Isolation and Characterization of the 160,000-Da Phosphotyrosyl Protein, a Putative Participant in Insulin Signaling*

(Received for publication, March 11, 1991)

Susanne R. Keller, Kouichiro Kitagawa, Ruedi Aebersold, Gustav E. Lienhardt, and Charles W. Garner

From the Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire 03756, §The Biomedical Research Centre and Department of Biochemistry, University of British Columbia, Vancouver V6T 1W5, Canada, and the §Department of Biochemistry and Molecular Biology, Texas Tech University Health Sciences Center, Lubbock, Texas 79430

The 160,000-Da protein (pp160) which is rapidly phosphorylated on tyrosine in response to insulin and thus is a putative participant in signaling from the insulin receptor has been purified to homogeneity from 3T3-L1 adipocytes. Isolation of this protein was accomplished by chromatography on an immobilized monoclonal antibody against phosphotyrosine, followed by gel electrophoresis. Sufficient protein was obtained to allow the determination of the sequences of several peptides, which in turn enabled the development of anti-peptide antibodies that specifically recognize pp160. Immunoblotting of 3T3-L1 adipocyte lysates, together with the purified pp160 as a standard, indicate that an insulin-treated 3T3-L1 adipocyte possesses about 230,000 copies of tyrosine-phosphorylated pp160 and that this amount is approximately 25% of the total pp160 in the cell. The number of tyrosine-phosphorylated pp160s per cell is approximately the same as that of insulin receptor β subunits. These results provide further evidence for a role of pp160 in insulin signaling. Moreover, the availability of purified protein and knowledge of peptide sequences will allow the elucidation of the structure and function of this protein.

Insulin triggers a variety of cellular events upon binding to its receptor on the cell surface. The pathways of signaling from the receptor are still largely unknown. The insulin receptor is a tetramer consisting of two α and two β subunits. The α subunits are entirely extracellular and the β subunits are transmembrane. When insulin binds to the extracellular domain, a protein tyrosine kinase intrinsic to the intracellular part of the β subunit is activated, with concomitant autophosphorylation on tyrosine (1-3). A reasonable hypothesis is that signaling from the insulin receptor then proceeds by the phosphorylation of certain target proteins upon tyrosine, since the appearance of several phosphotyrosyl polypeptides has been detected upon exposure of various cells to insulin (4-8). Prominent among these is a protein of 160,000-185,000 Da, which was first described by White et al. (9) (hereafter designated pp160).

Considerable circumstantial evidence exists to suggest that pp160 is involved in signaling from the insulin receptor. First, in several cell lines and tissues this protein has been found to be the major polypeptide, in addition to the β subunit of the insulin receptor phosphorylated on tyrosine in response to insulin. These are rat (9-13) and 3T3-L1 (13-15) adipocytes, human epidermoid carcinoma cells (16), Fao (9, 15, 17), H4 (18), and H35 hepatoma cells (19), mouse neuroblastoma cells (20), Chinese hamster ovary cells (17, 21), 3T3 fibroblasts (22, 23), L6 myoblasts (24), FRTL5 thyroid cells (25), and rat liver (26). Second, the phosphorylation of pp160 occurs rapidly at about the same rate and at the same concentrations of insulin as does the phosphorylation of the β subunit of the insulin receptor in 3T3-L1 adipocytes (14) and other cell types (9-11, 16, 20, 25). Third, an insulin receptor which is mutated from tyrosine to phenylalanine at position 960 and expressed in Chinese hamster ovary cells is not able to elicit either phosphorylation of pp160 or the typical cellular responses upon exposure to insulin, even though the receptor is still functional as an insulin-activated tyrosine kinase against itself and exogenous substrates (27). Last, partly purified pp160 is further phosphorylated on tyrosine by the purified insulin receptor (19).

In addition to its potential role in insulin signaling, pp160 may also function in signaling from the insulin-like growth factor I (IGF I) receptor. Exposure of 3T3-L1 adipocytes (14), kidney cells (28), human epidermoid carcinoma cells (16), mouse neuroblastoma cells (20), and FRTL5 thyroid cells (25) to physiological levels of IGF I leads to the rapid phosphorylation on tyrosine of a protein of this size. In contrast, platelet-derived growth factor and epidermal growth factor do not appear to elicit the tyrosine phosphorylation of pp160 (14, 16, 22).

Despite this intensive investigation of pp160, the purification of this protein has not been reported. 3T3-L1 adipocytes are relatively abundant in pp160 (13, 14), and we describe here the purification of the protein from this cell type. A sufficient amount was obtained to enable determination of the amino acid sequences of several peptides from the protein. On the basis of one of these, anti-peptide antibodies which specifically recognize pp160 were developed. By immunoblotting 3T3-L1 lysates, we have found that in response to insulin the cellular content of tyrosine-phosphorylated pp160 appears to be approximately the same as that of the insulin receptor β subunit.
EXPERIMENTAL PROCEDURES

Materials—Mouse monoclonal antibody 1G2 against phosphorylated Tyr(P) was obtained from Oncogene Science, Inc. (Uniondale, NY). Goat anti-rabbit IgG was the product of Du Pont-New England Nuclear.

Cell Culture—3T3-L1 fibroblasts were cultured and induced to differentiate into adipocytes as described in Ref. 29. Adipocytes were used between the 8th and the 12th day after differentiation. The amount of pp160 detected by immunoblotting SDS lysates of insulin-treated cells with antibodies against Tyr(P) was unchanged during this 5-day period.

Purification of pp160—10-cm plates of 3T3-L1 adipocytes were placed in 10 ml of serum-free Dulbecco's modified Eagle's medium for 2 h before use. Cells were then treated with insulin (300 nM) for 2–4 min, a time period during which the amount of pp160 detected by immunoblotting for Tyr(P) is maximal (14). The medium was aspirated, and each dish was immediately frozen in liquid nitrogen. The frozen cells were overlaid with 2 ml/dish of homogenization buffer (20 mM Tris-HCl, 50 mM NaCl, 1 mM NaVO₄, 1 mM ethylenediamine, 30 mM sodium pyrophosphate, 1 mM phenylmethanesulfonyl fluoride, pH 7.6) and scraped with a rubber policeman from the dish as a slush. Upon thawing, the cells were broken with 10 hand-driven strokes in a 55-ml glass homogenizer with a Teflon pestle (Arthur H. Thomas 3431D94). The suspension was then centrifuged at 45,000 rpm in a Beckman type 70T rotor for 1 h at 200,000 × g. The infranatant solution was carefully removed with a pipette in order to leave as much of the lipid layer on the walls of the tubes as possible and then filtered through a 0.65-µm Millipore filter, type PVDF 047 00, to remove the remaining lipid. In a typical preparation 100 10-cm plates of 3T3-L1 adipocytes were processed through these steps in two 50-plate batches.

The clarified extract was then subjected to immunoaffinity chromatography at 4 °C by passing it through a 2-ml column of goat IgG immobilized on a 1 ml column of Sepharose 4B (Pharmacia) against Tyr(P) antibody (1G2) immobilized on agarose. After all of the extract was applied, the goat IgG column was disconnected and the 1G2 column was washed with 200 ml of 20 mM Tris-HCl, 150 mM NaCl, 1 mM NaVO₄, pH 7.6. The Tyr(P) proteins were then eluted with 3 ml phenyl phosphate in the wash buffer and collected in 2-ml fractions. Since the protein concentration of the eluate is very low, loss of pp160 through adsorption to the collecting tubes was initially a problem. It was found that this loss did not occur with polyethylene tubes designed for low protein adsorption (4.0-ml Minisorp tubes from Nunc, catalog no. 443901), and these were then used. Flow rate was maintained throughout at 10 ml/h with a peristaltic pump. The column was washed by generating with a 1-ml wash of 1 mM NaCl, 20 mM Tris-Cl, 6 mM NaN₃, pH 7.5. They have been used for 10 preparations over a period of 6 months without any noticeable loss in the yield of pp160.

Final separation of pp160 from other proteins was accomplished by reverse-phase high pressure liquid chromatography system (Waters Peptide Analyzer; column, Vydac C4 2.1 x 150 mm, developed with a acetonitrile gradient in 0.1% trifluoroacetic acid). The collected peptides were subjected to sequence analysis in an Applied Biosystems model 477 sequencer.

Generation of Antibodies—Peptides were synthesized, conjugated to keyhole limpet hemocyanin, and then used to immunize rabbits, as described in Ref. 31. Antibodies were affinity-purified from serum by chromatography on the immobilized peptide, as described previously (31).

Gel Electrophoresis and Immunoblotting—SDS-polyacrylamide gel electrophoresis was carried out on minigels by the method of Biely et al. (32) (data in Fig. 1) or the larger gels described above (data in Fig. 2). The polypeptides were electrophoretically transblotted onto nitrocellulose (Schleicher and Schuell, BA83, 0.2 µm) at 300 mA for 2 h (minigels) or at 200 mA for 16 h (large gels). The nitrocellulose was either stained for protein with colloidal gold (Bio-Rad) or immunoblotted with antibodies against Tyr(P) or pp160. In the case of Tyr(P) blots were first blocked for 2 h with 5% bovine serum albumin in Tris-buffered saline (150 mM NaCl, 20 mM Tris-Cl, pH 7.6) and then treated with 2.0 µg/ml affinity-purified rabbit anti-Tyr(P) antibodies, prepared by the method of Pang et al. (32), in the buffer with 2 µg/ml albumin overnight. Subsequently, the blots were washed 5 times with buffer and then incubated for 2 h with 125I-labeled goat anti-rabbit IgG (0.68 µCi/ml buffer containing 2 µg/ml albumin and 0.3% Tween 20). The blots were washed with buffer/Tween and autoradiographed. In the case of pp160, blots were blocked with 5% Carnation nonfat dry milk in 150 mM NaCl, 10 mM sodium phosphate, pH 7.4, washed at each stage with 1% Triton X-100 in this buffer, and treated with the affinity-purified antibodies against the peptide at 50 ng/ml and the 125I-labeled second antibody in 1% milk in this buffer. Quantitation of radioactivity on the immunoblots was done by cutting out the area of the nitrocellulose corresponding to the band on the autoradiogram and measuring the radioactivity in a γ counter; the label in an appropriate blank area of the blot was used to correct for background.

RESULTS AND DISCUSSION

Purification of pp160—pp160 was purified from the soluble proteins of insulin-treated 3T3-L1 adipocytes by a combination of immunoaffinity chromatography with a monoclonal antibody against Tyr(P) and SDS-gel electrophoresis. The details of the purification procedure are given under "Experimental Procedures." Fig. 1 presents the analysis of the fractions from the immunoaffinity chromatography for total polypeptides by protein stain (Fig. 1A, fractions 11–5) and for Tyr(P) polypeptides by immunoblotting with antibodies against Tyr(P) (Fig. 1B, fractions 11–5). The prominent, slightly broad band at 160 kDa detected by protein stain in fractions 12 and 3 is coincident with the Tyr(P) polypeptide in both mobility and relative intensity, and therefore is considered to be purified pp160. Further evidence for the absence of significant impurities in this band is the finding that this polypeptide was entirely absent in the fractions obtained when the purification procedure was carried out with the soluble proteins from basal 3T3-L1 adipocytes (Fig. 1, A and B, fractions B1–5). These results, of course, do not rigorously exclude the possibility that the pp160 band includes more than one protein. The yield of pp160 was estimated by visual comparison of its protein staining intensity on a Coomassie Blue-stained gel with several known amounts of the standards, myosin, and β-galactosidase (data not shown). Approximately 4 µg were isolated from about 108 3T3-L1 adipocytes (100 10-cm plates, 1 g of protein).

Peptides from and Antibodies against pp160—Sequences of portions of four tryptic peptides from pp160 were obtained: (a) XXVFAAADSM*AMQDSE*YQALLQ, (b) XXTIPGANLGTS-PALPGDEAAGAADL, and (c) XIPGANLGTSPALPGDEAAGAADL, and (d) XVP*APQQINPX (X indicates unidentified residue; * indicates some uncertainty). None of the sequences is identical to ones in the Protein Identification Resources data bank, and so pp160 is unlikely to be a previously sequenced protein.

Antisera were raised against synthetic peptides correspond-
The 160,000 Da Protein in Insulin Action

Fig. 1. Purification of pp160. The procedure for the purification of pp160 was carried out separately with the soluble proteins from basal (B) and insulin-treated (I) 3T3-L1 adipocytes (in each case 60 10-cm plates). Fractions 1-5 eluted from the matrix of anti-Tyr(P) antibody with phenyl phosphate were analyzed through electrophoresis on 6% gels and transblotting to nitrocellulose. Each lane contained the proteins from one-twentieth of the fraction. A, total protein by staining with colloidal gold; B, immunoblot with antibodies against Tyr(P); C, immunoblot with antibodies against pp160 peptide. The markers on the right show the positions of protein standards of 200, 116, 97, and 66 kDa.

Fig. 2. Amounts of Tyr(P) and total pp160 in 3T3-L1 adipocytes. SDS lysates of cells, either basal or treated with 300 nM insulin for 3 min, were prepared by scraping 10-cm plates in 3 ml of SDS sample buffer with protease inhibitors (31), 20 mM dithiothreitol, and 1 mM sodium orthovanadate and shearing the DNA by passage of the lysate through a syringe needle. The pp160 standards were prepared from fraction 2 of a preparation (see Fig. 1A); their content of pp160 protein was determined by visual comparison of its intensity with that of several amounts of myosin, β-galactosidase, and phosphorylase on a Coomassie Blue-stained gel. Gels were 7% polyacrylamide. A, immunoblot with antibodies against Tyr(P); B, immunoblot with antibodies against the pp160 peptide. For each blot the lanes contained: 1 and 2, lysate of basal cells (2 and 1% of a 10-cm plate); 3 and 4, lysate of insulin-treated cells (2 and 1% of a 10-cm plate); 5-7, purified pp160 (20, 10, and 5 ng). Aliquots of the same samples were applied to both gels. The markers on the right show the positions of the protein standards of 205, 117, and 80 kDa. A repetition of this experiment with the lysates from a second set of basal and insulin-treated 3T3-L1 adipocytes gave similar results.

immunoblotting of the Tyr(P) form, which is present only in the insulin-treated cells.

Even with this complication it was still possible to estimate the total amount of pp160 in the cells in the following way (illustrated with one set of data). With the SDS lysate of insulin-treated cells, the pp160 signal (cpm) (Fig. 2B, lane 3, 3030 cpm) on the immunoblot consists of contributions from both the Tyr(P) and non-Tyr(P) forms, whereas the pp160 signal from the SDS lysate of basal cells (lane 1, 2410 cpm)
is given by the same total amount of entirely non-Tyr(P) form. The contribution of the Tyr(P) form to the total signal in the insulin lysate (1340 cpm) can be calculated from the amount of this form (9.5 ng), given by the data in Fig. 2A, and the specific activity of this form (cpm/ng), given by the standards (Fig. 2B, lanes 5–7, 141 cpm/ng), which are entirely the Tyr(P) form. The remaining portion of the total pp160 signal in the insulin lysate (3030 cpm) is due to the non-Tyr(P) form, and this cpm value divided by that of the purified pp160 used as the standard gives the percentage of non-Tyr(P) form present after insulin treatment (70%). The difference between this percentage and 100% is in turn the percentage of Tyr(P) form after insulin treatment (30%). The percentage of non-Tyr(P) form present after insulin treatment (50%), from which value and the ng of this form (given by Fig. 2A) the total amount of pp160 is calculated.

The determination of the amount of the Tyr(P) form of pp160 and therefore also of the total amount of pp160 assumes that the Tyr(P) form of pp160 in the cell lysate and in the purified preparation is immunoblotted with equal efficiency by the antibodies against Tyr(P). There is suggestive evidence that pp160 may be phosphorylated on more than one tyrosine residue (19). Consequently, it remains possible that the Tyr(P) content of the purified pp160 used as the standard differs from that of pp160 in the lysate of insulin-treated cells, even though the purified pp160 was isolated from identically stimulated cells. Thus, until this assumption can be examined, the values given above should be considered as estimates.

Conclusions—The purification of microgram amounts of pp160 has allowed the determination of the sequences of several peptides from the protein and the development of anti-peptide antibodies that recognize pp160. These should enable the cloning of the cDNA encoding this protein, an effort now under way in our laboratory. The findings that the amount of the Tyr(P) form of insulin-treated pp160 in 3T3-L1 adipocytes appears to be approximately the same as the amount of the insulin receptor β subunit and that probably a substantial percentage of pp160 is phosphorylated on tyrosine in response to insulin provide further evidence for a role of pp160 in insulin signaling.

Acknowledgments—We are deeply indebted to Susan Brown for expert culture of the 3T3-L1 adipocytes. We are grateful to Dr. Gwyn Gould and Joe Franklin for preparation of the antibodies against Tyr(P), to Dr. Thomas Ciardelli for the synthesis of peptides, and to Edward Bures for peptide sequence analysis.

REFERENCES
1. Rosen, O. M. (1987) Science 237, 1452-1458
2. Kahn, C. R., and White, M. F. (1988) J. Clin. Invest. 82, 1152–1156
3. Yarden, J., and Ulrich, A. (1988) Annu. Rev. Biochem. 57, 443–478
4. Kasuga, M., Izumi, T., Tobe, K., Shiba, T., Monomura, K., Tashiro-Hashimoto, Y., and Kadowaki, T. (1990) Diabetes Care 13, 317–326
5. Boulton, T. G., Yancopoulos, G. D., Gregory, J. S., Slaughter, C., Moon, C., Hau, J., and Cobb, M. H. (1990) Science 249, 947–957
6. Ruderman, N. B., Kapeller, R., White, M. F., and Cantley, L. C. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 1411–1415
7. Endemann, G., Yonezawa, K., and Roth, R. A. (1990) J. Biol. Chem. 265, 396–400
8. Margolis, R. N., Schell, M. J., Taylor, S. L., and Hubbard, A. L. (1990) Biochem. Biophys. Res. Commun. 166, 562–566
9. White, M. F., Maron, R., and Kahn, C. R. (1986) Nature 318, 183–186
10. Momomura, K., Tobe, K., Sayama, Y., Takaku, F., and Kasuga, M. (1986) Biochem. Biophys. Res. Commun. 155, 1181–1186
11. Moseley, R. A., Borwell, K. L., Lukowik, J. S., and Cassellie, J. E. (1989) Endocrinology 124, 422–429
12. Del Vecchio, R. L., and Pilch, P. F. (1989) Biochim. Biophys. Acta 986, 41–46
13. Gibbs, E. M., Allard, W. J., and Lienhard, G. E. (1986) J. Biol. Chem. 261, 16897–16603
14. Maddox, D. H., Martensen, T. M., and Lane, M. D. (1988) Biochem. 252, 7–15
15. Witters, L. A., Watts, T. D., Gould, G. W., Lienhard, G. E., and Gibbs, E. M. (1989) Biochim. Biophys. Res. Commun. 163, 992–998
16. 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
17. White, M. F., Staggman, E. W., Dull, T. J., Ulrich, A., and Kahn, C. R. (1987) J. Biol. Chem. 262, 9769–9777
18. Tornquist, H. E., Gunsalas, J. R., Nemenoff, R. A., Frackelton, A. R., Pierce, M. W., and Avrch, J. (1988) J. Biol. Chem. 263, 350–359
19. Tashiro-Hashimoto, Y., Tobe, K., Kashio, O., Izumi, T., Takaku, F., Akamuna, Y., and Kasuga, M. (1989) J. Biol. Chem. 264, 6879–6885
20. Shemer, J., Adamo, M., Wilson, G. L., Heffner, D., Zick, Y., and LeRoith, D. (1987) J. Biol. Chem. 262, 15476–15482
21. Chou, C. K., Dull, T. J., Roberston, K. S., Gherzi, R., Lebwohl, D., Ulrich, A., and Rosen, O. M. (1987) J. Biol. Chem. 262, 1842–1847
22. Pasquale, E. B., Maher, P. A., and Singer, S. J. (1988) J. Cell. Biol. 107, 147–156
23. Bride, N. P. J., Tavare, J. M., Dickens, M., Wittaker, J., and Siddle, K. (1990) Biochim. J. 268, 615–620
24. Burdet, E., Mills, G. B., and Klip, A. (1990) Am. J. Physiol. 258, C99–C108
25. Condorelli, G., Formisand, P., Villone, G., Smith, R. J., and Beinert, F. (1989) J. Biol. Chem. 264, 12653–12658
26. Tobe, K., Kashio, O., Tashiro-Hashimoto, Y., Takaku, F., Akamuna, Y., and Kasuga, M. (1990) Diabetes 39, 528–533
27. White, M. F., Livingston, J. N., Backer, J. M., Larins, V., Dull, T. J., Ulrich, A., and Kahn, C. R. (1988) Cell 54, 641–649
28. Izumi, T., White, M. F., Kadowaki, T., Takaku, F., Akamuna, Y., and Kasuga, M. (1987) J. Biol. Chem. 262, 1282–1287
29. Frost, S. C., and Lane, M. D. (1985) J. Biol. Chem. 260, 2646–2652
30. Aebersold, R. H., Levitt, J., Saavedra, R. A., Hood, L. E., and Kent, S. B. H. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 6970–6974
31. Tanner, L. I., and Lienhard, G. E. (1989) J. Cell Biol. 108, 1537–1545
32. Pang, D. T., Sharma, B. R., and Shafer, J. A. (1986) Arch. Biochem. Biophys. 242, 176–186
33. Rubin, C. S., Hirsch, A., Fung, C., and Rosen, O. M. (1978) J. Biol. Chem. 253, 7570–7578
34. Smith, P. J., Wise, L. S., Berkowitz, R., Wan, C., and Rubin, C. S. (1988) J. Biol. Chem. 263, 9402–9408