ErbB2 kinase domain is required for ErbB2 association with β-catenin

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To investigate the region of ErbB2 for the ErbB2-β-catenin interaction, a proteasome resistant-β-catenin and various ErbB2 constructs were transfected in COS7 cells. ErbB2 proteins were immunoprecipitated, and coimmunoprecipitated β-catenin was examined by Western blotting. β-catenin coimmunoprecipitated with full length ErbB2. Of the truncated ErbB2 proteins DT (1-1123), DHC (1-1031) and DK (1-750), the ErbB2 constructs containing the kinase domain, DT and DHC, precipitated together with β-catenin but DK containing no kinase domain did not. To further test the requirement of the kinase domain for β-catenin-ErbB2 interaction, the presence of β-catenin in the immunocomplex was examined following transfection with an ErbB2 mutant (Δ750-971) whose kinase domain is internally deleted and subsequent immunoprecipitation of the ErbB2 mutant. β-catenin was not detected in the immunocomplex. These results suggest that the ErbB2 kinase domain comprises a potential site for β-catenin binding to the receptor tyrosine kinase.

Key words – β-catenin, ErbB2, protein association

Introduction

β-catenin is a multifunctional protein that plays a central and essential role in the Wnt signaling and in maintenance of tight cell-cell-contacts [1-5]. Initially characterized in *Drosophila* as the product of the segment polarity gene Armadillo [6], β-catenin and its relative plakoglobin (γ-catenin) were subsequently shown to be components of adhesion junctions. Here, β-catenin links cadherins at the plasma membrane to α-catenin [7-9]. Recent studies demonstrated that the E-cadherin-β-catenin-α-catenin complex at the adherens junctions forms a dynamic, rather than a stable, link to the cytoskeleton [10,11]. In the Wnt signaling pathway, by contrast, β-catenin functions as a transcriptional activator in conjunction with LEF/TCF (lymphoid enhancer factor/T-cell factor) DNA binding proteins [3,4].

Although many studies have focused on mechanisms that abrogate the proteasome sensitivity of uncomplexed cytoplasmic β-catenin in tumor cells, less attention has been paid to the status and regulation of β-catenin-E-cadherin association in cells that either express GSK-3β resistant β-catenin or lack the APC protein. During development of carcinomas, cells undergo epithelial-mesenchymal transition (EMT), which is characterized by loss of cell-cell adhesion and increased cell motility [12]. The hallmark of EMT is the loss of function of E-cadherin and, thereby, the dissociation of the E-cadherin-β-catenin-α-catenin complex from the membrane [13,14]. Loss of E-cadherin-mediated cell adhesion correlates with increased β-catenin-dependent transcription of a number of growth-promoting genes, including c-myc and cyclin D [3,15]. The resultant nuclear accumulation of β-catenin is thought to play a pivotal role in tumorigenesis [16].

Dissociation of adherens junctions can be induced by phosphorylation of crucial tyrosine residues in components of the complexes for cell adhesion [17]. Overexpression of tyrosine kinases, and mutations in tyrosine phosphatase genes that might catalyze these phosphorylation events have been reported in tumors [18]. Interestingly, human epidermal growth factor receptor-2 (HER2/neu; erbB2), which belongs to a family of four transmembrane receptor tyrosine kinases involved in signal transduction pathways that regulate cell growth and proliferation, has been found to be associated with β-catenin-E-cadherin complexes in epithelial cells, suggesting opposing roles for these proteins in modulating β-catenin-E-cadherin association [19, 20]. ErbB2 interacts with the carboxyl terminus of β-catenin, at a site containing Tyr-654 [21]. Recently, Roura *et al.* [22] reported that the Tyr-654 residue of β-catenin is preferentially phosphorylated under conditions that disrupt β-catenin-E-cadherin association and, furthermore, the phosphorylation status of the Tyr-654 residue of β-catenin regulates β-catenin association with E-cadherin. Considering the

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previous reports, it is very likely that intervening in the ErbB2-β-catenin association is a useful strategy to deter tumor progression. Localizing the region for protein-protein interaction provides basic information for rational drug design of a drug intervening in the interaction. However, there is no report on the binding region of β-catenin in ErbB2 although it is known that carboxyl terminus of β-catenin interacts with ErbB2. Therefore, we investigated the region of ErbB2 for the ErbB2- catenin interaction using a mutant β-catenin resistant to proteasome degradation and various ErbB2 constructs. Our data demonstrate that the β-catenin coimmunoprecipitated with only ErbB2 constructs containing the ErbB2 kinase domain in COS7 cells transfected with β-catenin, plus full-length, truncated or internal-deleted ErbB2 constructs. Thus, these results suggest that the ErbB2 kinase domain comprises a potential site for β-catenin binding to the receptor tyrosine kinase.

Materials and Methods

Cells and Antibodies

COS-7 cells were purchased from American Type Culture Collection (Rockville, MD). Anti-ErbB2 immunoprecipitating anti-ErbB2 antibodies (Aβ-2, 5), which were premixed before use, were from Oncogene Science Inc. (Cambridge, MA), and Western blotting anti-ErbB2 antibodies Aβ-3 [clone 3B5] for the intracellular domain and AB-3 [clone L87] for the extracellular domain) were from Oncogene Science Inc. and NeoMarkers (Fremont, CA), respectively. Anti-β-catenin polyclonal antibody was from Invitrogen (Carlsbad, CA).

Preparation of Plasmid Constructs

pcDNA3-*ErbB2* were described previously [23]. Truncated ErbB2 proteins (DT (1-1123), DHC (1-1031) and DK (1-750)) were made by joining a fragment cut out of pcDNA3-*ErbB2* with *Hin*dIII/*Sph*I with another fragment amplified by polymerase chain reaction. The polymerase chain reaction was performed with the shared 59-end primer 59-G ATGAGGAGGGCGCATGCCAGCCTT-39 and the 39- end primer 59-GCGCTCGAGTTACTCAGAGGGCAGGGGTAC TG-39 for ErbB2/DT, 59-GCGCTCGAGTTAGAAGAAGCC CTGCTGGGGTA-39 for ErbB2/DHC or 59-GCGCTCGAGT TATCTCCGCATCGTGTACTTCC-39 for ErbB2/DK. The products of polymerase chain reactions were digested with *Sph*I and *Xho*I and, together with the first fragment, were ligated into the pcDNA3 vector. An internal deletion mutant (Δ750-971) was constructed by inserting ErbB2 DNA fragments, amplified by PCR, into the pcDNA3 vector (Invitrogene). Two fragments were used for the mutant. The 5'-end primer for the N-terminal end fragment is AATACGA CTCACTATAGGGGAGA; the 3'-end primer for the C-terminal end fragment is GGCAACTAGAAGGCACAGTCGA GG. Additional primers are GGGCGTACGCAGTGGAATT TTCACATTCTCCCCA for the position ending at amino acid 750 and GGGCGTACGGAGTTGGTGTCTGAATTCTC C-CG for the position starting at amino acid. The two fragments were cut with BsiWI plus HindIII or XbaI, and ligated with HindIII-and XbaI-cut pcDNA3 vector.

Cell Culture and Transient Transfection

COS-7 cell culture medium contained 90% Dulbecco's modified Eagle's medium, 10% fetal calf serum, 2 mM glutamine, 1 mM Hepes, and 1 mM sodium pyruvate. For transient transfections, each plasmid, which was premixed with FuGene 6 (Roche Molecular Biochemicals), was added to cells at 50-70% confluency. Cells were continually cultured in the same medium for 24 h until lysis. β-catenin plasmids were gifted from Dr. Jane Trepel (NCI, USA)

Immunoprecipitation and Western Blotting

Cells were washed once with cold phosphate-buffered saline (pH 7.0) and lysed by scraping in lysis buffer (50 mM Tris-HCl (pH 7.5), 0.5% Nonidet P-40, 150 mM NaCl, and 1 mM sodium orthovanadate) supplemented with CompleteTM proteinase inhibitors (Roche Molecular Biochemicals). Cell lysates were clarified by centrifugation at 14,000 rpm (4°C) for 15 min, and protein concentration was determined by the BCA method (Pierce). For immunoprecipitation, 1 mg of lysate protein was incubated with 4 mg of mouse monoclonal antibodies at 4°C for 2 h, followed by the addition of protein-G-agarose beads (Invitrogen) and rotation at 4°C overnight. The beads were washed five times with lysis buffer, resuspended in SDS sample buffer (80 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 100 mM dithiothreitol, and 0.0005% bromphenol blue), and boiled for 5 min. Immunoprecipitated proteins were separated by 4-20% SDS-PAGE. Western blotting was performed as described previously[24].

Results

We prepared various ErbB2 truncation constructs in-

cluding ErbB2-DT (DT, deletion of 132 amino acids at the C-terminus), ErbB2-DHC (DHC, deletion of 224 amino acids at the C terminus), ErbB2/DK (DK, deletion of most of the intracellular domain) and ErbB2/750-971 (Δ750-971, an ErbB2 construct with an internal deletion of the kinase domain) as shown in Fig. 1A. The expression of the ErbB2 constructs was confirmed in COS7 cells (Fig. 1B). We also tested the expression of WT β-catenin and a mutant β-catenin (Ser-37Phe, a mutant resistant to proteasome degradation) in COS7 cells. As expected, transfection with the mutant accumulated greater amount of its gene product than that with WT β-catenin. Ectopical expression of WT β -catenin did not afford significant accumulation of β-catenin compared with the level of endogenous β-catenin (Fig. 1C). The mutant β-catenin was used for cotransfection with ErbB2 constructs to scrutinize the region for ErbB2-β-catenin interaction more efficiently. To examine which region of ErbB2 is responsible for the interaction with β-catenin, cells were transfected with the mutant β-catenin and either full length ErbB2 or ErbB2 constructs progressively truncated from the C-terminus, DT, DHC or DK and the cells were lysed 24 h later and ErbB2 proteins were immunoprecipitated. Coimmunoprecipitation of β-catenin and ErbB2 proteins was examined by Western blotting. As shown in Fig. 1D, consistent with previous reports [20,21], full length ErbB2 coprecipitated with either ectopically expressed β-catenin or endogenous β-catenin although endogenous one was detected very weakly. Further, while the truncated ErbB2 constructs DT and DHC containing the kinase domain precipitated together

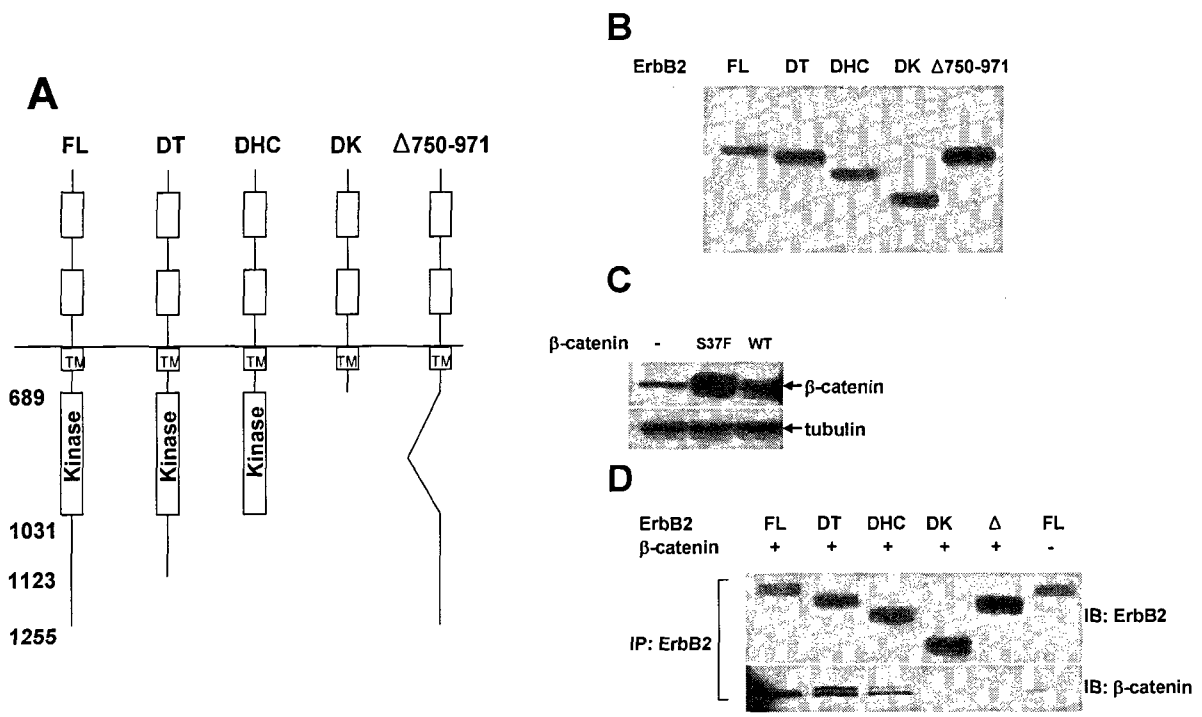

Fig. 1. The kinase domain of ErbB2 comprises a potenial binding site of β-catenin. A. Schematic description of ErbB2 constructs used in the experiment B COS7 cells in 6-well plates were transfected with full-length (FL), truncated (DT, DHC and DK) or internal-deleted ErbB2 (Δ750-971) constructs. Twenty-four hours after transfection, cells were lysed. Cell lysates (40 μg) were mixed with 5 x SDS sample buffer and separated by 4-20% SDS-PAGE. Blots were probed with anti-ErbB2 antibodies (AB-3, Neomarkers) C. COS7 cells in 6-well plates were transfected with wild type β-catenin or a mutant β-catenin (S37F, a mutant resistant to proteasome degradation) constructs. Twenty-four hours after transfection, cells were lysed. Cell lysates (20 μg) were mixed with 5 x SDS sample buffer and separated by 4-20% SDS-PAGE. Blots were probed with anti-β-catenin polyclonal antibody. D. COS7 cells in 10 cm dishes were transfected with a mutant β-catenin (S37F), plus full-length (FL), truncated (DT, DHC and DK) or internal-deleted ErbB2 (Δ750-971) constructs. Twenty-four hours after transfection, cells were lysed. For immunoprecipitaion (*IP*), 1 mg of cell lysate was incubated with anti-ErbB2 mAb (Ab2/Ab5), and the immunocomplex were precipitated with protein-G-agarose beads. Immunoprecipitated proteins were solubilized in SDS sample buffer and separated by 4-20% SDS-PAGE. Blots were probed with anti-ErbB2 (AB-3, Neomarkers) or anti-β-catenin antibody.

with β-catenin, DK containing no kinase domain failed to coprecipitate with β-catenin suggesting that the kinase domain may be the region for the association of the two proteins. To further test the requirement of the kinase domain, the same experiment was done using an ErbB2 mutant whose kinase domain is internally deleted. As shown in Fig. 1D, the ErbB2 construct did not coprecipitate with β-catenin.

Discussion

In this study, by demonstrating that the ErbB2 proteins with its kinase domain but not those without it coprecipitated with β-catenin, we suggest that ErbB2 kinase domain comprises a potential binding site of β-catenin. β-catenin-E-cadherin association/dissociation, which is crucial for E-cadherin-mediated cell adhesion and β-catenin-dependent transcription of a number of growth-promoting genes, including c-myc and cyclin D, seems to be regulated, at least in part, by tyrosine phosphorylation/dephosphorylation of β-catenin [25], inasmuch as tyrosine phosphorylated β-catenin associates poorly, if at all, with E-cadherin [22]. ErbB2 interacts with the carboxyl terminus of β-catenin, at a site containing Tyr-654, preferentially phosphorylated under conditions that disrupt β-catenin-E-cadherin association [21]. Furthermore, ErbB2 is responsible for constitutive tyrosine phosphorylation of β-catenin in melanoma cells [26]. These suggest that, together with Wnt signal, tyrosine phosphorylation of β-catenin via ErbB2-β-catenin interaction plays an important role in cell-cell interaction and cell proliferation.

Consistent with previous reports [26,27], a mutant β-catenin whose GSK-3β amino terminal phosphorylation site is mutated (Ser-37Phe) was greatly accumulated in cells, which reflects the cellular situation where the proteasome sensitivity of uncomplexed cytoplasmic β-catenin is abrogated by mutation of APC and/or GSK-3β. Our data showing that only ErbB2 constructs with the kinase domain was able to associate with β-catenin strongly suggest that the kinase domain contains a potential binding site of β-catenin. Although we did not access whether the β-catenin binding to the region is required for the Tyr (654)- phsophorylation of β-catenin, considering that the β-catenin region including Tyr (654) binds to the kinase domain of ErbB2 [21], it is very likely that the association induces phosphorylation of the tyrosine residue. In cells that are able to degrade efficiently uncomplexed cytoplasmic β-catenin, deregulation of β-catenin tyrosine phosphorylation may not cause an elevation in the cytoplasmic free pool of the protein, but the lack of association of β-catenin with E-cadherin in such cells would still result in enhanced motility and decreased cell-cell contacts [28]. In cells unable to efficiently degrade free cytoplasmic β-catenin, increased tyrosine phosphorylation of the protein should augment the level of the free pool and result in the increased nuclear accumulation of β-catenin. Thus, pharmacological abrogation of β-catenin tyrosine phosphorylation, even in cells expressing aberrantly stable β-catenin, may antagonize the tumor promoting activity of nuclear-localized β-catenin while simultaneously enhancing the tumor suppressive activity of plasma membrane β-catenin-E-cadherin complexes. Therefore, our results may be of particular use in structure based design for a drug intervening in the interaction between ErbB2 and β-catenin, thus preventing ErbB2-mediating Tyr (654)-phosphorylation of β-catenin.

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초록 : ErbB2의 kinase 영역이 β-catenin과 ErbB2의 결합에 필요하다

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β-catenin과 결합하는 ErbB2의 부위를 조사하기 위하여 proteasome에 의하여 분해되지 않는 β-catenin과 다양한 ErbB2 construct를 COS7 세포에 transfection 한 후 ErbB2 단백질을 그것의 항체로 가라앉혔다. 이 때 공침한 β-catenin을 Western blot으로 분석하였다. C 말단에서부터 잘려진 ErbB2 단백질 중에 kinase 영역을 가지고 있는 것들만 β-catenin과 공침하였다. kinase 영역의 필요성을 확인하기 위하여 kinase 영역이 내부에서 제거된 ErbB2 construct를 β-catenin과 transfection 한 후 동일한 실험을 실시하였다, 이 실험에서 β-catenin는 kinase 영역이 내부적으로 제거된 ErbB2 단백질과 공침하지 않았다. 이 결과는 β-catenin과 결합하는 ErbB2의 위치는 kinase 영역내에 있음을 제시한다.