Arch Virol (2005) 150: 1857–1870
DOI 10.1007/s00705-005-0530-0

Archives of Virology
Printed in Austria

Effects of deletions of *kelch*-like genes on cowpox virus biological properties

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Received November 17, 2004; accepted February 17, 2005
Published online April 14, 2005

Summary. Cowpox virus (CPXV) strain GRI-90 contains six genes encoding *kelch*-like proteins. All six proteins contain both, the *N*-terminal BTB domain and the *C*-terminal *kelch* domain. We constructed mutant variants of a CPXV strain with targeted deletions of one to four genes of the *kelch* family, namely *D11L*, *C18L*, *G3L*, and *A57R*. As *kelch* genes are located in terminal variable regions of the CPXV genome, we studied the relationship of these genes with integral biological characteristics such as virulence, host range, reproduction *in vitro* and *in ovo* (in chicken embryos). It was demonstrated that the following effects occurred in a gene dose dependent manner with an increase of the number of genes deleted: (1) range of sensitive cells altered – deletion mutants lacking three genes displayed a considerably decreased ability to reproduce in MDCK cells; mutants lacking four genes lost this ability completely; (2) analysis of pocks formed by mutants with deletion of three and four *kelch*-like genes on chorioallantoic membranes of chicken embryos demonstrated that pock size and virus yield were significantly decreased; (3) light microscopic analysis of the pocks revealed impaired proliferation and reduced vascularisation in the pock region. More alterations were detected by electron microscopic analysis: the reproduction of mutants results in a reduction of the number of mature virions formed, and in many cells this process was arrested at the stage of assembly of immature virions; and (4) the evaluation of LD_{50} and body weight loss in BALB/c mice infected intranasally with CPXVs revealed a reduction of the virulence of the deletion mutants, which became statistically significant when four *kelch*-like genes were excised.

Introduction

The family of genes encoding the proteins homologous to the *Drosophila* kelch protein was revealed in genomes of members of the *Orthopoxvirus* genus [22].

Characteristics of the proteins belonging to this family are the presence of a BTB domain, capable to form homo- and heterodimers, in the *N*-terminal part [1, 24] and a kelch domain, showing an affinity for actin filaments of the cell, in the *C*-terminal part of the molecule [15]. In orthopoxvirus genomes, genes of the *kelch* family are located in terminal variable regions and show species-specific differences in the organization of the proteins they potentially encode [21]. Vaccinia virus (VACV) encodes three full-size copies of *kelch*-like genes [9]. Six such genes were detected in the genome of cowpox virus (CPXV) [20]. The genome of ectromelia virus (ECTV) encodes four full-size genes of *kelch*-like proteins [2]. Only one such gene was detected in the genome of monkeypox virus [23]. All genes of the *kelch* family were damaged in the genome of variola virus (VARV), which is highly pathogenic for its only host, man [13, 19]. The distinctions in the number and structure of orthopoxvirus *kelch*-like genes suggest that these genes are important as they manifest species-specific features of orthopoxviruses.

Although there are numerous *kelch*-like proteins of orthopoxviruses, their functions still are unclear. It has been demonstrated that *kelch*-like genes of VACV are inessential for growth in cell culture [9, 14], however, the VACV mutant lacking the *C2L* gene has a different plaque morphology due to an altered cytopathic effect on infected cells. Mutant-infected cells showed a reduction in the formation of VACV-induced cellular projections and in the VACV-induced Ca^{2+}-independent cell–extracellular matrix adhesion phenotype. In an intradermal mouse model, the VACV *C2L kelch*-like gene product reduced the cellular infiltration of VACV-infected ears and reduced the pathology associated with VACV infection, promoting the healing of lesions [4]. All these researches used VACV, whose natural reservoir and origin are still unknown; therefore, we concluded it would be preferable to study the properties of the *kelch*-like genes of orthopoxvirus isolated from natural sources.

In our opinion, CPXV is highly suitable for this purpose, as it has the largest number of *kelch*-like genes in the genome and, in its overall properties, may be considered as the ancestor of other orthopoxvirus species pathogenic for humans [18, 20]. It is reasonable to use CPXV for detecting and investigating the genes determining its features *in vivo*, because this virus has a very wide host range, including small laboratory animals [11].

For the work, the CPXV strain GRI-90, isolated in 1990 from a 4.5-year-old child who had contracted the disease from an infected mole, was chosen. The passage history of this strain is well known and short, indicating its relationship with the naturally circulating human pathogenic CPXV strains [10]. *Kelch*-like proteins of CPXV GRI-90 have a length of about 500 amino acids, 22–26% of which are identical. All six proteins contain both, the *N*-terminal BTB domain and the *C*-terminal kelch domain [20]. Earlier, we constructed mutant CPXVs with one *kelch*-like gene deleted (*D11L*, *G3L*, or *C18L* – single mutants), two genes deleted (*D11L/G3L*, *D11L/C18L, G3L/C18L* – double mutants), and three genes deleted (*D11L/G3L/C18L* – triple mutant); in the last case, all three genes located in the left variable genomic region were excised sequentially [8]. Deletion of one or two CPXV genes of the *kelch* family failed to cause reliable changes in virus

reproduction *in vitro* and *in ovo*. The mutants with three *kelch*-like genes deleted differed essentially from the wild type virus in the ultrastructural characteristics of the infection as well as in the size and morphology of the pocks they induced on the chorioallantoic membranes (CAMs) of chicken embryos [8].

In this paper we report the construction of CPXV GRI-90 with deletion of the *kelch*-like genes *D11L*, *G3L*, *C18L* and *A57R* and the alteration of the biological properties of this quadruple mutant in comparison with single (including A57R deletion mutant), double and triple deletion mutant viruses.

Materials and methods

Plasmid construction

The integration plasmid pΔA57R, intended for deleting the *A57R* gene from the CPXV genome, was assembled as follows. Plasmid pUC19gpt [8] cleaved with *Bam*HI and *Hin*dIII restriction endonucleases and harboring the *E. coli* selective gene *gpt* under the control of the VACV 7.5K promoter (isolated from plasmid pTK61-gpt kindly provided by Dr. B. Moss) was used as vector. The fragments of the CPXV genome flanking the *A57R* gene from the right (R fragment) and left (L fragment) were inserted into this vector. The fragments were PCR amplified using the plasmid pCB64 from the CPXV genomic library as a template [17]. Primers were calculated so that the target gene was deleted to a maximum without damaging the adjacent ORFs. The right fragment with a length of 930 bp was amplified using the following primers:

5′-CCTCC**GGTTACC**TGCGAACCGATTAAACATAAATATCC-3′ (A57R2F), which contains the *Psp*EI restriction site (underlined), and
5′-GCG**GGATCC**ACTACCGCCTACAGTAGTTGGTGGT-3′ (A57R2R), which contains the *Bam*HI restriction site (underlined).

The L fragment with a length of 917 bp was amplified using the following primers:

5′-GGG**AAGCTT**TAAACATGGATTTTTGATGGTGG-3′ (A57R1F), containing the *Hin*dIII site (underlined), and
5′-GGTTG**GGTAACC**AATTCACTGCTGTTATTCATGATGGC-3′ (A57R1R), containing the *Psp*EI site (underlined).

The resulting amplicons were digested with *Hin*dIII/*Psp*EI (L) and *Psp*EI/*Bam*HI (R) restriction endonucleases and cloned jointly in the vector pUC19gpt to form the integration plasmid (pΔA57R), carrying both, the *E. coli* selective gene *gpt* under the control of the VACV 7.5 promoter and the CPXV genomic sequences flanking the gene to be deleted.

Virus strains and cells

African green monkey kidney cells (CV-1), rhesus macaque kidney cells (LLCMK-2), human cervical carcinoma cells (HeLa), human lung cells (MRC-5), human embryo lung fibroblasts (L-68), porcine kidney cells (PK-15), canine kidney cells (MDCK), adult normal bovine kidney cells (MDBK), rabbit kidney cells (RK-13), human osteosarcoma cells (HOS), hamster kidney cells (BHK-21), and cells of murine embryonic musculocutaneous tissue (3T3) were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS). Cell cultures were obtained from the SRC VB "Vector" collection.

The stock of cowpox virus strain GRI-90 was received from the collection of Prof. S.S. Marennikova. All deletion mutants were derived from the CPXV-5 cloned variant, which has

the same properties as the parental stock CPXV strain GRI-90. Earlier constructed single mutants with *D11L*, *G3L* or *C18L* genes deleted, double mutants with targeted deletion of *kelch* genes Δ*D11L/C18L* and Δ*D11L/G3L* (clone 27 – parental variant for triple mutants construction) and triple mutants Δ*D11L/G3L/C18L* (clones 52 and 73) [8] were used in the work. The viruses were grown in CV-1 cells or in CAMs of 11-day-old Leghorn chicken embryos (GPPZ "Novosibirsky", Koltsovo, Novosibirsk region, Russia).

Cloning was done by plaquing on CV-1 cell monolayers by means of agar overlay. Virus plaques were isolated, passaged and recloned twice to receive a homogenous population. Cloned variants were propagated once in CV-1 cells, aliquoted and stored at −70 °C. For the deletion mutants aliquots from each clone were characterized for the presence of deletion(s) examined. The same aliquots were used for investigation of the biological properties.

To study cell sensitivity and virus replication characteristics the monolayer of different cells was infected with 0.01 PFU of wild or mutant CPXV per cell, incubated at 37 °C for 24 and 48 h, and then frozen and thawed three times. The virus suspension obtained was sonicated and titrated by plaque technique on CV-1 cell monolayer.

Analysis of pocks induced by CPXV variants on chicken embryo CAMs

Chicken embryos were inoculated with 30–50 PFU per CAM of CPXVs to obtain clear and detached pocks, which were studied visually and microscopically. The size of the pocks was measured 48 h after infection. To determine the virus yield, individual pocks were cut out from CAM, placed into microtubes, ground with sterile disposable pestles in 0.5 ml of Hanks' solution, treated with ultrasound, and frozen. The virus suspensions obtained were titrated by plaque technique on CV-1 cell monolayer.

For microscopic examination, pieces of CAMs with pocks were dissected 48 h after infection and fixed in 4% paraformaldehyde for 48 h at a temperature of 4 °C. The samples were postfixed in 1% osmium tetraoxide Hank's solution for 2 h, dehydrated in increasing alcohol concentrations and embedded in epon-araldit mixture [3]. Ultrathin sections were stained with uranyl acetate and lead citrate and examined using a Hitachi H-600 electron microscope.

Infection of mice

Male BALB/c mice were 4 to 5 weeks old and weighed between 11 and 14 g. Groups of 8–10 mice were inoculated intranasally with 30 μl of tenfold dilutions of a virus in Hank's buffered saline. Infectious doses ranged from 10^4 to 10^7 PFU per mouse. Mice inoculated with Hank's buffered saline served as controls. The mice were examined daily for mortality and morbidity, and the body weight was recorded over 21 days. A dose causing a 50% fatality rate (LD_{50}) was determined as previously described [7].

Statistical analysis

Student's *t*-test was used to determine the significance of the results. A P value of <0.05 was considered significant.

Results

Construction of CPXV variant with targeted deletion of four kelch family genes (D11L, G3L, C18L and A57R)

The construction of deletion mutants of CPXV strain GRI-90 lacking one (single mutants), two (double mutants), or three (triple mutants) genes belonging to the *kelch* family was described earlier [8]. All genes examined were situated at the

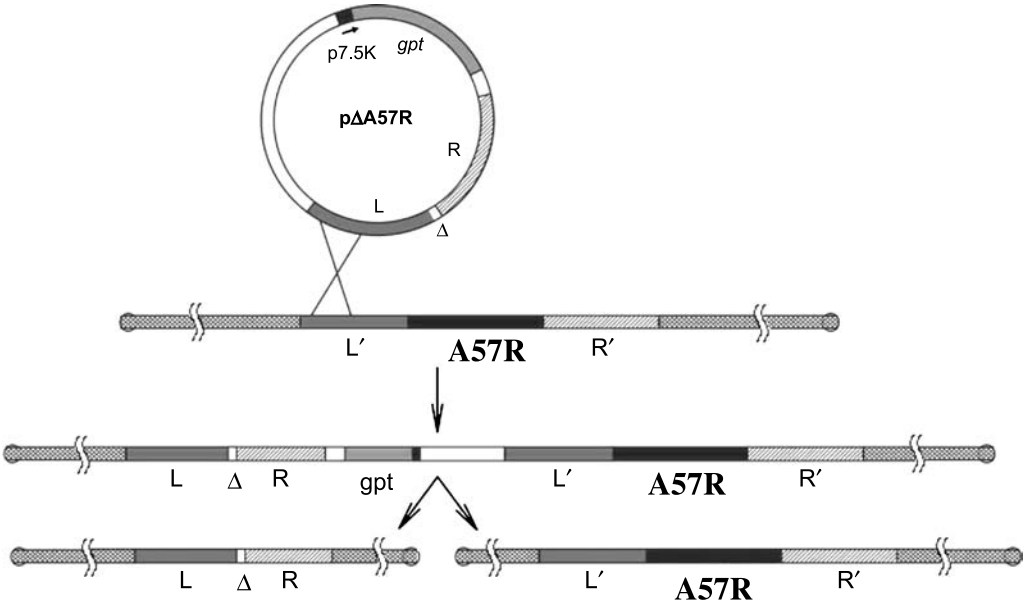

Fig. 1. Scheme of *A57R* gene targeted deletion introduction into the genome of CPXV

left terminal variable region of virus genome. The group consisting of three other CPXV *kelch* genes (*A57R*, *B9R* and *B19R*) was mapped within the right terminal variable region of the virus genome [20]. One of these genes (*A57R*) was investigated. Figure 1 shows the layout for introducing the *A57R kelch* gene deletion into the CPXV genome. The deletion mutants with one (Δ*A57R*) or four *kelch*-like genes excised (Δ*D11L/G3L/C18L/A57R* – quadruple mutant) were formed by homologous recombination between the transfected plasmid pΔA57R and the CPXV-5 clone DNA or the CPXV triple mutant (Δ*C18L/D11L/G3L*) DNA inside the infected CV-1 cells. Recombinant viruses were selected by means of the transient dominant selection system [5]. The mono- or tetra-deletion structure of selected mutants was confirmed by PCR using the following primers:

For the *C18L* gene

5′-GGAACGTCACGCTCTCCACCTT-3′ and
5′-GAAGAGTTAGGATTGGCTACTGAATATTTA-3′;

For the *G3L* gene

5′-CACCGCCTCCTATATCAATGCCTTT-3′ and
5′-TGCGATTTCGCATGTTTGATGTTTA-3′;

For the *D11L* gene

5′-TCACGGATCCATCTTCTATCTGCG-3′ and
5′-CAGATGAAGCTTGACTAAGATAAAATTG-3′; and

For the *A57R* gene (used for testing of single or quadruple mutants)

5′-CCATGCGGATACTTTGGTGGAATA-3′ and
5′-GGGCTCCTTATACCAAGCACTCATAA-3′.

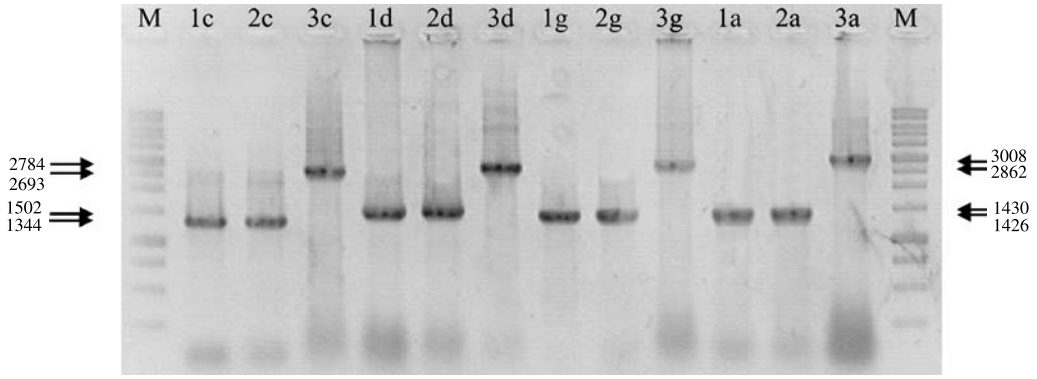

Fig. 2. PCR assay of CPXV mutants 216 and 337 with four deleted *kelch*-like genes (electrophoretic pattern of PCR amplified CPXV DNA fragments in 1% agarose gel): *1* and *2*, mutants 216 and 337, respectively; *3*, CPXV-5 clone; *c*, primers for *C18L* gene; *d*, primers for *D11L* gene; *g*, primers for *G3L* gene; *a*, primers for *A57R* gene; and *M*, 1 Kb marker

The presence of the deletion in each particular region of the mutant virus DNA was detected according to the length of the fragments amplified. The amplified fragment of the full-size *A57R* gene has a length of 3008 bp; *D11L* gene, 2784 bp; *C18L* gene, 2693 bp; and *G3L* gene, 2862 bp. In quadruple mutants, the length of the corresponding amplified fragments changed as follows: Δ*A57R*, 1430 bp; Δ*D11L*, 1502 bp; Δ*C18L*, 1344 bp; and Δ*G3L*, 1426 bp. The PCR assay of two independently selected clones of the quadruple mutant, designated as 216 and 337, is shown in Fig. 2. Both of these mutants were used in parallel in subsequent studies to exclude an influence of possible spontaneous mutations in the virus genomes on the viral properties. The single mutant marked Δ*A57R* was also studied as a control.

Cell sensitivity to CPXV variants

Eleven cell cultures of various origins were studied to assess the influence of *kelch*-like gene deletions on a range of sensitive cells and on the replication characteristics of CPXVs. The results of these experiments are presented in Table 1. The experiments did not reveal any significant difference between the wild type CPXV strain GRI-90, the CPXV-5 clone, the single (Δ*D11L*, Δ*G3L*, Δ*C18L* or Δ*A57R*), and double mutants (Δ*D11L/G3L* and Δ*C18L/D11L*). Data on single and double mutants have been omitted from Table 1 to make it more compact and informative.

It is evident that all CPXVs either reproduced weakly or failed to reproduce in MDBK cells. The quadruple and triple mutants showed delayed replication in L-68, MRC-5, and PK-15 cells at 24 h of incubation. Replication of the quadruple mutants in 3T3 and LLCMK-2 cells was significantly lower than that of the wild type CPXV. The viruses with four deleted genes completely lost their ability to reproduce in MDCK cells.

Table 1. Growth of mutants and corresponding wild type CPXVs in cell cultures

Name of cell line	Post infection time (hours)	Virus growth titer (log_{10}PFU/ml)* Strains				
		Uncloned CPXV	Clone CPXV-5	Clone 73 (triple mutant)	Clone 216 (quadruple mutant)	Clone 337 (quadruple mutant)
CV-1	24	6.2 ± 0.2**	6.1 ± 0.2	5.4 ± 0.5	6.0 ± 0.2	6.2 ± 0.3
	48	6.9 ± 0.3	7.13 ± 0.2	7.0 ± 0.2	7.4 ± 0.3	7.2 ± 0.2
L-68	24	5.5 ± 0.3	5.1 ± 0.2	5.0 ± 0.4	4.4 ± 0.2	4.2 ± 0.3
	48	6.6 ± 0.2	6.9 ± 0.3	6.8 ± 0.2	6.5 ± 0.3	6.8 ± 0.2
HOS	24	6.4 ± 0.3	6.3 ± 0.2	5.7 ± 0.4	5.8 ± 0.3	5.9 ± 0.2
	48	7.1 ± 0.2	7.1 ± 0.3	7.4 ± 0.2	7.2 ± 0.2	6.8 ± 0.4
LLCMK-2	24	4.5 ± 0.2	4.4 ± 0.3	3.8 ± 0.3	3.6 ± 0.2	3.4 ± 0.4
	48	5.4 ± 0.2	5.3 ± 0.2	5.3 ± 0.3	4.8 ± 0.2	4.4 ± 0.3
MDBK	24	2.3 ± 0.3	$\leq 1.0 \pm 0.2$	2.3 ± 0.3	$\leq 1.0 \pm 0.2$	$\leq 1.0 \pm 0.2$
	48	$\leq 1.0 \pm 0.2$	$\leq 1.0 \pm 0.2$	$\leq 1.0 \pm 0.2$	$\leq 1.0 \pm 0.2$	$\leq 1.0 \pm 0.2$
RK-13	24	5.7 ± 0.2	5.6 ± 0.3	5.1 ± 0.4	5.3 ± 0.3	5.4 ± 0.4
	48	7.0 ± 0.1	6.7 ± 0.2	6.7 ± 0.1	6.7 ± 0.2	6.9 ± 0.3
HeLa	24	6.4 ± 0.2	6.4 ± 0.3	6.0 ± 0.3	6.1 ± 0.4	6.2 ± 0.3
	48	7.2 ± 0.3	7.3 ± 0.2	6.8 ± 0.3	7.0 ± 0.2	6.9 ± 0.3
MDCK	24	4.5 ± 0.2	3.8 ± 0.4	3.6 ± 0.2	$\leq 1.1 \pm 0.2$	$\leq 1.1 \pm 0.2$
	48	5.0 ± 0.3	5.1 ± 0.2	4.4 ± 0.2	$\leq 1.0 \pm 0.1$	2.5 ± 0.2
MRC-5	24	5.0 ± 0.4	5.4 ± 0.2	4.6 ± 0.3	4.5 ± 0.3	4.2 ± 0.4
	48	7.0 ± 0.2	7.1 ± 0.3	6.9 ± 0.2	6.9 ± 0.2	6.5 ± 0.2
3T3	24	5.0 ± 0.2	4.8 ± 0.2	4.1 ± 0.4	4.0 ± 0.4	3.8 ± 0.4
	48	5.7 ± 0.3	5.7 ± 0.2	4.6 ± 0.2	–	4.5 ± 0.2
PK-15	24	5.5 ± 0.3	5.0 ± 0.4	4.5 ± 0.4	4.4 ± 0.3	–
	48	6.5 ± 0.4	6.2 ± 0.4	5.8 ± 0.3	5.9 ± 0.2	6.3 ± 0.4

*Virus concentrations in suspensions were determined by plaque technique on CV-1 cell monolayer grown in six-well plates

**The mean values ± SD were obtained in three independent experiments, $P < 0.05$

Comparative study of CPXV induced pocks

We did not detect any prominent differences between the pocks induced by the CPXV-5, single and double deletion mutants. Deletion of three (*D11L*, *C18L*, *G3L*) or four (*D11L*, *G3L*, *C18L*, *A57R*) *kelch*-like genes resulted in detectable changes in the pocks' morphology (Fig. 3). After 48 h, CPXV-5 induced large red pocks with a diameter of 2.1 ± 0.33 mm on the CAMs, whereas the pocks induced by triple and quadruple mutants were whitish and significantly smaller in diameter – 0.96 ± 0.38 mm and 0.84 ± 0.27 mm, respectively.

The virus yield obtained from the pocks induced by the quadruple mutants was significantly lower than from the pocks generated by the parental strain (Table 2).

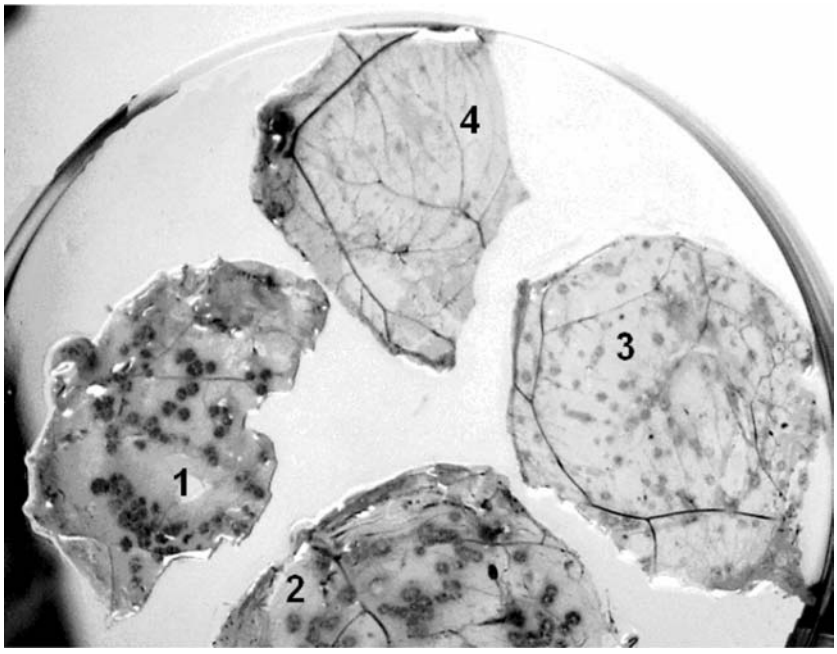

Fig. 3. Pocks formed on chicken embryo CAMs by clone CPXV-5 and deletion mutants derived from CPXV-5: *1*, CPXV-5; *2*, double mutant; *3*, triple mutant; and *4*, quadruple mutant 216

Table 2. Characteristics of infection with CPXV-5 and derived deletion mutants *in ovo* and *in vivo*

Virus strains	Log_{10}PFU/pock*	$Log_{10}LD_{50}$ (PFU/mouse)**	Mice body weight loss (%)***
CPXV-5	6.3 ± 0.4	5.03 ± 0.07	22.4 ± 7.0
27 (double mutant)	6.6 ± 0.3	4.59 ± 0.55	22.3 ± 14.5
52 (triple mutant)	5.8 ± 0.3	ND	ND
73 (triple mutant)	5.3 ± 0.3	5.58 ± 0.41	12.9 ± 12.5
216 (quadruple mutant)	5.1 ± 0.2	5.68 ± 0.34	1.6 ± 10.7
337 (quadruple mutant)	5.2 ± 0.2	5.94 ± 0.18	0.5 ± 11.2

*Viral yield in individual pock on chicken embryo CAM. Number of pocks tested was in no case less than 30, $P < 0.05$

**The mean values $\pm$ SD were obtained in three independent experiments, $P < 0.05$

***Body weight of the mice was determined 9 days post-infection in groups of mice infected with 10^4 PFU of corresponding virus. Weight loss was calculated as percent of difference between simple averages of initial body weight and body weight of infected mice. The mean values $\pm$ SD were obtained in three independent experiments (the same as for LD_{50} study), $P < 0.05$

ND: not done

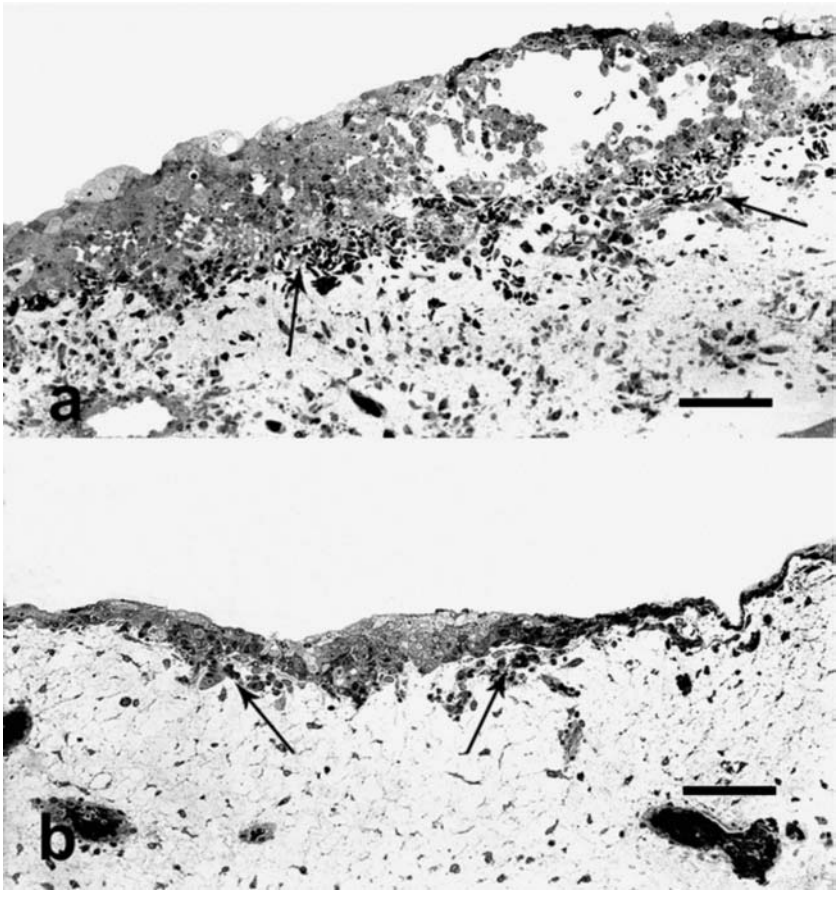

Fig. 4. Cross sections of pocks induced by clone CPXV-5 (**a**) and quadruple mutant 216 (**b**). Note the large cavity in the chorion epithelium of the pock induced by clone CPXV-5. Blood vessels are shown by arrows. The magnification of both photos is identical. Bars represent 130 μm. Light microscopy (semi thin sections stained with azur-2)

Microscopic investigations

Light microscopy revealed pronounced differences in the structure of the pocks induced by the CPXV-5 and the quadruple mutants 216 and 337. The pocks of strain CPXV-5 displayed a distinct disk shape. The chorion epithelium in the region of a pock was stratified; the number of cell layers increased from periphery to center, reaching 13–15. A destruction zone in the center of the pock contained a cavity, bordered by surface epithelium layers, where destroyed cells were localized (Fig. 4a).

The pocks induced by the mutants 216 and 337 were devoid of a distinct zone of central necrosis; the number of cell layers did not exceed three to five, even in the pock center, indicating an impaired proliferation of the epithelial cells in the region of virus replication (Fig. 4b). Inflammatory cell reaction was poor in the pocks induced by both, the parental virus CPXV-5 and the quadruple mutants.

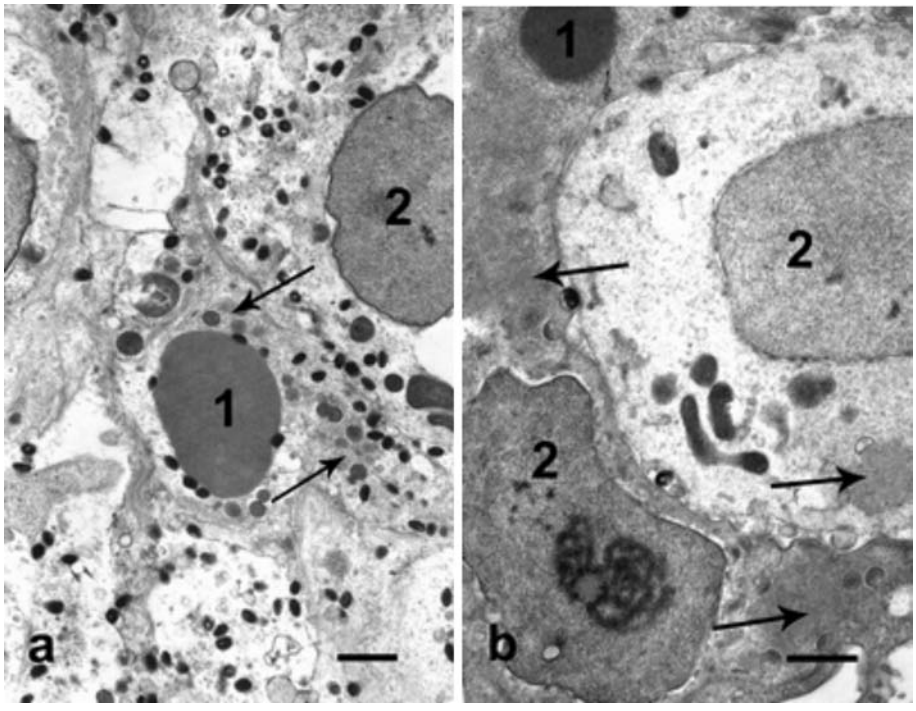

Fig. 5. Virus-specific structures in chorion epithelial cells, infected by clone CPXV-5 (**a**) and quadruple mutant 216 (**b**). Round immature and dense ovoid mature virions are visible inside and outside of cells. A-type inclusion body – *1*; cellular nucleus – *2*. Arrows show viral factories. Bars represent 850 nm

Numerous blood vessels filled with blood elements were located under the basal membrane of the pocks induced by the CPXV-5, giving evidence for an extensive vascularisation. The vessels about each other, forming an additional "layer" (Fig. 4a). Obviously, it was this vessel "layer" that caused the red color of the pocks on CAM (Fig. 3). Vascularisation of the pocks induced by the quadruple mutants was poor: an all-over vascular "layer" was absent, and the vessels were separated by wide interlayers of connective tissue (Fig. 4b), explaining the whitish color of the pocks (Fig. 3).

Electron microscopic examination showed plenty of infected cells and a high reproduction level of the CPXV-5 strain: numerous immature and mature virions, and A-type inclusion bodies (Fig. 5a). The number of infected cells in the pocks induced by quadruple mutants was considerably lower. The total viral yield was evidently lower than in cells infected with the wild type virus. Many cells showed a blockage of virus reproduction and contained only small viroplasm loci and a small number of immature and mature virions. A-type inclusion bodies were either small or completely absent (Fig. 5b).

Studies of CPXV infection in BALB/c mice

Visual signs of disease were noted on days 4–6 depending on the infectious dose. Mice lost their appetite and activity, showed ruffled haircoats, tachypnea,

cringing, huddling and conjunctivitis. The mice infected with the deletion mutants showed no signs different from those in the mice infected with the wild type virus. However, a one to two day earlier onset of the illness and weight loss were observed in the mice infected with CPXV-5, single and double mutants in comparison with those infected with triple and quadruple mutants. Mice infected with CPXV-5, single and double mutants at doses from 10^4 to 10^7 PFU developed symptoms of the disease 6-3 days after infection, respectively. Mice infected with CPXV-5, single and double mutants at a dose of 10^4 PFU did not die and showed symptoms of the disease and weight loss four days longer than those infected with triple and quadruple mutants. Only mice infected with 10^4 PFU of the quadruple mutants 216 and 337 exhibited a significant difference in weight loss (1.6 ± 10.7 and $0.5 \pm 11.2\%$) ($P < 0.05$) on day 9 after infection (time of maximal weight loss detected) in comparison with those infected with wild type virus ($22.4 \pm 7.0\%$) (Table 2). No statistically significant difference was detected between wild type virus and triple mutants, although a decreasing tendency of the weight loss level was evident (Table 2). Mice infected with CPXVs at doses $\geq 10^5$ PFU died on days 4 to 10 (40–100% of lethality depending on the dose), which did not allow us to follow up on the changes in body weight for the representative period of time after infection.

No visible lesions of the skin and mucosa were noted during the entire observation period (21 days). As is evident from Table 2, the triple and quadruple mutants displayed a significantly ($P < 0.05$) decreased virulence for BALB/c mice.

Discussion

Genes of *kelch*-like proteins are located in variable terminal genomic regions of CPXV, which are proposed to determine the host range and the level of virulence for sensitive hosts [20, 23]. However, no data confirming the relationship of these genes with integral biological characteristics such as virulence, host range, reproduction *in vitro* and *in ovo*, have been published.

We examined 11 cell cultures to compare the range of reproductive ability of the wild type CPXV and the deletion mutants lacking two, three, or four genes encoding *kelch*-like proteins. It appeared that all the CPXV variants tested, including the wild type virus, either reproduced weakly or virtually failed to reproduce in MDBK cell culture. Presumably, this may be further used as a differential characteristic when identifying CPXV. A delay in the reproduction of triple and quadruple mutants was observed in L-68, MRC-5, and PK-15 cells. Reproduction of quadruple mutants was significantly reduced compared to that of the wild type CPXV in 3T3 and LLCMK-2 cells. Thus, the data obtained showed that CPXV *kelch*-like genes may be related to the group of host range genes. As the effect evidently emerges when only three or four *kelch*-like genes are deleted, we suppose that the effect is formed in a gene dose dependent manner.

Formation of pocks on chicken embryo CAM and their visual and microscopic examination are a good model for studying orthopoxviruses. Size and appearance of the pocks vary depending on the species, representing a classification parameter

for orthopoxviruses [11]. The pock morphology of individual poxviruses is determined by the intensity of virus reproduction, epithelium proliferation, inflammation and destruction [16]. Analysis of pock size and viral yield revealed a dose-dependent effect with regard to the number of *kelch*-like genes deleted, similar to the pattern described above. Deletion of three and four genes (the mutants 73, 216, and 337) resulted in a significant decrease of the analyzed parameters. Microscopic analysis of the pocks induced by quadruple mutants confirmed the changes reported earlier for triple mutants [8], and demonstrated their further intensification. Impaired proliferation and reduced vascularisation in the region of pocks induced by quadruple mutants were revealed by light microscopy. Electron microscopic comparison of the cells infected with the quadruple mutants and the wild type CPXV-5 demonstrated that reproduction of the mutants resulted in a decrease of the number of mature virions formed; moreover, in many cells the process was arrested at the stage of assembly of immature virions.

A similar gene dose dependent effect was observed in experiments on the LD_{50} evaluation in a well-known CXPV laboratory model – BALB/c mice [12]. As is evident from Table 2 the virulence decreased with the number of *kelch*-like genes deleted. The most significant difference was registered in quadruple mutants. The dynamics of the body weight loss observed in the mice also confirmed the weakening of the pathogenic properties of quadruple mutants.

In summary, our studies demonstrated that a deletion of *kelch*-like genes caused a change of the sensitive cell's host range; a decrease of the viral yield in some cell types and in the pocks induced on chicken embryo CAM; a decrease of the size and morphological changes of the pocks; and a decrease of virulence in BALB/c mice.

Acknowledgements

We are grateful to Drs. P.F. Safronov and S.V. Seregin for assistance in manuscript preparation; to Mr. O.S. Taranov for help in computer processing of electron microscopic images. This work was supported by the Russian Foundation for Basic Research, Grant No. 03-04-48432.

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