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Sequence analysis, expression, and paratope characterization of a single-chain Fv fragment for the eukaryote ribosomal P proteins☆

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Abstract

The variable genes of monoclonal antibody (mAb) B10, specific for the C-terminal region of the eukaryotic ribosomal P protein, have been cloned as a single-chain Fv fragment (scFv) and expressed in *Escherichia coli*. The primary sequence of the variable regions of the B10 antibody, together with a detailed characterization of the reactive residues of the antigen, allowed the construction of a model of the paratope–epitope interaction, giving a first insight into the binding mechanisms of anti-P autoantibodies to their target peptides. The mAb and scFv could be useful for extensive P protein detection since both recognize the highly conserved motif DDxGF.

Keywords: Ribosomal P protein; *Trypanosoma cruzi*; Autoantibody; Single-chain Fv fragment; Paratope–epitope modelling

Ribosomal P proteins are located on the stalk of the large subunit of the ribosome and play a critical role in prokaryote and eukaryote protein synthesis [1]. Despite their intracellular location, antibodies (abs) against ribosomal P proteins in humans are markers of systemic lupus erythematosus (SLE) [2]. In chronic Chagas heart disease (cChHD), antibodies against the ribosomal P proteins of *Trypanosoma cruzi*, the haemoflagellate that causes the infection, are also detected [3]. For many years it was assumed that anti-P antibodies from SLE patients were similar to those found in cChHD, since both recognized the 13 C-terminal residues (EEEDDDMGFGLFD, R13 peptide) of the *T. cruzi* ribosomal P2β (TcP2β), as well as the human motif EESDDDMGFGLFD. However, fine epitope mapping demonstrated that the antibodies induced in these two pathological disorders are different. Anti-P abs from patients infected with *T. cruzi* or mice immunized with the *T. cruzi* ribosomal P2β protein, also known as anti-R13 abs, bind the *T. cruzi*-specific motif ExDDxGF (residues 3, 5, 6, 8, and 9 of R13), whereas true anti-P abs from SLE patients recognize the 10 residue-long eukaryotic P consensus sequence, DDDMGFGLFD, and bind ribosomal P proteins exposed on the surfaces of different human cell lines. Recent progress in this field has demonstrated that mice immunized with autologous P protein, as well as in certain mice immunized with TcP2β, also developed anti-P autoantibodies, reacting with the DDxGF motif exhibited by the P consensus sequence and by R13 (residues 5, 6, 8, and 9 of R13) [4–6].

A monospecific mAb with anti-DDxGF specificity named B10, derived from BALB/c mice immunized with TcP2β, was used in the present work to study the antigen binding site of anti-P antibodies. As part of our ongoing studies on the structure of antibodies reacting with the C-terminal regions of the *T. cruzi* ribosomal P proteins, we sequenced the variable region of the genes

☆ *Abbreviations:* TcP2β, *Trypanosoma cruzi* ribosomal P2β; mAb, monoclonal antibody; abs, antibodies; CDR, complementarity-determining region; FR, framework region; scFv, single-chain Fv fragment; VL, light chain variable region; VH, heavy chain variable region.

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encoding B10 mAb, and cloned and expressed its scFv version. The modelling of the paratope–epitope interactions of B10 mAb with the motif DDMGF gives a first insight into the binding mechanisms of anti-P autoantibodies to the eukaryotic consensus sequence. Moreover, due to the striking similarity of motifs DDxGF and ExDDxGF, the model presented here might allow a better understanding of the pathological features of anti-P antibodies present in cChHD patients.

Materials and methods

Amplification of the V genes and construction of the scFv. Total RNA was isolated from B10-expressing hybridoma cells using the RNeasy kit (Qiagen). cDNA synthesis was performed with Moloney murine leukemia virus reverse transcriptase, using random hexamers as primers (Promega). PCR amplification was carried out using a 3:1 mixture of *Taq* DNA polymerase (Amersham-Pharmacia) and *Pfu* polymerase (Stratagene). The heavy and light chain variable regions (VH and VL) were amplified in a three-step PCR as described [7] using the primers VH1Back (5′-AGGTSMARCTGCAGSAGTCWGG-3′), VH1For (5′-TGAGGAGACGGTGACCGTGGTCCCTTGGCCCC-3′), Vk2Back (5′-GACATTGAGCTCA CCCAGTCTCCA-3′), and Vk2For (5′-CCGTTTBAKYTCCARCTTKGTSCC-3′). The linker and restriction sites sequences were added in a second PCR using the primers SCVHFor (5′-AGAGCCACCTCCGCCTGAACCGCCTC CACCTGAGGAGACGGTGACCG-3′) and SfiVHBack (5′-TACTC GCGGCCCAACCGGCCATGGCCCAGGTSMARCTGCAGSAG TC-3′) for VH and NotVkFor (5′-GATATGAGATACTGCGG CCGCCCG TTTBAKYTCCARCTTK GTSCC-3′) and SCVkBack (5′-GGCGGAGGTGGCTCTGGCGGTGGCGGATCCGACATTGA GCTCACCCAG-3′) for VL. PCR products were joined in a third reaction by using primers SfiVHBack and NotVkFor and cloned into the *Not*I and *Nco*I sites of the pSW1 vector, provided by Dr. J.L. Teillaud. Plasmid pSW1 is based on pUC119 and contains a multiple cloning site preceded by a pelB leader sequence, followed by a c-myc epitope and a six-histidine tag. Double-stranded nucleotide sequencing was performed by the dideoxy chain-termination method from multiple independent clones. Analysis of the nucleotide sequence was performed using Ig-Blast and the IMGT databases (Fig. 2). The VL and VH nucleotide sequences of B10 have been deposited in the GenBank Database under Accession Code AF522288.

Expression and purification of scFv B10. Expression of the scFv was induced by adding 1 mM IPTG to the culture during 12 h at 30 °C [7]. The scFv was purified from the periplasmic fraction using His-Bind Resin (Qiagen) according to manufacturer's instructions.

Competitive binding of scFv to TcP2β. Inhibition of binding of the B10 mAb to TcP2β in the presence of scFv was measured by ELISA. Polystyrene immunoplates were coated overnight at 4 °C with 50 μl of 2 μg/ml GST-TcP2β recombinant protein in 0.05 M bicarbonate–carbonate buffer (pH 9.6). GST was used as control. Different amounts of the scFv were added to the wells overnight at 4 °C. Plates were washed and the B10 mAb (0.2 μg/ml) was added 1 h at 37 °C. Binding of the mAb to TcP2β was measured from the absorbance at 405 nm after the addition of alkaline-phosphatase-conjugated anti-mouse Ig (Sigma), followed by *p*-nitrophenyl phosphate (Sigma). The specificity of the scFv for the C-terminal portion of the ribosomal P protein was assessed by adding 30 μM of peptide R13 (EEEDDDFGMGLFD) to the plates together with the scFv. The non-related 13 amino acid-long peptide TMVP (AEAALUKMALMKV), derived from tobacco mosaic virus coat protein, was employed as a negative control. Peptides were from Neosystem (Strasbourg, France). The B10 mAb was purified from ascitic fluids by using HiTrap IgM purification columns (Amersham-Pharmacia).

Alanine scanning mutagenesis. The fine specificity of the reaction of mAb and scFv B10 against the C-terminal portion of TcP2β was performed using the SPOTs kit (Genosys Biotechnologies, UK). A set of 14 peptides representing the R13 sequence and its 13 alanine-replacement analogs were synthesized. Strips containing the 14 peptides were incubated 1 h at 37 °C with the mAb or overnight at 4 °C with the scFv. After incubation with the scFv, strips were incubated 1 h with an anti-6× His antibody (Qiagen), followed by peroxidase-conjugated anti-mouse IgG (Sigma) and developed using the ECL technique (Amersham). Washes were performed as indicated by the suppliers.

Model building. Modelling was performed using the InsightII, Biopolymer, Homology, Docking and the Discover MSI software modules (Accelrys, San Diego, CA). Starting with the 3D structure of a mutated anti-lysozyme antibody (PDB 1A7N) and an anti-ouabain antibody (PDB 1IBG), a chimeric construct was obtained from the VH of the former and the VL of the latter in which the amino acids differing between B10 and the corresponding Fv fragments of the matrix abs were manually replaced. A first minimization procedure using the steepest descent method was performed to relax the chimeric protein (1000 steps). Peptide DDMGF was built in silico, the N-terminal was acetylated, and the C-terminal was amidated to avoid non-specific charge interactions. The peptide, charged as at pH 7.4, was then relaxed at a dielectric constant of 80 by the conjugation method until the RMS was 0.001 kcal/mol Å. After charging the chimeric Fv corresponding to B10 at pH 7.4, the minimized peptide was manually docked in the combining site until negative values for intermolecular energy were obtained. The complex was assembled, framework residues were fixed, the peptide and the hypervariable region were minimized first by steepest descent using a distance-dependent dielectric constant, and afterwards with conjugated steps until an RMS of 0.001 kcal/mol Å was obtained. To explore the conformational space of the epitope–paratope interactions, molecular dynamics were performed for 110 ps (10 ps equilibrium, 100 ps dynamics) at 300 K in a distance-dependent dielectric constant. Analysis of the potential energy as a function of time allowed the selection of a conformation with minimal potential energy which was again submitted to conjugated minimization until an RMS of 0.001 kcal/mol Å was reached.

Results and discussion

The VH and VL domains of B10 mAb were PCR-amplified and cloned in the form of a scFv by introducing the 15-residue linker $(Gly_4Ser)_3$, connecting the C-terminus of VH to the N-terminus of VL. The recombinant protein also carries a hexahistidine tail in a C-terminal position to VL, to facilitate purification and as a tag for detection of the scFv in immunological assays. The scFv was expressed in a soluble form in *E. coli* as a 33-kDa protein and purified to near homogeneity by immobilized metal affinity chromatography.

The recombinant scFv B10 construction retained the property of binding the ribosomal P protein, since it inhibited the binding of the mAb to GST-TcP2β in a dose-dependent manner (Fig. 1A). This effect was not observed when the scFv was preincubated with the peptide R13, demonstrating the specificity of the scFv to the C-terminal end of the P protein. Preincubation of the scFv with the control peptide TMVP did not affect the binding of the mAb to GST-TcP2β (Fig. 1A). The fine specificity of the scFv was assessed by alanine-scanning mutagenesis of peptide R13. This assay

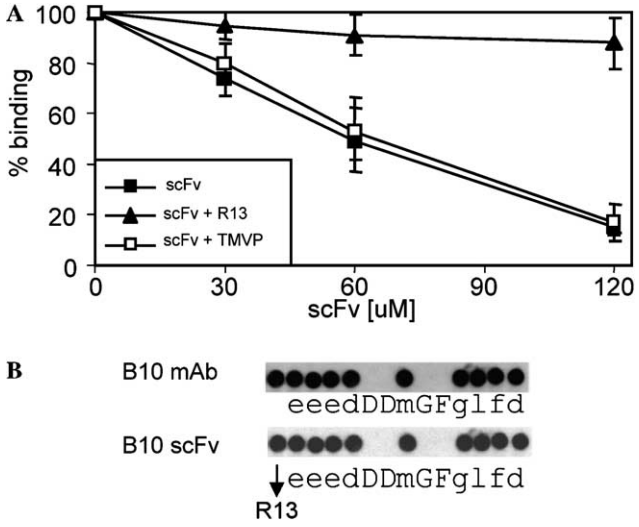

Fig. 1. Reactivity of mAb and scFv B10 against TcP2β and R13. (A) Inhibition of the binding of mAb to TcP2β by scFv. TcP2β-coated plates were incubated with different concentrations of scFv alone, scFv plus R13, or scFv plus TMVP. The degree of reactivity of the scFv was assessed indirectly by adding B10 mAb to the plate. Results were plotted as the percentage of the binding obtained in the absence of scFv. (B) Reactivity of mAb and scFv B10 against R13 alanine-replaced peptides. The indicated amino acids of R13 were replaced by Ala and the reactivity of the mutated peptides was assayed using B10 mAb or B10 scFv. Amino acids recognized by B10 are in capital letters.

confirmed that the specificity of the scFv mimics that of the parental antibody, both recognizing the DDxGF motif (Fig. 1B). Since the DDMGF sequence is present in all members of the P protein family (i.e., P1, P2, and P0) and is also highly conserved throughout eukaryotes [8], it is possible to anticipate that the B10 mAb and scFv version will be useful tools for detection of P proteins in different biological systems.

Following confirmation of the anti-P reactivity of the scFv, the VH and VL domains of B10 were sequenced. Comparison of the sequences with the closest known germ-line genes allowed us to carry out the identification of the V, D, and J gene segments used during somatic recombination to originate genes coding for the B10 mAb. The nucleotide sequence of B10 was compared with the closest known germ-line genes together with the deduced protein sequence of B10. The amino acid residues were numbered according to Kabat et al. [9] and the complementarity-determining regions (CDR) were assigned using the IMGT database [10]. Analysis of the VH region (Fig. 2) showed that it belongs to the mouse H-chain family subgroup IB of Kabat et al. [9] and that it shares 92.8% sequence identity with the germ-line gene Vox-1, a member of the Q52 VH subfamily. The H-CDR3 comprises nine codons contributed by the D gene segment DSP2.4, used in reading frame 1, and the 5′ end of the JH3 segment (Fig. 2A) (GenBank Database Accession Code AF522288). Sequence analysis of the VL region showed that it belongs to the mouse chain family X, subgroup III of Kabat et al. [9] (Fig. 2B). Finally, the VL gene segment of B10 exhibits 97.1% homology with the MUSIGKVR1 germ-line sequence, belonging to the Vk21 family and was productively rearranged with the germ-line JK2 gene (Fig. 2B).

Of the 44 mutations spanning VH and VL, 18 were silent and 26 led to amino acid replacements (Fig. 2). However, the ratio of replacement vs. silent (*R*/*S*) mutations in B10 was much higher in the CDRs than in the framework regions (FRs) (Table 1). The probability that such distribution of replacement residues could have arisen in a germ-line gene by a random mechanism is 9.2×10^{-5} as calculated by the method of Chang and Casali [11]. These results strongly suggest that differences in B10 arise from selection of replacement mutations in the CDRs under the persistent influence of antigen. This is a relevant finding, since B10 is an IgM mAb, and it was necessary to rule out the possibility that it was obtained in an early step of the affinity maturation process.

The existence of 3D structures of antibody fragments with close similarity to the heavy chain (PDB 1A7N) and the light chain (PDB 1IBG) (Fig. 3A) allowed us to construct a 3D model of the B10 scFv (Fig. 3B).

To identify the critical residues of the antigen-combining site, peptide DDMGF, previously identified as the target for B10 mAb, was docked into the B10 structural model (Fig. 3B).

Docking the epitope into the combining site was considered to correspond to a realistic model for the following reasons: (1) contact residues in the combining site with the peptide were limited to the CDR regions of both VL and VH, (2) nine of the ten contact residues of VH and four of the eight contact residues of the VL are mutated compared to the matrix mAbs. In particular, Gly53H was replaced by Ser allowing the formation of a hydrogen bridge with the peptide through its OH group. Similarly His36L and Leu100L were both replaced by Tyr, once again allowing hydrogen bond formation between the peptide and the OH groups, (3) the two δ-carboxyl groups of Asp 1 and Asp 2 from the peptide which have been shown to be essential for antibody recognition are both anchored into the combining site by hydrogen bonding and electrostatic interactions with L-CDR1 and L-CDR3, (4) the Met 3, which has been proved to be non-essential for antigen binding, was directed out of the combining site, (5) the unfavourable, negatively charged Asp97L in the CDR3 region of the light chain has been mutated to Ala97L, allowing the formation of a hydrogen bond and a salt bridge between the first Asp of the epitope and Ser95L and Arg96L, respectively, and (6) the Phe 5 is linked by its CO group to the Ser53H of the H-CDR2 (mutated from a Gly in the 1A7N heavy chain) and the aromatic ring embedded between the H-CDR2 and H-CDR3 in which the

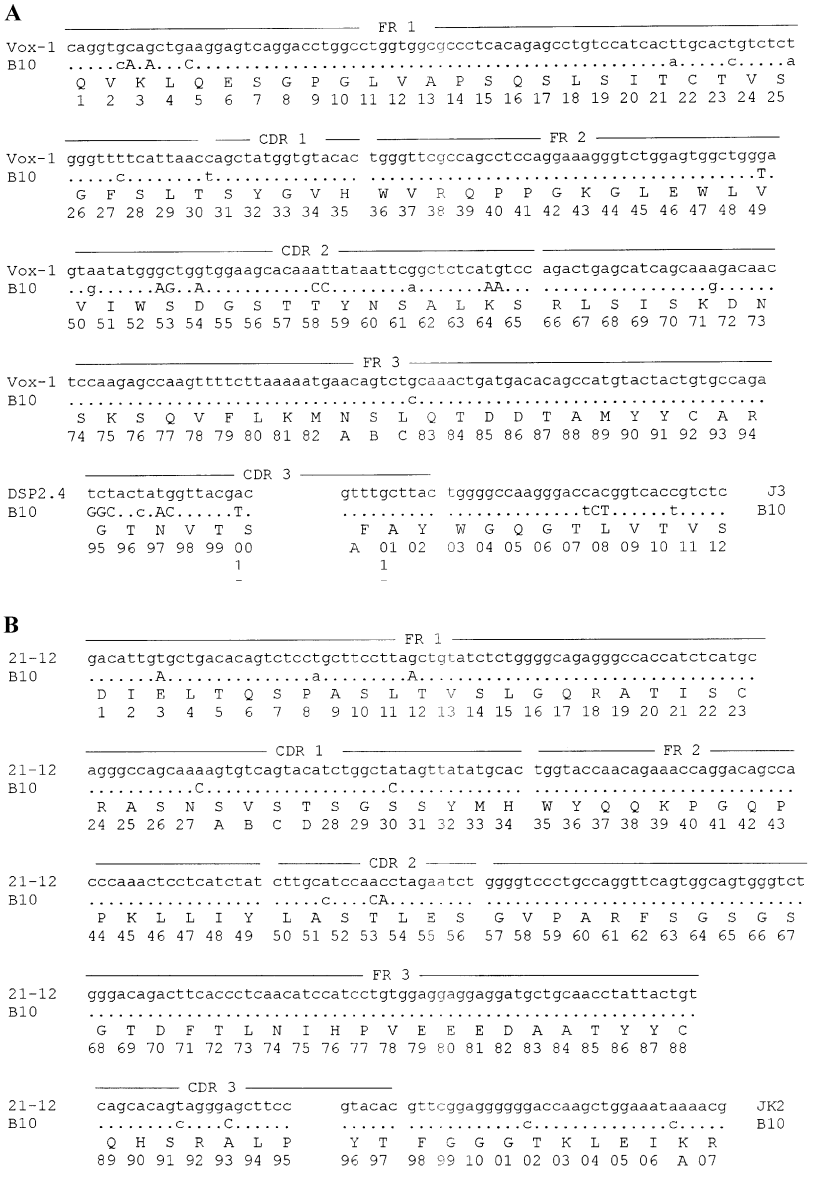

Fig. 2. Sequence of the heavy (A) and light (B) chain variable regions of B10. The nucleotide sequence of B10 mAb was compared with the closest germ-line genes. Identities are indicated by dots. Capital and small letters indicate replacement and silent mutations, respectively. The deduced amino acid sequences of the rearranged H and L chains of the B10 mAb are also shown. The CDRs and FRs are according to Kabat et al.

Table 1
Silent and replacement mutations in B10 mAB

	FRs			CDRs		
	R^a	S^b	R/S	R	S	R/S
H	6	8	0.75	13	5	2.60
L	2	3	0.66	5	2	2.50
H + L	8	11	0.72	18*	7	2.57

Silent and replacement mutations were arranged according to their position on either the FRs or CDRs of the H and L chains of B10 (see Fig. 2). The high R/S quotient on CDR regions indicates that affinity maturation of B10 mAb took place under the persistent influence of antigen.

[a] Replacement mutations.

[b] Silent mutations.

* p, 9.2×10^{-5}.

charged residues of CDR3 of the matrix VH, normally unfavourable for anchoring a hydrophobic group, were replaced by uncharged amino acids.

The high degree of compatibility of the model with experimental data from alanine scanning mutagenesis allowed the identification of residues Ser53H, Thr31L, Tyr36L, Ser95L, Arg96L, and Tyr100L as critically involved in antigen binding. Paratope modelling as described above does not replace experimental evidence but does constitute a useful tool for designing future site-directed mutagenesis studies on critical residues of the scFv directed toward uncovering the participation of each of them on the ligand binding properties of the monoclonal antibody.

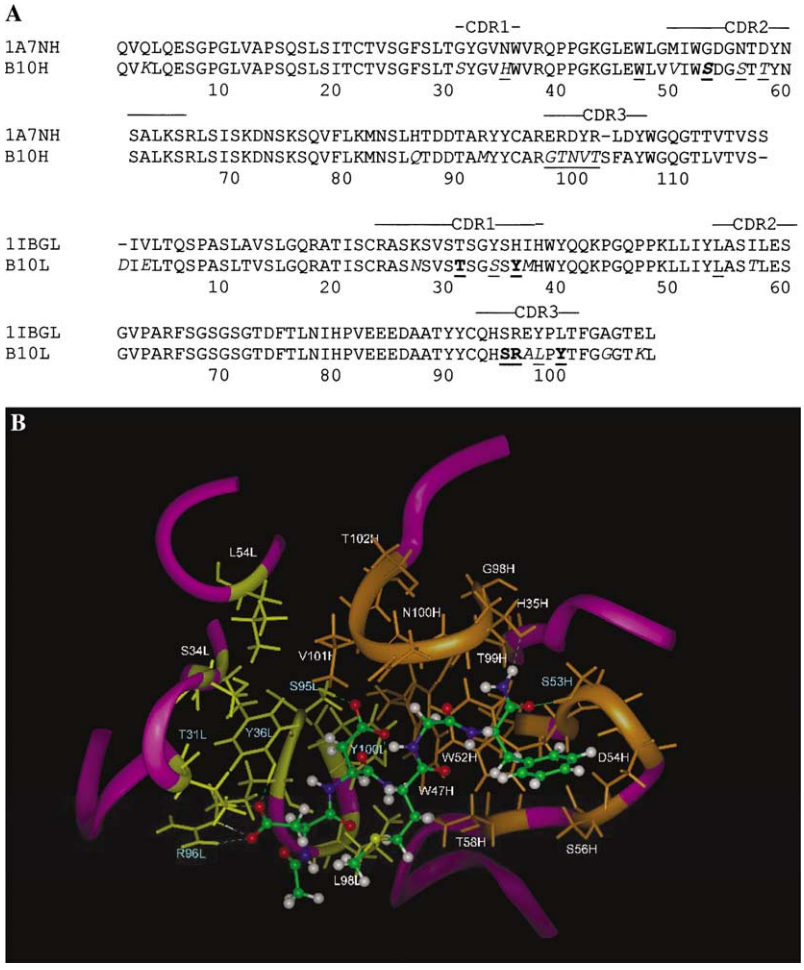

Fig. 3. Model of the paratope–epitope interaction between B10 and peptide DDMGF. (A) Comparison of the sequences of B10 with the H chain of PDB structure 1A7N and the L chain of PDB structure 1IBG. CDRs are depicted; mutations are shown in italics, while contact residues with the peptide are underlined. Bold characters show residues involved in hydrogen bonding with the epitope. (B) Structural model of the epitope–paratope interaction. The three CDRs of the L chain (left) and H chain (right) are drawn as ribbons. The epitope is coloured by atoms (hydrogen, white; carbon, green; nitrogen, blue; oxygen, red; and sulphur, yellow) and shown as balls and sticks. The contact residues of the paratope (all residues with a distance less than 10 Å from the geometrical centre of the epitope) are shown: yellow for the L chain and orange for the H chain. Numbering is as in (A). Those residues involved in hydrogen bonding with the epitope are shown in light blue.

The response to the *T. cruzi* P ribosomal proteins has proved to be extremely complex since abs against the closely related motifs ExDDxGF (in TcP2β), DDxGF (in both *T. cruzi* and mammalian P proteins), and ExDxDxDF (in TcP0) have been described to have differential pathogenic properties [5,6]. Antibodies against ExDxDxDF are linked to the induction of sinusal arrythmia and repolarization and conduction abnormalities, whereas anti-ExDDxGF abs display an exclusive β1-adrenergic stimulating activity whose appearance strictly correlated with the recording of sinusal tachycardia and sudden death [5].

Since B10 abs are not linked by any pathological alteration but react with the DDxGF sequence including the critical Phe residue at the end of the motif, which is also recognized by other two anti-P receptor stimulating-antibodies, it is tempting to hypothesize the fact that the B10 CDRs may share structural characteristics with these abs, as it has been established for anti-DNA abs [12]. This possibility deserves to be further explored by sequence and structural analysis of the other anti-P abs, a study that is currently underway in collaboration with other groups [13].

In conclusion, the present study reports for the first time the sequence of the variable genes of an anti-P autoantibody, providing the first clues to the characterization of its antigen binding sites. Since these antibodies have been linked to a typical autoimmune disease such as SLE and to the pathogenesis of chronic infections by *T. cruzi*, structural knowledge of the paratope–epitope interactions as documented here may be useful in the comprehension of different aspects of these pathologies, as well as to understand the subtle differences in anti-P antibody specificity.

Acknowledgments

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