Journal of Cell Science 111, 347-357 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
JCS7163

Long-range and selective autoregulation of cell-cell or cell-matrix adhesions by cadherin or integrin ligands

Shulamit Levenberg[1], Ben-Zion Katz[2], Kenneth M. Yamada[2] and Benjamin Geiger[1]

[1]Department of Molecular Cell Biology, The Weizmann Institute of Science, Rehovot 76100, Israel
[2]Craniofacial Developmental Biology and Regeneration Branch, National Institute of Dental Research, National Institutes of Health, Bethesda, MD 20892-4370, USA

Accepted 11 November 1997: published on WWW 15 January 1998

SUMMARY

In this study we demonstrate that local stimulation of cell surface cadherins or integrins induces a selective enhancement of adherens junction or focal contact assembly, respectively, throughout the cell. N-cadherin transfected CHO cells (CHO-Ncad) were incubated with different ligands including N-cadherin extracellular domain (NEC), anti-N-cadherin antibodies, fibronectin and concanavalin A (ConA), conjugated to synthetic beads. Electron microscopic examination indicated that both cadherin- and integrin-reactive beads bound tightly to the cell surface and were apparently endocytosed after several hours of incubation. The ConA-beads remained largely at the cell surface. Immunofluorescence labeling of the cells with antibodies to different adhesion-associated molecules indicated that both NEC- and anti-N-cadherin-conjugated beads induced a major increase in the level of junction-associated cadherin and β-catenin labeling and a modest increase in junctional vinculin labeling, compared to untreated cells or cells bound to ConA-beads. FN-conjugated beads, on the other hand, significantly enhanced vinculin labeling at focal contacts and suppressed cadherin and β-catenin staining in cell-cell junctions. The cadherin-reactive beads specifically stimulated tyrosine phosphorylation at cell-cell junctions, while the FN-beads increased the levels of focal contact-associated phosphotyrosine, as shown by immunofluorescence labeling of the cells for phosphotyrosine. Inhibition of this phosphorylation by genistein resulted in a complete suppression of the effects of both types of beads. These findings indicate that specific cadherin- and integrin-mediated surface interactions can trigger positively cooperative long-range signaling events which lead to the selective assembly of cell-cell or cell-matrix adhesions, and that these signals involve tyrosine phosphorylation.

Key words: Adhesion-mediated signaling, Cadherin, Cell adhesion, Integrin, Tyrosine phosphorylation

INTRODUCTION

Cell adhesion to external surfaces such as the extracellular matrix (ECM) or the membrane of neighboring cells has pleiotropic effects on cell morphogenesis, motility, regulation of growth and differentiation, wound healing and malignant transformation (Folkman and Moscona, 1978; Vasiliev and Gelfand, 1981; Geiger et al., 1987, 1990; Burridge et al., 1988; Gumbiner, 1996). Some of these effects can be directly attributed to the physical interaction between individual cells, leading to the formation of multicellular tissues and organs, while others involve the transduction of adhesion-mediated signals that have long-range effects on cell behavior and fate (Meredith et al., 1993; Doherty and Walsh, 1994; Williams et al., 1994; Garratt and Humphries, 1995).

Over the past several years, much information has accumulated on the molecular basis for cell adhesion, shedding light both on the adhesive interactions per se, which include binding specificity and association with the cytoskeleton (Geiger et al., 1995), and on the mechanisms of adhesion-related signaling (Juliano and Haskill, 1993; Yamada and Miyamoto, 1995). ECM adhesion occurs via specific surface receptors such as different members of the integrin family, which are attached at their cytoplasmic aspects to submembrane anchor proteins (i.e. vinculin, paxillin, talin and α-actinin) which in turn link them to the actin cytoskeleton (Hynes, 1992; Garratt and Humphries, 1995). Similar structures mediating cell-cell interactions contain adhesion molecules of the cadherin family (Takeichi, 1990; Geiger and Ayalon, 1992), as well as a submembrane plaque, which consists of anchor proteins such as catenins, vinculin and α-actinin that link actin filaments to the junction (Critchley, 1993; Geiger, 1993a,b; Kemler, 1993).

Recent studies indicate that in addition to the proteins that participate directly in the transmembrane linkage between the external surface and the cytoskeleton, there are many enzymes known to be involved in transmembrane signaling, which are associated with cell-cell junctions, matrix adhesions or both (Geiger et al., 1995; Miyamoto et al., 1995b). It was further postulated that the adhesion-triggered clustering and/or activation of these enzymes could have local effects on junction assembly, as well as long-range effects on

cell activity (Volberg et al., 1992; Behrens et al., 1993; Michalides et al., 1994).

Cell adhesion (mainly to the ECM) has been shown to involve several interdependent temporal steps, starting with the establishment of initial cell-substrate contacts, extension of these adhesions and finally formation of focal adhesions and spreading on the matrix (Grinnell, 1978; Grinnell and Hays, 1978; Duval et al., 1988).

The development of early adhesions into mature focal contacts is still poorly understood at the molecular level, though it appears that the process is driven by highly cooperative interactions. This cooperativity is promoted by clusters of surface receptors which interact with external ligands, as well as by the retention or recruitment of such adhesion molecules by components of the submembrane plaque and the associated cytoskeleton (Miyamoto et al., 1995a).

Recent studies indicate that the assembly of adhesion sites may also be controlled by post-translational modification of their various components. Both cell-cell adherens junctions and focal contacts were shown to host a wide variety of protein kinases (Rohrschnieder, 1980; Geiger et al., 1992; Dong et al., 1993; Barry and Critchley, 1994; Schaller and Parsons, 1994; Van Etten et al., 1994) and to be primary targets for tyrosine-specific protein phosphorylation (Behrens et al., 1993; Hamaguchi et al., 1993; Lo et al., 1994; Turner, 1994). Moreover, it was established that these modifications have a profound effect on the assembly and stability of the adhesion sites. A certain basal level of phosphorylation was found to be essential for cell adhesion (Volberg et al., 1992; Tsukita et al., 1991), while over-phosphorylation resulted in deterioration of adhesion sites and cell detachment (Volberg et al., 1991; Matsuyoshi et al., 1992; Hamaguchi et al., 1993).

In the present study, we have examined the long-range effect of adhesive interactions mediated through cadherins or integrins on the adhesive behavior of cells. Specifically, we have incubated N-cadherin-transfected cells with cadherin- or integrin-reactive beads, and have determined the effect of this treatment on intercellular or cell-ECM adhesion. We show here that cadherin-reactive beads can induce a major increase in cadherin, β-catenin and phosphotyrosine levels at adherens junctions. Fibronectin-coated beads, on the other hand, increased phosphotyrosine and vinculin levels in focal contacts rather than in cell-cell contacts. Both effects appeared to depend on tyrosine phosphorylation and could be blocked by genistein. It thus appears that surface interactions with cadherin or integrin can trigger long-range phosphotyrosine-dependent signaling events which selectively affect cell-cell and cell matrix adhesions. This selective autoregulatory process provides a feedback mechanism to reinforce selectively one of these two forms of adhesion in each cell.

MATERIALS AND METHODS

Cells

Chinese hamster ovary (CHO) cells were obtained from the American Type Culture Collection (Rockville MD, USA). These cells were transfected with cDNA encoding chicken N-cadherin (kindly provided by M. Takeichi, Kyoto University) and subcloned into the pECE eukaryotic expression vector (Ellis et al., 1986). Of the various CHO-Ncad clones available we have used here a line (FL4) expressing relatively high levels of N-cadherin (Geiger et al., 1992). The cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum (Bio-Labs, Israel). Cells were cultured at 37°C in a humidified incubator under an atmosphere of 5% CO_2 and 95% air.

Production and purification of N-cadherin ectodomain (NEC)

To cluster N-cadherin, we have used a bacterially expressed polypeptide corresponding to nearly the entire ectodomain of chicken N-cadherin (NEC). The cDNA encoding the ectodomain of chicken N-cadherin (from GAC 166 to GAC 1,720; GenBank accession number X07277) was generated by the polymerase chain reaction (PCR) using full-length chicken N-cadherin cDNA as a template and inserted into the pET3d vector. The oligonucleotide primers contained a 5′ *Nco*I restriction site and a 3′ *Bam*HI restriction site, to facilitate cloning into the bacterial vector.

The sequences of the two oligonucleotides were as follows: N-terminal primer: 5′-TCTATACCATGGACTGGGTTATCCCT-3′; C-terminal primer: 5′-TCTAGCGGATCCTAGTCAACATCAGTACA-3′. To produce the cadherin ectodomain, competent BL21 cells were transformed with the pET3d construct, and the expression of NEC was induced with IPTG using the T7 polymerase-inducible pET system (Studier et al., 1990). The inclusion bodies were isolated and the protein extracted using GuHCl followed by renaturation in DTE/glutathione solution (Buchner et al., 1992). The typical yield of NEC obtained from 500 ml of bacterial cultures was 10-20 mg.

Preparation and application of cadherin- and integrin-reactive beads

Polybead amino microspheres (mean diameter 6 μm, Polysciences, Inc.), were coated with either NEC polypeptide, purified monoclonal anti-N-cadherin (BE) antibodies (Volk and Geiger, 1986), human plasma fibronectin purified as described by Miekka et al. (1982) or ConA (Worthington Biochemical, USA), by washing 10^8 beads with PBS at pH 7.4, incubating with 8% glutaraldehyde for 16 hours with gentle mixing, washing with PBS followed by incubation with 500 μg/ml of NEC, FN, ConA or antibody for 5 hours. The beads were then incubated with 0.5 M ethanolamine in PBS for 30 minutes followed by incubation with 10 mg/ml BSA for 30 minutes and resuspended in a storage buffer containing 10 mg/ml BSA, 0.1% NaN_3 and 5% glycerol in PBS, pH 7.4.

Immunochemical reagents and procedures

Cells grown on glass coverslips were fixed/permeabilized in a solution containing 0.5% Triton X-100 and 3% paraformaldehyde in PBS for 3 minutes, postfixed with 3% paraformaldehyde for 20 minutes and stained with the various antibodies. The antibodies used in this study included: Pan-cadherin antibody CH19 and anti-human vinculin (Sigma Chemical Co.); anti-P-Tyr 4G10 (UBI, Lake Placid, NY, USA); and anti-β-catenin (94.5), a gift from Dr M. Wheelock (Dept of Biology, University of Toledo). The secondary antibody was rhodamine-labeled goat anti-mouse IgG (Jackson Laboratories). The cells were indirectly immunolabelled and mounted in Elvanol (Mowiol 4-88, Hoechst, Frankfurt, Germany) on glass slides and examined using either a conventional fluorescence microscope (Zeiss Axiophot, Oberkochen, Germany) or a digital fluorescence microscope (see below).

Digital immunofluorescence microscopy

The computerized microscopic system used here was based on the design of Agard and Sedat (Agard et al., 1988, 1989), consisting of a Zeiss Axioscope and Micro VAX III workstation which controls image acquisition, light shutters, filter wheels and focus. Images were recorded with a cooled, scientific-grade, charge-coupled device (CCD) camera (Photometrics, Tucson, AZ, USA). Images were recorded into an array processor (Mercury Computer Systems, Lowel, MA, USA)

which calculated on-the-fly pixel-per-pixel correction for illumination and CCD sensitivity, and scaled and deconvoluted images, essentially in real-time (Chen et al., 1990; Hiraoka et al., 1990).

For pseudo-color quantitative representation of fluorescence intensities, we randomly selected (using phase contrast) microscopic fields with bead-associated cells and measured the image intensities. Background fluorescence was routinely subtracted from the experimental values. Sets of images (all obtained from the same experiment) were displayed using the same color scale (mostly linear), and the exact locations of the beads (based on the phase contrast image) were marked. Images were photographed on Ektachrome 160 film using a Focus 4700 Imagerecorder (Focus Graphics, Foster City, CA).

Calculation of fluorescence intensities

Cell-cell adherens junctions and focal adhesions (identified by labeling for associated molecules, i.e. cadherin, β-catenin and vinculin) were manually marked by polygons enclosing the entire relevant area, and the net intensity of labeling (minus background) in them was determined. The average intensity values of the enclosed pixels displaying signal above the threshold level were then calculated.

The effect of cadherin- and integrin-reactive beads on cell adhesions, using different combinations of cells and beads, was examined in 12 independent experiments. Full quantitation (namely addition of the four types of beads and labeling for the four antigens) was repeated 3 times, and measurement of effects on cadherin and catenin levels was performed in two additional experiments. Variations between experiments were typically lower than 20%. In each experiment data were collected from 15-20 microscopic images containing a total number of about 50 marked cell-cell adhesions and 250 marked focal contacts, all in selected cells containing bound beads. To compare the results obtained following treatment with cadherin- and integrin-reactive beads to those found in control cells (associated with ConA-beads), Student's *t*-test was performed, and significance values were calculated for each pair.

Scanning electron microscopy

Cells were cultured on glass coverslips for 24 hours, and then beads were added for 30 minutes or 4 hours. The cells were fixed for 1 hour with Karnovsky's fixative (3% paraformaldehyde, 2% glutaraldehyde, 5 mM $CaCl_2$ in 0.1 M cacodylate buffer, pH. 7.4, containing 0.1 M sucrose), rinsed and postfixed for 1 hour with 1% osmium tetroxide in 0.1 M cacodylate buffer followed by incubation with 1% tannic acid in water and than with 1% uranyl acetate in water. The slides were rinsed, dehydrated with a graded ethanol series, critically point dried (Pelco CPD2) and sputter coated with gold (S150 Edwards, USA). The specimens were examined at an accelerating voltage of 20-25 kV using a JEOL GMC 6400 scanning electron microscope.

Transmission electron microscopy

Cells were seeded in 35 mm Falcon dishes, for 24 hours and then beads were added for 4 hours. The cells were fixed in Karnovsky's fixative, postfixed with 1% osmium tetroxide, 0.5% potassium dichromate and 0.5% potassium hexacyanoferrate in 0.1 M cacodylate buffer. The cells were stained en bloc with 2% aqueous uranyl acetate, followed by ethanol dehydration. The dishes were embedded in Epon 812 (Tuosimis, MD, USA). Sections were cut using diamond knife (Diatome, Biel, Switzerland) and examined using a Philips CM-12 transmission electron microscope at an accelerating voltage of 100 kV.

RESULTS

Production of specific ligands to cadherin and integrin receptors

In order to modulate specific binding to the cadherin receptor, cells were exposed to the external domain of N-cadherin (NEC). For that purpose, NEC was over-expressed in bacteria, purified from inclusion bodies and renatured (Fig. 1A). The purified 66 kDa polypeptide, which reacted with monoclonal antibodies specific for the extracellular domain of N-cadherin (Fig. 1B), was covalently linked to 6 μm Polybead microspheres. Similarly, anti-N-cadherin monoclonal antibodies (BE) covalently linked to the beads were also used to stimulate cadherin receptors. In addition, fibronectin (FN) or ConA covalently linked to the 6 μm microsphere beads were used to stimulate integrin receptors or to mediate non-specific binding, respectively.

The specificity of binding of NEC and BE ligands to cadherin receptors was determined by comparing CHO cells (which express very low levels of cadherins) and N-cadherin transfected CHO (CHO-Ncad) cells (Geiger et al., 1992). As shown in Fig. 2A, the binding of the cadherin-reactive beads (NEC, BE) to CHO-Ncad cells was considerably higher (3- to 6-fold) than to CHO cells. Moreover, when FN and ConA were

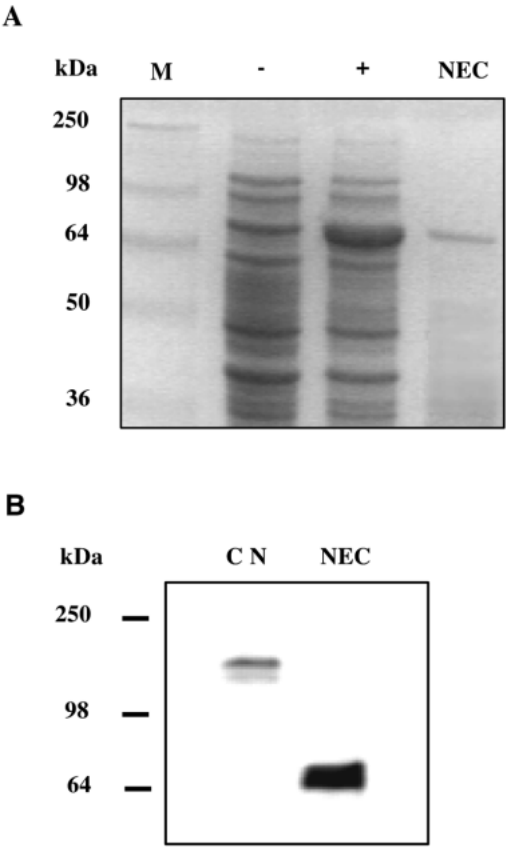

Fig. 1. Bacterial expression and purification of N-cadherin extracellular domain (NEC). cDNA encoding the chicken N-cadherin extracellular domain was cloned into the pET3d plasmid and expressed in BL21 cells following IPTG induction. (A) Coomassie blue-stained polyacrylamide gel, showing protein extracts from transformed BL21 bacteria, with (+) or without (–) IPTG induction as well as the purified NEC. M, molecular mass markers. (B) Western blot analysis of protein extract from CHO-Ncad cells expressing full length chicken N-cadherin cDNA (CN), and N-cadherin extracellular domain (NEC) after purification from inclusion bodies. Immunodetection used monoclonal antibody ID-7.2.3 reactive with the extracellular domain of chicken N-cadherin (Volk and Geiger, 1986).

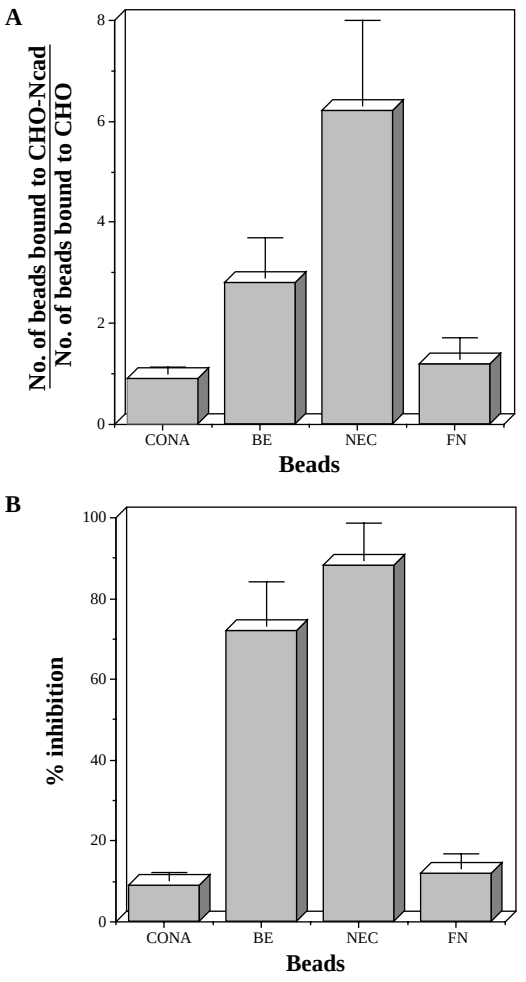

Fig. 2. Specificity and Ca^{2+} requirement of bead binding to CHO-Ncad cells. (A) Four types of beads (coated with Concanavalin A (CONA), with monoclonal anti-N-cadherin antibodies (BE), with N-cadherin extracellular domain (NEC) and with fibronectin (FN)) were added for 15 minutes to CHO cells or CHO-Ncad cells 24 hours after plating. The cells were washed, fixed, and stained with DAPI and the number of beads bound to the cells was counted. The ratio between number of beads bound to CHO-Ncad and CHO is shown. (B) The various beads were added to CHO-Ncad cells for 15 minutes, with or without 2 mM EGTA. The number of beads bound to the cells was counted and results expressed as the percentage of total binding inhibitable by EGTA.

similarly analyzed, they showed no binding preferences toward CHO or CHO-Ncad cells, as expected (Fig. 2A).

The interaction of NEC and BE with the cell surface was found to be highly calcium dependent, in contrast to the interaction of FN and ConA (Fig. 2B). This finding is consistent with the known calcium-dependence of cadherin-mediated interactions (Geiger and Ayalon, 1993) and further suggests that the monoclonal antibody used here reacts with a calcium-dependent epitope of N-cadherin.

Characterization of the interaction between CHO-Ncad cells and the cadherin- and fibronectin-coated beads

The binding of the various bead-conjugated ligands to the cell surface and their fate at different time points after binding were examined by scanning electron microscopy. As shown in Fig. 3, both the NEC-, and BE-coupled beads were attached to the cell surface via extensive membrane protrusions which were apparent within 30-40 minutes of binding (Fig. 3A,B). Upon longer incubation (up to several hours), most of the beads were apparently engulfed by the cells (data not shown). The fibronectin-coated beads were also found to be attached to the cells, and they were apparently endocytosed by the cells in a manner similar to the cadherin-reactive beads (Fig. 3C). In contrast, the ConA-coated beads, while avidly binding to the cell surface, were not engulfed by the cells and remained largely exposed at the cell surface even after prolonged incubation (Fig. 3D). Transmission electron microscopy provided some additional insight into the mode of binding of beads to the surface and the fate of the beads after prolonged incubation. Examination of the interface between the cells and the cadherin- or integrin-reactive beads showed a very close association of the beads with the cell surface (Fig. 3E-H). Moreover, careful serial sectioning indicated that many of the apparently internalized beads were not fully enclosed in an endocytic or phagocytic vesicle but were located instead in deep, unsealed membrane folds (Fig. 3E′). This notion was further corroborated by fluorescence labeling of non-permeabilized cells that were previously incubated for several (2-4) hours with anti-N-cadherin (BE)-coated beads with fluorescent goat anti-mouse IgG. Fluorescence microscopic examination of these specimens indicated that most of the beads were accessible to the antibody even after 4 hours of incubation (data not shown).

Long-range changes in cell-cell junctional proteins induced by cadherin receptor stimulation

The intensity of immunolabeling for cadherin at cell-cell junctions dramatically increased following 20 minutes of cadherin receptor stimulation using either NEC- or BE-coated beads (Fig. 4). This increase in cadherin levels (in cells associated with the cadherin reactive beads) occurred at cell-cell junctions irrespective of their proximity to the site of cadherin receptor stimulation (i.e. the bead binding site). Interestingly, enhanced levels of cadherin staining (usually 1.5- to 2-fold higher than the levels measured in control cells) were commonly detected in cells neighboring those associated with cadherin-reactive beads (Fig. 4**).** Occasionally, cadherin was also detected at the bead-cell interface, but at lower levels than those detected at cell-cell junctions, and the labeling was often heterogeneous, reflecting the variable modes of cell-bead interaction apparent from the electron microscopic data in Fig. 3 (see Discussion). It is noteworthy that soluble NEC or anti-cadherin antibodies did not induce a similar enhancement of adherens junctions (not shown), while NEC immobilized on the flat tissue culture substratum induced a generalized increase in cadherin localization at cell-cell junctions (unpublished data). In contrast, integrin receptor stimulation, using FN-coated beads, did not induce an increase in cadherin levels at cell junctions and even a slight reduction was observed (Fig. 4 ‘FN’ and Fig. 8). In addition, ConA beads had no apparent effect on cadherin levels in cell-cell junctions as expected (Fig. 4 ‘ConA’).

To evaluate the extent of enhancement of cadherin labeling at cell junctions following cadherin receptor stimulation using

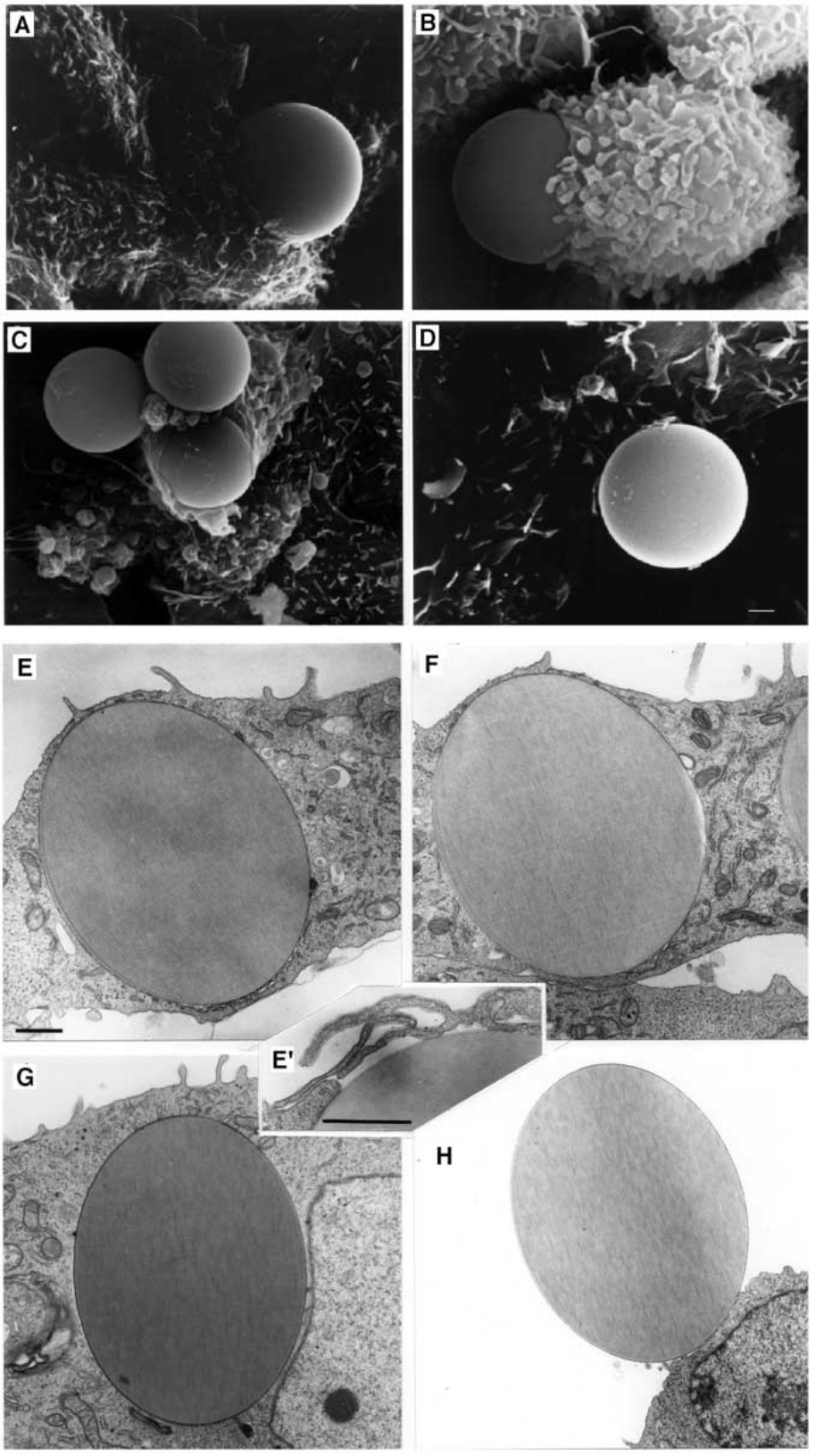

Fig. 3. Scanning (A-D) and transmission (E-H) electron microscopy of CHO-Ncad cells, following 30 minutes (A-D) or 4 hours (E-H) incubation with the different types of beads. The beads were conjugated with monoclonal anti-N-cadherin antibodies (BE) (A and F), with N-cadherin extracellular domain (NEC) (B, E and E′), with fibronectin (C and G) or with ConA (D and H). The insert E′ shows a section revealing the lack of a complete membrane seal around the bead, which was commonly observed for each of the endocytosed beads. It is noteworthy that the ConA-coated beads were firmly but only superficially associated with the cell surface. Bars, 1 μm (A-D and E-H are at the same magnification).

either NEC- or anti-N-cadherin (BE)-coated beads, we quantitated the local immunofluorescence intensities associated with these sites using a digital microscopic system (see Materials and Methods). As shown in Fig. 8A, both types of cadherin-reactive beads induced a highly significant (3- to 4-fold) increase in the intensity of labeling of junctional

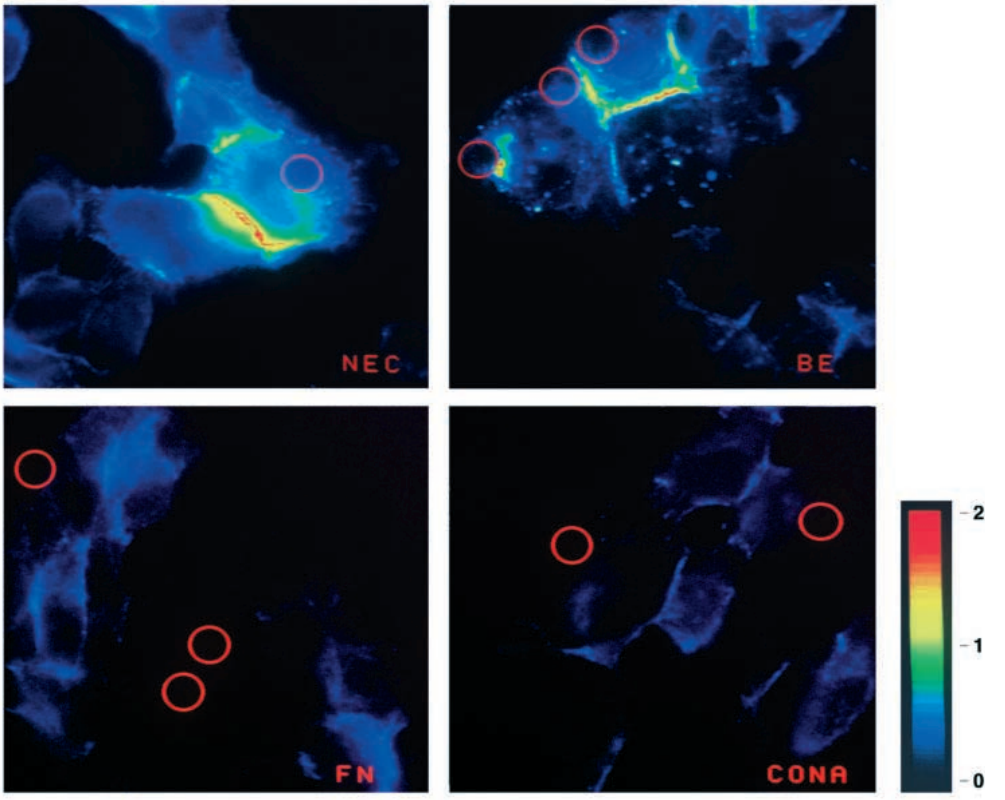

Fig. 4. The effect of cadherin- and integrin-reactive beads on the distribution and level of endogenous N-cadherin in CHO-Ncad cells. Cells were seeded on glass coverslips, and 24 hours later beads coated with the various ligands were added to the cells. The ligands included: N-cadherin extracellular domain (NEC), monoclonal anti-N-cadherin antibodies (BE), fibronectin (FN) and ConA (CONA). Following 20 minutes of incubation at 37°C, the cells were fixed and stained with anti-N-cadherin antibodies. The labeled cells were examined by digital microscopy, using identical exposure times (1 second). Net fluorescence was calculated and represented as pseudocolors (see scale at the lower left corner in arbitrary intensity units). Red circles indicate the location of beads.

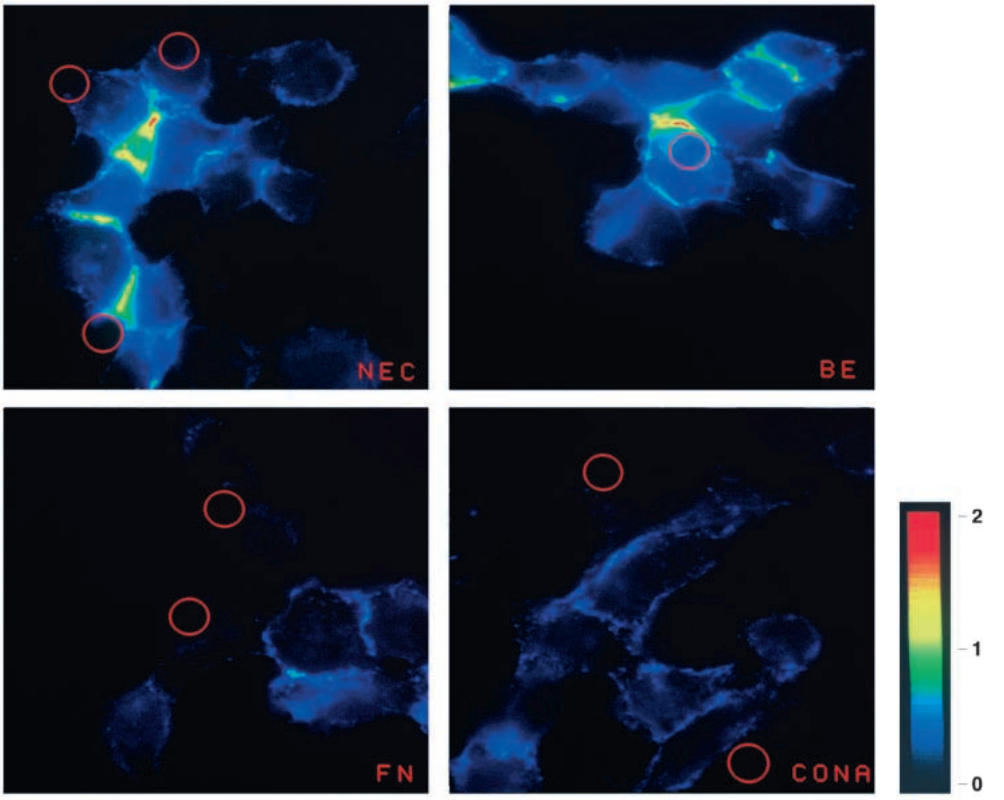

Fig. 5. The effect of cadherin- and integrin-reactive beads on the distribution and level of endogenous β-catenin in CHO-Ncad cells. Cells were seeded on glass coverslips; 24 hours later, beads coated with the various ligands indicated were added to the cells as described for Fig. 4 above.

cadherin, compared to ConA-beads (P<0.001). In contrast integrin-receptor stimulation reduced cadherin at cell junctions by 1.5-fold (P<0.02).

In order to determine whether the high levels of junctional cadherin described above were induced by the associated cadherin-reactive beads or whether these beads merely bound

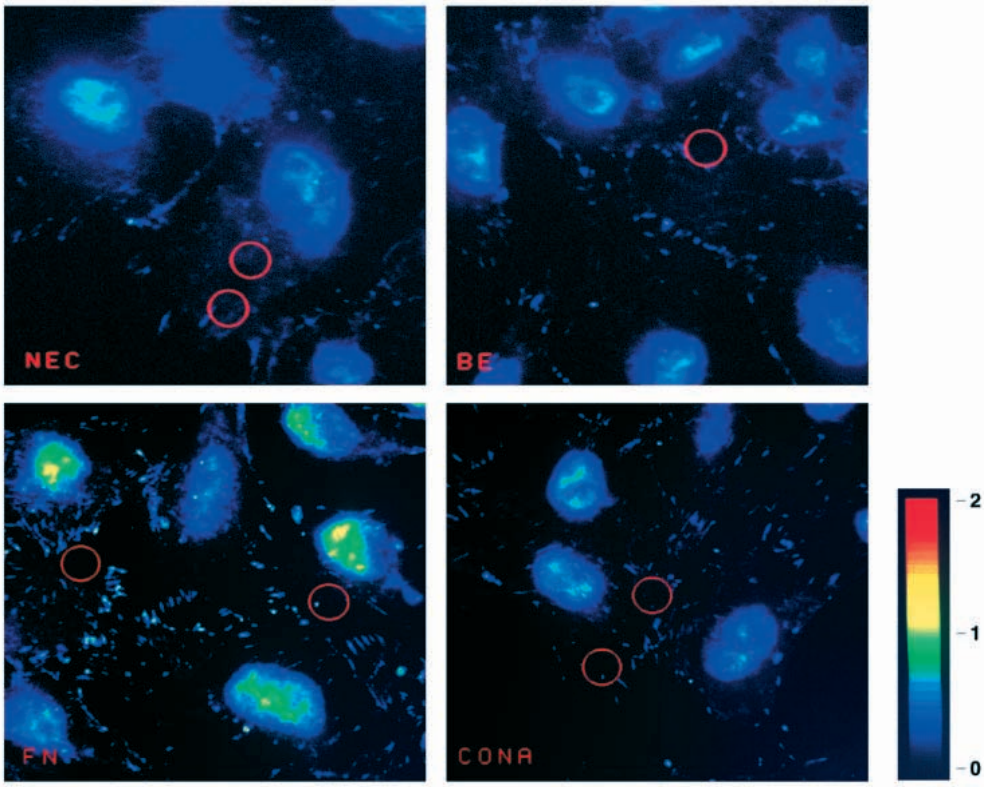

Fig. 6. The effect of cadherin- and integrin-reactive beads on the distribution and level of endogenous vinculin in CHO-Ncad cells. Cells were seeded on glass coverslips; 24 hours later, beads coated with the various ligands indicated were added to the cells as described for Fig. 4 above.

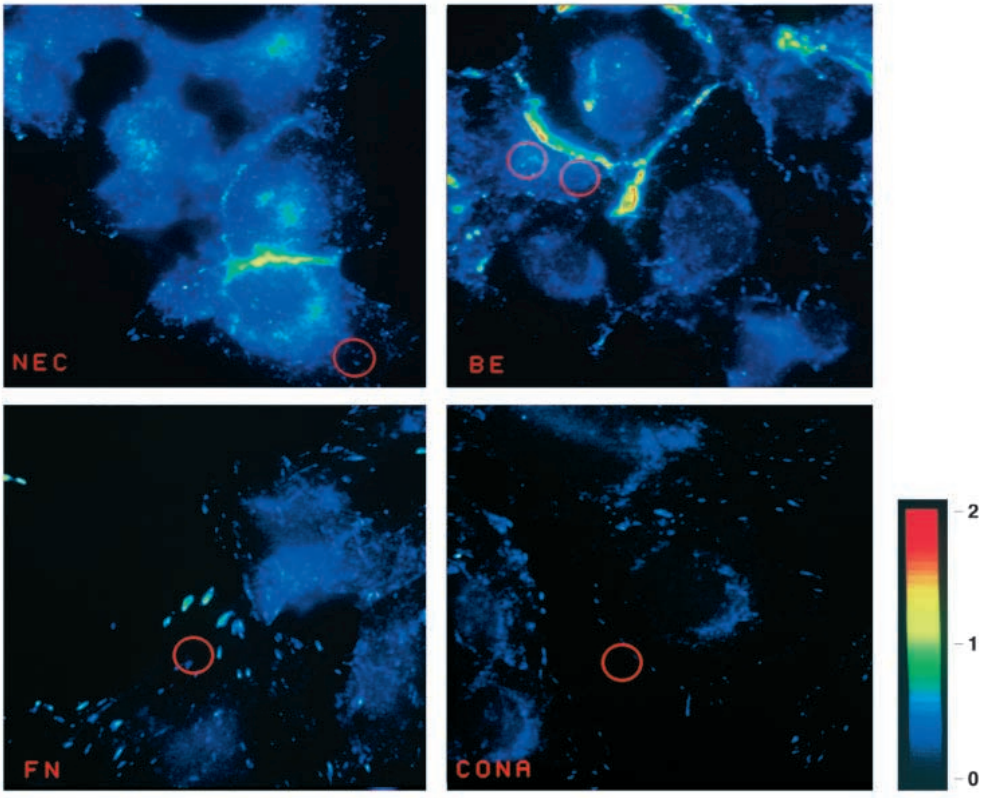

Fig. 7. The effect of cadherin- and integrin-reactive beads on the distribution and level of phosphotyrosine in CHO-Ncad cells. Cells were seeded on glass coverslips; 24 hours later, beads coated with the various ligands indicated were added to the cells as described for Fig. 4 above.

to cells that had intrinsically high cadherin levels, we compared the frequency of cells with high cadherin labeling in NEC-bead treated and control cultures. For each culture, 15-20 fields were randomly selected and about 100 adhesion sites marked and analyzed. The selection of fields was performed irrespective of the presence or absence of beads. In the non-treated cells

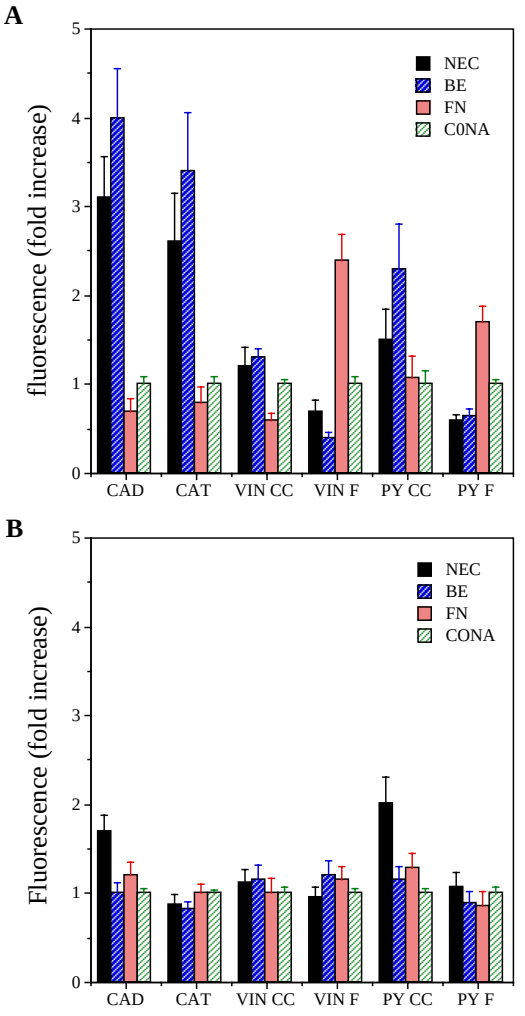

Fig. 8. Quantitative analysis of the effect of cadherin- and integrin-reactive beads (NEC and BE, see legend to Fig. 4), as well as ConA-coupled beads, on the levels of cadherin (CAD), β-catenin (CAT), vinculin (VIN CC) and phosphotyrosine (PY CC) in cell-cell contacts, as well as vinculin and phosphotyrosine in focal contacts (VIN F and PY F, respectively). The experiment was conducted in the presence (B) or absence (A) of 100 μg/ml of the broad-spectrum tyrosine kinase inhibitor genistein. The bars represent the change in immunofluorescence intensity relative to the labeling for the same component in cells treated with ConA-coupled beads. Note that genistein effectively abolished the changes in cadherin, catenin and phosphotyrosine intensities induced by each of the various beads.

culture a rather narrow distribution of junctional labeling intensity was obtained, with a maximum of 5% of the cells exhibiting intensity levels comparable to those commonly found in cells attached to the cadherin-reactive-beads. In cultures incubated with NEC-beads, on the other hand, over 30% of the total cell population exhibited elevated cadherin levels, and these cells were essentially all attached to the beads. In typical experiments, 30-50% of the cells were associated with the beads.

In addition to the increase in junctional cadherin levels, a similar enhancement (2.5- to 3.5-fold) of the submembrane adherens junctional protein, β-catenin, was observed following cadherin receptor stimulation by either NEC- or anti-N-cadherin (BE)-coated beads (Figs 5 and 8). This increase was found to be highly significant (P<0.001). Some increase in β-catenin levels was also observed in neighboring cells. Similarly to cadherin, β-catenin levels were slightly reduced following integrin receptor stimulation, by FN-coated beads, while ConA-coated beads had no apparent effect on the cells (Figs 5 and 8).

Changes in vinculin and phosphotyrosine levels following cadherin- and integrin-receptor stimulation

To examine further, the differential effects of specific cadherin- and integrin-receptor stimulation on cell-cell versus cell-matrix adhesions, we analyzed the levels of vinculin, the submembranous protein that is associated with both types of adhesion sites. As shown in Figs 6 and 8, there was small, yet reproducible, enhancement of junctional vinculin following cadherin-receptor stimulation (P<0.05), accompanied by a modest, yet highly significant (P<0.001), reduction in vinculin staining at focal contacts when compared to incubation of cells with ConA-coated beads. Integrin receptor stimulation, on the other hand, moderately reduced vinculin levels at cell-cell adhesions and induced a major and highly significant (P<0.001) increase (2.5-fold) in vinculin levels at focal contacts (Figs 6 and 8).

In view of the involvement of tyrosine-specific protein phosphorylation in both cell-cell and cell-matrix adhesion, we examined the subcellular distribution of proteins containing phosphotyrosine following cadherin- and integrin-receptor stimulation. Specific stimulation of cadherin receptor using NEC- and BE-coated beads induced high levels of junctional phosphotyrosine labeling (1.5- to 2.5-fold increase), and decreased the level of phosphorylation in focal contacts to half the control value (Fig. 7 'NEC' and 'BE' and Fig. 8A). Both effects were highly significant (P<0.001). On the other hand, specific stimulation of integrin receptor by fibronectin-coated beads enhanced phosphotyrosine levels in focal contacts (1.5- to 2-fold increase, P<0.001; see Fig. 7 'FN' and Fig. 8A 'PY F'). Similar effects were induced by beads coated with RGD peptides (data not shown).

To determine whether the changes in the organization of cadherin, β-catenin and vinculin induced by the various beads depend on tyrosine phosphorylation, the broad-spectrum tyrosine kinase inhibitor genistein (100 μg/ml) was added to the cultures together with the beads. This treatment essentially abolished the effects of the different beads on the distribution of the three junctional proteins (Fig. 8B).

Taken together, cadherin receptor stimulation using NEC- or BE-coated beads caused a major increase in cadherin and β-catenin at cell-cell junctions. Vinculin and phosphotyrosine staining in these sites also increased, while their levels at focal contact sites were significantly reduced (Fig. 8A). Integrin receptor stimulation, on the other hand, reduced cadherin and β-catenin at cell-cell junctions, and significantly increased vinculin and phosphotyrosine in focal contacts. This reorganization appeared to depend on tyrosine phosphorylation.

DISCUSSION

In this study, we have identified a novel set of mutually

exclusive and reciprocally regulated changes in the structure and molecular organization of cellular adhesion sites, which are triggered by cadherin- or integrin-mediated interactions. These apparent autoregulatory events involve transmembrane signaling systems. One system selectively enhances the assembly of cell-cell adherens junctions following stimulation via the cadherins, and is accompanied by a reduction in cell-substrate focal adhesions. The other system reciprocally enhances the assembly of focal adhesions while reducing adherens junctions.

In other words, interactions mediated through each major adhesion system, cadherin or integrin, specifically and selectively enhance the formation of only the same type of adhesive structure. Each process involves long-range and apparently positively cooperative effects, since contact at a single point on the cell surface triggers enhancement of the homologous adhesive structure throughout the cell. In addition, we provide evidence for roles of site-selective tyrosine-specific protein phosphorylation in each of these processes.

Adhesive interactions characteristically involve forms of molecular synergism that reflect the complex nature of cell contact. On the exterior of the cell, local aggregation of adhesion receptors synergizes with occupancy by ligand to mediate accumulations of certain cytoskeletal molecules (Miyamoto et al., 1995a). Conversely, such adhesion receptors appear to be stabilized further or even recruited to primordial adhesions by a submembrane mesh of plaque proteins (Geiger et al., 1990; Geiger, 1993a; Yamada and Geiger, 1997). A complex hierarchy of cytoskeletal and signaling molecules accumulate in local transmembrane complexes near the site of contact with an integrin ligand such as fibronectin (e.g. see Miyamoto et al., 1995b).

The autoregulatory mechanism described here is conceptually different, because it involves long-range (rather than local) induction of junction assembly. In the following, we would like to discuss the capacity of cell adhesion to both induce such signals and to respond to them. The concept that adhesion to different surfaces can activate specific signaling cascades is based on several lines of evidence. Interactions with the extracellular matrix are known to be essential for normal cell growth (Stoker et al., 1968; Folkman and Moscona, 1978) and often for blocking apoptosis (Meredith et al, 1993; Frisch and Francis, 1994; Re et al., 1994; Boudreau at al., 1995), a phenomenon referred to as ‘anchorage dependence’.

It has further been demonstrated that modulation of signaling processes, such as inhibition or stimulation of tyrosine phosphorylation or changes in the levels or activity of small G-proteins of the Rho family has a profound effect on the structure, dynamics and stability of cell adhesions (Ridley and Hall, 1992; Volberg et al., 1992; Hamaguchi et al., 1993; Tokman, 1997). Moreover, it has been shown that adhesion to specific surfaces can induce tyrosine phosphorylation in cells (Burridge et al., 1992; Kornberg et al., 1992) and that both adherens junctions and focal contacts are major tyrosine phosphorylation sites in cells (Kinch et al., 1997). In addition, at least two distinct types of MAP kinase activation, calcium influx, and pH changes are triggered by integrin-mediated cell interactions with extracellular matrix (Clark and Brugge, 1995; Miyamoto et al., 1995b; Schwartz et al., 1995). Finally, it has been shown that many transformed cells display reduced contact responsiveness, deteriorated adhesion sites and deregulated growth (Behrens et al., 1989; Shimayoma et al., 1992; Van Roy and Mareel, 1992; Tsukita et al., 1993; Becker et al., 1994; Watabe at al., 1994).

These many types of signaling events triggered by cell adhesion can modulate a variety of general cellular processes. The mutually exclusive autoregulation of adherens junctions or focal contacts described in this study appears to be a novel phenomenon. Despite the fact that both integrin- and cadherin-mediated effects involve tyrosine phosphorylation, the targets for the two processes are distinctly different. One speculation is that the integrin-reactive beads activate the integrin cytoskeletal and signaling pathway (Curtis et al., 1992; Schlaepfer et al., 1994; Miyamoto et al., 1995b) in treated cells, and that a soluble ‘second messenger’ produced following this stimulation is targeted to focal adhesions, upregulating local tyrosine phosphorylation and thus inducing increased assembly of these structures. The nature of the cadherin-mediated signal involved in the stimulation of adherens junction assembly is presently unclear, since so little is known about cadherin-mediated signaling.

Another noteworthy observation is the reciprocity between the level of organization of cell-cell and cell-matrix contacts. These inter-relationships could be interpreted as reflecting competition for molecular ‘building blocks’ shared by the two types of adhesion. Thus, stimulation of junction assembly by cadherin beads might cause a shortage of components such as vinculin, leading to deterioration of focal contacts, and vice versa. The other alternative, however, is that the signals induced by each adhesion system have, in fact, direct inhibitory activity on the heterologous system. To distinguish between these two possibilities, we have examined whether NEC-coated beads affect focal contacts in individual cells. In a series of preliminary experiments we did not observe a significant reduction in focal contacts, suggesting that the primary effect is the augmentation of cell-cell junctions and that the effect on focal contacts is secondary.

An interesting and intriguing observation was the low and inconsistent level of cadherin labeling at the cell-bead interface, compared to the higher cadherin levels detected at the junctions of the same cells. While the basis for such differences is still not clear, it may reflect the capacity of the native junctions to grow by recruiting additional cadherin molecules to sites of adhesion between neighboring cells, in contrast with the more limited capacity of cadherin molecules to pack into the cell-bead contact area. The enhanced accumulation of cadherin molecules at sites of contact with the adjacent cell might trigger a propagated signal in that second cell as well. In line with this view, one might expect to observe some augmentation of junction formation in beads-free cells which are directly neighboring bead-associated cells. As shown in Figs 6 and 7, this indeed was the case. This ‘neighbor’ effect raises another interesting question: why are the bead-associated cadherin ligands so effective in inducing junction assembly, compared to neighboring cells which carry on their surface the same cadherin? While we have no direct explanation for this difference, it appears possible that a certain minimal surface density of cadherins is essential for initiating the long-range response. Such threshold density might be present on the bead surface but not on the CHO-Ncad cells used here. This possibility is also in line with previous findings that transfected CHO cells expressing different levels of N-

cadherin differ significantly in their morphology and capacity to form stable junctions (Geiger et al., 1992). Attempts to test this possibility by adding to the CHO-Ncad cells beads containing different densities of the cadherin ligands at their surface, or reacting the beads with epithelial cells that contain prominent junctions, are currently underway.

In summary, these studies establish a novel phenomenon of autoregulatory stimulation of adhesion sites that is specific and mutually exclusive for adherens junctions and focal adhesions after local clustering and/or immobilization of surface integrins or cadherins. The effects are transmitted throughout the cell, are both dependent on tyrosine phosphorylation, and yet are reciprocal. Future studies could focus on the possible intracellular signaling mechanisms involved. Whatever the mechanisms that underlie these events, the phenomenon provides a new means for feedback augmentation of a particular adhesive mechanism, whether mediating cell-cell or cell-substrate adhesion and interaction.

We thank Dr Anat Yarden for her critical comments and helpful discussions and Prof. Zvi Kam for his devoted guidance with the digital microscopy. This study was supported by grants provided by the Minna Heinemann Foundation and by the Minerva Foundation, Munich. B.G. is the Erwin Neter Professor in Cell and Tumor Biology. This paper was written while B.G. was a Scholar-in-Residence at the Fogarty International Center for Advanced Study in the Health Sciences, NIH, Bethesda, MD.

REFERENCES

Agard, D. A., Hiraoka, Y. and Sedat, J. W. (1988). Three-dimensional light microscopy of diploid Drosophila chromosomes. *Cell Motil. Cytoskel.* **10**, 18-27.

Agard, D. A., Hiraoka, Y., Shaw, P. and Sedat, J. W. (1989). Fluorescence microscopy in three dimensions. *Meth. Cell Biol.* **30**, 353-377.

Barry, S. T. and Critchley, D. R. (1994). The RhoA-dependent assembly of focal adhesions in Swiss 3T3 cells is associated with increased tyrosine phosphorylation and the recruitment of both $pp125^{Fak}$ and protein kinase C-δ to focal adhesions. *J. Cell Sci.* **107**, 2033-2045.

Becker, K. F., Atkinson, M. J., Reich, U., Becker, I., Nekarda, H., Siewert, J. R. and Hofler, H. (1994). E-cadherin gene mutations provide clues to diffuse type gastric carcinomas. *Cancer Res.* **54**, 3845-3852.

Behrens, J., Mareel, M. M., Van Roy, F. M. and Birchmeier, W. (1989). Dissecting tumor cell invasion: epithelial cells acquire invasive properties after the loss of uvomorulin-mediated cell-cell adhesion. *J. Cell Biol.* **108**, 2435-2447.

Behrens, J., Vakaet, L., Friis, R., Winterhager, E., Van Roy, F., Mareel, M. M. and Birchmeier, W. (1993). Loss of epithelial differentiation and gain of invasiveness correlates with tyrosine phosphorylation of the E-cadherin/β-catenin complex in cells transformed with a temperature-sensitive v-SRC gene. *J. Cell Biol.* **120**, 757-766.

Boudreau, N., Sympson, C. J., Werb, Z. and Bissell, M. (1995). Suppression of ICE and apoptosis in mammary epithelial cells by extracellular matrix. *Science* **267**, 891-893.

Buchner, J., Pastan, I. and Brinkmann, U. (1992). A method for increasing the yield of properly folded recombinant fusion proteins: single-chain immuno-toxins from renaturation of bacterial inclusion bodies. *Anal. Biochem.* **205**, 263-270.

Burridge, K., Fath, K., Kelly, T., Nuckolls, G. and Turner, C. (1988). *Annu. Rev. Cell Biol.* **4**, 487-525.

Burridge, K., Turner, C. E. and Romer, L. H. (1992). Tyrosine phosphorylation of paxillin and $pp125^{FAK}$ accompanies cell adhesion to extracellular matrix: a role in cytoskeletal assembly. *J. Cell Biol.* **119**, 893-903.

Chen, H., Sedat, J. W. and Agard, D. A. (1990). Manipulations, display, and analysis of three-dimensional biological images. In *Handbook of Biological Confocal Microscopy* (ed. J. B. Pawley), pp. 141-150. Plenum Press, NY.

Clark, E. A. and Brugge, J. S. (1995). Integrins and signal transduction pathways: the road taken. *Science* **268**, 233-239.

Critchley, D. R. (1993). α-Actinins. In *Guidebook to the Cytoskeletal and Motor Proteins* (ed. T. Kreis and R. Vale), pp. 22-23. Oxford University Press, New York.

Doherty, P. and Walsh, F. S. (1994). Signal transduction events underlying neurite outgrowth stimulated by cell adhesion molecules. *Curr. Opin. Neurobiol.* **4**, 49-55.

Dong, L., Stevens, J. L. and Jaken, S. (1993). Transformation-sensitive localization of alpha-protein kinase C at cell-cell contacts in rat renal proximal tubule epithelial cells. *Cell Growth Differ.* **4**, 793-798.

Duval, J. L., Letort, M. and Sigot-Luizard, M. F. (1988). Comparative assessement of cell/substratum static adhesion using an in vitro organ culture methods and computerized analysis system. *Biomaterials* **9**, 155-161.

Ellis, L., Clauser, E., Morgan, D. O., Endery, M., Roth, R. A. and Rutter, W. J. (1986). Replasment of insulin receptor tyrosine residues 1162 and 1163 compromises insulin-stimulated kinase activity and uptake of 2-deoxyglucose. *Cell* **45**, 721-732.

Folkman, J. and Moscona, A. (1978). Role of cell shape in growth control. *Nature* **273**, 345-349.

Frisch, S. M. and Francis, H. (1994). Disruption of epithelial cell-matrix interactions induces apoptosis. *J. Cell Biol.* **124**, 619-626.

Garratt, A. N. and Humphries, M. J. (1995). Recent insight into ligand binding, activation and signalling by integrin adhesion receptors. *Acta Anat.* **154**, 34-35.

Geiger, B., Volk, T., Volberg, T. and Bendori, R. (1987). Molecular interactions in adherens-type contacts. *J. Cell Sci.* **8**, 251-272.

Geiger, B., Ginsberg, D., Salomon, D. and Volberg, T. (1990). The molecular basis for the assembly and modulation of adherens-type junctions. *Cell Differ. Dev.* **32**, 343-353.

Geiger, B. and Ayalon, O. (1992). Cadherins. *Annu. Rev. Cell Biol.* **8**, 307-332.

Geiger, B., Ayalon, O., Ginsberg, D., Volberg, T., Rodriguez Fernandez, J. L., Yarden, Y. and Ben-Ze'ev, A. (1992). Cytoplasmic control of cell adhesion. *Cold Spring Harbor Symp. Quant. Biol.* **57**, 631-642.

Geiger, B. (1993a). Cytoskeletal anchor proteins. In *Guidebook to the Cytoskeletal and Motor Proteins* (ed. T. Kreis and R. Yale), pp. 215-218. Oxford University Press, New York.

Geiger, B. (1993b). Vinculin. In *Guidebook to the Cytoskeletal and Motor Proteins* (ed. T. Kreis and R. Yale), pp. 243-244. Oxford University Press, New York.

Geiger, B., Yehuda-Levenberg, S. and Bershadsky, A. D. (1995). Molecular interactions in the submembrraane plaque of cell-cell and cell-matrix adhesions. *Acta Anat.* **154**, 46-62.

Grinnell, F. (1978). Cellular adhesiveness and extracellular substrata. *Int. Rev. Cytol.* **53**, 65-144.

Grinnell, F. and Hays, D. G. (1978). Induction of cell spreading by substratum- adsorbed ligands directed against the cell surface. *Exp. Cell Res.* **116**, 275-284.

Gumbiner, B. M. (1996). Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell* **84**, 345-357.

Hamaguchi, M., Matsuyoshi, N., Ohnishi, Y., Gotoh, B., Takeichi, M. and Nagai, Y. (1993). $p60^{v\text{-}src}$ causes tyrosine phosphorylation and inactivation of the N-cadherin-catenin cell adhesion system. *EMBO J.* **12**, 307-314.

Hiraoka, Y., Sedat, J. W. and Agard, D. A. (1990). Determination of three-dimensional imaging properties of a light microscope system. Partial confocal behavior in epifluorescence microscopy. *Biophys. J.* **57**, 325-333.

Hynes, R. O. (1992). Integrins: versatility, modulation and signaling in cell adhesion. *Cell* **69**, 11-25.

Juliano, R. L. and Haskill, S. (1993). Signal transduction from the extracellular matrix. *J. Cell Biol.* **120**, 577-585.

Kemler, R. (1993). From cadherins to catenins: cytoplasmic protein interactions and regulation of cell adhesion. *Trends Genet.* **9**, 317-321.

Kinch, M. S., Petch, L., Cuiling, Z. and Burridge, K. (1997). E-cadherin engagement stimulates tyrosine phosphorylation. *Cell Adhes. Commun.* **4**, 425-437.

Kornberg, L., Earp, H. S., Parsons, J. T., Schaller, M. and Juliano, R. L. (1992). Cell adhesion or integrin clustering increases phosphorylation of a focal adhesion-associated tyrosine kinase. *J. Biol. Chem.* **267**, 23439-23442.

Lo, S. H., Weisberg, E. and Chen, L. B. (1994). Tensin: A potential link between the cytoskeleton and signal transduction. *BioEssays* **16**, 817-823.

Matsuyoshi, N., Hamaguchi, M., Taniguchi, S., Nagafuchi, A., Tsukita, S. and Takeichi, M. (1992). Cadeherin-mediated cell-cell adhesion is

perturbed by v-src tyrosine phosphorylation in metastatic fibroblasts. *J. Cell Biol*. **118**, 703-714.

Meredith, J. E., Fazeli, B. and Schwartz, M. A. (1993). The extracellular matrix as a cell survival factor. *Mol. Biol. Cell* **4**, 953-961.

Michalides, R., Volberg, T. and Geiger, B. (1994). Augmentation of adherens junction formation in mesenchymal cells by co-expression of N-CAM or short-term stimulation of tyrosine-phosphorylation. *Cell Adhes. Commun.* **2**, 481-490.

Miekka, S. I., Ingham, K. C. and Menache, D. (1982). Rapid method for isolation of human plasma fibronectin. *Thromb. Res*. **27**, 1-14.

Miyamoto, S., Akiyama, S. K. and Yamada, K. M. (1995a). Synergistic roles for receptor occupancy and aggregation in integrin transmembrane function. *Science* **267**, 883-885.

Miyamoto. S., Teramoto, H., Coso, O. A., Gutkind, J. S., Burbelo, P. D., Akiyama, S. K. and Yamada, K. M. (1995b). Integrin function: molecular hierarchies of cytoskeletal and signaling molecules. *J. Cell Biol.* **131**, 791-805.

Re, F., Zanetti, A., Sironi, M., Polentarutti, N., Lanfrancone, L., Dejana, E. and Colotta, F. (1994). Inhibition of anchorage-dependent cell spreading triggers apoptosis in cultured human endothelial cells. *J. Cell Biol.* **127**, 537-546.

Ridley, A. J. and Hall, A. (1992). The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* **70**, 389-399.

Rohrschneider, L. R. (1980). Adhesion plaques of Rous sarcoma virus-transformed cells contain the src gene product. *Proc. Nat. Acad. Sci. USA* **77**, 3514-3518.

Schaller, M. D. and Parsons, J. T. (1994). Focal adhesion kinase and associated proteins. *Curr. Opin. Cell Biol.* **6**, 705-710.

Schlaepfer, D. D., Hanks, S. K., Hunter, T. and Van der Geer, P. (1994). Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. *Nature* **372**, 786-791.

Schwartz, M. A., Schaller, M. D. and Ginsberg, M. H. (1995). Integrins: emerging paradigms of signal-transduction. *Annu. Rev. Cell Dev. Biol.* **11**, 549-599.

Shimoyama, Y., Nagafuchi, A., Fujita, S., Gotoh, M., Takeichi, M., Tsukita, S. and Hirohashi, S. (1992). Cadherin dysfunction in a human cancer cell line: possible involvement of loss of α-catenin expression in reduced cell-cell adhesiveness. *Cancer Res.* **52**, 5770-5774.

Stoker, M., O'Neill, C., Berryman, S. and Waxman, V. (1968). Anchorage and growth regulation in normal and virus-transformed cells. *Int. J. Cancer* **3**, 683-693.

Studier, F. W., Rosenberg, A. H., Dunn, J. J. and Dubendorff, J. W. (1990). Use of T7 RNA polymerase to direct expression of cloned genes. *Meth. Enzymol*. **185**, 60-89.

Takeichi, M. (1990). Cadherins: a molecular family important in selective cell-cell adhesion. *Annu. Rev. Biochem.* **59**, 237-252.

Tokman, M. G. (1997). Regulation of cadherin-mediated adhesion by the small GTP-binding protein Rho in small cell lung carcinoma cells. *Cancer Res.* **57**, 1785-1793.

Tsukita, S., Oishi, K., Akiyama, T., Yamanashi, Y., Yamamoto, T. and Tsukita. S. (1991). Specific proto-oncogenic tyrosine kinases of src family are enriched in cell-to-cell adherens junctions where the level of tyrosine phosphorylation is elevated. *J. Cell Biol.* **113**, 867-879.

Tsukita, S., Itoh, M., Nagafuchi, A., Yonemura, S. and Tsukita, S. (1993). Submembranous junctional plaque proteins include potential tumor suppressor molecules. *J. Cell Biol.* **123**, 1049-1053.

Turner, C. E. (1994). Paxillin: a cytoskeletal target for tyrosine kinases. *BioEssays* **16**, 47-52.

Van Etten, R. A., Jackson, P. K., Baltimore, D., Sanders, M. C., Matsudaira, P. T. and Janmey, P. A. (1994). The COOH terminus of the c-Abl tyrosine kinase contains distinct F- and G-actin binding domains with bundling activity. *J. Cell Biol.* **124**, 325-340.

Van Roy, F. and Mareel, M. (1992). Tumor invasion: effect of cell adhesion and motility. *Trends Cell Biol.* **2**, 163-169.

Vasiliev, J. and Gelfand, I. (1981). *Neoplastic and Normal Cells in Culture*. Cambridge University Press, Cambridge, London, New York.

Volberg, T., Zick, Y., Dror, R., Sabanay, I., Gilon, C., Levitzki, A. and Geiger, B. (1992). The effect of tyrosine-specific protein phosphorylation on the assembly of adherens-type junctions. *EMBO J.* **11**, 1733-1742.

Volk, T. and Geiger, B. (1986). A-CAM:A 135-kD receptor of intercellular adherans junctions. II. Antibody-mediated modulation of junction formation. *J. Cell Biol*. **103**, 1451-1464.

Watabe, M., Nagafuchi, A., Tsukita, S. and Takeichi, M. (1994). Induction of polarized cell-cell association and retardation of growth by activation of the E-cadherin-catenin adhesion system in a dispersed carcinoma line. *J. Cell Biol*. **127**, 247-256.

Williams, E. J., Furness, J., Walsh, F. S. and Doherty, P. (1994). Activation of the FGF receptor underlies neurite outgrowth stimulated by L1, N-CAM and N-cadherin. *Neuron* **13**, 583-594.

Yamada, K. M. and Miyamoto, S. (1995). Integrin transmembrane signaling and cytoskeletal control. *Curr. Opin. Cell Biol.* **5**, 681-689.

Yamada, K. M. and Geiger, B. (1997). Molecular interactions in cell adhesion complexes. *Curr. Opin. Cell Biol*. **9**, 76-85.