Regulation of E-cadherin/Catenin Association by Tyrosine Phosphorylation

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Alteration of cadherin-mediated cell-cell adhesion is frequently associated to tyrosine phosphorylation of p120- and β-catenins. We have examined the role of this modification in these proteins in the control of β-catenin/E-cadherin binding using in vitro assays with recombinant proteins. Recombinant pp60src efficiently phosphorylated both catenins in vitro, with stoichiometries of 1.5 and 2.0 mol of phosphate/mol of protein for β-catenin and p120-catenin, respectively. pp60src phosphorylation had opposing effects on the affinities of β-catenin and p120 for the cytosolic domain of E-cadherin; it decreased (in the case of β-catenin) or increased (for p120) catenin/E-cadherin binding. However, a role for p120-catenin in the modulation of β-catenin/E-cadherin binding was not observed, since addition of phosphorylated p120-catenin did not modify the affinity of phosphorylated (or unphosphorylated) β-catenin for E-cadherin. The phosphorylated Tyr residues were identified as Tyr-86 and Tyr-654. Experiments using point mutants in these two residues indicated that, although Tyr-86 was a better substrate for pp60src, only modification of Tyr-654 was relevant for the interaction with E-cadherin. Transient transfections of different mutants demonstrated that Tyr-654 is phosphorylated in conditions in which adherens junctions are disrupted and evidenced that binding of β-catenin to E-cadherin in vivo is controlled by phosphorylation of β-catenin Tyr-654.

E-cadherin is the predominant cadherin in epithelial tissue and, as a member of this family, is responsible for the correct establishment and maintenance of adherens junctions, through Ca2+-dependent homophilic interactions with adjacent cells (1). The adhesive function of E-cadherin requires its attachment to the actin cytoskeleton (2–5). In adherens junctions, or perhaps p120, to the cadherin/catenin complexes that implicate the dissociation of their components (14). Since a direct relationship of β-catenin phosphorylation to E-cadherin loss of function has not been established yet, it is also possible that, as proposed by some authors, E-cadherin dissociation from the cytoskeleton after cell transformation is not directly related to tyrosine phosphorylation of catenins.

Several hypotheses have been proposed to explain the adhesive changes of cadherin complexes in response to catenin tyrosine phosphorylation. These hypotheses include 1) alterations in the affinity of p120- and β-catenin for the cytoplasmic region of E-cadherin; 2) conformational changes in these components that result in the disruption of linkage within cadherin and the cytoskeleton; or 3) recruitment of unknown proteins, or perhaps p120, to the cadherin/catenin complexes that implicate the dissociation of their components (14).

We were particularly interested to evaluate the different possibilities that led to the disassembly of adherens junctions upon tyrosine phosphorylation of β-catenin. We have studied the ability of β-catenin and p120-catenin to bind E-cadherin in vitro, and the modulation of this binding by tyrosine phosphorylation.

EXPERIMENTAL PROCEDURES

Expression of Recombinant p120- and β-Catenins and Cytosolic E-cadherin (cytoEcad)—Full-length murine β-catenin (provided by Dr. R. Kemler, University of Freiburg, Freiburg, Germany) was inserted in the BamHI restriction site of pGEX-6P3 plasmid (Amersham Pharmacia Biotech, Uppsala, Sweden) and expressed in Echerichia coli as a glutathione S-transferase (GST) fusion protein. After purification by

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affinity chromatography on glutathione-Sepharose columns, the fusion protein was cleaved with PreScission protease (Amersham Pharmacia Biotech). A DNA fragment corresponding to amino acids 196 to 268 of murine p120-catenin was obtained from pDNA3-Cas1 plasmid (provided by Dr. Albert Reynolds, Vanderbilt University, Nashville, TN) by digestion with BglII and EcoRI at their 5'-ends, respectively. The 0.5-kilobase amplification fragment was digested with BglII and EcoRI and cloned in the same sites of pGEX-6P3 plasmid. The absence of mutations in this fragment was verified by sequencing. GST-ctoEcad fusion proteins were prepared and isolated as above. Purified proteins were aliquoted and stored at 50% (v/v) glycerol at −40 °C until use.

**Generation of β-Catenin Mutants—** β-Catenin mutants 1–106 and 1–575 were generated cutting pGEX-6P3-β-catenin with restriction enzymes SphI and EcoI-CRI, respectively, filling in and ligation. An entire C-terminal mutant (575–783) was obtained cloning the EcoI-CRI-BamHI fragment into the SmaI site of pGEX-6P3; 575–696 mutant was generated by cutting pGEX-6P3 (575–783) with BglII and NotI, filling in, and ligation. 696–783 mutant was constructed cutting pGEX-6P3-β-catenin SmaI, filling in, and ligating the entire NotI fragment in SmaI site of pGEX-6P2. Pink mutant Tyr-86 → Glu, Tyr-86 → Phe, Tyr-654 → Glu, and Tyr-654 → Phe were obtained using the QuickChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA). A polymerase chain reaction was performed with Pfu polymerase, pGEX-6P3-β-catenin as template, and oligonucleotide primers containing each mutation. Sense primers used for generation of Tyr-86 → Glu and Tyr-86 → Phe mutants, respectively, 5'-GCTGATATTGGCGGCACGGCAGGAAAGCAATGACTAGG-3' and 5'-GCTGATATTGGCGGCACGGCAGGAAAGCAATGACTAGG-3', and for generation of Tyr-654 → Glu and Tyr-654 → Phe, 5'-GGCGTGACAAAAAGCAACGCGTCTGCTGCTGCC-3' and 5'-GGCGTGACAAAAAGCAACGCGTCTGCTGCTGCC-3', respectively. Changes are indicated in bold. After amplification, the product was treated with DpnI, which digests the parental construct. Finally, the nicked plasmid was transformed and sequenced. A double mutant Tyr-86 → Phe/Tyr-654 → Phe was also obtained using this procedure with Tyr-86 → Phe-β-catenin. All β-catenin mutants were expressed as GST fusion proteins, purified, and cleaved as indicated above.

**In Vitro Phosphorylation Assay—** In vitro assays were performed in a final volume of 30 μl in the following conditions: 25 mM Tris-HCl, pH 6.25, 25 mM MgCl2, 0.5 mM CaCl2, 0.5 mM dithiothreitol, 1 mM [γ-32P]ATP (1000 cpm/pmol), 1 μM of either β-catenin or p120-catenin, and 0.5 units of recombinant pp60 © src protein kinase (from Upstate Biotechnology, Inc.). Reactions were performed at 22 °C for 0.5–4 h. Samples were analyzed by SDS-polycrylamide gel electrophoresis in standard 10% polyacrylamide gels. After electrophoresis, gels were fixed in 25% methanol, 10% acetic acid, dried, and exposed to x-ray film at −80 °C for 12 h. Quantitation of phosphorylated bands was done using a phosphorimager with 10 μg/ml protein, 20 μg/ml leupeptin, 1 μM PMSF, and 0.5 μM Na3VaO4. Lysates were centrifuged at 13,000 rpm in a microcentrifuge for 5 min at 4 °C. 250 μg of extract were incubated in a final volume of 0.2 ml with 15 μl of a 50% (w/v) suspension of nickel-NTA-agarose (Qiagen, Hilden, Germany), for 30 min at 4 °C. Beads were washed with RIPA buffer, and proteins were eluted with electrophoresis sample buffer. Samples were analyzed by SDS-PAGE and Western blot using antibodies against β-catenin (from Transduction Laboratories). In order to reprobe the membranes, blots were stripped as described (15) and re-analyzed with mAbs against Tyr(P)-specific inhibitor (16). The absence of signal after stripping was always checked incubating with the correspondent secondary antibody and ECL reagent.

**RESULTS**

Full-length recombinant β-catenin or a fragment of p120-catenin comprising all the arm repeats were produced as GST fusion proteins and purified as indicated under “Experimental Procedures.” The purity of these preparations is shown in Fig. 1A. Both catenins were phosphorylated in vitro by recombinant pp60 © src (Fig. 1B); only Tyr residues were modified (data not shown). β-catenin or p120 phosphorylation (Fig. 1B) was totally blocked by addition of herbimycin, a pp60 © src specific inhibitor (16). Similar results were obtained with a purified preparation of pp60 © src (data not shown).

The stoichiometry of these phosphorylations was investigated. 1 unit of pp60 © src was able to incorporate up to 2.4 nmol of P/1.6 nmol of β-catenin or 2.7 nmol of P/1.3 nmol of p120-catenin, when incubated at 25 °C. At 30 °C, the reaction proceeded faster but performed to a lesser extent, probably because pp60 © src was being inactivated (data not shown). These stoichiometries of phosphorylation, 1.5 and 2 nmol of P/nmol of β- or p120-catenin, respectively, indicated that, in both cases, more than one site was being modified.

To determine the relevance of this modification, the abilities of Tyr-phosphorylated catenins to bind E-cadherin were analyzed and compared with those of the unmodified proteins. In
order to perform this assay, the cytosolic domain of E-cadherin (cytoEcad) was prepared and purified as a GST fusion protein (Fig. 1A). It has been previously described that a limited fragment of this E-cadherin-domain is sufficient to bind β-catenin in vivo as well as in vitro (17). Different amounts of recombinant p120- and β-catenins, from 0.12 to 12 pmol, were incubated separately with 1.2 pmol of GST-cytoEcad. The complexes were bound to glutathione-Sepharose and the presence of the catenins associated to the beads was analyzed by Western blot with specific mAbs (Fig. 2). The results were compared with the signal obtained from known amounts of standards that were included in the Western blot analysis. Scatchard analysis were carried out and the association constant ($K_a$) was determined from the slope of the regression line obtained. $K_a$ for E-cadherin/β-catenin or E-cadherin/p120-catenin were estimated to be 1.0 ± 0.2 × 10^8 and 3.6 ± 0.5 × 10^8 M^-1, respectively (Table I). The value of this constant for β-catenin/E-cadherin binding was similar to that obtained by Obama and Ozawa (18) for the association of this same catenin to α-catenin. p120-catenin presented a lower affinity, as has been suggested previously (14). Phosphorylation of β-catenin by pp60^src significantly decreased the affinity by E-cadherin (Fig. 2A); the association constant was 5-fold lower (Table I). On the other hand, phosphorylation of p120-catenin promoted the opposite effect; association of this catenin to E-cadherin was increased by a factor of 4 (Fig. 2B and Table I).

It has been reported previously that p120-catenin does not bind to the same region of the cytosolic domain of E-cadherin than β-catenin. However, a possibility to be considered consisted in that the association of p120-catenin could hamper β-catenin binding only when both proteins were tyrosine-phosphorylated. Therefore, β-catenin binding assays were performed in the presence of p120-catenin. The addition of increasing amounts of this last protein (in a molar excess up to 4), either phosphorylated or not, did not modify the binding of control or Tyr-phosphorylated β-catenin to cytoEcad (Fig. 2C). The association constants measured in these conditions were not substantially different from those calculated for β-catenin in the absence of p120 (Table I).

Since β-catenin phosphorylation presented a functional relevance for E-cadherin activity, the identification of the modified Tyr residues was pursued. Phosphorylation of several β-catenin deletion mutants demonstrated the existence of Tyr residues capable to be phosphorylated in two different parts of the molecule (Fig. 3). A short N-terminal mutant, comprising amino acids 1–106, was efficiently phosphorylated, incorporating approximately 1 pmol of phosphate pmol of protein. No significant differences were detected between the amount of phosphate incorporated by this form and a longer N-terminal mutant (amino acids 1–575). This result suggests that amino acids placed between 106 and 575 were not phosphorylated by pp60^src. The C-terminal tail of the molecule (575–783) was also substrate of this tyrosine kinase, although in a lower extent than the N terminus (approximately 0.5 pmol of phosphate/pmol of protein). Another shorter C-terminal fragment (575–693) was phosphorylated identically to the complete C-tail, indicating that the modified residue (or residues) was confined to this short sequence. In accordance, a fragment comprising residues 693–783 was not phosphorylated (Fig. 3).

The Tyr residues present in the 1–106 and 575–693 fragments were analyzed and compared with sequences of well known substrates of Src protein kinases. None of the Tyr residues present in the N-terminal 575–693 fragment fit well to the optimal phosphorylation sequence of this kinase, but among the three Tyr, Tyr-654 showed the best match. Tyr-654 presents the best features: Arg at position +1 and +2, and a Lys at +7, all characteristics repeated found in Src substrates. Tyr-654 is also a Tyr residue on the N-terminal side (position –5), two Ala residues at position +1 and +2, and a Lys at +7, all characteristics repeated found in Src substrates.

Three Tyr residues are also present in the 1–106 β-catenin fragment. Among these, Tyr-86 shows the best features: Arg at position +7, Ala at position +1 and, especially, two Asp residues upstream (–3 and –6). In addition, it contains a Gln at –1, another feature found in pp60^src substrates (19, 20). None, or only one, of these characteristics was observed in the sequences surrounding other Tyr residues.

Therefore, the best candidates, Tyr-654 and Tyr-86, were mutagenized to Phe residues and mutant β-catenins were expressed and purified (Fig. 4A). Phosphorylation experiments revealed that our predictions were correct; both Tyr-86 → Phe and Tyr-654 → Phe were significantly less phosphorylated by pp60^src than the wild-type β-catenin (Fig. 4). The phosphorylation of the double mutant was undetectable (Fig. 4). As expected from the experiments performed with the deletion mutants, that suggested a higher stoichiometry of phosphorylation for Tyr-86 than for Tyr-654 (Fig. 3), the presence of the muta-
The capacity of both \( \beta \)-catenin mutants to bind E-cadherin was also assayed and compared with that of wild-type \( \beta \)-catenin. As shown in Fig. 5A, Tyr-654 → Phe and Tyr-654 → Glu did not alter the binding of Tyr-654 → Phe wild-type \( \beta \)-catenin but did it much worse (Fig. 5C). This fact is probably due to the incomplete phosphorylation of Tyr-654 in our \textit{in vitro} assays.

The functional relevance of Tyr-654 phosphorylation was also evidenced \textit{in vivo}. Caco-2 cells were transiently transfected with wild-type \( \beta \)-catenin or the two Tyr-654 mutants (Tyr-654 → Phe, Tyr-654 → Glu) labeled with a polyhistidine tag, to facilitate their purification. After 24 h, cells were incubated with the Tyr(P) phosphatase inhibitor Na\textsubscript{3}VO\textsubscript{4}, a compound that increases \( \beta \)-catenin phosphorylation in Tyr residues, at the same time that dissociates adherens junctions (15, 22, 23). Transfected forms of \( \beta \)-catenin were purified by Ni\textsuperscript{2+}-agarose chromatography, and their phosphorylation status was analyzed. As shown in Fig. 6A, phosphorylation of wild-type \( \beta \)-catenin was remarkably increased by incubation with Na\textsubscript{3}VO\textsubscript{4}. On the other hand, the levels of Tyr(P) present in the two Tyr-654 mutants were not increased by this compound. Curiously, the

### TABLE I

| Binding of cytoEcad to \( \beta \)-catenin and p120-catenin | \( K_a \) (x 10\textsuperscript{-5}) |
|----------------------------------------------------------|----------------------------------|
| \( \beta \)-catenin                                       | 10 ± 2.0                         |
| \( \beta \)-catenin (pp60\textsuperscript{src}-phosphorylated) | 1.8 ± 0.4                        |
| p120-catenin (pp60\textsuperscript{src}-phosphorylated)     | 0.36 ± 0.05                      |
| p120-catenin (4-fold molar excess)                         | 1.2 ± 0.3                        |
| \( \beta \)-catenin (4-fold molar excess)                  | 8.2 ± 0.5                        |
| \( \beta \)-catenin (phosphorylated) + p120-catenin (4-fold molar excess) | 1.4 ± 0.2                        |
| \( \beta \)-catenin (Y65F)                                 | 10.2 ± 0.2                       |
| \( \beta \)-catenin (Y68F) (pp60\textsuperscript{src}-phosphorylated) | 1.6 ± 0.3                        |
| \( \beta \)-catenin (Y65F)                                 | 10.4 ± 0.2                       |
| \( \beta \)-catenin (pp60\textsuperscript{src}-phosphorylated) | 10.2 ± 2.2                       |
| \( \beta \)-catenin (Y68E)                                 | 8.6 ± 0.7                        |
| \( \beta \)-catenin (Y65E)                                 | 0.77 ± 0.14                      |
basal Tyr(P) content of the three forms was very similar. This experiment suggests that Na₃VaO₄ induces phosphorylation of Tyr-654, but this residue is not phosphorylated in basal conditions.

As previously mentioned, Na₃VaO₄ effects on tyrosine phosphorylation are accompanied by a lower number of E-cadherin/β-catenin cellular complexes. E-cadherin was retained by Ni²⁺-agarose only when β-catenin was bound to this resin (Fig. 6A). After addition of Na₃VaO₄, the amount of E-cadherin that copurified with β-catenin was substantially reduced. Similar levels of E-cadherin were observed in the beads when wild-type β-catenin or Tyr-654→Phe mutant were expressed (Fig. 6A). However, the amount of E-cadherin that copurified with β-catenin Tyr-654→Phe was not modified by Na₃VaO₄ as this form is not phosphorylated.

The association of E-cadherin to β-catenin mutants Tyr-654→Glu and Tyr-86→Glu was also analyzed as these forms behave as constitutively phosphorylated. The amount of E-cadherin associated to Tyr-654→Glu mutant was substantially lower than to the wild-type form (Fig. 6, A and B) and was not modified by addition of Na₃VaO₄. Unlike Tyr-654→Glu, Tyr-86→Glu interacted with E-cadherin identically than wild-type β-catenin (Fig. 6B).

All these results indicate that Tyr-654 phosphorylation is relevant for the modulation in vivo of the interaction β-catenin-E-cadherin.

**DISCUSSION**

Several authors have determined that de-assembly of adhesion junctions was frequently associated to augmented tyrosine phosphorylation of proteins present in the complex (14, 22–24). Among these proteins, the most relevant changes have been detected in β-catenin; it has been assumed that increased tyrosine phosphorylation of this protein was the cause of E-cadherin-loss-of-function. However, a conclusive relationship had not been established so far, and some authors have proposed alternative explanations (25). In this article we show that tyrosine phosphorylation of β-catenin decreases its binding to E-cadherin in assays using purified proteins, evidencing a direct cause-effect relationship. We have identified the Tyr involved in regulation of this binding as Tyr-654 and have demonstrated that modification of this residue alters the modulation of E-cadherin/β-catenin binding in vivo.

In our in vitro assays, β-catenin phosphorylation was performed by pp60⁰⁺⁰src tyrosine kinase. This protein kinase, or its viral homologue pp60⁰⁺⁰src, has been reported to promote de-assembly of adherens junction, loss of function of E-cadherin, and phosphorylation of β-catenin in Madin-Darby canine kidney cells. Our results suggest that pp60⁰⁺⁰src might be the tyrosine kinase directly responsible for the phosphorylation and, thus, the inactivation of β-catenin. However, it cannot be discarded that another protein-tyrosine kinase, activated by pp60⁰⁺⁰src, is the direct β-catenin kinase, or contributes to the complete tyrosine phosphorylation of this protein. At this respect, it should be remembered that the two Tyr residues modified in pp60⁰⁺⁰src, other plausible candidates to be responsible for Tyr-654 phos-
Phosphorylation are the Fer kinase and the EGF receptor, two tyrosine kinases that are associated to β-catenin (26, 27). In this respect, both EGF receptor and its homologue the proto-oncogene c-erb-2 have been shown to associate to the last three armadillo repeats of β-catenin (10), precisely the region of this protein where Tyr-654 is located.

Altered cell adhesion has been reported to be also associated

![Figure 4](image1.png)

**FIG. 4.** Phosphorylation of β-catenin point mutants by pp60src protein kinase. Panel A, approximately 2 μg of the four β-catenin forms were analyzed by SDS-PAGE and stained with Coomassie Blue. Panel B, 100 ng (1.1 pmol) of the four β-catenin forms were phosphorylated with recombinant pp60src protein kinase as described and analyzed by SDS-PAGE and autoradiography. WT, wild-type β-catenin; Y654F, β-catenin mutant Tyr-654 → Phe; Y654F, β-catenin mutant Tyr-654 → Phe; Y86F, Y654F, double mutant. Panel C, the four β-catenin forms as GST fusion proteins were phosphorylated by pp60src and analyzed as described in Fig. 4C. No radioactivity was detected associated to the resin when β-catenin (Y86F, Y654F) was used as substrate.

![Figure 5](image2.png)

**FIG. 5.** Binding of β-catenin point mutants to the cytosolic domain of E-cadherin. Panel A, 0.32 pmol (30 ng) of wild-type β-catenin (WT) or Tyr-654 → Phe mutant (Y654F) were phosphorylated by pp60src and incubated with 1.2 pmol of either cytoEcad-GST or GST, in a final volume of 200 μl as described under “Experimental Procedures.” Analysis of β-catenin bound was performed as previously indicated. The numbers below the lanes indicate the amount of β-catenin bound; these values were calculated comparing the result of the scanning of the corresponding lanes with known amounts of each catenin included as reference in the same blot (St). Panel B, the purity of the preparations of the different Tyr to Glu mutants as estimated by SDS-PAGE and Coomassie staining is shown. Panel C, a binding assay similar to that described in panel A was also performed with equivalent amounts of Tyr-86 → Glu (Y86E) or Tyr-654 → Glu (Y654E) mutants. No signal was detected when GST was used as bait. Although not shown, the anti-β-catenin mAb used in our assays reacted identically with all the β-catenin forms analyzed.

![Figure 6](image3.png)

**FIG. 6.** In vivo phosphorylation and binding to E-cadherin of β-catenin point mutants. Wild-type or mutant β-catenin cDNAs were inserted into pcDNA3 plasmid, labeled with a polyhistidine tag, and transfected to Caco-2 cells as described under “Experimental Procedures.” After 30 h, cell extracts were prepared from mock-transfected cells or from cells where the indicated mutants have been introduced. When mentioned, cells were incubated with Na3VaO4 (250 μM) for the last 6 h. Expressed β-catenin forms were purified by chromatography on nickel-agarose, and proteins bound to the column were subjected to SDS-PAGE and Western blot anti-β-catenin mAb to check that similar levels of expression were obtained in all the cases. Membranes were stripped and re-analyzed with mAbs against Tyr(P) or E-cadherin.
to disruption of α-catenin/β-catenin binding (28). Tyrosine phosphorylation of unknown β-catenin residues seems to be responsible of this effect. Dissociation of these proteins is probably required for β-catenin to be transported to the nucleus and work as a transcriptional cofactor (reviewed in 29). We have tested the ability of our mutants to bind recombinant α-catenin. No changes in the in vitro association of these two proteins were observed after phosphorylation of β-catenin by pp60c-src or when binding to α-catenin of Tyr-86 → Glu and Tyr-654 → Glu mutants were compared with the wild-type form. These results suggest that pp60c-src is not directly responsible for the modification of the Tyr residue that modulates α-catenin/β-catenin association and that neither Tyr-654 nor Tyr-86 are involved in this process. A more plausible candidate for this role is Tyr-143, the only Tyr residue present in the β-catenin domain necessary for α-catenin binding (30). In any case, the physiological relevance of tyrosine phosphorylation in the control of β-catenin/α-catenin binding is likely to be dependent on the cell system, since in Caco-2 cells treatment with Na3VO4 did not modify the amount of α-catenin/β-catenin binding. No differences were observed in the amount of α-catenin that copurified with transfected β-catenin in the experiment described in Fig. 6 after treatment with Na3VO4 (data not shown).

p120-catenin was initially characterized as a substrate of the activated pp60c-src kinase and only recently has been described its binding to E-cadherin (14). Our group (15) and others (24) have reported that this protein is tyrosine-phosphorylated in its binding to E-cadherin (14). Our group (15) and others (24) have tested the ability of our mutants to bind recombinant β-catenin but does not compete with the binding of p120-catenin to E-cadherin. This protein still has to be demonstrated. The description by 2 J. Piedra, A. García de Herreros, and M. Duñach, unpublished observations.


dominated by ion pairs over an extended region, more than a close steric complementarity on a more limited area. It is easy to speculate that the introduction of a phosphate group, and thus, a negative charge, in the armadillo domain might affect this interaction, either diminishing the number of ion pairs or partially closing the long groove. The fact that the modified residue is not in the central part of this structure may help to explain the armadillo domain is still able to associate to other proteins after phosphorylation, as adenomatous polyposis coli (30, 34–35).

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