20. Resident colonic lamina propria T cells did not respond to CD3 stimulation in vitro nor did they respond to int280 alone over a dose range of 1 to 25 μg/ml, but they did give a proliferative response to Con A (5 μg/ml). This response was not enhanced by int280. However, int280 strongly costimulates T cells from the colonic lymphoid follicles. In response to CD3 activation [details as in (9)], colonic lymphoid follicle T cells gave 9097 ± 1327 cpm, but in the presence of int280 the CD3 response increased to 30,252 ± 4678 cpm.
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Genetic Selection of Peptide Inhibitors of Biological Pathways

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Genetic selections were used to find peptides that inhibit biological pathways in budding yeast. The peptides were presented inside cells as peptamers, surface loops on a highly expressed and biologically inert carrier protein, a catalytically inactive derivative of staphylococcal nuclease. Peptamers that inhibited the pheromone signaling pathway, transcriptional silencing, and the spindle checkpoint were isolated. Putative targets for the inhibitors were identified by a combination of two-hybrid analysis and genetic dissection of the target pathways. This analysis identified Ydr517w as a component of the spindle checkpoint and reinforced earlier indications that Ste50 has both positive and negative roles in pheromone signaling. Analysis of transcript arrays showed that the peptamers were highly specific in their effects, which suggests that they may be useful reagents in organisms that lack sophisticated genetics as well as for identifying components of existing biological pathways that are potential targets for drug discovery.

Peptide-protein interactions have critical roles in biology. Many signals are transmitted by the binding of peptides to cell-surface receptors, and many protein-protein interactions inside cells are dominated by the binding of a peptide on one protein to a pocket on another. These interactions have inspired methods to select members of random peptide libraries that bind to known protein targets displayed on the outside of viruses (phage display) (*1*) or within cells (*2*). An alternative strategy is to select peptides whose binding to unknown targets produces a phenotype in the same way that mutations produce phenotypes by inactivating genes (*3*). Like mutations, peptides can be used to probe the function and mechanism of biological pathways as well as to identify their in vivo protein targets.

We developed methods to express peptamers, peptides displayed as an exposed loop on the surface of an inert carrier protein, at high concentrations in budding yeast cells. This approach protects the peptides from proteolytic degradation and imposes some conformational rigidity (*4*). A catalytically inactive version of staphylococcal nuclease (*5*) was used as a carrier protein because it is small, folds spontaneously without chaperones, has a prominently exposed loop on its surface (*6*), and can be strongly expressed as a soluble protein in eukaryotes and prokaryotes. The peptamer libraries contained 16 random amino acids inserted into the staphylococcal nuclease open reading frame (ORF) in place of the carrier's most exposed surface loop (*7*).

Because the extent of pathway inhibition depends on inhibitor concentration, we maximized expression of the peptamers. A high-copy vector was made that contains a strong constitutive promoter driving the expression of a staphylococcal nuclease gene that uses optimal codons for efficient translation and epitope tags for immunological detection and protein purification (*8*). Cells containing this vector expressed the peptamers as one of the most abundant proteins in the cell (Fig. 1).

We developed selections for inhibitors of two signal transduction pathways, the spindle checkpoint (*9*) and the mating pheromone response pathway (*10*). The spindle checkpoint arrests cells in mitosis in response to chromosomes that fail to attach to the mitotic spindle (*11*) and the pheromone pathway arrests cells in G_1 in response to a peptide mating factor. Both pathways are good targets for inhibitor selection because neither is essential for viability, and activation of either pathway prevents cell proliferation, creating a selection for peptamers that inhibit the pathway.

The spindle checkpoint is evolutionarily conserved and is defective in many human tumor cell lines (*12*). Selecting for inhibitors of the spindle checkpoint requires genetic trickery. In normal cells, the checkpoint is activated by improperly aligned chromosomes, and overriding the checkpoint in these cells leads to errors in chromosome segregation and cell death (*13*). However, overexpression of the checkpoint protein Mps1 activates the checkpoint in cells that have normal spindles (*14*). In this situation, inactivating the checkpoint allows cells to divide and form viable colonies. Thus, we engineered the selection strain to overexpress Mps1 when grown on galactose (*15*). We identified inhibitors of the spindle checkpoint by transforming the peptamer library into this strain and selecting for the rare transformants that formed colonies on galactose-containing medium (*16*). From a pool of 6.5×10^6 transformants, we identified three peptamers that allow cells to proliferate on galactose (Fig. 2A). Two of the

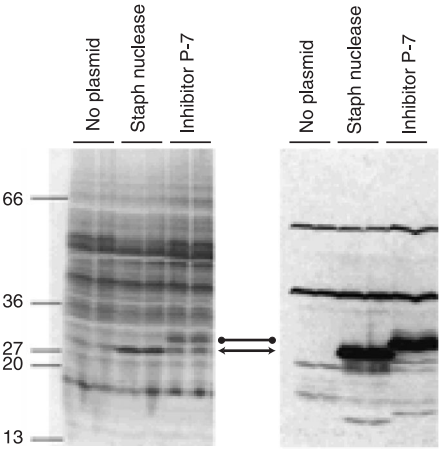

Fig. 1. Peptamer expression in yeast. Measurement of peptamer expression in yeast by Coomassie staining (left) and antihemagglutinin immunoblotting (right). Double-headed arrow denotes staphylococcal nuclease; double-headed closed circle denotes peptamer P-7. Numbers on left are kilodaltons.

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peptamers reduced the amount of the Mps1 protein (Fig. 2B). This effect appeared to be on the folding or stability of Mps1, because neither inhibitor reduced the amount of β-galactosidase or another protein kinase (Cdc28) expressed from the same promoter (*17*).

We tested whether the putative checkpoint inhibitors could overcome the arrest caused by two other perturbations that activate the spindle checkpoint: depolymerization of mitotic spindle (Fig. 2C) and the presence of short linear minichromosomes (*17*). Only the inhibitor that did not alter the amount of Mps1 expression overcame the mitotic arrest caused by these other perturbations, which suggests that it alone is a general inhibitor of the spindle checkpoint.

The phenotypes of peptamers likely depend on binding to a protein target. We used the two-hybrid technique, which detects protein-protein interactions in yeast, as one approach to identify possible targets of inhibition (*18*). We fused the spindle checkpoint inhibitor to a DNA binding domain and tested it for interactions with a panel that expresses more than 85% of yeast genes fused to a transcriptional activation domain. In this assay, the inhibitor interacted strongly with an ORF of unknown function (*YDR517W*), which is the closest yeast homolog of GRASP65, a mammalian protein that is associated with the Golgi apparatus (*19*). Cells lacking Ydr517w have defects in the spindle checkpoint (Fig. 2D). We believe that the peptamer that binds to Ydr517w also interacts with other proteins because the peptamer overcomes the effects of Mps1 overexpression more strongly than the deletion of Ydr517w, even when the peptamer is expressed in *ydr517wΔ* cells. Green fluorescent protein fused to the COOH-terminus of Ydr517w produces punctate cytoplasmic fluorescence. Although the role of a cytoplasmic protein in the spindle checkpoint is not immediately obvious, our analysis shows that identifying proteins that peptamers bind to can uncover additional members of well-studied pathways.

We also isolated inhibitors of the pheromone response pathway. Budding yeast exist in two mating types, **a** and α, which can mate with each other only when both cells are in the G_1 phase of the cell cycle. The α cells secrete α factor, which arrests **a** cells in G_1 (Fig. 3A). Two types of peptamers allow **a** cells to form colonies on plates containing α factor: those that interfere with pheromone signaling directly and those that interfere with transcriptional silencing (*20*). The latter class cause haploid **a** cells to behave as pheromone-insensitive **a**/α diploids by allowing them to express a copy of the α genes (*HMLα*) that is present in **a** cells but is normally transcriptionally silent. Deleting *HMLα* restores α factor sensitivity in strains that carry silencing inhibitors but has no effect on the phenotype of signaling inhibitors.

We isolated 29 peptamers that allowed **a** cells to proliferate in the presence of α factor, of which 20 are silencing inhibitors (Table 1). Selecting for cell proliferation demands a minimum specificity of peptamers, because those that strongly inhibit essential processes are not recovered. To test the specificity of the silencing inhibitors more stringently, we performed a global analysis of peptamer effects on transcription. Using whole genome DNA microarray analysis, we compared the pattern of transcription of strains expressing two of the silencing inhibitors to that of a dominant-negative *SIR4* mutant ($SIR4^{DN}$), which disrupts repression at the silent mating-type loci and at telomeres. The transcriptional effects of the peptamers were highly correlated with those of $SIR4^{DN}$ (Fig. 3B).

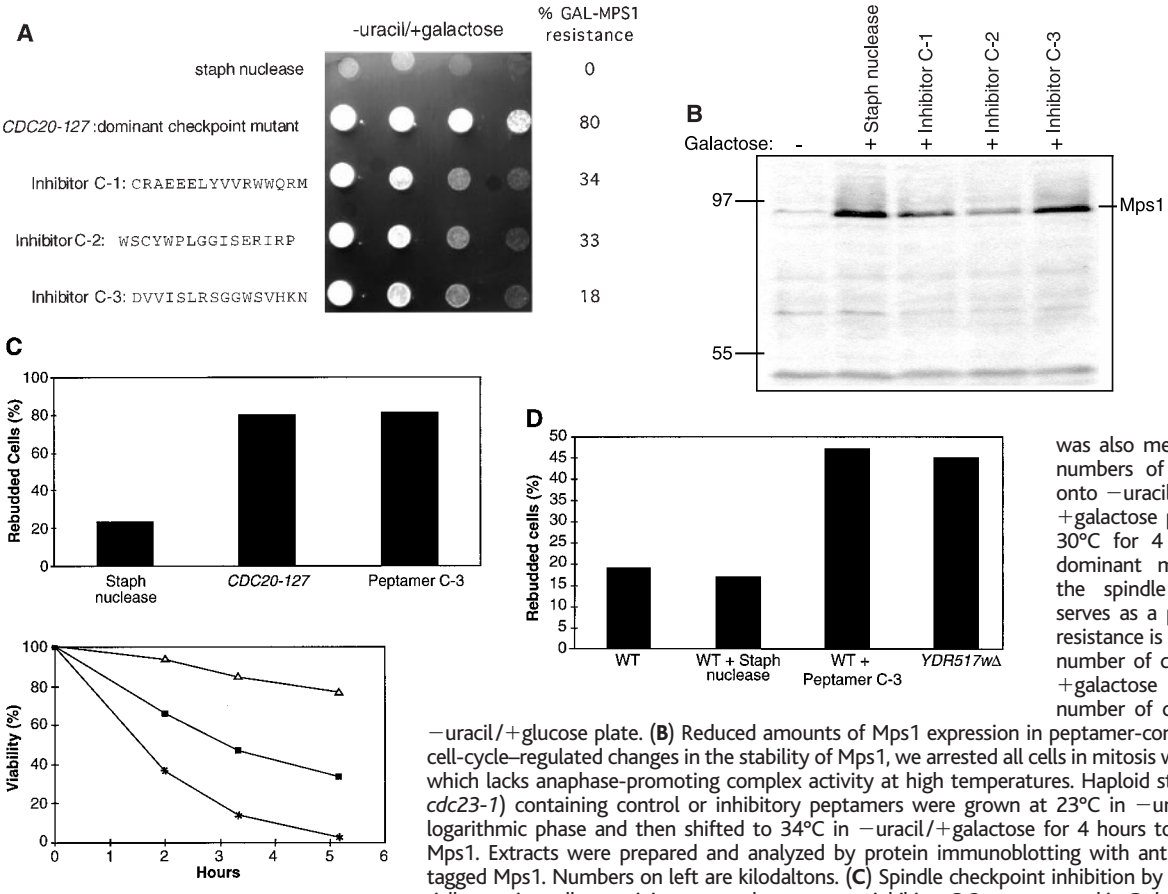

Fig. 2. Identification of an inhibitor of the spindle checkpoint. (**A**) Effects of three peptamers on the spindle assembly checkpoint activated by overexpression of Mps1. Cultures of logarithmic-phase controls and inhibitor-containing strains (*15*) were equalized for cell density, serially diluted by a factor of three, spotted onto −uracil/+galactose plates, and incubated at 30°C. *GAL-MPS1* resistance was also measured by plating equal numbers of logarithmic-phase cells onto −uracil/+glucose and −uracil/+galactose plates and incubating at 30°C for 4 days. *CDC20-127* is a dominant mutant that inactivates the spindle checkpoint (*33*) and serves as a positive control. Percent resistance is defined as 100 times the number of colonies on the −uracil/+galactose plate divided by the number of colonies growing on the −uracil/+glucose plate. (**B**) Reduced amounts of Mps1 expression in peptamer-containing strains. To avoid cell-cycle–regulated changes in the stability of Mps1, we arrested all cells in mitosis with the *cdc23-1* mutant, which lacks anaphase-promoting complex activity at high temperatures. Haploid strains (*GAL-MPS1::URA3*, *cdc23-1*) containing control or inhibitory peptamers were grown at 23°C in −uracil/+raffinose to early logarithmic phase and then shifted to 34°C in −uracil/+galactose for 4 hours to induce transcription of Mps1. Extracts were prepared and analyzed by protein immunoblotting with antibodies to Myc epitope-tagged Mps1. Numbers on left are kilodaltons. (**C**) Spindle checkpoint inhibition by peptamer C-3. Exponentially growing cells containing a control peptamer or inhibitor C-3 were arrested in G_1 by treatment with α factor for 4 hours. After α factor was washed out, cells were (i) sonicated briefly, incubated on −uracil/+benomyl (20 μg/ml) plates, and scored for the percentage of cells that rebud after 5 hours at 16°C; or (ii) treated with nocodazole (15 μg/ml) for 0, 2, 3.3, and 5 hours and plated for viability. Δ, Staphylococcal nuclease; ×, *CDC20-127*; ■, peptamer C-3. (**D**) Effect of deleting the *YDR517w* gene on the spindle checkpoint. Wild-type (WT) cells, wild-type cells plus a control peptamer or peptamer C-3 (which binds Ydr517w), and *YDR517w*Δcells were tested for their ability to rebud in the presence of benomyl as in (C).

Many *MAT***a**-specific genes (for example, *STE2* and *BAR1*) and haploid-specific genes (for example, *GPA1*) showed correlated decreases in the abundance of their mRNAs, whereas the amount of *MATα2* mRNA increased 1.5- to 2-fold, consistent with derepression of the silent loci. Furthermore, transcription of a number of genes located very near telomeres, including *COS12*, *YER188W*, and *YAL069W*, increased 1.6- to 2.5-fold, consistent with derepression of telomeric silencing. The absence of other widespread changes shows that the peptamers specifically inhibit silencing rather than causing general perturbations of cellular transcription.

We used the two-hybrid method to find proteins that interacted with five of the silencing inhibitors. Four of the five peptamers interacted strongly with specific proteins. One interacted with Asf1, whose overexpression or removal diminishes silencing (*21*). Another peptamer bound to Sfh1, an essential chromatin remodeling factor that interacts physically with components required for nucleosome restructuring (*22*).

Two silencing inhibitors that contain tryptophan-rich sequences (Table 1) interacted with two proteins, Tec1 and Dig1, and one of these peptamers also interacted weakly with Ste12. Both Tec1 and Dig1 interact with Ste12, a transcription factor required for the pheromone response; Dig1 was originally characterized as a negative regulator of Ste12 (*23*), and Tec1 interacts with Ste12 to stimulate pseudohyphal growth in diploids and invasive growth in haploids (*24*). Both peptamers blocked pseudohyphal growth in diploids, and one blocked invasive growth in haploids (*25*), phenotypes shared by both *tec1*Δ and *ste12*Δ mutants. The ability of these peptamers to inhibit silencing does not appear to depend on their interaction with Dig1, Tec1, or Ste12 because mating type silencing is maintained in a *dig1*Δ *tec1*Δmutant and telomeric silencing is maintained in a *ste12* mutant (*25*).

To characterize the nine pheromone signaling inhibitors, we used genetic tests to dissect the signaling pathway (Table 2). All the peptamers appeared to interfere downstream of Ste4, the G protein that interacts with the pheromone receptor, and upstream of Far1 the cyclin-dependent kinase inhibitor that directly induces cell cycle arrest (*26*). Five peptamers blocked a constitutively active allele of *STE11* (*27*), a mitogen-activated protein (MAP) kinase kinase kinase, from inducing transcription of a pheromone-responsive reporter gene, suggesting that they interfere with the MAP kinase cascade. We analyzed these inhibitors in more detail. The responses to pheromones and high osmolarity share a MAP kinase kinase kinase (Ste11) and certain other components (Ste20 and Ste50) but use different MAP kinases to produce their final output (*28*). A single peptamer increased the osmotic sensitivity of strains that depend on Ste11, Ste20, and Ste50 for osmotic signaling (Table 2), which suggests that this peptamer inhibits Ste11, Ste20, or Ste50. Two-hybrid experiments supported this conclusion: this peptamer interacted strongly with Ste50 and more weakly with Ste11, which binds to Ste50 (*29*). We believe that Ste50 is the target of the peptamer, because *ste50*Δ cells show only modest α factor resistance, even in the presence of the peptamer, whereas expressing the peptamer in wild-type cells conferred strong resistance (Fig. 4). Other peptamers caused strong pheromone resistance in *ste50*Δ, showing that these cells can become fully pheromone resistant. This result suggests that Ste50 has positive and negative functions in signaling and that the peptamer stimulates the activity of Ste50 that interferes with signaling, possibly by stimulating adaptation of the signaling pathway (*30*). One peptamer interacted with two MAP kinases (Fus3 and Kss1), either of which can transmit the pheromone signal, but failed to block the response to high osmolarity, which depends on the MAP kinase Hog1 (Table 2). This observation shows that peptamers are capable of discriminating between members of a family of homologous proteins.

Genetic selections can identify peptamers that inhibit biological pathways. Both of our selections produced two classes of peptamers, those that interfered indirectly with the target pathway and those that did so directly.

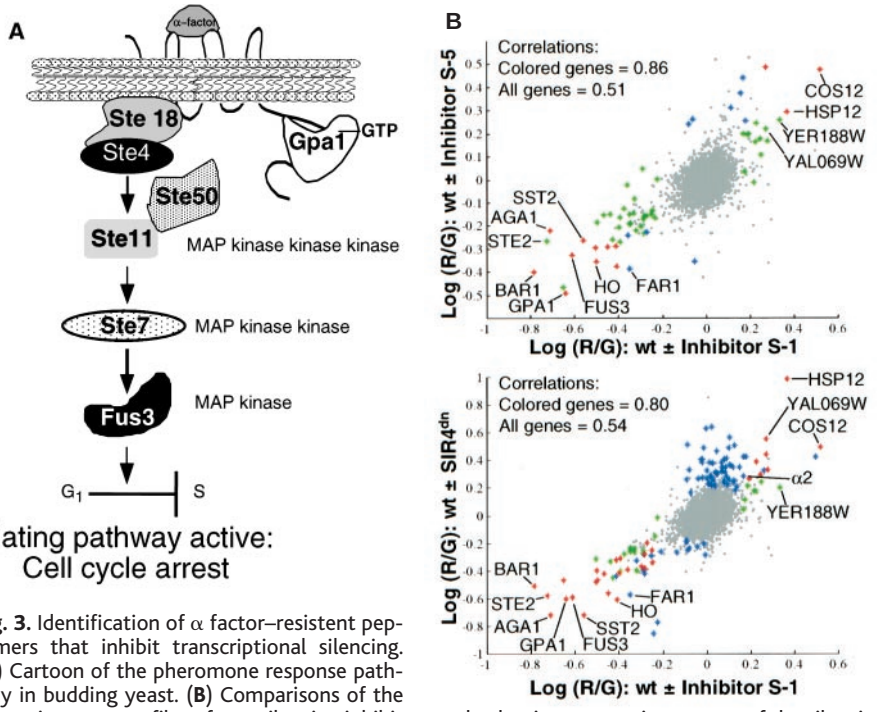

Fig. 3. Identification of α factor–resistent peptamers that inhibit transcriptional silencing. (**A**) Cartoon of the pheromone response pathway in budding yeast. (**B**) Comparisons of the transcript array profiles of two silencing inhibitors and a dominant-negative mutant of the silencing protein Sir4. RNA samples were prepared from wild-type cells and from cells expressing peptamer S-1, S-5, or a dominant-negative allele of *SIR4* and were analyzed by competitive hybridization to DNA microarrays containing >6200 yeast ORFs (*34*). Correlation plots comparing the results of these hybridizations are shown (*35*). (**Top**) Log_{10} of the expression ratio of ORFs from cells containing or lacking peptamer S-1 [(log_{10}(R/G)] plotted versus the log_{10} ratio of ORFs from cells containing or lacking peptamer S-5 expression. (**Bottom**) Log_{10}(R/G) ratios of ORFs from cells containing or lacking peptamer S-1 expression plotted versus ORFs from cells with or without $SIR4^{DN}$ expression. For each plot, >5600 ORFs (91 to 96% of total spots) for which reliable data were measured are plotted. ORFs whose expression did not change in either experiment are plotted as gray dots; ORFs that changed significantly ($P \leq 0.01$) in expression in both experiments are plotted as red stars; ORFs that changed significantly ($P \leq 0.01$) only in the competitive hybridization plotted on the *x* or *y* axis are plotted as green or blue stars, respectively. Specific subtelomeric, *MAT***a**-, and haploid-specific genes are labeled. Supplementary material is available at: www.sciencemag.org/feature/data/1041079.shl

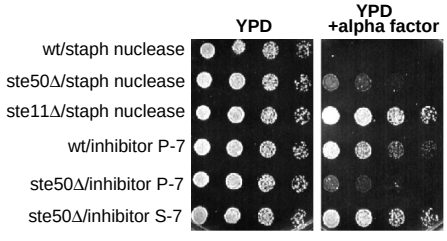

Fig. 4. Genetic identification of a peptamer target. Cultures of logarithmic-phase *MAT***a**, *ste11*Δ *MAT***a**, or *ste50*Δ *MAT***a** cells expressing staphylococcal nuclease, peptamer P-7, or peptamer S-7 were equalized for cell density, serially diluted by a factor of five, and spotted onto plates containing yeast extract, peptone, and dextrose (YPD) and α factor (1 μg/ml) and incubated at 30°C for 3 days.

Thus we obtained inhibitors of Mps1 expression as well as those that directly inhibit the spindle checkpoint and inhibitors of transcriptional silencing and those that interfere directly with pheromone signaling. These results highlight the importance of secondary tests that provide independent information about how each inhibitor passed the original selection.

We used three different methods to gain information about the targets of the peptamers: two-hybrid analysis, transcript arrays, and pathway dissection. Each approach has strengths and weaknesses, and a combination of methods is likely to be the most reliable way to identify targets. Two-hybrid analysis is simple, reports on physical interactions, and can identify additional components of existing pathways. The major drawbacks are false positives and false negatives: 7 of the 15 peptamers we tested failed to give a reproducible interaction with any protein, 3 peptamers showed interactions with more than one protein, and, in two cases, genetic tests suggested that none of the interacting proteins was the physiological target of the peptamer. Analyzing transcript arrays tests whether peptamers produce a similar response to known genetic perturbations and is the most powerful way of analyzing the specificity of inhibition. However, if mutations in multiple genes produce a similar transcriptional response, the product of any one of these genes could be the target of a peptamer. Genetic tools that dissect a pathway into separate modules are good reagents for directly identifying the targets of peptamers, but they are available for only a small number of well-studied pathways. Other methods that may identify targets include screening for proteins whose overexpression overcomes the inhibitory phenotype and mass spectrometry-based identification of proteins that bind to peptamers.

Genetic selection of inhibitory peptamers has four possible applications to drug discovery. First, by selecting for proliferation of the cells in which the target pathway has been inhibited, it provides a useful minimum of specificity. Second, it identifies new targets for drug discovery by finding new components of the target pathway. Third, a protein whose function can be inhibited with a short peptide is likely to be a good candidate for inhibition by small organic molecules. Finally, genetic selections make it easy to isolate peptamers with higher and lower potencies, and correlations between structure and activity could be used to guide combinatorial or peptidomimetic chemistry.

Table 1. Amino acid sequence (*31*) and characterization of 20 silencing inhibitors. NA, not applicable; NI, no interaction detected; NT, not tested.

Gene on plasmid	Amino acid sequence	α-Factor Resistance (%)*	Induces sporulation†	Two-hybrid interaction‡
Staph nuclease	—	0	No	NA
*MAT*α	—	100	Yes	NA
SIR4^{DN}	—	100	Yes	NA
Inhibitor S-1 (7 aa)	VCLGGVP	82	Yes	NT
Inhibitor S-2 (15 aa)	RFFWNPWTRVMQRAP	66	Yes	NT
Inhibitor S-3 (4 aa)	WVNW	43	No	NT
Inhibitor S-4 (15 aa)	RRTGGWGGNTCIIKFD	9	No	NT
Inhibitor S-5 (4 aa)	WVGW	70	No	DIG1, TEC1, STE12
Inhibitor S-6	VYLRKFSKVVPITWGW	59	No	NT
Inhibitor S-7 (9 aa)	VVWLDCW	100	Yes	DIG1, TEC1
Inhibitor S-8 (6 aa)	GRMEPGAAPRDSKCNA	49	No	NT
Inhibitor S-9	SLLATRSAKLALCSAR	91	Yes	NT
Inhibitor S-10	ILIKSKMHQRTLFSAL	100	Yes	SFH1
Inhibitor S-11	VYWRGQSLYATLSTSE	94	Yes	ASF1
Inhibitor S-12	VPSLRALWAYAGLGDS	79	Yes	NT
Inhibitor S-13	PCLVSSGPAGRSPSAW	11	Yes	NT
Inhibitor S-14	VYRCGPGGVLYPPACR	72	No	NT
Inhibitor S-15	PLLDPQQHAAPVAAGP	92	No	NI
Inhibitor S-16	ILLTRVHLRRSYMGAT	18	Yes	NT
Inhibitor S-17	FVFARRGYHLASTVHT	59	No	NT
Inhibitor S-18	CVACGLKLAGRLVGYL	85	No	NT
Inhibitor S-19	LLWSSVVKNPKFGGLF	100	No	NT
Inhibitor S-20	RLMSWRDSLWSYARLS	83	Yes	NT

*Equal numbers of logarithmic-phase cells were plated on −uracil/+glucose plates either lacking or containing α factor (1 μg/ml) and incubated at 30°C for 3 days. Percent resistance is 100 times the number of colonies on medium with α factor divided by the number of colonies growing on the plate lacking α factor. †A *MAT***a**/*MAT***a** strain was transformed with control or inhibitory peptamers, grown to saturation in −uracil/+glucose, and transferred to sporulation medium (2% KOAc, 0.02% raffinose) and incubated at 23°C for 3 days. Cells were analyzed under a microscope for the presence of tetrads. ‡Five peptamers were fused to a DNA binding domain and tested for two-hybrid interactions against a panel containing >85% of the ORFs in yeast fused to a transcriptional activation domain (*32*).

Table 2. Amino acid sequence and characterization of nine pheromone signaling inhibitors.

Gene on plasmid	Amino acid sequence	α-factor Resistance (%)*	At or below Ste4†	From Ste11 to Fus1‡	At Ste11, Ste20, or Ste50§	At or below Far1¶	Two-hybrid interaction‖
Staph nuclease	—	0	NA	NA	NA	NA	NA
*MAT*α	—	100	NA	NA	NA	NA	NA
SIR4^{DN}	—	100	NA	NA	NA	NA	NA
Inhibitor P-1	LYATRGLVRSHVCLGL	44	Yed	Yes	No	No	FUS3, KSS1
Inhibitor P-2	LLWSSVVKNPKFRHLF	100	Yes	No	No	No	NI
Inhibitor P-3	WWVRREIWFGAVISYE	42	?	No	No	No	NT
Inhibitor P-4	CRSVKEALVVFRRMLQ	100	Yes	Yes	No	No	NI
Inhibitor P-5	RIKGRYLAFVRQVGGF	51	Yes	No	No	No	NT
Inhibitor P-6	CWVCVPRVLRQRLLGI	76	Yes	No	No	No	NI
Inhibitor P-7	VLDVKDASDESILLSW	100	Yes	Yes	Yes	No	STE11, STE50
Inhibitor P-8	HGGVPGRPPSFILWKM	75	Yes	Yes	No	No	NI
Inhibitor P-9	EIRRWVQATYPLFASS	76	Yes	Yes	No	No	ARG80

*See Table 1 for determination of percent α factor resistance. †Ability of peptamers to overcome the cell-cycle arrest generated by galactose-induced overexpression of Ste4 (pGAL-STE4). ‡Ability of peptamers to overcome the signaling of a constitutively active *STE11-1* mutant. Cells containing a *FUS1-HIS3* reporter induced by *STE11-1* were transformed with control or inhibitory peptamers and tested for their ability to grow on −uracil/−histidine plates. §Osmotic sensitivity of a strain that depends on Ste11, Ste20 and Ste50 for osmotic signaling. A haploid *MAT***a** *ssk1* strain (*28*) that uses the Sho1 osmosensor and Ste11, Ste20, and Ste50 for osmoresistance was transformed with control or inhibitory peptamers. Transformants were tested for osmosensitivity by streaking onto −uracil/0.9 M NaCl plates. ¶Ability of peptamers to overcome the cell-cycle arrest generated by galactose-induced overexpression of a dominant *FAR1-22* mutant. ‖See legend to Table 1.

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7. The library in *Escherichia coli* contained 1.7×10^8 members, of which more than 1.14×10^8 directed the synthesis of peptamers. The synthetic staphylococcal nuclease gene was assembled in two cloning steps: 15 oligonucleotides were annealed and ligated to form double-stranded NH_2-terminal (177 bases) and COOH-terminal (330 bases) fragments that were ligated separately to vector (pTCN13) cut with Bst EII and Eco RI and with Eco RI and Sal I, respectively. The Eco RI and Sal I sites allow insertion of a loop that replaces amino acids 19 to 27 of the mature staphylococcal nuclease. The resulting constructs were digested with Eco RI and Xba I and ligation of the staphylococcal nuclease gene-containing fragments assembled the full ORF fused to a hemagglutinin epitope tag at the NH_2-terminus and to a stretch of six histidine residues (pTCN22) at the COOH-terminus. The random peptide insert was prepared by self-annealing the following oligonucleotide: 5′-CCC-GAATTCTTCGGTGGT$(NNS)_{16}$GGTGGTGTCGACAC-3′ [N = A:T:G:C (1:1:1:1), S = G:C (1:1)]. Annealed DNA was extended by using the Klenow fragment of DNA polymerase I, and the double-stranded product was cut with Eco RI and Sal I to create two equivalents of library DNA that were ligated to the staphylococcal nuclease construct (pTCN22) cut with Eco RI and Sal I, installing library DNA in-frame.
8. The expression vector pTCN23 carried the replicator of the 2-μm circle, an endogenous yeast high-copy plasmid, the *PGK1* promoter [A. Chambers, C. Stanway, A. J. Kingsman, S. M. Kingsman, *Nucleic Acids Res.* **16**, 8245 (1988)], and had codon usage optimized according to Sharp and Li [P. M. Sharp and W. H. Li, *Nucleic Acids Res.* **15**, 1281 (1987).
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15. Unless otherwise indicated, all experiments in this study were done in strains isogenic to W303. A single selection strain, DA2050A (*a/a bar1/bar1 mec1-1/mec1-1 CDC28VF::LEU2/CDC28-VF::LEU2 GAL1-MPS1/GAL1-MPS1*), was engineered to identify inhibitors of the spindle checkpoint or of the pheromone response pathway. The *bar1* mutation increases sensitivity to α factor by removing a protease that degrades the pheromone, and the *CDC28-VF* mutation blocks the ability of cells to adapt to overexpression of Mps1 from the *GAL1* promoter. The *mec1-1* mutation, which abolishes the DNA damage checkpoint, is irrelevant to the experiments reported here.
16. For both selections, transformants were grown on uracil-deficient plates for 2 days. Cells were scraped from these plates, and aliquots were plated onto selection plates [lacking uracil and containing galactose (−uracil/+galactose) for the spindle checkpoint selection; lacking uracil and containing α factor (−uracil/+α factor) (1 μg/ml) for the pheromone pathway selection] and grown at 30°C for 2 or 3 days. Plasmid DNA was recovered from the entire population of colonies that grew on these plates, purified, amplified by transformation into *E. coli*, and retransformed into DA2050A to enrich for peptamers that conferred resistance as opposed to genomic mutations that caused resistance. Cell populations harboring active peptamers typically grew as lawns on selection plates, and individual plasmids were isolated from these plates and retested to confirm that they gave plasmid-dependent resistance.
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31. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
32. A yeast ORF-Gal4 activation domain fusion array has been assembled that expresses about 85 to 90% of the predicted ORFs of *S. cerevisiae* in strain pJ69-4a [P. James, J. Halladay, E. Craig *Genetics* **144**, 1425 (1996)]. In this strain, *GAL4* is absent and the reporters *HIS3* and *ADE2* are under Gal4 control. To probe the Gal4-AD fusion array for protein-protein interactions, we mated the array to a strain expressing a Gal-4–DNA binding domain fusion. After selecting for diploids, we identified two-hybrid positives by testing diploids on plates containing different concentrations of 3-aminotriazole.
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35. We performed the following competitive hybridizations: wild-type cells with or without peptamer S-1, with or without peptamer S-5, and with or without expression of $SIR4^{DN}$. Each comparison was done on four arrays composed of two pair of hybridizations done with reversal of the fluorophore labelings to eliminate biases of fluorophore incorporation. We used two different sources of mRNAs for labeled cDNA production, including a total RNA protocol (http://www.nhgri.nih.gov/DIR/LCG/15K/HTML/) and twice-purified polyadenylated mRNAs [(M. J. Marton *et al. Nature Med.* **4**, 1293 (1998)].

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Neuronal Protection in Stroke by an sLe^x-Glycosylated Complement Inhibitory Protein

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Glycoprotein adhesion receptors such as selectins contribute to tissue injury in stroke. Ischemic neurons strongly expressed C1q, which may target them for complement-mediated attack or C1qRp-mediated clearance. A hybrid molecule was used to simultaneously inhibit both complement activation and selectin-mediated adhesion. The extracellular domain of soluble complement receptor–1 (sCR1) was sialyl Lewis x glycosylated (sCR1sLe^x) to inhibit complement activation and endothelial-platelet-leukocyte interactions. sCR1 and sCR1sLe^x colocalized to ischemic cerebral microvessels and C1q-expressing neurons, inhibited neutrophil and platelet accumulation, and reduced cerebral infarct volumes. Additional benefit was conferred by sialyl Lewis x glycosylation of the unmodified parent sCR1 molecule.

Interrupting blood flow to the brain, even for relatively short periods, can trigger inflammatory events within the cerebral microvasculature that can exacerbate cerebral tissue injury. The tissue damage that accrues is amplified by activation of both inflammatory and coagulation cascades within postischemic cerebral microvessels, impairing recovery of blood flow and causing collateral damage to bystander neurons. In a murine model of stroke, increased expression of the glycoprotein adhesion receptors P-selectin (*1*) and ICAM-1 (*2*) promotes leukocyte recruitment. Mice lacking these adhesion receptors exhibit

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