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Analysis of protein expression by mammalian cell lines stably expressing lactate dehydrogenase-elevating virus ORF 5 and ORF 6 proteins

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Abstract

Lactate dehydrogenase-elevating virus (LDV) has a strict species-specificity. Because only a subset of mouse primary macrophages have been identified that can support LDV replication in vitro, the precise molecular mechanism of viral entry and replication remains unclear. To analyze the LDV envelope proteins, which probably mediate viral attachment to the host cell, we developed a mammalian system for stable co-expression of LDV open reading frame (ORF) 5- and ORF 6-encoded proteins (ORF 5 and ORF 6 proteins), which correspond to envelope VP-3 and M/VP-2, respectively, and compared these expressed proteins to the native ones. Western blotting analysis combined with *N*-glycanase digestion revealed that ORF 5 and ORF 6 proteins were similar in size to native VP-3 and M/VP-2, and that ORF 5 protein was N-glycosylated, like the native VP-3. Immunofluorescence microscopy revealed that both ORF 5 and ORF 6 proteins were distributed throughout the cytoplasm and were colocalized in most cells. Moreover, ORF 5 protein was localized both in the perinuclear region and the Golgi complex and transported to the cell surface. This mammalian expression system in which the exogenously expressed proteins closely resemble the native proteins will provide the experimental basis for further studies of the interactions between LDV envelope proteins and host cells.

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Résumé

Le virus d'élévation de la lactate déshydrogénase (LDV) a une spécificité d'espèce très stricte. La reproduction in vitro du LDV n'étant pour l'instant possible que dans les macrophages primaires d'un sous-groupe de souris, le mécanisme moléculaire de pénétration et de reproduction virale précis est mal connu. Pour analyser les protéines de l'enveloppe du LDV, qui fixe probablement une médiat viral sur la cellule hôte, nous avons développé un système mammifère pour une co-expression stable des protéines codées 5 et codées 6 du cadre ouvert de lecture du LDV (protéines de l'ORF 5 et de l'ORF 6), correspondant respectivement aux enveloppes VP-3 et M/VP-2, puis nous avons comparé les protéines ainsi exprimées aux protéines natives. L'analyse du transfert de type Western combinée à la digestion du *N*-glycanase a montré que les protéines de l'ORF 5 et de l'ORF 6 étaient de taille similaire aux protéines natives VP-3 et M/VP-2 et que la protéine de l'ORF 5 était *N*-glycosylée, comme la VP-3 native. L'examen microscopique immunofluorescent a montré que les protéines de l'ORF 5 et de l'ORF 6 étaient distribuées dans tout le cytoplasme et colocalisées dans la plupart des cellules. De plus, la protéine de l'ORF 5 a été localisée à la fois dans la région périnucléaire et dans l'appareil de Golgi, puis transportée à la surface. Ce système d'expression mammifère dans lequel des protéines à expression exogène ressemble de très près aux protéines natives permet de fournir une base expérimentale pour les futures études des interactions entre les protéines de l'enveloppe du LDV et les cellules hôtes.

Mots-clé: Virus d'élévation de la lactate déshydrogénase (LDV); ORF5; ORF6; VP-3; M/VP-2; Expression stable

1. Introduction

The *Arteriviridae* are enveloped positive-stranded RNA viruses that comprise a variety of animal pathogens, including lactate dehydrogenase-elevating virus (LDV), equine arteritis virus (EAV), simian hemorrhagic fever virus (SHFV), and porcine reproductive and respiratory syndrome virus (PRRSV) [1]. The ability to replicate in a variety of cell lines is characteristic of EAV, SHFV, and PRRSV, but not of LDV, which has a strict host cell specificity. In mice, it is known that only a subpopulation of peritoneal macrophages and other macrophages support LDV replication [2]. To date, no cell lines in which LDV can replicate or LDV receptors responsible for cell tropism have been identified; therefore, the mechanism underlying LDV susceptibility restriction remains unclear.

Because of an essential role in attachment to the plasma membrane of receptive host cells, it is important for studying the biological features of arterivirus envelope proteins. Arterivirus acquire their envelope by budding into the lumen of smooth membranes of the exocytic pathway, probably including those of the Golgi complex [3–7]. The specific roles of the various envelope proteins in arterivirus assembly and infectivity have not yet been reported, however, the recent development of infectious cDNA clones for arteriviruses [8–10] has opened the possibility of studying arterivirus assembly by modifying the expression of envelope proteins.

LDV has two major envelope proteins. The smaller of the two is an 18–19-kilodalton (kD) nonglycosylated protein M/VP-2, encoded by open reading frame (ORF) 6. It is close to the N-terminal end of three potential adjacent transmembrane segments that mimic sequences in the corona virus M protein [11]. The larger protein is the envelope glycoprotein VP-3, encoded by ORF 5. It is generally heterogenous in size (25–40 kD) due to varying amounts of *N*-glycosylation [12]. Li et al. [13] have postulated that LDV VP-3 may be the virus attachment protein. Their studies have shown that the neutralization epitope is located in the short envelope glycoprotein ectodomain and is associated with polylactosaminoglycan chains, which may affect neutralizing antibody binding to LDV virions.

LDV M/VP-2 and VP-3 are present in virions as heterodimers that are covalently linked by disulfide bonds, probably between single cysteine residues in the protein ectodomains [14]. Because disulfide bond breakage causes viral infectivity loss, linkage between M/VP-2 and VP-3 appears to be required for host cell entry and is perhaps achieved by generating the virion receptor attachment site. Further analysis of the interaction between LDV VP-3-M/VP-2 heterodimer envelope proteins and host cells will require an expression system that includes glycosylation and cell surface localization. Using transient mammalian expression systems based on Sindbis and vaccinia virus-based expression vectors, the non-structural proteins 2 and 3 (nsp2 and 3) of EAV and ORF 2, 4, 5, 6, and 7 products of PRRSV have been reported [15–17]. Although in vitro translation of LDV ORF 5 and ORF 6 transcripts has been reported in a rabbit reticulocyte lysate system [18], a mammalian cell system expressing LDV proteins remains unestablished. While experiments with the transient expression systems described above are convenient for studying cytotoxicity [17], cellular immune responses [15], and viral processing [16], there is considerable variability between individual experiments. Cell lines stably expressing viral envelope proteins would be useful for detailed molecular analysis of interactions between viral and host cell proteins and mechanisms underlying LDV susceptibility restriction. In this study, we established a stable mammalian cell system expressing both the LDV ORF 5- and ORF 6-encoded proteins (ORF 5 and ORF 6 proteins) as viral envelope proteins and analyzed the expressed proteins by immunological methods.

2. Materials and methods

2.1. Cells and transfection

Monkey kidney Cos7 cells [19] were obtained from Riken Cell Bank (RCB0539, Tsukuba, Japan) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine (conditioned medium). For transfection, approximately 5×10^6 cells were suspended in 0.5 ml K-PBS (30 mM NaCl, 120 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 5 mM $MgCl_2$) and electroporated with 25 μg DNA with a Bio-Rad gene pulser (Bio-Rad Laboratories, California, USA). The electrical parameters were 220 V, 975 μF, and 100 Ω resistance.

2.2. Virus and antibodies

Purified LDV type C (LDV-C) was prepared with 4 and 8-week-old SJL/J mice (Jackson Laboratory, Maine, USA) as described previously [20]. A polyclonal antibody (#36) against LDV M/VP-2 was obtained after immunizing rabbits with a synthetic polypeptide corresponding to the LDV-C ORF 6 C-terminal region coupled with the keyhole limpet hemocyanin (KLH) [21]. A monoclonal antibody against LDV VP-3 (MAb no. 36) has been described previously [22].

2.3. Expression plasmids

To prepare expressed LDV-C ORF 5 and ORF 6 proteins, plasmid pcDNA3.1-VP3 and pcDNA6-VP2 were constructed as shown in Fig. 1. Briefly, the entire LDV ORF 5 coding region plus a Kozak consensus sequence that is the ATG initiator codon [23] were isolated from LDV-C infected mouse sera by the reverse-transcription-polymerase chain reaction (RT-PCR). Viral RNA was isolated using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany). The RNA was reverse transcribed by murine leukemia virus reverse

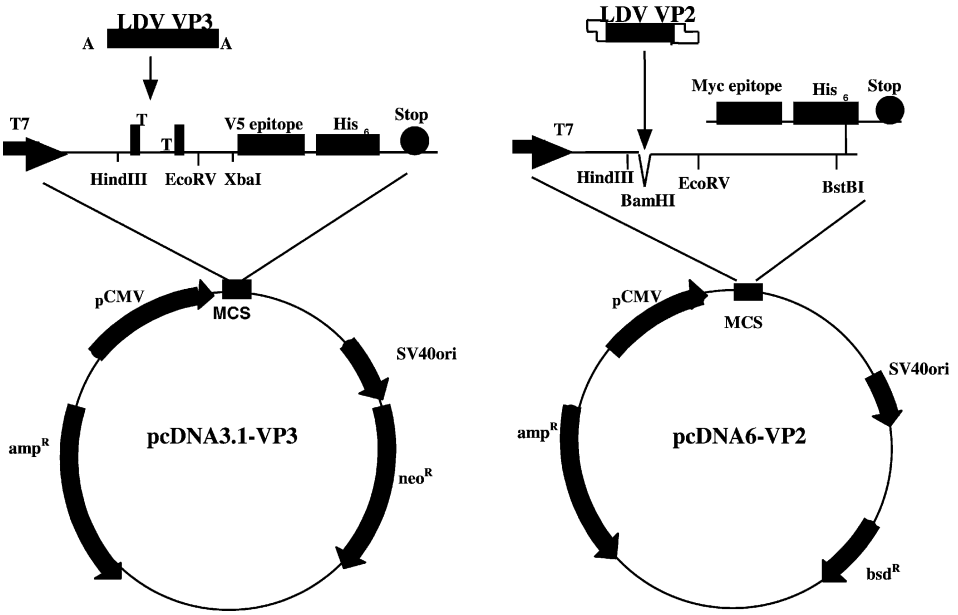

Fig. 1. Schematic diagram of vectors pcDNA6-VP2 and pcDNA3.1-VP3 for the expression of LDV-C ORF 6 and ORF 5, respectively. In both vectors, expression is under the control of the human cytomegalovirus (CMV) immediate-early promoter/enhancer element ($_P$CMV). pcDNA-VP2 expresses the predicted ORF 6 protein as a fusion protein with the C-terminal *myc* epitope (derived from *c-myc*; EQKLISEEDL) and the 6 × His-tag (HHHHHH–COOH). pcDNA3.1-VP3 expresses the predicted ORF 5 protein as a fusion protein with the C-terminal V5 epitope (derived from the paramyxovirus P and V proteins; SV5, GKPIPNPLLGLDST) and the 6 × His-tag. $_P$CMV, CMV promoter; MCS, multiple cloning site; SV40ori, SV40 promoter and origin; bsd^R, Blasticidin resistance gene; neo^R, Neomycin resistance gene; amp^R, Ampicillin resistance gene.

transcriptase (PERKIN ELMER, New Jersey, USA) according to the manufacturer's instructions. For ORF 5 cDNA amplification, the forward primer VP3F 5′-AAATTATGG GGGACGGTTATAACCTTGGTTTTGGCC-3′ corresponding to nt 1–31 of LDV ORF 5 with a Kozak consensus sequence (underlined) and reverse primer VP3R 5′-TTTCTTATCGTCATCGTCGGCCTCCCATTTTTCGGC-3′ which was complementary to nt 625–642 of ORF 5 with enterokinase recognition site (underlined) were utilized. The RT-PCR product was cloned into cDNA3.1/V5-His-TOPO (Invitrogen, NV Leek, The Netherlands) to yield pcDNA3.1-VP3. This expression vector has a single, overhanging 3′ deoxythymidine (T) residue and a neomycin resistance cassette. ORF 5 cDNA may be expressed as a C-terminal fusion to the V5 epitope derived from the P and V proteins of the paramyxovirus, SV5, and the polyhistidine metal-binding tag (6 × His-tag).

Plasmid pcDNA6-VP2 construction for LDV ORF 6 expression was carried out with the expression vector pcDNA6/*Myc*-His (Invitrogen), which contains the blasticidin resistance gene. The LDV-C ORF 6 coding region plus a Kozak consensus sequence was derived from infected sera by RT-PCR, as described above. For amplification of ORF 6 cDNA, the forward primer VP2F 5′-CGGGATCCATTATGGGAGGCCTAGAATTTTG-3′ corresponding to nt 1–20 of LDV ORF 6 with a BamHI site (underlined) and a Kozak consensus sequence (double underlined) and reverse VP2R 5′-CGGGATCCTTTTGAGACATATTT CAAAA-3′ which was complementary to nt 494–513 of ORF 6 with a BamHI recognition site (underlined) were utilized. The amplified ORF 6 cDNA was inserted into the pcDNA6/*Myc*-His BamHI sites. The inserted ORF 6 cDNA may be expressed with a C-terminal peptide encoding the *Myc* epitope derived from *c-Myc* and the 6 × His-tag.

2.4. Generation of stable LDV envelope protein-expressing cell lines

Cos7 cells stably expressing LDV envelope proteins were generated by cotransfection of linearized plasmids pcDNA-VP2 and pcDNA-VP3, followed by subsequent selection with both 400 μg/ml Geneticin (G418; Gibco BRL, Rockville, MD, USA) and 5 μg/ml blasticidin S (Kaken Seiyaku Co., Ltd, Japan). Resistant colonies were typically visible within 2–3 weeks under selection. Individual colonies were picked and amplified to confluence in 6-well plates for testing protein expression. At this stage, selection pressure was lifted, but conditioned medium described above was still utilized. Forty clones were screened for expression of the 6 × His-tagged proteins by Western blotting, as described below. Clones expressing high levels of proteins were selected and expanded for further analysis.

2.5. Western blotting

For screening cell clones stably expressing LDV ORF 5 and ORF 6 proteins, the 40 individual clones expanded as described above were analyzed by Western blotting. Confluent cell monolayers collected from each clone were harvested at 72 h after passage, and the cells were lysed with lysis buffer composed of 8 M urea, 2% TritonX-100, 5% 2-mercaptoethanol, and EDTA-free protease inhibitor cocktail (Roche Diagnostics Co., Ltd, Germany). Lysates were separated by sodium dodecyl

sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5% polyacrylamide gels and electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Millipore Corporation, Bedford, USA). Blots were reacted with a monoclonal antibody raised against 6 × His-tagged proteins (diluted 1:1000, Qiagen) followed by incubation with HRP-conjugated sheep anti-mouse IgG (diluted 1:5000, Amersham Biosciences, Buckinghamshire, UK). Immunoreactions were visualized with the ECL Western blotting analysis system (Amersham Biosciences).

To analyze co-expression of LDV ORF 5 and ORF 6 proteins in the established clones, each expanded clone was harvested within five passages of the initial establishment and lysed as described above. Cell lysates were purified by histidine-tagging HiTrap chelating columns charged with Ni^{2+} ions (Amersham Biosciences) according to the manufacturer's instructions with a few modifications. In brief, lysates were loaded onto columns equilibrated with start buffer composed of 20 mM phosphate, 0.5 M NaCl, and 8 M urea. Then, the loaded column was washed with start buffer. Although the manufacturer recommends using start buffer containing 10 mM imidazole to prevent nonspecific binding of cellular proteins, buffer without imidazole was used in this study because of the inhibition of specific binding and decreased recoveries of 6 × His-tagged specific proteins in response to imidazole (data not shown). Bound protein was eluted with 20 mM phosphate buffer containing 0.5 M NaCl, 8 M urea, 0.5 M imidazole, and 50 mM EDTA.

Purified LDV virion protein prepared as previously described [24,25] and expressed protein purified as described above were separated by SDS-PAGE on 12.5% polyacrylamide gels and electroblotted onto a PVDF membrane. Blots were reacted with rabbit antibody #36 (diluted 1:100) against LDV-C ORF 6 as reported previously [21] or monoclonal antibody no. 6 against LDV VP-3 [22]. The secondary antibody was HRP-conjugated donkey anti-rabbit IgG (diluted 1:10000, Amersham Biosciences) or HRP-conjugated sheep anti-mouse IgG (1:5000, Amersham Biosciences) as described above.

2.6. *N*-glycanase treatment

LDV ORF 5 protein extracted from established stable clones was analyzed for N-glycosylation by treatment with *N*-glycanase. In brief, affinity-purified protein extracted from each clone was desalted with PD-10 desalting columns (Amersham Biosciences) and concentrated. The protein was then diluted to a final volume of 50 μl with buffer containing 50 mM sodium phosphate (pH 6.7), 10 mM EDTA, 1% (v/v) Nonidet P-40, 1% (v/v) 2-mercaptoethanol, and protease inhibitor cocktail as described above. The protein was incubated with and without 50 U of PNGase F/ml (New England BioLabs, Beverly, USA) at 37 °C for 18 h and analyzed by Western blotting with monoclonal antibody no. 6 against LDV VP-3 as described above.

2.7. Immunofluorescence analysis

To examine the cellular localization of expressed ORF 5 and ORF 6 proteins in stable clones, immunofluorescence analysis was performed as described previously [22,25], with some modifications. In brief, cell clones within five passages of initial establishment were harvested at 72 h after passage and fixed for 10 min with 2% paraformaldehyde in

phosphate-buffered saline (PBS) at 4 °C with or without permeabilization by Triton X-100 for intracellular or cell surface staining [26]. Permeabilized and intact cells were incubated with LDV-C ORF 6 peptide-specific rabbit antibody #36 (diluted 1:100) and monoclonal antibody no. 6 against LDV VP-3 (not diluted) at 4 °C overnight. After washing with PBS, the cells were incubated with tetra-methylrhodamine isothiocyanate (TRITC)-conjugated swine anti-rabbit IgG (H + L) (1:10, DAKO Co. Ltd, Denmark), fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG (H + L) (1:10, DAKO Co. Ltd), and 4′,6-diamino-2-phenyl-indole (DAPI; 2 μg/ml, Roche Diagnostics Co. Ltd) which stains DNA 1 h at room temperature. The cells were washed again with PBS, and mounted preparations were observed with an LSM510 laser-scanning confocal microscope equipped with an Axiovert-100M (Carl Zeiss, Oberkochen, Germany).

3. Results

3.1. Generation of a stable 6 × His-tagged protein-expressing cell line

To establish cell lines stably expressing LDV ORF 5 and ORF 6 proteins, Cos7 cells were cotransfected with pcDNA-VP2 and pcDNA-VP3. Forty clones resistant to both G418 and blasticidin were selected and screened for 6 × His-tagged protein expression. Four of the 40 clones expressed the 6 × His-tagged protein, of which two were high-level expressers (clones 4-D and 4-H). These two clones were shown to stably express ORF 5 and 6 proteins, however, expression was reduced significantly by the 20th cell passage, even in the continuous presence of selective antibiotics (data not shown).

3.2. Western blot analysis of ORF 5 and ORF 6 proteins in established cell lines

Because they express high levels of 6 × His-tagged protein, clones 4-D and 4-H were used for analysis of LDV ORF 5 and ORF 6 protein expression. After extraction and purification of the expressed proteins in clones 4-D and 4-H by histidine-tagging HiTrap chelating columns as described in Section 2, the expression rate levels and molecular

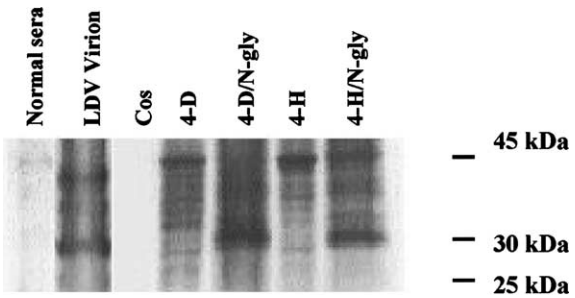

Fig. 2. Western blotting analysis of expressed ORF 5 protein in clones 4-D and 4-H with Mab no. 36 against LDV VP-3. Samples are as follows: LDV virion control from normal SJL/J mouse sera (Normal sera), LDV virion protein purified from infected mouse sera (LDV virion), mock-transfected Cos7 cells (Cos), cell clone 4-D treated without (4-D) and with *N*-glycanase (4-D/N-gly), clone 4-H treated without (4-H), and with *N*-glycanase (4-H/N-gly).

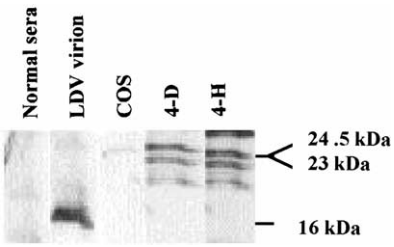

Fig. 3. Western blotting analysis of expressed ORF 6 protein in clones 4-D and 4-H with polyclonal antibody #36 against LDV M/VP-2. Samples are normal SJL/J mouse sera (Normal sera), LDV virion protein (LDV virion), mock-transfected Cos7 cells (COS), clones 4-D (4-D), and clone 4-H (4-H).

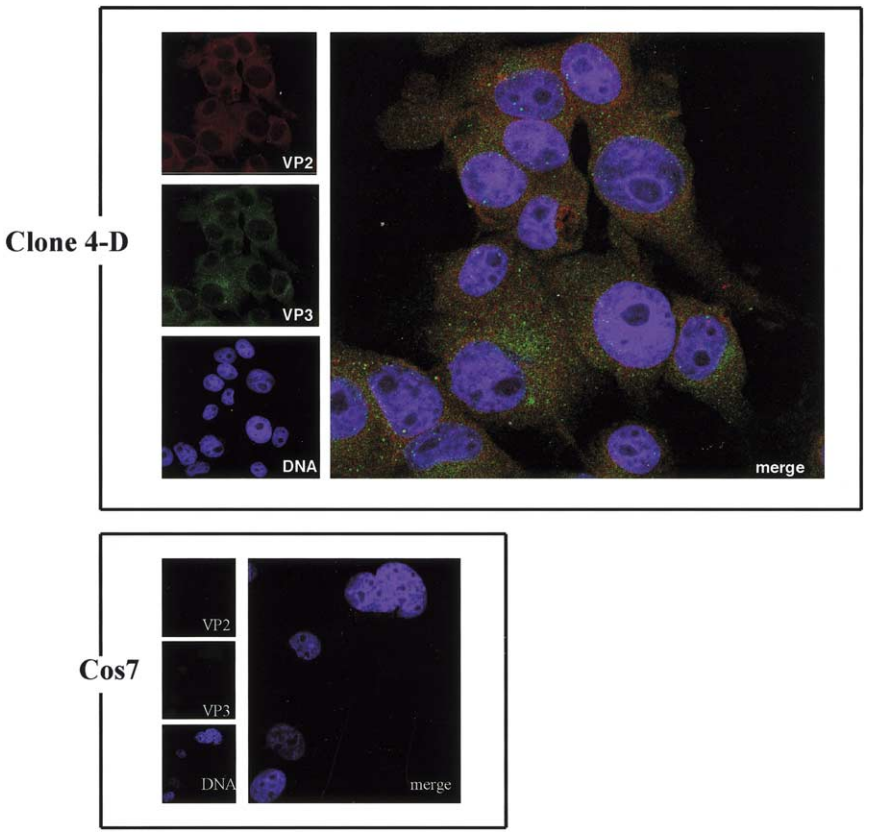

Fig. 4. Intracellular localization of LDV ORF 5 and ORF 6 proteins in clone 4-D and mock-transfected Cos7 cells. Permeabilized cells were triple labeled with polyclonal anti-peptide antibody #36 to LDV ORF6 (VP2), monoclonal antibody no. 6 against LDV VP-3 (VP3), and DAPI (DNA). Separate images of the same field were merged (merged). Magnification, × 640.

weight of each protein in the clones were determined by Western blotting. The peak levels of 6 × His-tagged ORF 5 and ORF 6 protein expression were observed 72 h after passage (data not shown). As shown in Fig. 2, purified His-tagged ORF 5 protein was detected as a cluster of bands approximately 30–45 kD (lanes 4 and 6). These bands comprise full-length ORF 5 plus the V5 epitope and 6 × His-tag sequences. In addition, 23 and 24.5 kD bands, derived from full-length ORF 6 plus, the *c-myc* epitope and 6 × His-tag sequences, were also detected (Fig. 3, lanes 5 and 6).

3.3. Identification of glycosylated ORF 5 protein

To examine whether expressed ORF 5 protein was glycosylated, the effects of *N*-glycanase were investigated. After *N*-glycanase digestion, the protein band shifted to 28 kD, which represents full-length ORF 5 plus the V5 epitope and 6 × His-tag sequences (Fig. 2, lanes 5 and 7). These results suggest that the size of unglycosylated ORF 5 protein is 28 kD and that the other protein bands were due to different degrees of glycosylation.

3.4. Intracellular distribution of ORF 5 and ORF 6 proteins

Indirect immunofluorescence analysis with permeabilization of clone 4-D revealed that 100% of the cells of the clone were fluorescence-positive, albeit at different relative intensities, whereas the parent Cos7 cells were negative except for nuclei of all cells stained with DAPI (Fig. 4). LDV ORF 5 protein was distributed throughout the cytoplasm, showing characteristic localization around the perinuclear region and Golgi complex (Fig. 4, VP3). ORF 6 protein was also distributed throughout the cytoplasm (Fig. 4, VP2). The nuclei were detected by DAPI staining (Fig. 4, DNA). In addition, ORF 5 and ORF 6 proteins appeared to be colocalized in most cells (Fig. 4, merge). The clone 4-H staining data was identical to that of clone 4-D (data not shown).

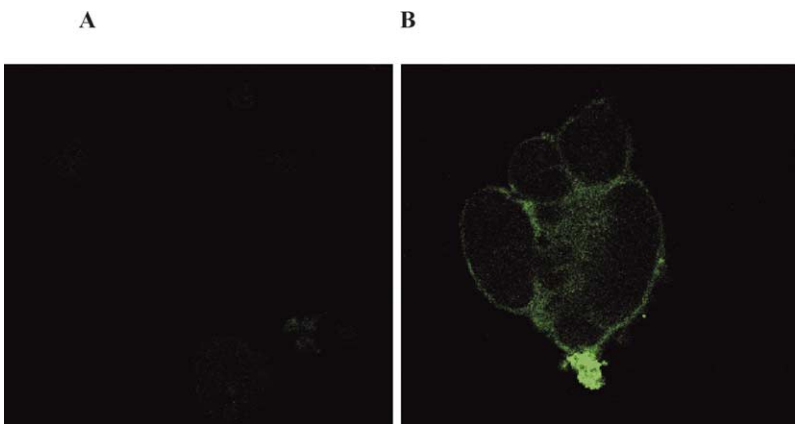

Fig. 5. Detection of expressed LDV VP-3 protein on the clone 4-D cell surface. Mock-transfected Cos-7 cells were labeled as a negative control (A). Unpermeabilized cells were labeled with monoclonal antibody no. 6 against LDV VP-3 (B). Magnification, ×640.

3.5. Cell surface expression of ORF 5 protein

To examine ORF 5 protein cell surface expression, clone 4-D was analyzed by immunofluorescence analysis without permeabilization. As shown in Fig. 5, fluorescence was observed on the edge of unpermeabilized cells, suggesting that expressed ORF 5 protein is present on the cell surface. The clone 4-H staining data was identical to that of clone 4-D (data not shown). In contrast, ORF 6 protein cell surface expression was unverified because there are no antibodies that react with cell surface M/VP-2. In our previous study [21], a rabbit antibody that reacted consistently with virion M/VP-2 in infected macrophages was generated, however, the antibody reacts only with the M/VP-2 cytoplasmic domain.

4. Discussion

In this report, a mammalian expression system that expresses proteins that closely resemble the native LDV envelope proteins was described. Western blotting analysis combined with *N*-glycanase digestion revealed that the expressed ORF 5 protein was similar in size to native VP-3 and was N-glycosylated like native VP-3. This result is consistent with the native virion VP-3 size reported previously [12]. ORF 6 protein tagged 6 × His residues was 23 and 24.5 kD, which is similar in size to native M/VP-2, but the reason for the two bands was unclear. It is possible that one band represents an ORF 6 plus 6 × His-tag sequences degradation product. Moreover, Western blotting consistently yielded unidentifiable bands. These bands may comprise a 6 × His-tagged specific protein cluster that was not relaxed sufficiently by 8 M urea treatment or a mammalian protein present due to incomplete purification.

Immunofluorescence analysis revealed that the localization pattern of expressed ORF 5 and ORF 6 proteins was similar to that of LDV VP-3 and M/VP-2 in infected macrophages [20,21]. In addition, ORF 5 and ORF 6 proteins were colocalized in most cells. Because M/VP-2 appears to be associated with VP-3 in virions [14,27], colocalization of ORF 5 and ORF 6 proteins was expected. Therefore, an association of ORF 5 and ORF 6 proteins expressed in our established cell lines is necessary to be verified, however, we failed to detect the association by a co-immunoprecipitation assay using antibodies against LDV ORF 5 [22] and ORF 6 proteins [21]. Since the antibodies were unable to immunoprecipitate expressed ORF 5 and ORF 6 proteins solubilized in the lysis buffer described in Section 2, detailed experiments are required to select the suitable lysis buffer and condition for immunoprecipitation assay.

In general, the Western blotting and immunofluorescence analysis verified that the exogenously expressed forms of both ORF 5 and ORF 6 proteins were similar in size and form to the native virion proteins. We have established a mammalian expression system that expresses proteins closely resembling the native LDV envelope proteins. Application of this expression system will be useful for studies of the interaction between LDV envelope proteins and host cells.

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