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 \( \alpha \)-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 c-Jun 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 \( \alpha \)-sarcin, both of which catalyze sequence-specific RNA damage in the 28S rRNA. Consistent with our hypothesis, ricin A chain and \( \alpha \)-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 \( \alpha \)-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 c-Jun 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\(_3\)\(^{0+}\) (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 c-Jun (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\(^{\ast}\)P\(^{\ast}\)Y\(^{\ast}\) by the dual-specificity protein kinase SEK1/MKK4 (12, 26, 40). The protein kinase M\(\text{E}K\)K1 (25), in turn, activates SEK1/MKK4 through phosphorylation of serine 219 and threonine 223 (55). The mode of regulation of M\(\text{E}K\)K1 (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 GT Pases 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 \( \alpha \)-sarcin, catalyze sequence-specific RNA damage within a conserved loop, the \( \alpha \)-sarcin/ricin (S/R) loop, of the 28S rRNA (53). We asked whether ricin A chain and \( \alpha \)-sarcin could activate SAPK/JNK1 and its activator, SEK1/MKK4, and found that this was indeed the case. Like anisomycin, ricin A chain and \( \alpha \)-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.

Antibiotics. Puromycin, diphtheria toxin, gougerotin, Pseudomonas exotoxin A, and diphtheria toxin were from Sigma Chemical Company, St. Louis, Mo. Ptcadamycin 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 H2O. Anisomycin, emetine, pactamycin, T-2 toxin, and gonergitin were dissolved in (HCl)2SO. α-Sarcin, Pseudomonas exotoxin A, and diphtheria toxin were reconstituted in double-distilled H2O. All antibiotics were from Calbiochem, except for emetine and pactamycin, which were obtained from the Sigma Chemical Company. Blasticidin S was used as a 10 mg/ml solution in dimethyl sulfoxide (DMSO). Anisomycin, puromycin, cycloheximide, emetine, pactamycin, and T-2 toxin were added directly to the cultures at the stated concentrations.

Northern blot analysis of rRNA. Reverse transcription of rRNA by primer extension. Reverse transcription of rRNA was performed as described in reference 20, with modifications. Crude oligonucleotide primer (5′-CACATACACAAATGTC-3′; Genosys Biotechnologies, Inc., The Woodlands, Tex.) was end labeled with T4 polynucleotide kinase (New England Biolabs, Inc., Beverly, Mass.) in the presence of [γ-32P]ATP (3000 Ci/mmol; Amersham) and T4 polynucleotide kinase. The labeled primer was annealed to 100 ng of total RNA with 5 U of RNase H and 10 U of RNase T1 (New England Biolabs, Inc.) at 60°C for 10 min. The reaction was quenched by addition of EDTA to 100 mM, and the samples were loaded on 7% non-denaturing polyacrylamide gels. The gel was dried and exposed to film and/or a PhosphorImager screen. The sequence of the primer-extended transcript was determined by using 70% formamide, 10% glycerol, 1 mM EDTA, and 0.5 mg/ml methylene blue as the separating gel and 90% formamide, 10% glycerol, 1 mM EDTA, and 0.5 mg/ml methylene blue as the running gel.

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 [3H]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-E1k (38) (Fig. 1; see also Fig. 2, 3b, 4b, 6, and 7) or GST-c-Jun (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 [3H]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-
Activation of SAPK/JNK1 by 28S rRNA Damage

To test 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 (ii) inhibition of translational elongation by pretreatment with emetine, which results in disintegration of the active polysomes into 60S and 40S ribosomal subunits and a proteolytic fragment of the latter that contains the Elk-1 phosphoacceptor sites and the recognition sequence for the binding of SAPK/JNK1.

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 R-at-1 cells (data not shown). However, the observed polysomes were nonfunctional, as the [3H]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.

Activity 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 APNA 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 R-at-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
abolished the activation of SAPK/JNK1 by either blastcidin S or gougerotin (data not shown), just as it did to the activation of SAPK/JNK1 by anisomycin (Fig. 6c). 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 in intact ribosomes 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 SAPK/JNK1 (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.

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Cells were then stimulated as indicated with either anisomycin \( (\text{Aniso}) \) or IL-1 \( \alpha \). Cells (in triplicate) were left untreated (control) or were treated with emetine \( (100 \mu\text{m}) \). 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 \( (\text{T}2; 10 \mu\text{g/ml}) \) for 30 min. Cells were then stimulated as indicated with either anisomycin \( (\text{Aniso}) \) or IL-1 \( \alpha \) 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 \( (100 \mu\text{m}) \) and then immediately exposed to a pulse of \( [\text{H}]\text{leucine} \) for 1, 2, 3, 4, or 5 min. SD, standard deviation. Lower panel: cells were treated as indicated with anisomycin \( (\text{Aniso}) \) or IL-1 \( \alpha \) as described for panel b. Emetine \( (100 \mu\text{m}) \) was given as indicated either 2 min before \([\text{Em} (-2)]\) or 2 min after \([\text{Em} (+2)]\) the respective agonist. Just as with GST-ELK1 (see the legend to Fig. 1b), GST-\( \text{cJun} \) preparations display a second phosphorylated band \(+1\) which most likely represents a proteolytic fragment containing the SAPK/JNK1 recognition sequence and the phosphoacceptor sites (serines 63 and 73 [19]) of \( \text{cJun} \).

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 pretranslational ribosome and cannot catalyze the ribosomal translocation, thus leading to cessation of translation (43) (Fig. 2a). A's 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 \( \alpha \)-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 in the pretranslational ribosome and thus 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 \( \alpha \)-sarcin, but not 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 \( \alpha \)-sarcin require functional ribosomes in order to activate SAPK/JNK1. If ricin A chain and \( \alpha \)-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 \( \alpha \)-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 \( \alpha \)-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 \( \alpha \)-sarcin-induced SAPK/JNK1 activation is that the inactivated ribosomes were not susceptible to RNA damage by ricin A chain or \( \alpha \)-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 \( \alpha \)-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).
Using eight antibiotic ribosomal inhibitors and induced their accumulation when applied together with epidermal growth factor (EGF), could induce and superinduce the expression of c-fos and c-jun mRNAs. Northern blot analyses in Rat-1 cells showed that the ricin A chain induced the accumulation of c-fos and c-jun mRNAs 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 [3H]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 28S rRNA serves as a sensor for ribotoxic stress.
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 SA PK/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 SA PK/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. A thorough investigation of these intermediates remains unidentified, our data provide insight into some of their properties. For instance, treatment 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 pre-translocational 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. 7c). This phosphorylation resulted from either (i) phosphorylation of SEK1/MKK4 by SAPK/JNK1 in response to ricin A chain or α-sarcin (Fig. 7c). This phosphorylation 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.

Evolutionary conservation of the stress-sensing functions of ribosomes. Although to our knowledge this is the first report that describes the ability of eukaryotic ribosomes to sense
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). A similar example of ribosome-mediated stress signaling is the selective upregulation 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 285 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 RCA60 (10 μg/ml), EGF (40 ng/ml), or EGF (40 ng/ml) following a 1-h pretreatment with RCA60 (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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