Signaling from E-cadherins to the MAPK Pathway by the Recruitment and Activation of Epidermal Growth Factor Receptors upon Cell-Cell Contact Formation*

Salvatore Pece and J. Silvio Gutkind‡

From the Oral and Pharyngeal Cancer Branch, NIDCR, National Institutes of Health, Bethesda, Maryland 20892-4330

E-cadherins are well characterized cell surface molecules expressed in epithelial cells, which play a major role in cell adhesion through the establishment of calcium-dependent homophilic interactions at sites of cell-cell contacts. They are also integral components of morphogenetic programs controlling the maintenance of the structural and functional integrity of epithelia. Accumulated evidence indicates that the E-cadherin-mediated cell adhesion system is highly regulated from inside the cells by a number of intracellular signaling pathways. Recently available information suggests that E-cadherins may also play a role in the transduction of signals from the outside of the cell to the cytoplasm. However, the nature of the biochemical routes regulated by E-cadherins is still largely unknown. In this study, we set out to explore the possibility that E-cadherins may regulate the activity of MAPK, a key signaling pathway involved in cell fate decisions, upon the formation of cell-cell contacts among neighboring cells. By using an immortalized non-tumorigenic keratinocyte cell line, HaCat, as a model system, we provide evidence that the assembly of calcium-dependent adherens junctions leads to a rapid and remarkable increase in the state of activation of MAPK and that this event is mediated by E-cadherins. Furthermore, we found that E-cadherins stimulate the MAPK pathway through the ligand-independent activation of epidermal growth factor receptors and the consequent activation of a biochemical route leading to the stimulation of MAPKs. These findings suggest that E-cadherins can initiate outside-in signal transducing pathways through the engagement of tyrosine kinase receptors for epidermal growth factor, thus providing a novel molecular mechanism whereby these cell adhesion molecules may ultimately control the fate of normal and transformed epithelial cells.

E-cadherins are cell surface molecules that play a major role in cell adhesion in epithelial cells through the establishment of calcium-dependent homophilic interactions at sites of cell-cell contacts (1). They also connect the extracellular environment to the contractile cytoskeleton inside the cells by their ability to bind through their short intracytoplasmic tail a set of related proteins collectively known as catenins, which, in turn, bind actin filaments and participate in the activation of certain nuclear responses (2, 3).

E-cadherins are major determinants of the epithelial cell phenotype. Indeed, E-cadherin-mediated cell-cell adhesive interactions, together with cell-extracellular matrix (ECM) contacts and growth factor signaling, are regarded as an integral part of morphogenetic programs controlling the maintenance of the structural and functional integrity of epithelia (reviewed in Ref. 4). Furthermore, misregulated E-cadherin expression or function can alter the pattern of epithelial cell growth and differentiation (4), and a strong correlation between loss of E-cadherin at the level of the cell surface and enhanced cell invasiveness in vitro or tumor progression in vivo has been described (reviewed in Ref. 5). These events often have been viewed as resulting from disrupted cell-cell adhesion rather than as a consequence of the loss or subversion of E-cadherin-dependent regulatory events. Most recently, however, it has been demonstrated that the E-cadherin-mediated adhesion system is subject to regulation from the cytoplasmic side (inside-out signals) (6), and an increasing body of evidence suggests a role for E-cadherins in the transduction of signals from the outside of the cell to the cytoplasm (outside-in signaling) (7, 8). However, despite the obvious importance of E-cadherins in epithelial cell fate determination, the nature of the E-cadherin-mediated signaling events is still largely unknown.

Of interest, the kinase cascade regulating the activity of p42/p44 mitogen-activated protein kinases (MAPKs) is one of the best characterized intracellular signaling pathways, which integrates signals emanating from a wide array of cell membrane receptors (9). For example, the MAPK pathway has been found to play a critical role in the control of cell survival, proliferation, and differentiation when activated by both polypeptide growth factor receptors and surface adhesion molecules, such as integrins, in a variety of cellular settings (10, 11). In epithelial cells, and in particular in keratinocytes, additional mechanisms controlling the activity of MAPK may also exist, such as those substituting for the loss of integrin-ECM interactions or compensating for the decreased cell surface expression of growth factor receptors under both physiological and pathological conditions, including the stepwise formation of normal stratified epithelia and tumor development and metastasis. In this study, we set out to explore the possibility that E-cadherins may regulate the activity of MAPKs upon the formation of cell-cell contacts among neighboring cells. By us-

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Oral and Pharyngeal Cancer Branch, NIDCR, Bldg. 30, Rm. 211, National Institutes of Health, 9000 Rockville Pike, Bethesda, MD 20892-4330. Tel.: 301-496-6259; Fax: 301-402-0823; E-mail: sg39v@nih.gov.

1 The abbreviations used are: ECM, extracellular matrix; MAPKs, mitogen-activated protein kinases; EGF, epidermal growth factor; DME/M, Dulbecco’s modified Eagle’s medium; MBE, myelin basic protein; poly-HEMA, poly-2-hydroxyethylmethacrylate; PBS, phosphate-buffered saline; DECA, extracellular domain of E-cadherin molecule; EGFRe, EGF receptors; BSA, bovine serum albumin; Pipes, 1,4-piperazinediethanesulfonic acid.
ing a human immortalized non-tumorigenic keratinocyte cell line, HaCat, we provide evidence that the assembly of calcium-dependent adherens junctions leads to a rapid and remarkable increase in the state of activation of MAPK and that this event is mediated by E-cadherins. Furthermore, in search for the mechanism(s) underlying MAPK activation by E-cadherins, which are devoid of any intrinsic enzymatic activity, we found that E-cadherins can induce the ligand-independent activation of EGF receptors (EGFRs), and the consequent activation of a biochemical route leading to the stimulation of MAPKs. Taken together, these findings suggest that E-cadherins can transduce outside-in signals through the engagement of tyrosine kinase receptors for EGF and provide an attractive mechanism whereby these cell-adhesion molecules can affect cell fate decision upon cell-cell contact formation.

**EXPERIMENTAL PROCEDURES**

**Cells and Culture Conditions**—HaCat cells are a spontaneously immortalized, non-tumorigenic keratinocyte cell line widely utilized as a model system for the study of structural and regulatory aspects of epithelial cell physiology and pathology (12, 13). Early passage HaCat cells were routinely maintained at 37 °C in a humidified atmosphere of 5% CO₂ and 90% air in Dulbecco’s modified Eagle’s medium (DMEM, Life Technologies, Inc.) supplemented with 10% fetal calf serum (HyClone) and 100 units/ml penicillin and 100 µg/ml streptomycin. For all the experiments, cells were grown as a confluent monolayer to optimize cell-cell contacts as well as to minimize the influence of integrin-ECM interactions (7).

**Reagents, Antibodies, and Immunologic Detection Methods**—Human recombinant EGF was from Upstate Biotechnology Inc. (Lake Placid, NY). Myelin basic protein (MBP) and poly-2-hydroxyethylmethacrylate (poly-HEMA) were from Sigma. Typhostin AG 1478 was purchased from Calbiochem. Antibodies raised against the intracytoplasmic tail of E-cadherins (Transduction Laboratories, Lexington, KY) were used for immunoprecipitation and Western blotting. Antibodies to the extracellular domain of E-cadherin molecule (DECAM-1 clone, Sigma) were used for immunostaining as well as for antibody inhibition and clustering experiments. An anti-extracellular signal-regulated kinase/mAPK polyclonal antibody (C-16, Santa Cruz Biotechnologies, Inc.) was used for in vitro kinase assay and Western blotting. Mouse monoclonal anti-phosphotyrosine (anti-Tyr(P), 4G-10 clone) and rabbit polyclonal anti-human Shc antibodies were from Upstate Biotechnology, Inc. Monoclonal antibodies recognizing EGFRs or specifically their tyrosine-phosphorylated (activated) forms (clone 14 and 74, respectively, Transduction Laboratories) were used in immunoprecipitation, Western blotting, and in immunofluorescence experiments, as appropriate. Anti-mouse and anti-rabbit secondary antibodies coupled to hors eradish peroxidase were from Cappel Laboratories (Durham, NC). Fluoro chromo-tagged antibodies for immunoprecipitation and immunofluorescence were obtained from Jackson ImmunoResearch Laboratories, Inc. Co-immunoprecipitation and Western blotting experiments with appropriate antibodies were performed as described previously (8). When necessary, blots were stripped (100 mM β-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl, pH 7.6) for 1 h at 50 °C, washed in PBS, 0.05% Tween-20 (PBST) before reprobing with specific antibodies.

**MAPK Assay**—To investigate the possible influence of adherens junction formation on MAPK activity in HaCat cells, cells were serum-starved overnight in DMEM containing 10% Heps, and the next day E-cadherin-mediated cell-cell contacts were disrupted by treatment with EGTA to a final concentration of 4 mM in DMEM for 30–45 min at 37 °C. Thereafter, intercellular interactions were allowed to recover in the presence of fresh, Ca²⁺-containing medium (1.8 mM). At different time points after calcium restoration, cells were harvested, lysed on ice, and tested for MAPK activity in immunocomplex kinase assays using MBP as a substrate in the presence of γ-32P[ATP, as described previously (8).

**EGF Receptor Kinase Assay**—A similar approach based on calcium switch experiments was also used to evaluate the fluorescence of E-cadherins in the intrinsic EGF kinase activity in HaCat cells. After serum starvation, cells were treated as described above and extracted in lysis buffer containing 1% Triton X-100, 15% glycerol, 150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 10 µg/ml aprotinin and leupeptin, 1 mM phenylmethylsulfonyl fluoride, 20 µM NaF, 1 mM Na₃PO₄, and 1 mM Na₂VO₄. After removing cell debris by centrifugation, endogenous EGF kinase was immunoprecipitated using anti-EGFR antibodies, and

EGFR kinase activity was assessed by the ability of the immunoprecipitates to phosphorylate MBP (1.5 µg/ml) in an appropriate kinase buffer (20 mM Pipes, pH 7.4, 10 mM MgCl₂, 10 mM MnCl₂, 1 mM dithiothreitol, 100 µM Na₂VO₄, 5 µM ATP, and 10 µCi of [γ-32P]ATP) at 30 °C for 5 min. Reactions were terminated by adding 2x Laemmli buffer and phosphorylated proteins were separated on 15% SDS-polyacrylamide gel, transferred onto polyvinylidene difluoride membranes, and exposed to detect phosphorylated MBP. Resulting autoradiograms were quantified with a PhosphorImager using ImageQuant software (Molecular Dynamics) densitometer. Equal immunoprecipitation and loading of proteins was assessed by immunoblotting membranes with anti-EGFR antibodies.

**Immunofluorescence Microscopy**—HaCat cells were grown on glass coverslips, washed with PBS, and fixed for 20 min at room temperature in 4% paraformaldehyde. After blocking aldehydes with 100 µM glycine in PBS for 20 min, cells were permeabilized with 0.5% Triton X-100 for 10 min at room temperature. Cells were washed with PBS, blocked with 1% BSA in PBS for 1 h, and incubated with the appropriate primary antibodies for 1 h at room temperature. Rat anti-E-cadherin (1:1000) and mouse anti-activated EGFR (1:500) antibodies were used. As secondary antibodies, fluorescein isothiocyanate anti-rat and Cy3 anti-mouse antibodies were used (1:250 working dilution), which are available as antibody preparations preadsorbed to minimize cross-reactivity with mouse or rat, respectively. Finally, cells were incubated with DAPI (Molecular Probes, Eugene, OR), and examined under a Zeiss Axiopt microscope at a ×63 magnification using appropriate filters. Images were captured by using an HRD060-NIK CCD camera (Diagnostic Instruments, Inc.) and processed by Adobe photoshop.

**Antibody Immobilization and Clustering Assays**—For antibody immobilization experiments, cells grown at confluence were serum-starved for 18–24 h, detached with 5 mM EDTA in phosphate-buffered saline (PBS), washed with PBS, and plated on tissue culture plates previously coated overnight at 4 °C with 10 µg/ml goat anti-rat IgG (Sigma), post-coated with bovine serum albumin (BSA) for 1 h at 37 °C, and further incubated with anti-E-cadherin DECMA-1 antibody (5 µg/ml) for 2 h at 37 °C. The optimal concentration of DECMA-1 antibody for this in situ immobilization assay was determined by measuring cell adhesion and MAPK activity in a set of preliminary dose-response experiments. For antibody clustering experiments, HaCat cells at confluence were detached as described above, washed, resuspended in prewarmed DMEM, and plated onto cell culture dishes precoated with poly-HEMA (0.4% in ethanol). Cells in suspension were incubated for 50–60 min at 4 °C with DECMA-1 antibodies (90 µg/ml), and surface clustering of monomeric E-cadherin receptors was induced by further incubation in the presence of 5 µg/ml goat anti-rat IgG for 30 min at 37 °C. At the end of the corresponding incubation periods, cells were washed with a stop solution containing 5 mM EDTA, 10 mM NaF, 10 mM Na₃PO₄, and 0.5 mM Na₂VO₄ in PBS, collected by pipetting, and detergent-extracted in lysis buffer for MAPK assay.

**RESULTS**

**E-cadherin-mediated Cell Adhesion Triggers MAPK Activation**—The calcium-dependent homophilic interactions among E-cadherins on adjacent cells are critical for the formation and maintenance of adherent junctions in areas of cell-cell contact (4). Thus, the ability to switch the extracellular concentration of calcium represents a simple and yet powerful tool for investigating different aspects of E-cadherin biology (14, 15). Therefore, we initially used a calcium switch approach in HaCat cells to explore the possibility that E-cadherins may signal intracellularly to the MAPK cascade upon adherens-type junction formation. HaCat cells were serum-starved for 18–24 h, and cell-cell contacts were disrupted by EGTA treatment. Subsequently, adherens junctions were allowed to reform in the presence of medium containing standard concentration of CaCl₂ (1.8 mM). As shown in Fig. 1, whereas the treatment with EGTA reduced the activity of MAPK below its basal levels, the reassembly of adherens junctions induced a remarkable elevation of MAPK activity, following a temporal pattern characterized by a rapid peak of activation as early as 5–15 min after calcium restoration, which decreased thereafter and declined to near basal levels after 1 h. Thus, these data suggest that the calcium-dependent formation of adherens junctions may stimulate intracellular signaling pathways leading to the activation of MAPKs.
E-cadherins Activate MAPK through EGFR

MAPK Activation following Cell Adhesion Requires the Engagement of E-cadherin—To assess the role of E-cadherins in MAPK activation upon the re-establishment of cell-to-cell contacts, HaCat cells were first preincubated for 30 min with a vast excess of DECMA-1 antibodies (50 μg/ml) prior to calcium restoration. Under these conditions, DECMA-1 antibodies act as blocking, function-perturbing agents likely by virtue of their ability to maintain E-cadherin in a monomeric, inactive state. As shown in Fig. 2A, hindering homophilic interactions with an excess of anti-E-cadherin antibodies prevented the elevation of MAPK activity in response to calcium restoration. In an attempt to confirm further the specificity of E-cadherin-mediated signaling, antibody immobilization and clustering experiments were also performed. As shown in Fig. 2B, plating HaCat cells onto plates precoated with anti-E-cadherin antibodies was sufficient to provoke a remarkable activation of MAPK, whereas cells plated on BSA, as a control, did not display any detectable increase of MAPK activity. Furthermore, antibody-induced clustering of E-cadherins in HaCat cells kept in suspension in poly-HEMA-coated plates also provoked a rapid and sustained activation of MAPK (Fig. 2C), thus further confirming that E-cadherin oligomerization by antibodies is able per se to induce this phenomenon. Of note, when an antibody against surface HLA-1 antigen was used in clustering experiments as a control, no increase in the state of activation of MAPK was observed (data not shown). Together, these findings indicate that the engagement of E-cadherins in calcium-dependent homophilic interactions in areas of cell-cell contact is sufficient to stimulate intracellular signaling pathways leading to MAPK activation.

Role of EGF Receptor in E-cadherin-dependent Activation of MAPK—Cell adhesion and growth factor receptor signaling are closely linked processes involved in the determination of cell fate. Of interest, receptor tyrosine kinases, including EGFR, have been found to localize to adherens-type junctions (16). Thus, in search for a mechanism underlying MAPK activation by E-cadherins, which do not exhibit any intracellular enzymatic activity, we asked whether EGFR could be involved in signaling to the MAPK cascade upon E-cadherin-dependent cell-cell contact formation. To address this issue, we took advantage of the availability of the potent EGFR kinase-specific inhibitor, such as tyrphostin AG 1478 (17). Indeed, as shown in Fig. 3A, preincubation of HaCat cells for 30 min with tyrphostin AG 1478 (250 nM) completely abolished the enhancement of MAPK activity following calcium restoration.

The mechanism of EGFR activation by cognate ligands implies a rapid activation of its intrinsic kinase activity resulting in increased phosphorylation of tyrosine residues on its intracellular domain (18). Thus, to investigate further the involvement of EGFR in signaling downstream of E-cadherin-mediated cell-cell contacts, the receptor kinase activity was evaluated on EGFR immunoprecipitates using MBP as an exogenous substrate. As shown in Fig. 3B, the state of activation of EGFR kinase increased in response to calcium-induced formation of intercellular boundaries mediated by E-cadherins. As a complementary approach, the accumulation of hyperphosphorylated, active forms of EGFR was also monitored using specific anti-phospho-EGFR antibodies. By using this approach, we found that the kinetic pattern of tyrosine phosphorylation of EGFR correlated closely with its elevated enzymatic activity in in vitro kinase assays.

Adhesion-induced Activation of EGFR Leads to Tyrosine Phosphorylation of Shc—A number of cytoplasmic proteins propagate signals emanating from EGFRs upon their activation. They include the adaptor molecule Shc, which is a well-known downstream effector of the EGFR-initiated pathway that leads to MAPK activation (20). Thus, we decided to test whether Shc underwent tyrosine phosphorylation as a consequence of E-cadherin-dependent enhancement of EGFR kinase activity. As shown in Fig. 3C, the p52 Shc isoform was effectively tyrosine-phosphorylated in response to calcium restoration of EGTA-treated HaCat cells, as judged by probing anti-Shc immunoprecipitates with specific anti-phosphotyrosine antibodies. Of note, the profile of Shc phosphorylation mirrored that of EGFR kinase activity, thus suggesting that Shc phosphorylation in response to cell adhesion was functionally related to the state of activation of EGFR kinase.

E-cadherin and EGFR Form a Higher Order Complex upon Assembly of Adherens-type Junction—In light of the involvement of EGFR in E-cadherin-mediated activation of MAPK, we decided to investigate the molecular bases of their interaction. As an approach, we examined whether E-cadherin and EGFR can associate physically and whether dynamic changes of this interaction can occur in relation to the stepwise organization of adherens junctions when performing calcium switch experiments. As shown in Fig. 4A, upper panel, EGFR was effectively co-immunoprecipitated with E-cadherins, thus indicating that they form higher order protein complexes in HaCat cells. The amount of EGFR co-immunoprecipitating with E-cadherins increased as a function of time upon calcium restoration, and reprobing the same blots with antibody directed against E-cadherins confirmed that the differences in EGFR association were not due to differences in immunoprecipitated E-cadherins. The interaction of EGFR and E-cadherins was specific, as immunodepletion of EGFR from cell extracts resulted in the disappearance of the corresponding band (Fig. 4, bottom panel), and no co-immunoprecipitated EGFR was detected when using an antibody against HLA-1 as a control. Of note, by comparing the amount of EGFR associated with E-cadherin immunoprecipitates with that detected in the corresponding total cell...
lysates, it appears that the fraction of EGFR associated with E-cadherin represents a small subset of the total cellular EGFR molecules.

These findings suggested a physical interaction between E-cadherin and EGFR and prompted us to investigate further their functional relationship by immunofluorescence experiments. As illustrated in Fig. 4B, even in serum-starved cells (untreated), it was possible to detect a basal level of activated EGFR associated with sites of cell-cell contacts, as suggested by the overlapping distribution of tyrosine-phosphorylated EGFR with that of E-cadherins. Following chelation of extracellular calcium (EGTA), cell-cell contacts were disrupted, and E-cadherins were hardly detectable at the level of the cell surface and appeared to be diffusely distributed, with the concomitant disappearance of phosphorylated EGFR from cell-cell adhesion sites. After calcium restoration, the dissociated cells gradually formed cell-cell adhesions over time, starting as early as 5 min, a process that was accompanied by the accumulation of both E-cadherin and EGFR, in its active form, at cell-cell adhesion sites (Fig. 4C). Interestingly, upon stimulation with a saturating dose of EGF (100 ng/ml), along with a diffuse pattern of staining for active EGFR throughout the cells, a scattered

FIG. 2. E-cadherin-mediated adhesion is necessary and sufficient for enhanced MAPK activity. A, HaCat cell monolayers were left untreated or treated with 4 mM EGTA and function-perturbing anti-E-cadherin antibodies (DECMA-1 clone, 50 μg/ml) prior to calcium restoration, as indicated. As a control, serum-starved cells were treated with 100 nM EGF for 5 min. Cellular lysates were processed for immunocomplex MAPK assays. B, HaCat cells, grown at confluence and serum-starved overnight, were detached with 5 mM EDTA and then plated for the indicated time onto anti-E-cadherin DECMA antibodies (5 μg/ml) previously immobilized in tissue culture plates, as described under “Experimental Procedures.” Cells plated for 30 min on BSA alone or BSA plus anti-Rat IgG-coated dishes were used as controls. Attached and suspended cells were collected, and cellular lysates were processed for MAPK assays. C, HaCat cells at confluence were detached as above and kept in suspension in cell culture dishes precoated with poly-HEMA (0.4%). After 1 h at 4 °C in the presence of a saturating concentration anti-E-cadherin DECMA antibodies (50 μg/ml), surface clustering of E-cadherin receptors was induced by further incubation in the presence of 5 μg/ml goat anti-rat IgG for the indicated time at 37 °C. In all cases, MAPK activity was assessed by in vitro kinase assays using MBP as an exogenous substrate. 32P-Labeled substrate (MBP) is indicated. Total MAPK in immunoprecipitates was assessed by Western blotting (WB). Values shown are the average ± S.D. from three to five independent experiments and are expressed as fold induction relative to the corresponding controls.
E-cadherins Activate MAPK through EGFR

**DISCUSSION**

Cadherins constitute a family of key morphoregulatory molecules whose role in cell adhesion is crucial for the regulation of cell fate and the maintenance of tissue integrity (1). The ability of cadherins to act also as signal transducing molecules has just begun to be recognized. For example, recent work has indicated that the assembly of E-cadherin-mediated adherens junctions is sufficient to trigger the activation of the phosphatidylinositol-3′-OH kinase/Akt cascade in epithelial cells (8) and that signals initiated by VE-cadherin play a pivotal role for endothelial cell survival and vascular morphogenesis in vivo (23). In this study, we provide evidence that one of the earliest molecular events resulting from the engagement of E-cadherins in areas of cell-cell contacts in epithelial cells is the rapid and sustained activation of the MAPK cascade, a key signaling pathway involved in both cell proliferation and differentiation (9).

Initially, we took advantage of a simple and powerful experimental approach for investigating E-cadherin function, which involves the use of calcium-chelating agents to disrupt cell-cell adherens junctions in epithelial cells, followed by the formation of new cell-cell contacts upon restoration of normal calcium concentration. By using this “calcium-switch” approach, we observed the rapid activation of the MAPK pathway upon reformation of cell adhesion complexes. A key role for E-cadherins in MAPK stimulation was supported by several lines of evidence as follows. 1) Preventing the formation of E-cadherin-mediated adhesion by the use of function-perturbing antibodies abolished the activation of MAPK in response to calcium restoration. 2) Antibody-induced clustering of E-cadherins by platelets on anti-E-cadherin immobilized antibodies or by the use of dimerizing antibodies in suspended cells provoked the rapid activation of MAPK. These findings indicate that oligomerization of E-cadherins molecules is necessary and sufficient to signal to the MAPK pathway upon cell contact formation and strongly support the concept that formation of E-cadherin dimers is a prerequisite for their functional activation (24). Interestingly, disruption of E-cadherin-dependent intercellular contacts also diminished the activity of MAPK below control levels, thus suggesting that the persistent occupancy of E-cadherins by their recruitment to areas of cell-cell contact may contribute to the basal activity of MAPK in epithelial cells.

Of note, as E-cadherins do not exhibit any enzymatic activity in their short intracytoplasmic tail, it is conceivable that their ability to function as signal transducing receptors may depend on their short intracytoplasmic tail. It is conceivable that their ability to function as signal transducing receptors may depend on their short intracytoplasmic tail, it is conceivable that their ability to function as signal transducing receptors may depend on their short intracytoplasmic tail.
on their physical interaction with other signal transduction systems. Indeed, we found that during the stepwise formation of adherens junctions after calcium restoration, clustering of E-cadherins is able per se to induce a ligand-independent activation of the EGFR kinase. This process appears to involve the formation of higher order molecular complexes between E-cadherin and EGFRs, which increase over time after calcium restoration. Consistent with this finding, we also provide evidence that EGFR is co-localized to adhesive structures with E-cadherins even at the very early stages of the formation of cell-cell adhesion complexes. Remarkably, both immunoprecipitation and immunofluorescence studies demonstrated that a fraction of these EGFRs detected at sites of cell-cell contacts is present in the phosphorylated, active form, thus strongly supporting the concept that E-cadherins are able to transactivate EGFR by a yet to be identified mechanism, likely as a result of their co-clustering (16). Furthermore, the use of tyrphostin AG 1478, which specifically inhibits EGFR kinase by competing for ATP (17), further confirmed that E-cadherin and EGFR cooperate directly to activate signaling pathways downstream of adherens junctions, thus leading to the phosphorylation of Shc and an increase in the enzymatic activity of MAPK. These findings may have significant implications in our understanding of epithelial cell biology, as the MAPK pathway plays a critical role in many cellular processes, including proliferation, apoptosis, differentiation, and senescence (9). Similarly, EGFRs transduce proliferative signals and also play a key role in cell differentiation by promoting skin maturation and multilayered thickening of normal epidermis (25, 26). In both cases, it is conceivable that the duration and the amplification of this signaling cascades are controlled by the ability of E-cadherins to transactivate EGFR.

on their physical interaction with other signal transduction systems. Indeed, we found that during the stepwise formation of adherens junctions after calcium restoration, clustering of E-cadherins is able per se to induce a ligand-independent activation of the EGFR kinase. This process appears to involve the formation of higher order molecular complexes between E-cadherin and EGFRs, which increase over time after calcium restoration. Consistent with this finding, we also provide evidence that EGFR is co-localized to adhesive structures with E-cadherins even at the very early stages of the formation of cell-cell adhesion complexes. Remarkably, both immunoprecipitation and immunofluorescence studies demonstrated that a fraction of these EGFRs detected at sites of cell-cell contacts is present in the phosphorylated, active form, thus strongly supporting the concept that E-cadherins are able to transactivate EGFR by a yet to be identified mechanism, likely as a result of their co-clustering (16). Furthermore, the use of tyrphostin AG 1478, which specifically inhibits EGFR kinase by competing for ATP (17), further confirmed that E-cadherin and EGFR cooperate directly to activate signaling pathways downstream of adherens junctions, thus leading to the phosphorylation of Shc and an increase in the enzymatic activity of MAPK. These findings may have significant implications in our understanding of epithelial cell biology, as the MAPK pathway plays a critical role in many cellular processes, including proliferation, apoptosis, differentiation, and senescence (9). Similarly, EGFRs transduce proliferative signals and also play a key role in cell differentiation by promoting skin maturation and multilayered thickening of normal epidermis (25, 26). In both cases, it is conceivable that the duration and the amplification of this signaling cascades are controlled by the ability of E-cadherins to transactivate EGFR.

FIG. 4. Adherens junction assembly induces the recruitment of active EGFRs to E-cadherin-containing complexes. A, HaCat cells were serum-starved overnight and treated with 4 mM EGTA for 30–40 min, and then calcium was restored in serum-free medium for the indicated time. E-cadherins were immunoprecipitated from total cell lysates with anti-E-cadherin-specific monoclonal antibodies (DECMA-1). The association of the EGFR receptor in anti-E-cadherin immunoprecipitates was detected with specific anti-EGFR monoclonal antibodies (α-EGFR). As controls, untreated (control) or EGF-treated cells were also included. Membranes were stripped and re-probed with specific monoclonal antibodies against the active, phosphorylated form of EGFR receptor. Bands corresponding to EGFR and phospho-EGFR are shown. The amount of E-cadherin and EGFR, respectively, in total cell lysates is also indicated. B, cell extracts from untreated or EGF-treated HaCat cells were immunoprecipitated with anti-E-cadherin antibodies or anti-HLA-1 antibodies as a control (lane 4), and immunoblotted with anti-EGFR antibodies. One-mg aliquot of HaCat extracts was subjected to immunoprecipitation (IP) with anti-EGFR antibodies prior to immunoprecipitation with anti-E-cadherin monoclonal antibodies and then immunoblotted against EGFR, leading to immunodepletion of EGFR from cell extracts and consequent disappearance of the 170-kDa EGFR protein (lane 3). C, HaCat cells were grown on a glass coverslip and treated with EGTA, and calcium was restored for the indicated time in serum-free or EGF-containing medium, as a control. After fixation and permeabilization, E-cadherin and EGFR were immunostained with monoclonal rat anti-E-cadherin and mouse monoclonal antibodies anti-activated, phosphorylated form of EGFR, respectively. As secondary antibodies, fluorescein isothiocyanate anti-rat and Cy3 anti-mouse antibodies were used. Microscope analysis was performed at ×63 magnification.
tude of signals determine the nature of the final biological responses. Thus, we can speculate that upon adherens junction formation, signals emanating at the level of cell-cell contacts as a result of the E-cadherin-EGFR interaction, may be involved in maintaining the functional and structural integrity of quiescent epithelia and, as a function of the adhesion status of the cells, possibly in promoting epithelial cell differentiation rather than proliferation.

In conclusion, E-cadherins appear now to play multiple functional roles in addition to their contribution to cell adhesion. Many of these functions likely involve the well known interactions of E-cadherins with cytoskeletal proteins, as well as the activation of biochemical routes involved in intracellular signaling pathways. In this case, the co-clustering of E-cadherins with EGFRs may provide an attractive mechanism whereby cell-cell adhesion can initiate the activity of tyrosine kinase growth factor receptors. Further studies will be necessary to elucidate fully how engagement of E-cadherins results in the transactivation of EGFRs, and how signaling pathways downstream of these cell adhesion-growth factor receptor complexes participate in normal epithelial cell proliferation and differentiation, as well as in certain pathophysiological conditions, including cancer.

REFERENCES
1. Takeichi, M. (1991) Science 251, 1451–1455
2. Ozawa, M., and Kemler, R. (1992) J. Cell Biol. 116, 989–996
3. Aberle, H., Schwartz, H., and Kemler, R. (1996) J. Cell. Biochem. 61, 514–523
4. Gumbiner, B. M. (1996) Cell 84, 345–357
5. Takeichi, M. (1993) Curr. Opin. Cell Biol. 5, 806–811
6. Braga, V. M. (1999) Mol. Pathol. 52, 197–202
7. Kinch, M. S., Petch, L., Zhong, C., and Burridge, K. (1997) Cell Adhes Commun. 4, 425–437
8. Pece, S., Chiarie, M., Murga, C., and Gutkind, J. S. (1999) J. Biol. Chem. 274, 19347–19351
9. Seger, R., and Krebs, E. G. (1995) FASEB J. 9, 726–735
10. Howe, A., Aplin, A. E., Alsabri, S. K., and Juliano, R. L. (1998) Curr. Opin. Cell Biol. 10, 220–231
11. Zhu, A. J., Haase, I., and Watt, F. M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 6728–6733
12. Boukamp, P., Petrussevska, R. T., Breitkreutz, D., Hornung, J., Markham, A., and Fusenig, N. E. (1998) J. Cell Biol. 106, 761–771
13. Breitkreutz, D., Schoop, V. M., Mirenska, N., Baur, M., Stark, H. J., and Fusenig, N. E. (1996) Eur. J. Cell Biol. 70, 273–286
14. Völlberg, T., Geiger, B., Kartenbeck, J., and Franke, W. W. (1986) J. Cell Biol. 102, 1832–1842
15. Kartenbeck, J., Schmelz, M., Franke, W. W., and Geiger, B. (1991) J. Cell Biol. 113, 881–892
16. Hoschuetzky, H., Aberle, H., and Kemler, R. (1991) J. Cell Biol. 127, 1375–1380
17. Levička, A., and Gazit, A. (1993) Science 267, 1782–1788
18. Schlessinger, J. (1994) Curr. Opin. Genet. & Dev. 4, 25–30
19. Moro, L., Venturino, M., Bozzo, C., Silengo, L., Altruda, F., Beguinot, L., Tarone, G., and Delilippi, P. (1996) EMBO J. 17, 6622–6632
20. Pelici, G., Lanfrancone, L., Crignani, F., McGlade, J., Cavollo, F., Forini, G., Nicoletti, I., Pawson, T., and Pelicci, P. G. (1992) Cell 70, 93–104
21. Shinohzaki, H., Kadowaki, T., Doki, Y., Inoue, M., Tamura, S., Oka, H., Iwashawa, T., Matsui, S., Shimoya, K., Takeichi, M., and Mori, T. (1995) Br. J. Cancer 71, 250–258
22. Kemler, R. (1993) Trends Genet. 9, 317–321
23. Carmeliet, P., Lampugnani, M. G., Moons, L., Breviario, F., Compagnone, V., Bon, F., Balconi, G., Spagnuolo, R., Oostwyk, B., Dewerchin, M., Zani, A., Angellino, A., Mattot, V., Nuyens, D., Lutgens, E., Clotman, F., de Ruiter, M. C., Gittenberger-de Groot, A., Poelmans, R., Lupu, F., Herbert, J. M., Collen, D., and Dejana, E. (1999) Cell 98, 147–157
24. Steinberg, M. S., and McNutt, P. M. (1999) Curr. Opin. Cell Biol. 11, 554–560
25. Carpenter, G., and Cohen, S. (1979) Annu. Rev. Biochem. 48, 193–216
26. Miettinen, P. J., Berger, J. E., Menneses, J., Phung, Y., Pedersen, R. A., Werb, Z., and Derynck, R. (1995) Nature 376, 337–341