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Reconstitution of a yeast protein kinase cascade *in vitro*: Activation of the yeast MEK homologue STE7 by STE11

(mating response/mitogen-activated protein kinase/MEK kinase/*Saccharomyces cerevisiae*)

AARON M. NEIMAN AND IRA HERSKOWITZ*

Department of Biochemistry and Biophysics, Program in Genetics, University of California, San Francisco, CA 94143-0448

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ABSTRACT **The mating-factor response pathway of *Saccharomyces cerevisiae* employs a set of protein kinases similar to kinases that function in signal transduction pathways of metazoans. We have purified the yeast protein kinases encoded by *STE11*, *STE7*, and *FUS3* as fusions to glutathione *S*-transferase (GST) and reconstituted a kinase cascade in which STE11 phosphorylates and activates STE7, which in turn phosphorylates the mitogen-activated protein kinase FUS3. GST–STE11 is active even when purified from cells that have not been treated with α-factor. This observation raises the possibility that STE11 activity is governed by an inhibitor which is regulated by pheromone. We also identify a STE11-dependent phosphorylation site in STE7 which is required for activity of STE7. Conservation of this site in the mammalian STE7 homologue MEK and other STE7 relatives suggests that this may be a regulatory phosphorylation site in all MAP kinase kinases.**

Haploid *MAT***a** and *MATα* cells of the budding yeast *Saccharomyces cerevisiae* each secrete small peptide pheromones, **a**- and α-factor, respectively, to which the opposite cell type responds (1, 2). The signal transduction pathway mediating response to pheromone is initiated by a membrane-bound receptor that is coupled to a heterotrimeric guanine nucleotide-binding regulatory protein (G protein). Dissociation of the G-protein α subunit from the βγ subunit complex in response to ligand binding allows free βγ to trigger downstream events (3, 4). The *STE5* and *STE20* gene products are thought to link βγ to three protein kinases, encoded by *STE11*, *STE7*, and *FUS3* (4–9). These kinases are structurally related to mammalian protein kinases: FUS3 is a mitogen-activated protein kinase (MAP kinase) (10); STE7 is a kinase for FUS3 and is a MEK homologue (11, 12); STE11 is a MEK kinase (MEKK) homologue (13).

Functional studies indicate that these kinases act in the order STE11 → STE7 → FUS3. In particular, phosphorylation of FUS3 in response to α-factor requires both *STE11* and *STE7* (8). Furthermore, constitutively active alleles of *STE11* require *STE7* and *FUS3* for function (6, 7). This putative yeast kinase cascade is analogous to two kinase cascades proposed to function downstream of various growth factor receptors in mammalian cells: Raf → MEK → MAP kinase and MEKK → MEK → MAP kinase (14).

Our studies focus on STE11, which has been demonstrated to have protein kinase activity in immunoprecipitates (15). Physiologically relevant substrates of STE11 have not yet been defined. Here we show that this yeast kinase cascade can be reconstituted *in vitro* with purified glutathione *S*-transferase (GST)–STE11, –STE7, and –FUS3 fusion proteins and that STE7 is a substrate of STE11.

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MATERIALS AND METHODS

Media, Strains, and Plasmids. Standard media and genetic methods were used (16, 17). Strains AN1012 (*ste7*Δ), AN1016 (*ste11*Δ), and mating tester IH1793 have been described (18). The plasmids used were constructed as follows. pRD-STE7-RI was made by cloning an *Eco*RI fragment coding for the carboxyl-terminal 381 amino acids of STE7 into pGEX3 (Pharmacia) and then moved as an *Xma* I–*Hin*dIII fragment into pRD56 (19). A 1.4-kb *Bgl* II–*Hin*dIII fragment of pRD-STE7-RI was replaced with the same fragment of pYGD7-A220 (7) to create pRD-STE7-RI-A220. pRD-STE7-RI-V363 was made by site-directed mutagenesis (20) of pRD-STE7-RI with the oligonucleotide 5′-CTATCGCTGACGTCTTTGT-TGGAACG-3′. pRDSTE11-RI was made by replacing the *STE7* sequences of pRD-STE7-RI with an *Eco*RI–*Xho* I fragment coding for the carboxyl-terminal 606 amino acids of STE11. A 2.2-kb *Hin*dIII–*Cla* I fragment of pRD-STE11-RI was replaced with the same fragment of pNC245-R444 (15) to create pRD-STE11-RI-R444. A *Hin*dIII–*Xho* I fragment carrying the carboxyl-terminal 354 amino acids of STE11 was cloned into pRD56 to create pRD-STE11-H3. pRD-STE11-ATG was made by first cloning a 1.2-kb *Nhe* I–*Hin*dIII fragment of pAIS-STE11 (from S. Marcus, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) into *Xba* I/*Hin*dIII-cut pUC18 to create pUC-STE11-NH_2 and then moving a 1.2-kb *Bam*HI–*Hin*dIII fragment of pUC-STE11-NH_2 into pRD-STE11-H3. GST–FUS3 was expressed from pGEX-FUS3, which contains the entire *FUS3* coding region in plasmid pGEX3 (Pharmacia).

Mating Tests and β-Galactosidase Assays. Transformants were patched onto an SD plate selective for the plasmid, then replica plated to a plate containing galactose as the sole carbon source. After overnight incubation at 30°C, the patches were replica plated to a minimal-medium plate spread with a lawn of the mating tester strain IH1793 (*MATα lys1*). β-Galactosidase assays were performed essentially as described (18).

Protein Purification and Kinase Assays. STE11 and STE7 fusion proteins were purified as follows. Cells carrying the appropriate plasmid were grown to OD_{600} of 1.0 in galactose medium, pelleted, suspended in lysis buffer [20 mM Hepes, pH 7.6/200 mM KCl/2 mM EGTA/2 mM EDTA/0.1% Nonidet P-40, 10% (vol/vol) glycerol/1 mM phenylmethanesulfonyl fluoride/1 mM benzamidine hydrochloride/1 mM leupeptin], and ground with glass beads. The extract was spun at 12,000 × *g* for 1 hr. The supernatant was mixed with 1 ml of glutathione-agarose for 30 min, and then the agarose was pelleted and poured into a column. The column was washed with 40 ml of wash buffer 1 (20 mM Hepes, pH 7.6/500 mM KCl/0.1% Nonidet P-40/10% glycerol) and 10 ml of wash buffer 2 (20 mM Hepes, pH 7.6/200 mM KCl/10%

Abbreviations: GST, glutathione *S*-transferase; MAP kinase, mitogen-activated protein kinase; MEKK, MEK kinase.

*To whom reprint requests should be addressed.

glycerol). The fusion protein was eluted in 3 ml of elution buffer (20 mM Hepes, pH 7.6/200 mM KCl/10% glycerol/10 mM glutathione). The eluate was concentrated and exchanged into storage buffer (20 mM Hepes, pH 7.6/50 mM KCl/10% glycerol) in a Centricon-30 concentrator (Amicon). For purification, all STE11 and STE7 fusions were expressed in strain IH2361 (*ste5*Δ) without pheromone treatment. The FUS3 fusion protein was purified from *Escherichia coli* by a similar protocol except that cells were lysed by addition of lysozyme to 1 mg/ml. For kinase assays, the appropriate proteins were mixed and brought to a final volume of 20 μl in kinase buffer (10 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$). Reactions were started by addition of ATP to a final concentration of 100 μM plus 10 μCi of [γ-^{32}P]ATP. Reactions were stopped after 20 min by addition of SDS sample buffer. For quantitation of kinase activity, samples were electrophoresed in an SDS/polyacrylamide gel and electrophoretically transferred to a nitrocellulose membrane. Intensity of the bands was quantified on a PhosphorImager (Molecular Dynamics), and the blot was probed with anti-GST antibodies (from D. Kellogg, University of California, San Francisco) to ensure that equal amounts of protein were loaded.

Phospho Amino Acid Analysis and Tryptic Digests. For both analyses, GST–STE7$^{134-515}$ was phosphorylated by GST–STE11$^{111-717}$ *in vitro* and separated from the reaction mix as described above. For phospho amino acid analysis, the protein was then blotted to an Immobilon membrane (Millipore). The region of the membrane containing STE7 was excised, and two-dimensional phospho amino acid analysis was performed (21). Two-dimensional tryptic phosphopeptide mapping was performed as described (21) except that the electrophoresis buffer was acetic acid/formic acid/water, 3:1:16 (vol/vol), and that a Kodak 13255 thin-layer chromatography sheet was used.

RESULTS

Reconstitution of a Yeast Kinase Cascade. In order to simplify the purification of the kinases, the *STE11*, *STE7*, and *FUS3* genes were fused to a sequence which encodes a GST (22). Neither the short amino-terminal deletions nor the addition of the GST moiety appeared to interfere with the function of STE11 or STE7 *in vivo*, as judged by their ability to complement the mating defect of *ste11* or *ste7* deletion strains, respectively (Fig. 1). The GST–STE11 and GST–STE7 fusion proteins expressed in *E. coli* had little or no protein kinase activity. Therefore, both fusion proteins were purified from *S. cerevisiae*.

To test for kinase activity, the purified fusion proteins were mixed with [γ-^{32}P]ATP and assayed by PAGE for the presence of radioactively labeled protein. GST–STE11$^{111-717}$ was an active kinase as judged by the observed autophosphorylation (Fig. 2*B*), which produced multiple, slower migrating forms of GST–STE11$^{111-717}$ (Fig. 2*A*, lane 1). In contrast, the GST–STE7$^{134-515}$ fusion exhibited little autophosphorylation activity (Fig. 2*A*, lane 2). When GST–STE11$^{111-717}$ and GST–STE7$^{134-515}$ were mixed, however, GST–STE7$^{134-515}$ was phosphorylated (Fig. 2*A*, lane 3). Mixture of GST–STE11$^{111-717}$ or GST–STE7$^{134-515}$ with the GST–FUS3$^{1-353}$ fusion protein (purified from *E. coli*) produced no significant phosphorylation of GST–FUS3$^{1-353}$, but when all three kinases were mixed, GST–FUS3$^{1-353}$ was phosphorylated (Fig. 2*A*, lanes 4–6). The simplest interpretation of these results is that GST–STE11$^{111-717}$ phosphorylates GST–STE7$^{134-515}$, which is then competent to phosphorylate GST–FUS3$^{1-353}$. This interpretation is consistent with the genetic epistasis analyses (6, 7, 9) and with the observation that STE7 immunoprecipitated from yeast can phosphorylate and activate FUS3 produced in *E. coli* (11).

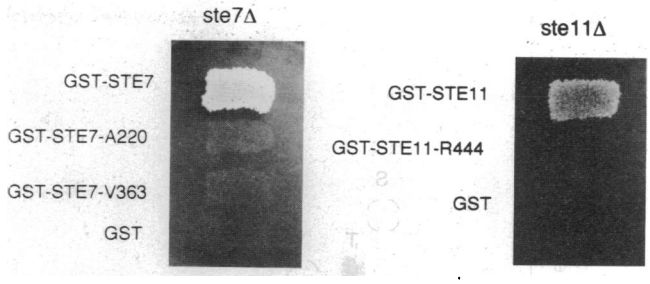


FIG. 1. Complementation by GST fusion proteins in mating tests of *ste7* and *ste11* mutant strains carrying various GST plasmids. AN1012 (*ste7*Δ) and AN1016 (*ste11*Δ) were transformed with plasmids pRD56 (a *CEN ARS* vector expressing GST under control of the *GAL1* promoter), pRD-STE7-RI (expressing a fusion of the *GST* gene to the wild-type *STE7* gene), pRD-STE7-RI-A220 (*GST–STE7* carrying a mutation of lysine-220 to arginine), pRD-STE7-RI-V363 (*GST–STE7* carrying a mutation of threonine-363 to valine), pRD-STE11-RI (expressing a fusion of *GST* to the wild-type *STE11*), or pRD-STE11-RI-R444 (*GST–STE11* carrying a mutation of lysine-444 to arginine).

If this *in vitro* system is faithfully recapitulating what occurs *in vivo*, then the phosphorylated GST–FUS3$^{1-353}$ protein should now be an active kinase. Activation of FUS3, as with all MAP kinases, is associated with phosphorylation on a threonine and a tyrosine residue (8, 23). Phospho amino acid analysis of GST–FUS3$^{1-353}$ phosphorylated *in vitro* demonstrated that it contained equal amounts of phosphothreonine and phosphotyrosine but no phosphoserine (data not shown). Although we have not shown that the phosphor-

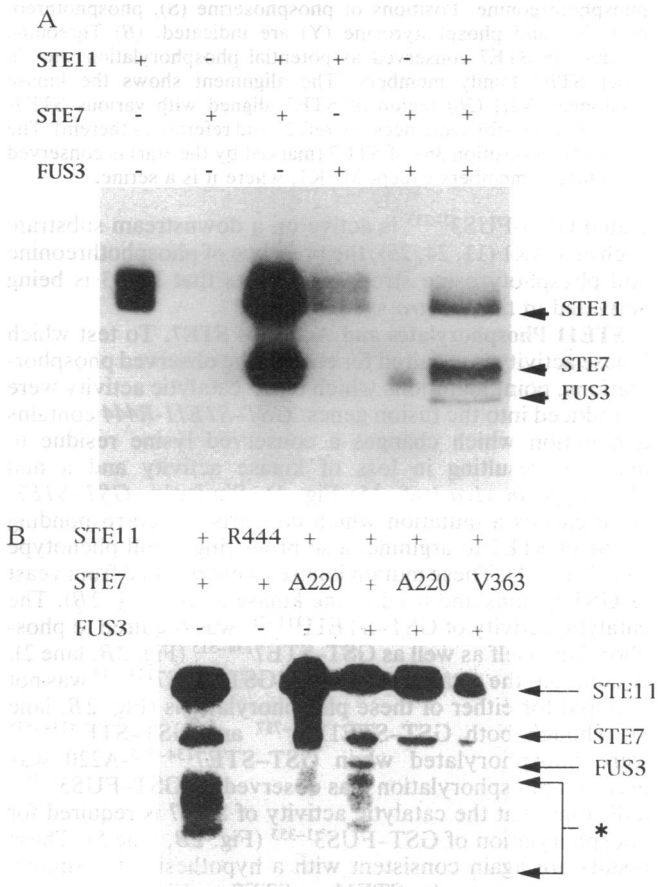
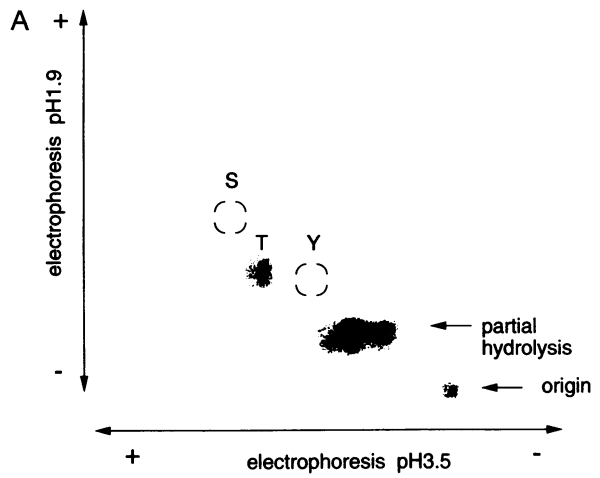


FIG. 2. Reconstitution of the kinase cascade *in vitro*. (*A*) Kinase assay with purified GST fusion proteins. (*B*) Kinase assay with mutant GST fusion proteins. Asterisk indicates GST–STE7$^{134-515}$ degradation products present in the wild-type preparation.



B

STE7	359	S I A D T F V G T S T Y M S P E	374
MEK1	218	S M A N S F V G T R S Y M S P E	233
Byr1	214	S V A Q T F V G T S T Y M S P E	229
PBS2	514	S I A K T N I G C Q S Y M A P E	529
Wis1	469	S I S K T N I G C Q S Y M A P E	484
MKK1	377	S L A T T F T G T S F Y M A P E	392
MKK2	370	S L A M T F T G T S F Y M A P E	385

FIG. 3. Identification of threonine-363 as a potential STE11 phosphorylation site on STE7. (*A*) Phospho amino acid analysis of GST–STE7$^{134-515}$ phosphorylated by GST–STE11$^{111-717}$ *in vitro* indicates that the activated GST–STE7$^{134-515}$ contains exclusively phosphothreonine. Positions of phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y) are indicated. (*B*) Threonine residues in STE7 conserved as potential phosphorylation sites in other STE7 family members. The alignment shows the kinase subdomain VIII (26) region of STE7 aligned with various STE7-family kinases (for sequences see ref. 27 and references therein). The threonine at position 363 of STE7 (marked by the star) is conserved in all family members except MEK1, where it is a serine.

ylated GST–FUS3$^{1-353}$ is active on a downstream substrate such as FAR1 (11, 24, 25), the presence of phosphothreonine and phosphotyrosine strongly suggests that FUS3 is being activated in the *in vitro* system.

STE11 Phosphorylates and Activates STE7. To test which kinase activity is required for each of the observed phosphorylations, point mutations which block catalytic activity were introduced into the fusion genes. *GST–STE11-R444* contains a mutation which changes a conserved lysine residue to arginine, resulting in loss of kinase activity and a null phenotype *in vivo* (ref. 15; Fig. 1). Similarly, *GST–STE7-A220* carries a mutation which converts the corresponding lysine of STE7 to arginine, also producing a null phenotype (ref. 7; Fig. 1). These mutant kinases were purified from yeast as GST fusions and used in the kinase assay (Fig. 2*B*). The catalytic activity of GST–STE11$^{111-717}$ was required to phosphorylate itself as well as GST–STE7$^{134-515}$ (Fig. 2*B*, lane 2). In contrast, the catalytic activity of GST–STE7$^{134-515}$ was not required for either of these phosphorylations (Fig. 2*B*, lane 3). Though both GST–STE11$^{111-717}$ and GST–STE7$^{134-515}$ were phosphorylated when GST–STE7$^{134-515}$-A220 was used, no phosphorylation was observed on GST–FUS3$^{1-353}$, indicating that the catalytic activity of STE7 is required for phosphorylation of GST–FUS3$^{1-353}$ (Fig. 2*B*, lane 5). These results are again consistent with a hypothesis of a simple, linear kinase cascade: STE11 → STE7 → FUS3.

STE7 Is Phosphorylated on a Conserved Site. We next used this *in vitro* system to identify a phosphorylation site required for GST–STE7$^{134-515}$ activation. Phospho amino acid analysis indicated that the activated GST–STE7$^{134-515}$ contained exclusively phosphothreonine (Fig. 3*A*). An alignment of STE7 with a number of other STE7 family kinases was used to

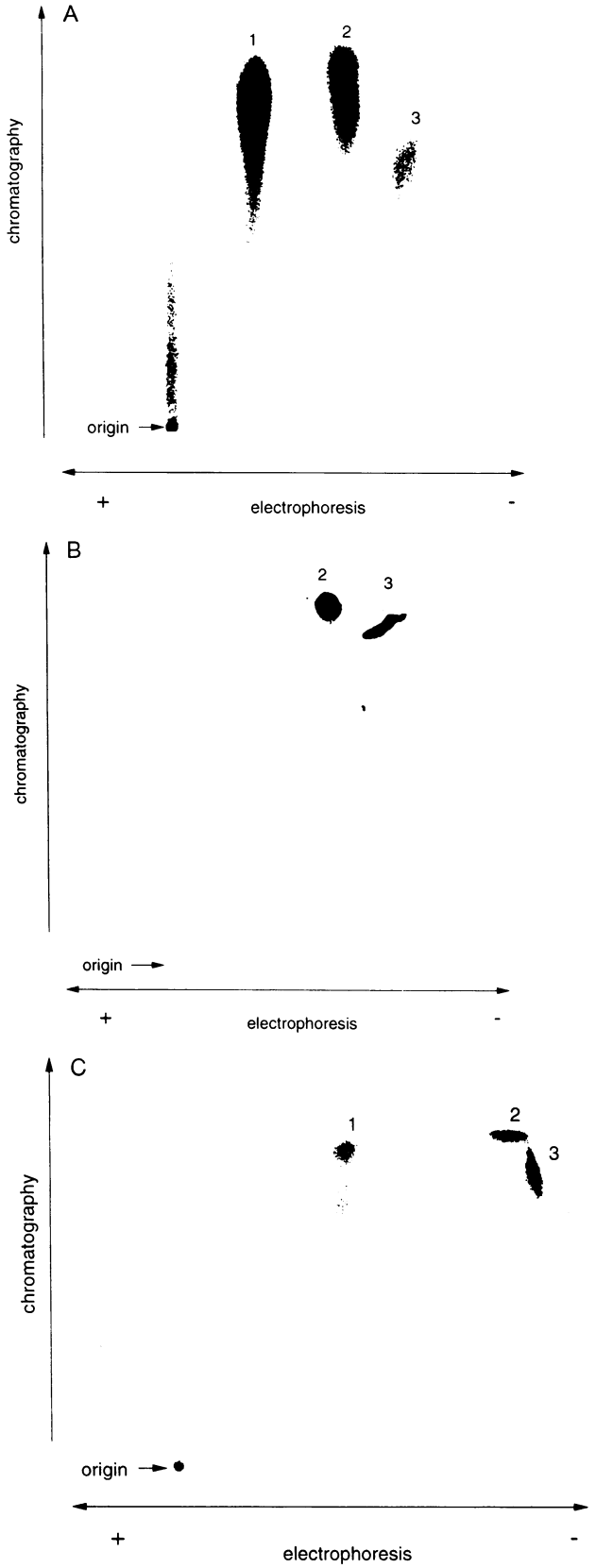


FIG. 4. Threonine-363 of STE7 is a phosphorylation site for STE11. (*A*) Tryptic phosphopeptide map of wild-type GST–STE7$^{134-515}$ phosphorylated by STE11$^{111-717}$ *in vitro*. (*B*) Tryptic phosphopeptide map of GST–STE7$^{134-515}$-V363 phosphorylated by STE11$^{111-717}$ *in vitro*. (*C*) Mixture of the two samples used in *A* and *B*.

identify likely target threonine residues. There is only one threonine in STE7 which is conserved as serine or threonine in all members of the STE7 family (Fig. 3*B*); it is located at position 363 in the STE7 primary sequence (28). A 14-amino acid peptide spanning this region of STE7 (residues 360–373) was an efficient substrate for GST–STE11$^{111-717}$ in the kinase assay and was phosphorylated by GST–STE11$^{111-717}$ on the threonine residue corresponding to position 363 (data not shown).

To determine whether phosphorylation of threonine-363 was important for STE7 function, valine was substituted at this position in the STE7 fusion protein. *In vivo*, the *GST–STE7-V363* allele behaved as a null mutation of *STE7* (Fig. 1). *In vitro*, the purified mutant protein was still phosphorylated by GST–STE11$^{111-717}$, though at a reduced level. The GST–STE7$^{134-515}$-V363 protein was, however, unable to phosphorylate GST–FUS3 (Fig. 2*B*, lane 6). Tryptic digests of GST–STE7$^{134-515}$ and GST–STE7$^{134-515}$-V363 phosphorylated *in vitro* revealed that there was one major phosphorylation on the wild-type protein which was absent on the V363 mutant (Fig. 4). Tryptic digests of GST–STE7$^{134-515}$ and GST–STE7$^{134-515}$-V363 labeled *in vivo* also showed an α-factor-dependent phosphorylation on the wild-type protein which was missing or reduced in the V363 mutant (data not shown). Taken together, these data indicate that phosphorylation of threonine-363 of STE7 by STE11 is necessary to activate STE7.

STE11 Activity Is Not Regulated *in Vitro*. To investigate the regulation of STE11 activity, two further GST–STE11 fusions were constructed: one joined the entire coding region of STE11 to GST (GST–STE11$^{1-717}$); the second removed the amino-terminal 363 amino acid residues (GST–STE11$^{364-717}$) and was expected to create a constitutively active kinase (7). These kinases behaved as expected when expressed *in vivo*: the GST–STE11$^{1-717}$ conferred α-factor-inducible expression on a *FUS1::lacZ* reporter, whereas GST–STE11$^{364-717}$ caused constitutive, high-level expression (Table 1). A strikingly different result was observed *in vitro*: the proteins exhibited equivalent kinase activities (Table 1), suggesting that the GST–STE11$^{1-717}$ had induced levels of activity *in vitro*. No significant differences between kinase activities of the full-length and truncated STE11 were detected in time-course or titration experiments (data not shown). In addition, there was no detectable difference in the activity of GST–STE11$^{1-717}$ whether it was purified from α-factor-treated or untreated cells (data not shown), as has been reported previously for a Myc-tagged version of STE11 assayed in immunoprecipitates (15).

Table 1. Activities of full-length and truncated STE11

Protein expressed	*In vivo* β-galactosidase activity*		*In vitro* kinase activity†
	− α-factor	+ α-factor	
GST–STE11$^{1-717}$	0.9	29	199
GST–STE11$^{364-717}$	91	85	193
GST	<0.1	<0.1	

Plasmids producing the indicated protein were expressed in *ste11*Δ strain AN1016. Plasmids used were pRD-STE11-ATG, pRD-STE11-H3, and pRD56.

*Calculated as described in ref. 18.

†Values represent the amount of phosphorylation of GST–STE7$^{134-515}$ (arbitrary units) per nanogram of GST–STE11 protein, determined as described in *Materials and Methods*.

DISCUSSION

Given the structural similarity of STE11 and mammalian MEKK and the physiological observations made in yeast (6, 7, 9), we anticipated that STE7 would be a substrate for STE11. Our results demonstrate that this is the case and thus argue against more complex scenarios such as STE11 working in conjunction with another kinase to phosphorylate STE7. We show that the phosphorylated form of STE7 is now functional for phosphorylation of FUS3. We have thus described the reconstitution of two steps of the yeast mating factor response pathway *in vitro*.

We have shown that phosphorylation of the threonine residue at position 363 is required for STE7 activity both *in vivo* and *in vitro*. It is not clear whether phosphorylation of this site is sufficient to activate STE7. A mutant fusion protein with a substitution of aspartate for threonine at this position did not exhibit constitutive kinase activity but rather was inactive (data not shown). The conservation of threonine-363 (Fig. 3*B*) suggests that phosphorylation of this site may be necessary for activation of other members of the STE7 family. If so, then we predict that activation of MEK1 will require phosphorylation of serine-222 (Fig. 3*B*).

GST–STE11 appears to be constitutively active when purified from yeast. There are a number of possible explanations for this observation; for instance, the amino-terminal domain of STE11 may be unable to fold properly *in vitro*. A more interesting possibility is that an inhibitory factor is lost during the purification of the protein. If so, then this suggests a role for STE5 and STE20, which function upstream of STE11: they might regulate the activity of an inhibitor of STE11. It should be possible to use our *in vitro* system to search for this inhibitor.

The findings reported here are consistent with previous work demonstrating that partially purified MEKK (the mammalian STE11 homologue) can phosphorylate and activate MEK (13), reinforcing the parallels between the metazoan and yeast cascades. Given the similarity of the yeast and mammalian kinase cascades, the mating-factor response pathway should continue to be instructive for understanding signaling systems in metazoans, perhaps including upstream regulators of STE11/MEKK.

Note. Gotoh *et al.* (Y. Gotoh and E. Nishida, personal communication) have shown that phosphorylation of serine at position 222 of *Xenopus* MAP kinase kinase (MEK) by STE11 or *Xenopus* MAP kinase kinase kinase (MEKK) is required to stimulate activity of the MAP kinase kinase. These results are consistent with our observations on STE11 and STE7.

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1. Marsh, L., Neiman, A. M. & Herskowitz, I. (1991) *Annu. Rev. Cell Biol.* **7**, 699–728.
2. Kurjan, J. (1992) *Annu. Rev. Biochem.* **61**, 1097–1129.
3. Whiteway, M., Hougan, L., Dignard, D., Thomas, D. Y., Bell, L., Saari, G. C., Grant, F. J., O'Hara, P. & MacKay, V. L. (1989) *Cell* **56**, 467–477.
4. Blinder, D., Bouvier, S. & Jenness, D. (1989) *Cell* **56**, 479–486.
5. Leberer, E., Dignard, D., Harcus, D., Thomas, D. Y. & Whiteway, M. (1992) *EMBO J.* **11**, 4815–4824.
6. Stevenson, B. J., Rhodes, N., Errede, B. & Sprague, G. F., Jr. (1992) *Genes Dev.* **6**, 1293–1304.
7. Cairns, B. R., Ramer, S. W. & Kornberg, R. D. (1992) *Genes Dev.* **6**, 1305–1318.
8. Gartner, A., Nasmyth, K. & Ammerer, G. (1992) *Genes Dev.* **6**, 1280–1292.
9. Zhou, Z., Gartner, A., Cade, R., Ammerer, G. & Errede, B. (1993) *Mol. Cell. Biol.* **13**, 2069–2080.

10. Boulton, T. G., Yancopoulos, G. D., Gregory, J. S., Slaughter, C., Moomaw, C., Hsu, J. & Cobb, M. H. (1990) *Science* **249**, 64–67.
11. Errede, B., Gartner, A., Zhou, Z., Nasmyth, K. & Ammerer, G. (1993) *Nature (London)* **362**, 261–264.
12. Crews, C. M., Alessandrini, A. & Erikson, R. L. (1992) *Science* **258**, 478–480.
13. Lange-Carter, C. A., Pleiman, C. M., Gardner, A. M., Blumer, K. J. & Johnson, G. L. (1993) *Science* **260**, 315–319.
14. Neiman, A. M. (1993) *Trends Genet.* **9**, 390–394.
15. Rhodes, N. L., Connell, L. & Errede, B. (1990) *Genes Dev.* **4**, 1862–1874.
16. Mortimer, R. K. & Hawthorne, D. C. (1969) in *The Yeasts*, eds. Rose, A. H. & Harrison, J. S. (Academic, New York), pp. 385–460.
17. Hicks, J. B. & Herskowitz, I. (1976) *Genetics* **83**, 245–258.
18. Neiman, A. M., Stevenson, B. J., Xu, H.-P., Sprague, G. F., Jr., Herskowitz, I., Wigler, M. & Marcus, S. (1993) *Mol. Biol. Cell* **4**, 107–120.
19. Park, H.-O., Chant, J. & Herskowitz, I. (1993) *Nature (London)* **365**, 269–274.
20. Kunkel, T. A., Bebenek, K. & McClary, J. (1991) *Methods Enzymol.* **204**, 125–139.
21. Peter, M., Nakagawa, M., Doree, M., Labbe, J. C. & Nigg, E. A. (1990) *Cell* **60**, 791–801.
22. Smith, D. B. & Johnson, K. S. (1988) *Gene* **67**, 31–40.
23. Crews, C. M., Alessandrini, A. & Erikson, R. L. (1992) *Cell Growth Differ.* **3**, 135–142.
24. Peter, M., Gartner, A., Horecka, J., Ammerer, G. & Herskowitz, I. (1993) *Cell* **73**, 747–760.
25. Elion, E. A., Satterberg, B. & Kranz, J. E. (1993) *Mol. Biol. Cell* **4**, 495–510.
26. Hanks, S. K. & Quinn, A. M. (1991) *Methods Enzymol.* **200**, 38–62.
27. Irie, K., Takase, M., Lee, K. S., Levin, D. E., Araki, H., Matsumoto, K. & Oshima, Y. (1993) *Mol. Cell. Biol.* **13**, 3076–3083.
28. Teague, M. A., Chaleff, D. T. & Errede, B. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 7371–7375.