Sequence motifs and prokaryotic expression of the reptilian paramyxovirus fusion protein

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Summary. Fourteen reptilian paramyxovirus isolates were chosen to represent the known extent of genetic diversity among this novel group of viruses. Selected regions of the fusion (F) gene were sequenced, analyzed and compared. The F gene of all isolates contained conserved motifs homologous to those described for other members of the family *Paramyxoviridae* including: signal peptide, transmembrane domain, furin cleavage site, fusion peptide, N-linked glycosylation sites, and two heptad repeats, the second of which (HRB-LZ) had the characteristics of a leucine zipper. Selected regions of the fusion gene of isolate Gono-GER85 were inserted into a prokaryotic expression system to generate three recombinant protein fragments of various sizes. The longest recombinant protein was cleaved by furin into two fragments of predicted length. Western blot analysis with virus-neutralizing rabbit-antiserum against this isolate demonstrated that only the longest construct reacted with the antiserum. This construct was unique in containing 30 additional C-terminal amino acids that included most of the HRB-LZ. These results indicate that the F genes of reptilian paramyxoviruses contain highly conserved motifs typical of other members of the family and suggest that the HRB-LZ domain of the reptilian paramyxovirus F protein contains a linear antigenic epitope.

Introduction

Paramyxoviruses are recognized as important pathogens of reptiles [14, 20, 21, 37]. Beginning with the initial reports [10, 15] of the Fer-de-Lance virus (FDLV), reptilian paramyxoviruses have been isolated from outbreaks associated with severe sickness and mortality in collections of viperid-, colubrid-, boid- or elapid snakes and lizards held in venom farms, herpetariums and zoological parks in Europe and North America [17, 20]. Analysis of the full genome of the Fer-de-Lance virus (FDLV), the proposed type species virus for the group,

suggests these viruses are *bona fide* members of the family *Paramyxoviridae*, but have unique properties including the presence of a novel gene not found in other members of the family [26]. Sequence analysis of a portion of the polymerase (L) and hemagglutinin-neuraminidase (HN) genes was used by Ahne et al. [2] to assess the extent of genetic diversity among 16 isolates of reptilian paramyxoviruses. In that study, and in an additional analysis by Kindermann et al. [24], most isolates fell into one of two distinct subgroups (genogroups), designated 'a' and 'b', that differed by more than 20% at the nucleotide level with a few isolates falling into 'intermediate' positions. A similar study of 18 reptilian paramyxovirus isolates by Franke et al. [16] confirmed these findings of genetic diversity and provided a preliminary characterization of portions of the fusion (F) gene.

Studies of mammalian and avian paramyxoviruses have shown that the fusion (F) protein of paramyxoviruses is a key determinant of virulence [25]. The F protein is synthesized as a biologically inactive precursor (F0) that is post-translationally cleaved by cellular proteases into the disulfide-linked, fusio-genically active F1 and F2 subunits [39]. The newly released N-terminus of the F1 subunit contains a fusion peptide that aids insertion of the virus into the target membrane of the new host cell [5]. The carboxy-terminus anchors the viral transmembrane domain. The F protein motifs are highly conserved among paramyxoviruses [28]. However, activation of the F protein can be influenced by amino acid changes within several of the F protein motifs such as the cleavage site, the fusion peptide, heptad repeats A and B, or the leucine zipper [7, 8, 18, 22, 33, 36, 40]. In 2001, Junquiera de Azevedo et al. [23] reported a partial F gene sequence of a reptilian paramyxovirus, found as an expressed sequence tag clone from a wild-caught Fer-de-lance snake. In the same year, analysis of partial F gene sequences from 18 reptilian paramyxovirus isolates by Franke et al. [16] showed the presence of a predicted furin cleavage site having an amino acid sequence R-E-K-R within the F protein. This site was highly conserved among the isolates and homologous to the paramyxovirus consensus sequence Arg-X-Arg/Lys-Arg (R-X-R/K-R). Franke et al. [16] also compared the sequences for a 25-amino-acid region of the predicted fusion peptide and reported an 11 amino acid region (TSAQITAGIAL) was identical among the isolates and was homologous to the conserved domain described for certain genera of the subfamily *Paramyxovirinae*.

In the present study, the full-length F gene sequence from FDLV [26] was used to identify a more complete set of predicted motifs including, in addition to the furin cleavage site and fusion peptide, the complete heptad A and heptad B regions, that included a leucine zipper in the HRB. Portions of the F gene containing these predicted motifs were sequenced for 14 reptilian paramyxovirus isolates that were chosen from among those characterized by Ahne et al. [2] to represent the known extent of genetic diversity among this novel group of viruses. With the exception of the type strain FDLV and the isolate Gono-GER85, the isolates in the present study are different from those described by Franke et al. [16]. The purpose of this portion of the work was to characterize and compare additional sequence motifs within the F protein, and to describe the extent of conservation or variation among genetically diverse isolates of reptilian paramyxoviruses. In addition, prokaryotic

expression of various portions of the F protein was used to verify the function of the furin cleavage site and to investigate the presence of antigenic determinants within the F protein of the reptilian paramyxoviruses.

Materials and methods

Viruses

Fourteen reptilian paramyxoviruses (Table 1) were selected from among the isolates analyzed by Ahne et al. [2]. These isolates were chosen to encompass the range of genetic sequence types identified in that study and included three isolates from subgroup 'a' (Crot1-VA95, Trim-MD97, Crot3-CA98), six from subgroup 'b' (More-GER86, Biti-GER87, Pyth-GER88, Ela2-GER93, Ela1-GER94, Lamp-MD96), and five designated as 'intermediate' (FDLV, Gono-GER85, Call-GER88, Crot2-OH90, Ela-FL93) that did not fall within either subgroup. The set included the proposed type strain, Fer-de-Lance virus (FDLV; ATCC VR-895), the only reptilian paramyxovirus for which the sequence of the entire genome is known [26], and Gono-GER85, which has been further characterized in biological and genetic studies [1–4, 16, 31]. All isolates were grown and harvested as previously described [1, 3].

PCR amplification and sequencing

Degenerate primers (Table 2) were used to amplify two portions of the coding region of the fusion gene from each virus isolate. The first region was 295 nucleotides (nt) in length (nt 327–621 of the F gene) and contained the predicted cleavage site, fusion peptide, and heptad repeat A. The second region of 120 nt (1420–1539) included heptad repeat B that contained a leucine zipper. The degenerate primers were chosen based upon alignment of the GenBank sequences of FDLV (AY141760), *Sendai virus* (M30202) and other representative

Table 1. Reptilian paramyxovirus isolates used in this study

Isolate name	Subgroup ^a	Isolation year	Host	Location
Crot1-VA95	a	1995	<i>Crotalus sp.</i>	Virginia USA
Trim-MD97	a	1997	<i>Trimeresurus sp.</i>	Maryland USA
Crot3-CA98	a	1998	<i>Crotalus mitchelli</i>	California USA
More-GER86	b	1986	<i>Morelia argus</i>	Germany
Biti-GER87	b	1987	<i>Bitis nasicornis</i>	Germany
Pyth-GER88	b	1988	<i>Python regius</i>	Germany
Ela2-GER93	b	1993	<i>Elaphe guttata</i>	Germany
Ela1-GER94	b	1994	<i>Elaphe schrenkii</i>	Germany
Lamp-MD96	b	1996	<i>Lampropeltis sp.</i>	Maryland USA
FDLV	i	1972	<i>Bothrops atrox</i>	Switzerland
Gono-GER85	i	1985	<i>Gonosoma oxycephala</i>	Germany
Call-GER88	i	1988	<i>Callopistes maculatus</i>	Germany
Crot2-OH90	i	1990	<i>Crotalus durissis</i>	Ohio USA
Ela-FL93	i	1993	<i>Elaphe guttata</i>	Florida USA

^aAs in Ahne et al. [2] where 'a' and 'b' indicate two clearly defined genogroups and 'i' indicates intermediate isolates that fell between the two subgroups in phylogenetic analyses

paramyxoviruses. Primers listed in Table 2 were also used to determine the sequence of the F gene of isolate Gono-GER85 (GenBank AY725422) for development of a prokaryotic expression system. Amplification of viral RNA using RT-PCR and sequencing of the PCR products was performed as described by Ahne et al. [2].

Nucleotide sequence analyses, alignments and predictions for amino acid sequences were performed using *Mac Vector 6.0* software (International Biotechnologies, New Haven, CT, USA). Protein motifs such as signal peptides and transmembrane domains were predicted

Table 2. Primers used to amplify portions of the F gene of 14 reptilian paramyxovirus isolates. The primer binding-site corresponds to the nucleotide number of the open reading frame of the full-length fusion protein of FDLV (AY141760) where the adenine in the initial methionine codon is nucleotide 1

a. Degenerate primers used to amplify and sequence the cleavage site, fusion peptide, HRA and HRB. Normal first round rt-PCR was performed with primers 1701/1718. Second round primers were also used as sequencing primers. To amplify the HRA and HRB regions of certain isolates, the annealing temperature was reduced from 50 °C to 35 °C during both rounds of amplification. Some isolates required other primer combinations: the HRB region of subgroup 'a' isolates required 1716/1718 in first round (35 °C), with 1799/1800 in second round (35 °C). The HRA region of 'intermediate' isolate Ela-FL93 was amplified with 1703/1718 in the first round (35 °C), then 1723/1715 in second round (35 °C)

Primer name	PCR round	Binding site	Nucleotide sequence
1701 Ext For	1	109–128	5' AAR TYM CTG AAG ATM RCK GG 3'
1718 Ext Rev	1	1542–1525	5' GMT GAT SAT CMC TAT RAT 3'
1703 Ext For	1	236–254	5' TS TTA RTM CCW YTG AGG GA 3'
1724 Cleavage For	2	244–263	5' CCT CTR AGR GAC ACC ATA AA 3'
1710 Cleavage Rev	2	397–380	5' T CCC NGC YGT TAT YTG TG 3'
1723 HRA For	2	319–338	5' AGA GAR AAG MGR TTT GTY GG 3'
1715 HRA Rev	2	596–577	5' GT RTT TTT RGC RAC ATC RCA 3'
1716 HRB For	2	1306–1328	5' AAC GTA ACT GCN AAY TTY GGN AA 3'
1797 HRB Rev	2	1508–1489	5' AC AGA RAC YAC RAT YAA TGC 3'
1799 HRB (subgp. a)	2	1339–1355	5' GGG CCG GTG GTA TCA GT 3'
1800 HRB (subgp. a)	2	1495–1477	5' T CAA TGC AAT AGA TGT CGG 3'

b. Primers used for cloning selected regions of the F gene into a prokaryotic expression vector. "RES" stands for a specific overhang sequence containing a restriction site

Primer name	PCR round	Binding site	Nucleotide sequence
Exp compl For	1	1–21	5' RES A ATG ACT AGG ATA ACT ATC CTT 3'
Exp compl Rev	1	1618–1638	5' RES TTA TTT TAT AAA GCC TAG ATT 3'
1905 Exp 1+2 For	1	75–96	5' RES T CTT GAA CAG GTA GGA GTT ATG 3'
1870 Exp 1 Rev	1	1470–1450	5' RES TAG ATT CTT ATT ATT GAT TCC 3'
1885 Exp 2 Rev	1	330–310	5' RES TCT CTT CTC TCT CTT GTT TGG 3'
1906 Exp 3 For	1	330–351	5' RES A TTT GTC GGG ATA GCA ATT GCA 3'
1888 Exp 3 Rev	1	1380–1360	5' RES CTC TGC CGA TAA GTC CAG ATC 3'

using *Signal P V2.0 version b2* and the *TMHMM server version 2.0* software programs (Center for Biological Sequence Analysis, Lyngby, Denmark). Oligopeptide repeats were determined by *Mac Vector 6.0* and by the *2ZIP* program (Max Planck Institute for Molecular Genetics, Berlin, Germany). Prediction of glycosylation sites was carried out using *NetNGlyc 1.0* software (Center for Biological Sequence Analysis, Lyngby, Denmark).

Prokaryotic expression of the F gene

A prokaryotic expression system was used to create a set of recombinant proteins based upon the nucleotide sequence of isolate Gono-GER85 (AY725422). The two longest recombinant proteins were designed to contain either the entire F0 protein (residues 1–546, not shown) or a truncated form (residues 26–490) lacking much of the signal peptide and the entire transmembrane anchor and cytoplasmic domain (Fig. 1). Two shorter recombinant proteins were designed to be analogous to the F protein subunit F1, excluding the heptad B motif and the transmembrane and cytoplasmic domains (residues 111–460), and subunit F2, excluding most of the signal peptide (residues 26–110). To generate the expression constructs, RNA was amplified with fragment-specific primers (Table 2) and the DNA fragments were cloned into the pET-30a (+) expression vector (Novagen, Madison, WI, USA). The vectors were propagated in the *NovaBlue E. coli* strain (Novagen) and expressed in *Rosetta (DE3) E. coli* strain (Novagen). As a positive control, the 120 kD β -galactosidase was expressed in *E. coli* strain *BL21* (Novagen), and as negative control, *E. coli* was transfected with plain vector. Expression was induced at 37 °C after 2 h incubation with 1 mM isopropyl- β -D-thiogalactopyranoside

Fig. 1. Generalized model of the F protein of reptilian paramyxoviruses. The F protein is divided into the F2 and a F1 subunits and the locations of the signal peptide (SP), cleavage site (CS), fusion peptide (FP), heptad repeat A (HRA), heptad repeat B containing a leucine zipper (HRB LZ), three N-glycosylation sites (N-G), and transmembrane anchor (TM) are shown. The amino acid numbers of the motifs within the F protein sequence are indicated. Three selected regions of the F protein were synthesized as recombinant proteins (rp 1–3) in a prokaryotic expression system. The rp1 was digested with the endopeptidase, furin, and the resulting cleavage products compared to rp2 and rp3. The C-terminal portion of rp1 included a region of 30 amino acids (LTKVQSDLKEAQDKLDESNAI LQGINNKIL) within the HRB LZ motif containing an antigenic epitope that reacted with antiserum in a western blot

(IPTG). Recombinant proteins containing a His-Tag were purified via affinity column using a Ni-NTA resin and the manufacturer's protocol (Novagen).

Furin protease digestion

The ability of furin to cleave the recombinant protein construct rp1 that included the furin cleavage site was tested *in vitro*. The recombinant protein was dialyzed against a furin buffer solution and 100 ng of protein was digested for 3 h at 30 °C with 2 units of furin per the manufacturer's product manual (Biolabs, Beverly, MA, USA).

Western blotting

For analysis of the recombinant proteins, equal amounts of crude bacterial cell lysates, the Ni-NTA purified recombinant proteins, or the dialyzed, digested proteins were separated using SDS-PAGE in 12% gels. Following electrophoresis, the proteins were transferred to 0.45 µm pore size nitrocellulose membranes for Western blotting (Bio-Rad, Hercules, CA, USA). An anti-His-tag monoclonal mouse-antibody (MAb *Anti-His*; Novagen) was used to detect the recombinant proteins. A virus-neutralizing polyclonal rabbit antiserum (PAb *Anti-RPMV*), made against isolate Gono-GER85, was used to detect the presence of antigenic domains. Secondary antibodies were detected using an alkaline phosphatase immunoblot kit (Bio-Rad).

Results

Model of the reptilian paramyxovirus F protein

The full-length sequence of the F gene of FDLV (GenBank AY141760) and Gono-GER85 (GenBank AY725422) were used to predict a model of the reptilian paramyxovirus F protein as presented in Fig. 1. The predicted N-terminal hydrophobic domain at amino acids 1–33 is believed to represent the signal peptide (SP) while the C-terminal hydrophobic domain at amino acids 503–520 is believed to represent the transmembrane anchor (TM). A predicted furin cleavage site (CS) is located at residues 107–110 at the C-terminal end of the F2 subunit and adjacent to the predicted fusion peptide (FP) at residues 111–135. Cleavage at this predicted site would divide the F protein into the F1 and F2 subunits. Two heptad repeats (HR) were predicted at residues 145–186 (HRA) and at residues 461–502 (HRB). Each heptad unit consisted of seven amino acids (positions a, b, c, d, e, f, g) with conserved hydrophobic or mostly non-polar residues occurring at positions a and d. The heptad units in FDLV were found to repeat six times (Fig. 2a, b). A helical wheel-model (Fig. 3) was used to illustrate the cross section of an alpha-helix into which the heptad repeats are believed to fold creating two opposing faces, a nonpolar-neutral face and a charged face. The HRB sequence also was predicted to encode a leucine zipper (LZ), with the hydrophobic amino acids leucine or isoleucine lining up in position a as shown in Fig. 3. Three potential amino acid-linked glycosylation sites (N-G) were identified at the asparagine residues located at positions 102, 436 and 443 of the F protein (Fig. 1); however, it was not determined if any of these sites were actually glycosylated.

a)	CS	Fusion Peptide	Heptad Repeat A Region	%*					
Viruses									
FDLV ('i') ^a	REKR	FVGIAVGAVALATSAQITAGTALSNTIKNAEA	IESIKSS IQASNQA IQKVIDA QGRVTTV INGIQDH INSVINP	100					
Gono-GER85 ('i') ^b				98					
Crot2-OH90 ('i')	G.			100					
3 Subgroup 'a' & 2 'i'				96					
6 Subgroup 'b' ^c				44					
Mumps (NC_002200)				40					
Measles (NC_001498)				40					
Hendra (NC_001906)				38					
NDV (AY325799)				34					
NDV avirulent (M24692)				36					
Sendai (NC_001552)				45					
Tupaia (NC_002199)									
b)									
Viruses									
FDLV ('i') ^a	DLDSAE	ITKVQSD	LKEAQDK	LDESNAI	LQGINNK	ILTAPTS	IALIVVS	VWVILL	%*
Gono-GER85 ('i') ^b									96
Calli-GER88 ('i')									96
Ela-FL93 ('i')									96
Crot2-OH90 ('i')			D.	N.					89
2 Subgroup 'a' ^d									96
Crot3-CA98 ('a')									95
6 Subgroup 'b' ^c									89
Mumps (NC_002200)	PI.I.T.	S.NAS	QN.VKY	IK...HQ	SV.VN	SKIGAI	V.AL.L.	ILS.II	33
Measles (NC_001498)	R..VGN	GNAIAK	ED.KEL	ES.DQ.	IRSMKGL	SS.SIVY	LFA.CL	GGL.GI	22
Hendra (NC_001906)	KV.I.SQ	ISSMNQS	QQS.K.Y	IK.AQK.	DTV.PS	ITSMLSM	I.Y.L.	TALCI	22
NDV (AY325799)	N..I.T.	GN.NNS	ISN.L.R	A..GK	DKV.VR	LTSTSL	.TY..LT	IISLVC	31
NDV avirulent (M24692)	N..I.T.	GN.NNS	ISN.L..	E...SK	DKV.V.	LTSTSL	.TY.FLT	ISLVC	35
Sendai (NC_001552)	PV.I.LN	AAATDF	QDSRAE	EKARK.	SEVGRW	YNSGA.L	.TI...M	I..IIV	25
Tupaia (NC_002199)	PI.V...	GNIELE	MDKT.KA	R..K.K.	DSMITE	VTPDKFG	.L.LWLF	G.SYYA	27
^a Complete genome sequence submitted as Genbank accession number AY141760.									
^b Fusion protein nucleotide sequence submitted as Genbank accession number AY725422.									
^c All isolates in subgroup 'b' had identical amino acid sequences.									
^d Crot1-VA95 and Trim-MD97 had identical amino acid sequences.									

^aComplete genome sequence submitted as Genbank accession number AY141760.

^bFusion protein nucleotide sequence submitted as Genbank accession number AY725422.

^cAll isolates in subgroup 'b' had identical amino acid sequences.

^dCrot1-VA95 and Trim-MD97 had identical amino acid sequences.

Fig. 2. Amino acid alignments of partial F protein amino acid sequences of 14 reptilian paramyxoviruses compared to representatives of other paramyxovirus genera. **a)** Residues 107–186 containing the cleavage site (CS), the fusion peptide at the start of F1 protein, and the six repeats of the heptad repeat A. **b)** The heptad repeat B that includes a leucine zipper. *Percentage amino acid identity with FDLV sequence. Dots represent identical residue as in FDLV. Reptilian paramyxovirus isolates are identified as members of subgroup 'a', subgroup 'b' or intermediate ('i')

Fig. 3. A helical wheel model representing a cross-section of the alpha-helix formed by the HRA (residues 145–186) and HRB (residues 461–502) of the F protein of a reptilian paramyxovirus. Each heptad motif repeats 6 times in a clockwise direction. Hydrophobic or uncharged amino acids are located in positions *a* and *d* while both charged and neutral residues occur in positions *b*, *c*, *e*, *f*, and *g*. The charge of each amino acid is written below or to the right. The resulting faces of the motif are either charged (+, –), or neutral (0). The leucine zipper is indicated in bold

Genetic diversity within the F gene motifs

To assess the extent of genetic diversity within the predicted motifs of the F gene of reptilian paramyxoviruses, the nucleotide sequences were determined for two selected regions of the F gene of 12 additional snake paramyxoviruses. The first region included the CS, FP, and HRA motifs (Fig. 2a) and the second region contained the HRB-LZ motif (Fig. 2b). Comparisons among the 14 isolates showed a high degree of similarity, but not identity, in these regions. In general, isolates identified by Ahne et al. [2] as members of subgroup ‘a’ had a conserved sequence for each of the motifs while isolates identified as subgroup ‘b’ had a conserved sequence that differed slightly from that of subgroup ‘a’. Those isolates identified as members ‘intermediate’ to the two subgroups showed inconsistent results, sometimes having the conserved sequence of subgroup ‘a’, sometimes subgroup ‘b’, or occasionally for certain motifs, differing from the sequence of members of either subgroup ‘a’ or ‘b’ (Fig. 2a, b).

The amino acid sequence of the predicted furin cleavage site of the 14 reptilian paramyxoviruses is shown in Fig. 2a. The conserved sequence, R-E-K-R was present in 13 of the isolates in agreement with the results reported by Franke et al. [16], while one ‘intermediate’ isolate, Crot2-OH90, had the sequence R-G-K-R. All the sequences were consistent with the paramyxovirus consensus furin cleavage site, R-X-R/K-R [28]. In the 10-amino acid sequence upstream of the CS at residues 97–106, virus isolates of subgroup ‘a’ differed from those of subgroup ‘b’ by 60% (6 of 10 aa) and with the ‘intermediate’ isolates by 20–40% [2–4 of 10 aa; data not shown).

Downstream of the CS was a 25 amino acid sequence identified as the fusion peptide (FP). The amino acid sequence of this motif at the start of the F1 protein was identical among the 14 isolates examined. The conserved domain of 11 amino acids (TSAQITAGIAL) within this motif was also identical to that of the reptilian paramyxovirus isolates examined by Franke et al. [16] and shared a high degree of similarity to FP sequences of members of other genera of the subfamily *Paramyxovirinae* (Fig. 2a).

Following the FP, the HRA motif was easily located (Fig. 2a). For this motif within the F gene of the 14 reptilian isolates, all isolates in subgroup 'a' and all but one of the 'intermediate' isolates had an identical amino acid sequence consisting of six heptad repeats. The six isolates in subgroup 'b' shared an identical sequence in this region that differed from subgroup 'a' and 'intermediate' isolates at 2 out of 42 amino acids. These substitutions were conservative such that the two HRA sequences had 100% amino acid similarity.

In the region of the F protein between amino acids 461–502 was the HRB motif (Fig. 2b). This motif possessed the characteristics of a leucine zipper (LZ) with a series of heptad repeats containing leucine or isoleucine in position a. The LZ motif was six L/I repeats in length for the reptilian sequences, which is longer than for Sendai virus, measles virus, simian virus 5, and Tupaia virus, and shorter than for Newcastle disease virus and Hendra virus. Within the 42 amino acids comprising HRB, isolates in subgroup 'a' and four of the 'intermediate' isolates had an identical sequence except for the last amino acids of the sixth repeat. One 'intermediate' isolate had a unique sequence with two additional amino acid replacements, while all of the subgroup 'b' isolates had a sequence that again differed by 2 of 42 amino acids. In general, the amino acid differences were substitutions with similar amino acids and occurred within the charged face of the repeat.

The overall amino acid sequence identity within the F protein motifs of the various reptilian paramyxovirus isolates was high, ranging from 93–100%. Overall amino acid sequence identity for the F protein motifs of the reptilian isolates compared to viruses representing other paramyxovirus genera was typically less than 45%. This level of sequence divergence within portions of the F gene was similar to levels of difference determined for the partial HN, L, and F sequences [2, 16]. For the entire F protein of FDLV (545 aa) the range of amino acid identity with representatives of other paramyxovirus genera was 22–32% [26].

Prokaryotic expression

The isolate Gono-GER85 was selected for prokaryotic expression because a polyclonal antibody raised against this virus has been previously shown to neutralize the virus [31] and to detect viral proteins in Western blots (Fig. 4). The PCR amplicons from selected regions of the F gene of the Gono-GER85 isolate were used to produce various constructs of the F protein in a prokaryotic expression system. A construct designed to produce the full length F0 protein did not express detectable levels of recombinant protein and was not considered further. However,

Fig. 4. The left column shows the western blots using a monoclonal anti-His antibody (MAb *Anti-His*) and the right column shows the corresponding western blots using the virus-neutralizing polyclonal rabbit serum (PAb *Anti-RPMV*). The blots in section **A** contain complete bacterial proteins including the recombinant protein (*rp*), section **B** contains His-purified *rp*, and section **C** contains His-purified, dialysed *rp* after digestion with furin. Lanes 1: *rp*1 (55 kD); 2: *rp*2 (14 kD); 3: *rp*3 (42 kD); 4: control (plain vector, expression induced); 5: control protein (120 kD) not induced; 6: control protein induced; 7: gradient-purified viral proteins; 8: IgH-2 cell proteins; 9: N-terminal portion of furin-digested *rp*1 (41 kD). Estimated molecular masses of the proteins are given on the sides of the blots as estimated from molecular weight standards (M^1 , M^2)

three other constructs shown as recombinant protein 1, 2, and 3 in Fig. 1 were expressed at levels that were easily detectable on Western blots developed using a monoclonal antibody to the terminal His-Tag on each recombinant protein (Fig. 4). The recombinant proteins were approximately 30 aa larger than the native partial proteins due to the His-Tag and the multiple cloning site derived from the expression vector. The largest of the three recombinant proteins (*rp*1) had an estimated molecular mass of 55 kD, the predicted size for the F0 protein

without the signal peptide or the transmembrane and cytoplasmic domains. The recombinant protein designed to mimic the F2 subunit (rp2) had an estimated molecular mass of 14 kD, while the recombinant protein designed to mimic a truncated F1 subunit (rp3) had an estimated molecular mass of 42 kD.

Furin cleavage of the recombinant protein

Of the three recombinant proteins that were expressed at high levels, only the 55 kD rp1 was designed to contain the predicted furin cleavage site. After digestion of purified rp1 by furin, a new protein fragment of approximately 41 kD was seen in gels that was the predicted size of the F1 subunit less the transmembrane and cytoplasmic domains (Fig. 4), confirming that the predicted furin cleavage site was active.

Antigenic analysis

Western blots developed using a neutralizing, polyclonal rabbit antiserum made against the Gono-GER85 isolate revealed that only the longest recombinant expression product, rp1, was recognized by the antibody (Fig. 4). The antibody also recognized the 41 kD cleavage product following digestion of rp1 by furin (Fig. 4). This cleavage fragment differed from rp3 only in that it contained 30 additional C-terminal amino acids that included most of the HRB-LZ motif (LTKVQSD LKEAQDK LDESNAI LQGINNK IL). These results indicated that the HRB-LZ domain of the reptilian paramyxovirus F protein contained at least one antigenic epitope. While other antisera were not available for this study, binding of the antiserum to Ni-NTA purified recombinant proteins following separation by SDS-PAGE indicated the epitope located within the HRB-LZ motif was likely not conformation dependent.

Discussion

The features of avian and mammalian paramyxovirus F proteins responsible for fusion of the virus with the host cell have been described as being quite conserved [25, 28], and include an enzymatic cleavage site, a fusion peptide, and two heptad repeat motifs, one of which is a leucine zipper [27]. Those motifs represent key determinants for cell infection and virulence [25, 28]. Our results extend the presence of these conserved F protein motifs to paramyxoviruses of reptile hosts as well. The F protein cleavage site of the reptilian paramyxoviruses was identified by sequence homology at the C-terminal end of the F2 subunit next to the N-terminal end of the F1 subunit fusion peptide (Fig. 1) as reported for other paramyxovirus F proteins [28]. With the exception of a single isolate, the reptilian viruses exhibited the same multibasic furin recognition site (R-E-K-R) reported by Franke et al. [16]. In the present study, we showed that a recombinant partial F protein of the Gono-GER85 isolate was cleaved by furin *in vitro*.

Proteins inducing membrane fusion in the families *Paramyxoviridae*, *Orthomyxoviridae*, *Retroviridae*, and *Filoviridae* generally require cleavage

modification to become biologically active [5, 22]. For the paramyxovirus, Newcastle disease virus (NDV), the nature of the CS was shown to be a major virulence factor [12, 33, 41]. Following conversion of the amino acid sequence of the CS of a low virulence (lentogenic) recombinant LaSota strain of NDV (G-G-R-Q-G-R||L) to the multibasic (G-R-R-Q-R-R||F) furin motif, the virus became highly pathogenic [33]. Also, passage of a lentogenic wild type strain of NDV in domestic chickens resulted in the emergence of a highly virulent (velogenic) pathogen. Sequence analysis of the strains showed the conversion of the cleavage site amino acid sequence, E-R-Q-E-R||L, to K-R-Q-K-R||F, the consensus motif for furin cleavage [41]. Furin occurs ubiquitously in the Golgi apparatus of host cells [47] and cleavage of the F0 protein by furin enables virus replication in a wide range of tissues and organs leading to fatal systemic infection and death [33, 42]. In contrast, tryptase Clara, a protease cleaving the lentogenic NDV strains and Sendai virus, is only secreted from epithelial cells present in respiratory and intestinal tracts [43]. The presence of a furin cleavage site is consistent with the ability of the reptilian paramyxoviruses to replicate in many tissues and to cause severe sickness and mortalities among infected snakes [14, 15, 17, 19–21].

In generalized paramyxovirus F protein models, the fusion peptide is the 25-amino acid, post-cleavage N-terminus of the F1 protein, which is known to be a highly hydrophobic and conserved domain [18] having an amino acid identity of up to 90% among various paramyxoviruses [28]. In reptilian paramyxoviruses, the FP was identified next to the cleavage site on the N-terminal end of the F1 subunit, and a conserved region of 11 amino acids was identical among the 14 isolates used in this study and in the isolates reported by Franke et al. [16]. This completely conserved domain consisted of the exclusively hydrophobic residues F, V, G, I, and A. To enable fusion with a host cell membrane, the hydrophobic fusion peptide also requires the previous attachment of the viral envelope to the host cell [34]. In the reptilian paramyxoviruses, as for most other members of the family, this may be provided by the presence of the HN protein [2, 26]. High fusion activity leads to successful viral replication, formation of syncytia and greater pathogenicity of the viruses [28].

The F protein sequences of the reptilian paramyxoviruses revealed two heptad repeats which proved to be nearly identical among isolates. An F protein model of SV5 paramyxovirus shows the HR motifs are located in the F1 protein following the N-terminal FP (HRA) and before the C-terminal transmembrane anchor (HRB). The two motifs are located more than 250 residues apart [22, 28], similar to the organization for FDLV as shown in Fig. 1. An important attribute of a HR is to facilitate self-assembly into an alpha-helix. In the native form, the F protein is shaped as a hairpin with the HRA and HRB forming anti-parallel alpha-helices [6, 8] and alpha-helical coiled coils form the backbones of the viral glycoproteins of paramyxoviruses and other enveloped viruses such as HIV [5, 22]. Additionally, the HRB is a leucine zipper with the amino acid leucine or isoleucine in position a [7, 50]. The leucine zipper motif is present in all paramyxovirus F proteins, coronavirus spike proteins and many retrovirus envelope proteins [6]. The amino acids in the key positions a and d generate a hydrophobic face

on the helix. Those faces enable proteins to oligomerize, which was shown to be essential for the paramyxoviral F protein [9]. This property allows several F protein monomers to form homo-oligomers [6, 13, 36]. Oligomer-formation is a characteristic described for fusion proteins of enveloped viruses such as member of the families *Paramyxoviridae* (SV5), *Orthomyxoviridae* (influenza virus), *Retroviridae* (HIV), and *Filoviridae* (Ebola virus) [5, 22], and the oppositely charged faces of the helix as shown in Fig. 3 are consistent with the structure needed for self assembly of reptilian paramyxovirus F protein monomers into oligomers. Mutation analysis of the leucine zipper motif in the measles virus (MV) and NDV showed that substitution of leucine residues abolished the fusigenic activity of the protein. However, protein expression, oligomerization, and cellular transport seemed to be unaffected [6, 36]. Young et al. [50] reported disruption of the secondary structure through replacement of leucine residues with non-polar alanine residues. For the HRB sequence of reptilian paramyxoviruses, leucine was found more often in position a and isoleucine in position d (Fig. 3) suggesting the oligomers are composed of a four-stranded assembly [11].

As described for the F protein of other paramyxoviruses [28], the trans-membrane anchor was located near the carboxy-terminus of the F protein of FDLV where it fastens the end of the protein within the viral envelope. Adjacent to the TM anchor in the N-terminal direction was the HRB as described by Chambers et al. [8] for fusion glycoproteins. From an immunological standpoint, the highly exposed position of viral glycoproteins on the surface of infected cells makes them targets for the host immune response. Both linear and conformation-dependent neutralizing epitopes have been identified on the F protein of several paramyxoviruses including human respiratory syncytial virus (HRSV), human parainfluenza virus 3, Sendai virus, and NDV [30, 32, 35, 44–46, 48, 49]. Sequence analysis of neutralization escape mutants selected by neutralizing MAbs showed the presence of unique epitopes on either the F1 or F2 subunits of the F protein as well as epitopes that mapped to both the F1 and F2 subunits [32, 38] indicating that, following cleavage, the two subunits are folded together and joined by disulfide linkages in a manner that gives rise to a number of complex, conformation-dependent epitopes. These analyses also revealed that many of the neutralizing and fusion-inhibiting epitopes mapped to the cysteine-rich region (amino acids 300–420) of the paramyxovirus F1 subunit located between the two heptad repeats. Langedijk et al. [29] mapped a highly conserved neutralizing epitope of HRSV to a site within HRA.

The present study showed the reaction of a virus-neutralizing polyclonal antiserum with a recombinant partial F protein of a snake paramyxovirus. The reacting epitope was located within a 30 amino acid region of the HRB containing the leucine zipper motif, a domain located outside of the viral membrane and therefore exposed to the host immune system. Because the antiserum reacted with the recombinant protein in Western blots following SDS-PAGE, we assume it is a linear epitope; however, the antiserum also reacted with several other denatured proteins from purified virus (Fig. 4). Thus, further studies will be needed to search for the presence of conformation-dependent epitopes and to

determine if the linear epitope we identified on the F protein is associated with neutralization.

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