MOLECULAR AND CELLULAR BIOLOGY, Sept. 2002, p. 6393–6405 Vol. 22, No. 18
0270-7306/02/$04.00+0 DOI: 10.1128/MCB.22.18.6393–6405.2002
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# I-mfa Domain Proteins Interact with Axin and Affect Its Regulation of the Wnt and c-Jun N-Terminal Kinase Signaling Pathways

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Received 29 April 2002/Returned for modification 23 May 2002/Accepted 12 June 2002

**I-mfa has been identified as an inhibitor of myogenic basic helix-loop-helix transcription factors, and a related human I-mfa domain-containing protein (HIC) also has been identified as a protein that regulates Tat- and Tax-mediated expression of viral promoters. HIC and I-mfa represent a family of proteins that share a highly conserved cysteine-rich domain, termed the I-mfa domain. We show here that both I-mfa domain proteins, HIC and I-mfa, interacted in vivo with the Axin complex through their C-terminal I-mfa domains. This interaction inhibited Axin-mediated downregulation of free levels of cytosolic β-catenin. I-mfa and HIC also both directly interacted with lymphocyte enhancer factor (LEF); however, I-mfa but not HIC significantly inhibited reporter constructs regulated by β-catenin. The overexpression of HIC but not I-mfa decreased the inhibitory effects of Axin on β-catenin-regulated reporter constructs, while both HIC and I-mfa decreased Axin-mediated c-Jun N-terminal kinase (JNK) activation. These data reveal for the first time that I-mfa domain proteins interact with the Axin complex and affect Axin regulation of both the Wnt and the JNK activation pathways. Interestingly, HIC differs from I-mfa in that I-mfa affects both Axin function and T-cell factor- or LEF-regulated transcription in the Wnt signaling pathway while HIC affects primarily Axin function.**

The human I-mfa domain-containing protein (HIC) has been identified as a protein that differentially regulates Tat- and Tax-mediated expression of viral promoters: enhancing expression from the human T-cell leukemia virus type 1 long terminal repeat and repressing expression from the human immunodeficiency virus type 1 long terminal repeat (30). HIC contains a cysteine-rich C-terminal domain with a high degree of homology to the C-terminal domain of I-mfa, termed an I-mfa domain (30), and the regulatory properties of HIC depend on this C-terminal domain. The I-mfa domain is also required for I-mfa inhibition of MyoD and other related myogenic basic helix-loop-helix (bHLH) proteins by preventing nuclear localization and DNA binding (6, 30). This similarity suggests that HIC and I-mfa may have similar protein interactions and properties (30). Indeed, I-mfa and XIC, the *Xenopus* orthologue of HIC, were both found to bind to the HMG box transcription factor Xtcf3 and to inhibit its DNA binding activity (29). Thus, HIC and I-mfa likely represent a family of proteins that share the I-mfa domain and regulate both bHLH-dependent myogenic and Wnt signaling pathways.

The stabilization of β-catenin is a key regulatory step during development and tumorigenesis. Several proteins, including glycogen synthase kinase 3 (GSK-3), the adenomatous polyposis coli gene product (APC), disheveled (Dvl), and Axin, are known to affect free β-catenin protein levels within the cell (10, 11, 13, 14, 17, 22, 25). In the Wnt pathway, the Wnt ligand protein binds the seven-membrane-spanning receptor protein, frizzled, which then inhibits GSK-3-dependent phosphorylation of β-catenin, resulting in the stabilization of β-catenin (5, 25). The stable β-catenin is transported to the nucleus, where it interacts with transcription factors, including T-cell factor (TCF) and lymphocyte enhancer factor (LEF) 1 (LEF-1), and stimulates the expression of many Wnt target genes (4, 5). It is unclear how Wnt signaling prevents β-catenin phosphorylation by GSK-3, although Dvl is implicated in this process (16). Several mechanisms have been suggested, including a direct inhibition of GSK-3 activity, inhibition of GSK-3 through the interaction with GBP/Frat1, and dephosphorylation and destabilization of Axin (19, 31). The scaffolding protein, Axin, forms a complex with GSK-3, β-catenin, APC, and Dvl through distinct binding sites (14, 22, 25). Within this complex, GSK-3 efficiently phosphorylates β-catenin, and this phosphorylation leads to the ubiquitination and proteasome-dependent degradation of β-catenin (9–11, 13). Thus, Axin negatively regulates the Wnt signaling pathway (14, 15).

In addition to its role in Wnt signaling, the overexpression of Axin also stimulates the c-Jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK) signaling pathways (35). This effect may require Axin homodimer formation and the direct physical interaction of Axin with MEKK1 (34). GSK-3β inhibits Axin-mediated JNK activation, and this inhibition does not require GSK-3β kinase activity. It has been suggested that GSK-3β binding to Axin affects the Axin conformation, resulting in a block in MEKK binding (36).

In this report, we show that the I-mfa domain proteins HIC and I-mfa interact in vivo with the Axin complex through their I-mfa domains. This interaction requires the Axin GSK-3 binding site and results in increased levels of free β-catenin. I-mfa and HIC also directly bind to the human LEF-1 protein. I-mfa and Axin both block the activation of TCF-mediated transcription by β-catenin, while HIC only slightly affects the activation of TCF by β-catenin but does partially reverse the inhibition by Axin. In addition, both I-mfa and HIC decrease Axin-induced

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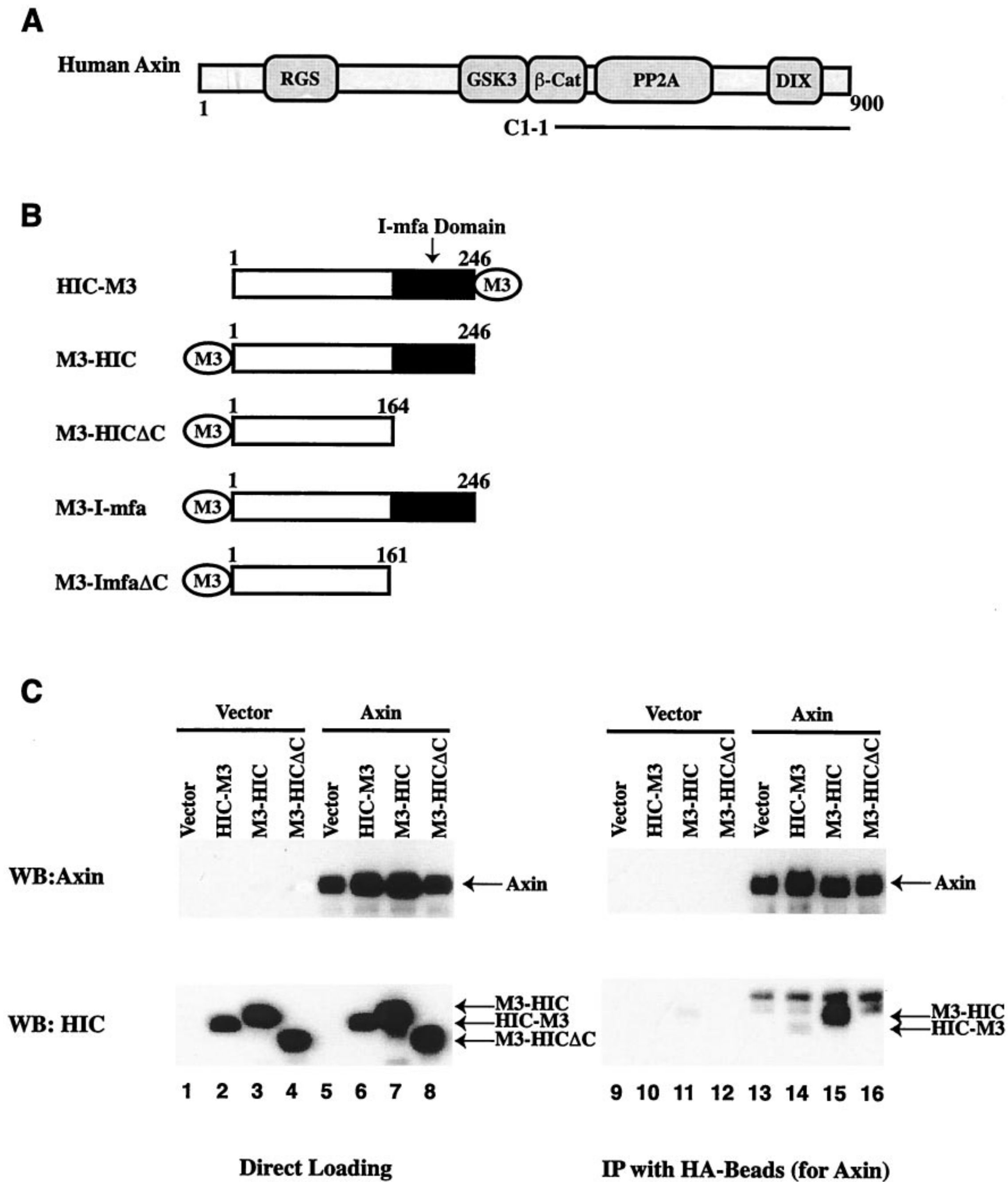



FIG. 1. HIC interacts with Axin in vivo. (A) Schematic representation of the Axin clone obtained from the yeast two-hybrid screening. Clone C1-1, containing the middle of the β-catenin binding site and encompassing the C-terminal portion of human Axin, interacted with the I-mfa domain of HIC in a yeast two-hybrid system. The structural motifs of Axin are indicated as follows: RGS, the RGS domain; GSK3, GSK-3α/β binding region; β-Cat, β-catenin binding region; PP2A, PP2A binding region; DIX, the DIX domain. (B) Schematic representation of the HIC and I-mfa expression constructs with the three myc epitope tags at either the amino or the carboxy terminus and the deletion mutants lacking the carboxy-terminal I-mfa domain. (C) Coimmunoprecipitation of HIC and Axin in vivo. COS-1 cells were transiently transfected with 0.5 μg of plasmid encoding HA-Axin and 0.5 μg of plasmid encoding HIC-M3, M3-HIC, or M3-HICΔC. Immunoprecipitation (IP) was performed with HA antibody-conjugated beads. (Left panel) Immunoblot analysis of total cell lysates with HA antibody to identify Axin or with anti-myc antibody to identify HIC-M3, M3-HIC, or M3-HICΔC. (Right panel) Immunoblot analysis of immunoprecipitated complexes with HA antibody to identify Axin or with anti-myc antibody to identify HIC-M3, M3-HIC, or M3-HICΔC. WB, Western blot. IP, immunoprecipitation.

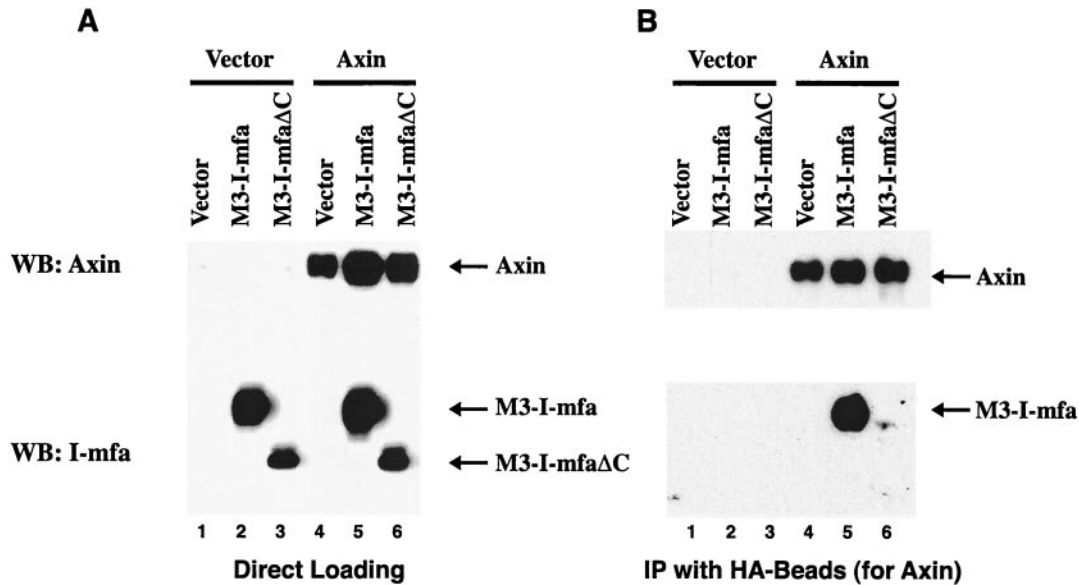



FIG. 2. I-mfa binds to Axin in vivo. COS-1 cells were transiently transfected with 0.5 μg of plasmid encoding HA-Axin and 0.5 μg of plasmid encoding M3-I-mfa or M3-I-mfaΔC. This step was followed by immunoprecipitation (IP) for Axin with HA antibody-conjugated beads. (A) Immunoblot analysis of total cell lysates with HA antibody to identify Axin or with anti-myc antibody to identify M3-I-mfa or M3-I-mfaΔC. (B) Immunoblot analysis of immunoprecipitated complexes with HA antibody to identify Axin or with anti-myc antibody to identify M3-I-mfa or M3-I-mfaΔC.

JNK activation. These findings indicate that I-mfa domain proteins affect Axin regulation of both the Wnt and the JNK signaling pathways and that HIC and I-mfa may differentially affect these pathways.

## MATERIALS AND METHODS

**Materials.** Antibody to β-catenin was purchased from Transduction Laboratory, antibodies to GSK-3α and GSK-3β were purchased from StressGen, and antibodies to myc (A-14), hemagglutinin (HA) (Y-11), JNK1 (FL), and phosphorylated JNK (G-7) were purchased from Santa Cruz Biotechnology. Western blotting analysis was performed with Super Signal West Pico chemiluminescent substrate (Pierce). myc epitope-tagged *Xenopus* β-catenin, myc epitope-tagged *Xenopus* GSK-3β (Xgsk3β), and HA epitope-tagged human LEF-1 plasmids were kindly provided by R. Moon (Howard Hughes Medical Institutes, University of Washington). The I-mfa cDNA was a gift from S. Tapscott (Fred Hutchinson Cancer Research Center, University of Washington), and the TOP-flash and FOP-flash reporter plasmids were obtained from Upstate Biotechnology. PCR amplification was performed with Platinum *Pfx* DNA polymerase (Life Technologies, Inc.) by using synthetic oligonucleotides synthesized at the Nucleic Acids Core Facility of the University of North Carolina, Chapel Hill. The amplified human lung cDNA library used for yeast two-hybrid screening was generously provided by S. Milgram (University of North Carolina, Chapel Hill). All of the DNA constructs were sequenced at the Automated DNA Sequencing Facility of the University of North Carolina, Chapel Hill.

**Construction of expression plasmids.** HIC cDNA fragments were amplified by PCR from a human lung cDNA library and cloned into pcDNA3 with three myc epitopes at either the N terminus or the C terminus (Invitrogen). A deletion mutant that lacked the C-terminal 82 amino acids representing the I-mfa domain of HIC was constructed by *Kpn*I-*Eco*RV digestion of the N-terminally tagged HIC plasmid and cloned into *Kpn*I-*Eco*RV-digested pcDNA3. The I-mfa domain of the HIC cDNA fragment was amplified by PCR from the HIC plasmid and cloned into pAS2-1 (Clontech) for expressing an in-frame fusion protein with a GAL4 DNA binding domain. An N-terminal myc epitope-tagged form of I-mfa was amplified by PCR from the I-mfa plasmid and cloned into pcDNA3. The I-mfa domain deletion form was amplified by PCR from the N-terminal myc epitope-tagged I-mfa cDNA and cloned into pcDNA3. N-terminal HA epitope-tagged Axin (HA-Axin) cDNA was amplified by PCR from the human lung cDNA library and cloned into pcDNA3.1+/Zeo (Invitrogen). A set of C-terminally truncated HA-Axin mutant cDNAs were amplified by PCR from the Axin cDNA plasmid and cloned into pcDNA3.1+/Zeo.

To express Axin and I-mfa domain proteins under inducible conditions for the E-cadherin and free catenin assay, HA-Axin and myc-tagged I-mfa domain proteins were cloned into pMEP4 (Invitrogen). Protein expression was induced by 2.5 μM CdCl2 for 12 h.

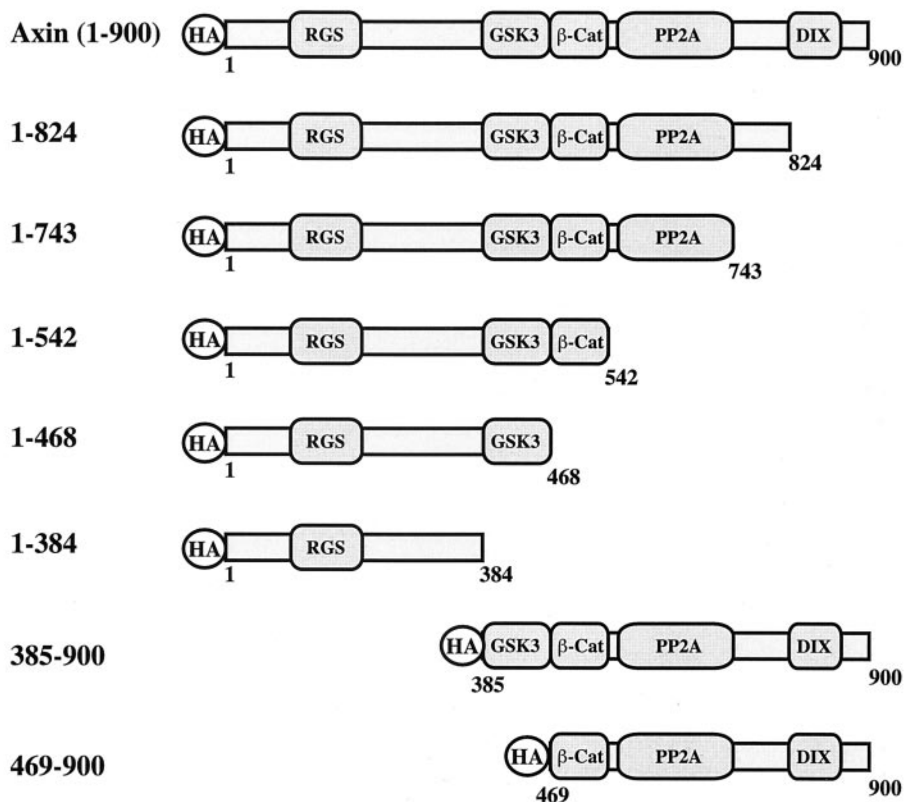
To express a glutathione *S*-transferase (GST)–E-cadherin fusion protein in *Escherichia coli*, the β-catenin binding region of human E-cadherin cDNA was amplified by PCR from the human lung cDNA library and cloned into pGEX-2TK (Amersham Pharmacia Biotech) as described previously (2). To express a GST–I-mfa domain of HIC, the I-mfa domain of HIC was amplified by PCR from the HIC cDNA and cloned into pGEX-3X.

**Yeast two-hybrid screening.** Yeast two-hybrid screening was performed as previously described (18) by using the I-mfa domain of HIC (amino acids 165 to 246) as bait for human lung cDNA library screening.

**Cell culturing and transfection.** COS-1 and HEK-293 cells were maintained in Dulbecco's modified Eagle's medium containing penicillin and streptomycin and supplemented with 10% fetal bovine serum (Gibco BRL). COS-1 or HEK-293 cells at $1 \times 10^6$ (for immunoprecipitation or phosphorylated JNK analysis) or $5 \times 10^5$ (for luciferase assay) were plated on 60-mm cell culture plates. Various amounts of DNA were transfected by using Fugene 6 (Roche Molecular Biochemicals) as suggested by the manufacturer.

**Immunoprecipitation and Western blotting.** At 48 h posttransfection, COS-1 cells were harvested and lysed in 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) lysis buffer containing a protease inhibitor cocktail (Sigma). Protein concentrations were determined as previously described (18). Lysates containing equal amounts of proteins were precleared with protein A-Sepharose beads (Amersham Pharmacia Biochemical) for 1 h at 4°C and immunoprecipitated with anti-HA affinity matrix (Roche Molecular Biochemical) for HA epitope-tagged proteins or anti-myc antibody 9E10-prebound protein A-Sepharose beads for myc epitope-tagged proteins. After 8 h at 4°C, immune complexes were collected, washed three times in CHAPS lysis buffer, and eluted in Laemmli sodium dodecyl sulfate (SDS) sample buffer. Proteins were separated on SDS-polyacrylamide gels and then transferred to Immobilon-P (Millipore). Western blotting was carried out with various antibodies as previously described (18).

**In vitro translation and GST binding.** GST or GST–I-mfa domain proteins were expressed in DH5α induced with 0.2 mM isopropyl-β-D-galactopyranoside (IPTG) at room temperature. The cells were pelleted and lysed by sonication in phosphate-buffered saline (PBS) containing 1% Triton X-100, 1 mM phenyl-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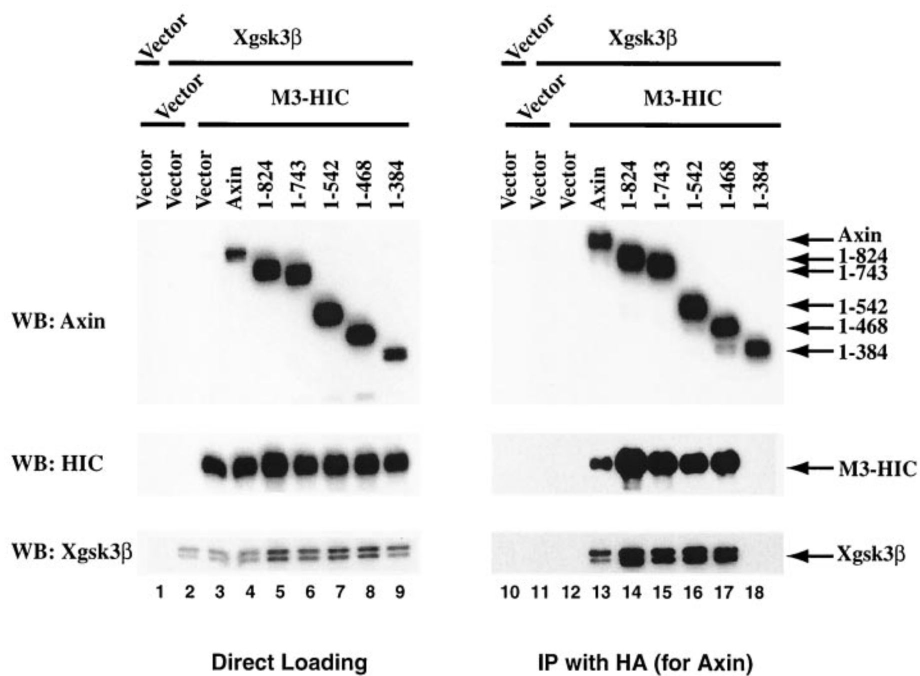
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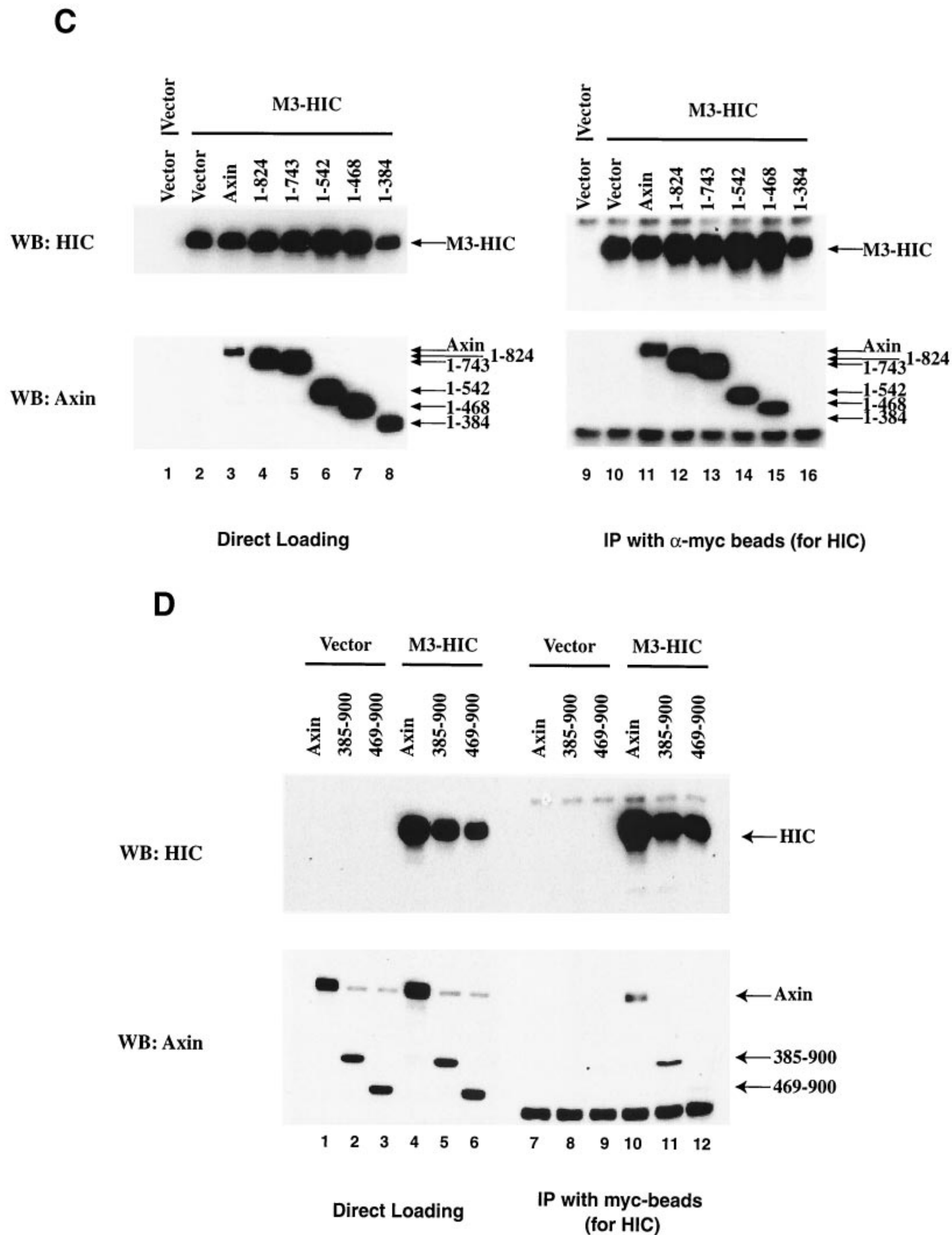


FIG. 3. The interaction of HIC with Axin requires the GSK-3 binding domain of Axin. (A) Schematic representation of the truncated Axin mutants. Structural motifs of Axin are indicated as described in the legend to Fig. 1. (B) Coimmunoprecipitation of HIC and C-terminally truncated Axin mutants in vivo. COS-1 cells were transiently transfected with 0.03 μg of plasmid encoding Xgsk3β, 1 μg of plasmid encoding M3-HIC, and 0.6 μg of plasmid encoding each of the HA epitope-tagged C-terminally truncated Axin mutants. Immunoprecipitation (IP) was carried out with HA antibody-conjugated beads. (Left panel) Immunoblot analysis of total cell lysates with anti-myc antibody to identify Xgsk3β and M3-HIC or with HA antibody to identify the C-terminally truncated Axin mutants. (Right panel) Immunoblot analysis of immunoprecipitated Axin complexes with anti-myc antibody to identify Xgsk3β and M3-HIC or with HA antibody to identify the C-terminally truncated Axin mutants. WB, Western blot. (C) Reciprocal coimmunoprecipitation of HIC and C-terminally truncated Axin mutants in vivo. COS-1 cells were transiently transfected with 0.4 μg of plasmid encoding M3-HIC and 1 μg of plasmid encoding each of the HA epitope-tagged C-terminally truncated Axin mutants. Immunoprecipitation was carried out with myc antibody-prebound protein A-Sepharose. (Left panel) Immunoblot analysis of total cell lysates with anti-myc antibody to identify M3-HIC or with HA antibody to detect the C-terminally truncated Axin mutants. (Right panel) Immunoblot analysis of immunoprecipitated HIC-containing complexes with anti-myc antibody to identify M3-HIC or with HA antibody to identify the C-terminally truncated Axin mutants. (D) Coimmunoprecipitation of HIC and N-terminally truncated Axin mutants in vivo. COS-1 cells were transiently transfected with 0.5 μg of plasmid encoding M3-HIC and 1 μg of plasmid encoding each of the HA epitope-tagged N-terminally truncated Axin mutants. Immunoprecipitation was carried out with myc antibody-prebound protein A-Sepharose. (Left panel) Immunoblot analysis of total cell lysates with anti-myc antibody to identify M3-HIC or with HA antibody to identify the N-terminally truncated Axin mutants. (Right panel) Immunoblot analysis of immunoprecipitated HIC-containing complexes with anti-myc antibody to identify M3-HIC or with HA antibody to identify the N-terminally truncated Axin mutants.

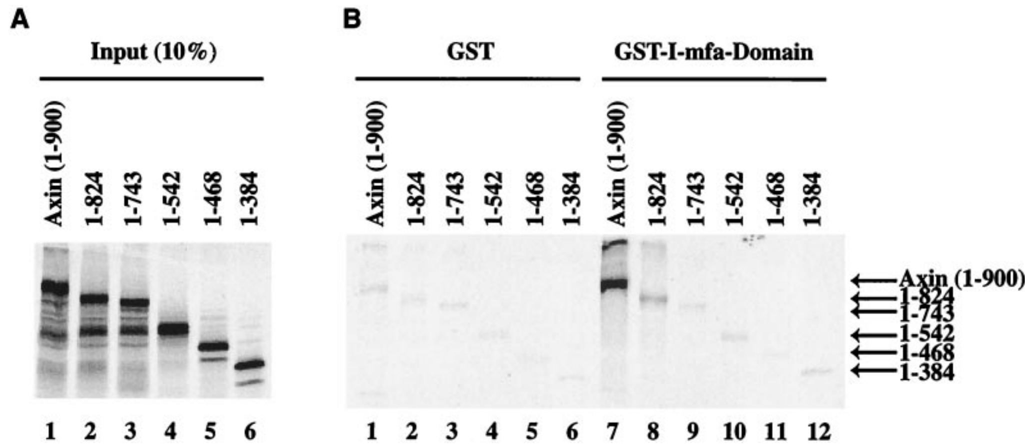


FIG. 4. The direct interaction of in vitro-translated Axin with a GST–I-mfa fusion protein requires C-terminal amino acids. Plasmids (1 μg each) encoding Axin and mutants were transcribed and translated in vitro in the presence of [$^{35}$S]methionine. (A) In vitro-translated products (3 μl) were subjected to SDS-PAGE. The gel was dried and exposed to film. (B) In vitro-translated products (20 μl) were diluted in 330 μl of PBS containing 1% Triton X-100 and incubated with GST- or GST–I-mfa-bound beads for 2 h at 4°C. The beads were washed three times with PBS containing Triton X-100. The proteins were eluted from the beads into SDS sample buffer and subjected to SDS-PAGE.

methylsulfonyl fluoride, and 5 μg of aprotinin/μl on ice. Bacterial lysates were clarified by centrifugation, and the GST or GST–I-mfa domain proteins were purified by binding to glutathione-Sepharose beads (Amersham Pharmacia Biotech) for 1 h at 4°C. The beads were washed three times and then resuspended in PBS containing 1% Triton X-100. Axin and C-terminally truncated Axin mutants were transcribed and translated in vitro by using a TNT T7 coupled reticulocyte lysate system (Promega) as recommended by the manufacturer. The in vitro-translated products were diluted in PBS containing 1% Triton X-100 and mixed with GST or GST–I-mfa. After incubation for 2 h at 4°C, the beads were collected by centrifugation and washed three times. Proteins were eluted in Laemmli SDS sample buffer, subjected to SDS-polyacrylamide gel electrophoresis (PAGE), and analyzed by using a phosphorimager.

**GST–E-cadherin pull-down assay.** GST or GST–E-cadherin protein was expressed in DH5α induced with 0.5 mM IPTG at 37°C. Cells were pelleted, resuspended in ice-cold PBS, and lysed by sonication in E-cadherin lysis buffer (10 mM sodium phosphate [pH 7.4], 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 5 μg of aprotinin/ml) on ice. Bacterial lysates were clarified by centrifugation, and GST or GST–E-cadherin was purified by binding to glutathione-Sepharose beads for 1 h at 4°C. The beads were washed three times and resuspended in E-cadherin lysis buffer. At 48 h posttransfection, COS-1 cells were harvested and lysed in E-cadherin lysis buffer containing a protease inhibitor cocktail for 30 min at 4°C. Protein concentrations in lysates were quantitated. Protein lysates (100 μg) were precleared with GST-bound Sepharose beads for 45 min at 4°C and incubated with Sepharose beads bound to GST–E-cadherin. After 1 h of incubation at 4°C, the beads were collected and washed three times by centrifugation. Proteins were eluted in Laemmli SDS sample buffer, separated on SDS-polyacrylamide gels, and then transferred to Immobilon-P. Western blotting was carried out with various antibodies as previously described (18).

**Luciferase assay.** At 72 h posttransfection, COS-1 cells were harvested and lysed in Glo lysis buffer (Promega). Protein concentrations in lysates were determined. Luciferase activities in equal volumes of lysates were analyzed as suggested by the manufacturer. Luminescence intensities were normalized against protein concentrations.

**Western blotting of active JNK.** At 48 h posttransfection, HEK-293 cells were harvested and lysed in radioimmunoprecipitation buffer containing a protease inhibitor cocktail and phosphatase inhibitor cocktail I (Sigma). Protein concentrations of lysates were determined as previously described (18). Equal amounts of proteins were subjected to SDS-PAGE and then transferred to Immobilon-P. Levels of activated JNK were analyzed with the phosphorylated JNK antibody G-7. Total JNK levels were analyzed by using the same membrane and a JNK1 antibody (FL) following stripping of the phosphorylated JNK antibody.

## RESULTS

**HIC interacts with Axin through its I-mfa domain.** I-mfa was identified as a protein that interacts with and inhibits the activity of MyoD and related myogenic bHLH proteins through its C-terminal I-mfa domain (6). Although HIC also regulates transcription, it does not directly interact with myogenin or MyoD (unpublished data). To identify a cellular target protein of HIC, a human lung cDNA library was screened by using the yeast two-hybrid method with the I-mfa domain of HIC (amino acids 165 to 246) as bait. Approximately $6 \times 10^5$ transformants were tested for growth on selection medium and β-galactosidase induction. Sequence analysis of a positive clone revealed that the cDNA represented the C-terminal portion of human Axin (amino acids 505 to 900) (33). This clone contained part of the β-catenin binding site and spanned the C terminus of human Axin (12) (Fig. 1A).

To test the interaction between the I-mfa domain of HIC and Axin in vivo, HA-Axin and N-terminal myc epitope-tagged HIC (M3-HIC), N-terminal myc epitope-tagged truncated HIC with a deletion of the I-mfa domain (M3-HICΔC), or C-terminal myc epitope-tagged HIC (HIC-M3) were coexpressed in COS-1 cells (Fig. 1B). Immunoblotting of the total lysates indicated equivalent expression of all constructs (Fig. 1C). However, immunoprecipitation with HA antibody-conjugated beads to precipitate Axin revealed that M3-HIC was efficiently coimmunoprecipitated with HA-Axin (Fig. 1C, lane 15) and that deletion mutant M3-HICΔC was not coimmunoprecipitated (Fig. 1C, lane 16). The carboxy-terminal tagged form, HIC-M3, was less efficiently precipitated, possibly due to the effects of the C-terminal epitope tag on the protein-protein interaction between Axin and the C-terminal I-mfa domain of HIC. Immunoprecipitation of M3-HIC with myc antibody-conjugated beads also coprecipitated HA-Axin, while that of M3-HICΔC did not (data not shown). These observations revealed that HIC binds to Axin in vivo and that the I-mfa domain of HIC is necessary for this interaction.

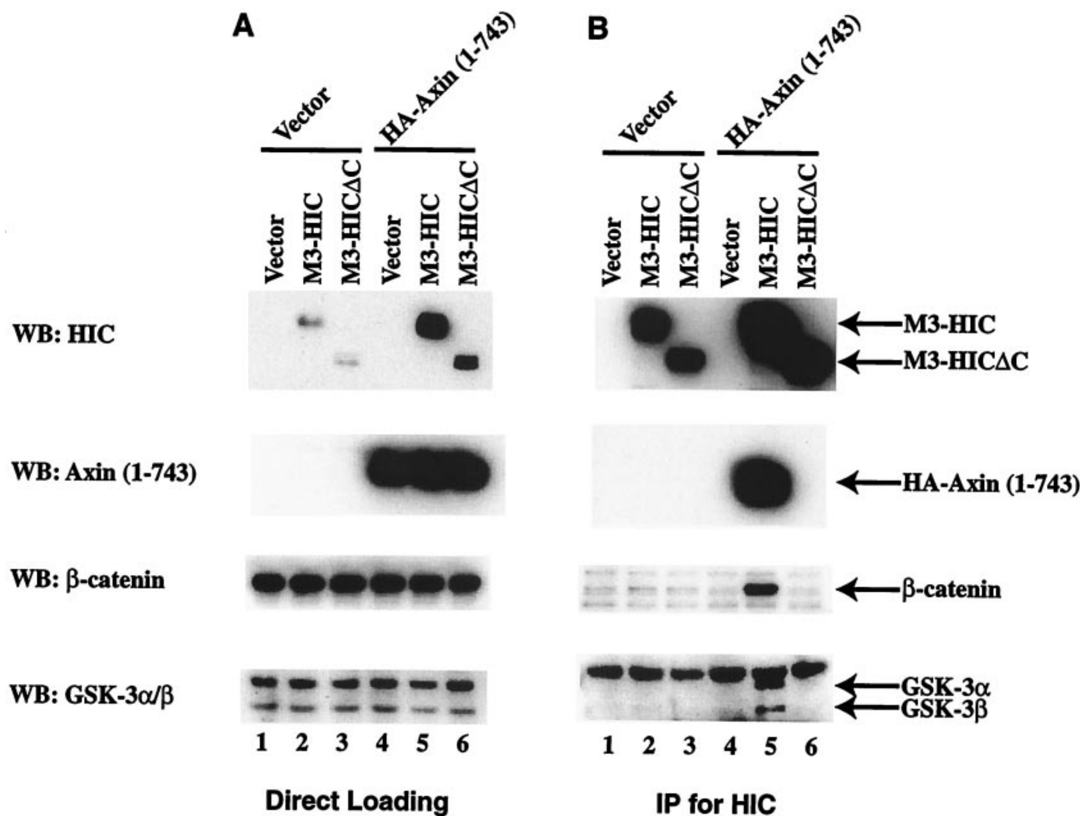


FIG. 5. C-terminally truncated Axin complexes interact with HIC in vivo and contain GSK-3 and β-catenin. COS-1 cells were transiently transfected with 1 μg of plasmid encoding the 1–724 Axin mutant and 1 μg of plasmid encoding M3-HIC or MC-HICΔC. (A) Immunoblot analysis of total cell lysates with HA antibody to identify the 1–724 Axin mutant, with anti-myc antibody to identify M3-HIC and MC-HICΔC, with β-catenin antibody to identify endogenous β-catenin, or with GSK-3 antibody to identify endogenous GSK-3α and GSK-3β. (B) Immunoblot analysis of precipitated material to identify Axin, β-catenin, GSK-3α, and GSK-3β in the HIC-containing complexes.

**I-mfa also binds to Axin.** As the I-mfa domains of HIC and I-mfa are highly homologous, with 77% identity and 81% similarity (30), the possible interaction between I-mfa and Axin was tested in vivo. I-mfa with three myc epitope tags at the amino terminus (M3-I-mfa) or I-mfa with a deletion of the I-mfa domain (M3-I-mfaΔC) was coexpressed with HA-Axin in COS-1 cells. Immunoprecipitation analysis with HA antibody-conjugated beads revealed that M3-I-mfa but not M3-I-mfaΔC was efficiently coimmunoprecipitated with HA-Axin (Fig. 2B, lanes 5 and 6). This result indicated that I-mfa also interacts with Axin in vivo through its I-mfa domain.

**Axin and I-mfa domain proteins interact through the GSK-3 binding domain in vivo.** Axin is known to a make a multiprotein complex with APC bound to the N-terminal RGS domain and GSK-3, β-catenin, and protein phosphatase 2A (PP2A) binding to the central part of Axin. The C-terminal DIX domain is responsible for binding to Dvl and enabling homodimer formation (28) (Fig. 3A).

C-terminally or N-terminally truncated mutants of human Axin were synthesized and tested for binding to HIC in COS-1 cells. Although all of the proteins were efficiently expressed, immunoblot analysis of immunoprecipitated full-length or C-terminally truncated mutants of Axin complexes indicated that the the Axin mutant with a deletion of amino acids 1 to 384 (1–384 Axin mutant) was not coprecipitated with HIC (Fig. 3B, lane 18), although full-length Axin and other C-terminally truncated Axin mutants were coprecipitated with HIC (Fig. 3B, lanes 13 to 17). These results suggested that amino acids 385 to 468 of Axin, which are thought to represent the GSK-3 binding domain, are required for the interaction with HIC. To confirm that amino acids 385 to 468 of Axin represent the GSK-3 binding domain, the Axin mutants were tested for interactions by coprecipitation with Xgsk3β. Similar to the interaction with HIC, the 1–384 Axin mutant was not coprecipitated with Xgsk3β (Fig. 3B, lane 18), while full-length Axin and other C-terminally truncated mutants of Axin efficiently interacted (Fig. 3B, lanes 13 to 17). These results indicated that amino acids 385 to 468 of Axin are required for the interaction between the Axin complex and HIC and for the interaction between Axin and GSK-3. The interaction between I-mfa and Axin mutants was also tested. Similar to the results obtained with HIC, I-mfa did not bind to the 1–384 Axin mutant but did bind to full-length Axin and other C-terminally truncated Axin mutants (data not shown). These observations indicated that the interaction of I-mfa domain proteins with the Axin complex requires the GSK-3 binding-site of Axin.

The interaction between HIC and C-terminally truncated mutants of Axin was confirmed in a reciprocal immunoprecipitation with myc antibody-prebound protein A-Sepharose beads to precipitate HIC. Immunoblot analysis of immunoprecipi-

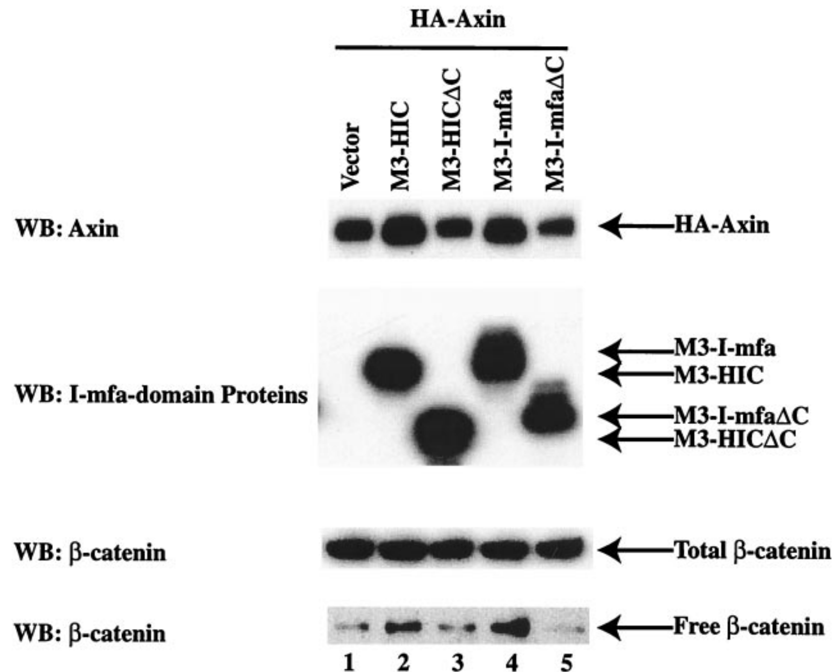


FIG. 6. I-mfa domain proteins HIC and I-mfa increase the free levels of β-catenin in the presence of Axin. COS-1 cells were transiently transfected with 1.0 μg of plasmid pMep4 encoding HA-Axin and 1.0 μg of plasmid encoding M3-HIC, M3-HICΔC, M3-I-mfa, or M3-I-mfaΔC. Cytosolic free β-catenin was precipitated from cytosolic cell lysates with a GST–E-cadherin fusion protein. Immunoblot analysis of 20 μg of cytosolic cell lysates was done with HA antibody to identify Axin, with anti-myc antibody to identify HIC and I-mfa, or with β-catenin antibody to identify the total levels of β-catenin and free β-catenin bound to the GST–E-cadherin fusion protein.

tated HIC-containing complexes confirmed that the 1–384 Axin mutant was not coprecipitated with HIC (Fig. 3C, lane 16), while full-length Axin and other C-terminally truncated Axin mutants were efficiently coprecipitated (Fig. 3C, lanes 11 to 15).

Although amino acids 385 to 468 of Axin were clearly required for the interactions of HIC and I-mfa with Axin in vivo, these sequences were not contained in the cDNA clone, C1-1, which was isolated from the yeast two-hybrid screening (Fig. 1A). This result suggested that the C terminus of Axin may contain an additional site for interactions with HIC. Therefore, N-terminally truncated mutants of Axin were also tested for interactions with HIC. Immunoblot analysis of immunoprecipitated HIC-containing complexes revealed that full-length Axin and the 385–900 Axin mutant were efficiently coprecipitated with HIC (Fig. 3D, lanes 10 and 11), while the 469–900 Axin mutant was very faintly detected (Fig. 3D, lane 12). These results confirmed that amino acids 385 to 468 of Axin not only represent the GSK-3 binding site but also are required for significant interactions between the Axin complex and I-mfa domains in vivo. The weak interaction of the 469–900 Axin mutant with HIC suggested a possible secondary binding site. This region of Axin was contained within the clone identified in the yeast two-hybrid screening.

**Direct interaction between HIC and Axin.** The Axin complex contains binding sites for multiple proteins, and the binding of some proteins has been suggested to affect the Axin conformation. To determine if there was a direct interaction between Axin and the I-mfa domain of HIC, the I-mfa domain of HIC was fused to GST, and Axin and the C-terminally truncated forms of Axin (Fig. 3A) were translated in vitro. Axin and the deletion mutants were translated with equivalent efficiencies and bound at trace levels to GST (Fig. 4). The GST–I-mfa domain fusion protein bound approximately 20% of the total input of full-length Axin (amino acids 1 to 900), while the 1–824 Axin mutant showed some residual binding. The 1–724 Axin mutant and other C-terminally truncated forms bound GST–I-mfa at levels equivalent to the level of nonspecific binding to GST. These results confirmed that, in vitro, Axin directly interacts with the I-mfa domain through the C-terminal 157 amino acids of Axin, sequences that were present in the clone isolated from the yeast two-hybrid screening.

As immunoprecipitation of full-length HIC did not immunoprecipitate C-terminally truncated mutants of Axin that did not interact in vitro with the I-mfa domain (Fig. 3C), the interactions of HIC and HIC lacking the I-mfa domain (HICΔC) with the 1–724 Axin mutant, which lacks the I-mfa interaction domain identified in vitro, were tested in vivo by immunoprecipitation (Fig. 5). The 1–724 Axin mutant could interact only with full-length HIC and not with HICΔC. These data confirmed that, in vivo, HIC can form a complex with Axin that lacks the sequences that directly interact with the HIC I-mfa domain in vitro and that this interaction requires the HIC I-mfa domain. These data suggested that the conformation of the Axin complex in vivo, when it is bound to additional proteins, enables the binding of the HIC I-mfa domain to the Axin complex and that this binding requires the GSK-3 binding site. Identification of other proteins in the HIC-Axin complex revealed that endogenously expressed GSK-3α, GSK-3β, and β-catenin were also present in the immunoprecipitated

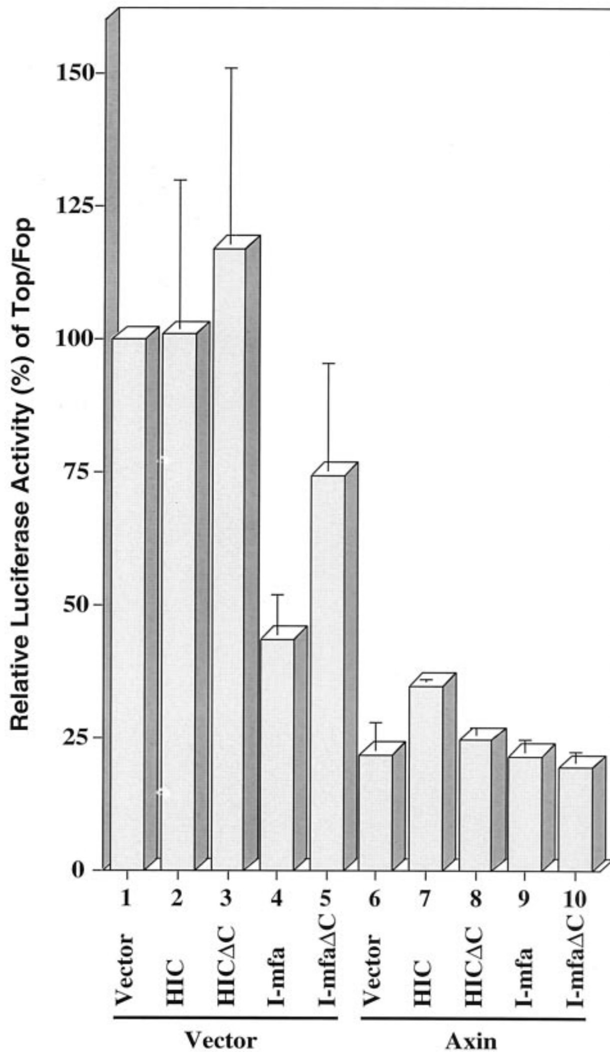


FIG. 7. I-mfa but not HIC inhibits β-catenin-responsive synthetic TCF promoter activity. COS-1 cells were transiently transfected with 1 μg of TOP-flash or FOP-flash plasmid; 0.2 μg of plasmid encoding *Xenopus* β-catenin, 0.3 μg of plasmid encoding HA-Axin, or vector pcDNA3.1+/Zeo; and 0.7 μg of plasmid encoding M3-HIC, M3-HICΔC, M3-I-mfa, or M3-I-mfaΔC or vector pcDNA3. At 72 h after transfection, cells were lysed and luciferase activities were determined. The TOP-flash/FOP-flash activity ratio is represented as 100% for vector plus β-catenin. The average relative value from four experiments is presented for M3-HIC, M3-HICΔC, M3-I-mfa, and M3-I-mfaΔC; error bars indicate standard deviations.

HIC-containing complex but not in the immunoprecipitated complex with HICΔC (Fig. 5). These proteins were not immunoprecipitated with HIC in the absence of Axin, indicating that GSK-3 and β-catenin do not directly interact with HIC, as their presence in the immunoprecipitated complex required Axin. The simultaneous binding of HIC and GSK-3 also apparently does not result from Axin dimerization, as the 1–743 Axin mutant has a deletion of the dimerization domain. These data suggested that HIC and GSK-3 may bind simultaneously to the same site of Axin or that the interaction with the Axin complex in vivo is indirect and possibly requires GSK-3 in the complex.

**HIC increases free β-catenin levels in the presence of Axin.** Axin is known to enhance the phosphorylation of β-catenin by GSK-3 and to decrease the level of free β-catenin (14). To detect free β-catenin, the C-terminal (β-catenin binding) domain of E-cadherin fused to GST was used to bind free β-catenin in cell lysates (24). The overexpression of Axin decreased the level of free β-catenin as previously described (data not shown). The coexpression of HIC or I-mfa with Axin increased the level of free β-catenin (Fig. 6), while the noninteracting HICΔC and I-mfa with a deletion of the I-mfa domain (I-mfaΔC) did not affect the level of free β-catenin. Although the data presented in Fig. 5 suggested that HIC apparently does not displace β-catenin from the Axin complex, HIC apparently can affect the function of the Axin complex, resulting in an increase in the level of free β-catenin.

**HIC and I-mfa differentially regulate Wnt signaling.** Free β-catenin activates the expression of Wnt-regulated genes through its interaction with the TCF and LEF transcription factors. However, I-mfa has been shown to inhibit the activity and DNA binding of the *Xenopus* TCF homologue, Xtcf3 (29). To determine the effects of the I-mfa domain proteins HIC and I-mfa on the Wnt signaling pathway, the ratio of the activity of a β-catenin-responsive promoter containing TCF binding sites (TOP-flash) to the activity of a promoter containing mutated TCF binding sites (FOP-flash) was determined in the presence of I-mfa or HIC. The expression of β-catenin increased the ratio of TOP-flash activity to FOP-flash activity approximately 10-fold, and this ratio was considered 100% activity (Fig. 7). As previously shown, Axin or I-mfa significantly decreased β-catenin activation of TCF-mediated transcription (Fig. 7, lanes 6 and 4). In contrast, HIC did not consistently decrease β-catenin activation of the TCF promoter (Fig. 7, lane 2) but did partially block the ability of Axin to repress β-catenin activation of TCF-mediated transcription (Fig. 7, lane 7). This effect was dependent on the I-mfa domain of HIC, suggesting that a direct protein-protein interaction is required. Importantly, these data revealed that the I-mfa domain proteins HIC and I-mfa have distinct effects on Wnt signaling.

**I-mfa binds to LEF-1 more efficiently than HIC in vivo.** In agreement with the data presented here, it was previously shown that I-mfa inhibits the activity and DNA binding of the *Xenopus* homologue of TCF, Xtcf3 (29). To determine whether HIC and I-mfa directly interact with human TCF or LEF, HA-tagged human LEF-1 (HA-LEF-1) and M3-HIC, M3-HICΔC, M3-I-mfa, or M3-I-mfaΔC were coexpressed in COS-1 cells. Immunoblotting of the total cell lysates indicated that all of the constructs were expressed, with approximately equal levels of HIC and I-mfa (Fig. 8A), and approximately equal amounts of HIC and I-mfa were present in the immunoprecipitated complexes (Fig. 8B). Immunoblotting of the immunoprecipitated I-mfa and HIC protein complexes revealed that HIC and I-mfa both interacted with LEF-1 (Fig. 8B, lanes 7 and 9), while the deletion forms, HICΔC and I-mfaΔC, did not (Fig. 8B, lanes 8 and 10). These observations are consistent with the previous finding that I-mfa binds to Xtcf3 through its I-mfa domain (29). However, the amount of LEF-1 detected in the precipitated I-mfa complex was considerably larger than that detected in the HIC-containing complex (Fig. 8B, lanes 7 and 9). These results suggested that both I-mfa domain proteins, HIC and I-mfa, bind to LEF-1 in vivo

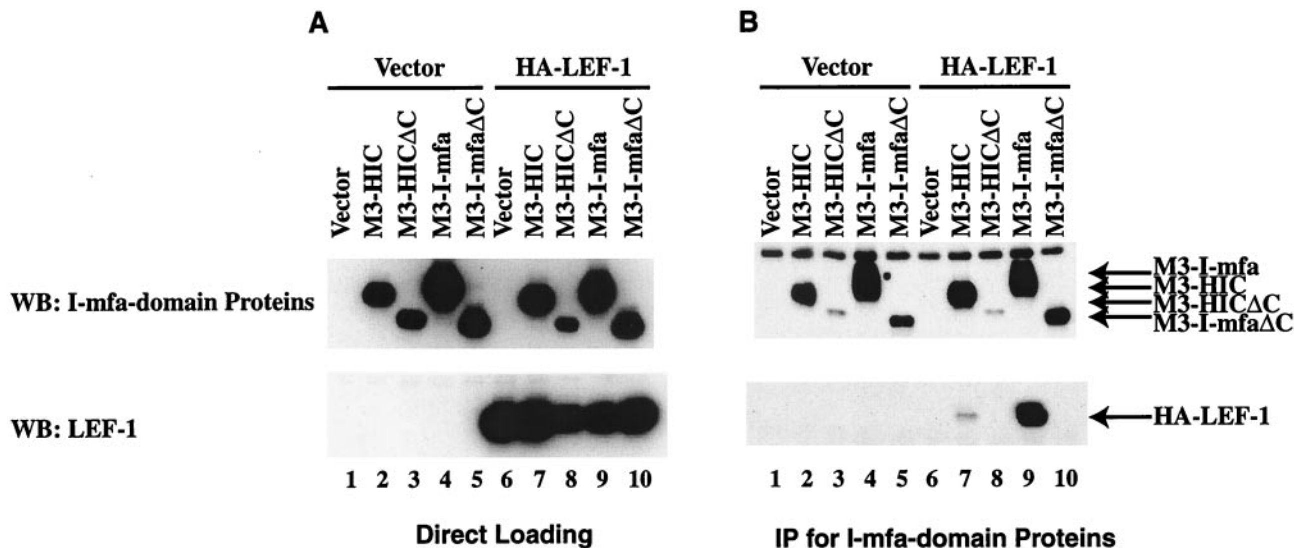


FIG. 8. I-mfa binds LEF-1 more effectively than HIC in vivo. COS-1 cells were transiently transfected with 0.8 μg of plasmid encoding HA-LEF-1 and 0.8 μg of plasmid encoding M3-HIC, M3-HICΔC, M3-I-mfa, or M3-I-mfaΔC. Immunoprecipitation (IP) of 300 μg of total cell lysates was performed with myc antibody-prebound protein A-Sepharose. (A) Immunoblot analysis of 20 μg of total cell lysates with HA antibody to identify HA-LEF-1 or with anti-myc antibody to identify M3-HIC, M3-HICΔC, M3-I-mfa, or M3-I-mfaΔC. (B) Immunoblot analysis of immunoprecipitated I-mfa and HIC-containing complexes with anti-myc antibody to identify M3-HIC, M3-HICΔC, M3-I-mfa, or M3-I-mfaΔC or with HA antibody to identify HA-LEF-1.

but that the binding affinity of I-mfa is apparently greater than that of HIC.

**I-mfa domain proteins inhibit Axin-mediated JNK activation.** It was previously shown that Axin activates the JNK pathway and that GSK-3β inhibits Axin-mediated JNK activation (34, 35). This inhibition of JNK activation by GSK-3β does not require GSK-3β kinase activity but does require the binding of GSK-3β, apparently blocking the binding of MEKK to Axin (36). As HIC and I-mfa interact with the GSK-3β binding domain of Axin, it was of interest to determine whether either I-mfa domain protein affected Axin-mediated JNK activation. The expression of HA-Axin in HEK-293 cells efficiently stimulated the phosphorylation of JNK1 and JNK2 (Fig. 9B, lanes 1 and 6). However, the coexpression of HIC or I-mfa with Axin decreased the levels of activated JNK1 and JNK2 (Fig. 9B, lanes 7 and 9), while the coexpression of HICΔC or I-mfaΔC with Axin did not affect the levels of activated JNK1 and JNK2 (Fig. 9B, lanes 8 and 10). Reprobing of the same membrane with an antibody that recognizes both phosphorylated JNK and unphosphorylated JNK indicated that the levels of total JNK1 and JNK2 in the lysates were identical (Fig. 9C). These results indicated that the presence of the I-mfa domain proteins HIC and I-mfa in the Axin complex decreases Axin-mediated JNK activation and that the I-mfa domains are necessary for this effect.

## DISCUSSION

Axin was first identified as the product of the mouse gene *Fused* and plays a critical role in the regulation of embryonic axis formation (33). Mutations of Axin have been reported for hepatocellular carcinoma, suggesting that Axin also plays an important role in the tumor development process in addition to its role in embryonic axis formation (27). Axin is a scaffolding protein that binds to GSK-3, β-catenin, APC, Dvl, and PP2A and enhances GSK-3-dependent phosphorylation of β-catenin (10–14, 16, 17, 22). The phosphorylation of β-catenin targets it for ubiquitination and proteasome-dependent degradation, resulting in low levels of cytosolic free β-catenin and no activation of TCF- or LEF-regulated transcription (Fig. 10) (1). The mechanism by which Wnt ligand binding inhibits GSK-3 is unknown; however, the Dvl protein, which also binds Axin, affects this process, resulting in the accumulation of free β-catenin and the activation of transcription of target genes through β-catenin interactions with the TCF and LEF transcription factors (3, 7, 8, 19, 21, 26, 32).

The data presented here reveal for the first time that the I-mfa domain proteins HIC and I-mfa directly interact with Axin in vitro and that the I-mfa domain of each protein is necessary for these interactions. The identification of the GSK-3 binding site sequences as the specific Axin sequences required for the interactions with HIC and I-mfa in vivo was surprising, as these sequences were not contained in the Axin cDNA clone (containing the C-terminal portion of Axin) that was isolated from the yeast two-hybrid screening with the I-mfa domain of HIC. However, the C-terminal region of Axin was required for the direct interaction of in vitro-translated Axin with the I-mfa domain. These data suggested that the conformation of Axin is altered in vivo when Axin is present in complexes with additional proteins. The binding of HIC and I-mfa to the Axin complex may be indirect. The clear requirement for the GSK-3 binding site may indicate that the appropriate conformation of the Axin complex for interactions with HIC and I-mfa requires GSK-3 in the complex. The finding that the expression of either HIC or I-mfa did not affect the binding of GSK-3 to Axin is similar to that of a recent study indicating that low-density lipoprotein receptor-related protein 5 also in-

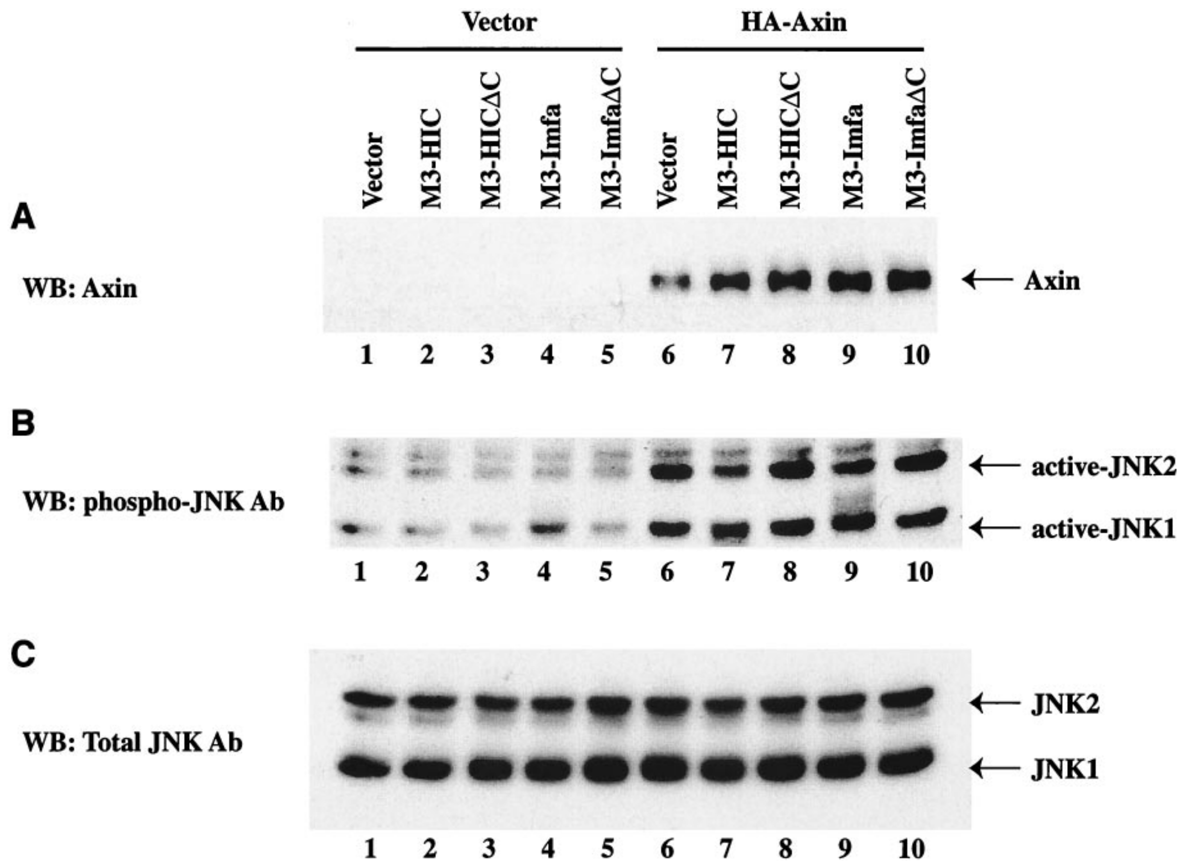


FIG. 9. HIC and I-mfa decrease Axin-mediated JNK activation. HEK-293 cells were transiently transfected with 1 μg of plasmid encoding HA-Axin and 1 μg of plasmid encoding M3-HIC, M3-HICΔC, M3-I-mfa, or M3-I-mfaΔC. (A) Immunoblot analysis of 20 μg of total cell lysates with HA antibody to determine the expression of transfected HA-Axin. (B) Immunoblot analysis with antibody (Ab) to phosphorylated JNK (phospho-JNK) to determine the levels of activated JNK1 and JNK2. (C) Immunoblot analysis of the same membrane as that used in panel B after stripping of the phospho-JNK antibody and reprobing with JNK1 antibody to identify the levels of total JNK1 and JNK2.

teracts with the GSK-3 binding domain of Axin without stoichiometric effects on the Axin–GSK-3β complex (20).

I-mfa was previously shown to inhibit TCF-mediated transcription through its interaction with TCF and its inhibition of TCF DNA binding (29). In this study, I-mfa but not HIC expression decreased β-catenin activation of TCF transcription (Fig. 7). These data suggested that HIC primarily targets and blocks Axin function, while I-mfa can negatively regulate both Axin and TCF and LEF activities. The negative regulation by I-mfa of Wnt signaling in development has been demonstrated in vivo by injection of I-mfa into *Xenopus* embryos, blocking Wnt-mediated axis duplication (29). I-mfa may primarily negatively regulate Wnt signaling through its inhibition of TCF- or LEF-mediated transcription, while HIC may have positive effects on the Wnt pathway through its inhibitory effects on Axin-mediated repression (Fig. 10).

An additional property of Axin is its stimulation of JNK/SAPK signaling through its effects on MEKK1 function, and it is known that the stimulation of JNK signaling is inhibited by the binding of GSK-3 to Axin (35). As this inhibition does not require GSK-3 kinase activity, the binding of GSK-3 to Axin apparently induces a conformational change in Axin that does not permit MEKK1 binding to Axin and the resultant MEKK1 activation (36). This finding suggested that distinct Axin complexes either can activate MEKK1 and JNK or can negatively regulate Wnt signaling (Fig. 10). The data presented here indicate that the I-mfa domain proteins HIC and I-mfa decrease Axin-mediated JNK activation through their interaction with the GSK-3 binding site of Axin. These observations may indicate that I-mfa domain proteins possibly induce or preserve the conformational changes in Axin that prevent MEKK1 binding to Axin and possibly also repress the function or formation of the Axin–GSK-3–β-catenin complex. Consequently, the I-mfa domain proteins increase the cytosolic levels of free β-catenin in the presence of Axin and decrease Axin-mediated JNK activation.

Axin has been shown to induce apoptosis in certain cell types due to its effects on β-catenin stability and JNK activation (23). Therefore, the I-mfa domain proteins HIC and I-mfa could protect from Axin-induced apoptosis, possibly indicating a role in cell survival. However, I-mfa has complex properties and can also inhibit both myogenic bHLH proteins and LEF and TCF transcription factors. In contrast, HIC does not bind to myogenic bHLH proteins and does not have significant inhibitory effects on synthetic TCF promoter activity. These results suggest that the primary functions of HIC may be to regulate Axin and possibly to contribute to cell survival.

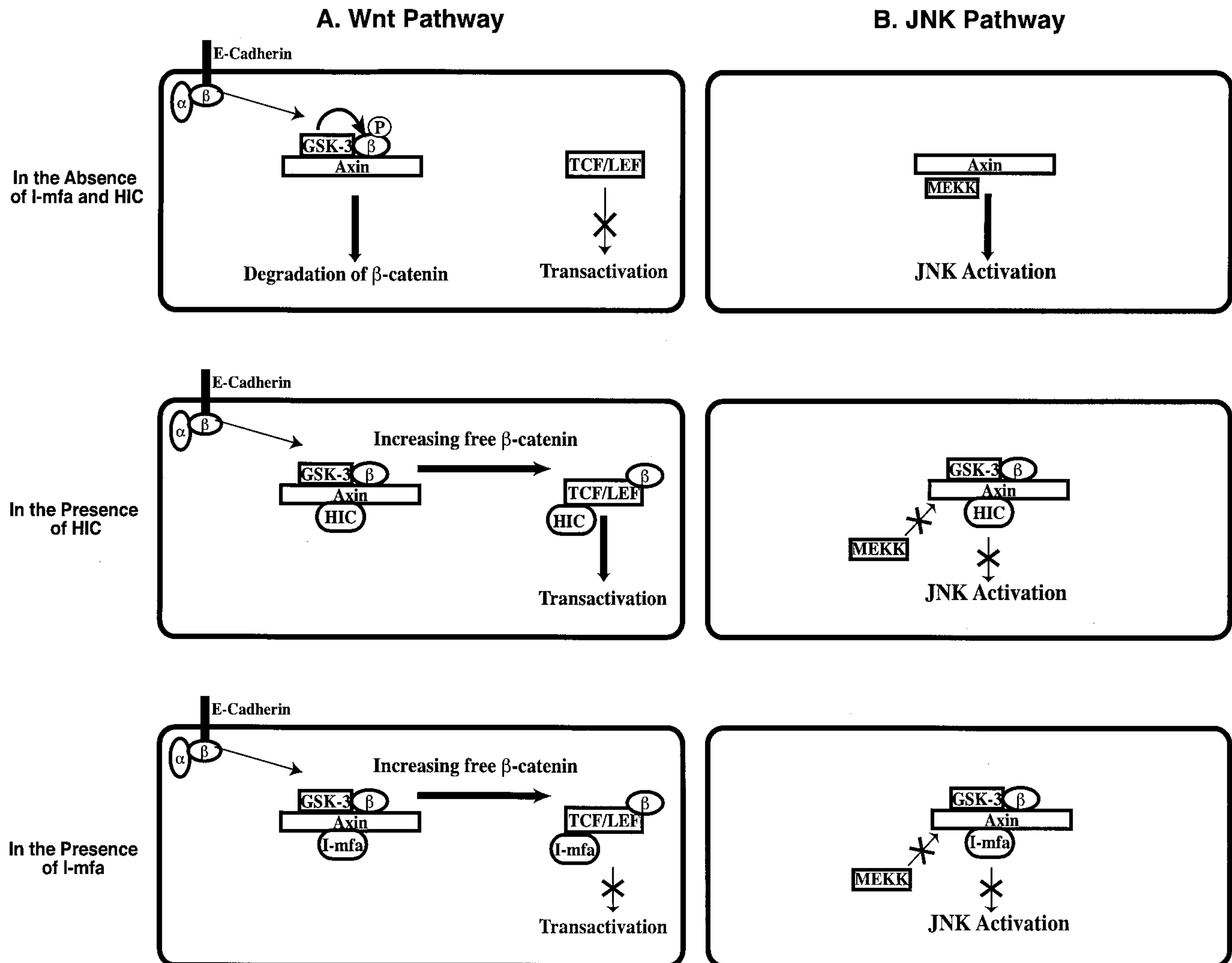


FIG. 10. Diagram of the effects of HIC and I-mfa on Axin-mediated signaling. (A) Effects on the Wnt Pathway. (Top panel) In the absence of HIC or I-mfa, Axin binds free β-catenin or β-catenin that is released from E-cadherin and promotes phosphorylation of β-catenin by GSK-3β and β-catenin degradation. TCF- or LEF-mediated transcription is not activated. (Middle panel) In the presence of HIC, the Axin-mediated decrease in the free β-catenin level is inhibited due to effects on Axin complex formation or GSK-3β activity. Axin inhibition of β-catenin activation of TCF- or LEF-mediated transcription is decreased. (Bottom panel) In the presence of I-mfa, the Axin-mediated decrease in the free β-catenin level is inhibited, and β-catenin activation of TCF- or LEF-mediated transcription is inhibited. (B) Effects on the JNK pathway. (Top panel) In the absence of GSK-3β, Axin binds MEKK, leading to activation of the JNK pathway. (Middle panel) HIC binding to Axin does not affect the binding of GSK-3β, and the activation of JNK is decreased. (Bottom panel) I-mfa binding to Axin does not affect the binding of GSK-3β, and the activation of JNK is decreased.


## ACKNOWLEDGMENTS

We thank R. Moon (Howard Hughes Medical Institutes, University of Washington) for myc epitope-tagged *Xenopus* β-catenin, myc epitope-tagged *Xenopus* GSK-3β (Xgsk3β), and HA epitope-tagged human LEF-1 plasmids; S. Tapscott (Fred Hutchinson Cancer Research Center, University of Washington) for I-mfa cDNA; and S. Milgram (University of North Carolina, Chapel Hill) for the amplified human lung cDNA library.

This study was supported by grant CA32979 awarded to N.R.-T. and by the Yamanouchi Foundation for Research on Metabolic Disorders and the Ryoichi Naito Foundation for Medical Research (support given to S.K.).


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