Bayerische Julius-Maximilian Universität Würzburg

THE CD23 RECEPTOR-
REGULATION OF EXPRESSION AND SIGNAL TRANSDUCTION

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SUMMARY

INTRODUCTION

1. General principles of transmembrane signalling

The B-cell represents one of the two major types of lymphocytes in the immune system, responsible for the humoral immune response. The antigen receptor on B-cells is a cell-surface immunoglobulin. After encountering the antigen B-cells differentiate into cells producing antibody molecules of the same antigen-specificity as their receptor.

B-cells communicate with the environment through a variety of cell-surface receptors that recognize and bind molecules present in the extracellular environment. Beside the antigen receptor, which is the most important in the response to antigens, a variety of other surface molecules contribute to coordinate growth, differentiation, metabolism and survival of the B-lymphocyte. These receptors convert extracellular ligand binding into an intracellular signal and activate intracellular pathways, which transmit the signal. This process is known as signal transduction. The final destination of receptor signalling is the nucleus, where the activation of transcription factors modifies gene expression.

Cell-surface receptors are transmembrane proteins that undergo conformational changes after ligand binding. This change can enable them for instance to associate with and activate a trimeric G protein (G protein-coupled receptor) or a protein-tyrosine kinase (tyrosine kinase linked receptors), or to activate their own intrinsic protein kinase activity (receptor tyrosine kinases). The cytosolic signal may activate a cascade of protein kinases or act through increasing the concentration of intracellular signalling molecules named second messengers (cAMP, cGMP, small lipid molecules and Ca^{2+}). These signal transduction pathways do not only transmit the signal, but also provide means for its amplification. Another important feature of signal transduction pathways is that they both converge- several receptors can activate the same signalling cascade or transcription factor- and diverge- one receptor or given transduction protein can have more than one effector. This provides means for specific, fine–tuned or complex responses to a variety of stimuli.

In B-cell signalling, tyrosine phosphorylation of membrane receptors is an important way to activate receptors. Amino acid motifs called ITAMs (immunoreceptor tyrosine based activation motifs) or ITIMs (immunoreceptor tyrosine based inhibitory motifs) are found in the cytoplasmic tails of Fc receptors, accessory chains of the B-cell receptor and other receptors. Phosphorylated tyrosine residues recruit intracellular signalling molecules to an activated receptor by binding a protein domain known as SH2 domain (Src homology domain 2). SH2 domains are phosphotyrosine binding motifs implicated in the regulation of protein-protein interactions and are thought to function as molecular adhesives facilitating the formation of protein complexes. SH2 domain binding to specific phosphotyrosine containing sequences may transmit intracellular signals by inducing conformational changes that alter an enzyme's catalytic activity or by altering the subcellular localization of a protein. Another protein domain involved in protein-protein interactions is the SH3 domain (Src homology domain 3), which binds proline-rich regions in diverse proteins to recruit them to signalling pathways. These binding domains can be found alone or in various combinations in proteins containing catalytic domains. These combinations provide great potential for complex interplay and cross-talk between different signalling pathways.

1.1 The Ras / MAPK pathway

The Ras/MAPK pathway is one of the best characterized pathway initiated by receptor tyrosine kinases. Ras is a small monomeric G protein with a central role in cell growth. In the active state it is bound to GTP while in the inactive state it is bound to GDP. Ras possesses an intrinsic GTPase activity that renders it inactive. In mammalian cells SOS, a guanine nucleotide exchange factor, controls the conversion of Ras from the normal, inactive state to the active state. SOS is brought to the membrane and activated by Grb2, an adaptor protein, in response to the phosphorylation of the receptor. Ras activates Raf, a serine/threonine kinase, which in turn activates MEK. In the MAP kinase pathway, this enzyme provides a convergence point, as it can be also activated by signals coming from G protein-coupled receptors. MEK is an enzyme with dual specificity, which can phosphorylate both threonine and tyrosine. Its target is Erk MAP kinase, which than activates target transcription factors directly into the cytoplasm or after its translocation to the nucleus. In the B-cell, MAP kinase pathways from the B-cell receptor or co-receptor combine to regulate the expression of many genes involved in cell growth [92,93].

1.2 Second messengers

Cyclic AMP is a classic second messenger. The initial step in the pathway is the activation of adenylate cyclase at the plasma membrane by an activated G protein associated with the receptor. cAMP binds to the regulatory subunit of PKA (protein kinase A) and releases the catalytic subunit of this enzyme, which is than free to translocate to the nucleus or to phosphorylate targets in the cytosol. One of the major substrates for PKA is CREB (cAMP response element binding protein). Phosphorylated CREB binds to CRE elements in the promoter of genes that are sensible to cAMP [94].

The inositol-lipid pathway is a common pathway for many types of receptors and involves second messengers derived from phosphatidylinositol. The enzyme phospholipase C-γ can be recruited through a SH-2 domain to the site of receptor-associated tyrosine kinase activity at the cell membrane. Phosphorylation of a tyrosine residue in PLC-γ activates the enzyme, which then cleaves the membrane phosphatidylinositol biphosphate (PIP_2) into inositol triphosphate (IP_3) and diacylglycerol (DAG). Diffusion of IP_3 away from the membrane causes the release of Ca^{2+} from intracellular storage sites into the cytosol, raising the intracellular Ca^{2+} level several times. The signal is sustained by the opening of Ca^{2+} channels into the plasma membrane. Ca^{2+} binds and activates a small cytosolic enzyme called calmodulin, which in turn binds to and regulates other enzymes or transcription factors like NF-AT. The signal eventually reaches the nucleus. DAG remains associated to the inner surface of the plasma membrane where it activates protein kinase C. Increased Ca^{2+} levels further activate this enzyme, which also initiate pathways leading to the nucleus [92,94].

1.3 Transcription factors with an important role in B-cell signalling

1.3.1 The JAK-STAT pathway

In contrast to signal transduction pathways that use a large number of components, like the MAPK and the inositol-lipid pathways, JAK-STAT pathway is much simpler. It is often activated by cytokine receptors that do not possess tyrosine activity. Binding of the ligand causes the receptor to dimerize and associate with and activate a JAK kinase. These are tyrosine kinases that phosphorylate transcription factors named STATs (signal transducer and activator of transcription). There are several JAK kinases and more than 7 STATs. Each STAT is phosphorylated by a particular set of JAK kinases. STAT phosphorylation leads to

the formation of homo- and heterodimers, which translocate to the nucleus and bind specific recognition elements in target genes. The activation of STATs is transient and can be terminated by the action of a phosphatase. STATs play important roles in numerous cellular processes including immune responses, cell growth and differentiation, cell survival and apoptosis [92,94].

1.3.2 The NF-kB family of transcription factors

The NF-kB transcription factors are important for B-cell activation. NFkB exists in the cytoplasm mainly as homo- or heterodimers with a family of structurally related proteins, called the Rel or Rel/NF-kB proteins. In non-stimulated cells, NF-kB complexes are sequestered in the cytoplasm in an inactive form via interaction with an inhibitory protein called IkB, which itself belongs to a structurally- and functionally-related family of proteins. When cells are stimulated by a variety of stimuli, like lipopolysaccharide (LPS) or CD40L, a kinase cascade leads to the phophorylation of two kinases, named Ikkα and Ikkβ, which form a dimer that in turn phosphorylates IkB. When phosphorylated, IkB dissociates from the complex and is rapidly degraded by proteosomes. NF-kB migrates to the nucleus where it is involved in regulating many aspects of the immune cell function, like cell survival, processing and presentation of antigen, responses to antigen recognition, aspects of the inflammatory response as well as responses against bacterial and viral infections [51]. The NF-kB transcription factor family consists of heterodimers or homodimers of the subunits NF-kB1 (p50), NF-kB2 (p52), c-REL, RELA (p65) and RELB. Different pairs of these subunits function at different stages of B cell development [57].

2. Pax-5 - the B-cell-specific activator protein

BSAP/Pax-5 is a member of the Pax (paired box) family of transcription factors, which constitute a small group of conserved developmental control genes. BSAP (B-cell-specific activator protein) was identified as the mammalian homologue of a sea urchin protein (TSAP), which is involved in the developmental regulation of two pairs of nonallelic histones H2A-2 and H2B-2 [3]. It is the only member of the family that is expressed in B-

lymphocytes. The other nine members of the Pax family identified so far have been associated with mouse developmental mutants and human syndromes. Deletion of the paired domain of Pax-3 is associated with the Splotch mutation in mice, characterized by spina bifida and exencephaly and with the Waardenburg's syndrome in humans, an autosomal dominant combination of deafness and pigmentary disturbances [20,80]; mutations in the Pax-6 gene are associated with congenital aniridia (lack of iris) in humans and the small eye (Sey) phenotype in mice- a semidominant mutation that in the homozygous condition results in the complete lack of eyes and nasal primordial [29].

2.1 Expression pattern

BSAP is encoded by the *pax-5* gene and is expressed at all stages of B-cell development except in terminally differentiated plasma cells [3]. In addition to all B-lymphoid organs, Pax-5 can also be found in the developing midbrain and adult testis of the mouse [1]. In accordance to this expression pattern, gene inactivation in the mouse germline revealed that Pax-5 plays an important role in B-lymphopoiesis and midbrain development. [84].

2.2 Molecular structure and DNA binding site

All members of the Pax protein family have a C-terminal transactivation domain and a N-terminal paired domain (the DNA binding domain). The transactivation domain is located in the 55 C-terminal amino acids of the molecule and contains a distinct serine/threonine/proline-rich sequence. This domain exerts its activating function from a promoter as well as an enhancer position and is subject of a strong negative regulation by adjacent sequences from the extreme C-terminus [16].

The paired domain consists of a stretch of 128 amino acids that has been well conserved in evolution and shows no obvious resemblance to other known DNA-binding motifs. Detailed mutational analysis of Pax-5 revealed the bipartite structure of a paired domain and lead to the identification of a nonpalindromic consensus recognition sequence [12].

In the model for the paired domain-DNA interaction (Fig. 1) the paired domain binds to its recognition sequence from one side of the DNA helix and interacts with two successive major grooves. The Pax-5 recognition sequence is divided in two halves, each corresponding

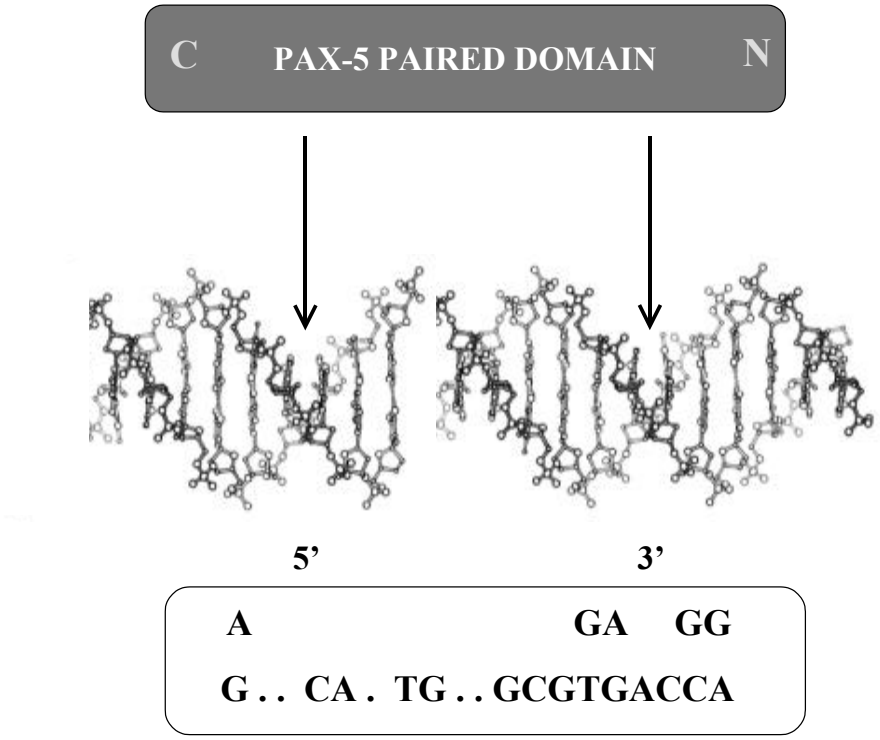


Fig.1 ***Model of the paired domain-DNA interaction***. The amino- and carboxi-terminal regions of the paired domain contact two successive major grooves from the same side of the DNA helix.

to one major groove contact site. The amino-terminal subdomain recognizes the more extensive 3' consensus motif of the Pax-5 binding site, whereas the carboxy-terminal part interacts with the 5' consensus motif.

One important observation of this model is that sites with a complete match to the consensus motif possess a very high affinity for Pax-5, which apparently is not required *in vivo*. All naturally occuring binding sites identified so far deviate from the consensus sequence and Pax-5 is able to interact with a panel of degenerate recognition sequences [12].

2.3 Role of Pax-5 in B-cell lineage commitment

Pax-5 is an essential B lineage commitment factor. Normal expression of E2A and EBF, two transcription factors implicated in the myeloid versus lymphoid lineage decision and located

upstream of Pax-5 in the B-cell developmental process, is not sufficient to commit B-cell progenitors to the B-lymphoid lineage in the absence of Pax-5. In Pax-5$^{-/-}$ mice B-cell development is arrested at early pro-B-cell stage in the bone marrow. These Pax-5$^{-/-}$ pro-B-cells still retain a broad lymphomyeloid development potential characteristic of uncommited hematopoiectic progenitors [60,67]. Upon appropriate cytokine stimulation, Pax-5$^{-/-}$ pro-B-cells are able to differentiate *in vitro* into functional NK cells, dendritic cells, macrophages, osteoclasts and granulocytes [60]. In addition, Pax-5$^{-/-}$ pro-B-cells possess extensive in vivo self-renewal potential and long-term reconstitution potential, which are features of hematopoietic stem cells (HSC), yet they fail to reconstitute the hematopoietic system of lethally irradiated mice [72].

Binding sites for Pax-5 have been identified in promoters of several genes. While activating CD19, mb-1, RAG-2 and BLNK [43,59,39] Pax-5 acts as a repressor for the XBP-1, the M-CSF-R, the immunoglobulin heavy-chain 3'C∝ enhancer and the J-chain gene [64,66,75]. The conversion to a repressor function appears to be possible by recruitment of corepressors of the Groucho family to selected target genes [19].

At lineage commitment, Pax-5 has a dual role by repressing "lineage-inappropriate" genes and simultaneously activating B-cell-specific genes, which leads to the consolidation of the B-lymphoid gene expression program. This role is best illustrated by the regulation of M-CSF-R and BLNK genes. By repressing M-CSF-R gene, Pax-5 renders B-cell precursors unresponsive to M-CSF, and prevents them to differentiate to monocytes under the influence of this cytokine. On the other hand, by activating the BLNK promoter, Pax-5 enables the expression of a central adaptor protein in BCR signaling [73].

2.4 Role of Pax-5 in late B-cell development

Pax-5 functions also at later stages of B cell development. The generation of a mouse strain in which the Pax-5 gene can be conditionally inactivated enabled the analysis of Pax-5 function in mature B cells [31]. Loss of Pax-5 resulted in a change of B cell subpopulations in the periphery with downregulation of several mature cell surface B cell markers. Considering that Pax-5 is also repressing XBP-1, a transcription factor essential for plasma-cell differentiation, it proves its important role for maintaining the identity and function of mature B cells.

Furthermore, Pax-5 might play a role in isotype class switching by regulating germline transcription from the downstream constant region gene, which appears to be a prerequisite for subsequent class switching. IgH gene expression and rearrangement are regulated by multiple *cis*-acting elements within the IgH locus, including the intronic enhancer (Eμ), the I regions of the constant region genes and the complex regulatory region 3' of Cα. Pax-5 was reported to bind at multiple sites in the IgH gene cluster, including regions located upstream of switch regions, like Sγ2a [46] and Sμ [86] and at sites within the 3' control region [52]. Repression or activation of these regulatory sites appears to require a concerted effort involving additional factors, such as octamer binding proteins and NF-kB-like complexes.

3. CD23- the low affinity receptor for IgE

CD23 was described as a low-affinity receptor for IgE (FcεRII) expressed on mature peripheric B-cells. The same molecule, expressed at high levels on Epstein-Barr virus-transformed cells was independently described as a B-cell activation marker.

The CD23 molecule is a type II membrane glycoprotein exhibiting substantial homology with several Ca^{2+}-dependent animal lectins. In humans it is expressed in two isoforms (CD23a and CD23b). CD23 has a functional role as a transmembrane receptor as well as a soluble receptor derived from the cell-bound form. CD23 is an important player in allergic, autoimmune and lymphoproliferative diseases.

In humans, the expression of CD23 is increased in allergic disorders, in terms of membrane expression on B-cells and monocytes but also in terms of sCD23 production [24]. The number of circulating CD23-bearing B-cells is also increased in patients with rheumatoid arthritis. In type II collagen-induced arthritis in mice, a model for human rheumatoid arthritis, antibody neutralisation of CD23 significantly ameliorated the disease, proving the involvement of the molecule in inflammatory processes [63]. In B-CLL patients, high levels of sCD23 in the serum are correlated with the clinical stage of the disease and can be used as prognostic marker. The accumulation of sCD23 results from an increased number of CD23-bearing B-cells but also from the overexpression of CD23 on the surface of malignant cells [69].

3.1 Cellular expression and its regulation

On normal human B-cells CD23 expression is restricted to mature peripheric B-cells co-expressing IgM and IgD. After switching to IgG, IgA and IgE B-lymphocytes cannot be induced to reexpress CD23. The receptor is comparably expressed on $CD5^+$ and $CD5^-$ circulating or tonsilar B-cells.

CD23 is also found on T-cells, monocytes, macrophages, platelets, eosinophils, Langerhans cells and follicular dendritic cells [13].

Normal human B-lymphocytes from peripheral blood express both CD23 antigen and CD23 mRNA. Still the molecule is not constitutively expressed since after 48 hours of incubation in the absence of a stimulant, highly purified B-cells loose both CD23 antigen and CD23 mRNA.

The major inducer of CD23 on B-cells is IL-4, which triggers the expression of both isoforms. The peak effect is observed after 36-48 hours. Signals delivered via CD40 synergize with IL-4 for the induction of CD23 on mature peripheral B-cells and interactions between B and T cells (presumably dependent on CD40) result in upregulation of CD23. EBV-transformation of B-cells leads to CD23 expression, which plays a role in the immortalization of the cell [8].

IFN-γ and IFN-α inhibit IL-4 induced expression of CD23 on normal B-cells at the protein and mRNA level [15].

IL-4 is also the main CD23 inducer on all the other CD23 bearing cell types.

3.2 Structure of the molecule

Human CD23 is a 45 kDa glycoprotein member of the C-type animal lectin family, with a long C-terminal extracellular domain, a short cytoplasmic N-terminus and is anchored by a single transmembrane region [13].

The extracellular part of the molecule consists of three regions (Fig. 2):

- the leucine zipper sequence near the transmembrane domain has a seven-amino-acid motif beginning with Leu or Ile that is repeated five times in the case of human CD23 and forms an α-helical coiled coil stalk region [5]. This region mediates the formation of trimers;

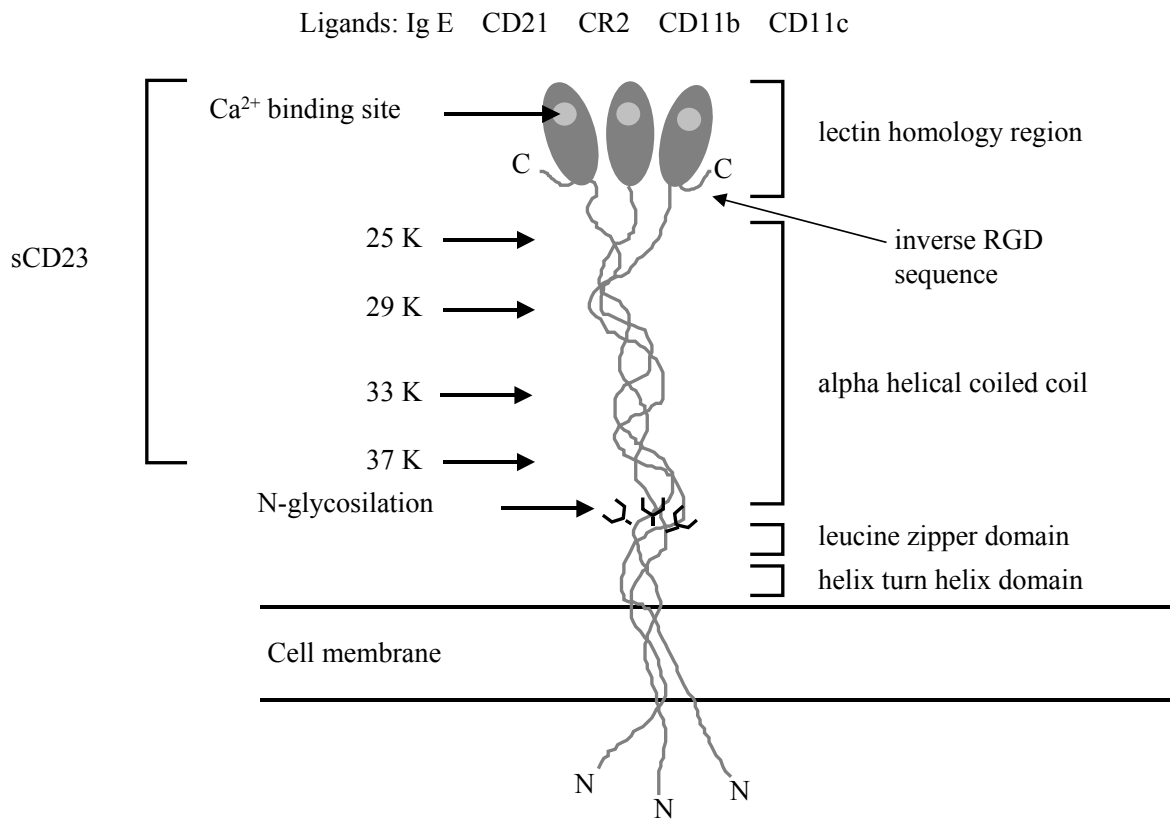


Fig. 2 ***Schematic representation of the CD23 receptor***

- the lectin head, a motif which mediates the binding of carbohydrates in a Ca^{2+} dependent manner. It consists of four highly conserved and two partially conserved cystein residues which interact by disulphide bonds and contains Ca^{2+} and sugar –binding amino acids;
- the inverse RGD sequence, a common recognition site for integrins, is located near the C-terminus of the molecule.

The intracytoplasmic part is very short, containing only 21-22 amino acids for Cd23a and CD23b respectively.

The membrane form gives rise to soluble fragments by an autocatalytic process involving a matrix metalloprotease. Cleavage at different sites gives rise to fragments of 37, 33, 29, 25 and 17 kDa, all retaining the binding capacity to IgE, although the smaller fragments with lower affinities. Soluble forms bigger than 25kDa upregulate IgE production.

Binding of IgE and IgE-IC protects and stabilizes the stalk from proteolyses in this way inhibiting the release of soluble fragments from the membrane form.

3.3 Ligands for CD23

The first ligand to be described for CD23 was IgE. Although IgE is highly glycosilated, the lectin homology region of CD23 appears to bind the protein moiety of the molecule, independently of carbohydrates. However, the binding is Ca^{2+} dependent and the correct folding of the lectin domain is critical, since deletion of conserved cysteins has a deletorious effect on IgE binding [6]. The CD23-binding site was mapped in the Cε3 constant region domain of IgE, in close proximity of the high-affinity receptor (FcεRI) binding site. The oligomerization of CD23 is an important factor in enabling high affinity binding to IgE.
The other ligands described for CD23 are CD21 (CR2), CD11b/CD18 (CR3) and CD11c/CD18 (CR4) - two members of the LFA-1 family.
Fucose-1-phosphate has been described as a competitive inhibitor of both IgE and CD21 binding for CD23 [26].

3.4 Biologic activity

Membrane CD23 and its soluble forms have been implicated in different functions, ranging from cellular adhesion, antigen presentation, growth and differentiation of B and T cells, rescue from apoptosis, release of cytotoxic mediators and regulation of IgE synthesis.
A well-characterized function of membrane-bound CD23 in B-cells is the enhancement of IgE-dependent antigen presentation to T-cells [23,27,35]. This requires the binding of antigen-IgE immune complexes to CD23, internalization of the complexes and transport to compartements of the endosomal network containing proteolytic enzymes and major histocompatibility complex class II antigens. CD23 is spatially associated with MHC class II DR on B-cells [34].
CD23 functions also as an adhesion molecule. Antigen presentation involves interaction between CD23 and CD21 at points of contact between B and T cells [2,7].
Human CD23 plays also a regulatory role in the IgE production with positive and negative effects. Crosslinking CD23 at the cell surface by IgE inhibits the release of sCD23 and delivers a negative feedback for IgE production. In contrast, sCD23 fragments larger than 25

kDa that retain part of the stalk region promote IgE production. Two possible mechanisms are discussed: (1) sCD23 possibly stimulates IgE production through CD21 triggering; (2) sCD23 traps IgE in the medium and prevents the negative feedback through membrane-bound CD23 [83].

There is evidence that soluble CD23 fragments exert other important roles except the regulation of IgE synthesis. In synergy with IL-1, sCD23 acts as a differentiating factor for early thymocytes [55] and induces proliferation of human bone marrow derived myeloid precursors [56]. It is also involved in the rescue of germinal center B-cells. In the presence of recombinant 25 kDa sCD23 and IL-1α centrocytes are rescued from apoptosis and can differentiate into plasmocytoid cells [49]. This is supported by the high density of CD23 on the surface of follicular dendritic cells in the light zone of the germinal center.

On monocytes, eosinophils and platelets, CD23 is involved in IgE dependent cytotoxicity against some parasites and in the IgE induced release of different mediators of inflammation [10,17]. CD23 also mediates the phagocytosis of IgE coated particles. Finally, sCD23 ligation of CD11b/CD11c on monocytes is able to promote release of inflammatory mediators such as IL-1β, IL-6 and TNF [45].

3.5 Isoforms of human CD23

Two isoforms of human CD23 (CD23a and CD23b) have been described. They differ by only 6-7 residues in the extremity of the cytoplasmic tail. CD23a contains a unique tyrosine residue while CD23b does not. CD23a is restrictively expressed on B-cells and EBV-transformed B-cell lines while CD23b is inducible on B-cells, as well as monocytes, eosinophils, macrophages and a variety of other cell types [87].

The two isoforms seem to be correlated to different functions associated with CD23. CD23a appears to be the isoform associated with endocytosis of IgE IC and mediating Ag presentation on B-cells. Yokota *et al*. identified a five amino-acids sequence in which the first residue is aromatic (Tyr-Ser-Glu-Ile-Glu) and that is particularly critical for endocytosis of coated pits [88]. CD23b has a phagocytosis motif (asparagine and proline in positions 2 and 3). Although the function of this isoform on B-cells is unclear, on myeloid cells it seems to be involved in the phagocytosis of IgE-coated particles, cytokine release and the generation of superoxides. There is solid circumstancial evidence that the two isoforms connect to different signalling transduction pathways.

CD23 expressing cells	CD23a	CD23b
B cell	+	+
T cell	-	+
Follicular dendritic cells	-	+
Langerhans cells	-	+
Monocytes	-	+
Macrophages	-	+
Eosinophils	-	+
Platelets	-	+
Thymic epithelial cells	-	+

Fig.3 ***The two isoforms of human CD23 are differentially expressed on cells of the hematopoietic lineage.***

3.6 Genomic structure of the human CD23 gene and analyses of its transcriptional regulation

CD23 is encoded within the human genome by a single gene located on chromosome 19. It consists of 11 exons, with a good correlation between the exon/intron structure and the corresponding domains of the protein. Exons 5-7 seem to have arisen by a triplication of an exon coding for an exact number of heptads and encode the stalk region. Exons 9-11, which are separated by a large intron from the rest of the gene, encode for the soluble forms of CD23.

The two isoforms, which differ by six aminoacids at the cytoplasmic amino terminus, are generated by using different transcriptional start sites and alternative RNA splicing (Fig. 4). Given the genomic sequence of CD23a as a reference, CD23b mRNA is lacking the first two exons and starts with an optional exon that is located within intron II. The two mRNAs share

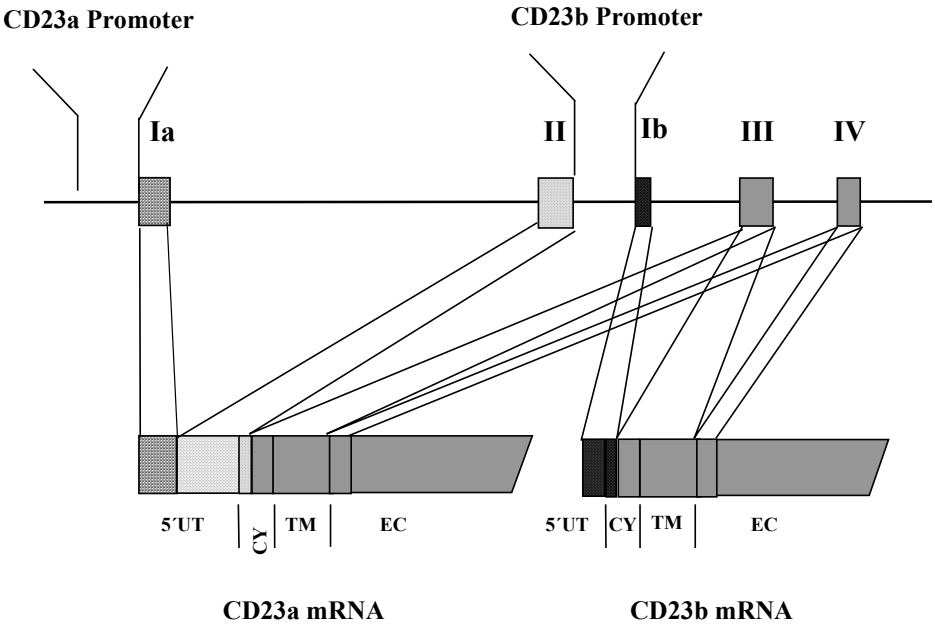


Fig.4 ***Genomic organization of the CD23 gene***

the part of the molecule incoded starting with exon 3- the extracellular, transmembrane and partially the intracellular domain [87].

The CD23a promoter sequence was first described by Suter *et al.* [79] and the CD23b promoter was identified by Yokota [87]. The first studies of the CD23a promoter identified a **CCAAT** motif, as well as four Alu sequences and repeat elements that form an extensive inverted repeat surrounding the promoter. Later studies of both promoters identified several transcription factors binding sites.

Both CD23a and CD23b promoters contain canonical STAT6 binding sites (**TTC-N_4-GAA**) and at least one of these elements is a defined IL-4 responsive element [21,41]. Similarly, sequences containing NF-kB binding sites (**GGGRNNYYCC**) are also found in both CD23a and CD23b upstream regions [40]. Binding sites for NF-AT transcription factors and candidate AP-1 binding sites (**TGASTCA**) have been characterized in the CD23b promoter [21,40].

EBNA-2 targets the CD23a promoter through a DNA-binding protein, CBF-1, which binds a recognition sequence with the common core motif **GTGGGAA** [48,85]. EBNA-2 is a transcriptional activator that modulates Epstein-Barr virus latency gene expression as well as the expression of cellular genes. CD23 expression is upregulated by EBNA-2 along with CD21 [11]. Activation of CD23 might be particularly important, since only EBV-infected B-cells expressing this marker become immortalized. Notch-2 also can regulate the CD23a promoter by binding to CBF1 sites. The Notch family genes encode transmembane receptors that modulate differentiation and proliferation. Notch-2 activation of the CD23a promoter through CBF1 responsive elements may play a role in the immortalisation of the cell and the pathogenesis of B-CLL [32].
Emerging data seem to lead to the conclusion of differential regulation of the CD23a and CD23b promoters, with CD23a showing less sensitivity to external stimuli than the CD23b promoter, at least in B-cells [21]. This would be in agreement with the idea of the two isoforms having different functions.

3.7 The murine CD23 receptor

The mouse CD23 shares only 57% aminoacid sequence homology with the human molecule. The protein is lacking the RGD motif by a naturally occuring truncation and the sCD23 fragments bind IgE with a much lower affinity [4]. There are also differences in the cellular distribution between species, with the mouse CD23 expressed only on B-cells, follicular dendritic cells and some T-cells. All these differences in structure and cellular expression may account for the differences in functions between mice and humans. From the study of $CD23^{-/-}$ mice, murine CD23 may not have the regulatory effects ascribed to human sCD23. $CD23^{-/-}$ mice display normal lymphocyte development, normal B-cell proliferation and germinal center formation. However, antigen-specific IgE-mediated enhancement of antibody responces was severely impaired, suggesting the role of murine CD23 in antigen-presentation [23,27]. Regarding the role of mouse CD23 in IgE production, some studies did not find modifications of IgE levels in the serum of $CD23^{-/-}$ mice [23,77], while other studies of heterozygous and transgenic mice suggest that the murine CD23, in particular the membrane-bound form, exerts an inhibitory role on IgE production [81,89]. There are no expressed isoforms described yet and the mouse CD23 seems to be more related to the CD23a human isoform, by distribution and functions: the cytoplasmic tail of the mouse CD23 contains a Tyr residue, part of an **YSGT** sequence and the mouse promoter also

displays homologies to the CD23a promoter. IL-4 is also the main inducer of mouse CD23. STAT6 and NF-kB binding sites have been described [82].

3.8 Signal transduction through the CD23 receptor

The intracellular signal transduction pathway activated through CD23 has been studied by Kolb and coworkers [42]. In human activated B-cells cross-linking of CD23 provokes a rapid increase in Ca^{2+}, which results from the generation of inositol (1,4,5) tri-phosphate following phospholipase C-dependent hydrolysis of phosphatidylinositol (4,5)-biphosphate. It has been suggested that G protein couples membrane CD23 to a PLC and it is possible that a tyrosine kinase is also involved. Evidence was provided by an experiment in which the product of the transfected CD23 gene in a NK cell line was found associated with p59fyn -a member of the src family of protein kinases [78]. Cross-linking of CD23 on the surface of resting or IL-4

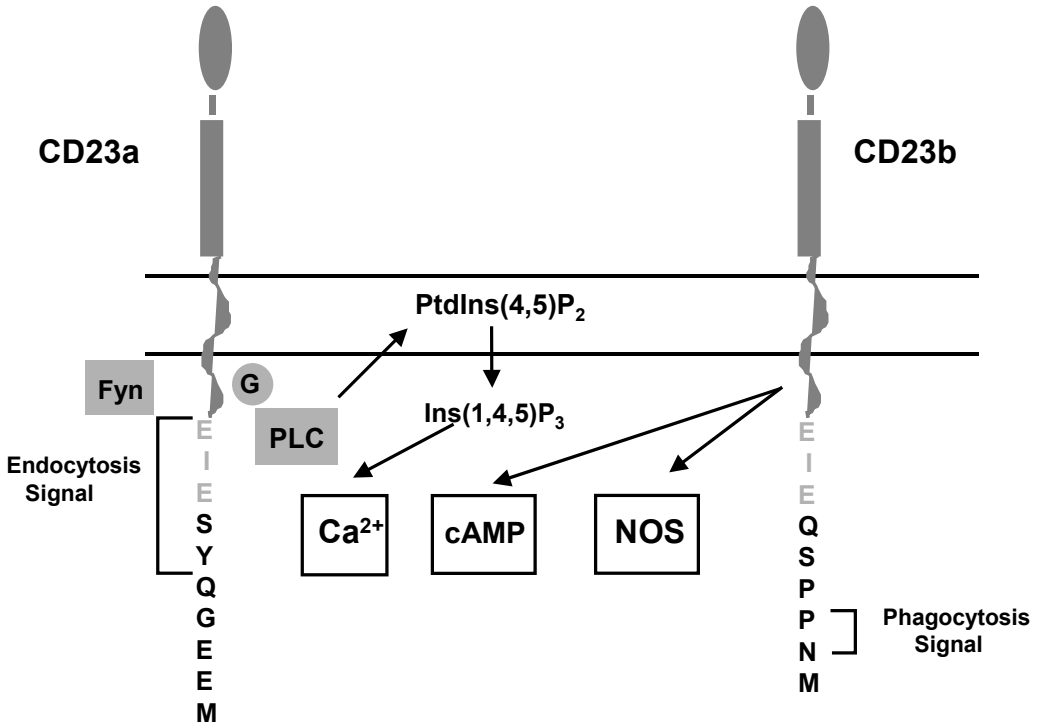


Fig.5 ***Schematic diagram of the CD23 receptor and known signal transduction pathways in murine and human B cells.***

stimulated B-cells resulted in a slow accumulation of intracellular cAMP, although it was unclear to what extent increased Ca^{2+} levels were dependent upon the prior activation of PLC. However, since this change could be observed in resting B-cells, where neither Ca^{2+} mobilization nor inositol (1,4,5) tri-phosphate production are significantly altered on engaging CD23, suggests that the elevation in cAMP levels may proceed independently of PLC activation. Moreover, cAMP accumulation was observed in monocytes, where the phosphoinositide pathway is clearly not involved [26]. In human monocytes CD23 is additionally coupled with the activation of inducible nitric oxide synthase (iNOS) pathway [18].

This would indicate that the B-cell specific, CD23a isoform and the non-lineage restricted CD23b isoform have distinct signalling mechanisms. The divergence in the signalling pathways must relate to the first 6-7 aminoacids of their cytoplasmic N-termini.

4. Two-hybrid systems

Two-hybrid systems provide a powerful technique to screen large libraries of genes and to identify new protein-protein interactions within the cell. A certain number of yeast, bacterial and mammalian two-hybrid systems have been developed in the last years.

4.1 CytoTrap Two Hybrid System

The CytoTrap® Two Hybrid System (Stratagene) is based on generating fusion proteins whose interaction in the yeast cytoplasm induces cell growth by activating the Ras signaling pathway.

The yeast strain used by the system- called cdc25H- contains a point mutation in the cdc25 gene, which is the homologue of the human SOS. The gene encodes a guanyl nucleotide exchange factor for Ras. The mutation prevents host growth at 37°C, with a permissive

temperature of 25°C. The system is based on the ability of the human SOS to complement the defect and activate the yeast Ras signalling pathway.

DNA encoding the bait protein is cloned into a vector, which will express it as a fusion protein with human SOS. DNA encoding the library (target) is cloned in the pMyr vector. The genes of the cDNA library will be expressed as fusion proteins to the myristilation signal, which will anchor them to the membrane. If the bait and target physically interact, hSOS is recruted to the membrane, activating the Ras signalling pathway and allowing the yeast to grow at 37°C (Fig. 6).

This system offers some advantages over the traditional two-hybrid systems:

1. it provides a better control of the activation;
2. it does not involve protein transport to the nucleus;
3. it can be used for proteins which are transcriptional activators or repressors.

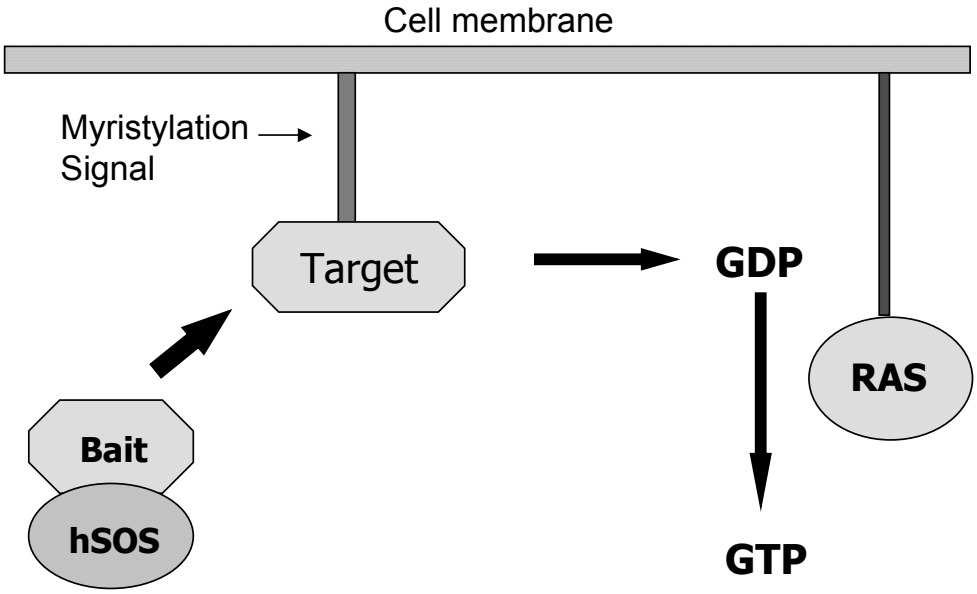


Fig.6 ***Schematic diagram of the Ras signalling pathway used in the CytoTrap Two Hybrid System***

In order to express fusion proteins in the yeast cytoplasm, the system provides two expression vectors.

The pSos vector (Fig. 18) contains the hSOS gene cloned upstream of the multiple cloning site and under the control of the ADH1 promoter which is constitutively active. It is designed with replication origins for propagation in yeast and bacterial strains and selectivity markers for transforming in yeasts (the auxotrophic marker LEU2) and bacteria (the ampicillin resistance gene).

The pMyr vector (Fig. 18) contains the gene coding for the myristilation signal cloned upstream of the target gene and under the control of the GAL1 promoter, which is inducible by adding galactose into the medium. It is designed with replication origins for propagation in yeast and bacterial strains and selectivity markers for transforming in yeasts (the auxotrophic marker URA3) and bacteria (the chloramphenicol resistance gene).

The different antibiotic resistance genes used by the two vectors allow to rapidly distinguish between the bait and the target vector when recovering plasmids from yeasts.

As controls, four different expression vectors are provided by the system: pSosMAFB, pMyrMAFB, pSosColl and pMyrLamin C. pSosMAFB and pMyrMAFB express the SOS protein or the myristilation signal as fusion proteins with full-lengh MAFB, a transcription factor that can form homodimers via its leucine zipper domain. pSosColl expresses the SOS protein as a fusion protein with the murine 72 kDa type IV collagenase (aa 148-357) and pMyrLamin C expresses the myristilation signal as fusion a protein with human lamin C (aa 67-230). These plasmids are used in pairwise combinations as positive and negative controls for the rescue of the temparature-sensitive phenotype of cdc25H. pSosMAFB and pMyrMAFB protein products interact in vivo. Co-transformation of these two control plasmids permits growth of the cdc25H mutants at the restrictive temperature of 37°C. The pSosMAFB + pMyrLamin C plasmid pair and the pSosColl + pMyrMAFB plasmid pair are negative controls whose protein products do not interact in vivo. These proteins do not allow the growth of cdc25H mutants at 37°C.

4.2 MATCHMAKER GAL4 Two-Hybrid System 3

This system (provided by Clontech) is a GAL4- based two-hybrid that provides a transcriptional assay for detecting protein interactions *in vivo* in yeasts. The bait gene is expressed as a fusion protein with the GAL4 DNA-binding domain (DNA-BD), while another gene or cDNA is expressed as a fusion protein to the GAL4 activation domain (AD).

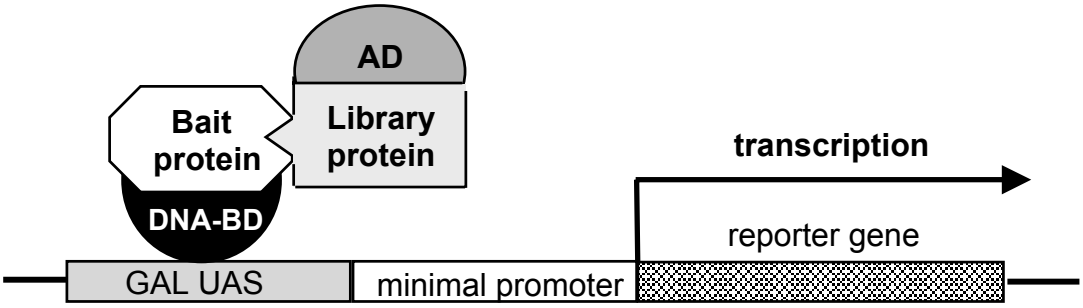


Fig.7 ***Schematic diagram of the principle employed by the MATCHMAKER3 GAL4 Two Hybrid System***

When bait and library fusion protein interact, the DNA-BD and AD are brought into proximity, thus activating transcription of four reporter genes: ADE2, HIS3, MEL1 and lacZ (Fig. 7). The system can be used to identify novel protein interaction as well as to confirm or define suspected interacting domains.

The system uses four different reporter genes under the control of distinct GAL4 upstream activating sequences (UASs) and TATA boxes. ADE2 and HIS3 reporters allow strong nutritional selection and the control of the stringency of selection. MEL1 and lacZ, which incode for α-galactosidase and β-galactosidase respectively, allow the employment of blue/white screening.

The vectors of the system- pGBKT7 and pGADT7 are designed to express different bacterial transformation markers- kanamycin and ampicillin, different yeast selection markers- -TRP1 and LEU2, c-Myc and hemagglutinin (HA) epitope tags for convenient identification of the fusion proteins and T7 promoters to allow in vitro transcription and translation of epitope-tagged fusion proteins.

The positive controls of the system are pGBKT7-53 and pGADT7-T vectors, which incode fusion proteins between GAL-4 DNA-BD and AD and murine p53 and SV40 large T-antigen. p53 and large T-antigen interact in a two-hybrid assay. Additionally, pCL1 encodes the full-length wild-type GAL4 protein and provides a positive control for α-galactosidase assays.

The negative control of the system is pGBKT7-Lam vector, which encodes for a fusion of the DNA-BD with human lamin C. This protein neither forms complexes nor interacts with most other proteins.

The yeast strains provided by the system- AH109 and Y187 are $gal4^-$ and $gal80^-$ in order to prevent the interference of native regulatory proteins with the regulatory elements of the two-hybrid system. AH109 usage is recommended for library screens using HIS3, ADE2 and MEL1. Y187 usage is recommended for testing interactions between two known proteins using the lacZ reporter only.

AIMS OF THE PROJECT

Two isoforms of human CD23 (CD23a and CD23b) have been described. They differ by only 6-7 residues in the extremity of the cytoplasmic tail. CD23a is restrictively expressed on B-cells while CD23b is inducible on B-cells, as well as monocytes, eosinophils, macrophages and a variety of other cell types after IL-4 stimulation.

The two isoforms seems to have different functions. CD23a appears to be the isoform associated with endocytosis of IgE IC and mediating antigen presentation on B-cells. CD23b has a phagocytosis motif and seems to be involved in the phagocytosis of IgE-coated particles, cytokine release and the generation of superoxides.

Previous studies indicate that the two isoforms connect to different signalling transduction pathways. The comparison of events taking place in cells that express only one or both CD23 isoforms would suggest that CD23b is involved in upregulating cAMP and iNOS, whereas CD23a mediates an increase in intracellular calcium. Additionally, recent observations show that there is distinct regulation of the two promoters.

Two questions regarding the biology of the CD23 receptor were addressed in this study:

1) How is the B-cell specific expression of CD23a isoform regulated? In particular, is the B-cell specific activator protein BSAP/Pax-5 implicated in the control of CD23a expression?

2) Who are the direct interaction partners of the two CD23 isoforms? In particular, can yeast two-hybrid systems be used in order to look for cytoplasmic interaction partners for the CD23 receptor?

MATERIALS AND METHODS

1.Gene cloning

1.1 Plasmid constructs

- The following plasmids were used for in vitro transcription:

pBS-23A contains a 299 bp cDNA fragment of CD23a cloned into the SmaI site of the pBlueScriptSK+ vector (Stratagene).

pBSβ–actin contains a 540 bp cDNA fragment cloned in the pBlueScriptSK+ vector (Stratagene).

- The following plasmids were used for in vitro transcription and translation:

pCR-Pax-5 –contains the full length human Pax-5 gene cloned in the MCS of the pCR-Zero Blunt vector (Invitrogen).

- The following plasmids were used in mammalian cell transfections/reporter gene assays:

pcDNA$_3$-Pax-5 contains the human Pax-5 gene cloned in the EcoRI site of the pcDNA$_3$ vector (Stratagene).

pEGZ –Pax-5 contains the human Pax-5 gene inserted between the EcoRI and SmiI sites of the pEGZ vector; pEGZ vector was provided by Dr. I. Berberich (Institute for Virology, Würzburg).

pLuc+ACP and **pLuc+AP** contain the CD23a core promoter (-203 to +83) and the CD23a promoter (-1216 to +211) cloned in the SalI site of the pLuc+ vector; pLuc+ vector was provided by Dr. J. Altschmied and all the pLuc+ constructs have been previously made in our lab.

pXM-STAT6 was kindly provided by Dr. E. Pfitzner (Frankfurt).

- The following plasmids were used in two-hybrid systems:

pSosCD23a and **pSosCD23b** contain the intracytoplasmic part of the CD23 isoforms cloned in the MCS of the pSos vector. The constructs have been previously made in our lab.

pSosCD23a+Linker and **pSosCD23b+Linker** contain the intracytoplasmic part of the CD23 isoforms and a linker region cloned in the HindIII site of the pSos vector, upstream of the human SOS gene.

pSosCD23a-Glu represents the construct pSosCD23a+Linker in which the tyrosine in position 6 of the cytoplasmic tail of CD23a has been replaced with a glutamic acid using site directed mutagenesis.

pMyr-fyn contains the fyn gene cloned in the MCS of the pMyr vector.

pGBKCD23a and **pGBKCD23b** contain the intracytoplasmic part of the CD23 isoforms cloned between the EcoRI and XhoI sites of the pGBK vector.

pGAD1/12 constructs contain different clones (spleen library genes) transferred from the pMyr library and cloned between the NcoI and BamHI site of the pGAD vector.

1.2 Oligonucleotides

The following annealed oligonucleotides (MWG-Biotech) were cloned inside the MCS of the pSos vector:

- CD23a –intracytoplasmic part

M191- 5'-GGC CAA GCT TCC ACC ATG GAG GAA GGT CAA TAT TCA GAG ATC GAG GAG CTT CCC AGG AGG CGG TGT TGC AGG CGT GGG GGA TCC CG-3'

M192- 5'-CGG GAT CCC CCA CGC CTG CAA CAC CGC CTC CTG GGA AGC TCC TCG ATC TCT GAA TAT TGA CCT TCC TCC ATG GTG GAA GCT TGG CC-3'

- CD23b- intracytoplasmic part

M193- 5'-GGC CAA GCT TCC ACC ATG AAT CCT CCA AGC CAG GAG ATC GAG GAG CTT CCC AGG AGG CGG TGT TGC AGG CGT GGG GGA TCC CG-3'

M194- 5'-CGG GAT CCC CCA CGC CTG CAA CAC CGC CTC CTG GGA AGC TCC TCG ATC TCC TGG CTT GGA GGA TTC ATG GTG GAA GCT TGG CC-3'

- Linker region

M195-5'-CGG GAT CCG GCG GTG GCG GTT CTG GTG GCG GTG GCT CCG GCG GTG GCG GTT CTG AAG CTT CGG G-3'

M196-5'-CCC GAA GCT TCA GAA CCG CCA CCG CCG GAG CCA CCG CCA CCA GAA CCG CCA CCG CCG GAT CCC G-3'

1.3 Annealing reaction

The standard reaction by which two complementary oligonucleotides were annealed in a double stranded DNA fragment was:

2 nmol oligonucleotide 1

2 nmol oligonucleotide 2

H_2O up to 50 µl

The reaction was incubated for 5 min at 95°C and left in a 100°C water bath to cool till it reached room temperature.

1.4 RT-PCR

The reaction was performed using Titan One Tube RT-PCR System (Roche) a sensitive technique that allows cloning of RNA messages in one step reaction.

a. The following combinations of primers (Gibco) were used:

Pax-5 Fwr	M130 - 5'-TTC CCT GTC CAT TCC ATC AA-3'
Pax-5 Rev	M131- 5'-TCA TGG GCT CTC TGG CTA-3'
CD23a Fwr	5'-GCCATGGAGGAAGGTCAATATTCA-3'
CD23a Rev	5′-GACTTGAAGCTGCTCAGATCTGCT-3'
β–actin Fwr	5'-GTGGGGCGCCCCAGGCACCA-3'
β–actin Rev	5'-CTCCTTAATGTCACGCACGATTTC-3'
p59 fyn Fwr	5' –AGA GGA CCA TGT CAG TGG GCT- 3'
p59 fyn Rev	5' –TCA CAT GCA ATC TGA TCC TGG- 3'

b. Reaction components (all reagents were provided by Roche, except RNase Inhibitor RNAguard^{TM}, which was purchased from Amersham Pharmacia):

Master Mix 1 (total of 25µl)	Master Mix 2 (total of 25µl)
1µl dNTP (10mM each)	10 µl 5xRT-PCR Buffer
1µl downstream primer (10pmol/µl)	1 µl enzyme mix
1µl upstream primer (10pmol/µl)	14 µl H_2O
1µl RNA template (1µg/µl)	
2.5 µl DDT (100mM)	
0.25 µl RNase Inhibitor (32 U/µl)	
18.25 µl H_2O	

Master Mix 1 and Master Mix 2 were mixed gently.

c. The conditions for the RT-PCR reaction were:

1 cycle-	elongation	30 min at 50°C
	denaturation	2 min at 94°C
10 cycles -	denaturation	15 sec at 94°C,
	annealing	30 sec at (*)°C
	elongation	(‡) sec, at 68°C
25 cycles -	denaturation	15 sec at 94°C,

	annealing	30 sec at (*)°C
	elongation	(‡) sec at 68°C + cycle elongation of 5 sec for each cycle
1 cycle -	elongation	7 min at 68°C

Amplification product	(*) Annealing temperature	(‡) Elongation time
CD23a	61°C	45 sec
Pax-5	66°C	60 sec
β-actin	66°C	45 sec
p59 fyn	53°C	80 sec

1.5 Site directed mutagenesis

The site-specific mutagenesis by overlap extension [28,30] uses two sets of mutagenic primers (R2/FM and RM/F2) and two successive PCR reactions in order to introduce a mutation into a desired location. The mutagenesis product is designed to contain restriction enzyme sites, which allow it to be cloned back into the vector.

a. The mutagenic primers used were (MWG-Biotech):
   - Set of mutagenic primers for introducing mutations into the CD23-1 site within the CD23 promoter (pLuc+ACPmu)

   M256-R2 – 5'-TGT ATC TTA TCA TGT CTG GAT CTC GAA GCT TGC-3'

   M257-FM – 5'-CAC GCA CAA CTT ATA CTGGCACTTCCCACACCC-3'

   M258-RM – 5'-GTG TGG GAA GTG CCA GTA TAA GTT GTG CGT GTA AT-3'

   M259-F2 –5'- TTT ACC AAC AGT ACC GGA ATG CCA AGC TCA G-3'.

   - Set of mutagenic primers for Tyr to Glu residues replacement in the pSosCD23a+Linker

   M320-FM- 5'-GGA GGA AGG TCA AGA ATC AGA GAT CGA GGA GCT T-3'

   M321–R2 - 5'-CCC AAC CAG CTT TAA AAT GTC TGC AGA AAT GTA TTC-3'

   M321–F2 - 5'-AAC GAG TTT ACG CAA TTG CAC AAT CAT GCT GAC-3'

   M323–RM -5'-AAG CTC CTC GAT CTC TGA TTC TTG ACC TTC CTC C-3'

b. Reaction components (total of 100 µl):

   2 µl template pLuc+ACP (50ng/µl)

   3 µl downstream primer (10pmol/ml)

   3 µl upstream primer (10pmol/ml)

2 µl dNTP (10 mM each)

1 µl Pfu DNA Polymerase (Stratagene)

10 µl 10xPfu Buffer (Stratagene)

79 µl H_2O

c. The conditions for all PCR reactions were:

25 cycles - denaturation 1 min at 94°C,

annealing 1 min at 67°C (CD23-1 mutant); 68°C (Tyr to Glu mutant)

elongation 1 min at 72°C

1 cycle - denaturation 1 min at 94°C

annealing 10 min at 72°C

1.6 Nucleic acid cleaning and purification procedures

PCR and RT-PCR products were either directly purified using QIAquick® PCR Purification Kit (Qiagen) or the bands were extracted from the agarose gel using QIA®quick Gel Extraction Kit (Qiagen) and MinElute™ Gel Extraction Kit (Qiagen).

DNA fragments resulted from enzymatic restriction were purified either by using QIAquick® Nucleotide Removal Kit (Qiagen) and MinElute™ Nucleotide Removal Kit (Qiagen) or by gel extraction.

1.7 Polishing of PCR products

In order to increase the efficiency of blunt-ended cloning reactions, PCR generated fragments were polished using PCR Polishing Kit (Stratagene) according to manufacturer's recommendations.

1.8 Subcloning of PCR products

Purified PCR and RT-PCR products have been cloned using the Zero Blunt™ PCR Cloning Kit (Invitrogen). This kit is designed to clone blunt PCR fragments (or any blunt DNA fragment) with a low background of recombinants. The pCR®-Blunt vector allows direct selection of recombinants via desruption of a lethal *E. coli* gene, *ccd*B. Ligation of the insert into the linearized pCR-ZeroBlunt vector and transformation into the One Shot™ Top 10 competent cells was done according to manufacturer's instructions. The efficiency of ligation was assessed by restriction enzyme digestion with EcoRI or by a control PCR reaction following the protocol of the kit, except that bacterial colonies picked directly from the plate

were used instead of template DNA. Taq polymerase and Buffer Y (PeqLab) were used in this specific PCR reaction.

1.9 DNA digestion with restriction enzymes

The procedure was generally used to cut DNA fragments from one plasmid in order to insert it in the multiple cloning site of another plasmid or to analyse insertion or orientation of a DNA fragments into a vector.

Reaction components:

1 µg plasmid DNA

2 µl 10x restriction enzyme Buffer

5 U restriction enzyme

H_2O up to 20 µl

The enzymatic reaction was incubated at 37°C for 1-4 hours. Enzymatic activity was stopped by heat inactivation, addition of 1xLoading dye or by freezing at –20°C.

The following restriction enzymes (New England Biolabs) were used in different application: EcoRI, SalI, BamHI, HindIII, XhoI, XbaI, HpaII, AvrII, NaeI, etc.

1.10 Klenow Fill-in reaction

DNA Polymerase I, Large (Klenow) Fragment is a proteolytic product of *E. coli* DNA Polymerase I, which retains polymerization and 3'to 5' exonuclease activity, but has lost 5' to 3' exonuclease activity. It was used for 3'-end labeling of DNA (described elsewhere), fill-in of 5' overhangs and removal of 3' overhangs to form blunt ends.

Reaction:

0.1-4 µg DNA in 1x Klenow Reaction Buffer or 1x NEBuffer (NE Biolabs)

1 µl dNTPs 0.5 mM each

1µl (5U) Klenow (USB Corporation)

The reaction was incubated for 60 minutes at 37°C.

1.11 Dephosphorylation of DNA

Alkaline phosphatase or Calf Intestinal Phosphatase (CIP) catalyses the removal of 5' phosphate groups from DNA. Since 5' phosphoryl termini are required by ligases, CIP treatment was used to prevent recircularization of vectors and thus to decrease the vector background in cloning strategies.

Reaction:

DNA suspended in 1xNEBuffer

CIP (NE Biolabs) 0.5 unit / 1 µg DNA

The reaction was incubated for 60 minutes at 37°C.

1.12 Ligation

T4 DNA Ligase joins blunt and cohesive ends by catalyzing the formation of phosphodiester bonds between 5' phosphate and 3' hydroxyl termini. It was used for cloning restriction fragments into vectors.

In all ligation reactions, an optimum insert: vector ratio of 5:1, expressed in pmol ends, was used. The following formula was used in order to assess the number of pmol ends in each case.

pmol ends/ µg DNA=$2x10^6$ / bp x 660

The typical ligation reaction (where x:y respect the above described ratio) was:

x µl insert DNA

y µl vector

1 µl 10x T4 DNA Ligase Reaction Buffer (NE Biolabs)

1 µl T4 DNA Ligase (NE Biolabs)

H_2O up to 10 µl

The reaction was incubated at 16°C overnight.

1.13 Transformation of bacteria

Several *Escherichia coli* strains were used for transformation of different cloning vectors, in order to propagate, multiply or store different constructs.

A standard transformation reaction is presented here:

- competent cells were thawed on ice; 100 µl competent cells were aliquated in prechilled 15 ml polypropylene tubes;
- 1-50 ng of DNA per trasformation reaction was added;
- the reaction was incubated on ice for 30 minutes;
- the reaction was heat-pulsed in a 42°C water bath for 20-90 sec, depending on the strain used; the duration of the heat pulse is critical for transformation efficiency;
- the reaction was incubated on ice for 2 minutes;
- 900 µl SOC medium or LB medium per trasformation reaction were added;
- the reaction was incubated at 37°C for 1 hour with shacking at 225-250 rpm;

- the cells were plated on LB agar plates containing the appropriate antibiotic;
- the plates were incubated overnight at 37°C.

1.14 Plasmid extraction

Plasmid extraction was performed using Nucleobond^{R} AX (Macherey-Nagel), GenElute Plasmid Mini-prep Kit (Sigma) and Wizard^{R} Plus SV Minipreps DNA Purification Systems (Promega).

1.15 Visualisation of DNA

DNA fragments were visualised by running 1-1.5% agarose gels, depending on the size of the expected bands. 50 Base-Pair Ladder (Amersham Pharmacia) and peqGold 1 kb DNA-Ladder (PeqLab) were used as markers.

1.16 Sequencing

Sequencing was mainly used to check the correct insertion of constructs or mutagenesis.

a. The following sequencing primers (Gibco BRL) were generally used:
   M13F (universal primer)– 5' –GTA AAA CGA CGG CCA G-3'
   M13R (universal primer) – 5'-CAG GAA ACA GCT ATG AC-3'
   M163 (pLuc+ reverse primer) –5'-CTT TAT GTT TTT GGC GTC TTC C-3'
   M216 (pLuc+ forward primer) –5'-GCA TTC TAG TTG TGG TTT GTC C-3'
   M78 (pSos 5' primer)- 5' –CCA AGACCA GGT ACC ATG-3'
   M79 (pSos 3' primer)- 5' –CGC AGG GTT TTC CCA GT-3'
   M215 (upstream of human Sos 5' primer)- 5' –CGT TCC CTT TCT TCC TTG-3'
   M110 (pMyr 5'primer)- 5' –ACT ACT AGC AGC TGT AAT AC-3'
   M111 (pMyr 3'primer)- 5' –CGT GAA TGT AAG CGT GAC AT-3'
b. Reaction components:
   4 µl ABI Prism®BigDye™ Terminator (Applied Biosystems)
   0.5 µl forward or reverse primer (10nmol/µl)
   500 ng DNA
   H_2O up to 20 µl
c. PCR conditions:

1 cycle-	denaturation	3 min at 95°C
25 cycles -	denaturation	30 sec at 94°C,
	annealing	1 min at 50°C

	elongation	3 min 60°C
1 cycle -	elongation	5 min at 72°C

d. Purification of PCR products was performed using Auto-Seq™G-50 columns (Amersham Pharmacia) according to manufacturer's instructions.
e. DNA precipitation: 20µl PCR product were precipitated with 80µl isopropanol 75%, vortexed and incubated for 15 min at room temperature, followed by centrifugation at top speed at room temperature for 20 minutes. After the removal of the supernatant the DNA pellet was washed with 250µl isopropanol 75%, mixed gently and centrifuged for another 5 minutes, top speed, at room temperature. The pellet was left to dry for 10-15 minutes at room temperature.
f. Resuspention of DNA: the DNA pellet was resuspended in 18µl Template Suppression Reagent (Applied Biosystems)
g. Denaturation of DNA: 3 minutes at 95°C in a heating block.

2. Protein extraction

2.1 Protein extraction from mammalian cells

$1x10^8$ cells were resuspended in 200µl Roti®-Load1 (Roth) and heated at 80°C for 5 min. The lysate was then sonicated 4 x 10 sec, 50% power, followed by another 3 min at 80°C. 3 µl of the lysate were used in Western Blot analyses.

2.2 Protein extraction from yeasts

5 ml overnight yeast culture with the O.D. >1 were pelleted at 4000xg, 10 min, 4°C and resuspended in 200 µl Cell Lysis Buffer for Protein Isolation with freshly added proteases. The cells were mixed with an equal volume of 0.5 mm glass beads (Roth) and vortexed for 5 min at 4°C. After centrifuging for 5 min at 12000xg and 4°C the supernatant was transferred to a new 1.5 ml Eppendorf tube and kept on ice. The procedure was repeated with 100 µl of Cell Lysis Buffer for Protein Isolation and the supernatants were combined. 15-20 µl were used in Western Blot analyses.

3. Western Blot Analyses

Western Blot analyses were performed in order to detect and quantify proteins by using polyclonal or monoclonal antibodies.

a. Denaturing SDS-Polyacrylamide Gel Electrophoresis

SDS-PAGE under denaturing conditions (0.1% SDS) separates protein based on molecular size as they move through a polyacrylamide gel matrix toward the anode. The polyacrylamide gel is cast as a separating gel topped by a stacking gel and secured in an electrophoresis apparatus.

The final acrylamide concentration in the stacking gel is 4%, while the acrylamide concentration in the separating gel had to be adjusted according to the protein size. A 10% gel was used for Pax-5 (50 kDa) and a 6% gel for human SOS (170 kDa). The samples were solubilized in Roti®-Load1 (Roth) and loaded on the gel together with a prestained SDS-PAGE Standard marker (Bio-Rad Laboratories). The electrophoresis was performed at 200V in an electrophoresis chamber (Hoefer) filled with Running Buffer.

b. Immunoblotting

After being separated by SDS-PAGE, the proteins were transferred using a semidry system (Panther Semidry Electroblotter- OWL) to a transfer nitrocellulose membrane (Hybond™ ECL™- Amersham Pharmacia) and stained by polyclonal or monoclonal antibodies.

After disassembling the PAGE gel and discarding the stacking gel, the separating gel was equilibrated for 15 min in Cathode Buffer on an orbital shaker. The nitrocellulose membrane was prewet on distilled water and equilibrated for 15 minutes in Anode buffer II on an orbital shaker.

The transfer stack was assembled as follows:

Cathode electrode plate
3 sheets of Whatman 3MM filter paper saturated with Cathode Buffer
Gel
Transfer membrane
1 sheet of Whatman 3MM filter paper saturated with Anode Buffer II
2 shees of Whatman 3MM filter paper saturated with Anode Buffer I
Anode electrode plate

The proteins were transferred for maximum 2 h at 0.8 mA/cm^2.

c. Immunodetection

Immunodetection was performed using the following protocol:

- Blocking of the membrane- non-specific binding sites were blocked by incubating the membrane 1 hour at room temperature or overnight at 4°C in 5% dried milk, 0.1% Tween 20 in PBS (Blocking Buffer) on an orbital shaker;
- Rincing of the membrane- 3 times for 5 minutes with Wash Buffer on an orbital shaker;
- Incubation with the primary antibody- the membrane was incubated with the primary antibody diluted in Blocking Buffer for 1 hour at room temperature on an orbital shaker. Anti Pax-5 and the anti SOS antibodies (BD Biosciences) were diluted 1:250;
- Rincing of the membrane- 1x 15 minutes and 3x 5 minutes with Wash Buffer;
- Incubation with the second antibody- the membrane was incubated with the secondary antibody –goat anti-Mouse IgG (BD Biosciences) diluted 1:2000 in Blocking Buffer for 1 hour at room temperature on an orbital shaker;
- Rincing of the membrane- 1x 15 minutes and 3x 5 minutes with Wash Buffer;
- Visualisation of proteins –the presence of proteins was detected using ECL Plus™ Detection Kit (Amersham Pharmacia) and Hyperfilm™ ECL™ chemiluminescence film (Amersham Pharmacia).

4. Electrophoretic Mobility Shift Assays (EMSAs).

This is a rapid and sensitive method for the detection of interaction between DNA-binding proteins and specific sequences of DNA. Proteins that bind specifically to an end-labeled DNA fragment retard the mobility of the fragment during electrophoresis, resulting in discrete bands corresponding to the protein-DNA complexes.

4.1 Oligonucleotides

The following annealed oligonucleotides (MWG-Biotech) were used as probes or as unlabeled competitors in direct binding or competition assays:

- Pax-5 high affinity binding site from the sea urchin H2A-2.2 gene
  M118- 5' -CAG GGT TGT GAC GCA GCG GTG GGT GAC GAC TGT-3'
  M119- 5' -GCC ACA GTC GTC ACC CAC CGC TGC GTC ACA ACC-3'
- putative Pax-5 binding site CD23-1 (-87 to -47)
  M304 - 5' -GGG TGT GGG GAG CAC CAG GAG AGG CCA TGC GTG TAA TGT TA-3'

M305 - 5’ -GGA TAA CAT TAC ACG CAT GGC CTC TCC TGG TGC TCC-3’

- putative Pax-5 binding site CD23-2 (-112 to -71)

  M306 - 5’–CGG ACT TCA CCC GGG TGT GGG GAG CA-3’

  M307 - 5’ –GGT GCT CCC CAC ACC CGG GTG AAG T-3’

- putative Pax-5 binding site CD23-3 (-238 to -209)

  M308 - 5’–GTG GTA TGA TTC AGT GTG CAG TAA CAG TGG TTC-3’

  M309 - 5’-GTG AAC CAC TGT TAC TGC ACA CTG AAT CAT A-3’

- CD23-1mu1 -mutated nucleotides are underlined

  M310 - 5’- GGG TGT GGG AAG TGC CAG TAT AAG TTG TG-3’

  M311- 5’-ACG CAC AAC TTA TAC TGG CAC TTC CCA C-3’)

- CD23-1mu2 -mutated nucleotides are underlined

  M265 - 5’- GTG TGG GGA GAA CCA GTA GAG GCC ATG CGT G-3’

  M266 - 5’- CAC GCA TGG CCT CTA CTG GTT CTC CCC A-3’

- A-1- CD23a promoter (-212 to –173)

  M37 - 5’- GGT TCA CAT CTT GAC GCT ACC ACT CAC CTC CTT CAG CCC-3’

  M38 - 5’- AGG GCT GAA GGA GGT GAG TGG TAG CGT CAA GAT GTG-3’

- A-2- CD23a promoter (-178 to -140)

  M39 - 5’- AGC CCT GTG GGA ACT TGC TGC TTA ACA TCT CTA GT-3’

  M40 - 5’- GAG AAC TAG AGA TGT TAA GCA GCA AGT TCC CAC AGG-3’

- A-3- CD23a promoter (-147 to -107)

  M41 - 5’- TAG TTC TCA CCC AAT TCT CTT ACC TGA GAA ATG GAG A-3’

  M42 - 5’- GTT ATC TCC ATT TCT CAG GTA AGA GAA TTG GGT GAG AA-3’

- A-4- CD23a promoter (-115 to -75)

  M43 - 5’- GGA GAT AAT AAT AAC ACG GAC TTC ACC CGG GTG TGG G-3’

  M44 - 5’- GCT CCC CAC ACC CGG GTG AAG TCC GTG TTA TTA TTA CT-3’

- A-5- CD23a promoter (-83 to -44)

  M45 - 5’- GTG GGG AGC ACC AGG AGA GGC CAT GCG TGT AAT GTT A-3’

  M46 - 5’- GGA TAA CAT TAC ACG CAT GGC CTC TCC TGG TGC TCC-3’

- A-6- CD23a promoter (-51 to -9)

  M47 - 5’- TGT TAT CCG GGT GGC AAG CCC ATA TTT AGG TCT ATG AAA-3’

  M48 - 5’- GTA TTT TCA TAG ACC TAA ATA TGG GCT TGC CAC CCG GAT A-3’

- A-7- CD23a promoter (-17 to +25)

  M49 - 5’- TGA AAA TAG AAG CTG TCA GTG GCT CTA CTT TCA GAA GA-3’

  M50 - 5’- GCT TTC TTC TGA AAG TAG AGC CAC TGA CAG CTT CTA TTT-3’

- A-8- CD23a promoter (+16 to +56)

  M51 - 5'- GAA GAA AGT GTC TCT CTT CCT GCT TAA ACC TCT GTC TC-3'

  M52 - 5'- GTC AGA GAC AGA GGT TTA AGC AGG AAG AGA GAC ACT TT-3'
- A-9- CD23a promoter (+49 to +85)

  M53 - 5'- GTC TCT GAC GGT CCC TGC CAA TCG CTC TGG TCG AC-3'

  M54 - 5'- GGG GTC GAC CAG AGC GAT TGG CAG GGA CCG TCA GA-3'

4.2 Radioactive labelling of annealed oligonucleotides

Double stranded DNA fragments were radioactively labelled at the 3'-end by a typical Klenow Fill-in reaction in which dCTP was replaced by ^{32}P-dCTP (Amersham Pharmacia and Hartmann Analytics).

Reaction components (for a total of 25 µl):

1 µl DNA (100ng/µl)

1µl d(TGA)TP, 5 mM each

5 µl α-^{32}P-dCTP (10µCi/µl)

1 µl (5 U) Klenow Fragments (USB Corporation)

2.5µl 10x Klenow Fill-in Buffer (USB Corporation)

13.5 µl H_2O

The reaction was incubated at 37°C for 30 min. Non-incorporated nucleotides were washed with the QIAquick® Nucleotide Removal Kit (Qiagen). The product was eluted in 80µl elution buffer and the incorporation of radioactive nucleotides was assessed using a scintillation counter.

4.3 In vitro Transcription and Translation

Human Pax-5 protein was obtained by in vitro transcription and translation using TNT®Quick Coupled Transcription/ Translation Systems (Promega) appropriate for vectors containing a promoter for T7 RNA polymerase. pCR-Pax-5 was used as template.

4.4 Direct Binding and Competition Assays

The method can be used to visualize protein-DNA interaction by direct binding assay, in which the protein binds a labeled oligonucleotide, or by competition assay, in which cold competitors are able to inhibit the formation of a complex between the protein and a labeled oligonucleotide. The specificity of the complexes formed is determined by supershifts, in

which antibodies added to the reaction bind to the protein–DNA complexes and retard the migration of the specific band.

The basic reaction in direct binding assays was:

2 µl 10x Pax-5 Binding Buffer

2 µl in vitro translated Pax-5 protein

1 µg poly[d(I-C)]

40000 cpm ^{32}P-labelled oligonucleotide (approximatively 0,5-1 ng)

1 µg anti-Pax-5 antibody (BD Biosciences) – only in supeshift samples

H_2O up to 20 µl

The reaction was incubated for 15 min at room temperature before adding:

2 µl of 10x Loading Buffer.

The basic reaction in competition assays was:

2 µl 10x Pax-5 Binding Buffer

2 µl in vitro translated Pax-5 protein

1 µg poly[d(I-C)]

2 µl of unlabeled oligonucleotides (1, 10 and 100ng/µl)

H_2O up to 20 µl

The reaction was incubated for 10 min at room temperature before adding:

40000 cpm ^{32}P-labelled oligonucleotide (approximatively 0,5-1 ng)

The reaction was incubated for 15 min at room temperature before adding:

2 µl of 10x Loading Buffer.

In all case the samples were run on nondenaturating 5% polyacrylamide gel at 15V/cm for 2 hours. The gels were covered with plastic wrap, dried under vacuum and then exposed on BioMax^{TM} MS (Kodak scientific imagining film) at –80°C overnight.

5. Ribonuclease Protection Assay

The RPA is an extremely sensitive procedure for the detection and quantitation of mRNA in a complex sample mixture of total cellular RNA.

In our case 5x10^6 U-937 cells where stimulated with IL-4 (50ng/ml), PMA (3ng/ml) or both for 48h. Total RNA was prepared in 1 ml TRIZOL reagent (Gibco) following manufacturer's instructions.

5.1. Linearization of template DNA

Plasmid DNA must be linearized with a restriction enzyme downstream of the insert to be transcribed, as circular plasmids will generate extremely long, heterogenous RNA transcripts. pBS-CD23a, the template plasmid for CD23 transcripts was linearized with EcoRI restriction enzyme; pBS-β-actin, the template plasmid for β-actin, which was used as an internal control, was linearized with XbaI restriction enzyme. The linearized plasmids were observed on a 1 % agarose gel and the bands were extracted with QIAquick® Gel Extraction Kit (Qiagen).

5.2. In vitro Transcription

In vitro transcription was performed using [α-^{32}P] UTP (Amersham Pharmacia) as radiolabeled nucleotide and MAXIscript ™ T7/T3 (Ambion) In vitro Transcription Kit following manufacturer's instructions. Transcription products were purified using MicroSpin™S-200 HR Columns (Amersham Pharmacia) and run on a denaturing 5 % polyacrylamide/urea gel at 200V for 1 hour. The disassembled gel was kept on one plate, covered with plastic wrap and exposed for 4 min on Sterling film (Diagnostic Imaging). Using the film as a template, the full length RNA band was excised from the gel. The RNA transcripts were eluted overnight, at 37°C in elution buffer from the HybSpeed ™ RPA Kit (Ambion). The incorporation of radioactive nucleotides was assessed using a scintillation counter. The probes gave rise to a 299 bp band for CD23a, a 184 bp band for CD23b and a double band around 130 bp for β-actin.

5.3. Hybridization reaction

Hybridization reaction was carried on using HybSpeed ™ RPA Kit (Ambion) according to manufacturer's instructions except hybridization was extended overnight. Results were assessed by autoradiography on BioMax™ MS (Kodak Scientific Imaging Film) at –80°C for 24-72 hours.

6. Transfection and reporter gene assay

Transient transfection assays are used for analysing mammalian gene expression in vivo. Fusion genes constructs consisting of promoter or enhancer sequences under study attached

to a gene directing the synthesis of a reporter molecule are used to assess gene expression within 48 hours after introduction of the DNA. Luciferase assay use the *luc* gene of the firefly as a reporter gene and yields light signals that can be detected using an automated injection luminometer.

6.1 Transfection

$3x10^5$ 293 cells were transfected with 100 ng of the pLuc+ vectors and 500 ng of the pcDNA3-Pax-5 and pXM-STAT6 vectors. DNA concentration was brought to a total of 2μg DNA / transfection using salmon sperm DNA (Amersham Pharmacia). Transfection was performed in 1 ml trasfection volume using GenePORTER™ Transfection Reagent (PeqLab) according to manufacturer's instructions, except that growth medium was completely renewed 24 hours post transfection. Four hours after transfection cells were stimulated with IL-4 to a final concentration of 50ng/ml (rIL-4 provided by Prof. Sebald, Biozentrum, University of Würzburg) and PMA to a final concentration of 3ng/ml (Sigma).

6.2 Cell Lyses and Luciferase Assay

Cells were removed by pipetting up and down, collected in 15 ml tubes and washed 2 times with cold PBS followed by centrifugation at 1200xg, 10 min, 4°C. Pellets were left to dry at room temperature and then resuspended in 1x Reporter Lysis Buffer (Promega). The luciferase activity was assessed following manufacturer instructions- Luciferase Assay System (Promega) using a luminometer (Berthold). The results were normalized for equal concentration of total protein.

7. Transfection/ Infection Assays

Transfection of U-937 cells with the recombinant vector pEGZ-Pax-5 has been performed by Dr. I. Berberich (Institute for Virology and Immunobiology, Würzburg). Recombinant retroviral particles were generated using the pHIT packaging system as described by Soneoka *et al* [47].

Briefly, 293T cells were transiently cotransfected using the standard calcium phosphate method with 5 μg of each of the packaging vectors pHIT456 (which codes for the amphotropic env protein) and 5-7 μg of the retroviral construct pEGZ/MCS and EGZ/Pax-5. Sixteen hours later the transfection solution was replaced by DMEM. Viral supernatants were

harvested 48h later and filtered (0.45µm). Polybrene (Sigma) was added to a final concentration of 10µg/ml. U-937 cells were mixed with the retroviral supernatant and centrifuged for 3 hours at 1000 x g. Thereafter cells were replated in RPMI. Transduced cells were selected with Zeocin (250 µg/ ml).

The cell line selected for experiments stained positive for EGZ in 97% of the cells, as revealed by FACS analysis.

8. Nucleic acid extraction

8.1 DNA extraction

DNA was extracted from 1x 10^8 cells using QIAamp^R DNA Blood Kit (Qiagen) following manufacturer's instructions.

8.2 RNA extraction

- Homogenisation- 1x10^7 mammalian cells were pelleted by centrifugation and lysed in 1.5 ml Trizol Reagent by repetitive pipetting.
- Phase separation- The homogenized samples were incubated for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. 0.3 ml chloroform was added and tubes were shacked vigorously by hand for 15 seconds and incubated at room temperature for 2 to 3 minutes. Then samples were centrifuged at 12,000 x g for 15 minutes at 4°C. Following centrifugation, the mixtures separated into a lower red, phenol-chloroform phase, an interphase and a colourless upper aqueous phase. RNA remains exclusively in the aqueous phase.
- RNA precipitation- The aqueous phase was transferred to a new tube and the RNA precipitated by mixing with 0.75ml isopropanol. Then samples were incubated at room temperature for 10 minutes and centrifuged at 12000x g for 10 minutes at 4°C. The RNA precipitate was visible as a gel-like pellet.
- RNA wash- After removal of the supernatant the pellet was washed once with 1.5ml of 75% ethanol. The samples were mixed by vortexing and centrifuged at 7,500x g for 5 minutes at 4°C.
- Redissolving the RNA- After discarding the ethanol the RNA pellet was air-dried for 5-10 minutes and dissolved in RNase-free water by passing the solution a few times through a pipette tip and incubating for 10 minutes at 55 °C.

9. Spectrophotometric measurements

9.1 Nucleic acids

To assess the concentration of nucleic acids in a sample absorbtion is measured at 260 wavelenght. Absorbance at 280 wavelengths is measured to assess the purity of the sample. The A_{260}/A_{280} ratio is used to estimate the purity of the sample and should be between 1.6 and 2. To determine the nucleic acid concentration (in µg/ml) the A260 reading has to be multiplied with a factor of 50 for double stranded DNA, 33 for single stranded DNA and 40 for RNA.

9.2 Proteins

For measuring the protein concentration 10 µl protein solution were diluted in 190 µl H_2O and 800 µl 1x Roti^R- Nanoquant (Roth). The absorbtion of the sample is measured at 590 and 450 wavelenghts. The A_{590}/A_{450} ratio was introduced into a linear regression program in order to determine the total protein concentration in the sample.

10. Two Hybrid Systems

10.1 Cytotrap^{TM} Two Hybrid System (Stratagene)

This is a novel method for detecting in vivo protein-protein interaction, which is based on generating fusion proteins whose interaction in the yeast cytoplasm induces cell growth by activating the Ras signaling pathway.

The system provides:

- the host strain cdc25H
- the vectors: the bait vector (pSos), the target vector (pMyr) and control vectors (pSos MAFB, pSos Coll, pMyr MAFB, pMyr Lamin C)

The human spleen cDNA library cloned in the pMyr vector was purchesed from Stratagene and amplified following manufacturer's instructions.

All protocols and procedures for the CytoTrap Two Hybrid System were carried on following manufacturer instruction, with a few modifications, as noted:

- Preparation and Trasformation of Yeast Competent Cells- was performed using the following protocol [91]:

- 5 ml of liquid YPAD were inoculated with one colony of yeast and shacked at 250 rpm, overnight, at 25°C.
- The overnight culture was used to inoculate 50 ml of YPAD and incubated up to a cell density of 0.7 OD.
- The culture was harvested in a sterile 50 ml tube at 3000xg for 5 minutes, at room temperature.
- The cells were resuspended very gently in 25 ml sterile water and centrifuged again.
- The pellet was resuspended in 1 ml 100mM lithium acetate (LiAc) and transferred to a 1.5 ml centrifuge tube.
- The cells were centrifuged for 5 sec at top speed in a table-top centrifuge and the LiAc was removed with a pipette.
- The cell were resuspended in a final volume of 500 µl 100mM LiAc
- 50 µl of cell suspension were pipetted in labeled microfuge tubes and centrifuged for 5 sec at top speed; LiAc was removed with a pipette.
- A "transformation mix" was assembled as follows, strictly respecting this order:

  240 µl PEG (50%w/v)

  36 µl 1.0 M LiAc

  25 µl single-stranded carrier DNA (2mg/ml) previously boiled for 5 min and placed directly on ice

  50 µl H_2O and plasmid DNA (1-3 µg)
- Each tube was vortexed vigorously until the cell pellet was completely mixed and incubated at room temperature for 30 minutes.
- Tubes were heat-shocked for 20 minutes in a water bath at 42°C.
- Tubes were centrifuged at 6000-8000 rpm for 15 sec and the transformation mix with a pipette.
- The pellets were resuspended in 250µl sterile water by gently pipetting up and down and plated onto selective plates.

• Isolation of pMyr cDNA plasmids from Yeasts –was performed using a RPM Yeast Plasmid Isolation Kit (Bio101).

The chart flow of a screening episode is schematically presented in Figures 8-11.
Briefly, the screening steps were as follows:
- pSosCD23a/b and pMyr spleen library were co-transformed in the cdc25H yeast strain;

- transformation products were plated on Glucose(-Ura, -Leu) 150 mm plates and incubated at 25°C for 58-60 hours;
- the yeast colonies were replica plated on Galactose(-Ura, -Leu) plates and incubated for 6-10 days at 37°C;
- every colony which appeared after replica plating was tested on Glucose(-Ura, -Leu) and Galactose(-Ura, -Leu) at 37°C in order to distinguish between temperature revertants and real interactions;
- the phenotype was checked: colonies that grew on glucose at 37°C were considered "temperature revertants" (the presence of glucose in the medium inhibits the GAL1 promoter which controls the expression of the fusion protein in the pMyr construct, in such a way that library proteins are not expressed when the yeast host is maintained on glucose). Colonies that grew at 37°C on galactose but not on glucose reflected a real interaction (Fig. 8);
- plasmids were extracted from yeasts, transformed in bacteria and plated on LB agar plates containing chloramphenicol, the selection for the pMyr vector (Fig.9);
- plasmids extracted from bacteria were analysed by enzymatic restriction with XbaI (two XbaI sites flank the MCS of the pMyr vector);
- every pMyr-library profile obtained by XbaI digestion in yeasts was co-transformed together with pSosCD23a/b (Fig.10);
- the interaction was checked on Glucose(-Leu,-Ura) and Galactose(-Leu,-Ura) selective plates at 37°C;
- the phenotype was checked (Fig.10). By-standing library constructs that co-transform together with those pMyr-library plasmids which cause a real interaction were excluded at this stage;
- pMyr-library clones were co-transformed in yeasts together with pSos or pSosColl (Fig.11);
- the transformants were patched on Glucose(-Leu,-Ura) and Galactose(-Leu,-Ura) selective plates at 37°C;
- the phenotype was checked: transformants that grew on Galactose(-Leu,-Ura) at 37°C were considered "false positive" (the interaction involves human SOS protein, not the bait). Transformants that didn't grow on Galactose(-Leu,-Ura) at 37°C were considered "true positive" (they could be subjected to further analyses).

10.2 MATCHMACKER GAL 4 Two-hybrid System 3 (Clontech)

This system is an advanced GAL4-based two-hybrid system that provides a transcriptional assay for detecting protein interactions *in vivo* in yeasts.

The system provides:

- the host strains: AH109 and Y187
- the vectors: the bait vector (pGBKT7), the target vector (pGADT7) and control vectors (pGBKT7-T, pGBKT7-53, pGBKT7-Lam, pCL1)

All protocols and procedures for the MATCHMACKER GAL 4 Two-hybrid System 3 were carried on following manufacturer instruction.

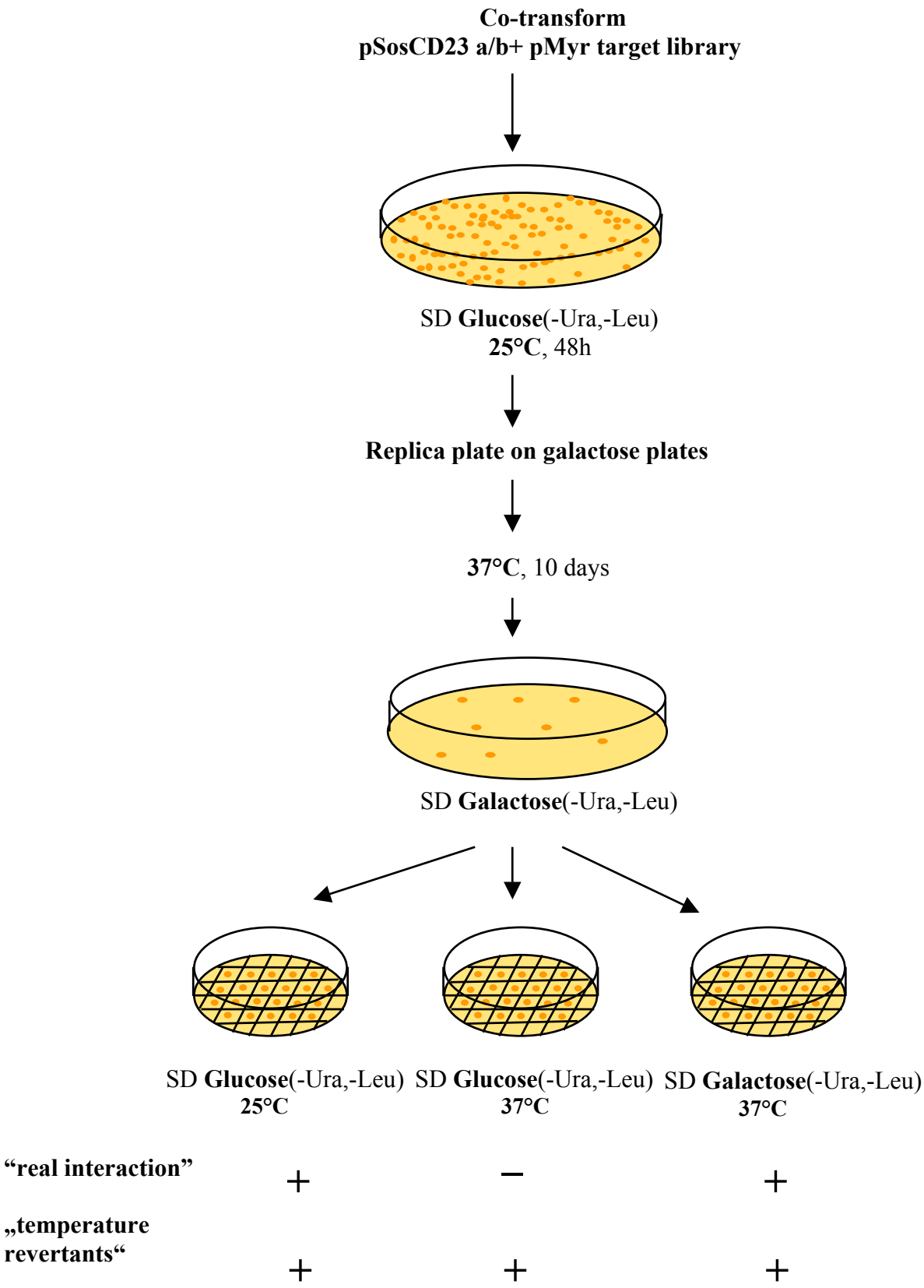


Fig.8 ***The chart flow of the CytoTrap screening: eliminating the temperature revertants***

Plasmid extraction (pSos and pMyr) from selected clones –"real interaction"

↓

Transform in E. coli competent cells

↓

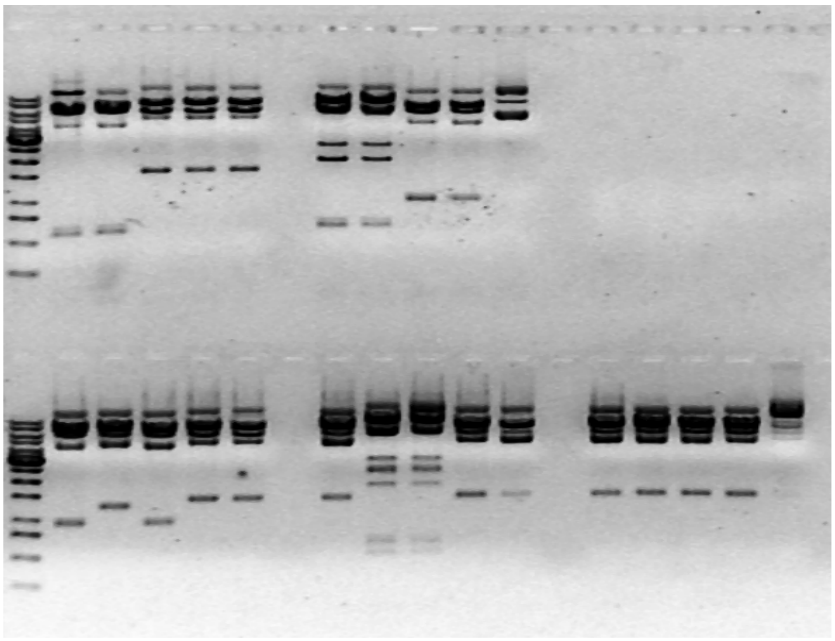
Select transformants on Chloramphenicol
(selective antibiotic marker for pMyr target plasmids)

↓

Extract plasmids from bacteria

↓

Check the clone profile by XbaI restriction enzyme digestion in order to assess the homogeneity of every clone



↓

Select every profile for re-transformation in yeasts in order to assess which one caused the interaction

Fig.9 ***The chart flow of the CytoTrap screening: isolating the pMyr yeast target DNA***

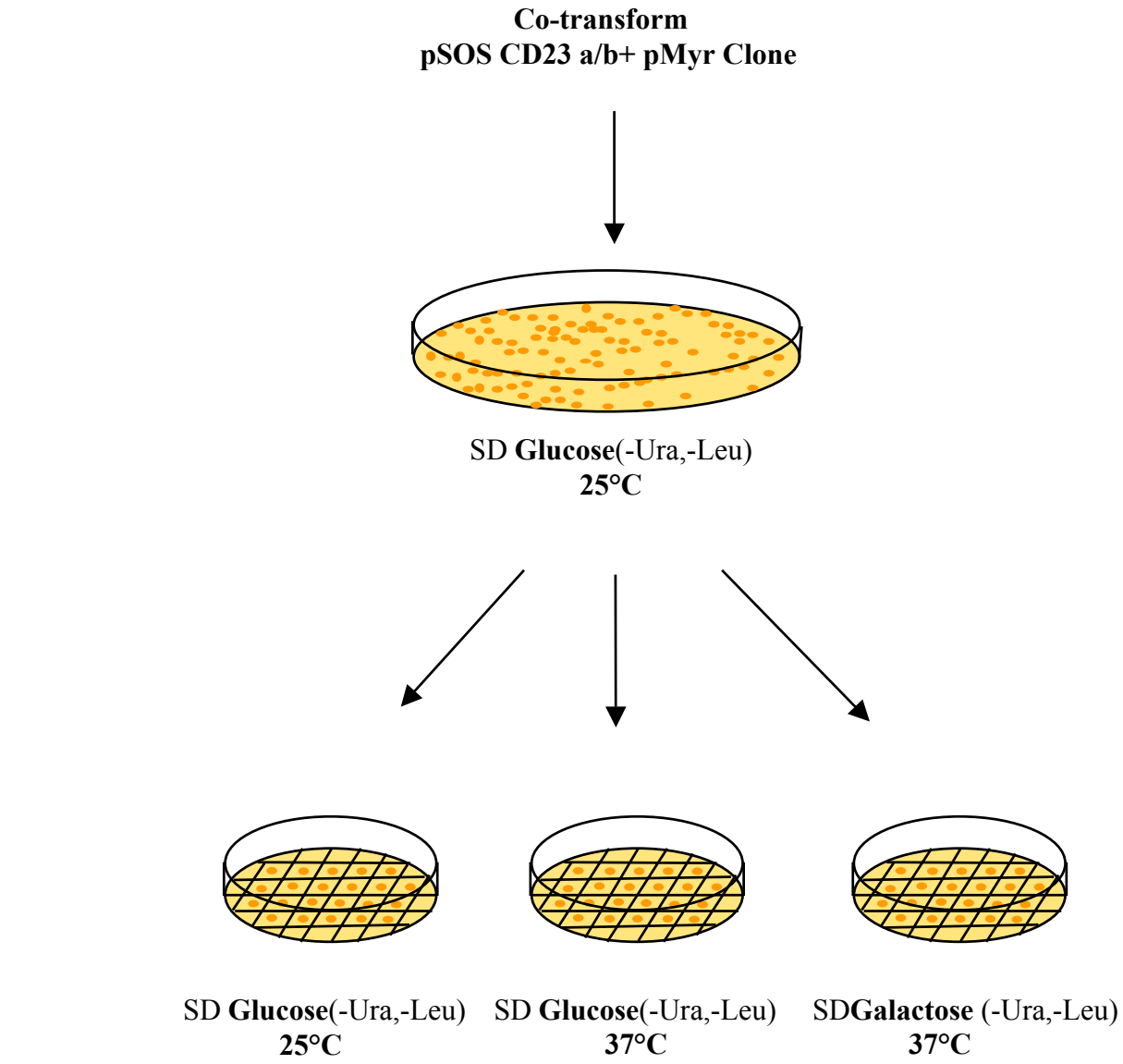


Fig.10 ***The chart flow of the CytoTrap screening: finding the putative positive clones***

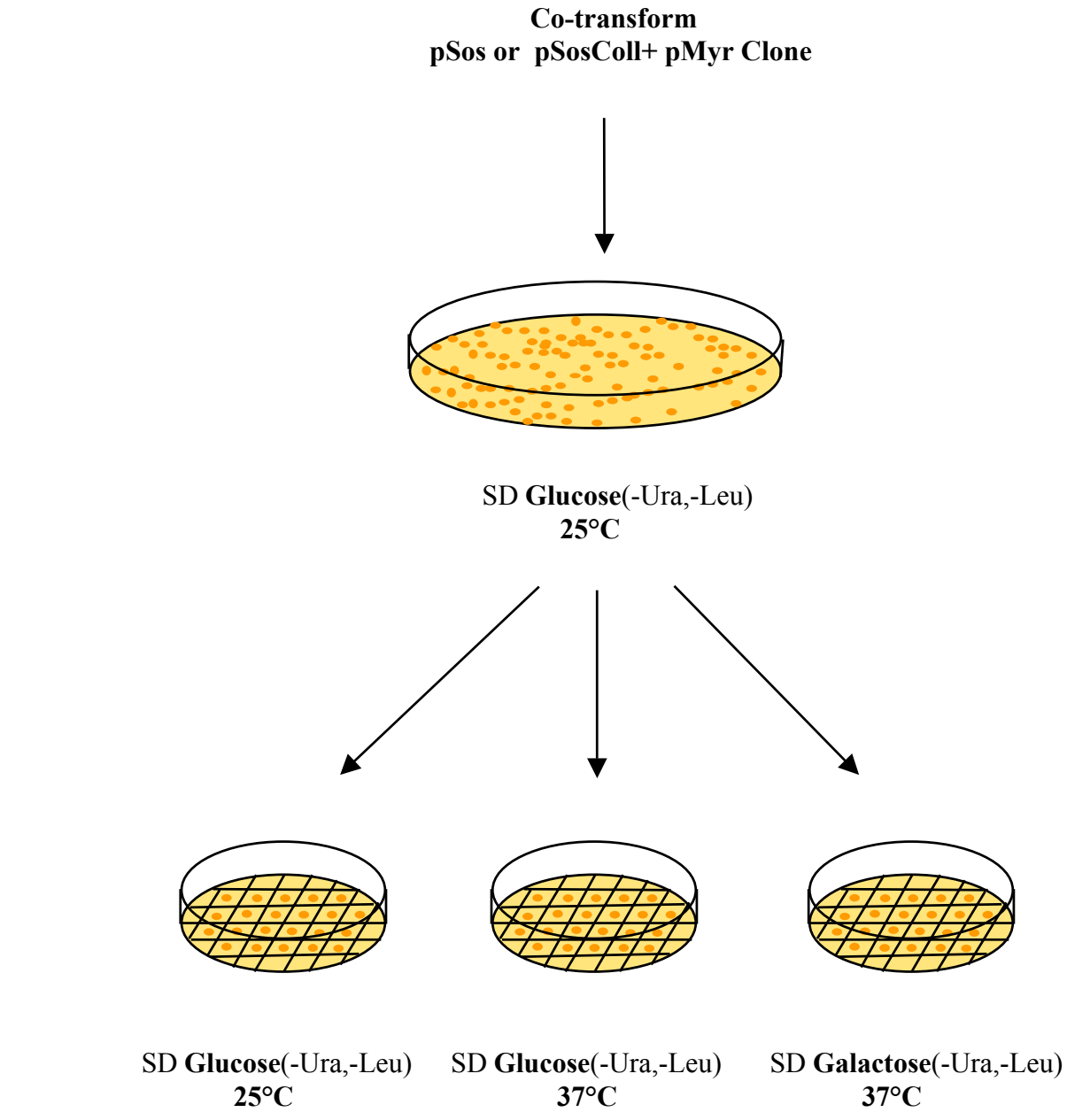


Fig.11 ***The chart flow of the CytoTrap screening: eliminating the false positive clones***

11. Materials

11.1 Buffers

10x Pax-5 Binding Buffer	100mM HEPES pH 7,9	
	1M KCl	
	40% Ficoll	
	10mM EDTA	
	10mM DTT	
Cell Lysis Buffer for Protein Isolation	140 mM NaCl	
	2.7 mM KCl	
	10mM Na_2HPO_4	
	1.8 mM KH_2PO_4	
	1% Triton X	
	+ Protease inhibitors	1mM PMSF
		10µg/ml aprotinin
		1µM pepstatin A
		100µM leupeptin
		1µg/ml chymostatin
Running Buffer for SDS-PAGE	3.03 g Tris	
	14.4 g glycin	
	1 g SDS	
	H_2O up to 1 liter	
Anode Buffer I	0.3 M Tris	
	10% methanol	
Anode Buffer II	25 mM Tris	
	10 % methanol	
Cathode Buffer	25mM Tris	
	40mM 6- amino n- caproic acid	
	10 % methanol	
Wash Buffer	100 ml PBS (10x)	
	1 ml Tween 20 (0,1%)	
	900 ml H_2O	

Blocking Buffer	1x PBS
	0.1% Tween 20
	5% dry milk
10 x Loading Dye (EMSA)	20% Ficoll
	0,1 M Na_2EDTA pH 8,0
	1 % SDS
	0.25 % bromphenolblue
	0.25 % xylene cyanol
6 x Loading Dye (Agarose gel)	0.25 % bromphenol blue
	0.25 % xylen cyanol
	30 % glycerol in water
TAE electrophoresis buffer (50x)	242 g Tris base
	57.1 ml glacial acetic acid
	37.2 g $Na_2EDTA{\cdot}2H_2O$
	H_2O up to 1 liter
TBE electrophoresis buffer (10x)	108 g Tris base
	55 g boric acid
	40 ml 0.5 M EDTA, pH 8.0
	H_2O up to 1 liter

11.2 Gels

Laemmli Gel (PAGE)

	Separating gels		*Stacking gels*
	6%	10%	4%
Acrylamyde 30%	1.99 ml	3.32 ml	0.67 ml
Tris 1M, pH 8,8	3.75 ml	3.75 ml	
Tris 1M, pH 6,8			0.625 ml
10% SDS	0.1 ml	0.1 ml	0.05 ml
H_2O	4.12 ml	2.78 ml	3.625 ml
10% ammonium persulphate	50 µl	50 µl	25 µl
TEMED	3.3 µl	3.3 µl	2.5 µl

Nondenaturing 5 % polyacrylamide gel (for EMSA)

Acrylamide 30 %	6.67 ml
10 x TBE	2 ml
H_2O	31.33 ml
TEMED	40 µl
10% ammonium persulphate	400 µl

Denaturing 5 % polyacrylamide/urea gel (for RPA)

Acrylamide 40 %	6.3 ml
10 x TBE	5 ml
Urea	24 g
H_2O	up to 50 ml
10 % ammonium persulphate	300 µl
TEMED	34 µl

Agarose gel

Agarose UltraPure (Gibco BRL) 1-1.5 %

1 x TAE

Ethidium bromide (0.5 µg /ml)

11.3 Media for bacteria and yeasts

LB Broth Base(Gibco BRL)

LB Agar (Gibco BRL)

Ampicillin (USB Corporation)

Chloramphenicol (Roche)

Kanamycin (USB Corporation)

DOB-Dropout Base (BIO 101)

DOBA-Dropout Agar base (BIO 101)

DOBA, 2% Gal, 1% Raf (BIO 101)

YPD Broth (BIO 101)

Agar-Agar (Roth)

CSM-Leu-Ura (BIO 101)

CSM-His-Leu-Trp (BIO 101)

CSM-His-Leu-Trp-Ura (BIO 101)

Adenine (Sigma)

L-Histidine (Sigma)

L-Leucine (Sigma)

L-Tryptophan (Sigma)

Uracil (Sigma)

X-α-Gal (Clontech)

11.4 *E.coli* competent cell strains

DH5α

Epicurian Coli® BL21-Codon Plus™ (Stratagene)

One Shot™ Top 10 (Invitrogen)

11.5 Cell lines

- The **293** cell line is a permanent line of primary human embryonal kidney transformed by sheared human adenovirus type 5 (Ad 5) DNA. The cells were maintained in DMEM (Gibco) supplemented with L-Glutamine (2mM), antibiotics (Penicillin 100U / Streptomycin 100µg/ml) and 10% FCS.
- The **U-937** cell line is a monoblastoid cell line. The cells were maintained in RPMI 1640 (Gibco) supplemented with L-Glutamine (2mM), antibiotics (Penicillin 100U / Streptomycin 100µg/ml) and 10% FCS.
- The **WIL2** cell line is an Epstein-Barr virus infected B cell line. The cells were maintained in RPMI 1640 (Gibco) supplemented with L-Glutamine (2mM), antibiotics (Penicillin 100U / Streptomycin 100µg/ml) and 10% FCS.

11.6 Software and websites

- DNAsis
- JellyFish
- pDRAW
- Kodak Imager
- Miscrosoft Office
- WebPrimer- http://genome-www2.stanford.edu/cgi-bin/SGD/web-primer

The Web Primer provides tools for the purpose to design primers. Current choices are limited to sequencing and PCR.

- NCBI Entrez Nucleotide- http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide

The Entrez Nucleotides database is a collection of sequences from several sources, including GenBank, RefSeq, and PDB.

- NCBI BLAST- http://www.ncbi.nlm.nih.gov/BLAST/

BLAST® (Basic Local Alignment Search Tool) is a set of similarity search programs designed to explore all of the available sequence databases regardless of whether the query is protein or DNA. The scores assigned in a BLAST search have a well-defined statistical interpretation, making real matches easier to distinguish from random background hits.

11.7 Lab devices

GeneAmp PCR System 2400 (Perkin Elmer)

ABI PRISM™ 310 Genetic Analyser (Applied Biosystems)

Digital camera

Ultrospec 1000 spectrophotometer (Amersham Pharmacia)

MicroLumat LB 96P luminometer (EG&G Berthold)

Optimax Developer (Protec)

Scintillation counter (Beckman LS 1801)

Vacuum dryer

Sonicator

Incubators

Shackers

Autoclaves

Centrifuges

Heating blocks

Water bath

RESULTS

1. The CD23a promoter- role of Pax-5 in the B-cell specific expression of CD23a isoform

1.1 The CD23a core promoter contains three putatitive binding sites for Pax-5

The consensus motif for Pax-5 has been characterised by Czerny *et al*. in an extended comparison between all known natural ligands of Pax-5, including the recognition sequences which originates from the sea urchin H2A-2.2 gene and the human CD19 gene [12].

Our approach to test the hypothesis of Pax-5 regulating the expression of CD23a isoform was to identify putative binding sites for Pax-5 in the CD23a promoter. We performed multiple sequence alignments between the CD23a core promoter (Fig. 12A) and the Pax-5 consensus sequence. Three putative Pax-5 binding sites were identified in positions -61 to -79, -78 to -96 and -215 to -233 of the CD23a promoter (Fig. 12) and named CD23-1, CD23-2 and CD23-3 respectively.

All naturally occurring Pax-5 binding sites identified so far deviate from the consensus sequence and Pax-5 is able to interact with a panel of seemingly degenerate recognition sequences. This makes the prediction of Pax-5 binding sites quite difficult, when only relating to the sequence of the promoter. When chosing the putative Pax-5 binding sites, we considered only the sites with at least 50% identity to the consensus sequence. Fig. 12B shows the sequence alignment of these three putative binding sites with the consensus motif and other previously identified Pax-5 binding sites from the sea urchin H2A-2.2 and CD19 genes. The CD23-3 putative binding site displayed the highest homology with the consensus motif (89%). A gap had to be introduced in the putative binding site CD23-1 in order to reconstitute the symmetry of the recognition sequence.

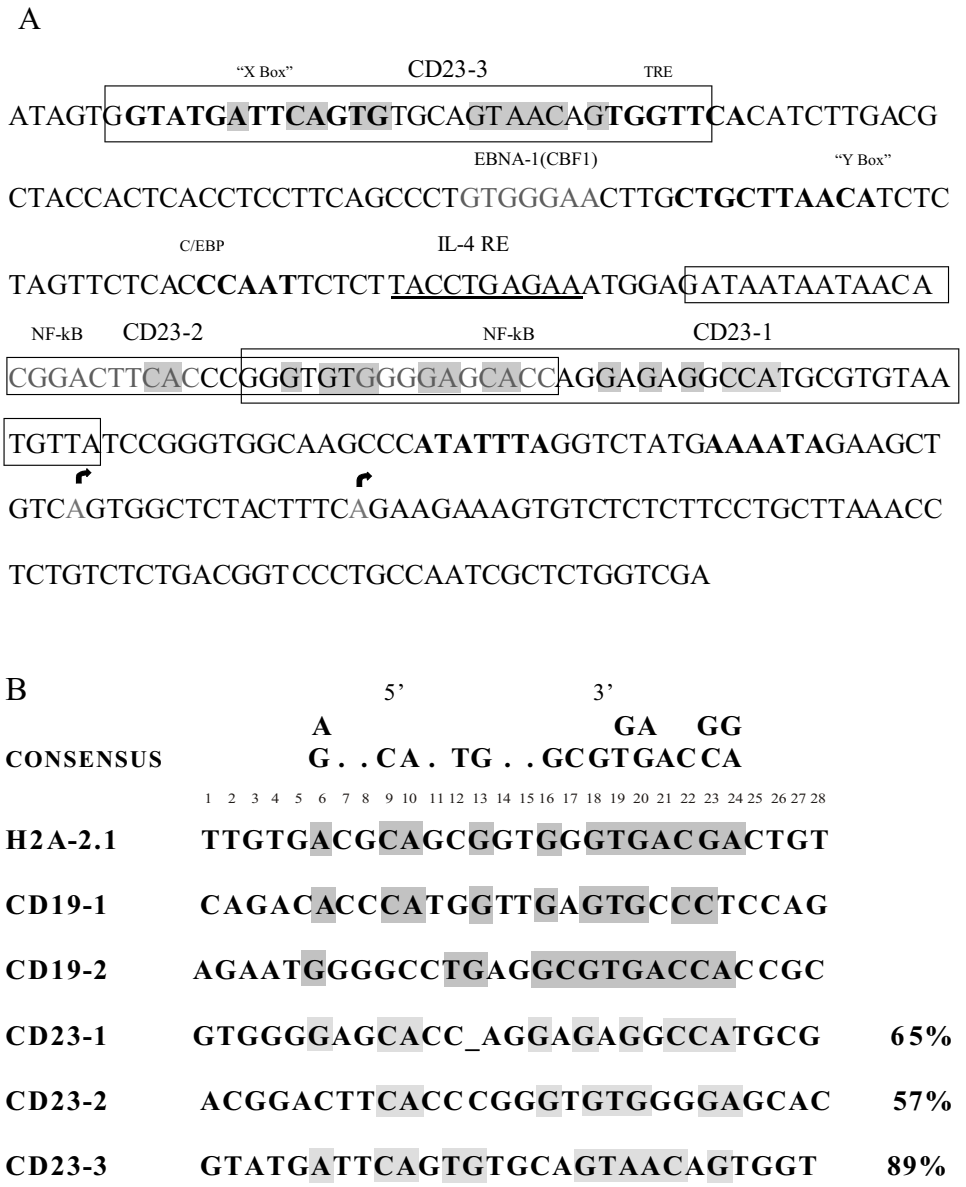


Fig.12 ***CD23a core promoter and consensus recognition sequences for Pax-5.*** **(A)** CD23 core promoter (-243 to +82): The three putative Pax-5 binding sites are shown with the homologous nucleotides shaded. The boxes represent the synthetic oligonucleotides containing the three putative binding sites: CD23-1, CD23-2 and CD23-3. The STAT6 binding site (IL-4 responsive element) is underlined; the localization of NF-kB binding sites is indicated by analogy with the murine CD23 promoter. The arrows (position 1 and 16) show the beginning of exon Ia (5' UTR). **(B)** Pax-5 recognition sequences: The three putative Pax-5 binding sites - CD23-1, CD23-2, CD23-3 are shown in comparison with Pax-5 binding sites from the sea urchin H2A-2.2 and the human CD19 promoters. The orientation of the sequences corresponds to the natural orientation of the Pax-5 binding sites. Consensus sequences are shaded and the deduced consensus sequence is shown above.

1.2 Pax-5 protein interacts with the CD23-1 binding site from the CD23a core promoter *in vitro*

1.2.1 CD23-1 binding site competes a high affinity Pax-5 binding site

We tested the three putative binding sites for their ability to bind Pax-5 protein. In a first stage synthetic oligonucleotides were used in EMSA competition experiments as cold competitors. The oligonucleotides were designed to cover regions in the CD23a promoter ranging from -84 to -47 for CD23-1, -112 to -71 for CD23-2, and -238 to -209 for CD23-3 putative sites. Their localization within the CD23a core promoter is represented in Fig. 10A as rectangles. As a labelled probe, an oligonucleotide representing the high affinity Pax-5 binding site from the sea urchin H2A-2.2 gene was used (Fig. 12B). Pax-5 was synthesized by in vitro transcription and translation. The plasmid construct used for Pax-5 translation generated an additional unspecific band (Fig.13A, lane 1). Under these conditions, supershift experiments using a monoclonal Pax-5 antibody verified the correct DNA-protein complex (Fig. 13A, lanes 2-3).

Complex formation was inhibited by a 10-fold excess of the oligonucleotide CD23-1, but not by an excess of oligonucleotides CD23-2 and CD23-3. For comparison, we also competed the high affinity Pax-5 binding site H2A-2.2 with itself (Fig. 13A, lanes 4-6). CD23-1 showed approximatively a 10 times lower affinity than the H2A-2.2 binding site. Both CD23-2 and CD23-3 oligonucleotides failed to compete the high affinity binding site.

1.2.2 CD23-1 binding site interacts with Pax-5 protein directly

To confirm the results obtained in competition assays, the same oligonucleotides representing the putative CD23-1, CD23-2 and CD23-3 sites were appropriately labeled and checked for direct binding (Fig. 13B). The specificity of the complexes formed was confirmed by supershifts using a monoclonal antibody against Pax-5. CD23-1 was the only site where we observed a specific complex formation with Pax-5 (Fig. 13B, lanes 4-5). Both competition experiments and direct binding approaches indicated CD23-1 putative site as a Pax-5 binding site.

1.3 Mutations of the CD23-1 binding site prevent Pax-5 binding

To further analyse the CD23-1 site we constructed oligonucleotides in which the Pax-5 binding site was mutated and tested again their ability to interact with Pax-5 in EMSAs.

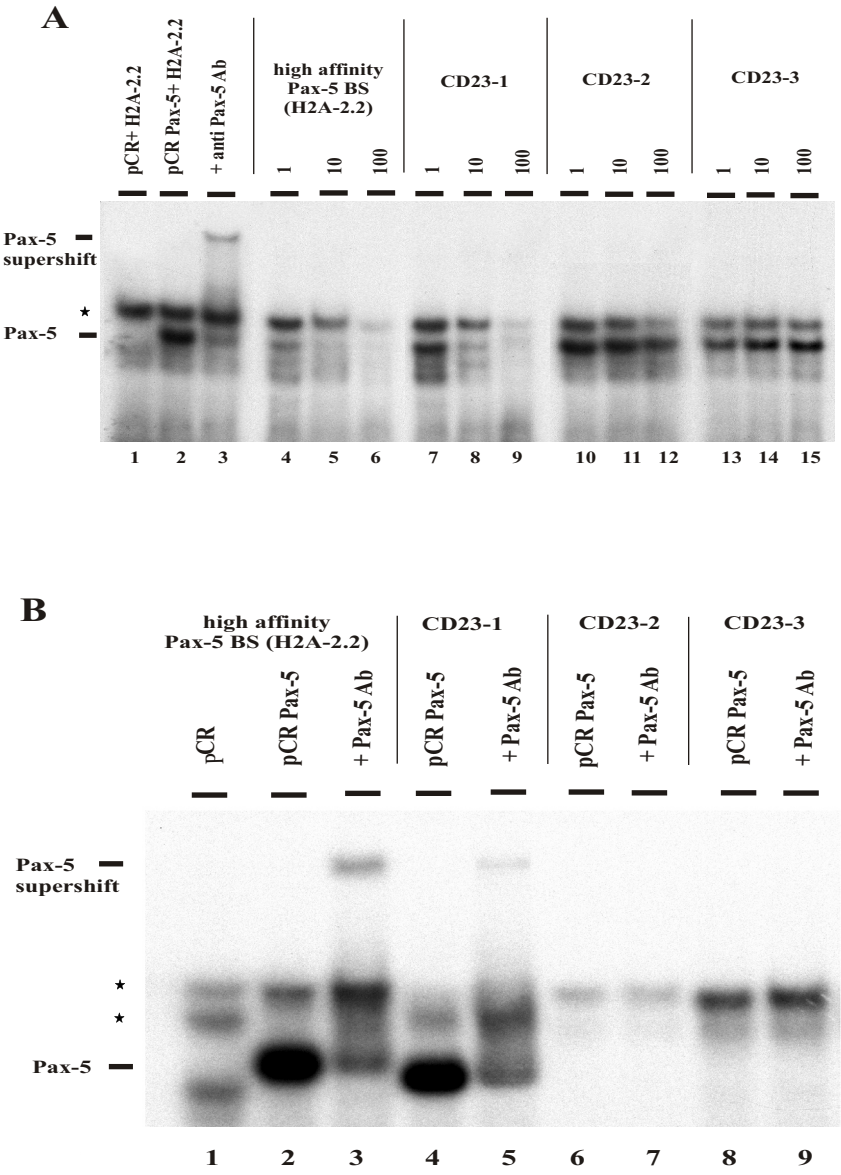


Fig.13 ***CD23-1 binding site interacts with Pax-5.*** **(A)** An oligonucleotide containing the optimal Pax-5 binding site from the sea urchin H2A-2.2 gene was radioactively labeled and incubated with Pax-5 protein obtained by in vitro transcription and translation. Unlabeled oligos representing the optimal Pax-5 binding site H2A-2.2 (lanes 4-6) or containing the three putative Pax-5 binding sites from the CD23a promoter (lanes 7-15) were used as competitors (ratios of 1:1,1:10 and 1:100 labeled H2A-2.2 to unlabeled competitor). Lane 1 shows the empty vector (pCR) used for in vitro transcription and translation of the Pax-5 protein incubated with the labeled oligonucleotide, which forms an unspecific complex (⋆). Lane 2: specific Pax-5 complex. Lane 3: Pax-5 supershift with anti Pax-5 antibody. **(B)** Oligonucleotides containing the optimal Pax-5 binding site from the sea urchin H2A-2.2 gene (lanes 2-3) and the three putative Pax-5 binding sites from the CD23a promoter (lanes 4-9) were radioactively labeled and incubated with Pax-5 protein obtained by in vitro transcription and translation. Pax-5 antibody was added to check the specificity of the bands (lanes 3, 5, 7, 9). Lane 1 shows the empty vector (pCR) used for in vitro transcription and translation of the Pax-5 protein incubated with the labeled high affinity site; (⋆)-unspecific complex.

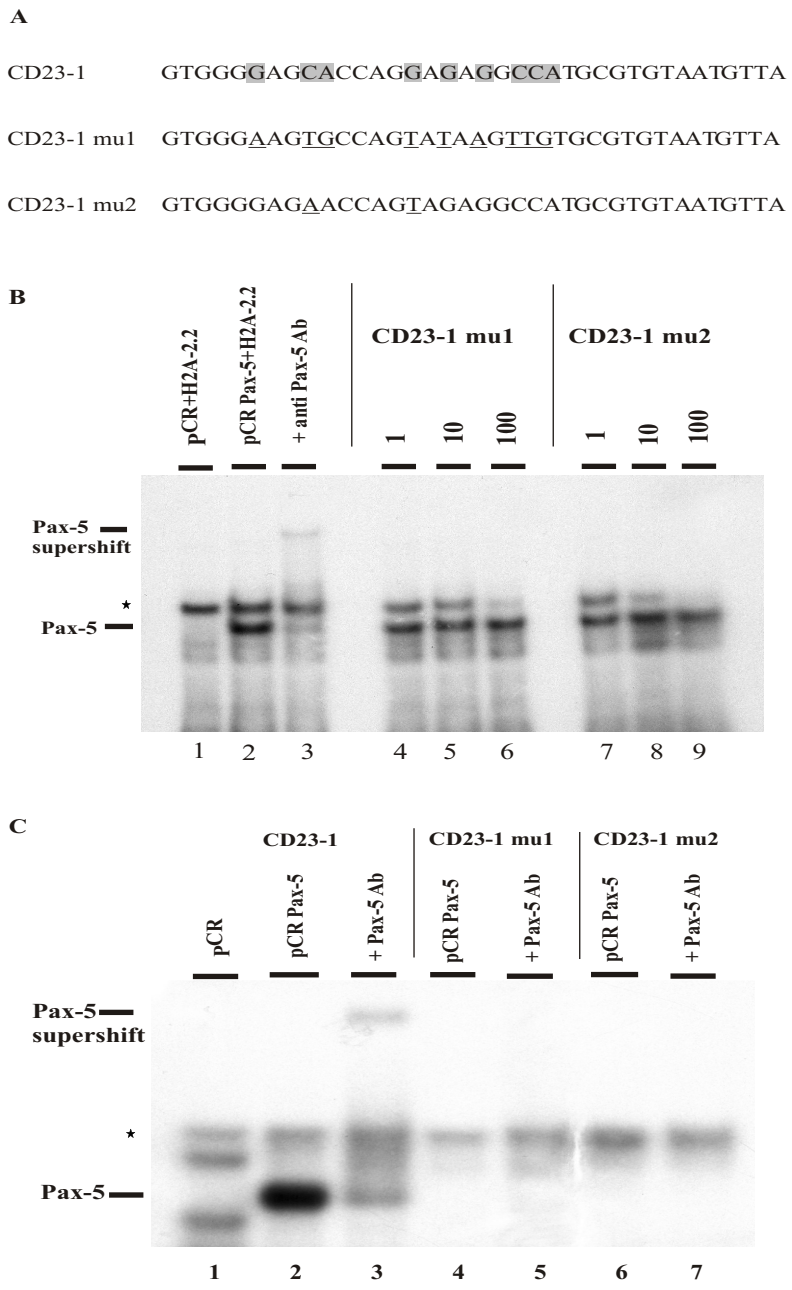


Fig. 14 ***Mutations in the CD23-1 binding site prevent Pax-5 binding.*** **(A)** CD23-1mu1 and CD23-1 mu2 oligonucleotides contain mutated variants of the CD23-1 putative Pax-5 binding site. Mutated nucleotides are underlined. **(B)** An oligonuclotide containing the high affinity Pax-5 binding site from the sea urchin H2A-2.2 gene was radioactively labeled and incubated with Pax-5 protein obtained by in vitro transcription and translation. Unlabeled oligos containing mutated variants of the CD23-1 putative binding sites (lanes 4-9) were used as competitors (ratios of 1:1,1:10 and 1:100 labeled H2A-2.2 to unlabeled competitor). Lane 1 shows the empty vector (pCR) used for in vitro transcription and translation of the Pax-5 protein incubated with the labeled oligonucleotide, which forms an unspecific complex (⋆). Lane 2: specific Pax-5 complex. Lane 3: Pax-5 supershift. **(C)** Oligonucleotides containing the putative CD23-1 binding site (lanes 2-3) and mutated sites CD23-1mu1 and CD23-1mu2 (lanes 4-7) were radioactively labeled and incubated with Pax-5 protein obtained by in vitro transcription and translation. Pax-5 antibody was added to check the specificity of the bands (lanes 3, 5, 7). Lane 1 shows the empty vector (pCR) used for in vitro transcription and translation of the Pax-5 protein incubated with the labeled CD23-1 site; (⋆)-unspecific complex.

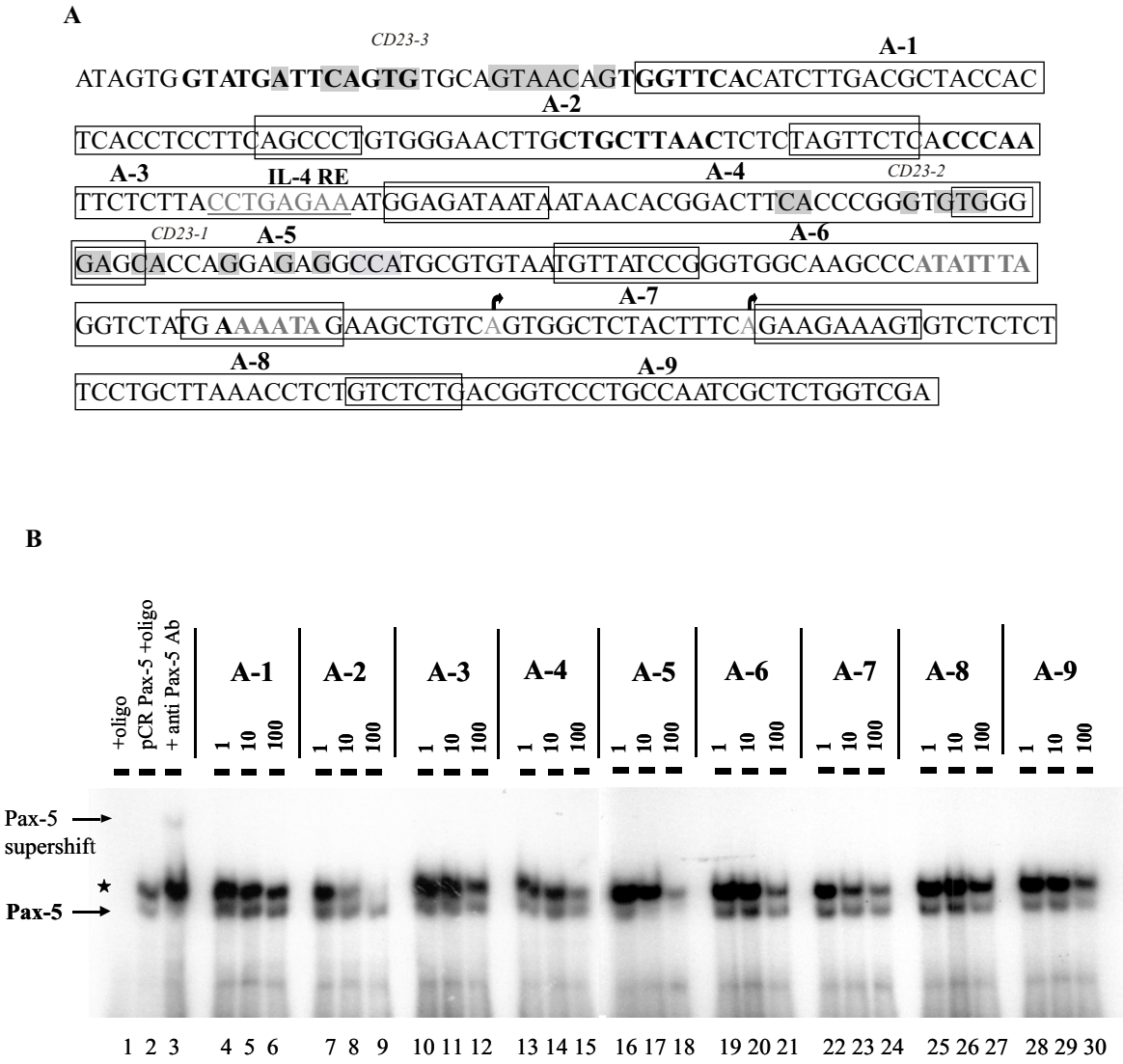


Fig.15 ***CD23-1 is the only site in the CD23a core promoter that directly binds Pax-5.*** **(A)** CD23 core promoter (-243 to +82): The boxes represent the overlapped synthetic oligonucleotides spanning the promoter: A-1 to A-9. The STAT6 binding site (IL-4 responsive element) is underlined; the arrows (position 1 and 16) show the beginning of exon Ia (5' UTR). **(B)** An oligonucleotide containing the optimal Pax-5 binding site from the sea urchin H2A-2.2 gene was radioactively labeled and incubated with Pax-5 protein obtained by in vitro transcription and translation. Unlabeled synthetic oligonucleotides A-1 to A-9 (lanes 4-30) were used as competitors (ratios of 1:1,1:10 and 1:100 labeled H2A-2.2 to unlabeled competitor). Lane 1 shows the empty vector (pCR) used for in vitro transcription and translation of the Pax-5 protein incubated with the labeled oligonucleotide, which forms an unspecific complex. Lane 2: specific Pax-5 complex. Lane 3: Pax-5 supershift with anti Pax-5 antibody. (⋆)-unspecific complex.

Since Pax-5 is able to interact with a panel of seemingly degenerate recognition sequences it is quite difficult to predict which nucleotides are essential in the protein-DNA interaction [12]. For this reason we created two different mutated variants of the CD23-1 putative site (Fig. 14A). In CD23-1mu1 all 9 nucleotides considered to be responsible for creating the Pax-5 binding site were mutated. CD23-1mu2 contains only two substitutions (C to A in position 4 and G to T in position 11 of the consensus motif), as these nucleotides seem to act like key points for Pax-5-DNA interaction [3,12].

The ability of the two mutant oligonucleotides to bind to Pax-5 protein was subsequently tested in EMSAs. As seen in Fig. 14B, both oligonucleotides CD23-1mu1 and CD23-1mu2 containing the mutated CD23-1 binding site were unable to compete, at any concentration, the sea urchin high affinity H2A-2.2 Pax-5 binding site. Direct binding assays showed that the oligonucleotides containing the mutated sites failed to bind Pax-5 protein anymore (Fig. 14C). These results give additional evidence that CD23-1 is a Pax-5 binding site since mutations introduced in this site completely abrogate the binding of the protein.

1.4 CD23-1 is the only site which directly binds Pax-5 protein

We considered the possibility that other putative sites, which displayed less than 50% homology with the consensus sequence, might bind Pax-5 anyway. Nine pairs of overlapping oligonucleotides, named A1-A9, covering the whole lentgh of the CD23a core promoter (Fig. 15A) were synthesized and used as competitors for the sea urchin H2A-2.2 binding site in EMSAs. As seen in Fig. 15B, the oligonucleotide A5, which covers the putative binding site CD23-1, was the only one to compete the high affinity binding site. This approach confirmed previous results, pointing for CD23-1 as the only site within the CD23a core promoter that can directly bind Pax-5.

1.5 Pax-5 mediates activation of the CD23a promoter *in vitro*

To examine the effect of Pax-5 on the activation of the CD23a promoter a luciferase reporter construct containing the CD23a core promoter (from position -203 to position +83) cloned in the pLuc+ vector was made and named pLuc+ACP (CD23a core promoter). Another construct, pcDNA$_3$-Pax-5 was used for ectopic expression of Pax-5. 293 cells were transfected with the luciferase construct pLuc+ACP alone or together with pcDNA$_3$-Pax-5. Luciferase activity was determined after 40 hours under non-stimulating conditions or after

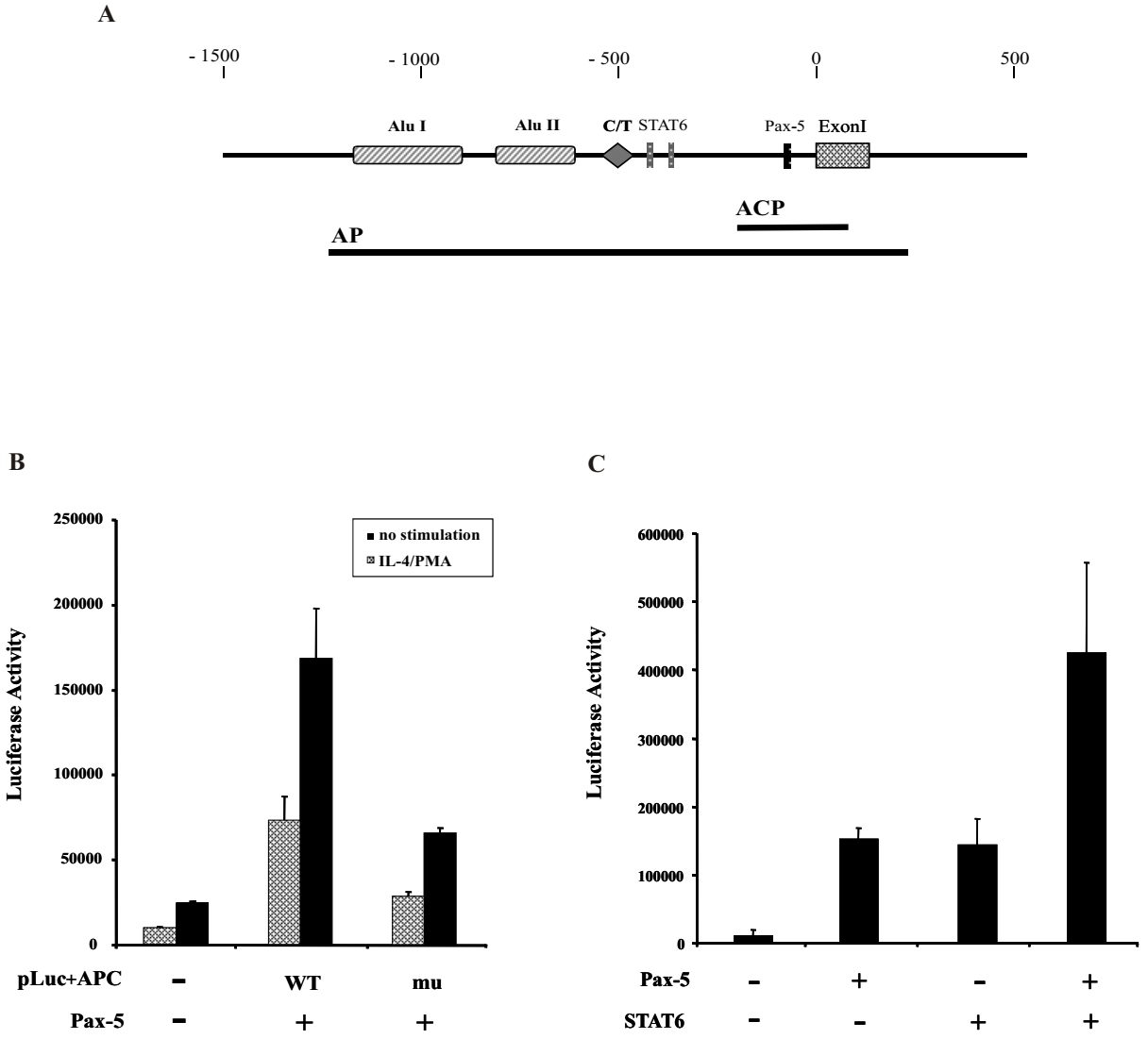


Fig.16 ***Pax-5 mediates the activation of the CD23a promoter.*** **(A)** Schematic representation of the reporter constructs used. **(B)** 293 cells were transfected with the vector containing the Cd23a core promoter (pLuc+ACP WT, positions -203 to +83) and the vector containing the CD23a core promoter in which the nucleotides of the putative Pax-5 binding site CD23-1 were mutated (pLuc+ACPmu). Where indicated cells were co-transfected with pcDNA3-Pax-5. Luciferase activity was assayed after 40 hours under nonstimulatory conditions or after stimulation with IL-4 (50ng/ml) and PMA(3ng/ml). The average luciferase value of 3 independent experiments is shown. Error bars indicate st andard deviation of the mean. **(C)** 293 cells were transfected with the vector containing the CD23a promoter (pLuc+AP, positions -1216 to +211) together with pcDNA3-Pax-5 and pXM-STAT6 as indicated. Luciferase activity was assayed after 40h.The average luciferase value of three independent experiments is shown. Error bars indicate standard deviation of the mean.

stimulation with PMA and IL-4. As shown in Fig. 16B, Pax-5 activates the CD23a core promoter (pLuc+ACP) about 7 fold when compared with the activity of the promoter without Pax-5. This is in line with our hypothesis that the CD23-1 binding site behaves as a functional Pax-5 site.

By site directed mutagenesis, we have mutated the CD23-1 putative binding site within the pLuc+ACP vector. The mutant construct, named pLuc+ACPmu contains the same nucleotide substitutions as the oligonucleotide CD23-1mu1 (Fig. 14A). The pLuc+ACPmu construct containing the CD23a core promoter with the mutated Pax-5 binding site failed to activate as strongly as the pLuc+ACP contruct containing the wild-type promoter. The remaining activation may be due to the recruitment of Pax-5 into protein complexes that can bind with lower efficiency but can still lead to a slight activation of the promoter.

A recent study [21] identified IL-4 as the main activator of the CD23a promoter, whereas the CD23b promoter shows a much wider range of responsiveness to extracellular stimuli. The authors described two STAT6 binding sites located close to each other in the –500 to -350 region of the CD23a promoter. Therefore we investigated if Pax-5, besides controlling the specificity of CD23a expression in B-cells, also cooperates with STAT6 in inducing a strong expression of the isoform.

For this purpose, another luciferase reporter construct (pLuc+AP) that includes these STAT6 binding sites was made by cloninig the whole CD23a promoter (from position –1216 to position +211) into the pLuc+ vector (Fig. 16A). pcDNA$_3$-Pax-5 and pXM-STAT6 were used for ectopic expression of Pax-5 and STAT6 respectively in 293 cells. As shown in Fig. 16B, Pax-5 and STAT6 each induced an 11-14-fold activation of the CD23a promoter respectively. When the cells are co-transfected with both transcription factors, however, a 40-fold activation of the CD23a promoter is induced. These results suggest that Pax-5 not only stimulates CD23a expression, but also cooperates with STAT6 in enhancing the level of CD23a transcription.

1.6 Pax-5 mediates CD23a expression *in vivo*

In order to further investigate whether the B-cell restricted expression pattern of CD23a is determined by lineage specific Pax-5 expression *in vivo* we used the monocytic cell line U-937 to determine the effects of ectopic Pax-5 expression on CD23a induction. U-937 cells, like other myeloid cells, regularly express only CD23b after appropriate stimulation with IL-4.

Human Pax-5 cDNA was stably inserted into the genome of U-937 cells by means of the recombinant retrovirus pEGZ [47]. The Pax-5 protein was coordinately expressed with the chimeric selection marker composed of the enhanced green fluorescent protein (EGFP) and the Zeocin (Zeo) resistance protein. We isolated a Pax-5 expressing population (U-937/EGZ Pax-5) using Zeo selection. As a control, we generated populations expressing only the selection marker at equivalent levels (U-937/EGZ). Western Blot analysis of protein extracts from Pax-5 transfected U-937 cells showed positive Pax-5 protein expression with a band located around 40 kDa (Fig. 17B). In addition we verified the correct transduction of the Pax-5 gene by sequencing. The full length Pax-5 gene was successfully integrated in the U-937 genome without deletions or point mutations (data not shown). The smaller size of the Pax-5 expressed in U-937 cells might be due to splicing differences.

Since available anti-CD23 antibodies are directed against the extracellular region of the receptor, which is shared between the two isoforms, the expression of CD23a cannot be analysed by FACS staining or Western Blot analyses. Therefore we performed RNase protection assays using a single RNA probe that is fully homologue to the CD23a mRNA and 2/3 homologue to CD23b mRNA. The analysis within one sample allows a reliable quantification of CD23a and CD23b isoforms on the transcriptional level.

Fig. 17A shows the results of the RNase protection assay performed with RNA extracts from U-937 cells, U-937 expressing only the selection marker EGZ and U-937 transfected with Pax-5. RNA extracts from WIL2 cells (B-cell line) were used as control for CD23a expression. CD23a/b isoforms expression was assessed under non-stimulating conditions, as well as after 48 hours stimulation with IL-4, PMA and IL-4+PMA.

As expected, IL-4 and PMA stimulations alone induce readily detectable amounts of CD23b mRNA in U-937, U-937/EGZ and U-937/ Pax-5 cells (Fig. 17A, lanes 2-3, 6-7 and 10-11). CD23b expression was further increased when cells were stimulated with a combination of the two stimuli (PMA and IL-4).

However, most importantly, U-937 cells transduced with Pax-5 exibited a strong and reproducible expression of CD23a mRNA when stimulated with PMA plus IL-4 (Fig. 17A, lane 12). IL-4 alone, as well as PMA stimulation alone were unable to induce CD23a expression in this monocytic cell line, providing evidence that other factors are required together with Pax-5. This experiment provides strong evidence that Pax-5 may be a limiting factor for enabling CD23a expression in vivo.

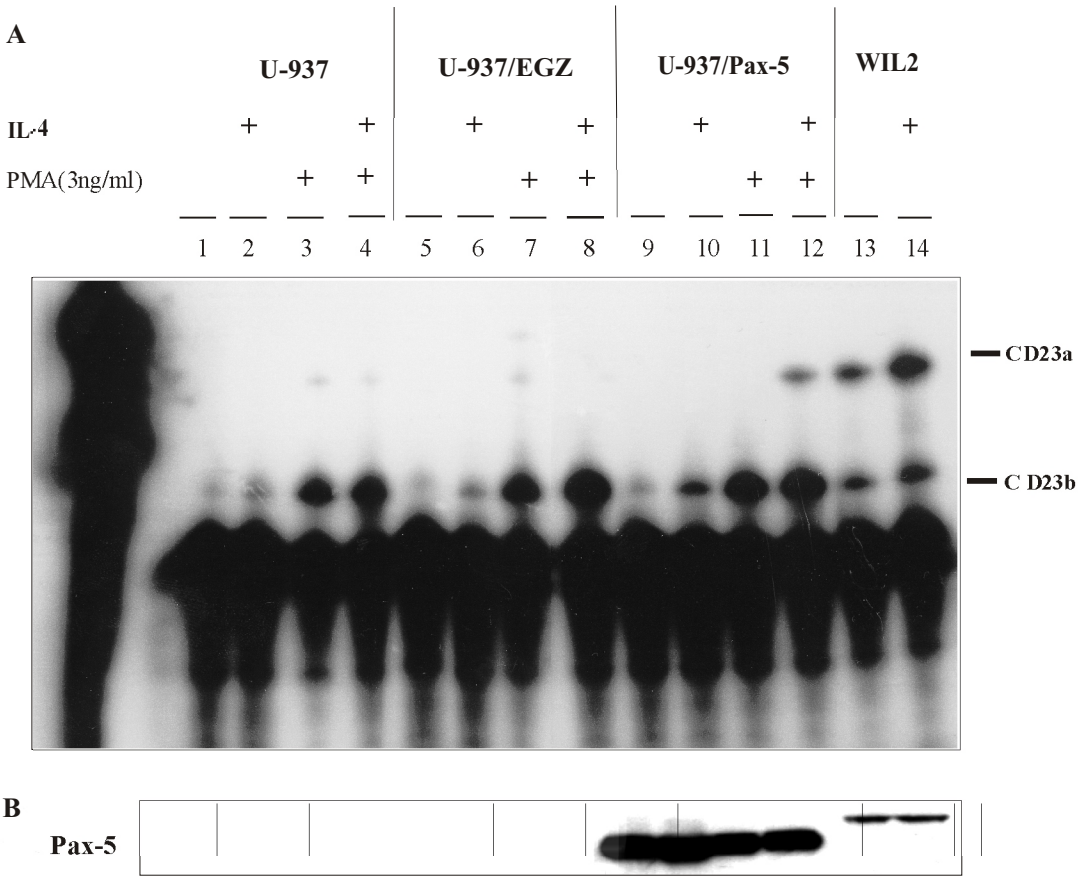


Fig.17 ***CD23a is expressed in U-937 cells transduced with Pax-5.*** **(A)** RNase protection assays were performed with RNA extracts from wild-type U-937 cells (lanes 1-4), U-937 transfected with the selection marker EGZ (lanes 5-8) and U-937 transfected with Pax-5 (lanes 9-12). Cells were stimulated with 50ng/ml IL-4 and 3ng/ml PMA for 48h as indicated. WIL2 cells (B cell line) express CD23a and CD23b constitutively and were used as controls (lanes 13-14). The extracted RNA was hybridized with a single CD23 mRNA probe for both CD23a and CD23b isoforms. **(B)** Western Blot analysis to assess Pax-5 expression.

2. Using a Two Hybrid System to find a cytoplasmic interaction partner for the CD23 receptor

The question of the transduction pathways involved in CD23 signalling is one of great importance since CD23 plays important roles in several diseases. No direct interaction partners for the cytoplasmic domain of membrane receptor CD23 have been found till now. We used a yeast two-hybrid system to address this question since it provides a powerful technique to screen large libraries of genes and identify new protein-protein interactions within the cell.

2.1 Establishing the system

The CytoTrap® Two Hybrid System (Stratagene) is based on generating fusion proteins whose interaction in the yeast cytoplasm induces cell growth by activating the Ras signaling pathway.

2.1.1 Bait constructs

pSos functions as bait vector by cloning the gene of interest -here CD23- in the MCS of the plasmid (Fig.18). 60-63 bp long synthetic oligonucleotides incoding for the 21-20 amino acids of the CD23a and CD23b intracytoplasmic tails were cloned in the MCS of the pSos vector. These constructs, named pSosCD23a and pSosCD23b, express CD23a and CD23b cytoplasmic domains as fusion proteins to the C-terminal part of human SOS protein.
pMyr functions as target vector. In order to screen for an interaction partner for CD23 a human spleen library cloned in the pMyr vector was purchased from Stratagene (Fig.18).

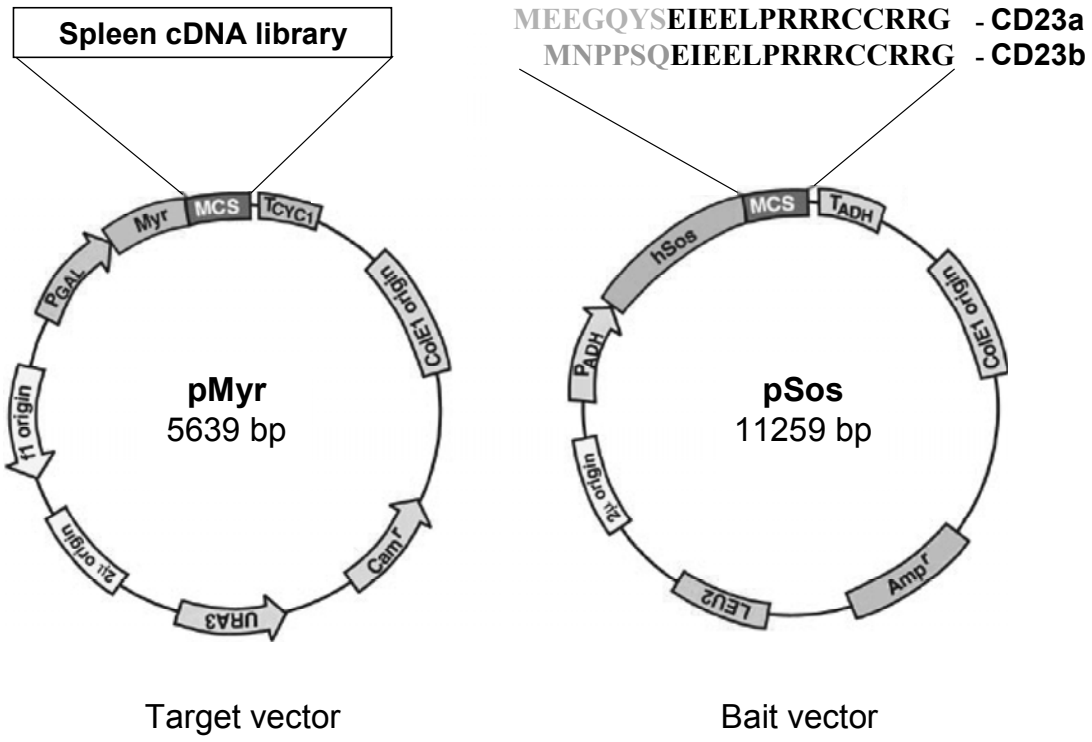


Fig.18 ***Schematic diagram of the bait and target constructs used in the CytoTrap Two Hybrid System***

2.1.2 Phenotype control

In order to verify the mutant phenotype, the yeast strain cdc25H was tested for its ability to grow on reach media (YPAD-Yeast extract, Peptone, Dextrose medium) and on dropout media, as presented in Table 1:

Table.1

Strain	**YPAD**		**Glucose(-Ura, -Leu)**		**Galactose(-Ura,-Leu)**	
	25°C	**37°C**	**25°C**	**37°C**	**25°C**	**37°C**
cdc25H	+	-	-	-	-	-

2.1.3 Control reactions

In order to test the reliability of the system, the pSos-bait constructs and the control plasmids were co-transformed in pairwise combinations into the cdc25H strain, plated on selective media and assayed for growth at 37°C and 25°C. The results are presented in the following table. Expression of the pMyr fusion protein is induced by adding galactose to the growth media and repressed by glucose.

Table.2

Control plasmids		SD(-Ura,-Leu)/25°C		SD(-Ura,-Leu)/37°C	
Sos fusion	**Myr fusion**	**Glucose**	**Galactose**	**Glucose**	**Galactose**
MAFB	MAFB	+	+	-	+
Collagenase	MAFB	+	+	-	-
MAFB	Lamin C	+	+	-	-
CD23a	Lamin C	+	+	-	-
CD23b	Lamin C	+	+	-	-

The transformation of pSosCD23a/b with pMyrLamC verifies that the constructs do not interact with the myristilation signal of the target fusion protein. The test of the control plasmids was permanently used during the screening and checking of the upcoming interactions as a control of the reliability of the system.

2.1.4 Establishing a high efficiency transformation protocol

Another important point to establish an efficient Two Hybrid System screening was to obtain a high efficiency of transformation in cdc25H yeast strain. Using the protocol described in Materials and Methods plasmid transformation in this yeast strain achieved efficiencies of 5000 to10 000 colonies per plate (150 mm diameter).

2.2 Screening results with pSosCD23a and pSosCD23b

The screening procedure is described in Material and Methods.

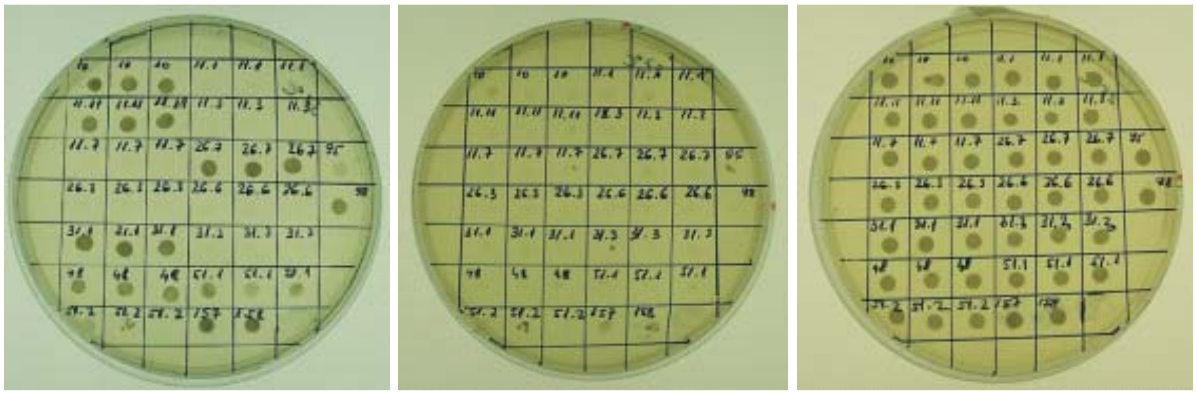
The two constructs described above (pSosCD23a and pSosCD23b) were used to screen the human spleen library cloned in the pMyr vector. The results are presented in Table 3.

Table.3

Two Hybrid System Screen	CD23a	CD23b
Number of clones screened	1,1 x 10^6	7 x 10^5
Number of clones growing on SD galactose (-Ura, -Leu) at 37°C (possible "temperature revertants")	305	518
Number of "putative positive" clones	17	34
Number of "true positive" clones	-	-

More than 1 million clones have been screened with the pSosCD23a construct and aproximatively 700 000 clones with the pSosCD23b construct. 305 and 518 clones for the CD23a or CD23b screening respectively were growing at 37°C on galactose medium after the replica plating step. Each of these colonies was individualy tested for the temperature revertant phenotype. Roughly 95 % of them have lost the point mutation and only ~5% of them hosted a real interaction. The 17 clones identified in the CD23a screening and the 34 clones identified in the CD23b screening were all tested in order to see if they are "true positive"- they host an interaction between a library protein and the bait (CD23), or they are "false positive" –they host an interaction between a library protein and the fusion protein (human SOS). In order to make the distinction, the library clones were retransformed in yeasts together with the pSos vector, a construct that only express the human SOS protein, without the CD23 bait (Fig.11). In all cases, the resulting colonies maintained the ability to grow on selective galactose media at 37°C. That showed that human SOS fusion protein, instead of the bait protein (CD23) was involved in all interactions. All clones resulted in the screening were considered "false positives".

False positive clones are an intrinsic of the system, as SOS is an adaptor molecule and it will interact with a large number of proteins encoded by the human spleen library.



A **B** **C**

SD **Galactose** (-Leu,-Ura) **37°C**

SD **Glucose**(-Leu,-Ura) **37°C**

SD **Glucose** (-Leu,-Ura) **25°C**

Fig.19 ***Selecting the „putative positive" clones***. **(A)** Growing the transformants at 37°C, on Galactose medium allows selection of clones that host an interaction. **(B)** Growing the transformants on Glucose medium at 37°C checks the „temperature revertant" phenotype. **(C)** Growing the transformants on Glucose medium at 25°C provides the back up for further analyses.

On the other hand, using SOS as a fusion protein could mask interactions between CD23 and i.e. small G proteins, because these could bind to SOS directly. As we had obtained quite a big number of false positive clones, we decided to check this hypothesis by using a two-hybrid system based on a different principle.

2.3 Using the MATCHMAKER GAL4 Two-Hybrid System to verify the screening results

This system is a GAL4- based two-hybrid that provides a transcriptional assay for detecting protein interactions *in vivo* in yeasts.

2.3.1 Establishing the system

a) Bait and target constructs

CD23 was cloned in the MCS of the pGBKT7 plasmid. 60-63 bp long synthetic oligonucleotides incoding for the 21-20 amino acids of the CD23a and CD23b intracytoplasmic tails were cloned between the NcoI and BamHI sites of the vector. The

constructs would express CD23a and CD23b cytoplasmic domains as fusion proteins with GAL4 DNA-BD.

Screening with the CytoTrap Two-Hybrid System resulted in a total of 17 clones when CD23a was used as bait and 34 clones when CD23b was used as bait. There was a high probability that certain genes of the library came up more than once in the screening process and that a certain number of duplicates would be found in the total of 51 clones.

In consequence, they were subjected to restriction enzyme analyses with HpaII, an enzyme that cuts CCGG motifs. The digest profile was used to compare and clasify the clones (Fig.20). The 51 clones resulted in 12 distinct profiles representing distinct genes. These genes were then cloned between the EcoRI and XhoI sites of the pGADT7 plasmid. The constructs, named pGADT7-1/12, would express them as fusion proteins with GAL4 DNA-AD.

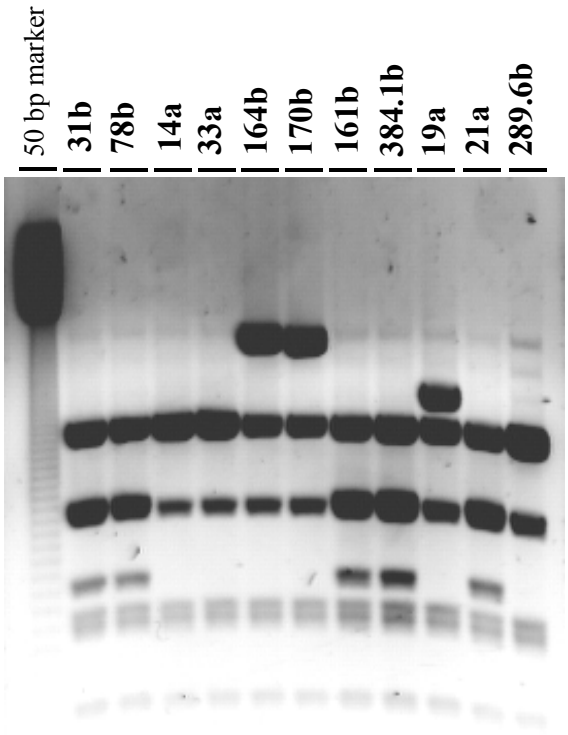


Fig.20 ***HpaII digest of false positive clones resulted from the Cytotrap Two Hybrid System screen***

b) Control of the yeast phenotype

In order to verify the phenotypes, the two yeast strains provided by the system were tested on dropout media as follows.

Table.4

Strain	SD/-Ade	SD/-Met	SD/-Trp	SD/-Leu	SD/-His	SD/-Ura	YPDA
AH109	-	+	-	-	-	-	+
Y187	-	-	-	-	-	-	+

c) Control reactions

In order to test the general reliability of the system and if DNA-BD and AD fusion constructs do not autonomously activate reporter genes, the pGBKT7-CD23a/b constructs, pGADT7-1/12 and the control plasmids were co-transformed in various combinations into the AH109 strain, plated on selective media and assayed for growth and blue/white phenotypes. The results are presented in the Table 5.

These results also show how one can modulate the stringency of the screening by plating the transformants on different selective media. The use of high-stringency media, SD/-Ade/-His-Leu/-Trp/X-α-Gal, virtually eliminates false positive interaction. The use of low-stringency media, SD/-Leu/-Trp, favours the detection of weak or transient interactions.

Also, the independent transformation of pGBKT7-CD23a/b and pGADT7-1/12 into AH109 verifies that the constructs do not activate reporter genes by themselves.

Table.5

Vectors	SD Minimal Medium	Phenotype	
		Mel1/lacZ	**His/Ade**
pCL1	-Leu/X-α-Gal	Blue	+
pGADT7	-Leu	White	+
pGBKT7-53	-Trp	White	+
pGADT7 + pGBKT7-53	-Leu/-Trp	White	+
	-Leu/-Trp/ X-α-Gal	Blue	+
	-Ade/-His-Leu/-Trp	White	+
	-Ade/-His-Leu/-Trp/X-α-Gal	Blue	+
pGADT7	-Leu	White	+
pGBKT7-Lam	-Trp	White	+
pGADT7 + pGBKT7-Lam	-Leu/-Trp	White	+
	-Leu/-Trp/ X-α-Gal	White	+
	-Ade/-His-Leu/-Trp		-
	-Ade/-His-Leu/-Trp/X-α-Gal		-
pGBKT7-CD23a/b	-Trp	White	+
	-Trp/ X-α-Gal	White	+
pGADT7-1/12	-Leu	White	+
	-Leu/ X-α-Gal	White	+

2.3.2 Results of the testing

Finally, pGBKT7-CD23a and pGBKT7-CD23b were separately cotransformed with each of the pGADT7-1 to pGADT7-12 constructs. In order to detect a plausible interaction, medium and low-stringency media were used. The results are presented in the following table.

Table.6

Vectors	SD Minimal Medium	Phenotype	
		Mel1/lacZ	His/Ade
pGBKT7-CD23a + pGADT7-1/12	-Leu/-Trp	White	+
	-Leu/-Trp/ X-α-Gal	White	+
	-His/-Leu/-Trp		-
	-His/-Leu/-Trp/ X-α-Gal		-
pGBKT7-CD23b + pGADT7-1/12	-Leu/-Trp	White	+
	-Leu/-Trp/ X-α-Gal	White	+
	-His/-Leu/-Trp		-
	-His/-Leu/-Trp/ X-α-Gal		-

Testing the interaction into the MATCHMAKER system verified that there was no true interaction between CD23 cytoplasmic tail and the clones resulted in the CytoTrap screening.

2.4 New constructs for the CytoTrap Two Hybrid System bait vectors

The fact that CD23 was cloned in the MCS of the pSos vector caused the intracytoplasmic part of CD23 to be expressed at the carboxyterminal end of SOS, implying also that the free end of CD23 was the C-terminal end. However, CD23 is a type II receptor, with the N-terminal end into the cell.

In order to change the orientation of the bait in the fusion protein we decided to clone CD23a/b upstream of the hSOS gene. This new constructs would express the bait at the N terminal end of human SOS protein implying the same orientation for the cytoplasmic tails of CD23a and CD23b as it occurs *in vivo* (Fig.21).

In addition, there is also a considerable difference in size between the human SOS proteins, which is a multidomain adaptor protein of 170 kDa and the short cytoplasmic tail of CD23, which has a total of 20-21 amino acids. In order to make this small amino acid tail more obvious into the fusion protein, we considered using a linker region. A 15 amino acids long synthetic hinge region containing a glycine and serine repetitive motif was used as linker between CD23a/b and the SOS protein. The amino acid sequence of this flexible oligopeptide is presented in the Fig. 21B.

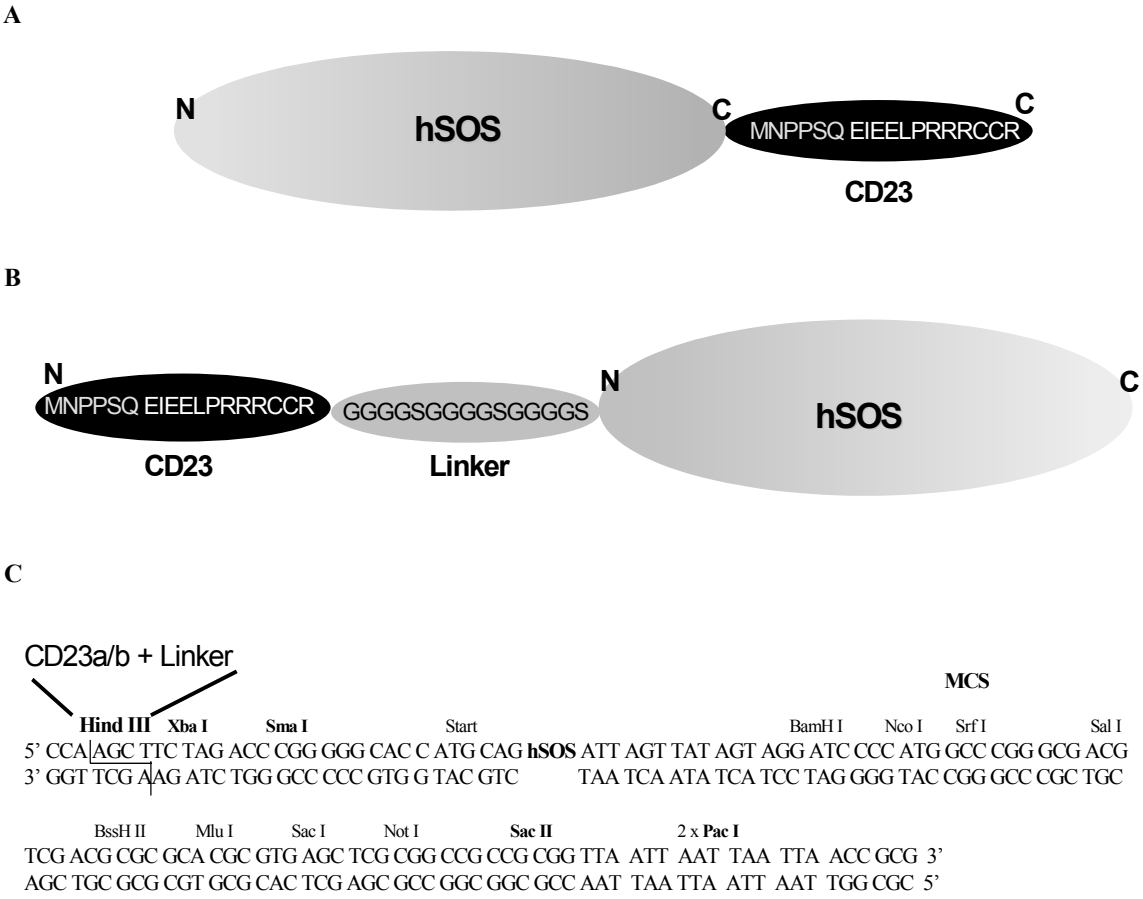


Fig.21 ***Schematic representation of the CD23-SOS fusion proteins expressed as bait in the CytoTrap Two Hybrid System.*** **(A)** The pSosCD23 construct expresses the cytoplasmic domain of CD23 receptor at the C terminal end of human SOS. **(B)** The pSosCD23+Linker construct expresses the CD23 cytoplasmic domain at the N terminal end of human SOS. A synthetic linker region is interposed between the two proteins. **(C)** In the pSosCD23+Linker construct the DNA encoding the cytoplasmic domain of CD23 and a linker region were cloned in a unique HindIII site located upstream of the hSOS gene.

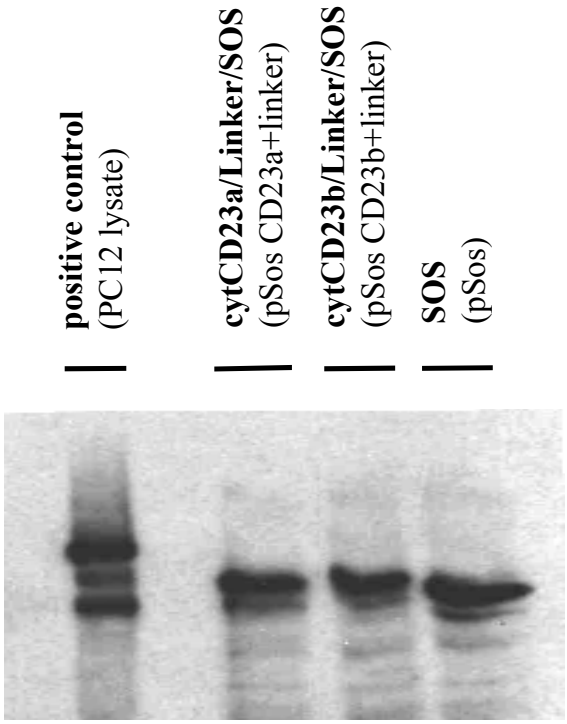


Fig.22 ***CD23-SOS fusion protein is expressed in the yeast cytoplasm.*** Protein extracts from yeasts transformed with pSos, pSosCD23a+Linker and pSosCD23b+Linker constructs were analysed by Western Blot using an anti-hSOS antibody.

Synthetic oligonucleotides representing the cytoplasmic tails of the two CD23 isoforms and the linker region were cloned into a Hind III unique restriction site situated 20 bases upstream of the starting point of SOS. The oligonucleotides were designed to contain a new START codon, a Kozak sequence in front of the START codon and restriction enzyme sites that would allow cloning without causing a frameshift in the hSOS gene located now downstream of the bait gene. The new constructs were named pSosCD23a+Linker and pSosCD23b+Linker.

In order to verify the expression of the fusion protein in the yeast cytoplasm, Western Blot analyses were performed using yeast lysates. As none of the available anti-CD23 antibodies work in Western Blot analyses, we used an anti-human SOS antibody to assess the expression of the fusion protein. As shown in Fig. 22, the CD23-linker-human SOS fusion protein was expressed in the yeast cytoplasm.

2.5 Screening results with pSosCD23a+Linker and pSosCD23b+Linker

These new bait constructs (pSosCD23a+Linker and pSosCD23b+Linker) were used to screen again the human spleen library cloned in the pMyr vector. The results are presented in Table 7.

Table.7

Two Hybrid System Screen	**CD23a**	**CD23b**
Number of clones screened	$5{,}8 \times 10^5$	$3{,}5 \times 10^5$
Number of clones growing on SD galactose (-Ura, -Leu) at 37°C (possible "temperature revertants")	344	464
Number of "putative positive" clones	14	13
Number of "true positive" clones	-	-

Around half a million clones have been screened with each of the pSosCD23a+Linker and pSosCD23b+Linker constructs. 278 and 391 clones for the CD23a or CD23b screening respectively were growing at 37°C on galactose medium after replica plating step. Each of these colonies was individually tested for the temperature revertant phenotype. Roughly 95-97 % of them have lost the point mutation and only ~5% of them hosted a real interaction. However, the 27 clones identified for CD23a and CD23b screening were all "false positive" since they interact with human SOS.

2.6 New construct for the CytoTrap Two Hybrid System CD23a bait vector

One known problem of yeast two hybrid systems for finding protein-protein interactions is that, if these interactions require phosphorylation in mammalian cells, they will not be detected. Yeast hosts might not express the kinases or they might not have the same phosphorylation patterns as the mammalian kinases.

The tyrosine residue in position 6 of the CD23a cytoplasmic tail could be a key player in recruiting other signalling molecules. The lack of phosphorylation at this site could have been a major problem for detecting an interaction. In consequence, we considered replacing the tyrosine residue with a glutamic acid residue. This strategy can be used in experimental settings when tyrosine phosphorylation cannot be achieved, as glutamic acid can mimic phosphorylated tyrosine in certain situations.

By site directed mutagenesis, using pSosCD23a+Linker as template, we constructed a new bait vector, named pSosCD23a-Glu. This new construct also contains the CD23a cytoplasmic tail expressed at the N-terminal part of human SOS and the linker between the two fusion proteins. In addition, the tyrosine from position 6 of the cytoplasmic tail of CD23a was replaced with a glutamic acid residue.

The pSosCD23a-Glu will be used in future screenings.

2.7 Verifying interactions between two known proteins

The CytoTrap Two Hybrid System can also be used to verify interactions between two known proteins. p59fyn (a member of the src family of protein kinases) was claimed to associate with the product of a transfected CD23 gene in a NK cell line.

In order to verify if this interaction can be detected and reproduced in the CytoTrap two-hybrid system, fyn cDNA amplified by RT-PCR from B-cell total RNA was cloned in the pMyr vector. pSosCD23a+Linker, pSosCD23b+Linker and pSosCD23a-Glu were co-transformed with pMyrfyn. There was no detectable interaction between the two proteins in the environment provided by the CytoTrap two-hybrid system (Table 8).

Table.8

Plasmids		**SD(-Ura,-Leu)/25°C**		**SD(-Ura,-Leu)/37°C**	
pSos	**pMyr**	**Glucose**	**Galactose**	**Glucose**	**Galactose**
Coll	fyn	+	+	-	-
CD23a+Linker	fyn	+	+	-	-
CD23b+Linker	fyn	+	+	-	-
CD23a-Glu	fyn	+	+	-	-

2.8 Perspectives

pSosCD23a-Glu represents a bait construct substantially improved. It has the advantage to express CD23 cytoplasmic tail as it occurs *in vivo*, with the N-terminus free as bait and contains a glutamic acid residue that can substitute a phosphorylated tyrosine in position 6. Using this construct for screening an improved potential to find an interaction partner for the CD23 receptor is to be expected.

DISCUSSION

High CD23 expression is associated with various chronic diseases such as B-CLL, rheumatoid arthritis and lupus erythematosus. It is assumed that signalling through CD23 contributes to the pathogenesis of these diseases. In humans there are two CD23 isoforms that are differentially expressed. Whereas CD23b is widely detected on lymphocytes and myeloid cells, CD23a is restricted to B-lymphocytes. The isoforms differ only in the short amino terminal intracytoplasmic part [87] and seem to be connected to different signal transduction pathways. The CD23a isoform specifically mediates endocytosis of bound ligands and can therefore influence B-cell mediated antigen presentation [41]. CD23b was shown to be associated with phagocytosis of IgE coated-particles [88].

Distinct promoters regulate the two CD23 isoforms expressed in humans. So far, IL-4 has been described as being the most important activator of CD23a and CD23b expression and binding sites for STAT6 have been characterized in both promoters. [21,41,87]. In addition to the IL-4 responsive element, NF-AT binding sites have been characterized in the CD23b promoter [40]. There are very limited data concerning the regulation of the human CD23a promoter. Studies of the mouse CD23 promoter, which is closely related in sequence to the human CD23a promoter, revealed functional STAT6, NF-kB and C/EBP binding sites [82]. Since the mouse and human CD23a promoter are 72% homologous in the core region, most of the binding motifs in the human promoter were characterized by comparison with the mouse promoter. Richards *et al* [65] observed that the homologous nucleotides appear in clusters, suggesting that common response elements have been conserved in the two promoters. As shown in Fig. 10A, STAT6, NF-kB and C/EBP binding sites are localized in the –70 to –140 region of the CD23a core promoter. Additional STAT6 binding sites were characterized in the extended CD23a promoter, in the –300 to –500 region. Multimerized STAT6 binding sites, as well as their association with NF-kB sites, seem to be a common trait of IL-4 regulated promoters [14, 54]. In addition, EBNA2 and Notch-2 can regulate the CD23a promoter by binding to CBF1 sites [32,48]. Both activatory elements may play a role in the immortalisation of the B-cell by enhancing CD23a expression.

The question of how the B-cell specific expression of CD23a isoform is regulated has not been experimentally addressed so far. Among B-cell specific transcription factors, Pax-5/BSAP binding sites have been very recently predicted in the CD23a core promoter [21,32]. Pax-5 is a critical modulator of early B-cell differentiation. Its expression is restricted to B cells, embryonic brain and testis. Binding sites for Pax-5 have been identified in promoters of several genes. While being a positive regulator of CD19, mb-1 and RAG-2 [39,43,59] Pax-5 acts as a repressor for the immunoglobulin heavy-chain 3'C∝ enhancer and the J-chain [66,75]. The importance of Pax-5 for development was demonstrated by knock-out experiments. Pax-5 is important in early and late B-cell development. Most of Pax-$5^{-/-}$ mice die within 3 weeks and B-cell development is blocked at the pro-B-cell stage [84]. The loss of Pax-5 in mature B-cells severely impairs B-cell identity [31].

Here we demonstrate the presence of a functional Pax-5 binding site in the CD23a core promoter that is able to induce and enhance CD23a expression. We also advance the hypothesis that Pax-5, being a B-cell specific protein, plays a central role in regulating the specific expression of CD23a on B-cells. CD23 is expressed later in the B-cell development than Pax-5. Additional transcription factors must be involved in modulating CD23a expression. The association of Pax-5 with other transcription factors in regulatory complexes has been documented so far. For example, Pax-5 associates with Oct-1 (octamer binding proteins) and NF-kB in modulating the 3'α-hs4, a distal 3' enhancer that regulates the IgH gene cluster at multiple stages of B-cell development. While Oct-1 and kB act as positive regulators at all stages of differentiation, Pax-5 acts as a repressor at the pre-B-cell stage and as a positive regulator in mature B-cells [52, 53]. Furthermore, both CD23 and Pax-5 expression are lost when differentiation to plasma cells occurs. This is in line with Pax-5 being a prerequisite factor for CD23a expression.

Another interesting observation comes from the comparison of the CD23 promoter with the germline immunoglobulin Cε and Cγ1 promoters, which are also regulated by IL-4. The transcription of these germline immunoglobulin genes is necessary for class switching to IgE and IgG1. Different combinations of cytokines and mitogens have been shown to stimulate class switching to particular isotypes. IL-4 promotes a class switch from IgM to IgE and IgG1 and STAT6 binding sites have been identified in both ε and γ1 germline genes. In addition, NF-kB and C/EBP binding sites have been described [14], while Pax-5 was shown to bind close to the STAT6 site in the germline ε promoter. Studies by Liao *et al.* show that Pax-5 is essential for germline ε transcription and therefore essential for class switching and

IgE production [46]. Thus, it seems that an IL-4 response in B-cells involves the cooperation of at least four transcription factors: C/EBP, STAT6, NF-kB and Pax-5. A high degree of sequence homology exists between the CD23 and germline ε promoters in both human and mouse [65]. This high homology in the promoter sequences is also reflected by the fact that both CD23 and IgE are upregulated by IL-4 in combination with LPS or anti-CD40. Since C/EBP, STAT6, NF-kB binding sites have been described in the CD23 promoter [82, 65], we were interested to verify if CD23a, as an IL-4 responsive promoter, is Pax-5 sensitive.

The identification of putative binding sites for Pax-5 in the CD23a core promoter was initially done by sequence alignment with the Pax-5 consensus sequence. This consensus motif has been characterized by Czerny *et al*. in an extended comparison between natural targets of Pax-5, including the recognition sequence which originates from the sea urchin H2A-2.2 gene and the human CD19 gene [12]. As seen in Fig. 1B, the Pax-5 recognition sequence is divided in two halves – a more extensive 3'-consensus motif recognized by the amino-terminal subdomain of the paired domain and a 5'-consensus motif recognized by the carboxy terminal part of the paired domain. One important observation was that all naturally occurring binding sites identified so far deviate from the consensus sequence and that Pax-5 is able to interact with a panel of seemingly degenerate recognition sequences. In testing the Pax-5 binding sites, we considered all putative binding sites with at least 50% identity to the consensus sequence. This resulted in three putative Pax-5 binding sites, which we designated as CD23-1, CD23-2 and CD23-3.

The putative binding site CD23-1 was the only one able to compete the high affinity H2A-2.2 binding site and to directly bind the Pax-5 protein (Fig. 11). This was further confirmed by the fact that oligonucleotides containing a mutated CD23-1 binding site completely lost the ability to bind the Pax-5 protein (Fig. 12). Interestingly, a gap had to be introduced in the CD23-1 binding site in order to reconstitute the symmetry of the site (Fig. 10B). Another putative binding site (CD23-3), which had 89% homology to the consensus sequence failed to bind Pax-5 in the same assays (Fig. 11). These observations confirm the difficulty to predict Pax-5 binding sites using sequence comparison alone. It appears that all naturally occurring binding sites identified so far deviate from the consensus sequence by base changes in their 5' or 3' half sites. In consequence, Pax-5 protein possesses a high flexibility to recognize quite degenerate recognition sequences as a result of the bipartite structure of the paired domain. Furthermore, binding sites with a complete match to the consensus

sequence would possess an exceptionally high affinity for Pax-5, which apparently is not required *in vivo*.

Another approach to identify Pax-5 binding sites in the CD23a core promoter was to construct overlapping oligonucleotides covering the whole length of the promoter and to test their ability to compete the sea urchin H2A-2.2 binding site in EMSAs. Both strategies revealed CD23-1 as the only site in the CD23a core promoter able to bind directly the Pax-5 protein (Fig. 13).

It is interesting to note that in our hands none of the two Pax-5 sites predicted in a recent study by Hubmann et al. [32] interacts directly with the Pax-5 protein. As this study used nuclear extract from B-CLL cells, it is possible that Pax-5 was recruited by another factor into a protein complex, which interacts with these putative sites.

To test the function of the identified Pax-5 binding site in vitro we performed luciferase assays in 293 cells linking the CD23a core promoter or an extended CD23a promoter to a luciferase reporter gene. We chose this approach since previous work has shown that promoters of Pax-5 regulated genes, like CD19, are weakly active in transiently transfected B-cells and can only be stimulated by ectopic Pax-5 expression in heterologous cell types [9]. Overexpression of Pax-5 in 293 cells led to a 7-10 fold activation of both CD23a promoter constructs (Fig. 14). However, reporter constructs with the mutated CD23-1 site were still slightly active. The remaining activation may be due to the recruitment of Pax-5 into protein complexes that can bind with lower efficiency but can still lead to a slight activation of the promoter. This may be indeed another mechanism by which Pax-5 regulates the CD23a promoter and which needs to be approached in future studies. Furthermore, in experiments using the extended promoter which includes two reported STAT6 sites, Pax-5 acted together with STAT6 in further enhancing CD23a expression above the level of the induction provided by the two factors alone. These results suggest that Pax-5 not only determines the B-cell specificity of CD23a, but also potentiates the STAT6 mediated stimulatory effect.

Previous studies seem to lead to the scenario that STAT6, even when multimerized, is not sufficient for the induction of IL-4 regulated genes [14, 54]. The cooperation of other transcriptional activators, like NF-kB, appears to be necessary, for example, in order to activate the transcription of the germline Cε promoter. The interaction with NF-kB appears to enhance the DNA binding affinity and the transactivating affinity of STAT6 [74]. It may also be possible that Pax-5 synergically acts and perhaps also directly associates with STAT6 (and NF-kB) in order to activate the CD23a promoter.

To further investigate the ability of Pax-5 to induce CD23a expression in vivo, we selected the monocytic cell line U-937, which normally expresses only CD23b after appropriate stimulation [87]. Using a retroviral infection system we ectopically expressed Pax-5 in U-937 cells. Western Blot analyses demonstrated a strong expression of Pax-5 with a band located around 40 kDa (Fig. 15B). The B cell line WIL2 used as a CD23a positive control showed a Pax-5 band located around 45 kDa, somewhat lower than the expected 53kDa. As sequence analyses of the integrated Pax-5 construct revealed no partial deletions or point mutations creating a STOP codon we suppose that the size difference is due to splicing differences or post-translational modifications. Zwollo *et al.* described several Pax-5 isoforms expressed in different pro-, pre- and mature B-cell lines. They are generated by the usage of a second distal start codon located downstream of exon 1 or by differential splicing. The expression of these isoforms seems to vary during B-cell development whereas the standard Pax-5 isoform is stably and relatively highly expressed at all B-cell stages. Their function is not yet characterized, but they seem to retain their capacity to interact with Pax-5 binding sites or to interact with other regulatory factors necessary for initiation of transcription [90].

The expression of Pax-5 in the U-937 cell line enabled a clear CD23a expression after appropriate stimulation with IL-4 and PMA. In comparison to B-cells, where the ratio of CD23a: CD23b expression is around 3-4:1 independent of the applied stimulus [25], CD23b exceeds CD23a expression in U-937/Pax-5 cells. This is likely due to the difference in the cellular environment between B-cells and macrophages. The U-937 cells have the phenotype of a mature, fully differentiated macrophage. One can expect different conditions compared to a B-cell, like the availability of the promoter or the presence of specific transcription factors. In any case, the CD23a induction in Pax-5 transduced U-937 cells was consistent and highly reproducible. Neither IL-4 nor PMA stimulation alone were able to induce CD23a expression in Pax-5 transfected U-937 cells. This would indicate IL-4 as being necessary but not sufficient for Pax-5 mediated expression of the CD23a isoform. Pax-5 seems to be a prerequisite transcriptional activator, which cooperates with other transcription factors during B-cells specific expression of the CD23a isoform.

In conclusion, we identified a functional Pax-5 binding site within the CD23a core promoter. In combination with other transcription factors, Pax-5 is able to mediate CD23a expression even in cells that normally do not express CD23a. Therefore our results indicate Pax-5 being a key regulator of B-cell specific expression of CD23a.

The question of the transduction pathways involved in CD23 signaling is one of great interest as CD23 plays important roles in several diseases. In B-CLL, for example, CD23a expression is associated with a state of cell survival. In contrast to normal B-cells where after 24 hours of incubation CD23a transcription is completely lost, accompanied also by a down-modulation of the antiapoptotic gene bcl-2, the levels of CD23a and bcl-2 expression remain high in B-CLL cells [32]. Overexpression of CD23a is associated with enhanced cell viability.

The two isoforms have different expression patterns and apparently quite distinct functions. Comparison between the events taking place in cells that express only one or both isoforms seem also to lead to the idea of different signal transduction pathways for CD23a and CD23b. Studies done by Kolb and co-workers show that cross-linking of CD23 on B-cells provokes a rapid increase in Ca^{2+} resulted from inositol (1,4,5) triphosphate generation and a slow accumulation of cAMP. Ligation of CD23 on the surface of monocytes results in cAMP generation and is additionally coupled with activation of iNOS. The difference is most likely due to the 6-7 amino acids at the N-terminal end of the cytoplasmic tail.

CD23a only contains a Tyr residue in position 6 and a Ser residue in position 7 that are predicted sites for phosphorylation. The tyrosine in CD23a cytoplasmic tail exists as a member of the YSEI sequence. The YXXL sequence is known as an ITIM motif, and if Leu can substitute for Ile, than theYSEI in the human CD23a could be seen as a variant of an ITIM motif [36]. As the suppression of IgE production was released in CD23-knockout mice, it would be reasonable to suppose that CD23 contains an inhibitory signal within the molecule. The meaning of this sequence remains unclear, although it may provide a base for the difference in signalling between the two isoforms.

Our approach to address the question of the transduction pathways involved was to look for direct interaction partners for CD23. Preliminary data suggested that a pertussis toxin-insensitive G protein couples membrane CD23 to the PLC. It is also possible that a protein tyrosine kinase is involved, since the product of the transfected CD23 gene in a NK cell line was found associated with p59fyn [78].

We used a yeast two-hybrid system to look for interaction partners for the CD23 receptor. These systems provide a powerful technique to screen large libraries of genes and to identify new protein-protein interactions within the cell. When compared with more traditional methods for studying protein-protein interactions, the two-hybrid system seems to offer several advantages. As the interaction occurs *in vivo,* the need for the detailed and laborious manipulation of conditions necessary for *in vitro* biochemical binding assays is abrogated.

Also, in at least some cases, the two-hybrid system is more sensitive than co-immunoprecipitation in detecting weak interactions. The system we employed –the CytoTrap® Two Hybrid System from Stratagene- offers additional advantages over the traditional two hybrid systems. As the interaction takes place in the cytoplasm, it does not involve protein transport to the nucleus. It also provides a better control of the activation.

There are, of course, several limitation and disadvantages, which are common to all yeast two-hybrids: some hybrid proteins may not be stably expressed in yeast or bacteria; fusion proteins may occlude the normal site of interaction or impair the proper folding; conditions in yeast may not allow the proper folding or posttranscriptional modifications.

One problem that is commonly encountered with library screens is achieving optimal transformation efficiency. Most protocols are a variation of standard lithium acetate transformation protocols. After testing several of these protocols we achieved good co-transformation efficiencies allowing us to screen approximately 3 million colonies. The quality of the carrier DNA and the freshness of the plated yeast streak are two critical factors that influence greatly the efficiency of the transformation.

Another major problem with two-hybrid screens is the appearance of false-positive clones. In the classic two-hybrid system, they are inherent in any transcriptional readout. In the CytoTrap system, the human SOS, as an adaptor protein, will interact with a number of other proteins. Any candidates from the primary screen must be retested in several configurations before they can be considered 'true' positives. First, the library plasmid must be separated from the bait plasmid. This is usually done by recovering the plasmid from yeast and transforming into bacteria, which are then grown on medium containing the same antibiotic for which the plasmid encoding the candidate gene contains a resistance gene. Once a plasmid has been recovered, it should be tested in the absence of the bait. Normally, a false positive will produce an interaction with the human SOS protein alone. The collagenase I expressed by the pSos Coll negative control plasmid can also be a useful control, as this protein does not generate interactions with most of the proteins.

As we obtained quite a high number of false positives, we also considered the possibility that SOS would mask an interaction between the bait (CD23) and a potential target. If the interaction partner would be a small G protein, for example, SOS could also interact with it in the absence of the bait. We tested some of the clones we obtained in the screening with CytoTrap system in another two-hybrid, the MATCHMAKER GAL4 (Clontech), which is

based on the classical priciple of the transcriptional activation. This additional control proved them to be false positive indeed.

In the case of our particular bait construct, a limiting factor for finding an interaction partner could be the size of the CD23 intracytoplasmic tail. The principle of the Cytotrap system is that, when bait and target interact, the SOS molecule is recruited to the membrane where it can activate Ras. Since CD23 is a transmembrane receptor we could only use the cytoplasmic domain as bait. Cloning the transmembrane region into the pSos vector would permanently anchor SOS to the membrane. The cytoplasmic tail of CD23 has only 21 amino acids for CD23a and 20 amino acids for CD23b. Nevertheless, successful screening with baits as small as 22 aminoacids have been previously described [50].

Another limiting factor for a successful screening could be the big size (170 kDa) and the tridimensional conformation of the human SOS. In order to partially overcome this problem we introduced a linker region between the bait and the hSOS in the second bait construct. This linker, represented by a 15 amino acids long synthetic hinge region, was designed to make the bait more obvious in the context of the fusion protein.

The expression of the fusion proteins between human SOS and CD23 was assessed using an anti-SOS antibody, as available anti-CD23 antibodies against the cytoplasmic tail of CD23 do not work in Western Blot. Two different bait constructs (pSosCD23a/b and pSosCD23a/b+Linker) were used for screening. In the first construct the cytoplasmic tail of CD23 was cloned into the MCS of the pSos vector, which expressed it at the C-terminal end of the human SOS. In the second construct the cytoplasmic tail was cloned upstream of the human SOS gene (with this construct we intended to create a fusion protein in which the free end of the bait to be the N-terminal region of CD23, as it occurs *in vivo*). In order to insure the expression of this fusion protein, we created a new START codon with a Kozak sequence situated upstream. Both fusion proteins were successfully expressed in the yeast cytoplasm, as assessed by Western Blot (Fig.22).

The CD23 receptor is expressed as a trimer on the surface of the cells. This could also raise potential problems since the fusion protein expresses only one copy of the CD23 cytoplasmic domain. A previous study involving chimeric proteins between the CD23 and CD69 molecules indicate that this is not the case [68]. Monomeric chimeras containing the cytoplasmic domain of CD23 fused with the extracellular domain of CD69 could efficiently transduce signals into the cell and provoke Ca^{2+} mobilization. The described chimeras demonstrated the modular nature of the molecule, with the ligand-binding and signal transduction domains independent of each other. The experiment also confirmed that the

cytoplasmic domain is fully responsible for signal transduction. On the other hand, oligomerization seems to be quite an important factor for ligand binding. CD23 affinity for IgE decreases in monomeric CD23 or in the case of small soluble CD23 fragments, which don't retain the helical coiled coil [38].

Another way in which a two-hybrid system can be used is to test an interaction between two known proteins. The analysis of the amino acid sequence in the cytoplasmic tail of CD23 and previous experiments pointed out several potential targets for this membrane receptor. As mentioned already, the product of a transfected CD23 gene in a NK cell line was found associated with fyn, a member of the src-family of protein kinases [78]. Also, if the tyrosine residue in the CD23a isoform is part of a functional ITIM motif, phosphatases like SHP and SHIP could be recuited through their SH2 domains. The gene for fyn has been cloned as cDNA into the pMyr vector and the ability to interact with CD23a and CD23b was tested in the system. Similar experiments were performed for SHP-1 and SHP-2 (results not shown). Anyway, as all these interaction would normally involve the phosphorylation of the tyrosine residue, yeast two-hybrid may not be the appropriate system to assess them. Yeast hosts might not express the kinases or they might not have the same phosphorylation patterns as the mammalian kinases. To overcome this problem one can, for example, express the required kinases in the yeast cytoplasm.

Another approach to overcome this problem would be to replace the tyrosine residue with a glutamic acid residue. This strategy can be used in experimental settings when tyrosine phosphorylation cannot be achieved. By site directed mutagenesis, we constructed a new bait vector, named pSosCD23a-Glu, in which the tyrosine from position 6 of the cytoplasmic tail of CD23a was replaced with a glutamic acid residue. We used this new construct in order to test the interaction between CD23a and fyn, SHP-1 and SHP-2. With this construct also no interaction was found. We also plan to use this new bait construct for future screening of the spleen library.

In conclusion, we used a yeast two-hybrid system in order to identify a direct interaction partner for CD23. This involved first the direct testing of interactions between the bait and possible or predicted interaction partners and secondly the intensive screening of a spleen library using different bait constructs. Other experimental approaches – like immunoprecipitation assays- were also tested (results not shown). These strategies did not result in the identification of an interaction partner for CD23 up to now.

To summarize, we demonstrate that Pax-5 regulates the CD23a promoter, proving to be a key regulator of B-cell specific expression of CD23a isoform. The establishment of a two-hybrid system represents a solid base for future investigations of the signaling mechanisms through the CD23 receptor.

ABSTRACT

Two isoforms of human CD23 (CD23a and CD23b) have been described. They differ by only 6-7 residues in the N-terminal cytoplasmic tail. CD23a is restrictively expressed on B-cells while CD23b is inducible on B-cells, as well as monocytes, eosinophils, macrophages and a variety of other cell types, after IL-4 stimulation.

The two isoforms seems to have different functions. CD23a appears to be the isoform associated with endocytosis of IgE immune complexes and mediating antigen presentation on B-cells. CD23b has a phagocytosis motif and seems to be involved in the phagocytosis of IgE-coated particles, cytokine release and the generation of superoxides.

Previous studies indicate that the two isoforms connect to different signal transduction pathways. Comparing the cells that express only one or both CD23 isoforms suggests that CD23b is involved in upregulating cAMP and iNOS, whereas CD23a mediates an increase in intracellular calcium.

In the main part of the study we investigated how the CD23a B-cell specific expression is regulated. Pax-5 is a B-cell restricted transcription factor with an essential role in early and late B-cell development. Putative Pax-5 binding sites have been predicted in the CD23a proximal promoter. Analyses of the CD23a promoter revealed three putative Pax-5 binding sites with more than 50% homology to the consensus sequence. One of these sites, named CD23-1 can compete a high affinity Pax-5 binding site or can directly bind Pax-5 protein in electrophoretic mobility shift assays. Introducing mutations into this site abrogates the binding. A different approach, in which overlapping peptides covering the length of the CD23a promoter were tested in competition assays against a high affinity binding site, also revealed CD23-1 as the only site that directly binds Pax-5 protein.

Expression of Pax-5 in 293 cells resulted in a 7-fold activation of a CD23a core promoter construct. Co-transfection together with STAT6 showed that Pax-5 cooperates with this transcription factor in enhancing the level of transcription of a CD23a extended promoter construct. Most importantly, ectopic expression of Pax-5 in the monocytic cell line U-937 that regularly expresses only the CD23b isoform enabled a significant CD23a expression after stimulation with IL-4 and PMA.

Our results suggest that Pax-5 is a key regulator of the B-cell restricted expression of the CD23a isoform.

In the second part of the project, we used a yeast two-hybrid system (CytoTrap™ from Stratagene) in order to look for cytoplasmic interaction partners for the CD23 receptor. The system was established in order to reach a high efficiency of transformation and different bait vector constructs were made. The screening was performed using a human spleen library cloned in the target vector of the system. The first bait constructs used (pSosCD23a and pSosCD23b) expressed the very short (22 amino acids) cytoplasmic tails of the isoforms at the C-terminal end of the fusion protein (human SOS). Improved bait constructs, (pSosCD23a+Linker and pSos CD23b+Linker) expressed the cytoplasmic tail of CD23a/b at the N-terminal side of the human SOS and had in consequence the N-terminal part free as a bait, as it occurs *in vivo*. A flexible linker region separated the fusion proteins in order to make the small amino acid bait chain more obvious. Approximately three million library clones were screened with these various constructs. No "true positive" interaction was detected. A relatively high number of "false positive" clones were obtained and checked in another two-hybrid system. A new bait construct, in which the tyrosine residue in the cytoplasmic tail of CD23a was replaced by a glutamic acid residue will be used for future screening.

The system was also used in order to test the interaction between CD23 and p59fyn, a member of the Src family of protein kinases that was mentioned to associate with CD23a. No interaction was detected by using the CytoTrap two-hybrid system.

In conclusion, the key result of the study demonstrates that Pax-5 is a main regulator of the B-cell specific expression of the CD23a isoform. In addition, a two-hybrid system was established and employed in order to look for cytoplasmic interaction partners for CD23.

ZUSAMMENFASSUNG

Bisher sind zwei Isoformen des humanen CD23 (CD23a und CD23b) beschrieben. Beide unterscheiden sich lediglich in 6-7 Resten im N-terminalen, zytoplasmatischen Anteil. CD23a wird ausschließlich auf B-Zellen exprimiert, während CD23b sowohl auf B-Zellen als auch auf Monozyten, eosinophilen Granulozyten, Makrophagen und zahlreichen anderen Zelltypen durch Stimulation mit IL-4 induziert werden kann.

Die beiden Isoformen vermitteln wahrscheinlich unterschiedliche Funktionen. CD23a gilt als Isoform, welche vornehmlich mit der Endozytose von IgE-Immunkomplexen und der Vermittlung von Antigen-Präsentation auf B-Zellen assoziiert ist. CD23b besitzt ein Phagozytose-Motiv und scheint bei der Phagozytose IgE besetzter Partikel, der Freisetzung von Zytokinen und der Bildung von Peroxiden eine Rolle zu spielen.

Frühere Untersuchungen legen die Vermutung nahe, dass die beiden Isoformen zwei getrennte Signalübertragungswege miteinander verbinden. Die Gegenüberstellung von Ereignissen, welche in Zellen, die nur eine einer oder beide Isoformen von CD23 besitzen, stattfinden, legt die Vermutung nahe, dass CD23b cAMP und iNOS hochreguliert, wohingegen CD23a einen Anstieg des intrazellulären Kalziums vermittelt.

Im ersten Teil unserer Untersuchungen haben wir die Regulation der B-Zell-spezifischen Expression von CD23a analysiert. Pax-5 ist ein auf B-Zellen beschränkter Transkriptionsfaktor, welcher für die frühe und späte B-Zellentwicklung von entscheidender Bedeutung ist. Mögliche Pax-5 Bindungsstellen wurden in den proximalen Abschnitten des CD23a Promotors vermutet. Die Analyse des CD23a Promotors ergab drei mutmaßliche Pax-5 Bindungsstellen mit mehr als 50% Homologie zur Konsensus-Sequenz. Eine dieser Bindungsstellen, namens CD23-1, kann mit einer hochaffinen Pax-5 Bindungsstelle konkurrieren oder direkt das Pax-5 Protein in Elektromobilitäts Experimenten (EMSA) binden. Das Einfügen von Mutationen an dieser Stelle verhindert die Bindung. Ein weiterer Versuch, bei dem die gesamte Länge des CD23a Promotors durch überlappende Peptide in einem kompetitiven Verfahren gegenüber hoch affinen Bindungsstellen getestet wurde, zeigt ebenso CD23-1 als die einzige Stelle, welche direkt Pax-5 binden kann.

In weiteren Experimenten führte die Expression von Pax-5 in 293 Zellen zu einer 7fachen Aktivierung eines CD23a Kernpromotor Konstrukts. Die Kotransfektion zusammen mit

STAT6 zeigte, dass Pax-5 mit diesem Transkriptionsfaktor kooperiert, indem es die Transkriptionsrate eines vergrößerten CD23a Promotorkonstrukts erhöht. Von besonderer Bedeutung ist die Tatsache, dass die ektope Expression von Pax-5 in der monozytären Zelllinie U-937, die normalerweise nur die CD23b Isoform exprimiert, dann zu einer Expression von CD23a nach Stimulation mit IL-4 und PMA führte. Unsere Ergebnisse legen nahe, dass Pax-5 in der auf B-Zellen beschränkten Expression der CD23 Isoform eine Schlüsselrolle zukommt.

Im zweiten Teil des Projekts haben wir ein "Zwei-Hefen-Hybrid-System" (Cyto-Trap von Stratagene) verwendet, um nach zytoplasmatischen Interaktionspartnern für den CD23 Rezeptor zu suchen. Das System wurde modifiziert um eine hohe Effizienz an Transformation zu erzielen. Unterschiedliche „Köder"-Vektorkonstrukte wurden hergestellt. Das Screening wurde mittels einer humanen Milzbibliothek mit dem Zielvektor des Systems durchgeführt. Die anfangs benutzten Konstrukte –pSosCD23a und pSosCD23b – exprimierten sehr kurze (22 Aminosäuren) zytoplasmatischen Reste der Isoformen am C-terminalen Ende des Fusionsproteins (humanes SOS). Verbesserte Konstrukte (pSos CD23a+Linker und pSosCD23b+Linker) exprimierten den zytoplasmatischen Anteil von CD23a/b am N-terminalen Ende des humanen SOS und hatten folglich den N-terminalen Anteil als Andockstelle frei, entsprechend den Bedingungen in vivo. Eine flexible Verbindungsregion trennte die Fusionsproteine, um auf diese Weise die kurze Aminosäurekette deutlich „sichtbar" werden zu lassen. Annähernd drei Millionen Klone wurden mittels der verschiedenen Konstrukte untersucht. Dabei konnte keine tatsächlich positive Interaktion gefunden werden. Stattdessen fand sich eine vergleichsweise hohe Zahl falsch-positiver Klone. Diese wiederum wurden in einem zweiten "Zwei-Hefen-Hybrid-System" getestet.

In Zukunft wird ein neues Konstrukt als Köder verwendet werden. Hierbei wurde ein Tyrosin-Rest im zytoplasmatischen Anteil von CD23a durch Glutamat ersetzt. Das System wurde bereits dazu verwendet, die Interaktion zwischen CD23 und p59fyn - einem Mitglied der Src-Familie von Proteinkinasen, welches mit CD23a assoziiert sein soll – zu testen. Jedoch konnte im CytoTrap "Zwei-Hefen-Hybrid-System" keine Wechselwirkung nachgewiesen werden.

Zusammenfassend zeigt das zentrale Ergebnis der Arbeit, dass Pax-5 der Schlüsselregulator ist, der die B-Zell-spezifische Expression von CD23a ermöglicht. Zusätzlich wurde ein "Zwei-Hefen-Hybrid-System" etabliert, mit dem zytoplasmatische Interaktionspartner für die CD23 Isoformen gefunden werden können.

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ABREVIATIONS

A_{260}	absorbance at 260 nm
aa	amino acids
AD	activation domain
Ag	antigen
B-CLL	chronic lymphocytic leukemia
BCR	B cell receptor
BLNK	B cell Linker
BSA	bovine serum albumine
BSAP	B-cell-specific activator protein
bp	base pairs
3'C∝	immunoglobulin heavy-chain enhancer
Ca^{2+}	calcium
cAMP	adenosine 3',5'-cyclic-monophosphate
CBF1	C promoter binding factor 1
C/EBP	CCAAT/ enhancer binding protein
CD	cluster of differentiation
CD40L	CD40 ligand
cDNA	complementary DNA
CIP	calf intestinal phosphatase
cpm	counts per minute
CR2/CR3/CR4	complement receptor 2/3/4
CRE	cAMP response element
CREB	cAMP response element binding protein
CY	cytoplasmic domain
Cε	immunoglobulin ε chain constant region exon
Cγ1	immunoglobulin γ1 chain constant region exon
DAG	diacylglycerol
dCTP	deoxycytidine triphosphate
DMEM	Dulbeccos minimum essential medium

DNA	deoxyribonucleic acid
DNA-BD	DNA-binding domain
dNTP	deooxynucleoside triphosphate
d(TGA)TP	deoxy(thymidine/guanosine/adenosine/) triphosphate
DTT	dithiothreinol
EBF	early B-cell factor
EBNA-2	Epstein-Barr virus nuclear antigen-2
EBV	Epstein-Barr virus
EC	extracellular domain
EDTA	ethylenediaminetetraacetic acid
EMSA	Electrophoretic Mobility Shift Assay
Erk	extracellular-regulated kinase
Eμ	Ig heavy chain intronic enhancer
FACS	fluorescence-activated cell sorter
FCS	fetal calf serum
FcεRI	high affinity IgE receptor
FcεRII	CD23, low affinity IgE receptor
Fwr	forward primer
Gal(-Ura,-Leu)	galactose medium without uracil and leucine
GDP	guanosine 5'-diphosphate
Glu	glutamic acid
Glu(-Ura,-Leu)	glucose medium without uracil and leucine
GTP	guanosine 5'-triphosphate
H2A-2/ H2B-2	histone genes
HA	hemagglutinin
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
3'α-hs4	DNase hypersensitivity site 4 in the α gene enhancer
HSC	hematopoietic stem cell
hSOS	human SOS
IC	immune complexes

IFN-α	interferon-α
IFN-γ	interferon-γ
IgA/ IgD/ IgE/ IgG/ IgM	immunoglobulin A / D / E / G / M
Ikkα/β	inhibitor of NF-kB kinase α/β
IkB	inhibitor of NF-kB
IL-1/ IL-4/ IL-6	interleukins
Ile	isoleucine
INOS	inducible nitric oxide synthase
IP_3	inositol 1,4,5-triphosphate
JAK	Janus Kinase
ITAM	immunoreceptor tyrosine based activation motifs
ITIM	immunoreceptor tyrosine based inhibitory motifs
LB	luria broth
Leu	leucine
LFA-1	lymphocyte-function-associated antigen-1
LiAc	lithium acetate
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MCS	multiple cloning site
M-CSF-R	macrophage colony-stimulating factor receptor
Met	methionine
MHC	major histocompatibility complex
mRNA	messenger RNA
N (nucleotide)	any nucleotide
NF-AT	nuclear factor of activated T cells
NK	natural killer cells
NOS	nitric oxide synthase
OD	optical density
Oct-1	octamer binding protein-1
PAGE	Polyacrylamide Gel Electrophoresis
PBS	phosphate-buffered saline

PCR	polymerase chain reaction
PEG	polyethylene glycol
PIP_2	phosphatidylinositol biphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC /PLC-γ	phospholipase C-γ
PMA	phorbolemyristatacetate
PMSF	phynilmethylsulfonyl fluoride
poly[d(I-C)]	poly-deoxy-inosinic-deoxy-cytidylic acid
R (nucleotide)	A or G
RAG-2	recombination-activating genes-2
RGD	arginine-glutamine-aspartic acid
rIL-4	recombinant IL-4
RNA	ribonucleic acid
RNase	ribonuclease
RPA	RNase protection assay
rpm	rotations per minute
RPMI	
RT-PCR	reverse-transcriptase polymerase chain reaction
S (nucleotide)	C or G
Sγ2a	switch region for immunoglobulin γ2a chain constant region
Sμ	switch region for immunoglobulin μ chain constant region
Ser	serine
sCD23	soluble CD23
SDS	sodium dodecyl sulphate
SH2	Src homology domain 2
SH3	Src homology domain 3
SHP-1	SH2-domain-containing protein tyrosine phosphatase 1
SHP-2	SH2-domain-containing protein tyrosine phosphatase 1

SHIP	SH2-domain-containing inositol polyphosphate 5’ phosphatase
SOS	Son of Sevenless
Src/ src	Rous sarcoma
STAT6	signal transducer and activator of transcription
SV40	simian virus 40
TAE	Tris acetate electrophoresis buffer
TBE	Tris borate electrophoresis buffer
TEMED	N,N,N’,N’-tetramethylethylenediamine
TM	transmembrane domain
TNF	tumor necrosis factor
Tris	tris(hydroxymethyl)aminomethane
TSAP	tissue specific activator protein
Tyr	tyrosine
UAS	upstream activatory sequence
Ura	uracil
UTP	uridine-5’-triphosphate
XBP-1	X-box-binding protein 1
Y (nucleotide)	C or T
YPAD	Yeast extract, Peptone, Dextrose medium

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PUBLICATIONS

1. Original articles

- **Visan I., Goller M., Berberich I., Kneitz Ch. and Tony H.-P.** Pax-5 is a key regulator of the B cell restricted expression of the CD23a isoform. Eur. J. Immunol. In press.

2. Published abstracts

- **Visan I., Goller M., Berberich I., Kneitz Ch., Avots A., Serfling E., Tony H.-P.** 2001. Pax-5 and NF-ATp are modulators of high CD23 expression. Scand. J. Immunol. 54 (Suppl.1)
- **Kneitz Ch., Goller M., Visan I., Simon A., Stibbe C., Avots A., Serfling E., Tony H.-P.** 2001. The CD23b promoter is a target of NF-AT transcription factors. Arthritis and Rheumatism 44, S9.
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3. Poster and oral presentations at Congresses and Symposia

- **Visan I., Goller M., Kneitz Ch., Chang C., Tony H.-P.** Looking for cytoplasmic interaction partners of CD23. 12th Protein Kinase Symposium: NO/cGMP and Protein Kinase Signaling, 8/2000, Bad Bruckenau
- **Visan I.** Using two hybrid Systems to find interaction partners of CD23. Joint Retreat of the Graduate Colleges 520 and 592, "Immunomodulation meets Lymphocyte Activation" 7/2001, Zeilitzheim
- **Visan I., Goller M., Berberich I., Kneitz Ch., Avots A., Serfling E., Tony H.-P.** Pax-5 and NF-ATp are modulators of high CD23 expression. 11th International Congress of Immunobiology, 7/2001, Stockholm, Sweden

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EIDESSTATTLICHE ERKLÄRUNGEN

Hiermit erkläre ich ehrenwörtlich, dass die vorliegende Dissertation „***The CD23 receptor-regulation of expression and signal transduction***“ selbständig an der Medizinischen Poliklinik der Universität Würzburg angefertigt wurde und dass ich keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

Würzburg, 31 Januar 2003
(Dipl Biologin Ioana Andreea Visan)

Hiermit erkläre ich ehrenwörtlich, dass die vorliegende Dissertation „***The CD23 receptor-regulation of expression and signal transduction***“ in gleicher oder ähnlicher Form noch nicht in einem anderen Prüfungsverfahren vorgelegen hat.

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Hiermit erkläre ich ehrenwörtlich, dass ich bisher noch keine akademische Grade erworben oder zu erwerben versucht habe.

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