GAG-binding variants of tick-borne encephalitis virus


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A B S T R A C T

Previously different authors described various flavivirus mutants with high affinity to cell glycosaminoglycans and low neuroinvasiveness in mice that were obtained consequently passages in cell cultures or in ticks. In present study the analysis of TBEV isolates has shown existence of GAG-binding variants in natural virus population. Affinity to GAG has been evaluated by sorption on heparin-Sepharose. GAG-binding phenotype corresponds to such virus properties, like small plaque phenotype in PEK cells, absence of hemagglutination at pH 6.4, and low neuroinvasiveness in mice. Mutations increasing charge of E protein were necessary but not sufficient for acquisition of GAG-binding phenotype. Molecular modeling and molecular dynamics simulation have shown that the flexibility of E protein molecule could bear influence on the phenotypic manifestation of substitutions increasing charge of the virions.

Introduction

For wide host range viruses, like arboviruses, the first stages of virus–cell interaction remain unclear. It is obvious, that there are at least three possibilities for such viruses: to use as receptors common molecules represented on various cell types, to use distinct receptors on different cell types or to entry cell by receptor-independent mechanism.

Glycosaminoglycans (GAGs) are polydispense mixture of linear polysaccharides consisting primarily of N-acetylated and N-sulphated disaccharides arranged mainly in segregated negatively charged domains. GAGs could be divided into: hyaluronan, chondroitin sulfate and dermatan sulfate, heparan sulfate and heparin, and keratan sulfate (Alberts et al., 2008). Heparan sulphate is presented on the cell surface in the form of heparan sulphate proteoglycans (HSPGs) of syndecan family (Gallagher et al., 1992). All ever studied cells (excluding B-stem cells), and organs express at least one syndecan family member (Kim et al., 1994). It has been shown previously that heparan sulfate GAGs serve as a receptor for various viruses (reviewed in Schneider-Schaullies, 2000). GAGs can serve as a virion concentration initial attachment molecule on the cell surface (Haywood, 1994).

In most cases HSPGs are not the unique receptors involved in viral attachment and entry processes, and virus is still able to infect cells deficient in GAG production or after GAGs removal from the cellular membrane (Byrnes and Griffin, 1998; Kroschewski et al., 2003; Martínez-Barragán and del Angel, 2001). However, in some cases switch of the receptor molecule (from ICAM-1 to GAG) could change the course of infection (Khan et al., 2007). Laboratory obtained variants with increased affinity to GAGs were shown for several viruses (Bernard et al., 2000; Heil et al., 2001; Hulst et al., 2000; Klimstra et al., 1998; Sa-Carvalho et al., 1997). These variants were attenuated for various animals.

Flavivirus genus gathers arthropod-transmitted viruses with virion surrounded by lipid bilayer, which contains multiple copies of proteins E and M, but only E protein forms outer surface. Thus, E glycoprotein serves for viral attachment, entry and other stages of viral penetration into the cell; besides, it’s the main target for neutralizing antibodies (Lindebach et al., 2007). E protein includes ectodomain (sE) that is connected to the membrane by stem-anchor region. The structure of ectodomain was first solved by Rey and co-authors (1995) for tick-borne encephalitis virus (TBEV) and then were obtained structures for other genus members (Zhang et al., 2003, 2004; Nybakken et al., 2006; Volk et al., 2006). The sE consists of 3 distinct domains: central structural domain I, domain II involved into the process of pH-dependent membrane fusion, and receptor attachment domain III (Rey et al., 1995).
Flaviviruses, like West Nile virus (WNV) and Japanese encephalitis virus (JEV), incorporate into the target cell by clathrin-mediated endocytosis (Chu and Ng, 2004a; Nawa et al., 2003). A lot of molecules were described as receptors for flaviviruses: integrins (Chu and Ng, 2004b; Protopopova et al., 1997), 37/67-kD high affinity laminin receptor (Protopopova et al., 1997; Thepparit and Smith, 2004), ICAMs (Navarro-Sanchez et al., 2003; Tassaneentrithep et al., 2003), GAGs (Goto et al., 2003; Kroschewski et al., 2003; Lee and Lobigs, 2000; Mandl et al., 2001; Martinez-Barragán and del Angel, 2001), etc.

It has been shown on TBEV that flaviviruses have 2 types of receptors on the cell surface: high- and low-affinity (Maldov et al., 1992). The role of each receptor type remains unclear, but GAGs seem to be a widespread low-affinity flavivirus receptor.

GAG-binding variants were described for various Flavivirus genus members: Yellow fever virus (YFV), Murray valley encephalitis virus (MVEV), WNV, JEV, and TBEV. These variants were obtained during laboratory passages in cell lines: BHK-21 (Goto et al., 2003; Mandl et al., 2001), SW-13 (Lee and Lobigs, 2002; Lee et al., 2004), Neuro-2 (Chiou and Chen, 2007), or in ticks (Romanova et al., 2007). Previously, about 2 dozen of increasing charge substitutions in all 3 domains of soluble part of E protein molecules of TBEV variants, obtained during cell line passages, leading to the appearance of GAG-binding phenotype and specific properties, were identified (Mandl et al., 2001). The main properties were small plaque phenotype in porcine kidney cell lines and low neuroinvasiveness for laboratory mice. The real impact of such mutations in the E protein structural changes has remained unknown. The existence of such variants in natural population of flaviviruses also remains unclear. TBEV strains with mutations increasing surface charge of the virion were deposited earlier (Ecker et al., 1999; Khassatinov et al., 2005; Lu et al., 2008), but their affinity to cellular GAGs and other characteristics have not been studied properly. It is natural to think that the presence of such mutations leads to appearance of GAG-binding phenotype.

During the present work we tried to solve two main problems: to describe affinity to GAGs of natural TBEV isolates and to elucidate whether every mutation increasing charge of E protein leads to appearance of GAG-binding phenotype. First, we analyzed properties of 13 pre-existing collection strains isolated in various parts of Russia and Baltics, including sequences of E protein, plaque phenotype, binding to heparin-Sepharose (HS), etc. Viral characteristics corresponded with increased sorption on heparin-Sepharose–GAG-binding phenotype—have been identified. It has been shown that GAG-binding variants with complex of specific properties do exist among field isolates. Second, simulation of E proteins dynamics of 3 viruses has shown that the phenotypic manifestation of substitution increasing charge of E protein molecule depends on amino acid context, and not every such mutation is sufficient for the appearance of the GAG-binding phenotype. Furthermore, we have shown that structural inflexibility of E protein molecule is highly involved in appearance of GAG-binding phenotype and complex of relevant properties.

### Results

#### Plaque phenotype of studied TBEV strains

Previously, it was shown that one of the features of GAG-binding variants was small plaque phenotype in porcine kidney cell lines (Mandl et al., 2001; Romanova et al., 2007). First we described plaque size in PEK cells under agar overlay on the 8th day of infection for all collection strains (Table 1). All results are summarized in Table 2.

| Strains Absettarov, 256, LK-138, YuK 4/13, SofjinKGG, 205KGG, DV 936k, EK-328, Lesopark, formed large plaques. Strain 80k formed small plaques, less than 1mm in diameter. Strain PK-36 formed plaques of middle size, 3–6 mm. The large and middle plaques appeared on 3–4 days p.i. and reached 7–10 mm in size by the 7–8 days of infection. In contrast, small plaques appeared only on days 6–7, and practically did not increase in size during the period of observation.

| Strain Ya10/89 formed plaques heterogeneous in size. Possibly, this was due to mixing of different viruses from 10 different ticks prepared in one single isolate or true heterogeneity of the isolate. For following investigations we isolated from plaques clones 115 and 125 with plaque sizes of 8 and 1 mm in diameter, respectively.

| Strain Absettarov formed plaques from 8 to 10 mm in diameter. From this population was plaque-puriﬁed clone 18A. The clone 18A showed plaques a bit smaller than parental strain ones: 7.3 ± 0.7 mm vs. 9.5 ± 1 mm (p<0.0001).

#### Analysis of TBEV strains and clones sorption on heparin-Sepharose

Small plaque size could be explained by several features, including virus inability to reproduce efﬁciently in the cell culture (low rate or...
level of reproduction), virus susceptibility to cell produced IFN, virus susceptibility to heparan sulphate GAGs we used common model of the process—binding to heparin-conjugated Sepharose beads. Virus was incubated with HS and S beads and the amount of unsorbed virus was determined by plaque assay, then the ratio HS-sorbed to control S-unsorbed virus was calculated.

Both small plaque forming TBEV variants (80k and Ya10/89 clone 125) showed significantly increased affinity to HS (over 90% of sorbed virions) (Table 2 and Fig. 1) in comparison to large plaque strains (0–25%). Thus, all small plaque virus variants had GAG-binding phenotype. Strain PK-36 with middle plaque size had middle HS sorption activity (about 60%).

Table 2
Biological, immunochemical and virological properties of TBEV strains.

<table>
<thead>
<tr>
<th>TBEV strain/clone</th>
<th>Plaque size (mm)</th>
<th>Virions bound to HS</th>
<th>Number of experiments</th>
<th>Sorption in RIEd</th>
<th>Cathode precipitate in RIE</th>
<th>Amino acid substitution in E protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absettarov</td>
<td>9.5 ± 1.0</td>
<td>2</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>Asp62 → Gly</td>
</tr>
<tr>
<td>Absettarov 18A</td>
<td>7.3 ± 0.7</td>
<td>4</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>Gly122 → Glu</td>
</tr>
<tr>
<td>256</td>
<td>8.3 ± 0.9</td>
<td>3</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>Asp62 → Gly</td>
</tr>
<tr>
<td>LK-138</td>
<td>9.2 ± 0.8</td>
<td>1</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>EK-328</td>
<td>7.0 ± 0.5</td>
<td>3</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>Lesopark</td>
<td>8.0 ± 1.5</td>
<td>3</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>YuK4/13</td>
<td>11.3 ± 1.8</td>
<td>1</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>Ya10/89</td>
<td>1.0±2.5</td>
<td>nd</td>
<td>nd</td>
<td>+</td>
<td>−</td>
<td>nd</td>
</tr>
<tr>
<td>(45:1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ya10/89 c.115</td>
<td>8.0 ± 0.5</td>
<td>1</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>Ya10/89 c.125</td>
<td>1.0 ± 0.5</td>
<td>1</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>Glu122 → Gly</td>
</tr>
<tr>
<td>SoljinKGG</td>
<td>8.0 ± 0.9</td>
<td>2</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>Gly122 → Glu</td>
</tr>
<tr>
<td>205KGG</td>
<td>9.0 ± 1.5</td>
<td>4</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>Asp62 → Gly</td>
</tr>
<tr>
<td>80k</td>
<td>1.0 ± 0.5</td>
<td>4</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>Thr91 → Ala</td>
</tr>
<tr>
<td>DV 936k</td>
<td>10.0 ± 0.5</td>
<td>1</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>Gly122 → Asp</td>
</tr>
<tr>
<td>PK-36</td>
<td>4.5 ± 2.0</td>
<td>1</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>Thr91 → Ala</td>
</tr>
</tbody>
</table>

a HS—heparin-Sepharose.

b +—sorption on HS is over 90%; −—sorption on HS is less than 70%.

c RIE—rocket immunoelectrophoresis; ‘+’—presence of cathode-pointed precipitate of virions with antibodies, ‘−’—absence of cathode-pointed precipitate of virions with antibodies.

d Mutation was marked in comparison with relevant genotype consensus, with parental strain Absettarov for Absettarov 18A, with Ya10/89 clone 115 for Ya10/89 clone 125, in comparison with Far-Eastern consensus for strain 80k; ‘−’ is used to mark the absence of such mutation.

e The ratio of small plaques (1mm) to bigger ones (2.5mm).

Rocket immunoelectrophoresis (RIE) behavior of studied TBEV strains and clones

Previously, TBEV virions behavior in RIE was described by Liapustin et al. (1987), Dzhivianian et al. (1991) and Romanova et al. (2007). In RIE buffer system TBEV virions have weak negative charge, and should remain still or slowly drift towards anode in electric field. Upon high endosmosis in agarose virions move towards the cathode and form cathode-pointed rocket of precipitate with antibodies—virion antigen. The oligomeric forms of secreted NS1 protein in culture fluid have strong negative charge and small size in comparison with the virion. So, in spite of endosmosis, NS1 forms an anode-pointed rocket—non-virion "soluble" antigen (Fig. 2).

For RIE experiments viruses were normalized by titer (6–7 log10 PFU) in infected cell culture fluid. RIE results are summarized in Table 2 and Fig. 2. Virions of all large and middle plaque strains formed cathode-pointed precipitate with antibodies in RIE. Virions of the small plaque strains did not produce such precipitate.

Increased affinity to agarose sulphopolysaccharide heads could explain the inability of virions of GAG-binding variants to form cathode-pointed rocket in RIE. Precipitation of virions by antibodies requires dense arrangement of virions at a distance comparable with the size of the immunoglobulin molecule, which could be achieved in the case of dense motion of virions towards the cathode in electric field. An increase of the charge of virions can promote their binding to agarose sulphogroups in the gel. In this case, virions would move not as a dense front, but as a diffuse smear, which would prevent a sufficient concentration of virions in any part of the gel, and subsequently avert formation of immunoprecipitate.

Hemagglutinating activity (HA) of studied TBEV strains and clones

Previously, described GAG-binding variants did not provide noticeable HA in pH 6.4 or the optimal pH was moved to pH 6.6.
(Goto et al., 2003; Romanova et al., 2007). We carried out hemagglutination of goose erythrocytes with 2-fold dilutions of infected cells supernatant aliquots in range pH 5.7–7.0 (pH 5.7, 6.0, 6.2, 6.4, 6.6, 6.8, 7.0). Results are summarized in Table 3.

Hemagglutinating activity of investigated TBEV strains.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Titer (lgPFU/ml)</th>
<th>Allowed of pH range</th>
<th>Optimal pH</th>
<th>Titer HA in optimal pH (lgPFU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absettarov</td>
<td>6.7</td>
<td>5.7–6.4</td>
<td>6.4</td>
<td>6</td>
</tr>
<tr>
<td>Absettarov 18A</td>
<td>7.2</td>
<td>5.7–6.4</td>
<td>6.0</td>
<td>5</td>
</tr>
<tr>
<td>256</td>
<td>5.2</td>
<td>5.7–6.8</td>
<td>6.0</td>
<td>5</td>
</tr>
<tr>
<td>LK-138</td>
<td>7.1</td>
<td>5.7–6.4</td>
<td>6.4</td>
<td>4</td>
</tr>
<tr>
<td>EK-328</td>
<td>7.8</td>
<td>5.7–7.0</td>
<td>6.4</td>
<td>9</td>
</tr>
<tr>
<td>Ya10/89*</td>
<td>6.9</td>
<td>nd</td>
<td>nd</td>
<td>5</td>
</tr>
<tr>
<td>Ya10/89 c.115</td>
<td>7.4</td>
<td>6.4–7.0</td>
<td>6.4</td>
<td>3</td>
</tr>
<tr>
<td>Ya10/89 c.125</td>
<td>7.5</td>
<td>7.0</td>
<td>7.0</td>
<td>1</td>
</tr>
<tr>
<td>SofjinKGG</td>
<td>6.3</td>
<td>6.2–6.6</td>
<td>6.4</td>
<td>10</td>
</tr>
<tr>
<td>205KGG</td>
<td>7.7</td>
<td>6.2–6.4</td>
<td>6.2–6.4</td>
<td>3</td>
</tr>
<tr>
<td>80k</td>
<td>6.9</td>
<td>nd</td>
<td>nd</td>
<td>–</td>
</tr>
<tr>
<td>DV 936k</td>
<td>8.1</td>
<td>6.2–7.0</td>
<td>6.4–6.8</td>
<td>3</td>
</tr>
<tr>
<td>PK-36</td>
<td>6.0</td>
<td>5.7–6.6</td>
<td>6.4</td>
<td>8</td>
</tr>
</tbody>
</table>

Titer of HA was determined in culture fluid of infected POK cells.

Table 3

Virulence of TBEV strains and clones in mice

Various authors showed that GAG-binding variants of flaviviruses had low neuroinvasiveness (Goto et al., 2003; Lee and Lobigs, 2002; Lee et al., 2004; Mandl et al., 2001; Romanova et al., 2007).

To evaluate the neuroinvasiveness BALB/c mice were chosen as susceptible to TBEV infection (Pletnev et al., 2000). To estimate the reliability of the neuroinvasiveness test differences for distinct viruses we had to know the variability of results from experiment to experiment. For this purpose we chose 2 of investigated viruses—strain Absettarov and strain EK-328 for detailed study and processed obtained results statistically. For strain Absettarov series of 16 independent experiments was provided. LD50 after i.p. inoculation was 0.4±0.2 log10PFU (SD=1.0 log10PFU). For strain EK-328 were obtained results statistically. For strain Absettarov series of 16 independent experiments was provided. LD50 after i.p. inoculation was 0.5 log10PFU (SD=0.8 log10PFU). We decided to use 0.5 log10PFU as standard error for all other obtained results.

Neuroinvasiveness on BALB/c mice differed a lot among studied TBEV strains from —2 log10PFU for strain 256 to 3.8 log10PFU for strain 80k (Table 4A). Neuroinvasiveness of both small plaque GAG-binding variants—80k and Ya10/89 clone 125 was significantly lower in comparison with other strains. The difference was the most visible in comparison of viruses of one subtype, like 80k vs. SofjinKGG; Ya10/89 clone 125 vs. clone 115, strains EK-328 and Lesopark. The neuroinvasiveness of clone Absettarov 18A was lower than the parental strain one (Absettarov 18A vs. Absettarov).

Low neuroinvasiveness could be due to inability of virus to invade CNS and/or to kill CNS cells. To elucidate this we obtained i.p. and i.c. values for several strains on outbred mice less susceptible for TBEV than BALB/c mice (Table 4B). Strains significantly differed in neurovirulence from —2.7 log10PFU for strain Absettarov to 0.7 log10PFU for strain EK-328. Neuroinvasiveness results in outbred mice corresponded to the ones in the BALB/c mice. Strain 80k and clone Absettarov 18A were the least virulent for outbred mice upon peripheral inoculation than other investigated strains. Clone 18A virulence upon i.c. and i.p inoculation was 5000-fold and 4000-fold lower than parental strain ones, respectively. Thus, clone 18A neurovirulence and neuroinvasiveness both decreased on the same level in comparison to the parental strain. Strain 80k neuroinvasiveness was 40-fold lower than the one of strain SofjinKGG, and neurovirulence was only 16-fold lower than the one of SofjinKGG.

Sequencing of E protein genes of TBEV strains and clones

We obtained nucleotide sequences of part of viral genome encoding E glycoprotein (or sE part of the glycoprotein) of collection TBEV variants. Using these sequences we analyzed amino acid substitutions leading to increase of the surface charge of the E protein molecule in comparison with consensus sequence of the relevant genotype.

Investigated TBEV strains represented all three subtypes: European (Absettarov, 256, LK138), Siberian (EK-328, Lesopark, YuK4/13, clones of Ya10/89), and Far-Eastern (SofjinKGG, 80k, 205KGG, DV936k, PK-36).

Both GAG-binding viruses showed amino acid substitutions increasing surface charge of the E protein molecule (Table 2). Strain 80k had Asp77→Asn, in comparison to the consensus of Far-Eastern genotype. Besides, strain 80k carried additional mutation in Thr95→Ala. Small plaque clone 125 of strain Ya10/89 carried Glu122→Gly in comparison with clone 115 and Siberian genotype consensus. In addition, sequencing revealed 2 nt synonymous substitutions in 1600 nt between clones of strain Ya10/98. Moreover, we found out the clone Absettarov 18A surprisingly carried Asp97→Gly mutation, similar to strain 80k, in comparison to the parental strain.

All other TBEV variants didn’t show any charge increasing substitutions in comparison to the genotype’s consensus sequences.
Homology modeling and molecular dynamics

Finally, we decided to elucidate why clone 18A of strain Absettarov with substitution increasing charge of the E protein molecule hadn’t changed its GAG affinity. To solve the problem we chose for further investigations viruses Absettarov 18A, carrying charge-increasing substitution in 67 amino acid position of E protein that didn’t affect the virus properties, strain 80k, as GAG-binding variant carrying analog substitution in the same position, and strain SofjinKGG, as a strain of Far-Eastern subtype.

We built models of soluble domain of E protein molecule for Absettarov 18A, SofjinKGG and 80k on the basis of strain Neudorl (PDB access code 1SVB). The difference between the template and target proteins was subtle (Fig. 3); no insertions or deletions occurred in the alignment and only local substitutions were present. 12 substitutions differed in Western and Far-Eastern genotype strains (47, 88, 115, 120, 178, 206, 260, 267, 277, 317, 331, 363 residues). Strain Absettarov differed from strain Neudorl in only 1 amino acid in position 167 (Ile to Val), and clone 18A differed from parental strain in position 67 (meant above), 2 amino acids in addition to 2 mutations marked above differed strain 80k from strain SofjinKGG (Thr4 → Ile, Ala153 → Val). Although, the main difference between strains occurred around position 67. Strains Neudorl and SofjinKGG contained Asp67, clone 18A carried Gly67, and strain 80k had Asn67, and additionally Thr86 → Ala.

The charge distribution for the mean structure of modeled proteins is shown in Fig. 4A. One could easily see that the surface charge distribution for the protein E of all strains was very similar. It was obvious for the strains 80k and Absettarov 18A due to the character of the amino acid substitutions, but for SofjinKGG such observation was less expected. Namely, net charge of the protein from strains Absettarov 18A and 80k equaled −2, whereas for the protein from strain SofjinKGG it was equal to −4. The negative charge of the latter protein was located mainly at the membrane side of the protein (data not shown). Although the ridge formed by amino acid side chains exposed on the external surface across 2 subunits of the E protein dimer slightly differed on the surface charge distribution map. These amino acids could be possibly involved into electrostatic interactions with molecules on cellular membrane, like GAGs.

Nevertheless, static representation of the proteins obtained by modeling was not sufficient to explain the differences in properties of Absettarov 18A and strain 80k.

We collected 10 ns trajectories for TBEV variants under consideration—Absettarov 18A, SofjinKGG and 80k. Principal component analysis revealed two main components of the protein movement during molecular dynamics simulation: (1) twist around the longest dimer axis I, and (2) bending around the axis II (Fig. 4B). For all cases the movement of the protein could be characterized by its bending. According to this observation, we had chosen the bending angle (b.a.) instead of root-mean-square distance (RMSD) as the main variable describing the conformation of the protein during dynamics. We had also built correlation maps with the aim to understand the possible correlated motions of the protein parts.

The simulation started from the planar conformation (b.a. ca. 180°) corresponded to the crystal structure of the template, and then during the first nanosecond bending was initiated by the thermal motion of the molecule. The bending angle dependence on time is presented in Fig. 4C. There were no significant differences between the strains which could illuminate the distinction between the HS-binding properties of three proteins.

We had built correlation maps (Fig. 4D) with the aim to describe correlated motions of the proteins. These maps were rather similar for all the proteins, but notable differences did exist. The domains showed strongly correlated movements inside the domain and negative correlation between domain II (residues 52–136 and 190–284) and other domains. The movement of domain I (residues 1–51, 137–189 and 285–302) possessed moderate correlation with the movement of domain III (residues 303–395). For clone Absettarov 18A and strain SofjinKGG a stronger correlation was observed inside the domain II compared to the strain 80k. The movement of domain I was more distorted for strain 80k, with regions of correlation and anti-correlation being more noticeable than for two other strains, especially in the region of residues 137–189.

Summarizing our simulation studies, we could say that the dynamic behavior of the strain 80k E protein differed from that of the clone Absettarov 18A and strain SofjinKGG. The difference was rather small due to very high identity of amino acid sequences of these proteins, but it was marked enough to be a working hypothesis for explanation of the experimental results.

Discussion

Interest for GAG-binding variants derives from several facts: (1) one amino acid substitution corresponds to complex of peculiarities typical for GAG-binding phenotype; (2) there could be several sites on the one protein responsible for GAG-binding phenotype appearance, and position of the mutation could influence the intensity of display of obtained variant properties; (3) change of affinity to GAGs could modulate virus cell tropism; (4) low neuroinvasiveness; (5) high potential for emergence of genetic variability for virulent variants, as set of various mutations compensating increased charge of E protein and restoring virions low affinity to cellular GAGs and, thus, increasing virulence, could appear. The existence of such variants for wide range of viruses indicates that different viruses could use the same mechanism to change population properties.

Previously, laboratory obtained GAG-binding variants were described for various flaviviruses, including TBEV (Chiou and Chen, 2007; Goto et al., 2003; Lee and Lobigs, 2002; Lee et al., 2004; Mandl et al., 2001; Romanova et al., 2007). During the present study we monitored 13 collection TBEV strains and found two containing GAG-binding variants. Therefore, we have provided evidence that GAG-binding variants do exist in natural TBEV population using the virus (clone 125 of Ya10/89) with short laboratory passage history and strain (80k), and could be isolated from different origins, like tick suspension or blood of patient with acute TBE. In the present work on natural strains we have supported our data obtained earlier for laboratory virus variants, that GAG-binding phenotype, i.e. increased sorption on HS, corresponded to such virus properties, like small plaques in PEK cells, absence of HA activity at pH 6.4 and cathode-pointed precipitate of virions with antibodies in RIE, low neuroinvasiveness in mice (Romanova et al., 2007). In the case the properties like HS-sorption, HA and precipitate forming in the RIE are determined only by E protein, and the properties like plaque size or neuroinvasiveness could be due to involvement of some other viral proteins activities. Previously, by methods of reverse genetics was shown that mutation Glu22 Gly, the same as in Ya10/89 clone 125, and Asp67 Gly, analogous to the one in strain 80k, defined the GAG-binding phenotype (Khasnatinov et al., 2009; Mandl et al., 2001). Summarizing all the data we can consider that the complex of properties indicates the GAG-binding variants.

Previously, the set of various mutations increasing virion charge have been described TBEV during virus propagation in cell culture (Goto et al., 2004; Mandl et al., 2001) or in ticks (Labuda et al., 1994; Romanova et al., 2007). Besides, it has been shown that TBEV can exist as stable heterogeneous population containing variants with different affinity to GAGs (Romanova et al., 2007). Both GAG-binding variants (strain 80k and Ya10/89 clone 125) described in the present study were cloned from primary isolate to investigate exactly the small-plaque variants: original strain 80 was described by the author as large plaque and parental strain Ya10/89 exhibited heterogeneous plaque phenotype. According to the data presented here and published previously of the existence of TBEV strains with E protein charge increasing mutations (Ecker et al., 1999; Khasnatinov et al.,
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Fig. 3. Alignment of E protein ectodomain of studied viruses (strain Absettarov, clone Absettarov 18A, strain SofjinKGG, strain 80k) and strain Neudorf, used as a template for modeling structures.
2009; Lu et al., 2008) or with complex of specific properties described above (Chunikhin et al., 1986; Pogodina et al., 1992) we can assume that GAG-binding variants exist like an admixture in natural TBEV population that is hardly seen.

They showed that GAG-binding variants could appear during TBEV adaptation to ticks: European strain to *Ixodes ricinus* ticks (Labuda et al., 1994), or Siberian strain to *Hyalomma marginatum marginatum* ticks (Romanova et al., 2007), or several strains (including Far-Eastern strain)
to *Hyalomma anatolicum* ticks (Chunikhin et al., 1986). According to the data of reproduction rate of GAG-binding variants (Romanova et al., 2007) or of genetically constructed variants with charge increasing mutation in E protein (Khasnatinov et al., 2009) in ticks we can consider that such variants have selective advantage during reproduction in tick organism, apparently, regardless of tick species.

Several cell lines, like BHK-21, SW-13, Neuro-2, used for virus isolation, were predisposed to select virus variants with increased affinity to cellular GAGs (Lee and Lobigs, 2002; Lee et al., 2004; Mandl et al., 2001). Thus, GAG-binding variants appearing even after short-term passages in these cultures could be novel adaptive or preexistent. However, mutants with decreased affinity to cellular GAGs could appear in population of GAG-binding virus during infection in mammalian host or in PEK cells (Chunikhin et al., 1986; Kaluzová et al., 1994; Liapustin et al., 1987; Romanova et al., 2007). Only one substitution has been sufficient to switch the phenotypic characteristics of GAG-binding variants (Romanova et al., 2007). So, passages in laboratory animals or some cells could lead to practically total elimination of GAG-binding variants from natural isolate. In the present work both GAG-binding variants were propagated in PEK cells that allowed us to assume that they preexisted in natural isolate because it was shown previously the cell line didn’t lead to appearance of adaptive mutations increasing affinity to GAGs (Romanova et al., 2007). Strain 80k didn’t change through long-term laboratory passaging and never produced plaques of heterogeneous phenotype (data not shown). Probably, this could be explained by specific composition of two neighboring mutations (Asp389 → Asn, Thr398 → Ala) in the E protein that make specific efficient conformation of the molecule, and mutations would be crucial for virus viability.

Amino acid mutations in domain II of E protein could affect on HA and fusion activities changing low-pH-triggered dimer–trimmer conformational switch (Allison et al., 1995; Heinz and Allison, 2000). In the present study both GAG-binding variants, carrying substitution in the domain II of E protein, lost their hemagglutinating activity. All large and middle plaque viruses, excluding clone Absettarov 18A, hemagglutinate erythrocytes at optimal pH 6.2–6.4. Gaining of mutation in E protein domain II of viruses meant above led them to narrow the range of allowed pH for HA and drastically moved the optimal pH. Strain 80k absolutely lost ability to HA, Ya10/89 clone 125 was able to agglutinate erythrocytes in minimal dilution at pH 7.0. In contrast to strain 80k, Absettarov 18A with analogous mutation sustained HA property, although optimal pH was a bit shifted. Lately the strain Yar 46-2 with the same mutation as the one of clone 18A was described and the strain had no ability to HA (Khasnatinov et al., 2009). It is obvious that HA decreasing or absence associates exactly with GAG-binding phenotype, rather than with just presence of mutation increasing charge or hydrophobicity of E protein.

In our work both GAG-binding variants Ya10/89 clone 125 and strain 80k showed lowered neuroinvasiveness. Previously, we described the tick-adapted variant M, obtained from strain EK-328, with the same mutation increasing charge or hydrophobicity of E protein (Khasnatinov et al., 2009) in ticks we can consider that such variants have selective advantage during reproduction in tick organism, apparently, regardless of tick species.

In contrast to strain 80k, Absettarov 18A with analog mutation sustained HA property, although optimal pH was a bit shifted. Lately the strain Yar 46-2 with the same mutation as the one of clone 18A was described and the strain had no ability to HA (Khasnatinov et al., 2009). It is obvious that HA decreasing or absence associates exactly with GAG-binding phenotype, rather than with just presence of mutation increasing charge or hydrophobicity of E protein.

The present work provides evidence that not every mutation increasing net charge of the E protein molecule leads to appearance of the new virus with GAG-binding phenotype and all complex of specific properties. The analysis of electrostatic potential distribution on the surfaces of the E proteins of Absettarov 18A, 80k and Sofjin revealed only slight differences. According to Mandl and co-authors (2001) most of the mutations increasing affinity to GAGs lay on the surface of domains I and II of E protein. Similarly, there is a visible ringe formed by side chains of amino acids of domains I and II of 2 connected subunits in dimer on electrostatic potential maps, obtained during homology modeling. In case of SofjinKGG this ringe include more negatively charged sites than in case of Absettarov 18A or 80k. Although, surface charge distribution difference does not explain distinct properties of clone Absettarov 18A and strain 80k.

Correlation maps based on MD trajectories showed striking differences between E proteins of these 3 viruses. The comparison of SofjinKGG and Absettarov 18A maps in common shows the genotype differences of correlated movement inside E protein molecule.

Comparison of the correlation map of the 80k with 2 other maps could explain the dramatic contrast of its properties. The protein of strain 80k seems being divided into small grains, whereas domains of the protein of strains Absettarov 18A and SofjinKGG move as a whole. Consequently, the protein of strain 80k is less tight than the one of two other strains, and higher mobility of small parts of the protein can be the reason for higher HS binding affinity. Because distinct surface residues of 80k E protein move more independently from each other that, probably, allows them to interact freely with HS and cellular membrane molecules.

Previously, all described variants with GAG-binding mutation were selected by exhibited phenotype, like plaque size. That could explain why variants, like Absettarov 18A, remain unnoticed. Although, such variants with pre-existing mutation that in some circumstances could lead to formation of novel GAG-binding variants may store in virus population for long periods of time and through virus transmission lifecycle. As far as Absettarov 18A is one of the plaque-purified clones of strain Absettarov, so we can propose it to long-term maintenance through laboratory passaging of the whole strain. Thus, such variants could survive silently, hiding its pre-existing mutation, in the virus population.

Thus, GAG-binding variants (with increased affinity to HS) with complex of peculiarities, like small plaque phenotype in PEK cells, absence of HA at pH 6.2–6.4, absence of cathode-pointed precipitate in RIE, low neuroinvasiveness, do exist in natural TBEV population. Even though, not every amino acid mutation increasing surface charge of E protein molecule leads to appearance of GAG-binding phenotype. According to obtained data we can suppose that the flexibility of E protein molecule could influence virion–GAG interactions.

**Materials and methods**

**Cells and viruses**

Pig embryo kidney (PEK) cell line was maintained at 37 °C in medium 199 (PIPVE, Russia) supplemented with 5% bovine serum (Furo, Russia).

TBEV strains used in the work are summarized in Table 1. Strain Lesopark and the virus containing tick suspension from where strain Ya10/89 was isolated were kindly provided by Dr. N.G. Bochkova and
Infection. The virus titer was calculated and expressed as the log_{10} containing 7.5% FBS and 0.015% neutral red. After incubation at 37 °C, PCR using overlapping sets of TBEV specific sequences available upon request. Sequencing was carried out according to the Kerber method (Lorenz and Bogel, 1973). Reverse transcription was carried out using M-MLV reverse transcriptase (Promega, Madison, WI) according to the manufacturer’s instructions. Viral genome cDNA was amplified by using a dimeric soluble domains were built for viruses Absettarov 18A, Absettarov 18B, SofijnKG, and 80k, and the simulated annealing procedure was applied to every model. The N-acetyl-D-glucosamine moiety attached to Asn154 was kept intact. The best models based on the MODELLER DOPE score and PROCHECK (Laskowski et al., 1993) validation score were chosen for subsequent optimization. Further optimization was performed for the models in SYBYL 8.0 (Tripos Inc., 1699 South Hanley Rd., St. Louis, Missouri, 63144, USA). All hydrogen atoms were added, and then 150 steps of the Powell minimization in the Tripos force field were performed. Operations of the molecules fitting and comparison were performed with SYBYL 8.0. The final structures of the models were used for molecular dynamics studies.

Molecular dynamics (MD) simulation

Molecular dynamics simulation was performed by means of the program AMBER 10 (Case et al., 2008). For the protein molecule force field AMBER ff99SB (Hornak et al., 2006) was used and GLYCAM06 (Kirschner et al., 2008) was used for the carbohydrate moiety. Atomic charges were automatically assigned in LeAP module of the AMBER suite. The SHAKE algorithm (Ryckaert et al., 1977) was applied to the lengths of the bonds containing hydrogen atoms to reduce computational time. Generalized Born solvent model (Tsui and Case, 2001) was utilized to mimic solvent effects.

The energy of system was minimized prior to the production dynamics run with 1500 iterations of steepest descent method and 1000 iterations of conjugated gradients method. Production MD run was performed with sander on 256 processors of the supercomputer SKIF MSU (Moscow State University Research Computing Center). The following parameters were used: time step=2 fs (femtoseconds), number of iterations=5 million, system temperature was kept equal to 300 K by Langevin thermostat with collision frequency of 1 ps^{-1} (picoseconds).
Visual analysis of trajectories was performed with VMD (Humphrey et al., 1996); statistical analysis was made in ptraj module of the AMBER suite. The principal component analysis of the trajectories was also independently done with DYNAMO web server (Barrett et al., 2004).

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