The mechanism of cell adhesion by classical cadherins: the role of domain 1

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Summary

The mechanism by which classical cadherins mediate cell adhesion and, in particular, the roles played by calcium and Trp2, the second amino acid in the N-terminal domain, have long been controversial. We have used antibodies to investigate the respective contributions of Trp2 and calcium to the stability of the N-terminal domain of N-cadherin. Using a peptide antibody to the βB strand in domain 1, which detects a disordered structure, we show that both Trp2 and calcium play crucial parts in regulating stability of the domain. The epitope for another antibody, mAb GC4, has been mapped to the base of domain 1. Binding of GC4 to this epitope was shown to depend on intramolecular 'docking' of Trp2 into the domain 1 structure. Using this property, we provide evidence that calcium regulates a dynamic equilibrium between docked and undocked Trp2. Finally, a novel technique has been developed to test whether Trp2 cross-intercalation between cadherin molecules from adjacent cells (strand exchange) is central to cadherin-mediated cell adhesion. Guided by crystal structures showing strand exchange, we have introduced single cysteine point mutations into N-cadherin domain 1 in such a way that a disulphide bond will form between opposing N-cadherin molecules during cell adhesion if strand exchange occurs. The bond requires complementary cysteines to be precisely juxtaposed according to the strand exchange model. Our results demonstrate that the disulphide bond forms as predicted. This provides compelling evidence that strand exchange is indeed a primary event in cell adhesion by classical cadherins.

Key words: Cadherin, Adhesion, Strand exchange

Introduction

Classical cadherins are a family of cell surface adhesion molecules that are essential for maintaining the structural integrity of all vertebrate solid tissues. Cadherins determine cell-cell recognition during morphogenesis and have signalling functions that influence cell migration and differentiation (Cavallaro and Christofori, 2004; Hirano et al., 2003; Thiery, 2003; Wheelock and Johnson, 2003). Adhesive interactions by cadherins are mostly, but not exclusively, homophilic and cadherin type-specific. Classical cadherins comprise five extracellular β-barrel-like domains, a transmembrane domain and a cytoplasmic domain. Adhesion requires the presence of calcium bound in the interdomain junctions and it is known that this rigidifies the cadherin molecule into a curved rod-like structure projecting from the cell (Boggon et al., 2002; He et al., 2003; Miyaguchi, 2000; Pokutta et al., 1994). Despite more than a decade of research, the mechanism by which cadherin extracellular domains form adhesive contacts remains controversial.

Insights into the process of adhesion have come mainly from four experimental strategies: observations of the effects of point mutations or domain deletions on cell adhesion; co-immunoprecipitation of epitope-tagged cadherin molecules in adhesive complexes between cells; structural studies of cadherins by NMR or X-ray crystallography; and physical studies, including measurements of intermolecular forces between cadherin molecules and direct observation of cadherins by electron microscopy. Cumulatively, these techniques have led to several alternative models for adhesion.

Amino acids that coordinate calcium in the junction between the first and second domains, ECD1 and ECD2, have been shown to play an essential role in adhesion (Corps et al., 2001; Klingelhofer et al., 2002) and structural studies have suggested that calcium will instigate dimerization of the recombinant protein ECD1-ECD2 via contact surfaces in the domain junction and ECD1 (Haussinger et al., 2002; Pertz et al., 1999). This effect of calcium has been demonstrated by physical measurements and electron microscopy (Alattia et al., 1997). Scanning mutagenesis in the N-terminal domain (ECD1) has shown that tryptophan 2 (Trp2), the second amino acid of the mature cadherin molecule, and amino acids lining an adjacent hydrophobic pocket are also indispensable for adhesion (Kitagawa et al., 2000; Tamura et al., 1998). The importance of Trp2 has been confirmed by immunoprecipitation studies, which have demonstrated that this residue is required for the formation of both adhesive (trans) dimers and lateral (cis) dimers (Laur et al., 2002; Ozawa, 2002). A possible explanation for the significance of Trp2 has been provided by three X-ray crystallography studies which have revealed a mechanism for dimerization in which Trp2 in β strand A of ECD1 docks into a hydrophobic pocket in ECD1 of its neighbour, a mutual process which holds the two ECD1

protomers together (Boggon et al., 2002; Haussinger et al., 2004; Shapiro et al., 1995). In principle, this interaction (strand exchange) could mediate dimerization in either cis- or trans-alignment. A recent immunoprecipitation study that was designed to discriminate between strand exchange and a calcium-mediated mechanism for dimerization is consistent with the strand exchange model (Troyanovsky et al., 2003).

A different perspective has emerged from measurements of intermolecular forces between recombinant cadherin molecules. The data here suggest that contact surfaces on two or more cadherin domains are required for adhesion and that opposing cadherin molecules can engage in several alternative anti-parallel alignments (Chappuis-Flament et al., 2001; Sivasankar et al., 2001; Zhu et al., 2003). This idea is at variance with direct observation, by electron microscopy, of purified recombinant cadherin molecules and cadherins in junctional complexes. These images suggest that both cis- and trans-dimerization takes place exclusively via ECD1 (Ahrens et al., 2003; Ahrens et al., 2002; He et al., 2003; Pertz et al., 1999). A central issue in these conflicting models is whether Trp2 serves only to stabilise an adhesive contact surface in domain 1 or whether strand exchange is the primary event in adhesion.

In the present report we have used antibodies to detect conformational changes in ECD1 of N-cadherin, prepared as an Fc-fusion protein, to investigate the effect of Trp2 and Ca^{2+} on the stability of this domain. In addition, we have investigated the effect of calcium on the propensity of Trp2 to dock into a hydrophobic pocket in its own domain. Finally, we provide persuasive evidence for strand exchange as the primary event in adhesion. A novel strategy has been used involving the formation of a 'reporter' disulphide bond that captures mutant cadherin molecules in trans-alignment as cells undergo adhesion. This bond can form only if the molecules are orientated by strand exchange.

Materials and Methods

Antibodies to N-cadherin

A polyclonal sheep antiserum was prepared by standard methods against the synthetic peptide PQELVRIRSDRDK, which spans the βB strand of chicken N-cadherin. The peptide was conjugated to keyhole limpet haemocyanin for immunisation. Preliminary experiments established that this antibody did not react with wild-type N-cadherin-Fc in its native conformation but gave a strongly positive result with N-cadherin-Fc that had been partially denatured by direct adsorption to a plastic surface. The rat mAb NCD-2, specific for an epitope in the BC loop of domain 1 of chicken N-cadherin, was obtained from R & D Systems (code BTA6). Mouse mAb 8C11, specific for domain 4 of human N-cadherin, was a gift from M. J. Wheelock (Puch et al., 2001) and mouse mAb GC4 (also known as GB-9) was obtained from Sigma (code C2542). A rabbit pan anti-cadherin antiserum specific for a conserved sequence of 24 amino acids in the cytoplasmic domain (Sigma, code C3678) was used for immunoblotting.

Antibody binding tests

Antibody binding to N-cadherin-Fc fusion proteins was detected by enzyme-linked immunosorbent assay (ELISA) based on a previously described method (Corps et al., 2001). Briefly, assay plates were coated with varying levels of monomeric or dimeric N-cadherin Fc-fusion proteins via rabbit or goat anti-human Fc. Assay plates were then pre-equilibrated for 7 minutes with varying levels of calcium chloride added to calcium-free Hanks balanced salt solution (HBSS), containing 0.075% Tween 20. Antiserum K7 (1:75) or mAb GC4 (2 μg/ml) was then added in HBSS containing the appropriate level of calcium and incubated for 1 hour at room temperature. Antibody binding was detected with anti-sheep or anti-mouse HRP-labelled secondary antibody. Assays were conducted in duplicate or triplicate and results are presented as mean±s.e.m.

Design of cysteine point mutations

Predictions of disulphide bond formation were based on the strand exchange structure PDB 1NCI (Shapiro et al., 1995). It was viewed and manipulated using Swiss PDB Viewer (http://www.expasy.org/spdbv/). Two extra amino acids at the N-terminus of this structure were removed to give the correct sequence. Alternative pairs of mutations, D1C,R25C or D1C,D27C, were introduced so that either pair would form a disulphide bond between the two domain 1 protomers; numbering refers to the mature cadherin protein. Formation of the C1-C25 disulphide bond required torsion, within Ramachandran limits, of psi angles in the α-carbon backbone of the βA strand in the vicinity of Val3, while maintaining the side chain of Trp2 in an unchanged position. Formation of the C1-C27 disulphide bond required only rotation of the side chain of C1. Adjustments were also made to the side chains of C25 and C27. After energy minimisation, the β carbon atoms of the paired cysteines for both bonds were within 4Å, which is optimal for disulphide bond formation. There were no amino acid clashes in either case.

DNA construction and transfection

Full-length chicken N-cadherin cDNA in pcDNA3.1 was obtained from P. Doherty (King's College, London). The point mutations D1C, R25C and D27C were introduced using a QuikChange mutagenesis kit (Stratagene). Mutant and wild-type constructs were stably transfected into the human myeloid leukaemia cell line K562 by electroporation and selection in G418 (1 mg/ml G418 in DMEM + 10% FCS). Clonal cell lines were obtained by limiting dilution and were matched for equal expression of N-cadherin by cell surface immunofluorescent staining and flow cytometry. Chicken N-cadherin-Fc fusion protein linked by disulphide bonds at the Ig hinge region to form a dimer was prepared as follows: cDNA for the five extracellular domains of N-cadherin, coding up to the amino acid sequence GLGT, was isolated by PCR and cloned into the vector pIgSig (R&D Systems). This vector provides a signal sequence from CD33 and adds the CH2 and CH3 domains and the hinge region of human IgG1 heavy chain. The construct was modified to produce monomeric N-cadherin-Fc by introducing the mutations F405A and Y407A into the CH3 domain of Fc (Dall'Acqua et al., 1998), which prevent dimerization of this domain. The fidelity of all DNA constructs was verified by sequencing.

Soluble N-cadherin-Fc fusion protein was obtained by transient transfection of Cos7 cells as previously described (Corps et al., 2003). Wild-type monomeric N-cadherin-Fc and the double mutant W2G,D134A were checked for molecular size by gel filtration on a Superdex-200 PC3.2/30 column equilibrated with 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$ and were shown to be monodisperse and monomeric. Soluble N-cadherin-Fc was routinely quantified using an ELISA for Fc, standardised against purified cadherin-Fc fusion proteins.

Cadherin-mediated cell adhesion tests

96-well immunoassay plates (Costar) were coated overnight with affinity-purified goat anti-human Fc (Sigma, code I 2136) at 5 μg/ml in PBS, then blocked with 1% BSA for 2 hours at room temperature. Monomeric or dimeric N-cadherin-Fc fusion protein in Cos cell supernatants was added as described for the ELISA assay. DX3

cells, a human melanoma cell line that expresses N-cadherin, were dissociated with cell dissociation solution (Sigma, code C5789), resuspended in HBSS with 2% FCS and assessed for adhesion to wild-type or mutant N-cadherin-Fc as previously described (Corps et al., 2001). Microscopic examination established that the cells were present as a single cell suspension as they settled onto the plate. For assays conducted in reducing conditions, 10 mM DTT was present during the adhesion and washing steps. Determinations were conducted in triplicate and results are presented as mean±s.e.m.

Formation of reporter disulphide bonds during cell adhesion

Monomeric N-cadherin-Fc (1 μg/ml) bearing the three alternative cysteine point mutations, D1C, R25C or D27C, was immobilised on 96-well plates with goat anti-human IgG Fc as previously described for E-cadherin-Fc (Corps et al., 2001). Unbound cadherin was removed by washing with HBSS, 0.1% BSA, followed by HBSS alone. The plates were not blocked with additional protein. K562 transfectants expressing N-cadherin bearing complementary or non-complementary point mutations (6×10^4 cells in 100 μl HBSS containing 10 mM DTT) in single cell suspension, were added to the coated wells containing 100 μl HBSS. The final concentration of DTT was therefore 5 mM during the adhesion stage. The cells were allowed to settle for 10 minutes at room temperature before incubation at 37°C for 30 minutes to complete adhesion. Microscopic examination of the wells before washing showed that the cells were not clumped but remained as a carpet of single cells on the surface of the plate. Non-adherent cells were then removed by washing with HBSS without DTT to restore oxidising conditions (4-6 washes over 15-20 minutes). Adherent cells from a pool of four wells for each experimental condition were then solubilised in sample buffer for SDS-PAGE and analysed on NuPAGE Novex gradient gels, 3-8% or 4-12% (Invitrogen), under non-reducing or reducing conditions. Cellular cadherin was detected by immunoblotting using rabbit pan anti-cadherin antiserum specific for the cytoplasmic domain. Alternatively, N-cadherin-Fc fusion protein was detected with rabbit anti-human IgG, Fc-specific (Pierce, code 31142). The secondary antibody for both was peroxidase-conjugated AffinPure goat anti-rabbit IgG, $F(ab')_2$ fragment-specific (Jackson ImmunoResearch Labs, code 111-035-006).

In an alternative protocol, Dynabeads (Dynal) coupled to Protein A were coated with dimeric N-cadherin-Fc (1 μg/ml), bearing the mutation D27C, for 1 hour at room temperature in the presence of 0.1% Tween 20 and 4 mM EGTA (to prevent aggregation). The beads were then washed with HBSS. K562 transfectants expressing cell surface N-cadherin with the mutations D1C, R25C or D27C, were treated with 10 mM DTT for 15 minutes at 37°C, then washed and resuspended in HBSS without DTT. Cells (2.4×10^5) were mixed with 3 μl of beads coated with mutant N-cadherin-Fc in a final volume of 100 μl HBSS. Cells and beads were incubated together at room temperature for 2 hours with slow rotation to allow adhesion. Approximately five beads became attached to each cell and there was some clumping of attached and unattached beads. 2 μl iodoacetamide (1.0 M), was then added to alkylate free sulphydryl groups. The samples were then spun down at 1500 ***g*** and the cells were lysed in HBSS, 0.075% SDS, 1% NP40, 0.2 mM AEBSF, for 4 minutes on ice. Beads were then isolated with a magnet and washed twice with lysis buffer. Disulphide-bonded complexes between cellular cadherin and the Fc-fusion protein were analysed by SDS-PAGE and immunoblotting for cadherin cytoplasmic domain as described above. To ensure equal loading, membranes were stripped and re-assayed with anti-Human Fc.

Atomic force imaging

N-cadherin-Fc was purified using Protein A Sepharose as previously described (Corps et al., 2001). Samples were centrifuged at 100,000 ***g*** for 45 minutes to remove any aggregates and diluted to 1 μg/ml in 5 mM HEPES, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.5, supplemented with 5 mM $NiCl_2$. A volume of 50 μl was pipetted onto freshly cleaved mica (Goodfellow, Huntingdon, UK) and incubated at room temperature for 10 minutes. Unattached protein was then washed away with the same buffer. The protein molecules were examined in the presence of 30 μl fresh buffer. AFM imaging was performed using a Nanoscope IIIa Multimode atomic force microscope (Veeco/Digital Instruments, Santa Barbara, CA) equipped with a J scanner. The N-cadherin-Fc molecules were imaged using oxide-sharpened silicon nitride probes (DNP-S; Digital Instruments) with a spring constant of 0.32 N/m operating in tapping mode at a drive frequency of ~7-9 kHz.

Results

Conformation of dimeric N-cadherin-Fc fusion protein

Electron microscopic examination of cadherin molecules as pentamers fused to cartilage oligomeric matrix protein (COMP) or as dimers linked to immunoglobulin-Fc, has revealed 'ring' and 'spectacle-like' structures deemed to represent cis- and trans- (adhesive) dimerization, respectively (Ahrens et al., 2003; Pertz et al., 1999). As a first step in the current series of experiments, we examined our preparation of N-cadherin-Fc protein by atomic force microscopy (AFM) in the presence of 5 mM calcium to see whether similar structures were detectable. All wild-type molecules had a Y-shaped form with the cadherin domains curving away from each other and the Fc region clearly distinguishable as a structure forming the 'stem' of the Y (Fig. 1). No N-cadherin domain 1 interactions were seen. The orientation of the curved cadherin domains suggests that the contact surfaces for dimerization, identified in crystal structures (Boggon et al., 2002; Pertz et al., 1999), could not easily be juxtaposed, and it is possible that the Fc hinge region in our construct imposes mechanical constraints which militate against dimerization of the N-terminal cadherin domains. Although we cannot completely rule out the possibility that contact with the mica substratum may have disrupted some dimers, the results suggest that at least a majority of the molecules in our preparations had this Y shape.

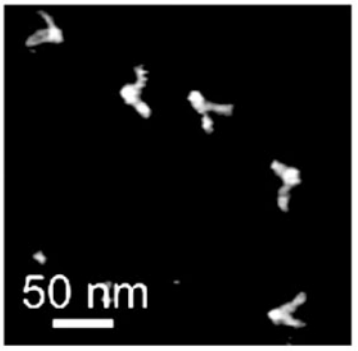

Fig. 1. Atomic force image of purified dimeric N-cadherin-Fc fusion protein adsorbed to a mica surface. The two curved 'arms' in each molecule are the five extracellular domains of N-cadherin, which are joined to the Fc region. The thickness of the deposited molecules is reflected in their shading and approximate values were obtained; 5 nm for the more intensely white Fc region and 3 nm for the cadherin domains.

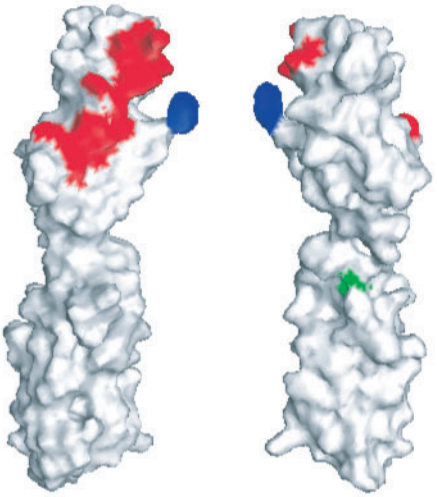

Fig. 2. The two-domain structure of N-cadherin, PDB 1NCJ (Tamura et al., 1998). The location of the 13-mer peptide in domain 1, against which antiserum K7 was prepared, is shown in red. Trp2 is not integrated into the domain fold and is coloured blue. The position of the D134A mutation is shown in green. The two images show opposite faces of the domains.

The absence of adhesive dimers (spectacle-like structures) previously seen by electron microscopy in cadherin-Fc preparations (Ahrens et al., 2003) could be attributable to technical differences. Our preparation did not contain glycerol and the protein concentration used to coat the mica in our experiments was approximately 100-fold lower than that used for previous electron microscopy studies.

Relationship between Trp2 and interdomain calcium in the structural integrity of N-cadherin domain 1

A polyclonal antibody, K7, was prepared against a synthetic linear peptide in the βB strand of N-cadherin domain 1 (Fig. 2). Efficient antibody binding required that the peptide epitope be released from structural constraints of the domain fold. K7 was tested against N-cadherin-Fc containing or lacking the mutation W2G or the junctional mutation D134A, which prevents coordination of the third calcium atom, Ca3, in the ECD1-ECD2 junction (Nagar et al., 1996). The two mutations were tested singly or in combination in an assay containing 1.25 mM calcium. Alternatively, calcium was added to the assay at varying levels (Fig. 3). The N-cadherin-Fc used was the normal dimeric form as depicted in Fig. 1. Antibody K7 failed to bind to wild type N-cadherin or to the D134A mutant, and reactivity with the W2G protein was low (Fig. 3a). Nevertheless, the two mutations in combination gave a strongly positive result. The single mutation W2G was tested at a range of calcium levels, and antibody binding decreased to background as the calcium concentration reached 0.75 mM (Fig. 3b). These results suggest that calcium and Trp2 act in conjunction to stabilise the structure of domain 1. An alternative explanation could be that calcium-dependent interactions occur between the two cadherin units in the dimeric fusion protein, which could prevent access of the antibody to the K7 epitope. Despite indications from our AFM scans that such interactions do not occur in our preparations,

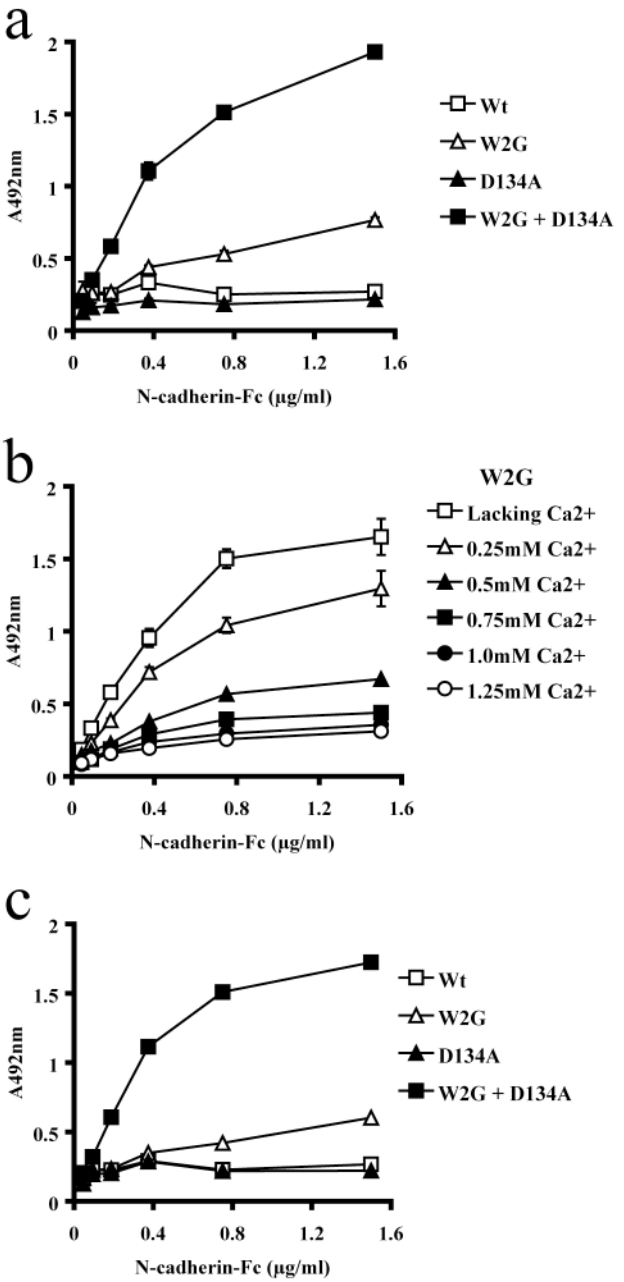

Fig. 3. Binding of peptide-specific antibody K7 to the wild type and mutant N-cadherin-Fc fusion protein. (a) The mutations W2G and D134A in N-cadherin Fc, dimerised via Fc, were tested singly or in combination and compared with results from the wild-type (Wt) molecule. Calcium was present in the assay at 1.25 mM. (b) N-cadherin-Fc mutant W2G was pre-equilibrated with varying levels of Ca^{2+}, which were maintained throughout the assay. (c) Monomeric N-cadherin-Fc was tested in the presence of 1.25 mM Ca^{2+}.

we tested monomeric N-cadherin-Fc to eliminate this possibility (Fig. 3c). In these molecules, dimerization via Fc had been prevented by mutations in the CH3 domain. The titrations were closely similar to those in Fig. 3a, showing that epitope masking could not explain our results. Because Trp2 is not part of the peptide epitope, the structural effect of this amino acid is almost certainly due to its integration into a

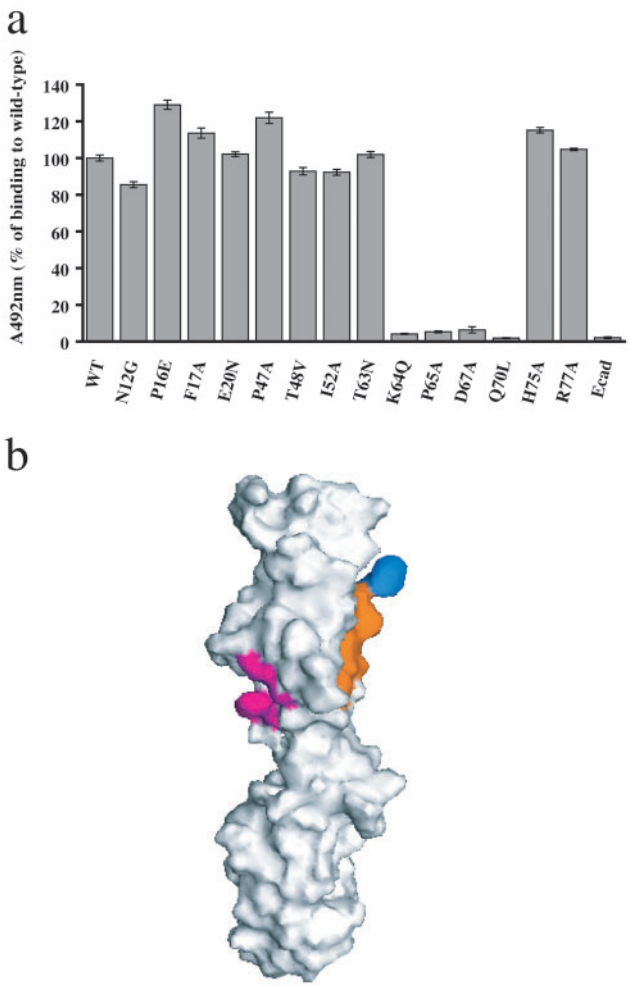

Fig. 4. Epitope mapping of antibody GC4. (a) Binding of mAb GC4 to dimeric N-cadherin Fc bearing point mutations, in the presence of 1.25 mM calcium. (b) Amino acids K64, P65, D67 and Q70 (purple) are seen in relation to the position of non-intercalated Trp2 (blue) and the βA strand (orange).

hydrophobic pocket in the domain structure, as previously observed by NMR (Haussinger et al., 2004) and X-ray crystallography (Pertz et al., 1999).

Antibody GC4 detects docking of Trp2

The epitope for a hitherto uncharacterised commercial N-cadherin antibody, GC4 (Volk and Geiger, 1984) was mapped using a panel of N-cadherin-Fc fusion proteins bearing point mutations (Fig. 4a,b). The epitope was found to include residues K64, P65, D67 and Q70. Mapping results were similar in the presence or absence of calcium. The GC4 epitope lies opposite the βA strand, far from Trp2. Binding of GC4 was prevented by the mutation W2G, regardless of the presence (Fig. 5a) or absence (Fig. 5b) of calcium. This is persuasive evidence that GC4 binding requires Trp2 to be docked into the domain structure. Antibody binding to wild type N-cadherin was greater in the absence of calcium (compare Fig. 5a,b,c). This could be due to modulation of Trp2 docking by calcium, with low levels of calcium favouring integration of Trp2. Alternatively, calcium may have a local effect on the epitope or its accessibility; both propensities could operate. To explore this issue further, we tested the hydrophobic pocket mutation A78M, which would hinder, but not necessarily preclude, Trp2 docking. This mutation is known to inhibit adhesion (Tamura et al., 1998). A78M inhibited binding of GC4 in the presence of 1.25 mM Ca^{2+}, compared to the wild type (Fig. 5d). The result is consistent with impaired Trp2 docking. In contrast, when calcium was removed, both the wild type and the A78M mutant gave equally high binding (Fig. 5e). This supports the explanation that calcium modulates Trp2 docking. In an alternative strategy to compromise insertion of Trp2 into the hydrophobic pocket, the N-terminus of N-cadherin was extended by three amino acids, MDP. An extension would be expected to have a negative effect on integration of Trp2 into the domain (Haussinger et al., 2004) but, again, would not necessarily preclude docking (Pertz et al., 1999; Schubert et al., 2002). In keeping with results for the A78M mutation, the MDP mutation strongly inhibited binding of GC4 in the presence of calcium (Fig. 5f). As with the A78M mutant, removal of calcium from the MDP extension mutant restored binding of GC4 to levels obtained with the wild-type molecule (Fig. 5g). We next showed that the effect of calcium could be attributed to its coordination in the ECD1-ECD2 junction. The mutation D134A, which disrupts coordination of Ca3 in this position, increased binding of GC4, compared with the wild type (Fig. 5h), despite the presence of calcium in the assay buffer. The result was similar with the MDP extension mutant (Fig. 5i), showing that the D134A junctional mutation had the same effect as removing calcium from the medium. As GC4 binding requires Trp2 to be docked, regardless of calcium, these results argue strongly that calcium modulates a dynamic equilibrium between docked and undocked Trp2 so that depletion of calcium favours more stable integration of Trp2 into the domain structure.

Trp2 cross-intercalation (strand exchange) is a primary event in cadherin-mediated cell adhesion

Elegant structural studies have demonstrated cadherin dimerization by strand exchange (Boggon et al., 2002; Haussinger et al., 2004; Shapiro et al., 1995). The question remains whether this happens in a physiological context between opposing cadherin molecules during cell adhesion. Strand exchange would orientate the molecules so that specific amino acids near Trp2 and its hydrophobic pocket are brought into close apposition. We reasoned that if complementary cysteine point mutations were located in these positions they should generate a ‘reporter’ disulphide bond during cell adhesion if strand exchange occurs. Using the strand exchange structure PDB 1NCI (Shapiro et al., 1995), we modelled formation of two alternative disulphide bonds in these circumstances using the complementary mutations D1C-D27C (Fig. 6a) and D1C-R25C (Fig. 6b). The bonds could be formed in silico equally well using other strand exchange structures (Boggon et al., 2002; Haussinger et al., 2004) and also when Trp2 was docked into its own domain (Pertz et al., 1999; Schubert et al., 2002).

To test for the formation of disulphide bonds during cell adhesion, K562 cells were transfected with N-cadherin bearing a single cysteine point mutation and allowed to adhere, in

reducing conditions, to an assay plate coated with monomeric N-cadherin-Fc bearing the complementary mutation. Oxidising conditions were then restored and the formation of a disulphide bond between cellular cadherin and cadherin-Fc was detected by immunoblotting. Initially, essential parameters of the experimental strategy were validated by testing adhesion of N-cadherin-positive DX3 melanoma cells to N-cadherin-Fc molecules bearing the cysteine point mutations. Monomeric N-cadherin-Fc, mutated to prevent Fc-Fc interaction, was used for most of our experiments in order to avoid the complication of disulphide-bonded dimerization in the hinge region of the Fc-fusion protein. Monomeric and dimeric N-cadherin-Fc supported adhesion of DX3 cells equally well throughout a range of coating concentrations (Fig. 7). It is possible that cis-

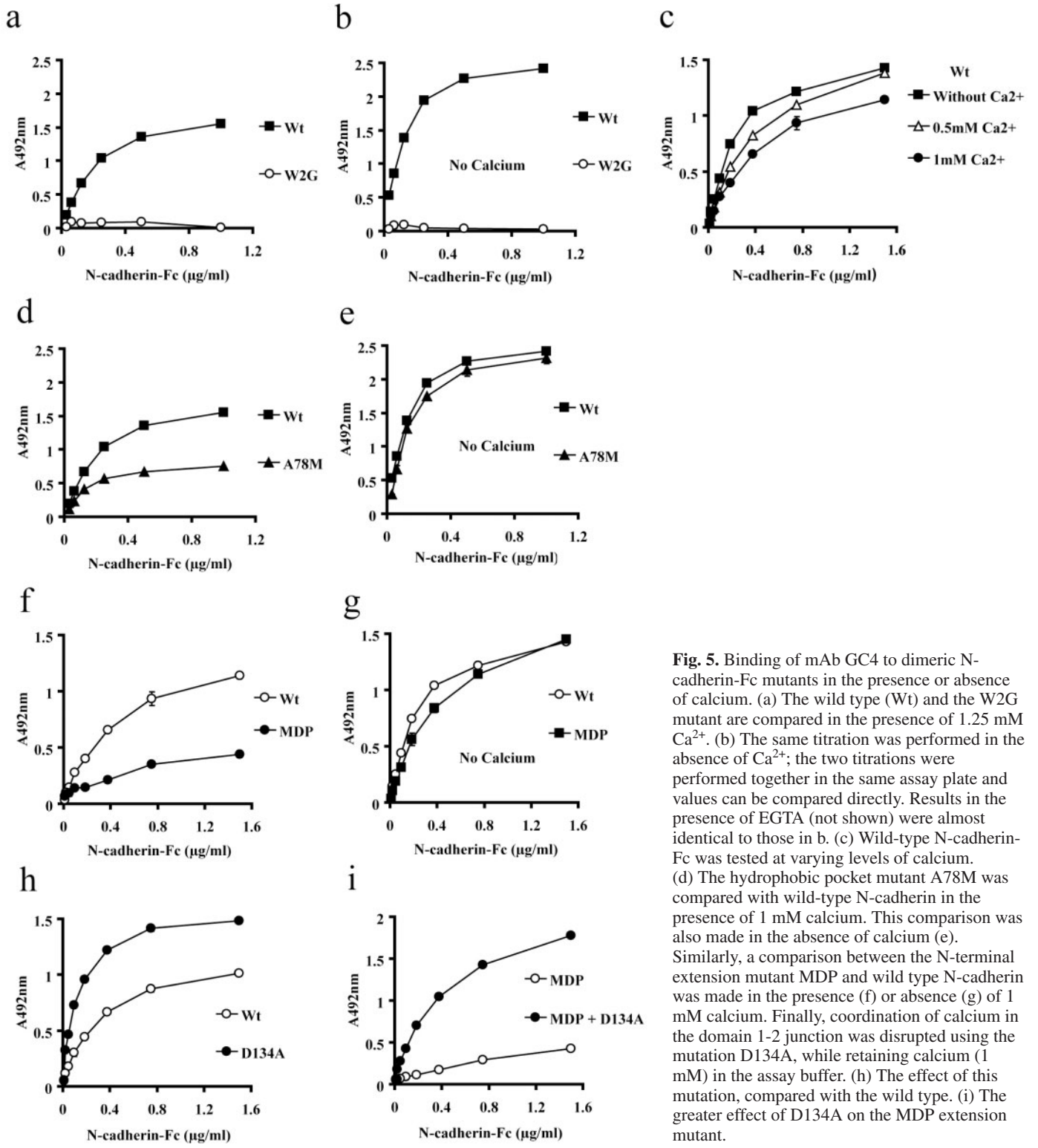

Fig. 5. Binding of mAb GC4 to dimeric N-cadherin-Fc mutants in the presence or absence of calcium. (a) The wild type (Wt) and the W2G mutant are compared in the presence of 1.25 mM Ca^{2+}. (b) The same titration was performed in the absence of Ca^{2+}; the two titrations were performed together in the same assay plate and values can be compared directly. Results in the presence of EGTA (not shown) were almost identical to those in b. (c) Wild-type N-cadherin-Fc was tested at varying levels of calcium. (d) The hydrophobic pocket mutant A78M was compared with wild-type N-cadherin in the presence of 1 mM calcium. This comparison was also made in the absence of calcium (e). Similarly, a comparison between the N-terminal extension mutant MDP and wild type N-cadherin was made in the presence (f) or absence (g) of 1 mM calcium. Finally, coordination of calcium in the domain 1-2 junction was disrupted using the mutation D134A, while retaining calcium (1 mM) in the assay buffer. (h) The effect of this mutation, compared with the wild type. (i) The greater effect of D134A on the MDP extension mutant.

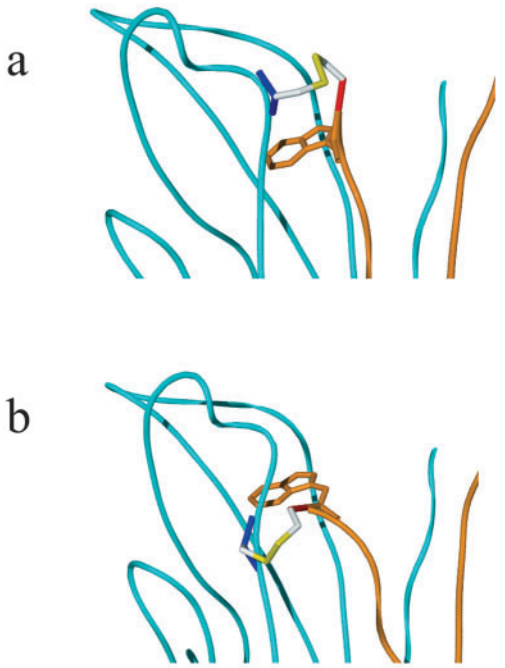

Fig. 6. Modelling the formation of 'reporter' disulphide bonds formed during strand exchange in domain 1. Structures are derived from PDB 1NCI (Shapiro et al., 1995). The two opposing N-cadherin domains are shown in pale blue and brown respectively. The side chain of Trp2 (brown) is shown located in the hydrophobic pocket of the blue domain. (a) The backbone of C1 is shown in red and the side chain forms a disulphide bond (yellow) with C27 (dark blue). (b) The disulphide bond is formed between C1 (red) and C25 (dark blue).

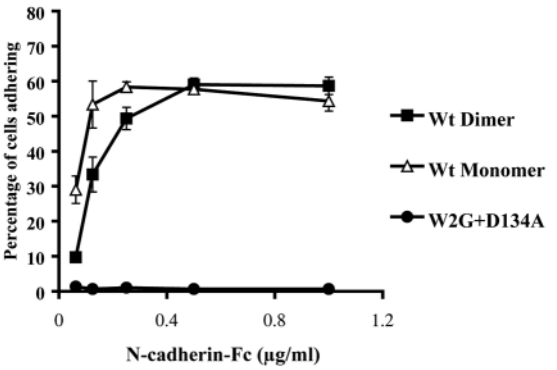

Fig. 7. Comparison of monomeric and dimeric N-cadherin-Fc in supporting adhesion of DX3 melanoma cells. Cadherin preparations, shown to be monodisperse, were titrated onto an assay plate coated with goat anti-human Fc. DX3 cells were applied and adhesion was assessed as described. Dimeric N-cadherin-Fc containing the mutations W2G and D134A provided a negative control.

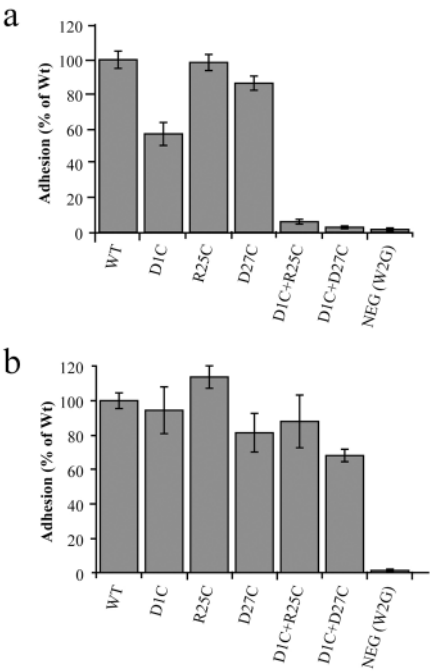

Fig. 8. Effect of cysteine point mutations on the adhesive capacity of monomeric N-cadherin-Fc. (a) The assay was conducted in HBSS + 2%FCS (oxidising conditions). (b) Reducing conditions were established by adding 10 mM DTT. Approximately 65% of wild-type cells adhered to the plate in both assays. Reduction restored adhesive capacity of the double mutants D1C,R25C and D1C,D27C.

dimerization of the monomer may occur at the highest coating levels but this could not happen as the monomer is diluted out on the plate. The single mutations, D1C, R25C and D27C had little or no effect on adhesion of DX3 cells in normal (oxidising) adhesion buffer, but the double mutation D1C,R25C and D1C,D27C abolished adhesion completely (Fig. 8a). It is to be expected that in these molecules Trp2 would be 'locked' into its own domain by an adjacent disulphide bond and would be unavailable for strand exchange. Reducing conditions largely restored the function of the double mutants (Fig. 8b).

K562 transfectants were matched for equal expression of wild-type and mutant chicken N-cadherin (Fig. 9a). Untransfected K562 cells lacked natural expression of human N-cadherin whereas DX3 cells showed strong expression (Fig. 9b). Assurance that K562 cells do not naturally express any classical cadherin was obtained by western blotting using pan cadherin antibody, which gave negative results (not shown). The molecular size of cellular cadherin from the transfectants was compared with that of the monomeric Fc-fusion protein. The cellular material was found to have a slightly higher molecular size than the fusion protein (Fig. 9c).

The K562 transfectants were allowed to adhere to N-cadherin-Fc monomer bearing complementary or non-complementary cysteine mutations. Trans-dimers were detected by immunoblotting, either for cellular cadherin using pan cadherin antibody to the cytoplasmic domain (Fig. 10a), or for the fusion protein using anti-Fc (Fig. 10b). The results show that disulphide-bonded trans-dimers formed only when complementary cysteine mutations were apposed, i.e. D1C-R25C or D1C-D27C (Fig. 10a,b, upper panels). In addition to trans-dimers, D1C-D1C and D27C-D27C cis-homodimers formed both on the cells and in the coating layer of Fc-fusion protein on the assay plate (indicated in Fig. 10). This did not happen with the R25C mutation. The trans-dimers, which consisted of N-cadherin-Fc monomer linked to a molecule of

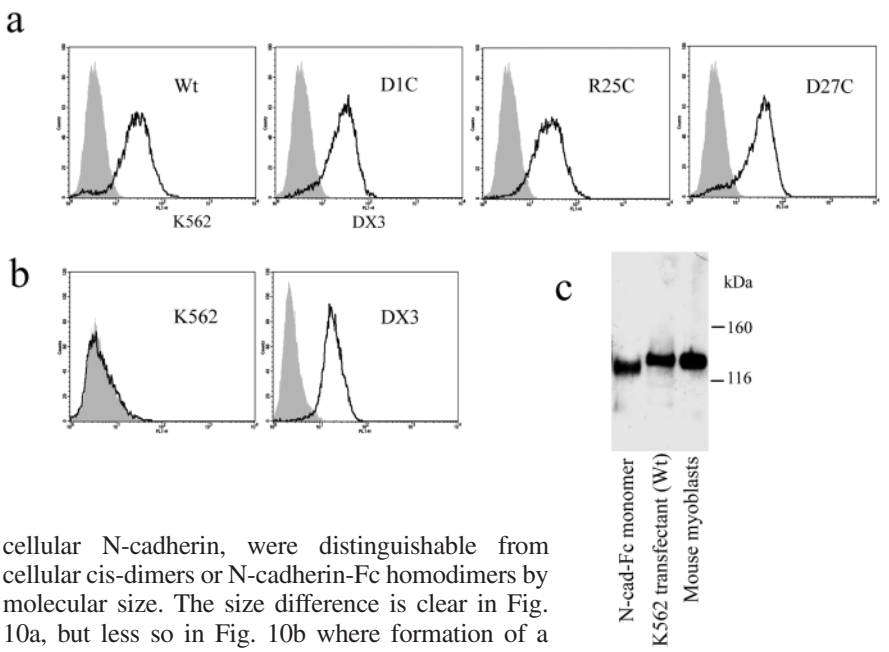

Fig. 9. Expression of cell surface N-cadherin by K562 transfectants and by DX3 melanoma cells. (a) Stable K562 transfectants were stained with mAb NCD-2 to chicken N-cadherin. The four panels show comparable levels of expression. (b) Untransfected K562 cells were tested with mAb 8C11 to human N-cadherin and the first panel verifies that these cells do not naturally express N-cadherin. The final panel shows DX3 cells stained with 8C11, showing strong expression of human N-cadherin. (c) The molecular size of cellular N-cadherin from K562 transfectants (Wt) is compared with that of monomeric N-cadherin Fc fusion protein (Wt) bearing the mutations F405A,Y407A in the Fc region to prevent dimerization. N-cadherin extracted from normal myoblast cells is also shown for comparison. The gel was run under non-reducing conditions and blotting was conducted using a mixture of pan cadherin antibody to the cytoplasmic domain and anti-Fc.

cellular N-cadherin, were distinguishable from cellular cis-dimers or N-cadherin-Fc homodimers by molecular size. The size difference is clear in Fig. 10a, but less so in Fig. 10b where formation of a trans-dimer is seen clearly with the combination D1C-R25C (left panel), but is not apparent with D1C-D27C (adjacent track). A weak trans-dimer band in this case would be largely obscured by the strong cis-dimer signal. The N-cadherin-Fc cis-dimers that formed on the assay plate are seen in the right hand panel. The major bands running slightly above 97 kDa in all the blots represent cadherin molecules that either have not formed an adhesive contact or have failed to produce the disulphide bond. Dimer bands were not detectable when samples were run under reducing conditions (Fig. 10a,b, lower panels).

A second protocol was used to test for the formation of the disulphide bond in trans-alignment. It was designed to avoid detection of cis-dimers. In this strategy, mutant N-cadherin-Fc (the conventional fusion protein dimerised via Fc) was coated to magnetic beads, which were allowed to adhere to the K562 transfectants. The cells were then lysed and the beads were separated from the lysate to extract the disulphide-bonded species in trans-alignment from the remainder of the cellular cadherin. Fig. 11 (left panel) shows results using K562 transfectants expressing wild-type N-cadherin or the three cysteine mutations adhering to beads coated with N-cadherin-Fc fusion protein bearing the mutation D27C. The blot was developed using antibody to cadherin cytoplasmic domain. As before, the disulphide-bonded species formed only with the complementary pair D1C-D27C, giving a major band at approximately 300 kDa. This represents one molecule of dimeric Fc-fusion protein, approximately 200 kDa, disulphide-bonded to one molecule of cellular cadherin. Other bands were also present representing higher order assemblies. The lower panel shows a loading control and the right hand panel shows monomeric cellular cadherin, for comparison.

These results taken together provide persuasive evidence that strand exchange occurs during cell adhesion. Further, the observation that disulphide-bonded homodimers between molecules bearing the same cysteine mutation, D1C or D27C, are produced in cis-, but not trans-, orientation argues that the molecular alignments here must differ from those that form the adhesive dimer.

Discussion

In this study, we have used two antibodies to investigate the stability of domain 1 in relation to the roles of calcium and Trp2 and have demonstrated a major effect of both factors acting in concert. The data complements recent NMR studies (Haussinger et al., 2004) and provides a perspective that is not available from crystal structures. It is possible that antibody binding could itself direct conformational change, but this would be subject to the varying constraints imposed by calcium and Trp2 in our experiments and would not affect our conclusions. The published crystal structures of cadherins all show a full complement of calcium atoms in the domain 1-2 junction, with or without intercalation of Trp2 into the domain structure. The α-carbon backbone is closely similar in all cases. In contrast, the original NMR structure of domain 1 of E-cadherin (Overduin et al., 1995) shows neither intercalated Trp2 nor correctly coordinated calcium atoms, and here the α-carbon trace shows significant displacement compared with that in the crystal structures. Our present data suggest that this NMR structure would be relatively unstable and the βB strand readily displaced from the domain. Results with the peptide antibody K7 show that Trp2 and calcium act in concert to stabilise domain 1, each limiting flexibility of the βB strand and constraining the overall conformation.

Binding of antibody GC4 showed an absolute requirement for Trp2, regardless of the presence or absence of calcium. Because this amino acid is located on the opposite side of the domain, 30 Å away from the GC4 epitope, the result argues persuasively that reactivity with GC4 requires Trp2 to be located in the hydrophobic cavity in domain 1. In these circumstances, Trp2 would impose structural constraints on the GC4 epitope, either via the core of the domain or by limiting movement of the βA strand at its base. Our data show that reduction of calcium in the domain 1-2 junction increased GC4 binding. The effect was modest with wild-type N-cadherin but greater with the mutant A78M or the N-terminally extended version MDP; each of these modifications would compromise

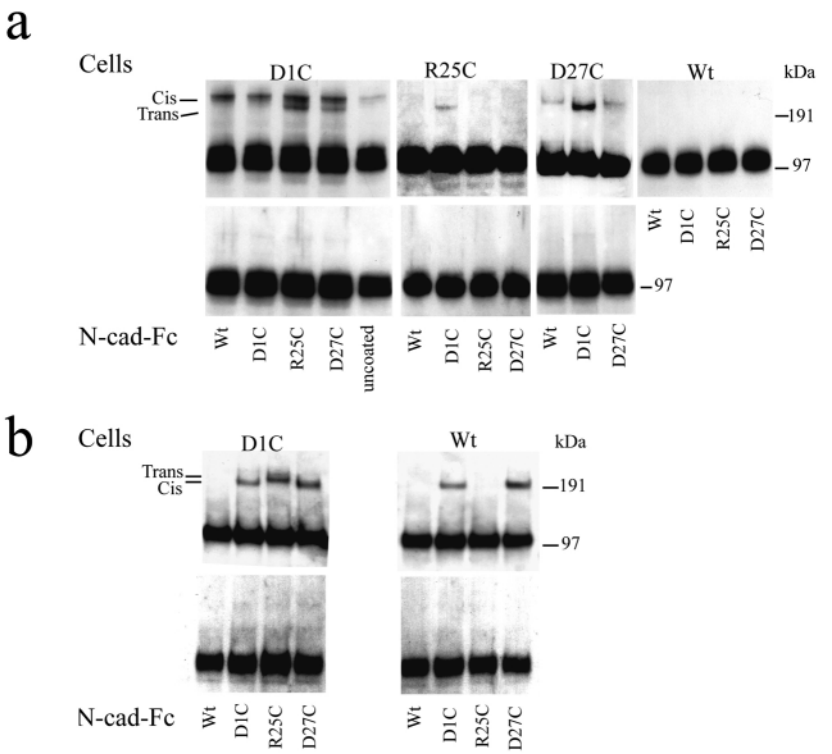

Fig. 10. Formation of 'reporter' disulphide bonds during cadherin-mediated cell adhesion. K562 cells expressing wild-type or mutant N-cadherin were allowed to adhere, under reducing conditions, to a panel of monomeric N-cadherin-Fc molecules bearing the same set of mutations. Oxidising conditions were then restored and the formation of disulphide bonds was assessed by SDS-PAGE and immunoblotting as described. (a) The blot was developed with antibody to cellular cadherin cytoplasmic domain. The upper panels show gels run under non-reducing conditions. Disulphide-bonded trans-dimers form only when cadherin molecules bearing complementary cysteine point mutations were apposed. In contrast, the D1C and D27C mutations allowed formation of disulphide-bonded cis-dimers on the cell surface. With wild-type cells (Wt, right panel), no disulphide-bonded species are seen. The track labelled 'uncoated' in this series reflects a small degree of background adhesion to wells lacking cadherin and shows the position of the cis-dimer. The lower panel shows the same preparations run under reducing conditions. (b) In a similar experiment, the blot was developed with anti-Fc. Again the trans-dimer can be seen when mutations D1C and R25C were apposed. As in (a), cis-dimers were formed by D1C and D27C mutants but not by R25C. Cis-dimers formed by monomeric N-cadherin-Fc are seen to run slightly below the trans-dimer in contrast to the situation in (a) where the cellular cis-dimer runs above the trans-species.

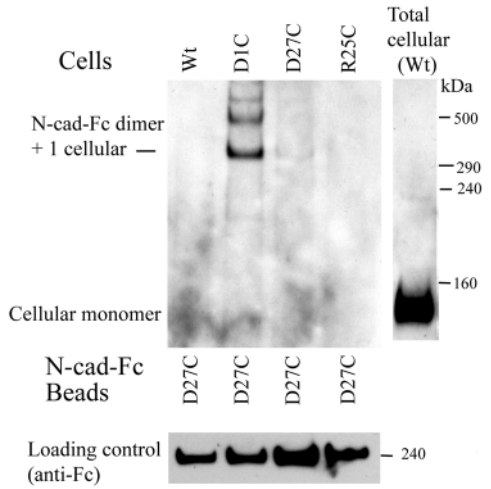

Fig. 11. Isolation of disulphide-bonded trans-dimers. Magnetic beads coated with N-cadherin-Fc bearing the mutation D27C were allowed to stick to K562 cells expressing wild-type N-cadherin or the mutants D1C, R25C or D27C. After formation of disulphide bonds, the trans-dimers attached to the beads were isolated from cis-dimers and non-involved cell surface N-cadherin. The trans-dimers were detected by immunoblotting for cellular N-cadherin cytoplasmic domain. The upper panel shows that trans-dimers formed only with the combination D27C beads adhering to D1C cells. The main band at about 300 kDa represents N-cadherin-Fc (dimerised by the Fc hinge region) disulphide-bonded to one cellular cadherin molecule. Higher order assemblies are also seen. The right-hand panel shows total cellular cadherin for comparison. The lower panel shows a gel loading control.

intercalation of Trp2. The results taken together argue persuasively that calcium modulates a dynamic equilibrium between docked and undocked Trp2. Thus, at low calcium levels Trp2 is more firmly integrated than at physiological levels. Dimerization by strand exchange requires that Trp2 swaps from insertion in its own domain to that of its neighbour, overcoming an activation barrier (Haussinger et al., 2004). The present results are consistent with recent NMR data showing that calcium facilitates this process (Haussinger et al., 2004). Our interpretation of the effect of calcium predicts that dimerization by strand exchange requires calcium but, once formed, the dimer can be isolated from the cell surface in buffers lacking calcium. This is consistent with empirical evidence (Chitaev and Troyanovsky, 1998; Klingelhofer et al., 2002).

Our data with GC4 reflect intramolecular docking of Trp2 rather than strand exchange because we obtained closely similar results (not shown) with monomeric N-cadherin-Fc over a wide titration range where, at lower coating levels, N-cadherin monomers would be widely spaced on the assay plate. We have not yet tested whether GC4 detects cross-intercalation of Trp2, as well as intramolecular docking. It is notable that our cell adhesion experiments demonstrate that monomeric N-cadherin coated to an assay plate, over a range of concentrations, supports cell adhesion equally as efficiently as the normal fusion protein dimerised at the IgG heavy chain hinge region. This dispels a widely held view that cis-dimerization is an obligatory stage in the formation of the adhesive complex (Brieher et al., 1996; Ozawa, 2002; Takeda et al., 1999; Tomschy et al., 1996).

Recently, Troyanovsky and colleagues (Troyanovsky et al., 2003) used a bifunctional sulphydryl cross-linking reagent to

determine the orientation of cadherin molecules in cis- and trans- dimers and concluded that a strand exchange mechanism provided the best explanation for both types of dimer. The present report addresses this issue by a more direct strategy. The formation of a disulphide bond during cell adhesion using complementary, but not identical, cysteine point mutations on opposing cadherin molecules provides compelling evidence for strand exchange. This degree of specificity in the formation of the bond demands that during adhesion Trp2 is either inserted into the hydrophobic pocket of the opposing cadherin molecule or is poised very close to it. By similar reasoning, the cis-dimers we detected between adjacent cadherin molecules bearing the same mutation, D1C or D27C, could not be formed by the mutual strand exchange mechanism depicted in current crystal structures (Boggon et al., 2002; Haussinger et al., 2004; Shapiro et al., 1995). This does not rule out the possibility that strand-exchange cis-dimers may occur on the cell surface; they would not be disulphide-linked and would escape detection on our gels. It is important to emphasise that disulphide bonded cis-dimers were formed with the D1C and D27C mutations, but not with the R25C mutation. This demonstrates that these bonds were not a consequence of random contacts between cadherin molecules. Specificity of the bond for D1C and D27C limits the possible orientations that the molecules can adopt in making the cis-contact. A favourable orientation to achieve this discrimination is for adjacent cadherin molecules to be aligned in parallel, similar to the calcium-dependent C2-symmetric E-cadherin dimer recently determined by NMR (Haussinger et al., 2002). Alternatively, cross-intercalation of one Trp2 residue, as opposed to mutual exchange, may allow sufficient rotation of the domains to bring two D27C mutations into apposition. This arrangement can be seen in a hypothetical structure (PDB 1Q5C) for the orientation of desmosomal cadherins, based on electron tomography (He et al., 2003).

The present results provide the most direct evidence so far that strand exchange is a primary event in cadherin-mediated cell adhesion. This conclusion must be reconciled with three controversial outstanding issues: the questions of cadherin type-specificity (Klingelhofer et al., 2000; Niessen and Gumbiner, 2002; Nose et al., 1990); the role of the conserved HAV motif (Makagiansar et al., 2001; Renaud-Young and Gallin, 2002; Williams et al., 2000; Williams et al., 2002); and the contribution of domains 2-4 to cell adhesion (Chappuis-Flament et al., 2001; Zhu et al., 2003). We envisage that Trp2 exchange is the initial event in cadherin-mediated adhesion; the HAV motif is not directly involved and the interaction is not cadherin type-specific. Subsequently, secondary interactions that require other regions of the cadherin molecule follow. These contacts facilitate clustering, provide specificity or initiate intracellular signalling. We suggest that our present strategy of using ‘reporter’ disulphide bonds to reveal adhesive surfaces in a physiological setting may be a powerful tool to investigate these interactions.

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