Vol. 171, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 31, 1990 Pages 75-83

ACTIVATION OF A MANGANESE-DEPENDENT MEMBRANE PROTEIN KINASE BY SERINE AND TYROSINE PHOSPHORYLATION

Toolsee J. Singh

Department of Biology, University of Waterloo, Waterloo, Ontario, Canada N2L 3G1

Received July 9, 1990

SUMMARY: A Mn^{2+}-dependent serine/threonine protein kinase from rat liver membranes copurifies with the insulin receptor (IR) on wheat germ agglutinin (WGA)-sepharose. The kinase is present in a nonactivated form in membranes but can be activated 20-fold by phosphorylating the WGA-sepharose fraction with casein kinase-1 (CK-1), casein kinase-2 (CK-2), or casein kinase-3 (CK-3). The activated kinase can use IR β-subunit, myelin basic protein, and histones as substrates. Activation of the kinase seems to proceed by two or more steps. Sodium vanadate and Mn^{2+} are required in reaction mixtures for activation to be observed, whereas the tyrosine kinase-specific substrate , poly (glu, tyr), completely inhibits activation. These observations suggest that, in addition to serine/threonine phosphorylation by one of the casein kinases, activation of the Mn^{2+}-dependent protein kinase also requires tyrosine phosphorylation. Such phosphorylation may be catalyzed by the IR tyrosine kinase.

The insulin receptor (IR) is a tyrosine kinase that is activated by autophosphorylation when it binds insulin. Studies with IR kinase mutants have shown that the tyrosine kinase activity of the receptor is required to mediate different physiological effects of insulin (1). Presumably this occurs by the direct phosphorylation of key regulatory proteins by the IR tyrosine kinase. One or more of these target proteins may be serine/threonine protein kinases which would become activated. These activated kinases can then activate other serine kinases in turn.

Several serine protein kinases have been shown to be activated after insulin stimulation of cells. These include S6 kinase (2), casein kinase-1 (CK-1) (3), casein kinase-2 (CK-2) (4), glycogen synthase kinase-3 (5), microtubule-associated protein-2 (MAP-2) kinase (6) Mn^{2+}-dependent protein kinase (7), and membrane-bound serine kinases (8, 9). MAP-2 kinase (6) and one of the membrane-bound serine kinases(9) are apparently phosphorylated on tyrosine after insulin stimulation of cells.

In addition to being phosphorylated on tyrosine, IR has also been shown to be phosphorylated on serine and threonine in both intact cells and *in vitro* using partially purified IR (8). The significance of such serine/threonine phosphorylation of IR is not fully

0006-291X/90 $1.50

understood. However, after phorbal ester stimulation of hepatoma cells an increase in serine phosphorylation of IR was correlated with a decrease in IR tyrosine kinase activity (10). A similar decrease in tyrosine kinase activity was observed after the phosphorylation of IR in vitro with protein kinase C (11) cyclic AMP-dependent protein kinase (A-kinase) (12), and CK-2 (13).

The present study was undertaken with the aim of further evaluating the role of serine phosphorylation of IR on its tyrosine kinase activity. Towards this end, IR that was partially purified from rat liver membranes by wheat germ agglutinin (WGA)-sepharose was phosphorylated by CK-1, CK-2, and casein kinase-3 (CK-3) (14), and its subsequent autophosphorylation evaluated. Although IR (auto)phosphorylation was enhanced several fold, surprisingly, the phosphorylation of only serine and threonine, not tyrosine, was observed. Our results suggest that a Mn^{2+}-dependent serine/threonine protein kinase, present in a nonactivated form in membranes, is activated by tyrosine phosphorylation. The IR tyrosine kinase may be involved in this activation process.

EXPERIMENTAL PROCEDURES

MATERIALS: CK-1, CK-2, and CK-3 were prepared from bovine kidneys as described previously (14). The catalytic subunit of A-kinase, myelin basic protein, mixed histones (type 2A), porcine insulin, poly (glu, tyr), heparin, Triton X-100, and N-acetylglucosamine were purchased from Sigma. Wheat germ agglutinin (WGA)-sepharose was from Pharmacia whereas [γ-^{32}P] ATP was from ICN.

METHODS: To prepare membranes, rat liver (60 gm) was homogenized in 3 volumes Buffer A (25 mM Tris, pH 7.5, 0.25 M sucrose, 5 mM EDTA, 5 mM iodoacetamide, 10 mM benzamidine, 2 mM PMSF) at 4°. The homogenate was centrifuged at 12,000 x g for 30 min. The crude extract was then centrifuged at 100,000 x g for 1 hr. The microsomal pellet was washed 3 times with Buffer A and resuspended in Buffer A minus iodoacetamide at a final protein concentration of 10 mg/ml. The membranes were either used immediately or frozen at -70°. Triton X-100 (1%) was added to the membranes (100 mg) and the mixtures stirred slowly at 4° for 1 hr followed by centrifugation at 100,000 x g for 1 hr. The supernatant was applied slowly to a WGA-sepharose column (10 ml). The flow-through fraction was reapplied 3 times to the column. The latter was then washed with 10 bed volumes of Buffer B (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 10 mM benzamidine, 2 mM PMSF, 0.1% Triton X-100). The column was eluted with Buffer B containing 0.3 M N-acetylglucosamine. Fractions containing the highest protein content were pooled and diluted with Buffer B to reduce the concentration of N-acetylglucosamine to 10 mM. After concentration by ultrafiltration, IR was stored in small aliquots at -70°.

IR (0.30 mg.ml) was phosphorylated by serine kinases in a reaction mixture containing Tris-HCl (pH 7.5), 25 mM; 2 merceptoethanol, 10 mM; $MgCl_2$, 10 mM; EGTA, 0.5 mM; NaF, 5 mM; ATP, 0.1 mM; and kinases. Reactions were initiated with ATP at 30° and incubated for 30 min. The serine phosphorylated IR was then used immediately to study IR autophosphorylation. Sodium vanadate (0.1 mM) was added to the reaction mixtures. When required, insulin (10^{-6} M) was added and reaction mixtures incubated for 10 min at 30° to allow for binding of the hormone. Autophosphorylation of IR was initiated by addition of $MnCl_2$ (4 mM) and [γ-^{32}P] ATP. After a further 30 min at 30°, reactions were

quenched with SDS-stop buffer and analyzed by SDS-PAGE. Gels were stained, destained, and exposed to Kodak X-OMAT AR film for autoradiography. The β-subunit of IR was then excised from the gels and ^{32}P incorporation determined by Cerenkov counting. To phosphorylate exogenous substrates such as myelin basic protein (0.5 mg/ml) or mixed histones (0.5 mg/ml) IR was autophosphorylated in the presence of unlabelled ATP as described above. [γ-^{32}P] ATP was then added and the reactions initiated by the addition of the exogenous substrates. Aliquots of the reaction mixtures were removed at different times and the reactions quenched by SDS-stop buffer. ^{32}P-labelled proteins were separated by SDS-PAGE and processed as described above.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) was performed as described by Laemmli (15). Protein was determined by the method of Bradford (16) using bovine serum albumin as a standard. Phosphoamino acid analysis was performed as described previously (17).

RESULTS

To evaluate the role of serine phosphorylation of IR on its intrinsic tyrosine kinase activity, phosphorylation of IR by the casein kinases (Fig. 1) was studied. Reactions were carried out in the absence of Mn^{2+} (0.5 mM EGTA present) and insulin but in the presence of 10 mM Mg^{2+}. Under these conditions only minimal phosphorylation of IR β-subunit was observed in the absence of any added kinase (Fig. 1, lane 1). Addition of CK-1 (lane 2), CK-2 (lane 3), and CK-3 (lane 4) increased phosphorylation 6.7, 3.8, and 4.9-fold, respectively. In the presence of heparin (5 μg/ml) phosphorylation of the β-subunit catalyzed by the three kinases is inhibited 90%, 93% and 85%, respectively, compared to controls (lanes 5-7). Further, the inclusion of Mn^{2+} (1 mM) was also found to inhibit phosphorylation of the β-subunit by CK-1, CK-2, and CK-3 by 82%, 100% and 84%,

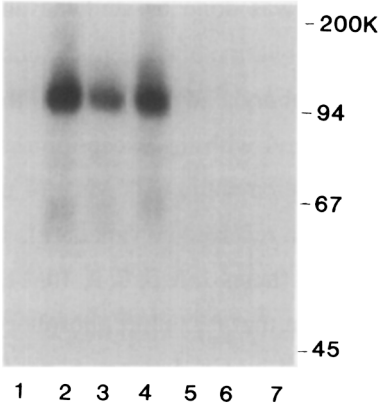
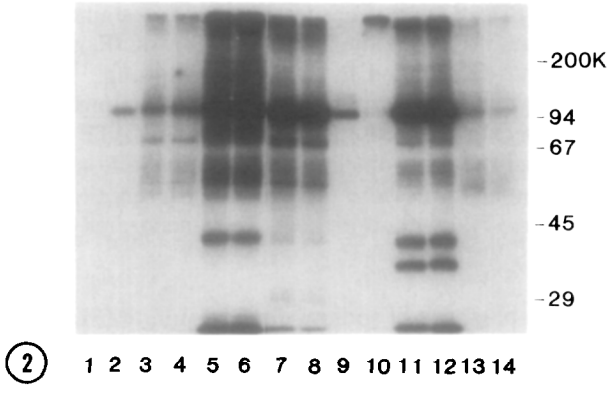


FIG. 1. Phosphorylation of IR by CK-1, CK-2, and CK-3. WGA-sepharose eluate (0.3 mg/ml) was phosphorylated in the absence of any added kinase (lane 1) or in the presence of 9 munits of CK-1 (lane 2), 6 munits CK-2 (lane 3), or 5 munits CK-3 (lane 4). Lanes 5, 6, and 7 are the same as lanes 2, 3, and 4, respectively, except that heparin (5 μg/ml) was present in phosphorylation mixtures.



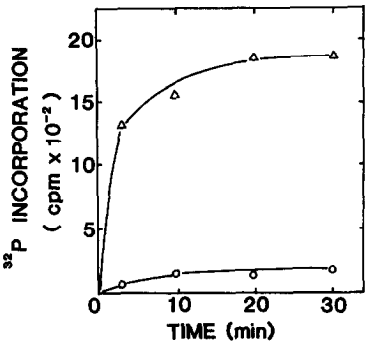


FIG. 2. Autophosphorylation of IR previously incubated in the absence and presence of different kinases. WGA-sepharose eluate (0.3 mg/ml) was phosphorylated in the absence of any added kinase (lanes 1-4) or in the presence of 9 munits of CK-1 (lanes 5-6), 6 munits CK-2 (lanes 7-10), 5 munits CK-3 (lanes 11-12), and 10 μg/ml of the catalytic subunit of A-kinase (lanes 13-14). Lanes 1 and 2 reaction mixtures also received no ATP in addition to no kinase. After 30 min at 30° autophosphorylation of IR was initiated as described in "Methods". Autophosphorylation of IR was carried out both in the absence (lanes 1, 3, 5, 7, 9, 10, 11, 13) and presence (lanes 2, 4, 6, 8, 12, 14) of 10^{-6}M insulin. Lane 9 contained no vanadate whereas lane 10 contained 0.6 mg/ml poly (glu, tyr).

FIG. 3. Activation of a Mn^{2+}-dependent myelin basic protein kinase by CK-1. WGA-sepharose eluate (0.3 mg/ml) was phosphorylated either in the absence (○) or presence (Δ) of 18 munits of CK-1. This step was then followed successively by an autophosphorylation step and the phosphorylation of myelin basic protein as described in "Methods".

respectively (not shown). The inhibition of CK-1, CK-2, and CK-3 by heparin and divalent metal ions using substrates other then IR was studied previously (14).

The consequence of serine/threonine phosphorylation of IR on its intrinsic tyrosine kinase activity was assessed. This was done by studying autophosphorylation of the β-subunit of IR (Fig. 2) or the phosphorylation of exogenous substrates (Fig. 3). To undertake these experiments IR was first phosphorylated for 30 min in the absence or presence of the different kinases as described in Fig. 1 with the exception that unlabelled ATP was used. All reaction mixtures were then supplemented with Mn^{2+} (4 mM) and vanadate (0.1 mM) (the exception in fig. 2 in lane 9 which received no vanadate). IR autophosphorylation (Fig. 2) was studied in either the absence (lanes 1, 3, 5, 7, 9, 10, 11, 13) or presence (lanes 2, 4, 6, 8, 12, 14) of insulin. It can be seen that β-subunit phosphorylation was greatly enhanced when IR was initially phosphorylated by CK-1 (lanes 5, 6), CK-2 (lanes 7, 8), and CK-3 (lanes 11, 12) compared to controls (minus kinase, lanes 3, 4). In the absence of insulin, β-subunit phosphorylation was enhanced 6.3, 7.5, and 6.5-fold as a result of prior phosphorylation by CK-1, CK-2, and CK-3, respectively. Since IR β-subunit was previously shown to be a substrate for A-kinase when very high concentrations of this kinase were used

TABLE I

Autophosphorylation of IR β-subunit under different conditions. WGA-sepharose eluate (0.3 mg/ml) was phosphorylated by CK-2 (6 munits) in the presence of 0.1 mM ATP and 10 mM Mg^{2+} for 30 min at 30°. Mn^{2+} (4 mM), vanadate (0.1 mM), and [γ-^{32}P] ATP were then added and the incubation continued for a further 30 min. The reaction mixtures were analyzed by SDS-PAGE. The β-subunit of IR was excised from the gel and ^{32}P-incorporation determined by Cerenkov counting

Autophosphorylation condition	β-Subunit phosphorylation
standard (10 mM Mg^{2+}, 4 mM Mn^{2+}, 0.1 mM vanadate)	100
- Mg^{2+}	112
- Mn^{2+}	15
- vanadate	17
- Mg^{2+} - Mn^{2+} + 4 mM EDTA	0
+ poly (glu, tyr) (0.6 mg/ml)	6
+ MAP-2 (0.4 mg/ml)	24

(12) we have also evaluated prior phosphorylation of IR by A-kinase on its subsequent autophosphorylation (lanes 13, 14). No enhancement of IR autophosphorylation was found.

Autophosphorylation of IR previously phosphorylated by CK-2 was further characterized (Table I). The reaction is highly specific for Mn^{2+}: less than 15% activity is observed with 10 mM Mg^{2+} compared with 4 mM Mn^{2+}. Activity with both Mg^{2+} and Mn^{2+}

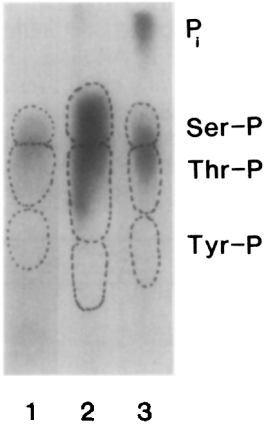


FIG. 4. Phosphoamino acid analysis of phosphorylated IR and myelin basic protein. WGA-sepharose eluate was phosphorylated by CK-1 (lane 1) and the reaction quenched with SDS-stop buffer. Alternatively, this step was followed by IR autophosphorylation (lane 2) and the phosphorylation of myelin basic protein (lane 3). ^{32}P-labelled IR β-subunit (lanes 1 and 2) and myelin basic protein (lane 3) were excised from SDS-polyacrylamide gels, the proteins eluted, and phosphoamino acid analysis performed. Dotted lines mark the positions of ser-P, thr-P and tyr-P standards as located by ninhydrin staining.

was less than that observed with Mn^{2+} alone. In the absence of the phosphotyrosine protein phosphatase inhibitor, vanadate, the level of autophosphorylation observed is the same as minus kinase controls (compare lanes 3 and 9 in Fig. 2). The tyrosine kinase specific substrate, poly (glu, tyr), inhibits β-subunit autophosphorylation by 94% (Table 1 and Fig. 2, lanes 7 and 10). Similarly MAP-2, which has been shown to be a substrate for IR tyrosine kinase (18) as well as a serine kinase (6), also inhibits β-subunit phosphorylation. These results suggest that β-subunit autophosphorylation (which occurs on serine/threonine, see Fig. 4 below) requires the participation of a tyrosine kinase, possibly IR tyrosine kinase.

The effect of insulin on IR autophosphorylation was evaluated (Table II). It is well known that insulin stimulates IR autophosphorylation (10). This observation is readily reproduced here. A 7.5-fold stimulation of β-subunit autophosphorylation by insulin was observed with insulin for IR that was not phosphorylated on serine/threonine prior to autophosphorylation (Fig. 2, lanes 1, 2). Prior incubation of IR in the presence of ATP alone (no added kinase) decreased the stimulation with insulin to only 1.2-fold (Table II and Fig. 2, lanes 3, 4). Similarly, autophosphorylation of IR previously phosphorylated by CK-1, CK-2, CK-3, and A-kinase was only minimally or not stimulated by insulin (Table II and Fig. 2).

The phosphorylation of exogenous substrates by the IR preparation was examined. A preliminary survey of different proteins indicated that, in addition to IR β-subunit, myelin

TABLE II

Effect of insulin on autophosphorylation of IR β-subunit. IR was incubated either in the absence of any added kinase or in the presence of 9 munits of CK-1, 6 munits CK-2, 5 munits CK-3, and 10 μg/ml catalytic subunit of A-kinase. Mg^{2+} and ATP were present at 10 mM and 0.1 mM, respectively. After 30 min at 30°, insulin (10^{-6} M) and vanadate (0.1 mM) were added and the incubation continued for 10 min to allow for insulin binding to IR. Autophosphorylation of IR was initiated by addition of 4 mM Mn^{2+} and [γ-^{32}P] ATP. After 30 min, reactions were quenched and ^{32}P-incorporation into the β-subunit of IR analyzed as in "Methods"

Activating kinase	β-Subunit phosphorylation (+/- insulin)[a]
- kinase - ATP[b]	4.48
- kinase + ATP[b]	1.20
+ CK-1	1.20
+ CK-2	0.96
+ CK-3	0.92
+ A-kinase	0.92

[a]Values shown are the average of four experiments.

[b]The presence or absence of ATP refers only to the activating step. All reactions contain 60 μM ATP in the subsequent autophosphorylation step.

basic protein and mixed histones were also good substrates for the activated kinase(s). Myelin basic protein was phosphorylated by IR preparation that was previously incubated in the presence or absence of CK-1 (Fig. 3). It can be seen that prior activation of kinase(s) by CK-1 is clearly required to observe appreciable phosphorylation of myelin basic protein. A 20-fold increase in the rate of phosphorylation of myelin basic protein as a result of prior activation by CK-1 is observed (Fig. 3). In a similar experiment a 1.6-fold activation was observed using mixed histones as a substrate (not shown).

The amino acids phosphorylated on the β-subunit of IR and on myelin basic protein were analyzed (Fig. 4). CK-1 phosphorylated the β-subunit on both serine and threonine (lane 1). Similar results were obtained with CK-2 and CK-3 (not shown). Surprisingly, CK-1-activated phosphorylation of the β-subunit (Fig. 2, lane 5) and of myelin basic protein (Fig. 3) occurred exclusively on serine and threonine (Fig. 4, lanes 2 and 3). Similar results were obtained when either CK-2 or CK-3-activated IR preparation autophosphorylated its β-subunit (Fig. 2, lanes 7 and 11) or myelin basic protein (not shown). These results indicate that prior incubation of the IR preparation with CK-1, CK-2 or CK-3 is required to activate a Mn^{2+}-dependent serine/threonine protein kinase which can then phosphorylate the β-subunit of IR or myelin basic protein.

DISCUSSION

In this study, we have identified a protein kinase that can phosphorylate IR, like CK-1, CK-2, and CK-3, on serine and threonine. The kinase is present in membranes in a nonactivated form. Its activation is a complex process that may involve multiple steps and both serine and tyrosine kinases. Besides IR, the kinase can also phosphorylate myelin basic protein, histones, and possibly MAP-2. With both IR and myelin basic protein as substrates, the kinase prefers Mn^{2+} over Mg^{2+} as a metal cofactor. Like IR, this Mn^{2+}-dependent protein kinase binds to WGA-sepharose and hence may be a glycoprotein.

Activation of the Mn^{2+}-dependent protein kinase is via a cascade comprised of two or more steps. This cascade can be initiated by the addition of one of the casein kinases (CK-1, CK-2 or CK-3) in the presence of ATP/Mg^{2+} to the glycoprotein fraction from WGA-sepharose. A-kinase is ineffective in initiating activation of the kinase. Another step in the activation process apparently involves a tyrosine kinase. Three observations support such a hypothesis. First, sodium vanadate is required in reaction mixtures for activation to be observed (Fig. 2, lane 9). Vanadate is a well known inhibitor of phosphotyrosine protein phosphatases. Hence its presence is required to prevent these phosphatases (present in the glycoprotein fraction from WGA-sepharose) from dephosphorylating phosphotyrosyl proteins that are required in the activation process. Second, the tyrosine kinase specific substrate,

poly (glu, tyr), inhibits activation (Fig. 2, lane 10). It probably acts as a competitive inhibitor of the tyrosine kinase thereby preventing the phosphorylation on tyrosine of proteins involved in the activation process. Third, Mn^{2+} is apparently required in the activation process itself, in addition to being required as a cofactor for the activated serine/threonine kinase. It is well known that for all tyrosine kinases Mn^{2+} is a better cofactor than Mg^{2+}. Although the identity of the tyrosine kinase involved in the activation process remains unknown a likely candidate is the IR tyrosine kinase.

The exact details of the activation of the Mn^{2+}-dependent protein kinase remains unknown presently. A likely scenario, however, is that the casein kinases are required to activate the tyrosine kinase. The activated tyrosine kinase may in turn directly phosphorylate the Mn^{2+}-dependent protein kinase thereby activating it. Alternatively, the tyrosine kinase may be required at a more distal step leading to the activation of the Mn^{2+}-dependent protein kinase. Although we have demonstrated in this study that IR β-subunit is phosphorylated by CK-1, CK-2, and CK-3 (Fig. 1) evidence that such phosphorylation leads to activation of the intrinsic IR tyrosine kinase is presently lacking.

Besides the Mn^{2+}-dependent protein kinase reported here, direct tyrosine phosphorylation has been implicated in the activation of two other serine/threonine kinases. A MAP-2 kinase was isolated from the cytosol of 3T3-L1 adipocytes and shown to be phosphorylated on tyrosine (6). A membrane-bound serine kinase that could phosphorylate histone and kemptide was activated after insulin treatment of rat adipocytes (9). A fraction (22%) of this kinase was found to be adsorbed by antiphosphotyrosine antibodies coupled to agarose. Phosphotyrosine, but not phosphoserine or phosphothreonine, was found to inhibit adsorption of the kinase to the affinity column suggesting that the kinase was phosphorylated on tyrosine (9). The identities of the tyrosine kinases that phosphorylated the MAP-2 kinase and the membrane kinase remain unknown; the IR tyrosine kinase was suggested as a likely candidate. Since MAP-2 kinase is found in the cytosol of cells and utilizes Mg^{2+} (6) it is not too likely that it may be identical to the Mn^{2+}-dependent protein kinase studied here. On the other hand it is possible that our kinase may be similar or identical to the membrane-bound serine kinase identified in rat adipocytes (9). Further studies are required to resolve this issue.

ACKNOWLEDGMENTS

This work was supported by a grant from the Natural Sciences and Engineering Research Council (NSERC) of Canada. T.J.S. is an NSERC University Research Fellow. I would like to thank Shelley Stobo for expert secretarial assistance.

REFERENCES

1. Zick, Y. (1989) in Critical Reviews in Biochemistry and Molecular Biology. (G.D. Fasman, ed.), Vol. 24, pp. 217-269. CRC Press, Boca Ratin, Florida.
2. Cobb, M. (1986) J. Biol. Chem. 261, 12994-12999.
3. Cobb, M.H., and Rosen, O.M. (1983) J. Biol. Chem. 258, 12472-12481.
4. Sommercorn, J., Mulligan, J.A., Lozeman, F.J., and Krebs, E.G. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 8834-8838.
5. Yang, S.D., Ho, L.T., and Fung, T.J. (1988) Biochem. Biophys. Res. Commun. 151, 61-69.
6. Ray, L.B., and Sturgill, T.W. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 3753-3757.
7. Yu, K.-T., Khalaf, N., and Czech, M.P. (1987) J. Biol. Chem. 262, 16677-16685.
8. Lewis, R.E., Wu, G.P., MacDonald, R.G., and Czech, M.P. (1990) J. Biol. Chem. 265, 947-954.
9. Yu, K.-T., Khalaf, N., and Czech, M.P. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 3972-3976.
10. Takayama, S., White, M.F., and Kahn, C.R. (1988) J. Biol. Chem. 263, 3440-3447.
11. Bollag, G.E., Roth, R.A., Beaudoin, J., Mochly-Rosen, D., and Koshland, Jr., D.E. (1986) Proc. Natl. Acad. U.S.A. 83, 5822-5824.
12. Roth, R.A., and Beaudoin, J. (1987) Diabetes 36, 123-126.
13. Grande, J., Perez, M., and Itarte, E. (1988) FEBS Lett. 232, 130-134.
14. Singh, T.J. (1989) FEBS Lett. 243, 289-292.
15. Laemmli, U.K. (1970) Nature (London) 227, 680-685.
16. Bradford, M.M. (1976) Anal. Biochem. 72, 248-254.
17. Hunter, T., and Sefton, B.M. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 1311-1315.
18. Kadowaki, T., Fujita-Yamaguchi, Y., Nishida, E., Takaku, F., Akiyama, T., Kathuria, S., Akanuma, Y., and Kasuga, M. (1985) J. Biol. Chem. 260, 4016-4020.