The role of the IL-15Rα cytoplasmic domain in the IL-15 signal transduction pathway

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hiermit bestätige ich, dass die Dissertation von Frau Mojgan Drasdo mit dem Titel "The role of the IL-15R alpha cytoplasmic domain in the IL-15 signal transduction pathway" in korrektem Englisch geschrieben ist.

Mit freundlichen Grüßen

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Für Jens, meinen Vater

und

in Gedenken meiner Mutter

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SUMMARY

Interleukin 15 (IL-15) is a pleiotropic cytokine with many IL-2-overlapping activities in the immune system and a potent anti-apoptotic function. IL-15 binds to a heterotrimeric receptor complex, which shares the IL-2 receptor beta (IL-2Rβ) and IL-2 receptor gamma (IL-2Rγ/γc) chains, but has a unique IL-15Rα chain. IL-15 functional specificity is insured by cytokine binding to its high affinity receptor chain, the IL-15Rα. Therefore, the goal of this work was to study the involvement of the IL-15Rα cytoplasmic domain in signal transfer and to identify interacting proteins, which are able to bind the IL-15Rα cytoplasmic domain.

In order to investigate the role of IL-15Rα in intracellular signalling, a mouse IL-3 dependent pro B-cell line, BA/F3, which does not express IL-15Rα was used. Here it was shown that in IL-3 deprived but IL-15Rα-transfected BA/F3 cells, the IL-15Rα mediates mitogenic and anti-apoptotic signals. The role of the cytoplasmic domain was investigated via generation of two mouse chimeric receptors, the IL-2Rα/**IL-15Rα** and the reverse construct IL-15Rα/**IL-2Rα**. It was shown that the cytoplasmic domain of IL-15Rα was able to transfer the mitogenic and anti-apoptotic signals in IL-3 deprived IL-2Rα/**IL-15Rα** transfected cells in the presence of IL-2. In contrast, the cells, which express the chimeric receptor IL-15Rα/**IL-2Rα**, responded neither to IL-15 nor IL-2.

In the second part of this work, two different fragments of the human IL-15Rα cytoplasmic domain (65 and 86 C-terminal amino acids) were used as "bait" to fish the IL-15Rα interacting proteins by yeast two-hybrid screening of a human bone marrow cDNA-library. Five interacting proteins were identified: lipocalin 2, importin α, BAT3, ICAM3 and ASNA1. The most frequently isolated protein was lipocalin 2. Lipocalin 2 due to its biological function as a cell specific apoptosis inducer, and importin α, as nuclear transporter, are the most important interacting partners for IL-15Rα. In addition, other alternative spliced isoforms of the IL-15Rα cytoplasmic domain were identified.

Taken together, the results of this work show that the IL-15Rα cytoplasmic domain plays a key role in signal transduction pathways. For the first time it was also shown that lipocalin 2 and importin α bind to the IL-15Rα cytoplasmic domain, which provides new insights in IL-15 signal transduction pathways. In addition, identification of alternatively spliced isoforms of the cytoplasmic domain reveals a new aspect of the diversity of IL-15Rα mediated signalling pathways stimulated by the pleiotropic cytokine IL-15.

1 INTRODUCTION

1.1 IL-15

Intracellular communications, essential for regulatory and effector functions during immune responses, are mediated by soluble factors termed cytokines. Cytokines exhibit a high degree of redundancy and pleiotropy, controlling a wide range of functions in various cell types. Interleukin 15 (IL-15) is a 14- to 15-kDa cytokine and like IL-2 belongs to the four α-helix bundle cytokine family. IL-15 was discovered through its capacity to replace IL-2 in supporting the growth of the murine IL-2-dependent CTLL cell line (Grabstein 1994, Burton 1994).

Analysis of the predicted secondary/tertiary structure revealed similarities to IL-2, despite no significant sequence homology between IL-2 and IL-15 (Grabstein 1994). IL-15 shares a number of biological activities with IL-2: IL-15 is a potent growth factor for T cells, B cells and NK cells (Armitage 1995, Grabstein 1994), acts as a T cell chemoattractant (Wilkinson 1995) and facilitates the production of IFN-γ and tumor necrosis factor-α (Carson 1994).

An additional function of general biological importance was described for IL-15: its property as a potent inhibitor of anti-Fas and growth-factor-deprivation-induced apoptosis (Lindner 1998, Bulfone-Paus 1997).

Dramatic differences exist between IL-15 and IL-2 in terms of their expression and the level of control of their synthesis and secretion. IL-15 production is controlled at the levels of transcription, translation, and intracellular trafficking (Kurys 2000, Onu 1997).

Unlike IL-2 mRNA, which is restricted to activated T cells, IL-15 mRNA is expressed by a variety of tissues and cell types, including monocytes/macrophages, keratinocytes, fibroblasts, nerve cells, placenta, skeletal muscles, and the heart (Satoh 1998, Bamford 1996, Grabstein 1994). IL-15 also has unique functions on an array of non-immunological cells, muscle cells, brain microglia and epithelial cells (Quinn 1997, Tagaya 1996 and 1996a).

Two isoforms of IL-15 are described, which differ in the length of their leader peptides. The long form contains 48 and the short form 21 amino acids (aa) (Onu 1997, Meazza 1996). The short signal peptide-IL-15 (SSP-IL-15) is not secreted but rather stored intracellularly in nuclear und cytoplasmic compartments, whereas long signal peptide-IL-15 (LSP-IL-15) is secreted at low levels (Kurys 2000, Tagaya 1997).

1.2 The IL-15 receptor alpha (IL-15Rα)

Biological effects of IL-15 are mediated after binding to its widely expressed heterotrimeric receptor complex, which consists of three subunits – the IL-15Rα, IL-2Rβ and IL-2Rγ chains (Giri 1994, Leonard 1994). The IL-15Rα chain is the specific receptor for IL-15. The IL-2Rβ subunit is shared between IL-2 and IL-15, while the γ_c chain serves as a common component of the receptors for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 (Parrish-Novak 2000, Lehours 2000, Sugamura 1996).

The cytokine specificity is determined by the unique IL-15Rα (Lehous 2000, Giri 1995), which binds IL-15 with high affinity (Kd ~10^{-11} M) (Giri 1995). This is contrasted by a low affinity (Kd ~10^{-9} M) association between IL-2Rα and IL-2, making it reasonable to speculate that the IL-15Rα subunit might be responsible for the differential functional effects of IL-15 and IL-2 on cells of the same type.

The human IL-15Rα is a type I transmembrane protein with a signal peptide of 32 amino acids (aa), an extracellular domain of 173 aa, a transmembrane domain of 21 aa, a 37-aa cytoplasmic tail, and multiple N- or O-linked glycosylation sites (Dubois 1999, Giri 1995). Comparison of the IL-2Rα and the IL-15Rα revealed the presence of a conserved protein binding motif (the sushi domain or GP-1 motif) and a similar intron/exon structure, placing IL-2Rα and IL-15Rα as the founding members of a new receptor family (Giri 1995).

The sushi domain, a common motif in protein-protein interaction, contains four cysteines forming two disulfide bounds in a 1-3 and 2-4 pattern and is critical for the binding of IL-15 (Wei 2001). The IL-2Rα contains two sushi domains, whereas IL-15Rα contains only one (Fig. 1).

IL-2 and its specific IL-2Rα chain expression is restricted to lymphoid cells. In contrast, IL-15 and its specific IL-15Rα are transcribed by a broad variety of different tissues and cells, including activated macrophages, bone marrow, keratinocytes, muscle cells, kidney, endothelial and neural cells (Rückert 2000, Waldmann 1999, Anderson 1995).

Eight splice variants of the human IL-15Rα have been identified (Dubois 1999, Anderson 1995a). One main class of transcripts does not contain the exon 2-coding sequence (Δ2 isoforms) (Fig. 2A). All isoforms are expressed in numerous cell lines and tissues and respective receptor forms display numerous glycosylation states, reflecting differential usage

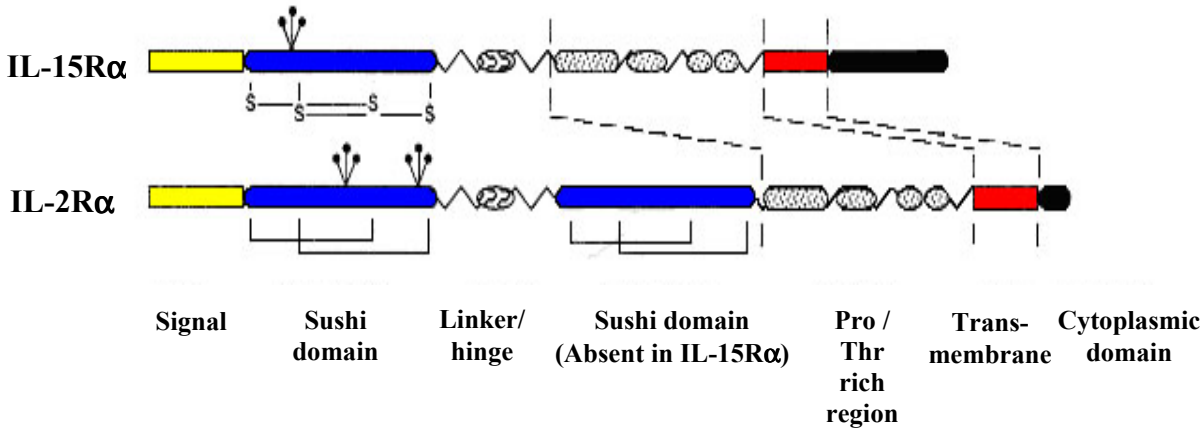


Figure 1. Structural comparison between IL-15Rα and IL-2Rα.
Both receptors are type I transmembrane receptors consisting of an extracellular, transmembrane and cytoplasmic domain. Structural comparison between IL-15Rα and IL-2Rα revealed, that the IL-15Rα contains only one short consensus "sushi" domain, whereas the IL-2Rα contains two. Potential N-linked glycosylation sites are indicated by ball-and-stick symbols (adapted from Giri 1995).

of a single N-glycosylation site as well as extensive O-glycosylation. Δ2IL-15Rα isoforms are unable to bind IL-15, thus revealing the indispensable role of the exon 2-encoded domain for cytokine binding (Fig. 2B).

IL-15Rα is expressed at low density on the cell surface (~1000 sites/cell). Confocal immunofluorescence studies and analysis of subcellular fractions showed that most of the IL-15Rα is associated with the nuclear membrane. A large proportion of this nuclear receptor is heavily O-glycosylated, suggesting that it is routed to the nuclear membrane through the Golgi. Due to the relatively large size (~60 kDa) of the glycosylated receptor, this observation suggests that an active mechanism is involved in its nuclear translocation, rather than passive diffusion through the nuclear pores. The presence of a putative nuclear localization signal (NLS) was found within the human IL-15Rα sequence. This sequence consists of two clusters of polycationic residues separated by a spacer (RERYICNSGFKRK, amino acids 24-36) (Andeson 1995). The possible involvement of this putative NLS in the nuclear routing of IL-15Rα is supported by the fact that the exon 2-truncated receptor, which does not contain this putative NLS motif located in the sushi domain, does not show nuclear localization

(Dubois 1999). It is suggested that exon 2 splicing of human IL-15Rα is a natural process that plays an important role in the receptor post-translational routing and also might have regulatory roles at different levels (Dubois 1999).

Recently our group has shown the expression of three novel IL-15Rα isoforms in murine mast cells (Bulanova 2003). These isoforms result from an alternative splicing of IL-15Rα mRNA and correspond to the deletion of exon 4, exons 3 and 4 and exons 3, 4, and 5 (Fig. 2A). These data show, contrary to the previously held concept about mast cells (Tagaya 1996a), the functional importance of IL-15Rα in mast cells.

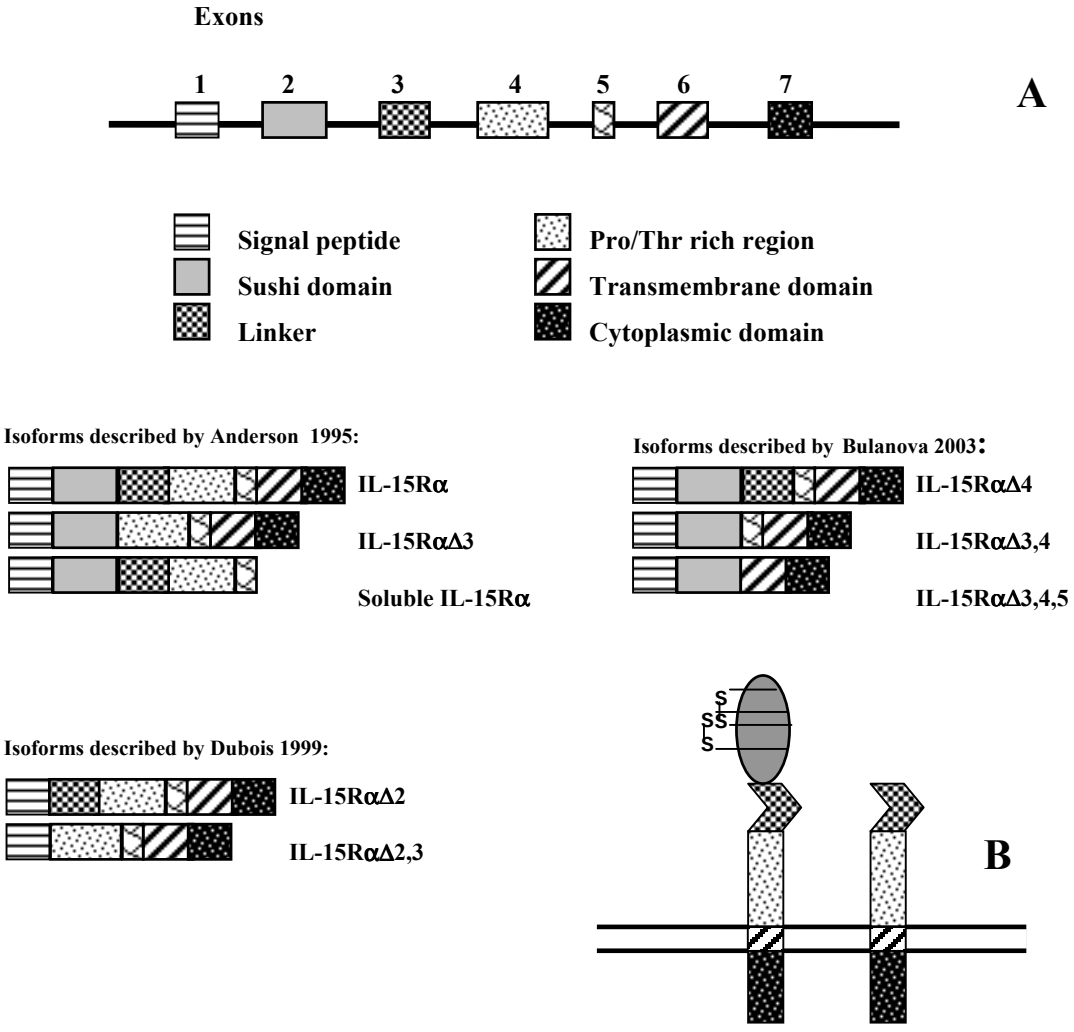


Figure 2. Schematic diagram of the IL-15Rα gene, transcription products and receptors. *A) The mouse IL-15Rα gene consists of seven exons. Recently, three novel isoforms of IL-15Rα were cloned from murine mast cells (Bulanova 2003). The human isoforms of IL-15Rα were described by Andeson and Dubois. B) Schematic structure of the IL-15Rα protein with and without exon 2, which codes for the Sushi domain.*

1.3 Signal transduction pathway of IL-15/IL-15Rα

1.3.1 Signalling via heterotrimeric IL-15Rα/IL-2Rβ/IL-2Rγ complex

IL-15 interacts with a receptor complex that is composed of three chains, IL-15Rα, IL-2Rβ and IL-2Rγ (Fig. 3). The IL-2Rβ and IL-2Rγ are shared with the IL-2R complex.

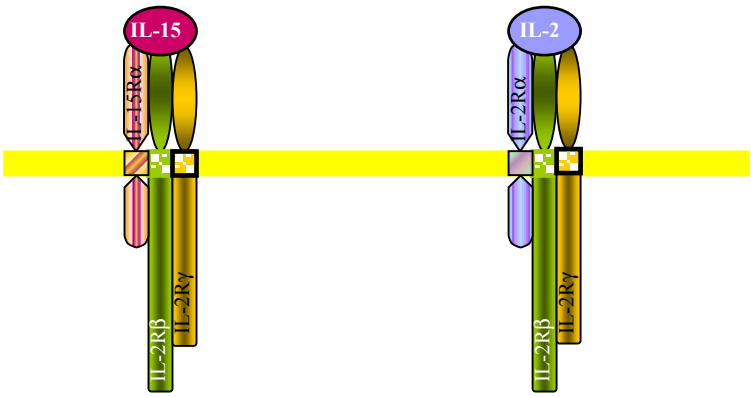


Figure 3. IL-15R and IL-2R heterotrimeric receptor complex.
Both IL-15 and IL-2 bind to a heterotrimeric receptor, which shares IL-2Rβ and IL-2Rγ, but has a unique α-chain that recognizes only its cognate cytokine.

Because IL-2 and IL-15 share two signalling components (IL-2Rβγ), they induce similar signalling pathway in various cell types, including activation of the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway (Ihle 1995).

The cytokine receptors belong to a receptor family characterized in part by the absence of intrinsic kinase activity in their intracellular domain and by their selective activation of the JAK/STAT pathway.

IL-2Rβ is associated with JAK1 and IL-2Rγ is associated with JAK3, resulting in STAT3 and STAT5 phosphorylation, respectively, after IL-15 binding (Lin 1995, Miyazaki 1994).

The Janus kinase family contain four members: JAK1, JAK2, JAK3 and Tyk2, all of which are large enzymes. Janus kinases comprise FERM, SH2, pseudokinase, and kinase domains.

The FERM domain mediates receptor association. Both the FERM and pseudokinase domains regulate catalytic activity (Hilkens 2001, Zhou 2001).

Despite the gaps in our knowledge, there is a general agreement on the overall scheme of cytokine signalling (Fig. 4): After cytokine-induced receptor aggregation, JAKs are activated by auto- and transphosphorylation. Subsequently, the JAKs phosphorylate the tyrosine residues within the cytoplasmic tail of the cytokine-receptor, providing recognition and docking sites for SH2 (Src homology 2) containing proteins. One class of SH2 bearing proteins are STATs, latent cytosolic transcription factors, which contain the following domains: N-terminal, coiled-coil, SH2, linker, DNA binding, and transcriptional activation domains.

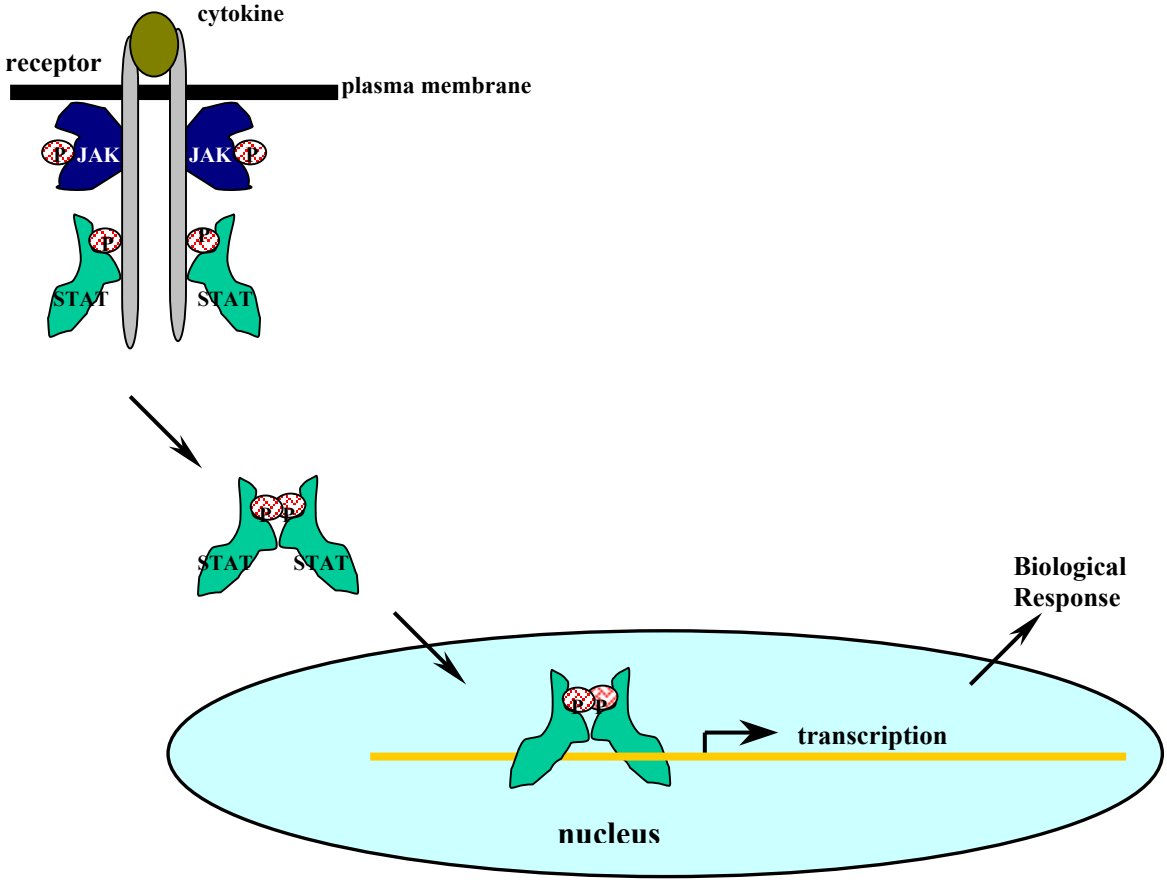


Figure 4. Cytokine signal transduction pathway.
Cytokine binding to the cytokine-receptors results in association of Janus kinases (JAKs) to the receptor. JAKs are activated via transphosphorylation and they in turn phosphorylate cytokine receptors allowing STATs to bind via SH2-phosphoserine interactions. STATs themselves are phosphorylated, permitting STAT dimerization and translocation to the nucleus, where they bind DNA and regulate gene expression (O'Shea 2002).

The receptor-bound STATs are then phosphorylated by JAKs on a conserved tyrosine residue and SH2 domain, resulting in dimerization through reciprocal phosphotyrosine/SH2 interactions. The phosphorylated STAT dimer translocates to the nucleus and binds to specific DNA sequences of target genes and regulates gene expression (Fig. 4) (O'Shea 2002).

Additional signalling pathways of IL-15 through the IL-2Rβγ complexes include the src-related tyrosine kinases, induction of Bcl-2, and stimulation of the Ras/Raf/MAPK pathway that ultimately results in fos/jun activation (Miyazaki 1995).

In neutrophils, IL-15 has been shown to activate NF-κB but not AP-1, whereas IL-15 stimulation of human peripheral blood lymphocytes activates both transcription factors (McDonald 1998).

1.3.2 IL-15Rα/Syk signalling

Our group has shown, that the IL-15Rα mediates signalling in activated B cells (Bulanova 2001). To analyse this, Raji cells, a human B-lymphoblastoid cell line was used, which expresses IL-15Rα and IL-2Rγ but lacks the IL-2Rβ chains. IL-15 induces proliferation of Raji cells and rescues them from C2-ceramide-induced apoptosis, which is abrogated by anti-IL-15Rα Ab treatment, but not with anti-IL-15Rγ Ab. Immunoprecipitation and Western blotting indicates, that the intracellular domain of IL-15Rα specifically associates with Syk upon IL-15 stimulation. Syk is a tyrosine kinase of the Syk/Zap70 family, involved in antigen receptor signalling in B and T cells (Chu 1996, Cheng 1995). Syk carries two src homology (SH2) domains capable of interacting with tyrosine-phosphorylated proteins (Law 1994).

Syk binds IL-15Rα perhaps via its SH2 domain, and phosphorylates the Tyr^{227} of IL-15Rα. Additionally, Syk phosphorylates phospholipase C-γ1 (PLCγ1), a substrate for the Syk kinase (Law 1996). The activated PLC-γ isoforms hydrolyse the phosphoinositides into diacylglycerol and inositol phosphates, resulting in activation of protein kinase C and increasing the concentration of intracellular free Ca^{2+} (Berridge 1993). Our group has also shown that Syk mediates IL-15 induced Ca^{2+} influx in Raji cells.

Thus, the PLCγ1 is a downstream signalling molecule after IL-15 stimulation in Raji cells.

1.3.3 IL-15Rα/TRAF2 signalling

A very important biological function of IL-15 is its potent inhibition of apoptosis in activated T and B cells *in vitro* and *in vivo,* thus protecting mice from anti-Fas induced lethal hepatic failure and multi-system apoptosis and from chemotherapy-induced epithelial cell apoptosis (Lindner 1998, Bulfone-Paus 1997).

Apoptosis is triggered in multiple cell types by signalling via members of the tumor necrosis factor (TNF) receptor family, including the TNF-α receptor type 1 (TNFR1) (Wallach 1997, Nagata 1997). TNFR1 induces apoptosis via an intracellular "death domain" (DD), a ~80 amino acid domain found in the cytoplasmic region. Oligomerization of TNFR1 by the trimeric ligand orients the DD in a conformation, that allows the recruitment of adaptor proteins. The adaptor proteins also contain a DD and associate with the TNFR1 through a homotypic DD interaction (Chinnaiyan 1996, Kitson 1996). The downstream signal-transduction and receptor-associated proteins that couple the TNFR1 have been identified: TRADD (TNFR1-associated death domain protein) (Wallach 1997), FADD (Fas-associated protein with death domain) (Chinnaiyan 1995), RIP (receptor interacting protein) (Hsu 1996), and TRAF2 (TNFR-associated factor 2) (Baker 1996, Rothe 1994). Presumably, two TNFR1-signaling cascades bifurcate at TRADD: one induces NF-κB activity, which may promote cell survival (Beg 1996); the other induces apoptosis via FADD and the caspase machinery (Fig. 5A). RIP is recruited to the TNFR1 complex with bifunctional activities, since it may promote either cell death or NF-κB activation (Chinnaiyan 1996). TRAF2 interacts with TRADD and RIP through homotypic TRAF domain interactions and is involved in the TNF-dependent activation of NF-κB (Hsu 1996a). NF-κB complexes are sequestered in the cytosol bound to one or more inhibitor proteins, of which the prototype is IκBα. Upon stimulation, IκBα dissociates from NF-κB as a result of phosphorylation and proteolytic degradation, thus permitting NF-κB to translocate to the nucleus (Baldwin 1996).

In our group, the ability of IL-15 to block TNFR1-mediated pro-apoptotic signalling was investigated in a murine fibroblast cell line (L929), which is highly sensitive to TNF-α-induced apoptosis (Xie 1993). L929 expresses IL-15Rα, only marginally IL-2Rβ, but not IL-2Rγ. IL-15 blocks TNF-α-induces apoptosis in L929 cells via IL-15Rα signalling (Bulfone-Paus 1999). IL-15Rα deflects TRAF2 from the TNFR1 complex and recruits it for NF-κB activation (Fig. 5B).

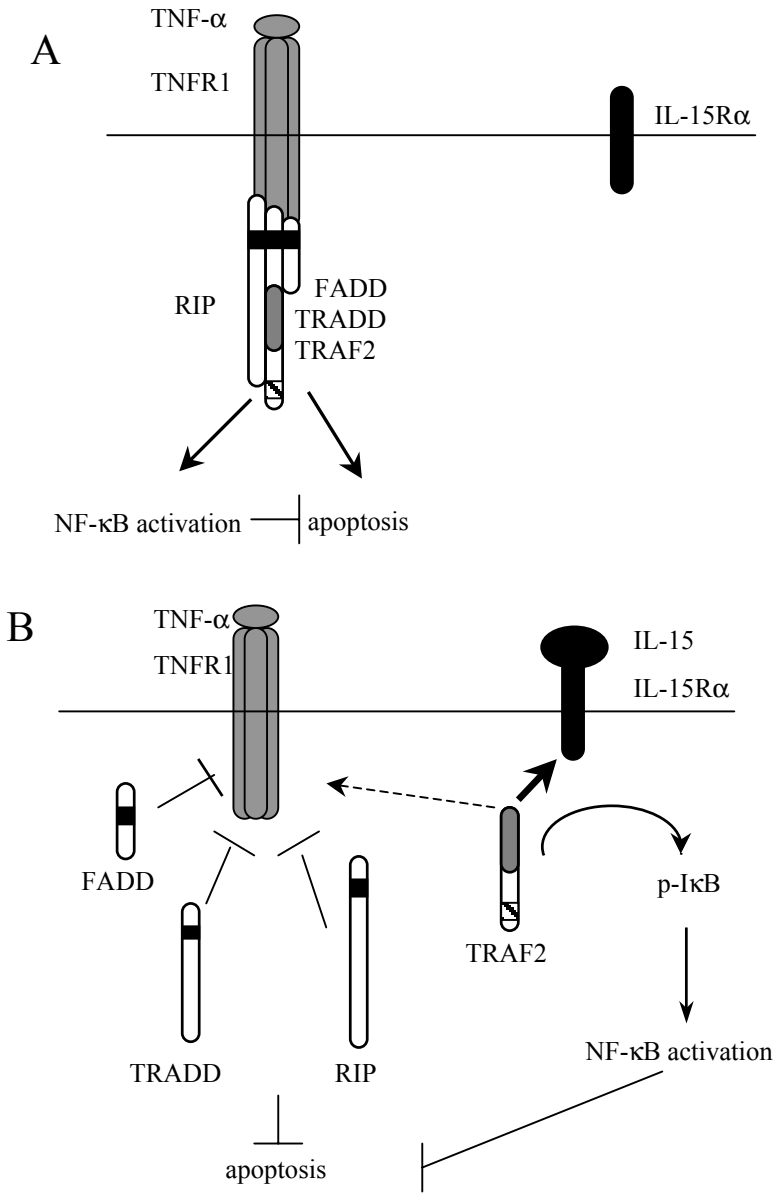


Figure 5. IL-15Rα mediates anti-apoptotic signals via binding to TRAF2.
A) Ligand binding to TNFR1 induces recruitment of the adaptor proteins TRADD, FADD, and RIP to the cytoplasmic domain of the receptor via homotypic death domain interactions. In addition, TRAF2 association with TRADD and RIP mediates NF-kB activation, which may inhibit apoptosis. In contrast, FADD association with TRADD is a key positive signal for undergoing apoptosis. B) TNF-α and IL-15 costimulation of the respective receptors is postulated to inhibit the association of TRADD, FADD, RIP, and TRAF2 with the TNFR1 cytoplasmic region, whereas the IL-15Rα chain, which displays a TRAF2 binding motif, directly interacts with TRAF2. This "depletes" TRAF2 from the ligand-stimulated TNFR1 complex, thus preventing assembly of the adaptor protein complex that normally signals for induction of apoptosis. In addition, the NF-κB antagonist IκB is phosphorylated upon IL-15Rα stimulation, possibly with participation of TRAF2, thus activating NF-κB. This rescues cells from TNFR1-mediated apoptosis (Bulfone-Paus 1999).

TRAF2 binds IL-15Rα via its cytoplasmic VEVET motif. In addition, stimulation of L929 cells with TNF-α and IL-15 induces IκBα phosphorylation, resulting in NF-κB activation, which may be used by IL-15-stimulated IL-15Rα as a second pathway for inhibiting TNFR1-mediated apoptosis in this fibroblasts (Bulfone-Paus 1999).

Additional interactions between IL-15Rα and TRAF2 and activation of NF-κB have been also shown in two human-melanoma cell lines, MELP and MELREO (Pereno 2000). In MELP cells, inhibition of endogenous IL-15 causes two distinct events: loss of co-localization between TRAF2 and the IL-15Rα chain and loss of the NF-κB nuclear localization, suggesting that association of TRAF2 and IL-15Rα is controlled by endogenous secreted forms of IL-15. Treatment with anti-IL-15Rα and TNF-α triggers deflection of TRAF2 from IL-15Rα towards TNFR1, restoring the activation of the NF-κB pathway through the TNF receptor and increasing the phosphorylation of Iκ-Bα.
In contrast, in MELREO cells, inhibition of endogenous IL-15 causes the loss of NF-κB activation, but cannot dissociate TRAF2 from the IL-15Rα chain. Thus, in these cells the use of anti-IL-15 Ab inhibits Iκ-Bα phosphorylation and NF-κB nuclear localization, but these events cannot be restored by the addition of TNF-α, since TRAF2 is not deflected from IL-15Rα to TNFR1 chain.
Moreover, there is a different IL-15/IL-15Rα intracellular trafficking in these two cell lines. In MELP cells only IL-15Rα is detected inside the nucleus, whereas IL-15 and IL-15Rα assemble at the cell surface and are internalised. In contrast, MELREO cells display IL-15Rα and IL-15 nuclear localization but only a partial co-localization of these molecules on the cell surface (Pereno 2000). Thus, IL-15Rα controls NF-κB activation, however differences in the intracellular trafficking of the IL-15 and/or IL-15Rα suggest a different biological role for this complex in MELP versus MELREO cells.
Recently, a novel IL-15Rα/TRAF2 mediated NF-κB activation pathway was identified, triggered by the IL-15Rα/IL-2Rγ/TRAF2 complex (Giron-Michel 2003).

1.3.4 IL-15Rα endosomal internalisation pathway

A common event following receptor engagement in many systems is the internalisation of the ligand/receptor complex. IL-15Rα belongs to this category of receptors. After endocytosis, many ligand/receptor complexes are transported to late endosomal compartments to be degraded in the lysosomes (Mukherjee 1997).

In contrast, IL-15Rα is not targeted to the degradation pathway after interaction with IL-15 (Dubois 2002). Consistent with the high-affinity binding of IL-15 to IL-15Rα, these two molecules form stable complexes on the cell surface and are internalised by endosomes. Later the IL-15/IL-15Rα complexes reappear again on the cell surface. Therefore, formation of IL-15/IL-15Rα complexes on cell surfaces induces a trans-endosomal recycling of IL-15 leading to the persistence of surface-bound IL-15 due to the constant reappearance of IL-15 on plasma membranes (Dubois 2002). The IL-15Rα cytoplasmic domain appears to be crucial for the recycling process since its deletion abrogated IL-15 recycling.

The IL-15Rα-mediated IL-15 recycling process, which leads to the persistence of IL-15 on the plasma membrane, contributes to the long survival of T cells expressing IL-15Rα after IL-15 withdrawal from the intercellular environment. Furthermore IL-15 associated with IL-15Rα on some types of cells (such as monocytes, dendritic, and stromal cells) can stimulate in trans the target cells (such as T cells) upon cell-cell interactions (Dubois 2002).

1.4 Yeast two-hybrid system

Protein- protein interaction is a central event in regulating many different cellular processes such as DNA synthesis, transcriptional activation, protein translation, protein localization and signal transduction. Fields and Song (Fields 1989) were the first who developed a molecular genetic screen to detect protein-protein interactions. This screen, performed in the yeast *Saccharomyces cerevisiae*, is commonly referred to as the ”yeast two-hybrid system”, and is now a standard procedure in molecular biology.

Since publication of this technique, the robust nature and far-reaching utility of yeast two-hybrid systems for functional expression library cloning has led to the identification of many novel proteins in all areas of biological life science research.

The yeast two-hybrid system offers a number of advantages. It is relatively inexpensive since it avoids costly procedures such as antibody production and protein purification (Sobhanifar 2003, Van-Crieknige 1999). cDNA expression libraries can easily be screened to isolate proteins interacting with the protein of interest. In this way, not only are the interacting proteins identified, but also the cloned cDNAs encoding them become available, so simplifying further studies. The system is often more sensitive than many *in vitro* techniques, and may be more suited for the detection of weak or transient interactions. Proteins expressed *in vitro*, or in bacterial cells, often lack key post-translational modifications that may be important for certain protein-protein interactions. In addition, the proteins may not fold correctly or may not be stable in the buffer conditions used. The yeast two-hybrid system simplifies mutational analyses, facilitating the mapping of motifs or residues required for protein-protein interaction (Sobhanifar 2003, Van-Crieknige 1999).
Over the 13 years since the first report of the system, the yeast two-hybrid has been modified in numerous ways and has been adapted for the study of not only protein-protein interactions but also DNA-protein interactions (the yeast one-hybrid system) and RNA-protein interactions (the yeast three-hybrid system; reviewed by Brent and Finley, 1997). Yeast cells offer a convenient system for these types of interaction studies, however, the system has also been adapted to use bacterial and mammalian cells.

1.4.1 The principle of the two-hybrid system

The early two-hybrid system is based on the fact that many eukaryotic transcriptional activators consist of two physically separable domains: one acts as DNA-binding domain, while the other functions as transcriptional activation domain.
The DNA-binding domain localizes the transcription factor to specific DNA sequences present in the upstream region of genes that are regulated by this factor, while the activation domain contacts the transcription machinery. Both domains are required for the normal function. Critical for the development of the two-hybrid system was the discovery that DNA-binding and transcriptional activation act independently. It was demonstrated, that if the DNA-binding domain of bacterial LexA is fused to the transcriptional activator domain of yeast GAL4, the LexA-GAL4 fusion protein actives transcription in yeast containing a reporter gene under the control of LexA operator sequences (Brent 1985). In the case of this

fusion protein, the DNA-binding activity is provided by the LexA component and the activation function by the GAL4 component (Fig. 6A).

It was then formally demonstrated that virtually any pair of proteins that interact with each other might be used to bring separate activation and DNA-binding domains together to reconstitute a transcriptional activator (Fields 1989). In a typical application, a favourite protein X is expressed as fusion protein with a specific DNA-binding domain (DBD); this fusion protein is termed as "bait". The protein is expressed fused to an activation domain (AD) termed as "prey". If bait and prey interact, the transcription machinery will be activated and the reporter gene will be expressed (Fig. 6B).

The most commonly used systems are the GAL4 system (in which the DNA-binding and activation domains of the yeast GAL4 protein are used; Fields 1989) and the LexA system (DNA-binding domain of the bacterial repressor protein LexA used in combination with the Escherichia coli B42 activation domain; Gyuris 1993). The LexA system is generally known as the interaction trap system.

The two hybrid system consist of three components:

a) An expression vector, encoding the favourite protein "bait", fused in frame to a DBD.
b) An expression vector, encoding the interacting protein "prey", or a pool of proteins encoded by a cDNA library, fused to an AD.
c) A reporter gene, which contains an operator or upstream activation sequences (UAS) specially recognized by the DBD.

In theory, one should be able to utilize any reporter gene that functions in yeast to monitor transcriptional activation. Mostly, lacZ has been utilized, however, prototrophic markers like His3 are also commonly used. These reporters have the advantage that they allow the selection of colonies, in which transcription has been activated.

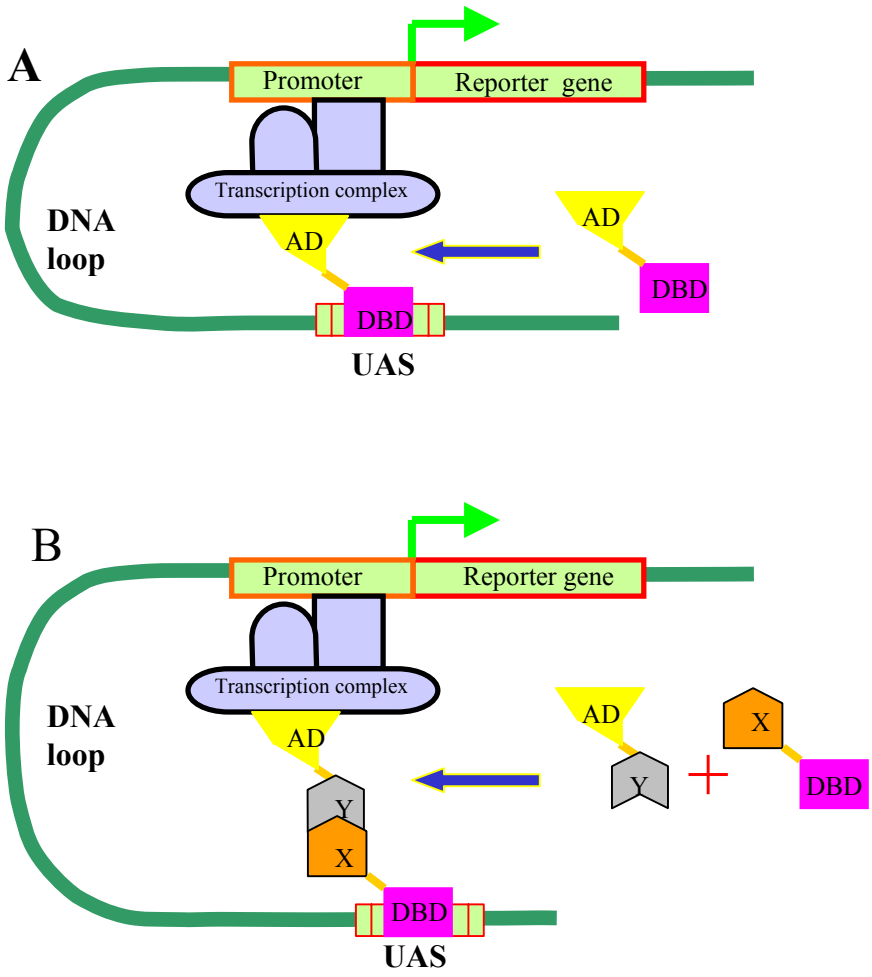


Figure 6. Schematic diagram of transcriptional activation in a typical eukaryotic system and in the two-hybrid system.
A) Activation of transcription by a typical eukaryotic transcriptional activator with a separable DNA binding domain (DBD) and activation domain (AD). The operator or upstream activation sequence (UAS) is specifically recognized by the DB. B) Activation of transcription by fusion proteins in the two-hybrid system. X represents a given protein fused to a specific DBD. This protein is termed the "bait". Y represents a given protein, or a pool of proteins encoded by a cDNA library, fused to a transcriptional activation domain. This fusion protein is often termed "prey". If X and Y bind to each other, AD is brought to the vicinity of the DNA bound DB and transcription is activated from the adjacent promoter (adapted from Luban 1995).

1.4.2 The two-hybrid system used for the identification of IL-15Rα interacting proteins

The major methods to study protein-protein interactions are: two-hybrid, pull-down *in vitro* assay, immunoprecipitation, mass spectrometry and fluorescence resonance energy transfer. Comparing advantages and limitations of these methods (Vollert 2003, Causier 2002, Uetz 2000, Cagney 2000, Mendelsohn 1999,Van-Criekinge 1999, Phizicky 1995) reveals that, the two-hybrid assays have been very successful at identifying real interactions. It has been estimated that more than 50% of all protein interactions described in the literature have been detected using the yeast two-hybrid system (Vollert 2003). The article from Fields et al., which first described the technique, has been cited nearly 2500 times, demonstrating its high value (Sobhanifar 2003). Therefore, the yeast two-hybrid system was applied here as an appropriate choice to identify proteins, interacting with IL-15Rα. The two-hybrid system has been also used for identification of interacting partners for the cytoplasmic domains of other cytokine receptors, such as IL-5Rα and IL-9Rα, and proved to be efficient for this application (Geijsen 2001, Sliva 2000).

I employed a version of the two-hybrid system, that is a combination of LexA- and Gal4-systems (Gisler 2001, Van-Criekinge 1999). The IL-15Rα cytoplasmic domain is expressed as "bait" in fusion with DBD of LexA. The cDNA-library, which provides a pool of "prey", is expressed as fusion protein with the Gal4 activation domain. The yeast strain L40 is used as host, which carries the lacZ reporter gene and the selection reporter gene His3 (Vojtek 1993). Both genes are integrated into the yeast genome under control of the LexA binding site, the UAS. This strain is mutated in the chromosomal copy of the His3 gene. Only those transformants that express interacting hybrid protein can survive in media lacking histidine nutrient. His3 codes for imidazole-glycerolphosphat-dehydratase, an enzyme involved in the histidine biosynthetic pathway. Because the His3 promoter expresses His3-protein at low level in the absence of any two-hybrid interaction, the background cell growth is reduced by growing the cells in the presence of 3-amino-triazole (3-AT), a competitive inhibitor of the imidazole-glycerolphosphat-dehydratase enzyme (Durfee 1993).

Bacterial lacZ, the second reporter gene of L40, allows screening of colonies that express interacting hybrid proteins by detection of β-galactosidase expression. The enzymatic activity of given colonies can be assessed on nitrocellulose filter replicas soaked in X-gal. This

method is preferred for screening libraries because the blue colour of an individual colony is easily seen amid a 'sea' of white colonies. L40 has two other nutritional marker genes, TRP1 and LEU2, which allow selection of plasmids encoding the bait and prey hybrid proteins (Fig. 7).

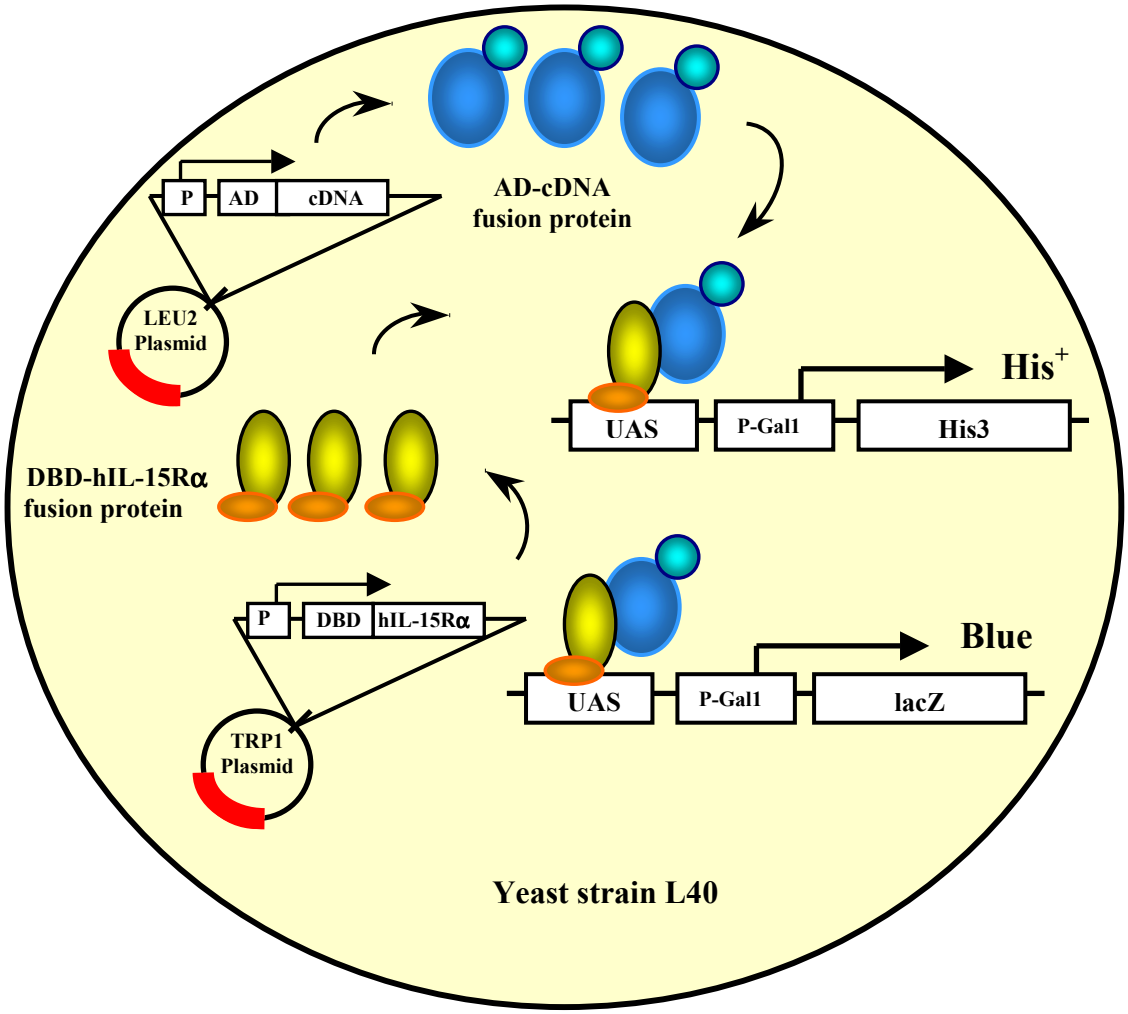


Figure 7. The yeast two-hybrid system used for identification of IL-15Rα interacting proteins.
The yeast cell (L40 strain) contains two plasmids, one encodes the DNA binding domain (DBD) of LexA fused to the hIL-15Rα cDNA and expressed from promoter P. This plasmid carries the TRP1 gene for selection in yeast and produces the DBD-hIL-15Rα fusion protein. The other plasmid encodes the Gal4 transcription activation domain (AD) fused to a cDNA sequence expressed from promoter P. This plasmid carries the LEU2 gene for selection in yeast and produces the AD-cDNA fusion protein. The yeast strain L40 contains two integrated reporter gene constructs. These reporter genes consist of Gal1 promoter (P-Gal1) fused to the selectable yeast gene (His3) and the E.coli lacZ gene. The DBD of DBD-hIL-15Rα fusion protein binds to the upstream activating sequence (UAS). Interaction between DBD-hIL-15Rα and AD-cDNA fusion proteins stimulates transcription of the reporter genes, indicated by arrows. Transcription and translation of His3 allows selection in synthetic complete medium

lacking histidine. These His^+ yeast colonies will also have β-galactosidase activity and turn blue in the presence of X-Gal (modified after Gietz 1997).

For quantitative analysis of protein-protein interactions, I used another reporter system using GFP (green fluorescence protein), which is provided by the yeast strain YRN974 (Mancini 1997). The GFP gene from the jellyfish *Aequorea Victoria* was chromosomally integrated down stream of the LexA binding site. Production of GFP is monitored and quantified by flow cytometry, which proved to be a fast and very sensitive technique. YRN974 contains also the TRP1 and LEU2 nutritional marker genes.

1.4.3 Advantages and limitations of the yeast two-hybrid system

The yeast two-hybrid system has a clear advantage over classical biochemical or genetic methods, in that it is an *in vivo* technique that uses the yeast cell as a living test-tube. This yeast system mimics higher eukaryotic reality better than most *in vitro* approaches or techniques based on bacterial expression.

With regard to classical biochemical approaches, which can require high quantities of purified proteins or good quality antibodies, the two-hybrid system has minimal requirements to initiate screening, since only the cDNA of the gene of interest is needed.

Weak and transient interactions, often the most interesting in signalling cascades, are more readily detected in the two-hybrid system since the genetic reporter gene strategy results in a significant amplification.

Apart from the ability to screen libraries, the two-hybrid system also allows the analysis of known interactions. This can be achieved by modifying crucial residues for interaction or by a functional characterization of the entire subdomain. By doing semi-quantitative experiments one can even interpret affinities from two-hybrid experiments. It was demonstrated that the strength of interaction as predicted by the two-hybrid approach generally correlates with that determined *in vitro*, permitting discrimination of high-, intermediate- and low-affinity interactions (Estojak 1995).

Although the two-hybrid assay was predicted to be limited to the study of cellular proteins, given that extracellular proteins often undergo modifications such as glycosylation or

disulfide links, not expected to occur in the yeast nucleus, there have been various reported successes with extracellular receptor/ligand complexes (Kajkowski 1997, Young 1995).

It should be noted, however, that the two-hybrid system does not provide a solution for all protein-protein problems. For various experimental reasons some proteins are not suited for this approach. Since the two-hybrid assay measures reporter activity in response to transcriptional activation, an obvious problem would arise if the proteins of interest are able to activate transcription on its own (auto-activation). It is, therefore, imperative that an initial experiment is done to test for the transcriptional activity of the protein of interest itself (Causier 2002).

Another critique concerns the extensive use of hybrid proteins. The use of artificial fusion proteins always embodies a potential risk. The fusion might change the actual conformation of the bait and/or prey and consequently alter functionalities. This misconformation might result in a limited activity or in the inaccessibility of binding sites (Van-Criekinge 1999).

A major drawback of testing protein-protein interactions in a heterologous system such as the yeast is that interactions may depend on certain post-transcriptional modifications, such as disulfide bridge formation, glycosylation, or phosphorylation, which may not occur properly or at all in the yeast system (Sobhanifar 2003, Van-Criekinge 1999, Fields 1994).

Since only reporter gene activity is measured, it is impossible to exclude the possibility that a third protein Z is bridging the two interacting partners. Although this possibility is rather unlikely and might even be considered as "specific," it holds for many of the conventional biochemical techniques.

Certain proteins, when expressed in the yeast system or targeted to the nucleus, may become toxic. Other proteins may degrade essential yeast proteins or proteins whose presence are required for the assay. Such genes may be counter-selected for during growth and may result in problems (Sobhanifar 2003 Causier 2002). As typical for all exhaustive screening assays, the identification of false binding partners presents itself as a disadvantage in the two-hybrid assay.

Due to the so-called time/space constraints it is potentially possible that both proteins, although able to interact, are never in close proximity to each other within the cell. The two proteins could be expressed in different cell types, or even when found in the same cell they could be localized in distinct sub-cellular compartments. Moreover, interacting proteins can be expressed at different points during embryogenesis or during homeostasis (e.g. at different time points in the cell cycle). So if two interacting partners are identified, the biological relevance of this interaction remains to be determined (Van-Criekinge 1999).

Although there are certain disadvantages involving the two-hybrid assay, the most convincing argument for its use is the speed and ease by which the molecular mechanisms of many signalling cascades have been defined using this technique.

1.4.4 Variation of the two-hybrid system

The two-hybrid system became an essential genetic tool for investigation of protein-protein interactions. Over the past several years numerous variations of two-hybrid paradigm have been developed to overcome the limitation of the classical system. (Vollert 2003, Sobhanifar 2003, Causier 2002,Van-Criekinge 1999)

Three-hybrid system

The yeast three-hybrid system has been developed to study the formation of ternary complexes (Egea-Cortines 1999, Zhang 1996). Via this system, protein complex interactions can be investigated, in which the stable interaction between X and Y may rely on the presence of a third protein Z. Protein Z either mediates the interaction or induces a conformational change in one of the proteins (e.g. X) so that it promotes interaction with protein Y. The kinase three-hybrid system (or tri-brid), one subclass of this system, can detect protein-protein interactions that depend on post-translational modifications (Osborne 1995). Some crucial post-translational modifications (such as tyrosine phosphorylation) do not occur in *Saccharomyces cerevisiae.* Osborne and co-workers solved this problem by introducing a third component, a cytosolic tyrosine kinase, which then phosphorylates substrates in the yeast cell (Osborne1995). Sengupta (1996) developed a three-hybrid system to detect and analyse RNA-protein interactions in which the binding of a bifunctional RNA molecule links the DBD and AD hybrid-proteins and activates transcription of the reporter gene. This system is known as RNA three-hybrid system.

Reverse two-hybrid system

The identification of mutations in each partner of an interacting pair of proteins, which disrupt the interactions, can be useful not only for probing the structural components of an interaction, but also as a way to generate genetic tools for characterizing *in vivo* function. This

can be particularly important for proteins that have multiple interaction partners. Expression of variants that interact *in vivo* with only a subset of partners can provide information about which interactions are important to mediate specific activities in the cell.

The "reverse" two-hybrid system has been invented to select for disrupted two-hybrid interactions e.g. by mutations, drugs or competing proteins (Vidal 1996). In this system the interaction of X and Y proteins induces the transcription of a reporter gene that confers toxicity to the yeast. One of the most widely used markers in the reverse-hybrid system is URA3, which encodes orotidine-5'-phosphate decarboxylase, an enzyme required for the biosynthesis of uracil (Vidal 1996 and 1996a). However, the URA3-encoded decarboxylase can also catalyse the conversion of a non-toxic analogue, 5-fluoroorotic acid (FOA) into a toxic product, 5-fuorouracil (Boeke 1984). Interaction of bait and prey in the presence of FOA is lethal. Therefore, dissociating mutations in interacting proteins can be isolated from a library of randomly generated mutants by selection for 5-FOA-resistant colonies (Vidal 1996a). Other reverse two-hybrid systems, based on different reporter genes, have also been developed for use in yeast (Brent 1997, Leanna 1996).

Ras recruitment system (RRS)

RRS was developed for identification of protein–protein interactions involving membrane proteins by Aronheim (1997a). The method, designated reverse RRS, is based on the fact that Ras localization to the plasma membrane is crucial for its function (Broder 1998).

When Ras localized at the plasma membrane, the yeast Ras guanyl nucleotide exchange factor cdc25 stimulates GDP/GTP exchange on Ras and promotes downstream signalling events that ultimately lead to cell growth. A mutant yeast strain harbouring the temperature sensitive cdc25-2 allele is still able to grow at 25°C but fails to grow at 36°C. Expression of cytoplasmic Ras in yeast does not complement mutations in the Ras guanyl nucleotide exchange factor, CDC25-2 (Petitjean 1990). However, membrane-bound mammalian Ras can efficiently complement CDC25-2 mutations (Aronhem 1997). Ras membrane translocation can be achieved via protein–protein interaction, which can be readily monitored in a Cdc25-2 yeast strain by cell growth at the restrictive temperature 36°C (Hubsman 2001).

USPS system

The ubiquitin-based split-protein sensor system (USPS), a cytoplasmic two-hybrid assay was developed by Johnsson (1998). Ubiquitin is a small protein of 76 amino acids, which acts as a "tag" for protein degradation. Proteins fused to ubiquitin are rapidly cleaved *in vivo* by ubiquitin-specific proteases (UBPs). If the carboxy terminal of ubiquitin (Cub) is fused to a reporter protein and co-expressed with the amino-terminal fragment (Nub), the two halves will reconstitute the native ubiquitin, resulting in the cleavage of the reporter protein. For its adaptation to detect protein-protein interactions a mutant Nub, unable to interact with Cub on its own, was fused to one protein and a Cub reporter hybrid was fused to its prospective interaction partner. Interaction between the two proteins allows ubiquitin to be reconstituted, leading to cleavage and release of the reporter gene.

1.4.5 Applications of yeast two-hybrid on proteomics

Proteomics is a new research area of the post-genomic era that aims at the analysis and identification of the entire proteins present in one cell, tissue or organism, and of functions and linkages of these proteins. Protein-protein interactions are characteristic of cellular activities and of course an important part of proteomics (Uetz 2001).

The two-hybrid assay can be used to establish protein linkage maps (PLM). These maps consist of all possible protein interactions that occur during the entire lifespan of a cell. PLMs might provide new functions of well-studied proteins by identification of unexpected interactions, clarify cross-connections between pathways, resulting in new drug-targets and the identification of new functions and gain some insight into overall cell complexity (Van-Criekinge 1999). In this approach, initially random libraries are fused both to the BD and AD and exhaustively screened for all possible interactions. The first genome-wide two-hybrid study was carried out on bacteriophage T7, in which a library of random T7 protein fragments was screened against random libraries of T7 activation domain fusions (Bartel 1996).

Development of "array" technology transformed not only the field of genomics but also proteomics. However, two-hybrid screening can also be done in a colony array format, in which each colony expresses a defined pair of proteins (Cagney 2000). Arrays allow a systematic approach to test all possible protein pairs for interaction (Uetz 2000), and also

facilitate the identification of false positives by comparing results of many screens (Uetz 2001).

2 Aims

IL-15 is a pleiotropic cytokine that supports innate and adaptive immune cell development and homeostasis. Biological effects of IL-15 are mediated by a widely expressed heterotrimeric receptor complex, consisting of three subunits IL-15Rα, IL-2Rβ and IL-2Rγ (Giri 1994, Leonard 1994).

It had been claimed that the IL-15Rα chain, like IL-2Rα, is not capable of signalling when it is expressed in the absence of IL-2Rβ or γc (Anderson 1995, Giri 1995, Leonard 1994, Giri 1994 Carson 1994). Nevertheless, it has been shown by a number of publications that IL-15Rα is involved in signal transfer (Giron-Michel 2003, Bulanova 2001, Pereno 2000, Bulfone-Paus 1999, Stevens 1997).

Our group presented evidence that TRAF2 and Syk kinase can associate with IL-15Rα (Bulanova 2001, Bulfone-Paus 1999). Although previous work showed phosphorylation of the single tyrosine residue in the cytoplasmic domain of IL-15Rα via Syk in the human B cells the structural and biochemical mechanism of IL-15Rα mediated signalling and also the downstream signal molecules in many different cells are still unknown.

In this context, it is important to understand how IL-15Rα transduces an intracellular signal and how this relates to biological functions.

Usually in transmembrane receptors, ligand binding induces modification in the cytoplasmic domains, interactions with downstream molecules and transmission of the signals. Since no conserved motif was identified in the small cytoplasmic domain of IL-15Rα, the signal transfer process via the cytoplasmic domain is still unclear. Therefore, the aim of this work is the study of the role of the IL-15Rα cytoplasmic domain in signal transduction pathways. In the first part, the involvement of the cytoplasmic domain in signal transfer was investigated. The second part deals with the search of new interaction partners, which are able to bind the cytoplasmic domain of IL-15Rα.

The results arising from these studies help us to clarify the complex and diverse IL-15 signalling network and give also new insights into the regulating pathways of IL-15 in immune responses, which provide novel strategies in scientific and clinical research.

3 MATERIAL AND METHODS

3.1 Material

All solutions, chemicals and biochemical reagents, of which the source is not specifically mentioned in this work, were obtained either from Merck (Darmstadt, Germany), Sigma-Aldrich (Diesenhofen, Germany), Roche Molecular Biochemical (Mannheim, Germany) or GibcoBRL (Eggenstein, Germany).

3.1.1 Cell lines

Name	Description	Culture media
Raji	Human B-lymphoblastoid cells	Complete RPMI medium*
BA/F3	IL-3 dependent murine pro B-cells	Complete RPMI medium + 10%WEHI-3 conditioned medium
L929	Murine fibroblast cells	Complete RPMI medium
CTLL-2	IL-2 dependent murine T-cells	Complete RPMI medium + 2β-mercaptoethanol 50µM sodium pyruvate 1mM rhIL-2 2.7ng/ml (Biotest Pharma, Dreieich Germany)
J558	Murine lymphoblasts	Complete RPMI medium
WEHI-3	IL-3 dependent murine myelomonocites	Complete RPMI medium

*Complete RPMI medium: RPMI 1640, 10% FCS (Biochrom, Berlin, Germany), 2 mM L-glutamine, 100 U/ml penicilin and 100 µg/ml streptomycin (PAA, Linz, Austria).

3.1.2 Yeast strains

S. cerevisiae	Genotype	Source
L40	MATa *his3*D200*trp*1-901*leu*2-3,-112*ade*2 LYS::(lexAop)4-*HIS3*, URA3::(lexAop)8-lacZgal4	Prof. K. Harbers (HPI, Hamburg)
YRN974	MATa *leu*2-3,112, *Trp* 1-289, *HIS3*-Δ1, MAL 2-8c, Suc2 URA3 ::(lexAop) EGFP	Prof. K. Harbers (HPI, Hamburg)

3.1.3 Yeast media

Medium	Compounds
YPD	Bacto-pepton 20 g/l Yeast-extract 10 g/l Glucose 2%
SD/-Trp	DOB 27 g/l CSM –Trp 0.75 g/l
SD/-Leu	DOB 27 g/l CSM –Leu 0.69 g/l
SD/-Trp-Leu	DOB 27 g/l CSM –Trp-Leu 0.64 g/l
SD/-His/-Trp/-Leu/-Lys/-Ura	DOB 27 g/l CSM –His-Trp-Leu-Lys-Ura 0.55 g/l

SD/plates were prepared with 43.7 g DOBA instead of DOB. All media were autoclaved for 15 min at 121°C, 1 bar. Sterile-filtered glucose was added to YPD medium after autoclaving. All SD/medium compounds were obtained from Q. Biogene (Heidelberg, Germany).

3.1.4 Bacterial strains

E.coli strain	Usage	Source
XL1-Blue	Cloning of different cDNA-fragments	Stratagene (La Jolla, USA)
DH5α	Cloning of different cDNA-fragments	Stratagene (La Jolla, USA)
HB101	Specific selection of pACT2 plasmids in Leu-free medium	Clontech (Heidelberg, Germany)

3.1.5 Bacterial Media

Medium	Compounds	Usage
LB (Luria Broth)	Bacto-trypton (Difco laboratories, Augsburg, Germany) 10 g/l Yeast-extract 5 g/l NaCl l5 g/l If required ampicillin 100 µg/ml	*E.coli* strains XL1-Blue and DH5α
M9 /-Leu minimal medium	HCM-Leu (Q. BIOgene, Heidelberg, Germany) 1.15 g/l 50× PART A solution (Q. BIOgene) 2% 50× PART B solution (Q. BIOgene) 2% Glucose 0.3 % Thiamine 0.002% (freshly prepared) Ampicillin 100 mg/ml	*E.coli* strain HB101

For preparation of LB- as well as M9-plates 15 g/l bacto-agar was added to the respective fluid media before autoclaving. The media were autoclaved for 15 min at 121°C, 1 bar. Media were supplemented with sterile-filtered glucose and thiamine solutions after autoclaving.

3.1.6 Antibodies

Antibody	Clone	Final concentration	Source
PE labelled anti-mouse CD132, cytokine common γ chain	TUGm2	0.2 µg/ml	BD Pharmingen (Heidelberg, Germany)
PE labelled anti-mouse CD25, IL-2 receptor α chain	PC61	0.2 µg/ml	BD Pharmingen (Heidelberg, Germany)
PE labelled anti-mouse CD122, IL-2 receptor β chain	TM-b1	0.2 µg/ml	BD Pharmingen (Heidelberg, Germany)
Biotin labelled goat anti-mouse IgG2b	Polyclonal	2.5 µg/ml	Southern Biotechnolgy Associates (Birmingham, USA)
PE labelled donkey anti goat IgG	Polyclonal	0.5 µl/100 µl	Dianova, (Hamburg, Germany)
Mouse anti- LexA	Monoclonal	1 µg/ml	Santa Cruz Biotechnology, (Santa Cruz, USA)
Rabbit anti IL-2Rα	M-19, polyclonal	1 µg/ml	Santa Cruz Biotechnology (Santa Cruz, USA)
Goat anti IL-15Rα	N-19, polyclonal	1 µg/ml	Santa Cruz Biotechnology (Santa Cruz, USA)
Fluorescent labelled IRDye 800 donkey anti-goat IgG	Polyclonal	0.4 µg/ml	Biotrend Chemikalien (Köln, Germany)
Fluorescent labelled IRDye 800 goat anti-rabbit IgG	Polyclonal	0.4 µg/ml	Biotrend Chemikalien (Köln, Germany)
Fluorescent labelled Alexa Fluor 680 goat anti-mouse IgG	Polyclonal	0.8 µg/ml	MoBiTec (Göttingen, Germany)
Horseradish peroxidase sheep anti-mouse IgG	Polyclonal	0.0002 µl/ml	Amersham Bioscience (Freiburg Germany)
Horseradish peroxidase donkey anti-rabbit IgG	Polyclonal	0.0002 µl/ml	Amersham Bioscience (Freiburg Germany)
Horseradish peroxidase rabbit anti-goat IgG	Polyclonal	0.2 µg/ml	Perbio Science (Bonn, Germany)

3.1.7 Plasmids and cDNA-libraries

Plasmid	Description	Source
pBTM116	Yeast vector: expression of "bait fusion proteins"	Prof. K. Harbers (HPI, Hamburg)
pBTML	Yeast vector: pBTM116- derivate	Prof. K. Harbers (HPI, Hamburg)
pACT2	Yeast vector: expression of "prey fusion proteins"	Clontech (Heidelberg, Germany)
pcDNA 3.1(+)	Mammalian expression vector	Invitrogen (Karlsruhe, Germany)
Bluescript KS	Bacterial cloning vector	Stratagen (La Jolla, USA)
PCRII-TOPO	Bacterial cloning vector	Invitrogen (Karlsruhe, Germany)

cDNA-library	Description	Source
Human bone marrow MATCHMARKER	Vector: pACT2 Cloning site: EcoRI/XhoI No. of clones: 3.5×10^6	Prof. H. Will (HPI, Hamburg)
Human placenta MATCHMARKER	Vector: pACT2 Cloning site: EcoRI/XhoI No. of clones: 3.5×10^6	Clontech (Heidelberg, Germany)

3.1.8 PCR-primers

Lab-name	5´-3´Sequence	Purpose
mIL-2Rα-1	CCCGGATCCTTGGCCATGTGCCAGGAAGATGGA	Cloning of mouse IL-2Rα in pcDNA 3.1 (+)
mIL-2Rα-2	GCTGGTACCAGGTGAGCCCGCTCAGGAGGAG	Construction of mouse IL-2Rα/IL-15Rα chimeric receptor
mIL-2Rα-3	GGGGAATTCCTAGATGGTTCTTCTGCTCTT CCTCCATCTGTGTTGCCAGGTCAGGAAAGCC ATCACAACCCCT	Construction of mouse IL-15Rα/IL-2Rα chimeric receptor
mIL-2Rα-4	GGGGAATTCCTAGATGGTTCTTCTGCT	Cloning of mouse IL-2Rα in pcDNA 3.1(+)
mIL-2Rα-5	GGATCCAAGATGGAGCCACGCTTGCTGACG	Amplification of mouse IL-2Rα
mIL-2Rα-6	AAGCTTTCAATACTCCATAGTGAGCACAAATGT CACC	Amplification of mouse IL-2Rα
mIL-2Rβ-up	GTCGACGCTCCTCTCAGCTGTGATGGCTACCAT A	Amplification of mouse IL-2Rβ
mIL-2Rβ-low	GGATCCCAGAAGACGTCTACGGGCCTCAAATTC CAA	Amplification of mouse IL-2Rβ
mIL-2Rγ-up	GTCGACAGAGCAAGCACCATGTTGAAACTA	Amplification of mouse IL-2Rγ
mIL-2Rγ-low	GGATCCTGGGATCACAAGATTCTGTAGGTT	Amplification of mouse IL-2Rγ
mIL-15Rα-1	CTTGGATCCAATTGGCCATGGCCTCGCCGCAGC TC	Cloning of mouse IL-15Rα
mIL-15Rα-2	CTTGAATTCGTGTGGTTAGGCTCCTGTGTCTT	Cloning of mouse IL-15Rα
mIL-15Rα-3	GTGGGTACCTCAAATCAAGGCAGCCTTCTCAG	Construction of mouse IL-2Rα/IL-15Rα chimeric receptor
mIL-15Rα-4	AACATCCACCCTGATTGAGTGT	Amplification of mouse IL-15Rα
mIL-15Rα-5	GTTTCCATGGTTTCCACCTCAA	Amplification of mouse IL-15Rα
hIL-15Rα-1	CTCGAATTCAAGAACTGGGAACTCACAGCA	Cloning of L-fragment of human IL-15Rα
hIL-15Rα-2	CTCGAATTCGACACCACTGTGGCTATCTCC	Cloning of M-fragment of human IL-15Rα
hIL-15Rα-3	CTCGAATTCGTGTCTCTCCTGGCATGCTAC	Cloning of S-fragment of human IL-15Rα
hIL-15Rα-4	CTCGAATTCTCATAGGTGGTGAGAGCAGTT	Cloning of human IL15Rα-fragments
pACT-D1	GAGATGGTGCACGATGCACAGTTGAAGTGA	Amplification of insert of pACT2-cDNA-library
pACT-U1	ACCACTACAATGGATGATGAT	Amplification of insert of pACT2-cDNA-library
pACT-U2	GGCTTACCCATACGATGTTCCA	Amplification of insert of pACT2-cDNA-library
pBTM116-1	GTTGCCAGAAAATAGCGAGT	Sequencing
pcDNA 3.1(+)-U776	GGGCGGTAGGCGTGTACGGTG	Sequencing
pcDNA 3.1(+)-D1072	CGGGGGAGGGGCAAACAACAG	Sequencing
β-actin up	GTGGGGCGCCCCAGGCACCA	Amplification of β-actin
β-actin low	CTCCTTAATGTCACGCACGATTTC	Amplification of β-actin

Underlined nucleotides indicate the site-specific sequences of restriction enzymes. All oligonucleotides were ordered from Metabion (Martinsried, Germany).

3.1.9 Buffers and solutions

Buffers and solutions	Compounds
10× PBS pH 7.2	Na_2HPO_4 0.58 M NaH_2PO_4 0.17 M NaCl 1.37 M
10× TE	Tris-HCl 100 mM EDTA 10 mM pH 7.5
Tris-glycine buffer	Tris-base 20 mM Glycine 250 mM pH 8.3 SDS 0.1%
Z-buffer pH 7	Na_2HPO_4 60 mM NaH_2PO_4 40 mM KCl 10 mM $MgSO_4$ 1 mM
Protein lysis buffer	Tris-HCl 20 mM pH 8 NaCl 75 mM EDTA 1 mM Na-vanadate 1 mM Pepstatin A 1 µg/ml Leupeptin 1 µg/ml NP40 1%
Transfer buffer	Tris-base 24 mM Glycine 192 mM Methanol 20%
SDS-loading buffer	Tris-HCl 50 mM pH 6.8 DTT 100 mM SDS 2% Bromophenol blue 1% Glycerol 10%
TBST (Tris-buffer saline/ with tween-20) pH 7.4	Tris-HCl 10 mM NaCl 150 mM Tween-20 0.5%

3.2 Methods

3.2.1 Molecular biology methods

Basic molecular biology techniques were performed as described in the “Molecular Cloning” (Sambrook, 2001).

3.2.1.1 Isolation of RNA and synthesis of first strand cDNA

Total extraction of RNAs form cells (Trizol reagent), elimination of genomic DNA contaminations (DNase I-kit) as well as first strand cDNA synthesis (SuperSript^{TM} II RNase H$^-$ Reverse transcriptase kit) were performed according to the protocol of supplier (All from Invitrogen, Karlsruhe, Germany).

3.2.1.2 PCR-cloning of murine IL-2Rα and IL-15Rα

The single strand cDNA derived from conA activated lymphnode cells was objected to PCR with primers "mIL-2Rα-1" and "mIL-2Rα-4" to amplify the whole coding region of murine IL-2Rα (See table 3.1.8). To clone murine IL-15Rα the single strand cDNAs from the fibroblast cell line L929 was amplified with primers "mIL-15Rα-1" and "mIL-15Rα-2 respectively. To prevent the misamplification of coding sequences generally caused by application of common Taq-DNA polymerases, we used always Pfu-DNA polymerase. The blunt-end PCR products were directly cloned into EcoRV-digested Bluescript-KS vector and sequenced. Using their flanking BamHI/EcoRI sites (see underlined sequences of primers in the table 3.1.8), the inserts were recloned into the mammalian expression vector pcDNA 3.1(+).

3.2.1.3 Construction of murine IL-2Rα/IL-15Rα and IL-15Rα/IL-2Rα chimeric receptors

To construct the chimeric receptor with IL-2Rα extracellular and transmembrane domains fused to the cytoplasmic domain of IL-15Rα, IL-2Rα/**IL-15Rα**, respective cDNA coding sequences were amplified using following primer pairs: "(BamHI) mIL-2Rα-1" and

"mIL-2Rα-2 (KpnI)" amplify amino acids 1-261 of IL-2Rα, and "(KpnI)mIL-15Rα-3" and "mIL-15Rα-2 (EcoRI)" amplify amino acid 227-263 of IL-15Rα (Fig. 8).

Due to the incorporation of the KpnI restriction site into the "mIL-15Rα-3" primer, which was necessary for cloning of chimeric receptor, Ile 228 (ATC) of mIL-15Rα was converted into Leu (CTC). The amplified cDNA fragments were ligated at KpnI sites and cloned into the BamHI/EcoRI sites of pcDNA 3.1(+).

To construct the second chimeric receptor with IL-15Rα extracellular and transmembrane domains fused to the cytoplasmic domain of IL-2Rα, IL-15Rα/**IL-2Rα**, the cDNA coding sequence of IL-15Rα was amplified using primer "(BamHI) mIL-15Rα-1" and "mIL-2Rα-3 (EcoRI)". The primer "mIL-2Rα-3 (EcoRI)" contained additional 33 nucleotides at 5´end coding for 11 amino acids (227-263) of IL-2Rα cytoplasmic domain (Fig. 8). This cDNA fragment was cloned into the pcDNA 3.1(+) using BamHI/EcoRI restriction sites.

(The restriction enzymes were obtained from MBI Fermentas, St. Leon-rot, Germany)

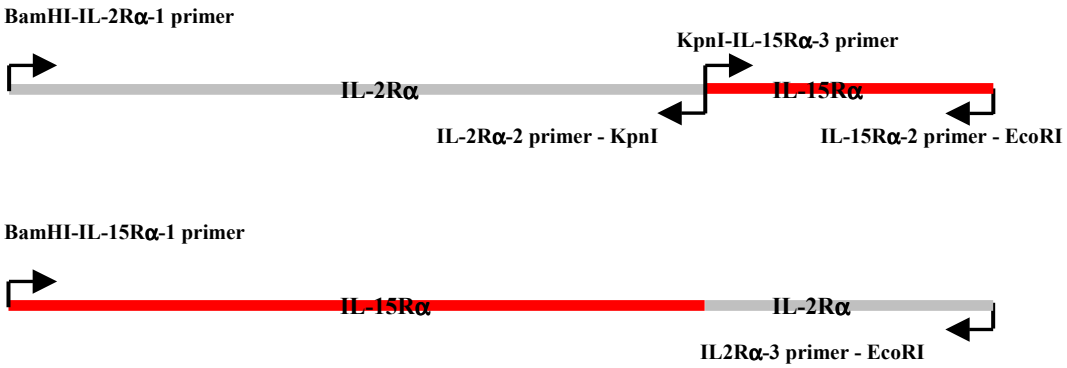


Figure 8. Construction of two murine chimeric receptors.

3.2.1.4 Cloning the different cDNA-fragments of human IL-15Rα containing cytoplasmic domain

To identify hIL-15Rα interacting proteins, 3 different cDNA fragments each encoding the cytoplasmic domain of human IL-15Rα but with different N terminal extensions were

constructed (Fragments: **S**hort, **M**edium and **L**ong). To this end whole population of cDNA from Raji-cells were amplified with common down stream primer: "(EcoRI) hIL-15Rα–4" in combination with 3 different up stream primers: hIL-15Rα–1", hIL-15Rα–2"and hIL-15Rα–3". Respective cDNA fragments (S, M and L) were cloned in pCRII-TOPO vector and sequenced. Subsequently the inserts were excised with EcoRI (MBI Fermentas, St. Leon-rot, Germany) and subcloned into the pBTM116 and pBTML plasmids (see attachments 7.4 and 7.5) resulting in the following 6 different cloned constructs: pBTM116/hIL-15Rα**L**, pBTM116/hIL-15Rα**M**, pBTM116/hIL-15Rα**S**, pBTML/hIL-15Rα**L**, pBTML/hIL-15Rα**M** and pBTML/hIL-15Rα**S**.

3.2.1.5 Generation of the insert free vector pACT2

Insert free pACT2 vector (attachment 7.6) was utilized in two-hybrid system as a negative control. Since this expression vector was not available commercially, we recovered it from MATCHMARKER bone marrow derived cDNA-library (Table 3.1.7) according to the following procedure:

Inserts were completely released from the pACT2 through digestion with BamHI and XhoI restriction enzymes in a 20 µl reaction volume**.** To fill in the sticky ends of vector pACT2, digestion reaction was supplemented with 0.05 U/µl Klenow enzyme (MBI Fermentas, St. Leon-rot, Germany) and 200 µM end concentration of dNTPs and incubated for 20 min at RT. Self ligation of pACT2 was performed in a standard 10 µl ligation reaction using 2 µl of blunt ended vector. In the successfully recovered insert-free pACT2 the recognition sequence of BamHI was destroyed whereas XhoI restriction site was restored.

```
GGATC      +   TCGAG    =   GGATCTCGAG
CCTAG          AGCTC        CCTAGAGCTC

                            XhoI
```

(in red: filled in sequences)

3.2.1.6 DNA-sequencing

Sequencing of the cloned- and PCR amplified-cDNA fragments were accomplished using BigDye terminator mix (Applied Biosystems, Weiterstadt, Germany) in an ABI 377 sequencing device (Applied Biosystems, Weiterstadt, Germany) according to the protocol of the provider.

3.2.2 Protein analysis methods

3.2.2.1 Protein extraction from mammalian cells

7×10^6 cells (Table 3.1.1.) were washed twice with PBS and the cell pellet resuspended in 100 µl protein lysis buffer. The lysate was incubated on ice for 15 min. The cell debris was spun down in a desk-centrifuge at 16000×g for 10 min at 4°C. The protein-containing supernatant was stored at -20°C.

3.2.2.2 Protein extraction from yeast cells

10 ml overnight culture of yeast cells was centrifuged at 1000×g for 5 min at RT. Pelleted yeast cells were resuspended in 100 µl SDS-loading buffer and then mechanically disrupted by adding of glass beads (the tip of a spatula) and vortexing for 3-4 min. The cell suspension was then boiled for 10 min. Proteins were separated from debris by centrifugation at 16000×g for 20 min at 4°C. The protein-containing supernatant was stored at -20°C.

3.2.2.3 SDS-PAGE

SDS-PAGE was accomplished according to protocols described in "Molecular Cloning" of Sambrook (Sambrook 2001) with the following modification:
Prior to electrophoresis, the protein lysate was mixed with an equal volume of 2× SDS loading buffer and denatured by boiling for 3 min. The proteins were separated in the electrophoresis device (the Mini-PROTEAN 3 cell, Biorad, Munich, Germany), by 8 V/cm in the Tris-glycine electrophoresis buffer.

3.2.2.4 Immunoblotting

Electrophoretic transfer of proteins from polyacrylamide gel to PVDF-membrane was performed by Mini Trans-blot cell (Biorad, Munich, Germany) in the presence of transfer buffer and according to the instruction manuals.
To prevent unspecific binding of antibodies (Abs) membranes were routinely blocked by incubation in the blocking buffer (5% low-fat dry milk in the TBST buffer) for 1 h at RT. Blocked membranes were then incubated with protein specific primary Abs (diluted in the blocking buffer as described in the table 3.1.6) for 1 h at RT and washed 4×5 min in the TBST buffer. Membranes were then incubated with the secondary Abs at the same conditions described for the primary Abs. Two different types of secondary Abs were used. One of them was horseradish-peroxidase-labelled Ab (Table 3.1.6), which was detected with ECL (Amersham Bioscience, Freiburg Germany) via a chemiluminescence signal on the X-ray film. The other type of secondary Ab was a fluorescent-labelled antibody (Table 3.1.6), which is optimised for the "Odyssey Infrared Imaging system" developed by LI-COR Bioscience.

3.2.3 Yeast two-hybrid system

The yeast two-hybrid system is a sensitive molecular genetic approach for studying protein-protein interactions *in vivo*.

3.2.3.1 Yeast transformation

The LiAc-mediated method was applied for the transformation of yeast cells. The yeast cells were grown overnight in 50 ml YPD at 30°C with shaking at 230 rpm to a stationary phase (OD_{600}> 1.5). The overnight culture was diluted with 300 ml of YPD to bring the OD_{600} down to 0.2 - 0.3 and incubated at 30°C for 3 h with shaking at 230 rpm until the OD_{600} reached 0.4 - 0.6. The cells were pelleted at 1000×g for 5 min at RT. After washing once with 1×TE the cell pellet was resuspended in 1.5 ml 1×TE/100 mM LiAc.
0.1 µg of each plasmid DNA and 100 µg of herring testis carrier DNA (Clontech, Heidelberg Germany) were mixed in a fresh 1.5 ml tube. 100 µl of yeast cells was added to each tube and mixed thoroughly. 600 µl 40% PEG/100 mM LiAc/1×TE solution were added to each tube, vortexed for 10 sec and incubated at 30°C for 30 min with shaking at 200 rpm. To increase

the transformation efficiency 70 µl DMSO was added to each tube. The mixture was then heat shocked for 15 min in a 42°C water bath and chilled immediately on ice for 2 min. The cells were centrifuged for 5 sec at 16000×g at RT. The pelleted yeast cells were resuspended in 0.5 ml 1×TE and were plated on the SD minimal plates that selected for the desired transformants.

3.2.3.2 Yeast transformation with human bone marrow cDNA-Library

The large scale library transformation was set up with an overnight culture of a bait-transformed L40 yeast strain in the SD/-Trp medium. The overnight culture was diluted with 1 liter SD/-Trp medium to bring OD_{600} down to 0.2-0.3. The culture was incubated at 30°C with shaking at 230 rpm until it reached OD_{600} = 0.5-0.6. The cells were centrifuged at 1000×g for 5 min and washed with 500 ml water. Cells were pelleted by centrifugation, resuspended in 20 ml 100mM LiAc/0.5× TE and incubated at RT for 10 min. 350 µg human bone marrow cDNA-library (MATCHMAKER, Clontech), 2.5 ml herring testis carrier DNA (10 mg/ml) and 140 ml 100mM LiAc/40% PEG/1×TE were added to the yeast cells and incubated at 30°C for 30 min with shaking at 200 rpm. To increase transformation efficiency 17.6 ml DMSO was added and mixed. A heat shock was performed for 15 min in a 42°C water bath and rapidly cooled on ice. The cells were pelleted and washed with 500 ml water. To recover the cells from transformation stress the cell pellet was resuspended in 1 liter YPD medium and shaken at 30°C for 1 h, at 230 rpm. The washing step was repeated. To select the co-transformants, the cells pellet were resuspended in 1 l SD/-Trp/-Leu and incubated for 6 h at 30°C with shaking at 230 rpm.
To remove the residual of selection medium cells were washed twice with 500 ml water and resuspended in 10 ml water. To screen for His3 expression the transformed yeast cells were plated on SD/HTLLU plates containing 10 mM and 20 mM 3-Amino triazole (3-AT).

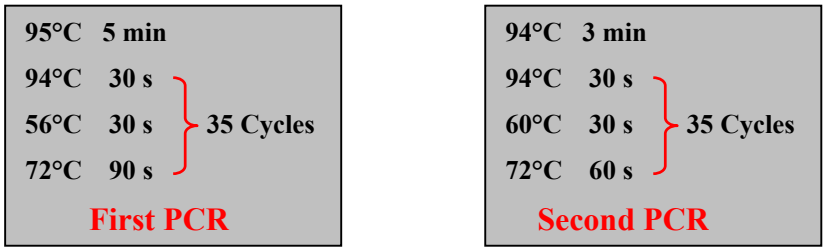
3.2.3.3 ß-Galactosidase filter assay

Colony-lift filter assay was used to measure the β-galactosidase activity, which monitors protein-protein interactions.
The yeast colonies were transferred to a nitrocellulose filter, the filter was then soaked in liquid nitrogen for 10 sec, removed and allowed to thaw at RT. To detect the ß- galactosidase

activity, nitrocellulose filter were placed on a wet Whatman #2 (soaked with 340 µg/ml X-gal/Z buffer). The filter was then incubated at 30°C until appearance of blue colour.

3.2.3.4 Yeast colony PCR

To analyse the cDNA-sequence of His^+ transformants, the direct yeast colony PCR was used. For PCR, a fresh yeast colony was picked and resuspended in 100 µl water. 5 µl of the cell suspension was amplified with pACT U1 und pACT D1 primers.
A nested PCR was then performed to verify the specificity of the first amplification as following: Amplicons of the first round of PCR were diluted 1/100 and reamplified with pACT D1 and pACT U2 primers according to the following programs:



The product of the second PCR was purified using PCR purification column (Qiagen, Hilden, Germany) and sequenced.

3.2.3.5 Isolation of library plasmid from yeast

Plasmids from His^+ transformants were isolated using "YEASTMAKER™ yeast plasmid isolation kit" (Clontech, Heidelberg, Germany) and recloned into the *E.coli* BH101 strain.
Selection for pACT2-plasmids encoding potential IL-15Rα interacting proteins was accomplished on selective M9 plates lacking the amino acid leucine.

3.2.3.6 Flow cytometry of yeast cells

The yeast strain YRN974, in which the reporter gene encodes the green fluorescent protein (GFP), was used for the quantitative detection of interactions between IL-15Rα fragments and potential interacting proteins. The transformed colonies were grown in 2 ml of the

corresponding SD medium at 30°C with shaking at 230 rpm for 16-18 h. 100 µl of overnight culture was diluted in 1 ml PBS. The amount of GFP in living cells was quantified by flow cytometry (Becton Dickinson, Heidelberg, Germany).

3.2.4 Cell biology methods

3.2.4.1 Flow cytometric analysis

Expression of common γ chain - IL-2Rγ (CD132), IL-2Rβ (CD122) and IL-2Rα (CD25) were analysed by flow cytometry after surface staining with specific monoclonal Abs (see section 3.1.6) according to standard protocols. Briefly, cells were washed twice with FACS-buffer (2% newborn calf serum, 0.1% NaN_3, 10 mM EDTA in PBS pH 7.4) and then stained with appropriately diluted Abs for 30 min on ice. Expression of IL-15Rα was detected by staining with IL-15 agonist, recombinant fusion protein IL-15-IgG2b (Bulfone-Paus 1997) or polyclonal goat anti IL-15Rα Abs (N-19, Santa Cruz). Binding of fusion protein was detected by subsequent staining of cells with biotinylated polyclonal Abs against mouse IgG2b (Southern Biotechnology Associates, Birmingham, USA) and streptavidin-phycoerythrin (PE) conjugates (Dianova, Hamburg, Germany). To exclude the unspecific binding of IL-15-IgG2b by Fc-Receptors or common γ chain alone, IL-15-IgG2b fusion protein was used as a negative control. Binding of goat anti-mouse IL-15Rα Abs was detected by staining of cells with secondary PE-labelled donkey anti-goat IgG Abs. In order to prevent unspecific binding, all samples were preincubated with Fc-block or unlabeled, isotype-matched unspecific Abs (BD Pharmingen, Heidelberg, Germany). Samples were analysed on a FACS-Calibur flow cytometer (BD Biosciences, Heidelberg, Germany). Gates on viable cells were set according to the exclusion of propidium iodide staining.

3.2.4.2 Proliferation assay

Proliferation of BA/F3 cells was measured by [^{3}H]-thymidine incorporation. 10^6 cells/ml were cultured for 48 h in 96-well flat-bottom plates (Greiner, Hamburg, Germany) in complete RPMI-1640 medium. Cells were stimulated with recombinant murine IL-3 (TEBU), recombinant human IL-15 (R&D Systems, Minneapolis, USA) or recombinant human IL-2 (Biotest Pharma, Dreieich Germany)) for 48 h. In previous studies it has been demonstrated

that these human cytokines are recognised by mouse receptors (Eisenman 2002**,** Bulfone-Pause 1999). [^{3}H]-thymidine (AP Biotechnology) (0.25 µCi /well) was added to the cells for the last 16 h of culture. Cells were harvested and [^{3}H]-thymidine uptake was determined by liquid scintillation counting (Wallac/PerkinElmer, Freiburg, Germany). All assays were prepared in triplicates.

3.2.4.3 Assays for measuring of cell viability

BA/F3 cells (2×10^5/ml) were incubated with or without IL-3, IL-15 or IL-2. In some experiments binding of cytokines to the common γ chain was blocked with monoclonal anti-common γ chain Abs or isotype matched control Abs of irrelevant specificity (α-CD4, clone L3T4) (both from BD Pharmingen, Heidelberg, Germany) added to a final concentration of 1µg/ml. After 24 h and 48 h, the numbers of the living and dead cells was determined by Annexin V-FITC or Trypan blue staining. Annexin V binds to phosphatidylserins expressed on the surface of apoptotic cells. Simultaneous staining with Propidium Iodide (PI) allows to discriminate between early apoptotic (Annexin V positive, PI-negative), late apoptotic (Annexin V positive, PI-positive) and necrotic (Annexin V negative, PI-positive) cells. For Annexin V-FITC staining appropriate amounts of cells were washed with PBS, resuspended in binding buffer (10 mM HEPES/NaOH, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) and stained with recombinant human Annexin V-FITC and PI (Annexin V/PI kit, Bender MedSystems, Vienna, Austria) according to manufacturers instruction. Binding of Annexin V-FITC was detected by FACS. 0.05% Trypan blue (Seromed) in PBS was used to count dead cells microscopically.

3.2.4.4 Stable transfection of BA/F3 cells by electroporation

1×10^7 BA/F3 cells/ml were washed twice with PBS. 10 µg DNA was pipetted into a prechilled 0.4 cm cuvette. 500 µl of the cell suspension was added. The electroporation was performed by Gene Pulser (Bio-Rad, Munich, Germany) at 350 V and 950 µF. After electoporation the cells were incubated on ice for 15 min and transferred into the flasks containing cell culture medium. After two days neomycin (G418) (PAA Laboratories, Coelbe, Germany) in final concentration 1mg/ml was added. Cells were cultivated in the presence of G418 for at least 14 days to select the stable transfectants.

3.2.5. Statistical analysis

Results are presented as mean ± SD (standard deviation) from pooled data of 3-4 identical experiments, all of which gave comparable results. FACS data from one representative experiment of at least three are shown. The student's t-test for unpaired samples was used for the determination of statistical differences (* $p \leq 0.05$; ** $p \leq 0.01$).

4 RESULTS

4.1 Studies investigating the role of IL-15Rα in signalling

The IL-15 receptor is a heterotrimeric complex, including β and γ subunits, which are shared with IL-2, and an IL-15 specific receptor subunit, IL-15Rα. Previously IL-15Rα had not been considered to be a component of signal transduction (Anderson 1995) but some data from our group showed that IL-15Rα indeed is involved in signal transfer (Bulfone-Paus 1999, Bulanova 2001). At the moment, the mechanism of signal transfer via IL-15Rα is not clear.
In order to investigate the function of IL-15Rα, we studied the ability of IL-15Rα to transfer signals in a IL-15Rα-free cell line, BA/F3. In addition, we constructed two chimeric receptors from mouse IL-15Rα and IL-2Rα and used them to study the function of the IL-15Rα-cytoplasmic domain in signal transfer. Chimeric receptor studies have contributed significantly to the mapping of receptor signalling pathways, for example the IL-6R complex (Schmitz 2000), gp130 (French 2002), Mer receptor (Guttridge 2002) and GM-CSF receptor (Wantanabe 2000).

4.1.1 Analysis of BA/F3 cells stably expressing IL-15Rα

4.1.1.1 Generation of BA/F3 cell lines stably transfected with mouse IL-15Rα

In order to investigate the role of IL-15Rα in cell signalling, cell lines stably transfected with a cDNA for mouse IL-15Rα have been generated. For this purpose, transfection of the cytokine dependent pro-B cell line BA/F3 was performed. BA/F3 is a mouse bone marrow derived pro-B cell line, which depends on IL-3 for growth and survival in culture (Rodriguez-Tarduchy 1990). As described previously, BA/F3 cells express the IL-2Rα and IL-2Rγ subunits, but do not express the IL-2Rβ chain (Nelson 1994). For cytokine receptors like IL-2Rβ, IL-4R, or IL-21R, stable transfection into BA/F3 cells and response to their corresponding cytokines has been demonstrated, resulting in the generation of transfectants in which the growth-promoting effect of IL-3 could be substituted by other cytokines (Nelson 1994, Deutsch 1998 and Habib 2002).

For the analysis of the receptor expression profile in BA/F3 cells, RT-PCR, FACS and Western blotting techniques were used. Expression of mRNA for IL-2Rα and IL-2Rγ, but not IL-2Rβ and IL-15Rα was demonstrated by RT-PCR and amplification with specific primers (Fig. 9A). Staining with specific Abs against receptors confirmed the well-known finding, that BA/F3 cells express IL-2Rα and the IL-2Rγ chain, but not IL-2Rβ on the cell surface. Additionally FACS analysis revealed that BA/F3 cells do not express IL-15Rα (Fig. 9 B). The absence of IL-15Rα expression has was also confirmed by Western blotting with anti-IL-15Rα Abs (Fig. 9C).

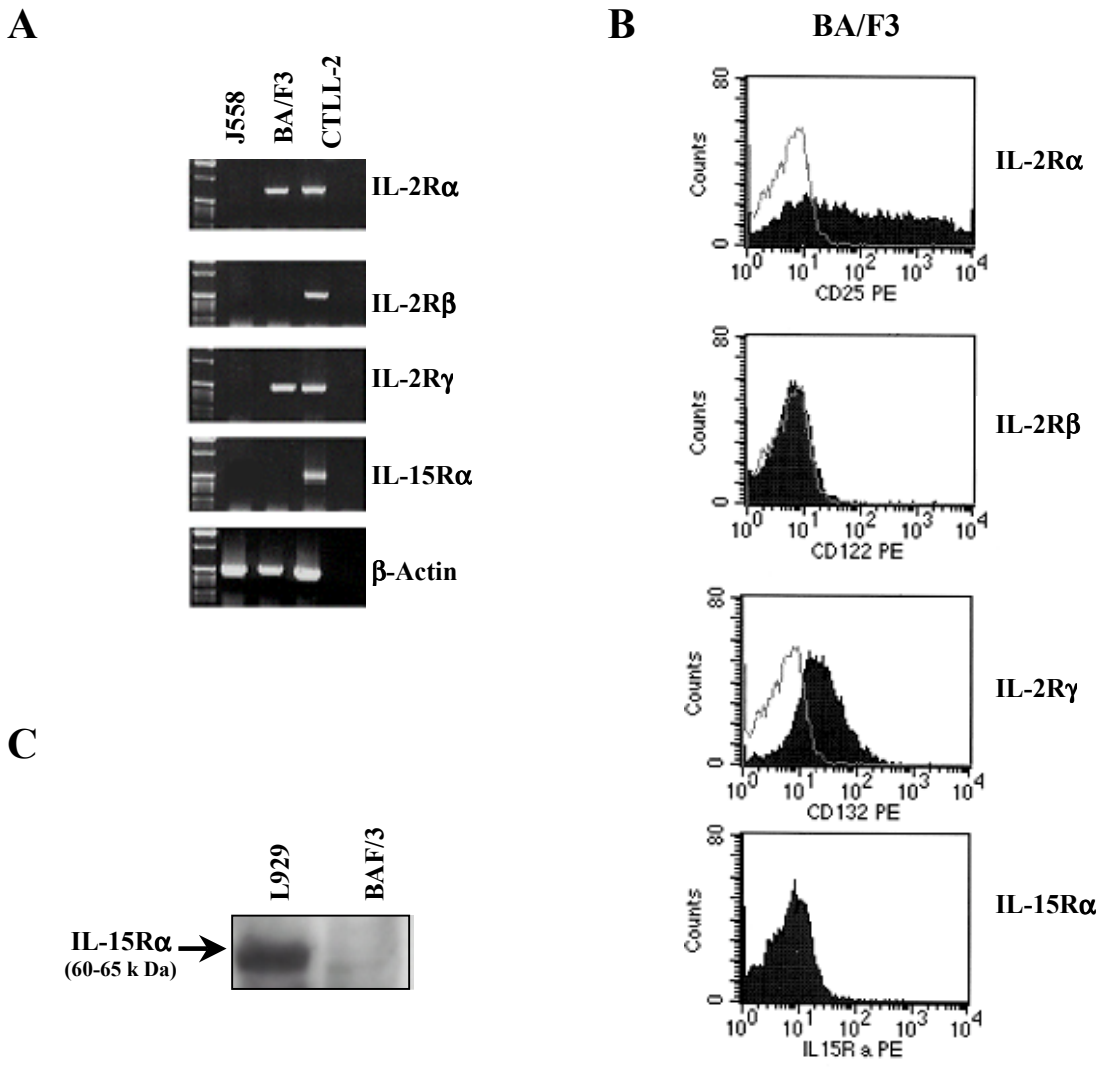


Figure 9. Expression of IL-2/IL-15 receptor complex in BAF/3 cells.
A) RT-PCR analysis of IL-2/IL-15 receptor expressions in BA/F3 cells. Total RNA was extracted from BA/F3 cells, CTLL-2 cells (as positive control) and J558 cells (as negative control), was reverse transcribed and subjected to PCR amplification using specific primers. β-actin message was used to normalize the cDNA amount. B) Surface expression of IL-15Rα,

IL-2Rα, IL-2Rβ and IL-2Rγ on the BAF/3 cells by FACS analysis. C) Western blotting analysis of IL-15Rα expression in BA/F3 cells using anti IL-15Rα Abs. L929 cells were used as positive control for IL-15Rα expression.

These data suggest that neither the endogenous IL-15Rα chain nor the IL-2Rβ chain are expressed by BA/F3 cells. Expression of IL-2Rα and IL-2Rγ was confirmed.

As a next step, BA/F3 cells were transfected stably with either a vector encoding for mouse IL-15Rα or an empty vector. Expression of IL-15Rα in transfected BA/F3 cells was analysed by RT-PCR and Western blotting. RT-PCR of transfected cells confirmed the presence of a mRNA for IL-15Rα in transfected BAF3 cells (Fig. 10A). Western blot analysis with an Abs against IL-15Rα confirmed also the presence of IL-15Rα at the protein level (Fig. 10B). Transfected cell lines show no differences in their growth ability, morphology or IL-3 dependence as compared with control-transfected (pcDNA3.1 plasmid without insert) cell lines.

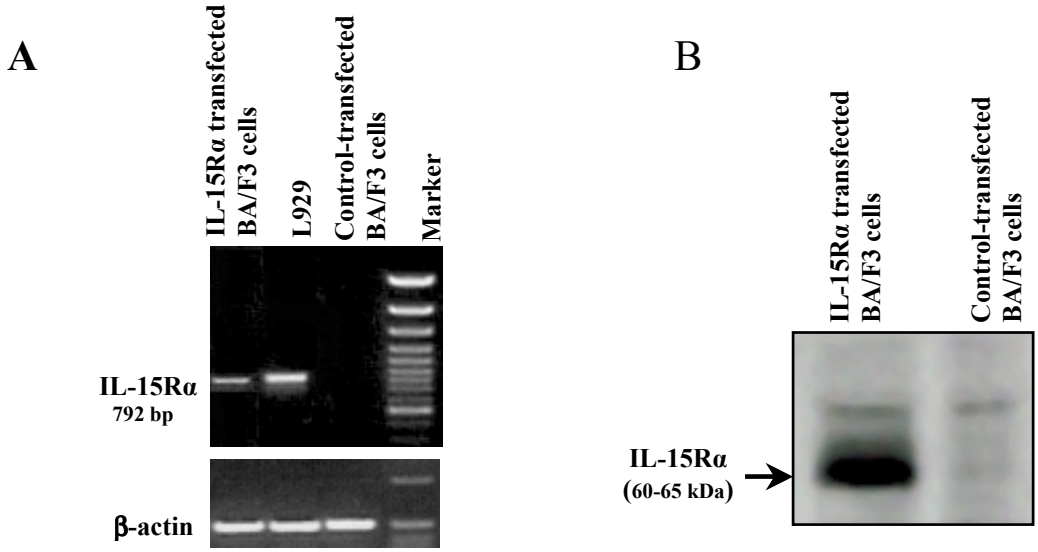


Figure 10. Expression of IL-15Rα in BA/F3 stable transfectants.
A) Total RNA from transfected BA/F3- and L929-cells were isolated and analysed by RT-PCR using specific primers. The amount of cDNA analysed was equalized by PCR amplification of β-actin. L929 cells were used as a positive control, control-transfected BA/F3 cells as a negative control. B) Protein lysates from IL-15Rα transfected BA/F3 cells were analysed in 10% SDS-PAGE using anti IL-15Rα Abs.

These data show not only the successful transfection of IL-15Rα cDNA into the BA/F3 cells but also the expression of IL-15Rα at the protein level in the transfected BA/F3 cells.

4.1.1.2 Functional characterisation of transfected IL-15Rα

After generation of cell lines stably expressing IL-15Rα, functionality of the receptor in the context of BA/F3 cytokine-dependent growth and cytokine-deprivation-induced apoptosis was investigated.

In order to show whether IL-15 is able to replace IL-3 for proliferation of IL-15Rα transfected BA/F3 cells, analysis of proliferation of stably transfected cell lines was performed. As shown in Fig. 11A, IL-15 induces a dose-dependent effect on proliferation of BA/F3 cells in the absence of IL-3.

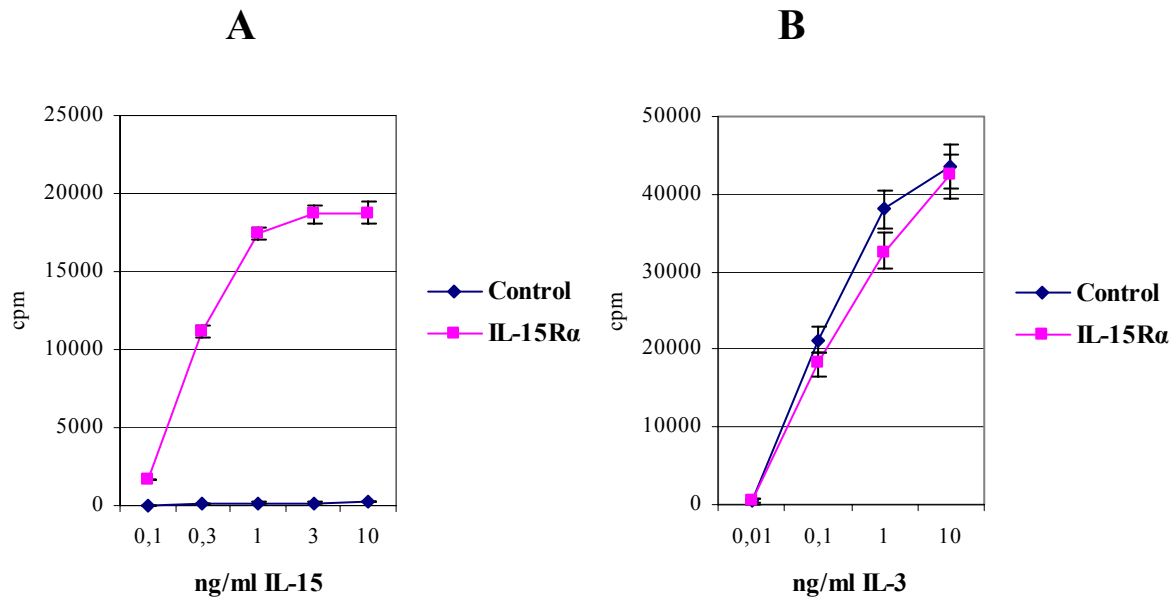


Figure 11. IL-15 induces proliferation of IL-15Rα transfected BA/F3 cells.
A) 10^5 cells of transfected BA/F3 cells expressing IL-15Rα were incubated in presence of different concentration of IL-15. The proliferation rates of the cells were calculated by the measurement of thymidine incorporation. The control-transfected BA/F3 cells were used as a negative control. B) Proliferation in presence of IL-3.

Increasing the concentration of IL-15 enhanced the thymidine incorporation. Already at the level of 1 ng/ml (= ~ $6x10^{-11}$ M) of IL-15, the curve approaches its plateau phase, which shows the saturation of the binding sites for IL-15. This result is in accordance with the high affinity binding of IL-15 to IL-15Rα (K_d ~ 10^{-11} M). As expected, the control-transfected BA/F3 cells did not show any proliferation in presence of IL-15. As a control the proliferative response of transfected cell lines to IL-3 was evaluated. Both IL-15Rα transfected and control-transfected BA/F3 cells demonstrated comparable proliferative response to the IL-3. Both transfectants showed high proliferation rates with a linear dependence of proliferation from 0.01 till 10 ng/ml of IL-3 (Fig. 11B).

These results indicate that IL-15Rα is able to transduce the proliferation inducing signals in a model of stably transfected BA/F3 cell line. In these BA/F3 cells IL-3 could be replaced by IL-15. IL-15 addition to these cells leads to sustained proliferative response.

One of the important functions of IL-15 is the inhibition of apoptosis (Bulfone-Paus 1997, Lindner 1998). As the next step, the ability of IL-15 to block the apoptosis, which was induced in the IL-3 dependent BA/F3 cells by deprivation of IL-3, has been investigated.
In order to prove, if IL-15 can inhibit the apoptosis in IL-15Rα transfected BA/F3 cells, IL-15 was added to the IL-3 deprived IL-15Rα- and control-transfected BA/F3 cells. After different time points (24, 48 and 72 h) the viability of the cells was analysed by Annexin-FITC and Propidium Iodide staining. In parallel the number of dead cells was analysed microscopically. As shown in Fig. 12, IL-15 significantly enhanced the survival rate of BA/F3 cells expressing IL-15Rα, keeping 80-90% of the cells alive, which is similar to the level in the presence of IL-3 (Fig. 12A), and significantly diminished the percentage of apoptotic cells (Fig. 12B). The same results were obtained by staining the cells with trypane blue (data not shown).
Survival of BA/F3 cells in the presence of IL-15 was IL-15Rα dependent and occurs only in IL-15Rα transfected cell lines, because the control-transfected cells died even in the presence of IL-15. IL-3 rescued both control-transfected and IL-15Rα transfected cells from apoptosis. These data show that IL-15 is able to inhibit the apoptosis induced by IL-3 deprivation in a model of stably transfected BA/F3 cell line.

Taken together, the stably transfected IL-15Rα was fully functional and successfully transduced the signals responsible for cell growth and proliferation and apoptosis inhibition.

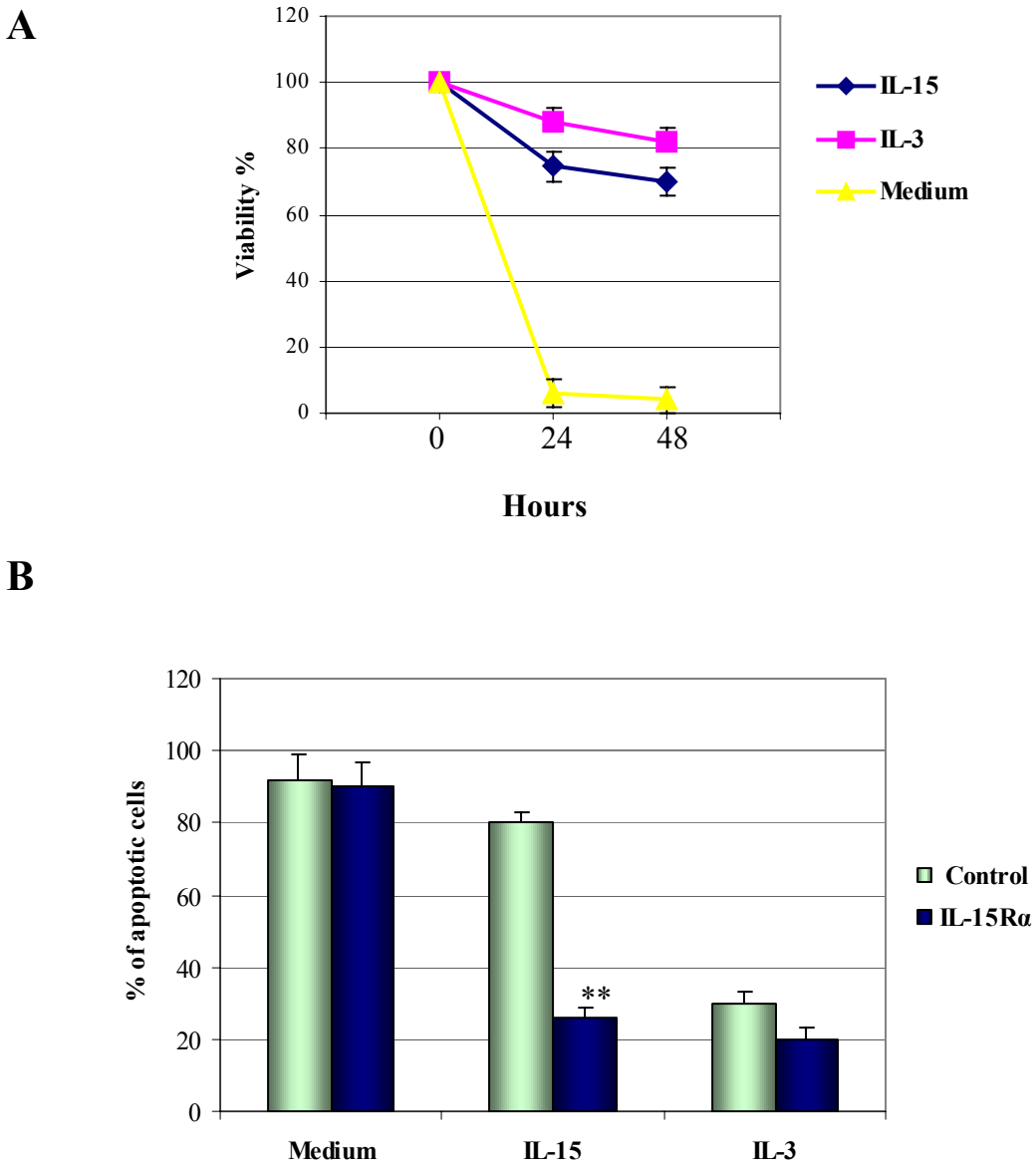
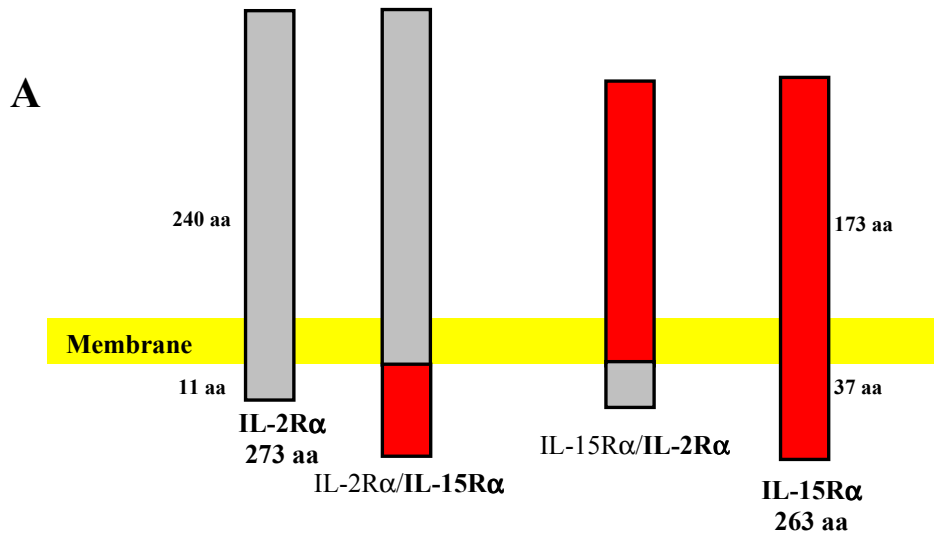


Figure 12. IL-15Rα mediates survival and prevents apoptosis of transfected BA/F3 cells. *A) IL-15Rα and control-transfected BA/F3 cells were incubated in the medium in the absence of IL-3, but in the presence of IL-15 (10 ng/ml ◆). Cell viability was analysed after 24 h and 48 h by propidium iodide staining via FACS. Incubation of the cells in the medium with (■) or without (▲) IL-3 was used as a control. B) The apoptotic cells were stained with Annexin-FITC and percentage of apoptotic cells was measured by FACS after 48 h. The value is the mean of three independent experiments. The significance was calculated using Student' t-test (** p≤0.01).*

4.1.2 Analysis of BA/F3 cells stably expressing IL-2Rα/IL-15Rα chimeric receptors

4.1.2.1 Generation of BA/F3 cell lines stably transfected with murine chimeric IL-2Rα/IL-15Rα receptors

Cytosolic domains of receptors play a pivotal role in signal transduction. In order to investigate the role of the cytoplasmic domain of IL-15Rα in signal transfer, two chimeric receptors from murine IL-15Rα and IL-2Rα were generated (Fig. 13A). Both IL-15Rα and IL-2Rα are type I transmembrane receptors (respective cDNA- and amino acid sequences are included in the attachment 7.2 and 7.3). For generating the mIL-2Rα/**IL-15Rα** chimeric receptor (cytosolic domains indicated by bold letters), a DNA fragment encoding amino acids (aa) 1-261 of mIL-2Rα, corresponding to the extracellular- and transmembrane domain of mIL-2Rα, was ligated to a fragment that encodes the cytosolic domain of mIL-15Rα (aa 227-263). For the appropriate connection between these two domains, a KpnI restriction site was created (Fig. 13B). This created a point mutation from Ile 228 (ATC) of mIL-15Rα into Leu (CTC). The mIL-15Rα/**IL-2Rα** chimeric receptor was constructed *vice versa* and contained extracellular and transmembrane domains of mIL-15Rα (aa 1-226) as well as cytosolic domain of mIL-2Rα (aa 262-272) (Fig. 13).



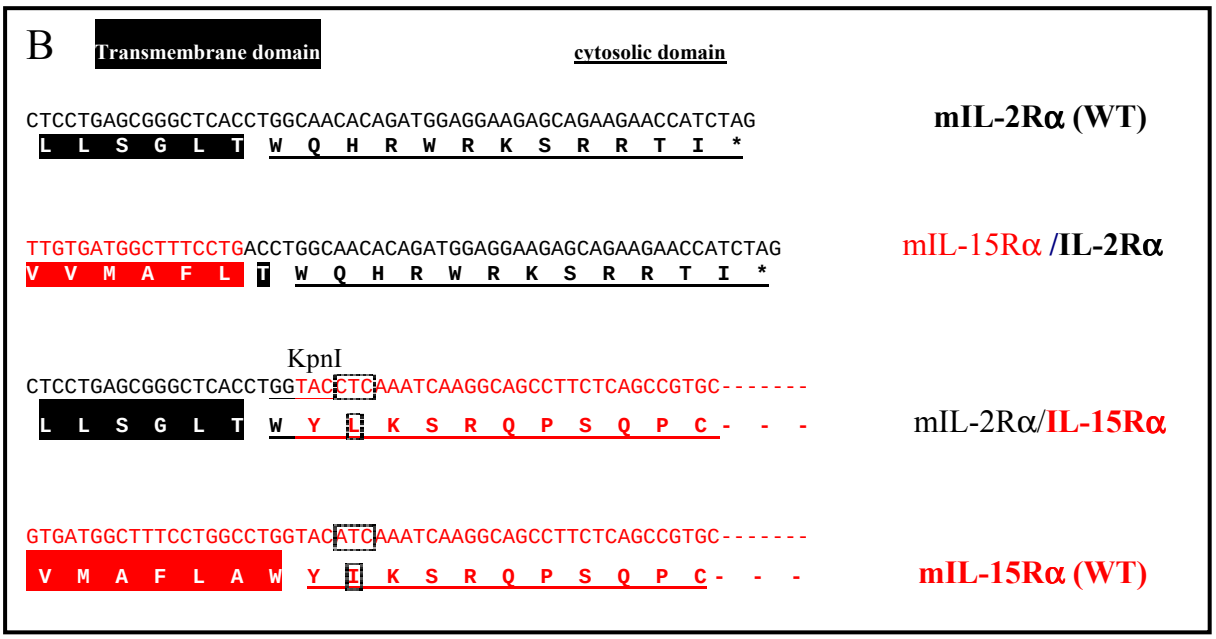


Figure 13. Generation of two murine chimeric IL-15Rα/IL-2Rα receptor proteins.
*A) Schematic representation of wild type (WT) and the two chimeric receptors of IL-15Rα and IL-2Rα. In the IL-15Rα/**IL-2Rα** chimeric receptor, the intracellular domain of IL-15Rα is replaced by the corresponding sequences in IL-2Rα and vice versa, in IL-2Rα/**IL-15Rα** chimeric receptor, the extracellular domain of IL-2Rα is connected to the cytoplasmic domain of IL-15Rα. B) C-terminal nucleotide- and encoded amino acid sequences of WT and chimeric IL-15Rα/IL-2Rα receptor proteins including the site of junction between the cytoplasmatic domain and transmembrane domain. Note that due to the incorporation of a KpnI-site in the IL-2Rα/**IL-15Rα** chimeric receptor ^{682}ATC is mutated to CTC, which is shown by dotted boxes. The IL-15Rα/**IL-2Rα** receptor was constructed by oligonucleotide primers (see materials and methods).*

In order to investigate the role of the IL-15Rα cytoplasmic domain in the signalling process, BA/F3 cells were stably transfected with vectors containing the chimeric receptors IL-15Rα/**IL-2Rα** or IL-2Rα/**IL-15Rα**. Receptor expression was analysed by RT-PCR and Western blotting. For each of the chimeric receptors, the presence of message was shown by RT-PCR of transfected cells (Fig. 14A). Western blot analysis with antibodies against IL-15Rα and IL-2Rα confirmed the presence of the chimeric receptors in transfected BA/F3 cells (Fig. 14B and C).

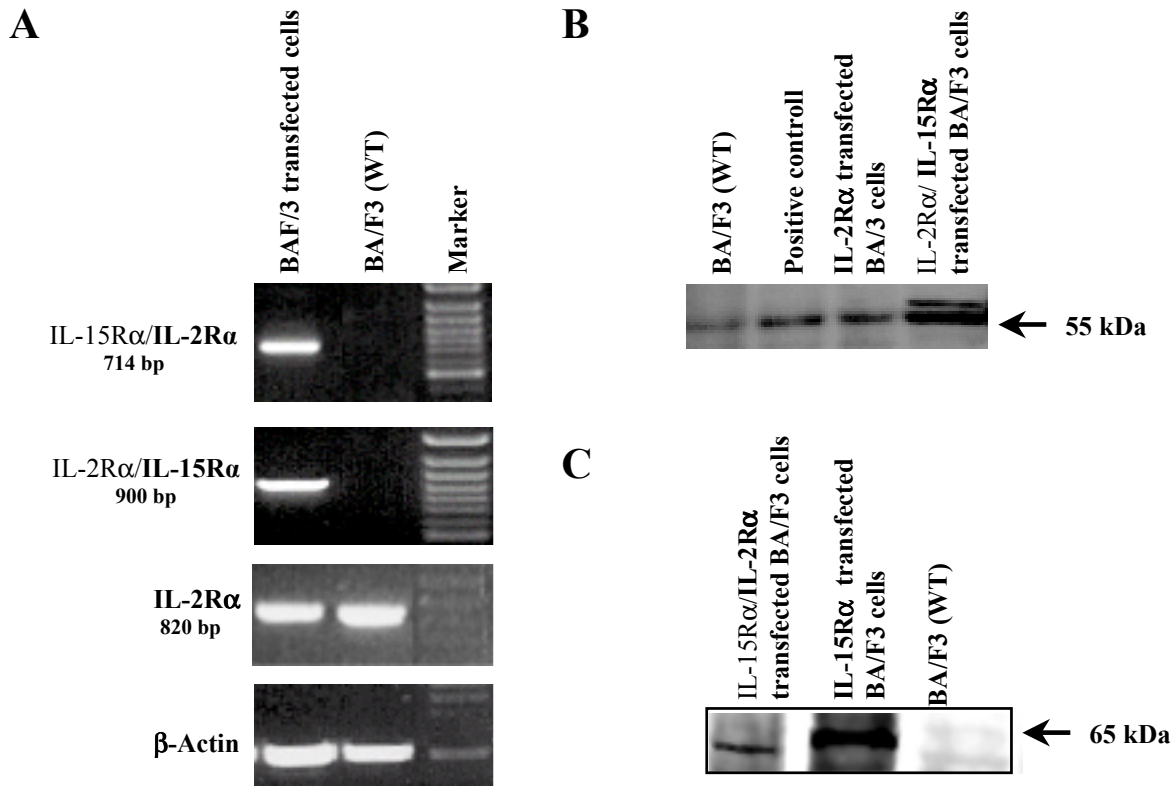


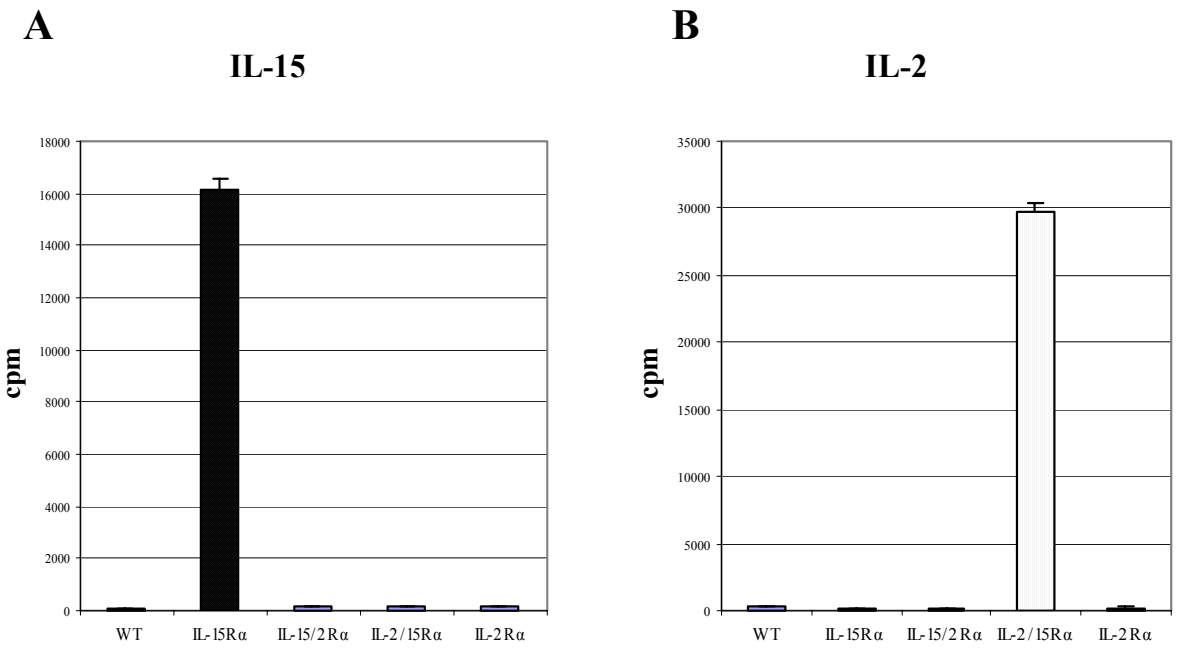
Figure 14. Expression of IL-2Rα WT and chimeric receptor of IL-15Rα /IL-2Rα in BA/F3 stable transfectants.
A) Total RNA from transfected BA/F3 cells was isolated and analysed by RT-PCR using specific primers. The amount of cDNA analysed was equalized by PCR amplification of β-actin. B) Protein lysates from transfected and WT BA/F3 cells were analysed in 10% SDS-PAGE using anti- IL-2Rα Abs. IL-2Rα is 55 kDa. C) Western blot analysis of transfected cells using anti-IL-15Rα Abs. IL-Rα is 60-65 kDa.

These data show the expression of chimeric receptors at the protein level, which is required for functional analysis of IL-15Rα cytoplasmic domain.

4.1.2.2 Functional characterisation of transfected chimeric receptors IL-2Rα/IL-15Rα and IL-15Rα/IL-2Rα

The role of the IL-15Rα cytoplasmic domain in signal transduction was analysed by using two chimeric receptors. Justly, it was investigated whether the IL-15Rα cytoplasmic domain is responsible for mediating the signals leading to cell proliferation. BA/F3 cells expressing

one of the following receptors - IL-15Rα, IL-15Rα/**IL-2Rα**, IL-2Rα/**IL-15Rα** and IL-2Rα - were stimulated with IL-2 and IL-15. The proliferation rate was determined in three independent experiments. As a control, proliferation in the presence of IL-3 or medium without cytokine was analysed. As shown in Fig. 15, only the cells that express IL-15Rα cytoplasmic domain proliferate in response to IL-15 (Fig. 15A) or IL-2 (Fig. 15B). The IL-15Rα/**IL-2Rα** and IL-2Rα transfected cells responded neither to IL-15 nor to IL-2 stimulation. Apparently, the cytoplasmic domain of IL-2Rα is not able to mediate the appropriate signal to induce cell proliferation. In contrast, the cytosolic domain of IL-15Rα is able to mediate signal-inducing proliferation in BA/F3 cells which express the IL-2Rα/**IL-15Rα** chimeric receptor after binding of IL-2. The level of induced proliferation was comparable to those levels observed after IL-15 stimulation of IL-15Rα transfected BA/F3 cells.



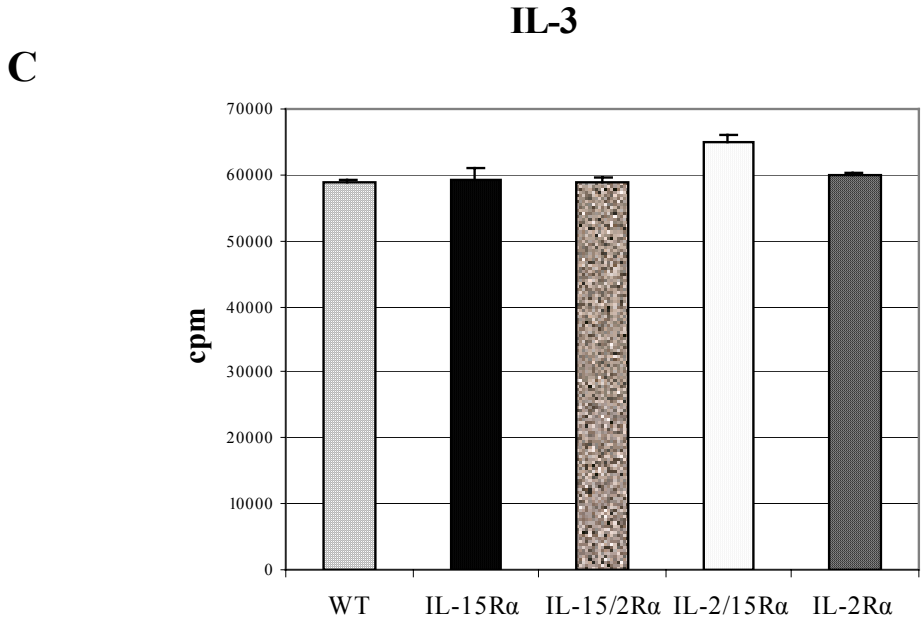


Figure 15. Cytoplasmic domain of IL-15Rα mediats mitogenic signal.
*5× 10 [5] BA/F3 cells, untransfected (WT) or transfected with indicated constructs, were incubated with 10 ng/ml of IL-15 (A), IL-2 (B) or IL-3(C) for 40 h. The proliferation rates of the cells were calculated by the measurement of thymidine incorporation. The value is the mean of three independent measurements. (IL-2/15Rα = IL-2Rα/**IL-15Rα** and IL-15/2Rα = IL-15Rα/**IL-2Rα**).*

These data indicate that in contrast to the cytoplasmic domain of IL-2Rα, the IL-15Rα cytoplasmic domain plays an essential role in the transfer of the mitogenic signal in transfected BA/F3 cells. Additionally, it can be assumed that the extracellular domain of IL-15Rα is not involved directly in signal transfer.

The results shown above indicate that the IL-15Rα is capable of triggering intracellular signalling upon IL-15 binding. IL-15 binding delivers an effective anti-apoptotic signal in IL-3 deprived IL-15Rα transfected BA/F3 cells (Fig. 11). In order to test this ability for chimeric receptors, the inhibition of apoptosis by stimulation with IL-2 and IL-15 was investigated. Transfected BA/F3 cells expressing the complete IL-15Rα and IL-2Rα were used for comparisons. The IL-3-deprived transfectants were maintained in the presence of IL-15 and IL-2 (10 ng/ml) for 2 days. The number of apoptotic cells was evaluated by using FITC-

Annexin V and Propidium Iodide staining. Cell cultures in the presence of IL-3 were used as a positive control. In parallel the number of the living and dead cells were counted with trypane blue staining for comparison with FACS analysis. Both evaluation methods showed similar results. As shown in Fig. 16, IL-15 significantly enhanced the survival rate of BA/F3 cells expressing IL-15Rα. In contrast, IL-15 was not able to inhibit apoptosis in IL-15Rα/**IL-2Rα** transfected cells. Cells expressing IL-2Rα/**IL-15Rα** could survive in the presence of IL-2. However, the survival rate was reduced as compared with cells transfected with IL-15Rα in the presence of IL-15 (Fig. 16).

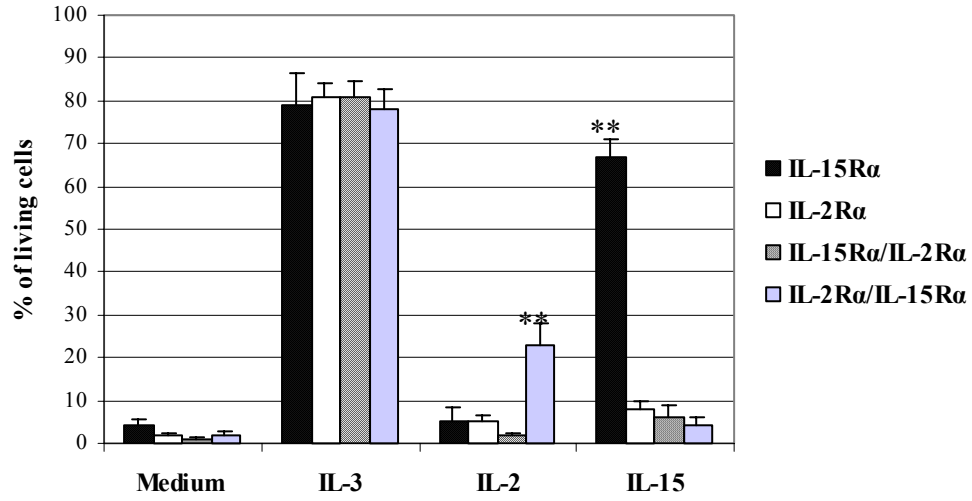


Figure 16. Cytoplasmic domain of IL-15Rα mediates the anti-apoptotic signal.
5×10^5 cells were incubated in the presence of 10 ng/ml of each cytokine. After 48 h the number of apoptotic- and dead-cells were measured by FACS after staining with Annexin-FITC and PI. IL-3 was used as positive control and medium without cytokine as negative control. The experiment was repeated three times and the significance was analysed using Student' t-test ($p\leq0.05$, ** $p\leq0.01$).*

Taken together these data demonstrate that the cytoplasmic domain of IL-15Rα is essential for transduction of signals responsible for proliferation and inhibition of apoptosis of BA/F3 cells transfected with chimeric receptor IL-2Rα/**IL-15Rα.** In BA/F3 cells expressing IL-15Rα/**IL-2Rα** chimeric receptor, binding of IL-15 does not induce cell proliferation or inhibition of apoptosis. This suggests that the cytoplasmic domain of IL-15Rα can induce downstream signal transduction events. One potential mechanism to explain this might be the recruitment, binding and activation of different adaptor proteins.

4.2 Identification of hIL-15Rα interacting proteins via the "yeast two-hybrid system"

The high affinity chain of IL-15Rα might contribute to the mechanisms of IL-15 pleiotropy. Given a widespread role of IL-15/IL-15Rα in diverse cellular processes within and beyond the immune system, the identification of potential IL-15Rα interactors may provide particularly helpful insights to better understand IL-15 signalling pathways. The yeast two-hybrid system is widely used as a sensitive molecular screening approach for studying protein-protein interactions *in vivo*. This methodology was used to investigate in more detail which additional intracellular signalling molecules may interact with the IL-15Rα chain. To this end, two separate expression vectors were applied. The first incorporated a sequence of the target protein (here the cytoplasmic part of IL-15Rα) fused in frame to the LexA DNA-binding domain (DBA) and served as "bait" construct. The second consisted of the Gal4 activation domain (AD) coupled to sequences from a bone marrow cDNA-library and was used as a "prey". Then, both constructs were transfected into yeast, leading to the production of respective hybrid proteins (Gisler 2001). If the "bait" protein would have a capacity to associate with a potential interactor encoded by a sequence from the cDNA-library ("prey"), this might bring "bait" and "prey" hybrid proteins in close proximity and reconstitute the transcriptional activation, resulting in the expression of integrated reporter genes in yeast strain L40 (Vojtek 1993, Bartel 1995, Gisler 2001). This strain is commonly used as a host for two-hybrid screening and carries two reporter genes, LacZ and His3, both of which are under control of LexA. His3 expression provides a nutritional selection for the two-hybrid association, whereas LacZ expression results in β-galactosidase activity, which can be detected by a blue colour assay in the presence of X-gal.

4.2.1 The "yeast two-hybrid system" twelve steps screening

The following steps in performing a two-hybrid screening of potential interactors with IL-15Rα-cDNA (bait):

(1) Construction of the DBD-IL-15Rα fusion plasmid. (2) Transformation of the DBD-IL-15Rα fusion plasmid into yeast. (3) Testing the DBD-IL-15Rα fusion plasmid for

IL-15Rα expression. (4) Testing the DBD-IL-15Rα fusion plasmid in yeast for auto-activation of reporter genes. (5) Choosing a cDNA-library and assessment of transformation efficiency for the AD-cDNA fusion library plasmid. (6) Transformation of the AD-cDNA fusion plasmid library into the yeast strain carrying the DBD-IL-15Rα fusion plasmid and selection for His3 activation. (7) Screening positives colonies for the activation of the lacZ reporter gene. (9) DNA sequence analysis of AD-cDNA fusions. (10) Reconstruction of two hybrid positive clones. (11) Identification of false positives. (12) Analysis of true positives.

These twelve steps are necessary for the identification of proteins, which may interact with the cytoplasmic domain of the IL-15Rα. Below, the results obtained at each step are explained in more detail.

4.2.2 The bait coding for hIL-15Rα cytoplasmic domain: generation of six different constructs

The human IL-15Rα is a type I transmembrane protein with a signal peptide of 30 amino acids (aa), an extracellular domain of 175 aa, a transmembrane domain of 21 aa and a 41 aa cytoplasmic domain (see section 7.1). The project focused on the identification of proteins that could bind to the cytoplasmic domain of the human IL-15Rα. Therefore, the hIL-15Rα cytoplasmic domain served as bait in the two-hybrid system to fish the putative interacting proteins.

The protein conformation is very important for the protein-protein interactions. The use of artificially made fusion proteins always embodies a potential risk. The fusion might change the actual conformation of the bait or prey and consequently alter functionalities. This misconformation might result in a limited activity or in the inaccessibility of binding sites (Van-Criekinge 1999). However, the use of tagged proteins, in general has been very successful in many biological approaches. This success might rely on the fact that protein domains can fold rather independently, enabling the co-existence of different, even artificially introduced, modules in the same protein (Van-Criekinge 1999).

Since it cannot be predicted, how the hybrid-protein id folded, different bait-constructs, characterized by a different IL-15Rα length, were created. Three different fragments of hIL-15Rα were amplified by PCR. All fragments contain the cytoplasmic domain of hIL-15Rα exsisting of 41 amino acids (227-267). They, however, differ in their N-terminus (Fig. 17A).

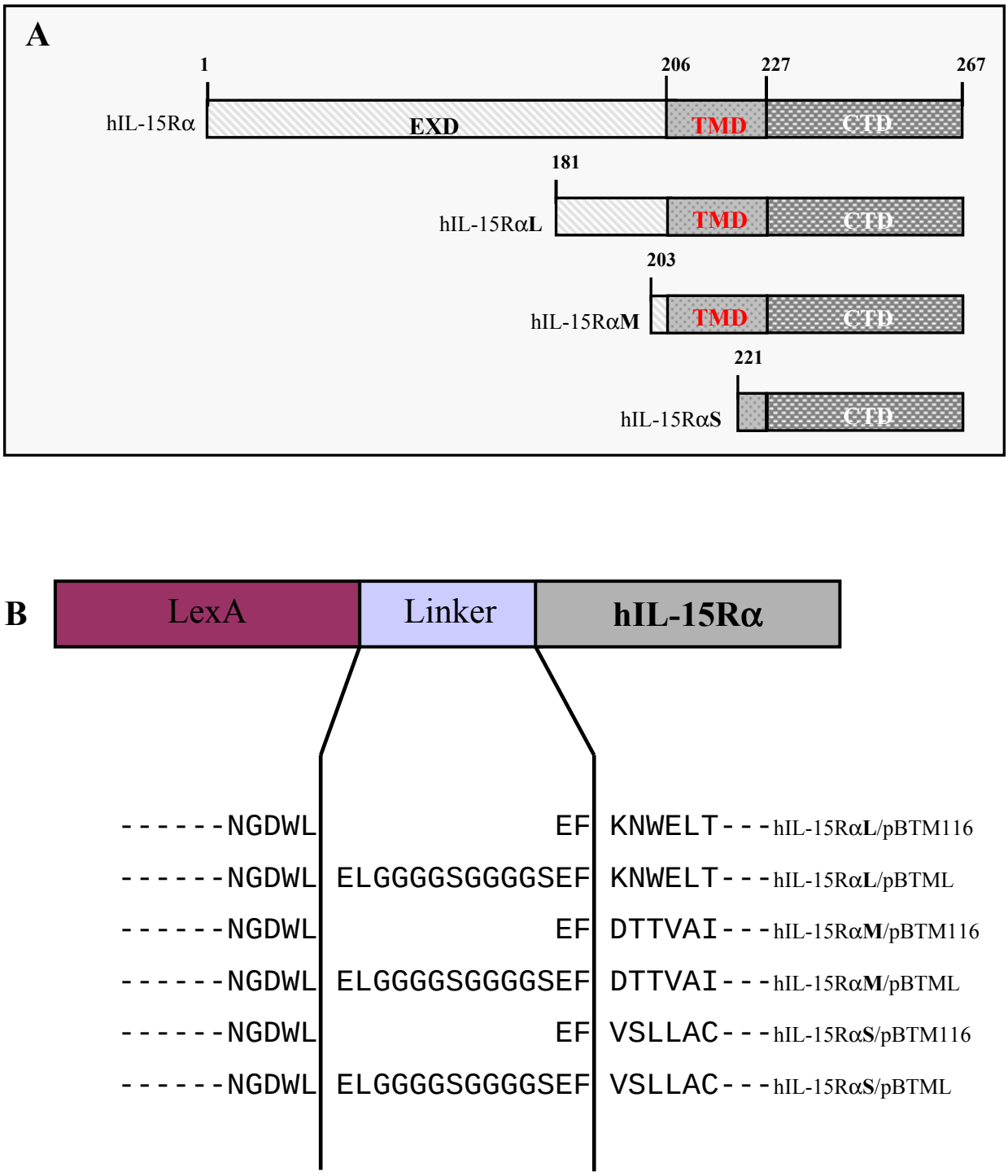


Figure 17. Structure of hIL-15Rα fragments using as "bait".
A) Schematic structure of small (S), middle (M) and large (L) fragment of hIL-15Rα. All fragments contain the cytoplasmic domain (CTD). In addition both L and M fragments carry the transmembrane domain (TMD) and a part of the extracellular domain (EXD). The numbers represent the amino acid positions in the full-length hIL-15Rα. B) Comparison of amino acid sequences of the linker region between different LexA/hIL-15Rα hybrid proteins. The cDNA- and amino acid sequences of hIL-15Rα are depicted in the attachment 7.1.

The smallest fragment, designated as fragment S, contained six additional aa of the transmembrane domain (221-267); the medium sized fragment, denoted as fragment M, had 24 additional aa (203-267), whereas fragment L, the largest of all three, has 45 additional aa (181-267) (Fig. 17A).

The fragments were cloned into the pBTM116 and pBTML yeast expression vectors, which serve as "bait" expression vectors and code for LexA protein as DBD (see section 7.4 and 7.5). In both plasmids the hIL-15Rα fragments were inserted downstream of LexA cDNA. Therefore they will be expressed as fusion proteins, which carry the LexA protein at the N-terminus (Fig. 17B). These vectors differed in the length of a linker that was inserted between the LexA and hIL-15Rα proteins. In the pBTM116 vector, the linker is only two aa in length. In contrast, in pBTML constructs 12 additional aa separate the LexA protein from the IL-15Rα fragments (Fig. 17B).

Successful cloning of IL-15Rα fragments into two yeast expression vectors, pBTM116 and pBTML, was validated by standard DNA sequencing, which allowed us to proceed to the next step of two-hybrid screening.

4.2.3 M and S fragments are expressed only in presence of a linker but expression of the L fragment is independent of linker

In the yeast two-hybrid system, it is critical to ensure that respective "bait" is expressed at the protein level after transformation of yeasts with the "bait" expression vector. Thus, the above described yeast expression vectors coding for six LexA-hIL-15Rα fusion proteins were transformed into the yeast strain L40 and selected on Trp-free medium. Next, the protein-containing cell lysates of Trp^+ transformed yeast cells were analysed by Western blotting using anti LexA Abs (Fig. 18).

As shown in Fig. 18, the S fragment of hIL-15Rα was expressed in the yeast cells transformed by the hIL-15Rα**S**/pBTML construct, in which a 14 amino acids linker separated the LexA and hIL-15RαS proteins. However, the expression of hIL-15Rα**S**/pBTM116 construct, which contains two aa linker, did not result in the appearance of the encoded hybrid protein. Alternatively, the resulting protein was instable and quickly degraded.

The medium-sized fragment M, displayed the identical expression pattern, i.e., expression was also only observed in cells transformed by the construct containing the 14 aa linker. In striking contrast, fragment L was detected in cells expressing both constructs (Fig. 18). Cells expressing the LexA protein alone were used as a positive control, whereas untransformed L40 served as a negative control. The molecular mass of the hIL-15Rα-fragments was calculated using the DNAstar program. The predicted molecular mass of the L fragment is ~9 kDa, M fragment ~7 kDa and S fragment ~5 kDa. The resulting hybrid proteins also contain the LexA, which is about 22-24 kDa.

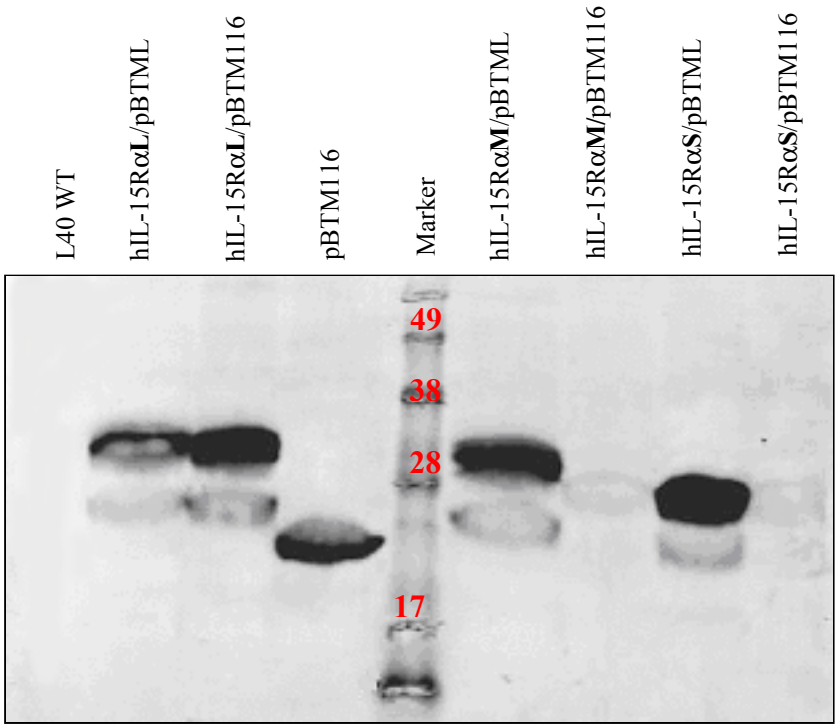


Figure 18. Expression of hIL-15Rα hybrid proteins.
Protein lysate from L40 yeast cells transformed with different hIL-15Rα bait-plasmids were isolated and analysed in 12,5% SDS-PAGE using anti-LexA Ab. The pBTM116 transformed yeast cells, which encodes only for LexA were used as positive control and untransformed yeast cells (WT) as negative control.

Taken together, these results provide evidence that the 14 aa linker has a positive effect upon the expression of constructs containing S and M fragments, but does not affect the expression vectors coding for L fragment. In addition, these data suggest that only four of the "bait"-containing vectors can be used for the next step.

4.2.4 Auto-activation of the hIL-15RαS fragment

Given that the two-hybrid system is based on reporter gene expression in response to transcriptional activation, an obvious problem would arise if the bait-protein were able to activate transcription on its own (auto-activation) (Sobhanifar 2003). Generally, some proteins fused to the DNA-binding domain may possess a transcription activation domain (Causier 2002). Initiation of transcription, due to some latent activating activity, is present in approximately 5% of all proteins (Van-Criekinge 1999). Indeed, up to 10% of randomly generated cDNAs inserted into a DNA-binding domain vector has been shown to auto-activate the reporter gene (Fashena 2000). Auto-activation would give rise to large number of false positives (El Housni 1998). Therefore, it is imperative that the bait fusion proteins are tested for auto-activation prior to the actual library screening (Sobhanifar 2003, Causier 2002, Van-Criekinge 1999).

The auto-activation test was performed only for the transformants carrying the following four plasmids: hIL-15Rα**S**/pBTML, hIL-15Rα**M**/pBTML, hIL15Rα**L**/pBTM116 and hIL-15Rα**L**/pBTML. As already mentioned above, yeast strain L40 has two reporter genes, His3 and lacZ. The activation of the lacZ reporter gene may be assessed by β-galactosidase filter assay. By this assay, the appearance of blue colour in the presence of X-gal confirms the expression of β-galactosidase encoded by the lacZ reporter gene. The ability of yeast to grow in the histidine-free media is indicative for the activation of the His3 reporter gene.

Trp^+ transformants, which contain one of the above bait-plasmids, were tested by β-galactosidase filter assay. As shown in Fig. 19, the construct bearing hIL-15Rα**S** fragment alone induces expression of β-galactosidase, and results in the appearance of blue colour on the filter indicative of an auto-activation effect (Fig. 19, first row). Constructs containing fragment M and fragment L exhibited no lacZ auto-activation properties. As a next step, the Trp^+ transformants were transferred to the plates and cultured in the absence of tryptophan and histidine (-Trp-His). By this technique, auto-activation of His3 reporter gene was tested.

Remarkably, only transformants expressing S fragment were able to grow in the histidine-free medium (Fig. 19 second row).

Bait-construct / Reporter gene activation test	hIL-15Rα**L** pBTML	hIL-15Rα**L** pBTM116	hIL-15Rα**M** pBTML	hIL-15Rα**S** pBTML
β-Galactosidase filter assay of Trp^+ transformants				
Activation of His3 reporter gene with Trp^+ transformants	−	−	−	+
β-Galactosidase filter assay of Trp^+ Leu^+ co-transformants				
Activation of His3 reporter gene with Trp^+ Leu^+ co-transformants	−	−	−	+

Figure 19. The hIL-15RαS construct exhibits auto-activation properties.
Yeasts were transformed by hIL-15Rα bait constructs in the absence or presence of pACT2 vector, and the cells were analysed for β-galactosidase activity in presence of X-gal. The blue colonies indicate expression of β-galactosidase, as a result of activation of lacZ reporter gene. In addition, all transformants were grown in histidine free medium in order to detect the activation of His3 reporter gene. Ability to grow in the absence of histidine served as an indicator for activation of His3 reporter gene.

The next important step was to determine, whether the constructs bearing hIL-15Rα-fragments are able to activate the reporter genes in the presence of empty library vector encoding only the activation domain (AD) of Gal4. Thus, four co-transformations were performed using each of previously selected four bait-vectors and an empty vector (pACT2) used in the generation of the cDNA-library. The L40 yeast cells were transformed by these constructs and the transformants were selected on SC-Trp-Leu medium. Next, the Trp^+ Leu^+ co-transformants were tested for the ability to activate the lacZ reporter gene. The filter assay revealed that only yeasts bearing the small fragment of hIL-15Rα developed blue colour, thus

reproducing the results obtained in the experiments using single transformants (Fig. 19 third row). In parallel, the $Trp^{+}Leu^{+}$ co-transformants were assessed for the capacity to induce activation of the His3 reporter gene by testing cell growth in the histidine-free medium. These experiments revealed that transformants that express the medium- (M) or large-sized fragments (L) of hIL-15Rα did not grow in the medium lacking histidine. The hIL-15Rα**S**/pBTML transformants could grow in the histidine-free medium (Fig. 19 fourth row), supporting the idea that the construct containing the small fragment can alone activate the His3 reporter gene.

In summary, the smallest fragment of hIL-15Rα fragment S, is able alone, as well as in presence of AD, to activate both lacZ and His3 reporter genes, whereas vectors containing M and L fragments did not show auto-activation properties. Therefore, the plasmid encoding the S fragment was excluded from the following screening, and the subsequent experiments were performed using the M and L constructs.

4.2.5 Choice of cDNA-library and the transformation efficiency

The choice of the cDNA-library is very important for the successful "fishing" of potential protein-protein interactors. Obviously, the cDNA should stem from a cell line or tissue, in which the expression of target protein is known to be biologically relevant. Such choice will reduce the number of false-positive colonies (Causier 2002, Van-Criekinge 1999). Important is also the relative strength of activation domains, indicating their ability to initiate transcription. Gal4 is known to be a strong activator making the system more sensitive. This might be needed for the detection of weak interactions (Van-Criekinge 1999). In addition, there are major differences in the promoter strength of different library plasmids. In the most commonly used plasmids of the Gal4 system, fusion proteins are weakly and constitutively expressed. A truncated weakly expressing version of the ADH1-promoter is also used in pACT2 (see attachment 7.6). However, since this promoter is adjacent to a section of pBR322 that acts as transcriptional enhancer in yeast, a medium expression level is obtained (Van-Criekinge 1999).

Based on the high expression of IL-15Rα in the bone marrow (Dubios 1999, Anderson 1995), the human bone marrow cDNA-library (Clontech) was used for identification of hIL-15Rα interacting partners. In this library the cDNAs (primed for the reverse transcription by

oligo-dT) had been cloned into the pACT2 expression vector, and the potential interactors were linked in frame to the 3´ site of yeast the Gal4 activation domain.
The expression of the IL-15Rα cytoplasmic domain was verified in the bone marrow cDNA-library by PCR using hIL-15Rα-3 and hIL-15Rα-4 primers (See section 4.3).
A commonly encountered problem in the library screens is the achievement of optimal transformation efficiency (Luban 1995). The co-transformation efficiency is determined by the number of colonies growing on SD medium that allows selection for both plasmids (bait and prey) and should be ~10^4 cfu/μg DNA. However, for screening of a library, the transformation efficiency must exceed 10^6 clones (Clontech protocol).
The standard lithium acetate transformation protocol was used for the yeast transformation. In order to improve the efficiency, rather than performing a co-transformation, first the yeast was transformed with the "bait" expression plasmid, and then subsequently transformed with the cDNA-library plasmid (sequential transformation).
The Trp^+ transformants containing hIL-15Rα**L**/pBTM116 or/and hIL-15Rα**M**/pBTML bait-plasmid were used for the library transformation. Prior to performing the library-scale transformation, the transformation efficiency was determined using small-scale transformation with the bone marrow library, which was about 7×10^3 cfu/μg.

These data show that the choice of human bone marrow cDNA-library was relevant in regard to the hIL-15Rα cytoplasmic domain expression and also indicate that the transformation efficiency lies in an optimal range, which is absolutely necessary for library screening.

4.2.6 Identification of the hIL-15Rα interacting proteins using two-hybrid screening of the bone marrow cDNA-library

The identification of hIL-15Rα interacting molecules was attempted by yeast two-hybrid screening of the human bone marrow cDNA-library.
The library transformation was performed in the transformed yeast strain L40 expressing L and M fragments using 350 μg cDNA-library. The co-transformants were plated on the high stringency SC plates without His, Trp, Leu, Lys and Ura (-HTLLU) containing 10 mM or 20 mM 3-amino-triazole (3-AT). The 3-AT is a histidine analogue molecule and was added in order to reduce the unspecific activation of the His3 reporter gene.

To determine the total number of co-transformants, an aliquot was plated on the media lacking Trp and Leu, the selection marker for bait- and library plasmid, which resulted more than 8.5×10^6 colonies. From these, 500 were capable of growing on the high stringency histidine-free medium, 155 in presence of 20 mM AT and 345 with 10 mM AT.

The next step was to analyse the colonies, which grew on the high stringency histidine-free medium. According to the Clontech protocol, analysis of the cDNAs from the grown colonies followed after isolation of the library plasmid from the yeast. Since isolation of 500 plasmids was time-consuming, the "yeast-colony-PCR" was performed first to analyse cDNAs (Schenk 1996). The optimal conditions for yeast-colony-PCR were determined using different primer pairs and/or cycling parameters. The best results were obtained with pACTU1 and pACTD1 primers and the following program:

95°C	5 min	
94°C	30 s	
56°C	30 s	**35 Cycles**
72°C	90 s	

Of 500 colonies, 220 were analysed by PCR. 70% of the colonies showed an amplified band. After a second PCR amplification, 81 of them were sequenced. Blast search in the NCBI data bank returned hits for only 51 of the sequenced PCR-products. As a result, 12 different proteins were identified as proteins interacting with hIL-15Rα. They are listed below (Table 1).

Sequenced cDNA	Number
Lipocalin 2	20
Ribosomal protein, large P1 (RPLP1)	15
HLA-associated transcript 3 (BAT3) = human Scythe	1
Karyopherin alpha 2 (Rch1, importin alpha 1)	1
Intracellular adhesion molecule 3 (ICAM3)	2
Similar to hypothetical protein MGC10526	2
TFIID subunits TAF20 and TAF15	1
FLJ36391 fis, high similar to ribonucleoprotein	1
Arsenite translocating ATPase (ASNA1)	2
Heterogeneous nuclear nucleoprotein H3 (HNRPH3)	3
Hemoglobin, beta (HBB)	1
Hemoglobin alpha 2	2

Table 1: The list of potentially interacting partners of hIL-15Rα identified by a two-hybrid screening.
The numbers on the right indicate the number of colonies expressing respective proteins.

Taken together, twelve potentially interaction partners for hIL-15Rα were identified by yeast two-hybrid screening of human bone marrow cDNA-library. Next, these results were subjected to rigorous verification to confirm true- and exclude false-positive interacting proteins.

4.2.7 Verification of hIL-15Rα interacting proteins

To verify the above results, it is important to check whether the interacting proteins are able to interact with hIL-15Rα after retransformation of plasmids encoding the two proteins.
For this purpose, the pACT2 library plasmids encoding lipocalin 2, intracellular adhesion molecule 3 (ICAM3), importin α1 and HLA-associated transcript 3 (BAT3) were isolated from the yeast cells and retransformed with hIL-15Rα**L**/pBTM116, hIL-15Rα**M**/pBTML and pBTM116 into the yeast strain L40. The interactions were analysed by β-galactosidase filter

assay. As shown in Fig. 20, the dark blue colour resulting from lacZ activation indicates that a strong interaction occurs between lipocalin, importin and BAT3 proteins with both hIL-15Rα fragments. In contrast, the ICAM3 appears to interact strongly with hIL-15Rα**M** fragment, whereas only a week association is detected using hIL-15Rα**L** fragment, as shown by a very light blue colour on top of the colony (Fig. 20). Empty vector pBTM116 was used as control. As shown in Fig. 20 (third row), no interactions were observed in the presence of pBTM116 and plasmids encoding lipocalin, importin, BAT3 and ICAM3. The interactions were also tested in the absence of histidine, which shows the activation of His3 reporter gene. Only the co-transformants containing pBTM116 alone as bait did not grow in the absence of histidine.

BAT3 + hIL-15Rα**L**	ICAM3 + hIL-15Rα**L**	Importin + hIL-15Rα**L**	Lipocalin + hIL-15Rα**L**
BAT3 + hIL-15Rα**M**	ICAM3 + hIL-15Rα**M**	Importin + hIL-15Rα**M**	Lipocalin + hIL-15Rα**M**
BAT3 + pBTM116	ICAM3 + pBTM116	Importin + pBTM116	Lipocalin + pBTM116
	Positive control	Negative control	

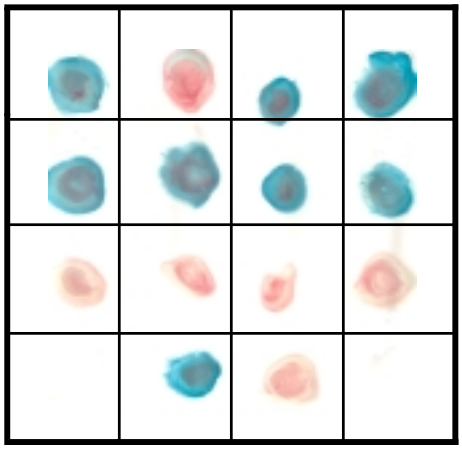


Figure 20. Interaction of hIL-15Rα with importin, lipocalin, ICAM3 and BAT3.
The constructs encoding L or M fragments of hIL-15Rα were cotransformed with plasmids encoding importin, lipocalin, intracellular adhesion molecule 3 (ICAM3) and HLA-associated transcript 3 (BAT3) into the L40 yeast cells. The co-transformants colonies were selected on selective medium and transferred on a nitrocellulose filter. After freezing and thawing in N_2, the β-galactosidase activity was detected in the presence of X-gal (visible as blue colour). In the right table, blue colour shows the β-galactosidase activity resulting from activation of lacZ reporter gene, which is inactive in light brown colonies. The left table describes the experimental set up of the cotransformation assay.

In addition, the β-galactosidase filter assay with Leu^+ transformed yeast containing only the pACT2 library plasmid encoding these four proteins showed no activation of the lacZ reporter gene (data not shown).

These results indicate that lipocalin, importin, ICAM3 and BAT3 are interacting partners of IL-15Rα cytoplasmic domain.

4.2.8 Quantitative analysis of interaction in the YRN974 yeast strain

In the last row of experiments, it was demonstrated that lipocalin, importin BAT3 and ICAM3 interact with IL-15Rα cytoplasmic domain. To further confirm these interactions, another system was used: the two-hybrid analysis using GFP as reporter gene in yeast strain YRN974 (Mancini 1997).

YRN974 contains a reporter gene encoding the GFP, which is chromosomally integrated downstream of a LexA binding site. The advantage of this system is the measurement of GFP production in living yeast cells. In addition, the measurement of the amount of GFP production in a yeast cell can be quantitated by flow cytometry and corresponds in general to the expression level of a specific promoter.

In the two-hybrid analysis using the GFP gene as reporter gene, an inactivate reporter gene shows only an auto-fluorescence peak, as indicated in Fig. 21A, by FACS analysis. The interaction between bait and prey results in the activation of the GFP reporter gene, leading to the increase of the fluorescence intensity, as illustrated Fig. 21B. Such an increase in fluorescence can be measured by flow cytometry.

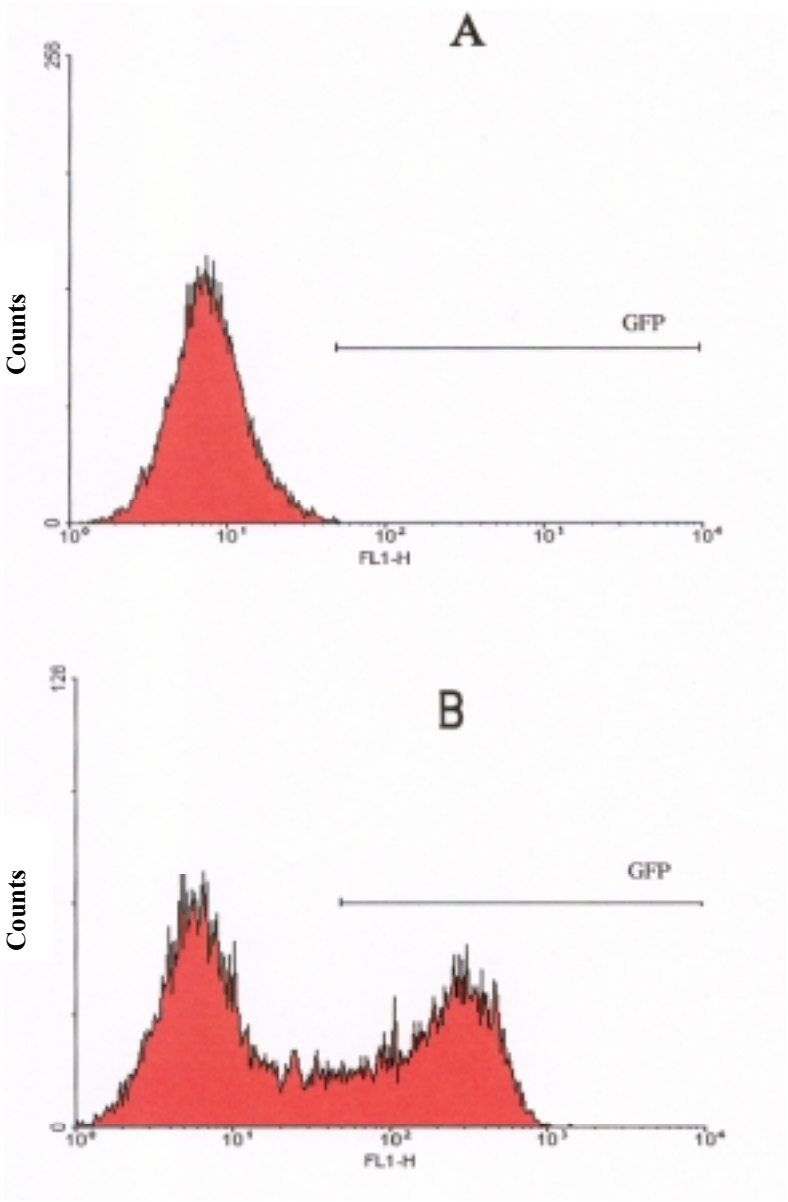
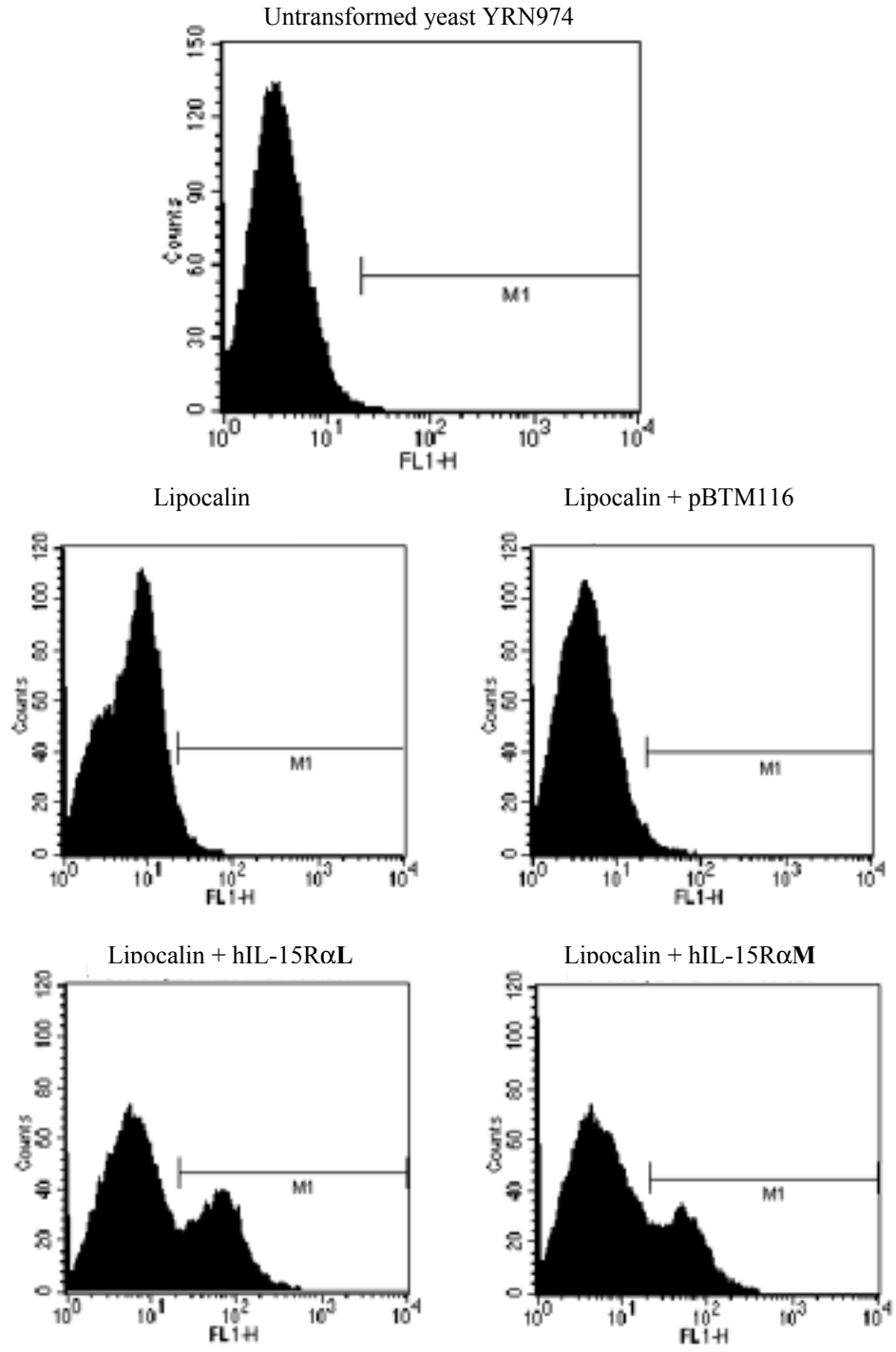


Figure 21. Flow cytometric analysis of living yeast cells expressing GFP.
A) The WT yeast strain shows auto-fluorescence, in which GFP is not active. B) Increasing of fluorescence intensity after activation of GFP gene. Count: number of cells showing a given fluorescence. FL1-H: fluorescence filter 1.

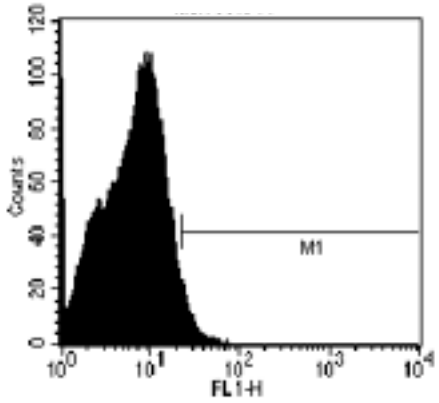
To verify the results showing interaction between the hIL-15Rα fragments and lipocalin, importin, ICAM3 and BAT3 using GFP as a reporter, the vectors coding for these proteins were transformed into the yeast strain YRN974 and analysed by flow cytometry (Fig. 22A). The fluorescence intensity of wild type yeast strain was set to zero and the mean

values of the fluorescence intensities of the transformed yeast cells were calculated relative to this, yielding a number called “relative green fluorescence” (Fig. 22B).

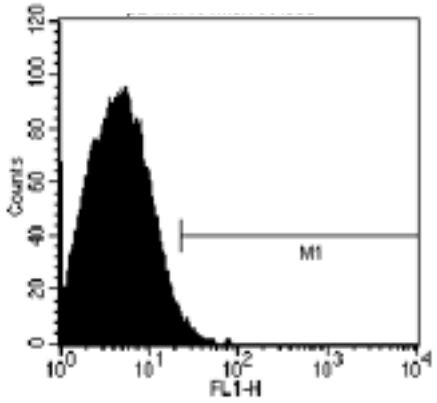
A



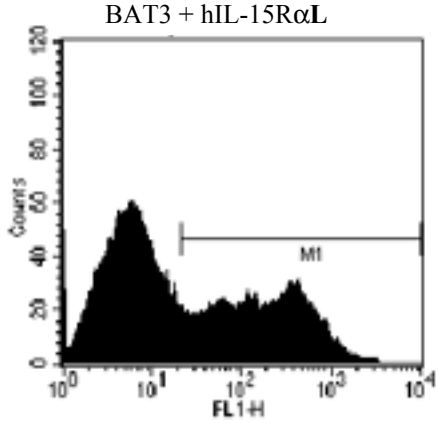
BAT3



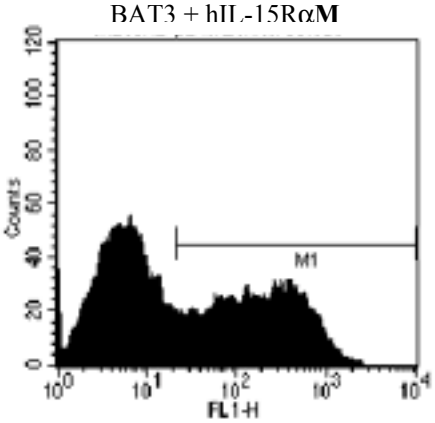
BAT3 + pBTM116



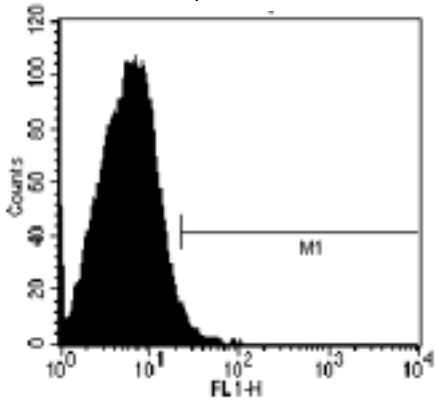
BAT3 + hIL-15Rα**L**



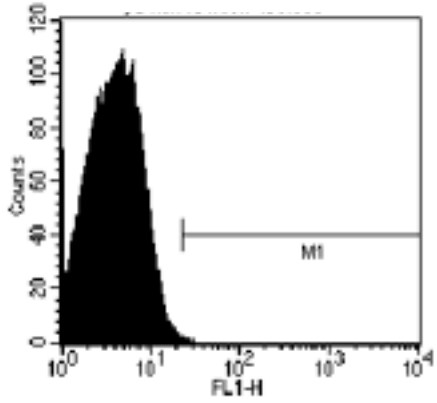
BAT3 + hIL-15Rα**M**



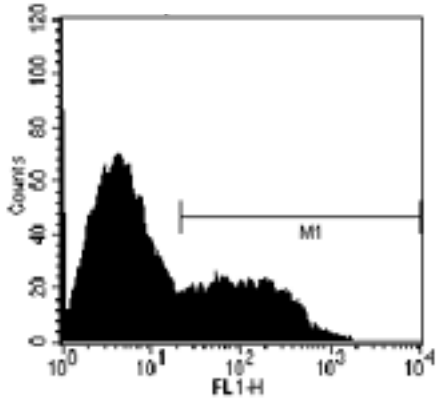
Importin



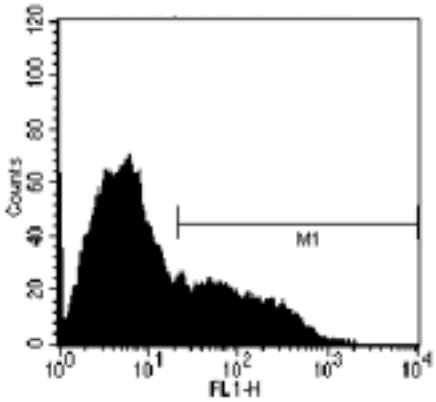
Importin + pBTM116



Importin + hIL-15Rα**L**



Importin + hIL-15Rα**M**



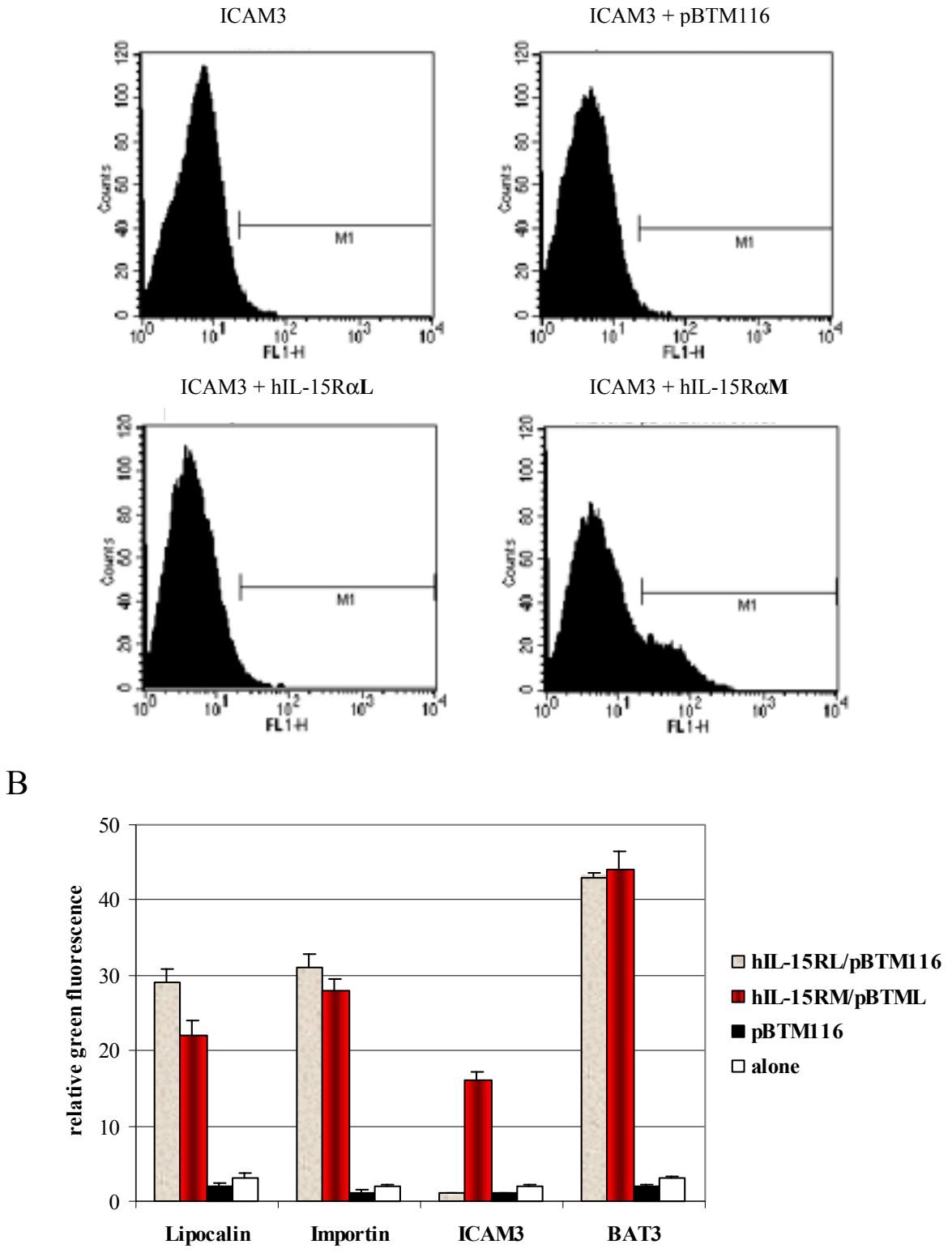


Figure 22. Quantitative analysis of interaction between hIL-15Rα fragments and the four interacting proteins, importin, lipocalin, BAT3 and ICAM3.

A) The protein-protein interaction measured by the yeast two-hybrid analysis using the GFP gene as a reporter. 20000 cells of three independent transformants clones were analysed for fluorescence intensity using a Becton Dickinson FACS sort flow cytometer. The fluorescence

intensity of untransformed yeast used for comparison. Shown is one representative out of 3 independent clones. B) The fluorescence intensities of transformed yeast cells were calculated relative to the untransformed yeast cells, which were arbitrarily set at zero. Vertical bars indicate the standard deviation based on the three sets of data.

All four interacting partners interact with the hIL-15Rα**M** fragment. Lipocalin, importin and BAT3 also interact with the large fragment of hIL-15Rα, but ICAM3 does not bind to the hIL-15Rα**L** (Fig. 22). In addition, BAT3 shows a high fluorescence intensity, indicating perhaps a higher binding-affinity than other proteins for hIL-15Rα. Yeast cells expressing hIL-15Rα L and M alone showed only auto-fluorescence (data not shown).

The FACS analysis confirm that lipocalin, importin, ICAM3 and BAT3 are true interacting partners for the hIL-15Rα cytoplasmic domain and also show that BAT3 bind IL-15Rα with higher affinity.

4.2.9 Identification of false positive interactions proteins of IL-15Rα

In order to verify the interactions of other potentially interacting candidates that are listed in Table 1, the ribosomal protein large P1 (RPLP1, which was found 15 times), the Arsenite translocating ATPase (ASNA1), the ribonucleoprotein (FLJ36391) and the hypothetical protein MGC10526 were chosen for analysis in two-hybrid system using GFP as reporter gene. Activation of GFP was quantified by flow cytometric analysis. As shown in Fig. 23, the hypothetical protein MGC10526 is a false positive candidate, because it actives the reporter gene with pBTM116 (the bait vector without insert). The RPLP1 is also a false positive candidate, because the negative controls, RPLP1 alone and with pBTM116, produce the same fluorescence intensity as with the hIL-15Rα fragments. ASNA1 and FLJ36391 associate with hIL-15Rα fragments, but it seems that FLJ36391 has a low affinity for hIL-15Rα fragments (Fig. 23).

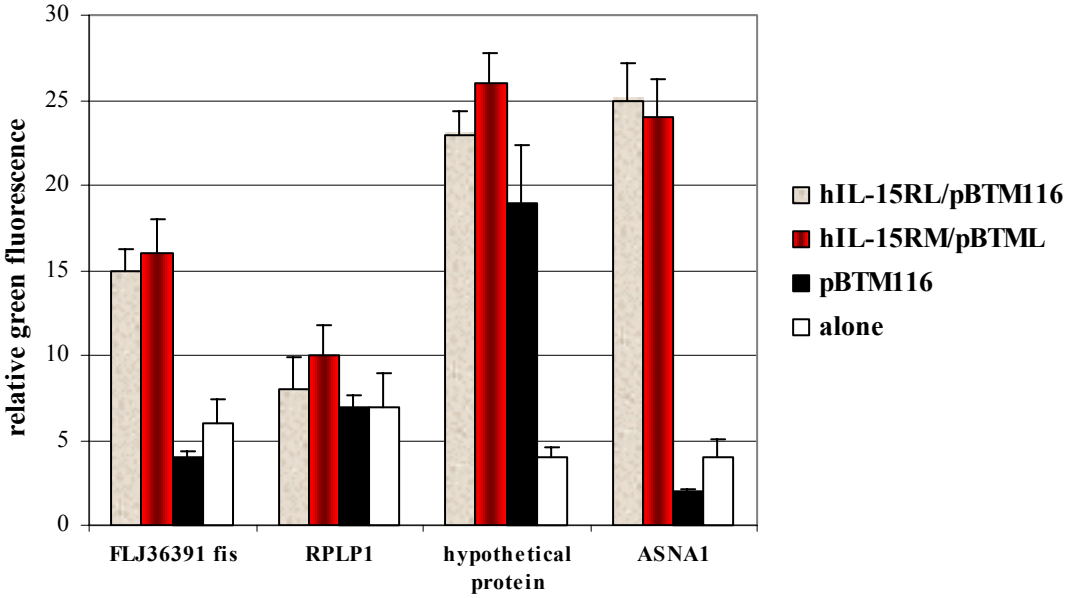


Figure 23. Analysis of protein-protein interactions by the yeast two-hybrid system using GFP as a reporter.
The flow cytometric quantification analyses of two-hybrid interactions of hIL-15Rα fragments with Ribosomal protein, large P1 (RPLP1), Arsenite translocating ATPase (ASNA1), ribonucleoprotein (FLJ36391) and the hypothetical protein MGC10526 were calculated from three independent experiments. The analysis of interacting proteins alone and with pBTM116 was performed as a control.

These data show that RPLP1and MGC10526 are false positive interacting partners, but ASNA1 and FLJ36391 can bind with a low affinity to IL-15Rα fragments.

4.2.10 DNA-sequence analysis of lipocalin expressing clones

In previous experiments, it was shown that lipocalin is a true interacting partner for hIL-15Rα. 40% of identified clones expressed lipocalin as interacting protein, which indicates a high value of correct screening and a high score hit.
DNA-sequence analysis was performed to answer the following questions:
(1) Which part of lipocalin was fished as a interacting region? (2) Are the cDNAs encoding lipocalin in the correct reading frame? cDNA-libraries are generally directionally cloned into

the relevant vector. Directional cloning ensures that one out of three cDNAs will be in-frame with the appropriate fusion partner compared with non-directionally cloned libraries where only one out of six cDNAs will be in-frame (Causier 2002).

(3) Are the cDNA fragments encoding lipocalin independent clones? It is important to ensure that the lipocalin expressing colonies are not the result of the amplification of a single yeast transformant.

In order to answer these questions, we analysed the cDNA sequences of the lipocalin encoding fragment via bioinformatic tools.

The agarose-gel electrophoresis of yeast-colony-PCR products showed that the size of inserts in clones expressing lipocalin are in the range of 800-1200 base pairs (bp) (Fig. 24A).

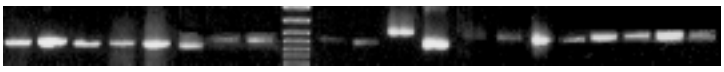
According to information from the Genbank, lipocalin mRNA is composed of 822 bp with a short 3´-UTR of ~150 bp and a coding region for a small protein of 198 amino acids (aa) (Fig. 24B). The alignment of identified sequences with the Genbank sequence showed that all inserts encode for the full-length protein.

In addition, the coding region of the lipocalin cDNA was in the correct reading frame in all clones, 2 of them are shown in Fig. 24C and D. Interestingly, in some clones the insertion was not correct, but by nucleotide deletion or insertion in the non-coding region, the reading frame was restored. Fig. 24D demonstrates one of these compensations.

Moreover, sequence analysis of identified clones expressing lipocalin indicated that they ere independent clones.

In summary, sequence analysis revealed that the all 20 clones expressing lipocalin were independent and code for the full-length protein in the correct reading frame.

This is additional evidence to confirm previous results that lipocalin is a true interacting protein of IL-15Rα.



A

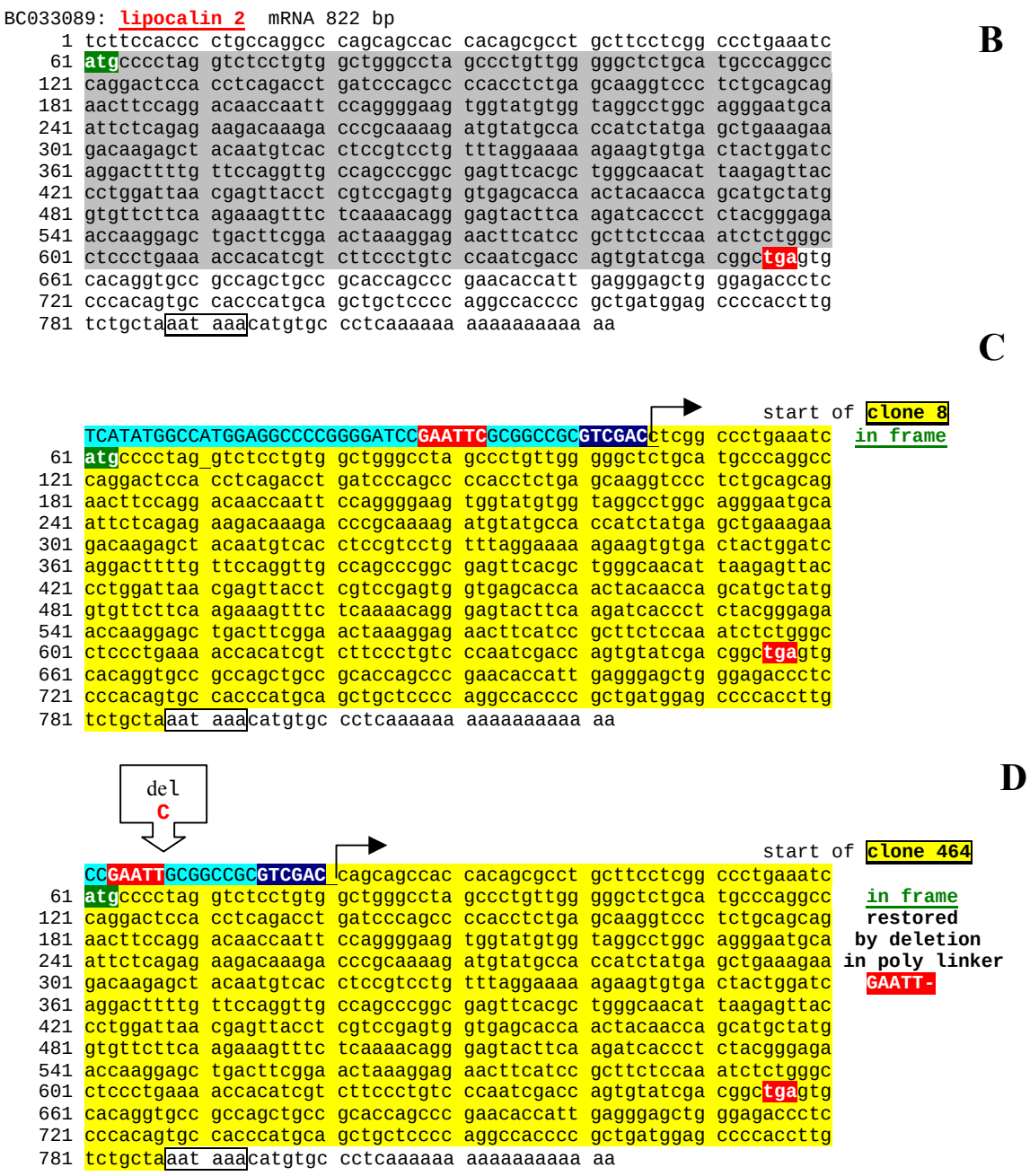


Figure 24. Sequence analyse of lipocalin expressing clones.
A) 1,5% agarose gel electrophoresis of direct colony-PCR products of 20 clones, which express lipocalin. B) DNA sequence of lipocalin, accession number BC033089. The mRNA is 822 bp and coding region contains 597 bp (61-658). C) DNA sequence of lipocalin insert in clone 8, the yellow labelled sequences show the lipocalin DNA sequence and the blue labelled sequence the plasmid. GAATTCGCGGCCGCGTCGAC *represents the adaptor sequence between plasmid and insert. The insertion is in frame. D) DNA sequence of clone 464. The insertion is not in frame, but a deletion in the adaptor sequence restores the reading frame of the lipocalin coding region.*

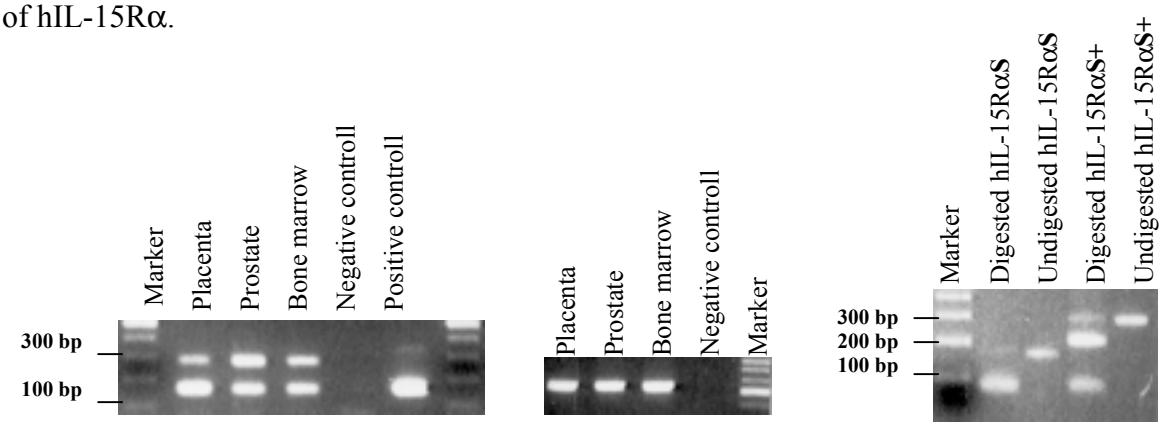
4.3 Identification of alternative spliced isoforms encoding the hIL-15Rα cytoplasmic domain

While screening the bone marrow cDNA by PCR for the presence of the hIL-15Rα cytoplasmic domain a fragment of 276 bp was found in addition to the expected 156 bp fragment. In the following the expected 156 bp fragment will be referred as hIL-15Rα**S** and the additional fragment as IL-15Rα**S+**. To confirm the presence of the additional fragment three different human cDNA libraries (from placenta, bone marrow and prostate) were analysed using hIL-15Rα-3 and hIL-15Rα-4 as PCR primers. As shown in Fig. 25A, the additional hIL-15Rα**S+** band was detected in all three libraries.

The DNA sequence of the human IL-15Rα gene was studied via bioinformatic tools for alternative splicing. The human IL-15Rα gene is composed of 8 exons. The cytoplasmic tail of hIL-15Rα WT is coded from exon7. The hIL-15Rα-3 primer localized in exon 6 and hIL-15Rα-4 in exon7.

Via gene analysis computer program, we found an alternative splice form within exon 7 of the hIL-15Rα gene, which we called 7U-A (Fig 25D). The exon 7U-A contains 120 bp and it can code for 26 aa. The presence of this 120 bp in exon 7 can explain the presence of the additional 276 bp band by PCR amplification. To confirm this speculation, both DNA fragments were isolated from the agarose gel and cut with the NcoI restriction enzyme. As we expected, digestion with NcoI results only in one visible band (~74 bp) for the hIL-15Rα**S** band but two for hIL-15Rα**S+**, ~ 74 and 202 (Fig. 25C). In order to support of these results, the isolated DNA-fragments were sequenced, confirming our hypothesis.

These data show the presence of two alternative spliced isoforms for the cytoplasmic domain of hIL-15Rα.



A **B** **C**

Figure 25. Alternative spliced isoforms of the human IL-15Rα cytoplasmic domain.
*A) The analysis of expression of hIL-15Rα cytoplasmic domain in cDNA-libraries by agarose gel electrophoresis of PCR-products. The lower band was called hIL-15Rα**S** and higher band hIL-15Rα**S+**. As positive control the plasmid encoding of IL-15Rα WT was used and water as negative control. B), The amount of cDNA was equalized by PCR amplification of b-actin. C) Digestion analysis of hIL-15Rα**S** and hIL-15Rα**S+** with NcoI. D) DNA- and encoded amino acid sequences of exon 6 and exon 7 of human IL-15Rα gene. For this alignment the cDNA of human IL-15Rα (GenBank accession No. U31628, 1610 bp) was used as query and the human IL-15Rα gene (GenBank accession No. AL137186, 191607 bp) as the subject. The numbers on the left site represent the nucleotide number in the cDNA or the gene.*

5 DISCUSSION

5.1 Role of the IL-15Rα intracellular domain in signalling

In this study the role of IL-15Rα in signal transduction was analysed in BA/F3 cells, a bone marrow derived IL-3-dependent mouse pro B-cell line. This cell line has the advantage that it does not express IL-15Rα and IL-2Rβ, but the IL-2Rα and γ-chain. Culture of these cells in the absence of IL-3 induces apoptosis (Leverrier 1997). It was demonstrated here, that in the absence of IL-3, transfected BA/F3 cells expressing IL-15Rα could be rescued from apoptosis when cultured in presence of IL-15. Thus, IL-15Rα mediate anti-apoptotic signalling. In addition, IL-15Rα can also mediate mitogenic signals in the IL-3 deprived transfected BA/F3 cells in the presence of IL-15. These results show that IL-15 via IL-15Rα not only rescues the transfected cells from apoptosis but also stimulates cell proliferation. The mediation of the anti-apoptotic effect of IL-15 via IL-15Rα clearly confirmed the postulated direct involvement of IL-15Rα in the IL-15-mediated signal transduction cascade in mice (Bulanova 2001, Bulfone-Paus 1999).

To understand the role of the IL-15Rα cytoplasmic domain in signalling, we performed studies using two chimeric receptors, made up of either the extracellular and transmembrane domains of mouse IL-2Rα joined to the cytoplasmic tail of mouse IL-15Rα (IL-2Rα/**IL-15Rα**) or the inverse combination (IL-15Rα/**IL-2Rα**).

I could show that transfected BA/F3 cells that express IL-2Rα/**IL-15Rα** can survive in the presence of IL-2 but the survival rate is reduced compared to IL-15Rα cells in presence of IL-15. Furthermore, the chimeras carrying the cytoplasmic domain of IL-15Rα (IL-2Rα/**IL-15Rα**) were also able to support proliferation of the IL3-dependent BA/F3 pro-B cells when IL-2 was substituted for IL-3, to the same degree as observed with the IL-15Rα expressing BA/F3 cells in the presence of IL-15. These data demonstrate that the intracellular part of IL-15Rα is absolutely necessary for transduction of signals responsible for proliferation and inhibition of apoptosis in a model of transfected BA/F3 cells. In contrast, in transfected BA/F3 cells, expressing IL-15Rα/**IL-2Rα** chimeric receptor, binding of IL-15 did not induce any cell proliferation or inhibition of apoptosis. This suggests that the cytoplasmic

domain of IL-15Rα induces completely different downstream signal transduction events. One potential mechanism to explain this might be the recruitment, binding and activation of different adaptor proteins.

It has also been shown that IL-4Rα, which normally signals when coupled to the γ-chain as a heterodimer, can signal as homodimer in the absence of the γ-chain (Fujiwara 1997, Kammer 1996). It may therefore be likely that IL-15Rα as homodimer interacts with some yet unknown signalling molecule and transfers a signal.

Another possible explanation of IL-15Rα-mediated signal transfer in BA/F3 cells is the cross talk between receptors of different cytokine families, as recently reported for IL-15 and GM-CSF, in human $CD34^+$ cells (Giron-Michel 2003).

Apoptosis is an autonomous cell suicide pathway, which is characterised by specific morphological changes of cells. The cellular machinery necessary for the death programe is present in most cell types in an inactivated form (Raff 1993). This state of inhibition is dependent on survival signals delivered by exogenous signals such as growth factors or adhesion molecules. Two major gene families are involved in the regulation of apoptosis: the caspases and the Bcl-2 family. Over-expression of Bcl-2 or Bcl-X can inhibit apoptosis induced by removal of surviving signals or by the delivery of death signals (Oltvai 1994). The level of Bcl-X in BA/F3 cells is tightly regulated by IL-3. Interestingly, apoptosis induced by IL-3-deprivation is inhibited on two levels: short-term inhibition by growth factors is mediated at the post-translational level and is not dependent on the Bcl-X induction and long-term inhibition of apoptosis depending on Bcl-X transcription and activation of the MAP-kinase pathway (Leverrier 1997).

Recently it has been shown by our group in mouse fibroblasts, the IL-15Rα binds to the receptor tyrosine kinase Axl and, via up regulation of Bcl-2 and Bcl-X, rescue the cells from TNFα-induced apoptosis (submitted for publication). Thus, signalling via binding to Axl is another potential pathway for the IL-15 mediated anti-apoptotic effect in BA/F3 cells.

5.2 Identification of IL-15Rα interacting proteins by the yeast two-hybrid system

Signalling of IL-15 via IL-15Rα is involved in different biological processes such as cell proliferation, prevention from apoptosis, chemoatraction, differentiation and many other signal transduction pathways, which has been reviewed by many groups (Shurin 2003, Fehniger 2002, Waldmann1999).

Although some of the IL-15Rα mediated signal transduction pathways are already described, the different effects of IL-15 lead to the assumption that there are new, unknown signalling players. The different biological activities of the receptor can be explained by the existence of specific, up to now unidentified interaction proteins of IL-15Rα

The second part of this work was concerned with the search for interacting proteins with the cytoplasmic domain of the human of IL-15Rα. The "yeast two-hybrid system" was employed for identification of IL-15Rα interacting proteins from a human bone marrow library. Despite its many drawbacks, which are invariably encountered when using high through-put screening techniques in a heterologous test system, this kind of "interaction trap" is still state of the art for identifying protein-protein interactions, for which this screen provides the very first step. Bone marrow is the most important hematopoietic organ. The high expression of IL-15Rα in bone marrow stromal cells indicates its involvement in hematopoiesis (Anderson 1995).

The two most appealing features of two-hybrid screen are 1) the detection of weak and transient interactions (often the most interesting and relevant in signalling cascades) since the genetic reporter gene strategy results in a significant amplification and 2) that it simultaneously provides the cloned cDNA of the potential interacting protein (or the binding domain thereof), which can directly be used in obligatory successive rounds of verifications.

As there are countless excellent articles in the scientific literature, in which the drawbacks of the two-hybrid system are discussed exhaustively (Causier 2002, Van-Criekinge 1999), only the aspects most relevant to the work presented in this thesis will be discussed below.

The first aspect concerns the chances of the correct folding of full-length proteins or functional protein domains when expressed as artificial fusion products. So far there is no reliable method to predict the conformation of proteins from their primary sequence. For this reason, three bait cDNA inserts were generated, each encoding the cytoplasmic domain (41 aa) of the human hIL-15Rα but carrying N-terminal extensions of increasing length

(covering also the transmembrane domain and part of the extracellular domain in the longest version). Moreover, each insert was cloned in two vectors that differed only by the presence or absence of an artificial linker following the DNA-binding domain but preceding the multiple cloning site. As demonstrated by a Western blot (Fig. 18), the presence of the linker sequence seemed to positively influence the efficiency of fusion-protein expression in the yeast host strain, at least in the case of the two smaller cDNA inserts.

Since the two-hybrid system is dependent on reconstitution of a functional transcription factor, auto-activation would give rise to large number of false positives. Therefore it is necessary that every bait construct is tested for auto-activation. The surprising result that only the smallest bait version, could activate the reporter gene on its own, clearly demonstrates the unpredictable folding properties of a protein domain within different sequence contexts.

In the literature it is suggested to reduce the size of the chosen fragment for elimination of self-activation (Van-Criekinge 1999). In this work the small sized fragment indicated auto-activation, which was eliminated by increasing the size.

The cytoplasmic domain of hIL-15Rα consists of only 41 amino acids. Maybe its small size results in an unspecific binding to some transcription factors or their related proteins. Increasing the size lead to stable folding and, therefore, eliminates unspecific binding. It seems that the size is an important factor for selection of the bait. Very big or very small baits could be optimised via reducing or increasing the size.

The second aspect deals with the choice of the cDNA library to be used in a two-hybrid screen. Obviously, the prey-cDNA should stem from a cell line or tissue, in which the target protein is known to be biologically relevant.

In addition, the results of screening depends on the quality of the library. An important quality parameter of libraries is the number of independent transformed clones. To screen a mammalian cDNA library until saturation $5\text{-}10\times10^6$ yeast transformants need to be screened (Causier 2002, Van-Criekinge 1999). Since the number of independent clones in our screening was $\sim8\times10^6$, the probability for identification of IL-15Rα interacting protein was expected to be adequate. Additionally, isolation of overlapping or independent clones for an identified interaction protein, such lipocalin in our screening, is also an indicator for the quality of two-hybrid screening.

When screening libraries by the yeast two-hybrid system proteins such as ribosomal subunits or heat shock proteins are often picked up that are not true interacting partners (Serebriiskii

2000). Our analysis showed that more than 60% of our identified proteins were specific binding proteins and the remaining clones were false positive.

Another point, rarely addressed, is that a prerequisite for obtaining real interaction partners in an expression cDNA library is not only the correct reading frame (1 out of 3 in a directionally cloned library) of the cDNA insert, but also that it covers the coding region at all. Many mRNAs carry 3' non-translated regions (3'-UTRs) that are exceeding the average insert sizes of cDNA expression libraries, which is roughly 2 kb. Since the two-hybrid screen relies on the synthesis of proteins, encoded by the bait-cDNA inserts, and the majority of commercially available cDNA libraries are generated by oligo-dT priming, the translation products of mRNAs with short 3'-UTRs will be over-represented, whereas those encoded by mRNAs with unusually long 3'-UTRs are very likely not present at all. In other words, the identification of interacting partners will be biased not only towards small protein-encoding mRNAs, but also towards those ending shortly after the translation stop codon. And this is indeed what was observed.

For instance, one of the most frequently selected open reading frames was the RPLP1 (15×). The corresponding mRNAs encode a short protein of 114 amino acids, and have unusually short 3'-UTRs of only about 40 nt.

The above considerations might well be the reason for the absence of TRAF2 and Syk-encoding cDNA clones among the two-hybrid selected clones obtained in this screen of a human bone marrow cDNA-library, although the former is known to be expressed in bone marrow (Turner 1997 and Hsu 1997).

The roughly 800 nt long 3'-UTR of the human TRAF2 mRNA, and the fact that the C-terminal 240 amino acids (720 nt) that make up the so-called "TRAF-C domain", are necessary for TRAF2-binding, would have required an insert size of about 1,5 kb to ensure expression of the TRAF-C binding domain. Moreover, the generally low basal expression of TRAFs in non-activated bone marrow cells and the notion that statistically only one out of three AD-fusion proteins are in the correct reading frame, further reduced the likelihood of selecting TRAF2 in the screen, performed in this thesis.

There is the similar problem for Syk. The 3´non-coding region of human Syk mRNA is ~ 600 nt. It was speculated that Syk bind to IL-15Rα via its SH2 domain, which is localized in the first 300 amino acids of the N-terminal end of the protein. The full lengh Syk mRNA is ~1900 nt. Therefore, the presence of inserts contaning more than 2500 nt is required for expression of the binding domain.

In retrospect, it is clear that a library, in which oligo-dT as well as random primers had been employed for first strand synthesis of a prey cDNA library would have been the better choice for fishing potential interaction partners of hIL-15Rα.

Via two-hybrid screening of a human bone marrow cDNA library, six potential interactors for cytoplasmic domain of human IL-15Rα were identified. The interaction between these proteins and IL-15Rα were verified not only via multiple reporter genes in yeast strain L40 but also in yeast strain YRN974 using GFP as reporter gene. They are lipocalin 2 (GenBank accession No. NM_005564), Karyopherin alpha 2 (RAG cohort 1, Rch1, importin alpha 1) (GenBank accession No. BC053343, NM_002266), HLA-associated transcript 3, BAT 3 (GenBank accession No. NM_080703), Intracellular adhesion molecule 3, ICAM 3 (GenBank accession No. NM_002162), Arsenite translocating ATPase, ASNA 1(GenBank accession No. AF047469) and FLJ36391 fis, high similar to ribonucleoprotein (GenBank accession No. AK093710).

Among the six candidates, only lipocalin and importin and there potential role will be discussed in more detail.

Lipocalin:

Lipocalin was the most frequently selected protein in the yeast two-hybrid system. This is not surprising, because besides being expressed in bone marrow (Cowland 1997), it has a role in apoptosis, described for pro B cell lines. There is extensive apoptosis taking placed in bone marrow, most notably during maturation of pre-B cells, only about 10% survive (Goldsby 2000). Another reason for the high frequency of lipocalin cDNAs selected in this screen is also its short 3'-UTR (~ 150 nt) and short complete coding region (597 nt), which taken together are well within the average size range of cDNA library inserts.

Lipocalin 2, [also called neutrophil gelatinase-associated lipocalin, NGAL, 24p3 (highly conserved mouse homologe) and uterocalin] is a member of the lipocalin family (same name), which are typically small secreted proteins (Flower 1996). In connection with the anti-apoptotic function of IL-15, lipocalin is the most interesting identified interaction partner for the IL-15Rα cytoplasmic domain. 24p3 is an inducible secretory protein, whose concentration is dramatically increased in mouse serum and liver via “acute phase response and induces apoptosis in a wide variety of leukocytes and primary bone marrow cells (Liu 1995, Devireddy 2001). The apoptosis sensitivity of a cell line for lipocalin correlates with the

presence of a putative receptor on the cell surface. Therefore, it was assumed that 24p3 induces the apoptosis through an autocrine pathway (Devireddy 2001). Deprivation of IL-3 lead to activation of transcription, synthesis and secretion of 24p3 in IL-3 dependent murine FL5.12 pro-B cells and thus induction of apoptosis. In this system it was shown, that ATFx, a member of ATF/CREB transcription factor family, is a target of 24p3 (Persengiev 2002). 24p3 repressed the expression of ATFx factor even in presence of IL-3. In contrast, cells, which ectopically express ATFx, are resistant against lipocalin. It was shown that Bcl-X in contrast to Bcl-2 is the sensitive and effective antagonist of 24p3 induced apoptosis (Devireddy 2001).

The acute phase proteins are produced by the liver and other tissue in response to inflammation or maintenance of homeostasis. Lipocalin is one of these proteins (Liu 1995). The secreted lipocalin is involved in immune system homeostasis, which requires that expanded cell populations are rapidly eliminated after their functions being completed.

Lipocalin has an important role in the innate immune response to bacterial challenge (Cowland 2003, Mallbris 2002, Friedl 1999, Nielson 1996). Binding to the bacterial ferric siderophores showed that lipocalin is a potent bacteriostatic agent and participates in the antibacterial iron depletion strategy of the innate immune system (Goetz 2002). Lipocalin was also identified as a novel iron carrier (Yang 2003).

Lipocalin as an acute phase protein and apoptosis inducer also plays an important role in "involution" (diminishment of tissue mass) in mammary gland and uterus (Nilson-Hamilton 2003).

BA/F3 cells are among the cell lines, which have been shown to be sensitive towards the apoptotic action of lipocalin (Devireddy 2001). Though not actually reported to react with lipocalin induction after IL-3 withdrawal, it is very likely that they do, as another mouse pro B cell line, FL5.12, was the one, in which lipocalin and its apoptotic role in IL-3 deprived cells was first uncovered (Devireddy 2001). Interestingly, the observed lipocalin sensitivity of cells correlated with the presence of a putative high-affinity receptor (K_d ~92 pM) on their surface.

Although BA/F3 cells seem not to express IL-15Rα endogenously, other lipocalin sensitive cells do (primary bone marrow cells, peripheral blood lymphocytes and neutrophils) (Girard 1998). Therfore, the BA/F3 cells, in which the IL-15Rα was expressed in full-length or as the cytoplasmic part of a truncated IL-2Rα receptor (chimera), might well be a meaningful test

system for providing a first clue towards the possible importance of a direct interaction between lipocalin and IL-15Rα.

Among the factors, which can substitute for IL-3 in proB-cell survival, is IGF1 (Insulin-like growth factor 1) which was shown to act via blocking the transcriptional activation of lipocalin, but being unable to prevent apoptosis resulting from direct addition of lipocalin to the medium of FL5.12 cells (Devireddy 2001). This raises the intriguing, though highly speculative possibility that IL-15Rα could do both: cause a direct inactivation of lipocalin, for instance by directing the lipocalin via internalisation to proteosomal degradation and at the same time prevent its synthesis. The latter possibility would be in agreement with frequent reports of a nuclear localization of IL-15Rα.

In addition, lipocalins as biochemical markers of disease have been used extensively. The clinical indications relate to almost any field of medicine, such as inflammatory disease, cancer, lipid disorders, liver and kidney functions (Xu 2000). Since IL-15 is involved in some disease such rheumatoid arthritis, cancer (Taylor 2003, Gonzalez, 2003, Kuniyasu 2003, Gangemi 2003, Waldmann 2002, Fehinger 2002), the study of interaction between IL-15Rα and lipocalin provides new aspects in clinical application.

Importin:

Importin α1 (also called as RAG cohort 1, Rch1 or karyopherin α 2) is the second potentially interacting partner of IL-15Rα, which belongs to the importin α subfamily.

Importin plays a key role in the nuclear import of proteins with a classical nuclear localization signal sequences (NLS). Importin, as a part of an importin α-β heterodimer, recognizes proteins carrying the NLSs and directly binds to the NLS. Importin β is responsible for the translocation of the importin α-substrate complex through the nuclear pore complex.

Since the localization of IL-15Rα in nucleus has been reported (Bulanova 2003, Pereno 2000, Pereno 1999, Dubois 1999), the interaction with importin may explain the nuclear transport mechanism of IL-15Rα. In addition IL-15Rα contains a NLS motif, localized into the sushi domain, which is encoded in exon 2 (Dubois 1999). Surprisingly, the importin (140 C-terminal amino acids of importin in fusion with Gal4 activation domain) was fished by the cytoplasmic region of IL-15Rα, where the NLS motif of IL-15Rα could not play a role in this connection. Interestingly, it was reported that importin also localizes at the plasma membrane and participates in a signal transduction pathway involving the shuttling of karyophilic

proteins from the plasma membrane to the nucleus (Andrade 2003). The subcellular localization of importin at the plasma membrane raises the possibility of a novel role for importin, not only in nuclear import across the nuclear envelope but also in providing a direct link in signal transduction of extracellular stimuli from plasma membrane to the nucleus. The involvement of importin in signalling also is confirmed by the fact, that the "Tec-family tyrosine kinase Itk binds importin and leads to phosphorylation of importin in human T cells (Perez-Villar 2001). Moreover, Itk lacks NLS und binds to importin via its SH3-domain.
Most likely there also are different function-dependent nuclear import mechanisms for the transfer of IL-15Rα into the nucleus, one via the NLS motif and another via an unknown importin α binding motif. This is in accordance with previous reports, showing that the different intracellular trafficking is involved in the nuclear localization of IL-15Rα in two melanoma cells (Pereno 2000 and 1999).
In agreement with the above observations, the finding of an open reading frame for importin alpha was considered as possibly very meaningful, despite the fact that the coding region of importin occurred only once among the sequenced two-hybrid selected clones. This rare occurrence could well be explained with the notion that the bone marrow contains resting or inactivated lymphocytes. Only activated lymphocytes show high amounts of importin-mRNA and -protein in the cell, whereas at basal level it is almost undetectable (Andrade 2003).

Another phenomenon that started to emerge in the scientific literature within the last years is the observation that also membrane spanning receptors others than the glucocorticoid receptor, might also act as activator of genes involved in their own signalling cascade. These modulations take place by induction of their respective ligands independent of known NLS motifs (Stachowiak 2003, Myers 2003, Nanba 2003, Xu 2003, Carpenter 2003, Boerner 2003)

5.3 **The alternative spliced isoforms of IL-15Rα cytoplasmic domain**

IL-15Rα, as well as IL-2Rα are type I transmembrane proteins. They do not belong to the hemopoietin receptor family but instead contain in their extracellular domains one (IL-15Rα) or two (IL-2Rα) "sushi" domains, protein-binding motifs. The close linkage of their genes in mice and man, suggests that they arose by a gene duplication event, which occurred before the separation between mouse and man (Anderson 1995).

Up to this point, two different cytoplasmic domains have been identified for the human IL-15Rα chain, arising from the alternate usage of two successive 3' splice sites in intron 6 of the hIL-15Rα gene, giving rise to two alternative last exons joined to exon 6, designated as ex7U-A and ex7U-B as shown in Fig. 25D, whereby ex7U-B makes up the 3' terminal part of ex7U-A. (Note that "ex7" in what is generally referred to as IL-15Rα mRNA (acc-Number: U31628, corresponds ex7U-B)). The existence of ex7U-A was first described by Anderson et al. (1995) as "a 120 bp insertion at the position of intron 6" and erroneously depicted as a full alternative exon (ex7') in the Dubois paper (1999). Using primer pairs, in which the downstream primer included the stop codon of the IL-15Rα coding region, two RT-PCR products have been repeatedly obtained in all tissues tested in this work (bone marrow, placenta and prostate). Sequence analyses revealed that both products corresponded indeed to either of the two alternative last exons.

A search in the database with IL-15Rα as query revealed another, this time indeed full length last exon joined to exon 6, again resulting from usage of two alternative 3' splice sites located about 4 kb downstream of ex7U-A and-B (Fig. 26 and 27, Karin Wiebauer, personal communication). Alignment of those sequences (called ex7D-A and-B, D for downstream) with the corresponding genomic DNA, suggests that there must have occurred another gene duplication event of a region including ex 7D-A/B, but this time, it apparently has taken place after separation of mice and men, since no such 3' terminal isoforms of IL-15Rα mRNA have been detected in mice.

The striking feature of this observation, further underlining the proposed role of IL-15Rα in signal transduction, is, however, that each of the four alternative last exons encode different amino acids, giving rise to four different IL-15Rα isoforms, each carrying a different cytoplasmic tail (Fig. 28).

Among them, hIL-15Rα-cytD-IV is the most interesting one, as it contains seven tyrosines, three of which are part of an SH2-binding motif, that, upon tyrosine phosphorylation, act as docking sites for SH2-groups containing kinases.

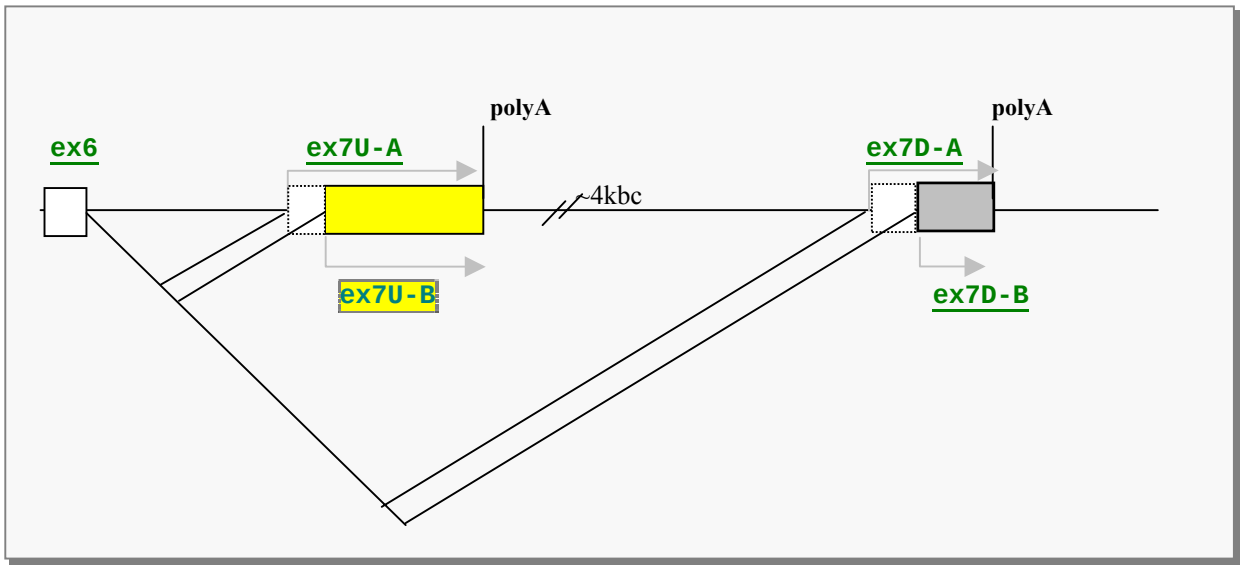


Figure 26. Schematic diagram of alternative splicing for isoforms of the human IL-15Rα cytoplasmic domain.
Via alternative splicing, the exon 6 of the IL-15Rα gene can be connected to four different alternatives of exon 7 coding for four the different cytoplasmic domains: ex7U-A, ex7U-B, ex7D-A and ex7D-B. The exon7U-B (shown in yellow) encodes for the WT isoforms of IL-15Rα cytoplasmic domain.

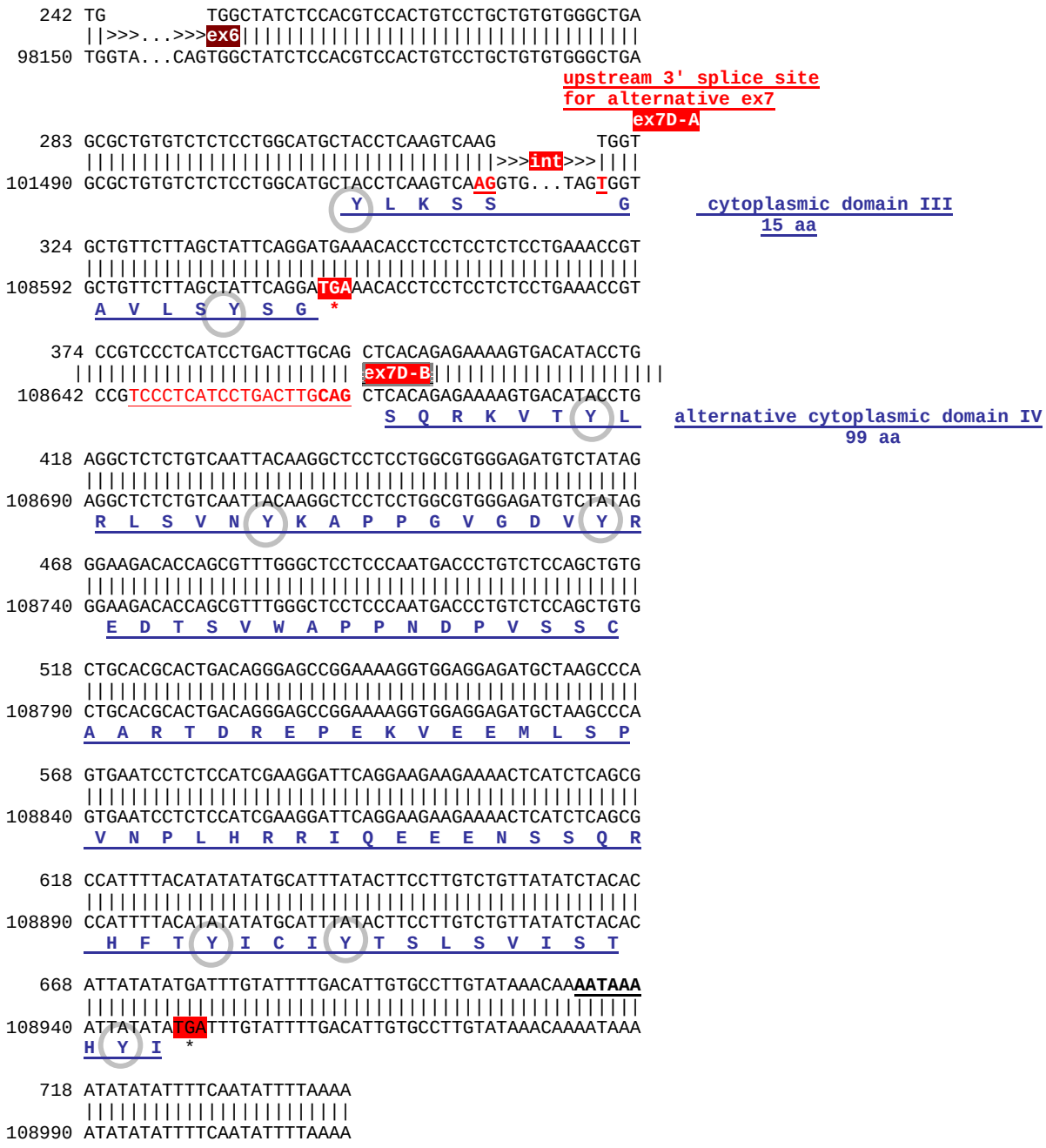


Figure 27. DNA- and encoded amino acids sequences of exon7D in the human IL-15Rα gene.

The alignment of the two sequences of human IL-15Ra gene (GenBank accession No. BX399036, 998 bp and AL137186, 191607 bp). The numbers on the left site represent the nucleotide number of DNA sequences. An 15 amino acids cytoplasmic domain (III) will be encoded by alternative splicing of exon 6 and exon 7D-A. Via splicing of exon7D-B, a mRNA can be generated, which encodes an 99 amino acids cytoplasmic domain (IV) with seven tyrosine residues (Y).

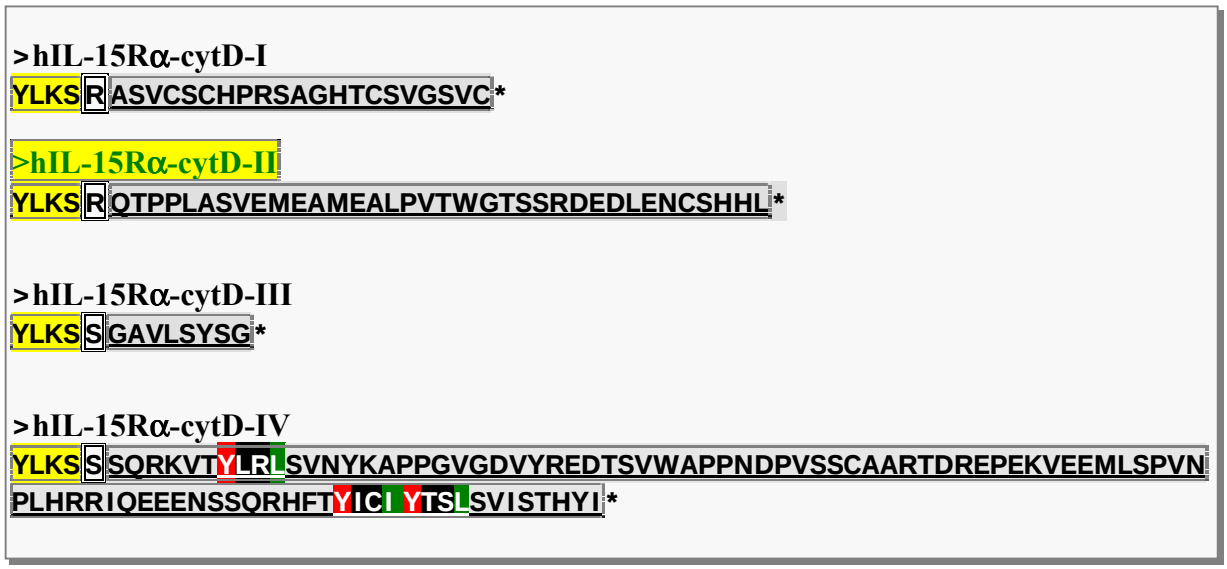


Figure 28. The amino acid sequences of four different isoforms of human IL-15Rα cytoplasmic domain encoded by exon 7.
The yellow-labelled amino acid sequence is encoded by exon 6 and double line boxes show amino acid encoded at the border between exon 6 and exon 7. The grey-labelled amino acid sequence is encoded by exon 7. The hIL-15Rα-cytD-II demonstrates the WT isoforms. The labelled amino acids in hIL-15Rα-cytD-IV indicate the SH2 motif (cytD = cytoplasmic domain).

Though the true biologically relevance of this finding has to await experimental evidence, the fact that human IL-15Rα can exist in these four isoforms that were not detected by the commonly used primer pairs in RT-PCR reactions and, moreover, that are not conserved in mice, might in part explain the often contradictory data in the IL-15Rα literature, and moreover demonstrates that data obtained with one organism are not necessarily true for the other.

Since the IL-15Rα is expressed in a wide variety of cells and mediates the variety effects of IL-15, the different cytoplasmic domains may be responsible for these diversities. It has been shown that alternatively spliced isoforms with distinct cytoplasmic domains are involved in differently signalling cascades or intracellular trafficking (Hermey 2003, Tabuchi 2002, Stepp 1999).

Taken together, the results of this work show that the IL-15Rα cytoplasmic domain plays a key role in signal transduction pathways. For first time was also shown here that lipocalin and importin bind to the IL-15Rα cytoplasmic domain, which provides new insights in IL-15 signal transduction pathways. In addition, identification of alternative spliced isoforms of the cytoplasmic domain reveals a new aspect of IL-15Rα mediated signalling pathways after stimulation by the pleiotropic cytokine IL-15.

The results presented in this thesis raise some interesting questions that should be further investigated. One important question concerns the biological processes triggered by the interaction of IL-15Rα with lipocalin and/or importin. Interestingly, IL-15Rα and lipocalin act as antagonist in apoptosis. To understand this phenomenon the biological significance of the IL-15Rα/lipocalin interaction should be studied in more detail. The cell line BA/F3, which is sensitive to lipocalin-induced apoptosis, can be used as a model system for these studies. Results obtained in this cell line should then be supported by a mouse model.

6 ABBREVIATIONS

Abs	antibodies
AD	activation domain
3-AT	3-amino-1, 2, 4-triazole
aa	amino acid
BD	binding domain
bp	bais pairs
CSM	complete supplement mixture
DBD	DNA binding domain
DEPC	diethylpyrocarbonate
DNA	deoxynucleic acid
dNTP	2'-deoxynucleoside 5'-triphosphate
DOB	drop out base
DOBA	drop out base with agar
DMSO	dimethyl sulfoxide
DTT	dithriothreitol
E. coli	Escherichia coli
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmatic reticulum
FACS	fluorescence activated cell sorter
FERM	four-point-one, ezrin, radixin, and moesin
FITC	fluorescein-5-isothiocyanate
IFNγ	interferon γ
IgG	immunoglobulin G
JAK	janus kinase
IL	interleukin
IL-15Rα	IL-15 receptor alpha
kDa	kilo dalton
LB	laurian broth
LPS	lipopolysacaride
MCS	multi cloning site
NF-κB	nuclear factor κB
nt	nucleotide
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PEG	polyethylene glycol
PI	propidium iodide
PVDF	polyvinylidene fluoride
RT	room temperature
SD	synthetic dropout medium
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SH2	Src homology 2
STAT	signal transducer and activator of transcription
TE	tris EDTA
TNF-α	tumor necrosis factor-α
TRAF2	TNF-α receptor associated factor 2
Tris	tris-hydroxymethyl-aminoethane
UAS	upstream activation sequence
WT	wild type
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactoside

7 ATTACHMENTS

7.1 The cDNA and amino acids sequences of the human IL-15Rα

```
     M  A  P  R  R  A  R  G  C  R  T  L  G  L  P  A  L  L  L  L  20
   1 ATGGCCCCGCGGCGGGCGCGCGGCTGCCGGACCCTCGGTCTCCCGGCGCTGCTACTGCTG

     L  L  L  R  P  P  A  T  R  G  I  T  C  P  P  P  M  S  V  E  40
  61 CTGCTGCTCCGGCCGCCGGCGACGCGGGGCATCACGTGCCCTCCCCCCATGTCCGTGGAA

     H  A  D  I  W  V  K  S  Y  S  L  Y  S  R  E  R  Y  I  C  N  60
 121 CACGCAGACATCTGGGTCAAGAGCTACAGCTTGTACTCCAGGGAGCGGTACATTTGTAAC

     S  G  F  K  R  K  A  G  T  S  S  L  T  E  C  V  L  N  K  A  80
 181 TCTGGTTTCAAGCGTAAAGCCGGCACGTCCAGCCTGACGGAGTGCGTGTTGAACAAGGCC

     T  N  V  A  H  W  T  T  P  S  L  K  C  I  R  D  P  A  L  V  100
 241 ACGAATGTCGCCCACTGGACAACCCCCAGTCTCAAATGCATTAGAGACCCTGCCCTGGTT

     H  Q  R  P  A  P  P  S  T  V  T  T  A  G  V  T  P  Q  P  E  120
 301 CACCAAAGGCCAGCGCCACCCTCCACAGTAACGACGGCAGGGGTGACCCCACAGCCAGAG

     S  L  S  P  S  G  K  E  P  A  A  S  S  P  S  S  N  N  T  A  140
 361 AGCCTCTCCCCTTCTGGAAAAGAGCCCGCAGCTTCATCTCCCAGCTCAAACAACACAGCG

     A  T  T  A  A  I  V  P  G  S  Q  L  M  P  S  K  S  P  S  T  160
 421 GCCACAACAGCAGCTATTGTCCCGGGCTCCCAGCTGATGCCTTCAAAATCACCTTCCACA

     G  T  T  E  I  S  S  H  E  S  S  H  G  T  P  S  Q  T  T  A  180
 481 GGAACCACAGAGATAAGCAGTCATGAGTCCTCCCACGGCACCCCCTCTCAGACAACAGCC

     K  N  W  E  L  T  A  S  A  S  H  Q  P  P  G  V  Y  P  Q  G  200
 541 AAGAACTGGGAACTCACAGCATCCGCCTCCCACCAGCCGCCAGGTGTGTATCCACAGGGC

     H  S  D  T  T  V  A  I  S  T  S  T  V  L  L  C  G  L  S  A  220
 601 CACAGCGACACCACTGTGGCTATCTCCACGTCCACTGTCCTGCTGTGTGGGCTGAGCGCT

     V  S  L  L  A  C  Y  L  K  S  R  Q  T  P  P  L  A  S  V  E  240
 661 GTGTCTCTCCTGGCATGCTACCTCAAGTCAAGGCAAACTCCCCCGCTGGCCAGCGTTGAA

     M  E  A  M  E  A  L  P  V  T  W  G  T  S  S  R  D  E  D  L  260
 721 ATGGAAGCCATGGAGGCTCTGCCGGTGACTTGGGGGACCAGCAGCAGAGATGAAGACTTG

     E  N  C  S  H  H  L  *     267
 781 GAAAACTGCTCTCACCACCTATGA
```

The numbers on *the right site* demonstrates the positions of nucleotides and the numbers on *the left site* indicate the amino acid positions. The signal peptide is shown in a dotted yellow box, the transmembrane domain by a dark blue box, and the cytoplasmic domain by a red solid underline. The grey amino acids in the extracellular part designate the "sushi" domain (GenBank accession number U31628).

7.2 The cDNA and amino acids sequences of the murine IL-15Rα

```
     M  A  S  P  Q  L  R  G  Y  G  V  Q  A  I  P  V  L  L  L  L  20
1    ATGGCCTCGCCGCAGCTCCGGGGCTATGGAGTCCAGGCCATTCCTGTGTTGCTGCTGCTG

     L  L  L  L  L  L  P  L  R  V  T  P  G  T  T  C  P  P  P  V  40
61   CTGTTGCTACTGTTGCTCCCGCTGAGGGTGACGCCGGGCACCACGTGTCCACCTCCCGTA

     S  I  E  H  A  D  I  R  V  K  N  Y  S  V  N  S  R  E  R  Y  60
121  TCTATTGAGCATGCTGACATCCGGGTCAAGAATTACAGTGTGAACTCCAGGGAGAGGTAT

     V  C  N  S  G  F  K  R  K  A  G  T  S  T  L  I  E  C  V  I  80
181  GTCTGTAACTCTGGCTTTAAGCGGAAAGCTGGAACATCCACCCTGATTGAGTGTGTGATC

     N  K  N  T  N  V  A  H  W  T  T  P  S  L  K  C  I  R  D  P  100
241  AACAAGAACACAAATGTTGCCCACTGGACAACTCCCAGCCTCAAGTGCATCAGAGACCCC

     S  L  A  H  Y  S  P  V  P  T  V  V  T  P  K  V  T  S  Q  P  120
301  TCCCTAGCTCACTACAGTCCAGTGCCAACAGTAGTGACACCAAAGGTGACCTCACAGCCA

     E  S  P  S  P  S  A  K  E  P  E  A  F  S  P  K  S  D  T  A  140
361  GAGAGCCCCTCCCCCTCTGCAAAAGAGCCAGAAGCTTTCTCTCCCAAATCAGATACCGCA

     M  T  T  E  T  A  I  M  P  G  S  R  L  T  P  S  Q  T  T  S  160
421  ATGACCACAGAGACAGCTATTATGCCTGGCTCCAGGCTGACACCATCCCAAACAACTTCT

     A  G  T  T  G  T  G  S  H  K  S  S  R  A  P  S  L  A  A  T  180
481  GCAGGAACTACAGGGACAGGCAGTCACAAGTCCTCCCGAGCCCCATCTCTTGCAGCAACA

     M  T  L  E  P  T  A  S  T  S  L  R  I  T  E  I  S  P  H  S  200
541  ATGACCTTGGAGCCTACAGCCTCCACCTCCCTCAGGATAACAGAGATTTCTCCCCACAGT

     S  K  M  T  K  V  A  I  S  T  S  V  L  L  V  G  A  G  V  V  220
601  TCCAAAATGACGAAAGTGGCCATCTCTACATCGGTCCTCTTGGTTGGTGCAGGGGTTGTG

     M  A  F  L  A  W  Y  I  K  S  R  Q  P  S  Q  P  C  R  V  E  240
661  ATGGCTTTCCTGGCCTGGTACATCAAATCAAGGCAGCCTTCTCAGCCGTGCCGTGTTGAG

     V  E  T  M  E  T  V  P  M  T  V  R  A  S  S  K  E  D  E  D  260
721  GTGGAAACCATGGAAACAGTACCAATGACTGTGAGGGCCAGCAGCAAGGAGGATGAAGAC

     T  G  A  *  263
781  ACAGGAGCCTAA
```

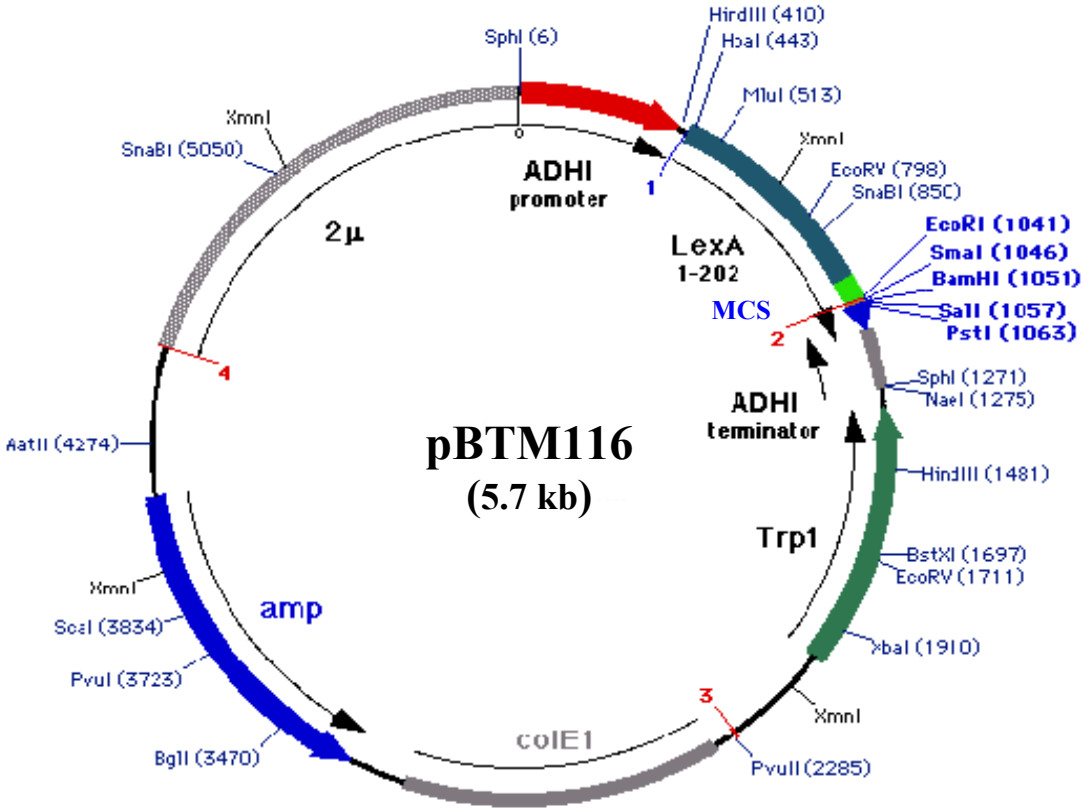
The numbers on *the right site* demonstrates the positions of nucleotides and the numbers on *the left site* indicate the amino acid positions. The signal peptide is shown in a dotted yellow box, the transmembrane domain by a dark blue box, and the cytoplasmic domain by a red solid underline. The grey amino acids in the extracellular part designate the "sushi" domain (GenBank accession number NM_008358).

7.3 The cDNA and amino acids sequences of the murine IL-2Rα

```
     M  C  Q  E  D  G  A  T  L  L  M  L  G  F  L  S  L  T  I  V   20
   1 ATGTGCCAGGAAGATGGAGCCACGTTGCTGATGTTGGGGTTTCTCTCATTAACCATAGTA

     P  S  C  R  A  E  L  C  L  Y  D  P  P  E  V  P  N  A  T  F   40
  61 CCCAGTTGTCGGGCAGAACTGTGTCTGTATGACCCACCCGAGGTCCCCAATGCCACATTC

     K  A  L  S  Y  K  N  G  T  I  L  N  C  E  C  K  R  G  F  R   60
 121 AAAGCCCTCTCCTACAAGAACGGCACCATCCTAAACTGTGAATGCAAGAGAGGTTTCCGA

     R  L  K  E  L  V  Y  M  R  C  L  G  N  S  W  S  S  N  C  Q   80
 181 AGACTAAAGGAATTGGTCTATATGCGTTGCTTAGGAAACTCCTGGAGCAGCAACTGCCAG

     C  T  S  N  S  H  D  K  S  R  K  Q  V  T  A  Q  L  E  H  Q  100
 241 TGCACCAGCAACTCCCATGACAAATCGAGAAAGCAAGTTACAGCTCAACTTGAACACCAG

     K  E  Q  Q  T  T  T  D  M  Q  K  P  T  Q  S  M  H  Q  E  N  120
 301 AAAGAGCAACAAACCACAACAGACATGCAGAAGCCAACACAGTCTATGCACCAAGAGAAC

     L  T  G  H  C  R  E  P  P  P  W  K  H  E  D  S  K  R  I  Y  140
 361 CTTACAGGTCACTGCAGGGAGCCACCTCCTTGGAAACATGAAGATTCCAAGAGAATCTAT

     H  F  V  E  G  Q  S  V  H  Y  E  C  I  P  G  Y  K  A  L  Q  160
 421 CATTTCGTGGAAGGACAGAGTGTTCACTACGAGTGTATTCCGGGATACAAGGCTCTACAG

     R  G  P  A  I  S  I  C  K  M  K  C  G  K  T  G  W  T  Q  P  180
 481 AGAGGTCCTGCTATTAGCATCTGCAAGATGAAGTGTGGGAAAACGGGGTGGACTCAGCCC

     Q  L  T  C  V  D  E  R  E  H  H  R  F  L  A  S  E  E  S  Q  200
 541 CAGCTCACATGTGTAGATGAAAGAGAACACCACCGATTTCTGGCTAGTGAGGAATCTCAA

     G  S  R  N  S  S  P  E  S  E  T  S  C  P  I  T  T  T  D  F  220
 601 GGAAGCAGAAATTCTTCTCCCGAGAGTGAGACTTCCTGCCCCATAACCACCACAGACTTC

     P  Q  P  T  E  T  T  A  M  T  E  T  F  V  L  T  M  E  Y  K  240
 661 CCACAACCCACAGAAACAACTGCAATGACGGAGACATTTGTGCTCACAATGGAGTATAAG

     V  A  V  A  S  C  L  F  L  L  I  S  I  L  L  L  S  G  L  T  260
 721 GTAGCAGTGGCCAGCTGCCTCTTCCTGCTCATCAGCATCCTCCTCCTGAGCGGGCTCACC

     W  Q  H  R  W  R  K  S  R  R  T  I  *     272
 781 TGGCAACACAGATGGAGGAAGAGCAGAAGAACCATCTAG   819
```

The numbers on *the right site* demonstrates the positions of nucleotides and the numbers on *the left site* indicate the amino acid positions. The signal peptide is shown in a dotted yellow box, the transmembrane domain by a dark blue box, and the cytoplasmic domain by a red solid underline. The grey amino acids in the extracellular part designate the "sushi" domain (GenBank accession number NM_008367).

7.4 Yeast expression vector pBTM116



LexA

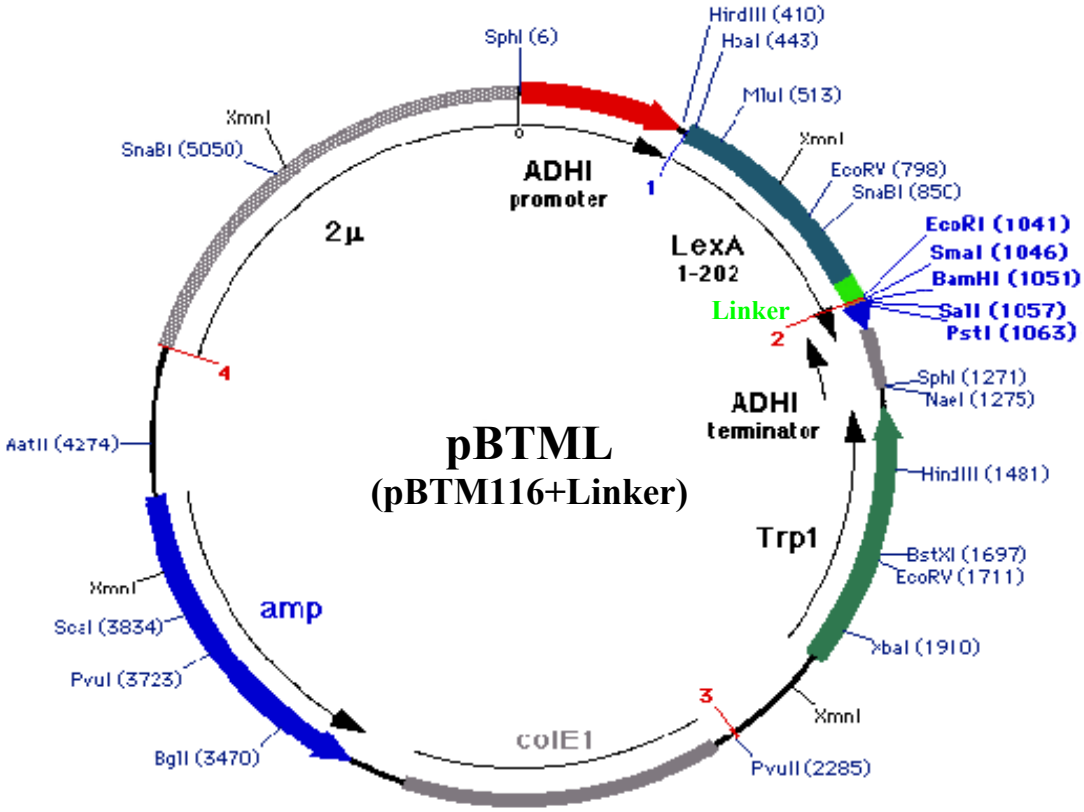
Multiple cloning site (MCS)

```
    Asp Trp Leu Glu Phe Pro Gly Ile Arg Arg Pro Ala Ala
3´  CTG ACC CTC CTT AAG GGC CCC TAG GCA GCT GGA CGT CGG  5´
5´  GAC TGG CTG GAA TTC CCG GGG ATC CGT CGA CCT GCA GCC  3´
```

EcoRI SmaI BamHI SalI PstI

pBTM116 (Bartel 1993), used in our “yeast two-hybrid system", generates a fusion protein of the bacterial LexA protein (amino acids 1-202) and the hIL-15Rα cloned into the MCS in the correct orientation and reading frame. The hybrid protein is expressed at high level in yeast host cells from the constitutive ADH1 promoter. Transcription terminated at the ADH1 terminator. pBTM116 replicates autonomously in both *E.coli* and *S. cerevisiae* and carries the bla gene, which confers ampicillin resistance in *E.coli*. pBTM116 also contains the TRP1 nutritional gene, that allows yeast auxotrophs to grow on the synthetic media lacking tryptophane.

7.5 Yeast expression vector pBTML

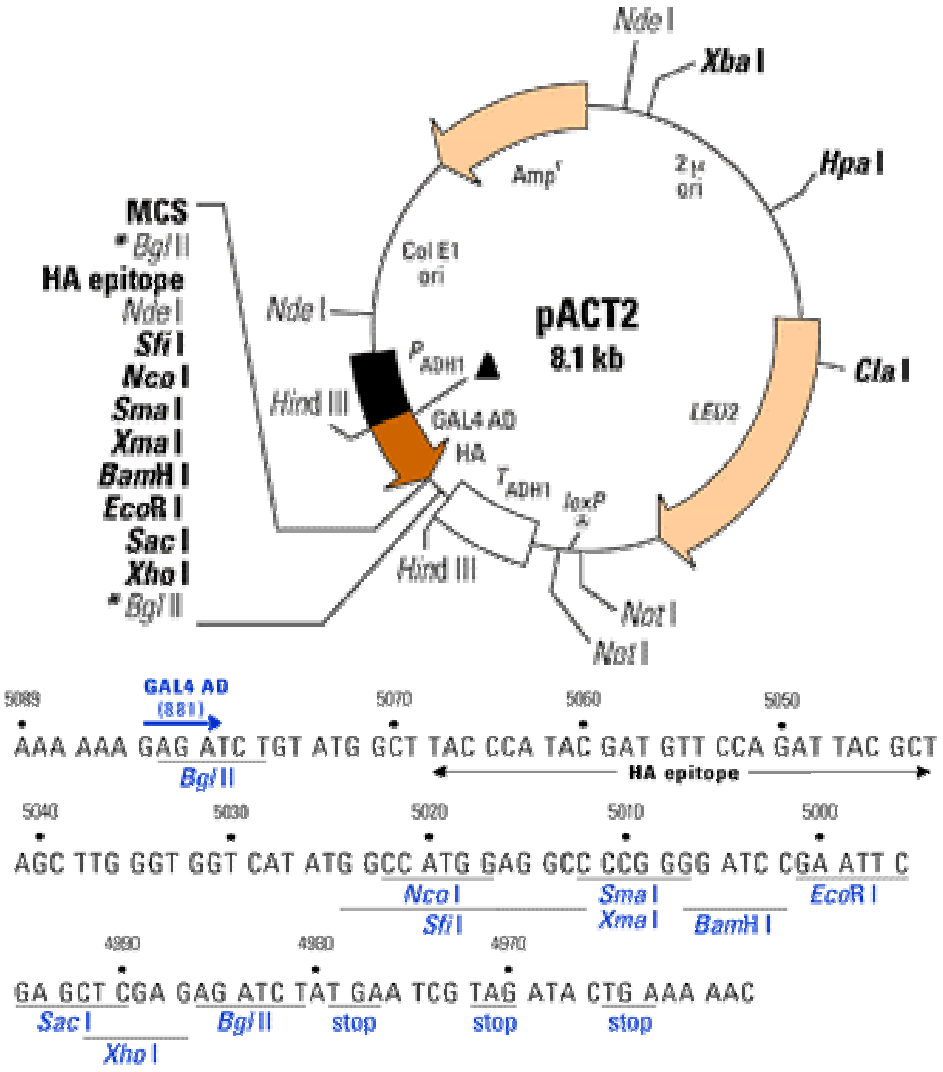


LexA **Linker** **Multiple cloning site (MCS)**

```
    Asp Trp Leu Glu Leu Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Glu Phe Pro Gly Ile Arg Arg
3´  CTG ACC CTC CTT AAC CCA CCT CCA CCT TCA CCT CCA CCT CCA AGC CTT AAG GGC CCC TAG GCA GCT
5´  GAC TGG CTG GAA TTG GGT GGA GGT GGA AGT GGA GGT GGA GGT TCG GAA TTC CCG GGG ATC CGT CGA
                                                              EcoRI  SmaI  BamHI  SalI
```

pBTML, a derivate of pBTM116 (section 7.4), contains an additional DNA fragment coding for a 12 amino acids linker peptide.

7.6 Yeast expression vector pACT2



pACT2, used in our “yeast two hybrid system", generates a fusion protein of the Gal4 AD (amino acids 768-881), an HA epitope tag, and a protein encoded by a cDNA in a fusion library (here the human bone marrow cDNA-library) cloned into the MCS in the correct orientation and reading frame. The hybrid protein is expressed at high level in yeast host cells from the constitutive ADH1 promoter (P). Transcription terminated at the ADH1 transcription termination signal (T). The protein is targeted to the yeast nucleus by the nuclear localization sequence from SV40 T-antigen, which has been cloned into the 5´end of the Gal4 AD sequence. pACT2 is a shuttle vector that replicates autonomously in both *E.coli* and *S. cerevisiae* and carries the bla gene, which confers ampicillin resistance in *E.coli*. pACT2 also contains the LEU2 nutritional gene, that allows yeast auxotrophs to grow on the synthetic media lacking leucine. Transformants with AD/library plasmids can be selected by complementation by the LEU2 gene by using HB101 *E.coli* strain that carries a leuB mutation. (Clontech, Heidelberg, Germany).

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