Simultaneous expression of recombinant baculovirus-encoded chicken anaemia virus (CAV) proteins VP1 and VP2 is required for formation of the CAV-specific neutralizing epitope

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Chicken anaemia virus (CAV) expresses three proteins, VP1, VP2 and VP3, but its capsid contains only the VP1 protein. In this paper, we report that for production of the neutralizing epitope, co-synthesis of (recombinant) VP1 and VP2 has to take place. We show via immunofluorescence that recombinant-baculovirus-infected Sf9 cells synthesizing VP1 (or VP2) alone react very poorly with CAV-specific neutralizing antibodies. In contrast, Sf9 cells co-infected with VP1- and VP2-recombinant baculoviruses, or infected with a single recombinant baculovirus co-expressing both VP1 and VP2, react strongly with the neutralizing antibodies. Furthermore, immunoprecipitation assays show that VP1 and VP2 interact directly with each other, which indicates that the non-structural protein VP2 might act as a scaffold protein in virion assembly. Recombinant baculovirus expressing VP1 and VP2 is, therefore, a potential production system for a subunit vaccine against CAV infection.

Introduction

Chicken anaemia virus (CAV) has been recognized as an important avian pathogen worldwide (reviewed in McNulty, 1991; Noteborn & Koch, 1995). In young chickens, it results in clinical signs such as anaemia and immunodeficiency caused by the destruction of erythrocyte precursors and thymocytes via the induction of apoptosis (Jeurissen et al., 1992; Noteborn et al., 1994). In older chickens, its effects are subclinical, but it does cause severe economic problems here also (McNulty, 1991; McLroy et al., 1992).

CAV is a small virus of a unique type; its capsid consists of a single protein, VP1 (Todd et al., 1990; G. Koch, unpublished results), and a circular single-stranded (minus-strand) DNA (Gelderblom et al., 1989; Noteborn et al., 1991; Todd et al., 1990). The major transcript from the CAV genome is an unspliced, polycistronic and polyadenylated mRNA of about 2100 nucleotides (Noteborn et al., 1992; Phenix et al., 1994). Several groups have shown that in CAV-infected cells the viral CAV transcript specifies three proteins of about 52 (VP1), 24 (VP2) and 14 (VP3; apoptin) kDa (Noteborn & Koch, 1995; Douglas et al., 1995). Despite the fact that the capsid contains only VP1, co-expression of VP2 is required for the induction of neutralizing antibodies. Thus, immunization with (recombinant) VP1 and VP2 synchronously synthesized in the same cells elicits a protective immune response, whereas separate expression of VP1 (and also VP2) does not (Koch et al., 1995).

In the present study, we have analysed the molecular biological aspects of this required co-production. Baculovirus-encoded VP1 binds to a CAV-specific neutralizing antibody optimally only when co-synthesized with recombinant VP2. We observed a (temporary) direct interaction between VP1 and VP2, indicating that VP2 might act as a scaffold protein that would allow VP1 to assume a conformation exposing its neutralizing epitope.

Methods

Baculovirus and insect cells. The recombinant baculovirus AcRP23-lacZ was obtained from R. D. Possee, NERC Institute of Virology, Oxford, UK, and its DNA was purified as described by Summers & Smith (1987). The VP1- and VP2-recombinant baculoviruses Ac-VP1 (expressing the CAV protein VP1), and Ac-VP2 (expressing VP2), are described by Koch et al. (1995). Spodoptera frugiperda (Sf9) cells were obtained from the ATCC (CRL 1711). Baculovirus stocks were...
grown in confluent monolayers or suspension cultures in TC-100 medium (GIBCO/BRL) containing 10% foetal calf serum, as described by Summers & Smith (1987).

*Cloning of CAV DNA.* All CAV DNA sequences described are derived from plasmid p1c-20H/CAV-EcoRI (Noteborn et al., 1991). Cloning steps with plasmid DNA were performed as described by Maniatis et al. (1982). DNA transformations were carried out in *E. coli* HB101. All plasmids were multiplied in large-volume cultures under agitation, and purified on CsCl gradients and Sephacryl-S500 columns.

**Construction of the recombinant-VP1/VP2 transfer vector.** The coding sequences for the CAV proteins VP1 and VP2 were cloned into the baculovirus transfer vector pAcUW51 (PharMingen; cat. no. 21205P).

Plasmid pET-16b-VP2 (M. H. M. Noteborn and others, unpublished) carries a CAV DNA fragment corresponding to bp 380–1512 (Noteborn et al., 1991). This fragment contains the coding region for VP2 flanked by 484 bp 3'-noncoding CAV DNA. Plasmid pET-16b-VP2 was treated with NdeI and Nhel, and the resulting sticky ends were filled with Klenow polymerase. A 0.8 kb CAV DNA fragment was isolated. Plasmid pAcUW51 was linearized with BamHI, the ensuing sticky ends filled by means of Klenow polymerase and finally treated with alkaline phosphatase (CIP). The 0.8 kb CAV DNA fragment was cloned into linearized pAcUW51 DNA. The orientation of VP2 in pAcUW51 was determined by restriction enzyme analysis. This construct was called pUW-VP2.

Plasmid pET-16b-VP1 (M. H. M. Noteborn and others, unpublished) contains CAV DNA from bp 853–2319 (Noteborn et al., 1991). This insertion contains the complete coding region for protein VP1 flanked by 117 bp of 3'-noncoding CAV DNA sequences. Plasmid pET-16b-VP1 was treated with the restriction enzymes Ndel and EcoRI, and the resulting sticky ends were filled with Klenow polymerase. A 1.47 kb CAV DNA fragment was isolated. Plasmid pUW-VP2 was linearized with EcoRI, the sticky ends filled by means of Klenow polymerase and finally treated with CIP. The 1.47 kb CAV DNA fragment was ligated at the linearized pUW-VP2. The orientation of VP1 with respect to the p10 promoter unit was checked by restriction enzyme analysis, and the final construct was called pUW-VP1/VP2.

**Construction and selection of recombinant-VP1/VP2 baculovirus.** AcRP23-lacZ DNA was digested with *Bsa*II, extracted with phenol and precipitated with ethanol. Sf9 cells were transfected with mixtures of 1 µg purified linearized AcRP23-lacZ DNA and 5 µg pUW-VP1/VP2 by the calcium phosphate method of Graham & Van der Eb (1973) as modified by Smith et al. (1983). The recombinant-VP1/VP2 baculoviruses were selected as described by Noteborn et al. (1990).

**Immunofluorescence assay.** Sf9 cells were infected with Ac-VP1 and/or Ac-VP2 baculoviruses with an m.o.i. of 5–10 per virus species, which guarantees optimal co-synthesis of VP1 and VP2, or with Ac-VP1/VP2 with an m.o.i. of 1–2. Cells were fixed with 80% acetone and permeabilized with 0.1% Triton X-100, 250 mM NaCl, 50 mM NaF and 5 mM EDTA. Extracts were incubated with MAb CAV-CVI-111.1 directed against CAV protein VP2 for 2 h at 4 °C and washed with IP-1 buffer (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 1% NP40, 1% sodium deoxycholate, supplemented with protease inhibitors). IP-1 has been optimized to co-precipitate heterodimeric complexes (B. M. Hagmeyer, personal communication). Finally, the proteins were separated on a 14% polyacrylamide (PAA)–SDS gel.

**Results**

**Role of VP2 in the formation of a conformational neutralizing epitope on VP1**

We examined whether co-synthesis of VP2 with VP1 results in the formation of the neutralizing VP1-epitope. Sf9 cells were infected with recombinant-CAV baculoviruses expressing VP1 or VP2, or co-infected with both of these recombinant baculoviruses. The infected cells were harvested 3 days after infection and analysed by indirect immunofluorescence assays using the CAV-specific neutralizing MAb CVI-CAV-132.1.

Cells containing VP2 only did not react at all with MAb 132.1, whereas the VP1-positive cells gave only a very poor immunofluorescence signal. Insect cells co-infected with both Ac-VP1 and Ac-VP2 reacted strongly with neutralizing MAb 132.1 (Fig. 1). To exclude possible differences in the levels of VP1 and/or VP2 among the various lysates as the cause, parallel [3H]leucine-labelled lysates of insect cells expressing VP1, VP2, or VP1 plus VP2, were examined by PAA–SDS gel electrophoresis. The autoradiographs showed that VP1 and VP2 are expressed at the similar level when synthesized alone or together (Fig. 2A). These results prove that co-synthesis of VP1 and VP2 is required for the optimal establishment of CAV-specific neutralizing epitopes.

**VP1 and VP2 interact with each other**

The conformational neutralizing epitope of VP1 is formed only when VP2 and VP1 are co-synthesized, but the CAV capsids consist only of VP1. This suggests that VP1 and VP2 associate with each other for a limited time-period.

By immunoprecipitation, we examined whether VP1 could associate with VP2. Sf9 cells were infected separately with recombinant baculovirus expressing VP1 or VP2, or cells were co-infected with both recombinant-VP1 and -VP2 baculoviruses (Fig. 2A). The lysates were immunoprecipitated with the VP2-specific MAb 111.1. The results shown in Fig. 2(B) clearly reveal that MAb 111.1 precipitates VP2 when VP2 is synthesized alone or in the presence of VP1. In cells co-expressing VP1 and VP2, VP1 co-precipitated. These immunoprecipitation results were obtained only when VP1 and VP2 were co-synthesized, and not when crude lysates containing VP1 or VP2 were simply mixed (data not shown). MAb 111.1 did not detectably precipitate VP1 when VP1 was synthesized alone.

Immunoprecipitation of lysates containing VP1 and VP2 with a MAb directed against blood-clotting factor VIII (negative control; Hoebe et al., 1990) did not result in VP1 and/or VP2-specific products (data not shown). These results
CAV neutralizing epitope

Fig. 1. Indirect immunofluorescence staining of Sf9 cells infected with recombinant baculovirus encoding VP1 (A), VP2 (B), or co-infected with VP1- and VP2-baculoviruses (C). The cells were fixed 3 days after infection and stained with the CAV-specific neutralizing MAb CVI-CAV-132.1 and FITC-conjugated second antibodies.

Fig. 2. (A) PAA–SDS electrophoresis analysis of crude lysates from [3H]leucine-labelled Sf9 cells infected with VP1-baculovirus (1), VP2-baculovirus (2), or co-infected with VP1- and VP2-baculoviruses (1/2). (B) Co-immunoprecipitation assay of lysates from [3H]leucine-labelled non-infected Sf9 cells (N) and of cells infected with recombinant VP1-baculovirus (1), VP2-baculovirus (2) or co-infected with both VP1- and VP2-baculoviruses (1/2) after PAA–SDS gel electrophoresis. All lysates were immunoprecipitated with MAb CVI-CAV-111.1, which is directed specifically against VP2, and then analysed by PAA–SDS gel electrophoresis.

Ac-VP1/VP2 baculovirus expresses both VP1 and VP2 resulting in the formation of the CAV-specific neutralizing epitope

Next, we examined whether a single baculovirus vector could be constructed that is able to produce both VP1 and VP2, thus resulting in the formation of the neutralizing epitope. Simultaneous expression of recombinant VP1 and VP2 in Sf9 cells infected with recombinant baculovirus Ac-VP1/VP2 was analysed by [3H]leucine labelling and PAA–SDS gel electrophoresis (Fig. 3). As comparative controls, cells were infected with recombinant baculovirus expressing either VP1 or VP2. The cells were harvested 1, 2, or 3 days after infection. As negative controls, mock-infected cells were used or cells infected with recombinant baculovirus AcRP23 expressing β-galactosidase (data not shown).

As expected, lysates of insect cells infected with recombinant baculovirus Ac-VP1 contained the CAV-specific 52 kDa imply that VP1 can be precipitated by MAb 111.1 only via association to VP2.

Thus VP1 and VP2 can associate physically and so create the VP1 conformation that results in the neutralizing epitope.
Fig. 4. Indirect immunofluorescence of Sf9 cells infected with the recombinant baculovirus Ac-VP1/VP2 co-expressing both VP1 and VP2. The cells were fixed 3 days after infection and stained with the CAV-specific neutralizing MAb 132.1 (A), MAb 111.1 directed against VP2 (B) or with a MAb against the blood-clotting factor VIII (C; Hoeben et al., 1990; negative control) and subsequently with FITC-conjugated goat-anti-mouse antibodies.

Discussion

The results obtained with baculovirus-encoded synthesis of recombinant VP1 and/or VP2 proteins indicate that for formation of the CAV-neutralizing epitope both VP1 and VP2 have to be synthesized synchronously. Most likely, VP1 gets its correct conformation by direct interaction with VP2, as was shown by our immunoprecipitation data. These data are strengthened by the observations of Douglas et al. (1995) that VP1 and VP2 are partially present in the same structures of CAV-infected MDCC-MSB-1 cells.

The neutralizing epitope is most likely situated on VP1. Immune-fluorescence data showed that neutralizing MAb 132.2 did not react at all with VP2, and weakly with VP1, when expressed alone. After VP1 gets its correct conformation via association with VP2, the VP1 will get its functional neutralizing epitope. Furthermore, under native conditions CAV-specific neutralizing antibodies react only with VP1 and not with VP2. The fact that neutralizing MAb 132.1 does not react with, for example, SDS-denatured VP1, indicates that the VP1 neutralizing epitope is a conformational one (G. Koch, unpublished results).

Since CAV capsids contain only VP1 (Todd et al., 1995; G. Koch and others, unpublished), and VP1 and VP2 interact with each other, it is probable that VP2 is a non-structural protein with a scaffold-like activity. Examples of viral scaffold proteins are the IVa2 and 39 kDa proteins of adenovirus (D’Halluin et al., 1978; Gustin et al., 1996; Persson et al., 1979) and the herpes simplex virus protein pre-VP22a (Trus et al., 1996). These proteins act as scaffold for the formation of the so-called light capsid, but are removed in the next step.

The information that the CAV-specific neutralizing epitope is formed only when VP1 and VP2 are synthesized synchronously is essential for the development of vaccines against CAV infections. Koch et al. (1995) have reported that only chickens inoculated with Sf9 cells co-infected with two recombinant baculoviruses expressing VP1 and VP2 could mount a protective immune response. The present report provides the molecular-immunological proof for this requirement for co-synthesized VP1 and VP2. In addition, we have created a new generation of CAV-recombinant baculoviruses that express VP1 and VP2 and thereby produce the essential neutralizing epitope. Preliminary data indicate that hens injected with crude lysates of insect cells infected with Ac-VP1/VP2 can make neutralizing antibodies against CAV (data not shown). Therefore, Sf9 cells infected with the single baculovirus Ac-VP1/VP2 constitute a potential production system for a subunit vaccine against CAV infections. Another conclusion from the present study is that CAV-recombinant live-virus vector vaccines, such as those based on Marek’s disease virus, have to express both VP1 and VP2 in order to elicit the required (neutralizing) immune response.
The development of both baculovirus and live-virus vectors expressing VP1 and VP2 for the production of vaccines against CAV infections is actively preferred in our institute.

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References


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