Cross-regulation of tyrosine kinase based signalling by serine/threonine kinases and their role in the pathomechanism of type II diabetes

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Final report

by András Zeke, MD PhD

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Introduction and aims of the project

Type II diabetes: a global burden of the developed countries

Although the 21th century has often been hailed as the “age of biology”, many significant medical challenges remain. Having defeated most of the infectious diseases and malnutrition, societies of the developed world are increasingly falling victim to conditions associated with the “western” lifestyle. As such, the incidence of cardiovascular diseases and diabetes has risen sharply between 1990 and 2020. In 2015, approximately 8.7% of population in the USA (more than 27 million people) suffered from diabetes [1]. Woldwide, it has been estimated that almost 425 million people suffer from this condition [2]. Most predictions agree that in the upcoming decades, the number of type II diabetes patients will continue to rise across the globe

Type II diabetes is a metabolic disease and essentially triggered by overweight and obesity in the absolute majority of cases. It is generally agreed that fat cells produce a number of substances (mostly cytokines) that cause insulin resistance in peripheral tissues. As insulin signalling becomes less and less efficient, the pancreas beta cells are forced to produce an increased amount of insulin to maintain the same level of blood sugar. Eventually, beta cells will become exhausted: this happens especially rapidly if their secretory capacity is inherently low, as genome-wide association studies (GWAS) show [3]. Finally, a fully-blown diabetes manifests itself, that might require lifelong administration of insulin. However, many different types of medicines have been developed to treat early-stage diabetes as well. Generally referred to as oral antidiabetics, they encompass insulin secretagogues (e.g. sulphonylureas), DPP4 protease inhibitors (“gliptins”), PPARγ receptor agonists (thiazolenediones) and other insulin sensitizers (e.g. metformin) or anti-glucosurics (“glifosins”), to maintain the metabolism of patients. However, there is not much evidence that these agents are efficient in reverting the manifest disease itself. Therefore there is a clear medical need for agents that could act in early-stage impaired glucose tolerance. But without deeply understanding the molecular-level details of type II diabetes, there can be little hope for novel, more efficient medicines. While an extensive research has been devoted to diabetes for almost 100 years, many critical linchpins are still missing from disease models. This is especially obvious at the molecular level, where mechanisms of insulin receptor desensitization are still largely unexplained. While the earlier studies tended to focus at cell-level or even organism-level pathomechanisms, there is an obvious need for more exact, biochemically sound, almost atomic-level diabetes models. My current project had exactly such a purpose: To decipher more of those molecular regulatory mechanisms, that act at the IRS1/2 (insulin receptor substrate protein) level.

Insulin signalling pathways in a nutshell

Insulin receptor signaling has been studied extensively in the last decades, thus we have very precise knowledge of the main molecular events after insulin receptor (InsR) engagement (see **Figure 1**). This dimeric tyrosine kinase is known to suffer a considerable conformation change upon extracellular ligand engagement, allowing for its intracellular, catalytic domains to allosterically activate each others, leading to activation loop tyrosine phosphorylation and stabilization of catalytic activity. InsR kinase domains then phosphorylate the juxtamembrane segment of the receptor (at an NPxY motif), leading to recruitment of insulin receptor substrate (IRS) proteins to this membrane-adjacent motif. While ISR1 and ISR2 are both major components and signalling hubs of insulin receptor pathway, the role of the more divergent IRS4 appears to be less critical. IRS proteins contain an array of phosphorylatable YxxM motifs that serve as anchor points for the phosphatidyl-inositol-3-kinase (PI3K) p85 regulatory subunits. The latter protein carries two, tandem SH2 domains that mediate rapid and high affinity binding to tyrosine

phosphorylated IRS proteins. In addition, engagement of the C-terminal SH2 domain of p85 also regulates catalytic activation of PI3K and generation of 3-phosphorylated phosphatidyl-inositol lipids in the membrane. These lipids not lead to recruitment of PDK1 and AKT1 protein kinases into the membrane and their subsequent activation, but also increase recruitment of IRS proteins (through their PH domains) into the membrane, as a positive feedback loop. The PDK1-activated AKT kinases are the main effectors of this pathway, phosphorylating both important transcription factors (such as FOXO-family proteins) as well as the exocytosis-regulating AS160 protein, controlling GLUT4 glucose transporter exocytosis. As a minor arm of the inculin receptor pathway, Ras small G-proteins and ERK1/2 kinases are also activated, through recruitment of GRB2 to phosphorylated IRS proteins (at YxN motifs). However, the latter event is mostly considered dispensable for insulin-dependent metabolic regulation.

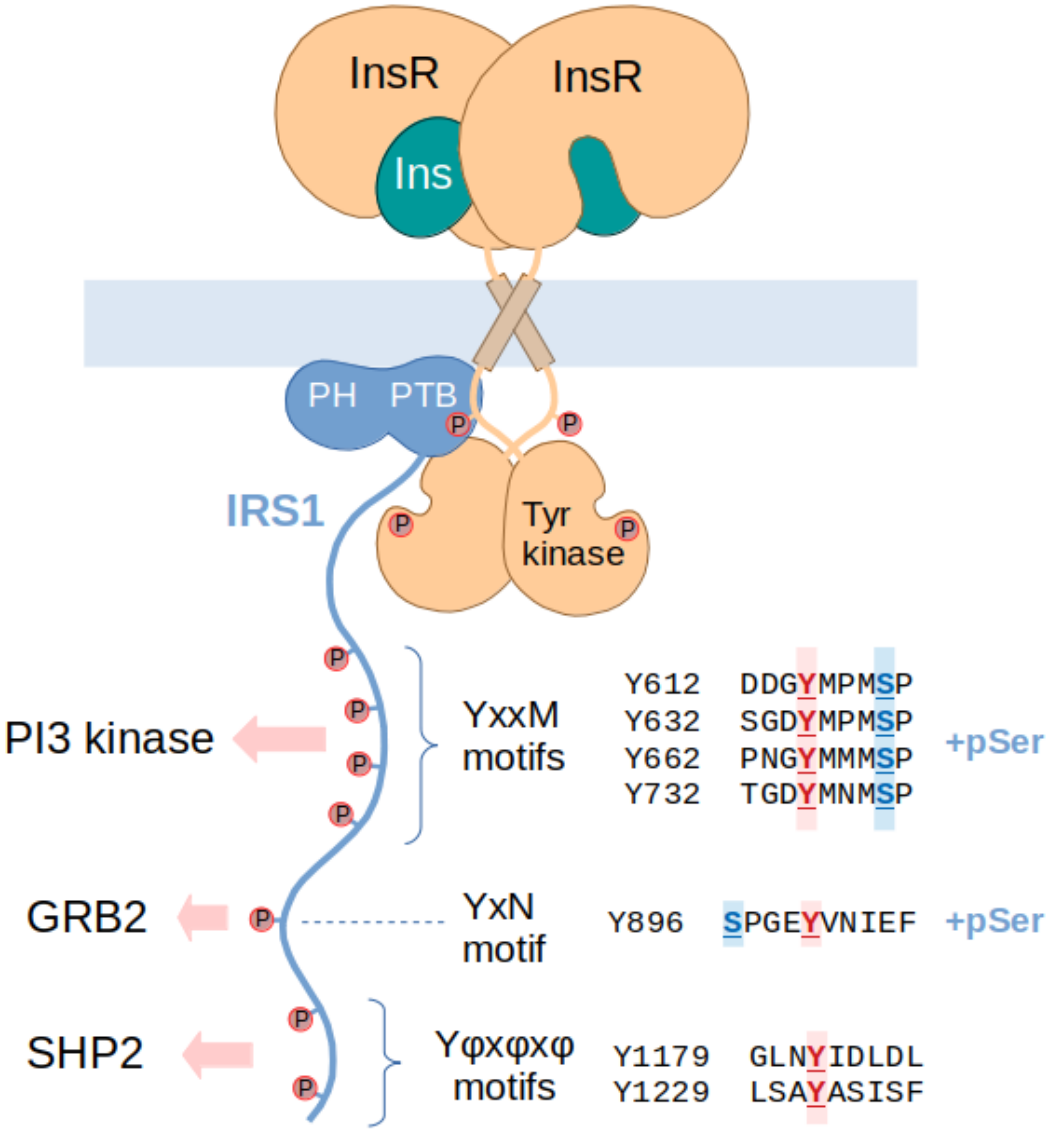


Figure 1: Schematic representation of the insulin receptor complex, with insulin (Ins, turquoise), insulin receptor (InsR, orange) and insulin receptor substrate 1 (IRS1, blue) together. Different phosphotyrosine motifs of IRS1 attract different targets, including PI3K, GRB2 and SHP2. Many of them can also be modulated by Ser/Thr kinases through adjacent phosphorylation sites (+pSer).

Negative regulation of insulin signalling and the role of IRS serine phosphorylation

Despite our extensive knowledge about insulin signalling, side pathways, and crucially, negative regulation are substantially less well understood. Protein tyrosine phosphatases and ubiquitin ligases are thought to be the most important to abolish insulin signaling at the receptor and/or the IRS level. On the other hand, IRS1 and IRS2 proteins were also found to be extensively phosphorylated on various serine residues, conferring an altered (mostly decreased) insulin sensitivity [4, 5, 6, 7]. However, the exact biochemical connection between serine phosphorylation and core insulin signaling events is poorly known. Since almost all of these phosphorylation events happen on the huge, disordered C-terminal segment of IRS proteins, previously proposed allosteric effects on phosphotyrosine binding (by the N-terminal PTB domain) can safely be excluded. However, a simple evolutionary conservation analysis points to them being part of linear motifs (**Figure 2A**). A careful alignment of vertebrate IRS1 and IRS2 proteins show that many of these well-established Ser phosphorylation sites are part of conserved RxRxxS[STAP] motifs, that match the phosphorylation consensus of AGC-family protein kinases (AKT, PKC, p70RSK, etc.). They also strongly resemble 14-3-3 protein binding sites, due to the small amino acid following the phosphoserine [8]. Another, even more characteristic subset of serine phosphorylation sites directly follows the YxxM tyrosine phosphorylation sites, forming a joint consensus YxxMSP. These sites are likely targeted by proline-directed protein kinases, such as MAPKs (like JNK, that has a specific, conserved recruitment site on IRS-family proteins) or the mTOR kinase. Since many proline-directed kinases (especially JNK) can also be activated by cytokine pathways, the pTyr-flanking pSer sites are potentially linked to cross-regulation of insulin signalling by inflammatory cytokines [9]. As we already know that low-level inflammation can cause insulin resistance and it is one of the main culprits in type II diabetes, these pSer sites are of a prime interest. In our current study, we exclusively focused on the latter IRS1 (and IRS2) phosphorylation sites flanking pTyr residues (YxxMSP motifs). IRS1 alone carries four different motifs of this type; and three of them are also found in the related paralog IRS2.

Correlation between YxxMSP motifs and SHP2 recruitment in IRS proteins

Evolutionary conservation analyses show that the dually-phosphorylated YxxMSP motifs have already emerged in non-vertebrate chordates (e.g. in the single IRS protein of the lancelet *Branchiostoma floridae*, see **Figure 2 B** and **C**). The evolutionary branching pattern of vertebrate-specific IRS paralogs indicate that while IRS1 and IRS2 faithfully preserves most of these ancestral motifs, they are missing from the third paralog, IRS4 [10]. The loss of all 4 pTyr-flanking Ser-Pro (SP) sites suggest that whatever this regulatory system is, it is selectively and completely missing from IRS4. The latter, divergent IRS protein also lacks another pair of important, conserved motifs from IRS1/2: the C-terminal SHP2-binding motifs. These linear motifs are responsible for the tyrosine-phosphorylation-dependent recruitment of the SHP2 tyrosine phosphatase to the IRS1 and IRS2 proteins (through the tandem SH2 domains of SHP2). However, as SHP2 is not required for metabolic regulation by insulin receptors, its exact role is still quite obscure (other than being a highly conserved IRS partner in most multicellular animals). SHP2 is a “picky” phosphatase that selectively dephosphorylate a limited number of substrates, thereby enhancing cell division and oncogenic transformation of cells. Therefore it is in stark contrast to most other Tyr phosphatases, acting as more generic negative regulators of tyrosine kinase pathways (including cytoplasmic tyrosine phosphatases, like PTP1B, or receptor-type phosphotyrosine phosphatases, e.g. PTPRε).

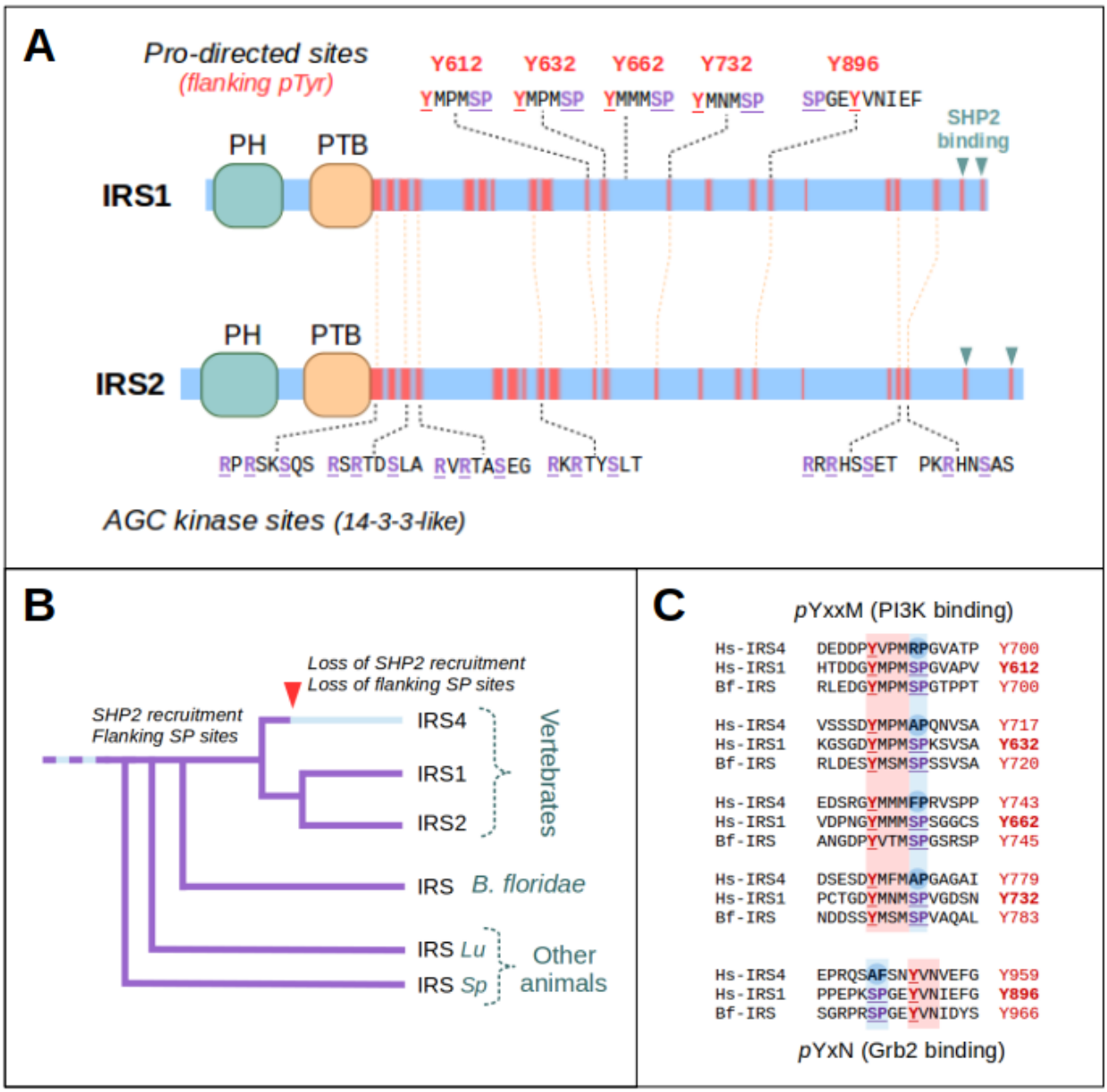


Figure 2: Conserved proline-directed Ser phosphorylation sites on both IRS1 and IRS2 proteins, flanking important phosphotyrosines (**A, upper side**) and other conserved Ser phosphorylation sites targeted by AGC type kinases (PKA, PKC, AKT, S6K and their ilk) (**A, lower side**). Matching motifs between human IRS1 and IRS2 are indicated by red bars and dotted lines. The evolutionary tree of vertebrate, chordate (*Branchiostoma floridae* lancelet) and other animal (*Lingula unguis* brachiopod, *Stylophora pistillata* coral) IRS proteins (**B**). Vertebrate IRS4 proteins appear to have lost all phosphotyrosine-flanking SP sites, at roughly the same time they lost the SHP2 binding motifs, hinting at a functional linkage between these two features. The lack of these SP sites in IRS4 is clearly secondary, as indicated by the fact that the single *Branchiostoma floridae* IRS protein (prior to divergence of IRS1, IRS2 and IRS4) already carries all these regulatory SP sites (**C**).

Our goals: Identifying molecules that mediate the effect of Ser phosphorylation on IRS1
Since the interactome of IRS1/2 proteins has been studied extensively, and even the tyrosine-phosphorylation-dependent interactors have been explored, it is a reasonable idea that proteins mediating the effect of pSer modifications, especially those adjacent to pTyr sites are already among the known partners of IRS1 [11]. Our goal with current project was to take these pTyr-dependent interacting molecules one-by-one, and try to identify those, whose binding or catalytic activities are profoundly altered by the presence of an additional phosphate group nearby. This prior knowledge of interactors entirely justified a "low-throughput" approach, listing, cloning, and analyzing these proteins one-by-one. The first explorative measurements were to be done using the fluorescence polarization method mostly with recombinant proteins cloned, expressed and purified in-house. Hits were then to be examined more thoroughly, with respect to their in vitro properties, as well as cell-based experiments. Finally, I planned to explore if any detected mechanism could be generalized to other proteins beyond IRS1. Besides, we attempted to gain atomic-level information about these effector molecules. These bits of date were aimed at exploring the possibility, if these effector(s) are useful as pharmaceutical targets. Since Ser phosphorylation of IRS1/2 is considered to be part of a powerful negative regulatory system desensitizing insulin signalling, I hoped to uncover therapeutically useful targets.

Planned and actual activities during the course of the project

Planned schedule:

- 1st year: In vitro studies using purified recombinant proteins, target identification
- 2nd year: Continuation of in vitro studies, establishment of cell-based assays
- 3rd year: Finalization of in vitro results, structural & bioinformatic studies

Actual progress:

- 1st year: Cloning & production of all relevant recombinant proteins, affinity and activity assay establishment. Biologically relevant target not yet encountered
- 2nd year: In vitro target identification successful (using extra hypotheses not included in the original plan); studies were re-focused at tyrosine phosphatases, most notably SHP2
- 3rd year: Phosphatase - tandem phosphopeptide complex successfully crystallized, molecular mechanisms generalized and clarified. Cell-based GLUT4 assay development unsuccessful. Publication draft prepared.

Project funding and use of resources
The PD-OTKA grant provided salaries for the lead scientist only. Other scientists (Péter Sok, Péter Egri, Krisztina Németh and Ádám Póti) also helped, with a very low amount of working hours allocated to this project. My MSc student Tamás Takács also aided in achieving these results. As research materials, reagents and other expenses were not covered by the PD-OTKA grant, they were provided directly by the research institution (Research Centre For Natural Sciences) and the Hungarian Academy of Sciences.

Detailed research results and their scientific impact

Selecting candidates for initial testing

As detailed in the introduction, we had the following initial assumptions of the putative effector of IRS1 serine phosphorylation events next to pTyr sites: (1) This protein is an already-known interactor of IRS1, (2) it binds to the pTyr residue and (3) its affinity towards Tyr phosphorylated IRS1 is altered in the presence of the additional pSer modification. In accordance with these properties, my initial set of candidates included the phosphatidyl-inositol 3 kinase (PI3K), the insulin receptor kinase (InsR) itself, the tyrosine phosphatase PTP1B as well as the ubiquitin ligase suppressor of cytokine signalling 3 (SOCS3). Interacting domains of all these proteins were cloned from a human HEK293-T cDNA pool (courtesy of Anita Alexa), inserted into modified PET vectors encoding a His6 tag (pBH4) or an MBP+His6 tag (pET-MBP). All proteins could successfully be expressed in E. coli (T1 phage resistant BL21-DE3 strain and/or Rosetta strain), except of the insulin receptor kinase domain. Since the latter could only be produced in Sf9 cells with a poor yield, we decided to buy it in an activated, GST-tagged form for later experiments (from SinoBiological Inc). I have produced and purified all the other proteins using affinity chromatography (Ni-NTA) and ion exchange chromatography (Äkta explorer equipped with a Resource “Q” column). Protein quality was routinely assessed using SDS-PAGE.

Fluorescence polarization based assays for affinity comparisons

First, I set up an assay to detect the effect of Ser phosphorylation on the affinity of these proteins and measured their affinity to “singly phosphorylated” versus “doubly phosphorylated” model peptide pairs. Due to technical reasons (stability and solubility), out of the four tandem motifs of IRS1, the second one was chosen as the basis of our model system (model #2, M2 peptide). This 15-amino acid peptide stretch (GRKGSGD{pTyr}MPM{pSer}PKS) incorporated the Tyr632 and Ser636 phosphorylation sites of human IRS1 (internal name: ppM2 peptide). These sites are among the most highly phosphorylated ones in IRS1 according to the PhosphoSitePlus database and literature data [4]. To be able to use the fluorescence polarization method, the doubly phosphorylated peptide had to be labelled with a carboxyfluoresceine (CF) moiety. First, we synthesized an N-terminally labeled CF-ppM2 peptide, and attempted to use it as a reporter. Unfortunately, it gave a rather small polarity change in titrations, presumably because of the large distance from last surface-bound amino acid. Thus we had to change our labelling strategy. The C-terminally labelled CF-ppM2 peptide as well as the unlabelled, dually phosphorylated ppM2 and singly phosphorylated p0M2 peptides (pTyr only) were ordered from GeneScript Inc. These peptides made it possible to assess binding affinity increase of protein partners upon having an extra Ser phosphorylated side chain. Fluorescence polarization (FP) assays were always performed in at least triples; the direct titration was done with changing protein concentration against CF-ppM2 (the reporter peptide). The dually-singly phosphorylated unlabelled pairs were subsequently used in competitive FP titrations with fixed CF-ppM2 and protein concentrations. Whenever possible, the titrations were fitted by exact equations representing a 1:1 protein-peptide complex (in Origin).

PI3 kinase binding is unaffected by flanking serine phosphorylation sites

One of the first major results obtained from these initial titrations was the observation that PI3K – the main effector of IRS1 and IRS2 tyrosine phosphorylation – is insensitive to serine phosphorylation events nearby the pTyr residue (**Figure 3**). In our titrations, the N-terminal and C-terminal SH2 domains of the p85 protein (the regulatory subunit of PI3K) were measured separately, due to analytical reasons (to make the titration stochiometry 1:1). While these domains

had very different affinities to the nSH2 and cSH2 domains of PI3K (with cSH2 binding much tighter to the model peptide), it did not change much between the doubly and singly phosphorylated peptides. With this result, I could safely discard earlier claims (based on cell biologic studies) that Ser phosphorylation of IRS1 on these sites would decrease PI3K binding [12]. Whatever mechanism has led to detecting less PI3K in immunoprecipitation with IRS1, was not due to reduced binding. Structural analysis of the SH2 domains of PI3K (PDB codes for nSH2: 5GJI, cSH2: 5GAUL) has also suggested that the pSer residue was neither clashing, nor contacting the protein surface, reinforcing our conclusions.

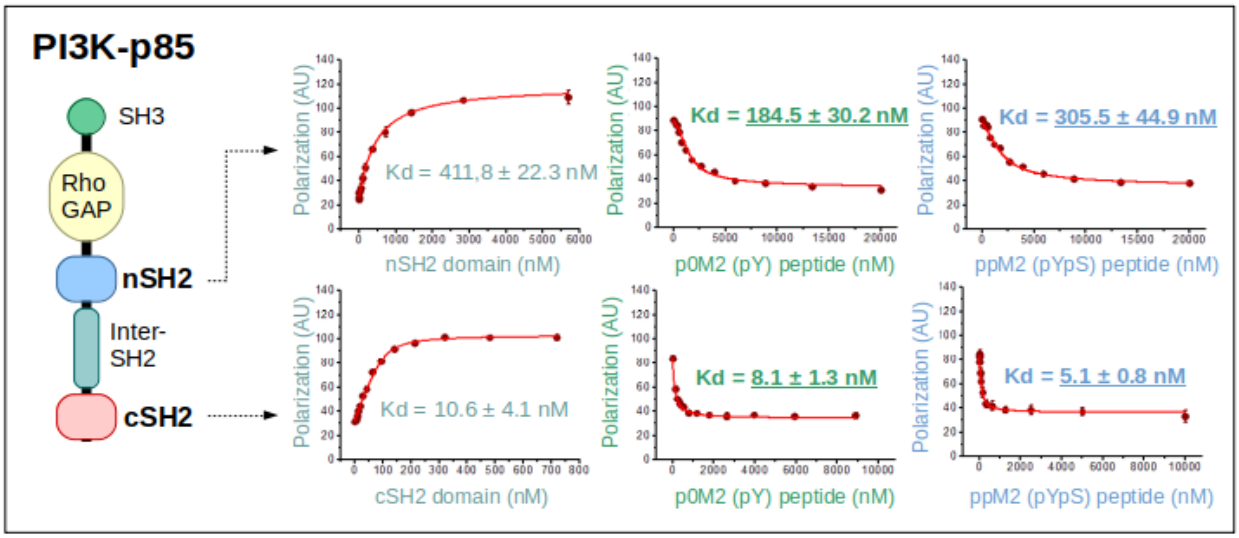


Figure 3: Fluorescence polarization (FP) titrations with isolated SH2 domains of the p85 regulatory subunit of phosphatidylinositol-3-kinase (PI3K). The direct titration (first column) as well as competitive titrations (second and third columns) indicate that albeit cSH2 and nSH2 bind the same peptide (pY632-IRS1) with different affinities, the effect of Ser phosphorylation is negligible.

Insulin receptor kinase is relatively insensitive to flanking Ser phosphorylation

As our next step, we also assessed the ability of the insulin receptor tyrosine kinase (InsR) to phosphorylate substrates with or without a “guide” phosphoserine residue. The idea of these studies was suggested by earlier publications on InsR substrate recognition [13]. For this purpose, a separate set of substrate peptides was synthesized, one carrying a pSer residue, and another completely unphosphorylated (also representing the Tyr632 - Ser636 region of human IRS1). For the activity assays (phosphorylation or dephosphorylation), we set up a novel analytical method, with the help of Krisztina Németh. Reactions were done in minimal buffers and samples were immediately run on capillary electrophoresis (CE) at pre-determined time points. As the peaks of phosphorylated and unphosphorylated peptides could easily be separated with CE, integration of peaks provided us quantitiative kinetic data. We typically used substrate decrease instead of product increase for quantitation (due to treshold/sensitivity issues) Unfortunately, the activity difference of InsR between the unphosphorylated and pSer pre-phosphorylated peptide were small in all concentrations tested. Therefore we had to conclude that InsR is unlikely to mediate the effect of serine phosphorylation, if the latter is located on the C-terminal side of the tyrosine residue.

Despite these negative results, structural studies on the InsR kinase domain suggested that the residues lying N-terminally from the Tyr to be modified are typically negatively charged to match opposing charges on InsR. While these are typically glutamic or aspartic acid residues in substrates, a similar role for the phosphoserine could not be excluded by us. The Tyr896 site of human IRS1 is flanked N-terminally by a well-established, conserved serine phosphorylation site (Ser892). Therefore we re-focused our efforts on the latter module (HPPEPK{pSer}PGE{pTyr}VNIEFGS). Called internally as the M+ peptide, we prepared an unphosphorylated variant in-house, and ordered a matching, serine phosphorylated version to be synthesized as well. With this new pair, we measured the phosphorylation reaction by InsR again. However, the kinase displayed very limited effects on the latter system as well (maximum ~2-fold difference at reaction rates). To sum up: in spite of the matching surface charge densities, a critical role for serine phosphorylation in modulating InsR activity could not be established.

Tyrosine phosphatases are positively modulated by flanking Ser phosphates

Having discarded the tyrosine kinase and its effector molecules from our list of pSer-modulated targets, we turned to miscellanous negative regulators involved in insulin signalling. First, I tested the ubiquitin ligase SOCS3, that was reported to bind (alongside with its relative, SOCS1) to IRS1. SOCS3 was chosen because of its uniquely highly positively charged SH2 domain, as well as its reported sensitivity to doubly tyrosine phosphorylated epitopes [14]. However, the purified, MBP-tagged recombinant SOCS3 SH2 domain completely failed to bind to our CF-ppM2 reporter peptide (results not shown). Therefore we directed our attention to tyrosine phosphatases instead of ubiquitin ligases. The well-known “reader-writer-eraser” model suggested that if a modification of the substrate neither affects the “writer” (the tyrosine kinase), nor the reader (the effector protein, in our test system, the PI3 lipid kinase), then it must alter the function of the “eraser” (the tyrosine phosphatase) in order to generate a biological effect. Testing the binding abilities of PTP1B to our model peptides lended credence to this hypothesis: We could detect an approximately ~17-fold enhanced binding to the inactive, recombinant PTP1B phosphatase domain upon introduction of the serine phosphorylation. This result gave us the first hint where the effectors of Tyr-flanking Ser phosphorylation events should be searched for: among tyrosine phosphatases.

The tyrosine phosphatase SHP2 uniquely strongly relies on Ser phosphorylation

After the discovery that the binding of PTP1B to key IRS1 tyrosine phosphorylation sites is affected by nearby phosphoserines, we sought for other phosphatases that could be similarly affected. Literature data suggested that – although PTP1B and the very closely related TCPTP are the main phosphatases implicated in InsR and IRS1 dephosphorylation, other phoshatases also play a role [15]. SHP2, an evolutionarily very conserved partner of IRS proteins could also play similar roles under certain conditions; and a distantly related receptor tyrosine phosphatase, PTPRε was also found to be an important regulator of insulin signalling [16]. We also aimed to test the atypical tyrosine phosphatase LMW-PTP, but could neither obtain a working clone, nor amplify it from our cDNA pool. However, SHP2 (courtesy of Anita Alexa) and PTPRε were successfully cloned, mutated to inactive forms and had their isolated phosphatase domains expressed recombinantly. Testing of these phosphatases (alongside with the previously mentioned PTP1B) revealed a varying dependence on flanking site serine phosphorylation. While PTPRε was barely sensitive to the latter modification, PTP1B displayed a modest, and SHP2 an extensive dependence on pSer sites. Binding assays with catalytically inative (Cys to Ser mutated) SHP2 phosphatase domains revealed an almost 50-fold affinity increase upon introducing the phosphoserine in addition to the phosphotyrosine residue (see **Figure 4**).

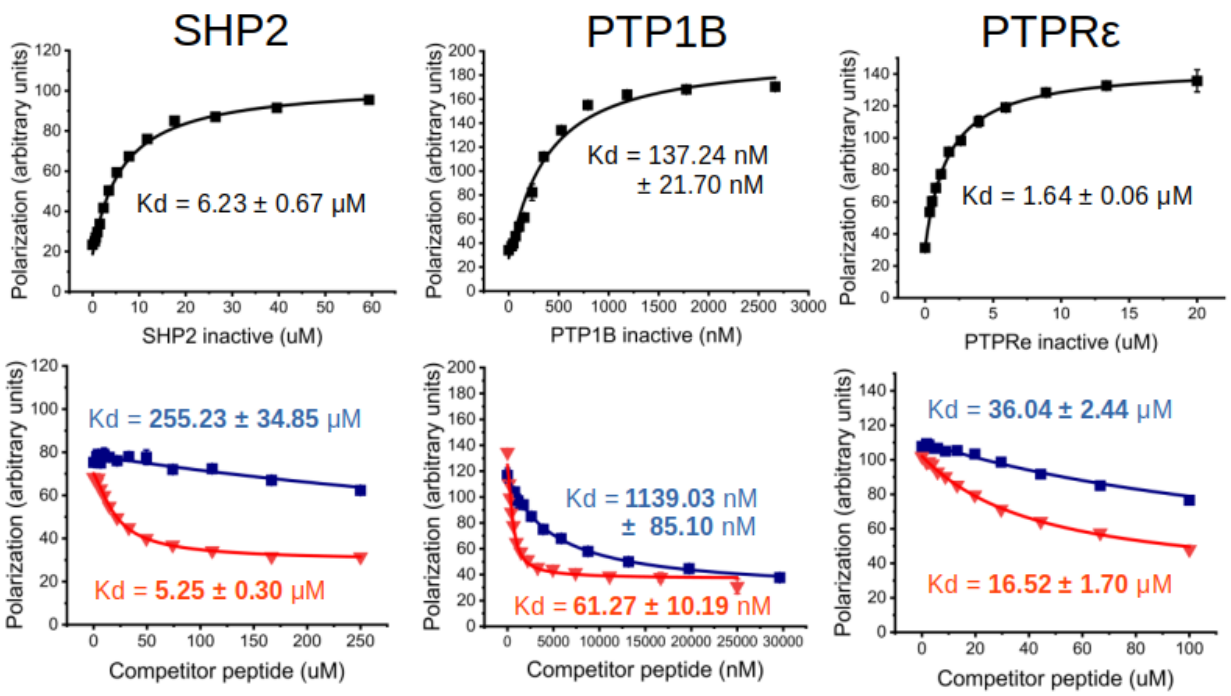


Figure 4: Affinity measurements between inactive (Cys->Ser) mutant tyrosine phosphatase domains and IRS1 phosphopeptides. The **upper lane** indicates the direct fluorescence polarization titrations against a CF-labelled pYpS-IRS1 peptide (Y632+S636), while the **lower lane** shows competitive titrations against the matching unlabelled pTyr or pTyr+pSer peptides. The effect of serine phosphorylation is absolutely major on SHP2 binding, while smaller on PTP1B. The last examined phosphatase, PTPRε does not seem to be affected by the presence of pSer.

To validate these results, we turned to a capillary electrophoresis based dephosphorylation assay (**Figure 5 A** and **B**). The dephosphorylation rates of PTPRε were completely identical on the singly and doubly-phosphorylated peptides. PTP1B, on the other hand, did show a certain difference, albeit not at high substrate concentrations. We suspected that – due to the unusually tight binding of PTP1B to the substrate – the system might get saturated easily. Therefore, a separate kinetic assay was developed for PTP1B. In this method, the dephosphorylation reaction took place at very low substrate concentrations (from 50 to 500 nM) in Falcon tubes. Reaction was stopped by heat inactivation (70C for 10 min) and tubes were immediately frozen on liquid nitrogen and liophilized. Samples were subsequently reconstituted in 40 ul doubly distilled water, and run on capillary electrophoresis, along with standards. To validate that PTP1B has been thoroughly heat inactivated, we tested the activity of samples againts the small-molecule chromogenic substrate DIFMUP. While native PTP1B was highly active on DIFMUP even after excessive dilutions, the reconstituted samples displayed no activity. This assay showed that the dephosphorylation kinetics of PTP1B differs on the two substrates, although not as much as the binding affinity difference would suggest.

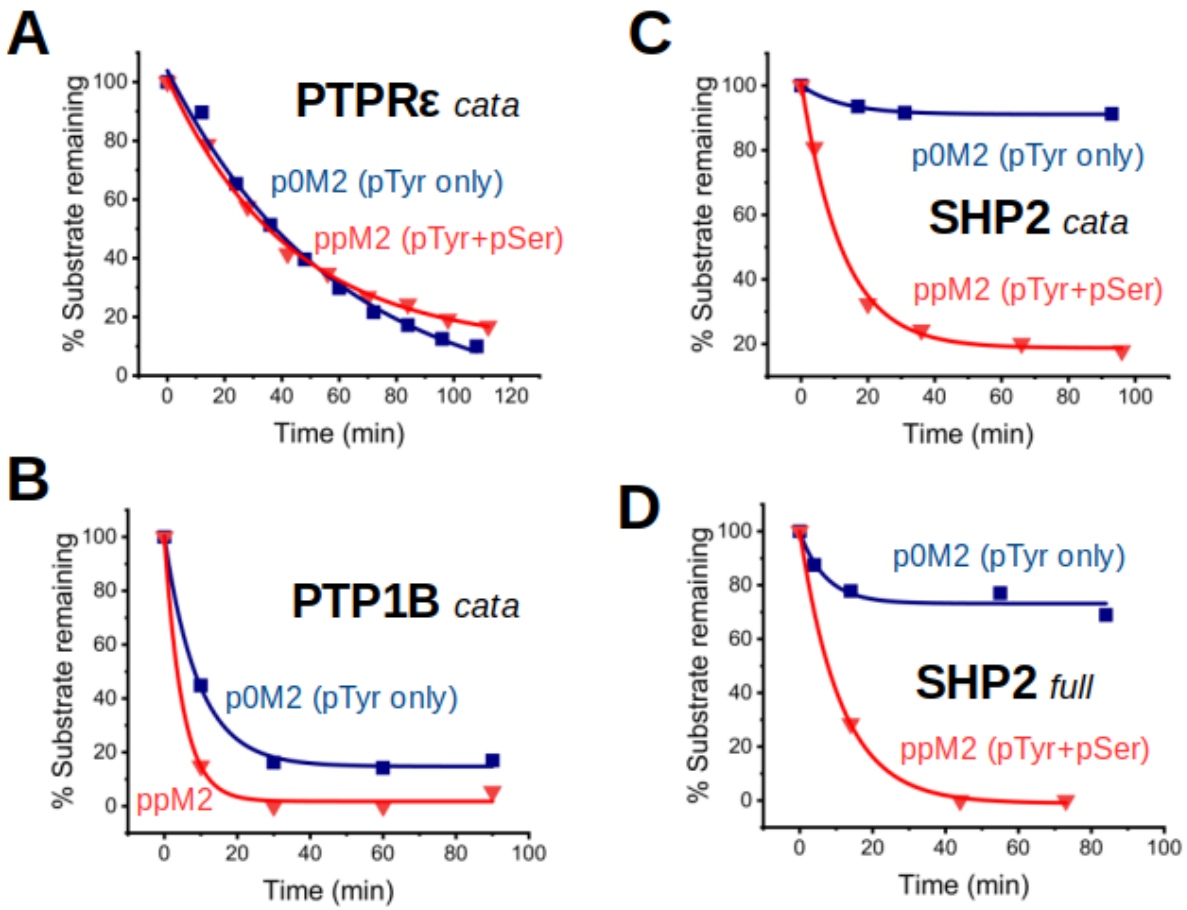


Figure 5: Sample activity assays with either isolated tyrosine phosphatase domains (SHP2, PTP1B, PTPRε), or full-length phosphatases (SHP2) carrying activating mutations. While the activity of PTPRε is similar on a dually-phosphorylated (pTyr+pSer) IRS1 peptide (**A**), PTP1B does show a modest activity enhancement by the flanking pSer on its pTyr substrate (**B**). The catalytic domain of SHP2 - on the other hand - only accepts the dually-phosphorylated peptide as its substrate (**C**). The same phenomenon can be seen with the full-length SHP2 protein, showing that the effect is not due to protein truncation (**D**).

Finally, the isolated, catalytically active phosphatase domain of SHP2 was also tested in capillary electrophoresis based dephosphorylation assays (**Figure 5 C**). Our results clearly show that SHP2 does not accept the singly-phosphorylated peptide as a substrate, only if the second, serine phosphorylation was also present. To demonstrate that this very strong dependence on the pSer site was not an artifact due to the truncation of SHP2, another round of experiments was also performed. We created an activated form of full-length SHP2 by disrupting its autoinhibition by a single point mutation (E76K). This full-length, activated SHP2 was expressed as a recombinant, GST-tagged protein, was purified, and used in a very similar activity assay. Despite its somewhat lower activity, the full-length SHP2 behaved surprisingly similar to the isolated phosphatase domain (**Figure 5 D**). It dephosphorylated the pTyr-pSer peptide (ppM2) at a high rate, while barely catalysed the dephosphorylation of the pTyr-only peptide (p0M2). These experiments demonstrate beyond doubt, that SHP2 strongly depends on the presence of the +4 serine phosphorylation site in our system.

Recognition of CD28 by SHP2 is also enhanced by threonine phosphorylation

Dually-phosphorylated motifs with the consensus YxxM[ST]P are not restricted to IRS proteins. As a next step in our research, I performed proteome-wide searches for similar motifs. This enabled us to identify the CD28 protein of leukocytes, where T-cell receptor associated kinases (Lck and ZAP70), effectors (GRB2, PI3K and Src-family kinases) as well as SHP2 meet each other. While tyrosine phosphorylation of this epitope (SRLLHSD{pTyr}MNM{pThr}PRR) has been studied extensively, large-scale proteomics data (PhosphoSitePlus) suggests that the neighbouring threonine residue is also highly phosphorylated. While the perpetrator kinase is currently unknown, the sequence surrounding the pThr site suggests involvement of cyclin-dependent kinases (CDKs). What is more intriguing that very recently, the same threonine residue was identified as a mutational hotspot in T-cell lymphomas [17]. Although mutation of this residue clearly disables its phosphorylation, the functional impact has been obscure, as it only impacts effector (GRB2) binding affinity to a small extent (with an approximately 2-fold increase). This is in clear contrast to our findings, where SHP2 is modulated very extensively by pSer (or pThr) residues located at the +4 position respective to the original pTyr residue. Crucially, CD28 is also a target of the SHP2 phosphatase in T-cells, and it is the main substrate whose dephosphorylation impacts T-cell receptor complex activation [18]. SHP2 is recruited to this complex by the PD1 protein – whose blockade by antibodies offers an important immunomodulatory treatment in cancer [19]. Due to the high therapeutic importance of CD28 signalling, we decided to examine if it behaves similarly to IRS1.

To enable affinity measurements, a pair of phosphopeptides representing the dually-phosphorylated (ppCD28) or the singly Tyr-phosphorylated (p0CD28) epitope were ordered. I repeated the same titrations performed with the IRS1 model peptide before, and came to comparable results. The Thr phosphorylation of CD28 did not alter PI3K recruitment to any relevant degree. On the other hand, it modulated the recruitment of phosphatases, depending on the identity of the enzyme. While binding of the inactive PTPRε catalytic domain was not altered, PTP1B binding was augmented by a factor of 2, while SHP2 binding increased approximately 7-fold. These results suggest that the modulatory effect of flanking Ser/Thr phosphorylation on SHP2 binding is not limited to IRS1.

Serine/threonine phosphorylation of -4 and +4 positions affect SHP2 activity equally

To get a clearer picture of SHP2 substrate preferences, after an extensive literature curation, I compiled a set of highly reliable substrate sites. Although mostly measured on proteins and peptides carrying Tyr-phosphorylation only, they still offered valuable insight into the charge preferences of SHP2, relative to the pTyr residue (position 0). Most Tyr phosphatases are known to preferentially phosphorylate substrates carrying one or more negative charges N-terminally from the pTyr residue [20]. The same was also observable on the 25 best characterized SHP2 substrate sites. However, these substrates also pointed to a similar preference of SHP2 for aspartic acid or glutamic acid residues C-terminally from the phosphotyrosine, at positions +4 or +5 (**Figure 6 A**). Not only does this match perfectly with the modulatory +4 phosphorylation sites that we identified, but also with the free carboxy terminus of other substrates (notably, Src-family kinases). Hence our theorem of SHP2 modulation by flanking Ser/Thr phosphorylation gained further reinforcement from these meta-analyses. At the same time, I observed that there exists a conserved motif in IRS proteins, where the serine phosphorylation site is located N-terminaly (at the -4 position) instead of C-terminally (corresponding to the surroundings of Tyr896 in human IRS1).

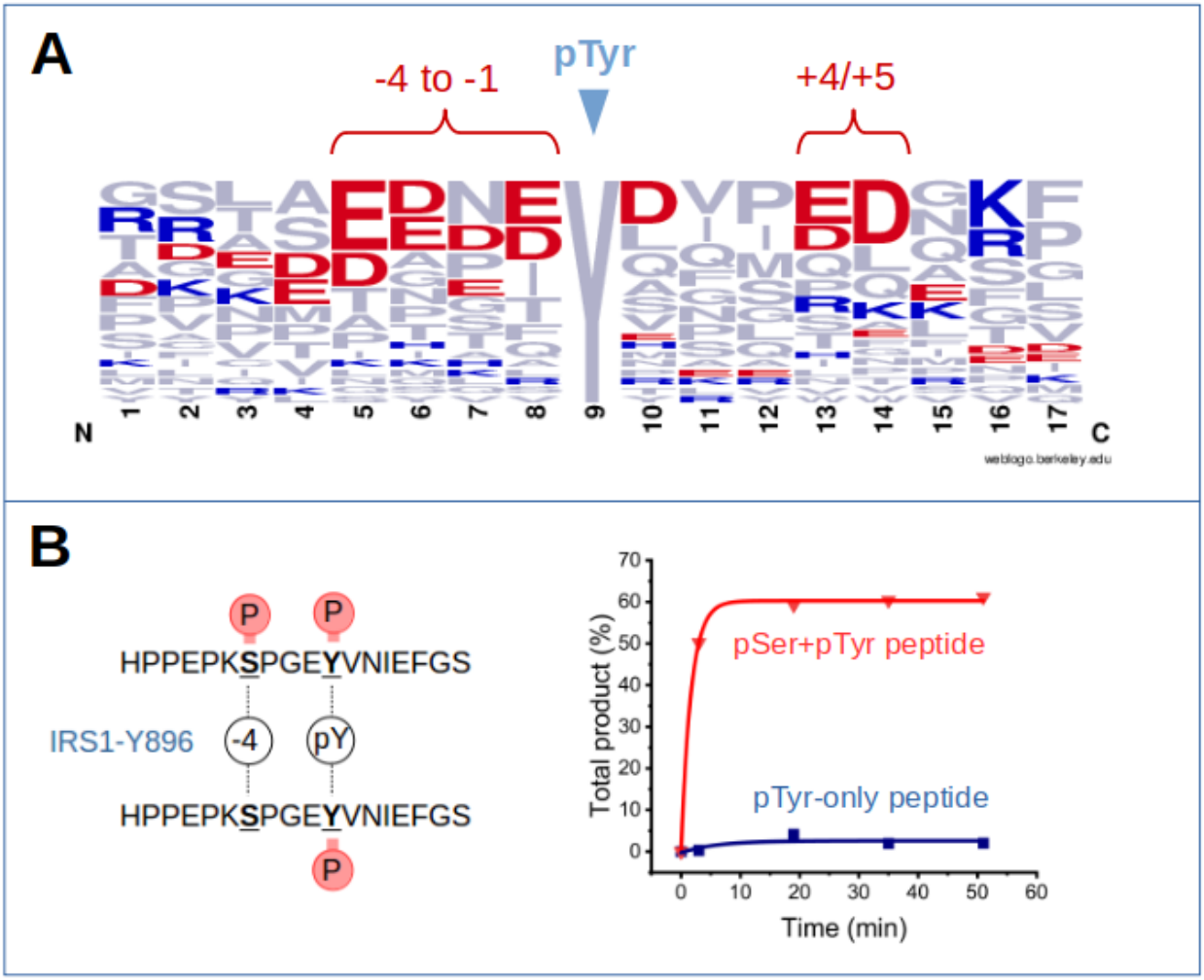
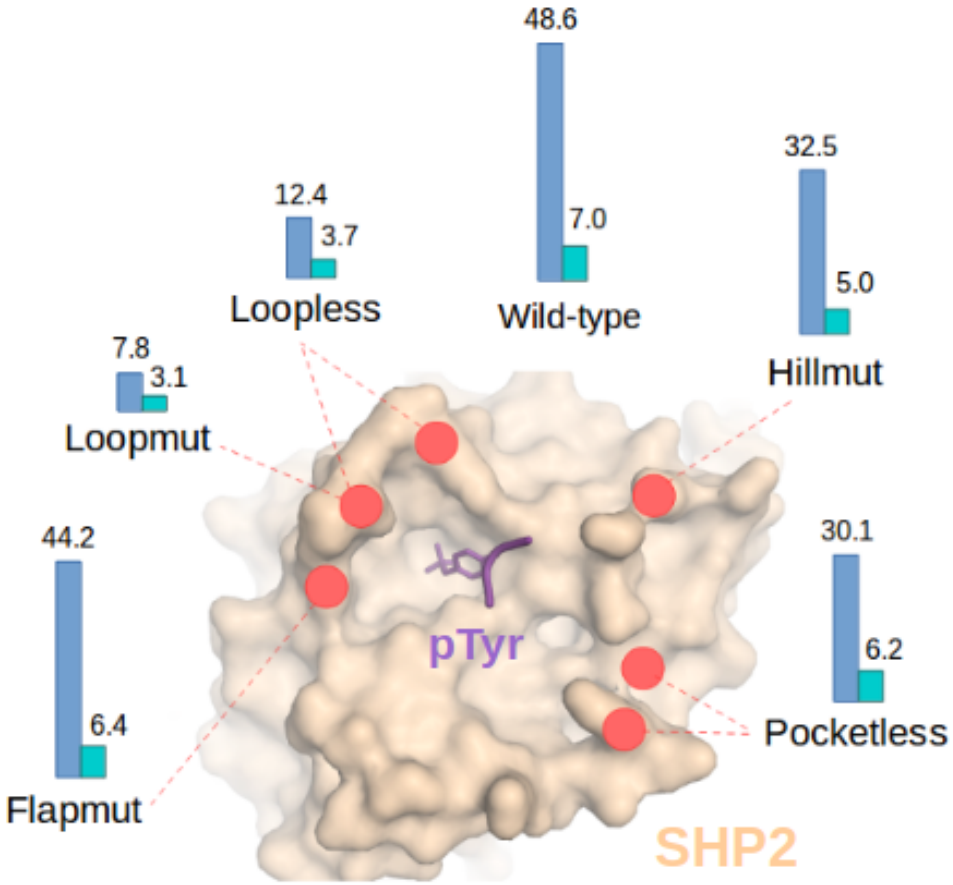


Figure 6: Logo of 25 different, experimentally validated SHP2 substrate sites from the literature (**A**). Negatively charged amino acids written in red, while positively charged ones are in blue. Two regions show enrichment of negative charges: one at +4/5 and another at -4 to -1. The Y896 site of IRS1 serves as an example of a phosphotyrosine motif that is flanked at the -4 position by a pSer modulatory site (contrasted to the previously studied motifs). However, the latter serine phosphorylation site also confers a greatly enhanced substrate recognition by SHP2, as dephosphorylation assays with the appropriate phosphopeptides indicate (**B**).

As the motif around Tyr896 of IRS1 (HPPEPK{pSer}PGE{pTyr}VNIEFGS) also matched exquisitely well with the SHP2 substrate consensus (a pSer residue substituting for negatively charged amino acids), we decided to test the latter in a functional assay. To create the Tyr-phosphorylated peptides, a semi-synthetic approach was used: the previously synthesized M+ and pSer-M+ peptides were treated with InsR kinase domain and an excess of ATP. Completion of phosphorylation was proven by capillary electrophoresis of products. These phosphopeptides (after removal of InsR activity) were then dephosphorylated by SHP2 in a capillary electrophoresis-based kinetic assay. The time curves of the dephosphorylation reaction shows a very large difference in the rates of catalysis, in favour of the dually-phosphorylated ppM+ peptide. In fact, the dephosphorylation rate was so rapid, for quantification we had to use much less phosphatase than what was needed in previous assays. This finding reinforces the idea that SHP2 can bind negative charges at both -4 as well as the +4 positions, and both enhance dephosphorylation incrementally (see our results on **Figure 6B**).



SHP2 surface mutant	Kd (p0M2)	Kd (ppM2)	Kd (p0CD28)	Kd (ppCD28)
Wild-type (inactive)	255.23 ± 34.85 μM	6.23 ± 0.67 μM	39.66 ± 1.81 μM	5.66 ± 0.29 μM
Loopmut (R362E)	23.33 ± 0.90 μM	3.01 ± 0.42 μM	20.46 ± 1.53 μM	6.66 ± 0.38 μM
Loopless (R362G+K364S)	195.35 ± 20.98 μM	15.76 ± 2.33 μM	94.31 ± 4.32 μM	25.79 ± 1.94 μM
Hillmut (K274E)	83.87 ± 2.58 μM	2.23 ± 0.14 μM	54.64 ± 5.12 μM	10.96 ± 0.57 μM
Flapmut (H430F)	171.80 ± 4.89 μM	3.89 ± 0.19 μM	63.68 ± 3.10 μM	9.96 ± 0.47 μM
Pocketless (K260E+R265S)	191.21 ± 15.49 μM	6.36 ± 0.28 μM	34.83 ± 2.25 μM	5.63 ± 0.29 μM

Figure 7: The effect of SHP2 surface mutations on phosphoserine recogntion. The surface figure indicates the positive charges altered in each mutant, while the bars and numbers indicate the relative binding strength of the two peptides ($Kd_{(pSer)}$ / $Kd_{(pTyr+pSer)}$). The blue bars refer to the IRS Y632-S636 peptide, while the green bars show the behaviour of the CD28 Y191-S195 peptide. For a detailed analysis, all the dissociation constants are tabulated below. The largest effect is always seen with mutations involving Arg632.

Flanking phosphorylations enhance substrate binding using SHP2-specific surface features

Our findings regarding the SHP2 phosphatase were largely unexpected, given its relative similarity to other related phosphatases. The reason why +4 phosphorylation events elicit similar effects to the -4 phosphorylation events (whose effect is more trivial, given the surface charge densities) was even more mysterious, begging for a structural explanation. As the first step, all substrate-bound tyrosine phosphatase structures were collected from the PDB. Comparison of different peptides suggested a broad surface area, where the +4 phosphoserine/threonine residue can theoretically contact the phosphatase domain. At the designated region, five different mutants were designed, removing or inverting the positively charged amino acids on the surface of SHP2. All these mutant, catalytically inactive phosphatase domains were expressed and purofied as recombinant proteins, and finally subjected to fluourescence polarization titartions. We argued that mutants, in which the key phosphate-binding amino acids are removed, should bind the singly- and doubly-phosphorylated model peptides similarly. From the results of all these titrations, it became clear that the “loop” reagion of SHP2, containing Arg362 and Lys364 must be part of the phosphate binding site (see **Figure 7**). Charge inversions (“loopmut”: R362E) and other mutants (“loopless”: R362G+K364S) clearly decreased the ratio of Kd(p0M2) / Kd(ppM2). Althogh this is very close to the pTyr binding site, the fact that some mutants (R362) actually bind stronger than the wild-type, argue against their involvement in pTyr binding. To corroborate our results, I repeated all competitive titrations with the CD28 peptides as well. Although the relative ratio of Kd(p0CD28)/Kd(ppCD28) was not as large as with the IRS1 peptide, it displayed the same tendencies with the SHP2 surface mutants. Together, these measurements would support a model, where the SHP2-specific Arg362 residue would contact the phosphoserine.

Crystallization and X-ray structure determination of SHP2 with the IRS1 peptide

To get even better insight into how the substrate recruitment of SHP2 works, we decided to crystallize a protein-peptide complex between the inactive SHP2 catalytic domain and pepIRS1. After several unsuccessful crystallization attempts with the native SHP2 phosphatase domain, I decided to optimize the construct for crystallization. Helical segment Thr219 to Gln245 (TRINAAEIESRVRELSKLAETTDKVKQ) was removed entirely and the highly flexible loop from Glu315 to Pro323 (ETKCNNSKP) was substituted with a short Gly-Ser linker (GSSG) to rigidify the surface. In addition, three extra amino acids (SGS) were inserted between the hexa-His tag and the phosphatase domain to facilitiate cleavage with TEV protease. This optimized construct was produced in E coli using an overnight expression after induction with IPTG. Purification was done on a Ni-NTA column, using the same procedure as with our other proteins. After TEV clevage, the protein was further purified with ion exchange (Äkta explorer, GE healthcare) on a resource Q column. Finally, a gel filtration was performed on a HiLoad 16/600 Superdex 75 column. The protein, which eluted as a single peak, was concentrated to ~9 mg/ml and mixed with the 15aa long IRS1-pTyr632-pSer636 peptide (ppM2) at a 3:2 molar excess. Crystallization was done in a vapour diffusion system, with the hanging drop method using NaCl as reservoir at room temperature. Best crystals were grown at pH 7.5 (HEPES buffer) and 5% PEG 20,000. Glycerol 20% (end concentration) was applied to crytals before flash freezing them on liquid N2. X-ray diffraction data was collected at Hamburg (PETRA III DESY facility) on a thick, prism-shaped crystal which diffracted to 1.53 A. It corresponded to the orthorhombic space group 20 (C222-1) with a unit cell of 53.54, 81.30, 146.29 and 90-90-90 degrees. Sturcture was solved using molecular replacement with Protein Data Bank structure 3ZM0 as a template. For initial phasing and subsequent refinement, standard crystallographic softwares (Phenix and Coot) were used.

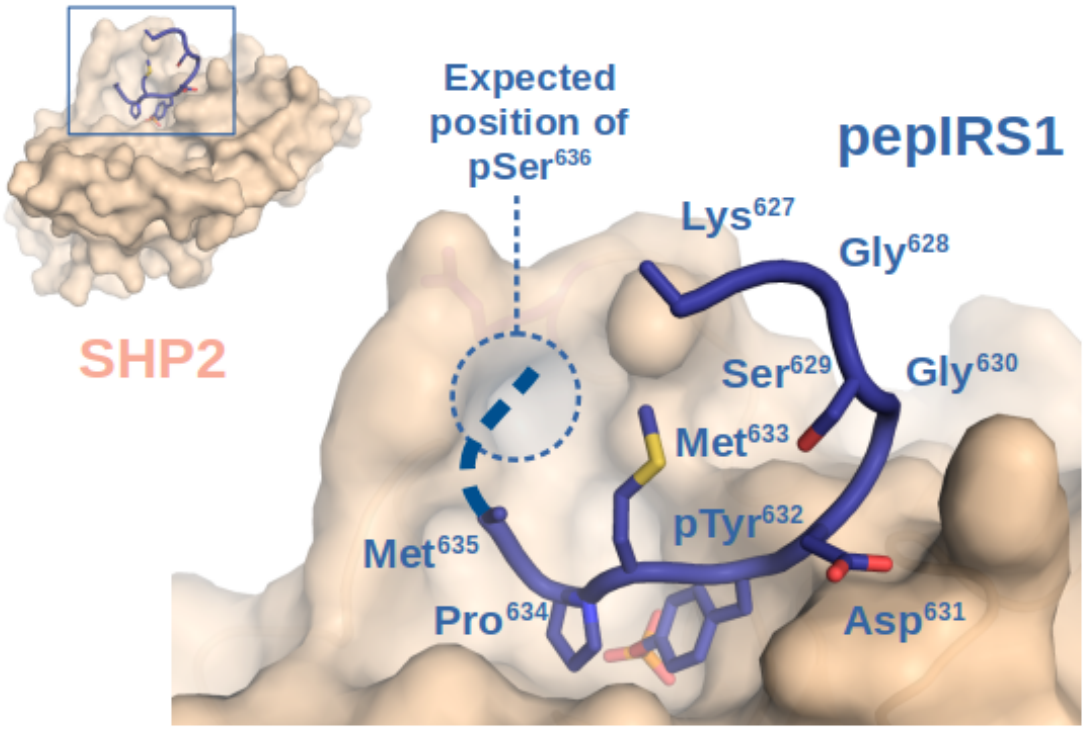


Figure 8: X-ray structure of the SHP2-pepIRS1 complex (upper left corner) and the details of phosphopeptide coordination (magnified). The phosphatase is shown in beige, while the IRS1 phosphopeptide is in blue. Although the pSer residue itself cannot be discerned, its position matches well with the one expected from the mutagenesis results (dotted line and circle)

The crystal structure clearly presents SHP2 with an open WPD loop, despite the catalytic site being occupied by a phospho-tyrosine peptide. This "open" conformation differs from most other tyrosine phosphatases, whose "apo" structures (i.e. without any ligand) presents the "open", and the peptide-loaded structures present a "closed" conformation. In the latter state, an aromatic amino acid from the top of the WPD loop (mostly His) moves to cover the phosphotyrosine with pi-pi-stacking interaction. However, in our SHP2 structure, the same position appears to be occupied by a proline amino acid (Pro634) from the substrate itself. The pTyr632 and Asp631 are very clearly visible from the electron density map; and are located in a conformation similar - although clearly not identical - to that of substrates in PTP1B, CD45, HePTP or other related enzymes. The observed dislocation of pTyr can likely be explained by its unusual stacking with a Pro residue. Although it is unclear whether this arrangement represents a true catalytic state, earlier mutagenesis experiments suggest that SHP2 does not absolutely rely on the WPD loop closure for catalysis, unlike other tyrosine phosphatases [20, 21]. Despite our best attempts, the phosphoserine could not be located on the density map; This suggests that the C-terminal end of the peptide is highly flexible, and exists as an ensemble of different conformations even at its phosphatase-bound state.

Major scientific impact: A new paradigm of phosphorylation-dependent regulation

Our results have delivered an unexpected result when inquiring about the mechanistic impact of IRS serine phosphorylation. It turned out that sites directly flanking tyrosine phosphorylation points are strong positive modulators of SHP2-dependent tyrosine dephosphorylation. A phosphorylation event modulating phosphatase recruitment to neighbouring sites appears to be an entirely novel concept in molecular biology. This new paradigm of phosphotyrosine pathway modulation will definitely help to understand regulation of many receptors, out of which insulin receptor and CD28 are just a few examples. In addition to the novelty of mechanism, it also offers a very elegant model for insulin receptor pathway desensitization upon IRS1/2 serine phosphorylation. Normally, IRS proteins associate with the effectors upon Tyr phosphorylation, and this effect is relatively long-lived. However, upon introduction of the flanking Ser phosphate, the dephosphorylation rate increases rapidly, so that signalling becomes less efficient. Thus the serine phosphate (which remains on IRS1/2 after removal of pTyr) acts almost like a catalytic switch to deteriorate insulin signalling at the IRS level. While I did not have the opportunity during the current project, in the near future, we intend to prove this mechanism in cellular assays.

It has not evaded our attention, that very recently (2019) an article has been published by scientists from the Howard Hughes Medical Institute (Texas, USA) examining the effects of the very same Tyr-flanking ser phosphorylation events in insulin signaling. [22] Those experiments suggested that dephosphorylated IRS motifs are efficient endocytosis trigger signals (due to their structural match with Yxxφ motifs). Their finding that SHP2 appears to be a major mediator of this dephosphorylation process clearly corroborates our results as well. Morover, they also examined the in vitro dephosphorylation rate of a model peptide different from ours (corresponding to human IRS1 Ser612-Tyr616), and found that SHP2 dephosphorylation rate is highly enhanced upon serine phosphorylation (similarly to our findings). What is more, an animal study was performed to examine the effects of an allosteric SHP2 inhibitor on insulin sensitivity. This preclinical study has shown that SHP2 inhibition can indeed ameliorate insulin resistance in test animals. These findings are in full accordance with ours, and - together with our structural insights - might provide a new therapeutic approach against type II diabetes.

Ongoing activities related to the current research project

Direct funding for the current project with András Zeke MD PhD as a PI has terminated with the expiry of this grant. Yet some activities are still in progress, before the final publication of results. As the crystal structure of the SHP2-pepIRS1 complex did not provide the details of the serine phosphate coordination, I am still engaged in a bioinformatic procedure, to model the peptide with the use of appropriate software tools (e.g. the Rosetta-based FlexPepDock and the HADDOCK suite). At the same time, efforts are ongoing to crystallize the SHP2 protein with another IRS1 peptide as well (representing the coordination of the -4 positioned phosphate group). Some cell based assays are still under development: to measure the effect of SHP2 manipulation on glucose uptake. But first and foremost, the most important activity is scientific writing, in order to prepare the manuscript for submission, as a peer-reviewed, open-access international publication.

Broader economic, technological and social impact of results

SHP2 inhibitors for the treatment of diabetes and other conditions.
Pharmaceutical inhibitors of tyrosine phosphatase SHP2 have recently entered clinical development with a major potential to treat a varietly of cancers. These agents act through multiple mechanisms: One is an immunomodulatory effect through the T-cell receptor complex; The other is a direct mechanism, inhibiting phospho-Ras dephosphorylation and hence a multitude of receptor tyrosine kinase pathways [23, 24]. The immunomodulatory effects (synergizing with PD1 inhibitors) might actually stem from the same molecular mechanism we uncovered [25]. But this is not the only potential medical application of SHP2 inhibitors. Our molecular-level observations, together with independently published results suggest that these agents also have the potential to ameliorate insulin resistance. This is corroborated by observations in human genetic diseases associated with SHP2 loss-of-function mutations as well as animal experiments with SHP2 inhibitors [22, 26]. Although the current project did not have the aim to develop new SHP2 inhibitors, or to test them in a preclinical or clinical setting, our observations shall definitely help the pharmaceutical industry to develop the right drugs for the treatment of early-stage diabetes.

Additional benefits for the host institution
On the top of the scientific achievements, a number of added benefits were also provided to the institution and my colleagues involved. It opened many new reserch horizons for the Reményi lab (my host lab). Together with the Péter Kele lab, we were also able to calibrate simple, new analytic assays for various protein kinases and phosphatases. My former student, Tamás Takács was also able to secure a PhD student position within the institution, and (under the supervision of László Buday) is now enrolled in a follow-up project. His goal shall be to explore the structural background of Ras dephosphorylation by SHP2, thereby directly using many of the tools, reagents and knowledge established during this project.

Publication of results
In order to make our results available to the broader scientific community, a draft article is currently in preparation, intended to be submitted in next year (2020). Since not all studies have completed fully until the end of the grant-supported period (2016.10.01 - 2019.09.31), I could not publish the findings beforehand. We intend to wait until the new cell-based studies and structural explorations bear final result (expected in the first half of 2020). Therefore I kindly ask the evaluation committee to take this into account when delivering the final verdict on the successfulness of this grant.

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