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Homophilic adhesion of E-cadherin occurs by a co-operative two-step interaction of N-terminal domains

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Cluster formation of E-cadherin on the cell surface is believed to be of major importance for cell–cell adhesion. To mimic this process the extracellular part of mouse E-cadherin (ECAD) was recombinantly fused to the assembly domain of rat cartilage oligomeric matrix protein (COMP), resulting in the chimeric protein ECAD–COMP. The COMP domain formed a five-stranded α-helical coiled-coil. This enabled the formation of a pentameric ECAD with bundled C-termini and free N-termini. The pentameric protein construct ECAD–COMP and the monomeric ECAD were expressed in human embryonal kidney 293 cells. Electron microscopy, analytical ultracentrifugation, solid phase binding and cell attachment assays revealed that pentamers showed strong self-association and cell attachment, whereas monomers exhibited no activity. At the high internal concentration in the pentamer the N-terminal EC1 domains of two E-cadherin arms interact to form a ring-like structure. Then the paired domains interact with a corresponding pair from another pentamer. None of the four other extracellular domains of E-cadherin is involved in this interaction. Based on these results, an *in vivo* mechanism is proposed whereby two N-terminal domains of neighbouring E-cadherins at the cell surface first form a pair, which binds with high affinity to a similar complex on another cell. The strong dependence of homophilic interactions on C-terminal clustering points towards a regulation of E-cadherin mediated cell–cell adhesion via lateral association.

Keywords: E-cadherin/homophilic interaction/model for cell–cell adhesion

Introduction

The classical cadherins (E-, N-, P-cadherin, L-CAM) are single pass transmembrane proteins that are involved in calcium dependent selective cell recognition and adhesion (Takeichi, 1988; Rimm and Morrow, 1994). These processes play a dominant role during embryonic morphogenesis and are responsible for initiation and maintenance of tissue architecture. E-cadherin/uvomorulin is already expressed on blastomeres of mouse pre-implantation embryos and is indispensable for cell compaction at the morula stage (Hyafil *et al.*, 1980; Vestweber *et al.*, 1987). Investigations on E-cadherin negative embryos clearly showed that the abundance of E-cadherin at cell–cell contact sites is crucial in providing cell polarity, tightness and integrity of the trophectoderm epithelium (Larue *et al.*, 1994). In differentiated epithelial cells it is preferentially localized in the zonula adherens between polarized cells. In many carcinomas a non-functional E-cadherin is the reason for disturbance of the integrity of intercellular junctions leading to loss of epithelial differentiation. A higher mobility and invasiveness of the tumour cells is the consequence of the reduced intermolecular adhesion (Birchmeier and Behrens, 1994).

Classical cadherins consist of five repeating domains (EC1–EC5) with internal homology in their extracellular segment, a membrane spanning region and a highly conserved cytoplasmic part. The cytoplasmic domain is linked to actin filaments by catenins, proteins homologous to plakoglobin and vinculin (Kemler, 1993; Aberle *et al.*, 1994). Cytoplasmic deletion mutants in which the binding to catenins is disturbed fail to form stable cell–cell contacts (Ozawa *et al.*, 1990). A regulation of the E-cadherin–catenin interactions by phosphorylation of the catenin-binding domain is proposed (Stappert and Kemler, 1994). Cytoplasmic interactions control clustering of E-cadherin to cell contact sites which leads to the formation of tight intercellular connections. Mutation analysis and construction of chimeric molecules suggest that the binding specificity resides in the N-terminal cadherin domain (Nose *et al.*, 1990).

The structural basis of the extracellular interaction was recently illuminated by solving the structure of the N-terminal domain (EC1) of E-cadherin by NMR (Overduin *et al.*, 1995), and of N-cadherin by X-ray structure analysis (Shapiro *et al.*, 1995a). Very interestingly, the domains formed two types of contacts in the crystal. A contact between parallel arranged domains was designated 'strand dimer interaction', and an antiparallel contact was attributed to the adhesion interaction between cells.

In contrast to the suggested interactions within crystals, self-association of E-cadherin extracellular fragments investigated by analytical ultracentrifugation was not observed in solution studies (Pokutta *et al.*, 1994). It was therefore not possible to study the homophilic interactions between the entire extracellular segments of cadherins in solution. This implies that such intrinsically weak binding is inadequate to ensure cell–cell adhesion. One mechanism whereby adhesion could be enhanced is if alignment of E-cadherin molecules is promoted by clustering at the cell surface.

In order to mimic cluster formation, the extracellular part of E-cadherin was fused at its C-terminus to the assembly domain of cartilage oligomeric matrix protein (COMP) by recombinant technology. The COMP domain forms a five-stranded α-helical coiled-coil (Efimov *et al.*,

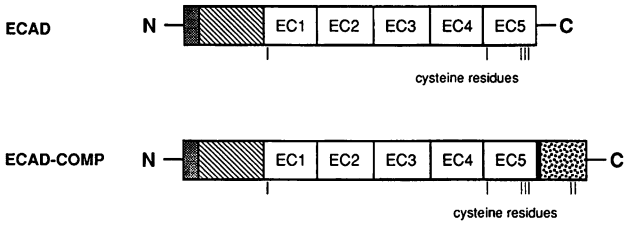

Fig. 1. Schematic drawing of the polypeptide regions of mouse E-cadherin and rat cartilage oligomeric matrix protein (COMP) which are included in the ECAD or ECAD–COMP construct. Grey box: signal peptide (aa 1–27); hatched box: propeptide (aa 28–156); white box: the five extracellular domains of E-cadherin (aa 157–699); black box: oligopeptide taken from the flanking region of the coiled-coil domain of human thrombospondin 4 (aa 914–922); stippled box: coiled-coil domain of COMP (aa 27–83). Cysteine residues are indicated as bars.

1994) and connected five cadherin extracellular fragments in the recombinantly expressed chimeric protein. The oligomers exhibited strongly enhanced self-association as compared with monomers. Electron microscopy revealed pair formation of N-terminal domains within the same pentamers. This pair combined with a similar pair of another pentamer, thus forming a tetrameric complex of two parallel and two antiparallel oriented N-terminal cadherin domains. These findings support a two-step model for E-cadherin mediated cell–cell adhesion via the N-terminal domains (Nose *et al.*, 1990; Shapiro *et al.*, 1995a), but argue against the model of multiple interactions involving the other four domains in the extracellular region of cadherins (Ozawa *et al.*, 1991; Shapiro *et al.*, 1995a).

Results

Expression of the extracellular fragment ECAD and the chimeric protein ECAD–COMP

Two different expression vectors were constructed to express the recombinant proteins (Figure 1). The first was designed for the expression of the complete extracellular part of E-cadherin. In the second vector the assembly domain of COMP was connected to the ECAD fragment by a linker coding for a peptide Ser-Glu-Pro-Ala-Ala-Ala-Thr-Gly. This sequence constitutes the N-terminal flanking region of the coiled-coil domain in thrombospondin 4 (Lawler *et al.*, 1993). Transfection of human embryonal kidney 293 cells with the expression vectors resulted in both cases in stable clones that synthesized and secreted large amounts of recombinant protein (30–40 μg/ml medium) (Figure 2). Morphological differences to non-transfected cells were not observed during expression time. Both recombinant proteins were purified from serum-free culture medium by successive column chromatography on DEAE–cellulose and Superose 12 or Sephacryl S300.

SDS–PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) of the purified proteins under reducing and non-reducing conditions gave rise to main bands of the expected molecular mass. The pattern of bands for ECAD–COMP under non-reducing conditions may be attributed to partially incomplete disulfide linkage of the five chains which are most likely stabilized by non-covalent interactions. Similar patterns were observed for the recombinantly prepared COMP fragment (Efimov

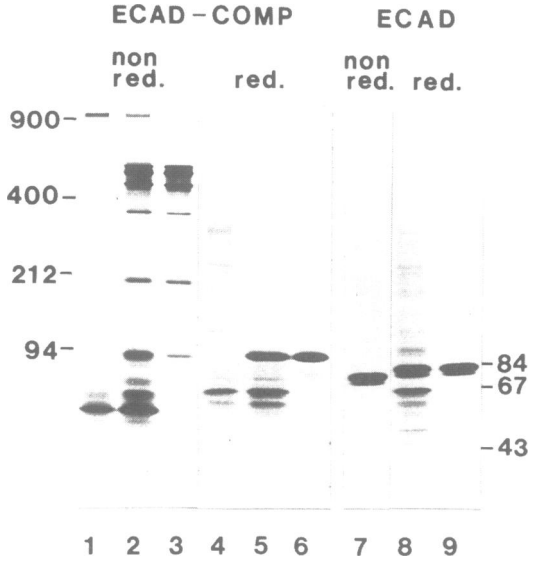

Fig. 2. Purification of the recombinantly expressed ECAD–COMP and ECAD proteins. A silver stained 3–15% SDS–polyacrylamide gel is shown. Probes were applied onto the gel under non-reducing and reducing conditions. Lanes 1 and 4: secreted proteins from medium of human embryonal kidney 293 cells (negative control); lanes 2 and 5: secreted recombinant ECAD–COMP from medium of transfected cells; lanes 3 and 6: purified ECAD–COMP after anionic-exchange and gel filtration chromatography; lane 8: secreted recombinant ECAD from medium of transfected 293 cells; lanes 7 and 9: purified ECAD after anionic-exchange and gel filtration chromatography.

et al., 1994), for which it was shown by native PAGE gels that it was a pure pentamer. The pentameric α-helical coiled-coil domain of COMP shows high thermal stability (thermal melting at 60°C) even in the absence of disulfide bonds (Efimov *et al.*, 1994). The main band for ECAD was accompanied by a slightly faster small band which was already present at the time of medium harvesting, as demonstrated by SDS–PAGE. Both bands contained ECAD with the same correct N-terminal sequence. The ECAD species which ran faster on SDS–polyacrylamide gel may be either incompletely glycosylated during expression or may lack a C-terminal part. N-terminal sequencing of ECAD and ECAD–COMP also demonstrated complete removal of the propeptide by the cells. ECAD was monomeric even under non-reducing conditions in spite of the four cysteine residues in the fifth domain of E-cadherin (EC5; Figure 1). It was not possible to form disulfide linked oligomers of ECAD (0.5 mg/ml) by reoxidation experiments at 22°C in a 10 mM mixture of reduced and oxidized glutathione (molar ratios 9:1 and lower) (data not shown).

Self-association of ECAD and ECAD–COMP

Analytical ultracentrifugation demonstrated a monomeric state for ECAD up to a concentration of 0.2 mg/ml (2 μM) under non-reducing conditions in the presence of 2 mM Ca^{2+}. The sedimentation coefficient of $s_{20,w}$ = 3.7S and the molecular mass from sedimentation equilibrium of 85 kDa were close to the values previously measured for similar native and recombinant fragments (Herrenknecht and Kemler, 1993; Pokutta *et al.*, 1994). In contrast, a wide range of molecular masses ranging from 500 kDa, which was expected for one pentamer, to 1500 kDa were observed for ECAD–COMP under similar conditions. A

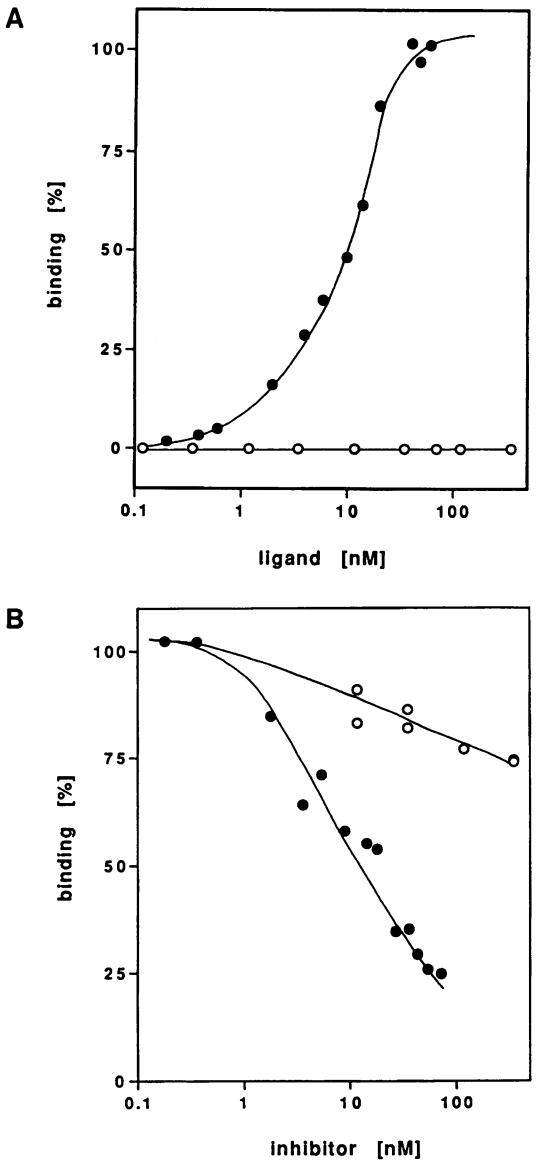

Fig. 3. Solid-phase binding and inhibition assays. ECAD–COMP was coated at concentrations of 10 nM and biotinylated bound protein was detected with horseradish peroxidase-streptavidin complex. (**A**) Binding of biotinylated ECAD–COMP (filled circles) or ECAD (open circles) to immobilized ECAD–COMP. (**B**) Inhibition of binding of biotinylated ECAD–COMP. Labelled protein at a constant concentration of 14 nM was used in competition with unlabelled ECAD–COMP (filled circles) or ECAD (open circles).

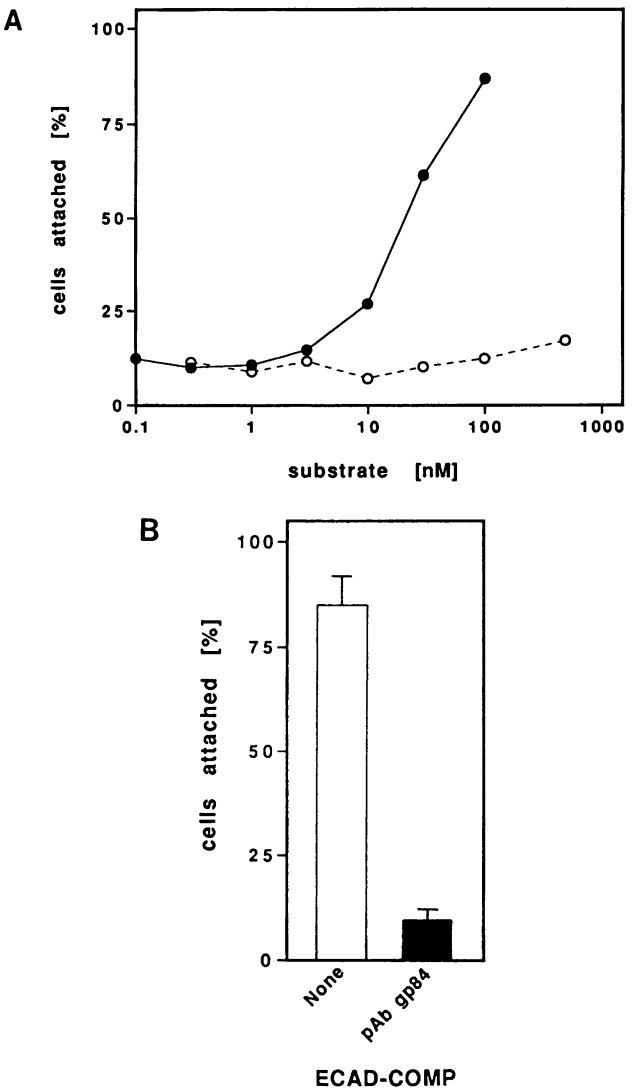

Fig. 4. Cell attachment assays. (**A**) Dose–response profiles of murine epithelial CMT-93 cells adhering to ECAD–COMP (filled circles) and ECAD (open circles). CMT-93 cells were allowed to attach to wells coated with increasing concentration of ECAD–COMP and ECAD. One representative experiment out of three is shown. (**B**) Effect of polyclonal antibodies gp84 (anti-E-cadherin) on adhesion of CMT-93 cells to ECAD–COMP. Wells were coated with a 100 nM ECAD–COMP solution. Cells were pre-incubated without antibodies (white bar) and with pAb gp84 (black bar; 5 µg/ml) before plating. Results are given as mean per cent of attached cells out of three independent experiments ± standard deviation.

broad sedimentation profile indicated self association to higher aggregates. Three populations with sedimentation coefficients of 10S (66%), 25S (20%) and 42S (14%) could be distinguished. Numbers in brackets indicate weight fractions estimated from the areas of the profiles. Only a single profile with 9S could be detected in the control experiment in which calcium was removed by the addition of 1 mM excess of EDTA. A sedimentation coefficient of 9–10S may be assigned to non-associated ECAD–COMP molecules.

Association between ECAD–COMP molecules was further substantiated by a solid phase binding and inhibition assay (Figure 3). Biotinylated ECAD–COMP bound with high affinity to immobilized ECAD–COMP with half maximum binding at 10 nM. In contrast, no binding was observed for biotinylated ECAD in the same system (Figure 3A). Inhibition of ECAD–COMP binding by unlabelled ECAD–COMP was very efficient (Figure 3B). The measured IC_{50} value (concentration at half maximum inhibition) of about 20 nM was close to the applied concentration of labelled protein (14 nM) indicating that the binding activity of the protein was not reduced by the biotinylation procedure. Unlabelled ECAD showed a much weaker inhibition indicated by an IC_{50}-value increased at least 100-fold. The COMP domain (Efimov *et al.*, 1994) showed no inhibitory effect up to 850 nM (data not shown).

Cell adhesion to ECAD and ECAD–COMP

The functionality of ECAD–COMP was further demonstrated in a more physiological context by cell attachment

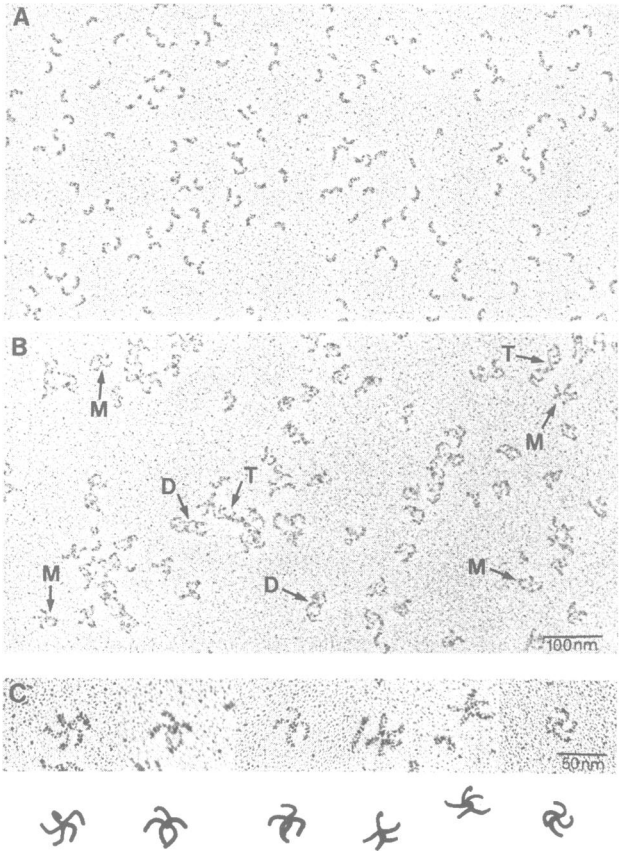

Fig. 5. Electron micrographs of recombinant ECAD and ECAD–COMP. Purified proteins were sprayed onto mica and rotary shadowed. Representative overviews of ECAD (**A**) and ECAD–COMP (**B**) are shown. Arrows in (B) point to monomeric (M), dimeric (D) and trimeric or higher (T) complexes of ECAD–COMP. (**C**) Selected single ECAD–COMP molecules.

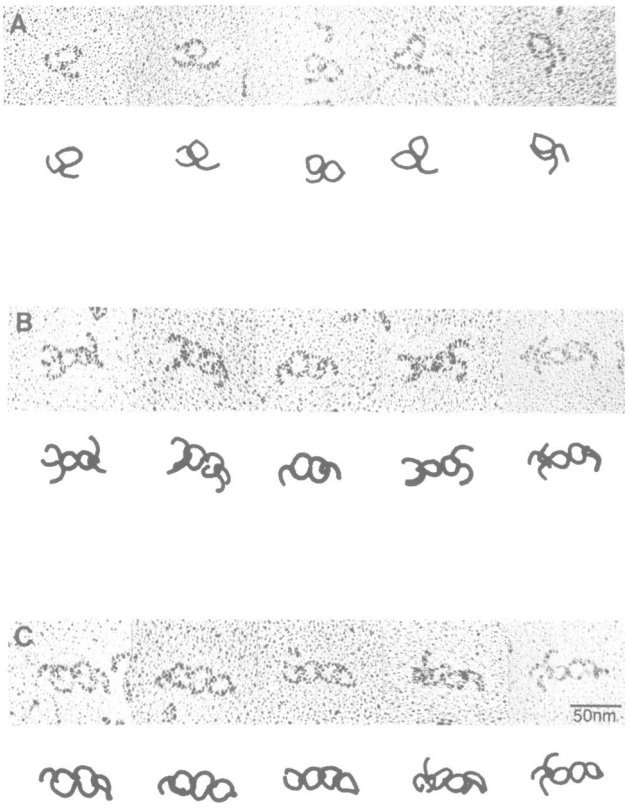

Fig. 6. Electron micrographs of rotary shadowed ECAD–COMP. Single or dimeric complexes of ECAD–COMP are selected and manually outlined. (**A**) Single molecules which form at most two intramolecular ring-like structures. Ring formation occurs by the interaction of the N-terminal regions of two E-cadherin arms. (**B**) Two molecules which are connected via their ring-like structures leading to a double ring-like structure. The intermolecular interactions seem to be restricted to the N-terminal ends of the four involved E-cadherin arms. (C) Associated molecules similar to those in (B) but with additional intramolecular ring-like structures.

assays (Figure 4). Murine epithelial CMT-93 cells adhered strongly to ECAD–COMP in a dose-dependent manner (Figure 4A). Only weak non-specific cell attachment was observed using ECAD as a substrate (Figure 4A). In control experiments the coiled-coil domain of COMP and BSA showed a similar non-specific cell adhesion (data not shown). Cell attachment could be blocked specifically with polyclonal antibodies against the extracellular fragment of murine E-cadherin (Figure 4B). Cells were pre-incubated with the antibodies in order to inhibit primarily the E-cadherin at the cell surface. In addition, the minimal amount of inhibitory antibodies was evaluated by dose-dependent cell attachment assays. The possibility that the antibodies also interact with the substrate cannot however be completely excluded.

Electron microscopic visualization of ECAD and ECAD–COMP

All electron microscopic experiments were performed with protein solutions at concentrations of 0.1–0.6 μM in the presence of 2 mM Ca^{2+}. The appearance of ECAD (Figure 5A) was indistinguishable from that of a similar fragment studied previously (Pokutta *et al.*, 1994). It exhibits a flexible rod-like structure with a total length of 26 ± 2 nm after rotary shadowing. This corresponds to a length of 22 ± 2 nm after correction for the length increase from metal decoration (Engel, 1994). Many particles showed a prominent bend in the central region. The electron micrographs confirmed the results of analytical ultracentrifugation experiments that ECAD is essentially monomeric.

For ECAD–COMP a large variety of aggregates is seen in Figure 5B and selected species are shown in Figure 6. Only a small fraction of the particles appeared as isolated stars with five arms (Figure 5C). The COMP domain located in the centre of the stars could not be distinguished in the electron micrographs because of its small size and/or orientation. Also, the COMP domain was not visible by negative staining (Figure 7) which provides a somewhat higher resolution than the rotary shadowing technique. In some cases not all of the five arms could be seen, presumably due to electron microscopic artifacts.

An analysis of the different aggregates of ECAD–COMP revealed a number of characteristic shapes (Figures 6 and 7). In a fraction of non-aggregated pentamers two E-cadherin arms were found to be connected at their distal ends, leading to a ring-like structure (Figures 6A and 7B). An average circumference of the rings of 54 nm ± 3 nm was determined. Frequently pentamers were seen with two intramolecular ring-like structures. In a large fraction of complexes two pentamers were connected via their ring-like structures leading to a double ring-like structure

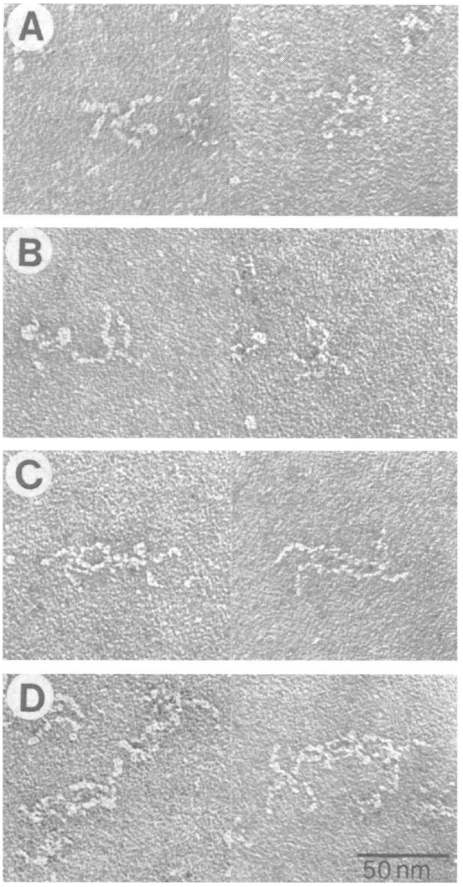

Fig. 7. Electron micrographs of negatively stained recombinant ECAD–COMP. (**A** and **B**) Single molecules, but in (B) two E-cadherin arms form an intramolecular ring-like structure by interacting via their N-terminal ends. The following panels show complexes existing of two (**C**) or more (**D**) ECAD–COMP molcules.

(Figures 6B and 7C). Associated pentamers often possess additional intramolecular ring-like structures which implicate aggregation of more than two pentamers in a chain-like manner (Figure 6C). Longer chain-like structures were also seen (Figures 5B and 7D), but could not be resolved in detail. In all ECAD–COMP associations the intermolecular interactions seem to be exclusively restricted to the N-terminal EC1 domains of the four involved E-cadherin arms (see Discussion).

Aggregation via the COMP domain was never observed. The coiled-coil fragment of COMP itself did not show any tendency to self-association, even at very high concentrations (Efimov *et al.*, 1994).

Discussion

ECAD monomers do not associate

Monomeric ECAD expressed by human embryonal kidney 293 cells did not associate in solution and no aggregation was observed by electron microscopy. It thus resembled a biochemically isolated extracellular fragment (Vestweber and Kemler, 1985) and a recombinant fragment obtained from insect cells (Herrenknecht and Kemler, 1993; Pokutta *et al.*, 1994). ECAD is longer than these fragments and contains the complete domain 5 (EC5) of E-cadherin. It terminates at residue 699 close to the beginning of the membrane spanning domain. Electron microscopy revealed a total length of the extracellular region of 22 nm. By X-ray crystallography the length of the N-terminal domain of N-cadherin was determined to be 4.5 nm (Shapiro *et al.*, 1995a). Assuming a similar shape for all five domains and a high structural relatedness between the domains in E- and N-cadherins (Shapiro *et al.*, 1995b), a total length of $5 \times 4.5 = 22.5$ nm is predicted in agreement with electron microscopy. It was suggested (Shapiro *et al.*, 1995a) that domain 5 may be involved in disulfide linked dimerization of N-cadherin monomers. Experimentally, however, dimerization was not observed for ECAD even under optimized reoxidation conditions. Lack of intermolecular disulfide linkage is in agreement with data for intact E-cadherin (Peyriéras *et al.*, 1983; Vestweber and Kemler, 1984). The four cysteine residues of domain 5 apparently form internal disulfide bonds, explaining the slightly faster mobility in SDS–PAGE under non-reducing conditions. Solid phase binding and inhibition assays revealed no or only a very weak interaction of ECAD with ECAD–COMP.

Self-association of ECAD–COMP pentamers

For ECAD–COMP strong association was observed by analytical ultracentrifugation, solid phase binding and inhibition assays and by electron microscopy. The calcium dependent self-aggregation of ECAD–COMP was demonstrated well by analytical ultracentrifugation experiments. Removal of calcium led to dissociation of aggregates and resulted in non-associated ECAD–COMP molecules.

Self-association was analysed in more detail by electron microscopy. Formation of rings and the interaction of two rings occurred only by association of N-terminal domains (EC1). The average circumference of the rings (54 ± 3 nm) is actually 10 ± 7 nm longer than the sum of the length of two arms (44 ± 4 nm). The large ring size may arise by two arms interacting at their N-termini taking into account the length of the two linker peptides of nine residues each. A suggestion which was made for N-cadherin was that other domains of the extracellular part may also be involved in complex formation (Shapiro *et al.*, 1995a) was not supported by the electron microscopic evidence for E-cadherin. It is unlikely that the lack of association between domains 2–5 may be explained by an unfavourable directionality imposed by the COMP domains because of the connection of the cadherin part to COMP by a flexible linker region.

Two types of interactions between N-terminal domains were observed. In the same pentamer two domains bound to each other leading to the formation of a ring (Figure 8B). Two rings from different pentamers were connected by a complex of four N-terminal domains (Figure 8C). This was the only type of connection between pentamers and no complexes of the type in Figure 8A were observed. Electron microscopy did not provide information on the sequence of these events but it is likely that species B contains a precursor pair which actively associates with another active pair to complex C resulting in a tetrameric assembly of N-terminal domains. Formation of B is favoured by the high local concentration of domains in pentamers. A lower limit of this concentration is estimated by dividing the number of N-terminal domains (five) by the volume of the sphere defined by the length of ECAD arms of 22 nm. An upper limit follows from using the

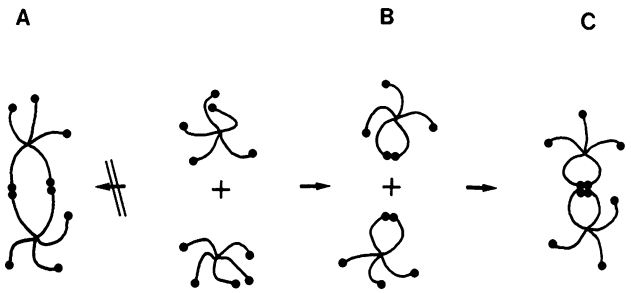

Fig. 8. Schematic drawing of complex formation between two ECAD–COMP molecules. (**A**) Association by single E-cadherin arms has not been observed. (**B**) One ECAD–COMP molecule forms an intramolecular ring-like structure. Only the N-terminal parts of two E-cadherin arms are connected. (**C**) Two ECAD–COMP molecules are associated by their preformed intramolecular ring-like structures. Four N-terminal domains are involved in complex formation.

volume of the outer 4.5 nm thick shell of the sphere, assuming that the domains are preferentially located in this shell because of the stiffness of the arms. The concentration range thus estimated is 0.1–2 mM. By comparison, in analytical ultracentrifugation experiments with the monomers at which no association was observed (see above) the concentration was only 6 μM. Formation of pairs in species B may co-operatively enhance the affinity and favour binding of other N-terminal pairs at a much lower concentration (0.1 μM in the electron microscopic experiments), leading to species C.

A zipper-like association as proposed on the basis of contacts in the crystal structure of the N-terminal N-cadherin domains (Shapiro *et al.*, 1995a) was not observed for ECAD–COMP. The present data support the conclusion drawn from X-ray structure analysis that two different contacts are important for the homophilic interaction. In the tetrameric contact region of two rings (Figure 8) two interacting domains have to be arranged in a parallel and an antiparallel orientation. Although details of this interaction cannot be seen by electron microscopy, it follows from the geometry of the tetramers that at least one of the contacts does not coincide with the crystallographic contact and that a transition to the zipper-like structure would require a rearrangement of one of the contacts.

The suggestion of a two step mechanism is further supported by the results gained from solid phase assays. High binding affinity was only observed for pentameric ECAD–COMP but not for monomeric ECAD. This may be explained by the presence of active pairs of two N-terminal domains in ECAD–COMP which are missing in ECAD.

In addition, it could be demonstrated that epithelial CMT-93 cells containing E-cadherin on their cell surface (Vestweber, 1985) attach strongly to ECAD–COMP but not to ECAD. This interaction can be specifically inhibited by polyclonal antibodies against E-cadherin. Again the ability of cell attachment may be correlated with the presence of active pairs in ECAD–COMP.

A model for the in vivo situation

Cross-linking of the extracellular regions of E-cadherin at the C-terminus by the COMP domain mimics an increase of surface concentration which under physiological conditions may arise through changes in cytoskeletal arrangement. In the framework of the proposed mechanism this will lead to formation of the active E-cadherin pairs (similar to species B) which may then combine with pairs from another cell. The mechanism is in accordance with the observed dramatic inhibition of homophilic interactions between cells expressing mutant molecules lacking the extracellular domains, together with endogenous cadherins (Kintner, 1992). Dilution of intact cadherin by these mutants may prevent internal associations at the cell surface and therefore formation of active species.

The importance of clustering of E-cadherin for its adhesive function is underlined by studies on the connection of E-cadherin to actin filaments mediated by different catenins and other cytoskeletal proteins. Deletions in the cytoplasmic domain led to a loss of function, although the mutated proteins are still expressed at the cell surface (Nagafuchi and Takeichi, 1988; Ozawa *et al.*, 1989; Kintner, 1992). Assembly and disassembly of cells during mitosis and development or invasion of tumour cells are dynamic processes in which lateral association of E-cadherin has to be modulated. Assembly requires clustering of E-cadherin while disassembly requires dissociation of the clusters. Clustering is supposed to be controlled by kinases acting on the cadherin–catenin complex. Phosphorylation of the serine residues at the catenin binding site of E-cadherin seems to be crucial for cell assembly (Stappert and Kemler, 1994), and probably also for blastomere compaction (Winkel *et al.*, 1990). Disassembly of cells, however, appears to be regulated by phosphorylation of tyrosine residues of catenins, and to a minor extent of cadherins (Behrens *et al.*, 1993).

Recently, three morphologically distinct sequential steps in epithelial cell recognition and adhesion of motile MDCK cells were described (McNeill *et al.*, 1993). The formation of stable cell–cell contacts characterized by an accumulation of E-cadherins at the lateral cell surface may arise by the tetrameric adhesion complex that we described. A zipper-like hyperstructure recently proposed by crystallographic data (Shapiro *et al.*, 1995a) may represent a final step in the adhesion mechanism.

Materials and methods

DNA constructions

Recombinant DNA techniques were based on Sambrook *et al.* (1989). Oligonucleotides were synthezised with a 380A DNA syntheziser (Applied Biosystems) and purified on a PD-10 gel filtration column (Pharmacia). *Escherichia coli* JM83 (Yanisch-Perron *et al.*, 1985) was used as a host strain. Polymerase chain reaction (PCR) was carried out in a volume of 100 μl, using ~20 ng of template DNA and 100 pmol of each primer.

Two DNA fragments coding for the extracellular part of mouse E-cadherin including signal peptide and propeptide (aa 1–699) were amplified using Pfu-polymerase and the c-DNA clone pSUM1 of mouse E-cadherin (Ozawa *et al.*, 1989) as template DNA. The primers for fragment 1 were designed to obtain a *Hin*dIII restriction site at the 5′ end (PE1: 5′-GCGTCTGCCGGGAAGCTTATGGGAGCCCGGTGC-CGC-3′) and a *Not*I restriction site at the 3′ end (PE2: 5′-CTGCTGCGGCCGCCTACTTCATGCAGTTGTTGACCG-3′). In this and all following sequences restriction sites are underlined. In addition, the artificial stop codon TAG was introduced after the triple base pairs coding for lysine 699. Fragment 2 should later be connected to the DNA sequence coding for the assembly domain of rat cartilage oligomeric matrix protein (COMP). Therefore primer PE2 was exchanged by PE3 which contains a *Not*I restriction site within an additional 18 base sequence coding for one part of a nine amino acid region flanking

the coiled-coil domain of human thrombospondin 4 used as linker peptide (PE3: 5′-TCCTGCGGCCGCGGGTTCCGACTTCATGCAGT-TGTTGAC-3′). Both fragments were inserted via their restriction sites in the eukaryotic expression vector pRc/CMV (Invitrogen), where expression is controlled by enhancer/promoter sequences from human cytomegalovirus. The resulting plasmids are pECAD1 for fragment 1 and pECAD2 for fragment 2.

The 171 bp fragment coding for the assembly domain of rat COMP (aa 27–83) was amplified by *Taq* polymerase (Boehringer Mannheim) using the c-DNA clone p3b-COMP of the N-terminal region of COMP (Efimov *et al.*, 1994). The PCR primer PC1 at the 5′ end was designed to obtain a *Not*I restriction site within an additional 18 base sequence coding for the second part of the human thrombospondin 4 sequence (PC1: 5′-CAGGCGGCCGCCACGGGGACTGGAGACCTAGCCCCA-CAG-3′). PCR primer PC2 ended up with a stop codon and the restriction site *Xba*I at the very 3′ end (PC2: 5′-GCGCTCTAGACTACACGCTCA-GACCGGGGGTG-3′). The amplified DNA-fragment was then inserted into the expression vector pECAD2 via the restriction sites *Not*I and *Xba*I, leading to plasmid pECAD-COMP.

Recombinant plasmid DNA of pECAD1 and pECAD–COMP was purified from *E.coli* JM83 cloning host strain using a QIAprep spin plasmid kit (Qiagen) and sequenced. In the following, the protein encoded by pECAD1 will be named ECAD, and the protein encoded by pECAD-COMP will be named ECAD–COMP (Figure 1).

Transfection and expression

Human embryonal kidney cells 293 (ATCC; American Tissue Culture Collection) were stably transfected with pECAD1 and pECAD-COMP by the calcium phosphate method using the Cell Phect Transfection Kit protocol (Pharmacia). Dulbecco's medium F12 (Gibco) with 10% fetal calf serum (FCS) was used for transfection as well as for subsequent cell cultures. After 16 h of transfection, the cells were washed with fresh medium and incubated for an additional day. Then medium was replaced by selection medium containing 0.7 mg/ml G-418 (Calbiochem). After 1 week the medium was changed and after an additional week the first clones were picked.

To test the presence of secreted recombinant E-cadherin each clone was grown on a 96-well plate until confluency was reached. Then the cells were left in FCS free selection medium for 2 days. Harvested media were diluted 1/5, 1/25 and 1/125 with FCS-free medium and 50 µl of each dilution were filtered through nitrocellulose BA85 (Schleicher & Schuell). Adsorbed recombinant E-cadherin was detected by the emitting chemiluminescence (ECL) immunodetection method (Amersham), using the DECMA1 rat antibody against the extracellular fragment of mouse E-cadherin and the second antibody conjugated with horseradish peroxidase. Positive clones that showed a clear signal in all three dilutions were subcloned and the screening procedure was repeated until all subclones were positive for E-cadherin secretion.

For large scale production of recombinant protein stably transfected cells were seeded in 6×500 ml flasks (Falcon). When cells had formed a regular layer they were washed once with PBS and overlayed with a small volume of FCS-free medium. After 3 days of expression the media were collected and stored at –20°C. To let the cells recover from starvation they were left in FCS containing medium overnight. This procedure was repeated 4–5 times.

Isolation of the recombinant proteins and sequence analysis

To 180 ml harvested media a cocktail of protease inhibitors was added (final concentrations 0.1 mM PMSF, 1 µg/ml aprotinin, 0.1 µg/ml chymostatin, 0.1 µg/ml leupeptin). After 45 min of DNase I digestion at 37°C in the presence of 5 mM $MgCl_2$ the medium was centrifuged (27 000 *g*, 30 min, 4°C) to eliminate cell debris. The supernatant was then dialysed 3 times against 5 l 20 mM Tris–HCl, pH 7.5 containing 2 mM $CaCl_2$ (standard buffer) at 4°C. The dialysate was subjected to anionic exchange chromatography on DEAE–cellulose (DE52; 1.6×2 cm column, standard buffer, flow rate 30 ml/h) and eluted with 500 mM NaCl. Final purification of ECAD was reached by Superose 12 gelfiltration (Pharmacia; 1×30 cm column, standard buffer plus 100 mM NaCl, flow rate 30 ml/h). ECAD–COMP was purified by Sephacryl S300 gel filtration column (1.6×65 cm, standard buffer plus 100 mM NaCl, flow rate 15 ml/h). Purity of the protein containing fractions was tested by SDS–PAGE (8% or 3–15% acrylamide gels), followed by silver staining. For storage small portions of the preparation were frozen in liquid nitrogen and kept at –70°C.

For sequence analysis samples were transfered from SDS–polyacrylamide gels (3–15% or 8%) onto nitrocellulose membranes and stained with Coomassie Blue G250. Sequence determinations were kindly performed by Dr Paul Jenö (Biozentrum, Universität Basel).

Analytical ultracentrifugation

Sedimentation velocity and sedimentation equilibrium experiments were performed in a Beckman XLA analytical ultracentrifuge equipped with an AN-60Ti rotor using UV scanning at 230 nm and 278 nm in 20 mM Tris–HCl, pH 7.5, 2 mM $CaCl_2$, 100 mM NaCl in the presence or absence of 3 mM EDTA. Equilibrium experiments were carried out at protein concentrations of 0.2–0.5 mg/ml and rotor speeds of 12 000 and 17 000 r.p.m. (ECAD) and 4200 and 6800 r.p.m. (ECAD–COMP). The partial specific volume was assumed to be 0.73 ml/g.

Sedimentation coefficients were evaluated at rotor speeds of 56 000 r.p.m. (ECAD) and 32 000 and 48 000 r.p.m. (ECAD–COMP) and corrected to standard conditions (water, 20°C; Schachman, 1968).

Solid phase binding/inhibition assay

Assays were carried out on Immulon 2/U-plates (Dynatec). Protein concentrations were determined by UV absorption using absorption coefficients 59.4×10^3 cm^{-1} M^{-1} for ECAD and 298.6×10^3 cm^{-1} M^{-1} for ECAD–COMP calculated using Wetlaufer's (1962) formulae. One hundred millilitres per well of 10 nM of the ECAD–COMP protein in 50 mM Tris–HCl, pH 7.5, 2 mM $CaCl_2$ (assay buffer) were adsorbed for 17 h at 4°C. The wells were then blocked for 2 h at room temperature with 3% bovine serum albumin (BSA; Boehringer Mannheim) in assay buffer. All subsequent washing and binding steps were performed in assay buffer with 0.04% TWEEN 20. Soluble ligands (ECAD, ECAD–COMP) biotinylated according to the manufacturer's instructions (Boehringer Mannheim) were incubated in coated wells for 2 h at room temperature. For the inhibition assay biotinylated ECAD–COMP at a concentration of 14 nM was incubated with non-labelled ECAD–COMP or ECAD. Biotinylated protein was visualized after 1–2 h incubation with horseradish peroxidase–streptavidin complex (Bio-Rad) with 2,2′-azino-di-[3-ethylbenzthiazidine-6-sulfonic acid] (ABTS; Bio-Rad). The colour reaction was stopped with 2% oxalic acid and the plates were evaluated in an ELISA reader at 405 nm.

Cell attachment assay

CMT-93 cells, murine epithelial cells from a mouse rectum carcinoma (American Tissue Culture Collection) were cultured in Dulbecco's medium F12 with 10% FCS. Cell attachment assays were performed on Immulon 2/U-plates. The substrates ECAD–COMP, ECAD, coiled-coil domain of COMP (0.1–500 nM) and BSA as negative control (75, 750 nM) were coated as described for the solid phase assays. Substrates were blocked with 1% heat-denatured BSA for 2 h at room temperature (100 µl/well). Cells were detached with 0.05% (w/v) trypsin and 0.02% (w/v) EDTA, resuspended to 10^6/ml in serum free medium, and allowed to recover for 20 min at 37°C. One hundred microlitre aliquots of cells were added to wells and incubated for 15 min at 37°C. Cell adhesion to ECAD–COMP was also determined in the presence of affinity purified polyclonal antibodies against the extracellular fragment of murine E-cadherin. In this case cells were pre-incubated with 5 µg/ml of pAb gp84 for 20 min at 37°C. Unbound cells were aspirated off and attached cells were fixed with 100 µl 5% glutaraldehyde (20 min, room temperature). Crystal violet staining was carried out according to Mould *et al.* (1994). To estimate the reference value of 100% attachment, the cell aliquots were fixed by direct addition of 10 µl of 50% (w/v) glutaraldehyde. The corresponding absorption value was set 100%.

Each sample was assayed in triplicate and corrected for the background signal which was estimated as the mean absorbance of six non-coated wells with no cells attached. Values were normalized to the absorption obtained for maximal number of cells per well (100%).

Electron microscopy

Samples (0.3–0.5 mg/ml) in 20 mM Tris–HCl, pH 7.5 containing 2 mM $CaCl_2$ were 5–10-fold diluted with the same buffer. This solution was mixed 1:1 (v/v) with 80% (v/v) glycerol and sprayed onto freshly cleaved mica. Rotary shadowing with platinum/carbon at an angle of 9°, carbon shadowing at 90°, replica formation and electron microscopy followed earlier protocols (Engel, 1994). For negative staining, ECAD–COMP protein (5 µg/ml) in 20 mM Tris–HCl, pH 7.5, 2 mM $CaCl_2$ was adsorbed to a glow discharged formvar/carbon support film and stained with 2% uranyl formiate.

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