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Characterization of Ankyrin Repeat Domain 54 (ANKRD54) and its role on the regulation and subcellular localization of Bruton's Tyrosine Kinase (BTK)

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Cover: Immunofluorescence microscopy image of stimulated Cos-7 cells. Global tyrosine phosphorylation labeled with red, actin-green (GFP) and nucleai-blue (DAPI). By Manuela Gustafsson, experiment from 2011.

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Department of Laboratory Medicine, Clinical Research Center

Characterization of Ankyrin Repeat Domain 54 (ANKRD54) and its role on the regulation and subcellular localization of Bruton's Tyrosine Kinase (BTK)

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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"Forget what hurt you, but never forget what it taught you"

Unknown

Dedicated to inspire the young generation

ABSTRACT

Bruton's tyrosine kinase (BTK) is an important cytoplasmic signaling protein, where the kinase activity plays a pivotal role in the development, proliferation and differentiation of B-cell lineages. Ankyrin repeat domain 54 (ANKRD54) is a nuclear-resident adaptor protein, where the ankyrin domain repeats are critical for specific protein-protein interaction, while the NLS and NES motifs control the nucleo-cytoplasmic shuttling ability.

We have identified and characterized ANKRD54 as a novel functional (paper I), interaction-partner for BTK using proteomics analysis. ANKRD54 is the first protein identified that specially influences the nuclear export of both BTK and TXK/RLK, in a Crm-1 dependent manner. Further, we mapped the interaction site to the C -terminus of BTK-SH3 domain, by using a synthetic peptide of BTK, covering the following region: C-ARDKNGQEEGYIPSNYVTEAEDS. In addition, tyrosine phosphorylation of BTK was investigated in the presence of increased amount of ANKRD54 and selectively the phosphorylation of BTK was down regulated.

We have presented a second novel interaction-partner and regulator of BTK (paper II), the 14-3-3 ζ protein, which is also identified by proteomics strategy. In this work, we have mapped the interaction sites on BTK to phospho-serine pS51 in the (RGRRGpS)-motif in the PH-domain and phospho-threonine pT495 in the (RHRFQpT)-motif in the kinase domain. Additionally, a newly characterized 14-3-3 inhibitor (BV02) interfered binding with BTK and siRNA knockdown of 14-3-3ζ increased the nuclear translocation of BTK, while overexpression of 14-3-3ζ resulted in accumulation of BTK in the perinuclear region.

We have generated single ankryin domain deletions of ANKRD54 and subsequently characterized their binding capacity and also their influence on the sub-cellular localization of BTK (paper III). In this work, we report that three out of four ankyrin repeats are required for the interaction and nucleo-cytoplasmic shuttling of BTK. Inhibition of Crm-1 nuclear export pathway influences differently the nuclear shuttling; rapid-ANKRD54 versus slow-BTK nuclear accumulation. Furthermore, we have determined that the interaction between BTK and ANKRD54 establishes in the nuclear compartment.

We have classified ANKRD54 as a prime interactor to the SH3-domain of BTK (paper IV). In this study, we utilized a screening strategy based on phage display libraries of the complete human “SH3-domainome” as a possible binding-target for ANKRD54. The aim is to identify the target spectrum and specificity of ANKRD54 for SH3 domain library, containing all the 296 human SH3 domains. The novel finding is that BTK is not only binding to ANKRD54, but also stands out as the preferred interactor, being highly dominant over all other human SH3 domains. However, other lower colony-score candidates for SH3-domain interactions were found, but without any further *in vivo/in vitro* validation.

REZUMAT POPULAR

Corpul uman are aproximativ 100 bilioane de celule. Fiecare celulă umană posedă ADN – material genetic ereditar, bine împachetat în nucleul celulei. Genomul nuclear este împărțit în 23 de perechi de molecule ADN liniar, numite cromozomi, din care ultima pereche conţine cromozomii sexuali, şi anume XX la femei şi XY la barbaţi. ADN-ul deține informația din care iau naştere proteinele, care de fapt sunt esenţa tuturor organismelor vii.

Celulele au diferite funcţii şi forme, iar durata lor de viaţă variază, ca de exemplu: celulele din muşchi trăiesc aproximativ 15 ani, celulele roşii din sânge au o durată de viaţă de aproximativ 120 zile, iar celulele pielii trăiesc în medie 14 zile.

Celulele mature B, aşa numitele limfocite, joacă un rol important în acţiunea sistemului imunitar, cu efect de autoapărare a corpului împotriva microbilor. Celulele mature B dau naştere la celule B cu memorie şi plasmocite. Funcţia de bază a plasmocitelor este de a produce anticorpi. În cazul unei infecţii, o singură celulă poate produce o cantitate imensă de anticorpi, aproximativ 2000 anticorpi/secundă.

De asemenea, în prezenţa antigenilor, celulele B se pot activa şi transforma în celule B cu memorie, care circulă pe traseul sânge – organ limfoid – sânge, pe o perioadă de câţiva ani. La o eventuală întalnire cu un microb, ele pot acţiona sistemul imunitar – ca exemplu bolile copilăriei nu vor mai fi contactate a doua oară. Dacă limfocitele nu ar circula, atunci ele nu ar avea posibilitatea de a întâlni şi de a declanşa un răspuns imun cu scopul de a distruge bacteriile, de a inactiva virusurile sau de a neutraliza microbii.

Un număr redus de pacienţi, cu precădere de sex bărbătesc, sunt diagnosticaţi cu Agamaglobulinemie legată de cromozomul X (XLA), o imunodeficienţă ereditară. Acestor pacienţi le lipsesc limfocitele B şi de aceea este blocată formarea de plasmocite, care sunt responsabile pentru producerea de anticorpi. Drept urmare, aceşti pacienţi sunt extrem de susceptibili la diferite tipuri de infecţii recurente sau severe de genul: sinuzitelor, otitelor, conjuctivitelor, rinitelor, bronşitelor, pneumoniilor şi infecţiilor de piele.

În primele şase luni de viaţă ale bebeluşilor, în special la sugari, această boală nu poate fi diagnosticată deoarece în sistemul nou-născuţilor se regăsesc anticorpii transmişi de la mamă pe durata sarcinii. Laptele matern conţine combinaţia perfectă de proteine, grăsimi, vitamine şi carbohidraţi, suplimentată cu anticorpi şi leucocite care protejează împotriva unor infecţii sau alergii.

Bruton Tirosin Kinaza (BTK) este proteina responsabilă pentru dezvoltarea normală a limfocitelor B. Pacienţii cu XLA prezintă mutaţii genetice la nivelul acestei gene, situate pe cromozomul X, aşa după cum îi sugerează şi numele.

Această boală nu poate fi transmisă, ci doar moştenită în cadrul familiei. Rudele de sex feminin din familie pot fi purtătoare ale bolii, ele neprezentând niciun simptom. Există totuşi 50% şanse de a transmite boala fiilor lor. La ora actuală nu există niciun tratament pentru vindecarea acestei boli. Singura încercare de ameliorare este administrarea intravenoasă sau subcutanată a anticorpilor lipsă, combinată cu utilizarea zilnică a antibioticelor. Este interzisă efectuarea de vaccinuri cu virusuri vii. Cu acest tratament, pacienţii cu XLA sunt capabili să îşi continue viaţa în condiţii relativ normale [1,2].

Proteina BTK joacă un rol important în maturitatea finală a celulelor B. Lipsa acestor celule şi a altor proteine, presupuşi parteneri ai proteinei BTK, este cauza şi a altor boli imunodeficitare bine-cunoscute. De asemenea, cancerul limfatic este cauzat de divizarea şi multiplicarea necontrolată a celulelor B.

Sub îndrumarea profesorului Edvard Smith, grupul de cercetare din care fac parte şi eu, grup numit "Molecular Cell Biology and Gene Therapy Science", se axează pe două programe de cercetare paralele care studiază pe de o parte *semnalizarea intercelulară* în limfocite – având drept scop proteina Bruton Tirosin Kinaza – iar pe de altă parte *terapia genetică* – cu scopul de a dezvolta noi tratamente. Printre acestea se numără şi terapia bazată pe oligo-nucleotide sintetice, care reprezintă bucăţi scurte de material genetic.

Obiectivul principal al tezei mele de doctorat este de a înţelege procesul de semnalizare intercelulară în limfocite. Fiecare celulă are capacitatea de a recunoaşte, interpreta şi răspunde la semnalele din mediul înconjurător. Semnalizarea intercelulară implică semnale preluate de receptori din exteriorul celulei şi transmise mai departe prin căi biochimice direct în nucleu, unde se efectuează un răspuns celular.

Un alt obiectiv al tezei de doctorat se referă la identificarea de parteneri noi ai proteinei BTK, investigarea relaţiilor structură-funcţie dintre ele şi descoperirea rolului biologic al proteinei BTK în nucleu. Totodată, studiind şi înţelegând componentele implicate în semnalizarea celulară, se va deschide o nouă arie de cercetare pentru dezvoltarea tratamentelor aplicate bolilor cauzate de disfuncţiile asociate cu proteina BTK.

ANKRD54 şi 14-3-3 (zeta) sunt două proteine identificate ca fiind noi parteneri ai proteinei BTK şi care influenţează în mod diferit BTK.

Cercetarea ştiinţifică nu-şi ia niciodată...pauză de cafea. Aventura descoperirii şi a cunoaşterii împinge cercetătorii de la spate, iar rezultatele cercetării din întreaga lume sunt publicate lunar în jurnalele ştiinţifice recunoscute pe plan internaţional.Eu sper ca munca efectuată pe parcursul acestei teze de doctorat – aceste câteva piese de puzzle – să fie utile şi să îşi găsească locul potrivit în imaginea de ansamblu referitoare la semnalizarea intercelulară a proteinei BTK.

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CONTENTS

LIST OF ABBREVIATIONS

ABL	Abelson murine leukemia viral oncogene homolog
AKT	Activated serine/threonine kinase
ANKRD54	Ankyrin Repeat Domain 54
BCAP	B-cell cytoplasmic adaptor protein
BCR	B-cell receptor
BLNK	B-cell linker protein
BMX	Bone marrow tyrosine kinase chromosome X
BTK	Bruton's tyrosine kinase
CD	Cluster of differentiation
CRM1	Chromosome maintenance region 1
DAG	Diacylglycerol
DARPins	Design ankyrin repeat proteins
ER	Endoplasmic reticulum
GFP	Green fluorescent protein
GSK3β	Glycogen synthase kinase 3 beta
Ig	Immunoglobulin
IP3	Inositol (1, 4, 5)- triphosphate
ITAM	Immunoreceptor tyrosine-based activation motif
ITK	IL-2 inducible T cell kinase
KO	Knockout
LYN	LCK/YES novel tyrosine kinase
NES	Nuclear export signal
NFAT	Nuclear factor of activated T cells
NFκB	Nuclear factor kappa B
NLS	Nuclear localization signal
PEI	Polyethylenimine
PI3K	Phosphatidylinositol-3 kinase
PIN-1	Peptidyl-propyl-cis/trans isomerase-1
PIP2	Phosphatidylinositol (4,5)-biphosphate
PIP3	Phosphatidylinositol (3, 4, 5) triphosphate

PKB	Protein kinase B
PKC	Protein kinase C
PLCγ	Phospholipase-C gamma
PPI	Protein Protein Interaction
PH	Pleckstrin homology
PHSCs	Pluripotent hematopoietic stem cells
RLK	Resting lymphocyte kinase
SDS	Sodium dodecyl sulphate
SHIP1	SH2 domain containing inositol 5-phosphatase-1
SYK	Spleen tyrosine kinase
TFK	TEC family kinase
TH	TEC homology
TLR	Toll like receptor
T_H	T_{HELPER} cell
TXK	T-cell expressed kinase
WT	Wild type
Xid	X-linked immunodeficiency
XLA	X-linked agammaglobulinemia

1 INTRODUCTION

Protein-protein interactions (PPI) are required for the most cellular functions; they are building signal transduction networks starting from cell-membrane receptors through cytoplasmic compartment until specific responses are reached, such as transcriptional activation, which control many aspects of the cell biology. Therefore, understanding the molecular and cellular mechanisms, as well the specific roles of proteins will definitely provide us with a deeper insight into biological processes. Still, these aspects remain greatly challenging due to the diversity of how proteins interact and, as well, the involvement of various post-translational protein modifications.

1.1 B-CELL RECEPTOR (BCR) SIGNALING

1.1.1 B-lymphocytes (B-cells)

Pluripotent hematopoietic stem cells (HSCs) are immature and very specialized cells that have the ability of both self-renewal and differentiation to all lineages of blood cells, including B-cells. The latest reports indicated that the hematopoietic system could be divided in four separate compartments: hematopoietic stem cells, hematopoietic progenitor cells, precursor cells and mature blood cells [3,4].

Since the discovery and characterization of B-cells in the mid-1960s a lot has been learnt about B- and T-cell development [5,6]. Except all critical checkpoints [7], B-lymphocyte development requires involvement of several different transcription factors and a network of cytokines that coordinate with each other [8]. B-cells play an essential role in the humoral immune response, which includes antibody production, isotope switching (IgM to IgG/IgA/IgE) or memory cell generation. First part of B-cell development takes place in the bone marrow and compromises the pro-B-cell to pre-B-cell to immature-B-cell stages. During these differentiations steps occurs the expression of surface pre-B-cell receptor and mature B-cell receptor (Figure 1). However, these cells are still immature and can be easily eliminated in contact with self-antigen. After exit from the bone marrow, the cells migrate to secondary lymphoid tissues, where after two transitional steps, become mature B-cells [9,10].

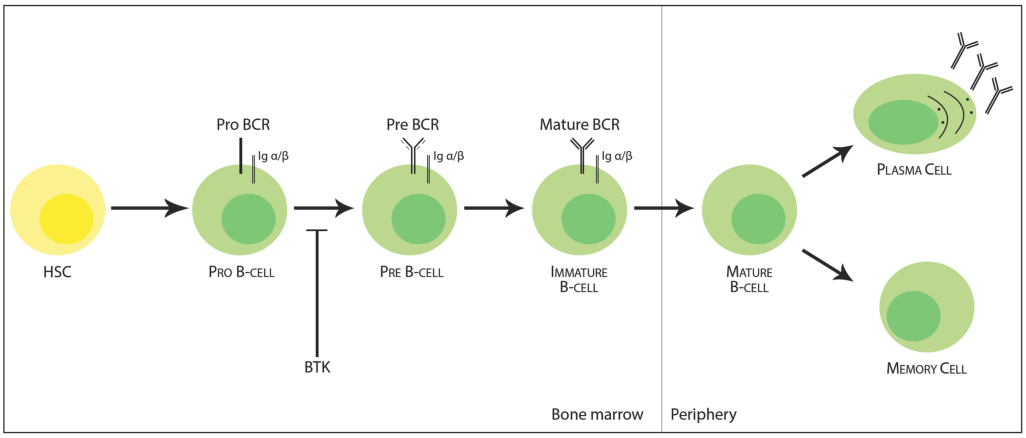

Figure 1. Schematic overview of B-cell differentiation, whereby the pre B-cell developmental arrest in XLA patients is indicated.

B-cell activation starts in spleen and lymph node when an antigen binds to the B-cell receptor (BCR), leading to the further differentiation into plasma cells or memory B-cells. When B-cells fail to successfully complete the development process, it will undergo programmed cell death, apoptosis. Memory B-cells are circulating through the body ready to initiate a rapid and strong response, have a long life span and express membrane bound antibodies. Opposite of memory B-cells, the plasma cells secrete about 2000 antibody molecules per second and the mature forms reside in the bone marrow for many years [11,12]. The quantity and quality of the circulating antibodies in our body are the result of a complex system, still it is not completely understood [13].

Following the latest results within B lymphocyte field, the basic role of B-cells is to recognize and bind antigen, to present antigen, to generate antibodies and cytokines. Except these, B-cells can receive help from a helper T-cell (T-cell dependent B-cell response) and generate high affinity antibodies during several days. As well, antigens can activate B-cells, without T-cell help (T-cell independent B-cell response) and generate a rapid response, but with low-affinity antibodies [14].

1.1.2 B-cell abnormalities

B-cell disorders [15] are divided into defects within the B-cell development/immunoglobulin production (Figure 1), known as immunodeficiencies (X-linked agammaglobulinemia, XLA), into defects in the B-cell regulation, called autoimmune diseases (multiple sclerosis [16] systemic lupus erythematous [17], rheumatoid arthritis [18], type 1 diabetes [19]) and into defects due to the uncontrolled proliferation causing lymphoma/leukemia (Hodgkin's [6] and non-Hodgkin's lymphoma [20], CLL, chronic [21]- or ALL acute lymphocytic leukemia [22]). Other B-cell malignancies, where BTK activity or inhibition is crucial, are: ABC-DLBLC, activated B-cell-like diffuse large B-cell lymphoma [23,24]; MCL, mantle cell lymphoma; WM, Waldenström's macroglobulinaemia [25] or MM, multiple myeloma [26].

1.1.3 B-cell receptor (BCR)

A mature BCR is a transmembrane receptor protein, composed of two parts. A membrane bound antibody of one isotype (IgM, IgG, IgA or IgE), which recognizes/binds antigen and a heterodimer co-receptor Ig-α/β (also known as CD79A/B) that can mediate signal transduction through its cytoplasmic tail that contains an immunoreceptor tyrosine-based activation motif (ITAM). The receptor binding site is unique and randomly determined by heavy and light chain [V(D)J] rearrangements [8,27].

1.1.4 The BCR signalosome

When an antigen binds to the BCR it results in receptor aggregation in the lipid rafts. Because the cytoplasmic tails of the receptor immunoglobulins are too short they are not signaling directly, but they covalently associate with the Ig-α/β heterodimer, which further transduces signals into the interior of the cell. The SRC–family kinases phosphorylate the ITAM motifs, which in turn recruit other cytoplasmic tyrosine kinases, described below. The activation of BCR results in the formation of a "micro-signalosome" in which BTK plays a necessary role. Subsequently, different signaling cascades will be activated, that ultimately will lead to the B-cell survival, proliferation and differentiation [28,29].

1.2 THE KINOME

Approximately, 2% of the human genes code for protein kinases, that plays a fundamental role in various signal transduction pathways, as well as involved in other processes like, cell-cycle regulation, differentiation, apoptotsis or metabolism. The human kinome (whole set of kinases) consists of 518 putative protein kinase genes, divided into groups and sub-groups [30,31]. The largest group is represented by protein tyrosine kinases (PTKs), which is subdivided into 58 trans-membrane receptor kinases and 32 cytoplasmic kinases [32]. Taken together only the cytoplasmic Tec family kinases (TFKs, where BTK belongs) and Src family kinases (SFKs, where LYN belongs), are covering about 45% of all non-receptor PTKs [33]. Customized creation of the human kinome tree, are available via a web-based tool, Kinome Render [30]. Protein kinases, as mentioned earlier are playing a central role in many cellular processes, especially in cancer development, therefore they become (and still are) a popular therapeutic target in development of new kinase inhibitors [34–37].

1.3 TYROSINE KINASES

1.3.1 Bruton's Tyrosine Kinase (BTK)

Bruton's tyrosine kinase (BTK) is a non-receptor tyrosine kinase belonging to the TEC family kinases (TFKs) together with the additional four mammalian members: tyrosine kinase expressed in hepatocellular carcinona (TEC), Interleukin 2 inducible T cell kinase (ITK/EMT/TSK), bone-marrow expressed kinase (BMX/ETK) and resting lymphocyte kinase (RLK/TXK). The structures of these tyrosine kinase family members are 50-60% conserved and they share domain structures, even if especially RLK/TXK differs from the others. They together constitute the second largest family of cytoplasmic tyrosine kinases in humans and play a key role in signaling and maturation of hematopoietic cells [38,39]. In addition, these kinases are found in other species, including *Drosophila melanogaster* [40], skate and zebra fish [41].

1.2.1.1 Structure of BTK

Bruton's Tyrosine Kinase (BTK) is expressed in all hematopoietic cells except T-lymphocytes and plasma cells. Btk is a 659 amino acid protein with a molecular weight of 77 kDa. Human and mouse Btk share 98.3% sequence homology [42–44].

The BTK protein has a conserved multi-domain architecture comprised of five domains, necessary for proper function. From the N-terminus, Pleckstrin homology (PH) domain, Tec homology (TH) domain, Src homology 3 (SH3), Src homology 2 (SH2) and Kinase (SH1) domain [45]. Each of the BTK domains has the capacity to interact with various molecules, enabling BTK to carry out diverse biological processes (Figure 2).

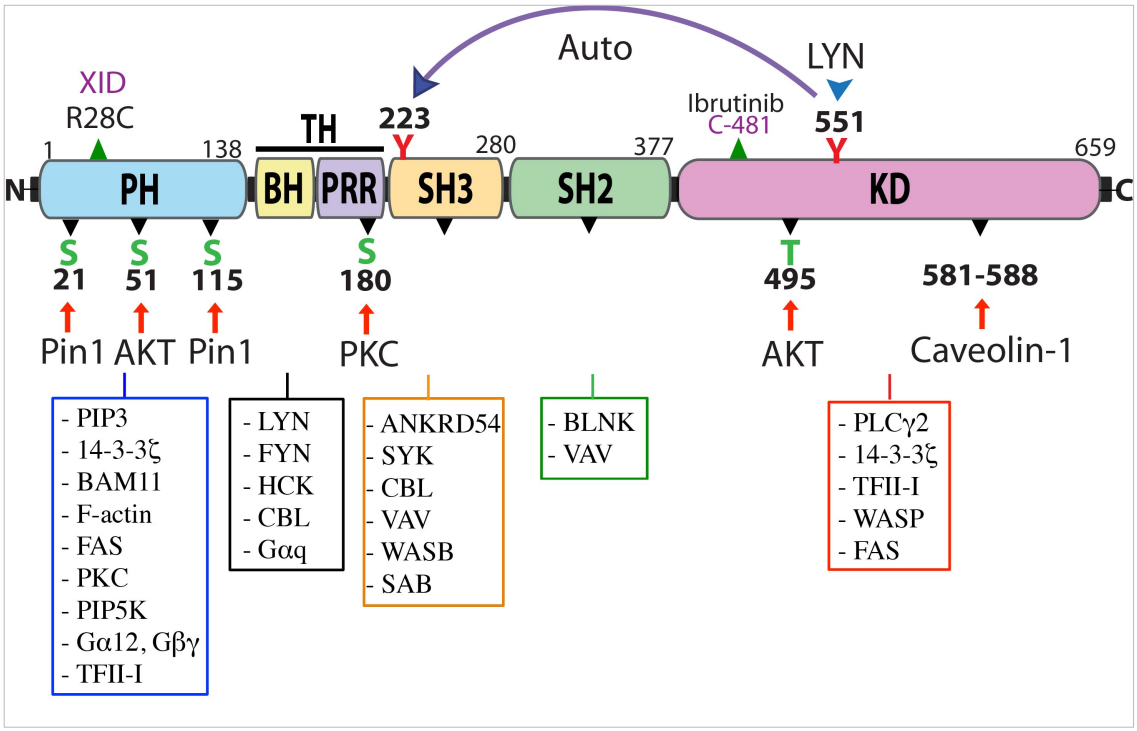

Figure 2. Domain structure of Bruton's tyrosine kinase and interaction partners**.**

The PH domain, comprising 138 amino acids is important for membrane translocation/anchoring during BCR signaling [46,47]. It has the capacity to bind to phosphatidylinositol lipids facilitating membrane recruitment for further protein-protein interactions [46,48]. The TH domain consist of two regions of approximately 80 amino acids; a BTK motif characterized by a zink-binding sequence important for optimal protein activity and stability; and a proline rich region, which can bind to other SH3 containing proteins [49,50]. The SH3 domain is involved in protein-protein interaction, its known to interact with proline-rich regions, but not only that. This domain comprises about 60 amino acids and includes the autophosphorylation site tyrosine, Y223. The SH2 domain is consisting of about 100 amino acids and the main function is to interact with phosphorylated tyrosine residues. The largest domain of BTK is the kinase domain [51], consisting of approximately 250 amino acids and it contains the conserved tyrosine residue Y551 [52], located in the activation loop [53]. This domain is catalytically active and important as substrate binding domain [29,43,54].

1.2.1.2 Activation of BTK

Through the years many biochemical experiments have contributed to elucidation BTK's activation mechanism. Upon BCR stimulation, SRC-family kinases such as LCK/YES novel tyrosine kinase (LYN) are activated, which enables them to phosphorylate the tyrosine in the

ITAMs [55], thereby providing docking sites for spleen tyrosine kinase (SYK) [54]. At the same time, B-cell adapter protein BCAP [56] and B-cell co-receptor protein CD19 recruits and enables activation of phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) at the plasma membrane [57]. Activated PI3K catalyzes the conversion of membrane-associated phosphatidylinositol (4,5)-bisphosphate (PIP2) [58] to phosphatidylinositol (3,4,5)-trisphosphate (PIP3) [59], that is important for the plasma membrane recruitment of two kinases: tyrosine kinase BTK and serine/threonine AKT/PKB. The PH domain of BTK will bind to its principal lipid ligand, PIP3 in the membrane and become fully activated by SYK kinase [60].

The adaptor protein B-cell linker (BLNK/SLP-65) [61] is recruited to phosphorylated the Ig-α/β, to the plasma membrane, where is activated by SYK. Tyrosine phosphorylated BLNK serves as a scaffold protein that recruits and binds to BTK and phospholipase Cγ2 (PLCγ2). In this close proximity, BTK is phosphorylated by SRC kinase at Tyr551 [62], followed by an auto-phosphorylation at Tyr223 [63,64]. BTK activates PLCγ2 by phosphorylation [65], which leads to cleavage of PIP2 into secondary messengers, inositol (1,4,5) triphosphate (IP3) and diacylglycerol (DAG). IP3 binds to its receptor on endoplasmic reticulum (ER) where it activates Ca^{2+} channels, resulting in intracellular Ca^{2+} influx [29] thereby activation of nuclear factor of activated T cells (NFAT), respectively DAG mediates activation of protein kinase C (PKC) [66], which further activates the NF-κB pathways [67–69] that will promote cell survival and cell cycle entry [70,71].

BTK is expressed also in other hematopoietic lineages, and can be activated through various receptor types like: chemokine receptor [72] CXCR4 and CXCR5 signaling important for tissue homing, homeostatis or B-cell trafficking [73] and toll-like receptor 4 (TLR) [74,75] in Btk-deficient B-cells shows reduced proliferation potential [76].

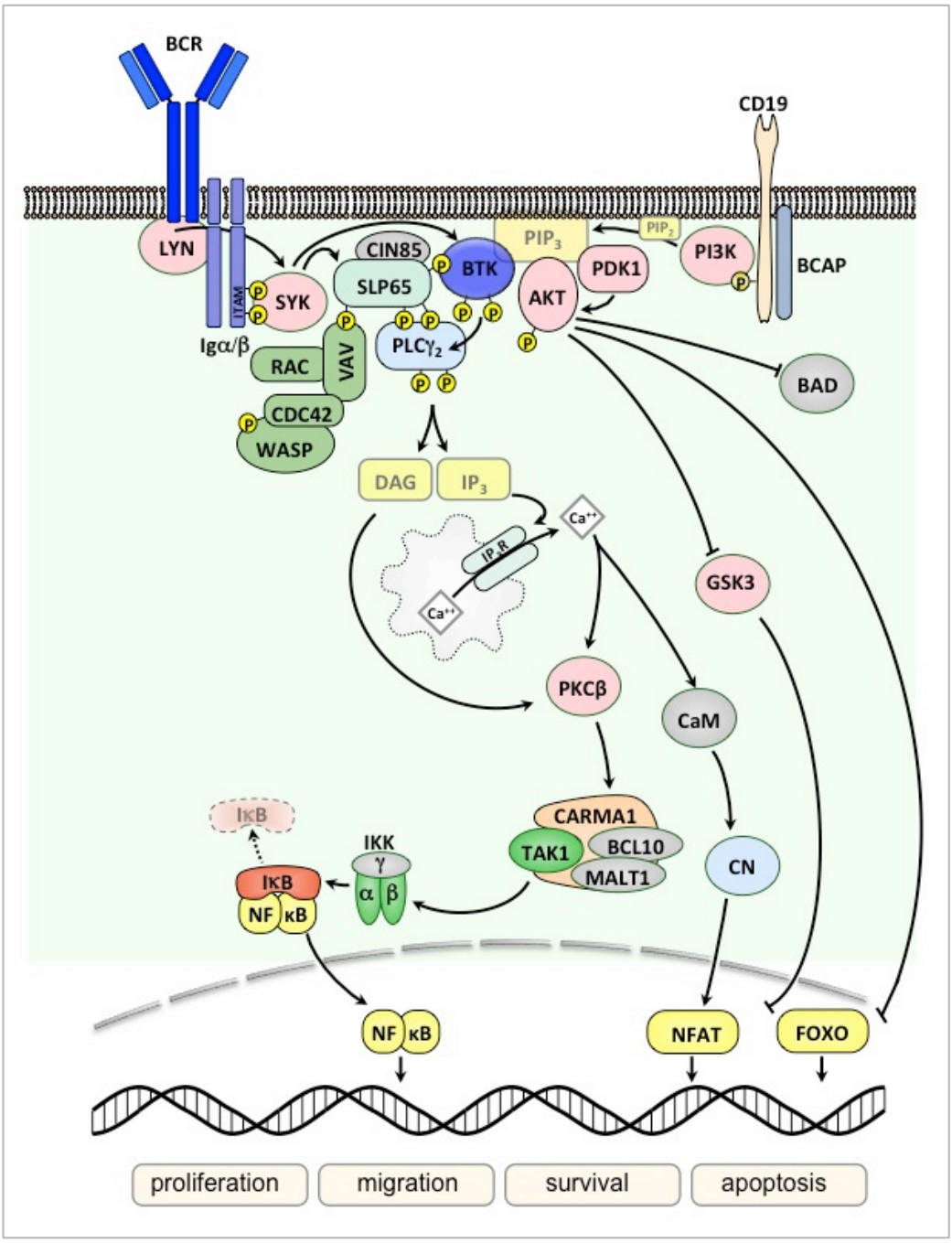

Figure 3. Model for Bruton's tyrosine kinase involvement in B-cell receptor signaling.

1.2.1.3 Down-regulation of BTK

In B-cells, BTK activation can be regulated by various phosphatases. Both, SH2 domain containing inositol 5-phosphatase-1 (SHIP) [77] and the dual-specific phosphatase and tensin homologue (PTEN) [78,79] can dephosphorylate PIP3 and thereby inhibit BTK association to the membrane. Association whit Caveolin-1 [80], PKC [49], AKT [81] or Peptidylpropyl *cis/trans* isomerase PIN-1 [82] proteins have been found to decrease BTK kinase activity.

1.2.1.4 BTK deficiency in human

1952, Dr. Odgen Carr Bruton was first describing the immunodeficiency symptoms in an 8-years boy, suffering of recurrent bacterial (respiratory tract and gastrointestinal) and enteroviral infections [83]. Dr Bruton was able to determine the absence of gamma globulin in patient blood serum by electrophoretic analysis [84], method developed by Arne Tiselius in Uppsala, Sweden, in 1937 and for which he awarded the Nobel Prize in chemistry in1948.

In 1986, the gene for X-linked agammaglobulinemia (XLA) was mapped on the long arm of the X chromosome Xq22.1, region 3-22 [85,86] and in the subsequent years later, these results were further confirmed [87,88]. Meanwhile it has been speculated the possibility of an enzyme defect, responsible for the disease[89,90]. Later, in 1993 two independent groups, characterized *ATK* (agammaglobulinemia tyrosine kinase) [91] as the defective gene in XLA, respectively *BKP* (B-cell progenitor kinase) [92] as candidate for XLA. From there on, a common name *BTK* (Bruton's tyrosine kinase) was adopted and first used when a similar defect in mice X-linked immunodeficiency (Xid), was reported [93,94].

X-linked agammaglobulinemia (XLA), is characterized by severe defects in B-cell development. The defect is caused by a partial block at the transition step from the pro-B-cell to the pre-B-cell stage (Figure 1), followed by a complete block from the pre-B-cell to mature B-cell stage in the bone marrow. XLA patients have <1 % of the normal number of B-cells in their circulation and the symptoms normally appear only after the first 6-9 mounts of life. Due to the X- chromosome linked inheritance essentially only boys are affected, whereas their mothers are healthy carriers [95–98]. Database BTKbase contains a collection of more than 800 *BTK* mutations [99].

1.2.1.4 BTK deficiency in mice

X-linked immunodeficiency (Xid) [94] is the corresponding XLA disease in mice [100–103]. It is caused by a single point mutation in the PH domain of Btk, where a highly conserved arginine at position 28 is replaced by a cysteine (R28C) [72,104]. In contrast with XLA, the Xid phenotype is a less severe form of B-cell deficiency and the affected mice still have 50% of the normal values of B-lymphocytes. Compensatory role for Btk has been studied in Tec/Btk double-deficient mice [105].

1.2.1.4 BTK inhibitors

Chemical compounds like LMF-A13 or terric acid have been shown to inhibit BTK catalytic activity, *in vitro* [106,107]. Ibrutinib (PCI-32765/ Imbruvica) [108] is the first-in-human FDA-approved inhibitor and binds irreversibly to cysteine 481 near ATP binding domain of BTK [66]. Several Phase II and III clinical trials are ongoing to valuate BTK inhibition in patients with various B-cell malignancies [109–112].

Acalaibrutinib (APC-196) is the second generation of BTK inhibitor designed to have more selectivity, still irreversible with less side effects. This novel inhibitor is already in clinical trials in patients with chronic or more aggressive B-cell malignancies [113]. While BTK inhibitors remain highly efficient, several cases of relapses or disease progression on therapy with BTK inhibitors have been reported [66,114,115].

1.3.2 Resting lymphocyte kinase (RLK)

RLK, also known as T-cell expressed kinase (TXK) [116], is a TEC family member expressed predominantly in T-cell lineage, especially $T_{Helper}1$ cell and mast cells. RLK kinase has a nuclear localization signal (NLS) sequence and is structurally similar to BTK, except that this kinase lacks the PH-domain, but instead consists of a distinctive cysteine-string motif, causing a constitutive membrane association [117]. Upon receptor stimulation, a high proportion of RLK molecules shuttles to nucleus, where it enhances interferon INF-γ production [118].

1.3.3 LCK/YES novel tyrosine kinase (LYN)

LYN is a member of SRC family non-receptor kinases that compromises 9 members [119] and is expressed in all blood cells, neural, liver and adipose tissues [120]. Upon B-cell activation, LYN undergoes rapid phosphorylation that will initiate a series of signaling events [121]. SRC tyrosine kinases share conserved domain structure and consists of a myristoylated N-terminal domain followed by SH2, SH3, kinase domain and a C-terminal tail. LYN has been reported to play an important role in Epo-induced erythroid differentiation, as well in involved in signal transduction from various hematopoietic receptors [122].

1.4 SERINE/THREONINE KINASES

1.4.1 Protein kinase AKT

AKT, also known as protein kinase B (PKB) is a specific serine/threonine kinase involved in several signaling pathways divided through the three AKT isoforms; cell growth, proliferation, differentiation (AKT1/PKBα); glucose metabolism, insulin signaling (AKT2/PKBβ) and brain development (AKT3/PKBγ) [123].

AKT isoforms have similar domain structure consisting of an N-terminal PH domain, required for membrane recruitment where it binds to PIP3. AKT carries a central kinase domain containing Thr308 site important for partial kinase activation and a C-terminal hydrophobic regulatory motif containing Ser473 site critical for fully activation and cellular relocation of the AKT kinase [124] (Figure 4). One of the downstream effects of PI3K-AKT relocation is further phosphorylation of target proteins containing RXRXXS/T motif [81]. Because many of these substrates are shared with other kinases, the PI3K/AKT signaling is intersecting with other signaling pathways affecting in this way many other downstream effects [125].

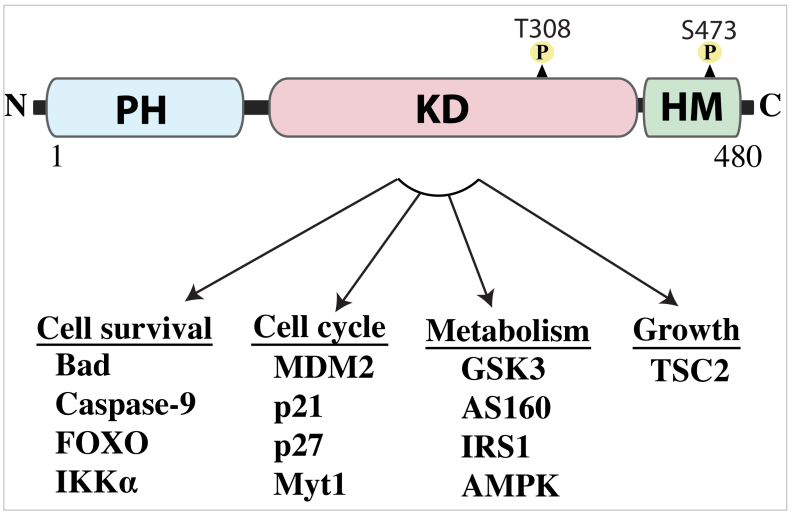

Figure 4. Domain structure of protein kinase AKT and regulatory partners.

1.5 ADAPTOR PROTEINS

1.5.1 Ankyrin repeat domain 54 protein (ANKRD54)

ANKRD54, also known as Liar, LYN-interacting ankyrin repeat, is a 300 amino acid protein consisting of 4 ankyrin repeats, flanked by a nuclear localization signal (NLS) and a nuclear export signal (NES), and is expressed in various tissues.

The structure of ANKRD54 displays a highly conserved structure among rat, mouse and human [126] (Figure 5). ANKRD54 is a nuclear/cytoplasmic shuttling protein and three of four ankyrin repeats are important for the interaction with BTK and LYN, interactions independent of the proline-rich regions in the SH3 domain. The direct interaction with BTK influences its nucleo/cytoplasmic shuttling in B-cells [126] and association of ANKRD54 with LYN and HS1 in a complex influences erythropoietin induced differentiation of erythrocytes [122].

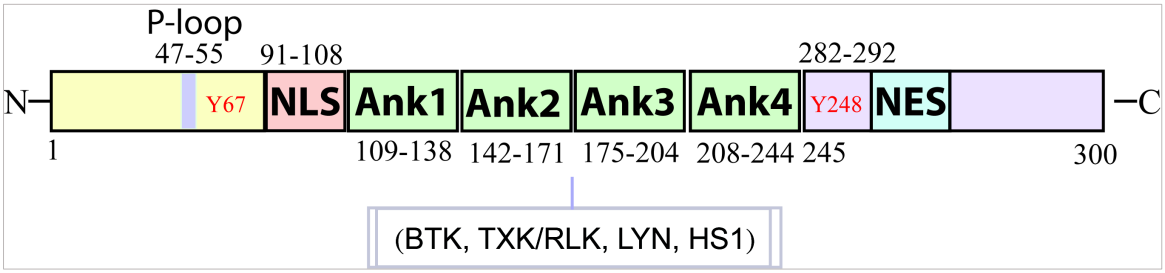

Figure 5. Schematic representation of ANKRD54 domains and interacting partners.

Ankyrin repeat domains are considered the most common sequence motif in nature [127], found in a plethora of proteins with a wide range of cellular function, they are involved in: protein transport, cell-cell signaling and development (Notch) [128], cytoskeleton integrity, cell-cycle regulation/tumor suppressor (family of INK4 tumor suppressors, p15, p16, p18, and p19 and 53BP2, a regulator of the tumor suppressor p53) [129] or transcription factors (NFκB or Swi6) [130]. In addition, the three-dimensional structure of some of these ankyrin repeat proteins has been determined [127]. Except predominant existence in eukaryotes, ankyrin repeats have been found in viruses, bacteria and archaea [131].

Each internal ankyrin repeats consist of 33 amino acids residues, composed of a β-hairpin structure followed by two antiparallel α-helices and a variable loop [132,133]. A unified trait among ankyrin repeats is the conserved GxTPLHLA motif [134–136] (Figure 6), found in all internal and C-terminal ankyrin repeats within a protein [137]. Analyses of databases show that the number of ankyrin repeats in a protein varies greatly [136,138], but the majority of proteins contains two to six ankyrin repeats [137], tightly packed in a well-folded structure [139,140]. Both theory and experiments agree that ankyrin repeats folding and structure is favorable for binding to targets proteins despite the lack of "long-range contacts", like globular protein for example [141].

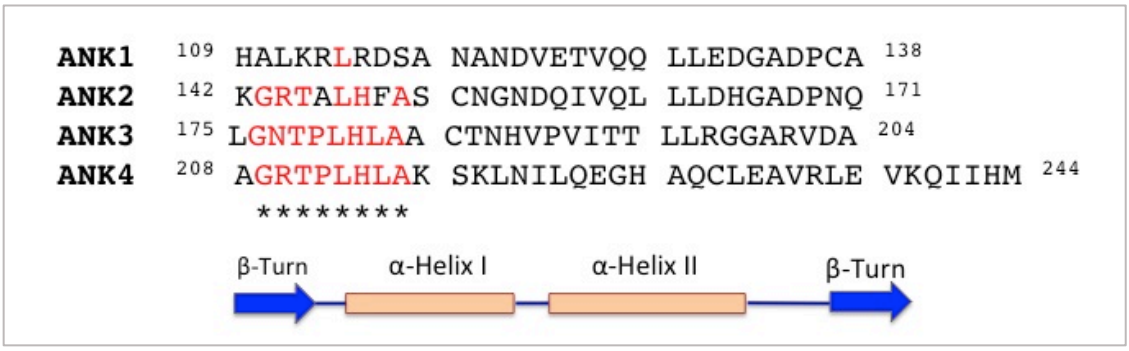

Figure 6. Conserved motif features and secondary structural composition of ANKRD54 ankyrin domains.

In addition, designed ankyrin repeat proteins (DARPins) have a great potential as antibody mimetic proteins, already used in clinical and preclinical development [142]. DARPins have high specificity binding potential to their target proteins, consists of 2 to 5 very stable repeats, designed after natural repeats, which as previously mentioned, are involved in protein-protein interaction in relatively all species, and are found in all extra-/intracellular environments [143,144]. A unifying trait of the ankyrin repeat proteins is that they are lacking enzymatic activity and typically function as universal scaffold or adaptor molecules [139].

1.5.2 14-3-3 protein

The 14-3-3 family of acidic soluble proteins consists of 7 distinct members named with Greek letters (β, γ, ε, ζ, η, θ and σ) after their elution profile. The 14-3-3 are present in all eukaryotic cells and are involved in regulation of a large spectra of cellular proceses, including metabolic pathway signaling, cell cycle, cytoskeleton organization, cellular traffiking, apoptosis. These proteins have the ability to bind to a plethora of signaling molecules, including transmembrane receptors, kinases or phosphateses [145]. Except abundant localization in the cytoplasm, 14-3-3 proteins can also be detected in the nucleus, Golgi apparatus or at the plasma membrane [146].

Phospho-bindning motifs like RSXpSXP and RXXXpSXP (where pS is phosphoserine and can be replaced by pT Phosphothreonine) has been reported as 14-3-3 binding sites [147], as well as an AKT phosphorylation site RXRXXpS/T [81].

14-3-3 proteins are able to form homo- and heterodimeric [148] U shaped structures and they were classified [149] according to their mode of binding:

(i) conformational changes directly on the target protein, e.g. catalytically inefficient Serotonin-N-acetyltransferase due to phosphorylation on two separates sites [150].
(ii) inhibition by protein interaction and modulation of subcellular localization of target protein, for example cytoplasmic sequestration of class II histone deacetylases [151] or high nuclear export rate of Cdc25 [152].
(iii) acting as a scaffold molecule, by bringing in close proximity two different proteins, e.g. BCR/RAF [153] or GSK3β to tau [154].

14-3-3 are small proteins (27.7 kDa), mainly localized into the nucleus [155], but can rapidly translocate to the cytplasm, in a chromosome maintenance region 1 (Crm1)-dependent manner, when its C-terminal NES becomes exposed. 14-3-3 proteins are exported from the nucleus together with their targets [156].

1.6 SH3-DOMAINS

Src-homology 3 (SH3) domains are small interaction modules found in many signaling proteins, including non-receptor tyrosine kinases. Evolutionary they are well conserved mediating protein-protein interactions important for a variety of signal transduction processes. The human genome encodes almost 300 SH3 domains [157].

1.6.1 SH3 Domain structure and function

Generally, this domain is sharing many common structural features; a β-sandwich consisting of five stands connected by three loops and a short 3_{10} helix, where the amino acids are arranged in a right-handed helical structure. Each SH3 domain consists of approximately 60 amino acids. The three-dimensional structures of a number of SH3 domains [158,159] are well described and cataloged [160–162], predicting different modes of binding. Numerous low- and

high affinity-binding partners have been found [163], characterized by their biological function [157] or stored into peptide libraries [164], as well as accessible databases [165]. BTK, RLK and LYN contain a single SH3 domain, but several other proteins can contain up to six SH3 domains. The significant role of the SH3 domain is mediation of protein-protein interaction.

Following the reports about the composition and solution structure of BTK-SH3-domain (Figure 5), it has been determined that three tyrosine (Y223, Y225 and Y268), one aspartate (D232) and one tryptophan (W251) are highly conserved surface residues [166,167]. These amino acids are important for fully activation of BTK (Y223 for auto-phosphorylation) or as key binding site with other proteins (ANKRD54 and Y268) (Figure 2). Surface representation of amino acids forming BTK SH3-domain, places P265 in an inner cleft and Y268 very exposed and available on the surface [166].

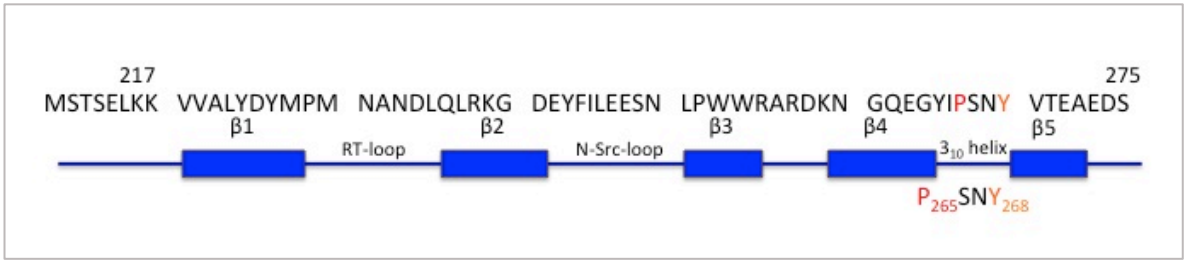

Figure 7. Secondary structure elements in the BTK SH3-domain,

1.6.2 SH3 Domain in signal transduction

As like TEC-family tyrosine kinases, many SH3-containing proteins are involved in signal transduction pathways, including SRC-family kinases, PLCγ, RasGAP and PI3K self-phosphorylated residues, within the SH3 domain, are important for the full activation of these molecules and subsequent downstream transduction of signals [168,169].

2 AIMS OF THE THESIS

The focus of this study was to explore futher the signaling of Tec family kinases, with the main focus for BTK kinase. Along that line, we have investigated the signalosome complex downstream of the BCR using proteomics approach, aiming to identify new bindings partners of BTK, in order to elucidate their mode of binding and biological function. Owing to that BTK shuttles between the cytoplasm and the nucleus, elucidating the role of BTK in the nucleus is of great interest. In particular, we have characterized two novel interaction-parteners ANKRD54 and 14-3-3ζ proteins. In summary, we have:

AIM 1: Characterized a novel interaction-partner for BTK, the ankyrin repeat domain 54 protein, ANKRD54 and studied its role in the nucleo-cytoplasmic shuttling of BTK.

AIM 2: Characterized a second novel interaction-partner for BTK, the 14-3-3ζ protein, and investigated its role in the regulation of the intracellular signaling of BTK.

AIM 3: Characterized the critical ankyrin-repeat domains of ANK54RD responsible for the BTK interaction. The site of intracellular binding was determined to be in nucleus.

AIM 4: Characterized the SH3 domain binding specificity with ANKRD54 through screening of a complete human SH3-domain library.

3 MATERIALS & METHODS

Many more details of the experimental procedures use in this thesis are available in the published papers and manuscripts. Briefly, I describe some of the general principles.

3.1 CELLS

3.1.1 Cell lines

Namalwa and Ramos (human Burkitt B-cell lymphoma), Nalm-6 (pre-B-cell leukemia), K562 (human myelogenous leukemia), A20 (mouse B-cell lymphoma), Jurkat (human T-lymphocyte), Cos7 (African green-monkey fibroblast-like kidney), Phoenix GP and PG13 (retrovirus producer lines based on a human-embryonic kidney, HEK293T cells) and NIH 3T3 (mouse embryonic fibroblast) cell lines were obtained from the American Type Culture Collection (ATCC). The cell lines Phoenix GP, Cos7 and NIH3T3 were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat inactivated Fetal Bovine Serum (FBS) (Invitrogen). All other hematopoietic cells were cultured in RPMI1640 medium with supplements. All cells were cultivated at 37 °C in a humidified 5% CO_2 incubator.

3.1.2 Primary cells

Purified mouse splenic B-cells or total cell suspensions prepared from spleens obtained from BTK wild type and BTK KO mice were immediately used. Stockholm South animal ethics committee approved the use of mouse models in this study.

3.2 MASS SPECTROMETRY

Mass spectrometry (MS) is a chemical analytical technique with remarkable improvements and is applied to proteins, peptides, DNA and other biological molecules. This powerful method combined with chromatographic separation methods allows detection of proteins in a mixture. Proteins are fragmented into small peptides, then converted to ions that are easier to manipulate by electric and magnetic fields. Ions are directed in a mass analyzer where they are separated by mass to charge ratio. The resulting spectra were analyzed by using the MASCOT program against the human Ensembl Database, thereby providing the molecular mass and other structural information. Further, the proteins of interest were biochemically validated by other methods used in our lab, co-IP and Western blot and/or immune-fluorescence analysis. Modern MS allows high-confidence identification of protein-protein interactions and it is often used in large-scale studies [170].

3.3 PHAGE-DISPLAY SCREENING

Phage-display technology allows *in vitro* high-throughput screening of protein-protein interaction with help of bacteriophages [171]. We have screened through a library containig a complete collection of all human Src homology-3 (SH3) domains expressed on the surface of the M13-derived phagemid vector fused to pVIII gene. As mentioned before, some proteins can contain more that one SH3 domain. In this library each SH3 domain, together with six

splice variants were counted as individual domains, which all jointly comprises of 296 SH3 domains [172]. Detalied bioinformatic information and sequences of all these SH3- domains are avalaible online [173].

3.4 TRANSFECTION METHODS

Transfection is the process of deliberately inserting genetic material into the cells. In this work different, transfection protocols have been used. At 48 h post-transfection, all cells were collected and analyzed.

3.4.1 Electroporation

The hematopoietic cells were transfected by electroporation using a Neon Electroporator (Life Technologies) and the 100 µL tip kit with the following settings: 1350V (pulse-voltage); 20ms (pulse-width); 2 (pulse-number).

3.4.2 RNA interference

Small interfering RNAs (siRNA), or short interfering RNA is a class of double-stranded RNA molecules (20-25 base pair in lenght) that interfiers with the expression of a certain gene, that displays complementary sequence, by inducing degradation of the messenger RNA (mRNA) [174]. The synthetic RNA duplex induces a short-term silencing of protein coding genes.

3.4.3 Plasmid transfection

For the transient transfections of all the adherent cells, we frequentuely used the cationic polymer polyethyleneimine (PEI) (Polyscience Inc.). The standard calcium phosphate precipitation method [175] was used in isolated cases for the ectopic expression in HEK293T cells.

3.5 PROTEIN ANALYSIS

3.5.1 Immuniprecipitation

Individual protein Immunoprecipitation (IP) or Complex protein Immunoprecipitation (Co-IP) are one of he most widely used techniques to precipitate and purify a desired protein (antigen), or a target protein from a complex, or to determine posttranslational modifications of proteins, based on antigen-antibody interaction.

3.5.2 Biotin-streptavidin pull-down assay

The biotin-streptavidin pull-down assay is a comparable method to Co-IP, used to study protein-protein interactions as well. The interaction between biotin and streptavidin has been explored in many detection/ purification methods and is cataloged as a robust and sensitive assay. The principle of IP is based on using specific antibodies against a target protein forming an immune complex, which then is captured onto Protein A or G beads. In a similar fashion the biotinylated proteins are pulled down with Streptavidin beads. The advantage of the biotin-streptavidin pull-down assay is that there are no heavy or light chains (from antibodies) which interfere during the immunoblotting step.

3.5.3 Western blott (WB)

Electrophoretic transfer of proteins, later renamed as Western blot [176] is an important technique to detect proteins in native or phosphorylated state. Isolated proteins from cells are solubilized with sodium dodecyl sulfate and separated by gel electrophoresis, SDS-PAGE, followed by protein transfer onto nitrocellulose membrane. The subsequent employment of primary and secondary antibodies is used to detect specific proteins, by chemiluminescence or by Odyssey infrared imaging system (LiCOR Bioscience GmbH).

3.6 MICROSCOPY

3.6.1Immunohistochemisty and Immunofluorescence

Immunohistochemisty is a powerfull method, very often used in the laboratories, that reveals the existance and the localization of the target protein, in different cell types and tissues, in different biological conditions. Antibody-mediated antigen detection is a multi-step process that requires carefully optimization in order to obtain a accurate and specific signal detection. Vizualization of antibody-antigen can be acomplishmed by chromogenic or fluorescence detection and requirers light microscope, respectively fluorescence microscope.

An alternative method to study dynamic of protein localization, is by vizualization of exogenous fusion protein using GFP (green) or RFP (red), which can be fused to the protein of interest. This approach is faster, less sensitive and allows to monitor the protein movement and localization directly in living cells.

3.6.2 Confocal microscopy

High-resolution optical images of the transfectd cells were captured on a LeicaDMRXA confocal microscope eguipped with a 3D digital microscopy work station (©Leica Microsystem, Weltzar) and analyzed using “Slide Book” program (Intelligent imaging Innovation Inc., CO, USA)

3.6.3 Fluorescence microscopy

Transfected cells were analyzed in a motorized Olympus IX-81 inverted-fluorescence microscope equipped with an XM10-monochrome-camera and narrow band-filter cubes for UV (DAPI), green (GFP) and red (Cy3) excitation.

4 RESULTS & DISCUSSIONS

4.1 PAPER I

Regulation of Nucleocytoplasmic Shuttling of Bruton's Tyrosine Kinase (Btk) through a Novel SH3-Dependent Interaction with Ankyrin Repeat Domain 54 (ANKRD54)

In this study, we have identified ANKRD54 as a novel interacting partner to BTK and found that ANKRD54 is the first identified protein that specifically influences the nuclear export of BTK.

The groundwork of this investigation was to identify more partners within the signalosome complex downstream of the BRC under different cellular conditions (starvation, activation or inhibition), by using bottom-up "shotgun" proteomics approaches, where affinity purified samples were subjected to proteolytic digest by trypsin, followed by nano-liquid chromatography separation coupled to mass spectrometry. We have established a stable Namalwa B-cell line expressing Flag-tagged BTK and the purified BTK-Flag complexes were prepared for gel-free, bottom-up proteomics. From the resulting tandem mass spectra we have selected ANKRD54 and 14-3-3ζ (paper II) for further studies.

Combination of biochemical techniques and microcopy were used to confirm the direct SH3-domain dependent interaction between ANKRD54 and BTK. With help of peptide motif-finder databases, the ANKRD54 motifs were identified. The core region consists of four ankyrin domains, important for the interaction with other proteins, flanked by N-terminal NLS and C-terminal NES motifs, while the N-terminal and C-terminal part of the protein are unique.

Remarkably, we found that ANKRD54 co-localizes with BTK in the cytoplasm and more interestingly even when BTK was tagged with a synthetic NLS. In similar experiments we demonstrated that ANKRD54 does not affect localization of two different nucleus-resident proteins; estrogen receptor β and transcription factor T-bet or the nucleus-trapped tyrosine kinase c-ABL. Unexpectedly, the cellular localization of another TEC family kinase, namely TXK/RLK was affected by ANKRD54.

Since a mutant of BTK lacking the entire SH3 domain failed to interact with ANKRD54, we designed a peptide covering the C-terminal of BTK'S SH3 domain (ARDKNGQEGYIPSNYVTEAEDS) that was sufficient to pull-down endogenous ANKRD54.

Additionally, we showed that the BTK-ANKRD54 interaction was independent of kinase activity and that an increased amount of ANKRD54 down regulates tyrosine (Y551) phosphorylation of active BTK.

Moreover, we demonstrated that both proteins utilized the Crm-1 dependent export pathway and upon Leptomycin B treatment, complete ANKRD54 nuclear localization

occurred after only 3 hours, compared with BTK that required more than 12 hours, suggesting that the physical interaction may occur in the nuclear compartment.

In conclusion, we suggest a model of nuclear export of BTK, mediated by an SH3-domain dependent interaction with ANKRD54.

4.2 PAPER II

Dual phosphorylation of Btk by Akt/Protein Kinase B Provides Docking for 14-3-3 ζ, Regulates Shuttling, and Attenuates both Tonic and Induced Signaling in B cells

This paper represents a parallel study to our previous publication (paper I). Here, we investigated the interaction between BTK and its novel partner, 14-3-3ζ, which was identified from the proteomic data.

14-3-3 proteins are recognized to bind to RXRXXPpT/S consensus region sequences. Two such motifs were identified in BTK, one located in the PH domain (46-RGRRGS-51) and the second located in the kinase domain (490-RHRFQT-495).

Furthermore, we revealed that pS51 and pT495 sites on BTK were phosphorylated by the AKT/PKB, and found to be critical for the interaction with 14-3-3, since both BTK and 14-3-3 inhibitors hindered the interaction. Surprisingly, in the presence of 14-3-3 inhibitor, the BTK, phospholipase Cγ2 and nuclear factor-κB protein phosphorylation increased strongly, suggesting its implication in the B- cell receptor mediated tonic signaling.

In addition, the loss-of-function BTK mutant (S51A/T495A) failed to interact with 14-3-3ζ and oppositely the phosphomimetic BTK mutant (S51D/T495D), exhibited strong tyrosine phosphorylation, which ultimately triggers ubiqutination and degradation of active BTK. Another inhibitor targeting PI3-K abrogated BTK phosphorylation and bindning to 14-3-3ζ.

Co-localization studies revealed that both BTK and 14-3-3ζ resided in the cytoplasmic and perinuclear regions, while depletion of 14-3-3ζ, by siRNA led to more nuclear accumulation of BTK.

Taken together, we present AKT as a novel negative regulator of BTK, via 14-3-3ζ that binds to phosphorylated BTK and prevents it's translocation to the nuclear compartment. The fate of active BTK is now dictated by ubiquitination and subsequently proteasome degradation, followed by termination of BCR signaling.

4.3 PAPER III

Domain Structure Characterization of ANKRD54 and Subcellular Localization of Bruton's Tyrosine Kinase (BTK)

In this work we have extended our initial observation regarding co-localization between ANKRD54 and BTK. By site-directed mutagenesis and combined with co-lokalization studies of ANKRD54 wt and various mutants, we characterized that three out of four ankyrin domain are responsible for the interaction with BTK.

ANKRD54 contains four ankyrin repeats domain and accordingly with the litterature [137] the first 10 residues of each middle and C-terminal repeats are highly conserved consensus regions (GxTPLHLA).

We generated single domain deletions of each ankyrin domain, mutation of NLS and NES consensus, together with two point mutation of possible important tyrosine sites. Ankyrin repeat domain ANK2, ANK3 and ANK4 were able to influence the cellular localization of nuclear targete BTK.

Owing the fact that Leptomycin B influences diffrently the nuclear accumulation of BTK vs ANKRD54, we explored futher and determined that the interaction between BTK and ANKRD54 establishes in the nuclear compartment.

The Crm-1 dependent nuclear accumulation of BTK suggests that the protein has a functional NLS, but the mechanism utilized by BTK to shuttle to the nucleus still remains enigmatic.

4.4 PAPER IV

ANKRD54 preferentially selects Bruton's Tyrosine Kinase (BTK) from a Human Src-Homology 3 (SH3) Domain Library

In this study, we aimed to indentify the spectrum of an ANKRD54 SH3-interactome. From our previous work we knew that ANKRD54 interacts with BTK in an SH3 domain dependent manner and that a 22-aa short peptide covering the C-terminal of BTK SH3 domain, was sufficient for binding.
Owing this knowledge, we visualy inspected the C-terminal of other kinases and found certain similarities; the 265-**P**SN**Y**-268 motif, were SNY ammino acids represents a 3.10 helix between the beta sheet 4 and 5 [159,166]. In addition, single or double point mutation of proline or tyrosine residues demonstratrate the importance of tyrosine, Y268.

After three independent screenings, with a complete SH3-domain library, we identified 15 different, positive hits. Only two of them were previously reported in literature as ANKRD54 interactors, namely BTK and LYN. Importantly, the BTK-SH3 domain was predominantly selected in each screening round.

Further, we have explored the posibility of competition between BTK and LYN toward ANKRD54 and we determined that there is no enhanced binding between LYN and ANKRD54 in the absence of BTK.

Taken together, our findings suggest that interaction between BTK and ANKRD54 is highly selective, therefore we identify BTK as being the preferred partner to ANKRD54.

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVE

Proteins and proteomics studies remains a key topic in modern cell biology. Proteins are coming in all sizes and shapes and are “the workers” that together with an increasing number of RNA species control all biological systems in a cell. Even if some proteins can function independently, the majority of them need other proteins for proper biological activities. In addition, proteins are dynamic, constantly co- and post-translationally modified and allows crosstalk of different cellular pathways.

Signal transduction is the information flow within eukaryotic cells, relies on both stable and transient protein-protein interactions. Due to the complex nature of the proteome, modern technologies have been developed, together with the improvement of the old one, to investigate the proteome in depth. Neverless, the greatest challenge remains to understand and determine function/-s of proteins in their proper biological context, in order to be able to combat diseases and/or develop new treatments.

Two bona-fide interaction partners to BTK have been identified and studied in this work, namely ANKRD54 and 14-3-3ζ, a nuclear-export mediator respectively, a nuclear-import preventor.

The adaptor protein, ANKRD54 preferentially associates with BTK in the nuclear compartment, where ANKRD54-mediated export is induced, independent of BTK kinase activity.

14-3-3ζ interaction with BTK is facilitated by AKT phosphorylation on two different sites on BTK, thereby preventing its nuclear translocation. Furthermore, this serine/threonine activation leads to proteosomal degradation of BTK and thereby termination of BCR signaling.

Seemingly, our findings will contribuite to further understanding the regulation and subcellular localization of BTK. However, parallel proteomic work is ongoing whitin the scientific world that hopefully will expand todays knowledge about the molecular mechanisms underlying regulation, sub-cellular localization and signaling of BTK.

6 ACKNOWLEDGEMENTS

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