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Searching for the initiating site of the major capsid protein to generate virus-like particles for a novel laboratory mouse papillomavirus

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ABSTRACT

Correctly folded virus-like particles (VLPs) of papillomavirus (PV) display conformationally dependent epitopes that are type specific, maintained on authentic virions, and induce neutralizing antibodies. Alignment of the L1 amino acid (aa) sequences of 84 PVs revealed that the lengths of their N-termini are diverse and that multiple, possible initiation methionine (met) codons exist. The L1 gene of MusPV (MmuPV1), that naturally infects immunodeficient laboratory mouse strain (NMRI-*Foxn1*^{nu}/*Foxn1*^{nu}), has four met codons at the 1st, 2nd, 28th, and 30th aas from its N-terminus. Of these, the 3rd and 4th mets, that are at the 28th and 30th aa position from the N-termius, respectively, are located at the position where most PVs have their first met. These two mets, located at the 9th and 11th from the YLPP conserved aas of most PVs, should be considered as consensus initiation codons of PV L1s. Three L1 proteins of MusPV, starting from the 2nd, 3rd, and 4th mets, were expressed using a baculovirus expression system and characterized for their ability to self-assemble into VLPs. While MusPV L1 proteins starting from the 2nd met expressed an L1 protein that did not fold into VLPs, the L1s starting from the 3rd and 4th mets generated correct VLPs in abundant quantities. We now conclude that the highest quantity and best quality VLPs are made from the consensus L1 met of MusPV.

Introduction

Papillomaviruses (PVs) are small non-enveloped DNA viruses that are species-specific and cause benign and malignant tumors in their natural host (Baker, 1987; De Villiers, 1989; Pfister, 1984; Schiller et al., 2010). To date, more than 120 human PVs (HPVs) and 64 nonhuman PVs have been identified (Bernard et al., 2010; De Villiers et al., 2004), including, most recently, the mouse PV (MusPV, also designated as MmuPV1) (Ingle et al., 2011). HPVs cause more than 5% of all solid human cancers, including virtually all cervical cancers, most anal cancers, and at least 25% of head and neck cancers (Bosch et al., 2013; Howley and Lowy, 2007; Psyrri and DiMaio, 2008; Stanley, 2012; Sudhoff et al., 2011).

MusPV, which was recently isolated from florid papillomatosis that arose in a laboratory nude mouse colony (NMRI-*Foxn1*^{nu}/*Foxn1*^{nu}) (Ingle et al., 2011), offer an ideal animal model to reveal insights into the pathogeneity of human PVs and also the therapeutic measures that can be utilized against PV-induced disease with existing animal models or other rodent PVs (*Micromys minutus* PV, MmiPV; *Mastomys natalensis* PV 1, MnPV1; *Mastomys coucha* PV 2, McPV2; *Rattus norvegicus* PV 1, RnPV1; *Mesocricetus auratus* PV 1, MaPV1; *Erethizon dorsatum* PV 1, EdPV1) (Bernard et al., 2010; Sundberg et al., 1996). The MusPV genome consists of double-stranded circular DNA, 7.5 kb in size. The genomic DNA, which is divided into early, late and non-coding regions, has seven open reading frames (ORFs) (Joh et al., 2011), as has been observed in other PVs.

PVs are approximately 55 nm in diameter (Baker et al., 1991; Crawford and Crawford, 1963; Pfister, 1987) and have a $T = 7$ icosahedral lattice (Baker et al., 1991; Bishop et al., 2007; Finch and Klug, 1965). PVs have two capsid proteins, major and minor. The major capsid (L1) proteins of PVs have molecular mass (mm) of between 55 and 57 kDa (Pfister, 1984). The predicted mm of MusPV L1 protein was 61 kDa, and the minor capsid protein was 57 kDa (Joh et al., 2011). PV capsids are composed of 72 pentameric capsomeres, each of which is folded together with five L1 proteins (Baker et al., 1991). Recombinant L1 proteins expressed in eukaryotic expression systems, including vaccinia virus, yeast, and baculovirus expression systems (Christensen et al., 1994; Hagensee et al., 1994; Kirnbauer et al., 1992; Rose et al., 1994), are known to assemble into virus-like particles (VLPs) that mimic the pristine ultrastructure of intact virions (Bishop et al., 2007; Finch and Klug, 1965).

Previous research has demonstrated that the neutralizing immune response for PVs targets mainly type-specific conformational epitopes on the surface of virus particles and the epitopes are targeted by high-titred, type-specific, neutralizing antibodies to PV (Christensen et al.,

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1994; Ghim et al., 1994, 1996; Kirnbauer et al., 1992; Rose et al., 1994). Further, since it has been proven that the PV VLPs reproduce type-specific neutralizing epitopes of each PV type (Cowsert et al., 1987; Kirnbauer et al., 1992; Rose et al., 1994) and are antigenically similar to intact PV virions (Christensen et al., 1994; Ghim et al., 1991, 1996; Rose et al., 1994), recombinant VLPs as prophylactic vaccines against HPV infection resulting in cervical cancer have been developed. The VLPs also have been proven to be the best candidates for immunological reagents for sero-epidemiological studies (Rose et al., 1994; Wideroff et al., 1999). Prophylactic VLP vaccines of HPV types predominantly found in cervical cancer and/or venereal warts were approved by the U.S. Food and Drug Administration (FDA) in 2006 and 2009.

By using the diverse N-terminal structures of MusPV L1, this study examined the folding efficacy of PV vaccine proteins that are composed of the VLPs presenting conformational epitopes on authentic virions. Consensus met from N-terminus of L1 ORF that yielded good quantity and the correct size VLPs were first defined in this study.

Materials and methods

Alignment of papillomavirus L1 sequences and cloning of MusPV L1 genes

The genomic DNA sequences of 23 HPVs and 61 nonhuman PVs, including seven rodent PVs, were collected from the database of GenBank (Table 1). Amino acid (aa) sequences of full-length L1s starting from initiation codons of the open reading frame (ORF) were then deduced using an EditSeq program (Dnastar). These L1 sequences were aligned using MegAlign Program (Dnastar) to determine conserved aa sequences and consensus initiation met codons.

To clone MusPV-L1 genes starting from potential initiation mets at different positions, three sets of primers specific to MusPV L1 were employed for polymerase chain reaction (PCR) cloning. Proteins starting from the 2nd met at the N-terminus of MusPV L1 ORF were cloned using L1M2-f and r primer set (L1M2-f: TGGATG CTCGAGATGACTTTGCTGA, and L1M2-r: GTTCAGGAATTCTTATTTGC TTCCC) and those starting from the 28th aas were cloned using L1M28-f and r set (L1M28-f: TTTCAGCT CGAGATGGCAATGTGGAC, and L1M28-r: CACCAGGAATTCTTCAGTTATTTGCTTC). L1 gene cloning for the expression of L1 protein starting with met at the 30th aa was described in our previous paper (Joh et al., 2012). The primer set used for met at the 30th aa protein was P1 and 2. For site directed cloning, *Xho*I restriction enzyme site was added to forward primers, and the *Eco*RI site was added to reverse primers.

For the PCR reactions, 3 μl (0.1 μg/μl) of the MusPV genomic DNA cloned to pUC19 was added as template into 17 μl of PCR mixture [0.5 μM of forward and reverse primers, 0.5 mM of dNTP, 2 mM $MgSO_4$, 1 unit of Platinum High Fidelity Taq polymerase (Life Technologies), 1× HiFi PCR buffer]. The amplification was conducted by preheating for 1 min at 94 °C followed by 45 cycles of 45 s at 94 °C, 45 s at 58 °C, and 2 min at 68 °C. The final extension was accomplished by incubation for 10 min at 68 °C. PCR products were loaded onto a 1% agarose gel (UltraPure Agarose; Life Technologies), DNA bands corresponding to L1 genes were excised from the gel, and amplicons were purified using a gel extraction kit (QIAquick gel extraction kit; Qiagen). Purified L1 genes were cloned into pCR-XL-TOPO according to the manufacturer's instructions (TOPO XL PCR cloning kit, Life Technologies).

Expression of MusPV proteins in insect cells and purification of virus-like particles (VLPs) of MusPV

To generate recombinant baculovirus transfer vectors containing MusPV L1 genes, L1 gene cloned into pCR-XL-TOPO vector was excised and subcloned into the *Xho*I and *Eco*RI sites of pBlueBac4.5 (Life Technologies). The resulting recombinant baculovirus maintaining L1 gene was recombined with linearized baculovirus DNA in Sf9 insect cells using a Bac-N-Blue linear transfection kit according to the manufacturer's recommendation (Life Technologies). The resulting recombinant baculovirus stocks were plaque purified under semi-solid medium containing 1.25% agarose (Seaplaque; Lonza). Plaque-purified viruses were tested for the presence of inserted L1 genes by PCR using a set of primers (PH-f: TTTACTGTTT TCGTAACAGTTTTG, and PH-r: CAACAACGCACAGAATCTAGC) annealing to polyhidrine promoter and downstream of multiple cloning sites, respectively, as well as for the expression of L1 protein with a rabbit polyclonal antibody raised against disrupted canine cutaneous papillomavirus type 2 (CfPV2, also designated as CPV2) virions (Joh et al., 2012) by immunofluorescence (IF). VLPs were purified from Sf9 insect cells infected with recombinant baculovirus maintaining these MusPV L1 genes. Sf9 insect cells were collected at 72 h post-infection by centrifugation at 400 g for 10 min. The cell

Table 1
Eighty-four papillomaviruses in public database.

PV	Accession no.	PV	Accession no.	PV	Accession no.	PV	Accession no.
BPV1	NC_001522	EEPV	M15953.1	PcPV-1	AY904723.1	HPV-4	X70827.1
BPV2	M20219	EQPV	AF394740.1	PePV	AF420235.1	HPV-5	M17463.1
BPV3	NC_004197	FcPV-1	AY057109.1	PlpPV-1	AY904724.1	HPV-6	AF092932.1
BPV4	X05817	FdPV-1	AF480454.1	PlPV	AY763115.1	HPV-9	X74464.1
BPV5	NC_004195	HaOPV	E15111	PpPV	X62844.1	HPV-11	M14119.1
BPV6	AJ620208	LrPV-1	AY904722.1	PsPV-1	AJ238373.1	HPV-16	NC_001526.1
BPV7	NC_007612	McPV-2	DQ664501	PtPV	AF020905.1	HPV-18	NC_001357.1
BPV8	NC_009752	MfPV-1	EF028290.1	RaPV-1	DQ366842.1	HPV-31	J04353.1
BPV9	AB331650	MfPV-3	EF558839.2	RhPV-1	M60184.1	HPV-33	M12732.1
BPV10	AB331651	MfPV-4	EF558841.1	RnPV-1	GQ180114.1	HPV-35	M74117.1
ChPV1	DQ091200.1	MfPV-5	EF558843.1	ROPV	AF227240.1	HPV-42	M73236.1
COPV	D55633	MfPV-6	EF558840.1	RtPV	AF443292.1	HPV-43	AJ620205.1
CPV2	AY722648.1	MfPV-7	EF558838.1	SsPV-1	EF395818.1	HPV-44	U31788.1
CPV3	DQ295066.1	MfPV-8	EF558842.1	TmPV-1	AY609301.1	HPV-45	X74479.1
CPV5	FJ492743.1	MfPV-9	EU490516.1	TtPV-1	EU240894.1	HPV-48	U31789.1
CPV6	FJ492744.1	MfPV-10	EU490515.1	TtPV-2	AY956402.1	HPV-51	M62877.1
CPV7	FJ492742.1	MmiPV	NC_008582	TtPV-3	EU240895.1	HPV-52	X74481.1
CRPV	K02708.1	MnPV	NC_001605	UmPV-1	EF536349.1	HPV-56	X74483.1
DPV	M11910.1	MusPV	GU808564	UuPV-1	DQ180494.1	HPV-58	D90400.1
EcPV1	AF498323.1	OvPV-1	U83594.1	HPV-1	U06714.1	HPV-63	X70828.1
EdPV1	NC_006951	OvPV-2	U83595.1	HPV-2	X55964.1	HPV-101	DQ080081.1