Immobilized dimers of N-cadherin-Fc chimera mimic cadherin-mediated cell contact formation: contribution of both outside-in and inside-out signals

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SUMMARY

Cell adhesion receptors of the cadherin family are involved in various developmental processes, affecting cell adhesion and migration, and also cell proliferation and differentiation. In order to dissect the molecular mechanisms of cadherin-based cell-cell adhesion and subsequent signal transduction to the cytoskeleton and/or cytoplasm leading to adapted cell responses, we developed an approach allowing us to mimic and control cadherin activation. We produced a dimeric N-cadherin-Fc chimera (Ncad-Fc) which retains structural and functional properties of cadherins, including glycosylation, Ca$^{2+}$-dependent trypsin sensitivity and the ability to mediate Ca$^{2+}$-dependent self-aggregation of covered microbeads. Beads covered with either Ncad-Fc or anti-N-cadherin antibodies specifically bound to N-cadherin expressing cells. Both types of beads induced the recruitment of N-cadherin, β-catenin, α-catenin and p120, by lateral mobilization of preexisting cell membrane complexes. Furthermore, cadherin clustering elicited by Ncad-Fc beads triggered local accumulations of tyrosine phosphorylated proteins, a recruitment and redistribution of actin filaments, as well as local membrane remodeling. Beads covered with either Ncad-Fc or anti-N-cadherin antibodies specifically bound to N-cadherin expressing cells. Both types of beads induced the recruitment of N-cadherin, β-catenin, α-catenin and p120, by lateral mobilization of preexisting cell membrane complexes. Consequently, cadherin clustering elicited by Ncad-Fc beads triggered local accumulations of tyrosine phosphorylated proteins, a recruitment and redistribution of actin filaments, as well as local membrane remodeling. Additionally, bead-cell binding was altered by agents promoting microfilament and microtubule depolymerization or microtubule acetylation and tubulin cytoskeletal sequestration, indicating a possible regulation of the adhesive properties of the extracellular domain of N-cadherin by intracellular factors (inside-out signaling).

Key words: Juxtacrine cell interaction, Catenin, Cytoskeleton, Signal transduction, Tyrosine phosphorylation

INTRODUCTION

The widespread and highly conserved cell adhesion molecules of the cadherin family constitute one of the major classes of molecules mediating intercellular adhesion and juxtacrine cell interactions between animal cells. Their contribution to cell aggregation and segregation in conjunction with coordinated cell growth, motility and differentiation is essential for embryogenesis and histogenesis. Thus, cadherins are key morphoregulatory molecules in developmental processes as well as essential contributors to cell-cell cohesion within adult tissues and organs (see for review Edelman, 1986; Takeichi, 1988). In addition, cadherin expression is also regulated by normal cell and tissue functions, while a deregulation of cadherin-mediated adhesion has been associated with alterations of tissue homeostasis (Mareel et al., 1997). Cadherins are transmembrane glycoproteins that mediate calcium-dependent cell-cell adhesion through homophilic Ca$^{2+}$-dependent interactions of their N-terminal extracellular domain (Yap et al., 1997). The conserved cytoplasmic domain of cadherins forms, with catenins α, β, γ and p120, a multimolecular actin-associated complex which links cadherins to the cytoskeleton (Kemler, 1993). Thus, cadherins establish a direct mechanosensing link between the adjacent cell, the plasma membrane and the cytoskeleton (Mège et al., 1988; Volk and Geiger, 1986). Moreover, beside their well-established function in cell adhesion, cadherins may play a role in signal transduction in response to cell adhesion (Yap et al., 1997).

The cadherin extracellular region (ectodomain) is organized in 5 structural domains (EC1 to EC5). The three-dimensional structure of the most N-terminal of these domains, bearing the adhesion site (EC1) has been resolved for N-cadherin (Shapiro et al., 1995) and E-cadherin (Overduin et al., 1995) suggesting a possible antiparallel association of these domains reflecting the homophilic adhesive interactions formed between opposing cells. In addition, these structural data suggested a possible dimerization of cadherin ectodomains in the plane of the membrane, resulting from an association between adjacent parallel EC domains. Based on these structural data, a two...
step model for cadherin interactions has been proposed in which cadherin ectodomains first dimerize then interact with dimers presented by the adjacent cell. In favor of this hypothesis, mutation of residues predicted to be important for lateral dimerization abrogates the ability of N-cadherin to mediate adhesion (Tamura et al., 1998). Furthermore, ectodomain dimerization in vitro has been reported for recombinant E-cadherin and C-cadherin ectodomains (Tomschy et al., 1996; Brieher et al., 1996). Dimerization of E-cadherin in the plane of the membrane has also been demonstrated in living cells using recombinant molecules (Norvell and Green, 1998; Chitaev and Troyanovsky, 1998) or a cross-linking approach (Takeda et al., 1999).

Yet, the molecular mechanisms of cadherin ectodomain interactions and their modulation by intracellular events remain unclear. The most carboxy-terminal part of the cytoplasmic domain of cadherin directly interacts with either β-catenin or plakoglobin (γ-catenin) two closely related and mutually exclusive armadillo-type proteins (Butz et al., 1992; Aberle et al., 1994). α-Catenin, interacting directly with β-catenin or plakoglobin, further links the cadherin/catenin complex to actin filaments directly or through α-catenin (Nieset et al., 1997; Knudsen et al., 1995). While the ectodomains of C-cadherin (Brieher et al., 1996) or N-cadherin (Levenberg et al., 1998) were able to retain some degree of functional activity in vitro, integrity of the whole cadherin/catenin complex and its correct linkage to actin filaments network is required for cadherin optimal adhesive interactions in living cells (Fujimori and Takeichi, 1993; Brieher et al., 1996; Nagauchi and Takeichi, 1988). However, Yap et al. (1998) were able, by laminar flow assay and aggregation assay, to detect significant adhesive activity of C-cadherin deleted of the β-catenin-binding site, while C-cadherin deleted of its whole cytoplasmic domain did not show any activity. A fourth catenin, the phosphoprotein p120, originally described as an src substrate (Reynolds et al., 1992), has been also shown to interact with cadherin/catenin complexes (Staddon et al., 1995; Reynolds et al., 1994). It has been suggested that p120 binds to a juxtamembrane region of cadherin cytoplasmic domain distinct from the β-catenin/plakoglobin binding site, influencing dimerization (Yap et al., 1998) and modulating cadherin adhesive functions (Ohkubo and Ozawa, 1999; Aono et al., 1999). Thus, there are strong evidences for a direct involvement of catenins in the regulation of cadherin function, even if the mechanisms of action of these cytoplasmic molecules remain unclear.

In order to further dissect the molecular mechanisms of cadherin-based adhesion and subsequent signal transduction to the cytoskeleton and/or cytoplasm, we set up a model system allowing us to mimic and control cadherin-mediated interactions. We expressed a chimera containing the whole chicken N-cadherin ectodomain dimerized by the mouse IgG2b Fc domain. Chimera-loaded latex beads self aggregate and specifically bind, in a Ca2+-dependent manner, to N-cadherin-expressing cells. We further show that chimera or anti-N-cadherin coated beads fully mimic cadherin mediated cell-cell interaction, inducing the accumulation of N-cadherin, β-catenin, α-catenin and p120 at the bead-cell contact. Furthermore, coated beads induced a recruitment of tyrosine phosphorylated proteins, the recruitment and redistribution of F-actin and membrane remodelling at the bead/cell contacts.

These results further support a model where adhesive interactions of cadherin ectodomains induce the mobilization in the plane of the membrane of cadherin/catenin complexes, followed by a strong anchorage to actin filaments leading to cytoskeleton redistribution and activation of intracellular signaling (outside-in signals). However, as cadherin-mediated bead/cell binding was inhibited by agents promoting microfilament and microtubule depolymerization or tyrosine phosphorylation, we also propose the existence of a complex regulation ‘en retour’ of the adhesive properties of cadherin by intracellular factors (inside-out signals).

**MATERIALS AND METHODS**

**Plasmids construction**

The 864-bp PstI-EcoRV fragment of the mouse IgG2b cDNA (EMBL MM1GG7) corresponding to the Fc fragment was inserted between the PstI-EcoRV sites of pBluescript (Stratagene, La Jolla, CA) to generate pBSgamma. A PCR fragment, corresponding to the C-terminal end of the chicken N-cadherin ectodomain, between PstI and NsiI sites at nt 2122 and 2190, respectively, was prepared using the oligonucleotides: 5’- CACCTGCAGCTAGCACTT 3’ and 5’- CACATGCATTCAAATCGAGT 3’ and the plasmid pNA18-53 containing the full length chicken N-cadherin cDNA as a template (Matsuzaki et al., 1990). To construct the cDNA coding for the entire chicken N-cadherin ectodomain, including the leader peptide and propeptide, linked to the Fc fragment (Ncad-Fc; Fig. 1A), the 72-bp long PstI-NsiI PCR fragment was co-inserted with the 2780-bp long Xbal-NsiI fragment of pNA18-53, between the Xbal and PstI sites of pBSgamma, to generate pBSCH. The 3716-bp Xbal-EcoRV fragment of pBSCH was then subcloned into the pCEP4 vector (Invitrogen, Leek, Netherlands) to generate pCECH.

**Cell culture, transfection and production of the chimeric Ncad-Fc protein**

The S180 mouse sarcoma cell line and the C2 mouse myogenic cell line (Yaffe and Saxel, 1977) were cultured in Dulbecco modified Eagle’s medium (DMEM) containing 10% fetal calf serum (FCS) at 37°C in 7.5% CO2. S180Ncad (S180 transfected cell line expressing the chicken N-cadherin; Matsuzaki et al., 1990) and S180Edcad ($180 cell line expressing L-CAM, the chicken homologue of E-cadherin; Mège et al., 1988) were cultured at 37°C in 7.5% CO2 in the same medium plus 0.4 mg/ml geneticin. The PC12 rat pheochromocytoma cell line (Greene and Tischler, 1976) was cultured in DMEM containing 5% FCS and 10% horse serum (HS) at 37°C in 5% CO2.

The human embryonic kidney HEK-293 cell line transfected with the EBNA-1 gene (Invitrogen) was cultured in DMEM containing 10% FCS and 0.4 mg/ml geneticin at 37°C in 5% CO2. HEK-293 cells plated at 5x10^3 cells/cm^2 for 2 days were transfected with 2 μg of pCECH DNA, using 6.5 μl Lipofectamine (Life Technologies) following the manufacturer’s protocol. Thirty six hours after transfection, medium was changed and hygromycin (Sigma, St Louis, MO) was added to the medium at 0.5 mg/ml. The medium was changed every 2 days during the 10 day-long selection. Then, the medium was changed to the serum-free AIMV synthetic medium (Life Technologies). The AIMV production media were collected every 2-3 days and assayed for the presence of the fusion protein by western blot analysis.

**Chimeric Ncad-Fc protein purification**

The AIMV production medium was centrifuged to remove floating cells and debris and concentrated 10-fold on BioMax-100 membrane (Millipore Corporation, Bedford, MA). The medium was then incubated overnight under gentle agitation at 4°C with Protein
Protein extracts were then separated on 8% SDS-polyacrylamide gels. Living cells were directly scraped and boiled in Laemmli buffer. Protein extraction and western blots were then analyzed by western blotting with a HRP-conjugated goat anti-mouse IgG (1/10000 dilution, Sigma), a goat anti-mouse Fc fragment-specific antibody with gentle mixing (1/5000 dilution, Dako, Copenhagen, Denmark). Molecular masses of proteins of interest were determined graphically on a log molecular mass versus relative mobility (Rf) calibration curve, obtained with a high molecular mass standard mixture (29 to 205 kDa) and a crosslinked phosphorylase b SDS molecular mass standard (Sigma).

Trypsin digestion
Beads loaded with chimera proteins were incubated in PBS alone or in PBS containing 0.005% trypsin (Life Technologies) for 30 minutes at 37°C either in the presence of 2 mM Ca^2+ or in the presence of 2 mM EGTA. Untreated or trypsin-digested beads were directly boiled in sample buffer (Laemmli, 1970) and proteins separated on 8% SDS-polyacrylamide gels either in non-reducing or reducing conditions.

G-Sepharose (Protein G-Sepharose 4 fast flow, Pharmacia). Protein G-Sepharose was collected by centrifugation, transferred to an empty chromatography column and extensively washed with PBS. Protein G-bound proteins were then eluted with a 0.1 M glycine buffer, pH 2.8. Eluted fractions were immediately neutralized by addition of 1 M Tris-HCl, pH 9, and their protein content was determined by dot blot analysis against a mouse Fc standard. Chimera-containing fractions were then pooled, snap-frozen and stored at –80°C.

Ncad-Fc chemical crosslinking
About 20 μg of Protein G-purified chimeric Ncad-Fc protein were dialyzed against phosphate buffer saline, pH 7.4 (PBS) on 6-8000 kDa Spectra/Por membrane (Spectrum Medical Industries, Los Angeles, CA). Crosslinking reaction was performed in the presence of 1 mM ethylene glycolbis(succinimidylsuccinate) (EGS, Pierce, Rockford, IL) for 30 minutes at room temperature according to manufacturer’s instructions. The reaction was quenched for 15 minutes with 1 M Tris-HCl, pH 7.5. Proteins were then separated on 5% SDS-polyacrylamide gels and western blot analysis was performed using successively the GC4 anti-chicken N-cadherin monoclonal antibody (1/10000 dilution, Sigma), a goat anti-mouse Fcγ fragment-specific antibody (1/2000 dilution, Jackson Immunoresearch, West Grove, PA) and a HRP-conjugated anti-goat immunoglobulin antibody (1/5000 dilution, Dako, Copenhagen, Denmark). Molecular masses of proteins of interest were determined graphically on a log molecular mass versus relative mobility (Rf) calibration curve, obtained with a high molecular mass standard mixture (29 to 205 kDa) and a crosslinked phosphorylase b SDS molecular mass standard (Sigma).

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Protein extraction and western blots
Living cells were directly scraped and boiled in Laemmli buffer. Protein extracts were then separated on 8% SDS-polyacrylamide gels and transferred to 0.45 μm nitrocellulose membranes. Membranes were blocked in 5% milk in 12 mM Tris-HCl, pH 7.4, 160 mM NaCl, 0.1% Triton X-100 for 1 hour and probed in 1% milk in the same buffer with either a polyclonal anti-α-catenin antibody (1/10000 dilution; Sigma), a monoclonal anti-β-catenin antibody (1/500 dilution; Transduction Laboratories, Lexington, KY), or a monoclonal anti-p120 catenin antibody (1/1000 dilution, Transduction Laboratories). Membranes were then probed with either HRP-conjugated anti-mouse or anti-rabbit immunoglobulin antibodies (both at 1/5000 dilution; Dako) and developed using the ECL method (Amersham, Amersham, UK). Western blotting analysis of the chimeric Ncad-Fc protein was performed in similar conditions.

Preparation of coated beads
Latex-sulfate microspheres (3x10^6, diameter 6.2 μm; Interfacial Dynamics Corporation, Portland, Or) were washed, resuspended in 0.2 ml of 0.1 M borate buffer, pH 8, and then incubated with 100 μg goat anti-mouse Fcγ fragment-specific antibody with gentle mixing for 18 hours at room temperature. Beads were then centrifuged at 16,000 g for 10 minutes at 4°C and washed 3 times with 2 ml PBS. Beads were then incubated with PBS containing 5 mg/ml bovine serum albumin (BSA) for 1 hour at room temperature. Aliquots of 200 μl bead suspension (6x10^7 microspheres) were then charged with each protein of interest. Bead aliquots were sonicated, then incubated with either 4 ml of AIMV production medium, 30 μg of the GC4 monoclonal mouse anti-chicken N-cadherin antibody, 30 μg of purified Fc fragment (Jackson Immunoresearch), or 3 μl of mouse non-immune serum in 200 μl of PBS/BSA. Beads were then washed 3 times in 1 ml PBS/BSA and resuspended in 200 μl PBS/BSA. Alternatively, microspheres were loaded in one step with poly-DL-ornithine (8 μg/200 μl, Sigma) following the same procedure.

Bead aggregation and bead-cell adhesion assays
Sonicated beads (1 μl, diluted in 250 μl of DMEM, 10% FCS) were placed on a three-well glass slide (Super-Teflon, 14 mm diameter well, Polylabo, France) in the absence or in the presence of 2.5 mM EGTA. Slides were then placed at 37°C in the cell incubator (5% CO2) and the aggregation evaluated under the microscope at regular time points. For quantification, after 45 minutes of aggregation, bead suspensions were randomly photographed under the microscope and the number and size of bead aggregates were scored. The aggregation index was determined as (the total number of counted beads minus the number of aggregates) divided by the total number of counted beads.

Drug treatments
Cells were pretreated for 10 minutes either with 10 μM nocodazole directly added to the culture medium from a 10 mM stock solution in DMSO, or with 1 μg/ml cytochalasin D, also directly added to the culture medium from a 2 mg/ml stock solution in DMSO. In some experiments, cells were either pretreated for 10 minutes with pervanadate (50-100 μM pervanadate from a 100 mM vanadate/1.5 mM H2O2 aqueous stock solution) or for 1 hour with brefeldin A-5 (μg/ml from a 5 mg/ml stock solution in methanol, Sigma). Alternatively, cells were pretreated for 1 hour with brefeldin A plus bafilomycin A1 (1 μM from a 100 μM stock solution in DMSO; Sigma). The action of brefeldin A alone or in conjunction with bafilomycin A1 on S180Ncad cells was verified by the absence of N-cadherin cell surface recovery and cell-cell contact reformation after cell surface N-cadherin trypsinisation in the presence of Ca2+ (not shown).

Immunofluorescent staining, conventional and confocal microscopy
Cell cultures were fixed for 10 minutes at room temperature with 3% formaldehyde in PBS and then washed 3 times 5 minutes with PBS, 0.1 M glycine. Preparations were then permeabilized with Triton X-100 0.5% in PBS for 5 minutes. After 1 hour of blocking in PBS, BSA 1.5%, cells were incubated in the same buffer with either a polyclonal anti-pan-cadherin antibody (1/200 dilution, Sigma), a polyclonal anti-α-catenin antibody (1/5000 dilution, Sigma), a polyclonal anti-β-catenin antibody (1/500 dilution, Sigma), a polyclonal anti-p120 catenin antibody (1/500 dilution, Sigma), a polyclonal anti-p120 antibody (1/1000 dilution, Transduction Laboratories), a polyclonal anti-phosphotyrosine (anti-P-tyr) antibody (1/200 dilution, Upstate Biotechnology, NY) or a monoclonal anti-P-tyr antibody (1/200 dilution, Transduction Laboratories). The preparations were washed 3 times 5 minutes with PBS and incubated for 1 hour at room temperature with a FITC-conjugated anti-rabbit IgG (Jackson Laboratories) or a FITC-conjugated anti-mouse IgG (Biosys, Compiègne, France) both diluted 1/500 in PBS/BSA. For F-
RESULTS

Biochemical analysis of the Ncad-Fc chimera produced in eukaryotic cells

In order to mimic and control cadherin-mediated adhesion, a recombinant chimeric protein (Ncad-Fc) comprising the chicken N-cadherin extracellular domain fused to the Fc domain of the mouse IgG2b was produced in HEK-293 cells (Fig. 1A). The production of chimera by the transfected cells, evaluated by western blots, was close to 2 µg/10^6 cells/day of culture (data not shown). The secreted chimera migrated as a single 130 kDa band on reducing SDS-PAGE gels (Fig. 1B, lane 1). In non-reducing conditions, anti-mouse IgG antibodies revealed a band of about 240-300 kDa suggesting that the chimera was indeed secreted as a dimer (Fig. 1B, lane 2). A minor band of about 160 kDa was also revealed. This band which was almost undetectable in some batches of production medium, never exceeded 5% of the total immunoreactive material, and may correspond to partially degraded molecules.

In order to demonstrate that the band migrating at 240-300 kDa in non-reducing gels was actually a dimer, we performed chemical crosslinking experiments on Protein G-purified Ncad-Fc chimera (Fig. 1C). In order to evaluate with more accuracy the apparent molecular mass of the non-crosslinked monomer and of the crosslinked oligomeric form of the chimera, the reaction products were separated on 5% acrylamide gels in the presence of β-mercaptoethanol followed by western blotting. The incubation of Ncad-Fc chimera with the amine-reactive crosslinker EGS resulted in the formation of only two species (lane 2). The lower band, very faint, ran at a molecular mass of 130 kDa, consistent with the molecular mass of a monomer. This band was the only one detected in the absence of crosslinker (lane 1). In contrast, the upper band was the major one detected after crosslinking. This band with an apparent molecular mass of 300 kDa, ran slightly higher than the expected size for a dimer (260-270 kDa), but no other intermediate band was detected, indicating that this band corresponds to a dimer. Notice the substantial increase in electrophoretic mobility of the dimer in the absence of β-mercaptoethanol (Fig. 1C, lane 3) corresponding to a calculated apparent molecular mass of 270 kDa.

Analysis of the trypsin sensitivity of the recombinant Ncad-Fc protein

To further characterize the biochemical properties of the Ncad-Fc protein we first asked whether this fusion protein had conserved the specific properties of classical cadherins towards trypsin digestion (Fig. 2). The chimera was submitted to

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Scanning electron microscopy

Cells were cultured on glass coverslips for 24 hours, then coated beads were added for 45 minutes before cells were fixed in 2.5% glutaraldehyde, 0.1 M Na cacodylate at pH 7.4 for 1 hour at 4°C. Samples were dehydrated in graded ethanol baths before critical-point drying with liquid CO₂, coated with gold-palladium and observed with a Jeol JSM 840A SEM operated at 17 kV.

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Fig. 1. Construction, production and biochemical analysis of the Ncad-Fc dimeric chimera. (A) Schematic representation of the Ncad-Fc construct cDNA and of the mature secreted protein. pp: leader peptide and propeptide of the chicken N-cadherin; N-cad extra: chicken N-cadherin extracellular domain (ectodomain; amino acids 1-720); h and Fc: hinge and Fc domain of the mouse IgG2b immunoglobulin heavy chain, respectively (amino acids 83-336). The mature secreted Ncad-Fc chimeric protein is expected to dimerize by disulfide bridges in the IgG hinge domain. (B) Recombinant proteins were immunoprecipitated from 30 µl of HEK-293 transfected cell culture medium on goat anti-mouse Fc antibody beads, and directly processed for SDS-PAGE either in the presence (+bME) or in the absence of β-mercaptopoethanol (-bME). Anti-mouse IgG antibody immunoblotting revealed a protein of 130 kDa in reducing conditions (lane 1) and a major band of 240-300 kDa in non-reducing conditions (lane 2) suggesting that the chimera is indeed secreted as a dimer. A faint band running at a molecular mass of about 160 kDa was also detected, which may correspond to a degradation product. (C) Protein G-purified Ncad-Fc chimera (10 µg in PBS) was incubated for 30 minutes in the absence (NT) or in the presence (CL) of 1 mM ethylene glycolbis(succinimidylsuccinate) (EGS). Crosslinking reaction products were then separated on 5% SDS-PAGE in the presence (+bME) or in the absence of β-mercaptopoethanol (-bME), and immunoblotted with the GC4 anti-chicken N-cadherin monoclonal antibody, and a goat anti-mouse Fcγ fragment specific antibody. In the absence of crosslinker (lane 1), the reduced monomeric form had an apparent molecular mass of 130 kDa. After crosslinking (lane 2), the monomer was barely detectable compared to the major band migrating at 300 kDa, corresponding to the crosslinked dimer. Note the increased electrophoretic mobility of the dimer in non-reducing conditions (lane 3).
trypsin digestion in the presence or in the absence of Ca\(^{2+}\) and the digestion products were separated by non-reducing SDS-PAGE and analyzed by immunoblotting with an anti-mouse IgG antibody (Fig. 2A). The untreated immunoreactive material migrated as a single band of about 270 kDa (lane 1). Trypsin treatment in the presence of Ca\(^{2+}\) only led to a mild reduction in the intensity of this band attributed to the dimer (lane 2). In contrast, this 270 kDa band had totally disappeared after the incubation of the protein in the presence of trypsin and EGTA (lane 3), indicating that the dimer was largely protected from trypsin digestion by Ca\(^{2+}\). Mild trypsin digestion in the presence of Ca\(^{2+}\) led to the appearance of two additional bands running at a molecular mass of 160 kDa and 50 kDa, respectively. In contrast, after trypsin digestion in the presence of EGTA, the 160 kDa species, likely comprising a full length monomer linked to a cleaved Fc fragment, had totally disappeared and there remained only a band migrating around 50 kDa likely corresponding to dimers of cleaved Fc fragments.

To confirm that the chimera was preserved from trypsin digestion in the presence of Ca\(^{2+}\), the same trypsin digestion products were separated by reducing SDS-PAGE and immunoblotted with the GC4 anti-N-cadherin monoclonal antibody and anti-mouse Fc fragments (Fig. 2B). In these conditions the undigested Ncad-Fc chimera migrated as a 130 kDa band (lane 1). The chimera was largely preserved from trypsin digestion in the presence of Ca\(^{2+}\) (lane 2), while it was totally degraded in the presence of EGTA (lane 3). An additional 80-90 kDa band was generated in the presence of trypsin plus Ca\(^{2+}\), which was specifically labelled by the GC4 antibody (lane 2). This band which was not recognized by anti-mouse immunoglobulin antibodies (not shown), likely corresponds to cadherin ectodomains, previously shown to be resistant to further trypsin digestion in these conditions (Gallin et al., 1983). Altogether, these observations indicate a protective effect of Ca\(^{2+}\) ions on the Ncad-Fc chimera against trypsin digestion.

**Immobilized Ncad-Fc retains Ca\(^{2+}\)-dependent adhesive properties of native N-cadherin**

In order to determine the adhesive properties of the Ncad-Fc chimera, the molecule was immobilized on 6 \(\mu\)m latex beads. The quality and quantity of the proteins retained by immunoaffinity on the beads was tested by western blot analysis. Its density was evaluated to be about 7 \(\times\)10^5 dimers/bead. The aggregation properties of the Ncad-Fc-coated beads were then investigated and compared to the aggregation of beads presenting at their surface either Fc fragments (Fc), pre-immune mouse IgGs (PI), an anti-chicken N-cadherin monoclonal antibody (GC4) or anti-Fc antibodies (IgG). The Ncad-Fc beads rapidly self-aggregated while all the other types of beads remained in majority unaggregated, as illustrated by the quantitative analysis of the number of Ncad-Fc-coated or Fc-coated beads engaged in aggregates after 45 minutes of incubation (Fig. 3A). While the aggregation index ranged from 25±2% to 32±1% and 38±7% for Fc, PI and GC4 coated beads, respectively, this index reached 63±10% for Ncad-Fc beads (significantly different from the formers, \(P<0.01, n=3\)). Furthermore, the bead aggregation induced by immobilized dimeric N-cadherin ectodomain was dependent on the presence of free Ca\(^{2+}\) since the aggregation index of the Ncad-Fc beads dropped down to 22±2% in the presence of 2 mM EGTA (see also Fig. 3A).

**Immobilized Ncad-Fc allows Ca\(^{2+}\)-dependent bead-cell binding**

We then asked whether the Ncad-Fc chimera could also induce specific binding of coated beads to N-cadherin-expressing...
cells. As shown in Fig. 3B, Ncad-Fc beads bound to transfected S180 cells expressing the chicken N-cadherin (S180Ncad) but also to C2 mouse myogenic cells and PC12 rat neuron-like cells expressing endogenous rodent N-cadherins. After 45 minutes of incubation, 60±2% of the S180Ncad cells, 42±1% of the C2 cells and 58±19% of the PC12 cells presented Ncad-Fc beads bound at their surface. This binding was highly specific since the binding of control beads (PI, Fc and IgG beads) in the same conditions never exceeded 3±1%. Further supporting a specific N-cadherin mediated bead-cell binding, Ncad-Fc-coated beads did not bind to cells expressing no cadherin (S180 cells) or to cells expressing E-cadherin (S180Ecad). In this bead-cell assay, as well as in further experiments, we compared the properties of the immobilized chimera to those of the immobilized anti-chicken N-cadherin monoclonal antibody GC4 (Fig. 3B). GC4 beads attached to S180Ncad cells almost as efficiently as Ncad-Fc beads (48±6% of cells with at least one GC4 bead bound), but did not bind to S180 and S180Ecad cells. Moreover, they did not bind either to C2 or PC12 cells expressing mouse and rat N-cadherins respectively. Thus, the Ncad-Fc chimera, as well as anti-N-cadherin antibodies, induce the specific binding of coated beads to N-cadherin-expressing cells. In addition, the binding of Ncad-Fc beads was prevented in the presence of 2 mM EGTA, whereas the binding of GC4-coated beads was not affected in the absence of free Ca2+ (data not shown).

**Ncad-Fc bead-cell binding is dependent on both microtubule and microfilament integrity and tyrosine phosphorylation**

Since cadherin-mediated cell-cell adhesion has been reported previously to depend on the integrity of the cadherin cytoskeleton linkage (Matsuzaki et al., 1990; Nagafuchi and Takeichi, 1988), we asked whether the adhesive properties of the Ncad-Fc chimera would be affected by cytoskeleton disorganization. Previous to bead-cell binding assay, S180Ncad and C2 cells were pretreated with either nocodazole or cytochalasin D, inducing a depolymerization of microtubules and microfilaments respectively. As shown in Fig. 4, Ncad-Fc bead binding to N-cadherin-expressing cells was dramatically inhibited following both nocodazole and cytochalasin D treatments. Since, protein phosphorylation on tyrosine has also been proposed to affect cadherin-mediated adhesion (Ozawa and Kemler, 1998), the effect of increased tyrosine phosphorylation by treatment with the tyrosine phosphatase inhibitor pervanadate was evaluated on bead-cell interactions. Pretreatment of S180Ncad or C2 cells with 500 µM pervanadate dramatically increased the whole intracellular anti-phosphotyrosine immunostaining and furthermore totally prevented Ncad-Fc bead binding (data not shown). Thus, the constitutive homophilic adhesion of immobilized N-cadherin ectodomains appears to depend, in the bead-cell configuration, on intracellular factors such as microtubule or microfilament integrity and the level of protein phosphorylation on tyrosine.

**N-cadherin and catenins α and β are all recruited at the Ncad-Fc bead-cell contact sites**

In order to determine to what extent Ncad-Fc chimera could mimic cadherin-mediated cell-cell interactions, we asked whether coated beads could induce the recruitment of the various molecular components of the cadherin-catenins...
complex. The subcellular distribution of N-cadherin, revealed with a pan-cadherin antibody directed against the cytoplasmic domain of N-cadherin, was determined in S180Ncad cells on which Ncad-Fc beads had been allowed to bind for 45 minutes (Fig. 5A). Accumulations of N-cadherin immunostaining were indeed detected both at the cell-cell contacts and at the Ncad-Fc bead-cell contacts. Similar or even stronger accumulations of N-cadherin immunostaining were also detected at the sites of contact between the GC4 anti-N-cadherin-loaded beads and S180Ncad cells (Fig. 5B). In contrast, no accumulation of cadherin was detected at the very rare sites of contact between Fc-coated beads and S180Ncad cells (not shown). In order to determine if catenins were also recruited at the sites of bead-cell contact similar preparations were stained with either anti-β-catenin or anti-α-catenin antibodies. Accumulations of both β-catenin and α-catenin staining were detected in S180Ncad cells both at the cell-cell contacts and at the Ncad-Fc bead-cell (Fig. 5C,E) or GC4 bead-cell contacts (Fig. 5D,F). In addition, strong accumulations of β-catenin and α-catenin immunostainings were also detected at the sites of Ncad-Fc bead-cell contact in another cell system, the C2 cells (not shown). In order to ensure that this recruitment was a specific response to cadherin-mediated interaction, rather than a non-specific artifact induced by any bead-cell surface interaction, S180Ncad and C2 cells were incubated with poly-ornithine loaded beads. Such beads bound very efficiently to the cells. However, accumulations of catenin immunostaining were never detected at the sites of contact between poly-ornithine beads and S180Ncad and C2 cells (inset in Fig. 5D, and data not shown).

We next asked whether this recruitment was due to a local incorporation in the cell membrane of newly synthesized proteins or to a lateral recruitment of cadherin/catenin complexes already present in the plasma membrane. To address this question, Ncad-Fc and GC4 beads were applied on S180Ncad cells treated with brefeldin-A, a blocker of protein transfer from the Golgi apparatus to the plasma membrane. Strong accumulations of β-catenin immunostaining were still detected at the bead-cell contacts after brefeldin-A treatment (not shown), suggesting that β-catenin recruitment was independent of the addressing of newly synthesized proteins to the plasma membrane. Furthermore, pretreatment of the cells with brefeldin A and bafilomycin A1, reported to inhibit both transferrin receptor and E-cadherin recycling back to the cell membrane (Luan Le et al., 1999) did not prevent either the recruitment of β-catenin at the bead-cell contacts (not shown), indicating that this recruitment is also independent of recycling and likely results from a lateral diffusion of complexes already present at the plasma membrane.

The catenin p120, relocalized upon cadherin expression, is differently recruited at the bead-cell contact sites

We then evaluated the effect of bead-induced N-cadherin mobilization on the localization of the catenin p120. First, the steady state levels of this catenin in untransfected and N-cadherin-transfected S180 cells were determined by western blots and compared to those of α- and β-catenins. We observed very low, albeit detectable levels of α and β-catenins in untransfected S180 cells, and a strong elevation of the steady state levels of these two catenins in S180Ncad (Fig. 6A,B) and S180Ecad (not shown) transfected cells. In contrast p120 protein expression was already high in S180 cells and no further increase was observed in S180Ncad cells (Fig. 6B). However, while p120 immunostaining appeared exclusively cytosolic in S180 cells (Fig. 6C), it was mainly membranous with strong accumulations at the cell-cell contacts in S180Ncad cells (Fig. 6D). Thus, upon N-cadherin expression, while β-catenin and α-catenin cell contents were strongly increased and

![Fig. 4. Inhibition of Ncad-Fc bead-cell binding by cytochalasin D and nocodazole. Bead-cell binding assays were performed on S180Ncad (A) and C2 cells (B) treated with 10 μM nocodazole or 1 μg/ml cytochalasin D. The effect of these drugs on microtubule and microfilament depolymerization was followed by anti-α-tubulin immunostaining and phalloidin-Alexa staining, respectively (not shown). Ncad-Fc bead binding on treated cells was compared to Ncad-Fc bead binding on vehicle-treated cells (0.1% DMSO) and to unspecific binding of Fc coated beads. Data represent mean values ± standard deviation. Nocodazole (Nc) and cytochalasin D (CD) treatments strongly inhibited Ncad-Fc beads binding to both cell types (P<0.05 and P<0.01 versus control for nocodazole and cytochalasin treatment, respectively).]
proteins recruited at the membrane, p120 appeared only relocated to the membrane.

In order to determine whether Ncad-Fc and antibody-coated beads were also able to recruit p120 at the bead-cell contacts, S180Ncad cells bearing coated beads were immunolabeled with an anti-p120 polyclonal antibody (Fig. 6E,F). Surprisingly, no accumulation of p120 was detected at the sites of contact between Ncad-Fc beads and S180Ncad cells, while strong accumulations of p120 immunostaining were detected in the same cell type at GC4-loaded bead-cell contacts (Fig. 6F). However, in C2 cells, strong accumulations of p120 staining were observed with Ncad-Fc beads (insert Fig. 6E), indicating that, at least in these latter cells immobilized Ncad-Fc chimera was able to induce the recruitment of p120.

N-cadherin-mediated binding triggers tyrosine phosphorylated molecules accumulations at the sites of cell-cell and bead-cell contact

Another signature of cell-cell contacts, also shared with cell-substratum contacts, is the accumulation of tyrosine phosphorylated molecules (Kinch et al., 1997). Thus, the accumulation of anti-P-tyr immunostaining at the sites of cell-cell and bead-cell contact was evaluated in the S180 as well as C2 cell model systems (Fig. 7). In both S180 and S180Ncad cells, strong anti-P-tyr staining was associated with focal contacts (not shown). In addition, strong anti-P-tyr staining was also found associated with the numerous and extensive cell-cell contacts established between S180Ncad cells (Fig. 7B). However, accumulations of anti-P-tyr staining were also found in S180 cells at the few cell-cell contacts established between these cells (Fig. 7A). In order to determine whether the accumulation of tyrosine phosphorylated molecules may, however, be directly related to cadherin-mediated adhesion, the ability of Ncad-Fc and GC4-coated beads to induce the accumulation of tyrosine phosphorylated molecules was evaluated first in the S180Ncad cells. Indeed, accumulations of anti-P-tyr staining were detected at the sites of Ncad-Fc or GC4 loaded bead-cell contacts (Fig. 7C-D). Similarly, accumulations of anti-P-tyr staining were also detected at the cell-cell contact (Fig. 7E) as well as at the Ncad-Fc bead-cell contacts in the C2 cells (Fig. 7F). Moreover, P-tyr recruitment appears as a specific response to cadherin-mediated bead-cell binding since no accumulation of anti-P-tyr immunostaining was detected at the sites of contact between poly-ornithine coated beads and S180N-cad (insert in C) or C2 cells (insert in F).

Immobilized Ncad-Fc induces the recruitment of actin filament at the bead-cell contacts

Since all the known components of the cadherin/catenin complex were recruited following the initial N-cadherin-mediated adhesion mimicked by immobilized N-cadherin ectodomains, we speculated that cytoskeleton modifications could also be induced under the coated beads. Actin
filaments were immunofluorescently labeled with Alexa-conjugated phalloidin on S180Ncad cells on which Ncad-Fc (Fig. 8A) or GC4 (not shown) loaded beads had attached. In these cells, presenting only poorly organized microfilaments, phalloidin only diffusely stained the cell cortex and no stress fibres were visualized. There was only a weak or no accumulation of phalloidin staining at the bead-cell contact. Nevertheless, phalloidin-labeled filopodia or lamellipodia were often observed at the bead-cell contact. Microfilament modifications triggered by N-cadherin-mediated adhesion were further investigated in C2 cells (Fig. 8B), where phalloidin staining revealed the presence of numerous stress fibres. In these cells, strong accumulations of F-actin were detected around the bound Ncad-Fc beads as well as numerous stress fibres ending at the bead-cell contact site. Yet, actin cytoskeleton remodeling was not observed at the sites of contact between poly-ornithine beads and C2 cells (not shown). Thus, Ncad-Fc-coated beads fully mimic the formation of N-cadherin-mediated cell-cell contacts, not only recruiting all the components of the cadherin/catenin complex but also inducing a remodeling of the actin cytoskeleton.

**Ncad-Fc bead-cell binding elicits cell membrane extension and bead internalization**

To further characterize the bead-cell contacts, S180Ncad cells bearing either Ncad-Fc or GC4-loaded beads were examined by scanning electron microscopy (Fig. 9). After 45 minutes of incubation, both types of beads appeared intimately associated with cell membranes. In most cases, cells had extended long filopodia (up to 2-3 µm long) over the bead surface, reminiscent of the numerous intermingled filopodia observed at cell-cell contacts (Fig. 9A,C). Some beads were even more intimately associated with the cells which had sent large lamellipodia tending to envelop the beads (Fig. 9B,D). Bead internalization was observed after a longer period of incubation (15 hours) by direct immunofluorescent staining with an anti-mouse Fc fragment labeling the Ncad-Fc proteins loaded on the beads (not shown). Actually, while free or cell surface-bound beads were intensely labeled, internalized beads were totally negative, as a result of the degradation of bead-bound proteins. Both types of beads were internalized, although Ncad-Fc bead internalization was 2- to 3-fold more efficient than that of antibody-coated bead.
To dissect the molecular mechanisms of cadherin-based adhesion and growth, allowing adapted cell responses in terms of cell-matrix or cell-cell interactions, we worked out a model system allowing us to study the subsequent signal transduction to the cytoskeleton or other intracellular factors. Further supporting this hypothesis, we observed that soluble Ncad-Fc did not bind to N-cadherin-expressing cells, as expected from the restricted species specificity of the antibody. Although the actual binding was largely underestimated by our quantification method, we found that Ncad-Fc induced at least a 50-fold increase of bead-cell binding over background, while Levenberg et al. (1998) only obtained a 3-6 fold increase in a similar assay, using a bacterial recombinant N-cadherin ectodomain. This result further shows the efficiency of our reagent due to either the dimerization and/or the glycosylation of the ectodomain, or alternatively, to the method of immobilization by immobilization affinity that may ensure a high density of correctly oriented, active molecules. Moreover, we showed that Ncad-Fc induced strong Ca\(^{2+}\)-dependent self-aggregation of beads, indicating that the N-cadherin ectodomain alone had retained adhesive properties. The immobilization of the cadherin ectodomain dimers may mimic an ectodomain presentation or dimerization and/or the glycosylation of the ectodomain, or structure usually provided in the cells by cytoskeleton anchorage or other intracellular factors. Further supporting this hypothesis, we observed that soluble Ncad-Fc did not bind to N-cadherin-expressing cells, while Ncad-Fc pre-aggregated by an anti-mouse antibody did (unpublished observations).

One of the major contributions of this work was to show that immobilized Ncad-Fc not only mediates the initial bead-cell binding but also induces the recruitment of N-cadherin as well as the other components of the cadherin/catenin complex. The absence of effect of brefeldin-A treatment on β-catenin recruitment, in conditions where recycling was also inhibited.
expression, likely as a result of protein stabilization (Ozawa et al., 1998). Indeed, while the steady state levels of $\alpha$- and $\beta$-catenins were increased upon cadherin expression on one hand and $p120$ on the other hand. However, in contrast to $\alpha$- and $\beta$-catenins, $p120$ was not recruited at sites of contact between S180Ncad cells and Ncad-Fc beads. Nevertheless, strong accumulations of $p120$ were detected at the contact sites between S180Ncad cells and antibody-loaded beads and between C2 cells and Ncad-Fc beads. These differences in $p120$ recruitment, despite the fact that the two cell lines contain similar levels of protein (data not shown), reveal the existence of so far unidentified factors associated with N-cadherin ectodomain activation and/or intracellular environment which are essential for the recruitment of adhesion complexes. In the future, the identification of these critical factors will be of major importance to further progress in the understanding of the molecular basis of cadherin-mediated adhesion. Even if such discrepancies are not understood so far, these results clearly show that conditions exist where N-cadherin, $\alpha$-, and $\beta$-catenins can be recruited without substantial recruitment of $p120$. These results may suggest that $p120$ can be recruited as a consequence of clustering of cadherin/catenin complexes instead of being actively involved in this process as previously proposed (Brieher et al., 1996; Ohkubo and Ozawa, 1999). $p120$ recruitment may constitute one of the first steps of a signaling cascade initiated in response to cell adhesion.

Cadherin-mediated adhesion has been proposed to influence various cellular responses such as cell growth arrest and elevation of p27 (Levenberg et al., 1999), as well as activation of muscle cell differentiation (Goichberg and Geiger, 1998). The immobilized chimera developed here appears particularly well adapted to the search for either early or later intracellular responses elicited by cadherin activation and leading to these various cellular responses. We show here that Ncad-Fc-mediated cadherin activation elicits intracellular signaling leading to the relocation and/or production of tyrosine phosphorylated proteins, the modification of actin cytoskeleton as well as the induction of local cell membrane remodeling. We confirmed that cadherin expression induces the mobilization at the cell-cell contact of a pool of tyrosine phosphorylated proteins (Kinch et al., 1997). Interestingly, anti-phosphotyrosine immunoreactive proteins were present at contact sites occasionally formed between S180 cells that do not express cadherins. Nevertheless, the recruitment of at least some of these phosphoproteins is directly under the dependence of cadherin-mediated adhesion, since proteins phosphorylated on tyrosine were specifically recruited at contact sites occasionally formed between S180Ncad cells and Ncad-Fc beads. These differences in $p120$ recruitment, despite the fact that the two cell lines contain similar levels of protein (data not shown), reveal the existence of so far unidentified factors associated with N-cadherin ectodomain activation and/or intracellular environment which are essential for the recruitment of adhesion complexes. In the future, the identification of these critical factors will be of major importance to further progress in the understanding of the molecular basis of cadherin-mediated adhesion. Even if such discrepancies are not understood so far, these results clearly show that conditions exist where N-cadherin, $\alpha$-, and $\beta$-catenins can be recruited without substantial recruitment of $p120$. These results may suggest that $p120$ can be recruited as a consequence of clustering of cadherin/catenin complexes instead of being actively involved in this process as previously proposed (Brieher et al., 1996; Ohkubo and Ozawa, 1999). $p120$ recruitment may constitute one of the first steps of a signaling cascade initiated in response to cell adhesion.

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Fig. 8. Recruitment and redistribution of actin at bead-cell contact sites. S180Ncad (A) and C2 (B) cells were incubated for 45 minutes with Ncad-Fc beads before fixation and immunofluorescently stained with phalloidin-Alexa. Bead positions at the cell surface are indicated by asterisks. S180Ncad cells (A: 1 μm confocal optical section) showed only poorly organized F-actin and only weak or no accumulations under the bead. In C2 cells (B: conventional light microscope view), strong F-actin accumulations (arrowhead) were detected under the beads as well as stress fibres ending at bead-cell contacts (arrow). Bars, 5 μm.

Fig. 9. Cell membrane remodeling elicited by Ncad-Fc coated beads. S180Ncad cells incubated with Ncad-Fc beads were fixed and processed for scanning electron microscopy as described in Materials and Methods. Both cell-cell and bead-cell contacts were characterized by the presence of extensive cell membrane filopodia extensions (A,C). Some beads were more intimately associated with cell surface and covered by lamellipodia extensions (B,D). Similar observations were made with GC4 beads. Bar, 1 mm.
dynamics, N-cadherin-mediated bead-cell binding induced also local modifications of the cell membrane, characterized by the formation of filopodia and lamellipodia. The lamellipodia covering in some cases the beads may represent the first step in the process of bead internalization observed at longer times of incubation in the present and previous studies (Levenberg et al., 1998). Since a differential internalization of chimera and antibody-coated beads was noticed, we propose that bead internalization is a specific cell response to cadherin activation in the absence of an active cell partner. Interestingly enough, it has been shown that the interaction between the L. monocytogenes protein internalin and cellular E-cadherin promotes bacterium entry into the cells (Mengaud et al., 1996) and that internalin-coated beads interacting with E-cadherin-expressing cells, are specifically internalized (Lecuit et al., 1997). Altogether, these observations show that direct cadherin-receptor triggering leads to complex cellular responses including cytoskeleton redistribution and intracellular signaling. Although N-cadherin ectodomain alone induced strong bead-binding, we also observed that perturbations of either microfilament or microtubule networks alter N-cadherin-mediated bead-cell interactions. If the requirement of a linkage of cadherin/catenin complexes to actin filaments has already been documented (Matsuzaki et al., 1990; Nagafuchi and Takeichi, 1988), this study demonstrates for the first time the necessity of an intact microtubule network for cadherin-mediated adhesion. Thus, the entire cytoskeleton and probably the whole cell architecture, or contractility, influence cadherin adhesion. The adhesive properties of N-cadherin ectodomain observed in a cell free assay were also altered in the cells by pervanadate treatment. It has been proposed recently that an increased tyrosine phosphorylation of β-catenin destabilizes the β-catenin/α-catenin association leading to the dissociation of the complex from the actin network (Ozawa and Kemler, 1998). The observed dependence of cadherin-mediated adhesion upon intracellular events, such as the integrity of the microfilament or microtubule networks, or the level of phosphorylation on tyrosine, further supports that the intrinsic adhesive properties of the N-cadherin ectodomain may be regulated by intracellular signals.

Altogether our results show that immobilized N-cadherin ectodomains are sufficient to mimic cadherin-mediated cell-cell contact and subsequent signal transduction. They support a model where adhesive interactions of dimeric cadherin ectodomain induce a rapid recruitment of cadherin/catenin complexes, followed by a strong anchorage to actin filaments, leading to cytoskeleton redistribution and activation of intracellular signaling. The model system developed here, allowing us to mimic and tightly control cadherin-mediated adhesion, will allow us now to study in detail the molecular mechanism associated with cell-cell contact establishment and regulation.

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