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Insertion of foreign epitopes in HBcAg: how to make the chimeric particle assemble

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Summary. Hepatitis B core antigen is one of the most promising protein carriers of foreign epitopes of various human and animal pathogens. Chimeric HBcAg particles can be used as effective artificial immunogenes. Unfortunately, not all chimeric proteins are able to be particulated. The dependence of correct or incorrect folding of chimeric proteins on physical and chemical properties of inserts was studied with the help of ProAnalyst, SALIX and QSARPro computer programs. We have found that insertion of amino acids with high hydrophobicity, large volume, and high β-strand index prevent self-assembling chimeric proteins. These factors are most important for the C-termini of inserts. Recommendations for obtaining correct folding of chimeric HBcAg particles have been given.

Keywords: Amino acids HBcAg – Foreign epitopes – Self-assemble – Core particles

Introduction

The design of molecular vaccines on the basis of peptides that are epitopes of infection agents is a promising trend in vaccinology. Unfortunately, such peptides exhibit rather low immunogenicity and therefore require coapplication of adjuvant to induce strong immune response. Immunogenicity can be increased via presenting target sequences in several copies on the surfaces of recombinant virus or virus-like particles (Lomonossoff and Johnson, 1995; Ulrich et al., 1998).

Hepatitis B core protein (HBcAg) is one of the most promising delivery vehicles of foreign epitopes suitable for designing highly immunogenic vaccines (Clarke et al., 1987; Pumpens et al., 1995; Schodel et al., 1996). HBcAg consists of identical 21-kDa protein subunits, which are able to spontaneously assemble into a core particle. Recent electron 3D microscopy

suggests that there are two kinds of core particle: 34 and 30 nm in diameter, having T = 4 and T = 3 symmetry and containing 240 and 180 protein subunits, respectively (Crowther et al., 1994).

There are several reasons to use the HBc protein as a carrier of foreign epitopes. First, this can be readily availability in large amounts and is able to spontaneously assemble into a perfect core particle, whether expressed in pro- or eucaryotes. HBc-protein self assembly does not require other viral components. Secondly, HBcAg is highly immunogenic, it induces strong B cell, T cell and CTL responses in human and immunized animals. HBcAg may act either as a T-dependent or as a T-independent immunogen, which directly activates B cells (Milich and McLachlan, 1986). Furthermore, chimeric HBcAg particles enhance the immune response to the inserted foreign epitope, which is presumably because the epitope is presented in many copies on the surface of core particles each containing 180–240 HBc subunits (Clarke et al., 1987).

Foreign epitopes were inserted into HBcAg in various protein regions, including the N-, C-termini and the immunodominant e1 loop (Pumpens et al., 1995). It has been demonstrated that the loop in the main determinant of the core antigen is the most promising insertion site from the immunological point of view (Schodel et al., 1991; Karpenko and Ilyichev, 1998). Epitopes inserted there possess higher antigenicity and immunogenicity than anywhere else. Unfortunately, not all of chimeric proteins are able to be particulated. The ability of chimeric HBcAg to self-assemble is therefore most likely to depend on the physical and chemical properties of the amino acid residues forming the inserted foreign peptide. Although the problem is well known, the dependence has not been studied in detail. Herein we report correlation between various properties of amino acids inserted in the e1 loop and the ability of chimeric HBcAg to assemble into virus particles.

Material and methods

Peptides were inserted in the immunodominant e1 loop of HBcAg. For convenience, those not precluding particle assembly are referred to as "positive"; those doing so as "negative"; the chimeric particles that can still assemble as "viable"; throughout.

Analysis of amino acid sequences of the inserts was performed using the programs ProAnalyst (Eroshkin et al., 1995), SALIX and QSARPro (Ivanisenko, 1998), of which the latter two are our recent developments.

ProAnalyst is a software tool for studying the structural and functional organization of proteins and the correlation of structure and activity. Also, it predicts functionally important amino acid substitutions in peptide alignments or protein alignments, calculates a large number of physical and chemical properties of sites in primary and tertiary protein structures, specifies those important with respect to activity and plots the structure/activity dependencies.

QSARPro handles ProAnalyst output data on structure/activity relationships and generates a list of amino acid substitutions deemed optimal for the user to attain the desired modification of protein activity.

ProAnalyst and QSARPro have statistical data processing capabilities thereby enabling multiple regression, discriminant and variance analyses.

SALIX performs multiple structure alignment of protein sequences, calculates structural parameters and outputs physical and chemical profiles and multiple alignment data in a handy format. SALIX performs multiple alignments of primary and secondary structures, physical and chemical profiles.

We used these programs to calculate the correlation between the physical and chemical properties of the inserts and the viability of chimeric particles. Statistical data processing was performed using multiple linear regression, discriminant and variance analyses. Both methods yielded similar results, we will only refer to those of regression analysis throughout. To enable regression analysis, 1 was assigned to the ability to assemble, and 0 to failure to do so.

The study was conducted on a large variety of physical and chemical properties of amino acids, namely hydrophobicity, volume and polarity (Bogardt, 1980), Chou-Fasman parameters (propensity for α-helices, β-strands, β-turns) (Chou and Fasman, 1978), charge and others. Physical and chemical properties of the inserts were calculated by averaging those of their amino acid sequences.

Results

Analysis of relationships between amino acid factors and the viability of chimeric core proteins

All the information on positive and negative inserts available to us was gathered into a database (Table 1).

As can be seen, the lengths of positive inserts vary between 4 and 111 amino acid residues, which suggests that this parameter is not a factor in the dilemma "to fold or not to fold". In the face of it, length was not included in the analysis.

The search for a relationship between insert structure and core particle viability was conducted using a sliding frame of a length ranging from 1 to the full length of the inserts in the course of analysis of the physical and chemical properties of both full-length sequences and their fragments.

Analysis of full-length inserts revealed a correlation of viability with the β-strand index, hydrophobicity and volume of the amino acids in the inserts. Where the values of these insert parameters are high, the chimeric HBcAg particles are normally not viable. The correlation coefficients fall within an interval between 0.4 and 0.53.

The most convincing correlation was revealed for region 1–7 of the C-terminus (see Table 1). From among all the correlates found, the most statistically significant are the β-strand index, hydrophobicity and volume. Not only does the same refers to the full-length sequences, but their respective dependencies behave alike: there higher the values, the lower the viability of chimeric proteins (see Fig. 1).

These data provide further support to the hypothesis about an important role of the hydrophobicity and β-strand index of the insert in the preservation of the native conformation of chimeric proteins (Gren and Pumpen, 1988; Makeeva et al., 1995).

Noteworthy, no statistically significant correlation were found for the N-termini of the inserted peptides. It is likely that the structure of a native protein imposes by far less harsh structural requirements on the N-terminus than on the C-terminus of the inserts.

Table 1. Sequences of inserts into immunodominant loop of HBcAg

No	Name#	Refs.	Sequence of inserted peptide	Viability
*	e1 loop of HBcAg	Argos et al. (1988)	**(74)**VNLEDPASRDLVVSYVNTN**(92)**	1
1.	pnsd	Nassal (1988)	VNLED**pnsd**PASRDLVVSYVNTN	1
2.	HPV 16 ET	Tindle et al. (1994)	VNLEDPAS**eymldas**RDLVVSYVNTN	1
3.	HBV HBsAg	Karpenko (1998)	VNLEDPI**Sictkptdgncs**RDLVVSYVNTN	1
4.	HBsAg preS2	Schodel et al. (1990)	VNLEDPASR**dprvrglyfpa**DLVVSYVNTN	1
5.	HRV2 VP2	Clarke et al. (1991)	VNLEDPAS**vkaetrlnpdlqptecas**RDLVVSYVNTN	1
6.	HIV gp41	Clarke et al. (1991)	VNLEDPAS**drpegieeeggerdrdrsas**RDLVVSYVNTN	1
7.	HPV 16 ET	Tindle et al. (1994)	VNLEDPAS**edeidgpagqaepdrahynias**RDLVVSYVNTN	1
8.	HPV 16 ET	Tindle et al. (1994)	VNLEDPAS**eymldagidgpagqaepdrahynias**RDLVVSYVNTN	1
9.	HBV preS1	Makeeva et al. (1995)	VNLEDPIS**rvgvdpafradprvrglyfpardpis**RDLVVSYVNTN	1
10.	HBV preS1	Makeeva et al. (1995)	VNLEDPIS**rvgvpastnrqsgrqpdprvrglyfpardpis**RDLVVSYVNTN	1
11.	HBV HBsAg	Borisova et al. (1993)	VNLED**gfpgssttstgpcrtcttpaqgtsmypsccctkpsdgnctc**RDLVVSYVNTN	1
12.	SPAG-1	Boulter et al. (1995)	VNLEDPAS**lvkdvseehqvgigdlsdpssrtpnakpaelgpslviqnvpsdpskvtptqpsnlp qvpttgpgngtdgtttgpggngeggkdlkegekkeglfqkiknkllgsgfevaas** RDLVVSYVNTN	1
13.	FMD(A22)V VP1	Karpenko (1993)	VNLEDPIS**rhkqkiiaps**RDLVVSYVNTN	0
14.	VEE E2	Karpenko (1998)	VNLEDPIS**indkwvynss**RDLVVSYVNTN	0
15.	LCMV gp272–293	Schodel (unpubl.)	VNLEDPASR**lsdssgvenpggycltkwmila**DLVVSYVNTN	0
16.	HPV 16 ET	Tindle (unpubl.)	VNLEDPAS**edeidgpagqaepdrahynivtfcckas**RDLVVSYVNTN	0
17.	HBsAg preS1	Schodel et al. (1991)	Vn**dhqldpafgansnnpdwdfnpvkddwp**SRDLVVSYVNTN	0
18.	HBV preS2	Makeeva et al. (1995)	VNLEDPIS**rvgvdprvrglyfpadprvrglyfpardpis**RDLVVSYVNTN	0
19.	FMDV(A22)VP1	Nekrasova (unpubl.)	VNLED**hldpngtgkysaggmgrrgdleplaarvaaqlpwyvlvd**PISRDLVVSYVNTN	0
20.	HBV preS1 × 3	Makeeva et al. (1995)	VNLEDPIS**rvgvpastnrqsgrqppastnrqsgrqppastnrqsgrgpdprvrglyfpardpis** RDLVVSYVNTN	0

#Source of foreign epitope; *1* viable insert; *0* not viable inserts; *the region of HBcAg loop to be inserted. Capital letters indicate HBc sequence; small letters correspond to inserted sequences. C-terminal parts of inserts are underlined.
HBV hepatitis B virus; *HBcAg* core antigen of HBV; *HBsAg* surface antigen of HBV; *HPV* human papilloma virus; *HIV* human immunodeficiency virus; *VEE* Venezuelan equine encephalomyelitis virus; *FMDV* foot and mouth disease virus; *SPAG* theileria annulata sporozoite antigen; *CMV* cytomegalovirus; *HRV* human rhinovirus.

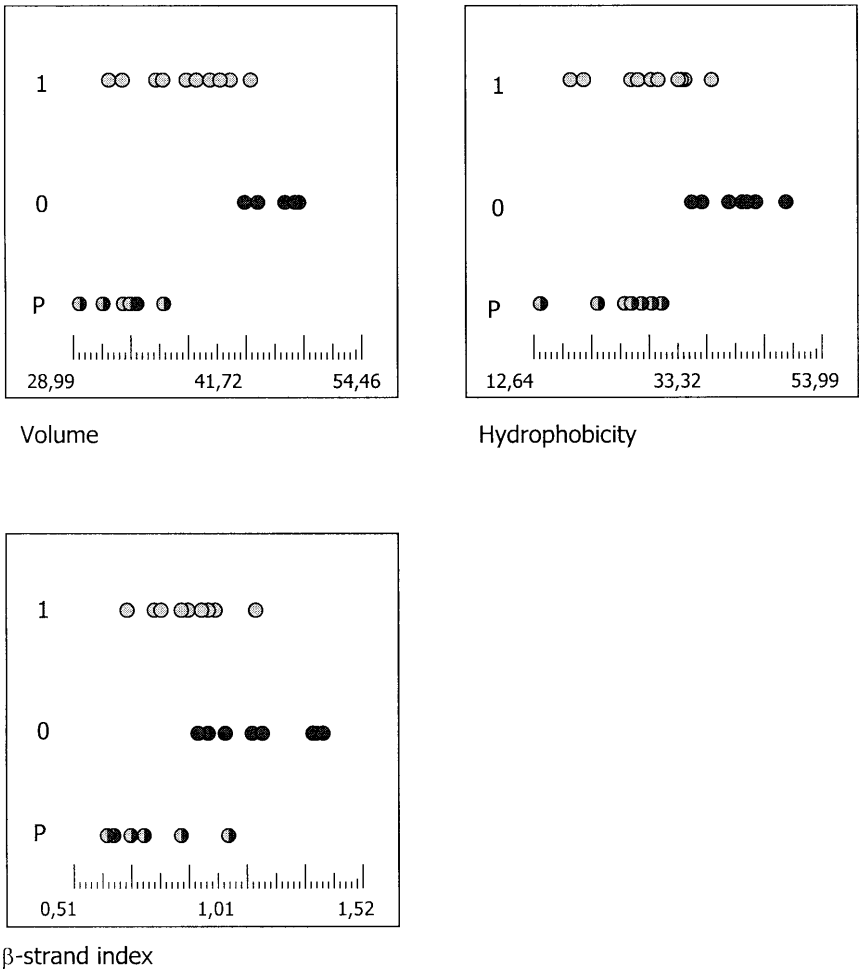

Fig. 1. The relationship between physical and chemical properties of inserts and ability of chimeric HBcAg proteins to self-assemble (by computer program ProAnalyst, SALIX, QSARPro). *1* positive insertions (◎); *0* negative insertions (●); *P* predicted negative inserts (◑) with GSGDEGG spacer at the C-terminus

Discussion

Analysis of the carrier protein structure
How to make the chimeric particle assemble

There are different ideas of what the tertiary structure of the hepatitis B core protein is like. Argos and Fuller (Argos and Fuller, 1988) describe HBcAg as mostly β-strand protein, whereas Crowther et al. (Crowther et al., 1994) see it mostly α-helical. Despite this difference, the authors agree that spontaneous assembly is preceded by the formation of the HBc dimers. Furthermore, the 76–87 fragment of the main antigen determinant of HBcAg is a loop under either model.

According to the Crowther et al. (Crowther et al., 1994), the loop region, into which peptides were inserted, has high conformational flexibility. The

result of it, we believe, should be a higher probability of the loop being in the contact region of two dimer subunits, provided that the inserts have high hydrophobicity. Obviously, the probability of a perfect dimer is much lower under these circumstances. If the contact region include two loop regions with hydrophobic inserts within, a perfect dimer is yet less probable. The fact that one of the factors influencing the viability of chimeric particles is the hydrophobicity of the inserts does corroborate the hypothesis (Fig. 1).

We propose that the problem of assembly in the situation as described above can be solved by using mosaic particles. Any such particle would consist of two types of dimer, natural and chimeric. Any chimeric dimer, in turn, would consist of different molecules, one native and one chimeric. In this case, a preference for the contact region would only be attributable to the loop region of the subunits carrying the insert, because the loop of the native core protein is hydrophilic.

This hypothesis was verified on a negative insert, which was the Venezuelan equine encephalomyelitis (VEE) E2 epitope. According to our calculations, the VEE epitope has high hydrophobicity, which may account for the inability of chimeric HBcAg-VEE to self-assemble. To produce mosaic capsids, E.coli cells carrying the plasmid that codes for the chimeric HBc-VEE protein were infected with a bacteriophage carrying the native HBcAg gene. Following simultaneous expression of the two genes in one E.coli cell, viable mosaic particles were produced (Loktev et al., 1996).

As was demonstrated above, particle viability also depends on the tendency of inserts to form β-strand. As is known, spatially close linear regions in proteins with high β-strand index can form β-sheets. Naturally, we wanted to see if the HBcAg sequence contains β-strands indeed. As was noted, HBcAg secondary structure is a point at issue (Argos and Fuller, 1988; Crowther et al., 1994). The prediction we did using the PHD program (Rost and Sander, 1994) favours the α-helicity hypothesis (see Fig. 3). As to the location of β-strand regions, one of them (Fig. 2) adjoins loop region 76–85. When the peptide is inserted at position 81, its C-terminus adjoins this region too. If the C-terminus has a high preference for β-strand, there is a possibility of insertion regions and carrier regions forming a β-sheet. We assume that this structural element is capable of preventing assembly. The fact that most of the negative inserts has high β-strand in the C-terminal region provides further support to this idea (Fig. 1).

In our opinion, if the β-strand index is high, there are two solutions to the problem. For instance, β-sheet can be prevented by removing the β-stranded fragment of HBcAg. This was verified by experimenting with a negative insert carrying the epitope of the VP1 protein of the foot and mouth disease virus (FMDV) (see Table 1). Insertion of the FMDV epitope renders the chimeric core protein insoluble and detectable only in inclusion bodies. When region 80–90 of HBcAg (presumably, β-stranded) was removed, the chimeric HBcAg-FMDV protein became soluble and able to self-assembly (Karpenko, 1993).

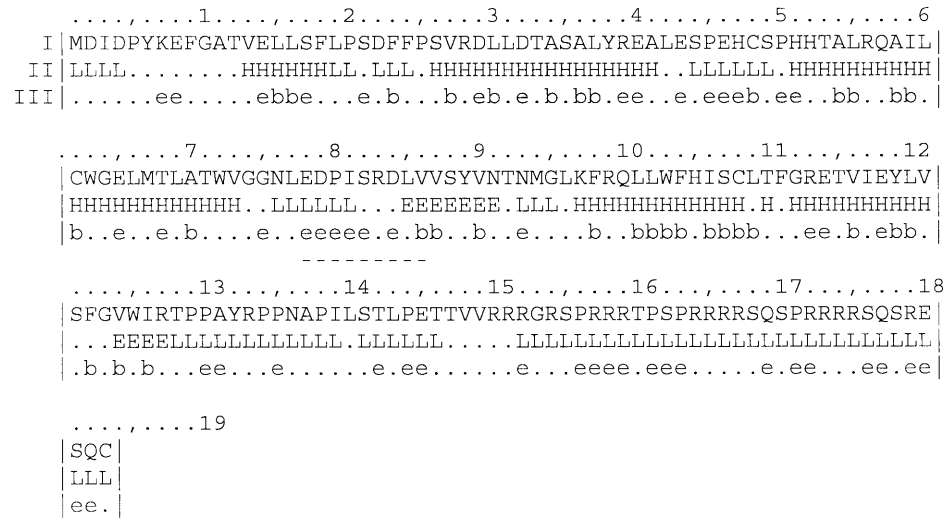

Fig. 2. The secondary structure of HBcAg predicted by PHD program. *I* HBcAg amino acids sequence; *II*, secondary structure of HBcAg. Loop signed by *L*; helices by *H*; beta strand by *E*; *III* buried amino acids signed by *b*; exposed ones by *e*

Alternatively, the problem can be solved by using a spacer between the C-terminus of the inserted peptide and the native HBcAg protein. As was mentioned above, it is conditional on such spacers not containing large and hydrophobic amino acids or those with high β-strand index. With these restrictions in mind, we attempted to predict the optimal sequence of a spacer peptide on the basis of the relationships found. The prediction was based on regression equations and classification by Mahalanobis distances, all the procedures were run by QSARPro and ProAnalyst. The spacers selected as eligible by both programs were qualified. In essence, the procedure was a step-by-step filtering of candidate peptides. Because it was the 7 aa C-terminal fragment that proved most significant for spontaneous assembly, the sample comprised random peptides of the indicated length and homologous to the C-terminal fragments of positive inserts. Viability was calculated by regression equations (1–3) for each of these peptides. The candidates were checked for conformity to two requirements: first, viability in excess of 0.8 (which corresponds to viable proteins) following calculations by no matter which equation; second, the physical and chemical properties close to those of the positive inserts.

Eventually, we came up with few peptides, and one of them is GSGDEGG. Our calculations suggest that the fusion of this peptide to the C-termini of negative inserts imparts viability to chimeric proteins (see Fig. 1). Similar results were obtained for other predicted spacer peptides. The one we disclose can be helpful in designing chimeric proteins carrying foreign epitopes.

We hope that our approaches will be helpful to investigators interested in chimeric HBcAg engineering.

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