JYU DISSERTATIONS 63

Lina Antenucci

Biochemical and Structural Comparison of Spleen Tyrosine Kinase Interaction with Integrin and the Immunoreceptor Tyrosine-Based Activation Motif

UNIVERSITY OF JYVÄSKYLÄ
FACULTY OF MATHEMATICS
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"Ti sogno in blu
Vestita di un abbraccio.

Ti sogno di un bacio ideale
Consumato in un secondo.

Scie scomparse,
scie dipinte
nella mente
di entrambi e di ognuno
contemporaneamente."

Manuel Iallonardi

"But in the end it's only a passing thing, this shadow;
even darkness must pass."

J.R.R. Tolkien, The Lord of the Rings

ABSTRACT

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Integriinien ja immunoreseptoreiden rooli pernan tyrosiinikinaasin säätelyssä
Diss.

Spleen tyrosine kinase (Syk) is a non-receptor tyrosine kinase involved in many different signalling pathways activated by immunoreceptors and integrins. The Syk activation mediated by phosphorylated Immunoreceptor Tyrosine-based Activation Motif (pITAM) receptors involves Src homology 2 (SH2) regulatory domains leading to Syk structural rearrangements. Differently, integrin cytoplasmic domains bind to the regulatory domain of Syk and the interaction does not require the phosphorylation, but the molecular mechanism is still unknown. This work focussed on describing the mechanism of integrin-Syk interaction and on how integrins activate Syk. First, using a fluorescent-based kinetic assay we showed that the soluble integrin peptide had no detectable effect on Syk activity, whereas Syk was activated by clustered integrin peptides. This suggests that autophosphorylation is involved in the integrin-induced activation process. Clustered integrins had also a synergic effect combined with pITAM. Next, we wanted to elucidate the molecular mechanism of Syk-integrin interaction. Surface plasmon resonance techniques was used to measure the binding affinities of different Syk constructs towards the integrin peptide. The N-terminal SH2 domain (N-SH2) plus the interdomain A (IA) segment had a comparable binding affinity to the two SH2 domains, whereas N-SH2 was a weak binder. Using nuclear magnetic resonance spectroscopy, we solved the structure of the N-SH2 domain of Syk and analysed the changes on the chemical environments of the N-SH2 domain induced by the integrin peptide and pITAM. The results indicate that integrin and pITAM binding induce changes on the same surface of the protein. In line with this, surface plasmon resonance experiments showed that the integrin and pITAM peptides compete for binding to the regulatory domain of Syk. We compared the binding affinity of pITAM to N-SH2 with C-terminal SH2 (C-SH2) domains observed that N-SH2 had a very low binding affinity compared to C-SH2. These studies suggest that integrin and pITAM-mediated Syk activation are independent of each other but may cause synergistic activation responses at the cellular level.

Keywords: Syk kinase, integrin, ITAM, Nuclear Magnetic Resonance (NMR).

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ABSTRACT IN FINNISH

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Pernan tyrosiinikinaasi (engl. *spleen tyrosine kinase)*, Syk, on solunsisäinen entsyymi, joka osallistuu immuunireseptoreiden ja integriiniperheen solun tarttumisreseptoreiden välittämään solun toiminnan säätelyyn. Immuunireseptoreiden aiheuttama Syk-entsyymin aktivaatio perustuu reseptoreihin liittyvään fosforyloituvaan proteiinialueeseen nimeltään pITAM (engl. *phosphorylated Immunoreceptor Tyrosine-based Activation Motiv*). Immunoreseptoreiden kiinnittyessä solun ulkoisiin vastinmolekyyleihinsä, Syk kiinnittyy pITAM-alueeseen reseptorin solunsisäisessä osassa. Tämä kiinnittyminen aiheuttaa Syk-entsyymissä rakennemuutoksen ja aktivoi sen. Tämän tutkimuksen tarkoituksena on ollut selvittää, miten integriinit saavat aikaan Syk:n aktivaation. Tutkimuksessa käytettiin yhdistelmä-DNA tekniikoilla tuotettua ja puhdistettua Syk-proteiinia sekä sen säätelyosan paloja. Entsyymiaktiivisuusmittaukset osoittivat, että liukoinen integriinipeptidi ei aktivoinut Syk-entsyymiä, mutta pITAM-peptidi aktivoi. Sen sijaan analyysikammion pintaan kiinnitetty integriinipeptidi aktivoi Syk-entsyymiä ja pystyi tehostamaan liukoisen pITAM-peptidin aiheuttamaan aktivaatiota. Tästä pääteltiin, että pITAM ja integriinit aktivoivat Syk entsyymiä eri mekanismeilla: pITAM sitoutumisen aiheuttaman rakennemuutoksen kautta ja integriinit klusteroimalla Syk-entsyymiä niin, että se pystyy fosforyloimaan itseään ja näin aktivoitumaan. Toinen lähestymistapa tutkimuksessa oli integriini- ja pITAM-peptidien sitoutumispaikkojen tarkka kartoitus Syk:n säätelyalueessa. Tähän käytettiin pintaplasmoniresonanssiin perustuvaa menetelmää ja ydinmagneettista resonanssispektroskopiaa, NMR-spektroskopiaa (engl. *Nuclear Magnetic Resonance*). Tutkimuksia varten myös laskettiin NMR-mittauksiin perustuva 3-ulotteinen proteiinimalli Syk:n N-SH2-domeenista. Tulokset osoittivat, että pITAM-alue sitoutuu ensisijaisesti Syk:n C-SH2-domeeniin ja toissijaisesti N-SH2-domeeniin. Integriini sen sijaan sitoutuu ensisijaisesti domeenien väliseen alueeseen ja toissijaisesti N-SH2-domeeniin. Yllätys oli, että N-SH2-domeenissa integriinin sitoutuminen aiheutti kemiallisia muutoksia lähellä pITAM:n sitoutumiskohtaan ja että pITAM-peptidi pystyi inhiboimaan Syk:n säätelyosan sitoutumista integriinipeptidiin. Kokonaisuudessaan nämä tutkimukset osoittavat, että integriinien ja pITAM-reseptoreiden aiheuttama Syk-entsyymin aktivaatimekanismit ovat osittain toisistaan riippumattomia ja voivat siten voivat siten solutasolla toimia yhteistyössä.

Avainsanat: Syk kinaasi, integriini, ITAM, ydinmagneettinen resonanssispektroskopia

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original papers, which will be referred to in the text by their Roman numerals:

I Lina Antenucci, Vesa P. Hytönen, and Jari Ylänne. 2018. Phosphorylated immunoreceptor tyrosine-based activation motifs and integrin cytoplasmic domains activate spleen tyrosine kinase via distinct mechanisms. *Journal of Biological Chemistry* 293(13): 4591-4602

II Lina Antenucci, Helena Aitio, Maarit Hellman, Jari Ylänne, and Perttu Permi. Binding studies of N- and C- terminal Src homology 2 (SH2) domains of spleen tyrosine kinase Syk with pITAM. (Manuscript)

III Lina Antenucci, Maarit Hellman, Vesa P. Hytönen, Perttu Permi, and Jari Ylänne. Integrin cytoplasmic domain and pITAM compete for spleen tyrosine kinase binding. bioRxiv doi: 10.1101/524447. (Submitted manuscript)

In addition to these some unpublished data are also presented in the thesis.

ABBREVIATIONS

BCR	B-Cell Receptor
CLEC	C-type lectin
C-SH2	C-terminal SH2 domain
CSP	Chemical Shift Perturbation
EGFR	Epidermal Growth Factor Receptor
Fc	Fragment crystallizable
FERM	Four-point-one, Ezrin, Radixin, Moesin
IA	Interdomain A
IB	Interdomain B
ITAM	Immunoreceptor Tyrosine-based Activation Motif
NMR	Nuclear Magnetic Resonance
NRTK	Non-Receptor Tyrosine Kinase
N-SH2	N-terminal SH2 domain
PH	Pleckstrin Homology domain
pITAM	Phosphotyrosine ITAM
PLC	Phospholipase C
PTB	Phospho-Tyrosine Binding
pTyr	Phosphotyrosine
RTK	Receptor Tyrosine Kinase
SH2	Src Homology 2 domain
SH3	Src Homology 3 domain
SHP	Src Homology 2 domain-containing Phosphatase
SPR	Surface Plasmon Resonance
Src	Sarcoma related kinase family
Syk	Spleen Tyrosine Kinase
TCR	T-Cell Receptor
Zap-70	ζ-chain of T-cell receptor Associated Protein kinase of 70 kDa

CONTRIBUTIONS TO THE WORK

I I cloned full-length Syk in baculovirus expression vector, designed and optimised the expression protocol of the kinase and the Y348F/Y352F mutant in insect cells and purified them. I designed and optimised the fluorescence-based kinetic activity measurement of Syk and analysed the data. I expressed and purified tandem Src homology 2 (tSH2) domains protein construct. I designed and performed the surface plasmon resonance (SPR) analysis with Integrin β_3 cytoplasmic domain peptide and analysed the data with the guidance of Prof Vesa Hytönen. I wrote the manuscript together with Prof Vesa Hytönen and Prof Jari Ylänne.

II I cloned, expressed and purified ^{15}N and ^{15}N-^{13}C labelled the N-terminal SH2 (N-SH2) and C-terminal SH2 (C-SH2) domain of Syk for Nuclear Magnetic Resonance (NMR) data collection. Prof Perttu Permi collected the NMR data for N-SH2 assignment and structure determination. I assigned the protein chemical shift of backbone and side chains of N-SH2. I calculated and refined the N-SH2 structure with the help of Drs Helena Aitio and Drs Maarit Hellman. I designed the titration with double and hemi phosphorylated Immunoreceptor Tyrosine-based Activation Motif (ITAM) peptides with both N-SH2 and C-SH2. Prof Perttu Permi collected the spectra. I analysed the titration data. I wrote the manuscript with my co-authors and supervisors.

III I cloned, expressed and purified all the Syk constructs. I designed and conducted the SPR experiments and the thermofluor analysis and analysed the data. I designed and analysed the NMR titration experiments with hemi-phosphorylated ITAM peptide and Integrin β_3 cytoplasmic domain peptide. Prof. Perttu Permi collected the NMR data. I analysed the data with the guidance of Prof. Perttu Permi. I wrote the manuscript together with the co-authors and supervisors.

1 INTRODUCTION

1.1 Tyrosine kinases and cell signalling

1.1.1 Protein kinases

Phosphorylation is the most common way to regulate biological processes. Phosphate is very stable in water at physiological pH and has a large hydrated ionic shell. In cells, phosphate ions are stored in ATP, so they can be used whenever needed. The hydrolysis of the phosphoanhydride bond in the ATP chemical structure is a fundamental chemical reaction to push forward biological processes. Phosphate ions stored in ATP are also used to covalently modify proteins and to regulate their activity. When phosphorylated, proteins are *new* entities with an 'altered' chemical surface and therefore easy to recognised compared with those not phosphorylated (Hunter 2012). For this reason, it is not surprising that ~ 2 % of the total human genome encodes for protein kinases (Manning *et al.* 2002, Taylor and Kornev 2011) including Serine/Threonine kinases and Tyrosine kinases; the latter are the focus of the present work. There are also smaller kinase families, such as the protein Histidine-kinases, Cysteine-kinases and protein -aspartyl or -glutamyl kinase (Hunter 1991, 2012, Wolanin *et al.* 2002, Woo *et al.* 2004, Taylor and Kornev 2011, Adam and Hunter 2017).

The first kinase to be crystallised was the cyclic AMP-dependent protein kinase A (PKA), which is a Ser/Thr kinase (Knighton *et al.* 1991, Moore *et al.* 2002, Huse and Kuriyan 2002). In 1997 (Xu *et al.* 1997), the structure of Rous Sarcoma Virus transforming tyrosine kinase (Src) was elucidated (though its kinase activity had already been demonstrated in 1981) (Barbacid *et al.* 1981), and it was then used as a prototype for other tyrosine kinases. Many crystal structures of protein kinases are now available, and, even though there are huge differences in their regulation and domain composition, the structural principles of kinase catalysis and the most important structural components involved in the active site regulation are well conserved ((Robinson *et al.* 2000),

more detailed information is provided in Section 1.1.2). Of the 518 human protein kinases, 90 are classified as tyrosine kinases based on sequence similarity (Manning *et al.* 2002).

Tyrosine kinases, in turn, are divided into two main classes: receptor and non-receptor tyrosine kinases (Bradshaw 2010, Lemmon and Schlessinger 2010). Receptor tyrosine kinases (RTKs)(Section 1.1.3) are trans-membrane proteins which are activated by the binding of extracellular ligands and transduce the extracellular signal in the cytoplasm through auto-phosphorylation and phosphorylation of other proteins (Lemmon and Schlessinger 2010). In contrast, non-receptor tyrosine kinases (NRTKs) are mainly located in the cytoplasm and are activated by diverse intracellular signalling pathways after the activation of specific receptors. Tyrosine kinases are important mediators of cell signalling; thus their activity is finely regulated via diverse activation and degradation mechanisms (Hunter 2007, Lemmon and Schlessinger 2010). Significantly, dysregulation and mutations of tyrosine kinases are often associated with a variety of diseases including cancer, auto-immune diseases and inflammation (Xu and Huang 2010, Lemmon and Schlessinger 2010, Krisenko and Geahlen 2015).

The present work is focussed on Spleen tyrosine kinase (Syk), a non-receptor tyrosine kinase, and its activation mechanism. Therefore, in Sections 1.1.4-6, I review the classification, structural features, and regulation of NRTKs, before moving to the details of Syk in Section 1.2.

1.1.2 The protein kinase domain: a common feature of all kinases

The main group of eukaryotic protein kinases catalyses the reaction of transferring the γ phosphate group of ATP to the hydroxyl (-OH) group of Ser, Thr or Tyr residues. The catalytic domain of protein kinases is normally kept in an inactive or closed conformation by the presence of regulatory domains in the kinase sequence or by the phosphorylation state of specific sites in the kinase sequence (Huse and Kuriyan 2002). The kinase domain is divided into two lobes called the N-lobe and the C-lobe based on the position in the sequence (Fig. 1, Panel A). The N-lobe is formed by a 5-stranded β-sheet and an α-helix (called the αC-helix) which place a crucial role in the activation process. Conversely, the C-lobe is predominantly an α-helical structure and contains only a β-sheet. The two lobes are anchored to each other through the αC-β4 loop (Kornev *et al.* 2008, Shudler and Niv 2009, McClendon *et al.* 2014). Based on sequence alignments of kinase domains, conserved sequences have been identified which are important for kinase activity (Kornev *et al.* 2006). The most important conserved sequences are the Gly loop or Gly-rich loop, the AxK motif, the catalytic loop and the activation loop. The **Gly loop** (containing the sequence GxGxxG) connects the strands β1 and β2 in the N-lobe (Fig. 1, Panel A). It makes contact with ATP and is responsible for putting the γ phosphate group in the right position for the catalysis. At the same time, in the N-lobe, specifically in the β3 strand, Lys in the conserved **AxK motif** interacts with ATP, keeping it correctly oriented towards the αC-helix (Johnson *et al.* 2001,

Huse and Kuriyan 2002, Taylor and Kornev 2011). On the other hand, Glu in the αC-helix interacts directly with the Lys in the AxK motif in the protein active conformation only. The salt bridge formed between the Glu and Lys is the fingerprint of the active kinase (Taylor and Kornev 2011). The αC-helix is a flexible part of the inactive kinase, but its movements are stabilised in the active kinase (Fig. 1, Panels A and B) (McClendon *et al.* 2014)).

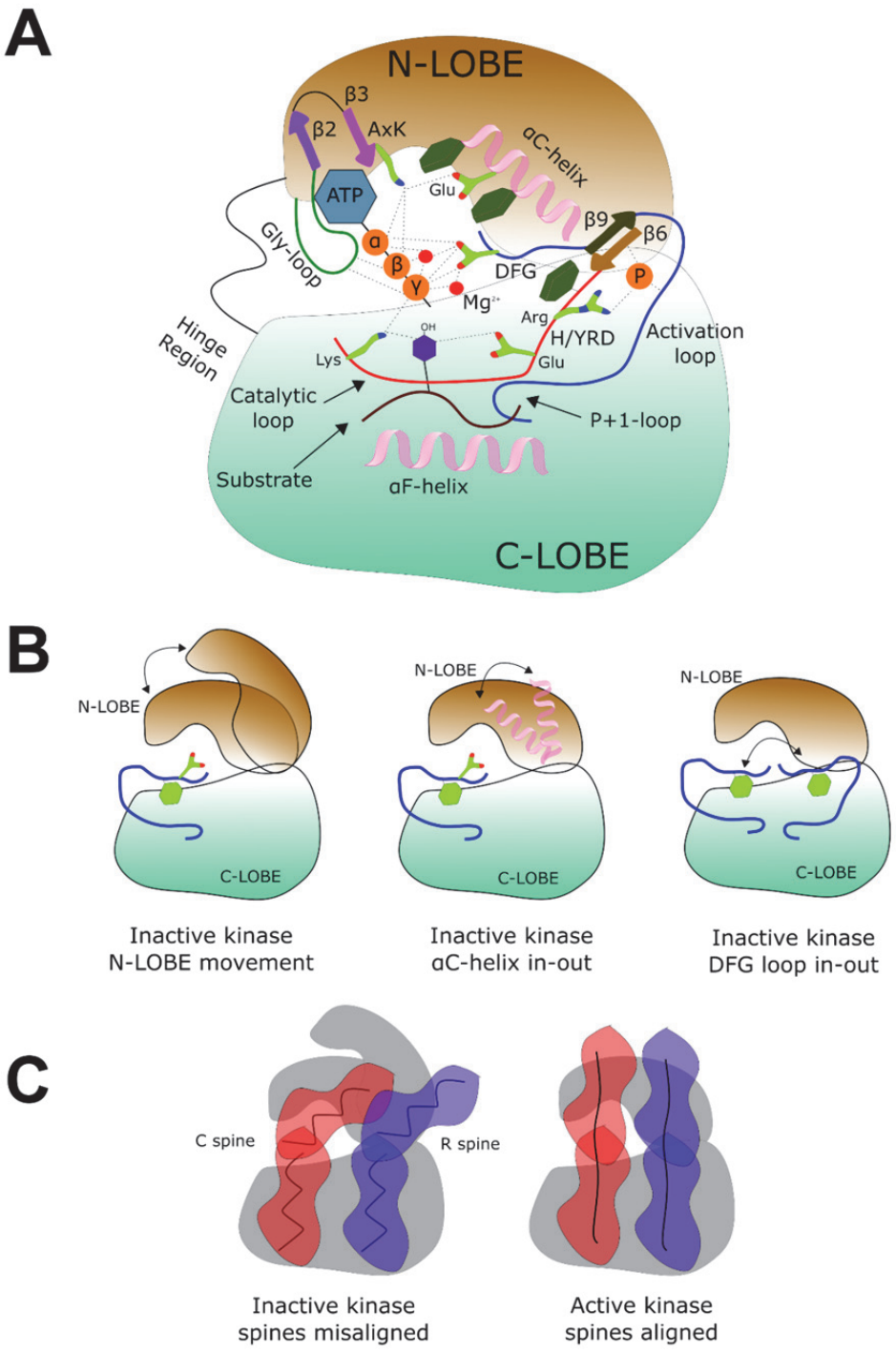

FIGURE 1 **A-** Graphical representation of active conformation of the kinase domain. The N-lobe is represented in brown and the C-lobe in light green. ATP and substrate are kept in the correct orientation for catalysis through a complex

interaction pattern. In the N-lobe, a conserved Gly loop (dark green) is responsible for orienting the ATP γ phosphate group for catalysis. The Lys in the β3 strand AxK motif interacts with a conserved Glu in the αC helix. This interaction helps to keep the αC helix in a stable conformation conducive to catalysis. The C-lobe contributes to catalysis, supporting the incoming substrate; the C-lobe has a very stable hydrophobic core, and the αF helix is the region most crucial for stability and catalysis. Moreover, the C-lobe contains the catalytic (red) and the activation loop (blue). The latter contains critical residues which are phosphorylated during kinase activation (labelled with red P). The phosphorylation permits the interaction with conserved residues (H/YRD) in the catalytic loop. As a consequence, the catalytic loop adopts the right conformation to stabilise the substrate residue to be phosphorylated (a Tyr in the figure represented in purple). **B-** Schematic representation of subdomain movements in the inactive kinase movements, described as the breathing of the kinase domain: the N-lobe (left) and its αC helix (middle) are moving. The DGF - containing activation loop in blue (Phe and Glu are represented in the figure) is moving in and out (right). **C**- Orientation of the catalytic spine (C-spine) and the hydrophobic spine (R spine) in the inactive and active kinase.

The main role of the C-lobe is to form the hydrophobic core of the kinase and create a support for the incoming substrate. Two of the most important conserved segments of the kinase belong to the C-lobe. The loop between the β6 and β7 strands is the catalytic loop (Fig. 1, Panel A). The **catalytic loop** contains the conserved sequence HRD (or YRD in tyrosine kinases) which is involved in maintaining the correct orientation of the activation loop after its phosphorylation during the kinase activation process (Scheeff and Bourne 2005, Kannan and Neuwald 2005). The conserved Arg counteracts the negative charge added by the phosphorylation and blocks the activation loop in a fixed conformation. In the **activation loop**, there is a conserved DFG motif which is points directly towards the active site, keeping ATP in the correct orientation. In particular, the Asp coordinates the Mg^{2+} ions in the active site, whereas the Phe creates hydrophobic interactions with residues nearby (Fig. 1, Panel A). In the inactive conformation the activation loop is quite loose and moves in-and-out (Fig. 1, Panel B). It has been shown that the presence of the Gly in the DFG motif allows a rotation of the activation loop so that it can exist in a permissive or not permissive conformation for catalysis (Nolen *et al.* 2004, Chen *et al.* 2007, Kornev *et al.* 2008, Masterson *et al.* 2010, McClendon *et al.* 2014). Although not identified by its conserved sequences, the αF helix of the C-lobe has a major role. It contacts the catalytic and the activation loop, as well as the catalytic spine (see below) (Kornev *et al.* 2008).

In addition to the conserved sequence motifs discussed above, further conserved structural patterns of kinase domains have been identified via local spatial pattern alignment of several protein kinase crystal structures. These features are important in keeping the N- and C- lobes in the correct orientation for catalysis and they are called **regulatory or hydrophobic** and **catalytic spines** (Fig. 1, Panel C). As suggested by its name, the hydrophobic spine is formed by two hydrophobic residues from the N-lobe and two from the C-lobe (Kornev *et al.* 2008). This spine is assembled and disassembled in a very dynamic fashion and, since it involves the αC-helix, the activation and the catalytic loop, it is a

crucial regulator of the kinase activity. The catalytic spine is also formed by hydrophobic residues, but it is completed by the ATP adenine ring. It is important for the correct orientation of ATP in the active site (Huse and Kuriyan 2002).

As mentioned at the beginning of this Section, the protein kinases include a huge variety of regulatory domains which are important for the regulation of the kinase domain activity. The tyrosine kinases, already divided into RTKs and NRTKs, are also classified in families based on the domain composition and their regulation. The next Sections are focussed on a more detailed description of those mechanisms in RTKs and NRTKs.

1.1.3 Receptor tyrosine kinases

The human genome contains 58 RTKs which are divided into 20 different families. RTKs include receptors for insulin, epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and nerve growth factor (NGF) (Hubbard and Till 2000). They all share a similar molecular architecture which includes an extracellular ligand-binding domain, a transmembrane helix and an intracellular region formed by the tyrosine kinase domain and the juxtamembrane regulatory regions (Fig. 2). The extracellular parts vary greatly from one kinase to another. In contrast, the conserved features are the single transmembrane domain and an intracellular tyrosine kinase domain (Nolen *et al.* 2004, Schlessinger 2014).

The inactive state of RTKs can be monomeric or oligomeric, but the active form always requires dimerisation or oligomerisation. The activation procedure is triggered by the binding of a ligand to the extracellular parts of the receptor. Three examples are given here to illustrate the variability in the role of the extracellular ligand-binding parts in kinase activation (Fig. 2). The first one is the tropomyosin receptor kinase A (TrkA), also known as nerve growth factor receptor, which has high affinity for its ligand. The inactive TrkA (tropomyosin receptor kinase A) is monomeric, and the extracellular domains do not contact each other in the absence of a ligand. NGF (nerve growth factor) binds to two TrkA (tropomyosin receptor kinase A) molecules and, the interaction of the extracellular parts is solely mediated by NGF (nerve growth factor) (Wehrman *et al.* 2007) (Fig. 2, Panel B). The inactive epidermal growth factor receptor (EGFR) is also monomeric, but EGF (epidermal growth factor) binding causes a huge conformational change in the extracellular domain and reveals a dimerisation arm which is completely buried by intramolecular interactions in the inactive state (Burgess *et al.* 2003). Thus, in the EGF (epidermal growth factor) -bound EGFR dimer, the contact between the extracellular parts is mediated via the receptor only, not by the growth factor (Fig. 2, Panel A). The third example is mast/stem cell growth factor receptor (SCFR, also known as c-Kit or CD117). The binding of the ligand does not alter the monomers association but, instead, induces reorientation of two domains next to the membrane with a consequent activation of the intracellular kinase domain

(Yuzawa *et al.* 2007) (Fig. 2, Panel C). Even though the active form of the kinase domain of different RTKs is the same, there are remarkable differences in the structure of the inactive conformation of the kinase domains. This reveals that there are different activation mechanisms for the kinase domain (Lemmon and Schlessinger 2010). As an example, the kinase domain of the insulin receptor (InsR) is kept closed by the conformation of the activation loop. After insulin stimulation, Tyr residue in the activation loop becomes trans-phosphorylated, leading to kinase activation (Hubbard 2013). On the other hand, the juxtamembrane region interacts with the kinase domain in the resting PDGF (platelet-derived growth factors) receptor and keeps it in an autoinhibited, inactive conformation. This autoinhibition can be released by phosphorylation of Tyr residues at the juxtamembrane region, leading to displacement of the juxtamembrane region from the active site (Hubbard 2004, Nolen *et al.* 2004). In the EGFRs, the kinase domain is activated via an allosteric effect. In this case, receptor dimerisation causes contacts between the C-lobe of one kinase domain and the N-lobe of the other kinase domain (Fig. 2, Panel A). These interactions induce conformational rearrangements in the N-lobe which lead to a displacement of the activation loop from the active site (Zhang *et al.* 2006).

When the RTKs are active, they can promote intracellular signalling which involves many different proteins. The most important and common feature in RTK signalling is the intracellular signal propagation via Tyr phosphorylation as mentioned at the beginning of this thesis. Phosphorylated Tyr (pTyr) are the binding site for many proteins – like adaptor proteins, kinases and phosphates- which create a complex intracellular signalling circuit. This corresponds to a second phase of RTKs signalling (Lemmon and Schlessinger 2010). Different positive and negative feedback are needed to maintain a delicate equilibrium between all the involved signalling components. Positive feedback is mainly mediated by sustaining the phosphorylated status of the intracellular domain of the RTKs and their binding partners, as well as by keeping the activity of recruited phosphatases as low as possible. Downregulation of RTKs involve the same machinery and same mechanisms of the positive ones. One good example is the regulation of EGFR. The activated EGFR recruits the phosphates SHP-1 and SHP-2. At the same time, EGFR promotes the activation of Protein Kinase C (PKC) which in turn phosphorylate the juxtamembrane domain of the receptor leading to its inactivation (Lemmon and Schlessinger 2010). On the top of that, internalization of RTKs through clathrin-dependent and independent mechanisms are important for RTKs downregulation (Hunter 2007). The internalisation may lead to a complete degradation of the receptor in the lysosomes or, eventually, to a recycling process of the RTKs (Lemmon and Schlessinger 2010).

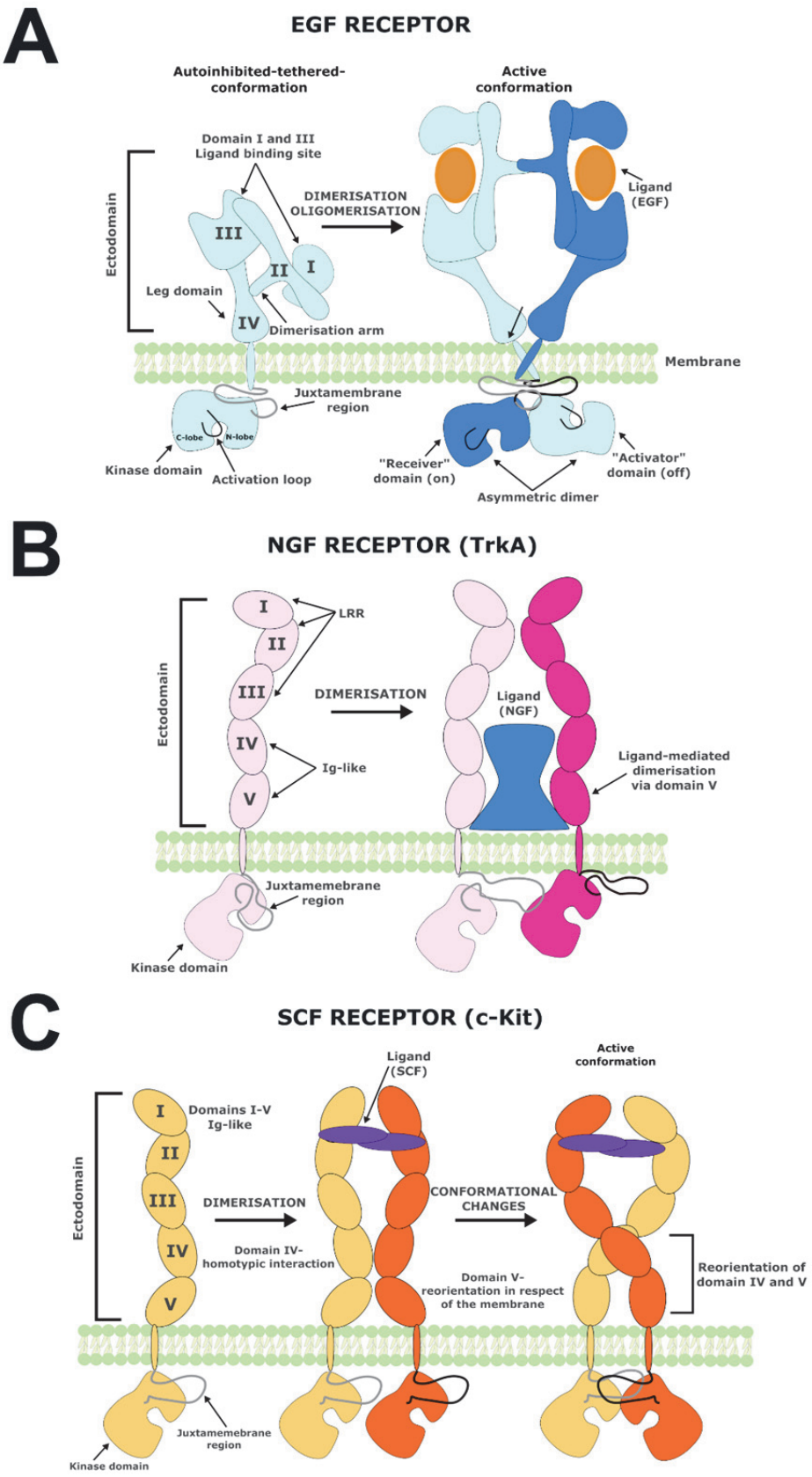

FIGURE 2 Schematic representation of structure and activation mechanism of RTKs. **A-**Structure of the EGFR in the auto-inhibited form (left side) and the active form (right side). The extracellular part is composed of 4 different domains,

indicated with Roman numerals from I to IV. The ectodomain is connected to the intracellular kinase domain by a transmembrane region. The binding of the EGF induces the switch of the ectodomain from inactive to active conformation and the dimerisation via the "dimerisation arm". The dimerisation is completely mediated by the EGFR and not by the ligand. The dimerisation also induces the reorientation of the transmembrane and the interaction of the kinase domains. The activation of the kinase activity is mediated by allosteric regulation of one kinase domain (receiver) on the other (activator). **B-** Activation mechanism of the TrkA receptor. The ectodomain is formed by the three Leucine-Rich Repeat (LRR) (domains I, II and III) and two Immunoglobulin-like (Ig-like) domains (IV and V). The activation is induced by ligand-mediated dimerisation (no contacts between receptors). **C-** The c-Kit receptor ectodomain is formed by 5 Ig-like domains, indicated with Roman numerals in the figure. Domains I, II and III are responsible for ligand binding, the domain IV for homotypic interaction and domain V for reorientation during activation. In this case, the activation is mediated by both the ligand and the receptor itself.

1.1.4 Non-receptor tyrosine kinases

There are 32 human NRTKs, which can be divided into 10 subfamilies: ABL, ACK, CSK, FAK, FES, JAK, SRC, TEC and SYK ((Bradshaw 2010, Siveen *et al.* 2018), Table 1). The SRC family is the largest and the SYK family is one of the smallest, including just Spleen Tyrosine kinase (Syk) and ζ-chain associated kinase of 70 kDa (Zap-70) (Bradshaw 2010). NRTKs are intracellular proteins and, thus, compared with RTKs, lack the extracellular ligand binding domain and the transmembrane domain. However, some NRTKs are anchored to the membrane via myristylation or palmitoylation in the N-terminus. In addition to this, all NRTKs contain special protein-protein, protein-lipid or protein-DNA binding domains (Siveen *et al.* 2018) (Fig. 3). The most common protein-protein interaction domains are the SH2 and SH3 domains. The only NRTK subfamily which does not contain either SH2 or SH3 domains is the focal adhesion kinase (FAK) family (Mitra *et al.* 2005). A more detailed description of certain domains NRTKs is given below since they are known to be involved in the regulation of kinase activity and they will be further mentioned in the next sections.

TABLE 1 Classification of NRTKs.

Family		Members
ABL	Abelson murine leukaemia-related kinase	ABL1, ABL2
ACK	Activated Cdc42-associated kinase	ACK1/TNK2, ACK2, DACK, TNK1, ARK1, DPR2
CSK	C-terminal Src kinase	CSK, CHK
FAK	Focal adehesion kinase	FAK, PYK2
FES	Feline sarcoma-related kinase	FER, FES
JAK	Janus kinase	JAK1, JAK2, JAK3, TYK2
SRC	Sarcoma-related kinase	BLK, FGR, FRK/RAC, FYN, HCK, LCK, LYN, c-SRC, c-YES, YRK, SRM
SYK	Spleen tyrosine kinase	SYK, ZAP-70
TEC	Transient erythroblastopenia of childhood related kinase	BMX/ETK, BTK, ITK/EMT/TSK,TEC,RLK/TXK

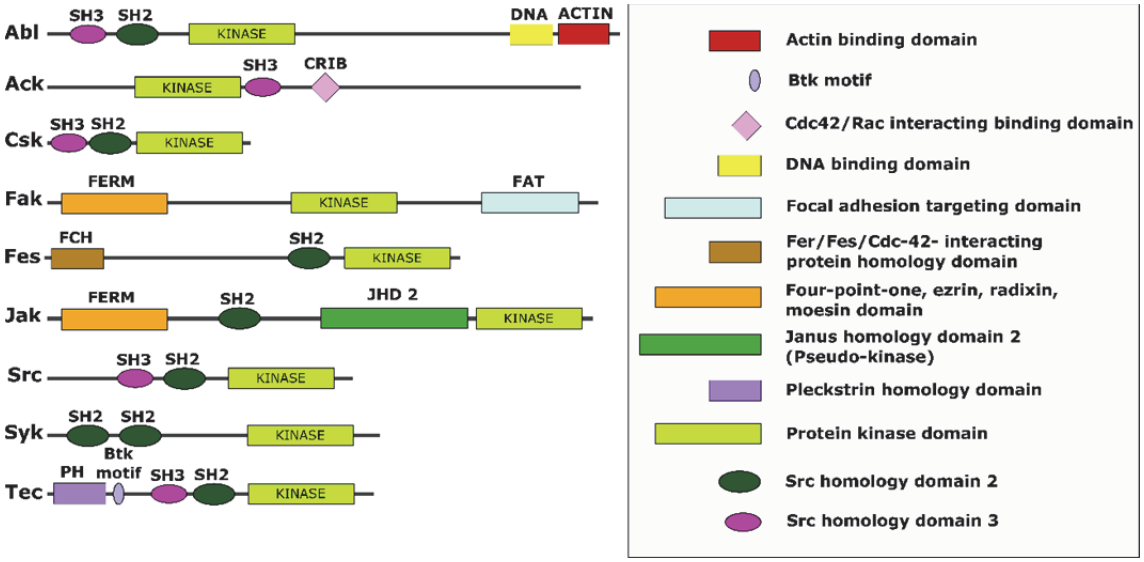

FIGURE 3 NRTK families' classification and domains organisation. *Left*- simplified representation of the structural architecture of all NRTK families. Each domain is represented with a specific colour. *Right*- extended names of each domain.

The SH2 domains are approximately 100 amino acids long and their main function is to bind to sequences containing phosphorylated Tyr residues. Since the SH2 domains are the main focus of this thesis, their structure and binding mechanism are described in detail in the next paragraph.

SH3 domains are normally approximately 60 amino acids long, and they recognise proline-rich sequences with the conserved motif PxxP which typically, but not always, adopt a polyproline type II helical conformation (Musacchio 2002, Saksela and Permi 2012, Kurochkina and Guha 2013). The structure of SH3 domains is formed by five to eight β-strands which can pack in the conformation of two antiparallel β-sheet or, alternatively, in a β-barrel (Musacchio 2002, Kurochkina and Guha 2013). The binding is mediated by the structured parts and some of the loops which confers a high specificity for the binding.

Pleckstrin homology (PH) domains were initially characterised as phosphoinositide binding domains. The PH domain is formed by seven antiparallel β-strands and a terminal α-helix, and its architecture resembles the phosphotyrosine binding (PTB) domain found in many signalling proteins (Scheffzek *et al.* 2012). Since their discovery, many structures of PH domains in complex with partners have been solved showing that they act mainly as protein-protein interactions mediators and that they have adapted, for instance, to interact with polyproline sequences, pTyr, small GTP-binding proteins and transcription factors (Scheffzek *et al.* 2012).

Four-point-one, ezrin, radixin, moesin (FERM) domains are present in diverse mammalian proteins, including those mentioned in the acronym FERM and also, for instance, talin, kindlin, guanine nucleotide exchange factors (GEFs), kinases and phosphatases. FERM domains are formed by three globular lobes indicated as F1, F2 and F3 oriented in the shape of a 'clover leaf' (Ceccarelli *et al.* 2006). F1 has a ubiquitin-like fold, F2 resembles an acyl-CoA binding domain, while F3 is similar to PH-PTB. FERM domains are generally regarded as membrane lipid and membrane protein interaction domains (Bosanquet *et al.* 2014) . In focal adhesion kinase (FAK) the FERM domain is involved in the auto-inhibition of FAK kinase (Frame *et al.* 2010).

1.1.5 Structure and interactions of Src homology 2 (SH2) domains

The SH2 domains have a very conserved structure formed by two α-helices flanking a central antiparallel β-sheet (Fig. 4) (Wagner *et al.* 2013). The SH2 domains are pTyr-binding domains which contain a positively charged binding pocket formed by αA helix and one side of the central β-sheet to allocate the negative charge of the pTyr . Specifically, Arg βB5 (strand B position 5 in β-sheet) and Arg αA2 contribute to counteract the pTyr negative charge. Instead, His βD4 coordinates the oxygen atoms of the pTyr. Mutations of those critical residues abolish the pTyr binding (Bibbins *et al.* 1993, Fütterer *et al.* 1998, Liu *et al.* 2012). The interaction of the pTyr provides half of the ligand binding energy (Liu *et al.* 2012). The other half depends on 1) a hydrophobic binding pocket defined by the helix αB and the opposite face of the β-sheet, and 2) interactions mediated by loop regions, also called the specificity pocket (Zhou and Abagyan 1998, Liu *et al.* 2012, Wagner *et al.* 2013).

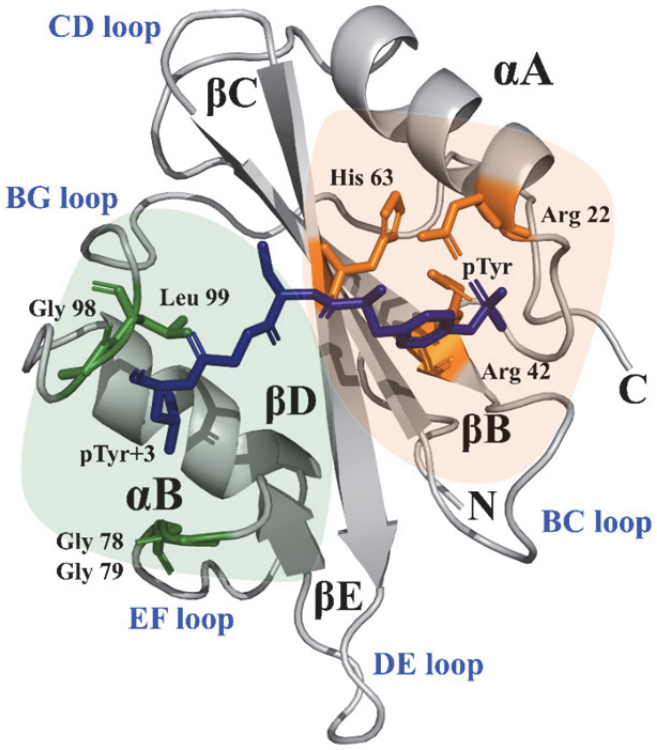

FIGURE 4 Crystal structure of N-SH2 domain of Syk (PDB: 1A81). In the figure, the formal names of conserved secondary structure components are indicated with black, while light blue is used for the loop regions. Orange is used to highlight the amino acids directly involved in the pTyr binding pocket (two Arg residues one from αA helix and βB strand, and an His in βD). Green is used for the amino acids in the hydrophobic pocket (Gly in EF loop and Gly plus Leu in BG loop). The phosphopeptide is represented in dark blue with stick visualisation mode; pTyr and pTyr+3 are marked.

A typical example of a SH2 binding ligand is the motif pYxxI/L, where a pTyr is followed by any two other residues plus a hydrophobic one (see also Section 1.2.2). The pTyr is accommodated in the positive charged pocket and the Ile/Leu residue in the hydrophobic pocket. In the case of the Syk N-terminal SH2 domain (N-SH2) (Fig. 4), side chains of Arg22 and Arg42 directly counteract the negative charge of the pTyr and His63 is responsible for hydrophobic interaction with the pTyr aromatic ring. Leucine in the position +3 in the motif is completely buried in the hydrophobic pocket formed by Gly78, Gly79 , Gly98 and Leu 99 (Fütterer *et al.* 1998). These residues are located in the loops after the βE strand and αB helix (Fütterer *et al.* 1998). Generally, in SH2 domains, further specificity for the interaction is provided by residues at positions -1/+1 to +7 from the pTyr. The interaction sites of these residues constitute the so called "specificity pocket" even though they form a rather large surface (Waksman and Kuriyan 2004, Filippakopoulos *et al.* 2009, Kaneko *et al.* 2010, 2012).

Even if SH2 domains can sometimes make low affinity interactions with non-phosphorylated Tyr (Poy *et al.* 1999), pTyr is normally needed for the

binding. However, the hydrophobic residue next to the pTyr is not that strictly conserved. An example is the growth factor receptor-bound protein 2 (Grb2) SH2 domain, which preferentially binds to the pYxNx because the Asn makes an interaction with a Trp in the DE loop (Luzy *et al.* 2008, Jadwin *et al.* 2018). Similarly, SH2 domains of the Crk adaptor protein and of Bruton's tyrosine kinase (Btk) bind the permissive sequence pYxxP. In this case, the amino acids between the pTyr and the Pro act as regulators of the binding. Some of them may act as non-permissive factors, which blocks the interaction of one of them and vice versa (Liu *et al.* 2012). It is interesting to note that proteins such as Syk, Zap-70 and Src homology 2-containing protein tyrosine phosphatase 2 (SHP-2 also known as tyrosine-protein phosphatase non-receptor type 11 PTPN11) contain two SH2s in tandem. As a consequence, they can bind two successive pTyr containing motifs resulting in a very high-affinity binding, at least 20 times higher compared with one SH2 domain bound to a single pTyr (Fütterer *et al.* 1998, Folmer *et al.* 2002, Kaneko *et al.* 2012, Grädler *et al.* 2013).

A recent study conducted with 76 different human SH2 domains demonstrated that SH2 domains can act as lipid-binding modules through alternate cationic patches (ACPs), which are electropositive surface areas with neighboring hydrophobic or aromatic residues (Park *et al.* 2016). These binding sites do not overlap with the pTyr binding sites and are often located on the opposite site or perpendicular to it. Interestingly, approximately 90 % of the tested SH2 domains bound plasma membrane lipids and 60 % showed binding specificity to phosphatidyl inositol lipids with K_D comparable to the specific lipid-binding PH domains (Park *et al.* 2016). It is likely that the ability of SH2 domains to bind phosphorylated lipids helps in their localisation near the membrane. Relevant to the present study, the lipid-binding motif in the C-terminal SH2 domain of Zap-70 is required for effective T-cell receptor (TCR) signalling in the cells (Park *et al.* 2016), and Syk has a similar motif (Park *et al.* 2016).

1.1.6 Regulation of non-receptor tyrosine kinases

The regulation of NRTKs activity can happen at many different levels. The kinase is normally kept in the inactive conformation and is only activated by specific intracellular signals. The activation steps are different based on the domain composition, the intra-domain interactions and the phosphorylation status of specific residues of the kinase. The first kinase to be discovered was Src and, since then, it has been acknowledged as the 'prototype' for understanding the regulation of other kinases (Boggon and Eck 2004). In Src family kinases, the kinase is kept closed through intramolecular interactions which involve both SH2 and SH3 domains. Specifically, the SH2 domain interacts with a C-terminal pTyr, whereas the SH3 domain is bound to a highly conserved proline-rich region in the linker between the SH2 domain and the kinase domain (Fig. 5, Panel A). As a consequence, the activation loop is in a non-permissive conformation for catalysis (Xu *et al.* 1997). The removal of that phosphorylation by protein phosphatase (Fig. 5, Panel A - step 1) initiates the

protein activation since the overall structure becomes more flexible and able to interact with binding partners through SH2 and SH3 domains (Bradshaw 2010). Furthermore, the phosphorylation of the activation loop is also needed for catalysis (Boggon and Eck 2004, Bradshaw 2010) (Fig. 5, Panel A - Step 2) . The activation of the Src kinase has been described as 'turned on by touch' (Boggon and Eck 2004) or 'graded switch' activation (Bradshaw 2010). Turning the Src kinase on by touch means that many different interactions are known to influence the activation of the kinase. The interaction the C-terminal pTyr with the Src SH2 domain can be competed for by other pTyr containing sequences. For instance, Src can interact with the pTyr of the PDGF (platelet-derived growth factors) receptor, and this interaction competes with the intramolecular SH2/C-terminal pTyr interaction and leads to the activation of Src (Kypta *et al.* 1990, Alonso *et al.* 1995) (Fig. 5, Panel A - Step 3). On the other hand, Src family kinases can be activated by displacement of their SH3 domain from the proline-rich linker, for instance by other proline-rich sequences, as found in the case of the HI-virus protein Nef (Moarefi *et al.* 1997) (Fig. 5, Panel A - Step 4). The graded activation concept of Src family kinases means that full biochemical activation only takes place when all of these interactions and modifications have been completed (Bradshaw 2010) (Fig. 4). In a cellular context, it is possible, however, that even partial activation can lead to an activity level which is over the threshold of biological function.

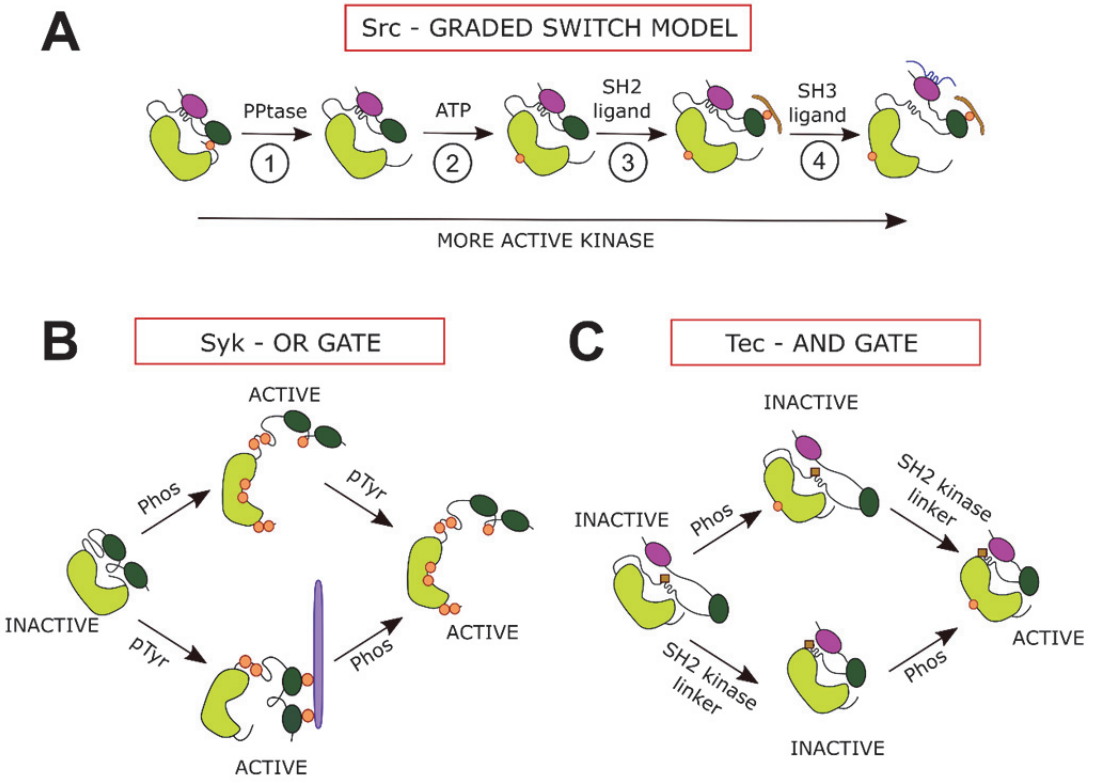

FIGURE 5 Proposed mechanisms for NRTK activation. SH2 domains are represented in dark green, SH3 in pink and kinase domain in light green. Phosphorylation sites are marked with orange circles. **A-** The 'graded

switch' model is proposed for Src family kinases; the kinase passes through different partially active states before being fully active. Each step is marked with a number in the figure. **B-** In the 'OR gate' model, the phosphorylation of specific sites along the structure (mediated by other kinases or by auto/transphosphorylation) or the interaction with pTyr in the receptor lead to a fully active kinase. **C-** In the 'AND gate' model both the phosphorylation and the structural rearrangements should take place to activate the kinase.

As in Src family kinases, in Syk family kinases the inactive, closed conformation is maintained by intra-molecular interactions between linker sequences and the kinase domain (Grädler *et al.* 2013, Yan *et al.* 2013). Differently from Src kinases, the Syk kinases do not contain a SH3 domain which would interact with proline-rich sequences in the linker between the regulatory domains and the kinase domain. Instead, two Syk linker sequences are involved in interactions between the regulatory domain and the kinase domain. The activation mechanism of Syk is described as the 'OR gate' switch (Bradshaw 2010). This means that either interaction between the two SH2 domains with double phosphorylated Tyr or phosphorylation of the linker and kinase domain in Syk can lead to full activation of the kinase (Papp *et al.* 2007, Tsang *et al.* 2008) (Fig. 5). Significantly, the phosphorylation of the activation loop tyrosine seems not to be required for Syk activity (Papp *et al.* 2007). Further structural details of Syk family kinases will be discussed in the next chapter.

Differently, the Tec family kinases show another activation mechanism, which is described as the 'AND gate' model. Both 'OR gate' and 'AND gate' mechanisms imply that the kinase switches from active to inactive conformation in an 'all-or-none' way, but the main difference is that in the 'OR gate' model, just one of the stimuli is needed to switch on the kinase, while in the 'AND gate' model more than one stimulus should simultaneously occur to make the kinase active (Bradshaw 2010). In fact, in Tec kinases both phosphorylation of the activation loop and a proper docking of a specific linker region to the kinase domain are needed (Fig. 5); this activation mechanism appears still partially unclear since the structure of the full-length Tec kinase is not available.

The above described activation mechanism suggests at least two alternative routes for NRTK activation. The first is mediated by allosteric interactions to the back of the kinase domain, and these interactions keep the kinase in an inactive state. The second mechanism is represented by the phosphorylation of the kinase. The regulatory domains of the kinase can participate in these two routes either by contributing to the allosteric interaction or by recruiting the kinase in complexes where it can be phosphorylated.

Interestingly, in same cases, the SH2 and SH3 domains involved in the activation procedure, might regulate the kinase activity (Filippakopoulos *et al.* 2009). The SH2 domain in the Src kinase has no interactions with the kinase domain, and deletion of both SH2 and SH3 have very little influence on the kinase activity (Xu and Miller 1996). Similarly, the deletion of tandem SH2 domains from Syk kinase does not affect the kinase activity (Papp *et al.* 2007). Conversely, the same deletion in the kinase Csk has a drastic effect on the

enzymatic activity. This is due to the interactions between the SH2-kinase linker, SH2, SH3 and the kinase domain (Shekhtman *et al.* 2001; Cole *et al.* 2003). The presence of SH2 seems to be important also for the kinase activity of all members of the Tec family (Joseph *et al.* 2007).

1.2 Spleen tyrosine kinase

1.2.1 Spleen tyrosine kinase structure and activation

Spleen tyrosine kinase, Syk, is a NRTK discovered in bovine thymus (Yang *et al.* 1994). It is a 72 kDa protein formed by two SH2 domains and a kinase domain. The SH2 domains are named N-SH2 and C-SH2 based on the position in their protein sequence; they are connected by a partially flexible region called Interdomain A (IA). The second SH2 domain, C-SH2, is linked to the kinase domain via a second linker region called Interdomain B (IB) (Grädler *et al.* 2013) (Fig. 6, Panel A). There is a short version of Syk kinase called Syk B, which differs from the long one due to the lack of 23 amino acids in the IB sequence (Fig. 6, Panel A). The role of Syk B is still to be discovered, but it is interesting to note that comparing the Zap-70 and Syk B sequences shows that they both lack amino acids in the same region. They also share a more limited cell type distribution compared with Syk (Latour *et al.* 1998).

A general description of the Syk autoinhibited form has already been given in Section 1.1.6. In the 'closed' Syk conformation, the two SH2 domains keep the IA and IB regions packed towards the kinase domain (Fig. 6, Panel B). Specifically, Tyr630 and Tyr631 of the kinase domain form hydrogen bonds to Ile148 and Ser149 in the second α-helix (αL2) of IA; conversely Tyr629 is not involved in any interaction thus indicating that the phosphorylation of Tyr 630 and 631 are critical in Syk activation (Grädler *et al.* 2013) (Fig. 6, Panels C and D). Moreover, Tyr348 and Tyr352 located in IB stabilise the interactions with IA and the kinase domain (Grädler *et al.* 2013). Tyr348 forms a hydrogen bond with the carbonyl group of Ala142, which is located in IA, and Tyr352 forms a hydrogen bond with Arg359, located in the kinase domain. Tyr348 and Tyr352 play a critical role in Syk activation (Tsang *et al.* 2008, Grädler *et al.* 2013) (Fig. 6, Panel D), and they are phosphorylated in the early stages of Syk activation. Mutation of these two Tyr to Phe causes a defect in the Syk activation (Grädler *et al.* 2013). In a similar fashion, the closed conformation of Zap-70, a paralogue of Syk, is sustained by similar intra-molecular interactions. Tyr315 and Tyr319, which correspond to 348 and 352 in the Syk sequence, are involved in an aromatic π-stacking interaction, and mutation of those Tyr to Phe stabilised the inactive conformation (Jin *et al.* 2004, Au-Yeung *et al.* 2009, Yan *et al.* 2013). Moreover, the critical Pro147 in IA region keeps Zap-70 in a closed conformation, locked in a cleft between Tyr597 and Tyr598 in the C-lobe of the kinase domain. There is one marked difference between the closed conformation of Syk and Zap-70. In Syk the IA segment makes a hydrogen

bond with the non-phosphorylated tyrosine residue close to the C-terminus of the kinase domain. This interaction is not seen in the structure of Zap-70 (Au-Yeung *et al.* 2009, Grädler *et al.* 2013).

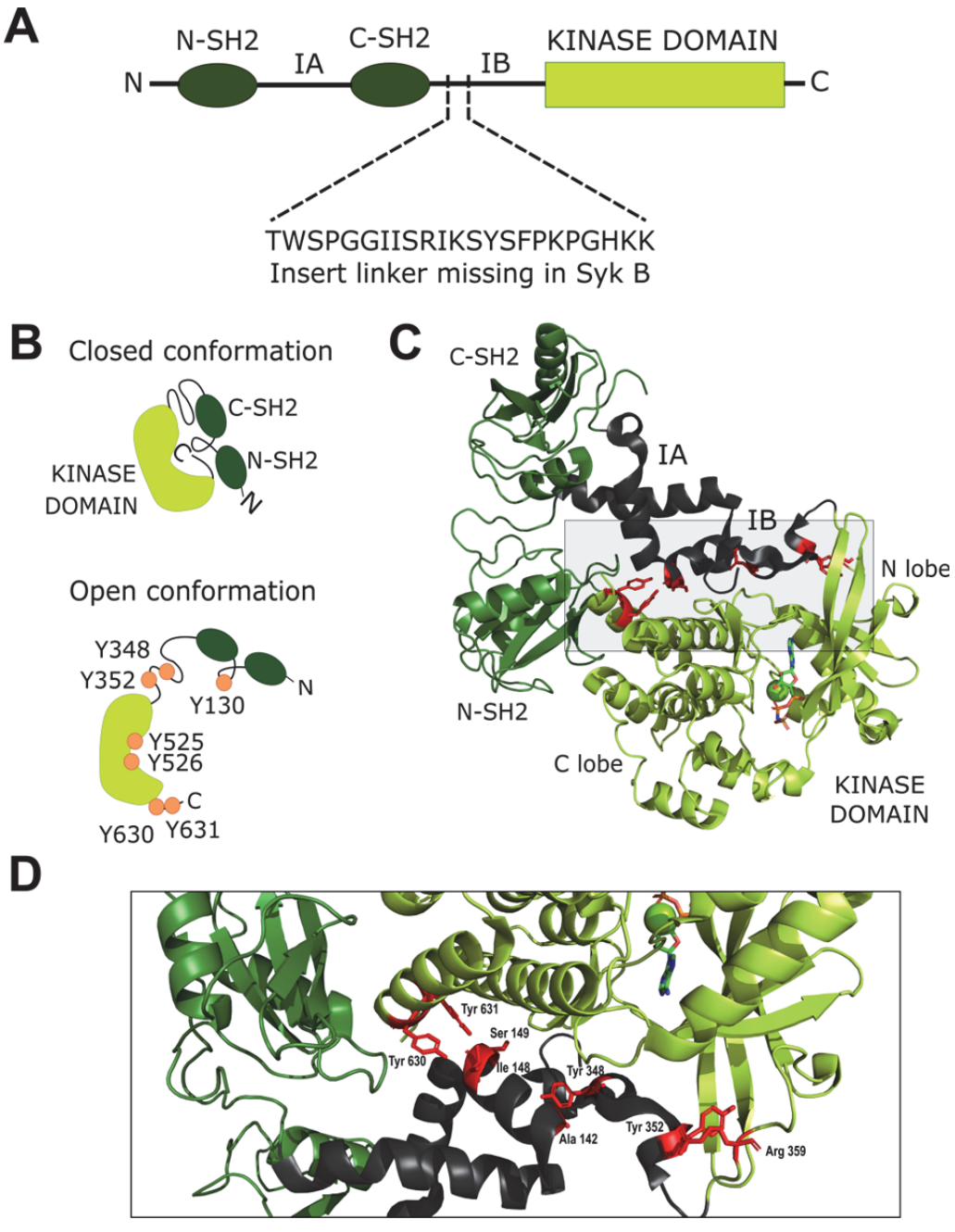

FIGURE 6 **A-** Schematic representation of Syk structure. Syk is formed by two tandem SH2 domains (called N-SH2 and C-SH2) represented in dark green and a kinase domain in light green. Tandem SH2s are connected via a partially structured region called Interdomain A (IA). The C-SH2 is connected to the kinase domain via a second flexible region called Interdomain B (IB). A shorter version of Syk, called SykB, lacks 23 amino acids in the IB sequence. **B-** Syk domains orientation in closed and open conformation. The closed

conformation is kept via intramolecular interactions which involve the C-terminus and the linker regions IA and IB. In the open Syk, seven important phosphorylation (orange circles) sites are indicated. **C**- Crystal structure of Syk closed conformation (PDB: 4FL2) (Grädler *et al.* 2013). The same colours are used to indicate the Syk structural components; all structures are well defined except for the IB. The kinase domain is also divided into a N-lobe and a C-lobe. Red is used to mark the residues involved in the intramolecular interaction which keep Syk in the closed conformation. The grey square indicates the zoomed-in region visible in the next Panel. **D**- Intramolecular interaction in Syk closed conformation. C-terminal Tyr 630 and Tyr 631 interact with Ile 148 and Ser 149. Tyr 348 forms a hydrogen bond with the carbonyl group of Ala 142, while Tyr 352 interacts with Arg 359.

The idea of the 'OR-gate' activation mechanism of Syk is based on the fact that the interaction of the IA and IB segments with the kinase domain can be disrupted either by interaction of the SH2 domains with pITAM sequences or by phosphorylation of the linker and kinase domain Tyr residues. The activation mediated by interaction with pITAM implies large structural rearrangements which reorient the SH2 domain and IA so that they can no longer block the kinase domain. The outcome of phosphorylation of several Tyr residues is expected to be very similar to changes in the SH2 orientation: the pTyr residues block the packing of IA and IB segments on the kinase domain (Papp *et al.* 2007, Tsang *et al.* 2008, Falet *et al.* 2010) (Figure 5, Panels B and D). There are at least 6 important Tyr residues which are phosphorylated during the activation (Fig. 6, Panel B). In particular, Tyr348 and Tyr352 in IB, as well as Tyr630 and Tyr631, are phosphorylated at an early stage during the activation process since they are involved in intra-molecular interactions to keep the protein in an inactive conformation (see above) (De Castro *et al.* 2010, Grädler *et al.* 2013). Instead, the phosphorylation of Tyr526 and Tyr526 in the activation loop occurs later, and it has been shown that kinase activity is not directly dependent on the phosphorylation of those sites (Tsang *et al.* 2008). Both Syk and Zap-70 have the ability of auto/trans phosphorylation, but, at the same time, it is known that they can be phosphorylated by Src family kinases which are recruited together with them (Au-Yeung *et al.* 2009, Mócsai *et al.* 2010).

Tyr 130, together with Tyr323, plays an important role in the regulation of Syk activity. The mutation of Tyr130 to Glu, a phospho-mimicking mutation, reduced the interaction time between tandem SH2 and pITAM. It resulted in partially impaired cell signalling compared with the Syk wild type (Keshvara *et al.* 1997, Schwartz *et al.* 2017). NMR studies also showed that this mutation alters the mutual orientation of SH2 domains, and they proposed that the phosphorylation of this site induces the release of Syk from pITAM (Yuzawa *et al.* 2007, Feng and Post 2016). This is in line with the fact that Syk, after its activation, should be released into the cytosol in the active form and bind to its partners. Tyr323 appears to be important in Syk degradation. Phosphorylated Tyr 323 is recognised by E3 ubiquitin ligase CBL which is responsible for Syk degradation (Yankee *et al.* 1999, Hunter 2007).

In addition to the role in Syk regulation, phosphorylation of specific tyrosines along the Syk structure is also important for recruitment of binding partners (Furlong *et al.* 1997, Zhang *et al.* 2008, De Castro *et al.* 2010, Mócsai *et al.* 2010). Phosphorylated Tyr323 is recognized by the C-terminal SH2 domain of PI3K regulatory subunit p85α, and the N-terminal SH2 domain of p85α binds to pTyr348 and pTyr352 (Seda and Mraz 2015). Tyr348 is also recognised by the SH2 domain of the adaptor protein VAV1 (Schymeinsky *et al.* 2006). Phosphorylated Tyr630 of Syk mediates the interaction with the adaptor protein SLP-76. It is thought that the binding to SLP-76 acts as an allosteric regulation of Syk activation, since the binding of SLP-76 prevents the Tyr630 from being dephosphorylated and mediates the closed, autoinhibited conformation (De Castro *et al.* 2010).

1.2.2 Syk and ITAM signalling

The ITAM sequence was introduced in Section 1.1.5. Here a more detailed description of ITAM containing receptors and the signalling pathway is given.

ITAM was identified in 1989 based on sequence comparison (Reth 1989). ITAM is present in the cytoplasmic tail of membrane receptors (such as FcγRIIA) (Fig. 7), in a separate transmembrane adaptor associated with the receptor (such as a T cell receptor) (Fig. 7), or in a membrane-associated cytoplasmic protein (such as the ERM family) (Underhill and Goodridge 2007). ITAM receptors are expressed in many hematopoietic cells such as T and B cells, natural killer (NK) cells, macrophages, dendritic cells and platelets (Love and Hayes 2010). The TCR is formed by two subunits, either TCRα and TCRβ, or TCRδ and TCRγ, which lack the intracellular signalling region. This dimer associates with signal transducing subunits CD3γ, CD3δ, CD3ε and TCRζ (Love and Hayes 2010). CD3γ, CD3δ and CD3ε contain one ITAM sequence and TCRζ contains three. Therefore, the TCR complex contains 10 ITAM sequences in total (Love and Hayes 2010). Based on the accepted model for TCR activation, ITAM signalling is initiated by interaction with the ligand and subsequent clustering of the receptors (Love *et al.* 2000). This rapidly results in the Src kinase family-mediated phosphorylation of the ITAM motif in the transducing subunits which enables the recruitment of other signalling components, such as Syk kinase and Zap-70 (Chan *et al.* 1992, Latour *et al.* 1997). Zap-70 has a role restricted to T and B cells and NK cells different from Syk which is widely expressed throughout the hemopoietic system (Chan *et al.* 1992, Mócsai *et al.* 2010, Wang *et al.* 2010).

Syk and Zap-70 phosphorylate different proteins in the cytosol, such as the linker of activation of the T-cells (LAT) and the SH2 containing leukocyte protein of 76 kDa (SLP-76) which mediate the activation of phosphatidylinositol 3-kinase (PI3K), phospholipase Cγ (PLCγ) and other pathways (Chan *et al.* 1992, Underhill and Goodridge 2007) (Fig. 7). The presence of many chains containing an ITAM sequence in TCR is not redundant since each chain is preferentially recognised by different proteins. For example, the adaptor protein Grb2 and the

SHC-transforming protein 1 (Shc1) binds to CD3γ and δ but not to ε (Osman *et al.* 1996).

In the B cell receptor (BCR), as in TCR, ITAMs are present in the transducing transmembrane subunits. Signalling mediated by phosphorylation of ITAM is important for B-cell maturation and differentiation. Syk is required for BCR signalling and Syk-deficient mice lack mature B cells (Cheng *et al.* 1995, Turner *et al.* 1995). Studies with conditional knock out mouse models show that Syk is also required for BCR signalling in mature B cells (Ackermann *et al.* 2015) and that Zap-70 can partially substitute for Syk function in mature B cells (Fallah-Arani *et al.* 2008).

Different from TCR and BCR, a receptor for the immunoglobulin constant region (FcR), FcγRIIA, has ITAM in its cytoplasmic domain. Other Fc receptors such as FcεRI associate with transmembrane ITAM adaptors. FcRs are responsible for antibody-dependent cell-mediated cytotoxicity in natural killer cells and activation signalling in myeloid cells inducing phagocytosis, ROS and cytokine production and degranulation (Underhill and Goodridge 2007). FcRs are good examples of ITAM sequences which can behave as activators or inhibitors of cell signalling. FcαRI mediates degranulation in mast cells in the case of receptor clustering, but in the presence of a monomeric ligand it has an inhibitory response (Pasquier *et al.* 2005). An opposite behaviour is observed for FcεRI (Xiao *et al.* 2005).

In the case of inhibitory stimuli, the ITAM sequence has been identified as an Immunoreceptor tyrosine-based inhibitory (ITIM) motif (Barrow and Trowsdale 2006). One example is the association of protein phosphates, such as SHP-1, SHP-2 and SHIP, to ITIM sequences with FcRs (Kimura *et al.* 1997, Tamir *et al.* 2000). The mechanisms which cause the FcRs to initiate activating or inhibitory responses are still mostly unclear, but it has been shown that inhibitory response are associated with low levels of Syk activity and activation of extracellular signal-regulated kinases (ERKs) and mitogen-activated protein (MAP) kinases (Pasquier *et al.* 2005).

Syk also interacts with single pTyr ITAM motifs, called hemITAMs which are normally found in C-type lectin (CLEC) receptors (Bauer and Steinle 2017). The interaction between Syk and CLEC most likely occurs through the involvement of two CLEC receptors, each one providing one pTyr (stoichiometry 2:1). Those receptors participate in innate immunity responses in dendritic cells, macrophages and B cells (Chiffoleau 2018). Syk activation is important for recruitment of caspase-recruitment domain (CARD) which lead to nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) response. Moreover, it seems that the Syk-mediated signalling downstream CLEC2 receptors in platelets might relate to the Syk $^{-/-}$ fatal phonotype. The possible explanation is that the foetuses have a deficiency in separation between lymphatic and blood vessels (Abtahian *et al.* 2003, Suzuki-Inoue *et al.* 2006, Futosi and Mócsai 2016).

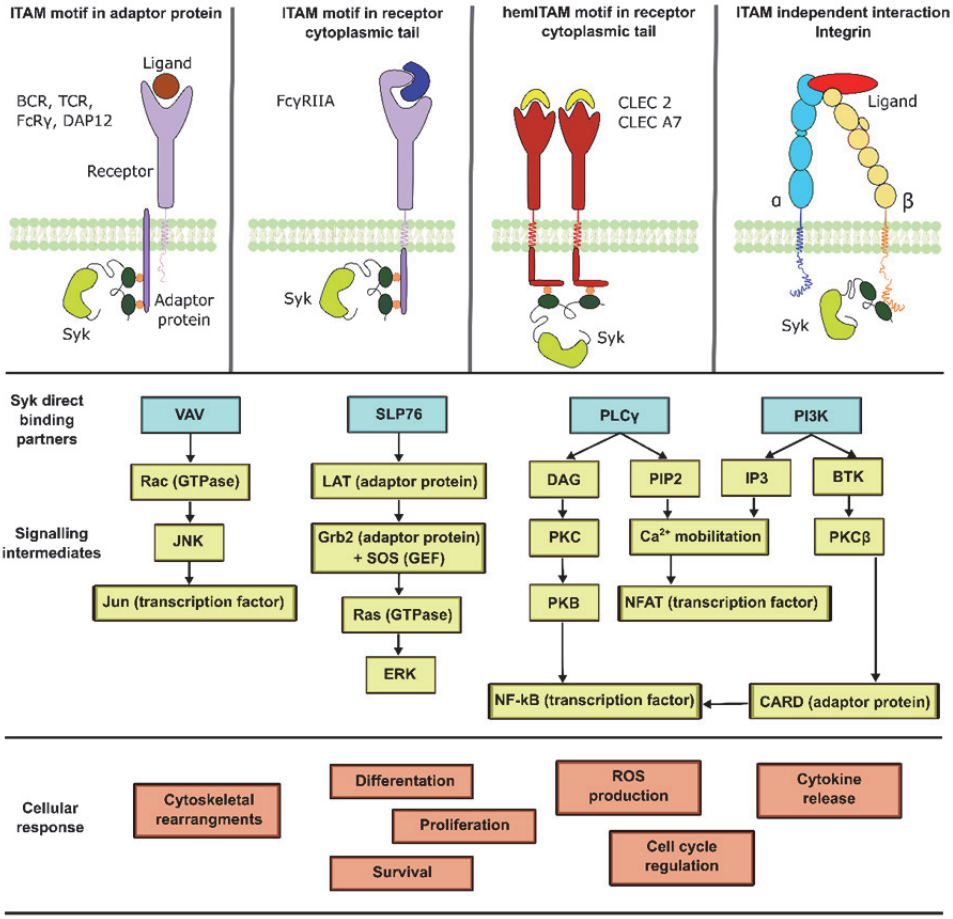

FIGURE 7 Syk activation mechanism mediated by ITAM and integrin receptors. Syk is recruited by the ITAM motif in the cytoplasmic domain of the receptor or in adaptor proteins associated with the receptors. Alternatively, Syk can interact with the hemi-phosphorylated immunoreceptor tyrosine-based activation motif (hemITAM) which includes just one pYxxL/I motif, so that two receptors are needed to make the complex with Syk. Conversely, the ITAM-independent interaction is mediated by the integrin β cytoplasmic domain and it is phosphorylation independent. Syk activation is mediated by structural rearrangement and phosphorylation of specific sites along its structure. Src family kinases are recruited together with Syk to enforce the activation process. When active, Syk has four known direct binding partners: VAV1 and 3, PLCγ, PI3K and SLP76. These interactions lead to different cellular responses which are finely regulated through the recruitment of different components (signalling intermediates). BCR, B-Cell Receptor; BTK, Bruton Tyrosine Kinase; CARD, Caspase-Recruitment Domain; CLEC, C-type Lectine; DAG, diacylglycerol; DAP12, DNAX activating protein of 12 kDa; ERK, Extracellular signal-Regulated Kinase; FCR, FC Receptor; Grb2, Growth factor Receptor-Bound protein 2; JNK, c-Jun N-terminal Kinase; IP3, Inositol triphosphate; LAT, Linker for Activation of T-cells; NFAT, Nuclear Factor of Actived T-cells; NF-kb, Nuclear Factor k-light-chain-enhancer of activated B-cells; PI3K, Phosphoinositide 3-kinase; PIP2, Phosphatidylinosoitol biphosphate; PKB, Protein Kinase B; PKC, Protein Kinase C; PKCβ, Protein Kinase C β; PLCγ, Phospholipase Cγ; Rac, Ras-related C3 botulinum toxin substrate; Ras, Rat Sarcoma protein; ROS, Reacting Oxygen Species; SLP76, SH2 domain-containing leukocyte protein 76; TCR, T-Cell Receptor.

1.2.3 Integrin adhesion receptors and Syk

Integrins are a family of transmembrane cell adhesion receptors (Anthis and Campbell 2011, Durrant *et al.* 2017). They are formed by two non-covalently associated glycoprotein subunits called α and β (Hynes 1987, 2002, Harburger and Calderwood 2009, Morse *et al.* 2014). Humans have 18 α and 8 β subunit which combine to form 24 distinct heterodimeric integrins. Each subunit contains an ectodomain, a transmembrane helix and, typically, a short cytoplasmic domain (from 20 to 60 amino acids, with the exception of the β_4 cytoplasmic domain, which is approximately 1000 amino acids long). The cytoplasmic domains of the α subunits are quite divergent and contain only a short conserved GFFKR motif near the membrane. Instead, most β subunit cytoplasmic domains are highly similar and contain two copies of a conserved NPxY motif. Despite their short sequence length, at least 40 direct interactors have been reported for integrin tails, and they can be classified as activators, inactivators, signalling adaptors and endosomal trafficking regulators (Morse *et al.* 2014)

Two of the main activators of integrin function, the FERM-domain containing cytoplasmic proteins talin and kindlin, bind to the conserved NPxY motifs of the integrin β tails (Calderwood *et al.* 2003, Li *et al.* 2017). These two proteins are involved in two phases of integrin function, called the 'inside-out' and 'outside-in' signalling. The 'inside-out' phase describes the intracellular events which lead to a higher affinity of integrins towards their extracellular ligands. Integrins are known to reside in a bent, inactive conformation, and they acquire active, straightened conformation when cytoplasmic tails separate in response to intracellular interactions (Hynes 2002). Integrin 'outside-in' signalling refers to responses initiated by the attachment of integrin to their extracellular ligands. This is associated with integrin clustering, assembly of intracellular adhesion complex, actin cytoskeleton organisation and generation of intracellular signals (Morse *et al.* 2014).

Kinases play a crucial role in integrin 'outside-in' signalling. Src family kinases bind directly to the integrin β_3 chain cytoplasmic domain via an interaction between the last seven amino acids of the integrin (including the second NPxY motif) and the polyproline-binding surface of the Src SH3 domain (Eckstein and Tsafriri 1986, Arias-Salgado *et al.* 2005, Katyal *et al.* 2013, Wu *et al.* 2015). Similar to polyproline-containing sequences, interaction with integrin tails can activate Src, even though integrin binding has a relatively low affinity and apparently clustering and further scaffolding interactions are involved (Wu *et al.* 2015). The importance of the role of Src family kinases in many integrin functions is demonstrated by a lack of cell adhesion-induced tyrosine phosphorylation in cells derived from mice lacking Src, Yes and Fyn kinases (Klinghoffer et al., 1999). However, the assembly of cell adhesion complexes is not inhibited in the absence of these three Src-family kinases (Klinghoffer *et al.* 1999) or in the presence of Src-inhibitors (Horton *et al.* 2016). One of the major substrates of Src in integrin adhesion complexes is the NRTK called the focal

adhesion kinase (FAK) (see Chapter 1.1.4), and its close homologue proline-rich tyrosine kinase 2 (PYK2), which is activated downstream of integrin function (Schaller 2010). Src- and FAK- family kinases are involved in several signalling events downstream of integrins, including cell cytoskeleton organisation, cell metabolism, proliferation, inhibition of apoptosis, and differentiation (Humphries *et al.* 2006). During cell spreading and migration, FAK is specifically involved in the disassembly of adhesion complexes (Ilić *et al.* 1995). The molecular mechanism of FAK kinase recruitment in the integrin transduction has been controversial. Specifically, it has been proposed that the FAK kinase interacts with the integrin cytoplasmic domain via talin-dependent and -independent mechanisms (Serrels and Frame 2012). Moreover, FAK recruitment might be different when considering the early or late stage of focal adhesion machinery assembly (Lawson *et al.* 2012). An important regulator of FAK recruitment, as well as of other signalling proteins such as talin, is the phosphatidylinositol- 4,5 bisphosphate (PIP2) (Goñi *et al.* 2014). It seems that FAK can interact with its FERM domain to PIP2, and this triggers FAK activation via the decoupling of the FERM domain and the kinase domain. Moreover, PIP2, in high concentration, can mediate the FAK activation by a clustering effect (Goñi *et al.* 2014). The FAK kinase represents a critical step in integrin signaling, especially considering that it acts as a kinase as well as an adaptor protein. One possible mechanism for integrin adhesion complex disassembly is phosphorylation of the two NPxY motifs in the integrin β tail. Phosphorylated integrin is unable to bind talin, kindlin or Src (García-Alvarez *et al.* 2003, Wu *et al.* 2015, Li *et al.* 2017).

Syk kinase is also involved in integrin signalling by direct interaction with the integrin β subunit cytoplasmic tail (Woodside *et al.* 2001, 2002). Syk and Zap-70 can bind to β_1, β_2 and β_3 with high affinity. The binding is mediated by N-SH2 plus the IA in both Syk and Zap-70. This is confirmed by IA domain-swapping experiments. The protein phosphates SHP-1 and 2, which contain tandem SH2, do not bind to integrin, most likely because the linker between the two SH2 domains in SHP 1 and 2 is much longer than in Syk and Zap-70. The substitution of the SHP linker with the one from Syk makes SHP able to bind the integrins (Woodside *et al.* 2002). The last four C-terminal residues of the integrin tail are needed for the binding, as well as both NPxY motif sequences (Woodside *et al.* 2001). Syk interaction with the β3 cytoplasmic tail is impaired if the Tyr747, located in the N-terminal NPxY motif, in integrin sequence is mutated to Ala (Woodside *et al.* 2002). It is worth noting that the last three amino acids of the β_3 cytoplasmic domain are needed for the interaction with c-Src and Syk in osteoclasts (Woodside *et al.* 2002, Zou *et al.* 2007). Moreover, in contrast to pITAM binding, the interaction of Syk and Zap-70 with the integrin tail does not require the phosphorylation.

The functional role of Syk-integrin interaction has been mostly studied in platelets because of the high expression of Syk in hematopoietic cells and because platelets express a very high density of $\alpha_{IIb}\beta_3$ integrin on their surface and the signalling related to $\alpha_{IIb}\beta_3$ is one of the most well characterised (Clark *et*

al. 1994, Shattil and Newman 2004, Durrant *et al.* 2017). The main platelet function, aggregation, is mediated by the interaction of the integrin $\alpha_{IIb}\beta_3$ with fibrinogen. This is initiated by complex signalling mediated by G-protein coupled receptors, von Willebrand factor binding receptors, collagen binding receptors (including $\alpha_2\beta_1$ integrin and FcRγ) and, for instance, the FcγRIIA receptor (which contains ITAM). In some cases, $\alpha_{IIb}\beta_3$ alone can initiate the final platelet activation response, but typically in vivo activation requires several signalling steps (Estevez and Du 2017). Src, Lyn and Fyn kinases are involved in the $\alpha_{IIb}\beta_3$ receptors downstream signalling in platelet in concert with the Syk kinase. Clearly, Syk-/- platelets spread poorly on fibrinogen; this is probably due to a compromised outside-in signalling from $\alpha_{IIb}\beta_3$ and the lack of a coordinated response between $\alpha_{IIb}\beta_3$ and FcγRIIA receptors and between Syk and Src kinases (Boylan *et al.* 2008). This has been tested in transgenic mice where FcγRIIA was expressed and compared with wild type mice which do not normally express this receptor. The outcome was that platelets from transgenic mice spread more efficiently on fibrinogen with a robust activation of Syk kinase compared with the wild type (Zhi *et al.* 2013). Moreover, the Syk null phenotype was also associated with the lack of Vav1, Vav3 and SLP-76 recruitment, demonstrating that Syk plays a role in coordinating the integrin response (Law *et al.* 1999, Obergfell *et al.* 2002). Vav1/3 are needed for activation of PLCγ2, which is important for then activating the protein kinase C (PKC) for Ca^{2+} mobilisation in platelets (Shih *et al.* 2014, Durrant *et al.* 2017). Syk is also activated downstream of the collagen-binding integrin $\alpha_2\beta_1$ and it induces activation of PLCγ2. This process also involves the ITAM receptor FcRγII (Keely and Parise 1996). On the other hand, Syk signalling downstream of $\alpha_{IIb}\beta_3$ integrins does not require pTyr binding to Syk as pTyr binding blocking mutations in Syk do not inhibit Syk activation downstream $\alpha_{IIb}\beta_3$ even though they block FcR signalling (Hughes *et al.* 2015).

In neutrophils integrin signalling involves Syk activation. Integrins take part in the late stage of neutrophil adhesion to the endothelial cells and their transmigration in the inflammation site (Futosi and Mócsai 2016). Neutrophils express integrins $\alpha_L\beta_2$ (LFA-1) and $\alpha_M\beta_2$ (Mac-1) which bind to the intercellular adhesion molecule 1 (ICAM-1) present on the endothelial surface (Mócsai *et al.* 2015). Normally, neutrophils are recruited to the inflammatory site via a multi-step process. In the early stage, the neutrophils rolling in the intravascular space is slowed down by the interaction with L- or P- selectin and pITAM receptors. The early activation induces the inside-out integrin signalling which increases the affinity of the integrins towards ICAM-1. Subsequently, the complete activation of the integrin permits a stable adhesion of neutrophils on the endothelium surface and the transmigration in the inflammatory site, as well as the granules release and respiratory burst (Futosi and Mócsai 2016). Integrin signalling in neutrophils involves the Syk kinase and the Src-family kinases Hck, Fgr and Lyn (Mohn *et al.* 1995, Yan *et al.* 1995). Syk $^{-/-}$ neutrophils do not spread on fibrinogen and ICAM-1, and they show defects in granules release and respiratory burst. This phenotype is similar to that observed in

absence of Hck, Fgr and Lyn (Vines *et al.* 2001, Mócsai *et al.* 2002). The integrin signalling requires the recruitment of SLP-76, Vav1/3 and Src-family kinases; knock-out of each of those proteins resulted in a similar phenotype with impaired integrin-mediated adhesion and cellular response (Newbrough *et al.* 2003, Volmering *et al.* 2016). As for platelets, Syk is important for coordinating integrin outside-in signalling. Interestingly, knock-out of FcRγ and DAP12 pITAM receptors result in an impaired integrin signalling showing a close correlation between these two pathways (Mócsai *et al.* 1999, 2006). Syk is also activated by integrin β_1. For example, in monocytic cells lacking Syk, stimulation of β_1 with antibodies results in an impaired respiratory burst and activation of NF-kB (Tsung *et al.* 1995; Mócsai *et al.* 2002).

Syk kinase is involved in osteoclasts signalling. Osteoclasts are bone-resorbing cells. To initiate the process of bone-resorption, osteoclasts need to make contact with the bone matrix and establish the resorption compartment (Xu and Teitelbaum 2013). To do that, integrin $\alpha_v\beta_3$ interacts with different ligands in the bone matrix, such as osteopontin, vitronectin and sialoprotein (Nakamura *et al.* 2007). $Syk^{-/-}$ osteoclasts look smaller and show a crenated appearance because of defects in actin ring formation which are needed to isolate the resorption compartment (Zou *et al.* 2007). This is probably due to the lack of Syk association with guanine-exchange factor Vav3 which, in turn, activates the GTPase Rac. Rac is expressed in the osteoclasts and it is required for the cytoskeleton organization mediated by integrins (Zou *et al.* 2007). Syk kinase interacts with SLP-76 in the osteoclasts, and it is important for successive recruitment of Vav3 (Reeve *et al.* 2009). Remarkably, the lack of Syk causes similar effect as the lack of c-Src and $\alpha_v\beta_3$ suggesting the fact that those work together in mature osteoclasts (Zou *et al.* 2007). Moreover, in osteoclasts Syk activation mediated by integrin seems to be dependent on DAP12 and FcRγ - which contain ITAM motif - as Syk was not phosphorylated in cells lacking those receptors (Mócsai *et al.* 2004). These observation lead to the hypothesis that in the osteoclasts Syk might act as a bridge between integrin and pITAM and its activation is promoted by the presence of the c-Src kinase (Zou *et al.* 2007). This model is also supported by the fact that substitution of Syk with Zap-70 in osteoclasts resulted in abnormalities in cytoskeleton organization under $\alpha_v\beta_3$ stimulation (Zou *et al.* 2013). Most likely, this is due to the less flexible structure of Zap-70 compared with Syk kinase. It is worth mentioning that the lack of DAP12 and FcRγ in the osteoclasts is associated with the absence of osteoclasts maturation. This phenotype is different from the one observed when integrin $\alpha_v\beta_3$ is absent, suggesting that integrins are not involved in the osteoclasts differentiation (Zou *et al.* 2007). Interestingly, the double mutation of Tyr747 and Tyr759 to Phe in integrin β_3 cytoplasmic domain alters the platelets function, but has no effect on osteoclasts (Feng *et al.* 2001). This suggests that Syk activation might have a different mechanism in platelets and osteoclasts.

2 AIMS OF THE WORK

It is well established that Syk can be activated by ITAM receptors and by integrins and that ITAM- and integrin-mediated Syk activation is physiologically relevant. While the molecular details of Syk activation by ITAM receptors are well known, the details of Syk-integrin interactions are unclear. Furthermore, in some systems, Syk interaction with both ITAM and integrins is required for proper signalling, whereas in other systems integrins can activate Syk independently of ITAM. To clarify these open questions the aims of this thesis were:

- to find out if Syk is activated by integrins via a similar mechanism as by ITAM receptors,
- to compare the binding site of integrin on Syk with that of pITAM, and
- to define integrin-induced structural changes on the N-SH2 domain of Syk and to show whether Syk can simultaneously bind integrin and pITAM at the same time.

3 SUMMARY OF THE METHODS

Method	Publication
Conventional cloning	I, II
Ligation-independent cloning	I, II
Site-directed mutagenesis	I, II
Bacterial protein expression	I, II
Insect cell protein expression	I
Protein purification	I, II
NMR measurements	II, III
NMR data analysis	II, III
NMR data assignment	II, III
NMR structure determination and refinement	II
Thermal stability assay	III
Surface plasmon resonance	I, II
Fluorescence-based kinetic measurements	I

4 RESULTS

4.1 Syk kinase activation is mediated by integrin cytoplasmic domain through a pITAM-independent mechanism

It is known that Syk is one of the proteins involved in downstream signalling of integrins (see Section 1.2.3). At the same time, in many different cells integrin signalling is concomitant with the pITAM signalling, so it appeared quite difficult to clearly distinguish and isolate the two pathways using in cell or in vivo experiments (Kahn and Koretzky 2006). For this reason, we used in vitro kinetic measurement with heterologous expressed and purified Syk.

To measure Syk activity in the presence of integrin β_3 cytoplasmic domain peptide, I utilised a fluorescence-based kinetic assay with SOX ((S)-2-amino-N^{α}-(9-fluorenylmethyloxycarbonyl)-3-[8-hydroxy-5-(N,N-dimethylsulfonamido)quinoline-2-yl]propionic acid) peptide (I) (Shults and Imperiali 2003, Shults *et al.* 2006). Our purified Syk was in the inactive state compared with the data available in the literature (Tsang *et al.* 2008) and showed an initial lag phase with low phosphorylation activity toward the peptide substrate (Fig. 8 - basal Syk) as reported earlier (Shults *et al.* 2006). In the first minutes of the reaction, the kinase is activated by auto/trans phosphorylation and then the SOX peptide is phosphorylated (Shults and Imperiali 2003, Shults *et al.* 2006, Tsang *et al.* 2008). This was confirmed by the absence of the lag phase with the pre-activated Syk through incubation with ATP (Fig. 8 - active Syk). As expected, the addition of pITAM peptide (from the CD3ε receptor) in the reaction mix reduced the lag phase compared with the inactive enzyme (Fig 8 - Syk+pITAM). This is probably due to the fact that pITAM activates the enzyme by structural rearrangements as suggested also from Tsang *et. al.* (Tsang *et al.* 2008, Grädler *et al.* 2013). This is supported by the data obtained with the Tyr 348/352 mutant to Phe presented below. However, even though we used 10 µM pITAM, which should be a saturating concentration, we saw a short lag phase in the reaction compared with the active Syk (Fig. 8 - Syk+pITAM). This suggests that, in the presence of pITAM, the enzyme was not in the full active state, but further activation took place in the

presence of ATP. Moreover, the addition of the pITAM peptide to a pre-activated enzyme did not affect the kinetics (I; Fig. 4, Panels C and D). These results do not completely match with the 'OR gate' switch model, or the lag phase would be completely abolished in the presence of pITAM as observed for the pre-activated enzyme (I; Fig. 4, Panels C and D).

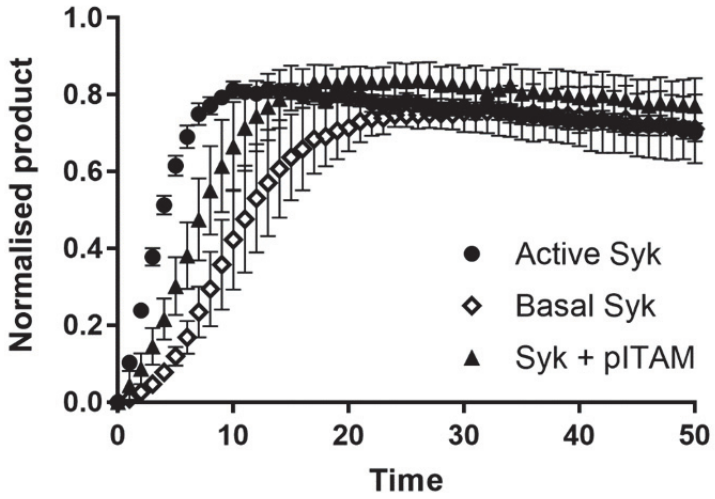

FIGURE 8 Fluorescence-based kinetic measurements of Syk kinase. Syk activity is expressed as normalised product formation over time (min). The data are obtained from six independent experiments. Basal Syk (*open diamonds*) is compared with Syk in the presence of 10 μM of pITAM peptide (*black triangles*) and with active protein (*black circles*). The Syk concentration was 17 nM. Protein activation was done by incubating the basal Syk with 100 μM ATP at room temperature for 30 min. The presence of pITAM peptide determines a reduction of the initial lag phase, while the preactivation of the kinase results in an abolishment of the lag phase. This figure is compiled from the data presented in Figure 2, Panel A and Figure 4, Panel A in I.

The effect of integrin β_3 cytoplasmic domain peptide on Syk activity was tested using the same protocol as with pITAM. As shown in Figure 4, Panels A and B (I), the soluble integrin peptide had no effect on Syk activation. Taking into account that one of the most crucial steps for integrin activation is represented by integrin clustering (see Section 1.2.3), we performed the kinetic measurements after coupling the integrin peptide on the plate surface. We assumed that the integrin peptides were 1) in a local high density, and 2) in a fixed position. These two conditions artificially mimic the effect of integrin clustering on the membrane. The result was a clear reduction of the lag phase in the presence of the integrin coupled on the surface compared with the soluble peptide (I; Fig. 5, Panels A and B). The addition of soluble pITAM peptide together with surface-bound integrin further reduced the lag phase (I; Fig. 5, Panels A and B)

To further analyse the integrin effect on Syk activation, we decided to test the Tyr348Phe/Tyr352Phe mutant. This mutant has very low intrinsic activity

compared with the wild type enzyme (Grädler *et al.* 2013). Tyr348Phe/Tyr352Phe Syk was activated by the soluble pITAM peptide but not by soluble integrin (I; Fig. 6, Panels A and B). As observed for the Syk wild type, the mutant was also activated by clustered integrin peptides (I; Fig. 6, Panels C and D). The activation kinetics with the Tyr348Phe/Tyr352Phe mutant were always much slower than in the case of wild type Syk. The presence of integrin peptide probably helps the activation by auto/transphosphorylation involving different phosphorylation sites than Tyr348 and Tyr352.

4.2 Quantitative measurement of Syk-integrin interaction

Interaction between Syk and integrin is mediated by N-SH2 plus the IA segment of Syk (Woodside *et al.* 2002). To confirm this and to obtain quantitative information about the contribution of different Syk regions in the interaction, we measured the affinity of different Syk constructs using SPR, with the integrin peptide as ligand and proteins as analyte. In our experimental, settings the K_D of full-length Syk to the integrin peptide was 391 ± 24.2 nM (Fig. 9). This is a somewhat lower value than the 24 nM published earlier (Woodside et al., 2002). We found that the K_D of tSH2 towards the integrin was 4.44 ± 1.55 μM and that of N-SH2 plus IA was 8.33 ± 0.83 μM (III; Fig. 2, Table 1).

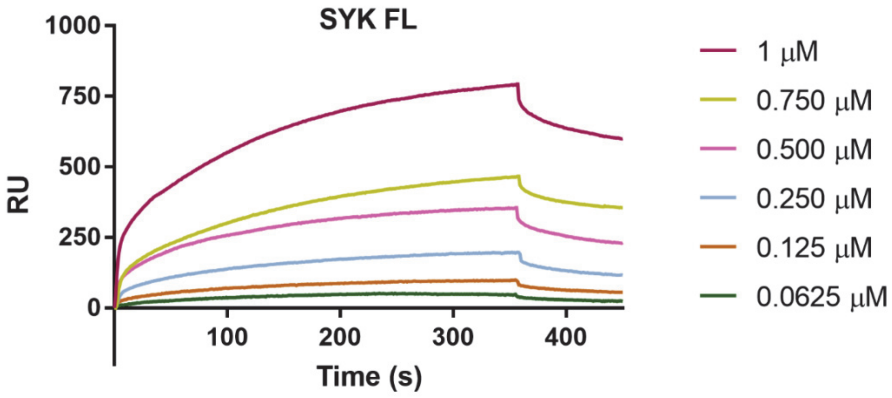

FIGURE 9 SPR measurement of the Syk full-length binding with integrin β_3 cytoplasmic domain peptide (for experimental procedure, see III).

Instead, the affinity of the single N-SH2 was~20 times less than tSH2. Moreover, the IA plus the C-SH2 showed ~ 5 times less affinity compared with tSH2 (III-Fig 2 and Table 1). Unfortunately, the isolated C-SH2 could not be tested with SPR due to technical problems. These results confirmed that the IA plays a major role in Syk-integrin binding and, is probably the principal binding site since the C-SH2 plus IA is still able to bind the integrin with higher affinity compared with isolated N-SH2; on the other hand, the available data do not exclude the possibility that the C-SH2 might be involved in the binding.

We also conducted a thermal stability analysis to show the effect of the integrin peptide on the IA region. Interestingly, the presence of the integrin peptide showed a minor destabilisation of isolated N-SH2 and a more prominent effect on N-SH2 plus IA (Fig. 10). On the other hand, no effect was observed in the presence of pITAM with the same constructs. It is likely that, the integrin peptide binds N-SH2 plus IA in a region where the IA contacts the domain, thus decreasing the stability of the entire protein construct. This effect is not observed in the presence of pITAM since it makes contact only with the N-SH2.

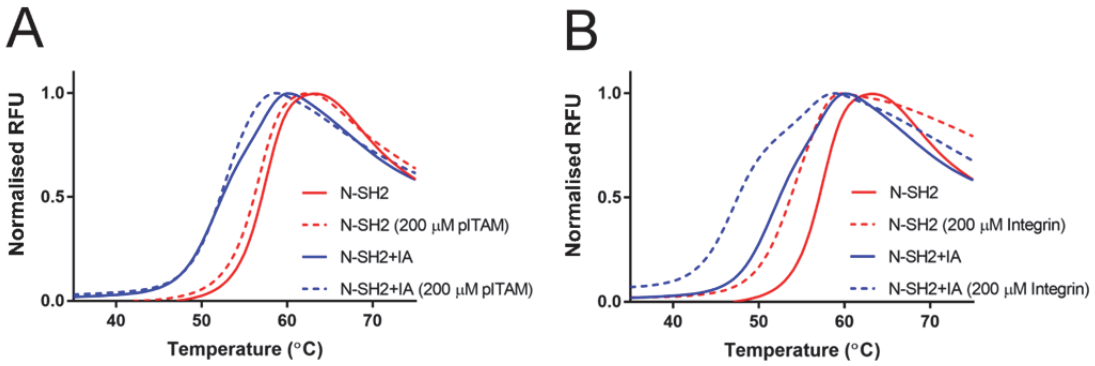

FIGURE 10 Thermal stability analysis expressed as the variation of the normalised fluorescence versus the temperature (°C) of N-SH2 plus IA (blue solid line) and N-SH2 (red solid line) in the presence of the pITAM peptide (dotted lines - Panel A) and integrin peptide (dotted lines - Panel B). The proteins were used at 4 μM and the peptides at 200 μM (for experimental procedure, refer to III).

4.3 N-SH2 shows a lower affinity for phosphorylated ITAM compared with C-SH2

We determined the N-SH2 structure using solution-state NMR spectroscopy (II; Fig. 1). Even if the Syk crystal structure was available, we wanted to ensure that the N-SH2 domain maintained the same structural features if isolated from the rest of the protein. The NMR sample of N-SH2 gave a well dispersed ^{1}H, ^{15}N-HSQC (heteronuclear single quantum coherence) correlation spectrum indicative of a properly folded domain. This allowed us to assign most of the observable peaks (Table 1 in III). Comparing the obtained structure with the N-SH2 in Syk full-length crystal structure we could not detect any major changes in domain folding. Moreover, comparing the N-SH2 domain with other SH2s in other proteins, we observed that the secondary structure elements and the domain folding are very well conserved despite the low sequence similarity (II; Fig. 3). Interestingly, we observed a double set of cross peaks for the Tyr39

aromatic ring in the (HB)CB(CGCD)HD/(HB)CB(CGCDCE)HE spectra as well as in the aromatic ^{1}H, ^{13}C-HSQC (heteronuclear single quantum coherence) spectra (II; Fig. 2). We deduced that Tyr39 exists in two conformations owing to slow aromatic ring flipping around the Cβ-Cγ bond. Therefore, one of the two sets of peaks were used to obtain the final 15 structures.

We also titrated the N-SH2 and C-SH2 in the presence of single and double pTyr ITAM peptide and observed that 1) the affinity of N-SH2 for the C-terminal pTyr is very low, being 5.7 ± 0.76 mM while the affinity of C-SH2 for N-terminal pTyr is 62 ± 4.1 μM; 2) both SH2 domains can bind to both pTyr in the CD3ε ITAM sequence; and 3) the affinity of C-SH2 for C-terminal pTyr is higher than the affinity of N-SH2 for that site, and, therefore, N-SH2 has lower affinity for both sites compared with C-SH2. Considering that, our results are consistent with the model that in Syk the N-SH2 is a secondary binding site for pITAM and only occupied when C-SH2 is first bound (Grädler *et al.* 2013).

4.4 Integrin- and pITAM-responding surfaces on Syk N-SH2 are partially overlapping

To further understand the relationship between integrin and pITAM interaction on the regulatory domain of Syk, we next located the peptide interaction surfaces using NMR chemical shift perturbation (CSP) mapping (III; Fig. 3). Titration with hemi-phosphorylated ITAM peptide with N-SH2 was carried out at two different pH, 7.5 and 5.0, and the results obtained were comparable. The interaction of integrin peptide with the N-SH2 + IA fragment was tested with pH 5.0, because the integrin peptide was poorly soluble at neutral pH.

Titration with the hemi-phosphorylated pITAM (C-ter pTyr) peptide confirmed that the binding interface of pITAM in the N-SH2 domain involves a positively charged pocket formed by side chains of Arg22, Arg42 and Asn46 interacting with the phosphate group of pITAM. On the other hand, major changes in the chemical environment were observed for Gly98 and Val100 involved in hydrophobic contacts with the Leu in position +3 in relation to the pTyr (III; Fig. 3, Panels A, B, C and D). This is all consistent with the general pTyr peptide binding mode of SH2 domains and the crystal structure of Syk tSH2 with the same CD3ε peptide used here (Fütterer *et al.* 1998).

Titration with the integrin β_3 cytoplasmic domain peptide revealed a compact surface, where the Ser44 and Leu48 displayed most prominent CSPs (III; Fig. 3, Panels E and F). Moreover, chemical shift of the side chain NH group of Asn46 and the backbone NH of Arg45 changed significantly with integrin, much as they did with pITAM. However, the side chains of Arg22 and Arg42 did not show CSPs with integrin, suggesting that the changes were near the phosphate binding pocket but not in the pocket. A second but closely located cluster of integrin responsive residues included Ile20, Arg22 and Glu23 (III; Fig. 3, Panels E and F). This cluster is adjacent to the pITAM interaction site. In

addition to the cross peaks corresponding to residues in the N-SH2 binding site, we observed several integrin-binding induced chemical shift changes in non-assigned cross peaks which are apparently derived from the IA segment (III; Supporting information figure 1).

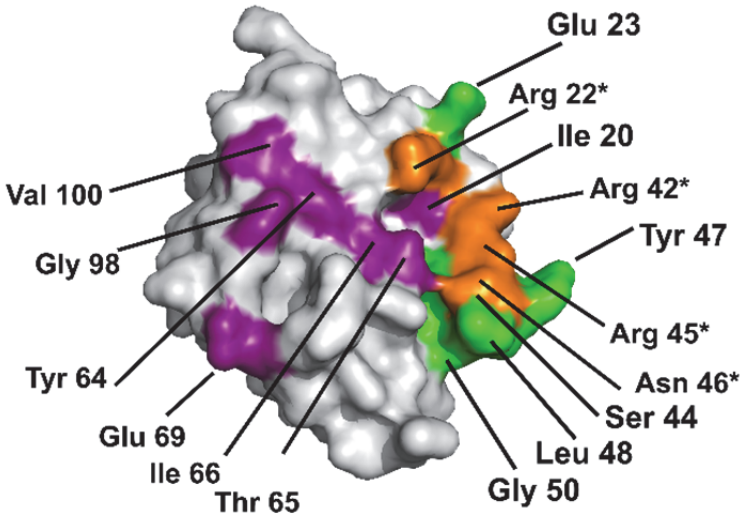

FIGURE 11 Surface representation of N-SH2 structure (PDB: 4FL2). The amino acids whose environment change only upon the integrin peptide titration are highlighted in green, while those responding to pITAM are in purple. The amino acids which respond in both titrations are highlighted in orange. The asterisk (*) refers to the shift of the NH in the side chain. Shifts higher than 0.05 ppm in the integrin titration and higher than 0.1 ppm in hemi-phosphorylated pITAM titration were considered significant and they are named in Figure 3 in III. The figure summarises the results shown in Figure 3 in III.

These titrations revealed that residues highlighting chemical shift changes upon integrin and pITAM peptide binding are partially the same on the N-SH2 of Syk (Fig. 11). Furthermore, it is possible that a part of the observed CSPs, induced by the integrin peptide association, do not stem from the direct interaction with integrin but rather are caused by the change of IA segment orientation upon binding. Particularly, residues ranging from Ser44 to Leu48 are very close to the IA segment in the crystal structure of full-length Syk (Grädler *et al.* 2013), and they are not surface accessible in that conformation (III; Fig. 5). The hypothesis that the binding interfaces are overlapped is supported by the SPR competition assay (III; Fig. 4). The addition of pITAM to tSH2 reduced the binding of the protein to the integrin peptide in a concentration-dependent fashion (III; Fig. 4).

5 DISCUSSION

The idea behind the present work was to study whether the interaction of Syk with integrin cytoplasmic domain affects the protein activation and the interplay with other pathways simultaneously occurring, such as pITAM-mediated Syk activation. We showed that Syk is activated by integrins via a mechanism different from the one previously described involving pITAM receptors. We also mapped the amino acids directly and indirectly involved in integrin β cytoplasmic domain binding and compared them with those involved in pITAM binding. Based on this and on competition experiments, integrin and pITAM compete for Syk N-SH2 binding. These findings are discussed below and, at the end a model which can accommodate these results is presented.

As described in the introduction, both integrins and immunoreceptors can activate Syk. Examples of that can be found in platelets, osteoclasts and immune cells (Mócsai *et al.* 2010). In some cases, integrin-mediated Syk activation is independent of ITAM signalling, and in other cases these pathways are dependent on each other (Sections 1.2.2 and 1.2.3). How can these results be fitted in the 'OR-gate' switch model of Syk activation? More specifically, does integrin-mediated Syk activation use the same arm of the 'OR-gate' as ITAM or the other arm which is dependent on Syk phosphorylation? The present work aimed to delve into the molecular details of Syk integrin binding, considering that Syk-pITAM interaction is already well known at the molecular level (Fütterer *et al.* 1998, Grädler *et al.* 2013), and to try to solve the mystery behind the regulation of Syk activity mediated by integrin cytoplasmic domain and pITAM whenever these pathways are both activated.

Using in vitro fluorescence-based kinetic assay, we demonstrated that the soluble integrin peptide cannot activate Syk, even though it binds to the Syk regulatory domain. This demonstrates that integrin does not act on the same arm of the 'OR-gate' switch as pITAM. On the other hand, clustered integrin cytoplasmic domain clearly activated Syk. This suggests to us that Syk autophosphorylation is involved in the process and integrin clustering enhances the activation through a proximity effect. We also observed that, in

the presence of both pITAM and integrin, Syk does not behave as a fully active enzyme during the first minutes of catalysis. Accordingly, clustered integrin and pITAM together had an additive effect on Syk activation. Thus, the activation state induced by integrin, as well as by pITAM, might correspond to a partially-active enzyme which is then fully activated by the auto/transphosphorylation. Taking that into consideration, Syk could belong to the category of a gradually-activated enzyme as Src family kinases (see Section 1.1.5). Our findings were also confirmed using the Syk Tyr348Phe/Tyr352Phe mutant. This mutant was less active than wild type Syk, but it showed similar behaviour in the presence of integrin compared with the wild type protein. Tyr348 and Tyr352 are the first to be phosphorylated, together with Tyr630 and Tyr631 in the C-terminal part, and mutation in these residues affects Syk autophosphorylation ability (Papp *et al.* 2007, Tsang *et al.* 2008). Thus, our results fit with a model in which at least Tyr630 or Tyr631 phosphorylation can increase Syk activity when Tyr348 and Tyr352 are mutated to Phe. Perhaps all these four sites need to be phosphorylated to turn the enzyme fully active. In that scenario, it would be interesting to test the effect of the integrin peptide on other Syk mutants to check whether different phosphorylation sites might play an important role in integrin-mediated Syk activation process. (Zeitlmann *et al.* 1998, Papp *et al.* 2007).

As previously mentioned, Syk binds to integrin β cytoplasmic domain via the N-SH2 plus IA (Woodside *et al.* 2002). We quantitatively characterised the interaction measuring the K_D for different Syk constructs towards integrin β_3 cytoplasmic domain peptide. Our data confirmed that the N-SH2 binds to integrin with the contribution of the IA segment. Moreover, the IA has a crucial role in the process since the tSH2 showed a comparable binding affinity to the N-SH2+IA fragment. This is also in accordance with the fact that IA+C-SH2 retained the ability to bind integrin, considering that C-SH2 seems not to have a role in integrin binding (Woodside *et al.* 2002). On the other hand, all short constructs of Syk regulatory domain had a lower affinity to integrin than the full-length Syk. The most apparent explanation for this is that the IA and its orientation related to N-SH2 can be stabilised in the full-length protein - as seen in the crystal structure (Grädler *et al.* 2013) - and this stabilised conformation is favourable for integrin binding. On the other hand, it has been shown that the IA segment and both SH2 domains can tumble in variable orientations when expressed separately (Zhang *et al.* 2008).

The other main aims of this study were to compare the Syk residues which are involved in integrin binding with those involved in pITAM binding and to study the relationship between these two interactions. To do that, we focussed on the N-SH2 domain. Therefore, N-SH2 chemical shifts were assigned and structure models were calculated. The overall fold of the domain was comparable to the available crystal structures, and model quality parameters indicate that the quality of the structure was good (Table 1 - II).

The assignments of the backbone and the side-chains of N-SH2 made it possible to compare the CSPs induced by the integrin and pITAM. Integrin-

induced CSPs were located in a compact area close to the location of the IA segment in the full-length Syk crystal structure. Thus, it is apparent that many of the CSPs may be caused by reorientation of the IA segment because of integrin binding. CSPs of unassigned peaks in the spectra were indicative of integrin-induced changes within the IA segment. In our thermal stability experiments, we clearly observed a destabilisation of the N-SH2+IA construct, probably because integrin is binding in the interface between N-SH2 and IA segment (Fig. 10). However, we believe that some of the CSPs in the N-SH2 must be caused by direct interaction of the integrin peptide with the N-SH2 domain, because the domain alone was able to bind the integrin peptide in the SPR experiment and because the presence of the integrin peptide altered the stability of the N-SH2 domain in thermal unfolding experiments.

We then tested the pITAM binding with N-SH2. As expected, the pITAM binding-induced CSPs described two separated pockets: one for the pTyr, including positive charged amino acids, and one for the hydrophobic residue next to the pTyr. This result is in line with the available crystallography data (Fütterer *et al.* 1998, Grädler *et al.* 2013). Comparing the two titrations, we noticed that in the presence of the integrin peptide there were significant shifts in the backbone of Arg22 and Glu23, which are located close to the pTyr binding pocket, and the CSPs of Arg45 and Asn46 were common to both titrations (Fig. 11). These results showed that CSPs induced by the two peptides are partially overlapping. We also found that pITAM inhibited tSH2 binding to the integrin in the SPR assay. We suggest that the orientation of the IA segment and the C-SH2-domain make a major contribution in this inhibition since pITAM binds to both SH2 domain and it also changes the orientation of these domains in relation to each other. In the crystal structure of tSH2 bound to pITAM the integrin-responsive surface of N-SH2 is mostly hidden in the interphase between the N-SH2 and C-SH2 domains (Fig. 12) and may not be accessible for integrin binding.

Currently, we do not have any NMR data regarding the integrin β subunit residues which interact with Syk, but based on the data available in the literature, the last four amino acids (759-762) in the C-terminus are required, as well as amino acids between the positions 733 and 740 (Woodside *et al.* 2001). As the integrin β subunit cytoplasmic tail structure is mostly unfolded and flexible (Vinogradova *et al.* 2002) and it can take various conformations upon interaction (García-Alvarez *et al.* 2003, Kiema *et al.* 2006, Takala *et al.* 2008, Li *et al.* 2017) it is likely that the interaction surface can be rather extended. Further NMR experiments using labelled peptide could be done to identify the binding epitope on the integrin structure and the orientation of the integrin peptide in relation to the N-SH2 plus IA. Eventually, NMR will allow us to identify possible conformation changes in the integrin cytoplasmic domain.

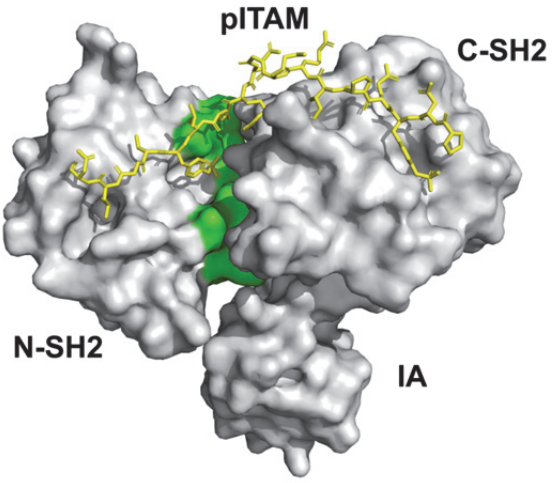

FIGURE 12 Bound form of tandem SH2 domains with pITAM peptide (PDB: 4FL2). The N-SH2 is represented using surface visualisation (light grey), the aminoacids shifting in the presence of the integrin peptide are in green and the pITAM peptide is in yellow with stick visualisation.

Given that the pITAM and integrin physically compete in binding the N-SH2 domain, we also wanted to measure the affinity of N-SH2 and C-SH2 for hemi-phosphorylated ITAM peptides. Surprisingly, N-SH2 bound the single pTyr with very low affinity, while C-SH2 showed an affinity comparable to the data available in the literature (Bu *et al.* 1995, Narula *et al.* 1995). Moreover, both SH2 domains showed a binding promiscuity towards both pTyr residues in the pITAM peptide and preferred the N-terminal pTyr in the tested pITAM. The fact that N-SH2 and C-SH2 can bind to both sites is not surprising considering that, in contrast to other SH2 domains, the Syk SH2s have no sequence specificity except for the conserved hydrophobic residue after the pTyr (pYxxI/L) (Narula *et al.* 1995, Liu *et al.* 2012). This was also observed for SH2 in Zap-70 (Folmer *et al.* 2002). Our data confirmed that the C-SH2 must be the first to bind to pITAM as already proposed by Grädler *et al.* on the basis of the available crystallography data (Grädler *et al.* 2013). Moreover, C-SH2 participates in the pITAM binding by interacting with the pTyr as well as with most of the amino acids between the two pTyr residues (Fütterer *et al.* 1998).

In conclusion, we can state that our experimental data does not fit very well with the 'OR gate' model previously proposed for Syk activation, but it is possible to fit it into gradual activation model (Bradshaw 2010). In this model, Syk is recruited near the plasma membrane interacting with the integrin β cytoplasmic domain. The clustering of the integrins creates a proximity effect, thus favouring the Syk transphosphorylation activity. A similar mechanism has been already proposed for FAK kinase activation involving PIP2 (Goni *et al.* 2014). The proximity effect, which is not a new regulation mechanism by itself, is similar to the dimerisation in the activation process of RTKs (Lemmon and

Schlessinger 2010). As for pITAM receptors, integrin signalling involves Src family kinases which are recruited to the membrane. In our model, the presence of other kinases may enforce the phosphorylation of Syk and help to sustain the signalling as long as necessary. Based on this model, the Syk activation mechanism mediated by the integrin can be independent from pITAM. On the other hand, it is possible that in some cells, integrin- or Src-family kinase - induced activation may not be enough for full activation of Syk. For this, further activation via pITAM may be required. In some systems, pITAM-mediated activation alone may be sufficient.

Our quantitative measurements showed that Syk N-SH2 has an affinity for integrin comparable to the C-SH2 for the hemi-phosphorylated pITAM. Thus, at a cellular level, when the integrin and pITAM receptors are co-clustered, Syk might bind them simultaneously. This model was already proposed by Zou *et al.* to explain their results regarding the interplay between integrin $\alpha_V\beta_3$ and FcRγIIA receptors in osteoclasts (Zou *et al.* 2007). Moreover, this model clearly brings to mind the binding of Syk also proposed for platelet CLEC-2 receptors which contain a single ITAM motif where Syk gathers together two different receptors via its SH2 domains (Suzuki-Inoue *et al.* 2006, Bauer and Steinle 2017).

A recent publication from Park *et al.* showed that SH2 domains can also act as lipid-binding domains (Park *et al.* 2016). Interestingly, Syk and Zap-70 C-SH2 domains bind PIP3 and PIP2 with a K_D comparable to the one measured for pTyr (Park *et al.* 2016). Moreover, in cells, the mutation of the lipid-binding site in Zap-70 affects the binding and the downstream signalling apparently because it destabilises the Zap-70/receptor interaction (Park *et al.* 2016). Considering that Syk-integrin binding is mediated by the N-SH2 plus the IA, it might be possible that the C-SH2 is involved in membrane interaction, thus stabilising the entire complex. As mentioned before, the importance of the PIP2 has been already shown in the case of the FAK kinase since it promotes the kinase activation via structural destabilization of the FAK inactive conformation and via clustering effect (Goni *et al.* 2014).

In conclusion, with the present work, we partially clarified how Syk interacts with integrin and the impact of this interaction on the Syk activity regulation at the molecular level. We have also developed good clues about the possible interplay between the integrin and pITAM binding pathways, which open the possibility of interesting follow-up experiments. Considering that the intracellular signalling is quite complex and that many proteins are engaged at the same time, our biochemical and structural studies with isolated proteins have been useful for the understanding of molecular details.

6 CONCLUSIONS

The main conclusions of the present thesis project are:

1- Syk is activated by integrins via a different mechanism compared with pITAM. Syk activity measurements in the presence of integrin β_3 cytoplasmic domain peptide showed that Syk is activated by clustered integrins. Comparable results were obtained with Syk Tyr348 and Tyr352 to Phe mutant. We defined a new Syk activation model in which Syk is activated by auto/transphosphorylation and integrin clustering is a crucial step. Moreover, comparing the kinetics of pre-activated Syk and Syk in the presence of pITAM, we proposed that the Syk activation mechanism is more realistically described by a 'graded' instead of an 'OR-gate' switch model.

2- The solution structure of the N-SH2 domain, obtained with NMR spectroscopy, revealed the same overall fold compared with the corresponding crystal structure of N-SH2 available in the literature.

3- The NMR titrations of N-SH2 with the integrin β cytoplasmic domain and pITAM peptides showed that they induced chemical shift changes on partially overlapping surfaces of the Syk N-SH2 domain and, thus, they at least partially compete for binding.

4- The quantitative measurements of the affinity of SH2s for integrin and hemi-phosphorylated ITAM peptides allowed us to partially clarify the interplay between integrin and pITAM at the molecular level.

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REFERENCES

Abtahian F., Guerriero A., Sebzda E., Lu M.M., Zhou R., Mocsai A., Myers E.E., Huang B., Jackson D.G., Ferrari V.A., Tybulewicz V., Lowell C.A., Lepore J.J., Koretzky G.A. & Kahn M.L. 2003. Regulation of blood and lymphatic vascular separation by signaling proteins SLP-76 and Syk. *Science* 299: 247–251.

Ackermann J.A., Nys J., Schweighoffer E., McCleary S., Smithers N. & Tybulewicz V.L.J. 2015. Syk tyrosine kinase is critical for B cell antibody responses and memory B cell survival. *The Journal of Immunology* 194: 4650–4656.

Adam K. & Hunter T. 2017. Histidine kinases and the missing phosphoproteome from prokaryotes to eukaryotes. *Nature Publishing Group* 98: 233–247.

Alonso G., Koegl M., Mazurenko N. & Courtneidge S.A. 1995. Sequence requirements for binding of Src family tyrosine kinases to activated growth factor receptors. *Journal of Biological Chemistry* 270: 9840–9848.

Anthis N.J. & Campbell I.D. 2011. The tail of integrin activation. *Trends in Biochemical Sciences* 36: 191–198.

Arias-Salgado E.G., Haj F., Dubois C., Moran B., Kasirer-Friede A., Furie B.C., Furie B., Neel B.G. & Shattil S.J. 2005. PTP-1B is an essential positive regulator of platelet integrin signaling. *Journal of Cell Biology* 170: 837–845.

Au-Yeung B.B., Deindl S., Hsu L.-Y., Palacios E.H., Levin S.E., Kuriyan J. & Weiss A. 2009. The structure, regulation, and function of ZAP-70. *Immunological Reviews* 228: 41–57.

Barbacid M., Donner L., Ruscetti S.K. & Sherr C.J. 1981. Transformation-defective mutants of Snyder-Theilen feline sarcoma virus lack tyrosine-specific protein kinase activity. *Journal of virology* 39: 246–254.

Barrow A.D. & Trowsdale J. 2006. You say ITAM and I say ITIM, let's call the whole thing off: the ambiguity of immunoreceptor signalling. *European Journal of Immunology* 36: 1646–1653.

Bauer B. & Steinle A. 2017. HemITAM: a single tyrosine motif that packs a punch. *Science Signaling* 10: eaan3676.

Bibbins K.B., Boeuf H. & Varmus H.E. 1993. Binding of the Src SH2 domain to phosphopeptides is determined by residues in both the SH2 domain and the phosphopeptides. *Molecular and cellular biology* 13: 7278–7287.

Boggon T.J. & Eck M.J. 2004. Structure and regulation of Src family kinases. *Oncogene* 23: 7918–7927.

Bosanquet D.C., YE L., Harding K.G. & Jiang W.G. 2014. FERM family proteins and their importance in cellular movements and wound healing. *International Journal of Molecular Medicine* 34: 3–12.

Boylan B., Gao C., Rathore V., Gill J.C., Newman D.K. & Newman P.J. 2008. Identification of FcγRIIa as the ITAM-bearing receptor mediating αIIbβ3 outside-in integrin signaling in human platelets. *Blood* 112: 2780–2786.

Bradshaw J.M. 2010. The Src, Syk, and Tec family kinases: distinct types of molecular switches. *Cellular Signalling* 22: 1175–1184.

Bu J.Y., Shaw A.S. & Chan A.C. 1995. Analysis of the interaction of ZAP-70 and syk protein-tyrosine kinases with the T-cell antigen receptor by plasmon resonance. *Proceedings of the National Academy of Sciences* 92: 5106–5110.

Burgess A.W., Cho H.S., Eigenbrot C., Ferguson K.M., Garrett T.P.J., Leahy D.J., Lemmon M.A., Sliwkowski M.X., Ward C.W. & Yokoyama S. 2003. An open-and-shut case? Recent insights into the activation of EGF/ErbB receptors. *Molecular Cell* 12: 541–552.

Calderwood D.A., Fujioka Y., Pereda J.M. de, García-Alvarez B., Nakamoto T., Margolis B., McGlade C.J., Liddington R.C. & Ginsberg M.H. 2003. Integrin beta cytoplasmic domain interactions with phosphotyrosine-binding domains: a structural prototype for diversity in integrin signaling. *Proceedings of the National Academy of Sciences of the United States of America* 100: 2272–2277.

Castro R.O. De, Zhang J., Jamur M.C., Oliver C. & Siraganian R.P. 2010. Tyrosines in the carboxyl terminus regulate Syk kinase activity and function. *Journal of Biological Chemistry* 285: 26674–26684.

Ceccarelli D.F.J., Hyun K.S., Poy F., Schaller M.D. & Eck M.J. 2006. Crystal structure of the FERM domain of focal adhesion kinase. *Journal of Biological Chemistry* 281: 252–259.

Chan A.C., Iwashima M., Turck C.W. & Weiss A. 1992. ZAP-70: A 70 kd protein-tyrosine kinase that associates with the TCR ζ chain. *Cell* 71: 649–662.

Chen H., Ma J., Li W., Eliseenkova A. V, Xu C., Neubert T.A., Miller W.T. & Mohammadi M. 2007. A molecular brake in the kinase hinge region regulates the activity of receptor tyrosine kinases. *Molecular Cell* 27: 717–730.

Cheng A.M., Rowley B., Pao W., Hayday A., Bolen J.B. & Pawson T. 1995. Syk tyrosine kinase required for mouse viability and B-cell development. *Nature* 378: 303–306.

Chiffoleau E. 2018. C-type lectin-like receptors as emerging orchestrators of sterile inflammation represent potential therapeutic targets. *Frontiers in Immunology* 9: 1–9.

Clark E. a, Shattil S.J., Ginsberg M.H., Bolen J. & Brugge J.S. 1994. Regulation of the protein tyrosine kinase pp72syk by platelet agonists and the integrin alpha IIb beta 3. *The Journal of Biological Chemistry* 269: 28859–28864.

Cole Ã P.A., Shen K., Qiao Y. & Wang D. 2003. Protein tyrosine kinases Src and Csk: a tail's tale. *Current Opinion in Chemical Biology* 7: 580–585.

Durrant T.N., Bosch M.T. Van Den & Hers I. 2017. Integrin αIIbβ3outside-in signaling. *Blood* 130: 1607–1619.

Eckstein B. & Tsafriri A. 1986. The steroid c-17,20-lyase complex in isolated graafian follicles: effects of human chorionic gonadotropin. *Endocrinology* 118: 1266–1270.

Estevez B. & Du X. 2017. New concepts and mechanisms of platelet activation signaling. *Physiology* 32: 162–177.

Falet H., Pollitt A.Y., Begonja A.J., Weber S.E., Duerschmied D., Wagner D.D., Watson S.P. & Hartwig J.H. 2010. A novel interaction between FlnA and Syk regulates platelet ITAM-mediated receptor signaling and function. *The Journal of Experimental Medicine* 207: 1967–1979.

Fallah-Arani F., Schweighoffer E., Vanes L. & Tybulewicz V.L.J. 2008. Redundant role for Zap70 in B cell development and activation. *Eur. J. Immunol.* 38: 1721–1733.

Feng X., Novack D. V., Faccio R., Ory D.S., Aya K., Boyer M.I., McHugh K.P., Ross F.P. & Teitelbaum S.L. 2001. A Glanzmann's mutation in β3 integrin specifically impairs osteodast function. *Journal of Clinical Investigation* 107: 1137–1144.

Feng C. & Post C.B. 2016. Insights into the allosteric regulation of Syk association with receptor ITAM, a multi-state equilibrium. *Physical Chemistry Chemical Physics* 18: 5807–5818.

Filippakopoulos P., Müller S. & Knapp S. 2009. SH2 domains: modulators of nonreceptor tyrosine kinase activity. *Current Opinion in Structural Biology* 19: 643–649.

Folmer R.H.A., Geschwindner S. & Xue Y. 2002. Crystal structure and NMR studies of the Apo SH2 domains of ZAP-70: two bikes rather than a tandem. *Biochemistry* 41: 14176–14184.

Frame M.C., Patel H., Serrels B., Lietha D. & Eck M.J. 2010. The FERM domain: organizing the structure and function of FAK. *Nature Reviews Molecular Cell Biology* 11: 802–814.

Furlong M.T., Mahrenholz A.M., Kim K.-H., Ashendel C.L., Harrison M.L. & Geahlen R.L. 1997. Identification of the major sites of autophosphorylation of the murine protein-tyrosine kinase Syk. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* 1355: 177–190.

Futosi K. & Mócsai A. 2016. Tyrosine kinase signaling pathways in neutrophils. *Immunological Reviews* 273: 121–139.

Fütterer K., Wong J., Grucza R.A., Chan A.C. & Waksman G. 1998. Structural basis for Syk tyrosine kinase ubiquity in signal transduction pathways revealed by the crystal structure of its regulatory SH2 domains bound to a dually phosphorylated ITAM peptide. *Journal of Molecular Biology* 281: 523–537.

García-Alvarez B., Pereda J.M. De, Calderwood D.A., Ulmer T.S., Critchley D., Campbell I.D., Ginsberg M.H. & Liddington R.C. 2003. Structural determinants of integrin recognition by talin. *Molecular Cell* 11: 49–58.

Goni G.M., Epifano C., Boskovic J., Camacho-Artacho M., Zhou J., Bronowska A., Martin M.T., Eck M.J., Kremer L., Grater F., Gervasio F.L., Perez-Moreno M. & Lietha D. 2014. Phosphatidylinositol 4,5-bisphosphate triggers activation of focal adhesion kinase by inducing clustering and conformational changes. *Proceedings of the National Academy of Sciences* 111: E3177–E3186.

Grädler U., Schwarz D., Dresing V., Musil D., Bomke J., Frech M., Greiner H., Jäkel S., Rysiok T., Müller-Pompalla D. & Wegener A. 2013. Structural and biophysical characterization of the Syk activation switch. *Journal of Molecular Biology* 425: 309–333.

Harburger D.S. & Calderwood D.A. 2009. Integrin signalling at a glance. *Journal of Cell Science* 122: 1472–1472.

Horton E.R., Humphries J.D., Stutchbury B., Jacquemet G., Ballestrem C., Barry S.T. & Humphries M.J. 2016. Modulation of FAK and Src adhesion signaling occurs independently of adhesion complex composition. *Journal of Cell Biology* 212: 349–364.

Hubbard S.R. 2004. Juxtamembrane autoinhibition in receptor tyrosine kinases. *Nature Reviews Molecular Cell Biology* 5: 464–470.

Hubbard S.R. 2013. The insulin receptor: both a prototypical and atypical receptor tyrosine kinase. *Cold Spring Harbor Perspectives in Biology* 5: 1–12.

Hubbard S.R. & Till J.H. 2000. Protein tyrosine kinase structure and function. *Annual Review of Biochemistry* 69: 373–398.

Hughes C.E., Finney B.A., Koentgen F., Lowe K.L. & Watson S.P. 2015. The N-terminal SH2 domain of Syk is required for (hem) ITAM , but not integrin , signaling in mouse platelets. *Blood* 125: 144–155.

Humphries J.D., Byron A. & Humphries M.J. 2006. Integrin ligands at a glance. *Journal of Cell Science* 119: 3901–3903.

Hunter T. 1991. Protein kinase classification. *Methods in Enzymology* 200: 3–37.

Hunter T. 2007. The age of crosstalk: phosphorylation, ubiquitination, and beyond. *Molecular Cell* 28: 730–738.

Hunter T. 2012. Why nature chose phosphate to modify proteins. *Philosophical Transactions of the Royal Society B: Biological Sciences* 367: 2513–2516.

Huse M. & Kuriyan J. 2002. The conformational plasticity of protein kinases. *Cell* 109: 275–282.

Hynes R.O. 1987. Integrins: a family of cell surface receptors. *Cell* 48: 549–554.

Hynes R.O. 2002. Integrins: bidirectional, allosteric signaling machines. *Cell* 110: 673–687.

Ilić D., Furuta Y., Kanazawa S., Takeda N., Sobue K., Nakatsuji N., Nomura S., Fujimoto J., Okada M. & Yamamoto T. 1995. Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature* 377: 539–544.

Jadwin J.A., Curran T.G., Lafontaine A.T., White F.M. & Mayer B.J. 2018. Src homology 2 domains enhance tyrosine phosphorylation in vivo by protecting binding sites in their target proteins from dephosphorylation. *Journal of Biological Chemistry* 293: 623–627.

Jin L., Pluskey S., Petrella E.C., Cantin S.M., Gorga J.C., Rynkiewicz M.J., Pandey P., Strickler J.E., Babine R.E., Weaver D.T. & Seidl K.J. 2004. The three-dimensional structure of the ZAP-70 kinase domain in complex with staurosporine: implications for the design of selective inhibitors. *Journal of Biological Chemistry* 279: 42818–42825.

Johnson D.A., Akamine P., Radzio-Andzelm E., Madhusudan & Taylor S.S. 2001. Dynamics of cAMP-dependent protein kinase. *Chemical Reviews* 101: 2243–2270.

Joseph R.E., Min L. & Andreotti A.H. 2007. The linker between SH2 and kinase domains positively regulates catalysis of the Tec family kinases. *Biochemistry* 46: 5455–5462.

Kahn M.L. & Koretzky G.A. 2006. Integrins and ITAMs: more than just good neighbors. *Nature immunology* 7: 1286–1288.

Kaneko T., Huang H., Zhao B., Li L., Liu H., Voss C.K., Wu C., Schiller M.R. & Li S.S.C. 2010. Loops govern SH2 domain specificity by controlling access to binding pockets. *Science Signaling* 3: ra34.

Kaneko T., Joshi R., Feller S.M. & Li S.S.C. 2012. Phosphotyrosine recognition domains: the typical, the atypical and the versatile. *Cell Communication and Signaling* 10: 32.

Kannan N. & Neuwald A.F. 2005. Did protein kinase regulatory mechanisms evolve through elaboration of a simple structural component? *Journal of Molecular Biology* 351: 956–972.

Katyal P., Puthenveetil R. & Vinogradova O. 2013. Structural insights into the recognition of β3 integrin cytoplasmic tail by the SH3 domain of Src kinase. *Protein Science* 22: 1358–1365.

Keely P.J. & Parise L. V. 1996. The α2β1integrin is a necessary co-receptor for collagen-induced activation of Syk and the subsequent phosphorylation of phospholipase Cγ2 in platelets. *Journal of Biological Chemistry* 271: 26668–26676.

Keshvara L.M., Isaacson C., Harrison M.L. & Geahlen R.L. 1997. Syk activation and dissociation from the B-cell antigen receptor is mediated by phosphorylation of tyrosine 130. *Journal of Biological Chemistry* 272: 10377–10381.

Kiema T., Lad Y., Jiang P., Oxley C.L., Baldassarre M., Wegener K.L., Campbell I.D., Ylänne J. & Calderwood D.A. 2006. The molecular basis of filamin binding to integrins and competition with talin. *Molecular Cell* 21: 337–347.

Kimura T., Sakamoto H., Appella E. & Siraganian R.P. 1997. The negative signaling molecule SH2 domain-containing inositol- polyphosphate 5-phosphatase (SHIP) binds to the tyrosine-phosphorylated β subunit of the high affinity IgE receptor. *Journal of Biological Chemistry* 272: 13991–13996.

Klinghoffer R.A., Sachsenmaier C., Cooper J.A. & Soriano P. 1999. Src family kinases are required for integrin but not PDGFR signal transduction. *EMBO Journal* 18: 2459–2471.

Knighton D.R., Zheng J.H., Eyck L.F. Ten, Ashford V.A., Xuong N.H., Taylor S.S. & Sowadski J.M. 1991. Crystal structure of the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. *Science* 253: 407–414.

Kornev A.P., Haste N.M., Taylor S.S. & Eyck L.F. Ten. 2006. Surface comparison of active and inactive protein kinases identifies a conserved activation mechanism. *Proceedings of the National Academy of Sciences* 103: 17783–17788.

Kornev A.P., Taylor S.S. & Eyck L.F. Ten. 2008. A helix scaffold for the assembly of active protein kinases. *Proceedings of the National Academy of Sciences* 105: 14377–14382.

Krisenko M.O. & Geahlen R.L. 2015. Calling in SYK: SYK's dual role as a tumor promoter and tumor suppressor in cancer. *Biochimica et Biophysica Acta - Molecular Cell Research* 1853: 254–263.

Kurochkina N. & Guha U. 2013. SH3 domains: modules of protein-protein interactions. *Biophysical Reviews* 5: 29–39.

Kypta R.M., Goldberg Y., Ulug E.T. & Courtneidge S.A. 1990. Association between the PDGF receptor and members of the src family of tyrosine kinases. *Cell* 62: 481–492.

Latour S., Fournel M. & Veillette A. 1997. Regulation of T-cell antigen receptor signalling by Syk tyrosine protein kinase. *Mol Cell Biol* 17: 4434–4441.

Latour S., Zhang J., Siraganian R.P. & Veillette A. 1998. A unique insert in the linker domain of Syk is necessary for its function in immunoreceptor signalling. *The EMBO Journal* 17: 2584–2595.

Law D.A., DeGuzman F.R., Heiser P., Ministri-Madrid K., Killeen N. & Phillips D.R. 1999. Integrin cytoplasmic tyrosine motif is required for outside-in αIIbβ3 signalling and platelet function. *Nature* 401: 808–811.

Lawson C., Lim S.-T., Uryu S., Chen X.L., Calderwood D.A. & Schlaepfer D.D. 2012. FAK promotes recruitment of talin to nascent adhesions to control cell motility. *The Journal of cell biology* 196: 223–232.

Lemmon M.A. & Schlessinger J. 2010. Cell signaling by receptor tyrosine kinases. *Cell* 141: 1117–1134.

Li H., Deng Y., Sun K., Yang H., Liu J., Wang M., Zhang Z., Lin J., Wu C., Wei Z. & Yu C. 2017. Structural basis of kindlin-mediated integrin recognition and activation. *Proceedings of the National Academy of Sciences* 114: 9349–9354.

Liu B.A., Engelmann B.W. & Nash P.D. 2012. The language of SH2 domain interactions defines phosphotyrosine-mediated signal transduction. *FEBS Letters* 586: 2597–2605.

Love P.E. & Hayes S.M. 2010. ITAM-mediated signaling by the T-cell antigen receptor. *Cold Spring Harbor perspectives in biology* 2: 1–11.

Love P.E., Lee J. & Shores E.W. 2000. Critical relationship between TCR signaling potential and TCR affinity during thymocyte selection. *The Journal of Immunology* 165: 3080–3087.

Luzy J.P., Huixiong Chen, Gril B., Liu W.Q., Vidal M., Perdereau D., Burnol A.F. & Garbay C. 2008. Development of binding assays for the SH2 domain of Grb7 and Grb2 using fluorescence polarization. *Journal of Biomolecular Screening* 13: 112–119.

Manning G., Plowman G.D., Hunter T. & Sudarsanam S. 2002. Evolution of protein kinase signaling from yeast to man. *Trends in Biochemical Sciences* 27: 514–520.

Masterson L.R., Cheng C., Yu T., Tonelli M., Kornev A., Taylor S.S. & Veglia G. 2010. Dynamics connect substrate recognition to catalysis in protein kinase A. *Nature Chemical Biology* 6: 821–828.

McClendon C.L., Kornev A.P., Gilson M.K. & Taylor S.S. 2014. Dynamic architecture of a protein kinase. *Proceedings of the National Academy of Sciences* 111: E4623–E4631.

Mitra S.K., Hanson D.A. & Schlaepfer D.D. 2005. Focal adhesion kinase: in command and control of cell motility. *Nature Reviews Molecular Cell Biology* 6: 56–68.

Moarefi I., LaFevre-Bernt M., Sicheri F., Huse M., Lee C.H., Kuriyan J. & Miller W.T. 1997. Activation of the Src-family tyrosine kinase Hck by SH3 domain displacement. *Nature* 385: 650–653.

Mócsai A., Abram C.L., Jakus Z., Hu Y., Lanier L.L. & Lowell C.A. 2006. Integrin signaling in neutrophils and macrophages uses adaptors containing immunoreceptor tyrosine-based activation motifs. *Nature Immunology* 7: 1326–1333.

Mócsai A., Humphrey M.B., Ziffle J.A.G. Van, Hu Y., Burghardt A., Spusta S.C., Majumdar S., Lanier L.L., Lowell C.A. & Nakamura M.C. 2004. The immunomodulatory adapter proteins DAP12 and Fc receptor -chain (FcR) regulate development of functional osteoclasts through the Syk tyrosine kinase. *Proceedings of the National Academy of Sciences* 101: 6158–6163.

Mócsai A., Ligeti E., Lowell C.A. & Berton G. 1999. Adhesion-dependent degranulation of neutrophils requires the Src family kinases Fgr and Hck. *J Immunol* 162: 1120–1126.

Mócsai A., Ruland J. & Tybulewicz V.L.J. 2010. The SYK tyrosine kinase: a crucial player in diverse biological functions. *Nature Reviews Immunology* 10: 387–402.

Mócsai A., Walzog B. & Lowell C.A. 2015. Intracellular signalling during neutrophil recruitment. *Cardiovascular Research* 107: 373–385.

Mócsai A., Zhou M., Meng F., Tybulewicz V.L. & Lowell C.A. 2002. Syk is required for integrin signaling in neutrophils. *Immunity* 16: 547–558.

Mohn H., Cabec V. Le, Fischert S. & Maridonneau-Parini I. 1995. The src-family protein-tyrosine kinase p5ghck iS located on the secretory granules in human neutrophils and translocates towards the phagosome during cell activation. *Biochem. J* 309: 657–665.

Moore M.J., Kanter J.R., Jones K.C. & Taylor S.S. 2002. Phosphorylation of the catalytic subunit of protein kinase A: autophosphorylation versus phosphorylation by phosphoinositide-dependent kinase-1. *Journal of Biological Chemistry* 277: 47878–47884.

Morse E.M., Brahme N.N. & Calderwood D.A. 2014. Integrin cytoplasmic tail interactions. *Biochemistry* 53: 810–820.

Musacchio A. 2002. How SH3 domains recognize proline. *Advances in Protein Chemistry* 61: 211–268.

Nakamura I., Duong L.T., Rodan S.B. & Rodan G.A. 2007. Involvement of αvβ3integrins in osteoclast function. *Journal of Bone and Mineral Metabolism* 25: 337–344.

Narula S.S., Yuan R.W., Adams S.E., Green O.M., Green J., Philips T.B., Zydowsky L.D., Botfield M.C., Hatada M., Laird E.R., Zoller M.J., Karas J.L.

& Dalgarno D.C. 1995. Solution structure of the C-terminal SH2 domain of the human tyrosine kinase Syk complexed with a phosphotyrosine pentapeptide. *Structure* 3: 1061–1073.

Newbrough S.A., Mocsai A., Clemens R.A., Wu J.N., Silverman M.A., Singer A.L., Lowell C.A. & Koretzky G.A. 2003. SLP-76 regulates Fcgamma receptor and integrin signaling in neutrophils. *Immunity* 19: 761–769.

Nolen B., Taylor S. & Ghosh G. 2004. Regulation of protein kinases: controlling activity through activation segment conformation. *Molecular Cell* 15: 661–675.

Obergfell A., Eto K., Mocsai A., Buensuceso C., Moores S.L., Brugge J.S., Lowell C.A. & Shattil S.J. 2002. Coordinate interactions of Csk, Src, and Syk kinases with αIIbβ3 initiate integrin signaling to the cytoskeleton. *Journal of Cell Biology* 157: 265–275.

Osman N., Turner H., Lucas S., Reif K. & Cantrell D.A. 1996. The protein interactions of the immunoglobulin receptor family tyrosine-based activation motifs present in the T cell receptor ζ subunits and the CD3 γ,δ and ε chains. *European Journal of Immunology* 26: 1063–1068.

Papp E., Tse J.K.Y., Ho A., Wang S., Shaw D., Lee S., Barnett J., Swinney D.C. & Bradshaw J.M. 2007. Steady state kinetics of spleen tyrosine kinase investigated by a real time fluorescence assay. *Biochemistry* 46: 15103–15114.

Park M.J., Sheng R., Silkov A., Jung D.J., Wang Z.G., Xin Y., Kim H., Thiagarajan-Rosenkranz P., Song S., Yoon Y., Nam W., Kim I., Kim E., Lee D.G., Chen Y., Singaram I., Wang L., Jang M.H., Hwang C.S., Honig B., Ryu S., Lorieau J., Kim Y.M. & Cho W. 2016. SH2 domains serve as lipid-binding modules for pTyr-signaling proteins. *Molecular Cell* 62: 7–20.

Pasquier B., Launay P., Kanamaru Y., Moura I.C., Pfirsch S., Ruffié C., Hénin D., Benhamou M., Pretolani M., Blank U. & Monteiro R.C. 2005. Identification of FcαRI as an inhibitory receptor that controls inflammation: dual role of FcRγ ITAM. *Immunity* 22: 31–42.

Poy F., Yaffe M.B., Sayos J., Saxena K., Morra M., Sumegi J., Cantley L.C., Terhorst C. & Eck M.J. 1999. Crystal structures of the XLP protein SAP reveal a class of SH2 domains with extended, phosphotyrosine-independent sequence recognition. *Molecular Cell* 4: 555–561.

Reeve J.L., Zou W., Liu Y., Maltzman J.S., Ross F.P. & Teitelbaum S.L. 2009. SLP-76 couples Syk to the osteoclast cytoskeleton. *The Journal of Immunology* 183: 1804–1812.

Reth M. 1989. Antigen receptor tail clue. *Nature* 338: 383.

Robinson D.R., Wu Y.M. & Lin S.F. 2000. The protein tyrosine kinase family of the human genome. *Oncogene* 19: 5548–5557.

Saksela K. & Permi P. 2012. SH3 domain ligand binding: what's the consensus and where's the specificity? *FEBS Letters* 586: 2609–2614.

Schaller M.D. 2010. Cellular functions of FAK kinases: insight into molecular mechanisms and novel functions. *Journal of Cell Science* 123: 1007–1013.

Scheeff E.D. & Bourne P.E. 2005. Structural evolution of the protein kinase-like superfamily. *PLoS Computational Biology* 1: e49.

Scheffzek K., Welti S., Sudol M., Cesareni G., Superti-Furga G. & Just W. 2012. Pleckstrin homology (PH) like domains - versatile modules in protein-protein interaction platforms. *FEBS Letters* 586: 2662–2673.

Schlessinger J. 2014. Receptor tyrosine kinases: legacy of the first two decades. *Cold Spring Harbor Perspectives in Biology* 6: 1–13.

Schwartz S.L., Cleyrat C., Olah M.J., Relich P.K., Phillips G.K., Hlavacek W.S., Lidke K.A., Wilson B.S. & Lidke D.S. 2017. Differential mast cell outcomes are sensitive to FcεRI-Syk binding kinetics. *Molecular Biology of the Cell* 28: 3397–3414.

Schymeinsky J., Sindrilaru A., Frommhold D., Sperandio M., Gerstl R., Then C., Mócsai A., Scharffetter-Kochanek K. & Walzog B. 2006. The Vav binding site of the non-receptor tyrosine kinase Syk at Tyr 348 is critical for β2integrin (CD11/CD18)-mediated neutrophil migration. *Blood* 108: 3919–3927.

Seda V. & Mraz M. 2015. B-cell receptor signalling and its crosstalk with other pathways in normal and malignant cells. *European Journal of Haematology* 94: 193–205.

Serrels B. & Frame M.C. 2012. FAK and talin: who is taking whom to the integrin engagement party? *Journal of Cell Biology* 196: 185–187.

Shattil S.J. & Newman P.J. 2004. Integrins: dynamic scaffolds for adhesion and signaling in platelets. *Blood* 104: 1606–1615.

Shekhtman A., Ghose R., Wang D., Cole P.A. & Cowburn D. 2001. Novel mechanism of regulation of the non-receptor protein tyrosine kinase Csk: insights from NMR mapping studies and site-directed mutagenesis. *Journal of Molecular Biology* 314: 129–138.

Shih C.-H., Chiang T.-B. & Wang W.-J. 2014. A critical role for the regulation of Syk from agglutination to aggregation in human platelets. *Biochemical and Biophysical Research Communications* 443: 580–585.

Shudler M. & Niv M.Y. 2009. Blockmaster: partitioning protein kinase structures using normal-mode analysis. *Journal of Physical Chemistry A* 113: 7528–7534.

Shults M.D., Carrico-Moniz D. & Imperiali B. 2006. Optimal Sox-based fluorescent chemosensor design for serine/threonine protein kinases. *Analytical Biochemistry* 352: 198–207.

Shults M.D. & Imperiali B. 2003. Versatile fluorescence probes of protein kinase activity. *Journal of the American Chemical Society* 125: 14248–14249.

Siveen K.S., Prabhu K.S., Achkar I.W., Kuttikrishnan S., Shyam S., Khan A.Q., Merhi M., Dermime S. & Uddin S. 2018. Role of non receptor tyrosine kinases in hematological malignances and its targeting by natural products. *Molecular Cancer* 17: 31.

Suzuki-Inoue K., Fuller G.L.J., García Á., Eble J.A., Pöhlmann S., Inoue O., Gartner T.K., Hughan S.C., Pearce A.C., Laing G.D., Theakston R.D.G., Schweighoffer E., Zitzmann N., Morita T., Tybulewicz V.L.J., Ozaki Y. & Watson S.P. 2006. A novel Syk-dependent mechanism of platelet activation by the C-type lectin receptor CLEC-2. *Blood* 107: 542 LP-549.

Takala H., Nurminen E., Nurmi S.M., Aatonen M., Strandin T., Takatalo M., Kiema T., Gahmberg C.G., Ylänne J. & Fagerholm S.C. 2008. Beta2 integrin phosphorylation on Thr758 acts as a molecular switch to regulate 14-3-3 and filamin binding. *Blood* 112: 1853–1862.

Tamir I., Dal Porto J.M. & Cambier J.C. 2000. Cytoplasmic protein tyrosine phosphatases SHP-1 and SHP-2: regulators of B cell signal transduction. *Current Opinion in Immunology* 12: 307–315.

Taylor S.S. & Kornev A.P. 2011. Protein kinases: evolution of dynamic regulatory proteins. *Trends in Biochemical Sciences* 36: 65–77.

Tsang E., Giannetti A.M., Shaw D., Dinh M., Tse J.K.Y., Gandhi S., Ho H., Wang S., Papp E. & Bradshaw J.M. 2008. Molecular mechanism of the Syk activation switch. *Journal of Biological Chemistry* 283: 32650–32659.

Tsung H. L., Rosales C. , Mondal K., Bolen J. B., Haskill S. J.R.L. 1995. Integrin-mediated tyrosine phosphorylation and cytokine message induction in monocytic cells. A possible signaling role for the Syk tyrosine kinase. *Journal of Biological Chemistry* 270: 16189–16197.

Turner M., Joseph Mee P., Costello P.S., Williams O., Price A.A., Duddy L.P., Furlong M.T., Geahlen R.L. & Tybulewicz V.L.J. 1995. Perinatal lethality and blocked B-cell development in mice lacking the tyrosine kinase Syk. *Nature* 378: 298.

Underhill D.M. & Goodridge H.S. 2007. The many faces of ITAMs. *Trends in Immunology* 28: 66–73.

Vines C.M., Potter J.W., Xu Y., Geahlen R.L., Costello P.S., Tybulewicz V.L., Lowell C.A., Chang P.W., Gresham H.D. & Willman C.L. 2001. Inhibition of β2 integrin receptor and Syk kinase signaling in monocytes by the Src family kinase Fgr. *Immunity* 15: 507–519.

Vinogradova O., Velyvis A., Velyviene A., Hu B., Haas T.A., Plow E.F. & Qin J. 2002. A structural mechanism of integrin αIIbβ3 "Inside-Out" activation as regulated by its cytoplasmic face. *Cell* 110: 587–597.

Volmering S., Block H., Boras M., Lowell C.A. & Zarbock A. 2016. The neutrophil Btk signalosome regulates integrin activation during sterile inflammation. *Immunity* 44: 73–87.

Wagner M.J., Stacey M.M., Liu B.A. & Pawson T. 2013. Molecular mechanisms of SH2- and PTB-domain-containing proteins in receptor tyrosine kinase signaling. *Cold Spring Harbor Perspectives in Biology* 5: 1–20.

Waksman G. & Kuriyan J. 2004. Structure and specificity of the SH2 domain. *Cell* 116: S45–S48.

Wang H., Kadlecek T.A., Au-Yeung B.B., Goodfellow H.E.S., Hsu L.Y., Freedman T.S. & Weiss A. 2010. ZAP-70: an essential kinase in T-cell signaling. *Cold Spring Harbor perspectives in biology* 2: 1–17.

Wehrman T., He X., Raab B., Dukipatti A., Blau H. & Garcia K.C. 2007. Structural and mechanistic insights into nerve growth factor interactions with the TrkA and p75 receptors. *Neuron* 53: 25–38.

Wolanin P.M., Thomason P.A. & Stock J.B. 2002. Protein family review histidine protein kinases : key signal transducers outside the animal kingdom. *Genome Biology* 3: 1–8.

Woo M.S., Ohta Y., Rabinovitz I., Stossel T.P. & Blenis J. 2004. Ribosomal S6 kinase (RSK) regulates phosphorylation of filamin A on an important regulatory site. *Molecular and cellular biology* 24: 3025–3035.

Woodside D.G., Obergfell A., Leng L., Wilsbacher J.L., Miranti C.K., Brugge J.S., Shattil S.J. & Ginsberg M.H. 2001. Activation of Syk protein tyrosine kinase through interaction with integrin β cytoplasmic domains. *Current Biology* 11: 1799–1804.

Woodside D.G., Obergfell A., Talapatra A., Calderwood D.A., Shattil S.J. & Ginsberg M.H. 2002. The N-terminal SH2 domains of Syk and ZAP-70 mediate phosphotyrosine-independent binding to integrin β cytoplasmic domains. *Journal of Biological Chemistry* 277: 39401–39408.

Wu Y., Span L.M., Nygren P., Zhu H., Moore D.T., Cheng H., Roder H., DeGrado W.F. & Bennett J.S. 2015. The tyrosine kinase c-Src specifically binds to the active integrin αIIbβ3 to initiate outside-in signaling in platelets. *Journal of Biological Chemistry* 290: 15825–15834.

Xiao W., Nishimoto H., Hong H., Kitaura J., Nunomura S., Maeda-Yamamoto M., Kawakami Y., Lowell C.A., Ra C. & Kawakami T. 2005. Positive and negative regulation of mast cell activation by Lyn via the Fc RI. *The Journal of Immunology* 175: 6885–6892.

Xu W., Harrison S.C. & Eck M.J. 1997. Three-dimensional structure of the tyrosine kinase c-Src. *Nature* 385: 595.

Xu A.M. & Huang P.H. 2010. Receptor tyrosine kinase coactivation networks in cancer. *Cancer Research* 70: 3857–3860.

Xu B. & Miller W.T. 1996. Src homology domains of v-Src stabilize an active conformation of the tyrosine kinase catalytic domain. *Molecular and cellular biochemistry* 158: 57—63.

Xu F. & Teitelbaum S.L. 2013. Osteoclasts: New Insights. *Bone Research* 1: 11–26.

Yan Q., Barros T., Visperas P.R., Deindl S., Kadlecek T.A., Weiss A. & Kuriyan J. 2013. Structural basis for activation of ZAP-70 by phosphorylation of the SH2-kinase linker. *Molecular and Cellular Biology* 33: 2188–2201.

Yan S.R., Fumagalli L. & Berton G. 1995. Activation of p58c-fgr and p53/56lyn in adherent human neutrophils: evidence for a role of divalent cations in regulating neutrophil adhesion and protein tyrosine kinase activities. *Journal of inflammation* 45: 297–311.

Yang C., Yanagi S., Wang X., Sakai K., Taniguchi T. & Yamamura H. 1994. Purification and characterization of a protein-tyrosine kinase p72syk from porcine spleen. *European journal of biochemistry / FEBS* 221: 973–978.

Yankee T.M., Keshvara L.M., Sawasdikosol S., Harrison M.L. & Geahlen R.L. 1999. Inhibition of signaling through the B cell antigen receptor by the protooncogene product, c-Cbl, requires Syk tyrosine 317 and the c-Cbl phosphotyrosine-binding domain. *Journal of immunology (Baltimore, Md. : 1950)* 163: 5827–5835.

Yuzawa S., Opatowsky Y., Zhang Z., Mandiyan V., Lax I. & Schlessinger J. 2007. Structural basis for activation of the receptor tyrosine kinase KIT by stem cell factor. *Cell* 130: 323–334.

Zeitlmann L., Knorr T., Knoll M., Romeo C., Sirim P. & Kolanus W. 1998. T cell activation induced by novel gain-of-function mutants of Syk and ZAP-70. *Journal of Biological Chemistry* 273: 15445–15452.

Zhang X., Gureasko J., Shen K., Cole P.A. & Kuriyan J. 2006. An allosteric mechanism for activation of the kinase domain of epidermal growth factor receptor. *Cell* 125: 1137–1149.

Zhang Y., Oh H., Burton R.A., Burgner J.W., Geahlen R.L. & Post C.B. 2008. Tyr130 phosphorylation triggers Syk release from antigen receptor by long-distance conformational uncoupling. *Proceedings of the National Academy of Sciences of the United States of America* 105: 11760–11765.

Zhi H., Rauova L., Hayes V., Gao C., Boylan B., Newman D.K., Steven E., Cooley B.C., Poncz M., Newman P.J., Dc W. & Mckenzie S.E. 2013. Cooperative integrin / ITAM signaling in platelets enhances thrombus formation in vitro and in vivo. *Thrombosis and haemostasis* 121: 1858–1867.

Zhou Y. & Abagyan R. 1998. How and why phosphotyrosine-containing peptides bind to the SH2 and PTB domains. *Folding and Design* 3: 513–522.

Zou W., Croke M., Fukunaga T., Broekelmann T.J., Mecham R.P. & Teitelbaum S.L. 2013. Zap70 inhibits Syk-mediated osteoclast function. *Journal of Cellular Biochemistry* 114: 1871–1878.

Zou W., Kitaura H., Reeve J., Long F., Tybulewicz V.L.J., Shattil S.J., Ginsberg M.H., Ross F.P. & Teitelbaum S.L. 2007. Syk, c-Src, the αvβ3 integrin, and ITAM immunoreceptors, in concert, regulate osteoclastic bone resorption. *The Journal of Cell Biology* 176: 877–888.

ORIGINAL PAPERS

I

PHOSPHORYLATED IMMUNORECEPTOR TYROSINE-BASED ACTIVATION MOTIFS AND INTEGRIN CYTOPLASMIC DOMAINS ACTIVATE SPLEEN TYROSINE KINASE VIA DISTINCT MECHANISMS

by

Lina Antenucci, Vesa P. Hytönen, and Jari Ylänne, 2018

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JBC ARTICLE

Phosphorylated immunoreceptor tyrosine-based activation motifs and integrin cytoplasmic domains activate spleen tyrosine kinase via distinct mechanisms

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Spleen tyrosine kinase (Syk) is involved in cellular adhesion and also in the activation and development of hematopoietic cells. Syk activation induced by genomic rearrangement has been linked to certain T-cell lymphomas, and Syk inhibitors have been shown to prolong survival of patients with B-cell lineage malignancies. Syk is activated either by its interaction with a double-phosphorylated immunoreceptor tyrosine-based activation motif (pITAM), which induces rearrangements in the Syk structure, or by the phosphorylation of specific tyrosine residues. In addition to its immunoreceptor function, Syk is activated downstream of integrin pathways, and integrins bind to the same region in Syk as does pITAM. However, it is unknown whether integrins and pITAM use the same mechanism to activate Syk. Here, using purified Syk protein and fluorescence-based enzyme assay we investigated whether interaction of the integrin β_3 cytoplasmic domain with the Syk regulatory domain causes changes in Syk activity similar to those induced by pITAM peptides. We observed no direct Syk activation by soluble integrin peptide, and integrin did not compete with pITAM-induced activation even though at high concentrations, the integrin cytoplasmic domain peptide competed with Syk's substrate. However, clustered integrin peptides induced Syk activation, presumably via a transphosphorylation mechanism. Moreover, the clustered integrins also activated a Syk variant in which tyrosines were replaced with phenylalanine (Y348F/Y352F), indicating that clustered integrin–induced Syk activation involved other phosphorylation sites. In conclusion, integrin cytoplasmic domains do not directly induce Syk conformational changes and do not activate Syk via the same mechanism as pITAM.

Spleen tyrosine kinase (Syk) is a 72-kDa cytoplasmic signaling protein discovered in bovine thymus (1) and forms a small family of non-receptor tyrosine kinases with ζ chain-associated protein kinase of 70 kDa (ZAP-70) (2). Knockout studies in mouse show that Syk is required for B-cell differentiation (3). $Syk^{-/-}$ mice are not viable because of severe perinatal hemorrhage due to lack of separation of lymphatic and venous vasculature (4). Even though $Syk^{-/-}$ stem cells can give rise to all other hematopoietic cells than B-cells, Syk seems to have a wide function in many hematopoietic cell types, whereas the function of ZAP-70 is more restricted to T-cell and NK cells (2).

Syk has an important role in cancer. In hematological malignancies Syk is mainly considered as a tumor promoter (5). A chromosomal translocation has been found in T-cell leukemias leading to fusion of interleukin 2 inducible T-cell kinase (*ITK*) and *SYK* genes and to an aberrantly active Syk kinase (6). This fusion has been validated as an oncogenic driver in a mouse model (7). In many other hematological malignancies high Syk activity has been detected (5), although, to our knowledge, no human point mutations have been validated as oncogenic driver mutations. Small molecule Syk inhibitors have been designed and they were shown to prolong survival of patients (8). At the moment, Syk inhibitors have not been approved for clinical use because they are rather nonspecific and many side effects are observed (9). In solid tumors, the role of Syk is less clear than in hematological tissues. In many cases *SYK* expression is reduced during malignancy (*e.g.* see Ref. 10, reviewed in Ref. 5). Many somatic mutations have been found in *SYK* genes (cancer.sanger.ac.uk/cosmic)[2] and epigenetic modification can also be responsible for alterations in the expression (11). There is some evidence that Syk can act as a tumor suppressor in breast carcinomas because it reduces cell growth when re-expressed in a breast carcinoma cell line and tested in a xenotransplantation model (12).

Syk has a C-terminal tyrosine kinase domain and an N-terminal regulatory domain formed by two Src homology 2 (SH2)[3] domains connected by a flexible linker region called interdomain A (IA). This tandem SH2 region (tSH2) is connected to the kinase domain via a second linker region called interdomain B (IB) (13). The Syk activation mechanism is mostly understood. The inactive kinase can interact through SH2 domains

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[3] The abbreviations used are: SH2, Src homology 2; tSH2, tandem SH2; IA, interdomain A; IB, interdomain B; pITAM, phosphorylated immunoreceptor tyrosine-based activation motif; SPR, surface plasmon resonance; TEV, tobacco etch virus; RU, response unit; ANOVA, analysis of variance.

with phosphorylated immunoreceptor tyrosine-based activation motif (pITAM) associated with TCR, BCR, and FcR receptors. Upon pITAM binding the tSH2 of Syk changes its orientation in relationship to the kinase domain and allows kinase activity (13). In addition, Syk is phosphorylated by Lyn and Lck kinases and it also has an auto/transphosphorylation activity that can lead to Syk activation independently from pITAM (14). Considering that, an "OR gate switch" model for Syk activation has been proposed (14, 15). This means that an initial activation by either pITAM binding, OR, by a phosphorylation event can ultimately lead to full activity of the enzyme.

In many different biological processes ITAM-based receptor signaling is concomitant with integrin signaling. The classical example of this kind of collaboration is immunological synapse, where both β_2 integrins and TCR are required (16, 17). In neutrophils FcR signaling is linked to both integrin β_2 and β_3 function (18, 19). During platelet adhesion to damaged vascular cell wall the collagen receptor GPVI–FcRγ complex works in concert with β_1 and β_3 integrins (20). Clearly there are multiple crossing points between pITAM/Syk and integrin signaling pathways (reviewed in Ref. 2). There is also a direct interaction between Syk and integrins: Syk interacts with integrin β_1, β_2, and β_3 cytoplasmic tails (21) and the interaction is mediated mainly by N-SH2 domain and IA, whereas the C-SH2 has only a marginal role in the binding (22). On the integrin side, the last 23 amino acids of β_3 are sufficient for binding to Syk and deletion of four C-terminal residues abolished the binding (21). There are two tyrosine residues in the Syk interacting sequence of integrin β_3, but the interaction does not require integrin phosphorylation. Rather, tyrosine-phosphorylated integrin tails fail to interact with Syk (22). Although Syk has been shown to be required for normal $\alpha_{IIb}\beta_3$ integrin function in platelets (23), a recent study has shown that integrin-induced Syk signaling is independent on pITAM-induced Syk activation (24). In this study, mutation of the Syk N-SH2 domain that prevented phosphotyrosine binding blocked ITAM-dependent signaling in mouse platelet, but did not alter integrin-dependent Syk activation (24).

Even though integrins were found to activate Syk signaling more than two decades ago (25), it is still unclear how this activation works. More specifically, can integrins activate Syk via a similar conformational mechanism as pITAM? Are other integrin-associated kinases required for the activation? Here we used *in vitro* kinetic fluorescence-based assay to study the effect of the integrin β_3 cytoplasmic domain peptide on the activity of Syk. Our results show that soluble integrin peptides do not directly activate Syk as pITAM peptide does. On the other hand, we show that clustered integrin peptides can induce Syk activation independently of other kinases, presumably via an auto/transphosphorylation mechanism.

Results

Syk activity measurement using real-time fluorescence-based assay

To study the regulation of Syk *in vitro*, we first characterized the enzymatic properties of our purified full-length Syk protein (Fig. 1). To monitor kinase activity, we used a substrate peptide with SOX fluorophore ((*S*)-2-amino-N^{α}-(9-fluorenylmethyloxycarbonyl)-3-[8-hydroxy-5-(*N*,*N*-dimethylsulfonamido) quinoline-2-yl]propionic acid), whose fluorescence is modulated by phosphorylation (13, 25–27). As shown before (14), purified full-length Syk has low initial activity, which is enhanced in the presence of ATP after a lag period (Fig. 2*A*). This indicates that Syk has an intrinsic autophosphorylation or transphosphorylation activity that can initiate protein activation by phosphorylation of specific tyrosines residues along the structure (14). Because calculation of initial reaction velocity v_i is meaningless for the inactive or partially active enzyme, we analyzed the kinetic parameters of the activated Syk that was first incubated for 30 min with ATP (Fig. 2, *B–D*). The data were fitted to the ternary model equation because it has been previously shown that Syk kinase substrate binding fits better with ternary complex equation instead of the ping-pong model (28). K_m for SOX peptide was estimated to be 6 ± 1 μM and K_m for ATP was 29 ± 3 μM. The k_{cat}, calculated considering the initial velocity, was 22 ± 3 min^{-1}.

Syk can be activated by pITAM but not by soluble integrin β_3 tails

The interaction between the integrin cytoplasmic tail and Syk is mediated by tSH2 (22). We used surface plasmon resonance (SPR) to confirm the interaction between tSH2 and the integrin β_3 cytoplasmic tail peptide. The integrin peptide was coupled on the surface and increasing concentrations of tSH2 were injected on the sensor. The interaction had a fast, concentration-dependent association phase and concentration-independent dissociation phase (Fig. 3*A*). When the experimental data were fitted with Langmuir equation, the kinetic parameters were: $k_{on} = 398 \pm 87$ M^{-1} s^{-1}, $k_{off} = (3.22 \pm 0.12)\ 10^{-3}$ s^{-1}, and $K_D = (8.1 \pm 1.5)\ 10^{-6}$ M. Because the association phase was very fast and the Langmuir model is mainly based on the equilibrium levels, we also calculated the kinetic parameters assuming a single binding site and using the association and dissociation data only (Fig. 3, *B–E*) (see "Experimental procedures") (29, 30). With this method, the calculated kinetic parameters were: $k_{on} = 604 \pm 212$ M^{-1} s^{-1}, $k_{off} = (2.68 \pm 0.20)\ 10^{-3}$ s^{-1}, and $K_D = (4.44 \pm 1.55)\ 10^{-6}$ M. As expected, the k_{off} values were comparable, whereas the k_{on} calculated with the Langmuir model is half of the one calculated using first-rate reaction equation. We reason that the kinetic model should in this case be more accurate. On the other hand, the calculated K_D are comparable. Thus we consider that the K_D value of 4.44 ± 1.55 μM is the best estimate from our data.

With β_3 integrin/Syk interaction parameters in hand, we next compared the effect of saturating concentrations of pITAM and β_3 integrin tail peptides on Syk activity. As expected, 10 μM pITAM significantly reduced the lag phase on Syk activation. However, 30 μM β_3 tail peptide failed to do so (Fig. 4*A*). When both peptides were present, the integrin peptide did not change the effect of pITAM. When active Syk was used in this assay in the presence of a saturating concentration of SOX peptide neither pITAM nor integrin showed any effect (Fig. 4*B*).

To further analyze the effect of integrin β_3 cytoplasmic tail, we investigated the possible activity modulation of active Syk.

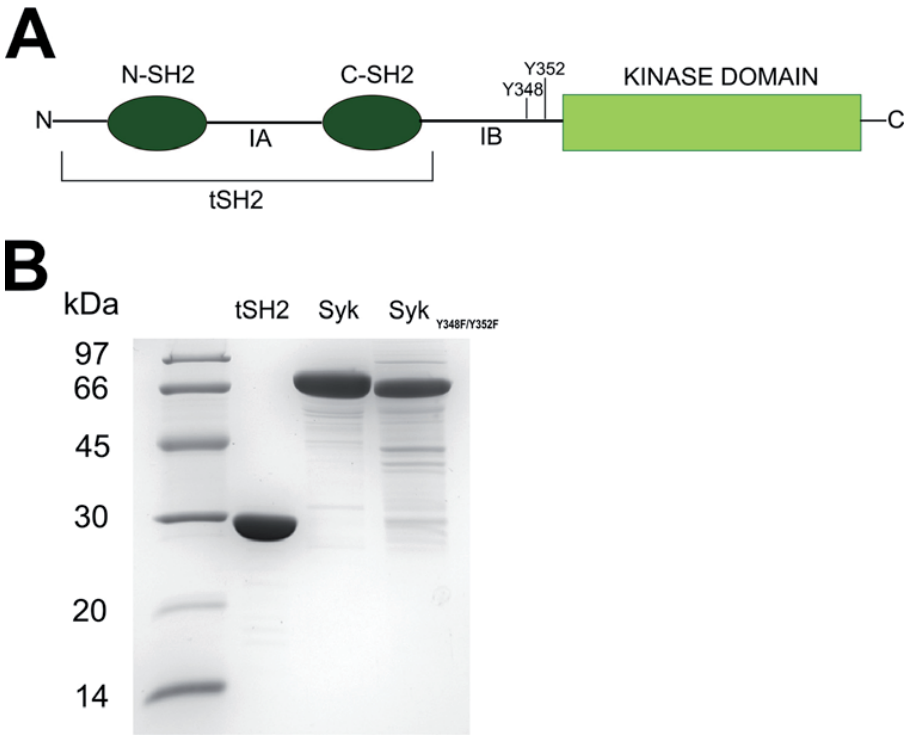

Figure 1. *A*, schematic representation of the Syk kinase structure. The N-SH2 and C-SH2 are the regulatory domains that are connected to each other through IA; they are named tandem SH2 (*tSH2*) together. tSH2 are connected to kinase domain via IB. Tyrosines 348 and 352 get phosphorylated during protein activation and in this study were mutated to phenylalanine. *B*, SDS-PAGE of purified proteins. For each protein preparation 5 μg of protein was loaded on the gel.

To assess the integrin effect on K_m for the SOX peptide, the ATP concentration was kept in saturating concentration and different concentrations of SOX peptide ranging from 1.5 to 30 μM were tested in the presence of integrin peptide (from 0 to 100 μM). The same experiment was done to test the effect of integrin on K_m for ATP: SOX peptide was in saturating concentration and ATP was tested from 10 to 100 μM. The double-reciprocal plots (Fig. 4, *C* and *D*) are consistent with integrin being a weak competitive inhibitor for the SOX peptide and non-competitive inhibitor for ATP. However, the inhibition was only observed at high integrin concentrations and the K_i values could not be accurately calculated, possibly due to poor solubility of the integrin peptide at 100 μM concentration. This is all consistent with the integrin peptide containing two tyrosine residues and being able to act as a substrate at high concentrations even though both tyrosines are not preceded by negatively charged amino acids that are required for good Syk substrates (31). Taken together, these data show that the integrin β_3 tail peptide does not directly activate Syk, but it may be a substrate or competitive inhibitor at high concentrations.

Clustered integrin β_3 tails can enhance Syk activation

In contrast to our *in vitro* findings, assays with platelets or cultured cells have shown that integrin ligation enhances Syk activity (21, 25). This could either be mediated by other kinases activated by integrin, such as Src family kinases, or by integrin clustering. To mimic integrin clustering *in vitro*, integrin β_3 peptide was coupled through the thiol group of the N-terminal cysteine on the assay plate at a high concentration. The clustered integrin peptide caused a significant reduction of the lag phase of Syk activity (Fig. 5*A*). Moreover, this effect was additive to that of the soluble pITAM peptide. This is consistent with the hypothesis that recruitment of Syk to integrin clusters will enhance its transphosphorylation and thus lead to increased local activity of the enzyme. As expected, clustered integrin did not have any effect on the pre-activated Syk (Fig. 5*B*).

Syk mutant (Y348F/Y352F) can be activated by clustered integrin β_3 tails

There are two tyrosine residues in the linker peptide of C-SH2 and the kinase domain Syk (Tyr-348 and Tyr-353 in human and Tyr-342 and Tyr-346 in mouse) that have been reported to be the main phosphorylation sites required for activation of Syk via Src-family kinases (Fig. 1). To find out if these residues are required for integrin clustering-induced activation of Syk, we studied the activation of double mutant Syk (Y348F/Y352F). Consistently with literature (13), this mutant has a low basal activity that can be enhanced by the pITAM peptide (Fig. 6*A*). Surprisingly, Syk (Y348F/Y352F) activation was enhanced by the clustered integrin in a similar way as that of wildtype Syk. This suggests that Syk clustering by integrins causes transphosphorylation of Syk activation tyrosine residues other than Tyr-348 and Tyr-352.

Discussion

Syk plays a crucial role in diverse types of cells and it is involved in many biological processes. In this paper we have

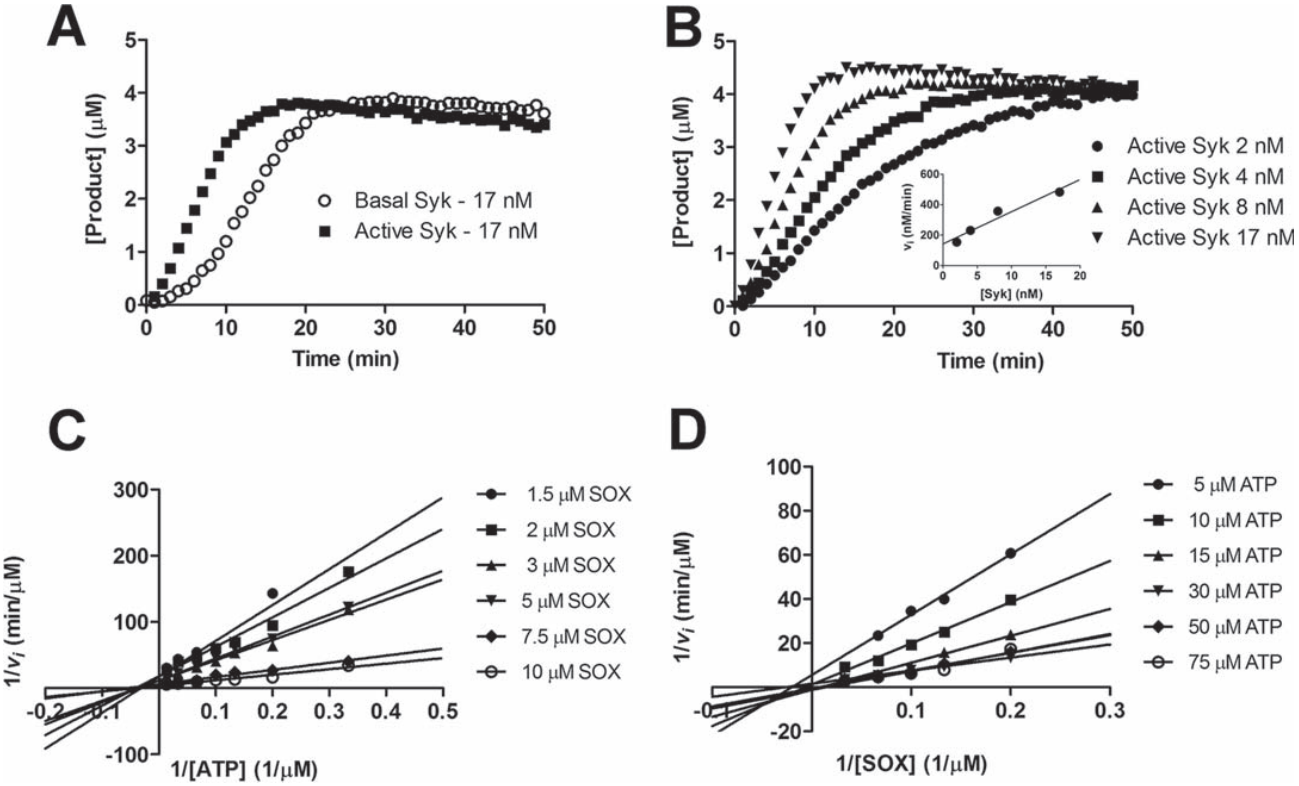

Figure 2. Kinetic characterization of Syk kinase. *A*, time course (min) of SOX peptide phosphorylation (μM) for basal Syk (*open circles*) and active Syk (*squares*). Syk was activated through incubation with 100 μM ATP for 30 min at room temperature. Basal Syk and active Syk were used at the same concentration of 17 nM; basal Syk showed a ~5 min lag phase, which was abolished in the active Sky. *B*, kinetic measurements of active Syk at different concentrations (2, 4, 8, and 17 nM). The *inset* shows the initial reaction speed (nM/min) calculated using linear regression fit from 5 to 15 min plotted against the active Syk concentration (nM). The calculated k_{cat} in this experiment was 22 ± 3 min^{-1}. *C* and *D*, Lineweaver-Burk plot generated from two-substrate analysis measurements of $1/v_i$ *versus* 1/[ATP] (*C*) and 1/[SOX] (*D*). Experimental data were fit with a ternary complex equation and the calculated K_m was 6 ± 1 μM for SOX peptide and 29 ± 3 μM for ATP.

studied the consequences of integrin cytoplasmic domain binding to the enzymatic activity of Syk. Although it is well established that pITAM containing receptors induce conformational changes in the regulatory domain of Syk and thus cause direct activation of the kinase domain, it has not been studied before whether integrin cytoplasmic domain peptides binding to the same region as pITAM can do the same. We found that soluble integrin peptides were not able to induce direct activation of Syk. Instead, integrin clustering could induce autophosphorylation of Syk and thus enhance its activation. These two main findings are discussed below.

There are two pathways for the initialization of Syk activation. This property has been named the OR gate switch model of Syk activation (15) meaning that Syk activation can either be initialized via pITAM binding to tSH2 or via phosphorylation. The molecular details of the pITAM/tSH2 interaction are known (32). Briefly, the N-SH2 interacts with the C-terminal pY*XX*L/I motif in ITAM receptor and the C-SH2 with the N-terminal pY*XX*L/I motif. The structures of two SH2s are very similar and they contain a positively charged pocket to accept the phosphotyrosine and a hydrophobic pocket to accommodate the leucine/isoleucine. It seems that for pITAM binding both SH2s are needed even if the C-SH2 seems to be more flexible and unstable compared with N-SH2 (32). As a consequence of pITAM binding, the orientation of tSH2 in relationship to the kinase domain changes and this releases the hinge between two globes of the kinase leading to enhanced activity. The other arm of the OR gate switch model implies that at least Tyr-348, Tyr-352, and Tyr-630 can be phosphorylated directly by Src family kinases such as Lyn, or auto/transphosphorylated by Syk itself. This can also lead to full activation of the enzyme independent of the interaction with pITAM (15).

Here our aim was to relate the OR gate switch model to integrin-mediated Syk activation. Syk interacts with integrins through tSH2 and, specifically, the N-SH2 and IA are mostly involved, whereas the C-SH2 has a marginal contribution (22). We confirmed the interaction between isolated tSH2 and integrin β_3 cytoplasmic tail using SPR experiments; the measured K_D was 4.44 ± 1.55 μM, which indicates a medium-low affinity binding. Woodside *et al.* (22) reported a higher affinity (K_D 24 nM) using different peptides and different Syk preparations. This discrepancy in the determined affinities should not affect our enzyme activation assays as we used 30 μM concentration of integrin peptide, which should be close to saturating concentration. We found that soluble integrin cytoplasmic domain peptides did not change the lag phase of Syk activation in the same way as pITAM peptides. Furthermore, integrin did not inhibit pITAM-induced activation. This is consistent with pITAM and integrins having distinct binding sites (21, 22). Our

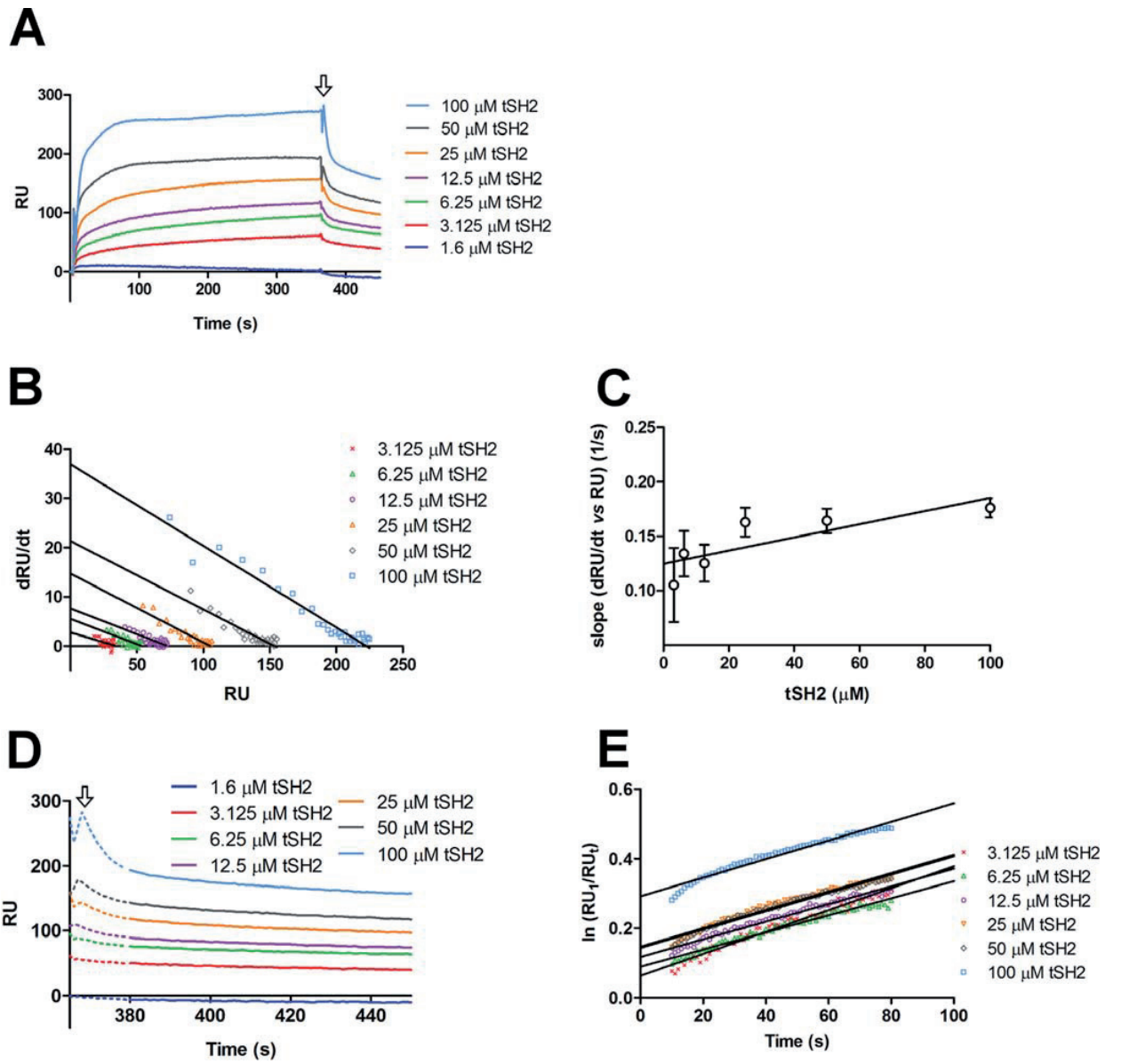

Figure 3. Interaction between tSH2 and integrin β_3 tail. *A*, SPR experiment where integrin β_3 tail peptide was coupled on the surface (ligand) and different concentrations of tSH2 (from 1.3 to 100 μM) were injected (analyte). In the graph is plotted the variation of the RU in the time (s) for each tSH2 concentration tested. The *arrow* indicates the stop of the injection and, therefore, the beginning of the dissociation phase. *B*, dRU/dt (variation of RU in time) is plotted against RU (from 10 to 30 s of the association curve, see *panel A*). *Solid lines* correspond to the best linear regressions calculated for each tSH2 concentration. *C*, calculation of association rate constant (k_{on}) of the tSH2–integrin β_3 tail peptide complex formation. *Error bars* are derived from linear regression analysis of *panel B* in this figure (see "Experimental procedures"). *D*, variation of RU in the time (s) of the dissociation phase. The *arrow* indicates the end of injection as in *panel A*. The *dotted line* corresponds to the part of dissociation curve that was not used for the determination of the dissociation rate constant (see *panel E*), whereas the *solid line* indicates the part that was used. *E*, variation of $\ln(RU_1/RU_t)$ in time (s), the best linear fits are represented with *solid lines*. RU_1 indicates the response units at the beginning of dissociation phase, and RU_t indicates the response units at the time *t*. The slope of the linear regression corresponds to the dissociation rate constant (k_{off}) (see "Experimental procedures").

results thus indicate that integrins do not induce similar conformational changes in tSH2 as pITAM.

We also performed competition experiments using the β_3 integrin cytoplasmic domain peptide with the active Syk kinase and its ATP substrates and the SOX peptide. In this assay integrin β_3 peptide acted as a competitive inhibitor toward the SOX peptide and showed characteristics of uncompetitive inhibition toward the ATP. In both cases the inhibition was observed at considerably higher concentrations than the measured K_D for the integrin binding to the tSH2 of Syk. Due to technical problems related to low inhibitory activity, we were not able to calculate K_i values using these data. The simplest explanation of the observed effect is that the tyrosine-containing sequences in the integrin peptide can compete with the substrate peptide. Similar behavior has been observed earlier with ITAM peptides: even though they do not contain the optimal Syk substrate recognition sequences, at high concentrations they reduce Syk activity

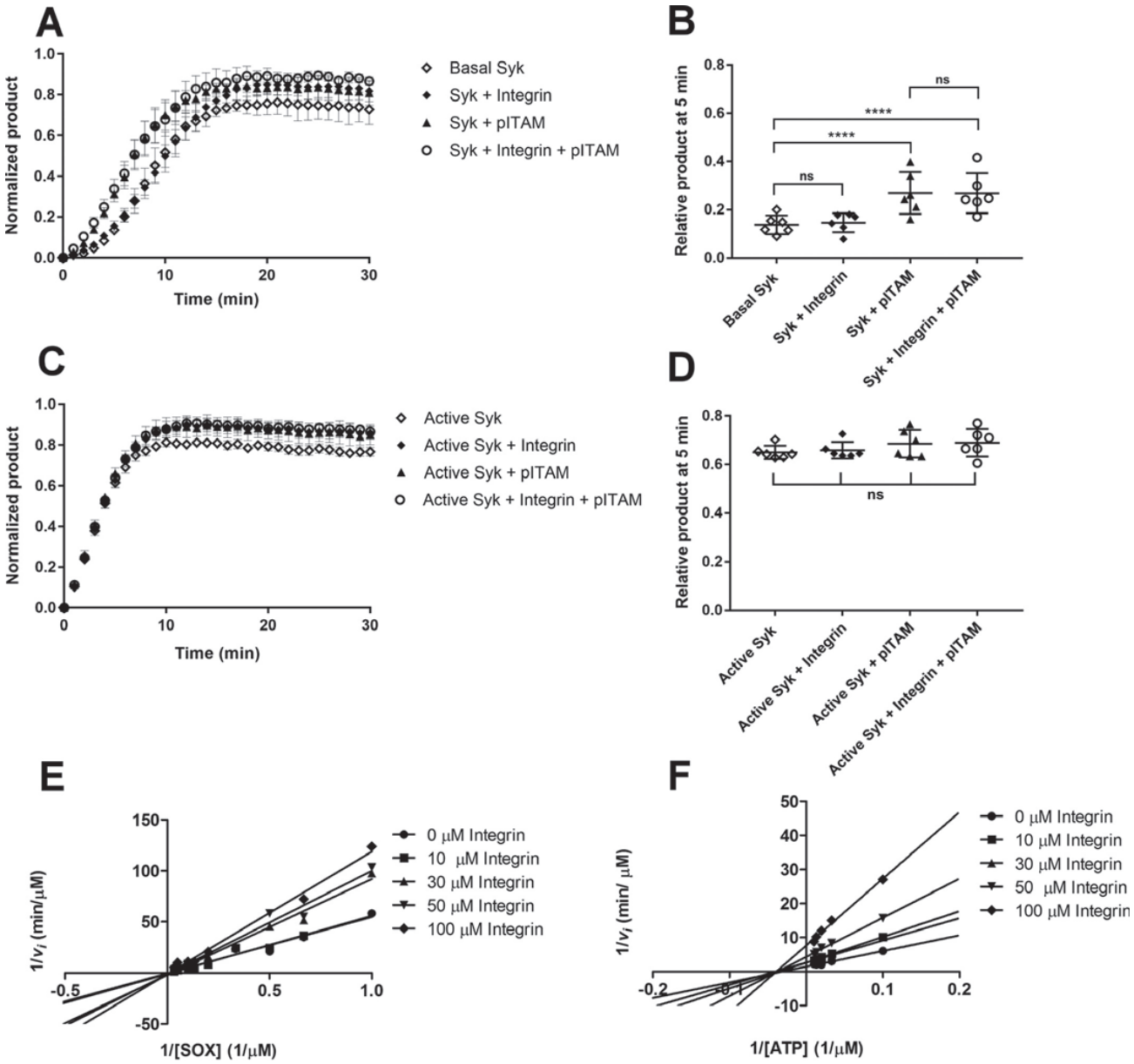

Figure 4. Effect of pITAM and integrin β_3 tail on Syk wildtype activity. *A,* kinetic measurement of Basal Syk in the absence of peptide (*open diamonds*), in presence of 30 μM integrin β_3 cytoplasmic tail peptide (*diamonds*), 10 μM pITAM peptide (*triangles*), and a combination of both (*open circles*). The activity is expressed in terms of normalized product formation in time (min); basal Syk was used at concentration of 17 nM. *Error bars* correspond to the standard deviation calculated from six different experiments. *B,* ANOVA of the 5-min time point in the experiment shown in *panel A*. The data indicate that the presence of soluble pITAM activates the protein reducing the lag-phase of basal Syk. *p* value for basal Syk *versus* Syk + pITAM and basal Syk *versus* Syk + pITAM + integrin were both <0.0001. Comparing the basal Syk *versus* Syk + integrin and the Syk + pITAM *versus* Syk + integrin + pITAM with the ANOVA test resulted in nonsignificant (*ns*) *p* values thus showing that the soluble integrin β_3 cytoplasmic tail peptide had no effect. *C,* kinetic measurement of active Syk in the absence of peptide (*open diamonds*), in the presence of 30 μM integrin β_3 cytoplasmic tail peptide (*diamonds*), 10 μM pITAM peptide (*triangles*), and a combination of both (*open circles*). The activity is expressed in terms of normalized product formation in time (min). Active Syk was used at 17 nM. *D,* the ANOVA of the 5-min time point in *panel C*; it resulted in a nonsignificant (*ns*) *p* value suggesting that the pre-activated Syk is not modulated by the presence of either of two peptides. *E* and *F,* Lineweaver-Burk plots showing the competitive inhibition toward SOX peptide (*E*) and uncompetitive inhibition toward ATP (*F*) by the integrin β_3 cytoplasmic tail. In each case $1/v_i$ was plotted against 1/[SOX] (*E*) or 1/[ATP] (*F*). The experiments were carried out using active Syk at 4 nM.

(14). Whether this phenomenon has any relevance to physiological Syk function, remains unclear.

To study the possible contribution of integrin binding to the other arm of the OR gate switch model, we mimicked integrin clustering *in vitro* by coupling the β_3 cytoplasmic domain peptide via its N terminus to the surface of the enzymatic assay plate. Integrin clustering is the basis of many, if not all, integrin adhesion-induced signaling events (33). Integrin clustering is partly mediated by extracellular ligands, partly by adhesome components such as talin and kindlin, and partly by actin cytoskeleton (34). Integrin clusters are dynamic structures where several components are exchanged rapidly (34, 35). We found

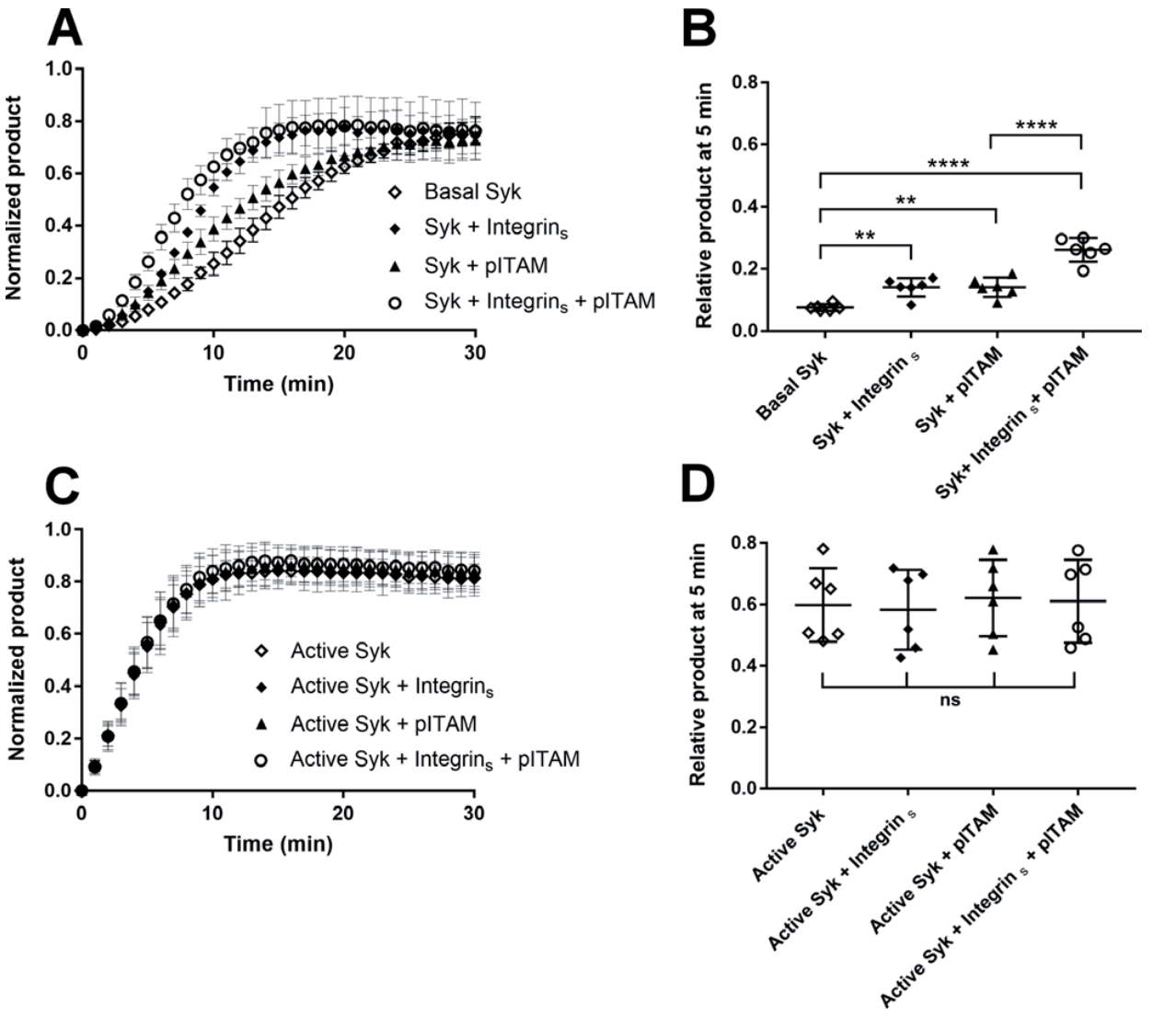

Figure 5. Effect of clustered integrin β_3 cytoplasmic tails on Syk activation. *A,* time course (min) of the normalized product formation in the absence of peptides (*open diamonds*) and in the presence of the integrin β_3 cytoplasmic tail peptide coupled on the assay plate surface (*diamonds*), 10 μM pITAM (*triangles*), and combination of the two peptides (*open circles*). Basal Syk was used at 17 nM. *Error bars* correspond to the standard deviation calculated using six different experiments. *B,* ANOVA of the 5-min time point in *panel A*. The experiment shows that surface-bound integrin peptide reduces the lag phase of basal Syk; *p* value for basal Syk *versus* Syk + integrin$_s$ was 0.0014 and was comparable with the *p* value for basal Syk *versus* Syk + pITAM, which was 0.0013. A combination of two peptides further accelerated the process (*p* value <0.0001). Integrin β_3 cytoplasmic tail bound on the plate surface also has a synergic effect in the presence of pITAM peptide, in fact comparing the Syk + pITAM *versus* Syk + pITAM + integrin$_s$ the ANOVA test gave a *p* value <0.0001. *C,* time course (min) of the normalized product formation in the absence of peptides (*open diamonds*) and presence of the integrin β_3 cytoplasmic tail peptide coupled on the assay plate surface (*diamonds*), 10 μM pITAM (*triangles*), and a combination of two peptides (*open circles*). Active Syk was used at 17 nM. *D,* ANOVA of the experiment shown in *panel C*. *p* values were calculated using a ANOVA test including six separate experiments using the relative product formed at the 5-min time point as described under "Experimental procedures." *p* values were nonsignificant (*ns*).

that clustered integrin peptides significantly reduced the lag phase of Syk autoactivation. This effect was additive with the pITAM-induced activation. We take this as evidence that integrin clustering rapidly induces Syk transphosphorylation by bringing several kinase molecules in close proximity, leading to increased local concentration (Fig. 7). We also found that the Syk (Y438F/Y352F) mutant could be activated by clustered integrins. This shows that in addition to the main regulatory tyrosines 348 and 352, there are other residues that can be transphosphorylated by the Syk itself and that activate the kinase. Although it has been shown that activation loop tyrosines 525 and 526 have little effect on Syk activity, it is well established that the C-terminal tyrosines 630 and 631 can stabilize the closed, inactive conformation of the enzyme (13, 36), and thus phosphorylation of these could induce the activity of even the Y438F/Y352F mutant. In mouse, Syk residues corresponding to Tyr-630 and Tyr-631 have been found to be autophosphorylated or transphosphorylated (37).

In conclusion, enzyme kinetic studies presented here imply that integrin cytoplasmic domain peptides do not

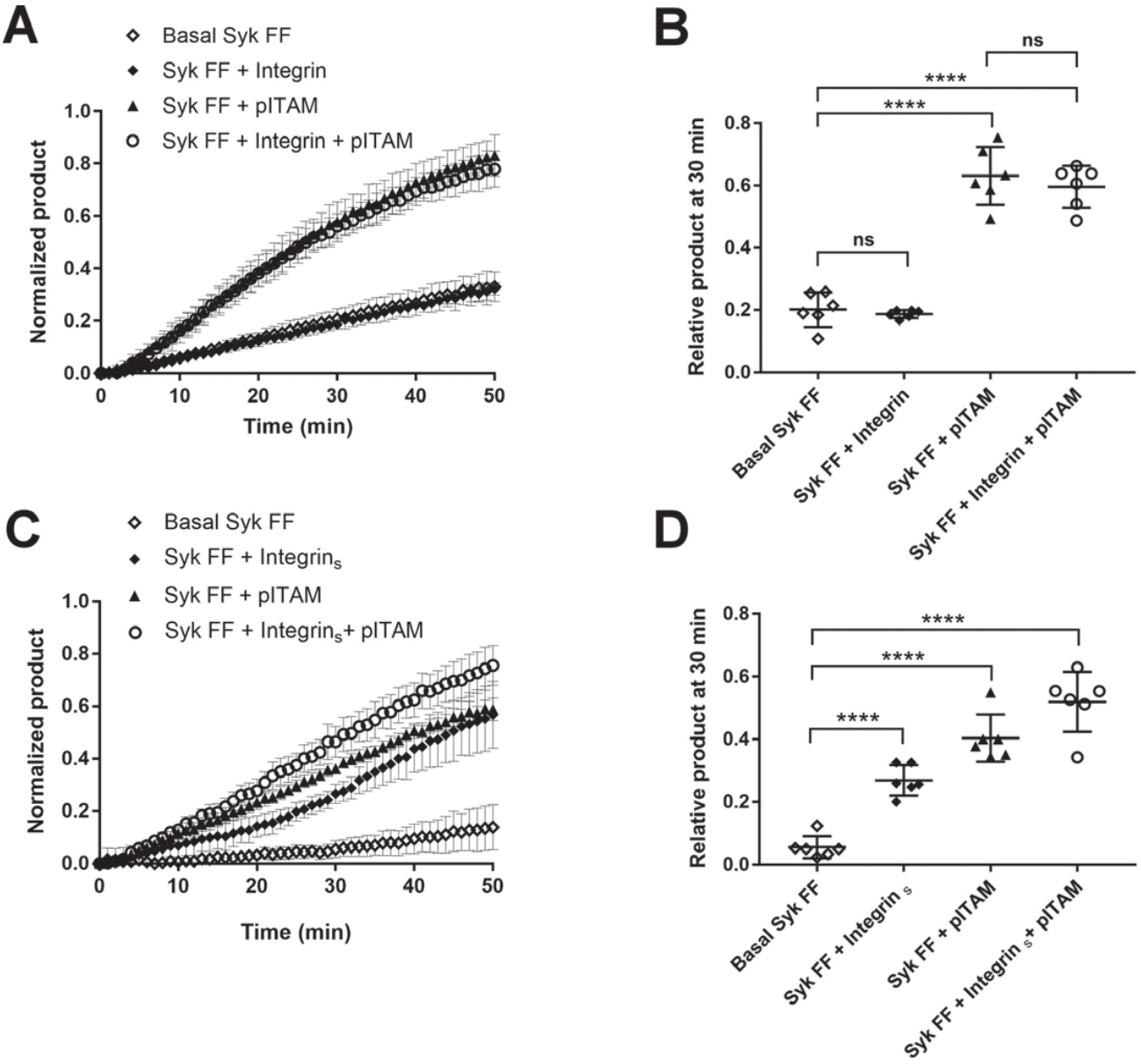

Figure 6. Effect of pITAM and clustered integrin β_3 tails on Syk Y348F/Y352F mutant activation. *A,* kinetic measurement of Syk mutant activity in the absence of peptides (*open diamonds*), 30 μM integrin β_3 tail peptide (*diamonds*), 10 μM pITAM (*triangles*), and a combination of both peptides (*open circles*). Syk FF was used at 17 nM concentration and the *error bars* are derived from the calculated standard deviation from six different experiments. *B,* ANOVA of the 30-min time point in *panel A*. Comparison of basal Syk FF *versus* Syk FF + integrin and the Syk FF + pITAM *versus* Syk FF + integrin + pITAM revealed that the soluble integrin peptide does not activate Syk FF (*p* value non-significant, *ns*) in contrast to pITAM (*p* values <0.0001). *C,* the same experiment described in *panel A*, except the integrin β_3 tail peptide, which was coupled on the plate surface. *D,* ANOVA of the 30-min time point in *panel C*. All *p* values were <0.0001.

induce direct conformational changes in Syk and do not lead to its activation with a similar mechanism as pITAM. Instead, we propose that integrin clusters may increase the local concentration of Syk and enhance its activity via increased transphosphorylation.

Experimental procedures

Syk full-length expression and purification

Spleen tyrosine kinase (*SYK*) (EC 2.7.10.2) sequence cDNA (UniProt ID: P43405) was obtained from Origene Technologies Inc. (Rockville, MD) and a fragment containing amino acids 1–635 was expressed with the cleavable N-terminal His_6 tag (MSGSHHHHHHGSSGENLYFQ ↓ SL, where ↓ denotes a tobacco etch virus (TEV) cleavage site) with pFastBac NT-TOPO (Bac-To-Bac expression system, Invitrogen, Thermo Fisher Scientific Inc.). The sequence of the expression expression construct was verified by Sanger sequencing. *Spodoptera frugiperd*a (Sf9) insect cells were transfected using Cellfectin II reagent (Thermo Fisher Scientific Inc.) using the manufacturer's protocol. Recombinant protein

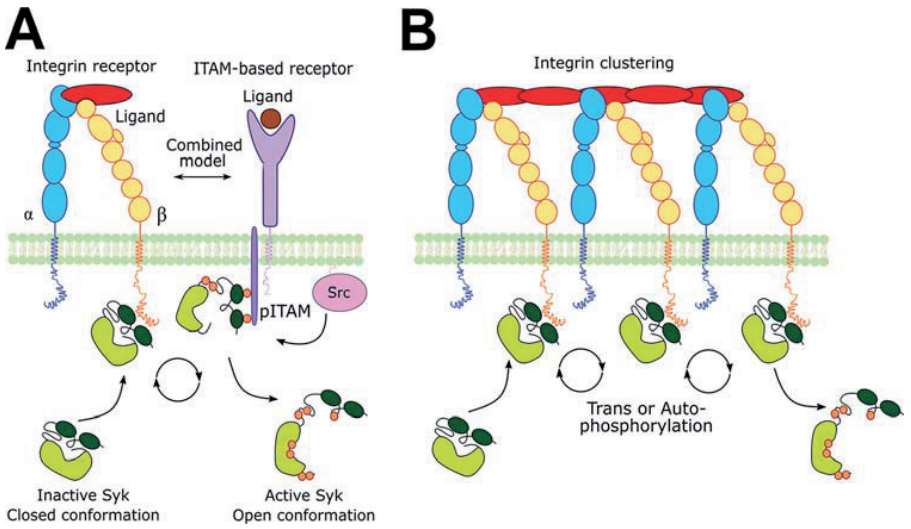

Figure 7. A model of Syk activation mediated by clustered integrins. Integrin receptor subunits α and β are represented in *blue* and *yellow*, respectively. ITAM containing receptor is in *purple*. Src family kinase is represented in *pink*; Lyn kinase is the Src kinase, which is mainly involved in the Syk activation procedure. Syk is represented in two conformations (*closed* and *open*). The kinase domain is *light green*, whereas the SH2 domains are in *dark green*, *black lines* indicate the linker regions. The *small orange circles* represent the phosphorylations along the Syk structure. *A*, combined model of Syk activation mediated by integrin and ITAM-containing receptors. Syk is recruited near the membrane by the interaction with integrin tail and phosphorylated ITAM. Syk is first activated by interaction with pITAM through conformational rearrangement in the kinase structure. Then it can be phosphorylated by other kinases recruited via the integrin pathway. Src-family kinases may be responsible for the phosphorylation of both ITAM receptor and Syk. *B,* Syk activation model mediated by integrin clustering. Inactive Syk can interact with the integrin tail. The proximity effect enhances Syk trans/autophosphorylation activity leading to Syk activation. Importantly, this pathway can occur independently of pITAM and Src family kinases.

was expressed as described previously (38). Cells were harvested 72 h after infection (multiplicity of infection = 1) by centrifugation at 500 × *g* and resuspended in cold lysis buffer (50 mM sodium phosphate, 500 mM NaCl, 5 mM $MgCl_2$, 0.1% Nonidet P-40, 1 mM dithiothreitol (DTT), pH 8); Pierce™ Universal Nuclease for Cell lysis (ThermoFisher) 25 units/ml and protease inhibitors tablet (cOmplete™ Protease Inhibitor mixture, Sigma) were added separately to resuspended cells. 5 ml of lysis buffer was used for grams of wet mass. Cells were sonicated (10 times, 1-s pulse) in ice, and then the cells suspension was clarified by centrifugation at 100,000 × *g* for 1 h, 4 °C. Supernatant was loaded on a pre-equilibrated (50 mM sodium phosphate, 500 mM NaCl, 10 mM imidazole, pH 8) His Trap FF, 1-ml prepacked column (GE Heathcare Inc.) with peristaltic pump at a flow rate of 0.5 ml/min, 4 °C. The column was washed with binding buffer and protein was eluted by stepwise imidazole gradient (10, 20, 50, 100, 250, and 500 mM imidazole in 50 mM sodium phosphate, 500 mM NaCl, pH 8). Fractions containing Syk were pooled and concentrated using Amicon® Ultra 15-ml Centrifugal Filter, 30-kDa cutoff (Merck KGa). Protein was then loaded on HiLoad 10/30 Superdex 200 column (GE Heathcare Inc.) equilibrated with 10 mM Hepes, 150 mM NaCl, 10% glycerol, 10 mM methionine, 1 mM DTT, pH 7.5. Protein was concentrated to final concentration of 2 mg/ml; aliquots were frozen in liquid nitrogen and stored in −80 °C.

Syk mutant (Y348F/Y352F) was obtained with a QuikChange Multi-Site-directed Mutagenesis Kit (Agilent Technologies Inc.) using wildtype Syk as template. Sf9 insect cells were harvested after 48 h infection (multiplicity of infection = 3) and lysed as described for Syk wildtype. Protein was purified as the wildtype except that the affinity purified protein was incubated with TEV overnight at 4 °C to remove the His_6 tag. Protein was then concentrated and loaded for gel filtration as described for Syk wildtype. The final protein preparation was concentrated to 1 mg/ml. The final protein sequence contains the Leu–Ser sequence remaining from the TEV recognition site.

Tandem SH2 domains expression and purification

Tandem SH2 (amino acids 9–264) was cloned in plasmid pGTvL1-SGC (Structural Genomics Consortium, University of Oxford) using a ligation-independent method (39). The final construct was sequence verified. The final protein sequence contains a Ser–Met sequence that remained from the vector. tSH2 were expressed using *Escherichia coli* BL21 gold cells (Agilent Technologies Inc.) at 30 °C for 4 h with 0.4 mM isopropyl β-D-1-thiogalactopyranoside. The bacterial pellet was lysed using a French press. The protein was captured with on a glutathione-agarose column (Protino glutathione-agarose 4B, Macherey-Nagel Gmbh) equilibrated with 20 mM Tris, 100 mM NaCl, 1 mM DTT, pH 8, and then eluted using same buffer containing 50 mM glutathione. Glutathione *S*-transferase–tSH2 complex was cut using TEV protease (Invitrogen), and further purified by size exclusion chromatography with a HiLoad 26/60 Superdex 75 column (GE Healthcare) in 20 mM Tris, 100 mM NaCl, 1 mM DTT, pH 8. The final protein preparation was concentrated using a Amicon® Ultra Centrifugal Filter, 30-kDa cut off to 30 mg/ml. Aliquots were frozen in liquid nitrogen and stored at −80 °C.

MS data acquisition and processing

Purified proteins were analyzed by mass spectrometry using a Q-TOF type instrument (Waters Synapt G1) connected to an

Acquity (Waters) UPLC chromatography system equipped with a C4 reversed phase column (Waters BEH300 C4 1.7 μm, 2.1 × 100 mm). Protein samples were diluted 10-fold with 0.1% trifluoroacetic acid (TFA) and 3-μl aliquots were injected. The eluents were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The gradient was from 3 to 60% B over 8 min. The mass spectrometer was tuned for detection of proteins to take 1-s lock mass (Leu encephalin) corrected scans in the mass range from 500 to 2000 m/z. Combined mass spectra for the chromatographic peaks were deconvoluted using the MaxEnt 1 algorithm as part of Masslynx (Waters). For tSH2 the measured molecular mass was 29,160 Da (expected 29,163 Da), for Syk WT it was 74,443 Da (expected: 74,525 Da), and for Syk FF it was 72,230 Da (expected 72,234 Da).

Surface plasmon resonance

The SPR experiment was done using a Biacore *X* instrument (GE Healthcare Inc.). Integrin β_3 cytoplasmic tail peptide (UniProt ID: P05106 CKFEEERARAKWDTANNPLYKEATSTFTNITYRGT, ProteoGenix, Schiltigheim, France) was coupled onto CM5 (GE Healthcare) chips using thiol coupling. The immobilization step was done at a 5 μl/min flow rate with a single flow cell. The carboxymethyl– dextran surface was activated using 1:1 ratio of 0.4 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and 0.1 M *N*-hydroxysuccinimide for 10 min. Then, 0.1 M ethylenediamine, 0.1 M sodium borate, pH 8.5, for 7 min, and 1 M ethanolamine, pH 7, for 10 min were injected. To introduce thiol-reacting groups the surface was coupled with 50 mM Sulfo-GMBS (*N*-[γ-maleimidobutyryloxy]succinimide) in 0.1 M sodium borate, pH 8.5, for 4 min. The peptide was diluted using 10 mM Hepes, pH 7, at 50 μg/ml and injected for 10 min to maximize the reactivity with sulfo-GMBS. Finally, the surface was blocked with 50 mM cysteine, 1 M NaCl, 0.1 M sodium acetate, pH 4, for 4 min. The experiment with tSH2 was carried out with 10 mM Hepes, 150 mM NaCl, pH 7.5, as running buffer and the flow rate of 10 μl/min using two flow cells. 2-Fold dilutions of tSH2 were prepared in the running buffer starting from 1.6 to 100 μM. Each sample was injected for 6 min with a delayed wash of 150 s. No surface regeneration was done between injections. Each measurement was done at least three times using different chips. The final resonance units (RU) were obtained by subtracting the binding response obtained with the nonfunctionalized sensor from that obtained from the integrin β_3 cytoplasmic tail peptide-coupled cell. The original response curves were fit using a Langmuir model with Biacore evaluation software 3.1 provided by the manufacturer. The data were then analyzed following the method previously described (29, 30). Briefly, to calculate the association rate k_{on} of complex integrin peptide–tSH2 the data from 10 to 30 s after injection were used. First a plot of dRU/dt (variation of RU in time) *versus* RU was done, and then the slopes obtained from linear regressions were replotted against the tSH2 concentrations used. The k_{on} is calculated based on equation,

$$\text{Slope}\left(\frac{d\text{RU}}{dt} vs \text{RU}\right) = k_{on} \times C + k_{off} \quad \text{(Eq. 1)}$$

where k_{on} is the association constant, C is the protein concentration injected, and k_{off} is an estimation of the dissociation rate of the complex. Because the dissociation rate is not dependent on protein concentration it can be obtained using a first-rate reaction equation,

$$\ln\left(\frac{R_1}{R_t}\right) = k_{off} \times t \quad \text{(Eq. 2)}$$

where R_1 is the RU at the end of sample injection, R_t is the RU at the time t, and k_{off} is the dissociation constant. The K_d is equal to k_{off}/k_{on}. Data analysis was performed using linear regression.

Fluorescence-based kinetic assay

Kinetic measurements of Syk were done using SOX fluorescent peptide (26, 27). The SOX peptide used was Ac-EEEEYIQ-[DPro-Sox-G]-NH_2 (Sigma) (28). The assay was performed in SOX assay buffer (20 mM Hepes, 0.1 mM EGTA, 1 mM $MgCl_2$, 10 μM sodium orthovanadate, 5 mg/ml of BSA, 1 mM DTT, pH 7.15) in a final volume of 50 μl. The fluorescence was detected with Victor™ X4 2030 Multilabel Reader (PerkinElmer Life Sciences) using 355 nm as excitation wavelength and 460 nm as emission wavelength in 96-well black plates at 30 °C. Data were collected every 30 s for at least 50 min. Each experiment was repeated six times and each measurement within an experiment was done in quadruplicate. Standard deviation was calculated using the data from six different experiments. Data were analyzed using repeated measurements one-way ANOVA (RM-ANOVA) followed by Tukey's multiple comparison test using GraphPad Prism 7. To conduct the ANOVA analysis data were first normalized to the highest measured product concentration in the presence of pITAM and integrin and then arcsine transformed.

Syk auto/transphosphorylation

Syk autophosphorylation was performed as previously described with some modifications (14). Syk wildtype, 2.7 μM, was incubated for 30 min at room temperature in the presence 100 μM ATP, in a buffer containing 15 mM Hepes, 20 mM NaCl, 10 mM $MgCl_2$, 1 mM EGTA, 10 μM sodium orthovanadate, 10% glycerol, pH 7.4, in a final volume of 2 ml. After incubation, excess ATP was washed away using a PD10 desalting column (GE Healthcare Inc.) using the same buffer and the protein was concentrated to 0.2 mg/ml with Amicon® Ultra Centrifugal Filter, 30 kDa cutoff.

Kinetic measurements of Syk activity and two-substrate analysis

Syk kinetic measurements were performed following the increase of the peptide fluorescence in the time as above described in the presence of 10 μM SOX peptide and 25 μM ATP. Basal Syk and active Syk were used at 17 nM, except for two substrate analysis where active kinase was used at 4 nM; the reaction was started by adding ATP as the last ingredient. Each measurement was done at least in triplicate; a blank without Syk was recorded also and it was subtracted from the fluorescence measured in the presence of the kinase. The two-substrate analysis was used to determine the K_m for ATP and the SOX peptide. Hence, in the case of K_m for ATP, the ATP concentration

was varied from 5 to 75 μM in the presence of a constant concentration of SOX peptide (from 1.5 to 30 μM). Likewise, K_m for SOX was determined changing the SOX peptide concentration from 1.5 to 10 μM in the presence of a constant concentration of ATP (from 5 to 75 μM). The data were analyzed assuming that the ternary complex model describes the Syk kinetic mechanism (28),

$$v = \frac{v_{max}[ATP][SOX]}{[ATP][SOX] + [ATP]K_m^{SOX} + [SOX]K_m^{ATP}K_m^{ATP}K_a^{SOX}} \quad \text{(Eq. 3)}$$

where K_m^{SOX} and $K_{m\ m}^{ATP}$ are Michaelis-Menten constants of SOX and ATP, respectively, and K_a^{SOX} is the association constant of SOX for Syk.

K_m for ATP and SOX peptide were calculated using the following equations derived from Equation 3 assuming that one of the two substrates is kept in a constant concentration,

$$\frac{1}{v_{max(app)}} = \frac{K_{mSOX}}{V_{max}}\frac{1}{[SOX]} + \frac{1}{V_{max}} \quad \text{(Eq. 4)}$$

$$\frac{1}{v_{max(app)}} = \frac{K_{mATP}}{V_{max}}\frac{1}{[ATP]} + \frac{1}{V_{max}} \quad \text{(Eq. 5)}$$

where $V_{max(app)}$ corresponds to the apparent rate of the reaction in presence of a constant concentration of the other substrate.

Effect of pITAM and integrin β_3 cytoplasmic tail on kinase activation and on Syk activity

Basal and active Syk were tested in the presence of the pITAM peptide from the CD3ϵ chain (UniProt ID: P07766, NPDpYEPIRKGQRDLpYSGLNQR, ProteoGenix) and in the presence of the integrin β_3 cytoplasmic tail peptide. Both peptides were dissolved in water and added to the reaction mixture. The assay was performed as described above. pITAM peptide and integrin β_3 cytoplasmic tail peptide were used at concentrations of 10 and 30 μM, respectively; both basal and active Syk were 17 nM. Differently, the inhibition analysis was done using active Syk at 4 nM. Hence, to test the effect of the integrin β_3 cytoplasmic tail peptide on Syk affinity for the SOX peptide, the ATP concentration was kept constant to 250 μM (saturating concentration), SOX was varied from 1 and 30 μM, and the integrin peptide from 0 to 100 μM. Similarly, to assess the integrin peptide effect on Syk affinity for ATP, the SOX peptide concentration was kept constant equal to 30 μM (saturating concentration) and ATP concentration ranged from 10 and 100 μM.

Effect clustered integrin β_3 cytoplasmic tails on kinase activation

Integrin β_3 cytoplasmic tail peptide was coupled on the surface of the assay plate through thiol coupling. Pierce™ maleimide-activated plates (Thermo Scientific) were used for the coupling reaction following the methods suggested from the manufacturer with minor changes. The plate was first washed with washing buffer (0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2); peptide was diluted with coupling buffer (0.1 M sodium phosphate, 0.15 M NaCl, 10 mM EGTA, pH 7.2) at 5 μM and incubated for 2 h on a plate shaker. Then, the not-reacted groups were blocked with 10 μg/ml of cysteine·HCl in coupling buffer. Finally, the plate was washed and dried by centrifugation upside-down at 1000 × *g*. The SOX peptide-based assay was performed as described before except for the absence of DTT in the assay buffer.

Effect of pITAM and integrin β_3 cytoplasmic tail on Syk mutant (Y348F/Y352F)

To assess the effect of pITAM and integrin β_3 cytoplasmic tail peptide on Syk mutant activity, the same procedure described for Syk wildtype was used. Syk mutant was used at 17 nM and pITAM and integrin peptide at 10 and 30 μM, respectively. The same procedure was also used in the presence of the integrin β_3 cytoplasmic tail peptide coupled on the surface.

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References

1. Yang, C., Yanagi, S., Wang, X., Sakai, K., Taniguchi, T., and Yamamura, H. (1994) Purification and characterization of a protein-tyrosine kinase $p72^{syk}$ from porcine spleen. *Eur. J. Biochem.* **221,** 973–978 CrossRef Medline
2. Mócsai, A., Ruland, J., and Tybulewicz, V. L. (2010) The SYK tyrosine kinase: a crucial player in diverse biological functions. *Nat. Rev. Immunol.* **10,** 387–402 CrossRef Medline
3. Cheng, A. M., Rowley, B., Pao, W., Hayday, A., Bolen, J. B., and Pawson, T. (1995) Syk tyrosine kinase required for mouse viability and B-cell development. *Nature* **378,** 303–306 CrossRef Medline
4. Abtahian, F., Guerriero, A., Sebzda, E., Lu, M.-M., Zhou, R., Mocsai, A., Myers, E. E., Huang, B., Jackson, D. G., Ferrari, V. A., Tybulewicz, V., Lowell, C. A., Lepore, J. J., Koretzky, G. A., and Kahn, M. L. (2003) Regulation of blood and lymphatic vascular separation by signaling proteins SLP-76 and Syk. *Science* **299,** 247–251 CrossRef Medline
5. Krisenko, M. O., and Geahlen, R. L. (2015) Calling in SYK: SYK's dual role as a tumor promoter and tumor suppressor in cancer. *Biochim. Biophys. Acta* **1853,** 254–263 CrossRef
6. Streubel, B., Vinatzer, U., Willheim, M., Raderer, M., and Chott, A. (2006) Novel t(5;9)(q33;q22) fuses ITK to SYK in unspecified peripheral T-cell lymphoma. *Leukemia* **20,** 313–318 CrossRef Medline
7. Dierks, C., Adrian, F., Fisch, P., Ma, H., Maurer, H., Herchenbach, D., Forster, C. U., Sprissler, C., Liu, G., Rottmann, S., Guo, G. R., Katja, Z., Veelken, H., and Warmuth, M. (2010) The ITK-SYK fusion oncogene induces a T-cell lymphoproliferative disease in mice mimicking human disease. *Cancer Res.* **70,** 6193–6204 CrossRef Medline
8. Friedberg, J. W., Sharman, J., Sweetenham, J., Johnston, P. B., Vose, J. M., Lacasce, A., Schaefer-Cutillo, J., De Vos, S., Sinha, R., Leonard, J. P., Cripe, L. D., Gregory, S. A., Sterba, M. P., Lowe, A. M., Levy, R., and Shipp, M. A. (2010) Inhibition of Syk with fostamatinib disodium has significant clini-

cal activity in non-Hodgkin lymphoma and chronic lymphocytic leukemia. *Blood* **115,** 2578–2585 CrossRef Medline
9. Liu, D., and Mamorska-Dyga, A. (2017) Syk inhibitors in clinical development for hematological malignancies. *J. Hematol. Oncol.* **10,** 145 CrossRef Medline
10. Moroni, M., Soldatenkov, V., Zhang, L., Zhang, Y., Stoica, G., Gehan, E., Rashidi, B., Singh, B., Ozdeirli, M., and Mueller, S. C. (2004) Progressive loss of Syk and abnormal proliferation in breast cancer cells. *Cancer Res.* **64,** 7346–7354 CrossRef Medline
11. Yuan, Y., Mendez, R., Sahin, A., and Dai, J. L. (2001) Hypermethylation leads to silencing of the *SYK* gene in human breast cancer hypermethylation leads to silencing of the *SYK* gene in human breast cancer. *Cancer Res.* **61,** 5558–5561 Medline
12. Coopman, P. J. P., Do, M. T., Barth, M., Bowden, E. T., Hayes, A. J., Basyuk, E., Blancato, J. K., Vezza, P. R., McLeskey, S. W., Mangeat, P. H., and Mueller, S. C. (2000) The Syk tyrosine kinase suppresses malignant growth of human breast cancer cells. *Nature* **406,** 742–747 CrossRef Medline
13. Grädler, U., Schwarz, D., Dresing, V., Musil, D., Bomke, J., Frech, M., Greiner, H., Jäkel, S., Rysiok, T., Müller-Pompalla, D., and Wegener, A. (2013) Structural and biophysical characterization of the Syk activation switch. *J. Mol. Biol.* **425,** 309–333 CrossRef Medline
14. Tsang, E., Giannetti, A. M., Shaw, D., Dinh, M., Tse, J. K. Y., Gandhi, S., Ho, H., Wang, S., Papp, E., and Bradshaw, J. M. (2008) Molecular mechanism of the Syk activation switch. *J. Biol. Chem.* **283,** 32650–32659 CrossRef Medline
15. Bradshaw, J. M. (2010) The Src, Syk, and Tec family kinases: distinct types of molecular switches. *Cell Signal.* **22,** 1175–1184 CrossRef Medline
16. Monks, C. R. F., Freiberg, B. A., Kupfer, H., Sciaky, N., and Kupfer, A. (1998) Three-dimensional segregation of supramolecular activation clusters in T cells. *Nature* **395,** 82–86 CrossRef Medline
17. Bromley, S. K., Burack, W. R., Johnson, K. G., Somersalo, K., Sims, T. N., Sumen, C., Davis, M. M., Shaw, A. S., Allen, P. M., and Dustin, M. L. (2001) The immunological synapse. *Annu. Rev. Immunol.* **19,** 375–396 CrossRef Medline
18. Mócsai, A., Zhou, M., Meng, F., Tybulewicz, V. L., and Lowell, C. A. (2002) Syk is required for integrin signaling in neutrophils. *Immunity* **16,** 547–558 CrossRef Medline
19. Yan, S. R., Huang, M., and Berton, G. (1997) Signaling by adhesion in human neutrophils: activation of the p72^{syk} tyrosine kinase and formation of protein complexes containing p72^{syk} and Src family kinases in neutrophils spreading over fibrinogen. *J. Immunol.* **158,** 1902–1910 Medline
20. Auger, J. M., Kuijpers, M. J., Senis, Y. A., Watson, S. P., and Heemskerk, J. W. (2005) Adhesion of human and mouse platelets to collagen under shear: a unifying model. *FASEB J.* **19,** 825–827 CrossRef Medline
21. Woodside, D. G., Obergfell, A., Leng, L., Wilsbacher, J. L., Miranti, C. K., Brugge, J. S., Shattil, S. J., and Ginsberg, M. H. (2001) Activation of Syk protein tyrosine kinase through interaction with integrin β cytoplasmic domains. *Curr. Biol.* **11,** 1799–1804 CrossRef Medline
22. Woodside, D. G., Obergfell, A., Talapatra, A., Calderwood, D. A., Shattil, S. J., and Ginsberg, M. H. (2002) The N-terminal SH2 domains of Syk and ZAP-70 mediate phosphotyrosine-independent binding to integrin β cytoplasmic domains. *J. Biol. Chem.* **277,** 39401–39408 CrossRef Medline
23. Law, D. A., Nannizzi-Alaimo, L., Ministri, K., Hughes, P. E., Forsyth, J., Turner, M., Shattil, S. J., Ginsberg, M. H., Tybulewicz, V. L., and Phillips, D. R. (1999) Genetic and pharmacological analyses of Syk function in αIIbβ3 signaling in platelets. *Blood* **93,** 2645–2652 Medline
24. Hughes, C. E., Finney, B. A., Koentgen, F., Lowe, K. L., and Watson, S. P. (2015) The N-terminal SH2 domain of Syk is required for (hem) ITAM, but not integrin, signaling in mouse platelets. *Blood* **125,** 144–154 CrossRef Medline
25. Clark, E. A., Shattil, S. J., Ginsberg, M. H., Bolen, J., and Brugge, J. S. (1994) Regulation of the protein tyrosine kinase pp72^{syk} by platelet agonists and the integrin αIIbβ3. *J. Biol. Chem.* **269,** 28859–28864 Medline
26. Shults, M. D., Janes, K. A., Lauffenburger, D. A., and Imperiali, B. (2005) A multiplexed homogeneous fluorescence-based assay for protein kinase activity in cell lysates. *Nat. Methods* **2,** 277–283 CrossRef Medline
27. Shults, M. D., Carrico-Moniz, D., and Imperiali, B. (2006) Optimal Sox-based fluorescent chemosensor design for serine/threonine protein kinases. *Anal. Biochem.* **352,** 198–207 CrossRef Medline
28. Papp, E., Tse, J. K., Ho, H., Wang, S., Shaw, D., Lee, S., Barnett, J., Swinney, D. C., and Bradshaw, J. M. (2007) Steady state kinetics of spleen tyrosine kinase investigated by a real time fluorescence assay. *Biochemistry* **46,** 15103–15114 CrossRef Medline
29. Natsume, T., Koide, T., Yokota, S., Hirayoshi, K., and Nagata, K. (1994) Interactions between collagen-binding stress protein HSP47 and collagen: analysis of kinetic parameters by surface plasmon resonance biosensor. *J. Biol. Chem.* **269,** 31224–31228 Medline
30. Yan, B., Calderwood, D. A., Yaspan, B., and Ginsberg, M. H. (2001) Calpain cleavage promotes talin binding to the β3 integrin cytoplasmic domain. *J. Biol. Chem.* **276,** 28164–28170 CrossRef Medline
31. Schmitz, R., Baumann, G., and Gram, H. (1996) Catalytic specificity of phosphotyrosine kinases Blk, Lyn, c-Src and Syk as assessed by phage display. *J. Mol. Biol.* **260,** 664–677 CrossRef Medline
32. Fütterer, K., Wong, J., Grucza, R. A., Chan, A. C., and Waksman, G. (1998) Structural basis for Syk tyrosine kinase ubiquity in signal transduction pathways revealed by the crystal structure of its regulatory SH2 domains bound to a dually phosphorylated ITAM peptide. *J. Mol. Biol.* **281,** 523–537 CrossRef Medline
33. Ye, F., Petrich, B. G., Anekal, P., Lefort, C. T., Kasirer-Friede, A., Shattil, S. J., Ruppert, R., Moser, M., Fässler, R., and Ginsberg, M. H. (2013) The mechanism of kindlin-mediated activation of integrin αIIbβ3. *Curr. Biol.* **23,** 2288–2295 CrossRef Medline
34. Morse, E. M., Brahme, N. N., and Calderwood, D. A. (2014) Integrin cytoplasmic tail interactions. *Biochemistry* **53,** 810–820 CrossRef Medline
35. Maartens, A. P., and Brown, N. H. (2015) The many faces of cell adhesion during *Drosophila* muscle development. *Dev. Biol.* **401,** 62–74 CrossRef Medline
36. de Castro, R. O., Zhang, J., Jamur, M. C., Oliver, C., and Siraganian, R. P. (2010) Tyrosines in the carboxyl terminus regulate Syk kinase activity and function. *J. Biol. Chem.* **285,** 26674–26684 CrossRef Medline
37. Furlong, M. T., Mahrenholz, A. M., Kim, K.-H., Ashendel, C. L., Harrison, M. L., and Geahlen, R. L. (1997) Identification of the major sites of autophosphorylation of the murine protein-tyrosine kinase Syk. *Biochim. Biophys. Acta* **1355,** 177–190 CrossRef
38. Fitzgerald, D. J., Berger, P., Schaffitzel, C., Yamada, K., Richmond, T. J., and Berger, I. (2006) Protein complex expression by using multigene baculoviral vectors. *Nat. Methods* **3,** 1021–1032 CrossRef Medline
39. Gileadi, O., Burgess-Brown, N. A., Colebrook, S. M., Berridge, G., Savitsky, P., Smee, C. E., Loppnau, P., Johansson, C., Salah, E., and Pantic, N. H. (2008) High throughput production of recombinant human proteins for crystallography. *Methods Mol. Biol.* **426,** 221–246 CrossRef Medline

II

BINDING STUDIES OF N- AND C- TERMINAL SRC HOMOLOGY 2 (SH2) DOMAINS OF SPLEEN TYROSINE KINASE SYK WITH pITAM

by

Lina Antenucci, Helena Aitio, Maarit Hellman, Jari Ylänne and Perttu Permi, 2019

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III

INTEGRIN CYTOPLASMIC DOMAIN AND pITAM COMPETE FOR SPLEEN TYROSINE KINASE BINDING

by

Lina Antenucci, Maarit Hellman, Vesa P Hytönen, Perttu Permi, and Jari Ylänne, 2019

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Integrin cytoplasmic domain and pITAM compete for spleen tyrosine kinase binding

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Running title: Competition of integrin and pITAM for Syk binding

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Keywords: Syk, pITAM, SH2 domain, phosphotyrosine, NMR

ABSTRACT

In hematopoietic tissues cell-cell communication involves immunoreceptors and specialized cell adhesion receptors that both mediate intracellular signals. Spleen tyrosine kinase (Syk) is a non-receptor tyrosine kinase involved in the downstream signaling of both immunoreceptors tyrosine activation motif (ITAM) receptors and integrin family cell adhesion receptors. Both phosphorylated ITAM (pITAM) and integrins bind to the regulatory domain of Syk composed of two Src homology 2 (SH2) domains. The interaction with pITAM is mediated by binding of a specific phosphotyrosine to each of the SH2 domains, leading to conformational changes and Syk kinase activation. Integrins bind to the interdomain A segment between the two SH2 domains and to the N-terminal SH2 domain, but the detailed binding site is not known. In order to map the binding site, we performed NMR titration experiments. We found that integrin cytoplasmic domain peptide induced chemical shift changes near the IA segment and at the phosphotyrosine binding site of the N-terminal SH2 domain of Syk. These changes were distinct, but partially overlapping with those induced by pITAM peptide. We were also able to show that pITAM peptide inhibited integrin binding to Syk regulatory domain. These results suggest that ITAM receptors and integrins cannot bind simultaneously to Syk, but provide two distinct routes for Syk activation.

Tyrosine kinases are an important class of cellular signaling enzymes and main targets of several cancer drugs (1). Understanding the regulation of tyrosine kinases is a key for understanding the function of cellular signaling networks. All non-receptor tyrosine kinases share a similar enzymatically active kinase domain but their regulatory domains allow their classification to 10 functional groups (2). Spleen tyrosine kinase (Syk) is a non-receptor tyrosine kinase that belongs to a distinct kinase subfamily whose regulatory domain is composed of two phosphotyrosine-binding Src homology 2 (SH2) domains (3).The other member of this subfamily in mammals is the zeta-chain associated kinase of 70 kD (ZAP70) (3). Gene depletion studies in mouse have shown that Syk is specifically required for the development of B-cells (4, 5) and ZAP70 for T-cell maturation (6, 7). Syk-deficient mice die newborn because of failure to separate blood vessels and lymphatic vessels (4, 5), a defect that may reflect the role of platelet adhesion in

this process (3). In addition to platelets and lymphocytes, Syk has essential function in many other cells of hematopoietic origin as well as in many non-hematopoietic tissues, for instance in breast cancers (8). This reflects its function in mediating signal transduction from adaptive immune receptors, innate immune receptors and certain cell adhesion receptors.

The mechanism of Syk activation downstream of most of the above listed receptors is rather well understood: Src family kinases (Fyn and Lck) are activated upon immune receptor clustering and phosphorylate two Tyr residues in the cytoplasmic domains of the immune receptor complex (reviewed in (3)). These residues are found in a conserved sequence motif called the immunoreceptor tyrosine-containing activation motif (ITAM) that has a consensus sequence: YxxL/Ix_{6-12}YxxL/I. ITAM is found either in the main transmembrane spanning polypeptide of the immune receptor (as in FcγRIIA) or in a separate transmembrane adaptor in immune receptors (as in TCR, BCR and FcεRI), innate immune pattern-recognizing receptors (in certain C-type lectin receptors as CLEC4E, CLEC6A, CLEC5A), or cell adhesion receptors (in platelet glycoprotein IV, GPIV, and osteoclasts-associated receptor, OSCAR). In addition, C-type lectin receptors exists where the full ITAM motif is formed by clustering of receptors containing single phosphorylated tyrosine motifs, so called hemITAM motifs (CLEC2, CLEC7A, CLEC9A) (reviewed in (3)). Phosphorylated ITAM (pITAM) sequences are able to interact with the two SH2 domains of Syk so that each of the pTyr residues binds to one SH2 domain (9). This interaction changes the relative orientation of these two domains resulting in the detachment of interdomain A (IA) and interdomain B (IB) segment from the kinase domain leading to kinase domain activation (9, 10).

In contract to phosphorylation-dependent interaction with transmembrane receptors or adaptors, described above, Syk interacts with integrin family cell adhesion receptors independently on receptor phosphorylation (11, 12). Direct interaction has been shown at least with β_1, β_2 and β_3 integrin subunit cytoplasmic domains (12, 13). Integrin cytoplasmic domains contain Tyr residues, but they do not form ITAM motifs and Tyr phosphorylation of integrin tails inhibits Syk interaction (12, 13).

In many cell adhesion events ITAM signaling and integrin signaling happens simultaneously and sometimes even in the same adhesion complex. For instance, during platelet adhesion to vessel wall, ITAM-dependent signaling from the GPIV collagen receptor may happen simultaneously with the function of the integrin $\alpha_2\beta_1$ collagen receptor (14) and is immediately followed by the function of integrin $\alpha_{IIb}\beta_3$, which is the major fibrinogen receptor mediating platelet aggregation but also interact with several other extracellular matrix proteins (15). In immunological synapses TCR and $\alpha_L\beta_2$ integrins function simultaneously in specific adhesion zones and signaling from these zones or cluster is regulated by diverse kinases and phosphatases (16). During osteoclast adhesion to bones, ITAM-linked OSCAR and integrin $\alpha_V\beta_3$ signal parallelly (17).

While the pITAM-Syk interaction mechanism is well known, molecular details of integrin-Syk interaction are still unclear. Even though there are variations of relative affinities of various pITAM sequences binding to individual SH2 domains (18), present evidence shows that pITAM interaction with SH2 domains mostly follow the general mode of pTyr/SH2 interaction (9, 19). The main interaction is mediated by the phosphate group of the pTyr residue and in addition hydrophobic interaction with the +3 position Ile or Leu residue determines the specificity of the interaction (19). The exact residues of integrin tail mediating the contact with Syk regulatory domain are not known, but truncation studies have shown that the deletion of four C-terminal residues in β_3 abolish the interaction (12) and that the minimum β_3 cytoplasmic tail peptide is Arg734-Thr762 (29 residues) (12). On the Syk side, the main interaction sites have been mapped to the interdomain A (IA) segment and the N-terminal SH2 domain (13)

We have earlier shown that binding of soluble β_3 integrin cytoplasmic domain does not directly activate Syk as in the case of soluble pITAM, but clustered integrin peptides can activate purified Syk (20). This fits well with the idea that integrins do not bind to the two SH2 domains in a similar way as pITAM and thus they cannot induce similar reorientation of Syk SH2 domains as pITAM.

In this study we set to test the hypothesis that integrin and pITAM binding to Syk are independent on each other. We used surface plasmon resonance (SPR) and nuclear magnetic resonance (NMR) spectroscopy methods to compare the binding surfaces of pITAM and β_3 integrin cytoplasmic domain on Syk regulatory domains. We find that the Syk IA segment is the main binding site of integrin, but on the N-terminal SH2 domain, binding of integrin and pITAM peptides induce NMR chemical shift perturbations (CSPs) on overlapping areas. Interestingly, the integrin responsive surface on N-SH2 is close to the location of the IA in published structures. We also show that soluble pITAM inhibits Syk regulatory domain binding to integrin-coated surfaces. We believe these findings are important for understanding the cross-talk between integrins and ITAM receptors in Syk signaling.

Results

Mapping of pITAM and integrin binding surfaces on the N-terminal SH2 domain of Syk

To be able to study the possible cross-talk between integrin cytoplasmic domain and pITAM binding, we first set up to verify the binding site of integrin β_3 peptide on Syk. We purified four fragments of the regulatory domain of Syk. The tandem SH2 (tSH2) fragment contained both SH2 domains and the intervening interdomain A (IA) segment. We also used either N-terminal SH2 (N-SH2) or the C-terminal SH2 (C-SH2) domains with the IA segment or N-SH2 alone (Figure 1). A 32-residue peptide from the C-terminus of integrin β_3 subunit was coupled to the SPR sensor chip via an N-terminal Cys residue and the protein fragments were injected to the soluble phase. We found that the tSH2 and N-SH2+IA fragments bound integrin with similar dissociation constants (K_D:s) of 3 μM and 8 μM, respectively (Figure 2A,B). The N-SH2 domain bound remarkably weaker (K_D = 130 μM) and IA-C-SH2 fragment somewhat weaker (K_D=25 μM) to the integrin β_3 peptide (Figure 2C, D). The K_D, k_{on} and k_{off} values are shown in Table 1. We were not able to test the C-SH2 domain alone due to protein aggregation. The results are consistent with earlier observations (13) i.e. the IA segment of Syk is the major interaction site for β_3 integrin tail and the N-SH2 domain contributes to the interaction.

We further analyzed the binding site on pITAM and the β_3 integrin peptide using solution state NMR spectroscopy. We carried out the chemical shift assignment of N-SH2 fragment (Antenucci et al., manuscript in preparation) and transferred the assignments to the ^{15}N, ^{1}H correlation spectrum (^{15}N-HSQC) of N-SH2+IA. Hemi-phosphorylated pITAM peptide was titrated to the ^{15}N-labeled N-SH2 fragment and the binding induced CSPs were analyzed (Figure 3A, B). The major changes mapped to the crystallographically determined binding site of pITAM (Figure 3C). To map the integrin binding surface on Syk, the ^{15}N-labeled N-SH2+IA fragment was used instead, given that the integrin peptide was not well soluble in concentrations above 500 μM and the K_D for its binding to N-SH2 alone was estimated to be 130 μM. On the contrary, K_D with N-SH2+IA was 8 μM, as determined using SPR. NMR titration with the β_3 integrin peptide revealed distinct CSPs for residues 20-23 and 44-51 (Figure 3E,F). In addition to the observed CSPs for the assigned peaks in the N-SH2 domain, some unassigned peaks were also shifted upon interaction (Supporting information figure). These unassigned peaks were apparently derived from the IA segment indicating that they are involved in binding of the β_3 integrin peptide. Interestingly, some of the residues in the N-SH2 domain showed CSPs both in the presence of β_3 integrin and pITAM. Particularly only Arg22 main chain NH resonance was shifted by integrin, whereas

only its side chain N^{ε}-H^{ε} was shifted by pITAM. This is in good accordance with the crystal structure of the tSH2-pITAM complex, where N^{η} of Arg22 interacts with phosphate group of pITAM (9). Arg45 main chain NH and Asn46 side chain NH_2 were shifted by both integrin and pITAM. Visualization these shifts on the surface of the domain (Figure 3A,E) suggests that the interaction sites of pITAM and integrin overlap partially on the N-SH2 domain of Syk.

pITAM inhibits integrin binding to N-SH2 domain of Syk

To test if pITAM and integrin β_3 cytoplasmic peptide compete for binding to the regulatory domain of Syk, we performed SPR competition experiments. pITAM was able to inhibit tSH2 binding to integrin-coated SPR surface with IC50 value of 15 μM (Figure 4A). To make sure that pITAM concentrations used in this experiment were high enough for saturation binding, we measured the K_D of pITAM to the same tSH2 preparation using thermofluor methods. We found that pITAM stabilized the melting temperature of tSH2 and this stabilization could be fitted to a single site binding equation with K_D of 4.2 ± 0.68 μM (Figure 4B,C). These data suggest that, contrary to our initial hypothesis, pITAM and integrin cytoplasmic domain compete for binding to the regulatory domain of Syk.

Discussion

In this study we wanted to test the hypothesis that the integrin binding surface on the regulatory domain of Syk is different from the pITAM binding site. We were able to confirm that the main binding site on integrin β_3 subunit cytoplasmic domain was the IA segment of Syk, but the N-terminal SH2 domain was also found to contribute to the binding. This is clearly a different binding mode than for pITAM. However, contrary to our initial hypothesis, we found that the NMR chemical shift changes on Syk N-terminal SH2 domain induced by integrin partially overlapped with those of pITAM. Furthermore, pITAM competed for β_3 integrin binding to the regulatory domain of Syk. These three main findings are discussed below.

The role of the interdomain A (IA) segment of Syk and ZAP70 in integrin binding has been reported before (13). Our results are consistent with these results. However, the more detailed binding site of integrin on Syk has not been mapped before. We used NMR spectroscopy to map the chemical shift changes caused by soluble β_3 peptide on the N-SH2 domain. As the affinity of the β_3 integrin peptide to N-SH2 alone is rather low and because of limited solubility of the peptide, the experiment could not be done with the N-SH2 alone. Instead, we used the N-SH2+IA fragment that has higher affinity for integrin. Even though we were not able to assign the IA segment residues in the NMR spectra, we detected several chemical shift changes in the unassigned NMR signals, presumably arising from the IA segment. In addition to this, we saw clear chemical shift changes on the assigned part of the spectra and these changes partially overlapped with to the pITAM binding surface in the available crystal structure Syk N-SH2 (9). To verify this, we also performed NMR titrations with single-phosphorylated pITAM peptide on N-SH2 and verified the interaction site of pITAM. Particularly, it is interesting that integrin titration induced chemical shift changes at residues Ser44, Arg45, Asn46 and Tyr47 that are at edge of the pTyr binding pocket. The main positively charged side chains interacting with the phosphate group of the pITAM peptide are those of Arg22 and Arg42. Asn46 may also have a polar interaction with the phosphate and thus it is interesting to note that its side chain NH**δ** also shifted by integrin binding. Earlier it has been shown that mouse Syk Arg41Ala (corresponding to Arg42 in human) mutation disrupts pTyr binding, but does not affect integrin signaling in platelets (21), nor the direct interaction with β_3 (12). This is in line with our result that the environment of Arg42 does not change upon integrin titration. We observed a change in the main chain NH chemical shift of Arg22 associated with the integrin binding, but not in its side chain $N^{\varepsilon}H^{\varepsilon}$ resonance. This suggest that the positively

charged side chains of Arg22 and Arg42 do not contribute to integrin interaction. Similarly, β_3 peptide did not cause CSPs at the hydrophobic interaction site of pITAM (Gly98 and Val100), further demonstrating the specificity of our titration.

The observed integrin-responsive surface on Syk N-SH2 is located close to the IA segment in the available structure of the Syk regulatory domains (Figure 5A). This fits with IA and N-SH2 being both involved in the interaction with a linear integrin peptide. Interestingly this surface is largely masked by the C-SH2 domain in the pITAM Syk tandem SH2 complex crystal structure (Figure 5B). On the other hand, in the structure of full-length Syk (10) this surface is mostly available in a groove between the IA segment and the N-SH2 domain (Figure 5A). This suggests that when pITAM binds to the regulatory domain of Syk, either pTyr interaction on N-SH2 or the movement of C-SH2 domain and IA segment might hinder integrin binding on N-SH2.

In line with the NMR titration experiments, we found that soluble pITAM peptide inhibit the interaction of Syk regulatory domain with the surface coupled integrin in the SPR assay. The apparent IC50 value was 15 μM, which is much higher concentration than the measured K_D between pITAM and tSH2 (about 100 nM; (10, 18)). However, the measured IC50 value was close to the K_D value that we estimated using a thermal stabilization assay. K_D values of pITAM-Syk interaction vary considerably depending on the assay and proteins used, for instance Grädler et al. reported K_D of 100 nM by isothermal titration calorimetry and 50 μM by SPR (10). This may reflect the conformational flexibility of Syk.

In contrast to our current result, Woodside and collaborators observed no cross-inhibition between pITAM and β_3 integrin peptides in an enzyme-linked immunosorbent assay where His6-integrin peptide was allowed to bind surface immobilized GST-Syk(6-370), or in pull-down experiments between His6-integrin coupled to beads and binding of soluble GST-Syk(6-370) (12). Although we cannot explain the cause of the different results, it is possible that differences in pITAM peptides used in the assay explain the variation. Woodside et al., used FcεRIγ pITAM at 10 μM, we used CD3ε pITAM at 1-100 μM.

In conclusion, our results suggest that Syk cannot bind to integrins and pITAM at the same time. This fits with the finding that in platelets integrin-mediated Syk signaling has been found to be independent on pITAM signaling (21). On the other hand, in neutrophils fibrinogen and β_3 -induced oxidative burst is dependent on ITAM signaling (22). Similarly in neutrophils and macrophages, integrin-induced Syk phosphorylation also requires ITAM signaling (3). Our current results imply that in those cases where ITAM and integrin-induced activation of Syk are both required, they need to occur spatially or temporally separated.

Experimental procedure

Proteins expression and purification

Tandem SH2 (amino acids 9-264) and shorter constructs (Figure 1) were cloned in plasmid pGTvL1-SGC (Structural Genomincs Consortium, University of Oxford) using a ligation-independent method (23). The final construct was sequence verified. The final proteins contain N-terminal Ser-Met sequence derived from the vector. Proteins were expressed using *Escherichia coli* BL21 gold strain (Agilent Technology Inc.) cultured in Terrific Broth (TB) at 25 °C for 20 hours and induced at OD600=0.8 using 1 mM isopropyl-β-D-1-thiogalactopyranoside. Cells were lysed in cold using a French press. Proteins were captured using glutathione-agarose column (Protino glutathione-agarose 4B, Macherey-Nagel Gmbh) equilibrated with 20 mM Tris HCl, 100 mM NaCl, 1 mM DTT, pH ranging from 7 to 8 depending on the isoelectric point of the specific protein construct. Glutathione-*S*-Transferase-protein complex was cut using TEV protease (Invitrogen), and further purified with size-exclusion chromatography with HiLoad 26/60 Superdex 75 column (GE Healthcare). The final protein preparations were concentrated to 1 mM using Amicon® Ultra 10 or 3 kDa cut off centrifugal filters (Merck

KGaA). Aliquots were frozen in liquid nitrogen and stored in -80 °C.

Surface Plasmon Resonance (SPR)

The SPR experiment was done using Biacore X instrument (GE Healthcare Inc.). Integrin β_3 cytoplasmic tail peptide (Uni-Prot ID: P05106 CKFEEERARAKWDTANNOLYKEATSTFTN ITYRGT, ProteoGenix, Schiltgheim, France) was coupled onto CM5 chip (GE healthcare) using thiol coupling). The coupling procedure was done as previously described (20). The experiments were carried out using 10 mM HEPES, pH 7.5 as running buffer, with a flow rate of 10 µl/min using two flow cells. 60 µl of proteins dilutions were injected followed by a 150 s delayed wash. 2-fold protein dilutions were prepared from 100 to 1.6 µM, except for N-SH2 where 300, 200 and 150 µM were added due to the low affinity binding. No surface regeneration was done between the measurements. The plotted response units (RU) were obtained by subtraction of the response obtained from the non-coupled reference sensor from the signal obtained from integrin-functionalized sensor. The curves were fitted using Langmuir binding model with Biacore Evaluation software 3.1 provided by the manufacturer.

pITAM-Integrin competition assay with SH2 constructs

pITAM competition assay was performed using SPR. Protein concentration was kept constant equal to 25 µM throughout the experiment and different concentrations of pITAM peptide from CD3ε chain (UniProt ID: P07766, NPDpYEPIRKGQRDLpYSGLNQR, ProteoGenix) ranging from 6.25 to 100 µM were added to the protein, incubated for 5 min at room temperature and, then, injected on the sensor chip. A sample without peptide was also run. The final response units (RU) were obtained by subtraction of the response obtained from the non-functionalized sensor from the response obtained from the integrin-functionalized sensor. The inhibition effect was calculated considering the reduction of the RU at the end of the injection compared to the RU obtained in the absence of pITAM. Each measurement was repeated three times, the average and the standard deviation were calculated. Data were plotted using the reduction of RU expressed as percentage of the response obtained without the pITAM *versus* the pITAM concentration used. Data were fit with GraphPad Prism 7 software using "[Inhibitor] *vs.* normalized response" model to obtain the IC50.

Nuclear Magnetic Resonance (NMR) spectroscopy

Expression of ^{15}N-labelled or ^{15}N,^{13}C-labelled N-SH2 or N-SH2+IA was done using *Escherichia coli* BL21 gold strain cultured in M9 medium supplemented with ^{15}N-NH_4Cl, ^{13}C-Glucose (Cambridge Isotope Laboratories, Inc) and trace elements at 25 °C for 20 hours using 1 mM isopropyl-β-D-1-thiogalactopyranoside. The purification was done as described above. For the NMR experiments the sample was concentrated to 50 µM buffered with 50 mM Na-Phosphate pH 5 and supplemented with 4% v/v D_2O. The double resonance ^{15}N-HSQC was recorded at 25 °C using Bruker Avance III HD 800 MHz NMR spectrometer, equipped with cryogenically cooled TCI ^{1}H, ^{13}C, ^{15}N triple resonance probe head. The peptide titrations were performed by adding increasing amount of peptide to the protein preparation. Integrin β_3 peptide was titrated with N-SH2+IA protein construct using protein:peptide ratios 1:1, 1:2.5, 1:5 and 1:10. N-SH2 was used in the titration of CD3ε single pTyr ITAM peptide (NPDYEPIRKGQRDLpYSGLNQR, CASLO) with protein:peptide ratios 1:40 and 1:80.

Thermal shift assay

The thermal shift assay was performed using thermal cycler C1000 Touch (BioRad) in 96 well plate (Hard-Shell ® PCR plates, BioRad). 4 µM of all studied protein constructs were mixed with different concentrations of peptides ranging from 1 to 200 µM. Sypro Orange protein gel staining 5000x concentrated (Invitrogen) was used as fluorescent dye at the concentration suggested from the manufacturer; the buffer used in the assay mix was 10 mM Hepes, 20 mM NaCl, 10 mM $MgCl_2$, 1 mM EGTA, pH 7.4. The final volume of the mixture was 25 µl. A blank

containing the buffer was run as well as a sample containing the protein without the peptides and one with the peptides alone. The temperature was raised from 20 to 95 °C with steps of 0.5 °C and the temperature was kept constant for 30 seconds before recording the fluorescence signal. Each measurement was run in quadruplicate. The final fluorescence curve was obtained subtracting the signal obtained from the buffer in the case of the proteins without the peptide and subtracting the buffer and the peptides signal from the sample where the proteins were mixed with the peptides. The fluorescence curves were then fitted with Boltzmann isotherm (24) using GraphPad Prism 7 to calculate the melting temperature (T_m). T_m was plotted *versus* the pITAM concentrations used and the data were fitted using models "One-site-specific binding" and "Two site-specific binding" with GraphPad Prism 7 to calculate the dissociation constant K_D.

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Conflicts of interests
The authors declare that they have no conflicts of interest with the contents of this article.

References

1. Hunter, T. (2014) The Genesis of Tyrosine Phosphorylation. *Cold Spring Harb. Perspect. Biol.* **6**, a020644
2. Taylor, S. S., and Kornev, A. P. (2011) Protein kinases: evolution of dynamic regulatory proteins. *Trends Biochem. Sci.* **36**, 65–77
3. Mócsai, A., Ruland, J., and Tybulewicz, V. L. J. (2010) The SYK tyrosine kinase: a crucial player in diverse biological functions. *Nat. Rev. Immunol.* **10**, 387–402
4. Cheng, A. M., Rowley, B., Pao, W., Hayday, A., Bolen, J. B., and Pawson, T. (1995) Syk tyrosine kinase required for mouse viability and B-cell development. *Nature*. **378**, 303–306
5. Turner, M., Mee, P. J., Costello, P. S., Williams, O., Price, A. A., Duddy, L. P., Furlong, M. T., Geahlen, R. L., and Tybulewicz, V. L. (1995) Perinatal lethality and blocked B-cell development in mice lacking the tyrosine kinase Syk. *Nature*. **378**, 298–302
6. Chan, A. C., Kadlecek, T. A., Elder, M. E., Filipovich, A. H., Kuo, W. L., Iwashima, M., Parslow, T. G., and Weiss, A. (1994) ZAP-70 deficiency in an autosomal recessive form of severe combined immunodeficiency. *Science*. **264**, 1599–1601
7. Negishi, I., Motoyama, N., Nakayama, K., Nakayama, K., Senju, S., Hatakeyama, S., Zhang, Q., Chan, A. C., and Loh, D. Y. (1995) Essential role for ZAP-70 in both positive and negative selection of thymocytes. *Nature*. **376**, 435–438
8. Coopman, P. J. P., Do, M. T. H., Barth, M., Bowden, E. T., Hayes, A. J., Basyuk, E., Blancato, J. K., Vezza, P. R., McLeskey, S. W., Mangeat, P. H., and Mueller, S. C. (2000) The Syk tyrosine kinase suppresses malignant growth of human breast cancer cells. *Nature*. **406**, 742–747
9. Fütterer, K., Wong, J., Grucza, R. A., Chan, A. C., and Waksman, G. (1998) Structural basis for syk tyrosine kinase ubiquity in signal transduction pathways revealed by the crystal structure of its regulatory SH2 domains bound to a dually phosphorylated ITAM peptide1. *J. Mol. Biol.* **281**, 523–537
10. Grädler, U., Schwarz, D., Dresing, V., Musil, D., Bomke, J., Frech, M., Greiner, H., Jäkel, S., Rysiok, T., Müller-Pompalla, D., and Wegener, A. (2013) Structural and biophysical characterization of the Syk activation switch. *J. Mol. Biol.* **425**, 309–333
11. Gao, J., Zoller, K. E., Ginsberg, M. H., Brugge, J. S., and Shattil, S. J. (1997) Regulation of the pp72syk protein tyrosine kinase by platelet integrin alpha IIb beta 3. *EMBO J.* **16**, 6414–6425
12. Woodside, D. G., Obergfell, A., Leng, L., Wilsbacher, J. L., Miranti, C. K., Brugge, J. S., Shattil, S. J., and Ginsberg, M. H. (2001) Activation of Syk protein tyrosine kinase through interaction with integrin β cytoplasmic domains. *Curr. Biol.* **11**, 1799–1804
13. Woodside, D. G., Obergfell, A., Talapatra, A., Calderwood, D. A., Shattil, S. J., and Ginsberg, M. H. (2002) The N-terminal SH2 domains of Syk and ZAP-70 mediate phosphotyrosine-independent binding to

integrin beta cytoplasmic domains. *J. Biol. Chem.* **277**, 39401–39408
14. Poole, A., Gibbins, J. M., Turner, M., Vugt, M. J. van, Winkel, J. G. J. van de, Saito, T., Tybulewicz, V. L. J., and Watson, S. P. (1997) The Fc receptor γ-chain and the tyrosine kinase Syk are essential for activation of mouse platelets by collagen. *EMBO J.* **16**, 2333–2341
15. Cimmino, G., and Golino, P. (2013) Platelet biology and receptor pathways. *J Cardiovasc. Transl. Res.* **6**, 299–309
16. Dustin, M. L. (2014) The Immunological Synapse. *Cancer Immunol. Res.* **2**, 1023–1033
17. Zou, W., Kitaura, H., Reeve, J., Long, F., Tybulewicz, V. L. J., Shattil, S. J., Ginsberg, M. H., Ross, F. P., and Teitelbaum, S. L. (2007) Syk, c-Src, the avb3 integrin, and ITAM immunoreceptors, in concert, regulate osteoclastic bone resorption. *J. Cell Biol.* **176**, 877–888
18. Bu, J. Y., Shaw, A. S., and Chan, A. C. (1995) Analysis of the interaction of ZAP-70 and syk protein-tyrosine kinases with the T-cell antigen receptor by plasmon resonance. *Proc. Natl. Acad. Sci.* **92**, 5106–5110
19. Bradshaw, J. M., and Waksman, G. (1999) Calorimetric examination of high-affinity Src SH2 domain-tyrosyl phosphopeptide binding: fissection of the phosphopeptide dequence dpecificity and coupling energetics. *Biochemistry*. **38**, 5147–5154
20. Antenucci, L., Hytönen, V. P., and Ylänne, J. (2018) Phosphorylated immunoreceptor tyrosine-based activation motifs and integrin cytoplasmic domains activate spleen tyrosine kinase via distinct mechanisms. *J. Biol. Chem.* **293**, 4591–4602
21. Hughes, C. E., Finney, B. A., Koentgen, F., Lowe, K. L., and Watson, S. P. (2015) The N-terminal SH2 domain of Syk is required for (hem)ITAM, but not integrin, signaling in mouse platelets. *Blood*. **125**, 144–154
22. Abtahian, F., Bezman, N., Clemens, R., Sebzda, E., Cheng, L., Shattil, S. J., Kahn, M. L., and Koretzky, G. A. (2006) Evidence for the requirement of ITAM domains but Not SLP-76/Gads interaction for integrin signaling in hematopoietic cells. *Mol. Cell. Biol.* **26**, 6936–6949
23. Gileadi, O., Burgess-Brown, N. A., Colebrook, S. M., Berridge, G., Savitsky, P., Smee, C. E. A., Loppnau, P., Johansson, C., Salah, E., and Pantic, N. H. (2008) High throughput production of recombinant human proteins for crystallography. *Methods Mol. Biol. Clifton NJ*. **426**, 221–246
24. Soon, F.-F., Suino-Powell, K. M., Li, J., Yong, E.-L., Xu, H. E., and Melcher, K. (2012) Abscisic Acid Signaling: Thermal Stability Shift Assays as Tool to Analyze Hormone Perception and Signal Transduction. *PLOS ONE*. **7**, e47857

Tables and Figures

Table 1: Binding constants of tSH2 constructs calculated from the surface plasmon resonance experiments, where the biosensor surface was functionalized with integrin β_3 cytoplasmic tail peptide

	k_{on}(1/M*s)	k_{off}(1/s) *10^{-3}	K_D (M) *10^{-6}
tSH2 wild type	64.0±1.81	0.20±0.13	3.13±0.71
N-SH2+IA	60.5±1.76	0.50±0.11	8.33±0.83
N-SH2	123±18.5	16.1±1.28	131±3.17
IA+C-SH2	236±5.79	6.11±0.09	25.9±0.09

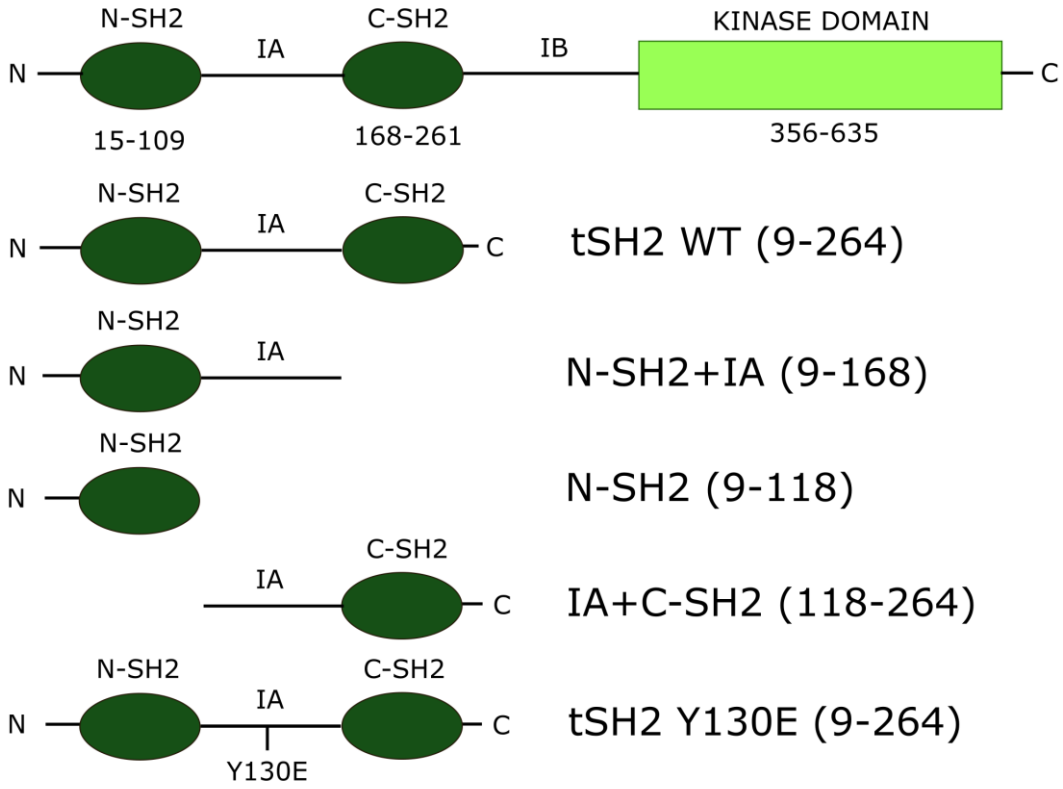


Figure 1: **Schematic representation of tSH2 constructs used in this study**. The SH2 domains are represented in dark green and the kinase domain in light green. The scheme of full length Syk is present as reference, but it was not used in the current study.

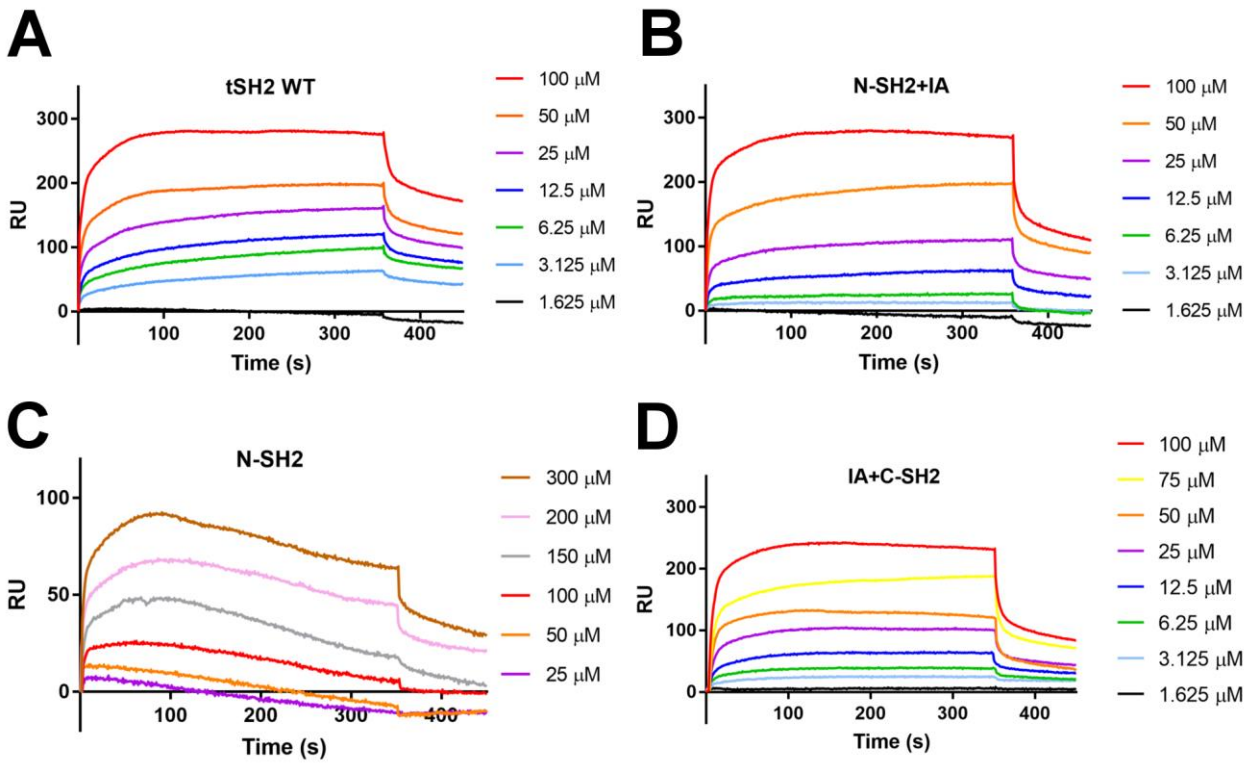


Figure 2: Interaction between integrin β_3 cytoplasmic domain with tSH2 and shorter tSH2 constructs. Surface plasmon resonance experiments where integrin β_3 cytoplasmic domain peptide was coupled on the surface (ligand) and different protein concentrations (from 1.625 to 300 μM) have been injected (analyte). In the graphs, for each construct, the variation of Response Units (RU) in the time (s) is plotted. The injection/association phase starts at time 0 seconds and ends in the time point marked with an *arrow;* the end of the injection coincides with the beginning of the dissociation phase. The graphs where fitted with Langmuir model using Bia Evaluation software to calculate the K_D and kinetic constants .

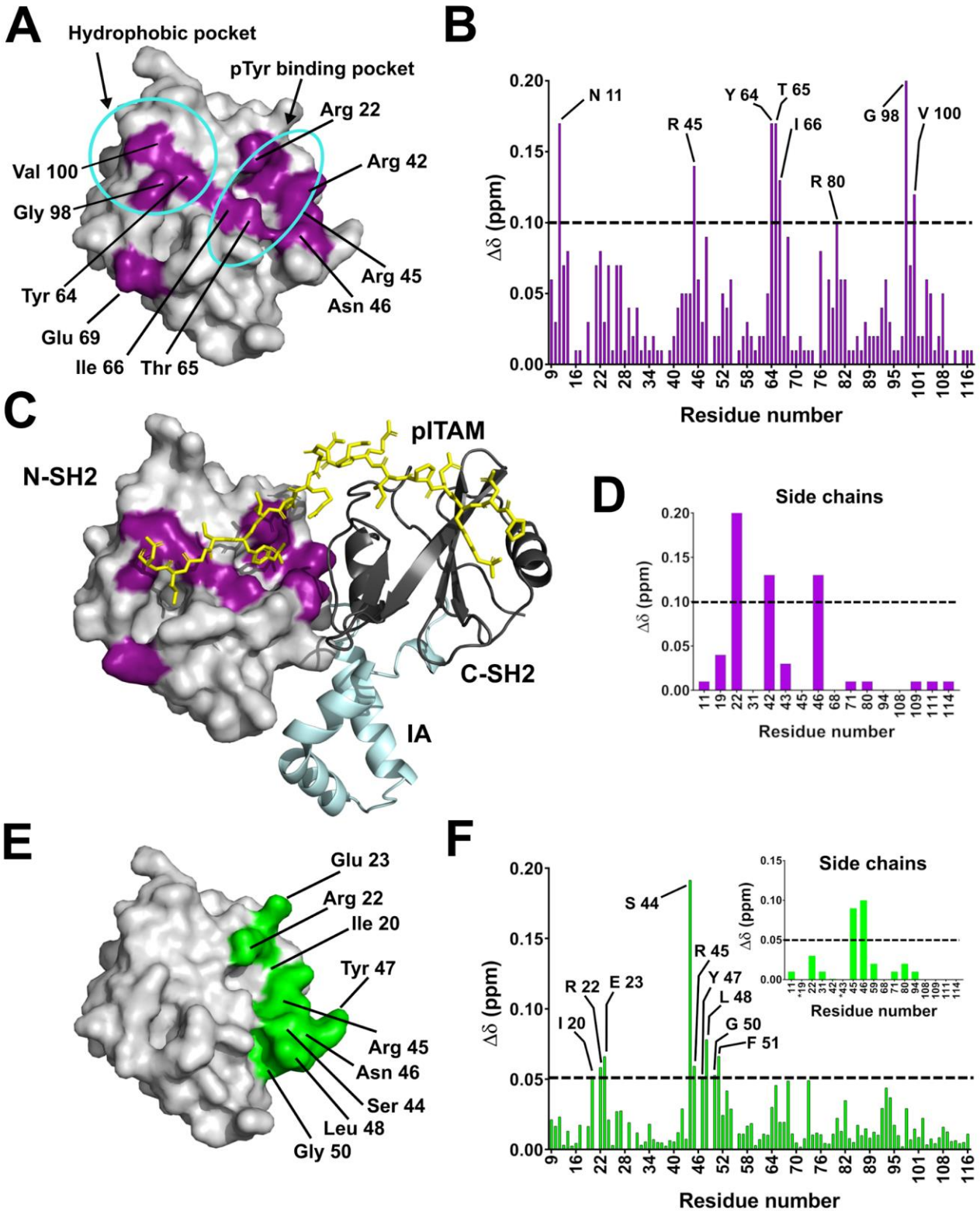


Figure 3: NMR chemical shift perturbations induced by ppITAM and integrin peptides

A. Surface representation of N-SH2 structure (PDB: 4FL2). The amino acids, whose chemical shifts changed upon addition of ppITAM peptide are highlighted in purple. **B.** Bar graph showing the chemical shift perturbations (Δδ, ppm) observed in ^{15}N-HSQC spectra of the N-SH2 in presence of ppITAM (1:80, protein:peptide ratio). The shifts higher than 0.1 were considered significant and the cut off line is indicated. **C**. Comparison of the crystal structure

of ppITAM-tSH2 complex (PDB: 1A81) with the chemical shift perturbations shown in A. **D.** Chemical shift perturbations in the ^{15}N-HSQC spectra of the N-SH2 side chains in from the same titration as A. **E.** Amino acids showing a shift higher than 0.05 with the integrin peptide mapped on the same structure as in A and shown in green. **F.** Bar graph showing the chemical shift change (Δδ, ppm) observed in ^{15}N-HSQC spectra of the N-SH2 backbone in presence of integrin peptide (protein:peptide ratio 1:20). The cut off line at 0.05 is indicated. The side chain shifts are shown in the insert.

B.

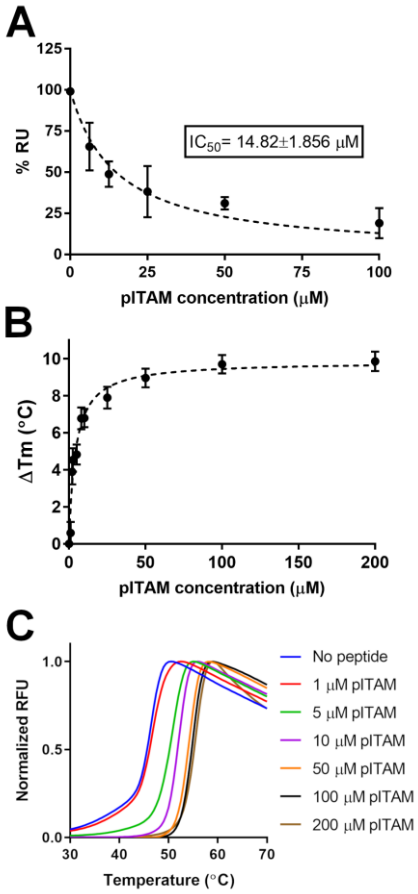


Figure 4: pITAM-Integrin competition experiment. A. Competition experiment was carried out using Surface Plasmon Resonance. The integrin β_3 cytoplasmic domain peptide was coupled on the surface and a fixed Syk tSH2 protein concentration (25 μM) mixed with different pITAM concentrations (from 6.25 to 100 μM) was injected. The competition was evaluated considering the reduction of the Response Units (RU) at the end of the injection. The results were plotted as percentage (%) of the RU obtained in absence of pITAM peptide *versus* the pITAM concentrations (μM) used. The error bars in the graphs represent differences between three separate experiments. **B and C**. An estimation of pITAM binding to Syk tSH2 based on thermal denaturation experiment. B shows a the change of Tm in the presence of different concentrations of pITAM. C shows the actual fluorescence thermal denaturation data.

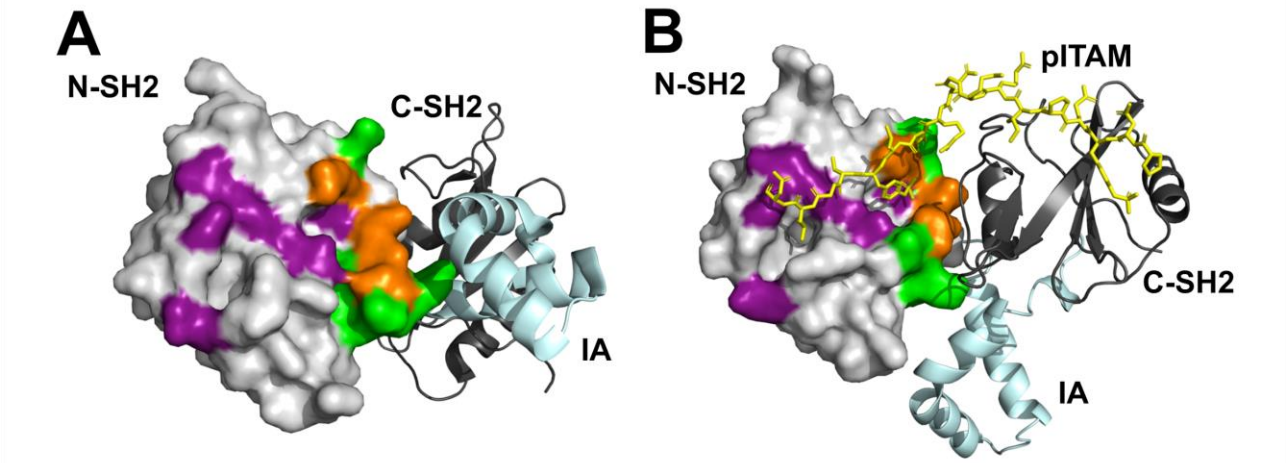
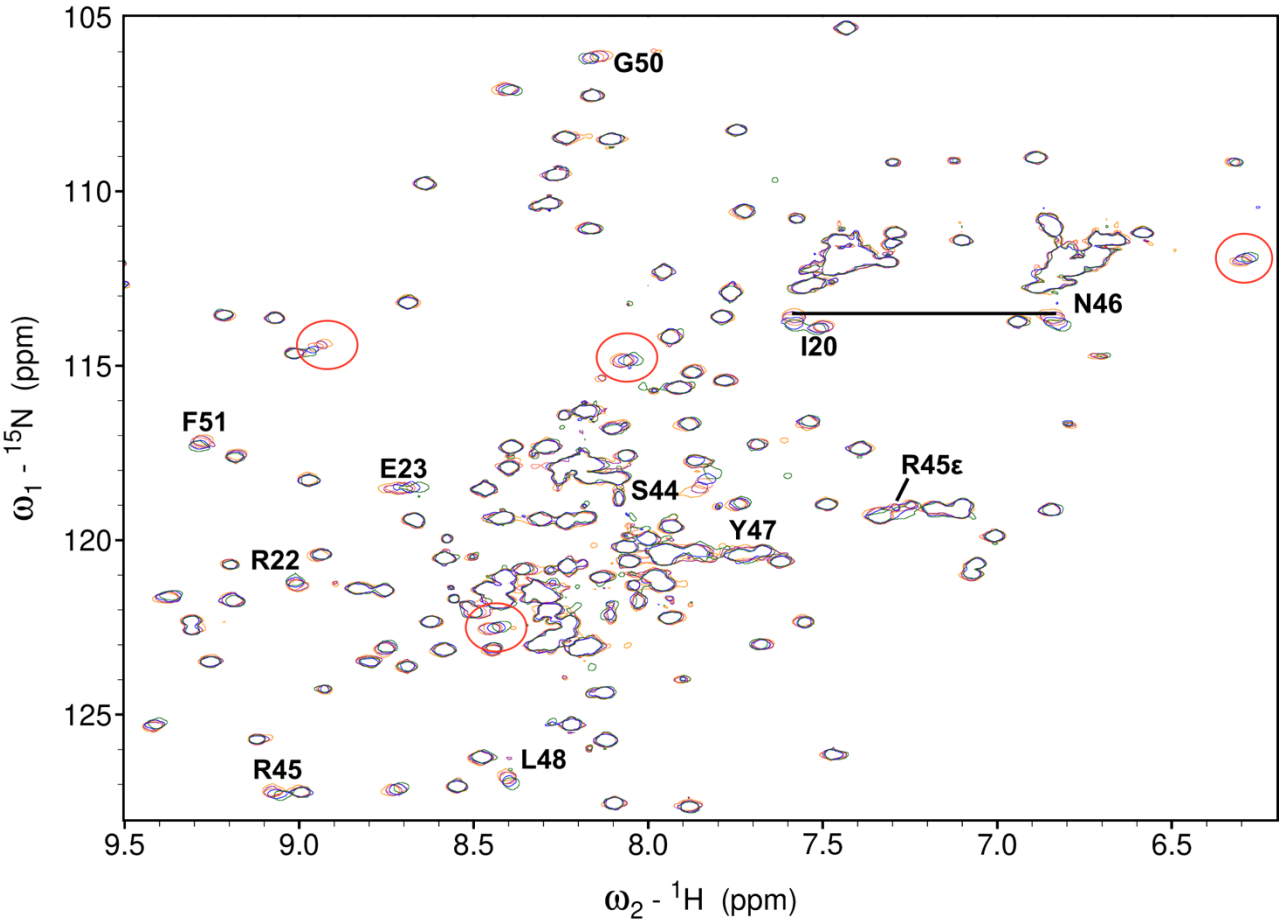


Figure 5: Location of the IA segment and C-SH2 in relation to the integrin-responsive surface of N-SH2. Comparison of the tSH2 structure from the full length Syk (PDB 4FL2) (**A**) and in the pITAM-tSH2 complex (PDB 1A81) (**B**). The residues whose chemical shifts changes upon both integrin on pITAM titration are coloured orange, those changed by only integrin green, and only pITAM purple. Note that in the conformation found in the full length Syk (A), the IA segment is located very close to the integrin-responsive surface, suggesting that the IA segment may be partly contributing to to the changes. On the other hand, in the presence of pITAM the surface is mostly covered by the C-SH2 and pITAM peptide (B).



Supporting Information figure. Integrin titration NMR spectra. A region of the ^{15}N-HSQC spectrum of Syk N-SH2+IA fragment without integrin peptide (red) and in the presence of 1x (orange), 2.5x (yellow) and 10x (blue) excess of the integrin peptide. The x-axis corresponds to proton chemical shift and and the y-axis to ^{15}N chemical shift. The peaks indicated with red circles are unassigned peaks that change upon titration and apparently derived from the IA segment. The assigned peaks from N-SH2 that show most prominent changes are labelled. The two peaks from the Asn46 side chain are connected with a horizontal line.