A New Tyrosine-phosphorylated 97-kDa Adaptor Protein Mediates Interleukin-2-induced Association of SHP-2 with p85-Phosphatidylinositol 3-Kinase in Human T Lymphocytes*

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Interleukin (IL)-2 is a major cytokine that controls differentiation and proliferation of T lymphocytes. In this report we characterize an as yet unidentified 97-kDa protein that is a major tyrosine kinase substrate in IL-2-stimulated cells. pp97 was found to associate with the p85-p110 phosphatidylinositol 3-kinase complex, the Src homology 2 (SH2) domain-containing tyrosine phosphatase SHP-2, and the adaptor molecules CrkL and Grb2. We demonstrate that these interactions are directly mediated through the SH2 domains of CrkL, p85, and SHP-2 and through the SH3 domains of Grb2. pp97 was found to mediate the IL-2-induced interaction between p85 and both a phosphorylated and a non-phosphorylated form of SHP-2. In this study we show that pp97 behaves as a docking protein and associates with at least CrkL, p85, and SHP-2 in the same multimolecular complex. We thus characterized pp97 as a new tyrosine kinase substrate in human T lymphocytes which might play a central role in the regulation of several pathways activated by IL-2.

Interleukin (IL)1-2 is a major cytokine that controls the transition from G1 to the S phase of the cell cycle and thus the proliferation of antigen-activated T lymphocytes. The high affinity IL-2 receptor (IL-2R) is composed of three subunits, α, β, and γ, the latter being shared with the IL-4, -7, -9, and -15 chains. It has been shown that the first conformational changes induced either by the phosphorylation of α or by association of the p85-SH2 domains with non-receptor tyrosine kinases that are physically associated with the IL-2Rβ and -γ chains. It has been shown that the first steps of IL-2 signaling depend upon the activation of several tyrosine kinases of the Jak, Syk, and Src families (3–6). The IL-2 receptor itself becomes phosphorylated on tyrosine residues, which creates docking sites for a number of SH2 or phosphotyrosine binding domains containing signaling molecules (7, 8). Among these, the adaptor protein Shc is recruited on a tyrosine residue located at position 338 of IL-2Rβ (9). Shc is then tyrosine-phosphorylated leading to the recruitment of the Grb2-Sos complex responsible for the activation of the mitogen-activated protein kinase pathway via Ras (10). Grb2 is a signaling adaptor molecule composed of one SH2 and two SH3 domains, the latter mediating the constitutive association with Sos, the guanosine nucleotide exchange factor for Ras (11).

IL-2 has also been reported to increase the activity of phosphatidylinositol 3-kinase (PI3K) (12–15). PI3K activated in response to IL-2 is a type I PI3K composed of two subunits, a p85 regulatory subunit (p85) and a p110 catalytic subunit. p85 contains one SH3 domain, two proline-rich motifs, and two SH2 domains (16, 17). Through its multiple possibilities of protein-protein interactions, p85 is responsible for the recruitment of p110 to the membrane and its activation. The lipid kinase activity of PI3K leads to the phosphorylation of phosphatidylinositol on the D3 position of the inositol ring. PI3K was found to be critical for the transduction of anti-apoptotic and proliferative signals as it regulates Bel-2 expression and c-Myc activation, possibly through a pathway involving Akt/PKB and p70S6 kinases (18, 19). PI3K activation appears to result from conformational changes induced either by the phosphorylation of p85 (20) or by association of the p85-SH2 domains with tyrosine residues located in a YXXM (Y indicates tyrosine, X indicates any amino acid, and M indicates methionine) environment (21–23). This type of association recruits PI3K to the membrane receptor complex, a required and sufficient event to mediate activation, as recently emphasized by the observation that direct targeting of p110 to the cell membrane results in constitutive activation of PI3K (24). However, the mechanism of activation of PI3K in response to IL-2 remains to be determined since p85 tyrosine phosphorylation is not seen in most cells, in particular in the Kit 225 cell line used in this study, and since the IL-2 receptor does not contain a YXXM motif suggesting the involvement of intermediary molecules.

Adachi et al. (25) reported the tyrosine phosphorylation of the SH2 domain-containing tyrosine phosphatase SHP-2 in response to IL-2. SHP-2 (also called Syp, PTP1D, PTP2C, or SHPPT) is a ubiquitously expressed 70–72-kDa cytosolic phosphatase that contains two SH2 domains in tandem (26–28). The mechanism of SHP-2 activation may be quite similar to those described for PI3K as they seem to involve either tyrosine phosphorylation (29) or SHP-2 association with other tyrosine-phosphorylated proteins (30, 31). SHP-2 has been described to transduce positive signals by binding directly to various receptor protein tyrosine kinases such as PDGF-, EGF-, or insulin receptors or to adaptor molecules such as GST, glutathione S-transferase; PI3K, phosphatidylinositol 3-kinase; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; IRS, insulin receptor substrate.
IL-2-stimulated Tyrosine Phosphorylation of 97-kDa Protein

IRS-1 or Grb2 leading to activation of Ras (32, 33). IL-2-induced activation of SHP-2 was shown to be a Jak-independent mechanism probably mediated through Src kinases or a new type of kinase and is strictly dependent upon the expression of the IL-2Rβ and -γ chains (25, 34). The identification of SHP-2-interacting proteins will be of interest in order to understand its function in IL-2 signaling and to identify potential downstream pathways.

We recently reported that IL-2 induces the tyrosine phosphorylation of the proto-oncogene product CrkL and regulates its association with p120Cbl and with the p85 regulatory subunit of PI3K (35). CrkL is a member of the Crk family, a group of SH2 and SH3 domains containing adaptor proteins, comprising the two splice products Crk-I and Crk-II and the product of a separate but related gene, Crkl (36–38). During the course of this previous study we observed a p97–100-kDa protein associated with CrkL and phosphorylated on tyrosine residues in response to IL-2. In this report, we demonstrate that pp97 phosphoprotein participates in the formation of a multimolecular complex containing the signaling molecules CrkL, Grb2, SHP-2, and the PI3K regulatory p85 subunit. pp97 is one of the major early tyrosine kinase substrates in IL-2 signaling, and it directly associates with CrkL, p85, and SHP-2 through their respective SH2 domains. We also provide evidence that pp97 mediates the interaction between p85 and SHP-2 that occurs in response to IL-2. Given its characteristic features, as described here, it is possible that pp97 may be related to phosphoproteins that display similar molecular mass and migration properties and that have been recently described. Indeed, three independent reports have described 97- and 100-kDa phosphoproteins in Bcr-Abl-transformed cells or in response to M-CSF or IL-3 (39–41). Similar to the pp97 described in our study, these phosphoproteins directly associate with SHP-2 and p85. The apparently central role of pp97 in the formation of a large and unique complex containing CrkL, SHP-2, and PI3K suggests that pp97 might exert an important function at the crossroad of three major signaling pathways triggered by IL-2.

MATERIALS AND METHODS

Cell Lines and Culture Conditions—The human T-lymphocytic leukemia-derived, IL-2-dependent Kit 225 cell line was kindly provided by Dr. T. Hori (Kyoto University, Japan (42)). Cells were maintained in culture medium (RPMI 1640, 10% fetal calf serum, and antibiotics) supplemented with 0.5 mM recombinant human IL-2 (generously provided by P. Ferrara, Sanofi, France). Kit 225 cells were deprived of IL-2 by washing three times and resuspending the cells in culture medium without IL-2 at 5 × 10^6/ml for 48 h. For stimulation, IL-2-deprived Kit 225 cells were resuspended in culture medium at 2 × 10^7/ml and incubated at 37 °C without (control) or with 1 nM recombinant IL-2 for various periods as specified. Cells were harvested by centrifugation and lysed at 5 × 10^6/ml in cold 0.5% Triton lysis buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 0.5% Triton X-100, 10 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM vandate, and 5 μg/ml each leupeptin, aprotinin, and pepstatin). Lysis was performed for 30 min on ice. Lysates were clarified by centrifugation at 15,000 × g for 20 min at 4 °C.

Human peripheral blood mononuclear cells were obtained from volunteer anonymous healthy donors. Mononuclear cells were separated by Ficoll-Hypaque gradient centrifugation. Non-adherent lymphocytes were stimulated with 1 nM recombinant human IL-2 supplemented with 0.5 nM recombinant human IL-2 (genetically and sterically pure, Upstate Biotechnology) and antibiotics) supplemented with 0.5 nM recombinant human IL-2 and incubated for 0.5 h at room temperature with either 5% dry milk or 3% bovine serum albumin in Tris-buffered saline (TBS), 0.5% Tween (TBS-T). Filters were washed 4 times in TBS-T and incubated for 1.5 h with optimal concentrations of primary antibodies diluted in TBS, 0.1% Tween. Following 4 additional washes in TBS-T, the filters were further incubated for 45 min with horseradish peroxidase-conjugated secondary antibodies (sheep anti-mouse Ig from Amersham Pharmacia Biotech or goat anti-rabbit Ig from Dako). Visualization was performed using Amersham Pharmacia Biotech ECL reagents and autoradiographic films.

GST Fusion Proteins—A plasmid encoding for GST-Grb2 full-length fusion protein was kindly provided by Dr. P.-O. Couraud (ICGM, Paris, France) and was used as a template to amplify the Grb2-SH2 domain in a BamHI-EcoRI-linearized PGEX-4T. For this purpose we used the following pair of primers: AGA CGG ATC CAT GAA ACC ACA TCC GTG GTT T' (sense) and AGA CAG ATT CTC AGA CCT ATG TCG GCT GTG G (antisense). GST-CrkL constructs (CrkL full-length, SH2, and SH3N) were a kind gift from B. J. Druker (Oregon Health Sciences University, Portland, OR). Plasmids encoding for the SHP-2 SH2 (N-terminal, C-terminal, and tandem) were kindly provided by J. C. Cambier (National Jewish Institute, Denver, CO) and A. Kazlauskas (Schepps Eye Research Institute, Boston, MA). A polyclonal antibody was also raised in the laboratory against a GST fusion protein containing the N-terminal half (amino acid residues 1 to 343) of human p85α. Isoform-specific antiserum directed against p110α were a kind gift from A. Roche (CNRS EP612, SS 18274, Villiers-le-Bel, France). The monoclonal anti-GST antibody was raised and purified in the laboratory. The Tyr-317 phosphopeptide (PSypYMDMSKDESID) derived from the human Shc protein was provided by C. Garbay (INSERM Unit 266, Paris, France). The Tyr-740 phosphopeptide (GGpYMDMSKDESID) derived from the PDGF receptor was synthesized and Genoeye. Ecdysoglycosidase F/N-glycosidase F mix was obtained from Boehringer Mannheim.

Immunoprecipitation and Immunoblotting—Cell lysates (2.5 to 5 × 10^7 cell equivalent) were precleared with protein G-Sepharose beads. For immunoblotting the PVDF membranes (Amersham Pharmacia Biotech) were blocked for 2 h at room temperature with either 5% dry milk or 3% bovine serum albumin in Tris-buffered saline (TBS), 0.5% Tween (TBS-T). Filters were washed 4 times in TBS-T and incubated for 1.5 h with optimal concentrations of primary antibodies diluted in TBS, 0.1% Tween. Following 4 additional washes in TBS-T, the filters were further incubated for 45 min with horseradish peroxidase-conjugated secondary antibodies (sheep anti-mouse Ig from Amersham Pharmacia Biotech or goat anti-rabbit Ig from Dako). Visualization was performed using Amersham Pharmacia Biotech ECL reagents and autoradiographic films.

For Western Blotting—Proteins were immunoprecipitated, separated, and transferred to PVDF membrane as described above. A first
incubation of the membrane was performed with 5 nM of indicated fusion proteins in TBS, 0.1% Tween, 3% bovine serum albumin for 1 h. The membrane was washed 4 times and incubated with a monoclonal anti-GST antibody for 1.5 h. After 4 washes the membrane was further incubated 45 min with an anti-mouse antibody coupled to horseradish peroxidase. Visualization was performed using Amersham Pharmacia Biotech ECL reagents and autoradiography.

**Glycosylation Analysis—**Proteins were immunoprecipitated from 5 × 10^7 cells and washed four times with lysis buffer. Immunoprecipitates were eluted by boiling 5 min in 20 μl of phosphate buffer, 0.5% SDS, 1% Triton X-100, 0.5 mM sodium phosphate, pH 7.2; 1 mM EDTA, 0.5% SDS, 1% β-mercaptoethanol. Eluates were diluted in 80 μl of digestion buffer (50 mM sodium phosphate, 1 mM EDTA, 0.6 unit of endoglycosidase F) and incubated at 37 °C for 18 h. Reaction was stopped by adding 1 volume of Laemmli’s sample buffer and boiling 5 min.

### RESULTS

**pp97 Associates with p85, SHP-2, CrkL, and Grb2 in Response to IL-2—**We have previously described the IL-2-dependent tyrosine phosphorylation of CrkL in T lymphocytes and its association with the proto-oncogene product Cbl and the p85 regulatory subunit of PI3K (35). CrkL also associates with several unidentified tyrosine-phosphorylated proteins including a relatively faint band at 70–72 kDa, an 85-kDa protein that is constitutively phosphorylated in Kit 225 cells, and a major IL-2-inducible signal at 97–100 kDa. As shown in Fig. 1A pp97 is one of the major phosphoproteins coprecipitated with CrkL and p85 in an IL-2-dependent manner. pp97 is also present in Grb2 immunoprecipitates from IL-2-stimulated cell lysates, albeit at a lower intensity than in p85 immunoprecipitates. The p70–72-kDa tyrosine-phosphorylated protein detected in p85 and Grb-2 immunoprecipitates (also observed in CrkL immunoprecipitates upon longer exposure) was formally identified as the cytosolic tyrosine phosphatase SHP-2 with a specific anti-SHP-2 antiserum. By using the same antibody we demonstrated that pp97 was also coprecipitated with SHP-2 (Fig. 1A, lanes 7 and 8). Upon IL-2 stimulation, p85 and Grb2 become detectable in SHP-2 immunoprecipitates (Fig. 1A, lower panels, lanes 7 and 8). As reported recently, and probably due to a very low stoichiometry of the complexes described, CrkL was not detectable in any co-immunoprecipitation experiment (35). Unexpectedly, SHP-2 immunoblotting revealed two specific bands coprecipitated with p85, whereas only the slower migrating species was detected in association with Grb2. Comparison of the anti-phosphotyrosine (4G10) and SHP-2 immuno- 

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**Fig. 1.** A tyrosine-phosphorylated pp97 interacts with SHP-2, p85, Grb2, and CrkL in IL-2-stimulated Kit 225 cells and normal T-cell blasts. A, 5 × 10^7 Kit 225 cells were untreated (−) or stimulated 5 min with 1 nM IL-2 (+), lysed, and immunoprecipitated with the indicated antibodies. Immunocomplexes were washed, separated on SDS-9% PAGE, and transferred on PVDF. The membrane was immuno- 

![Image](https://via.placeholder.com/150)

**B** 5 × 10^7 T-cell blasts were untreated (−) or stimulated 5 min with 1 nM IL-2 (+). Cells were lysed and immunoprecipitations were performed with pre-immune serum (Pre-I) or anti-p85 (p85). Immunocomplexes were washed, separated, and transferred as above.

becomes detectable by anti-phosphotyrosine immunoblotting in p85 and SHP-2 immunoprecipitates as soon as 30 s following IL-2 stimulation of Kit 225 cells. After 2 min of stimulation pp97 displays a slower migration profile and is detected as a higher molecular weight protein, whereas its level of tyrosine phosphorylation decreases steadily but remains detectable after 60 min of stimulation. As shown in Fig. 2A, pp97 appears to be the main common tyrosine-phosphorylated protein associated with p85 and SHP-2 in IL-2-stimulated cell lysates. Control blots with anti-p85 or anti-SHP-2 (not shown) confirmed the equal loading of all lanes, and also evidenced, as illustrated above in Fig. 1A, that p85 was associated with both a tyrosine-phosphorylated form and a non-phosphorylated form of SHP-2. This result suggests that p85, which is not tyrosine-phosphorylated in IL-2-stimulated human T lymphocytes, may interact indirectly with SHP-2, and the presence of pp97 in both types of immunoprecipitates suggests that pp97 might function as an intermediary molecule between these two proteins.

To analyze further these interactions, GST fusion proteins containing the various domains of p85 and SHP-2 were used in pull-down experiments from IL-2-stimulated cell lysates (Fig. 2B). pp97 was easily detected using the p85(N + C)SH2 con-
domains.

A level of pp97 indicating that IL-2R

stimulated 5 min with 1 nM IL-2, were incubated with 5

micrograms of the indicated fusion proteins. Precipitates were washed and separated as described in A. The membranes were immunoblotted with anti-phosphotyrosine antibody (4G10). The membrane corresponding to the left panel was dehybridized and reprobed with the anti-IL-2Rβ antibody.

pp97 does not correspond with the missing adaptor protein that should link p85 or SH2 to the IL-2R. In agreement with our previously published data, pp97 binds to the full-length construct but did not bind to either p85 SH3 or p85 proline-rich region (PBP). It is not clear why in these experiments pp97 binding to the full-length construct appeared rather weak. This may be due to steric hindrance or conformational factors of the full-length protein that would favor interaction with the p85 tandem SH2 in vitro in the absence of the other p85 domains. In addition, another phosphoprotein, with a broad migration between 75 and 80 kDa also bound GST-p85(ΔN+C)SH2 as well as GST-p85 and was formally identified as the IL-2Rβ chain by specific Western blotting (Fig. 2B, left panel). As observed on this figure the presence of IL-2Rβ does not correlate with the level of pp97 indicating that IL-2Rβ is not likely to mediate the interaction between pp97 and p85. Conversely, pp97 probably does not represent the missing adaptor protein that should link p85 to the IL-2R. In agreement with our previously published data, the tyrosine-phosphorylated protein detected in the p85-SH3 pull-down was identified as p120Cbl (not shown). On the other hand, pp97 also associated with SHP-2 SH2 domains, and this interaction appears to require that both domains be present in tandem in the GST protein, as either SH2 domain alone was quite inefficient. Two additional phosphoproteins are detected that bind to SHP-2 SH2 and remain to be identified.

Since it has been suggested that pp85 may be present in cells in molar excess to the p110 catalytic subunit, and may thus exist as a free form, we investigated whether pp97 could be associated with p85-p110 complexes. To address this question, immunoprecipitation experiments were performed with specific anti-p110α and β antibodies (Fig. 3). pp97 was clearly detected in association with both p110α and p110β, which indeed coprecipitated p85. This result demonstrates that pp97 associates with p85-p110 complexes and might therefore function in the regulation of PI3K activity by IL-2.

p85-SHP-2 Interaction Is Mediated Through p85-SH2 Domain—The SH2-mediated interactions described above were further characterized by peptide competition assays in either p85 or SHP-2 immunoprecipitates. A phosphorylated tyrosine 740-containing peptide (YMDM) derived from the PDGF receptor, which competes with the p85-SH2 domains, was used in p85 or SHP-2 immunoprecipitates. A phosphorylated tyrosine 317-containing peptide derived from Shc (YVNV), which competes with the Grb2 SH2, provided a specificity control in these experiments. As shown in Fig. 4A, the PDGF-R-phosphopeptide displaced pp97 and SHP-2 from a p85 immunoprecipitate. On the other hand, the PDGF-R-phosphopeptide efficiently competed with p85 but did not decrease pp97 in SHP-2 immunoprecipitates (Fig. 4B). The apparent simplicity of the anti-phosphotyrosine pattern, as compared with Fig. 1A, results from a shorter exposure being shown in Fig. 4 to better evidence the effects of peptide competition on pp97 association. Altogether these results show that p85 interacts with pp97 and SHP-2 through its SH2 domains recognizing tyrosine-phosphorylated residues probably located in the consensus YXXM environment. On the other hand the SHP-2-p85 interaction depends upon this YXXM consensus motif but is independent of the phosphorylation of both partners. Additionally, SHP-2-pp97 interaction is mediated via SHP-2 SH2 domains and is not affected by the phosphopeptides used in these experiments. These observations reinforce the hypothesis that p85 and SHP-2 might interact together through a common tyrosine-phosphorylated intermediary molecule.

CrkL Associates with pp97 in Response to IL-2—We recently described the association of CrkL with multiple signaling molecules in response to IL-2 (35 and Fig. 1A). Kinetic experiments shown in Fig. 5 indicate that tyrosine phosphorylation of pp97 is detectable more rapidly in CrkL immunoprecipitates than in p85 or SHP-2 immunoprecipitates (Fig. 2A). It reaches a maximum between 0.5 and 2 min of stimulation, whereas the tyrosine phosphorylation of CrkL was maximum around 10 min. This observation would indicate that the presence of tyrosine-phosphorylated pp97 associated with CrkL is not dependent on the tyrosine phosphorylation of CrkL itself. It is of interest to note that CrkL immunoprecipitates also contain p120Cbl, as described previously, and that pp70–72 SHP-2 protein associates with CrkL with a kinetics similar to that of pp97 (open arrowheads, Fig. 5A).

To characterize further the interactions between CrkL and pp97, CrkL SH2 and SH3 domains were used as GST fusion proteins in pull-down experiments (Fig. 5B). Multiple tyrosine-
phosphorylated proteins are precipitated when using the full-length GST-CrkL, with pp97 representing a major species that appears in IL-2-stimulated cell lysates. A number of these proteins parted between the SH2 and SH3 domains, but pp97 was detectable on both. The lower intensity of pp97 on the domains, as compared with the full-length CrkL, was observed in multiple experiments and suggests that cooperative interactions might occur between CrkL SH2 and SH3 domains for pp97 association (Fig. 5B). These experiments were also performed in the presence of 50 mM phenyl phosphate which should prevent recognition of phosphotyrosine residues by SH2 domains. Indeed, phenyl phosphate inhibited association of all phosphoproteins to the SH2 domain, including pp97, but unexpectedly also inhibited association to the SH3 domain (Fig. 5B, lanes 9 and 10). Western blotting of the membrane with p85 was performed as a control and showed that the effect of phenyl phosphate was fairly specific as p85 association with CrkL-SH2 domains was only reduced to a level consistent with that of the direct association with p85 proline-rich motifs as described previously (35). These results thus suggest that association of pp97 with CrkL may involve a possibly direct interaction with the SH2 domain, whereas association with the SH3 domain is likely to be indirect and mediated by a phosphotyrosine-dependent interaction with an other protein.

Association of pp97 with Grb2 Involves Grb2 SH3 Domains—As shown in Fig. 1A, pp97 was also observed in association with the adaptor molecule Grb2. pp97 associated with Grb2 was easily recognizable by its pattern of phosphorylation and migration, despite a relatively low signal intensity. The time-dependent phosphorylation of pp97 in Grb2 immunoprecipitates (Fig. 6A) appeared to be superimposable to the kinetics observed with CrkL (Fig. 5A). As shown in Figs. 1A and 6A, multiple phosphoproteins are present in Grb2 immunoprecipitates including p120Cbl, an unidentified pp75 and p52 and p46Shc as described previously. In addition a pp70–72 band was detectable just below pp75 and was identified as SHP-2, as shown above in Fig. 1A, and by SHP-2 immunoblotting of the membrane (not shown).

Full-length GST-Grb2 was able to bind pp97, but unexpectedly, using GST fusion proteins of the Grb2 domains; the pp97-Grb2 interaction was found to be specifically mediated through the Grb2 SH3 domains (Fig. 6B). Indeed, pp97 was undetectable in pull-down experiments using a Grb2 SH2 GST fusion, whereas a GST-Grb3–3 fusion protein corresponding to a naturally occurring mutant form of Grb2 expressing a deleted,
nonfunctional SH2 domain could coprecipitate pp97 in response to IL-2. Consistent with our previous findings, Grb3–3 also bound p120Cbl, seen as a doublet at approximately 115–120 kDa. Furthermore, it was observed that when the experiment was performed in the presence of 50 mM phenyl phosphate, which indeed was efficient in displacing the unidentified pp75 from the SH2 domain, pp97 association with Grb3–3 was unaffected, suggesting that this interaction was independent of tyrosine phosphorylation, and might be direct (Fig. 6B).

pp97 Interacts Directly with p85-, SHP-2-, and CrkL-SH2 Domains—At this stage of the study we have characterized pp97 as a protein that interacts with the SH2 domains of CrkL, p85, and SHP-2 and with the SH3 domains of CrkL and Grb2. We were then interested in determining which of these interactions was direct. For this purpose we turned to the far Western technique which is currently one of the most reliable approaches to evidence direct protein-protein interactions between bacterially expressed and whole cell lysate-derived proteins. As described under “Materials and Methods,” these experiments were performed using a constant concentration of 5 nM for each recombinant protein, a concentration determined in preliminary experiments to give negative results with the GST moiety alone. Far Western experiments performed with the p85-SH2 domains fusion protein indicate that p85 binds directly to the tyrosine-phosphorylated pp97 present in p85, SHP-2, CrkL, or Grb2 immunoprecipitates (Fig. 7B). It has to be noticed that the level of the signals obtained in far Western blots followed exactly the level of signals obtained in anti-phosphotyrosine blots (compare with Fig. 1A) with Grb2 giving the lowest intensity signal, thus providing an additional indication that pp97 as seen in far Western is probably the same protein that associates with all four molecules. SHP-2 immunoprecipitates contain the largest amount of pp97 and were therefore used to investigate whether GST-SHP2 or GST-CrkL-SH2 recognized pp97 in far Western experiments (Fig. 7, B and C). The results indicate that both SHP-2 and CrkL could interact directly with pp97 through their SH2 domains. However, the CrkL SH2, which indeed recognized phosphorylated Cbl quite efficiently in Cbl immunoprecipitate from IL-2-stimulated cell lysates (Fig. 7C), gave a lower signal than SHP-2 SH2, possibly due to a lower affinity or to a smaller number of its putative phosphotyrosine target residues in pp97. In parallel experiments no direct interaction could be demonstrated using the Grb2 or CrkL SH3 domains (not shown).

Additionally, results obtained from the experiment shown in Fig. 7A indicated that p85-SH2 domains did not recognize either SHP-2 or CrkL in far Western. This observation is consistent with the hypothesis made from competition peptide experiments and suggests that interactions seen in immunoprecipitations are indirect.

pp97 Simultaneously Associates to p85, SHP-2, and CrkL—To investigate whether p85, SHP-2, and CrkL associate with pp97 in the same complex, we performed immunodepletion experiments using either anti-p85 or anti-SHP2 rabbit sera followed by a final CrkL immunoprecipitation. When p85 was depleted from stimulated lysates, the level of CrkL-associated pp97 was dramatically reduced (Fig. 8A), whereas the phosphorylated Cbl signal, migrating with the 112-kDa marker, was largely unaffected, as described previously (35). These experiments also evidenced that when p85 was depleted, the CrkL-associated SHP-2 became undetectable, suggesting that these three molecules are part of the same complex. Conversely, when SHP-2 immunodepletions were performed, pp97 association with CrkL was also decreased to a similar extent. In addition we have previously reported that CrkL and p85 interacted constitutively via the CrkL SH3 domain and that IL-2 stimulation induced an increased association involving tyrosine-phosphorylated residues. In the experiments shown here, the IL-2-mediated increased association of p85 with CrkL (Fig. 8B, lanes 9 and 10) was no longer detectable (lanes 12...
verse 10) in the absence of pp97 that had been removed as a consequence of SHP-2 depletion, whereas the constitutive association remained unaffected (Fig. 8B, lanes 9 and 11). These results led us to conclude that pp97 may mediate the IL-2-dependent association between p85, SHP-2, and CrkL in a large complex.

**pp97 Is Not Glycosylated**—The typical migration profile displayed by pp97 may be due to post-translational modifications such as glycosylation. To investigate this point, in vitro deglycosylation experiments were undertaken using a mixture of endoglycosidase F/endoglycosidase F. This treatment was shown to be effective in deglycosylating the IL-2Rβ. Indeed, the treatment of IL-2Rβ chain immunoprecipitates resulted in a shift of migration, and the immunoprecipitated proteins appeared to migrate as a lower apparent molecular weight species in comparison with untreated immunoprecipitates (Fig. 9). Under the same conditions however, the p85-associated pp97 did not reveal any modification of its migration profile, whereas the efficiency of deglycosylation can be evidenced by the faster migration pattern of deglycosylated immunoglobulin heavy chains. Taken together these results indicate that pp97 is probably not a N-glycosylated protein.

**DISCUSSION**

Binding of IL-2 to its high affinity receptor leads to a rapid and important increase in tyrosine kinase activity, responsible for the phosphorylation of various substrates which results in the activation of downstream effectors such as PI3K and SHP-2. In this report, we demonstrate that a common 97-kDa protein that is tyrosine-phosphorylated in IL-2-stimulated T lymphocytes coprecipitates with the PI3K p85p110 complex, the tyrosine phosphatase SHP-2, and the SH2-SH3-containing adaptor molecules CrkL and Grb2. As evidenced by pull-down and far Western experiments, pp97 interacts directly with the SH2 domains of p85, SHP-2, and CrkL, suggesting that pp97 may be phosphorylated on multiple tyrosine residues located in the specific environments required for recognition by these SH2 domains. Additionally, pp97 was shown to bind to the SH3 domains of CrkL and Grb2, through interactions which seem to be mediated by different mechanisms as the CrkL-SH3-pp97 association can be disrupted by phenyl phosphate whereas the Grb2-SH3-pp97 interaction cannot. These results indicate that the CrkL SH3 domains do not directly recognize pp97 and that this interaction requires an intermediary molecule that itself associates with pp97 through a phosphotyrosine-dependent mechanism. On the other hand Grb2 interacts with pp97 independently of phosphotyrosine residues. This interaction may be direct, although the presence of an intermediary molecule between Grb2-SH3 domains and pp97 could not be excluded since far Western blotting with the Grb2-SH3 domains did not evidence a direct interaction with pp97. Furthermore, pp97 is only detected through its phosphorylation, and the lack of specific antibodies against pp97 does not allow investigations of a possible constitutive association of non-phosphorylated pp97 with SH3 domains containing proteins.

Since the identity of pp97 has not yet been established, the question of its relationship with other known proteins can only be addressed indirectly. In particular, the recently described SIRP/SHPS SHP-2-associated transmembrane glycoproteins share similar molecular mass and are heavily phosphorylated in response to growth factor stimulation (44–46). In cell fractionation experiments, however, we found that pp97 is mainly observed in the cytosol and thus is unlikely to be a transmembrane protein (not shown). Furthermore, unlike the SIRP/SHPS-glycosylated
transmembrane protein family, the migration profile of pp97 was not modified by treatment with N-glycosidase. Finally, pp97 is not recognized, in Kit 225 cells, by two different antibodies kindly provided by A. Ullrich against murine SIRP (not shown). Gab1, another adaptor protein involved in EGF and insulin signaling has also been recently identified that shares some properties with pp97 (47). In our investigations and in agreement with the data previously published by Holgado-Madruga et al. (47), Gab-1 migrates at a significantly higher apparent molecular mass, closer to 115 kDa, than pp97 does and appears poorly expressed in lymphoid tissues. Additional experiments clearly excluded the possibility that pp97 be one of the already known tyrosine kinase substrates involved in IL-2 signaling such as Vav or STAT5 (not shown).

Between 30 s and 2 min of IL-2 stimulation pp97 is maximally tyrosine-phosphorylated and is detected as a broad band of 90–95 kDa apparent molecular mass and undergoes a migration shift after 2 min. Taken together with its association with the SH2 domains described above, this characteristic migration pattern is reminiscent of similar molecular weight phosphoproteins recently described in other systems (39–41). Carlberg and Rohrschneider (39) and Craddock and Welham (41) described the tyrosine phosphorylation of an unidentified p100 protein associated with SHP-2 in response to M-CSF or IL-3, respectively, and Gu et al. (40) characterized a similar p97 phosphoprotein in Bcr-Abl-transformed and in IL-3-stimulated murine hematopoietic cells. It is thus possible that the pp97 described here, in human IL-2-stimulated cells, may be related to these. However, an answer to this question will have to wait until pp97 is identified or specific antibodies obtained.

As shown in several figures in this report, the tyrosine phosphorylation of pp97, as seen in anti-phosphotyrosine Western blotting, is maximum around the 2-min time point, then declines progressively. In parallel experiments we have also observed that when cells were stimulated by IL-2 in the presence of the phosphatase inhibitor pervanadate, the phosphorylation of pp97 was greatly increased (not shown). Although we did not address this question specifically, it is tempting to speculate that SHP-2, which associates with pp97, might be responsible for its dephosphorylation. Another characteristic feature of pp97 is a migration shift detectable after 2 min of stimulation. This shift is unlikely to be dependent on tyrosine phosphorylation since it persists while the amount of phosphotyrosine residues in pp97 decreases. The modifications of pp97 migration profile may be due to additional post-translational modifications such as ubiquitination or serine phosphorylation that require further investigation.

We also provide evidence, by peptide competition assays and immunodepletion experiments, that a large proportion of pp97 is simultaneously associated with CrkL, p85, and SHP-2. Furthermore, p85 and SHP-2 were found to associate directly with pp97 through their SH2 domains, whereas the interaction between p85 and SHP-2 is likely to depend upon an intermediary molecule. The observation that this p85-SHP-2 interaction is nevertheless disrupted by a pYXXM peptide, and thus depends upon the p85 SH2, and is only observed in the presence of pp97, led us to propose a hypothetical model (Fig. 10) in which pp97 mediates the interaction between p85 and SHP-2. The tyrosine phosphorylation of SHP-2 has been shown recently, but it is the first time that SHP-2 is reported to interact with other signaling molecules in response to IL-2. The function of SHP-2 tyrosine phosphorylation is not yet understood and may lead to SHP-2 activation or may be a required event to mediate protein-protein interactions. Regarding IL-2 signaling, it is not yet known if SHP-2 phosphatase activity is required or if it functions solely as an adapter molecule. We provide evidence that p85 interacts with both a phosphorylated and a non-phosphorylated form of SHP-2 through a YXXM motif-dependent mechanism. YXXM motifs are present in SHP-2, but there are no indications as to whether they are phosphorylated or not in response to IL-2, and we have found no evidence that such motifs, if phosphorylated, are involved in p85/SHP-2 association. Rather the model would be consistent with our data showing that p85 can interact with SHP-2 independently of its tyrosine phosphorylation. This model also depicts the direct association of pp97 with CrkL and accounts for its presumed role in the association of CrkL with SHP-2. Since we have observed in far Western experiments that CrkL-SHP2 did not bind pp97 very efficiently, CrkL may primarily associate with SHP-2 via a very indirect mechanism involving p85 as well as pp97, and the size of this complex probably explains at least in part the low amount of SHP-2 detectable in CrkL immunoprecipitates. On the other hand pp97 interacts with Grb2, and the scheme shown in Fig. 10 only illustrates the Grb2-SH3-pp97 interaction, as supported by our pull-down experimental data. However, Grb2 may also participate in this complex in several other ways, recognizing p85 proline motifs or phosphorylated SHP-2 (48, 49). Grb2/SHP-2 association has already been reported in response to PDGF and was shown to mediate the recruitment of Grb2 to the PDGF receptor leading to the activation of Ras and the mitogen-activated protein kinase pathway (32, 33). The scheme shown in Fig. 10 is based upon pull-down and immunodepletion experiments performed at 5 min following IL-2 stimulation. It is possible, although this has not been tested, that the complex behaves in a dynamic way, with a stoichiometry that could vary with time. Such an hypothesis would be consistent with our observation that at a later stimulation time pp97 appears to be present in larger amounts on p85 and SHP-2 than on CrkL and Grb2 immunoprecipitates.

The multiple protein interaction domains present in this...
large complex provide several possible means by which it could be recruited to the cell membrane and the IL-2R. Although pp97 is largely a cytosolic protein, a significant proportion can be found in membrane fractions after IL-2 stimulation (not shown). It is of interest that PI3K activity has been reported to associate with IL-2 receptor in response to IL-2 despite the absence of XXM motifs in either chains of the IL-2R (14). IRS-1 and IRS-2 proteins are large, heavily tyrosine-phosphorylated docking proteins primarily described in insulin receptor signaling (50). IRS-2, also called 4PS, was initially reported to be tyrosine-phosphorylated in response to IL-4 in hematopoietic cells. The tyrosine phosphorylation of these proteins has recently been described in response to stimulation of the cyto kinase receptors that share the common γ chain (i.e. IL-2, -4, -7, and -15) (51). Indeed, we observed a pp180 associated to p85 in response to IL-2 that may represent IRS-1 or IRS-2 (Fig. 1, lane 4 and Fig. 8a, lane 8). Additionally it has been demonstrated that IRS-2 may serve as an alternate pathway to activate PI3K in response to erythropoietin (52). Notwithstanding the possible role of IRS-1 or IRS-2, it is hypothesized that pp97 may play a central role in the recruitment of PI3K to the IL-2 receptor or to the membrane.

In vitro experiments, fusion proteins containing the p85-SH2 domains precipitated a large amount of IL-2Rβ. However, IL-2Rβ was not evidenced in p85 immunoprecipitates and pp97 was not detectable in IL-2Rβ immunoprecipitates (not shown). This may be related to relatively labile interactions due to indirect interactions or to a low stoichiometry of these complexes in vivo. Alternatively, PI3K may be recruited to the membrane, where its preferred substrate phosphatidylinositol 4,5-bisphosphate is located, via CrkL interaction with cytoskeletal proteins, which would be consistent with the rapid phosphorylation of the CrkL-associated pp97. Consistent with this is the recent observation that CrkL plays a central role in β1 integrin-mediated adhesion mechanisms and has been shown to tightly interact with focal adhesion-associated proteins (53).

Our data thus suggest that pp97 may represent a novel example of large docking proteins such as IRS-1, IRS-2, or Gab-1 and may play important functions in several of the signal transduction pathways activated by IL-2. The formal identification of pp97 or the generation of specific antibodies should help in understanding these functions. Furthermore, the identification of pp97 should help in providing new insights in the respective functions of CrkL and SHP-2 in IL-2 signaling.

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