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Axin-Induced Apoptosis Depends on the Extent of Its JNK Activation and Its Ability to Down-Regulate β-Catenin Levels

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Axin is a multidomain protein that coordinates a variety of critical factors in Wnt signaling and JNK activation. In this study, we found that overexpression of Axin leads to apoptosis in several cell lines. A mutant Axin (Axin-ΔMID) that does not contain the MEKK1-interacting domain and is not capable of activating JNK, has less apoptotic effect. Together with the observations that dominant-negative forms of MEKK1 and JNK1 can attenuate Axin-induced apoptosis, we suggest that JNK activation is required for Axin-mediated apoptosis. Wild-type Axin proteins that can lead to destabilization of β-catenin are more effective at causing cell death than those constructs (Axin-ΔGSK/β-cat, Axin-ΔRGS/GSK/β-cat) that are defective in regulation of β-catenin but still fully capable of JNK activation. Furthermore, enhanced β-catenin signaling by coexpression of β-catenin or PP2Cα attenuate cell death. Taken together, we suggest that the ability of Axin to induce apoptosis is determined by its ability to activate JNK and destabilize β-catenin.

Key Words: **axin; apoptosis; JNK; MEKK1; β-catenin.**

Axin is recently recognized to be an important component in the Wnt signaling pathway that plays critical roles in development, including directing cell fates during embryogenesis, cell proliferation in adult tissues, and oncogenesis (1–4). Originally identified as the product of the mouse *Fused* locus, Axin acts as a negative regulator of Wnt signaling in that its mutation results in axis duplication (5, 6). Axin binds directly to adenomatous polyposis coli (APC), glycogen synthase-3β (GSK-3β), and β-catenin, serving as a scaffold protein to coordinate the regulation of β-catenin levels for Wnt signaling. In the absence of Wnt signal, Axin links these components together and facilitates the phosphorylation of β-catenin by GSK-3β, leading to the degradation of β-catenin. Stimulation of the Wnt signaling pathway activates Dishevelled, resulting in inhibition of GSK-3β and stabilization of β-catenin. The accumulated β-catenin is then translocated into the nucleus where it associates with the lymphoid enhancer factor (LEF) or T-cell factor (TCF) transcription factors (7, 8) to stimulate expression of genes such as c-*MYC* (9). Axin possesses a regulator of G protein signaling (RGS) homologous domain for APC binding, a GSK-3β binding site, a β-catenin binding domain, and a Dishevelled homologous (DIX) domain (6, 10–13), all of which are necessary for Wnt signaling. In addition, Axin also binds to protein phosphatases 2A (PP2A) and 2C (PP2C), which modulates the phosphorylation of APC and Axin, respectively (14–16). However, the precise mechanism of by which Axin negatively regulates Wnt signal for axis formation is not yet clear.

We have recently reported that apart from its well-characterized role in Wnt signaling, Axin has a novel functional role that serves to activate JNK (17). Domains on Axin known to be required for Wnt signaling, i.e., the RGS homologous domain for APC binding and regions for binding GSK-3β and β-catenin are not involved in this JNK activation. An MEKK1-interacting domain (MID) flanked by the APC- and GSK-3β binding sites, and the C-terminal region of Axin which includes the oligomerization domain, are essential for JNK activation. We have also shown that Dishevelled and Axin utilize different mechanisms in JNK activation. In order to understand and compare biological consequences of JNK activation by the two factors, we examined if Axin activation of JNK could lead to apoptosis, which is an intrinsic feature associated with development and homeostasis. Here we report that Axin overexpression in CHO cells can lead to cell death, which can be attenuated by dominant-negative

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forms of MEKK1 and JNK1. We also show that lowered β-catenin signaling due to overexpressed Axin increases the cellular vulnerability to apoptosis.

MATERIALS AND METHODS

Construction of plasmids. A series of different constructs of mouse Axin were created as previously described (17). Expression vectors for MEKK1, MEKK1-K1255M, and MEKK1-C were gifts from Dr. M. Karin (University of California, San Diego, CA). Plasmids of FLAG-JNK1 and its mutant form FLAG-JNK1-APF, and HA-ASK1-K709M have been described (17, 18). β-catenin and PP2Cα were kindly provided by Drs. X. He (Harvard Medical School, MA) and S-H. Shen (Biotechnology Research Institute, National Research Council, Canada) respectively. pCG-LEF1 and pGL3-fos-7LEF-luciferase were gifts from from Dr. L. T. Williams (Chiron Corporation, CA), while pCMV-β-galactosidase was contributed by Dr. V. Yu (IMCB, Singapore).

Hoechst staining and fluorescence microscopy analysis. Chinese hamster ovary (CHO) cells maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 IU penicillin, 100 μg/ml streptomycin, and 2 mM glutamine, were grown on glass coverslips in six-well culture dishes. Cells were transiently transfected with 0.5 μg of green fluorescent protein (GFP)-expressing vector pEGFPN1 (Clontech), together with 1.5 μg of either empty vector pCMV5, HA-Axin, HA-Axin-ΔMID, Axin-ΔGSK/β-cat, Axin-ΔRGS/GSK/β-cat, HA-Dvl2, or HA-MEKK1-C using DOSPER according to the manufacturer's instructions (Boehringer Mannheim). For coexpression studies, cells were cotransfected with 0.5 μg of GFP vector and either 1 μg of HA-MEKK1-K1255M, HA-ASK1-K709M, FLAG-JNK1-APF, β-catenin, PP2Cα, or empty vector in the presence or absence of 0.5 μg of HA-Axin. At 32 h post-transfection, cells were rinsed in PBS, and fixed for 30 min in 4% paraformaldehyde in PBS at room temperature. After three rinses in PBS, the cells were stained with the DNA binding dye Hoechst 33442 (2 μg/ml) for 15 min at room temperature. Cells were washed in PBS three times, mounted in GelMount (Biomeda Corp., Foster City, CA) and kept at 4°C. Slides were examined under Axioplan fluorescence microscope for GFP fluorescence and Hoechst 33342 staining (excitation/emission for these fluorophores, 488/507 nm and 360/460 nm, respectively). For statistical analysis, 100 cells were counted in five different fields. Apoptotic cells positive for both GFP fluorescence and chromatin condensation and nuclear fragmentation were expressed as a percentage of cells positive for GFP fluorescence. The results were replicated in 5–6 independent experiments performed in duplicate, and the data are presented as mean ± S.E.

LEF1-luciferase reporter gene assay. COS-7 cells maintained in DMEM medium supplemented with 10% fetal bovine serum, 100 IU penicillin, 100 μg/ml streptomycin, and 2 mM glutamine, were seeded in 6-well culture plates at 1×10^5 24 h prior to transfection. Cells were transfected with 0.2 μg of pGL3-fos-7LEF-luciferase, 0.05 μg of pCG-Lef1, 0.1 μg of pCMV-β-galactosidase, 0.2 μg of Dvl-2, together with 0.5 μg of either vector or each of the Axin constructs using DOSPER according to the manufacturer's instructions (Boehringer Mannheim). At 32 h post-transfection, cells were lysed and divided into two portions and measured for luciferase and β-galactosidase activities (Promega). The ratio of luciferase activity to β-galactosidase activity varied less than 10% among the samples. Results of luciferase activities are expressed as relative percentage of luciferase activity compared with samples transfected with Dvl2 in the absence of Axin, which is assigned a value of 100%. Data are presented as means ± S.E. from three separate experiments performed in triplicate.

Annexin V staining. CHO cells transfected with HA-Axin for 32 h were pretreated with 20 μM DEVD or ZVAD (Enzyme Products, CA), washed in PBS and resuspended in 1× binding buffer (Annexin V binding buffer, Pharmingen) at a concentration of 1×10^6 cells/ml. The cells were incubated according to the manufacturer's instructions for 15 min with Annexin V which is conjugated to the fluorochrome phycoerythrin (Annexin V-PE; Pharmingen), before analysis on a FACScan (Becton Dickinson). Data were analyzed using Winmdiv2.8 software (University of Massachusetts, Amherst) on a univariate plot.

Immunokinase assays. Human embryonic kidney 293T cells were transfected with 1 μg of FLAG-JNK1 and 1 μg of either empty vector pCMV5 or HA-Axin using Superfect according to the manufacturer's instructions (Qiagen). At 40 h post-transfection, cells were lysed, FLAG-tagged JNK1 was immunoprecipitated using mouse monoclonal anti-FLAG M2 beads (Sigma), and the kinase activities were determined as described previously using 1 μg GST-c-Jun (aa 1–79; Stratagene) as substrate (17).

RESULTS

Overexpression of Axin Leads to Apoptotic Cell Death in Certain Cells

One of the biological consequences of JNK activation is the induction of apoptosis, at least in certain systems (19, 20). The robust activation of JNK by Axin prompted us to study if Axin could play a role in apoptosis. We examined the morphological characteristics of CHO cells after 32 h transfection with either wild-type Axin, mutant Axin without the MID domain (Axin-ΔMID), C-terminal kinase domain of MEKK1 (MEKK1-C), or the vector alone. Cells were stained with Hoechst 33342 and observed for the blue stained nuclei under a fluorescence microscope; transfection efficiency was assessed using the GFP expression plasmid. The expression levels of the transfected proteins were relatively similar, as determined by Western blotting. As shown in Fig. 1A, in cells overexpressing wild-type Axin, majority of the nuclei (~55%) appeared shrunken and/or fragmented, similar to those observed in cells overexpressing MEKK1-C (~53%) or Bax (data not shown), both of which are known to cause apoptosis (21). In contrast, cells overexpressing either the vector, Axin-ΔMID, or Dvl2, showed a higher proportion of round blue nuclei and the percentage of cell death was comparatively less at 12%, 26%, and 28% respectively (Fig. 1A). We also examined the proportion of apoptotic CHO cells transfected with either vector, wild-type Axin, Axin-ΔMID, or MEKK1-C over a time course of 46 h using Hoechst staining. As shown in Fig. 1B, cells transfected with Axin-ΔMID showed a relatively low percentage (~26%) of cell death over the course of 46 h. By contrast, cells overexpressing Axin or MEKK1-C exhibited a significantly higher percentage of apoptotic cells at 18 h (~46%), and the proportion of cell death increased over 46 h to ~60%. Similar findings were also obtained when the same experiments were performed in PC12 cells (data not shown).

Axin-Induced Cell Death Involves MEKK1 and JNK

We have previously shown that unlike wild-type Axin which robustly activates JNK, Axin-ΔMID loses

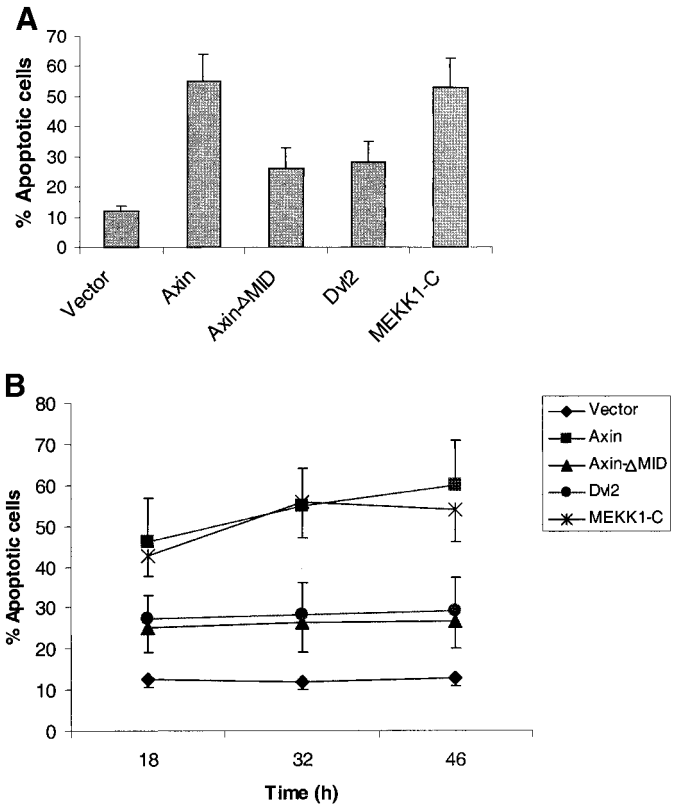

FIG. 1. Overexpressed Axin causes apoptotic cell death in CHO cells. (A) Percentage of apoptosis in CHO cells induced by Axin, Axin-ΔMID, Dvl2, or MEKK1-C. Cells were cotransfected for 32 h with GFP vector together with either HA-Axin, HA-Axin-ΔMID, HA-Dvl2, HA-MEKK1-C, or empty vector. Cells were examined by fluorescence microscopy following staining with Hoechst 33342. The values represent means ± S.E. from 5–6 independent experiments performed in duplicate. (B) Time-dependent induction of apoptosis in CHO cells. CHO cells were transfected as in (A) and cell death was assessed at the indicated times. The percentage of apoptotic cells is presented as in (A).

its ability to activate JNK (17). Since Axin-ΔMID does not cause apoptosis as does wild-type Axin (Fig. 1), we asked whether JNK activation is necessary for apoptosis induced by overexpressed Axin. We transfected CHO cells with wild-type Axin in the presence or absence of the kinase-inactive forms of MEKK1 (HA-MEKK1-K1255M), ASK1 (HA-ASK1-K709M), or JNK1 (FLAG-JNK1-APF), stained the cells with Hoechst 33342 and scored the cells for apoptotic morphology. Figure 2 shows that cells overexpressing Axin were highly apoptotic (~55%) compared to vector-transfected cells (~10%). Coexpression of kinase-deficient MEKK1 or kinase-inactive JNK1 in Axin-transfected cells reduced the proportion of cell death to ~26%, suggesting that MEKK1 and JNK are involved in the Axin-mediated apoptosis. In contrast, coexpression of dominant-negative HA-ASK1-K709M did not appear to affect apoptotic cell death caused by overexpressed Axin, consistent with our previous observation that ASK1 is not involved in Axin-mediated JNK activation (17).

Lower β-Catenin Signaling Facilitates Axin-Induced Cell Death

Axin possesses multiple functional domains, including the RGS homologous domain which binds APC, distinct binding sites for GSK-3β and β-catenin, and a DIX domain (5, 6). In addition of JNK activation, we next examined whether any other region(s) on the Axin protein is involved in apoptosis. CHO cells were separately transfected with different Axin mutants and analysed with Hoecst 33342. Figure 3A (left panel) shows that cells overexpressing wild-type Axin exhibited significant cell death (~54%) compared to vector-transfected cells (~11%). Deletion of the GSK-3β/β-catenin binding site alone (Axin-ΔGSK/β-cat), or together with the RGS homologous domain (Axin-ΔRGS/GSK/β-cat) reduced the level of cell death to ~28%, while removal of the MID domain (Axin-ΔMID) reduced cell death to ~24%. We also determined the effect of different Axin mutants on LEF1-luciferase activity. We transfected COS-7 cells with each of the various Axin constructs, together with Dvl2 to mimic Wnt signaling and the reporter LEF1-luciferase, and measured the LEF1-luciferase activities. As shown in Fig. 3A (right panel), expression of Dvl2 with LEF1-luciferase in the absence of Axin resulted in high luciferase activity, consistent with the importance of Dvl2 in activating β-catenin mediated LEF1-signaling (22, 23). We assigned a value of 100% to this high luciferase activity. Coexpression with wild-type Axin significantly reduced LEF1-luciferase activity (Fig. 3A), in agreement with its role as a negative regulator of Wnt

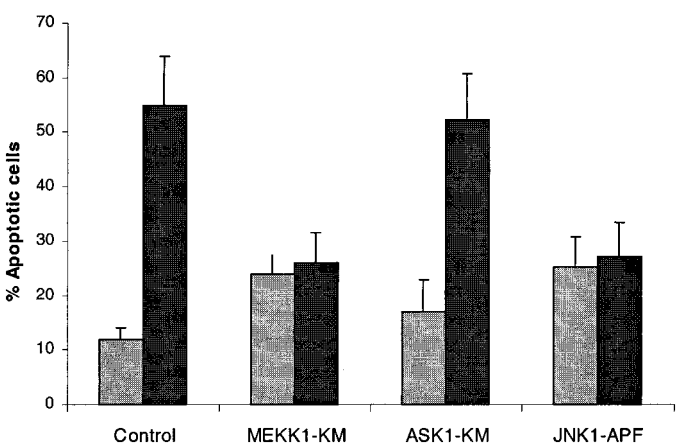

FIG. 2. Axin-mediated apoptosis is inhibited by kinase-inactive forms of MEKK1 and JNK1. CHO cells were cotransfected for 32 h with 0.5 μg of GFP vector and either 1 μg of HA-MEKK1-K1255M, HA-ASK1-K709M, FLAG-JNK1-APF, or empty vector in the presence (dark columns) or absence (light columns) of 0.5 μg of HA-Axin. The percentage of apoptotic cells is presented as in the legend to Fig. 1.

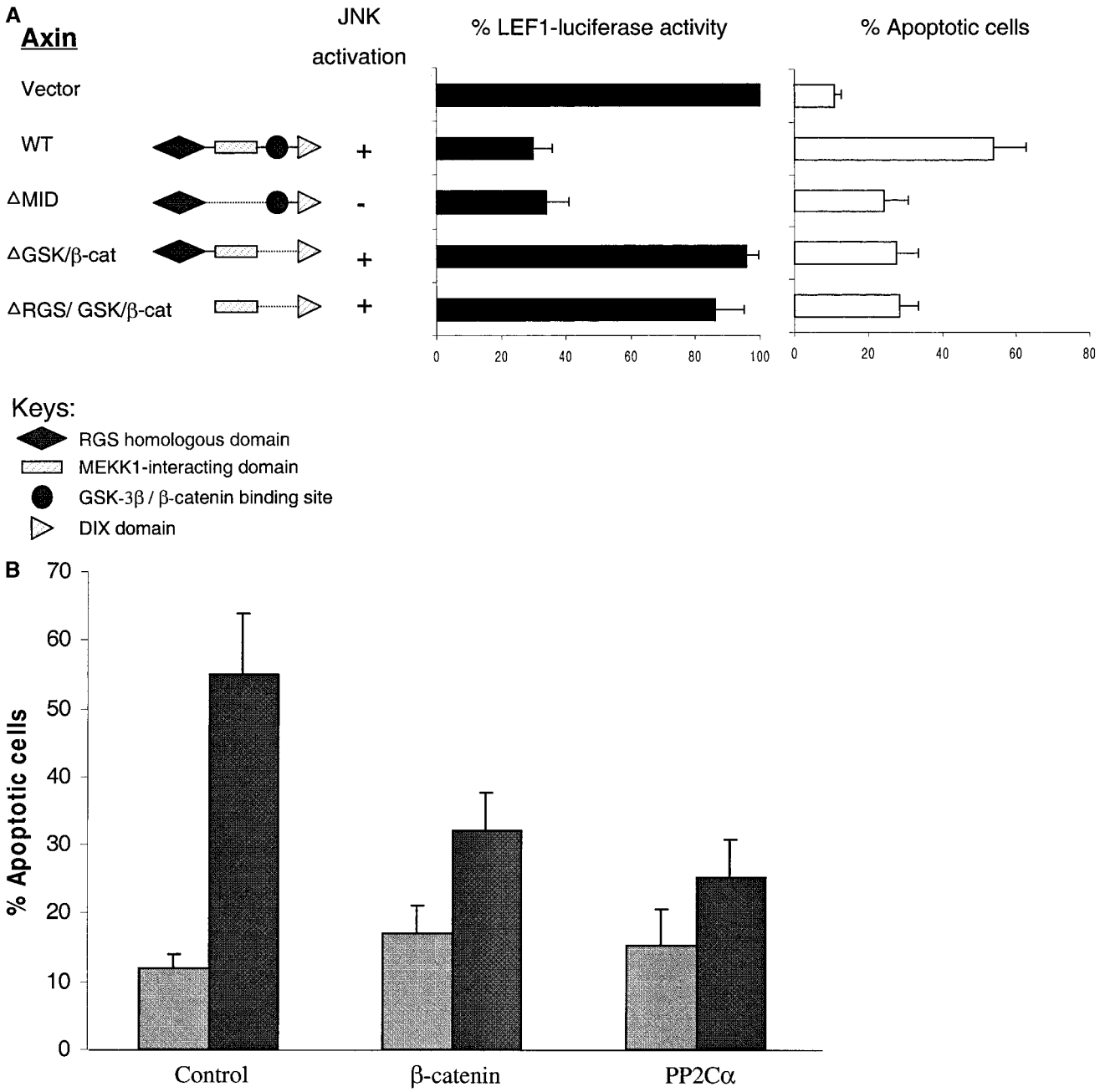

FIG. 3. Apoptosis caused by Axin depends on its ability to attenuate β-catenin signaling. (A) Domains on Axin critical for attenuating LEF1 reporter activity are necessary for Axin-induced apoptosis. Schematic representation indicates the Axin constructs used, and the RGS homologous domain, MEKK1-interacting domain (MID), binding sites for GSK-3β and β-catenin, and the Dishevelled homologous domain (DIX) are indicated. The extent of JNK activation are summarized as "+" for high activities and "−" for low activities. The LEF1-luciferase activity is expressed as relative percentage luciferase activity compared with activity produced in cells transfected with Dvl2 in the absence of Axin, which is assigned a value of 100%. The values represent the means ± S.E. from three separate experiments performed in triplicate. The apoptotic cell death was assessed as described in the legend to Fig. 1. (B) Inhibition of Axin-induced apoptosis by β-catenin and PP2Cα. Cells were cotransfected for 32 h with 0.5 μg of GFP vector and either 1 μg of β-catenin, PP2Cα or empty vector in the presence (dark columns) or absence (light columns) of 0.5 μg of HA-Axin. The percentage of apoptotic cells is presented as in the legend to Fig. 1.

signaling (13, 24). Coexpression with Axin-ΔGSK/β-cat and Axin-ΔRGS/GSK/β-cat did not affect the ability of Dvl2 to induce LEF1-luciferase activity, indicating the importance of the RGS domain and GSK-3β/β-catenin binding sites on Axin in regulating β-catenin levels for Wnt transduction. However, coexpression with Axin-ΔMID exhibited low LEF1-luciferase activity, suggesting Axin-ΔMID is still capable of inhibiting Wnt signaling. It appeared that the domains on Axin affecting β-catenin signaling are involved in facilitating Axin-

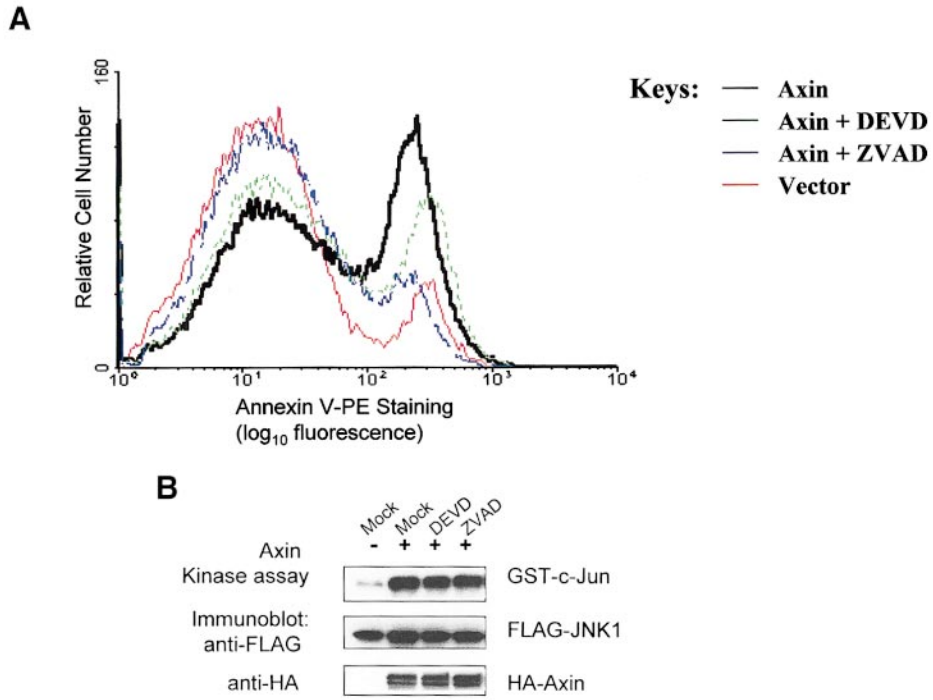

FIG. 4. Effect of DEVD and ZVAD on Axin-mediated apoptosis and JNK activation. (A) Flow cytometry analysis of Axin-overexpressing CHO cells pretreated with DEVD or ZVAD. CHO cells pretreated with 20 μM DEVD or ZVAD were transfected with HA-Axin, harvested at 32 h post-transfection, labelled with annexin V-PE, and analysed by flow cytometry. Each histogram profile is representative of results obtained in five independent experiments. (B) DEVD or ZVAD pretreatment does not affect Axin-activation of JNK. 293T cells transfected with 1 μg of FLAG-JNK1 and 1 μg of HA-Axin were treated with either 20 μM DEVD or ZVAD. Immunokinase assays were performed and the kinase activation was normalized for their expression levels. The data shown here is representative of results obtained in three separate experiments.

induced cell death. To test this, we transfected CHO cells with wild-type Axin in the presence or absence of β-catenin, and scored the cells for apoptotic features using Hoechst 33342 staining. Figure 3B shows that coexpression of the active forms of β-catenin reduced Axin-induced cell death. As PP2Cα is reported to activate LEF1-dependent transcription (16), we also transfected cells with PP2Cα and found that PP2Cα also inhibited Axin-induced cell death.

Effects of DEVD and ZVAD on Axin-Mediated Apoptosis and JNK Activation

Caspases are critical components in driving apoptosis pathways (25). In particular, MEKK1 has been shown to be activated by caspase cleavage of its N-terminus (26). We therefore asked whether apoptosis caused by Axin could be affected by caspase inhibitors DEVD (a caspase-3 inhibitor) and ZVAD (a general caspase inhibitor). CHO cells pretreated with either DEVD or ZVAD were transfected with or without Axin, and analysed by FACS using annexin V-PE for the portion of cells undergoing early apoptosis. As shown in Fig. 4A, overexpression of Axin in CHO cells resulted in high percentage of positive staining for annexin V-PE (~33%), compared with vector-transfected cells which showed a low level background of ~10%. Incubation with DEVD partially reduced annexin V-PE positive cells to 20%, while treatment with ZVAD further decreased the apoptotic cell percentage to levels close to those of vector-transfected cells (~12%). We were also interested in examining the effect of these caspase inhibitors on JNK activation mediated by Axin, and assayed the kinase activities in Axin-transfected 293T cells pretreated with DEVD or ZVAD. As shown in Fig. 4B, we found that neither DEVD nor ZVAD altered JNK activation by Axin. These results suggest that Axin activation of JNK does not require MEKK1 cleavage, and that caspases act downstream of JNK in Axin-induced apoptosis.

DISCUSSION

In this study, we found that overexpression of wild-type Axin resulted in significantly high levels of apoptosis in certain cells, while Axin-ΔMID showed less apoptotic cell death. Since overexpressed wild-type Axin but not Axin-ΔMID robustly activates JNK (17), it is likely that JNK activation plays a role in Axin-

induced cell death. The JNK signal transduction pathway is well-established to be a mechanism used by cells in response to stress and in the regulation of many normal physiological processes including tissue morphogenesis, cell proliferation and survival or cell death (19–21, 27). While there are many reports implicating JNK activation in the events leading to apoptosis, there is also considerable evidence for JNK in mediating cell survival, depending on the cell type and the context of activation of other signaling regulatory pathways in the cell (19, 20). For instance, the dual deficiency of JNK1 and JNK2 causes early embryonic lethality and severe dysregulation of apoptosis during brain development, indicating the importance of JNK1 and JNK2 in the regulation of regional specific apoptosis during early brain development (28). Overexpression of MEKK1 and its activation via caspases results in apoptosis in MDCK cells (26). On the other hand, elevation of JNK activity enhances cell survival in response to stimuli such as UV radiation and TNF (29). Dishevelled, another important component in the Wnt signaling, has recently been reported to also cause apoptosis when overexpressed (16). It also activates JNK (22, 23, 30), although Axin and Dishevelled adopt different mechanisms in JNK activation (17, 22, 23). In our study, we observed that overexpression of Axin leads to a higher percentage cell death than Dvl2. This may be due to different extents of JNK activation by Axin and Dvl2, wherein Axin activates JNK to a higher degree than Dvl2. We also noted that kinase-deficient MEKK1 inhibits both Axin-mediated JNK activation and apoptosis. While JNK activation by Axin remains unaffected, caspase inhibitors can prevent cell death, suggesting that caspases act downstream of JNK and Axin activation of MEKK1 does not require cleavage of its N-terminus seen in the process of anoikis (21, 26). However, it remains possible that activated caspases can accelerate cleavage of MEKK1 in a positive feedback loop during late apoptosis (21).

Apoptosis caused by overexpressed Axin does not seem to be solely dependent on the JNK pathway. We observed that even though Axin-ΔRGS/GSK/β-cat and Axin-ΔGSK/β-cat possess high JNK activation activity comparable to wild-type Axin, they exhibit less cell death than wild-type Axin (Fig. 3A). Since both Axin-ΔRGS/GSK/β-cat and Axin-ΔGSK/β-cat are unable to inhibit LEF1 reporter activity, and wild-type Axin effectively blocks LEF1 reporter gene transcription, we suggest that LEF1-signaling mediated by β-catenin may also play a role in Axin-induced cell death. It has been demonstrated in Alzheimer's diseases that overexpression of mutant presenilin-1, a frequently mutated gene in early-onset of the disease, causes apoptosis. The mutated presenilin is thought to interfere with Wnt signaling cascade by increasing β-catenin degradation and reducing nuclear translocation of β-catenin (31, 32). Furthermore, β-catenin has been shown to be linked to neuronal cell death as reduced β-catenin signaling increased neuronal vulnerability to apoptosis (32). A recent report suggests that overexpression of Dishevelled results in apoptotic cell death which can be prevented by coexpression of PP2Cα (16). Our findings indicate that overexpressed Axin caused cell death to a higher extent than did Dvl2, possibly due to a combination of higher JNK activation and lower β-catenin-mediated LEF1 signaling. Relatively lower JNK activation together with higher β-catenin signaling by Dvl2 may provide a survival signal. Furthermore, we noted that coexpression of PP2Cα could prevent Axin-induced cell death. PP2C has been shown to not only dephosphorylate Axin and activate LEF1-dependent transcription (33), but also inhibit the JNK cascade (34, 35). It is conceivable that PP2Cα inhibition of Axin-induced JNK signaling, as well as relief of Axin-suppressed LEF1 transcription, may account for the protection of cells from Axin-induced cell death by co-expressed PP2Cα.

In conclusion, we demonstrate that overexpression of Axin can cause apoptosis. The biological significance of Axin-mediated apoptosis may be illustrated by a recent report that Axin mutations could lead to carcinogenesis due to a possible lack of apoptosis (36, 37). We also show that the apoptotic event requires JNK activation. This finding is consistent with the ubiquitous expression of Axin in adult tissues, which suggests Axin also plays important roles in maintaining homeostasis in addition to its role in development. We also provide evidence that vulnerability of cells to apoptosis is determined by death signals and cell survival signals conferred by the β-catenin signaling cascade.

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