p57KIP2 Modulates Stress-activated Signaling by Inhibiting c-Jun NH2-terminal Kinase/Stress-activated Protein Kinase*

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p57KIP2, a member of the Cip/Kip family of enzymes that inhibit several cyclin-dependent kinases, plays a role in many biological events including cell proliferation, differentiation, apoptosis, tumorigenesis and developmental changes. The human p57KIP2 gene is located in chromosome 11p15.5, a region implicated in sporadic cancers and Beckwith-Wiedemann syndrome. We here report that p57KIP2 physically interacts with and inhibits c-Jun NH2-terminal kinase/stress-activated protein kinase (JNK/SAPK). The carboxyl-terminal QT domain of p57KIP2 is crucial for the inhibition of JNK/SAPK. Overexpressed p57KIP2 also suppressed UV- and MEKK1-induced apoptotic cell death. p57KIP2 expression during C2C12 myoblast differentiation resulted in repression of the JNK activity stimulated by UV light. Furthermore, UV-stimulated JNK1 activity was higher in mouse embryonic fibroblasts derived from p57−/− mice than in the cells from wild-type mice. Taken together, these findings suggest that p57KIP2 modulates stress-activated signaling by functioning as an endogenous inhibitor of JNK/SAPK.

Cyclin-dependent kinases (CDKs) are a family of protein kinases that catalyze cellular reactions required for cell cycle progressions. Enzymatic activities of CDKs are positively regulated by specific cyclins and negatively by CDK inhibitors (1, 2). On the basis of structural and functional characteristics, CDK inhibitors can be grouped into two families (2). The INK4 family, which includes p16INK4a, p15INK4b, p18INK4a, and p19INK4b, inhibits the cyclin D-dependent CDKs, CDK4 and CDK6. The Cip/Kip family, which includes p57KIP2, p21CIP1/WAF1, and p27KIP1, can inhibit all CDKs that regulate the G1/S-phase transition (2, 3). p57KIP2 shares sequence homology with p21CIP1 and p21CIP1/WAF1 in the NH2-terminal domain, which is involved in the binding to cyclin-CDK complexes (4, 5). p57KIP2 and p27KIP1 also have unique carboxyl-terminal QT domains. The function of the QT domain is unclear. The human p57KIP2 gene, which encodes a 316-amino acid protein, is located in chromosome 11p15.5, a region implicated in sporadic cancers and Beckwith-Wiedemann syndrome, a familial cancer syndrome (5). Interestingly, p57KIP2 null mice show altered cell proliferation and differentiation, apoptosis, and many other phenotypes that can be observed in patients with Beckwith-Wiedemann syndrome (6, 7). Thus, p57KIP2 has been implicated in the modulation of many cellular events including cell cycle control, differentiation, apoptosis, tumorigenesis, and development. However, the mechanism by which p57KIP2 exerts its modulatory functions is not yet fully understood.

A variety of extracellular stimuli initiate intracellular signaling through the sequential protein phosphorylations leading to the activation of mitogen-activated protein kinases (MAPKs). The mammalian MAPK family includes several subgroups such as extracellular signal-regulated kinase (ERK), p38 MAPK, and c-Jun NH2-terminal kinase/stress-activated protein kinase (JNK/SAPK) (8–10). The JNK/SAPK signaling pathway is preferentially stimulated in cellular responses to a variety of stresses that include UV light, ionizing irradiation, DNA-damaging chemicals, reactive oxygen species, heat and osmotic shock, and metabolic inhibitors (9, 10). The JNK/SAPK signaling cascade is composed of JNK/SAPK and its upstream kinases, MAPK kinases such as SEK1/JNKK1/MKK4 and MAPK kinase kinases such as MEKK1. Once activated, JNK/SAPK phosphorylates its cellular substrates, which include several transcription factors such as c-Jun, ATF2, and TCF/Elk-1, thereby enhancing their transcriptional activities (11–13). Accordingly, JNK/SAPK has been implicated in the regulation of diverse cellular activities such as cell growth, transformation, survival, and death (9, 10).

JNK/SAPK activity has been shown to be modulated by other proteins through protein-protein interactions (14–16). We previously reported that overexpressed p21CIP1/WAF1 binds and inhibits JNK/SAPK (15). In the present study, we investigated
whether p57KIP2 can modulate the JNK/SAPK signaling pathway. Our findings indicate that p57KIP2 negatively regulates the JNK/SAPK signaling cascade through direct inhibition of JNK/SAPK, independently of its well known inhibitory function on CDKs. This new function of p57KIP2 on JNK/SAPK may be an important mechanism by which p57KIP2 can modulate the intracellular signaling events that mediate a variety of cellular activities including cell differentiation and survival.

EXPERIMENTAL PROCEDURES

Plasmid Construction—The full-length human p57KIP2 (ATCC 99411) was subcloned into pGEX-4T-3 (Amersham Biosciences). The plasmids encoding GST fusion proteins of p57-(1–79), p57-(1–158), p57-(126–316), and p57-(238–316) were created by PCR amplification from a human p57KIP2 cDNA template and cloning in-frame into pGEX-4T.

Cell Culture and Transfection—Human embryonic kidney HEK293, COS7, and HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in a 5% CO₂ atmosphere at 37 °C. Mouse embryonic fibroblast (MEF) cells from wild-type and p57KIP2 knockout mice were used between passage three and five (17), and they were in the same passage during the experiments. Myoblast C2C12 cells were routinely grown in Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum (growth medium). Where indicated, C2C12 cells were moved to a complete medium at 37 °C for 10 min and solubilized with 1% Triton X-100. The soluble fraction was applied to glutathione-agarose beads. GST-SEK1 was eluted from the beads and assayed for SEK1 activity using GST-SAPK as substrate. C. p57KIP2 does not affect MEKK1 activity. HEK293 cells were transiently transfected for 48 h with plasmids encoding MEKK1-FLAG and p57KIP2 as indicated. Cell lysates were subjected to immunoprecipitation with anti-FLAG antibody and the resulting immunoprecipitates were assayed for MEKK1 activity. These data represent the results from three independent experiments.

FIG. 1. p57KIP2 inhibits JNK/SAPK in intact cells. A, ectopic p57KIP2 inhibits JNK, but not ERK or p38 MAPK, in HEK293 cells. HEK293 cells were transfected with plasmid vectors encoding HA-JNK1, HA-ERK2, or p38-FLAG along with plasmid encoding p57KIP2 as indicated. After 48 h, the cells were exposed to either 60 J/m² UV light for JNK and p38 stimulation or 200 nM 12-O-tetradecanoylphorbol-13-acetate (TPA) for 30 min for ERK stimulation. Whereas exposed to UV light, the cells were further incubated for 1 h at 37 °C. After treatment, cells were lysed and cell lysates were subjected to immunoprecipitation with the use of anti-HA or anti-FLAG antibody. The resulting immunoprecipitates were examined for kinase activities of JNK1, p38, and ERK2 by immune complex kinase assay. Cell lysates were also subjected to immunoblot (IB) analysis with indicated antibodies. B, p57KIP2 does not affect SEK1 activity. HEK293 cells were transfected for 48 h with plasmids encoding GST-SEK1 and p57KIP2 as indicated. Where indicated, the cells were exposed to 60 J/m² UV light and then incubated for 1 more hour. Cells were lysed and the soluble fraction mixed with glutathione-agarose beads. GST-SEK1 was eluted from the beads and assayed for SEK1 activity using GST-SAPK as substrate. C, p57KIP2 does not affect MEKK1 activity. HEK293 cells were transiently transfected for 48 h with plasmids encoding MEKK1-FLAG and p57KIP2 as indicated. Cell lysates were subjected to immunoprecipitation with anti-FLAG antibody, and the resulting immunoprecipitates were assayed for MEKK1 activity. These data represent the results from three independent experiments.

Co-immunoprecipitation of JNK and p57KIP2—To test the physical association of endogenous p57KIP2 and JNK1 protein in intact cells, HeLa cells were lysed in buffer A containing 120 mM NaCl, 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol, 10 mM NaF, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μM aprotinin, and 1% digitonin. The lysates were then subjected to immunoprecipitation with the use of rabbit polyclonal anti-p57KIP2 antibody or rabbit pre-immune IgG. The resulting immunopellets were subjected to SDS-PAGE on 10% polyacrylamide gel and analyzed by immunoblot with mouse monoclonal anti-JNK1 antibody. To test the interactions of ectopically expressed proteins, HEK293 cells were transfected with pcDNA3 vectors encoding HA-JNK3/SAPK6 and p57KIP2. The transfected cells were lysed with buffer A, and the cell lysates were subjected to immunoprecipitation with the use of mouse monoclonal anti-HA antibody or mouse pre-immune IgG as the negative control. The immunopellets were analyzed by immunoblot probed with rabbit polyclonal anti-p57KIP2 antibody.
rabbit polyclonal anti-p57 KIP2 antibody. Cell lysates (10% of total) were subjected to immunoblot analysis with anti-p57 KIP2 antibody.

IB agarose beads. Bound 35S-labeled proteins were eluted, resolved by SDS-PAGE on a 10% polyacrylamide gel, and visualized by autoradiography.

Nonidet P-40, and 0.1 mM sodium orthovanadate. After the beads were in vitro translated with anti-FLAG antibody. Amounts of p57 KIP2, HA-JNK1, and c-Jun resulting immunoprecipitates were subjected to immunoblot (IB) analysis with mouse monoclonal anti-HA or mouse pre-immune IgG, respectively. The resulting immunoprecipitates were subjected to immunoblot (IB) analysis with rabbit polyclonal anti-p57KIP2 antibody. Cell lysates (10% of total) were also subjected to immunoblot analysis with anti-p57KIP2 antibody.

physical interaction between endogenous p57KIP2 and JNK proteins in HeLa cells. HeLa cell lysates were subjected to immunoprecipitation (IP) using mouse monoclonal anti-JNK1 antibody. Cell lysates were also subjected to immunoblot analysis with anti-JNK1 antibody.

FIG. 2. p57KIP2 physically associates with JNK/SAPK. A, in vitro binding of JNK to GST-p57KIP2. JNK1, JNK3/SAPKβ, p38, or ERK2 was translated in vitro in the presence of [35S]methionine. 35S-Labeled proteins were then mixed with GST or GST-p57KIP2 immobilized on glutathione–agarose beads. Bound 35S-labeled proteins were eluted, resolved by SDS-PAGE on a 10% polyacrylamide gel, and visualized by autoradiography. The input of 35S-labeled proteins (33%) is also shown. B, ectopic p57KIP2 physically associates with JNK1 in transfected HEK293 cells. HEK293 cells were transfected with plasmids expressing p57KIP2 and HA-JNK1. Cell lysates were subjected to immunoprecipitation (IP) using mouse monoclonal anti-HA or mouse pre-immune IgG, respectively. The resulting immunoprecipitates were subjected to immunoblot (IB) analysis with rabbit polyclonal anti-p57KIP2 antibody. Cell lysates (10% of total) were also subjected to immunoblot analysis with anti-p57KIP2 antibody.

FIG. 3. p57KIP2 inhibits an interaction between JNK1 and c-Jun. HEK293 cells were transfected for 48 h with expression vectors encoding c-Jun-FLAG (3 μg) and HA-JNK1 (3 μg) in the absence or presence of p57KIP2 construct (1 or 2 μg) as indicated. Cell lysates were subjected to immunoprecipitation (IP) with anti-HA antibody. The resulting immunoprecipitates were subjected to immunoblot (IB) analysis with anti-FLAG antibody. Amounts of p57KIP2, HA-JNK1, and c-Jun-FLAG present in cell lysates were also examined by immunoblotting with indicated antibodies. These data represent the results from two independent experiments.

In Vitro Binding Assay—GST fusion proteins were bacterially expressed using pGEX-4T vector. JNK1, JNK3/SAPKβ, p38, and ERK2 were in vitro translated in the presence of [35S]methionine using the Tnt reticulocyte lysate system (Promega). 35S-Labeled proteins were incubated at 4°C for 2 h with GST fusion proteins immobilized on glutathione–agarose beads in 400 μl of binding buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM dithiothreitol, 0.1% Nonidet P-40, and 0.1 mM sodium orthovanadate. After the beads were rinsed three times with washing buffer (1 mM EDTA, 1 mM dithiothreitol, 0.1% Tween 20, 150 mM NaCl, 50 mM HEPES, pH 7.5). 35S-labeled proteins were eluted from the beads and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography.

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RESULTS

p57KIP2 Inhibits JNK/SAPK Activity in Intact Cells—To investigate whether p57KIP2 could modulate the JNK signaling pathway, we examined the effect of p57KIP2 on JNK activity in intact cells. We transfected HEK293 cells with plasmids encoding p57KIP2 and JNK1 and induced JNK stimulation by exposing transfected cells to UV light (Fig. 1). Our data indicate that ectopic p57KIP2 inhibited the UV-stimulated activity of JNK1 (Fig. 1A) or SAPKβ/JNK3 (data not shown), whereas it did not affect either the UV-stimulated p38 MAPK activity or the phosphor-ester-stimulated ERK2 activity (Fig. 1A). Because the JNK signaling system comprises JNK and its upstream kinases, which include a MAPK kinase such as SEK1/MKK4/JNKK1 and a MAPK kinase kinase such as MEKK1, we decided to examine whether p57KIP2 would also inhibit SEK1 or MEKK1 activity. HEK293 cells were transfected with plasmid encoding GST-SEK1, and then the transfected cells were unexposed or exposed to UV light. UV irradiation enhanced the kinase activity of GST-SEK1 in the cells (Fig. 1B). Co-expressed p57KIP2 did not affect the UV-stimulated GST-SEK1 activity. Similarly, p57KIP2 also did not affect the UV-induced stimulation of MKK7, which is another MAPK kinase upstream of JNK (data not shown). We looked next for a possible effect of p57KIP2 on MEKK1 activity (Fig. 1C). HEK293 cells transfected with plas-
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GST-p57KIP2 fusion proteins as indicated. The diagram in cipitated HA-JNK1 (A) or CDK2 (B) were subjected to immunoprecipitation with anti-HA antibody, and the resulting immunoprecipitates were subjected to immunoblot analysis with anti-p57KIP2 antibody. The immunoblot data showed that HA-JNK1 physically associated with p57KIP2 in the transfected cells. We also examined the physical interaction between endogenous p57KIP2 and endogenous JNK1 in HeLa cells. HeLa cell lysates were subjected to immunoprecipitation with the use of anti-p57KIP2 antibody. Immunoblot analysis using anti-JNK1 antibody of the p57KIP2 immunoprecipitates revealed that endogenous JNK1 physically interacted with endogenous p57KIP2 (Fig. 2C).

Next, we examined the effect of p57KIP2 on the interaction between JNK1 and c-Jun, a substrate of JNK1, in intact cells. Co-immunoprecipitation data showed that the physical interaction between JNK1 and c-Jun markedly decreased in the cells expressing p57KIP2 (Fig. 3). These results thus suggest that p57KIP2, by binding JNK1, inhibits an interaction between JNK1 and c-Jun.

The QT Domain of p57KIP2 Is Involved in Inhibition of JNK—Next, we investigated the in vitro effects of p57KIP2 and p57KIP2 variants on JNK activity. An in vitro kinase study showed that recombinant GST-p57KIP2 inhibited JNK1 activity, whereas the GST control protein did not affect the kinase activity (Fig. 4A). GST-p57(126–316) and GST-p57(238–316) also inhibited JNK1 activity, whereas GST-p57(1–79) and GST-p57(1–158) did not. In comparison, GST-p57(238–316) did not inhibit CDK2 activity in vitro, whereas GST-p57(1–79) did (Fig. 4B). These results suggest that p57KIP2 inhibits JNK and CDK2 by distinct mechanisms and that the carboxyl-terminal QT domain of p57KIP2 is crucial for JNK inhibition. Transfection studies using HEK293 cells also showed that UV-stimulated JNK1 activity was inhibited by ectopically expressed p57(238–316) but not by p57(1–79) (data not shown). GST-p57KIP2 protein was not phosphorylated in vitro by JNK1 (data not shown).

p57KIP2 Suppresses JNK-involved Apoptosis—The JNK/SAPK signaling pathway has been shown to be involved in the mechanism of apoptosis (9, 18, 19). We therefore tested whether ectopic p57KIP2 could suppress the JNK-involved apoptosis (Fig. 5). First, we examined the effect of p57KIP2 on apoptosis induced by expression of ΔMEKK1, a constitutively active mutant of MEKK1 (Fig. 5A). Apoptotic cell death was enhanced in HEK293 cells expressing ΔMEKK1, and ΔMEKK1-induced apoptosis was reduced when SEK1(K129R) was co-expressed. These data suggest that ΔMEKK1 induces apoptotic cell death through the MEKK1–SEK1–JNK signaling cascade. ΔMEKK1-induced apoptosis was suppressed in cells expressing ectopic p57KIP2. Because p57KIP2 does not inhibit either MEKK1 or SEK1 activity (Fig. 1), these results suggest that JNK inhibition is the major mechanism by which p57KIP2 suppresses JNK-mediated apoptosis. Next, we examined the effect of p57KIP2 on UV-induced apoptosis. The DAPI staining data indicated that exposure of HEK293 cells to UV light resulted in a marked increase in apoptotic cell death (Fig. 5B). The UV-induced apoptosis was decreased in cells expressing SEK1(K129R), a dominant-negative mutant of SEK1, suggesting that the JNK signaling cascade was involved in the mechanism of UV-induced apoptosis. We found that cells transfected with plasmid encoding p57KIP2 became more resistant to UV radiation than cells transfected with control plasmid. TUNEL

mid expressing MEKK1 showed high enzymatic activity of MEKK1 even without any further treatment, and ectopic p57KIP2 did not alter the MEKK1 activity. A separate transfection study also indicated that p57KIP2 did not affect the UV-stimulated MEKK1 activity (data not shown). Taken together, these results suggest that p57KIP2 suppresses the JNK/SAPK signaling pathway through acting on a site or sites downstream of MAPK kinase, probably JNK itself.

p57KIP2 Physically Interacts with JNK—In the following experiments, we tested whether p57KIP2 could interact with JNK/SAPK. An in vitro binding study using in vitro translated \[^{35}S\]-labeled protein kinase proteins revealed that GST-p57KIP2 protein directly interacted with \[^{35}S\]-labeled JNK1 and SAPKβ/JNK3 but not with \[^{35}S\]-labeled ERK2 or p38 (Fig. 2A). Next we examined the physical association of p57KIP2 and JNK in HEK293 cells after co-transfecting the cells with plasmids encoding p57KIP2 and HA-JNK1 (Fig. 2B). The transfected HEK293 cells were subjected to immunoprecipitation with anti-HA antibody, and the resulting immunoprecipitates were subjected to immunoblot analysis with anti-p57KIP2 antibody. The immunoblot data showed that HA-JNK1 physically associated with p57KIP2 in the transfected cells. We also examined the physical interaction between endogenous p57KIP2 and endogenous JNK1 in HeLa cells. HeLa cell lysates were subjected to immunoprecipitation with the use of anti-p57KIP2 antibody. Immunoblot analysis using anti-JNK1 antibody of the p57KIP2 immunoprecipitates revealed that endogenous JNK1 physically interacted with endogenous p57KIP2 (Fig. 2C).

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Depletion of Endogenous p57KIP2 Enhances Kinase Activity of Endogenous JNK in Intact Cells—Recently it has been shown that expression of p57KIP2 is enhanced during C2C12 myoblast differentiation (20), which can be induced by withdrawing serum from the culture medium. We therefore tested whether the induction of p57KIP2 expression by serum withdrawal would result in suppression of JNK activity in C2C12 myoblast cells. We confirmed a dramatic induction of p57KIP2 expression in C2C12 cells after reducing the serum concentration from 20% (growth medium) to 2% (differentiation medium; data not shown). We then measured JNK1 activity in undifferentiated and differentiated C2C12 cells (Fig. 6). Our data show that the UV-stimulated JNK1 activity was inhibited in C2C12 cells in differentiation medium, compared with C2C12 cells in growth medium (Fig. 6A). The immunoblot data revealed the induction of p57KIP2 expression in the cells in differentiation medium. To further examine whether p57KIP2 induction was responsible for the inhibition of JNK activity during C2C12 cell differentiation, we constructed C2C12 cells that were stably transfected with p57KIP2 antisense vector. Expression of p57KIP2 protein was markedly reduced in the C2C12 cells harboring the p57KIP2 antisense compared with control cells that were stably transfected with the empty vector alone (Fig. 6B). Our data thus indicate that suppression of p57KIP2 expression prevents the inhibitory action of p57KIP2 on UV-stimulated JNK1 activity.

To further investigate the biological relevance of the inhibitory action of p57 on JNK activity, we examined the UV-stimulated activity of endogenous JNK1 in MEF cells derived from p57+/− and p57−/− mice. The JNK1 activity at the UV-stimulated state was higher in MEFp57+/− cells than in MEFp57−/− cells (Fig. 7). Thus, these results suggest that p57KIP2 is an endogenous inhibitor of JNK/SAPK.

**DISCUSSION**

We have demonstrated here that p57KIP2 can modulate stress-activated signaling through negatively regulating the JNK/SAPK pathway. p57KIP2 appears to suppress the JNK signaling pathway by targeting JNK. p57KIP2 did not affect other MAPK family members such as ERK and p38. p57KIP2 physically associates with and inhibits JNK in intact cells. Thus we show in this study that p57KIP2 functions as a natural inhibitory protein of JNK/SAPK, independently of its inhibitory action on CDKs.

p57KIP2 contains the NH₂-terminal CDK binding domain, the PAPA domain, and the carboxyl-terminal QT domain (4, 5). p57 shares sequence homology with other members of the p21 family, p21 and p27, in the NH₂-terminal CDK binding domain (4, 5). The carboxyl-terminal QT domain of p57KIP2 also exhibits its sequence similarity to that region in p27. Although the function of the QT domain of p57KIP2 remains unclear, this domain is thought to be involved in protein-protein interaction (5). Our present data show that a carboxyl-terminal p57KIP2 fragment containing the QT domain is crucial for the inhibition of JNK/SAPK. Interestingly, p27, which also contains the QT domain in its carboxyl-terminal region, failed to inhibit JNK/SAPK activity in vitro (data not shown). The difference be-
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REFERENCES

1. Morgan, D. O. (1995) Nature 374, 131–134
2. Sherr, C. J., and Roberts, J. M. (1995) Genes Dev. 9, 1149–1163
3. Harper, J. W., and Elledge, S. J. (1996) Curr. Opin. Genet. Dev. 6, 56–64
4. Lee, M. H., Reynolds, I., and Massague, J. (1995) Genes Dev. 9, 639–649
5. Matsuzaka, S., Edwards, M. C., Baim, C., Parker, S., Zhang, P., Baldini, A., Harper, J. W., and Elledge, S. J. (1995) Genes Dev. 9, 650–662
6. Zhang, F., Liegeois, N. J., Wong, C., Finegold, M., Hou, H., Thompson, J. C., Silverman, A., Harper, J. W., DePinho, R. A., and Elledge, S. J. (1997) Nature 387, 151–158
7. Yan, Y., Friisen, J., Lee, M. H., Massague, J., and Barbacid, M. (1997) Genes Dev. 11, 973–983
8. Cobb, M. H., and Goldsmith, E. J. (1995) J. Biol. Chem. 270, 14431–14446
9. Ip, Y. T., and Davis, R. J. (1998) Curr. Opin. Cell Biol. 10, 205–219
10. Minden, A., and Karin, M. (1997) Biochim. Biophys. Acta 1333, F85–F104
11. Gupta, S., Campbell, D., Derijard, B., and Davis, R. J. (1995) Science 267, 389–393
12. Derijard, B., Hibi, M., Wu, I. H., Barrett, T., Su, B., Deng, T., Karin, M., and Davis, R. J. (1994) Cell 76, 1025–1037
13. Wharton, A. J., Shore, P., Sharrow, A. D., and Davis, R. J. (1995) Science 269, 403–407
14. Park, H. S., Lee, J. S., Huh, S.-H., Seo, J.-S., and Choi, E.-J. (2001) EMBO J. 20, 446–456
15. Shim, J., Lee, H., Park, J., Kim, H., and Choi, E.-J. (1996) Nature 381, 804–806
16. Shim, J., Park, H. S., Kim, M. J., Park, J., Park, E., Cho, S.-G., Eom, S.-J., Lee,
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H.-W. Joe, C. O., and Choi, E.-J. (2000) J. Biol. Chem. 275, 14107–14111
17. Takahashi, K., Nakayama, K., and Nakayama, K. (2000) J. Biochem. (Tokyo) 127, 73–83
18. Xia, Z., Dickens, M., Raingeaud, J., Davis, R. J., and Greenberg, M. E. (1995) Science 270, 1326–1331
19. Chen, Y. R., Wang, X., Templeton, D., Davis, R. J., and Tan, T. H. (1996) J. Biol. Chem. 271, 31929–31936
20. Reynaud, E. G., Pelpel, K., Guillier, M., Leibovitch, M. P., and Leibovitch, S. A. (1999) Mol. Cell. Biol. 19, 7621–7629
21. Hatada, I., Nabetani, A., Morisaki, H., Xin, Z., Ohishi, S., Tonoki, H., Niikawa, N., Inoue, M., Komoto, Y., Okada, A., Steichen, E., Ohashi, H., Fukushima, Y., Nakayama, M., and Mukai, T. (1997) Hum. Genet. 100, 681–683
22. Hatada, I., Ohashi, H., Fukushima, Y., Kaneko, Y., Inoue, M., Komoto, Y., Okada, A., Ohishi, S., Nabetani, A., Morisaki, H., Nakayama, M., Niikawa, M., and Mukai, T. (1998) Nat. Genet. 14, 171–172
23. Behrens, A., Jochum, W., Shilina, M., and Wagner, E. F. (2000) Oncogene 19, 2657–2663
24. Zhang, P., Wong, C., Liu, D., Finegold, M., Harper, J. W., and Elledge, S. J. (1999) Genes Dev. 13, 213–224
25. Nagata, Y., and Todokoro, K. (1999) Blood 94, 853–863
26. Dong, C., Yang, D. D., Wysk, M., Whitmarsh, A. J., Davis, R. J., and Flavell, R. A. (1998) Science 282, 2092–2095