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The fusion protein of wild-type canine distemper virus is a major determinant of persistent infection

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Abstract

The wild-type A75/17 canine distemper virus (CDV) strain induces a persistent infection in the central nervous system but infects cell lines very inefficiently. In contrast, the genetically more distant Onderstepoort CDV vaccine strain (OP-CDV) induces extensive syncytia formation. Here, we investigated the roles of wild-type fusion (F_{WT}) and attachment (H_{WT}) proteins in Vero cells expressing, or not, the canine SLAM receptor by transfection experiments and by studying recombinants viruses expressing different combinations of wild-type and OP-CDV glycoproteins. We show that low fusogenicity is not due to a defect of the envelope proteins to reach the cell surface and that H_{WT} determines persistent infection in a receptor-dependent manner, emphasizing the role of SLAM as a potent enhancer of fusogenicity. However, importantly, F_{WT} reduced cell-to-cell fusion independently of the cell surface receptor, thus demonstrating that the fusion protein of the neurovirulent A75/17-CDV strain plays a key role in determining persistent infection.

Keywords: Canine distemper virus; Persistence; Fusogenicity; Envelope glycoproteins; SLAM

Introduction

Canine distemper virus (CDV) is a *Morbillivirus* closely related to measles virus and depending on the viral strain may cause encephalomyelitis in dogs. The wild-type virulent A75/17-CDV strain typically produces a chronic demyelinating disease associated with a persistent infection in the central nervous system (CNS) (Bollo et al., 1986; Higgins et al., 1989; Imagawa et al., 1980; Vandevelde et al., 1980). The antiviral immune response, which follows invasion of immune cells in the CNS, leads to viral clearance within the inflammatory lesions (Bollo et al., 1986). However, this viral clearance is restricted to certain lesions, and simultaneously, A75/17-CDV has been shown to further spread in astrocytes in other areas of the brain without eliciting an inflammatory response. Thus, A75/17-CDV persistent infection in the CNS was suggested to be in part determined by a defective immune response (Bollo et al., 1986; Johnson et al., 1988; Vandevelde et al., 1986). However, in primary dog brain cell culture, wild-type CDV persistence was also associated with selective spread and very little virus release when compared to the Onderstepoort (OP) CDV vaccine strain (Zurbriggen et al., 1995), suggesting an important role also for viral factors in the establishment of persistent infection.

A75/17-V is a Vero cell-adapted CDV strain producing a persistent infection characterized by a very limited cytopathic effect (CPE). The virus spreads in a cell-to-cell manner without obvious syncytium formation (Hamburger et al., 1991; Meertens et al., 2003; Plattet et al., 2004). In contrast, the Onderstepoort (OP) CDV vaccine strain, which

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has been extensively passaged in vitro, produces a pronounced CPE in many cell types accompanied by the formation of large syncitia (Appel and Gillspie, 1972; Haig, 1956; Rockborn, 1959). Therefore, the non-cytolytic A75/17-V strain represents a promising tool to study the genetic determinants underlying viral persistence.

Both cell culture-adapted A75/17-V and OP-CDV have very similar genome organization as compared to the A75/17 wild-type strain and consists of a single-stranded negative-sense RNA of 15690 nucleotides. While A75/17-V differs from the wild-type strain by only seven amino acids located in the P, V, M and L proteins (Plattet et al., 2004), OP-CDV is genetically more distant and presents about 9% amino acid differences.

The CDV envelope contains the attachment (H) and the fusion (F) glycoproteins. The H protein binds to the CDV receptor of the host cell membrane, and the F protein mediates the membrane fusion event, which allows the entry of the viral genome into the cytoplasm. The OP-CDV cytolytic strain was demonstrated to spread through cell cultures both by producing infectious extracellular particles and by lateral cell-to-cell fusion (Gassen et al., 2000; Schmid et al., 2000; von Messling et al., 2001; Zurbriggen et al., 1995). Interestingly, it was shown that the coexpression of the F and H glycoproteins was necessary and sufficient to induce cell fusion, and that the CDV H protein was the major factor determining cell tropism (Stern et al., 1995). In addition, reverse genetics technology based on the OP-CDV strain suggested that not only the CDV tropism, but also the cytopathogenicity, also termed fusogenicity, in Vero cells were mainly determined by the H protein (von Messling et al., 2001).

The observation that Vero cell adaptation of wild-type CDV does not necessarily require amino acid changes in the H protein (Nielsen et al., 2003; Plattet et al., 2004) is inconsistent with the idea that host cell specificity is determined exclusively by the H protein. Thus, the question remains as to whether the phenotype of infection observed with A75/17-V is mainly determined by the H protein, as described for OP-CDV (von Messling et al., 2001).

Fusogenicity was previously suggested to be mediated both by viral factors and by cell surface receptors (Takeuchi et al., 2002). The CDV receptor in Vero cells has not yet been identified, although the CD9 molecule was shown to influence CDV-induced cell-to-cell fusion, but not virus–cell fusion (Loffler et al., 1997; Schmid et al., 2000). In contrast, the expression of signaling lymphocytic activation molecule (SLAM; also known as CD150) in non-susceptible cell lines was reported to allow binding and entry of both wild-type and vaccine CDV strains (Seki et al., 2003; Tatsuo et al., 2001). In humans, SLAM expression is restricted to activated T and B lymphocytes, immature thymocytes (Cocks et al., 1995; Sidorenko and Clark, 1993) and mature dendritic cells (Ohgimoto et al., 2001). The SLAM cDNA of the dog has recently been isolated (Tatsuo et al., 2001) but the tissue distribution of SLAM in this species has not yet been determined.

In this study, genetic determinants of the persistent infection phenotype of A75/17-V, which exhibits identical F and H proteins compared to the wild-type strain, were characterized. Towards this end, the F and H proteins from wild-type and OP-CDV were analyzed with respect to their cell-to-cell fusion-inducing ability using a transient-expression system and a newly established A75/17-V-based reverse genetics technology (Plattet et al., 2004). The role of SLAM in fusogenicity was also evaluated. Our results showed that both the wild-type F and H proteins as well as the canine SLAM receptor act in concert to determine the phenotype of infection.

Results

Differences in fusogenicity between A75/17-V-CDV and OP-CDV

Vero and Vero-dogSLAMtag cells were infected with the two different CDV strains at an MOI of 0.001 and their respective CPE-inducing ability was examined 24 h after infection by phase contrast microscopy and immunofluorescence. Although no CPE was observed in Vero cells infected with A75/17-V (Fig. 1A), immunofluorescence analysis showed single cell staining of A75/17-V throughout the cell monolayer (Fig. 1E). In these cells, OP-CDV produced the expected pronounced level of CPE (Fig. 1B), which was also confirmed by immunofluorescence analysis (Fig. 1F).

In the presence of the canine SLAM receptor, the level of CPE of OP-CDV was even more pronounced than in SLAM-negative Vero cells (Figs. 1D, H). In contrast, A75/17-V produced only few and small syncytia (Figs. 1C, G). These results demonstrate that viral proteins are the major determinants of cell-to-cell fusion but that SLAM also acts as a fusion-enhancing molecule. Since both morbillivirus envelope glycoproteins have been described as important fusion modulators, we wished to dissect the role of the A75/17-V F and H proteins in determining the low CPE-inducing phenotype of infection.

Both viral surface glycoproteins and SLAM modulate cell-to-cell fusion

As wild-type A75/17 and A75/17-V present identical F and H proteins (Plattet et al., 2004), they were named in this study wild-type F (F_{WT}) and wild-type H (H_{WT}). In order to investigate the role of the two surface glycoproteins of OP-CDV and A75/17-V CDV in producing a cytolytic versus persistent infection, we first carried out a comparative analysis of the predicted amino acid sequences between F_{WT} and F_{OP} as well as of H_{WT} and H_{OP}. The sequence of F_{WT} has already been published (Cherpillod et

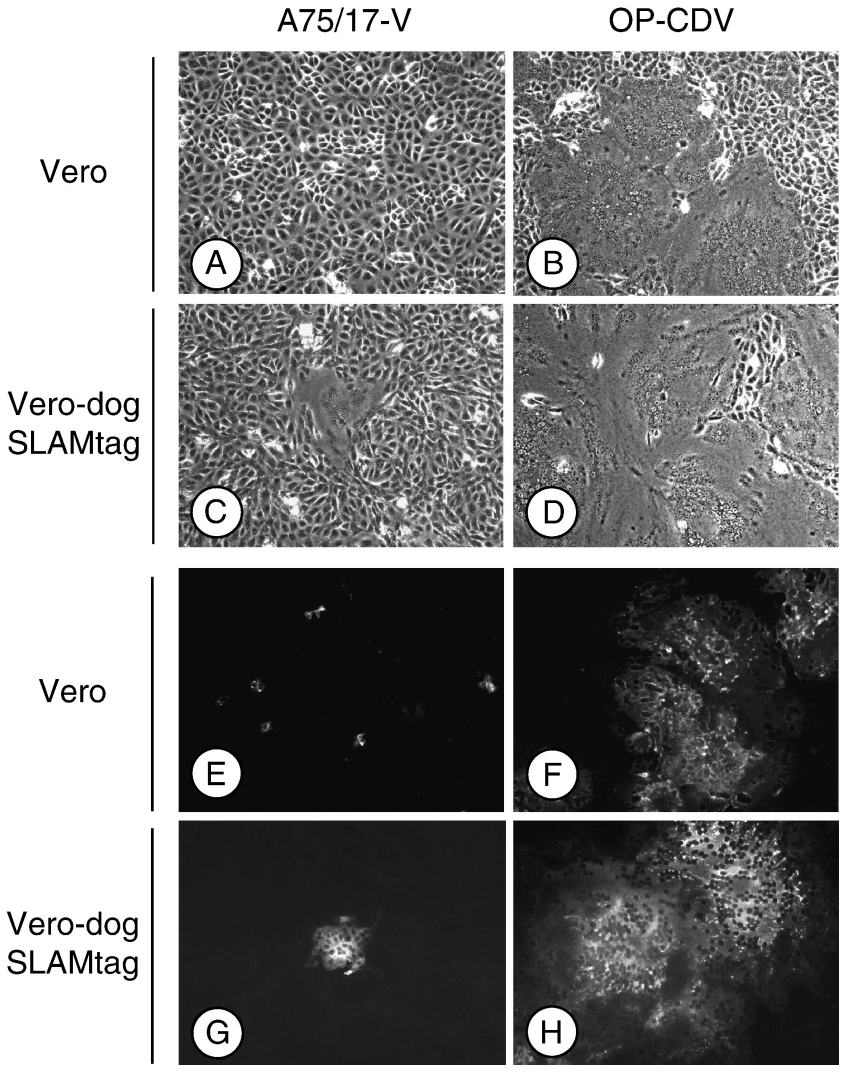

Fig. 1. Difference in cell-to-cell fusion between A75/17-V CDV and OP-CDV. Vero and Vero-dogSLAMtag cells were infected with A75/17-V CDV and OP-CDV at a MOI of 0.001 and observed after 24 h of infection by phase contrast microscopy (A-D). Representative fields of view are shown. Cells were also stained with an anti-CDV N protein MAb, followed by FITC-conjugated-labeled anti-mouse IgG and observed under a fluorescence microscope (E–H). Representative fields of view are shown.

al., 1999), and that of H_{WT}, which differs from the published sequence (Cherpillod et al., 1999) by 4 amino acids has been deposited in GenBank (accession no. AF164967). We have also sequenced the F and H genes of the OP-CDV strain used in our laboratory and found these sequences to be identical to those of the large plaque forming variant of OP-CDV (von Messling et al., 2001). The comparison of the F and H proteins between OP-CDV and A75/17-V revealed 58 and 55 amino acid differences, respectively (Table 1).

The efficacy of the F and H protein from wild-type (F_{WT}/H_{WT}) and OP-CDV (F_{OP}/H_{OP}) to induce cell-to-cell fusion was next examined. To this end, expression plasmids encoding the F and H proteins from the two strains were used in transfection studies in Vero cells and their activity was determined in a quantitative fusion assay (Nussbaum et al., 1994). Within 24 h, large syncytia were observed in Vero cells cotransfected with plasmids pCI-F_{OP} and pCI-H_{OP} (Fig. 2A) and the fusion activity was set at 100% in the quantitative fusion assay (Fig. 2B). Fusion activities obtained after transfection of only pCI-H_{WT} or pCI-H_{OP} were considered background levels. In cells transfected with the wild-type genes (pCI-F_{WT} and pCI-H_{WT}), no fusion activity was observed (Figs. 2A, B). When pCI-F_{OP} and pCI-H_{WT} were coexpressed, the fusion activity barely exceeded the background value after 24 h of transfection (Figs. 2A, B). However, 2–3 days post-transfection, few syncytia could be detected in the cell monolayer (data not shown). Thus, these results are consistent with the previously described findings demonstrating that the CDV H protein is a major determinant of the cell-to-cell fusion activity in Vero cells (von Messling et al., 2001). Surprisingly, when Vero cells were cotransfected with pCI-F_{WT} and pCI-H_{OP}, only few and small

Table 1
Amino acid differences between the F and H proteins of A75/17-CDV (WT)[a] and OP-CDV

Position	Amino acid in protein		
	F_{WT}	F_{OP}	
3	N	R	Pre
4	K	G	
8	R	S	
10	N	K	
12	R	Q	
13	K	T	
19	L	R	
23	H	P	
26	K	E	
27	S	L	
28	A	E	
31	K	R	
34	Q	R	
43	R	Q	
48	H	Y	
49	G	D	
51	L	R	
57	I	V	
58	H	S	
60	I	T	
64	I	T	
67	C	R	
70	A	T	
74	S	L	
75	D	K	
79	A	V	
82	D	N	
85	G	A	
88	H	Q	
90	T	I	
92	G	E	
96	Q	K	
99	G	R	
101	R	Q	
102	L	I	
103	K	E	
107	S	P	
110	T	I	
115	Q	H	
124	I	L	
126	I	M	
178	D	E	F0
216	V	L	
233	A	V	
317	K	R	
347	S	I	
366	N	S	
390	L	F	
431	V	I	
445	P	S	
513	G	S	
540	V	A	
556	S	G	
567	E	D	
616	I	S	
640	N	H	
641	A	T	
646	T	A	

Table 1 (*continued*)

Position	Amino acid in protein	
	H_{WT}	H_{OP}
3	S	P
9	S	G
21	S	T
29	E	G
30	Q	H
50	M	L
145	K	T
146	I	V
155	D	E
156	T	S
162	S	A
186	Y	H
197	R	K
216	I	V
238	Y	D
241	G	R
242	G	E
246	Q	R
247	K	E
276	E	K
298	D	E
309	D	S
324	G	W
330	Q	H
331	V	I
342	V	M
343	E	K
367	V	A
376	N	G
386	S	T
393	T	A
401	G	R
415	P	A
417	I	V
446	D	N
456	N	D
459	V	I
460	L	S
467	S	G
475	I	L
500	M	R
502	K	R
506	T	I
510	L	I
517	N	S
518	F	I
530	G	S
544	A	T
549	Y	H
572	D	N
585	S	I
586	T	A
605	S	
606	K	
607	P	

Pre: signal peptide.
F0: precursor of F1 and F2 subunits.
[a] A75/17 and A75/17-V CDV share identical F and H proteins.

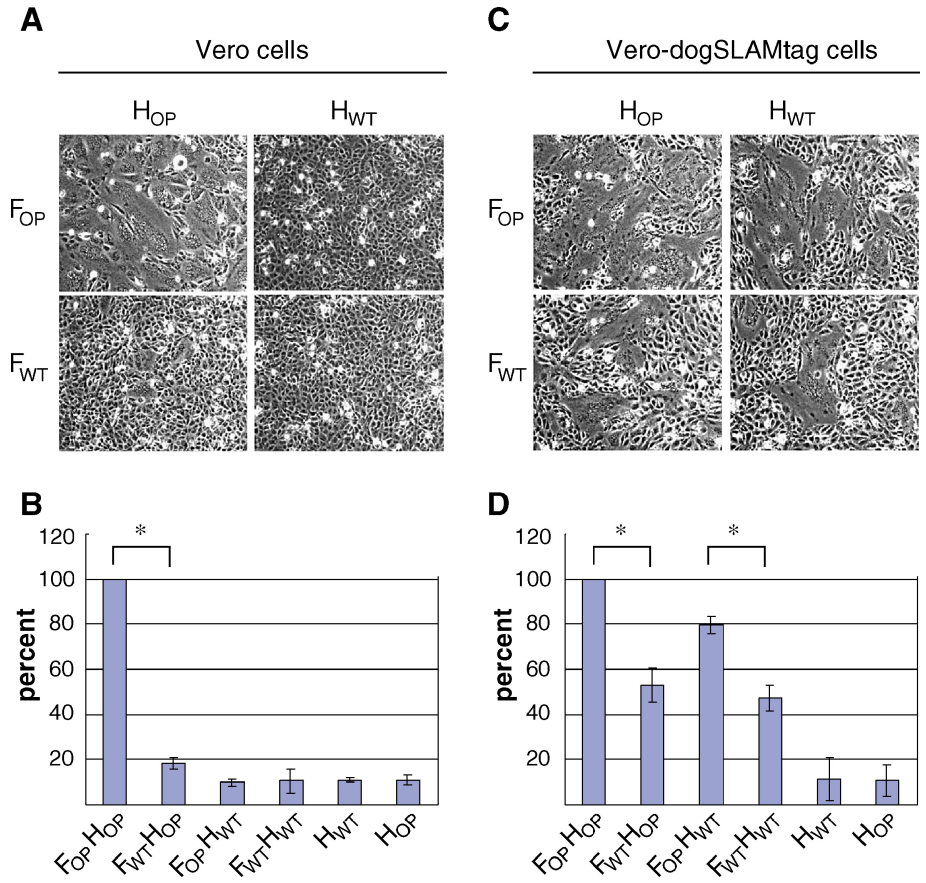

Fig. 2. The wild-type F protein reduces syncytium formation in a transient fusion assay. Representative fields of view of Vero (A) and Vero-dogSLAMtag cells (C) 24 h after transfection with different combinations of wild-type and OP-CDV F and H proteins. Quantitative fusion assays in Vero (B) and Vero-dogSLAMtag cells (D). One population of Vero and Vero-dogSLAMtag cells were infected with MVA-T7 (MOI, 1). Another population of Vero cells was transfected with different combinations of F and H proteins, and a plasmid containing the CAT reporter gene under the control of the T7 promoter. Transfected cells were further incubated in the presence of fusion-inhibiting antibodies (see Materials and methods). At 16 h after incubation at 37 °C, transfected cells were washed and overlaid onto MVA-T7 infected cells. Mixed cell populations were further incubated at 37 °C for 6 h and fusion was quantified by measuring the amount of CAT produced. For each experiment, the value measured for the F_{OP}/H_{OP} combination was set to 100%. Means and standard deviations for three independent experiments in duplicate are shown, $*P<0.05$.

syncytia were observed, consistent with the quantitative fusion assay showing a reduction of about 80% of fusion activity (Figs. 2A, B).

In order to study the involvement of the canine SLAM receptor in the fusion process, combinations of the wild-type and OP-CDV F and H proteins were expressed in Vero-dogSLAMtag cells. In the presence of SLAM, all homotypic and heterotypic combinations of F and H proteins were fusogenic, demonstrating that the cell surface receptor itself is a contributing determinant of cell-to-cell fusion (Fig. 2C). These results suggest that lack of fusogenicity of certain F/H combinations in Vero cells is probably due to the absence of SLAM, rather than impaired F/H interactions or inefficient cell surface targeting. In addition, the quantitative fusion assay confirmed that the wild-type F protein is less fusogenic than the OP-CDV F protein, also in the presence of SLAM (Fig. 2D). Taken together, these results showed that the H_{WT} protein modulates fusogenicity in a receptor-dependent manner, whereas the F_{WT} protein reduces cell-to-cell fusion independently of the cell surface receptor.

Recovery and characterization of recombinant CDVs with swapped F and H proteins

Despite the screening of a large number of cell lines, we were unable to find one in which A75/17-V formed syncytia, precluding the isolation of recombinant viruses. However, although only to a limited extent, A75/17-V induced a CPE in our newly established Vero-dogSLAMtag cells. Using this cell line, we were able to rescue a persistent A75/17-V from cDNA. A schematic representation of the genome of the recombinant virus is shown in Fig. 3A. We next demonstrated that rA75/17-V grew similarly (Fig. 3B) and exhibited an identical persistent phenotype of infection (Fig. 3C) in Vero cells as compared to the parental A75/17-V virus.

To confirm that the CDV wild-type F protein modulates fusogenicity, recombinant viruses bearing heterologous glycoproteins were generated. To facilitate the construction of recombinant viruses differing in the F and H glycoproteins, restriction sites previously introduced in the full-

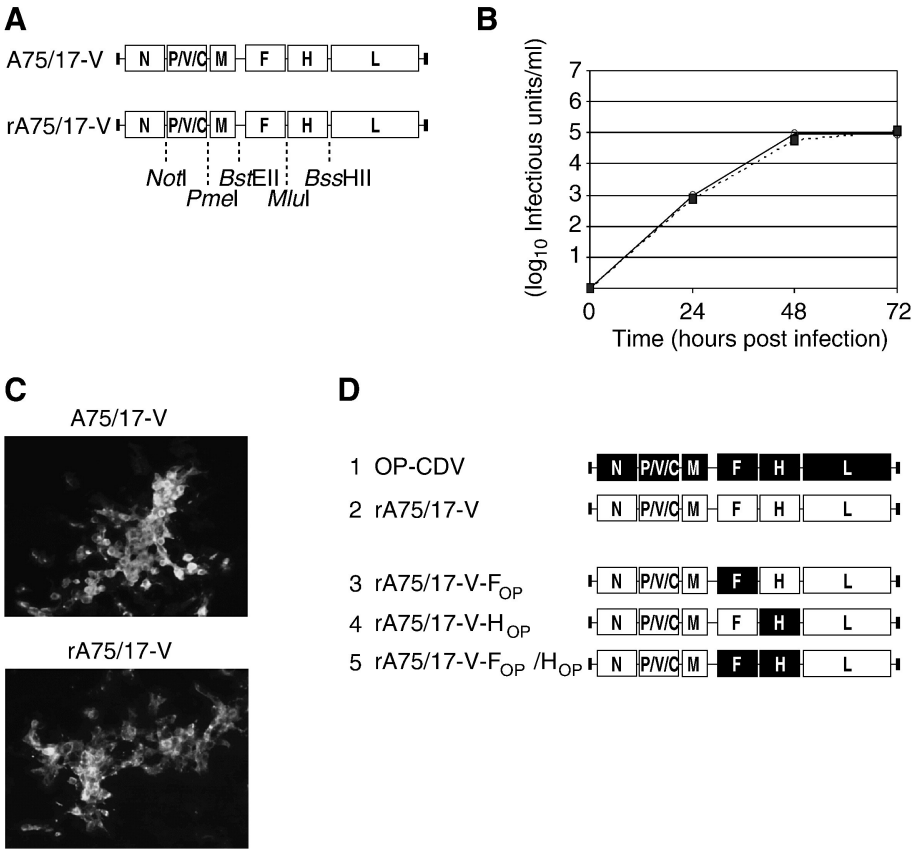

Fig. 3. Characterization of recombinant CDV viruses. (A) Schematic representation of the genomes of A75/17-V and rA75/17-V. Restriction sites and their positions that were engineered in the cDNA of rA75/17-V are shown. (B) Growth kinetics of parental A75/17-V (○) and rA75/17-V (■). Vero cells were infected at an MOI of 0.01. At the indicated hours post-infection, cells were lysed and cell-associated virus was titrated on Vero cells. (C) Immunofluorescence of Vero cells infected with the parental A75/17-V and the recombinant rA75/17-V viruses. Cells were stained 2 days post-infection with an anti-CDV N protein monoclonal antibody followed by a FITC-conjugated anti-mouse IgG. (D) Schematic representation of the genomes of OP-CDV (black boxes) and rA75/17-V (white boxes). The genomes of the chimeric recombinant viruses are also represented.

length plasmid pA75/17-V3 were used (Plattet et al., 2004). The H gene of A75/17-V was exchanged with that of OP-CDV, resulting in plasmid pA75/17-V-H_{OP}. The F gene of both pA75/17-V3 and pA75/17-V-H_{OP} was then replaced by the F_{OP} gene, resulting in plasmids pA75/17-V-F_{OP} and pA75/17-V-F_{OP}/H_{OP}. Recombinant viruses were then generated as described previously (Plattet et al., 2004) with minor modifications (see Materials and methods). The various rCDVs were designated as illustrated in Fig. 3D. In all viruses, the entire F and H genes were sequenced and no mutations were observed when compared to the transfected plasmids, thus confirming the genetic background of all chimeric viruses (data not shown).

Fusogenicity of recombinant viruses

We next examined the fusion efficiency and the spread of the recombinant viruses and of OP-CDV in Vero cells. OP-CDV induced large syncytia already 24 h post-infection (hpi) (Fig. 4A). In contrast, rA75/17-V produced a persistent infection (Fig. 4C). These different phenotypes were also seen by immunofluorescence analysis (Figs. 4B, D). Three days after infection, OP-CDV induced extensive CPE leading to cell death (data not shown), while rA75/17-V continued to spread within the cell monolayer without obvious syncytium formation (Fig. 4E). Infection with the chimeric viruses rA75/17-V-F_{OP} and rA75/17-V-H_{OP} was characterized by a very limited CPE at 24 hpi, as demonstrated both by phase contrast microscopy and immunofluorescence staining (Figs. 4F, G, I, J). At 72 hpi, immunofluorescence analysis revealed that more than 80% of the cells were infected but no obvious cell-to-cell fusion occurred, confirming that the chimeric viruses replicated and spread efficiently in Vero cells (Figs. 4H, K). When both F and H proteins of OP-CDV were introduced into the persistent virus (rA75/17-V-F_{OP}/H_{OP}), cell-to-cell fusion was highly efficient and the CPE was similar to the parental OP-CDV (Figs. 4L, M). Thus, in the A75/17-V genetic background, the presence of either F_{OP} or H_{OP} is not sufficient to yield efficient cell-to-cell fusion as seen in OP-CDV infection. Indeed, in order to achieve the fusogenicity of OP-CDV, both F_{OP} and H_{OP} glycoproteins are needed in the A75/17-V genome. These results thus showed that the wild-type CDV F and H proteins are implicated in persistent

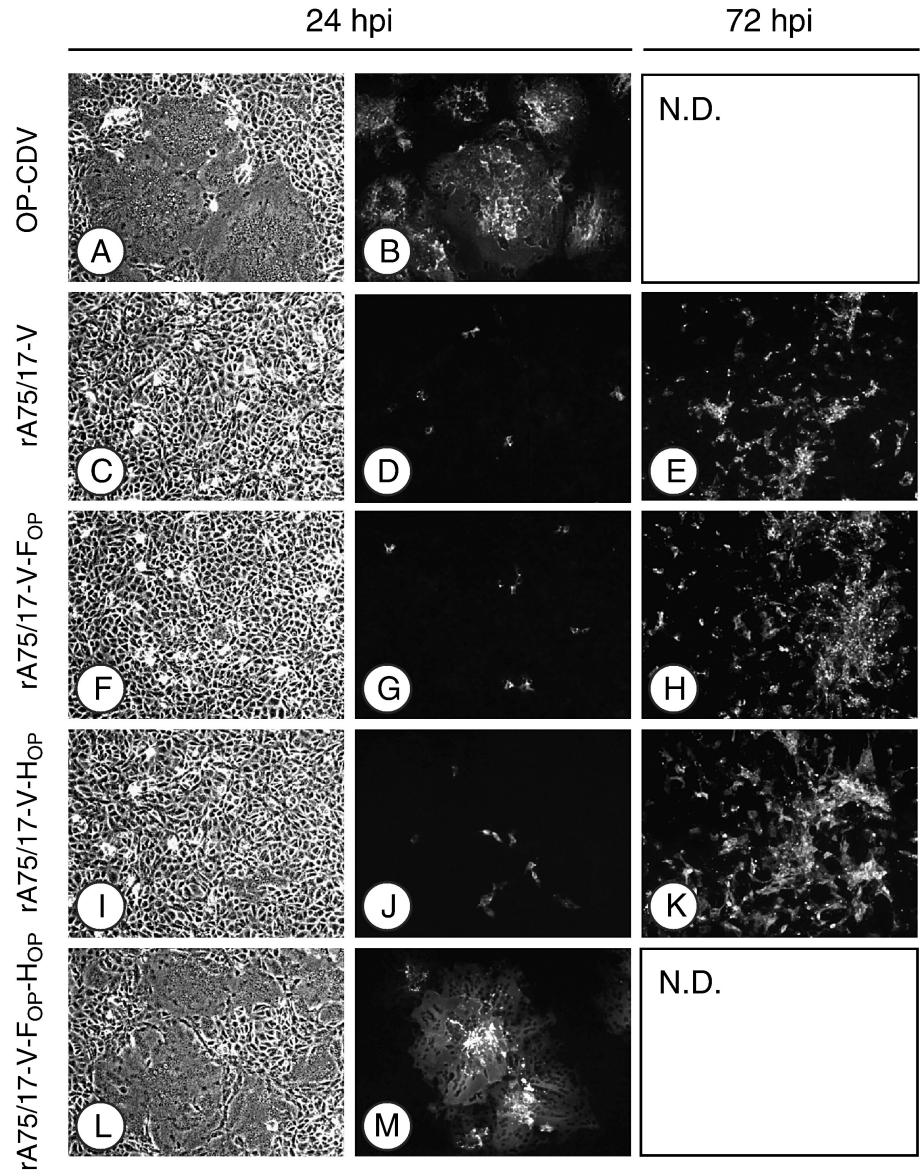

Fig. 4. Fusion efficiency and dissemination of recombinant CDV viruses and OP-CDV in Vero cells. Vero cells were infected with a MOI of 0.01 with OP-CDV (A, B) and rCDVs (C–M). Vero cells were observed after 24 h of infection by phase contrast microscopy (A, C, F, I, L) or stained with anti-CDV N protein MAb, followed by FITC-conjugated-labeled anti-mouse IgG (B, D, E, G, H, J, K, M). Representative fields of view are shown. Uninfected cell treated in the same way did not show any fluorescence. N.D., not determined due to extensive CPE.

infection in that they both have the potential to reduce cell-to-cell fusion.

The phenotype of the recombinant CDV viruses and OP-CDV were next examined in Vero-dogSLAMtag cells. Interestingly, in the presence of the receptor SLAM, all viruses gained the ability to induce cell-to-cell fusion, although their fusion-inducing capabilities varied. Indeed, OP-CDV and rA75/17-V-F$_{OP}$/H$_{OP}$ produced a very pronounced CPE at 24 hpi (Figs. 5A, B, O, P), whereas rA75/17-V and rA75/17-V-H$_{OP}$-infected cells only exhibited small and rare syncytia at this time point (Figs. 5C, E, K, M). Two days post-infection, the latter recombinant viruses produced a more pronounced CPE characterized by intermediate syncytia formation (Figs. 5D, F, L, N). Surprisingly, 24 h post-infection, rA75/17-V-F$_{OP}$ already induced efficient syncytia formation. However, the syncytia were smaller in size as compared to OP-CDV and rA75/17-V-F$_{OP}$/H$_{OP}$ (Fig. 5G, H, I, J). Taken together, these results show that recombinant viruses bearing the wild-type F protein produce a more limited CPE and that cellular surface proteins, such as demonstrated here for SLAM, play a crucial role in modulating the level of viral cell-to-cell fusion.

SLAM does not significantly influence virus grow kinetics

Since we clearly showed that the canine SLAM molecule acts as a host cell factor favoring cell-to-cell fusion, we wondered if virus production was also affected by the

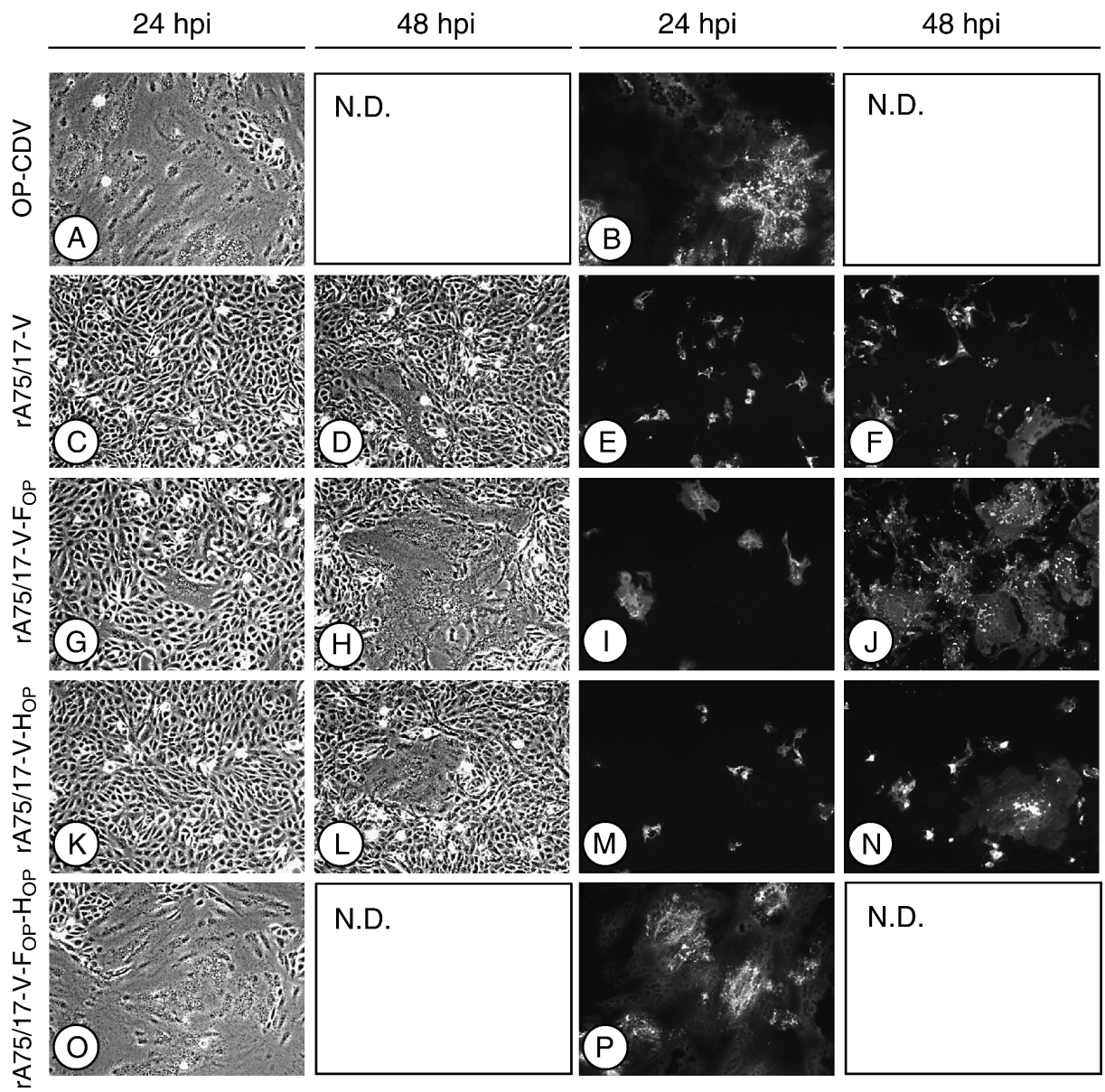

Fig. 5. Fusion efficiency and dissemination of recombinant CDV viruses and OP-CDV in Vero-dogSLAMtag cells. Vero-dogSLAMtag cells were infected with a MOI of 0.01 with OP-CDV (A, B) and rCDVs (C–P). Vero-dogSLAMtag cells were observed after 24 h and 48 h of infection by phase contrast microscopy (A, C, D, G, H, K, L, O) or stained with anti-CDV N protein MAb, followed by FITC-conjugated-labelled anti-mouse IgG (B, E, F, I, J, M, N, P). Representative fields of view are shown. Uninfected cell treated in the same way did not show any fluorescence. N.D., not determined due to extensive CPE.

presence of SLAM. Titration experiments showed that for all viruses, SLAM did not significantly alter the growth kinetics (Fig. 6A). However, viruses inducing limited CPE (rA75/17-V, rA75/17-V F_{OP}, rA75/17-V H_{OP}) showed a marked delay in virus production, which was independent of the presence of SLAM.

Efficiency of OP-CDV and rCDVs cell entry in the presence or absence of SLAM

To assess precisely the entry efficiency of the various viruses in the presence of SLAM, Vero and Vero-dogSLAM tag cells were infected with an MOI of 0.01 of the different viruses titrated in Vero cells. One day post-infection, immunohistochemistry was performed and stained cells in a defined area were counted. The number of infected Vero cells was arbitrary set to 100% for each virus (Fig. 6B). Whereas viruses bearing the OP-CDV H protein did not show any significant differences in cell entry efficiency between the two cell lines, viruses bearing the wild-type H protein clearly entered Vero cells expressing the canine SLAM receptor 6 to 10 times more efficiently than Vero cells. Thus, cell entry efficiency is mainly determined by the origin of the H protein. This result clearly show that virus–cell fusion and cell-to-cell fusion are closely related but different processes, since cell-to-cell fusion is regulated by both viral surface glycoproteins.

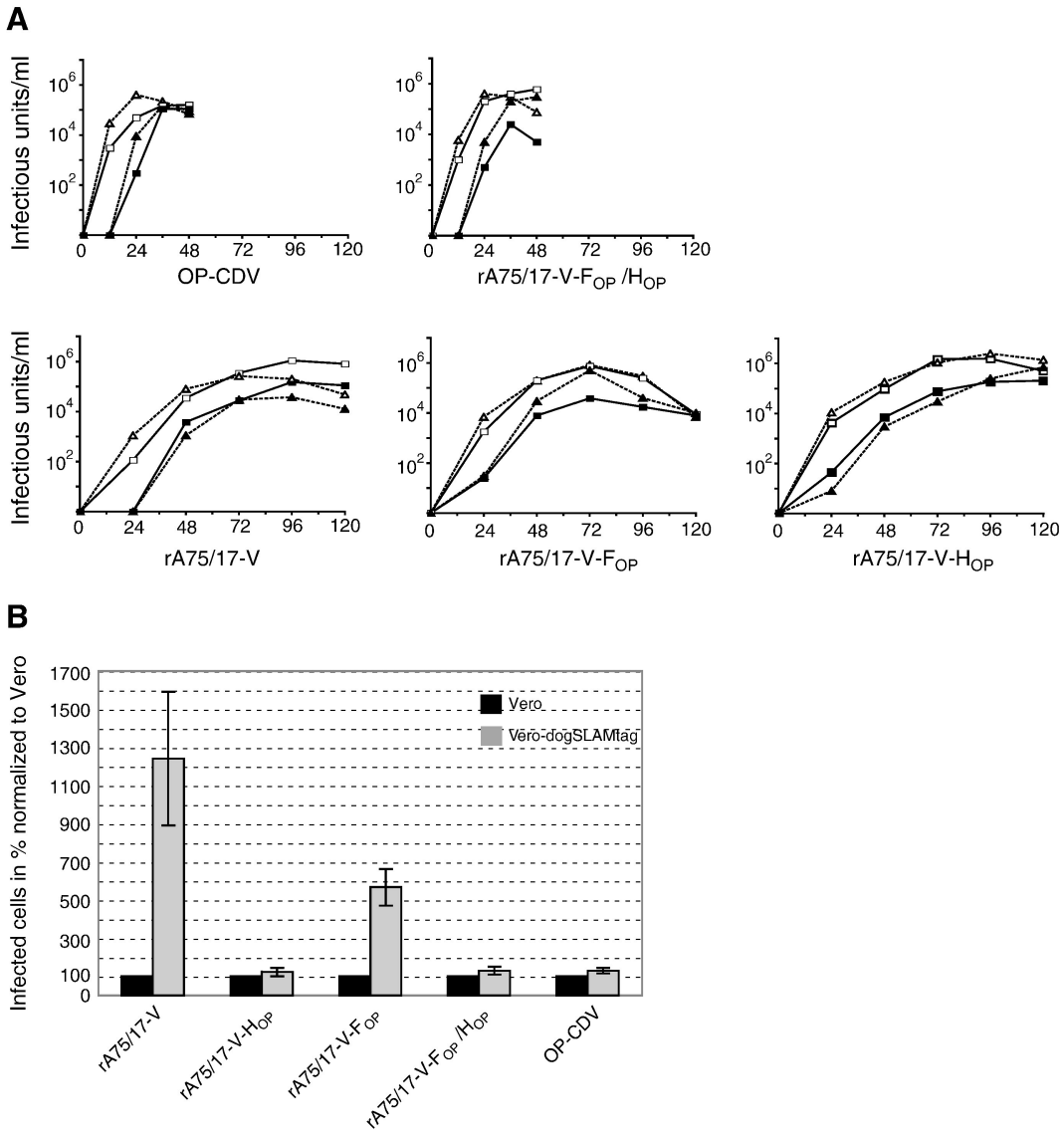

Fig. 6. (A) Growth kinetics of recombinant CDV viruses in the presence or absence of SLAM. Vero (□) and Vero-dogSLAMtag cells (△) were infected at an MOI of 0.01 with OP-CDV and the indicated rCDVs. Virus titers were determined at the indicated times post-infection by immunocytochemistry. White symbols represent the cell-associated virus and black symbols represent infectious progeny released into the cell culture medium. (B) Cell entry efficiency through different cell surface receptor. Vero (black histogram) and Vero-dogSLAMtag (grey histogram) cells were infected at an MOI of 0.01 with the indicated CDVs. 24 h post-infection, cells were fixed and stained by immunocytochemistry. Infected cells were counted in 5 separate defined areas of 2 infected cell cultures. The number of infected Vero cells was arbitrarily set at 100%. Mean values of infected cells ± the standard errors of the means from two experiments are shown.

Both surface glycoproteins reach the cell surface in cells infected with viruses producing a persistent infection

To investigate whether persistent infections correlate with impaired surface glycoproteins targeting, Vero cells were infected with the various CDVs at an MOI of 0.01. Prior to lysis, the infected cells were biotinylated at 4 °C to label the surface-exposed proteins. The total amount of F and H produced was studied by Western blot analysis of cell extracts (Figs. 7A, TL). Cell surface-exposed F and H proteins were analyzed by precipitation with agarose-coupled streptavidin followed by detection of the proteins by Western blotting (Figs. 7A, SF). As expected, the surface glycoproteins of the OP-CDV and rA75/17-V-F$_{OP}$/H$_{OP}$ viruses with a highly cytolytic phenotype of infection were readily expressed at the cell surface after 24 h of infection (Figs. 7A, SF), at a time when these viruses already exhibited extensive CPE (see Fig. 4). Interestingly, rA75/17-V, rA75/17-V-H$_{OP}$ and rA75/17-V-F$_{OP}$ also showed efficient F and H surface targeting (Fig. 7A, SF). The glycoproteins of these viruses were analyzed at 3 days post-infection, when most cells were infected (see Fig. 4) but when very limited CPE was evident. Western blotting was also performed with a monoclonal anti-P antibody as a

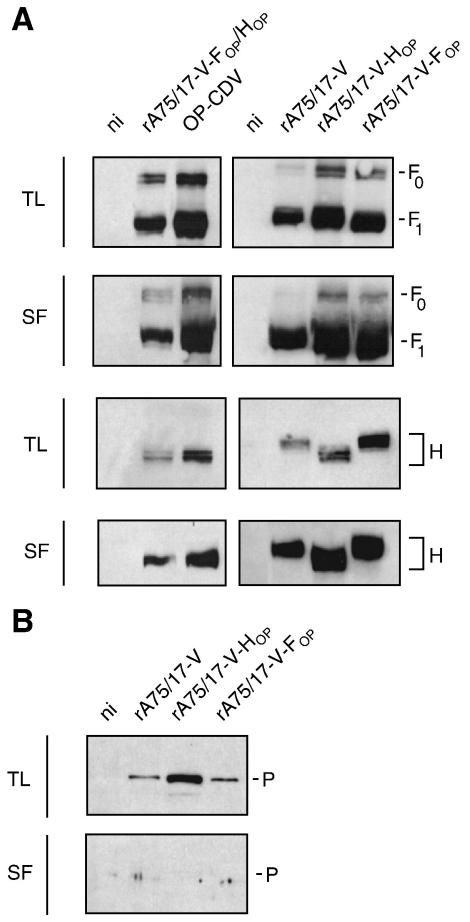

Fig. 7. Cell surface expression of F and H glycoproteins. Total proteins (TL) were extracted form Vero cells infected either with rCDVs or OP-CDV, separated by reducing SDS-PAGE and blotted onto nitrocellulose membranes. To determine cell surface expression of proteins (SF), duplicate cultures of infected Vero cells were shifted to 4 °C, biotin labeled, lysed, and immunoprecipitated overnight with streptavidin coupled to sepharose beads, separated by reducing SDS-PAGE and blotted onto nitrocellulose membranes. (A) Blots were probed with an anti-F, an anti-H antiserum. (B) Blots were probed with an anti-P antiserum, serving as a control. The position of the H, or of the F_0 and F_1 proteins is indicated at the right.

control. As expected, the P protein was readily detected in total cell extracts but only trace amounts were seen in Western blots of surface expressed proteins (Fig. 7B). Thus, the efficient surface expression of both glycoproteins of persistent viruses suggests an intrinsic defect of the wild-type F and/or H protein in mediating the fusion process rather than an impaired cell surface targeting.

Discussion

Functional F and H/HN interactions are well known to be important for promoting cell-to-cell fusion in paramyxoviruses (Tanabayashi and Compans, 1996; Tsurudome et al., 1998; Wild et al., 1994). For measles virus (MV), this interaction has been shown to occur already in the endoplasmic reticulum (Plemper et al., 2001). Furthermore, host cell molecules promoting or hindering viral–cell fusion or cell-to-cell fusion have been described for Sendai virus and Newcastle disease virus (Kumar et al., 1997; Okamoto et al., 1997). Thus, the fusion process seems to be very complex and not exclusively dependent on viral determinants, but also on the interaction between viral proteins and cellular factors. The current model of the paramyxovirus fusion mechanism proposes that after receptor binding, the H protein undergoes conformational changes, which also lead to changes in the tridimensional structure of the F protein, resulting in the insertion of the hydrophobic fusion peptide into the cell membrane and finally in membrane merging (Lamb, 1993; Lamb and Kolakofsky, 2001).

Our results obtained in the transient expression and infection experiments showed that in the absence of SLAM, the H protein plays an important role in fusogenicity, which is consistent with a previous study (von Messling et al., 2001). Indeed, whereas combinations involving the wild-type H protein induced very limited CPE, combinations containing the OP-CDV H protein produced more pronounced cell-to-cell fusion. While the precise mechanism by which interaction between the H protein and the cell surface receptor induces conformational changes is still unclear, it is tempting to speculate that the strength of the interaction is crucial for triggering H protein conformational changes. In this context, we showed that recombinant viruses containing the wild-type H protein entered Vero cells expressing SLAM 6–10 times more efficiently than Vero cells. This suggests that the wild-type H protein binds to a yet unknown Vero cell receptor with a reduced affinity compared to the OP-CDV H protein. As a result of this low binding affinity, cell-to-cell fusion will be impaired due to a defect of H_{WT} in supporting the fusion process. In contrast, our results obtained in Vero-dogSLAMtag cells suggest that both H_{WT} and H_{OP} can bind to SLAM with sufficiently high affinity to trigger fusion. This shows that both the H protein and the cell surface receptor affect cell-to-cell fusion, suggesting that the affinity of the H protein for its receptor is an important determinant of fusogenicity.

Surprisingly, our transfection and infection experiments clearly showed that the wild-type F protein also reduced syncytia formation. In a previous study, von Messling et al. (2001) demonstrated that fusogenicity was determined exclusively by the H protein. The apparent discrepancy between our work and the latter study may be due to the different origin of the F protein used. Indeed, von Messling et al. (2001) worked with F proteins obtained from cell culture-adapted CDV strains, which may be significantly different in their amino acid sequence with respect to the original wild-type protein. The F protein of the Vero cell-adapted virus used in our study is identical to that of the virulent dog isolate A75/17 (Plattet et al., 2004).

We clearly demonstrated that independently of the phenotype of infection, both F and H proteins were efficiently expressed at the cell surface. This strongly

suggests that the wild-type fusion protein is intrinsically poorly fusogenic and plays a key role in determining persistent infection. It has been shown that paramyxovirus fusion proteins can control fusion efficiency by different means. Indeed, mutations in the cytoplasmic tail, the signal peptide, and the F_1/F_2 subunits have been demonstrated to affect various steps in the fusion process (Cathomen et al., 1998b; Dutch and Lamb, 2001; Eichler et al., 2003; Moll et al., 2002; Seth et al., 2003, 2004; Tong et al., 2002; von Messling and Cattaneo, 2002; Waning et al., 2004). In addition, N-linked glycans, as well as lateral interactions with the attachment protein, have also been demonstrated to modulate fusogenicity (Corey et al., 2003; Gravel and Morrison, 2003; Ohuchi et al., 1997; Plemper et al., 2002; Takimoto et al., 2002; von Messling and Cattaneo, 2003). In our case, the precise mechanism by which the wild-type F protein induces a limited CPE is yet unknown. Nevertheless, while F_{WT} and F_{OP} proteins share identical potential N-glycosylation sites, it is interesting to note that both proteins present about 9% differences in their predicted amino acid sequence. Interestingly, most of these amino acid differences (71%) are located in the first 135 aa, which has been designated the pre sequence, whereas only 29% reside in the precursor F0. Shortening of the pre sequence was shown to increase fusogenicity (von Messling and Cattaneo, 2002). Thus, this hyper variable region between F_{WT} and F_{OP} could be involved in limiting the wild-type F protein-induced CPE. On the other hand, the fusion-support function of the H protein could be affected by impaired lateral interactions when combined with the wild-type F protein, which would in turn induce a persistent infection. Works is in progress in order to dissect the precise mechanism by which the F protein of the neurovirulent CDV strain reduces cell-to-cell fusion. Taken together, our fusion experiments obtained in the presence or absence of SLAM suggest that the H/receptor affinity is the key determinant of cell-to-cell fusion, the extent of which is modulated by the nature of the F protein.

In Vero cells, all rCDVs grew to similar titers, although their growth kinetics appeared to be dependent on the levels of CPE produced. Viruses producing a very limited CPE (r-A75/17-V, r-A75/17-V-F_{OP} and r-A75/17-V-H_{OP}) were characterized by a delay of infectious virus production, typically attaining a plateau only after 3–5 days post-infection. In contrast, viruses inducing a marked CPE (r-A75/17-V-F_{OP}/H_{OP} and OP-CDV) reached a maximum titer already at 36 h post-infection. As these viruses, except OP-CDV, differ only in their glycoprotein complexes, this suggests that the rate of virus release is directly correlated with the ability of a given virus to induce membrane fusion. On the other hand, cytolytic viruses spread through a cell culture both by producing infectious particles and by lateral cell-to-cell fusion. Thus, the delay of infectious virus production could simply reflect a slower spread of less fusogenic viruses through the cell monolayer by depending predominantly on secondary infections rather than rapid lateral transmission. Another intriguing observation was the fact that H_{WT}-expressing viruses, which showed a more efficient cell entry through the SLAM receptor, were characterized by only a small increase in the rate of virus production and yields of infectious virus. This observation remains unclear and needs further investigation. Nevertheless, it is possible that a step in the viral life cycle is modulated by the presence of SLAM, which in turn leads to a decrease in virus release.

An unexpected result was the limited CPE induced by rA75/17-V and rA75/17-V-H_{OP} in Vero-dogSLAMtag cells. This is in contrast to the transfection experiments, where expression of the same glycoproteins complexes in the presence of SLAM showed efficient cell-to-cell fusion. The most likely explanation for this difference is that in a virus infection, the M protein is present, which may limit the rA75/17-V- and rA75/17-V-H_{OP}-induced CPE. Indeed, it has been shown for MV that the M protein reduces cell-to-cell fusion by interacting with the cytoplasmic tails of the F and H proteins (Cathomen et al., 1998a, 1998b). On the other hand, despite the fact that rA75/17-V-F_{OP} and rA75/17-V-F_{OP}/H_{OP} harbor the same M gene, they induce syncytia formation. This could be explained by the fact that the two latter viruses express the highly fusogenic OP-CDV F protein. Moreover, as F_{OP} exhibits three amino acid differences in the cytoplasmic tail (CT) as compared to F_{WT}, and since the CT domain has been shown to regulate the fusion process (Bagai and Lamb, 1996; Caballero et al., 1998; Moll et al., 2002; Seth et al., 2003, 2004; Tong et al., 2002; Waning et al., 2004), one could speculate that the M/F_{OP} interaction is weaker than the M/F_{WT} interaction, thus allowing the intrinsic F/H-induced fusion efficiency to occur. Since the role of M in cell-to-cell fusion cannot be evaluated in transfection experiments (Plemper et al., 2002, our unpublished observations), recombinant CDVs with swapped M proteins are currently being generated.

We believe that persistence in cultured cells is based on similar mechanisms as persistence in animals. It is well known that wild-type CDV produces a persistent infection in the central nervous system (CNS). In contrast, in lymphoid organs, CDV infection is highly cytopathic, which corresponds to our results obtained in Vero and Vero-dogSLAMtag cells. Thus, it is reasonable to speculate that (i) the presence or absence of SLAM may also determine cytolytic versus persistent infection in vivo and (ii) a yet unidentified low-binding affinity CDV receptor could be present in the CNS of dogs. To test the first hypothesis, we are currently performing co-localization studies of SLAM and viral proteins in tissues of CDV infected dogs. Taken together, our studies performed with the A75/17-V virus, which is closely related to the neurovirulent A75/17 isolate, emphasize the importance of using viruses with limited cell culture adaptation to better understand the molecular basis of persistent infection in CDV.

Materials and methods

Cell culture, virus and infection

Vero cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma) containing 10% fetal bovine serum, penicillin and streptomycin (Gibco) at 37 °C and 5% CO_2. Vero-dogSLAMtag cells (see below) and Bsr-T7 cells stably expressing the phage T7 RNA polymerase (Buchholz et al., 1999) were maintained in DMEM containing 5% fetal bovine serum, penicillin and streptomycin at 37 °C and 5% CO_2 in the presence of 0.5 mg/ml and 1 mg/ml, respectively, of G418 (Sigma).

The A75/17-V strain (A75/17-V) was obtained after 17 blind passages of wild-type virus in Vero cells (Hamburger et al., 1991). The tissue culture-adapted OP-CDV strain (gift of U. Kihm, Federal Institute of Virus Diseases and Immune Prophylaxis, Switzerland) was propagated in Vero cells.

Cells were infected with the different viruses at the MOI indicated in the figure legends. After incubation at 37 °C for 2 h, the virus suspension was removed and the cells were washed with PBS. DMEM supplemented with 10% FCS was then added and the cells were again incubated at 37 °C.

Generation of a Vero cell line stably expressing the canine SLAM receptor

For the establishment of the Vero-dogSLAMtag cell line, the membrane-bound form of canine SLAM cDNA fused to the influenza virus HA tag (Tatsuo et al., 2001), a generous gift of Dr. Yanagi, Kyushu University, Japan, was amplified by PCR using the Expand High-fidelity system (Roche Biochemicals) and ligated to the *Hin*dIII–*Xho*I-cleaved pcDNA-Neo eukaryotic expression vector (Stratagene). Vero cells were then transfected with the pcDNA-dog-SLAMtag-Neo construct and selected in the presence of 2 mg/ml of G418. The resistant clones were screened by immunofluorescence staining using an anti-HA-tag FITC-conjugated antibody (Santa Cruz Biotechnology, Inc.). The clone, which expressed the highest level of canine SLAM, was selected for further experiments.

Construction of plasmids

The chlorenphenicol acetyl transferase (CAT) gene was cloned into the pTM-1 vector (Moss et al., 1990), in which the CAT gene is under control of the T7 promoter. The resulting plasmid was designated pTM-CAT.

The wild-type F and H proteins were cloned into the eukaryotic expression vector pCI (Promega), as described elsewhere (Cherpillod et al., 2000). To generate plasmids expressing the OP-CDV glycoproteins, RNA was extracted from OP-CDV-infected Vero cells using the RNeasy kit (Qiagen). Regions spanning the F and H genes were then reverse transcribed from viral genomic RNA using Superscript III (Qiagen) reverse transcriptase (RT) and gene-specific primers. PCR amplification was performed using the Expand High-fidelity system (Roche Biochemicals) with specific primers and cloned into the eukaryotic expression vector using standard methods. As already stipulated in Introduction, wild-type A75/17 and A75/17-V present identical F and H proteins and are termed in this study F_{WT} and H_{WT}, respectively.

Plasmid pA75/17-V3, carrying the full-length cDNA genome of A75/17-V, has been described previously (Plattet et al., 2004). To exchange the F and H genes of pA75/17-V3 with those of OP-CDV, the F_{OP} and H_{OP} genes were amplified from the eukaryotic expression plasmids pCI-F_{OP} and pCI-H_{OP} (see below), and subcloned into pGEM-Teasy (Promega). The PCR primers contained the transcription regulatory elements (EIS) of A75/17-V and the restriction sites *Bst*EII and *Mlu*I (F_{OP}) or *Mlu*I and *Bss*HII (H_{OP}). The fragment containing the H_{OP} gene was then excised from pGEM-T easy construct and ligated to *Mlu*I–*Bss*HII-cleaved pA75/17-V3, resulting in plasmid pA75/17-V-H_{OP}. The fragment containing the F_{OP} gene was excised from pGEM-T easy vector and ligated into pA75/17-V3 and pA75/17-V-H_{OP}, resulting in plasmids pA75/17-V-F_{OP} and pA75/17-V-F_{OP}/H_{OP}, respectively.

Transfections and quantitative fusion assay

Vero and Vero-dogSLAMtag cells, in 6-well plates at 90% confluency, were cotransfected with 2 μg of pCI-F_{OP}, pCI-H_{OP}, pCI-F_{WT} and pCI-H_{WT} in different combinations, using Lipofectamine 2000 (Gibco) according to the manufacturer's protocol. Pictures of transfected cells were taken 24 h after transfection with a microscope equipped with a DC 350FX digital camera (Leica).

The quantitative fusion assay was performed essentially as described previously (Nussbaum et al., 1994). For measles virus, the fusion inhibitory peptide (FIP) has been used to inhibit F/H-induced syncytia formation during the first period of incubation (Plemper et al., 2003). As the FIP did not have the desired effect in transfection experiments using the CDV F gene, Vero cells cotransfected with the F and H expression plasmids and 0.1 μg of pTM-CAT were incubated in the presence of a canine distemper antiserum (VMRD, Inc.) and a rabbit polyclonal anti-CD9 antiserum (Santa Cruz Biotechnology, Inc.). In parallel, separate 6-well plates of Vero or Vero-dog-SLAMtag cells at 30% confluency were infected with the recombinant vaccinia virus MVA-T7 expressing the T7 RNA polymerase (Sutter et al., 1995) at a multiplicity of infection (MOI) of 1. Sixteen hours post-transfection or post-infection, transfected Vero cells were subcultured in duplicate with the MVA-T7-infected Vero or Vero-dog-SLAMtag cells. After incubation at 37 °C for 6 h, cells were lysed, and the CAT production was determined using an ELISA kit (Roche Biochemicals).

Recovery, propagation and titration of recombinant CDV

Recombinant viruses were recovered using Bsr-T7 cells as described (Plattet et al., 2004). Briefly, Bsr-T7 cells were transfected with N, P and L expression vectors together with a DNA copy of the relevant CDV genome. Cells transfected with either pA75/17-V-F_{OP} or pA75/17-V3 were overlaid on Vero-dogSLAMtag cells, whereas cells transfected with either pA75/17-V-F_{OP}/H_{OP} or pA75/17-V-H_{OP} were overlaid on Vero cells. For each virus, four syncytia were isolated and incubated with fresh Vero cells in 6-well plates. The infected cells were expanded in 10-cm dishes. When severe cytopathic effect was observed or after 5 days, the culture medium was replaced by 2 ml of Opti-MEM (Gibco) and the cells were harvested and lysed by freeze–thaw. The cleared supernatants were used for further analysis. To verify the identity of recombinant viruses, total RNA was isolated from infected cells using RNeasy-kit (Qiagen), reverse transcribed and subjected to PCR amplification, as described previously (Plattet et al., 2004). The resulting PCR fragments were sequenced using an ABI Prism 3100 automated sequencer (Applied Biosystems). For all viruses, the titer was determined by limiting dilution of the viral stock followed by immunohistochemical staining using a CDV-N-specific monoclonal D110 antibody, as described previously (Bollo et al., 1986).

Western blotting

Proteins were separated by electrophoresis in a 10% reducing SDS-polyacrylamide gel and blotted onto nitrocellulose transfermembranes (Schleicher and Schuell). The membranes were incubated with a rabbit anti-F or an anti-H antiserum (Cherpillod et al., 1999) or an anti-P-specific monoclonal antibody (Martens et al., 1995). After incubation with peroxidase-conjugated goat anti-rabbit immunoglobulin G (DakoCytomation) or goat anti-mouse immunoglobulin G (Cell Signaling) for 45 min at room temperature, proteins were visualized by enhanced chemiluminescence (Amersham Biosciences).

Surface biotinylation

Biotinylation was performed with a biotinylation kit (Roche) according to the instructions of the manufacturer. Briefly, cells were infected with the various viruses at an MOI of 0.01 for 1 or 3 days, depending on their phenotype of infection. After a wash in cold PBS, cells were incubated in biotinylation buffer containing 0.05 mg/ml of biotin-7-NHS for 15 min at 4 °C. The reaction was terminated by adding 50 μl/ml of stop solution to yield a final concentration of 50 mM NH_4Cl and incubated for 15 min at room temperature. Cells were washed with PBS, and scraped into lysis buffer containing protease inhibitors. The lysates were cleared by centrifugation at 20 000 × *g* for 15 min at 4 °C. One hundred and fifty micrograms of biotinylated proteins were absorbed overnight to Immunopure immobilized streptavidin–agarose beads (Pierce) at 4 °C. Beads were pelleted by centrifugation at 8000 rpm for 30 s in a microfuge and washed three times with lysis buffer before 25 μl of 2× loading buffer containing 100 mM DTT was added. Western blot analysis using rabbit anti-F or anti-H antiserum, or an anti-P monoclonal antibody was performed as described above. To determine the total amount of H, F or P protein present in infected cells, 15 μg of total proteins of infected cells were used for Western blotting.

Indirect immunofluorescence assay

Vero and Vero-dogSLAMtag cells at 80% confluency were infected at an MOI of 0.01 for 2 h at 37 °C with the respective viruses. One day after infection (or as specified in the manuscript), the cells were fixed with 5% acetic acid: 95% ethanol (v/v), blocked with 5% goat serum and stained with the CDV-N-specific monoclonal antibody D110. Cells were then incubated with fluorescein isocyanate-conjugated goat anti-mouse immunoglobulin G (Sigma) and examined by fluorescence microscopy.

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