Biochimica et Biophysica Acta, 986 (1989) 41–46
Elsevier

BBAMEM 74627

Insulin stimulates the tyrosine phosphorylation of a 165 kDa protein that is associated with microsomal membranes of rat adipocytes

Robert L. Del Vecchio and Paul F. Pilch

Department of Biochemistry, Boston University School of Medicine, Boston, MA (U.S.A.)

(Received 24 January 1989)
(Revised manuscript received 13 June 1989)

Key words: Protein, 165 kDa; Insulin; Phosphotyrosine; Kinase; Insulin receptor; Phosphorylation; (Rat)

The β-subunit of the insulin receptor possesses an insulin-stimulatable protein tyrosine kinase activity. It has been widely postulated that this activity may mediate the transduction of the insulin signal by phosphorylation of cellular substrates involved in the mechanism of insulin action. We have identified, by immunoblotting with antiphosphotyrosine antibodies, a 165 kDa protein in rat adipocytes that is rapidly phosphorylated in response to insulin. Phosphorylation of this protein (pp165) occurs within 5–10 s of exposure to 10 nM insulin, suggesting that it may be a direct substrate for the insulin receptor. This protein was recovered in an intracellular membrane that fractionates with the low-density microsomes. Using discontinuous sucrose density-gradient centrifugation, pp165-containing vesicles were separated from other vesicles of the low-density microsomes including the glucose transporter-containing vesicles, indicating that pp165 is probably not a regulatory component of the vesicles that translocate glucose transporters in response to insulin. However, pp165 may be involved in conveying receptor activation at the cell surface to an intracellular site of insulin action.

Introduction

An initial cellular response to insulin exposure, following hormone binding, is the tyrosine-specific autophosphorylation of the β-subunit of the insulin receptor [1]. This autophosphorylation activates the receptor tyrosine kinase towards exogenous substrates in vitro [2,3]. Evidence has now accumulated supporting a role for the kinase activity in cellular insulin action. Recent analyses using cell lines expressing transfected, kinase-deficient insulin receptors provide evidence for the importance of the receptor kinase in insulin signal transduction. Mutation of the amino acids thought to be involved in ATP binding [4,5] and in autophosphorylation [6] compromise the ability of the receptor to mediate insulin-induced biological effects. Taken together, these findings imply that the insulin receptor mediates signal transduction via phosphorylation and further suggest that a search for a physiologically relevant, in vivo substrate for the insulin-receptor kinase is warranted. It is hoped that identification of such a substrate may lead to an understanding of the signaling pathway utilized by the insulin receptor.

A number of different approaches have been employed in the search for cellular substrates that are tyrosine-phosphorylated in response to insulin. In vitro substrates have been identified based on the ability of these proteins to be tyrosine-phosphorylated by purified insulin receptor [7,8]. Subsequent studies have shown that these proteins are also phosphorylated in insulin-stimulated cells [7,8]. Another approach is the use of two-dimensional gel electrophoresis to separate and identify tyrosine-phosphorylated proteins from ^{32}P-labeled, insulin-treated cells. In this way an 15 kDa tyrosine-phosphorylated protein was identified in 3T3-L1 adipocytes [9]. While there is evidence that this 15 kDa protein may be involved in insulin-stimulated glucose transport, the role of this and other possible substrates in insulin action is not known. Recent efforts

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DTT, dithiothreitol; EGF, epidermal growth factor; IGF-1, insulin-like growth factor, type 1; αPTYR, antiphosphotyrosine antibodies; pp165, 165 kDa phosphoprotein.

Correspondence: P.F. Pilch, Department of Biochemistry, Boston University School of Medicine, 80 East Concord St., Boston, MA 02118, U.S.A.

towards substrate identification have employed anti-phosphotyrosine antibodies (αPTYR). These antibodies, produced against either phosphotyrosine or a phosphotyrosine analog, are used to specifically immunoprecipitate phosphotyrosine-containing proteins. Using this methodology, the insulin-dependent tyrosine phosphorylation of a 180 kDa protein was observed in Fao hepatoma cells [10]. Phosphotyrosine proteins of a similar molecular weight have subsequently been isolated from other insulin-stimulated cell lines [11–15]. Whether or not these proteins are related has yet to be determined. In addition, a number of different proteins were precipitated from rat adipocytes with αPTYR [15]. In the present study, we have used αPTYR to immunoblot proteins from insulin-stimulated rat adipocytes, a major target cell for insulin action. Subcellular fractionation was performed prior to immunoblot analysis in order to partially purify, and therefore, potentially amplify the detection of scarce phosphotyrosine-containing proteins. Combining subcellular fractionation and immunoblotting with αPTYR we have identified an insulin-dependent tyrosine phosphorylated 165 kDa protein in an intracellular vesicle pool from rat adipocytes. We describe here the initial characterization of this protein and the vesicles with which it is associated.

Materials and Methods

Materials

Porcine monocomponent insulin was a gift from Dr. R.E. Chance, Eli Lilly. ^{125}I-Protein A was purchased from Dupont-New England Nuclear. Collagenase was from Cooper Biomedical. Electrophoresis apparatus and chemicals were from Bio-Rad. The electrophoretic transfer apparatus was from Idea Scientific. Molecular-weight standards were purchased from Bethesda Research Laboratories. All other chemicals were obtained from Sigma.

Preparation of adipocytes and subcellular fractionation

Adipocytes were isolated from epididymal fat pads of 175–200-g male Sprague–Dawley rats (Taconic Labs) by the method of Rodbell [16] as modified by Cushman [17]. All incubations were done in a modified Krebs-Ringer buffer containing 12.5 mM Hepes (pH 7.4)/120 mM NaCl/6 mM KCl/1.2 mM $MgSO_4$/1 mM $CaCl_2$/1 mM sodium phosphate/2.5 mM D-glucose/2% BSA (Sigma, radioimmunoassay grade). Fat pads were digested with crude collagenase and prepared for incubations as previously described [18].

Adipocytes were separated into the cytosol and various membrane fractions by the method of McKeel and Jarett [19] as modified by Simpson et al. [20]. Cells were washed three times in 12° C HESV (20 mM Hepes (pH 7.4)/255 mM sucrose/1 mM EDTA/0.1 mM sodium vanadate), then homogenized in HESV at 4° C. In order to inhibit phosphatase activity which may neutralize any insulin induced phosphorylation during the processing of the samples, orthovanadate, an inhibitor of phosphotyrosine-specific phosphatases, was added to the homogenization buffer. The addition of 0.1 mM sodium orthovanadate had no affect on the yield of total membrane protein. There was no effect on the distribution of the glucose transporter. The distribution of the characteristic enzyme markers was not determined. In experiments where rapid time points of insulin exposure were necessary, the cells were washed only one time in 4° C HESV and were then rapidly homogenized in the same buffer. All subsequent handling of the fractions was done at 4° C in HESV, except as noted. Further subfractionation of the low density microsomes by discontinuous sucrose gradient centrifugation was done as previously described [18]. Protein was assayed in each fraction using the bicinchoninic method [21], with regents from Pierce Chemicals.

Production and purification of antiphosphotyrosine antibodies

Antiphosphotyrosine antibodies (αPTYR) were prepared as described by Pang et al. [22] with some modifications. Sera produced in rabbits immunized with a Keyhole limpet hemocyanin-phosphotyramine conjugate were precipitated with ammonium sulfate prior to purification. Antibodies were then purified on a column containing a phosphotyrosine BSA conjugate, crosslinked with glutaraldehyde, and covalently bound to Affi-Gel 10 (Bio-Rad). After washing the column with 10 mM Tris (pH 7.6)/100 mM $MgCl_2$/0.1% TX-100, followed by phosphate-buffered saline, antibodies were eluted with 100 mM Glycine (pH 10). Eluate fractions were immediately neutralized and screened by an enzyme-linked-immunoadsorbant assay (ELISA) using the BSA-phosphotyrosine conjugate as the antigen. Immunoreactive fractions were pooled, concentrated and dialyzed overnight against phosphate-buffered saline.

Purified antibodies were screened by ELISA and by immunoblot for reactivity towards BSA-phosphotyrosine, BSA-phosphoserine, BSA-phosphothreonine and BSA. αPTYR only reacted with BSA-phosphotyrosine. The antibodies were able to immunoblot the phosphorylated form of the EGF receptor from EGF-stimulated A431 cells (not shown). They were also able to immunoprecipitate placental insulin and IGF-1 receptors that were phosphorylated in vitro (data not shown). Immunoprecipitation of these receptors could be inhibited by the addition of phosphotyrosine, but not by phosphoserine or phosphothreonine.

Immunoblotting

SDS-PAGE was carried out as described by Laemmli

[23]. Samples of each fraction were separated in the presence of 100 mM DTT, on either 5% or 3–15% gradient acrylamide gels. Proteins were transferred electrophoretically to nitrocellulose as described by Towbin et al. [24]. The nitrocellulose was blocked with 5% powdered, nonfat milk in 30 mM Hepes (pH 7.4) for 2 h at 22° C. αPTYR were added to the nitrocellulose at 6 μg/ml in 30 mM Hepes (pH 7.4)/150 mM NaCl/1% nonfat milk for 1 h at 37° C. After washing three times in 30 mM Hepes (pH 7.4)/150 mM NaCl/0.1% Triton X-100, the nitrocellulose was then incubated with ^{125}I-protein A (100000 cpm/ml), diluted in the same buffer as the primary antibody. The blot was incubated for 2 h at 22° C, then washed three times as before. The nitrocellulose was dried and autoradiography was performed at −70° C using Kodak X-Omat AR film and Cronex Lightning Plus enhancing screens. In some experiments, immunoblots were probed a second time with 1F8, a monoclonal antibody which recognizes an adipocyte/muscle specific glucose transporter [25]. The antibody-antigen complex was visualized using a biotinylated sheep anti-mouse second antibody followed by horseradish peroxidase conjugated streptavidin (Amersham) with 4-chloro-1-naphthol as the substrate.

Results

Insulin-stimulated tyrosine phosphorylation in subcellular fractions of rat adipocytes

In an attempt to characterize and localize phosphotyrosine-containing proteins that may be substrates for the insulin-receptor tyrosine kinase, we examined subcellular fractions of rat adipocytes by immunoblotting with αPTYR. The appearance of an insulin-dependent, 165 kDa phosphoprotein (pp165) was detected in the low-density microsomal fraction (Fig. 1) after 1 min of insulin exposure. A number of additional proteins were detected by immunoblotting as shown in Fig. 1, but no other insulin-stimulated tyrosine phosphorylation was seen in any fraction. A faint signal was detected in the high-density microsomes of insulin-stimulated cells at 165 kDa. This observation was not reproducible, and is probably due to contamination of the high-density microsomes by low-density microsomes. In the same experiment, cells were stimulated with insulin for 5 and 30 min (not shown). pp165 was the only insulin-dependent phosphotyrosine-containing protein seen at 5 min in any fraction, and no signal was observed after 30 min (see below). Interestingly, we were unable to consistently detect a band corresponding to the phosphorylated insulin receptor with our immunoblotting protocol. In some experiments a weak insulin-dependent signal was observed at 95 kDa, but we did not verify its identity as the insulin receptor. The lack of a signal for the insulin receptor could be due to low amounts of receptor in our preparations relative to pp165 or to a low affinity of αPTYR towards the receptor (see Discussion).

Many cellular responses to insulin occur rapidly, within minutes and possibly seconds of insulin binding [1]. Thus, we measured the time-course of pp165 phosphorylation to determine if it occurred rapidly enough to be involved in the early events of insulin action. Adipocytes were treated with insulin for the indicated times and the cells were processed for immunoblotting. Phosphorylation of pp165 occurs within 5 to 10 s of insulin addition (Fig. 2). This rapid time-course is consistent with a role for pp165 in the early cellular responses to insulin. After 10 s of insulin exposure, the

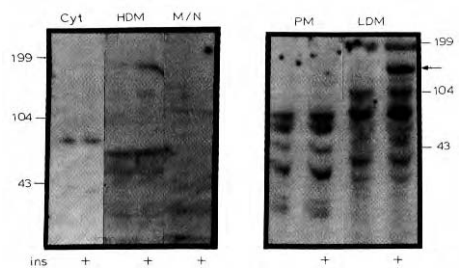

Fig. 1. Subcellular distribution of insulin-dependent phosphotyrosine proteins. Rat adipocytes were stimulated with 10 nM insulin for 1 min. Subcellular fractions were prepared as described in Materials and Methods. Proteins from the cytosol (Cyt), high-density microsomes (HDM), mitochondria and nuclei (M/N), plasma membrane (PM) and low-density microsomes (LDM) were separated using 5% SDS-PAGE and immunoblotted with αPTYR. The arrow indicates position of pp165; + indicates addition of insulin (ins). This figure is representative of three experiments.

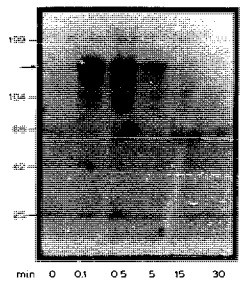

Fig. 2. Time-course of insulin-stimulated pp165 phosphorylation. Adipocytes were stimulated with 10 nM insulin for the times indicated, then rapidly washed (see text) and the LDM fraction was isolated. Microsomal proteins were separated using 3–15% gradient SDS-PAGE and immunoblotted with αPTYR. The arrow indicates the position of pp165. Numbers to the left indicate position of molecular-mass markers (values in kDa). This figure is representative of two experiments.

signal for pp165 decreases, suggesting a rapid dephosphorylation of the protein. As mentioned above, this reduction in pp165 seen in the low density microsomes cannot be accounted for by its movement to another subcellular fraction. After 15 min of insulin stimulation, pp165 could no longer be detected. In some experiments an insulin-dependent signal migrating just below pp165 was seen. This phenomenon was not reproducible, and is possibly due to proteolytic cleavage of pp165 during preparation of the membranes.

To ascertain that the specificity of our antibody with respect to pp165 was towards phosphotyrosine, soluble phosphoamino acids were used to inhibit antibody binding to immunoblots of low-density microsomes. Antibodies were mixed with the various competing amino acids (see Fig. 3) prior to incubation with the immunoblot. When phosphotyrosine was added, the pp165 signal was completely inhibited (Fig. 3), indicating that αPTYR is recognizing a phosphotyrosine residue in this protein. Addition of phosphoserine had little effect on the pp165 signal. Although phosphothreonine slightly decreased the pp165 signal, previous characterization of the αPTYR, as discussed in the Materials and Methods, demonstrated that they do not recognize threonine phosphorylated proteins. Together, these data indicate that the αPTYR are recognizing a tyrosine phosphorylated protein, pp165.

pp165 can be separated from low-density microsomal fractions containing glucose transporters using discontinuous sucrose-gradient centrifugation

To determine whether pp165 is associated with a

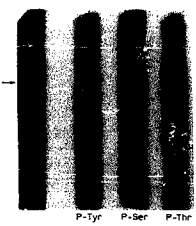

Fig. 3. Specificity of antiphosphotyrosine antibodies. Low-density microsomes isolated from insulin-stimulated rat adipocytes were run a 5% SDS-PAGE in the presence of 100 nM DTT and transferred to nitrocellulose. Strips of the nitrocellulose were blotted with αPTYR with the following additions: no addition, 10 mM phosphotyrosine (P-Tyr), 10 mM phosphoserine (P-Ser), or 10 mM phosphothreonine (P-Thr). The arrow indicates the position of pp165. This figure is representative of two experiments.

particular vesicle population within the low-density microsomes, we sedimented the membranes by rate-zonal centrifugation through a discontinuous sucrose gradient and immunoblotted the gradient fractions with antiphosphotyrosine antibodies. Our laboratory has used this density-gradient method to partially purify glucose transporter containing vesicles from low-density micro-

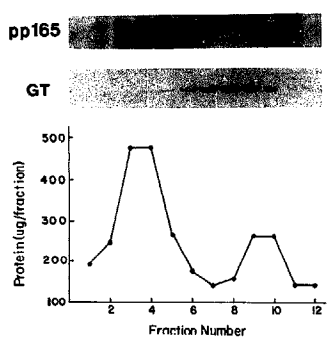

Fig. 4. Distribution of pp165 vesicles on sucrose gradients. Low-density microsomes (1–2 mg), isolated from adipocytes which had been stimulated with 10 nM insulin for 10 s, were loaded onto a discontinuous sucrose gradient. Aliquots (equal volumes) of each fraction were analyzed using a 3–15% gradient SDS-PAGE and immunoblotting with either αPTYR or 1F8 as described in the text. The position of pp165 is indicated on the upper panel; the glucose is on the middle panel. The lower panel shows the protein profile of the gradient. This figure is representative of three experiments.

somes [18]. The pp165-containing vesicles were resolved into one peak at fractions 4 and 5 (Fig. 4, top panel). In four separate experiments, the peak of pp165 vesicles always sedimented one fraction to the right of the first peak of protein (bottom panel, Fig. 4). pp165 could be recovered by centrifugation of gradient fractions 3, 4 and 5 at 180000 × g for 90 min, indicating that it was associated with membranes following sedimentation through sucrose (data not shown).

A major consequence of insulin stimulation of adipocytes is an increase in glucose transport and the translocation of glucose transporters from an intracellular vesicle pool (contained in the low-density microsomes) to the plasma membrane [27,28]. To determine whether pp165 and the glucose transporter are located on the same vesicle, we immunoblotted sucrose-gradient fractions of low-density microsomes with αPTYR and 1F8, a monoclonal antibody which recognizes an adipocyte/muscle specific glucose transporter [25]. The peaks of immunoreactivity for the two proteins were separated by two fractions (Fig. 4). We also performed the experiment shown in Fig. 4 after 3 min of insulin exposure, with identical results (data not shown). Thus, it seems that pp165 and the glucose transporter do not co-localize to the same intracellular vesicle following insulin stimulation. However, our results do not rule out an involvement of pp165 in an intermediate step between the insulin receptor and the glucose transporter.

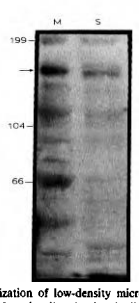

Fig. 5. High-salt solubilization of low-density microsomal proteins. Low-density microsomes from insulin-stimulated adipocytes were suspended in 0.8 M NaCl at 4°C for 15 min. The membranes were pelleted by centrifugation at 175000 × g for 90 min. The supernatant (S), containing proteins removed from the membranes by salt treatment, and the membrane proteins (M) were processed for immunoblotting with PTYR. The arrow indicates the position of pp165. Numbers to the left indicate the positions of molecular-mass standards in kDa. This figure is representative of two experiments.

To further characterize how pp165 is associated with the microsomal membranes, we attempted to strip proteins from the low-density microsomal fraction with 0.8 M NaCl. As shown in Fig. 5, this protocol removed only part of pp165 from the membrane. In addition, when low-density microsomes are extracted with Triton X-114, pp165 partitions into the aqueous phase while the glucose transporter, a hydrophobic integral membrane protein, partitions into the detergent phase (data not shown). Taken together, these results indicate that pp165 may be a tightly-associated peripheral membrane protein adherent to a specific pool of intracellular vesicles distinct from those containing the glucose transporter.

Discussion

In order to understand how the insulin receptor kinase is involved in signal transduction, there has been much effort devoted to identifying physiologically important phosphorylated substrates. While some candidate proteins have been described [7–15], neither their function nor their role in insulin action has yet been fully elucidated. We describe here a 165 kDa protein which is rapidly phosphorylated on tyrosine in response to insulin in isolated rat adipocytes. We see two important differences between the phosphorylation of pp165 shown here and previous reports describing similar insulin-dependent phosphoproteins [10–15]. First, there may be signal amplification with respect to receptor autophosphorylation (see below) and second, there is clear evidence that pp165 is tightly associated with a discrete, intracellular membrane pool. Using sucrose density-gradient centrifugation it was shown that these membranes are distinct from vesicles containing the insulin-regulatable glucose transporter. Previous characterization of the distribution of the Golgi marker galactosyltransferase indicates that pp165 sediments in a region of the gradient populated by Golgi-derived membranes and is well separated from membranes of the endoplasmic reticulum, as identified by the enzyme glucose-6-phosphatase. Due to the rapid time-course observed, we feel that phosphorylation of pp165 may represent an important early event in insulin action, possibly as a direct substrate for the insulin-receptor kinase.

The fact that we find pp165 entirely in an intracellular pool poses some interesting questions as to where potential enzyme–substrate interactions take place. Internalization of the insulin receptor is one of the initial events that follows insulin binding to its receptor, although it apparently occurs slower than phosphorylation of pp165 [29]. In any case, recent reports have shown that the insulin-receptor kinase is still active after insulin-induced internalization [30,31]. Thus, our finding of an intracellular, low-density

microsome-associated phosphotyrosine protein suggests the possibility that phosphorylation of pp165 may take place at an intracellular locus following receptor internalization. Alternatively, the localization of pp165 (the unphosphorylated form of the protein) within the cell could be such that, following insulin addition, there is a rapid interaction with the receptor leading to pp 165 phosphorylation, independent of receptor internalization. Further characterization of the spatial relationship between pp165 and the insulin receptor is needed. pp165 is probably not found on the same vesicle as the glucose transporter (Fig. 4). Therefore, while pp165 is not likely to be a component of the glucose transporter-containing vesicles, it may lie along the path to stimulation of glucose transport.

We typically see no immunoblotting of the phosphorylated insulin receptor in our adipocyte preparations (Fig. 1 and Results), whereas most reports of a similar nature see a substantial signal from receptor autophosphorylation (e.g., Refs. 10 and 11). As noted above, this may be due to signal amplification where the cascade of events responsible for insulin actions begins with the catalytic phosphorylation of pp165. This result may also arise from the fact that adipocytes are very sensitive to insulin and may therefore show exceptionally strong pp165 phosphorylation. Alternatively, antiphosphotyrosine antibodies are known to be idiosyncratic in their recognition of substrates. This is probably due to the fact that the epitopes recognized by the antibodies are likely to comprise several amino-acid residues in addition to the phosphotyrosine hapten [32]. Our antibody may therefore recognize the phosphotyrosine in pp165 with a significantly higher affinity than it recognizes the phosphorylated insulin receptor.

There have been other reports of insulin-dependent phosphotyrosine proteins of a size similar to that described herein. White et al. [10] have studied a 185 kDa protein that is tyrosine-phosphorylated in response to insulin in Fao hepatoma cells. Others have reported phosphotyrosine proteins in the same molecular-weight range in KB epidermoid cells [12], and 3T3-L1 adipocytes [14]. Phosphoprotein 185 from Fao cells may be either a soluble or weakly-attached membrane protein [15], while the pp160 observed in 3T3-L1 will associate with membrane under certain conditions [14]. Taken together, it seems likely that all these reports may be describing the same protein, or a related family of proteins that may serve a similar function. Our studies of the rat adipocyte may provide a useful system in which to study this class of proteins because of the well described subcellular fractionation schemes [18–20] that can be exploited to this end.

Acknowledgements

We wish to thank Dr. David E. James for help with experimental design, and Dr. Marianne Boni-Schnetzler for help in the production of the antiphosphotyrosine antibodies. This research was supported by grants DK-30425 and DK-36424 from the U.S.P.H.S. P.F.P. is a recipient of a Research Career Development award from the U.S.P.H.S.

References

1 Rosen, O.M. (1987) Science 237, 1452–1458.
2 Rosen, O.M., Herrera, R., Olowe, Y., Petruzzelli, L.M. and Cobb, M.H. (1983) Proc. Natl. Acad. Sci. USA 80, 3237–3240.
3 Yu, K.-T. and Czech, M.P. (1984) J. Biol. Chem. 259, 5277–5286.
4 Ebina, Y., Araki, E., Taira, M., Shimada, F., Mori, M., Craik, C.S., Siddle, K., Pierce, S.B., Roth, R. and Rutter, W.J. (1987) Proc. Natl. Acad. Sci. USA 84, 704–708.
5 Chou, C.K., Dull, T.J., Russell, D.S., Gherzi, R., Lebwohl, D., Ullrich, A. and Rosen, O.M. (1987) J. Biol. Chem. 262, 1842–1847.
6 Ellis, L., Clauser, E., Morgan, D.O., Edery, M., Roth, R.A. and Rutter, W.J. (1986) Cell 45, 721–732.
7 Rees-Jones, R.W. and Taylor, S.I. (1985) J. Biol. Chem. 260, 4461–4467.
8 Colca, J.R., DeWald, D.B., Pearson, J.D., Palazuk, B.J., Laurino, J.P. and McDonald, J.M. (1987) J. Biol. Chem. 262, 11399–11402.
9 Bernier, M., Laird, D.M. and Lane, M.D. (1987) Proc. Natl. Acad. Sci. USA 84, 1844–1848.
10 White, M.F., Maron, R. and Kahn, C.R. (1985) Nature 318, 183–186.
11 Kadowaki, T., Koyasu, S., Nishida, E., Tobe, K., Izumi, T., Takaku, F., Sakai, H., Yahara, I. and Kasuga, M. (1987) J. Biol. Chem. 262, 7342–7350.
12 Gibbs, E.M., Allard, W.J. and Lienhard, G.E. (1986) J. Biol. Chem. 261, 16597–16603.
13 Yu, K.T., Khalaf, N. and Czech, M.P. (1986) J. Biol. Chem. 161, 7865–7873.
14 Madoff, D.H., Martensen, T.M. and Lane, M.D. (1988) Biochem. J. 252, 7–15.
15 White, M.F., Stegmann, E.W., Dull, T.J., Ullrich, A. and Kahn, C.R. (1987) J. Biol. Chem. 262, 9769–9777.
16 Rodbell, M. (1964) J. Biol. Chem. 239, 375–380.
17 Cushman, S.W. (1970) J. Cell Biol 46, 326–341.
18 James, D.E., Lederman, L. and Pilch, P.F. (1987) J. Biol. Chem. 262, 11817–11824.
19 McKeel, D.W. and Jarett, L. (1970) J. Cell Biol. 44, 417–432.
20 Simpson, I.A., Yver, D.R., Hissin, P.J., Wardzala, L.J., Karnieli, E., Salans, L.B. and Cushmann, S.W. (1983) Biochim. Biophys. Acta 763, 393–407.
21 Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. (1985) Anal. Biochem. 150, 76–85.
22 Pang, D.T., Sharma, B.R. and Shafer, J.A. (1985) Arch. Biochem. Biophys. 242, 176–186.
23 Laemmli, U.K. (1970) Nature 227, 680–685.
24 Towbin, H., Staehelin, T. and Gordon, S. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
25 James, D.E., Brown, R., Navarro, J. and Pilch, P.F. (1988) Nature 333, 183–185.
26 James, D.E. and Pilch, P.F. (1988) Biochem. J. 256, 725–732.
27 Cushman, S.W. and Wardzala, L.J. (1980) J. Biol. Chem. 255, 4758–4762.
28 Suzuki, K. and Kono, T. (1980) Proc. Natl. Acad. Sci. USA 77, 2542–2545.
29 Marshall, S. (1985) J. Biol. Chem. 260, 4136–4144.
30 Klein, H.H., Freidenberg, G.R., Matthaei, S. and Olefsky, J.M. (1987) J. Biol. Chem. 1262, 10557–10564.
31 Khan, M.N., Savoie, S., Bergeron, J.J.M. and Posner, B.I. (1986) J. Biol. Chem. 261, 8462–8472.
32 Kamps, M.P. and Sefton, B.M. (1988) Oncogene 2, 305–315.