Phosphatase toward MAP kinase is regulated by osmolarity in Madin-Darby canine kidney (MDCK) cells

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Abstract **We have reported that MAP kinase and its activator were activated by increase in extracellular osmolarity in Madin-Darby canine kidney (MDCK) cells [J. Clin. Invest. 93 (1994) 2387–2392]. The activation of MAP kinase quickly disappeared when cells in hypertonicity were shifted to isotonicity. Present study was planned to elucidate the mechanism for the inactivation of MAP kinase when osmolarity decreased. Combination of two different phosphatase inhibitors, 10^{-6} M okadaic acid and 0.2 mM sodium orthovanadate, blocked the inactivation of MAP kinase after the decrease in osmolarity. We also demonstrated that phosphatase toward MAP kinase was activated in response to the decrease in osmolarity. These results suggest that MAP kinase is inactivated by phosphatase that is activated when osmolarity decreased.**

Key words: Osmotic stress; MAP kinase; Phosphatase; MDCK cell

1. Introduction

The renal medulla is the only tissue that physiologically undergoes large changes of osmolarity. These changes are part of the renal mechanism for producing a concentrated urine. Renal medullary cells adapt to an increase in extracellular osmolarity by accumulating organic osmolytes. Kidney-derived culture cells such as Madin-Darby canine kidney (MDCK) cells accumulate osmolytes by increasing in transcription of genes for osmolyte transporters and synthetases when they are subjected to hypertonic stress in vitro (for review, see [1,2]). Hypertonicity also increases the levels of mRNA encoding the early gene transcription factors, *Egr-1* and c-*fos* in MDCK cells [3]. Messenger RNAs for Na/K-ATPase and HSP70 are induced by hypertonic stress in renal culture cells [3,4]. These studies indicate that the change of extracellular osmolarity results in the generation of signals that transverse the cytoplasm and eventually reaches the nucleus. But the mechanism by which extracellular osmolarity regulates transcription of these genes remains to be determined.

As well as in the signal transduction of growth factors or cytokines, recent studies have revealed that MAP kinase family functions in the signaling cascade of osmolarity (for review, see [5,6]). In yeast, *HOG1*, a new member of MAP kinase family, is essential to grow normally in hypertonic medium and regulate the accumulation of glycerol that is an osmolyte for yeast [7]. We have demonstrated for the first time that extracellular signal-regulated kinase (*ERK*), a classical type of MAP kinase, and its activator are activated in response to hypertonic stress in mammalian cells [8]. A mammalian homologue of *HOG1* has been cloned as *p38* kinase [9]. It has been also reported that a distinct MAP kinase, *JNK/SAPK*, which is originally identified as c-jun kinase, is activated by hypertonic stress in mammalian cells [10–12]. Both *p38* kinase and *JNK/SAPK*, but not *ERK*, partially rescue yeast mutant lacking in *HOG1* activity, suggesting *p38* kinase and *JNK/SAPK* play a physiological role in the osmosensing pathway [9,12]. Furthermore, we have recently found that *ERK* is not essential for transcriptional stimulation of osmolyte transporter genes [13]. In result, the role of *ERK* is still unknown in the osmosensing pathway,

In our previous paper, we showed that *ERK* induced by hypertonic stress was quickly inactivated when MDCK cells in hypertonic medium were shifted to isotonic medium [8]. This suggested that the decrease in the extracellular osmolarity activated an inactivator for *ERK*. Phosphatase toward *ERK* (*ERK* phosphatase) may be a most probable candidate as an inactivator because *ERK* is activated by phosphorylation and is inactivated by dephosphorylation. To clarify this issue, we tried to determine whether *ERK* phosphatase was regulated by the extracellular osmolarity.

2. Materials and methods

2.1. Materials

MDCK cells were a generous gift from the Japanese Cancer Research Resources Bank. A recombinant MAP kinase, rat *ERK2* fusioned with glutathione *S*-transferase (GST-ERK2) was prepared as described previously [8]. Protein phosphatase inhibitors, okadaic acid and sodium orthovanadate, were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Other chemicals were from commercial sources.

2.2. Cell culture

MDCK cells were cultured and grown as described previously [8]. After preincubation with buffer A (10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfoni c acid (HEPES)·NaOH, pH 7.4/130 mM NaCl/5.4 mM KCl/1.2 mM $CaCl_2$/1.2 mM $MgSO_4$/0.1% dialyzed bovine serum albumin), MDCK cells were incubated with hypertonic buffer A that was made 500 mOsmol/kg H_2O by adding raffinose. In order to shift hypertonic cells to isotonic condition, hypertonic buffer A was changed to prewarmed isotonic buffer A 30 min after exposure to hypertonic stress.

2.3. Assay of MAP kinase (ERK) activity

Activity of *ERK* was measured as described previously with a slight modification [8]. Briefly, cells were directly lysed on culture dishes with Laemli's sampling buffer, and then boiled at 100°C for 5 min. Kinase activity in these samples were assayed by the in-gel kinase assay method using myelin basic protein as a substrate.

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Abbreviations: MDCK, Madin-Darby canine kidney; ERK, extracellular signal-regulated kinase; GST, glutathione *S*-transferase; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfoni c acid. Data similar to Fig. 1 was reported in: J. Clin. Invest. 93 (1994) 2387–2392.

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2.4. Preparation of cytosol fraction

Except for ^{32}P-labeling of GST-ERK2, the cytosol fraction was prepared as described previously with slight modifications. Briefly, cells were scraped off with 500 μl/10 cm dish of ice-cold homogenizing buffer (25 mM HEPES·NaOH, pH 7.4/5 mM EDTA/1 mM EGTA/2 mM dithiothreitol (DTT)/10 μM *p*-amidinophenyl methansulphonyl-fluoride/aprotinin (100 kIU/ml)). Homogenized in microtubes by brief sonication, the homogenate was centrifuged at 125,000 × *g* at 4°C for 30 min. The supernatant was collected as the cytosol fraction and stored at −80°C until use.

2.5. Preparation of ^{32}P-labeled GST-ERK2

An active form of crude MAP kinase-kinase was prepared from cells exposed to hypertonic stress for 10 min as described previously, which was able to phosphorylate and activate GST-ERK2 [8]. The phosphorylation of GST-ERK2 was performed as described previously with a slight modification [8]. Briefly, 50 μg of crude MAP kinase-kinase was incubated with 140 μg of GST-ERK2 in 500 μl of reaction mixture (20 mM Tris-HCl, pH 8.0/2 mM EGTA/10 mM $MgCl_2$/100 μM [γ-^{32}P]ATP (25 μCi/ml)) at 30°C for 30 min. After diluted with 10 volumes of ice-cold stopping buffer (20 mM Tris-HCl, pH 8.0/2 mM EGTA/10 mM $MgCl_2$/0.1 mM sodium orthovanadate), it was applied on a 8 × 40 mm column of glutathione Sepharose CL-4B (Pharmacia LKB). The column was extensively washed with 50 mM Tris-HCl, pH 8.0, ^{32}P-labeled GST-ERK2 was eluted with 2.5 ml of elution buffer (50 mM Tris-HCl, pH 8.0/2 mM reduced glutathione) and stored at −80°C until use. It was confirmed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by Coomassie brilliant blue protein staining and autoradiography, that GST-ERK2 was exclusively phosphorylated. Under this condition, 0.7 pmol of ^{32}P was incorporated per mol of GST-ERK2.

2.6. Assay of ERK phosphatase

Activity of *ERK* phosphatase was measured as described previously with slight modifications [14]. Briefly, 5 μl aliquot of the cytosol fraction was incubated with 400 nM of ^{32}P-labeled GST-ERK2 at 25°C in 50 μl of reaction mixture (25 mM HEPES·NaOH, pH 7.4/5 mM EDTA/10 mM DTT) in the presence or absence of okadaic acid (10^{-6} M) and sodium orthovanadate (0.2 mM). Twenty minutes later, 750 μl of charcoal mixture (0.9 M HCl/90 mM sodium pyrophosphate/2 mM NaH_2PO_4/8%(v/v) activated charcoal) was added and transferred on ice. After the centrifugation at 7000 × *g* for 2 min, the aliquot of the supernatant was transferred into a new microtube and ^{32}P released from labeled GST-ERK2 was measured by Cerenkov method. Using this protocol, 15% of the totoal ^{32}P was released maximally from ^{32}P-labeled GST-ERK2, where the enzyme activity was almost linear with time.

3. Results and discussion

Fig. 1 showed the time course of the activity of *ERK* in MDCK cells after medium osmolarity increased from isotonic (300 mOsmol/kg) to hypertonic (500 mOsmol/kg) at *Time 0*. Thirty minutes after exposure to hypertonic stress, some cells were switched back to isotonic buffer. As shown in Fig. 1, *ERK* was quickly inactivated and returned to near basal level in 60 min. On the contrary, kinase activity was maintained under persistent hypertonic condition. When the cells were re-exposed to hypertonic stress at *Time 90*, *ERK* was activated again. Thus, *ERK* is activated by the change in extracellular osmolarity. These results were consistent with our previous reports [8]. It is unlikely that degradation of *ERK* or permanent cell damage is involved in this drastic inactivation because *ERK* was activated again at *Time 90*.

To see if dephosphorylation of *ERK* was involved in the inactivation of *ERK* by decrease in osmolarity, we examined the effects of inhibitors for serine/threonine and tyrosine phosphatases. After cells were exposed to hypertonic stress for 30 min, hypertonic buffer A was switched back to isotonic buffer A supplemented with 10^{-6} M okadaic acid and/or

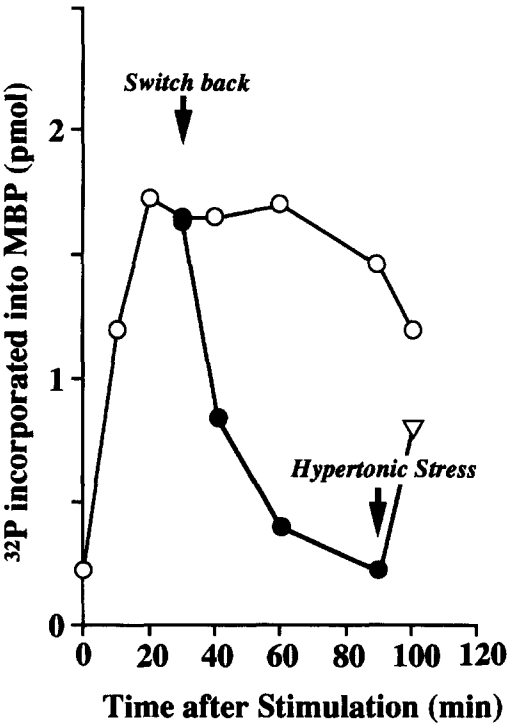

Fig. 1. Time course of ERK activity. *Time 0* = cells in isotonic buffer A were exposed to hypertonic buffer A (○); *Time 30* = some dishes of hypertonic cells were switched back to isotonic buffer (●); *Time 90* = some dishes of isotonic cells were shifted to hypertonic buffer again (▽). After the time indicated, cells were lysed and ERK activity was measured. Activity was expressed as ^{32}P incorporated into myelin basic protein (MBP) located at 49 kDa. The result was a representative time course of three independent experiments.

0.2 mM sodium orthovanadate. As shown in Fig. 2, neither of these inhibitors affected the inactivation of *ERK* after the decrease in extracellular osmolarity. However, when both of them were present, *ERK* was not inactivated but activated over the control level. We speculate that more than two kinds of phosphatases might be simultaneously activated because two different types of inhibitors were necessary to prevent the inactivation. Alternatively, an unknown type of phosphatase that was inhibited only in the presence of okadaic acid plus sodium orthovanadate might be activated. The overshooting of *ERK* in the presence of both inhibitors may come from reactivation of *ERK* kinase (MAP kinase-kinase) because *ERK* kinase is also regulated by its own phosphorylation (Fig. 2).

To determine whether *ERK* phosphatase is really activated by decrease in osmolarity, we directly measured the activity of *ERK* phosphatase. The cytosol fraction prepared after the decrease in extracellular tonicity was incubated with ^{32}P-labeled GST-ERK2, and the amount of released ^{32}P was measured. Release of ^{32}P was not due to degradation of GST-ERK2, because protein band of GST-ERK2 showed no remarkable change on SDS-PAGE even after 60 min incubation with the cytosol fraction (data not shown). This is the reason why ^{32}P released from labeled GST-ERK2 could be designated as the activity of *ERK* phosphatase. As shown in Fig. 3, *ERK* phosphatase was activated in response to the decrease in osmolarity. This activity in the cytosol fraction was partially suppressed by the addition of phosphatase inhibitors, which was expected

from the result shown in Fig. 2 (Fig. 3). We have shown that the activation of MAP kinase-kinase under hypertonicity was transient [8]. These and previous results suggested that the activation of *ERK* by hypertonicity is predominantly caused by the activation of MAP kinase-kinase, whereas the inactivation by the decrease in osmolarity is mainly due to the activation of *ERK* phosphatase. Thus, *ERK* could be a signal transducer of extracellular osmolarity, although its role in the osmosensing pathway remains unclear.

A candidate for an osmosensor located on plasma membrane, *SLN*1, has been reported in *Saccharomyces cerevisiae* [15,16]. Several phosphatases were identified as multicopy suppressor genes using yeast mutants of *SLN*1 [17]. These reports suggest that phosphatases are involved in the signal transduction of extracellular osmolarity. It has been demonstrated that phosphatases toward threonine/tyrosine residues function as MAP kinase inactivator in the signaling pathway of growth factors and stress response [18–25]. Although our assay system is likely to contain several phosphatases toward each step of MAP kinase cascade, our results strongly suggest that phosphatase toward *ERK* is regulated by the extracellular osmolarity. Further examination are necessary to clarify the role of *ERK* and *ERK* phosphatase in the osmosensing pathway.

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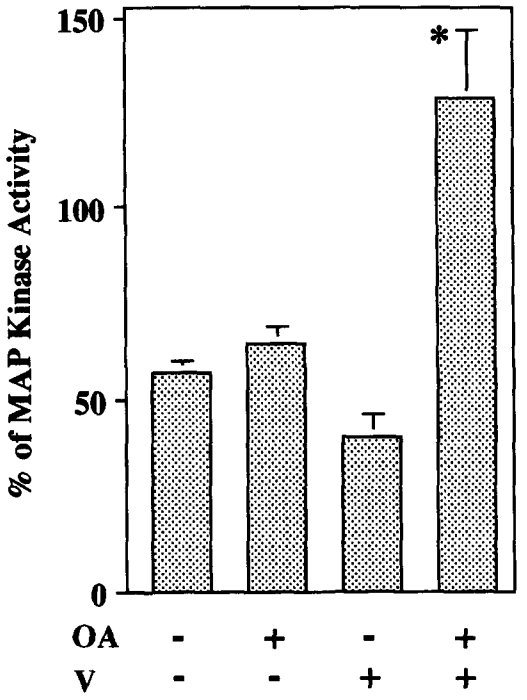

Fig. 2. The effects of okadaic acid and sodium orthovanadate on MAP kinase activity. Cells incubated with hypertonic buffer A for 30 min was switched back to isotonic buffer A supplemented with okadaic acid (OA; final, 10^{-6} M) and/or sodium orthovanadate (V; final, 0.2 mM). Ten minutes later, cells were lysed and ERK activity was measured. The activity 10 min after incubation with hypertonic buffer A was designated as 100% (Control). The results were expressed as means ± S.E.M. (n = 3). Data were compared by analysis of variance. *P < 0.05 vs. in the absence of phosphatase inhibitors.

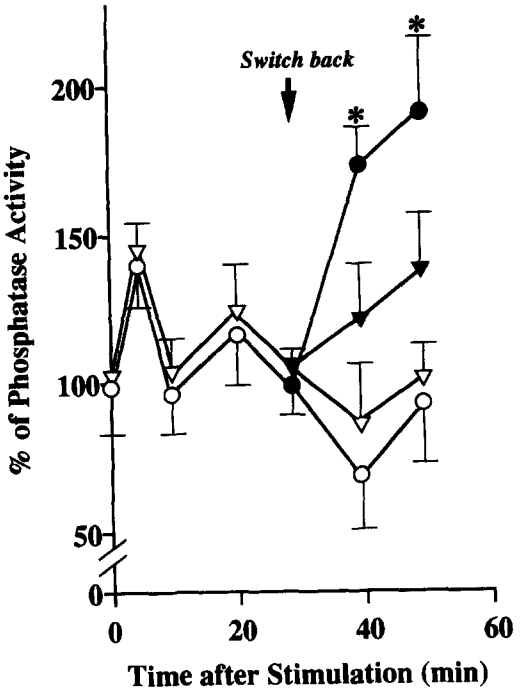

Fig. 3. Activity of ERK phosphatase. *Time 0* = cells in isotonic buffer A were exposed to hypertonic buffer A. *Time 30* = some dishes of hypertonic cells were switched back to isotonic buffer. The cytosol fraction was prepared at the time indicated, and the activity of ERK phosphatase was measured. (○); cells under hypertonicity in the absence of phosphatase inhibitors, (●); cells under hypertonicity followed by isotonicity in the absence of phosphatase inhibitors, (▽); cells under hypertonicity in the presence of phosphatase inhibitors, (▼); cells under hypertonicity followed by isotonicity in the presence of phosphatase inhibitors. The activity at Time 0 was designated as 100%, where 5.0 ± 0.9% (○) and 3.7 ± 0.6% (▽) of the total ^{32}P were released from the substrate, respectively. The results were expressed as means ± S.E.M. (n = 4). Data were compared by analysis of variance. *P < 0.05 vs. *Time 0*.

References

[1] Garcia-Perez, A. and Burg, M.B. (1991) Physiol. Rev. 7, 1081–1115.
[2] Kwon, H.M. (1994) Renal Physiol. Biochem. 17, 205–207.
[3] Cohen, D.M., Wasserman, J.C. and Gullans, S.R. (1991) Am. J. Physiol. 261, C594–C601.
[4] Yordy, M.R. and Bowen, J.W. (1993) Kidney Int. 43, 940–948.
[5] Davis, R.J. (1994) Trends Biochem. Sci. 19, 470.
[6] Blenis, J. (1993) Proc. Natl. Acad. Sci. USA 90, 5889–5892.
[7] Brewster, J.L., Valoir, T.D., Dwyer, N.D., Winter, E. and Gustin, M.C. (1993) Science 259, 1760–1763.
[8] Itoh, T., Yamauchi, A., Miyai, A., Yokoyama, K., Kamada, T., Ueda, N. and Fujiwara, Y. (1994) J. Clin. Invest. 93, 2387–2392.
[9] Han, J., Lee, J., Bibbs, L. and Ulevitch, R.J. (1994) Science 265, 808–811.
[10] Kyriakis, J.M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E.A., Ahmad, M.F., Avruch, J. and Woodgett, J.R. (1994) Nature 369, 156–160.
[11] Matsuda, S., Kawasaki, H., Moriguchi, T., Gotoh, Y. and Nishida, E. (1995) J. Biol. Chem. 270, 12781–12786.
[12] Galcheva-Gargova, Z., Dérijard, B., Wu, I.-H. and Davis, R.J. (1994) Science 265, 806–808.
[13] Kwon, H.M., Itoh, T., Rim, J.S. and Handler, J.S. Biochem. Biophys. Res. Commun. (in press).
[14] Streuli, M., Krueger, N.X., Tsai, A.Y.M. and Saito, H. (1989) Proc. Natl. Acad. Sci. USA 86, 8698–8702.

[15] Maeda, T., Wurgler-Murphy, S.M. and Saito, H. (1994) Nature 369, 242–245.
[16] Ota, I.M. and Varshavsky, A. (1993) Science 262, 566–569.
[17] Maeda, T., Tsai, A.Y.M. and Saito, H. (1993) Mol. Cell. Biol. 13, 5408–5417.
[18] Zheng, C.F. and Guan, K.L. (1993) J. Biol. Chem. 268, 16116–16119.
[19] Allessi, D.R., Smythe, C. and Keyse, S.M. (1993) Oncogene 8, 2015–2020.
[20] Sun, H., Charles, C.H., Lau, L.F. and Tonks, N.K. (1993) Cell 75, 487–493.
[21] Ward, Y., Gupta, S., Jensen, P., Wartmann, M., Davis, R.J. and Kelly, K. (1994) Nature 367, 651–654.
[22] Denu, J.M., Zhou, G., Wu, L., Zhao, R., Yuvaniyama, J., Saper, M.A. and Dixon, J.E. (1995) J. Biol. Chem. 270, 3796–3803.
[23] Duff, J.L., Monia, B.P. and Berk, B.C. (1995) J. Biol. Chem. 270, 7161–7166.
[24] Guan, K.L. and Butch, E. (1995) J. Biol. Chem. 270, 7197–7203.
[25] Misra-Press, A., Rim, C.S., Yao, H., Roberson, M.S. and Stork, P.J.S. (1995) J. Biol. Chem. 270, 14587–14596.