AGRICULTURAL BIOLOGY,
2015, V. 50, № 6, pp. 785-793
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

Causative agents of especially dangerous viral infections — molecular genetics, immunogenisity, cultivation

UDC 636.4:619:616.98:578:577.2.08:51-76

doi: 10.15389/agrobiology.2015.6.785rus
doi: 10.15389/agrobiology.2015.6.785eng

AFRICAN SWINE FEVER VIRUS GLYCOPROTEINS p54 AND CD2v IN THE CONTEXT OF IMMUNE RESPONSE MODULATION: BIOINFORMATIC ANALYSIS OF GENETIC VARIABILITY AND HETEROGENEITY

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Acknowledgements:
Supported by Russian Foundation for Basic Research, science project «Mol-a-ved» (15-34-20995).
Received June 30, 2015

Abstract

The African swine fever virus (ASFV) is a unique representative of Asfaviridae family, which still remains the sole member of genus *Asfarvirus*. ASF virus is the causative agent of one of the most dangerous diseases of the animals from *Suidae* family, and moreover, it is capable of infecting soft ticks of the genus *Ornithodoros*. Genetic and phenotypic heterogeneity of ASF virus is one of the main reasons for the lack of vaccines against this dangerous transboundary disease. In this work we present the analysis of structure and functions of the most variable glycoproteins ASFV p54 and CD2v using bioinformatics analysis and recombinant constructs expressed in mammalian cell cultures, the African green monkey cell culture COS-I and the human embryonal kidney cell culture HEK-293. The index of variability of amino acid sequences for P54 and CD2v proteins was calculated by Simpson's method. The CD2v protein has variable region (N-terminal domain), which is highly glycosylated (28-30 sites) and located in the outer surface of the cell membrane. This region also contains immunoglobulin domain (amino acids at positions 1-225), which is responsible for CD2v interaction with antibodies. The revealed differences in post-translational modifications and genetic variations of CD2v protein might explain the diversity of the hemadsorption phenomenon among ASF virus isolates. In contrast, p54 protein has variable glycosylated extracellular and intracellular parts. High level of differences in the nucleotide sequences of p54gene (*E183L*) for various ASFV isolates may be the result of random mutations during virus evolution. Characteristic antigenic properties of ASF virus isolates can obviously be due to found peculiar post-translational processing and genetic variations on CD2v protein. Herein we report the first bioinformatic analysis of post-traslation N- and O-glycosylation in most variable ASF virus proteins, p54 and CD2v. A transient expression of gene constructions used to obtain the recombinant products, p54-EGFP and CD2v-HA, allowed us to demonstrate the evidence for different localization of viral proteins p54-EGFP and CD2v-HA in the transfected cells. Particularly, the fluorescence caused by p54-EGFP was observed in the cytoplasm of the COS-I cells, transfected with recombinant plasmid p54-pEGFP-N1, whereas recombinant CD2v-HA protein was detected only in cell membrane. According to immunoblotting analysis, the CD2v molecular weight was 90 kDa against calculated 65 kDa indicating about 30 % of carbohydrate component in this surface glycoprotein. Moreover, 25 kDa and 90 kDa CD2v molecules, the probable differently glycosylated forms, were revealed in immonoblotting test that is in line with other published data. Thus, bioinformatic analysis and in vitro studies using transient expression in COS-I и HEK-293 cell cultures have shown that protein CD2v is the most likely candidate to define the interaction of ASF virus with the virus-specific antibodies.

Keywords: African swine fever, glycoprotein, variability, glycosylation, transient expression, immunotypes.

The African swine fever virus (ASFV) still remains the only known member of family *Asfarviridae* [1-3]. It is the causative agent of one of the most dangerous diseases of the *Suidae* family representatives, and moreover, it is capable of infecting soft ticks of the genus *Ornithodoros* [4-6]. The virus ge-

nome is a double stranded DNA of 170 to 190000 bp depending on the strain [7, 8]. Analysis of the complete nucleotide sequence of BA71V avirulent isolate identified at least 151 genes [9]. The function and the role of some genome-encoded proteins in pathogenesis have been identified [10-16], but a significant number of them have not been fully characterized yet.

The viral particle, being very similar to iridoviruses in morphology [17, 18], is about 200 nm in diameter and consists of several concentric domains [19, 20]. About 3 5% of the virion weight is made up by its major p72 capsid protein. The p150, p37, p34, and p14 structural proteins (their predecessor is polyprotein P220) account for up to 25 % of the particle weight [21, 22]. Of all known ASF viral proteins, glycoproteins ASF 54 and CD2v localized on the viral particle surface are the most variable ones. The p54 protein (encoded by *E183L* gene) contains a potential transmembrane domain close to the N-terminal region. This protein is involved in the early stages of viral infection and is responsible for attaching the ASF virus to the target cell. The molecular weight of the polypeptide part of p54 protein ranged from 24 to 28 kDa in various viral isolates [23]. CD2v protein determines the haemadsorbing properties of the virus and is encoded by the *EP402R* gene. CD2v is unimportant for virus replication in cell cultures [24, 25]. In natural isolates incapable of haemadsorbtion, the changes have been found in the *EP402R* sequence corresponding to the CD2v signal part which cause a shift in the reading frame. CD2v is a transmembrane protein with an N-terminal signal peptide and a transmembrane domain similar to the host cell CD2 extracellular protein domain [24, 26, 27].

Despite their functional differences, both glycoproteins (p54 and CD2v), according to some authors [24, 28], are responsible for the phenotypic characteristics of the ASF virus, showing the properties of serotype-specific antigens. Determination of isolate serotypes is extremely important for the study of ASF virus biology and in specific prevention against this disease.

In this study, we first analyzed the post-translational modification of the most variable ASF virus glycoproteins p54 and CD2v. The p54-EGFP and CD2v-HA recombinant chimeric proteins were produced in mammalian cell cultures by transient expression and their cellular localization was shown.

The purpose of this work was to analyze the structure and role of the most variable p54 and CD2v ASFV glycoproteins based on bioinformatic analysis, functional genomics, molecular cloning, and on recombinant construct expression.

Technique. African swine fever virus (ASFV) p54 and CD2v nucleotide sequences were obtained from the GenBank international database (the National Center for Biotechnology Information — NCBI): NC_001659.2, GQ410768.1, GQ410771.1, GQ410767.1, KC610537.1, KC610538.1, KJ671542.1, KC610535.1, KC610532.1, KF303309.1, KF303306.1, KC990883.1, EU620682.1, FR682468.1, FN557520.1, AM712240.1, AF481876.1, DQ026270.1, DQ026267.1, KM262845.1, KM262844.1, DQ026268.1.

Analysis of variable gene regions, domains, and p54 and CD2v protein structure was performed using the http://www.cbs.dtu.dk/services/ service. To determine N- and O-glycosylation level, the http://www.expasy.org/proteomics service was used, the amino acid sequence variability was analysed using the the http://imed.med.ucm.es/PVS/ service [29, 30].

When designing primers, the nucleotide sequence of strain Georgia 2007/wb ASF virus DNA (FR682468.1) was used as reference. Primers flanking the full-length copies of p54 and CD2v protein viral genes were constructed using the SerialCloner v. 2.6.1 (F. Perez, 2004), Oligo v. 6.71 (P. Rychlik, 2005) software. Specific hexameric sequences of restriction sites of endonuclease SacI,

EcoRI for p54 and BglII and EcoRI for CD2v were integrated in the oligonucleotide primers.

The p54 and CD2v nucleotide sequences were synthesized in polymerase chain reaction (PCR) using One Taq Hot start master mix (New England Biolabs, USA) and a Maxygene thermal cycler instrument (Axygen Scientific Inc., USA) according to manufacturer's recommendations. The amplification program included 30 sec initial denaturation at 94 °C and 40 PCR cycles (denaturation for 30 sec at 94 °C, primer annealing for 30 sec at 55 °C, elongation for 2 min at 72 °C). Target DNA was isolated from strain of Stavropol-08/01 ASF virus (obtained from the collection of the All-Russian Research Institute of Veterinary Virology and Microbiology) using QIAamp DNA Mini Kit (Qiagen N.V., Germany) according to manufacturer's instructions. Amplicons were separated by electrophoresis in 1.5 % agarose gel with 0.001 % ethidium bromide at a current of 50 mA. To purify the PCR products from agarose gel, QIAquick Gel Extraction Kit (Qiagen N.V., Germany) was used.

At the intermediate stage of cloning, PCR products were integrated into the pGEM-T-easy vector with ampicillin resistance marker (Pro-mega, USA) and specific endonuclease cleavage sites using the T_4 phage DNA ligase (NEB, USA). Transformation of *Escherichia coli* Dh5 cells by the obtained constructs was performed by electroporation using a Gene Pulser Xcell instrument (Bio-Rad, USA). Plasmid DNA was isolated from selected ampicillin resistant transformants using QIAGEN Plasmid Mini Kit (Qiagen N.V., Germany). The presence of specific recombinant plasmids inserts was confirmed by PCR using gene specific primers with checking its identity by restriction sites sequencing using an Applied Biosystems 3130xl genetic analyser (Applied Biosystems, USA). The presence of specific restriction sites in the constructed recombinant plasmid was also confirmed using SacI and EcoRI endonucleases in case of p54 gene cloning, and BglII and EcoRI in the case of CD2v gene cloning.

To control the functional integrity of the cloned target viral genome sites and to produce genetically engineered constructs allowing the expression of p54 and CD2v proteins in mammalian cells, the coding sequences of *E183L* (p54) and *EP402L* (CD2v) genes from recombinant pGEM-T-easy plasmids, where they were under the control of bacteriophage T_7 promoter, were recloned sequentially for the above restriction sites in acceptor vectors with a cytomegalovirus promoter. For p54, the pEGFP-N1 plasmid (Clontech, USA) with a kanamycin resistance gene and *eGFP* (enhanced green fluorescence protein) encoding a fluorescent marker protein, was this vector. pCMV-HA-C vector (Clontech, USA) with ampicillin resistance gene containing a short (29 nucleotide) sequence which encodes an influenza virus immunogenic portion of hemagglutinin (HA) was selected for CD2v. DNA of produced plasmid constructs was purified in ion exchange columns using QIAGEN Plasmid Maxi Kit (Qiagen N.V., Germany).

Transfection of African green monkey (COS-I) and human embryonic kidney (HEK-293) cell cultures with purified recombinant plasmids (2.5 μg per well) was performed in 6-well plates using Lipofectamine (Invitrogen, USA) according to manufacturer's instructions [31].

Then, 24, 48, 72 and 96 hours after transfection, cells were screened for the presence of reporter fluorescence using an Olympus MIT-2 (Japan) inverted fluorescence microscope; the cells were collected with a scraper and lysed in TBS buffer (0.1 % Tween 20) supplemented with Protease Inhibitor Cocktail Set V (Calbiochem, USA). To confirm the presence of recombinant proteins, the immunoblotting [32] was used with monoclonal GFP (FL) antibodies (Santa Crus Biotechnology, USA) against GFP reporter protein and with HA-

probe monoclonal antibodies (F-7) (Santa Crus Biotechnology, US) against the marker HA portion and secondary antispecies monoclonal antibodies labeled with horseradish peroxidase. Clarity Western ECL Substrate (Bio-Rad, USA), and DAB (Thermo Scientific, USA) kits were used as the substrates for detection, respectively.

Results. The Simpson method based on the estimation of the diversity index of amino acid sequence proportions was used for the analysis of variable gene regions. This index describes the probability of similarity of the two sequences with substitutions selected at random to other amino acid sequences. This index values are from 0 to 1, and the greater the sample variety, the greater index value is.

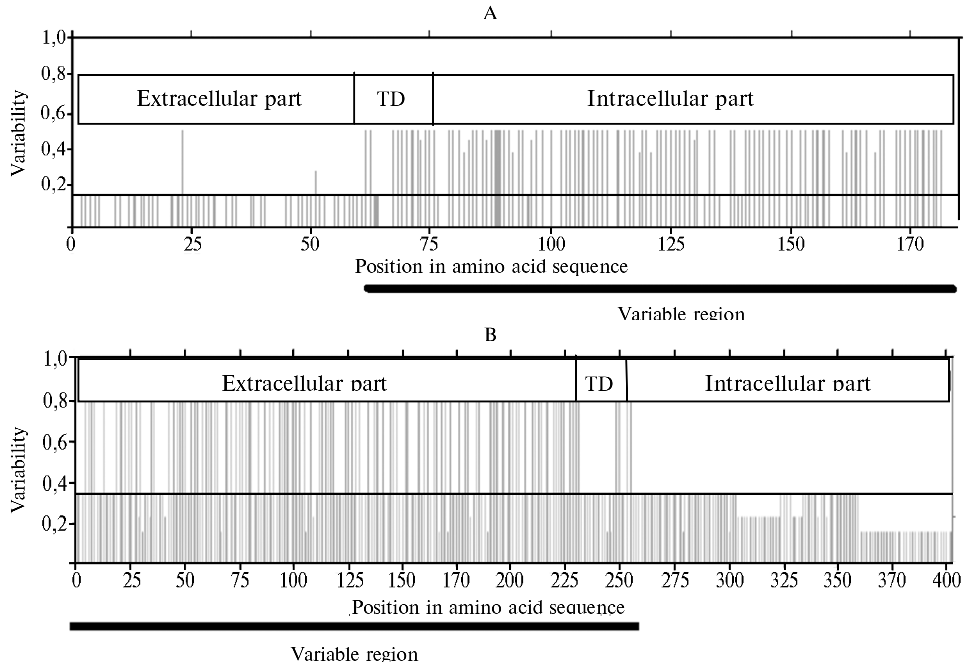

Fig. 1. Results of p54 (A) **and CD2v** (B) **capsid glycoprotein variability profile analysis in various strains of African swine fever** (ASF) **virus according to Simpson method:** TD — transmembrane domain. The regions for which the resulting values exceed the threshold (corresponding to the horizontal line) are considered variable. Amino acid sequences were obtained by the translation of nucleotide sequences of genes deposited in the GenBank (a total of 22 ASF virus isolates analyzed).

Amino acid sequences were estimated based on the gene nucleotide sequences deposited in the GenBank. Analysis of p54 (*E183L*) protein amino acid sequences in various isolates (n = 22) demonstrated that its C-terminal region which is localized on the inner side of the cell membrane is mostly variable (amino acids in positions 62-185) (Fig. 1, A). This region consists of tandem repeats of the four amino acids (Ile-Ile-Ile-Ile, Ala-Ala-Ala-Ala). The protein portion passing through the cell membrane, a transmembrane domain (amino acids 25-45), is localized close to the N-terminal region. CD2v (*EP402L*) protein has a variable N-terminal region localized on the outer side of the cell membrane (amino acids 1-225) (Fig. 1, B), and is fixed in the membrane by the transmembrane domain. According to the heterogeneity profiles, CD2v was more variable than p54.

Analysis of amino acid sequences for the presence of N- and O-glycosylation sites showed that p54 protein was characterized by the predominance of O-glycosylation, and the majority of potential glycosylation sites (18 sites) are localized at the C-end. In CD2v protein, N-glycosylation was more pronounced

predominantly at the N-terminal region (28-30 sites) (Fig. 2).

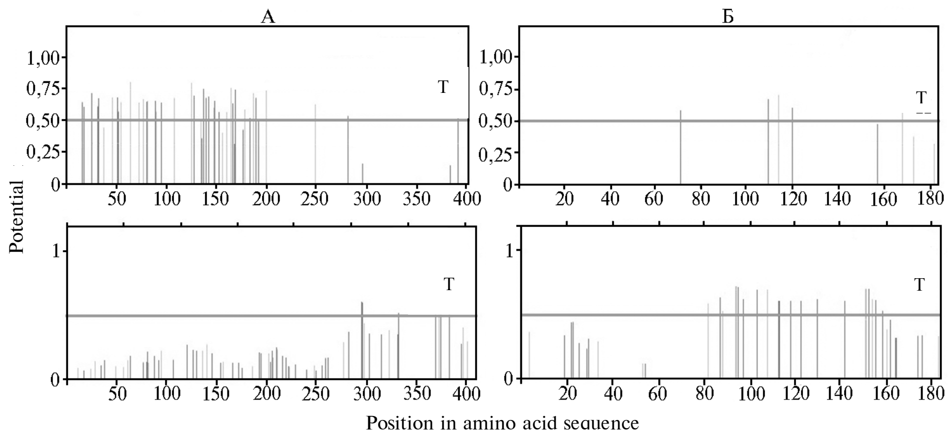

Fig. 2. Analysis of potential N- (top row) **and the O-glycosylation** (bottom row) **sites distribution in African swine fever virus CD2v** (A) **and p54** (B) **capsid glycoproteins.** The intersection of the horizontal threshold line (T) means that glycosylation is most likely in the relevant amino acid positions. N- and O- protein glycosylation was predicted based on the analysis of amino acid sequences and the presence of certain amino acid combinations potentially relevant of glycosylation sites. A totsl of 28-30 N-glycosylation sites and 5-7 O-glycosylation sites for CD2v, and 5 N-glycosylation sites and 18 O-glycosylation sites for p54 were identified.

It is noteworthy that the *EP402L* gene (CD2v) responsible for haemadsorbtion was identified in non-hemadsording strains as well [14, 24], but the open reading frame was violated in them. We have shown that the number of potential CD2v glycosylation sites did not differ in haemadsorbing and not capable of hemadsorbtion (L-50, LTsPP, Kongo-49, Mfuati-79, Mozambique-78, France-32, PPA, the PSA-1/NH, Lee, Uganda, TC7, Rodeziya-79) ASF virus strains. We managed to find that the signal peptide and the signal peptide cutting site were absent in the CD2v protein amino acid sequence in all non-hemadsorbing ASF virus strains. Therefore, CD2v is not glycosylated even in the presence of relevant potential sites.

Thus, the bioinformatic analysis led to the conclusion that the CD2v molecule variable region (N-terminal region) is heavily glycosylated and is localized on the outer surface of the cell membrane. This area contains an immunoglobulin domain as well, which is involved in the interaction of CD2v with antibodies. The differences found in post-translational modifications and genetic variations for CD2v protein may explain the diversity of antigenic properties of ASF virus isolates. In contrast, p54 protein has a variable region localized on the inner side of the cell membrane, and a glycosylated region located outside the cell. A considerable dissimilarity in the nucleotide sequences of the p54 gene (*E183L*) in various ASF virus isolates may be due to mutational variability during evolution.

The cultured COS-I and HEK-293 cells that carried the constructs derived from pEGFP-N1 and pCMV-HA-C plasmid vectors with cytomegalovirus (CMV) promoters and capable of being expressed in mammalian cells in extrachromosomal state were the producers of ASFVP recombinant p54 and CD2v proteins. The open p54 protein reading frame was linked to the reporter *eGFP* gene included in the acceptor vector. Target protein merging with EGFP makes it possible to detect the cellular localization of chimeric protein by fluorescence microscopy. To avoid structural changes in the case CD2v protein, the strategy has been selected for merging with the marker HA site in the molecule C-terminal region. HA sequences at the C-end of the recombinant protein does

not affect the immunological properties and makes it possible to detect the target protein in the native conformation, which is especially important in the presence of a considerable carbohydrate portion in the glycoprotein.

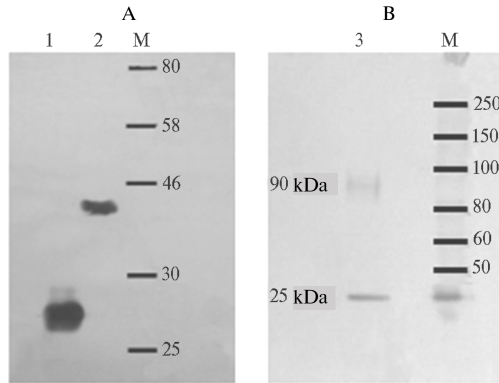

Fig. 3. Detection of African swine fever virus recombinant p54-EGFP (A) **and CD2v-HA** (B) **capsid proteins in cell culture lysates by immunoblotting:** 1 — EGFP (green fluorescent protein), 2 — p54-EGFP, 3 — CD2v-HA; M — molecular weight markers for p54-EGFP (Unstained Protein Ladder, Broad Range 10-250 kDa; NEB, USA) and CD2v-HA (Prestained Protein Marker, Broad Range 7-175 kDa; NEB, USA). COS-I HEK293 cell line cultures transfected with p54-pEGFP-N1 (A) and pCMV-CD2v (B) recombinant plasmids, respectively, with lipofectamine, were used. Monoclonal antibodies are conjugated with horseradish peroxidase; Clarity Western ECL Substrate (Bio-Rad, USA) and DAB (Thermo Scientific, USA) were used as the substrates for detection; 25 kDa and 90 kDa (B) bands are nonglycosylated and glycosylated CD2V-HA protein forms, respectively.

The p54-EGFP reporter fluorescence was observed in the cytoplasm of transfected COS-1 cells for 96 hours. The strongest signal was observed in the period from 24 and 48 hours after transfection, then (from 72 to 96 hours) it decreased. By contrast, recombinant CD2v expression was detected in cell membranes only. The p54 and CD2v recombinant proteins were also detected in lysates of transfected cells by immunoblotting with monoclonal antibodies against the GFP green fluorescent protein and immunogenic portion of influenza virus hemagglutinin (HA) (Fig. 3). Two isoforms of CD2v recombinant protein were present in transfected cells (Fig. 3), of which the high molecular weight (90 kDa) isoform corresponded to mature CD2v glycoprotein, while low molecular weight (25 kDa) isoform was not completely glycosylated.

Thus, the p54 and CD2v African swine fever (ASF) virus glycoproteins have pronounced structural and functional features, but their role in the formation of this pathogen immunotypes has not yet been revealed. Of all ASF virus structural proteins, p54 and CD2v are the most variable ones and are of interest for the study of its genetic and phenotypic variability in relation to its capability of inducing hemadsorbtion. In CD2v protein, N-glycosylation of the extracellular portion (N-terminal region) is predominant, while C-terminal region O-glycosylation is characteristic of p54. The CD2v variable portion is also at the N-terminal region and contains the immunoglobulin domain, which may indicate a relationship between glycosylation and the nature of the virus-antibody interaction. At the same, this study demonstrated that the lack of hemadsorbtion phenomenon in some ASF virus strains is associated with a change in the reading frame of the *EP402L* gene encoding CD2v, and does not depend on its glycosylation level. Fluorescence of labeled recombinant p54-EGFP protein was observed in the cytoplasm of COS-I cells transfected with recombinant p54-pEGFP-N1 plasmid. CD2v-HA protein was detected only in cell membranes. According to immunoblotting data, the CD2v molecular weight was 90 kDa (against calculated 65 kDa). Therefore, the carbohydrate component makes up about 30 % of this ASF virion surface glycoprotein. The 25 kDa and 90 kDa CD2v molecules, the probable differently glycosylated forms, were revealed in immonoblotting test that is consistent with other published data. Based on bioinformatic analysis and the studies of COS-I и HEK-293 transfected cells, CD2v

is the most likely candidate to define the interaction with the virus specific antibodies and formation of immune types for ASF in animals.

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