Erythropoietin Induces the Tyrosine Phosphorylation of Insulin Receptor Substrate-2

AN ALTERNATE PATHWAY FOR ERYTHROPOIETIN-INDUCED PHOSPHATIDYLINOSITOL 3-KINASE ACTIVATION**

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In this report, we demonstrate that insulin receptor substrate-2 (IRS-2) is phosphorylated on tyrosine following treatment of UT-7 cells with erythropoietin. We have investigated the expression of IRS-1 and IRS-2 in several cell lines with erythroid and/or megakaryocytic features, and we observed that IRS-2 was expressed in all cell lines tested. In contrast, we did not detect the expression of IRS-1 in these cells. In response to erythropoietin, IRS-2 was immediately phosphorylated on tyrosine, with maximal phosphorylation between 1 and 5 min. Tyrosine-phosphorylated IRS-2 was associated with phosphatidylinositol 3-kinase and with a 140-kDa protein that comigrated with the phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase, SHIP. Moreover, IRS-2 was constitutively associated with the erythropoietin receptor. We did not observe the association of IRS-2 with JAK2, Grb2, or PTP1D. Using BaF3 cells transfected with mutated erythropoietin receptors, we demonstrate that neither the tyrosine residues of the intracellular domain nor the last 109 amino acids of the erythropoietin receptor are required for erythropoietin-induced IRS-2 tyrosine phosphorylation. Altogether, our results indicate that erythropoietin-induced IRS-2 tyrosine phosphorylation could account for the previously reported activation of phosphatidylinositol 3-kinase mediated by erythropoietin receptors mutated in the phosphatidylinositol 3-kinase-binding site (Damen, J., Cutler, R. L., Jiao, H., Yi, T., and Krystal, G. (1995) J. Biol. Chem. 270, 23402–23406; Gobert, S., Porteu, F., Pallu, S., Muller, O., Sabbah, M., Dusant-Fourt, I., Courtois, G., Lacombe, C., Gisselbrecht, S., and Mayeux, P. (1995) Blood 86, 598–606).

Insulin receptor substrate-1 (IRS-1)** is a major substrate of the IGF-1 and insulin receptors (1). IRS-1 is a hydrophilic protein with a theoretical molecular mass of 131 kDa that migrates between 160 and 185 kDa on SDS-polyacrylamide gel electrophoresis partially because of a high serine phosphorylation state (2, 3). IRS-1 contains a pleckstrin homology domain, a PTB domain, and at least 20 potential tyrosine phosphorylation sites including nine YXXM motifs that are consensus binding sites for the SH2 domains of the regulatory subunit (p85) of PI 3-kinase (4). IRS-1 binds to the tyrosine-phosphorylated IGF-1 and insulin receptors through its PTB domain and becomes a docking protein for signaling proteins such as PI 3-kinase, Grb2, PTP1D, and Nck after tyrosine phosphorylation (5). Moreover, a recent report shows that the pleckstrin homology domain could also be involved in the association of IRS-1 with the insulin receptor (6). More recently, a second IRS protein that was identified that was designated IRS-2 (7). This protein corresponds to the previously identified 4PS protein (for IL-4-induced phosphotyrosine substrate) (8, 9). IRS-2 exhibits a high structural similarity to IRS-1, with a strong conservation of the PTB and pleckstrin homology domains. Moreover, tyrosine phosphorylation sites shown to bind PI 3-kinase, Grb2, and PTP1D in IRS-1 are also conserved in IRS-2.

Not only insulin and IGF-1 receptors, but also several cytokine and interferon receptors can induce tyrosine phosphorylation of IRS-1 and IRS-2 (9–18). IRS-1 and IRS-2 activation by IL-4 is well documented. Indeed, it has been shown that IRS-1 and IRS-2 bind to a peptidic sequence of the activated IL-4 receptor with a typical NPYX PTB domain-binding motif (19), and IRS-1 or IRS-2 expression appears to be required for IL-4-induced mitogenesis (10). However, other cytokine receptors that induce the tyrosine phosphorylation of IRS-1 and IRS-2 do not possess typical PTB domain-binding motifs, and the mechanism of IRS-1 and IRS-2 activation by these receptors remains unknown.

The erythropoietin (Epo) receptor also belongs to the cytokine receptor family (20, 21). Epo binding to its receptor activates the receptor-associated JAK2 tyrosine kinase (22) and induces the tyrosine phosphorylation of the Epo receptor (23–26) and other proteins. Several intracellular signaling pathways are subsequently activated, including mitogen-activated protein kinases (27, 28), STAT5 (29, 30), and PI 3-kinase (31–34). PI 3-kinase was shown to associate with phosphorylated Tyr479 of the Epo receptor (35), and removal of this tyrosine main; PI, phosphatidylinositol; IL, interleukin; Epo, erythropoietin; SCF, stem cell factor; GST, glutathione S-transferase; GM-CSF, granulocyte-macrophage colony-stimulating factor.
residue abrogates PI 3-kinase association with the Epo receptor (34–36). However, binding of PI 3-kinase to the Epo receptor does not appear to be required for Epo-induced PI 3-kinase activation since Epo receptors devoid of Tyr⁴⁷⁹ still activate PI 3-kinase (35, 36). These results strongly suggest that Epo could activate PI 3-kinase by several mechanisms. However, these alternate pathways for Epo-induced PI 3-kinase activation have not been identified up to date. We have previously shown that one of these mechanisms required only the first 127 amino acids of the Epo receptor intracellular domain and was not dependent on the phosphorylation of the single tyrosine residue (Tyr³⁴⁸) present in this region (36).

In this report, we show that erythropoietin induces the tyrosine phosphorylation of IRS-2. Epo-induced IRS-2 tyrosine phosphorylation does not require the tyrosine residues of the intracellular domain of the Epo receptor, and IRS-2 appears to be constitutively associated with the Epo receptor. After Epo-induced tyrosine phosphorylation, IRS-2 associates with PI 3-kinase and with a tyrosine-phosphorylated protein comigrating with SHIP (p140). In contrast, we did not detect the association of Grb2 with IRS-2 in Epo-stimulated cells. Thus, our results strongly suggest that IRS-2 binding could be an alternate mechanism for Epo-induced PI 3-kinase activation.

**EXPERIMENTAL PROCEDURES**

**Materials**—The highly purified recombinant human Epo (specific activity of 120,000 units/mg) used throughout this study was a generous gift from Dr. M. Brandt (Boehringer Mannheim). Recombinant SCF was kindly provided by Dr. A. Shimazaka (Kirin Brewery Co., Tokyo). Anti-IRS-1 (catalog number 06-248), anti-IRS-2 (catalog number 06-506), and anti-JAK2 (catalog number 06-255) antibodies were from Upstate Biotechnology, Inc. Anti-PI 3-kinase (p85 subunit; catalog number P13030) and anti-SHC (catalog number S14630) antibodies were purchased from Transduction Laboratories, and anti-Grb2 antibodies (catalog number C-20) were from Santa Cruz. Anti-phosphotyrosine antibodies (4G10) were a generous gift from Dr. B. Drucker. Anti-Epo receptor antibodies were produced by immunizing rabbits with a recombinant protein composed of the full intracellular domain of the human Epo receptor fused to GST. Anti-GST antibodies were also prepared in our laboratory using the same protocol. Whole anti-GST and anti-Epo receptor sera were used.

**Cell Lines and Cell Culture**—A subclone of the human leukemic cell line UT-7 (37) able to grow in SCF, GM-CSF, or Epo was established. These cells were cultured in α-minimal essential medium containing 5% fetal calf serum supplemented with 2 units/ml Epo, 2.5 mg/ml GM-CSF, or 50 mg/ml SCF. Before each experiment, the cells were serum- and growth factor-deprived by incubation overnight in Iscove’s modified Dulbecco’s medium (Life Technologies, Inc., catalog number 13190-029) containing 0.1% deionized bovine serum albumin. HCD57 cells (38), Mo7E cells (39), and TF-1 cells (40, 41) were cultured in α-minimal essential medium containing 5% fetal calf serum and supplemented with 2 units/ml Epo (HCD57) or 2.5 mg/ml GM-CSF (Mo7E and TF-1). BaF3 (42) and FDCP-1 (43) cells were cultured in the same medium supplemented with 2.5% WEHI-conditioned medium as a source of IL-3. WEHI-conditioned medium is the supernatant of end-logarithmic phase cultures of WEHI-3B3 cells (ATCC TIB-68). BaF3 cells were transfected with expression vectors encoding wild-type or mutated Epo receptors and selected for their ability to grow in Epo as described previously (36). Before each experiment, these cells were serum- and growth factor-deprived by incubation for 5 h in Iscove’s modified Dulbecco’s medium.

**IRS-1 and IRS-2 Detection in Whole Cell Extracts**—Exponentially growing hematopoietic cells were washed twice with phosphate-buff-ered saline and solubilized by boiling in electrophoresis sample buffer. Samples corresponding to 5 × 10⁶ cells (hematopoietic cells) or 2.5 × 10⁶ cells (NIH 3T3 cells) were separated through a 6.5% polyacrylamide gel and transferred to a nitrocellulose sheet, which was successively probed with anti-IRS-2 and anti-IRS-1 antibodies. The molecular masses of protein standards run in adjacent lanes are indicated.

**RESULTS**

**Epo-sensitive Hematopoietic Cell Lines Express IRS-2, but Not IRS-1**—To test for the possible involvement of IRS-1 or IRS-2 in the Epo mode of action, we first investigated whether these proteins were expressed in erythroid and other hematopoietic Epo-responsive cells. As a positive control, we used an NIH 3T3 cell lysate that was previously shown to express both IRS-1 and IRS-2 (18). Total cell lysates from these cells were analyzed by Western blotting. As shown in Fig. 1, all hematopoietic cells tested expressed IRS-2, although IRS-2 expression appeared to be relatively low in Mo7E cells. IRS-2 migrated as diffuse bands with apparent molecular masses of 160–175 kDa in the murine cells (T3Cl2, BaF3, FDCP-1, HCD57, and NIH 3T3) and 170–190 kDa in the human cells (UT-7, Mo7E, and TF-1). In contrast, IRS-1 expression was not detected in these cells. (The faint band in Fig. 1 (lower panel) was exactly at the same position as IRS-2, and its intensity varied as did the IRS-2 signal in Fig. 1 (upper panel). Thus, it most likely corresponds to some cross-reactivity of the anti-IRS-1 antiserum with IRS-2.) In another experiment, we immunoprecipitated UT-7 cell extracts with anti-IRS-1 antibodies, and we analyzed these immunoprecipitates by Western blotting using anti-IRS-1 antibodies. Again, no IRS-1 protein was detected in these cells (data not shown).

**Epo Induces the Tyrosine Phosphorylation of IRS-2 in UT-7 Cells**—Serum- and growth factor-deprived UT-7 cells were stimulated for 2.5 min with 25 ng/ml GM-CSF, 10 units/ml Epo, or 100 ng/ml SCF. To ensure maximal response to the cytokine, each cell population was previously cultured for at least 3 weeks in the corresponding growth factor. Cell lysates were immunoprecipitated with anti-IRS-2 antibodies and analyzed by Western blotting using anti-phosphotyrosine antibodies. As shown in Fig. 2A, Epo (but not GM-CSF or SCF) induced the tyrosine phosphorylation of two proteins of 180 and 140 kDa immunoprecipitated by anti-IRS-2 antibodies. Reprobing the blot with anti-IRS-2 antibodies confirmed that the 180-kDa protein was IRS-2. To control the efficiency of cytokine stimulation, the same extracts were immunoprecipitated with anti-JAK2 (Epo- or GM-CSF-stimulated cells) or with anti-ε-Kit (SCF-stimulated cells) antibodies and analyzed by anti-phosphotyrosine Western blotting. As shown in Fig. 2B, JAK2 was activated by GM-CSF, although with a lower intensity than by Epo. Moreover, a strong tyrosine phosphorylation of ε-Kit was detected in SCF-stimulated UT-7 cells. Thus, although GM-CSF and SCF receptors were also efficiently stimulated, only Epo induced the tyrosine phosphorylation of IRS-2 in UT-7 cells.

The tyrosine phosphorylation of IRS-2 was maximal after
Epo Induces Tyrosine Phosphorylation of IRS-2

Fig. 2. Epo induces the tyrosine phosphorylation of IRS-2 in UT-7 cells. UT-7 cells were serum- and growth factor-starved for 18 h and incubated for 2.5 min in the presence (+) or absence (−) of 25 ng/ml GM-CSF, 10 units/ml Epo, or 100 ng/ml SCF. The cells were then lysed using 1% Brij 96, and the lysates were cleared by centrifugation (27,000 × g, 15 min). Lysates from 10^7 cells were immunoprecipitated (IP) using anti-IRS-2 antibodies (A) and anti-JAK2 or anti-c-Kit antibodies (B). Immunoprecipitates were analyzed by Western blotting using anti-phosphotyrosine antibodies (anti-PY). In A, the blot was stripped and reprobed with anti-IRS-2 antibodies.

Fig. 3. Time course of Epo-induced tyrosine phosphorylation of IRS-2. Growth factor- and serum-deprived UT-7 cells were stimulated for 2.5 min with the indicated Epo concentrations. Cell lysates were immunoprecipitated with anti-IRS-2 antibodies, and immunoprecipitated proteins were immunoblotted with anti-phosphotyrosine antibodies (anti-PY). The blot was then stripped and reprobed with anti-IRS-2 antibodies. Assuming an equilibrium dissociation constant of 200 μM for the Epo receptor in UT-7 cells (49), receptor occupancy was 2% for 10 milliunits/ml Epo, 18% for 100 milliunits/ml, 70% for 1 unit/ml, 95% for 10 units/ml, and 99.5% for 100 units/ml.

Fig. 4. Epo-induced IRS-2 tyrosine phosphorylation correlates with Epo receptor occupancy. Growth factor- and serum-deprived UT-7 cells were stimulated for 2.5 min with the indicated Epo concentrations. Cell lysates were immunoprecipitated with anti-IRS-2 antibodies, and immunoprecipitated proteins were immunoblotted with anti-phosphotyrosine antibodies (anti-PY). The blot was then stripped and reprobed with anti-IRS-2 antibodies. Assuming an equilibrium dissociation constant of 200 μM for the Epo receptor in UT-7 cells (49), receptor occupancy was 2% for 10 milliunits/ml Epo, 18% for 100 milliunits/ml, 70% for 1 unit/ml, 95% for 10 units/ml, and 99.5% for 100 units/ml.

**IRS-2 Associates with PI 3-Kinase in Epo-stimulated UT-7 Cells**—We then tested the association of tyrosine-phosphorylated IRS-2 with signaling proteins after Epo stimulation of UT-7 cells. The blot presented in Fig. 3 was reprobed with anti-PI 3-kinase regulatory subunit (p85) antibodies. As shown in Fig. 5A, p85 was clearly detected in IRS-2 immunoprecipitants from Epo-stimulated cells. To confirm that PI 3-kinase associates with tyrosine-phosphorylated IRS-2, lysates from Epo-stimulated UT-7 cells were immunoprecipitated with anti-p85 antibodies and analyzed by Western blotting using anti-phosphotyrosine antibodies (Fig. 5B). The tyrosine phosphorylation of a band comigrating with IRS-2 was significantly increased in Epo-stimulated cells. Most likely, this band corresponded to IRS-2, although only a very faint band was detected by anti-IRS-2 antibodies in anti-p85 immunoprecipitates due to the relatively poor sensitivity of the anti-IRS-2 antiserum (data not shown). We then tested whether Grb2, SHC, or PTP1D also associated with IRS-2 in Epo-stimulated UT-7 cells. Lysates from Epo-treated UT-7 cells were immunoprecipitated with antibodies specific for these proteins and analyzed by Western blotting using anti-phosphotyrosine and anti-IRS-2 antibodies. No IRS-2 protein either tyrosine-phosphorylated or not was detected in these immunoprecipitates (data not shown). Moreover, Grb2, SHC, and PTP1D were not detected in anti-IRS-2 immunoprecipitates analyzed by Western blotting with antibodies specific for these proteins (data not shown). In contrast, IRS-2 was associated with two tyrosine-phosphorylated proteins of 140 and 70 kDa, respectively, in Epo-stimulated cells (see Fig. 3). In many cells, Epo induces the tyrosine phosphorylation of a 140-kDa SHC-associated protein that was identified as the PI-3,4,5-trisphosphate 5-phosphatase, SHIP (44–47). Since anti-SHIP antibodies are not yet commercially available, the ability of this protein to associate with SHC was used to identify SHIP (48). As shown in Fig. 6, the 140-kDa protein that was immunoprecipitated with anti-IRS-2 antibodies comigrated with the SHC-associated SHIP and most likely...
Resting UT-7 cells were incubated for 2.5 min with Epo (+) or with vehicle alone (−). Cleared cell lysates were immunoprecipitated (IP) with either anti-IRS-2 or anti-p85 antibodies. Immunoprecipitated proteins were immunoblotted with anti-phosphotyrosine antibodies (anti-PY).

Corresponds to this protein. The apparent molecular mass of the other IRS-2-associated protein suggests that it could be the tyrosine-phosphorylated Epo receptor. To test this hypothesis, anti-Epo receptor immunoprecipitates were probed with anti-phosphotyrosine antibodies. As shown in Fig. 7, only a faint band comigrating with IRS-2 was detected in extracts from Epo-stimulated cells. We next probed the same blot with anti-IRS-2 antibodies. Surprisingly, the IRS-2 protein was easily detected in anti-Epo receptor immunoprecipitates from both resting and Epo-stimulated UT-7 cells (Fig. 7, lower panel), showing that IRS-2 was constitutively associated with the Epo receptor. Control anti-GST antibodies did not immunoprecipitate any protein revealed by either anti-phosphotyrosine or anti-IRS-2 antibodies.

Tyrosine Phosphorylation of the Epo Receptor Is Not Required for Epo-induced IRS-2 Phosphorylation—BaF3 cells were transfected with the wild-type Epo receptor or with the Epo receptor devoid of tyrosine residues in its intracellular domain (ZERO) and selected for growth in the presence of Epo. These cells were stimulated for 2.5 min with 10 units/ml Epo.

Cellular extracts were immunoprecipitated with anti-IRS-2 antibodies and analyzed by Western blotting using anti-phosphotyrosine antibodies (Fig. 8). As shown in Fig. 8, Epo induced the tyrosine phosphorylation of IRS-2 in BaF3 cells expressing each type of Epo receptor, demonstrating that the tyrosine residues of the intracellular domain of the Epo receptor are not required for Epo-induced tyrosine phosphorylation of IRS-2. Reprobing the blot with anti-IRS-2 antibodies showed equal loading of each line (Fig. 8, right panel). Epo-induced tyrosine phosphorylation of IRS-2 was significantly higher in cells expressing the Epo receptor devoid of intracellular tyrosine residues. This result was consistently obtained in several experiments and could be related to a higher number of Epo receptors at the cell surface of BaF3 ZERO cells. Indeed, as previously reported, BaF3 ZERO cells express ~6000 Epo-binding sites/cell, whereas BaF3 cells transfected with the wild-type Epo receptor express only ~1500 Epo receptors/cell.

DISCUSSION

Expression of IRS-1 and IRS-2 in the hematopoietic system is relatively poorly documented. Both IRS-1 and IRS-2 were reported to be expressed and activated by IL-4 and IL-2 in human T lymphoblasts (13). In contrast, mast cells (18) or the murine myeloid progenitor cell line 32D (10) expressed neither IRS-1 nor IRS-2. Expression of IRS-2, but not IRS-1, was also detected in other IL-3-dependent myeloid progenitor cell lines such as FDCP-1 and FDCP-2 (10), murine macrophages, and murine B and T lymphocytes (18). Here, we studied the expression of these proteins in cells with erythroid and megakaryocytic characteristics. UT-7 cells express markers from different differentiation lineages depending on the stimulatory cytokines. Indeed, it has been shown that erythroid or megakaryocytic differentiation markers are expressed in UT-7 cells stimulated by Epo (49) or by thrombopoietin (50), respectively. TF-1 and HCD57 cells seem to be strictly committed in the erythroid differentiation pathway, whereas Mo7E cells exhibit megakaryocytic characteristics (38, 39, 41, 51). T3Cl2 cells are Friend virus-transformed cells that correspond to erythroid cells blocked at the colony-forming unit erythroid/proerythroblast stage (52). All these cell lines express IRS-2, whereas we did not detect IRS-1 expression in any hematopoietic cell line tested. Thus, our results confirm that hematopoietic cells express IRS-2 rather than IRS-1 and extend this observation to cells of the erythroid lineage.

We observed that Epo stimulation of UT-7 cells induced the rapid tyrosine phosphorylation of IRS-2 since maximal tyrosyl phosphorylation was detected between 1 and 10 min and decreased after this time. This kinetics was slightly different from that reported for growth hormone or prolactin, which maximally induced the tyrosine phosphorylation of IRS-1 and IRS-2 after 10–20 min (12, 15, 17). The time course of Epo-
induced IRS-2 tyrosine phosphorylation in UT-7 cells closely paralleled the kinetics of JAK2 activation in these cells (data not shown) and was superimposable to the activation kinetics of other intracellular signaling pathways such as PI 3-kinase (33) and mitogen-activated protein kinases (28). The rapid IRS-2-phosphorylation and its association with the Epo receptor (see below) strongly suggest that Epo-induced IRS-2 tyrosine phosphorylation is a direct event of Epo receptor activation.

Although UT-7 cells are sensitive to GM-CSF and SCF, we did not detect the tyrosine phosphorylation of IRS-2 in UT-7 cells stimulated with these cytokines. Using another cell line, Welham et al. (18) previously reported the stimulation of IRS-2 tyrosine phosphorylation by IL-3 and GM-CSF. This discrepancy could be due to the low number of high affinity GM-CSF receptors expressed in UT-7 cells. Indeed, we detected only a few hundred high affinity receptors for GM-CSF, whereas these cells express ~7000 Epo receptors (49). Moreover, as shown in Fig. 2B, JAK2 activation was also much less efficient using GM-CSF than Epo in these cells. In contrast, the inability of SCF to induce IRS-2 tyrosine phosphorylation is not due to a low number of SCF receptors since these cells express ~35,000 receptors for this cytokine. It should be noted that CSF-1, whose receptor shares strong similarity with c-Kit, also does not induce the tyrosine phosphorylation of IRS-2 (18).

Many cytokines whose receptors belong either to subclass 1 or 2 (interferon receptors) have now been reported to induce the tyrosine phosphorylation of IRS-1 and/or IRS-2, suggesting that this relay could be a common signaling pathway for this class of receptors. All these receptors mediate intracellular signaling through the activation of JAK kinases (reviewed in Ref. 53). Moreover, mutated ZERO receptors that essentially substitute for this signal. Our results show that PI 3-kinase activation by these different pathways could be additive and required to allow a cellular response to a low level of Epo

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Fig. 8. Tyrosine residues of the intracellular domain of the Epo receptor are not required for Epo-induced IRS-2 tyrosine phosphorylation. BaF3 cells expressing either the wild-type Epo receptor (WT) or the Epo receptor devoid of tyrosine residues in its intracellular domain (ZERO) were serum- and growth factor-deprived for 5 h. Starved cells were then incubated for 2.5 min with 10 units/ml Epo (+) or with vehicle alone (−). Cleared lysates were immunoprecipitated (IP) with anti-IRS-2 antibodies, and immunoprecipitated proteins were analyzed by Western blotting with anti-phosphotyrosine antibodies (anti-PY) (left panel). The blot was then stripped and reblotted with anti-IRS-2 antibodies to ensure equal loading of each lane. The structures of the wild-type and ZERO Epo receptors are indicated. To construct the ZERO Epo receptor, a stop codon was inserted at the HindIII restriction site (codon 374), and Tyr to Phe was modified (36).

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2 P. Mayeux, unpublished results.

3 S. Gobert and P. Mayeux, unpublished results.
stimulation. One of the signaling relays downstream of PI 3-kinase was recently shown to be the serine/threonine kinase AKT (see Ref. 59 for review). Interestingly, AKT is activated by PI 3,4-bisphosphate, but not by PI 3,4,5-trisphosphate (60, 61). One way to produce PI 3,4-bisphosphate is the dephosphorylation of the main product of PI 3-kinase, PI 3,4,5-trisphosphate, which could be performed by the PI-3,4,5-trisphosphate 5-phosphatase, SHIP. Our results show that a 140-kDa protein that most likely corresponds to SHIP also associates with IRS-2. Thus, the same protein complex appears to contain both PI 3-kinase and SHIP, and these associations could increase the efficiency of PI 3,4-bisphosphate production.

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