Activation of TrkA tyrosine kinase in embryonal carcinoma cells promotes cell compaction, independently of tyrosine phosphorylation of catenins

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SUMMARY

Cadherins are transmembrane receptors whose extracellular domain mediates homophilic cell-cell interactions, while their cytoplasmic domain associates with a family of proteins known as catenins. Although the mechanisms that regulate the assembly and functional state of cadherin-catenin complexes are poorly understood, current evidence supports a role for protein tyrosine kinase activity in regulating cell adhesion and migration. Tyrosine phosphorylation of catenins is thought to mediate loss of intercellular adhesion promoted by activation of receptor tyrosine kinases in embryonic cells. Here, we show that activation of ectopically expressed TrkA, the tyrosine kinase receptor for nerve growth factor (NGF), stimulates embryonal carcinoma P19 cells to develop extensive intercellular contacts and to assemble into closely packed clusters. Thus, activation of receptor tyrosine kinases can differentially regulate adhesiveness by cell-type-specific mechanisms. Furthermore, activation of TrkA in P19 and epithelial MDCK cells induces tyrosine phosphorylation of p120⁶⁶⁸ and of β-catenin, irrespective of the elicited cellular response. The selective Src tyrosine kinase inhibitor PP2, however, suppresses NGF- or HGF-induced tyrosine phosphorylation of catenins in both P19 and MDCK cells without interfering with the acquisition of a compacted or scattered phenotype. These findings provide a cogent argument for considering that tyrosine phosphorylation of catenins is dispensable for their interaction with cadherins and, ultimately, for the modulation of cadherin-based cell adhesion by receptor tyrosine kinases.

Key words: Tyrosine kinase, Catenin, Cadherin, NGF, Embryonal carcinoma cell

INTRODUCTION

Adhesive cell interactions play essential roles in the assembly of individual cells to generate and maintain tissue structure (Gumbiner, 1996). Deregulation of intercellular adhesion has often been observed in cancer cells and compelling correlations exist between the loss of tight cellular association and the invasive and metastatic properties of many tumours (reviewed in Takeichi, 1993; Birchmeier and Behrens, 1994). Among cell adhesion molecules, proteins of the cadherin family mediate the most important and ubiquitous type of adhesive contacts, and loss of E-cadherin function in epithelial cells results in progression to malignancy and induction of an invasive behaviour (Perl et al., 1998). Classical cadherins are Ca²⁺-dependent homophilic cell-cell adhesion receptors, which via their highly conserved cytoplasmic domain associate with the catenins, namely β-catenin, plakoglobin, p120⁶⁶⁸ and α-catenin (reviewed in Kemler, 1992; Yap et al., 1997). β-catenin and plakoglobin are vertebrate homologs of the Drosophila Armadillo (Arm) protein, an essential component of the Wingless signaling pathway (McCrea et al., 1991). The binding of β-catenin or plakoglobin to α-catenin mediates the tethering of cadherins to the actin cytoskeleton and is essential for optimal adhesion function of cadherins (Cowin and Durke, 1996). p120⁶⁶⁸ (hereafter referred to as p120) is structurally similar to β-catenin and plakoglobin (Reynolds et al., 1994), and binds to classical cadherins in a different domain from other catenins (Reynolds et al., 1994; Navarro et al., 1998; Yap et al., 1998), suggesting a specific role in modulating cell-cell adhesion (Yap et al., 1998; Ozawa and Kemler, 1998; Aono et al., 1999).

The current evidence supports a role for protein-tyrosine kinase activity in regulating cell adhesion and migration. Tyrosine phosphorylation of β-catenin correlates with decreased intercellular adhesion and has been implicated in the dissociation of the cadherin-catenin complex from the actin cytoskeleton (Kinch et al., 1995). Activation of a number of growth factor receptors with intrinsic tyrosine kinase activity (RTKs), including the epidermal growth factor (EGF) receptor and the hepatocyte growth factor (HGF) receptor, induces tyrosine phosphorylation of β-catenin and plakoglobin and correlates with increased cell dissociation and migration (Hoschuetzky et al., 1994; Shibamoto et al., 1994). Significantly, it has been reported that β-catenin directly binds
to the EGF receptor and the related c-erbB-2 receptor (Hoschuetzky et al., 1994; Ochiai et al., 1994). Cell transformation by v-src, a non-receptor tyrosine kinase, also results in the accumulation of phosphorylated tyrosine residues in β-catenin and p120, and in the attenuation of cadherin-mediated cell adhesion (Hamaguchi et al., 1993; Beherens et al., 1993). Moreover, it is becoming clear that a number of protein tyrosine phosphatases (PTPs) associate with cadherins and catenins in vivo and are, therefore, assumed to participate in regulating cell adhesion (Kypa et al., 1996; Brady-Kalnay et al., 1998; Muller et al., 1999). In spite of the evidence pointing towards phosphorylation as a regulatory event in intercellular adhesion, the data to date are largely correlative and it remains to be clarified how intracellular protein tyrosine phosphorylation events can regulate the functional state of the cadherin-catenin complex in a variety of cell contexts.

To delineate some of the mechanisms by which activation of an RTK might affect cadherin function, we expressed TrkA, the tyrosine kinase receptor for nerve growth factor (NGF) (Barbacid, 1995), in P19 cells. The pluripotent P19 embryonal carcinoma cells have been widely used as an in vitro model to investigate the role of intercellular adhesion during cell differentiation. Indeed, P19 cells can be induced to differentiate efficiently along the neurogenic or myogenic lineages by exposure to retinoic acid or DMSO, respectively, only when the cells are allowed to form extensive cell-cell contacts in aggregates (McBurney et al., 1988). The requirement of cell-cell adhesion is demonstrated by the observation that P19 cells ectopically expressing MyoD require aggregation in order to differentiate into skeletal muscle cells (Skerjanc et al., 1994), whereas the expression of v-src, which decreases cell-cell adhesion, inhibits neuronal differentiation of P19 cells (Schmidt et al., 1992). In the current study, we show that NGF activation of TrkA receptors in P19 cells leads to increased intercellular adhesion, resulting in the acquisition of a compacted phenotype. Since MDCK-TrkA epithelial cells scatter in response to NGF (Sachs et al., 1996), this finding introduces a novel perspective on the role of RTKs in modulating cell adhesiveness. Furthermore, we show that catenins are tyrosine phosphorylated in compacted cells as they are in scattering cells. Through the use of pharmacological inhibitors, we find that tyrosine phosphorylation of catenins can be divorced from their competence to associate with cadherins and from the distinct phenotypes induced by activation of RTKs in P19 and MDCK cells.

MATERIALS AND METHODS

Materials and antibodies

Highly purified NP-40 and Triton X-100 were from Boehringer. NHF was purified from mouse submaxillary glands and generously provided by D. Mercanti. The HAV decapeptides (LRAHVSDKVGG- and LRAHPVDVNG-amide) were kindly provided by F. Tatò. A rabbit polyclonal antiserum to TrkA, Trk (C-14), was from Santa Cruz Biotechnology. Rabbit polyclonal antisera against α-catenin and Shc, and mAbs to E-cadherin (Clone 36), β-catenin (Clone 14) and p120 (Clone 98), were purchased from Transduction Laboratories. Anti-phosphotyrosine mAbs PT-66 and 4G10 were purchased from Sigma and Upstate Biological Inc., respectively. The 13A9 monoclonal antibody to the intracellular domain of N-cadherin has been described previously (Knudsen et al., 1995). The 15F11 mAb to plakoglobin was kindly provided by M. J. Wheelock. Rat mAb DECA-1, used for immunofluorescent labeling of E-cadherin, was purchased from Sigma.

Cell culture and generation of TrkA-expressing cell lines

A TrkA expression vector (pcDNA3/TrkA) was constructed by subcloning the full-length human TrkA cDNA (Martin-Zanca et al., 1989; kindly provided by M. Barbacid) into the expression plasmid pcDNA3 (Invitrogen). The expression of TrkA cDNA is driven by the CMV promoter. P19-TrkA cell lines were developed by stable transfection of wild-type P19 with pcDNA3/TrkA plasmid using LipofectAMINE reagent. After transfection, neomycin-resistant clones were selected and analyzed for TrkA protein expression by immunoblotting analysis. MDCK epithelial cells were kindly provided by F. Tatò and by K. Simon. A subline of MDCK cells that expresses TrkA (T-12) (Sachs et al., 1996) was kindly provided by W. Birchmeier. MDCK and MDCK-TrkA cells were maintained in DMEM containing 10% FBS and P19-TrkA cells in α-MEM supplemented with 7.5% heat-inactivated newborn calf serum and 2.5% FBS.

Cell dissociation assay

For the cell dissociation assay, monolayer cultures of P19-TrkA cells were grown for 24 hours on 35 mm Petri dishes (Nunc) in the presence or absence of 50 ng/ml NGF. Cells were detached using a rubber policeman and the cell suspensions were forced 20 times through Pasteur pipettes. 3 mM EDTA was then added to cell suspension samples to obtain single cells. The extent of cell dissociation was represented by the index Np/Nc, where Np and Nc are the total number of particles and cells per dish, respectively. The mean value of three independent experiments was determined.

Cell extraction and immunoprecipitation

Subconfluent cultures were treated with NGF (50 ng/ml) for the indicated lengths of time and H2O2 (50 μM) and Na3VO4 (25 μM) were added to the medium for 5 minutes at 37°C. The treatment was terminated by washing with PBS containing 100 μM Na3VO4, followed by extraction in situ at 4°C. For co-immunoprecipitation analysis, cells were solubilized with a 0.5% NP-40 containing CSK extraction buffer (10 mM Pipes buffer, pH 6.8, 100 mM NaCl, 300 mM sucrose, 1 mM MgCl2, 1 mM EGTA, 1 mM Na3VO4, 10 mM NaF, 10 μg/ml aprotonin, 10 μg/ml leupeptin) for 30 minutes at 4°C and lysates were clarified by centrifugation at 10,000 g for 10 minutes at 4°C. To assess tyrosine-phosphorylated proteins, cells were solubilized in 1% SDS containing extraction buffer, heated at 100°C for 10 minutes and then diluted to 0.1% SDS with extraction buffer. Equal amounts of protein lysates were incubated at 4°C for 2 hours with the appropriate antibodies and the immunocomplexes were collected by binding to either protein A or protein G-Agarose beads (Boehringer), followed by three washes with 0.5% NP-40 containing extraction buffer. In immunoprecipitation experiments with mAb 13A9, agarose-conjugated goat-anti-mouse (Sigma) was used. Detergent-soluble and -insoluble fractions were obtained by lysing cells in CSK 0.5% NP-40 buffer for 5 minutes at 4°C. The supernatants were collected as detergent-soluble fractions. NP-40 extracted cells were washed once with PBS and resuspended in SDS-lysis buffer and this fraction was used as the detergent-insoluble fraction. For total protein analysis, cell lysates were alcohol-precipitated (ethanol, methanol, acetone and water, 4:2:2:2) and resuspended in Laemmli sample buffer (Laemmli, 1970). The protein concentration of clarified extracts was determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories).

Immunoblotting

Total cell extracts or washed immune complex pellets were boiled for 5 minutes in Laemmli sample buffer, resolved in a 7.5% SDS-polyacrylamide gel and electrophotically transferred to Hybond C-Super nitrocellulose paper (Amersham). For western immunoblot
analysis, filters were incubated in a Tris-buffered saline (TBS) solution containing 0.1% Tween 20 and 5% non-fat dried milk or 2% bovine serum albumin (BSA) fraction V (for phosphotyrosine immunoblots). Filters were then incubated for 2 hours at room temperature with the appropriate antibodies diluted in TBS also containing 0.1% Tween 20 and 2% non-fat dried milk or 1% BSA solution (for phosphotyrosine immunoblots). Following extensive washing in TBS solution, filters were incubated with either horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgGs (Amersham). The filters were washed as above and developed using the POD chemiluminescence detection system (Boehringer).

**Scanning electron microscopy**

Cells cultured on plastic coverslips, or cell aggregates, were washed twice with PBS and fixed with 2.5% glutaraldehyde in 0.1 M Millonig’s phosphate buffer (MPB; van der Horst et al., 1989) for 1 hour at 4°C. After washing in MPB, cell aggregates were applied to poly-lysine-coated coverslips. All samples were post-fixed with 1% OSO4 in the same buffer for 1 hour at 4°C and dehydrated through ascending acetone concentrations. The specimens were critical point-dried with liquid CO2 and sputter-coated with gold before examination under a Stereoscan 240 scanning electron microscope.

**Immunofluorescence**

Cells grown on 35 mm dishes were fixed at room temperature for 15 minutes with 3.7% formaldehyde and permeabilized for 10 minutes with 0.5% Triton X-100 when applying mAbs to E-cadherin (DECMA-1) and N-cadherin (13A9). Primary antibody detection was by reaction with FITC-conjugated rabbit anti-rat or goat anti-mouse IgGs (Sigma). Cells were visualized using a Leitz microscope equipped with 50× oil-immersion objectives.

**RESULTS**

**NGF stimulates adhesive strength and compaction of P19-TrkA cells**

We have previously shown that undifferentiated P19 cells do not express any of the Trk receptors (Salvatore et al., 1995), the high-affinity neurotrophin receptors with intrinsic tyrosine kinase activity (Barbacid, 1995). P19-TrkA clones were established by stable transfection of P19 cells with an expression vector containing the human TrkA cDNA and, out of 15 clones isolated independently, we chose three transfectants which express high levels of TrkA. As measured by immunoprecipitation and immunoblotting with specific anti-TrkA antibodies, we estimated that each of the selected P19-TrkA clones expresses approximately six- to tenfold more receptor than endogenous TrkA in PC12 cells (data not shown). Analysis of all three clones gave consistent results and data from clone CA-1 are shown.

By scanning electron microscopy, monolayer cultures of parental and TrkA-transfected P19 cells appeared morphologically indistinguishable (data not shown). Untreated P19-TrkA cells were flat and well spread, both in subconfluent and high density cultures (Fig. 1). High magnification views of the cultures clearly showed widely spaced cells with only restricted areas of the cell periphery involved in intercellular contacts (Fig. 1B). Time-lapse video microscopy of P19 and P19-TrkA cells growing in subconfluent cultures highlighted the disposition of both
types of cells to migrate away from one another and to rapidly remodel intercellular contacts.

The presence of NGF greatly modifies the pattern of distribution of P19-TrkA cells, while having no effect on the morphology of wild-type P19 cells. After 3-4 hours of NGF treatment, P19-TrkA cells rounded up or exhibited reduced spreading and, after approximately 8 hours, small groups of cells developed extensive and intimate contacts along adjacent cell surfaces. Following longer exposure to NGF (24-48 hours), P19-TrkA cells packed together and, instead of spreading onto the substrate, piled up on each other forming three-dimensional clusters in which individual cells were not easily distinguished (Fig. 1C-F). Despite the tight clustering, NGF-treated P19-TrkA cells continued to grow and retained the 14-hour doubling time typical of untreated cells, measured by countings of dissociated cells over a 48 hour time period. Time-lapse video microscopy showed that clustered cells break contacts with adjacent cells and round up just before dividing, and that daughter cells rapidly resume stable contacts with cells of the same and, more rarely, of a different cluster. Within the cluster, cells were motile and migrated as adherent cells, resulting in changes of shape and position of the clusters.

When cultured in suspension, P19-TrkA, as well as parental P19 cells (McBurney et al., 1988), spontaneously tend to cluster, forming large aggregates. We found that, under these culture conditions, NGF-stimulation results in the formation of highly compacted and smooth-surfaced cell aggregates, clearly distinguishable from those of untreated cells (not shown), indicating that a decreased cell-substrate interaction does not account for the phenotype observed in cells grown onto a substrate. Compaction of P19-TrkA cells required the continuous presence of NGF and it was a reversible process. Moreover, incubation of P19-TrkA cells with 100 nM K252a, a selective inhibitor of the tyrosine kinase activity of Trk receptors (Tapley et al., 1992), completely prevented the NGF-induced compaction (data not shown).

Quantification of the strength of cell-to-cell adhesion by a cell dissociation assay (see Materials and Methods) revealed that, following exposure to NGF for 24 hours, adhesion of P19-TrkA cells shifts from a weak state, \( N_p/N_c = 0.68\pm0.05 \) s.d., to an approximately threefold stronger state, \( N_p/N_c = 0.23\pm0.05 \) s.d. (\( N_p \) and \( N_c \) are the total number of particles and cells per dish, respectively).

NGF-induced cell compaction depends on cadherin activity

To understand the basis of the observed phenotypic changes in P19-TrkA cells, we first investigated whether NGF-stimulated compaction was sensitive to extracellular calcium levels. Consistent with the involvement of Ca\(^{2+}\)-dependent homophilic interactions between cadherins, we found that reduction of Ca\(^{2+}\) in the culture medium to 50 \(\mu\)M prevented NGF from inducing adhesiveness of P19-TrkA cells and let cells grow adherent to the substrate in an entirely dissociated pattern (not shown).

The involvement of cadherin-mediated cell-cell adhesion was assessed in a different way by culturing P19-TrkA cells for 24 hours in the presence of NGF and of a synthetic HA V-containing decapeptide, shown to inhibit cadherin-mediated cell adhesion when applied extracellularly (Blaschuk et al., 1990). HA V(histidine-alanine-valine) is a highly conserved sequence present in the EC1 domain of all cadherins and has been proved to be crucial for homophilic cadherin interactions. As shown in Fig. 2, incubation of the cultures with the HA V peptide (1 mg/ml), which affected neither the morphology nor the doubling time of P19-TrkA cells, significantly inhibited NGF-induced cell compaction, clearly indicating that this effect requires functional cadherins. A decapeptide in which the alanine residue within the HA V sequence was substituted with a proline, was without effect (not shown).

We also found that activation of endogenous TrkA receptors in PC12 cells, which is known to elicit neuronal differentiation (reviewed in Levi and Alemà, 1991; Barbacid, 1995) resulted in the calcium-dependent formation of cellular aggregates and in an increased strength of intercellular adhesion (not shown).
1605 TrkA induces compaction of P19 cells

Compaction of P19-TrkA correlates with an increased association of p120 and β-catenin to N-cadherin

To investigate further the involvement of cadherin complexes in mediating the NGF-stimulated compaction of P19-TrkA cells, we analyzed the steady-state levels and organization of proteins that take part in the assembly of cadherin-based adhesion complexes. For this analysis, a schedule of 24 hour NGF stimulation was chosen since at this time the NGF-promoted cell clustering was already fully evident (see Fig. 1C,D).

Western blot analysis of total cell extracts revealed that the overall amount of cadherins and catenins remained essentially unchanged in compacted P19-TrkA cells as compared to unstimulated or parental P19 cells (Fig. 3B). To analyze cadherin-associated catenins, P19-TrkA cells cultured in the absence or in the presence of NGF were solubilized in 0.5% NP-40 containing CSK buffer (see Materials and Methods) and the detergent-soluble protein fractions were immunoprecipitated with antibodies to E- or N-cadherin, β-catenin, p120 and plakoglobin (pg), as indicated. (B-C) Wild-type (WT) P19 cells and (TrkA) P19-TrkA cells, cultured for 24 hours in the absence or in the presence of NGF (50 ng/ml), were lysed in 1% SDS extraction buffer. Protein-equivalent portions of cell extracts diluted 1:10 were subjected to SDS-PAGE (B) or immunoprecipitated with a specific antibody to phosphotyrosine (PY) and analyzed by western blot with mAbs to E-cadherin, N-cadherin, plakoglobin, β-catenin and p120 and with antisera to TrkA (TrkA) and to α-catenin (α-cat), as indicated (C). Samples of the same extracts were immunoprecipitated with mAbs to β-catenin and p120 and probed with antibody to PY. Note that two bands of 120 and 100 kDa, corresponding to p1201 and p1202 isoforms of p120, are clearly resolved.

NGF increases the tyrosine phosphorylation levels of p120 and β-catenin

Cadherins and some catenins are targets of different tyrosine kinases and changes in the tyrosine phosphorylation level of these proteins have been correlated with changes in the functional state of cadherin adhesion complexes (reviewed in Daniel and Reynolds, 1997). To analyze changes in tyrosine phosphorylation of individual components of cadherin-catenin complexes during NGF-stimulated compaction, P19-TrkA cells cultured in the absence or in the presence of NGF were lysed in a buffer containing 1% SDS and equivalent amounts showed pronounced N-cadherin staining of extensive areas along intercellular surfaces in NGF-stimulated P19-TrkA cells. In contrast, E-cadherin staining was not significantly affected by NGF, remaining restricted to a few cell-cell contact areas as in unstimulated P19-TrkA cells (not shown).
of total cell protein were immunoprecipitated with a phosphotyrosine-specific antibody (anti-PY) and analyzed by immunoblotting with specific antibodies to TrkA, E-cadherin, N-cadherin, β-catenin, plakoglobin, p120 and α-catenin. Parental P19 cells were used as control. Analysis of tyrosine-phosphorylated proteins revealed that, compared to parental cells, P19-TrkA showed slightly increased basal levels of tyrosine-phosphorylated β-catenin, p120 and N-cadherin (Fig. 3C). Activation of TrkA by NGF notably increased the pools of tyrosine-phosphorylated β-catenin and, to a much greater extent, p120, without affecting the tyrosine phosphorylation level of E-cadherin, N-cadherin and plakoglobin (Fig. 3C). Phosphorylation of α-catenin was not detected (Fig. 3C). The aggregation of PC12 cells following NGF treatment was also paralleled by an increased association of p120 and β-catenin with N-cadherin complexes and correlated with the accumulation of tyrosine-phosphorylated p120 and β-catenin (data not shown; Kypta et al., 1996).

To quantify the fraction of β-catenin and p120 that undergoes tyrosine phosphorylation in response to TrkA activation, cell lysates from 24 hour NGF-treated P19-TrkA cells were subjected to sequential cycles of immunoprecipitation with anti-PY as previously described (Matoskova et al., 1995) and analyzed by western blot with antibodies to β-catenin and p120. We estimated that about 20% of the total p120 pool was phosphorylated under our experimental conditions, whereas tyrosine-phosphorylated β-catenin was ≤1% of the total pool (data not shown).

An increased association of tyrosine-phosphorylated p120 with E-cadherin has been observed in cells transformed by ras and src oncogenes (Kinch et al., 1995; Papkoff, 1997). In an attempt to understand the role of tyrosine-phosphorylated catenins in compacting cells, we first determined whether tyrosine-phosphorylated p120 was complexed with N-cadherin in NGF-stimulated P19-TrkA cells. Sequential immunoprecipitations were therefore carried out: whole-cell extracts were first immunoprecipitated with antibodies to N-cadherin. Immunocomplexes were analyzed by immunoblotting with mAbs to p120, β-catenin, α-catenin and N-cadherin, as indicated. (C) The Src kinase inhibitor PP2 inhibits tyrosine phosphorylation of catenins without affecting the association of catenins to N-cadherin. Cell lysates of P19-TrkA cells treated with NGF and PP2 for 24 hours were subjected to immunoprecipitation with antibodies to PY and to N-cadherin. Western blots were probed with antibodies to TrkA, p120, β-catenin and N-cadherin.
autophosphorylation of TrkA and tyrosine phosphorylation of Shc, an adapter protein linking growth factor receptors to downstream signaling molecules (Rozakis-Adcock et al., 1992), were detected within 10 minutes of NGF stimulation (not shown). Moreover, a time-resolved pattern of NGF-stimulated tyrosine-phosphorylated protein from cell extracts of MDCK cells treated with HGF (10 ng/ml) and PP2 (3 μM) as indicated. The blots were probed with antibodies to the indicated proteins. (C) Cell lysates of unstimulated MDCK cells or treated with HGF and PP2 as indicated were fractionated into a 0.5% NP-40-soluble (S) and -insoluble (I) fraction. Equal sample volumes were loaded and probed for β-catenin and E-cadherin.

**Tyrosine phosphorylation of p120 and β-catenin is not required for NGF-induced cell compaction**

To directly address the question of whether tyrosine-phosphorylated catenins play a role in mediating NGF-stimulated adhesiveness, we performed experiments aiming at selectively interfering with the activity of kinases putatively acting downstream of TrkA receptors. In agreement with recent evidence that nonreceptor tyrosine kinases of the Src family are implicated in catenin phosphorylation (Calautti et al., 1998), we found that exposure of NGF-stimulated P19-TrkA cells to PP2 (3 μM), a specific inhibitor of Src family kinases (Hanke et al., 1996), caused a reduction of tyrosine-phosphorylated β-catenin and p120 to basal levels within 30 minutes of treatment (not shown). The inability of PP2 to alter TrkA tyrosine kinase activity was verified by measuring the NGF-induced autophosphorylation of TrkA in P19-TrkA cells exposed to the inhibitor (Fig. 4C), at concentrations blocking NGF-induced phosphorylation of Arm catenins.

The concomitant treatment of P19-TrkA cells with PP2 and NGF for up to 24 hours completely prevented the NGF-stimulated tyrosine phosphorylation of β-catenin and p120, but neither impaired the increased association of p120 and β-catenin with N-cadherin (Fig. 4C), nor suppressed the formation of cell adhesive structures and cell compaction, as assessed by morphological analysis and immunofluorescence staining with anti-N-cadherin antibodies of the cultures (not shown). Since PP2 was equally ineffective when added to 24-hour NGF-stimulated P19-TrkA cells and assayed for maintenance of compacted phenotype (not shown), we conclude that tyrosine phosphorylation of catenins is not required for cadherin complex assembly/disassembly in NGF-compacted P19 cells.

**HGF-induced adherens junction disassembly in MDCK cells is independent of tyrosine phosphorylation of catenins**

Activation of TrkA by NGF appears to promote opposite responses in adhesion properties depending on the cellular context. We show that NGF induces clustering of P19-TrkA cells, whereas it has been reported that NGF promotes cell scattering of TrkA-expressing MDCK epithelial cells (Sachs et al., 1996). Biochemical analysis of the MDCK-TrkA, clone T-12 (Sachs et al., 1996), consistent with data obtained in HGF-treated MDCK cells (Potempa and Ridley, 1998), showed that NGF promoted disruption of adherens junctions as assessed by: (1) the dispersion of E-cadherin, β-catenin and plakoglobin from intercellular contacts within 4-6 hours of treatment (not shown); (2) a decreased association of plakoglobin with E-cadherin complex (Fig. 5A) and (3) a reduced amount of E-cadherin in the detergent-insoluble fraction of cell lysates (not shown). Cadherin solubility is considered to reflect its association with the actin cytoskeleton and to correlate with changes in the functional state of cadherins. In addition, we found that the loss of adherens junctions promoted in MDCK cells by NGF or HGF correlated with the accumulation of tyrosine-phosphorylated β-catenin, plakoglobin and p120 (Fig. 5A,B), starting as early as 10 minutes after stimulation and beginning to decline 6-8 hours after (not shown).

In order to establish whether stimulation of tyrosine phosphorylation of catenins and destabilization of cell adhesion are causally related events, we used PP2 to interfere with the action of NGF and HGF in MDCK cells. Fig. 5 shows that treatment with PP2 (3 μM) fully inhibited tyrosine phosphorylation increase of catenins (β-catenin, plakoglobin and p120) promoted by NGF in MDCK-TrkA cells (Fig. 5A).
and by HGF in parental MDCK cells (Fig. 5B). The selective increase of the basal levels of tyrosine-phosphorylated β-catenin and plakoglobin in unstimulated MDCK cells exposed to PP2, however, remained unresolved (Fig. 5A,B). Although affecting tyrosine phosphorylation of catenins, PP2 treatment did not prevent the scattering response of three different strains of parental MDCK to HGF. The disruption of cadherin-mediated adhesion was assessed morphologically by immunostaining with anti-E-cadherin antibodies (Fig. 6) and biochemically by the redistribution of E-cadherin and β-catenin from the NP-40-insoluble to the NP-40-soluble fraction (Fig. 5C) and dissociation of plakoglobin from E-cadherin complex (not shown and Fig. 6A), events associated with a shift from a strong to a weak state of intercellular adhesiveness, were not affected by PP2. The distinct behaviour of MDCK-TrkA cells is likely to be due to the clonal nature of these cells, which may have adopted a pattern of regulation of interaction with the substrate and of cell motility that is more dependent on the activity of Src family kinases. Taken together, these results provide evidence that tyrosine phosphorylation of catenins is not involved in the disassembly of adherens junctions promoted by RTKs in MDCK cells.

**DISCUSSION**

Much data to date correlate an increased tyrosine phosphorylation activity with a perturbed cadherin-mediated cell adhesion. Specifically, it has been reported that several RTKs, including TrkA, mediate cell dissociation and increased motility in epithelial cells (Sachs et al., 1996; Daniel and Reynolds, 1997). In this report we show that activation of ectopically expressed TrkA receptors stimulates P19 cells to assemble in closely packed three-dimensional clusters and promotes an increased strength of cell-to-cell adhesion by a mechanism involving cadherin function. Moreover, activation of TrkA, irrespective of the elicited effect on the adhesiveness, compaction of P19 cells or dissociation of MDCK cells, leads to increased levels of tyrosine-phosphorylated p120 and β-catenin. Therefore, these findings reveal a novel function for a receptor tyrosine kinase in sustaining a range of strengths of cadherin-mediated adhesion, thus lending support to the importance of tyrosine phosphorylation events for cell-cell adhesion. However, they implicate rich complexity in the molecular mechanism by which protein-tyrosine kinases modulate adhesiveness that was previously unappreciated, since they indicate that tyrosine phosphorylation of catenins per se may not suffice to determine the functional state of cadherin complexes and that cell-type-specific mechanisms or molecules participate in regulating adhesive activity.

Arm catenins (β-catenin, plakoglobin and p120) are major targets of protein tyrosine kinases within cadherin complexes, and an increased tyrosine phosphorylation of catenins correlates with a functional inactivation of cadherin-mediated cell adhesion in v-Src-transformed or growth factor-stimulated cells (reviewed in Daniel and Reynolds, 1997). On the basis of these findings, several models have been proposed to explain how tyrosine phosphorylation of catenins might affect cadherin/catenin complex assembly and perturb cadherin activity (Daniel and Reynolds, 1997; Yap et al., 1997). Attempts to draw firm conclusions on this issue, however, have been frustrated by conflicting evidence generated by largely correlational observations (Reynolds et al., 1994; Shibamoto et al., 1994; Calautti et al., 1998; Lampugnani et al., 1997).

A discerning finding that allows us to address the role of tyrosine-phosphorylated catenins in RTK-mediated remodelling of intercellular adhesion is that tyrosine phosphorylation of catenins following RTK activation appears to be under the control of Src family tyrosine kinases in both P19 and MDCK cells. Although not proving that catenins are direct substrates of Src kinases, PP2, a specific inhibitor of Src kinases, selectively erases the tyrosine phosphorylation signal from Arm catenins in response to NGF or HGF. Inhibition of tyrosine phosphorylation of catenins by PP2 does not affect cadherin/catenin complex assembly and, most importantly, compaction of NGF-stimulated P19-TrkA cells. Scattering of MDCK cells in response to HGF is also not affected by
treatment with PP2. In keratinocytes, however, inhibition of Fyn activity by a compound related to PP2 inhibits p120 tyrosine phosphorylation and also impairs cell-cell adhesion (Calautti et al., 1998). Therefore, additional Src family-regulated pathways, which are not operating in the cells used in this study, appear to be involved in regulating adhesiveness in different cell types.

The conclusion that tyrosine phosphorylation of catenins is not involved in the modulation of cadherin activity by NGF and HGF raises the question as to the purpose of catenin phosphorylation by non-receptor tyrosine kinases. Our results, although not revealing the role of tyrosine-phosphorylated catenins in cadherin-independent functions, leave open other potential roles for catenin phosphorylation, such as an additional control on cadherin function, for which we do not have yet an assay. In this context it is of interest that tyrosine phosphorylation of catenins in endothelial cells appears to be temporally regulated, being downregulated in fully established junctions (Lampugnani et al., 1997). Alternatively, tyrosine-phosphorylated catenins may provide potential docking sites for the recruitment to cadherin complexes of as-yet-unknown SH2 containing proteins, thereby initiating signalling pathways.

Concerning the molecular mechanism underlying cell compaction, the observation of specific recruitment of p120 to N-cadherin complexes deserves particular attention. Compaction of P19 cells does not appear to involve a regulated interaction of cadherin with the actin cytoskeleton, since no significant changes in cadherin-associated pools of α- and β-catenins are observed until very late in compacting cells. In addition, the fraction of detergent-insoluble N-cadherin, denoted as the cytoskeleton-associated pool of cadherin, does not change upon compaction of P19-TrkA cells (our unpublished data). In contrast, NGF-induced p120/N-cadherin association closely parallels all stages of the cell compaction process. We have also observed that inhibition of NGF-induced compaction of P19 cells by low calcium prevents the increased association of p120 to N-cadherin (our unpublished observation). This indicates that recruitment of p120 requires prior engagement of cadherin receptors through homophilic binding. At variance with other catenins, p120 binds to the membrane-proximal region of the cadherin cytoplasmic tail (Lampugnani et al., 1997; Yap et al., 1998), a domain that is probably involved in the regulation of lateral clustering of cadherins (Yap et al., 1998; Ozawa et al., 1998). Clustering activity, which results in a redistribution of adhesive binding sites at the cell surface and in their accumulation in clusters, generates adhesive strength that appears to be independent of the interaction of cadherins with the actin cytoskeleton (Yap et al., 1997). Since clustering of cadherins seems to require intermediary cadherin-binding proteins (Yap et al., 1998), p120 is an obvious candidate to perform this role. Here, we show that the increased association of p120 with N-cadherin is the most striking change occurring in cadherin complexes during NGF-stimulated compaction of P19 cells. Altogether, our findings introduce the possibility that TrkA promotes cell compaction by regulating cadherin clustering activity and that p120 is specifically targeted by TrkA in regulating this process.

Since this manuscript was submitted for publication, Thoreson et al. (2000) have reported that in a reconstructed cell system p120 is capable of regulating E-cadherin-mediated transition to tight cell adhesion, in agreement with the observations reported here.

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