We studied mitogen-activated protein kinase (MAPK) activities during the cell cycle of Chinese hamster ovary (CHO) cells using site-specific antibodies against extracellular signal-regulated kinase-1, a 44-kDa MAPK (Boulton, T. G., Yancopoulos, G. D., Gregory, J. S., Slauer, C., Moomaw, C., Hsu, J., and Cobb, M. H. (1990) Science 249, 64-67). These antibodies detected two distinct MAPKs (44- and 42-kDa MAPKs) in CHO cells. CHO cells were arrested at metaphase in the M phase by treatment with nocodazole, and activities of MAPKs were analyzed at specific time points after release from arrest. Immune complex kinase assay and renaturation and phosphorylation assay in substrate-containing gel revealed that both 44- and 42-kDa MAPKs had activities in the G1 phase and around the M phase. MAPKs were inactivated in metaphase-arrested cells. The amount of MAPKs did not change significantly in the cell cycle. In the G1, S, and G2/M phases, MAPKs were phosphorylated on both tyrosine and threonine residues and are activated biphasically: in G1 and around the M phase. MAPKs were inactivated in metaphase-arrested cells. Our data suggest that MAPKs may play some role in the cell cycle other than G0/G1 transition.

Mitogen-activated protein kinase (MAPK) is a kinase that is activated by various mitogens (1-6) and plays an important role in the kinase cascade that results in G1/S transition (7-9). It is a unique serine/threonine kinase that is activated through phosphorylation on its tyrosine and threonine residues (10, 11) and is believed to be the 42-kDa protein that is phosphorylated on stimulation with mitogens (12). Recently, a partial cDNA of the putative MAPK (or extracellular signal-regulated kinase-1 (ERK-1)) has been cloned from rat brain cDNA library (13). Subsequently, two related kinases have been cloned (14). Interestingly, there is a considerable homology between amino acid sequences of the ERK-1 and FUS3 or KSS1, which is a yeast gene involved in cell cycle control. In starfish and Xenopus oocytes, homologous kinase is activated in the M phase (15) and phosphorylated on tyrosine residue (16-19) (for review, see Refs. 20 and 21).

Therefore, we were interested in whether MAPKs of mammals are controlled in a cell cycle-dependent manner. In the present study, MAPK activity was measured in the cell cycle in proliferating Chinese hamster ovary (CHO) cells. To this end, we used specific antisera against two synthetic peptides corresponding to the regions of the predicted amino acid sequence of ERK-1, a putative MAPK (13). To synchronize the cells, reagents that arrest cells in specific points in the cell cycle were used in combination with a collection of metaphase cells with mechanical shakeoff (22). Two reagents were used in this study. Nocodazole, a microtubule disrupting reagent, is known to arrest cells in the M phase (23), and aphidicholin, an inhibitor of the synthesis of DNA, is known to arrest cells in the S phase (24). Since synchronization by nocodazole seemed to be more effective, this reagent was used in most of the experiments in this study.

In this study, we have found that both 44- and 42-kDa MAPKs have activities in the G1 phase and S phase and are activated biphasically: in G1 and around the M phase. We have also shown that activation of MAPKs in the cell cycle correlates with phosphorylation on both tyrosine and threonine residues as in stimulation with mitogens. Our data suggest that MAPKs may play some role in the cell cycle other than G0/G1 transition.

**EXPERIMENTAL PROCEDURES**

Materials—Myelin basic protein, histone H1, nocodazole, and aphidicholin were from Sigma. [γ-32P]ATP (111 TBq/mmol) was from Du Pont-New England Nuclear, [3H]orthophosphate (about 10.5 TBq/mg) was from ICN, [3H]protein-A (>1.1 GBq/mg) and [methyl-3H]thymidine (2.6-3.1 TBq/mmol) were from Amersham. Protein A-Sepharose CL-4B was from Pharmacia LKB Biotechnology Inc. Pansorbin was from Seikagaku Kogyo. Cell culture medium was either from GIBCO or Flow Laboratory.

**Cell Culture and Synchronization**—CHO cells were grown in minimal essential medium (GIBCO) supplemented with 10% fetal calf serum. To avoid contact inhibition, cells were plated in a 9-cm dish at a density between 5 x 10^5 and 2 x 10^6/dish. CHO cells were arrested at the metaphase/anaphase transition of the M phase by treating cells for 12-16 h with 0.5 μg/ml nocodazole. M phase-arrested cells were collected by gentle pipetting. Over 90% cells collected by this mechanical shakeoff method were in metaphase of the M phase (22). The cells were centrifuged at 1,000 rpm for 3 min, resuspended in fresh medium, and plated again. Replated cells were collected at the indicated time after replating. Cells were washed once with serum-free medium and were quickly frozen in liquid nitrogen and stored at -70 °C until assay. In S phase arrest, cells released from M phase

---

*Footnotes:
1. This work was supported by Juvenile Diabetes Foundation International Grant 190831 (to T. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
2. To whom correspondence should be addressed. Tel.: 03-3815-5411 (ext. 8296); Fax: 03-5684-3987.
3. The abbreviations used are: MAPK, mitogen-activated protein kinase; ERK-1, extracellular signal-regulated kinase-1; CHO cell, Chinese hamster ovary cell; HEPES, 4-(2-hydroxyethyl)-l-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; RT, room temperature.
Tris-buffered saline containing 1 M NaCl for 1 h at RT, and after collected with gentle pipetting and centrifugation, resuspended in polyvinylidene difluoride membrane (Immobilon P, Millipore) with roacetic acid-insoluble fraction was counted in Aquasol. Drying up, autoradiographed at -70°C with an intensifying screen.

concentrated Laemmli's sample buffer and boiled for 5 min. Samples cells/well). At the indicated times after replating, cells were washed with 20 mM NaF, 20 mM sodium pyrophosphate, 1 mM sodium vanadate, 1% Triton X-100, 100 units/ml aprotinin, and 1 mM PMSF. The cell lysates were centrifuged at 15,000 rpm for 15 min at 4°C. After washing in Tris-buffered saline for 30 min at RT, the samples was determined by Bradford method (26) and adjusted to 500 μl. The lysate was incubated with 5 pl of kinase buffer containing 50 mM HEPES pH 7.4, 100 mM NaCl, 1 mM EDTA, 20 units/ml aprotinin, and 1 mM PMSF. The cell lysates were centrifuged at 14,000 rpm for 15 min, and the supernatants were recovered as crude lysates. The protein concentration of the samples was determined by Bradford method (26) and adjusted before assay. To 450 μl of lysate, 3 μl of anti-cdc2 (αY21) or anti-MAPK (αC92) serum was added. When antisera αY91 was used for immunoprecipitation, 0.1-0.15% SDS was added to the lysate buffer. After incubation, αY91 immunoprecipitated MAPK was eluted poorly. After a 1-h incubation on ice, 40 μl of a 50%/v/v suspension of protein A-Sepharose was added and incubated for 3 h at 4°C with continuous agitation. Immunoprecipitated complex was washed twice with lysis buffer, once with 50 mM Tris, pH 7.4, 500 mM LiCl, twice with 50 mM Tris, pH 7.4, 10 mM MgCl₂. kinase assay was initiated by adding 50 μl of kinase buffer containing 50 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 2 mM ATP, 2 μCi of [γ⁻³²P]ATP, and substrate (final 0.2 mg/ml) to immune complex. As substrate, histone H1 and myelin basic protein were used for cdc2 kinase and MAPK, respectively. After a 15-min incubation at RT, the reaction was stopped by adding 15 μl of 5 × concentrated Laemmli's sample buffer (27) and boiling for 5 min. Thymidine Uptake—For thymidine uptake, cells arrested with nocodazole were collected and replated into six-well dishes (1 × 10⁵ cells/well). At the indicated times after replating, cells were washed once with serum-free medium and incubated in the same medium containing 2-6 × 10⁻⁸ M [methyl-³H]thymidine for 1 h at 37°C. At the end of incubation, cells were washed three times with ice-cold phosphate-buffered saline and then 10% trichloroacetic acid and solubilized in 0.4 N NaOH. The count incorporated into the trichloroacetic acid-insoluble fraction was counted in Aquasol.

Synchronization of the Cell Cycle—The synthesis of DNA assayed as [methyl-³H]thymidine uptake began at 6–8 h after the M phase, and the maximum level was attained at 12–14 h (Fig. 1A). Therefore, the S phase is thought to be 6–12 h from M phase arrest. At the point when nocodazole-arrested cells were collected, immunoprecipitated cdc2 kinase activity was very high, and this point was indicated as time 0. 0–16 h after release from M phase arrest by nocodazole, histone H1 kinase activity of immunoprecipitated cdc2 kinase was reactivated. This second peak of histone H1 kinase activity ranged from 14 to 20 h with its maximum at 16 h (Fig. 1B). Therefore, the second M phase is around 16 h from the release after M phase arrest.

Renaturation and Gel Phosphorylation Assay—When crude lysate was used, several bands were detected. Only the 44- and 42-kDa bands seemed to change their intensity during the cell cycle, apparently in a coordinated fashion. The peaks of kinase activities of the 44- and 42-kDa bands were detected at about 2–6 h and 14–20 h after release from the M phase (Fig. 2A, upper panel). The second peak was more apparent, and the first peak was variable in several experiments. When samples were immunoprecipitated with antisera αY91, the

The immune complex was precipitated by incubation with 70 μl of Pansorbin for 30 min on ice. The precipitated immune complex was washed five times with 500 μl of wash buffer (lysis buffer containing 0.1% SDS). The ³²P-labeled proteins were eluted by boiling in Laemmli's sample buffer and resolved through two-dimensional gel electrophoresis followed by autoradiography.

Renaturation and Phosphorylation in Substrate-containing Gel—Renaturation of the kinase after SDS-polyacrylamide gel electrophoresis was essentially as described (28). In brief, samples were resolved through a minigel containing 0.3–0.5 mg/ml myelin basic protein. After electrophoresis, the gel was immersed in 50 mM Tris, pH 8.0, 20% isopropl alcohol for 1 h at RT to remove SDS and then incubated in 50 mM Tris, pH 8.0, 5 mM β-mercaptoethanol for 1 h at RT. Subsequently, proteins in the gel were denatured completely in 6 M guanidine HCl for 1 h at RT. The denatured proteins were renatured slowly by incubating the gel in 50 mM Tris, pH 8.0, 5 mM β-mercaptoethanol, and 0.04% Tween 40 for 16 h at 4°C. The buffer was changed five times during this time. The renatured gel was preincubated in 50 mM HEPES pH 7.4, 2 mM dithiothreitol, and 10 mM MgCl₂ for 30 min at RT. The kinase assay was done by adding kinase buffer containing 50 mM HEPES pH 7.4, 2 mM dithiothreitol, 10 mM MgCl₂, 100 μM ATP, and 10 μCi/ml [γ⁻³²P]ATP for 1 h at RT. After phosphorylation, the gel was washed extensively in 7% acetic acid, with at least five 500-ml changes.

RESULTS

Synchronization of the Cell Cycle—The synthesis of DNA assays as [methyl-³H]thymidine uptake began at 6–8 h after the M phase, and the maximum level was attained at 12–14 h (Fig. 1A). Therefore, the S phase is thought to be 6–12 h from M phase arrest. At the point when nocodazole-arrested cells were collected, immunoprecipitated cdc2 kinase activity was very high, and this point was indicated as time 0. 0–16 h after release from M phase arrest by nocodazole, histone H1 kinase activity of immunoprecipitated cdc2 kinase was reactivated. This second peak of histone H1 kinase activity ranged from 14 to 20 h with its maximum at 16 h (Fig. 1B). Therefore, the second M phase is around 16 h from the release after M phase arrest.

Renaturation and Gel Phosphorylation Assay—When crude lysate was used, several bands were detected. Only the 44- and 42-kDa bands seemed to change their intensity during the cell cycle, apparently in a coordinated fashion. The peaks of kinase activities of the 44- and 42-kDa bands were detected at about 2–6 h and 14–20 h after release from the M phase (Fig. 2A, upper panel). The second peak was more apparent, and the first peak was variable in several experiments. When samples were immunoprecipitated with antisera αY91, the

FIG. 1. Thymidine uptake of synchronized cells. CHO-K1 cells arrested with nocodazole were replated in fresh medium. At the indicated time after release from M phase arrest, cells were pulse labeled with 5.8 × 10⁻¹⁴ cpm/well of [methyl-³H]thymidine for 1 h. Radioactivity incorporated into the trichloroacetic acid-insoluble fraction was counted.

20294 Biphase Activation of Two MAP Kinases
result was almost the same although kinase activities at time 0 were not detectable (Fig. 2A, lower panel). Since the band at time 0 in the crude lysate gel showed slightly faster mobility in duplicated experiments, this band may represent a distinct kinase. These results suggest that the 44- and 42-kDa kinase activities in crude lysate are the polypeptides recognized by this antisera.

**Immune Complex Kinase Assay-MAPK Activity in Comparison with cdc2 Kinase Activity**—The histone H1 kinase activity of immune complex precipitated with anti-MAPK C-terminal antiserum (αC92) was assayed at time 0 to check the contaminating cdc2 kinase activity. Although cdc2 kinase activity was very high at time 0, histone H1 kinase activity of the immune complex by anti-MAPK (αC92) was negligible (Fig. 2B), indicating that contamination with cdc2 kinase is very little, if any. Since αC92 precipitates the 44 kDa predominantly, the kinase activity detected in this assay is that of the 44-kDa MAPK. The MAPK activity had two broad peaks during the cell cycle; the first peak appeared at 2–6 h after release from the M phase, and the second peak appeared at 14–20 h after release (Fig. 2B). A summary of three independent experiments has revealed a biphasic activation of MAPK during the cell cycle (Fig. 2C). When cells were collected after release from S phase arrest, a peak of cdc2 kinase activity appeared at 5–7 h after release, and the MAPK activity seemed to be activated slightly earlier (data not shown). In contrast to cdc2 kinase, which declined rapidly to a negligible level after release, MAPK activity was detected through the cell cycle. The only exception was at time 0 when the activity of MAPK was at the least level.

It seems paradoxical that MAPK activities are very low in M phase-arrested cells and activated at around the second M phase. This phenomenon can be interpreted in two ways. By one hypothesis, MAPK is activated in the late M phase-arrested cells and activated at around the second M phase or in the early part of the M phase and then rapidly deactivated at the metaphase/anaphase transition of the M phase, at which point nocodazole-treated cells are arrested. An alternative hypothesis is that nocodazole per se interferes with MAPK.

To clarify which is the case, M phase cells were collected by mechanical shakeoff from CHO cells in the second M phase. After centrifugation, collected cells were solubilized as above and divided into two parts for immunoprecipitation with either antiserum αC92 or antisera against cdc2 kinase (αY21). Immunoprecipitated cdc2 kinase exhibited high histone H1 kinase activity; however, MAPK activity was very low (Fig. 2D). Since no nocodazole treatment was done in this experiment, the low activity of MAPK in nocodazole-arrested cells is not an artifact caused by the reagent.

**Amount of MAPKs during the Cell Cycle**—The amount of MAPK during the cell cycle was determined by Western blotting (Fig. 3). The protein concentrations of the cell lysates from each time points were adjusted and blotted with antisera αC92 or αY91. No significant change in the intensity

---

**Fig. 2. Activities of MAPK and cdc2 kinase during the cell cycle.** M phase-arrested cells were collected as in legend of Fig. 1. At the indicated time after release, cells were frozen in liquid nitrogen and kept at −70 °C until assay. Samples were treated as described under "Experimental Procedures." Panel A, gel phosphorylation assay. Upper panel, total cell lysates were analyzed; lower panel, samples immunoprecipitated with anti-ERK-1 antibody (αY91) were analyzed. Panel B, immune complex kinase assay. Samples were divided into two and immunoprecipitated either with anti-ERK-1 (αC92) or anti-cdc2 kinase antibodies. Immunoprecipitated kinase activities were analyzed using myelin basic protein (for MAPK) or histone H1 (for cdc2 kinase) as substrate. Panel C, densitometric scanning of three independent experiments as described in panel B. The optical densities of substrate bands were analyzed with a Bio-Rad model 620 video densitometer. The data from each experiment were normalized to the second peak level in the experiment. The average and the standard deviation are shown. Panel D, cells in metaphase were collected by mechanical shakeoff at several time points. Cells that remained on the dish, in which metaphase cells are decreased, were also collected. The protein concentration of the samples was adjusted before assay. Each sample was divided into two, and MAPK and cdc2 kinase were assayed as in B. M, cells in metaphase. R, cells remaining on the dish.
cells released from M phase arrest were incubated in medium with 1 µg/ml aphidicholin for 12 h and arrested in the S phase, then released from arrest by washing and replating with fresh medium. The cells were frozen at the indicated time after release and solubilized as above. Samples from each time points were blotted as described under "Experimental Procedures."

FIG. 3. Western blotting of MAPK during the cell cycle. The cells released from M phase arrest were incubated in medium with 1 µg/ml aphidicholin for 12 h and arrested in the S phase, then released from arrest by washing and replating with fresh medium. The cells were frozen at the indicated time after release and solubilized as above. Samples from each time points were blotted as described under "Experimental Procedures."

The phosphorylation state of MAPKs was analyzed by immunoprecipitation of MAPKs from cells labeled with 32P orthophosphate with or without nocodazole during the last 2 h of incubation at 37°C. Immunoprecipitates with anti-MAPK (αY91) were analyzed by two-dimensional gel electrophoresis. Left panel, M phase-arrested cells; right panel, 16 h after release from M phase arrest. Upper arrow, 44-kDa MAPK, lower arrow; 42-kDa MAPK. In isoelectric focusing (IEF), the left side is basic, and the right side is acidic.

of the bands was observed during the cell cycle.

Phosphorylation State of MAPK in the Cell Cycle—The phosphorylation state of the MAPKs was analyzed by immunoprecipitation of MAPKs from cells labeled with 32P in vivo. By two-dimensional gel electrophoresis, both 44- and 42-kDa phosphoproteins were resolved into two close spots (Fig. 4). The acidic spot contains phosphotyrosine and phosphothreonine, and the basic spot contains phosphotyrosine only (29). All four spots of MAPKs were detected in the G1, S, and G2/M phases. Only in nocodazole-arrested cells, phosphorylation of MAPKs was not detected (Fig. 4). Phosphopeptide mapping of the 44- and 42-kDa protein revealed that the phosphorylation pattern of these proteins in the M phase was very similar to that of the serum-stimulated proteins (data not shown).

DISCUSSION

In this study, two MAPK activities of 44 and 42 kDa were shown to be activated coordinately during the cell cycle in the gel phosphorylation assay. Although there are denaturation and renaturation procedures, this assay is at least semi-quantitative. The same pattern of activation was confirmed by an immune complex kinase assay at least for the 44-kDa MAPK. The two MAPKs were activated biphasically; the first peak was 2–6 h from release from the M phase, and the second was 14–20 h. Compared with the pattern of thymidine uptake and histone H1 kinase activity ofcdc2 kinase, the first peak was in the G1 phase, and the second peak was around the M phase. Although activities of MAPK were more apparent in the second peak, the level was lower compared with that of growth factor-stimulated MAPKs. The same pattern of activation was observed in the immune complex kinase assay. In contrast to cdc2 kinase whose activity is almost negligible during the G1 to G2 phase, MAPK activities of both 44- and 42-kDa proteins were clearly detected in the G1 and S phases, although the levels were lower compared with those in the G2 to M phase. In vivo labeling and two-dimensional electrophoresis revealed that phosphothreonine and phosphotyrosine are contained in both 44- and 42-kDa proteins throughout the cell cycle except for time 0 when the cells are arrested at metaphase by nocodazole treatment. Since phosphorylation of both threonine and tyrosine residues is believed to be required for the activation of MAPK, this result is consistent with the presence of activities of the kinases in the cell cycle except for time 0. In Xenopus oocytes, MAPKs are phosphorylated in meiosis (16–19). However, in nocodazole-arrested mammalian cells phosphorylation of the 42-kDa protein is not detectable (17). Our results are consistent with these results since the content of phosphotyrosine was least in the nocodazole-arrested cells. This phenomenon is not the result of a toxic effect of nocodazole because a similar result was obtained when metaphase cells were collected without nocodazole treatment. However, at 16 h after release from M phase arrest, clear phosphorylation of the tyrosine residue(s) is detectable. This result indicates that MAPKs are tyrosine-phosphorylated and activated at the G2/M transition or in the early part of the M phase. It seems likely that the mechanism for activation of MAPKs is almost the same in growth factor stimulation and in the M phase. In Xenopus oocytes, the time course of activation of histone H1 kinase (cdc2 kinase) and myelin basic protein kinase show periodic changes in a similar pattern, and the activation of cdc2 kinase appeared to precede the activation of myelin basic protein kinase (19). It is suggested from these data that MAPK is under control of cdc2 kinase. In our result, low but distinct MAPK activities are detected in the G1 and S phases, when cdc2 kinase activity is almost negligible. This result suggests that there could be an alternative mechanism(s) that controls activities of MAPKs during the cell cycle.

In cdc2 kinase, specific sites are phosphorylated in a cell cycle-dependent manner. It may be related to the complex formation of cdc2 with p13 and cyclinB, which is the prerequisite for the activation of cdc2 kinase in the M phase. In contrast to cdc2 kinase, MAPKs seems to have only two states: activated and deactivated. It seems that only the fractions of activated MAPKs to total MAPKs are changing in the cell cycle, and the MAPKs may be controlled under the dynamic balance of activator(s) and phosphatase(s). Activation of MAPK by okadaic acid (4) supports this hypothesis. Recently, Posada and Cooper (31) showed that kinase(s) that phosphorylate MAPK is (are) activated in the M phase of Xenopus oocytes. The identity of the activator(s) in the M phase remains to be clarified.

Recently MAPK in the Xenopus oocyte was reported to be activated in the M phase (18, 19) and to control microtubule reorganization in the cell cycle through phosphorylation of...
the microtubule-associated protein (19). MAPKs in the fibroblasts of the mammals are activated when quiescent cells are stimulated with a variety of growth factors (1–6, 20, 21) and are believed to be involved in the cascade of events resulting in the G<sub>2</sub> to G<sub>1</sub> transition (7–9). In this study, MAPKs were shown to be activated not only in the G<sub>2</sub>/G<sub>1</sub> transition but also in G<sub>1</sub> and in G<sub>2</sub>/M in mammalian somatic cells. It is probable that MAPKs are functioning in the G<sub>2</sub>/M transition or in the early M phase in a way similar to that in Xenopus oocytes.

**Acknowledgments**—We thank Dr. E. Nishida for many fruitful discussions and Drs. J. Maller and E. Erikson (University of Colorado) for critically reading the manuscript. We also thank Dr. Morrioka and personnel of the Radioisotope Center at the University of Tokyo, and Drs. T. Yamamoto and Y. Matsuzawa at the Institute of Medical Science, University of Tokyo, for support.

**Note Added in Proof**—After submission of this paper, three groups have reported purification of “MAPK activator” from mitogen-activated cells (Nakielny, S., Cohen, P., Wu, J., and Sturgill, T. (1992) *EMBO J.* 11, 2123–2129, Seger, R., Ahn, N. G., Munar, E. S., Jensen, A. M., Cooper, J. A., Cobb, M. H., and Krebs, E. G. (1992) *J. Biol. Chem.* 267, 14373–14381, or from Xenopus oocytes (Kosako, H., Gotoh, Y., Matsuda, S., Ishikawa, M., and Nishida, E. (1992) *EMBO J.* 11, 2903–2908). MAPK activator is a protein kinase that can phosphorylate inactive MAPK on threonine and tyrosine residues and reactivate it.

**REFERENCES**

1. Ray, L. B., and Sturgill, T. W. (1987) *Proc. Natl. Acad. Sci. U.S.A.* 84, 1502–1506
2. Ray, L. B., and Sturgill, T. W. (1988) *J. Biol. Chem.* 263, 12721–12727
3. Hoshi, M., Nishida, E., and Sakai, H. (1988) *J. Biol. Chem.* 263, 5386–5401
4. Ahn, N. G., Weisel, J. E., Chan, C. P., and Krebs, E. G. (1990) *J. Biol. Chem.* 265, 11487–11494
5. Gotoh, Y., Nishida, E., Yamashita, T., Hoshi, M., Kawakami, M., and Sakai, H. (1990) *Eur. J. Biochem.* 193, 661–669
6. Gotoh, Y., Nishida, E., and Sakai, H. (1990) *Eur. J. Biochem.* 193, 671–674
7. Sturgill, T. W., Ray, L. B., Erikson, E., and Maller, J. L. (1988) *Nature* 334, 715–718
8. Ahn, N. G., Seger, R., Bratlien, R. L., Diltz, C., Tonks, N. K., and Krebs, E. G. (1991) *J. Biol. Chem.* 266, 4290–4297
9. Ahn, N. G., and Krebs, E. G. (1990) *J. Biol. Chem.* 265, 11495–11501
10. Ray, L. B., and Sturgill, T. W. (1988) *Proc. Natl. Acad. Sci. U.S.A.* 85, 3753–3757
11. Anderson, N. G., Maller, J. L., Tonks, N. K., and Sturgill, T. W. (1990) *Nature* 343, 651–653
12. Cooper, J. A., Sefton, B. M., and Hunter, T. (1984) *Mol. Cell. Biol.* 4, 30–37
13. Boulton, T. G., Yancopoulos, G. D., Gregory, J. S., Slaughtier, C., Moorman, C., Hsu, J., and Cobb, M. H. (1990) *Science* 240, 64–67
14. Boulton, T. G., Nye, S. H., Robbins, D., Ip, N. Y., Radziejewska, E., Morgenbesser, S. D., DePinho, R. A., Panayotatos, N., Cobb, M., and Yancopoulos, G. D. (1991) *Cell* 65, 663–675
15. Sanghera, J. S., Padidon, H. B., Bader, S. A., and Pelech, S. L. (1990) *J. Biol. Chem.* 265, 52–57
16. Ferrell, J. E., Wu, M., Gerhart, J. C., and Martin, G. S. (1991) *Mol. Cell. Biol.* 11, 1965–1971
17. Cooper, J. A. (1989) *Mol. Cell. Biol.* 9, 3143–3147
18. Posada, J., Sanghera, J., Pelech, S., Aebi, H. B., and Cooper, J. A. (1991) *Mol. Cell. Biol.* 11, 2517–2525
19. Gotoh, Y., Nishida, E., Matsuda, S., Shiina, N., Kosako, H., Shikawa, K., Akiyama, T., Ohita, K., and Sakai, H. (1991) *Nature* 349, 251–254
20. Maller, J. L. (1990) *Biochemistry* 29, 3157–3166
21. Cobb, M. H., Boulton, T. G., and Robbins, D. J. (1991) *Cell Regul.* 2, 965–978
22. Tobey, R. A., Anderson, E. C., and Peterson, D. F. (1967) *J. Cell. Physiol.* 70, 63–68
23. Merla, A. O., Draetta, G., Beach, D., and Wang, Y. J. (1989) *Cell* 58, 193–205
24. Pines, J., and Hunter, T. (1989) *Cell* 58, 845–856
25. Tohe, K., Kadowaki, T., Tanemoto, H., Ikai, K., Hara, K., Koshin, O., Momomura, K., Gotoh, Y., Nishida, E., Akamura, Y., Yazaki, Y., and Kasuga M. (1991) *J. Biol. Chem.* 266, 24978–24983
26. Bradford, M. M. (1976) *Anal. Biochem.* 72, 248–254
27. Laemmli, U. K. (1970) *Nature* 227, 680–685
28. Kameshita, I., and Fujisawa, H. (1989) *Anal. Biochem.* 183, 139–143
29. Chatani, Y., Tanaka, E., Tohe, K., Hattori, A., Sato, M., Tanemoto, H., Nishizawa, N., Nomoto, H., Takayama, T., Kadowaki, T., Kasuga, M., and Kohno, M. (1992) *J. Biol. Chem.* 267, 9911–9916
30. Ando, A., Momomura, K., Tohe, K., Yamamoto-Honde, R., Sakura, H., Tamori, Y., Katamura, Y., Koshin, O., Akamura, Y., Yazaki, Y., Kasuga, M., and Kadowaki, T. (1992) *J. Biol. Chem.* 267, 12788–12796
31. Posada, J., and Cooper, J. A. (1992) *Science* 255, 212–215