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MECHANISMS OF DNA VIRUS INFECTION: ENTRY AND EARLY EVENTS

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1. ABSTRACT

The cellular components engaged in entry of viruses has been an area of intense investigation in recent years. We examine the entry and receptors used for well-studied and prevalent human DNA viruses adenoviruses, poxviruses and two herpesviruses- herpes simplex virus and Epstein-Barr virus. Little is yet known about the entry or early events for other human DNA viruses. Common themes that emerge for entry of these prevalent human DNA viruses include engagement of multiple receptors, use of cell surface molecules that are prominent and, in most cases, conserved on cells, and interactions with proteins that can alter morphology of the cytoskeleton or modulate intracellular signaling for gene expression. Where available, we provide evidence that entry not only transports the capsid and genome across a cell membrane, but that these events also can set up the cell for subsequent events that contribute to successful viral replication.

2. INTRODUCTION

Investigations in the last several decades provide some understanding of the proteins in viral particles and on cell surfaces that allow infection. Moreover, progress has been substantial in determining some of the molecular mechanisms by which these proteins mediate entry (1, 2). This review focuses on recent findings about receptors used by some prevalent DNA viruses for entry into the cell interior. We explore the premise that entry not only

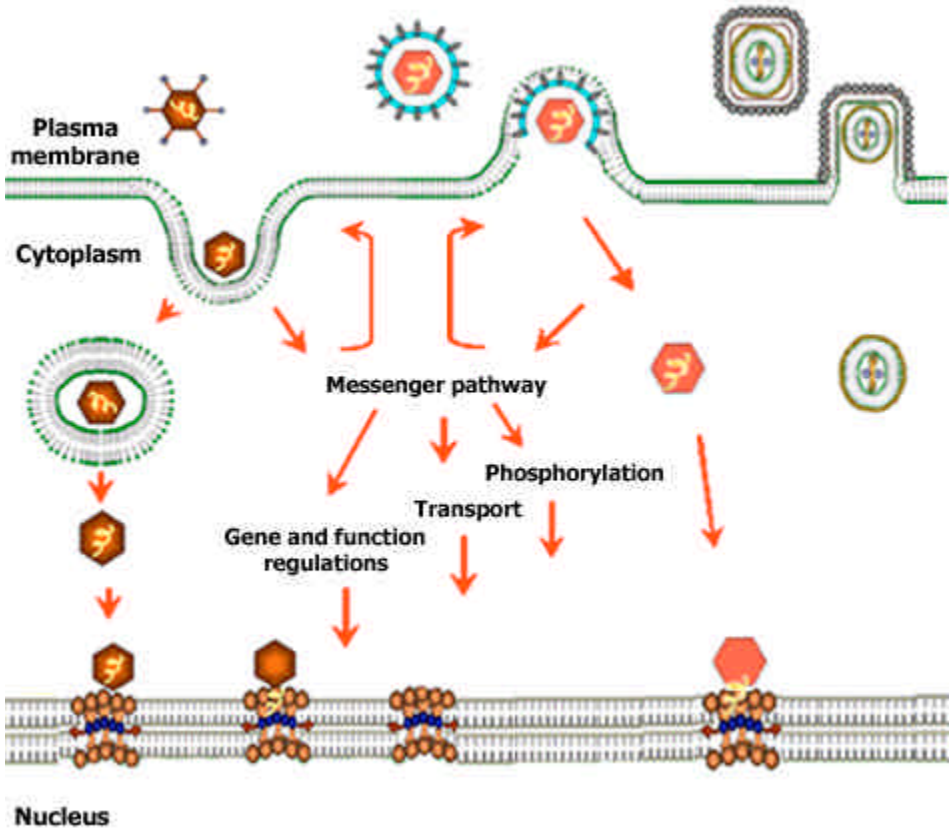

Figure 1. Entry and early events for some DNA viruses The diagram summarizes the entry pathways for prototypic adenovirus, poxvirus and herpesvirus. Binding to plasma membrane receptors may cause internalization in the endocytic pathway as with adenovirus (left). Subsequent uncoating from an intracellular vesicle allows capsid and genome transport to the nuclear pore. For the prototypic herpesvirus (center) and poxvirus (right), entry occurs after attachment through binding of receptors by direct fusion of the virus envelope with the plasma membrane. In entry of either of these viruses, effects of extracellular binding to cellular receptors can include induction of cell signaling pathways, recycling of cell surface molecules and protein changes such as oligomerization or phosphorylation of intracellular proteins. Other outcomes of virus engagement of cell surface receptors include alteration in the cell cytoskeleton and morpohology, changes in transport of ions and molecules and modification in regulation of cellular gene expression. Regardless of the number of cell surface receptors engaged or the mechanism by which penetration and uncoating occur, effects on the cell can be similar and set up viral gene expression and successful replication.

transports the capsid and genome across a cell membrane, but that these events also can set up the cell for subsequent events needed for successful viral replication.

Specific features of human DNA viruses that are relevant to this discussion include the following. *(i)* The structure of viral and cellular genetic material is similar. This allows the virus to selectively take advantage of some cellular enzymes and features of cellular transcriptional regulation. *(ii)* DNA viruses usually have a relatively large capacity genome that encodes viral specific factors. These factors provide a strategic advantage to the virus in its race to replicate and to evade destruction by the host cell. *(iii)* Because DNA viruses must go through the events required for all viruses as intracellular pathogens, entry is intricately connected with other events in replication.

Entry results in transit of virus capsids across the plasma membrane or that of an intracellular vesicle (Figure 1). Attachment and penetration also likely achieve other goals such as *(i)* facilitates uncoating of the virus genome so it is in the correct site and conformation for viral gene expression. For most DNA viruses, with the exception of poxviruses, this site is the cell nuclei. *(ii)* Entry can prime the cell for transcriptional activity, eg. activation of resting cells (B-cells or T-cells). *(iii)* Entry sets up events that may trigger or counter natural host defenses such as induction of apoptosis or production of cytokines. *(iv)* Attachment and penetration can down-regulate cellular molecules that participate in presenting viral proteins as foreign signals to activate specific host immune responses.

What are the cellular molecules used as receptors by DNA viruses? What is the impact or outcome of binding of these cellular proteins by viral ligands? Do virus binding and subsequent events of membrane fusion activate cellular pathways for signaling in transcription, transport of virus or virus components, or communication with adjacent cells? We examine receptor usage in entry for the more extensively studied DNA viruses, adenoviruses, poxviruses,

Table 1. Receptors indicated for these human DNA viruses

Virus	Cells infected in host	Receptor	Normal function\family
EBV	B-cells	CD21\CR2	CCP family
		HLA-DR	MHC molecule type II
		HLA-DQ	"
		HLA-DP	"
	Epithelial cells	IgA	Immunoglobulin
HSV	Epithelial cells, neurons	HS	Glycosaminoglycan
		HVEM or HveA	Tumor necrosis factor
		Nectin-1 or HveC	Immunoglobulin
		Nectin-2 or HveB	"
		3-OST-3	Sulfotransferase
		hfl-B5	Coiled coil membrane receptor
Adenovirus	Endothelial, epithelial and lymphoid cells	CAR	Superficial immunoglobulin
		Integrin αvβ3	Heterodimeric cell surface
		Integrin αvβ5	"
		HS	Glycosaminoglycan
		Sialic acid	
Poxvirus	Variety of cell types	Chemokine receptors	Cytokines receptor

and several human herpesviruses. Known receptors for these are summarized in Table 1.

Early events in entry and successful replication of each these viruses have some common features. Each virus must bind to one or more cell surface molecules, uncoat the genome and release any viral associated factors, transport these to the correct location in proximity of cellular transcriptional machinery and counter or prevent activation of normal cellular defense responses. For some, such as the human herpesviruses, Epstein Barr virus (EBV) and Kaposi's associated sarcoma virus (KSHV), a decision must be made between initiating lytic or latent replication to successfully establish infection. A single receptor, or multiple receptors, may be used independently or cooperatively to mediate entry. Frequently, receptor availability is one determinant of tissue and species tropism. Virus engagement of cellular receptors may affect their normal functions for cells. These may include activation of signaling pathways, up-regulation of transcriptional factors, changes in cell morphology (adherence loss, cell fusion, transformation etc) or effects on the cytoskeleton as measured by changes in ion transport such a Ca^{++} or H^{+}.

3. ADENOVIRUSES

Adenoviruses (Ad) are among the most well investigated DNA virus for entry and receptor usage. Structurally, they are nonenveloped DNA viruses that contain fiber proteins protruding from the 5-fold vertices of an icosahedral capsid. Each fiber, which terminates in a distal knob that binds to cell surface receptors, is attached to one of the 12 penton bases located at positions of five-fold symmetry in the capsid. The remainder of the shell is built from 240 additional subunits, the hexons, each of which is a trimer of viral protein. Formation of a capsid depends on nonequivalent interactions among subunits. The Ad particle contains at least seven additional proteins that play important structural roles and several small polypeptides that are produced by proteolytic cleavage of precursor proteins during maturation of newly assembled virus particles.

Human adenoviruses are classified into six subgroups (A to F) that contain over 49 serotypes. Criteria for this classification include oncogenic and transforming potential, genetic analyses of the viral genomes and hemagglutination patterns.

Ad are involved with a wide range of pathologies frequently associated in humans with acute respiratory, gastrointestinal and ocular infections. Multiple receptor binding events (Table 1) promote efficient cell entry of Ad into the natural range of susceptible tissue types.

3.1. Ad interactions with CAR

Ad infection is initiated by the formation of a high-affinity complex between the COOH-terminal knob domain from virus and a host cell surface protein. Most Ad types bind to cells through a membrane glycoprotein that is a member of the immunoglobulin superfamily referred to as the Coxsackie Adenovirus Receptor (CAR). Several studies reveal that CAR is an inducible cell protein (3, 4). Mediators of inflammatory processes and tissue regeneration are capable of inducing CAR expression in tissues normally devoid of this receptor. CAR expression decreases in mature tissues such as in adult skeletal muscle and increases in high-density culture of *rhabdomyosarcoma* cells (RD), that frequently are used as CAR-negative control) and in endothelial cells (reviewed in (5)). Although there is speculation about the functions of CAR, its natural role in cells is not yet known. Honda *et al.*(2000) suggest that CAR functions as a cell adhesion molecule in the developing brain (6). They showed that recombinant C6 cells transfected to express CAR formed cell-cell aggregates among themselves, but failed to aggregate with the parental cells. Okegawa *et al.* (2000) found an inverse relationship between the levels of CAR protein in prostate carcinoma cell lines, and thus suggested that CAR is a tumor inhibitor (7).

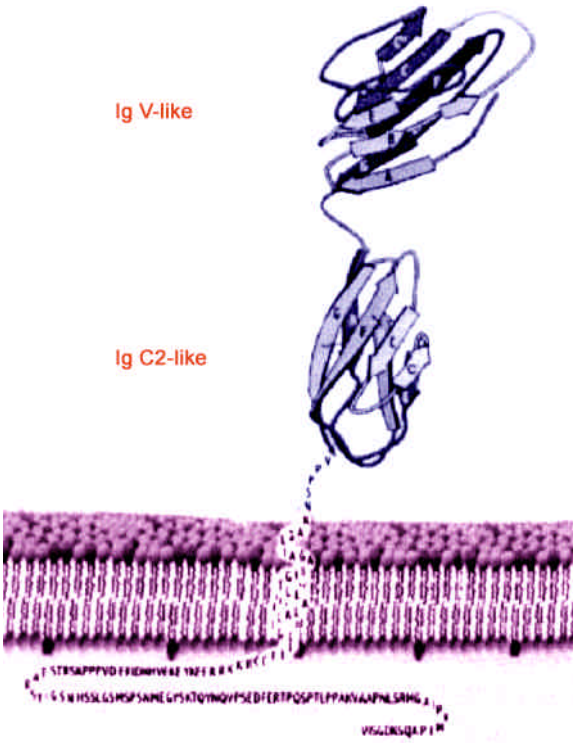

Figure 2. CAR molecule secondary structure from Carson, 2001. A diagram of the Coxsackie Adenovirus Receptor protein containing the extracellular domain with both V- and C2-like modules, a hydrophobic transmembrane domain and a cytoplasmic tail.

The CAR protein was isolated from HeLa cells by chromatography as a 46 kDa protein (10). A transmembrane domain separates the 216-residue extracellular domain from a 107-residue cytoplasm domain (Figure 2). Two prospective sites for N-glycosylation occur at Asn 87 and Asn 182. The CAR sequence predicts an immunoglobulin-like (Ig superfamily) structure with the extracellular domain consisting of two Ig-related regions, the amino-terminal IgV and IgC2 domains (Figure 2).

The primary binding of Ad to CAR proteins on cells is through the COOH-terminal knob of the adenovirus fiber coat protein. Soluble viral knobs can block this binding. Although the fiber knob amino acid sequences among different serotypes vary extensively, almost all of the Ad serotypes can recognize CAR. This implies a conservation of the amino acids or functional motifs involved in receptor binding. Through sequence analysis and mutagenesis, Roelvink *et al.* (1999) identified a conserved receptor-binding region on the fiber protein (11). This region consists of residues from the AB loop, the Bβ sheet, and the DE loop and includes residues S408, P409, K417, K420 and Y477. Kirby *et al.* (2000) demonstrated that Ser 408 and Pro 409 in the AB loop, Tyr 477 in the DG loop and Leu 485 in β strand F from Ad5 fiber protein are in direct contact with CAR (12).

A crystal structure of the CAR-recognizing Ad12 fiber knob in a complex with the D1 domain of the CAR protein has been analyzed (13). The results of this study confirm that the fiber AB loop plays an essential role in interaction with the CAR. Functional analysis of recombinant forms of CAR shows that the first immunoglobulin-like domain (D1) is sufficient for Ad binding (14).

3.2. Ad serotype differences in receptor binding

Several differences among Ad serotypes affect fiber binding to cells and indicate that Ad interacts with distinct cellular receptors. Difference in fiber length may contribute to serotype-specific variation in binding and tissue tropism. Comparative studies show that the shorter length of fiber 9 (Ad9) relative to fiber 2 (Ad2) permits independent binding of the fiber Ad9 penton base to αv-integrands. Both use the same cellular fiber receptor, but use different binding strategies for attachment (15).

Ad serotypes from subgroup B do not use CAR as a receptor to bind to the fiber knob (16). This was demonstrated using baculovirus expression clones of eight fiber knobs derived from serotypes representing the six groups. Competition experiments show that all Ad serotypes in the five subgroups A and C-F cross compete for CAR. One strain, Ad3 from subgroup B, does not compete for CAR binding. These results were confirmed by showing that other serotypes, but not Ad3, bind a soluble form of the CAR protein.

Recently, it has been shown that Ad37 from subgroup D also does not use CAR as a primary receptor. The fiber knob from Ad37 differs from the prototype strain fiber by two amino acids. Arnberg *et al.* (2000) have shown that Ad37 binds to sialic acids α-(2—3) linked to neighboring saccharides localized on cellular glycoproteins (17).

3.3. Interactions through the Ad penton base protein

The trimeric Ad fiber protein and the pentameric penton base protein each interact with separate and distinct host cell receptors. The generally accepted entry of Ad includes initial virus binding to target cells via interactions between the fiber knobs and CAR. Subsequently, association between the Ad penton base and αv-integrins mediates virus internalization and membrane permeabilization.

The Ad penton base protein can bind integrins, particularly αv integrins. Integrins are a large family of heterodimeric cell surface adhesion receptors that mediate biological processes as diverse as cell-cell comunication, cell movement and adhesion, leukocyte trafficking and binding of cell-surface and extracellular matrix ligands (18). There are 16 known α and 8 known β subunits that combine to form at least 22 distinct α-β heterodimers. Presence of these integrins along with CAR increases the susceptibility of cells to Ad infection (19, 20). The proximity and natural relationship in cells, if any, between CAR and these integrins remain to be determined. Recently Li *et al.* (2001) report that the association of penton base with αvβ3 or αvβ5 integrins promotes Ad entry (21). Penton-integrin association requires initial attachment to

the Ad virus through CAR. Assays in the presence or absence of antibodies that block the functions of specific integrins significantly blocked Ad infection.

The virus penton base consists of five polypeptide subunits. Each contains a tripeptide motif, Arg Gly Asp (RGD), which binds to αv-integrins. The penton proteins of adenovirus serotypes 2 and 5 from subgroup C, serotype 3 from subgroup B, serotype 4 from subgroup E and serotype 12 from subgroup A contain the RGD motif. All utilize αv integrins during viral entry into cells (22). The three-dimensional structure of soluble recombinant integrin αvβ5 bound to human Ad2 or Ad12 (23) suggests that the precise spatial arrangement of the five RGD protrusions on the penton base promotes integrin clustering. Such clustering is proposed to mediate the signaling events required for virus internalization.

Because integrins are associated with cell increment signaling and communication, their involvement in Ad penetration seems appropriate as receptors used to perform the events of entry.

Recently, it has been demonstrated that heparan sulfate glycosaminoglycans (HS GAGs) expressed on cell surfaces are involved in the binding and infection of Ad2/5 (24, 25). Cell surface heparan sulfate and glycosaminoglycans are ubiquitous attachment sites for many pathogenic microorganisms (26). For some, this interaction is not required but may enhance binding activity and increase the likelihood of finding required receptors. For other pathogens, HS interaction may be critical to alter conformation of viral surface proteins so they function in subsequent events of entry.

Some studies indicate that HLA class I molecules may participate in Ad 5 fiber binding to the cell surface (27). However, later studies demonstrate that the observed increase in attachment and permissiveness of Daudi HLA+ cells to Ad5 may not be due to a direct interactions of MHC class I molecules and Ad5 fiber. Instead, it may be due to increased accessibility of CAR as a consequence of expression of HLA class I molecule on the surface (28). This is one example of how receptors used in entry may influence, or be influenced by, events that up-regulate or down-regulate cellular gene expression.

3.4. Effects of Ad entry and internalization on cellular events

Virus protein and cell receptor interactions also facilitate virus internalization necessary for genome uncoating. Interactions that lead to penetration may set up the cell in other ways to support virus replication. Adenovirus has been useful in uncovering some of the signaling events involved in integrin-mediated viral endocytosis (Figure 1).

Wang *et al.* (1998) show that interaction of the Ad 2/5 penton base with αv integrins mediates virus internalization via the clathrin-coated pit pathway (29). Interaction of Ad penton with integrins induces activation of phosphatidylinositol- 3-OH kinase (PI3K), a member of the lipid kinase family of enzymes. These kinases catalyze phosphorylation of phospholipids that are thought to act as second messengers for a number of important cell processes (30). Recent studies using inhibitors of P13K activity demonstrate that interaction of Ad with cells can modulate actin assembly downstream of PI3K and involves the small GTPases Rac and CDC42 (31). Cytochalasin D, a fungal metabolite that disrupts cortical actin filament, was used to show that virus-induced activation of PI3K results in the assembly of actin filaments. Actin filament assembly is required for cytoskeleton movement in virus internalization and translocation to the nucleus. The Rho/Ras, Rac and CDC42 proteins belong to the family of small GTP binding proteins implicated in PI3K activation. Blocking virus uptake by pretreatment of cells with a specific inhibitor of Rho GTPases indicates that Rho GTPase also regulates Ad internalization. Thus, a principal role of Rho GTPases in Ad entry seems to be associated with the formation of actin-associated stress fibers in the cytoskeleton.

An effector domain mutant of Rac/CDC42 that prevents cytoskeletal reorganization also could block adenovirus uptake and gene delivery, while mutants that lack this capacity do not (31). Thus, for efficient adenovirus entry, reorganization of the actin cytoskeleton is also a required process. Since Ad is nonenveloped, this reorganization may facilitate passage of the capsid through the cell cytoskeleton by a means that differs from membrane fusion used by enveloped viruses (Figure 1). Once through the cytoskeleton and in the cytosol, the Ad capsid is deposited so the DNA genome can enter into the cell nucleus as the site of viral gene transcription.

Recent investigations with Ad2 indicate that the virus does not move through the cytoplasm by diffusion. When the microtubule (MT) network was depolymerized with nocodazole, virus infection was reduced thereby indicating some form of mediated transport (32). After restoring the MT network by removal of inhibitor, virus mobility was observed in the cell periphery or near MT organizing centers (MTOC). This suggests that cytosolic Ad uses both minus and plus end directed MT-dependent motors. MT-dependent transport also functions to direct incoming virus particles to the cell nucleus as the site of replication (32). Penetration and movement for Ad uncoating also seems to have other effects on the cell.

3.5. Ad entry and the modification of cellular immune responses

Adenovirus encodes potential immunomodulatory proteins. Although these may limit application of these viruses as vectors in gene delivery, the immunomodulatory potential contributes to successful infection of an immune competent host.

Ad infection in cell culture proceeds in well-regulated phases. Cells begin to lyse for release of new virus particles at 2 to 3 days post infection. Until then, the infected cells must remain intact and evade immune destruction during the extended period of replication. The immediate-early E1A proteins induce transcription of delayed-early genes in the E1B, E2, E3 and E4 ordered transcription units. The E19 protein, encoded by E3, has been known to bind HLA class I molecules and to retain

them in the endoplasmic reticulum. E19 also binds TAP (transporter associated with antigen processing) and acts as a tapasin inhibitor. This inhibits class I/TAP association to prevent antigen presentation and killing of infected cells by cytotoxic T lymphocytes (33). A tightly regulated and ordered process that is set in place at the time of genome uncoating controls the expression of adenovirus proteins such as E19. Entry and internalization must set up uncoating and regulation of gene expression so that E19 and other proteins critical to survival in the host are available to interact with host factors.

3.6. Ad induction and inhibition of apoptosis

Eukaryotic cells are able to counter foreign invasion or an aberrant event by inducing apoptosis. While viruses use cell surface molecules for entry, results of some of these interactions may trigger cell apoptosis. Pre-mature cell death before virus is made does not allow successful replication. Hence, many DNA viruses seem to encode inhibitors to block apoptosis that might be triggered inadvertently during entry or capsid transport for uncoating.

Three Ad proteins inhibit apoptosis induced by TNF-α and Fas (also known as CD95). TNF and Fas are members of the tumor necrosis factor superfamily. They contain conserved protein domains that participate in protein-protein interaction leading to activation of caspases that mediate apoptosis. The Ad E3 protein, designated as receptor internalization and degradation (RID), inhibits FAS mediated apoptosis (34). RID stimulates the internalization of cell surface Fas into endosomes, which are subsequently transported to lysosomes and degradated. Ad RID also can inhibit apoptosis induced by TNF (35). Other E3 proteins (E3-14.7 and E3-19K) have been reported to inhibit apoptosis through the TNF and Fas pathway (36, 37).

Recently, it has been shown that Ad proteins RID, E3-14.7 and E3-19K independently inhibit TRAIL-induced apoptosis (38). TRAIL is another member of TNF superfamily that is used by cells of the immune system to kill virus-infected and tumor cells. How this inhibition is mediated remains to be determined. However, it seems likely that Ad association with CAR, or with αv-integrins, might initiate events to induce apoptosis, or to block apoptosis induced by binding of virus to receptors for entry.

Ad engagement of cellular receptors is critical to virus replication and might also alter functions of cell surface molecules that trigger the immune system or that mediate apoptosis. Although the pathways and connections remain to be clearly elucidated, adenoviruses have evolved to engage in entry cellular receptors or molecules that perform a variety of roles in cell signaling and host immune defenses.

4. POXVIRUSES

Poxviruses are a family of large double-stranded DNA viruses that infect both vertebrate and invertebrate hosts. Most poxviruses are host-species specific, with vaccinia virus (VV) as a remarkable exception. The most prominent of the poxviruses, smallpox virus (vareola), caused a severe disease in human that was eliminated by vaccination.

The large double-stranded DNA genome of about 200 kbp encodes approximately 200 genes. The complex structure of a poxvirus particle contains a tightly compressed nucleoprotein with a core that is flanked by two lateral bodies with unknown roles (Figure 1). The extracellular form has two membranes with proteins important for entry embedded in the outer membrane.

4.1. Chemokines in entry of poxviruses

Recent studies indicate that both chemokines and chemokines receptors play critical roles in poxviruses infection and replication. A wide variety of cell types produce chemokines upon activation. The initial signal is emitted primarily by resident tissue cells with subsequent amplification by professional antigen-presenting cells and T cells. Chemokines and chemokines receptors play an important role in controlling the developmental and functional aspects of leukocytes, and are critical for defense against infectious pathogens.

Based on structural criteria, four families of chemokines have been described (39). Each shows a distinctive N-terminal cysteine motif. The CXC, (or α) family has a two-cysteine motif with an intervening amino acid. The CC (or β) family is characterized by two contiguous cysteine residues. The C family has a single-cysteine motif and the CX3C (or δ) family that presents two cysteine separated by three residues.

Based upon their function, two major groups of chemokines have been characterized. Housekeeping (HK) chemokines are expressed constitutively and function in development. Homeostasis and proinflammatory (PI) chemokines are inducible and participate in the innate and adaptative immune responses (reviewed in (40)).

The activity of chemokines is mediated by interaction with cell surface receptors that contain seven transmembrane spanning domains that are in the G-protein coupled receptor superfamily (GPcRs) (41). Signaling through chemokine receptors is mediated by heterotrimeric G-proteins that activate different cascades of intracellular signal conduction. Viruses have developed strategies to use both chemokines and chemokine receptors by hijacking them for entry into cells and reprogramming their normal function to aid immune system evasion.

Until recently, a relationship in entry between chemokines and viruses had been restricted to the RNA viruses. For instance, HIV engages CXCR4 and CCR5 as primary co-receptors for entry (reviewed in (42)). Recent studies show that poxviruses, as DNA viruses, also are able to engage chemokine receptors to infect specific cell subtypes. Allan *et al.* (1999a) found that co-expression of any of the human chemokine receptors CCR1, CCR5 or CXCR4 in normally non-permissive cells, conferred susceptibility to infection by myxoma virus, a rabbit poxvirus (43). Because of the wide array of tissue types and

species infected by poxviruses, receptors used for entry must be molecules that are highly conserved among different species. Alternatively, poxviruses may interact with many different cell surface proteins.

Poxvirus interactions with chemokines and chemokine receptors by several mechanisms have been shown as a strategy for viral immune evasion (44, 45).

One strategy used by poxviruses to modulate the host immune response is by encoding viral homologues to cellular chemokines (vCKs). For molluscum contagiosum virus (MCV), the *MC148R* gene encodes a chemokine antagonist with homology to the cellular CC chemokine CTAK (46, 47). The viral gene has a deletion in the amino-terminal domain that makes it incapable of chemotactic activity.

Another viral strategy adopted by poxvirus to counteract chemokine activities is attachment and inactivation of chemokines by specific viral encoded protein (vCkBPs). There are two types of these proteins based on the capacity of each protein to modulate chemokine activity by different mechanisms. The first type makes poxviruses the only virus family known to produce secreted versions of receptors for cytokines such as TNK,IL-1β, IFN-αβ and IFN-γ? These viral encoded homologues to chemokine receptors bind cytokines and block their normal activity by preventing interactions with receptors on the target host cell. These molecules were initially identified in vaccinia virus (VV). VV expresses a soluble form of the IFN receptor encoded by *B18R* as a 65 kDa glycoprotein (48). This receptor has high affinity for human IFN-α. The B18R protein is unique among host response modifications by poxviruses in that it appears to exist in both a soluble and membrane bound form. Thus, it should effectively block both autocrine and paracrine function of IFN (49).

Myxoma virus (MV) encodes an M-T7 molecule that is a soluble IFN-γ receptor homolog (50). This protein modulates the infiltration of leukocytes into infected tissue by binding to CC, CXC and C chemokines through their proteoglycan binding domain.

4.2. Chemokine receptors in entry of poxviruses

A second type of modulators is found in other poxviruses. Their mechanism of action involves direct inhibition by binding of chemokine receptors. This can limit the influx of inflammatory cells to infected tissues. Poxvirus expression of potent and soluble forms of vCkBP suggests an important role for chemokines in anti-viral defense.

Vaccinia virus expresses a 35 kDa soluble chemokine binding protein that binds CC chemokines with high affinity and inhibits their ability to induce signal transduction and cell migration in vitro (51). The T1/35 kDa proteins are a family of poxvirus-secreted proteins that bind CC and CXC *in vitro*. Infection with a rabbit poxvirus mutant that does not express the secreted 35 kDa chemokine binding protein shows alterations in the influx of leukocytes into virus-infected rabbit tissues in vivo. This suggests a role in modulation of the influx of inflammatory cells into virus-infected tissues *in vivo* (52). To understand the interaction between the soluble viral expressed CC-chemokine and cellular CC-chemokines, different mutant and wild type forms of human MCP-1 (monocyte chemoattractant protein-1) were interacted with VV-35KDa protein. Several residues were identified in cellular CC-chemokines that contribute to the high affinity interaction with VV-35 KDa. VV-35 KDa recognizes similar epitopes on MCP-1 that are important for binding and signaling through CCR2b. This demonstrates the use of common determinants by structurally distinct proteins (53, 54).

Later studies with myxoma virus identified the MT-1 glycoprotein that binds to a broad range of CC-chemokines (43). Results with rabbits infected with a recombinant myxoma virus deleted in the MT-1 gene indicate that MT-1 influences chemotaxis of inflammatory cells, especially macrophages, into infected tissue sites during the initial phases of virus infection.

Another strategy by which poxviruses counteract the host immune response is through production of virus-encoded homologs of chemokine-receptors on cells (vCkRs). These vCkRs retain functional competency. The virus can then use induction of cellular chemokines in infected cells to increase the efficiency of virus spread and to disseminate the infection. The first soluble poxvirus IFN-γ receptor (vIFN-γR) was described for myxoma virus (50). Subsequent studies have found vIFN-γR in other member of the poxvirus family.

Sequence analyses of the genomes of different poxviruses have revealed putative viral genes that encode G-protein coupled chemokine receptors (GCR) (55). In swinepox virus, two ORFs (C3L and K2R) have been detected. K2R ORF appears to encode an intact G-coupled receptor. However, C3L ORF is missing the N-terminal portion of the protein and may not be properly expressed, folded or translocated to the ER (55). In capripoxvirus the nucleotide sequence of the HindIII generated Q2 fragment has revealed two partial ORFs designated Q2/1L and Q2/3L and one complete ORF designated as Q2/2L (56).

Vaccinia virus (VV) produces a soluble IFN-γ receptor early after infection. The VV IFN-γR shows broad species specifity and is expressed from the gene *B8R*. Experiments with rabbits showed that VV lacking this protein was attenuated. This suggests a role for IFN-γR in virus virulence (57).

Evasion of the immune response of an infected cell by poxviruses may or may not be associated with the same molecules that are used for attachment and penetration in entry. While a plethora of homologues to cellular proteins are encoded by poxviruses, the receptors used by individuals in this family of viruses remain to be identified. It remains to be determined if receptors used for binding and penetration will be related to the chemokines and chemokine receptors encoded by poxviruses.

Hypotheses			
IR	SR	FR	Fmd/NT
HS	HveC,B,A 3-OST	B5/?	?/dynein
gB/gC	gD	gH/gL	gM,gK,gB gE/gI

Figure 3. Hypothesis for use of multiple receptors in viral entry. A hypothesis of events in virus entry that may be performed by any one of multiple receptors. The events that seem to lead to successful entry include: IR= initial attachment receptor; SR= stable attachment receptor; FR= fusion receptor; Fmd/NT= Fusion modulation or Nucleocapsid transport receptors. Identified receptors in the entry of herpes simplex virus are used as an example. These are HS= heparan sulfate; Hve C,B,A= herpesvirus entry receptors C, B or A; B5= putative colield coil containing receptor for fusion encoded by *hfl-B5;* dynein= implicated in HSV association with the cytoskeleton for nucleocapsid transport to nuclear pores. ?= other unknown cellular proteins are predicted to perform this function. HSV glycoproteins that are thought to participate in each process are indicated. In this model of multiple receptor usage, any one of several cell surface components may be engaged to perform the same function. Requirements are that each component can function with the preceding and subsequent virus-cell interaction so that the entry process is successfully completed.

5. HERPESVIRUSES

5.1. Herpes Simplex Virus

The herpesvirus family contains eight prevalent human pathogens: herpes simplex virus type 1 and type 2 (HSV-1 and HSV-2), varicella zoster virus (VZV), Epstein-Barr virus (EBV), human cytomegalovirus (HCMV), human herpes 6 and 7 (HHV-6, HHV-7) and Kaposi's sarcoma associated herpes virus (KSHV or HHV-8). A unique characteristic of all herpesviruses is establishment of latency in specific cell types for life-long infection.

The herpesvirus particle is composed of nucleocapsid, tegument and lipid membrane envelope. The linear DNA of 120 to 150 Kb is surrounded by a protein capsid formed by 162 capsomers (12 pentons and 150 hexons). The tegument is a protein layer between the envelope and the capsid. For HSV there are at least 12 different viral polypeptides for which most have functions are not yet well understood. The envelope contains at least 12 different membrane glycoproteins some of which are involved in receptor binding and fusion. Although studies are in progress to explore early replication events for HCMV and VZV, here we focus on entry of HSV and EBV for which more is known about interactions of viral components and cellular receptors.

Herpesviruses enter into susceptible cells by multiple binding events that lead to penetration (Figure 3). HSV, as the most studied prototype of this virus family, was shown to enter cells by fusion of the virus envelope with the plasma membrane of cells independent of pH change (58, 59). Entry involves binding of viral glycoproteins on the envelope with one or several of multiple cellular receptors. Although functions of the envelop glycoproteins have been focused upon extensively by many investigations (reviewed in (2, 60, 61)), the mechanisms by which fusion and penetration are initiated and how the cell receptors are involved are not yet understood. A chapter in this volume specifically focuses on entry and cell to cell spread of HSV.

5.1.1. HSV entry and viral proteins for attachment and penetration

The two serotypes of HSV (HSV-1 and HSV-2) infect a wide variety of cells in tissue culture and have co-linear envelope proteins with high levels of homology. Entry and spread in cells requires glycoproteins B (gB), gD, and the gH/gL complex (reviewed in (60)). The ability of HSV envelope glycoproteins to fuse cells is evident in polykaryocytes or syncytia found in infected cultured cells and in cells of infected host tissues.

Kinetics and efficiency of entry vary for the same virus strain on different susceptible cells (62). This was taken to indicate differences in type and possible amounts of cell receptors**.** In this study, HSV entry was shown to participate in at least two experimentally distinguishable types of attachments that lead to penetration and uncoating (Figure 3). While these distinctions are useful in defining events of entry and the components involved, how these attachment components are connected or how they cooperate in entry is not yet known. However, as with adenoviruses, entry of HSV includes an initial binding and a subsequent more stable type of binding that lead to penetration into susceptible cells.

Binding of wild type and mutant viruses lacking either essential protein gD or gH to susceptible human cells or to entry-defective porcine cells as measured by FACS showed that gD, but not gH, must be present in the HSV virion envelop for stable binding that engages cellular proteins present on susceptible human cells (63). In this study, absence of either gD from the virion, or of a functional human receptor protein on the cell prevented the heparin resistant stable binding of HSV that leads to entry. Viruses lacking gH could stably bind to human cells, but were incapable of penetration.

5.1.2. HSV interactions with heparan sulfate proteoglycan

Several human cell surface proteins that mediate HSV entry have recently been identified (Table 1) (60, Perez *et al*., 2001, submitted). So far, all except one seem to interact with the essential gD on the HSV envelope.

The glycosaminoglycan, heparan sulfate (HS) is involved in the initial interaction of HSV with cells (64, 65) through binding mediated by the virus glycoproteins gC and gB. Cells treated with heparinases, or altered by mutations that prevent HS biosynthesis, have reduced capacity to bind virus and are least partially resistant to virus infection (65, 66). While this initial attachment to HS has been shown to resist elution with neutral pH buffer, numerous studies show that virus bound to HS can be competed from the cell with soluble heparin. A subsequent

stable binding occurs that is resistant to elution by heparin. Stably bound virus has not yet penetrated because it remains sensitive to extracellular inactivation. These results also indicated that HSV binds to cellular receptors other than HS (62, 63, 67).

When susceptible cells were saturated for HSV binding that leads to entry, binding to HS was not saturated. This was interpreted to indicate presence of specific cell protein receptors that are engaged by HSV (68). Results with saturation of virus blocking, from characterizing interactions of gD null mutants with susceptible or non-permissive cells, or from superinfection interference by expression in cells of gD indicated presence of cellular proteins as receptors for HSV (63, 69-71). As discussed briefly below and in more details elsewhere (60), so far at least four human membrane receptor proteins have been isolated that seem to interact with gD (Table 1).

5.1.3. Cellular protein receptors for HSV entry

An experimental challenge to isolation of protein receptors for HSV was the broad tropism of HSV to infect almost all cultured cells. Chinese hamster ovary (CHO) cells, porcine testes or kidney (SK-6) cells and transformed baby hamster kidney (BHK)cells were shown as non-permissive for HSV infection due to a block at entry (63, 72-76). Porcine cells that are poorly susceptible to HSV were shown to have functional HS for HSV attachment, but to lack functional stable attachment receptors (63, 73, 74). Interestingly, they are competent for HSV replication since transfected viral DNA can result in virus progeny equal to that from viral genome transfected human cells (73).

The search for cellular receptors used by HSV was facilitated by entry-defective cell lines that are resistant to virus infection. CHO cells are resistant to HSV-1, but less so to HSV-2 (64). There is a defect at entry, and also subsequently at an event in viral replication. Porcine kidney (SK-6) cells that have a defect only at entry, are resistant to infection by both serotypes of HSV (73, 74). The approach of transfecting pools of plasmids from human cDNA libraries into CHO or SK-6 cells resulted in isolation of cDNAs that encode putative human receptor proteins that could render the cells susceptible to viral entry. With this procedure, some of the human proteins that can mediate entry of HSV and thus serve as cell receptors have been identified (Table 1). These are described briefly below and in more detail in another review in this volume.

Interestingly, the human molecules identified so far, with the one exception of HveA, are from different human protein families and share a broad prevalence consistent with the broad cell tropism of HSV (Table 1).

The first HSV protein receptor identified is a member of the TNF-receptor family that was designated as herpes virus entry mediator (HVEM), or HveA (72). The ability of HveA to mediate HSV entry is dependent on presence of glycoprotein D (gD) expressed on the virus envelope (77, 78). Recently, the structure of soluble forms of the gD-HveA complex has been examined by X-ray crystallography (79). These analyses indicate that interaction between gD and HveA is centered around a tyrosine of HveA that fits into a pocket on gD. That the gD protein shows a different conformation when it is examined in an unligated form suggests that a conformational change occurs during its interaction with HveA. This suggestion is consistent with previous findings that antibodies to gD can prevent infection by blocking penetration or attachment and that conformational dependent epitopes are critical to binding of neutralizing antibodies and to gD function in entry.

The role of HveA, as with other identified receptors, in HSV infection of human cells remains to be determined. In one approach to determine receptor involvement, polyclonal antibodies to HveA could completely block HSV entry into HveA expressing porcine cells, but they had no effect on infection of human cells in culture (Perez *et al*, in preparation). This indicates that HSV entry can occur through other cell surface proteins present on human HEp-2 and Vero cells and agrees with use of multiple entry receptors.

Other human protein receptors for HSV have been identified by similar functional cloning strategies to screen human cDNA libraries by transfer of susceptibility to infection. Two of these are members of the immunoglobulin family designated nectin-1 or HveC (80) and nectin-2 or HveB (81). Previously isolated as poliovirus related receptor proteins (PVRR), HveB and HveC as we refer to through out the discussion, serve as cell adhesion molecules that localize to sites of cadherin-based cell junctions (78, 82-85). HveC and HveB can serve as receptors that bind to gD (reviewed in (60)). For CHO cells, interaction of HSV with either gD receptor can lead to membrane fusion in viral entry (79, 86-90).

Expression of either human cell proteins nectin-1 and nectin2 (HveB or HveC) allows entry of HSV-2 strains and an HSV-1 mutant strain RID1 into CHO cells. In contrast, wild-type HSV-1 can use HveC, but not HveB (80, 81). By mRNA analyses, HveC seems to be broadly expressed in a range of human cells, while HveB expression is more restricted. The role of either in HSV infection of human cells also has not yet been demonstrated. It remains to be determined if these proteins are redundant as gD receptors, or if they differ in their functions during interactions with viral and cellular proteins.

The most recently reported gD receptor is an HSV-1 binding site that is generated in heparan sulfate (HS) by the action of specific glucosaminil-3-O-sulfotransferase (3-OST). In the absence of other receptors, 3-OST expression in HSV-resistant CHO cells makes the cells susceptible to HSV-1 entry, but not entry of HSV-2, PRV or BHV-1 (91). The fact that specific sites in heparan sulfate generated by 3-OST can serve as entry receptors for HSV-1 suggests that, viral binding and entry can be mediated entirely by heparan sulfate, provided that the appropriate sites for virus binding and gD binding are present (92).

Recent studies explore how several of these isolated human gD receptors affect entry of HSV-1, HSV-2

and Pseudorabies (PRV) (93). Previous studies indicated some overlap in receptor usage by these alpha herpesviruses (68). Martinez and Spear (2001) follow up previous findings that human and murine HveB and HveC differ in their ability to allow HSV-1 and HSV-2 entry into CHO cells. Minor amino acid changes in certain regions of human and murine HveB and HveC receptor proteins altered receptor interactions with viral components and changed entry efficiency of HSV or PRV.

5.1.4. Cellular receptors and viral proteins for membrane fusion

HSV glycoproteins B (gB), gD and gH/gL are essential for entry and also define a minimal set of viral glycoproteins required for HSV-induced cell fusion (94, 95). Recently, Browne et al. (2001) extended previous studies to show that gB, gD and gH/gL act in a coordinated and unidirectional fashion to mediated fusion. Moreover, they find that all four viral proteins must be expressed in the same membrane for fusion to occur (96).

Search for receptors that interact with glycoproteins, other than gD, gB and gC, are in progress. A new class of HSV receptor is encoded by a previously uncharacterized human gene that currently is designated as human fetal lung gene B5 *(hfl-B5)* (Perez *et al.*2001, submitted). *Hfl-B5* encodes a 43kd membrane protein, B5, which was isolated from a screen of a human fetal lung cDNA library for plasmids that confer susceptibility to porcine cells. *Hfl-B5* transcripts are broadly expressed in human cell line and are found in primary human tissue cells from brain, kidney, lung and muscle. For entry-defective swine kidney cells, presence of the B5 protein allows stable HSV binding that leads to membrane fusion and penetration.

The B5 protein has a unique structure in that it is a type II protein that contains heptad repeats that are predicted to form putative coiled coils. Coiled coils are found in the fusion proteins of several viruses (HIV, influenza virus, Ebola virus, respiratory syncytia virus) (97-100) and are common to cellular SNARE proteins important for targeting and membrane fusion in trafficking of cellular proteins (101, 102). Initial experiments provide evidence to suggest that B5 may function in membrane fusion and possibly interacts with the highly conserved viral glycoprotein complex gH/gL.

Perez *et al.* (2001) find that susceptible porcine cells that express only human B5 fuse extensively when exposed to a soluble form of gH/gL. In this complex, gH is truncated to lack its transmembrane region, but retains residues needed for association with gL (103). There was no cell fusion detected when B5 porcine cells were exposed to soluble gD or gE/gI in the same experiment. gH/gL addition to the media results in extensive fusion of porcine monolayers that express B5, but not of porcine cells expressing HveA. These results suggest interactions of gH/gL directly with B5 or indirectly through another porcine or viral protein and represent a new class of HSV receptor. If putative gH/gL interactions with B5 are confirmed, this interaction would likely function in some manner to initiate or facilitate the fusion of the viral envelop and cellular plasma membrane and serve as a new potential target for development of anti-virals that work at HSV entry.

Interestingly, the essential gH/gL complex is highly conserved among all known herpesviruses, while gD is found only in the alpha herpesviruses. Thus, the gH/gL complex seems likely to function for membrane fusion as an event that is required for all herpesviruses. HSV attachments to HS and gD receptors could lead to conformational changes in viral fusogenic proteins such as gH/gL to expose sequestered regions so they can engage the fusion machinery of cells. Putative coiled coils of B5 as a SNARE-like molecule fits this hypothesis as a strategy for HSV pH independent entry. We speculate that regulation of this process to fusion of a limited membrane area during virus entry might explain the roles of other HSV envelope proteins that have been shown to affect syncytia formation by the virus. These include viral proteins such as gK, gB, gM and involve other as of yet unknown cell proteins on highly susceptible human or animal cells.

Other human cellular molecules likely will be isolated that interact with one or another of the many HSV envelop glycoproteins involved in entry or spread of virus. This fits with life-long latency and ability to infect and replicate in a variety of human and animal tissues or cells even in the presence of a robust host immune response to HSV.

Recent findings with identified HSV receptors confirm implications from previous reports that HSV entry is a multi-step process that involves a plethora of viral components and numerous human proteins (Figure 3). The cellular receptor interactions with viral proteins may occur individually, or may be connected in a complex of proteins that mediate membrane fusion and subsequent events of uncoating and transport of the capsids to the cell nucleus for gene expression (Figures 1 and 3). How the neutral pH entry occurs, how limited fusion is regulated at the plasma membrane during entry, or how more extensive membrane fusion occurs during spread or syncytia formation, and how it affects later events in the cell are subjects of continuing studies.

5.1.5. HSV entry and subsequent cellular events

HSV encodes proteins that may affect signaling pathways in cell death (76). These may function in an attempt to counter effects of virus binding to receptors or formation of protein complexes that allow fusion during entry. Such cellular proteins could normally function in cellular signaling pathways.

Recently, gene array technology has been used as a strategy with HCMV to detect changes in cellular genes expressed at different stages after HCMV entry and replication (104). Such an approach may prove helpful in examining the impact of human herpesvirus entry on cellular gene expression.

5.2. EPSTEIN-BARR VIRUS

Epstein-Barr virus (EBV), also designated as human herpesvirus 4 (HHV-4), is a persistent human

gammaherpesvirus that is linked to a variety of human diseases including endemic Burkitt's lymphoma and undifferentiated nasopharyngeal carcinoma. In immunocompromised individuals the viral infection correlates with a variety of disorders such as oral hairy leukoplakia, Hodgkin's and non Hodgking's lymphomas and B cell lymphoma.

Although EBV entry *in vitro* is restricted to B cells, association of EBV with diseases of multiple tissue origins indicates that it gains access into a wide variety of host cells. In infection of the human host, mucosal epithelium is the first barrier that must be crossed for viral invasion prior to viral dissemination. Therefore, epithelial cells are thought to participate in the initial steps of primary EBV infection.

Strategies of EBV to enter its various target cells are complex and incompletely understood. However, evidence is accumulating that entry of EBV, like many other viruses, involves interactions between several viral glycoproteins and multiple cellular entry mediators.

5.2.1. EBV entry and the CD21 receptor

EBV readily infects human B cells *in vitro*. The initial events required for entry include binding to cellular CD21 (also known as CR2). CD21 is a member of B cell antigen receptor complex implicated in intracellular signaling pathways that modulate B cell activation, growth and differentiation. The natural ligand of CD21 is the C3d fragment of complement (105, 106). CD21 occurs on many human cell types other than B cells, i.e. folliculum dendritic and endothelial cells, thymocytes, T cells, epithelial cells and monocytes (107, 108). EBV binds to CD21 via a short primary sequence epitope in the major viral envelope glycoprotein (gp350/220). This epitope is homologous in sequence to the binding epitope in C3d (109, 110).

5.2.2. EBV interactions with other cell surface receptors

As with other herpesviruses, additional molecules are necessary for post binding events such as fusion of virus and cell membranes and capsid internalization (111, 112). Viral envelope fusion with the host cell membrane at penetration requires the additional interaction of the complex of EBV glycoproteins gp85-gp25-gp42 complex with its cellular receptor.

Gp85 and gp25 are the EBV homologs of gH and gL respectively in HSV. Gp42 has been shown to interact with the HLA class II protein HLA-DR that can serve as a coreceptor for EBV entry (113, 114). B cells lacking HLA-DR expression are not susceptible to superinfection unless the expression of HLA-DR is restored (115).

HLA class II antigens are α/β heterodimeric cell surface glycoproteins that function to present processed antigens to $CD4^+$ T lymphocytes. Recent studies, using transient transfection of different class II molecules into cells resistant to infection by EBV show that the virus also can use HLA-DP or HLA-DQ (MHC class II isotypes) as a coreceptor to mediate entry by substituting for HLA-DR (116). Analysis by flow cytometry using resistant cell lines expressing CD21 and electroporated with HLA-DR, HLA-DP or HLA-DQ revealed that expression of each MHC isotypes renders these cells susceptible to infection by EBV (116).

The specific site essential for EBV entry has been determined through mutational analysis of different HLA-DQ alleles as the glutamic acid at residue 46 of the HLA class II β chain (117). Interestingly, this site is homologous to the domain by which MHC class I binds to the c type-lectin-like natural killer receptor.

That EBV can enter cells via all three isotypes of HLA class II molecules emphasizes the feature common to many viruses that can enter cells through multiple entry mediators. Tissue specificity of HLA class II genes is typically restricted to immune cells, including B cells, dendritic cells, activated T cells, macrophages and thymic epithelial cells.

5.2.3. EBV binding activates cell signaling through NF-kb

In animal models or infected human hosts, EBV infection is accompanied by immune suppression and cytokine deregulation to transform and immortalize human B cells into continuously growing lymphoid cell lines (LCL). It has been found that the binding of EBV to B cells causes the secretion of interleukin-6 (IL-6). IL-6 is a pleiotropic cytokine that regulates many hematopoietic cell functions. In EBV infected cells, IL-6 is a paracrine and autocrine growth factor that increases immunoglobulin (Ig) production and promotes tumor formation (118). It has been shown that elimination of EBV-positive B cells in healthy individuals infected with the virus is initiated by NK cells and followed by activity of EBV-specific cytotoxic T cells.

The effects of binding to receptors on down stream signaling events are well-established for EBV. Several studies evaluate the intracellular signaling pathway initiated by EBV binding to CD21 to induce IL-6 secretion. Sugano *et al.*(1997) used EBV-genome positive immortalized cells to confirm the dependence of NF-$\kappa\beta$ activation on CD21-gp350/220 interaction (119). They used a soluble fragment of gp350 (gp105 fragment) and a soluble OKB7 monoclonal antibody to CD21. Both of these proteins could bind to CD21 and inhibited EBV-induced NF-$\kappa\beta$ activation. NF-$\kappa\beta$ activation is mediated by a process that involves IkB phosphorylation on specific serine residues, ubiquination and degradation. EBV-induced NF-$\kappa\beta$ activation was solely dependent on binding of viral ligand to CD21, and was associated with IkB degradation. Both NF-$\kappa\beta$ activation and IκB degradation, were inhibited by specific protein kinase C (PKC) inhibitors. Stimulation of PKC induces significant increases in IL-6 expression. Thus using PKC-specific inhibitors can prevent IL-6 induction following EBV infection (120). The increase in IL-6 secretion implies that NF-$\kappa\beta$ activation is triggered by EBV binding to CD21 on B cells.

Recently, D'Addario *et al.*(2001) demonstrated that EBV human B-lymphocytes stimulate binding of NF-

κβ proteins to the IL-6 promoter to induce gene activation (121). They have shown that purified recombinant gp350 can initiate events of infection in B cells. Binding by EBV particles or gp350 alone upregulated IL-6 mRNA synthesis and enhanced the binding of the NF-κβ transcription factor as determined by EMSA analyses.

EBV interaction with CD21 enhances the binding of transcription factors KF-kB and NF-IL6 to the IL-6 promoter. NF-IL6 is a protein that was initially found to regulate the expression of IL-6. It is now known to modulate the expression of other cytokine genes including TNF-α, IL-8 and G-CSF. Although EBV is able to induce NF-κβ and NF-IL6 binding to their respective oligonucleotides, only NF-κβ appears to be necessary for IL-6 gene activation (121). In summary, for EBV infection, increase in the level of IL-6 through interaction of gp350 with CD21/CR2, seems to be mediated by the NF-kB transcription factor through a PKC-dependent signal transduction pathway.

Other signaling pathways of the cell are involved in successful EBV infection as a result of expression of viral encoded proteins such the nuclear antigens EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C and EBNA-LP, the latent membrane proteins LMP1, LMP2 and LMP2B, two small non polyadenylated RNAs (EBERs) and the BARFO RNAs. These interactions and their roles in transformation are discussed in detail in other chapters of this volume. Although direct connections with events initiated at entry remain to be confirmed, these interactions demonstrate how viral encoded factors modify the cell to support virus latency and reactivation.

5.2.4. EBV entry into epithelial cells

Studies of EBV infection of epithelial cells have been limited because the virus does not readily infect these cells *in vitro*. A common property of epithelial cells is polar distribution of the proteins and lipids in the plasma membrane to create two distinct surface domains. Viruses may be restricted in entry to one surface domain thereby limiting the sites susceptible to infection. Since the EBV genome can be found in a variety of non lymphoid cancers, primarily epithelial and, most notably, undifferentiated nasopharyngeal carcinoma, at some frequency this virus infects non lymphoid cell types *in vivo*.

Little is known about EBV entry into epithelium. Attachment and penetration occur through interactions with as of yet unidentified receptors. Although CD21 RNA is detected in epithelial cells, including nasopharyngeal carcinoma cells, CD21 has not yet been shown to be the EBV receptor of epithelial cells. In studies using gastric carcinoma cells, it has been shown that CD21 negative cell lines could be infected with EBV (122). This implies occurrence of CD21-independent virus entry into the gastric epithelium. This observation can be extended to other epithelial cells of different tissue origin, which are efficiently infected by cell-to-cell contact. In these cells, expression of EBV genes in virus infected epithelial cells is exclusively restricted to expression of EBNA1, EBERs, LMP2A and BARF0 (123).

These results suggest that an unidentified epithelium-specific binding receptor(s) distinct from CD21 can mediate viral infection. HLA class II molecules are typically restricted to immune cells such as B cells, dendritic cells, activated T cells, macrophages and the thymic epithelial cells. This might seem to argue for other types of receptors for EBV entry into epithelial cells.

It has been shown that low levels of CD21 expression are sufficient for EBV infection. Fingeroth *et al.*(1999) used a human embryonic kidney cell line (293) persistently infected with EBV that had been genetically altered to carry a G418 resistance gene to explore whether EBV could stably infect cell lines of epithelial origin (124).

Other studies have shown that the epithelial polymeric immunoglobulin receptor (pIgR) can mediate in vitro CD21-independent EBV entry (125). Madin-Darby canine kidney (MDCK) cells expressing the IgR have been used to provide evidence that epithelial cell polarity is a key determinant in the infectious outcome of immunoglobulin A (IgA) mediated entry of EBV (126). Further studies were performed using MDCK cell stable transfected with the EBV receptor CD21 (127). The results showed preferential direction of viral entry and release in polarized cells. This seems to support the predictions that EBV trafficks between epithelial cells and lymphocytes at the time of primary infection. Such trafficking of virus among cells would be a critical and an expected link between initial exposure in the host of epithelial cells to EBV and subsequent tropism and long-term effects from infection of B-cells.

5. PERSPECTIVES

Entry through attachment, penetration and uncoating transports viral genetic material to the appropriate site in an infected host cell (Figure 1). It is the beginning of critical events that are carried out by diverse strategies for DNA viruses. Although mechanisms may vary, some of the strategies and outcomes are similar.

Functions of receptors, identity of other cell proteins involved, their interactions with viral components and their cooperation or independence are areas for intense investigations. Common themes that appear among the DNA viruses include engagement of multiple receptors, use of cell surface molecules that are prominent and, in most cases, conserved on cells, and interactions with proteins that can alter morphology of the cytoskeleton or modulate intracellular signaling for gene expression.

Figure 3 provides a model that includes currently identified entry receptors for HSV as one example of how multiple receptors might be used. The hypothesis proposes that several key receptor interactions are critical. For convenience of discussion, these are described as receptors for initial binding (IR), stable binding (SR), fusion initiation (FR) and capsid transport (CT) across the cytoskeleton eventually to a site of uncoating for viral gene expression. For HSV, we propose that any one of several receptor molecules, e.g. the gD receptors HveA, B, C or 3-

OST, may function in the same capacity (e.g. stable binding) as long as this interaction can cooperate with the cellular receptors available for the next event.

A question that arises with identification of receptors for HSV entry has been why any one of several human proteins that bind to gD can mediate entry into CHO or porcine cells. This might be explained if, in the presence of a human protein receptor, cooperation can occur with animal homologues of human HSV receptors naturally found on poorly susceptible cells. In the absence of a human protein receptor as the normal status of these animal cells, interaction of HSV proteins with a receptor homologue are not sufficient to trigger required changes in the virus needed for entry. Although many aspects remain to be investigated, this hypothesis that involves multiple binding events is consistent with current information for HSV and with that available for adenoviruses and EBV. Entry by this means is a logical process by which virus can remain stable in the extracellular environment, yet mediate change at the appropriate time and place to transport its capsid across the hydrophobic plasma or vesicle membrane.

Current and future research goals are to decipher the entry process for as many viruses as possible. How does engagement of viral proteins and cell receptor proteins cause conformational changes that allow membrane transit and eventual uncoating of the viral genome? Understanding mechanisms will lead to identification of specific sites for broad spectrum anti-viral therapeutics and will impact use and design of more efficient virus vectors to deliver foreign genes to cells.

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