Molecular interactions between desmosomal cadherins

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Desmocollins (Dscs) and desmogleins (Dsgs) are cell-adhesion molecules involved in the formation of desmosome cell–cell junctions and share structural similarities to classical cadherins such as E-cadherin. In order to identify and provide quantitative information on the types of protein–protein interactions displayed by the type 2 isoforms and investigate the role of Ca^{2+} in this process, we have developed an *Escherichia coli* expression system to generate recombinant proteins containing the first two extracellular domains, namely Dsg2(1–2) and Dsc2(1–2). Analytical ultracentrifugation, chemical cross-linking, CD, fluorescence and BIAcore have been used to provide the first direct evidence of Ca^{2+} binding to desmosomal cadherins. These studies suggest that Dsc2(1–2) not only exhibits homophilic interactions in solution, but can also form heterophilic interactions with Dsg2(1–2). The latter, on the other hand, shows much weaker

homophilic association. Our results further demonstrate that heterophilic interactions are Ca^{2+} -dependent, whereas the Ca^{2+} -dependence of homophilic association is less clear. Our data indicate that the functional properties of Dsc2(1–2) are more similar to those of classical cadherins, consistent with the observation that Dsc shares a higher level of sequence homology with classical cadherins than does Dsg. In addition to corroborating the conclusions of previously reported transfection studies which suggest the formation of lateral heterodimers and homodimers, our results also provide direct quantitative information on the strength of these interactions which are essential for understanding the adhesion mechanism.

Key words: Ca^{2+} binding, cell adhesion, desmocollin, desmoglein, dimerization.

INTRODUCTION

Desmosomes are major intercellular adhesive junctions found in all epithelial tissues and also in cardiac muscle, brain meninges and follicular dendritic cells [1,2]. They provide anchorage of the plasma membrane to the cytoskeleton by interacting with intermediate filaments on their cytoplasmic side, thus contributing to tissue integrity. In contrast with the adherens junctions, which usually contain only one type of cadherin, desmosomes invariably contain two types of transmembrane cadherin molecules, desmocollins (Dsc) and desmogleins (Dsg), as their adhesive glycoprotein components [3,4]. These proteins are present as different isoforms [4], Dsc1, Dsc2 and Dsc3, and Dsg1, Dsg2 and Dsg3, which are expressed in tissue-specific and differentiation-dependent patterns [5–8]. The type 2 desmosomal cadherins are the most widely expressed forms, being present in all desmosome-bearing tissues and cells, whereas expression of types 1 and 3 isoforms is restricted to certain tissues such as tongue, epidermis and stratifying epithelia [9–11]. Desmosomal cadherins share structural similarities with classical cadherins in having five extracellular repeats, a single transmembrane region and a cytoplasmic domain [3,12–14]. Various cytoplasmic plaque proteins, including plakoglobin [15], desmoplakin [16] and plakophilins 1 and 2 [17–19], link these proteins to the intermediate filaments.

Evidence for lateral homoassociation of classical cadherins has come from *in vivo* studies of chemical cross-linking of L cells

transfected with murine E-cadherin cDNA [20]. Ca^{2+} -dependent weak dimerization of the first two domains of E-cadherin observed by analytical ultracentrifugation [21], forced clustering of cadherin mutants lacking the cytoplasmic tail by the use of the FKBP–FK1012 protein oligomerization system (where FKBP is FK506 binding protein, and FK1012 is an FK506 analogue) [22,23] and electron microscopy of recombinant Ecad1–2 [24] and Ecad-COMP, a recombinant E-cadherin ectodomain pentamerized by the assembly domain of cartilage oligomeric matrix protein [23,25,26]. To date, the crystal structures of the first domain of N-cadherin (NC1) [27], of the first two domains of N-cadherin (NC1–2) [28], and of the first two domains of E-cadherin (Ecad1–2) [26,29], have been determined. On the basis of the observation of *cis* and *trans* interactions in the structure of NC1, a zipper model for cadherin adhesion was proposed to reflect physiologically relevant interactions. The *cis* interaction represents the formation of parallel dimers on the same cell, while the *trans* interaction involves antiparallel oriented molecules protruding from two opposing cells to form the adhesive contacts. Alternating *cis* and *trans* interactions then form a zipper-like superstructure. The putative adhesive contacts were not, however, observed in crystal structures of NC1–2 and Ecad1–2, while the nature of amino acid side-chain contacts in the *cis* interaction also differed from those observed in NC1. The closest contact within the parallel dimer was seen in the Ca^{2+} -binding region [29] instead of a mutual exchange of a β -strand, due to binding of Trp² from one molecule into a hydrophobic

Abbreviations used: Dsg, desmoglein; Dsc, desmocollin; NC1, first domain of N-cadherin; Ecad1–2, the two N-terminal domains of E-cadherin; Dsg2(1–2), the two N-terminal domains of desmoglein2; Dsc2(1–2), the two N-terminal domains of desmocollin 2; GST, glutathione S-transferase; DTT, dithiothreitol; IPTG, isopropyl β -D-thiogalactopyranoside; Caps, 3-(cyclohexylamino)propane-1-sulphonic acid; CAMs, cell-adhesion molecules; FXa, Factor Xa (or activated Factor X), a restriction protease from Roche; Ni-NTA-agarose, Ni^{2+} -nitrilotriacetate-agarose; BS³, bis(sulphosuccinimidyl) suberate.

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pocket of the partner molecule, as observed in the structure of NC1. Thus the exact details of intercadherin interactions remain unclear.

In the case of desmosomal cadherins, transfection experiments using mouse L cells have shown that co-expression of both types of desmosomal cadherin and plakoglobin is essential for conferring strong Ca^{2+} -dependent intercellular adhesion [30], suggesting the possibility of formation of side-by-side heterodimers. L cells expressing either one or both of Dsc2a and Dsg1 display weak Ca^{2+} -dependent homophilic and heterophilic interactions [30–33]. Further evidence for intercellular heterophilic interactions has come from studies involving transfection of Dsg2-expressing human fibrosarcoma HT-1080 cells with Dsc1a [34]; dramatic changes in the subcellular distribution of endogenous Dsg2 were observed. These data were further supported by co-immunoprecipitation and solid-phase binding assays using recombinant first two extracellular domains of Dsg and Dsc. These observations suggest that, despite the observed structural similarities between desmosomal and classical cadherins, and in particular among the extracellular repeat domains, distinctly different mechanisms of cell adhesion may be operating in the two subfamilies of cadherins. However, direct evidence for the contribution of extracellular regions of desmosomal cadherins to putative homophilic or heterophilic interactions suggested for these molecules is lacking at present. Furthermore, no quantitative information on the strength of these interactions, or indeed the affinity or stoichiometry of binding of Ca^{2+} to these molecules, is available to date.

We have developed an *Escherichia coli* expression system to express and purify sufficient quantities of recombinant type 2 isoforms containing the first two extracellular domains, Dsg2(1–2) and Dsc2(1–2), to identify and provide quantitative information on the types of interactions displayed by these proteins as well as investigate the role of Ca^{2+} in this process. A combination of techniques, including analytical ultracentrifugation, chemical cross-linking, CD, fluorescence and BIAcore (Biacore AB, Stevenage, Herts., U.K.) analysis, have been used. We provide direct evidence of Ca^{2+} binding to desmosomal cadherins. The data from sedimentation-equilibrium analysis, cross-linking and BIAcore studies suggest that Dsc2(1–2) displays both homophilic and heterophilic interactions. Dsg2(1–2), on the other hand, only undergoes heterophilic association. Our results further demonstrate that heterophilic interactions are Ca^{2+} -dependent, whereas the Ca^{2+} -dependence of homophilic association is less clear. In addition to corroborating the formation of lateral heterodimers and homodimers, our results also provide direct quantitative information on the strength of these interactions, which are clearly essential for understanding the adhesion mechanism.

MATERIALS AND METHODS

Plasmid construction

Full-length human cDNA of Dsc2a (clone p5B3) [35] and Dsg2 [11] were used as templates for PCR amplification of DNA sequences corresponding to the first two extracellular domains of these proteins for cloning into the fusion vector, pGEX-6P-1 with glutathione S-transferase (GST) as the fusion partner. Restriction sites for *Bam*H1 and *Xho*I were included in the 5' and 3' primers respectively. Recent evidence has suggested that, in the case of E-cadherin, the presence of extraneous residues at the N-terminus, either as a result of incorrect processing of the pro-sequence or as an artefact of cloning, leads to loss of adhesive function [36] and the observation of a disordered state of the N-terminus in the reported crystal structure of Ecad1-2 [29]. To

circumvent this, the 5' primers also included codons for the FXa cleavage site immediately 3' of the *Bam*H1 site directly followed by the desmosomal coding sequence [FXa is Factor Xa (or activated Factor X), a restriction protease from Roche]. Thus introduction of the FXa cleavage site immediately next to the protein N-terminus without the presence of a linker allows the production of a protein without any extraneous residues after cleavage of the GST fusion partner. For some studies, Dsg2(1–2)-His [Dsg2(1–2) with a hexahistidine tag at the C-terminus] was used. Codons for this tag were introduced into the 3' primer between the coding sequence and the *Xho*I site. Two stop codons (CTACTA) were also included in the 3' primers for efficient termination of translation. The sequences of primers optimized by the use of the program Primer Designer are as follows: FXaBEG1Dsc2, 5'-AAGGTCGTAGATGGGCTCCAATTCC-TTGT-3'; *Xho*IEND2Dsc2, 3'-TGCACTCGAGCTACTAA-AATGTTGGCAAGTGGTCATTTAC-5'; FXaBEG1Dsg2, 5'-CCCCTGGATCCATCGAAGGTCGTGCCTGGATCAC-CGCCCCGTGGCT-3'; *Xho*IEND2Dsg2, 3'-TGCACTCGA-GCTACTATACTACAGGTATATTGTCATTGACATC-5'.

All primers were obtained from Oswell DNA Service, University of Southampton, Southampton, U.K. Amplifications were performed in Stratagene buffer with 1 unit of Pfu, 20 ng of template, 3 mM MgCl_2 , 250 μM of each dNTP and 20 pmol of each primer in a total reaction volume of 50 μl . A total of 30 cycles with denaturation for 60 s at 94 °C, annealing for 30 s at 60 °C and extension for 3 min at 72 °C were followed by a final 10 min elongation at 72 °C. PCR products were digested with *Bam*H1 and *Xho*I in Sure-cut buffer B (Roche) at 37 °C for 2 h, gel-purified and directionally cloned into pGEX-6P-1 vector, which had similarly been digested and purified, using T_4 DNA ligase (New England Biolabs) according to a standard protocol. DH5 α cells (Life Technologies) were transformed with the ligated plasmids. ABI Prism, a sequencing kit from Applied Biosystems, was used to sequence both DNA strands in conjunction with the ABI 377 DNA sequencer (PerkinElmer). The entire DNA sequence corresponding to the first two extracellular domains was found to be error-free and in frame with the vector.

Expression and purification of desmosomal cadherins

Proteins were expressed in DH5 α cells by growing a culture to an attenuation (D_{600}) of 1.5 at 37 °C before induction with 0.1 mM isopropyl β -D-thiogalactopyranoside (IPTG) at 29 °C for 4 h. The whole process was scaled up to 80 litres to compensate for relatively low yields of soluble protein attributable to the presence of cadherin largely in the form of inclusion bodies. Cells were harvested and resuspended in ice-cold 50 mM Tris/HCl (pH 8) buffer containing complete protease cocktail, 5 mM CaCl_2 , 100 mM NaCl and 10 mM dithiothreitol (DTT). Cells were broken using a cell disruption system (Constant Systems, Ltd, Low March, Daventry, Northants, U.K.). The cell lysate was clarified at 50000 g for 1 h in a Beckman preparative ultracentrifuge before being applied to a 10 ml GSH-Sepharose affinity column pre-equilibrated with the above buffer, at a flow rate of 10–20 ml/h. The column was washed with at least 10 column volumes of FXa buffer [50 mM Tris/HCl (pH 8)/100 mM NaCl/5 mM CaCl_2]. In the case of Dsg2(1–2), FXa was used to carry out on-column cleavage of the fusion protein by circulating 5 ml of a solution containing 1 μg of protease/mg of fusion protein at 4 °C for 18–24 h. By contrast, Dsc2(1–2) fusion protein was first eluted from the column by passing 4 column vol. of a pH-adjusted solution of 20 mM GSH in FXa buffer. Proteins were concentrated by precipitation with $(\text{NH}_4)_2\text{SO}_4$ (80% saturation) and centrifugation at 27000 g for 15 min in a

Beckman model J2-21 centrifuge. The protein pellet was re dissolved in 50 mM Tris/HCl buffer, pH 8, containing 1 M NaCl and 5 mM CaCl₂, followed by dialysis against the same buffer for 24 h to displace all the tightly bound GSH. Proteins were extensively dialysed against FXa buffer before being digested by protease in solution for 24 h at 4 °C prior to second affinity purification on a 10 ml GSH-Sepharose column to remove the cleaved GST. Usually, some high- and low-molecular-mass impurities were removed either by gel filtration on a column of Sephadex G-75 or by ion-exchange chromatography on a Mono Q column by FPLC (Amersham). In the former case, a 150-cm-long × 2.5-cm-diameter column was run in 10 mM Hepes buffer, pH 7.5, containing 150 mM NaCl and 5 mM CaCl₂ at a flow rate of 10 ml/h, 1 ml fractions being collected. The Mono Q column was equilibrated with 20 mM Tris/HCl (pH 8)/20 mM NaCl/5 mM CaCl₂/5 mM DTT, and 3 ml of Dsc2(1-2) solution was loaded; the column was successively washed with equilibration buffer for 20 min followed by 200 mM NaCl for 10 min, prior to developing a gradient of 200–400 mM NaCl over a period of 40 min, 0.5 ml fractions being collected. Finally, the column was washed with 500 mM NaCl for 10 min. Fractions from Sephadex G-75 or Mono Q columns were analysed by SDS/PAGE with a 12.5 % resolving gel. The identity of the band corresponding to Dsc2(1-2) was further confirmed by immunoblotting using a polyclonal antibody against the entire extracellular portion of Dsc2 [37]. The relevant pooled fractions of Dsc2(1-2) and cleaved affinity-purified Dsg2(1-2) were concentrated by (NH₄)₂SO₄ precipitation and dialysed against 10 mM Hepes buffer containing 150 mM NaCl and 5 mM CaCl₂. These buffer conditions were used for all subsequent studies unless otherwise stated.

In the case of Dsg2(1-2)-His, cleaved protein eluted from the affinity column was further purified by metal-chelate chromatography. First, the protein was dialysed against 50 mM Tris/HCl (pH 8.0)/100 mM NaCl/10 mM imidazole and loaded on a 5 ml column of Ni²⁺-nitrilotriacetate (Ni-NTA)-agarose (Qiagen) which had been pre-equilibrated in the same buffer. The column was washed with 10 column vol. of equilibration buffer followed by elution of the protein by 250 mM imidazole. Purified protein was dialysed against several changes of 10 mM Hepes buffer, pH 7.5, containing 150 mM NaCl and 5 mM CaCl₂.

Characterization of proteins by N-terminal sequencing and MS analyses

Proteins were subjected to SDS/PAGE in a 12.5 %-(w/v)-polyacrylamide resolving gel [38], followed by electroblotting on to a ProBlott membrane for 1 h at a constant current of 1 mA/cm² using 10 mM Caps [3-(cyclohexylamino)propane-1-sulphonic acid], pH 11, and 10 % (v/v) methanol. The membrane was soaked in methanol and stained for 30 s with 0.1 % Coomassie Blue R-250 in 40 % (v/v) methanol/1 % (v/v) acetic acid. Destaining was performed with 50 % (v/v) methanol for 1–2 min before extensive rinsing with deionized water. Protein bands were excised for N-terminal-amino-acid sequencing by Edman degradation on an Applied Biosystems 477A Protein Sequencer.

Protein molecular masses were determined by electrospray MS on a single-platform quadrupole mass spectrometer (Micromass, Limited, Altrincham, Cheshire, U.K.). A 100–200 pmol portion of protein was desalted prior to analysis using a 2 mm × 2 cm column containing porous R2 [PerSeptive Biosystems (U.K.) Ltd., Foxholes Business Park, Hertford, U.K.]. Protein was eluted off the column into the mass spectrometer in 70 % (v/v) acetonitrile/0.1 % (v/v) formic acid.

Determination of protein concentration

The concentration of purified recombinant cadherins was determined by measurements of A_{280} using absorption coefficients calculated as described in [39]. The following relationship was used:

$$\epsilon_{280} = (nW \times 5500) + (nY \times 1490) + (nC \times 125)$$

where ϵ_{280} is the calculated molar absorption coefficient of the protein, nW , nY and nC are the number of tryptophan, tyrosine and cystine residues in the amino acid sequence respectively, and the numbers in the parentheses are the individually determined molar absorption coefficients of the above amino acids.

Immunoblotting

Total cell lysates or purified cadherins were treated with gel loading buffer under reducing conditions, boiled and separated by SDS/PAGE using 12.5 % polyacrylamide gels. Proteins were transferred to nitrocellulose filters (Sartorius) soaked in blotting buffer [2.72 mM Tris/HCl (pH 8.3)/19.2 mM glycine/0.05 % (w/v) SDS/20 % (v/v) methanol] using a Biometra (Göttingen, Germany) semi-dry blotter at 1 mA/cm² of gel for 2 h. Filters were blocked with 5 % (w/v) Marvel skimmed dried milk and then incubated with rabbit antiserum to the entire extracellular portion of Dsc2a [37], mouse monoclonal antibody to the pentahistidine tag (Qiagen) or rabbit polyclonal antibody against GST (Amersham). Membranes were washed four times (5 min each time) with PBS containing 0.05 % (v/v) Tween 20 and incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibody (Sigma) for 30–60 min at room temperature. Following extensive washing, filters were developed using an enhanced-chemiluminescence system (ECL[®], Amersham).

Analytical ultracentrifugation

Analytical ultracentrifugation was carried out on a Beckman Optima XL-A analytical ultracentrifuge equipped with a UV-absorption system. Protein absorbance was monitored at 280 nm. Sedimentation-equilibrium runs were performed at speeds of 18000, 21000 and 28000 rev./min at concentrations of 4.1, 12.5 and 21 μ M for Dsc2(1-2) and 11.4 and 64 μ M for Dsg2(1-2) in the presence of 5 mM CaCl₂ or 10 mM EDTA; the temperature was maintained at 20 °C. Analysis of absorbance profiles was carried out using both a global fit to the data for all speeds and concentrations and the fit to individual concentrations (XL-1 Origin v4.0); this analysis yielded an apparent average molecular mass. Sedimentation-velocity experiments were carried out at 20 °C under buffer conditions identical with those used above at 40000 rev./min for Dsc2(1-2) and 30000 rev./min for Dsg2(1-2). Apparent sedimentation coefficients were obtained by analysing data using the transport method (XLA/XL1 Origin version 4.0 Microcal analysis software) and then corrected to standard conditions (20 °C, water) [19]. The shape factor, f/f_0 , was calculated using the following relationships:

$$f = M(1 - \nu\rho)/Ns \quad (1)$$

$$f_0 = [6\pi\eta(3M\nu \times 10^{24}/4\pi N)^{1/3} + 2.8 \text{ \AA}] \times 10^{-8} \quad (2)$$

where f is the experimental frictional coefficient, f_0 is the calculated frictional coefficient of a sphere having the same volume as the particle, M is the molecular mass of the protein determined from sedimentation-equilibrium analysis, s is the apparent corrected sedimentation coefficient, ν is the partial specific volume, ρ is the density of the buffer solution, η is the

viscosity of solvent and N is Avogadro's constant. The additional term, $2.8 \text{ \AA}$ ($= 0.28 \text{ nm}$), in eqn (2) refers to the width of a monolayer of water of hydration around the protein. Values of molecular mass used were 27258 and 40023 Da for Dsg2(1-2) and Dsc2(1-2) respectively. The density of BIAcore buffer at 20°C is 1.005 g/ml , whereas the viscosity at this temperature is $0.001 \text{ Pa} \cdot \text{s}$ (0.01 P). The respective partial specific volumes of Dsg2(1-2) and Dsc2(1-2) calculated from their amino acid sequences were 0.739 and 0.729 ml/g . An estimation of particle asymmetry can be made by modelling the protein molecules as prolate ellipsoids of revolution. The axial ratio, a/b , can then be derived from a plot of f/f_0 versus a/b [40].

Chemical cross-linking studies

Dsc2(1-2) and Dsg2(1-2)-His ($5 \mu\text{M}$) were incubated with $10 \mu\text{M}$ of the cross-linker bis(sulphosuccinimidyl) suberate (BS^3) in the presence of either 5 mM CaCl_2 or 10 mM EDTA at 4°C for 90 min in a total reaction volume of $20 \mu\text{l}$. The reaction was stopped by addition of 50 mM (final concn.) Tris/HCl, pH 7.5. BSA and carbonic anhydrase at equivalent concentrations were also treated in an identical manner to act as positive and negative controls respectively. Cadherin samples were then analysed by Western blotting using a polyclonal anti-Dsc2 antibody and a monoclonal antibody against the hexahistidine tag of Dsg2(1-2)-His according to the procedure described above, whereas control samples were detected by SDS/PAGE and Coomassie Brilliant Blue staining.

Far-UV CD and fluorescence-emission spectroscopy

Far-UV CD spectra ($200\text{--}260 \text{ nm}$) in the presence of 5 mM CaCl_2 or 10 mM EGTA of a $36 \mu\text{M}$ solution of Dsg2(1-2) or a $8.6 \mu\text{M}$ solution of Dsc2(1-2) were recorded on a Jasco J-715 spectropolarimeter using a 2-mm -path-length quartz cuvette and an averaging of ten scans per spectrum. A scan rate of 100 nm/min , a time constant of 0.25 s and a bandwidth of 2 nm were used to minimize errors in CD measurements. To determine the dissociation constant, K_D , for Ca^{2+} binding, $2 \mu\text{l}$ additions of a buffered solution of $0.1\text{--}0.5 \text{ M}$ CaCl_2 to the Ca^{2+} -free protein solution were made and the averaged spectrum was recorded after each addition. The mid-point of inflection of a plot of change in CD signal at a particular wavelength versus Ca^{2+} concentration is a measure of the K_D for Ca^{2+} binding. Fluorescence-emission spectra of a $1 \mu\text{M}$ protein solution in the presence of either 5 mM CaCl_2 or 10 mM EDTA were measured on a SPEX FluoroMax fluorimeter (Jobin Yvon Horiba, Edison, NJ, U.S.A.) with an excitation wavelength of 280 nm using a 1-cm -path-length quartz cuvette. Ten scans per spectrum were recorded. All spectra were subjected to baseline subtraction, correction for dilution and smoothing. The temperature throughout all measurements was maintained at 25°C .

Fluorescence spectroscopy of urea-induced protein unfolding

A $1 \mu\text{M}$ solution of either protein solution containing 5 mM CaCl_2 prepared in buffer alone was equilibrated at 20°C and then titrated by addition of aliquots of freshly prepared $1 \mu\text{M}$ protein in buffered 10 M urea also containing 5 mM CaCl_2 . After each addition, a fluorescence emission spectrum was recorded (ten scans) as described above over the wavelength range $290\text{--}450 \text{ nm}$, with an excitation wavelength of 280 nm . The first derivative of each smoothed spectrum was used to accurately determine the emission maximum.

Studies of protein-protein interactions using BIAcore

Kinetic analysis of molecular interactions was performed with a BIAcore device, which uses surface plasmon resonance. Immobilization of Dsc2(1-2) ($5.2 \mu\text{M}$), Dsg2(1-2) ($4.1 \mu\text{M}$) and BSA ($1 \mu\text{M}$) to the surface of the sensor chip CM5 was carried out in sodium acetate buffer, pH 3.92, using amine coupling chemistry. Proteins were first buffer-exchanged on a PD-10 column into HBS-P BIAcore buffer [10 mM Hepes (pH 7.4)/ 150 mM NaCl/ 0.005% surfactant P20]. The sensor chip was equilibrated with this buffer throughout. Injections of protein ($50 \mu\text{l}$) were carried out and the signal was monitored for a period of about 10 min . Both on- and off-rate constants for the binding of Dsg2(1-2) were determined using the BIAevaluation software version 2.1. The dissociation phase of the sensorgrams could be readily fitted to a simple $A + B = AB$ model, whereas the association could only be analysed by using a two-independent-binding-sites model.

RESULTS

Expression, purification and characterization of Dsc2(1-2) and Dsg2(1-2)

The absence of a linker region between the restriction-protease-FXa cleavage site and the N-terminus of the cadherin leads to variability in the accessibility of this site in the case of the two recombinant fusion proteins. Cleavage of Dsg2(1-2) fusion protein is very efficient both in solution (Figure 1A) and on a GSH-Sepharose column (Figure 1B, lanes 3–6), yielding a band of approximate molecular mass 30 kDa . However, significant cleavage of the Dsc2(1-2) fusion protein could only be effected by performing the cleavage reaction for longer periods and using much larger amounts of protease (results not shown). As optimal cleavage of Dsc2(1-2) fusion protein occurred in solution, a second affinity purification on GSH-Sepharose was carried out to remove cleaved GST and a considerable level of uncleaved fusion protein. For this purpose, extensive dialysis against high-ionic-strength buffer (1 M NaCl) is essential to displace all the tightly bound GSH to facilitate complete subsequent removal of cleaved GST on the affinity column. Cleaved Dsc2(1-2) could be purified by ion-exchange chromatography on a Mono Q column (Figure 1D), with most of the fractions for Dsc2(1-2) being eluted in fractions 7–24 (Figure 1E), while free GST and FXa protease are eluted with fractions 5 and 6 (Figures 1D and 1E). Quite often an aggregate with a molecular mass of 81 kDa was seen (Figure 1E), which could be readily removed by size-exclusion chromatography on a Sephadex G-75 column (results not shown).

For some experiments, Dsg2(1-2)-His fusion protein was expressed. As with Dsg2(1-2) lacking the tag, efficient on-column cleavage of the protein could be achieved. On some occasions, high- and low-molecular-mass impurities were also eluted with the cleaved Dsg2(1-2)-His. These were effectively removed by metal-chelate chromatography on an activated Ni-NTA-agarose column (Figure 1C). The cleaved protein bound with high affinity in buffer containing 150 mM NaCl and a low concentration of imidazole (20 mM) to reduce non-specific binding and was eluted by 250 mM imidazole (Figure 1C, lane 4).

Both Dsc2(1-2) and Dsg2(1-2) were subjected to N-terminal sequence analysis. In both cases, the correct N-terminus was found without any extraneous residues. MS analysis was performed to rule out any post-translational modifications of the proteins by comparing the molecular mass calculated from the published protein sequence (DNA Star program) with the experimentally determined value. For both recombinant proteins,

Figure 1 Expression and purification of recombinant desmosomal cadherin domains

The details of cloning, expression and purification of cadherin fragments are given in the Materials and methods section. **(A)** Cleavage of Dsg2(1-2) with FXa in solution; lane 1, uncleaved fusion protein; lane 2, cleaved fusion protein. **(B)** On-column cleavage of Dsg2(1-2) with FXa; lane 1, uncleaved fusion protein; lane 2, eluate after binding of fusion protein to the affinity column; lanes 3–7, cleaved Dsg2(1-2) fractions after circulating FXa through the column; lane 8, elution of uncleaved fusion protein and cleaved GST. **(C)** Further purification of cleaved Dsg2(1-2)-His by metal-chelate chromatography on a Ni-NTA-agarose column; lane 1, partially purified cleaved Dsg2(1-2)-His before metal-chelate chromatography; lane 2, unbound fusion protein and cleaved Dsg2(1-2)-His; lane 3, eluate after washing the column with equilibration buffer; lane 4, elution of bound Dsg2(1-2)-His with buffer containing 250 mM imidazole. **(D)** Purification of FXa-cleaved Dsc2(1-2) by ion-exchange chromatography on a Mono Q column; fractions 17–24 contain Dsc2(1-2), whereas fractions 5 and 6 contain a GST contaminant which had not been removed by affinity chromatography **(E)**. Values on the left show molecular masses of markers in kDa.

Table 1 Determination of the molecular masses of Dsc2(1-2) by sedimentation-equilibrium analysis in the presence and absence of Ca^{2+}

Experimental details for the equilibrium run are identical with those given for Figure 2 and are described fully in the Materials and methods section. The values for molecular mass refer to fits for individual concentrations of the protein.

[Dsc2(1-2)] (μM)	Molecular mass (Da)	
	Dsc2(1-2) + Ca^{2+}	Dsc2(1-2) + EDTA
4.2	31 957	34 756
12.5	33 909	58 280
20.8	69 857	68 332

both sets of molecular masses showed agreement to within a few daltons. In order to accurately determine the protein concentration, the method of Pace et al. [39] was used to calculate a molar absorption coefficient for each protein. This yielded values

of $24000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ and $17400 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for Dsc2(1-2) and Dsg2(1-2) respectively.

Oligomeric state of desmosomal cadherin domains as studied by analytical ultracentrifugation

The oligomeric nature of both type 2 desmosomal cadherin fragments was investigated by sedimentation-equilibrium analysis. Relative molecular masses were derived from fitting absorption profiles to individual concentrations in the presence of 5 mM Ca^{2+} and 10 mM EDTA (Table 1). The K_D for the monomer $\leftrightarrow$ dimer equilibrium in the presence of 5 mM Ca^{2+} was obtained by performing a global fit of all concentrations and speeds by fitting to a model with two species in equilibrium with each other while keeping the molecular mass of the protein fixed for the monomer (25 kDa) (Figure 2). The relatively small residuals illustrated in Figure 2 and the absence of a systematic variation in their values are indicative of the validity of the fits. The global fit of the data resulted in a K_D of $4.2 \mu\text{M}$

Figure 2 Homophilic interactions of Dsc2(1-2) as studied by sedimentation-equilibrium analysis

The experimental details of sedimentation-equilibrium runs of Dsc2(1-2) are described in the Materials and methods section. A global fit to different concentrations of Dsc2(1-2) in the presence of Ca^{2+} at the three different speeds assuming a monomer $\leftrightarrow$ dimer equilibrium is shown.

Table 2 Apparent sedimentation coefficients (*s*) at 20 °C of Dsc2(1-2) and Dsg2(1-2) in the presence of 5 mM CaCl_2 and 10 mM EGTA

	Sedimentation coefficient (<i>s</i>) (S)			
	Transport method		Svedberg method	
	+ Ca^{2+}	+ EGTA	+ Ca^{2+}	+ EGTA
Dsc2(1-2)	3.76	—	4.24 ± 0.013	4.41 ± 0.014
Dsg2(1-2)	2.07	—	2.28 ± 0.01	2.33 ± 0.006

for Dsc2(1-2) in the presence of Ca^{2+} , but negligible association of Dsg2(1-2), even at a concentration of 64 μM . Values of apparent molecular mass for Dsg2(1-2) in the presence and absence of EDTA as a result of the global fit were 25.71 kDa and 27.25 kDa respectively. These values did not change significantly on application of fits to individual concentrations. The data clearly demonstrate that, whereas Dsg2(1-2) does not show any evidence of self-association, Dsc2(1-2) undergoes homophilic association either to dimers, as indicated by the K_D derived from the global fit, or to even higher order oligomers, possibly tetramers, as shown by fits to individual concentrations (Table 1). However, the data also show that the self-association of Dsc2(1-2) occurs to a large extent in a Ca^{2+} -independent fashion, albeit with a higher affinity of association in the presence of EDTA.

Apparent sedimentation coefficients of Dsc2(1-2) and Dsg2(1-2) in the presence of either 5 mM CaCl_2 or 10 mM EGTA, listed in Table 2, were derived by analysis of sedimentation-velocity data by using the transport method ORIGIN (Beckman Optima XL-A/XL-1 data analysis software, version 4.0) or SVEDBERG [43] analysis. Although these two methods give somewhat different *s* values, meaningful comparisons can still be made between values derived from the same method of analysis. For example, the transport method yields a higher value of

Figure 3 Evidence for homophilic and heterophilic interactions for Dsc2(1-2) and Dsg2(1-2)-His by chemical cross-linking in the presence and absence of Ca^{2+}

(A) Dsc2(1-2) (5 μM) detected by an anti-Dsc2 polyclonal antibody. Lane 1, no cross-linker plus Ca^{2+} ; lane 2, BS^3 plus Ca^{2+} ; lane 3, no BS^3 plus EDTA; lane 4, BS^3 plus EDTA; lanes 5–8, same as for lanes 1–4, but containing 5 μM each of Dsc2(1-2) and Dsg2(1-2)-His. (B) Cross-linking of 5 μM Dsg2(1-2)-His as detected by anti-His monoclonal antibody. The description of lanes is the same as for Dsc2(1-2). (C) BSA and carbonic anhydrase (5 μM of each) used as positive and negative controls respectively. The descriptions for lanes 1–4 and 5–8 are identical with those given for lanes 1–4 in (A).

3.76 S for Dsc2(1-2) as compared with a value of 2.07 S for Dsg2(1-2) in the presence of Ca^{2+} . Similarly, a higher value of 4.24 S for Dsc2(1-2) is obtained, in contrast with a value of 2.28 S for Dsg2(1-2) under identical conditions using the Svedberg analysis. Table 2 also shows that the apparent *s* values for both cadherin fragments do not significantly change in the absence of Ca^{2+} . These observations are consistent with Ca^{2+} -independent oligomerization of Dsc2(1-2) and the lack of it in the case of Dsg2(1-2) under the specified conditions. The respective shape factors for Dsc2(1-2) and Dsg2(1-2) derived by incorporation of *s* values obtained using the transport method into eqns (1) and (2) are 0.99 and 1.31, which lead to *a/b* values of 1.0 and 6.0 respectively. Using *s* values obtained from Svedberg analysis, shape factors for Dsc2(1-2) and Dsg2(1-2) are 0.858 and 1.176 respectively, which correspond to *a/b* ratios of 1.0 and 4.0. Despite the different axial ratios for Dsg2(1-2) derived from the two types of analyses, it can still be concluded that the likely shape of the Dsc2(1-2) fragment is symmetrical, while Dsg2(1-2) probably exists in an extended rod-like configuration. Furthermore, the axial ratio for Dsc2(1-2) is in agreement with the expected ratio of 1.5 for the *cis*-dimer of Ecad1-2 that can be derived from the reported crystal structure of this fragment [29]. Our data therefore support the conclusion that Dsc2(1-2) exists in the form of *cis*-dimers in solution as opposed to *trans*-dimers.

Evidence of homophilic and heterophilic interactions from chemical cross-linking

Chemical cross-linking, on the other hand, lends support to a Ca^{2+} -dependent component to the homophilic association of Dsc2(1-2). Lanes 1–4 in Figure 3(A) show that almost all of the Dsc2(1-2) undergoes complete cross-linking, indicating

Figure 4 Determination of the dissociation constant for binding of Ca^{2+} to Dsc2(1-2) and Dsg2(1-2) by monitoring changes in the far-UV CD spectrum

Solutions of Dsc2(1-2) (8.5 μM) or Dsg2(1-2) (36 μM) equilibrated at 20 $^{\circ}\text{C}$ were titrated with different concentrations of CaCl_2 . **(A)** CD spectra of Dsc2(1-2) at different concentrations of CaCl_2 (0–10 mM). The inset shows a plot of molar CD absorption coefficient, $\Delta\epsilon_M$, at 220 nm against Ca^{2+} concentration. The dissociation constant, K_D , equals the concentration of Ca^{2+} at which half of the protein is saturated. **(B)** CD spectra of Dsg2(1-2) with 5 mM CaCl_2 (curve 3), 5 mM CaCl_2 plus 10 mM EGTA (curve 2), no CaCl_2 (curve 1). The inset shows a plot of $\Delta\epsilon_M$ values at 210 nm versus Ca^{2+} concentration.

Figure 5 Ca^{2+} binding to Dsc2(1-2) and a comparison of the thermodynamic stability of Dsc2(1-2) and Dsg2(1-2) by fluorescence spectroscopy

(A) Fluorescence-emission spectrum of 1 μM Dsc2(1-2) in the presence of 5 mM CaCl_2 (broken line) or 5 mM CaCl_2 plus 10 mM EDTA (continuous line). **(B)** Plots of fluorescence emission maxima of Dsc2(1-2) (■) and Dsg2(1-2) (<>) in the presence of 5 mM CaCl_2 at different concentrations of urea.

dimerization in the presence of Ca^{2+} (lane 2), which is dramatically reduced in the presence of 10 mM EDTA (lane 4). The observed Ca^{2+} -dependence could alternatively be explained by different extents of exposure or proximity of the cross-linked side chains due to Ca^{2+} -dependent conformational changes. Furthermore, the inclusion of equimolar Dsg2(1-2)-His in the mixture led to significant inhibition of cross-linking of Dsc2(1-2) in the presence of Ca^{2+} (Figure 3A, lane 6), suggesting the formation of a heterophilic complex between Dsc2(1-2) and Dsg2(1-2). In

contrast with the sedimentation data, Dsg2(1-2) also undergoes cross-linking, but this occurs in a Ca^{2+} -independent manner and to a much lesser extent than Dsc2(1-2) (Figure 3B, lanes 1–4), suggesting a low-affinity Dsg2(1-2) homophilic interaction. Like Dsc2(1-2), this is abolished to a considerable extent by the presence of Dsc2(1-2) in the mixture (Figure 3B, lane 6). BSA, which acted as a positive control (Figure 3C), showed the formation of dimers and tetramers, as indicated by arrows in lanes 1–4. Carbonic anhydrase, which was included as a negative

control for cross-linking, did not show any evidence of oligomers (Figure 3C, lanes 5–8). Taken together, these data strongly suggest the specific formation of oligomers of Dsc2(1-2) and Dsg2(1-2) and that heterophilic interactions are preferred to homophilic ones.

Direct evidence of Ca^{2+} binding to desmosomal cadherins by CD and fluorescence spectroscopy

Owing to conflicting data on the Ca^{2+} -dependence of the homophilic association of Dsc2(1-2), we carried out direct determination of Ca^{2+} binding by CD and fluorescence spectroscopy. The far-UV CD spectra for Dsc2(1-2) and Dsg2(1-2) (Figures 4A and 4B respectively) are very similar in shape and consistent with the presence of a considerable amount of β -sheet. The absence of a significant shoulder at 222 nm in the case of Dsg2(1-2) indicates the relatively small contribution of α -helical secondary-structural elements to the spectrum. However, some contribution from α -helical structure is observed at 222 nm for Dsc2(1-2) and accounts for approx. 15% of the residues. Furthermore, a significant decrease in negative ellipticity is observed on addition of 5 mM Ca^{2+} in the case of both cadherin fragments. No changes in the near-UV spectra were observed due to Ca^{2+} addition (results not shown). Spectral changes were used to estimate the dissociation constants for the binding of Ca^{2+} (insets to Figures 4A and 4B), giving K_D values of ≈ 1 mM for Dsc2(1-2) and ≈ 5 mM for Dsg2(1-2) (Figure 4B) respectively.

Evidence for Ca^{2+} binding was also provided by the occurrence of a spectral shift from 330 to 337 nm in the fluorescence-emission maximum of Dsc2(1-2) upon Ca^{2+} addition (Figure 5A), suggesting that the single conserved tryptophan residue (the second residue in the mature protein sequence) becomes more accessible to the solvent, owing to a Ca^{2+} -induced conformational change. A similar shift was not observed for Dsg2(1-2) (results not shown). Furthermore, the observation of emission maxima near 330 nm in the presence of Ca^{2+} for both cadherins (Figure 5B) suggests a buried environment for the tryptophan residue.

It was observed that certain purified preparations of Dsg2(1-2), but not Dsc2(1-2), were significantly more susceptible to undergoing slow degradation in the presence of 10 mM EDTA than in the presence of 5 mM CaCl_2 (results not shown). Studies on classical cadherins have shown that binding of Ca^{2+} leads to an extended rigid structure and an increased resistance to proteolytic attack [29,41]. In the light of this, the relative thermodynamic stabilities of Dsc2(1-2) and Dsg2(1-2) in the presence of Ca^{2+} were investigated by following the shift in fluorescence-emission maximum to monitor urea-induced unfolding of both proteins (Figure 5B). Only Dsg2(1-2) undergoes complete unfolding in the presence of 5 M urea, as shown by the shift in emission maximum from 330 to 350 nm. Dsc2(1-2) is only partially unfolded, even in 5.5 M urea. This suggests a more stable and compact conformation, which is consistent both with the relatively greater resistance to proteolysis and the oligomerization displayed by this cadherin fragment.

Study of protein–protein interactions by use of surface plasmon resonance (BIAcore)

The technique of surface plasmon resonance using BIAcore was applied to provide evidence for Ca^{2+} -dependent heterophilic interactions between the two desmosomal cadherins (Figure 6). Both the ultracentrifuge and cross-linking data demonstrate that the final concentration of the protein used for coupling would be an important factor in determining whether the proteins undergo immobilization as monomers or homodimers. The concentration

Figure 6 Surface plasmon resonance (BIAcore) analysis of heterophilic interactions between Dsc2(1-2) and Dsg2(1-2)

Both the on- and off-rate constants for the binding of Dsg2(1-2) to immobilized Dsc2(1-2) were determined using BIAevaluation software version 2.1. (A) Binding curves for 6.8 μM (■), 13.7 μM (□) and 27.4 μM (◆) Dsg2(1-2) in the presence of 5 mM CaCl_2 , 27.4 μM Dsg2(1-2) in the presence of 10 mM EGTA (●) and 27.4 μM Dsg2(1-2) passed over immobilized Dsg2(1-2) (○). (B) A typical fit to the binding curve of Dsg2(1-2) to Dsc2(1-2) to obtain the 'on-rate constant' by use of a two-site model. Data for 13.7 μM Dsg2(1-2) are shown. The fit leads to superposition of the observed association curve and the fitted curve. Abbreviation: RU, response units.

of Dsc2(1-2) during coupling was 5.2 μM, which is slightly higher than the K_D value of 4.2 μM as determined by sedimentation-equilibrium analysis. Assuming that the pH of the coupling buffer does not substantially alter this equilibrium, then it is probable that significant amounts of homodimers of Dsc2(1-2) were immobilized. As shown in Figure 6(A), the initial binding of Dsg2(1-2) to immobilized Dsc2(1-2) is a function of protein concentration. The dissociation phase of the sensograms could be readily fitted to a simple $A + B \rightleftharpoons AB$ first-order kinetic model, whereas the association could only be analysed by using a model involving two independent binding sites. A typical fit to the binding curve is also shown (Figure 6B). The binding is completely abolished by inclusion of 10 mM EGTA in the injection (Figure 6A). Dsg2(1-2), on the other hand, shows very little binding to immobilized Dsg2(1-2) (Figure 6A). These data are fully consistent with inhibition of cross-linking of either Dsc2(1-2) or Dsg2(1-2) and the lack of Dsg2(1-2) self-association

in the ultracentrifuge. The data in Figure 6(A) yielded a K_D of 23.4 μM [an association constant, K_A , of $4.28 \times 10^4 \text{ M}^{-1}$ with rate constants, k_{on} and k_{off} , of $1240 \pm 89 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $0.029 \pm 1.37 \times 10^{-3} \text{ s}^{-1}$ (means $\pm$ S.E.M.) respectively], which strongly supports the presence of weak heterophilic interactions. Analysis of the dependence of these interactions on Ca^{2+} concentration was carried out by monitoring binding of Dsg2(1-2) to immobilized Dsc2(1-2) in the presence of various concentrations of Ca^{2+} (results not shown). An independent estimate of a combined K_D for simultaneous Ca^{2+} binding to Dsg2(1-2) and immobilized Dsc2(1-2) can be obtained by a plot of response signal at a particular time versus Ca^{2+} concentration using data which are normalised at zero time (results not shown). The estimated value of 4 mM is close to 4.25 mM, the value estimated from CD titrations (Figure 4).

DISCUSSION

We provide here the first evidence of Ca^{2+} binding to desmosomal cadherins. Data from sedimentation-velocity, sedimentation-equilibrium analysis, cross-linking and BIAcore studies suggest that Dsc2(1-2), in addition to participating in homophilic interactions, also undergoes heterophilic interactions. Dsg2(1-2), on the other hand, only undergoes heterophilic association. The sedimentation-coefficient values derived for both cadherin fragments in the presence of Ca^{2+} were used to obtain information on the shape of these molecules. The calculated axial ratios clearly show that Dsc2(1-2), which has an a/b ratio of close to 1.0, is symmetrical in shape, whereas Dsg2(1-2), which gives an a/b ratio of between 4.0 and 6.0, has an extended rod-like configuration. The agreement obtained between the axial ratio observed for Dsc2(1-2) and that estimated for the parallel dimer of Ecad1-2 [29] suggests that the desmosomal cadherin domains also undergo *cis*-interactions in solution. Our results further demonstrate that heterophilic interactions are Ca^{2+} -dependent, whereas Ca^{2+} -dependence of homophilic association appears to be less clear, as reflected in conflicting data obtained from cross-linking and analytical-ultracentrifugation studies. Binding of Ca^{2+} , which leads to changes in the secondary and tertiary structure of the cadherin fragment, could influence the accessibility of the relevant lysine residues. As a consequence, heterodimers of Dsc2(1-2) and Dsg2(1-2) may be formed in solution, but cannot be observed by cross-linking with the particular chemical reagent. The use of cross-linkers of different chain length could increase the likelihood of directly observing formation of heterodimers. Similarly, formation of homodimers of Dsc2(1-2) occurs in solution in the absence of Ca^{2+} , as shown by sedimentation-equilibrium analysis, but is not observed by cross-linking, perhaps due to the lack of close proximity of appropriate amino acid residues. Furthermore, the observation of homophilic interactions only in the case of Dsc2(1-2) is consistent with the urea-denaturation profiles (Figure 5B), which show that Dsc2(1-2) does not fully unfold, as compared with Dsg2(1-2), suggesting that the former has a more compact and thermodynamically stable structure.

Our results, while supporting the findings of other workers which suggest the formation of lateral heterodimers and homodimers [30,34], also provide direct quantitative information on the strength of these interactions. The affinity of protein-protein interactions, as indicated by the determined K_D values of 23.4 μM (BIAcore) for heterodimerization and 4.2 μM for homodimerization of Dsc2(1-2) (sedimentation equilibrium), are much higher than those observed for classical cadherins. For example, in the case of recombinant Ecad1-2, K_D values of 80 μM and 170 μM were obtained using equilibrium dialysis [21] and sedi-

mentation-equilibrium analysis [24] respectively. However, the affinities for both of the above cadherin families are similar to those reported for other cell-adhesion molecules (CAMs) such as the interactions of CD2-CD58 [42]. In addition, the cross-linking data suggest that the affinity of interactions for the formation of a heterodimeric complex in solution is probably greater than the above K_D value would indicate. Nevertheless, the relatively weak interactions of desmosomal cadherins can largely be attributed to a low k_{on} of $1240 \text{ M}^{-1} \cdot \text{s}^{-1}$ and a k_{off} of 0.029 s^{-1} , which is relatively high compared with the dissociation of antibody OX34 from its complex with CD2 ($2 \times 10^{-4} \text{ s}^{-1}$) or the dissociation of interleukin-1 from its receptor ($\approx 10^{-4} \text{ s}^{-1}$) [42]. Although the transient nature of these interactions is typical of CAMs at the monomeric level [42], the mechanism of cell-cell adhesion is eventually a highly co-operative and multimeric process involving a large number of interactions between CAMs of opposing cells, thus preventing the cells from spontaneously dissociating from each other.

Of particular interest is the type of analysis carried out for the binding of Dsg2(1-2) to immobilized Dsc2(1-2). The k_{on} could only be analysed by using a two-independent-binding sites model ($A + B1 + B2 = AB1 + AB2$) as opposed to a single-site model ($A + B = AB$). In this case, two independent rate constants representing the initial fast phase and a second slower phase are obtained. The K_D value of 23.4 μM has been calculated only for the initial binding of Dsg2(1-2). This analysis is consistent with the formation of heterodimers with *cis* and *trans* interactions, although the disparity between the two rate constants and the observation of a single k_{off} for the dissociation phase would suggest that only one of the two forms represents stable interactions. However, the presence of two different conformations of either of the two types of heterodimers cannot be ruled out on the basis of these observations. Studies involving transfection of L cells show that, although weak heterophilic interactions occur when Dsc and Dsg are co-expressed, these are significantly enhanced when both cadherins are co-expressed with plakoglobin [30]. This provides support to the generally held view that cytoplasmic plaque proteins strengthen adhesive interactions by binding to the cytoplasmic cadherin domains either through an inside-out signalling mechanism, which increases adhesive capacity of the extracellular domains, or by causing clustering of cadherins within the membrane, leading to an overall increase in adhesion. Thus our data do not preclude the importance of extracellular domains 3–5 in the adhesion mechanism.

Considerable evidence has accumulated for lateral homo-association of classical cadherins. However, unlike desmosomal cadherins, heterophilic interactions are not observed. Studies on Ca^{2+} binding to Ecad1-2 [21] give estimates for K_D using a three-site model as two similar sites with K_D of 330 μM and a third site with a much higher K_D of 2 mM. Electron-microscopic studies of recombinant Ecad-COMP at different Ca^{2+} concentrations [26] have shown that low Ca^{2+} concentrations ($< 50 \mu\text{M}$) stabilized the rod-like structure of E-cadherin, whilst *cis*-dimers formed at low-medium concentrations of Ca^{2+} (0.5–1 mM). Only at concentrations greater than 2 mM did two *cis*-dimers form a *trans*-interaction. Our estimates of K_D for Ca^{2+} binding to Dsc2(1-2) and Dsg2(1-2) (0.97 mM and 4.25 mM respectively) are in agreement with these values, although only the averaged K_D could be obtained. The lower K_D for Dsc2(1-2) suggests a higher affinity of Ca^{2+} binding to Dsc2(1-2) homodimers as opposed to Dsg2(1-2) monomers. Crystal structures of the first two extracellular domains of E and N-cadherin [26–29] reveal the presence of two seven-stranded β -barrel domains for each molecule of the homodimer; interestingly, no α -helix was observed. The structure

of Ecad1-2 also revealed how three Ca^{2+} ions complexed at the interface of the two consecutive domains stabilize the otherwise flexible hinge region between the domains and provide rigidity to the whole extracellular portion. The observed changes in secondary structure upon Ca^{2+} binding to desmosomal cadherins (CD and fluorescence) are consistent with these observations. This is not surprising, since a comparison of the amino acid sequences of the two N-terminal domains of Ecad1-2, Ncad1-2, Dsc2(1-2) and Dsg2(1-2) shows that the residues involved in the Ca^{2+} -binding motif are conserved in both the classical and desmosomal cadherins (results not shown). Although the CD spectra of the latter reported here are consistent with a structure containing large contributions from β -sheets, a noticeable shoulder at 220 nm is present in the spectrum for Dsc2(1-2), which suggests that approx. 15% of the residues are in an α -helical conformation, which is largely absent from the Dsg2(1-2) spectrum. The spectra as a whole are also very similar in shape and intensity to those originally reported for E-cadherin [21]. This suggests that the three-dimensional structure of desmosomal cadherins may be similar to those of classical cadherins and that it is quite likely that our strategy for expression and purification of recombinant desmosomal cadherins reported here leads to a correctly folded native conformation. However, the structure of Dsc2(1-2) containing a larger contribution from α -helical secondary-structural elements might also show some differences from the structures of Dsg2(1-2) and classical cadherins. It is plausible that the observed differences between the two desmosomal cadherins with regard to affinity of Ca^{2+} binding and self-association of Dsc2(1-2) could be the result of these structural differences.

The significance of a Ca^{2+} -independent *cis*-dimerization of Dsc2(1-2) reported here for the *in vivo* function of this molecule in the adhesion mechanism has recently been suggested by studies of human keratinocytes cultured in low Ca^{2+} (I. A. King, F. Henkler and I. Burdett, personal communication). Using immunofluorescent staining it was shown that Dsc2 expressed at the cell surface was initially present in novel punctate structures consisting of a single desmosomal cadherin that formed independently of the cytoskeleton and did not co-localize with E-cadherin, Dsg or desmoplakin. The expression of Dsc1 and Dsc3 was negligible under these conditions. These were not desmosomes and were not involved in cell-cell adhesion. It was concluded that these might represent early pre-desmosomal structures consisting of lateral aggregates of Dsc2 that might act as nucleation centres for the subsequent assembly of functional desmosomes. Although in our studies *cis*-dimers of Dsg2(1-2) were not observed, it is likely that the full-length molecules with the participation of cytoplasmic plaque proteins would be able to form *cis*-interactions within the cell membrane at very high concentrations of these molecules. Our findings, taken together, support the view that the *in vivo* function of both cadherins involves their participation in both *cis* and *trans* interactions in the cell-adhesion mechanism.

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