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Translocation of PKN from the cytosol to the nucleus induced by stresses

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ABSTRACT **Effects of environmental stresses on the subcellular localization of PKN were investigated in NIH 3T3, BALB/c 3T3, and Rat-1 cells. The immunofluorescence of PKN resided prominently in the cytoplasmic region in non-stressed cells. When these cells were treated at 42°C, there was a time-dependent decrease of the immunofluorescence of PKN in the cytoplasmic region that correlated with an increase within the nucleus as observed by confocal microscope. After incubation at 37°C following heat shock, the immunofluorescence of PKN returned to the perinuclear and cytoplasmic regions from the nucleus. The nuclear translocation of PKN by heat shock was supported by the biochemical subcellular fractionation and immunoblotting. The nuclear localization of PKN was also observed when the cells were exposed to other stresses such as sodium arsenite and serum starvation. These results raise the possibility that there is a pathway mediating stress signals from the cytosol to the nucleus through PKN.**

PKN is a fatty acid-activated serine/threonine protein kinase that has a catalytic domain highly homologous to that of protein kinase C in the carboxyl terminus and contains a unique regulatory region in the amino terminus (1–3). Recently, we demonstrated that Rho, a small GTPase protein, binds to PKN in a GTP-dependent fashion, and that this binding leads to the activation of PKN (4, 5), suggesting that PKN is one of the targets of Rho. Rho is implicated in the organization of cytoskeleton in response to growth factors such as the formation of stress fibers and focal adhesions. Rho regulates cytoskeletal rearrangements, such as cell morphology (6), platelet aggregation (7, 8), cell motility (9), and cytokinesis (10, 11). Rho also has roles in signaling to the nucleus and the regulation of transcriptional activation (12), cell-cycle progression (13), and cell transformation (14). Thus, the targets of the signaling pathway of Rho seem to be located within several cellular compartments. Signal transduction therefore requires the localization of Rho and Rho-regulated signaling molecules in each subcellular compartment that contains physiologically relevant roles mediated by Rho. We have reported that PKN associates and phosphorylates the intermediate filament proteins *in vitro*, indicating that the regulation of the cytoskeletal components was one of the possible functions of PKN (15).

Recently, increasing evidence indicates that there is overlapping of the growth factor- and stress-signaling pathways. Rac and Cdc42Hs, other members of the Rho family small GTPases, are activated not only by growth factors but by stresses such as proinflammatory cytokines and ultraviolet radiation, and contribute to activation of stress-activated mitogen-activating protein kinases (16–18). However, little is known about the Rho-mediated signaling pathways of stresses. In this report, we investigate the effects of various stresses on the subcellular localization of PKN in culture cells and present a possibility that PKN, one of the targets of Rho, is a part of a pathway mediating stress signals from the cytosol to the nucleus.

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MATERIALS AND METHODS

Antisera. Polyclonal antisera designated αN2 (1) and αC6 were prepared by immunizing rabbits with the bacterially synthesized fragments of amino acids 1–390 of rat PKN and 863–946 of rat PKN, respectively. A polyclonal antiserum designated αF1 was prepared, employing the full coding region of rat PKN purified from infected Sf9 cells as antigen (2). Epitope-specific reactions of αC6, αN2, and αF1 were confirmed by immunoblotting using amino- and carboxyl-terminal antigenic regions of PKN (1). The antiserum against protein phosphatase 2A (αPP2A) was kindly provided by T. Kuno (Kobe University).

Cell Line, Cell Culture, and Stress Treatment. NIH 3T3 and BALB/c 3T3 cells were grown in Dulbecco's modified eagle medium (DMEM) containing 10% calf serum. Rat-1 cells (kindly provided by T. Akiyama, Osaka University) were grown in DMEM containing 10% fetal calf serum. Cell lines were incubated in a humid 37°C chamber containing 5% CO_2, and experiments were performed using cells in subconfluent growing phase. Heat shock treatment was achieved by shifting replica dishes to an incubator containing 5% CO_2 at 42°C, and the time after the shift was designated as the time of heat treatment. Where indicated, sodium arsenite was added in the culture medium. Serum starvation was performed by changing medium to a serum-free medium containing 1 mg/ml bovine serum albumin (lipid free). The ultraviolet irradiation was performed by treatment with UV-C and incubated for 1 hr at 37°C (19).

Subcellular Fractionation and Immunoblotting. Cells were harvested, suspended in 1 ml of buffer A (10 mM Tris·HCl, pH 7.5/1 mM EGTA/1 mM EDTA/5 mM $MgCl_2$/1 mM phenylmethylsulfonyl fluoride/1 μg of leupeptin per ml), and homogenized with 30 strokes in a Dounce homogenizer. The protein contents of the total cell homogenates were determined by the method of Peterson (20), and equal amounts of protein were centrifuged at 500 × *g* for 7 min at 4°C to obtain the nuclear pellets and postnuclear fractions. The nuclear pellets were washed once with buffer A. The postnuclear fractions were further centrifuged at 100,000 × *g* for 1 hr at 4°C to give the cytosolic supernatants and plasma membrane pellets. These supernatant and pellet fractions were subjected to SDS/PAGE (21) and immunoblotting as described (22). Blots were developed by the enhanced chemiluminescence method.

Abbreviations: αPP2A, protein phosphatase 2A; SRF, serum response factor.

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Immunofluorescence. Cells grown on coverslips were washed twice with phosphate-buffered saline (PBS), fixed for 1 hr at 4°C in 4% paraformaldehyde, rinsed with PBS, and then blocked for 1 hr in PBS-T (PBS containing 0.05% Triton X-100) containing 5% normal goat serum. After washing with PBS-T, cells were incubated overnight at 4°C with each antiserum diluted with PBS-T at ≈10 μg/ml. Coverslips were rinsed with PBS-T, and incubated with fluorescein isothiocyanate isomer I-conjugated goat anti rabbit IgG (Medical and Biological Laboratories, Nagoya, Japan) for 60 min. Coverslips were rinsed with PBS-T followed by PBS, mounted with glycerol-containing 0.1% 1,4-diazabicyclo(2,2,2)octane (DABCO), and viewed on a Zeiss laser scan microscope. Nonspecific fluorescence, determined by incubation without primary antiserum, was negligible.

RESULTS AND DISCUSSION

Effects of Heat Shock on Subcellular Distribution of PKN. Immunoblotting was performed to determine the amounts of PKN in NIH 3T3, Rat-1, and BALB/c 3T3 cell lysates by using αN2, αC6, and αF1, that reacted specifically with PKN as indicated in Fig. 1. Treatment of the cells at 42°C for 90 min did not affect the total level of immunoreactive PKN in NIH 3T3 cells, BALB/c 3T3 cells, and Rat-1 cells (data not shown). We therefore assessed the effect of heat shock on the distribution of PKN in cytosolic, plasma membrane, and nuclear fractions (Fig. 2). PKN was predominant in the cytosolic fraction in untreated cells, and heat treatment increases PKN in the nuclear fraction, whereas, no significant change was observed in the plasma membrane fraction (Fig. 2).

We examined the immunofluorescence localization of PKN in NIH 3T3 cells by using αC6, αN2, and αF1. As shown in Fig. 3*A*, PKN was detected in the cytoplasmic region of untreated cells. Consistent with the results of immunoblotting, heat-shocked cells exhibited a pronounced increase in the immunofluorescence of PKN associated with the nucleus. The translocation of PKN was not due to the nonspecific effect of heat shock, since there was no difference in the subcellular distribution of immunoreactivity of αPP2A between heat-shocked and untreated cells. Fig. 3*B* shows that these phenomena were also observed in Rat-1 cells and BALB/c 3T3 cells. The translocation of PKN was reversible, because immunofluorescence of PKN redistributed to the perinuclear and cytoplasmic region when the cells were cultured for 4 hr at

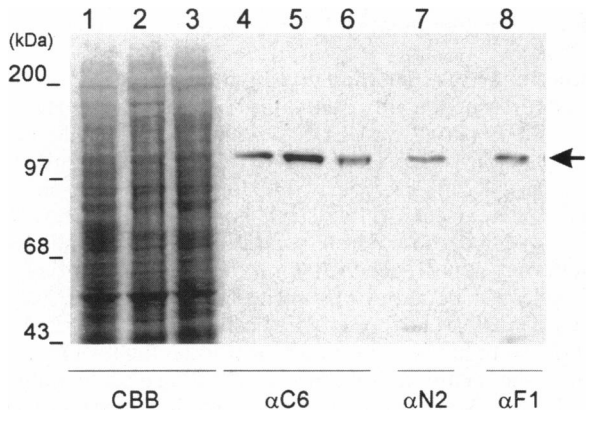

FIG. 1. Immunoblotting of PKN. Cell lysates (50 μg protein) from NIH 3T3 cells (lanes 1, 4, 7, and 8), Rat-1 cells (lanes 2 and 5), and BALB/c 3T3 cells (lanes 3 and 6) were subjected to SDS/PAGE and followed by immunoblotting. Proteins were stained with Coomassie brilliant blue (lanes 1–3). Immunostaining was performed with αC6 (lanes 4–6), αN2 (lane 7), and αF1 (lane 8). The positions of marker proteins are indicated in kDa, and the position of PKN is indicated by an arrow.

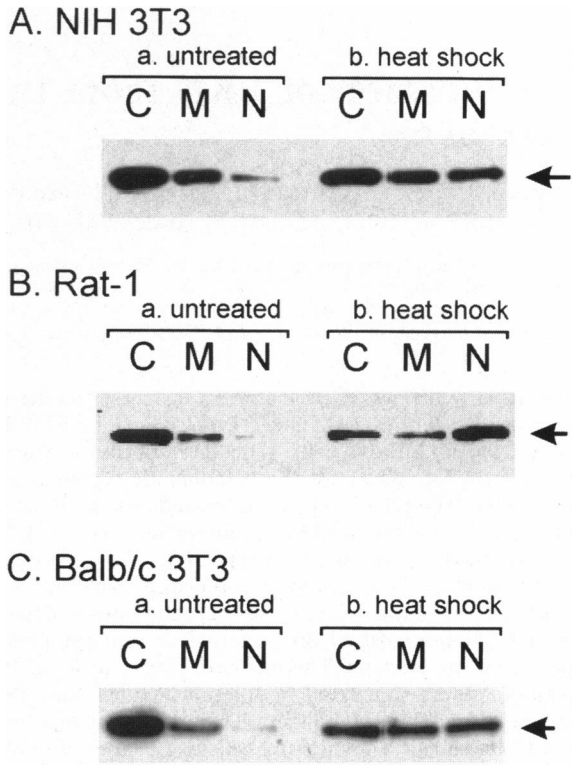

FIG. 2. Effects of heat shock on subcellular distribution of PKN. Cells were treated at 42°C for 90 min, homogenized, and fractionated into cytosolic (lanes C), plasma membrane (lanes M), and nuclear (lanes N) fractions. PKN was detected by immunoblotting by using αC6. The position of PKN in control untreated cells (*a*) and heat-shocked cells (*b*) is indicated by arrows. (*A*) NIH 3T3 cells. Each lane contains 19 μg of total protein. (*B*) Rat-1 cells. Each lane contains 11 μg of total protein. (*C*) BALB/c 3T3 cells. Each lane contains 11 μg of total protein.

37°C after heat shock (Fig. 3 *A* and *B*). The immunofluorescence of PKN resided within the nucleus rather than in the nuclear membranes in heat-shocked cells as shown by confocal microscopy (Fig. 3*C*).

Time Course of Heat Shock-Induced Translocation of PKN. As judged by immunofluorescence microscope, PKN underwent an almost complete change of distribution in heat-shocked cells, starting to move to the nucleus as early as 10 min, and becoming predominantly nuclear after 60–90 min of heat shock (Fig. 4). Exit of PKN from the nucleus was partial at 1 hr after shift back to 37°C from 42°C and was almost complete at 4 hr after shift back (data not shown).

Effects of Sodium Arsenite and Serum Starvation on Subcellular Distribution of PKN. Chemical poisoning and heavy metal toxicity also induce heat shock proteins and the stress responses in experimental systems (23). To test whether localization of PKN could be also influenced by chemical shock, Rat-1 cells were exposed to sodium arsenite, which is known to produce a cellular stress response similar to that induced by heat shock (24). As expected, the treatment with 50 μM sodium arsenite resulted in a shift of PKN to the nucleus as observed by microscopic examination (Fig. 5*A*). Similar results were obtained when NIH 3T3 cells and BALB/c 3T3 cells were treated with 80 μM sodium arsenite (data not shown). We further investigated whether the translocation of PKN to the nucleus could also be induced by other stresses such as serum starvation. The translocation of PKN to the nucleus was also observed when NIH 3T3 cells were subjected to serum starvation (Fig. 5*B*), and PKN gradually returned to the cytoplasmic region after addition of 10% fetal calf serum. It took at least 4 hr for PKN to return

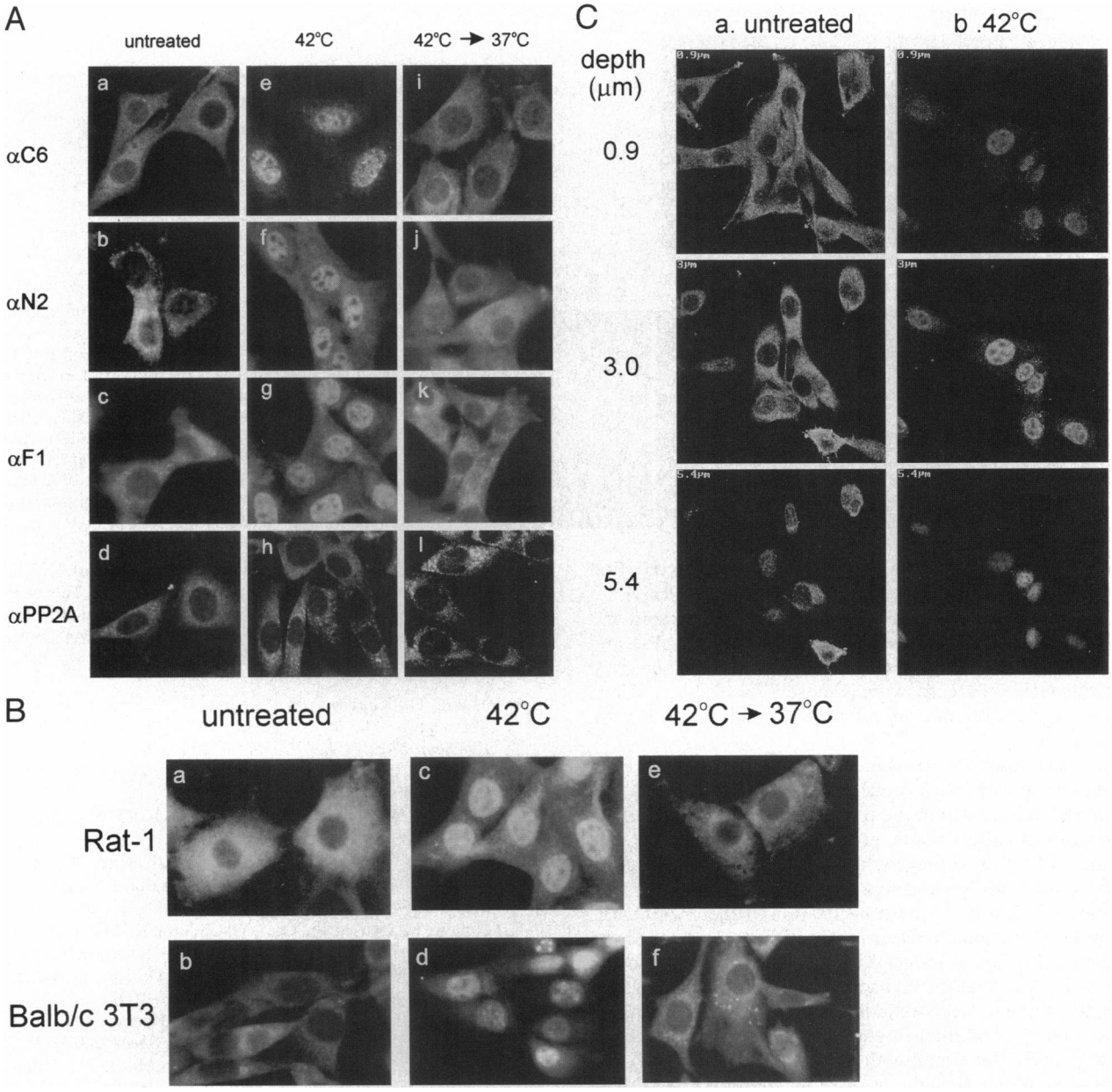

FIG. 3. Effects of heat shock on immunofluorescence staining of PKN. (*A*) Effects of heat shock on NIH 3T3 cells. Control untreated cells (*a–d*), cells treated at 42°C for 90 min (*e–h*), and cells incubated at 37°C for 240 min following 90 min-heat shock (*i–l*) were immunostained by each antiserum. The first antiserum was αC6 (*a*, *e*, and *i*), αN2 (*b*, *f*, and *j*), αF1 (*c*, *g*, and *k*), and αPP2A (*d*, *h*, and *l*). (*B*) Effects of heat shock on Rat-1 and BALB/c 3T3 cells. Rat-1 (*a*, *c*, and *e*) and BALB/c 3T3 (*b*, *d*, and *f*) cells were exposed to heat shock at 42°C. The first antiserum was αC6. (*a* and *b*) Control untreated cells; (*c* and *d*) cells after 90-min heat shock; and (*e* and *f*) cells after incubation at 37°C for 4 hr following 90 min-heat shock. (*C*) Localization of PKN in NIH 3T3 cells. Control untreated cells (*a*) and cells treated at 42°C for 90 min (*b*) were immunostained using αC6 and viewed on confocal laser scanning microscope. Optical sections from the bottom of the cells were performed at the indicated depths.

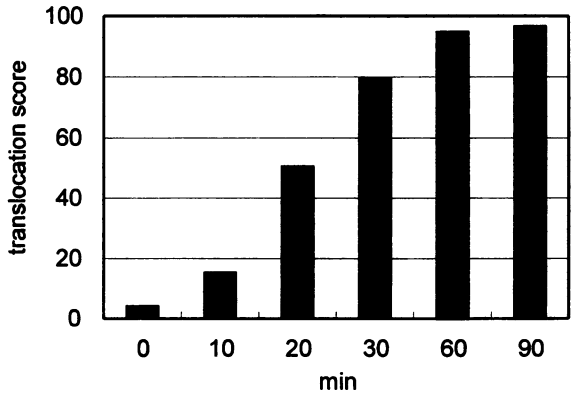

FIG. 4. Time course of heat shock-induced translocation of PKN in NIH 3T3 cells. Control, untreated cells (indicated as 0 min) and cells treated at 42°C for the indicated time were immunostained by αC6. Solid black bars respresent the level of translocation expressed as the percent of cell population (designated as translocation score) in which immunofluorescence intensity of PKN in nuclei are equal or stronger than that in the cytoplasms.

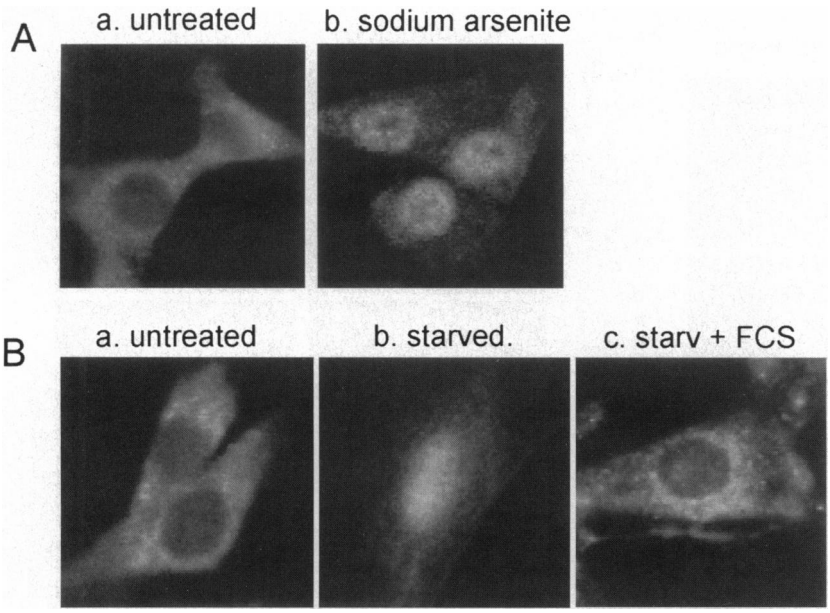

FIG. 5. Effects of sodium arsenite and serum starvation on the immunofluorescence staining of PKN. (*A*) Effects of sodium arsenite on Rat-1 cells. Control, untreated cells (*a*) and cells treated with 50 μM sodium arsenite in the culture medium at 37°C for 2 hr (*b*) were immunostained using αC6. (*B*) Effects of serum starvation on NIH 3T3 cells. Control untreated cells (*a*), cells serum starved for 24 hr at 37°C (*b*), and cells incubated with 10% fetal calf serum at 37°C for 4 hr following serum starvation (*c*) were immunostained using αC6.

to the nonstressed state (Fig. 5*B*). The ultraviolet irradiation was other means of stress in experiments, and ultraviolet response of mammalian cells is characterized by a rapid and selective increase in gene expression mediated by AP-1 and NF-κB (25, 26). However, the translocation was not observed when the NIH 3T3 cells were exposed to 40 J/m^2 UV-C irradiation (data not shown) which is enough for activation of JNK (27).

The mechanism of stress-induced translocation of PKN remains unknown because a nuclear localization sequence has not been identified within the primary structure of PKN. The following mechanisms can be proposed. (*i*) PKN has a cryptic nuclear localization sequence that is exposed to its surface by treatment of cells with stress. There is a possibility that activation of PKN drive the translocation of the enzyme. (*ii*) PKN lacks a functional nuclear localization sequence, and the nuclear localization is induced by the association with other proteins. PKN-activator Rho itself could be a candidate of such associate proteins. Recent studies indicate that Rho kinase/ROKα, another potential target of Rho, is recruited specifically to plasma membrane with activated RhoA (28, 29) and that Raf, a target protein of Ras, is recruited to plasma membrane with Ras (30–32). As in the case of Rho-Rho kinase/ROKα and Ras-Raf, PKN might be recruited into the nucleus with activated Rho.

Recently Hill *et al.* (12) reported that serum response factor (SRF) required for the activity of c-*fos* serum response element is a nuclear target of a novel Rho-mediated signaling pathway. SRF activation requires functional Rho for regulated c-*fos* transcription by lysophosphatidic acid, serum, and stresses such as arsenite and osmotic shock (12). SRF activation, however, does not correlate with activation of MEK (mitogen-activated protein kinase kinase/extracellular signal regulated kinase kinase), SAPK/JNK (stress-activated protein kinase/c-Jun amino-terminal kinase), or MPK2/p38 in NIH 3T3 cells (12). The existence of an appropriate kinase that correlates with SRF-linked signaling pathway has been postulated, analogous to the Ras/Raf/mitogen-activating protein kinase (MAPK) pathway leading to ternary complex factor activation (16–18), and the Rac or Cdc42Hs/PAK/JNK pathway leading to c-Jun activation. Since heat shock or serum starvation induces the translocation of PKN to the nucleus, PKN may be a candidate protein kinase involved in nuclear SRF-linked signaling by environmental stresses.

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1. Mukai, H., Kitagawa, M., Shibata, H., Takanaga, H., Mori, K., Shimakawa, M., Miyahara, M., Hirao, K. & Ono, Y. (1994) *Biochem. Biophys. Res. Commun.* **204,** 348–356.
2. Mukai, H. & Ono, Y. (1994) *Biochem. Biophys. Res. Commun.* **199,** 897–904.
3. Palmer, R. H., Ridden, J. & Parker, P. J. (1995) *Eur. J. Biochem.* **227,** 344–351.
4. Watanabe, G., Saito, Y., Madaule, P., Ishizaki, T., Morii, N., Mukai, H., Ono, Y., Kakizuka, A. & Narumiya, S. (1996) *Science* **271,** 645–648.
5. Amano, M., Mukai, H., Ono, Y., Chihara, K., Matsui, T., Okawa, K., Iwamatsu, A. & Kaibuchi, K. (1996) *Science* **271,** 648–650.
6. Paterson, H. F., Self, A. J., Garrett, M. D., Just, I., Aktories, K. & Hall, A. (1990) *J. Cell Biol.* **111,** 1001–1007.
7. Morii, N., Teru uchi, T., Tominaga, T., Kumagai, N., Kozaki, S., Ushikubi, F. & Narumiya, S. (1992) *J. Biol. Chem.* **267,** 20921–20926.
8. Tominaga, T., Sugie, K., Hirata, M., Morii, N., Fukata, J., Uchida, A., Imura, H. & Narumiya, S. (1993) *J. Cell Biol.* **120,** 1529–1537.
9. Takaishi, K., Sasaki, T., Kato, M., Yamochi, W., Kuroda, S., Nakamura, T., Takeichi, M. & Takai, Y. (1994) *Oncogene* **9,** 273–279.
10. Kishi, K., Sasaki, T., Kuroda, S., Itoh, T. & Takai, Y. (1993) *J. Cell Biol.* **120,** 1187–1195.
11. Mabuchi, I., Hamaguchi, Y., Fujimoto, H., Morii, N., Mishima, M. & Narumiya, S. (1993) *Zygote* **1,** 325–331.
12. Hill, C. S., Wynne, J. & Treisman, R. (1995) *Cell* **81,** 1159–1170.
13. Yamamoto, M., Marui, N., Sakai, T., Morii, N., Kozaki, S., Ikai, K., Imamura, S. & Narumiya, S. (1993) *Oncogene* **8,** 1449–1455.
14. Khosravi Far, R., Solski, P. A., Clark, G. J., Kinch, M. S. & Der, C. J. (1995) *Mol. Cell. Biol.* **15,** 6443–6453.
15. Mukai, H., Toshimori, M., Shibata, H., Kitagawa, M., Shimakawa, M., Miyahara, M., Sunakawa, H. & Ono, Y. (1996) *J. Biol. Chem.* **271,** 9816–9822.
16. Minden, A., Lin, A., Claret, F. X., Abo, A. & Karin, M. (1995) *Cell* **81,** 1147–1157.
17. Coso, O. A., Chiariello, M., Yu, J. C., Teramoto, H., Crespo, P., Xu, N., Miki, T. & Gutkind, J. S. (1995) *Cell* **81,** 1137–1146.
18. Zhang, S., Han, J., Sells, M. A., Chernoff, J., Knaus, U. G., Ulevitch, R. J. & Bokoch, G. M. (1995) *J. Biol. Chem.* **270,** 23934–23936.
19. Adler, V., Schaffer, A., Kim, J., Dolan, L. & Ronai, Z. (1995) *J. Biol. Chem.* **270,** 26071–26077.

20. Peterson, G. L. (1977) *Anal. Biochem.* **83,** 346–356.
21. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
22. Kitagawa, M., Mukai, H., Shibata, H. & Ono, Y. (1995) *Biochem. J.* **310,** 657–664.
23. Maytin, E. V. & Young, D. A. (1983) *J. Biol. Chem.* **258,** 12718–12722.
24. Welch, W. J. & Suhan, J. P. (1986) *J. Cell Biol.* **103,** 2035–2052.
25. Devary, Y., Rosette, C., DiDonato, J. A. & Karin, M. (1993) *Science* **261,** 1442–1445.
26. Devary, Y., Gottlieb, R. A., Lau, L. F. & Karin, M. (1991) *Mol. Cell. Biol.* **11,** 2804–2811.
27. Derijard, B., Hibi, M., Wu, I. H., Barrett, T., Su, B., Deng, T., Karin, M. & Davis, R. J. (1994) *Cell* **76,** 1025–1037.
28. Matsui, T., Amano, M., Yamamoto, T., Chihara, K., Nakafuku, M., Ito, M., Nakano, T., Okawa, K., Iwamatsu, A. & Kaibuchi, K. (1996) *EMBO J.* **15,** 2208–2216.
29. Leung, T., Manser, E., Tan, L. & Lim, L. (1995) *J. Biol. Chem.* **270,** 29051–29054.
30. Stokoe, D., Macdonald, S. G., Cadwallader, K., Symons, M. & Hancock, J. F. (1994) *Science* **264,** 1463–1467.
31. Leevers, S. J., Paterson, H. F. & Marshall, C. J. (1994) *Nature (London)* **369,** 411–414.
32. Marais, R., Light, Y., Paterson, H. F. & Marshall, C. J. (1995) *EMBO J.* **14,** 3136–3145.