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Short Communication

The P gene of Newcastle disease virus does not encode an accessory X protein

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Many paramyxoviruses encode non-essential accessory proteins that are involved in the regulation of virus replication and inhibition of cellular antiviral responses. It has been suggested that the P gene mRNA of Newcastle disease virus (NDV) encodes an accessory protein – the so-called X protein – by translation initiation at a conserved in-frame AUG codon at position 120. Using a monoclonal antibody that specifically detected the P and X proteins, it was shown that an accessory X protein was not expressed in NDV-infected cells. Recombinant NDV strains in which the AUG was changed into a GCC (Ala) or GUC (Val) codon were viable but showed a reduction in virulence, probably because the amino acid change affected the function of the P and/or V protein.

The family *Paramyxoviridae* includes important pathogens that can cause severe disease in humans as well as animals. Well-known examples of human pathogens are measles virus, mumps virus, Hendra virus and Nipah virus. Animal pathogens include rinderpest virus, bovine respiratory syncytial virus and Newcastle disease virus (NDV). The subfamily *Paramyxovirinae* consists of five genera, *Respirovirus*, *Morbillivirus*, *Rubulavirus*, *Henipavirus* and *Avulavirus* (Mayo, 2002a, b). Paramyxoviruses have a non-segmented negative-sense single-stranded RNA genome that encodes six to ten genes (Lamb & Kolakofsky, 2001). Characteristic of most, if not all, paramyxoviruses is the ability to generate multiple proteins from the P gene. These so-called accessory proteins are generated by an RNA-editing event and in some instances by the use of alternative open reading frames (ORFs) present within the P gene. For respiroviruses, morbilliviruses and avulaviruses, the P protein is encoded by an unedited transcript of the P gene, whereas the V and W proteins are the result of an mRNA-editing event in which one (V) or two (W) G residues are inserted at a specific position within the P gene mRNA. For rubulaviruses, the unedited mRNA generates the V protein while the P and W proteins are the result of mRNA-editing. Additional proteins derived from the P gene mRNA may be generated by translation initiation at different start points in the +1 reading frame (C proteins) or in the same reading frame (X protein) (Curran *et al.*, 1998). Morbilliviruses express at least one C protein, whereas some respiroviruses express two or more. Apart from the P protein, the Sendai virus P gene seems to encode a total of at least seven accessory proteins, i.e. V and W by RNA-editing, C, C′, Y1 and Y2 from the +1 reading frame, and X from an in-frame reading frame (Curran *et al.*, 1998). Expression of an accessory C protein, or X protein, has not yet been reported for rubulaviruses or avulaviruses. However, McGinnes *et al.* (1988) reported the existence of 38 and 29 kDa non-structural proteins derived from the P gene ORF of NDV (genus *Avulavirus*) and suggested that these proteins could have been generated by in-frame translation initiation at amino acid positions 82 and 120, respectively. Analysis of the P gene sequences of 23 different NDV strains showed that the AUG codon at position 82 was not conserved, whereas the one at position 120 was completely conserved in all strains (Locke *et al.*, 2000). These results led Locke and co-workers to suggest that – in addition to the P and V/W proteins – an additional protein, termed the X protein, could potentially be expressed by the P gene of NDV (Fig. 1).

To determine whether NDV encodes an accessory X protein, we tried to detect the X protein in NDV-infected cells using two different monoclonal antibodies (mAbs) against the P protein. To determine the specificity of these mAbs, we first expressed the individual P, V, W and X proteins in eukaryotic cells by means of an expression vector. The different ORFs were amplified by PCR using the Z-*Taq* system (Takara) with full-length NDV cDNA as a template (Peeters *et al.*, 1999; GenBank accession no. AF077761). The P ORF was amplified using primers pRT1 (5′-CAAAGAATTCAGAAAAAAGTACGGGTAGAAG-3′) and p2 (5′-GCAGTCTAGATTAGCCATTCACTGCAAGGCGC-3′). The X ORF was amplified using primers XpF (5′-GACGAATTCCGTCGACACACAGTTCAGG-3′) and p2. The X ORF provided with an optimized translation initiation site (Kozak, 1987) was amplified using primers XpFkz (5′-AACGAATTCGCCGCCATGCTTGACAAGCTAGCAATAAATCG-3′) and p2. The V ORF was amplified by means of fusion PCR using primer pRT1 as forward primer and

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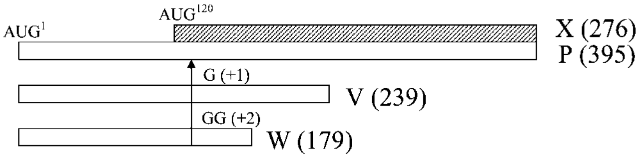

Fig. 1. Schematic diagram of the different proteins that can be expressed from the P gene of NDV. The P protein is the result of translation initiation at the first AUG start codon. The X protein (hatched) is the result of translation initiation at an in-frame AUG start codon at amino acid position 120. The V and W proteins are generated from edited P gene mRNAs, which are generated by the insertion of one (V) or two (W) G residues at a specific position (arrow) within the P gene mRNA. Numbers in parentheses refer to the total number of amino acid residues in each protein.

primer VpR (5′-GGGCTCGACCATGGGCCCCTTTTTAGCATTGGACG-3′) as reverse primer in PCR 1, and primer VpF (5′-CGTCCAATGCTAAAAAGGGGCCCATGGTCGAGCCC-3′) as forward primer and p2 as reverse primer in PCR 2. The products of PCR 1 and PCR 2 were combined and joined by fusion PCR using primers pRT1 and p2. Similarly, the W ORF was amplified using primers pRT1 and WpR (5′-GGGCTCGACCATGGGCCCCCTTTTTAGCATTGGACG-3′) in PCR 1 and primers WpF (5′-CGTCCAATGCTAAAAAGGGGGCCCATGGTCGAGCCC-3′) and p2 in PCR 2. The products of PCR 1 and PCR 2 were combined and joined by fusion PCR using primers pRT1 and p2. The different PCR fragments were subsequently cloned in the expression plasmid pCIneo (Clontech) between the *Eco*RI and *Xho*I sites behind the T7 promoter, yielding pCIneo-P, pCIneo-V, pCIneo-W, pCIneo-X and pCIneo-X$_{\mathrm{kz}}$. To test whether the P-specific mAbs 688 (Russell *et al.*, 1983) and P1a (McGinnes *et al.*, 1988) could be used to detect the putative X protein, their specificity was determined using Western blots after transient expression of the different proteins in QM5 cells (Antin & Ordahl, 1991). To this end, QM5 cells were seeded in six-well culture dishes and grown overnight to 80 % confluency. The monolayers were infected with Fowlpox-T7 (Britton *et al.*, 1996) at an m.o.i. of 1 and after 1 h the monolayers were washed once with Optimem (Gibco) and transfected with 2 μg plasmid DNA using 6 μl FuGENE 6 (Roche). After incubation for 48 h, lysates were prepared in lysis buffer [PBS containing 1 % Triton X-100, 0·5 % sodium deoxycholate, 0·1 % SDS and Complete protease inhibitor cocktail (Roche)]. Samples were analysed by 12 % SDS-PAGE followed by Western blotting on to Immobilon-P (Millipore). Proteins reacting with mAbs 688 and P1a were detected using horseradish peroxidase-labelled anti-mouse IgG antibodies and a chemiluminescence detection system (Supersignal; Pierce).

Fig. 2(a) shows that mAb 688 recognized the P protein as well as the V and W proteins but not the X protein. This indicated that the epitope recognized by mAb 688 was located within the N-terminal half of the P gene, i.e. before the RNA-editing site. Fig. 2(b) shows that mAb P1a recognized both the P protein and the X protein but not the V or W proteins, indicating that the epitope recognized by this antibody was located within the C-terminal half of the P gene, i.e. after the RNA-editing site. Expression of the X protein was not dependent on the presence of an optimized Kozak sequence. Apparently, the sequence upstream of the AUG codon at position 120 functions as an efficient translation initiation site. However, it was of interest to note that the X protein could not be detected in cells transfected with pCIneo-P. This indicated that, at least in this system, the AUG codon at position 120 in the P gene mRNA was not used for initiation of translation of the X ORF.

Next, we generated a knockout mutant in which expression of the X protein was abolished by changing the putative AUG start codon at position 120 into a GCC (Ala) codon. Mutagenesis of the AUG codon was accomplished using fusion PCR as described above. Primers p1356+ (5′-AAATCGGAGTCCTCACTGGG-3′) and KOXR (5′-AGAGAGTTGCTTGCTCCGGTCCTGAACTGTGTATCGACGGCTTCG-3′) were used for PCR 1 and primers KOXF3 (5′-TCAGGACCGGAGCAAGCAACTCTCTGCTGTTGGTCCTTGACAAGC-3′) and p2617− (5′-TGATAGTCAACTTTACTTAC-3′) were used for PCR 2. The products of PCR 1 and PCR 2 were combined and joined by fusion PCR using primers p1356+ and p2617−. The resulting

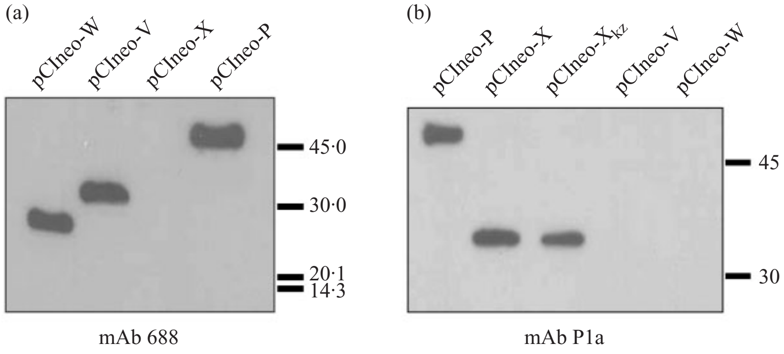

Fig. 2. Western blots showing proteins detected by mAb 688 (a) or mAb P1a (b) in lysates of transfected QM5 cells after transient expression of different NDV proteins for 48 h. The sizes of marker proteins (kDa) are indicated.

fragment was digested with *Sal*I and *Apa*I and used to replace the corresponding fragment in a plasmid containing the full-length cDNA of NDV (pNDFLtag). The resulting full-length cDNA was used to rescue virus by means of co-transfection with NP, P and L helper plasmids in QM5 cells as described previously (Peeters *et al.*, 1999). The rescued virus was designated NDFLtagKOX3. Sequence analysis showed that the desired mutation was present in the genome of NDFLtagKOX3 (data not shown). Strain NDFLtagKOX3 replicated to similar titres in embryonated specific-pathogen-free eggs as the parent strain NDFLtag (data not shown). However, the intracerebral pathogenicity index (ICPI) in 1-day-old chickens of NDFLtagKOX3 was 1·1, which was somewhat lower than that of NDFLtag (ICPI = 1·3).

To determine whether the X protein was expressed by NDFLtag but not by NDFLtagKOX3, the viruses were used to infect QM5 cells. After incubation for 48 h, lysates were prepared and subjected to 12 % SDS-PAGE followed by Western blotting as described above. As references, lysates of QM5 cells transfected with pCIneo-P and pCIneo-X were included. Fig. 3 shows that large amounts of P protein were present in infected cells. However, no X protein could be detected in NDFLtag-infected cells using mAb P1a. These results strongly suggested that NDV does not express an accessory X protein.

Accessory proteins encoded by paramyxoviruses are involved in regulation of viral genome expression (Curran *et al.*, 1992; Tapparel *et al.*, 1997; Tober *et al.*, 1998) and interference with cellular antiviral responses (Didcock *et al.*, 1999; Garcin *et al.*, 2001; Young *et al.*, 2001; Gotoh *et al.*, 2002). The V protein of NDV has been shown to act as an interferon antagonist (Park *et al.*, 2003; Huang *et al.*, 2003) and is involved in pathogenesis and host-range restriction (Mebatsion *et al.*, 2001; Park *et al.*, 2003). However, since an effect on viral genome expression has not been reported for the V protein of NDV, such a function – if present at all – might be exerted by (an)other accessory protein(s). The 38 and 29 kDa proteins observed by McGinnes *et al.* (1988) in NDV-infected cells might represent such proteins. However, we have shown here that the X protein, which is equivalent to the 29 kDa protein, is not expressed in NDV-infected cells. Furthermore, the observation that the AUG codon at position 82 (which would lead to the expression of the 38 kDa protein) is not conserved among 23 different NDV strains strongly argues against the existence of a 38 kDa accessory protein. Since we used the same mAb, the fact that we did not observe the 38 and 29 kDa proteins described by McGinnes *et al.* (1988) is rather puzzling. One explanation would be that these proteins are specific proteolytic degradation products derived from the P protein. However, other possibilities cannot be ruled out.

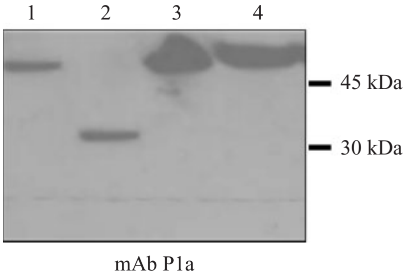

Fig. 3. Western blot showing proteins detected by mAb P1a in lysates of QM5 cells infected for 48 h with parental strain NDFLtag (lane 4) and strain NDFLtagKOX3 (lane 3). In the latter strain, the AUG codon at position 120 has been changed into a GCC codon. Lanes 1 and 2 were included for reference and contained lysates of QM5 cells transfected with pCIneo-P and pCIneo-X (as in Fig. 2b). Sizes of marker proteins are indicated.

Replacement of the AUG (Met) codon at position 120 by a GCC (Ala) codon in the P gene had no significant effect on replication of the corresponding NDFLtagKOX3 virus in embryonated eggs (data not shown). However, we did notice a reduction in virulence after intracerebral inoculation of 1-day-old chickens. Recently, we obtained similar results with KOX mutants in which the AUG codon was replaced by a GUC (Val) codon. In this case the ICPI was reduced further, to 0·7–0·9.

Since, as shown here, NDV does not produce an X protein, the amino acid replacement probably has an effect on the biological function of the P and/or V/W proteins. This is not unexpected, since the V protein of NDV has been implicated in pathogenesis (Mebatsion *et al.*, 2001). When we introduced the AUG→GCC mutation into a non-virulent vaccine strain, we observed that the 50 % embryo lethal dose in 18-day-old embryos was significantly higher than that of the parental strain (data not shown). Experiments to test whether such mutants can be used as *in ovo* vaccines are in progress.

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