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Original Article

CFTR: Effect of ICL2 and ICL4 amino acids in close spatial proximity on the current properties of the channel

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Abstract

Background: CFTR is the only ABC transporter functioning as a chloride (Cl^-) channel. We studied molecular determinants, which might distinguish CFTR from standard ABC transporters, and focused on the interface formed by the intracellular loops from the membrane spanning domains.
Methods: Residues from ICL2 and ICL4 in close proximity were targeted, and their involvement in the functioning of CFTR was studied by whole cell patch clamp recording.
Results: We identified 2 pairs of amino acids, at the extremity of the bundle formed by the four intracellular loops, whose mutation i) decreases the Cl^- current of CFTR (couple E267–K1060) or ii) increases it with a change of the electrophysiological signature (couple S263–V1056).
Conclusions: These results highlight the critical role of these ICL residues in the assembly of the different domains and/or in the Cl^- permeation pathway of CFTR.

Keywords: CFTR; ICL; Structure-function; Channel gating; ABC transporter

1. Introduction

Chloride (Cl^-) fluxes through cellular membranes are achieved by membrane proteins including Cl^- transporters and Cl^- channels that catalyze ion flow by two distinct mechanisms. Schematically, channels have only one gate that opens and closes in response to external stimuli, whereas the substrate translocation pathway in transporters is delimited by two gates which are never simultaneously open [1,2]. However, the separation between channels and transporters has become less clear: within the ClC family it was shown that the same basic architecture can support both Cl^- channel and Cl^-/H^+ antiporter functions [3,4]. Another example of functional versatility between channels and transporters, implying a unique structural scaffold, is the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) protein, which mainly controls the Cl^- transport in epithelial cells [5]. CFTR (ABCC7) belongs to the superfamily of ATP-binding cassette (ABC) transporters, however, among the 49 human ABC proteins, only CFTR is known to function as an ion channel [6]. Dysfunction of CFTR causes the fatal inherited genetic disorder cystic fibrosis (CF) [7]. Members of the ABC superfamily use an alternate access mechanism, with two gates that open alternatively to allow access to the substrate-binding site from the cytoplasm or from the extracellular milieu. The CFTR anion channel appears to have evolved from this ABC architecture.

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The mechanisms of Cl^- movement in the CFTR permeation pathway have mostly been explored in the transmembrane (TM) helices from the membrane-spanning domains (MSDs), proposed to form the channel pore [8]. Results deduced from comparative sequence analysis have highlighted some amino acids in TMs, which appeared to be involved in the evolutionary transition from transporter to ion channel, [9]. It has also been surmised that the cytoplasmic-side gate (inner gate) of CFTR has become atrophied, or uncoupled from the outer gate [1,10], in order to allow the passage of Cl^- in the ATP-bound conformation. This hypothesis is supported by analysis of the reactivity of intracellularly applied thiol-specific probes with cysteine residues substituted in TM12 [11].

Models of the 3D structure of outward-facing forms of the CFTR channel, assumed to correspond to the open configuration of the channel and in which the two nucleotide-binding domains (NBDs) are closely associated in a head-to-tail configuration, have been built based on the crystal structures of Sav1866 [12,13]. Models for closed conformations have been obtained more recently, based on the experimental 3D structures of MsbA and Pg-P in an inward-facing configuration [14]. Molecular dynamics (MD) simulations have been also conducted on outward-facing, Sav1866-based [15,16] and inward-facing models [17] to derive "channel-like" conformations. The MD simulations on outward-facing models led to the observation of a narrow region ("bottleneck") at the level of the proposed selectivity filter, separating the outer and inner vestibules [15,16], whereas those performed on the inward-facing model lead to observe a tight dimerization of the NBD upon ATP-binding and a MsbA "closed-apo"-like conformation in absence of ATP [17]. Experimental data generally agree with the models of CFTR open states facing outwardly and a cytoplasmic vestigial gate [11,18]. Cysteine-scanning studies indeed suggested the presence of a cytoplasmic gate and a scheme where the open state would face inwardly [19]. This last scheme is however hardly reconciled with the tight association of the NBDs in the open configuration.

In all the models of the CFTR, outward-facing open channel, the MSD intracellular loops (ICLs), located at the cytoplasmic side of the pore, come close together to form, through their internal helices, a four-helix bundle. As a passageway to the internal vestibule is observed in our Sav1866-based models at the level of this bundle, we hypothesized that the selected amino acids may participate in the inner "broken" gate of the channel, in addition to being involved in the tight association between the two halves of the protein. In particular, we focused on two pairs of amino acid predicted to line a narrow passageway, at the base of the four-helix bundle: residues E267 and K1060 (presumed to form an ICL2–ICL4 acid–base pair and located at the entry of the passageway) and residues S263 and V1056 (located just above the acid–base pair).

2. Methods

2.1. Homology modeling

Our model of the open form of human CFTR MSD:NBD assembly, based on the Sav1866 structure [20] has been described previously [12]. Molecular graphic images were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (http://www.cgl.ucsf.edu/chimera).

2.2. Construction and expression of CFTR mutants

Mutagenesis was performed in the expression vector pEGFP-CFTRwt as previously described [21]. HEK-293 cells were transiently transfected with the cDNA constructs using cationic lipids (JetPEI, QBiogene) with 0.2 μg/ml (patch-clamp method) and 1 μg/ml of plasmid (western blot analysis). Cells were used 48 h post-transfection.

2.3. CFTR protein detection

CFTR maturation was explored by western blot analysis. HEK293 cell lysates were separated by 5% polyacrylamide SDS-PAGE (25 μg protein per lane). Immunoblotting was done with a rabbit polyclonal anti-GFP primary antibody (clone A6455, Invitrogen). Horseradish peroxidase-conjugated sheep anti-rabbit (1:10,000; GE Healthcare) was used as secondary antibody and revealed with ECL Western Blotting Detection Reagent (Millipore). Core-glycosylated (B band) and fully-glycosylated (C band) levels were quantified by densitometry of scanned western blots using ImageJ software (Wayne Rasband, National Institut of Health, USA). Percentage of processing for each mutant was given by (band C/(band B + band C) × 100) and normalized using percentage value for each mutant over the same percentage for wt-CFTR run on the same gel.

2.4. Patch-clamp studies of CFTR mutants

Ionic currents were recorded using the whole-cell configuration of the patch-clamp method. Current/voltage (IV) relationships were built by clamping the membrane potential at −40 mV and stepping the voltage from −100 to +100 mV in 20 mV increments. The currents were digitized at 3 kHz and filtered at 2–5 kHz using a computer attached to a Digidata 1200 interface (Axon). The external bath solution contained (in mM) 145 NaCl, 4 CsCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 10 TES (titrated with NaOH to pH 7.4). The intrapipette solution contained (in mM) 113 L-aspartic acid, 113 CsOH, 27 CsCl, 1 NaCl, 1 $MgCl_2$, 1 EGTA, 1 TES, and 3 MgATP (ex *temporane*) (titrated with CsOH to pH 7.2). All experiments were conducted at room temperature (20–25 °C) and the mean values of membrane capacitance were 24.7 ± 2.6 pF (n = 97). In all experiments, CFTR channels were activated by elevating intracellular cAMP levels using 10 μM of the adenylate cyclase agonist forskolin (Fsk). Then the thiazolidinone blocker $CFTR_{inh}$-172 was used at 10 μM to systematically and selectively inhibit CFTR channels [21,22].

2.5. Statistics

Results are expressed as mean ± standard error of the mean (SEM) of n observations. Data were compared with Student's *t*-test. Differences were considered statistically significant at $p < 0.05$.