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Examining the Potential Modification of the Protein Tyrosine Kinase Pyk2 by SUMO-1

by

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ABSTRACT

Proline-rich tyrosine kinase 2 (Pyk2) is a non-receptor protein tyrosine kinase that is highly expressed in hematopoietic cells. Our lab generated two polyclonal antibodies to investigate the regulation of Pyk2 in macrophages and T cells. In macrophages the N-terminal antibody immunoprecipitated a higher molecular weight form of Pyk2. This shift was not due to differential phosphorylation or isoform expression. Since FAK, a close relative to Pyk2 undergoes a molecular weight shift due to SUMOylation, my thesis project was to investigate the potential SUMOylation of Pyk2. This study demonstrates that endogenous and exogenous Pyk2 associates with SUMO-1. The E3 ligase PIAS1 was shown to promote the association of Pyk2 with SUMO-1. Lysines 35, 145, and 646 were not the sites of Pyk2 SUMOylation, although SUMO-1 does associate with Pyk2 in the FERM domain. Direct Pyk2 SUMOylation was not confirmed, although SUMO-1 and PIAS1 overexpression increases Pyk2 protein levels.

ACKNOWLEDGEMENTS

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aa	amino acid
Ab	antibody
BMDM	bone marrow-derived macrophages
BSA	bovine serum albumin
CIAP	calf intestinal alkaline phosphatise
CT	C-terminal
DMEM	Dubelcco's modified Eagle's medium
DNA	Deoxyribonucleotide acid
ECL	enhanced chemiluminescence
E. coli	*Escherichia coli*
ECM	extracellular matrix
FAK	focal adhesion kinase
FRNK	FAK related non-kinase
FAT	focal adhesion targeting
FERM	Four point one, Ezrin, Radixin, Moesin
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FL	full length
GFP	Green fluorescent protein
HRP	horseradish peroxidase
IP	immunoprecipitation
ITAM	immune tyrosine activation motif
KO	knock-out
MDM2	mouse double minute 2 homolog
MTOC	microtubule organizing centre
MW	molecular weight
NEM	N-ethylmaleimide
NES	nuclear export signals
NLS	nuclear localization signal
NP-40	Nonidet P-40
NT	N-terminal
NTS	nuclear translocation signal

PBS	phosphate buffered saline
PCR	polymerase chain reaction
PIAS	protein inhibitor of activated STAT-1
PRNK	Pyk2 related nonkinase
PRR	proline rich region
PTK	protein tyrosine kinase
PTP	protein tyrosine phosphatase
Pyk2	proline-rich tyrosine kinase 2
RANGAP1	Ran GTPase-activating protein 1
RPMI	Roswell Park Memorial Institute
RSB	reducing sample buffer
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SFK	Src family kinase
SUMO	small ubiquitin-related modifier
TC	tissue culture

CHAPTER 1: INTRODUCTION

1.1 Macrophage migration and adhesion

1.11 Overview of macrophage function and motility

Macrophages are innate immune cells that play a central role in the immune response to pathogens. They reside in almost every tissue in the body and are extremely motile cells. Macrophages perform a number of roles in normal tissue development and immune surveillance, and also participate in development, inflammation, and activation of the adaptive immune system [2, 3]. They carry out these roles by secreting cytokines and growth factors and phagocytosing foreign materials and dead cells [3]. In order for macrophages to respond to pathogens they must migrate to the affected regions of the tissue.

Cell migration is a very complex process, which can be generally broken down into five steps: (i) extension of the leading edge toward stimulus; (ii) adhesion of the leading edge to the substrate; (iii) contraction of the cytoplasm toward the leading edge; (iv) release from contact sites at the lagging edge; and (v) recycling of membrane receptors from the lagging edge to the leading edge of the cell [4, 5]. This is a simplified cycle that relies heavily on the dynamic regulation of a variety of intracellular proteins involved in adhesion including: integrins, protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). Understanding and identifying the mechanisms that regulate macrophage migration and adhesion can lead to fully comprehending macrophage function and can potentially provide targets to modulate macrophage responses.

1.1.2 Integrins

Integrins are αβ heterodimers that bind to the extracellular matrix (ECM) through a large extracellular domain and links to the actin cytoskeleton through a short cytoplasmic tail [6]. Integrins relay information from the ECM to intracellular signalling pathways, informing the cell of its environment. This type of signalling is called “outside in signalling” [7]. In mammals there are eight β subunits that can combine with 18 α subunits to form 24 distinct integrins [8]. Integrins display no intrinsic enzymatic activity, so they require direct association of their tails with adaptor proteins, phosphatases, and kinases to signal [9].

The extracellular domain of integrins determines binding specificity to diverse matrix ligands including fibronectin, collagen, and laminin [6]. For an integrin to bind to its ligand it must first be activated through a conformational change that then allows for the integrin to bind with high affinity to its ligand [7]. The conformational change is triggered by intracellular activation pathways and is termed “inside out signalling” [7]. Binding of an integrin to its ligand induces integrin clustering leading to multiprotein complexes composed of adaptor molecules that connect to the actin skeleton [6, 10].

In order for a cell to migrate, integrin-mediated adhesions dynamically form and turn over, and the assembly and disassembly of adhesion is essential for direction and cell speed [6]. Integrin-mediated adhesion is not only important for cell migration but is also crucial for cell proliferation, differentiation, and environmental sensing [11].

1.1.3 Focal adhesions

Focal adhesions (FAs) are complex macromolecular assemblies that function to connect the actin cytoskeleton to integrins. FAs undergo different stages of maturation wherein they reorganize their protein composition to enable cells to respond to their various environments [12]. The stimuli that can trigger FA maturation can be supplied by biochemical or physical cues [12]. Focal complexes are considered the immature form of FAs, as they are smaller than FAs but contain some of the same cytoskeletal proteins found in FAs [3, 13]. Macrophages tend to form focal complexes instead of FAs since focal complexes support a stronger traction force than mature FAs [3].

FAs are highly dynamic and undergo constant remodeling and reorganization. Regulatory proteins are used to modulate FAs through their enzymatic activities including regulating protein phosphorylation states [12]. The activity of PTKs and PTPs trigger signalling cascades that control multiple FA dynamics [1, 3, 12, 14]. For instance Src family protein tyrosine kinases (SFKs) play key roles in regulating signal transduction that leads to cytoskeletal rearrangements, and focal adhesion kinase (FAK) can influence the disassembly of integrin-based adhesion sites [1, 14]. Another important adaptor protein that is prominent in phosphorylated adhesions is Paxillin [3]. Paxillin is a highly phosphorylated multidomain scaffold protein that functions in regulating adhesion signalling molecules, many of which control actin polymerization [3].

The complex association of PTPs and PTKs with other regulatory proteins produces the signalling that regulates the dynamics of FAs, and controls the

linkage between integrins and the actin cytoskeleton [12]. An understanding of how PTKs and PTPs can regulate FAs can lead to an understanding of cell migration in many biological processes and disease states.

1.2 The tyrosine kinases Pyk2 and FAK

Focal adhesion kinase (FAK; also known as $pp125^{FAK}$) is a 125 kDa non-receptor tyrosine kinase that is expressed in most mammalian tissues and cell types [15]. Proline rich tyrosine kinase 2 (Pyk2; also known as: CAKβ, RAFTK, or CADTK) is a 116kDa non-receptor tyrosine kinase and is primarily expressed in cells of the central nervous system and hematopoetic lineage [16-19]. Pyk2 is closely related to FAK [16-19]. Pyk2 and FAK share a very similar structural organization and share approximately 40% identity in both the N- and C- terminal domains and approximately 60% identity in the kinase domain (Figure 1.1) [16, 18, 20]. Due to the high sequence similarity between FAK and Pyk2, it is interesting to compare the regulation and function of Pyk2 with that of FAK.

Pyk2 and FAK are responsible for integrating signals from cell adhesion receptors, G-protein coupled receptors, and growth receptors leading to the activation of signaling pathways that can regulate cellular proliferation, survival, and migration from many different cell types [21-23] [reviewed in [24]]. Due to their roles in cellular proliferation and survival, FAK and Pyk2 have also been shown to be involved in a number of metastatic cancers [25-27]. For instance both FAK and Pyk2 have been found to be overexpressed in early-stage and invasive ErbB-2-Positive breast cancer [25]. The dual inhibition of both Pyk2 and FAK has

also been suggested to be a potential therapeutic target against certain metastatic cancers such as breast cancer [25], [reviewed in [28, 29]].

Although Pyk2 and FAK have similar structure and functions, they differ in many instances. For example, FAK is mainly localized to focal contacts whereas Pyk2 is mostly found in the cytoplasm and at the microtubule organizing center [1, 30]. Furthermore, FAK seems to be essential to cellular function and development, as a FAK knockout is embryonic lethal, whereas a Pyk2 knockout does not affect the viability or fertility of knockout mice [4, 31]. Furthermore, Pyk2 and FAK can interact with different proteins with their unique sequences, which gives them the ability to have a variety of distinct functions [21]. For instance FAK has been shown to uniquely bind to the protein talin, which has been suggested to mediate the activation and focal contact localization of FAK [32].

Pyk2 has been shown to be essential in macrophage function including: macrophage migration, adhesion, and polarization. Pyk2 is highly expressed in macrophages and the importance of Pyk2 was demonstrated by using Pyk2 KO mice. Macrophages from these mice demonstrated altered cell polarization and a diminished chemokine-induced migration [4]. F-actin localization was also impaired in these macrophages and these macrophages demonstrated morphological alterations [4]. Thus, these results suggest that Pyk2 is important in the cytoskeletal rearrangements and directional movement in macrophages.

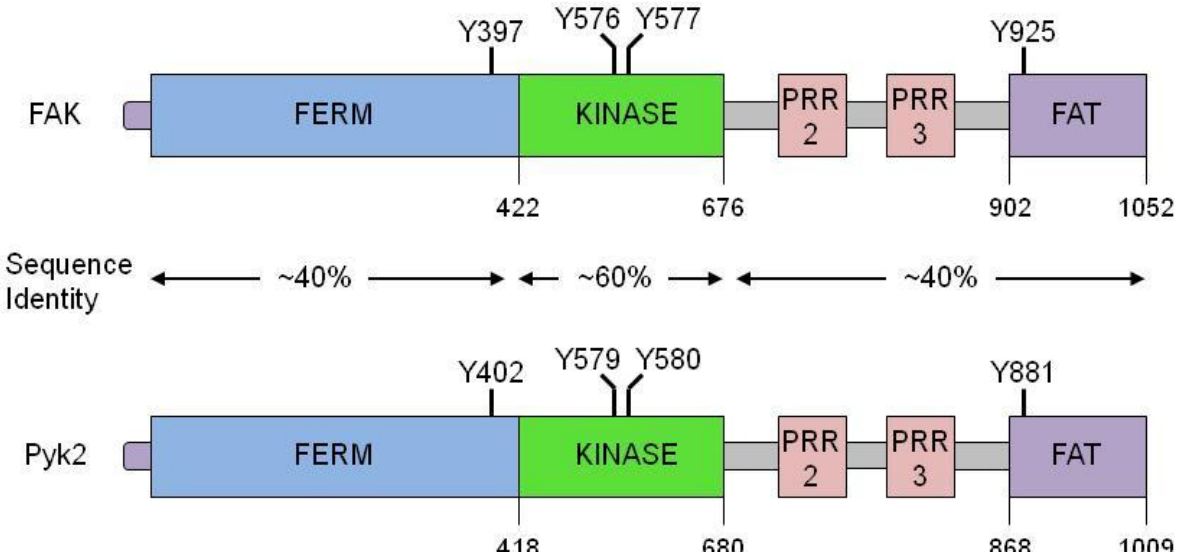


Figure 1.1: Pyk2 and FAK have a conserved domain structure. Pyk2 and FAK are composed of an N-terminal 4.1/ezrin/radixin/moesin (FERM) domain, a tyrosine kinase domain, proline rich regions (PRR), and a C-terminal focal adhesion targeting (FAT) domain. Pyk2 tyrosines 402, 579, 580 and 881 correspond to FAK tyrosines 397, 576, 577 and 925, respectively. Adapted from [1].

1.2.1 Structural and Functional domains of Pyk2 and FAK

1.2.1.1 The N-terminal domain

The Pyk2 and FAK N-terminal FERM (Four point one, Ezrin, Radixin, Moesin) domain consists of three globular subdomains or lobes (F1, F2 and F3) and appear as a compact cloverleaf structure [33]. The FERM domain of Pyk2 and FAK mediate protein-protein interactions in two different manners [28, 33]. First, FERM domains can mediate intermolecular interactions, primarily by providing docking sites for the cytoplasmic tails of transmembrane proteins [34]. Lastly, the FERM domain functions in intramolecular or homophilic intermolecular interactions [34]. The amino terminus of the FERM domain of Pyk2 is preceded by 38 amino acids that currently have no known function and have very little conservation with the corresponding residues of FAK (Figure 1.1) [28, 35].

Many classical FERM domain-containing proteins are regulated by FERM domain-mediated intramolecular associations [reviewed in [28, 36, 37]]. Structural studies found that the FERM domain of FAK binds directly to the kinase domain, which blocks access to the catalytic cleft and protects the activation loop of FAK from phosphorylation by Src [38]. Although the FERM domains of FAK and Pyk2 exhibit 40% sequence similarity, the FERM domain for Pyk2 has not been found to interact with the Pyk2 kinase domain and affect the autophosphorylation site Tyr 402 [1]. However, experimental results with chimeric proteins demonstrate that replacing the FERM domain of Pyk2 with the

FAK FERM domain increases Pyk2 catalytic activity and substrate phosphorylation [39]. These results support a regulatory role for the FERM domain of Pyk2 although, the molecular mechanism for this regulation remains uncertain.

One method for regulating Pyk2 has been proposed by Kohno *et al.* where Ca^{2+}/calmodulin binding to the Pyk2 FERM domain releases the Pyk2 kinase domain from autoinhibition through the formation of a homodimer [40]. Recently, a new model proposed by Daniel Riggsa *et. al.* may explain the regulation of Pyk2 through the FERM domain [24]. In this study they found that Pyk2 expressed in HEK 293 cells forms oligomeric complexes that is initially mediated through FERM domain interactions, and results in an increase in Pyk2 tyrosine phosphorylation [24]. They also found that the expression of the FERM domain as an autonomous fragment competes with Pyk2 preventing the formation of Pyk2 oligomeric complexes, which results in a reduction in the tyrosine phosphorylation of Pyk2 [24]. Further evidence supporting a regulatory role for the Pyk2 FERM domain also came from site-directed mutations in the FERM domain which inhibit Pyk2 phosphorylation [41, 42]. This effect could be due to changes in protein-protein interactions or changes in Pyk2 cellular location [28].

A number of receptor and protein interactions mediated by the FERM domain of FAK have been well characterized. For instance the FERM domain of FAK can bind to epidermal growth factor receptor, integrins, ezrin, and the Arp 2/3 complex [43-45]. Unlike FAK however, identifying proteins that interact specifically with the Pyk2 FERM domain has been limited [28].

Recently many FERM domain containing proteins, including FAK, and Pyk2 have been found in the nucleus and have nuclear functions [33]. Web-based software programs designed to locate nuclear export signals (NESs) and/or nuclear localization signals (NLSs) have been used to determine that many FERM domain containing proteins contain NESs and/or NLSs [33]. The F1 FERM subdomain of FAK contains one NES and one NLS, which are also conserved in Pyk2 [33, 46]. In the face of cellular stress signals or reduced integrin adhesion, FAK leaves focal contact sites and localizes to the nucleus through FAK FERM-mediated targeting [35]. Once inside the nucleus, FAK has been shown to act as a scaffold to stabilize a p53-Mdm2 complex, which results in the polyubiquitination of p53 and subsequent degradation of p53 by the proteosome [35]. It remains unknown how FAK manages to shuttle from focal adhesions to the nucleus and back. Although, it has been suggested that FAK uses its FERM interactions to mediate a "cortex to nucleus" communication shuttle that allows FAK to respond to events in the nucleus and be coordinated with events at the cell edges [33]. However, the NLS was found to be unnecessary for the regulated nuclear accumulation of Pyk2 and FAK [35, 47]. The FERM domain of Pyk2 has also been shown to have an important role in the nucleus. Upon FAK knockdown, the FERM domain of Pyk2 mediates nuclear translocation, p53 binding, and enhanced Mdm2-dependent p53 ubiquitination leading to p53 inhibition, which promotes cell proliferation and survival [47].

1.2.1.2 The Kinase domain

Pyk2 and FAK share 60% sequence identity in the kinase domain (Figure 1.1) [1]. Two tyrosine residues in Pyk2, Y579/Y580 and in FAK Y576/Y577 are located in the activation loop of the catalytic domain, and function to enhance catalytic activity upon phosphorylation [48-50].

Multiple studies have found ways to inhibit the catalytic activity of Pyk2 to investigate the function of Pyk2 within cells [16, 51, 52]. For instance it was found that an alanine substitution at lysine 457 in Pyk2 abolishes kinase activity [16]. Lysine 457 was later found to be the ATP binding site in Pyk2 [16]. Using the K457A mutant, it was discovered that Pyk2 is involved in interleukin-2 production in Jurkat T cells [51]. Furthermore, FIP200 (FAK family kinase-interacting protein of 200 kDa) has been found to bind to the kinase domain of Pyk2 and may function as an endogenous inhibitor of Pyk2 [28, 53].

1.2.1.3 The C-terminal domain

The C-terminal domains of FAK and Pyk2 contain proline rich regions (PRRs) (Figure 1.1). Neither Pyk2 nor FAK contain any SH2 or SH3 domains, but the PRRs function as binding sites for SH3-containing proteins [21]. For instance one protein that binds to these PRRs in FAK and Pyk2 is the adaptor protein $p130_{Cas}$, which is important in promoting cell migration [54].

Recently, the PRR of Pyk2 (aa 700-841) was shown to contain a nuclear translocation signal (NTS). An NTS is a different motif than a NLS and has been known to target non-nuclear proteins to the nucleus [20, 55]. The NTS of Pyk2 was found to play an accessory role in the nuclear import of full-length Pyk2 only

following the mutation of the NLS motif [55]. Furthermore, the PRR of FAK and Pyk2 contains an NES that is involved in nuclear export [55].

A focal adhesion targeting (FAT) domain is also contained in the C-terminal domain of Pyk2 (Figure 1.1). The resolution of the crystal structure of Pyk2 showed that the FAT domain of Pyk2 shares ~40% identity with the corresponding domain of FAK [28, 56]. However, only the FAT domain of FAK contains the sequence for focal adhesion localization [57]. The FAK FAT domain promotes the co-localization of FAK with integrins at focal adhesions, and allows for the direct interaction with the proteins talin and paxillin [32, 58]. Although the FAT domain of Pyk2 is similar to FAK and has also been shown to interact with paxillin, the Pyk2 FAT domain does not bind to talin [30, 32]. The FAT domain also contains tyrosine Y881 in Pyk2 (Y925 in FAK) which, when phosphorylated by Src, serves to recruit the adaptor protein Grb2, which couples Pyk2 to the MAP kinase signaling pathway [59].

1.2.2 Pyk2 Isoforms

There are three isoforms described for Pyk2 including full length Pyk2 (Figure 1.2). The first is the hematopoietic form of Pyk2 (Pyk2-H or Pyk2s), which is primarily expressed in hematopoetic cells, while the unspliced full length Pyk2 is predominantly expressed in brain [28, 60] [reviewed in [61]]. Pyk2-H arises from alternatively spliced RNA that is missing exon 23 that encodes for a 42 amino acid insert in the C-terminal domain found in full length Pyk2 (Figure 1.2) [28, 60]. This spliced region is enriched in serine, proline and threonine residues, which suggests that this region may mediate protein-protein interactions

or may lead to changes in the intrinsic tyrosine kinase of Pyk2 [61]. However, no changes in kinase activity, or structural changes of the C-terminal domain been observed [61]. Thus, it still remains unclear as to why certain cells express full length Pyk2 or Pyk2-H and other cells express both isoforms. However, a study by Kacena *et al.* found that megakaryocytes can mediate the change in the location, expression, and activity of both full length Pyk2 and Pyk2-H [62]. This change is thought to aid in regulating osteoblast function and megakaryocyte induced increase in osteoblast proliferation [62]. Furthermore, a recent study by Faure *et al.* discovered that GFP-Pyk2-H transfected into PC12 cells (model of sympathetic neurons) was found to have a predominately (80%) nuclear localization, whereas full length GFP-Pyk2 had a predominately cytoplamsic distribution [55]. GFP-Pyk2-H was also shown to be missing the NTS due to the alternative splicing in the PRR domain [55]. Additionally, full length GFP-Pyk2 was demonstrated to have a Ca^{2+}-dependent regulation of nuclear localization in neurons, but not Pyk2-H [55].

The second isoform of Pyk2 is referred to as PRNK (Pyk2 related nonkinase) (Figure 1.2). PRNK lacks both the N-terminal FERM domain and the kinase domain and encodes 228 residues of the C-terminal domain fused to nine unique N-terminal amino acids [63]. PRNK has been found to be present in the brain and spleen by alternative splicing [63]. The function of PRNK is still unclear, but it has been shown to interact with paxillin and not with p130Cas or Graf [28, 63]. Furthermore, PRNK is localized to focal adhesion whereas full length Pyk2 has a predominately cytoplasmic distribution [61].

The structural organization of PRNK is similar to that of FRNK (FAK related non-kinase), which shares the C-terminal region of FAK including the FAT domain [64]. FRNK is expressed in multiple tissues including embryonic tissue, and has been shown to play a role in FAK regulation [65]. For instance, FRNK can act as a dominant negative inhibitor to FAK [66]. Since FRNK does not contain an autophosphorylation site, it can disrupt FAK signalling by competitive binding to focal adhesions [65, 66]. FRNK has also been shown to co-immunoprecipitate with paxillin, and when FRNK was overexpressed there was a significant decrease in the amount of paxillin positive focal adhesion [66].

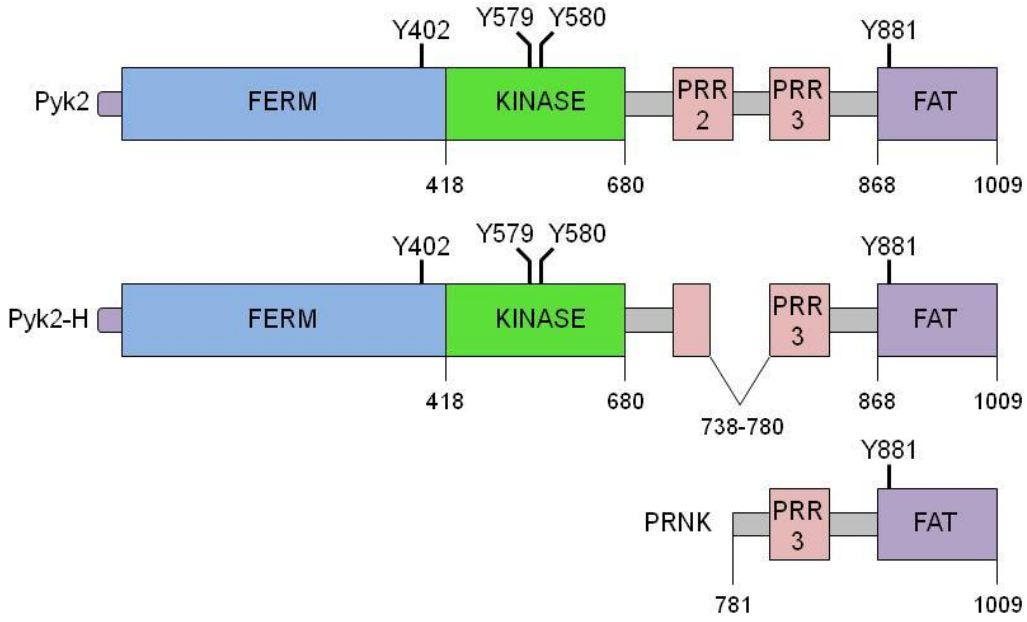


Figure 1.2: Isoforms of Pyk2. The Pyk2-H isoform contains a 42 amino acid deletion (amino acids 738-780) in the proline rich region that is normally seen in full length Pyk2. PRNK only contains a portion of the C-terminal end of full length Pyk2.

1.2.3 The regulation of Pyk2 and FAK by tyrosine phosphorylation

Pyk2 and FAK have four tyrosine residues that are conserved at analogous positions (Figure 1.1). The activity of Pyk2 begins with autophosphorylation at Y402 and once Y402 is phosphorylated, the SFK SH2 domain can bind to Pyk2, resulting in SFK recruitment and activation [21, 67]. The recruited SFK then phosphorylates Pyk2 at Y579 and Y580, which enhances the catalytic activity of Pyk2 and provides new docking sites for SH2 domain-containing proteins [21]. Even though the initial activation of Pyk2 still remains unclear, Pyk2 has been shown to be primarily activated in response to a variety of stimuli that increases intracellular calcium [16, 21, 68]. For instance, the loss of Calcium/calmodulin-dependent kinase kinase 2 (CaMKK2) greatly affects the activation of Pyk2 [69].

FAK undergoes similar steps in its activation by becoming autophosphorylated at Y397 and recruiting SFK that phosphorylates additional tyrosine residues that enhance the catalytic activity of FAK. Although the sequence of activation for FAK and Pyk2 are quite similar, the primary method of activation for FAK is different than Pyk2. FAK is primarily activated following integrin mediated adhesion to the ECM [1, 28].

1.2.4 Regulation of FAK by SUMOylation

A study by Kadaré *et al.* discovered a new mechanism for the regulation of FAK [70]. They found that SUMOylation could promote the catalytic activation of FAK [70]. A detailed explanation of the processes of SUMOylation will be provided detailed. GST-pulldown assays were used to show that FAK interacts

with the E3 SUMO ligase PIAS1 [70]. Furthermore, COS cells transfected with PIAS1, SUMO-1 and FAK were used to show that a higher molecular weight (MW) band of 125kDa corresponded to a FAK-SUMO conjugate, and that FAK was SUMOylated on Lys 152 in the FERM domain [70]. SUMOylation of FAK was also found to stimulate autophosphorylation *in vivo* and *in vitro*, and was suggested to promote the nuclear translocation of FAK [70]. Kadaré *et al.* found that Lys 152 did not exist in Pyk2 and thus, whether Pyk2 can also be regulated by SUMOylation remains to be investigated.

1.3 Small ubiquitin-like modifier (SUMO)

1.3.1 The SUMO conjugation cycle

SUMO is a reversible posttranslational protein modifier and SUMOylation is a multiple-step process similar but distinct to the ubiquitination process (Figure 1.3) [71]. SUMO proteins are approximately 10 kDa in size and resemble the 3-dimensional structure of ubiquitin, yet share less than 20% sequence identity with ubiquitin [71]. The SUMOylation pathway begins with the translation of SUMO as an immature precursor, similar to many other ubiquitin-like proteins (Ubls) [72]. The precursor is first processed by a protease to generate a mature form that contains a C-terminal diglycine motif [72]. The SUMO protein is then activated in an ATP-dependent fashion by the E1 activating enzyme SAE1/SAE2 and forms an E1-SUMO thioester [73, 74][reviewed in [75]]. The E1-SUMO is then transferred to a conserved cysteine on the E2 conjugating enzyme Ubc9 thereby creating a Ubc9-SUMO thioester [76-79]. Unlike ubiquitination, which has

multiple E2 enzymes, only one E2 enzyme exists for SUMOylation [80-82]. From there, the Ubc9 can directly catalyse the conjugation of SUMO to the binding partner through an isopeptide bond formed between the glycine residue of SUMO and an acceptor lysine residue of the binding partner [72]. However, in most cases, an E3 ligase is often needed to facilitate the process by catalyzing the transfer of SUMO from Ubc9 to a binding partner [72].There are many E3 ligases, but the largest group of E3 ligases are characterized by the presence of an SP-RING motif [71]. The Siz/PIAS family of E3 ligases contain the SP-RING and function in an analogous manner to the ubiquitin RING E3 enzymes by co-localizing and binding substrates and the Ubc9-SUMO thioester [72]. This brings them all into close proximity, which facilitates the SUMO transfer. Finally, SUMOylation is a highly dynamic and reversible process [72]. Once SUMO is conjugated to a target protein it can be deconjugated by SUMO-specific proteases (SENPs) (Figure 1.3) [72].

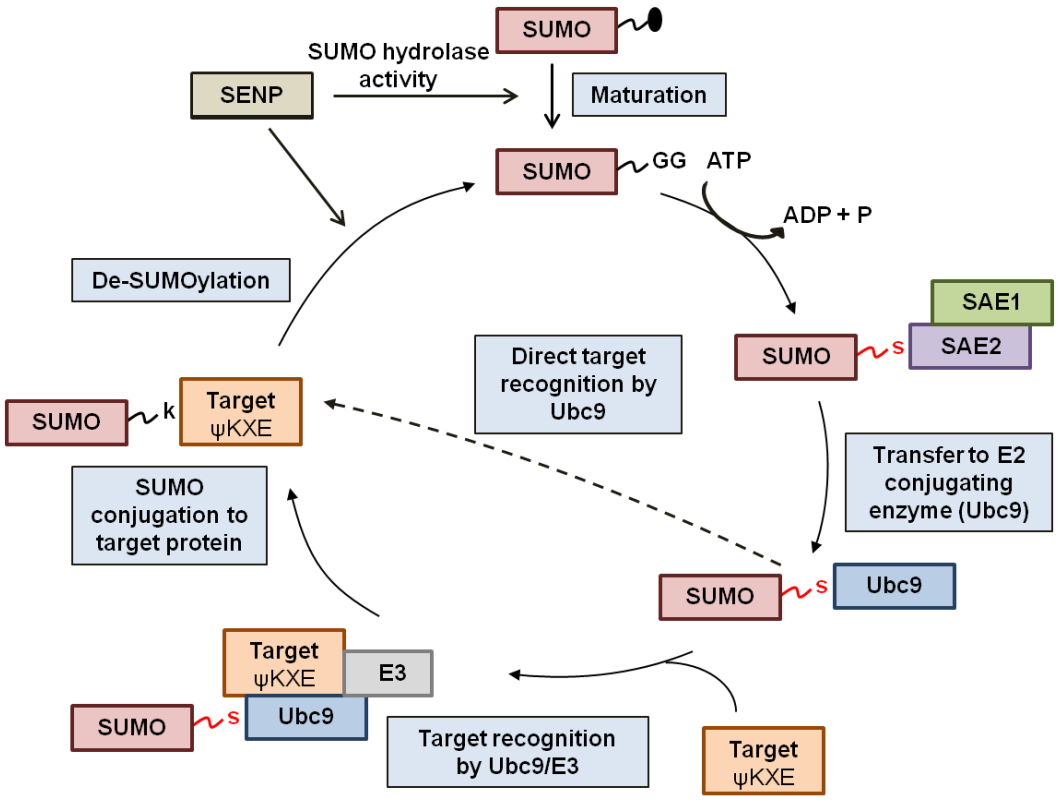


Figure 1.3 The SUMOylation pathway. SUMO is synthesized as a precursor and matured by hydrolase activity (cleaved propeptide sequence HSTVN as black dot) of SUMO proteases (SENP) that exposes a diglycine motif. SUMO is then activated by ATP which results in the formation of a thioester bond (red string) with SUMO activating enzyme (SAE1/SAE2). Ubc9 can directly recognize a target protein and catalyze the transfer of SUMO or conjugation can happen in conjunction with an E3 enzyme. An isopeptide bond is then formed between the target lysine residue (often in consensus motif ψKXE, where ψ is a large hydophobic residue, K is lysine, X is any amino acid, and E is glutamic acid) and SUMO. SUMOylation is reversible and SUMO can be removed by the isopeptidase activity of SENP. (Adapted from Stéphane Martin *et al.*[75])

1.3.2 SUMO isoforms

Vertebrates have four SUMO isoforms SUMO1-4, which are encoded by distinct genes, whereas yeast and invertebrates encode only one SUMO gene [72]. SUMO-2 and SUMO-3 share 97% identity and are often referred to as SUMO-2/3

since antibodies cannot distinguish between the two forms [72]. SUMO-1, on the other hand, only shares 50% identity with SUMO-2/3, and SUMO-1 and SUMO-2/3 serve distinct functions by conjugating to different proteins [72, 83]. For instance Vertegaal *et al.* found that SUMO-1 preferentially conjugated to RanGAP1, whereas the protein Sp100 preferentially conjugated to SUMO-2 [83]. Humans contain a fourth gene for SUMO-4 which has an 86% similarity to SUMO-2 [84]. SUMO-4 mRNA transcripts have a very limited expression compared to the other SUMO species and since no native SUMO-4 has been found expressed in tissues, SUMO-4 is thought to be expressed as a pseudogene [84]. Furthermore, SUMO-4 contains a proline instead of a glutamine at the C terminus which prevents the maturation of SUMO-4 by SENPs [84]. Another difference between SUMO-2/3 and SUMO-1 is that SUMO-2/3 can conjugate to target proteins in a chain-wise fashion *in vivo* due to internal SUMO conjugation motifs (SCMs), but SUMO-1 lacks this ability [85]. However, both SUMO-1 and SUMO-2/3 have been shown to form multiple SUMO-SUMO linkages *in vitro* [86].

1.3.3 Consensus motifs for SUMO conjugation

After comparing the sequences of multiple SUMO targets, a classical SUMOylation consensus motif ψKX(D/E) (where ψ is a large hydophobic residue, K is lysine, X is any amino acid, and D/E is aspartic and glutamic acid) was described [87]. These residues were found to play a critical role in regulating the stability and interaction of Ubc9 with the substrate protein [72]. Recently, four

different extensions of the classical consensus SUMO motif have been identified: the negatively charged amino-acid-dependent sumoylation motif (ψKXEX$_{(2-5)}$E/D)[88], the phosphorylation-dependent sumoylation motif (ψKXE/DXXpSP) [89], an inverted SUMO conjugation motif (E/DXKψ) [90] and a hydrophobic cluster SUMOylation motif [90]. It has also been discovered recently, using a proteomics approach, that SUMO targets can be SUMOylated at a non-consensus motif [91, 92]. Furthermore, a short motif has been indentified in proteins that interact non-covalently with SUMO called SUMO Interacting Motifs/ SUMO binding Motifs (SIM/SBM) [71, 93, 94]. Few proteins have been shown to possess this motif and the study of SIM/SBM-containing proteins is still early [71].

1.3.4 Functions of SUMOylation

It is difficult to predict the molecular consequences of SUMOylation for a target. It is generally thought that SUMOylation *in vivo* can affect a target protein inter and/ or intramolecular interactions and hence its localization, stability, or activity [85]. SUMOylation may cause changes to a substrate protein-protein interaction by simply revealing or destroying existing binding sites by causing conformational changes in the target [71].

There are many biological functions of SUMOylation, such as the maintenance of genome integrity, transcriptional regulation, promyelocytic leukemia protein-nuclear body (PML-NB) formation, subcellular localization, DNA repair, nuclear transport, ubiquitin-mediated proteolysis, signal

transduction, and tumorigenesis [71, 85, 95-98]. Interestingly, many proteins (over 165 identified) that undergo SUMO modification and/or contain a SIM can be dynamically targeted to PML-NBs [85]. Wimmer P *et al.* discusses how many intracellular pathogens use host cell SUMO machinery to either change essential components or use it to be targeted to PML-NBs themselves [85].

Although SUMOylation can have profound effects, only a small amount of a target protein is SUMO modified in relation to the total pool of the target protein [85, 99]. This discrepancy has been aptly named the SUMO enigma [85]. One possible explanation is that once a SUMOylated substrate's biological function has been activated, de-SUMOylation of the substrate does not affect the initiated biological activity [85].

1.3.5 SUMOylation and Phosphorylation

Since SUMOylation is important in multiple cellular processes it seems that protein phosphorylation could also be an important mechanism of SUMO regulation. Only just recently has it been found that phosphorylation networks can be influenced by posttranslational modification including SUMOylation [100], palmitoylation [101], and methylation [102] [reviewed in [103]]. In the study by Qi Yao *et al.* they demonstrated through immunoblotting techniques that protein tyrosine phosphorylation was positively correlated with SUMOylations [100]. Particularly, they found that SUMOylated proteins were specifically enriched in the ginkgolic acid-regulated phosphoprotein group, creating a direct link between SUMOylation and phosphorylation [100]. As previously stated, FAK is

SUMOylated and a decrease in FAK SUMOylation leads to a decrease in FAK phosphorylation at Y397 [70]. Qi Yao *et al.* found that HEK293T cells treated with ginkgolic acid (a compound recently shown to be a specific inhibitor of SUMOylation) demonstrated decreased FAK autophosphorylation compared with an untreated control group [100]. From this result they suggested that SUMOylation could ultimately affect the activity of tyrosine kinases like FAK, which in turn could cause changes in global protein tyrosine phosphorylation [100]. Since tyrosine phosphorylation plays a key role in cellular signalling in cancers, the cross talk between SUMOylation and phosphorylation may provide a new therapeutic target against cancer.

1.4 Pyk2-H exists as two distinct populations in macrophages

Our lab generated polyclonal antibodies to regions in the N-terminal (NT) and C-terminal (CT) domain of Pyk2 [104]. The NT F298 and the CT F245 antibodies were produced in rabbit by injection of a peptide corresponding to aa 2-12 and a GST fusion protein consisting of aa 720-826 of Pyk2, respectively (Figure 1.4) [104, 105]. In the study by J. St-Pierre *et al*., these antibodies were used to initially investigate the regulation of Pyk2 and paxillin in macrophages and T cells [30]. Using the F245 and F298 antibodies however, they found that there are distinct molecular species of Pyk2 in RAW 264.7 macrophages and T cells [30].

RAW 264.7 lysates immunobloted with F245 antiserum detected a doublet which contained a higher MW Pyk2 species and a lower MW Pyk2 species [30].

However RAW 264.7 lysates immunobloted with F298 only detected one higher MW Pyk2-H species. Interestingly when the RAW 264.7 lysates were immunoprecipitated with F245, the F298 antiserum was only able to detect the higher MW Pyk2 species. Furthermore the F245 antiserum was unable to recognize F298-recoved Pyk2 from RAW 264.7 lysates. Thus, the F245 antiserum does not recognize Pyk2 immunoprecipitated with F298 in RAW 264.7 cells, but F245 can immunoprecipitate a form of Pyk2 that is strongly recognized by F298 [30]. This led to the hypothesis that the F245 and F298 Pyk2 recognizes distinct populations.

To determine if the two Pyk2 populations from the F245 antiserum were distinct from the Pyk2 population from the F298 antiseum, J. St-Pierre *et al.* performed an immunodepletion experiment [30]. From this experiment they found that the F245 antiserum recognized two different MW species in the Pyk2 immunoprecipitates while the F298 antiserum preferentially recognized the higher MW species. It was also noted that the F245 antiserum was insufficient to deplete all of the F245-immunoreactive Pyk2. Furthermore, after immunodepleting F298 in T cells, the F245-reactive Pyk2-H corresponded to a slightly more rapidly migrating species, which may have represented a hyperphosphorylated Pyk2. This implied that there may be two distinct Pyk2-H species found within the higher MW band detected by F245. Thus in conclusion the F298 and F245 antiserum appear to bind overlapping but distinct populations of Pyk2-H [30].

J. St-Pierre *et al.* then went on to determine if the differences in the MWs of the Pyk2 populations were due to isoform expression [30]. Using RT-PCR and

mass spectrometry, J. St-Pierre *et al.* confirmed that the F245 Pyk2 populations and the F298 Pyk2 population were Pyk2-H and therefore the MW differences were not caused by alternative splicing. Furthermore, J. St-Pierre *et al.* used calf intestinal alkaline phosphatase (CIAP) to demonstrate that the difference in migration on an SDS-PAGE between the two F245 populations was not due to a difference in phosphorylation. However, F298-captured Pyk2-H did display higher overall serine/threonine phosphorylation levels in comparison to the F245-captured Pyk2.

The one striking difference that J. St-Pierre *et al.* determined about the two different antisera was that only the F245 antiserum was able to co-immunoprecipitate paxillin with Pyk2 [30]. Using NIH 3T3 cells, which only express the lower MW Pyk2-H population that is recognized by the F245 antiserum, J. St-Pierre *et al.* also found that only the lower MW Pyk2 species recognized by the F245 antiserum associates with paxillin. It still remains unclear as to why the F298-reactive Pyk2 is unable to bind paxillin as strongly as the F245-reactive Pyk2.

J. St-Pierre *et al.* further demonstrated that the F245 and F298 Pyk2 populations differed in cellular localization [30]. The F245 population was enriched with paxillin at the microtubule organizing centre (MTOC) whereas the F298 population enriched at the plasma membrane. These results together suggest that Pyk2 may be found in multiple conformational states and that macrophages may contain a reservoir of inactive Pyk2 that associates with paxillin and localizes to the MTOC. Furthermore, J St-Pierre *et al.* suggested that Pyk2 may be in an

autoinhibited conformation where the F298 epitope is not accessible to the F298 antiserum but is still bound by F245 and paxillin. Together these distinct populations may reflect different functions of Pyk2 in macrophages. A summary of the difference between the two F245 and F298 Pyk2-H populations can be seen in Figure 1.5.

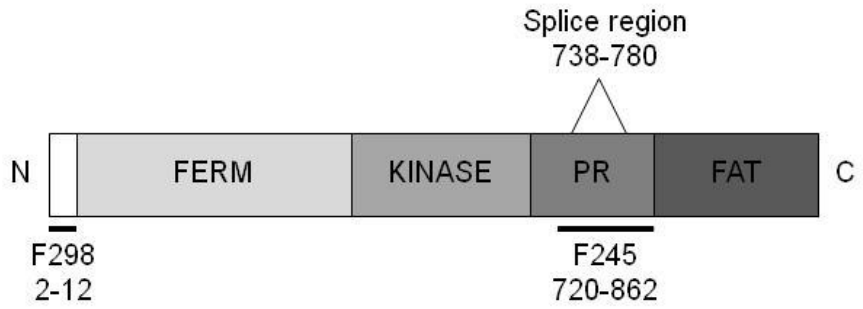


Figure 1.4 F245 and F298 Ab epitopes. Diagram of the domain structure of Pyk2 indicating the F245 and F298 Ab epitopes.

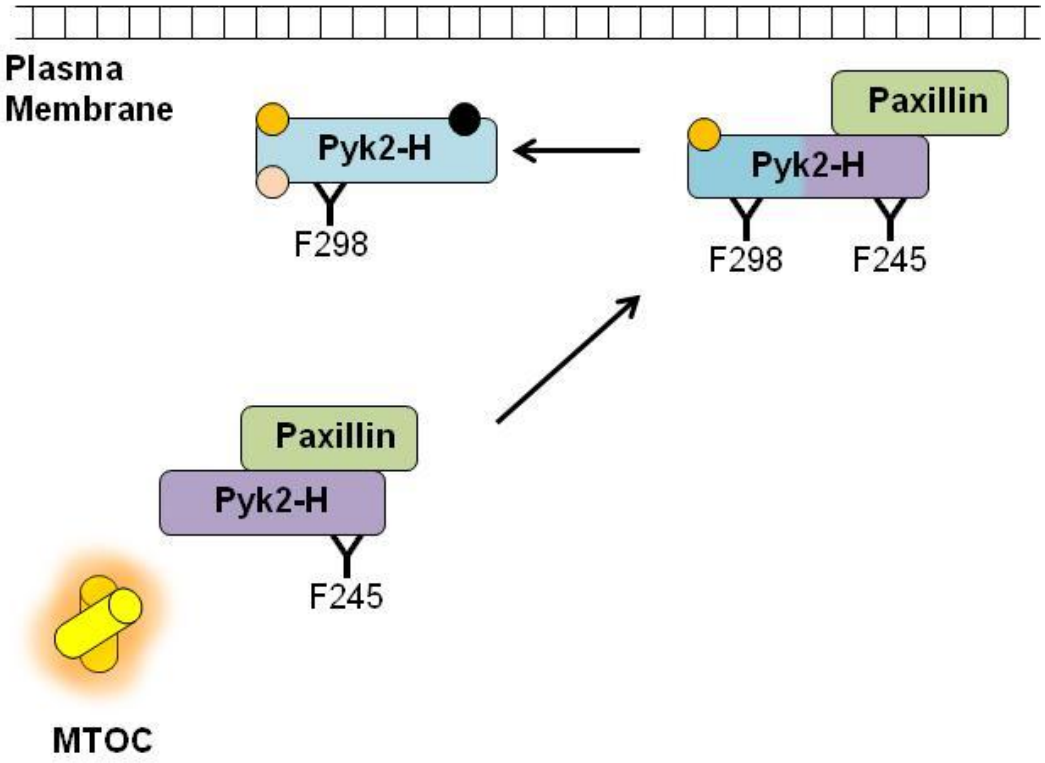


Figure 1.5: Proposed model explaining the differences between the F245 and the F298 Pyk2 populations in macrophages. Pyk2-H is expressed first in an autoinhibited conformation at the MTOC where the F298 epitope is not accessible but can bind to Paxillin and the F245 antiserum (purple Pyk2). Upon localization to the plasma membrane, a fraction of the Pyk2 molecules become tyrosine phosphorylated upon stimulation. This allows Pyk2-H to adopt a more open conformation and allows both the F245 and F298 antiserum to bind and preserves paxillin binding (blue and purple Pyk2). Pyk2-H then becomes phosphorylated on serine/threonine residues and/or potentially posttranslational modified in order to maintain an open and active conformation. The phsophorylated serine/threonine residues and the open conformation disrupt Paxillin and the F245 antiserum from binding (blue Pyk2).

1.5 Potential postranslational modification of Pyk2

J. St. Pierre went on to further examine the differences in the MWs of the F245 and F298 populations in her thesis [106]. Since the differences of the Pyk2 populations could not be explained through alternative splicing or differences in

phosphorylation, this led her to speculate that Pyk2 could be posttranslationally modified. As previously explained in section 1.2.4, FAK has been shown to be SUMOylated in COS cells co-transfected with SUMO-1, FAK, and PIAS1 [70]. This led J. St. Pierre to investigate whether Pyk2 could associate with SUMO-1 in RAW 264.7 macrophages [106]. She found that the protein Dynamin, which has been demonstrated to be directly SUMOylated was recovered in both Pyk2 and SUMO-1 IPs [107]. However, she was unable to determine whether Pyk2 was directly posttranslationally modified by SUMO-1, or if another SUMOylated protein such as Dynamin associated with Pyk2. Thus further investigation was needed into the possible SUMOylation of Pyk2.

1.6 Rationale for this project

Since J. St. Pierre found that the MW shift of Pyk2-H is not caused by phosphorylation or alternative splicing and because FAK has also been shown to be directly SUMOylated, it seems reasonable to consider that Pyk2 may also be directly SUMOylated. Since Pyk2 was shown to play an important role in the migration and cytoskeletal rearrangements of macrophages (section 1.2), it also seems possible that the SUMOylation of Pyk2 could play a role in macrophage motility and cytoskeletal rearrangements.

1.6.1 Hypothesis:

Pyk2 is a target of SUMOylation and the modifications of Pyk2 by SUMOylation affects Pyk2 function.

1.6.2 Specific aims:

1) **Determine if Pyk2 is directly modified by SUMO-1 or associates with SUMOylated proteins**
2) **Determine if the overexpression of SUMO-1 affects the protein expression or autophosphorylation of Pyk2-H**

CHAPTER TWO: MATERIALS AND METHODS

2.1 Antibodies

The F298 and F245 polyclonal antibodies were generated in our laboratory by injecting New Zealand White rabbits with a peptide corresponding to aa 2-12 (F298) or aa 720-826 fused to GST (glutathione-S-transferase) (F245) and have been described previously [104]. The F245 and F298 antisera names were chosen based on the numbers assigned to the New Zealand White rabbits used for generating the antibodies. The generation of polyclonal anti-GST (F307) was performed in our laboratory by injecting GST (glutathione-S-transferase) protein into rabbits. The purification and source of PY72.10.5 (anti-phosphotyrosine) has been described previously [104, 105]. Anti-Pyk2 (mouse monoclonal clone 11) was acquired from BD Biosciences (Mississauga, ON). A monoclonal anti-SUMO-1 (D-11) and polyclonal anti-Ubc9 were acquired from Santa Cruz Biotechnology (Santa Cruz, CA.). The SUMO-1 antibody used for the *in vitro* SUMOylation assay and anti-SUMO-2/3 were purchased from Enzo Life Sciences (Farmingdale, NY). Anti-actin was purchased from Sigma-Aldrich (Mississauga, ON). The rabbit monoclonal antibody to PIAS1 was acquired from AbCam (Cambridge, MA). The rabbit polyclonal Anti-GFP was purchased from Invitrogen (Camarillo, CA). Anti-phospho-Pyk2 Y^{402} (mouse monoclonal) was purchased from Upstate Cell Signaling Solutions (Charlottesville, VA). Anti-mouse IgG-HRP was acquired from Jackson ImmunoReserach Laboratories (West Grove, PA).

2.2 Cell Lines

NIH 3T3 cells were obtained from Dr. Jim C. Stone (University of Alberta, Edmonton, AB) and were maintained in DMEM with 10% CS and 200 mM L-glutamine. To subculture, NIH 3T3 cells at 80-85% confluency were trypsinized at room temperature for 2 minutes and passaged at 1:10 dilutions. The RAW 264.7 macrophage cell line was purchased from Sigma-Aldrich and maintained in RPMI with 10% FCS-gold (PAA Laboratories, Etobicoke, ON), and 200 mM L-Glutamine. RAW 264.7 cells reaching 70-80% confluency were passaged at 1:10 dilutions. All cell lines were incubated at 37°C under 5% carbon dioxide atmosphere.

2.3 Reagents

Protein A sepharose beads were purchased from Amersham Bioscience (Piscataway, NJ). Glutathione Sepharose 4B beads were acquired from GE Healthcare (Burlington, ON). Protein A conjugated to HRP was obtained from Pierce (Rockford, IL). N-ethylmaleimeide (NEM) was purchased from Sigma-Aldrich (Mississauga, ON). The protease inhibitor cocktail was obtained from Roche (Indianapolis, IN). The Pierce Silver Stain kit was purchased from Thermo Scientific (Rockford, IL).

2.4 Constructs

We obtained the Flag-mPIAS1 (Mus musculus) (Addgene plasmid 15206), and pcDNA3-HA-SUMO-1 (Homo sapiens) (Addgene plasmid 21154), constructs

from the plasmid repository at Addgene. They have been described previously [108, 109]. Tara Lysechko extracted murine Pyk2-H RNA from AB.1 cells, and performed first strand cDNA synthesis, amplification of DNA, ligation of Pyk2-H into the pC1-Neo vector, and sequencing. Full length Pyk2 and K457A-Pyk2-H was cloned into pEGFP-N1 by Samuel Cheung. NT-Pyk2, and CT-Pyk2 was ligated into pEGFP-C1 by Tara Lysechko. pGEX-NT-Pyk2 and pGEX-CT-Pyk2, were cloned intro the pGEX-4T-3 vector by Tara Lysechko. The RAN-GAP1-GST purified protein was provided with the SUMOylation assay kit from Enzo Life Sciences.

2.5 Generation of Pyk2-H and SUMO-1 mutants by site directed mutagenesis

Site-directed mutagenesis was performed to remove the predicted SUMOylation sites from Pyk2-H or SUMO-1. Mutations were introduced using PCR into the Pyk2-H PC1-Neo or pcDNA3-HA-SUMO-1 construct. All oligos were purchased from SIGMA Genosys and are listed in Table 2.1. Pfu turbo (Turbo Invitrogen) was used to insert the desired mutations. The Pyk2-H PCR reaction solution was subjected to temperature cycles consisting of one cycle of 3 min at 95°C; 40 cycles of 1 min at 95°C, 1 min at 52°C, and 16 min of 68°C; and one cycle of 68°C for 1 hour. The SUMO-1 (T95R) PCR reaction was subjected to temperatures cycles consisting of one cycle of 3 min at 95°C; 40 cycles of 30 sec at 95°C, 30 sec at 52°C, and 1 min of 68°C; and one cycle of 68°C for 5 minutes. PCR products were purified with the Qiaquick PCR purification kit (Qiagen, Mississauga, ON). 1µl of the PCR reaction was mixed with 4µl of dH_2O and transformed into *E. coli* DH5α library efficiency cells (Invitrogen, Camarillo,

CA). The mutation in each construct was verified by nucleic acid sequencing (McGill University, Génome Québec Innovation Centre, QC). The primers for sequencing are listed below in Table 2.2.

Table 2.1: List of primers used for site directed mutagenesis

Desired mutation	Primer
Pyk2-H K646R	Forward - 5'GGG GAC AGG CTG CCC AGG CCC GAA CTC TGT CCG CCT GTC CTT 3' Reverse- 5' CGG ACA GAG TTC GGG CCT GGG CAG CCT GTC CCC TTT CTC CAG 3'
Pyk2-H K35R	Forward - 5' CCA GTG GAT GTG GAG AGG GAA GAC GTG CGC ATC CTC AAG GTC 3' Reverse - 5'GCG CAC GTC TTC CCT CTC CAC ATC CAC TGG TAC CAC CAC 3'
Pyk2-H K145R	Forward -5' TTC ATG GAG AGC CTG AGA GAA GAC AGG ACC ACA TTG CTG TAC 3' Reverse- 5'CAA TGT GGT CCT GTC TTC TCT CAG GCT CTC CAT GAA GTC TTC 3'
SUMO-1 T(95)S	Forward - 5' GAA GTT TAT CAG GAA CAA AGG GGG GGT CAT TCA ACA GTT TAG 3' Reverse - 5' AAC TGT TGA ATG AAC CCC CCT TTG TTC CTG ATA AAC TTC AAT3'

Table 2.2: List of primers used for sequencing

Construct	Primer for sequencing
K646R	mPyk7 – 5' TACAAAGCCCTCGTGACAC 3'
K35R	mPyk2N TermF - 5' CATAGGAATTCGCAGTCTGAGAGGATGT 3'
K145R	mPyk2 – 5' CTGGCTGAATGCTATGG 3'
T(95)R	CMV – 5' CGC AAA TGG GCG GTA GGC GTG 3'

2.5 Transfection of NIH 3T3 cells

6×10^5 NIH 3T3 cells were plated and incubated for 24 hours prior to transfection. NIH 3T3 cells were then transfected using the Effectene kit from

QIAGEN (Mississauga, ON). Briefly, the expression plasmids were mixed with 300µl of Enhancer buffer and 16µl of Enhancer. After a 2-5 minute incubation at room temperature, 60µl of Effectene Transfection Reagent was added, and the solution was incubated for another 5-10 minutes at room temperature. During the incubation, the NIH 3T3 cells were washed once with warm 1X PBS and 7 ml of transfection medium (DMEM, 10% FCS Hyclone characterized serum, and 1% L-glut) was added. After incubation, 3 ml of transfection medium was added to the expression plasmid solution, and the solution was then immediately added in drops to the NIH 3T3 cells. The cell culture dish was then mixed gently to assure uniform distribution of the DNA complexes. The cells were then cultured for another 48 hours before lysis.

2.6 Cell lysis

1 x 10^7 RAW 264.7 cells were washed once with 1X PBS and with 10mM of NEM where indicated. Cells were lysed with lysis buffer containing 1% NP-40, 10mM Tris (pH 7.5), 5mM EDTA, 150mM NaCl, 1mM orthovanadate, protease inhibitor cocktail and in the presence of absence of 10mM of NEM and incubated for 20 minutes on ice with occasional agitation.

Two days post-transfection, 100 mm plates of transfected NIH 3T3 cells were washed with 1X PBS or 10mM NEM. 2 mls of 1% NP-40 lysis buffer containing 20 mM NEM was directly added to the cell culture plates and they were incubated on a rocker at 4°C for 20 minutes.

RAW 264.7 and NIH 3T3 cell lysates were transferred to 1.5 ml eppendorf tubes and centrifuged at 13 000 x g for 5 minutes to pellet out the nuclei. Post-

nuclear lysates were used for immunoprecipitation or loaded onto SDS-PAGE gels for subsequent Western Blotting.

2.7 Immunoprecipitation

Post nuclear lysates were incubated with Pyk2 antiserum (F245 or F298) or anti-GFP for 20 minutes on ice, or incubated with anti-SUMO-1, anti-SUMO-2/3, or anti- PIAS1 overnight at 4°C. Protein A Sepharose was then added (30 µl of 50% slurry) and the samples were rotated at 4°C for approximately 2 hours. Beads were pelleted and washed three times with 1% NP-40 lysis buffer three times before being resuspended in 60µl of 1X Laemmli reducing sample buffer and boiled for 3 minutes. Samples were then loaded onto SDS-PAGE gels.

2.8 SDS-PAGE and Western Blotting

Total cell lysates or immunoprecipitates were loaded onto SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked in 4% skim milk or 4% BSA in enhanced chemiluminescence (ECL) buffer and immunoblotted with the appropriate primary and HRP-conjugated antibodies and visualized by enhanced ECL (PerkinElmer Life Sceince Products, Boston, MA). When sequential Western Blots were preformed on the same membrane, the membrane was stripped with buffer containing β-mercaptoethanol, Tris-HCl (pH 6.7), and SDS at 56°C in between each blot. Quantification of Western blot bands was performed using the ImageJ software, version 1.43u (http://rsb.info.nih.gov/ij/).

2.9 Protein expression and purification

The NT and CT Pyk2-H domains cloned into the pGEX-4T-3 vector were expressed in BL-21 competent *Escherichia coli* after induction with 0.5mM IPTG for 2.5 hours. Cells were resuspended in 1X PBS and sonicated 5 x 30 sec and incubated with 1% Triton X-100 for 30 min. After centrifugation, the cell lysate was incubated with Glutathione Sepharose 4B beads for 45 minutes at room temperature. The beads were washed three times with 1x PBS and three times with mQH_2O. GST-NT-Pyk2 and GST-CT-Pyk2 protein was quantified by comparing dilutions of each GST fusion to known amount of bovine serum albumin (BSA) on an SDS-PAGE gel that was subsequently stained with Coomassie blue (Bio-Rad, Hercules, CA).

2.10 *in vitro* SUMOylation

In vitro SUMOylation was performed using the SUMOylation assay kit from Enzo Life Sciences. Each SUMOylation reaction contained 5 μM of NT or CT Pyk2-H or 1 μM of RanGAP1, 1 μM of E1 enzyme, 1μM of E2 enzyme, and 1 μM of SUMO-1 with 5 μl of SUMOylation buffer in a total of 20μl volume. In addition, 1 μM of Mg^{2+}-ATP and 1 μM of PIAS1 was added where indicated. The assay components were mixed and incubated at 37°C for two hours. The reaction was then quenched by adding 20 μl of 2X Laemmli reducing sample buffer. The samples were then run on an 8.5% SDS-PAGE.

2.11 Preparing samples for Mass Spectrometry

NIH 3T3 cells were transfected with Flag-PIAS1 (3μg), HA-SUMO-1(T95R) (2μg), and Pyk2-H (2μg) as described above. $1x10^7$ cells were washed

with PBS and lysed with 2mls lysis buffer and 50mM NEM. Lysates were pre-cleared with protein A sepharose for 1 hour at 4°C. Pre-cleared lysates were treated with 9 µl of F245 or F298 antiserum or appropriate isotype control for 20 minutes on ice and incubated with 70 µl protein A sepharose beads for 2 hours at 4°C. Immunoprecipitations were washed three times. Immunoprecipitations were resolved on a 7.5% SDS-PAGE gel and stained with the Pierce Silver Stain Kit. Stained gel pieces were washed, and in-gel tryptic digested. Mass spectrometry was conducted at the University of Alberta Department of Chemistry.

2.12 Liquid Chromatography – MS/MS

The resultant peptides were subject to LC-MS/MS analysis on a UPLC (Waters, Milford, MA) coupled with q-Tof premier mass spectrometer (Water, Milford, MA). 5 µL of the resultant peptide digests was loaded onto a nanoAcquity UPLC system with peptide trap (180µm x 20mm, Symmetry® C18 nanoAcquity™ column, Waters, Milford, MA) and an analytical column (75 µm × 150 mm, Atlantis™ dC18 nanoAcquity™ column, Waters, Milford, MA). Desalting on the peptide trap was achieved by flushing trap with 2% acetonitrile, 0.1% formic acid at a flow rate of 10µL/min for 1-3 minutes. Peptides were separated with a gradient of 2-60% solvent B (acetonitrile, 0.1% formic acid) over 35 minutes at a flow rate of 350 nL/min. The column was connected to a Q-Tof premier Mass spectrometer (Waters corporation) for ESI-MS/MS analysis.

2.13 Mascot search

Obtained MS/MS data were analyzed through proteomic software called Mascot (version 2.2, Matrix science). Settings for database search were as follows: parent ion and MS/MS tolerance were set to 0.1 Da and 0.2 Da respectively; semi-trypsin as enzyme was specified; carbamidomethylation on cysteine was selected as fixed modifications and oxidation on methionine and ubiquitination (GG) was selected as variable modification. Semi-trypsin was selected since this means that Mascot will search for peptides cleaved (KR not P) at one terminus, but may not necessarily be cleaved by trypsin at the other terminus. Peptides were searched through the NCBInr database.

Confidence of positive protein identification was judged by high protein and peptide scores in the search results. Manual inspection of the original MS/MS spectra were often performed to make sure major peaks in the MS/MS spectra were matched and explained.

In order to identify SUMOylation sites with the MASCOT search engine, the software program ChopNSpice was used [110]. The FASTA sequence for Pyk2-H was chopped into tryptic fragments, and the program putatively attached to any lysine residue within Pyk2-H the C-terminal SUMO-1 (T95R) sequence (GG) or allowing for one missed cleavage site (ELGMEEEDVIEVYQEQRGG). The search engine compares the *in silico*-generated Pyk2H peptides to the experimentally obtained fragments from the MS/MS [111]. The following parameters were used in the ChopNSpice software: the spice species was *Homo sapiens*; the spice sequence was custom: ELGMEEEDVIEVYQEQRGG; the

spice site was KX; the spice mode was once per fragment; include unmodified fragments in output; the enzyme was trypsin; allow up to three protein miscleavages; allow up to one miscleavage in the spice sequence; the output formatting was FASTA (single protein sequence); include unmodified fragments in output; the enzyme was trypsin-advanced model; allow up to three protein miscleavages; allow up to one miscleavage in the spice sequence; the output formatting was FASTA (single protein sequence); cleavage marker none; and retain comments in FASTA format; line breaks in FASTA output. For SUMOylated site identification with MASCOT, all MS/MS spectra were searched against a new FASTA file that was created by ChopNSpice with the following parameters: mass tolerance of 10 ppm in MS mode and 0.8 D in MS/MS mode; allow zero missed cleavages; consider methionine oxidation and cysteine carboxyamidomethylation as variable modifications.

2.14 Reproducibility of Results

Unless otherwise stated, all experiments were repeated at least 3 times and representative data is shown.

CHAPTER 3: RESULTS

3.1 NEM increases the association between endogenous Pyk2-H and SUMO-1

As introduced in section 1.4, Pyk2-H exists as at least two distinct populations in macrophages [30]. Immunoblotting RAW 264.7 macrophage cell lysates with F245 antiserum reveals two Pyk2 species; one of a higher MW and one of a lower MW. This was first reported by J. St-Pierre *et al.*, and can be seen in the F245 immunoblot in Figure 3.1A [30]. Figure 3.1B also demonstrates that only a higher MW Pyk2 species is seen in the RAW 264.7 lysates immunobloted with F298.

Since J. St-Pierre *et al.* had already shown that endogenous Pyk2-H from RAW 264.7 cells can be recovered in endogenous SUMO-1 immunoprecipitates, I wanted to see if the association between Pyk2-H and SUMO-1 could be enhanced by using the chemical N-ethylmaleimide (NEM) [106]. SUMO can be rapidly de-conjugated from its targets by SUMO proteases. Thus, NEM, a cysteine alkylating agent, is used to irreversibly bind and inhibit SUMO proteases [112, 113]. I also wanted to determine whether the F245 or F298 antiserum was better at detecting the association of Pyk2-H with SUMO-1. To test this I prepared RAW 264.7 lysates in the presence or absence of NEM and immunoprecipitated endogenous Pyk2-H and SUMO-1 using F245 or F298 antiserum and anti-SUMO-1. Immunoblots were then performed using F245 or F298 antiserum and anti-SUMO-1.

The F298 antiserum was not able to detect Pyk2 in the SUMO-1 IP in the absence of NEM (Figure 3.1B). However, once the RAW 264.7 lysates were

treated with NEM, Pyk2-H was pulled down with anti-SUMO-1 in the F298 immunoblot (Figure 3.1B). This suggests that normally there is a very small amount of the F298 Pyk2-H population that associates with endogenous SUMO-1. NEM however appears to stabilize and enhance the association of F298 Pyk2-H population with SUMO-1.

In contrast, the F245 antiserum was not able to detect any Pyk2-H species pulled down by anti-SUMO-1 in the presence of NEM (Figure 3.1A). However, in the SUMO-1 IP in the absence of NEM there is the appearance of a faint band that is a slightly higher MW than the high MW F245-reactive Pyk2-H band (Figure 3.1A). This band was also not seen in the IP control without NEM (Figure 3.1A). Since this band has not been confirmed to be Pyk2-H and has not been consistently seen in every replicate of this experiment, it seems possible that this band is due to non-specific detection of another protein pulled down by SUMO-1.

Interestingly, there is a decrease in the amount of the lower MW F245-reactive Pyk2-H detected in the F245 IP by the F245 antiserum when NEM is present compared to when NEM is absent (Figure 3.1A). This shift towards a higher MW species would be consistent with the conjugation of SUMO-1 to Pyk2-H.

Since I observed that NEM could increase the association of F298-reactive Pyk2-H with SUMO-1, I then wanted to explore the possibility that the increased molecular mass of the high MW F245-reactive species or the F298-reactive Pyk2-H was due to the posttranslational modification by SUMO-1. Immunoblotting with a polyclonal SUMO-1 Ab revealed no immunoreactive protein in the F245 or

F298 IPs in the presence or absence of NEM (Figure 3.1C). This suggested that the differences seen in the F298 Pyk2-H population may not be caused by the covalent attachment of SUMO-1 to Pyk2-H. Furthermore an approximately 80kDa unidentified protein was pulled down by SUMO-1 in the presence of NEM, which suggests that the SUMO-1 Ab is able to both IP and immunoblot SUMOylated proteins (Figure 3.1C).

These results together suggest that the endogenous F298 Pyk2-H population is associating with SUMO-1. However, it remains unclear as to whether the F298 Pyk2-H population is directly modified by SUMO-1, or if the F298 Pyk2-H population interacts indirectly with SUMO-1 by associating with another SUMOylated protein.

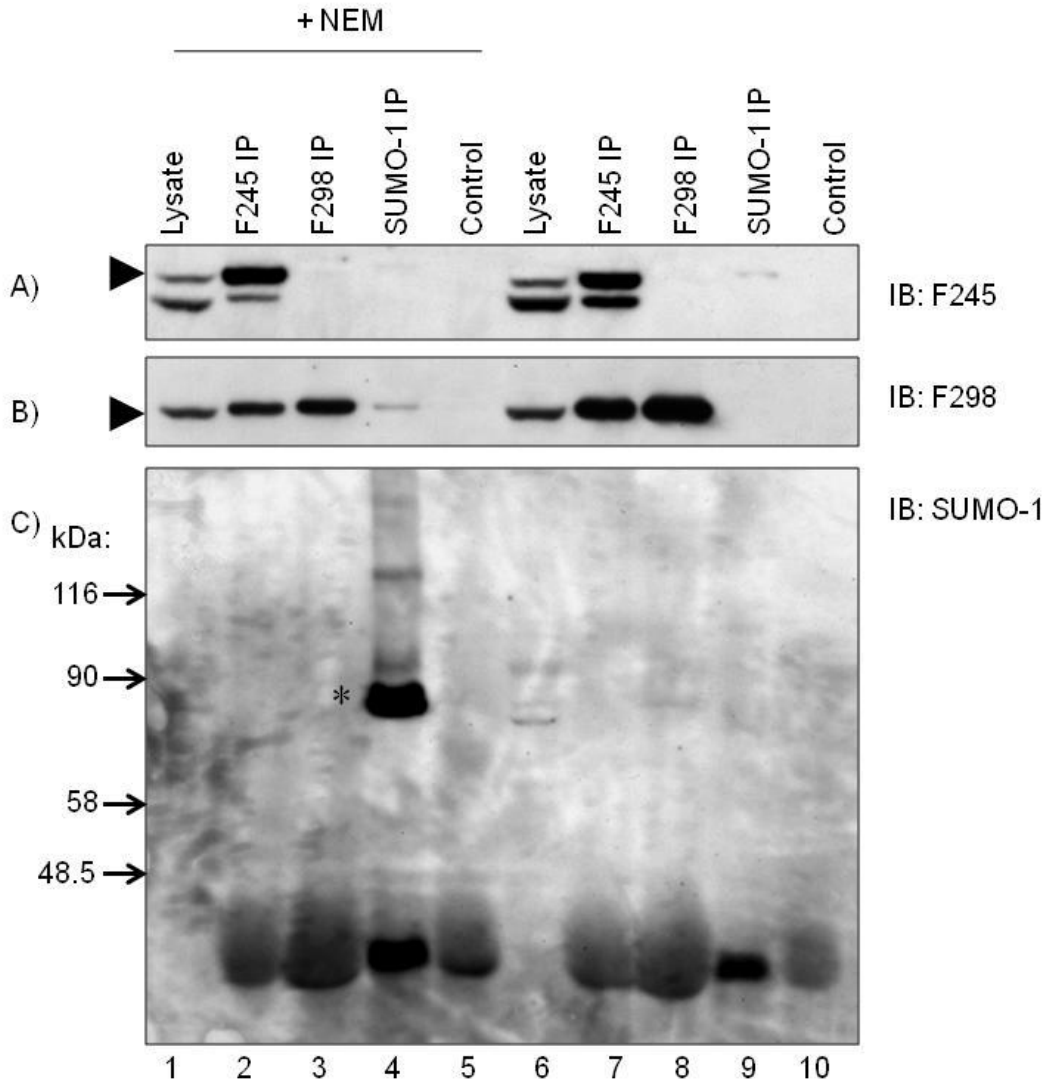


Figure 3.1: NEM increases the association of the F298-reactive Pyk2-H with SUMO-1 in RAW 264.7 cells. RAW 264.7 cell lysates treated with NEM, or left untreated were immunoprecipitated with F245, F298 and SUMO-1 antibodies. Immunoprecipitates were blotted with A) F245 B) F298 or C) SUMO-1. Control lanes represent IP controls with sepharose A beads and the appropriate isotype controls. The position of the arrowhead indicates the same position on the gel in each immunoblot. The arrows indicate the molecular weight markers. * represents an unknown protein detected by anti-SUMO-1.

3.2 Pyk2 does not associate with SUMO 2/3

Since SUMOylation can occur with any of the three SUMO isoforms (SUMO-1, SUMO-2, SUMO-3), I wanted to determine if SUMO-2 or SUMO-3 could also associate with the endogenous F298-reactive Pyk2-H. Since SUMO-1 only shares 50% identity with SUMO-2/3, anti-SUMO-2/3 antibodies are not able to detect SUMO-1 and can therefore be used to distinguish which SUMO isoform may interact with Pyk2-H [72, 83]. Thus RAW 264.7 lysates were immunoprecipitated with F245 or F298 antiserum, anti-SUMO-1 or anti-SUMO-2/3 in the presence or absence of NEM.

Neither Pyk2-H population was detected by the F245 or F298 Abs in the SUMO-2/3 IP in the absence or presence of NEM (Figure 3.2A, B). The F298-reactive Pyk2-H however was pulled down in the SUMO-1 IP in the presence of NEM as seen in Figure 3.1B, which was used as a positive control (Figure 3.2B). No immunorective bands were detected in the F245 and F298 IPs in the presence or absence of NEM in the SUMO 2/3 immunoblot (Figure 3.2C). Multiple unknown proteins were pulled down in the SUMO-2/3 IP in the presence of NEM, which suggests that the polyclonal SUMO-2/3 Ab is able to detect and immunoblot SUMOylated proteins (Figure 3.2C). Since SUMO-2/3 does not appear to associate with the F298 Pyk2-H population in the presence or absence of NEM, this suggests that the endogenous F298 population associates only with SUMO-1.

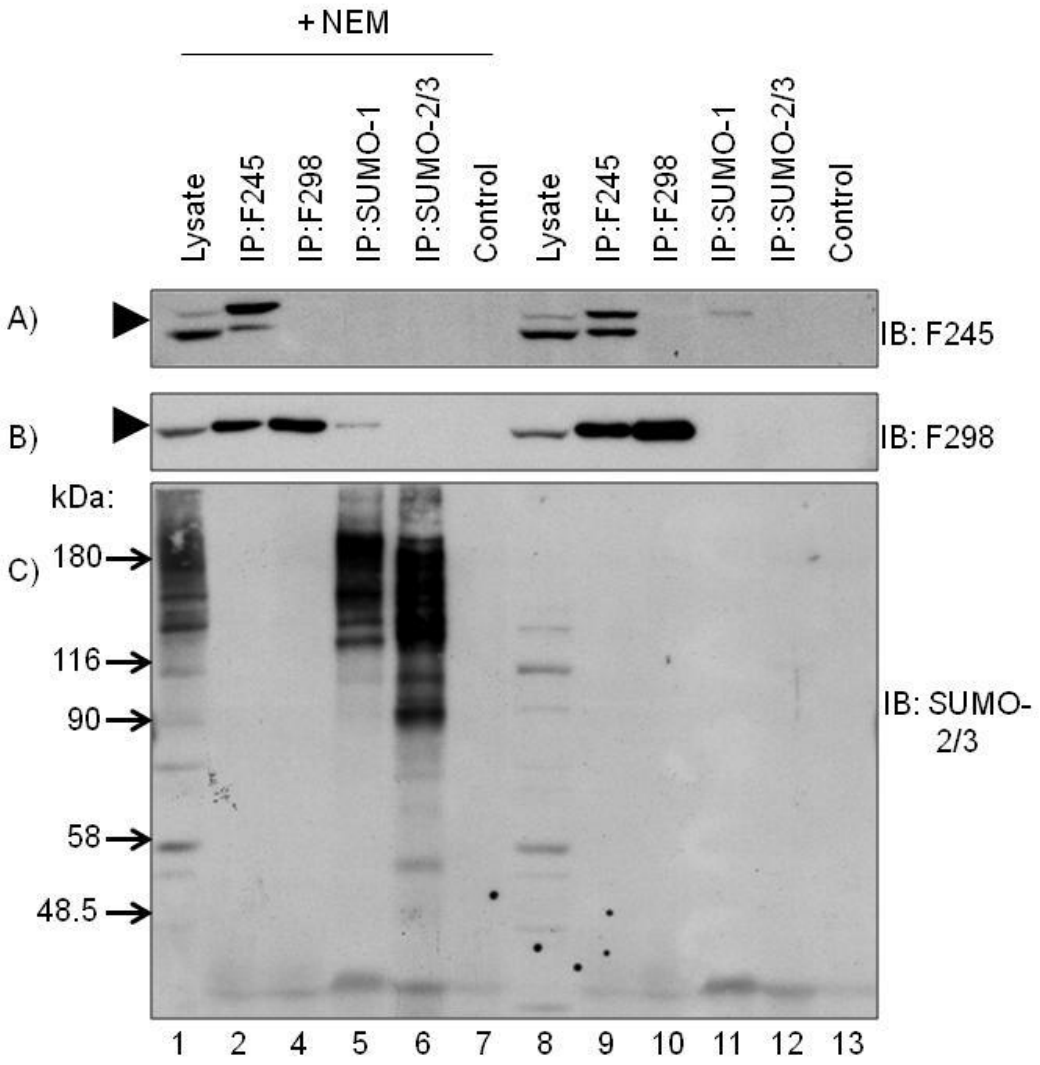


Figure 3.2: Pyk2 associates selectively with SUMO-1. RAW 264.7 macrophages were treated with NEM, or left untreated and immunoprecipitated with F245, F298, SUMO-1 or SUMO-2/3 antibodies. Immunoprecipitates were blotted with F298 (B) then stripped and reprobed for F245 (A) and SUMO-2/3 (C). Control lanes represent IP controls with appropriate isotype controls and sepharose A beads. The position of the arrowhead indicates the same position on the gel in each immunoblot.

3.3 Exogenous F298-reactive Pyk2-H associates with SUMO-1 in NIH 3T3 cells

In Figure 3.1C I was unable to detect the covalent attachment of SUMO-1 to Pyk2-H using the SUMO-1 mAb. Since only a very small amount of Pyk2-H may be SUMOylated, it was possible that the SUMO-1 mAb was unable to detect SUMOylated Pyk2-H. Therefore, to potentially increase the amount of Pyk2-H that may be SUMOylated I decided to overexpress Pyk2-H and SUMO-1 in NIH 3T3 cells. NIH 3T3 cells have been shown to contain only the lower MW F245-reactive Pyk2-H and not the F298-reactive Pyk2-H population [30]. Therefore, I could also determine if the exogenous F298-reactive Pyk2-H can associate with SUMO-1. NEM would also be used in these experiments since it was shown to increase the association of Pyk2-H with SUMO-1 in RAW 264.7 cells

Untransfected NIH 3T3 cell lysates contained the lower MW Pyk2-H recognized by the F245 antiserum, but also another faster migrating band that has not been confirmed to be Pyk2-H and is most likely a non-specific protein detected (Figure 3.3A and B lane 1). There was an increased detection of the lower MW F245-reactive Pyk2-H by the F245 antiserum when untransfected NIH 3T3 lysates were immunoprecipitated with anti-F245 (Figure 3.3A lane 5). No Pyk2-H was observed in the untransfected SUMO-1 IPs with either Pyk2-antiserum or a commercial Pyk2-H mAb (Figure 3.3A, B, C lane 13). This further suggests that the lower MW F245-reactive Pyk2-H does not associate with SUMO-1.

As seen with the RAW 264.7 macrophages, Pyk2-H was detected in the SUMO-1 IP with the Pyk2-H mAb when Pyk2-H was transfected into NIH 3T3 cells (Figure 3.3C). However, neither antiserum was able to detect the association of Pyk2-H with SUMO-1 when only Pyk2-H was transfected (Figure 3.3A, B lane 14). Therefore, it appears that exogenous Pyk2-H can associate with the endogenous SUMO-1 in NIH 3T3 cells.

The Pyk2 mAb detected a greater amount of Pyk2-H pulled down in the SUMO-1 IP when SUMO-1 and Pyk2-H were co-transfected in NIH 3T3 cells (Figure 3.3C lane 14). However, no Pyk2-H was detected by the F245 antiserum in the SUMO-1 IP and a faint band was detected by the F298 antiserum when NIH 3T3 cells transfected with SUMO-1 and Pyk2-H (Figure 3.3A, B lane 15). This result suggests that increasing the amount of SUMO-1 in a cell increases the association between Pyk2-H and SUMO-1.

Interestingly when NIH 3T3 cells were co-transfected with Pyk2-H and SUMO-1, there is an increase in the detection of the higher MW Pyk2-H recognized by the F245 antiserum in the F245 IP (Figure 3.3A lane 7). The F245 antiserum was also able to detect a greater amount of the F298-reactive Pyk2-H in the F298 IP when Pyk2-H and SUMO-1 were transfected (Figure3.3 A lane 11). Therefore, it appears that overexpressing SUMO-1 may affect the protein expression of Pyk2-H. Furthermore, when Pyk2-H and SUMO-1 are co-transfected, there is a great amount of the higher MW F245-specific Pyk2-H population detected by the F245 antiserum in the F245 IP (Figure 3.3A lane 7). This shift was not seen when Pyk2-H alone was transfected in NIH 3T3 cells,

however J. St-Pierre *et al.* has seen the higher MW F245-reactive Pyk2-H when Pyk2-H is transfected in NIH 3T3 cells (Figure 3.3A lane 6) [30]. Additionally the higher MW F245-specific Pyk2-H band in the F245 IP is the same MW as the F298-reactive Pyk2-H band in the F298 IP (Figure 3.3A lane 7 and 11). The MW difference in Figure 3.3A may be caused by a slight slant in the SDS-PAGE gel. The increase in the higher MW F245 specific Pyk2-H upon SUMO-1 transfection would be consistent with the increased mass due to a SUMO-1 conjugate.

Since the E3 ligase PIAS1 has been shown to enhance the SUMOylation of FAK in COS cells [70], I also wanted to investigate whether co-transfecting PIAS1 with SUMO-1 and Pyk2-H would enhance the association of SUMO-1 with Pyk2-H. Lysates from NIH 3T3 cells co-transfected with all three constructs contained greater amounts of the higher MW F245-reactive Pyk2-H and the F298-reactive Pyk2-H (Figure 3.3A and B lane 4). The F245 antiserum also detected a greater amount of the higher MW F245-reactive Pyk2 in the F245 IP (Figure 3.3A lane 8). The F298 antiserum and the Pyk2 mAb also detected a greater amount of the F298-reactive Pyk2-H in the F298 IP (Figure 3.3B and C lane 12). Co-transfecting all three constructs into the NIH 3T3 cells led to the detection of Pyk2-H in the SUMO-1 IP by the F245 and F298 antiserum, and the Pyk2-H mAb (Figure 3.3A, B, C lane 16). The Pyk2-H mAb also detected higher amounts of Pyk2-H in the SUMO-1 IP when all three constructs were co-transfected compared to the transfection of Pyk2-H and SUMO-1 (Figure 3.3C lane 16). These results together demonstrate that the association between Pyk2-H and SUMO-1 can be greatly enhanced by co-transfecting SUMO-1 and PIAS1 with

Pyk2-H into NIH 3T3 cells. Furthermore, the protein expression of Pyk2-H is greatly enhanced when all three constructs are co-transfected into NIH 3T3 cells.

However, even though I was able to increase the association of Pyk2-H and SUMO-1 by transfecting PIAS1, immunoblotting with the SUMO-1 Ab revealed no immunoreactive bands in the F245 or F298 IPs with Pyk2-H, SUMO-1, and PIAS1 (Figure 3.3D). Without being able to detect a SUMO-1 reactive protein of the appropriate MW in either the F245 or F298 IPs, I remained unable to conclude that Pyk2-H was being directly SUMOylated. For the next section of this thesis I will examine the association of Pyk2-H with SUMO-1 and PIAS1. The ability of Pyk2-H to be directly SUMOylated and potential Pyk2-H SUMOylation sites will be addressed later in this chapter.

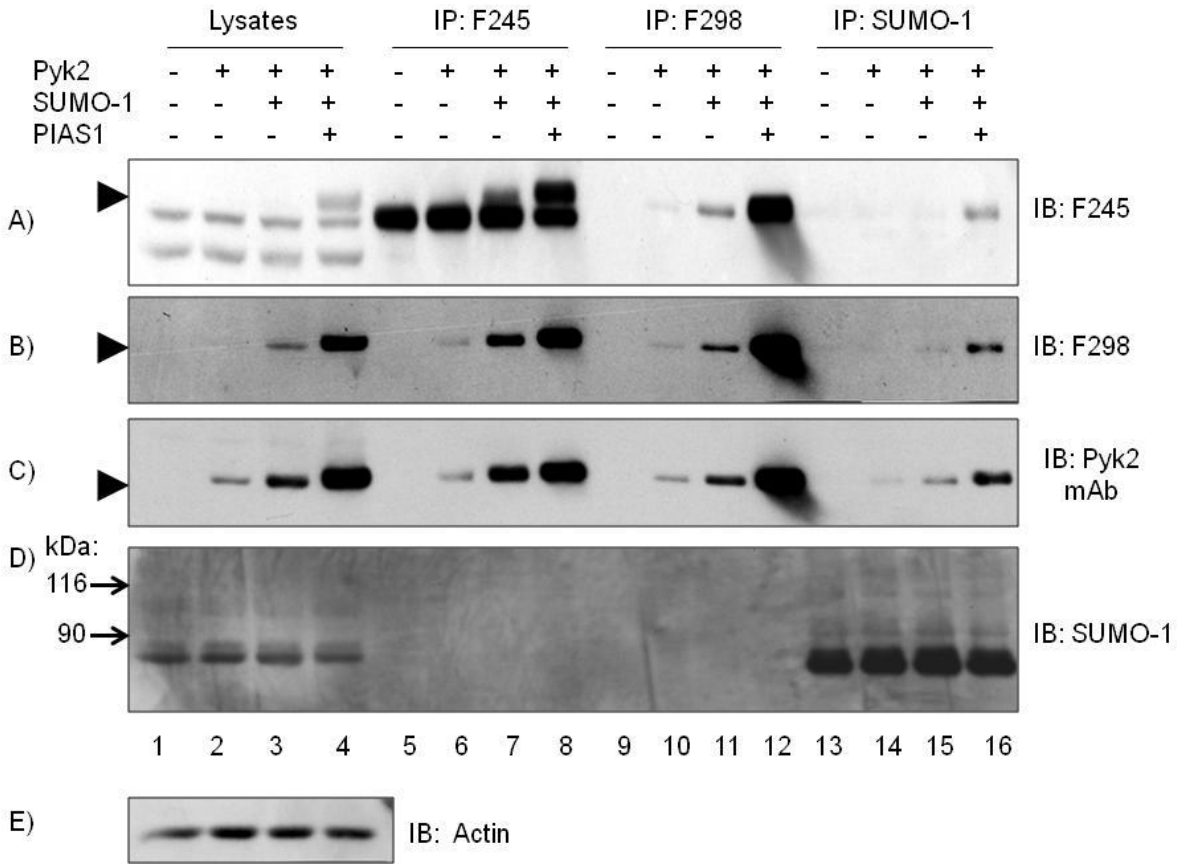


Figure 3.3 Exogenous F298-reactive Pyk2-H is able to associate with SUMO-1 when PIAS1 is co-transfected in NIH 3T3 cells. Pyk2 and SUMO-1 were immunoprecipitated from NIH 3T3 cell lysates treated with NEM and transfected with Pyk2 , SUMO-1, and PIAS1 or left untransfected. The membrane was probed with anti-SUMO-1 (D) and then stripped and reproped with F245 (A) and F298 (B) antiserum, and the Pyk2 mAb (C). Actin (E) was used as a loading control. The position of the arrowhead indicates the same position on the gel in each immunoblot.

3.4 PIAS1 promotes the association of Pyk2-H and SUMO-1

Since F298-reactive Pyk2-H was shown to associate with SUMO-1 when PIAS1 was present in NIH 3T3 cells, I next wanted to determine whether PIAS1 could enhance the association of Pyk2-H and SUMO-1 in a dose dependent manner. To test this I transfected NIH 3T3 cells with Pyk2-H and SUMO-1 and an increasing amount of PIAS1 plasmid. NIH 3T3 lysates treated with NEM were then immunoprecipitated with anti-F245, anti-F298, and anti-SUMO-1. Interestingly, the detection of the higher MW F245-reactive Pyk2 and the F298-reactive Pyk2-H with anti-F245, anti-F298 and anti-Pyk2 increased when the amount of co-transfected PIAS1 was increased in a dose-dependent manner (Figure 3.4A, B, C). The amount of the lower MW F245-reactive Pyk2-H appeared to remain constant whether SUMO-1 or an increasing amount of PIAS1 was transfected into NIH 3T3 cells (Figure 3.4A). Furthermore, there was an increase in the F298-reactive Pyk2-H recovered by anti-SUMO-1 when PIAS1 was co-transfected in a dose dependent manner (Figure 3.4A, B, C). Actin was used as a loading control (Figure 3.4D). Therefore, these results suggest that SUMO-1 and Pyk2-H associate in the presence of PIAS1, which may be functioning as an E3 ligase.

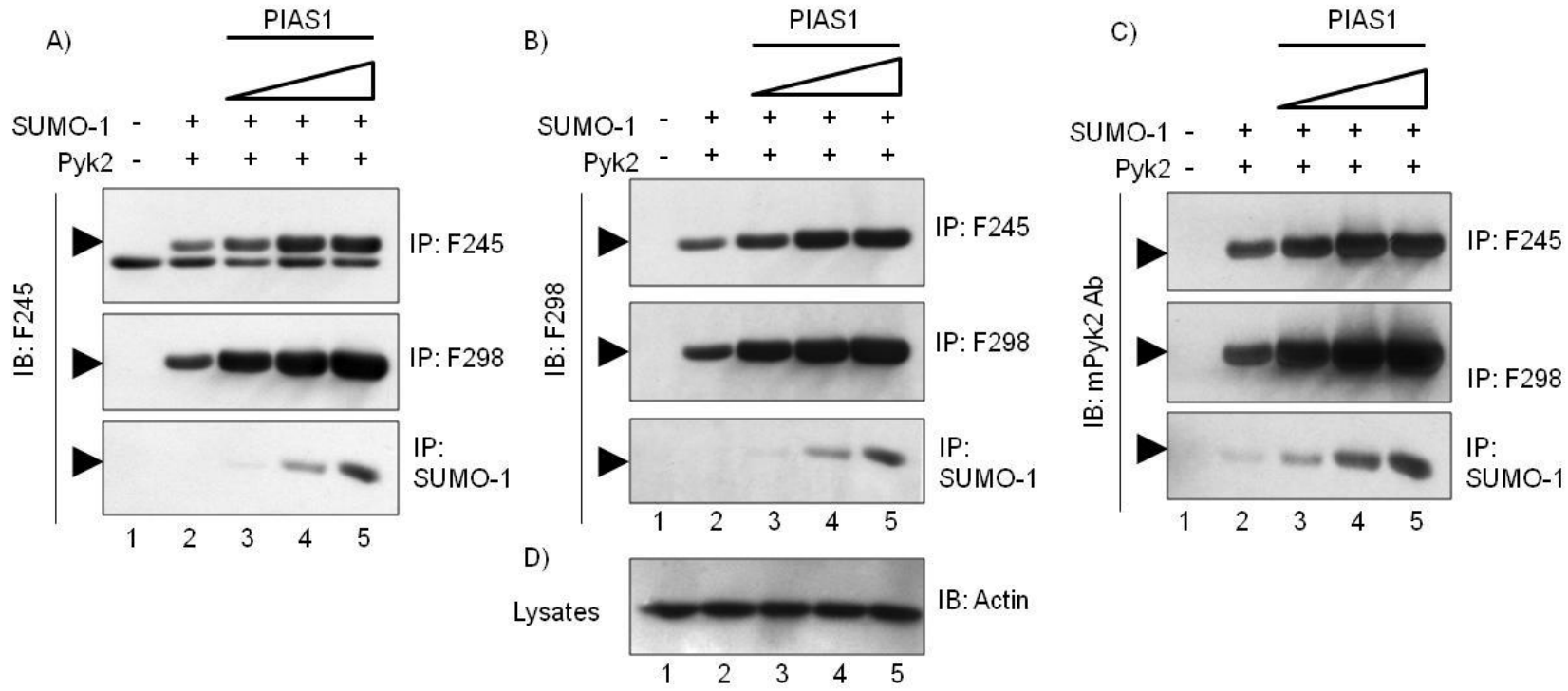


Figure 3.4: Increasing PIAS1 greatly enhances the amount of Pyk2-H in SUMO-1 immunoprecipitates. NIH 3T3 cells were transfected with Pyk2-H and HA-SUMO-1 (lanes 2-5) and increasing quantities of PIAS1 plasmid symbolized by a triangle (lanes: 3, 1µg DNA; 4, 2µg; 5, 3µg). Lysates were treated with NEM and immunoprecipitated with F245, F298, or SUMO-1. Immunpoprecipitiates were treated with NEM and immunoblotted with F245 (A) and then stripped and reprobed for F298 (B), and Pyk2 mAb (C). D) Lysates were immunoblotted for actin, which was used as a loading control. The arrowheads represent the same position on the gel in each immunoblot.

3.5 The E3 ligase PIAS1 co-immunoprecipitates with Pyk2-H

Since I found that PIAS1 enhances the association of Pyk2-H with SUMO-1, I then wanted to determine if Pyk2-H and PIAS1 can co-IP. To test if Pyk2 and PIAS1 are complexed I co-transfected NIH 3T3 cells with Pyk2-H, PIAS1, and SUMO-1 and immunoprecipitated the lysates treated with NEM with F245 or F289 antiserum, and anti-PIAS1.

Untransfected NIH 3T3 cells revealed that the presence of PIAS1 was not detected by anti-PIAS1 in either the F245 or F298 IPs (Figure 3.5A). However, when NIH 3T3 cells were co-transfected with Pyk2-H and PIAS1, PIAS1 was detected in the F245 and F298 IPs by anti-PIAS1 (Figure 3.5A). Similar levels of PIAS1 was detected in the F245 and F298 IPs when SUMO-1 was also co-transfected with Pyk2-H and PIAS1 (Figure 3.5A). Although less PIAS1 was detected in the F245 IP compared to the F298 IP (Figure 3.5A).Therefore, PIAS1 is able to be immunoprecipitated by both the F245 and F298 antiserum; however the F298 antiserum consistently recovers more PIAS1.

The F245 antiserum was unable to detect any Pyk2-H in the PIAS1 IP of NIH cells co-transfected with Pyk2-H and PIAS1 or co-transfected with all three constructs (Figure 3.5B). However the F298 antiserum did detect a faint band corresponding to the F298-reactive Pyk2-H in the PIAS1 IP when all three constructs were co-transfected (Figure 3.5C). The Pyk2 mAb was able to detect a F298-reactive Pyk2-H band in the PIAS1 IP of NIH 3T3 cells co-transfected with Pyk2-H and SUMO-1 (Figure 3.5D). Therefore these co-immunoprecipitation

studies of NIH 3T3 cells expression Pyk2-H, SUMO-1, and PIAS1 reveal that Pyk2-H robustly forms a complex with PIAS1.

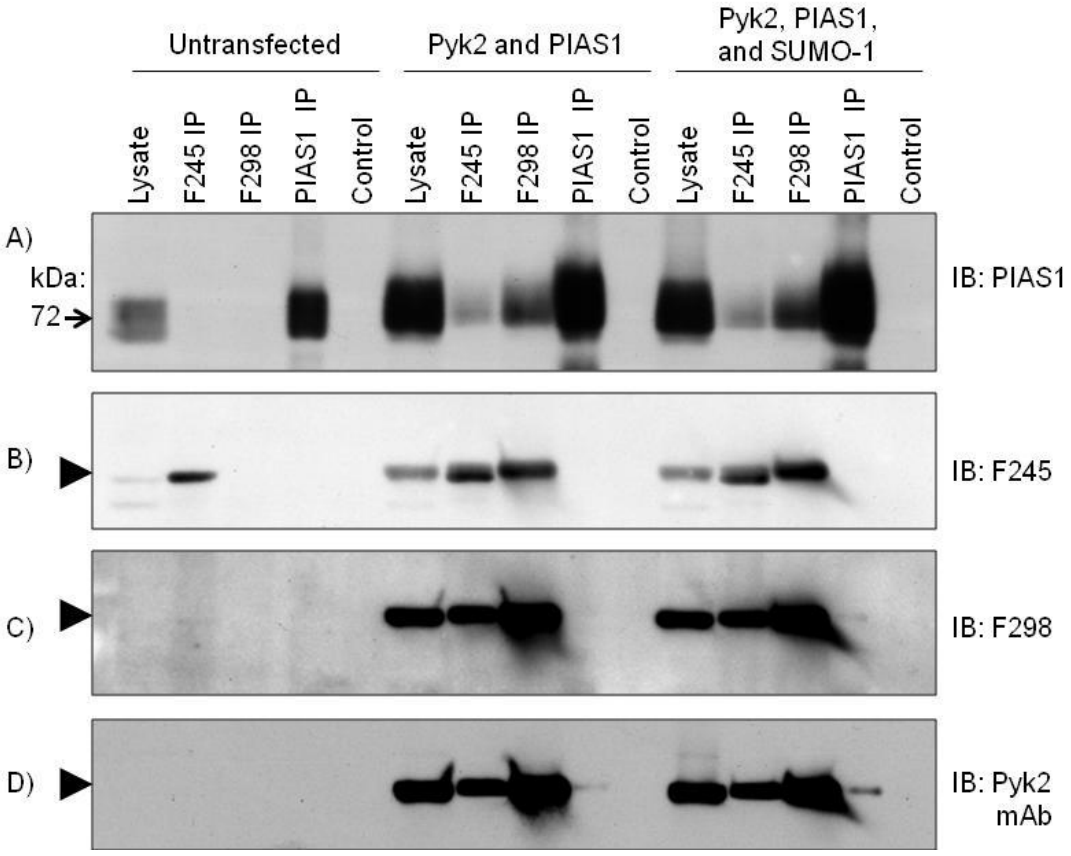


Figure 3.5: PIAS1 co-immunoprecipitates with F298-reactive Pyk2-H. NIH 3T3 cells were transfected with Pyk2H, SUMO-1, and PIAS1 or left untransfected. Lysates were treated with NEM and immunoprecipitated with PIAS1, F245, or F298. Immunpoprecipitates were immunoblotted with PIAS1 (A) and then stripped and reprobed for F298 (B), F245 (C)and Pyk2 mAb (D). Control lanes represent IP controls with appropriate isotype controls. Arrowheads represent the same position on the gel in each immunoblot.

3.6 The F298-reactive Pyk2 is preferentially tyrosine phosphorylated upon co-transfection of SUMO-1, PIAS1 and Pyk2-H

As previously stated in the introduction, FAK has been shown to be SUMOylated, and SUMOylation of FAK leads to an increase of phosphorylation at the site of autophosphorylation Y397 [70]. Since I have demonstrated that the F298-reactive Pyk2-H associates with SUMO-1 in a PIAS1 dose dependent manner, I next wanted to investigate whether the association of SUMO-1 with Pyk2-H would affect the tyrosine phosphorylation of Pyk2, particularly at the autophosphorylation site Y402. To investigate this possibility I co-transfected NIH 3T3 cells with a combination of Pyk2-H, SUMO-1, and PIAS1. Lysates treated with NEM were then immunoprecipitated with F298 of F245 antiserum.

Anti-phosphotyrosine (α-PY72) detected only the F298-reactive Pyk2-H and not the lower MW F245-reactive Pyk2-H (Figure 3.6C). When Pyk2-H and SUMO-1 were co-transfected in NIH 3T3 cells, the level of tyrosine phosphorylation for the F298-reactive Pyk2-H increased (Figure 3.6C). Additionally, co-transfecting PIAS1 with Pyk2-H and SUMO-1 further increased the levels of tyrosine phosphorylation (Figure 3.6C). However, the F298 antiserum also detected higher amounts of Pyk2-H when Pyk2-H, and SUMO-1 or all three constructs were co-transfected into NIH 3T3 cells (Figure 3.6B). Therefore, the increase in the protein expression of the F298-reactive Pyk2-H correlates with an increase in tyrosine phosphorylation.

To determine if SUMO-1 would also increase the level of phosphorylation of Y402 in Pyk2-H, I also immunoblotted using anti-PY402. An increased Y402

phosphorylation signal in both F245 and F298 IPs was detected for Pyk2-H when NIH 3T3 cells were co-transfected with Pyk2-H and SUMO-1, compared to NIH 3T3 cells transfected with Pyk2-H alone (Figure 3.6D). Additionally the levels of phosphorylation for Y402 further increased upon the co-transfecting all three constructs compared to co-transfecting Pyk2-H and SUMO-1 (Figure 3.6D). Thus the increase in Y402 autophosphorylation also correlates with an increase in the protein expression of the F298-reactive Pyk2-H when SUMO-1, Pyk2-H, and PIAS1 are co-transfected in NIH 3T3 cells.

These results together suggest that the F298-reactive Pyk2-H is preferentially phosphorylated when SUMO-1 and PIAS1 are overexpressed. However, it is difficult to determine if SUMO-1 directly increases the tyrosine phosphorylation of F298-reactive Pyk2-H, since SUMO-1 increases the protein expression of Pyk2-H.

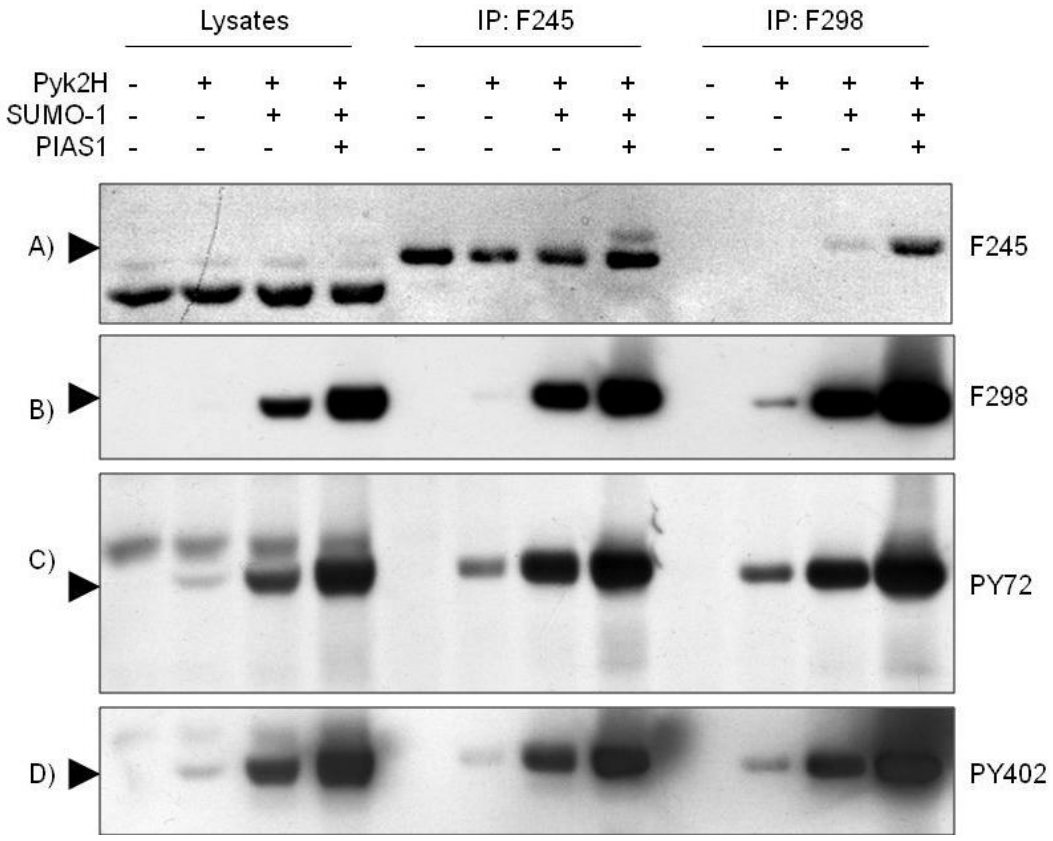


Figure 3.6: Increased tyrosine phosphorylation correlates with an increased protein expression of F298-reactive Pyk2-H. NIH 3T3 cells were co-transfected with Pyk2-H, SUMO-1, and PIAS1. Lysates were treated with NEM and immunoprecipitated with F245 and F298 antiserum. Immunoprecipitates were immunoblotted with F245 (A) and F298 (B) antiserum. Pyk2 tyrosine phosphorylation was detected with anti-PY72 (C) and Pyk2 autophosporylation was detected with PY402 (D). Arrowheads represent the same position on the gel in each immunoblot.

3.7 Tyrosine phosphorylation is not required for the association of Pyk2-H with SUMO-1

As previously discussed in section 1.3.5, SUMOylation may affect the phosphorylation of a target protein. Additionally it has also been found that SUMOylation can depend on the phosphorylation state of the target protein [114, 115]. Since I found that the association of SUMO-1 may influence the tyrosine phosphorylation of Pyk2-H, I next wanted to investigate whether the phosphorylation of Pyk2-H was an important factor for the immunoprecipitation of Pyk2-H with SUMO-1. To test this I decided to use a kinase-inactive form of Pyk2-H called Pyk2-K457A (lysine 457 was changed to alanine). This kinase-inactive form of Pyk2 has previously been shown to have a decreased level of phosphorylation [51]. I co-transfected NIH 3T3 cells with FL-Pyk2-GFP or the kinase-inactive Pyk2-K457A-GFP along with SUMO-1 and PIAS1. Lysates were treated with NEM and then immunoprecipitated with anti-GFP, SUMO-1, or the appropriate isotype control.

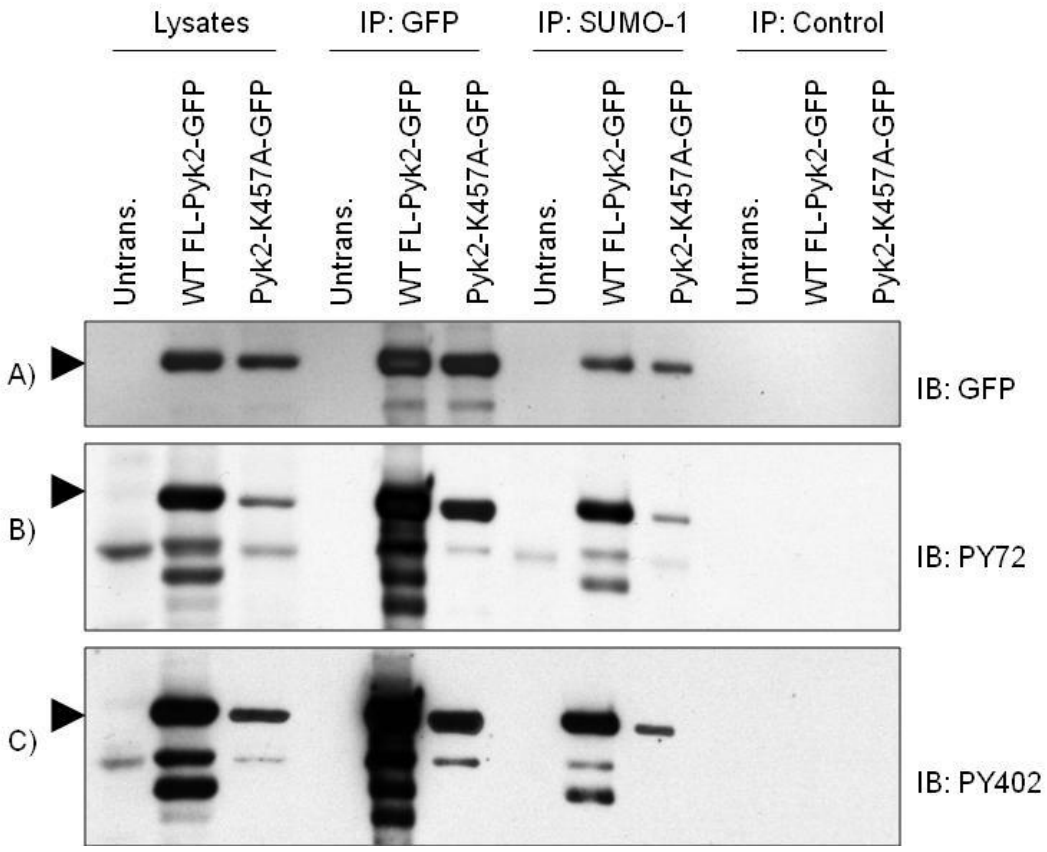
Immunoblotting with anti-GFP revealed that there was a slight decrease in the amount of Pyk2-K457A-GFP compared to FL-Pyk2-H-GFP pulled down by anti-SUMO-1 (Figure 3.7A). Using ImageJ software I quantified the band intensities, which are represented as a ratio of the Pyk2-H in the lysates to Pyk2-H in the SUMO-1 IP of both the FL-Pyk2-H-GFP and Pyk2-K457A-GFP (Figure 3.7D). I found that the Pyk2-K457A-GFP had a slightly less band intensity at 0.542 compared to the FL-Pyk2-H-GFP at 0.615 (Figure 3.7D). I then calculated the percent difference between the band intensities of the FL-Pyk2-H-GFP and

Pyk2-K457A-GFP in figure 3.7A to be 12.6%. The mean of the percent differences of four replicates of this experiment between the band intensities of the FL-Pyk2-H-GFP and Pyk2-K457A-GFP was 11.73% and the standard deviation was 3%. This suggests that there is a decrease in the amount of Pyk2-K457A-GFP associating with SUMO-1 compared with the FL-Pyk2-H-GFP associating with SUMO-1. However, this decrease is quite small and therefore does not appear that tyrosine phosphorylation of Pyk2-H strongly influences the amount of Pyk2-H recovered in the SUMO-1 IP.

However, even though Pyk2-K457A is kinase-inactive it has been previously shown that Pyk2-K457A-GFP can undergo tyrosine phosphorylation at Y402, Y881, and Y580 in Jurkat T cells [51]. To determine to what extent Pyk2-K457A-GFP can be phosphorylated in NIH 3T3 cells I immunoblotted with anti-PY72 (Figure 3.7B). Anti-PY72 detected a low level of tyrosine phosphorylation in the Pyk2-K457A-GFP lysate and in the GFP IP compared to the WT FL-Pyk2-GFP lysate and GFP IP (Figure 3.7B). The Pyk2-K457A-GFP lysate and GFP IP also contained a low level of Y402 phosphorylation compared to the WT FL-Pyk2-GFP lysate and IP GFP (Figure 3.7C). Therefore the level of tyrosine phosphorylation for the Pyk2-K457A-GFP is substantially reduced compared to the WT FL-Pyk2-GFP.

These results together suggest that although Pyk2-K457A associates slightly less with SUMO-1, since the decrease in tyrosine phosphorylation is substantially less then WT Pyk2, it appears that tyrosine phosphorylation is not

required for the association of Pyk2-H with SUMO-1 but may influence the association.



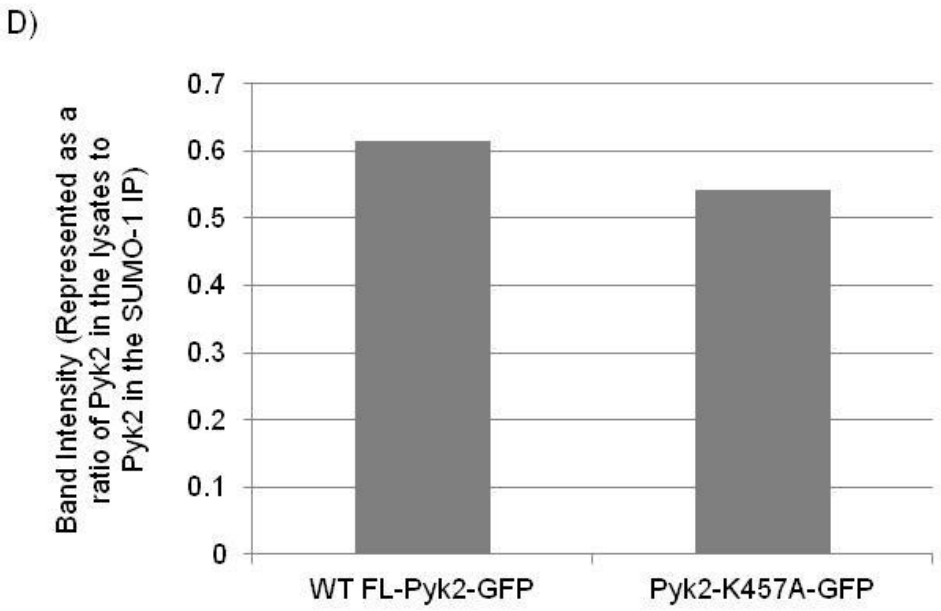


Figure 3.7: The association of F298-reactive Pyk2-H and SUMO-1 decreases as the tyrosine phosphorylation of Pyk2-H decreases. NIH 3T3 cells were transfected with FL-Pyk2-H-GFP or K457A-Pyk2-H-GFP, SUMO-1, and PIAS1. Lysates were treated with NEM and immunoprecipitated with GFP, SUMO-1 or appropriate isotype controles. Pyk2 tyrosine phosphorylation was detected using anti-PY72 and Pyk2 autophosphorylation was detected with PY402. The membrane was first probed with GFP (A) and was then stripped and reprobed for PY72 (B), and PY402 (C). The membrane was blocked in 4% BSA/ECL. Arrowheads represent the same position on the gel in each immunoblot. D) Quantification of band intensity, represented as a ratio of the Pyk2-H in the lysates to the Pyk2-H in the SUMO-1 IP band intensity from (A). Band intensity was calculated using ImageJ Software. The percent difference between the band intensity of WT FL-Pyk2-GFP and Pyk2-K457A-GFP was calculated at 12.6% and is representative of four independent experiments.

3.8 SUMOylation software predicts lysine 646 in Pyk2-H as a SUMOylation site

Since I was unable to detect SUMO-1 covalently attached to Pyk2-H in the SUMO-1 immunoblot (Figure 3.3D), I next sought to determine if Pyk2-H contained a SUMOylation consensus sequence. It has previously been shown that

the majority of proteins are SUMOylated at the consensus site ΨKXE (where Ψ is hydrophobic and X is any residue) [116]. SUMOylation prediction software has become a useful tool for predicting where a SUMOylation consensus site within a target protein may be located [117]. I used two computer programs: SUMOsp 2.0 and SUMOplot (http://www.abgent.com/doc/sumoplot) to predict potential SUMOylation sites in Pyk2 [117] (Table 3.1). SUMOsp 2.0 found that only lysine 646 was at the consensus site ΨKXE and gave this lysine a high probability of being a SUMOylation site (Table 3.1). However, the computer program SUMOplot found that lysine 646 had a low probability of being a SUMOylation site (Table 3.1). However, SUMOsp 2.0 has been shown to make more accurate predictions than SUMOplot [118]. Since Pyk2-H does contain a consensus SUMOylation site, this suggests that Pyk2-H may be directly SUMOylated.

Table 3.1: Prediction of Pyk2 SUMOylation sites. Prediction of SUMOylation sites with the computer programs SUMOsp 2.0 and SUMOplot. Lysines were scored from both computer programs as low (L) Medium (M) or high (H) probability of being a SUMOylation site. The type of consensus site was only defined by SUMOsp 2.0.

Position	Peptide	Score from SUMOsp 2.0	Score from SUMOplot	Type of SUMOylation site by SUMOsp 2.0
646	RLP**K**PEL	H	L	TypeI: Ψ-K-X-E
35	DVE**K**EDV	M	H	TypeII: Non-consensus
145	ESL**K**EDR	M	H	TypeII: Non-consensus

3.9 Mutating lysine 646 of Pyk2-H does not affect the association of Pyk2-H with SUMO-1

In order to test if lysine 646 was the SUMOylation site of Pyk2-H I created a Pyk2-H mutant construct that contained an arginine substitution of lysine 646 (K646R). A lysine to arginine substitution has been shown to abolish SUMO-1 conjugation to target proteins [70]. NIH 3T3 cells were co-transfected with PIAS1, SUMO-1 and wild type (WT) Pyk2-H or Pyk2-H K646R. Lysates were treated with NEM and immunoprecipitated with anti-F245, anti-F298, and anti-SUMO-1.

The K646R Pyk2-H did not appear to decrease the amount of Pyk2-H that was pulled down by anti-SUMO-1 compared to the WT Pyk2-H in both the F245 and F298 immunoblots (Figure 3.8). Furthermore K646R did not appear to affect the detection of the F245 or F298 Pyk2-H populations by F245 or F298 antiserum (Figure 3.8). These results demonstrate that SUMO-1 does not appear to interact with F298-reactive Pyk2-H at lysine 646. This argues that perhaps Pyk2-H interacts with SUMO-1 at another lysine residue or that Pyk2-H may not be directly SUMOylated.

It is also noteworthy to mention that I found that the consensus sequence at lysine 646 was only conserved in mice and rats (Table 3.2). Several SUMOylation sites found in other proteins have been shown to be conserved throughout multiple species [70, 119-121]. This finding supports that lysine 646 is not the SUMOylation site for Pyk2-H and that the putative SUMOylation consensus site may need to be conserved throughout multiple species.

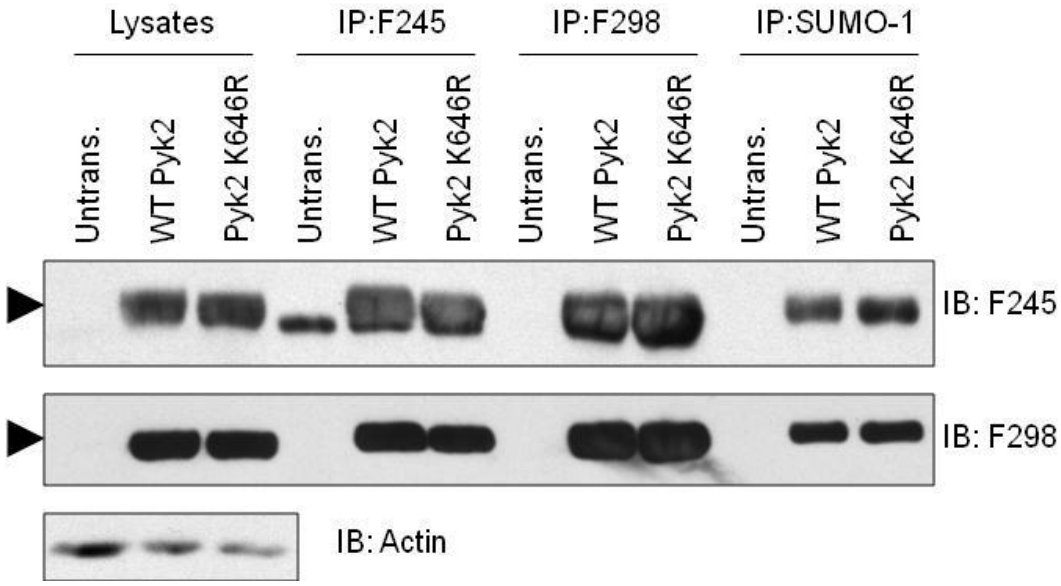


Figure 3.8: Lysine 646 of Pyk2-H is not required for Pyk2-H to complex with SUMO-1 IP. NIH 3T3 cells were transfected with either WT-Pyk2-H or K646R along with HA-SUMO-1 and FLAG-PIAS1. Lysates were treated with NEM and immunoprecipitated with F245, F298 or SUMO-1 and immunoblotted with indicated antibodies. Actin was used as a loading control. The position of the arrowhead indicates the same position on the gel in each immunoblot.

Table 3.2: The SUMOylation consensus site at lysine 646 is not conserved throughout different species. Alignments of the SUMOylation consensus site at lysine 646 of Pyk2 found in humans, cattle, mice, and rats. Conserved amino acids are highlight in grey and lysine 646 is bolded

Homo Sapiens	KDVIGVLEKGDRLP**K**PDLCPPVLYTLMT-60
Bos Taurus	KDVIGVLEKGDRLP**K**PDLCPPILYTLMT-60
Mus Musculus	KDVIGVLEKGDRLP**K**PELCPPVLYTLMT-60
Rattus Norvegicus	KDVIGVLEKGDRLP**K**PELCPPVLYTLMT-60
	ΨKXE

3.10 The Pyk2-H FERM domain associates with SUMO-1

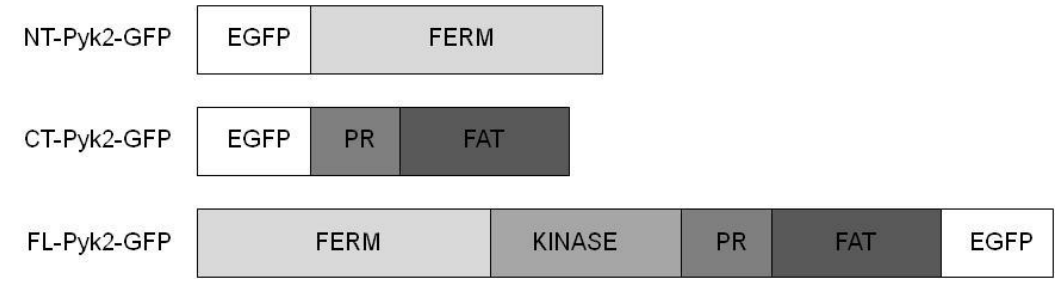
Following the finding that Pyk2-H associates with SUMO-1, but is not SUMOylated at lysine 646, I next sought to identify the domain of Pyk2-H with which SUMO-1 interacts. To determine this I used GFP constructs that contained either the N-terminal FERM domain of Pyk2-H, or the C-terminal domain containing both the PR and FAT region, or full length Pyk2 (Figure 3.9A). I then transfected these GFP-Pyk2 constructs into NIH 3T3 cells with SUMO-1 and PIAS1. The lysates were treated with NEM and immunoprecipitated with anti-GFP, and anti-SUMO-1.

The FL-Pyk2-GFP was pulled down by anti-SUMO-1 as expected (Figure 3.9B). Interestingly however, when I compared the ability of the NT region and the CT region to associate with SUMO-1, I found that only the NT region associated with SUMO-1 (Figure 3.9B). This suggests that SUMO-1 may be interacting with Pyk2-H in the FERM region and not in the PRR or FAT regions of Pyk2-H. This result was interesting because FAK has been shown to be SUMOylated on lysine 152 in the FERM domain [70]. Since Pyk2 and FAK are similar, this result does suggest that the SUMOylation site for Pyk2 may also reside in the FERM domain.

Another important result to mention is the multiple bands detected in the GFP IP in the FL-Pyk2-GFP, NT-Pyk2-GFP, and CT-Pyk2-GFP lanes. Since these bands are not present in the control IP or in the lysates, this suggests that these bands are due to GFP oligomerization. It is known that in high enough concentrations that GFP can dimerize [122]. Therefore, the bands that are

distinctly higher than the GFP-constructs bands could be caused by GFP oligomers.

A)



B)

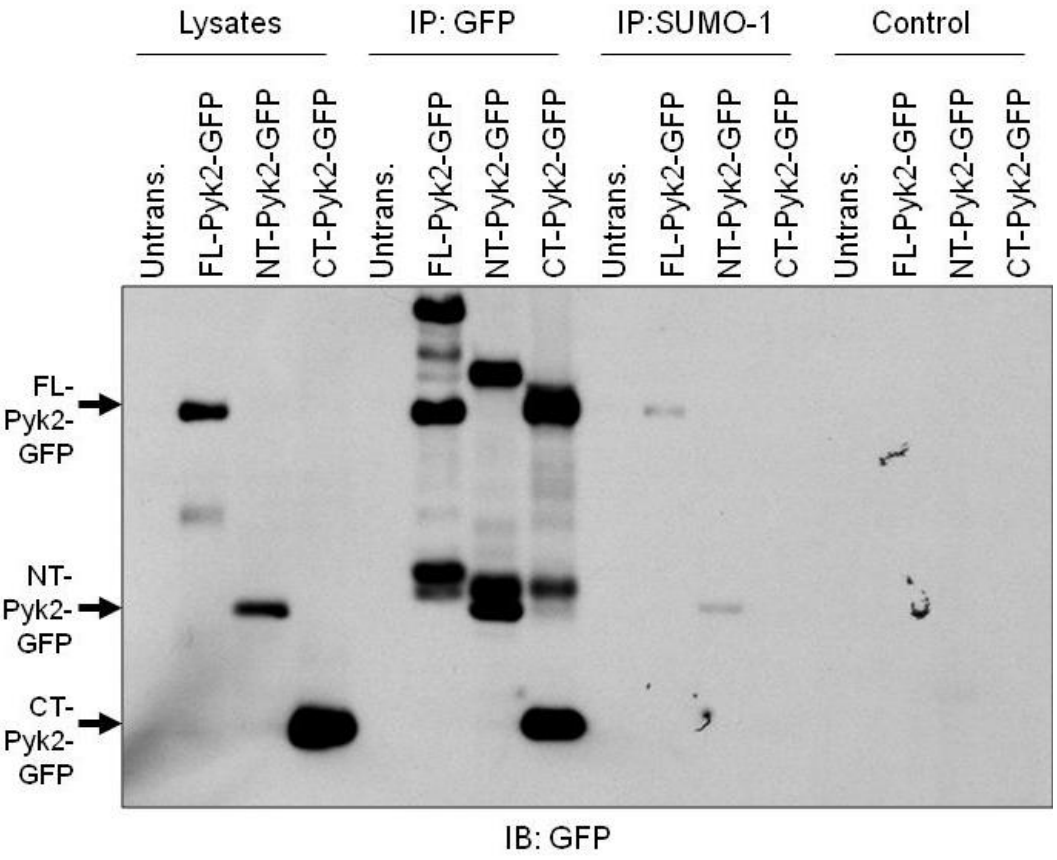


Figure 3.9: Pyk2 associates with SUMO-1 in the N-terminal region of Pyk2. A) Diagram of the domain structure of the Pyk2 GFP constructs. B) NIH 3T3 cells were untransfected or transfected with either FL-Pyk2-GFP, NT-Pyk2-GFP, or CT-Pyk2-GFP, along with SUMO-1 and PIAS1. Immunoprecipitates were probed with anti-GFP or anti-SUMO-1. Control lane represents IP controls using isotype matched Ab and sepharose A beads.

3.11 PIAS1 interacts with the FERM domain of Pyk2-H

Since SUMO-1 was found to associate with the FERM domain of Pyk2-H, I next wanted to investigate whether PIAS1 would also interact with the FERM domain of Pyk2-H. Up to this point it still remained unclear whether Pyk2-H was directly SUMOylated, and therefore it remained possible that PIAS1 and SUMO-1 could associate with completely separate domains of Pyk2-H. To investigate this I again used the Pyk2-H GFP constructs (Figure 3.10A), and transfected these GFP-Pyk2 constructs into NIH 3T3 cells with SUMO-1 and PIAS1. The lysates were treated with NEM and immunoprecipitated with anti-GFP, anti-PIAS1 and appropriate isotype controls.

Immunoblotting for PIAS1 revealed that the FL-Pyk2-GFP and the NT-Pyk2-GFP, but not the CT-Pyk2-GFP recovered PIAS1 (Figure 3.10A). Surprisingly however, there was a lower amount of PIAS1 detected in the lysates and in the PIAS1 IP for NIH 3T3 cells co-transfected with PIAS1, SUMO-1, and the NT-GFP (Figure 3.10A). At first it was thought that PIAS1 could be located in the nucleus, and therefore would not appear in the post nuclear lysates. However, Figure 3.10B demonstrates that there is still remarkably less PIAS1 in the total cell lysates when the NT-Pyk2-GFP is transfected. Therefore, it appears that overexpressing NT-Pyk2-GFP may contribute to a decreased amount of PIAS1 in NIH 3T3 cells. Although there is a lesser amount of PIAS1 when NT-Pyk2-GFP

is transfected, there appears to be a greater amount of PIAS1 pulled down with the NT-Pyk2-GFP than the FL-Pyk2-GFP. This strengthens the conclusion that PIAS1 is able to associate with the FERM domain of Pyk2-H

In the reverse experiment, immunoblotting for GFP reveals that PIAS1 pulls down the FL-GFP and the NT-GFP, but not the CT-GFP (Figure 3.10A). This suggests that PIAS1 is able to co-immunoprecipitate with the FERM domain of Pyk2-H. As is seen in Figure 3.9B there are GFP oligomers present in the GFP IP (Figure 3.10A). Furthermore the band located under the NT-Pyk2-GFP band in the PIAS1 IP is unknown and may be due to protein degradation.

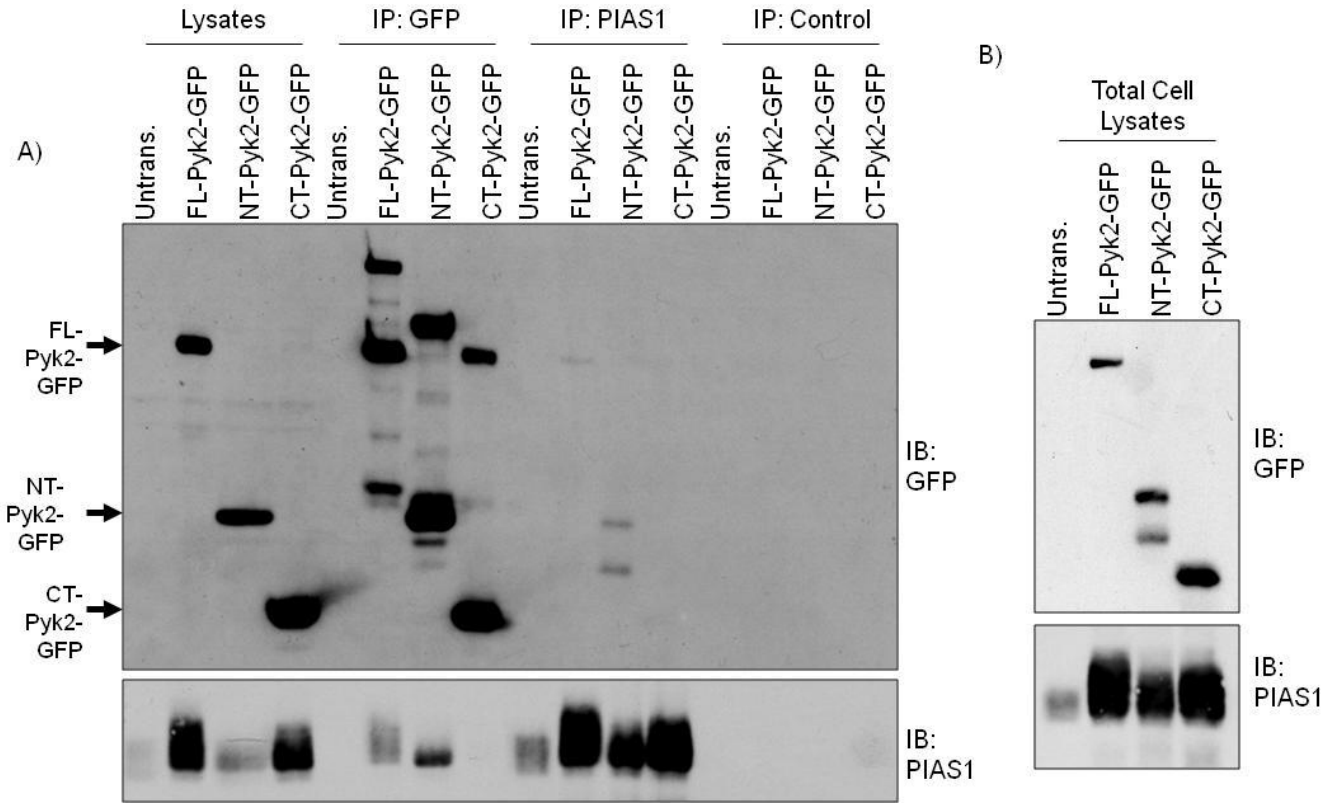


Figure 3.10: PIAS1 associates with the FERM domain of Pyk2-H. A). NIH 3T3 cells were untransfected or transfected with either FL-Pyk2-EGFP, NT-Pyk2-EGFP, or CT-Pyk2-EGFP, along with SUMO-1 and PIAS1. Immunoprecipitates were probed with anti-GFP or anti-PIAS1. Control lanes represents IP controls with appropriate isotype controls. Lysates represent post nuclear lysates. B) Total cell lysates of NIH 3T3 cell co-transfected with PIAS1, SUMO-1, and Pyk2-H. Lysates were immunoblotted with indicated antibodies.

3.12 K145 and K35 are not the exclusive sites of Pyk2-H SUMOylation

With the result that SUMO-1 may interact with Pyk2-H in the FERM domain, I decided to determine whether Pyk2-H contained any SUMOylation consensus sites within the FERM domain. However, the SUMOylation prediction software found no other consensus sites within Pyk2-H besides K646 (Table 2). SUMOylation normally occurs on the motif ΨKXE, however numerous non-consensus modification sites have been reported as described in the introduction section 1.3.3. To investigate the possibility of Pyk2 SUMOylation on a non-consensus site I again used the computer software programs SUMOsp 2.0 and SUMOplot. Since Pyk2 is SUMOylated in the FERM domain, I limited the search to only lysines in the FERM domain. Lysine 35 and lysine 145 were non-consensus sites but were identified to have either high or medium probability of being a Pyk2 SUMOylation site. Both lysine residues are also conserved throughout multiple species (Table 3.3).

To determine if lysine 35 or lysine 145 was the site of Pyk2 SUMOylation, I transfected NIH 3T3 cells with WT Pyk2, K35R and K145R along with HA-SUMO-1 and FLAG-PIAS1. Lysates were treated with NEM and immunoprecipitated with anti-F245, anti-F298, and anti-SUMO-1. The K35R mutation did not appear to affect the detection of the F298-reactive Pyk2-H in both F245 and F298 immunoblots (Figure 3.11). The same can also be said for the K145R mutation when compared to WT Pyk2-H (Figure 3.11). However, the high MW F245-reactive K35R Pyk2-H appears to be at a slightly greater MW than the higher MW WT Pyk2-H (Figure 3.11). This could perhaps be due to a slight

conformational change in Pyk2-H caused by the point mutation. This higher MW shift was also seen in 3 other independent experiments and is not caused by differences in the SDS-PAGE gel.

Although the K35R may have caused a MW shift, the mutant however did not appear to affect the amount of F298-reactive Pyk2-H that was recovered with anti-SUMO-1 in the F245 or F298 immunoblots (Figure 3.11). The K145R mutant also did not affect the amount of the F298-reactive Pyk2-H that was recovered with anti-SUMO-1 in the F245 and F298 immunoblots (Figure 3.11). Therefore, the molecular MW shift of the K35R mutant does not affect the recovery of Pyk2 in the SUMO-1 IP. All these results together show that neither lysine 646, lysine 35, or lysine 145 are exclusive sites of Pyk2 SUMOylation.

Table 3.3: The SUMOylation non-consensus sites at lysine 35 and 145 in Pyk2-H are conserved throughout multiple species. Alignments of the two SUMOylation non-consensus sites at lysine 145 and 35 of Pyk2 found in humans, cattle, mice, and rats. Conserved amino acids are highlight in grey and lysine 35 and 145 are bolded.

Homo sapiens	28-VPVDVE**K**EDVR-40	140-MESL**K**EDRT-150
Bos taurus	28-VPVDVE**K**EDVR-40	140-MESL**K**EDKT-150
Mus musculus	28-VPVDVE**K**EDVR-40	140-MESL**K**EDRT-150
Rattus norvegicus	28-VPVDVE**K**EDVR-40	140-MESL**K**EDRT-150

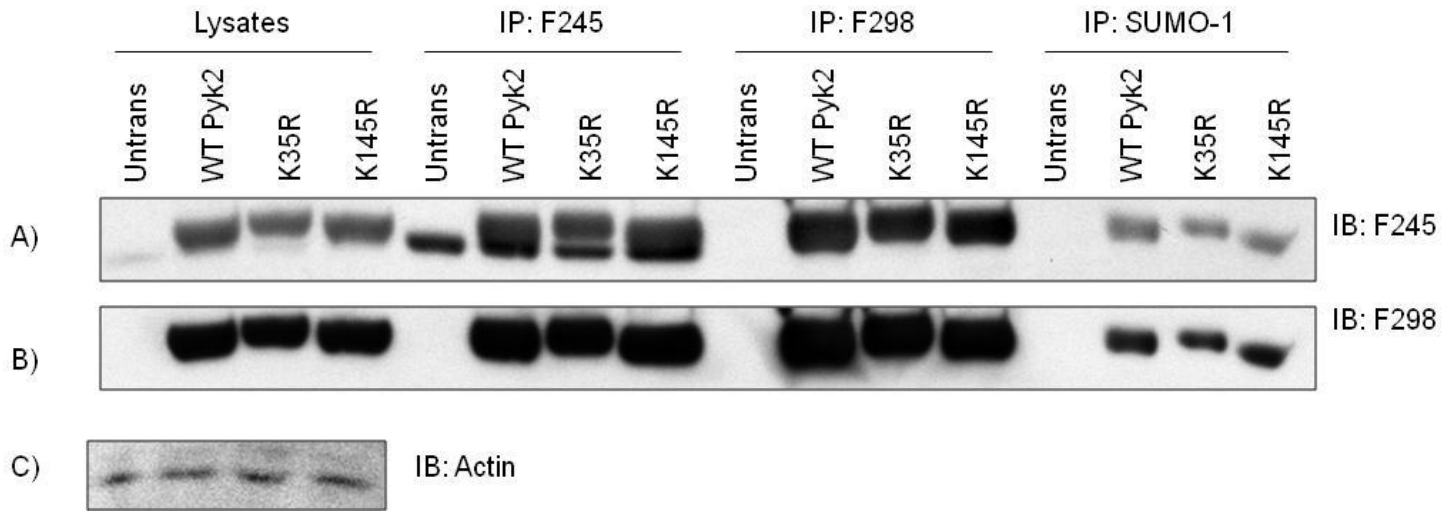


Figure 3.11: K35R and K145R do not affect the complex formation of Pyk2-H with SUMO-1 IP. NIH 3T3 cells were transfected with either WT-Pyk2-H, K145R, or K35R along with SUMO-1 and PIAS1. Lysates were treated with NEM and immunoprecipitated with F245 or F298 and immunoblotted with indicated antibodies.

3.13 The FERM domain of Pyk2-H is not SUMOylated *in vitro*

To determine if Pyk2-H is SUMOylated, I examined whether Pyk2-H could be directly SUMOylated in an *in vitro* assay system. *In vitro* SUMOylation has the advantage of eliminating the presence of SUMO-1 proteases that are normally found within eukaryotic cell lysates. Previous attempts at expressing full length Pyk2-H cloned into the pGEX-4T-3 vector was unsuccessful. The lack of expression could be due to the large size of the full length Pyk2-H vector, or full length-Pyk2-H may be toxic to the BL21 cells. Only the NT domain or CT domain of Pyk2-H cloned into the pGEX-4T-3 vector was successfully expressed. Since the *in vitro* SUMOylation assay requires purified protein, only the NT and the CT domain of Pyk2-H could be tested for SUMOylation. However Figure 3.9 demonstrated that the FERM domain of Pyk2-H associated with SUMO-1. Therefore, there is the possibility that the FERM domain alone could be SUMOylated. The CT domain of Pyk2-H could also be used as a negative control since it was shown not to associate with SUMO-1 (Figure 3.9).

NT -Pyk2-GST and CT-Pyk2-GST were incubated in the presence of SUMO-1-His, E1 activating enzyme SAE1/SAE2, E2 conjugating enzyme Ubc9, and ATP. Since PIAS1 was shown to enhance Pyk2 SUMOylation *in vivo* (Figure 3.12), I also tested whether PIAS1 could facilitate Pyk2 *in vitro* SUMOylation. As shown in Figure 3.12B, no immunoreactive band corresponding to the NT- Pyk2 or the CT-Pyk2 was detected by anti-SUMO-1 when ATP or ATP and PIAS1 was added to the reaction (Figure 3.12A). Furthermore, the immunoblot for GST demonstrates that there was no significant shift in the MW of the NT-Pyk2 or CT-

Pyk2 when ATP or ATP and PIAS1 was added to the reaction (Figure 3.12B). Also noteworthy is that the many bands detected below the NT-Pyk2-H or CT-Pyk2-H in the GST immunoblot are likely due to protein degradation since these bands are present in the samples with or without ATP.

RanGAP1-GST was used as a positive control since RANGAP1 is known to be SUMOylated by SUMO-1, 2, and 3 (Figure 3.12) [80]. As seen in Figure 3.12B, anti-GST detected a MW shift in RanGAP1, which would correspond to a RanGAP1-SUMO-1 conjugate when ATP or ATP and PIAS1 was added. Furthermore, anti-SUMO-1 detected the higher MW RanGAP1, confirming that the higher MW RanGAP1 is due to the covalent attachement of SUMO-1. (Figure 3.12A)

These results suggest that NT-Pyk2 is not SUMOylated *in vitro* with only SAE1/SAE2, Ubc9, SUMO-1, ATP, and PIAS1. This suggests that there may be more SUMOylation machinery required to SUMOylate Pyk2 *in vitro*, or full length Pyk2 is required for *in vitro* SUMOylation due to possible conformational constraints.

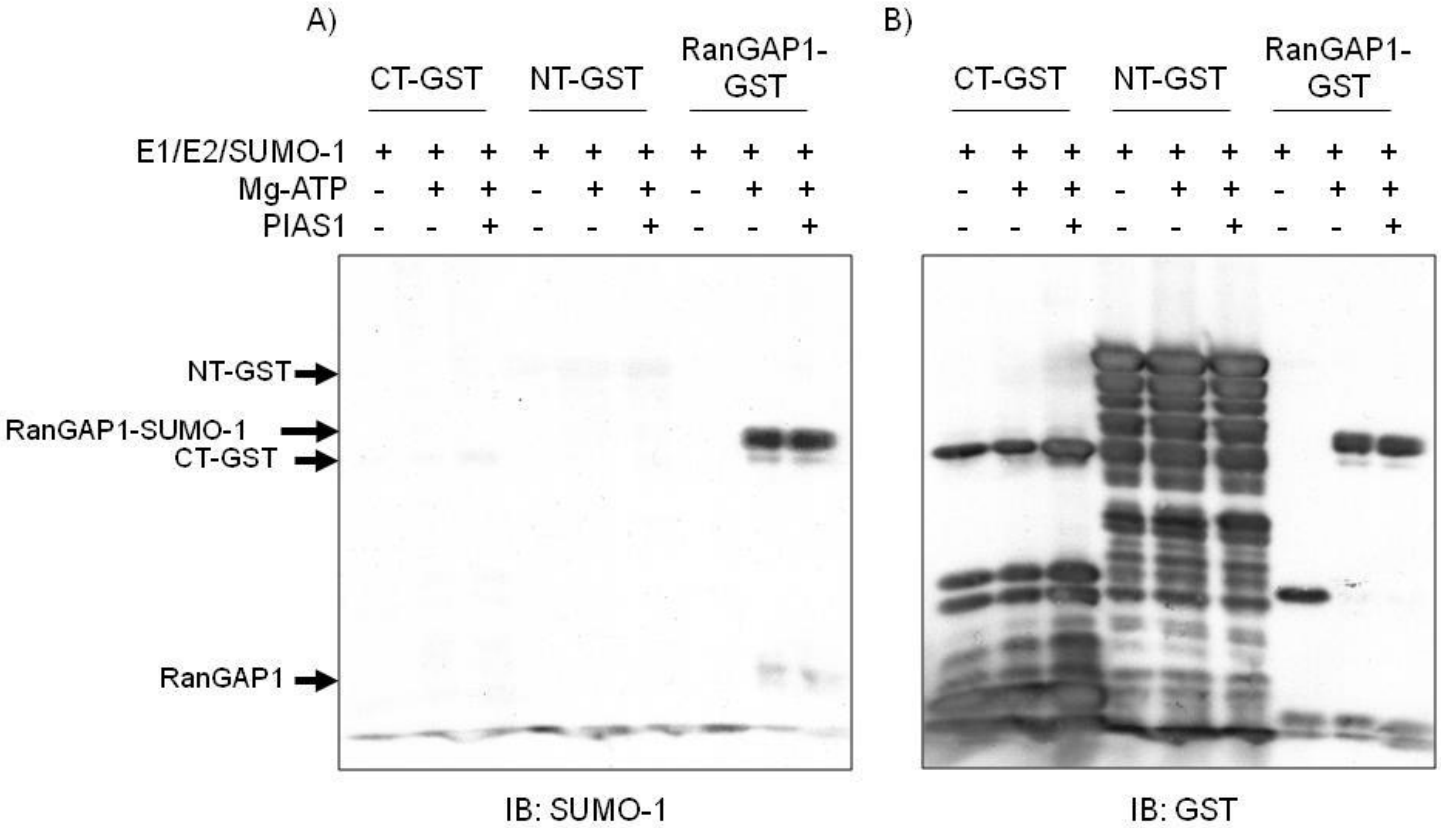


Figure 3.12 SUMO-1 does not modify the FERM domain of Pyk2-H through *in vitro* SUMOylation. *in vitro* SUMOylation of Pyk2 was carried out using the Enzo Life Science SUMOylation kit. The FERM domain of Pyk2 (NT-GST) and the CT-terminal domain of Pyk2 (CT-GST) were used as a substrate for SUMO-1. RanGAP1 was used as a positive control for experimental integrity. times 1µl of 20X PIAS1 an E3 ligase was added. Samples were incubated for 2 hours at 37°C and the reaction was stopped by adding 20µl of 2X reducing sample buffer.

3.14 Pyk2-H does not appear to be directly SUMOylated as assessed by mass spectrometry

Mass spectrometry has recently emerged to be a powerful tool in identifying and mapping sites of posttranslational modifications [123]. Identifying a SUMOylated protein by mass spectrometry can be quite difficult however. For instance digestion of a SUMOylated protein with trypsin creates a long amino acid sequence (-EQIGG) that remains on the modified lysine [124]. This is unlike ubiquitination of a protein where trypsin digestion results in a simple diglycine tag [124]. The long amino acid tag on the modified lysine results in a mass shift of +484 Da, which can be used to identify SUMOylated proteins [125, 126] [reviewed in [124]]. However, the large SUMO-conjugated peptides generated from the trypsin digestion impede identification by most database searches [123].

In order to avoid this problem I created a threonine – arginine substitution (T95R) on the CT side of SUMO-1. The arginine can be cleaved by trypsin and leaves a signature diglycine tag (114Da) on a modified peptide. This tag can be more easily identified by database searches and is the same tag that is used to identify ubiquitinated proteins. This mutation has also been shown not to affect SUMO-1 conjugation [91].

Usually, samples sent for mass spectrometry analysis have been *in vitro* SUMOylated, but Pyk2 SUMOylation *in vitro* was unsuccessful. This meant that I needed to perform Pyk2 SUMOylation *in vivo*, which can be problematic due to the low amount of Pyk2-SUMO-1 conjugates formed. To create samples for mass spectrometry analysis I transfected NIH 3T3 cells with Pyk2-H, SUMO-1 or

SUMO-1(T95R), and PIAS1. The lysates were treated with NEM and immunoprecipitated with F245 or F298, or with the proper isotype control. The immunoprecipitates were run on an SDS-PAGE and stained with silver stain. Using the lower MW F245-specific Pyk2-H band as comparison, the band corresponding to the F298-specific Pyk2-H was cut from the silver stain gel and sent for mass spec analysis (Figure 13). Figure 3.13 also demonstrates that the T95R mutation in SUMO-1 does not affect the appearance of the F245-reactive Pyk2-H or the F298-reactive Pyk2-H.

The mass spectrometry data was analysed through the NCBInr database which matched multiple peptides to the Pyk2-H sequence however, no SUMO-1 peptides were identified (Table 3.4). This experiment was also repeated two more times with similar results. The list of all proteins matched in the NCBInr database from the Pyk2-H samples from the third attempt is listed in Table 3.4. Furthermore, another database created to only contain the amino acid sequences of both SUMO-1 and Pyk2-H did not identify any SUMO-1 peptides.

Even though no SUMO-1 peptides were found by Mass Spectrometry I continued to analyze the mass spectrometry results to determine if I could locate a SUMOylation site within Pyk2-H. To do this I added ubiquitination (GG) as a variable modification into the MASCOT MS/MS Ion Search and used both SwissPro and NCBInr database to search for Pyk2 peptides. None of these databases was able to identify any Pyk2-H peptides with an added diglycine tag. Next I used the software program ChopNSpice to make a new database containing all potentially modified Pyk2 peptides by the SUMO-1 mutant (T95R). This new

database was then used to search the mass spec data, however again no modified Pyk2-H peptides were found. I also examined all Pyk2 peptides that contained a lysine misscleavage for a 114.1-Da increase in mass, which would be caused by the covalent attachment of the two glycine residues to a lysine residue. No peptides contained an increase in mass of 114.1-Da. Furthermore, lysine residues 35, 145, and 646 were confirmed not to be sites of Pyk2-H SUMOylation. These results together suggest that the higher MW Pyk2-H population may not be SUMOylated. However, the approach of using the T95R is challenging due to poor detection sensitivity and lack of specific purification techniques for the peptides containing the SUMOylation sites [91]. Therefore, Pyk2-H may be directly SUMOylated, but remains undetectable by mass spectrometry using these methods.

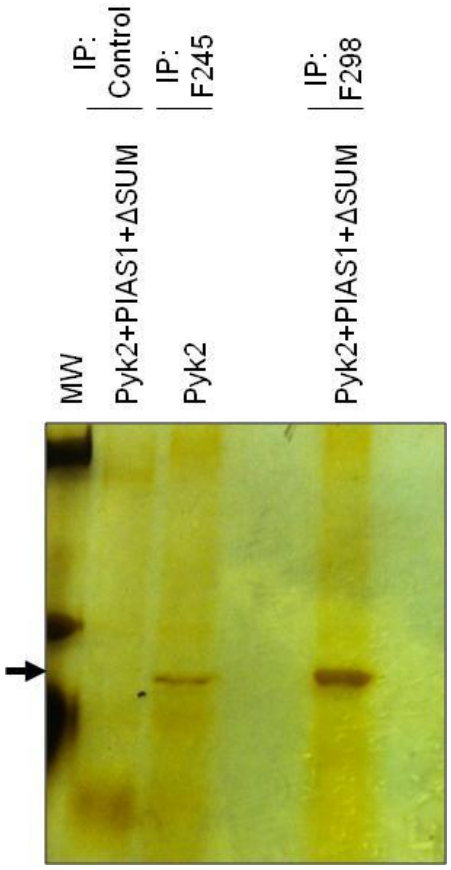


Figure 3.13: The threonine to arginine substitution in SUMO-1 (T95R) does not affect the F245 or F298 population. NIH 3T3 cells were transfected with Pyk2-H, PIAS1 and SUMO-1-T(95)S. Lysates treated with 40mM NEM were immunoprecipitated with F298, F245, or an appropriate isotype control were run on an SDS-PAGE. Bands were visualized with silver stain. The position of the arrowhead indicates the F298-reactive Pyk2-H. MW is the molecular weight marker.

Table 3.4: Representative data of the list of the protein family hits from the mass spectrometry analysis software MASCOT 2.0. Peptides were searched on the NCBInr database. The protein score is the sum of the highest ions score for each distinct sequence. For matches the first number is the total count while the number in parentheses is the count for matches above the significance threshold.

NCBInr #	Score	Mass	Matches	Sequence Coverage	Protein Name
gi|241982783	593	122286	57 (23)	51%	*Protein-tyrosine kinase 2-beta isoform 3 [Mus musculus]
gi|189054178	223	66151	11(5)	18%	** Unnamed protein product [Homo sapiens]
gi|181402	169	66110	3 (2)	8%	Epidermal cytokeratin 2 [Homo sapiens]
gi|435476	139	62320	4 (3)	9%	Cytokeratin 9 [Homo sapiens]
gi|2136983	101	20734	4 (1)	26%	Ig gamma heavy chain constant region- rabbit (fragment)
gi|1100745	76	25579	2 (2)	18%	Immunoglobulin kappa chain [Oryctolagus cuniculus]
gi|387400	83	23691	1 (1)	6%	Epidermal keratin type I [Mus musculus]
gi|223961	66	31323	1 (1)	5%	Complement C4d
gi|780372	21	48299	1 (1)	4%	Enolase [Oryza sativa Japonica Group]

* Protein matching the same set of peptides as protein tyrosine kinase 2-beta isoform 3 is proline rich tyrosine kinase 2 isoform 2 [Mus musculus]

**Protein matching the same set of peptides as Unnamed protein product is keratin 1 [Homo sapiens].

CHAPTER 4: DISCUSSION AND FUTURE DIRECTIONS

4.1 Thesis Summary

Previously J. St. Pierre *et al.* used two polyclonal antibodies, which recognize the N-terminal and C-terminal portion of Pyk2 (F245 and F298 respectively) to find that there exists two distinct Pyk2-H populations in macrophages. In this study, I investigated whether the differences in the MWs seen between the Pyk2-H populations was caused by the SUMOylation of Pyk2-H.

The data presented in this thesis supports that endogenous and exogenous F298-reactive Pyk2-H can be recovered in SUMO-1 immunoprecipitates. This association may depend on the level of tyrosine phosphorylation of Pyk2-H, and may influence the autophosphorylation of Pyk2-H. However, it remains unclear as to whether the association between Pyk2-H and SUMO-1 is direct. Additionally my results have shown that PIAS1 co-IPs with Pyk2-H. Furthermore, SUMO-1 and PIAS1 associate with the FERM domain of Pyk2-H. PIAS1 appears to increase the association of exogenous F298-reactive Pyk2-H with SUMO-1. The overexpression of PIAS1 and SUMO-1 in NIH 3T3 cells also appears to increase the protein expression of the F298-reactive Pyk2-H. In all, these results suggest that the association of Pyk2-H with SUMO-1 may influence the regulation of Pyk2-H in macrophages.

4.2 Pyk2-H associates with SUMO-1 *in vivo*

Using RAW 264.7 macrophages, I, as well as J. St. Pierre, found that endogenous Pyk2-H can associate with SUMO-1 in the presence of NEM. This result is quite novel since even when using NEM, which can prevent the de-conjugation of SUMO-1 to target proteins, there is normally a low abundance of total substrate that is detectably associating with SUMO-1 at any given time in a cell [127]. Furthermore, I specifically found that it was only the F298 Pyk2-H population that associated with SUMO-1 in RAW 264.7 macrophages (Figure 3.1A). This further substantiates the claim that the F245 and F298 Pyk2-H populations are distinct and may be regulated in different fashions.

However, I was unable to determine if the endogenous F298-reactive Pyk2-H could be directly SUMOylated, since I was unable to detect Pyk2-H with anti-SUMO-1 (Figure 3.1C). It has been previously been demonstrated that many commercial antibodies available for detecting SUMOylated proteins have limitations, mainly regarding specificity and designing proper negative and positive controls [128]. Therefore, due to these limitations and also to the low amounts of endogenous SUMOylated proteins within cells, it is possible that the SUMO-1 Abs that I used were not sufficient to immunoblot SUMOylated Pyk2-H.

Another possibility for being unable to detect Pyk2-H with anti-SUMO-1, is that Pyk2-H is not directly SUMOylated but rather complexes with another protein that is SUMOylated. J. St-Pierre demonstrated that the protein Dynamin directly interacts with Pyk2-H in RAW 264.7 macrophages [106]. Dynamin has

been previously shown to directly interact with SUMO-1, PIAS1, and Ubc9 [107]. In my results I also found that Pyk2-H specifically associates with SUMO-1 (Figure 3.2). Therefore, it could be possible that the endogenous F298-reactive Pyk2-H associates with SUMO-1 through the protein Dynamin, or another protein that directly interacts with SUMO-1.

4.3 Pyk2-H may be a target of PIAS1

As FAK has been shown to bind to the E3 SUMO ligase PIAS1, I investigated whether PIAS1 could increase the association between SUMO-1 and Pyk2-H [70]. Co-transfection of NIH 3T3 cells with Pyk2-H, SUMO-1, and PIAS1 demonstrated that PIAS1 co-IPs with the F298-reactive Pyk2-H (Figure 3.5). In addition, the expression of PIAS1 consistently enhanced the association of Pyk2-H and SUMO-1 (Figure 3.3 and 3.4). Therefore it appears that PIAS1 associates with Pyk2-H.

As previously stated in the introduction there are many E3 ligases and PIAS1 is a part of the PIAS family of E3 ligases. In humans there are five PIAS family members: PIAS1, PIAS3, PIASxα, PIASxβ, and PIASγ [72]. Therefore it would be interesting to determine if Pyk2-H interacts selectively with PIAS1 or if Pyk2-H can associate with the other members of the PIAS family.

Interestingly, when only the FERM domain of Pyk2-H was expressed, there was a decrease in the amount of PIAS1 (Figure 3.10). It is possible that the expression of the FERM domain of Pyk2-H may influence the protein stability of PIAS1 either through regulating translation or degradation of PIAS1. Therefore, future investigations should focus on the relationship between Pyk2-H and PIAS1.

The finding that PIAS1 interacts and enhances the association of Pyk2-H with SUMO-1 adds to the possibility that PIAS1 may be responsible for regulating the nuclear localization of Pyk2-H [47]. PIAS proteins have been shown to target their substrates to subnuclear structures [129]. Furthermore, SUMOylated FAK was found to be enriched in the nucleus, whereas exogenous FAK FERM domain expression is nuclear-localized [130, 131]. Interestingly however, FAK SUMOylation was found to be non-essential for nuclear translocation, but p53 regulation was dependent on FAK nuclear translocation [35]. Thus, the role of SUMOylation on nuclear FAK remains unclear. Since, like FAK, Pyk2-H is reported to function in the nucleus and contains an NLS and an NES in the FERM domain and another NES in the kinase domain, it would be interesting to determine if the association between Pyk2-H and PIAS1 allows for the nuclear translocation of the Pyk2-H to the nucleus [33, 47]. Therefore, future investigations should focus on the importance of the association of Pyk2-H and PIAS1 on Pyk2-H nuclear localization.

4.4 Pyk2-H and SUMO-1 may associate in a SUMOylation-independent manner

Even though I overexpressed Pyk2-H, SUMO-1, and PIAS1 in NIH 3T3 cells, I was unable to detect a band corresponding to a Pyk2-H-SUMO-1 conjugate with anti-SUMO-1 (Figure 3.3D). Again, the difficulty of possibly detecting SUMOylated Pyk2-H though western blotting may have been caused by the limitations of the SUMO-1 Abs as explained in section 4.1.

Without being able to identify a Pyk2-H SUMO-1 conjugate via western blot, I attempted to identify a site where SUMO-1 may conjugate to Pyk2-H. I used the prediction software SUMOsp and SUMOplot to find one potential SUMOylation motif (ψKX(D/E)) in Pyk2-H at lysine 646 (Table 3.1). Although I found that this site was not conserved throughout multiple species (Table 3.2), the majority of SUMOylated proteins are modified at this consensus motif and warranted investigation [132]. However, upon transfecting the Pyk2-H K646R mutant into NIH 3T3 cells with SUMO-1 and PIAS1, there was no decrease in the association of Pyk2-H and SUMO-1 detected. This suggested that lysine 646 of Pyk2-H is not the site of SUMO-1 conjugation. However it is also possible that Pyk2-H could be SUMOylated at multiple locations, or that the over-expression of SUMO-1 may force Pyk2-H to be SUMOylated at other lysine residues. It is also possible that another lysine residue located beside lysine 646 could be SUMOylated when lysine 646 is mutated.

Even with these limitations I investigated two other lysine residues, lysine 35 and lysine 145, that although were located in a non-consensus site, they did contain a high predictive SUMOylation score and were conserved throughout multiple species (Table 3.1 and 3.3 respectively). However, mutating these lysine residues did not affect the association between Pyk2-H and SUMO-1 (Figure 3.11). Again, these lysine residues may not be where SUMO-1 conjugates or may be subject to the same limitations listed above for lysine 646. Therefore, using point mutations to identify a SUMOylation site for Pyk2-H may not be the best approach. Since I was able to determine that SUMO-1 associates with the FERM

domain of Pyk2-H, future studies should focus on constructing deletion mutants of the FERM domain to narrow down the region in the FERM domain where SUMO-1 associates.

Since overexpressing SUMO-1 and using point mutants to identify if Pyk2-H is SUMOylated failed, I then attempted to use an *in vitro* approach. Since SUMOylation is a reversible process, *in vitro* SUMOylation has the advantage of examining the modification in the absence of SUMO proteases that prevent the deconjugation of SUMO. However, there was no increased MW of the Pyk2-H FERM domain band or no immunoreactive FERM domain bands detected by anti-SUMO-1 when ATP was added to the assay (Figure 3.12). SUMOylation of the Pyk2-H FERM domain was also not detected even after adding PIAS1, which was shown to increase the association of Pyk2-H and SUMO-1. Therefore it is possible that the FERM domain of Pyk2-H may not be modified by SUMO-1 *in vitro*. However, there are limitations to using the *in vitro* assay. For instance, I was not able to use full length Pyk2-H in the assay due to a lack of expression of full length Pyk2-H in BL21 cells. It is possible that SUMO-1 may only conjugate to Pyk2-H when Pyk2-H is in a specific conformation. Therefore, by removing the other domains of Pyk2-H, it may not be possible for SUMO-1 to efficiently conjugate to the FERM domain alone. I also found that the association of SUMO-1 with Pyk2-H may be facilitated by tyrosine phosphorylation (Figure 3.7). Since no kinases are available to phosphorylate Pyk2-H *in vitro*, it may not be possible for SUMO-1 to conjugate to Pyk2-H *in vitro*. It is also possible that Pyk2-H

requires other SUMOylation machinery present in a cell to be SUMOylated that is lacking in the *in vitro* assay.

Together, all of these results suggested that determining if Pyk2 is directly SUMOylated may be more complex than first anticipated. It is possible that Pyk2-H contains more than one consensus site for SUMOylation, since many other proteins have now been shown to contain more SUMOylation sites than first thought [133-135]. It has also been recently shown that SUMOylation motifs may be more difficult to find due to complex site specificity [136]. For instance a non-consensus lysine may exist within an α helix, however if the secondary structure of the protein is disrupted, SUMOylation may be directed to a neighboring consensus motif [136, 137]. Another possibility may involve E3 ligases. Multiple proteins have been shown to be modified by SUMO at nonconsensus sequences, and E3 ligases may be responsible for this modification [96, 138]. For instance, the E3 ligase Siz1 is required for the SUMOylation of PCNA at a nonconsensus site at lysine 164 [139].

To avoid the labor-intensive mutational analysis to find SUMOylation sites and to determine if a protein is multiply SUMOylated and/or SUMO conjugated in a nonconsensus region, one can use Mass Spectrometry [140]. Therefore, I attempted to use Mass Spectrometry to determine if Pyk2-H is SUMOylated and if Pyk2-H is SUMOylated, then determine the SUMOylation site or sites of Pyk2-H. I attempted to enhance my chances of finding whether Pyk2-H was directly SUMOylated using Mass Spectrometry by using a modified SUMO-1 in which the terminal –TGG was mutated to the ubiquitin sequence –

RGG [141]. This was done to allow for tryptic digestion of the SUMO conjugate to yield a signature diglycine remnant that would be attached to a target lysine and would allow for the rapid identification of the Pyk2-H SUMOylation site by Mass Spectrometry [141]. However, even with this modification and using a computational program called ChopNSpice, I did not find any SUMO-1 peptides or the SUMOylation site for Pyk2-H. For many SUMO substrates it appears that only a small proportion of the cellular pool is modified by SUMO [142]. Therefore, using *in vivo* SUMOylated Pyk2 may have made the detection of the SUMOylation site by Mass Spectrometry technically very challenging.

Together, these results suggest that the MW shift of Pyk2-H may occur in a SUMOylation-independent manner. Thus, it remains possible that Pyk2-H may be associating with another protein that is directly SUMOylated. For instance, PIAS1 has been demonstrated to be SUMOylated in COS-1 cells [143]. Since I have shown that Pyk2-H can complex with PIAS1 in NIH 3T3 cells, it is possible that Pyk2-H associates with SUMO-1 through PIAS1. Furthermore, I have found that the Pyk2-H FERM domain contains a SUMO binding Motif (SBM) using prediction software GPS-SBM 1.0 (Figure 4.1) [144]. This motif may allow for Pyk2-H to non-covalently associate with SUMO-1 [71, 93, 94]. Therefore, future investigations should determine if this SBM allows for the non-covalent association of SUMO-1 with Pyk2-H.

Table 4.1: Prediction of a SUMO binding motif (SBM) in the FERM domain of Pyk2-H. Results from the SBM prediction software GPS-SBM 1.0. Residues in bold represent the SBM in Pyk2-H. The threshold or cutoff selected was medium.

Position	Peptide	Score	Cutoff
38-41	VEKED **VRIL** KVCFY	4.739	4.3

4.5 Pyk2-H may be directly SUMOylated

With all limitations listed in the previous section it is impossible to conclude that Pyk2-H is not directly SUMOylated. Furthermore, I have shown that Pyk2-H can undergo a molecular weight shift in both RAW 264.7 and NIH 3T3 cells, as detected by the F245 and F298 Abs, which would be consistent with a SUMO-1 Pyk2-H conjugate. Overexpressing Pyk2-H and SUMO-1 in NIH 3T3 cells also increases the association of the F298-reactive Pyk2-H with SUMO-1. In addition, the overexpression of SUMO-1, PIAS1 and Pyk2-H in NIH 3T3 cells increases the protein expression of the F298 Pyk2-H population. Therefore, taking into account all the limitations previously mentioned and these results, it remains possible that Pyk2-H is directly SUMOylated with SUMO-1.

4.6 SUMO-1 and PIAS1 overexpression increases Pyk2-H protein expression

I found that co-transfecting NIH 3T3 cells with SUMO-1, Pyk2-H, and PIAS1 increased the amount of F298 Pyk2-H population (Figure 3.3 and Figure 3.4). It has previously been shown that SUMO modification or the overexpression of SUMO-1 can regulate the protein stability of a substrate protein [145, 146]. Therefore, I propose that the overexpression of SUMO-1 increases Pyk2-H

protein stability. The method in which SUMO-1 can affect the protein stability of Pyk2-H remains unclear.

One method where SUMOylation may increase the protein stability is by competing for the same consensus lysine residue on a substrate protein as ubiquitin [147]. This has previously been shown for the protein IκBα, where IκBα is both SUMOylated and ubiquitinated on the same lysine residue [147]. SUMO thus prevents ubiquitin from binding and prevents IκBα from undergoing proteasomal degradation [147]. Thus it is possible that SUMO-1 acts through the inhibition of ubiquitination to stabilize Pyk2-H.

It is also possible that SUMO-1 and PIAS1 may increase the expression of Pyk2-H at a transcriptional level. These methods should be further investigated as well as a functional role for increased Pyk2-H expression in a cell.

4.7 Increased Pyk2 protein expression correlates with increased tyrosine phosphorylation

There are many SUMOylation functions including subcellular localization, nuclear transport, and signal transduction [95, 97]. Yet, having neither determined whether Pyk2-H is directly SUMOylated or the SUMOylation site for Pyk2-H, finding a function for the association of SUMO-1 with Pyk2-H became difficult. If in future studies the SUMOylation site for Pyk2-H is found, it would be interesting to use mutated Pyk2-H SUMOylation site(s) to study the role of SUMOylated Pyk2 in macrophage adhesion and migration along with the cellular location of SUMOylated and non-SUMOylated Pyk2.

However, in this study I was able to investigate the role of SUMO-1 and Pyk2-H association on phosphorylation. SUMOylated FAK was shown to have increased autophosphosphorylation, and so I examined whether co-transfecting Pyk2-H and SUMO-1 into NIH 3T3 cells would affect the levels of phosphorylated Pyk2-H [70]. I found that the F298-reactive Pyk2-H is preferentially phosphorylated when SUMO-1 and PIAS1 are overexpressed (Figure 3.6). However, the increase in tyrosine phosphorylation correlated with an increase in the amount of Pyk2-H. Therefore it is difficult to say that SUMO-1 caused an increase in tyrosine phosphorylation, due to the increase in the amount of the F298-reactive Pyk2-H.

I also examined whether phosphorylation of Pyk2-H could affect the association of SUMO-1 with Pyk2-H. Although the kinase dead Pyk2-H (K457A) had a significantly lower level of tyrosine phosphorylation than WT Pyk2-H, there was only a slight decrease in the amount Pyk2-H-K457A recovered in the SUMO-1 IP. This suggests that the association of SUMO-1 with Pyk2-H does not depend on tyrosine phosphorylation, although tyrosine phosphorylation may influence the their association. Since it was demonstrated that inhibiting the SUMOylation of FAK using ginkgolic acid decreased the phosphorylation of Y397 of FAK, future investigation should examine the affects of ginkgolic acid on the phosphorylation of Pyk2-H and the association of SUMO-1 [100].

In vitro kinase assays with SUMOylated FAK have shown that the increase in phosphorylation is due to the intrinsic ability of FAK to autophosphorylate, potentially by inducing an “open” conformation of FAK [70].

This raises the question as to whether SUMO can also induce an "open" conformation of the Pyk2 molecule as well. In the paper by Joelle St-Pierre *et al.* they proposed that Pyk2 was in an autoinhibited conformation and the F298 epitope is not accessible and cannot be immunoprecipitated by the F298 antiserum, but can be bound by the F245 antiserum and by the protein paxillin. Upon stimulation a fraction of the Pyk2 molecules become tyrosine phosphorylated which allows for an "open" conformation where the F298 antibody is able to bind, while preserving F245 and paxillin binding.

I propose that upon stimulation a portion of Pyk2-H molecules complex with SUMOylated proteins (or becomes directly SUMOylated), which aids Pyk2-H in becoming further phosphorylated and activated and allows Pyk2-H to adopt an "open" conformation (Figure 4.1). The phosphorylation of Pyk2-H or the association with SUMOylated proteins may also prevent ubiquitin from binding to Pyk2-H, which would increase the protein expression of Pyk2-H.The increased amount of Pyk2-H would also correlate to the increased amount of tyrosine phosphorylated Pyk2-H. The open conformation also allows for both F298 and F245 binding and this would explain why both the F298 and F245 antiserum are able to detect larger amounts of Pyk2-H when SUMO-1 and PIAS1 are co-transfected into NIH 3T3 cells (Figure 3.3, 3.4, and 3.6).

4.8 Conclusions

This thesis project presents the novel finding that PIAS1 and SUMO-1 associate with the FERM domain of Pyk2-H. The hypothesis of this thesis was that the higher MW Pyk2-H found in RAW 264.7 cells is caused by the direct

SUMOylation of Pyk2-H. Although the results of this thesis support that Pyk2-H associates with SUMO-1 and that the overexpression of PIAS1 and SUMO-1 increases the expression of Pyk2-H, it is still unclear as to whether Pyk2-H is directly SUMOylated or associates with SUMOylated proteins. Therefore, it is possible that the higher MW Pyk2-H is due to another posttranslational modification other than SUMOylation. Future studies will need to be conducted to examine the potential roles of the independent-SUMOylation of Pyk2-H and the role of SUMO-1 and Pyk2-H in the regulation of macrophage adhesion.

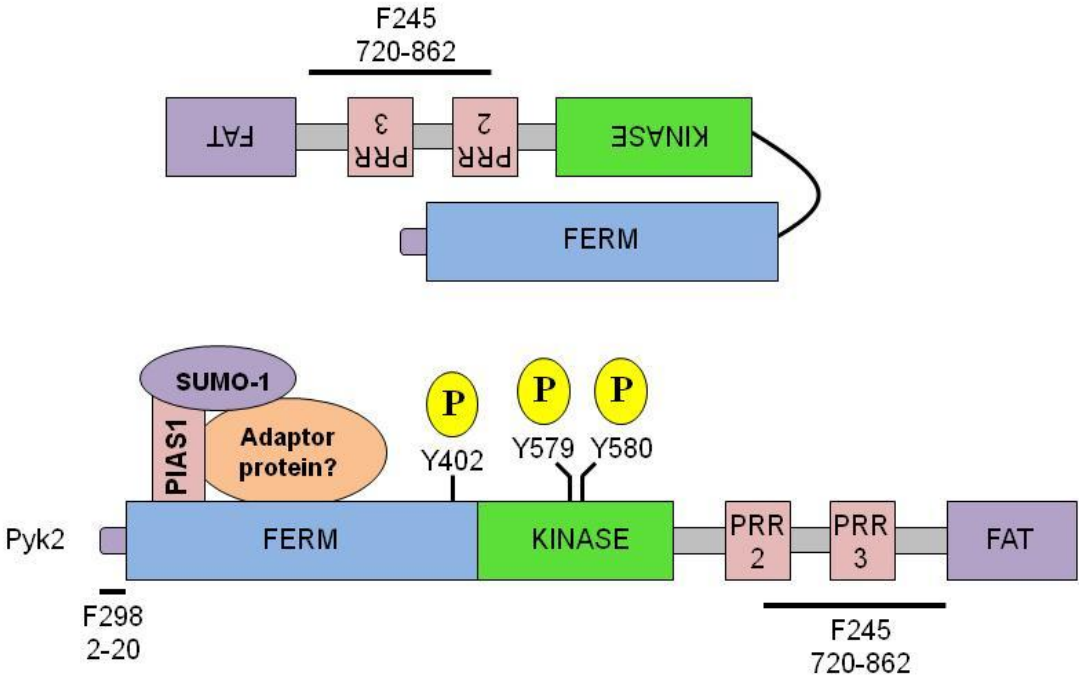


Figure 4.1: Proposed model for the activation of Pyk2 by SUMO-1. We believe that Pyk2-H adopts an autoinhibited conformation which prevents the F298 antibody from binding to the N-terminal region of Pyk2, but allows for F245 and paxillin binding. Upon stimulation Pyk2-H associates with PIAS1 and SUMOylated protein(s) or becomes SUMOylated, which allows for Pyk2 to adopt an "open" conformation. This allows for the autophosphorylation of Pyk2 at Y402

and further phosphorylation of Tyr 579 and Tyr 580 leading to Pyk2 activation and allowing for binding of the F298 antibody. The association of SUMO-1 or SUMOylated proteins may also maintain the “open” conformation of Pyk2-H or contribute to serine and or/theronine phosphorylation in the proline rich region.

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