A Novel 160-kDa Phosphotyrosine Protein in Insulin-treated Embryonic Kidney Cells Is a New Member of the Insulin Receptor Substrate Family*

(Received for publication, May 2, 1997, and in revised form, June 27, 1997)

Brian E. Lavan‡, Valeria R. Fantin, Ellen T. Chang, William S. Lane§, Susanna R. Keller, and Gustav E. Lienhard¶

From the Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire 03755 and Harvard Microchemistry Facility, Department of Molecular and Cellular Biology, Harvard University, Cambridge, Massachusetts 02138

We have previously identified a 160-kDa protein in human embryonic kidney (HEK) 293 cells that undergoes rapid tyrosine phosphorylation in response to insulin (PY160) (Kuhné, M. R., Zhao, Z., and Lienhard, G. E. (1995) Biochem. Biophys. Res. Commun. 211, 190–197). The phosphotyrosine form of PY160 was purified from insulin-treated HEK 293 cells by anti-phosphotyrosine immunoadsorption chromatography, the sequences of peptides determined, and its cDNA cloned. The PY160 cDNA encodes a 1257-amino acid protein that contains, in order from its N terminus, a pleckstrin homology (PH) domain, a phosphotyrosine binding (PTB) domain and, spread over the C-terminal portion, 12 potential tyrosine phosphorylation sites. Several of these sites are in motifs expected to bind specific SH2 domain-containing proteins: YXXM (7 sites), phosphatidylinositol 3-kinase; YVNM (1 site), Grb-2; and YIEV (1 site), either the phosphotyrosine phosphatase SHP-2 or phospholipase Cγ. Furthermore, the PH and PTB domains are highly homologous (at least 40% identical) to those found in insulin receptor substrates 1, 2, and 3 (IRS-1, IRS-2, and IRS-3). Thus, PY160 is a new member of the IRS family, which we have designated IRS-4.

The insulin receptor is a tyrosine kinase, which when activated by insulin binding phosphorylates cellular substrates. The most well characterized of these are two members of the IRS family, IRS-1 and IRS-2, and the protein Shc. Tyrosine phosphorylation of the IRS proteins creates binding sites for SH2 domain-containing signaling molecules, including PI 3-kinase, the adapter molecule Grb-2, and the protein-tyrosine phosphatase SHP-2. Docking of these proteins in turn activates specific signal transduction pathways (reviewed in Refs. 1 and 2). Recently, we have identified, by purification and cloning, a third member of the IRS family, called IRS-3, which in insulin-treated adipocytes is tyrosine-phosphorylated and associated with PI 3-kinase (3, 4). All three IRS family members possess a common domain structure that includes PH and PTB domains at the N terminus and, C-terminal to these, a number of potential tyrosine phosphorylation sites (1, 2, 4, 5). The presence of these features can therefore be viewed as defining an IRS. Previously, we have identified a 160-kDa protein in HEK 293 cells, termed PY160, which is rapidly tyrosine-phosphorylated in response to insulin but which is immunologically unrelated to IRS-1 (6). In the present study we have isolated PY160 from insulin-treated HEK 293 cells and cloned its cDNA. The predicted amino acid sequence shows that PY160 is a new member of the IRS family.

EXPERIMENTAL PROCEDURES

Cell Culture and Preparation of Lysates—HEK 293 cells were grown on 10-cm plates as described previously (6). Before use, confluent plates of cells were incubated in serum-free medium for 2 h and then incubated for 5 min further with either no addition or the addition of 1 μM insulin to activate fully the insulin and IGF-1 receptors present on these cells (7). Each plate was rinsed with phosphate-buffered saline and lysed by the addition of 1 ml of 3% SDS, 10 mM dithiothreitol in Buffer A (50 mM Hepes, 100 mM NaCl, 2 mM EDTA, 1 mM sodium vanadate, pH 7.4, with protease inhibitors (10 μM E64, 10 μM leupeptin, 10 μM aprotinin, 1 mM pepstatin A, 4 mM diisopropyl fluorophosphate)). The lysate was held at 100 °C for 5 min, and the DNA in it was sheared by repeated passage through a syringe needle. Finally, the lysate was diluted by the addition of 5 ml of 3% Triton X-100 in Buffer A; free sulphydryl groups were capped by the addition of N-ethylmaleimide to a final concentration of 6.7 mM; and the lysate was clarified by centrifugation at 150,000 × g for 1 h. Immunoadsorption of PY160—Aliquots of lysates (1 ml) from basal and insulin-stimulated 293 cells were incubated with anti-Tyr(P) antibodies (20 μl of 4G10 agarose from Upstate Biotechnology) for 4 h at 4 °C. The beads were washed twice with a wash buffer (50 mM Hepes, 100 mM NaCl, 1.5% Triton X-100, 0.25% SDS, 1 mM sodium vanadate with protease inhibitors, pH 7.4), and the Tyr(P)-containing proteins were eluted by the addition of 135 μl of 40 mM phenyl phosphate in the wash buffer. To estimate the recovery of PY160, samples containing the original extract, the depleted extract, and the phenyl phosphate eluate were immunoblotted for Tyr(P), as described (3). The yield of the Tyr(P) form of PY160 by immunoadsorption from the lysate of insulin-treated cells was approximately 15%.

Purification of PY160 and Sequencing of Peptides—PY160 was purified by anti-Tyr(P) affinity chromatography from an extract derived from a total of thirty-six 10-cm plates of insulin-stimulated HEK 293 cells. In a single purification, half of the extract (110 ml) was passed at 0.2 ml/min through a 1.0-ml column of immobilized anti-Tyr(P) antibody (4G10 agarose at 1 mg/ml). Once the extract was applied, the goat IgG column was disconnected, and the anti-Tyr(P) column was washed sequentially with a 30 ml of 1% Triton X-100, 0.25% SDS in wash buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM sodium vanadate, pH 7.4,
with protease inhibitors (2 μg/ml aprotinin, 2 μM leupeptin, 0.2 mM pepstatin A) at 1 ml/min, (b) 30 ml of 1% Triton X-100 in wash buffer with protease inhibitors at 1 ml/min, (c) 300 ml of 0.05% Triton X-100 in wash buffer with protease inhibitors at 0.3 ml/min, and (d) 7 ml of 0.015% sodium deoxycholate in wash buffer at 0.5 ml/min. Elution buffer (0.015% sodium deoxycholate in wash buffer) was run onto the column and the flow stopped for 10 min. Tyr(P)-containing proteins were then eluted at 0.5 ml/min, and 2-ml fractions were collected in low protein adsorption tubes (Nunc no. 443990). The purification was repeated with the other half of the lysate, and the two fractions from each preparation containing the bulk of the PVY product were concentrated by trichloroacetic acid precipitation as described in Ref. 3. The precipitates were resuspended in SDS sample buffer and separated in a single lane on a 7% acrylamide gel. Following transfer to ProBlott membrane (Applied Biosystems) and staining with Amido Black, the band corresponding to PY160 was excised (about 1.5 μg (10 pmol)). The protein band was subjected to in situ digestion with LysC; the resultant peptides were separated by microbore HPLC, selected fractions were screened by MALDI-TOF mass spectrometry and microsequenced by the methods described previously (4). By UV absorbance, approximately 1–5 pmol of peptides were present in the HPLC separation.

PY160 cDNA—Total RNA was obtained from HEK 293 cells using the Trizol reagent (Life Technologies, Inc.), and mRNA was subsequently isolated if using the Fast-Track Kit (Invitrogen). A 10-cm plate of confluent HEK 293 cells yielded about 3 μg of mRNA. A Marathon ReadyTM cDNA library from human fetal kidney was obtained from CLONTECH. The nucleotide sequence encoding the central portion of peptide a (see “Results and Discussion” for peptides) was obtained by PCR amplification. The sequences of the N and C termini of peptide a were used to design degenerate oligonucleotides; restriction sites for EcoRI (sense) and BamHI (antisense) were incorporated to facilitate cloning (‘GGCAATTCYNGARCGNCAG3’ and ‘GGGATCCGGCTTTCYCTAR3’, where Y is C or T, R is A or G, and N is A, C, G, or T; restriction sites are underlined). cDNA, produced by random hexamer primed reverse transcription of HEK 293 mRNA, was used as the template. A PCR product of the expected size (63 bp) was obtained and cloned into the EcoRI/BamHI site of pBluescript II (SK−). Several clones were sequenced and found to encode the central portion of the peptide (APARLE; nt 390–408). The 5′-end of the cDNA was obtained in two separate 5′-RACE reactions. In the first, a degenerate antisense primer derived from the C terminus of peptide a (‘5′TTNCNGNGRTYTYT3’ and ‘5′TTYCTNGCRTYT3’ mixed in a 2:1 molar ratio, respectively) was used to reverse transcribe HEK 293 mRNA. The resulting cDNA was tail-cut and amplified by PCR with an antisense primer derived from the sequence encoding the middle portion of a peptide (‘5′CTRTARTATTCCGGCGACG3’; exact sequence underlined, see above) and the abridged anchor primer of a 5′-RACE kit, according to the manufacturer’s instructions (Life Technologies, Inc.). The products were reamplified using a nested antisense primer (derived from the N terminus of peptide a (‘5′GGGGCGTCGTCGTC3’)) and the abridged universal amplification primer of the kit. A 330-bp product was purified and cloned into pCR-Script. The inserts of several clones were sequenced and were likely to arise due to internal priming by the oligo(dT) primer. The 1300- and 980-bp PCR products were sequenced directly (nt 1840–3052 and nt 2979–3939, respectively). To confirm the DNA sequence, overlapping fragments were generated by PCR amplification of cDNA obtained by random hexamer-primed reverse transcription of total RNA from HEK 293 cells and sequenced directly on both strands (nt 170–637, 598–1124, 1076–1704, 1651–2308, 2139–2889, 2729–3502, and 3200–3898). Sequencing was performed on the Applied Biosystems 373 DNA sequencing system using the Perkin-Elmer DNA sequencing kit; data were analyzed with the Applied Biosystems software. Homology searches were performed with the BLAST program (8).

RESULTS AND DISCUSSION

Identification and Purification of PY160—Treatment of HEK 293 cells with insulin elicits the tyrosine phosphorylation of a protein of approximately 160 kDa, which is immunologically distinct from IRS-1 (6). Immunoblotting of HEK 293 cell lysates with antibodies to IRS-2 detected a protein larger than PY160; this result indicated that PY160 was also not IRS-2 (data not shown).

To assess the feasibility of isolating PY160 by anti-Tyr(P) immunoadsorption chromatography, we performed immunoadsorptions with anti-Tyr(P) immobilized on agarose beads. Detergent extracts were prepared from basal and insulin-stimulated HEK 293 cells, incubated in the presence or absence of phenyl phosphate (a ligand competing with Tyr(P)), and then immunoadsorbed with anti-Tyr(P) beads. The adsorbed proteins were eluted with phenyl phosphate and analyzed by immunoblotting and protein staining. Fig. 1 (lanes 1–4) shows the eluted Tyr(P)-containing proteins that were detected by anti-Tyr(P) immunoblotting. Two major insulin-elicted Tyr(P) proteins were present. One had the size expected for the Tyr(P) form of PY160; the other, based on its size (about 100 kDa), is most likely a mixture of the tyrosine-phosphorylated β-subunits of the insulin and IGF-1 receptors; both receptors are present in HEK 293 cells, and the latter would be expected to be activated at 1 μM insulin (7). Protein staining of the eluates showed a single major protein, which co-migrated with the Tyr(P) form of PY160 (Fig. 1, lanes 5–8). The recovery of this protein from extracts of basal and insulin-treated cells paralleled the recovery of the Tyr(P) form of PY160; this indicates that the protein

![Fig. 1](Image 355x617 to 517x729)
**A New Member of the IRS Family**

was PY160 (Fig. 1, compare lanes 5 and 6 with lanes 1 and 2). The binding to the anti-Tyr(P) beads was specific; no proteins were present in the eluates from anti-Tyr(P) immunoprecipitates of lysates preincubated with phenyl phosphate (Fig. 1, compare lanes 3 and 4 and 7 and 8).

The results in Fig. 1 showed that it would be possible to purify PY160 from HEK 293 cells by anti-Tyr(P) chromatography in an amount sufficient to obtain peptide sequences. A large number of sequences were obtained by a slight modification of a method that we previously used to purify Tyr(P) proteins from insulin-treated adipocytes (3) (see “Experimental Procedures”). This yielded sufficient PY160 to allow determination of the sequences of five peptides: a, LETADAPARLEYYENARK; b, DYWQVIVK; c, RSYFGK; d, FLGRGLDK; and e, EVSYNWDPK (see Fig. 2). A search of the data base using the BLAST program revealed that peptides a and b had significant homology with sequences in IRS-1.

**Domains and Tyr(P) Motifs in PY160**—A comparison of the amino acid sequence of PY160 with the data base and an examination for the presence of potential tyrosine phosphorylation sites revealed that PY160 contains, in order from its N terminus, a PH domain, a PTB domain, and spread over the C-terminal portion, 12 potential tyrosine phosphorylation sites revealed that PY160 contains, in order from its N terminus, a PH domain, a PTB domain, and spread over the C-terminal portion, 12 potential tyrosine phosphorylation sites (Fig. 3A). This architecture is the same as that of the three known members of the IRS family (see the Introduction). Therefore, PY160 is a new member of the IRS family, and henceforth we refer to it as IRS-4.

IRS-4 is of a similar length (1257 aa) to both IRS-1 and IRS-2 (1242 aa for human IRS-1 and 1321 aa for mouse IRS-2 (5)). Overall IRS-4 displays limited sequence identity with IRS-1 and IRS-2 (27 and 29%, respectively). However, significant homology is found in the PH and PTB domains.

The PH domain of IRS-4 consists of 120 amino acids (residues 78–197) and exhibits a high degree of homology with the PH domain of IRS-1, IRS-2, and IRS-3 (49, 50, and 43% identity, respectively) (Fig. 3B). This high degree of conservation suggests a common function for the PH domain in IRS family members. In IRS-1, the PH domain is necessary for efficient tyrosine phosphorylation by the insulin receptor in vivo, al-
though it does not appear to interact directly with the receptor (11–13).

The PTB domain of IRS-4 consists of 101 amino acids (residues 231–331) and exhibits a high degree of homology with this domain in IRS-1, IRS-2, and IRS-3 (66, 62, and 43% identity, respectively). The crystal structure of the IRS-1 PTB domain complexed with a 9-residue Tyr(P) peptide modeled on the residues surrounding Tyr-960 in the insulin receptor has been determined (14). Of interest, 13 out of the 15 amino acids in IRS-1 that interact with the bound peptide are identical in IRS-4, including the two arginines whose guanidinium groups contact the phosphate of the Tyr(P) residue (Fig. 3B). Thus, it is likely that the PTB domain in IRS-4 will also be found to bind to the activated insulin receptor by association with the Tyr(P)960 segment.

Both IRS-1 and IRS-2 contain non-PTB domains, which are important for interaction with the insulin receptor. These are a domain immediately downstream of the PTB domain, referred to as the SAIN domain (residues 313–462 in human IRS-1, and IRS-3 (66, 62, and 43% identity, respectively). The crystal structure of the IRS-1 SAIN domain complexed with a 9-residue Tyr(P) peptide modeled on the residues surrounding Tyr-960 in the insulin receptor has been determined (14).

The PH domain of IRS-4 consists of 101 amino acids (residues 231–331) and exhibits a high degree of homology with this domain in IRS-1, IRS-2, and IRS-3 (66, 62, and 43% identity, respectively). The crystal structure of the IRS-1 PH domain complexed with a 9-residue Tyr(P) peptide modeled on the residues surrounding Tyr-960 in the insulin receptor has been determined (14).

The PH and PTB domains from human (h) IRS-1, mouse (m) IRS-2, rat (r) IRS-3, and human IRS-4 were aligned with the PILEUP program (GCG, Wisconsin). Positions where at least 3 out of 4 amino acid residues are identical are on a black background, and positions where 2 out of 4 amino acid residues are either identical or are conserved are on a gray one. The percentage values at the end of each sequence are the percentage of identical amino acids in a pairwise comparison of each to the IRS-4 sequence. The PH and PTB domains of IRS-1 and IRS-2 are 69 and 75% identical to each other, respectively (5). The two conserved Arg residues of the IRS-1 PTB domain that contact the Tyr(P) of the bound peptide similar in sequence to that around Tyr-960 of the insulin receptor (14) are marked with an asterisk.

**Implications**—This study, in conjunction with our recent discovery of IRS-3 (4), raises the question of whether there are additional members of the IRS family to be discovered. A major challenge now is to define the roles of each IRS in signaling from the insulin and IGF-1 receptors and possibly other receptors as well.

**Acknowledgments**—We thank Renee Robinson, John Neveu, and Terri Addona for expertise in the HPLC, peptide sequencing, and mass spectrometry, respectively.

---

2 V. R. Fantin, J. D. Sparling, G. E. Lienhard, and B. E. Lavan, manuscript in preparation.
REFERENCES

1. Myers, M. G., Jr., and White, M. F. (1996) Annu. Rev. Pharmacol. Toxicol. 36, 615–658
2. Waters, S. B., and Pessin, J. E. (1996) Trends Cell Biol. 6, 1–4
3. Lavan, B. E., and Lienhard, G. E. (1993) J. Biol. Chem. 268, 5921–5928
4. Lavan, B. E., Lane, W. S., and Lienhard, G. E. (1997) J. Biol. Chem. 272, 11439–11443
5. Sun, X. J., Wang, L-M., Zhang, Y., Yenush, L., Myers, M. G., Jr., Glasheen, E., Lane, W. S., Pierce, J. H., and White, M. F. (1995) Nature 377, 173–177
6. Kuhne, M. R., Zhao, Z., and Lienhard, G. E. (1995) Biochem. Biophys. Res. Commun. 211, 190–197
7. Beitner-Johnson, D., and LeRoith, D. (1995) J. Biol. Chem. 270, 5187–5190
8. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) J. Mol. Biol. 215, 403–410
9. Dalphin, M. E., Brown, C. M., Stockwell, P. A., and Tate, W. P. (1996) Nucleic Acids Res. 24, 216–218
10. Keller, S. R., Aebersold, R., Garner, C. W., and Lienhard, G. E. (1993) Biochim. Biophys. Acta 1172, 323–326
11. Myers, M. G., Jr., Grammer, T. C., Brooks, J., Glasheen, E. M., Wang, L-M., Sun, X. J., Blenis, J., Pierce, J. H., and White, M. F. (1995) J. Biol. Chem. 270, 11715–11718
12. Volkovitch, H., Schindler, D. G., Hadari, Y. R., Taylor, S. I., Accili, D., and Zick, Y. (1995) J. Biol. Chem. 270, 18083–18087
13. Yenush, L., Makati, K. J., Smith-Hall, J., Ishihashi, O., Myers, M. G., Jr., and White, M. F. (1996) J. Biol. Chem. 271, 24300–24306
14. Eck, M. J., Dhe-Paganon, S., Trub, T., Noite, R. T., and Shoelson, S. E. (1996) Cell 85, 695–705
15. Gustafson, T. A., He, W., Craparo, A., Schaub, C. D., and O’Neill, T. J. (1995) Mol. Cell. Biol. 15, 2500–2508
16. He, W., Craparo, A., Zhu, Y., O’Neill, T. J., Wang, L-M., Pierce, J. H., and Gustafson, T. A. (1996) J. Biol. Chem. 271, 11641–11645
17. Sawka-Verhelle, D., Tartare-Deckert, S., White, M. F., and Van Obberghen, E. (1996) J. Biol. Chem. 271, 5980–5983
18. Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W. G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R. J., Noel, B. G., Birge, R. B., Fajardo, J. E., Chou, M. M., Hanafuza, H., Schaffhausen, B., and Cantley, L. C. (1993) J. Biol. Chem. 268, 767–778
19. Songyang, Z., Shoelson, S. E., McGlade, J., Olivier, P., Pawson, T., Bustelo, X. R., Barbacid, M., Sube, H., Hanafuza, H., Yi, T., Ren, R., Baltimore, D., Ratnofsky, S., Feldman, R. A., and Cantley, L. C. (1994) Mol. Cell. Biol. 14, 2777–2785
20. Patschinsky, T., Hunter, T., Esch, F. S., Cooper, J. A., and Sefton, B. M. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 973–977
21. Sun, X. J., Crimmins, D. L., Myers, M. G., Jr., Miralpeix, M., and White, M. F. (1995) Mol. Cell. Biol. 13, 7418–7428