Characterization and Cloning of a 58/53-kDa Substrate of the Insulin Receptor Tyrosine Kinase*

(Received for publication, October 5, 1995, and in revised form, November 29, 1995)

Tammie C. Yeh‡, Wataru Ogawa‡, Anne G. Danielsen, and Richard A. Roth§

From the Department of Molecular Pharmacology, Stanford University School of Medicine, Stanford, California 94305

A monoclonal antibody has been produced which immunoprecipitates 58- and 53-kDa proteins which are rapidly tyrosine phosphorylated in insulin-treated cells. These proteins can also be tyrosine phosphorylated in vitro by the isolated human insulin receptor. Increased tyrosine phosphorylation of these proteins is also observed in cells expressing a transforming chicken c-Src (mutant Phe-527) and in cells with the activated tyrosine kinase domains of the Drosophila insulin receptor, human insulin-like growth factor I receptor, and human insulin receptor-related receptor. P58/53 did not appear to associate with either the GTPase activating protein of Ras (called GAP) or the phosphatidylinositol 3-kinase by either co-immunoprecipitation experiments or in Far Westerns with the SH2 domains of these two proteins. Since p58/53 did not appear, by immunoblotting, to be related to any previously described tyrosine kinase substrate such as the SH2 containing proteins SHC and the tyrosine phosphatase Syp, the protein was purified in sufficient amounts to obtain peptide sequence. This sequence was utilized to isolate a cDNA clone that encodes a previously uncharacterized 53-kDa protein which, when expressed in mammalian cells, is tyrosine phosphorylated by the insulin receptor.

In the last few years, there has been extensive progress toward an understanding of the mechanism whereby tyrosine kinases such as growth factor receptors elicit subsequent biological responses (1, 2). The identification and isolation of endogenous substrates for these molecules have revealed that various enzymes such as phospholipase Cγ are tyrosine phosphorylated and activated by this modification (3). In addition, other proteins have a sequence which is homologous to a region of c-Src (called the SH2 domain) that allows these proteins to bind to tyrosine-phosphorylated proteins (4). This complex formation can itself stimulate the enzymatic activity of the SH2 containing protein (for example, the binding of the phosphatidylinositol 3-kinase to insulin receptor substrate-1) or redirect it to another cellular compartment where its enzymatic activity is required (for example, the translocation of the GTPase activating protein of Ras to the plasma membrane, the site of its substrate) (4). In the case of the insulin receptor (IR), 5 several cytosolic substrates have been described. These include the most extensively characterized substrate, called insulin receptor substrate-1 (IRS-1), which is tyrosine phosphorylated and subsequently bound by the phosphatidylinositol (PI) 3-kinase as well as several other SH2 containing proteins (5). A variety of experimental approaches have implicated this substrate as playing a role in mediating several biological responses (6–9) although gene knockout mice which lack IRS-1 still exhibit most of their responsiveness to insulin (10, 11). In addition, recent studies have shown that growth hormone, interleukins 4 and 13, interferons-α and γ, and leukemia inhibitory factor can all stimulate the tyrosine phosphorylation of IRS-1 (12–14).

In addition to IRS-1, a number of other tyrosine-phosphorylated proteins have been observed after insulin-stimulation of cells. These include at least one 115-kDa protein which has been found to associate with the SH2 containing tyrosine phosphatase Syp (15, 16) and two 60-kDa proteins which have been found to associate with various SH2 containing proteins including the GTPase activating protein of Ras (called GAP) and the phosphatidylinositol 3-kinase (17–20). These two 60-kDa proteins appear to be distinct since some cells, such as adipocytes, contain predominantly the PI 3-kinase associated 60-kDa protein (17), whereas other cells, such as Chinese hamster ovary cells (CHO), contain predominantly the GAP-associated protein (19). At least one cell type (the rat hepatoma called HTC) appears to contain both of these proteins (20). In prior studies a monoclonal antibody has been generated against the tyrosine-phosphorylated 60-kDa GAP-associated protein in CHO cells and this antibody was found not to recognize the PI 3-kinase-associated 60-kDa protein, further indicating that these two proteins are distinct (19). In the present studies we have therefore sought to produce a monoclonal antibody that recognizes the PI 3-kinase-associated 60-kDa protein. A monoclonal antibody was generated against a 60-kDa tyrosine phosphorylated substrate from the HTC cells. However, this antibody was found to recognize a protein that appeared to be distinct from both the GAP and PI 3-kinase-associated 60-kDa tyrosine phosphorylated proteins. The sequence of this protein revealed that it was indeed unique and thus represents a new substrate for tyrosine kinases such as the IR.

EXPERIMENTAL PROCEDURES

Materials—The following were purchased: phorbol 12-myristate 13-acetate from Calbiochem; horseradish peroxidase-conjugated anti-phosphotyrosine antibody RC20 from Transduction Labs; monoclonal anti-GAP antibody from Santa Cruz Biotechnology; polyclonal anti-SH2.
Substrates of the Insulin Receptor Kinase

Fig. 1. Identification of a monoclonal antibody to p58/53. HTC-IR cells were treated with 0.5 mM vanadate and 1 µM insulin, lysed and the lysates were immunoprecipitated with either a polyclonal antibody to the 85-kDa subunit of PI 3-kinase (p58, a monoclonal antibody to the 60-kDa GAP-associated protein (p60), the new monoclonal antibody called H720, or control mouse immunoglobulin (NMG)). The immunoprecipitates were analyzed by SDS-PAGE and immunoblotted with either anti-phosphotyrosine antibodies (anti-tyr) or H720. Positions of prestained protein markers (in kDa) are indicated.

antibody, polyclonal anti-Syp antibody, and GAP amino-terminal SH2 domain.

Production of Monoclonal Antibodies—Forty 15-cm plates of HTC-IR cells were treated with 0.5 mM sodium vanadate for 1 h in serum-free Dulbecco's modified Eagle's medium and then stimulated with 1 µM insulin for 7 min. After washing with 20 mM Hepes, pH 7.5, 150 mM NaCl (HBS), cells were lysed with lysis buffer A (HBS containing 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 1 mM vanadate, 20 mM β-glycerophosphate) and the lysates were centrifuged at 100,000 g for 1 h. The supernatant was applied to a wheat germ agglutinin-agarose and the bound proteins eluted with HBST containing 100 mM NaCl, 5 mM MgCl2, 5 mM MnCl2, 0.5 mM ATP in 20 mM Hepes, pH 7.5, 0.1% Triton X-100 and analyzed as described above for immunoprecipitates. To prepare the partially purified IR, CHO.T cell lysates were treated with protein G-agarose and the bound proteins were eluted with HBS containing 0.1% Triton X-100.

For in vitro phosphorylation studies were carried out essentially as described previously (19). CHO cells which overexpress human IR (CHO.T) were as described previously (23). CHO cells overexpressing PKCθ alone (CHO-0) or together with the human IGF-I receptor, a chimeric human IRR, or a chimeric Drosophila IR were as described (24). A rat hepatoma cell line overexpressing human IR (HTC-IR) (20) and NIH3T3 cells expressing a mutant transforming c-Src (with phenylalanine replacing tyrosine 527) were treated for 30 min with 0.5 mM vanadate and then with 1 µM insulin.

Microchemistry Facility.

Purification and Amino Acid Sequence of p58—CHO.T cells (40 15-cm plates per preparation) were lysed with lysis buffer B, centrifuged at 100,000 g for 1 h, and the supernatant was designated as the cytosolic fraction. The pellet was then solubilized with lysis buffer A, centrifuged at 100,000 g for 1 h and this supernatant was designated as the particulate fraction. Each fraction was immunoprecipitated with either H720 or control IgG and then the beads were washed twice with each of the following buffers: 1) 20 mM Hepes, pH 7.5, 1 mM NaCl, 1% Triton X-100, 100 mM NaCl, 1% deoxycholate; 2) 20 mM Hepes, pH 7.5, 0.15 M NaCl, 0.1% Triton X-100. The washed beads were heated in the presence of SDS sample buffer and the eluted proteins were analyzed by SDS-PAGE and autoradiography.

Purification and Amino Acid Sequence of p58—CHO.T cells were lysed with lysis buffer B, centrifuged at 100,000 g for 1 h, and the supernatant was preabsorbed with control agarose beads for 3 h to remove proteins which bind to the resin nonspecifically. The adsorbed lysate was then incubated with H720 coupled to Affi-Gel 10 (Bio-Rad). After extensive washing, the adsorbed proteins were released by heating with SDS sample buffer, separated on a 15% sodium dodecyl sulfate-polyacrylamide gel, and electroblotted to nitrocellulose membranes. After staining with Ponceau S (Sigma), the p58 bands were cut out, digested with trypsin, and the resulting peptides were separated by high performance liquid chromatography and two peptides were subjected to microsequencing by the Harvard Microchemistry Facility.

Purification and Sequencing of the cDNA Encoding p53—Four degenerate primers were synthesized based on the sequences of the 14 and 22 amino acid cDNA tryptic peptides from p58 (P1 and P2 are from the 22-amino acid peptide; P3 and P4 are from the 14-amino acid peptide). These primers are as follows: P1: 5'-GGGGATCCATATCAGAACCGATCCCTCGGATTCG-3'; P2: 5'-GGGAATTCGGCGGCTCTAGATATGCTCCTCGGATTCG-3'; P3: 5'-GCGGAATTCCGCGGCTCTAGATATGCGGCTCTAGATATGCTCCTCGGATTCG-3'; P4: 5'-GCGGAATTCCGCGGCTCTAGATATGCTCCTCGGATTCGATCTCCCTCGGATTCG-3'; P5: 5'-GCGGAATTCCGCGGCTCTAGATATGCTCCTCGGATTCG-3'; P6: 5'-GCGGAATTCCGCGGCTCTAGATATGCTCCTCGGATTCG-3'; P7: 5'-GCGGAATTCCGCGGCTCTAGATATGCTCCTCGGATTCG-3'; P8: 5'-GCGGAATTCCGCGGCTCTAGATATGCTCCTCGGATTCG-3'; P9: 5'-GCGGAATTCCGCGGCTCTAGATATGCTCCTCGGATTCG-3'; P10: 5'-GCGGAATTCCGCGGCTCTAGATATGCTCCTCGGATTCG-3'. The underlined nucleotides are additional sequences.
added to include BamHI or EcoRI restriction sites to the PCR fragments. CHO cDNA (Clontech) was first amplified with P1 and P4 as well as P2 and P3. The resulting cDNA bands were individually purified from acrylamide gels and re-amplified with P1 and P2 and screened for the production of a 69-bp fragment. A 600-bp fragment, amplified originally with P2 and P3, was subcloned into pBluescript (Stratagene) using the PCR-generated BamHI and EcoRI sites. The PCR reaction mixtures contained 20 mM Tris, pH 8.4, 50 mM KCl, 1.5 mM MgCl₂, 5 μM each dNTP, 2.5 units of Taq polymerase (Life Technologies, Inc.), and 0.5 μM each primer. Thirty cycles were generally used for the reactions; the annealing temperatures ranged from 45 °C to 55 °C.

The cloned 600-bp PCR product was radiolabeled using [α-³²P]dCTP (Amersham) and the multiprime DNA labeling system (Amersham) and used to screen an oligo(dT)-primed Uni-ZAP XR CHO cDNA library (Stratagene) as well as a random and oligo(dT)-primed xgt11 CHO cDNA library (Clontech). Positive clones were subcloned into pBluescript and sequenced with the U.S. Biochemical Corp. Sequenase 2.0 kit (Amersham). A full-length clone was obtained by replacing the incomplete 5' end of the largest Uni-ZAP clone, an EcoRI-AatI fragment, with the 5' end of one of the xgt11 clones. The full-length p53 cDNA was sequenced on both strands and the sequences were analyzed using IntelliGenetics and BLAST. Both the non-redundant and expressed sequence tag data bases were searched.

Expression of the Full-length Clone—BamHI digestion of the full-length plasmid released a 1.8-kilobase fragment containing the entire open reading frame as well as about 200 bp of the 3' untranslated region and this fragment was cloned into the BamHI site of the pCLDN mammalian expression vector (a gift of Drs. John Trill and Dave Pfarr of SmithKline Beecham Pharmaceuticals). COS-7 cells were transiently transfected using the DEAE-dextran method with either the empty pCLDN vector or the vector containing the cloned cDNA. Briefly, 5 ml of DME-H21 media containing 0.1 mM chloroquine, 250 μg/ml DEAE-dextran, and 4–5 μg of either plasmid were added to 10-cm plates of cells at about 80% confluence. After 4 h, the cells were treated with 10% dimethyl sulfoxide in phosphate-buffered saline for 2 min at 37 °C, washed 2 × with phosphate-buffered saline and fresh media was added. Two days later, the cells were serum-starved, treated with 0.5 mM vanadate for 60 min, and then stimulated with 1 μM insulin for 10 min. The cells were lysed and p58/53 was immunoprecipitated with either H720 covalently coupled to Affi-Gel 10 (for the H720 Western blot) or H720 bound to protein A-agarose (for the anti-phosphotyrosine Western blot) and analyzed as described earlier.

RESULTS AND DISCUSSION

Generation of Hybridomas and Identification of p58/53—Tyrosine-phosphorylated proteins from the HTC-IR cells (which include both the GAP and PI 3-kinase associated 60-kDa proteins) were purified and injected into mice. The lymphocytes from these mice were fused to myeloma cells and the resulting hybridomas were screened for the production of antibodies to tyrosine-phosphorylated proteins by immunoprecipitation and Western blotting with anti-phosphotyrosine antibodies. Twenty-three hybridomas were identified as producing antibodies to tyrosine-phosphorylated proteins; however, only 3 of these antibodies appeared to recognize proteins whose tyrosine phosphorylation was stimulated by insulin treatment. One of these antibodies appeared to be directed against the 85-kDa subunit of the PI 3-kinase while another antibody associated with either of these two tyrosine-phosphorylated proteins.

Fig. 3. Effect of IR overexpression and vanadate on the insulin-stimulated tyrosine phosphorylation of p58/53. CHO or CHO-T cells were incubated with or without 0.5 mM vanadate for 15 min and then stimulated with 1 μM insulin for 7 min, as indicated. Cell lysates were immunoprecipitated with H720 and the bound proteins were eluted and analyzed by SDS-PAGE and Western blotting with anti-phosphotyrosine antibodies.

Fig. 2. Inability of tyrosine-phosphorylated p58/53 to be recognized by either the SH2 domains of GAP or p85 or by the SH3 domain of GAP. The 60-kDa tyrosine-phosphorylated PI 3-kinase-associated protein (immunoprecipitated from HTC-IR cells with antibodies to p85), the GAP-associated protein (immunoprecipitated from insulin-treated CHO.T cells with the p60 antibody), and p58/53 (immunoprecipitated from insulin and vanadate-treated CHO.T cells with monoclonal antibody H720) were analyzed by SDS-PAGE and blot with anti-phosphotyrosine antibodies (anti-ptyr), the p12 contained either GAP or p85 or the SH3 domain of GAP.
Associated 60-kDa protein or with antibodies to the PI 3-kinase (Fig. 1). The simplest explanation of these results was that H720 recognized two related tyrosine-phosphorylated proteins of 58 and 53 kDa and that both of these proteins were distinct from the previously described GAP and PI 3-kinase-associated 60-kDa tyrosine phosphorylated proteins.

To further test this hypothesis, p58/53 and the two 60-kDa tyrosine-phosphorylated proteins were immunoprecipitated from insulin-treated HTC-IR or CHO.T cells, electrophoresed on SDS gels, transferred to nitrocellulose, and blotted with either the SH2 domains of GAP or the 85-kDa subunit of PI 3-kinase or the SH3 domain of GAP. As expected, the GAP-associated p60 was recognized by the SH2 domain of GAP whereas the PI 3-kinase-associated 60-kDa protein was specifically recognized by the SH2 domain of the 85-kDa subunit of PI 3-kinase (Fig. 2), further indicating the distinct nature of these two proteins (19). In contrast, p58/53 was not recognized by either SH2 domain nor by the SH3 domain of GAP (Fig. 2). In other experiments, the SH2 domains of abl, phospholipase Cγ, Syp, Grb2, Src, and SHC were also found not to bind to tyrosine-phosphorylated p58/53 (data not shown). Further evidence for the distinct nature of p58/53 and the two 60-kDa proteins came from studies showing that the H720 antibodies did not immunoprecipitate either GAP or the PI 3-kinase in association with p58/53 (data not shown). Finally, p58/53 could be depleted from cell lysates with antibody H720 without affecting the levels of the GAP-associated p60 (data not shown).

All of these data argue for the distinct nature of p58/53.

Characterization of p58/53—To further test whether the IR was responsible for the tyrosine phosphorylation of p58/53, CHO cells and CHO cells overexpressing the IR (CHO.T) were treated with or without insulin in the presence or absence of vanadate, a tyrosine phosphatase inhibitor. The insulin-stimulated tyrosine phosphorylation of p58/53 was much more readily observed in the cells overexpressing IR and this phosphorylation was potentiated by the presence of vanadate (Fig. 3). The insulin-stimulated increase in p58/53 tyrosine phosphorylation was observed after only 1 min of insulin treatment (Fig. 4A) and a dose response with different concentrations of insulin showed that the insulin-stimulated increase in p58/53 tyrosine phosphorylation closely paralleled the activation of the IR kinase activity (Fig. 4B). These results were consistent with the hypothesis that the IR was phosphorylating p58/53. This hypothesis was directly tested by incubating H720 immunoprecipitates with or without purified IR in the presence of ATP. In the presence of IR, but not in its absence, both p58 and p53 were tyrosine phosphorylated, indicating that they could serve as direct substrates of the IR.
To test whether p58/53 were substrates of other tyrosine kinases, we first examined the ability of other members of the IR family to phosphorylate these proteins. In CHO-PKC cells overexpressing either the human IGF-I receptor or chimeric receptors with the cytoplasmic domains of the 
\textit{Drosophila} IR or the human insulin receptor-related receptor and the extracellular domain of the IR, insulin was found to stimulate the tyrosine phosphorylation of p58/53 (Fig. 5). In contrast, in the parental cells, no insulin-stimulated tyrosine phosphorylation of these proteins was observed, indicating that the insulin-stimulated increase in tyrosine phosphorylation required the presence of these expressed receptors. Since in prior studies activation of PKC was found to inhibit the \textit{in situ} tyrosine phosphorylation of several other substrates of the various members of the IR family (24), we also tested whether the pretreatment of these cells with an activator of PKC, phorbol 12-myristate 13-acetate, affected the ability of these receptors to tyrosine phosphorylate p58/53. In each case, PKC activation inhibited the insulin-stimulated tyrosine phosphorylation of p58/53 (Fig. 5). In studies of other cells, neither epidermal growth factor, nerve growth factor, nor platelet-derived growth factor were found to stimulate an increase in tyrosine phosphorylation of p58/53 although p58/53 was found to have increased tyrosine phosphorylation in cells expressing a transforming c-Src (the F527 mutant) (data not shown). Since this Src has previously been shown to activate endogenous IGF-I receptors (26), it is possible that the increased phosphorylation of p58/53 is mediated via this receptor.

To determine whether p58/53 was associated with other proteins in the cell, CHO.T cells were metabolically labeled with radioactive methionine and cysteine, treated with or without insulin, and analyzed by SDS-PAGE and immunoblotting with anti-phosphotyrosine.
Exactly the same pattern was observed if the cells were treated with insulin (data not shown). A small amount of a tyrosine-phosphorylated 45-kDa band was also observed in p58 precipitates from CHO.T cells (Figs. 2 and 4), suggesting that this lower molecular weight band may be either a proteolytic fragment of p58/53 or an alternate form of this protein. Interestingly, both p58 and p53 (as well as the 45-kDa band) appear to be primarily present in the particulate fraction of the lysate (Fig. 6). This location of the protein was also unaffected by the addition of insulin to the intact cells prior to lysis (data not shown).

To determine the tissue distribution of p58/53, lysates of different mouse organs were immunoprecipitated with H720 and the immunoprecipitates were phosphorylated in vitro with isolated IR. For comparison, the lysates were also immunoprecipitated with the antibody to the GAP-associated 60-kDa protein and these immunoprecipitates were also phosphorylated in vitro with the isolated IR. Of the tissues tested (brain, spleen, muscle, and liver), p58/53 was found to be highest in brain (Fig. 7). In contrast, the GAP-associated p60 was found to be most abundant in spleen (Fig. 7), further indicating the distinct nature of these proteins.

In addition to the GAP and PI 3-kinase-associated 60-kDa tyrosine-phosphorylated proteins, at least two other substrates of tyrosine kinases have been described which also have molecular masses close to p58. These include the tyrosine phosphatase Syp (27) and the linker protein SHC (28). Immunoprecipitates with antibodies to these two proteins showed that their tyrosine phosphorylation patterns were quite distinct from that of p58/53 and that the p58/53 proteins immunoprecipitated by monoclonal antibody H720 did not react with antibodies to either of these two proteins (Fig. 8). Thus, these studies suggested that p58/53 is a previously uncharacterized substrate for tyrosine kinases and further indicates that it does not associate with either of these two SH2-containing proteins.

Cloning a cDNA Encoding p58—

Since p58/53 appeared to be a new substrate for tyrosine kinases, sufficient protein was purified from CHO.T for sequence analysis by the use of H720 affinity columns. The p58 band was digested with trypsin and the resulting peptides isolated by high performance liquid chromatography. The sequences of two peptides were obtained (EL-GDMDVLFQMAGVHR and SNLVISDPIPGAKPLPVPPELA).
the complete coding region. In addition, the 3’-terminus contained the sequences of the two original tryptic peptides (underlined) and a poly(A) tail. A search of several databases did not reveal any substantial homology of the deduced p58/53 sequence with other known proteins although several expressed sequence tags were identified as having high identity with the isolated cDNA sequence, including one from PC12 cells and another from macrophages (29, 30).

To test whether the protein product of the isolated cDNA was recognized by the monoclonal antibody H720, the cDNA was subcloned into the mammalian expression vector pcCLDN and either this plasmid or the parental vector was transiently transfected into COS cells. The monoclonal antibody to p58/53 specifically immunoprecipitated a single H720 immunoreactive band from COS cells transfected with the isolated cDNA but not from COS cells transfected with the vector control (Fig. 10). To test whether the expressed protein could be tyrosine phosphorylated, the plasmids were transfected into either CHO.T cells or into COS cells either with or without a plasmid encoding the IR (23). The cells were treated with insulin and vanadate, lysed, and H720 immunoprecipitates from the lysates were immunoblotted with anti-phosphotyrosine antibodies. In the CHO.T cells transfected with the isolated cDNA, an increase in the tyrosine phosphorylation of only the p53 band was observed over that seen in the control cells transfected with the vector alone (Fig. 10). In the COS cells transfected with only the isolated cDNA, a single tyrosine-phosphorylated band was observed that co-migrated with the lower (p53) band in CHO.T cells (Fig. 10). In COS cells transfected with only the IR encoding plasmid, the tyrosine phosphorylation of the endogenous p58/53 was observed. In contrast, in the cells transfected with both the IR and the p53 plasmid, a much greater increase in the tyrosine phosphorylation of the p53 band was observed compared to the cells transfected with either plasmid alone (Fig. 10). In other studies, insulin treatment was found to stimulate a 2- to 3-fold increase in the tyrosine phosphorylation of p53 in COS cells transiently transfected with the isolated cDNA and the IR. These results indicate that the isolated cDNA encodes the 53-kDa tyrosine-phosphorylated band observed in CHO.T cells. Since the peptide sequence utilized for isolating this cDNA came from p58, these results further support the hypothesis that p58 and p53 are highly related, most likely due to alternative splicing of the mRNA or possibly due to post-translational modifications. The relative levels of these two different forms of the protein differed in various cells and tissues, with HTC cells having predominantly p58 whereas brain had predominantly p53.

**Fig. 10. Transient expression of p53 and its tyrosine phosphorylation by IR.** CHO.T or COS-7 cells were transiently transfected with control pcCLDN vector (V), pcCLDN containing the cDNA (p53), and/or a plasmid encoding the IR (IR). P58/53 was immunoprecipitated from the cell lysates and analyzed by SDS-PAGE and immunoblotting with either the monoclonal antibody to p58/53 or anti-phosphotyrosine (anti-pyr). For the tyrosine phosphorylation experiments, the cells were stimulated with 0.5 mM vanadate and 1 μM insulin prior to lysis.

CONCLUSIONS

In the present studies, a monoclonal antibody has been produced against 58/53 kDa proteins which rapidly become tyrosine phosphorylated in CHO.T and HTC-IR cells treated with insulin. Although this molecular mass is close to several previously described substrates for tyrosine kinases including a 60-kDa GAP-associated and a 60-kDa PI 3-kinase-associated protein, the 58/53-kDa proteins did not appear to be either of these two substrates since they did not associate with either GAP or PI 3-kinase in vitro or in vivo. Also, the monoclonal antibody to p58/53 did not react on Western blots with either of these two other substrates. The purification of the p58/53-kDa proteins allowed us to obtain the sequence of two peptides from p58 which was used to obtain a cDNA which encodes for p53. The deduced sequence of p53 indicates that this protein is a previously unidentified substrate for tyrosine kinases. Interestingly, this protein appears to be primarily in the particulate fraction of cell lysates, possibly suggesting that this protein is either membrane-associated or in a particular subcellular compartment. In this regard, it is of interest that the carboxyterminal 3 amino acids (A-R-F) of p53 fits a consensus sequence (SAGCN)-(RKH)-(LIVMAF) for targeting to peroxisomes although this is a relatively uncommonly utilized combination of amino acids (31). Also, a stretch of 46 amino acids in the carboxy tail of p53 (residues 408 to 454) were identified by BLAST and MOST as exhibiting homology with the SH3 domains of 2 yeast proteins, called BOB1 and BEB1 (32, 33). The presence of a possible SH3 domain in p53 would also be consistent with its location in the particulate fraction of cells since this motif is often found in cytoskeletal-associated proteins and would suggest a role for this protein in cell signaling (34).

Acknowledgements—We thank Dr. Roman Herrera for performing some of the SH2 blotting experiments, Dr. Ira Goldfine for the HTC-IR cells, Dr. Edgar Wood for the SH2 domain constructs, and Drs. John Trill and Dave Pfarr for the pcCLDN expression vector. We are also grateful to Dr. Christos Ouzounis for help in the analysis of the p58/53 sequence and Drs. Mark Rutherford and Lawrence Schook for their p53 expressed sequence tag plasmid.
REFERENCES

1. Lee, J., and Pilch, P. F. (1994) Am. J. Physiol. 266, C319–334
2. Fantl, W. J., Johnson, D. E., and Williams, L. T. (1993) Annu. Rev. Biochem. 62, 453–482
3. Wahl, M. I., Jones, G. A., Nishibe, S., Rhee, S. G., and Carpenter, G. (1992) J. Biol. Chem. 267, 10447–10456
4. Pant, W. J., Johnson, D. E., and Williams, L. T. (1993) Annu. Rev. Biochem. 62, 453–482
5. Quon, M. J., Butte, A. J., Zarnowski, M. J., Sesti, G., Cushman, S. W., and Taylor, S. I. (1994) J. Biol. Chem. 269, 27920–27924
6. Waters, S. B., Yamauchi, K., and Pessin, J. E. (1993) J. Biol. Chem. 268, 22231–22234
7. Rose, D. W., Saltiel, A. R., Majumdar, M., Decker, S. J., and Olefsky, J. M. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 797–801
8. Ellis, L., Clauser, E., Morgan, D. O., Edery, M., Roth, R. A., and Rutter, W. J. (1986) Cell 45, 721–732
9. Araki, E., Lipes, M. A., Patti, M. E., Bruning, J. C., Haag, B., Johnson, R. S., and Kahn, C. R. (1994) Nature 372, 182–186
10. Welham, M. J., Learmonth, L., Bone, H., and Schrader, J. W. (1995) J. Biol. Chem. 270, 12286–12296
11. Argetsinger, L. S., Hsu, G. W., Myers, M. G., Jr., Billestrup, N., White, M. F., and Carter-Su, C. (1995) J. Biol. Chem. 270, 14685–14692
12. Uddin, S., Yenush, L., Sun, X., Sweet, M. E., White, M. F., and Platania, L. C. (1995) J. Biol. Chem. 270, 15938–15941
13. Pliakis, K. L., and Saltiel, A. R. (1994) J. Biol. Chem. 269, 21239–21243
14. Javitch, J. A., and Lienhard, G. E. (1993) J. Biol. Chem. 268, 5921–5928
15. Kavacina, K. S., and Roth, R. A. (1993) Biochem. Biophys. Res. Commun. 192, 1303–1311
16. Hosono, Y., Shii, K., Ogawa, W., Matsuba, H., Yoshida, M., Okada, Y., Yokono, K., Kasuga, M., Baba, S., and Roth, R. A. (1994) J. Biol. Chem. 269, 11488–11502
17. Sung, C. K., and Goldfine, I. D. (1992) Biochem. Biophys. Res. Commun. 189, 1024–1030
18. Wood, E. R., McDonald, O. B., and Sahyoun, N. (1992) J. Biol. Chem. 267, 14136–14144
19. Gieny, J. R. (1991) Methods Enzymol. 201, 92–100
20. Ellis, L., Clauser, E., Morgan, D. O., Edery, M., Roth, R. A., and Rutter, W. J. (1986) Cell 45, 721–732
21. Danielson, A. G., Liu, F., Hosono, Y., Shii, K., and Roth, R. A. (1995) J. Biol. Chem. 270, 21600–21605
22. Kuhne, M. R., Zhao, Z., Rowles, J., Lavan, B. E., Shen, S. H., Fischer, E. H., and Lienhard, G. E. (1994) J. Biol. Chem. 269, 15833–15837
23. Peterson, J. E., Jelinek, T., Kaleko, M., Siddle, K., and Weber, M. J. (1994) J. Biol. Chem. 269, 27315–27321
24. Fing, G. S., Hui, C. C., and Pawson, T. (1993) Science 259, 1607–1611
25. Pellici, G., Lanfrancone, L., Grignani, F., McGlade, J., Cavallo, F., Forni, G., Nicoletti, I., Grignani, F., Pawson, T., and Pellici, P. G. (1992) Cell 70, 93–104
26. Lee, N. H., Weinstock, K. G., Kirkness, E. F., Earle-Hughes, J. A., Fuldner, R. A., Marmaaras, S., Glodek, A., Gocayne, J. D., Adams, M. D., Kerlavage, A. R., Fraser, C. M., and Venter, J. C. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8303–8307
27. Yang, S., Schook, L. B., and Rutherford, M. S. (1995) Mol. Immunol. 32, 733–742
28. de Hoop, M. J., and Ab, G. (1992) Biochem. J. 286, 657–669
29. Tatsus, R. L., Altschul, S. F., and Koonin, E. V. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 12091–12095
30. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) J. Mol. Biol. 215, 403–410
31. Mayer, B. M., and Eck, M. J. (1995) Curr. Biol. 5, 364–367

Substrates of the Insulin Receptor Kinase