Identification and location on syndecan-1 core protein of the epitopes of B-B2 and B-B4 monoclonal antibodies

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Abstract Using a phage display peptide library, we characterized the epitope of two monoclonal antibodies reacting with syndecan-1: B-B2 and B-B4. The identified epitopes QDIT, for B-B2, and LPEV, for B-B4, were found to align with residues 36–39 and 90–93 of the mature protein, respectively. In contrast to B-B4, the B-B2 epitope is close to a potential glycosaminoglycan attachment site. Since syndecan-1 is heavily glycosylated and post-translational modifications are cell type specific, these results might explain the differences observed in the reactivity pattern of B-B2 and B-B4 and suggest that these monoclonal antibodies are useful probes to study cell surface exposed syndecan-1.

Key words: B-B2 monoclonal antibody; B-B4 monoclonal antibody; Syndecan-1; Epitope characterization; Peptide phage display

1. Introduction

Two monoclonal antibodies (mAbs), B-B2 and B-B4, which specifically identify the human plasma cell among hemopoietic cells were previously developed [1]. These mAbs appeared to be useful tools for multiple myeloma pathology identification involving terminally differentiated B-cells [2,3]. B-B4 has also been used to isolate pure populations of myeloma cells from bone marrow samples of such patients [4,5]. This mAb does not react with acute lymphocytic leukemia and chronic lymphoblastic leukemia cells [2] which are less differentiated B-cells, but it reacts with Reed-Sternberg cells of classical Hodgkin's disease [6]. By expression cloning the antigen recognized by B-B4 was shown to be syndecan-1 [2], which defines the new CD138 cluster of leukocyte differentiation [7]. This molecule was originally cloned from mammalian epithelial cell lines of mouse and human origin [8,9]. For reviews of syndecan-1 see [10–15]. Briefly, in mature tissues syndecan-1 is constitutively expressed by epithelial cells. However, syndecan-1 expression is regulated during embryogenesis, wound healing and carcinogenesis. In response to wound healing, syndecan-1 expression is induced in sprouting and proliferating endothelial cells and in proliferating keratinocytes [16]. In malignancies its expression can also be altered. It is suppressed upon malignant transformation of human keratinocytes [17], and an association between syndecan-1 expression by squamous cell carcinoma of the head and neck and clinical outcome has been observed [18]. In mice, syndecan-1 is expressed by maturing B-cells and plasmocytes but not by circulating B-cells [19]. Different roles have been suggested for syndecan-1 such as in proliferation and adhesion.

To better characterize B-B2 and B-B4 mAbs reacting with syndecan-1 transfected COS-7 cells and displaying similar but not identical patterns of recognition with cell lines [2], it was attempted to identify their epitopes by using the phage display technology. This technology is a powerful approach to study protein-protein interactions. Originally, peptides were expressed as fusion proteins to the minor coat protein pIII of the bacteriophages, therefore enabling large libraries to be screened by affinity purification of the modified phage particles [20,21]. The display has been extended to proteins like antibody fragments [22–24]. Here we used the phage peptide approach to characterize the epitopes of B-B2 and of B-B4 mAbs. The results allowed us to localize exactly their interacting site on syndecan-1 core protein.

2. Materials and methods

2.1. MAbs and synthetic peptides

B-B2 and B-B4 mAbs were produced by ascitic fluid and purified on a protein A column. The isotype of B-B2 is IgG2b and that of B-B4 IgG1. The synthetic peptides were assembled automatically by stepwise Fmoc-*t*-butyl solid phase synthesis in a Synergy Appl. The purified materials were then characterized by amino acid composition, sequence analysis [25] and laser desorption [26].

2.2. Selection of phage peptides

The library (Biolabs) expressing random unconstrained heptapeptides fused to the N-terminus of pIII consists of 2×10^9 clones. This diversity is close to the theoretical number of combinations for an amino acid heptamer. For panning the mAbs were coated at 50 μg/ml in phosphate-buffered saline (PBS), overnight and at 4°C. After washing three times with 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% (v/v) Tween-20 buffer, wells were saturated at room temperature (RT) for 90 min with 5 mg/ml bovine serum albumin (BSA) in PBS for the first and third panning, and 2% non-fat dry milk powder in PBS for the second panning. Then 10^{11} phages in washing buffer containing 1% BSA were added for 2 h at RT. After washing 20 times bound phages were eluted with 0.1 M glycine-HCl pH 2.8 for 10 min at RT, and neutralized with 2.6 M Tris pH 8.8 (1/20 of the glycine volume). This procedure was applied for the first two rounds. Between each round, eluted phages were amplified in *Escherichia coli* strain ER2537 in liquid medium or on plates. For the third and last round, the phages were added to two wells, one coated with the selecting mAb and the other with an irrelevant control mAb. The elution was firstly done by competitive elution [27] by addition 10 μg of the selecting mAb, B-B2 or B-B4, to the microtiter well for 2 h at RT. Remaining phages were eluted with acidic condition, as for the two first rounds of selection.

2.3. Sequencing

Phages were randomly picked up after the third selection. Single strand DNA was prepared and used for sequencing performed with Sequenase enzyme (Amersham) using −28 gIII or −96 gIII sequencing primers (Biolabs).

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2.4. ELISA analysis

Wells were coated with peptides (0–100 ng/well) in PBS overnight at 4°C, and saturated with 5% BSA for 2 h at RT. Three washes were done between each step. The mAbs B-B2, B-B4 or isotype matched controls in PBS 1% BSA were added (10–100 ng/well) and incubated for 2 h at RT. MAb binding was detected with biotinylated goat anti-mouse Ig (Diaclone) which was incubated for 1 h at RT, then streptavidin horseradish peroxidase (Europa) incubated for 30 min at RT and finally with TMB substrate (Biosource). Optical density was measured at 450 nm.

3. Results and discussion

3.1. Epitope identification

By expression cloning, we previously characterized the antigen recognized by B-B4, a mAb specifically identifying terminally differentiated B-cells among human hemopoietic cells. Sequencing performed on the isolated clone (H13.7) revealed that the targeted molecule was syndecan-1. In order to determine the interacting site of B-B4 mAb, and also of B-B2 mAb which has a similar reactivity pattern as B-B4 and reacts with COS cells transfected with H13.f cDNA, we decided to study the epitopes recognized by B-B2 and B-B4 mAbs with a phage library displaying random unconstrained peptides composed of seven residues. Coated mAbs were used to select the phages which were then eluted with an acidic treatment, and amplified in bacteria for the next round of selection. After the third round of selection bound phages were first eluted by adding the selecting mAb as competitor, and then acidic conditions were applied to recover the remaining phages. Independently of the elution procedure used, the number of recovered phages was at least 30 times higher from the well coated with the selecting mAb, B-B2 or B-B4 than with an irrelevant control mAb. These results suggest that the selected phages were mAb specific. Sequencing was performed on randomly picked clones and the encoded peptide sequences deduced.

1	MRRAALWLWLCALALSLQLA	20
21	LPQIVATNLPPEDQDGSGDD	40
41	SDNFSGSGAGALQDITLSQQ	60
61	TPSTWKDTQLLTAIPTSPEP	80
81	TGLEATTASTSTLPAGEGPK	100
101	EGEAVVLPEVEPGLTAREQE	120
121	ATPRPRETTQLPTTHQASTT	140
141	TATTAQEPATSHPHRDMQPG	160
161	HHETSTPAGPSQADLHTPHT	180
181	EDGGPSATERAAEDGASSQL	200
201	PAAEGSGEQDFTFETSGENT	220
221	AVVAVEPDRRNQSPVDQGAT	240
241	GASQGLLDRKEVLGGVIAGG	260
261	LVGLIFAVCLVGFMLYRMKK	280
281	KDEGSYSLEEPKQANGGAYQ	300
301	KPTKQEEFYA	310

Fig. 1. Position of the B-B2 and B-B4 epitopes on the syndecan-1 amino acid sequence. The syndecan-1 hydrophobic sequences corresponding to the leader and transmembrane regions are boxed, the putative glycosaminoglycan attachment sites are underlined, and the B-B2 and B-B4 epitopes are boxed in gray.

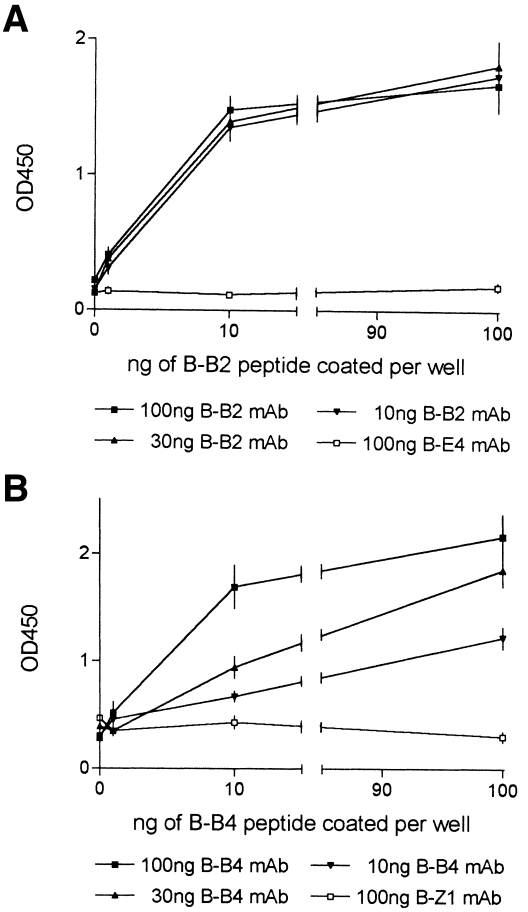


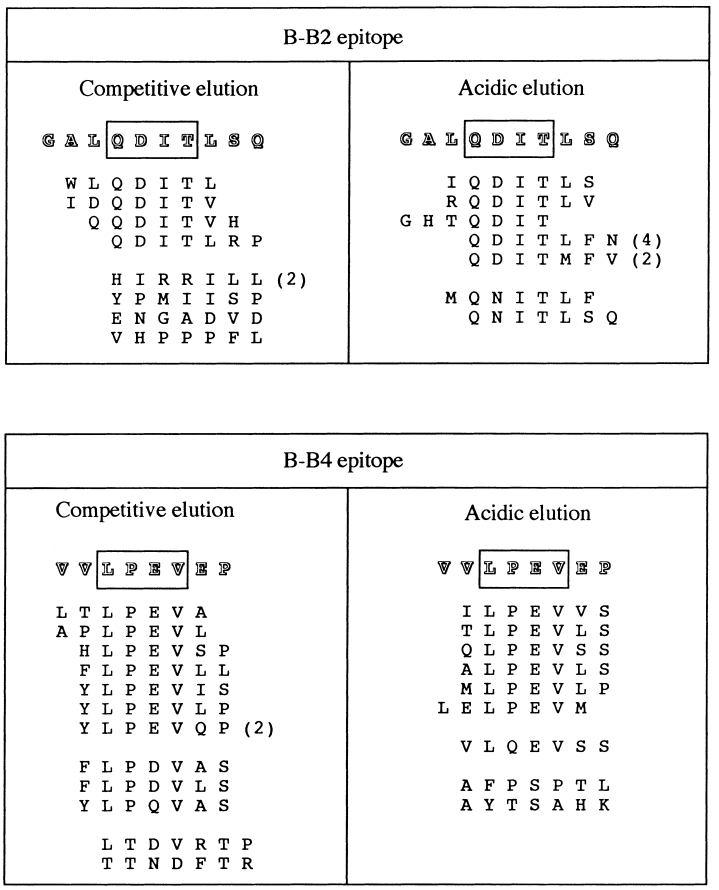
Fig. 2. ELISA analysis performed with the chemically synthesized peptides. Different amounts of B-B2 peptide (A) and B-B4 peptide (B) were coated on plates. The reactivity of the mAbs was tested with different concentrations of B-B2 mAb (IgG2b) and B-B4 mAb (IgG1), and with isotype matched control mAbs B-E4 (IgG2b) for B-B2 and B-Z1 (IgG1) for B-B4. The experiments were carried out in triplicate.

Most of the clones displayed a consensus sequence of four residues (Table 1), the B-B2 peptide consensus sequence (QDIT) being different from that of B-B4 (LPEV). Some clones align with three amino acids of the consensus sequence, in the case of B-B2 aspartic amino acid was replaced by its amide form, asparagine, and in the case of B-B4 glutamic amino acid was replaced by its amide form, glutamine, or by aspartic amino acid. The position of the consensus sequences in the phage displayed peptides is variable for B-B2. For B-B4, there is at least one amino acid in front of the consensus sequence.

3.2. Epitope location on syndecan-1 core protein

The comparison of the consensus peptide sequences to that of syndecan-1 enabled us to localize the two epitopes on the core protein. The B-B2 and B-B4 consensus sequences align with amino acids 36–39 and 90–93, respectively, of the mature core protein if the leader sequence is 17 amino acids long (Fig. 1). For some clones the homology is extended. This result is in agreement with previous experiments showing that B-B2 mAb did not compete with B-B4 mAb and vice versa for the binding to cell line U266 (not shown). The B-B2 and B-B4 epitope sequences have not been found in the known syndecan-1 se-

Table 1
Sequence of the peptides encoded by the phages



The shadded lines correspond to syndecan-1 sequences. A sequence found more than once is followed by the number of identical clones in brackets.

quences of other organisms, nor in the known sequences of other members of the human syndecan family.

3.3. Synthetic peptides

Peptides were chemically synthesized in order to confirm the mAb specificity. The B-B4 epitope contains three hydrophobic amino acids and is flanked by other hydrophobic residues in syndecan-1. To avoid possible problems with solubility, the chosen sequence for the central part (QLPEVSS) of B-B4 peptide (SGQLPEVSSGGGC) corresponds to the sequence of one of the isolated clones. B-B2 peptide (GALQDITLSQQTC) aligns with the syndecan-1 sequence except for the last cysteine residue. ELISA analysis showed that B-B2 peptide reacted with B-B2 mAb and B-B4 peptide with B-B4 mAb (Fig. 2). The reactivity of the mAbs for their respective peptides was dose dependent. B-B2 peptide did not react with B-B4 mAb (not shown), nor with an irrelevant isotype matched control mAb (B-E4: IgG2b, Fig. 2A), and B-B4 peptide did not react with B-B2 mAb (not shown), nor with an irrelevant isotype matched control mAb (B-Z1: IgG1, Fig. 2B). These results correlate with those obtained with the phages during the third round of selection. They show that the displayed peptide sequences on the phages interacted with the respective mAb and that phage environment was dispensable. It is difficult to compare the reactivity of B-B2 and B-B4 mAbs for their respective peptides since factors such as coating efficiency and conformations of the coated peptides, playing a role in the final results, are not known. Amino acid preferences for the peptide-plastic interaction are not excluded and could influence the results if such preference concerns amino acids of the consensus sequences. There are three hydrophobic residues in both peptides but all of them are located inside the consensus sequence for B-B4 and outside for B-B2. For example a preference for a hydrophobic interaction between the peptide and the plastic surface is expected to lower the affinity of B-B4 mAb for the B-B4 coated peptide.

These mAbs might find applications in the field of recombinant protein purification as such linear epitope sequences can be introduced into a protein of interest with the recombinant technology and the expressed proteins can be purified by affinity chromatography using the corresponding mAb. By PCR we introduced the B-B4 epitope at the C-terminus of the extracellular domain of the β chain of the interferon-γ receptor. Preliminary results showed that B-B4 mAb reacted with this modified protein in an ELISA test (not shown).

3.4. Conclusions

The results show how powerful the peptide phage display technology is and how specific the mAbs are, since the epitopes defined with a library of peptides unrelated to syndecan-1 could be localized on the protein. In both cases the epitope is linear. These results are in line with the fact that the protein is

expected to have an extended conformation deduced from its high content of proline residues [9] and its abnormal migration on SDS-PAGE [28]. Both mAbs do not react with post-translational modifications of syndecan-1 which is known to be heavily glycosylated. The B-B2 epitope is close to a potential glycosaminoglycan attachment site. Syndecan-1 glycosylation is cell type specific, therefore the B-B2 epitope might not always be as accessible as the B-B4 epitope and this could explain the differences observed in the reactivity patterns of the two mAbs [2]. This point has to be investigated to test the hypothesis.

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