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Human proteins incorporated into tick-borne encephalitis virus revealed by in situ proximity ligation

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ABSTRACT

Host proteins incorporated into virus particles have been reported to contribute to infectivity and tissue-tropism. This incorporation of host proteins is expected to be variable among viral particles, however, protein analysis at single-virus levels has been challenging. We have developed a method to detect host proteins incorporated on the surface of virions using the in situ proximity ligation assay (isPLA) with rolling circle amplification (RCA), employing oligonucleotide-conjugated antibody pairs. The technique allows highly selective and sensitive antibody-based detection of viral and host proteins on the surface of individual virions. We detected recombinant noninfectious sub-viral particles (SVPs) of tick-borne encephalitis virus (TBEV) immobilized in microtiter wells as fluorescent particles detected by regular fluorescence microscopy. Counting the particles in the images enabled us to estimate individual TBEV-SVP counts in different samples. Using isPLA we detected individual calnexin-, CD9-, CD81-, CD29- and CD59-positive SVPs among the viral particles. Our data suggests that a diversity of host proteins may be incorporated into TEBV, illustrating that isPLA with digital counting enables single-virus analysis of host protein incorporation.

1. Introduction

In the course of investigating mechanisms for viral infection, determining the protein composition of virus particles is of central importance. Viral structural and non-structural proteins encoded in viral genome have been studied extensively. Previous investigations have also uncovered several types of host proteins that become incorporated in enveloped viruses, such as human immunodeficiency virus (HIV) and Epstein-Barr virus [1,2]. For example, integrin complexes incorporated into HIV envelopes have been shown to contribute to tissue-homing of virions [3], and virions having incorporated CD59, a complement control protein, seem to play immune inhibitory roles for intravital viral propagation [4]. Accordingly, host proteins have been suggested as promising therapeutic targets for treatment of infectious diseases caused by enveloped virus [5].

Variable incorporation of the typical exosome protein markers CD9 and CD81 has been observed among individual influenza virus particles by electron microscopy [4], suggesting host protein diversity between individual virions. With respect to methodology, most investigations of virion-incorporated proteins have employed mass spectrometry or electron microscopy. The former is a method for bulk virus analysis while the latter may be used to analyze single virus particles, but not in a high-throughput and multiplex manner. Recently, using specialized flow cytometry (flow virometry), heterogeneity of both particle size and genomic content among thousands of individual virion was reported [6,7], however this method does not possess sufficient sensitivity to detect proteins on small virions, such as 20–40 nm. Thus, improved methodology is required for multi-parametric, high-throughput analysis of individual virus particles to characterize viral subsets defined by incorporated host proteins, and to explore the properties of these

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viral variants.

The approach we took to explore incorporation of host proteins in virions is based on in situ proximity ligation assays (isPLA). These assays convert protein information to DNA molecules, serving as reporters that can be amplified and quantified at single-molecule resolution via regular microscopy and flow cytometry [8,9]. Our approach uses the isPLA concept, which serves to visualize proximity between two or more proteins over distances estimated at less than 40 nm [8,10]. This dimension is similar to the sizes of exosomes and small virus particles. When using isPLA for detecting proteins immobilized on solid phases, proteins were detected at 10–100 times lower concentrations, compared to standard sandwich ELISAs [11].

In this study, we investigated tick-borne encephalitis virus (TBEV), a member of flaviviruses, as a model virus to explore the isPLA approach. TBEV causes the most medically important tick-borne infectious disease and the virion size is 30–50 nm [12]. TBEV assembly and maturation of viral particles, in which viral surface protein E is embedded into the virion, occurs in the host's endoplasmic reticulum (ER) and the Golgi complex. Host proteins may be incorporated into the virions in the course of viral assembly. Here, we observed noninfectious, recombinant subviral particles (SVPs) of TBEV by isPLA with regular fluorescence microscopy, and we detected human proteins incorporated into SVPs produced from human cell lines. The proportion of human protein-expressing SVPs was estimated from the resulting numbers of fluorescent particles, which were digitally recorded and identified as individual SVPs.

2. Materials and methods

2.1. Antibody

The mouse monoclonal antibody 1H4 and rabbit serum specific for TBEV-E and -M proteins were described in previous publications [13,14]. Rat anti-calnexin, rabbit anti-calnexin and mouse anti-StrepTag (StrepMAB-Classic) were purchased from BioLegend (USA), Abcam (UK) and IBA GmbH (Germany), respectively. Mouse anti-CD9 and CD81 antibodies, goat anti-CD29 and CD59 antibodies and sheep anti-giantin antibodies were purchased from R&D (USA).

2.2. Production and isolation of subviral particles

pCAG-TBEV-M-StrepE or pCAG-TBEV-M-E plasmid was transfected into HEK293T cells using Lipofectamine 2000 (ThermoFisher, USA), following the manufacturer's instructions, to produce TBEV (Oshima 5–10 strain) subviral particles that are noninfectious enveloped particles with Strep-tagged or native E proteins [15]. The cells were grown in DMEM (ThermoFisher) supplemented with 10% exosome-depleted fetal bovine serum (System Biosciences, USA) and 1% L-glutamine (ThermoFisher) at 37 °C for 36 h after the transfection. The culture supernatant containing SVPs was centrifuged at 300×*g* for 10 min to remove cells and cell debris, filtrated through MILLEX GP filter unit 0.22 μm (Millipore, USA), and centrifuged at 100,000×*g* for 2 h, followed by washing with filtrated phosphate buffered saline (PBS) and centrifugation at 100,000×*g* for 2 h. Pellets containing SVPs and extracellular vesicles were reconstituted in filtrated PBS, supplemented with protease inhibitors, Complete Mini (Roche, Germany) and stored at −80 °C.

Total protein concentrations of the purified samples including extracellular vesicles and SVPs were measured by Dot-it-Spot-it Total Protein Assay kit (Maplestone AB, Sweden). E proteins in the samples were confirmed by sandwich ELISA using 1H4, and rabbit serum specific for TBEV-E/-M and goat anti-rabbit IgG conjugated with horseradish-peroxidase (HRP) (R&D).

2.3. isPLA

To detect SVP in microtiter wells, SVP samples were diluted in 100 mM carbonate buffer (pH 9.9) and coated in a 96-well plate (675986, Greiner Bio-One, Germany) at 37 °C for 1 h, followed by three washes with Tris buffered saline (TBS). In some experiments, SVP samples were pre-treated with 2% Triton X-100 (Sigma-Aldrich, Germany) for 30 min before coating. The wells were blocked by 100 μl Odyssey Blocking Buffer in TBS (LICOR, USA) supplemented with 10% donkey serum (Jackson ImmunoResearch, UK) at 37 °C for 1 h and quickly washed with TBS and then with TBS supplemented with 0.05% Tween 20 (TBST, Sigma-Aldrich) twice at room temperature for 5 min. This washing protocol was used for all other steps, unless otherwise specified.

Primary antibodies were incubated overnight at 4 °C in 40 μl antibody diluent buffer (TBS supplemented with 33% Odyssey Blocking Buffer and 3% donkey serum). The wells were washed and subsequently incubated with 40 μl secondary antibody mix; Duolink anti-rabbit PLUS (1:10, Sigma-Aldrich) and anti-mouse MINUS (1:10, Sigma-Aldrich) or anti-goat MINUS (1:10, Sigma-Aldrich) at 37 °C for 1 h to detect primary antibody pairs positioned in proximity. After washes, the wells were incubated with 40 μl ligation mix (0.05 unit/μl T4 DNA ligase (ThermoFisher), 250 μg/ml bovine serum albumin (BSA, New England Biolab, USA) and 125 nM 5′-phosphorylated connector oligonucleotides; 5′ P-CTATTAGCGTCCAGTGAATGCGAGTCCGTCTAAGAGAGTAGTACAGCAGCCGTCAAGAGTGTCTA 3′ and 5′ P-GTTCTGTCATATTTAAGCGTCTTAA 3′ in 1 × T4 Ligase Buffer (ThermoFisher)) at 37 °C for 30 min, followed by the washing. Next, the ligated DNA circles were used to template replication reactions with 40 μl RCA mix (0.5 U/μl phi29 DNA polymerase (ThermoFisher), 250 μg/ml BSA, 250 μM dNTP (ThermoFisher) in 1 × Phi29 buffer (ThermoFisher)) at 37 °C for 2 h, followed by washes. We detected the amplified RCA products by hybridization with 25 nM Texas Red or Atto488-labeled oligonucleotide probes, Fluorophore 5′ CAGTGAATGCGAGTCCGTCTUUUU 3' (U indicates 2′-O methyl U) in TBS supplemented with 250 μg/ml BSA and 2.5 μg/ml sheared salmon sperm DNA (ThermoFisher). After the washes, 100 μl TBST was added to the wells and these were stored in the dark until imaging. All oligonucleotides were synthesized and HPLC-purified at Integrated DNA Technologies (USA).

To detect TBEV-SVP in cell lines by PLA, HEK293T cells were seeded in Lab-Tek II Chamber Slide System (ThermoFisher) and transfected as described above. The cells were fixed with 3.7% paraformaldehyde (Merck, Germany) for 15 min at room temperature 36 h after the transfection, washed three times with PBS and stored at 4 °C until staining. The cells were permeabilized using PBS supplemented with 0.1% Triton X-100 (Sigma-Aldrich) for 10 min, followed by washes. Antibody staining, ligation, RCA and detection steps were performed as described above except for using Duolink blocking and Duolink diluent buffers (Sigma-Aldrich) as the blocking and antibody diluent buffers, respectively. Nuclei were stained using Hoechst 33342 (ThermoFisher), and rat anti-calnexin and sheep anti-giantin were visualized using Alexa Fluor 594-conjugated anti-rat IgG (ThermoFisher) and Alexa Fluor 555-conjugated anti-sheep IgG (ThermoFisher) in the detection step. After the final washes, the slides were mounted with SlowFade Gold (ThermoFisher).

2.4. Imaging

Stained SVP samples were imaged by epifluorescence microscopy with DMi8 (Leica, Germany) equipped with DFC9000 sCMOS camera (Leica), excitation and emission filters for DAPI, FITC, Texas

Red, and objective lens, HC PL FLUOTAR L 20×/0.40 for the plate samples and HC PL APO 63×/1.40 for the cell samples. We collected two images per sample using the LAS X software (Leica), while avoiding obvious debris, and exported maximally projected z-stacks of 7–15 slices as tiff files.

2.5. Quantification of RCA products from images

We quantified numbers of RCA products in the tiff images using CellProfiler software [16]. We designed pipelines for automated analysis with the following modules: ColorToGray, CorrectIlluminationCalculate, CorrectIlluminationApply, EnhanceOrSuppressFeatures, IdentifyPrimaryObjects, and MeasureObjectSizeShape. We validated manual thresholds (0.06–0.12) and size restrictions (2 or 3 pixels as a minimum and 30 at maximum) to identify RCA products with the IdentifyPrimaryObjects module. The quantification data was exported with the ExportToSpreadsheet module for further analyses.

2.6. Electron microscopy

For quality check of SVP samples, transmission electron microscopy (TEM) was performed by Biotwin Tecnai G3 Spirit BioTWIN (Thermofisher) at the BioVis service facility in Uppsala, Sweden, using negative staining. Briefly, samples were fixed in 2% paraformaldehyde and deposited on a FORMvar/carbon coated grid (Ted Pella, USA) for 20 min. The grids were sequentially floated on a drop of PBS, PBS with 1% glutaraldehyde, distilled water, uranyl-oxalate solution (pH 7.0) for 3, 5, 8 and 5 min at room temperature, followed by floating on a drop of mixtures of 4% uranyl acetate and 2% methyl cellulose for 10 min on ice. Excess liquid was removed by blotting on filter paper. After drying for 5 min, the grids were examined at 80 kV.

2.7. Statistics

One-way ANOVA with Tukey's tests were conducted using GraphPad Prism version 7.04 (GraphPad Software, USA). Data in bar graphs is presented as mean ± SD. A P-value < 0.05 was considered statistically significant for all tests.

3. Results

3.1. TBEV detection using isPLA in cells and isolated from culture supernatant

Initially, StrepTagged TBEV-E protein was analyzed by isPLA in cell lines transiently expressing SVPs. The cells were probed with an antibody pair; rabbit polyclonal antibodies and a mouse monoclonal antibody specific for TBEV-E/-M and StrepTag, respectively. These two primary antibodies were recognized by anti-rabbit IgG and anti-mouse IgG, respectively, which were conjugated to oligonucleotides (Fig. 1a). Only when the pair of secondary antibodies were bound in proximity could single-stranded DNA (ssDNA) circles be created from two additional oligonucleotides that were joined by ligation, templated by oligonucleotides on the secondary antibodies. The DNA circles were then locally amplified by phi29 DNA polymerase in RCA reactions, followed by hybridization with fluorescence-conjugated oligonucleotides and imaging. SVPs were detected as fluorescent dots, representing one or more RCA products, in the cytoplasm of the cells, but not confined to the Golgi area, as evaluated using antibodies against giantin, a marker of the Golgi apparatus (Fig. 1b). No, or only very few fluorescent dots were observed in cells expressing non-StrepTagged SVP (Fig. 1b), in line with the requirement for binding by both primary antibodies to elicit isPLA reaction products.

Next, we applied the isPLA technique to visualize individual TBEV-SVPs immobilized directly in microtiter wells. One TBEV-SVP is formed by 30 E protein dimers on 30–50 nm-sized particles [12], and isPLA possesses the capacity to visualize proximity at single-molecule levels because of the strong localized signal amplification of the reporter DNA circles that form [8]. This does not mean, however, that the assays are capable of detecting every single molecule in the sample, meaning that the numbers recorded are minimal estimates of protein expression. We recorded the numbers of fluorescent dots in the wells as measures of the numbers of individual SVPs, using an imaging program (Fig. 1c). We detected about 5,000 fluorescent dots per microscopic field of view with a 20x magnifying lens (approximately 0.45 mm^2; Fig. 1d and e). When omitting either one of the primary antibodies, or the enzymes, oligonucleotides, or antigen, then less than 100 fluorescent dots were observed per field (Fig. 1d). Isotype control antibodies with irrelevant binding specificity used as alternatives to one of the primary antibodies also did not give rise to more than 50 fluorescent dots per field (Fig. 1f). These data indicate that only very low background signals are created by antibody cross-reactivity and nonspecific amplification from free-oligonucleotides. The numbers of fluorescent dots increased as more SVPs were immobilized per well (Fig. 1g), demonstrating proportionality to the numbers of particles in the investigated samples.

3.2. Visualization of proximity between host proteins and TBEV-SVPs

Calnexin is a marker of the ER and it assists folding of viral glycoproteins in flaviviruses, such as TBEV-E [17]. Therefore, it is conceivable that calnexin molecules may become incorporated into the SVPs. We observed that RCA products generated by isPLA reactions specific for StrepTagged SVPs, illustrated in Fig. 1, were mostly located in calnexin-positive regions as detected by a regular immunostaining, indicating that SVPs were located in the ER (Fig. 2a), as also observed previously [17]. We also detected proximity between calnexin and TBEV-E, mainly in the cytoplasm using isPLA with an antibody pair specific for calnexin and StrepTag (Fig. 2b). The proximity was also detected in purified SVPs deposited in microtiter wells (Fig. 2c). From our analysis we estimate that calnexin was incorporated into 40.8% of the secreted SVPs. However, as we did not investigate the efficiency with which host proteins were detected in the virions by isPLA, when comparing counts of calnexin-positive SVPs and SVPs detected by anti-TBEV-E/-M and anti-StrepTag, this percentage could be an underestimate of the true frequency, We next investigated other human proteins, previously reported to be incorporated in several envelope viruses [1,4], in purified SVPs in microtiter wells. CD9-, CD81-, CD29-, and CD59-positive SVPs were detected by isPLA with antibody pairs specific for TBEV-E/-M and each of the human proteins (Fig. 2d and e). When comparing counts for StrepTag-positive SVPs and ones also positive for the investigated proteins, we evaluated that 6.2%, 4.2%, 3.7%, 5.7% of SVPs expressed CD9, CD81, CD29, and CD59, respectively. SVPs pre-treated with the detergent TritonX-100 showed significantly less fluorescent dot counts (Fig. 2d and e), suggesting that these host proteins were incorporated in the fatty acid membrane of SVPs. By TEM of samples purified by ultracentrifugation we observed many SVPs and extracellular vesicles (Fig. 2f), supporting our interpretation that most signals were generated from StrepTag-positive SVPs, and not from protein aggregates. These data demonstrate that isPLA serves to visualize host protein incorporation into SVPs at the single-virus level, observable by regular fluorescence microscopy.

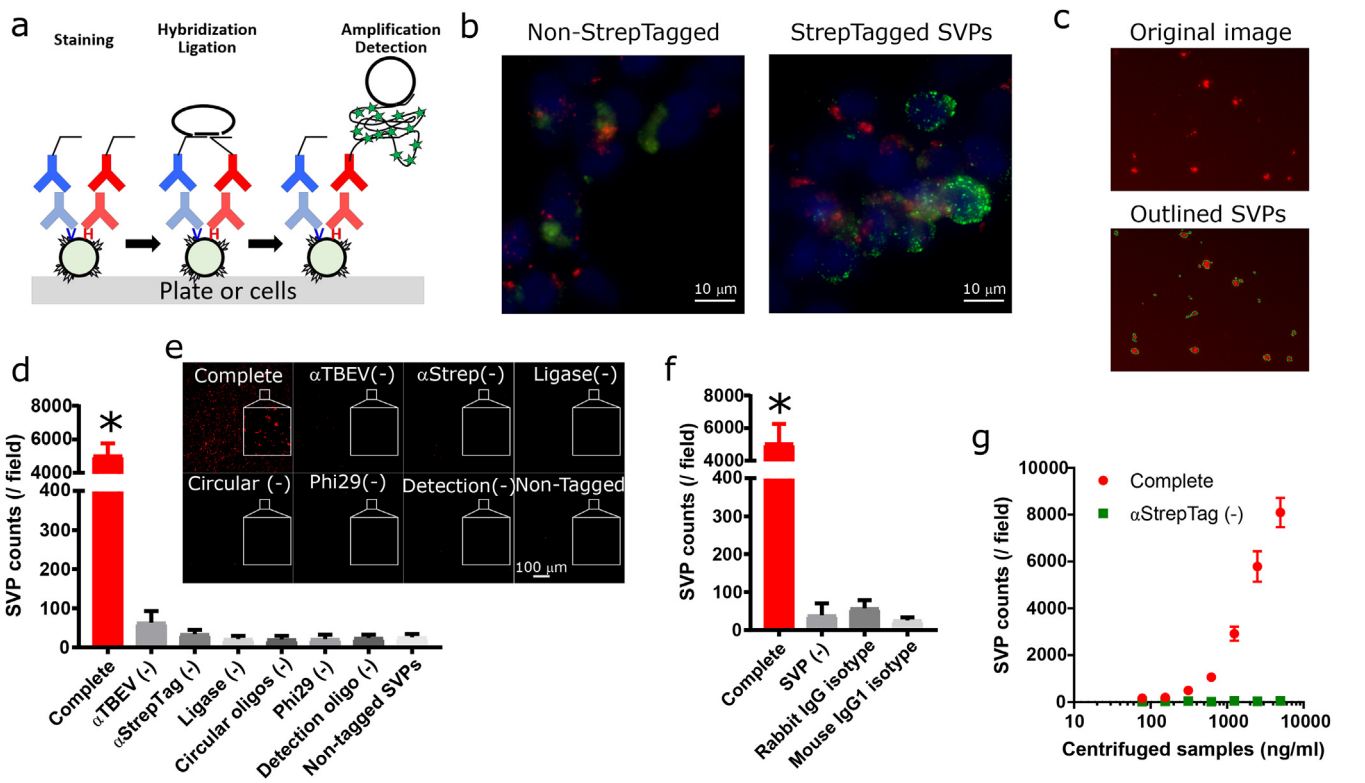

Fig. 1. Detection of TBEV-SVP by isPLA.
(a) Schematic illustration of isPLA for analysis of incorporation of human proteins in TBEV-SVP. Only upon proximal binding by a pair of secondary isPLA probes, specific for primary antibodies against viral proteins (V) and host proteins (H), can a circular DNA strand be formed by enzymatic ligation. The DNA circles are then locally amplified by RCA and detected by fluorophore-modified oligonucleotide probes (stars). (b) Cells transfected with SVP with or without StrepTag were stained with primary antibodies against TBEV-E/-M and StrepTag, and secondary isPLA probes against rabbit and mouse IgG. The resulting RCA products were labeled with Atto488 (green dots). Nuclei were stained with Hoechst 33342 (blue) and the Golgi marker giantin was revealed with anti-giantin antibodies, visualized with conventional secondary antibodies (red). (c) SVPs immobilized microtiter wells were detected by isPLA using primary antibodies against TBEV-E/-M and StrepTag and Texas Red-conjugated detection oligonucleotides (red). Detected SVPs (green outlines) were counted using CellProfiler imaging software (d and e). Bar graphs and contrast-enhanced images, respectively, of SVPs from samples (2500 ng/ml) performed with complete isPLA procedures and technical controls, omitting one of the primary antibodies, enzymes, oligonucleotides or StrepTag. (f) Bar graph representing control experiments omitting SVP immobilization or substituting primary antibodies with isotype controls. (g) Analysis of a 2-fold dilution series of centrifuged samples performed by isPLA (red circles) and a technical control omitting anti-StrepTag antibody (green squares). SVP counts were the mean of six replicates from three experiments with standard deviation. All images are representative of at least three experiments. Asterisks in bar graphs indicate statistical significance ($p < 0.05$) when comparing samples performed with complete isPLA procedures to all technical controls.

4. Discussion

It is well known that in the act of virus production proteins from the cellular host may become included in enveloped viruses, although at substantially lower frequency than the viral structural proteins [4,5]. We investigated the expression of CD9, CD81, CD29, and CD59 in TBEV-SVPs by both conventional immunoprecipitation and sandwich ELISA, but we were unable to detect signals over background (data not shown). Detection of host proteins therefore requires methods that can provide enhanced sensitivity. In the present study we demonstrated that isPLA offers sufficient sensitivity to detect the host proteins incorporated into virion envelopes at the level of individual virions. The incorporation was demonstrated by isPLA, where detection signals depend on simultaneous recognition of pairs of proteins [8,10], in this case TBEV-E/-M and host proteins positioned in proximity. The reduction of the isPLA signal by TritonX-100 treatment supported the position of these proteins in the fatty acid membrane of SVPs. We combined the isPLA with digital counting to enable single virus analysis in a high-throughput manner to allow estimation of proportions of virions having incorporated host proteins. This analysis can provide new insights of relevance for therapeutic approaches. For example, TBEV expressing CD29 (integrin β1) and CD59, a complement regulator, might possess altered tissue-tropism and immunoinhibitory ability as compared to CD29- and CD59-negative virions, therefore these proteins could be suitable targets for efforts to prevent inter-tissue propagation by the virions expressing these host proteins. The method does not require expression of recombinant proteins in cell lines or animals, or analysis using specialized instruments. Accordingly, the isPLA technology can be generally applied in virus research, provided only that suitable antibodies are available for the investigated viral and host proteins.

The recent development of multiplex protein detection technologies, such as mass cytometry resolving more than 30 features, have revealed new cell subsets and cell-transition stages, which would be difficult to identify by regular flow cytometry [18]. Given that more than 100 different human proteins have been shown by mass spectrometry to be incorporated in HIV [1], similar technical developments are also required to evaluate properties of viral subsets defined by protein composition. Simultaneous detection of several proteins at the single-virus levels will serve to identify viral subsets, such as a $CD59^+$ immunoinhibitory subset, a $CD29^+$ tissue-tropic subset or a double-positive one, and such markers of virus subsets could represent suitable therapeutic targets. An attractive feature of isPLA is the ability to detect multiple pairs or more proteins coexisting over short distances such as on individual viral particles, using sets of oligonucleotide-conjugated antibodies, as previously reported [10].

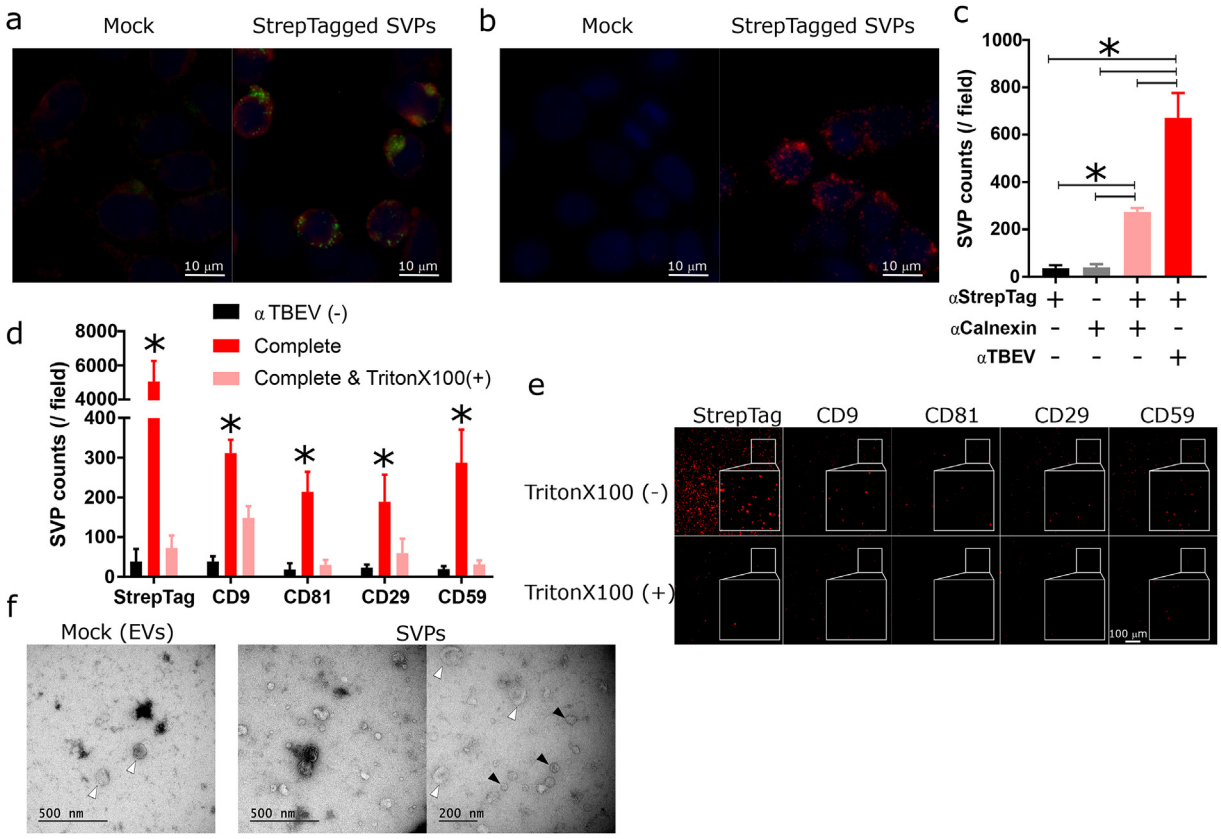

Fig. 2. Detection of host proteins incorporated into TBEV-SVPs.
(a, b) Cells transfected with mock or StrepTagged SVP were stained by isPLA using pairs of primary antibodies directed against TBEV-E/-M and StrepTag (a) or against StrepTag and the ER marker calnexin (b) and secondary oligonucleotide-conjugated isPLA antibody probes against rabbit and mouse IgG. RCA products were labeled with Atto488 (green dots in a) or Texas Red (red dots in b). Nuclei were detected by Hoechst 33342 (blue) and calnexin was visualized with anti-calnexin antibody and a secondary antibody (red in a). (c) Counts of calnexin-positive SVPs immobilized in microtiter wells (625 ng/ml) isPLA was performed using primary antibodies against StrepTag and calnexin or TBEV-E/-M and secondary isPLA probes against rabbit and mouse IgG. Asterisks indicate statistical significance ($p < 0.05$). (d) Bar graphs of counts of CD9, CD81, CD29 and CD59-positive SVPs immobilized in the microtiter wells (2500 ng/ml) is PLA was performed using primary antibodies against TBEV-E/-M (rabbit serum) in pair with antibodies for StrepTag, CD9 or CD81 (mouse IgG), CD29 or CD59 (goat IgG). The pair of secondary isPLA probes were directed against rabbit IgG and mouse or goat IgG. Results for samples omitting anti-TBEV-E/-M or pre-treated with Triton-X100 were included as controls. Asterisks indicate statistical significance ($p < 0.05$) when comparing samples performed with the complete isPLA procedure to controls. (e) Contrast-enhanced images of the reaction products plotted as bar graphs in (d). (f) Electron micrographs of ultracentrifuged samples from supernatants of mock and SVP-transfected cells. Black and white arrows indicate TBEV-SVPs and extracellular vesicles, respectively. SVP counts were mean of six replicates from three experiments. Images are representative of three experiments.

It can be difficult to distinguish extracellular vesicles and virions, since virus-infected cells are known to produce both extracellular vesicles containing viral proteins and/or nucleic acids and non-infectious virus particles that may be lacking nucleic acids, along with complete infectious virions [19]. There is therefore a possibility that in this study we detected a combination of virus and extracellular vesicles expressing both TBEV-E and one of the human proteins by isPLA. Simultaneous detection of viral protein, the RNA genome and host proteins in individual virions and extracellular vesicles might be a solution to this complex problem. Nonetheless, the ability to assess the presence of pairs of proteins on individual virus particles by isPLA provides a novel means to investigate the role of host proteins in virus biology and pathogenicity.

Author contributions

R.I., U.L. and M.K-M. designed experiments. R.I., K. Y. and E.M.D. contributed to SVP production. R.I. and A.I. performed experiments. R.I., E.M.D., L.L., A.A., L.C. and R.G. developed isPLA with fluorescence microscopy. R.I., A.I. and C.F. analyzed the data and performed statistical analysis. R.I., U.L. and M.K-M. wrote the manuscript. K.Y., U.L. and M.K-M. supervised the entire study.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Ulf Landegren is a founder, shareholder and board member of Navinci, having rights to the in situ PLA (isPLA) technology.

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