Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 9477–9481, August 1998
Immunology

T cell-independent type I antibody response against B cell epitopes expressed repetitively on recombinant virus particles

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Contributed by Rolf M. Zinkernagel, June 1, 1998

ABSTRACT **Recombinant viral or virus-like particles offer new tools for vaccine development. This study investigated hepatitis B core antigen (HBcAg) capsids and RNA phage Qβ coats as carriers of a foreign epitope to induce antibody responses in mice. HBcAg capsids were shown to induce T cell-independent (TI) antibodies. We found that these particles behave as antigen-specific TI type 1 (TI-1) Ag comparable to other rigidly structured viruses. When a 5-aa long epitope of the pre-S1 domain of hepatitis B surface antigen (HBsAg) was introduced into the optimal position of the HBc molecule, it also behaved as a TI-1 Ag. Best efficiency of the antibody response to the foreign epitope was achieved by a compensatory deletion after the epitope to retain the regular structure of the HBcAg capsid with a highly repetitive superficial exposition of the foreign epitope. For recombinant Qβ phage coats, a much more efficient antibody response to the foreign epitope was achieved when the foreign epitope was expressed repetitively on a particulate derivate of Qβ phage coats. Thus, recombinant virus particles are suitable vaccine carriers for the introduction of foreign B cell epitopes, if precise structural requirements are fulfilled.**

Specific neutralizing antibodies play a major role in protection of a host from primary and secondary infections with viruses and bacteria (1). Whereas some pathogens induce life-long protection against reinfection upon the first contact with the host [e.g., measles or poliovirus (2)], others induce and maintain protective antibody titers poorly. Whereas protective B cell memory may be impaired because of insufficient persistence of the antigen on follicular dendritic cells (3) or because of generation of antibody escape variants (4), low induction of specific antibodies sometimes results from lack or insufficient presentation of B and T helper cell epitopes during the first contact with the host.

B cell activation is known to involve two signals (5): an antigen-specific first signal delivered via cross-linking of surface Ig receptors and a second signal normally delivered by T helper cells. This model perfectly describes B cell responses to classical proteinaceous usually mono- or oligomeric T cell-dependent (TD) antigens. It suggests that, if a suitable class II-binding epitope is lacking or if arrangement of B cell epitopes does not allow cross-linking of surface Ig receptors, the B cell response will be weak. Several antigens that activate B cells independently of T helper cells have been described. They can be divided into two groups (6): T-independent type 1 (TI-1) antigens activate B cells without the need of second signal, either in a polyclonal (prototype LPS) or an antigen-specific fashion (several viruses as vesicular stomatitis virus (7, 8) or poliovirus); in contrast, T-independent type 2 (TI-2) antigens need residual noncognate T help for activation of B cells [polymeric antigens as dextran or bacterial polysaccharides (9)]. These two groups can be distinguished by immunization of neonatal mice or of mice carrying an x-linked immunodeficiency (*xid*) (10). TI-1 Ag are able to induce antibody responses in these mice, whereas TI-2 Ag are not. Analysis of haptenated polymers (11), *Salmonella* flagellin (12), hepatitis B virus (HBV) c vs. e antigen (13), and vesicular stomatitis virus (7) have shown that the degree of epitope repetitiveness and the spacing of the epitopes determine the degree of T cell independence of the primary IgM antibody response.

The observation summarized above suggested that, if a new antigenic determinant could be introduced into an optimally spaced array of identical antigenic determinants, B cell responses to the new epitope should be very efficient (14). We therefore studied antibody responses to a 5-aa long epitope of a TD antigen, namely the immunodominant epitope of the pre-S1 domain of HBsAg, expressed either within the icosahedral capsids of HBcAg, a known highly immunogenic TI antigen (15), or within particulate derivatives of Qβ phage coats.

MATERIALS AND METHODS

Mice. BALB/c, C57BL/6, and ICR nude mice were obtained from the breeding colony of the Institut für Labortierkunde, Veterinary Hospital, Zurich, Switzerland. CBA and CBA*xid* mice were purchased from Harlan Breeders, Indianapolis. Breedings and experiments were performed under specific pathogen-free conditions. Mice were used at 8–12 weeks of age.

Recombinant Particles and Immunization Procedures. Recombinant natural and chimeric HBcAg capsids and Qβ phage coats were generated as described (16–19). They were stored at −20°C. For immunization, protein solutions were diluted with balanced salt solution to inject 50 μg in a volume of 200 μl i.v. For immunization with adjuvants, protein solutions were diluted 1:1 with complete or incomplete Freund's adjuvant and injected s.c. at the base of tail (CFA) or i.p. (IFA). Usually, a secondary immunization with the same amount of protein and the same application route was performed after 12–14 days because IgG responses to the introduced foreign epitope were only detectable after a booster injection.

***In Vivo* CD4 Depletion.** *In vivo* CD4-depletion was performed by i.p. injection of two doses of 1 mg of anti-CD4 antibody YTS 191.6 (the hybridoma line was a generous gift of H. Waldmann, Oxford, UK) 3 days and 1 day before immunization. Efficiency of depletion was checked by FACS analysis

Abbreviations: Ag, antigen; HBcAg, hepatitis B core antigen; HBsAg, hepatitis B surface antigen; TD, T cell-dependent; TI-1/2, T cell-independent type 1/2.

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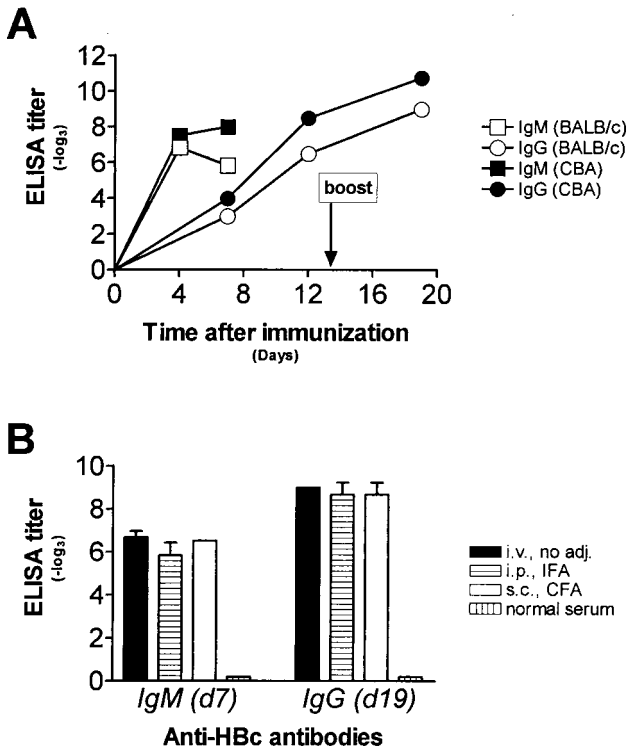

FIG. 1. Antibody response to HBcAg in different mouse strains and after various immunization procedures. (*A*) BALB/c (open symbols) and CBA (closed symbols) mice were immunized with HBcAg on day 0 and boosted with the same amount on day 13. IgM (squares) and IgG titers (circles) were determined in an ELISA assays on plates coated with HBcAg. Each data point represents the mean of three mice. (*B*) BALB/c mice were immunized with HBcAg as mentioned above either i.v. (black bars), i.p. in incomplete Freund's adjuvant (horizontally striped bars), or s.c. in complete Freund's adjuvant (open bars). Primary IgM titers on day 7 and secondary IgG titers on day 19 were determined by ELISA. Titers are indicated as $-\log_3$ of 20-fold prediluted sera. Each bar represents the mean of two to three animals; experiments were repeated twice.

from peripheral blood, and $CD4^+$ T cells were below detection level.

ELISA Assay. We used a sandwich ELISA with the following steps: (*i*) coating with natural or chimeric proteins (7 μg/ml) or with the preS1 peptide 21-PLGFFPDHQLDPAFRAN-TANPDWDFNP-47 derived from HBsAg (10 μg/ml), (*ii*) blocking with 2% BSA (Fluka) in PBS, (*iii*) 20-fold prediluted mouse serum titrated 1:3 over 8 dilution steps, (*iv*) isotype (IgM or IgG)-specific horseradish peroxidase-labeled goat anti-mouse antibodies (0.5 μg/ml; Southern Biotechnologies), (*v*) substrate ABTS (2.2′-Azino-di-[3-ethylbenzthiazoline-sulfonate]; Boehringer Mannheim) and H_2O_2 (Fluka). Plates were coated overnight at 4°C; all other incubations were for 60–90 min at room temperature. Between incubations, plates were washed three times with PBS containing 0.5 ml of Tween 20 per liter. OD was measured at 405 nm in an ELISA reader. All antibody titers are indicated as $-\log_3$ of 20-fold prediluted sera. Titers were read at the dilution step of half maximal OD.

RESULTS

HBcAg Capsids Are Potent Immunogens Under Various Conditions. HBcAg capsids have been shown earlier to be excellent B cell immunogens (15). To determine the kinetics of the immune response as well as the possible role of the mouse strain or application route, we immunized mice with 50 μg of natural HBcAg capsids on day 0 and boosted them with the same amount on day 13. Antibody titers were determined on days 4, 7, and 12 after primary immunization and on day 6 after secondary challenge and were analyzed in standard ELISA assays. The kinetics of the response to HBcAg were comparable in BALB/c ($H\text{-}2^d$), CBA ($H\text{-}2^k$; Fig. 1*A*), and C57BL/6 ($H\text{-}2^b$) mice (data not shown). A peak of the specific IgM response was reached between days 4 and 8. It efficiently switched to IgG after 7 days. Titers up to 1/20,000 were reached by one booster injection. We found titer differences of 3- to 10-fold between mouse strains, CBA ($H\text{-}2^k$) mice being the best, confirming earlier results (15). The role of adjuvants and different application routes was analyzed in BALB/c mice (Fig. 1*B*). No significant difference between immunizations with CFA s.c., IFA i.p., and without adjuvant i.v. were detected, so all of the following experiments used i.v. immunizations without adjuvants.

Generation of Chimeric HBcAg Capsids and Qβ Phage Coats. The technique for generation of chimeric HBcAg capsids (16, 17) as well as Qβ phage coats (18, 19) recently has been described elsewhere. Highly ordered repetitive antigens such as repetitive polymers (11) or rigidly structured viral envelope glycoproteins (20) are excellent B cell inducers. Also, HBcAg capsids are potent antibody inducers (15) because of their highly repetitive paracrystalline structure with regular spikes in a distance of about 10 nm (21). This was elucidated in detail by high resolution electron cryomicroscopy (22, 23). We therefore used chimeric particles, where the immunodominant 5-aa long epitope 31-DPAFR-35 of the pre-S1 domain of HBsAg was introduced into HBcAg (16) or Qβ phage (18) proteins (Table 1). In the case of *HBcAg*, the epitope was introduced at the position 78 that seems to be located on the tip of the spikes of the HBcAg capsids (22). Whereas in the chimeric HBcAg capsid II-116 the epitope was introduced into the normal HBcAg sequence without other changes, in the capsid S2–16 a compensatory deletion after the new epitope was introduced in addition (Table 1). As a result, in S2–16 capsids, the inserted epitope was indeed located on the tips of the spikes and therefore was accessible to a monoclonal

Table 1. Chimeric HBcAg capsids and Qβ derivatives used in this study

A HBcAg capsids*	Amino acid sequence starting from position 71								
	71	76				81	86		
Natural HBcAg	WVGGN	LED			PI	SRDLV	VSYVN		
II-116	WVGGN	LED		**PAFRAQD**	PI	SRDLV	VSYVN		
S2-16	WVGGN	**LED**	**HDHVDPAFRYVDH**		- -	- - - - -	- - - VN		
B Qβ derivatives†	**Amino acid sequence starting from position 146**								
	146	151	156	161	/	196	201	206	
Natural Qβ	KPDPV	IPDPP	IDPPP	GTGKY	/	QPREF	DVALK	D	LLGN
Qβ 18-25	KPDPV	IPDPP	IDPPP	GTGKY	/	QPREF	DVALK	D **PAFR**AQD	LLGN
Qβ Kpn	KPDPV	IPDPA **FR** (*Stop*)							

Bold, newly introduced sequences; DPAFR, immunodominant epitope derived from the preS1 domain of HBsAg; —, compensatory deletion.
*Refs. 16, 17, and 34.
†Refs. 18 and 19.

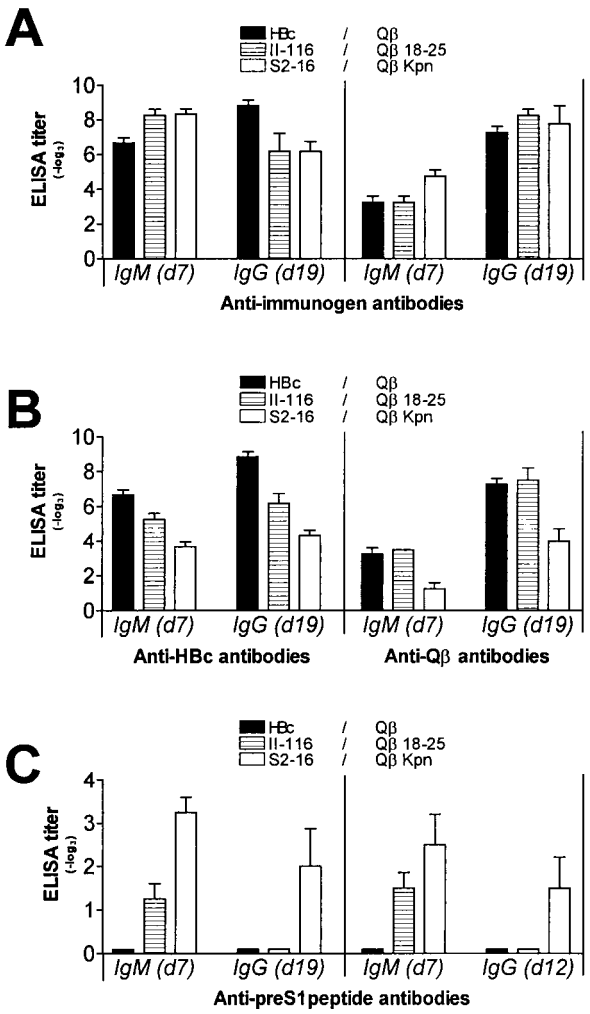

FIG. 2. Specificity of the antibody response to chimeric HBcAg and Qβ proteins. BALB/c mice were immunized twice (interval 12 days) with either natural HBcAg (black bars, left panels) or Qβ particles (black bars, right panels), chimeric proteins II-116 (striped bars, left), S2–16 (open bars, left), Qβ 18–25 (striped bars, right), or Qβ Kpn (open bars, right). Primary IgM (d7) and secondary IgG (d19) antibodies were measured on ELISA plates coated with the respective protein used for immunization (*A*), with natural HBcAg (*B*, *Left*) or Qβ (*B*, *Right*), or with the peptide preS1 peptide 21–47 (*C*) containing the immunodominant epitope DPAFR from HBsAg. Titers are indicated as $-\log_3$ of 20-fold prediluted sera. Each bar represents the mean of two to three animals; one of two experiments.

anti-preS1 antibody; in contrast, it was inaccessible to this antibody in II-116 capsids (16). In the case of *Qβ derivatives*, the epitope was introduced at various positions of the A1 extension of the Qβ coat (18, 19) (Table 1). The Qβ Kpn protein led to the formation of nonicosahedral regular particles differing in shape from natural icosahedral Qβ phage coats. In contrast, the Qβ 18–25 protein failed to form particles and exists as monomers.

Specificity of the Antibody Response to Chimeric HBcAg Capsids and Qβ Proteins. The specificity of the antibody response to recombinant natural and chimeric HBc and Qβ proteins was analyzed by ELISA assays. Mice were immunized i.v. with 50 μg of the respective proteins and boosted after 13 days with the same amount. IgM titers 7 days after primary (d7) and IgG titers 6 days after secondary (d19) immunization are shown in Fig. 2. The left half of the panels shows titers of mice immunized with various HBcAg capsids; in the right half, the titers of mice immunized with recombinant Qβ phage coats are shown. For the analysis in Fig. 2*A*, ELISA plates were coated with the same protein used for immunization of the respective mice (referred to as "immunogen"). The results show that overall immunogenicity of the recombinant proteins was comparable to the natural HBcAg and Qβ particles; a slight reduction of IgG titers by a factor 3 to 9 was found for chimeric compared with natural HBcAg capsids. To analyze the specificity of the antibody response, sera were analyzed on ELISA plates coated with either natural HBcAg or Qβ capsids, respectively (Fig. 2*B*) or on plates coated with the preS1 peptide 21–47, which contains the newly introduced preS1 epitope of HBsAg (Fig. 2*C*). In both cases (HBcAg and Qβ), induction of an antibody response to the new epitope was strictly correlated to a reduced response to the natural particle. This suggested that the foreign epitope was in fact placed at the best position for activation of B cells and that the new determinant was able to cross-link surface Ig receptors. Of interest, only the S2–16 capsids and Qβ Kpn particles were able to induce IgG antibodies to the foreign epitope, in contrast to II-116 capsids and nonparticulate Qβ 18–25 coats. In the case of S2–16 capsids, the equally long compensatory deletion after the new epitope may allow an appropriate folding of the altered HBcAg spikes that are responsible for the high immunogenicity of the particle. The superior immune response to the foreign epitope in the particulate Qβ Kpn compared with the nonparticulate Qβ 18–25 structures also fits the hypothesis that the capsid-like structure, but not the monomeric form, allows a highly repetitive arrangement of the new epitope.

T Cell Dependence of Antibodies to HBcAg and S2–16 Capsids. HBcAg capsids are known to be potent TI antigens (15). To examine whether this is also true for the chimeric capsids, we immunized ICR nude mice with natural HBcAg or chimeric S2–16 capsids and measured primary IgM and secondary IgG antibodies (Fig. 3*A*). The IgM and IgG antibody titers to HBcAg induced by S2–16 were $\approx$10-fold reduced compared with those induced by natural HBcAg in nude mice, but both IgM responses were T cell-independent. Also, the IgM reponse against the newly introduced foreign epitope assessed by peptide ELISA (Fig. 3*A*, right half) was TI, whereas the IgG response was clearly TD.

To further distinguish between TI-1 and TI-2 antibodies (10), we immunized CD4-depleted CBA mice carrying an x-linked immunodeficiency (*xid*) and compared them with normal CBA or CBA*xid* mice not depleted of T cells (Fig. 3*B*). Natural HBcAg, chimeric S2–16 capsids, and the new epitope introduced into S2–16 induced specific TI-1 IgM responses in these CD4-depleted CBA*xid* mice. As expected, IgG responses to S2–16 and the preS1 peptide 21–47 were TD, whereas those to natural HBcAg were largely TI-1; this TI-1 IgG response against HBcAg clearly was reduced by a factor of at least 100-fold in absolute titers. Similar results have been found with the paracrystalline virus surface determinants of polyoma virus (24) and vesicular stomatitis virus (7), where a noncognate interferon γ- and tumor necrosis factor α-dependent bystander T help was sufficient for IgG induction (25).

In summary, HBcAg capsids are TI-1 antigens for IgM and IgG antibody responses, whereas S2–16 particles and especially the newly introduced foreign epitope is a TI-1 antigen for IgM and a TD antigen for IgG responses.

DISCUSSION

There are several possibilities to improve the immunogenicity of an antigen or a specific epitope. Whereas in the past, different kinds of adjuvants were tested, molecular biology has now offered tools to construct recombinant proteins and even whole virus particles expressing foreign epitopes. As shown here, this allows us to augment immunogenicity of a certain epitope by putting it into the structural context that cross-links B cell receptors optimally.

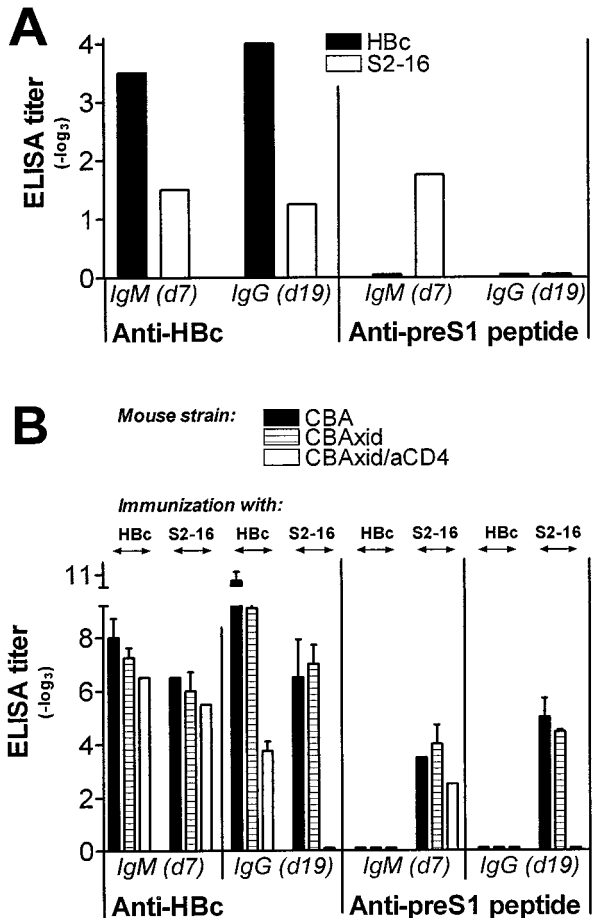

FIG. 3. T cell dependence of antibody responses against HBcAg und S2–16. (*A*) ICR nude mice were immunized twice (interval 13 days) with either HBcAg (black bars) or S2–16 (open bars) particles, and primary IgM and secondary IgG antibody titers against natural HBcAg (left) or preS1 peptide 21–47 (right) were determined by ELISA at the indicated time points. Each bar represents the mean of titers in three animals. Variations were within ±1 dilution step. One of two experiments. (*B*) CBA (black bars), CBA*xid* (striped bars), and $CD4^+$ T cell-depleted CBA*xid* (open bars) mice were immunized twice (interval 13 days) with either HBcAg or S2–16 capsids as indicated, and primary IgM or secondary IgG antibodies to natural HBcAg (left) or preS1 peptide 21–47 (right) were determined by ELISA on the indicated time points. Each bar represents the mean of titers of two to three animals.

HBcAg was shown to behave as a highly immunogenic TI and TD antigen (15), and therefore it was an attractive candidate for introduction of foreign epitopes (14, 26). The high immunogenicity of HBcAg relies on its structural qualities with repetitive spikes on the surface to induce TI responses (21) but also on the very efficient presentation of HBcAg by B cells to induce TD responses (27). The technical feasibility of the construction of chimeric HBcAg capsids was demonstrated 10 years ago (28). Several foreign epitopes from hepatitis B virus (16), HIV (29), Papillomavirus (30), and Plasmodium falciparum (31) have been introduced successfully into HBcAg capsids, and immunogenicity of the new epitopes has been demonstrated. Our results here add the following new findings: (*i*) HBcAg capsids behaved as antigen-specific TI-1 particles comparable to other viruses [as vesicular stomatitis virus, polyoma virus, poliovirus (32, 33)] but are different from polyclonal activators such as lipopolysaccharide; (*ii*) A known TD epitope (in our case from the preS1 domain of HBsAg) could be introduced into TI-1 HBcAg capsids and thereby could be changed into a TI-1 antigen, which induced an early IgM response within 4 days totally independent of T cell priming; (*iii*) In the case of HBcAg capsids as well as particulate Qβ phage coats, precise structural requirements allowing repetitive arrangement of the new epitope on the particle surface had to be fulfilled to induce TI antibody responses; for Qβ Kpn proteins, it was linked to the particulate structure of the phage coat, whereas for the chimeric HBcAg capsid S2–16 an equally long compensatory deletion had to be introduced after the new epitope to retain the overall structural integrity of the particle and to allow the optimal superficial positioning of the epitope on the tips accessible to B cell receptors. These results supplement two studies on chimeric HBcAg showing that the position for induction of new epitopes is crucial for immunogenicity (34, 35), and they confirm earlier studies with other viruses showing that a repetitive paracrystalline structure strongly facilitated induction of TI antibody responses (7). Many acute infectious agents exhibit highly repetitive antigen determinants in their envelope. At least in the case of cytopathic pathogens, a very early and efficient neutralizing IgM response seems to be important for survival of the host from acute infection (33).

We conclude that inert and therefore safe recombinant HBcAg or Qβ phage particles are attractive candidates for vaccine carriers that are able to augment the immunogenicity of a protective B cell epitope. However, precise structural requirements must be fulfilled for induction of efficient antibody responses.

We are very thankful to Galina Borisova and Olga Borschukova for chimeric HBcAg capsids as well as to Tatyana Kozlovska, Indulis Cielens, Inta Vasiljeva, and Regina Renhofa for chimeric Qβ proteins. We thank Uli Kalinke and Elmars Grens for helpful discussions and Alana Althage and Juris Ozols for excellent technical assistance. This work was supported by the Swiss National Science Foundation (Grant 31-32195.91 to R.M.Z.), the Kanton of Zürich, and the Latvian Council of Sciences (Grants 96.0731 and 96.0738).

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