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INHALT

Einleitung

1 Das Pseudorabies Virus

Die Klassifizierung der Herpesviren

Aufgrund biologischer Eigenschaften werden die Herpesviren der Säuger und Vögel in die drei Subfamilien *Alpha*-, *Beta*- und *Gammaherpesvirinae* eingeteilt (Roizman et al., 1992). Die Fisch- und Amphibienherpesviren weisen nur einen geringen Verwandtschaftsgrad zur Hauptlinie der Familie der *Herpesviridae* auf, zeigen aber dennoch Homologien zu elementaren Proteinen (Davison et al., 2002). Diese Viren werden vorrangig nach morphologischen Merkmalen klassifiziert (Minson et al., 2000).

Die Alphaherpesviren sind durch ein breites Wirtsspektrum, einen kurzen Infektionszyklus und eine schnelle Ausbreitung in Zellkultur mit effizienter Lyse der infizierten Zellen gekennzeichnet. Latente, reaktivierbare Infektionen werden häufig in sensorischen Ganglien des Wirts etabliert. Alphaherpesviren werden in vier Genera eingeteilt. Dem Genus *Simplexvirus* gehören die humanpathogenen Herpes Simplex Virus Typ 1 und 2 (HSV-1 und -2) an. Das humanpathogene Varizella-Zoster-Virus (VZV), sowie die Tierpathogene Pseudorabies Virus (PrV), Bovines Herpesvirus 1 (BHV-1) und Equines Herpesvirus 1 und 4 (EHV-1 und -4) werden dem Genus *Varicellovirus* zugeordnet (Roizman et al., 1992). Aufgrund von DNA-Analysen gehören außerdem die Genera *Marek´s Disease-like viruses (Mardivirus)* und *Infectious Laryngotracheitis-like viruses (Iltovirus)* den Alphaherpesviren an (Minson et al., 2000).

Betaherpesviren zeichnen sich durch ein engeres Wirtsspektrum und einen längeren Replikationszyklus aus. Sie zeigen eine langsamere Vermehrung in Zellkultur und eine hohe Zellassoziation, wobei es häufig zu einer abnormen Vergrößerung infizierter Zellen (Zytomegalie) kommt. Latente Infektionen kommen in sekretorischen Drüsen, lymphoretikulären Zellen, der Niere und anderen Geweben vor. Den Betaherpesviren werden die drei Genera Cytomegalovirus mit dem humanpathogenen Humanen Zytomegalievirus .(HCMV), Muromegalovirus, dessen

typischer Vertreter das Murine Zytomegalievirus (MCMV) ist, sowie Roseolovirus mit den Humanen Herpesviren Typ 6 und 7 (HHV-6 und HHV-7) zugeordnet.
Die Lymphozyten-assoziierten Gammaherpesviren weisen ein sehr enges Wirtsspektrum auf. Lymphoblastoide Zellen werden produktiv oder latent infiziert, Epithelzellen oder Fibroblasten zeigen lytische Infektionen. Dem Genus Lymphocryptovirus wird das humane Epstein-Barr-Virus (EBV), dem Genus Rhadinovirus das Humane Herpesvirus 8 (HHV-8, *Kaposi´s sarcoma associated herpesvirus* (KSHV)) zugeordnet.
Zu den „Herpes-like viruses“ gehören Viren, die genetisch wenig Ähnlichkeiten zu den Alpha-, Beta- und Gammaherpesviren aufweisen. Dazu gehören unter anderem das *Channel catfish virus* (CCV), das *Koi herpesvirus* (KHV) sowie das Salmonid Herpesvirus Typ 1 und Typ 2 (SalHV-1 und –2). Beispielhaft für Herpesviren der Amphibien sollen die Froschherpesviren RaHV-1 und RaHV-2 (*ranid herpesvirus*) erwähnt sein.

Die Morphologie der Herpesviren

Herpesviruspartikel setzen sich aus vier unterscheidbaren Komponenten zusammen (Abb. 1). Ein elektronendichter Kern enthält die doppelsträngige virale DNA, die als proteinassoziiertes lineares Molekül vorliegt (Furlong et al., 1972; Minson et al., 2000). Sie wird von einem ikosaedrischen Kapsid umgeben, das aus 162 Kapsomeren besteht und einen Durchmesser von circa 100 nm aufweist. Um das Nukleokapsid lagert das, amorph erscheinende, Tegument. Den äußeren Abschluss bildet eine Hüllmembran zellulären Ursprungs, in die virale (Glyko-) Proteine eingelagert sind (Gingsberg, 1988).

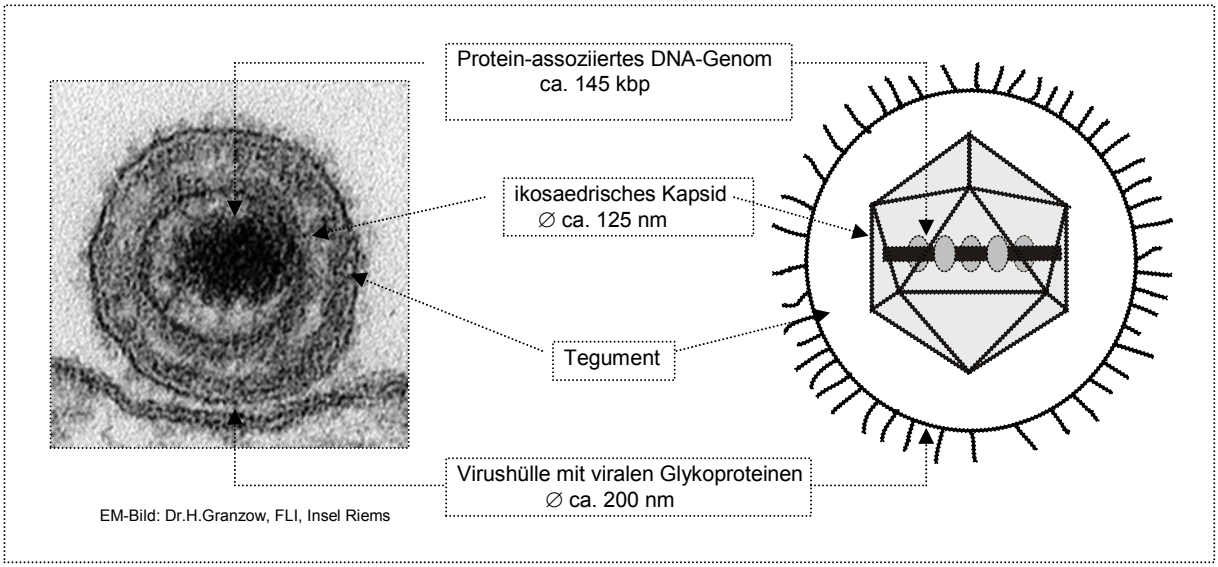

Abbildung 1: Aufbau eines Herpesviruspartikels

Der Aufbau eines PrV-Partikels ist anhand der elektronenmikroskopischen Aufnahme (links) und einer schematischen Übersicht (rechts) dargestellt.

Der Genomaufbau der Herpesviren

Die lineare doppelsträngige genomische DNA umfasst je nach Virus 125 (VZV) bis 240 (MCMV) kbp. Die Anordnung einzelner Gene variiert zwischen den verschiedenen Herpesviren. Die Genomformen werden aufgrund der Lokalisation repetitiver Sequenzen in sechs Gruppen von A bis F eingeteilt (Roizman & Pellett, 2001).

Die für das Genus *Varicellovirus* charakteristische Genomform der Gruppe D besteht aus einer “Unique Long“ (U_L)- und einer “Unique Short“ (U_S)-Region, die durch eine interne repetitive (IR)-Region voneinander getrennt sind, welche sich am 3´-Ende des Genoms in der terminalen repetitiven (TR)-Region wiederholt (Abb. 2). Durch die beiden repetitiven Regionen kann die U_S-Region in Relation zur U_L-Region in zwei antiparallelen Orientierungen auftreten, so dass zwei isomere Genomformen entstehen (Telford et al., 1992; Roizman & Pellett, 2001).

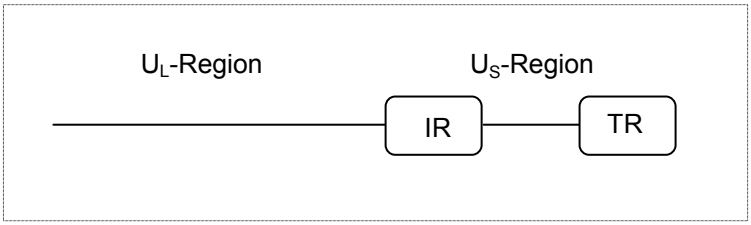

Abbildung 2: Schematische Darstellung eines herpesviralen Gruppe D-Genoms
Das PrV-Genom besteht aus der U_L- und der U_S-Region, die von repetitiven Sequenzen getrennt (IR) bzw. flankiert (TR) sind.

Das Genom von HSV-1 wird in die Gruppe E eingeteilt. Es unterscheidet sich in der Zahl und der Lokalisation der repetitiven Regionen sowie durch vier mögliche Isomere von den Gruppe D-Genomen. Die offenen Leserahmen von PrV und EHV-1 werden analog zu denen des HSV-1-Genoms bezeichnet. Sie sind aufsteigend beziffert und entsprechend der Lokalisation in einer der "Unique"-Regionen mit "U_L" oder "U_S" benannt (Roizman & Pellett, 2001).

Die Pathogenese der Pseudorabies Virus Infektion

Die vom Pseudorabies Virus hervorgerufene Aujeszky'sche Krankheit des Schweins äußert sich als fieberhafte Allgemeinerkrankung mit den neurologischen Symptomen einer Meningoenzephalitis, die häufig von Entzündungen des Respirationstrakts einschließlich der Lungen begleitet ist. Die Virusaufnahme erfolgt oronasal. Im weiteren Verlauf kommt es zur primären Virusreplikation in der Nasenschleimhaut bzw. im oberen Respirationstrakt und zu einer raschen Virusausbreitung innerhalb des zentralen Nervensystems.
PrV kann aufgrund seines breiten Wirtsspektrums die meisten Säugetiere infizieren, wobei die Mortalität nahezu 100 % beträgt (Wittmann, 1984). Ausnahmen sind Einhufer und höhere Primaten, einschließlich des Menschen, die gegenüber PrV-Infektionen resistent sind (Mettenleiter, 1994). Der natürliche Wirt des PrV ist das Schwein, das das einzige natürliche Reservoir für dieses Virus darstellt. Abhängig vom Alter des Tieres und der Virulenz des Stammes treten zwei verschiedene Krankheitsbilder auf (Wittmann & Rziha, 1989). Bei Saugferkeln verläuft die Infektion

meist letal, wobei hauptsächlich neurologische Symptome beobachtet werden. Die Tiere zeigen Erbrechen, Apathie und die charakteristischen zentralnervösen Symptome, die von Erregungszuständen und Koordinationsstörungen bis zu Ataxien, Lähmungen der Hintergliedmaßen („*dog sitting*“) sowie Krämpfen der Rückenmuskulatur und der Extremitäten mit Ruderbewegungen reichen (Pensaert & Kluge, 1989; Wittmann & Rziha, 1989; Wittmann, 1991). Nach ein bis zwei Tagen sterben die Tiere an den Folgen einer Gehirn- und Rückenmarksentzündung (Sabo et al., 1969). Der Krankheitsverlauf verzögert sich ab einem Alter von drei bis vier Wochen und die Mortalität der Ferkel sinkt auf 50-70 %. Ältere PrV-infizierte Tiere zeigen überwiegend respiratorische Symptome und überleben die Krankheit, allerdings unter Etablierung einer lebenslangen latenten Infektion.
Infektionen anderer empfänglicher Spezies führen rasch zu hohem Fieber und starkem Juckreiz (*„mad itch“*) an der Eintrittstelle des Virus (Aujeszky, 1902; Crandell, 1985), der die Tiere zu Kratz- und Scheuerbewegungen bis hin zur Selbstverstümmelung veranlasst. Die Tiere verenden dann meist nach ein bis zwei Tagen (Aujeszky, 1902).

Die Replikation von PrV

Elektronenmikroskopische Untersuchungen ergaben den folgenden Replikationsablauf, der typisch für eine lytische Herpesvirusinfektion ist. Der Infektionszyklus beginnt mit der Adsorption (*attachment*) der Viruspartikel an Oberflächenproteine der Zielzelle, die durch virale Glykoproteine vermittelt wird (Spear, 1993; Mettenleiter et al., 1990). Als Rezeptor-bindende Glykoproteine konnten bei PrV die Glykoproteine gC und gD identifiziert werden (Mettenleiter, 2000). Die Adsorption des PrV verläuft in mindestens zwei Phasen. Zunächst erfolgt eine schwache Bindung des nicht-essentiellen Glykoproteins gC an Heparansulfatproteoglykane der Zielzelle, die dann durch eine Bindung von gD an zelluläre Rezeptoren stabilisiert wird (Mettenleiter et al., 1990; Sawitzky et al., 1990; Karger & Mettenleiter, 1993).
Für Alphaherpesviren wurden unterschiedliche gD-Rezeptoren (*herpes virus entry mediators* HveA-HveD) identifiziert (Montgomery et al., 1996; Cocchi et al., 1998;

Warner et al., 1998). HveA gehört zur TNF (*tumor necrosis factor*)-Rezeptor-Superfamilie und vermittelt den Eintritt vieler HSV-1- und HSV-2-Stämme (Montgomery et al., 1996). Die Bindung von PrV wird von Rezeptoren der Superfamilie immunglobulinähnlicher, dem Poliovirus-Rezeptor verwandter Proteine, wie die Rezeptoren HveB, HveC und HveD, vermittelt (Geraghty et al., 1998; Warner et al., 1998; Nixdorf et al., 1999). Die Gruppe der 3-OS HS (*3-O-sulfated heparan sulfate*) fungiert nicht nur als gD-Rezeptor, sondern vermittelt auch Zellfusionen (Tiwari et al., 2004). Darüber hinaus existieren weitere Rezeptoren für PrV, die einen Viruseintritt in Abwesenheit von gD-Rezeptoren mit einer geringeren Effizienz ermöglichen, aber noch nicht identifiziert wurden (Schmidt et al., 1997; Karger et al., 1998; Nixdorf et al., 1999). Nach Adsorption des Virus fusioniert die virale Hüllmembran in einem direkten, pH-unabhängigen Prozess (Penetration) mit der Zytoplasmamembran. Dieser Prozess, bei dem die Nukleokapside und Tegumentbestandteile ins Zytoplasma freigesetzt werden, ist mechanistisch weitgehend unverstanden (Mettenleiter, 2000). Die viralen Hüllproteine gD, gB und ein Komplex aus gH und gL sind für die Penetration erforderlich, da entsprechende Negativmutanten zwar noch an Zellen binden, die Membranfusion jedoch unterbleibt (Spear, 1993; Mettenleiter, 2000; Mettenleiter & Spear, 1994).

Nach dem Eintritt ins Zytoplasma gelangen die Nukleokapside entlang von Mikrotubuli unter Beteiligung von Dynein zu den Kernporen, wo die Freisetzung von DNA in den Zellkern erfolgt (Granzow et al., 1997; Sodeik et al., 1997; Kaelin et al., 2000; Sodeik, 2000). Hier findet nach der Zirkularisierung der genomischen DNA die Transkription der viralen Gene, die Replikation der viralen DNA und die Neubildung von Nukleokapsiden statt.

Wie bei anderen Herpesviren ist die Transkription der PrV-Gene kaskadenartig reguliert und wird durch die zelluläre RNA-Polymerase II bewerkstelligt (Feldman et al., 1979). Zunächst werden die „immediate-early“-Gene (*ie*-, α-Gene) transkribiert. Die entsprechenden Proteine sind Transaktivatoren der nachfolgend exprimierten *early*-Gene. Bislang sind zwei PrV-Proteine bekannt, die mit einer *ie*-Kinetik exprimiert werden: IE180 (homlog zu HSV-1 ICP4) und RSp40 (homolog zu HSV-1 ICP22) (Cheung, 1989; Fuchs et al., 2000). Die Expression der *early*-Gene (*e*-, β-Gene) erfolgt vor Beginn der DNA-Replikation. Diese Proteine spielen eine Rolle im Nukleinsäurestoffwechsel (Thymidinkinase, dUTPase, Ribonukleotid-Reduktase) und

sind an der DNA-Replikation beteiligt (Helikase, Helikase/Primase, Primase und DNA-Polymerase) (Mettenleiter, 2000). Die *late*-Gene (*l*-, γ-Gene) werden nochmals in zwei Gruppen unterteilt. Die Transkription der *early-late*-Gene beginnt schon vor der Replikation der viralen DNA, die Transkription der *true-late*-Gene erfolgt erst nach DNA-Replikation. Letztere kodieren hauptsächlich für Strukturkomponenten des Virions (Ben-Porat & Kaplan, 1981). Die Strukturproteine verbleiben entweder im Zytoplasma, werden in den Kern transportiert oder reifen nach Passage des endoplasmatischen Retikulums im Golgi-Apparat zu modifizierten Proteinen. Die DNA-Replikation der Herpesviren verläuft ähnlich wie bei den Bakteriophagen T4 und λ nach dem rolling-circle-Mechanismus (Ben Porat, 1981). Danach werden DNA-Konkatemere während der Verpackung in die neugebildeten Kapside durch virale Endonukleasen so gespalten, dass eine Genomkopie in jedes Kapsid gelangt (Wu et al., 1986).

Während der Virusinfektion wird der zelluläre Proteinbiosyntheseapparat durch virale Proteine auf die Produktion vorwiegend viraler Genprodukte umgestellt. Insbesondere spielen dabei die Tegumentproteine eine Rolle. Das unter den Alphaherpesviren konservierte UL41-Tegumentprotein (VHS, *virion host shut off*), beispielsweise entfaltet zusammen im Komplex dem zellulären Translationsfaktor eIF4H RNase-Aktivität und hemmt so die Synthese zellulärer Proteine durch Degradierung von mRNAs zellulärer Gene (Kwong & Frenkel, 1989; Ward & Roizman, 1994; Elgadi et al., 1999).

Die Morphogenese von PrV

Der Zusammenbau der Nukleokapside erfolgt autokatalytisch im Zellkern (Newcomb et al., 1999). Das Kapsid wird um eine Gerüststruktur (scaffold), die aus dem UL26-Genprodukt besteht, geformt, welche zeitgleich mit der Einlagerung viraler DNA wieder entfernt wird. Der weitere Verlauf der Herpesvirusmorphogenese folgt dem *Envelopment-Deenvelopment-Reenvelopment*-Modell (Granzow et al., 2001; Skepper et al., 2001).

Die reifen Nukleokapside gelangen durch Knospung (*budding*) an der inneren Kernmembran in den perinukleären Spalt und erhalten dabei eine erste Hülle. Dieser

Prozess wird als primäre Umhüllung (*primary envelopment*) bezeichnet. Zwei konservierte herpesvirale Proteine, pUL31, ein nukleäres Phosphoprotein, und pUL34, ein Typ-II-Membranprotein, sind an der primären Umhüllung der Virionen beteiligt, wobei pUL34 vermutlich ein primäres Hüllprotein und pUL31 ein primäres Tegumentprotein darstellt (Chang & Roizman, 1993; Reynolds et al., 2001; Fuchs et al., 2002c; Klupp et al., 2002; Mettenleiter, 2002; Muranyi et al., 2002; Bjerke et al., 2003). Die virale Proteinkinase pUS3 ist sowohl in primär umhüllten als auch in reifen PrV- und HSV-1-Virionen Bestandteil des Teguments (Reynolds et al., 2002; Granzow et al., 2004). Die Abwesenheit dieses Tegumentproteins hat eine Akkumulation von primär umhüllten Virionen im perinukleären Spalt zur Folge, was auf eine Funktion des nichtessentiellen US3-Proteins während des Fusionsprozesses der Virionen im Verlaufe des *primary (de-) envelopments* schließen lässt (Klupp et al., 2001a; Reynolds et al., 2002; Ryckman & Roller, 2004). Wahrscheinlich sind bei diesem Prozess auch eine Reihe von zellulären Proteinen beteiligt, die eine Veränderung der Kernmembran bewirken. So ist das pUL34-Homologe des MCMV mit der Proteinkinase C (PKC) assoziert (Muranyi et al., 2002). Dieses Enzym ist an der Phosphorylierung von nukleären Laminen beteiligt und bewirkt vermutlich eine Auflockerung des Laminnetzwerks des Zellkerns während des budding-Prozesses. Perinukleäre Virionen verfügen im Vergleich zu reifen Virionen über einen geringeren Anteil an Tegument. Elektronenmikroskopische Aufnahmen zeigen weiterhin, dass sich die primäre Hülle von der reifer Virionen durch eine glatte Oberfläche unterscheidet, was vermutlich auf das Fehlen von Glykoproteinen zurückzuführen ist (Granzow et al., 1997).

Nach Fusion der primären Virushülle mit der äußeren Kernmembran und der Membran des endoplasmatischen Retikulums werden die Nukleokapside in das Zytoplasma freigesetzt. Die anschließende sekundäre Umhüllung (*secondary envelopment*) findet im trans-Golgi-Netzwerk statt. Dieser sehr komplexe Prozess schließt die Anlagerung von Tegumentproteinen und die Knospung in glykoproteinhaltige Vesikel ein (Whealy et al., 1991; Granzow et al., 1997). Die molekularen Mechanismen der sekundären Umhüllung sind Gegenstand zahlreicher Untersuchungen und erst in Ansätzen bekannt.

Der erste Schritt der Tegumentierung, die Komplexbildung zwischen den Tegumentproteinen pUL36 und pUL37, scheint innerhalb der Herpesviren konserviert

und konnte bei HSV-1, PrV und HCMV nachgewiesen werden (McGeoch et al., 1988; HARMON & Gibson, 1996; Zhou et al., 1999; Klupp et al., 2001b). Das UL3.5-Genprodukt, das in HSV-1 nicht vorhanden ist, erfüllt eine wichtige Funktion bei der sekundären Umhüllung, denn UL3.5-Negativmutanten zeigen eine Akkumulation von unbehüllten Nukleokapsiden im Zytoplasma (Fuchs et al., 1997a). Der weitere Ablauf der Virusmorphogenese ist noch nicht vollständig geklärt. Für einen geordneten Ablauf der Morphogenese dürften bei allen Einzelschritten Protein-Protein-Interaktionen eine entscheidende Rolle spielen. So interagiert zum Beispiel das UL48 Tegumentprotein des HSV-1 mit den Glykoproteinen gB, gD und gH (Zhu & Courtney, 1994; Gross et al., 2003). Die zytoplasmatischen Domänen der Glykoproteine gE und gM des PrV interagieren spezifisch mit dem C-terminalen Bereich des UL49 Tegumentproteins (Fuchs et al., 2002b). In Abwesenheit der Glykoproteine gE/gI und gM ist die sekundäre Umhüllung blockiert und nicht-umhüllte Kapside akkumulieren assoziiert mit den Tegumentproteinen im Zytoplasma (Brack et al., 1999; Brack et al., 2000). Nach Deletion des UL11 Proteins kommt es zu einer Akkumulation von Membranstapeln, Tegumentproteinen und nicht umhüllten Kapsiden im Zytoplasma infizierter Zellen (Kopp et al., 2003). Die Proteine pUL11 und gM sind vermutlich an verschiedenen Stadien der sekundären Umhüllung beteiligt, denn eine UL11/gM-Doppelmutante zeigt durch eine deutliche Beeinträchtigung der Virusreifung riesige Einschlüsse aus Kapsiden und Tegumentproteinen im Zytoplasma einen additiven Effekt der Einzeldefekte (Kopp et al., 2004).

Die umhüllten Virionen werden in Transportvesikeln zur Plasmamembran transportiert und durch Exozytose freigesetzt. Über virale Proteine, die in diesen Prozess eingreifen, ist bisher wenig bekannt. Das putative Membranprotein, pUL20 ist für den Transport der Pr-Virionen zur Zytoplasmamembran notwendig. In Abwesenheit dieses Proteins kommt es zur Akkumulation von umhüllten Viruspartikeln in zytoplasmatischen Vesikeln (Fuchs et al., 1997b). Für die Virusfreisetzung ist das Glykoprotein gK notwendig. Dieses Protein verhindert eine erneute Re-Fusion freigesetzter Viruspartikel mit der infizierten Zelle (Klupp et al., 1998).

2 Strukturproteine des PrV

Das etwa 143 kbp umfassende, vollständig sequenzierte PrV-Genom kodiert für 70 virale Proteine, die größtenteils bereits den Struktur- oder Nichtstrukturproteinen zugeordnet werden konnten (Klupp et al., 2004). Während Nichtstrukturproteine hauptsächlich regulatorische Funktionen im viralen Replikationszyklus erfüllen, sind Strukturproteine Bestandteile der gebildeten Virionen und werden je nach Lokalisation in Kapsid-, Tegument- und Glykoproteine eingeteilt.

Kapsidproteine des PrV

Kapsidproteine bilden durch Kopolymerisierung Proteinkomplexe, die sogenannten Kapside, die das Virusgenom enthalten. Das Hauptkapsidprotein VP5 (pUL19) bildet mit 960 Kopien (Steven & Spear, 1997) die 162 Kapsomere. VP19c (pUL38) und VP23 (pUL18) bilden eine Triplex-Struktur, die durch Interaktion angrenzende Kapsomere stabilisiert. Das VP26 (pUL35) begrenzt die Hexons zur außenliegenden Tegumentschicht. Das Portal-Protein pUL6 bildet einen axialen Tunnel zur Einschleusung der viralen DNA und wird auch den Kapsidproteinen zugeordnet.
Die beiden internen Proteine, das *scaffold-protein* VP22a (pUL26.5) und die Protease VP24 (pUL26), sind an der Ausbildung von Kapsid-Vorläufern beteiligt (Modrow et al., 2003). Dabei wird das Kapsid zunächst um das *scaffold* (Gerüst) geformt, welches mit Einlagerung der viralen DNA wieder entfernt wird.

Tegumentproteine des PrV

Etwa die Hälfte der Virusstrukturproteine bilden das Tegument, das Kapsid und Virushülle verbindet. Nach Penetration des Virus dissoziiert ein Großteil des Teguments vom Kapsid in das Zytoplasma. Diese Proteine sind für die Modifizierung der Zelle nach Virusinfektion und Synthese der *immediate early*-Proteine zuständig. Proteine, die mit dem Kapsid assoziiert bleiben, gewährleisten möglicherweise den

aktiven Transport entlang der Mikrotubuli zum Zellkern (Sodeik, 2000). Weiterhin dienen Tegumentproteine der Translokation der Kapside zum trans-Golgi-Netzwerk und der finalen Umhüllung der Nukleokapside während des Prozesses der sekundären Umhüllung. Die genauen Funktionen vieler Tegumentproteine, vor allem hinsichtlich der Virusmorphogenese, sind Gegenstand aktueller Untersuchungen. Einige für diese Arbeit relevante Tegumentproteine konnten bereits näher charakterisiert werden.

pUL36 stellt das größte Genprodukt der Herpesviren dar und bildet den innersten Teil der Tegumentschicht (McGeoch et al., 1988; Zhou et al., 1999; Klupp et al., 2001b). pUL36 ist sowohl für die HSV-1- als auch für die PrV- Replikation in Zellkultur essentiell. In Abwesenheit von pUL36 ist die sekundäre Umhüllung im Zytoplasma blockiert, nichtumhüllte Kapside akkumulieren und es werden keine Virusnachkommen gebildet (Desai, 2000; Fuchs et al., 2004). Durch Immunpräzipitation im Hefe-2-Hybrid System konnte eine Interaktion zwischen pUL36 und pUL37 nachgewiesen werden (Fuchs et al., 2004; Klupp et al., 2002).
pUL37 ist bei PrV nicht essentiell, Deletionsmutanten weisen jedoch eine deutliche Titerreduktion als Resultat akkumulierender Kapside im Zytoplasma auf (Klupp et al., 2002). Auch bei HSV-1 wurde eine Blockierung der Kapsidreifung im Zytoplasma infizierter Zellen beobachtet, was auf grundlegende Funktionen der Tegumentproteine pUL36 und pUL37 bei der Virusmorphogenese schließen lässt (Desai, 2000; Desai et al., 2001).
Die Untersuchung der Deletionsmutanten von **UL46** und **UL47** zeigte, dass beide Proteine, wie auch bei HSV-1, nicht essentiell für die Virusreplikation in Zellkultur sind (Kopp et al., 2002; Zhang et al., 1991; Zhang & McKnight, 1993). In Abwesenheit von pUL47 kommt es zu zytoplasmatischen Aggregationen von Kapsiden, die bereits von einzelnen Tegumentproteinen umgeben sind. PrV pUL47 entfaltet demnach seine Funktion bei der Virusmorphogenese im Zytoplasma (Kopp et al., 2002). Für HSV sind die Tegumentproteine pUL46 und pUL47 als Phosphoproteine beschrieben, die auch regulatorische Funktionen besitzen und die Aktivität von VP16 (α-TIF) modulieren (Zhang et al., 1991; Zhang & McKnight, 1993).
pUL48 (VP16) transkribiert die *immediate-early* (α-) Gene und wird daher auch als *alpha trans-inducing factor* (α-TIF) bezeichnet (Batterson & Roizman, 1983). HSV-1

UL48-Deletionsmutanten zeigen einen verminderten Einbau von DNA in die Kapside und nur eine minimale Anreicherung extrazellulärer Virionen (Mossman et al., 2000). Für PrV wurde in Abwesenheit von pUL48 ein Defekt bei der sekundären Umhüllung beschrieben, da nicht umhüllte Kapside im Zytoplasma akkumulieren (Fuchs et al., 2002a). pUL48 ist Teil mehrerer Protein-Protein-Interaktionen. Für HSV-1 konnten Wechselwirkungen mit den Tegumentproteinen pUL49 (Elliott & O'Hare, 2000), pUL41 (Smibert et al., 1994), sowie den Glykoproteinen gB, gD und gH (Zhu & Courtney, 1994; Gross et al., 2003) gezeigt werden.

pUL49-Deletionsmutanten zeigen bei PrV keine Auswirkungen auf die Virusreplikation, die Virulenz und die Neuroinvasion (del Rio et al., 2002; Fuchs et al., 2002b). Eine größere Bedeutung hat dieses Tegumentprotein für die HSV-1-Morphogenese (Elliott & Whiteley, 2001). HSV-1 pUL49 ist in der Lage, in Abwesenheit anderer viraler Proteine von einer exprimierenden Zelle in angrenzende Zellen zu gelangen (Elliott & O'Hare, 1997) und zeigt Eigenschaften eines Mikrotubuli-assoziierten Proteins (MAP), indem es Mikrotubuli bindet und stabilisiert (Elliott & O'Hare, 1998). PrV pUL49 interagiert mit den intrazytoplasmatischen Domänen der Glykoproteine E und M, was wiederum auf eine Funktion während des *secondary envelopments* hindeutet (Fuchs et al., 2002b).

pUS3 ist das bislang einzige bekannte Tegumentprotein, welches bei HSV-1 und PrV sowohl in primär umhüllten als auch in reifen Virionen enthalten ist (Reynolds et al., 2002; Granzow et al., 2004). In Abwesenheit von pUS3 akkumulieren umhüllte Viren im perinukleären Spalt, was auf eine Funktion dieses Proteins beim Fusionsprozess der Virionen während des *primary (de-) envelopments* schliessen lässt (Klupp et al., 2001a; Reynolds et al., 2002; Ryckman & Roller, 2004). Ein Substrat für die HSV-1 Proteinkinase pUS3 ist das primäre Membranprotein pUL34 (Purves et al., 1992; Ryckman & Roller, 2004), wobei die Phosphorylierung von PrV pUL34 nicht ausschließlich von pUS3 abhängig ist (Klupp et al., 2001a).

Die **pUL13**-Kinase ist wie pUS3 nicht essentiell für die HSV-1- und PrV-Replikation (Purves & Roizman, 1992). Substrate für HSV-1 pUL13 sind das ICP22 (*infected cell protein 22*) (Long et al., 1999), die Glykoproteine gE/I (Ng et al., 1998), das pUL49 (VP22) (Coulter et al., 1993) und das pUL41 (Overton et al., 1994).

Dem nicht essentiellen PrV **pUL11** wird eine Funktion während der sekundären Umhüllung der Kapside im Zytoplasma zugeschrieben. In Abwesenheit dieses

Proteins kommt es im Zytoplasma infizierter Zellen zu einer Akkumulation von Membranen, nicht umhüllten Kapsiden und Tegumentproteinen (Kopp et al., 2003). HSV-1 pUL11 Deletionsmutanten zeigen einen anderen Phänotyp. Akkumulierende Kapside wurden im Zytoplasma, aber auch im Zellkern infizierter Zellen entdeckt, was auf eine Funktion des HSV-1 pUL11 sowohl im Verlauf des *envelopments* im Kern als auch im Zytoplasma hindeutet (Baines & Roizman, 1992). Als viraler Interaktionspartner des HSV-1 pUL11 wurde das pUL16 identifiziert (Loomis et al., 2003).

pUL16 und **pUL21** stellen ebenfalls nicht essentielle Tegumentproteine dar. Für PrV konnte eine physikalische Interaktion von pUL16 und pUL21 gezeigt werden (Klupp et al., 2005). Ultrastrukturell wurden keine drastischen Defekte während der Replikation der PrV Deletionsmutanten von UL16 und UL21 nachgewiesen, bei gleichzeitiger Deletion beider Proteine ist jedoch eine clusterförmige Anlagerung von Kapsiden im Zytoplasma zu erkennen (Klupp et al., 2005). Die simultane Abwesenheit der Proteine pUL11, pUL16 und pUL21 führt zu einer Verformung und Akkumulation cytoplasmatischer Membranen (Klupp et al., 2005). Während die PrV- und HSV-1-UL11 Genprodukte membranassoziiert vorliegen, scheinen die PrV- und HSV-1-UL21 Proteine dem Kapsid angelagert zu sein (de Wind et al., 1992; Takakuwa et al., 2001). Die Hinweise auf die Lokalisation der Proteine sowie die identifizierten Interaktionen deuten darauf hin, dass der putative pUL11-pUL16-pUL21 Komplex das Kapsid und die Virushülle verbindet (Klupp et al., 2005).

Glykoproteine des PrV

Bei PrV wurden elf Glykoproteine identifiziert, die Homologien zu den entsprechenden HSV-1 Proteinen aufweisen. Sie werden als gB, gC, gD, gE, gG, gH, gI, gK, gL, gM und gN bezeichnet. Mit Ausnahme von gG sind alle Glykoproteine Bestandteile der Virushülle. Glykoprotein B liegt als Homodimer vor, während gE/gI, gH/gL und gM/gN heterooligomere Komplexe bilden, die funktionelle Einheiten darstellen. Die Glykoproteine sind sowohl an der Virusadsorption und Penetration, als auch an der Virusmorphogenese, der Virusfreisetzung und der Virusausbreitung

von Zelle zu Zelle (*cell-to-cell-spread)* beteiligt. Sie beeinflussen den Tropismus und die Virulenz des Virus und stellen Ziele der humoralen und zellulären Immunantwort des Wirtes dar (Ben Porat et al., 1986; Mettenleiter, 2000).

Virale Strukturproteine zellulärer Herkunft

Mit der Einführung neuer Techniken zur Identifizierung von Proteinen hat die Zahl der zellulären Proteine, die in Virionen gefunden wurden, stark zugenommen. Die Mehrzahl der bisher identifizierten Virionproteine zellulärer Herkunft sind an grundlegenden zellbiologischen Mechanismen beteiligt. Sie umfassen Proteine des endosomalen Vesikeltransports, Cyclophiline, Hitzeschockproteine oder Bestandteile des Zytoskeletts. Für einige dieser Proteine lässt sich eine Funktion im viralen Replikationszyklus ableiten. Es ist jedoch nicht ausgeschlossen, dass auch einige Proteine unspezifisch und ohne Funktion eingebaut werden (Cantin et al., 2005). Beispielhaft sollen im Folgenden einige dieser Virusstrukturpoteine zellulären Ursprungs erwähnt werden.
Eine Reihe von zellulären Proteinen wurde in den Herpesviren EBV, MCMV, HCMV und KSHV gefunden. Als abundante herpesvirale Strukturproteine gelten Aktin, Cofilin, Tubulin, HSP70 und HSP90, aber auch Myosin und Annexine (Johannsen et al., 2004; Kattenhorn et al., 2004; Varnum et al., 2004; Zhu et al., 2005). Aktin ist auch Teil der Tegumentschicht des PrV. In pUL49 negativen Pr-Virionen wurden erhöhte Mengen Aktin beobachtet (del Rio et al., 2005), offenbar als Resultat einer Kompensationsreaktion.

Einige umhüllte RNA-Viren nutzen den endosomalen Vesikeltransport der Zelle, was den Einbau von vesikulären Transportproteinen in solche Viren erklären könnte. Beispiele sind hier Ubiquitin in SIV (*simian immunodeficiency virus*), HIV-1 (*human immunodeficiency virus type 1*), MLV (*murine leukemia virus*) oder MMLV (Moloney MLV) (Gottwein et al., 2003; Ott et al., 1998; Ott et al., 2000). Verschiedene Bestandteile der drei ESCRT (*endosomal sorting complex required for transport*) - Komplexe werden von HIV-1- und SIV-Virionen acquiriert, vermutlich ebenfalls um

den zellulären Transportmechanismus zu nutzen (Stuchell et al., 2004; von Schwedler et al., 2003; Strack et al., 2003).

Influenza-, Ebola-, Masern- und HIV-1-Viren verlassen die infizierte Zelle über spezielle Membrandomänen, die Lipid Rafts, von denen einige Komponenten im Viruspartikel verbleiben (Chazal & Gerlier, 2003). So wurde in der Hüllmembran von Parainfluenza-, Influenza-, Epstein-Barr- und Herpes simplex -Viren das Gangliosid GM1, ein Lipid-Raft spezifisches Lipid nachgewiesen (Pickl et al., 2001). Aber auch die in den Lipid Rafts lokalisierten membranassoziierten Komplementregulatoren CD46, CD55 und CD59 sind Bestandteile der Hüllmembranen von HCMV, HTLV-1 (Humanes T-Zell-lymphotropes Virus Typ 1) und HIV-1 (Saifuddin et al., 1995; Saifuddin et al., 1997; Spear et al., 1995). Für das Vaccinia-Virus konnten Cantin und Mitarbeiter (Cantin et al., 2005) eine Unterdrückung der antiviralen Antwort der Zelle durch die Acquirierung dieser Komplementproteine zeigen.

Für das Verpacken genomischer RNA von Retroviren sind Interaktionen zwischen Wirtszellproteinen und viralem Genom erforderlich. Ein Beispiel ist das doppelstrang-RNA bindende Protein Staufen, welches in HIV-1-, HIV-2- und MMLV-Partikeln nachgewiesen wurde (Mouland et al., 2002). Herpesviren, Pockenviren und einige Retroviren kodieren für Uracil-DNA Glycosylasen (UNGs) oder dUTPasen, um den Uracil-Einbau in die virale DNA zu verhindern (Chen et al., 2002). Andere Viren, wie beispielsweise HIV-1, kodieren nicht für derartige Enzyme, bauen dafür aber zelluläre UNGs in die viralen Partikel ein (Willetts et al., 1999).

Umgekehrt haben Viren aber auch Strategien entwickelt, zelluläre Proteine gezielt von einer Inkorporation in das Viruspartikel auszuschließen. So verhindert HIV-1 die Inkorporation von APOBEC3G, einer zellulären antiviral wirksamen Cytosin-Deaminase (Vartanian et al., 1991), indem das zelluläre Protein an das virale vif-Protein gebunden und damit in der Zelle sequestriert wird (Sheehy et al., 2002; Kao et al., 2003).

3 Proteomanalyse

Das Proteom beschreibt das gesamte Proteinäquivalent eines Genoms unter definierten Bedingungen. Es umfasst damit die Gesamtheit der Proteinexpression einer Zelle oder eines Gewebes zu einem bestimmten Zeitpunkt. Im Gegensatz zum Genom, das in jeder Zelle eines Organismus unabhängig von Differenzierung oder Stoffwechsellage unverändert vorliegt, unterliegt das Proteom starken qualitativen und quantitativen Schwankungen. In Abhängigkeit von der Zelldifferenzierung und der damit verbundenen gewebe- bzw. zellspezifischen Genexpression haben Stoffwechsellage, hormoneller Status, Stress, die Einwirkung von Xenobiotika, Zell-Zell-Interaktionen, Temperatur oder Infektionen einen Einfluss auf den Proteinbestand. Die Proteomanalyse ist ohne die in den letzten Jahren gemachten Fortschritte auf den Gebieten der Proteinchemie, der Massenspektrometrie und der Bioinformatik undenkbar. In ihrer zur Zeit gängigsten Form erfolgt die Trennung des zu untersuchenden Proteingemisches mittels zweidimensionaler Gelelektrophorese, die eine isoelektrische Fokussierung mit einer SDS-Polyacrylamid-Gelelektrophorese kombiniert (O'Farrell, 1975; Klose, 1975). Das Verfahren erlaubt nicht nur die Trennung verschiedener Proteine, sondern oft auch die Darstellung einzelner modifizierter Formen desselben Proteins. Nach der Trennung erfolgt dann der enzymatische Verdau der Proteine und die massenspektrometrische Analyse der erhaltenen Peptide, der sogenannte *peptide mass fingerprint* (PMF). Durch Datenbankabfragen erfolgt dann die Identifizierung des Proteins anhand der Massenlisten des PMF.

Quantitative Proteomics

Während Prokaryoten auf Reize oft mit der Aktivierung von spezifischen Genclustern reagieren, nutzt die eukaryotische Zelle weitere Mechanismen wie posttranslationale Modifizierungen, um sich auf veränderte Umweltbedingungen einzustellen. Auf Proteomebene bedeutet dies, dass es weniger zu qualitativen als zu quantitativen Änderungen im Proteinmuster einer Zelle kommt. Um diese zu erfassen, wurden in den letzten Jahren verschiedene Analyseverfahren entwickelt. Besondere Bedeutung

haben Verfahren gewonnen, bei denen eine massenanalytische Quantifizierung durch den Einbau von stabilen Isotopen in Proben- oder Referenzmaterial ermöglicht wird. Die Verfahren unterscheiden sich im Wesentlichen durch den Zeitpunkt im Analysenverlauf, zu dem das Isotop eingebaut wird. Für Untersuchungen in Zellkultur eignet sich besonders ein als SILAC (*stable isotope labeling by amino acids in cell culture*) bezeichnetes Verfahren (Ong et al., 2002), bei dem die schweren Isotope metabolisch in Probe oder Referenz eingebaut werden. Durch Mischung von Proben und Referenzmaterial noch vor der 2D-Elektrophorese erreicht man die Einführung eines globalen inneren Standards zum frühestmöglichen Zeitpunkt und damit eine sehr hohe Robustheit gegen Analysenartefakte und daraus resultierend eine hohe Genauigkeit und Reproduzierbarkeit (siehe Abb.3).

In der hier verwendeten SILAC-Variante wurden Pr-Virionen auf Zellkulturen propagiert, bei denen die essentielle Aminosäure Leucin in der konventionellen Form (bei der Vermehrung der Mutanten) und in einer schweren Variante mit 3 Da Massenunterschied (bei der Vermehrung der Wildtyp-Virionen) vorlag. Nach Ernte und Reinigung der Virionen wurden diese gemischt und die Proteine elektrophoretisch dargestellt. Die Quantifizierung erfolgte nach Verdau der Proteine und Identifizierung durch PMF. Die Isotopenverhältnisse der einzelnen zu quantifizierenden Proteine wurden auf das Isotopenverhältnis des MCP142 (UL19) bezogen, das in jedem Viruspartikel in konstanter Kopienzahl vorliegt. Durch diese zusätzliche Normierung konnte eine sehr hohe Genauigkeit des Verfahrens erreicht werden.

Für Proben, bei denen eine metabolische Einführung der Isotope nicht möglich ist, haben sich Verfahren wie ICAT (*Isotope coded affinity tag*) oder iTRAQ (*Isobaric tags for relative and absolute quantitation*) durchgesetzt, bei denen die Massenmarkierung zwar chemisch erfolgen kann, dies allerdings zu einem späteren Zeitpunkt der Analyse. Das ICAT-Reagenz wird in seiner leichten und massenmarkierten Variante jeweils an reduzierte Cystein-Reste der zu analysierenden Peptide gekoppelt. Beide ICAT-Reagenzien und damit auch die zu vergleichenden Peptide weisen eine definierte Massenverschiebung auf (Gygi et al., 1999) (Abb.3; “chemische Modifizierung“). Der Vorteil der ICAT-Methode liegt in der Verringerung der Proben-Komplexität, da mit Cystein eine Aminosäure markiert wird,

die eine durchschnittliche Häufigkeit von nur 2-3% im Aminosäure-Pool aufweist und der Möglichkeit, spezifische Peptide über Affinitätsanker anzureichern.

Eine weitere Variante der Markierung ist die Durchführung der enzymatischen Hydrolyse der Proben in $H_2{}^{18}O$ (Schnolzer et al., 1996) (Abb.3; "gelabelte Peptide"). Die freiwerdenden carboxyterminalen Aminosäuren der Peptide werden mit den ^{18}O-Atomen markiert. Diese Peptide weisen einen Massenzuwachs von 2 Da gegenüber den korrespondierenden Peptiden einer konventionell hydrolysierten Probe auf. Beide Proben werden vereinigt und der MS-Analyse unterzogen. Korrespondierende Peptide erscheinen als Signalpaare mit einer Massendifferenz von 2 Da. Das Verfahren ist zwar allgemein anwendbar, nachteilig ist aber der nur geringe Massenabstand von 2 Da und der sehr späte Zeitpunkt der Markierung im analytischen Prozess aus Proteinextraktion, Proteintrennung, Hydrolyse und MS-Analyse.

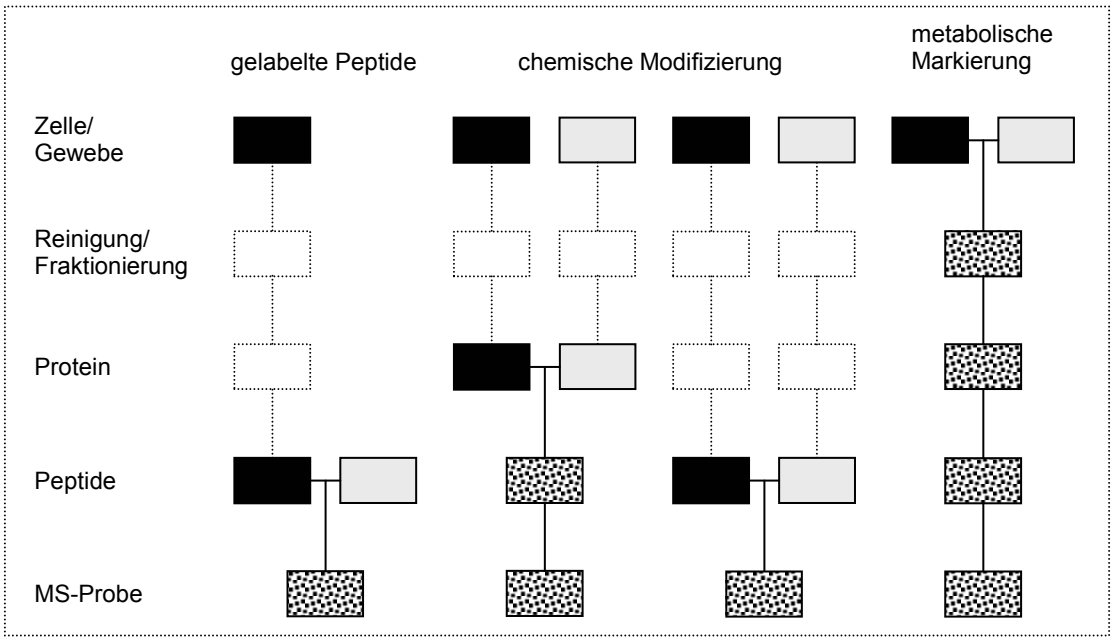

Abbildung 3: Möglichkeiten des Einbaus des isotopen-Labels während der Präparation quantitativ zu vergleichender Proteine. Gezeigt ist ein typisches Reinigungsprotokoll der quantitativen Proteomics von Zelle oder Gewebe über Reinigung und Protein-Verdau bis zur massenspektrometrischen Auswertung. Hell und dunkel hinterlegte Kästchen zeigen die verschieden markierten und zu vergleichenden Proben. Proben, die parallel aufgereinigt werden, sind mit gepunktetem Hintergrund gekennzeichnet. Die Abbildung stellt den Vorteil der metabolischen Markierung, die Markierung und Vereinigung des Probenmaterials zum frühestmöglichen Zeitpunkt, dar (modifiziert nach Ong & Mann, 2005).

Zielsetzung

Alphaherpesviren bestehen aus mehr als 30 Strukturproteinen, die in die morphologischen Untereinheiten Nukleokapsid, Tegument und Virushülle eingeteilt sind. Das Tegument, welches das Nukleokapsid und die Virushülle verbindet, besteht aus zahlreichen viralen und einigen zellulären Proteinen, die über ein Netzwerk von Protein-Protein-Interaktionen dem Nukleokapsid angelagert werden. Die Aufklärung der Funktion einzelner Tegumentproteine sowie die Identifizierung von Wechselwirkungen zwischen Strukturproteinen sind Gegenstand zahlreicher aktueller Untersuchungen. Im allgemeinen wird die Funktion viraler Proteine auf die Virusreplikation über die Mutation einzelner viraler Genprodukte analysiert. Funktionale Defekte des daraus resultierenden Phänotyps werden dem mutierten Protein direkt zugeschrieben.

Die funktionale Charakterisierung viraler Proteine durch Untersuchung von Deletionsmutanten setzt voraus, dass Produkte nicht betroffener Gene in gleicher Weise wie im Wildtyp-Virus exprimiert, modifiziert und, im Falle von Strukturproteinen, in das Virus inkorporiert werden. Beispiele aus der Literatur zeigen, dass dies nicht immer der Fall ist, sondern, dass Deletionen einzelner viraler Gene durchaus Veränderungen in anderen Virusproteinen nach sich ziehen können. Ziel dieser Arbeit war, einen analytischen Zugang zur Untersuchung solcher Sekundäreffekte nach Deletion einzelner Proteine bezüglich der Herpesvirus Morphogenese, bei der mehr als 30 verschiedene virale und zelluläre Proteine zu einem vollständigen infektiösen Partikel zusammengefügt werden, zu schaffen. Dazu sollte (1) die Zusammensetzung von Virionen des Alphaherpesvirus Pseudorabiesvirus (PrV) mittels hochauflösender zweidimensionaler Gelelektrophorese analysiert und virale und zelluläre PrV-Strukturkomponenten, einschließlich eventuell vorhandener Isoformen, massenspektrometrisch identifiziert werden, und (2) durch quantitative Massenspektrometrie unter Verwendung von stabilen Isotopen die Strukturkomponenten des PrV in Deletionsmutanten und verschiedenen Virusstämmen quantifiziert werden. Schwerpunkt der Untersuchungen waren Mutanten mit Deletionen von einzelnen Proteinen des Teguments, da dieses bei der Morphogenese eine herausragende Rolle spielt.

II Zusammenfassende Darstellung und Diskussion der Ergebnisse

Das Pseudorabiesvirus, der Erreger der Aujeszky´schen Krankheit beim Schwein, gilt heute nach Eindämmung der Krankheit als Modellvirus zur Erforschung der Herpesvirusinfektion auf molekularer Ebene. Von den mehr als 70 Genen des 143 kbp umfassenden PrV-Genoms wurden 31 als Strukturkomponenten identifiziert (Klupp et al., 2004). Annähernd die Hälfte der Strukturproteine ist Teil des Teguments, ein Indiz für die strukturelle und funktionale Bedeutung dieser herpesviralen morphologischen Untereinheit.
Das Ziel dieser Arbeit war die qualitative und quantitative Analyse der Zusammensetzung von Virionen des PrV.

(1) "Composition of pseudorabies virus particles lacking tegument proteins US3, UL47, UL49, or envelope glycoprotein E"

In Partikeln des PrV-Virusstammes Kaplan wurden nach ein- oder zweidimensionaler Elektrophorese und Identifizierung durch *peptide mass fingerprint* 27 Strukturproteine viraler und vier Strukturproteine zellulärer Herkunft (Annexin I und II, HSP70 und Aktin) identifiziert (Fig. 1,2). Die viralen Strukturproteine pUL37, pUL48, pUL18, pUL19, pUL29 (gB) und alle Strukturproteine zellulärer Herkunft wurden nach zweidimensionaler Elektrophorese in mehreren Isoformen nachgewiesen (Fig. 2). Das PrV Glykoprotein gB wurde als ungespaltene Vorstufe sowie in der proteolytisch prozessierten Form als N- und C-terminale Untereinheit nach 1D-Elektrophorese identifiziert. Im 2D-Gel erschien die größere, N-terminale Untereinheit in Form von mehr als 15 Ladungsvarianten. Das Hauptkapsidprotein pUL19 tritt mit dem erwarteten Molekulargewicht von 142 kDa ebenfalls in mehreren Ladungsvarianten auf. Daneben konnten sieben weitere Isoformen eines C-terminalen Fragments mit einem Molekulargewicht von 68 kDa nach 2D-Elektrophorese nachgewiesen werden. pUL26, das scaffold Protein, wurde lediglich als N-terminales Fragment und nicht in seiner full-length-Form nachgewiesen, ähnlich wie es für HSV gezeigt worden ist (Sheaffer et al., 2000).

Im zweiten Teil der Arbeit wurde die Zusammensetzung von Deletionsmutanten des PrV mit derjenigen von Wildtyp-Virionen verglichen. Ziel war hier die Analyse von Veränderungen in der Partikelzusammensetzung über den Verlust des deletierten Proteins hinaus, zum Beispiel als Folge einer dadurch nicht mehr möglichen Protein-Protein-Wechselwirkung oder einer abweichenden Morphogenese. Im Vordergrund stand dabei die Untersuchung der Tegumentproteine, da diese in eine Vielzahl von Protein-Protein-Interaktionen einbezogen sind und ihnen eine entscheidende Rolle während der Virusmorphogenese zukommt. Die quantitative Analyse von Mutanten mit Deletionen der Tegumentproteine pUS3, pUL47, pUL49, sowie des Glykoproteins E (Fig.4) (und aller im folgenden erwähnten Mutanten) erfolgte massenspektrometrisch mit der SILAC-Strategie.

Wie für PrV-ΔUL49 bereits beschrieben wurde (del Rio et al., 2005), hatte auch die Deletion der Tegumentproteine pUS3 und pUL47 einen teilweise drastisch vermehrten Einbau von Aktin in das Virion zur Folge (Fig.4B). Dieser Effekt zeigte sich am deutlichsten nach Deletion des sehr abundanten pUL47, was auf eine kompensatorische Akkumulation von Aktin im Viruspartikel hindeutet. Im Gegensatz dazu zieht die Deletion des US3 einen verminderten Einbau des pUL46 nach sich (Fig.4B). Eine stabilisierende Wirkung der Proteinkinase pUS3 auf das Tegumentprotein pUL46 wurde bereits für HSV-2 beschrieben (Matsuzaki et al., 2005). Eine bekannte Interaktion zwischen Virushülle und Tegument wird durch die Bindung des Glykoproteins E an pUL49 verursacht (Fuchs et al., 2002b). gE-negative PrV-Partikel zeigen eine stark verminderte Inkorporation des pUL49 in das Virion, wohingegen das Fehlen von pUL49 keine Auswirkungen auf den Einbau von Glykoprotein E hat (Fig.4B). Dies deutet auf eine Rekrutierung des pUL49 durch gE während der Morphogenese hin (Fuchs et al., 2002b).

Mehrere Ladungs- und Größenvarianten des Tegumentproteins pUL48, welches wegen seiner Funktion während der Transkriptionsregulation auch als *alpha trans-inducing factor* bezeichnet wird, sind Bestandteile von Pr-Virionen. In Abwesenheit von pUL47 und pUL49 konnte ein differentieller Effekt beim Einbau der zwei im 1D-Gel unterscheidbaren Größenvarianten des pUL48 festgestellt werden. Während der Gehalt der größeren UL48-Form (Bande 13) in beiden Mutanten im Vergleich zum PrV-Wildtyp konstant blieb, akkumulierte in beiden Mutanten die kleinere UL48-Form (Bande 14).

(2)“Pseudorabies virus particles lacking tegument proteins pUL11 or pUL16 incorporate less full-length pUL36 than wild-type virus but specifically accumulate a pUL36 N-terminal fragment “

Weiterhin wurde der Einbau des unter allen Herpesviren konservierten größten Genproduktes pUL36 in verschiedenen Präparationen von PrV-Wildtyp und ausgewählten Deletionsmutanten untersucht und mit Hilfe der SILAC-Methode verglichen. Die inkorporierten Mengen des kapsidnahen Tegumentproteins pUL36 erwiesen sich in den untersuchten PrV-Mutanten ΔUS3, ΔUL21, ΔUL49, ΔUL51 und ΔgE als sehr konstant (Fig.2A-C;3A), was auf eine strenge Stöchiometrie zwischen dem Kapsid und dem pUL36 hindeutet. Ausnahme waren ausschließlich die Deletionsmutanten ΔUL11 und ΔUL16 (Fig.2A-C;3A). In diesen Mutanten war der Einbau des pUL36 vermindert. Dieser Verlust wurde aber teilweise durch den Einbau eines C-terminal verkürzten pUL36 kompensiert, welches in Wildtyp-Virionen nur in sehr geringen Mengen vorlag (Fig.3B). Ob dieser Effekt im Zusammenhang mit einem bei HSV-1 nachgewiesenen Komplex aus pUL11 und pUL16 steht, bleibt dennoch unklar.

(3)“Efficient incorporation of tegument proteins pUS3, pUL46, and pUL49 into pseudorabies virus particles depends on the presence of pUL21“

In diesem Teil der Arbeit wurde das Proteinprofil von PrV-ΔUL21 mit dem des PrV-Impfstammes Bartha (PrV-Ba) unter Anwendung der SILAC-Strategie verglichen. PrV-Ba weist in seinem Genom neben Punktmutationen in den Genen UL10 (gM), UL21, UL44 (gC) und US3 eine Deletion in der U_S-Region auf, die die Gene US7 (gI), US8 (gI), US9 und US2 umfasst (Fig.1). Das Proteinprofil von PrV-Ba Virionen gleicht dem einer UL21-Deletionsmutante, indem die Tegumentproteine pUS3, pUL46 und pUL49 als Strukturkomponenten fast vollständig fehlen (Fig.2). So konnte das pUL21 als wichtiger Bestandteil des Teguments, welcher direkt oder über weitere Interaktionen an der Inkorporation von pUS3, pUL46 und pUL49 beteiligt ist, identifiziert werden. Damit wurde ausgeschlossen, dass die Deletion in der U_S-Region und die Mutationen im gC, gM oder US3 für den verminderten Einbau von pUS3, pUL46 und pUL49 in PrV-Ba verantwortlich sind (Lyman et al., 2003).

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Composition of Pseudorabies Virus Particles Lacking Tegument Protein US3, UL47, or UL49 or Envelope Glycoprotein E

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Proteins located in the tegument layer of herpesvirus particles play important roles in the replicative cycle at both early and late times after infection. As major constituents of the virion, they execute important functions in particular during formation of progeny virions. These functions have mostly been elucidated by construction and analysis of mutant viruses deleted in single or multiple tegument protein-encoding genes (reviewed in the work of T. C. Mettenleiter, Virus Res. 106:167–180, 2004). However, since tegument proteins have been shown to be involved in numerous protein-protein interactions, the impact of single protein deletions on the composition of the virus particle is unknown, but they could impair correct interpretation of the results. To analyze how the absence of single virion constituents influences virion composition, we established a procedure to assay relative amounts of virion structural proteins in deletion mutants of the alphaherpesvirus *Pseudorabies virus* (PrV) in comparison to wild-type particles. The assay is based on the mass spectrometric quantitation of virion protein-derived peptides carrying stable isotope mass tags. After deletion of the US3, UL47, UL49, or glycoprotein E gene, relative amounts of a capsid protein (UL38), a capsid-associated protein (UL25), several tegument proteins (UL36 and UL47, if present), and glycoprotein H were unaffected, whereas the content of other tegument proteins (UL46, UL48, and UL49, if present) varied significantly. In the case of the UL48 gene product, a specific increase in incorporation of a smaller isoform was observed after deletion of the UL47 or UL49 gene, whereas a larger isoform remained unaffected. The cellular protein actin was enriched in virions of mutants deficient in any of the tegument proteins UL47, UL49, or US3. By two-dimensional gel electrophoresis multiple isoforms of host cell-derived heat shock protein 70 and annexins A1 and A2 were also identified as structural components of PrV virions.

Pseudorabies virus (PrV) is a member of the *Alphaherpesvirinae* subfamily in the family *Herpesviridae*. Beyond its importance as the causative agent of Aujeszky's disease in pigs (33), it has been widely used as a model for the study of herpesvirus entry and morphogenesis (34, 35). Herpesvirus virions consist of four subviral structural components: the double-stranded genomic DNA in the core is enclosed in an icosahedral capsid which, in turn, is surrounded by a lipid bilayer called the envelope which contains virally encoded glycosylated and nonglycosylated proteins. Between envelope and capsid resides the tegument, which is the most complex structure of the virion, presumably linking the nucleocapsid and the envelope. Of the over 70 genes that were found within the 143-kbp PrV genome, 31 have been shown to code for structural components of the virion. Thirteen of them have been assigned to the tegument layer (24), underlining its importance for the structural and functional integrity of the virion. In keeping with its proposed role in virion formation, a number of interactions between tegument proteins among themselves and with other structural proteins have been described in PrV and other herpesviruses (reviewed in references 33 and 34). Electron microscopic studies of cells infected with mutants deleted in tegument proteins US3 (23), UL11 (27), UL16 (20), UL36 (10), UL37 (21), UL47 (28), UL48 (8), and UL51 (22) have documented the relevance of these tegument proteins for morphogenesis of PrV.

In addition to their structural function, catalytic activity has also been found in tegument proteins. The US3 protein is a protein kinase in PrV (58) and other alphaherpesviruses, e.g., Marek's disease virus (45) and herpes simplex virus type 1 (HSV-1) (7). The UL13 gene product of HSV-1 has also been reported to phosphorylate other viral proteins (42). A deubiquitinating protease activity has been assigned to the HSV-1 UL36 protein (19).

Due to the network of protein-protein interactions involving tegument components, it appears likely that deletion of single tegument proteins may lead to alterations in the overall composition of mutant virus particles beyond the loss of the targeted gene product. Higher amounts of actin were detected in virions of a UL49 deletion mutant of PrV than in wild-type virus particles (4). In the case of a UL20 deletion mutant of PrV, Dietz and coworkers (5) found that the UL53 gene product, glycoprotein K (gK), was not properly processed and localized. Deletion of the US3 gene in HSV-2 led to instability and to drastically decreased incorporation of the UL46 protein into the virus particle (32).

In this study we present a systematic approach to the quantitation of virus structural proteins. Highly purified virion preparations were analyzed by two-dimensional (2D) electrophoresis, and structural components were identified by peptide mass fingerprint analysis. Structural proteins from four PrV mutants deleted in a single gene each were quantified from one-dimensional (1D) gels. A method outlined by Ong and coworkers

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(39) and designated "stable isotope labeling with amino acids in cell culture" (SILAC) was used. The key feature of this procedure is the generation of a global internal standard by metabolic introduction of a stable isotope tag into either sample or reference, whereas the other is cultured under conventional conditions. Samples are then mixed, proteins are separated, and relative amounts of proteins are calculated after tryptic digestion from the intensity ratios of mass-tagged and conventional peaks from the mass spectra. Mutants with single deletions in genes coding for tegument proteins UL47, UL49, and US3 were analyzed, and a gE deletion mutant representing the deletion of a nontegument protein was also included.

MATERIALS AND METHODS

Cells and viruses. PrV mutants deleted in US3 (PrV-ΔUS3) (23), UL47 (PrV-ΔUL47) (28), UL49 (PrV-ΔUL49) (9), and glycoprotein E (PrV-ΔgE) (26) were derived from PrV strain Kaplan (PrV-Ka) (17). They were propagated on porcine kidney cells (Collection of Cell Lines in Veterinary Medicine, Insel Riems, Germany; RIE008). For titrations, 10-fold serial dilutions of virus suspensions were plated onto monolayers of Madin-Darby bovine kidney cells (MDBK cells, supplied by the Collection of Cell Lines in Veterinary Medicine) (31). Inocula were removed after 1 h, and cells were stained with crystal violet after further incubation at 37°C for 48 h.

Purification of pseudorabies virus virions. Twelve cell culture flasks with a 162-cm^2 growth area of confluent porcine kidney cells were infected at a multiplicity of infection of 1, and cell culture supernatants were harvested between 24 and 30 h after infection. Purification by sucrose gradient centrifugation was performed as described previously (18). Briefly, cellular debris was removed, and virus was purified by sedimentation through a 40% sucrose cushion followed by sucrose density gradient centrifugation. The quality of the virion preparation was verified by electron microscopy.

Stable isotope tagging. The SILAC procedure outlined by Ong and coworkers (39) was adapted. Dulbecco's modified Eagle's medium (DME)–nutrient mixture F12 Ham medium (1:1) (Sigma-Aldrich D-9785) was supplemented with 5% dialyzed fetal calf serum and with all missing amino acids (Sigma-Aldrich) except L-leucine. The medium was then divided and supplemented with either conventional L-leucine (Sigma-Aldrich) or deuterated L-leucine (L-leucine-5,5,5 d_3; Sigma-Aldrich catalog no. 486825; 99 atom% D) to yield ProLeu–DME/F-12 or DeuLeu–DME/F-12 cell culture medium, respectively. Porcine kidney cells were passaged in parallel in both media at a 1:6 ratio every 3 days. After four passages samples of the cell cultures were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and several protein bands were excised and tryptically digested. The isotope exchange in the deuterated cell batch was then controlled by mass spectrometry. Incorporation of L-leucine-5,5,5 d_3 increases the mass of leucine-containing peptides by 3 mass units per leucine residue. Virus stocks were propagated in porcine kidney cells adapted to ProLeu–DME/F-12 or DeuLeu–DME/F-12. In the latter case, complete exchange of conventional L-leucine with deuterated L-leucine in the virion proteins was ensured by an additional virus passage in porcine kidney cells grown in DeuLeu–DME/F-12.

One-dimensional gel electrophoresis. Samples containing 25 μg each of protonated and deuterated gradient-purified virions were separated in discontinuous SDS-12% polyacrylamide gels using either standard conditions (29) or a bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane-HCl–2-(*N*-morpholino) ethanesulfonic acid-buffered electrophoresis system (11) in a Protean II electrophoresis chamber (Bio-Rad, Munich, Germany). For convenience wild-type PrV was labeled with L-leucine-5,5,5 d_3, and the mutants were grown in medium containing conventional L-leucine. After Coomassie blue staining (37) samples were cut out of the centers of the protein bands and processed for peptide mass fingerprint analysis.

Two-dimensional gel electrophoresis. Samples containing 60 μg of purified PrV were sedimented (Beckman TLA45 rotor, 45 min, 45,000 rpm, 4°C) and extracted with rehydration buffer for isoelectric focusing (8 M urea, 2 M thiourea, 1% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, 20 mM dithiothreitol) (3) by sonication and shaking for 1 h at 20°C. Isoelectric focusing was performed on nonlinear 24-cm IPG strips (Amersham, Braunschweig, Germany) or ReadyStrips (Bio-Rad) in the pH range of 3 to 10 by using an isoelectric focusing cell (Bio-Rad). Thereafter, strips were sequentially equilibrated in buffers containing dithiothreitol and iodoacetamide as recommended by the manufacturers. For electrophoresis in the second dimension hand-cast 12% acrylamide gels were run in Dodeca Cell or Protean II electrophoresis chambers (Bio-Rad). Gels were fixed and stained overnight with colloidal Coomassie brilliant blue (37). Protein spots of interest were picked as gel plugs of 1.5 mm in diameter.

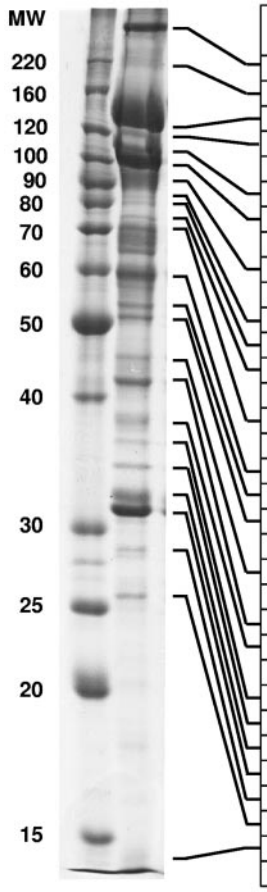
Peptide mass fingerprints. In-gel tryptic digestion (43) was carried out in 96-well V-bottomed polypropylene microtiter plates with 30 ng trypsin (Promega catalog no. V5111; Mannheim, Germany) per sample for 3 h at 37°C or overnight at 30°C. Mass spectra were registered on a Bruker Ultraflex instrument (Bruker Daltonics, Bremen, Germany) and processed by flexAnalysis 2.0 software (Bruker). For the quantitation of pairs of mass-tagged peptide peaks, it was crucial to choose the SNAP option as peak detection algorithm in the flexAnalysis software, which is robust with respect to overlapping peaks. For database queries following 2D electrophoresis carbamidomethylation was set as fixed modification. In some cases, an additional optional modification allowing a 3-Da mass shift corresponding to an exchange of leucine with deuterated leucine was included. The batch database search (MASCOT Server 2.0.0 software; Matrix Science Ltd., London, United Kingdom) (41) was launched by Biotools 2.2 (Bruker). Proteins were considered identified whenever statistically significant molecular weight search (MOWSE) scores were attained (40) using SwissProt, NCBI, or an in-house database covering the published PrV proteome (24). In some cases, MOWSE scores reached significant levels only after information on leucine content of isotope-labeled peak pairs was added in the query (47).

Quantitation of mass spectrometric data. Evaluation was carried out with an Excel (Microsoft, Redmond, Wash.) spreadsheet. After database search, peaks representing unmodified tryptic peptides of the hit with the highest MOWSE score were selected. The expected masses of the corresponding L-leucine-d_3-labeled peptides were calculated from the leucine content of the peptide. If the calculated masses were found in the peak list within error margins of 50 ppm, the peak pairs were selected for quantitative evaluation. After manual revision mean values of the intensity ratios of all qualified peak pairs representing the identified protein were normalized to the intensity ratio of the 142-kDa form of the UL19 major capsid protein from the same gel. By this normalization step, all protein ratios were referred to the relative content of the major capsid protein in both virus preparations, which reflects the ratio of virus particles, since the number of major capsid protein molecules is 960 for any virus particle (50).

RESULTS

Identification of PrV structural proteins. Proteins from highly purified PrV preparations were resolved by 1D (Fig. 1) or 2D (Fig. 2) electrophoresis and identified by peptide mass fingerprint analysis. Figure 1 shows a representative 1D gel of purified PrV virions indicating the major components of the 24 annotated bands as well as the experimentally derived and calculated molecular weights. In several bands, more than one structural component of PrV was identified, whereas several virion components were found in multiple bands. We did not identify any protein which had not previously been characterized as a structural virion component of PrV or of other alphaherpesviruses (24, 50). On the other hand, our analysis did not resolve all proteins which had been described as components of PrV virions, in particular constituents of the viral envelope. Whereas most of the 27 virally encoded structural proteins that were identified yielded the calculated molecular weight and isoelectric point, several results were noteworthy.

As expected, after 1D electrophoresis the major glycoprotein gB of PrV was detected primarily in three major bands which represent the uncleaved precursor as well as the N-terminal and C-terminal subunits of the disulfide-linked heterodimer (Fig. 1, bands 4, 9, and 11) (15, 30, 54), although gB-related peptides were also observed in adjacent bands possibly due to differential glycosylation resulting in a range of molecular masses. In the 2D gel the larger N-terminal subunit was present in a multitude of spots which most probably represent glycosylation isoforms exhibiting isoelectric points vary-



band	MWexp	gene	protein	location	MW calculated
1	>300	UL36		T	324.406
2	200	IE180	ICP4	?	148.600
3	137	UL19	major capsid protein	C	145.963
4	126	UL27	gB precursor	E	100.234
		US8	gE	E	62.410
5	110	UL46		T	75.555
6	103	UL47		T	80.379
		UL37		T	98.196
7	91	UL22	gH	E	69.024
		UL44	gC	E	51.234
8	83		HSP70	?	70.083
9	81	UL27	gB N-terminus	E	50.894
10	74	UL27	gB N-terminus	E	50.894
		UL17		C	64.255
11	69	UL21		T	55.233
		UL27	gB C-terminus	E	45.366
12	60	UL25		C	57.404
13	53	UL48	αTIF	T	45.116
14	51	UL48	αTIF	T	45.116
		US6	gD	E	44.338
15	46		actin	?	41.816
		UL10	gM	E	41.545
16	44	UL38		C	39.952
17	40	US3	protein kinase	T	36.879
		UL16		T	34.812
18	38		annexin A2	?	38.759
19	36	UL49		T	25.920
20	33	UL18		C	31.609
21	32	UL49		T	25.920
22	29	US2		T	27.757
23	26	UL26	VP24 homolog	C	25.514
24	14	UL35		C	11.474
		UL3.5		?	24.031

FIG. 1. Protein profile of purified wild-type PrV-Ka virions after one-dimensional analysis. Protein bands visualized by Coomassie blue staining (left) were numbered from 1 to 24 (first column), and components were identified by peptide mass fingerprint analysis. In several bands, more than one gene product was identified, and several proteins appeared in more than one band. Bands were annotated according to their major component (third column), but the presence of additional components is also indicated. Calculated and observed molecular weights (MW; in thousands) of the gene products (fourth column) are indicated (second and sixth columns). Predicted or confirmed subviral locations of the identified proteins are listed in the fifth column (C = capsid; T = tegument; E = envelope; ? = unclear). All proteins listed here were identified with statistically significant MOWSE scores.

ing from highly acidic to neutral values in a broad range (Fig. 2).

The identified UL26 gene product exhibited a molecular mass below 30 kDa, which is too small considering the calculated molecular mass of 54 kDa for the full-length translation product. In HSV-1 the incorporation into mature virions of only the N-terminal processing product of the UL26 primary translation product designated VP24 has been shown (48). The same apparently applies to the PrV UL26 protein since the tryptic peptides found in virus particles exclusively cover the N-terminal part of UL26.

The UL19 gene product was identified in multiple spots of the expected size of approximately 142 kDa after 2D analysis (Fig. 2). However, in addition seven protein spots which represent C-terminal fragments of 65 to 70 kDa were also found. Charge and/or molecular weight variants have also been observed for the UL18, UL37, and UL48 gene products.

The PrV immediate-early protein 180 (IE180) (16) has been identified in purified virions after 1D electrophoresis (Fig. 1, band 2). For PrV, the presence of this regulatory protein in the virion has not yet been reported, whereas its homolog ICP4 is part of the HSV-1 tegument (57).

In addition to virally encoded components, cellular proteins were also found as structural components of the virion. The presence of actin in the PrV particle (55) was confirmed. We also identified annexins A1 and A2 in PrV virions as well as heat shock protein 70 (HSP70) (Fig. 1 and 2). After 2D electrophoretic separation, three distinct spots were identified as HSP70, and two charge variants each were observed for annexins A1 and A2, respectively. In contrast, actin was represented by one isoform (Fig. 2).

Quantitation of PrV structural components in PrV mutants. To gain insight into the reproducibility of the virus purification procedure and the variation of relative protein contents between virus batches, preparations of mass-tagged and conventional wild-type PrV were mixed and analyzed (Fig. 3). After normalization to the 142-kDa form of the major capsid protein UL19 (MCP142), the relative abundance of all analyzed proteins was close to 1.0, indicating that the purification procedure yielded preparations with very little batch-to-batch variation in

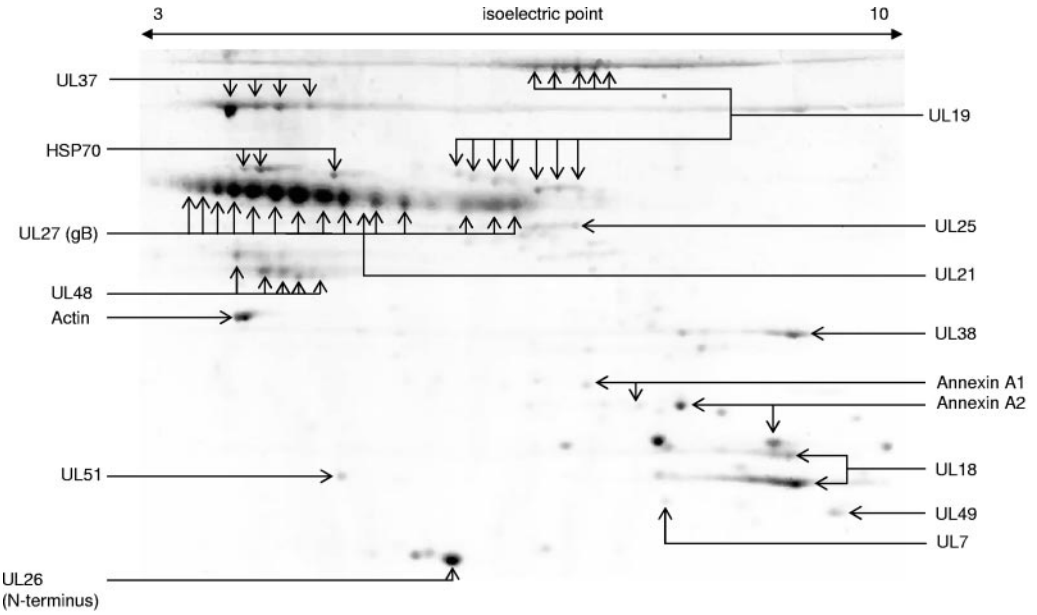


FIG. 2. Proteins from purified wild-type PrV-Ka virions were separated by two-dimensional electrophoresis, and the resulting protein spots were identified by peptide mass fingerprint analysis. Isoelectric focusing strips were used in the nonlinear gradient range of pH 3 to 10. A number of proteins were found in multiple size and/or charge modifications. Note that isoelectric points of gB isoforms vary from values close to 3 to up to 7. The UL48 protein appears in two horizontal chains of charge variants corresponding to bands 13 and 14 from the one-dimensional analysis (Fig. 1).

relative protein content. Margins of error which indicate the variation of the intensity ratios of the peptides used for quantitation rarely exceeded 15%.

In Fig. 4 data on the relative amounts of structural proteins located in or associated with the capsid (UL25 and UL38), tegument (UL36, UL46, UL47, UL48, UL49, and actin) or envelope (UL22/gH) are summarized. The results were obtained by analyses of two independent virus preparations (black or white bars) of PrV-ΔUS3, PrV-ΔUL47, PrV-ΔUL49, or PrV-ΔgE compared with mass-tagged wild-type PrV-Ka. Proteins to be analyzed were selected on the basis of an unambiguous identification in 1D gels and satisfactory data acquisition. In Fig. 4A, proteins are summarized which do not show quantitative variation in the mutant viruses. This had been expected for the capsid protein UL38 but was also found for the capsid-associated UL25 protein and tegument proteins UL36 and UL47 (if present). Glycoprotein H, the UL22 gene product, was also incorporated into virions in constant amounts (Fig. 4A). In contrast, variation of the other identified glycoproteins gB, gC, gD, and gE among preparations was so extensive that no consistent data could be acquired.

In contrast, the relative quantities of proteins represented in Fig. 4B changed drastically upon deletion of other proteins. Deletion of any of the tegument proteins US3, UL47, and UL49, but not deletion of glycoprotein E, led to a striking accumulation of actin which had previously been reported only for a UL49 deletion mutant of PrV (4). In fact, incorporation of actin was even more pronounced in the absence of UL47, resulting in a 5- to 10-fold increase in the virus particle. A decrease of tegument protein UL46 was observed after deletion of US3, which parallels an observed instability and decreased incorporation of the UL46 protein into HSV-2 particles in the absence of the US3 protein (32). Deletion of gE was accompanied by a marked decrease of UL49 content, which could be explained by the interaction of these two proteins (9).

In Fig. 4C, a differential effect of the deletion of UL47 or UL49 on the relative quantities of the two identified isoforms of the UL48 protein is demonstrated. Whereas the smaller isoform present in band 14 (Fig. 1) accumulated significantly, the amount of UL48 in band 13 remained constant.

DISCUSSION

A common strategy for elucidating the function of viral proteins is the construction and analysis of specific deletion mutants and corresponding rescuant viruses. However, the correct interpretation of the data and the assignment of a specific function to a specific viral gene product often rely on the assumption that the absence of a single protein does not impair any other viral function in *trans*. This is particularly relevant in studies of virion morphogenesis since virion formation in the herpesviruses entails a complex network of protein-protein interactions which are only incompletely understood (34, 35, 53). In this paper, we analyzed wild-type PrV virions by 1D and 2D electrophoresis and devised a strategy for exact relative quantitation of structural components of PrV particles using selective protein label with mass-tagged leucine.

By 1D electrophoresis 24 major protein bands derived from purified virions were identified which, by mass spectrometry, could be annotated to 25 different viral gene products. All of them had previously been characterized as structural components of virions of PrV or of other alphaherpesviruses (24, 50). We are aware that our analysis did not identify all virion constituents and that, in particular, viral envelope proteins

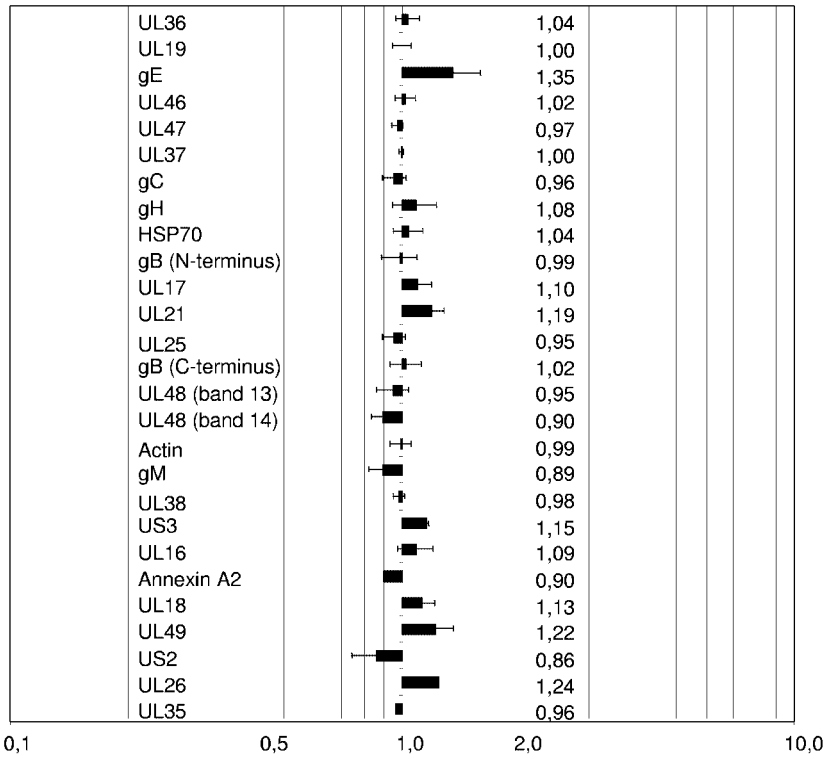


FIG. 3. Structural proteins from two independent preparations of wild-type PrV-Ka were quantitated as described in Materials and Methods. After normalization to the UL19 gene product MCP142, values for all identified proteins were near 1.0, indicating that the purification procedure is reliable. Numbers indicate the relative abundances of the respective proteins in the mass-tagged PrV-Ka preparation compared to the untagged preparation. Error bars indicate the variation of the intensity ratios of the peptides used for the quantitation.

were not adequately represented. By alterations in the gel systems this could possibly be overcome, if desired. Whereas most of the identified proteins exhibited the expected molecular weight and isoelectric points calculated from their primary amino acid sequence, variations did occur.

Perhaps most strikingly, the major glycoprotein B, which, after 1D separation, was identified as the uncleaved precursor as well as the amino- and carboxy-terminal cleavage products (15, 30, 54), produced a multitude of spots after 2D electrophoresis. They could all be assigned to the amino-terminal subunit and most likely represent glycosylation isoforms. The biological relevance of this striking variance resulting in isoelectric points between pH 3 and 7 is unclear at present. Differentially charged variants of glycoproteins gB, gC, and gD which were shown to be modified by sialic acid have also been found after infection of BHK cells with HSV-1 (14).

The major capsid protein UL19 also exhibited multiple isoforms after 2D electrophoresis (Fig. 2) which have not been described so far. Moreover, besides the 142-kDa form of the protein, seven protein spots which represent C-terminal fragments of 65 to 70 kDa were also identified. So far it is unclear whether these fragments were formed during the analytic process or are indeed present in the virion and serve a function there. Charge isoforms were also observed for the UL18, UL37, and UL48 gene products.

The incorporation of cellular proteins into viral particles is rather common. Actin has been detected in a number of other enveloped viruses, such as human immunodeficiency virus (1), human respiratory syncytial virus (51), or rabies virus (36), and actin-like filaments have been found located at the outer rim of the tegument in the vicinity of the viral envelope in a cryo-electron tomographic reconstruction of the HSV-1 particle (12). Actin has also previously been detected in PrV (55). By 2D analysis we also identified annexins A1 and A2 as constituents of purified PrV virions. The presence of annexins in herpesvirus particles has been observed before (52, 56), which in the light of their capacity to bind both actin and phospholipids is not surprising since both are present in herpesvirus virions. We also detected heat shock protein 70 in PrV virions, which was identified in three distinct spots after 2D gel electrophoresis. Due to the high homology between the highly inducible HSP70 and the constitutively expressed HSC70 protein, we were not able to reliably differentiate between these two proteins. Both are related to the life cycle of alphaherpesviruses as they are recruited into the nucleus after infection with HSV-1 (2). Kobayashi and coworkers (25) reported a drastic upregulation of HSP70 after infection with HSV-1 or HSV-2, but so far there has been no evidence for the incorporation of this protein into herpes simplex virus virions. In contrast, HSP70 has been reported as a structural component of human cytomegalovirus (52), rabies virus (44), and primate lentiviruses (13).

For exact quantitative comparison between wild-type and mutant PrV particles we used the SILAC technique. This procedure conveniently allows the quantitation of virus and host cell-derived structural components of purified virus particles

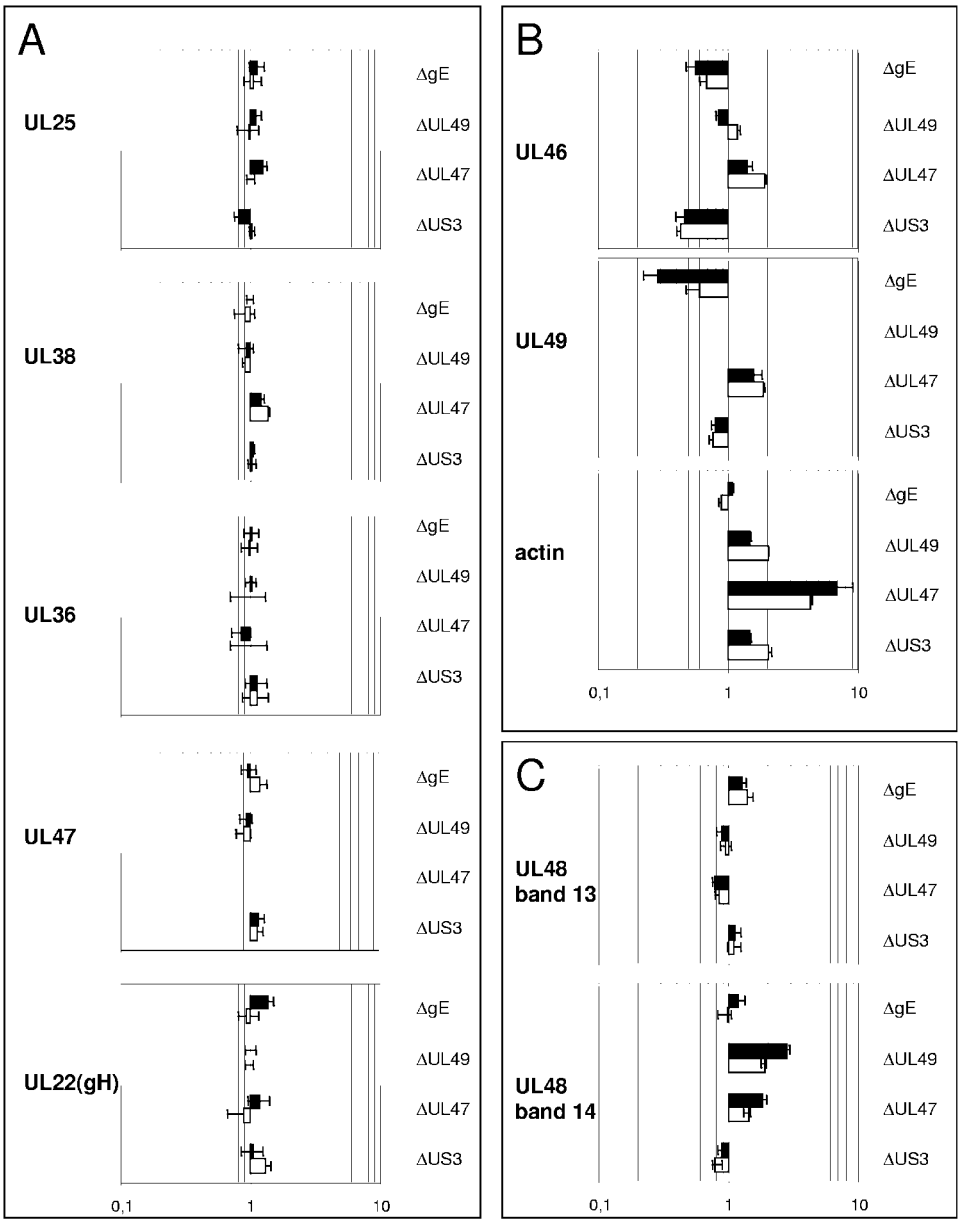


FIG. 4. Relative abundances of structural proteins in PrV mutants compared to wild-type PrV-Ka. Black and white bars represent two independent experiments. Error bars indicate the variation of the intensity ratios of the peptides from one spectrum selected for quantitation. Deleted genes are indicated on the right; the identity of the quantitated protein is given on the left. (A) Proteins with no significant changes in relative abundances after deletion of the US3, UL47, UL49, or gE genes. (B) Proteins that exhibit variations upon deletion of gE, UL49, UL47, or US3. (C) Differential effects on incorporation of two isoforms of UL48 are documented. After deletion of UL47 or UL49, the relative content of UL48 in the lower-molecular-weight band 14 (Fig. 1) increased, whereas the higher-molecular-weight form of UL48 present in band 13 remained stable.

independent of immunological reagents. The use of a global internal standard abolishes the influence of gel-to-gel variations, and in addition to the classical SILAC approach, the subsequent normalization to the 142-kDa major capsid protein encoded by the UL19 gene corrects for any variations in the amount of input virus. Taken together, this led to a very low variance in the measurement of relative protein contents so that even minor changes could be detected. Since data were selected from peptides reliably representing the protein to be evaluated, any disturbing background which is often present after 1D electrophoresis was reduced to a minimum. This allowed us to choose 1D electrophoretic gels as a starting point for the quantitation, taking into account the higher solubility of most proteins in SDS-containing sample buffer compared to the rehydration buffer used for first-dimension isoelectric focusing. The presence of more than one protein species in one band did not seriously impair identification or quantitation of a specific protein. Nevertheless, 2D analysis has been shown to be useful and two structural proteins (UL7 and UL51) could not be identified from 1D separations but were identified only after 2D electrophoresis.

The comparison of different batches of purified wild-type

PrV virions by the SILAC procedure demonstrated its superb reproducibility. The variation in the incorporation of a multitude of viral and the several identified cellular proteins was minor, and relative amounts of these proteins stayed close to 1.0 (Fig. 3). Thus, there was only little batch-to-batch variation, which provided the basis for subsequent analyses comparing wild-type and mutant viruses for their protein content.

To this end we analyzed PrV mutants lacking the tegument protein US3, UL47, or UL49 or glycoprotein gE for alterations in protein composition of viral particles. Using this novel approach, we were able to confirm the increased incorporation into PrV particles of actin in the absence of UL49 (4). In the absence of US3, UL46 incorporation decreased, which parallels data from HSV-2 in which deletion of US3 resulted in a concomitant loss of the UL46 protein due to instability (32). However, we did not assay whether the decrease of UL46 incorporation in the PrV US3 deletion mutant results from instability of the UL46 protein or is due solely to less efficient packaging of the protein into progeny virions. Together, these results were reassuring since they served to validate our approach. Interestingly, an accumulation of actin was also detected after deletion of US3 or UL47 but not after deletion of glycoprotein E. In fact, incorporation of actin was even more increased in the absence of UL47 than in the absence of UL49, whereas lack of US3 resulted in a similar level of actin incorporation as lack of UL49 did. However, an indirect effect of the US3 deletion on actin incorporation by reduction of the incorporation of the UL46 protein could not be excluded. These data may suggest that the tegument is replenished to a critical volume by cellular actin whenever the major viral component UL46, UL47, or UL49 is decreased or absent (4).

The decrease in the level of UL49 protein in PrV-ΔgE virions is in line with our previous observation that UL49 and gE interact. Thus, the presence of gE might support the recruitment of UL49 into the virion during morphogenesis, whereas UL49 is not required for incorporation of gE (9). We have previously shown that the PrV UL49 protein also interacts with gM and that deletion of both gE and gM resulted in a complete lack of UL49 incorporation (9). Thus, the residual amount of UL49 present in the gE deletion mutant might be recruited via gM. Again, the finding that UL49 incorporation is decreased in the absence of only gE demonstrates the power of our assay system.

Particularly striking was the result that not only the bona fide capsid protein UL38 and the capsid-associated UL25 but also components of the inner tegument, UL36, and the proposed outer tegument, UL47, remain unaffected by the deletion of US3, UL47, UL49, or gE. Moreover, gH was also present in invariant amounts in all the different virus mutants tested. Thus, there appears to be a strict molecular stoichiometry of these proteins in the virus particle which is independent of the other tegument and envelope components tested. This is not true for other envelope proteins such as gB, gC, gD, or gE, whose amount in virions varies extensively even between different batches of wild-type virus (data not shown). Therefore, in future experiments targeting specifically the envelope of mature virions, e.g., for analysis of the movement of glycoprotein-containing virions or vesicles, the visualization of gH might provide a more accurate picture than the observation of other virally encoded glycoproteins.

After two-dimensional electrophoresis (Fig. 4) bands 13 and 14 of 1D electrophoretic separations have been shown to be composed of a multitude of spots that are derived from the UL48 protein. The charge variants found within band 14 most probably represent different levels of phosphorylation which have been found in the UL48 homolog of HSV-1, the VP16 protein (38). Whereas the higher-molecular-weight species of UL48 (band 13) was present in invariant amounts in all mutant viruses tested, the lower-molecular-weight form of the protein (band 14) was specifically enriched in mutant viruses lacking UL49 or UL47. Further analyses will show if the observed compensation of the UL47 or UL49 proteins by UL48 protein is specific for single isoforms appearing in band 14. Thus, we found a differential regulation of incorporation of UL48 gene products into the PrV virion. The UL48 protein of HSV-1 has been demonstrated to physically interact with the UL36, UL41, UL46, UL47, and UL49 tegument proteins (6, 46, 49, 53). From our data it can be deduced that at least two different forms of the PrV UL48 protein exist which are differentially affected by mutations influencing virion composition. It would be interesting to analyze whether two different isoforms of the UL48 protein are also formed during HSV-1 infection and whether at least a part of the observed interactions may be isoform specific.

Finally, our approach for exact quantitation of virion constituents should be generally applicable to any other virus that can be propagated and isotope labeled in cell culture and highly purified in sufficient amounts and that contains a protein suitable for calibration. Thus, this method could become standard in the exact molecular analysis and comparison of virus particles derived from different parental strains, isolates, or mutants.

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Pseudorabies virus particles lacking tegument proteins pUL11 or pUL16 incorporate less full-length pUL36 than wild-type virus but specifically accumulate a pUL36 N-terminal fragment

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ABSTRACT

Proteins of the virion tegument of alphaherpesviruses are involved in protein-protein interactions which play important roles in viral morphogenesis. Seven single gene deletion mutants of pseudorabies virus were analyzed for alterations in the overall composition of the virion beyond the loss of the targeted protein. The UL36 protein (pUL36) was present in equal amounts in wild-type virions and mutants lacking pUL21, pUL49, pUL51, pUS3, or pUS8. Virions lacking pUL11 or pUL16 incorporated less full-length pUL36 than wild-type particles but contained increased amounts of an N-terminal fragment of pUL36 being present only in traces in wild-type virus or the other mutants.

MAIN TEXT

Homologs of the large tegument protein encoded by the UL36 open reading frame of herpes simplex virus are present in all members of the Herpesviridae analyzed so far. They constitute the largest herpesviral gene products and are proposed to be part of the capsid-proximal inner tegument, a structure which resides between nucleocapsid and envelope. PrV pUL36 (Klupp et al., 2002) is a 3.084aa protein which is strictly essential for viral replication (Fuchs et al., 2004). PrV pUL36 function is important for virion morphogenesis in the cytoplasm after nuclear egress. pUL36 interacts with pUL37, another conserved tegument protein. However, removal of the N-terminally located pUL37-binding domain did not result in a complete loss of function of pUL36 indicating that the essentiality of this protein is due to additional, still unknown functions (Fuchs et al., 2004). Recently, a deubiquitinating proteolytic activity was demonstrated to reside in the very N-terminus of herpesvirus pUL36 homologs (Kattenhorn et al., 2005). Thus, both hitherto assigned functional regions of pUL36

reside in the N-terminal part of the protein. It was shown in a previous study that PrV particles devoid of the tegument proteins pUS3, pUL47, pUL49 or pUS8 (the envelope glycoprotein E) incorporated the same amounts of several tegument proteins like pUL36, pUL37 and pUL47 as wild-type virions (Michael et al., 2006). This stoichiometric incorporation suggested that, like the assembly of the capsid, the architecture of the tegument might in part be strictly controlled. On the other hand, amounts of other tegument proteins like pUL46, pUL48, and pUL49 varied to some extent among the different mutants, indicating also some degree of flexibility in the tegument composition. The suggested interaction of pUL36 with pentonal sites on the capsid (Zhou et al., 1999) which are devoid of the small capsid protein pUL35 which decorates hexons may, in part, explain this strict stoichiometry.

In a study to analyze single protein deleted PrV mutants for secondary alterations in composition of the virion, we quantified structural proteins in virus particles by a procedure designated as ‚stable isotope labelling by amino acids in cell culture' (SILAC) developed by Ong and coworkers (Ong et al., 2002) as adapted for virions by our group (Michael et al., 2006).

Virus particles from infected porcine kidney cell cultures were purified by sucrose-density gradient centrifugation (Karger et al., 1998). While deletion mutants were propagated on cells maintained in DME/F12 cell culture medium (Sigma-Aldrich D-9785) supplemented with conventional leucine, wild-type PrV-Ka (Kaplan & Vatter, 1959) was harvested from cells which had been cultured in medium containing exclusively deuterated leucine (L-Leucine-5,5,5 d_3 , Sigma-Aldrich #486825, 99 atom % D) inserting a mass tag of 3 dalton per leucine residue. Mutant and mass tagged purified PrV-Ka were mixed at 1:1 protein ratios, and proteins were separated by

sodium dodecyl sulfate-polyacrylamid gel electrophoresis (SDS-PAGE, (Laemmli, 1970)). Protein bands of interest were cut from the Coomassie stained gels (Neuhoff et al., 1988) and digested with trypsin for peptide mass fingerprint (PMF) analysis (Rosenfeld et al., 1992).

Matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectra were registered on an Ultraflex Instrument (Bruker, Bremen, Germany) and the data was further processed by FlexAnalysis and BioTools software (Bruker). Database queries were performed with MASCOT (Matrixscience, London, UK, (Perkins et al., 1999)) software using an in-house database representing the PrV proteome (Klupp et al., 2004). Quantitative evaluation of the spectra was carried out by an in-house software. Mass lists were screened for peak pairs representing leucine containing pUL36 specific peptides in the conventional and the mass tagged variant, and an average intensity ratio reflecting the relative amounts of protein originating from wild-type PrV or mutant virions was calculated from all qualified peaks of one spectrum. Tryptic peptides derived from the major capsid protein which is present in 960 copies (Steven & Spear, 1997) in every intact virion were used as an internal standard (Michael et al., 2006). Values above 1.0 indicate an excess of the protein in the mutant particle and vice versa. Deletion mutants which were compared to their parental strain PrV-Ka have been described elsewhere (PrV-ΔUL11 (Kopp et al., 2003); PrV-ΔUL16, PrV-ΔUL21 (Klupp et al., 2005a); PrV-ΔUL49 (Fuchs et al., 2002), PrV-ΔUL51 (Klupp et al., 2005b), PrV-ΔUS3 (Klupp et al., 2001), PrV-ΔgE (Kopp et al., 2004)).

To assay for a potential variation in pUL36 incorporation in different wt PrV strains, virions of PrV-Ka, PrV-Becker (Robbins et al., 1984) or PrV-NIA-3 (Baskerville, 1973)

were analyzed for pUL36 incorporation. The protein migrating at the calculated molecular weight of 330 kDa for the full-length pUL36 (Fig.1) was identified as pUL36 by PMF and by immunoblot analysis (Towbin et al., 1979) with a pUL36 specific antiserum (Fig. 1B; (Klupp et al., 2002)). No difference was observed between the three different wild-type PrV strains in the level of incorporation as deduced from the gel or in the size of the packaged pUL36.

Analysis in SDS-PAGE of PrV mutants deleted in single tegument components as well as glycoprotein E (gE) produced an unexpected result. Whereas in mutants lacking pUS3, pUL21, pUL49, pUL51 or gE pUL36 appeared like in PrV-Ka, an additional protein of approximately 220 kDa appeared rather prominent in PrV-ΔUL11 and PrV-ΔUL16 virions in Coomassie stained gels (Fig. 2A). Silver staining with prolonged development (Fig. 2B) revealed that mutants lacking pUS3, pUL21, pUL49, pUL51 or gE contained only minute amounts of proteins migrating in this region of the gel (Fig. 2B). In an immunoblot analysis, the additional approximately 220 kDa protein found in PrV-ΔUL11 and PrV-ΔUL16 reacted with an pUL36 specific antiserum (Fig. 2C), but after prolonged film exposure faint reactivity in this region of the blot was also found in PrV-Ka and the other mutants, indicating that pUL36 derived proteins of the same approximate size were also present, although much less abundantly.

By PMF, the approximately 220 kDa protein could be identified as an N-terminal fragment of the pUL36. Tryptic peptides representing the pUL36 N-terminal fragment partially covered the pUL36 sequence (Swiss-Prot Q8UZ11, 3085 amino acids) from amino acids 118 to 2091, whereas tryptic peptides originating from the full-length form partially covered the sequence from amino acid 118 to 3081. The N-terminal

fragment of pUL36 found in PrV-ΔUL16 was slightly smaller than in PrV-ΔUL11, but this size difference did not translate into different peptide coverages in the PMF analysis. Thus, it remains unclear whether the different apparent molecular masses result from additional truncations of pUL36 found in PrV-ΔUL16 virions or from differential post translational modifications. Although present in PrV-Ka only in very small amounts, relative levels of the pUL36 N-terminal fragments in PrV-ΔUL11 and PrV-ΔUL16 could be determined by the SILAC technique from mixtures of mutant virus particles with mass tagged PrV-Ka. Levels of the pUL36 N-terminal fragment were elevated 3- to 4-fold in PrV-ΔUL11, and 5- to 6-fold in PrV-ΔUL16. Concomitant with the appearance of the N-terminal pUL36 fragment in the UL11 and UL16 deletion mutants, relative amounts of full-length pUL36 were reduced in both mutants but remained constant in all the other mutants tested (Fig. 3). Colorimetric evaluation of the Coomassie stained gels (AIDA software package, Raytest, Straubenhardt, Germany) confirmed the quantitative mass-spectrometric data. The pUL36 N-terminal fragment was found to represent 26.4 % (PrV-ΔUL11) and 32.9 % (PrV-ΔUL16) of the molar amounts of the respective full-length products and, thus, compensates a significant part of the the average loss of 37 % (PrV-ΔUL11) and 41 % (PrV-ΔUL16) of the full-length pUL36 (Fig. 3). In all other mutants tested, amounts of the pUL36 N-terminal fragments were minimal and ranged between 1.1 % and 4.0 % of the full-length product.

To assay whether the preferential incorporation of the pUL36 N-terminus in PrV-ΔUL11 and PrV-ΔUL16 resulted from overexpression in infected cells, expression levels were assayed by immunoblot analysis of rabbit kidney (RK13) cells infected with PrV-Ka, PrV-ΔUL11 or PrV-ΔUL16 (Fig. 2E). Cells were extracted 16 hours after infection with phosphate buffered saline containing 1 % Triton X-100 and a protease

inhibitor cocktail (Complete, Roche, Mannheim, Germany) for 10 minutes on ice with occasional shaking. Twenty micrograms of the clarified extract (15000 g, 15 min, 4°C) were analyzed after electrophoretic separation on 5% polyacrylamide gels (Laemmli, 1970). Expression patterns and ratios between the full length form and the N-terminal fragment (marked by arrows) were similar after infection of RK13 cells with either virus, and no excessive expression of the pUL36 N-terminal fragment was observed after infection with PrV-ΔUL11 or PrV-ΔUL16 indicating that incorporation of the pUL36 N-terminal fragment in mutant viruses did not simply reflect its amounts in infected cells. Immunoblot analysis of purified PrV-ΔUL11 or PrV-ΔL16 virions that had been propagated on pUL11 or pUL16 expressing recombinant cells, respectively (RK13-UL11, RK13-UL16 in Fig. 2D, (Kopp et al., 2003;Klupp et al., 2005a)) showed that the pUL36 N-terminal fragment was not incorporated into virus particles after phenotypic complementation of PrV-ΔUL11 and PrV-ΔUL16, although it was well expressed in the respective infected cells (Fig 2E). These results indicate that the presence of pUL11 and pUL16 is required to preclude any present pUL36 N-terminus from incorporation into the mature virion. The origin and potential function of the pUL36 N-terminal fragment is unclear. Since two functional motifs mediating pUL37 interaction and proteolytic deubiquitinating activity are located in the N-terminus of pUL36 (Fuchs et al., 2004;Kattenhorn et al., 2005), these domains are most likely still functional in the N-terminal pUL36 fragment. Thus, its incorporation into virions could be mediated in the network of protein-protein interactions by its complex formation with pUL37. However, most interesting is the specific correlation of incorporation of the N-terminal pUL36 fragment with the lack of pUL11 and pUL16. In either mutant, incorporation of the N-terminus of pUL36 into virions was accompanied by a significant loss of full-length pUL36, suggesting that full-length pUL36 was partly replaced by the N-terminal fragment. This could mean that the requirement for

constant amounts of pUL36 in an intact tegument can be accomplished by incorporation of a N-terminal fragment, and that the stoichiometric interaction of pUL36 with other virion components is rather mediated by the N-terminus than the C-terminus. Although neither UL11 nor UL16 are essential for the replication of PrV in cell culture, an impairment in secondary envelopment has been shown for the UL11 mutant (Kopp et al., 2003) in electron microsopic studies. Interestingly, absence of pUL36 also yields a defect in cytoplasmic virion morphogenesis, whereas neither deletion of UL36 nor of UL11, UL16 or both had an influence on the intranuclear stages of virus morphogenesis (Fuchs et al., 2004;Klupp et al., 2005a). Loomis and coworkers (Loomis et al., 2003) have demonstrated that pUL11 and pUL16 of HSV-1 form a complex, and that pUL11 of PrV can interact with pUL16 protein of HSV-1. Therefore, formation of a complex between pUL11 and pUL16 of PrV seems likely. From the data presented here, we hypothesize that pUL11 and pUL16 or a potential complex of both proteins plays an important role in the exclusive incorporation of the full length pUL36 into the tegument of mature PrV virions excluding any N-terminal fragments of pUL36 which are present in infected cells.

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Fig. 1. Protein analysis of different PrV strains. Protein patterns of PrV strains Kaplan (Ka), Becker (Be), and NIA-3 in the upper molecular weight range (A), and immunoblot analysis (B) with a pUL36 specific antiserum confirm that pUL36 (arrow) is present as a single protein band with the expected apparent molecular mass of 330 kDa. In standard immunoblots, no fragmentation of pUL36 was observed in purified PrV particles from either strain. Molecular weight standards are indicated in kDa.

Fig. 2. Biochemical characterisation of pUL36 in purified virions and infected cells. Preparations of PrV-Ka or the single protein deletion mutants as indicated in (A) containing 9 μg of total protein were separated by SDS-PAGE and stained with Coomassie brilliant blue (A), or silver stained and extensively developed (B). In (C), a replica of the gels represented in (A) and (B) was blotted to nitrocellulose, and reacted with a pUL36 specific antiserum. The full-length form of pUL36 is marked by an arrow. N-terminal fragments of pUL36 in PrV-ΔUL11 and PrV-ΔUL16 are highlighted by rectangles. Fragments of pUL36 in the other mutants were detected in immunoblots only after excessively prolonged film exposure. Molecular weight standards are given in kDa. Panel (D) shows immunoblots of virions purified from RK-13 or the respective UL11 or UL16 expressing recombinant RK13 cells (RK13-UL11 or RK13-UL16). Arrows indicate full-length pUL36 and the pUL36 N-terminal fragment of approximately 220 kDa. In (E), extracts from RK13 cells or recombinant cells constitutively expressing UL11 (RK13-UL11) or UL16 (RK13-UL16) and infected with the indicated virus were processed for immunoblot analysis with a pUL36-reactive antiserum. No significant differences in the ratios of the full-length and the N-terminal fragment of pUL36 were observed after infection with PrV-ΔUL11 or PrV-ΔUL16 in comparison to PrV-Ka. Molecular weights are given in kDa.

Fig. 3. Quantitation of full-length pUL36 and the N-terminal fragment. Relative amounts of the full-length form of pUL36 (A) and the N-terminal fragment (B) were assayed in two independent experiments (black and white bars). Results are expressed in multiples of the amount of the respective protein present in PrV-Ka, i.e. values above 1.0 indicate that higher amounts of the respective protein are present in the mutant than in PrV-Ka virions and values below 1.0 indicate lower amounts in the mutant viruses. In PrV-ΔUL11 and PrV-ΔUL16 a deficiency of full-length pUL36 and an increased incorporation of the N-terminal fragment was observed. Error bars represent the standard deviation of the isotope ratios of peptides that have been selected for quantitation.

Fig.1

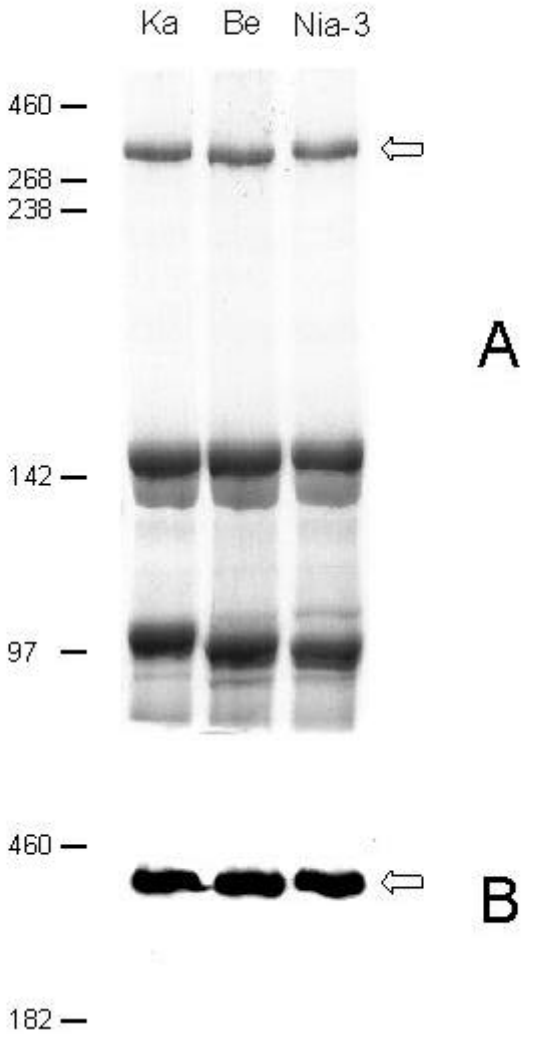
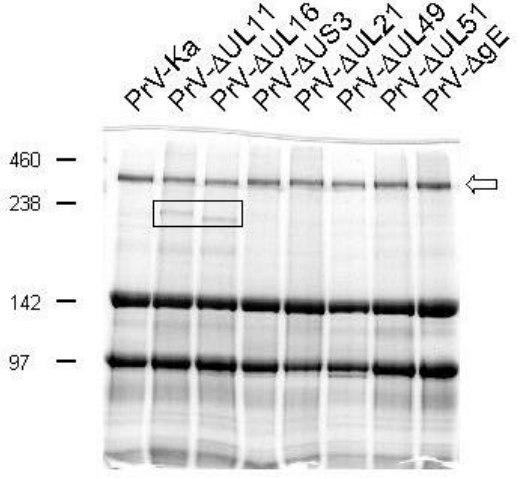
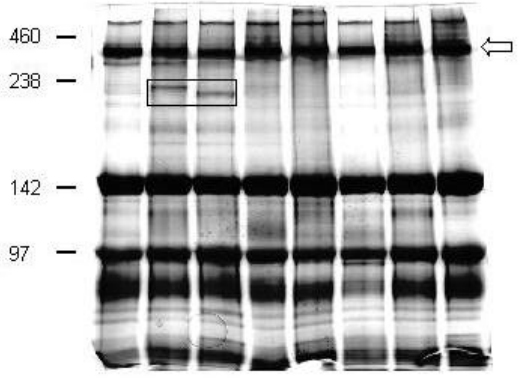


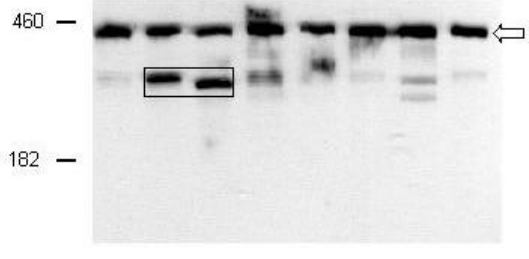
Fig. 2



A

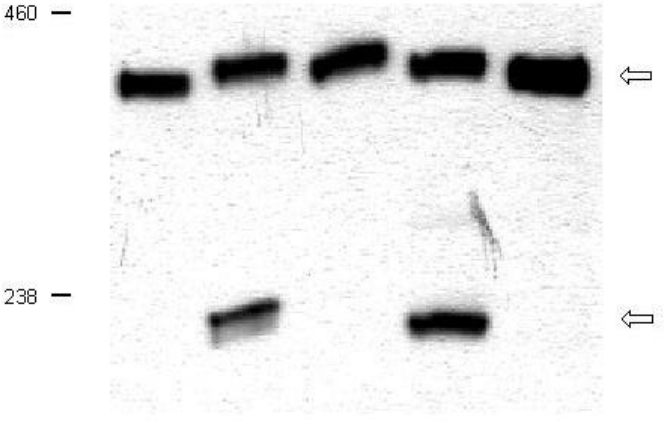


B



C

Virus	PrV-Ka	PrV-ΔUL11	PrV-ΔUL11	PrV-ΔUL16	PrV-ΔUL16
Cell	RK13	RK13	RK13-UL11	RK13	RK13-UL16



D

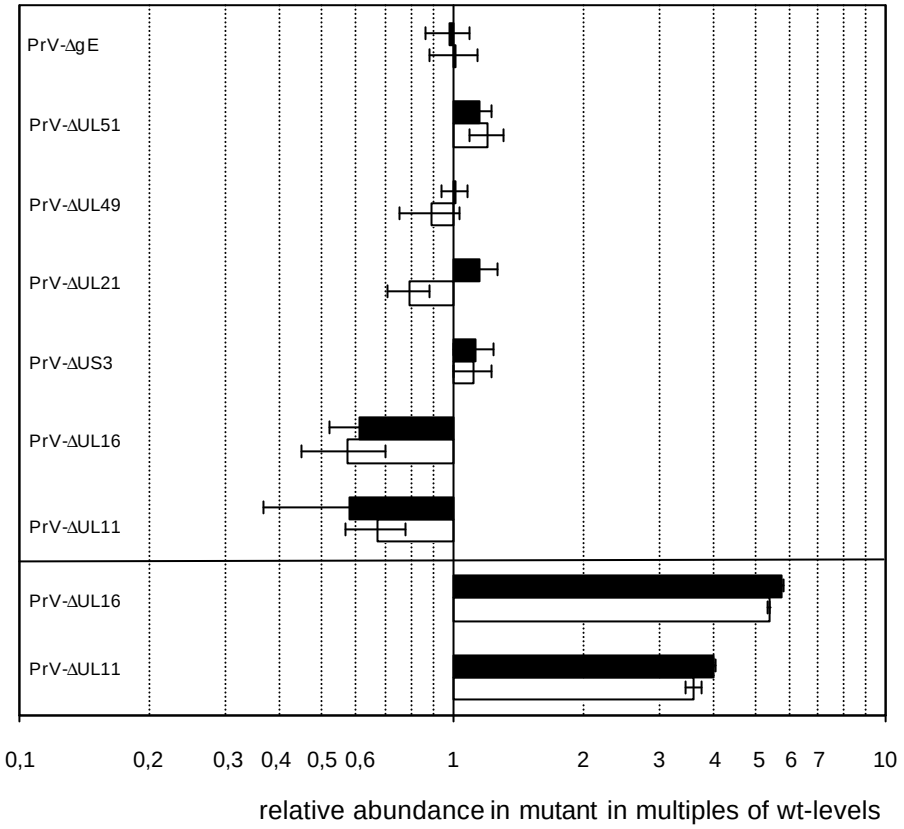
Virus	-	PrV-Ka	PrV-ΔUL11	PrV-ΔUL16	PrV-Ka	PrV-ΔUL11	PrV-Ka	PrV-ΔUL16
Cell	RK13	RK13	RK113	RK13	RK13-UL11	RK13-UL11	RK13-UL16	RK13-UL16

E

Fig. 3

A

B


PrV-ΔgE
PrV-ΔUL51
PrV-ΔUL49
PrV-ΔUL21
PrV-ΔUS3
PrV-ΔUL16
PrV-ΔUL11
PrV-ΔUL16
PrV-ΔUL11
0,1
0,2
0,3
0,4
0,5
0,6
1
2
3
4
5
6
7
10
relative abundance in mutant in multiples of wt-levels

Efficient Incorporation of Tegument Proteins pUL46, pUL49, and pUS3 into Pseudorabies Virus Particles Depends on the Presence of pUL21

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ABSTRACT

The mature virion of the alphaherpesvirus pseudorabies virus (PrV) contains a minimum of 31 structural proteins which are recruited into the virus particle by a network of protein-protein interactions which is only incompletely understood. We show here that deletion of the tegument protein pUL21 resulted in a drastic decrease in the incorporation of the pUL46, pUL49, and pUS3 tegument components into mature virions. Moreover, the attenuated PrV strain Bartha (PrV-Ba) which among other defects carries mutations in pUL21 also fails to package pUL46, pUL49 and pUS3 efficiently. By reconstitution of wild-type pUL21 expression to PrV-Ba and transfer of mutated PrV-Ba pUL21 into wild-type PrV we demonstrate that this phenotype is due to the mutated pUL21.

Alphaherpes virions contain in excess of thirty structural proteins which form the nucleocapsid, the tegument and constituents of the viral envelope. The tegument which links viral envelope and nucleocapsid is the least understood structure of the herpes virion. It consists of numerous viral and several cellular proteins which are recruited into the virus particle by a network of protein-protein interactions which is only incompletely understood (22,23). Generally, the importance of viral proteins on virus replication has been analyzed by mutating single viral gene products and ascribing the observed phenotypes directly to the mutated protein. However, both, functional redundancy which precludes a direct correlation of a phenotype to absence of one viral protein (2,6,15), and 'trans'-effects of mutations in a specific protein on other viral components have been observed (4,25). Thus, the assignment of a particular phenotype to a specific mutation requires an in-depth analysis of possible effects on other viral or cellular factors.

The pUL21 protein of the alphaherpesvirus pseudorabies virus (PrV) has been described as a capsid-associated protein involved in capsid maturation during cleavage and encapsidation of the viral genome (3,33). However, a pUL21 mutant of PrV is only slightly impaired in replication in cell culture (13,14) and in a murine intransal infection model (12), but strongly attenuated in PrV's natural host, the pig (13). This indicates that pUL21 is important for PrV replication in vivo. Although little is known about pUL21 function, interestingly the live attenuated PrV strain Bartha (PrV-Ba;1), besides containing a large deletion in the unique short (Us) region eliminating the genes for glycoprotein I (gI; pUS7), gE (pUS8), pUS9 and pUS2 (17,24,29), contains point mutations in signal sequence of gC (30), in gM (5), pUS3 (20) and pUL21 (13). Marker rescue experiments showed that the deleted genes as well as pUL21 are important for attenuation since their repair with the corresponding genes from PrV-Ka also restored to PrV-Ba the virulent phenotype of PrV-Ka (13,17,18). However, the molecular basis for this phenotype and which role pUL21 plays in PrV replication is still unclear.

Lyman and colleagues demonstrated by Western blot analysis of purified virions that PrV-Ba fails to package the tegument proteins pUS3 and pUL49 (VP22) (20). However, this has been challenged by Granzow et al., (9) who detected pUS3 and pUL49 in highly purified PrV-Ba virion preparations. Restoration of the large deletion in the Us region by the corresponding gene from the PrV strain Becker (PrV-Be) did not restore pUS3 packaging into virions (20), and restoration of gE expression did not restore pUL49 incorporation despite the known

interaction between the two (7). Thus, discrepancy still exists about the question whether PrV-Ba is indeed unable to package pUS3 and pUL49, and, if so, which Bartha component is responsible for this phenotype.

We recently adapted a highly quantitative method for the analysis of proteins in purified herpes virions (25). The original procedure has been designated as 'stable isotope labelling with amino acids in cell culture' (abbreviated 'SILAC'; 27). Here, samples to be compared were differentially labelled metabolically with either unmodified leucine or leucine carrying three atoms of the stable heavy hydrogen isotope deuterium. After gel electrophoresis and mass spectrometry, the ratio of each protein in the virus particle was determined with high accuracy using the constant amount of 960 copies per capsid of the pUL19 major capsid protein (32) as standard. Thus, we reanalyzed the protein composition of PrV-Ba in comparison to wild-type PrV strain Kaplan (PrV-Ka; 10), that of a pUL21 deletion mutant of PrV-Ka (PrV-ΔUL21; 14) as well as rescuants of PrV-Ba after marker rescue with the U_S-region of PrV-Ka (PrV-Ba 43/25a; 17), and, in addition, with Bam HI-fragment 4 of PrV-Ka which includes the pUL21 mutations (PrV-Ba 43/25aB4; 18). Moreover, the PrV-Ba UL21 gene was used to restore UL21 expression to PrV-ΔUL21 which carries a complete deletion of the UL21 gene (Fig. 1). Viruses were propagaged on porcine kidney cells (PK-15) and purified by sucrose-density gradient centrifugation (11). Purified PrV-Ka was propagated in cells cultured in medium DME/F12 (Sigma-Aldrich D-9785) containing exclusively deuterated leucine (L-leucine-5, 5, 5 d_3, Simga-Aldrich #486825, 99 atom % D) which resulted in the insertion of a mass tag of 3 dalton per leucine residue. PrV-Ka virions served as a global internal standard for the SILAC procedure. All other viruses were propagated on cells maintained in medium supplemented with conventional leucine. To compare the relative amounts of proteins in the different viruses in relation to PrV-Ka, purified virion preparations were mixed at a 1:1 protein ratio and mixtures were separated by standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 16). Thereafter, bands of interest were cut from the Coomassie stained gels (26). After tryptic digest (31), peptide mass fingerprint spectra were obtained on an Ultraflex mass spectrometer (Bruker, Bremen, Germany), and the data were further processed by FlexAnalysis and BioTools software (Bruker). Database queries were performed with MASCOT software (Matrixscience, London, UK; 28) using an in-house database representing the PrV proteome as compiled from the Swiss-Prot database (8). Quantitative evaluation of the spectra was carried out using a self-developed software. Mass lists were screened for peak pairs representing leucine containing

specific peptides of the respective protein in the conventional and the mass tagged variant, and an average intensity ratio reflecting the relative amounts of protein originating from PrV-Ka, PrV-Ba or the recombinant viruses was calculated from all qualified peaks of the spectrum. Intensity ratios of the tegument proteins were normalized to the intensity ratios of the major capsid protein (MCP) which is present in every intact virus particle in the capsid in 960 copies (32) to correct for any variations in protein input (25).

Deletion of pUL21 from PrV-Ka resulted in a decrease in the incorporation of the pUL46, pUL49, and pUS3 proteins into virions (Fig. 2) by 80-90%. A similar reduction in incorporation of these proteins compared to PrV-Ka was observed for PrV-Ba, indicating that PrV-Ba still packages pUL46, pUL49 and pUS3, but at strikingly lower levels. Thus, the differences observed in the assessment whether PrV-Ba packages pUS3 and pUL49 or not (9,20) can be explained by the nonquantitative nature of the immunoblot analysis and differences in the sensitivity of the immunological reagents used. Restoration of expression of gI, gE, pUS9 and pUS2 in PrV-Ba 43/25a did not correct the pUS3 incorporation defect (Fig. 2), which is in line with previous experiments (20), but increased incorporation of pUL46 and pUL49. The latter may be due to the interaction of gE with pUL49 (7). Interestingly, incorporation of pUL46, pUL49 and pUS3 was restored to nearly wild-type levels when, in addition to the repair of the U_S-deletion, an intact wild-type UL21 gene derived from PrV-Ka was present in the PrV-Ba genome. Thus, in an PrV-Ba background, PrV-Ka pUL21 aids in the incorporation of pUL46 and pUL49, and is required for efficient virion localization of pUS3. This is further corroborated after transfer of the mutated UL21 gene of PrV-Ba into PrV-Ka. In PrV-UL21(Ba), pUS3 incorporation is decreased by more than 70% and that of pUL49 by ca. 60%, whereas incorporation of pUL46 was only slightly affected. To allow a direct comparison of the obtained spectra, examples of relevant regions of the peptide mass fingerprints are compiled in Fig. 3. In all virus preparations, the amount of the capsid protein pUL18, and the capsid-associated pUL25 did not differ significantly from wild-type PrV-Ka as indicated by their values around 1. The same was observed for the inner tegument protein pUL36, and tegument proteins pUL16 and pUL21 (Fig. 2). PrV-Ka pUL21 has been shown to form a complex with pUL16 (14), which, in turn, may interact with pUL11 (19). Complex formation with pUL16 has also been observed for the PrV-Ba pUL21 (data not shown), so that the mutations apparently do not interfere with interaction between pUL16 and pUL21.

In summary, we demonstrate that PrV pUL21 is a virion component which is necessary for the efficient incorporation of pUL46, pUL49 and pUS3 into mature PrV virions. The serine/threonine kinase pUS3 and the major tegument protein pUL49 are not packaged efficiently into virions in the absence of pUL21, or in the presence of mutated PrV-Ba pUL21 which indicates that the point mutations in pUL21 of PrV-Ba interfere with incorporation of pUS3 and pUL49 but not pUL46. Thus, different domains of the protein may be important for this differential *trans* effect of pUL21 on virion localization of these PrV tegument proteins.

It has been described that absence of pUS3 results in inefficient virion localization of pUL46 in HSV-2 (21) and PrV-Ka (25). Thus, the defects observed in pUL46 incorporation in PrV-ΔUL21, PrV-Ba or PrV-Ba43/25a may be an indirect effect of the deficient pUS3 incorporation. However, PrV-UL21(Ba) packages nearly wild-type amounts of pUL46 while incorporation of pUS3 is strikingly decreased indicating that there is no direct interaction between the two.

Our data demonstrate another, hitherto unknown interaction between herpesvirus tegument proteins yielding another piece for the herpesvirus assembly puzzle (22). pUL21 is a virion protein conserved throughout the *Herpesviridae*, whereas pUS3, pUL46 and pUL49 are found only in the alphaherpesviruses. Thus, it is conceivable that pUL21 of beta- and gammaherpesviruses interacts with other, nonconserved tegument proteins during virion morphogenesis. Quantitation of virion components by SILAC and mass spectrometry should help to analyze whether this is indeed the case.

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FIGURE LEGENDS

FIG. 1. Diagram of viruses used in this study. (A) depicts a schematic map of the PrV genome with location of the long (U_L) and short (U_S) unique regions. (B) shows localization of the genes of interest in wild-type PrV-Ka. In (C) the defects in the analyzed virus mutants are indicated. Δ indicates deletion of the corresponding portion of the viral genome. * indicates presence of mutations within the respective open reading frames. R= repair with PrV-Ka sequences.

FIG. 2. Quantitation of capsid (pUL18), capsid-associated (pUL25) and tegument proteins (pUL36, pUL16, pUL21, pUL46, pUL49, pUS3) in purified virions. Results are expressed on a logarithmic scale in multiples of the amount of the respective protein present in wild-type PrV-Ka. Values below 1 indicate a decrease in incorporation. Error bars indicate the standard deviation between at least three different independent virus preparations.

FIG. 3. Peptide mass spectra from selected peak pairs. Selected peak pairs of proteins derived from purified virions which had been labeled by SILAC and analyzed by mass spectrometry are shown. Indicated are spectra for pUL49, pUS3, pUL46 and pUL25.

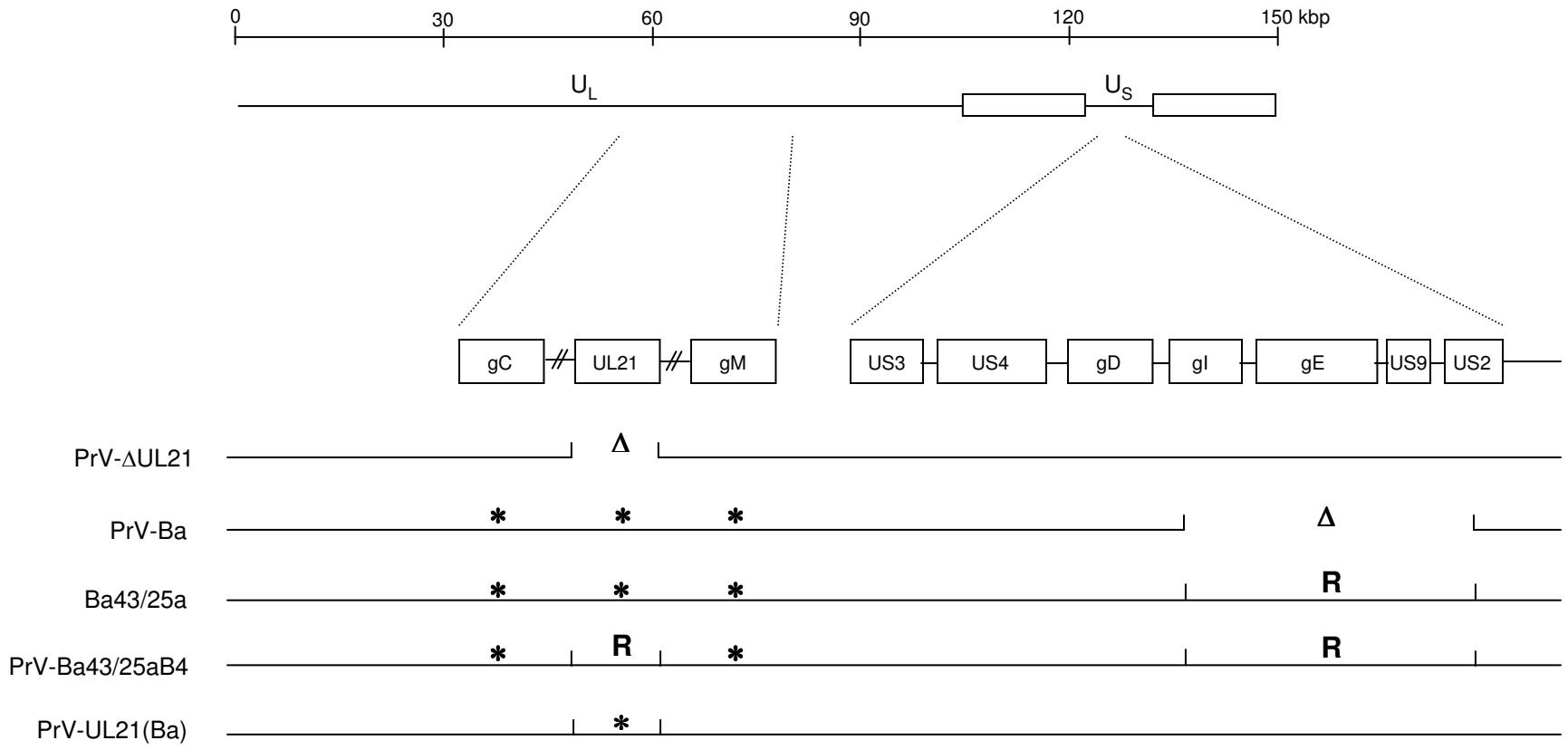
0
30
60
90
120
150 kbp
U_L
U_S
gC
UL21
gM
US3
US4
gD
gI
gE
US9
US2
PrV-ΔUL21
PrV-Ba
Ba43/25a
PrV-Ba43/25aB4
PrV-UL21(Ba)
Δ
R

Fig.1

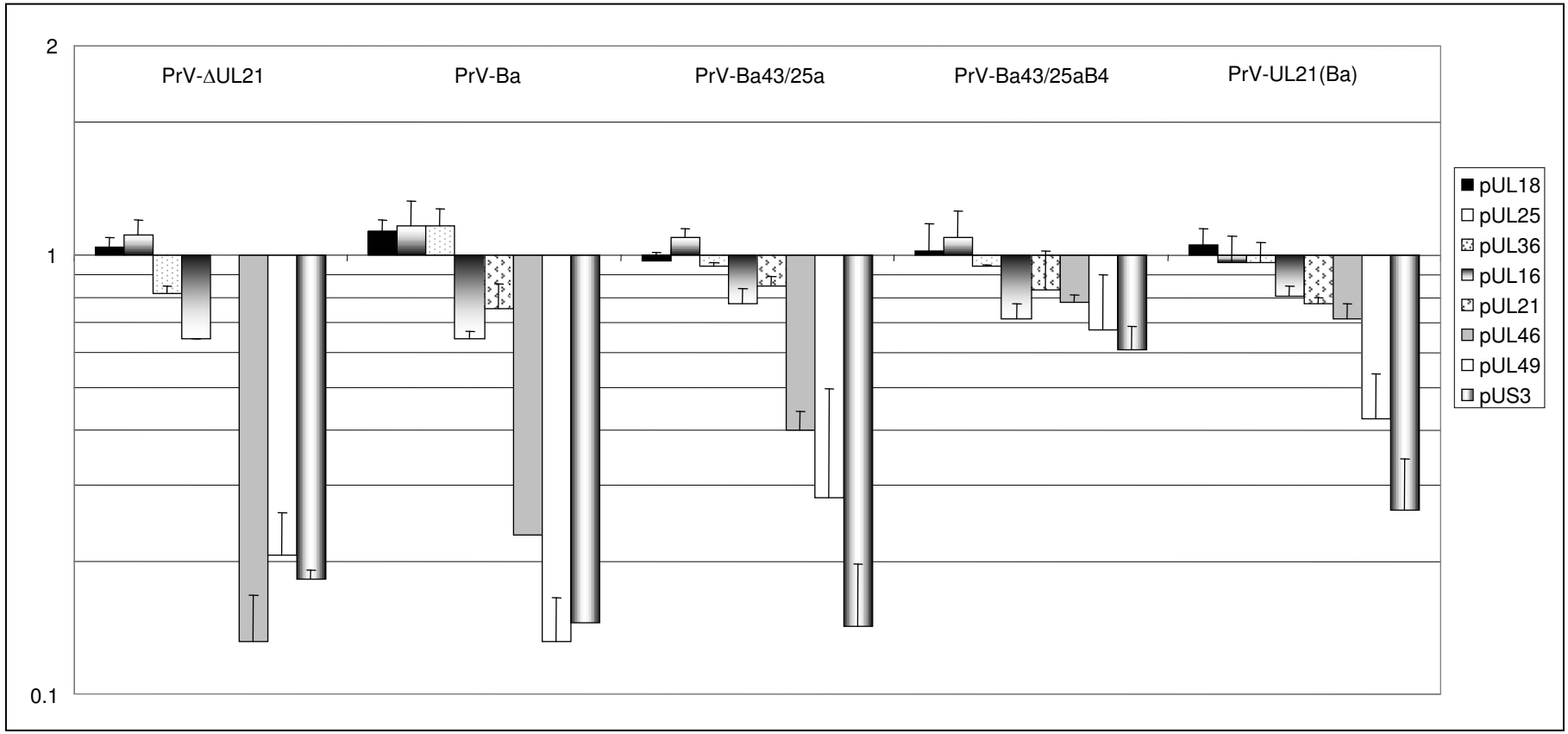
2
1
0.1
PrV-ΔUL21
PrV-Ba
PrV-Ba43/25a
PrV-Ba43/25aB4
PrV-UL21(Ba)
pUL18
pUL25
pUL36
pUL16
pUL21
pUL46
pUL49
pUS3

Fig.2

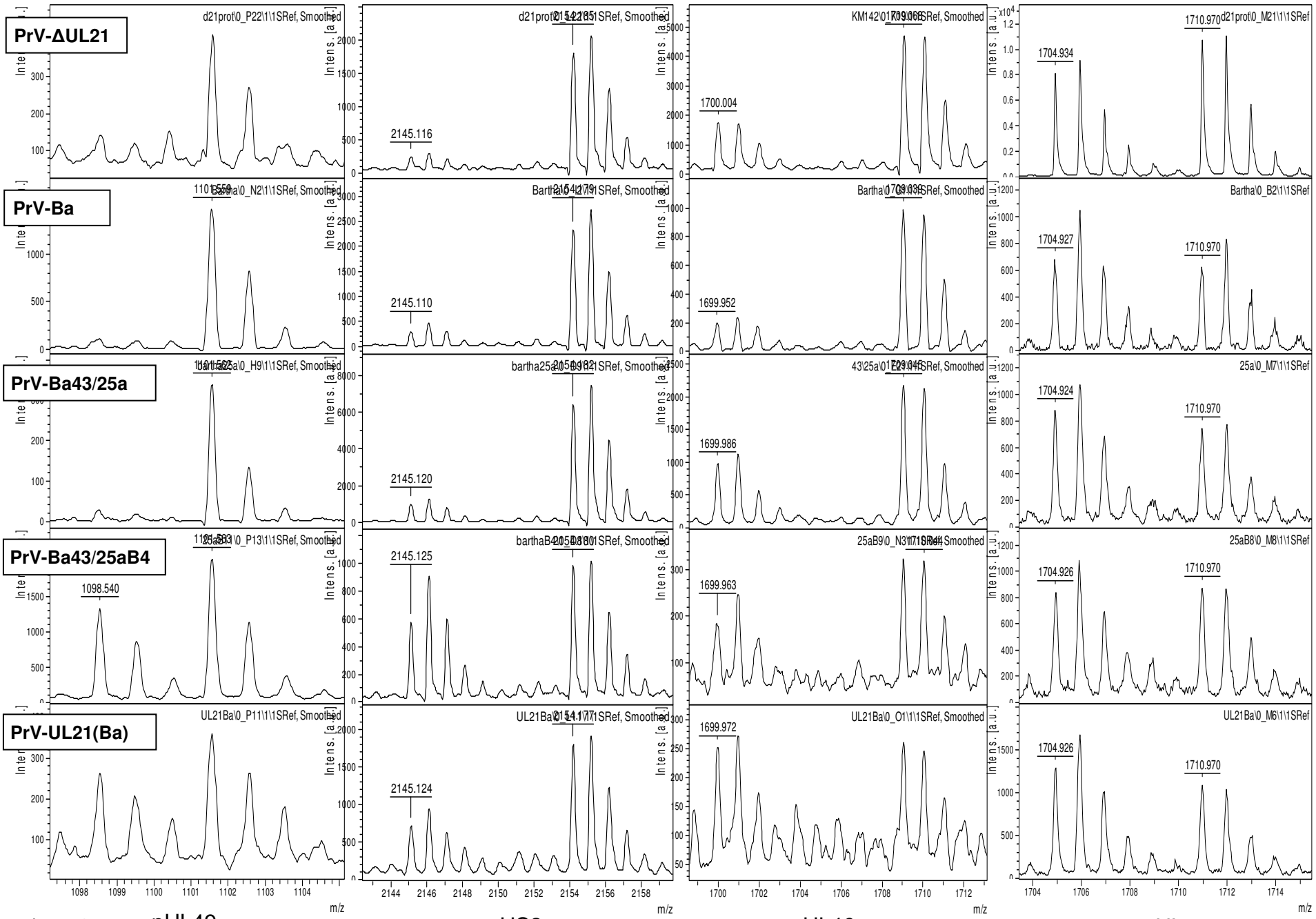

Fig.3

pUL49 pUS3 pUL46 pUL25

Zusammenfassung der Dissertation zum Thema:

Qualitative und quantitative massenspektrometrische Analyse von Virionen des Pseudorabies Virus

vorgelegt von

Kathrin Michael

Das Ziel dieser Arbeit war die qualitative und quantitative Analyse der Zusammensetzung von Partikeln des Pseudorabies Virus (PrV), des Erregers der Aujeszky'schen Krankheit beim Schwein.
In Partikeln des PrV-Virusstammes Kaplan wurden nach ein- oder zweidimensionaler Elektrophorese und Identifizierung durch *peptide mass fingerprint* 27 Strukturproteine viraler und vier Strukturproteine zellulärer Herkunft (Annexin I und -II, HSP70 und Aktin) identifiziert. Die viralen Strukturproteine pUL37, pUL48, pUL18, pUL19, pUL29 (gB) und alle Strukturproteine zellulärer Herkunft wurden nach zweidimensionaler Elektrophorese in mehreren Isoformen nachgewiesen.

Im zweiten Teil der Arbeit wurde die Zusammensetzung von Deletionsmutanten des PrV mit derjenigen von Wildtyp-Virionen verglichen. Ziel war hier die Analyse von Veränderungen in der Partikelzusammensetzung über den Verlust des deletierten Proteins hinaus, z.B. als Folge einer dadurch nicht mehr möglichen Protein-Protein-Wechselwirkung oder einer abweichenden Morphogenese. Im Vordergrund stand dabei die Untersuchung der Tegumentproteine, da diese in eine Vielzahl von Protein-Protein-Interaktionen einbezogen sind und ihnen eine entscheidende Rolle während der Virusmorphogenese zukommt. Die quantitative Analyse von Mutanten mit Deletionen der Tegumentproteine pUS3, pUL11, pUL13, pUL16, pUL21, pUL35, pUL41, pUL43, pUL47, pUL49, pUL51, sowie der Deletion eines C-terminalen Fragments des UL36-Gens und des Glykoproteins E erfolgte massenspektrometrisch mit der SILAC Strategie.

Nach Untersuchung der Strukturproteinprofile von allen oben genannten Deletionsmutanten lässt sich über die genannten Details hinaus generell folgendes feststellen:

(1) Kapsid- beziehungsweise kapsid-assoziierte Proteine (pUL18, pUL25, pUL35 und pUL38) werden in stöchiometrischen Mengen zum Hauptkapsidprotein MCP142 (pUL19) in die Viruspartikel eingebaut. Diese Stöchiometrie war robust gegen alle untersuchten Deletionen.
(2) Größere Flexibilität beim Einbau in das reife Virion zeigten Komponenten des Teguments. Kapsidnahe Tegumentproteine wie das pUL36 wurden meist stöchiometisch eingebaut. Größere Schwankungen beim Einbau in die verschiedenen untersuchten Deletionsmutanten zeigten die Tegumentproteine pUL11, pUL16, pUL21, pUL46, pUL48, pUL49 und pUS3. Deletionen in einzelnen Tegumentproteinen führten zu vermindertem Einbau anderer Proteine, was z.B. durch den Ausfall von Protein-Protein Wechselwirkungen erklärt werden kann, oder auf einen vermehrten Einbau anderer Tegumentproteine hindeutet.
(3) Virale Hüllglykoproteine zeigten die größten quantitativen Schwankungen im Einbau, was die Bewertung des Einbaus der Glykoproteine in die verschiedenen Deletionsmutanten erschwerte. Ausnahme war hier das essentielle Glykoprotein gH, dessen Einbau in die untersuchten Deletionsmutanten im Vergleich zum Wildtyp durchgängig unverändert war.

Publikationen

Michael K., B.G. Klupp, T.C. Mettenleiter, and A. Karger (2006)
Composition of pseudorabies virus particles lacking tegument proteins US3, UL47, UL49 or glycoprotein E; J. Virol. 80(3):1332-9.

Michael K., S. Böttcher, B.G. Klupp, A. Karger, and T.C. Mettenleiter (2006)
Pseudorabies virus particles lacking tegument proteins pUL11 or pUL16 incorporate less full-length pUL36 than wild type virus but specifically accumulate a pUL36 N-terminal fragment; J. Gen. Virol., (im Druck).

Michael K., B.G. Klupp, A. Karger, and T.C. Mettenleiter (2006)
Efficient incorporation of tegument proteins pUL46, pUL49, and pUS3 into pseudorabies virus particles depends on the presence of pUL21;
J. Virol., (eingereicht).

Böttcher S., B.G. Klupp, H. Granzow, W. Fuchs, K. Michael, and T.C. Mettenleiter (2006)
Identification of a 709 aa internal nonessential region within the essential conserved tegument protein (p)UL36 of pseudorabies virus; J. Virol., (im Druck).

Jung D., J.P. Teifke, A. Karger, K. Michael, S. Venz, W. Wittmann, K. Kindermann, K. Nöckler, and E. Mundt (2006)
Evaluation of baculovirus-derived recombinant 53 kDa protein of *Trichinella spiralis* for detection of *Trichinella*-specific antibodies in domestic pigs by ELISA;
Parasitol. Res. (im Druck).

Vortrag

Michael K Composition of Pseudorabies Virus Particles Lacking Tegument Protein US3, UL47, UL49 or Glycoprotein E; Gesellschaft für Virologie, Annual Meeting; 15.-18.03.2006; München.

Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbständig verfasst und keine anderen als die angegebenen Hilfsmittel benutzt habe.

Die Dissertation ist bisher keiner anderen Fakultät vorgelegt worden.

Ich erkläre, dass ich bisher kein Promotionsverfahren erfolglos beendet habe und dass eine Aberkennung eines bereits erworbenen Doktorgrades nicht vorliegt.

Lebenslauf

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