Regulation of β-Catenin Function by the IκB Kinases*

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Both the β-catenin and the nuclear factor κB (NF-κB) proteins are important regulators of gene expression and cellular proliferation. Two kinases, IKKa and IKKβ, are critical activators of the NF-κB pathway. Here we present evidence that these kinases are also important in the regulation of β-catenin function. IKKa- and IKKβ-deficient mouse embryo fibroblasts exhibited different patterns of β-catenin cellular localization. IKKβ decreases β-catenin-dependent transcriptional activity, while IKKa increases β-catenin-dependent transcriptional activity. IKKα and IKKβ interact with and phosphorylate β-catenin using both in vitro and in vivo assays. Our results suggest that differential interactions of β-catenin with IKKa and IKKβ may in part be responsible for regulating β-catenin protein levels and cellular localization and integrating signaling events between the NF-κB and Wingless pathways.

β-Catenin, the mammalian homologue of the Drosophila armadillo protein, is a ubiquitously expressed protein that has at least two distinct roles in the cell. First, it participates in cell-cell adhesion by mediating the association of E-cadherin with the cytoskeleton (1, 2). Second, it is a critical downstream component of the Wnt2 or Wingless signal transduction pathway (3–5). The Wnt family of secretory glycoproteins plays an important role in embryonic development, in the induction of cell polarity, and in the determination of cell fate. Deregulation of Wnt signaling disrupts axis formation in embryos (5–8) and is associated with multiple human malignancies (9).

The current model of Wnt signaling indicates that the binding of the Wnt proteins to their receptor, frizzled, stabilizes β-catenin by inhibiting the activity of a serine/threonine kinase glycogen synthase kinase-3 or GSK-3β (9). GSK-3β is associated with β-catenin in a multiprotein complex that includes the adenomatous polyposis coli tumor suppressor protein (APC), axin or conductin, protein phosphatase 2A, and dishevelled. GSK-3β phosphorylation of residues in the amino terminus of β-catenin results in APC-mediated β-catenin degradation via the ubiquitin-proteasome pathway (10, 11). Increased levels of β-catenin are frequently found in colon cancer due to mutations in either the APC gene (12–14) or at residues in the amino terminus of β-catenin that are phosphorylated by GSK-3β (15–17). In the nucleus, β-catenin forms a complex with members of the T-cell factor (TCF)/lymphocyte-enhancer factor (LEF) family and activates gene expression of a variety of target genes (18–23) including c-myc (24) and cyclin D1 (25, 26).

NF-κB comprises a family of transcription factors which are critical in activating the expression of genes involved in the immune and inflammatory response and in the regulation of cellular apoptosis (27, 28). NF-κB is sequestered in the cytoplasm by a family of inhibitory proteins known as IκB. Upon stimulation of this pathway by a variety of agents including IL-1 and TNFα, the kinases IKKa and IKKβ (29–33) in conjunction with the scaffold protein IKKγ/NEMO (34–36) leads to the phosphorylation of IκBa at serine residues 32 and 36. Gene disruption studies in mice indicate that IKKβ appears to be the critical kinase in activating the NF-κB pathway (37–39), while IKKα appears to be critical for other functions such as keratinocyte differentiation (40–42). IKKa and IKKβ can form homodimers and also heterodimerize with each other, and this process is critical for their kinase activity. IKK phosphorylation of IκBa leads to its ubiquitination and degradation by the 26S proteasome and the nuclear translocation of NF-κB (43).

Interestingly, the sequence DSGXXS, which is the target site in IκB for IKK phosphorylation, is also found in the amino terminus of β-catenin (11). Phosphorylation of this sequence in both β-catenin and IκB stimulates their interactions with the ubiquitin ligase β-TrCP leading to their degradation by the proteasome (10, 11, 44). It has also been demonstrated that the β-catenin/TCF complex increases β-TrCP levels by a post-transcriptional mechanism to result in opposite effects on β-catenin and NF-κB activity (45). In addition, disruption of either the murine GSK-3β and IKKβ genes results in a similar phenotype with embryonic lethality due to hepatic apoptosis from increased sensitivity to TNFα (46). These results suggest potential relationships between β-catenin and NF-κB signaling pathways.

Given the fact that both the NF-κB and β-catenin pathways are important in the control of cellular proliferation and are regulated by cellular kinases that lead to β-TrCP-mediated degradation (10, 11, 45), we explored potential similarities in their regulation. First, we addressed whether there were differences in the cellular localization of β-catenin in wild-type mouse embryo fibroblasts as compared with fibroblasts derived from IKKα- and IKKβ-deficient mice. Next, we analyzed inter-
actions between both IKKα and IKKβ and β-catenin and determined whether these kinases regulated β-catenin-dependent transcriptional activity. The results of this analysis indicate that IKKα can positively regulate β-catenin-dependent transcriptional activity while IKKβ negatively regulates this activity.

MATERIALS AND METHODS

Cells and Reagents—SW480 cells were purchased from American Tissue Culture Collection (Manassas, VA) and maintained in 1:1.5 medium supplemented with 10% fetal bovine serum (HyClone Laboratory, 2.1-glycolytic and antibiotics), COS, mouse embryo fibroblasts (MEFs, a kind gift of Xiaodong Wong), IKKα and IKKβ knock-out cells (39, 42) were maintained in Dulbecco’s modified Eagle’s medium and supplemented with the same components as above.

Antibodies—Polyclonal antibodies to IKKα (sc-7182), IKKβ (sc-7607), and β-catenin (sc-1486) were obtained from Santa Cruz Biotechnology. Monoclonal antibodies against β-catenin and TFIIIB (Transduction Laboratory), IKKα (PharMingen), the hemagglutinin epitope HA/12CA5 (Roche Molecular Biochemicals), and the FLAG-epitope-M2 (Sigma) were also used in immunoprecipitation and Western blot analysis. Donkey anti-rabbit, anti-mouse and anti-goat antibodies conjugated with either anti-FITC or Red-X rhodamine were obtained from Jackson Laboratory.

Plasmid Constructs—The pCMV5 expression vectors containing either FLAG-tagged IKKα and IKKβ including the constitutively active kinases (SS/EE) with substitutions at residues 176/180 for IKKα or 177/181 for IKKβ and the kinase defective (K/M) mutants at residue 44 in both IKKα and IKKβ were described previously (30, 47, 48). Wild-type and mutant IKKα and IKKβ cDNAs were each cloned into the baculovirus expression vector pAcHLT. The recombinant baculoviruses were used to infect SF9 cells to produce recombinant IKK proteins for in vitro kinase assays (48). The pCMV5 expression vectors containing the wild-type and the dominant negative NIK mutant in which lysine 781) bacterial expression vector was constructed by using polymerase chain reaction (PCR) to generate a fragment encompassing the full-length β-catenin, which was then cloned in frame with GST in the pGEX. The GST fusion protein containing the amino-terminal 91 amino acids of β-catenin was constructed by ScaI digestion and ligation of the GST fusion containing wild-type β-catenin. The constructs GSTβ-cat (130–751), GSTβ-cat (1–400), GSTβ-cat (130–400), and GSTβ-cat (G18–751) were constructed using PCR. The amino-terminal deleted form of β-catenin utilized PCR primers to generate a fragment containing amino acids 130–751, which was cloned into pCMV5 and contained a carboxyl-terminal HA-epitope. All constructs that were generated by PCR were subjected to DNA sequencing and cloned into pCDNA3.

Expression and Purification of GST β-Catenin Fusion Proteins—Recombinant GST β-catenin fusion proteins were expressed in bacterial strain BL21 and lysed in HMK buffer (50 mM Tris (pH 7.5), 0.1 mM NaCl, 1 mM phenylmethylsulfonyl fluoride), and the bacterial lysates were incubated with 0.5 ml of packed glutathione-conjugated-agarose beads (Sigma) for 2 h at 4 °C. After three washes, the GST fusion proteins were eluted with 10 mM glutathione in HMK buffer and dialyzed, and protein purity was assessed by SDS-polyacrylamide gel electrophoresis.

Luciferase Reporter Assays—COS cells and mouse embryo fibroblasts were plated at 50% confluence in 35-mm tissue culture wells. After 24 h, the cells were transfected using LipofectAMINE Plus with the indicated DNA constructs and either the TOPFLASH luciferase reporter containing LEF/TCF binding sites or the FOPFLASH luciferase reporter with mutated LEF/TCF sites. An NF-κB luciferase reporter containing the NF-κB binding sites upstream of a thymidine kinase minimal promoter was used to detect NF-κB-directed gene expression. An RSV-β-galactosidase reporter plasmid was included as an internal control for differences in transfection efficiency, and the pCMV5 plasmid was added to the transfection assays to standardize DNA quantities. Between 18 to 24 h posttransfection, the cells were washed twice with cold PBS, and the reporter activity was measured using the luciferase assay system (Promega). The results of this analysis indicate that IKKα can positively regulate β-catenin-dependent transcriptional activity while IKKβ negatively regulates this activity.

Fractionation of Cellular Extracts—Cytoplasmic extracts were prepared from 108 SW480 or COS cells as described previously (49) with slight modifications. Cells were washed twice with cold PBS, and cell pellets were resuspended in 5 volumes of buffer A (10 mM HEPES (pH 7.9), 1.5 mM MgCl2, 0.5 mM dithiothreitol, 0.2 mM EDTA) supplemented with phosphate buffer (10 mM NaF, 10 mM β-glycerophosphate, 0.5 mM o-iodoacetic acid, 1 mM sodium orthovanadate), and protease inhibitors (Roche Molecular Biochemicals). After incubation on ice for 10 min, the cells were lysed with 15 strokes of a Wheaton all-glass Dounce homogenizer (Tight pestle). Nuclei were pelleted by centrifugation for 5 min at 2000 rpm (Beckman bench-top centrifuge, CH5.7) and resuspended in buffer B (0.3 M HEPES (pH 7.9), 30 mM MgCl2, and 1.4 mM NaCl), and then centrifuged at 100,000 × g for 60 min at 4 °C.

Whole cell extracts were prepared from COS cells transfected with hemagglutinin-tagged β-catenin alone or β-catenin and FLAG-tagged IKKα and IKKβ as described (48) in lysis buffer containing 40 mM Tris, (pH 8), 500 mM NaCl, 0.1% Nonidet P-40, 6 mM EDTA, 6 mM EGTA, 5 mM β-glycerophosphate, 5 mM NaF, 1 mM Na3VO4, (pH 10.0), and protease inhibitors (Roche Molecular Biochemicals).

Cell Filtration Chromatography—S100 extracts prepared from the SW480 and COS cells were further fractionated on a Superdex-200 gel filtration column (Amersham Pharmacia Biotech) and equilibrated with buffer Z (50 mM HEPES (pH 7.9), 0.1 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 0.2 mM EDTA, 0.25 mM PMSF, and 2 mM protease inhibitors (Sigma) used for the calibration of the column). The S100 extracts were resuspended in 3 volumes of buffer F (0.3 M HEPES (pH 7.9), 30 mM MgCl2, 1.4 mM NaCl, and 25 mM KCl), and cytocrome c (12.5 kDa).

Protein Association and Western blot Analysis—For endogenous protein association studies, equal volumes of proteins (200–300 μl) from each of the Superdex-200 fraction were incubated overnight at 4 °C with 1 μg of indicated antibodies or normal mouse IgG followed by the addition of protein G-agarose (Sigma) for 2–3 h at 4 °C. For protein association studies using transfected IKK and β-catenin expression vectors, COS cells were transfected with FLAG-tagged IKKα or IKKβ and HA-tagged β-catenin cDNAs. Cells were harvested 18–24 h after transfection, extracted were prepared, and gel chromatography was performed as described above. Equal volumes of each column fraction were immunoprecipitated with 12CA5 antibody or anti-FLAG M2 antibody. Immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes (Amersham Pharmacia Biotech), and probed with specific antibodies. The membrane-bound immune complexes were analyzed with an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). In vitro association studies, 40 μl of the cytoplasmic fractions were incubated overnight at 4 °C with 40 μl of the glutathione-conjugated-agarose bound with indicated proteins. Following three washes with 10 volumes of cold PBS, the protein complexes were subjected to Western blot analysis as described above.

In Vitro Kinase Assays—Kinase assays were performed by Yeung et al. (48). A recombinant baculovirus-produced IKK proteins were purified by nickel-agarose chromatography and then immunoprecipitated with 12CA5 monoclonal antibody (48). The epo-tagged IKKα and IKKβ kinases were transfected into COS cells, and extracts were immunoprecipitated with the M2 monoclonal antibody directed against the FLAG-epitope. These kinases were added to kinase buffer containing 12.5 μCi of [γ-32P]ATP, 1 mM ATP, 1 mM dithiothreitol, 5 μM MgCl2, 100 mM NaCl, 50 mM Tris-HCl (pH 8.0), and then 1 μg of each of the substrates including wild-type or the S32A/S36A of GST-IκBα and GSTβ-catenin (1–91), 2 μg of each of these substrates was incubated with either FLAG-tagged IKKα or IKKβ, which were immunoprecipitated from COS cell extract with the M2 monoclonal antibody in kinase buffer containing 15 μCi of [γ-32P]ATP with a specific activity of 6000 Ci/mM (New England Nuclear) and either 0.01 mM, 0.01 mM, or 1.0 mM of cold ATP. The kinase reaction mixtures were subjected to electrophoresis on 10% SDS-polyacrylamide gels and autoradiography. The 32P-labeled IκBα and β-catenin substrates were then subjected to scintillation counting, and the moles of phosphate incorporated were calculated. Reactions were initiated by the addition of kinase buffer containing 60°C and stopped by the addition of protein loading buffer and heating to 90 °C.

Immunocytochemistry and Confocal Microscopy—Cells were cultured overnight on coverslips in Dulbecco’s modified Eagle’s medium without serum, washed twice with PBS, and fixed with 3.7% formaldehyde for 10 min followed by a brief permeabilization with 0.5% Triton X-100 in PBS. The cells were blocked for 30 min with 3% normal

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RESULTS

β-Catenin Localization in IKK-deficient Cells—First, the localization of IKKα and IKKβ in wild-type MEFs was compared with that seen in IKKα-deficient (IKKα-/-) and IKKβ-deficient (IKKβ-/-) cells using immunofluorescence analysis with confocal microscopy. Wild-type mouse embryo fibroblasts (Fig. 1A, panels A and B) and IKKα-/- (Fig. 1A, panel C and D) and IKKβ-/- (Fig. 1A, panels E and F) cells were plated on coverslips overnight and stained with rabbit polyclonal antibodies directed against either IKKα or IKKβ. In MEFs, IKKα localized in both the nucleus and the cytoplasm, while IKKβ localized predominantly in the cytoplasm (Fig. 1A, panels A and B). In IKKα-/- cells, IKKβ localized predominantly in the cytoplasm (Fig. 1A, panel D). In IKKβ-/- cells, there appeared to be increased IKKα present in the nucleus as compared with that seen in MEFs (Fig. 1A, panel E). There was no IKKα staining observed in IKKα-/- cells (Fig. 1A, panel C) or IKKβ staining seen in IKKβ-/- (Fig. 1A, panel F), thus confirming the identity of these cells.

Immunostaining of MEFs, IKKα-/-, and IKKβ-/-deficient cells with a polyclonal antibody directed against β-catenin demonstrated that β-catenin has a different pattern of staining in IKKα-/- and IKKβ-/- cells. β-Catenin was present in both the nucleus and the cytoplasm of MEFs with marked accumulation at cell-cell junctions (Fig. 1B, panel A). In IKKα-/- cells, there was reduced nuclear staining of β-catenin as compared with MEF cells (Fig. 1B, panel G). There was more β-catenin present in the nucleus and the perinuclear region of IKKβ-/- cells than in IKKα-/- cells (Fig. 1B, panel G). As a control, these cells were also stained with a monoclonal antibody directed against the basal transcription factor TFIIB, which is localized predominantly in the nucleus (Fig. 1B, panels B, E, and H). There was little background staining when the FITC-conjugated anti-goat secondary antibody was used with the mouse monoclonal antibody directed against TFIIB (Fig. 1B, panels C, F, and I). These results indicate that there is less β-catenin localized in the nucleus of IKKα-/- cells than in either IKKβ-/- cells or MEF cells.

β-Catenin Activity in IKK-deficient Cells—Next we addressed whether the differences in β-catenin distribution in the IKK-deficient embryo fibroblasts could alter its transcriptional activity. The IKK-deficient cells and the parental MEFs were transfected with a TOPFLASH reporter construct alone or with expression vectors encoding either LEF-1 or β-catenin. The TOPFLASH reporter is driven by four LEF/TCF binding motifs inserted upstream of a minimal c-fos promoter and a luciferase gene (15). As a control, the TOPFLASH reporter, which lacks LEF/TCF binding sites, was utilized. An RSV-β-galactosidase expression vector was included in these transfections to control for differences in transfection efficiency.

When the TOPFLASH reporter alone was transfected into IKKα-/- cells, there was consistently a 5-6-fold lower level of activity as compared with that observed in IKKβ-/- cells (Fig. 2). Transfection of an expression vector encoding LEF-1 into either IKKα-/- or IKKβ-/- cells markedly stimulated TOPFLASH activity as did transfection of expression vectors encoding both LEF-1 and β-catenin. There was no significant activity from the TOPFLASH reporter in either the absence or presence of β-catenin and LEF-1 (Fig. 2). Transfection of expression vectors encoding wild-type IKKβ and LEF-1 into IKKα-/- cells reduced TOPFLASH activity, while transfection of an IKKα expression vector with LEF-1 increased TOPFLASH activity in these cells (Fig. 2). When similar studies were performed in IKKβ-/- cells, transfection of an IKKβ expression vector reduced TOPFLASH activity, while transfection of an IKKα ex-
pression vector did not significantly alter TOPFLASH activity (Fig. 2). Transfection of both β-catenin and LEF-1 resulted in similar levels of TOPFLASH activity in the IKKβ−/− and IKKα−/− cells (Fig. 2). The parental MEF cells consistently gave somewhat higher TOPFLASH activity than seen in the IKK-deficient cells (Fig. 2). Again transfection of an IKKβ expression vector with LEF-1 into these cells reduced TOPFLASH activity, while transfection of an IKKα expression vector with LEF-1 resulted in little change in TOPFLASH activity (Fig. 2). Thus, the reduced levels of endogenous β-catenin in the nuclei of IKKα−/− cells are associated with decreased β-catenin activation of gene expression, and this defect could be complemented by transfection of an IKKα expression vector. IKKα does not increase gene expression in the IKKβ−/− and MEF cells, which have relatively abundant levels of nuclear β-catenin.

IKKβ and IKKα Have Differential Effects on β-Catenin Transactivation—The results presented in the previous section suggested that IKKβ and IKKα could potentially be involved in regulating the transcriptional stimulatory properties of β-catenin. Thus it was important to address whether either IKKα or IKKβ could alter β-catenin-mediated transcriptional activation in COS cells, which have low levels of endogenous β-catenin in the nucleus and relatively low levels of IKKα and IKKβ (data not shown). COS cells were transfected with either a TOPFLASH or FOPFLASH reporter, LEF-1 and β-catenin expression vectors, and increasing amounts of expression vectors encoding either the wild-type, constitutively active or kinase-defective mutants of IKKα and IKKβ. The constitutively active IKK proteins (IKKα SS/EE and IKKβ SS/EE) have glutamate substituted for serine residues in their T-loop so as to mimic phosphorylation of these residues and increase the activity of these kinases (30). The kinase-defective mutants (IKKα K/M and IKKβ K/M) contain a substitution of a lysine residue at position 44 with methionine (30).

As previously demonstrated, the coexpression of β-catenin and LEF-1 increased TOPFLASH but not FOPFLASH activity (Fig. 3A). When either wild-type IKKβ or the constitutively active kinase, IKKβ SS/EE, was cotransfected with β-catenin and LEF-1, TOPFLASH activity decreased in a concentration-dependent manner (Fig. 3A). In contrast, cotransfection of either wild-type IKKα or the constitutively active kinase, IKKα SS/EE, increased β-catenin-dependent transactivation in a concentration-dependent manner (Fig. 3A). Transfection of the IKKβ K/M mutant resulted in a modest decrease in β-catenin transactivation that was not concentration-dependent, while transfection of the IKKα K/M mutant did not significantly alter β-catenin transactivation (Fig. 3A).

The cotransfection experiments in COS cells indicated that IKKα increased β-catenin-dependent gene expression, while IKKβ decreased β-catenin-dependent gene expression. Thus, we investigated whether IKKα and IKKβ can alter β-catenin protein levels. In addition, we asked whether IKKα and IKKβ...
would affect the protein levels of a β-catenin mutant in which serine residues 33 and 37 were changed to alanine to result in increased β-catenin protein levels (15–17). Expression vectors encoding either the hemagglutinin-tagged wild-type or S33A/S37A mutant β-catenin were transfected into COS cells either alone or in the presence of either the constitutively active FLAG-tagged IKKα or IKKβ. Whole cell extracts were prepared from the transfected COS cells and analyzed by Western blot analysis using the 12CA5 and M2 monoclonal antibodies directed against the hemagglutinin and FLAG epitopes, respectively (Fig. 3B). IKKα expression increased the level of the epitope-tagged β-catenin protein (Fig. 3B, lane 2), while IKKβ reduced the amount of the epitope-tagged β-catenin protein (Fig. 3B, lane 3). In contrast, IKKα did not alter the level of the S33A/S37A β-catenin mutant (Fig. 3B, lane 5), while IKKβ reduced the level of this protein (Fig. 3B, lane 6). Transfection assays with the TOPFLASH reporter indicated that IKKα increased gene expression in the presence of the wild-type but not the mutant β-catenin, while IKKβ reduced gene expression in the presence of both of these β-catenin proteins (data not shown). These results suggest that IKKα either directly or indirectly may lead to increased levels of β-catenin to increase TOPFLASH activity, while IKKβ may reduce the levels of β-catenin to decrease β-catenin activity. The failure of IKKα to further increase the protein levels of the mutant β-catenin suggests that the structure of the amino terminus of β-catenin may be important in this process.

It was important to determine whether activation of the NF-κB pathway may be involved in the increased TOPFLASH activity seen in the presence of β-catenin and LEF-1. The TOPFLASH reporter was transfected with expression vectors encoding β-catenin, LEF-1, and the IκBα super-repressor (IκBα SS/AA) (Fig. 3C). The IκBα super-repressor protein, which contains substitutions of serine residues 32 and 36 with alanine, cannot be phosphorylated by IKK, and its resistance to degradation prevents the nuclear translocation of the NF-κB proteins in response to activators of this pathway (27). The transfection of the IκBα super-repressor did not alter activation of the TOPFLASH reporter in the presence of β-catenin and LEF-1 expression vectors, while it completely abolished the activity of an NF-κB reporter (Fig. 3C). These results suggest that NF-κB activation does not appear to be involved in the activation of TOPFLASH activity by β-catenin and LEF-1.

The Amino Terminus of β-Catenin Is Critical for IKKα but Not IKKβ Modulation of Gene Expression—Next we addressed whether the same or different domains in β-catenin were required for regulation by IKKα and IKKβ. The amino terminus of β-catenin is phosphorylated by GSK-3β leading to β-catenin degradation (50–52). Amino-terminal deletion mutants of β-catenin are very stable because they lack sequences that are involved in APC-mediated degradation (50–54). Furthermore, our results suggested that the amino terminus of β-catenin may be involved in IKKα-mediated regulation. To determine whether the amino terminus of β-catenin was critical for mediating the effects of IKKα and IKKβ, transfection of increasing amounts of a β-catenin expression vector deleted of its first 129 amino acids was transfected into COS cells along with LEF-1. There was increased TOPFLASH activity seen with this mutant similar to the results seen with wild-type β-catenin (Fig. 4). The expression of the constitutively active IKKα protein reduced activation of TOPFLASH reporter when transfected with this β-catenin mutant. In contrast, the expression of the constitutively active IKKα protein did not alter the ability of the amino-terminal deletion of β-catenin to activate the TOPFLASH reporter (Fig. 4). These results suggest that the decreased β-catenin transactivation observed with IKKα is not dependent on the amino terminus of β-catenin, while IKKα requires the presence of this domain to stimulate β-catenin transcriptional activity.
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β-Catenin Interacts with IKKα and IKKβ—To address whether the effects of IKKα and IKKβ on β-catenin-dependent gene expression may be mediated by direct interactions with β-catenin, we performed coimmunoprecipitation experiments of β-catenin and the IKK proteins using cytoplasmic extracts prepared from SW480 colon cancer cell line. SW480 cells express a truncated APC gene product and result in enhanced β-catenin levels. Following the incubation of SW480 cytoplasmic extract with the GST-β-catenin fusion proteins bound to glutathione-Sepharose beads, Western analysis was performed with antibodies directed against either IKKα or IKKβ. Each of the β-catenin fusion proteins, but not GST alone, was able to interact with IKKα and IKKβ (Fig. 6B). However, the GST-β-catenin fusion proteins extending between amino acid residues 1–400 and 130–400 consistently bound more IKKα and IKKβ (Fig. 6B). These results suggested that the region of β-catenin containing the first six armadillo repeats was probably critical for interaction with the IKK proteins. The data from the GST-pull down assays in conjunction with coimmunoprecipitation data of both endogenous and transfected proteins demonstrate that the IKK proteins and β-catenin can interact under a variety of different conditions.

IKKα and IKKβ Phosphorylate β-Catenin—Next we addressed whether IKK could phosphorylate the amino terminus of β-catenin and whether stimulation of IKK activity could result in increased β-catenin phosphorylation in an in vitro kinase assay. The amino terminus of β-catenin has been demonstrated to be a target for GSK-3β phosphorylation (9), while serine residues 32 and 36 in the amino terminus of GSK-IκBα are the target for IKK phosphorylation (29–33). HeLa cells were either untreated, treated with TNFα, or transfected with an expression vector encoding NIK (56, 57) to induce IKK kinase activity. The IKK complex was immunoprecipitated from extracts prepared from these cells and assayed for its fractional activity. The change in gene expression relative to the TOPFLASH vector alone was determined for each transfection and the average of three experiments (each in duplicate) is presented.

To further characterize the interactions of β-catenin with IKKα and IKKβ, in vitro binding of SW480 cytoplasmic extract with GST proteins fused to different domains of β-catenin was performed. To address whether stimulation of IKK activity could result in increased β-catenin phosphorylation in an in vitro kinase assay. The amino terminus of β-catenin has been demonstrated to be a target for GSK-3β phosphorylation (9), while serine residues 32 and 36 in the amino terminus of GSK-IκBα are the target for IKK phosphorylation (29–33). HeLa cells were either untreated, treated with TNFα, or transfected with an expression vector encoding NIK (56, 57) to induce IKK kinase activity. The IKK complex was immunoprecipitated from extracts prepared from these cells and assayed for its fractional activity. The change in gene expression relative to the TOPFLASH vector alone was determined for each transfection and the average of three experiments (each in duplicate) is presented.

Fig. 4. Differential effects of IKKβ and IKKα on transactivation of an amino-terminal truncated β-catenin. COS cells were cotransfected with the indicated plasmids including the TOPFLASH reporter, LIF-1, and either wild-type β-catenin (group 1) or an amino-terminal deletion of the first 129 amino acids of β-catenin (groups 2–4). The construct encoding the amino-terminal-deleted β-catenin was cotransfected at concentrations of 0.5 (group 2), 1.0 (group 3), and 2.0 μg (group 4) together with the constitutively active kinases IKKβ SS/EE (0.5 and 1.0 μg) and IKKα SS/EE (0.5 and 1.0 μg). An RSV-β-galactosidase expression vector was added to each transfection, and DNA quantities were standardized by addition of a pCMV5 expression vector. After 18 h, the cells were collected and luciferase activity was determined and normalized to correct for differences in β-galactosidase activity. The change in gene expression relative to the TOPFLASH reporter alone was determined for each transfection and the average of these three experiments (each in duplicate) is presented.
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ability to phosphorylate either GST-β-catenin-(1–91), GST-IκB-α (1–54), or GST-IκB-α(S9/AA)-1–54. IKK activity was induced by treatment with either TNFα or NIK and increased the phosphorylation of β-catenin (Fig. 7A, lanes 1–3) and IκB-α (Fig. 7A, lanes 4–6), but not the IκB-α mutant in which serine residues 32 and 36 were changed to alanine (Fig. 7A, lanes 7–9).

Recombinant baculovirus-produced IKKα and IKKβ were also tested in in vitro kinase assays using GST fusions with β-catenin or IκB-α. Both IKKα and IKKβ also phosphorylated the amino terminus of β-catenin and IκB-α, but not the IκB-α mutant (Fig. 7B). COS cells were next transfected with either epitope-tagged wild-type or mutant IKKα and IKKβ, and following immunoprecipitation with the M2 monoclonal antibody these kinases were assayed using in vitro kinase assays with β-catenin and IκB-α as substrates (48). Wild-type IKKα and IKKβ, but not the kinase-defective mutants, were able to phosphorylate β-catenin and IκB-α (Fig. 7C).

Finally, we addressed whether IKKα and IKKβ could also phosphorylate additional regions in β-catenin other than its amino terminus (Fig. 7D). Both kinases phosphorylated GST fusion proteins containing various portions of β-catenin (Fig. 7D, lanes 2–5). These GST fusions contained either the amino terminus of β-catenin, an amino-terminal-deleted form of β-catenin or full-length β-catenin (Fig. 7D). Similar results were obtained using IKKα and IKKβ preparations produced by baculovirus expression (data not shown). These results indicate that both IKKα and IKKβ phosphorylate multiple regions of β-catenin.

Stoichiometry of IKK Phosphorylation of IκB-α and β-Catenin—Next we compared the ability of IKKα and IKKβ to phosphorylase GST-IκB-α-(1–54) and GST-β-catenin-(1–91) substrates. In these in vitro kinase assays, we analyzed the phosphorylation of each of these substrates at specific points over a 120-min time course utilizing 0.01 mM, 0.1 mM, and 1.0 mM of cold ATP and 15 μCi of [γ-32P]ATP. Following SDS-PAGE and autoradiography (Figs. 8A and 8B, top panels), the 32P-incorporation into the β-catenin and IκB-α substrates was determined, and the number of the moles of phosphate incorporated per mole of...
substrate was calculated (Fig. 8, A and B, lower panels). Kinase assays performed with IKK\(\beta\) using 1 mM of cold ATP resulted in 0.05 mol of phosphate/mol of protein incorporated into the amino terminus of \(\beta\)-catenin as compared with 0.09 mol of phosphate/mol of protein incorporated into the amino terminus of I\(\kappa\)B \(\alpha\) after a 120-min reaction (Fig. 8A). Kinase assays performed with IKK\(\alpha\) using 1 mM of cold ATP demonstrated that there was 1.4 mol of phosphate/mol of protein incorporated into the amino terminus of \(\beta\)-catenin and 0.5 mol of phosphate/mol of protein incorporated in the amino terminus of I\(\kappa\)B \(\beta\) after 120 min (Fig. 8B). It is interesting to note that the phosphorylation of the \(\beta\)-catenin by IKK\(\beta\) may be biphasic in contrast to its phosphorylation of I\(\kappa\)B \(\alpha\) (Fig. 8B). Similar phosphate incorporation into these substrates was found using both baculovirus-produced and COS-transfected IKK\(\alpha\) and IKK\(\beta\) proteins (data not shown). In agreement with previous studies, this analysis indicates that IKK\(\alpha\) is a much weaker kinase than is IKK\(\beta\) in phosphorylating I\(\kappa\)B \(\alpha\) (58) and \(\beta\)-catenin. These results indicate that the IKK proteins result in relatively similar incorporation of phosphate into the amino terminus of \(\beta\)-catenin and I\(\kappa\)B\(\alpha\), although there are differences in the kinetics of this process.

**DISCUSSION**

In this study, we present data that IKK\(\alpha\) and IKK\(\beta\) can modulate \(\beta\)-catenin function. First, we observed the differential localization of \(\beta\)-catenin in mouse embryo fibroblasts derived from IKK\(\alpha\)- and IKK\(\beta\)-deficient cells. Second, the transcriptional activity of \(\beta\)-catenin was higher in IKK\(\beta\) cells as compared with IKK\(\alpha\) cells. Third, IKK\(\beta\) decreased \(\beta\)-catenin-dependent gene expression similar to the effects seen with GSK-3\(\beta\), while IKK\(\alpha\) increased this activity. Fourth, we found that IKK\(\alpha\) expression in COS cells increased the amount of \(\beta\)-catenin, while IKK\(\beta\) expression reduced the amount of \(\beta\)-catenin. Finally, we demonstrated that IKK\(\alpha\) and IKK\(\beta\) interacted with and were able to phosphorylate \(\beta\)-catenin. Experiments are underway to map the sites in \(\beta\)-catenin that are phosphorylated by IKK\(\alpha\) and IKK\(\beta\) in order to determine whether phosphorylation alters \(\beta\)-catenin function. Our preliminary results suggest that IKK\(\alpha\) phosphorylates different residues in the amino terminus of \(\beta\)-catenin than serine residues 33 and 37 that are phosphorylated by GSK-3\(\beta\).

Studies with an amino-terminal deletion of \(\beta\)-catenin indicated that IKK\(\alpha\) requires this region to increase \(\beta\)-catenin-de-
The effects of IKKβ on β-catenin activity are dependent on the region of β-catenin, which lacks a canonical nuclear localization signal. Although we demonstrate that the IKK proteins interact with β-catenin, it is possible that IKK interaction with other components of the Wnt pathway such as APC may also be involved in regulating β-catenin function.

Both IKKα and IKKβ can form heterodimers and homodimers, and dimerization of these kinases is essential for their activity. However, previous data has suggested that there is no synergy between IKKα and IKKβ in regulating their kinase activity. Given the wide disparity in their kinase activity, they may have other cellular targets in addition to IkB (48). The ability of these kinases to potentially associate with as yet unidentified cellular factors may alter their substrate specificity. Gene disruption studies indicate that IKKβ rather than IKKα is the critical kinase involved in the activation of the NF-κB pathway in response to treatment with either TNFα or IL-1β (37–39). The predominant cytoplasmic localization of IKKβ probably reflects the major role of this kinase in the phosphorylation of the IkB proteins that are localized in the cytoplasm bound to the RelA/p65 NF-κB protein (37–39). The results of our immunofluorescence studies

![Diagram](image-url)
FIG. 8. Stoichiometry of phosphate incorporation into the amino termini of IκBα and β-catenin by IKKα and IKKβ. A, FLAG-tagged IKKα and B, IKKβ were immunoprecipitated from COS extracts with the M2 monoclonal antibody and incubated with 2 μg of GST fusions containing either β-catenin-(1–91) or IκBα-(1–54) in kinase buffer containing 15 μCi of [γ-32P]ATP with a specific activity of 6000 Ci/mM and cold ATP at concentrations of 1 mM, 0.1 mM, and 0.01 mM. In vitro kinase reactions were performed for 0, 5, 15, 30, 60, and 120 min at 30 °C, and the samples were subjected to SDS-PAGE and autoradiography (A and B, top panels). Incorporation of 32P into these substrates was quantitated by scintillation counting and the moles of phosphate incorporated per mole of substrate was calculated (A and B, bottom panels).
suggest that IKKα is localized in both the nucleus and cytoplasm of MEFs and may be predominantly nuclear in the absence of IKKβ in IKKβ−/− cells. Consistent with these observations, Western blot analysis of extracts prepared from COS cells transfected with expression vectors encoding IKKα and IKKβ indicate that IKKβ is predominantly localized in the cytoplasm, while IKKα is present in both the nucleus and the cytoplasm. Additional studies are currently underway to better characterize the cellular localization of IKKα. Whether any of the effects of IKKα on skin and skeletal development may in part be mediated by either IKKα binding and/or phosphorylation of β-catenin remains to be determined.

IKK regulation of β-catenin activity differs from its activation of the NF-κB pathway. Cytokines such as TNFα stimulate IKK phosphorylation of IκB leading to its rapid degradation and the nuclear translocation of NF-κB. TNFα activation of an NF-κB reporter construct is blocked by transfection of an IKK dominant negative mutant. These results suggest that the effects of TNFα on β-catenin-dependent gene expression likely involve additional substrates and/or pathways other than IKKβ and β-catenin. Although our results support a role for IKKα and IKKβ on modulating β-catenin activity, the regulation of this pathway is different from that seen with TNFα-induction of IKK to activate the NF-κB pathway.

Several observations are also consistent with the potential for similar factors being involved in the regulation of the Wnt and NF-κB pathways. It has been demonstrated that β-catenin/TCP signaling increases β-TrCP levels by a posttranscriptional mechanism to result in increased degradation of both β-catenin and IκB (45). Thus, changes of β- TrCP levels can result in marked changes on both the β-catenin and NF-κB pathways. Additionally, GSK-3β, which is an important kinase involved in regulating β-catenin levels, has also been implicated in regulating NF-κB activation. Gene disruption studies have indicated that GSK-3β−/− mice have a phenotype similar to IKKβ−/− mice. Whether any of these factors being involved in the regulation of the Wnt and NF-κB pathways may alter the NF-κB pathway. Cytokines such as TNFα may alter the NF-κB pathway. It has been demonstrated that GSK-3β−/− mice have a phenotype similar to IKKβ−/− mice. Whether any of these factors being involved in the regulation of the Wnt and NF-κB pathways may alter the NF-κB pathway. Cytokines such as TNFα may alter the NF-κB pathway. It has been demonstrated that GSK-3β−/− mice have a phenotype similar to IKKβ−/− mice. Whether any of these factors being involved in the regulation of the Wnt and NF-κB pathways may alter the NF-κB pathway.
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