Osmotic Balance Regulates Cell Fusion during Mating in *Saccharomyces cerevisiae*

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Abstract. Successful zygote formation during yeast mating requires cell fusion of the two haploid mating partners. To ensure that cells do not lyse as they remodel their cell wall, the fusion event is both temporally and spatially regulated: the cell wall is degraded only after cell–cell contact and only in the region of cell–cell contact. To understand how cell fusion is regulated, we identified mutants defective in cell fusion based upon their defect in mating to a *fus1 fus2* strain (Chenevert, J., N. Valtz, and I. Herskowitz. 1994. *Genetics* 136:1287–1297). Two of these cell fusion mutants are defective in the *FPS1* gene, which codes for a glycerol facilitator (Luyten, K., J. Albertyn, W.F. Skibbe, B.A. Prior, J. Ramos, J.M. Thevelein, and S. Hohmann. 1995. *EMBO [Eur. Mol. Biol. Organ.] J.* 14:1360–1371). To determine whether inability to maintain osmotic balance accounts for the defect in cell fusion in these mutants, we analyzed the behavior of an *fps1Δ* mutant with reduced intracellular glycerol levels because of a

defect in the glycerol-3-phosphate dehydrogenase (*GPD1*) gene (Albertyn, J., S. Hohmann, J.M. Thevelein, and B.A. Prior. 1994. *Mol. Cell. Biol.* 14:4135–4144): deletion of *GPD1* partially suppressed the cell fusion defect of *fps1* mutants. In contrast, overexpression of *GPD1* exacerbated the defect. The fusion defect could also be partially suppressed by 1 M sorbitol. These observations indicate that the fusion defect of *fps1* mutants results from inability to regulate osmotic balance and provide evidence that the osmotic state of the cell can regulate fusion. We have also observed that mutants expressing hyperactive protein kinase C exhibit a cell fusion defect similar to that of *fps1* mutants. We propose that Pkc1p regulates cell fusion in response to osmotic disequilibrium. Unlike *fps1* mutants, *fus1* and *fus2* mutants are not influenced by expression of *GPD1* or by 1 M sorbitol. Their fusion defect is thus unlikely to result from altered osmotic balance.

T HE joining of two cells occurs during certain specialized cell–cell interactions such as sperm–egg fusion during fertilization, myoblast fusion during myotube formation, and gamete fusion during yeast mating. Intercellular fusion requires successful completion of a number of different events, the molecular details of which are poorly understood. The interacting cells must first recognize and adhere to each other. Extracellular material separating the interacting cells must then be removed. The zona pellucida surrounding the egg, extracellular matrix components separating myoblasts, and cell wall material separating haploid yeast cells must be removed to place the plasma membranes of the interacting cells into apposition. Finally, the plasma membranes of the two cells fuse, forming a single heterokaryon which can then undergo fusion of intracellular organelles.

The mating pathway of *Saccharomyces cerevisiae* culminates in the fusion of two haploid cells of opposite mating type (**a** and α) into an **a**/α diploid zygote. The events leading up to cell–cell contact are well characterized. Haploid cells secrete peptide pheromones (**a**-factor by **a** cells and α-factor by α cells) that are important for intercellular recognition and for preparing cells for fusion. These pheromones activate a G protein–coupled receptor on the surface of the opposite mating partner, which in turn activates a mitogen-activated protein (MAP)[1] kinase cascade, inducing a morphological response (shmoo formation), cell cycle arrest, and transcriptional induction (for reviews see Kurjan, 1992; Sprague and Thorner, 1992; Bardwell et al., 1994; Herskowitz, 1995). The mating pheromones prepare cells to fuse by inducing expression and localization of fusion components. In particular, synthesis of Fus1p and

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1. *Abbreviations used in this paper*: DAPI, 4′,6-diamidino-2-phenylindole; GFP, green fluorescent protein; MAP, mitogen-activated protein; ORF, open reading frame; YEPD, yeast extract/peptone/dextrose.

Fus2p, proteins required for cell fusion, is induced by pheromone (Trueheart et al., 1987; McCaffrey et al., 1987; Elion et al., 1995). These proteins are localized to the region of future cell contact (Trueheart et al., 1987; Elion et al., 1995). Cells polarize the actin cytoskeleton and secretory apparatus toward their selected mating partner by detecting a pheromone gradient (Jackson and Hartwell, 1990; Madden and Snyder, 1992; Segall, 1993). As a result, new membrane and cell wall material is deposited at the site of future cell contact (Field and Schekman, 1980; Adams and Pringle, 1984; Novick and Botstein, 1985; Hasek et al., 1987; Read et al., 1992), which may be important for localized cell wall modifications (Lipke et al., 1976; Tkacz and MacKay, 1979; Schekman and Brawley, 1979; Baba et al., 1989) and targeting of the fusion machinery.

Although pheromones activate cells for fusion, cell wall degradation does not begin until the mating partners contact each other. Initially, cell surface agglutinins mediate attachment of the mating partners (Lipke and Kurjan, 1992), which is reversible by sonication. The cell walls then become irreversibly attached. Once cell–cell contact occurs, a thinning of the cell wall is observed that begins in the center of the region of cell contact and proceeds toward the edges (Osumi et al., 1974). Cell wall degradation and remodeling normally occur quickly, so that few cells in a population of mating cells are adhered but not fused (Trueheart et al., 1987). In mutants defective in cell fusion, zygote formation is blocked after the cells have adhered but before the intervening wall has been degraded, producing a dumbbell-shaped structure called a prezygote. The persistence of the cell wall in these mutants creates a physical barrier between mating partners, preventing cytoplasmic mixing and nuclear fusion (Trueheart et al., 1987; McCaffrey et al., 1987).

Products of the *FUS1-FUS3*, *FUS5-FUS8*, and *CEF1* genes are required for cell fusion (Trueheart et al., 1987; McCaffrey et al., 1987; Elion et al., 1990, 1995; Kurihara et al., 1994; Elia and Marsh, 1996). Fus1p is a transmembrane protein with an intracellular SH3 domain (Trueheart et al., 1987; Trueheart and Fink, 1989). Fus2p has no similarity to known proteins (Elion et al., 1995). Despite their lack of homology, Fus1p and Fus2p have overlapping functions, as overexpression of one can partially suppress loss of the other. Absence of both proteins results in a synthetic fusion defect. In addition, the fusion defect is greatly enhanced when both mating partners are mutant, suggesting that at least some activities required for cell fusion can be provided by either partner (Trueheart et al., 1987). Unlike *FUS1* and *FUS2*, which are specifically required for cell fusion, the other *FUS* genes have additional functions during mating. For instance, *FUS3*, whose role in cell fusion is unknown, encodes a MAP kinase that functions in the pheromone response pathway (Elion et al., 1990). *FUS5*, *FUS8*, and *CEF1* genes correspond to *AXL1*, *RAM1*, and *STE6*, respectively. These genes were previously identified for their role in **a**-factor production (Adames et al., 1995; Powers et al., 1986; Kuchler et al., 1989), suggesting that high levels of pheromone may play a role in cell fusion (Elia and Marsh, 1996; Brizzio et al., 1996). It is also possible that these proteins (e.g., Ste6p, the **a**-factor transporter) are required for cell fusion independently of their role in pheromone production or secretion (Elia and Marsh,

1996). Whether the products of these genes play direct roles in cell fusion or are involved in regulating fusion is unclear.

Cell fusion requires that cell contact be sensed and that the cell surface be remodeled in response to this contact. To prevent cell lysis and to maintain cell integrity, cell wall degradation must be highly regulated, occurring only after cell–cell contact and only in the region of cell–cell contact. We show that the glycerol facilitator, Fps1p, is required for cell fusion. Our studies on Fps1p provide evidence that the osmotic state of the cell regulates cell fusion. We present additional studies suggesting that protein kinase C, previously recognized for its role in osmotic regulation (Davenport et al., 1995), negatively regulates cell fusion, further linking osmosensing pathways to regulation of cell fusion.

Materials and Methods

Yeast Strains and Media

Yeast strains are described in Table I. Standard yeast growth conditions and genetic manipulations are described in Rose et al. (1990). Cells were grown at 30°C in yeast extract/peptone/dextrose medium unless otherwise noted. DNA manipulations were performed as described in Sambrook et al. (1989).

Yeast Plasmids and Transformations

YEp*GPD1* is a 2 μ *URA3* plasmid (derived from YEplac195) containing the *GPD1* gene, as described in Albertyn et al. (1994*b*) (kindly provided by S. Hohmann, Katholieke University, Leuven, Belgium). pJP67 (YCp50-DS1) is a YCp50-derived plasmid containing the *PKC1-R398P* allele as described in Nonaka et al. (1995) (kindly provided by Y. Takai, Osaka University, Osaka, Japan). The 4.3-kb SphI fragment containing *PKC1-R398P* from pJP67 was cloned into BamHI and SalI sites of pRS306 to generate pJP72. This plasmid was used to integrate *PKC1-R398P* at its genomic locus, generating strain JP317. Plasmids containing *PKC1* under control of the *GAL1* promoter are pDL242 (p*GAL1[PKC1-R398A]*), pDL293 (p*GAL1[PKC1::HA]*), and pDL295 (p*GAL1[PKC1-K853R::HA]*) as described in Watanabe et al. (1994). They were kindly provided by D. Levin (Johns Hopkins University, Baltimore, MD). pJW192 codes for a RAS2–green fluorescent protein (GFP) fusion protein under control of the *GPD* promoter on a 2μ *TRP1* marked plasmid (kindly provided by J. Whistler, University of California, Berkeley). Yeast transformations were performed by the lithium acetate method (Ito et al., 1983).

Strain Construction

The *GPD1* gene was deleted from strains using pUC*gpd1*Δ::*TRP1*, a construct designed to replace *GPD1* with *TRP1* as described in Albertyn et al. (1994*b*) (kindly provided by S. Hohmann). Strains were confirmed to be *gpd1*Δ by their sensitivity to high osmolarity media and by PCR analysis. *fus1*Δ strains were constructed using pJP2, which contains a substitution of the *FUS1* open reading frame (ORF) by *TRP1*. This plasmid was generated by cloning the 1.9-kb PstI–KpnI fragment containing *FUS1* into the PstI–KpnI sites of pBluescript KS+. The *FUS1* ORF was removed by cloning a ClaI–HincII fragment containing *TRP1* into the AccI–HincII sites of *FUS1*. *fus2*Δ strains were generated using pKOFUS2, a plasmid in which the 1.6-kb HindIII fragment containing *FUS2* was replaced by the 1.1-kb HindIII fragment containing *URA3*. *fus1*Δ and *fus2*Δ strains were confirmed by their defective mating and by PCR analysis. *fps1*Δ deletion strains were generated using either pJP31 or pJP52. pJP31 replaces *FPS1* with *LEU2;* pJP52 replaces *FPS1* with *URA3* (see Fig. 1 *b*). The BglII–HindIII fragment containing *FPS1* was cloned into the BamHI–HindIII sites of pUC18. To generate pJP31, *LEU2* was removed from pUC18-LEU2 (Herskowitz collection), in which *LEU2* is cloned into the SalI site of pUC18. A PstI–XbaI fragment, containing *LEU2*, was cloned into the NsiI–AccI site of *FPS1*. pJP52 was constructed from a plasmid in which a 111-nucleotide fragment was inserted at the stop codon of *FPS1*, generating a BamHI site 70 nucleotides 3′ to the stop codon. This plasmid was cut with BamHI and NsiI and a BamHI–NsiI fragment containing *URA3* was

Table I. Yeast Strains and Plasmids Used in This Study

Strain	Genotype	Source
IH2350	*MATα ura 3-52 his4-34 trp1 Δ1*	IH collection
IH2351*	*MATα ura 3-52 trp1Δ1 fus1Δ1 fus2Δ3*	IH collection
IH2353*	*MATa ura 3-52 trp1Δ1 fus1Δ1 fus2Δ3*	IH collection
IH3160	*MATa*	IH collection
IH3179	*MATa fps1-1*	IH collection
IH3182	*MATa fps1-2*	IH collection
IH3186	*MATα*	This study
IH3190	*MATα trp1Δ99*	This study
IH3194	*MATα trp1Δ99 leu2Δ1*	This study
IH3196	*MATa leu2Δ1*	This study
JP52	*MATa fus1Δ::TRP1 trp1Δ99 leu2Δ1*	This study
JP147	*MATa fps1Δ::URA3 leu2Δ1*	This study
JP150	*MATa FPS1::FPS1-URA3*	This study
JP153	*MATa ura3-52::URA3*	This study
JP154	*MATa fps1-1::FPS1-URA3*	This study
JP157	*MATa fps1-1 ura3-52::URA3*	This study
JP158	*MATa fps1-2::FPS1-URA3*	This study
JP161	*MATa fps1-2 ura3-52::URA3*	This study
JP163	*MATa fps1Δ:: LEU2Δ1*	This study
JP165	*MATa fps1Δ:: URA3 gpd1Δ::TRP1 leu2Δ1 trp1Δ99*	This study
JP168	*MATa gpd1Δ::TRP1 leu2Δ1 trp1Δ99*	This study
JP226	*MATα fps1Δ::URA3 trp1Δ99*	This study
JP233	*MATα fps1Δ::URA3 gpd1Δ::TRP1 trp1Δ99*	This study
JP236	*MATα gpd1Δ::TRP1 trp1Δ99*	This study
JP257	*MATα fus2Δ::URA3 trp1Δ99*	This study
JP285	*MATa fus1Δ::TPR1 gpd1Δ::TRP1 trp1Δ99*	This study
JP287	*MATα fus2Δ::URA3 gpd1Δ:: TRP1 trp1Δ99*	This study
JP317	*MATa PKC1::PKC1-R398P-URA3 leu2Δ1*	This study
JP325[‡]	*MATa fps1Δ::LEU2*	This study
JP326[‡]	*MATa mpk1Δ:::TRP1*	This study
JP327[‡]	*MATα fps1Δ::LEU2 mpk1Δ::TRP1*	This study
JP328[‡]	*MATα*	This study

Strains containing plasmids:

JP199	IH3196 containing YEp*GPD1*	
JP200	JP163 containing YEp*GPD1*	
JP300	IH3196 containing pJP67	
JP301	IH3196 containing YCp50	
JP333	IH3194 containing pJW192	
JP400	IH3196 containing YCplac195	
JP401	JP163 containing YCplac195	

Plasmid name	Description	Source
pDL242	p*GAL1*[*PKC1-R398A*] (pBM743)	D. Levin (Watanabe et al., 1994)
pDL293	p*GAL1*[*PKC1::HA*] (pBM743)	D. Levin (Watanabe et al., 1994)
pDL295	p*GAL1*[*PKC1-K853R::HA*] (pBM743)	D. Levin (Watanabe et al., 1994)
pJP2	*fus1Δ::TRP1*	This study
pJP30	*FPS1-URA3* (pRS306)[§]	This study
pJP31	*fps1Δ::LEU2*	This study
pJP52	*fps1Δ::URA3*	This study
pUC*gpd1Δ::TRP1*	*gpd1Δ::TRP1*	S. Hohmann (Albertyn et al., 1994*b*)
YEp*GPD1*	2μ *GPD1* (YEplac195)[§]	S. Hohmann (Albertyn et al., 1994*b*)
pJP67 (YCp50-DS1)	*PKC1-R398P* (YCp50)[§]	Y. Takai (Nonaka et al., 1995)
pJP72	*PKC1-R398P* (pRS306)[§]	This study
pJW192	*RAS2-GFP*	J. Whistler
pKOFUS2	*fus2Δ::URA3*	IH collection

*Isogenic to IH2350.

[‡] Isogenic derivatives in the EG123 strain background, whose full genotype is *trp1 ura3 his4 leu2 can1;* all other strains are isogenic derivatives of IH3160, whose full genotype is *MATa ade2-101 ura3-52 met1-1 HMLa HMRa.*

[§] pRS306, YCplac195, and YCp50 are described in Guthrie and Fink (1991).

IH, I. Herskowitz.

inserted. The *URA3* fragment was obtained from pSM32 (Herskowitz collection), a pUC18 plasmid containing *URA3*. *fps1Δ* strains were confirmed by defective mating and by PCR analysis.

Mating Assays

Quantitative mating was as described in Valtz and Herskowitz (1996) except that 6×10^6 cells of each mating partner were mixed. Mating assays scored microscopically were performed by mixing equal numbers of log phase **a** and α cells (6×10^6), collecting cells on 0.45-μm filters (Millipore Corp., Bedford, MA), and incubating on YEPD plates for ∼4 h at 30°C. Cells were resuspended in 5 ml 70% ethanol by vortexing, washed, and resuspended in 50% glycerol + 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI; Sigma Chemical Co., St. Louis, MO). Samples were sonicated and viewed with a microscope at 100× (Axioskop; Carl Zeiss, Inc., Thornwood, NY). Percentage of prezygotes was defined as prezygotes/(prezygotes + zygotes). At least 100 partnered cells (zygotes + prezygotes) were counted per sample. Numbers represent the average of at least three experiments unless otherwise noted. Assays in which one partner contained the RAS2-GFP plasmid were performed in essentially the same manner, except that strains were grown in SD-TRP (Rose et al., 1990) to select for the plasmid. In this case, cells were resuspended from the filter into 5 ml YEPD by vortexing, sonicated, and viewed with a microscope at 100× (BX50; Olympus Corp., Lake Success, NY). Mating assays scored on plates were performed by spreading a lawn of 9×10^6 log phase *fus1 fus2* mating tester cells on a YEPD plate. Patches of cells grown on YEPD (or on minimal media to select for plasmids) were replica plated to the lawn. After incubation at 30°C for 3.5–4.5 h, plates were replica plated to media selective for growth of diploids. For cells expressing *PKC1* alleles under control of the *GAL1* promoter (pDL242, pDL293, pDL295), patches were grown on raffinose-URA plates to select for the plasmid but maintain the *GAL1* promoter inactive. They were replica plated to a lawn of an *MATα fus1 fus2* strain at 30°C, on either YEPD or YEPGalactose. Cells were allowed to mate for 3.75 h and then replica plated to select for diploids in the presence of glucose.

Cloning of FPS1

FPS1 was cloned by complementation of the mating defect of the M8 mutant (Chenevert et al., 1994). Five plasmids that rescued the mating defect were isolated from a 2μ YEp24-derived library (Carlson and Botstein, 1982) from 22,000 transformants screened. All contained a common 6.5-kb overlapping DNA segment. An EcoRI–SalI fragment was subcloned into pRS316, a CEN-ARS vector, and deletion analysis was performed to identify the complementing ORF (see Fig. 2 *c*). A plasmid containing a 2.2-kb XhoI–HindIII fragment (pJP27) fully restored mating to the M8 and M11 mutants. The 2.4-kb BglII–HindIII segment containing *FPS1* was cloned into pRS306, a *URA3*-marked, integrating vector to form pJP30, which was able to restore mating and fusion when integrated at the *FPS1* genomic locus. A wild-type strain was transformed with the integrating plasmid, marking the *FPS1* locus with *URA3* (JP150), and then crossed to M8 and M11 mutants, which were *ura3*. 12 of 13 complete tetrads from the M8 cross showed 2:2 segregation of Ura3⁺:Ura3⁻ and Mating⁺:Mating⁻ phenotypes. One tetrad showed 1:3 segregation of Ura3⁺:Ura3⁻ and 2:2 segregation of Mating⁺:Mating⁻, presumably because of gene conversion at *URA3*. 10 complete tetrads from the M11 cross showed 2:2 segregation of Ura3 and mating phenotypes. In all spores, the Ura3⁺ phenotype cosegregated with mating proficiency, indicating that the mutations responsible for the mating defects of M8 and M11 are linked to *FPS1*.

Analysis of fps1Δ mpk1Δ Double Mutants

Segregants obtained by crossing an *fps1Δ* strain with an *mpk1Δ* strain were allowed to germinate on YEPD containing 1 M sorbitol at 25°C. Cells were passaged on YEPD (except the *mpk1Δ fps1Δ* double mutant, which was grown on YEPD + 1 M sorbitol), streaked for single colonies on either YEPD or YEPD + 1 M sorbitol, and grown for 2 d at 25°C.

Assaying of Intracellular Glycerol

Intracellular glycerol was assayed enzymatically using a glycerol determination kit (Boehringer Mannheim Biochemicals, Indianapolis, IN) essentially as described (Albertyn et al., 1994*a*). Cells were grown to mid-log phase in YEPD unless selecting for plasmids, in which case they were grown in SD-URA (Rose et al., 1990). From each culture, three 10-ml aliquots were separately filtered onto 0.45-μm filters (Millipore Corp.), washed quickly with 5 ml ice-cold YEPD, and resuspended in 2 ml 0.5 M Tris-HCl, pH 7.5, by vortexing. Samples were heated to 95°C for 10 min, and cell debris was removed by centrifugation. Aliquots were pooled, and glycerol determinations were performed as per manufacturer's specifications. Protein concentrations were determined using the Bio-Rad protein assay (Bio Rad Laboratories, Hercules, CA). Glycerol concentrations were normalized to total protein. Fold increase in glycerol concentrations was determined by normalizing to the level of intracellular glycerol in wild-type cells. Data represent the average of at least three experiments.

Results

Identification of Mutants Defective in Cell Fusion

Mutants defective in cell fusion were identified based upon their defect in mating to a *fus1 fus2* mutant (Chenevert et al., 1994). *fus1 fus2* double mutants are mildly compromised in mating to a wild-type strain but are severely defective in mating to *fus1* or *fus2* strains (Trueheart et al., 1987; Elion et al., 1995). Hence, a screen to find mutants defective in mating to a *fus1 fus2* strain should identify mutants defective in *FUS1*, *FUS2*, and other genes required for fusion. To determine if any of the mutants were defective in *FUS1* or *FUS2*, plasmids containing these genes were tested for their ability to restore mating to the mutants, which identified one mutant as defective in *FUS2* (Chenevert et al., 1994).

To identify additional cell fusion mutants, mutants exhibiting a mating defect but normal shmoo morphology (class 4 mutants in Chenevert et al., 1994) were examined microscopically to ascertain if prezygotes accumulated when the mutants were mated to a wild-type strain. Prezygotes were scored as structures in which the nuclei of mating partners remained unfused, as evidenced by two distinct DAPI staining structures, and in which a septum was visible between adherent mating partners. 9 of 13 mutants exhibited increased frequency of prezygotes, indicative of a fusion defect (data not shown). The mutants were of two classes: five were **a** cell specific and four were non–cell type specific, exhibiting mating defects as both **a** and α cells. Two mutants of the latter class, designated M8 and M11 (B6 and J10, respectively, in Chenevert et al., 1994), were further characterized and are described here.

M8 and M11 exhibit normal pheromone signaling and events leading up to cell–cell contact. They produce and respond to pheromone normally, as assayed by cell cycle arrest and shmoo formation (Chenevert et al., 1994; Philips, J., unpublished observations). We observed that, in matings between M8 or M11 mutants and a wild-type partner, the percentage of partnered cells ([prezgotes + zygotes]/total cells) was normal, suggesting that the defect in mating is not due to inability of the partners to find or adhere to each other. Fig. 1 illustrates the aberrant morphological structures that accumulated in mating mixes in which one partner is M8 or M11. More than 30% of the partnered cells were prezygotes (Fig. 1, *b* and *c*). In contrast, in mating reactions between wild-type partners (Fig. 1 *a*), <1% of the partnered cells were prezygotes. Further evidence that cell fusion was blocked before plasma membrane fusion was obtained by mating cells to a partner that produces a RAS2–GFP fusion protein (kindly provided by J. Whistler), which localizes green fluorescence around the
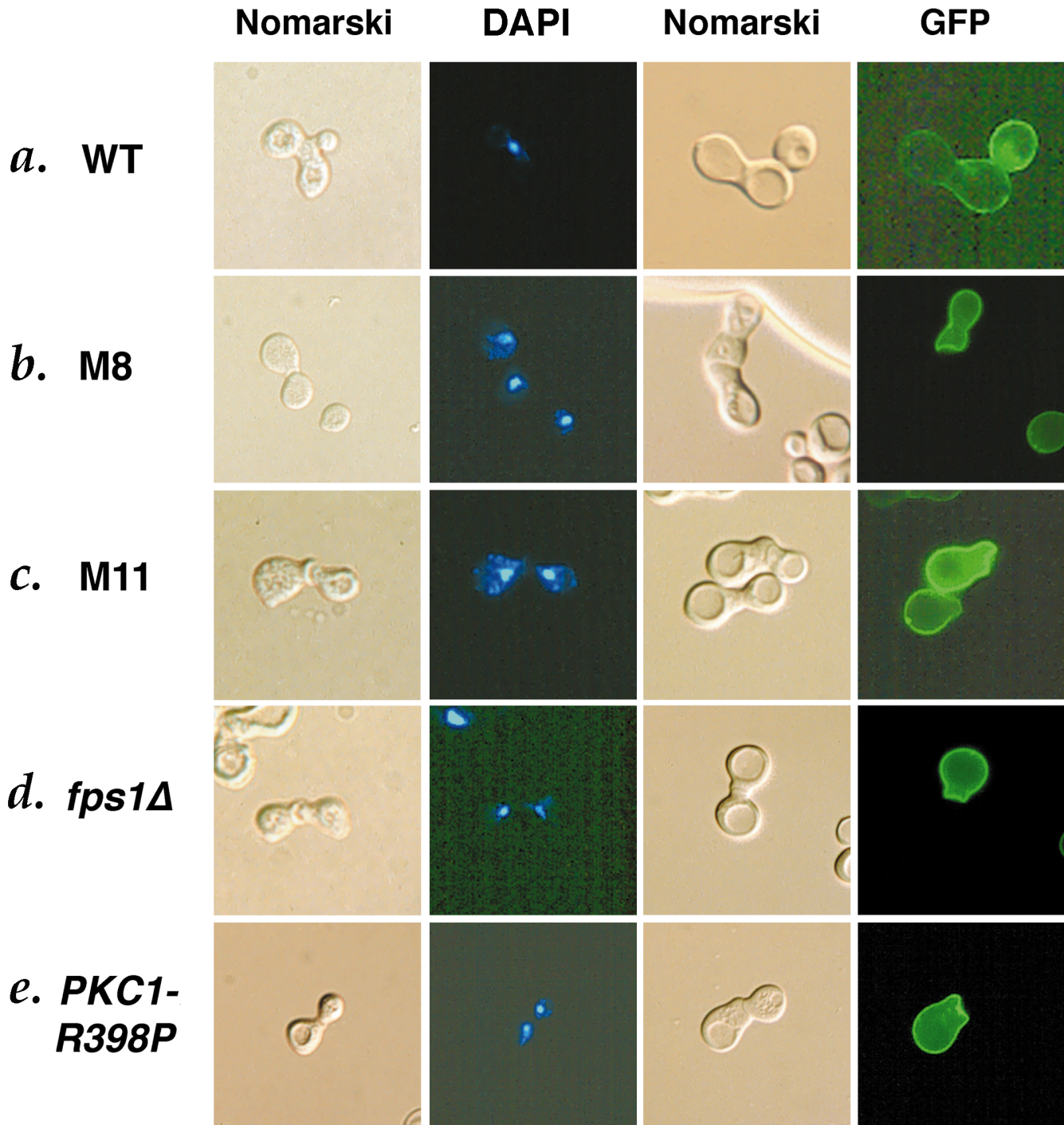
Nomarski	DAPI	Nomarski	GFP

Figure 1. Morphological phenotype of cell fusion mutants. (*a*) Wild-type (IH3160), (*b*) M8 (IH3179), (*c*) M11 (IH3182), (*d*) *fps1Δ* (JP147), and (*e*) *PKC1-R398P* (JP317) strains were mated on filters to wild-type strain IH3186 (*two left columns*) or to wild-type strain JP333 carrying the RAS2–GPF fusion plasmid (*two right columns*) as described in Materials and Methods. Nuclei were visualized by DAPI staining (*second column*). RAS2–GFP was visualized by fluorescence microscopy (*fourth column*).

periphery of the cell (Whistler, J., and J. Rine, personal communication). In mating reactions containing wild-type cells, zygotes showed the green fluorescent signal throughout the entire zygote (Fig. 1 *a*). In contrast, when mutants were mated to the wild-type partner containing RAS2–GFP, prezygotes were found in which the green fluorescent signal remained restricted to one cell, indicating a failure of plasma membrane fusion and cytoplasmic mix-

ing (Fig. 1, *b* and *c*). We conclude that M8 and M11 mutants are defective in cell fusion but are normal for earlier events of mating.

M8 and M11 Are Defective in the FPS1 Gene

To clone the gene defective in the M8 mutant, the strain was transformed with a high copy YEp24-derived library,
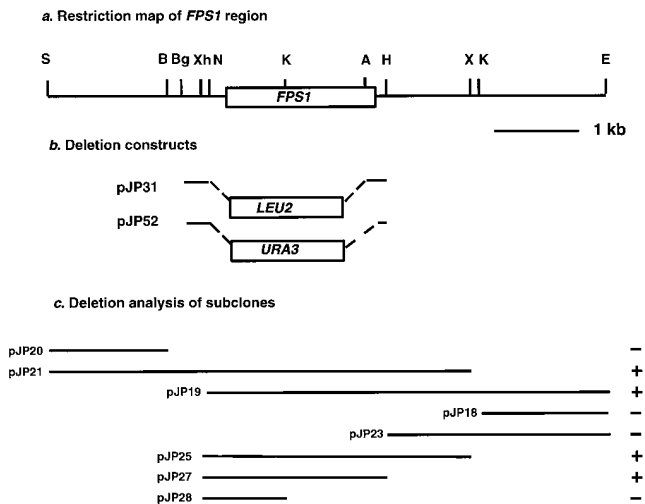
a. Restriction map of *FPS1* region

b. Deletion constructs

c. Deletion analysis of subclones

Figure 2. Restriction map (*a*), disruption constructs (*b*), and deletion analysis (*c*) of *FPS1* region. The ability of each plasmid to complement the mating defect of the M8 and M11 mutants is indicated on the right: +, complementation; −, no complementation. pJP are plasmids carrying the indicated segments. Restriction enzymes: *S*, SalI; *B*, BamHI; *Bg*, BglII; *Xh*, XhoI; *N*, NsiI; *K*, KpnI; *H*, HindIII; *X*, XbaI; *E*, EcoRI. Additional information is given in Materials and Methods and in the text.

Figure 3. The *FPS1* gene complements the mating defect of M8 and M11. (*Right*) WT (JP150), M8 (JP154), and M11 (JP158), all carrying *FPS1* plasmid pJP30 at the *FPS1* genomic locus; (*left*) WT (JP153), M8 (JP157), and M11 (JP161), all carrying the vector alone (pRS306). Strains were mated to a *MATα fus1 fus2* strain (IH2351) as described in Materials and Methods.

and 22,000 transformants were screened for ability to mate with a *fus1 fus2* strain. Five plasmids were identified that restored mating. All contained a common 6.5-kb overlapping DNA segment. This segment was subcloned into pRS316, a CEN-ARS vector, and further subcloning was performed to identify the minimal fragment capable of complementation (Fig. 2 *c*). A 2.2-kb XhoI–HindIII fragment (pJP27) restored mating to the M8 and M11 mutants. This fragment contains the *FPS1* ORF, which fully restored mating and cell fusion to M8 and M11 when integrated at its genomic locus (Fig. 3 and data not shown). The mutation responsible for the mating defect of M8 and M11 was demonstrated to be allelic to *FPS1* by following the segregation of M8 and M11 in crosses where the *FPS1* locus was marked (see Materials and Methods). The plasmid complementation and segregation analyses indicate that the mating defect of M8 and M11 strains is due to mutations in *FPS1*, which are designated *fps1-1* and *fps1-2*, respectively.

Mating Defect of fps1Δ Mutants

Analysis of strains deleted for *FPS1* confirmed that Fps1p is required for cell fusion. *FPS1* was deleted from the genome by replacing the *FPS1* coding sequence with either *LEU2* or *URA3* (Fig. 2 *b*; see Materials and Methods). *fps1Δ* strains, like *fps1-1* and *fps1-2* mutants, were defective in mating to *fus1 fus2* strains (Fig. 4) and accumulated prezygotes that were morphologically indistinguishable from those observed in mating reactions with *fps1-1* and *fps1-2* mutants (Fig. 1 *d*). In mating mixes of *fps1Δ* or *fps1-2* mutants to a wild-type partner, 30–45% of partnered cells were prezygotes. To determine if the *fps1-1* allele was quantitatively similar to the *fps1Δ* allele, *FPS1* was deleted in two different *fps1-1* strains. These *fps1Δ* deletion strains
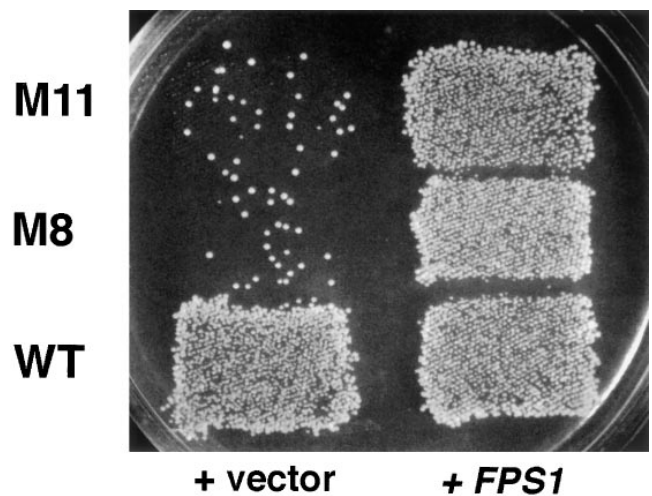
behaved like the *fps1-1* parent strains with respect to the percentage of prezygotes accumulated (data not shown). Thus, *fps1-1* and *fps1-2* appear to be null alleles of *FPS1*. *FPS1* deletion mutants in the EG123 strain background have a similar defect in cell fusion (data not shown).

Quantitative mating assays demonstrated that, like *fus1 fus2* strains, *fps1Δ* strains were mildly defective in mating, exhibiting a two- to threefold decrease in diploid formation (Table II). Unlike *fus1 fus2* mutants, which exhibit an enhanced defect when both mating partners are mutant (Trueheart et al., 1987; McCaffrey et al., 1987; Elion et al., 1995), *fps1Δ* mutants did not mate significantly worse or accumulate more prezygotes when mated to an *fps1Δ* partner than to a wild-type partner. In fact, when *fps1Δ* mutants are mated to wild-type cells, they yielded 61 ± 17% or 32 ± 5% prezygotes, depending upon the mating type of the mutant, whereas matings between two *fps1Δ* mutants yield 18 ± 9% prezygotes. Thus, unlike *fus1* and *fus2* mutants, matings between *fps1Δ* mutants may exhibit some suppression of the fusion defect. In contrast, *fps1Δ* mutants mated to *fus1 fus2* mutants at a much lower efficiency compared with wild type, a decrease of ∼25-fold (Table II).

Glycerol Accumulation in fps1Δ Mutants Is Correlated with Defective Cell Fusion

Fps1p is a member of the major intrinsic protein family, an evolutionarily conserved family of channel proteins that transport small molecules (for review see Reizer et al., 1993). It is most similar to the *Escherichia coli* glycerol facilitator, GlpF, and has been proposed to function as a glycerol transporter in yeast (Luyten et al., 1995). Consistent with its role as a glycerol transporter, *fps1Δ* mutants contain approximately twofold more intracellular glycerol than wild-type strains, suggesting that they are defective in glycerol efflux (Luyten et al., 1995). We observed a similar
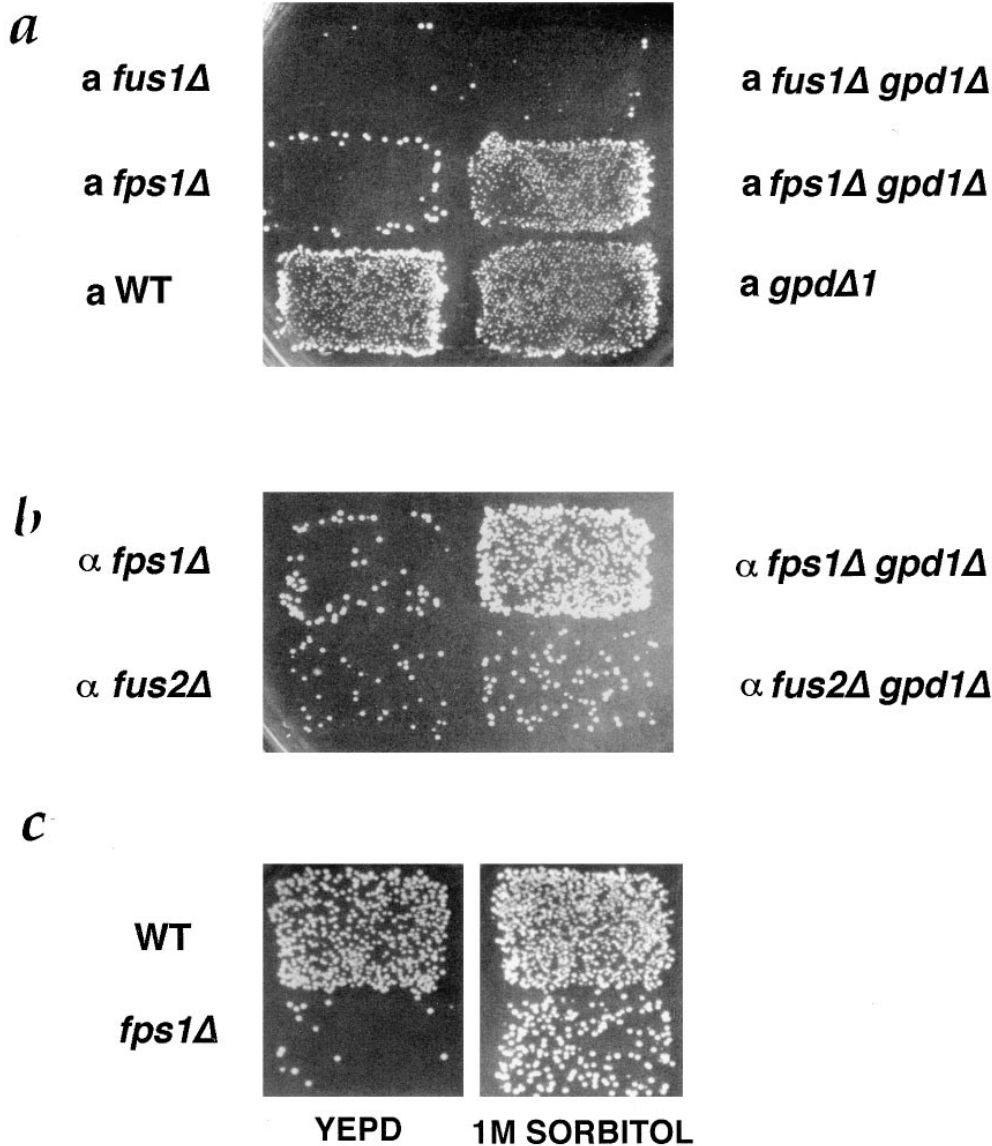
Figure 4. Suppression of mating defect of *fps1Δ* by *gpd1Δ* and 1 M sorbitol. (*a*) Patches of *MAT*a strains, WT (IH3160), *fps1Δ* (JP147), *fus1Δ* (JP52) (*left*) and *gpd1Δ* (JP168), *fps1Δ gpd1Δ* (JP165), and *fus1Δ gpd1Δ* (JP285) (*right*), were mated to a *MAT*α *fus1 fus2* strain (IH2351) as described in Materials and Methods. (*b*) Patches of *MAT*α strains, *fps1Δ* (JP226) and *fus2Δ* (JP257) (*left*) and *fps1Δ gpd1Δ* (JP233) and *fus2Δ gpd1Δ* (JP287) (*right*), were mated to a *MAT*a *fus1 fus2* strain (IH2353) as described in Materials and Methods. (*c*) Patches of *MAT*a strains, WT (IH3196), and *fps1Δ* (JP147), were mated on YEPD (*left*) or YEPD containing 1 M sorbitol (*right*) to a *MAT*α *fus1 fus2* strain (IH2351).

increase in intracellular glycerol in our *fps1Δ* mutant (Table III).

To ascertain whether the defect in cell fusion of *fps1Δ* strains results from elevated levels of intracellular glycerol, we determined whether reducing intracellular glycerol levels restored cell fusion. We reduced intracellular glycerol levels by deleting the *GPD1* gene, which encodes the NADH-dependent glycerol-3-phosphate dehydrogenase (GPDH) required for the first step of glycerol biosynthesis (Gancedo et al., 1968). Although yeast cells have a second NADH-dependent GPDH (Gpd2p), Gpd1p is responsible for ~95% of the NADH-dependent GPDH activity in the cell (Albertyn et al., 1994*b*). We found that the level of intracellular glycerol in the *fps1Δ GPD1* mutant was 2.1-fold higher than in the wild-type strain (*FPS1 GPD1*) and was only 1.1-fold higher than wild-type strains in the *fps1Δ gpd1Δ* mutant (Table III).

We next determined whether this decrease in intracellular glycerol level in *fps1Δ gpd1Δ* mutants suppressed the mating defect. We found that deletion of *GPD1* sup-

Table II. Quantitative Mating Defect of fps1Δ Mutants

a strain	α strain	Mating efficiency	Prezygotes
		%	%
1. WT (IH3196)	WT (IH3190)	100	0.8 ± 0.2
2. *fps1Δ* (JP147)	WT (IH3190)	46 ± 10	61 ± 17
3. WT (IH3196)	*fps1Δ* (JP226)	39 ± 8	32 ± 5
4. *fps1Δ* (JP147)	*fps1Δ* (JP226)	32 ± 15	18 ± 9
5. WT (IH3196)	WT (IH2350)	100	ND
6. WT (IH3196)	*fus1 fus2* (IH2351)	35 ± 10	ND
7. *fps1Δ* (JP147)	*fus1 fus2* (IH2351)	1.4 ± 1.0	ND

Mating efficiencies were calculated as described in Material and Methods. Mating efficiencies were normalized to the isogenic wild-type mating. For lines *2–4*, the values were normalized to the efficiency of IH3196 × IH3190 mating (line *1*). Values are the means of at least four experiments ± SD. For lines *6* and *7*, the values were normalized to the efficiency of IH3196 × IH2350 mating (line *5*) and are the mean of three experiments ± SD.

Percentage of prezygotes represents the number of prezygotes/(zygotes + prezygotes). At least 100 partnered cells were counted for each experiment. Values are the means of three experiments ± SD.

Table III. Intracellular Glycerol Concentrations in *fps1Δ* Strains

Strain	Fold change in intracellular glycerol
WT	1.0
fps1Δ	2.1 ± 0.2
gpd1Δ	1.3 ± 0.3
fps1Δ gpd1Δ	1.1 ± 0.2
WT + vector	1.0
WT + 2μ *GPD1*	2.2 ± 0.3
fps1Δ + vector	2.4 ± 0.5
fps1Δ + 2μ *GPD1*	4.2 ± 0.9

Strains were WT (IH3196), *fps1Δ* (JP147), *gpd1Δ* (JP168), and *fps1Δ gpd1Δ* (JP165). Fold change in intracellular glycerol was determined by normalizing to the glycerol level of the WT strain ($n = 5$). Strains containing plasmids were WT + vector (JP400), WT + YEp*GPD1* (JP199), *fps1Δ* + vector (JP401), and *fps1Δ* + YEp*GPD1* (JP200). Fold change in intracellular glycerol was determined by normalizing to WT + vector ($n = 3$). Glycerol concentrations were determined as described in Materials and Methods. Values are the means ± SD.

Table IV. Effect of Altering GPD1 Expression on the Defect in Cell Fusion of *fps1Δ* Mutants

Genotype	Percentage of prezygotes in matings to *	
	α *GPD1*	α *gpd1Δ*
a WT	0.75 ± 0.55	0.75 ± 0.55
a *gpd1Δ*	1.50 ± 0.75	1.75 ± 0.55
a *fps1Δ*	42.3 ± 6.7	55.5 ± 4.6
a *fps1Δ gpd1Δ*	14.0 ± 2.5	26.0 ± 7.2
	α WT + vector	α WT + 2μ *GPD1*
a WT + vector	2	<1
a WT + 2μ *GPD1*	5	<1
a *fps1Δ* + vector	20	7
a *fps1Δ* + 2μ *GPD1*	84	33

In the top four lines, α strains were WT (IH3186) and *gpd1Δ* (JP236). **a** strains were WT (IH3196), *gpd1Δ* (JP168), *fps1Δ* (JP147), and *fps1Δ gpd1Δ* (JP165). At least 100 partnered cells were counted for each experiment. Values are the means of four experiments ± SD. In the bottom four lines the α strain was IH3190. **a** strains were WT + vector (JP400), WT + YEp*GPD1* (JP199), *fps1Δ* + vector (JP401), and *fps1Δ* + YEp*GPD1* (JP200). More than 100 partnered cells were counted per sample. Data are from one representative experiment.
*Percentage of prezygotes represents the number of prezygotes/(zygotes + prezygotes).

pressed the mating defect of *fps1Δ* strains, whereas deletion of *GPD1* in *FPS1* strains had no effect on mating (Fig. 4). To determine if *gpd1Δ* suppressed the cell fusion defect of *fps1Δ* strains, we microscopically assayed the double mutant for accumulation of prezygotes: deletion of *GPD1* in an *fps1Δ* mutant decreased the number of prezygotes from 42% to 14% (Table IV). Again, we saw little effect of the *GPD1* deletion in an *FPS1* strain. Thus, deletion of *GPD1* partially restored both intracellular glycerol levels and mating to an *fps1Δ* mutant.

If increased intracellular glycerol is responsible for the cell fusion defect of *fps1Δ* mutants, we hypothesized that this defect would be exacerbated by further increasing intracellular glycerol levels. Overexpression of *GPD1* in *fps1Δ* strains raises intracellular glycerol levels (Luyten et al., 1995). We observed that overexpression of *GPD1* in *fps1Δ* mutant strain JP200 led to a 1.8-fold increase in glycerol levels in comparison with the *fps1Δ* strain carrying a control plasmid (JP401) (Table III, *seventh and eighth lines*). We observed that the glycerol-overproducing strain JP200 exhibited an enhanced defect in cell fusion, producing 84% prezygotes in comparison with strain JP401, which yielded 20% prezygotes when mated to a wild-type strain (Table IV). No significant increase in prezygotes was observed in the *FPS1* strain carrying the *GPD1* plasmid. We also examined the ability of these strains to mate with partners that overexpress *GPD1*. Once again, we observed that mating with JP200 yielded nearly fivefold more prezygotes than JP401: 33% vs 7% (Table IV, *second column, seventh and eight lines*). Overexpression of *GPD1* increased the levels of intracellular glycerol and exacerbated the defect in cell fusion of *fps1Δ* mutants. We conclude that the defect in cell fusion in *fps1Δ* mutants correlates with increased levels of intracellular glycerol.

In contrast with what was observed when *GPD1* was overexpressed in the *fps1Δ* mutant, we found that when *GPD1* was overexpressed in the mating partner there was a decrease in prezygotes formed, from 20 to 7% for the *fps1Δ* strain carrying the control plasmid (Table IV, *seventh line*) and from 84 to 33% for the *fps1Δ* strain carrying the *GPD1* plasmid (Table IV, *eighth line*). One explanation for this improvement in fusion is that overexpression of *GPD1* in the mating partner led to increased extracellular

glycerol, which restored osmotic balance to the *fps1Δ* mutant. Consistent with the idea that glycerol produced by the mating partner may osmotically stabilize the *fps1Δ* mutant, we found that deletion of *GPD1* in the partner somewhat exacerbated the defect of the *fps1Δ* mutant. *fps1Δ* mutants mated to *GPD1* strains produced 42% prezygotes, which increased to 55% when mated to a *gpd1Δ* partner (Table IV, *third line*). Additionally, *fps1Δ gpd1Δ* mutants mated to *GPD1* strains produced 14% prezygotes, which increased to 26% when mated to a *gpd1Δ* partner (Table IV, *fourth line*).

High Osmolarity Partially Suppresses the Cell Fusion Defect of fps1Δ Mutants

The correlation between increased intracellular glycerol levels and a defect in cell fusion suggests at least two possible causes of the cell fusion defect. Intracellular glycerol itself may inhibit cell fusion. Another possibility is that an imbalance between intracellular and extracellular solute levels inhibits fusion. To distinguish between these possibilities, we determined whether restoration of osmotic balance by 1 M sorbitol could suppress the cell fusion defect of *fps1Δ* mutants. If elevated intracellular glycerol per se inhibits cell fusion, then 1 M sorbitol should either have no effect or may exacerbate the cell fusion defect of *fps1Δ* mutants, since 1 M sorbitol induces *GPD1* expression (Hirayama et al., 1995). If the mating defect is due to osmotic imbalance, then mating in the presence of 1 M sorbitol may suppress the defect. We observed the latter: the mating defect of *fps1Δ* mutants was partially alleviated by 1 M sorbitol (Fig. 4 *c*). Because this mating assay involves mating to a *fus1 fus2* strain, it was possible that the improvement in mating resulted from an effect of the 1 M sorbitol on the *fus1 fus2* partner rather than on the *fps1Δ* partner. To address this issue, we examined the effect of 1 M sorbitol on *fps1Δ* mutants mated to a wild-type strain. We found that the fusion defect was suppressed in the presence of 1 M sorbitol; the number of prezygotes de-

Table V. 1 M Sorbitol Partially Suppresses the Cell Fusion Defect of fps1Δ Strains

	Percentage of prezygotes* in mating on YEPD	
Genotypes	Unsupplemented	+ 1 M sorbitol
a WT	0.3 ± 0.4	1 ± 0.7
a *fps1Δ*	37 ± 4	10 ± 2
a *fus1Δ*	12 ± 3	14 ± 2
α *fus2Δ*	7 ± 1	15 ± 2
α WT	1.7 ± 0.4	4.0 ± 3.1
α *fus1 fus2*	58.3 ± 19.0	90 ± 5.5

Cells were mated either on YEPD or YEPD supplemented with 1 M sorbitol. **a** strains were WT (IH3196), *fps1Δ* (JP147), and *fus1Δ* (JP52); they were mated to an α WT strain (IH3186). α strains were WT (IH2350), *fus2Δ* (JP257), and *fus1 fus2* (IH2351); they were mated to an **a** WT strain (IH3196). Values are the means of three experiments ± SD.
*Percentage of prezygotes represents the number of prezygotes/(zygotes + prezygotes).

clined from 37 to 10% (Table V). Moreover, the presence of 1 M sorbitol did not improve mating by *fus1 fus2* mutants (data not shown) or result in decreased levels of prezygotes (Table V). We conclude that the mating defect of *fps1Δ* mutants can be suppressed by restoring osmotic balance to these cells.

Additional evidence that the cell fusion defect of *fps1Δ* mutants is not due to an absolute increase in glycerol but rather to a difference between intracellular and extracellular glycerol comes from comparing the behavior of wild-type cells overexpressing *GPD1* and *fps1Δ* mutants. Both of these strains contained approximately twofold more intracellular glycerol than wild-type strains containing vector alone (Table III, *sixth and seventh lines*), yet the *fps1Δ* mutant exhibited a more severe cell fusion defect (Table IV, *sixth and seventh lines*). One explanation for this difference is that the wild-type cells, which contain a higher level of glycerol as a result of overexpression of *GPD1*, can efficiently release this glycerol, so that extracellular glycerol levels also increase. The *fps1Δ* mutant, which releases glycerol inefficiently, has higher intracellular glycerol and decreased extracellular glycerol (Luyten et al., 1995). These data suggest that osmotic imbalance, rather than the absolute level of intracellular glycerol, accounts for the cell fusion defect of *fps1Δ* mutants.

Activated Alleles of PKC1 Inhibit Cell Fusion

The protein kinase C pathway is induced by conditions in which intracellular solute is higher than extracellular solute (Davenport et al., 1995). Because *fps1Δ* mutants have higher intracellular glycerol concentrations than wild-type cells, we wondered whether the cell fusion defect of *fps1Δ* mutants was influenced or mediated by the *PKC1* pathway. If activation of the *PKC1* pathway is responsible for the defect in cell fusion of *fps1Δ* mutants, then activation of Pkc1p in an otherwise wild-type background should give a similar defect. We therefore examined the effect of expressing an activated allele of *PKC1* on mating and cell fusion.

A CEN-ARS plasmid containing such an allele of *PKC1* (*PKC1-R398P*) was introduced into a wild-type strain (IH3196) to generate strain JP300. *PKC1-R398P* alters the pseudosubstrate binding site of Pkc1p, creating a dominant, activated allele (Nonaka et al., 1995). Expression of

this allele under control of its own promoter had no detectable effect on cell viability (data not shown). The activated allele did, however, result in a mating defect similar to that of the *fps1Δ* mutant (Fig. 5 *a*). Assays of cell cycle arrest and shmoo formation indicated that JP300 responded normally to pheromone (data not shown). Furthermore, JP300 produced pheromone normally, as assayed by halo formation. When JP300 was mated to a wild-type strain, prezygotes accumulated. JP300 exhibited normal partnership, indicating that its defect did not result from inability to respond or adhere to its partner, but rather to a defect in cell fusion (Table VI, *first and second lines*; Fig. 1 *e*). We conclude that activation of the *PKC1* pathway is sufficient to cause a defect in cell fusion. We were able to activate the *PKC1* pathway at the time of mating by expressing an activated allele of *PKC1* (*PKC1-R398A*) under control of the *GAL1* promoter (Watanabe et al., 1994). Expression of this activated allele when cells were mating resulted in a mating defect, whereas no significant mating defect was seen when cells expressed wild-type *PKC1* or *PKC1-K853R*, an allele mutated in the kinase domain (Fig. 5 *b*). Hence, a constitutively active allele of Pkc1p expressed during mating causes a mating defect.

Pkc1p Does Not Act through Fps1p to Inhibit Cell Fusion

Activated alleles of Pkc1p and loss of function of *FPS1* caused quantitatively very similar cell fusion defects (Table VI), raising the possibility that Pkc1p negatively regulates cell fusion by inhibiting Fps1p. If the only target of Pkc1p for inhibiting cell fusion were Fps1p, then the activated allele of Pkc1p should not exacerbate the defect in fusion of an *fps1Δ* strain. However, we found that the *PKC1-R398P* allele increased the defect in cell fusion of the *fps1Δ* strain (Table VI, *third and fourth lines*). In addition, if Pkc1p negatively regulates Fps1p, then deletion of *GPD1* should suppress the *PKC1-R398P* allele, as it partially suppressed an *fps1Δ* mutant (Fig. 4 *a*; Table IV). Deletion of *GPD1* did not, however, suppress the *PKC1-R398P* allele, suggesting that Pkc1p acts downstream or parallel to glycerol accumulation (Table VI, *second and sixth lines*; Figs. 5 and 7).

fps1Δ mpk1Δ Double Mutants Require Osmotic Support for Viability

One explanation for the defect in *fps1Δ* mutants is that increased activity of the *PKC1* pathway accounts, at least in part, for the defect in cell fusion. Additional evidence that this pathway is active came from analysis of a mutant defective in both *FPS1* and *MPK1*, which codes for the MAP kinase regulated by Pkc1p (Lee et al., 1993; Torres et al., 1991). Although both *fps1Δ* and *mpk1Δ* mutants grow normally at 25°C (Van Aelst et al., 1994; Lee et al., 1993), we were unable to obtain double mutant segregants when spores were germinated at 25°C on YEPD in eight tetrads analyzed. The *fps1Δ mpk1Δ* double mutant was obtained, however, when spores were germinated in the presence of 1 M sorbitol: 14 double mutants were obtained from 18 tetrads; all were unable to grow on YEPD lacking sorbitol. The remaining 58 spores, none of which were double mutants, grew normally on unsupplemented YEPD at 25°C
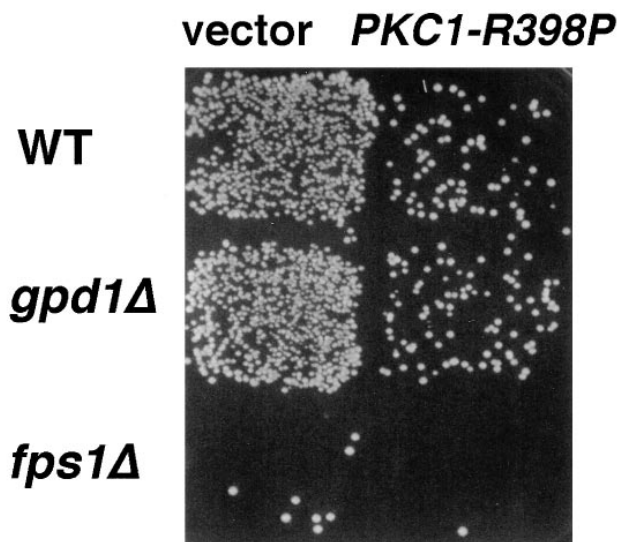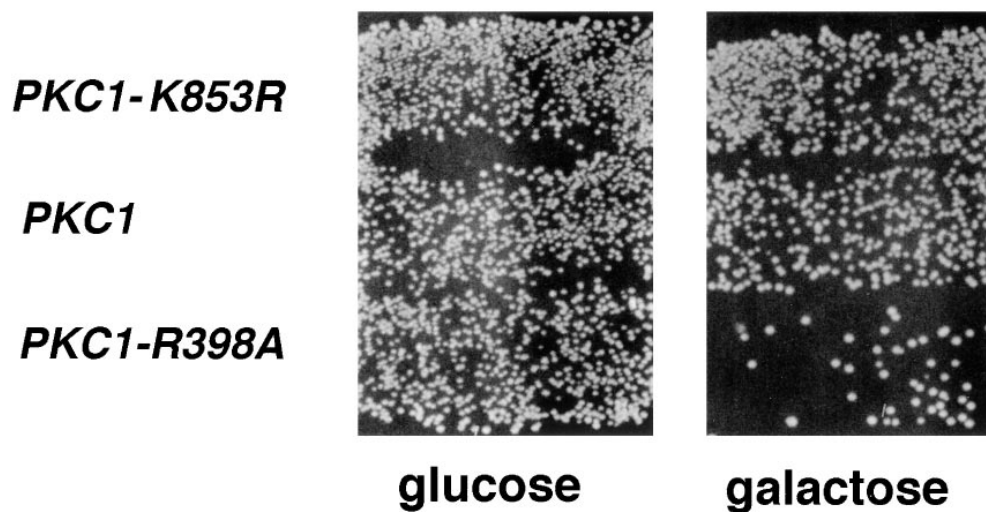
a

vector *PKC1-R398P*

WT

gpd1Δ

fps1Δ

b

PKC1-K853R

PKC1

PKC1-R398A

glucose   galactose

Figure 5. Inhibition of mating by activated *PKC1* alleles. (*a*) Patches of *MATa* strains, WT (IH3196), *gpd1Δ* (JP168), and *fps1Δ* (JP163), containing YCp50 (*left*) or YCp50-*PKC1-R398P* (pJP67) (*right*), were mated to a *MATα fus1 fus2* strain (IH2351). (*b*) A wild-type strain (IH3196) containing plasmids pDL295 (p*GAL1*[*PKC1-K853R::HA*]), pDL242 (p*GAL1*[*PKC1-R398A*]), or pDL293 (p*GAL1* [*PKC1::HA*]), was mated to a *MATα fus1 fus2* strain (IH2351) on either YEPD (*left*) or YEPGalactose (*right*) as described in Materials and Methods.

(Fig. 6). The inviability of the *fps1Δ mpk1Δ* strain demonstrates that the *fps1Δ* mutant, in the absence of osmotic stabilizing agents, requires *MPK1* for viability, and thus that Mpk1p is active in *fps1Δ* mutants.

Mutations in FUS1 and FUS2 Are Not Suppressed by Altering Osmotic Conditions

We have carried out a variety of analyses to determine whether the cell fusion defect of *fus1* and *fus2* mutants has a similar basis to that of *fps1* mutants. First, we determined whether deletion of *GPD1* restored mating and cell fusion to *fus1* or *fus2* mutants. Although deletion of *GPD1* improved mating in *fps1Δ* mutants, it did not improve matings of *fus1* and *fus2* mutants (Fig. 4, *a* and *b*). Similarly, microscopic examination of mating mixes did not reveal a significant change in prezygote accumulation in *fus1 gpd1*

or *fus2 gpd1* double mutants compared with *fus1 GPD1* or *fus2 GPD1* strains. Moreover, overexpression of *GPD1* did not exacerbate the cell fusion defect of *fus1* mutants (data not shown). We conclude that the cell fusion defect of *fus1* and *fus2* strains is unlikely to be due to an accumulation of intracellular glycerol. Additional evidence that the defect in cell fusion in *fus1* and *fus2* mutants differs from that in *fps1Δ* mutants came from analyzing the ability of mutants to mate in the presence of 1 M sorbitol. Accumulation of prezygotes was not detectably altered for *fus1Δ* mutants by mating in the presence of 1 M sorbitol, whereas *fus2Δ* and *fus1 fus2* strains showed an exacerbation of the fusion defect, a result opposite to that seen with *fps1Δ* mutants (Table VI). Thus, *fus1* and *fus2* mutants differ from *fps1Δ* mutants in their genetic interactions with *GPD1* and in their response to 1 M sorbitol. We conclude that it is unlikely that the cell fusion defect associated with

Table VI. The PKC1-R398P Allele Causes a Defect in Cell Fusion

Genotype	Plasmid	Percentage of prezygotes*	Percentage of partnership
a WT	vector	2.0 ± 0.7	41.6 ± 1.6
a WT	*PKC1-R398P*	16.3 ± 1.1	46.3 ± 3.9
a *fps1Δ*	vector	26.3 ± 6.4	40.0 ±2.5
a *fps1Δ*	*PKC1-R398P*	82.6 ± 4.9	44.0 ± 0.7
a *gpd1Δ*	vector	2.0 ± 0.7	40.7 ± 7.9
a *gpd1Δ*	*PKC1-R398P*	18.3 ± 1.5	41.0 ± 1.9

Percentage of partnership represents the number of (zygotes + prezygotes)/total cells. **a** strains were WT (IH3196), *fps1Δ* (JP163), and *gpd1Δ* (JP168). The strains were transformed with either YCp50 containing *PKC1-R398P* (pJP67) or YCp50 alone and were mated to a wild-type α strain (IH3186). More than 100 partnered cells were counted per experiment to determine the percentage of prezygotes. More than 200 total cells were counted per experiment to determined the percentage of partnership. Values are the mean of three experiments ± SD.
*Percentage of prezygotes represents the number of prezygotes/(zygotes + prezygotes).

mutations in *FUS1* and *FUS2* is due to an inability to maintain osmotic stability.

Discussion

During conjugation, haploid cells of opposite mating type reorganize their cell walls to allow cell fusion. The signals that control cell wall degradation and membrane fusion and the machinery that mediates these processes are not known. We have found that the *FPS1* gene, which codes for a glycerol transporter, is essential for efficient cell fusion. We identified *fps1* mutants based on their defect in mating to an enfeebled (*fus1 fus2*) mating partner and showed that they are specifically defective in cell fusion: they accumulated prezygotes during mating but were normal for pheromone signaling. The fusion defect of *fps1* mutants correlates with their increased level of intracellular glycerol relative to wild-type cells: the defect was partially suppressed by reducing intracellular glycerol and exacerbated by further increasing intracellular glycerol levels. The defect appears to result from osmotic imbalance rather than a high level of glycerol per se since extracellular 1 M sorbitol partially relieved the fusion defect. We propose that, during mating, cells monitor their osmotic state before committing to breaking down and remodeling their cell wall. In particular, under conditions of osmotic imbalance, such as in strains lacking Fps1p, cells interrupt cell wall breakdown. The *fps1* mutant thus reveals a checkpoint for cell wall breakdown during mating.

Because *fps1* mutants accumulate a high level of intracellular glycerol, we reasoned that they experience a situation analogous to that of wild-type cells exposed to hypotonic conditions, which would lead to activation of the *PKC1* pathway (Davenport et al., 1995; Kamada et al., 1995). We therefore anticipated that constitutive activation of the *PKC1* pathway, e.g., due to alteration of Pkc1p itself, would cause a fusion defect similar to that of *fps1* mutants. This prediction was borne out. Additional analyses of the constitutively activated *PKC1* mutant indicate that Pkc1p functions downstream of Fps1p and glycerol accumulation. We suggest that activation of Pkc1p couples sensation of hypoosmotic conditions to inhibition of cell fusion. Other genes required for cell fusion, in particular *FUS1* and *FUS2*, do not appear to participate in this osmo-

larity checkpoint, as mutants defective in these genes are not influenced by osmolarity.

Osmotic Balance Governs Cell Fusion

During vegetative growth, *fps1* mutants accumulate approximately twofold more intracellular glycerol than do wild-type cells, which does not cause any apparent growth defect (Van Aelst et al., 1991; Philips, J., unpublished observations). This increase in intracellular glycerol causes a defect in cell fusion during mating. In principle, Fps1p, which contains six putative membrane-spanning domains (Van Aelst et al., 1991), might play a role in mating that is distinct from its role in transport. Our analyses, however, indicate that its role in mating is a consequence of its function in glycerol transport and that, in its absence, the process of cell wall breakdown is inhibited. Our observations indicate that glycerol functions as an osmolyte during the mating process rather than being directly involved, e.g., in signaling between the mating partners. This conclusion comes from our observations that glycerol per se is not required during mating since the defect of *fps1* mutants can be suppressed by extracellular sorbitol or by deleting *GPD1*, which reduces intracellular glycerol levels. We thus favor the hypothesis that mating cells of yeast are exceptionally sensitive to osmotic disequilibrium before undergoing the potentially lethal morphogenetic changes required for cell fusion. Cells lacking *FPS1* sense that they are not osmotically balanced and respond by blocking cell wall breakdown. In other words, cells possess a checkpoint that ensures that they do not degrade their cell wall under hypoosmotic conditions.

It is unclear whether the osmotic imbalance perceived by the *fps1* mutant results from a difference between the mutant and its environment or a disparity between mating partners. If the osmolarity between mating partners were monitored, we might expect that an **a** *fps1* and α *fps1* mutant mated to each other would form zygotes at normal frequency. Although the number of prezygotes does not return to wild-type levels when *fps1Δ* mutants are mated with *fps1Δ* mutants, the reduction in the number of prezygotes compared with *fps1Δ* mutants mated to wild type may be significant (Table II), indicating that a difference in osmolarity between mating partners may be monitored.

The PKC1 Pathway May Mediate the Cell Fusion Checkpoint

Support for the hypothesis that osmosensing pathways regulate cell fusion was obtained by analyses of the *PKC1* pathway. This pathway is required for maintenance of cell integrity and cell wall construction (for review see Errede and Levin, 1993) and is induced when cells are subjected to conditions, such as hypoosmotic shock, that threaten cell integrity (Davenport et al., 1995; Kamada et al., 1995). We found that activation of Pkc1p using a constitutively activated allele of *PKC1* led to a fusion defect essentially identical to that of *fps1* mutants: *PKC1-R398P* and *fps1* strains accumulated prezygotes that were morphologically indistinguishable from each other.

One possibility is that Pkc1p blocks cell fusion by inhibiting *FPS1*. Two experiments suggest that this is not the case. First, the *PKC1-R398P* mutation has a more severe
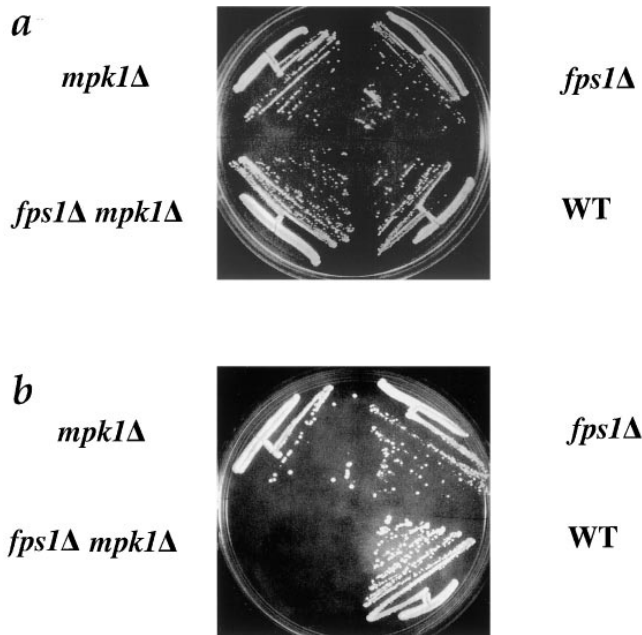
Figure 6. Synthetic lethality of *fps1Δ mpk1Δ*. (*a*) *mpk1Δ* (JP326), *fps1Δ* (JP325), *mpk1Δ fps1Δ* (JP327), and wild-type strains were grown on YEPD + 1 M sorbitol at 25°C for 2 d. (*b*) *mpk1Δ* (JP326), *fps1Δ* (JP325), *mpk1Δ fps1Δ* (JP327), and wild-type strains were grown on YEPD 25°C for 2 d.

Figure 7. Regulation of cell fusion by osmosensing pathways. (*a*) Increased intracellular glycerol levels due to deletion of *FPS1* and exacerbated by overexpression of *GPD1* activate the protein kinase C pathway, which responds to hypoosmotic shock. Pkc1p is part of a checkpoint that inhibits cell fusion if cells are hypoosmotically shocked. (*b*) Pkc1p is a negative regulator of cell fusion that is turned off or overridden in order for cells to fuse. When cells are osmotically stressed, because of deletion of *fps1Δ* and exacerbated by increased expression of *GPD1*, Pkc1p is activated, preventing cell fusion.

phenotype in an *fps1Δ* mutant than in a wild-type strain. Additionally, cell fusion is not restored to the *PKC1-R398P* mutant by reducing intracellular glycerol levels due to deletion of *GPD1*. These data, in conjunction with the previously demonstrated role of Pkc1p in responding to hypoosmotic shock, lead us to propose that Pkc1p lies downstream of Fps1p and Gpd1p and functions as a part of a checkpoint to monitor osmotic balance during mating (Fig. 7).

If activation of the *PKC1* pathway is responsible for the defect in cell fusion of *fps1Δ* mutants, then Pkc1p should be active in *fps1Δ* mutants and deletion of *pkc1* should suppress the mating defect. Because 1 M sorbitol suppresses the mating defect of *fps1Δ* mutants, and *pkc1Δ* mutants require 1 M sorbitol for viability (Levin and Bartlett-Heubusch, 1992; Paravicini et al., 1992), it is impossible to ask whether *pkc1Δ* suppresses the mating defect. However, evidence that the *PKC1* pathway is active in *fps1* mutants comes from the analysis of mutants defective in both Fps1p and Mpk1p, the MAP kinase in the *PKC1* pathway (Lee et al., 1993; Torres et al., 1991). We have observed that *fps1Δ mpk1Δ* mutants are inviable unless supported osmotically, indicating that Mpk1p is active in the *fps1* mutants. The *FPS1 mpk1* strains are inviable only at high temperatures (37°C) (Lee et al., 1993), whereas the *fps1 mpk1* strains are also inviable at low temperatures (25°C). One explanation for the inviability of the *fps1 mpk1* double mutant is that *fps1* mutants depend upon Mpk1 activity to respond to the osmotic imbalance caused by their inefficient release of glycerol.

At least two different models could explain the role of *PKC1* in cell fusion. According to one view, Pkc1p negatively regulates cell fusion as part of a checkpoint to en-
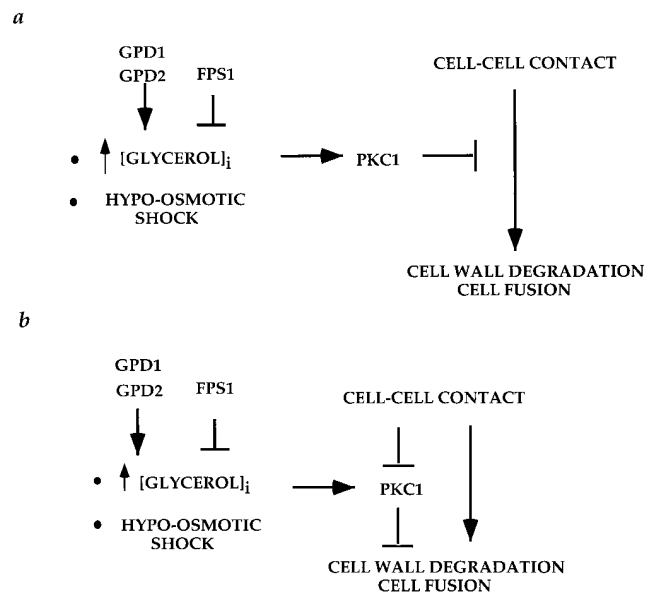
sure that cells are not osmotically vulnerable before fusion (Fig. 7 *a*). In this case, Pkc1p is used only under conditions, such as hypoosmotic shock, that make cell fusion particularly dangerous to cell integrity. According to a second view, Pkc1p constitutively inhibits cell fusion, so that cell fusion occurs only when Pkc1p is antagonized (Fig. 7 *b*). According to this model, Pkc1p is a key regulator of cell fusion, inhibiting fusion until a signal turns it off. In this case, the cell fusion defect of *fps1* mutants results from activation of the osmotic sensing pathway, which prevents the Pkc1p-dependent inhibition from being relieved during cell fusion.

Previous work has shown that Mpk1p is activated in response to mating pheromone (Errede et al., 1995; Zarzov et al., 1996), presumably because of activation of Pkc1p, and is necessary for viability of yeast cells under these conditions (Errede et al., 1995). Our observations indicate, in contrast, that activation of Pkc1p inhibits a late step in mating. To explain the apparently paradoxical actions of the *PKC1* pathway in mating, we suggest, as in the second model above, that Pkc1p is a negative regulator of cell fusion (Fig. 7 *b*) that, during normal mating, is activated and then subsequently inhibited. We propose that Pkc1p is first activated in response to pheromone and thereby inhibits cell wall degradation during initial stages of pheromone response and projection formation. When mating partners come in contact with each other, they generate a mechanical force as the cell walls become irreversibly adhered to each other, which generates a signal to turn off the *PKC1* pathway. We suggest that the signal to turn off

the *PKC1* pathway is a mechanical signal. It has been previously suggested that the *PKC1* pathway responds to membrane stretch due to various stimuli, such as low external osmolarity, high temperature, and drug-induced membrane stretch (Kamada et al., 1995).

Proteins Required for Cell Fusion: Regulators and Machinery

Genes required for cell fusion have been identified by a number of different strategies (Trueheart et al., 1987; Mc-Caffrey et al., 1987; Elion et al., 1990, 1995; Kurihara et al., 1994; Elia and Marsh, 1996). The studies presented here allow us to make a distinction between proteins that regulate the process of fusion (such as Pkc1p or, more indirectly, Fps1p) and potential participants in fusion itself (such as Fus1p and Fus2p). This distinction comes from the following observations. Fps1p is not directly required for cell fusion: its requirement can be relieved by deleting *GPD1* or by mating in the presence of high osmolarity. Rather, Fps1p is required to maintain osmotic balance and thereby avoid tripping a checkpoint that inhibits cell fusion. We have shown here that Pkc1p also governs fusion: cell expressing activated forms are defective in fusion presumably because they activate the cell fusion checkpoint. It is not clear whether Fus1p and Fus2p are regulators of fusion or play more direct roles in this process, or whether they are targets for the inhibition mediated by the *PKC1* pathway. The fusion defect of *fus1* and *fus2* mutants is not influenced by deletion of *GPD1* or osmotic stabilizers, and thus it is unlikely that these mutants exhibit a fusion defect by evoking the osmotic checkpoint.

Our findings on fusion during yeast mating may have implications for other examples of cell fusion. Maintenance of cell integrity is essential for all cell fusion processes. We therefore expect that processes such as sperm–egg and myoblast fusion may also be regulated by osmolarity. It would be striking if protein kinase C were involved in monitoring osmotic balance during sperm–egg or myoblast fusion. Eyster and McFarland (1995) have, in fact, reported that endogenous modulators of protein kinase C can regulate myogenesis. Perhaps activated versions of protein kinase C could block sperm–egg or myoblast fusion as they block fusion between mating partners in yeast. The targets of the inhibition triggered by the osmolarity-induced checkpoint in yeast remain to be determined. We are seeking to identify such targets by genetic strategies. These proteins might be directly involved in cell fusion during mating and illuminate mechanisms relevant to other cell fusion events.

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