was added and digested overnight at 37 °C. Peptides were extracted with 50% ACN/0.2% TFA (trifluoroacetic acid) and dried under vacuum for 2 h, followed by reconstruction with 3  $\mu\text{L}$  of CHCA ( $\alpha$ -cyano-4-hydroxycinnamic acid) matrix solution (8 mg of CHCA in 1 mL of 50% ACN/0.2% TFA).<sup>29</sup> One microliter of reconstructed sample was loaded onto a 96 $\times$ 2 MALDI plate. The peptide mass was acquired with the Voyager DE-PRO (Applied Biosystems, Framingham, MA) in reflector mode under 20,000 V of accelerating voltage, 76% grid voltage and 0.002% guide-wire voltage. The Cal Mix 2 of the MALDI-MS calibration kit (Applied Biosystems, Framingham, MA) was used for the external calibration, and autolysis fragments of trypsin were used for the internal calibration. Peptide matching and protein identification were performed with the Mascot peptide mass fingerprint.

**Analytical ultracentrifugation.** Sedimentation equilibrium measurements were performed at 25 °C on a Beckman Optima XL-A analytical ultracentrifuge, using a four-hole rotor with standard double sector cell at a rotor speed of 10,000 rpm. The values of the two variables, that is, the absorbances at 280 nm *versus* the radial positions, were obtained. The apparent molecular weight of SEDL was calculated by fitting data sets to a single-species model using BMPD-Nonlinear regression software. The partial specific volume of SEDL and the solvent density were calculated as described by Zamyatin.<sup>30</sup>

**BIAcore biosensor analysis.** Measurements of the apparent dissociation constants ( $K_D$ ) between CLIC1 and SEDL were carried out using a BIAcore 2000 biosensor (Biosensor, Sweden). CLIC1 (500  $\mu\text{g}/\text{mL}$  in 10 mM Sodium Acetate, pH 4.0) was covalently bound by an amine-coupling method to the carboxylated dextran matrix at a concentration corresponding to –1,200 response units (RU), as suggested by the manufacturer. A flow path involving two cells was employed to simultaneously measure the kinetic parameters from one flow cell containing the CLIC1-immobilized sensor chip to the other flow cell containing an underivatized chip. For kinetic measurements at room temperature, SEDL samples, ranging from 50 to 500 nM, were prepared by dilution with

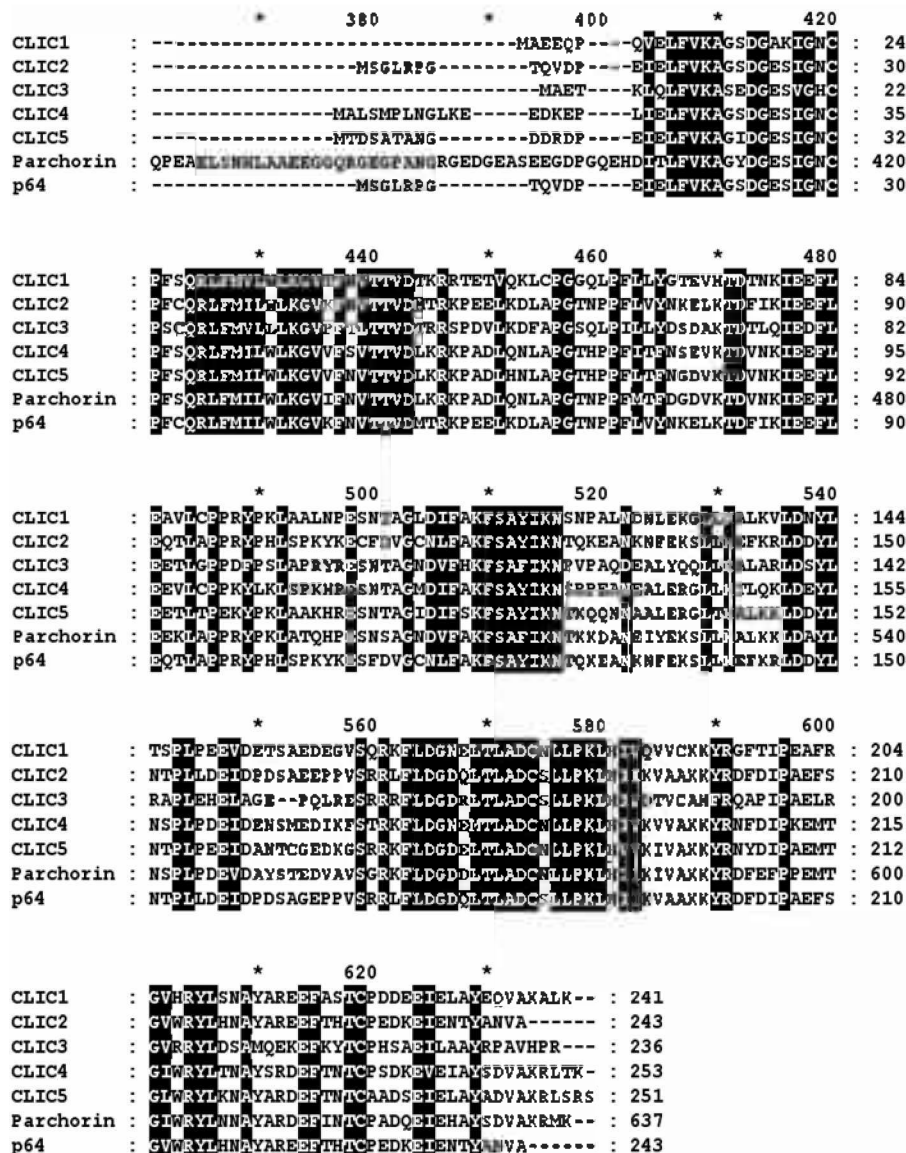
an HBS buffer (150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20, and 10 mM HEPES pH 7.4). Each sample was injected with 50  $\mu$ L of SEDL solution into the flow cells (association phase) at 10  $\mu$ L/min. Between cycles, the immobilized ligand was regenerated by injecting 30  $\mu$ L of 50 mM NaOH at 10  $\mu$ L/min.

**Fluorescence spectroscopy.** Fluorescence emission spectra were obtained on a Hitachi F-4500 Fluorescence Spectrometer using 1 cm path length cuvettes with excitation and emission slit widths of 20 nm. The fluorescence emission spectra of the CLIC1 and SEDL were obtained to identify the characteristic structures, namely the double bonds and aromatic groups, in molecules of varying complexities. The emission intensity was recorded from 280 to 400 nm, with an excitation at 295 nm. All of the spectra were obtained at a protein concentration of 50  $\mu$ g/mL at 24  $^{\circ}$ C. Ten spectra of each

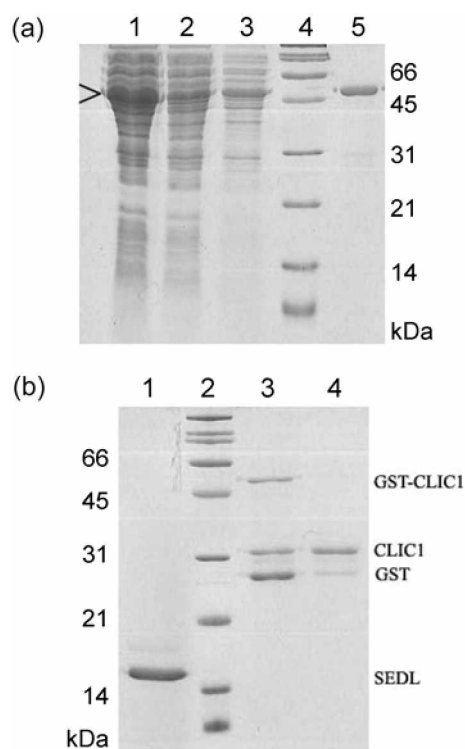
protein sample were accumulated, averaged, and subjected to baseline correction by subtracting the buffer spectrum.

**Results and Discussion**

The amino acid sequences of the seven CLIC family members are compared in Figure 1. The residues conserved are colored black. Five of the these proteins are each composed of about 240 residues, whereas the longer p64 and parchorin consist of distinct amino-terminal domains followed by the 240-residues CLIC module. This module has been shown to share a weak sequence homology with the GST superfamily.<sup>31</sup> CLIC1 (and the CLIC family) appears to contain the domains of a functional GSH-dependent redox protein structurally related to the GST superfamily, which raises the question as to whether CLIC1 is simply a



**Figure 1.** Sequence alignments of CLIC family [GenBank Accession No. CAG46868 (CLIC1), CAA03948 (CLIC2), BC007012 (CLIC3), CAG38532 (CLIC4), CAI21030 (CLIC5), AB035520 (Parchorin), CAA73228 (p64)]. The residues conserved in the seven species are colored black. Every 20 residues are indicated by asterisks (\*).



**Figure 2.** (a) Fractions of GST-tagged CLIC1: lane 1, supernatant; lane 2, unbound fraction; lane 3, washed fraction; lane 4, protein marker; and lane 5, eluted with 5 mM glutathione. (b) Purification using Superdex-75 column and concentration of SEDL. Cleavage of fusion tag by thrombin and SDS-PAGE analysis. Lane 1, purified SEDL (10 mg/mL); lane 2, protein marker; lane 3, CLIC1 after cleavage of GST-tag; and lane 4, purified CLIC1 (8 mg/mL).

regulatory element controlling an integral membrane ion channel. SEDL protein has been found to interact with 2 GST-fusion by immobilized GST-fusion proteins from rat cells (data not shown). These results show that SEDL can bind to the GST superfamily or to the CLIC superfamily.

Full-length CLIC1 was expressed at high levels in the pGEX-KG system. The CLIC1 protein was not expressed as a soluble in either the pGEX-4T-1 or the pET fusion system. A full-length human CLIC1 gene was constructed and over-expressed in *E. coli*. The construct was transformed into the expression host *E. coli* XL-blue, and protein expression was induced by 1 mM of IPTG at 37 °C. The overexpressed protein in soluble fraction was identified at the expected molecular mass of 56 kDa for GST-tagged CLIC1 (Fig. 2a). The CLIC1 protein was purified from the GST-column by its affinity to its fusion partner, the GST-tag. The column loading the GST-protein was washed with buffer A. Elution was carried out at 2.5 mL/min by monitoring the absorbance at 280 nm. The optimum elution for purification was found in the 5 mM glutathione concentration. This suggests that the GST-tag of the protein was less exposed to resin and thus was bound weakly to the resin in the GST column. The fractions were confirmed on SDS-PAGE gels and the protein was eluted in pure and homogeneous forms in Coomassie blue-stained gels, respectively. Five mg of GST-fusion protein in buffer A was treated with 5 units of thrombin (Novagen)

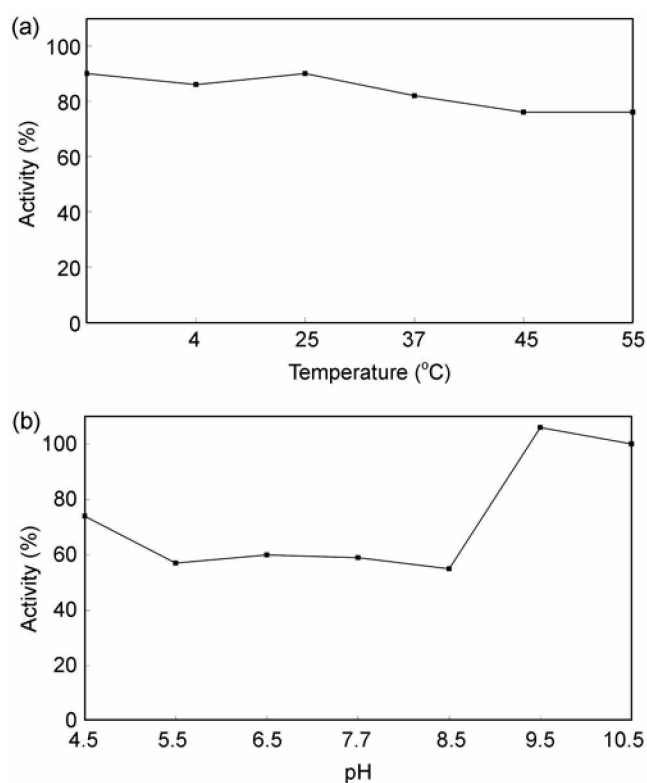
**Table 1.** Yield volumes and concentrations of CLIC1 proteins at each prep steps

GST-column	
Volume (mL)	70
Concentration (mg/mL)	0.3
Purity (%)	85
GST (thrombin)	
Volume	30
Concentration	0.5
Purity (%)	80
Q-Sepharose	
Volume	70
Concentration	0.3
Purity (%)	90
Superdex 75	
Volume	80
Concentration	0.2
Purity (%)	95

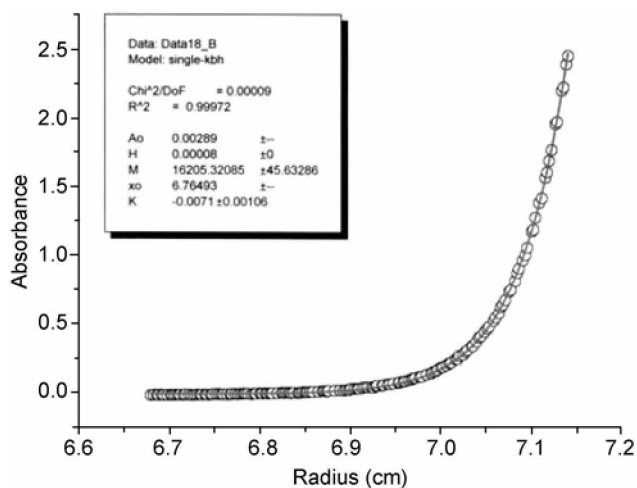
for 12 hrs at 4 °C, and the GST-tag was cleaved (Fig. 2b). The GST-tag is a relatively large fusion peptide having high flexibility as an effective crystal. Therefore, the GST-fusion protein was cleaved by specific proteases prior to crystallographic utilization. The fraction eluted from affinity chromatography was loaded onto a pre-packed column of Q-Sepharose-based strong anion-exchange and then eluted with a gradient of the NaCl solution. After affinity chromatography and ion-exchange chromatography, the protein was purified further using a superdex-75 column. In fact, the efficiency of this purification procedure could contribute greatly to crystallization and biochemical studies. The protein was then concentrated in soluble form to a final concentration of 8 mg/mL for CLIC1, using a quantification kit with bovine serum. Multi-step purifications of the protein under non-denaturing conditions were performed, as shown in Table 1. HPLC studies confirmed the purity of the recombinant proteins in that they exhibited a single peak at 280 nm (Fig. 2b).

The effects of temperature and pH on protein activity were examined (Fig. 3).<sup>22</sup> The protein exhibited optimal activity at 0-25 °C, and a decrease in activity took place at 37 °C or 45 °C. These results suggest that at those temperatures, the protein contains some disordered secondary structures. Those results also suggest that CLIC1 undergoes temperature-dependent conformational changes to a certain extent (Figure 3a). The biophotometer spectrum of the CLIC1 was recorded at various pH. The protein at pH 4.5, 9.5 and 10.5 exhibited an increase in activity, showing a similar degree of activity at pH 5.5-8.5 (Figure 3b).

SEDL was expressed in *E. coli* BL21(DE3)RIL strain and purified as described previously (26). In brief, SEDL was expressed as a His<sub>6</sub>-tagged fusion protein, and was obtained after cleavage by thrombin. Purification of SEDL required

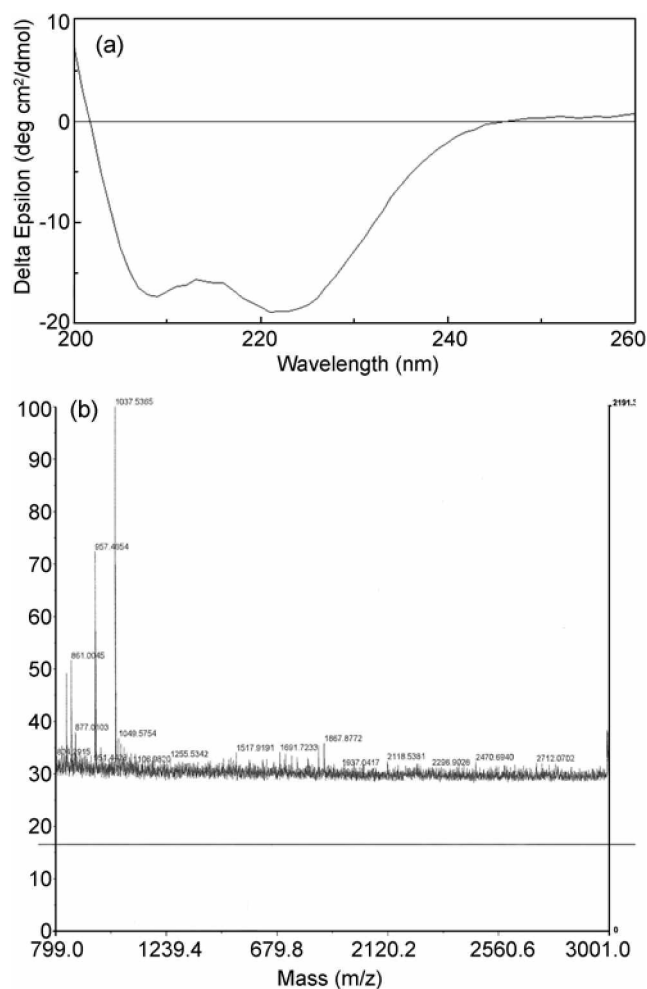


**Figure 3.** (a), (b) Effects of temperature and pH on the activity of the CLIC1 protein. The protein activity was assayed under the wavelength of 280 nm. Reactions were carried out for 5 min at various temperatures and pH.



**Figure 4.** Ultracentrifugation analysis of wild-type SEDL. The radial distribution of SEDL protein was precisely fitted to an ideal single species model in which the protein exists as a monomer in solution with a coincidence parameter of 0.99. The solid line and open circles indicate the calculated curve and the experimental curve, respectively. The concentration of SEDL was 0.05 mg/mL in 50 mM sodium chloride buffer (pH 7.5). Equilibrium was attained in 48 hrs.

the use of a Ni-NTA column (Qiagen), a Resource Q anion exchange column, and a Superdex 75 column (Amersham Biosciences). To determine the exact oligomerization state of the protein in solution, equilibrium centrifugation was performed. An ultracentrifugation analysis further evidenced

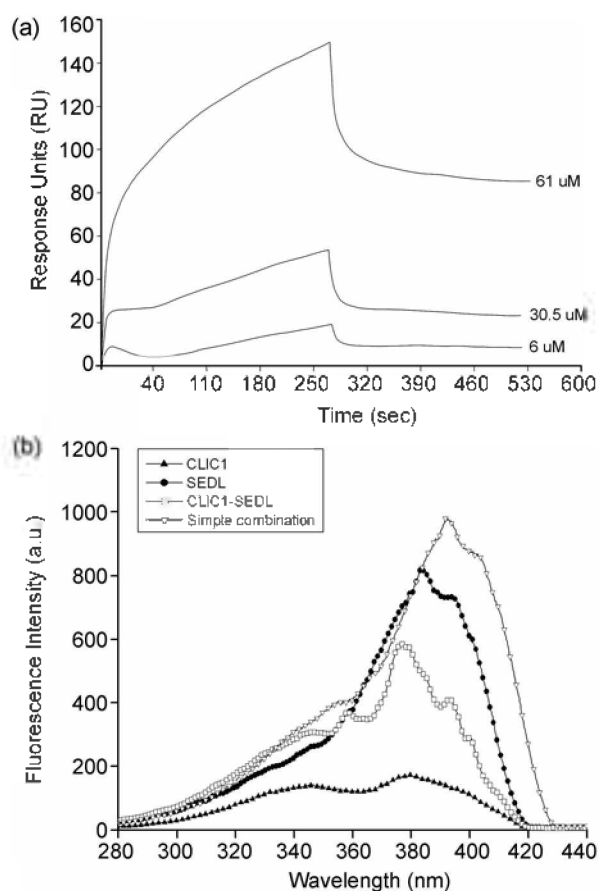


**Figure 5.** (a) Far-UV CD spectrum of CLIC1. The CD spectrum was measured from 200 to 260 nm using a 0.1 cm pathlength cell, and the CD signal was merged to CDNN. The spectrum was recorded typically as an average of five scans at 25 °C. (b) MALDI-TOF mass spectrum analysis. MALDI-TOF peptide spectrum of human CLIC1 protein.

the monomerization of SEDL (16205.3 Da) in solution, as shown in Figure 4.

The folding property of CLIC1 protein was characterized by CD spectroscopy. The circular dichroism spectrum of the CLIC1 showed the dominance of the  $\alpha$ -helix structure, exhibiting negative bands at about 208 nm and 222 nm (Fig. 5a). The CD signal was converted to mean residue ellipticity (MRE) using the following equation:  $MRE = \theta / (10 \cdot l \cdot C \cdot N_A)$ , where  $\theta$  is the ellipticity in mdeg,  $l$  is the light pathlength in cm,  $C$  is the molar concentration of the protein, and  $N_A$  is the number of CLIC1 residues. Deconvolution of the spectrum using the CDNN program indicated the following secondary structure contents: 96.3%  $\alpha$ -helices, 0.2%  $\beta$ -sheets, 3.0% turns, and 0.5% non-ordered form.

MALDI-TOF studies revealed the approximate molecular mass of the recombinant protein, which was in accordance with the theoretical mass prediction for the CLIC1 protein with the peptide mass tolerance (50 ppm) (Fig. 5b). Mass-fingerprinting analysis of the protein was carried out by



**Figure 6.** (a) BIAcore biosensor analysis of the binding of CLIC1 to SEDL at 25 °C. The sensorgrams for 6, 30.5, and 61  $\mu$ M human CLIC1 are shown, which were used to calculate the dissociation constants. The CLIC1 was immobilized to the dextran matrix, and SEDL samples at the three indicated concentrations were injected for 5 min. The kinetic parameters of the binding reactions were determined using the BIAevaluation version 2.1 software provided by the manufacturer. A control experiment using BSA did not show a detectable binding response to either of the enzymes. (b) Fluorescence analysis of the binding of CLIC1 to SEDL. Fluorescence spectra of the 1:1 mixture of CLIC1 and SEDL and the sum of the spectra of individual CLIC1 and SEDL. The CLIC1 and SEDL were preincubated together for 25 min at 25 °C. The fluorescence spectra were recorded with a wavelength of 295 nm to excite tryptophan and tyrosine. The concentration of each of the two proteins was 5  $\mu$ M. The sample buffer contained 50 mM of Tris-HCl (pH 7.5) and 200 mM of NaCl.

subjecting it to trypsin digestion. The monoisotopic masses obtained for the individual peptides were in the range of 800–3000 Da. The sequences of the digested peptides (No. 6/34, Accession No. O00299 and pI 5.09; molecular weight, 26922.9 Da) were matched with the protein sequences in the database using the PROFOUND program.

Establishing the link between SEDL and the chloride intracellular channel is necessary to understanding their functional interplay. Since both CLIC1 and SEDL proteins are membrane-associated proteins, their interactions and structural information might be important either to functions of the TRAPP complex or the proper targeting and functions of the chloride intracellular channels. To investigate the

interaction between CLIC1 protein and SEDL, the binding affinity of CLIC1 for SEDL was estimated by surface plasmon resonance spectroscopy (BIAcore) (Fig. 6a). Sensorgrams of SEDL binding to CLIC1 were used to calculate the kinetic binding constants.<sup>33</sup> Background sensorgrams were then subtracted from the experimental sensorgrams to yield representative specific binding constants. We found that SEDL indeed binds to CLIC1 with an apparent  $K_D$  of 261 nM.

The fluorescence emission spectra of the purified CLIC1 and SEDL proteins were confirmed, and the  $\lambda_{max}$  curve was found at 380 nm, suggesting a similarity in the conformation of the CLIC1 and SEDL proteins. The spectra represented the unusual emission consistent with the solvent-inaccessible environment of the tryptophan residue. The fluorescence intensity is about 750 a.u. at SEDL, but at CLIC1 it is 170 a.u. At 24 °C, an increase in the fluorescence intensity took place when the CLIC1 and SEDL were mixed together in 1:1 molar ratio, both at 5  $\mu$ M (Fig. 6b). The simple combination of the spectra of the CLIC1 and SEDL are not identical to the spectrum of the CLIC1-SEDL complex. Probably, the tight interaction of the two proteins is accompanied by more significant conformational changes of one or both of them, which is likely to be greatly facilitated at room temperature, because the residues of aromatic groups are buried in the three-dimensional structure of protein and the spectrum of the CLIC1-SEDL complex is much more decreased than in the simple combination. Also, the less rigid and hydrophobic environment required for the conformational change of CLIC1-SEDL can be caused by a decrease of fluorescent intensity.

The purified CLIC1 and SEDL were mixed in 1:1 molar ratio. After incubation for 12 hrs at 4 °C, the mixture was loaded onto a Superdex 200 HR 10/30 size-exclusion column (SEC) (Amersham Pharmacia Biotech). The fractions containing the CLIC1-SEDL complex were collected and boiled at 90 °C for 10 min to elute the proteins followed by loading onto a SDS-PAGE. However, the binding between the CLIC1 and SEDL proteins was too weak to be detected in SDS-PAGE gel. Comparison with the sequences of other CLICs indicated that the binding region was highly conserved, with an average 54% identity (Fig. 1). We also found, by BIAcore biosensor and fluorescence measurements, that CLIC1 interacts weakly with SEDL *in vitro*. These results suggest that full-length CLIC1 potentially blocks the SEDL binding surface by conformational changes of one or both of the two proteins. Such conformational changes of the CLIC1-SEDL complex might be caused by differences of pH and temperature in solution. To determine the multifunctional roles of CLIC and SEDL in membrane trafficking, we will conduct further structural studies of these complex proteins.

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