Creating a Protein-Based Element of Inheritance

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was impossible to collect a large number of data points from a single cell. We stopped using a cell when a change in the input impedance indicated either membrane breakdown or a loss of the tight seal between the recording pipette and the cell membrane. Most cells allowed one to three measurements [D. Axelrod, D. E. Koppel, J. Schlessinger, E. Elson, W. W. Webb, *Biophys. J.* **16**, 1055 (1976)].
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8. Whole-cell patch clamping was performed with a patch pipette intracellular solution consisting of the following: 150 mM KCl, 5 mM $MgCl_2$, 5 mM EGTA, and 10 mM Hepes, with an osmolality of 305 mOsm/kg and a pH of 7.3. Gigohm seals were formed at the base of the OHCs. After the whole-cell mode was entered, the series resistance was between 10 and 30 megohms. On-line series resistance compensation was 75 to 95%, and residual series resistance was not compensated.
9. Osmotic challenge experiments were performed using a lower NaCl concentration (130 mM). Glucose was added as needed to achieve osmolalities from 265 to 365 mOsm/kg. A 245 mOsm/kg solution was created by adding additional free water.
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Creating a Protein-Based Element of Inheritance

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Proteins capable of self-perpetuating changes in conformation and function (known as prions) can serve as genetic elements. To test whether novel prions could be created by recombinant methods, a yeast prion determinant was fused to the rat glucocorticoid receptor. The fusion protein existed in different heritable functional states, switched between states at a low spontaneous rate, and could be induced to switch by experimental manipulations. The complete change in phenotype achieved by transferring a prion determinant from one protein to another confirms the protein-only nature of prion inheritance and establishes a mechanism for engineering heritable changes in phenotype that should be broadly applicable.

Two genetic elements in *Saccharomyces cerevisiae*, [*PSI*$^+$] and [*URE3*], are widely believed to transmit phenotypes through proteins with self-perpetuating changes in conformation, rather than through altered nucleic acids (*1*). These elements are called yeast prions because of conceptual similarities between their modes of transmission and that postulated for the infectious agent in mammalian prion diseases (*2*). The yeast proteins, however, are unrelated to the mammalian prion protein and to each other. Moreover, they usually do not kill the organism, but produce cytoplasmically transmitted, heritable changes in phenotype (*3, 4*). For [*PSI*$^+$], the protein determinant is Sup35, a translation termination factor. In [*psi*$^-$] cells, Sup35 is soluble and functional (*5, 6*). In [*PSI*$^+$] cells, most Sup35 is insoluble and nonfunctional, causing a change in translation fidelity (*5, 6*). This phenotype is heritable because Sup35 protein in the [*PSI*$^+$] state influences new Sup35 protein to adopt the same state and passes from mother cell to daughter to perpetuate the cycle of conversion (*6–8*). [*PSI*$^+$] is, however, metastable: [*PSI*$^+$] cells occasionally give rise to [*psi*$^-$] cells and vice versa (*3*), as the [*PSI*$^+$] conformation is lost or gained.

Sup35 has three distinct regions (*9*). The NH_2-terminus (N) plays a critical role in Sup35's self-perpetuating change in state (*10–13*). The middle region (M) provides a solubilizing and/or spacing function (*14*). The COOH-terminus (C) provides translation-termination activity (*10, 15*). To test whether other proteins can be made to undergo a prion-like change in state, we fused N and M to a steroid hormone–regulated transcription factor, the rat glucocorticoid receptor (GR), and to a constitutive variant (GR^{526}) (*16*) that lacks the heat shock protein 90 (Hsp90) and hormone-binding domain (Fig. 1A) (*17*). The fusion did not block GR's inherent transcriptional activity: when NMGR and GR were expressed in [*psi*$^-$] cells over a broad range of induction levels, the activity of NMGR was in each case similar to that of GR (Fig. 1B, left). NMGR^{526} and GR^{526} also had similar activities in [*psi*$^-$] cells (*18*).

Three lines of evidence indicate that NM fusion proteins can interact with endogenous Sup35 to undergo prion-like changes in state. First, in contrast with [*psi*$^-$] cells, in [*PSI*$^+$] cells, newly synthesized NMGR had much lower activity than GR (Fig. 1B, right). Immunoblotting demonstrated that this was not due to a reduction in NMGR expression (*18*). Second, transient expression of NMGR, but not of GR, induced new heritable [*PSI*$^+$] elements in [*psi*$^-$] cells (Table 1). The NMGR^{526} variant also induced [*PSI*$^+$] elements, but not if the protein carried a small deletion of residues 22 through 69 in N (Δ22–69) (Table 1), which blocks [*PSI*$^+$] induction by Sup35 (*10*). Third, NMGR and NMGR^{526} exhibited the same unusual pattern of plasmid incompatibility as Sup35 (*10, 13*). High-copy Sup35 plasmids cannot be transformed into [*PSI*$^+$] cells because excessive Sup35 aggregation inhibits translational termination so severely that cells die. Cells are immune to the toxicity of the plasmid when the genomic copy of *SUP35* has its N and M regions deleted (Δ*NMSUP35*) (*10, 13*). Conversely, plasmids are not toxic if they carry the Δ22–69 deletion. When GR and NMGR were

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transferred to a high-copy vector with the yeast glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter, a strong, constitutive promoter, transformants were readily obtained in [*PSI*$^+$] cells with GR and GR^{526}, but not with NMGR and NMGR^{526} (Table 1). This transformation restriction was eliminated if the plasmid carried the Δ22–69 mutation or if the genome contained the Δ*NMSUP35* mutation (Table 1).

To determine if NMGR and NMGR^{526} can undergo self-perpetuating prion-like changes in activity on their own, we transformed the constitutive expression plasmids into a Δ*NMSUP35* strain (*19*). In addition to providing a test for the autonomy of NMGR conversions, this eliminated the potentially complicating toxicity of endogenous Sup35 inactivation. No changes in translational fidelity were detected in this background in our experiments.

Transformants were screened for different GR activity states by using the convenient blue/white colony color assay for a GR-regulated β-galactosidase reporter. With GR and GR^{526}, all transformants yielded blue colonies and gave rise only to blue colonies on restreaking. That is, the proteins were transcriptionally active and remained so. However, with NMGR and NMGR^{526}, white colonies were occasionally obtained (<1%) (*18*). On restreaking, blue NMGR and NMGR^{526} colonies primarily gave rise to blue colonies and white to white (Fig. 2), but each occasionally gave rise to colonies of the other color, and these in turn occasionally switched back. Thus, both NMGR and NMGR^{526} transformants exhibited heritable but metastable changes in their ability to activate the β-galactosidase reporter gene. Because NMGR^{526} assays did not require the addition of hormone, further work concentrated on this construct.

White colonies produced the same level of transcription factor as blue colonies (Fig. 2C), indicating that differences in GR activity were not due to differences in expression level. To determine if white colonies resulted from changes in the genome or reporter plasmid, white cells were streaked to media that caused them to lose the NMGR^{526} plasmid. When they were retransformed with GR^{526}, all new transformants were blue (*18*), indicating that both the reporter plasmid and the genome were fully capable of supporting β-galactosidase activity. When the same cells were transformed with NMGR^{526}, most colonies were blue, but a few

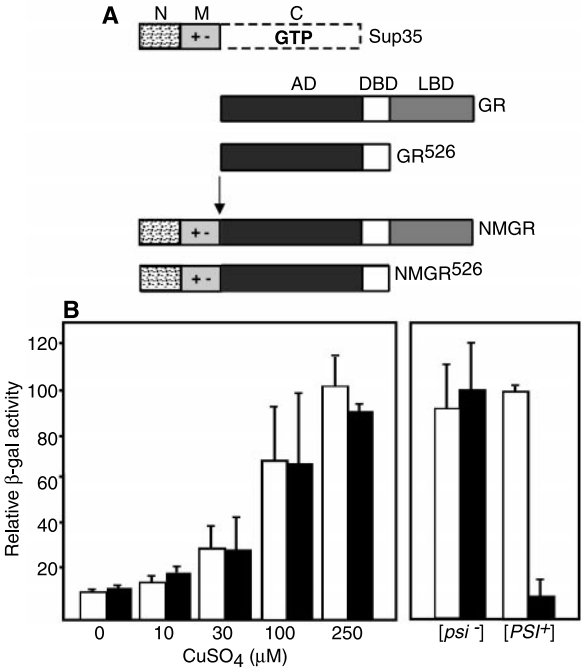

Fig. 1. (**A**) Derivation of NMGR and NMGR^{526} expression construct from Sup35 (N, M, and C regions) and GR (AD, activation domain; DBD, DNA-binding domain; and LBD, ligand-binding domain) (*9, 16*). NMGR was subcloned into both a single-copy, copper-inducible vector (*26*) (pCUP1-NMGR) and a multicopy (2μ) constitutive vector (*16*) (pG1-NMGR). NMGR^{526} was subcloned into the constitutive 2μ vector (pG1-NMGR^{526}). (**B**) GR and NMGR activities assessed (*27*) by activation of a β-galactosidase reporter construct, pL2/GZ (*16*). Three individual transformants were picked for each construct and duplicate samples were measured. Left: copper was added at the indicated concentrations to [*psi*$^-$] (74-D694) cultures to induce GR (open bar) and NMGR (filled bar). Right: GR and NMGR activities in isogenic [*psi*$^-$] and [*PSI*$^+$] cells (74-D694) at the same level of induction (100 μM $CuSO_4$).

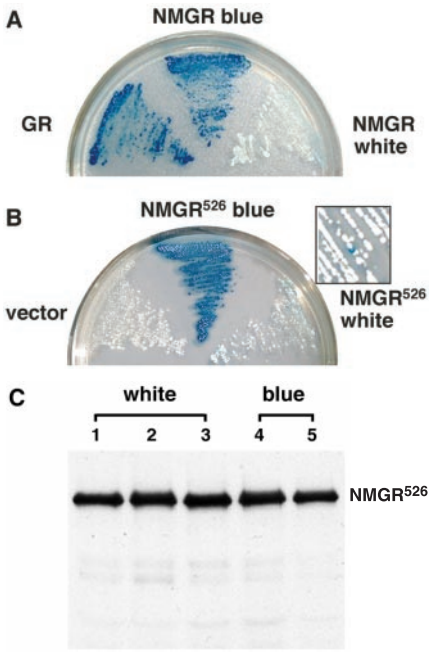

Fig. 2. NMGR and NMGR^{526} exist in distinct stable functional states. (**A**) Blue (active) and white (inactive) NMGR isolates (*28*) of 74-D694 Δ*NMSUP35* cells containing pL2/GZ, and pG1-NMGR (middle and right) or pG1-GR (left) were streaked onto selective medium (SC –trp, –leu). After 2 days of growth they were replicated onto selective medium containing 80 μg/ml of X-GAL. (**B**) Visualization of blue and white NMGR^{526} cells. Shown are 74-D694 Δ*NMSUP35* cells containing pG1 vector (left) or pG1-NMGR^{526} (middle and right). (**C**) Immunoblot analysis. Equal quantities of total cell proteins prepared from white and blue NMGR^{526} by ethanol lysis were separated by SDS-PAGE and immunoblotted with BuGR-2, a monoclonal antibody to GR (*29*).

Table 1. [*PSI*$^+$] induction and transformation incompatibility. Isogenic [*psi*$^-$], [*PSI*$^+$] and Δ*NMSUP35* (74-D694) were transformed with single-copy, copper-inducible plasmids or high-copy constitutive plasmids encoding GR, NMGR, or their COOH-terminal deletion derivatives with or without the Δ22-69 mutation. To test for [*PSI*$^+$] induction, [*psi*$^-$] transformants were grown in selective liquid media in the presence of 100 μM copper (for inducible plasmids) or without copper (constitutive plasmids) overnight. Five μl of the culture was then plated onto synthetic medium without adenine [SC (–ade)], which is selective for [*PSI*$^+$] cells but not for the plasmid. Representative [*PSI*$^+$] colonies were tested for curing by GdHCl. The (+) indicates at least 20 [*PSI*$^+$] colonies were obtained; (−) indicates no [*PSI*$^+$] colonies were obtained. To assess transformation competence, cells were plated onto medium selective for the plasmid. Each transformation was repeated at least three times. (+), 20 to 200 transformants per plate; (−), no transformants obtained. As expected, [*PSI*$^+$] cells induced from [*psi*$^-$] cells by high-copy, constitutive plasmids died during continuous selection for the plasmid and could only be recovered if selection for the plasmid was dropped.

	Single copy inducible		High-copy constitutive		High-copy constitutive		
	GR	NMGR	GR	NMGR	GR^{526}	NMGR^{526}	NMGR^{526} (Δ22–69)
[*PSI*$^+$] induction:	−	+	−	+	−	+	−
Transformation competence in:							
[*psi*$^-$]	+	+	+	+	+	+	+
[*PSI*$^+$]	+	+	+	−	+	−	+
Δ*NMSUP35*	+	+	+	+	+	+	+

were white. That is, in the absence of NMGR^{526}, all previous determinants of the GR activity state were lost and new NMGR^{526} transformants were as likely to be blue or white as were the initial transformants. We conclude that white NMGR^{526} colonies produced NMGR^{526} protein with a different heritable functional state than that of blue colonies, and the maintenance of this state depended upon continuous production of NMGR^{526}.

Next, we studied whether the inactive state of NMGR^{526} was "infectious." Cells expressing inactive NMGR^{526} (white colonies) were mated to cells expressing active NMGR^{526} or NMGR (blue colonies) from plasmids with different selectable markers. The resulting colonies were white and remained white even after the original NMGR^{526} plasmid was lost (*18*). We conclude that heritable differences in the activity of NM fusion proteins are due to their ability to form "infectious" protein-based genetic elements, or prions (*1*).

Other prions switch between active and inactive states spontaneously (as does NMGR) but can be induced to switch at much higher rates by experimental manipulation. NMGR functional states can be manipulated similarly. When cells from blue (active) NMGR^{526} colonies were transformed with an expression plasmid for the N domain and grown overnight in liquid media, ~10 to 20% yielded white colonies, and these remained white when the plasmid was lost (*18*). Virtually all colonies derived from vector-only transformants were blue. Thus, as for Sup35, transient overexpression of N can heritably convert NMGR^{526} to the inactive state. To determine if inactive NMGR^{526} could be induced to switch to the active state, we employed the protein denaturant guanidine hydrochloride (GdHCl) and the protein remodeling factor Hsp104, which can induce such changes in Sup35 (*20, 21*). When white colonies expressing NMGR^{526} were replica-plated to medium containing 5 mM GdHCl, most became blue (Fig. 3A). On restreaking in the absence of GdHCl, they remained blue. Similarly, white NMGR^{526} isolates transformed with an Hsp104 over-expression plasmid but not with the vector alone changed to blue (Fig. 3B) and remained blue when the Hsp104 plasmid was lost (*22*). As with the original transformants, blue colonies derived from GdHCl and Hsp104 treatments produced mostly blue colonies on restreaking but occasionally gave rise to white ones, indicating that the heritable changes they induced in NMGR^{526} activity were reversible. The effects of Hsp104 on NMGR^{526} also demonstrate that the critical determinant for Hsp104 interaction is within the NM domain of Sup35.

Our data clearly show that a prion-determining function is modular and transferable. When the prion determinant of Sup35 was deleted from its normal location and transferred to a completely unrelated protein, a new prion was created that maintained the epigenetic characteristics of the original prion and was subject to the same types of experimental manipulations (e.g., induction and curing). However, the phenotype changed completely, from one affecting the fidelity of protein synthesis to one affecting the transcription of a glucocorticoid-regulated promoter. Thus, the functional domain of the protein attached to NM is indeed the determinant of phenotype, affirming the protein-only nature of yeast prion inheritance.

Given the heterologous nature of the proteins employed here, it seems likely that this method for creating protein-based elements of inheritance will be broadly applicable. Of course, there will be exceptions. Indeed, fusing the prion-determining domain of Ure2 to β-galactosidase (*23*) and the NM regions of Sup35 to firefly luciferase (*24*) caused no change in β-galactosidase or luciferase activity. Even if the prion domains of these fusion proteins were to switch states, the functional domains might remain active because their substrates and products are freely diffusible. Expression levels would also impose another limitation: GR is expressed inefficiently in yeast; we were therefore only able to obtain heritable changes in activity with a high-copy vector. Nevertheless, the list of potential targets for prion manipulations is very large. It includes any protein that must be targeted to a particular location or assembled into complexes with other proteins in order to function. Here, it worked equally well with a transcription factor that requires hormone for activation and constantly cycles through interactions with the multicomponent Hsp90 chaperone machinery (GR) and a constitutive Hsp90-independent variant (GR^{526}). Preliminary results with a bacterial luciferase fusion suggest soluble enzymes might also be susceptible if substrates access or folding pathways can be altered by the prion domain (*24*). Protein-based genetic elements provide a method for engineering changes in phenotype that is fundamentally different from that of changes induced through the alteration of nucleic acids. Phenotypes switch spontaneously at rates that can be regulated by modifying the sequence of the prion determinant (*25*). Phenotypes can be induced to switch in opposite directions by transient over-expression of the prion determining domain or protein remodeling factors. Once switches occur they are stably inherited from generation to generation, but yet again, can be reversible by transient inducing stimuli. Perhaps most uniquely, prions provide a general mechanism for engineering loss-of-function phenotypes that are both dominant and infectious. Moreover, because they spread from one protein to others that share the same determinant, they can simultaneously inactivate diverse proteins in a single step.

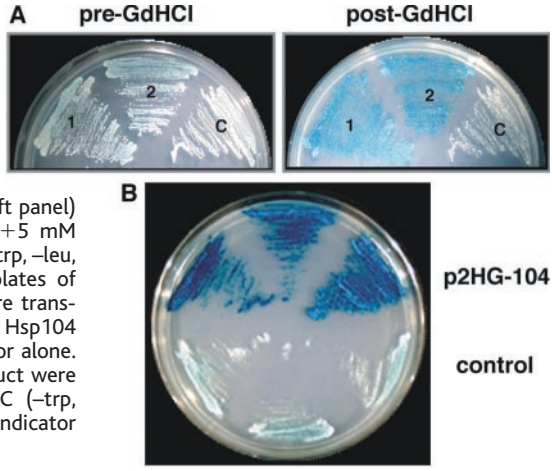

Fig. 3. Reactivation of NMGR^{526} by GdHCl and Hsp104. (**A**) Two white isolates (1, 2) of NMGR^{526} and a vector control (c) in 74-D694 Δ*NMSUP35* containing pL2/GZ were streaked onto a SC (–trp, –leu) plate. After incubation for 2 days at 30°C, colonies were directly replicated onto either SC (–trp,–leu, +X-GAL) (left panel) or first replicated to SC (–trp, –leu, +5 mM GdHCl), and then replicated onto SC (–trp, –leu, +X-GAL) (right panel). (**B**) White isolates of NMGR^{526} in 74-D694 Δ*NMSUP35* were transformed either with p2HG-104, a 2μ Hsp104 expression plasmid, or p2HG, the vector alone. Three transformants from each construct were randomly chosen and streaked on SC (–trp, –leu) then replica plated onto X-GAL indicator plates after 2 days.

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17. The GR-coding sequence was amplified by polymerase chain reaction (PCR) using plasmid pG1-N795 (*17*) as a template and primers, 5′-CGCCCGCGGATGGACTC-CAAAGAATCCT-3′ and 5′-CGCGAGCTCTCATTTTT-GATGAAACAGAAG-3′. The PCR fragment was digested with Sac I and Sac II and ligated in-frame with Sup35 NM coding sequence in a pRS316-based plasmid, pCNMG (T. R. Serio and S. Lindquist, unpublished data), that was digested. The resulting NMGR expression plasmid, pCUP1-NMGR, contained two additional codons, PR, between NM and GR sequences. It was digested

with Bam HI and Sac II and re-ligated after incubation with T4 DNA polymerase to make the control plasmid, pCUP1-GR. To create a 2μ expression plasmid for NMGR, pG1-NMGR, the NMGR DNA fragment was subcloned downstream of the GPD promoter in pG1 (*16*). To create pG1-NMGR526, two primers 5′-ATCAGGATCCAATGTCGGATTC-3′ and 5′-CGGGATCCTCATCCTGCAGTGGCTTGCTGAATC-3′ were used to amplify the $NMGR^{526}$ DNA fragment in a PCR reaction using pG1-NMGR as template. The PCR product was then digested with Bam HI, gel-purified, and subcloned into the Bam HI site of pG1.

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27. Each transformant was grown overnight in Synthetic complete media (SC) lacking uracil and leucine (–ura, –leu) at 30°C overnight then diluted into fresh SC (–ura, –leu) to a density of 2×10^6/ml. After 2 hours, deoxycorticosterone (DOC) was added to a final concentration of 10 μM and $CuSO_4$ to the concentrations as indicated in the Fig. 1 legend. Cells were harvested by centrifugation after overnight induction. Extracts were prepared by suspending the cell pellet (from a 3-ml culture) with 200 μl of lysis buffer containing 0.1 M potassium phosphate buffer (pH 7.8), 20% glycerol (v/v), 1 mM dithiothreitol, 2 μg/ml leupeptin, 2 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride. The cell suspension was mixed by vortexing 4 min at 4°C. After centrifugation at 1600*g* for 5 min, 5 μl of the lysate was used to measure β-galactosidase activity, using the Galacto-Light kit from TROPIX (Bedford, MA).
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30. We thank K. Yamamoto for BuGR2 antibody; S. Liebman for the N expression plasmid, pEMBL-Δbal2, and the control plasmid, pEMBL-Yex4; M. Patino for the primers of NMGR; Lindquist lab members for comments on the manuscript; and J.-J. Liu for assistance in manuscript preparation. Supported by the Howard Hughes Medical Institute and NIH.

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Coupling of Stress in the ER to Activation of JNK Protein Kinases by Transmembrane Protein Kinase IRE1

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Malfolded proteins in the endoplasmic reticulum (ER) induce cellular stress and activate c-Jun amino-terminal kinases (JNKs or SAPKs). Mammalian homologs of yeast IRE1, which activate chaperone genes in response to ER stress, also activated JNK, and *IRE1*$\alpha^{-/-}$ fibroblasts were impaired in JNK activation by ER stress. The cytoplasmic part of IRE1 bound TRAF2, an adaptor protein that couples plasma membrane receptors to JNK activation. Dominant-negative TRAF2 inhibited activation of JNK by IRE1. Activation of JNK by endogenous signals initiated in the ER proceeds by a pathway similar to that initiated by cell surface receptors in response to extracellular signals.

cJUN NH_2-terminal kinases [JNKs; also known as stress-activated protein kinases (SAPKs)] constitute a family of signal transduction proteins that are activated under a diverse set of circumstances (*1*). JNKs regulate gene expression through the phosphorylation and activation of transcription factors such as cJUN or ATF2 (*2*) or by regulating mRNA stability (*3*). The physiological significance of JNK signaling has been documented by genetic analysis in *Drosophila* and mice (*4*). Upstream activators of JNK signaling are arranged in a kinase cascade that is similar to that of the yeast pheromone mating pathway (*5*). However, only limited information is available about how proximal signals are coupled to activation of this kinase cascade. The best-characterized link is that between ligation of the tumor necrosis factor (TNF) receptor and activation of JNKs. This link depends on recruitment of adaptor proteins known as TRAFs to the cytosolic side of the ligated receptor (*6*). TRAF2 appears to be specifically important in this regard, because deletion of the gene abolishes JNK activation by TNFα (*7*). The TRAFs activate proximal kinases to initiate a kinase cascade, culminating in JNK phosphorylation and activation (*8*). The mechanistic details of the TRAF-dependent activation of the proximal kinases in the cascade are incompletely understood; however, TRAF effector function depends on the integrity of its NH_2-terminus (*9*).

Stress in the endoplasmic reticulum (ER), induced by perturbations that lead to accumulation of malfolded proteins in that compartment, also activates JNKs (*10*). However, coupling of ER stress to JNK activation is not understood. In yeast, IRE1p, the product of the inositol auxotrophy gene *IRE1*, serves to transduce stress signals from the ER that result in altered gene expression in a pathway known as the "unfolded protein response" (*11, 12*). Two mammalian homologs of yeast IRE1p have been identified: IRE1α (*13*) and IRE1β (*14*). These related transmembrane ER-resident protein kinases are believed to sense ER stress through their conserved lumenal domains. Signal transduction is associated with oligomerization and phosphorylation of the cytosolic portion of IRE1p and increased kinase activity of the protein (*11, 12*). Given their ability to transduce stress signals across the ER membrane and their similarity to classic transmembrane receptors, we examined the possibility that IRE1s also might contribute to JNK activation during ER stress.

Lysates from ER-stressed rat pancreatic acinar AR42J cells treated with thapsigargin (an agent that promotes ER stress by depletion of lumenal calcium stores), tunicamycin (which blocks protein glycosylation), or dithiothreitol (which interferes with disulfide bond formation) all exhibited increased JNK activity (Fig. 1A). Activation of ER stress is revealed by the shift in mobility of the PKR-like ER kinase (PERK), a convenient early marker of ER stress (*15*). Activation of JNKs by ER stress, although always present, varies in magnitude depending on cell type and is particularly pronounced in cells such as AR42J cells, which have a well-developed ER. It is consistently less than that observed in the same cells exposed to ultraviolet (UV) light or the protein synthesis inhibitor anisomycin.

Overexpression of IRE1p or its mammalian homologs leads to their activation independently of ER stress signaling (*13, 14, 16, 17*). Therefore, we overexpressed either form of mammalian IRE1 in cells and measured the kinase activity of a coexpressed exogenous JNK fused to a glutathione *S*-transferase tag (SAPK1β-GST). To limit the analysis of enzyme activity to that present in the transfected cells, the SAPK1β-GST fusion protein was purified by ligand affinity chromatography and then reacted in vitro with the recombinant GST-JUN substrate (*18*). Overexpression of either

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