BIOCHEMISTRY
including biophysical chemistry & molecular biology

Article

pubs.acs.org/biochemistry

Identification and Characterization of the X-Dimer of Human P-Cadherin: Implications for Homophilic Cell Adhesion

Shota Kudo,[†] Jose M. M. Caaveiro,[‡] Shuichiro Goda,[§] Satoru Nagatoishi,[‡] Keisuke Ishii,[∥] Tadashi Matsuura,[∥] Yukio Sudou,[∥] Tatsuhiko Kodama,[⊥] Takao Hamakubo,[⊥] and Kouhei Tsumoto*[,†,‡,#,▽]

[†]Department of Chemistry & Biotechnology, The University of Tokyo, Tokyo 108-8639, Japan

[‡]Department of Bioengineering, The University of Tokyo, Tokyo 108-8639, Japan

[§]Faculty of Engineering, Nagasaki University, Nagasaki 852-8521, Japan

[∥]Perseus Proteomics Inc., Tokyo 153-0041, Japan

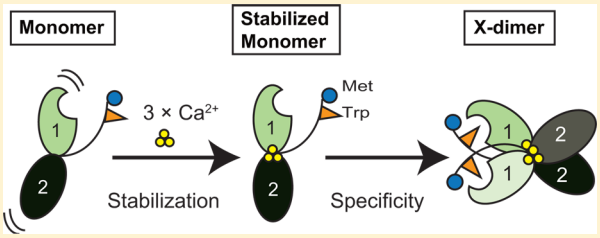
[⊥]Research Center for Advanced Science and Technology, The University of Tokyo, Tokyo 153-8904, Japan

[#]Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan

[▽]Department of Medical Genome Science, The University of Tokyo, Tokyo 108-8639, Japan

Supporting Information

ABSTRACT: Cell adhesion mediated by cadherins depends critically on the homophilic trans-dimerization of cadherin monomers from apposing cells, generating the so-called strand-swap dimer (ss-dimer). Recent evidence indicates that the ss-dimer is preceded by an intermediate species known as the X-dimer. Until now, the stabilized form of the X-dimer had only been observed in E-cadherin among the classical type I cadherins. Herein, we report the isolation and characterization of the analogous X-dimer of human P-cadherin. Small-angle X-ray scattering (SAXS) and site-directed mutagenesis data indicates that the overall architecture of the X-dimer of human P-cadherin is similar to that of E-cadherin. The X-dimerization is triggered by Ca^{2+} and governed by specific protein−protein interactions. The attachment of three molecules of Ca^{2+} with high affinity (K_d = 9 μM) stabilizes the monomeric conformation of P-cadherin (ΔT_m = 17 °C). The Ca^{2+}-stabilized monomer subsequently dimerizes in the X-configuration by establishing protein−protein interactions that require the first two extracellular domains of the cadherin. The homophilic X-dimerization is very specific, as the presence of the highly homologous E-cadherin does not interfere with the self-recognition of P-cadherin. These data suggest that the X-dimer could play a key role in the specific cell−cell adhesion mediated by human P-cadherin.



Cadherins are calcium-dependent cell adhesion proteins involved in selective cell−cell recognition, cell-sorting during morphogenesis, and the development and maintenance of solid tissues.[1−3] Defects in the adhesive properties of cadherins are associated with severe malignancies such as carcinogenesis and tumor invasion.[4−6]

The cadherin superfamily of proteins comprises more than a hundred different proteins. The best-characterized group, in terms of structure and function, are the classical cadherins,[7] which are divided into two groups (type I and II) according to their primary sequence and three-dimensional structure.[8] Classical cadherins display five extracellular cadherin (EC) domains, a single transmembrane domain, and an intracellular domain for the attachment to catenin.[9] Each EC domain (EC1−EC5) is composed of approximately 110 residues displaying an immunoglobulin-like fold. EC domains are connected by hinge regions containing binding sites for the essential Ca^{2+} ions.[9,10] The effect of the binding of Ca^{2+} on the function of cadherins has been previously studied at the molecular and cellular levels.[11−13] Mechanistically, classical cadherins promote cell−cell adhesion by trans-dimerization of their EC1 (N-terminal) ectodomains.[14,15] The so-called strand-swap dimer (ss-dimer) represents the stable dimerization complex of classical E- and N-cadherins. The ss-dimer is characterized by the exchange of a short N-terminal β-strand between the dimerizing cadherins. Binding is governed by the docking of Trp2 of EC1 in a well-defined hydrophobic pocket of the EC1 domain of the partner cadherin.

Recent evidence indicates that the ss-dimerization of classical E-cadherin is preceded by an intermediate known as the X-dimer.[14,16−20] The name of this intermediate dimerization state refers to its singular shape in the crystal form. The X-dimer can be prepared in a stable form by adding an extra residue at the N-terminus of the primary sequence of E-cadherin (e.g., a Met residue), by removing the side chain of residues involved in the stabilization of the ss-dimer (i.e., W2A or E89A), or in the presence of a competitive inhibitor of the ss-dimer such as the

Received: September 30, 2013
Revised: February 22, 2014
Published: February 23, 2014

   dx.doi.org/10.1021/bi401341g | *Biochemistry* 2014, 53, 1742−1752

amino acid Trp.[14,17,21] The X-dimer has only been observed in constructs containing at least the first and second EC domains. At the cellular level, the X-dimer is essential for the smooth remodeling of cell−cell adhesion junctions dependent on E-cadherin.[22] The X-dimer is also observed in the nonclassical T-cadherin.[23] However, it is currently unclear if other classical cadherins assemble in the X-dimer conformation or how the X-dimer contributes to their biological function.

P-cadherin is a member of the classical cadherin family (type I) expressed in basal epithelia.[1,6] Importantly, P-cadherin is overproduced in pancreatic, breast, gastric, and lung cancers.[24−26] These observations have prompted several therapeutic strategies seeking to inactivate its adhesive properties.[27−29] Until recently, and unlike other classical cadherins, P-cadherin had not been closely examined at the molecular level. In a recent study, we described, for the first time, the physicochemical properties of the ss-dimer of P-cadherin.[30] In that report, it was shown that Ca^{2+} greatly stabilizes P-cadherin, although this ion is not strictly necessary for ss-dimerization. However, it is not known if human P-cadherin attains the X-dimer conformation or what the thermodynamic and structural basis for self-dimerization are.

Herein, we brought together mutational, structural, thermodynamic, and spectroscopic methodologies to identify and characterize the X-dimer of P-cadherin. The data convincingly shows that X-dimerization is mediated by specific interactions, at the residue level, between the two protomers of P-cadherin. Moreover, the homophilic association of P-cadherin is not affected by the presence of the highly homologous classical E-cadherin, suggesting that the X-dimer is involved in the initial stages of cell−cell adhesion and recognition mediated by human P-cadherin.

EXPERIMENTAL PROCEDURES

Preparation of Protein Constructs. The sequence of MEC1 and MEC12 comprises residues 1−129 and 1−241 of the mature human P-cadherin, respectively. Both constructs contained an additional His_6-tag at the C-terminus and a Met residue at the N-terminus. MEC1 and MEC12 were amplified by PCR and cloned into a pET28 vector between the NcoI and XhoI restriction sites. Forward primer 5′-CATGCCATGGATTGGGTGGTTGCTCC-3′ and reverse primer 5′-CCGCTCGAGCTGCATCACAGAAGTACCTGG-3′ were used to clone MEC1 from a plasmid encoding the full-length protein (UniProt Code P22223). For MEC12, the forward primer was same as above, whereas the reverse primer was 5′-CCGCTCGAGCCTCTGCACCTCATGGC-3′. Site-directed mutagenesis was carried out with a Quik-Change kit following the instructions of the manufacturer. The complete list of primers is given in Supporting Information Table S1.

The sequence of EC1 (residues 1−129 of the mature human P-cadherin) was cloned into a Champion pET SUMO vector as described previously[30] using forward primer 5′-GATTGGGTGGTTGCTCCAATATC-3′ and reverse primer 5′-CTACTGCATCACAGAAGTACCTGG-3′.

The construct of human E-cadherin MEC12 corresponded to residues 1−240 of the mature protein. It also included a His_6-tag at the C-terminus and a methionine residue at the N-terminus. MEC12 of E-cadherin was cloned into pET28 using the forward primer 5′-CATGCCATGGACTGGGTTATTCCGC-3′ and the reverse primer 5′-CCGCTCGAGCGTAATCACTACATTGGC-3′.

Protein Expression and Purification. Overexpression of P-cadherin and E-cadherin (MEC constructs) was carried out in *Escherichia coli* Rosetta2 (DE3) cells. *E. coli* cells were transformed with expression vector, inoculated in 3 mL of LB medium containing 50 mg mL^{-1} of kanamycin and 34 mg mL^{-1} of chloramphenicol, and grown at 37 °C overnight. Cells were subsequently transferred to 1 L of fresh LB medium with the appropriate antibiotics and incubated at 37 °C until the OD at 600 nm reached a value of 0.45. Protein expression was induced with 0.5 mM IPTG followed by incubation at 20 °C for 18 h. Cells were harvested by centrifugation at 7000*g* for 15 min, resuspended in binding buffer (20 mM Tris-HCl, 300 mM NaCl, 3 mM $CaCl_2$, and 20 mM imidazole, pH 8.0), and lysed in an EmulsiFlex C-5 homogenizer (Avestin, Ontario, Canada). The lysate was centrifuged at 40 000*g* for 30 min at 4 °C. The supernatant was loaded onto a Ni-NTA agarose column pre-equilibrated with binding buffer (Qiagen, Valencia, CA). MEC12 was eluted using elution buffer (20 mM Tris-HCl, 300 mM NaCl, 3 mM $CaCl_2$, and 300 mM imidazole, pH 8.0). MEC12 was further purified by size-exclusion chromatography (SEC) in a Hiload26/60 Superdex-200 column (GE-healthcare, Piscataway, NJ) at 4 °C. Equilibration buffer contained 10 mM HEPES, 150 mM NaCl, and 3 mM $CaCl_2$, pH 7.5. The purity of MEC12 was at least 95%, as judged by SDS-PAGE. All muteins were purified following this protocol. When necessary, Ca^{2+} was removed from the protein samples by dialysis against Ca^{2+}-depleted buffer containing 1 mM EDTA at 4 °C overnight followed by a second dialysis in buffer without EDTA at 4 °C for 6 h (repeated three times).[31,32]

The constructs EC1 and EC12-K14E of P-cadherin were purified as described previously.[30] The final purification step was carried out in a Hiload26/60 Superdex-75 column (for EC1) or a Hiload26/60 Superdex-200 column (for EC12-K14E). All samples were dialyzed against the appropriate buffer for 12 h before analysis.

Analytical SEC. Analytical SEC was performed in a Superose12 10/300 GL column (GE Healthcare, Piscataway, NJ) at a flow rate of 0.5 mL min^{-1} at 25 °C. The equilibration buffer (SEC buffer) contained 10 mM HEPES, 150 mM NaCl, and 3 mM $CaCl_2$, pH 7.5. In a typical experiment, the protein was loaded at 50 μM into the column. Elution was monitored at 280 nm. The effect of the His_6-tag on the SEC elution profile is essentially negligible; the peak maxima with and without His_6-tag appear within 1% of each other (data not shown).

Intrinsic Fluorescence. The intrinsic fluorescence of P-cadherin was recorded in a F-2500 spectrofluorometer (Hitachi, Japan) at 25 °C. The protein sample was excited at 295 nm, and its emission was monitored from 300 to 400 nm. Protein was prepared at 3 μM in SEC buffer. Each measurement was performed in triplicate.

Small-Angle X-ray Scattering (SAXS). SAXS was performed at beamline BL-10C of the Photon Factory (Tsukuba, Japan). Concentration of MEC12 in a buffer with Ca^{2+} was 5.0 to 6.0 mg mL^{-1}. The detailed methodology used to collect and analyze the data is described in a previous report.[30] The calculation of the SAXS profile of the X-ray structure of E-cadherin (PDB codes 1FF5 and 1Q1P) and subsequent fitting to the experimental SAXS profile of P-cadherin were carried out with the program FOXS of the CHIMERA suite.[33]

Isothermal Titration Calorimetry (ITC). The binding of Ca^{2+} to P-cadherin was examined in an iTC200 instrument (GE Healthcare). In a typical experiment, the cell of the calorimeter

Table 1. Molecular Weight of Constructs of P-Cadherin Determined by FFF−MALS

protein	$MW_{construct}$ (kDa)	$MW_{measured}$ (kDa)	oligomerization state	concentration (mM)
MEC1	15.3	19.9 ± 0.6	monomer	0.63
EC1	14.2	36.3 ± 0.7	dimer	0.35
MEC12	27.6	46.6 ± 0.2	dimer	0.18
EC12	26.4	42.7 ± 0.3	dimer	0.22
MEC12-K14E	27.6	27.3 ± 0.1	monomer	0.26

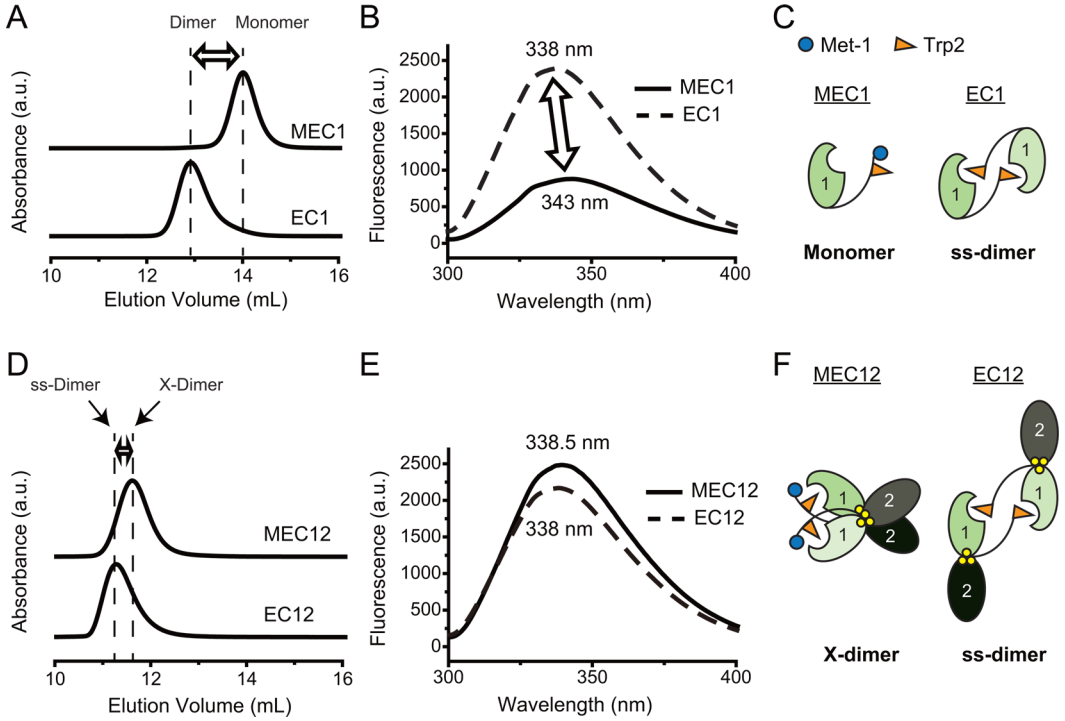


Figure 1. Dimerization of P-cadherin. The equilibrium monomer/dimer of P-cadherin was analyzed by SEC and intrinsic fluorescence. (A) Normalized elution profiles of samples containing only the first ectodomain of P-cadherin (EC1 or MEC1). (B) Intrinsic fluorescence of MEC1 (solid) and EC1 (dashed). The values of λ_{max} are indicated. (C) Schematic models of MEC1 and EC1 based on the experimental data. The yellow triangle and the blue circle represent Trp2 and the additional Met residue at the N-terminus of MEC1, respectively. (D) Normalized SEC profiles and (E) intrinsic fluorescence of P-cadherin comprising the first two extracellular domains. (F) Schematic models of dimerization complexes. The yellow circles represent Ca^{2+} ions.

was filled with Ca^{2+}-depleted protein (70 μM) in a buffer composed of 10 mM HEPES and 150 mM NaCl, pH 7.5. Protein was titrated with a solution containing 4.2 mM Ca^{2+} at 20 °C every 600 s at 1000 rpm. Data was analyzed with ORIGIN7 software using a one-site binding model.[34]

Differential Scanning Calorimetry (DSC). The thermal stability of P-cadherin was determined by calorimetry in a VP-DSC autosampler instrument (GE Healthcare). The cell was heated from 10 to 120 °C at a rate of 1 °C min^{-1}. The concentration of protein was 100 μM in SEC buffer. When necessary, the Ca^{2+} was removed from the protein samples prior to the experiment, as described above. Data analysis was carried out with the program ORIGIN7 using a two-state model.

Cross-Reactivity Assay. To evaluate the specificity of the X-dimer of P-cadherin, we mixed monomeric P-cadherin MEC12 and monomeric E-cadherin MEC12 monomer at 50 μM. The monomeric species were obtained by dialysis of the purified proteins in the presence of 1 mM EDTA. The mixture of P- and E-cadherin was subjected to a dialysis in a Ca^{2+}-free buffer (SEC buffer) at 4 °C overnight. To initiate the X-dimerization, the mixture was subjected to a dialysis in a buffer containing 3 mM Ca^{2+} at 4 °C overnight. The sample was examined by analytical SEC, as described above. Control experiments with each cadherin separately were performed under the same experimental conditions. The identity of the eluted fractions was examined by western blotting using specific antibodies against human P- or E-cadherin (BD Bioscience, Oxford, UK).

Field Flow Fractionation−Multiangle Light Scattering (FFF−MALS). The molecular weight of the proteins was determined with a Wyatt Eclipse instrument (Wyatt Technology, Santa Barbara, CA). Details are described elsewhere.[30,35,36] The molecular mass of the monomeric samples (MEC1 and MEC12-K14E) was determined at a concentration greater than that of the corresponding dimeric species (EC1, MEC12, and EC12). The protein concentration of each sample is given in Table 1. After a preliminary focusing step, the cross-flow decreased linearly from 3 to 0 mL min^{-1} in 15 min. The flow of the channel was kept at a constant rate of 1 mL min^{-1}.

RESULTS

Identification of X-Dimer of P-Cadherin. It was previously reported that the extension of the N-terminus of

dx.doi.org/10.1021/bi401341g | Biochemistry 2014, 53, 1742−1752

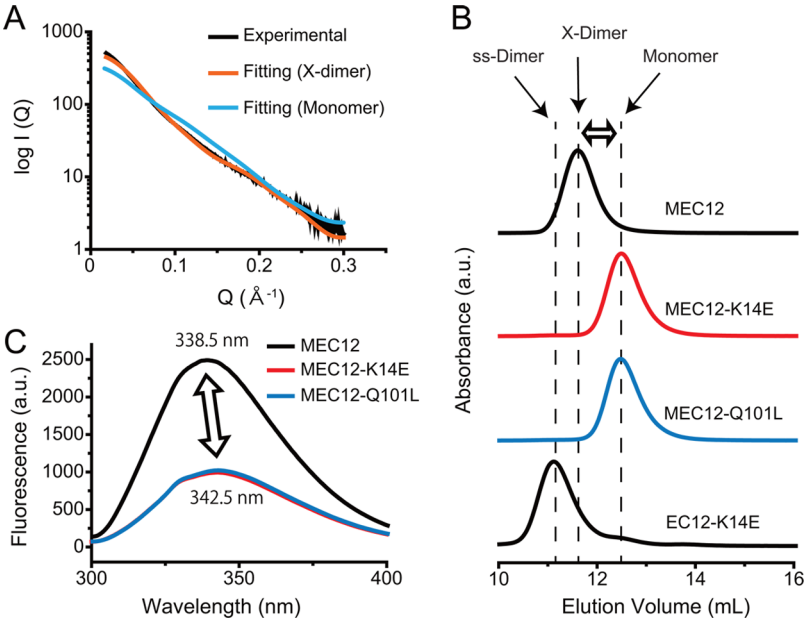


Figure 2. Identification of the X-dimer. (A) Experimental SAXS profile of MEC12 (black) and fitting of the predicted profile of the X-dimer (PDB code 1FF5, orange) and the monomer (PDB code 2O72, blue) of the highly homologous E-cadherin. The theoretical profile was calculated with the program FOXS.[33] (B) SEC profiles of MEC12 and muteins MEC12-K14E, MEC12-Q101L, and EC12-K14E. (C) Intrinsic fluorescence of MEC12 (black), mutein MEC12-K14E (red), and mutein MEC12-Q101L (blue). The excitation wavelength was set at 295 nm. The values of λ_{max} are indicated.

E-cadherin with a Met residue generates the X-dimer.[14,17−19,21] We first evaluated the effect of the additional Met residue on the dimerization of the first EC domain of P-cadherin by SEC, fluorescence spectroscopy (Figure 1A−C), and FFF−MALS (Table 1). The SEC elution profile of wild-type MEC1 (the letter M in MEC indicates methionine) is shifted ∼1.1 mL with respect to that of EC1, suggesting large differences between the hydrodynamic size of each construct. This observation is consistent with a dimeric species of EC1 and a noninteracting monomer of MEC1.[37] This interpretation was verified by FFF−MALS (Table 1). In addition, the wavelength at the emission maximum (λ_{max}) of EC1 decreases 5 nm compared to that of MEC1 (blue shift; Figure 1B), indicating that the key residue Trp2 of EC1 moves to a more hydrophobic environment like that in the ss-dimer (Figure 1C).[37−40] We note that the changes of the fluorescence spectra are unlikely to arise from the second tryptophan of P-cadherin (Trp59) because this residue is not located at the interface of either the ss-dimer or the X-dimer in the family of classical cadherins.[14,17] This experiment demonstrates that the additional Met residue at the N-terminus compromises the normal ss-dimerization of P-cadherin, in agreement with previous studies using E-cadherin.[17,41]

We next examined the two-domain constructs EC12 and MEC12 to evaluate the X-dimerization potential of P-cadherin specifically. These constructs were subjected to SEC (Figure 1D). The position of the chromatographic peaks suggests that MEC12 is a homodimer, although it is more compact than that of the ss-dimer of EC12. The dimeric state of MEC12 was corroborated by FFF−MALS (Table 1 and Supporting Information Figure S1). The effect of the protein concentration on the dimerization of MEC12 was also evaluated at 100 and 10 μM. The elution profile of both samples displays a single peak corresponding to the position of the dimer (Supporting Information Figure S2). This observation suggests that the X-dimer of P-cadherin MEC12 is more durable (higher affinity) than that of other homologous cadherins.[36,42] Moreover, compared with MEC1 (Figure 1B), the value of λ_{max} of MEC12 decreases (blue shift) and the intensity of the fluorescence band increases (Figure 1E), similar to what was observed with EC12. These data indicates that the Trp2 of MEC12 is located in a more hydrophobic environment (i.e., shielded from the solvent), as schematically indicated in Figure 1F.

Small-angle X-ray scattering (SAXS) was employed to corroborate the dimeric nature of the MEC12 dimer in solution (Figure 2A). The experimental SAXS profile and the theoretical SAXS profile of the X-dimer are nearly indistinguishable (in the absence of crystallographic data for P-cadherin, the calculated profile was obtained from the homologous E-cadherin, PDB code 1FF5). In contrast, the profile calculated from the crystallographic monomer of E-cadherin (PDB code 1Q1P) correlates poorly with the experimental profile. The SAXS profile of the ss-dimer was calculated using the crystal structure of human E-cadherin (PDB code 2O72) as surrogate (Supporting Information Figure S3). The profile of the ss-dimer also fits the experimental curve of MEC12, reflecting likely similarities between the three-dimensional structures of the dimers. From the comparison between experimental and calculated SAXS profiles, we conclude that MEC12 adopts a dimeric conformation in solution, as suggested in the SEC experiments earlier.

Site-directed mutagenesis was employed to reveal the nature of the MEC12 dimer. Muteins MEC12-K14E and MEC12-Q101L were prepared to validate the identity of the X-dimer. These two mutations, designed from a homology model built from E-cadherin, abrogate key stabilizing noncovalent forces present in the X-dimer but not in the ss-dimer (Supporting Information Figure S4).[14,22] Mutein EC12-K14E was employed as a negative control because the K14E mutation does not affect the equilibrium form of ss-dimer.[14,22] The three muteins were

dx.doi.org/10.1021/bi401341g | *Biochemistry* 2014, 53, 1742−1752

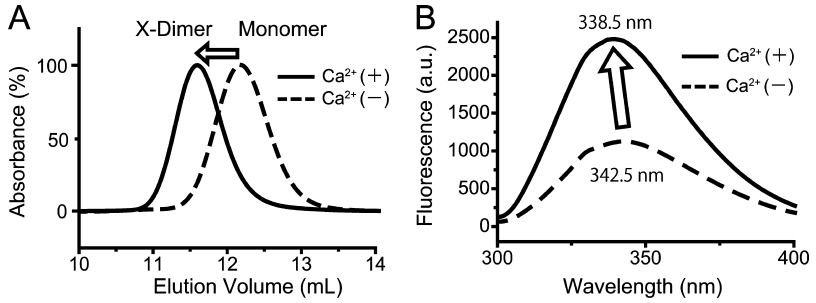


Figure 3. Effect of Ca^{2+} on the dimerization of P-cadherin. (A) SEC and (B) intrinsic fluorescence of MEC12 in the presence (solid line) or absence (dotted line) of Ca^{2+}. The concentration of Ca^{2+} was adjusted at 3 mM in the experiments in which the divalent cation was present.

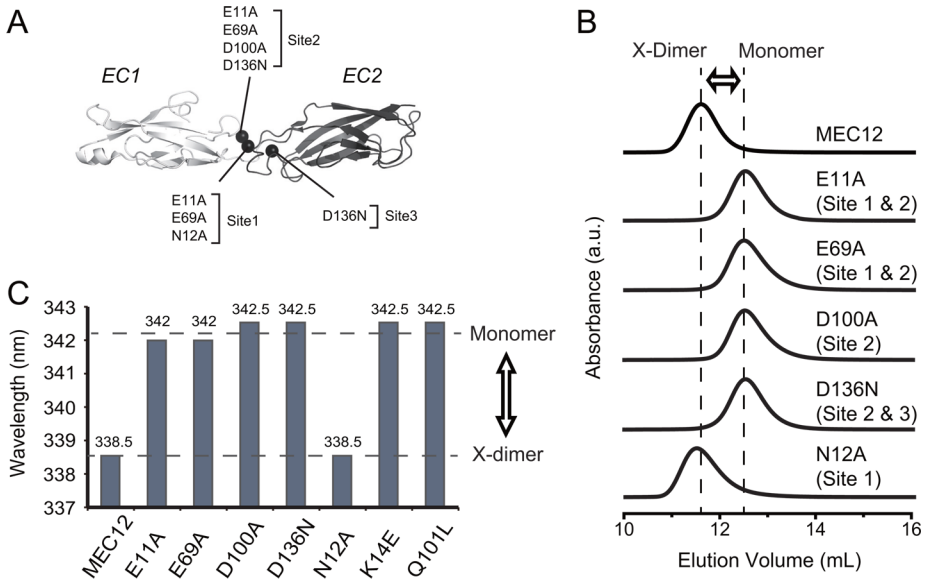


Figure 4. Evaluation of the Ca^{2+} binding sites. (A) Structure of the first two ectodomains of the highly homologous E-cadherin (PDB code 1Q1P). This structure was used as the basis for designing muteins of P-cadherin that targeted the Ca^{2+} binding sites. The black solid spheres depict Ca^{2+}. Only the mutated residues are indicated. (B) SEC profiles of MEC12 and muteins in the presence of 3 mM Ca^{2+}. (C) Values of λ_{max} obtained for MEC12 and muteins in the presence of 3 mM Ca^{2+}. The number above each bar indicates the value of λ_{max}.

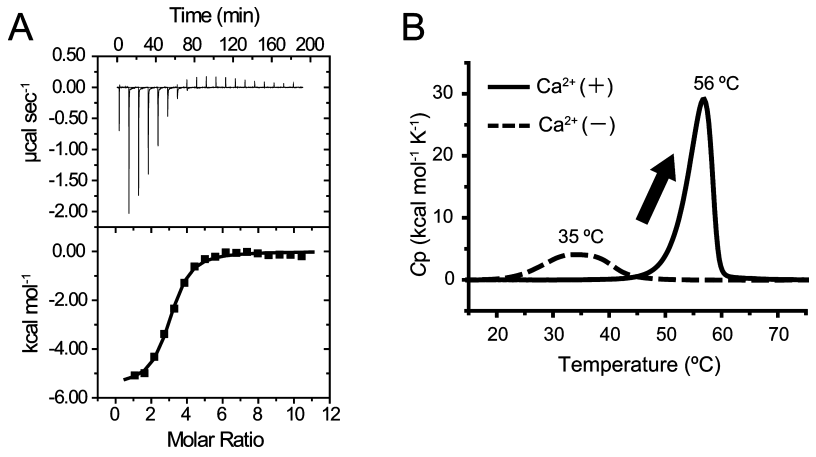


Figure 5. Thermodynamic analysis of the binding of Ca^{2+} to MEC12. (A) Titration of MEC12 (70 μM) with 4.2 mM Ca^{2+}. The top panel shows the titration kinetics, whereas the bottom panel represents the binding isotherm. Data analysis was performed with the program ORIGIN7. (B) Thermal stability of MEC12 in the presence and absence of Ca^{2+} (3 mM). The solid line and the dotted line represent the excess heat capacity of P-cadherin in the presence and absence of Ca^{2+}, respectively.

examined by SEC and fluorescence spectroscopy (Figure 2B,C). Whereas the SEC profile of EC12-K14E remains virtually unchanged with respect to the wild-type protein (compare it with that in Figure 1D), the elution peaks of muteins MEC12-K14E and MEC12-Q101L shifted to a higher elution volume like that of the monomeric form. The value of λ_{max} determined for both muteins of MEC12 is 4 nm greater (red shift) than that of MEC12, indicating that Trp2 moves to a

dx.doi.org/10.1021/bi401341g | *Biochemistry* 2014, 53, 1742−1752

Table 2. Ca^{2+}-Binding Parameters Obtained by ITC

protein		n	K_d (μM)	$\Delta H°$ (kcal mol^{-1})	$-T\Delta S°$ (kcal mol^{-1})[a]	$\Delta H°/\Delta G°$ (%)
MEC12	X-dimer	2.9 ± 0.1	8.7 ± 0.7	−5.5 ± 0.1	−1.3	81
MEC12-K14E	monomer	4.1 ± 0.1	17 ± 1.4	−2.8 ± 0.1	−3.6	44
MEC12-Q101L	monomer	3.7 ± 0.1	16 ± 1.3	−3.2 ± 0.1	−3.3	49
MEC12-D100A	monomer			N.D.[b]		
MEC12-D136N	monomer			N.D.[b]		
EC12	ss-dimer	2.7 ± 0.1	23 ± 1.5	−2.6 ± 0.1	−3.6	42

[a]The entropic term ($-T\Delta S°$) was calculated from the equation $\Delta G° = \Delta H° - T\Delta S°$. [b]N.D., binding not detected.

solvent-exposed region of the monomer upon mutation of these two key residues necessary for X-dimerization.[40] Moreover, the molecular weight of mutein MEC12-K14E in solution (determined by FFF−MALS) corresponds to that of the monomer (27 kDa, Table 1). Overall, we present compelling evidence about the existence of the X-dimer in human P-cadherin.

Ca^{2+} Is Essential for X-Dimerization. Binding of Ca^{2+} is an essential characteristic for the adhesive properties of cadherins.[12−14] Indeed, dimerization of MEC12 is not observed in the absence of Ca^{2+} (Figure 3). Both the SEC profile (Figure 3A) and the intrinsic fluorescence signal (Figure 3B) of Ca^{2+}-depleted MEC12 are virtually indistinguishable from the monomeric protein (also see Figure 2).

To investigate the role of Ca^{2+} in detail, key residues of the conserved Ca^{2+} binding sites of P-cadherin were mutated using the structure of E-cadherin as a guide.[10,13] SEC and intrinsic fluorescence were employed to examine the oligomeric state of five single mutations of MEC12 (E11A, E69A, D100A, D136N, and N12A) (Figure 4). All of the purified muteins except MEC12-N12A appear at high elution volumes in the SEC chromatogram (Figure 4B). In addition, the value of λ_{max} increases by 4 nm (red shift), and the fluorescence intensity decreases (Figure 4C and Supporting Information Figure S5). All of these observations are consistent with the predominance of monomeric species as shown above. In contrast, the mutation N12A does not abrogate dimerization, suggesting that negatively charged residues (Asp and Glu) play a more important role in the binding of Ca^{2+}.[11−13,43]

The thermodynamic basis of the binding of Ca^{2+} to MEC12 was examined by ITC (Figure 5A). The exothermic heat was integrated and fitted to a simple binding isotherm (Table 2).[30,44] Three Ca^{2+} ions (n = 2.9 ± 0.1) associate to one molecule of MEC12 with relatively low affinity (dissociation constant K_d = 8.7 ± 0.7 μM), a value comparable to that previously observed in other members of the cadherin family using other analytical techniques.[13,43] The binding reaction is driven by favorable changes of enthalpy ($\Delta H°$ = −5.5 ± 0.1 kcal mol^{-1}) and entropy ($-T\Delta S°$ = −1.3 kcal mol^{-1}). The value and sign of the thermodynamic parameters suggest that Ca^{2+} ions induce reorganization of the solvent and generate noncovalent interactions such as hydrogen bonds and van der Waals contacts.[45,46]

To understand the effect of Ca^{2+} on the structural stability of MEC12, we employed differential scanning calorimetry (DSC). The heat-denaturation curves of MEC12 in the presence and absence of Ca^{2+} are shown in Figure 5B. The heat-denaturation transition of the Ca^{2+}-depleted protein occurs at physiological temperature (T_m = 35 ± 0.1 °C) and entails a relatively small value of unfolding energy (ΔH_{DSC} = 59.1 ± 1.9 kcal mol^{-1}). In contrast, the thermal stability of MEC12 with Ca^{2+} bound is significantly higher: The transition peak is centered at higher temperature (T_m = 56.1 ± 0.1 °C, ΔT_m = 21 ± 0.2 °C) and gives rise to a larger value of unfolding energy (ΔH_{DSC} = 158 ± 0.9 kcal mol^{-1}, $\Delta\Delta H_{DSC}$ = 99 ± 3 kcal mol^{-1}) (Table 3). These results clearly indicate that Ca^{2+} stabilizes P-cadherin, probably by rigidifying the protein, as observed in other cadherins.[10,13]

Table 3. Effect of Ca^{2+} on the Thermal Stability of P-Cadherin MEC12

calcium	T_m (°C)	ΔH_{DSC} (kcal mol^{-1})
(+)	56.1 ± 0.1	158 ± 0.9
(−)	35.1 ± 0.1	59 ± 1.9
difference[a]	21 ± 0.2	99 ± 2.8

[a]The difference was calculated with respect to the Ca^{2+}-depleted sample.

Thermodynamic Scrutiny of Muteins. We have shown earlier that site-directed mutagenesis inhibits the X-dimerization of P-cadherin by two different mechanism depending on the nature of the mutation: (i) abrogating the binding of Ca^{2+} (mutations E11A, E69A, D100A, and D136N) or (ii) disabling key protein−protein interactions (mutations K14E and Q101L). We investigated the impact of these mutations on the thermodynamic parameters of MEC12 using ITC and DSC (Figure 6).

Binding of Ca^{2+} to muteins MEC12-D100A or MEC12-D136N was not detected by ITC (Supporting Information Figure S6). In contrast to the method used by Courjean et al. to study E-cadherin (ESI-MS),[43] ITC cannot detect the binding of each individual Ca^{2+} ion using our simple approach. However, on the basis of the assumption that neither Asp100 nor Asp136 interact simultaneously with all three Ca^{2+} ions, the detrimental effect upon mutation (muteins D100A or D136N) for all Ca^{2+} binding sites suggests that binding of Ca^{2+} to P-cadherin displays a high degree of cooperativity.

Titration of the second set of muteins, MEC12-K14E and MEC12-Q101L, with Ca^{2+} produces a robust exothermic signal (Figure 6A,B, Table 2, and Supporting Information Figure S6). The values of K_d for muteins MEC12-K14E and MEC12-Q101L were 17 ± 1.4 and 16 ± 1.3 μM, respectively. Similar values have been reported for N- and E-cadherin.[13,43] Intriguingly, an additional Ca^{2+} ion binds to the MEC12 muteins compared to the MEC12 parent construct. The exact location of the additional Ca^{2+} binding site will need to be investigated further with high-resolution structural techniques. Similar to MEC12, the driving force of binding arises from favorable changes of entropy and enthalpy. The relative impact of each thermodynamic component ($\Delta H°$ or $-T\Delta S°$) in the free energy ($\Delta G°$) is different from that of MEC12. The enthalpic component of MEC12 (evaluated from the ratio $\Delta H°/\Delta G°$) is twice as large as that of MEC12-K14E or MEC12-Q101L, whereas the entropic component (ratio

dx.doi.org/10.1021/bi401341g | Biochemistry 2014, 53, 1742−1752

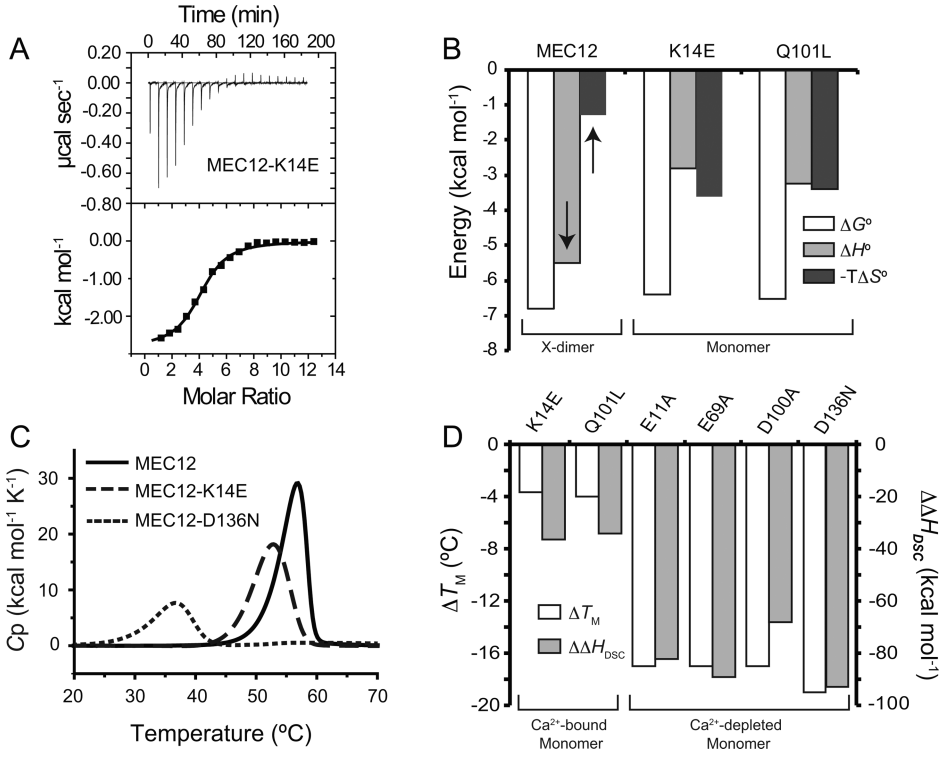


Figure 6. Thermodynamic analysis of the binding of Ca^{2+} to muteins of MEC12. (A) Binding of Ca^{2+} to muteins of MEC12 was examined by ITC. The top panel shows the titration kinetics of a representative example (MEC12-K14E), whereas the bottom panel represents its binding isotherm. Data analysis was performed with the program ORIGIN7. (B) Parameters of binding of Ca^{2+} to human P-cadherin (MEC12 and muteins). The white, gray, and black bars represent $\Delta G°$, $\Delta H°$, and $-T\Delta S°$, respectively. The vertical arrows highlight the increase or decrease of the energetic contributions with respect to the muteins. (C) Thermal stability of MEC12 (in the presence of Ca^{2+}) was examined by DSC. Data analysis was performed with the program ORIGIN7 using a two-state model. Representative data for MEC12, MEC12-K14E, and MEC12-D136N is shown. (D) Thermal stability of MEC12 and muteins (in the presence of 3 mM Ca^{2+}) examined by DSC. The values of ΔT_m and $\Delta\Delta H_{DSC}$ were calculated with respect to MEC12. The white and gray bars represent ΔT_m and $\Delta\Delta H_{DSC}$, respectively.

Table 4. Thermal Stability of Muteins of MEC12

protein	T_m (°C)	ΔH_{DSC} (kcal mol^{-1})	ΔT_m (°C)	$\Delta\Delta H_{DSC}$ (kcal mol^{-1})[a]
MEC12	56.1 ± 0.1	158 ± 0.9		
MEC12-K14E	52.5 ± 0.1	122 ± 0.8	−3.6 ± 0.2	−36 ± 1.7
MEC12-Q101L	52.1 ± 0.1	124 ± 0.6	−4.0 ± 0.2	−34 ± 1.5
MEC12-E11A	39.3 ± 0.2	76.2 ± 1.9	−17 ± 0.3	−82 ± 2.8
MEC12-E69A	38.9 ± 0.1	69.5 ± 1.4	−17 ± 0.2	−89 ± 2.3
MEC12-D100A	39.2 ± 0.1	89.8 ± 0.9	−17 ± 0.2	−68 ± 1.8
MEC12-D136N	37.4 ± 0.1	65.4 ± 1.3	−19 ± 0.2	−93 ± 2.2

[a] ΔT_m and $\Delta\Delta H_{DSC}$ were calculated with respect to MEC12.

$-T\Delta S°/\Delta G°$) only represents about one-third of the entropic contribution determined for the two muteins (Figure 6B and Table 2). Because the monomeric muteins do not form the X-dimer, the energetic differences with respect to MEC12 suggest that X-dimerization (i.e., Ca^{2+}-bound monomer → X-dimer) is driven by a favorable change of enthalpy (i.e., by favorable noncovalent interactions).

The thermal stability of the muteins was evaluated by DSC in the presence of Ca^{2+} (Figure 6C,D and Supporting Information Figure S7). The values of the thermodynamic parameters T_m and ΔH_{DSC} are given in Table 4. We distinguished between two groups of mutations based on their thermal stability. The first group comprises muteins MEC12-K14E and MEC12-Q101L (both bind Ca^{2+} but do not dimerize). These two mutations induce a modest destabilization of the protein with respect to MEC12 ($\Delta T_m \sim -4$ °C, $\Delta\Delta H_{DSC} \sim -30$ kcal mol^{-1}). In contrast, a second group of muteins (mutations E11A, E69A, D100A, and D136N) that do not bind Ca^{2+} displayed much diminished stability with respect to MEC12 ($\Delta T_m \sim -17$ to -19 °C, $\Delta\Delta H_{DSC} \sim -68$ to -93 kcal mol^{-1}). The data demonstrate that (i) both binding of Ca^{2+} and dimerization stabilize P-cadherin and (ii) the stabilization effect is more pronounced in the first step (i.e., the binding of Ca^{2+} to the monomer is the key event in the stabilization of human P-cadherin). In addition, the DSC data reinforce the conclusion that X-dimerization is driven by favorable changes of enthalpy, as suggested in the ITC analysis.

X-Dimerization Is Cadherin-Specific. The selectivity of the X-dimerization of human P-cadherin was investigated in the presence of the highly homologous human E-cadherin (Figure 7). To evaluate their cross-reactivity, the MEC12 construct of each cadherin was purified to homogeneity followed by

dx.doi.org/10.1021/bi401341g | Biochemistry 2014, 53, 1742−1752

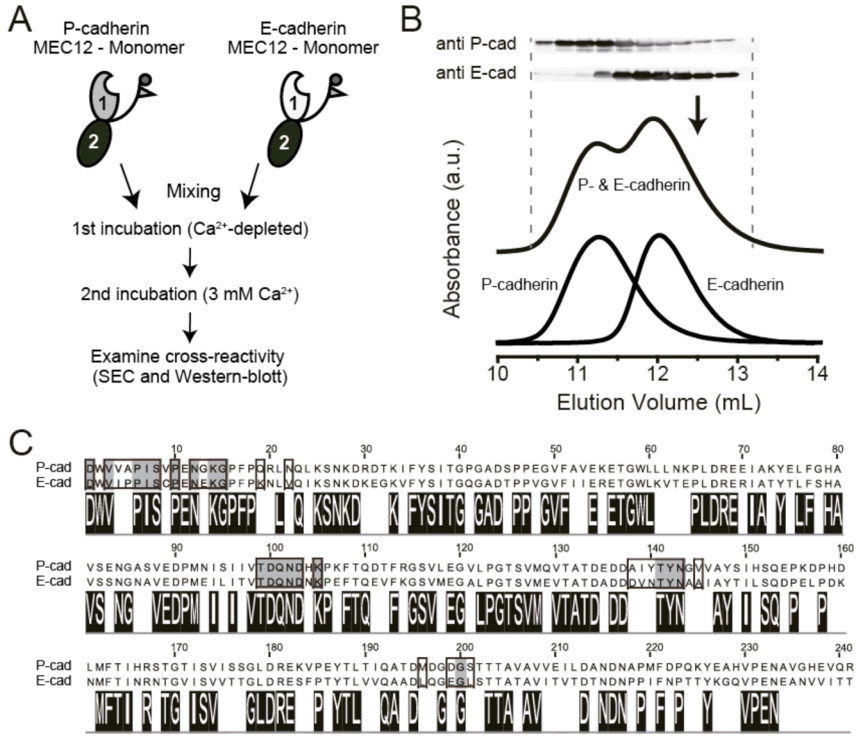


Figure 7. Specificity of the X-dimerization of human P-cadherin. (A) Experimental scheme to evaluate the cross-reactivity between P-cadherin and E-cadherin. The X-dimerization is initiated by addition of Ca^{2+} (second incubation). Proteins were incubated for 12 h in each step. (B) Upper chromatogram corresponds to the SEC profile of the mixture of P-cadherin and E-cadherin. Fractions were analyzed by western blot using specific antibodies against human P-cadherin or E-cadherin. The bottom chromatograms correspond to the SEC profiles of each cadherin assayed separately. The arrow indicates the position of the elution peak of the monomer of P-cadherin (MEC12-K14E). (C) Sequence alignment of the first two EC domains of human P-cadherin and E-cadherin using the T-coffee algorithm as implemented in the program Jalview.[53] The boxes indicate the residues participating in the interaction surface of the X-dimer as predicted from the crystal structure of E-cadherin (PDB code 1FF5).

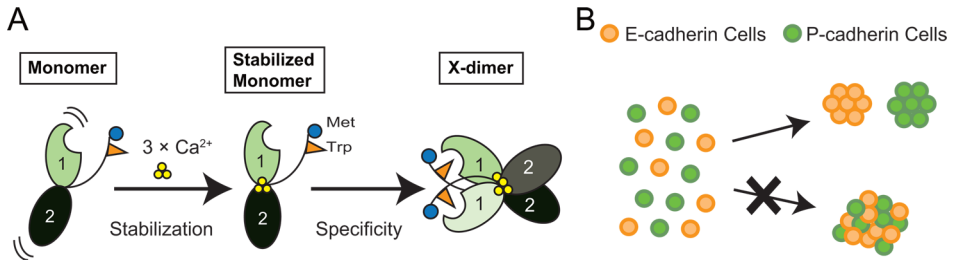


Figure 8. Mechanism of X-dimerization and model of homophilic adhesion. (A) X-dimerization of P-cadherin requires at least two sequential steps. First, the monomer is stabilized upon binding of three Ca^{2+} ions. Second, the stabilized monomer associates to form the specific and homophilic X-dimer. We found no significant evidence of cross-reactivity with the highly similar E-cadherin (Figure 7). (B) Cells expressing different types of cadherin aggregate in a homophilic manner.[49,50] Orange and green circles represent cells expressing E-cadherin and P-cadherin, respectively.

extensive treatment with EDTA to remove any residual Ca^{2+}. Proper folding of E-cadherin in the presence and absence of Ca^{2+} was confirmed by DSC (Supporting Information Figure S8). The cadherins were subsequently mixed while in the monomer state, dialyzed in the presence of 3 mM Ca^{2+}, and analyzed by SEC and western blot (Figure 7A). A control experiment using the individual proteins (not mixed) indicates that each cadherin appears in different elution peaks despite their similar sequence (>60% identity, Figure 7B,C). The elution peak of P-cadherin corresponds to the X-dimer, whereas that of E-cadherin appears at an intermediate position between the X-dimer and the monomer (Figure 7B). The separation between their elution peaks probably reflects differences of affinity between each homodimer.[36,42] In the experiment using the equimolar mixture of P-cadherin and E-cadherin, we observed two peaks at exactly the same positions as in the control experiments. The western blots reveal that the identity of each peak is maintained with respect to the control experiment, indicating minimal cross-reactivity between the otherwise highly homologous cadherins.

DISCUSSION

The basic tenet of cell adhesion mediated by cadherins is based on the trans-dimerization of apposing cadherins to form the so-called ss-dimer.[14,47] Recently, a second class of dimer, the X-dimer, has been identified and proposed as a key intermediate preceding the ss-dimer.[14,17−19,21] Until now, the X-dimer has been reported only for E-cadherin among the group I of classical cadherins.[9,14,22] It was unclear whether the X-dimer is a unique feature of E-cadherin or a characteristic shared among other members of the classical I cadherin family. Herein, we

dx.doi.org/10.1021/bi401341g | Biochemistry 2014, 53, 1742−1752

have elucidated the thermodynamic principle of the X-dimerization of human P-cadherin, and on the basis of these results, we have proposed a key role of the X-dimer in cell−cell adhesion.

We demonstrate that P-cadherin dimerizes in the X-conformation from multiple experimental approaches such as SAXS, SEC, fluorescence, calorimetry, and site-directed mutagenesis (Figures 1, 2, and 6). From the extensive data reported, we conclude that the overall architecture of the X-dimer and some protein−protein interaction hot spots are well-conserved between P-cadherin and E-cadherin.[14,16,17,23] Likewise, Ca^{2+} is essential for the formation of the X-dimer in both cadherins.[10,14,23] These findings reveal that the X-dimer is not limited to E-cadherin among the type I classical cadherins.

According to our data, the X-dimerization of P-cadherin is explained by two sequential (stepwise) processes, as schematically shown in Figure 8A. First, Ca^{2+} binds to the monomeric cadherin, a process governed by favorable changes of enthalpy and entropy, resulting in its thermal stabilization. Second, the stabilized monomers associate in the X-dimer conformation. The second step is driven by a favorable change of enthalpy but is opposed by a loss of entropy (reflecting the configurational entropy loss incurred upon dimerization). Biological interactions involving a favorable change of enthalpy generally indicate the establishment of specific noncovalent interactions such as hydrogen bonds and van der Waals contacts.[45,46,48] Indeed, the interaction surface of the X-dimer of the highly homologous E-cadherin establishes numerous hydrogen bonds and salt bridges between the protomers, such as between Lys14 and Gln140, Gln101 and Asp100, or Arg105 and Glu199.[14] These residues are well-conserved in P-cadherin (Figure 7C), and their mutation (K14E and Q101L) abolishes formation of the X-dimer (Figure 2B).

Although the characteristics of the X-dimer of P-cadherin are very similar to those of E-cadherin, such as overall structure (Figure 2A), interaction interface (Figure 2B,C), and Ca^{2+} binding ability (Figure 4), the affinity of P-cadherin X-dimer appears to be higher than that of E-cadherin and their cross-reactivity is very limited (Figure 7). From the favorable change of enthalpy of X-dimerization estimated by ITC ($\Delta H^{\circ} \sim -3$ kcal mol^{-1}), we suggest the existence of key noncovalent interactions in the X-dimer of P-cadherin not present in the X-dimer of E-cadherin.[45,46,48] We propose that a unique set of interactions present in the X-dimer of P-cadherin (but not in E-cadherin) contribute decisively toward its higher self-affinity and self-specificity. The distinct pattern of interactions results from differences at the primary sequence level and/or discrete changes in the three-dimensional structure and protein dynamics. A more definitive answer must wait for a comprehensive structural analysis by X-ray crystallography or other high-resolution structural techniques.

The high selectivity observed during the X-dimerization of P-cadherin has important biological implications for the understanding of cell sorting and tissue build-up because this key intermediate influences the formation of the long-lived ss-dimer.[14,17−19,21] Previous studies have demonstrated the lack of cross-reactivity between P-cadherin and E-cadherin at the cellular level, although at that time it was not known about the existence of the X-dimer.[49,50] Herein, we have demonstrated that cadherin-selectivity takes place at the X-dimer stage.

According to several studies, the specificity of cadherins depends on small differences in their expression levels and binding affinities.[51,52] Our results have revealed one more process governing the dimerization specificity between P-cadherin and other classical cadherins. Because the X-dimerization occurs before ss-dimerization, we propose that the X-dimer could act as the first checkpoint for cadherin-dependent cell sorting and segregation (Figure 8B). A careful and rigorous evaluation of this model in a biological context will be required to validate the implications of our findings for cell−cell adhesion.

CONCLUSIONS

In this study, we identified and characterized for the first time the X-dimer of the human adhesive protein P-cadherin. Dimerization is preceded by the binding of Ca^{2+} to monomeric P-cadherin, a necessary step that stabilizes the protein. In addition, the dimerization involves specific interactions at the residue level (favorable enthalpy) coupled with advantageous changes in the dynamics of the protein and solvent (favorable entropy). Importantly, human P-cadherin shows little cross-reactivity with the highly homologous E-cadherin. The high specificity may reflect unique interactions at the residue level on the dimerization interface.

ASSOCIATED CONTENT

Supporting Information

List of primers used; elution and scattering profile by FFF-MALS; SEC profile at two protein concentrations; calculated SAXS profile of ss-dimer; crystal structure of ss-dimer (PDB code 2O72) and X-dimer (PDB code 1FF5) of E-cadherin indicating mutational sites; intrinsic fluorescence signal; Ca^{2+} binding isotherms of muteins; thermal stability of muteins examined by DSC; and DSC profile of E-cadherin with and withour Ca^{2+}. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: tsumoto@bioeng.t.u-tokyo.ac.jp; Fax: +81-3-6409-2129; Tel: +81-3-5449-5316.

Funding

This work was supported by the Funding Program for World-Leading Innovative R&D on Science and Technology (FIRST) of the Japan Society for the Promotion of Science. This work was also supported by a grant-in-aid for JSPS fellows (to S.K.).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank the staff at the Photon Factory for excellent technical support. Access to beamline BL-10C was granted by the Photon Factory Advisory Committee (proposal nos. 2011G573, 2011G145, and 2011G535).

ABBREVIATIONS

EC, extracellular cadherin; ss-dimer, strand-swap dimer; SEC, size-exclusion chromatography; FFF, field-flow fractionation; MALS, multiangle light-scattering; SAXS, small-angle X-ray scattering; ITC, isothermal titration calorimetry; DSC, differential scanning calorimetry

REFERENCES

(1) Takeichi, M. (1988) The cadherins: Cell-cell adhesion molecules controlling animal morphogenesis. *Development 102*, 639−655.

(2) Takeichi, M. (1990) Cadherins: A molecular family important in selective cell-cell adhesion. *Annu. Rev. Biochem. 59*, 237−252.
(3) Gumbiner, B. M. (1996) Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell 84*, 345−357.
(4) Blaschuk, O. W., and Devemy, E. (2009) Cadherins as novel targets for anti-cancer therapy. *Eur. J. Pharmacol. 625*, 195−198.
(5) Bryan, R. T., and Tselepis, C. (2010) Cadherin switching and bladder cancer. *J. Urol. 184*, 423−431.
(6) Shimoyama, Y., Hirohashi, S., Hirano, S., Noguchi, M., Shimosato, Y., Takeichi, M., and Abe, O. (1989) Cadherin cell-adhesion molecules in human epithelial tissues and carcinomas. *Cancer Res. 49*, 2128−2133.
(7) Angst, B. D., Marcozzi, C., and Magee, A. I. (2001) The cadherin superfamily: diversity in form and function. *J. Cell Sci. 114*, 629−641.
(8) Patel, S. D., Ciatto, C., Chen, C. P., Bahna, F., Rajebhosale, M., Arkus, N., Schieren, I., Jessell, T. M., Honig, B., Price, S. R., and Shapiro, L. (2006) Type II cadherin ectodomain structures: Implications for classical cadherin specificity. *Cell 124*, 1255−1268.
(9) Brasch, J., Harrison, O. J., Honig, B., and Shapiro, L. (2012) Thinking outside the cell: How cadherins drive adhesion. *Trends Cell Biol. 22*, 299−310.
(10) Cailliez, F., and Lavery, R. (2005) Cadherin mechanics and complexation: The importance of calcium binding. *Biophys. J. 89*, 3895−3903.
(11) Prakasam, A., Chien, Y. H., Maruthamuthu, V., and Leckband, D. E. (2006) Calcium site mutations in cadherin: impact on adhesion and evidence of cooperativity. *Biochemistry 45*, 6930−6939.
(12) Ozawa, M., Engel, J., and Kemler, R. (1990) Single amino acid substitutions in one Ca^{2+} binding site of uvomorulin abolish the adhesive function. *Cell 63*, 1033−1038.
(13) Vunnam, N., and Pedigo, S. (2011) Sequential binding of calcium leads to dimerization in neural cadherin. *Biochemistry 50*, 2973−2982.
(14) Harrison, O. J., Bahna, F., Katsamba, P. S., Jin, X., Brasch, J., Vendome, J., Ahlsen, G., Carroll, K. J., Price, S. R., Honig, B., and Shapiro, L. (2010) Two-step adhesive binding by classical cadherins. *Nat. Struct. Mol. Biol. 17*, 348−357.
(15) Patel, S. D., Chen, C. P., Bahna, F., Honig, B., and Shapiro, L. (2003) Cadherin-mediated cell-cell adhesion: Sticking together as a family. *Curr. Opin. Struct. Biol. 13*, 690−698.
(16) Nagar, B., Overduin, M., Ikura, M., and Rini, J. M. (1996) Structural basis of calcium-induced E-cadherin rigidification and dimerization. *Nature 380*, 360−364.
(17) Pertz, O., Bozic, D., Koch, A. W., Fauser, C., Brancaccio, A., and Engel, J. (1999) A new crystal structure, Ca^{2+} dependence and mutational analysis reveal molecular details of E-cadherin homoassociation. *EMBO J. 18*, 1738−1747.
(18) Sivasankar, S., Zhang, Y., Nelson, W. J., and Chu, S. (2009) Characterizing the initial encounter complex in cadherin adhesion. *Structure 17*, 1075−1081.
(19) Leckband, D., and Sivasankar, S. (2012) Cadherin recognition and adhesion. *Curr. Opin. Cell Biol. 24*, 620−627.
(20) Li, Y., Altorelli, N. L., Bahna, F., Honig, B., Shapiro, L., and Palmer, A. G., 3rd (2013) Mechanism of E-cadherin dimerization probed by NMR relaxation dispersion. *Proc. Natl. Acad. Sci. U.S.A. 110*, 16462−16467.
(21) Rakshit, S., Zhang, Y., Manibog, K., Shafraz, O., and Sivasankar, S. (2012) Ideal, catch, and slip bonds in cadherin adhesion. *Proc. Natl. Acad. Sci. U.S.A. 109*, 18815−18820.
(22) Hong, S., Troyanovsky, R. B., and Troyanovsky, S. M. (2011) Cadherin exits the junction by switching its adhesive bond. *J. Cell Biol. 192*, 1073−1083.
(23) Ciatto, C., Bahna, F., Zampieri, N., VanSteenhouse, H. C., Katsamba, P. S., Ahlsen, G., Harrison, O. J., Brasch, J., Jin, X., Posy, S., Vendome, J., Ranscht, B., Jessell, T. M., Honig, B., and Shapiro, L. (2010) T-cadherin structures reveal a novel adhesive binding mechanism. *Nat. Struct. Mol. Biol. 17*, 339−347.
(24) Imai, K., Hirata, S., Irie, A., Senju, S., Ikuta, Y., Yokomine, K., Harao, M., Inoue, M., Tsunoda, T., Nakatsuru, S., Nakagawa, H., Nakamura, Y., Baba, H., and Nishimura, Y. (2008) Identification of a novel tumor-associated antigen, cadherin 3/P-cadherin, as a possible target for immunotherapy of pancreatic, gastric, and colorectal cancers. *Clin. Cancer Res. 14*, 6487−6495.
(25) Taniuchi, K., Nakagawa, H., Hosokawa, M., Nakamura, T., Eguchi, H., Ohigashi, H., Ishikawa, O., Katagiri, T., and Nakamura, Y. (2005) Overexpressed P-cadherin/CDH3 promotes motility of pancreatic cancer cells by interacting with p120ctn and activating rho-family GTPases. *Cancer Res. 65*, 3092−3099.
(26) Van Marck, V., Stove, C., Van Den Bossche, K., Stove, V., Paredes, J., Vander Haeghen, Y., and Bracke, M. (2005) P-cadherin promotes cell-cell adhesion and counteracts invasion in human melanoma. *Cancer Res. 65*, 8774−8783.
(27) Yoshioka, H., Yamamoto, S., Hanaoka, H., Iida, Y., Paudyal, P., Higuchi, T., Tominaga, H., Oriuchi, N., Nakagawa, H., Shiba, Y., Yoshida, K., Osawa, R., Katagiri, T., Tsunoda, T., Nakamura, Y., and Endo, K. (2012) In vivo therapeutic effect of CDH3/P-cadherin-targeting radioimmunotherapy. *Cancer Immunol. Immunother. 61*, 1211−1220.
(28) Zhang, C. C., Yan, Z., Zhang, Q., Kuszpit, K., Zasadny, K., Qiu, M., Painter, C. L., Wong, A., Kraynov, E., Arango, M. E., Mehta, P. P., Popoff, I., Casperson, G. F., Los, G., Bender, S., Anderes, K., Christensen, J. G., and VanArsdale, T. (2010) PF-03732010: A fully human monoclonal antibody against P-cadherin with antitumor and antimetastatic activity. *Clin. Cancer Res. 16*, 5177−5188.
(29) Park, J., Park, E., Han, S. W., Im, S. A., Kim, T. Y., Kim, W. H., Oh, D. Y., and Bang, Y. J. (2011) Down-regulation of P-cadherin with PF-03732010 inhibits cell migration and tumor growth in gastric cancer. *Invest. New Drugs 30*, 1404−1412.
(30) Kudo, S., Caaveiro, J. M., Miyafusa, T., Goda, S., Ishii, K., Matsuura, T., Sudou, Y., Kodama, T., Hamakubo, T., and Tsumoto, K. (2012) Structural and thermodynamic characterization of the self-adhesive properties of human P-cadherin. *Mol. BioSyst. 8*, 2050−2053.
(31) Urushihara, H., Ozaki, H. S., and Takeichi, M. (1979) Immunological detection of cell surface components related with aggregation of Chinese hamster and chick embryonic cells. *Dev. Biol. 70*, 206−216.
(32) Tong, K. I., Yau, P., Overduin, M., Bagby, S., Porumb, T., Takeichi, M., and Ikura, M. (1994) Purification and spectroscopic characterization of a recombinant amino-terminal polypeptide fragment of mouse epithelial cadherin. *FEBS Lett. 352*, 318−322.
(33) Schneidman-Duhovny, D., Hammel, M., and Sali, A. (2010) FoXS: A web server for rapid computation and fitting of SAXS profiles. *Nucleic Acids Res. 38*, W540−544.
(34) (2004) *Biocalorimetry 2. Applications of Calorimetry in the Biological Sciences* (Ladbury, J. E., and Doyle, M. L., Eds.) 2nd ed., John Wiley and Sons, Chichester, England.
(35) Yohannes, G., Wiedmer, S. K., Elomaa, M., Jussila, M., Aseyev, V., and Riekkola, M. L. (2010) Thermal aggregation of bovine serum albumin studied by asymmetrical flow field-flow fractionation. *Anal. Chim. Acta 675*, 191−198.
(36) Pollastrini, J., Dillon, T. M., Bondarenko, P., and Chou, R. Y. (2011) Field flow fractionation for assessing neonatal Fc receptor and Fcγ receptor binding to monoclonal antibodies in solution. *Anal. Biochem. 414*, 88−98.
(37) Shapiro, L., Fannon, A. M., Kwong, P. D., Thompson, A., Lehmann, M. S., Grubel, G., Legrand, J. F., Als-Nielsen, J., Colman, D. R., and Hendrickson, W. A. (1995) Structural basis of cell-cell adhesion by cadherins. *Nature 374*, 327−337.
(38) Trivedi, M., Davis, R. A., Shabaik, Y., Roy, A., Verkhivker, G., Laurence, J. S., Middaugh, C. R., and Siahaan, T. J. (2009) The role of covalent dimerization on the physical and chemical stability of the EC1 domain of human E-cadherin. *J. Pharm. Sci. 98*, 3562−3574.
(39) (2000) *Spectrophotometry and Spectrofluorimetry: A Practical Approach* (Gore, M. G., Ed.) 2nd ed., Oxford University Press, New York.
(40) Lakowicz, J. R. (2007) *Principles of Fluorescence Spectroscopy*, 3rd ed., Springer, New York.

(41) Harrison, O. J., Corps, E. M., and Kilshaw, P. J. (2005) Cadherin adhesion depends on a salt bridge at the N-terminus. *Journal of cell science 118*, 4123−4130.
(42) Philo, J. S. (2001) Overview of the quantitation of protein interactions, in *Current Protocols in Protein Science* (Coligan, J. E., Dunn, B. M., Speicher, D. W., and Wingfield, P. T.) pp 1−13, John Wiley & Sons, Hoboken, NJ.
(43) Courjean, O., Chevreux, G., Perret, E., Morel, A., Sanglier, S., Potier, N., Engel, J., van Dorsselaer, A., and Feracci, H. (2008) Modulation of E-cadherin monomer folding by cooperative binding of calcium ions. *Biochemistry 47*, 2339−2349.
(44) Ladbury, J. E. (2010) Calorimetry as a tool for understanding biomolecular interactions and an aid to drug design. *Biochem. Soc. Trans. 38*, 888−893.
(45) Ross, P. D., and Subramanian, S. (1981) Thermodynamics of protein association reactions: Forces contributing to stability. *Biochemistry 20*, 3096−3102.
(46) Ui, M., Tanaka, Y., Tsumuraya, T., Fujii, I., Inoue, M., Hirama, M., and Tsumoto, K. (2008) How protein recognizes ladder-like polycyclic ethers. Interactions between ciguatoxin (CTX3C) fragments and its specific antibody 10C9. *J. Biol. Chem. 283*, 19440−19447.
(47) Parisini, E., Higgins, J. M., Liu, J. H., Brenner, M. B., and Wang, J. H. (2007) The crystal structure of human E-cadherin domains 1 and 2, and comparison with other cadherins in the context of adhesion mechanism. *J. Mol. Biol. 373*, 401−411.
(48) Sinha, N., Mohan, S., Lipschultz, C. A., and Smith-Gill, S. J. (2002) Differences in electrostatic properties at antibody-antigen binding sites: implications for specificity and cross-reactivity. *Biophys. J. 83*, 2946−2968.
(49) Nose, A., Tsuji, K., and Takeichi, M. (1990) Localization of specificity determining sites in cadherin cell adhesion molecules. *Cell 61*, 147−155.
(50) Ahrens, T., Pertz, O., Haussinger, D., Fauser, C., Schulthess, T., and Engel, J. (2002) Analysis of heterophilic and homophilic interactions of cadherins using the c-Jun/c-Fos dimerization domains. *J. Biol. Chem. 277*, 19455−19460.
(51) Chen, C. P., Posy, S., Ben-Shaul, A., Shapiro, L., and Honig, B. H. (2005) Specificity of cell-cell adhesion by classical cadherins: Critical role for low-affinity dimerization through beta-strand swapping. *Proc. Natl. Acad. Sci. U.S.A. 102*, 8531−8536.
(52) Katsamba, P., Carroll, K., Ahlsen, G., Bahna, F., Vendome, J., Posy, S., Rajebhosale, M., Price, S., Jessell, T. M., Ben-Shaul, A., Shapiro, L., and Honig, B. H. (2009) Linking molecular affinity and cellular specificity in cadherin-mediated adhesion. *Proc. Natl. Acad. Sci. U.S.A. 106*, 11594−11599.
(53) Waterhouse, A. M., Procter, J. B., Martin, D. M., Clamp, M., and Barton, G. J. (2009) Jalview version 2−a multiple sequence alignment editor and analysis workbench. *Bioinformatics 25*, 1189−1191.