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Development and precise characterization of phospho-site-specific antibody of Ser^{357} of IRS-1: Elimination of cross reactivity with adjacent Ser^{358}

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ABSTRACT

Antibodies that recognize specifically phosphorylated sites on proteins are widely utilized for studying the regulation and biological function of phosphoproteins. The proposed strategy is a powerful, analytical tool allowing the generation of phospho-site specific antibodies albeit adjacent phosphorylation sites are present. Here, we demonstrate the assessment and elimination of cross reactivity of phospho-site-specific-Ser^{357} IRS-1 antibody. While determining the specificity of p-Ser^{357} antiserum we came across the cross reactivity of the antiserum with adjacent Ser^{358} which was successfully abolished by an improved immuno-purification method. The specificity of the purified antiserum was then verified by indirect ELISA, results of ELISA were also mirrored in the experiments carried out in BHK-IR cells using different mutants of IRS-1 carrying mutations at either Ser^{357}/Ser^{358}/Ser$^{357/358}$. Immuno-purified-p-Ser^{357} did not react with IRS-1 Ala^{357} and IRS-1 Ala$^{357/358}$. In conclusion, the present study describes generation and characterization of p-Ser^{357} IRS-1 antibody, which reacts with IRS-1 in site specific and phosphorylation state-dependent manner without showing cross reactivity to adjacent Ser^{358}. This antibody can be effectively used to further clarify the inhibitory role of Ser^{357} in insulin signal transduction.

Insulin resistance is a common pathological state wherein target tissues develop a less than normal response to circulating insulin. This plays a central role in the development of type 2 diabetes, the epidemic which is one of the main causes of morbidity and mortality worldwide [1]. The binding of insulin to its receptor initiates and activates the insulin signal transduction cascade. The discovery of insulin receptor substrate (IRS) proteins and their role to link cell surface receptors to the intracellular signaling cascades is a key step to understand insulin action [2]. IRS family members play a pivotal role in modulating and diversifying insulin signals and are absolutely required for normal insulin-stimulated glucose uptake [3]. While tyrosine phosphorylation of IRS-1 is definitely necessary for the activation of insulin action, IRS-1 serine phosphorylation mainly modulates the signal intensity and is a major mechanism in the physiological shut down. Therefore, dysregulated serine phosphorylation of IRS-1 is hypothesized to be a general molecular mechanism for insulin resistance [3–6].

A common tool to investigate the function of single or multiple phosphorylation sites is the generation of point mutations of the site(s) to be studied, i.e. the gain of function using an exchange of the site to Asp or Glu, and the loss of function by an exchange of Tyr to Phe, and Ser or Thr to Ala. The major limitation of this strategy is that solely on/off effects can be examined. Contrary to that, the most powerful tool to investigate slight regulations of distinct phosphorylation site(s), e.g. effect of insulin on a particular serine site in IRS-1, are phospho-site-specific antibodies. Over the last decade several specific Ser-phosphorylation sites of IRS-1 have been identified and their relative function in insulin signaling is established [7–10]. Most of such studies were based on the development of phospho-site-specific antibodies for a particular serine site of IRS-1. Development of phospho-site-specific-antibodies is crucial to demonstrate the physiological significance of a particular serine site in insulin action that cannot be resolved by more conventional methodologies. Of note, IRS-1, a sophisticated regulated multi-adapter protein, contains 34 tyrosine, 63 threonine and 181 serine residues at a total length of 1235 amino acid residues. Based on frequent nearby potential phosphorylation sites within IRS-1, the generation of phospho-site-specific antibodies requires a particular preparation strategy and proper validation to establish

Abbreviations: IRS-1, insulin receptor substrate-1; PTB, phosphotyrosine binding; PKC, protein kinase C; HPLC, high-performance liquid chromatography; BHKIR, baby hamster kidney cells stably expressing the human insulin receptor; GST, glutathione S-transferase; p-Ser, phospho-serine.

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that they do not cross react with unphosphorylated peptide, other phosphoproteins or especially other phospho residues of the same protein. By using a novel mass spectrometric approach for phosphopeptide screening [8,11], we recently identified Ser^{357}, located near PTB domain of IRS-1, as a PKC-δ-mediated phosphorylation site and demonstrated the biological significance of this site in insulin signal transduction [12]. During the course of our studies we observed a novel phenomenon of two functional serine sites of IRS-1 adjacent to each other i.e. Ser^{357} and Ser^{358}. Whereas, Ser^{358} had been postulated to be a potential phosphorylation site in prior studies using point mutated IRS-1 [5,13]. Here, we aimed to develop and characterize phospho-site-specific-Ser^{357} IRS-1 (p-Ser^{357}) antibody. The presence of the adjacent Ser^{358} was a challenge to develop a phospho-site specific antibody that can be specific enough to recognize the phosphorylation of Ser^{357} alone, without giving cross reactivity to adjacent Ser^{358} residue, so that single effect of Ser^{357} could be demonstrated in insulin signaling.

In this paper we describe for the first time, generation, characterization and validation of phospho-site-specific-Ser^{357} IRS-1 (p-Ser^{357}) antibodies. A complementary, generally applicable approach of epitope selection, phosphopeptide synthesis, multistep immuno-affinity-chromatography, and enzyme-linked immunosorbent assay using diverse synthetic peptides, and finally characterization and validation of the antibody in living cells to exclude cross reactivity of the phospho-site-specific antibody is demonstrated.

Materials and methods

Solid-phase peptide synthesis. The peptides were synthesized using standard Fmoc/tBu chemistry, performed on the multiple peptide synthesizer Syro II (MultiSynTech, Witten, Germany) on a 0.025-mmol scale using a 6-fold molar excess of Fmoc amino acids (MultiSynTech, Witten, Germany) on TCP-resin (PepChem, Reutlingen, Germany) or MAP-resin $(Lys)_4$-$(Lys)_2$-Lys-β-Ala-OR) as described in [14]. Crude peptides were purified using preparative reversed-phase high-performance liquid chromatography (RP-HPLC) and their identity was confirmed using MALDI-MS. Peptide purities were determined *via* analytical RP-HPLC and proved to be greater than 95%.

Generation of phospho-site-specific-Ser^{357} IRS-1 (p-Ser^{357}) antibodies. The free peptide AHRHRGpSSRLHPPLNHSRSI was coupled to keyhole limpet hemocyanin (KLH) by the glutardialdehyde method and was also synthesized as multiple antigen peptide (MAP) $(AHRHRGpSSRLHPPLNHSRSI)_8$-$(Lys)_4$-$(Lys)_2$-Lys-β-Ala-OH. The antiserum was obtained after repeated immunization of two rabbits with 1:1 mixture of the peptide-KLH and the MAP. This phospho-Ser^{357} antiserum was used to test the specificity of the antibody in the initial experiments (Fig. 1B and C).

Multistep immunoaffinity chromatography. The antiserum was purified by three step affinity purification. To achieve this 10 mg of each of the peptides AHRHRGSSRLHPPLNHSRSI (non-phosphorylated peptide I), AHRHRG-SpSRLHPPLNHSRSI (pSer358-peptide II), and the AHRHRGpSSRLHPPLNHSRSI (pSer357-peptide III) were coupled separately to 1 g of a CH-activated Sepharose (GE Healthcare, Freiburg, Germany) according to the manufactures instructions.

The antiserum was applied to the column (10 × 1 cm) loaded with the immobilized peptide I and recycled overnight at 4 °C. Afterwards, the serum was transferred to the column loaded with peptide II and again recycled overnight at 4 °C. Finally, the residual serum was affinity-purified on the immobilized peptide III overnight at 4 °C. After intensive washing with PBS, the antibody was eluted with 0.1 M glycine–HCl (pH 2.5) and neutralized with 1 M Tris–HCl pH 8.5. Appropriate fractions were collected and concentrated to 0.5 mg/ml on Amicon 20 kD ultra-filtration membrane. This immuno-purified-phospho-site-specific-Ser^{357} antibody was then used for all further experiments (Figs. 2 and 3).

Edman sequencing. The identity of peptide II and peptide III was confirmed by Edman degradation (Applied Biosystem, ABI 477). Both peptides showed the expected sequence and cross phosphorylation were not detected.

ELISA. The wells of microtiter plates (Nunc Brand Products, MaxiSorb surface, Wiesbaden, Germany) were coated with 5 μg of different peptides (listed in Table 1) in PBS in a final volume of 100 μl/well at 4 °C overnight. The plates were washed three times with 200 μl of washing buffer (PBS/0.05% Tween-20, pH 7.0) and blocked with blocking buffer (PBS/0.05% Tween-20, pH 7.0, containing 2% BSA) for 2 h at 37 °C. After washing, the plates were treated for 1 h at 37 °C with purified phospho-site-specific-Ser^{357} antibody (diluted in PBS/0.05% Tween-20, pH 7.0, containing 0.5% BSA) or the phospho-site-specific-Ser^{357} antiserum. After washing, the plates were incubated with HRP-conjugated goat anti-rabbit Ig (Dianova, Hamburg, Germany; 1:5000 diluted in PBS/0.05% Tween-20/0.5% BSA). 100 μl/well ABTS (2,2′-azino-bis/3-ethyl-benzthiazoline-6-sulfonic acid)/H_2O_2 in substrate buffer (citrate buffer 100 mM, pH 4.5) was added 100 μl/well and the color development analyzed at a wavelength of 405 nm [15].

Cell lines, reagents, and antibodies. C2C12 cells were from ATCC (Wesel, Germany). Baby hamster kidney cells, stably expressing the human insulin receptor (BHKIR), were kindly provided by R. Lammers (University Hospital Tuebingen). Oligonucleotides were synthesized by Invitrogen (Karlsruhe, Germany). Cell culture media, supplements, fetal calf serum (FCS) were purchased from Gibco (Eggenstein, Germany); the protease inhibitor mixture was product from Roche (Mannheim, Germany) and the antibodies against IRS-1 (C-terminus) from Upstate Biotechnology (Lake Placid, USA). The cytomegalovirus promoter-based expression vector for rat IRS-1 was described in [16].

Site-directed mutagenesis. Mutation of Ser^{357} of IRS-1 to alanine or glutamate, mutation of Ser^{358} to alanine and mutation of Ser^{357} and Ser^{358} to alanine was made by oligonucleotide-mediated mutagenesis. The mutagenic upstream primers used were IRS-1-Ala^{357} cc cac gcc cat cgg cat cga ggc gcc tcc agg ttg cac ccc cca ctc aac cac and IRS-1-Glu^{357} cc cac gcc cat cgg cat cga ggc gag tcc agg ttg cac ccc cca ctc aac cac, IRS-1-$Ala^{357/358}$ cc cac gcc cat cgg cat cga ggc gcc gcc agg ttg cac ccc cca ctc aac cac, IRS-1-Ala^{358} cc cac gcc cat cgg cat cga ggc agc gcc agg ttg cac ccc cca ctc aac cac with the wild-type IRS-1 expression vector serving as template. Positive clones were verified by sequencing.

Cell culture, transfection, and cell lysis. BHKIR cells were cultured in Dulbecco's modified Eagle's medium containing 25 mM glucose, 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. A minimum of 4.0×10^5 cells/well of a six-well plate were transfected using the $Ca_3(PO_4)_2$-DNA-co-precipitation method [17]. Cells were starved in Dulbecco's modified Eagle's medium (5.5 mM glucose) without fetal calf serum for 24 h and then stimulated with insulin (10 nM) or TPA (0.5 μM) as indicated. C2C12 cells were cultured in Dulbecco's modified Eagle's medium containing 25 mM glucose, 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. A minimum of 1.0×10^5 cells/well of a six-well plate were transfected using the $Ca_3(PO_4)_2$-DNA-co-precipitation method. Forty hours after transfection, the cells were starved in Dulbecco's modified Eagle's medium (5.5 mM glucose) without FCS for 3 h, and then stimulated with insulin (10 nM) or TPA (0.5 μM), as indicated. Cells were lysed with 200 μl of lysis buffer/well (50 mM Tris, pH 7.6, 150 mM NaCl, 1% Triton X-100, and containing protease and phosphatase inhibitors). Forty micrograms of protein of the total extracts were separated by SDS–PAGE (7.5%) and Western blot analysis was performed as described [8,18].

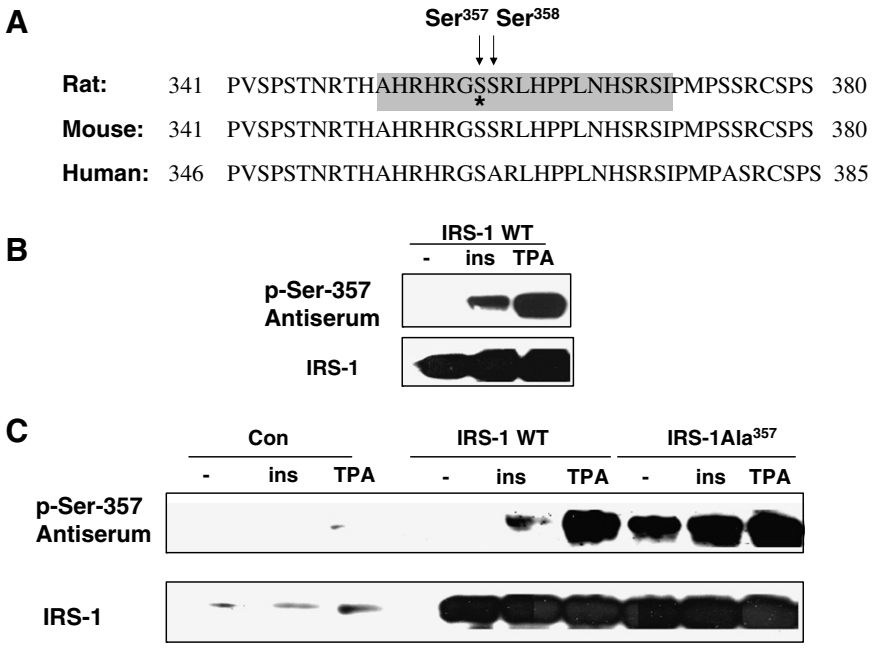

Fig. 1. Sequence alignment of the corresponding amino acid sequences around Ser^{357} and Ser^{358} of IRS-1 proteins from different specie and initial Characterization of the antiserum raised against phospho-Ser^{357}. (A) Sequence alignment of the corresponding amino acid around Ser^{357} for rat, mouse and human IRS-1 (amino acid numbers are indicated) is shown. Only the small region of rat IRS-1 containing the sequence AHRHRGpSSRLHPPLNHSRSI (antigenic peptide can be seen as shaded box, * shows phosphorylated residue) IRS-1 351–370 was used for generation of polyclonal phosphor-site-specific Ser^{357} antibody. (B) Characterization of the phospho-Ser^{357} antiserum in transfected C2C12 cells. Antibody characterization for phospho-Ser^{357} was performed in C2C12 cells transiently transfected with IRS-1 wild-type (WT) and stimulated with TPA and insulin for 30 min. The blot was reprobed to show the expressed IRS-1 protein levels. (C) C2C12 cells were transiently transfected with either IRS-1 wild-type (WT) or IRS-1 Ala^{357} and stimulated with insulin and TPA for 30 min. The blot was reprobed to show the expressed IRS-1 protein levels.

Statistical analysis. Results presented are derived from at least three independent experiments. Means ± SEM were calculated and groups of data were compared using Student's *t*-test. A *p*-value <0.05 was considered to be statistically significant.

Results and discussion

Initial characterization of antiserum raised against p-Ser^{357} showed cross reactivity with adjacent Ser^{358}

To identify novel Ser/Thr phosphorylation sites in IRS-1, *in vitro* phosphorylation assays were performed with the isolated N-terminal amino acid residues 2–304 and amino acid residues 265–522 of GST-IRS-1 protein fragments [8]. Upon incubation of recombinant IRS-1 fragments with various PKC-isoforms we identified Ser^{357} of rat IRS-1 (Ser^{362} in human IRS-1) as an *in vitro* PKC phosphorylation site (data not shown).

Next, we generated polyclonal phospho-site-specific antibodies (as described in Materials and methods) using a phosphopeptide sequence corresponding to the region of rat IRS-1 surrounding Ser^{357} (Fig. 1A). Previously, we and others [8,10,19] have successfully employed crude antiserum of the immunized rabbits for the detection of phospho-proteins. It was observed that phospho antibodies are more stable in the serum and show marked specificity for their respective phospho-proteins compared with their dephosphorylated versions. Therefore, in the initial experiments we used crude antiserum to test the specificity of the phospho-site-specific-Ser^{357} antibody.

Mice muscle cells, C2C12, overexpressing IRS-1 WT were stimulated with insulin or phorbol ester 12-*O*-tetradecanoyl 13-phorbol acetate (TPA), a pharmacological activator of classical and novel PKC-isoforms for 30 min (Fig. 1B). Before stimulation, antibody did not react with IRS-1, while insulin and TPA strongly stimulated phosphorylation of Ser^{357}, indicating that the antibodies recognize phosphorylation of Ser^{357}. To further verify that the antibody specifically recognizes phosphorylation of Ser^{357}, IRS-1 Ala^{357} mutant was generated by PCR site-directed mutagenesis, to prevent phosphorylation at Ser^{357}. C2C12 cells were transiently transfected with either IRS-1 WT or IRS-1Ala^{357} mutant and stimulated with insulin or TPA for 30 min. Surprisingly, the phosphorylation signal detected by antiserum was comparable between IRS-1 Ala^{357} and IRS-1WT expressing cells (Fig. 1C), indicating an unspecific binding of the antibody. This unspecific binding was possibly a cross reactivity with the adjacent Ser^{358} of IRS-1. Cross reactivity could be due to *in vivo* phosphorylation of Ser^{358}, as encountered by Sun et al. while developing anti-Bcr pSer-354 [20]. Moreover, we exclude the possibility of an exchange of the phosphate group between two serine sites during peptide synthesis, by performing Edman sequencing of the synthetic peptides. The results of Edman sequencing confirmed the identity of the peptides.

Since Ser^{358} had already been reported to be a functional site of IRS-1 we needed to eliminate the cross reactivity of the antibody with Ser^{358}, so that the single effect of Ser^{357} of IRS-1 in insulin signaling can be elucidated [21]. To address this problem we aimed to carry out immuno-purification of the antiserum raised against phosphorylation of Ser^{357} of IRS-1.

Immuno-purification of antiserum and determination of specificity of purified-phospho-site-specific-Ser^{357}antibody by indirect ELISA

The generated antibody was immuno-purified by multiple purification steps using non-phosphorylated Ser^{357}, p-Ser^{358}, and p-Ser^{357} peptide (as described in Materials and methods). To determine and compare the specificity and cross reactivity of the phospho-Ser^{357} antiserum and purified-p-Ser^{357} antibody, an indirect ELISA was performed using different phospho-synthetic peptides (Table 1). The results of indirect ELISA (Fig. 2A) revealed that the antibody specifically detected immunogenic phosphopeptide

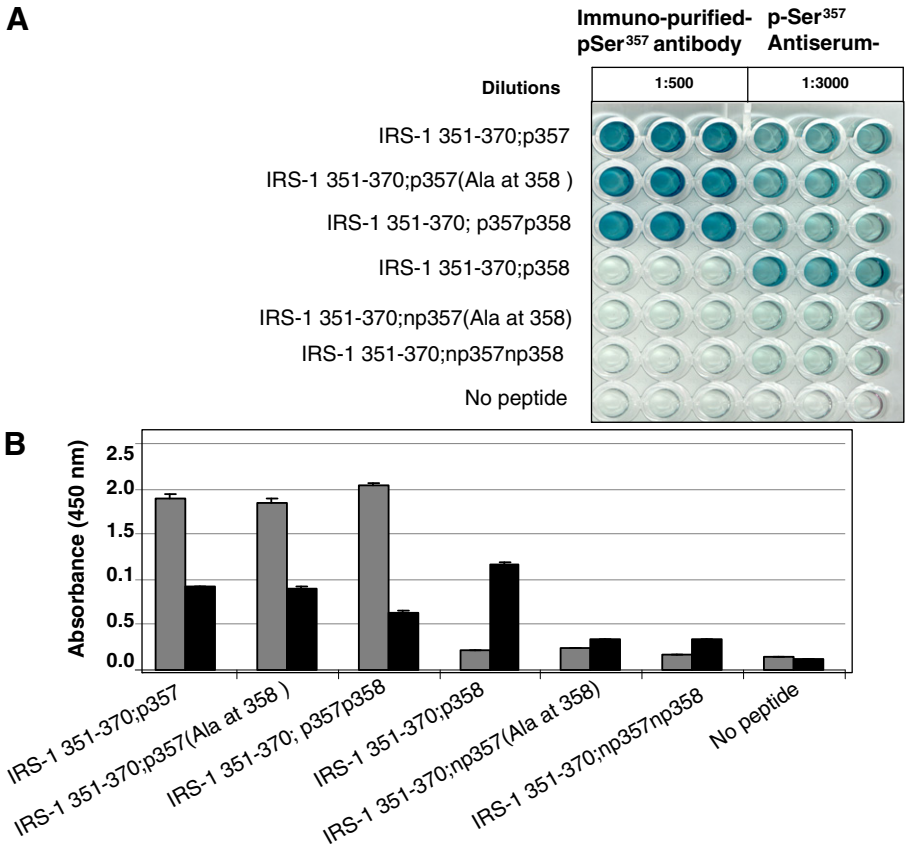

Fig. 2. Determination of specificity of polyclonal phospho-site-specific-Ser357 antibody by indirect ELISA. (A) The purified polyclonal-phospho-site-specific-Ser^{357} antibody specifically recognized immunogenic phosphopeptide (AHRHRGpSSRLHPPLNHSRSI) but not the non-phosphorylated form of the peptide or to other phosphopeptides. Synthetic peptides (5 μg) were incubated on an ELISA plate. Phospho-site-specific-Ser^{357} antiserum and purified phospho-site-specific-Ser^{357} antibodies were used for the detection, at dilutions of 1:3000 and 1:500, respectively. (B) Quantification of relative binding of each polyclonal antibody to the phospho-Ser^{357} of IRS-1 protein-based synthetic peptides (listed in Table 1) using the PEPSCAN-ELISA method.

(p-Ser^{357}) and shows a complete negative reaction towards non-phosphorylated form of the peptide or to other phosphopeptides (Fig. 2B) including the peptide phosphorylated at Ser^{358}.

Final determination of specificity of purified-phospho-site-specific-Ser^{357} antibody in living cells

Finally, in order to establish the specificity of the antibody against phosphorylation of Ser^{357} of IRS-1 in living cells, two more mutants of IRS-1 were generated; IRS-1Ala^{358} to prevent phosphorylation at Ser^{358} and a double mutant IRS-1Ala$^{357/358}$ to prevent phosphorylation simultaneously at both, Ser^{357} and Ser^{358}. BHKIR cells were transiently transfected with IRS-1 WT, IRS-1 Ala^{357}, IRS-1 Ala^{358}, and IRS-1 Ala$^{357/358}$ and stimulated with insulin or TPA for 30 min (Fig. 3A). The antiserum non-specifically recognized Ser^{358} phosphorylation upon stimulation with TPA and insulin in the cells overexpressing IRS-1 Ala^{357} comparable to the cells overexpressing IRS-1WT and IRS-1 Ala^{358} (Fig. 3A and B) .Whereas, with purified-phospho-Ser^{357} antibody very low signal of phosphorylation was detected in IRS-1 Ala^{357} and IRS-1 Ala$^{357/358}$ transfected cells, similar to the immunoblots obtained with control-transfected cells (Fig. 3B). The very low signal most likely depends on the endogenous IRS-1 in the transfected cells, which can be phosphorylated at Ser^{357}. These data evidently show that the immunopurified antibody specifically recognizes IRS-1, when it is phosphorylated on Ser^{357} without any cross reactivity with the adjacent putative phosphorylation residue Ser^{358}. Similar results have been obtained using C2C12 skeletal muscle cells (data not shown). Moreover, we also demonstrated *in vivo* phosphorylation of Ser^{357} using this immuno-purified-phospho-Ser^{357} antibody in our recent study [12] which further validate the specificity of the antibody. Additionally, we used double mutant IRS-1Ala$^{357/358}$ to confirm that the observed cross reactivity of the crude antiserum was against Ser^{358}. It is evident that a reduced phosphorylation signal was observed in IRS-1Ala$^{357/358}$ expressing cells, where phosphorylation at both sites is blocked, as compared to IRS-1 Ala^{357} where phosphorylation at only Ser^{357} is prevented (Fig. 3A upper panel). The very low signal in IRS-1Ala$^{357/358}$ expressing cells most likely depends on the endogenous IRS-1 in the transfected cells, which can be phosphorylated at Ser^{357}. These results support the conclusion that cross reactivity shown by the antiserum was against Ser^{358} phosphorylation.

The advantage of phospho-site-specific antibodies is that they permit rapid analysis of intracellular phospho-proteins without the need to employ complex structural protein analysis [20]. Generation of phospho-Ser-specific antibodies of IRS-1 provides a powerful tool to study regulatory function of Ser/Thr phosphorylation of IRS-1 in insulin signal transduction *in vivo*. The strength of our study is the generation and establishment of new phospho-site-specific-Ser^{357} antibodies, in the presence of adjacent Ser^{358}. We emphasize here that phospho antibodies require proper validation to determine that they do not cross react with adjacent or nearby serine residues. To achieve this goal, immuno-purification and validation of immunoreactivity of the phospho antibody with mutants of the serine site in question and adjacent sites is recommended, otherwise it can lead to erroneous interpretation of the data

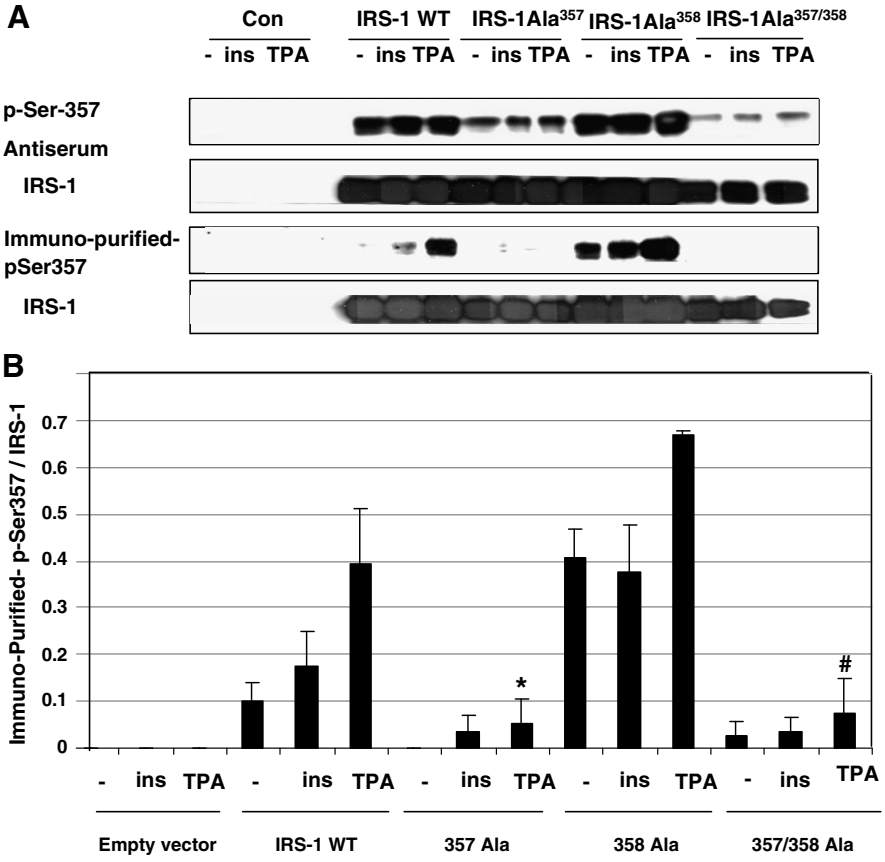

Fig. 3. Specificity of phospho-Ser^{357} antibodies in cultured cells. (A) Baby hamster kidney cells stably expressing the human insulin receptor (BHKIR-cells) were transfected with IRS-1 WT, IRS-1 Ala^{357}, IRS-1 Ala^{358}, and IRS-1 Ala$^{357/358}$ and treated with insulin (10 nM, 30 min) or TPA (0.5 μM, 30 min). After stimulation cells were lysed and analyzed by 7.5% SDS–PAGE and immunoblotted with phospho-Ser^{357} antiserum and immuno-purified-phospho-Ser^{357} antibodies. The same blots were stripped and reprobed with a polyclonal IRS-1 antibody. Representative results from three experiments are shown. (B) Phosphorylation intensity of Ser^{357} of IRS-1 detected by immuno-purified-phosho-Ser^{357} antibody was quantified based on scanning densitometry of immunoblots normalized for IRS-1 protein (means ± SE, $n = 3$, $^{*}p < 0.05$ IRS-1 Ala^{357} mutants vs. IRS-1 WT; $^{\#}p < 0.05$ IRS-1Ala$^{357/358}$ mutant vs. IRS-1 WT).

Table 1
Sequence of synthetic peptides

Peptide	Sequence
IRS-1 351–370; p357	AHRHRG+SRLHPPLNHSRSI
IRS-1 351–370; p357(Ala at 358)	AHRHRG+ARLHPPLNHSRSI
IRS-1 351–370; p357p358	AHRHRG++RLHPPLNHSRSI
IRS-1 351–370; p358	AHRHRGS+RLHPPLNHSRSI
IRS-1 351–370; np357(Ala at 358)	AHRHRGSARLHPPLNHSRSI
IRS-1 351–370; np357np358	AHRHRGSSRLHPPLNHSRSI

Plus (+) sign indicates a phosphorylated serine; p, phosphorylated; np, non-phosphorylated.

[20,22]. The proposed strategy accurately demonstrates the specificity of our generated antibody and rules out the likelihood of cross reactivity with adjacent sites. This is especially important in case of IRS-1 proteins because IRS-1 contains a number of potential serine phosphorylation residues and each serine phosphorylation site has a distinct and regulatory role in insulin signaling. Therefore, based on frequent side by side potential phosphorylation sites within IRS-1, we suggest that the generation of phospho-site-specific antibodies for IRS-1 in particular and other proteins in general require a particular preparation strategy.

The prepared phospho-Ser^{357} IRS-1 antibody is remarkably specific for the phosphorylated Ser^{357} epitope and works effectively in common applications such as western blot analysis and immunoprecipitation. Therefore, pSer^{357} IRS-1 antibody should facilitate efforts to further elucidate the *in vivo* inhibitory and regulatory role of PKC-induced Ser^{357} phosphorylation in insulin signal transduction and will be quite useful in better understanding the molecular basis of insulin resistance.

Acknowledgments

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