Agnoprotein 1a and agnoprotein 1b of avian polyomavirus are apoptotic inducers

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Avian polyomavirus (APV) causes an acute fatal disease in a variety of avian species. DNA laddering indicating apoptosis was demonstrated in APV-infected chicken embryo (CE) cells. DNA laddering, however, was not observed in Vero cells infected with mammalian polyomavirus simian virus 40. Expression of APV agnoprotein 1a and agnoprotein 1b induced apoptosis in insect cells and CE cells. An APV full-length plasmid transfected in CE cells induced apoptosis, and infectious virus was produced. After transfection of CE cells with a plasmid containing a mutated initiation codon for agnoprotein 1a and agnoprotein 1b, however, a considerably lower number of apoptotic cells was observed, and no infectious progeny was produced.

Introduction

Infections with avian polyomavirus (APV), originally described as budgerigar fledgling disease virus (BFDV), have now been recognized in a variety of avian species (Bernier et al., 1981; Bozemann et al., 1981; Johne & Müller, 1998). Whereas mammalian polyomaviruses typically cause innocuous persistent infections in their natural non-immunocompromised hosts, APV infections may cause acute lethal disease (Müller & Nitschke, 1986; Krautwald et al., 1989; Sandmeier et al., 1999). Peracute death with no premonitory signs, or acute infections with dehydration, subcutaneous haemorrhages, depression and dyspnoea are most common in psittacine birds (Clubb & Davis, 1984; Graham & Calnek, 1987; Ritchie et al., 1991). In fledgling budgerigars mortality rates may reach 100% (Krautwald et al., 1989; Müller & Nitschke, 1986). The induction of tumours, a common feature of mammalian polyomaviruses, was never observed after APV infections of new-born hamsters or mice (H. Müller, unpublished results).

APV virions are non-enveloped icosahedrons with a diameter of approximately 45 nm (Lehn & Müller, 1986; Müller & Nitschke, 1986). The circular double-stranded DNA genome, consisting of 4981 bp, is transcribed bidirectionally for the expression of early and late genes (Rott et al., 1988; Luo et al., 1995). Marked differences from mammalian polyomaviruses have been observed in the non-coding regulatory region, the region encoding the large tumour (T) antigen, and the agnoproteins (Luo et al., 1994, 1995). BFDV isolates from different species of birds can be grouped within one genotype and one serotype, and it has been suggested to adopt the name APV for all known isolates (Johne & Müller, 1998). Based on the unique molecular and biological characteristics of APV, it has been proposed to place this virus into a distinct subgenus Avipolyomavirus within the genus Polyomavirus (Stoll et al., 1993).

The functions of agnoproteins, small proteins of the primate polyomaviruses simian virus 40 (SV40), BK virus (BKV) and JC virus (JCV), still remain unknown (Jackson & Chalkley, 1981; Rinaldo et al., 1998). They are encoded by an open reading frame (ORF) located near to the 5′-end of the late bicistronic mRNA that also encodes the structural protein VP1. In APV, late gene expression is complicated by two putative promotor regions as well as partial and alternative splicing events. Seven mRNAs potentially encoding different agnoproteins have been observed during the infectious cycle (Luo et al., 1995). Recently, the two largest agnoproteins have been designated as agnoprotein 1a and agnoprotein 1b, respectively (Li, 1996; Liu, 1997). Agnoprotein 1a consists of 176 amino acids with no obvious sequence similarities to agnoproteins of mammalian polyomaviruses (Luo et al., 1995; Rott et al., 1988). By alternative splicing the ORF for agnoprotein 1b is created, which shares a common N- and C-terminus with agnoprotein 1a, but has an internal deletion of amino acids 69 to 132 (Luo et al., 1995).

In this communication, induction of apoptosis by APV infection of chicken embryo (CE) cells, and by the expression of agnoprotein 1a and agnoprotein 1b in insect cells and in CE
cells is described. The significance of these proteins for the induction of apoptosis and the production of infectious progeny was investigated by transfection of CE cells with APV full-length plasmids which either did express agnoprotein 1a and agnoprotein 1b, or did not.

Methods

Cells and viruses. Primary CE cell cultures and Vero cells, maintained in Dulbecco’s modified Eagle’s medium supplemented with 5% foetal calf serum, were infected with APV strain BFDV-1 (Rott et al., 1988; Stoll et al., 1993) and SV40, respectively, as previously described (Müller & Nitschke, 1986). SF9 cells were cultivated in TNM-FH insect medium (Sigma) supplemented with 10% foetal calf serum at 27 °C and used for propagation of recombinant baculoviruses and AcNPV wild-type baculovirus (PharMingen).

Expression of agnoprotein 1a and agnoprotein 1b in insect cells. Total RNA was isolated from APV-infected CE cells 5 days post-infection (p.i.) by proteinase K treatment and subsequent phenol–chloroform extraction (Sambrook et al., 1989). Agnoprotein 1a and agnoprotein 1b cDNAs were generated by RT–PCR (Titan–RT–PCR system, Boehringer Mannheim) using isolated RNA as template and primers 5′ GGA CAA CAT GTC TAC TCC AGC G 3′ and 5′ CGG GGG ATC CTT AGC GAG CC 3′. The 548 bp (agnoprotein 1a) and 356 bp (agnoprotein 1b) products were digested using AflIII and BamHI and ligated to Ncol/BamHI-restricted baculovirus transfer vector pLS46 (kindly provided by M. Pawlista, Heidelberg, Germany). The resulting plasmids were used to generate the recombinant baculoviruses agno1aa-baculo and agno1bbaculo. Generation and amplification of recombinant baculoviruses was carried out using the BaculoGold kit (Pharmingen) and SF9 cells as previously described (Pawlista et al., 1996).

Transient expression of agnoprotein 1a and agnoprotein 1b in CE cells. For transient expression in CE cells, the cDNAs of agnoprotein 1a and agnoprotein 1b were amplified as described above, but using primers 5′ CCT TTG GAT CCC TCT ACT C 3′ and 5′ CGG GGG ATC CTT AGC GAG CC 3′. The PCR products with 552 bp (agnoprotein 1a) and 300 bp (agnoprotein 1b) were digested with BamHI and ligated to the BamHI-restricted eukaryotic expression vector pSG5 (Stratagene), to generate plasmids agno1a-CE and agno1b-CE, respectively. Both plasmids were purified with the Qiagen Plasmid Midi kit (Stratagene) and used in a second PCR, together with primer 5′ GGA CAA CAT GTC TAC TCC AGC G 3′ and the same template. Both reactions were carried out with Pwo DNA polymerase (PeqLab). The PCR product was cleaved with EcoRI and XbaI. The resulting 670 bp cleavage product was ligated to an 8164 bp fragment of EcoRI/XbaI-digested pAPVinf, in order to generate pAPVinfmut. DNA sequencing of the inserted PCR product revealed this plasmid to be identical to the APV full-length plasmid pAPVinf, with the exception that GAT at position 289–291 was replaced by CTG. With this mutation the initiation codon ATG for agnoprotein 1a and agnoprotein 1b is exchanged for TGG.

Immunoblotting. Expression of viral structural proteins and agnoproteins was demonstrated by immunoblotting with antibodies elicited in rabbits immunized with APV strain BFDV-1 particles (Stoll et al., 1993). The antibodies cross-react with the structural polypeptides of SV40 (Stoll et al., 1993).

These antibodies can also be used for the detection of agnoprotein 1a and agnoprotein 1b, as these proteins have been observed in APV particles (R. Johne & H. Müller, unpublished data), in addition to the structural proteins VP1, VP2 and VP3 (Müller & Nitschke, 1986; Stoll et al., 1993). Their reactivity with agnoprotein 1b, however, is low, as only large amounts of agnoprotein 1b expressed in Escherichia coli can be detected (R. Johne & H. Müller, unpublished data).

Antibodies directed against a β-galactosidase–large T antigen fusion protein of BFDV-1 (Stoll et al., 1993) were used for the detection of large T antigen and small T antigen of APV.

DNA fragmentation analysis and TUNEL assay. Fragmented DNA indicating apoptosis was demonstrated by electrophoresis of isolated DNA or in situ using a terminal deoxynucleotidyl transferase–mediated dUTP nick end labelling (TUNEL) assay (Gavrieli et al., 1992). For DNA isolation, cells were treated with proteinase K at various times after infection. Cellular DNA was extracted with phenol–chloroform (Sambrook et al., 1989), treated with RNase A (Pharmacia), and subjected to electrophoresis on ethidium bromide-stained 2% agarose gels. For the TUNEL assay, CE cells were fixed with acetone for 15 min and 2% paraformaldehyde for 15 min at 4 °C. This was followed by permeabilization with 0·1% Triton X-100 for 30 min at 4 °C. DNA fragmentation was analysed using the in situ Cell Death Detection kit (Boehringer Mannheim). The percentage of apoptotic cells was determined by counting 200 cells in each experiment and averaging the results of three tests.

Results

Apoptosis is induced in APV-infected CE cells, but not in Vero cells infected with SV40

In CE cells infected with APV strain BFDV-1, DNA fragmentation was detected using electrophoresis of isolated DNA located within the coding region of VP1. As this led to a disruption of this gene, plasmid pAPV was digested with Hpal and EcoRI. The resulting 3852 bp fragment was circularized, linearized using BamHI, and ligated with a 4981 bp pAPV BamHI fragment to generate plasmid pAPVinf, capable of expressing all viral genes.

Site-directed mutagenesis. The megaprimer PCR technique (Barik, 1997) was used for site-directed mutagenesis at the initiation codon of agnoprotein 1a and agnoprotein 1b. The megaprimer was synthesized by PCR, with pAPVinf as template and primers 5′ GCC CTG AGA TCT GAA CAT TTC ACT TGT AA 3′ and 5′ TGG AGT AGA TCT GCC CCG 3′ (mutated nucleotides underlined), and used in a second PCR, together with primer 5′ GGA CAA CAT GTC TAC TCC AGC G 3′ and the same template. Both reactions were carried out with Pwo DNA polymerase (PeqLab). The PCR product was cleaved with EcoRI and XbaI. The resulting 670 bp cleavage product was ligated to an 8164 bp fragment of EcoRI/XbaI-digested pAPVinf, in order to generate pAPVinfmut. DNA sequencing of the inserted PCR product revealed this plasmid to be identical to the APV full-length plasmid pAPVinf, with the exception that GAT at position 289–291 was replaced by CTG. With this mutation the initiation codon ATG for agnoprotein 1a and agnoprotein 1b is exchanged for TGG.
DNA, beginning 2 days p.i. (Fig. 1A). No DNA fragmentation was observed in mock-infected cells. By immunoblotting, the expression of viral proteins VP1, VP2, VP3 and agnoprotein 1a was demonstrated in the infected cells (Fig. 1B).

Vero cells infected with SV40 showed no distinct fragmentation of DNA. A faint smear of DNA, visible after 2 days p.i., might be due to necrotic DNA degradation (Fig. 1C). At this time, an additional DNA band appeared in the infected cells, possibly representing viral DNA. No DNA degradation was observed in the mock-infected cells. By immunoblotting, the presence of SV40-specific polypeptides VP1, VP2 and VP3 was demonstrated in the infected cells (Fig. 1D).

**Agnoprotein 1a and agnoprotein 1b induce apoptosis in Sf9 insect cells**

Sf9 cells were infected with the recombinant baculoviruses agno1a-baculo and agno1b-baculo, respectively, at an m.o.i. of 10 p.f.u. per cell. Sf9 cells infected with either wild-type baculovirus or recombinant baculovirus expressing structural protein VP1 of APV (R. Johne & H. Müller, unpublished data) served as controls. Cytopathic changes observed in Sf9 cells infected with agno1a-baculo or agno1b-baculo were different from those observed in VP1-expressing and wild-type baculovirus-infected cells (not shown). Shrinkage and premature detachment of agno1a-baculo- and agno1b-baculo-infected cells from the surface were reminiscent of observations described for apoptotic cells. DNA fragmentation was not detected in cells infected with VP1-expressing baculovirus (Fig. 2A). Only weak DNA fragmentation was found in wild-type baculovirus-infected cells (Fig. 2C). However, distinct
Fig. 3. Immunoblot analysis of proteins in Sf9 cells infected with agnoprotein 1a- (A) or structural protein VP1- (B) expressing recombinant baculoviruses. Cells were analysed on day 1 (d1), 2, 3, 4, 5 and 6 p.i. Lane M, molecular mass markers labelled in kDa (biotinylated SDS–PAGE standards, low range; Bio-Rad).

Fig. 4. Demonstration of apoptosis by a TUNEL reaction (upper row) in CE cells after transient expression of agnoprotein 1a (A), agnoprotein 1b (B), or transfection of expression plasmid pSG5 as negative control (C). Cells were stained 3 days after transfection. The efficiency of transfection is indicated by GFP expression in the same cultures 24 h after transfection (lower row). For details, see text.

DNA laddering was observed in cells 4 days p.i. with agno1a-baculo (Fig. 2B) and 3 days p.i. with agno1b-baculo (Fig. 2D).

By immunoblotting, a double band with an apparent molecular mass of 32/33 kDa was demonstrated in cells infected with agno1a-baculo 3 days p.i. (Fig. 3 A), representing agnoprotein 1a with different post-translational modifications (Li, 1996; Liu, 1997). An additional band of 29 kDa, not characterized further until now, was also observed. Approximately the same amounts of protein were found from day 3 to day 5 p.i. Despite the apoptotic changes observed in the agno1b-baculo-infected cells, agnoprotein 1b was not detected (not shown). In cells infected with VP1-expressing recombinant baculovirus, increasing amounts of a 42 kDa protein corresponding to VP1 were observed after 2 days p.i. (Fig. 3 B). No proteins were detected in cells infected with baculovirus wild-type (not shown).

Agnoprotein 1a and agnoprotein 1b induce apoptosis after transient expression in CE cells

To investigate the induction of apoptosis in a host cell system permissive for APV replication, agnoprotein 1a and agnoprotein 1b were transiently expressed in CE cells under the control of the SV40 early promoter. In these experiments, CE cells transfected with the vector pSG5 were used as negative control. To determine the number of apoptotic cells,
a TUNEL assay was performed 3 days after transfection to detect the amount of cellular DNA fragmentation. A high proportion of cells transfected with agno1a-CE (16.8 ± 1.3%, Fig. 4A), or agno1b-CE (21.5 ± 2.5%, Fig. 4B), showed nuclear fluorescence under UV light, indicative of apoptotic DNA fragmentation. In contrast, fluorescence was observed only in a low number of cells transfected with the control plasmid pSG5 (7.8 ± 1.2%, Fig. 4C). The efficiency of transfection was 7.8 ± 0.3% in the case of cells transfected with agno1a-CE, 7.5 ± 0.5% in the case of cells transfected with agno1b-CE, and 8.5 ± 0.3% in the case of cells transfected with pSG5.

Proteins were analysed by SDS–PAGE and immunoblotting as described above. In CE cells transfected with agno1a-CE, a 33 kDa protein, in the same position in the gel as agnoprotein 1a, appeared 16 h after transfection (Fig. 5). No proteins could be detected in CE cells transfected with agno1b-CE or pSG5 (not shown).

Induction of apoptosis in APV infection correlates with expression of agnoprotein 1a and agnoprotein 1b

An infectious APV full-length plasmid (pAPVinf) was constructed. A similar plasmid, with a mutated initiation codon for agnoprotein 1a and agnoprotein 1b expression (pAPVinf.mut), was also constructed to confirm the significance of these proteins for replication and the induction of apoptosis. A scheme of these plasmids is shown in Fig. 6(A). This figure also shows the generated Pvull site, which allows identification of the mutation.

To test if infectious progeny was produced by these plasmids, CE cells were transfected as described. After 5 days of incubation at 38 °C, the cells were frozen and thawed three times, sedimented by centrifugation, and the supernatants were used to infect fresh CE cell cultures. These cells were incubated as before and the procedure was repeated once more. Then the sedimented cells were used to analyse the presence of APV-specific polypeptides by immunoblotting. Large T antigen as well as the structural proteins VP1 and VP3 were present in cells transfected with pAPVinf and pAPVinf.mut; a protein band corresponding to agnoprotein 1a, however, was found only in CE cells transfected with pAPVinf. No infectious progeny was produced after the transfection of pAPVinf.mut, since no APV-specific polypeptides were observed in the CE cells infected with the supernatants (Fig. 6B, C).

Further transfection experiments were performed to investigate induction of apoptosis by these plasmids. pBluescript II SK(+) was used as a negative control. Viral gene expression was determined by immunoblotting (data not shown) and induction of apoptosis was investigated by TUNEL, as described above, 4 days after transfection. As compared to CE cells transfected with pAPVinf.mut (Fig. 7A), nuclear fluorescence was observed in a considerably higher number of cells transfected with pAPVinf (Fig. 7B) (10.8 ± 2.8% and 29.3 ± 3.8%, respectively). The lowest number of apoptotic cells was observed in cells transfected with the negative
control (8.3 ± 0.8%; Fig. 7C). The transfection efficiency was 8.3 ± 0.8% in the case of pAPVin.mut, 7.8 ± 1.2% in the case of pAPVin, and 7.8 ± 0.8% in the case of the negative control.

**Discussion**

In APV, agnoprotein 1a consists of 176 amino acids, and agnoprotein 1b consists of 112 amino acids, with molecular masses as deduced from their gene sequences of 19.6 kDa and 12.3 kDa, respectively. In SDS–PAGE, however, apparent molecular masses of 32/33 kDa and 26 kDa, respectively, have been determined for these proteins (Li, 1996; Liu, 1997; Stoll et al., 1994). A profoundly elongated conformation and modification by phosphorylation at specific serine and threonine residues have been proposed to be responsible for these discrepancies (Liu, 1997).

After transfection of expression plasmids into CE cells, as well as after the infection of Sf9 cells with recombinant baculoviruses, agnoprotein 1b was not detected by immunoblotting, whereas agnoprotein 1a was regularly observed. As mentioned above, the antibodies used in this study only detect large amounts of agnoprotein 1b, for unknown reasons. As only low expression levels of agnoprotein 1a have been observed, it has to be taken into account that the amounts of agnoprotein 1b expressed in the experiments described are not sufficient to be detected by immunoblotting.

The data reported here demonstrate that these proteins induce apoptosis in Sf9 cells as well as in CE cells, the cell type commonly used for APV propagation. The amino acid sequence of agnoprotein 1b is identical to that of agnoprotein 1a, but with an internal deletion of amino acids 69 to 132 (Luo et al., 1995). Apoptosis is, therefore, induced by amino acids 1 to 68 and amino acids 133 to 176 of agnoprotein 1a. An extensive database search analysis with these sequences did not reveal significant similarities to currently known apoptotic inducers. Therefore, no conclusions can be drawn from the amino acid sequence as to the pathways by which apoptosis is induced. Transfection experiments employing cell lines with well-characterized mutations should provide some insight into these pathways.

As compared to plasmid agno1a-CE, apoptosis in CE cells was considerably stronger after transfection of plasmid agno1b-CE. When Sf9 cells were infected with agno1b-baculo, DNA fragmentation was observed about 24 h earlier as compared to infection with agno1a-baculo. The reasons for these differences are not known, but it may be speculated that amino acids 69 to 132, present in agnoprotein 1a and deleted in agnoprotein 1b, are involved. This region contains a potential nuclear localization signal (HRRRPYDR; Li, 1996) which might result in a decrease in apoptotic activity due to the transport of agnoprotein 1a to the nucleus.

There is increasing evidence that apoptosis plays a key role in the life-cycle of numerous viruses (Shen & Shenk, 1995; Teodoro & Branton, 1997). It has been reported recently that apoptosis is induced in SV40 T antigen-transformed fibroblasts (Tsao et al., 1998). To our knowledge, however, induction of apoptosis in the replicative cycle of mammalian polyomavirus has not been described. In this study we observed no distinct DNA fragmentation in Vero cells infected with SV40, but instead degraded DNA, which is more indicative of
necrotic than apoptotic processes. In contrast, apoptosis was clearly induced in APV-infected CE cells. It might be speculated that these conflicting observations could result from the different cell types used; they could, however, also be due to different properties of these viruses. To answer these questions, studies employing a broad range of different cell types are currently being performed.

Induction of apoptosis during APV infection correlates with the expression of agnoprotein 1a and agnoprotein 1b. Agnoproteins of the mammalian polyomaviruses SV40, BKV and JCV exhibit no obvious sequence similarities to agnoprotein 1a and agnoprotein 1b of APV. Various functions of SV40 agnoprotein have been discussed, such as effects on virus assembly (Margolskee & Nathans, 1983; Carswell & Alwine, 1986), maturation (Hou-Jong et al., 1987), regulation of transcription (Alwine, 1982; Hay & Aloni, 1985), and release of mature virus (Resnick & Shenk, 1986). In SV40, agnoprotein expression is not essential for virus replication (Hou-Jong et al., 1987; Resnick & Shenk, 1986). In contrast, as shown here, expression of agnoprotein 1a and agnoprotein 1b seems to play an essential role in the life-cycle of APV, because no infectious progeny was produced after pAPVinf.mut transfection. Further experiments will have to be performed to get an insight into the mechanism(s) responsible for blocking APV replication.

At present it may be assumed that agnoproteins of APV and agnoproteins of mammalian polyomaviruses exert different biological functions during virus replication. One of these functions of agnoprotein 1a and agnoprotein 1b is obviously the induction of apoptosis. Induction of apoptosis is known to be a mechanism to destroy cells in the absence of an inflammatory response. It could be speculated that this mechanism also plays a role in pathogenicity of APV, e.g. by allowing efficient virus release from the nucleus and rapid virus spread through the organism.

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