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Ribotoxic Stress Response: Activation of the Stress-Activated Protein Kinase JNK1 by Inhibitors of the Peptidyl Transferase Reaction and by Sequence-Specific RNA Damage to the α-Sarcin/Ricin Loop in the 28S rRNA

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Inhibition of protein synthesis per se does not potentiate the stress-activated protein kinases (SAPKs; also known as cJun NH_2-terminal kinases [JNKs]). The protein synthesis inhibitor anisomycin, however, is a potent activator of SAPKs/JNKs. The mechanism of this activation is unknown. We provide evidence that in order to activate SAPK/JNK1, anisomycin requires ribosomes that are translationally active at the time of contact with the drug, suggesting a ribosomal origin of the anisomycin-induced signaling to SAPK/JNK1. In support of this notion, we have found that aminohexose pyrimidine nucleoside antibiotics, which bind to the same region in the 28S rRNA that is the target site for anisomycin, are also potent activators of SAPK/JNK1. Binding of an antibiotic to the 28S rRNA interferes with the functioning of the molecule by altering the structural interactions of critical regions. We hypothesized, therefore, that such alterations in the 28S rRNA may act as recognition signals to activate SAPK/JNK1. To test this hypothesis, we made use of two ribotoxic enzymes, ricin A chain and α-sarcin, both of which catalyze sequence-specific RNA damage in the 28S rRNA. Consistent with our hypothesis, ricin A chain and α-sarcin were strong agonists of SAPK/JNK1 and of its activator SEK1/MKK4 and induced the expression of the immediate-early genes c-*fos* and c-*jun*. As in the case of anisomycin, ribosomes that were active at the time of exposure to ricin A chain or α-sarcin were able to initiate signal transduction from the damaged 28S rRNA to SAPK/JNK1 while inactive ribosomes were not.

The activity of the stress-activated protein kinases (SAPKs; also known as cJun NH_2-terminal kinases [JNKs]) is stimulated in response to certain kinds of cellular stress, including exposure of cells to short-wavelength UV radiation (11, 19), alkylating DNA-damaging agents (27), the tumor promoters As^{3+} (7) and palytoxin (23), hyperosmotic shock (16), proinflammatory cytokines (24), or withdrawal of a trophic factor (54). SAPKs/JNKs are members of the mitogen-activated protein kinase (MAPK) family of proline-directed serine/threonine protein kinases, which also includes the extracellular signal-regulated kinases (ERKs) and the p38/RK/HOG1 kinase (for a review, see reference 51). Upon activation, SAPKs/JNKs phosphorylate and activate transcription factors such as cJun (11), ATF-2 (17, 49), and Elk-1 (6, 52, 56), leading ultimately to the transcriptional activation of the immediate-early genes c-*fos* and c-*jun* (49, 56). The signal transduction cascades that lead to activation of SAPKs/JNKs and to subsequent gene induction are thought to be associated with stress responses that promote either cell recovery and survival after cellular damage (13, 18, 41) or, in some instances, apoptotic death (8, 54). The activity of SAPKs/JNKs is regulated through their phosphorylation on both threonine and tyrosine residues in the motif T*PY* by the dual-specificity protein kinase SEK1/MKK4 (12, 26, 40). The protein kinase MEKK1 (25), in turn, activates SEK1/MKK4 through phosphorylation of serine 219 and threonine 223 (55). The mode of regulation of MEKK1 (and of other potential SEK1/MKK4 kinases) by cellular stress remains unclear, but it is thought that the SEK1/MKK4-SAPK/JNK cascade is controlled through activation of small GTP-binding proteins, including Ras (11), Cdc42, Rac1 (9), and Rho (47). The set of GTPases that regulate SAPKs/JNKs seems to be cell type specific (47).

The antibiotic anisomycin inhibits the eukaryotic peptidyl transferase reaction (36, 50) and is a potent agonist of SAPKs/JNKs (56) and other cellular protein kinases, such as MAPKAP-2 (3). Efficient kinase activation was achieved with concentrations of anisomycin that inhibited protein synthesis by less than 50% (4, 56); it was therefore concluded that anisomycin activates protein kinases independently of its ability to inhibit protein synthesis (4). Here, we provide evidence that ribosomes that are functional at the time of contact with anisomycin are involved in the anisomycin-induced signal transduction to SAPK/JNK1. The binding site for anisomycin in the ribosome is located in the 28S rRNA (21, 39, 45) in a region that has been suggested to be part of the peptidyl transferase center (2, 39). It was our hypothesis, therefore, that the 28S rRNA may play a crucial role in initiating signal transduction from the ribosome to SAPK/JNK1. To directly test this hypothesis, we took advantage of the fact that two ribotoxic enzymes, ricin A chain and α-sarcin, catalyze highly specific RNA damage within a conserved loop, the α-sarcin/ricin (S/R) loop, of the 28S rRNA (53). We asked whether ricin A chain and α-sarcin could activate SAPK/JNK1 and its activator, SEK1/MKK4, and found that this was indeed the case. Like anisomycin, ricin A chain and α-sarcin required the presence of actively translating ribosomes in order to activate SEK1/MKK4 and SAPK/JNK1.

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MATERIALS AND METHODS

Cell culture. Rat-1 cells were maintained as previously described (28). All experiments presented here were performed with confluent, quiescent cultures obtained through serum deprivation for typically 48 h.

Chemicals and ribotoxins. Anisomycin, puromycin, cycloheximide, emetine, T-2 toxin, gougerotin, RCA_{60}, α-sarcin, *Pseudomonas* exotoxin A, and diphtheria toxin were from Sigma Chemical Company, St. Louis, Mo. Pactamycin was a generous gift from the Upjohn Company, Kalamazoo, Mich. Blasticidin S was from Calbiochem, San Diego, Calif. Before use, puromycin, cycloheximide, and blasticidin S were dissolved freshly in double-distilled H_2O. Anisomycin, emetine, pactamycin, T-2 toxin, and gougerotin were dissolved in $(H_3C)_2SO$. α-Sarcin, *Pseudomonas* exotoxin A, and diphtheria toxin were reconstituted in double-distilled H_2O at 1 mg/ml in accordance with the manufacturer's instructions. In all the cases in which $(H_3C)_2SO$ was used as a vehicle, corresponding control cells received the same amount of the vehicle alone (typically not more than 0.2% [vol/vol]). All radiochemicals were from DuPont NEN Research Products, Boston, Mass.

SAPK/JNK1 immunoprecipitation and immunocomplex kinase assay. Rat-1 cells from a 6-cm-diameter tissue culture dishes were harvested by lysis in a solution containing 20 mM HEPES-KOH (pH 7.4), 2 mM EGTA, 50 mM β-glycerophosphate, 1 mM dithiothreitol (DTT), 10% glycerol, 1% Triton X-100, 1 mM sodium vanadate, 1 μM microcystin, 1 mM phenylmethylsulfonyl fluoride, 1 μg of aprotinin per ml, and 1 μg of leupeptin per ml. SAPK/JNK1 was immunoprecipitated for 3 h at 4°C with an anti-JNK1 antibody (sc-474; Santa Cruz Biotechnology Inc., Santa Cruz, Calif.) precoupled to protein A-agarose (Santa Cruz Biotechnology). The immunoprecipitates were washed once with lysis buffer, once with a solution consisting of 100 mM Tris-HCl (pH 7.6), 500 mM LiCl, 1 mM DTT, and 0.1% Triton X-100, and once with a buffer containing 20 mM morpholinepropanesulfonic acid (MOPS; pH 7.2), 10 mM $MgCl_2$, 2 mM EGTA, 1 mM DTT, and 0.1% Triton X-100. For the kinase reaction, the immunoprecipitates were incubated with 1 μg of either glutathione *S*-transferase (GST)-Elk1 (38) or GST-cJun (24) fusion proteins in the presence of 10 mM MOPS (pH 7.2), 20 mM $MgCl_2$, 1 mM EGTA, 0.5 mM DTT, 0.05% Triton X-100 and 1 μCi [γ-^{32}P]ATP for 20 min at 30°C. After the reactions were stopped by adding 10 μl of 4× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer, the samples were resolved by SDS–13% PAGE. The phosphorylated GST-Elk1 was quantified from dried gels with a Molecular Dynamics PhosphorImager and IP Lab Gel software.

Western blot analysis of SEK1/MKK4. Rat-1 cells from a 10-cm-diameter tissue culture plate were lysed in 500 μl of boiling 2× SDS-PAGE loading buffer. The lysates were sheared by passing them five times through a 27-gauge needle, separated via SDS–13% PAGE, and transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, Mass.). The activation of SEK1/MKK4 was demonstrated in a Western blot with an antibody directed against the SEK1/MKK4 protein phosphorylated at Thr-223 (no. 9151S; New England BioLabs Inc., Beverly, Mass.) in accordance with the manufacturer's instructions.

Northern blot analysis of RNA. Rat-1 cells were harvested from two 10-cm-diameter tissue culture dishes per experimental point, and total cellular RNA was extracted, separated by electrophoresis, and analyzed by the Northern blotting procedure as previously described (28). The hybridization probes for c-*fos*, c-*jun*, and cyclophilin are described elsewhere (37).

Measurement of protein synthesis via [^{3}H]leucine incorporation. Rat-1 cells, grown in 12-well tissue culture plates, were leucine deprived by placing them in 1 ml of Dulbecco modified Eagle medium lacking leucine (DMEM/−Leu) for 1 h before treatment with different inhibitors of translation. At various times (as indicated in the text and in the figure legends), the cells were pulse-labeled for 5 min with 2 μCi of [^{3}H]leucine in 50 μl of DMEM/−Leu. The incorporation of [^{3}H]leucine was stopped by adding 1 ml of 10% trichloroacetic acid (TCA). After the cells were washed four times with 5% TCA, the TCA-insoluble proteins were solubilized in 250 μl of 88% HCOOH. A 200-μl aliquot of each specimen was transferred into a scintillation vial, 4 ml of aqueous scintillation liquid was added to each vial, and the samples were counted in a scintillation counter. In each experiment, three wells were used per experimental point.

Delivery of α-sarcin, diphtheria toxin, gougerotin, and blasticidin S into Rat-1 cells via lipofection. Lipofection was performed in 6-cm-diameter tissue culture dishes whenever SAPK/JNK1 activity was assayed (2 ml of lipofection mix) and in 12-well tissue culture plates whenever [^{3}H]leucine incorporation was measured (480 μl of lipofection mix). The following protocol was used for preparation of 480 μl of lipofection mix: 6 μl of Lipofectin Reagent (Gibco BRL/Life Technologies, Gaithersburg, Md.) was gently mixed with 104 μl of DMEM (serum and antibiotic free) in polystyrene tubes. Ten microliters of α-sarcin (1 mg/ml) or diphtheria toxin (1 mg/ml) or five microliters of gougerotin (30 mM) or blasticidin S (30 mM) was added, and after gentle mixing, the protein-lipid vesicles were allowed to form for 10 min at room temperature. The mixture was diluted with 360 μl (365 μl in the case of antibiotics) DMEM (serum and antibiotic free) and applied onto cells from which the old medium had been removed completely. Control cells were treated the same way except that the toxins and antibiotics were omitted from the lipofection mix. For the lipofection of cells in 6-cm-diameter dishes, the lipofection mix was scaled up to a final volume of 2 ml.

Reverse transcription of rRNA by primer extension. Reverse transcription of rRNA was performed as described in reference 20, with modifications. Crude oligonucleotide primer (5′-CACATACACCAAATGTC-3′; Genosys Biotechnologies, Inc., The Woodlands, Tex.) was end labeled with T4 polynucleotide kinase (Gibco BRL/Life Technologies) and then purified electrophoretically by using the Full-Lengther apparatus (BioKey/Cascade Biologics, Portland, Oreg.). For primer extension, a 10-μl mixture of 2 μg of total RNA plus 0.5 to 1.0 pmol of primer in 50 mM Tris-HCl (pH 8.3)–75 mM KCl–3 mM $MgCl_2$ was heated for 3 min at 90°C, placed on ice for 5 min, and then incubated at room temperature for 5 min before initiation of reverse transcription by addition of 10 μl of a mixture containing 2 mM deoxynucleoside triphosphates (dNTPs) and 30 U of reverse transcriptase (Superscript; Gibco BRL/Life Technologies) in 50 mM Tris-HCl (pH 8.3)–75 mM KCl–3 mM $MgCl_2$–10 mM DTT. Following 15 min of incubation at 48°C, the reactions were stopped by adding EDTA to 5 mM. Reaction products were precipitated in ethanol in the presence of 1 to 2 μg of glycogen, resuspended in formamide gel loading buffer, heat denatured, and electrophoresed in 8% acrylamide sequencing gels, which were subsequently dried and exposed to film and/or a PhosphorImager screen. The sequence of the primer-extended transcript was determined by carrying out 10-μl dideoxynucleotide sequencing reactions. rRNA and primer were annealed as indicated above but in half the volume. Primer extension for 15 min at 48°C included 0.1 mM dNTPs and either 0.5 mM ddGTP, 1.0 mM ddATP, 0.5 mM ddTTP, or 1.0 mM ddCTP; this was followed by a 5-min chase with 1.0 mM dNTPs.

RESULTS

Uncoupling of anisomycin-induced protein synthesis inhibition and activation of SAPK/JNK1. In order to understand the mechanism of the anisomycin-induced SAPK/JNK1 activation in Rat-1 cells, we did studies to determine whether this activation correlates with the inhibition of protein synthesis. To this end, two experimental approaches were chosen.

First, we treated Rat-1 cells with different concentrations of anisomycin and monitored both the inhibition of protein synthesis (as measured by incorporation of [^{3}H]leucine) and the degree of SAPK/JNK1 activation (Fig. 1). The activity of SAPK/JNK1 was examined in immunocomplex kinase assays using bacterially expressed GST-Elk1 (38) (Fig. 1; see also Fig. 2, 3b, 4b, 6, and 7) or GST-cJun (24) (see Fig. 3c) fusion proteins as substrates for phosphorylation (see Materials and Methods). Half-maximal activation of SAPK/JNK1 was achieved at a concentration of anisomycin that inhibited less than 10% of the protein synthesis (60 ng/ml [Fig. 1a]). Thus, in Rat-1 cells, anisomycin was able to activate SAPK/JNK1 significantly without severely affecting translation.

Second, we determined the extent to which inhibition of protein synthesis per se affects the activity of SAPK/JNK1. To this end, we treated Rat-1 cells with inhibitors of either translational initiation (pactamycin and T-2 toxin) or translational elongation (anisomycin, cycloheximide, puromycin, and emetine) and compared the abilities of these inhibitors to activate SAPK/JNK1. The modes of action of these translational inhibitors are summarized in Fig. 2a. At concentrations sufficient to inhibit [^{3}H]leucine incorporation by more than 95% (Fig. 2b), there was no correlation between the potentials of these agents to inhibit protein synthesis and their potentials to activate SAPK/JNK1. Of the ribosomal inhibitors tested, anisomycin was the most potent in activating the kinase (typically more than 20-fold activation [Fig. 2c]), and it was the only agent that activated SAPK/JNK1 significantly within 15 min of addition (>15-fold activation [Fig. 2c]). Cycloheximide, puromycin, and T-2 toxin appeared to be weaker activators (between three- and eightfold activation [Fig. 2c]). Most importantly, pactamycin and emetine completely failed to activate SAPK/JNK1 (Fig. 2c).

Functional ribosomes are required for anisomycin-induced activation of SAPK/JNK1. One possible explanation for the foregoing results was that the activation of SAPK/JNK1 by anisomycin is independent of the action of the drug on ribosomes. This question was addressed experimentally by deter-

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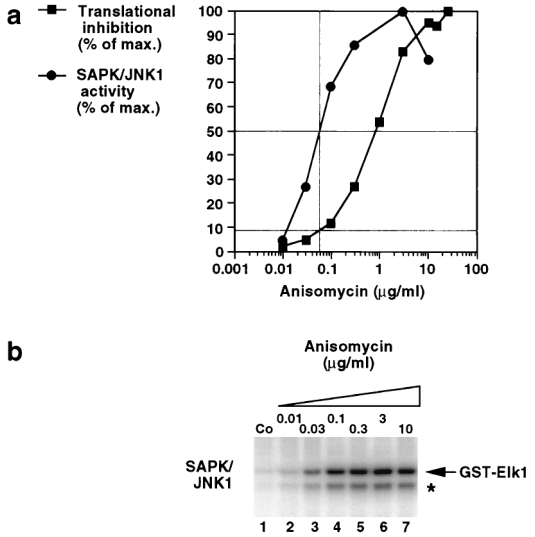

FIG. 1. Anisomycin-induced inhibition of translation and activation of SAPK/JNK1 in Rat-1 cells. Cells were treated with anisomycin at the indicated concentrations for 15 min and then either pulse-labeled with [^{3}H]leucine for 5 min or harvested for SAPK/JNK1 immunocomplex kinase assays as described in Materials and Methods. (a) Graphic representation of translational inhibition and SAPK/JNK1 activity as functions of the concentration of anisomycin. max., maximum. (b) SAPK/JNK1 immunocomplex kinase assay using GST-Elk1 fusion protein as a substrate for phosphorylation. The gel represents the data showed in panel a. The position of the full-length GST-Elk1 fusion protein (amino acid residues 307 to 428, representing the transcriptional activation domain of the human Elk-1 [38]) is marked by an arrow. The intensity of the lower phosphorylated band (marked ∗) varies in different preparations of GST-Elk1 (compare panel b with Fig. 4b). Since it is not present in preparations from bacteria expressing only GST (data not shown), and since its phosphorylation correlates with the phosphorylation pattern of the full-length GST-Elk1, it most likely represents a proteolytic fragment of the latter that contains the Elk-1 phosphoacceptor sites and the recognition sequence for the binding of SAPK/JNK1. Co, control.

mining whether a prior inactivation of the ribosomal function by another antibiotic (that does not activate SAPK/JNK1) could diminish the response of the kinase to anisomycin. Two distinct approaches were chosen for ribosomal inactivation: (i) inhibition of translational initiation by pretreatment of cells with pactamycin or T-2 toxin, which results in disintegration of the active polysomes into 60S and 40S ribosomal subunits and unprogrammed 80S monosomes; and (ii) inhibition of translational elongation by pretreatment with emetine, which results in preserved but inactive polysomes (36).

First we confirmed by sucrose gradient analysis (28) that treatment of Rat-1 cells with the inhibitors of translational initiation pactamycin (0.2 μg/ml for 30 min) and T-2 toxin (10 μg/ml for 30 min) resulted in disintegration of the active polysomes (data not shown). [^{3}H]leucine incorporation 30 min after treatment with either pactamycin or T-2 toxin was inhibited by 98% (data not shown). Pactamycin pretreatment completely abrogated the activation of SAPK/JNK1 by anisomycin (Fig. 3a; compare lanes 7 to 10 to lanes 12 to 15). In contrast, following pactamycin pretreatment, the activation of SAPK/JNK1 by the proinflammatory cytokine interleukin-1α (IL-1α) was not only preserved but was even prolonged in time (Fig. 3a; compare lanes 17 to 20 to lanes 22 to 25). This indicated that pactamycin pretreatment did not generally diminish the responsiveness of SAPK/JNK1 but specifically inhibited the response to anisomycin. Similarly, pretreatment of cells with T-2 toxin did not decrease the responsiveness of SAPK/JNK1 to IL-1α (Fig. 3b; compare lanes 5 and 6) but abrogated the activation of SAPK/JNK1 by anisomycin (Fig. 3b; compare lanes 3 and 4).

As expected, treatment of cells for various lengths of time (from 10 min to 2 h) with emetine (100 μg/ml), an inhibitor of ribosomal translocation, failed to change the polysomal profiles in Rat-1 cells (data not shown). However, the observed polysomes were nonfunctional, as the [^{3}H]leucine incorporation was inhibited by 98% (data not shown and Fig. 3c, upper panel). Importantly, emetine at 100 μg/ml completely blocked translation within 1 min after addition (Fig. 3c, upper panel). Treatment of cells with emetine for 2 min before addition of either anisomycin or IL-1α abrogated the responsiveness of SAPK/JNK1 to anisomycin (Fig. 3c; compare lanes 3 and 4) but did not diminish the activation of the kinase by IL-1α (Fig. 3c, bottom panel; compare lanes 5 and 6). If emetine was given 2 min after the treatment of cells with either anisomycin or IL-1α, SAPK/JNK1 responded well to IL-1α but the responsiveness of the kinase to anisomycin was restored as well (Fig. 3c, bottom panel; compare lanes 9 and 10 and lanes 11 and 12). These results indicate that the emetine-sensitive (presumably ribosomal) step of the anisomycin-induced signal transduction to SAPK/JNK1 occurs within the first 2 min after addition of anisomycin. Furthermore, these results suggest that the abrogation of anisomycin-induced activation of SAPK/JNK1 by pretreatment with ribosomal inhibitors probably did not result from a rapid turnover of a labile protein that participates in the signal transduction cascade used by anisomycin; if such a labile protein(s) exists, its level in the cell must decrease significantly within the first minutes after initiation of the translational block. On the basis of the ability of the ribosomal inactivators tested to inhibit the responsiveness of SAPK/JNK1 to anisomycin while not impeding the IL-1α-induced activation of the kinase, we concluded that the transduction of the anisomycin-initiated signal to SAPK/JNK1 requires the presence of ribosomes actively engaged in translation.

Activation of SAPK/JNK1 by APNAs. The binding site for anisomycin is located in domain V of the 28S rRNA (21, 39, 45) (Fig. 4a). (The nomenclature of the domains is derived from that of the *Escherichia coli* 23S rRNA as in reference 33). The same region has been proposed to be part of the ribosomal peptidyl transferase center (for reviews, see references 2 and 39). It is possible, therefore, that binding of anisomycin to its cognate sequence in the 28S rRNA causes alterations in the RNA molecule that interfere with the peptidyl transferase reaction on one hand and serve as a recognition signal for activation of SAPK/JNK1 on the other hand. In search of arguments in support of this hypothesis, we noticed that the same region of the 28S rRNA is also the ribosomal target for the aminohexose pyrimidine nucleoside antibiotic (APNA) blasticidin S (39) (Fig. 4a). Like anisomycin, blasticidin S and other members of the APNA family specifically inhibit the peptidyl transferase reaction (36, 50) (Fig. 2a). If alterations in the 28S rRNA, induced by binding of an antibiotic, could account for both the inhibition of translation and the activation of SAPK/JNK1, then treatment of cells with APNAs should also result in SAPK/JNK1 activation. Therefore, we treated cells with either blasticidin S or gougerotin, a structurally related APNA, and monitored the activity of SAPK/JNK1. Both antibiotics (300 μM each) appeared to require inclusion in lipid vesicles (lipofection; see Materials and Methods) in order to be efficiently delivered into Rat-1 cells (as measured by inhibition of translation) (data not shown). Like anisomycin, both blasticidin S and gougerotin potently induced an early (15 min after addition) and persistent activation of SAPK/JNK1 (Fig. 4b). Furthermore, pretreatment of cells with emetine

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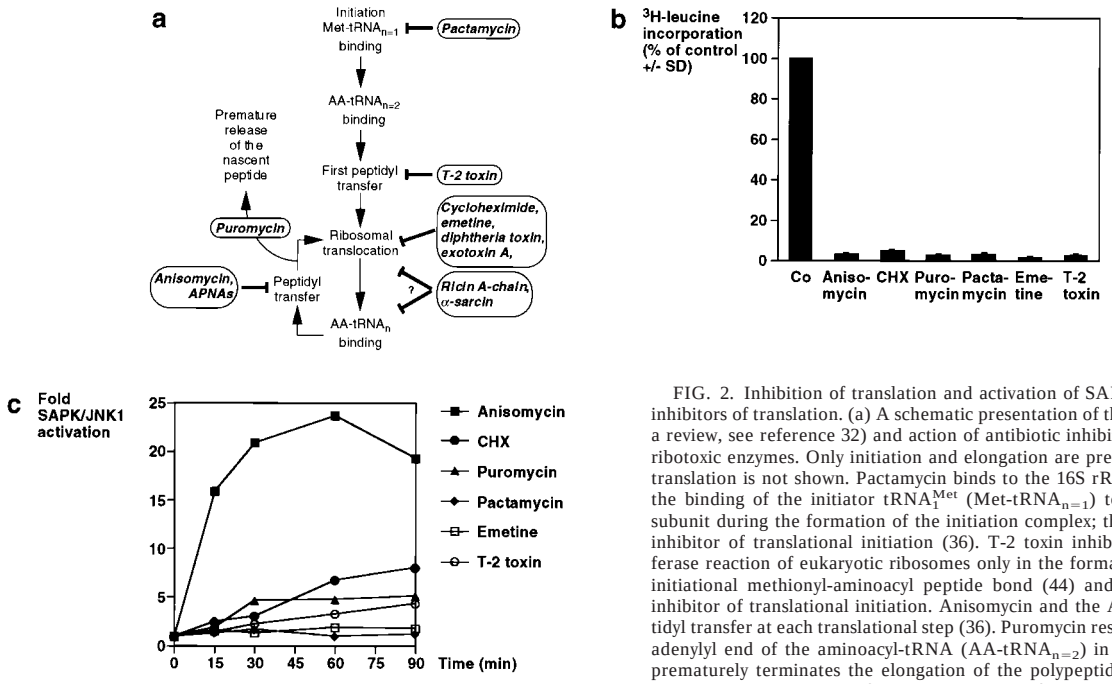

FIG. 2. Inhibition of translation and activation of SAPK/JNK1 by antibiotic inhibitors of translation. (a) A schematic presentation of the ribosomal cycle (for a review, see reference 32) and action of antibiotic inhibitors of translation and ribotoxic enzymes. Only initiation and elongation are presented; termination of translation is not shown. Pactamycin binds to the 16S rRNA (15) and prevents the binding of the initiator $tRNA_1^{Met}$ (Met-$tRNA_{n=1}$) to the small ribosomal subunit during the formation of the initiation complex; therefore, it is a potent inhibitor of translational initiation (36). T-2 toxin inhibits the peptidyl transferase reaction of eukaryotic ribosomes only in the formation of the first, post-initiational methionyl-aminoacyl peptide bond (44) and therefore acts as an inhibitor of translational initiation. Anisomycin and the APNAs block the peptidyl transfer at each translational step (36). Puromycin resembles the aminoacyl-adenylyl end of the aminoacyl-tRNA (AA-$tRNA_{n=2}$) in the A site. It thereby prematurely terminates the elongation of the polypeptide chain by competing with the aminoacyl-tRNA for the peptidyl transfer (36). Cycloheximide inhibits translation by preventing the release of the deacylated tRNA after the peptidyl transferase reaction has taken place, thus abrogating the translocation of the peptidyl-tRNA from the A to the P site (36). 80S ribosomes treated with emetine are deficient in translocation (36, 50). The modes of action of the ribotoxic enzymes used are explained in the text. (b) Cells were treated in triplicate for 10 min with anisomycin (10 μg/ml), cycloheximide (CHX; 25 μg/ml), puromycin (75 μg/ml), pactamycin (0.2 μg/ml), emetine (10 μg/ml), or T-2 toxin (10 μg/ml) and then pulse-labeled with [^{3}H]leucine for another 5 min. Co, control; SD, standard deviation. (c) Cells were treated for 15, 30, 60, or 90 min with each of the antibiotics used in the experiment shown in panel b and at identical concentrations. Cells were then harvested and processed for SAPK/JNK1 immunocomplex kinase assays as described for Fig. 1b. Quantitative representation is shown, obtained from PhosphorImager analysis.

abolished the activation of SAPK/JNK1 by either blasticidin S or gougerotin (data not shown), just as it did to the activation of SAPK/JNK1 by anisomycin (Fig. 3c). These findings are consistent with the notion that inhibitors of the peptidyl transferase reaction initiate signal transduction to SAPK/JNK1 via specific binding to a common cognate sequence in the 28S rRNA.

Activation of SAPK/JNK1 by ricin A chain and α-sarcin. We next tested the hypothesis that the 28S rRNA is involved in signaling to SAPK/JNK1 by using an experimental approach that is independent of the binding of antibiotic inhibitors of translation and that is based on two highly specific enzymatic reactions. The ribotoxic enzymes ricin A chain and α-sarcin specifically damage the S/R loop in the 28S rRNA (Fig. 4a). This fact enabled us to test experimentally whether RNA damage and/or conformational changes in the 28S rRNA may constitute an initiation event for a signal transduction to SAPKs/JNKs. Ricin A chain is an RNA N-glycosidase that depurinates a single adenosine at position A4324 of the 28S rRNA (see reference 52 and references therein) (Fig. 4a). The natural source of ricin A chain is the lectin RCA_{60} from the castor plant *Ricinus communis*. RCA_{60} is a dimer of the ricin A and B chains, the latter being required for the delivery of the active (ribotoxic) A chain to the cell interior (for a review, see reference 35). Therefore, RCA_{60} was used to deliver the toxin into Rat-1 cells. First, we developed an experimental assay to monitor the in vivo ricin A chain-induced depurination of A4324 based on lesion-induced arrest of a reverse transcriptase-driven primer extension (see Materials and Methods). The reverse transcription on ricin A chain-damaged 28S rRNA terminates abruptly at position G4323, which is located 5′ of the depurinated A4324. Treatment of cells with RCA_{60} resulted in substantial and precise damage to A4324 (Fig. 5; compare lane 6 to lanes 7 to 11) and potently inhibited protein synthesis as measured by [^{3}H]leucine incorporation (Fig. 6a). The specificity of the ricin A chain-induced lesion to A4324 was confirmed by using six primers that hybridize to different regions of the 28S rRNA; they failed to detect any other site of damage (data not shown). Consistent with our hypothesis, the ricin A chain strongly activated SAPK/JNK1 (Fig. 6b; Fig. 6c, lanes 2 to 5). This activation was contemporaneous with the ricin A chain-induced phosphorylation at threonine 223 of SEK1/MKK4 (Fig. 6d, lanes 2 to 5), a marker for MKK4/SEK1 activation (55). The depurination of A4324 became apparent 15 min after the RCA_{60} treatment and reached a maximum at 30 min posttreatment (Fig. 6e, upper panel). Both the phosphorylation of SEK1/MKK4 and the activation of SAPK/JNK1 were observed at 30 min but not at 15 min (Fig. 6e, middle and lower panels, respectively).

α-Sarcin is an RNA endonuclease from *Aspergillus giganteus* that in intact ribosomes selectively cleaves the phosphodiester bond on the 3′ side of position G4325, adjacent to the A4324 that is depurinated by the ricin A chain (see reference 52 and references therein) (Fig. 4a). The delivery of α-sarcin into Rat-1 cells required its inclusion in lipid vesicles (lipofection; see Materials and Methods). Lipofected α-sarcin proved to be an effective inhibitor of protein synthesis (Fig. 6a) and activated both SEK1/MKK4 (Fig. 6d, lanes 7 to 10) and SAPK/JNK1 (Fig. 6b; and Fig. 6c, lanes 8 to 11). Neither ricin A chain nor α-sarcin appeared to activate the extracellular signal-regulated kinase ERK1 (data not shown).

Based on the structural and the functional homologies between the *E. coli* 23S rRNA and the eukaryotic 28S rRNA, it is believed that elongation factor 2 (EF-2)-dependent ribosomal translocation is mediated by the binding of EF-2 to the

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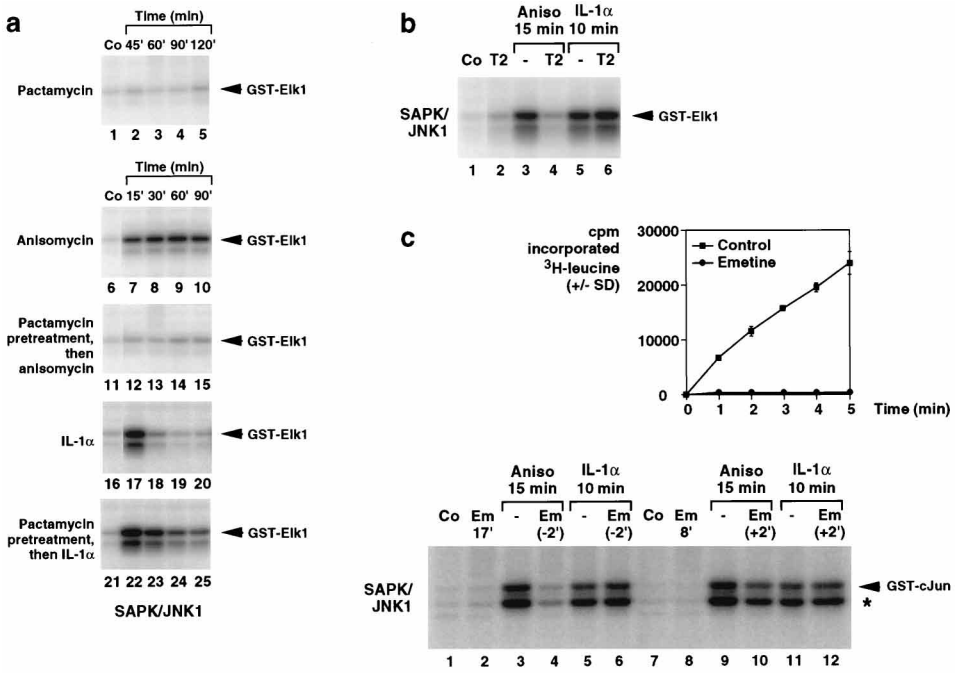

FIG. 3. Inhibition of the anisomycin-induced activation of SAPK/JNK1 by pretreatment of Rat-1 cells with pactamycin, T-2 toxin, or emetine. (a) Cells were left untreated or were pretreated, as indicated at the left side of each panel, with pactamycin (0.2 μg/ml) for 30 min and then stimulated as indicated with anisomycin (10 μg/ml) or IL-1α (25 ng/ml) for 15, 30, 60, or 90 min. The upper panel represents the effect of pactamycin alone on the activity of SAPK/JNK1 at all time points used in the lower panels. (Note that in Fig. 3a and 6c, some of the control lanes [Co] have been presented more than once for easier comparison. Each control lane, however, is matched to its properly corresponding experimental lanes.) (b) Cells were left untreated or were pretreated as indicated with T-2 toxin (T2; 10 μg/ml) for 30 min. Cells were then stimulated as indicated with either anisomycin (Aniso) or IL-1α at the concentrations used for the experiment shown in panel a for 15 min. (c) Upper panel: cells (in triplicate) were left untreated (control) or were treated with emetine (100 μg/ml) and then immediately exposed to a pulse of [^{3}H]leucine for 1, 2, 3, 4, or 5 min. SD, standard deviation. Lower panel: cells were treated as indicated with anisomycin (Aniso) or IL-1α as described for panel b. Emetine (100 μg/ml) was given as indicated either 2 min before [Em (−2′)] or 2 min after [Em (+2′)] the respective agonist. Just as with GST-Elk1 (see the legend to Fig. 1b), GST-cJun preparations display a second phosphorylated band (*) which most likely represents a proteolytic fragment containing the SAPK/JNK1 recognition sequence and the phosphoacceptor sites (serines 63 and 73 [19]) of cJun.

S/R loop (53), similar to the binding of EF-G, the *E. coli* homolog of EF-2, to the same loop in the 23S rRNA (31). The ribotoxic enzyme from *Corynebacterium diphtheriae* (diphtheria toxin) and exotoxin A from *Pseudomonas aeruginosa* inactivate EF-2 by causing its specific ADP-ribosylation (for a review, see reference 35). The ADP-ribosylated EF-2 loses its affinity for the pretranslocational ribosome and cannot catalyze the ribosomal translocation, thus leading to cessation of translation (43) (Fig. 2a). As the ADP-ribosylation of EF-2 interferes with the function of the S/R loop without causing RNA damage, we asked whether treatment of Rat-1 cells with diphtheria toxin and *Pseudomonas* exotoxin A could also activate SAPK/JNK1. Because mouse and rat cells lack functional diphtheria toxin receptors (30), we delivered the toxin into Rat-1 cells via lipofection, which effectively inhibited protein synthesis (Fig. 6a). *Pseudomonas* exotoxin A inhibited translation without requiring a vehicle for delivery (Fig. 6a). These two toxins differed significantly from ricin A chain and α-sarcin in their ability to activate SAPK/JNK1; *Pseudomonas* exotoxin A completely failed to activate the kinase (Fig. 6b; Fig. 6c, lanes 13 to 16), and diphtheria toxin reproducibly caused a detectable but only marginal activation (Fig. 6b; Fig. 6c, lanes 19 to 22; Fig. 7c, lanes 3 and 8). The ability of ricin A chain and α-sarcin, but not of diphtheria toxin and *Pseudomonas* exotoxin A, to strongly activate SAPK/JNK1 is consistent with our hypothesis that RNA damage to the S/R loop initiates signal transduction to SAPK/JNK1.

Ricin A chain and α-sarcin require functional ribosomes in order to activate SAPK/JNK1. If ricin A chain and α-sarcin activate SAPK/JNK1 through pathways similar to that of anisomycin, it would be expected that both ribotoxins would also require active ribosomes for the activation. Just as with anisomycin, in Rat-1 cells pretreated with pactamycin, the activation of SAPK/JNK1 by both ricin A chain and α-sarcin was severely reduced (Fig. 7a; compare lanes 4 and 5 and lanes 6 and 7). Furthermore, cells arrested in their elongation cycle by pretreatment with emetine were also unable to activate SAPK/JNK1 in response to ricin A chain and were significantly hampered in their ability to respond to α-sarcin (Fig. 7b; compare lanes 3 and 4 and lanes 7 and 8). A possible explanation for the ability of pactamycin and emetine to interfere with the ricin A chain- and α-sarcin-induced SAPK/JNK1 activation is that the inactivated ribosomes were not susceptible to RNA damage by ricin A chain or α-sarcin. The validity of this explanation was tested by exposing cells to either pactamycin or emetine prior to exposure to ricin A chain. As shown in Fig. 5, lanes 12 to 15, pretreatment of cells with pactamycin or emetine failed to inhibit the ricin A chain-induced A4324 depurination.

Consistent with the results obtained with pactamycin or emetine, in cells pretreated with diphtheria toxin, neither ricin A chain nor α-sarcin was able to activate SAPK/JNK1 substantially above the low level of activation induced by diphtheria toxin alone (Fig. 7c; compare lanes 3 to 5 and lanes 8 to 10), confirming that active ribosomes are required for the activation of SAPK/JNK1 by the two ribotoxins.

Activation of c-*fos* and c-*jun* expression by damage to the S/R loop. Potentiation of MAPK by anisomycin activates the transcription of the immediate-early genes c-*fos* and c-*jun* (56).

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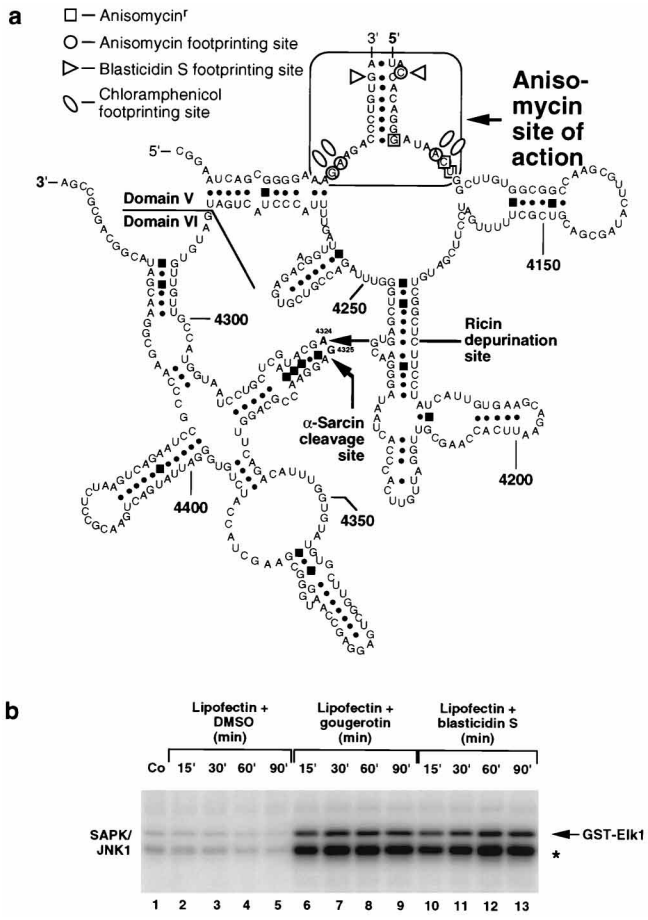

FIG. 4. (a) Secondary structure of the region of the 28S rRNA targeted by anisomycin (domain V) and by the ribotoxin enzymes ricin A chain and α-sarcin (domain VI) as presented in reference 53, with modifications reflecting recent data on the conformation of the S/R loop (46). Watson-Crick pairing is denoted by black dots; non-Watson-Crick pairing is denoted by black squares. Within the large shaded square is contained the portion of rRNA considered to be the site of action of anisomycin. The nucleotides subject to substitutions in the anisomycin-resistant mutants of *Tetrahymena* 28S rRNA and *Halobacterium* 23S rRNA (21, 45) are denoted by open squares. Open circles represent conserved nucleotides protected by anisomycin from chemical modifications in vitro (39). (b) Activation of SAPK/JNK1 by the APNAs gougerotin and blasticidin S. Rat-1 cells were treated with the antibiotics (300 μM each) for the indicated periods of time, and SAPK/JNK1 activity was assayed essentially as described in the legend to Fig. 2c except that the antibiotics were delivered via lipofection (see Materials and Methods). The asterisk indicates the same thing as in Fig. 1b. Co, control; DMSO, dimethyl sulfoxide.

When added in combination with activating growth factors, anisomycin augments and prolongs the accumulation of immediate-early gene mRNA, a phenomenon known as superinduction (14, 29). We tested whether RNA damage in the S/R loop, together with epidermal growth factor (EGF), could induce and superinduce the expression of c-*fos* and c-*jun* mRNA. Northern blot analyses in Rat-1 cells showed that the ricin A chain induced the accumulation of c-*fos* and c-*jun* mRNA when applied alone (Fig. 8, lanes 2 to 6) and strongly superinduced their accumulation when applied together with EGF (Fig. 8; compare lanes 7 to 9 with lanes 10 to 12).

DISCUSSION

Inhibition of protein synthesis per se does not activate SAPK/JNK1. Using eight antibiotic ribosomal inhibitors and four ribotoxic enzymes, we have demonstrated that at concentrations sufficient to impair [^{3}H]leucine incorporation by more than 90%, the inhibitors of translation differ significantly in their ability to activate SAPK/JNK1 (Fig. 2, 6, and 7) and that inhibition of protein synthesis per se cannot account for the SAPK/JNK1 activation. Recently, similar results demonstrating that anisomycin, but not cycloheximide and emetine, is a potent inducer of SAPK/JNK1 and SAPK/JNK2 activities in NIH 3T3 cells were reported (42). Therefore, the mechanisms by which some translational inhibitors activate SAPK/JNK1 are likely to be understood on the basis of the molecular alterations these agents cause in the ribosome. In an attempt to characterize one such mechanism, we concentrated on the binding of anisomycin to the 28S rRNA and on the 28S rRNA-damaging capacity of the ribotoxins ricin A chain and α-sarcin.

The 28S rRNA as a sensor for ribotoxic stress. Is there evidence to support the notion that the 28S rRNA is the sensor for anisomycin-induced ribotoxic stress and is implicated in the activation of SEK1/MKK4 and SAPK/JNK1 by anisomycin? We made use of APNAs, which, like anisomycin, inhibit the peptidyl transferase reaction (36 and 50) (Fig. 2a) and bind to the same region of domain V of 28S rRNA (39) (Fig. 4a). Blasticidin S and gougerotin, members of the APNA family, caused SEK1/MKK4 phosphorylation (data not shown) and activation of SAPK/JNK1 (Fig. 4b) as potently as anisomycin. Anisomycin and APNAs, although structurally dissimilar, bind to the same region of the 28S rRNA. Both require functional ribosomes to activate SAPK/JNK1 (Fig. 3 and data not shown). Therefore, it appears likely that the signal for activation of SAPK/JNK1 in response to either anisomycin or APNAs originates in their shared binding site in the ribosome.

The most convincing support for the hypothesis that the 28S rRNA serves as a sensor for ribotoxic stress came from experiments showing that nucleotide-specific RNA damage to the

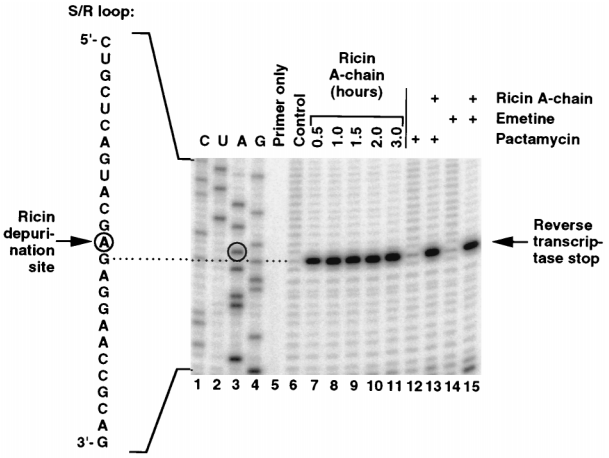

FIG. 5. Detection of ricin A chain-induced depurination of A4324 in vivo via reverse transcription of 28S rRNA. Rat-1 cells were left untreated or were treated with RCA_{60} (10 μg/ml) for the indicated periods of time. Lanes 6 to 11, cells were treated with RCA_{60} for up to 3 h; lanes 12 to 15, cells were either treated with RCA_{60} alone for 30 min or were pretreated with pactamycin (0.2 μg/ml) or emetine (10 μg/ml) for 30 min prior to exposure to RCA_{60} (as indicated). Total RNA was prepared and reverse transcriptase-driven primer extension was performed as described in Materials and Methods. The reverse transcriptase stop site is indicated by an arrow, and its exact position was determined via reverse transcriptase-mediated RNA sequencing (see Materials and Methods), shown in lanes 1 to 4 (dotted line). (The sequencing reactions G, A, T, and C (copy ribosomal DNA) are indicated as C, U, A, and G, respectively, to reflect the complementary rRNA sequence.) The sequence of the S/R loop is shown on the left side of the figure.

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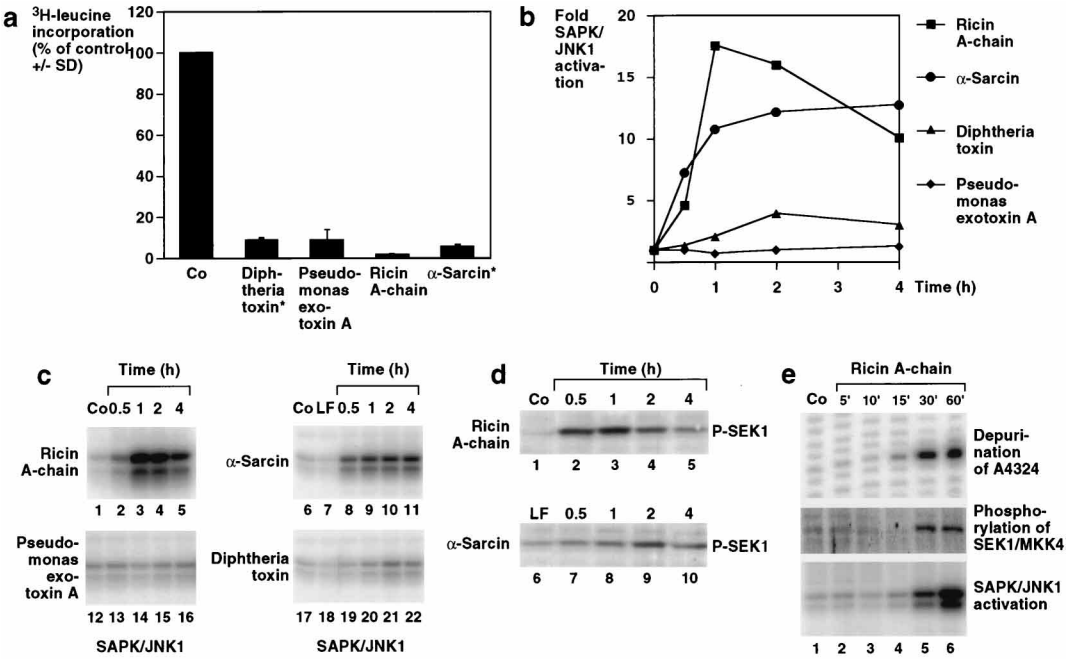

FIG. 6. Inhibition of translation, phosphorylation of SEK1/MKK4, and activation of SAPK/JNK1 by ribotoxic enzymes in Rat-1 cells. (a) Cells were treated in triplicate with *Pseudomonas* exotoxin A (5 μg/ml) without lipofection, or with diphtheria toxin (20 μg/ml) or α-sarcin (20 μg/ml) via lipofection (*), as described in Materials and Methods, and the levels of [^{3}H]leucine incorporation were determined 4 h after treatment. Appropriate control cells received lipofection mix without toxins, and the inhibition of protein synthesis by diphtheria toxin or α-sarcin was determined relative to these control cells. Error bars denote the standard deviations (SD) of independent dishes in triplicate. (b and c) Cells were left untreated or were treated with *Pseudomonas* exotoxin A, diphtheria toxin, RCA_{60}, α-sarcin, or (when required) lipofectin alone as for panel a for 0.5, 1, 2, or 4 h and were then harvested for determination of SAPK/JNK1 activity with GST-Elk1. The quantitative representation of the results shown in panel c is presented in panel b. Here, SAPK/JNK1 activity after lipofection alone is shown only for the 4-h time point (lanes 7 and 18), but see also Fig. 4b, lanes 2 to 5, for 15, 30, 60 and 90 min. (d) Cells were treated as described for panels b and c, and the phosphorylation of SEK1/MKK4 at threonine 223 was detected in a Western blot analysis as described in Materials and Methods. LF, lipofectin. (e) Cells were left untreated or were treated with RCA_{60} (10 μg/ml) for 5, 10, 15, 30, or 60 min. The depurination of A4324, the phosphorylation of SEK1/MKK4, and the activation of SAPK/JNK1 (using GST-Elk1) were determined from separate tissue culture dishes as described in Materials and Methods. Co, control.

S/R loop of the 28S rRNA, induced by the ribotoxic enzymes ricin A chain and α-sarcin, initiates a cellular response that involves phosphorylation of SEK1/MKK4, activation of SAPK/JNK1, and transcriptional induction of immediate-early genes such as c-*fos* and c-*jun* (Fig. 6 and 8). This response resembles the cellular reaction to anisomycin, as ribosomes that had been subjected to prior inactivation were unable to mediate the activation of SAPK/JNK1 in response to ricin A chain and α-sarcin (Fig. 7).

What are the intermediate signal transduction steps between the damage to 28S rRNA and activation of the SEK1/MKK4-SAPK/JNK1 cascade? Since cells containing translationally inactivated ribosomes fail to activate SAPK/JNK1 in response to anisomycin, ricin A chain, α-sarcin (Fig. 3 and 7), or APNAs (data not shown), it is possible that active ribosomes provide not only the sensor for ribotoxic stress but also the transduction machinery that translates the alterations in the 28S rRNA into a signal recognized by cellular components that lie upstream of SEK1/MKK4 and SAPK/JNK1. Although these intermediates remain unidentified, our data provide insight into some of their properties. For instance, pretreatment of Rat-1 cells with pactamycin or emetine did not prevent the 28S rRNA damage caused by ricin A chain but completely abrogated the activation of SAPK/JNK1 (Fig. 5 and 7a and b). Therefore, we conclude that ribosomes arrested in the pretranslocational state (e.g., by emetine pretreatment) or disintegrated into free subunits and unprogrammed monosomes by inhibitors of translational initiation (e.g., by pactamycin pretreatment) have lost their ability to transduce the signal from the 28S rRNA to SEK1/MKK4. This suggests that the binding of a signal-transducing molecule (such as a protein) may be restricted to a certain stage of the ribosomal cycle and that the binding of this transducing component may be abolished in arrested ribosomes. Examples of proteins that bind to the S/R loop in a ribosomal cycle-dependent manner are the elongation factors EF-Tu/EF-1 and EF-G/EF-2 (31, 32, 53). ADP-ribosylation of EF-2 inhibited the activation of SAPK/JNK1 by ricin A chain or α-sarcin (Fig. 7c). This inhibition may result from either (i) the arrest of the ribosomal cycle caused by ADP-ribosylation of EF-2 and subsequent prevention of the binding of a transducer protein different from EF-2 or (ii) inhibition of the binding of EF-2, which may itself be the transducer. Our experiments do not let us distinguish between these possibilities.

Once initiated, the signal from the damaged and/or conformationally altered 28S rRNA rapidly stimulates the pathway(s) leading to activation of SAPK/JNK1. SEK1/MKK4 became phosphorylated following treatment with anisomycin and APNAs (22) and after addition of ricin A chain or α-sarcin (Fig. 3 and 6d). SEK1/MKK4 becomes phosphorylated following IL-1α stimulation of Rat-1 cells as well (22). These data indicate that ribotoxic stress and growth factor- or cytokine receptor-activated signal transduction cascades leading to potentiation of SAPKs/JNKs have at least one common component upstream of SAPK/JNK1, i.e., SEK1/MKK4.

Evolutionary conservation of the stress-sensoring functions of ribosomes. Although to our knowledge this is the first report that describes the ability of eukaryotic ribosomes to sense

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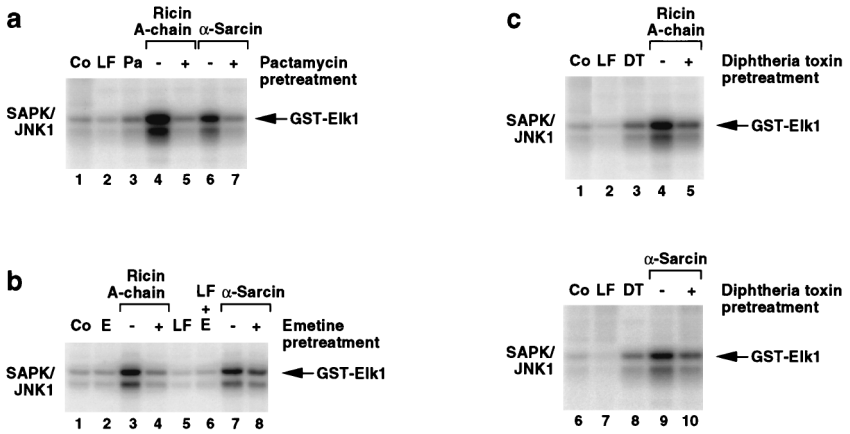

FIG. 7. Inhibition of ricin A chain- and α-sarcin-induced activation of SAPK/JNK1 by pretreatment of Rat-1 cells with pactamycin, emetine, or diphtheria toxin. (a) Cells were left untreated (control [Co]) or were treated with either RCA_{60} (10 μg/ml), α-sarcin (20 μg/ml, via lipofection), or lipofectin (LF) for 1 h, either alone or after pretreatment with pactamycin (0.2 μg/ml) for 30 min. SAPK/JNK1 activity was determined as described in Materials and Methods. (b) Cells were treated as described for panel a, except that some cells were pretreated with emetine (10 μg/ml). LF, lipofectin alone for 1 h; LF + E, pretreatment with emetine for 30 min followed by treatment with lipofectin plus emetine for 1 h (the proper control for lane 8). (c) Cells were treated with RCA_{60} (10 μg/ml; upper panel) or with α-sarcin (60 μg/ml, via lipofection; lower panel) for 1 h, either alone or following pretreatment with diphtheria toxin (20 μg/ml, via lipofection) for 3 h. LF, lipofectin alone for 4 h; DT, lipofectin plus diphtheria toxin for 4 h. The threefold-higher dose of α-sarcin (compared to previous experiments) used for the experiment shown in panel c was required to achieve sufficient SAPK/JNK1 activation, as in this experiment the cells were exposed to lipofectin twice. Pretreated cells were exposed either to lipofectin alone (lower panel, lanes 7 and 9) or to lipofectin plus diphtheria toxin (lower panel, lanes 8 and 10) for 3 h. This pretreatment with lipofectin appeared to decrease the uptake of α-sarcin-containing lipid vesicles into cells during the subsequent exposure to lipofectin.

cellular stress and to initiate cellular stress responses, similar functions for prokaryotic ribosomes have previously been reported. For example, prokaryotic ribosomes produce guanosine 3′,5′-bispyrophosphate (ppGpp) in response to stalling caused by amino acid starvation (5). The production of ppGpp results in abrupt transcriptional inhibition of genes encoding components of the translational apparatus (5). Another example of ribosome-mediated stress signaling is the selective up-regulation of the expression of stress proteins in response to antibiotic-induced inhibition of overall protein synthesis in *E. coli* (48). Interestingly, similar to the results from Rat-1 cells presented here, *E. coli* does not mount a general stress reaction to all translational inhibitors. Instead, the response is antibiotic specific. Chloramphenicol, erythromycin, fusidic acid, tetracycline, and spiramycin all induce cellular reactions indistinguishable from the bacterial cold shock response, whereas kanamycin, puromycin, and streptomycin induce the expression of the full complement of proteins characterizing the bacterial heat shock response (48). Our results demonstrate that the ribotoxic stress response in Rat-1 cells was blocked by pretreatment with some translational inhibitors. Similarly, pretreatment of *E. coli* with tetracycline, which produces a cold shock response, blocks the cellular response to subsequent heat shock (48). Interestingly, chloramphenicol and erythromycin (inducers of the cold shock response) bind to the same region of the 23S rRNA in prokaryotes as that to which anisomycin binds in the 28S rRNA of eukaryotic cells (Fig. 4a) (10, 39). This striking similarity in the ability of conserved regions of the 23S and 28S rRNAs to initiate cellular reactions in response to binding of inhibitors of the peptidyl transferase reaction in bacteria and in higher eukaryotes suggests the existence of a universal and evolutionarily conserved function of the ribosome in both sensing stress and directing the subsequent cellular responses.

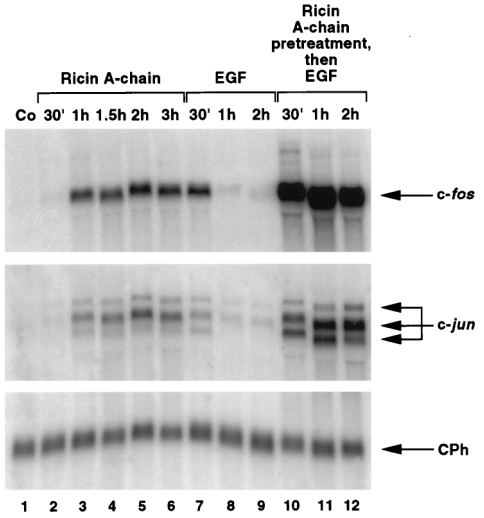

FIG. 8. Induction and superinduction of c-*fos* and c-*jun* mRNA accumulation by ricin A chain in Rat-1 cells. Cells were left untreated or were treated, as indicated, with RCA_{60} (10 μg/ml), EGF (40 ng/ml), or EGF (40 ng/ml) following a 1-h pretreatment with RCA_{60} (10 μg/ml) for the indicated periods of time. Total RNA was prepared and the accumulation of c-*fos* and c-*jun* mRNA was determined by Northern blot analysis as described in Materials and Methods. Three c-*jun* mRNA species are detectable (middle panel) as a result of alternative polyadenylation signals utilized in the processing of the primary c-*jun* transcript (1). A cyclophilin (Cph) cDNA probe (lower panel) was used to ensure that equal amounts of RNA (10 μg) were loaded in all lanes.

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