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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, Cys^{3240}. 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 Cys^{3240}Ala 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 Cys^{3240}-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.

Keywords: Cadherin 23; Glutathionylation; Conformational switch; Mechanosensation

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 NH_2-terminal cytoplasmic regions in Trp channels [14,15].

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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 Cys^{3240} 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 GAACCGTGGCTTCATTGACATCA (nt. 9321–9343 in exon 65: numbered according to GenBank Accession No. AF308939.1 [18]) and the reverse primer AGGCTGTACTGGGCGAAGGGCCG (nt. 10,128–10,106 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 pCMVMyc-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 proteinase 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 dithiothreitol (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].

Glutathione assay. Glutathione (GSH) levels in transfected cells were measured with Glutathione Assay Kit (Cayman Chemical Co.). Diethyl maleate (DEM; Cayman Chemical Co.), a reagent depleting GSH, was dissolved in dimethyl sulfoxide (DMSO) and added to culture medium at a final concentration of 1 mM. Transfected cells were harvested and analyzed at different intervals after DEM treatment at 18-h posttransfection. Cell extracts were deproteinized with 5% 5-sulfosalicylic acid [20], neutralized and subjected to assay.

Databases and analytical programs. Genome databases (http://www.ensembl.org/index.html; http://www.ncbi.nlm.nih.gov/Genomes/) were utilized to obtain genomic information on the TMCy(+68) region of Cdh23 from a variety of animals. PredictProtein [B. Rost, G. Yachdav, J. Liu, The PredictProtein Server, Nucleic Acids Res. 32 (Web Server issue) (2004) W321–W326] was used to analyze the secondary structures of proteins and to predict ambivalent sequences (ASP program).

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 dithiothreitol (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, Cys^{3137} in exon 66 region, and Cys^{3240} in exon 68 region. The observations suggest the latter to be responsible for the redox-sensitive phenomenon, and indeed, Cys^{3240}Ala substitution, but not Cys^{3137}Ala (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 Cys^{3240} (Fig. 1C). Most probably, Cys^{3240} 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 Cys, 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-