Identification of a 24 kDa protein expressed by chicken anaemia virus


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Antisera raised against oriented peptide conjugates were used to identify and partially characterize a 24 kDa protein product expressed by chicken anaemia virus (CAV). The peptides derived from the N and C termini of the protein were shown to react against the native protein, expressed within virus-infected cells, by immunofluorescence, immunoperoxidase and immunogold thin section electron microscopy techniques. The protein product was located by immunogold single labelling in intranuclear inclusions similar to those described previously for the 13 kDa CAV protein, which causes apoptosis. The 24 kDa protein was co-localized to the nuclear inclusions with the CAV 13 kDa protein by simultaneous dual labelling immunogold electron microscopy. Following isolation of the CAV proteins by nuclei isolation and SDS–PAGE, the antisera were used to probe for the protein by immunoblotting. The antisera recognized an expressed protein product of apparent molecular mass 30 kDa. An immunofluorescence time course study of CAV protein expression was carried out and the peptide antisera reacted against the protein at 12 h post-infection. Antisera against the 13 kDa protein reacted at similar times post-infection. This was in contrast to antisera raised against the 52 kDa capsid protein which is detectable by immunofluorescence only after 24 h. The 13 kDa and 24 kDa proteins thus appear to be early antigens produced by CAV during infection.

Introduction

Chicken anaemia virus (CAV) causes a disease in young chickens characterized by aplastic anaemia, lymphoid depletion, subcutaneous and intramuscular haemorrhages, and increased mortality (Yuasa et al., 1979). Infection with CAV appears to cause the destruction of erythroblastoid cells in bone marrow and depletion of cortical CD4+ lymphocytes in the thymus, resulting in acute anaemia and immunodeficiency (Jeurissen et al., 1989). In addition, infection of thymocytes in vivo and cell lines in vitro with the virus has been shown to cause fragmentation of the cellular DNA and cell death which is characteristic of that observed in cells undergoing apoptosis (Jeurissen et al., 1992).

CAV, which can be grown in a Marek’s disease virus-transformed chicken lymphoblastoid (MDCC-MSB 1) cell line, has recently been characterized as a 23.5 nm spherical or icosahedral virus containing a circular single-stranded DNA genome of 2.3 kb (Todd et al., 1990). The genomic structure of the virus is similar to two other small viruses, porcine circovirus (Tischer et al., 1982) and psittacine beak and feather disease virus (Ritchie et al., 1989); however, it appears to be morphologically distinct from and antigenically unrelated to either of these (Todd et al., 1991).

The CAV genome comprises three partially or completely overlapping open reading frames (ORFs) encoding proteins of 52, 24 and 13 kDa, which have been designated VP1, VP2 and VP3 (Noteborn et al., 1994). The protein product of the 52 kDa ORF (VP1) is associated with purified virus particles as shown by sucrose gradient analysis and Western blotting experiments using MAb 1H1 (Todd et al., 1990; Noteborn et al., 1992). The 13 kDa ORF expression product, designated VP3, has recently been characterized by Noteborn et al. (1994) and can be detected by Western blotting techniques using MAb 3B1 in nuclear isolates derived from CAV-infected MSB 1 cells with an molecular mass by SDS–PAGE of 16 kDa. Transient expression of this protein in a CAV-susceptible cell line resulted in apoptosis of the host cells. The protein was localized, using immunogold electron microscopy, to condensed nuclear structures which are morphologically similar to apoptotic bodies (Noteborn et al., 1994); however, the mechanism by which VP3 induces cell death is still unclear. The expression and distribution of the CAV 24 kDa ORF, designated VP2, is not currently known.

Detailed analysis of CAV mouse MAbs has shown that there are three distinct patterns of immunofluorescence associated with CAV infection (McNulty et
al., 1990a). Type 1 staining, characterized by fine granular nuclear staining, is indistinguishable from that described with chicken polyclonal antisera to CAV (McNulty et al., 1988). Type 2 staining is confined to large, spherical nuclear inclusions with a similar intensity to type 1. Type 3 staining is predominantly nuclear, with a fine and granular appearance similar to type 1, but differing from type 1 in being much more intense and occurring in a higher proportion of nuclei. No specific immunofluorescence related to VP2 has yet been described.

In the present paper we describe the identification and characterization of the protein expressed from the 24 kDa ORF using antisera raised to N- and C-terminally derived synthetic peptides. The peptides were conjugated to a carrier protein using a bifunctional crosslinker, allowing correct orientation of the sequences on the carrier with respect to the native CAV protein. The resulting mouse and rabbit antisera were used to confirm the identity and apparent molecular mass of the endogenous viral protein designated VP2, and characterize its cellular distribution and expression kinetics.

Finally, the intracellular distribution of VP2 was compared to that of the recently described VP3 using an anti-peptide antibody and a monoclonal antibody respectively.

**Methods**

**Peptide synthesis.** Peptide sequences were obtained from the published genomic sequences for the Cux-1 isolate of CAV (Meehan et al., 1992). The N- [MHGNGQPAAGGSEASREGQPGP(C)] and C- [CJGGDSGIVDELGRRPFTTPAPVRIV] terminal sequences from the 24 kDa ORF and the N-terminal sequence [MNALQEDTPPG(C)] from the 13 kDa ORF were synthesized on an ABI 431A automated synthesizer utilizing FASTMOC protocols. Cysteine residues were included to facilitate attachment of the peptides in the correct orientation to the carrier protein, keyhole limpet haemocyanin. The peptides were synthesized on HMP resin and cleaved with trifluoroacetic acid. Isolation of the synthetic product was achieved by diethyl ether precipitation followed by lyophilization from 5% acetic acid. The peptides were then purified by preparative C18 reverse phase chromatography using a Beckman System Gold HPLC and the chemical compositions of the sequences were confirmed by amino acid analysis and TOF/MS.

**Immunization.** The peptides were conjugated to keyhole limpet haemocyanin using maleimidobenzoic acid N-hydroxysuccinimide ester (MBS) following the procedure of Liu et al. (1979). Groups of two mice (BALB/c F1 hybrids) were then injected subcutaneously with Quil-A emulsions containing 75 μg of one particular peptide. In addition, a rabbit was immunized subcutaneously with an inoculum containing 200 μg of a mixture of the two 24 kDa ORF peptide conjugates. Three weeks after the initial injection, a booster injection of peptide in Quil-A was given. Animals were bled at 30 days subsequent to the primary injection. Further boosts were administered at monthly intervals.

**Solid phase ELISA for the detection of anti-peptide antibodies.** Microtitre plates (Immulon) were preactivated with 1% (v/v) glutaraldehyde for 1 h at 37 °C. The plates were then washed with water and the peptides were coated overnight with free peptide (5 μg per well) in carbonate–bicarbonate buffer containing 0.025% (v/v) glutaraldehyde. The plates were washed with PBS containing 0.05% (v/v) Tween 20 and preblocked for 1 h at 37 °C with 3% (w/v) BSA in PBS containing 2% (w/v) NaCl and 0.05% (v/v) Tween 80 (PTN buffer) prior to the addition of test sera. Sera were serially diluted from 1:200 to 1:3200 in PTN buffer and plates were incubated for 2 h at 37 °C and washed with PBS–Tween 20. Goat anti-mouse or goat anti-rabbit horseradish peroxidase IgG conjugate (Nordic Immunological Laboratories) diluted 1:5000 in PTN buffer was then added and the plates were incubated as before. After washing, the enzyme substrate tetramethylbenzidine (Sigma) was added in citrate–phosphate buffer pH 5.0 containing 0.3% hydrogen peroxide. The reaction was allowed to proceed for 5 min at room temperature after which time colour formation was terminated by the addition of 2.5 M-sulphuric acid. The absorbance was read at 450 nm. Four times the average of the negative control (mouse and rabbit prebleeds) was taken as the cut-off point for the titre of the anti-peptide sera.

**Monoclonal antibodies.** The MAb used in this study were previously described by McNulty et al. (1990a) and produced characteristic type 1 (MAb 2A9), type 2 (MAb 1H1) and type 3 (MAb 3B1) staining patterns with CAV-infected cells.

**Cells and virus.** The Cux-1 isolate of CAV was supplied by Dr V. von Bulow (Free University, Berlin, Germany). The isolate was propagated in MDCC-MSB 1 cells as previously described (McNulty et al., 1988).

**Indirect immunofluorescence and immunoperoxidase assay of peptide antisera.** The indirect immunofluorescence assay (IFA) for detecting antibody to CAV was performed with minor modifications to the method described by McNulty et al. (1988). In brief, IFA staining of MDCC-MSB 1 cells infected with the Cux-1 isolate of CAV was carried out using 1:100 and 1:500 dilutions of the anti-peptide antisera for 1 h at 37 °C. The multispots were washed in PBS for 10 min prior to the addition of anti-species FITC IgG conjugate diluted 1:80 with PBS for 1 h at 37 °C. The multispots were then washed with PBS for 10 min and visualized. The indirect immunoperoxidase assay was carried out using an antibody dilution of 1:1000 for 1 h at 37 °C, followed by use of the Histostain-SP kit (Zymed) for detecting mouse primary antibody.

**Viral protein expression kinetics.** Multispots of CAV-infected cells were prepared as above at 6 h intervals following infection. The slides were then probed with antisera specific to each of the viral proteins. Following extensive washing, the appropriate anti-species FITC conjugate was added. The slides were then visualized using a fluorescence microscope.

**Thin section electron microscopy.** MDCC-MSB 1 cells were infected with CAV as previously described and harvested at 48 h post-infection. Suitable matched uninfected cells were processed in a similar manner. Pellets of MDCC-MSB 1 cells were fixed in 2% (v/v) paraformaldehyde containing 0.05% (v/v) glutaraldehyde in PBS (pH 7.2) for 1 h at 4 °C. The pellets were washed and stored in PBS, embedded/encapsulated in 4% gelatine and processed through graded alcohols and embedded in Lowicryl HM20 by the progressive lowering of temperature method in a Leica CSAUTO. Thin sections were cut using an LKB Bromma ultramicrotome and mounted on 400 mesh nickel grids.

**Single gold labelling.** Grids were pre-wet in distilled water, then immersed in 1% BSA in Tris-buffered saline for 10 min, drained and immersed overnight at 4 °C in dilutions of primary antisera made in Tris-buffered saline containing 1% BSA. Grids were then washed, washed in 0.1% BSA in Tris-buffered saline and transferred to 1% BSA in Tris-buffered saline (BSA buffer) for 10 min. After labelling with the corresponding gold (G15)-labelled anti-species conjugate the grids were washed in 1% BSA buffer, followed by 0.1% BSA buffer.
and then water. The grids were contrasted with saturated uranyl acetate in methanol, washed in water and examined in a Hitachi H7000 transmission electron microscope.

**Dual gold labelling.** Simultaneous gold labelling of both 13 kDa and 24 kDa antigens in section was performed by diluting the appropriate antisera to half the normal dilution in 1% BSA buffer and mixing these in equal proportions. This mixture was applied to grids that had been pre-wet and blocked with 1% BSA buffer. After overnight incubation at 4°C the grids were drained, washed in 0.1% BSA buffer and transferred to 1% BSA buffer for 10 min. Gold-labelled conjugates (goat anti-mouse G15 and goat anti-rabbit G5) were then added and the grids washed and contrasted as before.

**Antigen isolation.** CAV-infected MDCC-MSB 1 cells were propagated for 48 h post-infection as described above. The cells were then isolated by centrifugation at 1200g and washed thoroughly in PBS. Nuclei were isolated by incubation for 30 min at 4°C in a buffer containing 0.01 M-TrisHCl, 0.01 M-NaCl, 1% (v/v) NP40, 1 mM-EDTA and 0.1 mM-PMSF, pH 7.4. Following centrifugation at 1200 g, the nuclei-rich pellet was dispersed in PBS using ultrasonication for 20 s at 0°C and solubilized by the addition of SDS (final concentration 2%). The solubilized extract was then fractionated by gel filtration through a column (45-1 x 5 cm) containing Sephacryl S-200 using PBS containing 0.05% SDS as eluant.

**SDS-PAGE and Western blotting.** Nuclear extracts obtained from CAV-infected and uninfected MDCC-MSB 1 cells were subjected to electrophoresis on SDS-polyacrylamide gels of 10% acrylamide according to the method of Laemmli (1970). The separated proteins were transferred electrophoretically onto nitrocellulose membranes and immunoblotted using the mouse 24 kDa antibody (1:1000 dilution) and the mouse MAb 3B1 (1:5000 dilution) described by McNulty et al. (1990a). Goat anti-mouse horseradish peroxidase conjugate (1:500 dilution) was then added. Bound conjugate was detected using diaminobenzidine (Sigma) as a substrate.

**Results and Discussion**

In this paper we describe the first characterization of a 24 kDa protein (VP2) expressed by CAV using specific peptide antisera raised against the N- and C-terminal portions of the predicted ORF. The antisera obtained following immunization of mice and rabbits with oriented conjugates were successful in recognizing both the corresponding peptide by ELISA and the native protein by immunofluorescence and immunoperoxidase. Typical titres for the N- and C-terminal portions of VP2 are shown in Table 1. Both peptides were strongly immunogenic, inducing significant ELISA and immunofluorescence titres in all of the animals used in this study. No cross-reaction was observed with either antisera between peptides or with secondary antibody conjugates. In addition, both antisera produced a similar staining pattern and titre by IFA suggesting that the N- and C-terminal portions of VP2 are exposed in the native conformation.

The indirect immunoperoxidase staining pattern produced by the VP2 antisera is shown in Fig. 1. The protein was mainly localized within the nuclei of infected cells in dense granules which are typically detected by type 2 CAV MAbs such as 1H1 (McNulty et al., 1990a); however, an additional diffuse pattern of nuclear staining which is typical of type 3 MAbs such as 3B1 (McNulty et al., 1990a) was also seen in the infected cells. Furthermore, the proportion of cells staining for VP2 was markedly higher than normally expected with type 2 MAbs such as 1H1 and was in fact more in keeping to that seen with type 3 MAbs. The immunoperoxidase staining pattern which typifies VP2 can thus be characterized as a hybrid type 2/type 3 pattern which has not been previously described for any CAV MAb.

The expression kinetics of the three CAV proteins was then investigated using indirect immunofluorescence. Multispot slides were prepared using MDCC-MSB 1 cells harvested at intervals of 6 h post-infection. Analysis of these slides using the VP2 peptide specific antisera, VP3-specific MAb 3B1 and VP1-specific MAb 1H1 (previously described by McNulty et al., 1990a) as

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**Table 1. Typical titres of antisera obtained with VP2 peptide conjugates**

<table>
<thead>
<tr>
<th></th>
<th>Rabbit</th>
<th>Mouse</th>
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<tr>
<td>Peptide ELISA titre</td>
<td>1:3200</td>
<td>1:4000</td>
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<tr>
<td>Immunoperoxidase titre</td>
<td>Not determined</td>
<td>1:1000</td>
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<tr>
<td>Immunofluorescence titre</td>
<td>1:400</td>
<td>1:500</td>
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* The titres for N- and C-terminal specific antisera were identical for each assay.
Table 2. *Viral protein expression kinetics*  

<table>
<thead>
<tr>
<th>Time (h)†</th>
<th>VP1</th>
<th>VP2</th>
<th>VP3</th>
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<tbody>
<tr>
<td>6</td>
<td>–</td>
<td>–</td>
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<td>12</td>
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<td>48</td>
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* Fluorescence results were judged subjectively based on cell number and intensity of staining.
† Time of harvesting post-infection.

probes is shown in Table 2. Antisera directed against VP2 and VP3 stained material within the infected cells from very early times post-infection. Positive immunofluorescence was first detected at 12 h post-infection for VP2 and VP3. This fluorescence was intense and showed no further detectable increase in intensity. At times after 12 h post-infection there was a general increase in the proportion of cells containing detectable antigen up to 30 h post-infection when it appeared that the number of infected cells had reached a maximum level. Thereafter it was not possible to discriminate differences between individual time points. In contrast, antisera specific for VP1 (MAb 1H1) showed a positive immunofluorescence after 30 h post-infection. The level of this protein reached a maximal value in terms of the number of cells containing antigen within 6 h of initial detection. Analysis of time-matched, uninfected cells showed no specific fluorescence associated with any of the antisera, indicating that the detected proteins were virus specific. It thus appears that the VP2 and VP3 proteins are preferentially produced very early in the infectious cycle and deposited in large amounts within the nucleus of infected cells. The presence of VP2 and VP3 at early time points may be required by the virus for replicative or transcriptional functions. This type of early protein expression is already well documented for other DNA viruses such as parvoviruses (Clemens & Pintel, 1988), adenoviruses (Horwitz, 1990) and polyomaviruses (Eckhart, 1990). However, in the case of CAV there is currently no direct evidence that the virus utilizes these proteins for any functions related to transcription or replication. The observation that two of the CAV proteins are expressed as early antigens has been confirmed by immunoblotting the three viral proteins from nuclear extracts obtained at sequential time points post-infection (Phenix, 1994).

Immunogold electron microscopy was carried out using mouse and rabbit antisera to VP2. The predominant features recognized by the antibodies were nuclear inclusions of the type previously described for CAV (McNulty et al., 1990b). The inclusions varied in size, shape and electron density (Fig. 2). Doughnut structures, as previously described by McNulty et al. (1990b), were prominent in the sections studied and were stained with the VP2 antisera. No correlation between inclusion morphology and the presence of VP2 could be made. A proportion of the inclusions visualized within infected cells were not stained with either of the VP2

Fig. 2. Immunogold labelling of electron-dense nuclear inclusions in CAV-infected MDCC-MSB 1 cells. Infected cells harvested 48 h post-infection were embedded in Lowicryl and allowed to react with antisera raised against the N-terminal peptide of VP2. An identical pattern was obtained with the C-terminal antisera. Bar marker represents 300 nm.
Chicken anaemia virus 24 kDa protein

Fig. 3. Immunogold labelling of electron-dense nuclear inclusions in CAV-infected MDCC-MSB 1 cells harvested 48 h post-infection. The cells were allowed to react against a mixture of antisera raised against VP2 and VP3. Gold conjugates (5 nm for VP2 and 15 nm for VP3) were added simultaneously. Bar marker represents 100 nm.

antisera. A similar pattern of staining was obtained using antisera raised to the N-terminal VP3 peptide (data not shown).

The intracellular distribution of VP2 and VP3 was studied by simultaneous double staining of electron microscopy sections using the VP2 rabbit sera and MAb 3B1. In this approach both antisera were added simultaneously to the section and allowed to react. The binding was then detected using a mixture of gold conjugates again added simultaneously. A typical pattern is shown in Fig. 3. The viral proteins were located in inclusions within the nucleus of infected cells. Inclusions staining for the individual proteins as well as double labelled structures were detected. In addition, structures were detected which apparently did not contain either protein. The distribution of the proteins was not correlated with either inclusion size or electron density. A similar pattern of protein distribution was obtained with rabbit antisera to the N-terminal VP3 peptide and mouse antisera to the N-terminal VP2 peptide (data not shown).

It has recently been shown that recombinant VP3 is capable of inducing apoptosis in CAV-susceptible chicken cell lines (Noteborn et al., 1994). The recombinant protein appears to be localized to nuclear inclusions, similar to those seen in natural CAV infections and which are assumed to be apoptotic bodies (Cohen, 1993). Various theories have been suggested for the mechanism of VP3-induced apoptosis (Jeurissen et al., 1992; Noteborn et al., 1994) including the interaction of the viral protein with host nucleic acids or with host proteins within the chromatin structure (Noteborn et al., 1994), leading to disorganization of the chromatin and thus rendering it susceptible to endonuclease fragmentation. Since nuclear structures containing only VP2 can be readily detected by simultaneous double labelling experiments, conducted in this current paper, it appears that the nuclear inclusions seen in CAV infections are unlikely to be exclusively VP3-induced apoptotic bodies. There is currently no evidence to suggest that VP2 can directly cause apoptosis by interaction with host nucleic acids, since the VP2 protein sequence does not contain the necessary interaction motifs. However, it may predispose the chromatin to attack or accelerate the actions of VP3. Preliminary electron microscopy studies with antisera to VP1 suggest that there may be an additional level of complexity in CAV protein distribution; however, this remains to be determined conclusively. Thus, the basis for CAV protein interaction with cellular structures still remains uncertain and is clearly a more complex process than previously thought (Noteborn et al., 1994).

The molecular mass of VP2 was determined by SDS–PAGE and immunoblotting. Nuclear extracts were prepared from CAV-infected cells using a modification of a method previously described for the extraction of lymphocytes (Davies & Brown, 1987). These extracts were then further separated by gel filtration and SDS–PAGE. Following electrottransfer and immunoblotting, a strong band was detected with the 24 kDa ORF peptide antisera at a molecular mass of 31 kDa (Fig. 4). In addition, the blot was probed with MAb 3B1 which is known to be specific for VP3 and this antibody detected a protein with a molecular mass of 17 kDa (as previously described by Noteborn et al., 1994). This band could also be detected by anti-peptide antibodies directed against the N terminus of VP3 (data not shown). Thus, it appears that both proteins are present in detectable amounts within nuclear extracts obtained from infected cells; however, neither of the proteins was detected in nuclear extracts obtained from corresponding uninfected cells. The apparent molecular masses obtained for both of the proteins were considerably larger than those predicted from the amino acid sequences and this observation may be consistent with some form of post-translational modification of the CAV proteins such as N-terminal modification or phosphorylation. Further studies would be warranted to resolve this matter. In addition, during gel filtration
Further detailed characterization of the CAV proteins and their interaction with host cell components (Phenix, 1994), suggesting that the proteins may be involved in complex interactions with other intracellular components, for example, nucleic acids and proteins. Molecular mass standards are also shown.

chromatography the VP2 and VP3 proteins were detected in fractions containing very high molecular mass components (Phenix, 1994), suggesting that the proteins may be involved in complex interactions with other intracellular components, for example, nucleic acids and proteins. Further detailed characterization of the CAV proteins and their interaction with host cell components will thus be required.

References


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