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Role of insulin receptor substrate-2 in interleukin-9-dependent proliferation

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Abstract Interleukin-9 (IL-9) stimulation results in JAK, STAT and IRS1/2 phosphorylation. The role of IRS adaptor proteins in IL-9 signaling is not clear. We show that IL-9 induces IRS2 phosphorylation and association with phosphatidylinositol-3 kinase (PI 3-K) p85 subunit in TS1 cells and BaF/9R cells, which proliferate upon IL-9 stimulation. We observed a PI 3-K-dependent phosphorylation of protein kinase B (PKB) in TS1 cells, but not in BaF/9R, nor in other IL-9-dependent cell lines. Finally, 32D cells that were transfected with the IL-9 receptor but lack IRS expression survived in the presence of IL-9. Ectopic IRS1 expression allowed for IL-9-induced proliferation, in the absence of significant PKB phosphorylation. **© 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Interleukin-9; Proliferation; Apoptosis; Insulin receptor substrate; Protein kinase B

1. Introduction

Interleukin-9 (IL-9) is secreted by T-helper lymphocytes and has pleiotropic activities on mast cells, eosinophils, hematopoietic progenitors, B- and T-lymphocytes [1,2]. In addition, IL-9 appears to play a role in certain murine and human lymphomas [3]. This is supported by the findings that IL-9 transgenic mice develop thymic lymphomas [4]. Most recently, genetic and functional studies have also implicated IL-9 and its receptor in regulating susceptibility to asthma and allergy [5–7].

The functional IL-9 receptor (IL-9R) consists of a heterodimeric complex that comprised an α chain (IL-9Rα), which binds IL-9 ligand directly, and the IL-2Rγ*C* chain, which is a common subunit of IL-2, IL-4, IL-7 and IL-15 receptor complexes [8]. It is thought that, upon receptor ligation, the juxtaposition of IL-9Rα and IL-2Rγ*C* chains results in *trans*-phosphorylation and activation of janus kinase (JAK) 1 and JAK3, which are constitutively bound to each chain, respectively. This event leads to phosphorylation of the tyrosine 367 in the cytoplasmic domain of the IL-9Rα chain, which has been reported to function as docking site for signal transducer and activator transcription factor (STAT) 1, STAT3 and STAT5 [9,10]. Subsequently, phosphorylated STAT molecules dimerize and migrate to the nucleus where they bind regulatory sequences for de novo gene expression [11], thus regulating survival and proliferation mediated by IL-9 [9,12].

Insulin receptor substrate (IRS) proteins have been implicated in cellular responses mediated by insulin, IGF-1 and IL-4 [13]. IL-9 can induce phosphorylation of both IRS1 and IRS2 in various hematopoietic cells [9,14], including the IL-9-dependent T-helper cell line TS1. The over-expression of IRS1 in TS1 cells has been reported to enhance their proliferative response to IL-9 [14], raising the possibility that IRS1/2 could play a role in IL-9-dependent proliferation. No data are available concerning downstream effectors of IRS1/2 function that are involved in IL-9 signaling.

Following JAK- or tyrosine receptor-mediated phosphorylation, IRS recruits signaling molecules containing Src homology 2 domains, such as the regulatory subunit of phosphatidylinositol-3 kinase (PI 3-K) p85, causing the activation of the PI 3-K catalytic subunit p110 [15]. The PI 3-K-mediated phosphorylation of phosphoinositide lipids triggers pleckstrin homology (PH) domain-mediated recruitment of protein kinase B (PKB) to the plasma membrane where it becomes activated via threonine and serine phosphorylation catalyzed by PDK1 [16] and by another yet undiscovered kinase referred to as PDK2 [17]. PKB protects cells from caspase-mediated apoptosis by phosphorylating Bad, and preventing the release of cytochrome *c* from mitochondria [18,19]. The PI 3-K/PKB pathway appears to be involved in most of the signal transduction processes that mediate cellular functions of different cytokines, including IL-2, IL-3, IL-4, IL-6, IL-7, stem cell factor (SCF) and PDGF [16,17].

With this study, we aimed to better define the role of IRS1/2 and downstream effectors in IL-9-elicited events involved in survival and proliferative responses, using IRS-null 32D cells and various cell lines that proliferate in response to IL-9.

2. Materials and methods

2.1. Cell culture and proliferation assay

TS1 cells were maintained in Dulbecco's modified Eagle medium (DMEM) (Gibco/BRL) containing 10% fetal bovine serum (FBS) (Gibco/BRL), non-essential amino acids (Gibco/BRL), 5.5×10^{-4} M 2-mercaptoethanol (Gibco/BRL) and 20 ng/ml of murine IL-9 (R&D Systems). Ba/F3, 32D and MC9 cells were maintained in DMEM

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Abbreviations: IL, interleukin; IL-9R, interleukin-9 receptor; JAK, janus kinase; STAT, signal transducer and activator transcription factor; IRS, insulin receptor substrate; PI 3-K, phosphatidylinositol-3 kinase; PKB, protein kinase B

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containing 10% FBS with the addition of 10 ng/ml of murine IL-3, whereas Mo7e cells were maintained in the presence of human IL-3 (hIL-3) (R&D Systems).

2.2. Transfection and plasmids

Baf/9R is a cell line expressing the hIL-9R that was derived from IL-3-dependent murine pro-B Ba/F3 cells as described [9]. 32D/9R cells were generated by neomycin selection of 32D cells electroporated with pEF-myc-cyto vector (Invitrogen), in which hIL-9R cDNA was cloned into the *Nco*I and *Not*I restriction sites. 32D/9R/IRS cells were generated by transfecting 32D/9R cells with pEF-BOS plasmid containing the human cDNA for IRS1 (kindly provided by Dr. Renato Baserga, Thomas Jefferson University, PA, USA) followed by selection with puromycin.

2.3. Analysis of STAT and PKB phosphorylation, and inhibitor treatment

Cells were washed with phosphate-buffered saline, resuspended in DMEM containing 0.5% bovine serum albumin, 20 mM HEPES, and incubated for 6 h at 37°C. Cells were stimulated in the same medium (10^6 cells/ml), centrifuged and resuspended in 250 μl of Laemmli buffer. 25 μl was loaded on 8% pre-cast SDS–PAGE and analyzed by Western blot using antibodies specific for phospho-serine 473 (Biosource) or phospho-threonine 308 (New England Biolabs) of PKB, an antibody specific to phospho-tyrosine 705 of STAT3 (New England Biolabs), or actin (Sigma). Both anti-phospho-PKB antibodies recognize all three isoforms of PKB. To determine PI 3-K dependence of PKB activation, TS1 cells were treated with 100 nM of either wortmannin or SB202190 (Calbiochem) for 4 h before cytokine stimulation.

2.4. IRS2 and PI 3-K co-immunoprecipitation

TS1 and BaF/9R cells were washed and cytokine-starved for 6 h. 50 million cells were stimulated with 50 ng/ml of IL-9 or IL-4 for 15 min, then washed and resuspended in lysis buffer (0.8% Brij96, 0.2% NP-40, 50 mM Tris pH 8, 150 mM sodium chloride, 1 mM EDTA, 1 mM sodium vanadate, 1 mM sodium fluoride, 1 mM PMSF, 10 μg/ml aprotinin and 10 μg/ml leupeptin). After 10 min, lysates were centrifuged ($13\,000 \times g$). 5 μg of anti-IRS2 antibody (Upstate Biotechnology Inc.) was added to supernatant and incubated for 2 h. Lysates were then incubated with protein-A-agarose overnight. The next day, beads were washed and resuspended in Laemmli buffer and boiled. Proteins were separated in a 6% SDS–PAGE gel (Novex) and analyzed by Western blot using 1 μg/ml of anti-phosphotyrosine 4G10, anti-p85/PI 3-K or anti-IRS2 antibodies (Upstate Biotechnology Inc.) as probes. Proteins were detected by chemiluminescence (ECL, Amersham).

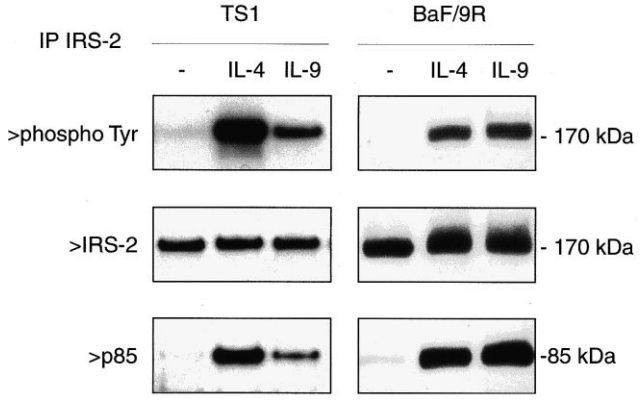

Fig. 1. IL-9 promotes IRS2 phosphorylation and recruitment of p85 PI 3-K subunit. TS1 and BaF/9R cells were cytokine-starved for 6 h and restimulated for 10 min with IL-4 (50 ng/ml) or IL-9 (50 ng/ml), or left untreated. IRS2 was immunoprecipitated from lysates using an anti-IRS2 antibody, and precipitated proteins were analyzed by Western blotting with anti-phosphotyrosine antibodies (upper panels) to detect IRS2 phosphorylation, or with anti-p85. As a control, membranes were reprobed with anti-IRS2 antibodies. Similar results were obtained in at least two independent experiments.

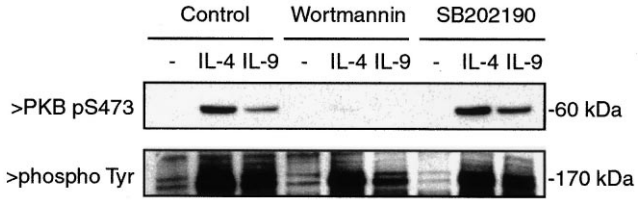

Fig. 2. PI 3-K-dependent phosphorylation of PKB by IL-9 in TS1 cells. TS1 cells were washed, starved for 6 h before stimulation with IL-4 or IL-9. When indicated, cells were pretreated for 4 h with wortmannin or SB202190 prior to stimulation. Total lysates were analyzed by Western blot with antibodies specific for phosphorylated serine 473 in PKB protein (upper panel) or with anti-phosphotyrosine antibodies (bottom panel). IRS1/2 proteins were identified as the major phosphorylated 170 kDa species after cytokine stimulation.

3. Results

3.1. IL-9 induces IRS2 phosphorylation and association to p85 PI 3-K subunit

TS1 is an IL-9-dependent T-helper cell line in which phosphorylation of IRS1 and IRS2 has been shown as a consequence of IL-9 stimulation [14,20]. Since IRS1 and IRS2 can associate with and activate PI 3-K upon insulin stimulation [15], we addressed the possibility that IL-9-induced activation of IRS1/2 may also lead to the formation of an IRS/PI 3-K complex. As shown in Fig. 1, both IL-4 or IL-9 stimulation resulted in the tyrosine phosphorylation of IRS2. In addition, p85 was found to co-immunoprecipitate with the phosphorylated form of IRS2 after stimulation with either cytokine (Fig. 1). IL-4 and IL-9 also induced IRS2 phosphorylation and association with p85 in Ba/F3 cells that were transfected with the IL-9R (Fig. 1, right panels).

3.2. IL-9 induces PKB phosphorylation in TS1 cells but not in BaF/9R, MC9 and Mo7e cells

As the IRS/p85 association has been shown to initiate a PI 3-K-dependent cascade, we tested the activation of PKB, a major downstream effector of PI 3-K. PKB phosphorylation at serine 473 by PDK1 has been reported to be critical for the activation of this kinase [17,21]. As shown in Fig. 2, treatment of TS1 cells with either IL-4 or IL-9 led to the phosphorylation of this residue. To further confirm that the IL-9-induced phosphorylation of PKB depends upon the activity of PI 3-K, we used the PI 3-K inhibitor wortmannin. As shown in Fig. 2, wortmannin completely inhibited the phosphorylation of PKB, but not the phosphorylation of IRS1/2, after IL-9 or IL-4 treatment of TS1 cells. The p38 mitogen-activated protein kinase (p38/MAPK) has also been shown to phosphorylate serine 473 in vitro [21]. The p38/MAPK inhibitor SB202190 did not affect PKB and IRS2 phosphorylation, demonstrating that p38/MAPK activity is not involved in this pathway (Fig. 2).

Surprisingly, in BaF/9R cells, IL-9 treatment did not result in phosphorylation of serine 473 of PKB (Fig. 3A), while IL-3 treatment did. Thus, IL-9-mediated IRS2 phosphorylation and p85 recruitment were not sufficient to induce PKB phosphorylation in these cells. We also used two other cell lines that proliferate in the presence of IL-9: the murine mast cell line MC9 and the human megakaryocytic leukemia Mo7e [1,22]. PKB phosphorylation was absent or barely detectable in MC9 and Mo7e cells treated with IL-9, in contrast to cells

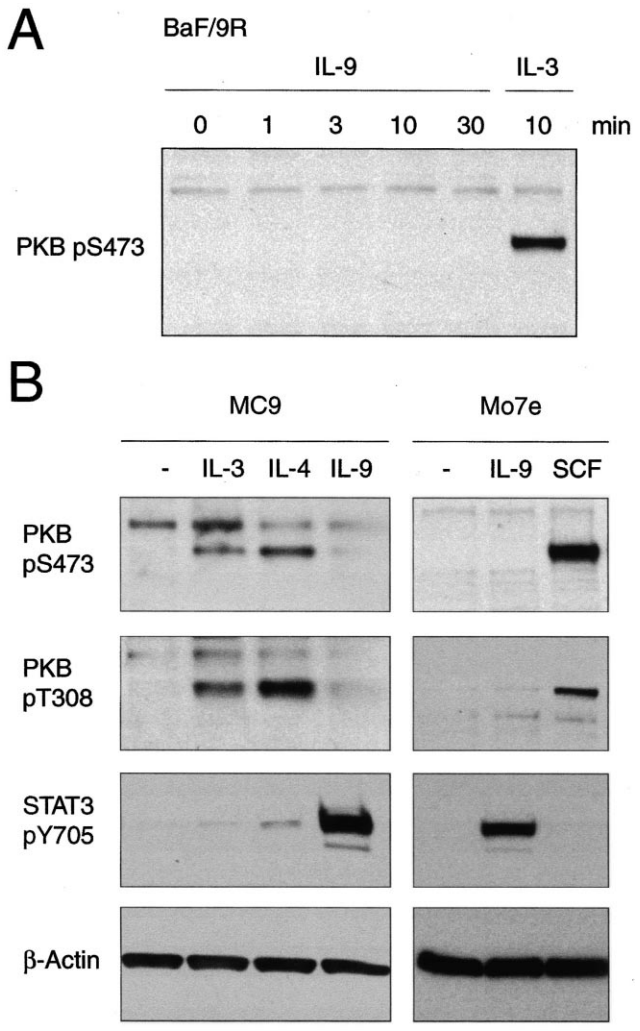

Fig. 3. PKB is not significantly phosphorylated in BaF/9R, MC9 and Mo7e cells treated with IL-9. (A) Cytokine-starved BaF/9R cells were restimulated with hIL-9 (100 ng/ml) for various periods of time or with IL-3 for 10 min. PKB phosphorylation was analyzed as above by Western blotting (10^5 cells/well). Similar results were obtained in three independent experiments. (B) The mast cell line MC9 was washed, starved and stimulated for 10 min with murine IL-9 (20 ng/ml), IL-4 (20 ng/ml), or IL-3 (500 U/ml). The human leukemia Mo7e was treated similarly and stimulated with hIL-9 or SCF (both at 100 ng/ml). Total lysates were analyzed by Western blot with antibodies recognizing phospho-serine 473 of PKB, phospho-threonine 308 of PKB, phospho-tyrosine 705 of STAT3, or actin. The results shown are representative of at least two experiments.

treated with IL-3, IL-4 or SCF (Fig. 3B). Similar results were obtained with antibodies recognizing the second important phosphorylated site of PKB, threonine 308, which is the target of an unidentified kinase referred to as PDK2 [17]. IL-9 induced the phosphorylation of this site in TS1 cells, but not in BaF/9R (data not shown). IL-9 activated STAT3 in all IL-9-dependent cells tested. In conclusion, IL-9-induced cell growth did not correlate with PKB phosphorylation.

3.3. Ectopic expression of IRS1 in 32D/9R cells causes proliferation in response to IL-9

To further understand the role of IRS1/2 in IL-9-dependent survival and proliferation, we used the IL-3-dependent murine myeloid cell line 32D, which lacks endogenous IRS1 and IRS2 [13,23], and has undetectable levels of IL-9R (data not shown). We transfected 32D cells with a hIL-9R expression construct and analyzed the effect of IL-9 on survival and proliferation of these cells (32D/9R) in absence of IRS1/2. As expected, parental 32D cells died even in the presence of high concentrations of IL-9 (Fig. 4). In contrast, treatment of 32D/9R cells with IL-9 resulted in a dose-dependent survival, but not cell growth. Hence, IRS1/2 is not required for IL-9-induced survival of 32D/9R cells. FACS analysis confirmed that the expression of IL-9R was comparable to the number of receptors expressed in BaF/9R cells, which proliferate in response to IL-9 [9], ruling out the possibility that a low receptor density is the cause for the non-proliferative effect of IL-9 on 32D/9R cells (data not shown).

Subsequently, we explored the possibility that expression of IRS1/2 in 32D/9R would result in the ability of IL-9 to induce proliferation of these cells. We transfected 32D/9R cells with an IRS1 expression vector and generated 32D/9R/IRS cells. Three independent electroporations were performed after which cells were maintained for 10 days in the presence of IL-3 and puromycin for selection. Puromycin-resistant cells were first tested for expression of IRS1 by Western blot (Fig. 5A) and IRS1 expressing cells were used for proliferation assays. Fig. 5B shows the effect of IL-9 treatment on proliferation of 32D/9R and 32D/9R/IRS cells. After 4 weeks, while the number of 32D/9R cells had increased to only about 2-fold, 32D/9R/IRS cells had expanded to over 600-fold, clearly demonstrating a positive effect of IRS1 on IL-9-induced proliferation. Noticeably, the proliferation rate of these cells in response to IL-9 remained quite low as compared to BaF/9R or TS1 cells.

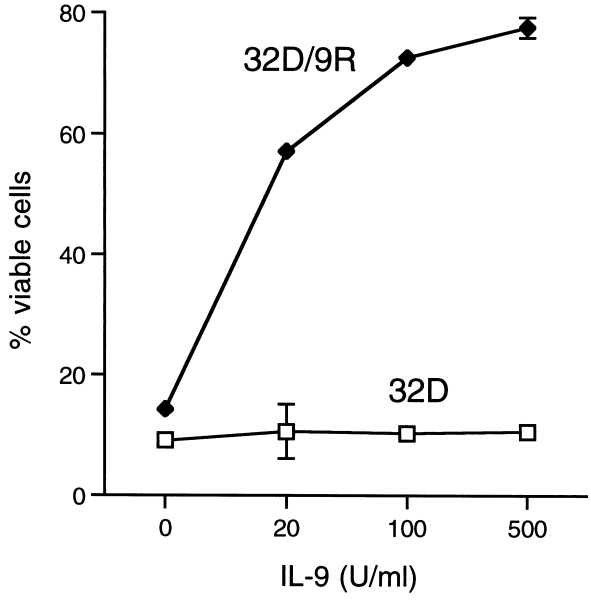

Fig. 4. 32D cells expressing IL-9R (32D/9R) survive in the presence of IL-9. Cells were washed and treated with increasing concentrations of hIL-9. After 48 h, the percentage of viable cell was measured by propidium iodide staining (125 μg/ml) followed by FACS analysis. In the presence of IL-3, viability of 32D and 32D/9R cells was 90.7 ± 0.6% and 90.1 ± 0.3%, respectively. The data represent the mean ± S.E.M. of triplicate samples. Similar results were obtained with four independent clones.

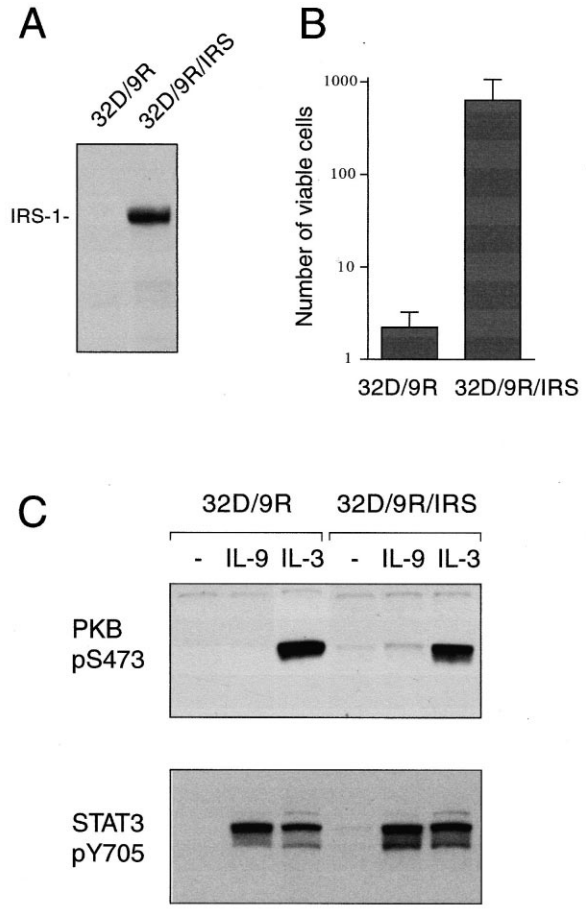

Fig. 5. Ectopic IRS1 expression in 32D/9R cells promoted proliferation in response to IL-9. (A) Expression of IRS1 was analyzed in 32D/9R and 32D/9R/IRS1 clones (10^5 cells/well) by Western blot with anti-IRS1 antibodies (UBI, 1 μg/ml). (B) 32D/9R cells were electroporated with an expression vector containing the IRS1 cDNA or with a control empty vector. Cells were then maintained for 10 days in the presence of IL-3 and puromycin for selection. At day 0, puromycin-resistant cells were washed and cultured in the presence of hIL-9. After 4 weeks, the average number of cells (±S.E.M.) from three independent transfections was obtained. No viable cells were obtained in the absence of cytokine. (C) 32D/9R and 32D/9R/IRS cells were cytokine-starved for 6 h in serum-free medium and then restimulated for 15 min with the appropriate cytokines. Western blots on total lysates were performed using antibodies against the phosphorylated serine 473 residue of the PKB protein (upper panel) or the phosphorylated tyrosine 705 residue of the STAT3 protein (lower panel). Detection of β-actin confirmed equal protein loading (not shown). Similar results were obtained for three independent clones in two independent experiments.

We next tested the activation of PKB in relation to IRS expression after treatment of 32D/9R and 32D/9R/IRS1 cells with IL-9. As expected, PKB was not phosphorylated upon IL-9 stimulation of 32D/9R cells (Fig. 5C), suggesting that it is not involved in IL-9-mediated survival. More surprisingly, PKB phosphorylation was only slightly detectable in 32D/9R/IRS1 cells treated with IL-9, based on the phosphorylation state of serine 473 (Fig. 5C). Similar results were observed when an antibody recognizing phospho-threonine 308 was used (data not shown). In contrast, IL-3 treatment of 32D/9R or 32D/9R/IRS1 cells produced a robust phosphorylation of PKB at both residues. STAT3 was found activated by either IL-9 or IL-3 in both cell lines, demonstrating that these cells responded to the cytokine treatment. Taken together, these data suggest that IRS1 expression promotes proliferation but is not sufficient to cause significant phosphorylation of PKB in response to IL-9.

4. Discussion

The present data demonstrate that IRS1/2 adaptor proteins play a prominent role in mediating the IL-9-induced proliferation in hemopoietic cells. This is based on the following observations: (i) IRS1 and IRS2 are phosphorylated upon IL-9 stimulation in various cell lines, (ii) 32D/9R cells, which lack IRS1/2 expression, survive but do not proliferate in response to IL-9, and (iii) IRS1 expression in these cells promotes IL-9-induced proliferation. Interestingly, IRS2 phosphorylation by IL-4 in Ba/F3 cells is not enough to promote proliferation in response to this cytokine (Fig. 1 and data not shown). In line with this result, IL-9 poorly stimulates the growth of Ba/F3 cells expressing a mutated IL-9R that does no longer activate STAT proteins but still allowed IRS2 phosphorylation [9,12], indicating that IRS1/2 is not sufficient to deliver a complete signal and that STAT function is necessary to complement the IL-9-induced proliferative response [12]. Thus, IL-9-mediated signal transduction seems to rely both on STAT and on IRS1/2 cascades to exert its effect on proliferation. IRS1 and IRS2 have both been shown to be important in the IL-4- and insulin-mediated proliferation and appear to function interchangeably [13,23]. Although IL-2 and IL-3 can induce phosphorylation of IRS1/2 proteins [24,25], IL-3-mediated proliferation of 32D cells does not require IRS1/2 activity and 32D cells expressing exogenous IL-2Rβ can proliferate in response to IL-2 [26], thereby via an IRS1/2-independent mechanism.

By contrast, survival of 32D/9R cells in the presence of IL-9 does not require IRS1/2 activation, although IRS1 is involved in apoptosis inhibition by insulin and IL-4 in these cells and various other models [27,28]. This is in line with our previous findings demonstrating that a constitutively active variant of STAT5 is sufficient to prevent apoptosis of the BW5147 T-lymphoma cell line [10].

An important aspect of IRS1/2 function is its interaction with the regulatory subunit of PI 3-K p85, which leads to activation of PI 3-K and its downstream mediator PKB. In this report, we show that IRS2 interacts with the p85 subunit of PI 3-K and this event correlates with a wortmannin-sensitive activation of PKB in TS1 cells. Remarkably, PKB phosphorylation upon IL-9 stimulation was barely detectable in any of the other IL-9-dependent cell lines analyzed in this study, although PKB could be activated by other cytokines. These data suggest that PKB function is not required for IL-9-dependent survival and proliferation. The cell-specific, IL-9-induced activation of PKB observed in TS1 cells may be the result of growth selection. Indeed, TS1 cells were derived from a T-helper clone that has been progressively selected for growth in the presence of IL-9 [3]. This selection may have altered the typical IL-9 signal transduction patterns.

Despite the fact that IRS1/2 appears implicated in the PI 3-K-dependent activation of PKB [15], our data demonstrate that activation of IRS1/2 does not always correlate with the activation of PKB. Conversely, IL-3-induced activation of PKB in 32D cells is PI 3-K-dependent but IRS1/2-independent (Fig. 5C and [29]). The signaling events triggered by IL-9

downstream IRS1/2 still remain unknown. We failed to detect association of SHP2 or GRB2 with IRS2 in response to IL-9 (data not shown). It is not clear whether IRS1/2-mediated IL-9 function depends on PI 3-K activity. The PI 3-K inhibitor LY294002 prevented the proliferation of BaF/9R cells that were grown in the presence of either IL-3 or IL-9 (data not shown). However, taking into account the many caveats associated with the use of inhibitors for functional analyses, more accurate studies using cells deficient in the different isoforms of PI 3-K will be required to uncover putative novel IRS1/2-dependent, PI 3-K-independent pathways that mediate cellular effects triggered by IL-9.

In conclusion, our data suggest that IL-9-mediated survival is independent of the phosphorylation PKB and IRS1/2, the latter protein being involved in promoting proliferation. This study also shows that p85 recruitment to IRS1/2 is not sufficient for PKB phosphorylation.

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