High Osmolarity Glycerol (HOG) Pathway-induced Phosphorylation and Activation of 6-Phosphofructo-2-kinase Are Essential for Glycerol Accumulation and Yeast Cell Proliferation under Hyperosmotic Stress*

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In response to changes in the environment, yeast cells coordinate intracellular activities to optimize survival and proliferation. The transductions of diverse extracellular stimuli are exerted through multiple mitogen-activated protein kinase (MAPK) cascades. The high osmolarity glycerol (HOG) MAPK pathway is activated by increased environmental osmolarity and results in a rise of the cellular glycerol concentration to adapt the intracellular osmotic pressure. We studied the importance of the short time regulation of glycolysis under hyperosmotic stress for the survival and proliferation of yeast cells. A stimulation of the HOG-MAPK pathway by increasing the medium osmolarity through addition of salt or glucose to cultivated yeast leads to an activation of 6-phosphofructo-2-kinase (PFK2), which is accompanied by a complex phosphorylation pattern of the enzyme. An increase in medium osmolarity with 5% NaCl activates PFK2 3-fold over the initial value. This change in the activity is the result of a 4-fold phosphorylation of the enzyme mediated by protein kinases from the HOG-MAPK pathway. In the case of hyperosmolar glucose a 5-fold PFK2 activation was achieved by a single phosphorylation with protein kinase A near the carboxyl terminus of the protein on Ser644 and an additional 5-fold phosphorylation within the same amino-terminal fragment as in the presence of salt. The effect of hyperosmolar glucose is the result of an activation of the Ras-cAMP pathway together with the HOG-MAPK pathway. The activation of PFK2 leads to an activation of the upper part of glycolysis, which is a precondition for glycerol accumulation. Yeast cells containing PFK2 accumulate three times more glycerol than cells lacking PFK2, which are not able to grow under hypertonic stress.

Cells of the yeast Saccharomyces cerevisiae possess rapidly responding, highly complex signaling pathways. These pathways allow the cells to quickly adapt to a changing environment (1). Prominent among yeast signaling pathways are the mitogen-activated protein kinase (MAPK) cascades.

In the yeast S. cerevisiae, a variety of external stimuli activate the MAPK pathways which convert these signals into appropriate metabolic responses (1). Five of these MAPK cascades have been characterized that respond to such diverse environmental conditions as the presence of mating pheromones, changes in osmotic pressure, heat stress, and nutrient availability (2). The yeast S. cerevisiae adapts to growth under conditions of increased external osmolarity through activation of the high osmolarity glycerol (HOG) MAPK pathway (3). The activation of this pathway ensures the accumulation of a high intracellular concentration of glycerol to reduce the transmembrane difference of osmotic pressure and to prevent the loss of water (4). The stimulation of glycerol synthesis is achieved by activating transcription of genes required for glycerol synthesis such as GPD1 encoding glycerol-3-phosphate dehydrogenase (5, 6).

The yeast monofunctional 6-phosphofructo-2-kinase (PFK2) catalyzes the synthesis of fructose 2,6-bisphosphate (Fru-2,6-P2), a signal molecule connecting environmental changes with glycolysis (7, 8). Fru-2,6-P2 is the most powerful activator of 6-phosphofructo-1-kinase, a key regulatory enzyme of glycolysis (9). A stimulation of the Ras-cAMP pathway by glucose addition to cultivated yeast cells leads to an in vivo activation of PFK2, which is accompanied by a rather complex phosphorylation pattern of the enzyme (10). The phosphorylation of the protein on Ser644 is the result of PKA stimulation, while the protein kinase(s) catalyzing the 5-fold phosphorylation of the peptide fragment T67–101 is (are) still unknown. PFK2 lacking this peptide T67–101 is inactive (10).

Recently we reported that under hyposmotic stress yeast PFK2 was found to be inhibited by in vivo phosphorylation on Ser652. The phosphorylation and hence inactivation of PFK2 are under control of the PKC MAPK pathway and reduce the rate of glycolysis, leading to an accumulation of glucose 6-phosphate (G-6-P) (11).

The present study examines the regulation of the glycolysis under hyperosmotic stress with the focus on the phosphorylation and activity change of PFK2. We also investigate the role of PFK2 in the control of the cellular glycerol concentration and survival of yeast cells under hyperosmotic stress.

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¶ The abbreviations used are: MAPK, mitogen-activated protein kinase; Fru-2,6-P2, fructose 2,6-bisphosphate; G-6-P, glucose 6-phosphate; HOG, high osmolarity glycerol; MALDI-TOF MS, matrix-assisted laser desorption ionization-time-of-flight mass spectrometry; PFK2, 6-phosphofructo-2-kinase; PKC, protein kinase C; PP2A, phosphoserine/phosphothreonine-specific protein phosphatase 2A; T67–101, tryptic peptide from amino acid 67 to amino acid 101.
EXPERIMENTAL PROCEDURES

Materials—Yeast nitrogen base and casamino acids were from Difco. The expression vector pMK11PFK2 was a gift from M. Kretschmer. [32P]Orthophosphoric acid, HiTrap affinity columns, and acetonitrile were purchased from MBI Fermentas. T4 ligase and phosphoserine/phospho-

Site-directed Mutagenesis of PFK2—The point mutations Ser<sup>644</sup>→Asp<sup>644</sup> and Asp<sup>652</sup>→Asn were introduced into cytosolic membranes. The antisera were probed with a rabbit antiserum raised against PFK2 and then incubated with horseradish peroxidase-

Whitfield et al. (10). The reaction was terminated by addition of loading buffer. After SDS-PAGE the PFK2 protein was purified by in-gel digestion using trypsin. The tryptic digest from the gel pieces was carried out with 50% acetonitrile/0.1% TFA. The resulting peptides were purified at 4°C. The extracts were pre-

Determination of Glycerol and Glucose 6-Phosphate—The extracts were prepared according to Ref. 26. The Fru-2,6-P<sub>2</sub> concentration was measured according to Ref. 27 and related to the cell dry weight.

RESULTS

Hyperosmolarity Induced in Vivo Phosphorylation and Activation of the PFK2

Hyperosmolarity with NaCl—To study the effect of hyperosmolarity on the regulation of glycolysis and the role of PFK2 in cell adaptation to the osmotic stress yeast cells were exposed to hypertonic shock as described under “Experimental Procedures.” Both the activity change and the phosphorylation status of PFK2 were monitored. Under hypertonic stress with 5% NaCl and in the presence of [32P]labeled inorganic phosphate the PFK2 was in vivo phosphorylated (Fig. 1, A, i). [32P] incorporation increased within the time of incubation without any augmentation of the PFK2 protein concentration (Fig. 1A, ii). Parallel to the phosphorylation an activation of the PFK2 was observed. The PFK2 activity increased about 3-fold compared with isosmotic conditions (Fig. 2A). The increase of the PFK2 activity after in vivo phosphorylation was reversed by dephosphorylation with PP2A (Table I).

To determine [32P] incorporation during the hyperosmotic stress, yeast cells were grown on YNB-EP after reaching the log phase the cells were gently centrifuged, resuspended in hyperosmotic medium (YNB-EP supplemented with 5% NaCl or 1 m glucose) with [32P]labeled inorganic phosphate, and incubated at 30°C for different times. After rapid cooling PFK2 was prepared, denaturated in loading buffer, and electrophoresed (SDS-PAGE). After the gels were stained and dried, the phosphate incorpo-

Yeast Cells and Culture Media—The S. cerevisiae strains FY658 (pHL2::LEU2, Fpb26::HIS3, leu2, his4, ura3, 12) and RS51-58A (Mata his4 leu2 trp1 ade2 his3 ADH1::TRP1) were used in this study. The plasmid pMK11PFK2His was a gift from M. Kretschmer.

Assay of Enzyme Activity—PFK2 activity was measured at 25°C in 50 mM Tris-HCl, pH 7.4, 6 mM ATP, 2 mM fructose 6-phosphate, 6.5 mM glucose-6-phosphate, 1 mM MgCl<sub>2</sub>, 2 mM K<sub>2</sub>HPO<sub>4</sub>, and 5 mM mercapto-

High Osmolarity Stress Experiments—The induction of hyperosmotic shock to yeast cells cultured in YNB-P was performed according to Luthey et al. (21). After reaching the log phase the cells were centri-

Western Blot Analysis—SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described previously (18) using a 10% acrylamide gel. After electrophoresis, proteins were transferred to nitrocellulose membranes. The blots were probed with a rabbit antiserum raised against PFK2 and then incubated with horseradish peroxidase-

S. cerevisiae

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6-Phosphofructo-2-kinase and HOG-MAPK Pathway

Yeast Glycolysis under Hypertonic Stress—The role of PFK2 in Glycerol Synthesis and for the Control of Key Enzymes of the HOG-MAPK Pathway

Hyperosmolarity with Glucose—Similar to hypertonic NaCl also osmotic stress exerted by hyperosmolar glucose causes a phosphorylation of the peptide fragment T67–101 with m/z 3755.8, which was phosphorylated up to four times. From mono- to tetraphosphorylation all phosphates could be removed by treatment with PP2A (Fig. 7B).

Hyperosmolarity with NaCl—Cells of the yeast strain DFY658 transformed with the plasmid pMK11PFK2His6 over-expressing His-tagged PFK2 were exposed to 5% NaCl. The identification of the in vivo phosphorylation site(s) of PFK2 was achieved by comparing the results of MALDI-TOF MS peptide mass fingerprinting of the tryptic digests of purified in vivo phosphorylated and in vitro dephosphorylated PFK2. The superposition of the mass spectra resulting from the two tryptic digests confirmed that the PFK2 was in vivo phosphorylated. This posttranslational modification affects the peptide fragments confirmed that the PFK2 was phosphorylated and activated in all yeast strains (Fig. 6).

MALDI-TOF MS Analysis of the Tryptic Digest of in Vivo Phosphorylated PFK2 after Hypertonic Shock

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Hyperosmolarity with Glucose—Similar to hypertonic NaCl also osmotic stress exerted by hyperosmolar glucose causes a phosphorylation of the peptide fragment T67–101 (m/z 3755.8, Fig. 8). However, with glucose this fragment was phosphorylated up the five times. An additional phosphorylation affects the fragment T642–654 carrying Ser644 (data not shown). To identify the phosphorylation site hyperosmolality experiments with the PFK2 Ser644 → Ala mutant were performed. In the mass spectrum of the tryptic digest obtained from the phosphorylated enzyme an additional peak ([MH+]+ P: m/z 1477.2) 80
Da larger than that of the unphosphorylated peptide T\textsubscript{642–654} (m/z 1397.2) was observed (Fig. 9A). The absence of this peak in the spectrum of dephosphorylated PFK2 (Fig. 9B) confirms that it results from the phosphorylation of Ser\textsuperscript{644}. In control experiments using the PFK2 Ser\textsuperscript{644}\textsuperscript{3}Ala mutant no phosphorylation of the peptide T\textsubscript{642–654} was observed (data not shown).

**Effect of Hyperosmolarity Stress with Glucose on PFK2 from RS13-58A—**Cells of the yeast strain RS13-58A, which lacks two catalytic subunits of PKA and is attenuated in the remaining catalytic subunit, were transformed with pMK11PFK2His6 and exposed to 1M glucose. Under this hypertonic stress and in the presence of \textsuperscript{32}P-labeled inorganic phosphate PFK2 was \textit{in vivo} phosphorylated (Fig. 10). The incorporation of \textsuperscript{32}P increased with the incubation time up to 20 min (Fig. 10A). The purified PFK2 was analyzed by SDS-PAGE before in-gel digestion with trypsin. MALDI-TOF MS analysis showed that the PFK2 was phosphorylated 4-fold on the peptide fragment T\textsubscript{67–101} (Fig. 10B), while the fragment carrying Ser\textsubscript{644} was not modified (data not shown).

**Effect of Hyperosmolarity on the NH\textsubscript{2}-terminal Deletion Mutant of PFK2—**To prove the functional significance of the peptide T\textsubscript{67–101} and its phosphorylation, a PFK2 mutant lacking this peptide was constructed, expressed in, and purified from, yeast strain DFY658. The in-gel digestion of the purified mutant protein followed by MALDI-TOF MS fingerprinting confirmed its identity (10). Activity tests in extracts from cells grown under isoosmotic conditions showed that this mutant was catalytically inactive. Also, after hyperosmolar stress with either NaCl or glucose no activity could be detected in cell-free extracts (data not shown).

**DISCUSSION**

**Osmotic Stress Induced in Vivo Phosphorylation and Activation of the PFK2—**An increase of the environmental osmolarity requires cellular reactions to counteract this stress. In the yeast \textit{S. cerevisiae}, the membrane proteins Sln1p and Sho1p have been described as sensors of the two upstream branches controlling the HOG-MAPK pathway (3, 28, 29). The activated MAPK cascade stimulates different processes, which act together to counterbalance the loss of water. The intracellular glycerol accumulation is one of the best known and well understood reactions of yeast cells on increased extracellular osmolarity (1, 5, 30). It occurs in a two-step process: immediate closure of plasma membrane channels to ensure retention of glycerol whose production is thereafter increased in the second phase (31). In our work we focus on the importance of the short time regulation of glycolysis for the glycerol production compensating hyperosmotic stress. Yeast PFK2 catalyzes the synthesis of Fru-2,6-P\textsubscript{2}, which is...
FIG. 5. Growth of yeast cells under hyperosmotic stress with 5% NaCl. ○, DFY658 cells overexpressing PFK2 after transfer to hyperosmotic medium; ○, DFY658 cells (lacking PFK2) after transfer to hypertonic medium.

FIG. 6. Effect of hyperosmolarity on PFK2 activity in mutant yeast strains deficient in key enzymes of the HOG-MAPK pathway. White bars, activity of the PFK2 in isosmotic medium; gray bars, activity of the PFK2 after exposing the mutants to high osmolarity with 1 M glucose; black bars, activity of the PFK2 after exposing the mutants to high osmolarity with 5% NaCl. Values represent the mean ± S.D. of three independent experiments.

The most powerful activator of glycolysis (9). The monofunctional PFK2 can be either activated or inactivated by in vivo phosphorylation (7, 11). We have shown earlier that phosphorylation at Ser652 by PKC under hyposmotic stress inactivates PFK2 (11). On the other hand, glucose induction of yeast cells activates PFK2 by a single phosphorylation at Thr-Pro (36) or Pro-Leu-Ser/Thr-Pro (37) is typical for MAPK.

by others (3) with yeast strains carrying deletions of the yeast homolog of the mammalian MAPK p38 (HOG1) or the MAPK kinase PBS2. These cells produce less glycerol and do not grow at high osmolarity (3). For glycerol synthesis during hyperosmotic stress the overexpression of the glycerol-3-phosphate dehydrogenase gene GPD1 is known to play an important role (5). Glycerol is produced from the glycolytic intermediate dihydroxyacetone phosphate in two steps catalyzed by NAD+-dependent glycerol-3-phosphate dehydrogenase and glycerol-3-phosphatase. This response is supported by the phosphorylation of the enzyme. Although the function of the glycerol-3-phosphate dehydrogenase is intact and the glycerol facilitator Fssp, which is responsible for glycerol uptake from the medium, is present, the yeast mutant lacking PFK2 was found incapable of glycerol accumulation and did not grow in hyperosmotic medium.

This finding supports the importance of PFK2 and the upper part of glycolysis for glycerol accumulation.

Yeast strains carrying mutations in key enzymes of HOG-MAPK pathway showed different reactions to hyperosmolarity. In the case of hyperosmotic NaCl the PFK2 from HOG-MAPK mutant strains was not activated. This proves that the phosphorylation and activation of PFK2 under high osmolarity with NaCl is mediated by protein kinases from the HOG-MAPK pathway. In the presence of hyperosmolar glucose a clear increase of the activity was measured. However, the cells do not grow at high osmolarity (14). This shows that PFK2 activation alone without an intact HOG-MAPK pathway and without increased expression of glycerol-3-phosphate dehydrogenase is not sufficient to balance the increased osmotic pressure.

Characterization of the in Vivo Phosphorylation of PFK2—The determination of protein phosphorylation sites is an essential step in the analysis of protein kinase-mediated signaling pathways. While a direct determination of individual phosphorylation sites of phosphoproteins in vivo has been difficult up to now, the combination of MALDI-TOF MS, radioactive labeling, and site-directed mutagenesis represents a reliable strategy for the identification of protein phosphorylation sites. MALDI-TOF MS analysis of the tryptic digest of PFK2 purified from yeast cells exposed to hypertonic stress showed a 4-fold (NaCl) or 6-fold (glucose) phosphorylation of the enzyme. Glucose causes a single phosphorylation of the Ser644 on the peptide fragment T642–654 and a 5-fold phosphorylation of the peptide T67–101. This peptide is 4-fold phosphorylated after hyperosmotic NaCl. An examination of the primary structure of PFK2 shows that the peptide T67–101 comprises several potential phosphorylation sites with different consensus sequences. Ser654 is a potential site for calmodulin-dependent protein kinase and PKA; both proteins are known to be involved in the regulation of glycolysis (9, 32–34). The yeast Rck2 protein kinase is a member of the family of calmodulin-dependent protein kinases. Its phosphorylation and activation through the HOG-MAPK pathway has been reported (35). This knowledge supports the assumption that PFK2 could be a substrate of Rck2. Ser70, Thr72, Ser86, and Ser92 are surrounded by proline residues. This kind of consensus sequence (Pro-X-Ser/Thr-Pro (36) or Pro-Leu-Ser/Thr-Pro (37)) is typical for MAPK. Thr658 is a potential substrate for casein kinase II, which is involved in the salt tolerance in bakers’ yeast (38).

It has been reported that PFK2 is activated by fermentable sugars via the RAS/cAMP pathway and that this activation is a result of the phosphorylation of the PFK2 by PKA and other protein kinases from the RAS/cAMP pathway (10). The phosphorylation pattern of PFK2 observed after hyperosmolar glucose is identical with the one we observed in earlier work after...
induction of the Ras-cAMP pathway with 2% glucose (10). The results of the experiment with hyperosmolar glucose and the PKA deficient yeast strain (RS13-58A) support the notion that the 4-fold phosphorylation of the peptide fragment T67–101 and in consequence the activation of the PFK2 are mediated by protein kinases from the HOG-MAPK pathway and not from Ras-cAMP pathway. Only the phosphorylation of the Ser644 and the phosphorylation of one residue on the T67–101 are presumably mediated by the Ras-cAMP pathway. In yeast cells a decrease in cAMP concentration was measured under hyper-

Fig. 7. Identification of the in vivo phosphorylation sites of PFK2 by MALDI-TOF MS. A, tryptic digest of PFK2 phosphorylated in vivo after incubation of the yeast cells under hypertonic conditions with 5% NaCl. The peak with m/z 3755.8 represents the unphosphorylated peptide T67–101; B, MALDI-TOF mass spectrum of the tryptic digest of in vivo phosphorylated and in vitro dephosphorylated PFK2.

Fig. 8. Identification of the in vivo phosphorylation sites of PFK2 by MALDI-TOF MS. A, tryptic digest of PFK2 phosphorylated in vivo after incubation of the yeast cells under hypertonic conditions with 1 M glucose. The peak with m/z 3755.8 represents the unphosphorylated peptide T67–101; B, MALDI-TOF mass spectrum of the tryptic digest of in vivo phosphorylated and in vitro dephosphorylated PFK2.
tonic conditions induced by NaCl (39) leading to an inhibition of PKA (40). This may explain the missing phosphorylation of Ser<sup>644</sup> in PFK2. At the peptide T<sub>67-101</sub> Ser<sup>70</sup> is also a potential phosphorylation site of the PKA. The missing fifth phosphorylation after hyperosmolar NaCl could also result from the inhibition of the PKA.

Depending on its concentration in the culture medium glucose can activate different pathways (41, 42). First, the glucose
induces the activation of the PKA through the membrane receptor Gpr1 of the Ras-cAMP pathway and adenylate cyclase. The activated PKA can then phosphorylate PFK2 at Ser644 and probably at Ser70. Second, glucose at high concentration activates the HOG-MAPK pathway and leads to the phosphorylation of the PFK2 by protein kinases of this pathway.

REFERENCES

1. Gustin, M. C., Albertyn, J., Alexander, M., and Davenport, K. (1998) Microbiol. Mol. Biol. Rev. 62, 1284–1300
2. Herskowitz, I. (1995) Cell 80, 187–197
3. Brewster, J. L., de Valoir, T., Dwyer, N. D., Winter, E., and Gustin, M. C. (1993) Science 259, 1766–1763
4. Blomberg, A., and Adler, L. (1992) Adv. Microbiol. Physiol. 33, 145–212
5. Albertyn, J., Hohmann, S., Thevelein, J. M., and Prior, A. (1994) Mol. Cell. Biol. 14, 4135–4144
6. Larsson, K., Ansett, R., Erikson, P., and Adler, L. (1993) Mol. Microbiol. 10, 1101–1111
7. François, J., van Schaftingen, E., and Hers, H. G. (1984) Eur. J. Biochem. 145, 187–193
8. Okar, D. A., and Lage, A. J. (1999) Biofactor 10, 1–14
9. Pilkis, S. J., Claus, T. H., Kurland, I. J., and Lage, A. J. (1995) Annu. Rev. Biochem. 64, 799–835
10. Dihazi, H., Kessler, R., and Eschrich, K. (2000) Biochemistry 42, 6275–6282
11. Dihazi, H., Kessler, R., and Eschrich, K. (2001) Biochemistry 40, 14669–14678
12. Kretschmer, M., Lange, C., and Prinz, W. (1993) Biochemistry 32, 11143–11148
13. Cameron, S., Levin, L., Zoller, M., and Wigler, M. (1987) Cell 53, 555–566
14. O’Rourke, S. M., and Herskowitz, I. (1998) Genes Dev. 12, 2874–2886
15. Rubin, G. (1973) J. Biol. Chem. 248, 3860–3875
16. Deleted in proof
17. Kessler, R., and Eschrich, K. (1996) FEBS Lett. 396, 225–227
18. Dihazi, H., Kessler, R., and Eschrich, K. (2001) Protein Expression Purif. 21, 201–209
19. François, J., van Schaftingen, E., and Hers, H. G. (1988) Eur. J. Biochem. 171, 599–608
20. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
21. Loyns, K., Albertyn, J., Skibbe, W. F., Prior, J., Ramos, J., Thevelein, J. M., and Hohmann, S. (1995) EMBO J. 14, 1360–1371
22. Levin, G. K., Peretz, T., Chikvashvili, D., Thornhill, W. B., and Lotan, I. (1995) J. Biol. Chem. 270, 14611–14618
23. Aasara, J. M., and Allison, J. (1999) J. Am. Soc. Mass Spectrom. 10, 35–44
24. Dai, Y., Whittal, R. M., and Li, L. (1999) Anal. Chem. 71, 1087–1091
25. Lang, G., and Michal, G. (1974) in Methods in Enzymatic Analysis (Bergmeyer, H. U., ed.) pp. 1235–1242, Academic Press, Inc., New York
26. Boles, E., Hinnen, J., and Zimmermann, F. K. (1993) Yeast 9, 761–770
27. Van Schaftingen, E., Lederer, B., Bartrons, R., and Hers, H. G. (1982) Eur. J. Biochem. 129, 191–195
28. Attfield, P. V. (1997) Nat. Biotechnol. 15, 1351–1357
29. Hohmann, S. (2002) Microb. Mol. Biol. Rev. 66, 309–372
30. Wojda, I., Alonso-Monge, R., Bebelman, J. P., Mager, W. H., and Siderius, M. (2003) Microbiology 149, 1193–1204
31. Tamás, M. J., Luyten, K., Sutherland, F. C., Hernandez, A., Albertyn, J., Valadi, H., Li, H., Prior, B. A., Kilian, S. G., Ramos, J., Gustafsson, L., Thevelein, J. M., and Hohmann, S. (1999) Mol. Microbiol. 31, 1087–1104
32. Schwerer, C. M., El-Maghrabi, M. R., Pilkis, S. J. and Soderling, T. R. (1985) J. Biol. Chem. 260, 13018–13022
33. Mieskes, G., Kadux, J., and Soling, H. D. (1987) Eur. J. Biochem. 167, 383–389
34. Bergmeyer, H. U. (1974) in Methods in Enzymatic Analysis (Bergmeyer, H. U., ed.) pp. 1235–1242, Academic Press, Inc., New York
35. Teige, M., Pouyssegur, J. (1997) Essays Biochem. 32, 1–16
36. Widmann, C., Gibson, S., Jarpe, M. B., and Johnson, G. L. (1999) Physiol. Rev. 79, 143–180
37. De Nadal E., Calero, F., Ramos, J., and Arino, J. (1999) J. Bacteriol. 181, 6456–6462
38. Marquez, J. A., and Serrano, R. (1996) FEBS Lett. 382, 89–92
39. O’Rourke, S. M., and Adler, L. (1992) Adv. Microbiol. Physiol. 33, 145–212
40. Siderius, M., Rots, E., and Mager, W. H. (1997) Mol. Biol. Cell. 15, 1564–1572
41. Oezcan, S., and Johnston, M. (1995) Mol. Biol. Cell. 6, 193–209
42. Goncalves, P. M., Griffioen, G., Bebelmann, J. P., and Planta, R. J. (1999) Mol. Microbiol. 25, 483–493
