2010

PROTEASOME-DEPENDENT ENTRY OF HERPES SIMPLEX VIRUS

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

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April 19th, 2010

ACKNOWLEDGMENTS

I am very grateful for my thesis advisor Anthony Nicola: for giving me the numerous opportunities, valuable guidance and the constant positive support throughout the years. I also want to thank the members of my thesis committee: Suzanne Barbour, Daniel Conrad, Phillip Hylemon, Kimberly Jefferson and Michael McVoy for their time and helpful input.

I am grateful to Gary Cohen, Richard J. Courtney, Prashant Desai, Roselyn Eisenberg, Roger D. Everett, Thomas Holland, Harvey Ozer, Priscilla Schaffer, Patricia Spear, and Stephen Straus for kindly providing reagents. I thank Carlos R. Siekavizza-Robles for assistance with viral preparations, Devin G. Roller for assistance with the fusion-from-without analysis and Farzaneh Rostami for assistance with the infection analysis. I would also like to thank current and past members of the lab and the McVoy lab, for their guidance, assistance and sometimes-erratic conversations.

I would like to thank and dedicate this work to the following family and friends for their love, support and prayers: Lydia and Frederick Delboy; Amanda Delboy, Dan Delboy, Fred Delboy, Ia Schomaker, Anna Schomaker, Kevin Heath, Jen Meadows, Stan Anders, Rosa Rios, Brandon Pace, Chris Osterhout, Ashley Foerster and those who are not mentioned but are still influential and important in my life.

ABSTRACT

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By Mark G. Delboy, B.S.

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Herpes simplex virus entry into cells is a multistep process that engages the host cell machinery. The proteasome is a large, ATP-dependent, multisubunit protease that plays a critical role in the maintenance of cell homeostasis. A battery of assays were used to demonstrate that proteasome inhibitors blocked an early step in herpes simplex virus entry that occurred after capsid penetration into the cytosol but prior to capsid arrival at the nuclear periphery. Proteasome-dependent viral entry was not reliant on host or viral protein synthesis. MG132, a peptide aldehyde that competitively inhibits the degradative activity of the proteasome, had a

reversible inhibitory effect on herpes simplex virus capsid transport. Herpes simplex virus can use endocytic or nonendocytic pathways to enter cells. These distinct entry routes were both dependent on proteasome-mediated proteolysis. In addition, herpes simplex virus successfully entered cells in the absence of a functional host ubiquitin-activating enzyme, suggesting that viral entry is ubiquitin independent.

Herpes simplex virus immediate-early protein ICP0 is a multifunctional regulator of herpes simplex virus infection. Late in infection ICP0 interacts dynamically with cellular proteasomes. ICP0 has a RING finger domain with E3 ubiquitin ligase activity that is necessary for its IE functions. The fundamental and functional properties of ICP0 that is present in the virion tegument layer have not been well characterized. For these reasons, I sought to characterize tegument ICP0 and determine the role of tegument ICP0 during proteasome-dependent entry of herpes simplex virus. Protein compositions of wild-type and ICP0 null virions were similar, suggesting that the absence of ICP0 does not grossly impair virion assembly. Virions with mutations in the RING finger domain contained greatly reduced levels of tegument ICP0, suggesting that the domain influences the incorporation of ICP0. Virion ICP0 was resistant to removal by detergent and salt and was associated with capsids, features common to inner tegument proteins. ICP0 mutations that resulted in the absence of ICP0 in the tegument layer, allow herpes simplex virus to enter cells independently of the proteasome activity. I propose that proteasomal degradation of virion and/or host proteins is regulated by ICP0 to allow for efficient delivery of incoming herpes simplex virus capsids to the nucleus.

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I. INTRODUCTION

A. HERPES SIMPLEX VIRUS

1. Background

Herpesviruses are a group of DNA viruses with a distinctive viral architecture (192). Assignment to the herpesviridae family is made on the basis of virion morphology. Virions are composed of a densely packed, linear double-stranded DNA core, which is enclosed by an icosahedral capsid. Surrounding the capsid is a proteinaceous or tegument layer. These two viral structures are bounded by a host-derived envelope with embedded glycoprotein spikes. More than 80 herpesviruses have been identified and 8 are known human pathogens (256). Biological criteria were used to assign viruses to three subfamilies, the *alpha-*, *beta-* or *gammaherpesvirinae* (192) (Table I.). A common feature of herpesviruses is the ability to persist for the lifetime of the host (256). Alphaherpesviruses have a broad host range but primarily infect sensory ganglia (224). Betaherpesviruses infect secretory glands, lymphoreticular cells, kidneys and other tissues. Gammaherpesviruses have a limited host range and usually infect either T or B lymphocytes. This grouping scheme correlates with evolutionary analysis of herpesviral genomes (192).

After herpes simplex virus (HSV) comes into contact with the skin or mucous membrane, the virus undergoes replication at the site of infection (65). The virus can then infect sensory

Table I. Human herpesviruses

Common Name	Subfamily	Common disease/Symptoms
Herpes simplex 1 (HSV-1)	α	Herpes labialis, keratitis
Herpes simplex 2 (HSV-2)	α	Herpes genitalis, neonatal encephalitis
Varicella-zoster virus (VZV)	α	Varicella (chicken pox), herpes zoster (shingles), postherpetic neuralgia
Epstein-Barr virus (EBV)	γ	Infectious mononucleosis, oral hairy leukoplakia, Burkitt's lymphoma, nasopharyngeal carcinoma
Human cytomegalovirus (HCMV)	β	Congenital CNS maldevelopment, mononucleosis, posttransplant and AIDS complications
Human B-lympotropic virus (HHV-6)	β	Infant exanthem subitum, posttransplant and AIDS complications
Human herpesvirus 7 (HHV-7)	β	None known
Kaposi's sarcoma-associated virus (KHSV)	γ	Kaposi's sarcoma, primary effusion lymphoma, multicentric Castleman's disease

Table modified from (232).

neurons that innervate the entry site (268). Some neurons undergo productive (lytic) infection while other neurons retain the virus in a dormant (latent) state (268). HSV can remain latent for the lifetime of the host. HSV may also be reactivated and transported to or near the initial site of infection (65). Lytic gene expression is repressed during latency (84). Viral-encoded proteins are essential for the efficient reactivation of HSV from latency (268).

HSV-associated diseases are among the most widespread infections (256). HSV type 1 (HSV-1) is the prototype alphaherpesvirus and primarily causes extragenital or oral infections (34). HSV type 2 (HSV-2) is primarily associated with infection of the anogenital region. Although a significant percentage of primary herpes infections are subclinical, HSV infections are still an important public health concern (256). HSV infection affects approximately 60% to 95% of adults worldwide (34). It is estimated that 50 million Americans are infected with HSV-2 (99). Recent studies have shown an increase in HSV-2 causing extragenital infections and in HSV-1 causing anogenital infection (34). HSV infection is normally localized to the mucosal sites and dorsal root ganglia but disseminated infection can be a threat to immunocompromised individuals and neonates (99). HSV is the common cause of sporadic encephalitis worldwide. HSV is the most common virus to infect the human brain. Ocular HSV infection is the second most common cause of blindness worldwide (34). HSV-2 neonatal infections have an incidence of 1 in 3,000 to 1 in 20,000 births per year in the United States.

Acyclovir, an inhibitor of HSV DNA replication, is the most widely used antiviral for HSV infections (34, 256). Acyclovir-resistant strains can develop in immunocompromised patients (34). Foscarnet and Cidofovir, also DNA replication inhibitors, are used for treatment of HSV infections resistant to Acyclovir. Management of HSV infections does not rid the host of virus. Current antivirals can only prevent transmission, suppress recurrence and attenuate clinical

course and viral shedding (256). There is currently no approved vaccine for general administration. Thus, HSV-1 and HSV-2 remain serious human pathogens that are important to study.

2. Virion structure

HSV is a multilayered particle with an overall diameter of approximately 200 nm. Virions are composed of four key structural components: a genome, capsid, tegument and envelope (Fig. I.). Virions are comprised of more than 40 distinct proteins (180).

The HSV genome is a linear double-stranded DNA molecule (Fig. II.). It contains 150 kilobase pairs with a G+C content of 67% (HSV-1) or 74% (HSV-2) (191). The DNA genome is divided into two covalently linked segments, designated long (L) and short (S) (190). Each segment contains unique sequences (U_L and U_S) flanked by a pair of inverted repeats. L and S segments can be inverted relative to one another, yielding four possible genome arrangements. The HSV genome has been sequenced and contains 74 different protein-encoding genes, although more have been suggested due to gene crowding (68, 190, 191, 231).

The capsid is a protein shell with a approximate thickness of 15 nm and 125 nm diameter (143). Seven different proteins can compose the capsid, arranged on a icosahedral surface lattice (247). VP5 is the major structural protein of the 12 pentons and 150 hexons. The capsid therefore is comprised of a total of 960 copies of VP5.

A proteinaceous layer resides between the capsid and the envelope. This tegument layer contains more than 20 viral proteins and is asymmetrically structured in extracellular

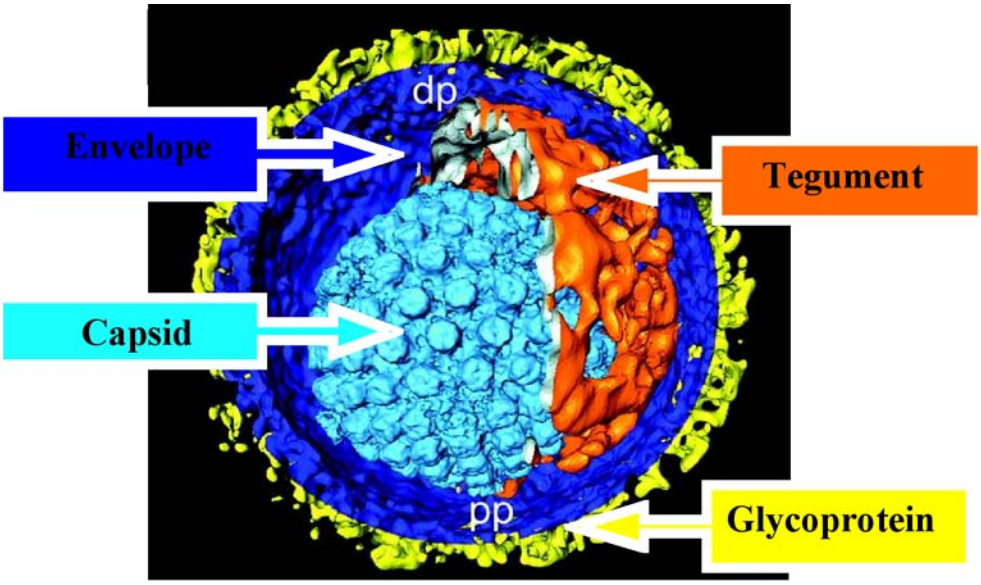

Figure I. Herpes simplex virus structural elements. Shown is the structure, as determined by cryo-electron tomography reconstruction, of the herpes simplex virion (122). The capsid, tegument, envelope and glycoprotein structures are indicated.

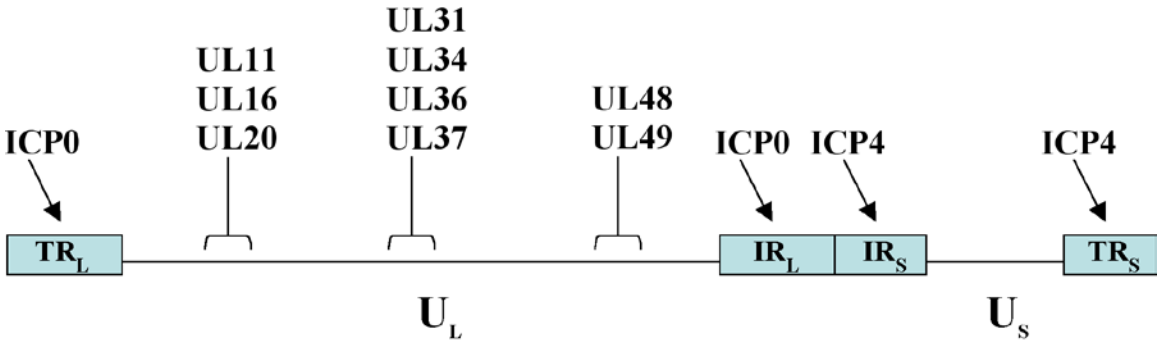

Figure II. HSV genome structure. The long and short unique regions (U_L and U_S) are flanked by inverted repeat regions (blue boxes). The approximate map locations of the genes encoding tegument proteins discussed in the thesis are shown.

virions (180). It is currently thought that these proteins act as viral regulators to mediate activities during viral entry and prior to viral gene expression (212).

The host cell-derived envelope consists of host lipids and polyamines and accommodates approximately 12 viral glycoproteins (113). The glycoproteins, visualized as spikes by cryo-electron microscopy, are arrayed in dense clusters around the tegumented region and are sparsely populated in other regions (122). They also vary in straightness, angles and whether they are organized as homo- or hetero-oligomers (122, 127).

3. Infectious cycle

The reproduction cycle of HSV has been extensively studied using cell culture models. Envelope glycoproteins mediate attachment, binding to cell receptors and fusion of the viral envelope with a host membrane (134) (Fig. III.). The virus can then penetrate into the cytoplasm of the cell. Capsids are rapidly transported to the nucleus via dynein interaction and movement on cellular microtubules (67, 252). Capsid docking at the nuclear pore complex (NPC) results in the release of the viral genome (12, 217). The nucleus is the site where viral transcription of genes, genome replication and capsid assembly occur.

HSV gene expression is a temporally regulated cascade of events. Gene expression begins when VP16 is released from the tegument and enters the nucleus to induce the transcription of immediate-early (IE) (α) genes (118, 169, 272). Several α proteins, including ICP4 and ICP0, act as transactivators for the transcription of early (E) (β) and late (L) (γ) HSV genes (58, 84). The β gene products include proteins that are involved in nucleic acid metabolism. Viral DNA synthesis is thought to proceed by a rolling circle mechanism (232). Several viral

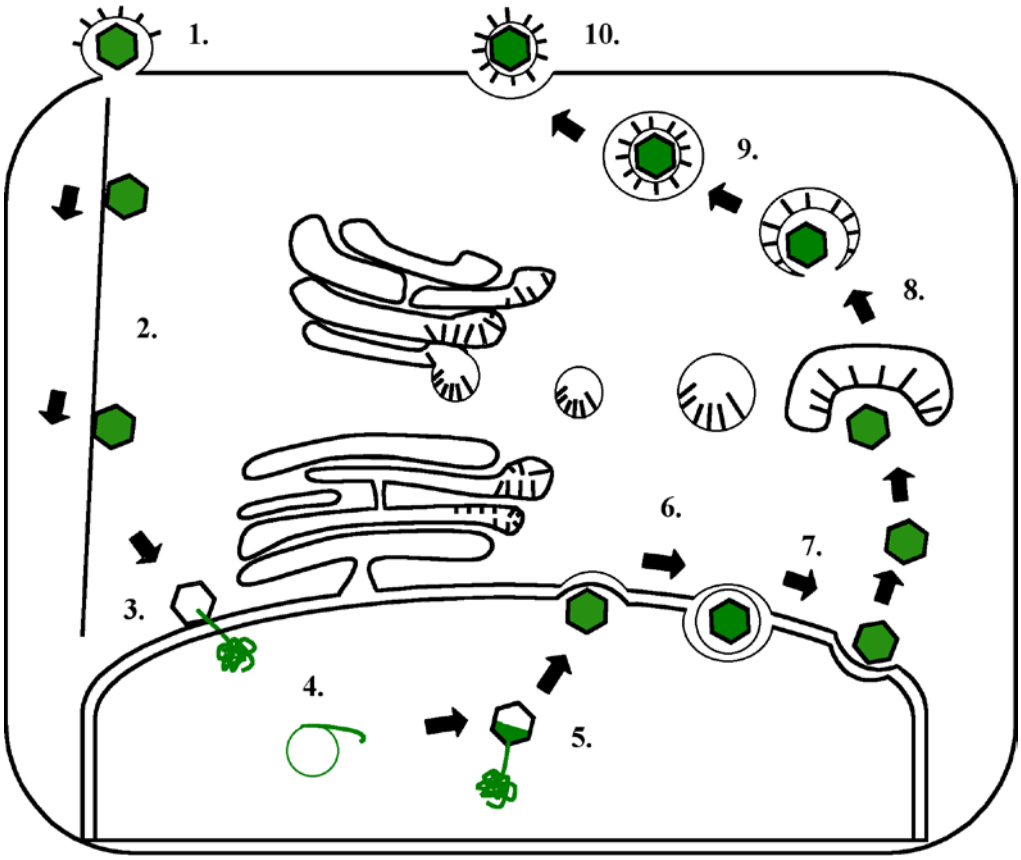

Figure III. HSV infection of a model neuronal cell. HSV attaches, binds and fuses with the cell membrane (1.). The capsid binds to microtubules and is transported to the nuclear pore (2.), where the virus releases its genome (3). Virus gene expression and DNA replication occurs in the nucleus (4.). Genomes are packaged into empty nucleocapsids (5.). Viruses bud into the inner nuclear membrane and form a primary envelope (6.). The virus then fuses with the outer nuclear membrane and releases the capsid into the cytosol (7.). The maturation of virions occurs when tegumented virions bud into glycoprotein containing vesicles of the trans-Golgi network (TGN) (8.) and form a secondary enveloped virion (9.). Mature virions are released at the cell surface (9.).

genes are necessary and sufficient for HSV DNA replication (218). These include a DNA polymerase (pU_L30), DNA polymerase cofactor (pU_L42), an origin binding protein (pU_L9), a single-stranded DNA-binding protein (ICP8) and a helicase-primase complex (pU_L5, pU_L8, and pU_L52) (49, 117). With the onset of DNA replication, the γ genes, encoding virion structural proteins, are expressed.

The newly synthesized viral glycoproteins localize to the cell surface and the inner nuclear membrane (INM) (232). Current studies have shown VP1-2 and VP16 presence and absence from the primary enveloped virions in the nucleus (24, 174, 201, 210). Whether tegument proteins are added to primary enveloped virions or solely to secondary enveloped virions is still unclear. The products of DNA synthesis are head-to-tail concatemers, which are cleaved into unit-length genomes and packaged into preformed nucleocapsids (147, 267).

In the 'envelopment-deenvelopment-reenvelopment' model, virions leave the nucleus by budding at the INM, forming a primary envelope, which is followed by virions fusing with the outer nuclear membrane (ONM) (198). After fusion, capsids are released into the cytoplasm, the site of virion maturation. Tegument is acquired and virions bud into glycoprotein containing vesicles of the TGN, forming a secondary enveloped virion. In permissive cells, the HSV life cycle, from entry to viral spread to neighboring cells, takes approximately 18 to 20 hours (237).

4. HSV entry

HSV entry is a multistep process that engages the host cell machinery in a coordinated fashion (254). Viral entry can be broadly defined as all events leading to the deposition of the

uncoated virus genome into the nucleus. HSV can enter cells by multiple routes (Fig. IV.). HSV can utilize either the endocytic or nonendocytic pathways for productive entry into host cells (214). Regardless of the entry pathway, glycoprotein B (gB), glycoprotein H (gH), glycoprotein (gL) and glycoprotein D (gD) are required for entry.

When introduced to target cells, HSV attaches to cell surface molecules. Both glycoprotein C (gC) and gB can mediate attachment to heparan sulfate moieties of cell surface proteoglycans, although this is not essential for entry (10, 136, 137).

gD binding to a cognate receptor is an essential step during HSV entry (253) (Fig. IV. 1.). gD can bind to Nectin-1 and Nectin-2, which belong to the immunoglobulin-like cell adhesion superfamily (111). gD can also interact with the tumor necrosis factor receptor-like herpesvirus entry mediator (HVEM) and specific sites on heparan sulfate generated by certain 3-O-sulfotransferases (202, 251). Upon receptor binding, it is suggested that the C-terminus of gD is displaced and activates the viral fusion machinery (107, 134, 280). Paired immunoglobulin-like type 2 receptor α (PILRα) is a cellular receptor of gB that appears to function in collaboration with gD receptors (245). Viral determinants, cellular gD-receptors, and the background of the target cell all seem to contribute toward the selection of entry pathway used by HSV (54, 70, 238).

gB and gH have been reported to play an important role in viral membrane fusion (30, 102, 262) (Fig. IV. 2.). Several studies suggest that gB functions as a fusion protein and the fusion loops at the base of gB protomers are important for its function (128, 129, 135). Recent studies suggest that low pH-induced conformational changes in gB, together with additional triggers, may be important for viral fusion during endocytosis (69). gB seems to require gH/gL interaction and function following gB fusion loop insertion into the host membrane (8).

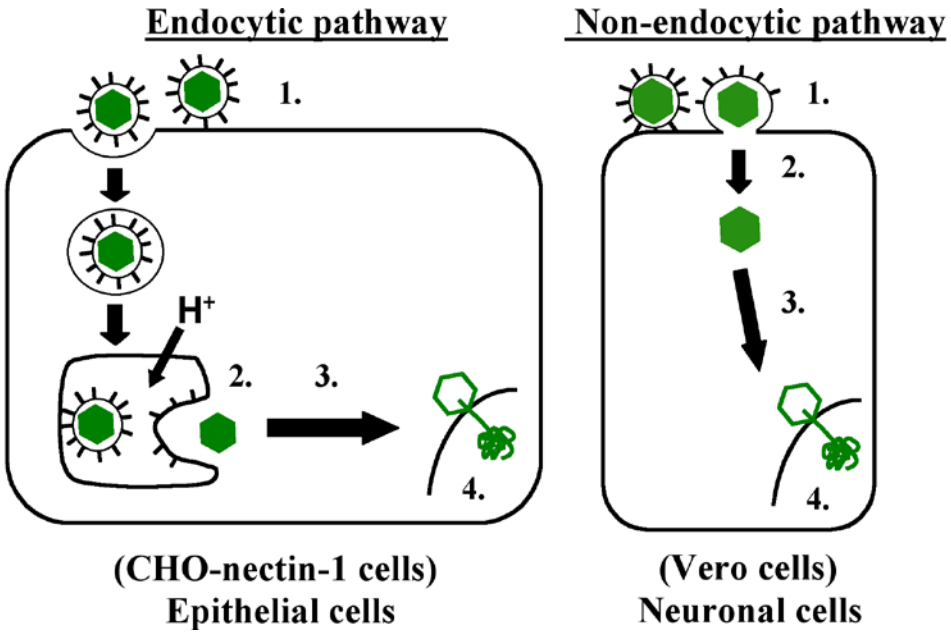

Figure IV. HSV entry pathways. On the left is the endocytic pathway or pH-dependent pathway. In this pathway, virions are taken up by cellular vesicles and then escape the endocytic pathway at a vesicle of the appropriate low pH. On the right is the nonendocytic pathway or pH-independent pathway. In this pathway, virions fuse directly with the plasma membrane. Once virions penetrate into the cytoplasm, they are transported to the nucleus, where they dock and release their genome. Model cell lines for each pathway are indicated at the bottom in parentheses. For HSV pathogenesis, our lab has proposed that the endocytic and nonendocytic pathways are used for distinct cell types, as indicated at the bottom (213). Numbers indicate the entry steps of HSV: 1. Binding, 2. Penetration/Fusion, 3. Capsid transport 4. Gene expression.

Endocytic uptake of virions is required for entry of HSV into v cells and epithelial cells, e.g. keratinocytes (213, 214) (Fig. IV. left). CHO cells are inherently resistant to HSV infection (215). Thus expression of gD receptors, such as HveA (HVEM), nectin-1, or nectin-2, are necessary for efficient entry and infection (111, 202). In CHO-nectin-1 cells, capsid uptake is rapid and half of input virus escapes the endocytic machinery by ~30 minutes (min) (215). Cellular phosphatidylinositol 3-kinase activity facilitates endocytic trafficking of HSV and notably is not required for HSV entry via the direct penetration at the cell surface.

In cell types such as neuronal or Vero cells, HSV penetrates directly through the plasma membrane and releases capsids into the cytosol (166, 213) (Fig. IV. right). In Vero cells, capsid penetration occurs within minutes and is complete by ~30 min (166, 214, 252)

After the viral envelope fuses, the bulk of the tegument appears to remain with the cell membrane (188). The HSV capsid that is released into the cytosol utilizes the minus-end-directed motor complex dynein/dynactin to travel on microtubules to the nuclear periphery (67, 252) (Fig. IV. 3.). Capsids then dock at the nuclear pore and release their DNA genome into the nucleus, the site of replication for herpesviruses (Fig. IV. 4.).

B. HSV TEGUMENT LAYER

All herpesviruses have the tegument layer between the capsid and envelope (198) (Table II.). The tegument layer is composed of mainly viral proteins. Based on the number of peptides detected by mass spectrometry, the tegument layer makes up more than half the virion (180). In extracellular herpes simplex virus, the tegument layer is asymmetrical (122, 212). At late times postinfection (p.i.), the HSV tegument undergoes a change from a symmetrical organized tegument layer that is sensitive to detergent removal to an asymmetrical tegument layer that is

Table II. Conservation of HSV-1 tegument proteins

HSV-1 Tegument protein	Gene conserved in Herpesvirinae subfamily		
	alpha	beta	gamma
pUL7	**YES**	**YES**	**YES**
pUL11	**YES**	**YES**	**YES**
pUL13	**YES**	**YES**	**YES**
pUL14	**YES**	YES	YES
pUL16	**YES**	**YES**	**YES**
pUL21	**YES**	YES	YES
pUL23	**YES**	No	**YES**
VP1/2	**YES**	**YES**	**YES**
pUL37	**YES**	**YES**	**YES**
VHS	**YES**	No	No
VP11/12	**YES**	No	No
VP13/14	**YES**	No	No
VP16	**YES**	No	No
VP22	**YES**	No	No
pUL50	**YES**	No	No
pUL51	**YES**	**YES**	**YES**
pUL55	**YES**	No	No
pUS2	**No**	No	No
pUS3	**YES**	No	No
pUS10	**No**	No	No
pUS11	**No**	No	No
ICP34.5	**No**	No	No
ICP0	**YES**	No	No
ICP4	**YES**	No	No

Bold indicates protein identified in virions by mass spectroscopy.

Table modified from (157).

resistant to tegument removal. This causes the capsid to be separated from the envelope by 30 to 35 nm of tegument on one side of the virion (122). It is currently thought that the virus carries these viral regulators to mediate activities prior to gene expression (212). Tegument proteins also have key roles in viral gene expression and egress of mature virions.

1. Composition and structure

In the mature herpes virion, there are 23 potential viral proteins and at most 49 host proteins in the tegument layer (180) (Table III.). The host proteins found in virions are expected to be in low abundance and their functions are currently unknown. The predominant viral proteins, more than 800 copies per virion, are VP16, VP22 and UL47 (131, 180). In contrast, VP1-2, UL37 (pUL37), ICP0 and ICP4 proteins are found in lower abundance, 100 copies or less, in virions (131, 180, 275, 276).

The tegument can be divided into inner and outer tegument proteins; a designation that is often based on the ease of experimental detergent removal of tegument proteins from mature virions (184, 203). By structural analysis, inner tegument proteins are icosahedrally arranged, centered on the capsid pentons (283). VP1-2 protein is suggested to be in the inner, capsid-associated part of the tegument layer due to the interaction with capsid bound pUL25 (48). Another inner tegument protein, pUL37 protein, interacts with the N-terminus of VP1-2 (200).

While VP1-2 and pUL37 are present in stoichiometric amounts, the amount of outer tegument proteins appears to vary (45). The link between the tegument layer and the virion envelope is thought to mainly depend on pUL11 and the internal domains of gD, gH and gE (97, 153, 278). Currently, VP16 is thought to be the link between inner and outer tegument proteins (206).

Table III. Properties and functions of HSV-1 tegument proteins

Tegument protein[a]	Required for growth[b]	Previously described[c]	Functions and potential roles
pUL7	NE	No	Regulates a mitochondrial function during egress
pUL11	NE	Yes	Secondary envelopment
pUL13	NE	Yes	Protein kinase, tegument release, regulates pUS3, inhibits IFN response
pUL14	NE	Yes	Nuclear import, regulates apoptosis, nuclear targeting of VP16 and capsids
pUL16	NE	Yes	Secondary envelopment
pUL21	NE	Yes	Secondary envelopment, regulates microtubule assembly
pUL23	NE	No	Thymidine kinase, viral DNA replication
VP1/2	E	Yes	Capsid transport, secondary envelopment, release of viral DNA, DUB activity
pUL37	E	Yes	Secondary envelopment, regulates viral replication
VHS	NE	Yes	Regulates translation and immune response
VP11/12	NE	Yes	Regulates pUL48-dependent transcription
VP13/14	NE	Yes	Secondary envelopment, regulates pUL48-dependent transcription
VP16	E	Yes	Secondary envelopment, regulates viral transcription
VP22	NE	Yes	Secondary envelopment, regulates microtubule assembly
pUL50	NE	No	dUTPase, viral DNA replication
pUL51	NE	Yes	Secondary envelopment
pUL55	NE	No	Unknown
pUS2	NE	Yes	Unknown
pUS3	NE	Yes	Protein kinase, primary deenvelopment, tegument release, regulates actin assembly and apoptosis
pUS10	NE	Yes	Unknown
pUS11	NE	Yes	Regulates host translation, capsid transport
ICP34.5	NE	Yes	Regulates host translation, viral DNA replication, and immune response
ICP0	NE	Yes	Regulates capsid transport
ICP4	E	Yes	Regulates viral transcription

[a] Identified HSV-1 in virions by mass spectroscopy (180).

[b] Essential (E) or Nonessential (NE).

[c] Based on studies prior to mass spectroscopy study (199, 237).

Table modified from (157).

2. *Functional properties*

Although knowledge of virion associated proteins has been broadened in recent years, the complete composition of the tegument layer awaits more comprehensive characterization (180). A few of the HSV tegument proteins have distinct functions during HSV infection, but the majority of the tegument proteins have potential functions or unclear mechanisms. Several studies suggest that tegument proteins play multiple roles throughout HSV infection.

a. Pre-immediate early events

Upon binding to a cellular receptor, there appears to be a transmission of a signal across the virus envelope causing tegument rearrangement (194). UL16 is capsid-associated in extracellular viruses and upon viral binding to cellular receptors UL16 is released from capsids (194, 198). This restructuring could prepare the tegument for release into the cytosol following viral fusion.

Virion host shutoff (VHS) protein of HSV is an RNase that degrades mRNA species (101, 168, 234). VHS is synthesized late in infection but is also found in the tegument layer of virions (101, 168). Thus VHS can immediately perform its functions following viral fusion.

In vitro and *in vivo*, inner tegument proteins promote capsid motility (183, 270). VP1-2 and pUL37 remain associated with capsids during the microtubule transport of capsids to the NPC. VP1-2 was shown to be required for capsid motility (183). While pUL37 is not essential for capsid transport, it is required for efficient transport (167, 183). However, no functional interaction between inner tegument proteins and cellular motor proteins have been identified (198).

After capsids are transported on microtubules, they bind to the NPC. VP1-2 has been suggested to play a role in the process due to the study of a temperature sensitive VP1-2 mutant, *ts*b7. At nonpermissive temperature, *ts*b7 capsids bind to the NPC but do not release their DNA (12). In a recent report, VP1-2 was shown to undergo cleavage after docking at the NPC, which was essential for DNA release (148).

b. HSV gene expression

Following viral penetration and release of tegument proteins, VP16 forms a complex with cellular cofactor HCF-1 (169). This allows for subsequent interaction with OCT-1 and assembly onto HSV IE promoters (272). OCT-1 is essential at low multiplicity of infection (MOI) while HCF-1 is key for IE gene expression (211, 216). The acidic C-terminal domain of VP16 is important for recruitment of host RNA polymerase II and initiation components (118). Viral transactivators and tegument proteins ICP0 and ICP4 may also aid in stimulation of IE gene expression but this has yet to be determined.

c. Primary envelopment and nuclear egress

After capsid assembly in the nucleus, the virus buds into the INM. HSV tegument pUL31 and pUL34 are required and sufficient for the budding process at the INM (164). Cellular and viral kinases are also recruited by pUL31 and pUL34 to disrupt nuclear lamins and allow for viral contact with the INM (207, 221). pUL34 is integrated into the primary envelope of virions interacting with tegument pUL31 but neither are included in mature virions (198).

The mechanism of fusion of the primary envelope at the ONM is currently unclear (198). pUL31 and pUL34 also appear to be involved in the deenvelopment stage (164). Mutagenesis

studies have also suggested that gB, gH and inner tegument protein pUS3 kinase may also be involved in HSV nuclear egress (98, 154). The phosphorylation of the cytoplasmic domain of gB seems to be important for this process (154).

d. Egress

Like entry, VP1-2 is required for capsid transport during egress (183). VP1-2 may also be involved in vesicular transport of secondary enveloped virions (250). In contrast to entry, UL37 is essential for egress and secondary envelopment since deletion of UL37 blocks formation of mature virions (61). How directionality is selected, dynein-mediated for entry and kinesin-mediated for egress, has yet to be determined.

pUL11, pUL20 and gK, all seem to play an important role in secondary envelopment. Deletion of UL11 results in cytoplasmic aggregates of capsids that contain tegument-like material (106). pUL11 localizes to TGN membranes (9). Due to this localization, the known interaction between pUL11 and tegument pUL16 might facilitate binding of tegumented capsids to TGN membranes (106, 177). pUL20 and gK are essential for cytoplasmic virion morphogenesis, since deletion of either protein results in cytoplasmic virions unable to acquire the secondary envelope (106). pUL11 functions are dependent on the functions of pUL20 and gK, that occur earlier in egress.

C. INFECTED CELL PROTEIN 0

HSV infected cell protein 0 (ICP0) is a 110-kDa multifunctional phosphoprotein expressed with IE kinetics (1, 84) (Table IV.). Although, ICP0 is not essential for infection, it is needed for efficient progression to lytic infection (84, 125, 242, 257). ICP0 null viruses exhibit a

Table IV. Functional properties of ICP0 proteins

Virus	Activation[a]	Growth Efficiency[a]	ND10 Disruption[a]	Colocalize Conjugated Ubiquitin *in vivo*[b]	E3 Activity *in vitro*[c]	ICP0 in Tegument[d]
17+	+++	+++	+++	+++	+++	+++
FXE	-	-	-	-	-	-
K144E	-	-	-	-	+++	-/+
N151D	-/+	-	+	+	+++	+++

[a] Activation was determined by measuring the induction of chloramphenicol acetyltransferase (CAT) activity after cotransfection of a UL39 promoter-CAT plasmid and a plasmid-expressing an ICP0 protein. Growth efficiency was measured using single-step growth curves. Cells were infected with the desired virus, harvested at various times and then titered. ND10 disruption ability was determined by transfection of plasmids-expressing ICP0 proteins and detecting PML by immunoflouresence microscopy (78).

[b] Colocalizing conjugated ubiquitin was the ability of the ICP0 protein expressed by plasmid to induce increased conjugated ubiquitin staining as detetected by immunoflouresence microscopy *in vivo* (83).

[c] E3 ligase activity was the ability of the ICP0 protein expressed by plasmid to stimulate the production polyubiquitin chains *in vitro* (21).

[d] ICP0 in the tegument was the ICP0 content of extracellular virus detected by Western blot (56).

reduction in infectivity and have similar particle numbers to wild type (WT) (257). This gives ICP0 null viruses a higher particle/PFU ratio than WT. ICP0 null viruses also exhibit a growth defect at low-multiplicity but can replicate efficiently at a high input multiplicity (242, 257). ICP0 is also needed for efficient reactivation from latency (229, 244, 257). It has been proposed that ICP0 might be a regulator in the switch between productive infection and latent infection (28, 175). ICP0 inhibits the antiviral response to cellular interferons and may help the virus bypass innate cellular repression pathways (125, 205). Bovine herpesvirus 1 (BHV-1), Equine herpes virus 1 (EHV-1), Pseudorabies virus (PRV) and Varicella Zoster virus (VZV) also express ICP0-related proteins (222).

1. Structural properties of newly expressed infected cell protein 0

Through mutagenesis studies, the N-terminal zinc-binding domain, a nuclear localization signal (NLS) and the C-terminal sequence were found to be important for many of the ICP0 functions (80, 82) (Fig. V.).

The structure of the C_3HC_4 motif or RING finger domain of equine herpes virus EICP0 was solved with two-dimensional H-NMR (11) (Fig. VI.). The RING finger domain contains an amphipathic α- helix that spans the length of the triple-stranded β-sheet. Four pairs of metal-binding residues sequester two zinc atoms and form an interleaving structure. Site-directed mutagenesis has shown that the α-helix is important for transactivation activities of ICP0 (11, 78). At the time, mutagenesis studies and the structure of the RING finger domain led investigators to believe that ICP0 family members bound DNA like classical RING domains (11). Later studies demonstrated that ICP0 belongs to the E3 ubiquitin ligase RING finger group (21).

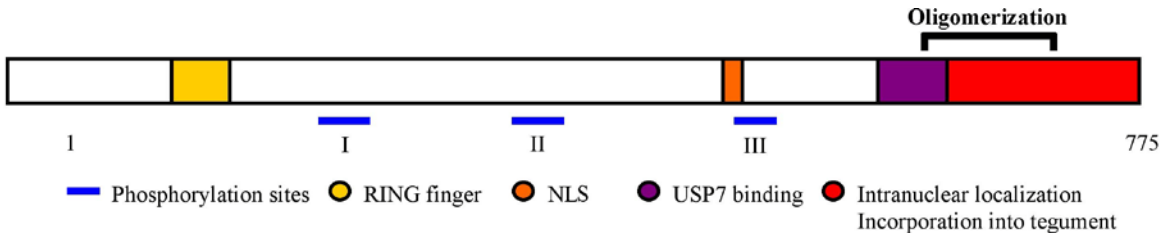

Figure V. ICP0 domains. A map of the 775-amino acid (aa) ICP0 protein, demonstrating the identified structural domains (84). Phosphorylation sites (blue) indicate three phosphorylated regions that have 11 putative phosphorylation sites (19, 52). RING finger domain (yellow) has the E3 ubiquitin ligase activity (11, 21). The NLS (orange) has the nuclear localization signal (80, 208). The USP7 binding domain (purple) is required for USP7 interactions (92, 196). The C-terminal sequence (red) is required for localization to ND10 and also required for incorporation into tegument of mature virions (80, 184, 196). Oligomerization sequences (upper bracket) indicate the domain that is required for self-multimerization (44, 196). The residue numbers of N-terminal and C-terminal portions of the protein are indicated.

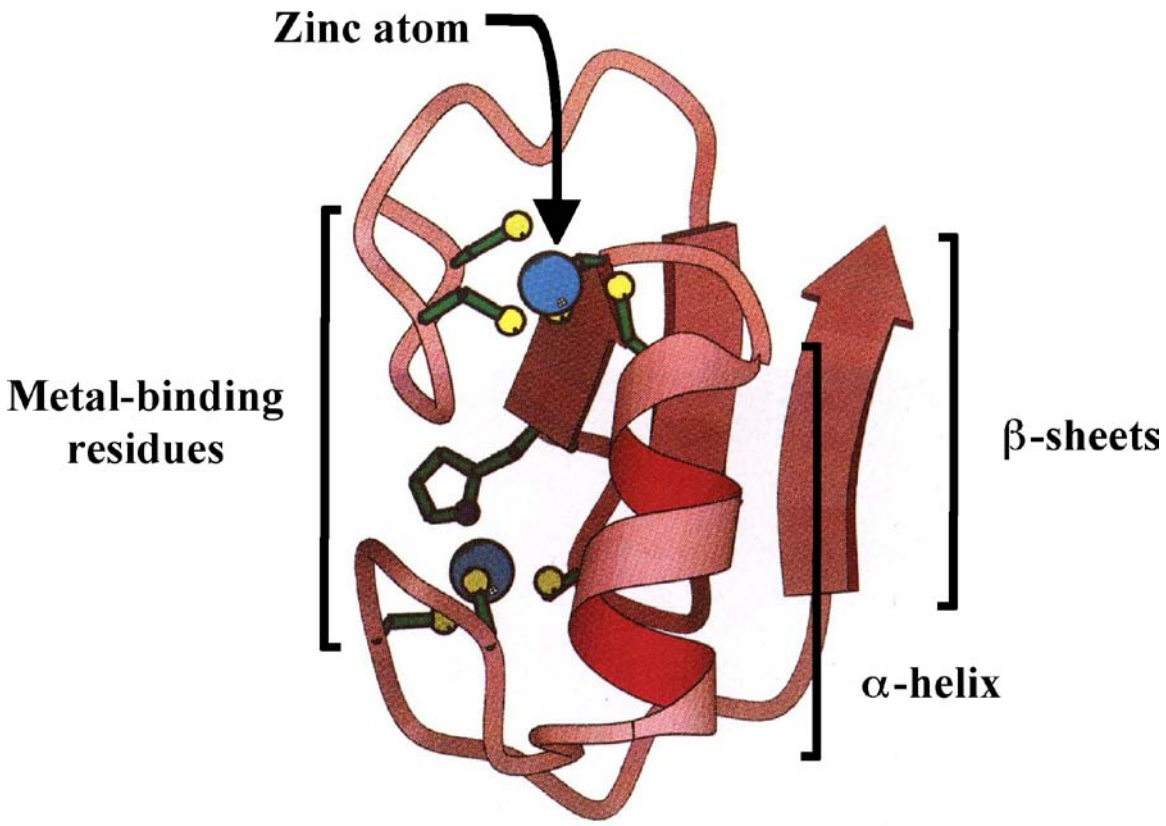

Figure VI. RING finger domain protein structure. A ribbon diagram showing the folding of the EICP0 RING finger domain of equine herpes virus. Zinc atoms are shown in blue. The zinc binding residues are represented as "ball-and-stick" (11).

Towards the center of the protein, a short 7-amino acid (aa) basic motif in ICP0 specifies the nuclear localization (80, 208). The last 125 aa of the ICP0 C-terminus function as an intranuclear localization site (80, 196). The C-terminal region of ICP0 is also important for ICP0s incorporation into the tegument layer (184).

Early studies suggested that ICP0 is dynamically phosphorylated (1). Three phosphorylation sites on ICP0 were identified and they were found to influence the localization, ND10 dispersal, stability, and transactivation characteristics of ICP0 (19, 52). However, the three phosphorylation sites do not appear to influence ICP0s E3 ubiquitin ligase activity *in vitro* (19).

During HSV infection, ICP0 strongly binds, using aa 593-633, to a cellular ubiquitin-specific protease enzyme USP7 (HAUSP) (92, 196). Interacting with USP7 protects ICP0 from auto-ubiquitination *in vitro* and proteasome-dependent degradation during infection (32). Although ICP0 and USP7 interaction can lead to ubiquitination and proteasome-dependent degradation of USP7, the stabilization of ICP0 seems to be the dominant function of the interaction (18). ICP0 can also form dimers and oligomers, using C-terminal aa 617-711, during HSV infection (44, 196). Both USP7 and oligomer formation seem to be important for HSV infection (196).

2. *Functional properties of newly expressed infected cell protein 0*

There are now numerous studies on IE ICP0 but the fundamental details of its functions are still unanswered. The link between *in vitro* ICP0 E3 ligase activity and *in vivo* activity has yet to be demonstrated. ICP0 E3 ligase activities have been suggested to repel the cellular response to HSV infection and help prepare for gene expression but still there is no direct

evidence. If ICP0 does have E3 ligase activity in vivo, then the identity of target substrates will have to found. Even so, ICP0 activities during infection and incorporation into the tegument layer have been extensively characterized.

a. Gene expression

ICP0 has been described as a promiscuous transactivator of viral and cellular genes (84, 85, 230). This is due to ICP0 being able to activate transcription from HSV or heterologous promoters independently of cis-elements (29, 93, 114, 125, 189). ICP0 appears to play a limited role in IE expression and a more prominent role in enhancing E and L expression (29). However, in the absence of viral proteins, ICP0 is required for maximum IE expression.

ICP4 is expressed with IE kinetics and is a major transactivator of early and late genes (58). ICP0 activates transcription of viral genes independently or in synergy with ICP4 (79, 85, 110, 230). ICP0 and ICP4 interact and this interaction is believed to have a synergistic effect on gene expression (110, 230).

b. E3 ligase activity

The ICP0 RING finger domain functions as an E3 ubiquitin ligase in vitro (21). The ICP0 RING finger is important for inducing the accumulation of conjugated ubiquitin at ND10 and centromeres *in vivo* (83). Newly synthesized ICP0 induces the proteasome-dependent degradation of ND10 components Promyelocytic leukemia (PML) and Sp100, centromere proteins CENP-A, CENP-B, and CENP-C, the catalytic subunit of DNA-PK, and p53 (20, 35, 88, 89, 176, 223). The induction of degradation is dependent on the RING finger domain of ICP0. This provides additional evidence that ICP0 may function as an E3 ubiquitin ligase *in vivo*

(20). Other alphaherpesvirus members have ICP0-related proteins that have E3 ubiquitin ligase activity *in vitro* and inhibit recruitment of ND10 proteins to HSV replication sites (86).

c. ND10 disruption

The cellular nucleus has an average of 10 subdomains, called ND10, with a diameter of 0.3 – 0.5 μm (7). PML and SP100 are major components of ND10 (108, 259). ND10 might be involved in such processes as cellular chromatin structure, transcription, and DNA repair (16). Early in infection, ICP0 can be found in discrete nuclear sites using immunofluorescence microscopy (82). The ICP0 foci are adjacent but separate from the replication compartments that include the HSV genome, ICP8, VP13/14 and VP22 (146, 187). These ICP0 foci colocalize with ND10, although, this colocalization is transient since the staining of ND10 is subsequently lost (186). The RING finger domain of ICP0 is required for the disruption of ND10 and degradation of PML; however, the C-terminal region is required for ND10 interaction (89, 185).

The disruption of ND10 structures is thought to facilitate HSV gene expression (187). The current hypothesis is that ND10 might be an intrinsic cellular antiviral response and have a repressive effect on gene expression (94). The depletion of PML and/or SP100 increases the infectivity of ICP0-null viruses (94, 95). However, ICP0-null virus is still not complemented to WT levels, suggesting that other factors may be involved in the repression mechanism. Interferon (IFN) is used as a cellular antiviral response and has been shown to increase PML expression (36, 171). ICP0-null virus is more sensitive than WT virus to IFN pretreatment of cells (130, 205).

3. *Properties of tegument infected cell protein 0*

Although initially defined as proteins expressed exclusively by the infected cell, ICP0 and ICP4 are detected in the virion tegument by Western blotting and mass spectrometry but not by protein staining (72, 74, 180, 219, 248, 255, 274-276, 282). Some properties of tegument ICP0 have been evaluated. Newly expressed ICP0 is localized in the nucleus early in infection (1, 90). Later in infection, IE ICP0 relocalizes to discrete cytoplasmic domains, where ICP0 may be incorporated into virions (74, 156, 178, 248). Cytoplasmic localization of ICP0 does not seem to be sufficient for incorporation into virions (184). In the absence of VP22, ICP0 is translocated to the cytoplasm but is not incorporated into virions (74, 184). Recently, it was found that ICP0 incorporation into virions correlates with the ability of ICP0 to form a complex with VP22 (184). IE protein ICP27 has also been suggested to be required for the cytoplasmic localization and incorporation of ICP0 and ICP4 into mature virions (248). Prior to the data presented here, the role of ICP0 in virion assembly was not known. The position of ICP0 in the tegument layer had not been characterized. Little was known about the ICP0 that is brought in with the infecting virion. It was not clear whether tegument ICP0 functions in a manner similar to its IE counterpart.

D. UBIQUITIN-PROTEASOME SYSTEM

Due to the high content of proteases, the lysosome was assumed to be the main site of proteolysis (53). Support for another proteolytic system came with the discovery of a cell extract containing an ATP-dependent and non-membrane-associated cellular proteolytic system that functioned at neutral pH (77). A component of the cell extract, ATP-dependent proteolysis factor-1 (APF1), was found to be conjugated to target substrates when incubated with the other

fractions and ATP (42, 139). APF1 was later shown to be ubiquitin, an 76 aa cellular protein that is added to substrates in order to target their degradation (41). The final component of this non-lysosomal system came with the discovery of a high molecular weight protease that degrades only ubiquitin tagged substrates in an ATP-dependent manner (144). This protease would later be known as the cellular proteasome. The ubiquitin-proteasome system (UPS) is currently known to be critical for maintenance of cell homeostasis, regulating processes such as cell cycle, apoptosis and antigen presentation (116, 163). The cellular proteasome also functions nonproteolytically during transcription, DNA repair, and chromatin remodeling (59).

1. Cellular proteasome

The 26S proteasome is an approximately 2.5 MDa cylindrical, multisubunit complex found in the cytosol and nucleus of cells (246) (Fig. VII.).

The cellular proteasome is responsible for degrading regulatory and misfolded proteins. It is composed of three complexes, a 20S catalytic core and two 19S regulatory complexes (Fig. VII.). The 20S catalytic core is composed of four heptameric rings that are stacked on top of each other, forming the hollow cylinder structure (120, 181). The two outer rings are comprised of seven different α-subunits while the inner rings contain seven different β-subunits (133). The four heptameric rings enclose three chambers, two fore chambers between the α-and β-rings and a central proteolytic chamber between the β-rings (120). The proteolytic active sites of the 20S core are on β-subunits and face the interior chamber of the cylinder structure. The entrance to the fore chamber is autoinhibited by the N-terminal tails of the α-ring subunits (119). Binding of the regulatory particle relieves the block imposed by the α-subunit N-terminal tails.

Three β-subunits demonstrate proteolytic activity: β1, β2 and β5 (Fig. VII.). Each of the

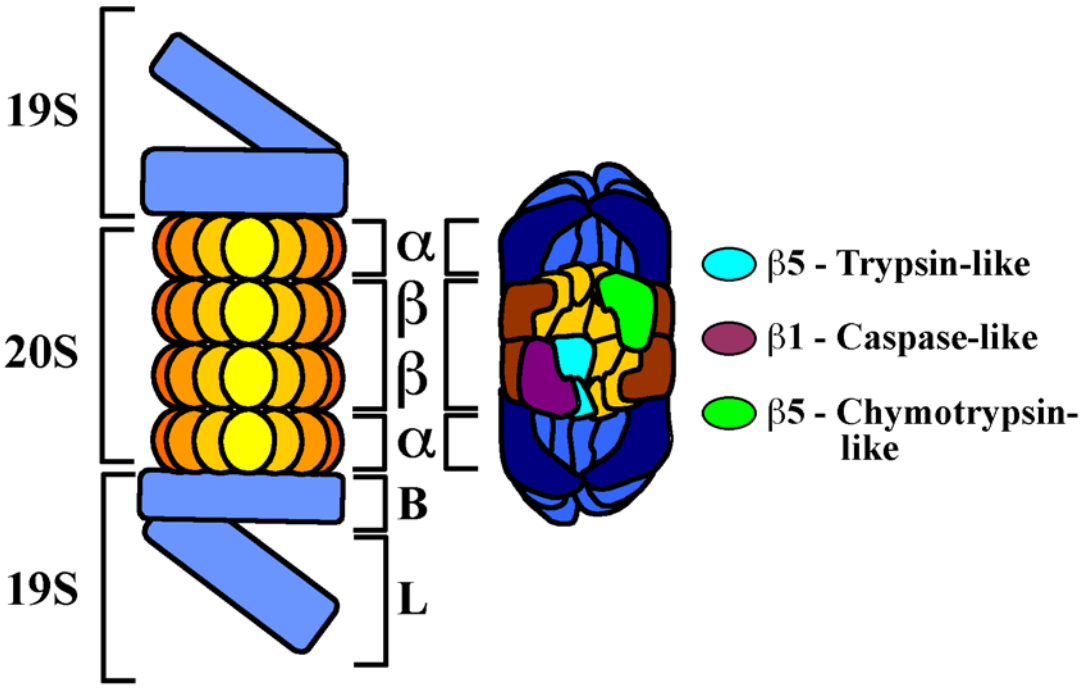

Figure VII. 26S and 20S proteasome structure. On the left, a representation of the 26S particle composed of a 20S catalytic core and two 19S regulatory complexes. The 20S catalytic core is composed of four rings of α-subunits and β-subunits stacked on top of each other. The 19S regulatory complex is composed of a base (B) and a lid (L). On the right, a cross-section of the 20S catalytic core showing the location of the three active sites.

three β-subunits has a different proteolytic activity (5, 132). β2 cleaves after hydrophobic residues and is described as having a chymotrypsin-like activity. β5 has a trypsin-like activity that preferentially cleaves after basic residues. β1 cleaves bonds after acidic residues. All of the proteolytic sites in the 20S catalytic core utilize a unique catalytic mechanism, where a N-terminal threonine is used for catalysis (249) (Fig. VIII.). Proteins degraded by the proteasome are released as oligopeptides, ranging between 3 and 24 residues (3, 161).

The 19S regulatory particle or PA700 is also a multisubunit complex (39). 19S seems to be involved in substrate recognition, unfolding and translocation of target substrates and controlling access to the proteolytic channel (22, 63, 119). The 19S subunit can be divided into base and lid components (115). The 19S base has a ring-like structure and has been shown to have ATPase activity. The ATPase component of 19S base, which regulates the opening to the proteasome, also controls the length of peptide products (165). Interferon-γ induces the formation of immunoproteasomes that have an alternate regulatory cap, 11S, and three different β-subunits (2, 13, 104, 235). This complex is believed to generate larger amounts of antigenic peptide for MHC class I presentation (15, 258).

2. Ubiquitin machinery

Ubiquitination is the covalent modification of cellular proteins with 8-kDa ubiquitin protein(s). Target substrates are modified with ubiquitin by a series of proteins, the El ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin-protein ligases (141) (Fig. IX.). The formation of the isopeptide bond between an ubiquitin molecule and the target

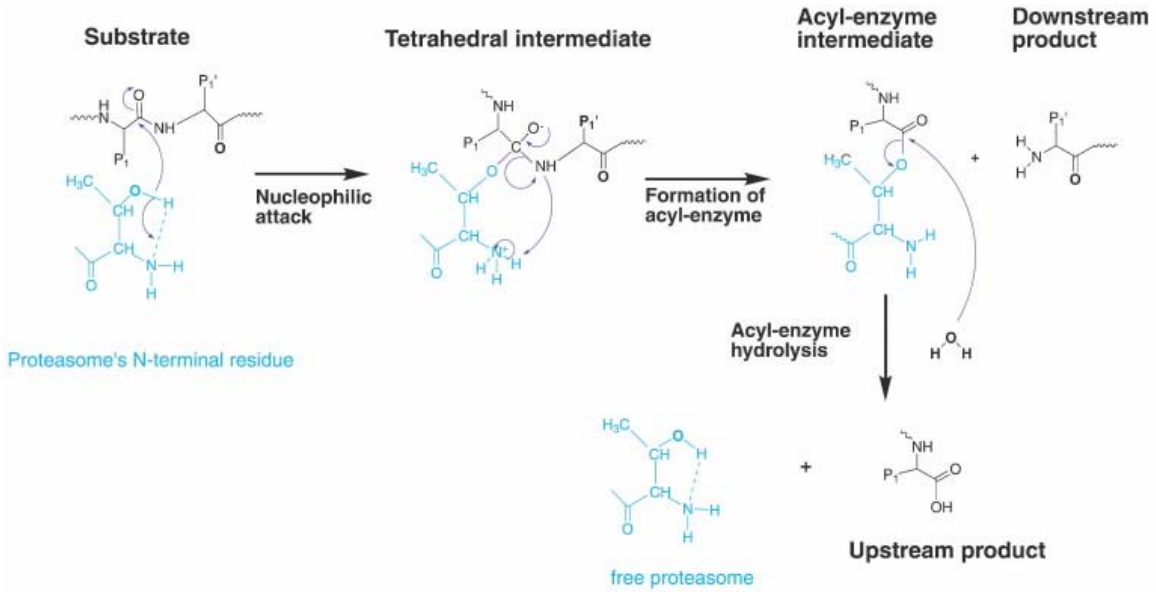

Figure VIII. 20S catalytic mechanism. Target substrate is in black, proteasome N-terminal residue is in light blue and bonds formed during catalysis are in purple (163).

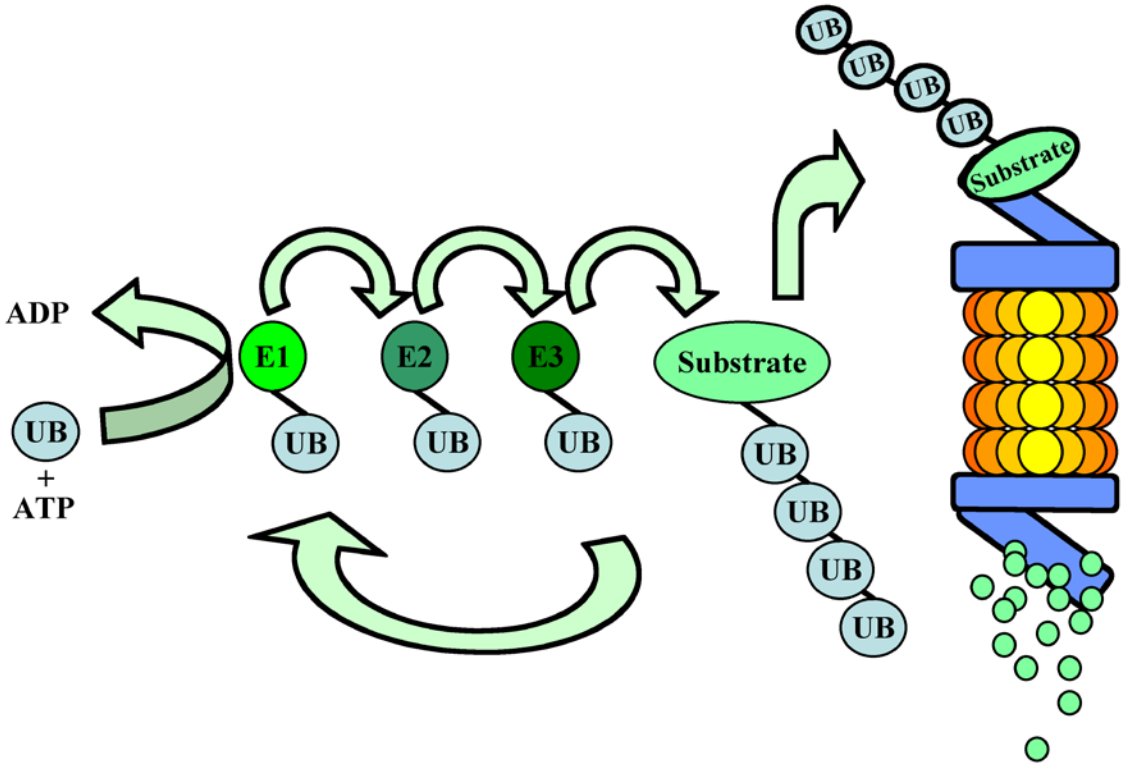

Figure IX. Overview of the ubiquitin-proteasome system. Three enzymes are required for adding ubiquitin (UB) to target substrates: E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme and E3 ubiquitin-protein ligating enzyme. E1 activates ubiquitin in an ATP-dependent manner while E3 recognizes the substrate. One ubiquitination cycle results in a monoubiquitinated substrate. The process can be repeated to form a polyubiquitin chain. The polyubiquitin chain allows for recognition by the 19S regulatory unit and subsequent degradation by the 20S catalytic core.

substrate requires ATP in order to activate the C-terminal carboxyl group of ubiquitin (43, 124). The activated ubiquitin molecule is transferred from E1 to thiol site of one of a family of E2 enzymes (226). The target substrate is bound by E3 ubiquitin ligases, which mediate the transfer of ubiquitin to a lysine residue on the substrate protein (141). E3 confers substrate specificity by recognizing and binding target proteins (142). There are thousands of cellular E3 ubiquitin ligases, each one specific for certain substrates (149). Some E2 enzymes, however, can transfer ubiquitin directly to target proteins, independently of E3 (37, 123). Deubiquitinating enzymes (DUBs) participate in the UPS by removing ubiquitin from inappropriately targeted proteins, recycling of ubiquitin from ubiquitin tagged proteins and disassembly of unanchored polyubiquitin chains (4, 170).

E3 ubiquitin ligases can be divided into two classes: Those homologous to the E6-AP Carboxyl Terminus (HECT) E3 ubiquitin ligases and Really Interesting New Gene (RING) E3 ubiquitin ligases (158). With the HECT ubiquitin ligase system, ubiquitin is transferred from E2 to E3 ligases and finally from E3 ligases to the target substrate. RING ubiquitin ligases play a more passive role, since ubiquitin is transferred directly from E2 to the target substrate. However, RING ligase activity can be regulated by phosphorylation, ubiquitination, and pseudosubstrates (62). C_3HC_4 or RING "finger" domains use conserved cysteine and histidine residues to bind to two zinc atoms and stabilize the structure (11, 17, 62). The zinc atoms in the RING domain are interleaved, hence "finger", to maintain a platform for protein-protein interaction.

The best understood function of ubiquitin modification is for selective protein degradation. The majority of proteins destined for proteasomal degradation are tagged with chains of ubiquitin (138). The minimal signal necessary for proteasomal recognition is a chain of

four ubiquitin molecules (260). The signal strength appears to increases with the number of tetraubiquitin units. E2 ubiquitin-conjugating enzymes seem to be at the heart of polyubiquitination by regulating the length and topology of the chain and processivity of chain formation (277). Typically, the α-carboxyl group of ubiquitin glycine-76 forms an isopeptide bond with the ε-amino group of lysine residue of the target substrate (26). Ubiquitin can also be added to the N-terminal residues or cysteines of cellular proteins (27, 40). In the classical view, multiubiquitin chains are formed with additional ubiquitin monomers being added to the lysine-48 of previous ubiquitin modifications (33, 140). However, each ubiquitin molecule has seven lysines, which allows for polyubiquitin chains to form through alternative linkages (243). Proteins can also be monoubiquitinated and this modification is important for regulation of histone function, in DNA repair pathways and in intracellular transport of membrane proteins (204).

3. Targeting a protein for the proteasome

Degrons are degradation signals on the target substrate that allow for UPS and/ or proteasome recognition (246).

The degrons necessary for ubiquitin machinery recognition involve N-degrons, post-translational modifications and protein folding. The N-degron involves those proteins that have an N-terminal destabilizing residue and an accessible lysine for ubiquitination (265). Post-translation modifications such as phosphorylation of specific serine and cysteine residues, hydroxylation of specific prolyl residues and glycosylation can target proteins for proteasomal degradation. Specific cellular chaperones can be used to recognize misfolded proteins and position them for ubiquitination.

The degrons necessary for proteasomal recognition and degradation involve the ubiquitin signal and unstructured regions. Ubiquitinated proteins are fairly stable unless they contain an unstructured region (228). The unstructured region in these proteins serves as an initiation site for proteasomal degradation. Typically the ubiquitin signal and the unstructured region are on the same protein, however, ubiquitination and unstructured regions can work *in trans* when separated onto different interacting proteins (227).

An ubiquitin-independent pathway can also degrade target proteins. In some circumstances, an unstructured region can serve as an initiation site by itself, in the absence of ubiquitin (246). Alternatively targeting can occur through a *trans* mechanism, where an ubiquitinated adaptor shuttles the substrate to the proteasome and the unstructured region of the substrate signals for degradation (14, 51, 227, 233). Although the adaptor would bind to the proteasome, it avoids degradation because the protein lacks a strong unstructured region (227).

4. Proteasomal inhibitors

Inhibitors have been a vital tool for understanding the unique proteolytic mechanism of the 20S catalytic core (249). Inhibitors of proteases are typically composed of a short peptide linked to a pharmacophore, a structure that interacts with enzymatic active sites and blocks its biological activity (163). The short peptide of these compounds associates with the substrate binding site. Inhibitors do not have to block all protease active sites in order to be effective. For example, inhibition or mutation of the chymotrypsin site causes a large reduction in protein breakdown while inhibition of the trypsin- or caspase-like sites has had little effect on overall proteolysis (5, 132, 160, 236).

Peptide aldehydes were the first group of proteasomal inhibitors and still are the most widely used (236, 266) (Fig. X.). Although these inhibitors bind slowly to the 20S chymotrypsin-like site, they still have a high degree of selectivity and potency for the chymotrypsin-like activity (266). These inhibitors can also inhibit cysteine and serine proteases. Due to fast dissociation rates and oxidation into inactive acids in cells, the effects of these inhibitors can be reversed upon removal of inhibitors (163, 173). MG132 (Z-Leu-Leu-Leu-al) is a commonly used peptide aldehyde. MG132 is a highly potent and selective inhibitor, since inhibition of calpains and cathepsins requires at least 10-fold higher concentrations than what is needed to block the proteasome (261).

Lactacystin is more selective in inhibition of the proteasome activity than peptide aldehyde inhibitors (Fig. X.). In contrast to synthetic peptide aldehydes, lactacystin is a *Streptomyces lactacystinaeus* metabolite. Lactacystin was found to irreversibly inhibit the proteasome chymotrypsin- and trypsin-like activities while reversibly inhibiting peptidyl glutamyl peptide hydrolyzing activity (100). Subsequent studies showed that lactacystin itself does not inhibit the proteasome but rather its neutral pH decomposition product, clasto-lactacystin-β-lactone, is responsible for inhibition (64). Lactacystin does not inhibit most serine proteases (109, 220)

Epoxyketones are the most selective inhibitors because of their unique mechanism of inhibiting 20S activity (Fig. X.). Since these compounds react with both the hydroxyl and amino group of N-threonine, they cannot inhibit cysteine or serine proteases (121, 195). The epoxyketone inhibitor, epoxomicin is an *Actinomycete* product (126).

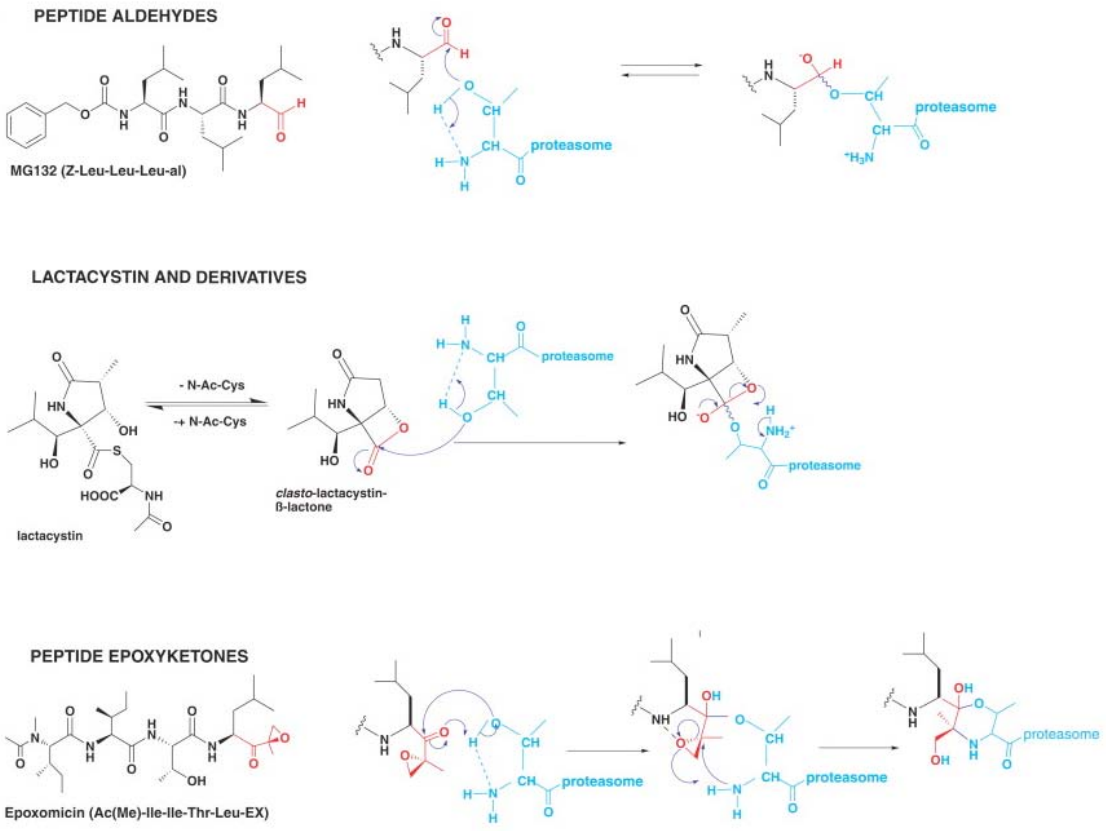

Figure X. Proteasome inhibitor mechanisms. Inhibitors are in black. Inhibitor pharmacophores are in red. The proteasome is in light blue, and newly formed bonds are purple (163).

E. AIM OF CURRENT WORK

The cellular proteasome activity has been shown to be important for the endocytic entry of several viruses. The overall goal of this research was to demonstrate a role for the cellular proteasome during HSV entry and then begin to detail the mechanism of proteasome-dependent entry. In the first section, I demonstrated that the proteasome activity was required early in HSV entry using β-galactosidase assays and time of addition experiments. Various proteasome inhibitors were used to determine which of the 20S β-subunits is required for HSV entry. A thermosensitive cell system was used to address whether the ubiquitin machinery is important for this process. I then sought to determine when the proteasome activity is required during HSV entry using binding, capsid transport, citrate inactivation, fluorescence protease protection, and fusion-from-without assays. Due to the IE ICP0's E3 ubiquitin ligase activity and dynamic interaction with the proteasome, I sought to address the fundamental properties of tegument ICP0 and determine the role of tegument ICP0 during proteasome-dependent entry of HSV. For the second section, I sought to characterize ICP0 in the virion tegument layer. The overall protein content of virions without tegument ICP0 was compared to WT to determine whether the absence of ICP0 influences assembly of mature virions. To address whether the RING finger domain of ICP0 is necessary for tegument ICP0 incorporation, the tegument ICP0 content of ICP0 mutant HSVs were analyzed via Western blot. A tegument release assay was used to discern whether ICP0 is an inner or outer tegument protein. In the last section, I begin to address ICP0's role in proteasome-dependent HSV entry using β-galactosidase reporter assays. The majority of the work presented in this thesis has been published or is in preparation for submission (55-57).

II. MATERIALS AND METHODS

A. CELLS AND MEDIA

Vero cells and U2OS cells (American Type Culture Collection, Rockville, MD) were propagated in Dulbecco modified Eagle medium (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (Gemini Bio-Products, West Sacramento, CA). CHO-nectin-1 cells (M3A cells) are CHO-K1 cells stably transformed with the human nectin-1 gene and the *Escherichia coli lacZ* gene under the control of the HSV ICP4 promoter (M3A cells provided by G. Cohen and R. Eisenberg, University of Pennsylvania) (111). The cells were propagated in Ham F-12 nutrient mixture (Invitrogen) supplemented with 10% fetal bovine serum, 150 g of puromycin (Sigma, St. Louis, MO)/ml, and 250 g of G418 sulfate (Fisher Scientific, Fair Lawn, NJ)/ml. Cells were subcultured in nonselective medium prior to use in experiments. Mouse ts20 cells (obtained from Harvey S. Ozer, University of Medicine and Dentistry of New Jersey) are derived from BALB/c 3T3 fibroblasts and contain a temperature-sensitive E1 enzyme. BALB/c 3T3 cells (American Type Culture Collection (ATCC), Manassas, VA) and ts20 cells were maintained at the permissive temperature of 35°C (38, 281). The nonpermissive temperature is 39°C.

B. VIRUSES

Parental WT HSV-1 strain KOS and its derivative 7134, which has the *lacZ* gene in place

of both inverted repeat copies of the IE ICP0 gene, were obtained from T. Holland (Wayne State University) and P. Schaffer (Harvard University) (31). HSV-1 KOS-tk12 (provided by Patricia Spear, Northwestern University) contains the lacZ gene under control of the ICP4 promoter (269). HSV-1 KOS K26GFP contains green fluorescent protein (GFP) fused to the N terminus of the VP26 capsid protein (60) (provided by Prashant Desai, Johns Hopkins University). HSV-1 strain ANG path was obtained from Thomas Holland, Wayne State University (150). WT HSV-2 strain G was obtained from ATCC (73). The additional parental WT HSV-1 and mutant strains were kindly provided by Roger D. Everett (MRC Virology Unit, Glasgow, U.K.) (Table V.). The additional WT HSV-1 virus used in this study was Glasgow strain 17 syn^+ (17^+) (23). The mutant *dl*1403 virus has a 2 kB lesion in ICP0 (257). The FXE virus has a defined lesion in the ICP0 RING finger domain (80-82, 103). Rescued *d1*1403 and FXE viruses were obtained by transfection of mutant virion DNA with a plasmid containing a fragment specifying the ICP0 gene (81, 241). The K144E and N155D viruses have single aa substitutions in the ICP0 RING finger putative alpha helix (78). M1 virus has a double aa substitution in ICP0 that reduces USP7 interaction while D12 has a deletion in the ICP0 C-terminus that inhibits USP7 binding (91, 196). Rescued M1 viruses were obtained by transfection of mutant virion DNA with fragment of the ICP0 gene that includes the locations of the M1 mutation (18). For Section III, viruses were propagated and titered on Vero cells. For Sections IV-V, viruses were propagated and titered on U2OS cells.

C. POLYCLONAL AND MONOCLONAL ANTIBODIES

Anti-p53 mouse monoclonal antibody (MAb) pab421 was purchased from EMD Chemicals, Inc. (Darmstadt, Germany). MAb 11060, which is specific for the exon 2 sequences of ICP0, was kindly provided by Roger D. Everett (MRC Virology Unit, Glasgow, U.K.) (87).

Table V. Details of ICP0 proteins expressed by viruses

Virus	ICP0 structure	Description
17+ [a]	1-775	Wild type
*dl*1403[b]	1-105	ICP0 null mutant
*dl*1403R[c]	1-775	ICP0 rescuant
FXE[d]	1-105::150-775	RING finger deletion
FXER[c]	1-775	RING finger rescuant
K144E [e]	aa 144 point mutation	Substitution in RING finger helix
N151D[e]	aa 151 point mutation	Substitution in RING finger helix
D12[f]	1-593::634-775	USP7 binding region deletion
M1[g]	R623L, K624I	USP7 binding negative substitution
M1R[h]	1-775	USP7 binding rescuant

[a] Parental virus HSV-1 Glasgow strain 17 syn$^+$ (17$^+$) (23).

[b] Has a 2 kb lesion in both inverted repeat copies of the ICP0 gene (257).

[c] Rescued virus was obtained by cotransfection with mutant virion DNA and a plasmid containing a fragment specifying the ICP0 gene (81, 241).

[d] Has a defined lesion in the ICP0 RING finger domain yielding a deletion of 45 amino acids (80-82, 103).

[e] Has a single amino acid substitution in the alpha helix of the ICP0 RING finger (78).

[f] Has a deletion in the ICP0 C-terminus USP7 binding domain (196).

[g] Has a double amino acid substitution in the USP7 binding domain (91).

[h] Rescued virus was obtained by cotransfection of mutant virion DNA with fragment of the ICP0 gene (18).

Anti-p53 MAb pab421 was purchased from EMD Chemicals, Inc. (Darmstadt, Germany). MAb 11060, which is specific for the exon 2 sequences of ICP0, was kindly provided by Roger D. Everett (MRC Virology Unit, Glasgow, U.K.) (87). Rabbit polyclonal antibody (RAb) is against the N-terminus of VP1-2 (R27B4, a gift of R. Courtney). Anti-actin MAb AC-74 and anti-alpha-tubulin MAb DM1A were purchased from Sigma (St. Louis, MO). Anti-ICP0 MAb H1A027, anti-gB MAb H1817, anti-ICP4 MAb HIA021, anti-VP16 MAb 1-21 and VP5 MAb HA018 were purchased from Virusys (North Berwick, ME). MAb 1-21 specific for VP16 was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, California) (193). Anti-gD MAb DL6 (47) and anti-gH-gL RAb R137 (225), were gifts of R. Eisenberg and G. Cohen.

D. CHEMICALS

Stocks of 0.5 M cycloheximide (Sigma), 1 mM epoxomicin (Peptides International, Louisville, KY), 4 mM MG132 (Sigma) and 10 mM N-tosyl-L-phenylalanine chloromethylketone (TPCK; Fisher Scientific) stocks were prepared in ethanol. 1 mM Lactacystin (Peptides International), 5 mM N-a-tosyl-L-lysinyl-chloromethylketone (TLCK; Sigma) and 0.2 mM YU102 (Biomol International, Plymouth Meeting, PA) stocks were prepared in dimethyl sulfoxide (Sigma). 0.5 mg of heparin (Sigma)/ml was prepared in water. All stocks were stored at 20°C.

E. PLAQUE ASSAY

Vero cells were grown in 24-well dishes overnight. Cells were mock treated or treated with MG132. HSV-1 KOS was added (100 PFU/well) in the presence or absence of MG132. At 3 hours (h) post-infection, medium was removed and sodium citrate buffer (135 mM NaCl, 40

mM $C_6H_5Na_3O_7$, 10 mM KCl [pH 3.0]) or phosphate-buffered saline (PBS) warmed to 37°C was added. At 18 to 24 h p.i., culture medium was removed, and cells were fixed with an ice-cold methanol-acetone solution (2:1 ratio) for 20 min at 20°C and air dried. Virus titers were determined by immunoperoxidase staining with anti-HSV polyclonal antibody HR50 (Fitzgerald Industries, Concord, MA).

F. CITRATE INACTIVATION ASSAY

Vero cells were grown in 24-well dishes overnight. Cells were mock treated or treated with MG132 for 30 min at 37°C. HSV-1 KOS was added (100 PFU/well) in the presence or absence of MG132 for various times at 37°C. Sodium citrate buffer or PBS warmed to 37°C was added for 1 min at 37°C. Culture medium was added, or cells were subjected to four 5 min washes with warmed medium. Infection proceeded for 18 to 24 h, and plaque formation was quantified.

G. β-GALACTOSIDASE REPORTER ASSAY

Confluent cell monolayers grown in 96-well dishes were treated with medium containing proteasome inhibitors or vehicle controls for 15 to 30 min at 37°C. HSV-1 KOS or KOS-tk12 (MOI of 4, unless otherwise indicated) was added. Cells were incubated in the constant presence of proteasome inhibitors for 6 to 7.5 h. 0.5% Nonidet P-40 (Sigma) cell lysates were prepared, chlorophenol red--D-galacto-pyranoside (Roche Diagnostic, Indianapolis, IN) was added, and the β-galactosidase activity was read at 595 nm with an ELx808 microtiter plate reader (BioTek Instruments, Winooski, VT). β-Galactosidase activity indicated successful entry. Similar results

were obtained at an MOI of 1. Mean results and standard errors were calculated for four replicate samples.

H. CELL VIABILITY

Cell cultures in 96-well dishes were treated with culture medium plus inhibitor or mock-treated for 7.5 h. Cell monolayers were treated with trypsin and resuspended in a 0.07% solution of trypan blue (Sigma). Cells were counted on a hemacytometer by using light microscopy. The 100% viability was equal to the number of cells that exclude trypan blue/total number of cells in mock-treated samples. Mean results and the standard error were calculated for four replicate samples.

I. CELL BINDING ASSAY

Vero cells were grown overnight on glass coverslips in 24-well dishes. Cells were treated with the indicated inhibitor for 15 min at 37°C. Cultures were then chilled on ice, and HSV-1 K26GFP was added at an MOI of 30. Dishes were centrifuged at 200 g for 1 h at 4°C to enhance virus binding. Cells were washed with ice-cold PBS (Invitrogen) and then fixed with 3% paraformaldehyde (Thomas Scientific, Swedesboro, NJ) in PBS prior to confocal microscopy.

J. SUBCELLULAR LOCALIZATION OF HSV BY CONFOCAL MICROSCROPY

Vero cells were grown overnight on glass coverslips in 24-well dishes. Cells were treated with proteasome inhibitor or vehicle control in the presence of 0.5 mM cycloheximide for 15 min at 37°C. Cultures were rapidly chilled on ice, and then a supernatant preparation of HSV-1 KOS K26GFP was added (MOI of 10) in the continued presence of agent. Virus was spinoculated onto

the cell surface by centrifugation at 200 g for 1 h at 4°C. Entry was initiated by shifting dishes to 37°C for 2.5 h in the continued presence of agent. Cells were washed three times with PBS and fixed in 3% paraformaldehyde in PBS prior to confocal microscopy.

Paraformaldehyde-fixed cells on cover-slips were permeabilized with 0.2% Triton X-100 (Fisher Scientific) in PBS for 8 min. Cells were then blocked with PBS containing 1% bovine serum albumin (Sigma) for 20 min. Nuclei were counterstained with DAPI (4,6-diamidino-2-phenylindole; Roche). Coverslips were washed with PBS, mounted with Fluoromount G (Electron Microscopy Sciences), and viewed with a Zeiss LSM 510 Meta microscope equipped with a 63 oil immersion objective lens. Digital images were processed with Adobe Photoshop CS2 version 9.

K. FLUORESCENCE PROTEASE PROTECTION ASSAY

Modified from (179). HSV entry into Vero cells proceeded in the presence of 25 μM MG132 for 2.5 h as described above for subcellular localization of GFP-tagged HSV. Cells were then chilled and washed twice with ice-cold KHM buffer (110 mM KC2H3O2, 20 mM HEPES, 2 mM MgCl2 [pH 7.2]). For protease treatment, 100 μg of ice-cold proteinase K (Invitrogen)/ml in KHM buffer was added to live cells for 2.5 min on ice. Proteolysis was halted with 4 mM phenylmethylsulfonyl fluoride (Sigma) and 1% bovine serum albumin in KHM buffer. For permeabilized samples, digitonin (EMD Chemicals, Inc.) in KHM buffer was added to cells for 3 min at 37°C prior to proteinase K treatment. Cells were washed twice with ice-cold PBS, fixed with 3% paraformaldehyde, and then visualized by confocal microscopy.

L. FUSION-FROM-WITHOUT ASSAY

As described previously (54), Vero cells were pretreated with growth medium containing 0.5 mM cycloheximide with or without 50 μM MG132 for 15 min. Cell-free supernatant preparations of HSV-1 ANG path were added to cells (MOI of 50) for 3 h at 37°C in the constant presence of 0.5 mM cycloheximide. Cells were rinsed with PBS and then fixed in 100% methanol. Monolayers were air dried, and then nuclei were stained with Giemsa (Sigma). Micrographs were taken with a Zeiss Axiovert 40C microscope equipped with a Canon PowerShot G6 digital camera. Digital images were processed with Adobe Photoshop CS2 version 9.0. To quantitate fusion, photomicrographs of random fields from triplicate wells (500 cells/well) were scored. The number of nuclei present in clusters of four or more divided by the total number of nuclei yielded the percent fusion.

M. THERMOSENSITIVE E1 UBIQUITIN-ACTIVATING ENZYME ASSAY

Modified from (38). Confluent cell monolayers grown in 96-well dishes of BALB/c 3T3 or ts20 cells were maintained at 35°C or transferred to 39°C for 18 h. HSV-1 KOS-tk12 was diluted in media and warmed at 39°C for 1 h. Virus was then added to cells and incubated in the constant temperature for 6 h. 0.5% Nonidet P-40 (Sigma) cell lysates were prepared, chlorophenol red-D-galacto-pyranoside (Roche Diagnostic, Indianapolis, IN) was added, and the β-galactosidase activity was read.

N. SODIUM-DODECYL POLYACRYLAMIDE ELECTROPHORESIS

Samples in Laemmli buffer were separated by sodium dodecyl sulfate (SDS)–10% polyacrylamide gel electrophoresis (PAGE), blotted onto nitrocellulose and probed for p53 or

alpha-tubulin. For the remaining experiments, samples in Laemmli buffer were separated by SDS–4-20% polyacrylamide gel electrophoresis. Nitrocellulose membranes were then incubated with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit immunoglobulin G (Pierce, Rockford, IL), developed with enhanced chemiluminescence detection reagents (Pierce), and exposed to X-ray film (Kodak).

O. PREPARATION OF UNINFECTED AND INFECTED CELL LYSATES

Modified from (38). BALB/c 3T3 or ts20 cells were maintained at 35°C or transferred to 39°C for 18 h. Lysates were prepared in radioimmunoprecipitation assay buffer (100 mM NaCl, 25 mM Tris [pH 7.5], 2% Nonidet P-40, 0.5% sodium deoxycholate, 0.2% SDS containing protease inhibitor cocktail (Roche).

U2OS cells were infected with the indicated virus (based on 17+ MOI of 1) for 18 h. Lysates were prepared in NP-40 buffer (150 mM NaCl, 50 mM Tris [pH 7.2], 1% Nonidet P-40, and 2 mM EDTA) containing protease inhibitor cocktail (Roche). Cell lysates were analyzed by SDS-PAGE followed by Western blotting with MAb H1A027 to ICP0.

P. TEGUMENT RELEASE ASSAY

Modified from (203). Extracellular 17+ virions (3×10^9 PFU) were resuspended in 0.2 ml lysis buffer (50 mM HEPES [pH 7.4] and 1% Triton X-100) containing 0.1, 0.5, or 1 M NaCl. The reactions were then incubated for 30 min at 37°C. The reaction mixtures were centrifuged at 14,000 rpm for 30 min through a 0.5 ml 35% sucrose cushion. The 200 µl supernatant above the sucrose cushion was recovered. Released proteins in the supernatant were heat precipitated and resuspended in Laemmli buffer. The sucrose cushion was removed, and the pellet, containing

capsids and capsid-associated tegument proteins, was resuspended in Laemmli buffer. Pelleted (pellet) and released (sup) fractions were analyzed by SDS-PAGE and immunoblotting with antibodies to ICP0, VP1-2, ICP4, VP16, gB, or VP5.

III. PROTEASOME ACTIVITY IS REQUIRED FOR HSV ENTRY AT A POST-PENETRATION STEP

A. INTRODUCTION

The major classes of cellular proteases include cysteine, serine, aspartic acid metalloproteases and the proteasome. Viruses take advantage of cellular proteases for viral entry and infection. Endosomal cysteine protease cleavage of Ebola virus glycoprotein facilitates viral interaction with cellular receptors (152). The 26S proteasome is important for the entry of several other viruses (159, 239, 240, 279), all of which utilize endocytic pathways. For studies with murine hepatitis and minute virus, pulse MG132 treatment of infected cells showed that proteasome activity is required early in viral entry (239, 240, 279). Influenza virus and murine hepatitis virus both require the proteasome activity prior to viral penetration (159, 279). MG132 traps influenza virus in what are thought to be sorting endosomes (159). Murine hepatitis virus is blocked in an endocytic compartment, prior to penetration (279). In contrast, MG132-arrested virions of the nonenveloped minute virus of mice appear in the nuclear periphery, after they have escaped or penetrated the endosomal membrane (239, 240). There have been several studies on the role of the proteasome in HSV infection of cultured cells (25, 35, 50, 76, 84, 89, 125, 178). These studies were carried out under postentry conditions and at times in which newly expressed viral gene products are active.

Our lab used a panel of protease inhibitors to evaluate whether HSV uses cellular proteases to facilitate entry into host cells. Only the proteasomal inhibitor lactacystin blocked HSV entry (unpublished data). The requirement for the proteasomal activity during HSV entry was then confirmed and characterized. Cell-type dependence on the proteasome activity during HSV entry was evaluated using model cell lines for endocytic and nonendocytic entry pathways. I also examined whether the proteasome-dependence during entry is strain- or serotype-dependent. I performed time of addition experiments with MG132 to demonstrate that the proteasome-dependence was with HSV entry and not with downstream events. The active site of the 20S proteasome required for HSV entry was examined. Potential interplay between the proteasome and ubiquitin machinery during HSV entry was also evaluated using a thermosensitive cell line.

The time of addition experiments suggested that proteasome activity is required for an early HSV step, either for binding to the cell surface, fusion or capsid transport. Although, the examples of virions that require the proteasome for entry do not appear to require the proteasome activity for binding, the possibility still remained and had to be tested. To define more precisely the step in viral entry that is influenced by proteasome activity, the effect of MG132 on steps prior to gene expression were analyzed by confocal microscopy. In confocal experiments, HSV was added to cells in the presence of cycloheximide, a protein synthesis inhibitor, to ensure that the entry steps being evaluated were independent of downstream events.

B. RESULTS

1. Early HSV infection is blocked by proteasomal inhibitors

The presence of MG132 during the first 3 h of infection inhibited HSV plaque formation by 60% (Fig. XI. A.). HSV-induced expression of β-galactosidase is frequently used as a measure of successful viral entry. HSV-1 KOS-tk12 entry into Vero cells, as measured by a β-galactosidase reporter assay, was inhibited by MG132 in a concentration-dependent manner (Fig. XI. B.). Epoxomicin also blocked HSV-induced β-galactosidase activity (Fig. XI. C.). The inhibitors were effective at concentrations that were not toxic to the cells, as measured by a cell viability assay (Fig. XI.). Since reporter gene activity is measured at 6 to 7.5 h p.i., it is important to note that steps up to and including IE gene expression might be affected in this assay. Together, the data suggested a role for the proteasome in HSV entry and infection.

2. Early HSV infection by endocytic or nonendocytic routes are proteasome-dependent

Vero cells support nonendocytic entry via direct fusion at the plasma membrane (105, 166, 214, 252). In contrast, CHO-nectin-1 cells are a prototype line that supports HSV entry via fusion with an internal membrane after endocytosis (214). Both MG132 and lactacystin impaired HSV KOS-tk12 infection of CHO-nectin-1 cells, as measured by HSV-induced β-galactosidase activity (Fig. XI. D.-E.). Lactacystin appears to have a greater effect than MG132 or epoxomicin

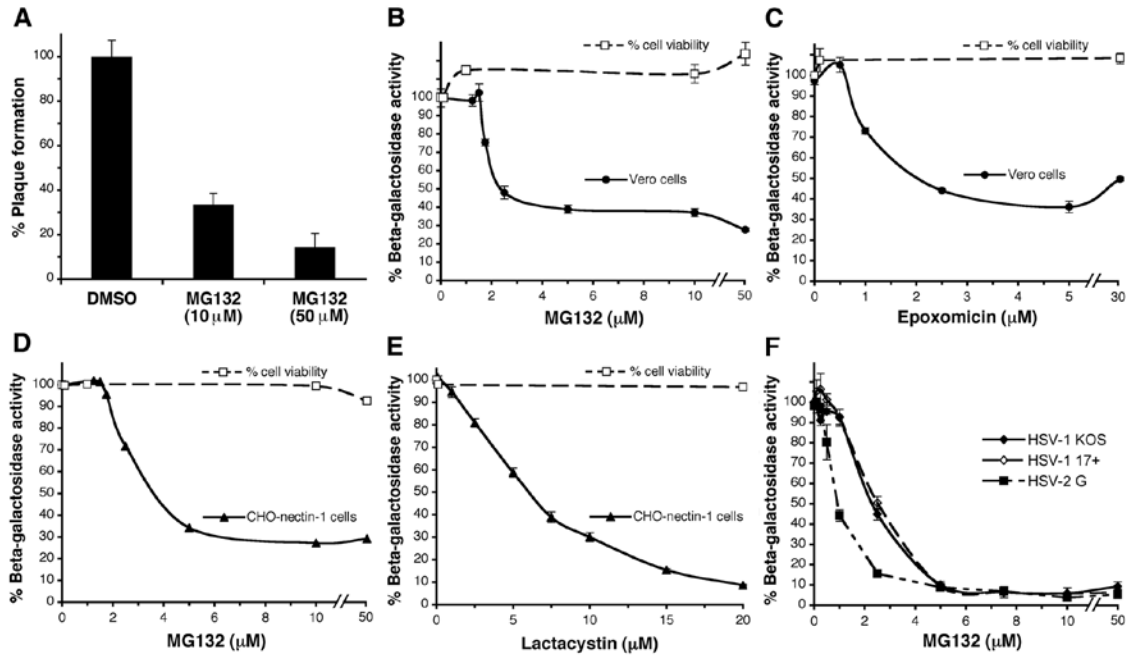

Figure XI. Effect of proteasomal inhibitors on early HSV infection. (A) Effect of MG132 on HSV plaque formation. HSV-1 KOS (100 PFU/well) was added to Vero cells in the presence of MG132. At 3 h post-infection, medium was removed, extracellular virus was acid-inactivated, and plates were incubated for 24 h. Plaques were detected by immunoperoxidase staining and quantified. Data are means of quadruplicate determinations with standard error. Vero cells (B, C) or CHO cells expressing nectin-1, containing a *lacz* gene cassette, (D, E, F) were treated with the indicated concentrations of inhibitor for 15 min. HSV-1 KOS-tk12, containing a *lacz* gene cassette, (B-E) was added for 7.5 h in the continued presence of inhibitor. HSV-1 KOS, HSV-1 17+, HSV-2 G (F) were added for 6 h in the continued presence of inhibitor. Entry was measured as the % of beta-galactosidase activity relative to that obtained in the absence of agent. Data are means of quadruplicate determinations with standard error. The effect of inhibitor on cell viability was measured by trypan blue exclusion. Viability in the absence of inhibitor was set to 100%. The mean viability and standard error of quadruplicate samples is shown.

in CHO-nectin-1 and Vero cells (Fig. XI. D.-E. and data not shown). This may be because the efficacy of a proteasome inhibitor can differ depending on the substrate being degraded (162). The data suggests that HSV infection is dependent on proteasomal degradation, regardless of whether endocytic or nonendocytic entry routes are utilized.

3. Proteasome-dependence is strain and serotype independent

The HSV-1 KOS strain was used to demonstrate a role for the cellular proteasome during HSV entry (55). For the ICP0-tegument studies, the HSV-1 17+ strain is the background for all of the ICP0 mutants. Before performing experiments with HSV-1 ICP0 mutants, I had to confirm that WT 17+ entered host cells via a proteasome-dependent pathway. KOS and 17+ were added to CHO-nectin-1 in the presence of MG132 and then β-galactosidase activity was measured at 6 p.i. KOS and 17+ were inhibited in a very similar concentration-dependent manner (Fig. XI. F.). Since HSV-2 G strain was also inhibited by MG132 (Fig. XI. F.), the data suggests that proteasome-dependent entry may be independent of HSV strain or serotype (55).

4. Inhibitors act directly on the cellular proteasome

Herpesviruses mimic a myriad of cellular functions (237). It was possible that in the previous experiments, the proteasome inhibitors were interfering with a virus-encoded function of HSV that is needed for entry. To determine whether this was the case, I took advantage of the known irreversible effect of lactacystin on the proteasome. Once lactacystin binds to 20S subunits, its inhibitory effect on the proteasome cannot be reversed by washing the cells. CHO-nectin-1 cells were pretreated with medium containing lactacystin. Medium was removed, and cells were washed prior to addition of HSV. Lactacystin inhibited HSV-induced β-galactosidase

activity in an equivalent manner, whether the cells were washed prior to infection or whether lactacystin was present for the duration of the assay (Fig. XII. A.). This result is consistent with the inhibitor acting directly on the proteasome itself and not on a function of HSV. If lactacystin were affecting HSV directly, then infection would have resumed after the removal and washout of lactacystin. This was not the case (Fig. XII. A.). In contrast to lactacystin, MG132 is a reversible inhibitor (173). Cells that were treated with MG132 and then washed prior to addition of virus were much more susceptible to HSV than the cells that were not washed (Fig. XII. B.). The effect of MG132 was 85% reversible in this cell system.

5. Proteasome activity is required at an early step of HSV entry

To begin to identify the viral process that involves proteasomal degradation, cells were infected with HSV, and then MG132 was added at different times postinfection. The later the MG132 was added, the less of an inhibitory effect there was on HSV infection of either Vero or CHO-nectin-1 cells (Fig. XIII.). MG132 was most effective at blocking virus-induced β-galactosidase activity when added to Vero cells during the 1st h p.i. or when added to CHO-nectin-1 cells during the first 2 h p.i. (Fig. XIII.). The results suggest that an early event in HSV infection, such as viral entry, requires the proteasome.

6. Chymotrypsin-like proteasome active site required for HSV entry

The 20S catalytic core contains three different proteolytic sites, chymotrypsin-, trypsin- and caspase-like active sites (162). The minute virus of mice, which also interacts with the proteasome for a post-penetration step, specifically requires the chymotrypsin-like site (239). Thus, I examined which of the 20S active sites was required for HSV entry. To address this

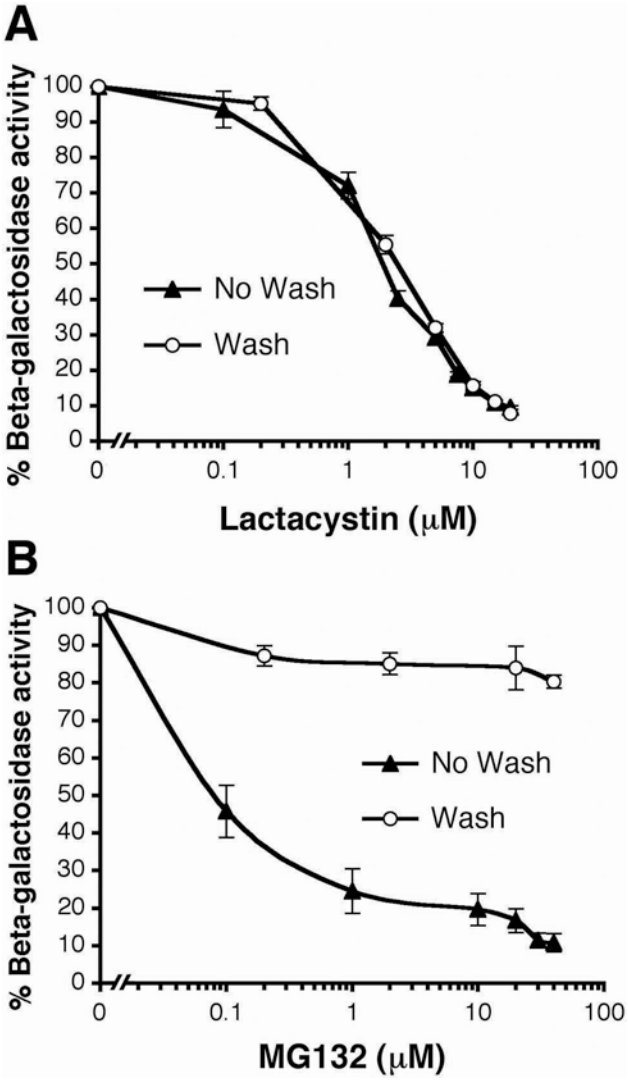

Figure XII. Lactacystin acts directly on the cell proteasome, not on an HSV-specific function. CHO-nectin-1 cells were treated with lactacystin (A) or MG132 (B) for 30 min at 37˚C. Medium was removed. Cells were either washed four times for 5 min with culture medium, and then HSV-1 KOS-tk12 (MOI of 1) was added in medium without inhibitor (Wash), or virus was added in the continued presence of agent (No Wash). Beta-galactosidase activity was measured at 6 h p.i. as an indication of viral entry (as in Fig. XI). Data are means of quadruplicate determinations with standard error.

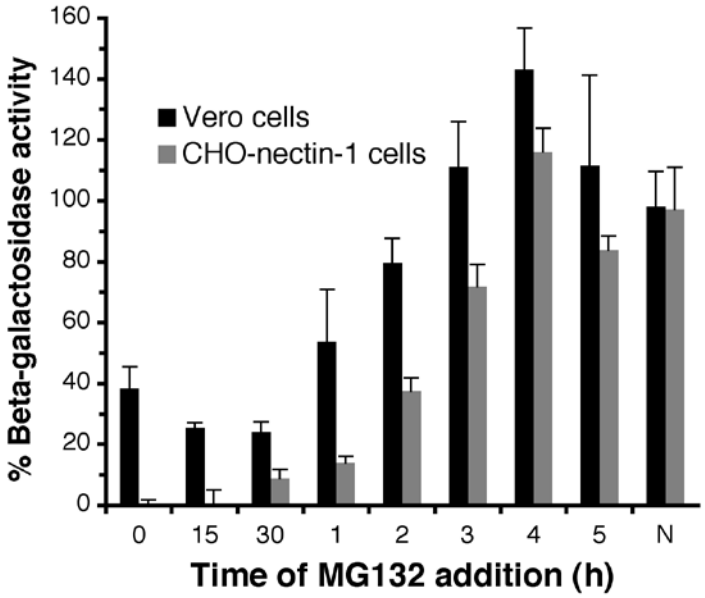

Figure XIII. MG132 is most effective when added early in infection. HSV-1 KOS-tk12 or KOS was bound to Vero or CHO-nectin-1 cells, respectively, for 1 h at 4˚C. Cells were rapidly warmed to 37˚C to initiate infection. At the indicated times, 25 μM MG132 was added to the cells. Beta-galactosidase activity was measured at 6 h p.i. as an indication of viral entry (as in Fig. XI). N, no MG132 added. Data are means of quadruplicate determinations with standard error.

question, the proteasomal chymotrypsin-, trypsin- and caspase-like activities were specifically inhibited with N-tosyl-L-phenylalanine chloromethylketone (TPCK), N-α-tosyl-L-lysinyl-chloromethylketone (TLCK) and YU102, respectively. Concentrations known to inhibit proteasome activity were used. TLCK and YU102 had no effect on early HSV infection (Fig. XIV.). Similar results were seen with leupeptin, a trypsin-like active site inhibitor (date not shown). A dose-dependent decrease in HSV-induced expression of β-galactosidase was only observed with TPCK (Fig. XIV.). Similar results were seen with the other chymotrypsin-like inhibitors MG132 (Fig. XI. B. and D.), lactacystin (Fig. XI. E.) and Epoxomicin (data not shown). These results suggest that the predominant active site of the proteasome required for HSV entry is the chymotrypsin-like site.

7. HSV entry is independent of the E1 ubiquitin-activating enzyme

I then determined whether the host ubiquitination machinery is required for HSV entry. To assess HSV entry under conditions where ubiquitin monomers are not activated, a mouse ts20 cell line was used (38), which is derived from BALB/c 3T3 fibroblasts. These cells have a temperature-sensitive E1 enzyme, which is nonfunctional at 39°C.

I first verified that E1 is nonfunctional in ts20 cells at the nonpermissive temperature by using a p53 stability assay. The transcription factor p53 is a regulator of cell proliferation, differentiation, and apoptosis (172). Wild-type p53 has a short half-life and is degraded by the UPS (38). p53 was stabilized in ts20 cells at the restrictive temperature (Fig. XV. A.), confirming inactivation of E1.

Notably, HSV-1 KOS-tk12 expressed β-galactosidase in ts20 cells at 39°C in a manner

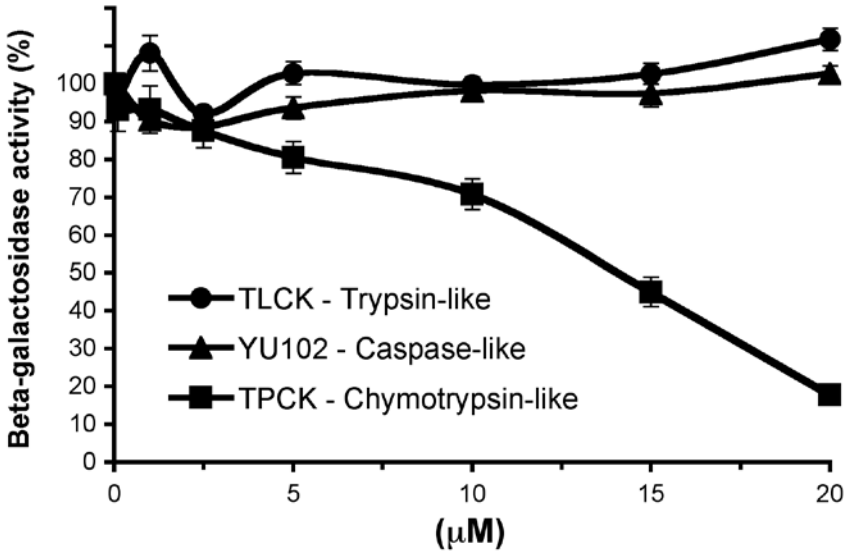

Figure XIV. Effect of inhibitors of the distinct proteolytic activities on the 20S β-subunit. Vero cells were treated with TLCK, YU102 or TPCK for 15 min at 37˚C. KOS-tk12 virus was added for 6 hr in the continued presence of inhibitor. The percent β-galactosidase activity relative to that obtained in the absence of agent is indicated. The data are means of quadruplicate determinations with the standard errors.

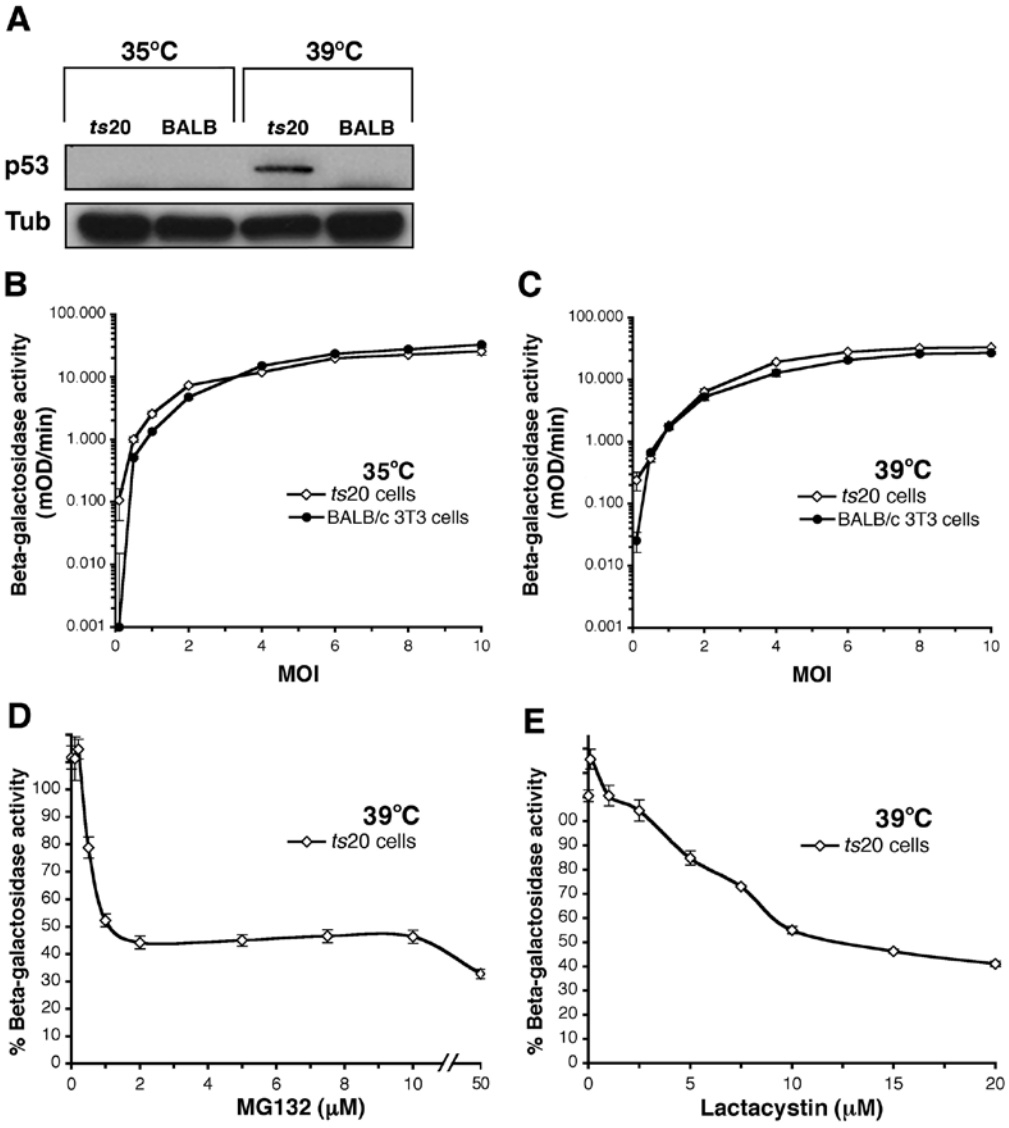

Figure XV. Role of a cellular E1 ubiquitin-activating enzyme in HSV entry. ts20 or BALB/c 3T3 cells were cultured at 35˚C or 39˚C for 18 h. (A) p53 is not degraded in ts20 cells at the non-permissive temperature. Lysates were prepared and analyzed by SDS-PAGE and immunoblotted for p53 or α-tubulin (Tub). (B-C) HSV enters cells with an inactive E1 ubiquitin-activating enzyme. HSV-1 KOS-tk12 was added to cells cultured at 35˚C (B) or 39˚C (C). Beta-galactosidase activity was determined at 6 h p.i. (D-E) E1 ubiquitin-activating enzyme-independent entry of HSV still requires proteasome activity. ts20 cells maintained at 39˚C were

treated with MG132 (D) or lactacystin (E) for 15 min. HSV-1 KOS-tk12 was added in the presence of inhibitor. Beta-galactosidase activity was measured at 6 h p.i. (as in Fig. 1). Data are means of quadruplicate determinations with standard errors.

similar to that seen at 35°C (Fig. XV. B.-C.). Thus, viral entry proceeded under conditions that inhibited a cellular E1 ubiquitin-activating enzyme (Fig. XV. A. and C.). HSV also infected control BALB/c 3T3 cells at both temperatures (Fig. XV. B.-C.). Virus-induced gene expression in ts20 cells at the nonpermissive temperature was inhibited by MG132 and lactacystin (Fig. XV. D.-E.), indicating that entry is still reliant on active proteasomes. Although MG132 and lactacystin seem to be slightly less effective in ts20 cells than CHO-nectin-1 cells (Fig. XI. D.-E. and Fig. XV. D.-E.). Together, the results suggest that HSV may enter cells in a proteasome-dependent, ubiquitin-independent manner.

8. Binding of HSV to the cell surface is independent of proteasome activity

First, I determined whether proteasome inhibitors disrupted HSV binding to Vero cells. Vero cells were treated with MG132, and then HSV-1 K26GFP was added for 1 h at 4°C. Each punctate fluorescent signal is an individual K26GFP virion. MG132 treatment had no discernible inhibitory effect on HSV binding to cells (Fig. XVI. A.-B.). In contrast, control treatment with 1 g of heparin/ml blocked virus binding to cells by more than 80% (Fig. XVI. C.-D.) (271). This suggested that the proteasome is not needed for virus binding to the cell surface.

9. MG132 affects an entry step prior to capsid transport

I investigated the role of the proteasome in the delivery of incoming HSV-1 K26GFP capsids to the nucleus during entry. In HSV-infected Vero cells, the bulk of the GFP signal is detected at or near the nucleus by 2.5 h p.i. (Fig. XVI. E.). In the continued presence of MG132, lactacystin, or epoxomicin, GFP-tagged capsids were not effectively transported to the nuclear periphery (Fig. XVI. F.-H.). Instead, the bulk of GFP-tagged viral particles appeared to be

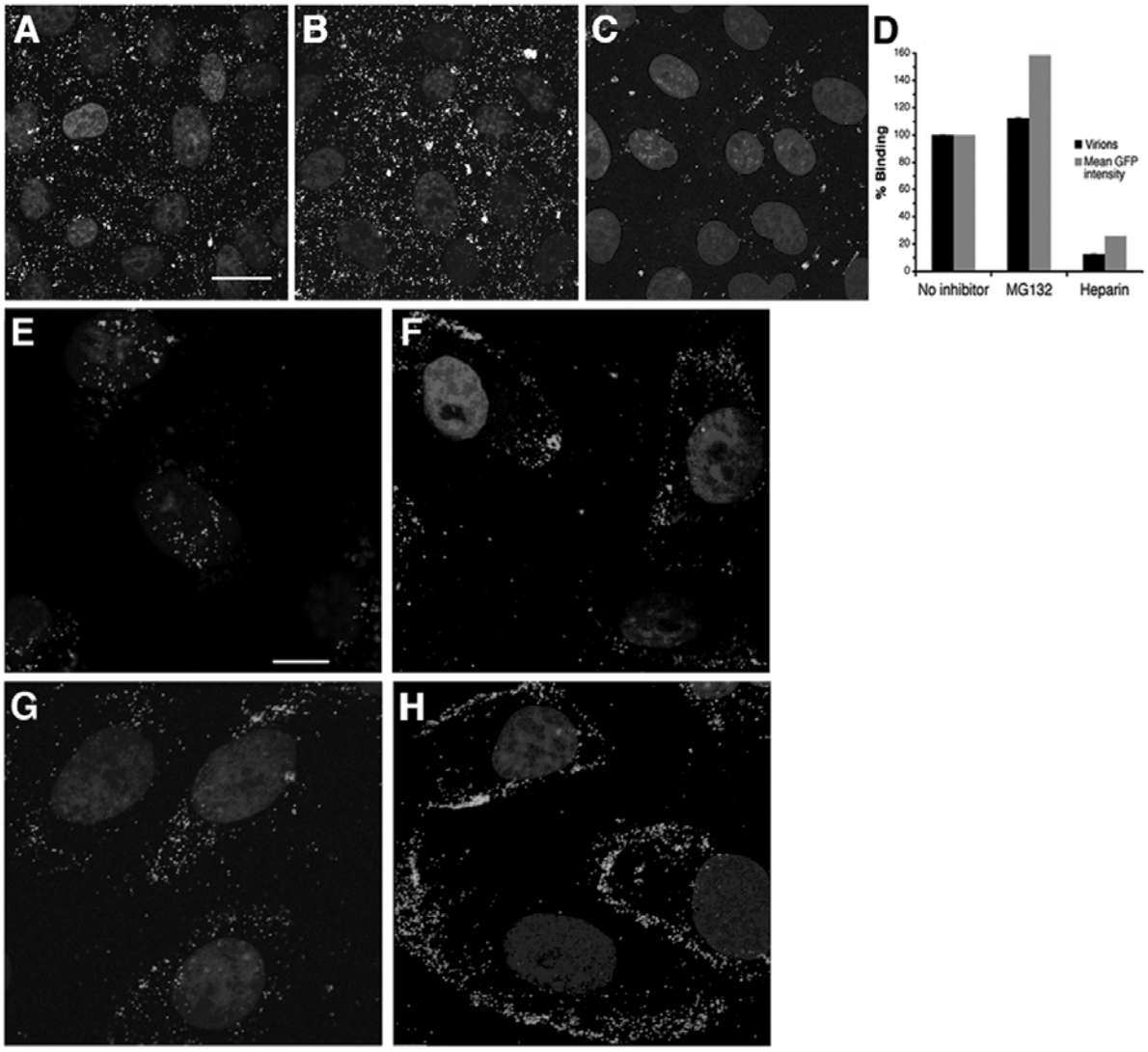

Figure XVI. Effect of proteasomal inhibitors on binding and capsid transport. (A-D) Effect of MG132 on virus binding to cells. HSV-1 KOS K26GFP (MOI of 30) was added to pre-chilled Vero cells for 1 h at 4^{o}C in the presence of no inhibitor (A), 25 μM MG132 (B), or 1 μg/ml heparin (C). Cells were fixed and confocal images were obtained. Bar, 100 microns. (D) The number of bound GFP-tagged virions was quantified by three observers blinded to experimental conditions (Virions). Data are the mean with standard error. In parallel, mean GFP intensity was measured by the Histogram function of Adobe Photoshop. Binding in the absence of inhibitor was set to 100%. (E-H) Effect of proteasome inhibitors on incoming capsid transport to the

nuclear periphery. Vero cells were mock-treated (E) or treated with 25 μM MG132 (F), 10 μM lactacystin (G), or 10 μM epoxomicin (H) for 15 min at 37°C. HSV-1 KOS K26GFP (MOI of 10) was added for 2.5 h in the constant presence of agent and 0.5 mM cycloheximide. Cells were fixed, and confocal images were obtained. Images are representative of cell population. Bar, 10 microns.

trapped at the cell periphery. Since these experiments were performed in the presence of cycloheximide, these results suggests that newly synthesized viral factors do not affect the proteasome dependence of entry. Whether our findings bear on downstream, proteasome-mediated events in HSV infection remains to be seen. Together, the results suggest that proteasome activity is needed for a step in HSV entry that occurs after cell binding but prior to capsid arrival at the nucleus.

10. MG132 block on capsid transport is partially reversible

MG132 can be washed out of cells prior to addition of virus, resulting in a much-reduced inhibitory effect (Fig. XII. B.). This is consistent with the reversible effect of MG132 on the proteasome. I then determined whether the block to capsid transport imposed by MG132 could be reversed by washout. Vero cells were infected with HSV-1 K26GFP for 2 h in the presence of MG132 (Fig. XVII. B.). After washout of MG132 and incubation for an additional hour, GFP-tagged capsids appeared to migrate toward the nucleus (Fig. XVII. C.). Punctate GFP signals were visualized at an intermediate site between the cell periphery and the nucleus and in the perinuclear region (Fig. XVII. C.). This is in line with the known reversible effect of MG132 on the 20S proteasome.

11. HSV entry relies on proteasome activity at a postpenetration step

From the fluorescence microscopy experiments (Fig. XVI. F. and XVII. B.), the location of MG132-arrested virions in Vero cells cannot be ascertained unambiguously. The GFP signal may represent either enveloped virions that have bound but not yet fused or capsids that are halted in the cytosol. I utilized multiple approaches to determine the location of these virions.

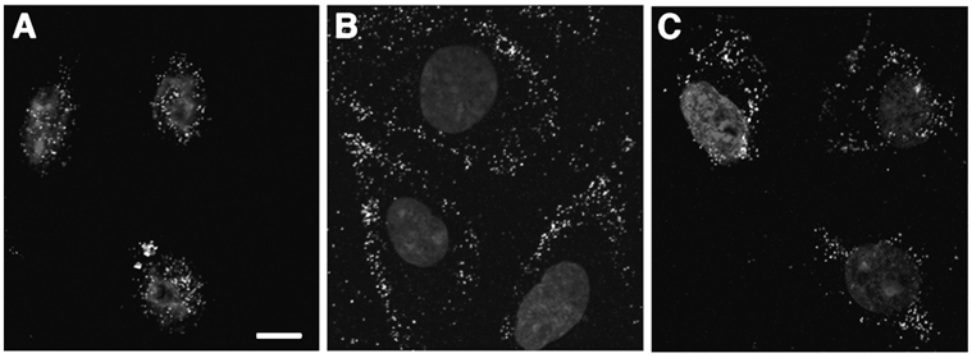

Figure XVII. Effect of washout on the MG132 block of capsid transport. Vero cells were mock-treated (A) or treated with 25 μM MG132 (B, C) for 15 min at 37°C. HSV-1 KOS K26GFP was allowed to infect cells for 2 h in the absence (A) or presence of MG132 (B-C). Cells were fixed (B) or were washed four times 5 min with culture medium, and returned to 37°C for an additional hour in normal culture medium prior to fixation (C). Bar, 10 microns.

First, a citrate inactivation assay was used to determine whether MG132-arrested virions were bound to the cell surface. Sodium citrate buffer (pH 3.0) inactivates cell-bound HSV that has not penetrated into the cytosol (145). For example, the infectivity of HSV that is bound to vero cells at 4°C is completely inactivated by citrate treatment (Fig. XVIII. A.). In these experiments, MG132 was washed out after citrate or PBS treatment to allow infection. When citrate buffer was added to the surface of infected cells treated with MG132, HSV infectivity was not significantly affected (Fig. XVIII. B.). Similar infectivity was observed in the control cells that received PBS treatment (Fig. XVIII. B.). The resistance to citrate inactivation supports the notion that HSV penetrates into the cytosol of Vero cells in the presence of MG132.

Second, a fluorescence protease protection assay was used to determine the accessibility of MG132-arrested virions to proteinase K. HSV that is bound to the cell surface can be removed by protease treatment; (215, 252). HSV-1 K26GFP was bound to Vero cells at 4°C (Fig. XVIII. C.). Proteinase K added to the cell surface effectively removed the GFP signal (Fig. XVIII. D.). In contrast, proteinase K did not effectively remove GFP-tagged HSV from MG132-treated cells (Fig. XVIII. F.), suggesting that arrested virions are not present on the cell surface. This is consistent with the inefficacy of citrate treatment (Fig. XVIII. B). When cells were first permeabilized with digitonin and then treated with protease, the GFP signal was removed more effectively (Fig. XVIII. G.). In this case, proteinase K may enter the permeabilized cell and act on cytosolic capsids such that they can be washed away or their GFP degraded. Digitonin treatment alone did not have an effect on retention of GFP signal (not shown). These results support the notions that capsids are halted in the cytosol of MG132-treated cells and that the proteasome is needed for a step in entry that follows penetration.

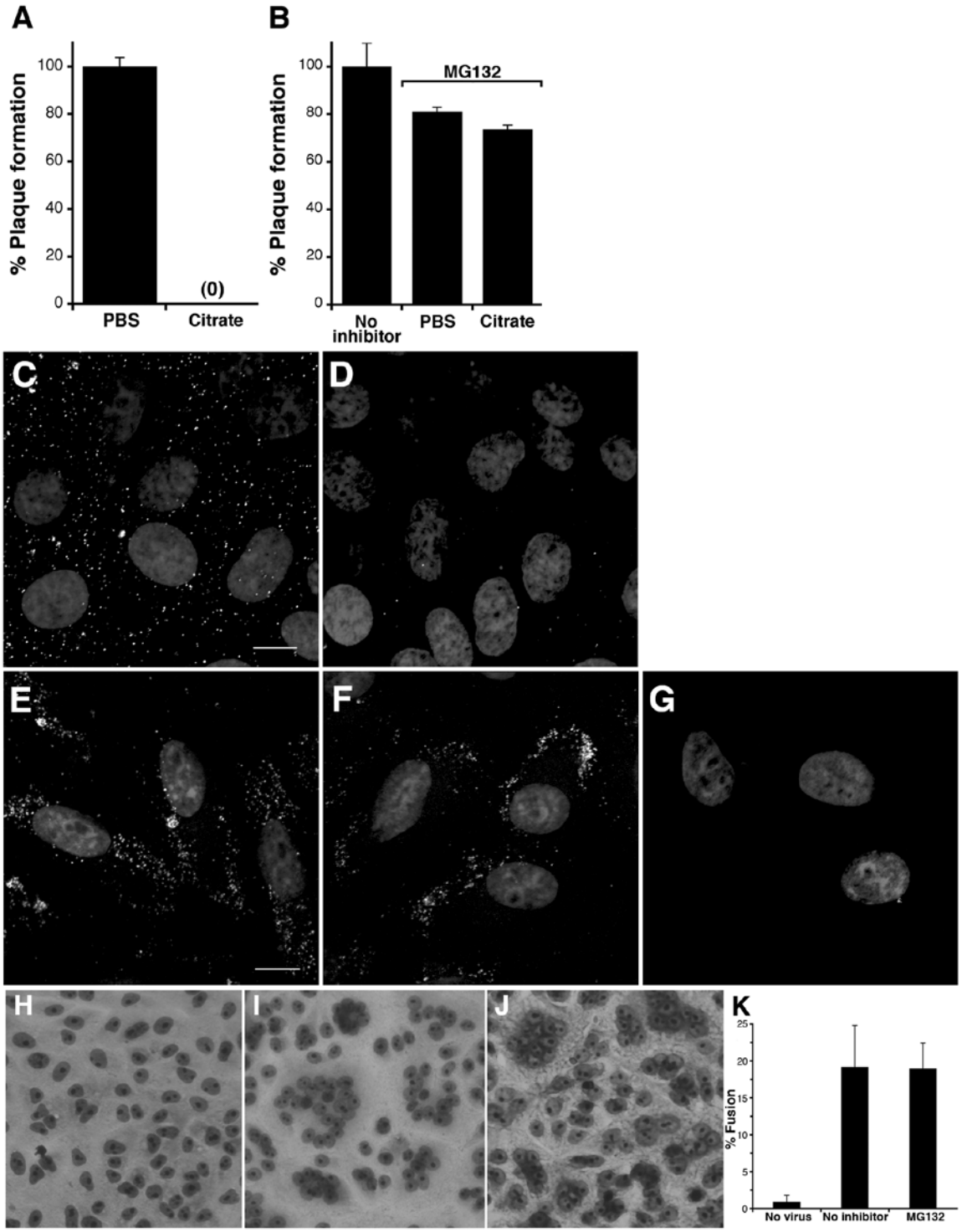

Figure XVIII. Effect of MG132 on pre-penetration and fusion steps. (A-B) Citrate treatment of the cell surface has no effect on the infectivity of MG132-arrested virions. (A) Vero cells were chilled on ice, and HSV-1 KOS (100 PFU/well) was added for 1 hr at 4° C. Cells were treated with warmed PBS or citrate buffer (pH 3.0). Plates were shifted to 37° C, and plaques were quantified at 24 h p.i. (B) Vero cells were mock treated (No inhibitor) or treated with 25 μM MG132 for 30 min at 37° C. Cells were chilled, and then HSV-1 KOS (100 PFU/well) was added for 1 h at 4° C to synchronize infection. Cultures were shifted to 37° C, and MG132 concentrations were maintained for 2.5 h at 37° C. Cells were then treated with PBS or citrate buffer (Citrate), and then extensively washed with culture medium to reverse the effect of MG132. Plaques were quantified at 18 h p.i. Data are the mean of triplicate samples with standard deviation. (C-H) Permeabilization of the plasma membrane followed by treatment with protease allows removal of MG132-arrested virions. HSV-1 KOS K26GFP (MOI of 10) was bound to pre-chilled Vero cells for 1 h at 4° C (C), and then 100 μg/ml Proteinase K was added for 2.5 min (D) prior to fixation and confocal microscopy. Bar, 10 microns. (E-G) HSV-1 KOS K26GFP (MOI of 10) was added to cells in the presence of 25 μM MG132 for 2.5 h p.i. at 37° C. MG132-treated cells received no additional treatment (E), were treated with Proteinase K (F), or were permeabilized with digitonin followed by Proteinase K treatment (G). Cells were fixed and analyzed by confocal microscopy. Bar, 10 microns. (H-K) MG132 has no effect on virion-induced fusion in a fusion-from-without assay. Vero cells were mock-treated (H, I) or treated with 50 μM MG132 (J) for 30 min at 37° C. Cells were left uninfected (H) or HSV-1 ANG path (MOI of 50) was added (I, J) for 3 h in the presence of cycloheximide. Cells were fixed and stained with Giemsa. (K) Fusion was quantitated from photomicrographs of random

fields as described in Materials and Methods. Data are means of triplicate determinations with standard error.

Third, I addressed more directly the possibility that proteasome activity was needed for membrane fusion itself. Virus-cell fusion (or penetration) during HSV entry is refractory to direct study. A fusion-from-without (FFWO) assay was used as a surrogate means to measure virion-induced fusion (54). FFWO is the induction of target cell fusion by addition of virions to the monolayer surface in the absence of viral protein expression (96). A subset of syncytial strains of HSV-1, including the ANG path strain has FFWO activity (150). Relative to uninfected (Fig. XVIII. H.), the addition of HSV-1 ANG path to Vero cell monolayers resulted in the clustering of nuclei, which is indicative of FFWO (Fig. XVIII. I.). I then assessed the effect of MG132 on FFWO. Cells treated with MG132 were susceptible to virion-induced FFWO (Fig. XVIII. J.) in a manner similar to untreated cells (Fig. XVIII. I. and K.), suggesting that the proteasome is not needed for the fusion activity of the virion. Together, the results are consistent with a role for the proteasome in delivering penetrated capsids to the nucleus.

IV. FUNDAMENTAL PROPERTIES OF TEGUMENT ICP0

A. INTRODUCTION

The function of tegument ICP0 during the earliest steps of HSV infection, prior to IE gene expression, has not been evaluated. IE ICP0 has known interactions with the cellular proteasome late in infection and also has E3 ubiquitin ligase activity. Thus ICP0 was a strong candidate for being involved in proteasome-dependent entry and merited analysis.

Prior to determining tegument ICP0's function, some fundamental questions about tegument ICP0 had to be addressed. If mutations in tegument ICP0 caused the loss of other structural proteins in mature virions, future experimental conclusions could not be attributed solely to mutations in tegument ICP0. If mutations caused ICP0 to no longer be incorporated into virions, then results in future experiments would be based on the absence of ICP0 and not on altered function of tegument ICP0. The position of a protein in the tegument layer, inner or outer, can be important for its function during early HSV infection. The position of ICP0 in the tegument could influence the interpretation of future experimental results. To address these questions, we looked at the protein and ICP0 content of WT compared to mutant ICP0 virions. We performed a tegument release assay in order to distinguish whether ICP0 is an outer or inner tegument protein.

B. RESULTS

1. Absence of ICP0 does not alter mature virion protein composition

I investigated whether ICP0 influences the protein composition of mature extracellular virions. One μg (Fig. XIX. A., left) or equivalent VP5 units (Fig. XIX. A., right) of virions of parental virus HSV-1 Glasgow strain 17+ syn (herein referred to as 17+) or virions of ICP0 null mutant *dl*1403 (Table V.) were analyzed by SDS-PAGE followed by Coomassie blue staining (23). For each structural protein of the 17+ virions that was detectable by Coomassie blue staining, there was a counterpart protein detected in *dl*1403. Although several proteins were detected at various levels, the overall protein profile of virions released from U2OS cells was not grossly altered by the absence of ICP0 (Fig. XIX. A.).

Equivalent VP5 units of extracellular virions were analyzed by Western blotting with antibodies to glycoproteins, tegument proteins and a major capsid protein. The envelope glycoproteins and tegument proteins tested did not appear to be reduced in the absence of ICP0 (Fig. XIX. B.). By these measures, ICP0 does not appear to grossly affect the protein content of mature virions.

2. Really interesting new gene domain influences tegument ICP0 incorporation

Next, I determined whether the RING finger domain influences ICP0 incorporation into the tegument layer. First, the relative amounts of VP5 in various ICP0 mutant virions (Table V.) propagated on U2OS cells were determined for each preparation of virus (Fig. XX. A.). The ICP0 contents of virions were then analyzed by SDS-PAGE followed by Western blotting with an antibody to ICP0. As expected, WT 17+ contained tegument ICP0 (Fig. XX. A., lane 17+)

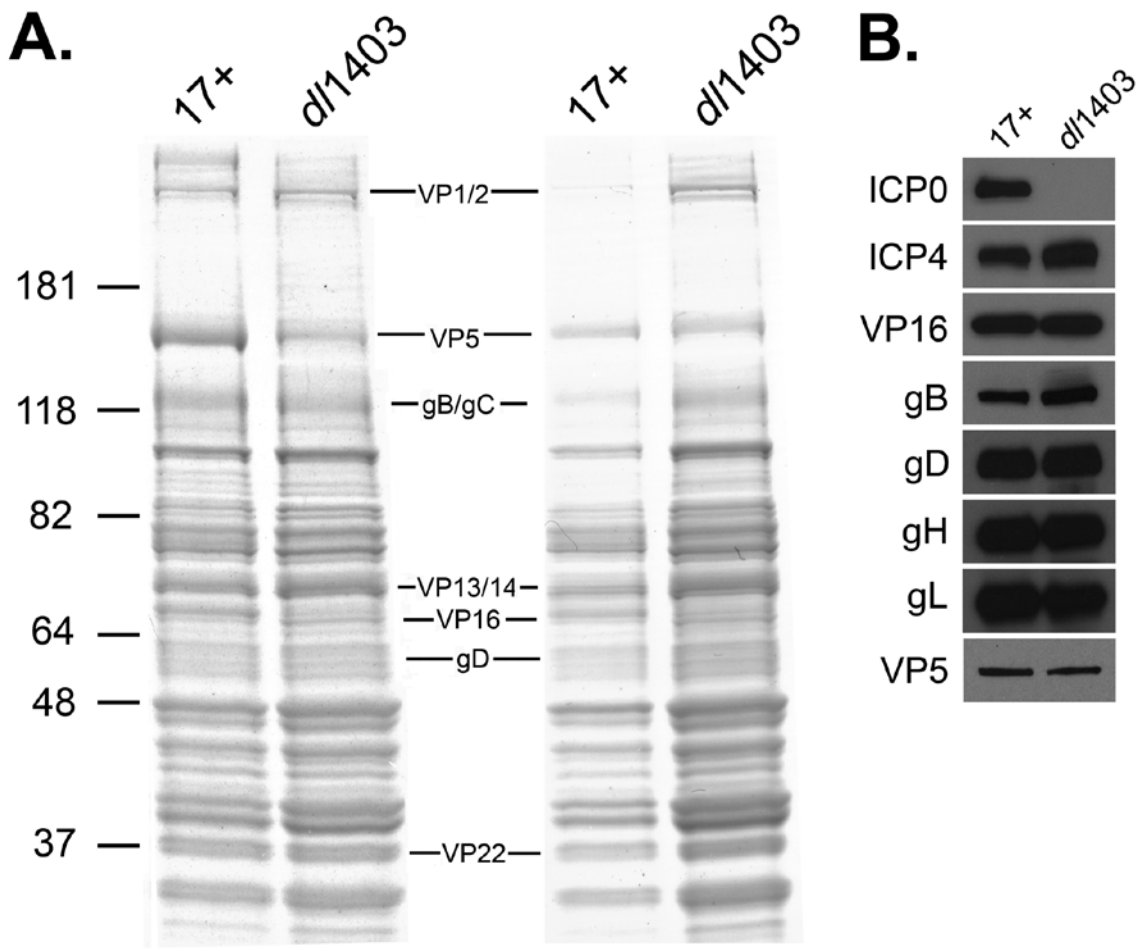

Figure XIX. Protein content of extracellular wild type and ICP0 null mature virions. (A) 1 μg (left) or equivalent VP5 units (right) of HSV-1 wild type (17+) or ΔICP0 (*dl*1403) were analyzed by SDS-PAGE followed by Coomassie blue staining. Locations of several HSV structural proteins are indicated at the right. (B) Equivalent VP5 units of extracellular virions were analyzed by SDS-PAGE followed by Western blotting with antibodies against the indicated tegument protein or glycoprotein.

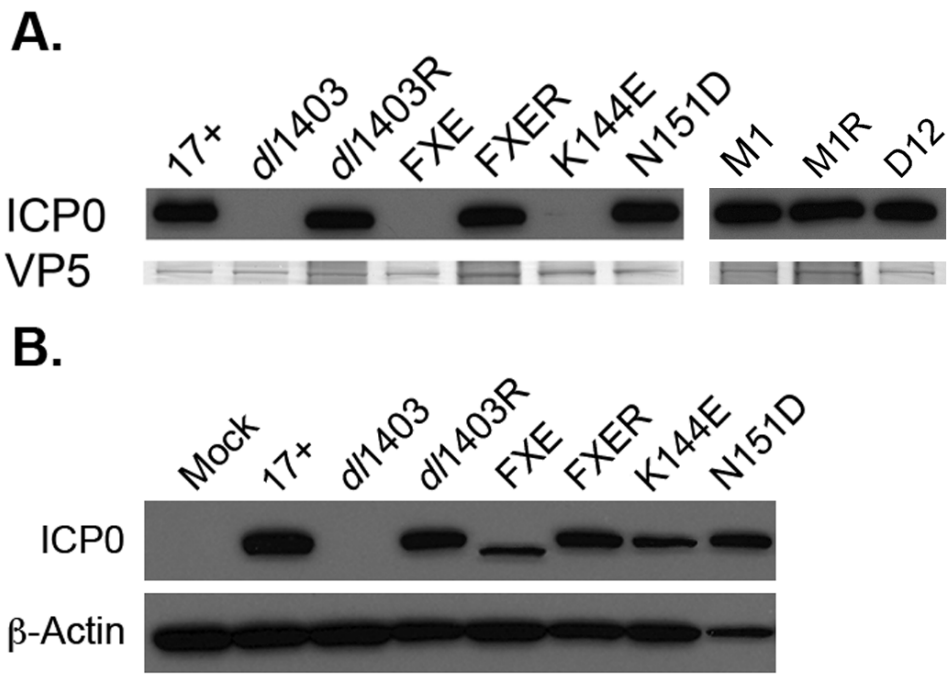

Figure XX. Effect of RING finger mutations on the incorporation of ICP0 into virions. Tegument ICP0 content of ICP0 mutants. (A) Incorporation of tegument ICP0 into HSV-1 mutants. The indicated extracellular virions were analyzed by SDS-PAGE followed by Western blotting with an antibody to ICP0. In parallel, VP5 was detected by Coomassie staining to demonstrate equivalent particle loading. (B) Expression of ICP0 in cells infected with WT or mutant viruses. U20S cells were infected with the indicated virus (based on MOI = 1 for 17+) for 18 hr. Cell lysates were analyzed by SDS-PAGE followed by Western blotting with an antibodies to ICP0 and β-actin.

while the *dl*1403 mutant did not (Fig. XX. A., lane *dl*1403). FXE virions did not contain ICP0 (Fig. XX. A., lane FXE), suggesting that the RING finger domain is important for the association of ICP0 with mature virions. ICP0 was detected in *dl*1403 and FXE rescuants (Fig. XX. A., lanes *dl*1403R and FXER), both of which bear the restored ICP0 gene. ICP0 was also detected in USP7 binding domain mutants M1, M1 rescuant and D12 (Fig. XX. A., lane M1, M1R and D12). This indicates that the USP7 binding region is not required for ICP0 incorporation, a result recently confirmed by Maringer and Elliot (184). Interestingly, K144E virions had greatly reduced levels of tegument ICP0 whereas N151D virions had WT levels (Fig. XX. A., lanes K144E and N151D). Trace amounts of ICP0 were reproducibly detected in K144E virions upon longer exposure to film. To confirm that the reduction in tegument ICP0 in FXE and K144E viruses was not due to lack of expression of mutant ICP0 in infected cells, the expression of ICP0 during mutant virus infection was compared to that during WT 17+ infection. Mutant ICP0 was readily detected in cells infected with FXE or the K144E mutant (Fig. XX. B.), albeit at lower levels as reported previously (78, 81). Thus, the results suggest that the RING finger plays an important role in the incorporation of ICP0 into virions.

3. Tegument infected cell protein 0 is capsid associated

To determine the position of ICP0 within the tegument layer, a tegument release assay was employed (6, 203, 270). This is a measure of how readily detergent and salt can remove a structural protein from the virion. Typically, outer tegument proteins are partially released from virions following treatment with Triton X-100 and up to 1 M NaCl, but capsid-associated or inner tegument proteins are resistant to such treatment. For these experiments, extracellular virions were resuspended in lysis buffer then layered onto a sucrose cushion and centrifuged.

The supernatant above the sucrose cushion was recovered, and the pellet, containing capsids and capsid-associated tegument proteins, was resuspended in Laemmli buffer. Pelleted and released samples were separated by SDS-PAGE and blotted onto nitrocellulose and probed with antibodies against envelope proteins, known inner and outer tegument proteins, ICP0 and ICP4.

As expected, gB was detected in the supernatant, indicating that it was removed along with the virion envelope (Fig. XXI.). In contrast, ICP0 remained in the pellet along with VP5, the major capsid component, even at high salt concentrations (Fig. XXI.). ICP0 was not detected in the supernatant even after longer exposures to X-ray film (data not shown). VP1-2 is a known inner tegument protein and remained associated with capsids in the pellet (Fig. XXI.) (203, 209, 270). In contrast, a fraction of VP16 was readily removed by 1% Triton X-100, consistent with the presence of VP16 in the outer tegument (203, 209, 270). Interestingly, detectable amounts of ICP4 were released by detergent and increasing salt concentrations (Fig. XXI.), suggesting that ICP4 is an outer tegument component.

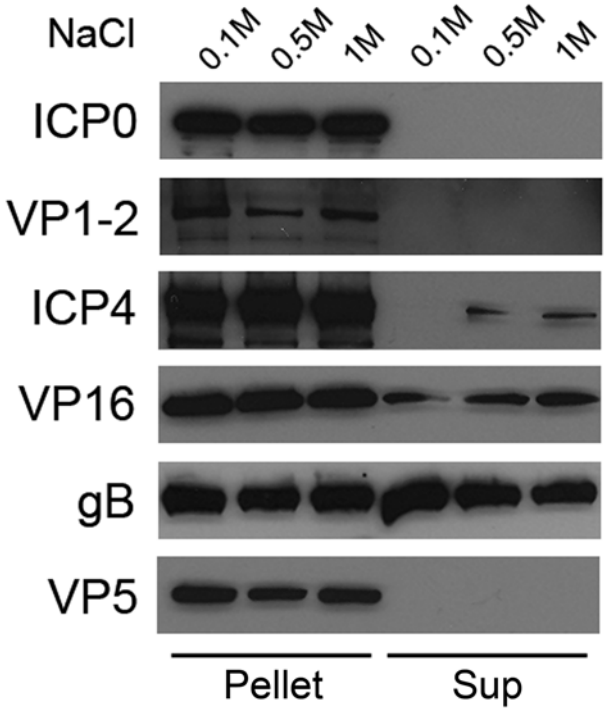

Figure XXI. Position of ICP0 in the virion tegument layer. Extracellular virions were treated with 1% Triton X-100 in the presence of 0.1, 0.5, or 1.0 M NaCl. The reactions were then centrifuged through a 35% sucrose cushion. Pelleted (Pellet) and released (Sup) fractions were analyzed by SDS-PAGE and immunoblotting with antibodies to ICP0, VP1-2, ICP4, VP16, gB, or VP5.

V. A PRE-IMMEDIATE EARLY ROLE FOR TEGUMENT ICP0

A. INTRODUCTION

For the first experimental section, the cellular proteasome is shown to be required for efficient capsid transport (55). In the second experimental section, ICP0 remains associated with capsids in the tegument release assay, a characteristic of inner tegument proteins (56). Since ICP0 is in the inner tegument, it may be transported with capsids on microtubules during HSV entry. Late in infection IE ICP0 interacts dynamically with cellular proteasomes (263). IE ICP0 has a RING finger domain with E3 ubiquitin ligase activity that is necessary for its IE functions (21). With these known ICP0 functions, it is possible that tegument ICP0 regulates proteasome-dependent capsid transport. Thus, in this last section I sought to determine the role of tegument ICP0 during proteasome-dependent entry of HSV. I began by looking at the effects of MG132 on the virus induced β-galactosidase expression or early infection of mutant ICP0 virions.

B. RESULTS

1. In the absence of tegument ICP0, early HSV infection is resistant to MG132

WT HSV-1 KOS entry into CHO-nectin-1 cells was inhibited by MG132 in a concentration-dependent manner (Fig. XXII. A.). In contrast, 7134 entry was refractory to inhibition (Fig. XXII. A.). Similar results at a lower MOI suggest that the phenotype seen is related to entry and not to infection (data not shown). Infections with WT 17+, *dl*1403 rescuant, FXE rescuant, N151D, M1 and D12 virions were inhibited by MG132 in a dose-dependent manner (Fig. XXII. B.-E.). Infections with *dl*1403, FXE and K144E viruses were inhibited to a lesser extent or not at all by MG132 (Fig. XXII. B.-D.). Recall, that FXE, K144E and *dl*2403 virions do not contain tegument ICP0 (Fig. XX.). Thus, these results suggest that virions deficient in tegument ICP0 appear to be less dependent on proteasome activity for initiation of infection. MG132 inhibited the efficiency of WT and N151D entry but had less of an effect on the efficiency of *dl*1403, FXE and K144E entry (Fig. XXII. F.-H.). Although at higher MG132 concentrations, the efficiency of entry appears to be similar (Fig. XXII. F.-H.). This suggests that any virions that are resistant to MG132 during WT and N151D entry may enter by a similar pathway as *dl*1403, FXE and K144E virions. Notably, HSV-1 *dl*1403 is capable of synthesizing β-galactosidase in the presence of up to 50 μM MG132 (Fig. XXII. B. and F.). This suggests that MG132 does not directly inhibit reporter gene expression in this assay, and is consistent with the notion that MG132 inhibits WT HSV at the level of capsid transport (55). Importantly, these results suggest a functional role for tegument ICP0 in proteasome-dependent entry.

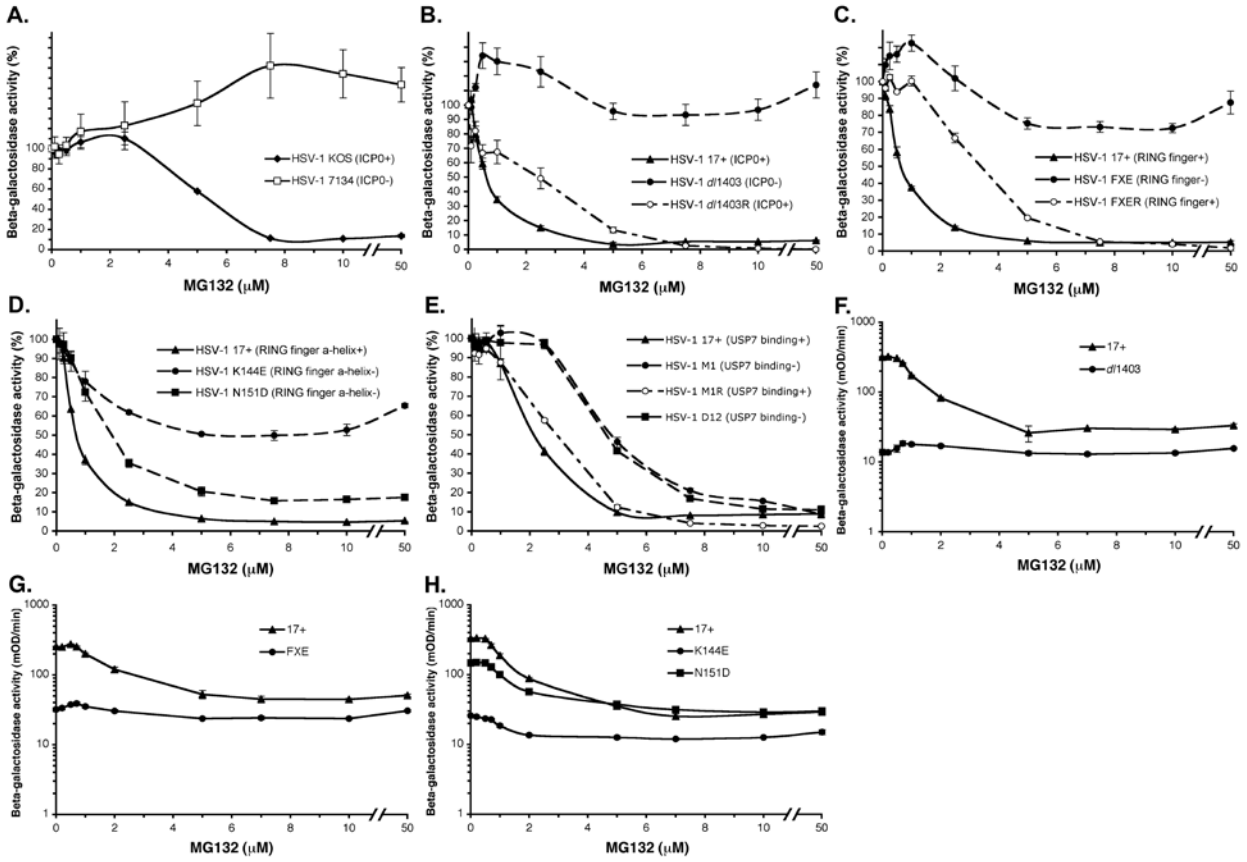

Figure XXII. Effect of MG132 on the entry of ICP0 mutants. (A-E) CHO-nectin-1 cells, containing a l*acZ* gene cassette, were treated with MG132 for 15 min at 37˚C. WT KOS (A), 7134, containing a l*acZ* gene cassette (A), WT 17+ (B-E), *dl*1403 (B), *dl*1403R (B), FXE (C), FXER (C), K144E (D), N151D (D), M1 (E), M1R (E), or D12 (E) mutant viruses were added for 6 hr in the continued presence of inhibitor. The percent β-galactosidase activity relative to that obtained in the absence of agent is indicated. MG132 decreases the efficiency of HSV entry in the presence of ICP0. (F-G) β-galactosidase activity was taken over 12 min during the reading of (B-D). The data are means of quadruplicate determinations with the standard errors.

VI. DISCUSSION

A. INTRODUCTION

Several virus-cell interactions have been identified during the initial infection of the host cell by HSV. Here I show that functional proteasomes are needed for efficient entry of HSV into cells. Interestingly, viral entry is not dependent on the ubiquitination activity of the cell. Proteasome inhibitors block HSV entry into cells that mediate either endocytic or nonendocytic entry pathways. Together, our studies suggest that a common step in both pathways, namely the delivery of newly penetrated capsids to the nuclear periphery, is proteasome-dependent.

B. CELL TYPE AND PROTEASOME-DEPENDENT ENTRY OF HSV

HSV entry into Vero cells appears to be the first example of a nonendocytic viral entry process to require cellular proteasome activity. The viruses known to require the UPS for viral entry, all utilize endocytic pathways (159, 239, 240, 279). Thus, the subcellular distribution of MG132-arrested HSV reported here appears to be novel. In total, proteasome-mediated proteolysis may play multiple roles in the intracellular trafficking of viruses.

Our results are consistent with the idea that the proteasome is also needed at a postpenetration step in CHO cell entry (Fig. XI.-XVIII.). However, this needs to be determined

conclusively. After endocytosis in CHO-nectin-1 cells, the HSV capsid penetrates from a low pH endocytic compartment (46, 112, 214, 215), but the precise site has not been established. Hence, Vero cells were more amenable to use in several aspects of our study because the penetration of capsid at the plasma membrane is synchronized easily and can be analyzed more directly.

The two major target cells for HSV in the human host are mucosal epithelial cells and sensory neurons of the peripheral nervous system. The distance that the capsid must travel in order to reach the nucleus in epithelial cells, and in the cultured cells used in the present study, is much shorter than the distance to be traveled down the axon of an innervating neuron (75). While proteasome inhibitors block 60 to 90% of HSV entry into Vero, CHO-nectin-1, BALB/c 3T3 (Fig. XI-XVIII), B78-nectin-1, HeLa, and HaCaT cells (data not shown), the proteasome may play an even more significant role in neuronal entry.

C. ASPECTS OF TARGET SUBSTRATE

Chymotrypsin-like active site inhibitors of the proteasome all inhibit HSV entry, while agents that primarily affect the trypsin-like or caspase-like catalytic sites have no effect on viral entry. Thus, the data suggests that the chymotrypsin-like active site is important for substrate proteolysis during HSV entry. To confirm the specificity of proteasome inhibitors I used, proteasomes isolated from inhibitor treated cells would have to be collected, and then the proteolytic activity of model substrates would have to be measured (162). Conclusions from such studies would have to be made with caution because the relative importance of a given active site varies with substrate.

Entry proceeds unimpeded in cells that lack a functional form of a cellular ubiquitin-activating enzyme, E1 (Fig. XV.). With the limitations of the ts20 cell model, I cannot rule out

that HSV might use a viral encoded E1 or a different cellular E1 ubiquitin-activating enzyme. The nature of the degradation signal involved in proteasome-dependent entry of HSV is currently not known. The target substrate could use a cellular or viral adaptor protein to target it to the proteasome. Alternately, the target substrate might be tagged with a ubiquitin-like modification. A number of ubiquitin-like modifications have been identified, such as sumoylation (162). Interestingly, PML and SP100 modified with SUMO are more sensitive to ICP0-mediated degradation (35, 89). It remains to be determined whether such ubiquitin-related moieties play a role in viral entry.

D. TEGUMENT ICP0

By the measures used, ICP0 does not appear to grossly affect the protein content of mature virions (Fig. XIX.). ICP4 is another transcriptional activator located in the tegument and it is known to interact with ICP0 during HSV gene expression. Western blot analysis was used to determine whether the absence of ICP0 affected the incorporation of ICP4. ICP4 was detected in *dl*1403 virions (Fig. XIX. B.). This suggests that ICP4 can be incorporated into virions independently of ICP0. It is important to note that these results do not rule out a role for ICP0 in proper virion assembly. Tegument assembly involves a complex series of protein-protein interactions, and individual tegument proteins may serve redundant functions (197). And based on the limitations of these analyses, it is still possible that one or more structural proteins in addition to ICP0 may be missing from the ICP0-null virus.

While K144E and N151D mutations in the RING alpha helix both decrease the ability of ICP0 to activate gene expression, the K144E phenotype is more pronounced (21, 78) (Table IV.). Also, K144E and N151D mutants both fail to induce the colocalization of conjugated ubiquitin,

but ICP0 with the N151D mutation may still interact with the ubiquitin machinery (84). It is not clear whether these activities relate to the inclusion of the N151D mutant protein and the exclusion of the K144E mutant protein from the tegument (Fig. XX.).

The RING finger of ICP0 plays a critical role in its incorporation into the tegument layer, a result that has recently been confirmed by Maringer and Elliot (184) (Fig. XX.). RING finger mutations cause ICP0 to be more readily retained in the nucleus and this would be a simple explanation as to why there is a reduction of tegument ICP0 (185). It is also possible that the reduction in ICP0 expression during FXE and K144E infection may influence ICP0 incorporation (Fig. XX. B.). ND10 disruption and gene expression have been shown to be requirements for translocation of ICP0 (151, 178). Thus RING finger mutations that inactivate these functions could also lead to nuclear retention. In the absence of VP22, ICP0 is still translocated to the cytoplasm but does not become incorporated into virions (74, 184). An ICP0 point mutant is unable to bind to cyclin D3, which might be involved in ICP0 translocation (264). This suggests that the RING finger mutations might block interaction with a cellular or viral factor important for ICP0 translocation (184). The RING finger-dependent incorporation of ICP0 also provides more evidence that ICP0 is specifically incorporated into viral tegument. Future studies could investigate the role that the RING domain plays in the relationship between the cellular localization of ICP0 and the incorporation of ICP0 into the tegument layer.

During infection, ICP0 is dynamically phosphorylated, so it is possible that one or more of these phosphorylation sites is important for ICP0's incorporation into virions (1). ICP0 is also able to autoubiquitinate itself, and such a signal could also target ICP0 to virions (32). Future studies could address whether ICP0 incorporated into virions is posttranslationally modified.

ICP0 was resistant to removal by detergent and high salt, a feature common to inner tegument proteins (Fig. XXI.), a result that has recently been confirmed (184). In contrast, ICP4 was sensitive to removal by detergent and high salt, a feature common to outer tegument proteins (Fig. XXI.). In contrast, ICP0 is capsid associated. Although specific functions have not yet been ascribed to virion ICP0 and ICP4 proteins, it is tempting to speculate that their different positions in the tegument layer reflect distinct roles in pre-IE events. The presence of tegument ICP4 near the viral envelope may facilitate rapid release into the cytoplasm upon viral penetration and allow for ICP4 to play a pre-IE role in HSV infection. Because ICP0 appears to be an inner tegument protein, tegument ICP0 may be transported with capsids on microtubules and may have a regulatory role at the proteasome-dependent step during HSV entry.

UL14 is a HSV protein that is expressed late in infection and is found in low amounts in the tegument of HSV (273). At low MOI, UL14 mutants exhibit an extended infection and have a defect in release of mature virions. This suggests that UL14 is important for efficient and productive infection. Recently, tegument UL14 has been shown to be required for efficient nuclear targeting of VP16 and capids during entry. UL14 in this case could be influencing the removal of tegument upon penetration, regulating the rate of capsid transport, or important for tegument-tegument interactions during capsid transport. The various correlations between UL14 and ICP0, suggest that ICP0 may have a similar role in entry or be a key player in the capsid transport mechanism.

Newly expressed ICP0 is suggested to play a role in regulating the efficiency of lytic and latent infection. The work presented in this thesis suggests that ICP0 also plays a role in regulating the efficiency of HSV entry. The current hypothesis is that HSV tegument ICP0 plays a pre-IE role in regulating proteasome-dependent entry at a step of capsid transport to the

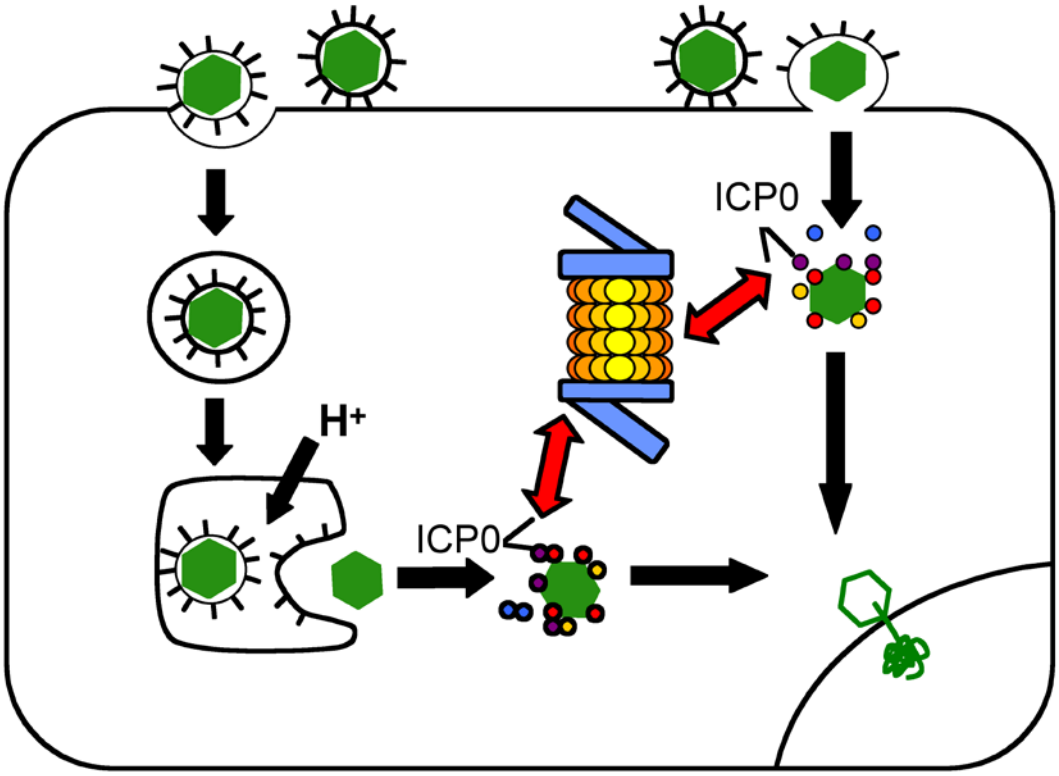

Figure XXIII. Potential mechanism of proteasome-dependent entry of HSV. This thesis proposes that independent of entry pathway, HSV tegument ICP0 plays a pre-immediate early role in regulating proteasome-dependent entry at a step during capsid transport to the nucleus.

nucleus (Fig. XXIII.). Ongoing studies are aimed at delineating the fate and function of tegument ICP0 during viral entry. As shown in this work, the effect of MG132 on the entry of ICP0 mutant virus was evaluated by β-galactosidase. HSV containing tegument ICP0 appears to enter cells more efficiently than virions that are deficient in tegument ICP0 (Fig. XXII. F.-H.). In addition, the majority of virions with capsid-associated ICP0 appear to enter cells via proteasome-dependent pathway (Fig. XXII. A.-E.), while entry of HSV in the absence of ICP0 is proteasome-independent (Fig. XXII. B.-D.). This suggests that when tegument ICP0 is present, it interacts with proteasomal machinery to facilitate and enhance the efficiency of HSV entry. In this case, MG132 blocks the majority of capsid transport and reduces WT entry efficiency due to a block in tegument ICP0 and proteasome interaction. In the absence of tegument ICP0, ICP0s interaction with the proteasome to facilitate HSV entry would not occur. Proteasome-independent HSV capsid transport is likely to be inefficient due to the absence of the enhancing effect of tegument ICP0. However, the lack of proteasome interaction would allow for capsid transport to occur in the presence of MG132.

E. FUTURE DIRECTIONS

Future studies will continue to evaluate the effect of MG132 on the entry of ICP0 mutants by looking at capsid transport by immunofluorescent microscopy. The particle to PFU ratio of ICP0 mutants will be addressed by infecting slides with equivalent VP5 units, based on WT 17+ MOI of 10. Future studies will also seek to complement *dl*1403 and 7134 virions with tegument ICP0. The goal of these studies would be to restore the WT phenotype in ICP0 null virus with cells expressing ICP0. The subcellular localization of tegument ICP0 during HSV entry will also be evaluated. By following input ICP0 by immunoprecipitation our lab hopes to

gain additional details to the mechanism of proteasome-dependent entry of HSV. There is also potential for functional interaction of ICP0 with the inner tegument protein VP1-2, which has an N-terminal DUB domain (155).

Efforts are under way to identify the polypeptide(s) that is degraded during HSV entry. Retrograde movement of herpes-virus capsids toward the nucleus likely involves the coordinated action of virus and cell components (66, 67, 71, 182, 183). HSV outer tegument or capsid-associated tegument proteins are candidate substrates for the 20S proteasome. Also, cytoskeletal elements and their associated motor proteins and regulatory molecules are host factors that may be targeted for degradation. Discovery of the substrate(s) will be critical for understanding the mechanism of proteasome-dependent viral entry.

VII. CONCLUDING REMARKS

Inhibitors and mutational analysis have been strong approaches for examining molecular processes. Proteasomal inhibitors have been valuable tools in discovering new substrates and implicating the proteasome in new aspects of viral infection. HSV interacts with many cellular systems during infection. The cellular 26S proteasome is a key player in HSV infection although its role in HSV entry is only starting to become clear. The mechanism of ICP0 regulation of entry will likely give further insight into the degradation signal and will aid in the discovery of the 26S substrate(s). Interestingly, the proteasome inhibitor, Bortezimib or Velcade®, has been approved for treatment of multiple myeloma (116). Identification of the substrate(s) during HSV entry could aid in the development of a potent proteasomal inhibitor and antiviral drug. The work presented in this thesis indicates that the proteasome has a role in facilitating capsid transport, identifies new aspects of tegument ICP0 and implicates tegument ICP0 as a regulator of this interaction during HSV entry.

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IX. VITA

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