β-Catenin Mediates the Interaction of the Cadherin–Catenin Complex with Epidermal Growth Factor Receptor

Heinz Hoschuetzky, Hermann Aberle, and Rolf Kemler
Max-Planck-Institut für Immunobiologie, D-79011 Freiburg, Germany

Abstract. Catenins mediate the linkage of classical cadherins with actin microfilaments and are part of a higher order protein structure by which cadherins are connected to other cytoplasmic and transmembrane proteins. The ratio of actin-bound to free cadherin–catenin complex, which varies depending on the type and growth rate of cells, is thought to be altered by cellular signals, such as those associated with mitosis, polarization of cells and growth factors during development. EGF induces an immediate tyrosine phosphorylation of β-catenin and γ-catenin (plakoglobin). We show here an association of the EGF-receptor with the cadherin–catenin complex. Using recombinant proteins we demonstrate the interaction of EGF-receptor and β-catenin in vitro kinase assays. This interaction is mediated by the evolutionarily conserved central "core" region of β-catenin. These results suggest that catenins represent an important link between EGF-induced signal transduction and cadherin function.
during changes of the developmental state of cells (McNeill et al., 1990; Năthke et al., 1994). It has been reported that α- and β-catenin become phosphorylated at tyrosine residues in cells expressing v-src and that this posttranslational modification correlates with changes of the epithelial phenotype (Matsuyoshi et al., 1991; Behrens et al., 1993; Kamaguchi et al., 1993). In addition, tyrosine phosphorylation of catenins was also observed in cells treated with hepatocyte growth factor and EGF (Shibamoto et al., 1994). This opened up the possibility that tyrosine phosphorylation of catenins might represent one mechanism which modulates the function of catenins. We have been particularly interested in the action of EGF on the cadherin–catenin complex. It is well established that EGF induces cell rounding and membrane ruffling and that the EGF-receptor (EGF-R) molecules are co-localized with catenins on the basolateral membrane of epithelial cells (Fukuyama and Shimiza, 1991).

We show here an association of the EGF-R with the cadherin–catenin complex and an EGF-induced tyrosine phosphorylation of β-catenin and plakoglobin. We provide evidence that β-catenin binds to the EGF-R and that this interaction is mediated by the conserved central "core" region of β-catenin.

Materials and Methods

Cell Lines

Human epidermoid carcinoma cells A431 (ATCC, CRL1555; American Type Culture Collection, Rockville, MD) and TRI46 (Kupniak et al., 1985), and porcine kidney epithelial cell line LLC-PK1 (ATCC, CCL10) were grown in DMEM supplemented with 10% (vol/vol) heat-inactivated FCS, at 37°C in a 10% CO2 atmosphere.

Antibodies

Affinity-purified antibodies against E-cadherin, α- and β-catenin, and plakoglobin have been described previously (Ozawa et al., 1989; Herrenknecht et al., 1991; Butz et al., 1992). The phosphotyrosine (p-tyr) specific monoclonal antibody 4G10 and the polyclonal anti-EGF-receptor (EGF-R) antibodies were obtained from Upstate Biotechnology Inc. (via Biomol, Hamburg, Germany). Peroxidase-labeled secondary antibodies were from Dianova GmbH (Hamburg, Germany).

Cell Extract Preparation

Cells were seeded at a density of 10⁶ cells per 90 mm dish, grown 24 h in DMEM, 10% FCS, and stimulated with 50 ng/ml EGF (human recombinant) (Sigma, Munich, Germany) for the indicated times. For metabolic labeling, cells were grown 4 h in methionine-free DME/FCS and then grown overnight in the presence of 50 µCi [³⁵S]methionine (3,000 Ci/mmol). Cells were washed twice with PBS and lysed at a cell density of 10⁷ cells/ml in 20 mM imidazole-HCl, pH 6.8, 100 mM KCl, 2 mM MgCl₂, 20 mM EGTA, 300 mM sucrose, 1 mM NaF, 1 mM Na-vanadate, 1 mM Na-molybdate, 0.2% (vol/vol) Triton X-100, 10 µg/ml leupeptin, 10 µg/ml soybean trypsin inhibitor, 10 µg/ml PMSF, and 0.1 U/ml a₂-macroglobulin. Crude cell extracts were clarified by centrifugation (14,000 g, 10 min), and the supernatant (insoluble fraction) and pellet (insoluble fraction) were separated. The insoluble, cytoskeletal fraction was washed with lysis buffer without Triton X-100 and solubilized in 0.1% SDS. After dilution to 0.02% SDS, immunoprecipitates were collected with anti-fl-catenin antibodies.

Immunoprecipitation

In Vitro Protein Kinase Assays

For in vitro kinase assays 20 µg total protein from insect cells lysates were incubated with 20 µg fusion proteins for 30 min at 37°C in 500 µl kinase buffer (20 mM Hepes-NaOH, pH 7.4, 100 mM KCl, 2 mM MgCl₂, 10 mM MnCl₂, 0.1% (vol/vol) Triton X-100, 0.1% Na-vanadate, 0.1 mM ATP). The GST-fusion proteins were isolated with glutathione-agarose, specifically bound antibodies were eluted with 2% SDS and separated by SDS-PAGE, and immunoblots were developed with p-tyr and EGF-R-specific antibodies.

Abbreviations used in this paper: aa, amino acids; EGF- and PDGF-R, EGF- and PDGF-receptor; p-tyr, phosphoryrosine.
Other Methods
Fluorography, SDS-PAGE and immunoblotting were done as described (Ozawa et al., 1989). SDS-PAGE and blots were standardized with pre-stained molecular weight markers (Sigma). Immunoblots were developed by peroxidase-labeled secondary antibodies followed by enhanced chemiluminescence and exposed to enhanced chemiluminescence–hyperfilm (Amersham, Braunschweig, Germany).

Results
In an initial series of experiments the effect of EGF on the cadherin–catenin complex in human epidermoid carcinoma cells A431 was analyzed. A431 cells have been studied extensively as a model system for the EGF/EGF-R induced signal transduction, and an association of the EGF-R with actin filaments has been reported in these cells (den Hartigh et al., 1992).

Immunoprecipitations from whole cell lysates of [35S]methionine labeled A431 cells with anti-E-cadherin antibodies detected a comparable relative ratio of the cadherin–catenin complex with or without EGF treatment (Fig. 1, lanes 1 and 2). Semi-confluent A431 cells were incubated with 50 ng/ml EGF, and at different times cadherin–catenin complexes were collected with antibodies against anti E-cadherin and probed with anti-p-tyr antibodies in immunoblots. As can be seen in Fig. 1 (lane 3) already at time 0 a trace amount of γ-catenin (plakoglobin) was tyrosine phosphorylated. By 3–5 min after EGF treatment, an increasing amount of tyrosine phosphorylation was already detected for both β- and γ-catenin (Fig. 1, lanes 4–6). These results clearly demonstrate that EGF treatment results in tyrosine phosphorylation of β- and γ-catenin. They differ from those obtained with v-src transformed cells where α- and β-catenin were the primary targets for tyrosine phosphorylation (Hamaguchi et al., 1993). This suggests that the EGF-induced and v-src-mediated phosphorylation are each specific for different catenins. Tyrosine phosphorylation of β- and γ-catenin seems to be specific for the EGF signal transduction pathway. In transfected Ltk− cells expressing both the platelet-derived growth factor receptor (PDGF-R) and the E-cadherin–catenin complex, PDGF had no detectable effect on the phosphorylation of catenins (not shown).

Cadherin–catenin complexes partition into both the non-ionic detergent-soluble and -insoluble cytoskeletal fractions (Ozawa et al., 1989). It was therefore of interest to examine whether tyrosine-phosphorylated catenins separate equally in both fractions. EGF-treated A431 cells (50 ng/ml EGF, 45 min) were solubilized and both the Triton X-100–soluble and -insoluble fractions (supernatant and pellet from 14,000 g centrifugation) were immunoprecipitated with anti-β-catenin antibodies. As can be seen in Fig. 2 (lanes 1 and 2) β-catenin was immunoprecipitated from the detergent-soluble (S) and -insoluble (P) fraction (to obtain comparable amounts of β-catenin 5–10 times more of the immunoprecipitate of the insoluble fraction was loaded on the gel). When these blots were stained with anti-p-tyr antibodies, tyrosine-phosphorylated β-catenin was exclusively found in the detergent-soluble fraction (Fig. 2, lanes 3 and 4). Anti-p-tyr antibodies also detected a phosphorylated protein of 180 kD in the cadherin–catenin complex immunoprecipitates (Fig. 1, lanes 3–6). As subsequent immunoblots with anti EGF-R antibodies revealed, this represents EGF-R (Fig. 1, lane 10). An association of EGF-R with the cadherin–catenin complex could also be demonstrated in A431 cells before treatment with EGF (Fig. 1, lane 8) and the receptor is weakly tyrosine-phosphorylated (Fig. 1, lane 3). Since the EGF-stimulation experiments were carried out on semi-confluent cell layers 24 h after plating, it is likely that cells received mitotic stimuli due to fetal calf serum components, which led to some activation of EGF-R. This would explain the weak tyrosine phosphorylation of plakoglobin and EGF-R already at time 0 of EGF stimulation and an association of EGF-R with the complex (Fig. 1, lane 3). However, an association of non-activated EGF-R with the cadherin–catenin complex can not be excluded from these experiments. Comparison of the entire EGF-R pool to EGF-R associated with the cadherin–catenin complex indicated that less than 10% of total EGF-R is associated with the complex (not shown). Since A431 cells express a high amount of EGF-R, it was of interest to see if tyrosine phosphorylation of catenins and an

were developed with a phosphotyrosine-specific (anti-p-tyr) antibody (lanes 3 to 6). A431 cells without (lanes 7 and 8) and with EGF stimulation (50 ng/ml, lanes 9 and 10) were immunoprecipitated with anti E-cadherin antibodies (lanes 7 and 9 are controls). Immunoprecipitates were blotted with anti-EGF-R antibodies, revealing the presence of EGF-R in the E-cadherin–catenin complex independent of EGF-treatment. Positions of catenin and catenins are indicated on the left; positions of prestained molecular weight markers are indicated on the right. The low molecular weight proteins in lanes 8 and 10 correspond to heavy chains of the precipitating antibody.

Figure 1. EGF-dependent phosphorylation of β- and γ-catenin in A431 cells and association of the EGF-R with the cadherin–catenin complex. Proteins were immunoprecipitated with an E-cadherin–specific antibody from lysates of metabolically labeled A431 cells treated with 50 ng/ml EGF (lane 2) or untreated (lane 1). The cadherin–catenin complex was collected from A431 cells treated with EGF for the indicated times, proteins were separated by SDS-PAGE and immunoblots were developed with a phosphotyrosine-specific (anti-p-tyr) antibody (lanes 3 to 6). A431 cells without (lanes 7 and 8) and with EGF stimulation (50 ng/ml, lanes 9 and 10) were immunoprecipitated with anti E-cadherin antibodies (lanes 7 and 9 are controls). Immunoprecipitates were blotted with anti-EGF-R antibodies, revealing the presence of EGF-R in the E-cadherin–catenin complex independent of EGF-treatment. Positions of catenin and catenins are indicated on the left; positions of prestained molecular weight markers are indicated on the right. The low molecular weight proteins in lanes 8 and 10 correspond to heavy chains of the precipitating antibody.
association of the EGF-R with the cadherin–catenin complex could also be observed in cells which express a more physiological amount of EGF-R. For this purpose we examined polarized proximal kidney epithelial cells, LLC-PK₁, grown as transfilter cultures (Costar Corp., Cambridge, MA), and the human squamous epithelial carcinoma cell line TR146. The cadherin–catenin complex was immunoprecipitated with anti-β-catenin antibodies from cell lysates of metabolically labeled LLC-PK₁ and TR146 cells (Fig. 3, lanes 1 and 3). Under these experimental conditions γ-catenin (plakoglobin) appears to be under-represented in the E-cadherin–catenin complex (Näthke et al., 1994). EGF, exposed basolaterally (50 ng/ml), also induces in both these cell types tyrosine phosphorylation of β-catenin and, interestingly, only in TR146 cells in addition tyrosine phosphorylation of γ-catenin (plakoglobin). These results suggest that β-catenin and plakoglobin are taken differently as substrates in LLC-PK₁ and TR146 cells after EGF-stimulation. Immunoblots with anti-β-tyr antibodies revealed a tyrosine phosphorylated high molecular weight protein (Fig. 3, lanes 2 and 4), as for A431 cells. Subsequent immunoblots of immunoprecipitates from TR146 cells with anti-EGF-R antibodies revealed, as for A431 cells, the presence of EGF-R in the complex (Fig. 3, lane 5). A possible association of the EGF-R with the cadherin–catenin complex could not be demonstrated for LLC-PK₁ cells since anti-EGF-R antibodies did not cross-react with the porcine EGF-R. Taken together, all these results demonstrate in three different epithelial cell types an EGF-induced tyrosine phosphorylation of β-catenin and plakoglobin and an association of the EGF-R with the cadherin–catenin complex.

To further elucidate the interaction of catenins with the EGF-R, β-catenin, and a conserved central β-catenin region (amino acid position 120-683, designated β-catenin core region) were expressed as glutathione-S-transferase fusion proteins in Escherichia coli. The core region of β-catenin is largely composed of hydrophobic repeats and exhibits the highest degree of homology to plakoglobin and the armadillo protein (Peifer et al., 1992; Rosenthal, 1993). Fusion proteins were purified by glutathione–agarose affinity chromatography (Fig. 4, lanes 1 and 2) and reacted with anti peptide antibodies in immunoblots (not shown). β-catenin fusion proteins and recombinant EGF-R, expressed in the baculovirus system, were subjected to in vitro kinase assays. Cell lysates from insect cells expressing wild-type baculovirus were included as controls. Glutathione–agarose purified proteins were subjected to immunoblot analysis with anti-β-tyr antibodies (Fig. 4). β-catenin, but not the β-catenin core region or the GST fusion partner, was phosphorylated in these assays (Fig. 4, lanes 4 and 5), while no tyrosine phosphorylated protein was detected with control cell lysates expressing wild-type baculovirus (not shown). These results indicate that the phosphorylated tyrosine residues are located either in the NH₂- or in the COOH-terminal domain of β-catenin. More importantly, β-catenin and the β-catenin core region were associated with a tyrosine phosphorylated protein which reacted with anti-EGF-R antibodies (Fig. 4, lane 6). Since no other tyrosine phosphorylated proteins were detected in these assays, these results suggest that β-catenin binds directly to EGF-R and that this interaction is mediated by the β-catenin core region.

**Discussion**

EGF acts on cells by binding to its receptor (EGF-R), thereby initiating a variety of cellular changes including rapid alteration in cell surface morphology, cytoskeletal reorganization and redistribution of the EGF-R (den Hartigh et al., 1992). On epithelial cells EGF counteracts the adhesive function of E-cadherin. While E-cadherin is of crucial importance for the biogenesis of an epithelium and mediates a tight association of these cells (Larue et al., 1994), EGF...
initiates cell rounding and membrane ruffling. Furthermore, EGF-R can be found co-localized with the cadherin–catenin complex at the lateral membrane of epithelial cells, and an association of EGF-R with the cytoskeleton has been reported (den Hartigh et al., 1992; van Bergen en Henegouwen et al., 1992). All this stimulated us to investigate a possible molecular interaction between EGF/EGF-R and the cadherin–catenin complex. We show here that EGF induces an immediate tyrosine phosphorylation of β- and γ-catenin cadherin-catenin complex. We demonstrated this association in different cell lines after EGF treatment, but these experiments did not allow us to distinguish whether the association was direct or indirect. To further clarify this point we then performed in vitro phosphorylation assays with recombinant proteins. Recombinant β-catenin has several structural features in common with native β-catenin, e.g., it is recognized by epitope-specific antibodies and associates with native α-catenin (Aberle et al., 1994). In in vitro kinase assays with recombinant β-catenin and EGF-R, tyrosine phosphorylation of β-catenin, and an association between β-catenin and EGF-R were demonstrated. It is generally accepted that proteins which interact with tyrosine kinase receptors become phosphorylated (Mustelin and Burn, 1993). If so, our results would indicate a direct interaction between EGF-R and β-catenin which appears to be mediated by the β-catenin core region. The core region exhibits, with about 80% of amino acid identity, the highest degree of homology to plakoglobin (γ-catenin) and the armadillo protein, which suggests that these proteins might also have EGF-R-binding properties. Our results indicate that tyrosine phosphorylation occurs at the amino- and/or carboxy-terminal regions of β-catenin. The in vitro kinase assays should allow us to determine more precisely which tyrosine residues are substrates for EGF-R or other kinases. Finally, β-catenin exhibits no obvious homology to SH2 domains, which are known to bind directly to tyrosine kinase receptors. It will be extremely important to identify the amino acid sequences on both EGF-R and β-catenin which mediate this interaction. β-catenin is also complexed to other members of the cadherin protein family and might also link these cell adhesion molecules to other members of the tyrosine kinase receptor family. If so, β-catenin may prove to be an important regulatory protein between receptor-mediated signaling and cadherin function.

We gratefully acknowledge Dr. M. Waterfield, Ludwig Institute für Cancer Research (London), for providing us with EGF-R expressed in the baculovirus expression system. We thank Andreas Rolke and Johann Wirsching for

Hoschuetzky et al. β-Catenin Interacts with EGF-Receptor

Figure 4. In vitro tyrosine phosphorylation and association of β-catenin with the EGF-R. Glutathione-S-transferase (GST) fusion proteins of β-catenin (lane 1) or the β-catenin core region (amino acids 120-683) were separated on SDS-polyacrylamide gels and stained with Coomassie brilliant blue (CBB, lanes 1 and 2). GST (lane 3), GST-β (lane 4) and GST-β-core (lanes 5 and 6) were phosphorylated in vitro with recombinant EGF-R. Fusion proteins were affinity purified and analyzed by SDS-PAGE. Subsequent immunoblots were developed with phosphotyrosine-specific (lanes 3 to 5) or EGF-R-specific antibody (lane 6). The low molecular weight bands in lane 1 represent degradation products of β-catenin as monitored by anti-β-catenin antibodies (not shown).
technical assistance, Lore Lay for preparing the photographs, and Rosemary Schneider for typing the manuscript. We are grateful to Dr. Randy Cassidy for critically reading the manuscript.

Received for publication 11 July 1994 and in revised form 1 August 1994.

References


