Redox-dependent structural ambivalence of the cytoplasmic domain in the inner ear-specific cadherin 23 isoform

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Abstract

Cadherin 23 (Cdh23), an essential factor in inner ear mechano-electric transduction, exists in two alternatively spliced forms, Cdh23(+68) and Cdh23(−68), depending on the presence and absence of exon 68. Cdh23(+68) is inner ear-specific. The exon 68-corresponding region confers an α-helical configuration upon the cytoplasmic domain (Cy) and includes a cysteine residue, Cys3240. We demonstrate here that Cy(+68) as well as the transmembrane (TM) plus Cy(+68) region is present in two different forms in transfected cells, reduced and non-reduced, the latter existing in more compact configuration than the former. The observed characteristic of Cy(+68) was completely abolished by Cys3240Ala substitution. Treatment of TMCy(+68)-transfected cells with diethyl maleate, a glutathione depleting reagent, resulted in conversion of the non-reduced to the reduced form of TMCy(+68), suggesting glutathione to be a Cys3240-binding partner. Multiple alignment of mammalian Cdh23Cy sequences indicated the occurrence of conformation-inducible Cys in Cdh23Cy of mammals, but not lower vertebrates. The implications of Cys-dependent structural ambivalence of Cdh23 in inner ear mechanosensation are discussed.

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Cadherin 23 (CDH23/Cdh23), the Usher syndrome type 1D factor (USH1D), is a transmembrane protein involved in adhesion and signal transduction in the inner ear. The protein has 27 extracellular cadherin repeats and bundles stereocilia that are sensory organelles projecting from the apical surfaces of hair cells. The cytoplasmic region of Cdh23 interacts with the multi-PDZ domain-bearing scaffold protein harmonin (USH1C), which also associates with other USH1 factors, myosin 7a (Myo7a; USH1B), protocadherin 15 (Pcdh15; USH1F), and Sans (USH1G), indicating formation of supercomplexes of plasma membrane/USH1 complexes/actin cytoskeleton to be essential in developmental differentiation of stereocilia for acquisition of hearing [1–3].

Cdh23 is also implicated as a main component of the ‘gating spring’ mechanosensation in inner ear. Mechanically sensitive stereocilia initiate opening/closing of transduction channels [4]. Each stereocilium is connected to its taller neighbor by an electron microscopically identifiable fine filament, tip link [5], that might be directly associated with transduction channels [6]. Recent studies have identified tip link to be made up of extracellular cadherin repeats of Cdh23 [7–9] and Pcdh15 [9,10]. Furthermore, the most reliable transduction channel is constituted by transient receptor potential (Trp)-family proteins [11–13], and the entity of the ‘gating spring’ is formed by ankyrin repeats of the NH2-terminal cytoplasmic regions in Trp channels [14,15].
Cdh23 exists in two alternatively spliced isoforms, Cdh23(±68), depending on the presence and absence of 35 exon 68-derived amino acids in the cytoplasmic region (Cy). Cdh23(+68) isoform is inner ear-specific and has one PDZ-binding interface (PBI) at the COOH-terminal. Cdh23(−68) additionally possesses an internally located PBI, and hence the occurrence of Cdh23(+68) in the inner ear implies effective clustering through harmonin–harmonin interactions [16]. We demonstrate here that Cys3240 in the exon 68-corresponding region is redox-sensitive and Cy conformation-inducible, suggesting the ambivalent nature of Cdh23 to be of great advantage for inner ear mechanosensation.

Materials and methods

The methods of RT-PCR, cell culture, transfection, site-directed mutagenesis, immunocytofluorescence, immunoblotting, and GST pull-down were as described earlier [17]. Neuro-2A, HEK293, and COS-1 cells were used for transfection experiments.

Constructs. In addition to FLAG-Cdh23TMCy(±68) constructs [17], Myc-tagged Cdh23Cy(±68) constructs were prepared. For this, DNA fragments were obtained by PCR using the forward primer GAACCCTGCGTTCAATGACATCA (nt. 9321–9343 in exon 65: numbered according to GenBank Accession No. AF308939.1 [18]) and the reverse primer AGGGTCTAGTCGACGAAAAGGC (nt. 10,128–10,143 in exon 69). The PCR products obtained were ligated with pCMVMyC (Amersham Pharmacia) and cloned. The pCMVMyc-Cy(+68; C3240A) construct was prepared by site-directed mutagenesis, and served as a template for preparing the pCMV-Cy+Cy(+68; C3240A, R3269C) construct. The nucleotide sequences of the prepared constructs were all examined and verified with an ABI PRISM 310 genetic analyzer (Applied Biosystems). The inserts of pCMVMyC-Cy(±68) constructs were transferred to the pGEX4T vector (Amersham Pharmacia) to prepare pGEX-Cy(±68) constructs. GST-fused harmonin PDZ1, PDZ2, and PDZ3 were prepared as described [17].

SDS–PAGE and native PAGE. Transfected cells were rinsed with phosphate-buffered saline (PBS) and lysed in PBS containing 0.5% Triton X-100 and protease inhibitors (10 μg/ml each of leupeptin, aprotinin and pepstatin, and 0.4 mM phenylmethylsulfonylfluoride) (lysis buffer), followed by sonication and centrifugation at 12,000 g for 15 min. The supernatants were used as samples for SDS–PAGE and native PAGE with or without diithiothreitol (DTT), co-immunoprecipitation, and GST pull-down. For DTT-free SDS–PAGE, DTT was omitted from the SDS–PAGE sample mixture. After electrophoresis, proteins were electrotransferred on PVDF membranes and immunostained [17].

For SDS-free native PAGE, cell extracts or purified GST-fused proteins were incubated with and without DTT at 37 °C for 10 min, prior to electrophoresis. Different concentrations of acrylamide gels (7.5% and 10%) were used to calculate retardation coefficients of the detected bands. Protein was determined by the Folin reagent method [19].

Results and discussion

Redox-sensitive conformational changes of Cdh23Cy(+68), but not Cdh23Cy(−68)

In a previous paper, we showed that FLAG-tagged TMCy of Cdh23(±68) is localized, when expressed in neuroblastoma cells, to filamentous actin-rich protrusions and the plasma membrane [17]. In an attempt to identify Cdh23Cy-interacting factors, we found that FLAG-TMCy(+68), but not FLAG-TMCy(−68), exists in two conformationally different forms, as revealed by diithiothreitol (DTT)-free SDS–PAGE (Fig. 1A). The slower-migrating band was comparable to the main band detected under reduced conditions, while the faster-migrating one was calculated as a mass seemingly just 4 kDa less than that of the reduced form. Similar results were obtained with Myc-tagged Cy(+68), which precludes the possibility of involvement of the helical TM structure in the redox-dependent phenomenon. The reduced forms of TMCy(+68) and Cy(+68), but not their non-reduced forms, were regularly accompanied by additional minor bands, which proved to be phosphorylated forms [17] (Supplementary Fig. 1).

There exist two cysteine residues in Cy, Cys317 in exon 66 region, and Cys3240 in exon 68 region. The observations suggest the latter to be responsible for the redox-sensitive phenomenon, and indeed, Cys3240Ala substitution, but not Cys317Ala (Supplementary Fig. 2), completely abolished the ambivalent characteristics (Fig. 1A). Native PAGE analysis revealed that Myc-Cy(+68) (Fig. 1B) as well as GST-fused Cy(+68) (Fig. 1C) migrated faster in the absence than in the presence of DTT, suggesting again that the reduced and non-reduced forms are discrete in conformation. Under denatured conditions, high molecular masses with GST signals were detected, which is indicative of a high reactivity of Cys3240 (Fig. 1C). Most probably, Cys3240 prefers, under denatured conditions, some free cysteine residue(s) of GST.

Conversion of the non-reduced to the reduced form of TMCy(+68)

If Cdh23Cy(+68) possesses a redox-sensitive and conformational change-inducible Cy, it is important to clarify its binding partner(s). The fact that non-reduced forms of TMCy(+68) and Cy(+68) are detected in more compact configuration than the respective reduced forms suggests the binding partner to be a small molecule. We tested the possibility of Cy(+68)-glutathione (GSH) association using diethyl maleate (DEM), a potent reagent depleting intracellular GSH. In transfection experiments with HEK293 or Neuro-2A cells, 1 mM DEM effectively decreased intracellular GSH levels, without significant changes in cellular protein levels (Fig. 2). Concomitantly, we detected conver-
version of the non-reduced to the reduced form of expressed TMCy(±68), strongly suggesting GSH to be a Cys\textsubscript{3240}-binding partner. We further attempted to identify a protein factor(s) associating with Cys\textsubscript{3240}, in cotransfection experiments followed by DTT-free SDS–PAGE, but so far, have found no clear Cys\textsubscript{3240}-involved interactions of TMCy(±68) with harmonin isoforms a1 (Arg\textsuperscript{10}-Phe\textsuperscript{348}, GenBank Accession No. AF228924.1 [21]) and b3 (Arg\textsuperscript{10}-Pro\textsuperscript{910}, GenBank Accession No. AF228925.1 [21]), a cytoplasmic domain of Pedh15 (Ile\textsuperscript{1420}-Leu\textsuperscript{1943}, GenBank...
Accession No. AF281899.1 [22]) or an NH2-terminal cytoplasmic region of Trpa1 (Met1-Arg719; GenBank Accession No. AC121789.2 [13]) (not shown).

**Conservation of Cys3240 in vertebrate Cdh23s and implications of the redox-sensitive ambivalence of Cy(+68)**

Genomic data on Cdh23 are now available for a variety of animals (Fig. 3 and Supplementary Fig. 3). In *Drosophila* and *Caenorhabditis elegans*, no CDH23Cy-related gene structures have been recognized. Alignment indicates that the presence of Cys3137, predicted to locate within an α-helical structure, is highly conservative in vertebrates. In contrast, Cys3240 is rather less conserved, replaced by three or four non-Cys residues in lower vertebrates. In mammals, Cys3240 is conserved from opossum to man, which is suggestive of acquisition in mammalian ancestors, but Ser3240 and Cys3269 have been found in the cow and four Cys residues, Cys3137, Cys3240, Cys3259, and Cys3269, in the horse. In this context, it is of interest that Arg3269Cys alteration in Myc-Cy(+68; C3240A) protein restores GSH-dependent structural ambivalence (Supplementary Fig. 4A). The ASP program (PredictProtein) predicts the occurrence of two conformational switches (CS) associated with the exon 68 region (Fig. 3). Based on the present findings, we believe that at least one of them does actually work redox-dependently in *vivo*. USH1D-associated CDH23TMCy(+68; 9626insC) [17,23] also behaves redox-sensitively in SDS–PAGE (Supplementary Fig. 4B). This may not be surprising, because the aberrant sequence accommodates an α-helical configuration and includes a Cys residue, like the exon 68 region, and because a conformational switch is predicted to exist just before the aberrant sequence (not shown). In zebrafish and *Xenopus* Cdh23(+68)'s, on the contrary, the redox-active Cys-lacking Cy regions are expected not to exhibit structurally ambivalent properties.

Given that the mammalian Cdh23Cy(+68) has a redox-sensitive Cys residue(s) which affects its conformation, the functional implications are intriguing. Most plausible is that the phenomenon provides an intermolecular relaxation factor against deflection-induced distortion stress. Force-mediated bundle deflection stretches tip link and simultaneously initiates ion influx via channel activation. Such mechanical bundle deflection could be expected to generate intermolecular distortion stress within plasma membrane/USH1 proteins/actin cytoskeleton supercomplexes. Myosin-1C, the adaptation motor [6], may not be useful for responding promptly to such intermolecular distortion. Rather, if ion influx alters the redox state micro-environmentally just beneath the plasma membrane and triggers induction of Cys-dependent conformational change of Cdh23Cy(+68), then it would be of great advantage in relieving deflection-associated distortion stress in Cdh23-involved supercomplexes. We found no significant differences between reduced and non-reduced TMCy(+68) forms in affinity towards harmonin PDZ2 (Fig. 4). This indicates that the conformational change of Cy does not affect Cdh23/harmonin interactions, suggesting that the ambivalent properties of Cdh23Cy(+68) work so as to transit harmonin-associating USH1 complexes, as a whole. In lower vertebrates, such a distortion stress-releasing function of Cdh23Cy may be executed by other USH1 factor(s).

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**Fig. 3.** Schematic illustration of animal Cdh23TMCy structures, with special reference to the presence of cysteine (C) and conformational switches (▲: CS in the human sequence). The secondary structures (α-helix, β-sheet) and CS were predicted by PredictProtein. The human structure is depicted as the representative of mammalian TMCys, with the exception of the cow and horse. Note the single cysteine in lower vertebrates and two or more in mammals. TM, transmembrane domain. For details, see the text and Supplementary Fig. 3.
There is another speculation that is much more attractive, associated directly with the ‘gating spring’ channel-activation mechanism. The most reliable candidates for inner ear transduction channels are now the Trp family proteins [11–13,24,25], and the Trpa1 channel also mediates transduction in nociceptors [26]. Interestingly, it relies containing NH2-terminal cytoplasmic region of Trpa1. Actions between TMCy(+68) and the ankyrin repeats-con

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Fig. 4. GST pulldown experiments to examine binding affinity of the reduced and non-reduced forms of FLAG-TMCy(+68) for GST-fused harmonin PDZ2. Procedures for GST pulldown have been described [17]. The amounts of GST-PDZ2 bound to 10 µl of GSH-Sepharose are indicated. SDS-PAGE was carried out without DTT. N2A, FLAG-TMCy(+68)-expressing Neuro-2A extracts. Blotted with anti-FLAG. Both the reduced and non-reduced forms bind to GST-PDZ2-adsorbed gels, dependent on the amount of coupled GST-PDZ2, indicating no significant difference in affinity towards GST-PDZ2.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbr.2007.11.102.

References


