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ARTICLE

Modeling of Main Characteristics of Bullous Pemphigoid Antigen-2 (BPAG2) Peptide Structure in Serological Recognition by Autoantibodies

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The serum level of autoantibodies against autoantigens of the bullous pemphigoid peptides 1 and 2 (BPAG1 and BPAG2) is a relevant diagnostic marker. Twelve representative sera of BP were tested against the RSILPYGDSMDRIEKDRLQMAP amino acid sequence that is an epitope fragment of the NC16A domain of BPAG2 (AC Q02802; 507-528) to find the most suitable antigenic form for specific detection of autoantibodies of BP patients' sera by quantitative ELISA system. The antigenic epitope sequence was presented as an antigen in a carrier free form of dimeric peptide (BP22), dimeric peptide fused to glutathione S-transferase (GST-BP22) or dimeric peptide chemically conjugated to polyLys(Ser-DL-Ala$_m$) (SAK-BP22). The intensity of ELISA reaction was highest against the recombinant fusion antigen GST-BP22; the chemically conjugated SAK-BP22 performed less well than the free dimeric form of the peptide. In the case of the GST-BP22 antigen, the (GST-BP22)-(GST)$_{492nm}$ optical density values were determined. There was no significant difference between the mean ODs of the GST-BP22 and the SAK-BP22 (0.888 vs. 0.892, p= 0.9726). Conjugating the epitope peptide with the synthetic carrier SAK was advantageous, as it abrogated cross-reactivity with GST carrier protein. Consequently, the SAK-BP22 conjugate appears to be the most reliable assay component, avoiding cross-reactivity with GST and simplifying the detection and evaluation of BP autoantibodies in routine ELISA diagnostic system. (Pathology Oncology Research Vol 10, No 1, 52–56)

Keywords: carrier effect, BPAG2 epitope, autoantibodies, immunological recognition, cross-reaction

Introduction

Synthetic peptides or recombinant antigenic peptides are widely used for the detection of autoantibodies reactive to self-antigens in a variety of diseases. However, the replacement of native antigens with shorter synthetic peptide fragments may impair the immunogenicity of the fragment.[2] Covalent bondage with protein carriers facilitates antibody binding to these peptides.[4] An alternative approach is to generate a branched core matrix and use it as a scaffold for subsequent epitope coupling. Recently, recombinant fusion epitopes expressed in bacteria have also been used for the immunological recognition of autoantigens in several assays. These peptides are often expressed as glutathione S-transferase (GST) fused proteins, which are easy to produce and purify. This arrangement approach may also imitate the proper conformation of the epitopes.[6] However, an important feature of all fusion systems is that the detected antibody should not show significant cross-reaction with the fusion protein, thus compromising the specificity of any diagnostic test.

Earlier we have found that recombinant production of BP epitopes in fusion with GST protein furnished an increased sensitivity of ELISA tests.[5] However, 7% of

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patients displayed non-specific reaction with the GST partner.

As the sera of patients with systemic autoimmune diseases frequently cross-react with the GST protein,[8,13] the main goal of our present work was to design and use an optimal epitope-carrier structure of the antigenic epitope of BP2, which abrogates the problematic cross-reaction, yet preserves the previously observed increased sensitivity of the BP ELISA test.

We did not find significant difference of immune sensitivity between the GST-BP22 recombinant fusion protein and the SAK-BP22 branched macromolecular complex. Therefore, it proved to be a good alternative for diagnostic evaluation.

Patients and methods

Twelve representative BP patients who proved to have BP by clinical, histological and immunohistological criteria, and 3 healthy individuals were studied.

ELISA

Microtiter plates (Nunc, USA) were coated with an even amount of antigens relative to the peptide component of each construct in 0.1 M bicarbonate buffer, pH 9.6 overnight at 4°C. The remaining protein binding sites were saturated with PBS-gelatin. The sera from BP patients and control persons were applied at a dilution of 1:100 and incubated for 1 hour at 37°C. Antibody binding was detected with HRPO labeled anti-human IgG (DAKO, Denmark). The reaction was developed using o-phenylene-diamine (Fluka, Germany) in 0.1 M citric acid buffer (pH 5.0) containing 0.02% H_2O_2. The reactions were blocked by sulfuric acid (4 M) and the color reaction was measured with a microphotometer (Dynatech MR 7000, USA) at 492 nm.

Antigens

BP22 and GST-BP22: cDNA coding the BP2 peptide epitope was hybridized together from chemically synthesized, overlapping 5'-phosphorylated oligonucleotides, the nicks were sealed by T4 DNA ligase *(Figure 1)*.[9] The sequence coding for the monomer epitope contained a GST-proximal *BamH*I and a GST-distal *Eco*RI restriction site, and a stop codon inserted between the C-terminal

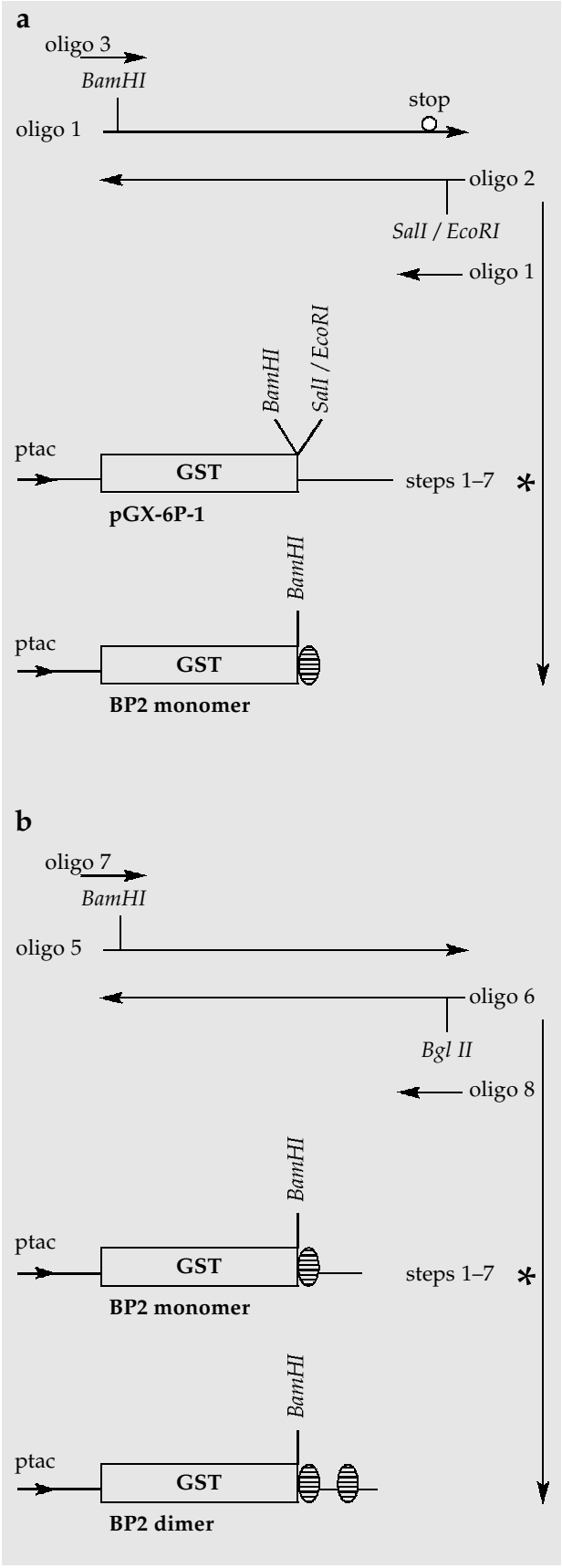


Figure 1. *Schematic diagrams of the monomeric* ***(a)*** *and dimeric* ***(b)*** *construct variants of the fusion-expression plasmids. For details see Patients and methods. (*Synthesis of annealing oligonucleotides, filled-in with Klenow polymerase, PCR amplification, digestion with restriction enzymes, sequencing.)*

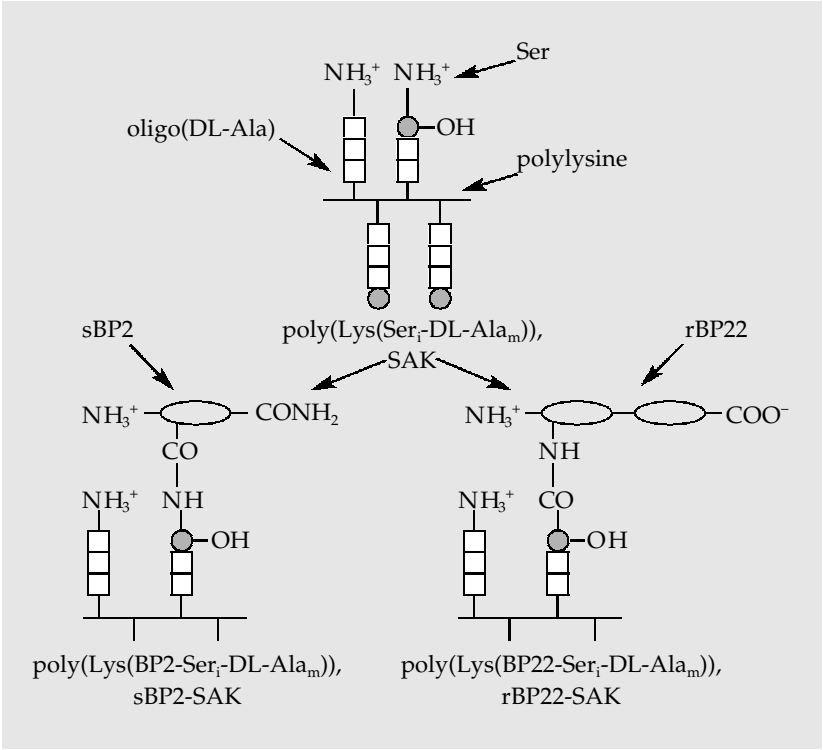


Figure 2. *Schematic presentation SAK and its BP22 dimer conjugates*

amino acid of the epitope and the *Eco*RI site. The synthetic DNA was digested with restriction enzymes and inserted into the expression plasmid pGEX-4T-2, in frame with GST through the *BamH*I site. As a result of the cloning strategy, a Gly-Ser dipeptide coded by the *BamH*I site connects the GST moiety and the epitope. In order to construct fusions with multiple blocks of the epitope, the cDNA coding the second epitope was produced as above, except that the C-terminal amino acid was followed by a Pro-Pro-dipeptide in place of the stop codon and a *Bgl*II recognition site. The *BamH*I and *Bgl*II cut insert was ligated into the *BamH*I cut plasmid coding the monomer fusion. In correct orientation of the insert, the *BamH*I site is regenerated while the hybrid *Bgl*II/*BamH*I site codes the Arg-Ser dipeptide. As a consequence, the two epitope peptides are separated by a Pro-Pro-Arg-Ser tetrapeptide. The expression and purification of recombinant fusion protein (GST-BP22) was performed as described earlier.[7] BP22 was prepared from the GST-BP22 product after enzymatic cleavage with thrombin and separated on a Superdex™ peptide column.

SAK-BP22: Branched polypeptide conjugate of BP22 was prepared by the covalent coupling of peptide to the -amino groups of the side chain terminal Ser residue of the branched polypeptide. Amide bonds were introduced in a random manner by water-soluble carbodiimide using the free COOH group(s) of sBP2 and rBP22 *(Figure 2)*.[3]

Results and Discussion

The autoantibodies in the sera of BP patients bind the BP2 peptide and its various complex variants. The sensitivity of the immune reaction for the BP22 peptide was as follows: GST-BP22 BP22 = SAK-BP22 *(Figure 3)*. Although there were some individual variations, the most intense reactivity (number of sera: 1, 3, 4, 6, 7, 8, and 12) was most often observed against the GST-BP22 construction *(Table 1)*.

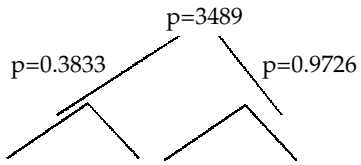
Table 2 presents the mean ODs and the standard deviations of the 12 BP sera and controls of the three different antigenic forms of BP22 peptide. The GST-BP22 and SAK-BP22 exhibited almost equal mean ODs without any significant difference (p= 0.9726). The GST-BP22 dimer

Table 1. **Reactivity of sera from BP patients (No. 1-12) and healthy individuals (No. 13-15) with dimer (BP22) constructs of BPAG2 fragment measured by ELISA**

Antigen	*Serum identification number*														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
BP22	1.082	0.907	0.199	1.006	0.536	0,452	0.788	0.726	0.682	0,854	1.265	0.833	0.023	0.009	0.010
GST-BP22*	1.270	0.941	0.2	1.082	0.478	0.771	0.802	1.078	1.130	0.664	1.225	1.017	0.04	0.005	0.004
SAK-BP22	1.148	1.190	0.180	1.059	0.582	0.662	0.800	0.963	1.145	0.866	1.153	0.963	0.015	0.006	0.029
GST	0.026	0.058	0.045	0.314	0.578	0.441	0.033	0.047	0.301	0.289	0.258	0.246	0.341	0.023	0.015

Abbreviations: GST: glutathione S transferase enzyme; SAK: name of carrier molecule; BP22: Bullosus pemphigoid antigen 2 dimer. * (GST-BP22)-(GST)$_{492nm}$ optical densities were considered.

***Table 2*. The results of the ELISA assays with three different coating antigens**



X Labels	*BP22*	*GST-BP22*	*SAK-BP22*	*NEG*	*GST*
Mean	0.7775	0.8882	0.8926	0.01567	0.2197
SD	0.2891	0.3197	0.2999	0.01247	0.1806

Values indicate the mean OD±SD for the positive (n=12), negative (n=3) sera for the BP22, GST-BP22 and SAK-BP22 antigen forms and also p values are indicated between the corresponding antigen pair. Microplates were coated 0.2 µg/well of BP22, 0.8 µg/well of GST, 1.0 µg/well of GST-BP22 and 0.2 µg/well of SAK-BP22 antigens and incubated with the sera at a dilution of 1:100. (SD: Standard deviation)

construction resulted in a higher sensitivity compared to the dimeric carrier-free epitope BP22, but no significant difference was observed (p=0.3833). The high efficiency of the GST fusion product might be a result of the avidity

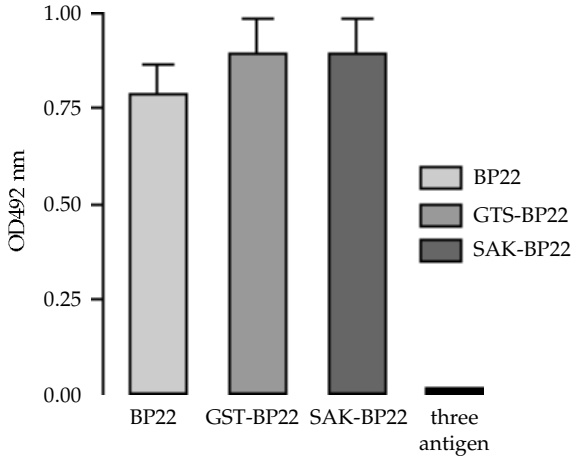


Figure 3. *Seroreactivities of the BP-positive (No. 12) and -negative controls (No. 3) with BP22, GST-BP22 or SAK-BP22 antigens. The fourth column represents the average of OD values of negative sera tested on all three antigens. OD values are mean±SEM of the corresponding group.*

effect of the GST dimer formation (BP22-GST-GST-BP22) rather than ligand affinity due to the tandem repeats.[6] The covalent coupling of the BP22 to SAK also resulted in higher antigenicity of the peptide than that of the free dimeric form, however, mean ODs of BP22 and SAK-BP22 did not show significant difference (p=0.3489). The immunogenicity of SAK-BP22 is similar to GST-BP22, although in this construction only one epitope of the dimeric peptide was accessible to autoantibodies (*Figure 2*). This observation suggests that the presentation of the peptide sequence in the SAK framework performs better than the GST background.

In summary, we have found the SAK-BP22 conjugate to be just as efficient as a diagnostic tool as the GST-BP22 fusion protein. Moreover, GST can be replaced with chemically synthesized carrier polypeptides to avoid the cross-reaction of the carrier molecule with human serum. Our data indicate that for routine diagnostic purposes the branched polypeptide form of the epitope is an attractive test antigen for the serological diagnostics of BP.

Acknowledgements

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