p150^{Ship}, a signal transduction molecule with inositol polyphosphate-5-phosphatase activity

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The production, survival, and function of monocytes and macrophages is regulated by the macrophage colony-stimulating factor (M-CSF or CSF-1) through its tyrosine kinase receptor Fms. Binding of M-CSF to Fms induces the tyrosine phosphorylation and association of a 150-kD protein with the phosphotyrosine-binding (PTB) domain of Shc. We have cloned p150 using a modified yeast two-hybrid screen. p150 contains one SH2 domain, two potential PTB-binding sites, an ATP/GTP-binding domain, several potential SH3-binding sites, and a domain with homology to inositol polyphosphate-5-phosphatases. p150 antibodies detect this protein in FDC-P1 myeloid cells, but the same protein is not detectable in fibroblasts. The antibodies immunoprecipitate a 150-kD protein from quiescent or M-CSF-stimulated FDC-P1 cells that hydrolyzes PtdIns(3,4,5)P₃ to PtdIns(3,4)P₂. This activity is observed in Shc immunoprecipitates only after M-CSF stimulation. Retroviral expression of p150 in FD-Fms cells results in strong inhibition of cell growth in M-CSF and a lesser inhibition in IL-3. Ectopic expression of p150 in fibroblasts does not inhibit growth. This novel protein, p150^{Ship} (SH2-containing inositol phosphatase), identifies a component of a new growth factor-receptor signaling pathway in hematopoietic cells.

[**Key Words:** Hematopoietic; signal transduction; inositol polyphosphate-5-phosphatase; PTB domain; Shc]

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Fms is a tyrosine kinase growth factor receptor closely related to the c-Kit, Flt3, and platelet-derived growth factor (PDGF) receptors (Rohrschneider 1995). The PDGF receptor is primarily expressed in fibroblasts and endothelial cells (Bowen-Pope et al. 1985), whereas Fms, Kit, and Flt3 are expressed in a lineage-dependent fashion in hematopoietic cells (Ullrich and Schlessinger 1990; Bernstein et al. 1991; Rosnet et al. 1991; Muench et al. 1995). These receptors control signals for growth, survival, differentiation, and expression of mature cell functions.

Fms and its signal transduction pathways provide a model for the understanding of the overall process of hematopoietic development. Binding of homodimeric macrophage colony-stimulating factor (M-CSF) to Fms results in receptor dimerization and *trans*-phosphorylation at specific tyrosine residues. SH2-containing molecules bind to these docking sites, and the receptor signal is transduced and amplified concurrent with tyrosine phosphorylation of additional cytoplasmic signaling proteins. For example, the SH2 domain of Grb2 binds the phosphorylated tyrosine Y697 of Fms (van der Geer and

Hunter 1993; Lioubin et al. 1994) and through its SH3 domains interacts with mSOS translocating this nucleotide exchange factor to the plasma membrane (Buday 1993; Egan et al. 1993; Li et al. 1993; Rozakis-Adcock et al. 1993). The SH2 domains of the p85 component of the phosphatidylinositol 3' kinase complex binds to the phosphorylated Y721 residue of Fms (Reedijk et al. 1992), and in addition, Src family members may interact with the phosphorylated Y559 residue in the juxtamembrane region of activated Fms (Alonso et al. 1995). Several other proteins are tyrosine phosphorylated after Fms activation (Lioubin et al. 1994), but it is unclear what additional kinases are involved in this process.

Recently, we identified a new protein that is tyrosine phosphorylated after M-CSF stimulation of Fms (Lioubin et al. 1994). A related 145-kD tyrosine-phosphorylated protein is detected after lymphokine stimulation of other receptors in a number of hematopoietic cell lines. Antigen receptor cross-linking in B cells (Saxton et al. 1994), IL-3, Steel factor, or erythropoietin stimulation of erythroid cells and megakaryocytes (Cutler et al. 1993; Damen et al. 1993), or M-CSF stimulation of monocytes and Fms-expressing erythroid cells, results in the tyrosine phosphorylation of a 145- to 150-kD protein that is found in a complex with Shc, Grb2, and mSOS (Lioubin

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et al. 1994; Liu et al. 1994). The nature of the associations between these proteins and the 145- to 150-kD protein is not understood, nor is the function of this protein in signaling known. An interaction between the SH3 domains of Grb2 and proline-rich sequences in the carboxy-terminal position of mSOS has been described, and similar interactions could occur with the 145- to 150-kD protein. SH2 domains are present in both Grb2 and Shc and could bind the tyrosine-phosphorylated 145- to 150-kD protein. Alternatively, a novel phosphotyrosine-binding (PTB) domain has been identified in the amino terminus of Shc (Blaikie et al. 1994) and could provide a strong link to the 145- to 150-kD protein. The PTB domain is distinct from SH2 domains and binds an NPXY motif in the epidermal growth factor (EGF) receptor tail (Blaikie et al. 1994).

The understanding of the signaling mechanisms by Fms requires a more complete knowledge of the proteins and components involved and how their interactions participate in this process. The p150 protein is a component of the signal transduction process by several hematopoietic growth factor receptors. We therefore sought to clone the cDNA for this protein for further analyses of the signaling mechanisms by the M-CSF receptor (Fms).

Results

We showed previously that p150 binds to Shc in M-CSF-stimulated Fms-expressing myeloid cells (Lioubin et al. 1994). The interaction of p150 with Shc was examined further in 32D-Fms cells with either antibodies to Shc or a glutathione *S*-transferase (GST) fusion protein containing the PTB domain of murine Shc (amino acids 48–209) (Fig. 1). Antibodies to Shc immunoprecipitated both Shc and p150, and this interaction was partially inhibited by

either 100 mM phenylphosphate, a phosphotyrosine analog, or the Shc PTB domain. When the GST fusion protein of the Shc PTB domain was incubated with cell lysates from M-CSF-stimulated hematopoietic cells expressing exogenous murine Fms, it readily bound tyrosine-phosphorylated p150 (Fig. 1). This association was partially interrupted by the addition of phenylphosphate, suggesting a high-affinity interaction between the Shc PTB domain and a phosphotyrosine on p150. Moreover, this complex contained very low levels of tyrosine-phosphorylated Shc, consistent with the observation that the predominant interaction of Shc with p150 occurs through the Shc PTB domain and not the Shc SH2 domain. The PTB domain of Shc has also been shown to bind a 145-kD tyrosine-phosphorylated protein in PDGF-stimulated fibroblasts (Kavanaugh and Williams 1994).

Mutations in Fms that abolish its interactions with PI3 kinase (Y721F) and Grb2/Sos (Y697F) do not abrogate p150 phosphorylation. Association of p150 with Shc and Grb2 is also unaffected by mutations of the known Fms autophosphorylation sites but does require Fms tyrosine kinase activity (Lioubin et al. 1994). This suggests that the p150-containing complexes interact with an as-yet-uncharacterized binding site on Fms, either through direct or indirect means.

A modified yeast two-hybrid system was designed to clone proteins that bind to the Shc PTB domain. The yeast two-hybrid system (Vojtek et al. 1993; Fields and Sternglanz 1994; Vojtek and Hollenberg 1995) includes a LexA fusion vector, pBTM116 (Bartel et al. 1993), which permits the in-frame fusion of LexA to a protein of interest (bait) and a VP16 fusion vector, pVP16, into which cDNA fragments encoding the target protein are ligated. Although this system has been utilized successfully to identify protein–protein interactions, it is limited in identifying protein–phosphotyrosine interactions because yeast does not express active tyrosine kinases (Dailley et al. 1990). Therefore, the pBTM116 vector was modified to include the expression of a tyrosine kinase that could phosphorylate the target fusion proteins (Fig. 2). To test the modified system, the PTB domain of Shc was fused to the LexA protein (LexA–PTB) as bait, and the EGF receptor carboxy-terminal tail (EGFRT) was fused to the VP16 protein as a target. We tested the ability of introduced kinases to phosphorylate the known PTB domain binding motif (NPXY) in the EGFRT domain, thus enabling it to bind the Shc PTB domain. No interaction was detected in the absence of the kinase or the PTB domain, but large dark blue colonies were obtained when yeast cells expressed the LexA–PTB domain along with the PDGF receptor kinase domain and the VP16–EGFRT (Fig. 3A). These results indicated the feasibility of such an approach, and a two-hybrid screen of a myeloid cell library was initiated.

The screen employed the pBTM116/PDGFR vector with the Shc PTB domain (amino acids 48–209) as the bait (Fig. 2), and a VP16 target library prepared from hematopoietic EML cells (Tsai et al. 1994). A total of 4.9×10^6 independent transformants were screened, resulting in 194 colonies that were positive for transcrip-

Figure 1. Shc interactions with p150 in murine myeloid cells. 32D cells expressing murine c-Fms were stimulated with M-CSF (5000 U/ml), and cell lysates were immunoprecipitated with Shc polyclonal antibodies in the presence (+) or absence (–) of 100 mM phenylphosphate (ϕPO_4) or 5 μg of soluble Shc PTB fused to GST (GST–PTB). Similar lysates were incubated with GST or GST–PTB fusion protein (5 μg) bound to agarose beads. The washed precipitates were Western blotted with anti-phosphotyrosine monoclonal antibodies.

Figure 2. Modified yeast two-hybrid system. (A) Schematic representation of the modified pBTM116 vector expressing the PDGF receptor tyrosine kinase pBTM116/PDGFR. Tyrosines Y1009 and Y1021 of the PDGF receptor were mutated to phenylalanine, F, to eliminate binding of PTP2C and PLC γ , respectively. (B) The pBTM116/PDGFR vector provides the LexA-Shc PTB fusion bait and the kinase (PDGFR). The pVP16 vector provides the library (target) as a fusion with VP16 that contains a nuclear localization signal (NLS). When both vectors are coexpressed in yeast, the kinase phosphorylates target fusion proteins on tyrosine residues enabling them to bind the bait. Such interactions form a bridge between LexA and VP16 and initiate transcription of the *his3* and *lacZ* genes through the *lexA* operator, allowing growth on histidine-depleted medium and β -galactosidase blue color selection, respectively.

tional activation. Forty-eight were cured, to allow loss of the pBTM116/PDGFR vector, and retested by mating with a panel of new LexA baits to confirm specificity (Fig. 3A). Forty-four, representing 14 distinct clones as determined by restriction enzyme analysis, were specific for Shc PTB domain binding. DNA sequence analysis indicated that the PTB motif (NPXY) was present in each of the 14 clones. Of the 14 clones, 10 were derived from the same gene (Fig. 3B). Northern blot analysis was performed on RNA from monocytic cells (Fig. 3C), with the cDNA insert (1.1 kb) from one of the clones (EML-11), and a single mRNA species of ~5.0 kb was identified.

The EML-11 cDNA insert was used as a probe to screen a cDNA library constructed from FDC-P1 clone 19 and Mac11 murine hematopoietic cells (Rohrschneider and Metcalf 1989; Gliniak and Rohrschneider 1990). One clone (150.8) containing an insert of ~5.0 kb was

subjected to DNA sequencing in both directions. This clone contained 4863 nucleotides with an open reading frame (ORF) encoding an 1190-amino-acid protein with a calculated molecular weight of 133,442 (Fig. 4A). In addition, we had previously isolated the tyrosine-phosphorylated p150 protein by anti-phosphotyrosine affinity chromatography and obtained amino acid sequence from Lysine C-generated peptides (Aebbersold et al. 1987). Eleven sequenced Lysine C peptides derived from the native protein were found in the same ORF, including a peptide (GRDYRDNTLP) 19 amino acids before a stop codon and an amino-terminal peptide, PAMVPGWN (residues 2–9) (Fig. 4A). Furthermore, a 150-kD tyrosine-phosphorylated protein was immunoprecipitated with antibodies made against peptide fragments of p150 (see below), and therefore, the nature of the translation product was verified by independent means.

Figure 3. Yeast two-hybrid screen. (A) Verification of positives. The EGF receptor carboxy-terminal tail (EGFRT) was fused to VP16 and used as a positive control. To verify specific interactions, the EML clones were cured of the pBTM116/PDGFR bait vector and tested in mating experiments with AMR70 yeast containing the LexA baits indicated at left. Clone 11 from the EML VP16 library (EML-11) is shown as an example. (B) Alignment of the two-hybrid screen clones. The yeast two-hybrid clones are indicated by a line. The nodes represent NPXY and NPLY sequences, respectively. (▼) A 15-nucleotide insertion (TCTCCCATGCTCCAG) that results in the translation of an additional 5 amino acids (amino acids 720 sknVSHAPGpgt). The significance of this is not yet known. (C) Northern blot analysis. Poly(A)⁺-selected RNA from FDC-P1 cells was hybridized with a 1.1-kb ³²P-labeled probe derived from the EML-11 clone. The size is indicated at left (in kb).

Figure 4. Amino acid sequence of p150 and its relationship to other proteins. (A) Amino acid sequence of the deduced translation product encoded by the cDNA clone 150.8. The SH2 domain is heavily underlined; (single underline) a region homologous (55% identity and 73% similarity) to 51C; (double underline) a region with homology (23%–30% identity and 44%–52% similarity) to sequences found in the inositol polyphosphate-5-phosphatases (IP5Pases), OCRL (IP5P), and IP5P2; (open box) the ATP/GTP-binding domain (P loop); (shaded boxes) the amino acid sequence obtained from Lysine C peptides of the native protein; (bold letters) the NPXY motifs. (B) Schematic representation of p150^{Ship} in comparison to 51C and IP5Pases showing the different domains. (Light shaded box) 51C homology domain; (dark shaded box) inositol polyphosphate-5-phosphatase domain; (insert boxes) NPXY motifs; (vertical cluster of lines) proline-rich motifs in agreement for SH3 domain binding. (C) Alignment of amino acid sequences conserved between IP5Pases and Ship. These subdomains are contained within the double underlined domain of p150 (Fig. 4A) and are considered to be signatures for IP5Pases. (SHIP) Murine p150 SH2-containing inositol phosphatase (GenBank accession no. U51742); (51C) human 51C gene product (L36818); (OCRL) human oculocerebrorenal gene product (P32019); (IP5P2) human inositol polyphosphate-5-phosphatase type II gene product (Q01968). The numbers at the left of each column denote amino acid position within each molecule. Data base accession numbers are indicated in parentheses. The single-letter amino acid code was used.

The amino terminus of p150 contains an SH2 domain belonging to the 1B class of SH2 domains (Songyang and Cantley 1995), and the carboxy-terminal region has two NPXY motifs (NPXY and NPLY) with potential PTB domain-binding ability. Of the 10 p150 clones identified in the two-hybrid screen, 3 contained only the NPXY motif

and 7 clones contained both the NPXY and NPLY motifs (Figs. 3B and 4A). This strongly suggests that at least the tyrosine within the NPXY motif is phosphorylated and binds to the Shc PTB domain. It is not known whether the tyrosine in the NPLY sequence is also phosphorylated and capable of binding to the Shc PTB domain. The

carboxy-terminal region of p150 also contains several proline-rich clusters with three in good agreement with motifs that are known to bind SH3 domains (Ren et al. 1993).

An appreciable proportion of ATP- or GTP-binding proteins share the conserved motif (A,G)X₄GK(S,T), or P loop, within a glycine-rich region (Walker et al. 1982; Saraste et al. 1990). It is believed that this motif interacts with one of the phosphate groups of the nucleotide. This motif (GqplhGKS) is also contained in the carboxyl terminus of p150, only 7 amino acids after the NPNY sequence.

The central portion of p150 exhibits a striking resemblance to the human 51C gene product (Fig. 4B). Interestingly, the murine 51C homolog was identified as one of the four additional positive clones obtained in the two-hybrid screen. The sequence of the murine 51C product indicated that it is clearly a distinct gene and the protein contains an NPAY motif as shown in Figure 4B. This suggests that like p150, 51C can be phosphorylated at the NPAY site during growth factor receptor stimulation and interact with the Shc or a related PTB domain.

The 51C cDNA was cloned previously on the basis of its ability to complement the Fanconi anemia group A complementation gene (FA-A) for hypersensitivity to DNA-damaging agents (Hejna et al. 1995), although it is not clear whether the cloned gene encodes that activity. The FA-C gene (for the C complementation group) encodes a 60-kD protein, and antibodies to this protein also recognize a 150-kD protein (Strathdee et al. 1992; Yamashita et al. 1994). The relationship of p150 to these FA-C proteins is not yet understood.

Significant amino acid homology is also shared between the central portion of p150 and proteins with inositol polyphosphate-5-phosphatase (IP5Pase) activity (Fig. 4). The X-linked Lowe's oculocerebrorenal syndrome gene (OCRL) was identified previously by a positional cloning strategy applied to a translocation occurring in a female patient (Attree et al. 1992). The OCRL transcript was absent, or of abnormal size, in most male patients with the disease. OCRL (IP5P) and IP5P2 (inositol polyphosphate-5-phosphatase type II) are involved in inositol metabolism, removing the 5-phosphate of inositol (1,4,5) triphosphate and/or inositol (1,3,4,5) tetrakisphosphate. Although p150 homology with the known IP5Pases is not as extensive as with 51C, numerous subdomains are conserved between p150, 51C, and IP5Pases (Fig. 4C) and may be critical for enzymatic activity.

The potential enzymatic activity of p150 was tested *in vitro* by use of PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ that had been phosphorylated in the D3 position by PI3K and [γ -³²P]ATP. p150 immunoprecipitated from quiescent or M-CSF-stimulated FDC-P1 cells hydrolyzed PtdIns(3,4,5)P₃ to PtdIns(3,4)P₂ with equal efficiency (Fig. 5). An increase in this 5-phosphatase activity was observed in Shc immunoprecipitates only after M-CSF stimulation, in agreement with the observation that p150 association with Shc occurs only after M-CSF stimulation. In contrast, PtdIns(3,4)P₂ was not a substrate for p150 (Fig. 5). The fact that tyrosine phosphorylation of p150 does

Figure 5. p150^{Ship} contains 5-phosphatase activity. Normal rabbit serum (NRS), anti-Ship (SHIP) or anti-Shc (SHC) immunoprecipitates from quiescent (–) or M-CSF stimulated (+) FDC-P1/Fms cells were incubated with equal amounts of either PtdIns(3*,4,5)P₃ or PtdIns(3*,4)P₂ in 5-phosphatase assay buffer. After 20 min at room temperature, the phospholipids were extracted with an equal volume of chloroform/methanol (1:1) and separated by TLC. Radioactive lipids were detected by autoradiography. The positions of PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂ are indicated at *left*. (*) Position of the inositol ring labeled with [³²P] phosphate.

not affect its catalytic activity suggests that localization after M-CSF stimulation might be important for its function. The enzymatic activity of p150 is in agreement with that observed for a gene (p145) that participates in erythropoietin signal transduction (Damen et al. 1996). Taken together, this indicates that p150, and possibly 51C, are IP5Pases. We propose that p150 be named SHIP for SH2-containing inositol phosphatase.

Antibodies raised against the unique carboxy-terminal region of p150^{Ship}, amino acids 889–1046, and corresponding to the full-length translation product of the EML-25 clone, recognized a major 150-kD protein in hematopoietic (32D–Fms) cells that comigrated with the tyrosine-phosphorylated protein observed after M-CSF stimulation (Lioubin et al. 1994) (Fig. 6A). The p150 band was the most prominent but often appeared as a doublet, and in each case, multiple minor bands (up to five), below the main p150 protein, were detectable. All of these Ship-related proteins contained phosphotyrosine. In addition, immunoprecipitation with p150^{Ship} antibodies and blotting with anti-Shc antibodies demonstrated that tyrosine-phosphorylated p52^{Shc} (and a lesser amount of p45^{Shc}) was associated with p150^{Ship} after M-CSF stimulation (Fig. 6A). The amount of tyrosine-phosphorylated p150^{Ship} was elevated after treatment with M-CSF and correlates with increased amounts of associated Shc. Moreover, Shc is eluted from such Ship immunoprecipitates with 100 mM phenylphosphate, indicating that such associations depend on p150^{Ship} phosphorylation (Fig. 6A). These results indicate that antibodies to the protein sequence of the EML-25 clone of p150^{Ship} recognize the same 150-kD protein that we described previously (Lioubin et al. 1994), and this protein becomes tyrosine phosphorylated and associated with Shc after

phenylphosphate and blotted with anti-Shc polyclonal antibodies. (B) Retroviral expression of p150^{Ship}. Psi2 cells were transfected with Ship containing LXS vector and were selected with 0.5 mg/ml of G418. Psi2/Ship supernatants containing virus were used to infect Rat2 cells. Lysates were normalized for total protein content and immunoblotted with Ship antibodies. Molecular weight markers are indicated at left (in kD); the positions of Ship and Shc are indicated at right.

M-CSF stimulation of 32D-Fms (Fig. 6A) or FD-Fms (not shown) cells.

Another striking feature of the p150-kD protein described previously is its lack of detection by phosphotyrosine antibodies in Fms-expressing fibroblast cells stimulated with M-CSF (Lioubin et al. 1994). This was re-examined in both Rat2 and Psi2 fibroblasts with the antibodies prepared to p150^{Ship}. In addition, p150^{Ship} was expressed in these cells by insertion of the entire clone 150.8 insert (nucleotide -91 to 4863), which includes both 5'- and 3'-untranslated regions including the poly(A) tail, into the retroviral vector LXS. This was transfected into the Psi2 cells and resultant retrovirus used to infect the Rat2 cells. Consistent with previous observations, the results in Figure 6B demonstrate that Ship antibodies do not recognize a 150-kD protein in fibroblasts containing the empty retroviral vector. Conversely, when the same cells expressing p150^{Ship} from the retroviral vector are examined, a prominent 150-kD protein of the same size as that in FDC-P1 cells is detected with the antibodies to Ship (Fig. 6B). Taken together, these results suggest that the 150.8 clone contains the full-length cDNA encoding a protein that is tyrosine phosphorylated in response to M-CSF and to a lesser extent in response to IL-3; it associates with Shc in a phosphotyrosine-dependent manner in myeloid cells, but the same protein is not expressed in fibroblasts.

The role of p150^{Ship} in Fms signaling was next examined by overexpression of the Ship protein in FD and FD-Fms cells with the same retroviral vector, pLXS, described above. FD and FD-Fms cells were infected by coculture over Psi2 cells expressing either the empty vector (Lx in Fig. 7) or the Ship-expressing vector (Ship). FD cells were selected ~2 weeks in G418 and analyzed for p150^{Ship} expression and growth in soft agar. No differences in Ship expression or cellular behavior were detected between cells infected with Lx and Ship. Under the assumption that negative selection for Ship-expressing cells occurred during the 2 weeks in G418, cells were analyzed either immediately after coculture over the

Psi2 cells by placement in agar assays with G418, or examination of growth in agar after a brief 3-day selection in a high concentration of G418. Both methods gave similar results, although overall there were fewer colonies formed in the presence of G418. FD-Lx and FD-Ship cells did not grow without added growth factor or with the addition of M-CSF because they lacked the M-CSF receptor (Fig. 7A,B). In the presence of IL-3, the

Figure 7. Soft agar colony formation. FD and FD-Fms cells were infected with the empty vector (Lx) or the same vector expressing Ship. (A) The size of the colonies grown without growth factor (-), with M-CSF, or with IL-3 added to the agar medium. (B) The total number of colonies (>10 cells) is shown for each of the growth conditions. The values are the average and standard deviation of quadruplicate determinations.

FD-Lx cells formed large colonies as expected (Rohrschneider and Metcalf 1989), and FD-Ship cells formed fewer, and in general, smaller colonies. FD-Fms/Lx cells again produced colonies of the size and number seen previously for FD-Fms cells (Rohrschneider and Metcalf 1989). In contrast, the FD-Fms/Ship cells formed fewer and significantly smaller colonies than the control FD-Fms/Lx cells in M-CSF. When the FD-Fms/Ship cells were grown in IL-3, the colony size was similar to the control FD-Fms/Lx cells but a reduction in colony number was observed (Fig. 7A,B). Although FD-Fms/Ship cells in M-CSF formed mainly small colonies in soft agar, a few were larger and compact like those formed in IL-3. Presumably, these larger colonies lacked the retrovirally expressed p150^{Ship} yet were G418 resistant and accounted for the outgrowth of normal cells and lack of p150^{Ship} overexpression after longer term culture. These results suggest that overexpression of p150^{Ship} in FDC-P1 cells results in a suppression of both M-CSF, and to a lesser degree, IL-3-dependent growth. This is not a non-specific effect because the same p150^{Ship} expressed in Rat2-Fms cells has no negative influence on M-CSF-dependent growth in soft agar; in fact, the p150^{Ship}-expressing cells grow slightly better than the cells expressing the empty vector (data not shown). Also, segments of the *ship* cDNA-expressing subdomains of the Ship protein can be expressed at high levels in FD cells by use of the same pLXSN vector (data not shown).

The expression level of p150^{Ship} was examined in both FD-Fms/Lx and FD-Fms/Ship cells after infection by 2-day growth over Psi2 cells followed by a 3-day selection in 0.75 mg/ml of G418. Immunofluorescence detection of the Ship protein with affinity-purified antibodies demonstrated that the FD-Fms/Ship cells expressed slightly higher levels of the protein than the endogenous p150^{Ship} detected in the FD-Fms/Lx cells (Fig. 8). The expression level for p150^{Ship} in the FD-Fms/Ship cells was uniformly higher than in the FD-Fms/Lx cells; however, this level of expression was not even two-fold higher. These results contrast sharply with the relative ease of p150^{Ship} overexpression in the Rat2 and Psi2 cells, and suggest that expression levels are very tightly regulated in the FDC-P1 cells, and overexpression may have deleterious effects.

Discussion

These results demonstrate that the M-CSF activation of Fms leads to downstream tyrosine phosphorylation of a 150-kD IP5Pase (p150^{Ship}). Phosphorylation within the carboxy-terminal NPNY motif (and probably at other sites) allows Ship to associate with the Shc PTB domain. The SH2 domains of both Shc and Ship suggest additional interactions with tyrosine-phosphorylated proteins. Proline-rich sequences in the carboxyl terminus of Ship may allow coupling to proteins containing SH3 domains. These interactions may either activate the enzymatic activity of Ship or transport Ship to a specific site for access to substrates. Although the two-hybrid assay required that the NPXY motif of p150 be tyrosine phos-

Figure 8. p150^{Ship} overexpression in FD-Fms cells. Immunofluorescence analysis of p150^{Ship} expression in FD-Fms cells infected with an empty retroviral vector (FD-Fms/Lx, thin-line histogram), or a retroviral vector expressing the p150^{Ship} protein (FD-Fms/Ship, thick-lined histogram). After infection, cells were grown in IL-3 and selected for 3 days in G418. The Ship protein was detected with affinity-purified rabbit antibodies and a secondary fluorochrome-labeled, affinity-purified antibody.

phorylated by the PDGF receptor kinase domain, it is not clear whether the Fms tyrosine kinase or another tyrosine kinase performs this task in vivo. The fact that a non-tyrosine kinase growth factor receptor, such as the EPO receptor, also stimulates the tyrosine phosphorylation of p150^{Ship} (see below) suggests that an additional tyrosine kinase may be involved.

The p150^{Ship} detected after M-CSF stimulation of Fms-expressing myeloid cells is identical to the p145 protein that is tyrosine phosphorylated after B-cell activation (M.T. Crowley and A.L. deFranco, pers. comm.) and the p145 protein that is tyrosine phosphorylated after activation of the EpoR and IL-3R (Cutler et al. 1993; Damen et al. 1993). Moreover, we have found that p150^{Ship} is tyrosine phosphorylated and associates with Shc after stimulation of erythroid cells with either Epo or M-CSF, and it participates in signaling by both IL-3 and M-CSF (and weakly by GM-CSF) in FD-Fms cells (M.N. Lioubin, unpubl.). Finally, Damen and colleagues using a PCR strategy, also isolated a cDNA for the p145 that is tyrosine phosphorylated after hematopoietic cytokine stimulation of myeloid progenitor cells and have obtained the same protein (Damen et al. 1996). This suggests a central role for Ship in diverse growth factor receptor signal transduction pathways in hematopoietic cells.

In contrast, the p145 Shc PTB protein observed in PDGF-stimulated fibroblasts (Kavanaugh and Williams 1994) was not tyrosine phosphorylated after M-CSF stimulation of Rat2-Fms fibroblasts (Lioubin et al. 1994) and our antibody to p150^{Ship} did not detect this protein in fibroblast cell lines. This suggests that the fibroblast p145 has a structure different than p150^{Ship} from he-

matopoietic cells, and neither can substitute for the other in Fms-expressing fibroblasts. This also would explain the lack of overt growth effects of ectopic p150^{Ship} expression in fibroblasts (M.N. Lioubin and L.R. Rohrschneider, unpubl.).

Ship-related genes (Fig. 4B,C) are linked to several disease states in humans, and each involves the metabolism of phosphatidylinositol (PI) polyphosphates. The 51C gene (now called INPLP1) (Hejna et al. 1995) exhibits the highest homology to Ship and was isolated by complementation of the DNA repair defect in cells from a Fanconi anemia patient, complementation group A (FA-A). Analysis of the gene from FA patients, however, indicated that 51C/INPLP1 was probably not responsible for FA-A. Whatever its function, the 51C/INPLP1 gene product probably acts in an analogous fashion to p150^{Ship} through tyrosine phosphorylation of the NPXY motif and binding of a PTB domain-containing protein, possibly Shc. These interactions likewise may effect the activation or localization of the PI5Pase activity. The OCRL protein also contained homology to Ship and IP5P in the central enzymatic domain (Attree et al. 1992). The mutated OCRL protein in humans is associated with congenital cataracts, mental retardation, and defective renal tubular function. Another rare hereditary syndrome, ataxia telangiectasia (AT) falls into the related category of gene products that may metabolize PI (Savitsky et al. 1995). Affected individuals exhibit an unsteady gait and dilated blood vessels, along with defects in growth, aging, and increased sensitivity to ionizing radiation. Part of the large AT gene has been identified as encoding a carboxy-terminal domain suggesting PI 3-kinase activity (Savitsky et al. 1995). TOR proteins represent other products with PI 3-kinase-like enzymatic domains, and TOR affects cell-cycle progression from G₁ to S phase through p70^{S6k} activation (Heitman et al. 1991). The TOR2 gene product is associated with a PI-4 kinase activity (Cardenas and Heitman 1995). The summation of these disease states, their structural relationship to Ship, and pleiotropic pathology resulting from defects in these genes suggest the widespread significance of inositol metabolism in multiple cellular regulatory processes.

The exact role of p150^{Ship} and its IP5Pase activity in Fms signaling is not presently understood, but the protein is tyrosine phosphorylated in FD-Fms cells within 30 sec of M-CSF stimulation (Lioubin et al. 1994) and a role in growth control is likely. FDC-P1 cells normally express abundant p150^{Ship}; however, elevation of the expression level of p150^{Ship} in FD-Fms cells by retroviral expression resulted in the strong inhibition of M-CSF-dependent growth and a weaker inhibition of growth in IL-3. On the other hand, p150^{Ship} was not detectable in Rat2 cells, and ectopic expression had no inhibitory influence on M-CSF or serum-dependent growth of Rat2 or Rat2-Fms cells (M.N. Lioubin and L.R. Rohrschneider, unpubl.). A nonspecific growth inhibition is an unlikely explanation for the above results, but the observations suggest that the Ship protein level is tightly regulated and that overexpression negatively controls the growth of cells in

which it functions. These results are reminiscent of other proteins that block cellular DNA synthesis and cell-cycle progression when overexpressed (Sherr and Roberts 1995).

The growth-inhibitory role of p150^{Ship} and its IP5Pase activity in Fms signaling (Fig. 7) is opposite to that reported for the positive mitogenic effects of PI-3 kinase activity (Valius and Kazlauskas 1993). These two lipid-modifying enzymes, however, may act in concert to regulate overall proliferation. This notion is supported by the finding that the p85/p110 PI-3 kinase enzyme forms a complex with a cytoplasmic inositol polyphosphate-5-phosphatase (Jackson et al. 1995). PI-3 kinase synthesizes the substrate PtdIns(3,4,5)P₃ for p150^{Ship}, and p150^{Ship} modifies the substrate further by removing the phosphate from the D5 position. Therefore, either the active lipid PtdIns(3,4,5)P₃ is removed and/or a new lipid product, PtdIns(3,4)P₂, is created for additional activities (Stephens et al. 1991; Hawkins et al. 1992). Thus, if PtdIns(3,4,5)P₃ is necessary for mitogenic stimulation, then growth inhibition by p150^{Ship} is easily understood as the degradation of this signal. In this regard, both PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ are known to stimulate protein kinase C family members (Toker et al. 1994), and growth stimulation could be affected by this activation.

Additional activities for p150^{Ship} may include modulation of the Akt/PKB kinase leading to p70^{S6k} activity (Burgering and Coffey 1995; Franke et al. 1995). PtdIns(3,4,5)P₃ binding to the pleckstrin homology domain of Akt/PKB may cause dimerization and activation, and p150^{Ship} could inhibit this pathway by metabolizing the phospholipid substrate. Other activities of an inositol 5-phosphatase may include the degradation of Ins(1,3,4,5)P₄. This lipid stimulates Ras GAP activity (Cullen et al. 1995) and p150^{Ship} expression and therefore, would be expected to decrease the amount of Ins(1,3,4,5)P₄ and stimulate growth. This effect is opposite to that observed and is therefore considered less likely to represent a major function for Ship.

Finally, the activity of p150^{Ship} as a negative regulator of cell growth, along with the observations that several checkpoint genes possess inositol lipid-modifying domains, suggests that p150^{Ship} could function in one of these pathways (a current discussion of these issues can be found at <http://www.fhcr.org/~lrr/>).

Materials and methods

Cells and culture

FDC-P1 cells were originally obtained from Don Metcalf (Duhren and Metcalf 1988) and a clone (clone 19) selected for its ability to differentiate along the macrophage lineage after retroviral vector expression of Fms and growth in M-CSF (Bourette et al. 1995). Both clone 19 FDC-P1 cells (FD) and the Fms-expressing cells (FD-Fms), as well as 32D-Fms cells, were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) and IL-3 from conditioned medium of either WEHI-3B cells or X63-IL-3 cells expressing the recombinant IL-3. When cells were shifted to M-CSF-containing medium, they were washed once in DMEM/10% FBS and either starved in this medium without added growth factor for a few hours, or were replated in M-CSF-containing medium. FD and

FD-Fms cells remained blast-like in IL-3, but the FD-Fms cells differentiated to macrophages in the presence of murine M-CSF (Bourette et al. 1995). Psi2 cells were maintained in DMEM with 10% calf serum, and Rat2 or Rat2-Fms cells in the same medium with 10% FBS.

Immunoprecipitation and Western blot (immunoblot) analysis

Cells were lysed in lysis buffer [20 mM Tris (pH 7.5), 10 mM EDTA, 100 mM NaCl, 1 mM ZnCl₂, 0.05% NaN₃, 2% polyoxyethylene 9 lauryl ether (C₁₂E₉), 2 mM orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 mM iodoacetamide, 5 µg of leupeptin per ml]. After 15 min on ice, lysates were spun at 25,000g for 30 min, and the pellet containing nuclei and other insoluble material was discarded. For Western blot analysis, samples were electrophoresed on SDS-7.5% or 8.75% polyacrylamide gels and transferred to nitrocellulose membranes for 4 hr at 0.8 mA/cm² with a semidry blotting apparatus (Ellard Instrumentation Ltd.). After transfer, the membranes were processed as described by Carlberg and Rohrschneider [1994]. Briefly, the membranes were blocked overnight at 4°C with block buffer [1% bovine serum albumin and 1% ovalbumin in rinse buffer (10 mM Tris-HCl at pH 7.5, 300 mM NaCl, 0.5% Tween 20)]. The nitrocellulose membranes were incubated for 1 hr with primary antibody diluted in block buffer, and the signal was visualized with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (ECL) reagents (Amersham).

For immunoprecipitations, lysates were incubated with the primary antibody and 10 µl of protein G-Sepharose beads (Pharmacia) for 1 hr at 4°C. The beads were washed five times with wash buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 0.1% C₁₂E₉, 200 µM orthovanadate], and proteins were eluted by the addition of 50 µl of 2× SDS-PAGE sample buffer and heating at 95°C for 5 min. Alternatively, cell lysates were incubated with the purified GST fusion proteins at 4°C for 2 hr, and GST fusion proteins were precipitated by addition of 20 µl of glutathione-agarose beads. The beads were washed five times with wash buffer, and proteins were eluted as described above for SDS-PAGE analysis.

GST-Shc PTB fusion proteins

The murine Shc PTB domain (amino acids 48–206) was PCR amplified from a murine full-length cDNA using the primers 5'-CGGAATTCAACAAGCTGAGTGGAGGCG-3' and 5'-GT-CGACTTCTTCCTCCTCATCCCAAG-3'. The amplified fragment was purified from an agarose gel, digested with *EcoRI* and *Sall*, and ligated in the same sites of pGEX-1B (pGEX-1 with the polylinker region exchanged for that of pBTM116). All GST fusion proteins were affinity purified from cell lysates of *Escherichia coli* XL-1 Blue (Stratagene) by adsorption to glutathione-agarose resin followed by elution with 5 mM glutathione as described previously [Smith and Johnson 1988].

Vector construction

Tyrosine kinases were inserted into the bait plasmid pBTM116 [Vojtek et al. 1993] as a cassette containing the *ADH1* promoter, kinase, and *ADH1* terminator provided by the pAD4 vector [Young et al. 1989; Keegan and Cooper 1996]. The cytoplasmic domain of the βPDGF receptor was PCR amplified from pRR6 Y1009F, Y1021F [Kashishian and Cooper 1993] with the following primers: GCCCAAGCTTACCATGGTTGAGTCTGTGAG and TATCAAATCGCCGCGCTACAAGGAAGCTAT. The PCR reactions were carried out using equal amounts of *Taq* polymerase (GIBCO BRL) and Extender supplement (Strata-

gene). The products were cut with *HindIII* and cloned into the *HindIII* and *SmaI* sites of pAD4. The *ADH1* promoter, kinase, and *ADH1* terminator cassette were excised as a *BamHI* fragment, end-filled with Klenow, and ligated into the *PvuII* site of the pBTM116 to produce the pBTM116/PDGFR plasmid. This insertion results in the loss of the BTM116 polylinker unique cloning sites except the *Sall* site. The Shc-PTB domain (amino acids 48–206) was PCR amplified with the primers 5'-GC-TACGCGTCGACCTGGGGTTTCCTACTTGTTTC and 5'-CGCGTCGACTTCTTCCTCCTCATCCCAAG, cut with *Sall*, gel purified, and inserted in the *Sall* site of the pBTM116 or pBTM116/PDGFR plasmid. pL[Ship]SN was constructed by ligating the *EcoRI*-*XhoI* fragment derived from clone 150.8 into the same restriction sites of the LXSN [Miller and Rosman 1989] retroviral vector. This construct contains the full-length clone 150.8 including the 5'- and 3'-untranslated regions and the poly(A) tail.

Two-hybrid screen

The two-hybrid screen was performed basically as described previously [Vojtek et al. 1993]. Briefly, L40 yeast containing the LexA-Shc PTB domain and PDGF receptor kinase (pBTM116/PDGFR/ShcPTB) was transformed with 500 µg of a VP16 cDNA library derived from EML cells [Tsai et al. 1994]. The transformed cells were grown for 14 hr at 30°C in liquid selective medium lacking uracil, tryptophan, and leucine to select for cotransformants and increase the efficiency of the *His3* reporter gene expression. The cells were washed twice with THULL media (lacking tryptophan, histidine, uracil, leucine, and lysine) and plated in the same media containing 50 mM 3-aminotriazol (3AT). After 3 days at 30°C, colonies were picked and grown in media lacking uracil and leucine but containing tryptophan to allow for loss of the bait-derived plasmid. The bait cured L40 yeast was mated with AMR70 yeast containing pBTM116, pBTM116/ShcPTB, pBTM116/PDGFR, or pBTM116/PDGFR/ShcPTB by cocultivation. Mated yeast was spotted on THULL containing 50 mM 3AT and X-gal, and grown for 3–4 days at 30°C. Clones that grow and induce β-galactosidase activity with the pBTM116/PDGFR/ShcPTB but not with the rest of the constructs, were considered positive for the interaction of the Shc PTB domain with a tyrosine-phosphorylated target and were analyzed further. Yeast miniprep plasmid DNA was prepared, and the VP16 library inserts were amplified by PCR with oligonucleotides 5'-GAGTTTGAGCAGATGTTTA and 5'-TGT-AAAACGACGGCCAGT. The gel-purified PCR products were digested with *HaeIII*, and the clones were grouped according to the fragment patterns. A representative insert DNA of each group was sequenced in both orientations. All DNA sequencing was performed with DyeDeoxy terminators (Applied Biosystems), and DNA sequencing data were analyzed by Sequencher software.

Library constructions

The EML library was constructed from factor-dependent lymphohematopoietic cells EML-C1 (CRL 11691 American Type Culture Collection). After second-strand synthesis, cDNAs >350 bp were ligated with *NotI* linkers and inserted into the *NotI* cloning site of pVP16. The FDC-P1/Mac11 library was constructed from equal amounts of FDC-P1 and Mac11 poly(A)⁺-selected RNA with a ZAP Express cDNA synthesis kit [Stratagene].

DNA hybridization

RNA or DNA transferred onto Nitran Plus membranes was cross-linked with a Stratalinker (Stratagene) UV irradiator. The

blots were prehybridized overnight [50% deionized formamide, 4× SSC, 5× Denhardt's, 50 mM NaPi (pH 7.0), 0.5 mg/ml of NaPPi, 0.1 mg/ml of sheared salmon sperm DNA, 1% SDS] and hybridized for 18 hr with QuickPrime (Pharmacia) ³²P-labeled DNA in hybridization solution (50% deionized formamide 4× SSC, 1× Denhardt's, 50 mM NaPi at pH 7.0, 0.5 mg/ml of NaPPi, 0.1 mg/ml sheared salmon sperm DNA, 1% SDS). The blots were washed numerous times at 55°C with 1× SSC, 0.1% SDS, and exposed to film at -70°C with intensified screens. Plaque lifts were autoclaved for 1 min before UV cross-linking and hybridized as above.

Antibodies

Polyclonal rabbit antibodies to the murine Shc SH2 domain were raised as a GST fusion protein. Ship antibodies were raised to GST fusion proteins containing either amino acids 670–868 (serum 5340) or amino acids 889–1046 (serum 5369). Serum 5369 antibodies were also affinity purified by antigen- affinity chromatography.

Protein purification

Phosphorylated p150 protein was purified from ~20 grams of packed 32D-Fms cells stimulated with excess murine M-CSF and frozen. Cells were thawed, lysed, and the p150 obtained by phosphotyrosine affinity chromatography and elution with 100 mM phenylphosphate. Peptides were generated by in situ Lysine C digestion of electroblotted p150 (Aebersold et al. 1987), and the HPLC-recovered peptides were subjected to automated Edman degradation sequencing.

Preparation of [³²P]PtdIns(3,4)P₂ and [³²P]PtdIns(3,4,5)P₃

PI3K was immunoprecipitated from Psi2 cells as described earlier with a rabbit polyclonal antibody (Upstate Biotechnology Inc., catalog 06-195). The immune pellets were washed six times with kinase buffer [50 mM HEPES (pH 7.25), 0.5 mM EDTA, 5 mM MgCl₂], and 25 µl portions were aliquoted in Eppendorf tubes. PtdIns(4)P or PtdIns(4,5)P₂ (100 µg) and phosphatidylserine (100 µg) in CHCl₃ were dried under argon and resuspended in 100 µl of kinase buffer by sonication for 5 min on ice in a batch sonicator (Branson 12). The lipid suspension was added to the immune pellet, and the reaction was initiated by the addition of [γ-³²P]-ATP (Dupont/NEN) (3000 Ci/mmol) to a final concentration of 150 nM. After 1 hr at room temperature, 100 µl of chloroform/methanol [1:1 (vol/vol)] was added to the reaction, and the organic phase containing the phospholipids was washed twice with 200 µl of 2 M KCl. The lipids were used immediately or stored under argon at -70°C. PtdIns(4)P, PtdIns(4,5)P₂, and Ptdserine were purchased from Sigma.

5-Phosphatase activity assays

Approximately 5×10⁵ cpm of ³²P-labeled phospholipid in chloroform/methanol was evaporated under argon and resuspended by sonication in 100 µl of 5-Pase buffer [50 mM HEPES (pH 7.25), 10 mM MgCl₂] before addition to immune pellets. The reactions were stopped after 20 min at room temperature by extraction of the phospholipids with 100 µl of chloroform/methanol and 100 µl of 2 M KCl. Assays with inositol polyphosphates were performed in the same 5-Pase buffer in a malachite green assay (Harder et al. 1994) with Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ as substrates purchased from Boehringer Mannheim.

Thin layer chromatography

Silica gel 60 TLC plates were treated with 1% potassium oxalate in 50% ethanol and allowed to dry at least 24 hr at room temperature before use. TLC plates were developed using a solvent mixture of chloroform/acetone/methanol/glacial acetic acid/water [80:30:26:24:14 (vol/vol)] (Traynor-Kaplan et al. 1988). Radiolabeled phospholipids were detected by autoradiography. The identity of the phospholipids was obtained by comparison of chromatographic mobility on the same TLC plate.

Soft agar assays

Factor-dependent growth was measured in 1-ml agar cultures with modifications of our previously described methods (Rohrschneider and Metcalf 1989). One hundred milliliters of a 2× Iscove's medium was prepared from 3.54 grams of powder (Sigma), 0.605 grams of sodium bicarbonate, 20 ml of FBS, and 200 U/ml of penicillin plus 200 µg/ml of streptomycin. One volume of 2× Iscove's was mixed with one volume of 0.62% agar at 42°C, the cells were added in a small volume of DMEM plus 10% FBS, and 1 ml was plated into a nontreated 35-mm plastic culture dish. Each assay was performed in quadruplicate. Appropriate growth factors were added to the bottom of the dishes before addition of the cells. After 7–10 days, the colonies were counted and cultures photographed. The average number of colonies for each culture condition (four culture plates) was calculated with the standard error.

Flow cytometry

Cells were collected by centrifugation, washed in PBS, and suspended in 1 ml of PBS. The cells were fixed in 80% ethanol at -20°C by injection into the ethanol solution at -20°C. Cells were fixed for 2 hr at this temperature, then collected by centrifugation, washed in sterile PBS-5% FBS, and resuspended for 5 min at room temperature in 2 ml of PBS containing 0.25% Triton X-100. The cells were washed twice with PBS-5% FBS, and after the last centrifugation the cell pellet suspended in 100 µl of affinity-purified antibody to p150^{Shp} diluted 1:10 in PBS-5% FBS. Incubation was overnight at 4°C. The cells were then washed once with cold PBS-5% FBS and incubated for 30–45 min on ice with a secondary FITC-labeled affinity-purified antibody to rabbit IgG (Jackson ImmunoResearch) diluted 1:200 in PBS-5% FBS. After two more washings with cold PBS-5% FBS, the cells were incubated for at least 30 min on ice with 300 µl of DAPI (1 µg/ml), and RNase (1 mg/ml) in PBS-5% FBS. Cells were finally diluted into 1- to 2-ml sterile filtered PBS-5% FBS before flow cytometry analysis on a Becton-Dickinson Vantage instrument. Analysis of sorted cells utilized two gates (forward scatter vs. side scatter, plus area vs. width of the DAPI/DNA fluorescence) to eliminate cellular multimers and any Psi2 cells that may have contaminated the FD cells.

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