Structure and Function of Cas-L, a 105-kD Crk-associated Substrate-related Protein That Is Involved in β1 Integrin–mediated Signaling in Lymphocytes

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Summary

Integrin/ligand binding evokes tyrosine phosphorylation of various proteins. We reported previously that a 105 kD protein (pp105) was tyrosine phosphorylated by the engagement of β1 integrins in T lymphocytes. We show here that pp105 is a novel p130Cas (Crk-associated substrate)-related protein. Deduced amino acid sequence revealed that pp105 contains conserved motifs with p130Cas, and both pp105 and p130Cas bind to focal adhesion kinase (pp125FAK) and Crk. However, pp105 has a clearly distinct structure from p130Cas, and pp105 is preferentially expressed in lymphocytes, whereas p130Cas is expressed in adherent cells. With these findings, we designate pp105 as Cas-L, lymphocyte-type Cas. Furthermore, we demonstrate that integrin/ligand binding results in the recruitment of Crk, Nck, and SHPTP2 to pp105. These findings further define the roles of pp105/Cas-L and pp125FAK in the integrin-mediated signaling pathways.

The β1 integrins play an important role in the interaction between T cells and the surrounding extracellular matrix (1) and may aid lymphocyte migration into tissues (2). In addition to their role in cell adhesion, recent studies have clearly shown that integrins transduce signals into the interior of T lymphocytes and other types of cells (3). We and others have shown that the binding of T cells to the extracellular matrix through β1 integrins provides costimulatory signals for T cell proliferation (4–9). We also identified protein tyrosine phosphorylation as a β1 integrin–mediated biochemical signal occurring before T cell proliferation. The engagement of β1 integrin molecules induced tyrosine phosphorylation of 140-, 120-, 110–105-, 80–70-, 60–55-, and 45-kD proteins in peripheral T cells (10–12). Several tyrosine-phosphorylated proteins have been identified, such as phospholipase C (PLC)-γ (pp140), focal adhesion kinase (pp125FAK [pp120])[1], paxillin (pp70), p59Fyn/p56Lck (pp60–55), and mitogen-activated protein (MAP) kinase (pp45) (10). However, a 105-kD tyrosine-phosphorylated protein (pp105) has not been identified.

pp105 is a protein that we first identified in T lymphoblastoid H9 cells as well as peripheral T cells (11). pp105 is a 105-kD protein that is tyrosine phosphorylated by the engagement of α4β1 integrin. Our previous studies demonstrated that pp105 is distinct from pp125FAK, although both pp105 and pp125FAK are tyrosine phosphorylated via similar kinetics by the engagement of α4β1 integrin in H9 cells (12). Given the fact that pp105 is one of the major proteins that is tyrosine phosphorylated by β1 integrin stimulation in peripheral T cells, pp105 may play an important role in β1 integrin–mediated signaling in T cells.

Recently, Sakai et al. (13) cloned the cDNA of p130Cas (Crk-associated substrate). p130Cas was originally identified as a tyrosine-phosphorylated 130 kD protein in the $p60^{v\text{-}Src}$- or $p47^{v\text{-}Crk}$-transformed fibroblasts. The deduced amino acid sequence of p130Cas revealed that p130Cas contained one Src homology (SH) 3 domain in the NH_2-terminal region and multiple putative binding sites of SH2 domains. Tyrosine phosphorylated p130Cas bound to Crk, and the deduced amino acid sequence of p130Cas contained multiple binding motifs of the Crk SH2 domain. Crk can bind to guanine nucleotide–releasing factors son of sevenless (SOS) and C3G by the SH3 domain of Crk (14, 15). SOS and C3G are involved in the activation of Ras

[1] *Abbreviations used in this paper:* Cas, Crk-associated substrate; CT, COOH-terminal domain; FAK, focal adhesion kinase; FN, fibronectin; GST, glutathione-S-transferase; PLL, poly-L-lysine; SH, Src homology; SOS, son of sevenless.

M. Minegishi, K. Tachibana, and T. Sato contributed equally to this work.

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and Rap1A, respectively (16, 17). These findings suggested that tyrosine-phosphorylated p130Cas is involved in the activation of Ras family proteins by the association with Crk. Moreover, we demonstrated that p130Cas is tyrosine phosphorylated by β1 integrin stimulation in fibroblasts (18). On the other hand, Polte and Hanks (19) reported that p130Cas bound to pp125FAK via its SH3 domain.

In this study, we demonstrate that pp105 is a novel Cas-related protein that is expressed in lymphocytes. We also show the predicted structure of pp105 based on an isolated cDNA. Furthermore, we show that pp105 binds to FAK and other signaling molecules, including Crk. These findings further suggest the role of pp105 in the β1 integrin–mediated signaling pathways.

Materials and Methods

Cell Culture. Rat 3Y1, SR-3Y1, T-47D, HL-60, K-562, U937, H9, HPB-ALL, Jurkat, Raji, and Cos-1 cell lines were obtained from American Type Culture Collection (Rockville, MD) and grown in DMEM or RPMI 1640 medium supplemented with heat-inactivated FCS, 2 mM L-glutamine, and gentamycin (50 μg/ml). Peripheral T cells and B cells were isolated from healthy donors as described previously (10). Fresh normal thymocytes were obtained from thymuses removed from patients at corrective cardiac surgery. A portion of thymus was finely minced, and a single-cell suspension was prepared by pressing the fragments through a stainless steel mesh. RBCs and debris were removed by centrifugation over Ficoll–Paque (Pharmacia LKB, Uppsala, Sweden), and then thymocytes were washed three times.

GST Fusion Proteins. Plasmids coding glutathione-S-transferase (GST) fusion proteins were constructed by the insertion of human FAK cDNA fragments into pGEX-3X or pGEX-2TK (Pharmacia LKB). GST–COOH-terminal domain (CT) contains FAK amino acid residues from 706 to 1,052, GST-ΔH contains amino acid residues from 706 to 904, and GST-H1 contains residues from 896 to 1,052 (20). GST-AblSH2 was provided by Dr. Wayne G. Haser (Dana-Farber Cancer Institute, Boston, MA) (21). GST-CrkSH2 and GST-NckSH2 were provided by Dr. Bruce J. Mayer (Children's Hospital, Boston, MA) (22). GST-LckSH2 and GST-ShcSH2 were provided by Dr. Gotz Baumann (Sandoz Pharma LTD., Basel, Switzerland) (23). GST-SHPTP2SH2 was obtained from Dr. Benjamin G. Neel (Beth Israel Hospital, Boston, MA) (21). GST-PI3KSH2 was obtained from Dr. Brian Schaffhausen (Tufts University School of Medicine, Boston, MA) (21). GST-Grb2SH2 was constructed from human Grb2 cDNA, which was obtained from Dr. Takeshi Urano (Tufts University School of Medicine, Boston, MA). GST-CskSH2 was generated from Csk cDNA, which was obtained from Dr. Masato Okada (Institute for Protein Research, Osaka University, Osaka, Japan). GST-PLCγSH2 was generated by reverse transcription PCR.

Immunoblotting and Immunoprecipitations. H9 cells were washed three times, resuspended in Iscove's serum-free media, incubated in plates coated with poly-L-lysine (PLL; Sigma Chemical Co., St. Louis, MO) or human plasma fibronectin (FN; GIBCO BRL, Gaithersburg, MD), and then solubilized in a modified NP-40 lysis buffer (1% NP-40, 0.5% deoxycholic acid, 150 mM NaCl, 50 mM Tris-HCl, 5 mM EDTA, 1 mM PMSF, 10 mM iodoacetamide, 1 μg/ml pepstatin A, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, and 0.4 mM sodium vanadate, pH 8.0) or 1% digitonin lysis buffer, which contained 1% digitonin (Wako Pure Chemical Industries Ltd., Osaka, Japan) instead of NP-40 and deoxycholic acid.

For immunoprecipitation, cell extracts were incubated with primary antibodies for 1 h at 4°C followed by additional incubation with goat anti–mouse IgG–conjugated agarose (Sigma Chemical Co.) or rabbit anti–mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) plus protein A–Sepharose (Pharmacia LKB) for 1 h at 4°C. Specific mAbs against Cas, Crk, pp125FAK, Nck, paxillin, and SHPTP2 were purchased from Transduction Laboratories (Lexington, KY). Otherwise, cell extracts were incubated with glutathione Sepharose (Pharmacia LKB), which was conjugated with GST–fusion proteins for 1 h at 4°C. Beads were washed with 1% NP-40 washing buffer (1% NP-40, 50 mM Tris-HCl, 140 mM NaCl, 2.5 mM EDTA) five times. For sequential immunoprecipitations, washed beads were boiled 5 min at 100°C in the presence of 2% SDS, and the supernatants were reprecipitated with antibodies in the 1% NP-40 lysis buffer containing 0.1% final SDS concentration. For immunoblotting, samples were fractionated by 7% SDS-PAGE under reducing conditions and electro-transferred onto nitrocellulose membranes. Membranes were analyzed by immunoblotting with a chemiluminescence reagent (DuPont NEN, Boston, MA) after incubation with primary Ab and horseradish peroxidase–conjugated anti–mouse IgG (Amersham Corp., Arlington Heights, IL). Otherwise, membranes were incubated with ^{125}I-labeled antiphosphotyrosine mAb (4G10; Upstate Biotechnology, Inc., Lake Placid, NY) (10).

Overlay Assay. For the overlay assay, pGEX-2TK fusion protein containing FAK residues 707–1,052 (GST-CT) was phosphorylated in vitro using bovine heart muscle kinase (Sigma Chemical Co.) and [γ^{32}P]ATP (DuPont NEN). Proteins transferred to the membrane were denatured in 6 M guanidine-HCl and subsequently renatured (24). This membrane was hybridized with ^{32}P-labeled GST–FAK fusion protein in buffer containing 20 mM Hepes, pH 7.7, 75 mM KCl, 0.1 mM EDTA, 2.5 mM $MgCl_2$, 1% nonfat dry milk, and 0.05% NP-40.

Molecular Biology. A λgt11 cDNA library constructed from human lymphoma Hut78 RNA (CLONTECH, Palo Alto, CA) was screened by anti-Cas mAb (Transduction Laboratory) and alkaline phosphatase–conjugated goat anti–mouse IgG (Sigma Chemical Co.) (25). DNA sequencing was performed by the dideoxy nucleotide method (25, 26). Isolated cDNA was inserted into pcDL-SRα for the transfection study in Cos-1 cells (27). pcDL-SRα-LckY505F was generated from DNA provided by Dr. Roger M. Perlmutter (University of Washington, Seattle, WA). pMT3-SrcY527F was provided by Dr. Takeshi Urano. pMT3-EE-CrkI and II were constructed from chicken Crk genes, which were provided by Dr. Bruce J. Mayer. Cos-1 cells were used for the transfection study by the DEAE–dextran method (25). For Northern blotting, a DNA fragment of isolated cDNA (nucleotides 1,446–1,773) was ^{32}P labeled and used as a probe (26).

Results

Association of pp105 with the COOH-terminal Domain of FAK. In our previous study, we demonstrated that pp105 and pp125FAK were tyrosine phosphorylated by β1 integrin stimulation via similar kinetics in H9 cells, although pp105 is a distinct molecule from pp125FAK (12). Because pp125FAK is an essential tyrosine kinase for β1 integrin–mediated protein tyrosine phosphorylation, we attempted to define the relationship between pp125FAK and pp105.

For this purpose, H9 cells were incubated with FN- or PLL-coated plates before cell lysis. H9 cell lysates were precipitated with GST fusion protein of the pp125FAK COOH-terminal domain and analyzed by immunoblotting with antiphosphotyrosine mAb (anti-pTyr). As shown in Fig. 1 *A*, a tyrosine phosphorylated 105-kD protein precipitated on the beads conjugated with GST-CT from FN-stimulated cell lysate (lane *6*), whereas this protein did not precipitate on GST-conjugated beads (lane *4*). This tyrosine phosphorylated protein migrated at the same position as pp105 in FN-incubated cell lysate (lane *2*) and was detected only minimally in PLL-incubated cell lysate (lane *5*). These results strongly suggest that pp105 binds to the CT of pp125FAK. Paxillin was also precipitated from H9 cell lysate and was detected by anti-pTyr as a 70-kD band (lane *6*), as we reported in HPB-ALL and T-47D cells (20). To determine whether pp105 binds to FAK or paxillin, H9 cell lysates were precipitated with deletion mutants of GST-CT. As shown in Fig. 1 *B*, pp105 was precipitated with GST-ΔH, but not with GST-H1. Conversely, Paxillin was precipitated with GST-H1 but not with GST-ΔH. These results demonstrate that pp105 binds to the FAK sequence that contains amino acid residues 706–904. This pp105-binding domain of FAK is distinct from the paxillin-binding domain of FAK, indicating that pp105/FAK binding is not mediated via paxillin.

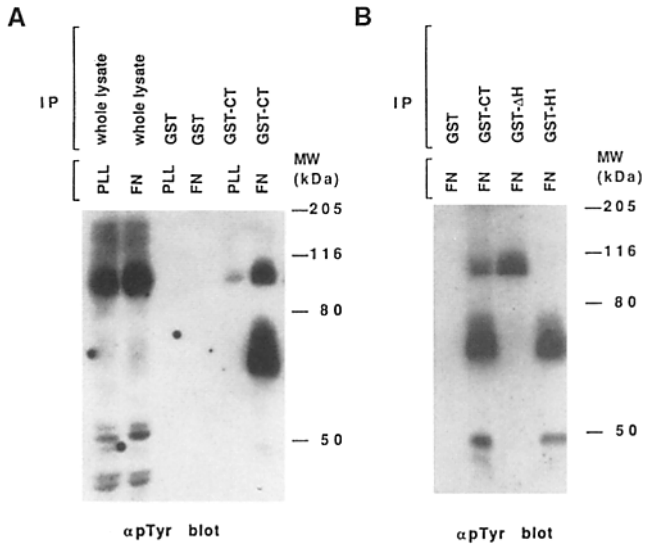

Figure 1. Binding of pp105 to the CT domain of FAK. (*A*) H9 cells were incubated in PLL- or FN-coated plates for 30 min (lanes *1*, *3*, and *5* and *2*, *4*, and *6*, respectively). After lysis, cellular lysates were precipitated by glutathione beads that were conjugated to GST or GST–FAK CT fusion protein (containing FAK residues 706–1,052, GST–CT beads). The whole lysate and the immunoprecipitates were analyzed by immunoblotting with antiphosphotyrosine mAb (anti-pTyr). (*B*) H9 cell lysates were precipitated with GST–FAK fusion protein–conjugated beads. GST-ΔH contains FAK residues 706–904, and GST-H1 contains FAK residues 896–1,052. Precipitates were analyzed by immunoblotting with anti-pTyr.

Identification of pp105 as a Cas-related Protein. pp105 was precipitated with the GST–FAK fusion protein from FN-stimulated H9 cell lysates and was detected by immunoblotting with anti-pTyr. A 130-kD tyrosine-phosphorylated protein was precipitated with the GST–FAK fusion protein from human breast cancer–derived T-47D cell lysates (Tachibana, K., and T. Sato, unpublished data). We identified this 130-kD protein as p130Cas using anti-Cas mAb (Transduction Laboratories) (Tachibana, K., and T. Sato, unpublished data). Recently, Polte and Hanks (19) reported that p130Cas bound to FAK by its SH3 domain. FAK residues 706–904, which were sufficient for pp105 binding, contained the reported p130Cas-binding site. We attempted to determine if pp105 was reactive with anti-Cas mAb. As shown in Fig. 2 *A*, a 105-kD protein was precipitated with the GST–FAK fusion protein and detected by immunoblotting with anti-Cas mAb (Transduction Laboratory) (anti-Cas blot, lanes *3* and *4*). This 105-kD protein showed the same migration in SDS-PAGE as pp105, which was precipitated by the GST–FAK fusion protein and detected by reblotting with anti-pTyr. We further performed a second immunoprecipitation with anti-Cas mAb after precipitation with the GST–FAK fusion protein. As shown in Fig. 2 *A*, a 105-kD Cas protein was precipitated first by the GST–FAK fusion protein and reprecipitated by anti-Cas mAb (anti-Cas blot). This 105-kD protein showed increased tyrosine phosphorylation by FN stimulation (Fig. 2 *A*, anti-pTyr blot, lanes *5* and *6*). To confirm that pp105 is the molecule recognized by anti-Cas mAb, H9 cell lysates were immunoprecipitated with anti-Cas mAb or anti-pTyr and analyzed by immunoblotting. As shown in Fig. 2 *B*, the 105-kD protein that was precipitated with anti-Cas mAb was tyrosine phosphorylated by the stimulation of FN (anti-pTyr blot, lane *2*). Moreover, a tyrosine phosphorylated 105-kD protein that was precipitated by anti-pTyr was detected by anti-Cas mAb (anti-Cas blot, lane *6*). To determine if this 105-kD protein recognized by anti-Cas mAb is the major tyrosine-phosphorylated protein among 105–110-kD proteins that are phosphorylated by the ligation of β1 integrins, we performed immunodepletion analysis using anti-Cas mAb. As shown in Fig. 2 *C*, a clear difference in the amount of tyrosine-phosphorylated 105-kD protein was observed with or without immunodepletion with anti-Cas mAb in the whole lysates (lanes *2* and *4*) and in the anti-pTyr precipitates (lanes *6* and *8*). These findings indicate that pp105, a 105-kD protein that is tyrosine phosphorylated by β1 integrin stimulation, is a Cas-related protein.

Differential Expression of 130- and 105-kD Cas Proteins. p130Cas has been reported to migrate as discrete species of 115 and 125 kD in SDS-PAGE (designated by Sakai et al. [13] as Cas-A and Cas-B, respectively) in rat fibroblast 3Y1 cells (also shown in Fig. 3). However, a decrease in the size of Cas-A and the simultaneous appearance of a broad 130-kD Cas band (designated Cas-C) were observed in both v-Src– and v-Crk–transformed 3Y1 cells. Because phosphorylated tyrosine residues were found predominantly in Cas-C, Cas-C appeared to be a modified form of Cas-A or Cas-B as the result of tyrosine phosphorylation (13).

We identified pp105 in H9 cells as a putative Cas-related protein. pp105 was originally identified as a 105-kD pro-

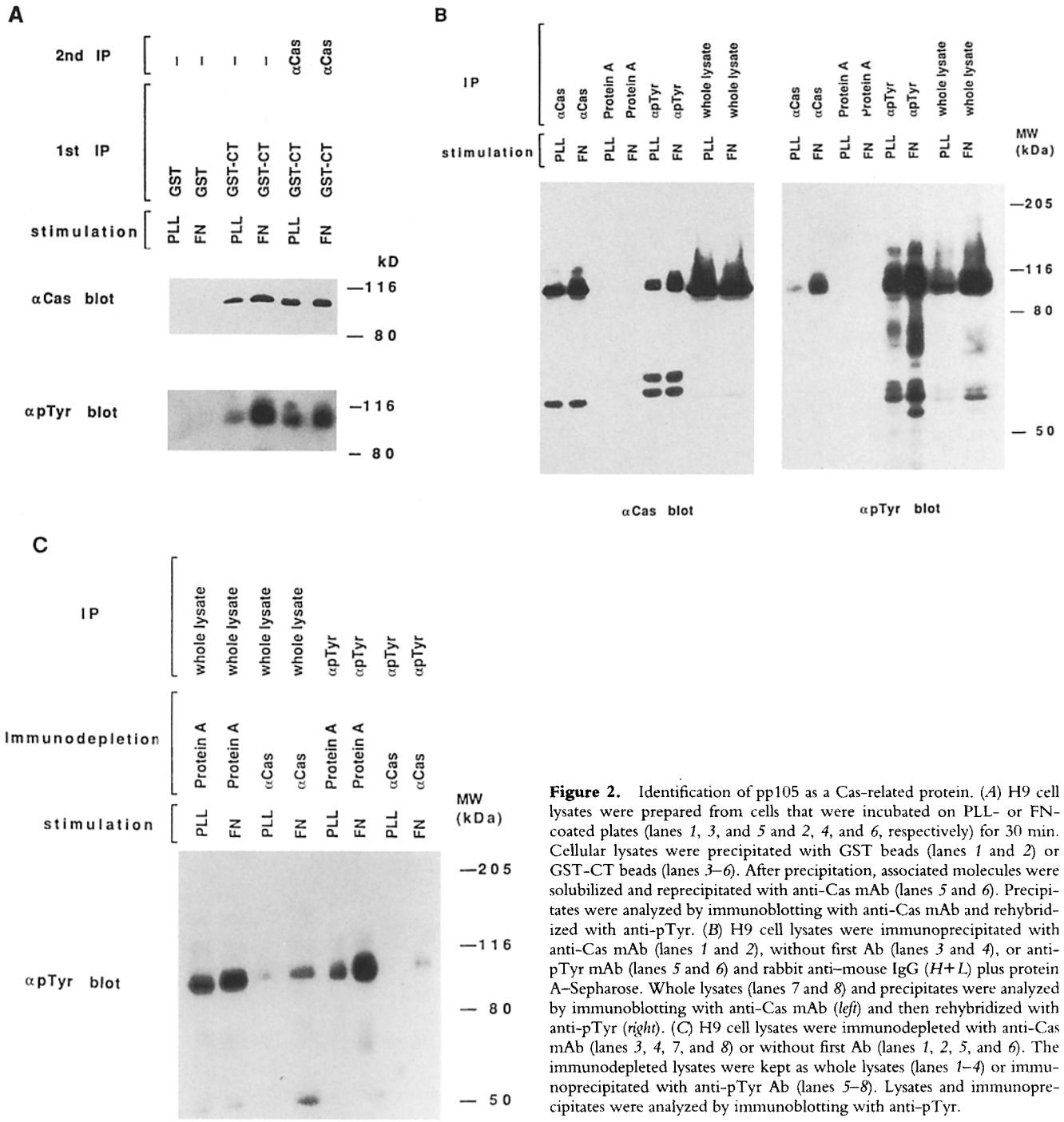

Figure 2. Identification of pp105 as a Cas-related protein. (*A*) H9 cell lysates were prepared from cells that were incubated on PLL- or FN-coated plates (lanes *1*, *3*, and *5* and *2*, *4*, and *6*, respectively) for 30 min. Cellular lysates were precipitated with GST beads (lanes *1* and *2*) or GST-CT beads (lanes *3–6*). After precipitation, associated molecules were solubilized and reprecipitated with anti-Cas mAb (lanes *5* and *6*). Precipitates were analyzed by immunoblotting with anti-Cas mAb and rehybridized with anti-pTyr. (*B*) H9 cell lysates were immunoprecipitated with anti-Cas mAb (lanes *1* and *2*), without first Ab (lanes *3* and *4*), or anti-pTyr mAb (lanes *5* and *6*) and rabbit anti-mouse IgG (*H+L*) plus protein A–Sepharose. Whole lysates (lanes *7* and *8*) and precipitates were analyzed by immunoblotting with anti-Cas mAb (*left*) and then rehybridized with anti-pTyr (*right*). (*C*) H9 cell lysates were immunodepleted with anti-Cas mAb (lanes *3*, *4*, *7*, and *8*) or without first Ab (lanes *1*, *2*, *5*, and *6*). The immunodepleted lysates were kept as whole lysates (lanes *1–4*) or immunoprecipitated with anti-pTyr Ab (lanes *5–8*). Lysates and immunoprecipitates were analyzed by immunoblotting with anti-pTyr.

tein that was tyrosine phosphorylated by the stimulation of β1 integrins. By immunoblotting with anti-pTyr, pp105 was detected predominantly in H9 cells as well as in peripheral T cells (11), whereas pp105 was not detected well in the other T cell lines. To determine the distribution of pp105 and p130Cas, we examined the expression and the mobility in SDS-PAGE of Cas proteins in various cell lines. As shown in Fig. 3, using the same amount of whole extract from each cell line, Cas proteins with mobilities similar to Cas-A and Cas-B from 3Y1 cells were detected in human breast cancer T-47D cells, although Cas proteins in T-47D cells showed slightly faster migration. Cas proteins of similar mobility to Cas-A protein of T-47D were also detected in human myelogeneous cell lines HL-60 and K562. On the other hand, pp105 that showed a distinct mobility from Cas-A and Cas-B was detected in human myelogeneous cell line U937 and in human T lymphoblastoid cell lines H9, HPB-ALL, and Jurkat, and in human B

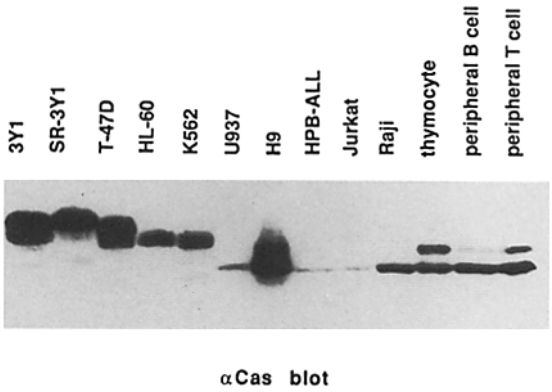

Figure 3. Differential expression of 130- and 105-kD Cas proteins. Expression of Cas protein in each cell line was analyzed by immunoblotting with anti-Cas mAb (lane *1*, rat 3Y1; lane *2*, SR-3Y1, v-Src transfected 3Y1 cell strain; lane *3*, T-47D; lane *4*, HL-60; lane *5*; K562; lane *6*, U937; lane 7, H9; lane *8*, HPB-ALL; lane *9*, Jurkat; lane *10*, Raji; lane *11*, whole thymocyte; lane *12*, peripheral B cell; lane *13*, peripheral T cell. 50-μg lysate per each lane).

lymphoid cell line Raji. pp105 was also detected in human thymocytes and in human peripheral T and B cells. The expression level of pp105 varied widely among cells. pp105Cas was significantly overexpressed in H9 cells, followed by peripheral T and B cells, thymocytes, and Raji cells. A 110-kD Cas protein that migrated more slowly than pp105 but faster than Cas-A of T-47D cells was also detected in H9 cells, thymocytes, and peripheral T cells. These findings indicate that pp105 is a Cas-related protein that is expressed in lymphocytes.

cDNA Cloning of pp105. To further determine the structure of pp105, we screened the λgt11 cDNA library derived from a human T lymphoblastoid cell line with anti-Cas mAb. Nucleotide sequences of three independent clones had homology with p130Cas. These three clones were cDNAs of an identical transcript, and the nucleotide sequences contained an open reading frame of 834 amino acids. The deduced amino acid sequence of this transcript showed conserved motifs with p130Cas, one SH3 domain in the NH_2-terminal region, and multiple putative binding sites of the SH2 domains (Fig. 4 *A*). Most of the SH2-binding motifs in the substrate domain are YXXP (YDXP), which are putative binding sites for Crk, Nck, and Abl SH2 domains (21). Despite the conserved motifs, homology between p130Cas and the deduced amino acid sequence of this cDNA is relatively low (Fig. 4 *B*, 78% in the SH3 domain, 32% in the substrate domain, 30% in the specific domain, and 52% in the CT). Homology with another Cas-related protein, Efs (28), is also relatively low (Fig. 4 *B*). These results indicate that the cDNA encodes a novel Cas-related protein.

To determine whether this cDNA of a novel Cas-related protein encodes pp105, the cDNA was inserted into an expression vector and transfected into Cos-1 cells. Cellular lysates from transfectants were analyzed by immunoprecipitation and immunoblotting. As shown in Fig. 5 *A*, anti-Cas mAb–reactive peptides that migrated at 105 and 110 kD in SDS-PAGE were detected (lanes *3* and *7*). pp105 from H9 cells (lanes *1* and *5*) comigrated with a 105-kD peptide detected in the lysate from the transfectant and a slightly slower migrating Cas protein of 110-kD in H9 cells comigrated with a 110-kD peptide. Both Cas proteins detected in H9 cells showed faster migration than Cas proteins in T-47D (lanes *4* and *8*) and Cos-1 cells (lanes *2* and *6*). This result strongly suggests that (*a*) the Cas-related gene encodes pp105 and (*b*) a 110-kD Cas protein detected in H9 cells is the protein product of the same transcript as pp105, despite a different mobility in SDS-PAGE (similar to that observed with p130Cas-A and Cas-B).

p130Cas was highly phosphorylated on tyrosine residues in v-Src– or v-Crk–expressing cells (13). To determine whether pp105 is tyrosine phosphorylated by these stimulations, an expression vector containing pp105 was cotransfected in Cos-1 cells, with expression vectors containing activated Src family kinases or Crk. pp105 was then precipitated by anti-Cas mAb and analyzed by immunoblotting with anti-pTyr. As shown in Fig. 5 *B*, pp105 was highly phosphorylated on tyrosine residues by the cotransfection of Src, Lck, CrkI, or CrkII (lanes *3–6*, respectively). Next, to identify whether tyrosine-phosphorylated pp105 binds to Crk, lysates of pp105-transfected Cos cells were immunoprecipitated with anti-Crk mAb and analyzed by immunoblotting with anti-Cas mAb. pp105 was coprecipitated with Crk only in Cos cells that coexpressed activated Lck (Fig. 5 *B*, lane *8*). Furthermore, pp105 was coprecipitated with EE-tagged CrkII (lane *9*). These results demonstrate that tyrosine-phosphorylated pp105 binds to Crk proteins in vivo.

Expression of the pp105 Transcript. Using anti-Cas mAb, we showed cell type–specific expression of pp105. To further investigate the expression of pp105 in different cell types, expression of pp105 mRNA was analyzed by Northern blotting. As shown in Fig. 6 *A*, 5.2- and 3.0-kb RNAs were detected in H9 cell polyA$^+$ RNA using ^{32}P-labeled pp105 cDNA fragment as a probe. This pp105 cDNA fragment expressed the lowest homology with p130Cas. The 5.2-kb RNA comigrated with 28S ribosomal RNA and was detected as a shifted band in Northern blotting with total RNA. A smaller amount of pp105 transcript was detected in peripheral T cells, and none was detected from 3Y1 and T-47D cells. A similar analysis was performed with RNAs from various tissues. As shown in Fig. 6 *B*, pp105 was expressed in most tissues including spleen and thymus, although pp105 was weakly detected in brain and liver. This result indicates that pp105 transcript is ubiquitously expressed, whereas the pp105 protein is detected only in lymphocytes among the cells investigated.

Identification of the pp105-binding Proteins. To further define the nature of pp105, we investigated pp105-binding proteins. As described previously, pp105 binds to FAK in vitro. To further characterize pp105–FAK binding, pp105 was precipitated from H9 cell lysates with anti-Cas mAb and analyzed by the overlay assay with a labeled GST–FAK

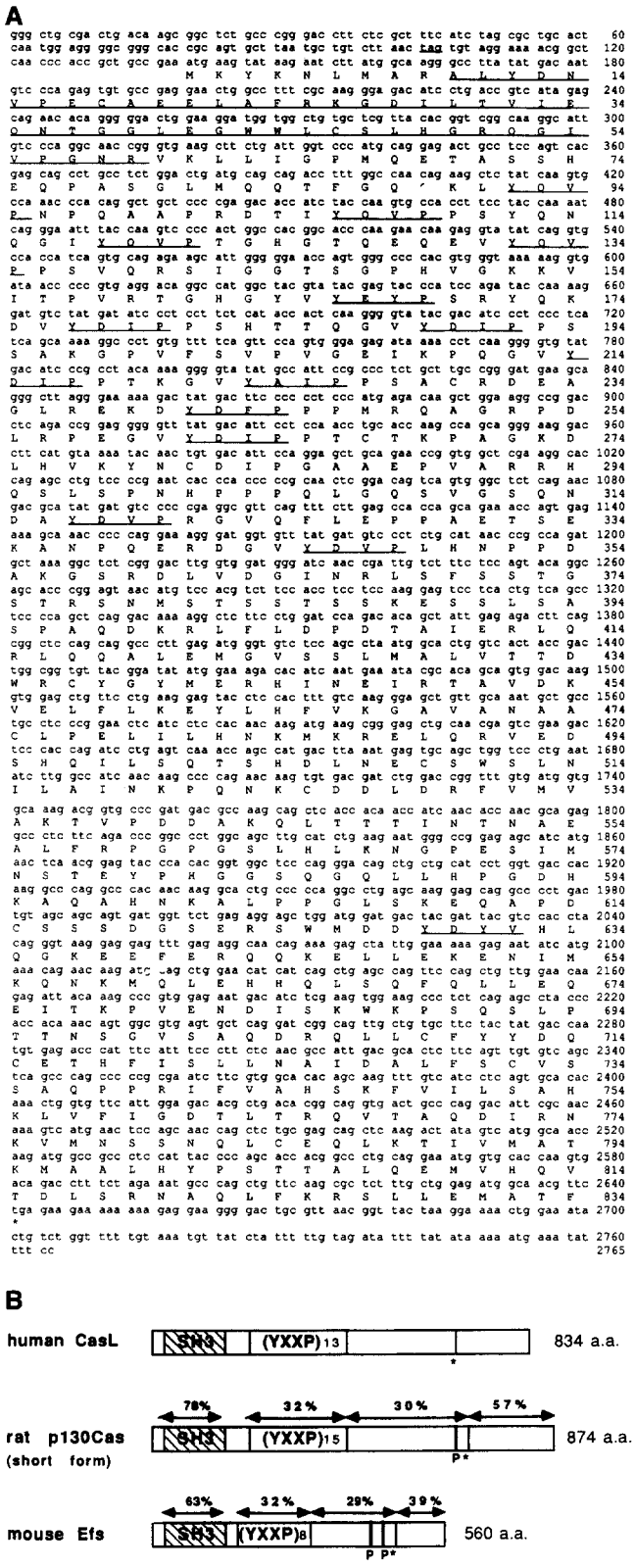

Figure 4. Structure of pp105/Cas-L. (*A*) Nucleotide and deduced amino acid sequences of pp105/Cas-L. The 2,765-nucleotide cDNA contains a single open reading frame that encodes a protein with 834 amino acids. An SH3 domain and putative SH2-binding sites are underlined. These sequence data are available from GenBank under accession number U64317. (*B*) Comparison of amino acid sequences among Cas proteins. Amino acid sequence homologies between Cas-L and the other Cas protein in the SH3 domain, substrate domain (Cas-L residues 92–348), specific domain (Cas-L residues 349–628), and CT (Cas-L residues 629–834) are shown above each domain. YDYVHL motifs are shown by asterisks and vertical lines. Proline-rich sequences are shown by "P" and heavier vertical lines.

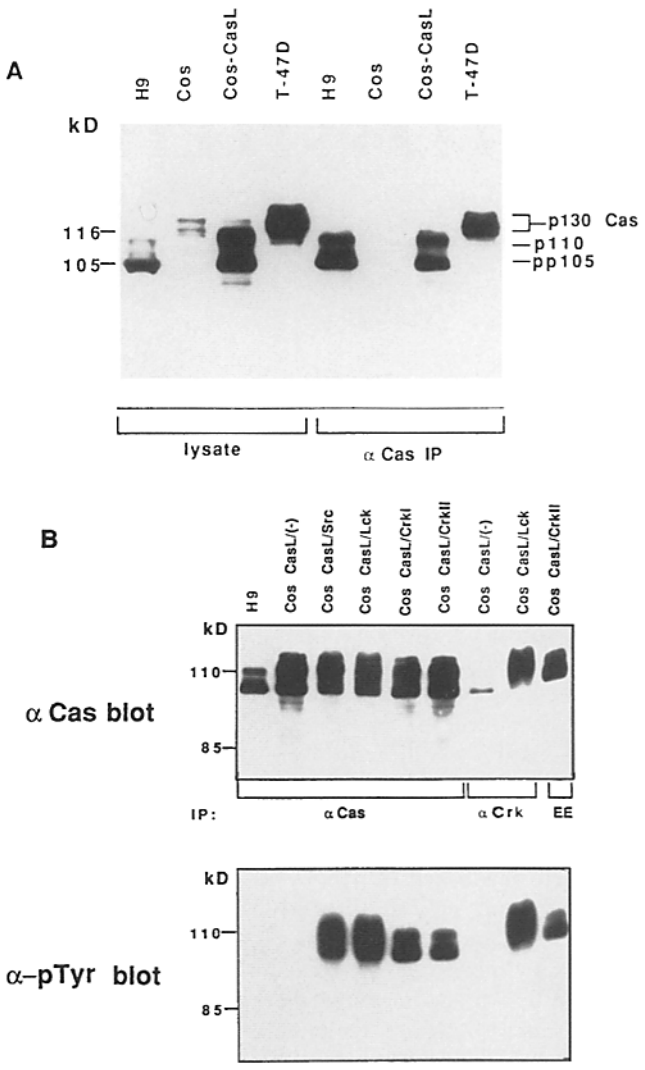

Figure 5. Transfection study of pp105. (*A*) Transient expression of pp105 in Cos cells. pcDL-SRα pp105 was transfected into Cos-1 cells. Cellular lysates of H9, T-47D, and Cos-1 cells were analyzed by immunoprecipitation and immunoblotting with anti-Cas mAb. (*B*) Tyrosine phosphorylation of pp105 by cotransfected Src-family kinases or Crk. pcDL-SRα pp105 was transfected into Cos-1 cells with pMT3 (vector, lanes *2* and *7*), SrcY527F (lane *3*), LckY505F (lanes *4* and *8*), EE-tagged Crk1 (lane *5*), and EE-tagged Crk2 (lanes *6* and *9*). After lysis, cellular lysates were immunoprecipitated with anti-Cas mAb (lanes *1–6*), anti-Crk mAb, anti-EE tag mAb (lane *9*) and analyzed by immunoblotting with anti-Cas mAb and anti-pTyr.

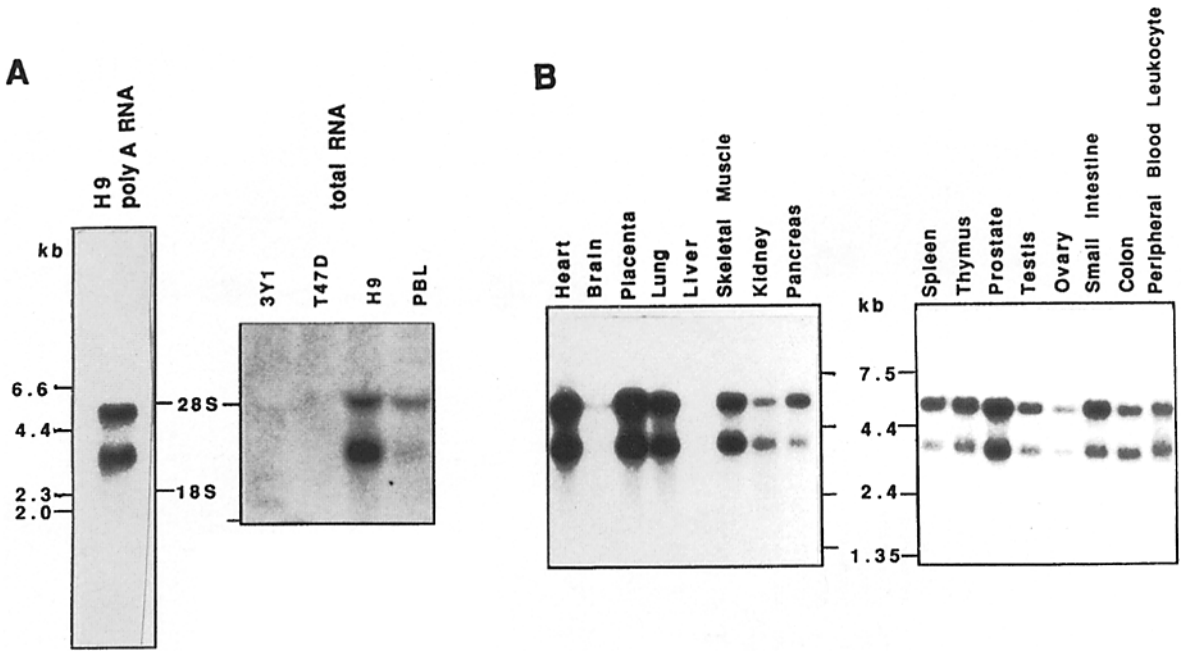

Figure 6. Expression of pp105 transcript. (*A*) H9 cell polyA$^+$ RNA was isolated by Fast Track 2.0 (Invitrogen, San Diego, CA), and total cellular RNAs were isolated by the method described (26). Northern blot analysis with a ^{32}P-labeled pp105 cDNA fragment was performed by the method described (26). (*B*) Tissue distribution of pp105 was determined by Northern blotting with human tissue blots (Clontech) and the pp105 probe described above.

fusion protein. As shown in Fig. 7 *A*, 105- and 110-kD proteins were detected with labeled GST–FAK fusion protein, indicating that both pp105 and p110-kD Cas protein directly bind to FAK. Another FAK-binding protein, paxillin, was also detected, as reported previously (20). To investigate the binding of pp105 to pp125FAK in vivo, coprecipitation analysis using anti-FAK mAb was performed. As shown in Fig. 7 *B*, lanes 7 and *8*, pp105 was coimmunoprecipitated with pp125FAK from H9 cell lysates, indicating in vivo association between pp105 and pp125FAK. This pp105–pp125FAK association appears to be unaffected by β1 integrin stimulation or subsequent tyrosine phosphorylation of both proteins, because coprecipitated pp105 showed no quantitative difference between FN-stimulated and unstimulated cells.

Next, we attempted to define the proteins that were recruited to pp105 in a phosphorylated tyrosine residue–dependent manner. For this purpose, lysates from FN-stimulated H9 cells were precipitated with GST fusion proteins that contained SH2 domains from various proteins and analyzed by immunoblotting with anti-Cas mAb and anti-pTyr. As shown in Fig. 7 *C*, 110–105-kD tyrosine-phosphorylated proteins were precipitated with GST fusion proteins of c-Abl, Crk, Csk, Grb2, Lck, Nck, and SHPTP2 SH2 domains (anti-pTyr blot). pp105 was precipitated by GST-AblSH2, GST-CrkSH2, and GST-NckSH2, whereas pp105 was weakly precipitated by GST-LckSH2, GST-SHPTP2SH2, and GST-CskSH2 (anti-Cas blot). To determine whether the binding of pp105 to these GST fusion proteins was induced by β1 integrin stimulation, a similar analysis was performed by using lysates of H9 cells that were incubated in PLL- or FN-coated plates. As shown in Fig. 7 *D*, enhancement of pp105 precipitation was observed in the lanes of GST–SH2 domain fusion proteins of c-Abl, Crk, and Nck, whereas increased but slight amounts of pp105 were detected in the lanes of the GST–SH2 domain fusion proteins of Lck and SHPTP2. These results indicated that tyrosine-phosphorylated pp105 binds to SH2 domains of c-Abl, Crk, Lck, Nck, and SHPTP2 in vitro.

To further determine if these pp105-binding proteins bind to pp105 in vivo, coimmunoprecipitation analysis of pp105 with these proteins was performed. As shown in Fig. 7 *E*, pp105 was coprecipitated with Crk and Nck, whereas pp105 was weakly coprecipitated with SHPTP2. Unlike pp125FAK–pp105 binding, pp105 that was coprecipitated with Crk, Nck, or SHPTP2 was increased by β1 integrin stimulation with FN. These results indicate that β1 integrin stimulation leads to the recruitment of various proteins, including Crk, Nck, and SHPTP2, to the tyrosine-phosphorylated pp105, in addition to stimulation-independent association with pp125FAK. These protein–protein interactions further suggest the putative function of pp105 in the β1 integrin–mediated signaling pathways.

Discussion

We reported previously that the engagement of β1 integrins induced tyrosine phosphorylation of a 105-kD protein (pp105) in H9 cells and peripheral T cells (10–12). pp105 was one of the major proteins in T cells that was tyrosine phosphorylated by β1 integrin stimulation. In this study, we identified pp105 as a tyrosine-phosphorylated

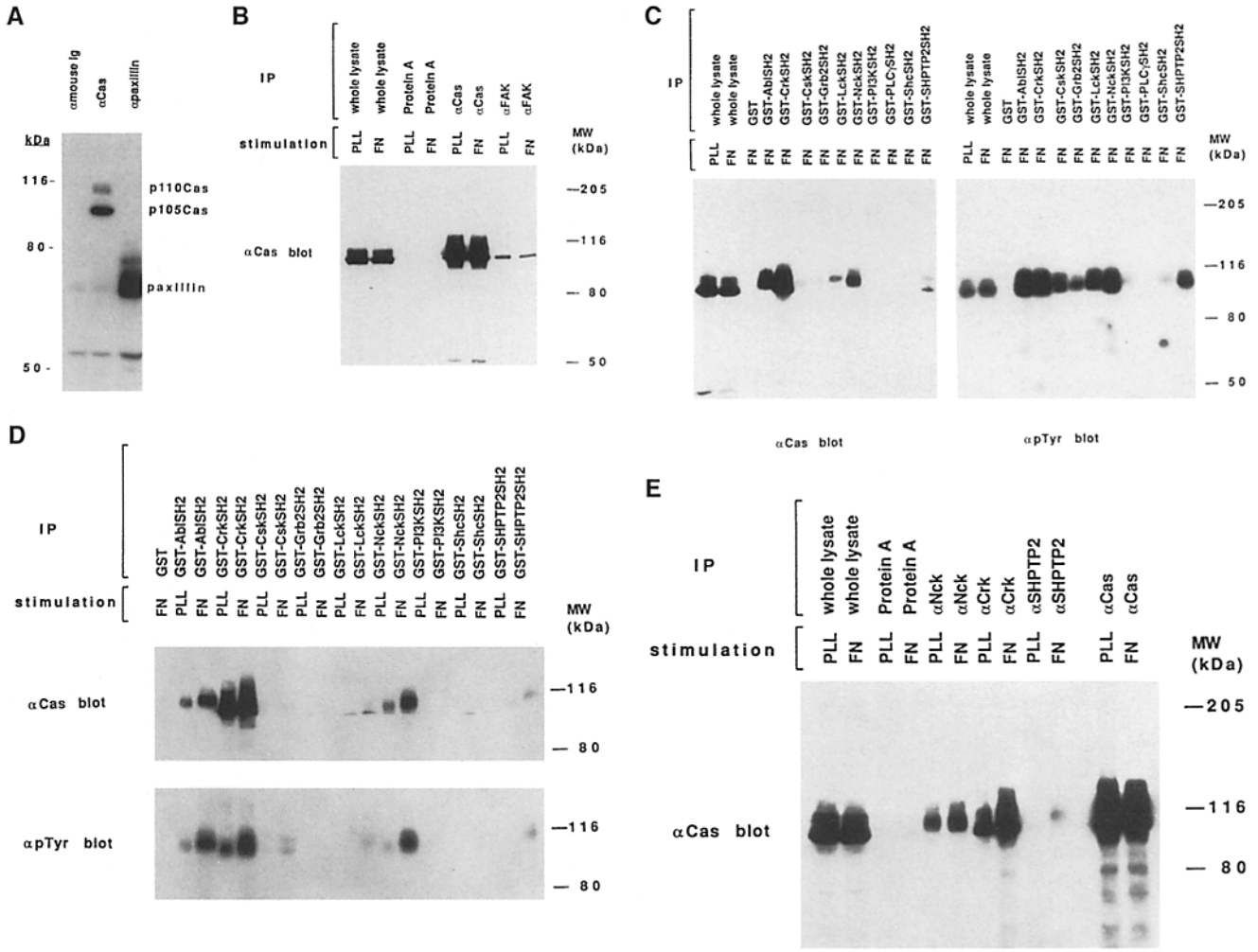

Figure 7. Identification of the pp105-binding proteins. (*A*) H9 cell lysates were immunoprecipitated without first Ab (lane *1*), with anti-Cas mAb (lane *2*), and with antipaxillin mAb (lane *3*), respectively. After fractionation by SDS-PAGE and electrotransfer, immunoprecipitates were denatured and renatured and were overlayed with ^{32}P-labeled GST–FAK fusion protein containing FAK residues 707–1,052. (*B*) H9 cells were incubated in PLL- or FN-coated plates (lanes *1*, *3*, *5*, and *7* and lanes *2*, *4*, *6*, and *8*, respectively) for 30 min. Cells were lysed in 1% digitonin lysis buffer, and cellular lysates were immunoprecipitated without first Ab (lanes *3* and *4*) or with anti-Cas mAb (lanes *5* and *6*) or anti-FAK mAb (lanes *7* and *8*). Whole lysates (lanes *1* and *2*) and immunoprecipitates were analyzed by immunoblotting with anti-Cas mAb. (*C*) Cellular lysates of FN stimulated H9 cells were precipitated with GST- (lane *3*), GST-AblSH2– (lane *4*), GST-CrkSH2– (lane *5*), GST-CskSH2– (lane *6*), GST-Grb2SH2– (lane *7*), GST-LckSH2– (lane *8*), GST-NckSH2– (lane *9*), GST-PI3Ka– (lane *10*), GST-PLCγSH2– (lane *11*), GST-ShcSH2– (lane *12*), and GST-SHPTP2SH2– (lane *13*) conjugated beads. Whole lysates (lanes *1* and *2*) and the precipitates were analyzed by immunoblotting with anti-Cas mAb (*left*) and rehybridized with anti-pTyr (*right*). (*D*) H9 cells were incubated in PLL- or FN-coated plates (lanes *2*, *4*, *6*, *8*, *10*, *12*, *14*, *16*, and *18* and lanes *1*, *3*, *5*, *7*, *9*, *11*, *13*, *15*, *17*, and *19*, respectively). After lysis, cellular lysates were precipitated with GST- (lane *1*), GST-AblSH2– (lanes *2* and *3*), GST-CrkSH2– (lanes *4* and *5*), GST-CskSH2– (lanes *6* and *7*), GST-Grb2SH2– (lanes *8* and *9*), GST-LckSH2– (lanes *10* and *11*), GST-NckSH2– (lanes *12* and *13*), GST-PI3KSH2– (lanes *14* and *15*), GST-ShcSH2– (lanes *16* and *17*), and GST-SHPTP2SH2– (lanes *18* and *19*) conjugated beads. Precipitates were analyzed by immunoblotting with anti-Cas mAb and rehybridized with anti-pTyr. (*E*) H9 cells were incubated in PLL- or FN-coated plates (lanes *1*, *3*, *5*, *7*, *9*, and *11* and lanes *2*, *4*, *6*, *8*, *10*, and *12*, respectively) for 30 min and lysed in 1% digitonin lysis buffer. Cellular lysates were immunoprecipitated without first Ab (lanes *3* and *4*) or with anti-Nck mAb (lanes *5* and *6*), anti-Crk mAb (lanes *7* and *8*), anti-SHPTP2 mAb (lanes *9* and *10*), or anti-Cas mAb (lanes *11* and *12*). Whole lysates (lanes *1* and *2*) and immunoprecipitates were analyzed by immunoblotting with anti-Cas mAb.

p130Cas-related protein, determined its structure and distribution, and further demonstrated the putative function of pp105 through its recruitment of SH2 domain–containing proteins.

pp105 and p130Cas share several similar characteristics. Both of them are tyrosine phosphorylated by the engagement of β1 integrins and both are pp125FAK-binding proteins (11, 12, 18, 19). Both pp105 and p130Cas are tyrosine phosphorylated by the cotransfection of activated Src or Crk, and both bind to Crk in a tyrosine phosphorylation–dependent manner (13). Structurally, the deduced amino acid sequence of each Cas protein contains one highly conserved SH3 domain and multiple binding sites for SH2 domains. The SH3 domain of Cas is the FAK binding site (19; Tachibana, K., unpublished data). pp105 contains 13 repeated YXXP motifs (7 YDXP motifs) in the substrate

domain, whereas p130Cas contains 15 repeated YXXP motifs (9 YDXP motifs) (13). The other Cas family protein, p83Efs, contains 8 repeated YXXP motifs (4 YDXP motifs) (28). These motifs are reported to be the putative binding sites of the SH2 domains of Crk, Nck, and Abl (21). In addition, each Cas protein contains one conserved YDYVHL motif in the CT. This motif is weakly homologous to the predicted binding sites of the Src SH2 domain and of the SHPTP2 SH2 domain, although the protein(s) that binds to this motif has not yet been determined. The CT is relatively conserved among these three Cas proteins, suggesting that this domain has an important function(s) common to Cas proteins.

Besides these conserved motifs, there is one motif that is not conserved in pp105. p130Cas and p83Efs contain proline-rich sequences (RPLPXPP) upstream from the YDYVHL motif, although pp105 does not contain these sequences. These sequences have been reported to be putative SH3 domain–binding sites, and p83Efs was originally cloned as a Fyn SH3 domain–binding protein (28). This difference in structure may result in the difference between protein interactions and in the difference in function between pp105 and the other Cas family proteins.

The largest difference between pp105 and p130Cas was the cell type distribution. We previously identified pp105 in human T lymphocytes (11), whereas Sakai et al. (13) identified p130Cas in fibroblasts. In this study, we demonstrated the expression of Cas proteins in various cell lines. p130Cas was predominantly expressed in a human breast cancer cell line, T-47D, as well as in rat fibroblast 3Y1. pp105 was predominantly expressed in human lymphocytes. However, Northern blotting with tissue RNAs showed ubiquitous distribution of pp105, suggesting that the expression of pp105 is not limited to lymphocytes. p130Cas transcript was reported to be ubiquitously expressed, especially in the brain, lung, intestine, kidney, and testis (13). On the other hand, another Cas family protein, p83Efs, was reported to be expressed in the embryo, placenta, brain, and skeletal muscle but not in the spleen (28). These results indicate that pp105 is the dominant Cas protein in lymphocytes, whereas p130Cas is the dominant Cas protein in fibroblasts. In addition to this reciprocal expression, pp105 RNA is not well expressed in brain and liver. This interesting observation may suggest the putative function and/or promoter specificity of pp105.

Based on the notion that pp105 is a p130Cas-related protein, we attempted to define the putative function of pp105. Because pp105 is tyrosine phosphorylated by β1 integrin stimulation in H9 cells and in peripheral T lymphocytes, it is conceivable that phosphorylated tyrosine residues of pp105 are involved in the transduction of β1 integrin–mediated signals. If so, how is pp105 tyrosine phosphorylated by the stimulation of β1 integrins? We reported previously that pp105 was tyrosine phosphorylated with similar kinetics to pp125FAK phosphorylation by FN stimulation in H9 cells (12). The presence of pp105–pp125FAK binding suggests the involvement of FAK in the β1 integrin–mediated tyrosine phosphorylation of pp105, because FAK itself is activated and autophosphorylated by β1 integrin stimulation (29). It has also been reported that autophosphorylated FAK bound to Src SH2 domain and formed a stable complex (30), and cotransfected Src family tyrosine kinases have been shown to phosphorylate pp105. Taken together, these observations suggest that pp125FAK itself, and/or FAK-associated Src-family kinases, are involved in integrin-mediated tyrosine phosphorylation of pp105.

The second question to be considered is how tyrosine-phosphorylated pp105 transduces downstream signals. Because one of the major functions of protein tyrosine phosphorylation is phosphorylated tyrosine–mediated protein–protein interaction, we investigated proteins that were recruited to pp105 by β1 integrin stimulation. We demonstrated that Crk and Nck were recruited to tyrosine-phosphorylated pp105 both in vivo and in vitro. SHPTP2 was also recruited, although this association was weak. These proteins, which are recruited to pp105, appear to be involved in β1 integrin signaling via their recruitment to pp105.

pp105 contains multiple putative binding sites for the Crk SH2 domain, and tyrosine-phosphorylated pp105 binds to Crk. Crk is an adapter protein composed of SH2 and SH3 domains. Crk binds to SOS and another guanine nucleotide exchange protein, C3G, through its SH3 domain. Recently, our and several other laboratories reported that β1 integrin stimulation induces activation of Erk (10, 31, 32). Crk was reported to be involved in Ras activation by the recruitment of SOS (14). Tyrosine-phosphorylated pp105 may be involved in the recruitment of SOS via Crk, and recruited SOS activates Ras and Erk (33). However, Crk has been reported to bind to C3G better than to SOS, and membrane-anchored C3G activated another small GTPase, Rap1A/K-rev1, which inhibited Ras-mediated signals by competition (34). These reports suggest that recruitment of Crk to tyrosine-phosphorylated pp105 may be involved in the regulation of Erk signals through Ras and Rap1A.

Nck is another adapter protein that contains three SH3 domains and one SH2 domain (35). It has been reported that overexpression of Nck resulted in oncogenic transformation in fibroblasts (35), although the native function of Nck has not yet been identified. However, pp105 contains multiple putative binding sites for the Nck SH2 domain, and tyrosine-phosphorylated pp105 recruited Nck. These findings strongly suggest the involvement of Nck in β1 integrin–mediated signaling.

SHPTP2 is a cytoplasmic tyrosine phosphatase with two SH2 domains (36). Recently, it was reported that a dominant-negative SHPTP2 mutant inhibited the insulin-dependent dephosphorylation of FAK (37). SHPTP2 is recruited to tyrosine-phosphorylated pp105 through its own SH2 domain, and the deduced amino acid sequence of pp105 contains a possible binding site for the SHPTP2 SH2 domain. This recruitment of SHPTP2 to pp105 could be involved in the dephosphorylation of FAK and pp105.

pp105, as well as p130Cas, is tyrosine phosphorylated by

the overexpression of Crk proteins. Since Crk itself does not contain a catalytic domain characteristic of protein tyrosine kinases, overexpressed Crk may recruit a tyrosine kinase(s) to pp105. c-Abl is known to bind to the Crk SH3 domain (38) and was reported to phosphorylate tyrosine residues of p130Cas in vitro (39). In addition, tyrosine-phosphorylated pp105 bound to the c-Abl SH2 domain in vitro. Unfortunately, we have been unable to demonstrate in vivo binding of pp105 to c-Abl. However, findings described above suggest that c-Abl may interact and phosphorylate pp105 directly or via the binding to Crk.

In summary, pp105, one of the major tyrosine phosphorylated proteins induced through the ligation of β1 integrin on the surface of lymphocytes, is identified as a novel p130Cas-related protein. pp105 is the major Cas protein in lymphoid cells, whereas p130Cas is the major Cas protein in fibroblasts. pp105 is directly associated with the FAK COOH-terminal region in an integrin stimulation–independent manner, and tyrosine phosphorylated pp105 binds to the SH2 domains of Crk, Nck, and SHPTP2 in T cells. These findings reveal a novel architecture of β1 integrin–mediated protein tyrosine phosphorylation and further suggest the involvement of Crk, Nck, and SHPTP2 in the downstream pathways of β1 integrin–mediated signaling.

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