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Generation of chimeric hamster polyomavirus VP1 virus-like particles harboring three tumor-associated antigens

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We have examined hamster polyomavirus VP1 virus-like particles (HaPyV VP1 VLPs) as potential carriers of tumor-associated antigens (TAAs). Three different HLA-*A–restricted human TAA epitopes: TRP – tyrosinase-related protein-2 epitope (amino acid sequence FVWLHYYSV), MAGE – the MAGE A family protein epitope (LVHPLLLKY) and HTERT – human telomerase reverse transcriptase epitope (ILAKFLHWT), were inserted into VP1 protein into positions #1 (81–88aa), #3 (244–246aa) and #4 (289-294aa) separately and into position #4 as one fused insert produced from all three TAAs. The constructed chimeric proteins were expressed in yeast *Saccharomyces cerevisiae* and after purification were capable to form VLPs. HaPyV-VP1 appeared as a suitable carrier of multiple TAAs. The insertion of separate hydrophobic peptides into different sites of VP1 VLPs has appeared to be a particularly promising approach, because insertion of a long fused hydrophobic peptide unfavorably influenced the VPL assembly and yield.

Abbreviations: HaPyV – hamster polyomavirus, VP1 – virus protein 1, VLPs – virus-like particles, TAA – tumor-associated antigen, CTL – cytotoxic T lymphocyte, TRP – tyrosinase-related protein-2 epitope, MAGE – the MAGE A family protein epitope, HTERT – human telomerase reverse transcriptase epitope

Key words: cancer, vaccine, virus-like particles (VLPs), HaPyV, VP1, TAAs, TRP, MAGE, HTERT

INTRODUCTION

Immunotherapy directed against cancer represents a fascinating perspective to improve the success of surgery, chemotherapy, radiology, pharmacology [1, 2]. Cancer immunotherapy is mainly focused on the induction of immune response against tumor-associated antigens (TAAs) presented on or over-expressed by tumor cells. When bound to MHC class I molecules, TAA peptides are recognized by CD8+ T cells. These activated CD8+ T cells may result in the destruction of different cancer cells and lead to tumor regression. Mainly, the anti-tumor vaccine has to activate the recognition of tumor cells presenting the TAAs by the immune system of a host.

Many of such human TAAs have recently been identified and molecularly characterized [3]. Unfortunately, a direct use of TAA peptides for vaccination faces a problem. The persistence of peptide antigen *in vivo* is limited by clearance and degradation. The presence of serum peptidases alters the antigenicity of peptides or rapidly inactivates them. To raise vaccine immunogenicity, peptides have been used with adjuvants, in liposomes, by direct attachment of lipids or proteins, but this strategy often failed to induce detectable immune responses. Although human tumors express multiple TAA epitopes that are recognized by T cells [4], some of the TAA epitopes can be lost or expressed at different times during tumor growth. A vaccine possessing multiple TAA epitopes might be more effective than a vaccine with a single epitope. TAA polypeptide vaccines may substantially increase the possibility of targeting the tumor cells that have lost one or more TAA epitopes but have retained at least one of the original epitopes [5].

Noninfectious (DNA-free, endotoxin-free) virus-like particles (VLPs) generated by the heterologous expression of viral structural proteins represent promising vaccine candidates. This is evidenced by the fact that the first recombinant vaccine for human use is based on VLPs derived from yeast-expressed surface antigen of hepatitis B virus. Previously, we have demonstrated that the yeast-expressed major capsid protein VP1 of hamster

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polyomavirus (HaPyV) spontaneously self-assembled to virus-like particles (VLPs) [6] and tolerated insertions of different-size foreign protein sequences without disruption of VLPs assembly. These chimeric VLPs were able to trigger an efficient antibody response and to activate T cells without any adjuvant [7, 8].

In this study, we have examined the capacity of hamster polyomavirus VP1 VLPs as a potential carrier of tumor-associated antigens. Three different HLA-*A-restricted human CTL epitopes were inserted into VP1 protein as separate copies or one fused peptide: TRP – tyrosinase-related protein-2 epitope (9 amino acid (aa): FVWLHYYSV) [9], MAGE – the MAGE A family protein epitope (9aa: LVHPLLLKY) [10], HTERT – human telomerase reverse transcriptase epitope (9aa: ILAKFLHWT) [11].

MATERIALS AND METHODS

Generation of expression plasmids. All DNA manipulations were performed according to the standard procedures. Recombinants were screened in *E. coli* K12 DH5α cells. The cloning and expression of the entire HaPyV-VP1-encoding sequence in yeast as well as the selection of target sites in the primary structure of HaPyV-VP1 between amino acid positions 81–88 aa (site #1), 244–246 aa (site #3) and 289–294 aa (site #4) for introducing foreign epitope sequences were described previously [6, 7, 12]. The insertion sites #1, #3, #4 were modified by the introduction of linkers encoding GSSG amino acids on both sides of BglII restriction site (Gedvilaite, unpublished observations).

Three CTL epitopes (TRP, MAGE, HTERT) 9 aa in length and one triple insert composed of TRP, MAGE and HTERT peptides encoding sequences fused in one polypeptide were inserted into VP1 molecule. All four inserts encoding oligonucleotide duplexes surrounded by cohesive ends for cloning were synthesized chemically (Metabion, Germany) (Fig.1A).

A scheme of the constructed chimeric proteins is shown in Fig.1B. Firstly, TRP epitope encoding oligonucleotide duplex was inserted as a single copy into the site #1, MAGE epitope encoding oligonucleotide into the site #3, HTERT epitope encoding oligonucleotide into the site #4 and TMH polypeptide encoding oligonucleotide into the site #4, thus resulting in plasmids pVP1/TRP-1, pVP1/MAGE-3, pVP1/HTERT-4, pVP1/TMH-4. Then a construct with two insertions (TRP at the position #1 and MAGE at #3) was generated by replacing the N-terminal encoding part of pVP1/TRP-1 with the corresponding part of pVP1/MAGE-3, resulting in plasmid pVP1/TRP-1/MAGE-3. The construct with three insertions (TRP at the position #1, MAGE at #3 and HTERT at #4) was generated by replacing the N-terminal encoding part of pVP1/TRP-1/MAGE-3 with the corresponding part of pVP1/HTERT-4, resulting in plasmid pVP1/TRP-1/MAGE-3/HTERT-4 (further pVP1/1T3M4H).

The chimeric VP1 proteins with CTL epitopes encoding genes were sequenced and subsequently subcloned into the unique XbaI site of yeast expression vector pFX7 under control of the hybrid GAL10-PYK1 promoter [6].

Expression and purification of VLPs. The pFX7-derived expression plasmids carrying the chimeric protein-encoding HaPyV-VP1

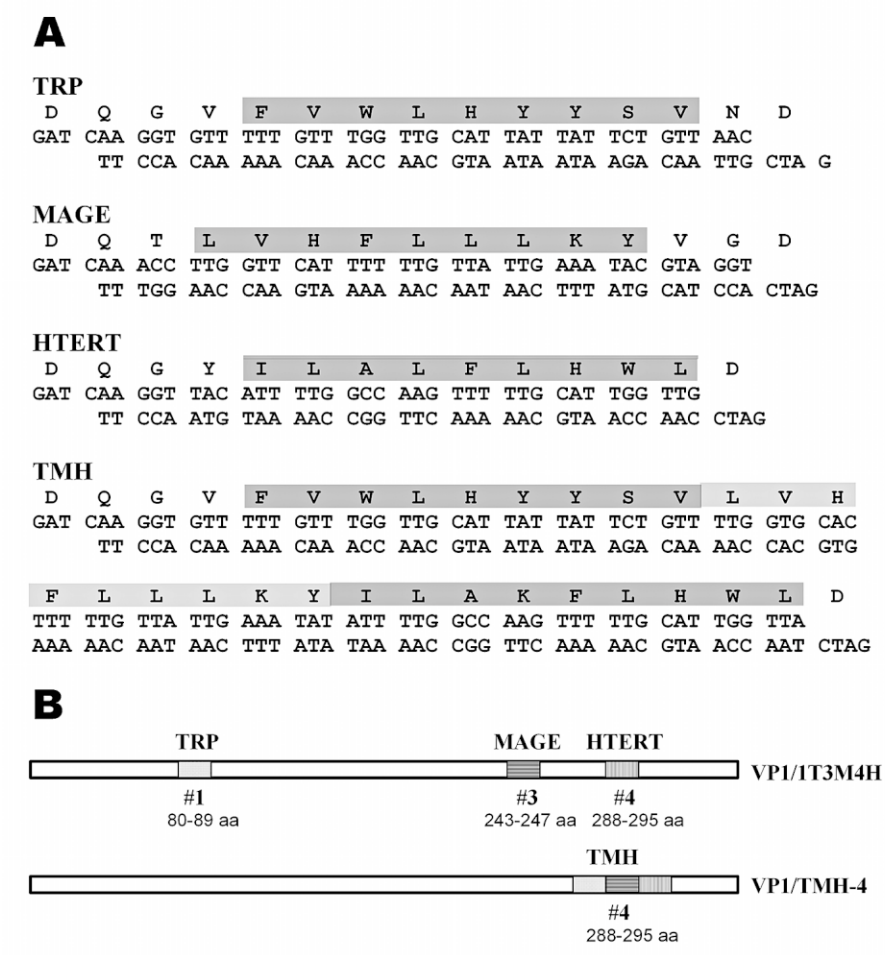

Fig. 1. The sequences of linkers encoding tumor CTL epitopes used as inserts into VP1 carrier protein (A) and the scheme of constructed chimeric proteins (B)
Three different HLA-*A-restricted human CTL epitopes were inserted into VP1 protein as separated or fused peptides: TRP – tyrosinase-related protein-2 epitope, MAGE – MAGE A family protein epitope, HTERT – human telomerase reverse transcriptase epitope, TMH – fused peptide. CTL peptides encoding amino acid sequences are given in grey boxes.

sequences with inserted CTL antigens were transformed into the yeast *S. cerevisiae* strain AH22 derivative 214 (MAT*a, leu2 his4*). As a control, plasmid pFX7-VP1/2-12 encoding the authentic HaPyV-VP1 was used [6]. Cultivation, harvesting and disruption of yeast cells as well as purification of VP1/1T3M4H, VP1/TMH-4 VLPs were accomplished according to the methods described earlier [6, 7]. All purified proteins were dialyzed against PBS buffer, lyophilized and stored at –20 ˚C until further use.

SDS-PAGE and Western blotting. Preparation of protein samples, SDS-PAGE, and Western blot analysis were performed according to the methods described previously [8]. For Western blot analysis, mouse monoclonal anti-VP1 6D11 antibodies [13] diluted 1:1000 were used, followed by incubation for 2 h with horse radish peroxidase (HRP)-labeled Goat Anti-Mouse-IgG conjugate (BioRad, USA). The HRP-mediated staining was performed by addition of 4-chloro-1-naphthol and H_2O_2.

Electron microscopy. Suspensions of recombinant particles purified by ultracentrifugation in cesium chloride gradient were placed on 400-mesh carbon-coated palladium grids. The samples were stained with 2% aqueous uranyl acetate solution and examined with a JEM-100S electron microscope.

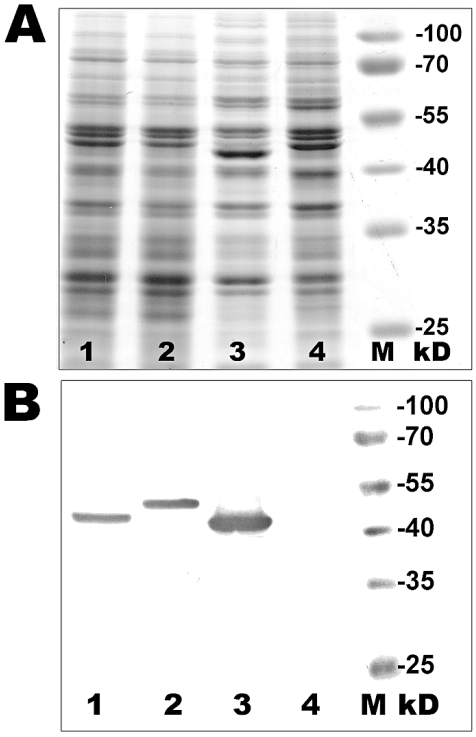

Fig. 2. Detection of VP1/1T3M4H and VP1/TMH-4 chimeric proteins and unmodified HaPyV-VP1 protein in Coomassie blue stained SDS-PAGE (A) and Western blot using anti-HaPyV-VP1 monoclonal antibodies 6D11 (B)
In (A) and (B), total lysates of yeast cells were loaded to a 12.5% SDS-PAGE: lane1 – VP1/TMH-4, lane 2 – VP1/1T3M4H, lane 3 – VP1, lane 4 – yeast cells transformed with pFX7 plasmid and lane M: prestained protein lader (#SM0671, Fermentas, Lithuania).

RESULTS

Three potential insertion sites (site #1 (81–88 aa), site #3 (244–246 aa) and site #4 (289–294 aa)) [7, 8] of the HaPyV major capsid protein VP1 were selected as a target for TRP, MAGE and HTERT TAAs insertions. Two genes encoding chimeric proteins were constructed. One construct had a TRP epitope inserted into the site #1, MAGE into the site #3 and HTERT into the site #4, and another construct had the same three TAAs fused in one polypeptide inserted into the site #4 (see Fig.1B). Both chimeric proteins were expressed in yeast, and lysates of yeast cells after induction were examined by SDS-PAGE and Western blot analysis. Figure 2 shows that both chimeric proteins were expressed in yeast cells, however, the expression level of these proteins was approximately half as low as compared with unmodified VP1 protein.

Purification of yeast-expressed proteins revealed that both chimeric proteins were less soluble in comparison to authentic VP1, tended to aggregate and sediment under protein concentration higher than 0.5 mg/ml. This caused some difficulties for purification. The CsCl gradient-purified unmodified VP1 was recovered as usual from CsCl fractions with densities 1.30–1.32 g/ml and effectively separated from other yeast proteins. The separation of VP1/1T3M4H protein was not enough, because the protein was found in fractions with CsCl densities ranging from 1.26 to 1.28 g/ml. Purification of VP1/TMH-4 protein encountered even more problems. VP1/TMH-4 protein was discovered in a number of different fractions with CsCl densities ranging from 1.21 to 1.28 g/ml with no pronounced peak of accumulation. EM analysis of samples from different fractions confirmed the occurrence of differently-sized VLPs and irregular aggregates simultaneously. The irregular aggregates were detected in CsCl fractions at densities of 1.21–1.25 g/ml (data not shown), whereas VLPs with the diameter of 40–45nm and the smaller VLPs (the diameter about 20 nm) were found at a density of 1.26–1.28 g/ml exclusively (Fig. 3). Only these 1.26–1.28 g/ml fractions containing 1/3 of total VP1/TMH-4 protein and 3/4 of total VP1/1T3M4H protein in all fractions were pooled and used for further purification in CsCl gradient.

After purification, the VLP assembly capacity of VP1/1T3M4H and VP1/TMH-4 chimeric proteins was evaluated by EM analysis. Both constructed proteins were capable to form VLPs similar to the authentic one in the diameter of 40–45 nm (Fig. 3). Because of the repeated centrifugation of VP1/TMH-4 protein fractions containing a mixture of differently-sized VLPs in CsCl gradient, VLPs in the diameter of 40–45 nm were effectively separated from those smaller in the diameter of 20 nm (Fig. 3).

DISCUSSION

It was shown that hamster polyomavirus VP1 VLPs tolerated foreign polypeptides of different origin and size exposed on VLP surface [7, 8]. In this study, we have

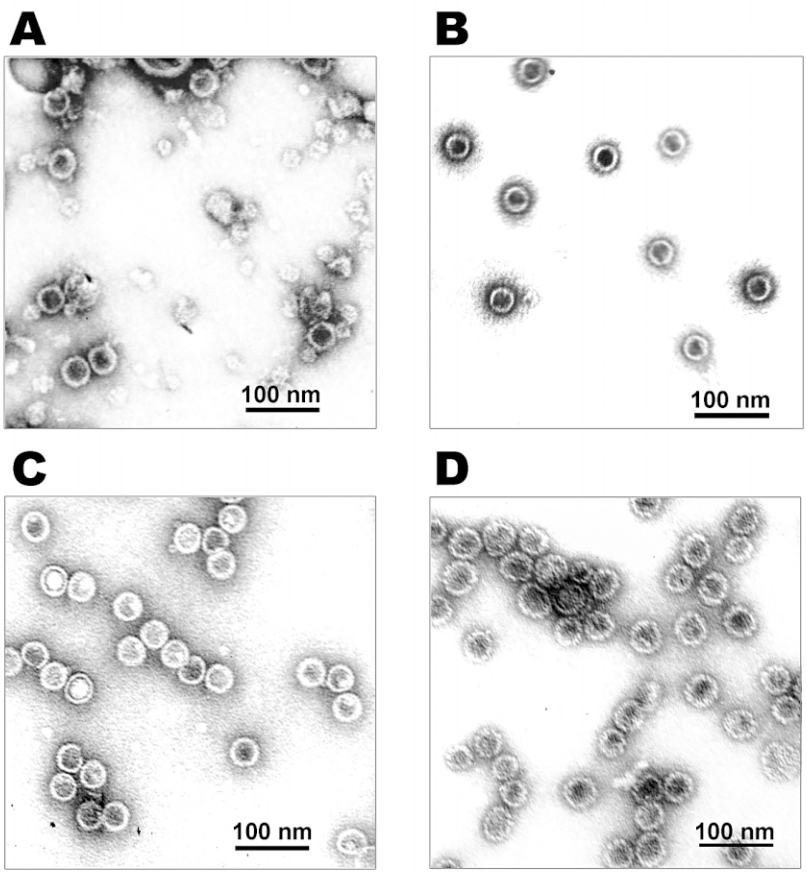

Fig. 3. Determination of VLP formation capacity of chimeric proteins by negative staining electron microscopy
VP1/TMH-4 VLPs after first (A) and second (B) centrifugation in CsCl gradient and VP1/1T3M4H VPLs after second centrifugation (C) in CsCl gradient were detected with negative staining electron microscopy. For comparison, preparation of unmodified HaPyV-VP1 VLPs is shown (D).

demonstrated that HaPyV-VP1 VLPs can be used as the carriers of three different CTL epitopes that are highly hydrophobic.

The evaluation of purified VP1/1T3M4H and VP1/TMH-4 VLPs confirmed the capability of HaPyV-VP1 to tolerate even very unfavorable hydrophobic inserts. However, it must be noted that in spite of a correct folding and assembly, the surface of chimeric VLPs had changed and gained a tendency to aggregate. Hydrophobic TRP, MAGE and HTERT peptides inserted into HaPyV-VP1 affected not only the solubility of VP1/1T3M4H and VP1/TMH-4 proteins but also caused some VLP assembly problems. Particularly, the folding of VP1/TMH-4 protein interfered with the hydrophobic TMH insert 30 aa long. During the purification procedure, only 1/3 of isolated VP1/TMH-4 protein was detected in CsCl fractions enriched with VLPs. The removal of 1/4 of isolated VP1/1T3M4H protein, and especially 2/3 of isolated VP1/TMH-4 protein found as irregular aggregates during the purification procedure led to a lower yield of purified VLPs as compared with the yield of unmodified VP1 VLPs. The failure to form VLPs of about 1/4 and 2/3 of isolated VP1/1T3M4H and VP1/TMH-4 proteins, respectively, might explain also the half as low expression levels of these proteins in yeast cells where incorrectly folded proteins probably are not tolerated.

In conclusion, HaPyV-VP1 is a suitable carrier of multiple tumor-associated antigens, but insertion of separate hydrophobic peptides into different sites of VP1 VLPs becomes a more promising approach, because the introduction of one long fused hydrophobic insert influences VPL assembly and yield. Direct insertion of antigens into the VLP sequence displays a very promising option of inducing tumor antigen specific cytotoxic T-cell responses.

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ŽIURKĖNO POLIOMOS VIRUSO VP1 BALTYMO PANAUDOJIMAS TRIMS SKIRTINGIEMS CTL EPITOPAMS ĮTERPTI

Santrauka

Navikinės ląstelės išskiria tam tikrus baltymus, kurių raiška normaliose ląstelėse nevyksta arba yra minimali. Tokių baltymų fragmentai, vadinami vėžiniais antigenais (VA), pateikiami MHC molekulių vėžinių ląstelių paviršiuje, čia juos turėtų atpažinti ir tokias vėžines ląsteles sunaikinti šeimininko imuninės sistemos T ląstelės. Pagrindinė užduotis kuriant priešvėžines vakcinas yra sustiprinti šį imuninį atsaką, o viena iš strategijų imuninės sistemos CTL atsakui prieš vėžines ląsteles sustiprinti yra vėžinių peptidų nešiklių, galinčių efektyviai transportuoti VA į antigenus pateikiančias ląsteles, paieška.

Šiame darbe buvo tikrinama galimybė kaip vėžinių peptidų nešiklį panaudoti žiurkėno poliomos viruso pagrindinį nukleokapsidės baltymą VP1, kuris efektyviai sintetinamas mielių ląstelėse ir savaime susirenka į virusus panašias daleles (VPD). Į žiurkėno poliomos viruso VP1 baltymą buvo įterpti trys skirtingi vėžiniai antigenai: TRP (FVWLHYYSV – su tirozinaze susijusio baltymo 2 antigenas), MAGE (LVHPLLLKY – MAGE A šeimos baltymo antigenas) ir HTERT (ILAKFLHWT – žmogaus telomerazės atvirkštinės transkriptazės antigenas), po vieną į tris skirtingas VP1 baltymo vietas (#1 (81–88aa), #3 (244–246aa) ir #4 (289–294aa) atitinkamai) arba visi trys sulieti į vieną peptidą #4 VP1 baltymo pozicijoje. Po ekspresijos mielių *S. cerevisiae* ląstelėse abu išgryninti chimeriniai baltymai formavo VPD. Palyginus šių chimerinių baltymų ekspresijos lygį, gryninimo lengvumą ir VPD išeigas, buvo nustatyta, kad trijų skirtingų ir labai hidrofobiškų vėžinių antigenų įterpimas po vieną į tris VP1 baltymo vietas turi mažesnę įtaką VPD surinkimo efektyvumui negu VA intarpas vieno ilgo peptido pavidalu. Sukurta vėžinių antigenų įterpimo į VPD sistema atveria galimybes panaudoti ją T limfocitų atsakui prieš vėžines ląsteles sukelti.