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Nearby Clusters of Hemagglutinin Residues Sustain SLAM-Dependent Canine Distemper Virus Entry in Peripheral Blood Mononuclear Cells

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Signaling lymphocytic activation molecule (SLAM, CD150) is the universal morbillivirus receptor. Based on the identification of measles virus (MV) hemagglutinin (H) amino acids supporting human SLAM-dependent cell entry, we mutated canine distemper virus (CDV) H and identified residues necessary for efficient canine SLAM-dependent membrane fusion. These residues are located in two nearby clusters in a new CDV H structural model. To completely abolish SLAM-dependent fusion, combinations of mutations were necessary. We rescued a SLAM-blind recombinant CDV with six mutations that did not infect ferret peripheral blood mononuclear cells while retaining full infectivity in epithelial cells.

The severe and long-lasting immunosuppression accompanying wild-type morbillivirus infections enhances an individual's susceptibility to secondary infections, which account for most of the high disease-related morbidity and mortality (3, 7, 41). Specific viral entry in lymphocytes through the universal Morbillivirus receptor SLAM (8, 14, 35, 36) is considered one possible immunosuppression determinant. Indeed SLAM expression on activated T cells, immature thymocytes, memory T cells, a proportion of B cells, activated monocytes, and dendritic cells overlaps largely with the dissemination of viral infection through the lymphoid system (5, 30, 38). Other receptors and postentry determinants also influence morbillivirus tropism (2, 4, 20, 23).

The lack of a small animal model has limited studies of the measles virus (MV) tropism determinants, but recently ferrets have been used to characterize the infection of the other Morbillivirus, canine distemper virus (CDV) (38, 39). To evaluate the importance of cell entry through SLAM for CDV infection of peripheral blood mononuclear cells (PBMC), we aimed at producing a recombinant virus unable to interact with this protein but maintaining entry through other receptors. Towards this we set up experiments to characterize residues on the CDV attachment protein necessary for SLAM-dependent cell fusion. We identified several amino acids and localized them on a novel CDV H protein structural model.

Identification of CDV H protein amino acids important for SLAM-dependent membrane fusion. There is no experimental Morbillivirus H-protein structure, but a three-dimensional model of the MV H protein based on the crystal structure of the Newcastle disease virus (NDV) hemagglutinin-neuraminidase (HN) protein (6) was generated (37). This model (Fig. 1A, top view and 1B, side view) suggests that the MV H protein has a globular ectodomain with six beta-sheets, each composed of four strands. The beta-sheets are arranged cyclically around an axis as the blades of a propeller (Fig. 1A, the four strands in each beta-sheet are shown in the same color). Four "anchor" residues with the strongest effect on human SLAM-dependent membrane fusion (Y529, D530, T533, and R553, shown in red) are located on beta-propeller sheet 5 (color coded blue); three other residues (T531, F552 and P554, shown in gold) are also important.

Since CDV and MV H are 36% identical (11, 40), they may share a similar structure and their SLAM-interacting surfaces may roughly correspond. Therefore we operated with the MV H model and mutagenized in CDV H all 23 residues corresponding to the MV amino acids for which alpha carbon atoms were predicted to be within 10 Å of the four MV H anchor residues and solvent exposed. Twenty-two of these residues are located between positions 483 and 543 in the CDV H protein sequence (Fig. 1E). In addition, residue 173 situated outside this segment was mutagenized. CDV numbering and MV H protein numbering are dephased by 4 (e.g., CDV residue 529 corresponds to MV residue 533; compare CDV with MV numbering in Fig. 1E, top).

The H protein of the large plaque-forming variant of the CDV vaccine strain Onderstepoort expressed from plasmid pCG-H_{OL} (40) was selected for initial analysis: this protein has strong SLAM-independent fusion support activity in Vero cells, in addition to SLAM-dependent fusion support activity. Two small amino acids, alanine to substitute charged and with polar residues and serine to replace apolar residues, were used to limit possible structural interferences (Fig. 1E, lowest line). To reduce the risk of reversion in subsequent phases of experimentation, at least two nucleotides were changed in each codon. We also confirmed that the sequence of the relevant H gene segments and the size of the H proteins estimated on denaturing gels were as expected (data not shown).

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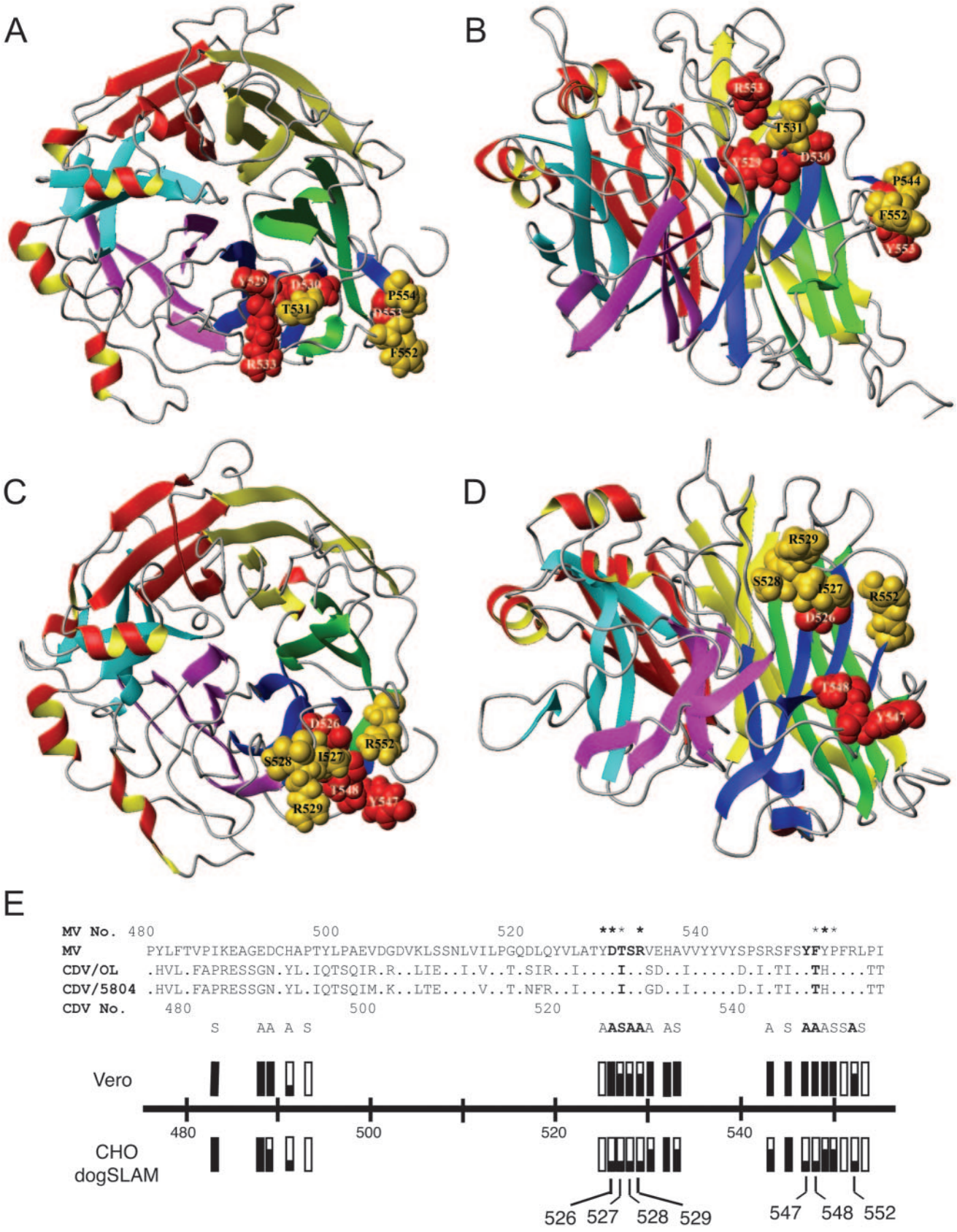

FIG. 1. Top and side views of the predicted structures of the MV H and CDV H proteins and localization of the residues necessary for SLAM-induced fusion. The CDV H model was generated based on the three-dimensional PSSM (16) alignment of the CDV H and the NDV HN

The function of all mutants was then tested in a fusion support assay based on the complementation of the homologous Onderstepoort F protein expressed from plasmid pCG-F_{OL} either on Vero or on SLAM-expressing CHO cells (CHOdogSLAMtag) (40). The degree of fusion was measured on a four-grade scale described in the legend to Fig. 1 (grades 0 to 3). At least three independent experiments were graded. The results are summarized graphically in the lower half of Fig. 1E. Since Vero cells have a higher intrinsic fusion propensity than CHOdogSLAMtag cells, the thresholds used to define fusion efficiency levels (high, intermediate, low, and none) in the two cell lines are different, as detailed in the legend to Fig. 1.

Proteins with mutations at positions 526, 547, and 548 lost two levels of fusion efficiency in CHOdogSLAMtag cells while maintaining standard activity in Vero cells (Fig. 1E, labeled positions). Proteins with mutations at positions 489, 527, 528, 529, 530, 533, 543, 549, 550, and 552 also lost their fusion activity preferentially in CHOdogSLAM cells. Four mutants completely lost function on both cell lines. The six remaining mutants maintained the same level of function in both cell lines. The seven mutations resulting in strongly reduced fusion activity in SLAM-expressing cells but with standard or near-standard activity in Vero cells are indicated with their ordinal number in Fig. 1E.

To illustrate the position of these amino acids, a new model of CDV H was produced using the same template structure and software suite previously used to model MV H (37). Like MV H, CDV H is predicted to fold as a propeller with six beta-sheets of four strands each. Top and side views of the CDV H model are shown in Fig. 1C and Fig. 1D, respectively. The seven residues necessary for efficient canine SLAM-dependent fusion are highlighted in red (strongest effect) or gold. Six relevant residues are located in two nearby clusters in beta-sheet 5, a situation similar to that of MV H (Fig. 1A and Fig. 1B). The locations of the two clusters vary slightly between CDV H and MV H, but their relative distance is similar: the distances of the alpha-carbon atoms are 12 to 21 Å between the two CDV H clusters and 13 to 22 Å between the two MV H clusters.

Combined mutations in both H residue clusters completely abolish SLAM-dependent fusion. To obtain an H protein that displays no residual SLAM-dependent fusion support, we generated seven combination mutants starting with individual mutations that strongly interfered with SLAM-dependent fusion. In a first mutagenesis round two neighboring mutations were combined, yielding 526/7, 528/9, and 547/8, all of which displayed low fusion support function (1.0, 0.8, and 0.5, respectively) in CHOdogSLAMtag cells. These fusion activities were lower than those of the single mutants (Fig. 1E), all in the 1.5 to 1.8 range. The further combinations of either 526/7 or 528/9 with 547/8, or of all six mutations (526-9 + 547/8), resulted in complete loss of syncytium formation (0 fusion), whereas the fourfold mutant 526-9 maintained some fusion activity. All proteins displayed near-wild-type function in Vero cells (>2.5 fusion), indicating that inefficient protein processing and cell surface transport were unlikely causes for the observed loss of SLAM-mediated fusion support activity. Thus, two clusters of H residues support the CDV H-(Onderstepoort attenuated strain) SLAM-dependent cell fusion. Mutations in both clusters are necessary to eliminate fusion-support function.

A SLAM-blind CDV replicates efficiently in epithelial but not in lymphoid cells. To assess the role of the CDV H-SLAM interactions in PBMC, the alanine or serine mutations in six residues necessary for efficient SLAM-dependent fusion identified in the H protein of the vaccine strain Onderstepoort were transferred to the H protein of the virulent wild-type strain 5804P (39). The Onderstepoort and 5804P H proteins have seven amino acids at variance between residues 480 and 560 (Fig. 1E), but none of these residues aligns with those influencing SLAM-dependent fusion.

To monitor virus recovery, we could not rely on syncytium formation because wild-type viruses are by far less proficient in fusing Vero cells than vaccine strains: cell rounding or very small syncytia dominate the cytopathic effect. Thus to facilitate the identification of cells supporting the replication of a SLAM-blind virus, we introduced the mutations into a cDNA copy of the genome of 5804P-eGFP/H, a virus that expresses enhanced green fluorescent protein (eGFP) from an additional transcription unit inserted between the H and L genes (38). Cells replicating this virus can be identified even if they do not

sequences and the crystal structure of NDV HN (6). The software suite MPACK was used (24, 26, 37), which was also recently used to model MV H and many other proteins (15, 22, 31). The illustrations of the models were prepared with MOLMOL (17). The four beta strands in each blade are coded with the same color: yellow, red, cyan, pink, blue, and green for propeller blades 1 to 6, respectively. The alpha-helical regions are shown in red and yellow. Relevant amino acids are highlighted in red or gold and indicated with their one-letter code and ordinal number. (E) Top: comparison of a relevant segment of the MV H protein sequence (second line) with the corresponding CDV/OL and CDV/5804 H protein sequences (third and fourth lines). Above the MV sequence, the seven residues with strong effects on SLAM-dependent fusion are indicated with asterisks; the MV numbering is also shown. Below the fourth line, the CDV numbering is shown and 22 DV/OL residues individually mutated to alanine (A) or serine (S) are indicated. The seven CDV H residues with the strongest effect on SLAM-dependent fusion are bolded. Bottom: graphic representation of the fusion support assay results obtained in Vero cells (upper row of bars) and CHOdogSLAMtag cells (lower row of bars). Each bar represents a single-residue mutant, positioned on the H sequence below the corresponding amino acid. Filled bars indicate wild-type fusion activity, open bars indicate no fusion activity, and two-thirds- and one-third-filled bars indicate intermediate fusion levels. Mutants with high receptor-mediated fusion differentials are indicated by the ordinal numbers corresponding to their positions on the CDV H sequence. For fusion assays, cells were seeded in 12-well plates at 70% confluency and transfected with equal amounts (1 μg) of the plasmids encoding the F and H genes, using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Fusion activity was analyzed 48 and 72 h posttransfection. The grading system was as follows: 0, syncytium formation at background levels; 1, syncytia found in some fields of view; 2, syncytia found in most fields of view; and 3, the majority of cells were either in syncytia or had already detached from the plate because of extensive fusion. An average fusion score was assigned to each mutant after at least three independent experiments. To take in account different levels of overall syncytium formation after transfection of Vero and CHOdogSLAM cells, the results were displayed using different fusion scales. Vero cells: high fusion (shown as completely filled box), fusion score >2.5; intermediate fusion (two-thirds-filled box), 1.5 to 2.5; low fusion (one-third-filled box), 0.5 to 1.5; no fusion (empty box), <0.5. CHOdogSLAMtag cells: high fusion, score >1.9; intermediate fusion, 1.6 to 1.9; low fusion, 1.3 to 1.6; no fusion, <1.3.

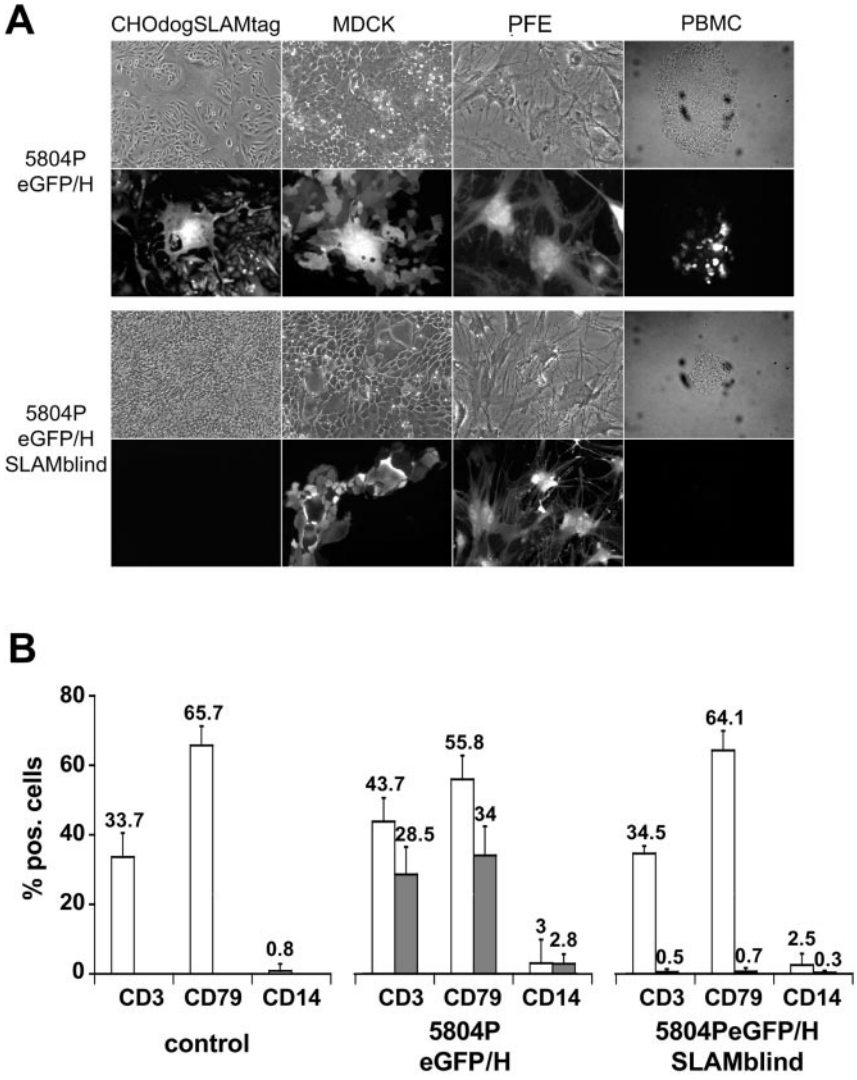

FIG. 2. Cell entry specificity of 5804P-eGFP/H-SLAMblind documented by microscopy and fluorescence-activated cell sorter (FACS) analysis. (A) Phase-contrast or fluorescent photographs of canine SLAM-expressing CHO cells (CHOdogSLAMtag), canine kidney epithelial cells (MDCK), PFE cells, and phytohemagglutinin (PHA)-stimulated ferret PBMC. CHO and CHOdogSLAMtag (cells expressing canine SLAM tagged with the HA epitope; kindly provided by Y. Yanagi [34]) were maintained in RPMI 1640 medium with 10% fetal calf serum (FCS) and G418 at the final concentration of 0.5 mg/ml. Madin-Darbin canine kidney (MDCK, ATCC CCL-34) cells were maintained in Dulbecco's modified Eagle's medium with 5% fetal calf serum. PFE cells were isolated from the kidneys of a 3-month-old male ferret following the method outlined for the isolation of primary bovine kidney cells (9). PBMC were isolated from three different 3- to 8-month-old male ferrets using the Ficoll gradient centrifugation method (Amersham Biosciences) according to the manufacturer's instruction. Cells were infected with 5804P-eGFP/H or 5804P-eGFP/H-SLAMblind at a multiplicity of infection (MOI) of 0.05. CHOdogSLAMtag cells and PBMC were photographed 3 days and MDCK and PFE cells 5 days after infection. (B) FACS analysis of PBMC mock infected (control) or infected with 5804P-eGFP/H or 5804P-eGFP/H-SLAMblind. For FACS analysis, Ficoll-purified ferret PBMC (5×10^6/well) were seeded into 24-well plates and stimulated with 15 μg/ml of PHA (Sigma) for 12 h prior to infection at an MOI of 0.05. Five days after the infection, the cells were washed with phosphate-buffered saline (PBS) and fixed with 1% paraformaldehyde. Aliquots (5×10^5 cells) were incubated with Alexa647-labeled antibodies against CD3 (Santa Cruz), CD79 (DAKO), CD14 (VMRD), or a corresponding isotype control for 45 min at 4°C. After two PBS washing steps, the cells were resuspended in 1% paraformaldehyde and subjected to FACS analysis. Data were analyzed using CellQuest (BD Biosciences). The percentage of cells reacting with the respective antibody is represented by a white column and that of eGFP-expressing cells reacting with the same antibody by the adjacent gray column. The graphs represent the mean and standard deviation of five independent experiments. The numerical value of the mean is indicated on the top of each column.

fuse because they emit green light upon stimulation. The mutations were introduced using the unique restriction sites RsrII and AscI (40). The corresponding fragment, which includes the last third of the H gene and the untranslated region between H and eGFP, was assembled by overlap extension PCR (13), yielding p5804SLAMblind-eGFP/H. We then attempted virus recovery using the wild-type CDV rescue system based on B95a cells (39). B95a cells were infected with a T7 polymerase-expressing, replication-deficient vaccinia virus (MVA/T7) (33) and transfected with the plasmid containing the full-length CDV genome as well as expression plasmids for the CDV nucleo-, phospho-, and polymerase proteins. Indeed we recovered a virus

that was named 5804P-eGFP/H-SLAMblind. This virus was propagated on VerodogSLAMtag cells, and its growth was analyzed on epithelial cells and PBMC, as shown in Fig. 2.

5804P-eGFP/H-SLAMblind readily infected MDCK cells, a canine epithelial kidney cell line, as well as primary ferret epithelial (PFE) kidney cells. In these cells, the cytopathic effects caused by the parental virus and the SLAM-blind virus 5 days postinoculation were similar (Fig. 2A, columns MDCK and PFE), with multiple small syncytia typical for wild-type strains. In contrast, infection with the SLAM-blind derivative was not detectable at the same time postinoculation in CHOdogSLAMtag cells and ferret PBMC (Fig. 2A; columns CHOdogSLAMtag and PBMC). Thus, the amino acids identified with the SLAM-dependent fusion support assay in cultured cells are essential also for the entry and propagation of a wild-type CDV strain in PBMC.

We then asked which types of PBMC support CDV replication. Using pan-species monoclonal antibodies, we separated ferret T cells ($CD3^+$), B cells ($CD79^+$), and macrophages ($CD14^+$). Figure 2B shows the percentages of each cell type (white columns) detected in cultivated PBMC five days after mock infection (control, left panel) or infection with the two recombinant viruses 5804P-eGFP and 5804P-eGFP-SLAM-blind (center and right panel, respectively). CDV infection resulted in a small but significant change in the distribution of T and B cells, possibly due to the preferential death of CD79-positive B lymphocytes. The wild-type virus but not its SLAM-blind derivative entered PBMC efficiently. In Fig. 2B the percentages of green fluorescent CD3, CD79, and CD14 cells five days after infection with the wild-type virus or its SLAM-blind derivative are represented with gray columns. More than half of the CD3, CD79, and CD14 cells emitted green light after wild-type virus infection, whereas GFP expression was minimal after infection with the SLAM-blind virus (Fig. 2B, right panel). Thus efficient CDV entry in all major PBMC types depends on efficient H protein interaction with SLAM.

Discussion. We have identified seven residues in the CDV H protein that are involved in SLAM-mediated fusion support and virus entry. Six of these residues are grouped in two clusters, both situated in propeller beta-sheet 5, and define areas analogous to those previously defined by the SLAM-interacting residues on MV H (37). A study of CDV adaptation to cell entry through marmoset SLAM resulted in the selection of a virus with mutations in residues 530 and 548, one residue in each cluster defined here (29). On the other hand, a mutagenesis study of charged MV H residues (21) identified arginine 533 and aspartates 505 and 507 as crucial for SLAM downregulation and SLAM-dependent fusion. This study did not cover residues downstream of position 537, and another study showed that aspartate 507 had a negative effect not only on SLAM-dependent but also on CD46-dependent fusion (37). It is possible that other amino acids support interactions with different receptors (25). It is remarkable that in CDV H multiple mutations are necessary to abolish canine SLAM-dependent fusion whereas in MV H single mutations strongly compromise human SLAM-dependent fusion (37).

We have shown here that a recombinant SLAM-blind wild-type CDV cannot infect PBMC but infects efficiently epithelial cells. This result and the recent observation in the ferret system that the parental wild-type CDV infects preferentially, if not exclusively, lymphocytes (38) are consistent with a major role of SLAM-dependent cell entry for morbillivirus-induced immunosuppression (35). The fact that in vivo only minimal macrophage infection by the wild-type virus was monitored (38), whereas macrophages are infected efficiently ex vivo, may be due to mitogen activation.

The SLAM-blind CDV retained efficient entry in primary ferret kidney epithelial cells and in canine MDCK cells. Which protein serves as a CDV receptor in these cells? Antibodies to CD9, a tetraspan transmembrane protein, inhibit CDV-induced fusion of primate cells (27), but the cellular molecule responsible for virus binding and uptake in epithelial cells of dogs and other carnivores has not yet been identified. In the case of the human morbillivirus MV, the ubiquitous regulator of complement activation CD46 acts as a cellular receptor for the attenuated and certain wild-type strains (19, 28); other alternative receptors have been sought but not yet identified (1, 12). CDV H does not efficiently interact with human or marmoset CD46 (10, 18, 32), and therefore it was suggested that CD46 may not be the ubiquitous morbillivirus receptor. However, lack of CDV H interaction is documented only for primate CD46, and studies based on canine CD46 cells are necessary to clarify the issue of the epithelial cell receptor for wild-type CDV.

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