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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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 c-Jun NH2-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 c-Jun NH2-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 Aβ3 (7) and palytoxin (23), hyperosmotic shock (16), proinflammatory cytokines (24), or withdrawal of a trophic factor (54). SAPK/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, SAPK/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 SAPK/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 SAPK/JNKs is regulated through their phosphorylation on both threonine and tyrosine residues in the motif T*+P*Y* by the dual-specificity protein kinase SEK1/MKK4 (12, 26, 40). The protein kinase M KK K1 (25), in turn, activates SEK1/MKK4 through phosphorylation of serine 219 and threonine 223 (55). The mode of regulation of M KK K1 (and of other potential SEK1/ M KK K4 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 SA PKs' 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/ M KK K4 and SAPK/JNK1.

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Reverse transcription of rRNA by primer extension. Reverse transcription of rRNA was performed as described in reference 20, with modifications. Crude oligonucleotide primer (5′-CATACATACACAAATGTC-3′; Genosys Biotechnologies, Inc., The Woodlands, Tex.) was end labeled with T4 polynucleotide kinase (Gibco BRL/Life Technologies) and then purified by using the Full-Longer apparatus (Bioyk Eycase Biologies, 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 MgCl₂ was heated for 2 min at 90°C, placed on ice for 2 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 MgCl₂. After 2.5 min of incubation at 48°C, the reactions were stopped by adding EDTA to 5 mM. The 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 diodeoxynucleotide sequencing reactions. rRNA and primer were annealed as indicated above in half the volume. Primer extension for 15 min at 40°C included 0.1 μM dNTPs and either 0.5 mM ddGTP, 1.0 mM ddATP, 0.5 mM ddCTP, or 1.0 mM ddTTP; 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/JNK. In order to understand the mechanism of the anisomycin-induced SAPK/JNK 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. Of the ribosomal inhibitors tested, anisomycin was the most potent in activating the kinase (typically more than 15-fold activation [Fig. 2c]). Cycloheximide, puromycin, and T-2 toxin appeared to be weaker activators (between three- and sevenfold activation at concentrations sufficient to inhibit more than 50% of protein synthesis). As shown in Fig. 2c, there was no correlation between the potentials of these agents to inhibit protein synthesis and their abilities 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 abilities 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, panfycin and emetine completely failed to activate SAPK/JNK1 (Fig. 2c).

Functional ribosomes are required for anisomycin-induced activation of SAPK/JNK. 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-
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). [3H]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 and 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 7 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 [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 (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
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. 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 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. 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 lecithin RCA60 from the castor plant Ricinus communis. RCA60 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, RCA60 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 RCA60 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 [3H]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 15 of SAPK/JNK1 (Fig. 6d; Fig. 6c, lanes 7 to 10), a marker for SAPK/JNK1 activation (53, 54). The depurination of A4324 became apparent 15 min after the RCA60 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 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 RCA60 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 [3H]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 15 of SAPK/JNK1 (Fig. 6d; Fig. 6c, lanes 7 to 10), a marker for SAPK/JNK1 activation (53, 54). The depurination of A4324 became apparent 15 min after the RCA60 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).

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Based on the structural and 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
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 ribotoxin 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). As the ADP-ribosylation of EF-2 interferes with the function of the S/R loop, 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 R-at-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 inactivate SAPK/JNK1: Pseudomonas exotoxin A completely failed to activate the kinase (Fig. 6b; Fig. 6c, lanes 13 to 16), and diphertheria 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 diphertheria 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 R-at-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-in-duced A4324 depurination. Consistent with the results obtained with pactamycin or emetine, in cells pretreated with diphertheria toxin, neither ricin A chain nor α-sarcin was able to activate SAPK/JNK1 substantially above the low level of activation induced by diphertheria 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).
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, induced by anisomycin or by the ribotoxic enzymes ricin A chain and α-sarcin, is able to substitute for the 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.

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...
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/J NK1 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 SA PK/J NK1 in response to anisomycin, ricin A chain, α-sarcin (Fig. 3 and 7), or A P NAs (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 SA PK/JNK1. A though 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 SA PK/J NK1 (Fig. 5 and 7a and b). Therefore, we conclude that ribosomes arrested in the pretranslational 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 SA PK/J NK1. SEK1/MKK4 became phosphorylated following treatment with anisomycin and A PNAs (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 SA PK/sJ NKs have at least one common component upstream of SA PK/sJ NK1, i.e., SEK1/MKK4.

Evolutionary conservation of the stress-sensing functions of ribosomes. A though to our knowledge this is the first report that describes the ability of eukaryotic ribosomes to sense
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 response 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).

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