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Advances in Brief

Functional Interaction between Retinoblastoma Protein and Stress-activated Protein Kinase in Multiple Myeloma Cells[1]

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Abstract

Previous studies have demonstrated that γ-irradiation (IR)-induced apoptosis in multiple myeloma (MM) is associated with activation of stress-activated protein kinase (SAPK). In the present study, we examined the molecules downstream of SAPK/C-Jun N-terminal kinase (JNK), focusing on the role of retinoblastoma protein (Rb) during IR-induced MM cell apoptosis. The results demonstrate that IR activates SAPK/JNK, which associates with Rb both *in vivo* and *in vitro*. Far Western blot analysis confirms that SAPK/JNK binds directly to Rb. IR-activated SAPK/JNK phosphorylates Rb, and deletion of the phosphorylation site in the COOH terminus domain of Rb abrogates phosphorylation of Rb by SAPK/JNK. Taken together, our results suggest that Rb is a target protein of SAPK/JNK and that the association of SAPK/JNK and Rb mediates IR-induced apoptosis in MM cells.

Introduction

Treatment of eukaryotic cells with IR[3] induces growth arrest, activation of DNA repair, and apoptosis. The finding that treatment with IR induces transcription of early response genes, such as c-jun and Egr-1, suggests the involvement of nuclear events (1, 2). To date, multiple protein kinases have been identified that transduce IR-induced signals to the nucleus. SAPK, also known as JNK, is related to the mitogen-activated protein kinases (3–7) and has been linked to apoptosis (8). Two serine residues (Ser^{63} and Ser^{73}) in the NH_2-terminal transactivation domain of c-Jun have been identified as substrates for SAPK/JNK (6, 7). Previous studies have shown that stress stimuli, *e.g.*, IR, tumor necrosis factor, sphingomyelinase, and UV light, activate SAPK/JNK (6–10). Our recent studies demonstrate that IR- and Fas-induced activation of SAPK/JNK in MM cells is associated with apoptosis (11, 12). To date, however, the mechanism whereby SAPK/JNK transduces apoptotic signals during MM cell apoptosis remains largely unknown.

The Rb protein is a nuclear phosphoprotein that regulates cell growth in the G_1 phase of the cell cycle (13, 14). Phosphorylation of Rb plays an important role in its growth-inhibitory function by modulating its interaction with other proteins, such as E2F (13, 14). Recent studies have also suggested a role for Rb in apoptosis (14, 15), in particular, during IR-induced apoptosis (16). Rb-mediated apoptosis has been linked to activation of serine proteases (14, 15). In addition, Rb protein may be a target of proteolysis by caspases, further suggesting its potential role during apoptosis (17).

In the present study, we characterized the signaling mechanisms during IR-induced apoptosis in a MM-derived cell line. The results demonstrate that IR-activated SAPK/JNK binds directly to Rb and phosphorylates Rb, suggesting that SAPK/JNK-Rb mediates IR-induced apoptosis in MM cells.

Materials and Methods

Cell Culture and Metabolic Labeling. The human MM cell line OCI-MY5 and Rb-deficient (Rb−/−) SAOS-2 and Rb-reconstituted (Rb+/+) SAOS-2 cell lines were cultured, and γ-IR was performed as described previously (11, 18). For labeling, cells (1×10^7) were resuspended in 1 ml of phosphate-free DMEM and incubated for 1 h before the addition of 5 mCi of [^{32}P]orthophosphate (carrier-free; ICN). The cells were then incubated for 5 h, treated with IR for 1 h, and harvested. Lysates were then subjected to immunoprecipitation with anti-Rb Ab.

Reagents and Abs. Anti-SAPK/JNK and anti-tubulin Abs were purchased from Santa Cruz Biotechnology (San Diego, CA); anti-Rb mAb (G3-245) was obtained from PharMingen (San Diego, CA). The GST-Rb (full length, 379–928) and various GST-Rb deletion constructs were kind gifts of Drs. William G. Kaelin, Jr. and Peter D. Adams (Dana-Farber Cancer Institute, Boston, MA).

Immunoblotting/Immunoprecipitation. Cell lysates were prepared as described previously (11, 12, 19). Equal amounts of proteins (250–300 μg) were subjected to immunoprecipitation with the indicated Abs, and immune complexes were precipitated with protein A-Sepharose. The resulting protein precipitates were washed three times with lysis buffer and resolved by SDS-PAGE. Proteins were then transferred to nitrocellulose filters, blocked by incubation in 5% dry milk in PBST (0.05% Tween-20 in PBS), and probed with the indicated antibodies. Blots were then developed by enhanced chemiluminesence (Amersham, Arlington Heights, IL).

***In Vitro* Immune Complex Assays.** Cell lysates were incubated with either PIRS or SAPK/JNK Abs for 2 h at 4°C before the addition of protein A-Sepharose. The immune complexes were washed three times with lysis buffer and once with kinase buffer and resuspended in kinase buffer containing [γ-^{32}P]ATP (3000 Ci/mmol; New England Nuclear, Boston, MA) and GST-Jun (2–100) or GST-Rb deletion constructs as substrates. The reaction was incubated for 15 min at 30°C and terminated by the addition of SDS sample buffer. Proteins were analyzed by SDS-PAGE, Coomassie Blue staining, and autoradiography.

Far Western Analysis. The lysates from IR-treated cells were subjected to immunoprecipitation with anti-SAPK Ab and PIRS. The proteins were resolved by 10% SDS-PAGE and transferred to nitrocellulose membrane. The filters were first incubated with purified GST-Rb (full length, 379–928) fusion protein (19) or with purified GST fusion protein alone (negative control) at room temperature for 2 h and then analyzed by immunoblotting with anti-GST mAb.

Results and Discussion

IR Induces SAPK/JNK Activation and Formation of SAPK/JNK-Rb Complexes. Many studies have demonstrated that IR induces apoptosis in various cell types. We and others have demon-

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[3] The abbreviations used are: IR, irradiation; SAPK, stress-activated protein kinase; JNK, c-Jun N-terminal kinase; Rb, retinoblastoma; MM, multiple myeloma; Ab, antibody; GST, glutathione S-transferase; mAb, monoclonal Ab; PIRS, preimmune rabbit serum.

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strated that anti-Fas mAb, dexamethasone, and IR trigger apoptosis in MM cells (11, 12, 20, 21). Multiple protein kinases have been shown to play an important role during apoptosis. For example, IR and UV trigger activation of SAPK/JNK and p38 kinase in MM cells as well as in other cell systems (6, 9, 11, 12). In the present study, OCI-MY5 MM cells were exposed to IR (20 Gy) for 1 and 3 h, and then assayed for activation of SAPK/JNK using an *in vitro* immune complex kinase assay with GST-Jun (2–100) as a substrate. Analyses of anti-SAPK/JNK immunoprecipitates demonstrated a transient 8–10-fold activation of SAPK/JNK at 1 h in MM cells without altering SAPK/JNK protein levels (Fig. 1*A*; data not shown). These results, coupled with our prior study (11), suggest a role of SAPK/JNK in IR-induced apoptosis in MM cells.

To determine whether IR-activated SAPK/JNK associates with partner proteins during apoptotic signaling, anti-SAPK/JNK immunoprecipitates from OCI-MY5 MM cells were analyzed for potential binding proteins. Coomassie Blue staining demonstrates that a M_r 110,000 protein coprecipitates with SAPK/JNK kinase, suggesting the possibility of an interaction with the Rb protein. Indeed, immunoblot analysis of anti-SAPK/JNK immunoprecipitates with anti-Rb Ab revealed increased reactivity after IR treatment (Fig. 1*B*). As a negative control, immunoblot analyses of PIRS immunoprecipitates with anti-Rb Ab demonstrated no reactivity with M_r 110,000 protein (data not shown). To provide additional support for an association between Rb and SAPK/JNK kinase, anti-Rb immunoprecipitates were analyzed for reactivity with anti-SAPK/JNK Ab. The results demonstrate that Rb associates with SAPK/JNK and that this association is increased by IR treatment (Fig. 1*C*). In these experiments, reprobing the blots either with anti-SAPK/JNK or anti-Rb Abs, respectively, demonstrated equal amounts of proteins in each lane (Fig. 1, *B* and *C*, *bottom panel*). To confirm the specificity of Rb binding, lysates from Rb-deficient SAOS-2 (Rb−/−) and Rb-reconstituted SAOS-2 (Rb+/+) cells (18) were immunoprecipitated with SAPK/JNK Ab and immunoblotted anti-Rb Ab. Rb was undetectable in Rb−/− cells; however, Rb coimmunoprecipitated with SAPK/JNK in Rb+/+ cells (Fig. 1*D*, *top panel*). Equal amounts of SAPK/JNK protein were present in both Rb−/− and Rb+/+ cells (Fig. 1*D*, *bottom panel*). These results confirm the specificity of the SAPK/JNK interaction with Rb. SAPK/JNK-Rb interaction was further examined by incubating lysates from control and IR-treated OCI-MY5 MM cells with a GST-Rb (full length, 379–928) fusion protein. Analysis of the adsorbates by immunoblotting with anti-SAPK/JNK Ab revealed increased reactivity with a M_r 46,000 protein (SAPK) in the IR-treated cells (data not shown). These findings suggest that SAPK/JNK interacts with Rb during IR-induced apoptosis in MM cells *in vivo*.

SAPK/JNK Phosphorylates Rb *In Vitro*. Rb is a nuclear phosphoprotein that plays an important role in cell cycle progression and differentiation (14). Recent studies have also suggested a role for Rb during apoptosis, in particular, IR-induced apoptosis (15, 16). Previous studies have shown that Rb harbors multiple phosphorylation sites and is a target of different kinases (14), suggesting that IR-activated SAPK/JNK may directly bind and phosphorylate Rb, thereby modulating its function. A Ser/Thr-Pro consensus sequence for phosphorylation by SAPK/JNK has been defined (5), and the COOH terminus

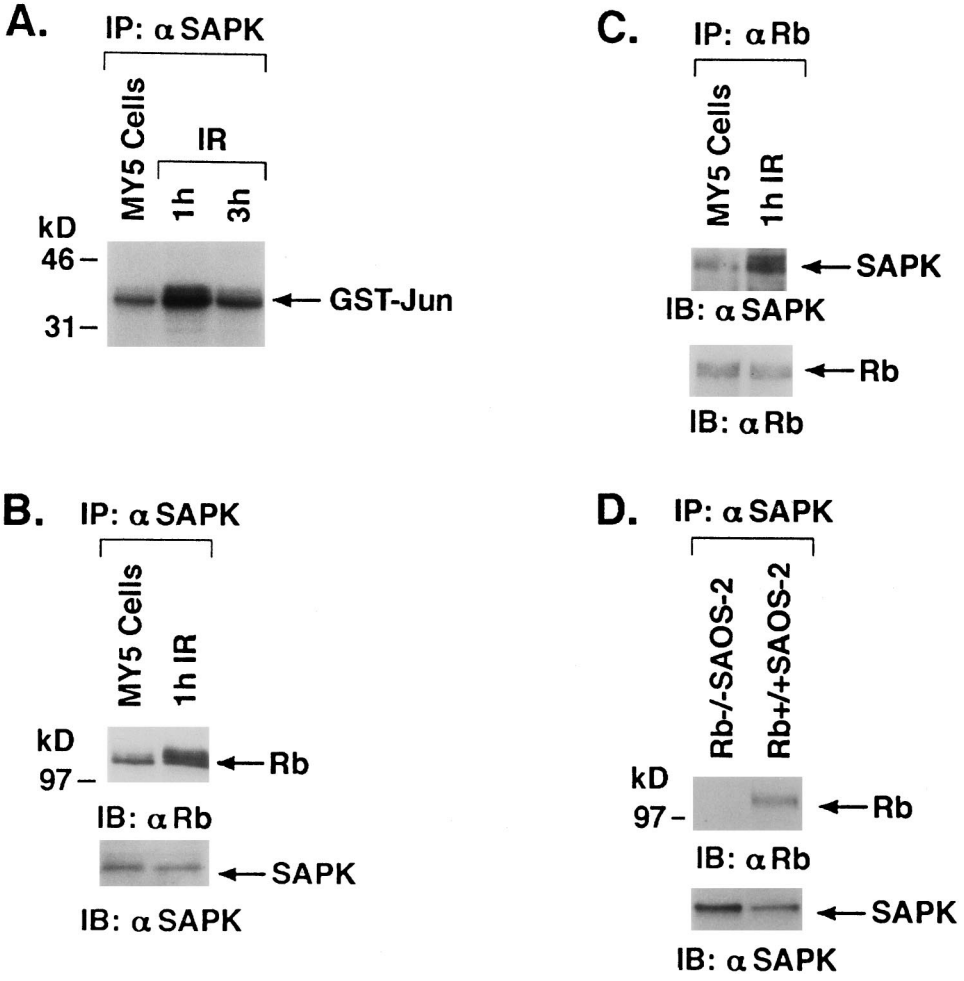

Fig. 1. IR induces SAPK/JNK activation and formation of SAPK/JNK-Rb complexes. *A*, cells were treated with IR (20 Gy) for 1 and 3 h, and harvested. Lysates from control and IR-treated OCI-MY5 MM cells were immunoprecipitated with anti-SAPK Ab. Immune complex kinase assays were performed by the addition of GST-Jun and [γ^{32}P]ATP and incubation for 15 min at 30°C. Phosphorylated proteins were resolved by 10% SDS-PAGE and analyzed by autoradiography. *B*, cells were treated with IR (20 Gy) for 1 h and harvested. Lysates from control and IR-treated OCI-MY5 MM cells were immunoprecipitated with anti-SAPK Ab. Equal amounts of proteins were resolved by 7.5% SDS-PAGE, transferred to nitrocellulose, and subjected to immunoblotting with anti-Rb Ab (*top panel*) or anti-SAPK/JNK Ab (*bottom panel*). *C*, anti-Rb immunoprecipitates were immunoblotted with anti-SAPK Ab (*top panel*) or anti-Rb Ab (*bottom panel*). *D*, anti-SAPK immunoprecipitates from Rb-deficient (Rb−/−) and Rb-reconstituted (Rb+/+) cells were subjected to immunoblot analyses with anti-Rb Ab (*top panel*) or anti-SAPK Ab (*bottom panel*).

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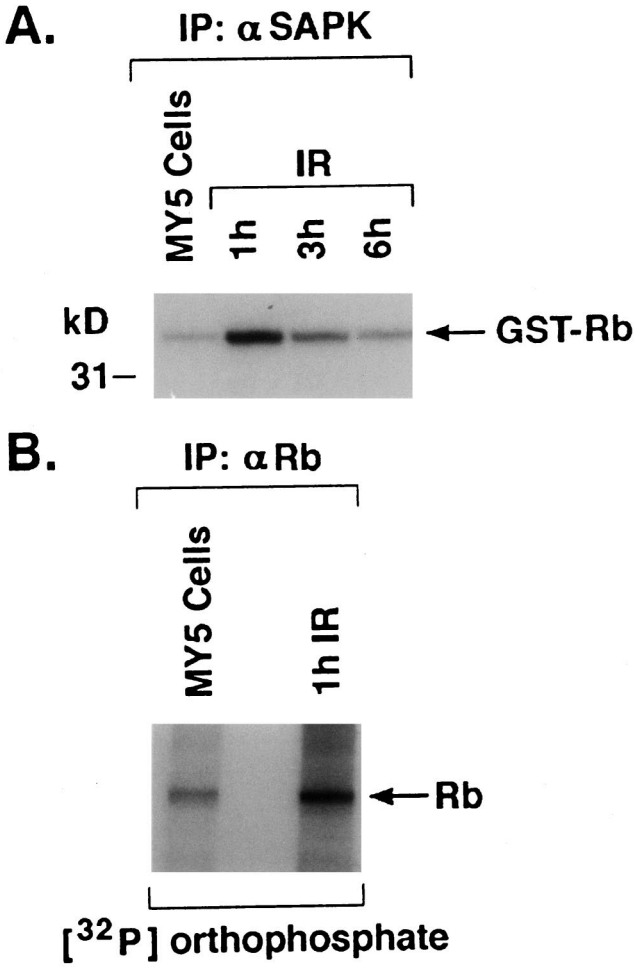

Fig. 2. SAPK/JNK phosphorylates Rb *in vitro*. *A*, cells were treated with IR (20 Gy) for 1, 3, and 6 h and harvested. Lysates from control and IR-treated OCI-MY5 MM cells were immunoprecipitated with anti-SAPK Ab. Immune complex kinase assays were performed by the addition of GST-Rb (379–928) and [γ^{32}P]ATP, followed by incubation for 15 min at 30°C. The phosphorylated proteins were resolved by 10% SDS-PAGE and analyzed by autoradiography. *B*, IR induces increased Rb phosphorylation *in vivo*. Cells were cultured for 5 h in the presence of [^{32}P]orthophosphate. Labeled cells were either left untreated or treated with IR (20 Gy) for 1 h and harvested. Lysates from control and IR-treated cells were immunoprecipitated with anti-Rb Ab. The phosphorylated proteins were resolved by 10% SDS-PAGE and analyzed by autoradiography and densitometric analyses.

domain of Rb has a Ser/Thr residue flanked by proline (Thr^{821}). To determine whether IR-activated SAPK/JNK can phosphorylate Rb, anti-SAPK/JNK immunoprecipitates were tested using *in vitro* immune complex kinase assays with GST-Rb (full-length, 379–928) as a substrate. The results demonstrated that SAPK/JNK phosphorylates Rb *in vitro* (Fig. 2*A*).

IR Induces Rb Phosphorylation *In Vivo*. Two recent studies show that Rb activation may be associated with apoptosis. First, IR-induced apoptosis in neonatal rat kidney cells is correlated with increased Rb expression (22). Rb phosphorylation is known to regulate the G_1-to-S-phase transition of cells, and a second study showed that tumor necrosis factor-treated adipocytes enter S phase; however, rather than progressing through the cell cycle, these cells undergo apoptosis (23). These two studies suggest that transient activation of Rb may be associated with tumor cell apoptosis. In the present study, we assayed for Rb phosphorylation *in vivo* in response to IR treatment. Cells were labeled with [^{32}P]orthophosphate before treatment with IR for 1 h. Lysates from both untreated and IR-treated cells were controlled for an equal amount of protein by Bradford assays (11) and immunoprecipitated with anti-Rb Ab; proteins were resolved on SDS-PAGE gel. Densitometric analyses demonstrated a threefold increase in phosphorylated Rb in IR-treated cells relative to Rb expression in untreated cells (Fig. 2*B*). Immunoblot analyses of the total cell lysates with anti-tubulin Ab demonstrated equal protein levels in untreated and IR-treated cell lysates (data not shown). These findings are consistent with other reports where Rb phosphorylation precedes cell death or apoptosis (22–24).

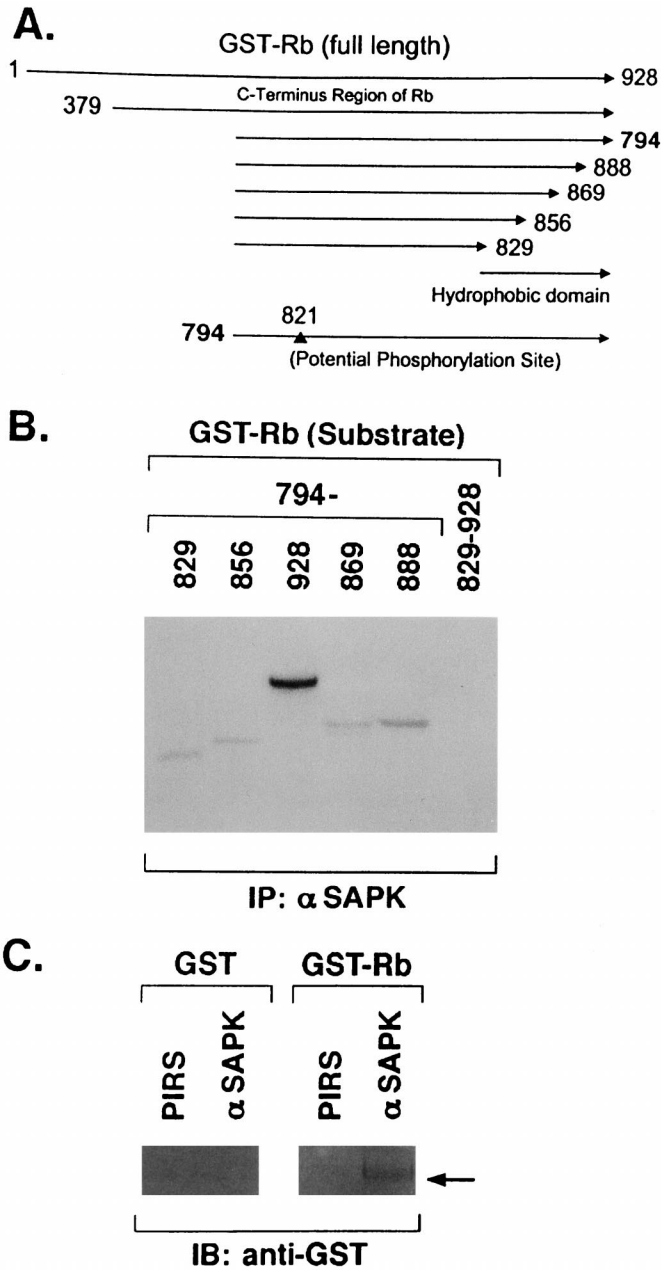

Fig. 3. *A*, schematic representation of GST-Rb fusion constructs. ▲, a potential phosphorylation site for SAPK/JNK in the Rb COOH terminus domain. *B*, differential phosphorylation of GST-Rb deletion constructs by IR-activated SAPK/JNK. Cells were treated with IR (20 Gy) for 1 h and harvested. Lysates from control and IR-treated OCI-MY5 MM cells were immunoprecipitated with anti-SAPK Ab. Immune complex kinase assays were performed by the addition of the indicated GST-Rb constructs and [γ^{32}P]ATP, followed by incubation for 15 min at 30°C. The phosphorylated proteins were resolved by 10% SDS-PAGE and analyzed by autoradiography. *C*, Direct association of SAPK with Rb. Lysates from IR-treated cells were subjected to immunoprecipitation with anti-SAPK Ab and PIRS. Proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membrane. The filters were incubated with purified GST-Rb (full length, 379–928) fusion protein or with purified GST alone (negative control) and then immunoblotted with anti-GST Ab. The *arrow* indicates the molecular weight of SAPK/JNK.

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IR-activated SAPK/JNK Differentially Phosphorylates the GST-Rb Deletion Construct. Having shown that IR-activated SAPK/JNK phosphorylates Rb, we next determined whether deletion of the hydrophobic domain or phosphorylation site in the COOH terminus domain of Rb can abrogate the ability of SAPK/JNK to phosphorylate Rb. OCI-MY5 MM cells were either untreated or treated with IR (20 Gy) for 1 h and harvested. Lysates were immunoprecipitated with anti-SAPK/JNK Ab, and *in vitro* immune complex kinase assays were performed utilizing various GST-Rb deletion fusion constructs as substrates (Fig. 3*A*). The results demonstrate that elimination of phosphorylation site Thr^{821} (GST-829–928) abrogates phosphorylation of Rb by activated SAPK/JNK (Fig. 3*B*). Moreover, sequential deletion of the hydrophobic domain in the COOH terminus of Rb also abrogates the ability of SAPK/JNK to phosphorylate Rb (Fig. 3*B*). Therefore, as with other protein kinases that phosphorylate Rb, SAPK/JNK requires a binding site in the COOH terminus of Rb to phosphorylate Rb. These findings confirm that the IR-induced association between SAPK/JNK and Rb is mediated primarily through the COOH terminus domain of Rb.

SAPK/JNK Directly Associates with Rb. To further examine the direct SAPK/JNK-Rb interaction, anti-SAPK/JNK and PIRS immunoprecipitates from IR-treated OCI-MY5 MM cell lysates were resolved on SDS-PAGE and transferred to nitrocellulose membrane. Two identical filters were obtained: (*a*) the first filter was incubated with purified GST-Rb fusion protein (full length, 379–928; Fig. 3*C*, *right panel*); and (*b*) the second filter was incubated with GST alone (as a negative control Fig. 3*C*, *left panel*). Both filters were then subjected to immunoblot analyses with anti-GST Ab. The appearance of a M_r 46,000 protein (consistent with the molecular weight of SAPK/JNK) in the filter incubated with GST-Rb confirmed the direct association between SAPK/JNK and Rb proteins (Fig. 3*C*). No reactivity was observed in the filter incubated with GST alone (Fig. 3*C*, *left panel*). These findings suggest that IR-activated SAPK/JNK may modulate Rb function by direct binding.

The results of the present study therefore demonstrate that IR-induced apoptotic signaling involves SAPK/JNK-Rb interaction and suggest a potential role of Rb in IR-triggered apoptosis both *in vitro* and *in vivo*. However, it remains unclear how binding of Rb to SAPK/JNK potentiates apoptotic signaling. For example, the interaction of Rb with SAPK may transiently enable cells to enter S phase, thereby increasing their susceptibility to undergo apoptosis. Given a recent report that overexpression of E2F-1 induces apoptosis (25), it is also possible that activated SAPK/JNK phosphorylates Rb, thereby increasing free E2F-1 and inducing apoptosis. Ongoing studies are delineating these and other mechanisms whereby Rb modulates apoptotic signaling.

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