BCR-ABL binds to IRS-1 and IRS-1 phosphorylation is inhibited by imatinib in K562 cells

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Abstract In the present study we used K562 cells to demonstrate that insulin receptor substrate 1 (IRS-1) is expressed and constitutively phosphorylated in BCR-ABL$^+$ cells. We observed association between BCR-ABL/IRS-1, IRS-1/phosphoinositide 3′-kinase (PI3-kinase), and IRS-1/Grb2 in the K562 cell line. Our findings demonstrate that imatinib treatment resulted in marked attenuation of BCR-ABL/IRS-1 association and of IRS-1-stimulated PI3-kinase activity in K562 cells. We concluded that the IRS-1 protein is involved in the signalling pathway of the BCR-ABL tyrosine kinase.

Key words: Imatinib; Chronic myeloid leukemia; BCR-ABL; IRS-1; K562 cells

1. Introduction

The signalling network controlled by BCR-ABL is complex and highly redundant [1]. The sum of these protein interactions translates into the altered phenotype of CML cells, which consists of constitutively active mitogenic signalling, defective adherence to stromal cells and extracellular matrix, and reduced response to apoptosis-inducing stimuli [2]. The tyrosine kinase activity of BCR-ABL is specifically inhibited by imatinib mesylate, formerly STI571 [3–6].

The insulin receptor substrate (IRS) signalling system mediates cell growth and metabolism during insulin/insulin-like growth factor 1 (IGF-1) and interleukin-4 (IL-4) stimulation [7]. The best characterized member of the family, IRS-1, contains multiple tyrosine phosphorylation sites, which during insulin stimulation are phosphorylated and act as docking sites for the SH2 domains of the p85 regulatory subunit of phosphoinositide 3′-kinase (PI3-kinase) [8,9], the adapter protein Grb-2 that links it to the Ras signalling cascade [10], SHP-2 phosphatase, and the Nck protein [11,12]. IRS-1 is detected in most cells and tissues, including lymphoid progenitor cells, B cells, carcinoma cells, fibroblasts, adipocytes, liver, skeletal muscle and brain.

To further extend our understanding of the mechanisms by which BCR-ABL transforms cells, we have attempted to identify more proteins that interact with BCR-ABL and which might direct BCR-ABL to various signal transduction pathways. In the present study we sought to determine whether BCR-ABL induces IRS-1 phosphorylation and whether this association requires BCR-ABL tyrosine kinase activity. We also thought of determining whether the function of the IRS-1 system is required for activation of PI3-kinase in BCR-ABL signalling and whether the IRS-1 phosphorylation leads to association with Grb-2. Therefore we evaluated the effects of imatinib on BCR-ABL/Grb2 association and on the levels of total extracellular signal-regulated kinase (ERK) and phospho-ERK expression in K562 cells treated or not with imatinib.

2. Materials and methods

2.1. Cell culture and imatinib treatment

The human leukemia cell lines K562 and HL60 were obtained from ATCC, Philadelphia, PA, USA. Cells were cultured in RPMI containing 10% fetal calf serum and glutamine with addition of penicillin/streptomycin and amphotericin B, they were maintained at 37°C, 5% CO_2. For experiments, cells were seeded at a density of 3×10^5 cells/ml and cultured for 7 days. K562 cells were incubated or not with 1 μM imatinib for 6 h. Imatinib was kindly provided by Dr. Elizabeth Buchdunger, Novartis Pharmaceuticals, Basel, Switzerland, and prepared as a 50 mM stock solution in sterile water. Samples containing 1×10^7 cells were pelletted and resuspended in lysis buffer (1% Triton X-100, 100 mM Tris (pH 7.4), 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM phenylmethylsulfonyl fluoride, and 0.1 mg/ml aprotinin) and incubated for 30 min at 4°C. Insoluble material was removed by centrifugation for 45 min at 12 000 rpm in a 70.Ti rotor (Beckman) at 4°C. Protein determination was performed by the Bradford dye method [13] using the Bio-Rad reagent and bovine serum albumin as the standard.

2.2. Western blotting analysis

Equal amounts of protein were used for total extracts or for immunoprecipitation with specific antibodies followed by SDS–PAGE and Western blot analysis with the indicated antibodies and [^{125}I]protein A as described [8,14], or ECL™ Western Blotting Analysis System (Amersham Pharmacia Biotech UK Limited). Quantitative analysis of the blots was performed using Scion Image Software. Monoclonal antibodies against phosphotyrosine (SC-508), Abl (SC-23), phospho-ERK (SC-7383) and polyclonal antibody against IRS-1 (SC-559), Grb2 (SC-255) and ERK1 (SC-93) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-PI3-kinase antiserum (06-195) was from UBI (Lake Placid, NY, USA). To confirm the IRS-1 presence, we also performed immunoblottings with a rabbit polyclonal antibody against IRS-1 that was kindly provided by Dr. Morris, F. White and X.J. Sun (Joslin Diabetes Center, Boston, MA, USA). To exclude unspecific interactions between the investigated

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proteins and protein A-Sepharose, control precipitations without the specific antibodies were performed, followed by immunoblotting with anti-phosphotyrosine, anti-IRS-1, anti-Abl and anti-PI3-kinase antibodies and no band was detected. The results indicated that the immunoprecipitations performed in the experiments described herein do not allow the detection of unspecific interactions.

2.3. PI3-kinase assay

Aliquots of supernatants containing equal amounts of protein were incubated overnight at 4°C using antibody against IRS-1, and the immunocomplexes were precipitated with a 50% solution of protein A-Sepharose 6MB. In vitro PI3-kinase assays were performed as described [8]. The ^{32}P-labeled 3-P-phosphatidylinositol was quantitated using Scion Image Software.

2.4. Statistical analysis

Experiments were performed by analyzing K562 cells treated and untreated with imatinib. For comparisons, Student's unpaired *t*-test was used. The level of significance was set at $P < 0.05$.

3. Results

3.1. BCR-ABL oncoprotein binds to IRS-1 and is constitutively phosphorylated

K562 cells were either left untreated or treated with imatinib for 6 h, and cell lysates were immunoprecipitated with an anti-Abl antibody and then immunoblotted with anti-phosphotyrosine. A 210 kDa protein band corresponding to tyrosine-phosphorylated BCR-ABL was detected. The level of phosphorylation of this band decreased after imatinib treatment. In the same anti-phosphotyrosine blots, in addition to the 210 kDa band, another band of 185 kDa was also de-

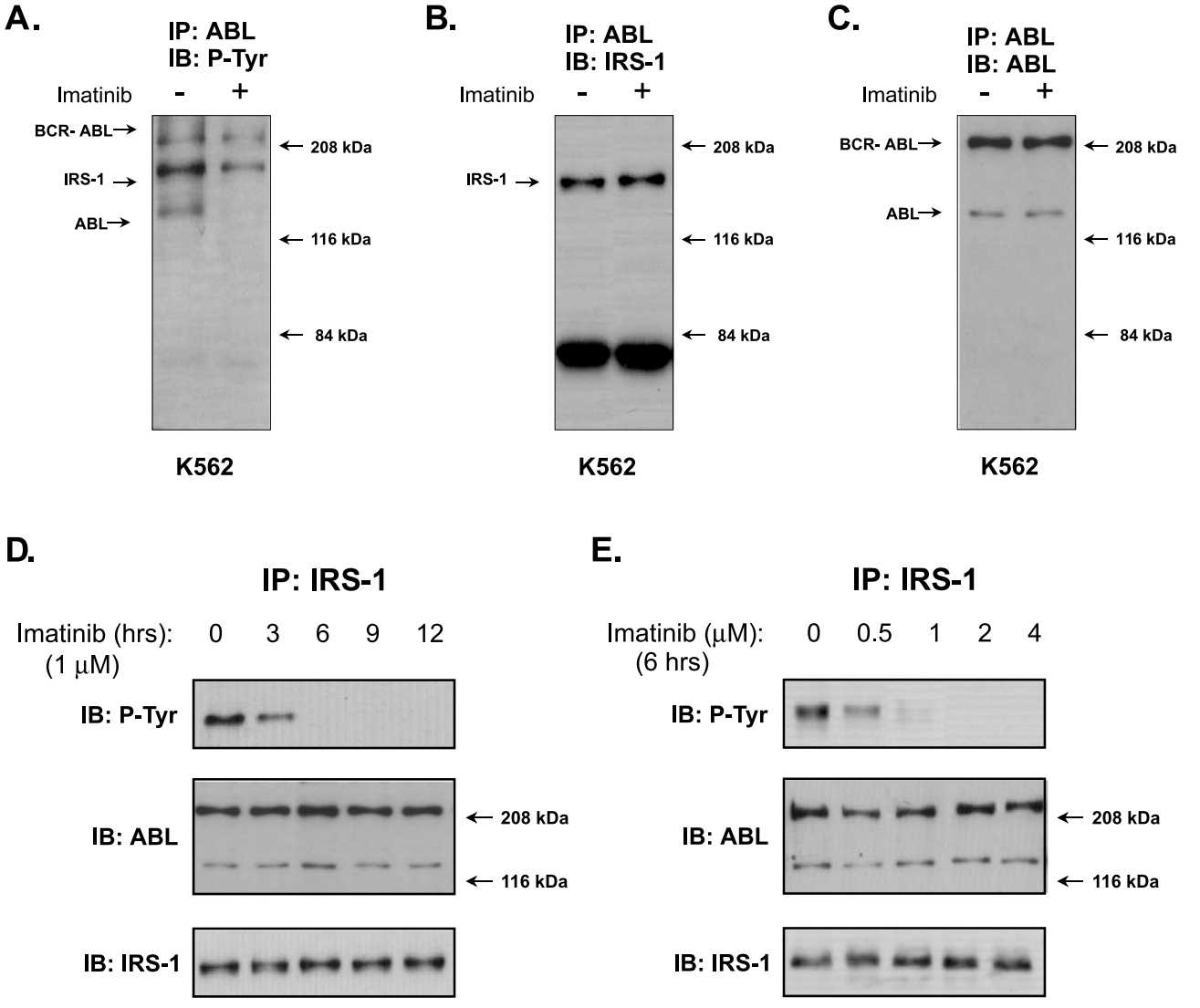

Fig. 1. IRS-1 is a substrate of BCR-ABL protein. IRS-1 is phosphorylated by BCR-ABL. A: K562 cells were exposed (+) or not (−) to 1 μM imatinib for 6 h. Lysates from K562 cells containing equal amounts of protein were immunoprecipitated (IP) with anti-Abl antibodies and immunoblotted (IB) with anti-phosphotyrosine antibodies (P-Tyr) as described in Section 2. The nitrocellulose membrane was stripped and reprobed with anti-IRS-1 antibodies (B), and with anti-Abl antibodies (C) ($n = 12$). Imatinib inhibits tyrosine phosphorylation of IRS-1. D: K562 cells treated with 1 μM imatinib at the time indicated were immunoprecipitated with anti-IRS-1 and blotted with anti-phosphotyrosine antibody (P-Tyr). The same membrane was stripped and reblotted with anti-Abl and anti-IRS-1 antibodies ($n = 4$). E: K562 cells treated for 6 h with different concentrations of imatinib, as indicated, were immunoprecipitated with anti-IRS-1 and blotted with anti-phosphotyrosine antibody (P-Tyr). The same membrane was stripped and reblotted with anti-Abl and anti-IRS-1 antibodies ($n = 4$). The Western blots were developed with the ECL Western Blotting Analysis System.

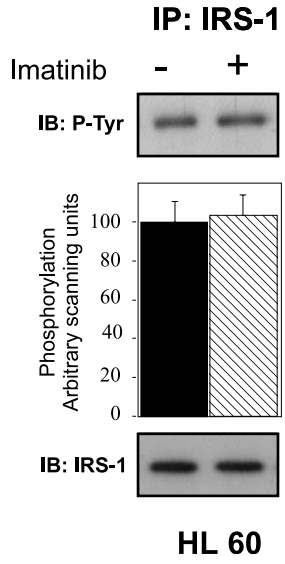

Fig. 2. The level of phosphorylation of IRS-1 in BCR/ABL$^-$ cells is not inhibited by imatinib. The HL60 cell line was treated (+) or not (−) with 1 μM imatinib for 6 h. Autoradiograms of SDS–PAGE of IRS-1 immunoprecipitates (IP) were immunoblotted (IB) with anti-phosphotyrosine antibodies (P-Tyr). Blots were stripped and re-probed with anti-IRS-1 antibodies. The results of a representative study are shown. The bar graphs represent the mean ± S.E.M. of the scanning densitometry of six experiments. The Western blots were developed with [^{125}I]protein A.

tected, and there was a very marked decline in this band after treatment (Fig. 1A). By reprobing this membrane with anti-IRS-1 antibody, we demonstrated that the 185 kDa band corresponds to IRS-1, and the level of this band was not modified by imatinib treatment, showing that the association IRS-1/BCR-ABL occurs independently of IRS-1 tyrosine phosphorylation (Fig. 1B). This result was confirmed by immunoblotting with another anti-IRS-1 antibody (kindly provided by Dr. Morris, F. White) (data not shown). As predicted, there were no changes in BCR-ABL protein levels (Fig. 1C).

We confirmed this result by Western blotting analysis of tyrosine-phosphorylated proteins in anti-IRS-1 immunoprecipitates before and after imatinib treatment. In order to estimate the rate of imatinib-induced inhibition of IRS-1 phosphorylation in K562 cells, we performed time course and dose–response experiments. The time course experiments were performed in K562 cells treated with 1 μM of imatinib and collecting samples at different time points. As shown in Fig. 1D (upper panel), IRS-1 phosphorylation is strongly inhibited after 3 h treatment with 1 μM of imatinib, with maximal inhibition occurring at 6 h and sustained for 12 h. The levels of BCR-ABL associated with IRS-1 were the same after imatinib treatment (Fig. 1D). There were no changes in IRS-1 protein levels (Fig. 1D, bottom panel). Subsequently, the dose–response curve was performed using K562 cells treated for 6 h with different concentrations of imatinib. As shown in Fig. 1E (upper panel), there was a dose-dependent decrease in IRS-1 phosphorylation after imatinib treatment. The IRS-1 phosphorylation almost disappeared with 1 μM imatinib treatment for 6 h. When the nitrocellulose membrane was stripped and immunoblotted with anti-Abl antibody, the BCR-ABL association with IRS-1 protein occurred independently of the imatinib treatment (Fig. 1E). Reprobing this nitrocellulose membrane with anti-IRS-1 antibody, there were no changes in IRS-1 protein levels (Fig. 1E, bottom panel).

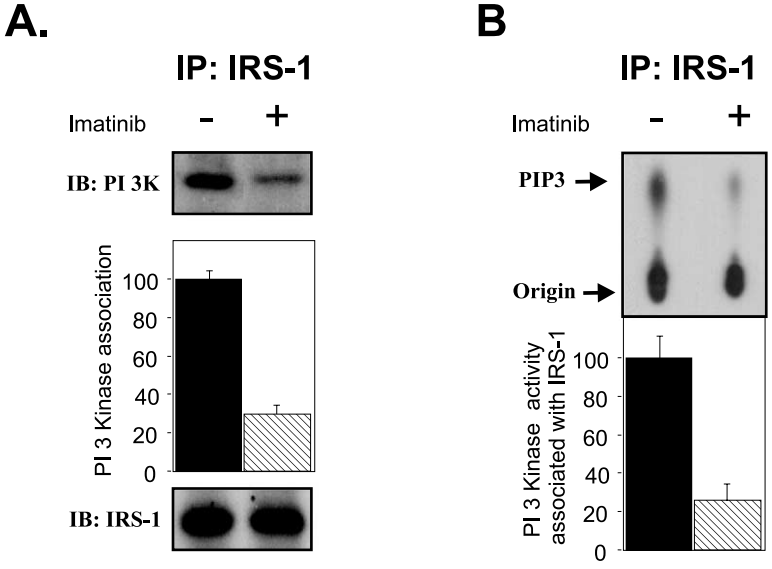

Fig. 3. IRS-1 associates and activates PI3-kinase in K562 cell line. A: K562 cells were exposed (+) or not (−) to 1 μM imatinib for 6 h. Autoradiograms of SDS–PAGE of IRS-1 immunoprecipitates (IP) were immunoblotted (IB) with anti-PI3-kinase antibodies. The nitrocellulose transfer was stripped and reblotted with antibodies to IRS-1. The results of a representative study are shown. The bar graphs represent the mean ± S.E.M. of the scanning densitometry of six experiments. The Western blots were developed with [^{125}I]protein A. B: Lysates from K562 cells exposed (+) or not (−) to 1 μM imatinib for 6 h were immunoprecipitated with anti-IRS-1 antibodies. PI3-kinase assays were performed as described. Fluorographs show the silica TLC plates of IRS-1-associated PI3-kinase activity. PIP3 indicates the migration position of phosphatidylinositol 3-phosphate. Bar graphs depict the relative incorporation of ^{32}P into PI3-P (mean ± S.E.M.) from six separate experiments.

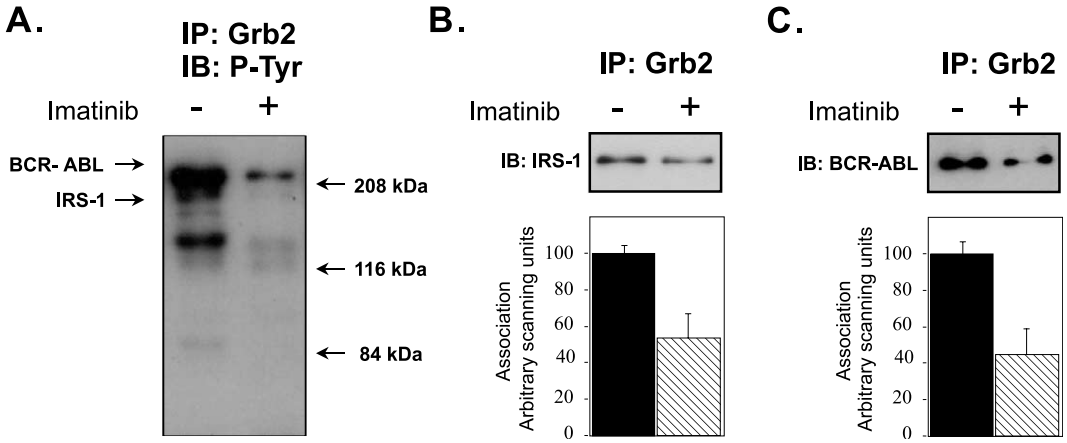

Fig. 4. Grb2, an adapter protein, interacts with IRS-1 in K562 cells. K562 cells were exposed (+) or not (−) to 1 μM imatinib for 6 h. A: Autoradiograms of SDS–PAGE of Grb-2 immunoprecipitates (IP) were immunoblotted (IB) with anti-phosphotyrosine antibodies (P-Tyr). The Western blot was developed with [^{125}I]protein A. Blots were stripped and reprobed with anti-IRS-1 antibodies (B) and with anti-Abl antibodies (C), and developed with the ECL Western Blotting Analysis System. The results of a representative study are shown. The bar graphs represent the mean ± S.E.M. of the scanning densitometry of eight experiments.

3.2. *IRS-1 phosphorylation is unaffected by imatinib in BCR/ABL$^-$ cells*

HL60 cells were treated or not with 1 μM imatinib for 6 h. Cell lysates were immunoprecipitated with anti-IRS-1, followed by immunoblotting with anti-phosphotyrosine antibodies. As expected, the levels of IRS-1 protein and IRS-1 tyrosine phosphorylation in HL60 cells remained unchanged with imatinib treatment (Fig. 2). These results demonstrated that IRS-1 tyrosine phosphorylation was not directly modulated by imatinib treatment in HL60 cells.

3.3. *IRS-1 associates and activates PI3-kinase in K562 cell line*

In samples from K562 cells previously immunoprecipitated with anti-IRS-1 antibody and immunoblotted with antibody directed against the 85 kDa subunit of PI3-kinase, a band with the expected molecular weight of the regulatory subunit of PI3-kinase (85 kDa) was present. The amount of PI3-kinase associated with IRS-1 was reduced to 30 ± 6% of the control values ($P < 0.05$) in K562 cells treated with imatinib (Fig. 3A), thus suggesting that IRS-1 associates with PI3-kinase in K562 cells and this association depends on the level of IRS-1 phosphorylation.

To determine if there is PI3-kinase activity in IRS-1 immunoprecipitates, K562 cells were prepared and immunoprecipitated with anti-IRS-1 antibodies. A basal PI3-kinase activity was present in anti-IRS-1 immunoprecipitates in control cells. After the treatment with imatinib, there was a dramatic decrease in PI3-kinase activity (Fig. 3B). Thus, compared with control cells, the level of PI3-kinase activity in anti-IRS-1 immunoprecipitates was reduced to 26 ± 10% ($P < 0.05$) in K562 cells treated with imatinib. These data suggest that in IRS-1 immunoprecipitates there is PI3-kinase activity, which is inhibited by imatinib.

3.4. *Grb2, an adapter protein, interacts with IRS-1 in K562 cells*

Cells were treated or not with imatinib and cell lysates were immunoprecipitated with anti-Grb2 antibody and then immunoblotted with anti-phosphotyrosine (Fig. 4A). A 210 kDa protein, corresponding to tyrosine-phosphorylated BCR-ABL, was present, as expected. A 185 kDa protein, corresponding to tyrosine-phosphorylated IRS-1, was clearly detectable in anti-Grb-2 immunoprecipitates in control cells. The levels of BCR-ABL phosphorylation and IRS-1 phos-

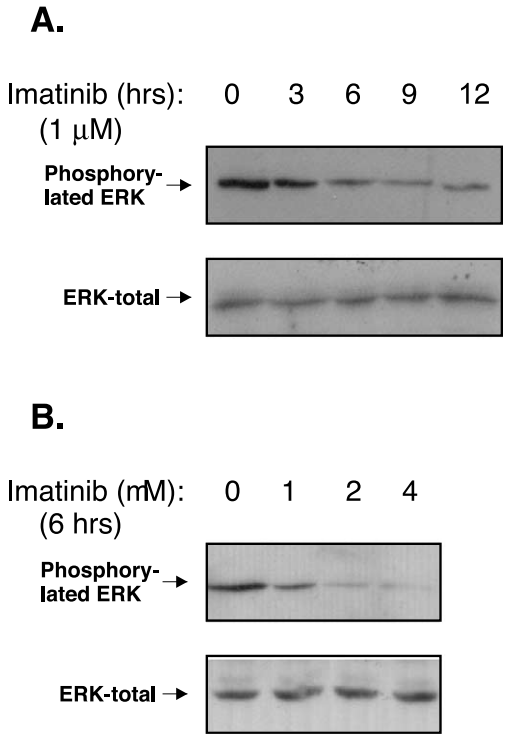

Fig. 5. Imatinib inhibits MAPK phosphorylation/activation in a time- and dose-dependent manner. A: K562 cells treated with 1 μM imatinib for the times indicated were lysed and the proteins were separated by SDS–PAGE on 12% gels and immunoblotted (IB) with antibodies directed against phospho-ERK1/2. Blots were stripped and reprobed with anti-total ERK1/2. B: K562 cells treated for 6 h with different concentrations of imatinib, as indicated, were lysed and the proteins were separated by SDS–PAGE on 12% gels and blotted with antibodies directed against phospho-ERK1/2. Blots were stripped and reprobed with anti-total ERK1/2. The results of a representative study are shown ($n = 4$). The Western blots were developed with the ECL Western Blotting Analysis System.

phorylation associated with Grb2 were significantly reduced in cells treated with imatinib (Fig. 4A). To assess the effect of imatinib on direct binding of Grb2 to IRS-1, this nitrocellulose membrane was stripped and immunoblotted with anti-IRS-1 and we observed a 50% decrease in the association between Grb2/IRS-1 after imatinib treatment ($P < 0.05$) (Fig. 4B). By reprobing this membrane with anti-Abl antibodies we confirmed that imatinib inhibits the Grb2/BCR-ABL association (Fig. 4C).

3.5. Imatinib inhibits MAPK phosphorylation/activation

To characterize the effects of imatinib on mitogen-activated protein kinase (MAPK) activation, expression of phospho-ERK and total ERK was determined by Western blot analysis of the total extracts of K562 cells incubated or not with imatinib at different times and doses. The time course experiments were performed in K562 cells treated with 1 μM of imatinib and collecting samples at different time points. As shown in Fig. 5A, ERK1/2 phosphorylation was inhibited after 6 h of 1 μM imatinib and this inhibition was sustained for 12 h. The level of ERK1/2 was the same (Fig. 5A, bottom panel). Subsequently, the dose–response curve was performed with K562 cells treated for 6 h with different concentrations of imatinib. As shown in Fig. 5B, the level of ERK1/2 phosphorylation had almost disappeared after 6 h of 2 μM imatinib treatment. The total ERK1/2 expression remained unaffected with imatinib treatment (Fig. 5B, bottom panel).

4. Discussion

In the present study we demonstrated the involvement of IRS-1 protein in BCR-ABL signal transduction. We discovered that the IRS-1 protein was phosphorylated in K562 cells previously immunoprecipitated with anti-Abl antibodies. Using a BCR-ABL tyrosine kinase inhibitor we discovered that there was a strong decrease in IRS-1 phosphorylation level, but independently of the IRS-1 tyrosine phosphorylation level, this protein interacted with BCR-ABL. These results, taken together, suggest that IRS-1 is constitutively phosphorylated and associated with BCR-ABL in K562 cells, and that IRS-1 phosphorylation is inhibited by imatinib. IRS proteins have been previously shown to be essential for the mitogenic effects of insulin/IGF-1 and IL-4 in myeloid hematopoietic cells [7,15], suggesting that IRS-1 regulates cellular pathways critical for cell metabolism and growth. Even though most of our knowledge regarding the molecular functions of IRS proteins has been derived from studies in the insulin signalling system, our data are the first evidence that this protein participates in BCR-ABL signalling pathway.

To clarify the role that IRS-1 protein plays in the BCR-ABL$^+$ cells, it is necessary to characterize the pathways of IRS-1 in response to the tyrosine kinase activity of the BCR-ABL oncoprotein. The role of PI3-kinase in transducing tyrosine kinase signals is well established [16,17], but the activation of PI3-kinase by tyrosine kinases may involve different mechanisms. Distinct domains from different proteins mediate the association of BCR-ABL/PI3-kinase and the activation of PI3-kinase [18]. IRS-1 contains about nine YMXM motifs, which bind to PI3-kinase [19]. Using anti-PI3-kinase antibodies in samples previously immunoprecipitated with anti-IRS-1 antibodies, we then demonstrated the association of IRS-1/PI3-kinase in K562 cells. We also demonstrated the IRS-1-associated PI3-kinase activity in this cell line. We observed a significant decrease in the association between IRS-1 and PI3-kinase and also in the IRS-1-associated PI3-kinase activity upon imatinib treatment, which suggests that the mechanism of activation of PI3-kinase by BCR/ABL involves binding to IRS-1 phosphorylated protein, and depends on the BCR-ABL tyrosine kinase activity.

In spite of the fact that imatinib can also inhibit c-kit and platelet-derived growth factor (PDGF) tyrosine kinase activity [20], evidence supports our hypothesis that the tyrosine phosphorylation of IRS-1 is mediated by BCR-ABL in K562 cell line. PDGF induces IRS-1 phosphorylation on serine/threonine residues, but not on tyrosine residues [21,22]. On the other hand, c-kit associates directly with PI3-kinase and this association depends on tyrosine 719 of the c-kit receptor [23,24]. As a point mutation in the c-kit receptor abolishes the PI3-kinase activation, it is unlikely that IRS-1 participates in the c-kit signalling pathway towards PI3-kinase activation. So, it is reasonable to speculate that the tyrosine phosphorylation of IRS-1, like the IRS-1-associated PI3-kinase activity, is mediated by BCR-ABL in K562 cells. Furthermore, we have excluded unspecific inhibition of IRS-1 phosphorylation by imatinib, since its phosphorylation was not inhibited in HL-60 human myeloid leukemia cells that do not express the BCR-ABL kinase activity (BCR-ABL$^-$ cells).

Several links between BCR-ABL and Ras have been defined. Autophosphorylation of tyrosine 177 in BCR-ABL provides a docking site for the adapter molecule Grb-2 [25,26]. Two other adapter molecules, Shc and Crkl, are substrates of BCR-ABL [27,28] and can also activate Ras. In this study we demonstrated that BCR-ABL might also recruit the IRS-1 protein, and once phosphorylated, IRS-1 recruits the Grb2 docking protein. The association between Grb2 and BCR-ABL has been well studied, and this association requires Y177 phosphorylation, a known autophosphorylation site on BCR-ABL (PMID: 7553858) [25,29]. Our results show that the association between Grb2 and BCR-ABL was inhibited by imatinib treatment. These data reinforce that BCR/ABL may induce the formation of multimeric complexes of signalling proteins, and in the case of Grb2 activation, IRS-1 participates in this multimeric complex, but is probably not essential for Grb2 activation in the BCR-ABL pathway. We also verified, in K562 cells, the effect of imatinib on the downstream proteins involved in the MAPK pathway. As previously described, imatinib is able to inhibit the MAPK pathway [20,30,31], and it seems to occur in a time- and dose-dependent manner.

In conclusion, we identified that IRS-1 is a new adapter protein in the BCR-ABL signalling pathway. IRS-1 phosphorylation depends on BCR-ABL tyrosine kinase activity, and is inhibited by imatinib. The IRS-1 protein, when phosphorylated by BCR-ABL, can activate the PI3-kinase pathway. Our results also demonstrated that IRS-1 can bind to Grb2 in K562 cells. In addition, the results suggest that IRS-1 is involved in the signalling pathway of the BCR-ABL tyrosine kinase and, by binding to PI3-kinase and Grb2, establishes a new link between BCR-ABL, Akt and p21^{ras}.

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