Vol. 205, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1994 Pages 857-863

EPIDERMAL GROWTH FACTOR TRIGGERS THE TRANSLOCATION OF INSULIN-RESPONSIVE GLUCOSE TRANSPORTER (GLUT4)

Kazuo Ishii, Seika Kamohara, Hideki Hayashi, Mikio Todaka[#], Fumihiko Kanai[##,*]
Takanobu Imanaka and Yousuke Ebina[¶]

Department of Enzyme Genetics, Institute for Enzyme Research, The University of Tokushima, 3-18-15 Kuramoto-cho, Tokushima 770, Japan

[##]The Second Department of Internal Medicine, Faculty of Medicine, University of Tokyo, 7-3-1 Tokyo 113,Japan

Received October 18, 1994

In a novel cell line we developed for direct, sensitive detection of insulin-responsive glucose transporter (GLUT4) on the cell surface, we considered that insulin-activated phosphatidylinositol 3-kinase (PI 3-kinase) may be involved in the signaling pathway of insulin-stimulated GLUT4 translocation.

We report here evidence that epidermal growth factor (EGF), which stimulates PI 3-kinase activity, also triggers GLUT4 translocation in Chinese hamster ovary (CHO) cells stably overexpressing the EGF receptor. The EGF-dependent GLUT4 translocation is possibly mediated by two independent pathways: one by PI 3-kinase and the other by protein kinase C (PKC); the PI 3-kinase-mediated pathway predominates. Triggering of the GLUT4 translocation is not specific for insulin, rather it may be a common property of growth factors which activate PI 3-kinase.

One of the major physiological effects of insulin is stimulation of glucose uptake in target cells (1), and any defect may be one major cause of non-insulin dependent diabetes mellitus (2). The glucose uptake in target tissues is due mostly to translocation of GLUT4 from an intracellular site to the plasma membrane (3-6). The molecular mechanism by which insulin induces this translocation of GLUT4 to the plasma membrane is not well understood. We earlier developed a simple, direct and sensitive method to detect GLUT4

[#]Present address: Department of Metabolic Medicine, Kumamoto University Medical School, 1-1-1 Honjo, Kumamoto 860, Japan.

[*]JSPS Research Fellow.

[¶] To whom correspondence should be addressed. Fax: 0886-33-1845.

Abbreviations: GLUT4, insulin-responsive glucose transporter; PI 3-kinase, phosphatidylinositol 3-kinase; EGF, epidermal growth factor; EGF•R, EGF receptor; CHO, Chinese hamster ovary; αPY, anti-phosphotyrosine; PKC, protein kinase C; PDBu, phorbol 12, 13-dibutyrate; PMA, phorbol 12-myristate 13-acetate; PLCγ, phospholipase Cγ; PDGF, platelet-derived growth factor.

0006-291X/94 $5.00

immunologically on the cell surface (7). cDNA containing GLUT4 with an insert of a c-myc epitope (14 amino acids) in the first ectodomain (GLUT4myc) was constructed. The chimeric GLUT4myc was stably expressed in 3T3-L1 adipocytes (3T3-L1-GLUT4myc) and in Chinese hamster ovary (CHO) cells (CHO-GLUT4myc). The translocation of GLUT4myc to the cell surface could be detected in 3T3-L1 adipocytes by binding assay with an anti-c-myc antibody, by immunoblot of subcellular fractions from the adipocytes with the anti-myc antibody, and by the stimulation of glucose uptake (7). The chimeric GLUT4myc is translocated to the cell surface in the same manner as GLUT4 (7). Although CHO cells do not express GLUT4 physiologically, the exogenously-expressed GLUT4myc is translocated to the cell surface, by insulin stimulation and this translocation was detected by binding assay with the anti-c-myc antibody and by the stimulation glucose uptake. The degree of the insulin-stimulated GLUT4myc translocation in CHO cells is lower than in the adipocytes, the physiological insulin-target cells. However, CHO cells seem to possess basic machinery for the translocation of exogenously-expressed GLUT4myc which mimics that of adipocytes, for the following reasons : 1) Exogenously-expressed GLUT4myc and GLUT1myc showed different intracellular distributions, and the GLUT4myc was translocated to the cell surface in response to insulin more efficiently than was GLUT1myc in CHO cells[1], as noted in adipocytes (8-11). 2) PMA, NaF and GTPγS besides insulin, which are able to trigger GLUT4 translocation in adipocytes (12-15), also trigger GLUT4myc translocation in CHO cells (7). 3) Wortmannin, an inhibitor of phosphatidylinositol 3-kinase (PI 3-kinase) inhibits PI 3-kinase activation, GLUT4myc translocation, and glucose uptake in response to insulin in our cell system (16) and in 3T3-L1 adipocytes (17). Therefore, our cell system (CHO-GLUT4myc) is useful to study mechanism related to GLUT4 translocation. The activation of PI 3-kinase may be involved in the signaling pathway of insulin-stimulated GLUT4 translocation. As epidermal growth factor (EGF) activates PI 3-kinase in mammalian cells (18,19), we asked whether EGF triggers GLUT4 translocation by PI 3-kinase activation. We report here evidence that GLUT4myc translocates in the CHO cell system, in response to EGF.

MATERIALS AND METHODS

Cells and materials — The parent cell line used in this study was CHO-GLUT4myc; Chinese hamster ovary (CHO) cells overexpressing the GLUT4myc were constructed by inserting the human c-myc epitope (14 amino acids) into the first ectodomain of GLUT4 (7). A mouse monoclonal antibody against human c-myc (9E10) was purchased from Oncogene Science, Inc. Wortmannin was purchased from Sigma. ^{125}I-labeled EGF, ^{125}I-labeled goat anti-mouse IgG and [γ-^{32}P]ATP were purchased from Amersham. A mammalian expression vector pCXN (20) was provided by Dr. J. Miyazaki, (University of Tokyo). All other reagents from commercial sources were of analytical grade.

Establishment of CHO-GLUT4myc cells overexpressing EGF receptors — The parent cells (CHO-GLUT4myc) were co-transfected with a mouse EGF receptor expression plasmid pCXN-EGF•R (20,21) and the hygromycin B phosphotransferase expression plasmid pSV2-

[1]Ishii, K., *et al.* under submission.

hph (22). The candidate clones were selected with hygromycin B. The number of wild type EGF•R was identified by the binding of ^{125}I-labeled EGF and by immunoblot using an anti-EGF•R antibody.

Immunoblot — Confluent CHO-GLUT4myc-EGF•R cells were treated with or without 50 ng/ml EGF for 10 min at 37° C and lysed in the buffer (140 mM NaCl, 20 mM Tris, pH8.0, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM DTT (dithiothreitol), 10% glycerol, 20 μM (p-amidinophenyl)methanesulphonyl fluoride hydrochloride, and 1% NP40). The cell lysates (30 μg each) were separated by SDS-PAGE, transferred onto nitrocellulose paper, followed by blocking with 3% bovine serum albumin and 1% ovalbumin, incubated overnight with an anti-phosphotyrosine (αPY) antibody PY20 (1:1,000 dilution). Protein bands were located by autoradiography after treatment with ^{125}I-labeled goat anti-mouse IgG antibody (23).

PI 3-kinase assay — The cell lysates (100 μg each) were immunoprecipitated with the αPY antibody and protein A-Sepharose. The immunoprecipitates were washed twice with buffer (i) 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 % NP40 and 1 mM DTT, and three times with buffer (ii)10 mM Tris-HCl, pH 7.4, 100 mM NaCl and 1 mM DTT, and subjected to the PI 3-kinase assay as described previously (23). We confirmed that the PI 3-kinase activity associated with EGF•R was specifically inhibited with 0.5% NP40.

Cell surface anti-c-myc antibody binding assay (GLUT4myc translocation) — CHO-GLUT4myc-EGF•R cells were grown overnight in 24-well plates and the subconfluent cells were incubated in 1 ml of KRH buffer (136 mM NaCl, 4.7 mM KCl, 1.25 mM $MgSO_4$, 1.25 mM $CaCl_2$, 20 mM Hepes, 2 mg/ml bovine serum albumin) for 30 min at 37° C, and then in 300 μl of KRH buffer with the indicated concentrations of EGF for 30 min at 37° C. They were then incubated with 300 μl of the anti-c-myc antibody, 9E10 (1:2,000 dilution) for 2 h on ice, washed with ice-cold KRH buffer, and incubated with 300 μl of ^{125}I-goat anti-mouse IgG (1: 300 dilution) for 2 h on ice. The wells were then washed four times with ice-cold KRH buffer. Bound ^{125}I-goat anti-mouse IgG was solubilized with 0.05 % SDS and radioactivity was determined in a γ-counter (7).

Down-regulation of PKC by PDBu and wortmannin treatment — CHO-GLUT4myc-EGF•R cells were grown overnight in 24-well plates and the subconfluent cells were incubated in the absence or presence of 100 ng/ml phorbol 12, 13-dibutyrate (PDBu) for 20 h at 37° C in medium for PKC down-regulation (24,25). For wortmannin treatment, the cells were incubated with or without 0.1 μM wortmannin for 30 min at 37° C. The cells were then incubated for 30 min at 37° C with 1 μM phorbol 12-myristate 13-acetate (PMA), 10^{-7} M insulin, 50 ng/ml EGF, or buffer alone for 30 min at 37° C. GLUT4myc translocation was determined by anti-c-myc antibody binding assay, as described above.

2-Deoxyglucose uptake measurements — Cells in 24-well plates were treated with or without 50 ng/ml EGF for 30 min and incubated with 0.1 mM 2-deoxy-D-[1,2-^{3}H]glucose (Du Pont-New England Nuclear) for 10 min at 37° C. Cells were washed, solubilized and the radioactivity was measured (7).

RESULTS AND DISCUSSION

PI 3-kinase activation and GLUT4myc translocation by EGF — To examine the effect of EGF on GLUT4 translocation, we used CHO cells stably expressing GLUT4myc which was inserted with a c-myc epitope (14 amino acids) in the first ectodomain of GLUT4, without disrupting GLUT4 function (7). When EGF receptors (EGF•R) were stably overexpressed in the parent CHO-GLUT4myc cells, EGF stimulated autophosphorylation of the overexpressed-EGF•R (Fig. 1A), and activated PI 3-kinase in the precipitates with an anti-phosphotyrosine (αPY) antibody (Fig. 1B) (18,19). There is controversy concerning the activation of PI 3-kinase by EGF (18,19,27,28). Since PI 3-kinase was more readily

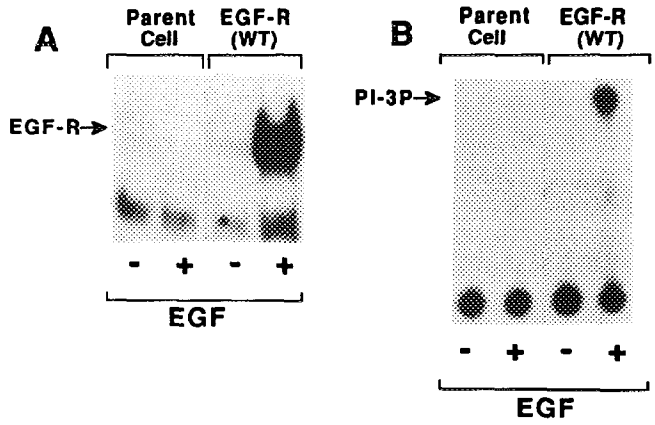

Fig. 1. EGF-dependent receptor autophosphorylation (A) and PI 3-kinase activation (B) in CHO-GLUT4myc and CHO-GLUT4myc-EGF•R cells. (A) EGF-dependent autophosphorylation of EGF•R. Quiescent CHO-GLUT4myc parent cells and those overexpressing EGF•R (CHO-GLUT4myc-EGF•R) were treated with 50 ng/ml EGF for 10 min. The cell lysates (30 μg each) were analyzed by immunoblotting with an anti-phosphotyrosine (pTyr) antibody, as described under MATERIALS AND METHODS. The arrow indicates the migrating position of EGF•R. (B) PI 3-kinase activity in anti-pTyr immunoprecipitates. The same lysates (100 μg each) as for (A) were immunoprecipitated with the anti-pTyr antibody, and subjected to PI 3-kinase assay, as described under MATERIALS AND METHODS. The arrow indicates the migrating position of PI 3-phosphate in thin-layer chromatography.

dissociated from EGF•R than from other receptors (for example PDGF receptor) *in vitro*, we washed the immunoprecipitate with αPY antibody, under mild conditions as described in "MATERIALS AND METHODS". This facilitated detection of activation of PI 3-kinase by EGF (Fig. 1B). The translocation of GLUT4myc was triggered by 1 ng/ml of EGF in cells stably overexpressing EGF•R (Fig. 2A).

We analyzed several independent CHO-GLUT4myc clones stably overexpressing EGF•R, and the results were much the same as shown in Figs. 1 and 2A. The EGF-triggered GLUT4myc translocation is not caused by co-migration with endogenous GLUT1 because the intracellular distribution and the degree of translocation of GLUT4myc differed from those of GLUT1, as based on analyses of exogenously-expressed GLUT4myc and GLUT1myc in CHO cells[1].

Stimulation of glucose uptake by EGF — To examine whether the GLUT4myc translocated by EGF contributes to glucose uptake by cells, we establised several clones of CHO cells expressing EGF•R (CHO-EGF•R) or both GLUT4myc and EGF•R (CHO-GLUT4myc-EGF•R), respectively. The clones with almost the same number of EGF•R were analyzed, using ^{125}I-EGF binding and immunoblotting and an anti-EGF•R antibody. Fig. 2B shows EGF-dependent glucose uptake in one of the typical clones. The translocated GLUT4myc on the cell surface in CHO-GLUT4myc-EGF•R resulted in a greater glucose uptake in response to EGF, compared to CHO-EGF•R. In addition, the EGF-stimulated

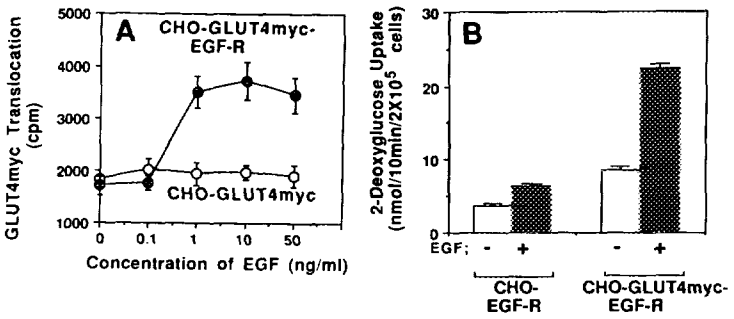

Fig. 2. Translocation of GLUT4myc (A) and 2-deoxyglucose uptake in CHO-EGF•R (CHO cells overexpressing EGF•R) and CHO-GLUT4myc-EGF•R cells (B). (A) The CHO-GLUT4myc parent cells (o) and those overexpressing EGF•R (•) were treated with various concentrations of EGF for 30 min. The amount of cell surface GLUT4myc was determined by anti-c-myc antibody binding assay, as described under MATERIALS AND METHODS. Values are means ± S.E. for three separate experiments done in triplicate. (B) Both cell lines had almost the same number of EGF•R and were treated with (+) or without 50 ng/ml EGF for 30 min. 2-Deoxyglucose uptake assay was done, as described under MATERIALS AND METHODS. Values are means ± S.E. of three determinations.

glucose uptake in CHO-GLUT4myc-EGF•R cells increased according to levels of expression of GLUT4myc[2]. Therefore, EGF triggers GLUT4myc translocation and stimulates glucose uptake by the translocated GLUT4myc (Fig. 2A, B).

Effects of phorbol 12, 13-dibutyrate (PDBu) and wortmannin on EGF-stimulated GLUT4myc translocation — PMA, which activates PKC (24,25), stimulates GLUT4myc translocation in CHO cells(7) and 3T3 L1 adipocytes (12,13). Since EGF activates PI 3-kinase (Fig. 1 B) (18,19) and PKC by stimulating PI-turnover(24-26), these two pathways may be involved in the GLUT4myc translocation by EGF. To examine the involvement of PI 3-kinase and PKC in the EGF-stimulated GLUT4myc, we used a PI 3-kinase inhibitor, wortmannin, and PDBu which down-regulates PKC (24,25). We examined GLUT4myc translocation by EGF following treatment with PDBu or (and) wortmannin (Fig. 3). As shown in Fig. 3A, the PDBu pretreatment almost completely inhibited the PMA-dependent GLUT4myc translocation, but only slightly inhibited EGF-dependent GLUT4myc translocation. This suggests that EGF-dependent PKC activation might be involved in the minor pathway for the GLUT4myc translocation. In addition, this finding indicated the existence of other pathway(s) besides PKC in EGF-dependent translocation. The PI 3-kinase inhibitor, wortmannin, completely inhibited insulin-dependent GLUT4myc translocation, and inhibited the EGF-dependent GLUT4myc translocation (Fig. 3B). However, the inhibition of EGF-dependent GLUT4myc by wortmannin was incomplete. The insulin-dependent GLUT4myc translocation may be mediated through PI 3-kinase activation only (7), and that the EGF-dependent GLUT4myc translocation was mainly mediated by PI 3-kinase activation and partially by other pathway(s). To confirm the presence of pathway(s) than PI 3-kinase in

[2]Kamohara, S., *et al.* unpublished observation.

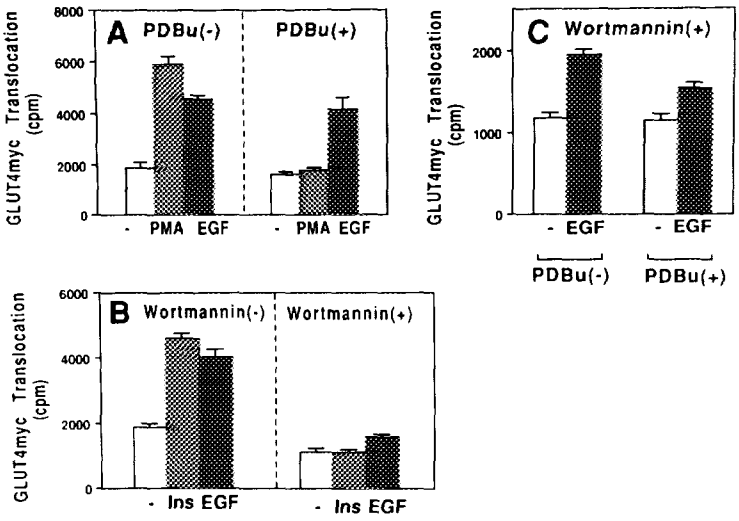

Fig. 3. Effects of PDBu (A), and wortmannin (B) and both (C) on EGF-stimulated GLUT4myc translocation in CHO-GLUT4myc-EGF•R cells. (A) PMA-, or EGF-stimulated GLUT4myc translocation after down-regulation of PKC by PDBu pretreatment. Cells stably overexpressing EGF•R (CHO-GLUT4myc-EGF•R) were treated with 0.1 μM PMA, 50 ng/ml EGF or buffer alone (-) for 30 min at 37° C, after pretreatment with 100 ng/ml PDBu (phorbol 12,13-dibutyrate) or medium alone (-) for 20 h at 37° C. The amount of cell surface GLUT4myc was determined. Values are means ± S.E. for three separate experiments done in triplicate. (B) Insulin-, or EGF-stimulated GLUT4myc translocation after pretreatment of a PI 3-kinase inhibitor, wortmannin. CHO-GLUT4myc-EGF•R cells were pretreated with 0.1 μM wortmannin or with buffer alone (-) for 30 min at 37° C. The cells were treated with 10^{-7} M insulin, 50 ng/ml EGF or buffer alone (-) for 30 min at 37° C, and the amount of cell surface GLUT4myc was determined by anti-c-myc antibody binding assay. Values are means ± S.E. for three separate experiments done in triplicate. (C) EGF-stimulated GLUT4myc translocation after pretreatment of both PDBu and wortmannin. CHO-GLUT4myc-EGF•R cells were pretreated with 100 ng/ml PDBu or medium alone (-) for 20 h at 37° C and treated with 0.1 μM wortmannin for 30 min at 37° C. The cells were treated with 50 ng/ml EGF or buffer alone (-) for 30 min at 37° C, and the amount of cell surface GLUT4myc was determined by anti-c-myc antibody binding assay. Values are means ± S.E. for three separate experiments done in triplicate.

the EGF-dependent GLUT4myc translocation, we examined the effects of PDBu pretreatment on the translocation in the presence of wortmannin (Fig. 3C). PDBu pretreatment decreased the EGF-dependent GLUT4myc translocation in the presence of wortmannin. This means that PKC activated by EGF-dependent PI turnover, mediated the EGF-dependent GLUT4myc translocation as well as PI 3-kinase, and that the PI 3-kinase-mediated pathway predominates over the PKC-mediated pathway. However, the possibility that other pathway(s) besides PI 3-kinase and PKC are involved in the EGF-dependent GLUT4myc translocation will need to be ruled out, because pretreatment with both PDBu and wortmannin did not completely inhibit the EGF-dependent GLUT4myc translocation (Fig. 3C).

We also observed that platelet-derived growth factor (PDGF), which activates PI 3-kinase, triggered GLUT4myc translocation in CHO-GLUT4myc cells stably overexpressing PDGF receptors, and that the pathway of the PDGF-stimulated GLUT4myc translocation

mediated by PI 3-kinase predominated over that mediated by PLCγ and PKC[3]. Thus, triggering of the insulin-responsive glucose transporter (GLUT4) translocation is not specific for insulin, rather it may be a common property of growth factors which activate PI 3-kinase.

ACKNOWLEDGMENTS. We thank T. Akiyama and S. Ishii for providing experimental materials, and M. Ohara for reading the manuscript. This work was supported by research grants from the Ministry of Education, Science, and Culture of Japan, by a grant for diabetes research from the Ministry of Health and Welfare, by the Foundation for Health Science (Japan), and by a grant for diabetes research from Otsuka Pharmaceutical Co., Ltd. Tokushima, Japan (to Y. E.).

REFERENCES

1. White, M. F., and Kahn, C. R. (1994) *J. Biol. Chem.* **269,** 1-4.
2. Taylor, S. I., Accili, D., and Imai, Y. (1994) *Diabetes* **43,** 735-740.
3. Cushman, S. W., and Wardzala, L. J. (1980) *J. Biol. Chem.* **255,** 4758-4762.
4. Suzuki, K., and Kono, T. (1980) *Proc. Natl. Acad. Sci. USA* **7,** 2542-2545.
5. James, D. E., Strube, M., and Mueckler, M. (1989) *Nature* **338,** 83-87.
6. Pessin, J. E., and Bell, G. I. (1992) *Annu. Rev. Physiol.* **54,** 911-930.
7. Kanai, F., Nishioka, Y., Hayashi, H., Kamohara, S., Todaka, M., and Ebina, Y. (1993) *J. Biol. Chem.* **268,** 14523-14526.
8. James, D. E., Brown, R., Navarro, J., and Pilch, P. F. (1988) *Nature* **333,** 183-185.
9. Gould, G. W., Derechin, V., James, D. E., Tordjman, K., Ahern, S., Gibbs, E. M., Lienhard, G. E., and Mueckler, M (1989) *J. Biol. Chem.* **264,** 2180-2184.
10. Haney, P. M., Slot, J. W., Piper, R. C., James, D. E., and Mueckler, M. (1991) *J. Cell Biol.* **114,** 689-699.
11. Piper, R. C., Hess, L. J., and James, D. E. (1991) *Am. J. Physiol.* **260,** 570-580.
12. Vogt, B., Mushack, J., Seffer, E., and Häring, H. U. (1991) *Biochem. J.* **275,** 597-600.
13. Mühlbacher, C., Karnieli, E., Schaff, P., Obermaier, B., Mushack, J., Rattenhuber, E., and Häring, H. U. (1988) *Biochem. J.* **249,** 865-870.
14. Obermaier-Kusser, B., Mühlbacher, C., Mushack, J., Rattenhuber, E., Fehlmann, M., and Häring, H. U. (1988) *Biochem. J.* **256,** 515-520.
15. Baldini, G., Hohman, R., Charron, M. J., and Lodish, H. F. (1991) *J. Biol. Chem.* **266,** 4037-4040.
16. Kanai, F., Ito, K., Todaka, M., Hayashi, H., Kamohara, S., Ishii, K., Okada, T., Hazeki, O., Ui, M., and Ebina, Y. (1993) *Biochem. Biophys. Res. Commun.* **195,** 762-768.
17. Okada, T., Kawano, Y., Sakakibara, T., Hazeki, O., and Ui, M.(1994) *J. Biol. Chem.* **269,** 3568-3573.
18. Hu, P., Margolis, B., Skolnik, E. Y., Lammers, R., Ullrich, A., and Schlessinger, J. (1992) *Mol. Cell. Biol.* **12,** 981-990.
19. Soler, C., Beguinot, L., and Carpenter, G. (1994) *J. Biol. Chem.* **269,** 12320-12324.
20. Niwa, H., Yamamura, K., and Miyazaki, J. (1991) *Gene* **108,** 193-200.
21. Velu, T. J., Beguinot, L., Vass, W. C., Willingham, M. C., Merlino, G. T., Pastan, I., and Lowy, D. R. (1987) *Sience* **283,** 1408-1410.
22. Sugden, B., Marsh, K., and Yates, J. (1985) *Mol. Cell. Biol.* **5,** 410-413.
23. Hayashi, H., Kamohara, S., Nishioka, Y., Kanai, F., Miyake, N., Fukui, Y., Shibasaki, F., Takenawa, T., and Ebina, Y. (1992) *J. Biol. Chem.* **267,** 22575-22580.
24. Chida, K., Hashiba, H., Sasaki, K., and Kuroki, T. (1986) *Cancer Res.* **46,** 1055-1062.
25. Nishizuka, Y. (1988) *Nature* **334,** 661-665.
26. Wahl, M. I., Nishibe, S., Suh, P. - G., Rhee, S. G., and Carpenter, G. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 1568-1572.
27. Kaplan, D. R., Whitman, M., Schaffhausen, B., Pallas, D. C., White, M., Cantley, L., and Roberts, T. M. (1987) *Cell* **50,** 1021-1029.
28. Otsu, M., Hiles, I., Gout, I., Fry, M. J., Ruiz-Larrea, F., Panayotou, G., Thompson, A., Dhand, R., Hsuan, J., Totty, N., Smith, A. D., Morgan, S. J., Courtneidge, S. A., Parker, P. J., and Waterfield, M. D. (1991) *Cell* **65,** 91-104.

[3]Kamohara, S., *et al.* under submission.