A Novel Phosphatidylinositol-3,4,5-trisphosphate 5-Phosphatase
Associates with the Interleukin-3 Receptor*

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To gain insight into the intracellular signaling cascades that are activated by the binding of interleukin-3 (IL-3) to its target cells, we have embarked on the identification of proteins that are associated with the IL-3 receptor (IL-3R). In a previous study we reported that a 110-kDa serine/threonine protein kinase is constitutively associated with the IL-3R and activated following IL-3 stimulation. We now report that a phosphatidylinositol-3,4,5-trisphosphate (PtdIns-3,4,5-P_3)-5-phosphatase (5-ptase) is also constitutively associated with the IL-3R. This 5-ptase is magnesium-dependent and removes the 5-position phosphate from PtdIns-3,4,5-P_3 but does not metabolize PtdIns-4,5-P_2, inositol (Ins)-1,3,4,5-P_4, or Ins-1,4,5-P_3. This substrate specificity distinguishes it from any previously characterized 5-ptase. Interestingly, it may be bound indirectly via phosphatidylinositol 3-kinase (PI 3-kinase), another enzyme that is constitutively bound to the IL-3R. However, unlike PI 3-kinase which becomes activated following IL-3 stimulation, this receptor-associated 5-ptase activity does not increase following IL-3 stimulation, and its primary function may be to keep the principal in vivo product of PI 3-kinase, PtdIns-3,4,5-P_3, at low levels in unstimulated cells, to terminate the PI 3-kinase signal following IL-3 stimulation or to metabolize PtdIns-3,4,5-P_3 to a metabolically active second messenger, i.e. PtdIns-3,4-P_2.

Interleukin-3 (IL-3)† is a potent hematopoietic cytokine that is produced primarily by activated T lymphocytes and stimulates the proliferation and differentiation of pluripotent stem cells and committed myeloid and lymphoid progenitors. It exerts its action by binding to specific cell surface receptors on its target cells (1, 2). These cell surface receptors are members of the hemopoietic receptor superfamily (2) and consist of two subunits, designated α and β (2). The 70-kDa α subunit is specific for IL-3 (3), while the 140-kDa β subunit (β_1) is shared by IL-3, GM-CSF, and IL-5 (4). In the mouse, there is a second β subunit called β_2 which shares 91% amino acid identity with β_1 and is specific for IL-3 (5). Although neither the α nor the β subunits possess intrinsic tyrosine kinase activity, the IL-3R β subunits, as well as several other intracellular proteins, rapidly become phosphorylated on tyrosine residues following IL-3 stimulation (6–14). This phosphorylation is mediated, at least in part, by a constitutively associated tyrosine kinase, Jak2 (15), that becomes activated following IL-3 binding (16). To gain further insight into the signaling pathways activated, and inactivated, by the binding of IL-3, we and others have recently set out to identify proteins that are intimately associated with the IL-3R itself. To date these studies have shown that the tyrosine-specific phosphatase, hematopoietic cell phosphatase, also known as PTP1C, SHP1, or SHPTP1, associates with the IL-3R β subunit following IL-3 stimulation (17, 18). This phosphatase is most likely responsible for the dephosphorylation of Jak2 and the IL-3R and for the subsequent down-regulation of the response of hemopoietic cells to IL-3 (17, 18). In addition, a novel 110-kDa Ser/Thr kinase has been found to constitutively associate with the IL-3R and become tyrosine-phosphorylated in response to IL-3 (19). This kinase may be involved in the extensive serine/threonine phosphorylation of the IL-3R that occurs following IL-3 stimulation (10).

Recently, we discovered that a phosphatidylinositol-3,4,5-trisphosphate (PtdIns-3,4,5-P_3)-5-phosphatase (5-ptase), which we have designated SHIP, for SH2-containing inositol phosphate phosphatase, becomes associated with Shc following IL-3 stimulation (20). Since Shc has been shown to bind to many cell surface receptors following growth factor stimulation (21–26), we investigated whether this 5-ptase could also be associated with the IL-3R following IL-3 stimulation. Unexpectedly, we found a 5-ptase was indeed associated with the IL-3R but it was not SHIP. Rather, it was a previously uncharacterized PtdIns-3,4,5-P_3-specific 5-ptase that associates constitutively with the IL-3R and is most likely bound to this receptor indirectly, via PI-3-kinase. This is the first demonstration of a 5-ptase being associated with a cell surface receptor, and, given its specificity for the major in vivo product of PI-3-kinase, it most likely plays an important role in regulating PI-3-kinase-mediated events.

MATERIALS AND METHODS

Reagents—The production and purification of COS cell-derived murine IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF) were as described previously (27). Rabbit antiserum to the human IL-3R β subunit was generated by immunizing animals with a gluta-
thione 5-transferase (GST) fusion protein containing the entire intracellular domain of the hIL-3R $\beta$ subunit. This antiserum is highly effective at immunoprecipitating the murine IL-3R $\beta$ and $\beta_{IL-3}$ subunits (10). The anti-phosphotyrosine (anti-Tyr(P)) mAb, 4G10, and rabbit polyclonal antiserum to phosphatidylinositol 3-kinase (PI 3-kinase) were purchased from UBI (Lake Placid, NY). Horseradish peroxidase-conjugated second antibodies were purchased from Jackson Immunoresearch (West Grove, PA). Protein-grade Nonidet P-40 was from Calbiochem. The enhanced chemiluminescence Western blotting reagents were obtained from Pierce. All other reagents were purchased from Sigma unless otherwise indicated.

**Immunoprecipitations and Western Blotting—**Murine B6SUtA1 cells, maintained in RPMI 1640 with 10% fetal calf serum and 5 ng/ml GM-CSF, were deprived of growth factors for 12 h at 37 °C in RPMI 1640 containing 1% fetal calf serum and then stimulated at 37 °C for 5 min with murine IL-3 (400 ng/ml). The cells were then washed once with phosphate-buffered saline solubilized at 2 $\times$ 10$^7$ cells/ml with 0.5% Nonidet P-40 in 4 °C phosphorylation solubilization buffer, i.e. 50 mM Hepes, pH 7.4, 100 mM NaF, 10 mM NaPPi, 2 mM Na$_3$VO$_4$, 4 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 2 μg/ml aprotinin, and subjected to immunoprecipitation and Western blotting as described previously (26).

**5-Ptase Assays—**PtdIns-3,4,5-P$_3$-5-Ptase was prepared using PtdIns-3,4,5-P$_3$-5-Ptase (1 μM/ATP (11 Ci/mmol), and recombinant PtdIns-3-kinase provided by Dr. L. T. Williams (Chiron Corp.) (28). 5-ptase activity was measured using sonicated vesicles containing 30,000 cpm of TLC-purified PtdIns-3,4,5-P$_3$-5-Ptase, with 150 μg of phosphatidylinositol in 50 μl Tris-Cl, pH 7.5. Reaction mixtures (25 μl) containing immunoprecipitate or 5-ptase II, 50 mM Tris-Cl, pH 7.5, 10 mM MgCl$_2$, and substrate were rocked for 30 min at 37 °C. Reactions were stopped, and the products were separated by TLC (29). Proof that the 5-P-label PtdIns-5-P$_2$ product was PtdIns-3,4,5-P$_3$ was obtained by incubation of the product with purified recombinant inositol phosphatase 4-ptase produced in Sf9 cells and separation of the subsequent products by TLC (29). Hydrolysis of $[^{3}H]$Ins-1,3,4,5-P$_4$ by immunoprecipitates was measured as above in 25 μl containing 16 μl of $[^{3}H]$Ins-1,3,4,5-P$_4$ (6000 cpm/mmol) under conditions where the reaction was linear with time (20 min, 37 °C) and enzyme amount (30). 5-ptase assays using PtdIns-4,5-P$_2$, Ins-1,3,4,5-P$_4$, and Ins-1,4,5-P$_3$ as substrates were carried out as described previously (20, 31, 32).

**PI 3-Kinase Assays—**Assays were performed as described previously (33) except that PtdIns-4,5-P$_2$ rather than PtdIns plus PtdIns-4,5-P$_2$ was used as the substrate.

FIG. 1. A PtdIns-3,4,5-P$_3$ 5-ptase is constitutively associated with the IL-3R. A, lysates from B6SUtA$_1$ cells, treated with (IL-3) or without (C) IL-3, were subjected to immunoprecipitation with either normal rabbit serum (NRS) or anti-Shc or anti-IL-3R antibodies. One-tenth of the immunoprecipitates (as well as recombinant 5-ptase II (PTII) and blank (BL) samples as positive and negative controls, respectively) were incubated with PtdIns-3,4,5-[32P]-P$_3$, under conditions where product formation was linear with time and the reaction products were separated by TLC.

**RESULTS**

Recently, we cloned the inositol polyphosphate 5-ptase, SHIP, from a hemopoietic, IL-3-dependent murine cell line, B6SUtA$_1$ (20). Of the 5-potases cloned to date (31, 32), SHIP was unique in that it possessed an SH2 domain, became phosphorylated on tyrosine residues, and associated with Shc following IL-3 stimulation (20). These properties suggested that it might play an important role in cytokine-mediated signaling. To determine at what site(s) within the cell that SHIP exerted its effects, we reasoned that since SHIP associates with many cell surface receptors following growth factor stimulation (21–26), it might bring SHIP to the IL-3R. To test this, B6SUtA$_1$ cells treated with and without IL-3 were lysed, and PtdIns-3,4,5-P$_3$-specific 5-potase assays were carried out with anti-Shc and anti-IL-3R immunoprecipitates. As can be seen in Fig. 1, normal rabbit serum immunoprecipitates did not contain any 5-potase activity while anti-Shc immunoprecipitates did, but, as shown previously (20), only after IL-3 stimulation. Anti-IL-3R immunoprecipitates, on the other hand, possessed 5-potase activity constitutively, and the level of this activity did not change with IL-3 stimulation.

**Fig. 2. The IL-3R-associated 5-potase is not SHIP.** A, lysates from B6SUtA$_1$ cells, treated with (IL-3) or without (C) IL-3, were subjected to immunoprecipitation with either anti-Shc or anti-IL-3R antibodies. Western analysis was then carried out with 4G10 (upper panel), and the blot was reprobed with anti-Shc antibodies (lower panel). The positions of SHIP (p145), the IL-3R β subunit (IL-3R), and the Shc isoforms (p56, p50) are indicated. B, 4 $\times$ 10$^7$ B6SUtA$_1$ cells, treated with (+) or without (−) IL-3, were lysed, and anti-IL-3, anti-Shc, and normal rabbit serum immunoprecipitates were incubated with $[^{3}H]$Ins-1,3,4,5-P$_4$ under conditions where product formation was linear with time.
PI3-kinase associates constitutively with the IL-3R and possesses PtdIns-3,4,5-P₃ 5-PTase activity. A, lysates from B6SUtA₁ cells, treated with (IL-3) or without (C) IL-3, were subjected to immunoprecipitation with antibodies to the 85-kDa subunit of PI 3-kinase (anti-p85) or with anti-IL-3R antibodies. Western analysis was then carried out with anti-p85 antibodies and treated as above to serve as a positive control. B, 4 x 10⁷ B6SUtA₁ cells, treated with IL-3, were immunoprecipitated with anti-p85 antibodies and treated as above to serve as a positive control. 

TLC was carried out as described previously under "Materials and Methods." Lysates from B6SUtA₁ cells, treated with IL-3, were immunoprecipitated with anti-p85 antibodies and treated as above to serve as a positive control. C, 4 x 10⁷ B6SUtA₁ cells, treated with (IL-3) or without (C) IL-3, were lysed and immunoprecipitated with either anti-p85 or anti-IL-3R antisera. One-tenth of the immunoprecipitates were incubated with PtdIns-3,4,5-[^32]P₃, under conditions where product formation was linear with time and the reaction mixture was fractionated by TLC. D, 1 x 10⁷ B6SUtA₁ cells, treated with IL-3 for 5 min at 37°C, were lysed and immunoprecipitated with either anti-p85, anti-IL-3R, or anti-SHIP (as a positive control) antiserum. The immunoprecipitates were incubated with PtdIns-3,4,5-[^32]P₃ for 30 min at 37°C and then purified 4-PTase was added, as indicated, and incubation continued for an additional 30 min at 37°C before the products were separated by TLC.
munoprecipitates were capable of hydrolyzing PtdIns-3,4,5-P_3. As can be seen in Fig. 3C, this was indeed the case and 5-ptase activity did not change with IL-3 stimulation, consistent with it being the IL-3R-associated 5-ptase. Moreover, the 5-ptase activity associated with the anti-p85 immunoprecipitates was substantially higher, on a per cell basis, than that associated with the IL-3R (Fig. 3C). This too was consistent with the possibility that the IL-3R-associated 5-ptase was binding indirectly to the receptor via PI 3-kinase since only a minor proportion of the total cellular PI 3-kinase is associated with the IL-3R (i.e. Fig. 3A).

To confirm that the ptases associated with the IL-3R and p85 were indeed 5-ptases and not 4-ptases, PtdIns-3,4,5-[γ-32P]P_3 5-ptase assays were carried with anti-p85, anti-IL-3R, and anti-SHIP immunoprecipitates from B6SuTα1 cells treated with IL-3, as usual, and the γ-32P-labeled PtdIns_P_3 product was incubated with or without purified inositol polyphosphate 4-ptase before separation by TLC (29). As can be seen in Fig. 3D, 4-ptase treatment of all three immunoprecipitates resulted in the appearance of a new γ-32P-labeled spot that comigrated with PtdInsP. Since the initial PtdIns_P_3 substrate was labeled in the 3-position, this confirmed that the IL-3R- and p85-associated enzyme was indeed a 5-ptase.

As a further test to see if the PI 3-kinase-associated 5-ptase was identical to the IL-3R-associated 5-ptase, substrate specificity comparisons were undertaken, and, as is shown in Table I, both 5-ptases only recognized PtdIns-3,4,5-P_3 as a substrate and both required Mg^{2+} as a cofactor. This last property distinguishes this 5-ptase from that reported in human platelets (35) and, taken together with its substrate specificity, suggests that the 5-ptase associated with PI 3-kinase is identical to that associated with the IL-3R, that it associates with the IL-3R via its constitutive interaction with PI 3-kinase, and that it is distinct from other 5-ptases reported to date.

DISCUSSION

The identification of receptor-associated proteins is an essential first step in elucidating downstream signals initiated by the binding of growth factors to their receptors. In the case of IL-3, our current results, coupled with previous data, suggest that Jak2, PI 3-kinase, a 110-kDa serine/threonine kinase, and a novel 5-ptase associate constitutively with the IL-3R while Shc and hematopoietic cell phosphatase bind only following IL-3 stimulation. Although our finding that PI 3-kinase associates constitutively with the IL-3R was somewhat of a surprise, it is consistent with a recent report by Rao and Mufson (42) in which they demonstrated that a GST fusion protein containing only the membrane proximal 67 amino acids of the IL-3R β_1 subunit cytoplasmic domain, and that was not detectably tyrosine-phosphorylated, was capable of binding PI 3-kinase in vitro (42). As to exactly how PI 3-kinase is binding to the IL-3R (which does not contain a consensus sequence for the SH2 domains of p85 on either its IL-3R α or β subunits), Jucker and Feldman (43) recently reported that a 76–85-kDa tyrosine-phosphorylated protein may link PI 3-kinase to the IL-3R β subunit in human TF-1 cells. This is of interest given that we recently identified a 72-kDa protein that was both constitutively associated with the IL-3R and constitutively tyrosine-phosphorylated in B6SuTα1 cells (19).

Our current results also suggest that the binding of 5-ptase to the IL-3R may be indirect, via PI 3-kinase. Although further studies will be required to confirm this, the finding that a novel 5-ptase binds to both PI 3-kinase and the IL-3R in B6SuTα1 cells is very intriguing given the growing evidence that PI 3-kinase plays a crucial role in mediating many growth factor-stimulated events, including mitogenesis, receptor internalization, membrane ruffling, and glucose transport (reviewed in Refs. 44 and 45). Most if not all of these events are likely mediated by its major phospholipid product, PtdIns-3,4,5-P_3, which has been shown recently to modulate Ca^{2+}-independent protein kinase C (49), protein kinase C-related kinase (49), phospholipase D (50), and Akt/protein kinase B (51) activity and to be capable of competing with tyrosine-phosphorylated proteins for binding to SH2 domains (52).

This has, in turn, drawn attention to the growing family of 5-ptases which hydrolyze PtdIns-3,4,5-P_3 to PtdIns-3,4-P_2. To date, this family consists of five members; the cloned human platelet-derived Type II 75-kDa inositol polyphosphate 5-ptase (ptase II) (32), which hydrolyzes PtdIns-3,4,5-P_3 (20, 35), PtdIns-4,5-P_2, PtdIns-1,4,5-P_3, and PtdIns-3,4,5-P_3 (32), and is related to the OCRL protein deficient in Lowe syndrome (31); the more recently cloned, murine myeloid cell-derived, Shc-associated, SH2-containing SHIP, which hydrolyzes both PtdIns-3,4,5-P_3 and PtdIns-1,4,5-P_3 (20, 53, 54); the human platelet-derived, magnesium-independent, PI 3-kinase-associated 5-ptase that hydrolyzes only PtdIns-3,4,5-P_3 (35); the rat brain-derived, 145-kDa 5-ptase that hydrolyzes both PtdIns-3,4,5-P_3 and PtdIns-4,5-P_2 (55); and lastly, the IL-3R- and PI 3-kinase-associated, magnesium-dependent, PtdIns-3,4,5-P_3-specific 5-ptase described herein. Whether the primary function of these 5-ptases is to terminate the PtdIns-3,4,5-P_3 signal or to generate another second messenger is still under investigation (46, 47, 56). In this regard, Toker et al. (56) recently reported some tantalizing data suggesting that PtdIns-3,4,5-P_3 may play a role in thrombin-stimulated plextrin phosphorylation in platelets. The specific 5-ptase we have identified appears to be constitutively associated with PI 3-kinase (i.e. its activity does not change in anti-p85 immunoprecipitates with IL-3 stimulation), and, since the major in vivo product of PI 3-kinase is the substrate for this 5-ptase, it is likely that one of its functions is to maintain low levels of PtdIns-3,4,5-P_3 in unstimulated cells. Following IL-3 stimulation, when PI 3-kinase activity is increased, its function may be to return PtdIns-3,4,5-P_3 levels back to resting levels after the various PI 3-kinase-activated cascades have been triggered and/or to generate sufficient PtdIns-3,4,5-P_3 to activate similar or as yet uncharacterized pathways.

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REFERENCES

1. Krystal, G., Alai, M., Cutler, R. L., Dickeson, Mui, L.-F., and Wognum, A. W. (1991) *Hematol. Pathol.* 5, 141–162
2. Miyajima, A., Mui, A. L.-F., Ogorochi, T., and Sakamaki, K. (1993) *Blood* 82, 1600–1604
3. Ikeda, T. and Miyajima, A. (1992) *EMBO J.* 11, 1875–1884
4. Gorman, D. M., Itoh, N., Kitamura, T., Schuurs, J., Yonehara, S., Yahara, I., Arai, K.-I., and Miyajima, A. (1990) *Proc. Natl. Acad. Sci. U. S. A.* 87, 5459–5463
5. Itoh, N., Yonehara, S., Schuurs, J., Gorman, D. M., Maruyama, K., Iehii, A., Yahara, I., Arai, K.-I., and Miyajima, A. (1990) *Science* 247, 324–327
6. Duronio, V., Clark-Lewis, I., Federspiel, B., Wieler, J. S., and Schrader, J. W. (1992) *J. Biol. Chem.* 267, 21856–21863
7. Isoft, R., Huhn, R. D., Frackelton, A. R., Jr., and Ible, J. N. (1988) *J. Biol. Chem.* 263, 19203–19209
8. Sorensen, P. H. B., Mui, A. L.-F., Murthy, S. C., and Krystal, G. (1989) *Blood* 73, 1192–1197

| Substrate | Anti-IL-3R IP | Anti-p85 IP |
|-----------|---------------|-------------|
| PtdIns-3,4,5-P_3 | + | + |
| PtdIns-4,5-P_2 | - | - |
| Ins-1,3,4,5-P_3 | - | - |
| Ins-1,4,5-P_2 | - | - |
| PtdIns-3,4,5-P_3 + 3 mm EDTA | - | - |

**TABLE I**
The IL-3R- and p85-associated 5-ptases have identical substrate specificities

Assays were carried out as described previously (20, 28–32).
