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

Jennifer Philips and Ira Herskowitz

Department of Biochemistry and Biophysics, Programs in Genetics and Cell Biology, *University of California, San Francisco, California 94143-0448*

**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.

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**The 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 (shmoos formation), cell cycle arrest, and transcriptional induction (for reviews see Kurjan, 1992; Sprague and Thorn, 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, Fus2p, Fus2p, and CEFI genes are required for cell fusion (Trueheart et al., 1987; McCaffrey et al., 1987; Elion et al., 1990, 1995; Kurihara et al., 1994; Elion 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 proteins with an intracellular SH3 domain (Trueheart et al., 1994; Elia and Marsh, 1996). Fus1p is a transmembrane protein with an intracellular SH3 domain (Trueheart et al., 1994; 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

YEpGPD1 is a 2 µ URA3 plasmid (derived from YEp24) containing the GPD1 gene, as described in Albertyn et al. (1994b) (kindly provided by S. Hohmann, Katholieke University, Leuven, Belgium). pJ7 (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 pJ7 was cloned into BamHI and Sall sites of pRS306 to generate pJ7P2. This plasmid was used to integrate PKC1-R398P at its genomic locus, generating strain J6817. Plasmids containing PKC1 under the control of the GAL1 promoter are pDL242 (pGAL1[PKC1-R398A]), pDL293 (pGAL1[PKC1::HA]), and pDL295 (pGAL1[PKC1::K53R::HA]) as described in Watanabe et al. (1994). They were kindly provided by D. Levin (Johns Hopkins University, Baltimore, MD). pWJ192 codes for a RAS2–green fluorescent protein (GFP) fusion protein under control of the GPD promoter on a 2 µ TRP1 marked plasmid (kindly provided by J. Whitster, 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 pUCgus1::TRP1, a construct designed to replace GPD1 with TRP1 as described in Albertyn et al. (1994b) (kindly provided by S. Hohmann). Strains were confirmed to be gus1A by their sensitivity to high osmolarity media and by PCR analysis. fus1Δ strains were constructed using pJ7P2, 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 Clal–HincII fragment containing TRP1 into the Accl–HincII sites of FUS1. fus2Δ strains were generated using pOKUFUS2, a plasmid in which the 1.6-kb HindIII fragment containing FUS2 was replaced by the 1.1-kb HindIII fragment containing URA3. fus2Δ and fus2Δ strains were confirmed by their defective mating and by PCR analysis. fps1Δ 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 pJPS1, LEU2 was removed from pUC18–LEU2 (Herskowitz collection), in which LEU2 is cloned into the Sall site of pUC18. A PstI–XbaI fragment, containing LEU2, was cloned into the NsiI–Accl 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 inserted into the NsiI–Accl site of FUS1. The resulting plasmid was used to transform the strain bearing pJPS1, creating strain J7107. To express the FUS1 gene under the control of the FUS1 promoter, a 2.0-kb HindIII–PstI fragment containing FUS1 was cloned into the PstI–HindIII sites of pML37, resulting in pML41.
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 trp1 Δ1 fus1 Δ1 fus2 Δ3                                    | IH collection           |
| IH2353*         | MATα ura 3-52 trp1 Δ1 fus1 Δ1 fus2 Δ3                                    | IH collection           |
| IH3160          | MATα                                                                    | IH collection           |
| IH3179          | MATα fps1-1                                                              | IH collection           |
| IH3182          | MATα fps1-2                                                              | IH collection           |
| IH3186          | MATα                                                                    | This study              |
| IH3190          | MATα trp1 Δ99                                                           | This study              |
| IH3194          | MATα trp1 Δ99 leu2 Δ1                                                    | This study              |
| IH3196          | MATα leu2 Δ1                                                            | This study              |
| JP52            | MATα fus1 Δ::TRP1 trp1 Δ99 leu2 Δ1                                       | This study              |
| JP147           | MATα fps1 Δ::URA3 leu2 Δ1                                                | This study              |
| JP150           | MATα FPS1::FPS1-URA3                                                    | This study              |
| JP153           | MATα ura3-52::URA3                                                      | This study              |
| JP154           | MATα fps1-1::FPS1-URA3                                                  | This study              |
| JP157           | MATα fps1-1 ura3-52::URA3                                               | This study              |
| JP158           | MATα fps1-2::FPS1-URA3                                                  | This study              |
| JP161           | MATα fps1-2 ura3-52::URA3                                               | This study              |
| JP163           | MATα fps1 Δ::LEU2 Δ1                                                    | This study              |
| JP165           | MATα fps1 Δ::URA3 gpd1 Δ::TRP1 leu2 Δ1 trp1 Δ99                           | This study              |
| JP168           | MATα gpd1 Δ::TRP1 trp1 Δ99                                               | This study              |
| JP226           | MATα fps1 Δ::URA3 trp1 Δ99                                               | This study              |
| JP233           | MATα fps1 Δ::URA3 gpd1 Δ::TRP1 trp1 Δ99                                   | This study              |
| JP236           | MATα gpd1 Δ::TRP1 trp1 Δ99                                               | This study              |
| JP257           | MATα fus2 Δ::URA3 trp1 Δ99                                               | This study              |
| JP285           | MATα fus1 Δ::TRP1 gpd1 Δ::TRP1 trp1 Δ99                                   | This study              |
| JP287           | MATα fus2 Δ::URA3 gpd1 Δ::TRP1 trp1 Δ99                                   | This study              |
| JP317           | MATα PKC1::PKC1-R398P-URA3 leu2 Δ1                                       | This study              |
| JP325           | MATα fps1 Δ::LEU2                                                        | This study              |
| JP326           | MATα mpk1 Δ::TRP1                                                       | This study              |
| JP327           | MATα fps1 Δ::LEU2 mpk1 Δ::TRP1                                           | This study              |
| JP328           | MATα                                                                    | This study              |

Strains containing plasmids:

JP199          | IHE196 containing YEpGPD1                                              | IHE196 containing YEpGPD1 |
JP200          | IHE163 containing YEgGPD1                                              | IHE163 containing YEgGPD1 |
JP300          | IHE196 containing pJP67                                               | IHE196 containing pJP67 |
JP301          | IHE196 containing YEpCp50                                              | IHE196 containing YEpCp50 |
JP333          | IHE194 containing pJPW192                                              | IHE194 containing pJPW192 |
JP400          | IHE196 containing YEpCp195                                             | IHE196 containing YEpCp195 |
JP401          | IHE163 containing YEpCp195                                             | IHE163 containing YEpCp195 |

| Plasmid name | Description          | Source                  |
|--------------|----------------------|-------------------------|
| pDL242       | pGAL1[PCK1-R398P] (pBM743) | D. Levin (Watanabe et al., 1994) |
| pDL293       | pGAL1[PCK1::HA] (pBM743)  | D. Levin (Watanabe et al., 1994) |
| pDL295       | pGAL1[PCK1::K583R::HA] (pBM743) | D. Levin (Watanabe et al., 1994) |
| pJ22         | fus1 Δ::TRP1          | This study              |
| pJ30         | FPS1-URA3 (pRS306)    | This study              |
| pJ31         | fps1 Δ::LEU2          | This study              |
| pJ32         | fps1 Δ::URA3          | This study              |
| pUCp7d1 Δ::TRP1 | gpd1 Δ::TRP1          | S. Hohmann (Albertyn et al., 1994b) |
| YEpGPD1      | 2μ GPD1 (YEplac195)   | S. Hohmann (Albertyn et al., 1994b) |
| pJ567        | PKC1-R398P (YCp50)    | Y. Takai (Nonaka et al., 1995) |
| pJ72         | PKC1-R398P (pRS306)   | This study              |
| pJW192       | RAS2-GFP              | J. Whistler              |
| pKFUS2       | fus2 Δ::URA3          | IH collection           |

* Isogenic to IH2350.
1 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 MATα ade2-101 ura3-52 met1-1 HMLα HMRa.
2 pRS306, YEpCp195, and YEpCp50 are described in Guthrie and Fink (1991).
IH, I. Herskowitz.
inserted. The URA3 fragment was obtained from pSM32 (Hershkowitz 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 Hershkowitz (1996) except that 6 × 10³ cells of each mating partner were mixed. Mating assays scored microscopically were performed by mixing equal numbers of log phase a and a cells (6 × 10³), collecting cells on 0.4-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% glycero + 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 + zygoetes. At least 100 partnered cells (zygoetes + 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 × 10⁴ log phase fps1 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 MATA 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 2a 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 confirm the presence of glucose.

**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., 1994a). 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 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 ([prezygotes + zygoetes]/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
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. 1a). 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 mixing (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,
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 Xhol–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 parent strains with respect to the mating defect of M8 and M11 mutants to a wild-type partner, 30–45% of partnered 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 fps1Δ 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
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., 1994b). 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-

| a strain | α strain | Mating efficiency | Prezygotes |
|----------|----------|-------------------|------------|
| 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.
pressed the mating defect of \(fps1\Delta\) strains, whereas deletion of \(GPD1\) in \(FPS1\) strains had no effect on mating (Fig. 4). To determine if \(gpd1\Delta\) suppressed the cell fusion defect of \(fps1\Delta\) strains, we microscopically assayed the double mutant for accumulation of prezygotes: deletion of \(GPD1\) in an \(fps1\Delta\) 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 \(fps1\Delta\) mutant.

If increased intracellular glycerol is responsible for the cell fusion defect of \(fps1\Delta\) mutants, we hypothesized that this defect would be exacerbated by further increasing intracellular glycerol levels. Overexpression of \(GPD1\) in \(fps1\Delta\) strains raises intracellular glycerol levels (Luyten et al., 1995). We observed that overexpression of \(GPD1\) in \(fps1\Delta\) mutant strain JP200 led to a 1.8-fold increase in glycerol levels in comparison with the \(fps1\Delta\) 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 eighth lines). Overexpression of \(GPD1\) increased the levels of intracellular glycerol and exacerbated the defect in cell fusion of \(fps1\Delta\) mutants. We conclude that the defect in cell fusion in \(fps1\Delta\) mutants correlates with increased levels of intracellular glycerol.

In contrast with what was observed when \(GPD1\) was overexpressed in the \(fps1\Delta\) 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 \(fps1\Delta\) strain carrying the control plasmid (Table IV, seventh line) and from 84 to 33% for the \(fps1\Delta\) 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 \(fps1\Delta\) mutant. Consistent with the idea that glycerol produced by the mating partner may osmotically stabilize the \(fps1\Delta\) mutant, we found that deletion of \(GPD1\) in the partner somewhat exacerbated the defect of the \(fps1\Delta\) mutant. \(fps1\Delta\) mutants mated to \(GPD1\) strains produced 42% prezygotes, which increased to 55% when mated to a \(gpd1\Delta\) partner (Table IV, third line). Additionally, \(fps1\Delta\) \(gpd1\Delta\) mutants mated to \(GPD1\) strains produced 14% prezygotes, which increased to 26% when mated to a \(gpd1\Delta\) partner (Table IV, fourth line).

### Table III. Intracellular Glycerol Concentrations in \(fps1\Delta\) Strains

| Strain          | Fold change in intracellular glycerol |
|-----------------|--------------------------------------|
| WT              | 1.0                                  |
| \(fps1\Delta\)   | 2.1 ± 0.2                            |
| \(gpd1\Delta\)   | 1.3 ± 0.3                            |
| \(fps1\Delta\ gpd1\Delta\) | 1.1 ± 0.2                          |
| WT + vector     | 1.0                                  |
| WT + 2\(\mu\) \(GPD1\) | 2.2 ± 0.3                           |
| \(fps1\Delta + vector\) | 2.4 ± 0.5                           |
| \(fps1\Delta + 2\(\mu\) \(GPD1\)\) | 4.2 ± 0.9                           |

Strains were WT (IH3196), \(fps1\Delta\) (JP147), \(gpd1\Delta\) (JP168), and \(fps1\Delta \ gpd1\Delta\) (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), \(fps1\Delta +\) vector (JP401), and \(fps1\Delta +\) YEp\(gpd1\Delta\) (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 \(fps1\Delta\) Mutants

| Genotype     | \(\alpha \ GPD1\) | \(\alpha \ gpd1\Delta\) |
|--------------|-----------------|----------------------|
| \(a\) WT     | 0.75 ± 0.55     | 0.75 ± 0.55          |
| \(a\) \(gpd1\Delta\) | 1.75 ± 0.55     |
| \(a\) \(fps1\Delta\) | 6.7 ± 0.6       |
| \(a\) \(fps1\Delta \ gpd1\Delta\) | 26.0 ± 7.2     |

In the top four lines, \(a\) strains were WT (IH3196) and \(gpd1\Delta\) (JP236). \(a\) strains were WT (IH3196), \(gpd1\Delta\) (JP168), \(fps1\Delta\) (JP147), and \(fps1\Delta \ gpd1\Delta\) (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 \(a\) strain was IH3190. \(a\) strains were WT + vector (JP400), WT + YEp\(GPD1\) (JP199), \(fps1\Delta +\) vector (JP401), and \(fps1\Delta +\) YEp\(gpd1\Delta\) (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.

**High Osmolarity Partially Suppresses the Cell Fusion Defect of \(fps1\Delta\) 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 \(fps1\Delta\) 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 \(fps1\Delta\) 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 \(fps1\Delta\) mutants was partially alleviated by 1 M sorbitol (Fig. 4 c). Because this mating assay involves mating to a \(fps1\Delta\) \(fus2\) strain, it was possible that the improvement in mating resulted from an effect of the 1 M sorbitol on the \(fus1\Delta\) \(fus2\) partner rather than on the \(fps1\Delta\) partner. To address this issue, we examined the effect of 1 M sorbitol on \(fps1\Delta\) 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-
Defect of fps1Δ

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

| Genotypes            | Percentage of prezygotes* in mating on YEPD |
|----------------------|---------------------------------------------|
|                      | Percentage of prezygotes* in mating on YEPD |
|                      | 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         |
| a fus2Δ              | 7 ± 1          | 15 ± 2         |
| a WT                 | 1.7 ± 0.4      | 4.0 ± 3.1      |
| a 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 a WT strain (IH3186), a strains were WT (IH2350), fus1Δ (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.

climbed 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 GDP1 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 GDP1, 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. 5a). 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. 1e). 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 mutared in the kinase domain (Fig. 5b). 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 GDP1 should suppress the PKC1-R398P allele, as it partially suppressed an fps1Δ mutant (Fig. 4a; Table IV). Deletion of GDP1 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 segregates 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.
The inviability of the $fps1\Delta$ $mpk1\Delta$ strain demonstrates that the $fps1\Delta$ mutant, in the absence of osmotic stabilizing agents, requires $MPK1$ for viability, and thus that Mpk1p is active in $fps1\Delta$ 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 $fus1\Delta$ and $fus2\Delta$ mutants has a similar basis to that of $fps1\Delta$ mutants. First, we determined whether deletion of $GPD1$ restored mating and cell fusion to $fus1\Delta$ or $fus2\Delta$ mutants. Although deletion of $GPD1$ improved mating in $fps1\Delta$ mutants, it did not improve matings of $fus1\Delta$ and $fus2\Delta$ mutants (Fig. 4, $a$ and $b$). Similarly, microscopic examination of mating mixes did not reveal a significant change in prezygote accumulation in $fus1\Delta$ $gpd1\Delta$ or $fus2\Delta$ $gpd1\Delta$ double mutants compared with $fus1\Delta$ $GPD1$ or $fus2\Delta$ $GPD1$ strains. Moreover, overexpression of $GPD1$ did not exacerbate the cell fusion defect of $fus1\Delta$ mutants (data not shown). We conclude that the cell fusion defect of $fus1\Delta$ and $fus2\Delta$ strains is unlikely to be due to an accumulation of intracellular glycerol. Additional evidence that the defect in cell fusion in $fus1\Delta$ and $fus2\Delta$ mutants differs from that in $fps1\Delta$ 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 $fus1\Delta$ mutants by mating in the presence of 1 M sorbitol, whereas $fus2\Delta$ and $fus1\Delta$ $fus2\Delta$ strains showed an exacerbation of the fusion defect, a result opposite to that seen with $fps1\Delta$ mutants (Table VI). Thus, $fus1\Delta$ and $fus2\Delta$ mutants differ from $fps1\Delta$ 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
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 (fps1 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 sorbitol or by deleting GDP1, 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 osmolality between mating partners were monitored, we might expect that an a fps1 and a fps1 mutant mated to each other would form prezygotes 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 osmolality 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 fps1Δ 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

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               |

Values are the mean of three experiments ± SD.

a Percentage of prezygotes represents the number of prezygotes/(zygotes + prezygotes).

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 GDP1, 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.
presses the mating defect of suppress the mating defect. Because 1 M sorbitol sup-
defect in cell fusion of (Fig. 7). of a checkpoint to monitor osmotic balance during mating
downstream of Fps1p and Gpd1p and functions as a part
such as hypoosmotic shock, that make cell fusion particu-
larly dangerous to cell integrity. According to a second
view, that activation of Pkc1p inhibits a late step in
activation of the osmotic sensing pathway, which prevents
the Pkc1p-dependent inhibition from being relieved during
cell fusion.

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 hypoos-
motically 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.

At least two different models could explain the role
of PKC1 in cell fusion. According to one view, Pkc1p nega-
tively 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 particu-
larly 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 fps1Δ mutants results from ac-
tivation 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 re-
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
matting. 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 fu-
sion (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 in-
hibits cell wall degradation during initial stages of phero-
mone response and projection formation. When mating
partners come in contact with each other, they generate a
mechanical force as the cell walls become irreversibly ad-
hered to each other, which generates a signal to turn off
the PKC1 pathway. We suggest that the signal to turn off

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 pck1 should
suppress the mating defect. Because 1 M sorbitol sup-
presses the mating defect of fps1Δ mutants, and pck1Δ
mutants require 1 M sorbitol for viability (Levin and Bar-
tlett-Heubusch, 1992; Paravicini et al., 1992), it is impossi-
to ask whether pck1Δ suppresses the mating defect.
However, evidence that the PKC1 pathway is active in
fps1Δ 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 ob-
served that fps1Δ mpk1Δ mutants are inviable unless sup-
ported osmotically, indicating that Mpk1p is active in the
fps1Δ mutants. The FPS1 mpk1Δ strains are inviable only
at high temperatures (37°C) (Lee et al., 1993), whereas the
fps1Δ mpk1Δ strains are also inviable at low temperatures
(25°C). One explanation for the inviability of the fps1Δ
mpk1Δ double mutant is that fps1Δ mutants depend upon
Mpk1 activity to respond to the osmotic imbalance caused
by their inefficient release of glycerol.
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 osmolality, 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; McCaffrey 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 Pck1p 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 GPDL 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 Pck1p also governs fusion: cell expressing active 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 PCK1 pathway. The fusion defect of fus1 and fus2 mutants is not influenced by deletion of GPDL 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 processes. 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.

We thank members of our laboratory and S. Doberstein for valuable discussion, S. Hohmann, D. Levin, Y. Takai, J. Chenevert, and J. Whistler for providing plasmids; and S. Hohmann for communicating results before publication. We also thank R. Tabtiang, L. Huang, and F. Banuett for helpful comments on the manuscript, as well as C. Boone for suggesting the RAS2-GFP plasmid.

This work was supported by Research and Program Project Grants from the National Institutes of Health (NIH) to I. Herskowitz. J. Philips was supported by a Medical Scientist Training Program grant from the NIH, supplemented by the Sussman Fund, the Markey Program in Biological Sciences, and the Herbert W. Boyer Fund.

Received for publication 21 March 1997 and in revised form 17 June 1997.

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Received for publication 21 March 1997 and in revised form 17 June 1997.

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