The Insulin Receptor Negatively Regulates the Action of Pseudomonas Toxin-Based Immunotoxins and Native Pseudomonas Toxin

Xiu Fen Liu, David J. FitzGerald, and Ira Pastan

Abstract

SS1P is a recombinant immunotoxin composed of an antimesothelin Fv fragment fused to a truncated portion of Pseudomonas exotoxin A. SS1P targets and kills mesothelin-expressing tumors, which include mesothelioma as well as ovarian, lung, and pancreatic cancers. SS1P is currently in clinical trials in mesothelioma. Because insulin acting through the insulin receptor is a survival factor for many cancer cell lines, we explored how lowering insulin receptor level would affect the cytotoxic action of SS1P. We show here that siRNA knockdown of the insulin receptor enhanced the cytotoxic action of native Pseudomonas exotoxin and enhanced SS1P toxicity on several human cell lines, but did not affect the response to other cytotoxic agents such as TRAIL, etoposide, and cycloheximide. To determine how insulin receptor knockdown enhances SS1P action, we analyzed various steps involved in cell killing. We found that insulin receptor knockdown increases the cleavage of SS1P by furin, which allows more toxin to reach the cytosol and inactivate elongation factor 2. These findings indicate that the insulin receptor negatively regulates immunotoxin action.

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Introduction

Monoclonal antibodies (mAb) are now widely used to treat cancer, but unfortunately many cancers are resistant to antibody treatment. To take advantage of the selective binding of antibodies to cancer cells, mAbs are now being used to deliver different types of cytotoxic agents to these cells (1). Immunotoxins are one such type of anticancer agent in which protein toxins are attached to mAbs (2, 3). We construct recombinant immunotoxins (RIT) by attaching a 38 kDa fragment of Pseudomonas exotoxin A (PE38) to the Fv portion of a mAb chosen to react selectively with cancer cells and not with essential normal tissues (3, 4). SS1P (anti-mesothelin Fv-PE38) is a RIT that targets mesothelin, a cell-surface protein that is highly expressed on mesothelioma cells, as well as pancreatic, ovarian, lung, and other cancer cells (5, 6). After binding to mesothelin, SS1P enters cells by endocytosis where the furin protease cleaves the Fv from the toxin (7). The toxin then is transferred in a retrograde fashion to the endoplasmic reticulum from which it is translocated into the cytosol, there the toxin catalyzes the ADP-ribosylation and inactivation of elongation factor 2, eventually leading to apoptosis (4, 7). Despite the effectiveness of SS1P and other immunotoxins in killing cancer cells (8, 9), many of the mechanistic steps in immunotoxin action have not been established. Understanding more about these mechanisms could be useful in developing strategies to make immunotoxins more effective in killing cancer cells.

Insulin is an important ingredient in many types of tissue culture media, because activating the insulin receptor promotes cell growth and protects against loss of viability and apoptosis (10–12). Likewise, the presence of insulin-like growth factor I (IGF-I), which also binds the insulin receptor, can promote growth and survival. We hypothesized that activities associated with the insulin receptor, including tyrosine kinase, could negatively regulate the ability of immunotoxins to kill target cells. Using siRNA technology to reduce expression, we show here that knockdown of the insulin receptor enhances the cytotoxic action of immunotoxin SS1P on several human cancer cell lines. We provide evidence that the insulin receptor acts as an early step in immunotoxin action and regulates the cleavage of the immunotoxin by furin, perhaps by regulating immunotoxin trafficking. Of further interest, we report that other toxic agents, unrelated to Pseudomonas exotoxin, were not enhanced by the silencing of the insulin receptor.

Materials and Methods

Reagents

Immunotoxins SS1P and HB21-PE40 were purified in our laboratory (13). AGL2263 was purchased from Santa Cruz...
Biotechnology. Rapamycin, PD98059, LY294002, anti-insulin receptor, anti-PARP, anti-cleaved caspase-3, anti-Bax, anti-Bak, anti-Bcl-xl, anti-Mcl-1 Abs were from Cell Signaling. Anti-furin Ab was from Invitrogen and insulin was purchased from Sigma. 3H-Leucine was purchased from GE Healthcare.

Cell culture
A431/H9 is a human mesothelin-transfected A431 cell (American Type Culture Collection) HAL-01 (DSMZ). KB31 cells were provided by Michael Gottesman (NCI, Bethesda, MD). M30 mesothelioma cell line is from Steven Albelda (University of Pennsylvania, Philadelphia, PA). A1847 is from Stuart Aaronson, (NCI). All cells are cultured in Dulbecco’s Modified Eagle’s Medium supplemented with 10% FBS and 1% penicillin-streptomycin in humidified atmosphere of 5% CO2 at 37°C. HAL-01, KB31, and A431/H9 cells were authenticated within 1 year by short tandem repeat (STR) analysis. The M30 and A1847 were analyzed in 1 month by STR and no known matches were found.

Transfection and cytotoxicity assays
To knockdown the insulin receptor, 5,000 cells were transfected via the addition of 3 μL of 20 μmol/L siRNA, 3.5 μL of DharmaFECT Transfection Reagent 3 (Dharmacon) in 125 μL final volume per well for 96-well experiments. After 48 hours of transfection, the cells were treated with SS1P or other toxic agents at the indicated concentration for a further 72 hours. Cell viability was then measured by the ATP levels using CellTiter-Glo luminescent cell viability assay (Promega). Viability is expressed as the percentage of luminescence with SS1P compared with control without SS1P treatment. All siRNA experiments used an unrelated luciferase siRNA (GL2) as a negative control.

Inhibitor study
Before experiments were initiated, 5,000 KB31 cells were seeded overnight in 96-well plates. Inhibitors were added and cells were incubated for 1 hour before the addition of SS1P. After 72 hours of incubation, cell viability was measured by ATP level. In some cases, inhibitors AGL2263 and rapamycin were also added and cells were incubated for approximately 18 hours before SS1P addition; however, the effects on inhibition of SS1P activity were similar to the 1 hour inhibitor incubation at 37°C in culture media. All experiments were done 3–4 times with reproducible results.

Western blot analysis
Cells were washed in PBS and disrupted by the addition of lysis buffer (50 mmol/L Tris HCl, 150 mmol/L NaCl, 5 mmol/L EDTA with 1% NP40, 5 μg/mL leupeptin, 5 μg/mL aprotinin, 10 μmol/L phenylmethylsulfonylfluoride) on ice for 30 minutes. After high-speed centrifugation, 20–40 μg supernatant protein was analyzed by SDS-PAGE, transferred to a polyvinylidene difluoride membrane and subjected to Western blotting with detection by enhanced chemiluminescence (ECL) or ECL plus (Amersham).

Internalization and FACS analysis
A431/H9 cells were transfected with siRNA for 48 hours in 6-well plates, then 1 μg/mL of SS1P-Alex-647 was added and cells were incubated at 37°C for the indicated times. After labeling, the cells were washed with PBS and stripped with glycine buffer containing 0.2 mol/L glycine (pH 2.5) and 1 mg/mL of bovine serum albumin to remove surface bound SS1P. Cells were then trypsinized, washed with fluorescence-activated cell sorting (FACS) buffer (PBS with 5% FBS, plus 0.1% NaN3), and analyzed by FACS Calibur.

SS1P cleavage
A431/H9 cells were transfected with siRNA for 48 hours in 6-well plates, 1 μg/mL of SS1P was added to cells and incubated on ice for 30 minutes to saturate SS1P binding. Cells were changed to fresh media and incubated at 37°C for the indicated time before making a total cell lysate.

Real-time PCR
RNA was isolated using the TRizol reagent (Invitrogen). Reverse transcription and cDNA synthesis were conducted using a Quantitect Reverse Transcription Kit following the manufacturer’s instructions (Qiagen). Primers are listed in Table 1. PCR was conducted using Quantifast SYBR Green PCR master kits (Qiagen).

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Results

Insulin receptor knockdown

Our strategy assumed that the insulin receptor would be activated in cells growing in serum-containing media because serum contains large amounts of IGF-I that, like insulin, can activate this receptor. To investigate its role in immunotoxin response, we used siRNA to knockdown the mRNA encoding the insulin receptor and then determined how effectively the immunotoxin SS1P killed various types of mesothelin-expressing cells. To measure cell viability we used the CellTiter-Glo assay, which measures cellular ATP levels. In early experiments we used KB31 cells, which express insulin receptor and are sensitive to killing by SS1P. We evaluated 4 different siRNA oligos targeting the insulin receptor: designated siIR-1, siIR-2, siIR-3, and siIR-4. As shown in Fig. 1A, we found that 3 of these (siIR-1, -3, and -4) increased the cytotoxic effect of SS1P (Fig. 1A) with siRNA-1 being the most effective. We then measured the levels of insulin receptor mRNA and protein after transfection with siIRs-1, -3, and -4 and found that siIR-1 caused the greatest reduction in both mRNA and protein levels, which also produced the greatest increase in SS1P cytotoxicity (Fig. 1A–C). We have seen siIR-1 knockdown insulin receptor protein level by 80% to 90% consistently and the IC50 value decreased 3- to 5-fold in KB31 cells. Transfection with siIR-2, which did not knockdown insulin receptor RNA or protein levels, did not increase SS1P toxicity (Supplementary Fig. S1). These results show that knockdown of the insulin receptor specifically increases the ability of SS1P to kill a mesothelin-expressing cell line.

To verify that the fall in ATP induced by SS1P in insulin receptor knockdown cells was associated with an increase in markers of apoptosis, we conducted Western blot analyses on siIR-1-treated cells. Figure 1D shows that the level of intact PARP was decreased and the levels of cleaved PARP and cleaved caspase-3 were increased relative to cells treated with SS1P alone (Fig. 1D). This result shows that apoptosis is being simulated. In addition, we observed that SS1P treatment of control cells decreased the insulin receptor level (Fig. 1D, lane 1 vs. 3). We believe this occurs because SS1P inhibits overall protein synthesis. In addition, we also found treatment of cells with HB21-PE40 that targets the transferrin receptor caused a fall of insulin receptor protein level (Supplementary Fig. S2). When SS1P was combined with siIR-1, insulin receptor protein was undetectable (Fig. 1D, lane 3 vs. 4).

Insulin receptor knockdown on other cancer cell types

To determine if lowering insulin receptor affected SS1P toxicity in other cancer cell types, we examined mesothelin-expressing cell lines: A431/H9 cells, which are an epidermoid carcinoma cell line transfected with mesothelin, A1847 ovarian cancer cells and M30 mesothelioma cells. Figure 2 shows that knockdown of the insulin receptor increased the cytotoxic effect of SS1P in all 3 cell lines as well as in KB31 cells (Fig. 2A–D). Insulin receptor knockdown did not enhance the killing by SS1P of cell lines, A431 and HAL-01, that do not...
express mesothelin. This indicates that insulin receptor knockdown does not promote nonspecific killing by SS1P (Fig. 2E–F). We also found that the growth rate of the cell lines was not reduced by insulin receptor knockdown indicating the effect on SS1P toxicity is independent of cell growth rate (data not shown).

**Immunotoxin specificity**

To determine if the insulin receptor knockdown effect was specific for an immunotoxin-targeting mesothelin, we examined the effects on a *Pseudomonas* exotoxin-containing immunotoxin (HB21-PE40) targeting the human transferrin receptor and found that its cytotoxic activity was also greatly increased (Fig. 3A). We also tested the effects of insulin receptor knockdown on immunotoxins that do not target receptors on KB31 cells and found that neither LMB9, which targets Lewis Y antigen, or BL22, which targets CD22, had any toxic activity (Fig. 3G and H), demonstrating that knockdown insulin receptor did not enhance nonspecific internalization.

Native *Pseudomonas* exotoxin receptor LRP1B is expressed in KB cells (Supplementary Fig. S3). We also investigated if insulin receptor knockdown affected the activity of native *Pseudomonas* exotoxin and found, as shown in Fig. 3B, that *Pseudomonas* exotoxin toxicity was increased 5-fold. This finding indicates that the effect of insulin receptor knockdown is not restricted to an immunotoxin-targeting mesothelin.

We then tested other toxic agents such as diphtheria toxin and cycloheximide, which inhibit protein synthesis, the extrinsic apoptosis inducer TRAIL, and etoposide, that acts through an intrinsic pathway. Silencing the insulin receptor inhibited cell killing by diphtheria toxin (Fig. 3C) and did not cause significant changes in the cell-killing activities of cycloheximide (Fig. 3D), TRAIL, or etoposide (Fig. 3E and F). These data indicate that the insulin receptor plays a specific regulatory role on immunotoxins containing *Pseudomonas* exotoxin or on native *Pseudomonas* exotoxin.

**Knockdown of insulin receptor affects SS1P processing**

To investigate the mechanism by which the insulin receptor controls SS1P toxicity, we measured the internalization of SS1P by exposing cells to SS1P labeled with Alexa-647 for 5–120 minutes and using flow cytometry to assess the amount of cell-associated SS1P in A431/H9 cells. Compared with the control, there was no increase in the rate or amount of SS1P taken up by
siIR-treated cells (Fig. 4A). We also showed that SS1P internalization did not increase in KB31 cells after siIR-1 treatment (Supplementary Fig. S4).

After internalization, the next step in SS1P action is processing by furin. Cleavage by furin separates the Fv from the toxin and generates a 35 kD toxin fragment. As shown in Fig. 4B, in insulin receptor knockdown cells there was an increase in the amount of the 35 kD fragment detected at 15, 60, and 150 minutes. By 150 minutes more than 90% of SS1P was degraded (Note about 4B—at 60 minutes there is more PE35 in insulin receptor knockdown but the amount of full sized SS1P is the same for either sample. This is consistent with interference of a degradation step—that is PE35 is not degraded but is stabilized. However, at 150 minutes there is less full sized SS1P in insulin receptor knockdown and more PE35 suggesting a precursor product relationship presumably with furin). To determine if an increase in furin levels was responsible for the increase in SS1P cleavage, furin levels were measured using Western blot analysis and were not found to increase after knockdown of insulin receptor (Fig. 4B). Because furin is the
only known cellular protease that cleaves Pseudomonas exotoxin-related immunotoxins and because furin cleavage is rate-limiting (see Discussion), these results indicate that low-ering insulin receptor expression increases the processing of SS1P by furin and generates an increased amount of the toxin fragment. As a consequence there should be more free toxin fragment available to move to the endoplasmic reticulum, translocate to the cytosol, inactivate elongation factor 2, and arrest protein synthesis (7). To evaluate if the rate of protein synthesis was affected we incubated cells for 20 hours with SS1P after insulin receptor knockdown and measured ³H-Leu incorporation into protein. Figure 4C shows that insulin receptor knockdown greatly enhanced the ability of SS1P to inhibit leucine incorporation.

**Insulin receptor knockdown did not change proapoptotic or antiapoptotic protein levels**

Because the insulin receptor plays an important role in cancer cell growth and apoptosis, we determined if knockdown of the insulin receptor would alter the levels of several proapoptotic or antiapoptotic proteins. As shown in Fig. 5, Mcl-1, which has been shown to play an important role in immunotoxin killing (14), was not changed in knockdown cells compared with control cells. Bax and xIAP (data not shown) also did not change. We observed a small but not significant reduction in Bcl-xl and Bak, but this small change would not explain the stimulation of SS1P-induced toxicity and the reduction in diphtheria toxin-induced toxicity. Together, these data indicate the effect of the insulin receptor is likely at the level of trafficking and not on proteins regulating apoptosis.

**Insulin receptor level, but not IGF-IR, correlates with SS1P toxicity**

Insulin receptor and IGF-IR are very homologous and use similar signaling pathways (12). Figure 6A shows that IGF-IR knockdown slightly decreases SS1P toxicity, an effect totally opposite to that of insulin receptor knockdown. However, when the 2 siRNAs were combined, there was still stimulation of SS1P activity although less than with insulin receptor knockdown alone.

To examine this finding further, we conducted Western blot analysis and found that treatment with siIGF-IR not only
Discussion

We show here that lowering insulin receptor levels in various cancer cell lines using siRNA knockdown increases the ability of immunotoxins containing Pseudomonas exotoxin or of native Pseudomonas exotoxin to kill target cells. This novel function of the insulin receptor affects Pseudomonas exotoxin-based toxins whether they enter the cell bound to an antibody to mesothelin or to an antibody to the transferrin receptor or through the native receptor for Pseudomonas exotoxin. We were surprised to find that insulin receptor knockdown did not enhance the cytotoxicity of diphtheria toxin, which like Pseudomonas exotoxin inactivates elongation factor 2, but instead protected cells from diphtheria toxin induced cell death. This is probably because Pseudomonas exotoxin and diphtheria toxin have different trafficking pathways and reach the cytosol by different mechanisms. Pseudomonas exotoxin must be cleaved by furin and transported to the endoplasmic reticulum before reaching the cytosol (15), whereas diphtheria toxin can be transferred directly to the cytosol from the endocytic compartment (7, 16). In addition, furin is the only cellular protease known to cleave Pseudomonas exotoxin and Pseudomonas exotoxin immunotoxins, suggesting insulin receptor knockdown is altering furin function but not other proteases.

It is known that one major action of insulin is the regulation of the transport of GLUT4 containing vesicles causing them to fuse with the plasma membrane and increase the amount of GLUT4 on the cell surface. Under conditions of low insulin, GLUT4 is sequestered in intracellular vesicles in muscle and fat cells. Insulin induces insulin receptor activation and through a complex cascade of signal transduction events increases the uptake of glucose by inducing the translocation of GLUT4 from these vesicles to the plasma membrane (17, 18). It is possible that some of the same proteins that control Pseudomonas exotoxin trafficking also control the trafficking of the insulin receptor. This area is under investigation.

Several compounds have been found to inhibit the action of insulin on target cells. We have investigated these agents to determine if they regulate the increase in Pseudomonas exotoxin toxicity produced by insulin receptor knockdown. As shown in Supplementary Fig. S5 and Supplementary Table S1, none of these agents enhanced SS1P cell killing and some of them actually protected against cell killing when combined with SS1P. The compounds investigated include the insulin receptor/IGF-IR substrate inhibitor AGL2263, the mTOR inhibitor rapamycin, the PI3K inhibitor LY294002, the MEK1 inhibitor PD98059, and a combination of both LY294002 and PD98059 (Supplementary Table S1). Furthermore, addition of insulin to the cells did not protect cells from killing by SS1P (Supplementary Fig. S1D). We have considered the possibility that the insulin receptor works through another pathway to regulate intracellular trafficking. Boucher and colleagues have presented evidence that the insulin receptor has a role in apoptosis regulation that is independent of its kinase activity (19). However, our findings here indicate the apoptotic protein levels did not change before or after insulin knockdown (Fig. 5). It is possible the insulin receptor function here also controls membrane trafficking. A kinase-independent mechanism of insulin receptor action could include interaction with a scaffolding protein that controls trafficking to lysosomes or retrograde trafficking of proteins leading to the accumulation of active PE35 in the cytosol.

Two splice variants of the insulin receptor exist in mammalian cells: insulin receptor-A, lacking exon 11, and full-length insulin receptor-B. Insulin receptor-A predominates in fetal tissues and is often upregulated in many cancer cells; insulin receptor-B is present in adult muscle and fat cells and is responsible for glucose regulation (20, 21). Our findings here indicate the apoptotic protein levels did not change before or after insulin knockdown (Fig. 5). It is possible the insulin receptor function here also controls membrane trafficking. A kinase-independent mechanism of insulin receptor action could include interaction with a scaffolding protein that controls trafficking to lysosomes or retrograde trafficking of proteins leading to the accumulation of active PE35 in the cytosol.

Figure 6. Knockdown IGF-IR did not increase SS1P toxicity. A, KB31 cells were transfected with control siRNA (siCo), siIR-1, siIGF-1R (siIGFR), or both siIR-1 and siIGF-IR (siDbl) for 48 hours. SS1P was added at indicated concentrations for 72 hours. Cell viability was measured by ATP assay. B, KB31 cells were transfected with siIR-1 or siIGF-IR for 48 hours. SS1P was added at indicated concentrations for 72 hours. Cell viability was measured by ATP assay. B, KB31 cells were transfected with control siRNA (siCo), siIR-1, siIGF-1R (siIGFR), or both siIR-1 and siIGF-IR (siDbl) for 48 hours. SS1P was added at indicated concentrations for 72 hours. Cell viability was measured by ATP assay. B, KB31 cells were transfected with control siRNA (siCo), siIR-1, siIGF-1R (siIGFR), or both siIR-1 and siIGF-IR (siDbl) for 48 hours. SS1P was added at indicated concentrations for 72 hours. Cell viability was measured by ATP assay. B, KB31 cells were transfected with control siRNA (siCo), siIR-1, siIGF-1R (siIGFR), or both siIR-1 and siIGF-IR (siDbl) for 48 hours. SS1P was added at indicated concentrations for 72 hours. Cell viability was measured by ATP assay. B, KB31 cells were transfected with control siRNA (siCo), siIR-1, siIGF-1R (siIGFR), or both siIR-1 and siIGF-IR (siDbl) for 48 hours. SS1P was added at indicated concentrations for 72 hours. Cell viability was measured by ATP assay.
We conclude both isoforms are important for the immunotoxin or *Pseudomonas* exotoxin toxicity. The insulin receptor is elevated and activated in several human malignancies including breast, colon, and lung cancer (20, 22) and functionally enhances tumor progression (23). Our finding of a novel insulin receptor function that regulates membrane trafficking may contribute to the understanding of insulin action and the insulin receptor in cancer and other human diseases.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

**Conception and design:** D.J. FitzGerald, I. Pastan  
**Development of methodology:** X.-F. Liu  
**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** X.-F. Liu  
**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** X.-F. Liu, D.J. FitzGerald, I. Pastan

**References**


**Writing, review, and/or revision of the manuscript:** X.-F. Liu, D.J. FitzGerald, I. Pastan  
**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** I. Pastan

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