Volume 258, number 1, 67-70 FEB 07838 November 1989

Glucose and nitrogen rapidly activate a Ca^{2+}-inhibitable, serine/threonine kinase activity toward microtubule-associated protein 2 in *Saccharomyces cerevisiae*

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Received 11 September 1989

Treatment of the glucose-starved yeast cells (*Saccharomyces cerevisiae*) with 1% glucose or 2-deoxyglucose induced a rapid increase in a protein kinase activity in cell extracts that phosphorylated microtubule-associated protein 2 (MAP2) in vitro. Addition of 0.5% ammonium sulfate to the nitrogen-starved yeast cells also stimulated the kinase activity toward MAP2. The stimulated MAP2 kinase activities had the following common properties: (i) Activation was rapid and transient in response to stimuli; (ii) The kinase activity was serine/threonine-specific; and (iii) The kinase activity was inhibited by micromolar concentrations of free Ca^{2+}. These properties are very similar to those of the mitogen-activated, Ca^{2+}-sensitive MAP2 kinase we have recently found in mammalian fibroblastic cells. The MAP2 kinase activation may be involved in initiation of proliferation of yeast cells.

Yeast; Microtubule-associated protein 2; Signal transduction; Protein kinase

1. INTRODUCTION

Recent studies have shown that stimulation of mammalian cultured cells with various growth factors activates several serine/threonine-specific protein kinases which may play important roles in the signal transduction [1-4]. We have found that in quiescent fibroblastic cells, a variety of mitogens stimulate in common a Ca^{2+}-sensitive protein kinase which catalyzes phosphorylation of microtubule-associated protein 2 (MAP2) in vitro [5]. Ray and Sturgill have reported a similar (but Ca^{2+}-insensitive) protein kinase toward MAP2 in insulin-stimulated 3T3-L1 adipocytes [6]. Interestingly, this MAP2 kinase is shown to phosphorylate and activate ribosomal protein S6 kinase in vitro [7]. Moreover, several data suggest that MAP2 kinase itself is activated by phosphorylation in cells [7,8]. These studies suggest a kinase cascade, i.e. sequential activation of protein kinases, as a likely mechanism for transmission of growth factor signals in mammalian cells.

In yeast, incubation in very low levels of glucose or nitrogen leads to the arrest of the cell cycle at the G_0/G_2 phase, and readdition of these nutrients results in initiation of cell proliferation. Furthermore, glucose and other nutrients induce inositolphospholipid turnover, Ca^{2+} mobilization, and increase in intracellular cAMP levels in a manner similar to the effect of various growth factors on mammalian cells [9,10]. Thus, it is of great interest to see whether these nutrients activate a specific protein kinase in arrested yeast cells. In this paper, we show that glucose and nitrogen activate in common a Ca^{2+}-inhibitable, serine/threonine-specific protein kinase which is similar to the mitogen-activated MAP2 kinase we have previously found in mammalian cells.

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2. MATERIALS AND METHODS

2.1. *Purification of MAP2*

MAP2 was purified from the heat-stable microtubule-associated protein fraction of porcine brains by DEAE-cellulose chromatography [11-13].

2.2. *Glucose or nitrogen stimulation of arrested yeast cells and preparation of extracts*

The *Saccharomyces cerevisiae* cells (SM201: MATα, Leu2, Ura3, Trp1) grown to the late logarithmic phase in minimal medium containing 0.67% yeast nitrogen base without amino acids (Difco) and 1% glucose supplemented with L-leucine, L-tryptophan and uracil to 30 mg/l each, were transferred to the minimal medium containing 0.02% glucose and further incubated for 48 h at 30°C. These glucose-starved cells were then exposed to 1% glucose or deoxyglucose for the indicated times. Nitrogen-starved cells were prepared by incubating the late-log cells in minimal medium containing 1% glucose without

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ammonium sulfate for 48 h at 30°C, and then subjected to 0.5% ammonium sulfate for various times. These cells (about 2×10^7 cells) were harvested by centrifugation and washed twice with 1 ml of ice-cold saline. The cells were suspended in 500 μl of an extraction buffer solution containing 20 mM Tris-Cl, pH 7.5, 5 mM EGTA, 0.5% Triton X-100, 50 mM β-glycerophosphate, 1 mM PMSF, 2% aprotinin, 6 mM DTT, and 1 mM sodium orthovanadate. Glass beads (diameter, 400 μm) were added to each sample, and the cells were broken by vortexing. Broken cells were centrifuged first at 1000 $\times$ g for 3 min and then at 400 000 $\times$ g for 15 min. The supernatant was used immediately as cell extracts.

2.3. *Assay of protein kinase activity*

Assays were performed at 25°C in a final volume of 50 μl containing MAP2 (60 μg/ml), 25 μM [γ-^{32}P]ATP, 40 mM Mes, 10 mM Hepes, 10 mM Tris, pH 7.0, 1.6 mM EGTA, 11 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 12 mM β-glycerophosphate, 0.25 mM PMSF, 0.5% aprotinin, 1.4 mM DTT, 0.25 mM sodium orthovanadate, and the cell extract (12.5 μl). The reaction was terminated by the addition of Laemmli's sample buffer [14]. The samples were boiled for 3 min and then electrophoresed in SDS-polyacrylamide gels followed by autoradiography. The bands corresponding to MAP2 were excised from the gels, and the radioactivity was counted using a scintillation counter.

3. RESULTS AND DISCUSSION

Yeast cells in late logarithmic phase were arrested at the G_0/G_1 phase by incubation in a very low level of glucose (0.02%) for two days, and then the arrested cells were stimulated by 1% glucose. The kinase activity of cell extracts prepared from these cells to phosphorylate exogenous MAP2 was examined. As shown in fig.1 (○), 1% glucose stimulated the kinase activity toward MAP2 by about 2-fold. The kinase activity reached a maximum 2 min after the addition of 1% glucose, and then decreased to the basal level after 15 min. It has been demonstrated that the addition of glucose to the arrested yeast cells triggers an increase in cAMP levels [9,15–18], inositolphospholipid turnover and Ca^{2+} mobilization [10]. The addition of non-metabolizable derivatives of glucose, such as 2-deoxyglucose, also triggers an increase in cAMP level, but does not trigger inositolphospholipid turnover or Ca^{2+} mobilization [10]. We have then examined whether 2-deoxyglucose activates the kinase activity toward MAP2. Treatment of the glucose-starved cells with 1% 2-deoxyglucose also activated the kinase activity toward MAP2 by about 2-fold (fig.1, △). The time course of the 2-deoxyglucose-induced MAP2 kinase activation was essentially the same as that of the glucose-induced activation. Therefore, the yeast MAP2 kinase activation does not seem to require inositolphosphate turnover or Ca^{2+} mobilization.

Next, we have examined whether nitrogen activates the MAP2 kinase activity in the nitrogen-starved yeast cells. As shown in fig.2 (○), the addition of 0.5% ammonium sulfate to the cells induced a rapid and transient activation of the MAP2 kinase activity in cell extracts.

The mammalian MAP2 kinase has a unique property of being inhibited by micromolar concentrations of free

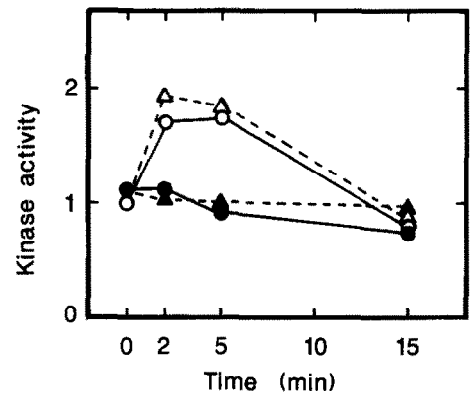

Fig.1. Time course of activation of the protein kinase activity toward MAP2 by glucose or 2-deoxyglucose. Glucose-starved yeast cells were treated with 1% glucose (○,●) or 1% 2-deoxyglucose (△,▲) for the indicated time at 30°C, and then cell extracts were prepared as described under section 2. MAP2 was phosphorylated by these extracts for 15 min at 25°C in the presence (●,▲) or absence (○,△) of 4 μM free Ca^{2+}. The kinase activities are expressed as -fold increases in ^{32}P radioactivity incorporated into MAP2. The kinase activity of untreated cell extracts in the absence of Ca^{2+} is expressed as 1.

Ca^{2+} [5,8]. We found that free Ca^{2+} at 4 μM inhibited the glucose- or 2-deoxyglucose-activated MAP2 kinase activity in yeast to the basal level (fig.1 ●, ▲). The nitrogen-activated MAP2 kinase activity was also inhibited strongly by Ca^{2+} (fig.2, ●). Phosphoamino acid analysis revealed that phosphorylation of MAP2 by the glucose- or nitrogen-activated kinase activity occurred mainly on serine and faintly on threonine residues (fig.3).

It is essential to distinguish the activated MAP2 kinase activity from cAMP-dependent protein kinase activity. When 7 μM cAMP was added to cell extracts from unstimulated cells, the kinase activity toward histone (type III-S, Sigma) was activated by about 4-fold (fig.4B). This kinase activity was inhibited to the

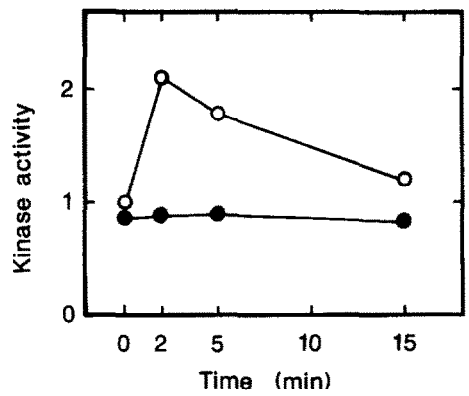

Fig.2. Time course of activation of the protein kinase activity toward MAP2 by nitrogen. Nitrogen-starved yeast cells were treated with 0.5% ammonium sulfate (○,●) for the indicated time at 30°C, and then cell extracts were prepared as described under section 2. MAP2 was phosphorylated by these extracts for 15 min at 25°C in the presence (●) or absence (○) of 4 μM free Ca^{2+}. The data are expressed as in fig.1.

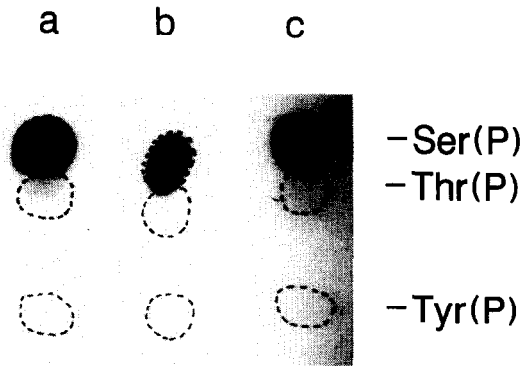

Fig.3. Phosphoamino acid analysis of MAP2 phosphorylated by extracts from stimulated yeast cells. Glucose-starved yeast cells were treated for 5 min at 30°C with 1% glucose or 1% 2-deoxyglucose. Nitrogen-starved yeast cells were treated for 5 min at 30°C with 0.5% ammonium sulfate. Then, extracts were prepared from these cells, and MAP2 was phosphorylated by the extracts from glucose- (lane a), 2-deoxyglucose- (lane b) or ammonium sulfate- (lane c) treated cells. Phosphoamino acid analysis of the MAP2 band excised from the SDS-polyacrylamide gels was performed as described previously [19,20].

basal level by 50 μg/ml synthetic peptide inhibitor of cAMP-dependent protein kinase (rabbit sequence, Sigma) (fig.4B, b and c). Therefore, this kinase activity represented the cAMP-dependent protein kinase. The cAMP-dependent protein kinase activity was insensitive to 4 μM free Ca^{2+}(fig.4B, b and d). On the other hand, extracts from nitrogen (3 min)-stimulated cells still had an about 2-fold higher MAP2 kinase activity than unstimulated cell extracts in the presence of 50 μg/ml synthetic peptide inhibitor of cAMP-dependent protein kinase (fig.4A, b and c). The nitrogen-activated MAP2 kinase activity was inhibited by 4 μM free Ca^{2+} to the basal level (fig.4A, b and d), as shown in fig.2. These results strongly suggest that the yeast MAP2 kinase is distinct from cAMP-dependent protein kinase.

In summary, we have found that yeast, glucose, 2-deoxyglucose and nitrogen activate in common a Ca^{2+}-inhibitable, serine/threonine-specific, MAP2 kinase, which is different from cAMP-dependent protein kinase. Activation of the yeast MAP2 kinase does not require inositolphosphate turnover or Ca^{2+} mobilization. The properties of the yeast MAP2 kinase so far revealed are very similar to those of the mitogen-activated MAP2 kinase we have found recently in mammalian fibroblastic cells [5], although further analysis of the yeast MAP2 kinase is difficult at the present time because of the instability of the kinase activity. It is possible that in yeast, the Ca^{2+}-sensitive MAP2 kinase plays a role in the mitogenic signal transduction.

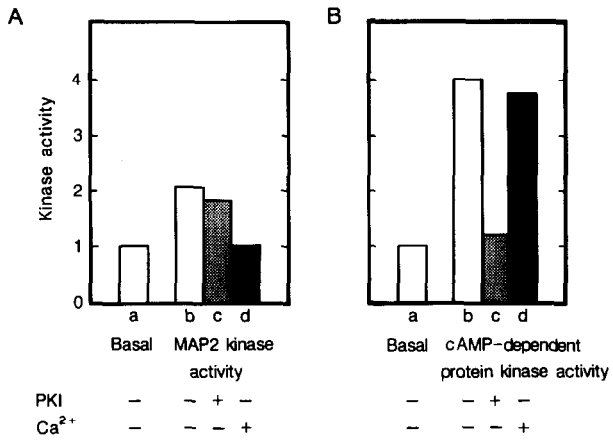

Fig.4. The nitrogen-activated protein kinase activity toward MAP2 is distinct from cAMP-dependent protein kinase. (A) Nitrogen-starved yeast cells were treated with 0.5% ammonium sulfate for 3 min, and then cell extracts were prepared as described under section 2. MAP2 (60 μg/ml) was phosphorylated by these extracts for 15 min at 25°C in the absence (lane b) or presence of 50 μg/ml synthetic peptide inhibitor of cAMP-dependent protein kinase (PKI, rabbit sequence, Sigma) (lane c), or in the presence of 4 μM free Ca^{2+} (lane d). The kinase activity of extracts from untreated cells is expressed as 1 (lane a). (B) In order to examine cAMP-dependent protein kinase activity in cell extracts, histone (type III-S, Sigma, 100 μg/ml) was phosphorylated by extracts from untreated cells in the absence (lane a) or presence of cAMP (7 μM) (lanes b,c,d). The synthetic peptide inhibitor of cAMP-dependent protein kinase (PKI, final 50 μg/ml) (lane c) or Ca^{2+} (final 4 μM) (lane d) was present in the kinase assay mixture. The kinase activity of untreated cell extracts in the absence of cAMP is expressed as 1 (lane a).

Acknowledgement: This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan (60065005).

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