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Mutational analysis of the J recombination signal sequence binding protein (RBP-J)/Epstein–Barr virus nuclear antigen 2 (EBNA2) and RBP-J/Notch interaction

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Epstein–Barr virus nuclear antigen 2 (EBNA2) and the Notch protein both function within the nucleus as transcriptional adaptor proteins. EBNA2 plays a key role during the immortalization of primary B-cells by Epstein–Barr virus (EBV). Notch proteins are involved in lymphomagenesis as well as in multiple cell fate decisions during tissue differentiation and development. Both, EBNA2 and Notch interact with the DNA binding protein RBP-J and thereby gain access to the promoter of their target genes. In order to identify regions within the J recombination signal sequence binding protein (RBP-J), that are relevant for either the Notch or the EBNA2 interaction, we have performed a mutational analysis of RBP-J. A library of RBP-J mutants was screened by a reverse two-hybrid system for alleles that fail to bind to either EBNA2 or Notch. The sequence analysis of these alleles reveals that a limited and particularly distinct number of amino-acid positions are relevant for either interaction only. Given the important role of RBP-J in B-cell immortalization, the EBNA2/RBP-J protein–protein interaction could be a candidate target for therapeutic intervention in EBV related diseases.

Keywords: protein–protein interaction; EBNA2; Notch; RBP-J; reverse yeast two-hybrid.

RBP-J is a ubiquitously expressed repressor protein, that binds to a specific DNA core sequence motif in the promoter of target genes. RBP-J recruits at least two co-repressor complexes to these promoters: SMRT/NcoR/HDAC1 and CIR/HDAC2/SAP30 [1–4]. Cellular, as well as viral, signalling pathways converge on the RBP-J protein. Epstein–Barr virus nuclear antigen 2 (EBNA2) is expressed in Epstein–Barr virus (EBV) immortalized B-cells and is essential for the proliferation of these B-cells [5–8]. EBNA2 is a transactivator of viral and cellular genes, that carries an intrinsic transactivation domain but cannot bind to DNA directly. EBNA2 can gain access to the promoters of target genes by interacting with DNA-binding proteins such as RBP-J. A direct protein–protein interaction between EBNA2 and RBP-J has been well documented in the past [9–16]. EBNA2 mutants, which cannot bind to RBP-J, no longer support the immortalization of primary B-cells by EBV indicating that this interaction drives a pathway essential for the immortalization process [9–16]. In fact, EBV targets the RBP-J pathway by more than one mechanism. EBNA3A, B, C and RPMS1 protein are additional viral proteins that interact with RBP-J and modulate EBNA2/RBP-J signalling [17–22]. The regions of RBP-J that mediate the EBNA3C and RPMS1 interaction have been mapped to its C-terminal domain [18,21].

The Notch pathway is a cellular pathway, that targets the RBP-J protein. The Notch proteins are a family of highly conserved transmembrane receptors. Upon ligand binding, the transmembrane protein is cleaved and an intracellular fragment of Notch translocates into the nucleus and, similarly to EBNA2, binds to RBP-J and activates target genes [23–25]. In mammals, four Notch genes are differentially expressed during development and control processes as diverse as neurogenesis, somite formation and hematopoeisis [4,26,27].

As EBNA2/RBP-J signalling is essential for the immortalizing function of EBNA2, the cellular protein RBP-J is a candidate target for therapeutic intervention in EBV associated diseases. On the other hand, we know that four mammalian Notch proteins also interact with RBP-J and we know or expect these interactions to be involved in a broad range of essential biological functions. The most desirable drug should thus interfere with EBNA2/RBP-J signalling, without impairing Notch/RBP-J signalling. A prerequisite for a specific interference with EBNA2/RBP-J signalling is the detailed knowledge of the respective protein–protein interaction.

Structural data on the RBP-J protein are not available. The primary sequence of the RBP-J protein has been highly conserved during evolution, but pattern and profile searches so far have only identified one conserved domain, called the IPT/TIG domain, which lies outside the minimal

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Abbreviations: HA, human infuenza virus hemagglutinin fragment (amino acids 75–110); EBNA2, Epstein–Barr virus nuclear antigen 2; EBV, Epstein–Barr virus; RBP-J, J recombination signal sequence binding protein; DB, DNA binding domain; FOA, 5-fluoroacidic acid; 3-AT, 3-aminotriazole; EMSA, electrophoretic mobility shift assay; AD, activation domain.

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domain of RBP-J required for Notch or EBNA2 binding [23,28–31]. The minimal domain of the RBP-J protein that mediates the direct interaction with EBNA2 and Notch has been mapped to the central portion of the protein, which also is involved in DNA-binding [31,32]. In order to better characterize the interaction of RBP-J with Notch and EBNA2 in more detail, we have performed a randomized mutagenesis of the *RBP-J* gene and analyzed phenotypically selected RBP-J mutant alleles.

MATERIALS AND METHODS

Plasmids and yeast strains

The yeast expression plasmids pKF3 and pKF2 were generated by adding HA and MYC tags to the ORF of the GAL4 activation (AD) and GAL4 DNA binding domain (DB) of PC86 and PC97-*CYH2,* respectively [33]. EBNA2 fragments encompassing amino acids 281–344 and mouse Notch1 fragments encompassing amino acids 1751–1850 were generated by PCR and subcloned into pKF2 generating plasmid pKF101 and pKF7. Mouse *RBP-J* and the mouse RBP-J encoding amino acids 205–389 were subcloned into pKF3 in order to generate pKF184 and pKF14, respectively. pKF186 expresses a *RBP-J* deletion mutant lacking the central domain (amino acids 205–387) from vector pKF3. EBNA2 fragments (amino acids 249–490) and the Notch fragment (amino acids 1751–2110) were subcloned into pKF3 in order to generate pKF12 and pKF16, respectively. The yeast strains MaV103 and MaV203 and all general procedures to detect protein–protein interactions have been described previously [33–35].

Screening conditions

RBP-J mutant alleles were generated by PCR amplification of 25 ng pKF14 using the following primers: CGCGTTTGGAATCACTACAGGG and GGAGACTTGACCAAACCTCTGGCG. The reaction was performed for 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C for 25 cycles. MaV 103 transformed with either pKF101 or pKF7. These transformants were then cotransformed with the PCR products and pKF3 digested with *Sal* I and *Not* I. Initially, 10 000 transformants were plated onto synthetic complete media (15-cm^2 plates) lacking leucine and tryptophan. For negative selection, these cells were replica plated onto solid media containing 0.2% 5-fluoroacidic acid (FOA). Surviving colonies were then replica plated onto cycloheximide containing complete synthetic media lacking tryptophan (0.5 $\mu g \cdot mL^{-1}$) in order to select for cells that had lost the EBNA2/DB or Notch/DB expression plasmids. MaV203 cells were transformed with pKF101 or pKF47 and subsequently mated with the colonies, that had survived the cycloheximide selection. Successfully mated colonies were isolated by growth on solid media lacking leucine and tryptophan and then tested for interaction with the second interaction partner, EBNA2 or Notch, respectively, by growth in the presence of 50 mM 3-aminotriazole (3-AT).

In vitro binding reactions

In vitro binding reactions of [^{35}S] methionine labelled *in vitro* translated protein were performed using the rat anti-HA Ig (Roche). Templates for *in vitro* translation were PCR products of the respective genes, that added T7 polymerase binding site and the appropriate tags to the *in vitro* translation products as described in [35].

Electrophoretic mobility shift assays (EMSAs)

In vitro translated HA-tagged RBP-J wild-type and mutant proteins were used for binding reactions in gel retardation assays using the 54-oligonucleotide derived from the *LMP2A* promoter as described in [36].

RESULTS

Genetic selection of RBP–J interaction-defective alleles

To define the RBP-J domain(s) involved in the interaction with either EBNA2 or Notch, we genetically selected for RBP-J mutant alleles which fail to bind EBNA2 while retaining the ability to bind Notch, or that fail to bind Notch while retaining the ability to bind EBNA2. To this end we used the reverse yeast two-hybrid system that allows selection for interaction-defective alleles using a negative selection strategy [33,34]. This system uses the counter-selectable reporter gene *URA3.* Yeast cells expressing Ura3p convert the compound 5-fluoroorotic acid (FOA) into the toxic derivative 5-fluorouracil. A two-hybrid interaction, that leads to activation of the *URA3* reporter gene can thus be counter selected in the presence of FOA and loss of function mutants can be specifically selected out of a large pool of wild-type alleles [33,34,37]. The initial negative selection step for loss of function mutants (e.g. loss of Notch binding) was followed by a positive selection step, in order to screen for the subset of mutants, that retain a second function (e.g. EBNA2 binding) as illustrated in Fig. 1. In order to generate a library of *RBP-J* mutant alleles, the RBP-J gene was randomly mutagenized by PCR amplification that allows misincorporation of single nucleotides. The PCR fragment was introduced into a yeast two-hybrid expression vector encoding the Gal4p activation domain (AD) by gap repair. The mutagenesis of the *RBP-J* gene was focused on the fragment encoding amino acids 205–387, a domain sufficient for both EBNA2 and Notch interaction. For two parallel screens, expression constructs for RBP-J/AD fusion proteins were cotransformed with the RBP-J binding domains of either Notch (amino acids 1751–1850) or EBNA2 (amino acids 281–344) GAL4 DNA binding domain (DB) expression constructs. Colonies of transformants were grown on permissive plates and then submitted to negative selection after replica plating onto media supplemented with FOA. As the DB expression construct also encodes the counter selectable CYH2, propagation of yeast colonies in the presence of cycloheximide will select for cells that have lost the DB expression construct, but retain the AD expression construct by segregation. Thus, after the FOA negative selection step the colonies were replica plated onto cycloheximide supplemented plates and loss of the respective DB expression construct was confirmed by

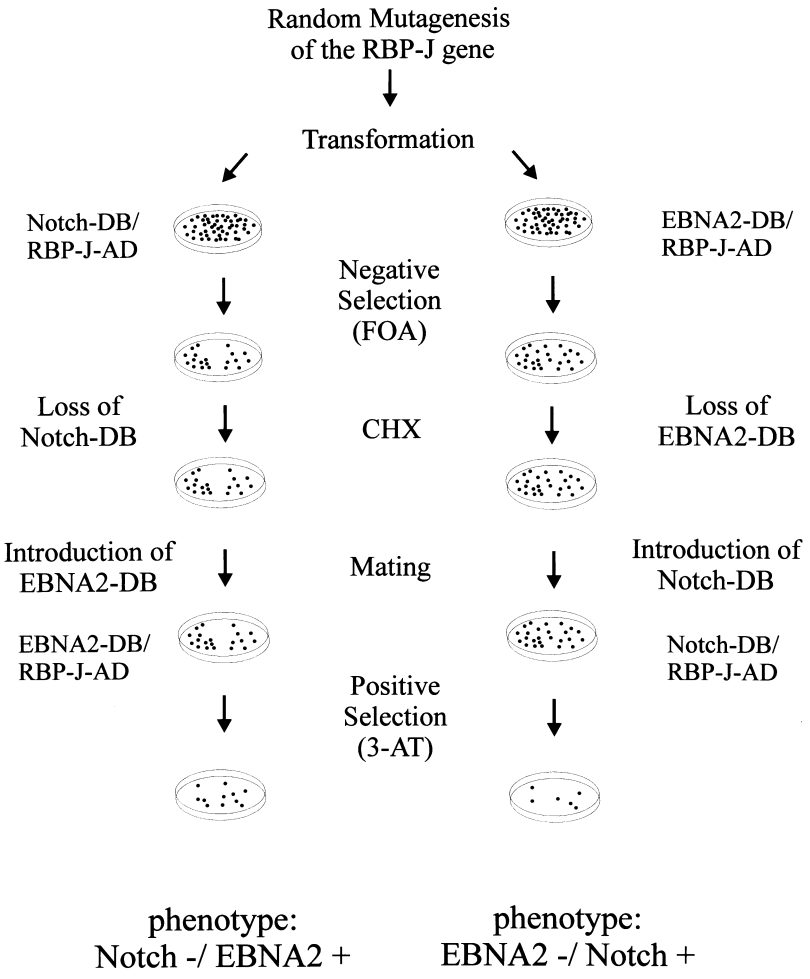

Fig. 1. The outline of a two-step selection protocol to isolate RBP-J mutants, that have lost the ability to bind to Notch (Notch$^-$) but retained the ability to bind to EBNA2 (EBNA2$^+$) and vice versa is schematically shown. Negative selection of Notch-DB/RBP-J-AD transformants on FOA containing media was performed in order to isolate Notch-mutants. This step was followed by cycloheximide (CHX) selection of segregants, which had lost the Notch-DB expression plasmid. EBNA2-DB was introduced into the colonies by mating and EBNA2-DB/RBP-J-AD were positively selected for expression EBNA2$^+$ phenotypes. In parallel RBP-J mutants, which had lost EBNA2 binding but retained Notch binding were isolated by a reciprocal protocol. All selection steps were performed on solid media by consecutive replica plating (see text for all details).

phenotypical analysis, i.e. by growth in the absence of tryptophan and the requirement for leucine in the medium (Leu$^-$/Trp$^+$).

In a second step the yeast colonies, expressing the potential loss of function RBP-J alleles were positively selected for retaining the second binding specificity. To this end the DB expression construct, either EBNA2 DB or Notch DB, was reintroduced by mating the yeast colonies, which had survived the negative selection step to yeast strain MaV203 pretransformed with either EBNA2 DB or Notch DB. Successfully mated colonies (Leu$^+$/Trp$^-$) were replica plated onto selective plates supplemented with the His3p inhibitor 3-AT and positively selected for the activation of the *HIS3* reporter gene. Out of 160 000 transformants screened for loss of Notch and maintenance of EBNA2 interaction (Notch$^-$/EBNA2$^+$), 69 transformants were isolated as potential candidates. The RBP-J/AD expression plasmids from these candidate transformants were isolated and cotransformed with either Notch/DB or EBNA2/DB into yeast strain MaV103. Initially we isolated 69 RBP-J expression plasmids and could confirm the expected phenotype Notch-/EBNA2$^+$ of 37 of these 69 RBP-J alleles (Fig. 2A). Out of 100 000 colonies screened for the phenotype Notch$^+$/EBNA2$^-$. A total of 68 candidate RBP-J alleles were isolated, of which 22 RBP-J alleles retained the expected phenotype after retransformation into MaV103 yeast cells (Fig. 2B).

Sequence analysis of the RBP-J alleles

Thirty-seven RBP-J alleles exhibiting the Notch$^-$/EBNA2$^+$ phenotype were sequenced and the deduced primary sequence of the respective alleles is illustrated in Fig. 3. Most of the mutations were repeatedly found in independent isolates as described in Table 1. We found amino-acid exchanges in four positions, D254, F261, K275, and A284, which were clustered within a stretch of 30 amino acids. Phenylalanine 261 was found to be substituted with either

Table 1. The phenotype of 37 Notch$^-$/EBNA2 + RBP-J and 22 EBNA2$^-$/Notch$^+$ mutant alleles were verified by retransformation and phenotypical analysis. The table lists amino-acid exchanges found in all alleles, which were sequenced and the number of independently picked colonies for each mutant.

Notch-/EBNA2$^+$	No. of colonies	EBNA2$^-$/Notch$^+$	No. of colonies
ΔD254	1	F246L	1
F261L	17	L326P	18
F261I	3	Q333L	3
K275M	7		
K275R	6		
K275Q	1		
A284V	2		

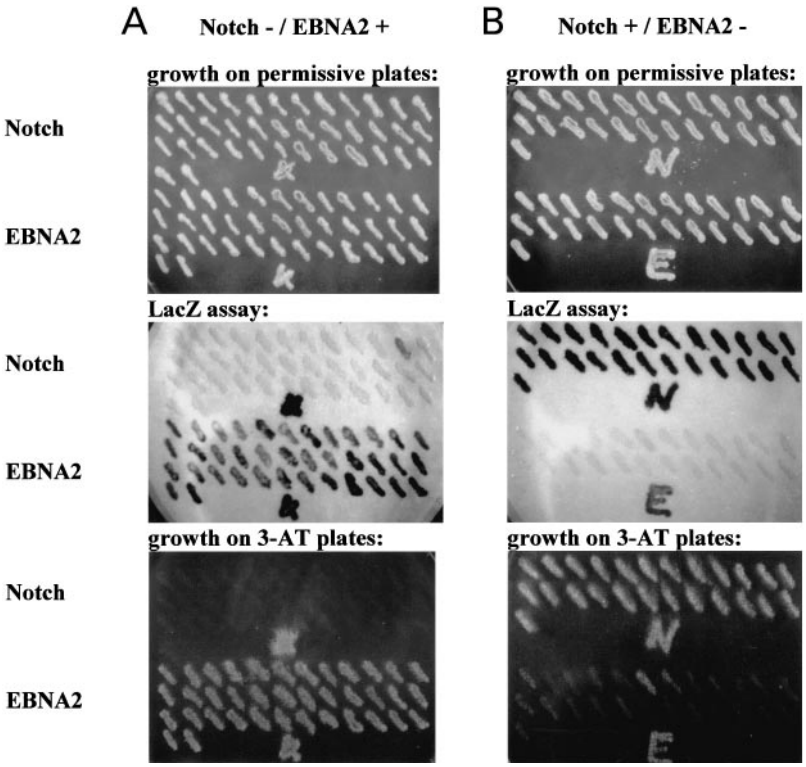

Fig. 2. Specific phenotype of the isolated RBP-J alleles. RBP-J AD mutants that showed the expected phenotype were coexpressed with Notch DB or EBNA2 DB. A representative single cell clone of each transformation was grown on a permissive master plate and then replica plated onto a LacZ assay test filter or a selective plate supplemented with 3AT (50 mM) and tested for interaction with Notch (upper panel) or EBNA2 (lower panel). (A) 37 RBP-J alleles showed the expected phenotype Notch$^-$/EBNA2$^+$. One clone scored as a false positive. Wild-type RBP-J transformed control yeast cells were drawn as K. (B) 23 RBP-J alleles showed the expected phenotype Notch$^+$/EBNA2$^-$. Within the panel of 25 clones, two false positives are visible. Wild-type RBP-J/Notch cotransfomants are drawn as N, wild-type RBP-J/EBNA2 cotransformants are drawn as E.

isoleucine or leucine and lysine 275 was changed to either methionine, arginine or glutamine. The amino-acid exchanges found in the 22 Notch$^+$/EBNA2$^-$ alleles were spread over a stretch of 87 amino acids of the primary sequence (F246, L326 and Q333 in Fig. 3). Similarily the same amino-acid exchanges, phenylalanine 246 to leucine and glutamine 333 to leucine were found repeatedly in independent isolates. The phenotype of one additional mutant (L285), which exhibited an intermediate phenotype, will be discussed below.

The phenotype of the RBP-J alleles *in vitro*

To biochemically characterize the RBP–J interaction-defective alleles, the most frequent mutant alleles were reconstituted in the context of the full-length protein. These constructs could then be *in vitro* translated and tested for interaction with hemagglutinin-tagged EBNA2 (a fragment containing amino acids 249–490) and Notch (a fragment containing amino acids 1751–2110) by coimmunoprecipitation using rat anti-HA Ig (Fig. 4A–D). F261I, K275 and A284 bound to EBNA2 like wild-type RBP-J, but had significantly lost the affinity for Notch. L326P and Q333L, in contrast, had retained the affinity for Notch but lost the ability to bind to EBNA2 almost completely. We then performed gel retardation assays to test whether the mutant proteins could still bind to DNA. All mutants could bind to the *RBP-J* oligonucleotide indicating that these mutants had retained their specific affinity for DNA (Fig. 4E,F). One additional mutant L285P had been originally isolated from the screen for the EBNA2$^-$/Notch$^+$ phenotype, but

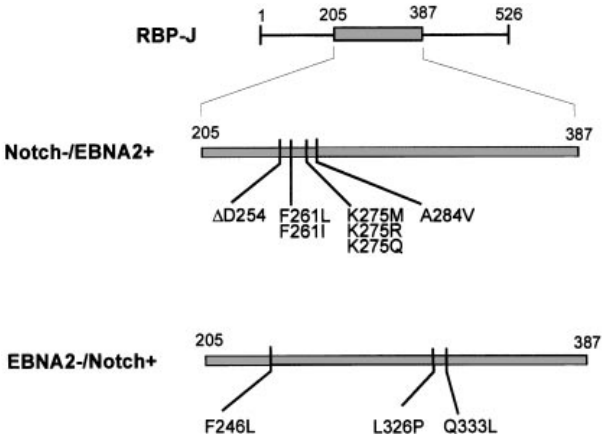

Fig. 3. Primary structure of the RBP-J mutant proteins. The central part of RBP-J (amino acids: 205–387) and the amino-acid exchanges are illustrated for RBP-J alleles exhibiting the phenotype Notch$^-$/EBNA2$^+$ or EBNA2$^-$/Notch$^+$. Mutations in Notch$^-$/EBNA2$^+$ alleles cluster within a narrow region of 33 amino acids between amino acids: 254 and 284, mutations in Notch$^+$/EBNA2$^-$ alleles are located in flanking regions between amino acids: 246 and 333.

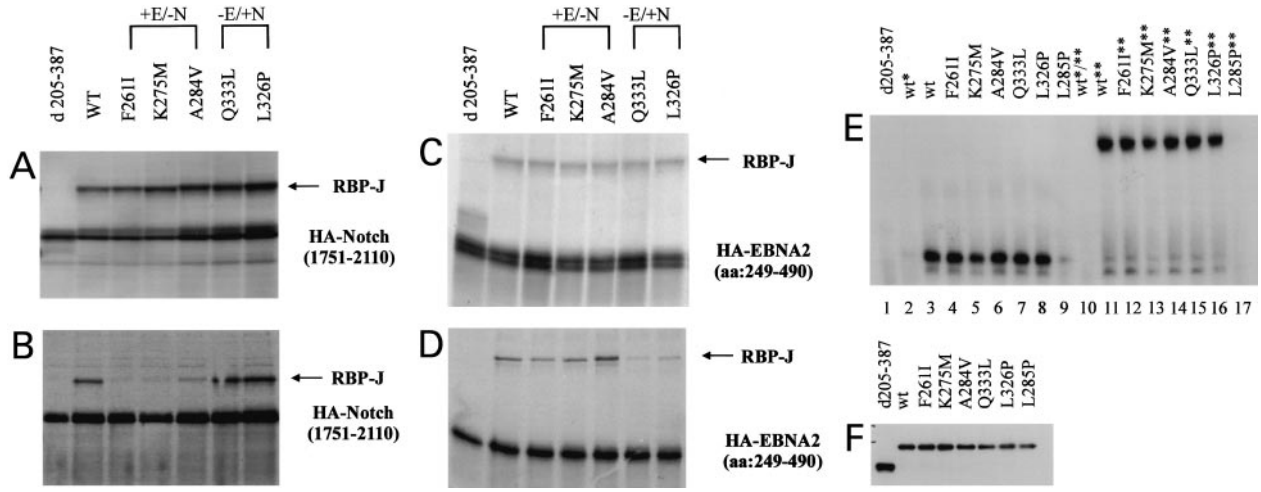

Fig. 4. The RBP-J alleles, that have lost either EBNA2 or Notch binding have retained their DNA binding specificity. [^{35}S]methionine labelled *in vitro* translated (A,B) HA tagged Notch (amino acids: 1751–2110) protein or (C,D) HA tagged EBNA2 (amino acids: 249–490) fragments were mixed with *in vitro* translated wild-type and mutant RBP-J proteins and immunoprecipitations were performed using the rat anti-HA Ig. Proteinfragments are shown before (A,C) and after (B,D) immunoprecipitation. (E) The *in vitro* translation products of HA-tagged RBP-J wild-type and mutant proteins as indicated were subjected to gel retardation assays. A deletion mutant of RBP-J (d205–387) lacking the central RBP-J region relevant for DNA binding was included as control. In order to show the specificity of the binding reactions unlabelled oligonucleotides were added to lanes 2 and 10 indicated by *. The anti-HA Ig was added to extracts in lanes 10–17 (**) in order to verify HA tagged RBP-J proteins by supershifts. (F) All *in vitro* translated HA-RBP-J proteins used in (E) show similar immunostaining in Western blots, using the rat anti-HA Ig.

we could not verify the phenotype in *in vitro* binding studies. When this *RBP-J* mutant was included in the gel retardation analysis it scored as a loss of DNA binding mutant. As several functions seem to be impaired by the L285P mutation, we cannot exclude the possibility that the L285P mutation causes a structural change in the RBP-J protein which abolishes the correct folding of the protein.

A sequence alignment of RBP proteins from different species (Fig. 5) revealed that all amino acids covered by our mutagenesis and selection protocol are conserved within the group of RBP-J proteins from different species. A more distant relative of RBP-J, the RBP-L protein has recently been described [38]. Even though a direct protein–protein interaction between EBNA2 or Notch and RBP-L could not been demonstrated, it could be shown that RBP-L can enhance transcriptional activation by EBNA2, but not by Notch. Comparison of the sequence alignment of all RBP proteins revealed that the mutants ΔD254, K275 and A284, which are important for the RBP-J/Notch interaction, are not conserved in RBP-L. The lack of sequence conservation within these three amino-acid positions might explain why RBP-L cannot interact with Notch.

DISCUSSION

Both, Notch and EBNA2 can interact with a central fragment of RBP-J encompassing amino acids 196–372. We, like others, have tried to minimize this region further in

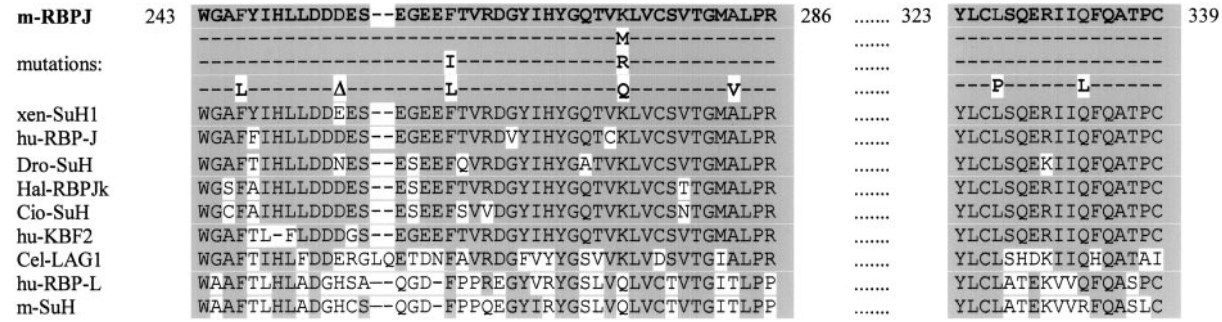

Fig. 5. Alignment of RBP-J and RBP-L orthologues. The deduced primary structure of the central region of RBP-J, which is essential for EBNA2, Notch and DNA binding is highly conserved across species. The alignment of the compiled RBP-J amino-acid sequences to human RBP-L revealed that D254, K275 and A284, which are mutated in Notch$^-$/EBNA$^+$ are not conserved in RBP-L. Abbreviations used in this figure are: m-RBPJ, mouse RBPJk (X17459); xen-SuH1, *Xenopus laevis* Supressor of hairless (U60093); hu-RBP-J, human RBP-J (L07872); Dro-SuH, *Drosophila melanogaster* Supressor of hairless (M94383); Hal-RBP-J, *Halocynthia roretzi* Supressor of hairless (AB003695); Cio-SuH, *Ciona intestinalis* Supressor of hairless (AF085173); hu-KBF2, human H2K binding factor 2 (L08904); Cel-LAG1, *Caenorhabditis elegans* LAG-1 gene (U49795); hum-RBPL, human RBP-L (AB026048.1); mus-SuH, mouse recombining binding protein Supressor of hairless like protein (NM_009036.1). All GenBank accession numbers are given in parenthesis.

order to identify smaller domains essential for either Notch or EBNA2 but were unable to do so [29–31]. A series of 23 mouse RBP-J replacement mutants and their phenotypes have recently been described [31,32]. Loss of Notch-binding was strictly correlated with loss of DNA binding in all examples analyzed in this study. A subset of these mutants carrying mutations in region amino acids 275–324 also showed reduced binding affinity for EBNA2 [31,32]. From these studies, the conclusion was drawn, that Notch and EBNA2 interaction involve overlapping but not identical regions of RBP-J. Another series of 12 human RBP-J replacement mutants have recently been generated and tested for Notch, EBNA2 and the CBF1 binding protein, CIR. CIR is one of the adaptor proteins that recruit histone deacetylase activity to RBP-J regulated promoters. One mutant (KLV249AAA) had lost the ability to bind to Notch and CIR, but had retained the ability to bind to EBNA2. This mutant showed for the first time, that Notch and EBNA2 binding can be uncoupled [29,30]. A second mutant EEF233AAA had lost the ability to interact with CIR but retained the ability to bind to EBNA2 [2]. To our knowledge no RBP-J mutant has been described, that had specifically lost EBNA2 binding but retained Notch binding.

We have undertaken a genetic screen which directly addresses the question whether and how many replacements mutants of RBP-J can be generated, that show a specific loss of phenotype.

The library of RBP-J mutants was generated by misincorporation of nucleotides in a PCR reaction. Formally, we cannot exclude that the PCR based mutagenesis protocol may constrain the complexity of the mutant library. A careful analysis of all mutants derived during this and other screens has however, not indicated a bias for specific nucleotide exchanges [33,34,39].

The selection strategy we have chosen to generate these mutants involved a two-step selection scheme; a negative selection step followed by a second positive selection step. First, by negative selection we isolated loss of function mutants. The majority of this set of loss of function mutants were uninformative deletion or structural mutants (data not shown). The second step selected positively for a minor subset of mutants, which had retained a specific characteristic functional feature, suggesting that the molecular structure of these RBP-J mutants has also been at least partially maintained. All but one mutant (L285P) had also retained the ability to bind to DNA. This result distinguishes our mutants from those isolated in previous studies and show that Notch, EBNA2 and DNA binding of RBP-J can be uncoupled.

The sequence analysis of all mutants revealed that most of the alleles were isolated independently during the screen more than once. Thus, our screen probably identified the majority of alleles of the desired phenotype and the number of clones screened was sufficiently high enough to cover the complexity of the library. Only four different amino-acid positions were found to be changed in the Notch$^-$/EBNA2$^+$ or EBNA2$^-$/Notch$^+$ set of mutants. Those mutations leading to loss of Notch binding form a tightly linked cluster within a stretch of 30 amino acids. In fact, two of these mutations (F261I and K275M) align to the region EEF233 and KLV249 in human RBP-J, which has been shown to be relevant for CIR binding [2]. When we tested F261L, K275M and A284V for CIR binding in yeast, all three mutants had lost CIR binding in contrast to Q333L and L226, which had lost EBNA2 but retained CIR binding in yeast (data not shown). Thus Notch and CIR target related regions on RBP-J, while the EBNA2 interaction relies on a diverse subregion.

The mutations which lead to loss of EBNA2 binding only, are found in regions flanking the Notch binding site stretching over 87 amino acids. As these positions were repeatedly changed, we conclude that the number of potential amino-acid exchanges leading to loss of only one interaction are very limited. Our screen did not address the question of which amino acids of RBP-J are involved in both EBNA2 and Notch binding and our results do not exclude the possibility that Notch and EBNA2 might interact with identical residues. The only mutant (L285P) isolated during this screen which had lost all EBNA2 binding and was impaired for Notch binding turned out to be a non-DNA-binding mutant and thus could as well be a structural mutant.

However, common binding sites for Notch and EBNA2 on RBP-J within the cellular context are expected, as recently, a novel RBP-J interacting protein, SKIP has been found. SKIP can interact with RBP-J as well as with Notch or EBNA2. It functions as an additional bridge within the Notch/RBP-J or EBNA2/RBP-J complex [3,40]. The RBP-J binding sites for SKIP have not yet been mapped but these binding sites should score as indirect binding sites for both Notch and EBNA2 in the context of the SKIP expressing mammalian cells.

According to the primary structure of the RBP-J protein, the cluster of mutations in Notch$^-$/EBNA2$^+$ alleles is embedded in the broader cluster of mutations found in EBNA$^-$/Notch$^+$ alleles. Whether this pattern reflects the spatial order of protein–protein interfaces within the complexes can only be solved by the crystal structure of the complexes.

Interference between EBNA2 and RBP-J is expected to have an anti-tumor activity in EBV-associated malignancies but such interference could be counterproductive if it would disturb the interaction between RBP-J and its cellular binding partner Notch. By using a genetic approach aiming at disrupting protein–protein interactions, we could identify for the first time regions within RBP-J that are important for EBNA2 but irrelevant for Notch binding.

The region within EBNA2 which directly interacts with RBP-J has been carefully mapped [38]. The region within RBP-J encompassing amino acid L326 and Q333 might thus be a prime target for generating EBNA2 specific inhibitors of EBNA2/RBP-J signalling. Eventually we will need an array of specific inhibitors which specifically target all relevant interacting surfaces of both binding partners in order to develop efficient thrapeutics for EBV associated diseases.

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