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MINIREVIEW

HIV-1 Gag Proteins: Diverse Functions in the Virus Life Cycle

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The Gag proteins of HIV-1, like those of other retroviruses, are necessary and sufficient for the assembly of virus-like particles. The roles played by HIV-1 Gag proteins during the life cycle are numerous and complex, involving not only assembly but also virion maturation after particle release and early postentry steps in virus replication. As the individual Gag domains carry out their diverse functions, they must engage in interactions with themselves, other Gag proteins, other viral proteins, lipid, nucleic acid (DNA and RNA), and host cell proteins. This review briefly summarizes our current understanding of how HIV-1 Gag proteins function in the virus life cycle.

INTRODUCTION

The HIV-1 Gag proteins are initially synthesized as a polyprotein precursor which is sufficient to produce noninfectious, virus-like particles in the absence of other viral proteins or packageable viral RNA (for reviews, see Wills and Craven, 1991; Hunter, 1994; Swanstrom and Wills, 1997; Vogt, 1997). In the case of HIV-1, this Gag precursor has been named, based on its apparent molecular weight, $Pr55^{Gag}$. During or shortly after virus budding from the host cell, the HIV-1 protease (PR) cleaves $Pr55^{Gag}$ into the mature Gag proteins p17 matrix (MA), p24 capsid (CA), p7 nucleocapsid (NC), and p6 (Fig. 1A). The proteolytic processing of Gag induces a major transformation in virion structure: MA remains associated with the inner face of the viral membrane, whereas CA condenses to form a shell around the viral RNA/NC complex. This rearrangement, known as maturation, produces a morphological transition to a particle with a conical core characteristic of an infectious virion (Fig. 1B). The general organization of the major Gag proteins (MA, CA, and NC) in the Gag precursor and within the virion is highly conserved among retroviruses (Wills and Craven, 1991; Hunter, 1994; Swanstrom and Wills, 1997; Vogt, 1997). Cleavage of $Pr55^{Gag}$ also generates two small spacer peptides, p1 and p2 (Fig. 1A). Although precise functions for these domains have not been established, their presence in $Pr55^{Gag}$ appears to regulate rates of cleavage at the individual PR recognition sites and thereby influences the ordering of processing and virion morphogenesis (Pettit *et al.,* 1994; Krausslich *et al.,* 1995; Wiegers *et al.,* 1998).

Retroviral Gag proteins face a major challenge in carrying out their functions. During virus assembly, the Gag proteins must assemble into higher-order, multimeric structures that are directed outward to the plasma membrane; in contrast, following virus entry, the same proteins must disassemble or be directed inward toward the nucleus. To some extent, this problem has been solved by a strategy in which the Gag precursor carries out assembly and membrane targeting functions while the mature proteins, produced after virion release from the cell, perform opposing functions of uncoating and disassembly. In theory, differing functions for Gag domains in the precursor versus the mature Gag proteins could have been achieved in several ways: (1) Gag domains could undergo major conformational (and thus functional) changes following PR processing of the Gag precursor, (2) functional domains in Gag could span cleavage sites such that they are altered by PR cleavage, (3) Gag proteins could undergo posttranslational modifications which alter their function following virion release from the cell, or (4) Gag proteins could associate with host cell factors during virus entry which direct the viral proteins along pathways not followed during assembly.

The HIV-1 life cycle can be considered as a sequence of steps, depicted schematically in Fig. 2, which are regulated by both viral and cellular proteins. Although the precise ordering of many of these steps has not been definitively determined and multiple steps are likely to occur in a concerted, simultaneous fashion, the life cycle can be divided into the following series of events: (1) The infection

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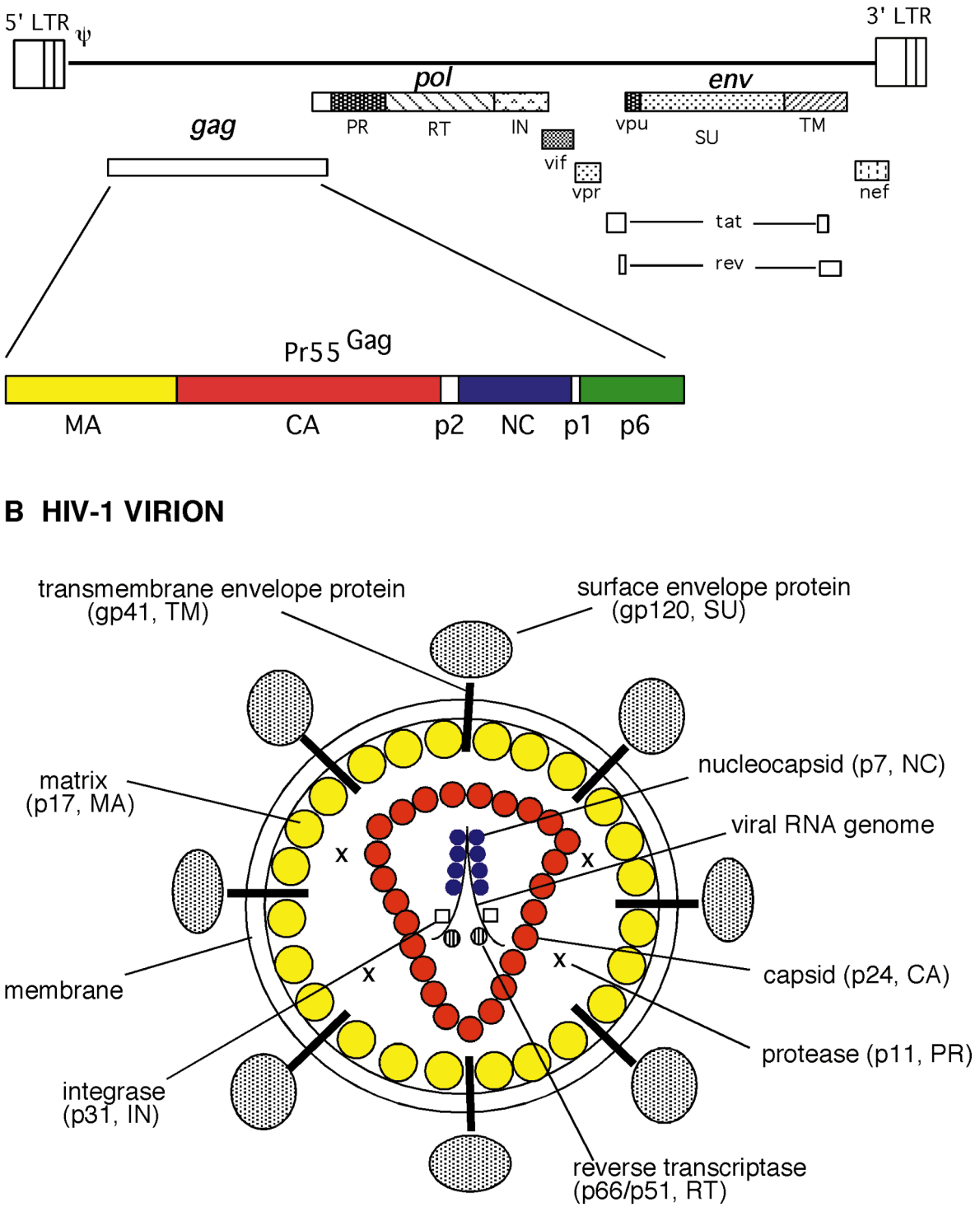

FIG. 1. Schematic representation of the HIV-1 genome and virion organization. (A) The location of the HIV-1 open reading frames is indicated. The RNA encapsidation signal, ψ, is shown near the 5′ end of the genome. The *gag* open reading frame is enlarged. (B) The HIV-1 virion, indicating the approximate location of Gag proteins, the Env glycoproteins, and the *pol*-encoded enzymes IN, RT, and PR. The location of $p6^{Gag}$ in the virions has not been definitively determined. Colors in the virion correspond to the location of the proteins in the Gag precursor. Details are provided in the text.

process begins when the surface (SU) envelope (Env) glycoprotein gp120 binds CD4 and interacts with coreceptor. (2) A membrane fusion reaction, induced by the transmembrane (TM) Env glycoprotein gp41, occurs between the lipid bilayer of the virion and the host cell plasma membrane, releasing the viral core into the cytoplasm. (3) A series of

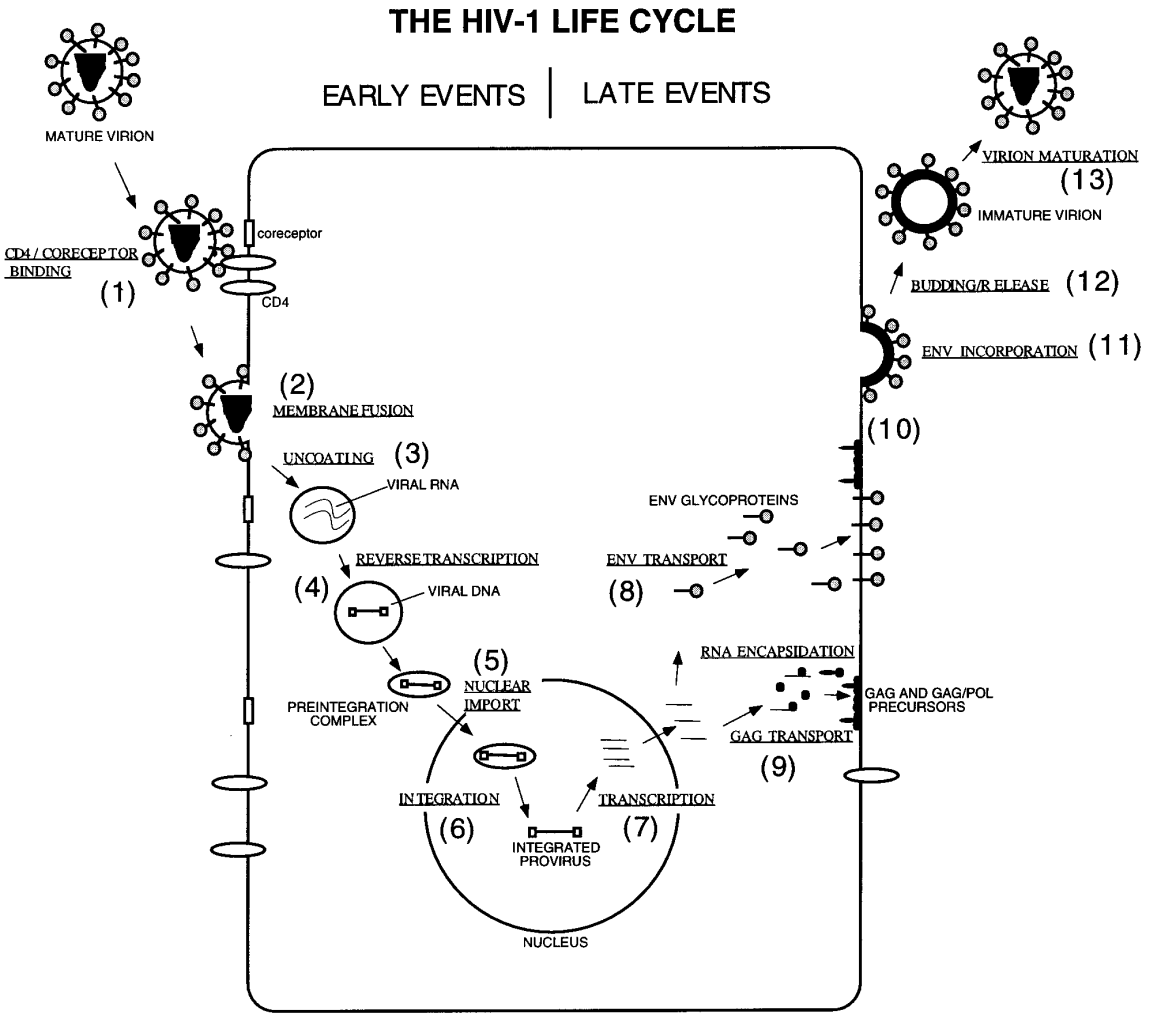

FIG. 2. The HIV-1 life cycle. Major steps in virus replication are indicated in a highly schematic form. The life cycle is subdivided into two stages: early and late. Details are provided in the text.

ill-defined steps, referred to collectively as uncoating, takes place. During this process, CA is lost, while at least some MA, as well as NC, the pol-encoded enzymes integrase (IN) and reverse transcriptase (RT), and the viral protein R (Vpr) are thought to be retained as part of a high-molecular-weight complex. (4) During uncoating, reverse transcription of the viral RNA to generate a double-stranded DNA copy is largely completed. (5) The high-molecular-weight complex, now referred to as the preintegration complex is transported across the nuclear membrane. (6) In the nucleus, integration of the viral DNA into the host cell chromosome is catalyzed by IN. (7) The integrated viral DNA, known as the provirus, serves as the template for the synthesis of viral RNAs, which are transported to the cytoplasm. (8) The Env glycoproteins are synthesized in the ER and are transported to the plasma membrane via the secretory pathway. (9) The Gag and Gag-pol polyprotein precursors are synthesized and transported by an unknown mechanism to the plasma membrane. During or after transport, the Gag precursor recruits two copies of the single-stranded viral RNA genome, interacts with the Gag-pol precursor, and assembles into structures visible by electron microscopy as dense patches lining the inner face of the plasma membrane. (10) The assembled Gag protein complex induces membrane curvature, leading to the formation of a bud. (11) During budding, the viral Env glycoproteins are incorporated into the nascent particles. (12) Budding is completed as the particle pinches off from the plasma membrane. And (13) during or immediately after budding, the viral PR cleaves the Gag and Gag-pol polyprotein precursors to the mature Gag and Pol proteins. PR cleavage leads to core condensation and the generation of a mature, infectious virion which is now capable of initiating a new round of infection.

MATRIX (MA)

MA, which forms the N-terminal domain of the $Pr55^{Gag}$ precursor, has been implicated in the targeting of Gag to the plasma membrane, Env glycoprotein incorporation

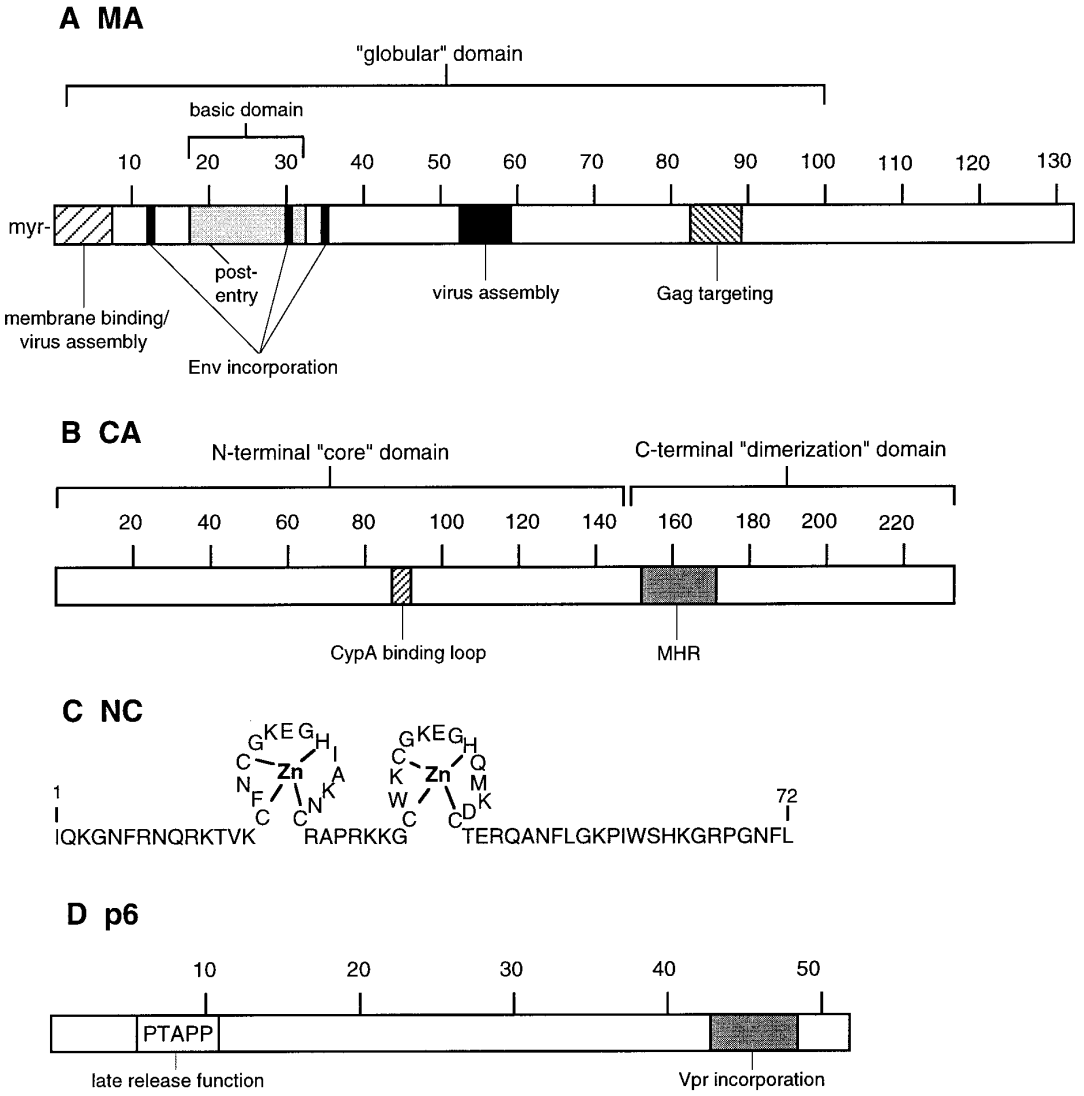

FIG. 3. Linear representation of the HIV-1 Gag genes, with some major domains indicated. Amino acid positions are shown over each diagram. (A) MA. Domains in which point mutations affect virus assembly, membrane binding, Gag targeting, postentry steps, and Env incorporation are shown. The region of MA which forms the main globular domain is indicated; the C terminus of MA projects away from the plane of the membrane. The position of the highly basic domain near the N terminus is shown. (B) CA. The two major domains visualized by structural analyses, the N-terminal "core" and C-terminal "dimerization" domains are indicated. The CypA binding loop and MHR are shown. (C) NC. The amino acid sequence is indicated and the two zinc-finger domains are shown. (D) p6. The two major currently recognized functional domains are shown: the N-terminal region involved in virus release and the C-terminal Vpr-interacting sequence. See text for additional details.

into virions, and early postentry events. The structure of the HIV-1 MA protein has been determined by both NMR spectroscopy and X-ray crystallography (Massiah *et al.,* 1994; Hill *et al.,* 1996; Conte and Matthews, 1998). The protein folds into a compact core domain, consisting largely of α-helices and a three-stranded β-sheet (Fig. 3A). The C-terminal ~30 residues of MA form an α-helix which may serve to connect MA and CA domains in $Pr55^{Gag}$. In the crystal structure, both HIV-1 and SIV MA are trimeric (Rao *et al.,* 1995; Hill *et al.,* 1996). The biological relevance of trimerization is currently unclear, although the formation of higher-order MA structures would have implications for virus assembly and Env glycoprotein incorporation into virions.

Membrane binding and Gag targeting

It is clear that a major function of the HIV-1 MA protein is to direct binding to, and assembly at, the plasma membrane. It was initially reported that mutation of the N-terminal Gly residue, which serves as the site of myristic acid attachment, blocked virus assembly and impaired binding of Gag to membrane (Gheysen *et al.,* 1989; Gottlinger *et al.,* 1989; Bryant and Ratner, 1990; Pal *et al.,* 1990; Freed *et al.,* 1994). Subsequently, it was observed that single amino acid changes near the N terminus of MA and in the vicinity of residues 55 and 85 also caused virus assembly defects (Freed *et al.,* 1994). A highly basic domain between MA residues 17 and 31

has been implicated in membrane binding (Yuan *et al.,* 1993; Zhou *et al.,* 1994); based on structural and mutational analyses of HIV-1 MA, it was proposed that this basic domain, in conjunction with other basic residues throughout the protein, forms a positively charged patch which promotes membrane binding by interacting with acidic phospholipids on the inner face of the lipid bilayer (Yuan *et al.,* 1993; Massiah *et al.,* 1994; Zhou *et al.,* 1994; Hill *et al.,* 1996). Interestingly, the structures of other retroviral MA proteins suggest that they too may interact with membrane by exposing a cluster of basic residues to the inner face of the lipid bilayer (Christensen *et al.,* 1996; Matthews *et al.,* 1996; Conte *et al.,* 1997; McDonnell *et al.,* 1998). The domains of retroviral MA proteins involved in membrane binding have been referred to as "M" domains (Parent *et al.,* 1995). Mutation of HIV-1 MA residue 6 impaired virus assembly and Gag membrane binding without affecting myristylation (Freed *et al.,* 1994; Ono *et al.,* 1997; Ono and Freed, unpublished results); analysis of a viral revertant of this mutant revealed that substitutions in the middle of MA or near its C terminus could reverse the virus assembly defect (Ono *et al.,* 1997). Thus it is apparent that binding of MA to membrane involves, directly or indirectly, at least several domains throughout the protein. The observation that MA itself binds membrane less efficiently than $Pr55^{Gag}$ (Zhou and Resh, 1996; Spearman *et al.,* 1997), together with the finding that deletions in the C terminus of MA (Zhou and Resh, 1996) or throughout the protein (Spearman *et al.,* 1997) enhance membrane binding, led to the proposal that in the Gag precursor the myristate moiety is highly exposed and thus free to interact with membrane, whereas in the mature MA protein, the N terminus is partially buried. This so-called "myristyl switch" model of membrane binding, which has been proposed in other systems (McLaughlin and Aderam, 1995), could help explain how a subset of MA protein might dissociate from the lipid bilayer and associate with the viral core and/or preintegration complex in the early steps of the virus life cycle (see below). It has been suggested that MA binding to membrane might be modulated by phosphorylation (Gallay *et al.,* 1995a,b; Bukrinskaya *et al.,* 1996). According to this model, cellular kinases, which are specifically incorporated into virions, induce phosphorylation of MA, thereby conferring a negative charge sufficient to induce release from the lipid bilayer. At this time, a biological role for MA phosphorylation needs to be assessed further, particularly in light of the observed equivalent degree of phosphorylation of MA which is bound or not bound to membrane (Spearman *et al.,* 1997).

Although progress has been made in delineating the domains of MA which promote binding of Gag to membrane, the mechanism by which the plasma membrane is specifically targeted, in preference to intracellular membranes, is unclear. Several lines of evidence suggest that MA plays an important role in this process. Membrane binding and virus assembly can still be achieved if substantial portions of MA are deleted; however, in many cases the proper targeting of assembly is disrupted. For example, deletion of a large region of MA retargeted virus assembly to the ER (Facke *et al.,* 1993; Gallina *et al.,* 1994) and replacement of the entire MA sequence with a heterologous membrane binding domain resulted in promiscuous assembly at both plasma and intracellular membranes (Reil *et al.,* 1998). Intriguingly, single amino acid changes between MA residues 84 and 88 redirect virus assembly to an intracellular compartment, apparently the Golgi apparatus or Golgi-derived vesicles (Freed *et al.,* 1994; Ono and Freed, unpublished). These point mutations may act by specifically disrupting a plasma-membrane targeting signal or by creating a novel signal that causes Gag to be retargeted. Studies conducted with the Mason–Pfizer monkey virus (M-PMV) demonstrated that mutations in MA could switch assembly from the type D (cytosolic) to a type C (plasma membrane) assembly pathway (Rhee and Hunter, 1990). Thus the targeting of virus assembly, as well as membrane binding itself, may be a common function of retroviral MA proteins.

Env glycoprotein incorporation

The Env glycoprotein complex, consisting of the surface (SU) and transmembrane (TM) glycoprotein, is incorporated into virions during virus budding from the plasma membrane (for review, see Freed and Martin, 1995a). The observation that HIV-1 virus particles which lack PR incorporate Env glycoproteins (Huang *et al.,* 1995) indicates that Env incorporation is mediated by $Pr55^{Gag}$. The close proximity of MA to the lipid bilayer has encouraged speculation that a direct interaction may take place between the cytoplasmic tail of TM and the MA domain of Gag. Support for an interaction between HIV-1 MA and the TM glycoprotein gp41 derives from studies demonstrating that deletions (Yu *et al.,* 1992b), multiple amino acid substitutions (Dorfman *et al.,* 1994b), and single residue mutations (Freed and Martin, 1995b, 1996; Ono *et al.,* 1997) in HIV-1 MA can block Env incorporation into virions. Furthermore, in HIV-1-visna Gag chimeras, the presence of the HIV-1 MA facilitated virion incorporation of HIV-1 Env (Dorfman *et al.,* 1994b). Finally, Env expression in polarized epithelial cells directs Gag release from the basolateral surface; this polarized budding is abrogated by mutations in MA and truncations in the gp41 cytoplasmic tail (Lodge *et al.,* 1994). In apparent contradiction, truncation of the entire cytoplasmic domain of gp41 does not block Env incorporation, and heterologous retroviral Env glycoproteins can be efficiently incorporated into HIV-1 virions (for review, see Freed and Martin, 1995a). This discrepancy is in part resolved by the finding that whereas single and multiple

amino acid substitution mutations in HIV-1 MA can block the incorporation of full-length HIV-1 Env into virions, these changes have no effect on the incorporation of truncated HIV-1 Env glycoproteins (Freed and Martin, 1995b, 1996; Mammano *et al.,* 1995). Thus it appears that while the incorporation of Envs with short cytoplasmic tails into HIV-1 virions is independent of MA, the incorporation of full-length Env requires a specific interaction with, or at least accommodation by, MA. Although a direct interaction between HIV-1 MA and gp41 has been detected *in vitro* (Cosson, 1996), it has been difficult to corroborate these findings in virus-expressing cells. One potential explanation for this difficulty is that Env–MA interactions *in vivo* may require that both proteins maintain their multimeric states in addition to their interactions with membrane and perhaps with host factors.

An appealing model for Env incorporation invokes an interaction between sequences within the long cytoplasmic tail of gp41 and holes present in the lattice-like MA structure formed upon MA trimerization (Hill *et al.,* 1996). In fact, the location of point mutations within MA which disrupt Env incorporation (Freed and Martin, 1995b, 1996) is consistent with this model. However, the biological relevance of MA trimerization awaits further investigation. It also remains to be determined when the putative Env–MA interaction takes place during virus assembly and whether this interaction plays a role in early events following membrane fusion.

Early postentry steps

In addition to the roles of the MA domain of $Pr55^{Gag}$ late in the virus life cycle, evidence supports a function for the mature MA protein in an early step postinfection. It has been reported that HIV-2 MA is present at low levels in the viral core (Gallay *et al.,* 1995b), and several laboratories have detected the presence of MA in the viral preintegration complex, which functions to transport the viral DNA into the nucleus (Bukrinsky *et al.,* 1993b; Gallay *et al.,* 1995a,b; Miller *et al.,* 1997). The association of MA with core structures suggests a role for MA following membrane fusion. In fact, mutations which impair virus replication without affecting assembly and release or Env incorporation have been described. It was observed that these mutations caused a defect in the amount of viral DNA synthesized postinfection (Yu *et al.,* 1992a; Reicin *et al.,* 1995, 1996; Casella *et al.,* 1997), suggesting a defect early in the life cycle. A recent study observed that specific MA mutations reduced the stability of viral DNA synthesized postinfection, implying an effect on the integrity of the high-molecular-weight complex in which reverse transcription takes place (Kiernan *et al.,* 1998). Consistent with an effect on core structure or stability, these MA mutations also reduced the efficiency of virion reverse transcription in "endogenous" RT assays (Kiernan *et al.,* 1998), which measure RT activity in gently disrupted virions using the viral RNA genome as a template. An additional phenotype of these mutants was observed: an increase in the apparent binding of Gag to membrane (Kiernan *et al.,* 1998). The increased membrane binding elicited by these mutations may cause MA to be retained in the lipid bilayer following membrane fusion, thereby ultimately leading to degradation of the reverse transcription complex. A role for MA early in the virus life cycle must be rationalized with results indicating that mutants lacking all or most of MA were still infectious in the presence of Env glycoproteins containing short tails (the MuLV Env, or truncated HIV-1 Env) (Wang *et al.,* 1993; Reil *et al.,* 1998). These results can be accommodated with current models for MA function by considering that mutations that disrupt normal roles for MA (for example, Gag membrane binding) may exert a negative effect not observed in the absence of MA. Furthermore, it must be emphasized that infectivity of MA-deleted mutants is observed only under restricted conditions: in the presence of a heterologous membrane binding domain, in specific cell types, and in the absence of the wild-type HIV-1 Env (Reil *et al.,* 1998).

In contrast to oncoretroviruses, which require that the host cell undergoes mitosis for a productive infection to be established, lentiviruses efficiently infect nondividing cells. This property has important biological consequences in allowing HIV-1 to infect terminally differentiated monocyte-derived macrophages, which constitute a major target for virus infection *in vivo* (Gartner *et al.,* 1986; Koenig *et al.,* 1986). These observations have led to the assumption that HIV-1 must encode protein(s) containing nuclear localization signals (NLSs), which serve to transport the viral preintegration complex to, and across, the nuclear membrane. Initially, it was postulated that MA provided this function: mutations in the highly basic domain of MA (discussed above in the context of membrane binding) were reported to specifically block infection of nondividing cells, and BSA conjugated with peptides derived from the highly basic domain localized to the nucleus (Bukrinsky *et al.,* 1993a). However, it was subsequently reported that defective infectivity was observed only in the context of virus isolates lacking a functional viral protein R (Vpr) gene (Heinzinger *et al.,* 1994; von Schwedler *et al.,* 1994), and a number of other studies demonstrated that basic domain mutations did not specifically block infection of nondividing cells, including primary macrophages (Freed and Martin, 1994; Freed *et al.,* 1995; Fouchier *et al.,* 1997; Nie *et al.,* 1998; Reil *et al.,* 1998). Furthermore, attempts to biochemically demonstrate the presence of an NLS in MA were unsuccessful (Fouchier *et al.,* 1997). As an extension of the model invoking MA as a major determinant of nuclear transport, it was proposed that phosphorylation of a Tyr residue located at the C terminus of MA induced the protein to dissociate from the plasma membrane and associate specifically with integrase. Mutation of this

C-terminal Tyr was reported to block infection of primary macrophages (Gallay *et al.,* 1995a); however, this result was not confirmed in other studies (Bukrinskaya *et al.,* 1996; Freed *et al.,* 1997). It is currently unclear what role MA plays in actively directing nuclear import of the viral preintegration complex in nondividing cells. MA either does not function in this regard or provides a weak signal that acts in conjunction with other viral proteins. Vpr (Heinzinger *et al.,* 1994) and integrase (Gallay *et al.,* 1997) have been implicated in this process.

CAPSID (CA)

In the mature virion, CA forms a shell surrounding the viral RNA genome and core-associated proteins. The CA domain of $Pr55^{Gag}$ plays an important role in virus assembly, and the mature CA protein functions in virion maturation and also appears to be involved in early postentry steps. The CA protein is composed of two domains: an N-terminal region (the so-called "core" domain, composed of residues 1–145), which functions in virion maturation and incorporation of the cellular protein cyclophilin A (CypA), and a C-terminal "dimerization" domain (residues 151–231), which contributes to Gag–Gag interactions (Fig. 3B). Structural data for HIV-1 CA have been obtained by both NMR spectroscopy (Gitti *et al.,* 1996) and X-ray crystallography (Gamble *et al.,* 1996; Momany *et al.,* 1996). At this time, the structure of the entire CA has not been resolved; rather, information is available separately for the two domains mentioned above. The core domain is highly helical, being composed of seven α-helices, two β-hairpins, and an exposed loop. The exposed loop serves as the binding site for CypA (Gamble *et al.,* 1996). The C-terminal CA domain is globular, also with a high helical content (Gamble *et al.,* 1997). This domain contains the only region of Gag that displays significant homology among different genera of retroviruses: the major homology region (MHR) (Wills and Craven, 1991).

Virus assembly and CypA binding

Mutational analysis of the CA domain of HIV-1 $Pr55^{Gag}$ has defined specific sequences that affect virus assembly. Specifically, mutations in the C-terminal third of CA have been reported by a number of groups to impair virus production (Jowett *et al.,* 1992; Hong and Boulanger, 1993; von Poblotzki *et al.,* 1993; Chazal *et al.,* 1994; Dorfman *et al.,* 1994a; Mammano *et al.,* 1994; Reicin *et al.,* 1995; Zhang *et al.,* 1996; Kattenbeck *et al.,* 1997). These results suggested that the C-terminal domain of CA might be involved in Gag–Gag interactions, a conclusion borne out by more recent structural analyses (Gamble *et al.,* 1997). Mutations in the HIV-1 MHR produce a broad array of phenotypes, with defects reported in virus assembly, maturation, and infectivity (Mammano *et al.,* 1994). Rather than being directly involved in forming the dimer interface, the highly conserved MHR residues form a network of hydrogen bonds in the crystal structure that stabilize the conformation of the entire domain (Gamble *et al.,* 1997). The importance of MHR residues to the overall structure of the C-terminal domain of CA helps explain the severity and diversity of phenotypes observed with MHR mutations, since these changes may induce global malfolding. *In vitro,* the mature HIV-1 CA protein forms large oligomers, consistent with its role in assembly (Ehrlich *et al.,* 1992, 1994; von Schwedler *et al.,* 1998).

CA has also been reported to be required for the incorporation of the Gag–Pol precursor into virions (Smith *et al.,* 1993; Srinivasakumar *et al.,* 1995; Huang and Martin, 1997), an event which is essential for the recruitment of RT, IN, and PR into particles. A specific region of CA required for the interaction of Gag with Gag–Pol has not been precisely mapped, perhaps because this interaction involves a series of noncontiguous domains within CA.

In a yeast two-hybrid screen for host cell proteins which interact with HIV-1 Gag, it was revealed that members of the cyclophilin family of proteins, which function in the cell as peptidyl-prolyl *cis-trans* isomerases, interact with HIV-1 Gag (Luban *et al.,* 1993). One of these family members, CypA, was found to be specifically incorporated into HIV-1 virions (Franke *et al.,* 1994b; Thali *et al.,* 1994). Mutational analysis indicated that the domain responsible for CypA incorporation resided in the vicinity of Pro 90 (Franke *et al.,* 1994b); this conclusion was confirmed by the cocrystallization of CA with CypA (Gamble *et al.,* 1996). The incorporation of CypA into HIV-1 virions can also be blocked by treatment of virus-producing cells with cyclosporine A or its nonimmunosuppressive analogs. Unfortunately, from the perspective of antiviral therapy, variants that are resistant to, or even dependent upon, the presence of these drugs rapidly emerge (Aberham *et al.,* 1996; Braaten *et al.,* 1996a). Interestingly, these resistant variants, which acquire mutations in CA in the vicinity of the CypA binding site, still bind CypA *in vitro.* Furthermore, CypA incorporation into drug-resistant virions is still disrupted by cyclosporine A (Braaten *et al.,* 1996a). Thus these mutants have not altered their ability to interact with CypA; rather, they no longer require this interaction for efficient infection. Although it is not clear what role CypA incorporation plays in the virus life cycle, CypA-deficient HIV-1 virions exhibit defects early postinfection (Thali *et al.,* 1994; Steinkasserer *et al.,* 1995; Braaten *et al.,* 1996b). In the virion, CypA packs with CA at a Gag:CypA ratio of ~10:1. The N-terminal CA domain forms continuous planar strips; it has been suggested that the intercalation of CypA in these strips might loosen CA–CA interactions, thereby facilitating uncoating after virus entry (Gamble *et al.,* 1996). This interesting hypothesis awaits confirmation.

Virion maturation and early postentry events

Unlike mutations in the C-terminal domain of CA which impair virus assembly, mutations in the N-terminal half of the protein generally do not affect the efficiency of particle production. However, virions with N-terminal CA mutations often display marked infectivity defects and aberrant core morphology by EM (Jowett *et al.,* 1992; von Poblotzki *et al.,* 1993; Wang and Barklis, 1993; Dorfman *et al.,* 1994a; Reicin *et al.,* 1995, 1996). After PR-mediated cleavage at the MA–CA junction, the N terminus of CA apparently undergoes a refolding to form a β-hairpin/helix structure that is stabilized by a salt bridge between Pro 1 of CA and an Asp residue at position 51 (von Schwedler *et al.,* 1998). This refolding creates a new CA–CA interface and may play a central role in core condensation (von Schwedler *et al.,* 1998). Consistent with this hypothesis, recombinant CA *in vitro* formed cylinders, while addition of as few as four or five C-terminal MA residues to the N terminus of CA resulted in the formation of spherical particles (Gross *et al.,* 1998; von Schwedler *et al.,* 1998). In addition, mutations which were specifically designed to disrupt the N-terminal interface markedly reduced virus infectivity and blocked normal core condensation in mutant virions (von Schwedler *et al.,* 1998). The correlation between infectivity defects and aberrant core morphology highlights the importance of proper core condensation in early events in the virus life cycle.

NUCLEOCAPSID (NC)

A distinguishing feature of HIV-1 NC proteins, both structurally and functionally, is the presence of two Cys-X_2-Cys-X_4-His-X_4-Cys (CCHC) domains reminiscent of the so-called zinc-finger motifs found in many cellular DNA binding proteins (Fig. 3C). Each of the two zinc fingers of HIV-1 NC (like those of other retroviruses) coordinates a zinc ion (Summers *et al.,* 1990, 1992; South *et al.,* 1990; Bess *et al.,* 1992; South and Summers, 1993). The existence of zinc-finger motifs in Gag NC proteins is highly conserved among retroviruses; one or two such domains are found in all retroviruses except the spumaviruses (Maurer *et al.,* 1988). In virions, NC is found in the core tightly associated with the viral RNA (Meric *et al.,* 1984). NMR analysis of HIV-1 NC indicates that the zinc-finger domains are located in a central globular domain, while the N and C termini of the protein are relatively disordered (Morellet *et al.,* 1992; South and Summers, 1993). The first finger is flanked on either side by highly basic sequences. The NC domains of retroviral Gag proteins were recognized early on as playing a role in RNA binding and encapsidation; however, like the other Gag domains, a variety of additional aspects of the virus life cycle are reportedly influenced by HIV-1 NC. These include RNA dimerization, Gag–Gag interactions, membrane binding, reverse transcription (tRNA incorporation and annealing to the primer binding site and strand transfer), and stability of the preintegration complex.

RNA encapsidation

During virus assembly, HIV-1 encapsidates two copies of the viral RNA genome per virion. Since PR-defective HIV-1 virions contain viral RNA (Fu *et al.,* 1994), encapsidation evidently involves contacts between the $Pr55^{Gag}$ precursor and viral RNA during assembly. The process of encapsidation strongly favors viral RNA over cellular RNA and full-length, unspliced viral RNA over spliced viral RNA. Mutations in the highly conserved Cys and His residues of the HIV-1 NC zinc-finger domains caused major defects in the specific encapsidation of full-length viral RNA and *in vitro* RNA binding activity (Aldovini and Young, 1990; Gorelick *et al.,* 1990; Dorfman *et al.,* 1993; Zhang and Barklis, 1995, 1997; Schwartz *et al.,* 1997). In some cases, these mutations reduced the specificity of encapsidation, resulting in an increased ratio of spliced to unspliced RNA in virus particles (Zhang and Barklis, 1995; Schwartz *et al.,* 1997). Mutations in basic residues flanking the zinc fingers also reduced RNA binding *in vitro* (Schmalzbauer *et al.,* 1996) and RNA encapsidation into virions (Poon *et al.,* 1996). Retroviral Gag proteins exhibit an additional level of specificity in that they encapsidate their cognate RNA genome more efficiently than the genomes of other retroviruses. This specificity, at least in some instances, is conferred by NC (Dupraz and Spahr, 1992; Berkowitz *et al.,* 1995; Zhang and Barklis, 1995).

The primary domain in the viral RNA that is responsible for specific RNA packaging, known as the ψ site, is located between the 5′ long terminal repeat and the beginning of the *gag* open reading frame (Aldovini and Young, 1990; Clever *et al.,* 1995; McBride and Panganiban, 1996; Fig. 1A). In HIV-1, this domain has been predicted to fold into four stem-loops (SL1–SL4) with SL3 being particularly well conserved among virus isolates (Clever *et al.,* 1995). Although NC binds single-stranded RNA relatively nonspecifically, it displays a strong preference for RNAs containing the ψ site; biochemical analyses using Gag proteins expressed *in vitro* confirmed the importance and specificity of HIV-1 NC in Gag–RNA interactions (Luban and Goff, 1991; Berkowitz *et al.,* 1993; Berkowitz and Goff, 1994; Dannull *et al.,* 1994; Clever *et al.,* 1995; Geigenmuller and Linial, 1996). Recently, the structure of HIV-1 NC in a complex with the SL3 region of HIV-1 ψ has been determined by NMR methods (De Guzman *et al.,* 1998). This structure provides some insights into the specificity of NC–RNA interactions: NC basic residues at positions 3 and 10 interact with the RNA major groove, while the zinc fingers hydrogen bond with exposed guanosines on the SL3 loop. The high-affinity interaction between NC and SL3 is apparently assisted by extensive

intramolecular interactions between the NC zinc-finger domains (De Guzman *et al.,* 1998).

HIV-1 NC has been reported to facilitate viral RNA dimerization (Darlix *et al.,* 1990; De Rocquigny *et al.,* 1992; Sakaguchi *et al.,* 1993) and to mediate the conversion of the dimeric RNA genome to a more stable form, a process known as "RNA maturation" (Feng *et al.,* 1996). This latter observation suggests that NC acts as an RNA chaperone (Herschlag, 1995) in that it catalyzes the conversion of RNA to a thermodynamically stable conformation.

Virus assembly

During the course of studies aimed at characterizing the role of NC in Gag function, it was observed that many HIV-1 NC mutations caused defects in virus release, suggesting that this domain of $Pr55^{Gag}$ plays a role in virus assembly (Dorfman *et al.,* 1993; Hong and Boulanger, 1993). Studies which analyzed HIV-1 Gag mutants containing C-terminal deletions indicated that those truncations which removed NC sequences abrogated efficient assembly and release. The N-terminal basic domain of NC, rather than the zinc fingers, appeared to be particularly important in this regard (Gheysen *et al.,* 1989; Hoshikawa *et al.,* 1991; Jowett *et al.,* 1992). Furthermore, NC appears to play a role in the tight packing of Gag in virions; deletions in NC led to the production of virions with a lighter-than-normal density (Bennett *et al.,* 1993). Interestingly, Rous sarcoma virus (RSV) mutants which produced low-density particles could be rescued to produce high-density particles by fusing small portions of NC from heterologous retroviruses, including HIV-1, to the RSV Gag (Bennett *et al.,* 1993). Again, the highly basic sequences rather than the zinc fingers appeared to be particularly important in the formation of high-density particles (Bennett *et al.,* 1993). Studies analyzing Gag–Gag interactions using yeast two-hybrid analysis supported a role for NC in mediating Gag multimerization (Franke *et al.,* 1994a), and HIV-1 CA–NC fusion proteins assembled into cylinders *in vitro* more efficiently than CA alone (Campbell and Vogt, 1995). Finally, it was recently demonstrated that a heterologous domain known to mediate protein–protein interactions could substitute for NC in the assembly of virus particles (Zhang *et al.,* 1998). The role of regions within retroviral NC proteins in mediating Gag contacts has led to their designation as interaction or "I" domains (Parent *et al.,* 1995). These results, together with the data discussed above implicating CA in virus assembly and Gag multimerization, argue that assembly of authentic HIV-1 particles involves cooperative interactions between multiple domains within $Pr55^{Gag}$.

It is currently not clear what role RNA plays in facilitating virus assembly. The importance of NC basic residues, which promote NC–RNA interactions (De Rocquigny *et al.,* 1992), suggests that RNA may form a scaffold along which Gag molecules align and pack. The contribution of RNA to Gag multimerization is supported by *in vitro* assembly studies in which treatment of purified CA–NC proteins with RNase abolished assembly (Campbell and Vogt, 1995). Reduced RNA encapsidation resulting from NC basic residue mutations correlated with defective core maturation, suggesting that NC–RNA interactions may also be required for proper core morphogenesis following PR-mediated Gag cleavage (Poon *et al.,* 1996).

It has been suggested that NC sequences are important for efficient binding of HIV-1 Gag to membrane and for specific Gag targeting to the plasma membrane. In a cell-free system, *in vitro* synthesized $Pr55^{Gag}$ bound membrane efficiently, whereas C-terminally truncated molecules lacking NC sequences were reduced in their membrane-binding ability (Platt and Haffar, 1994). More recently, Gag truncation mutations that removed a region near the N terminus of NC were reported to reduce plasma membrane targeting of $Pr55^{Gag}$ in a mammalian cell expression system (Sandefur *et al.,* 1998). Since the domain responsible for this phenomenon is also required for the production of dense particles, the role of NC in membrane binding may be indirect and result from effects on Gag–Gag interactions. Gag multimerization may optimize efficient membrane binding, a hypothesis supported by the analysis of high-molecular-weight complexes which form in the absence of efficient membrane binding (Lee and Yu, 1998). Alternatively, sequences in NC may influence the conformation of the N terminus of Gag (i.e., MA), thereby enhancing membrane binding and perhaps uncovering a plasma membrane targeting signal.

Early postentry steps and reverse transcription

It is well established that the NC domain of $Pr55^{Gag}$ plays a role in RNA encapsidation and virus assembly. However, NC mutations can block virus infectivity without affecting particle assembly and release or the levels of full-length genomic RNA in virions. Consistent with a role for the mature NC protein early in the virus life cycle, the nucleic acid annealing properties of NC assist in several steps required for efficient reverse transcription. The primer for HIV-1 reverse transcription is a $tRNA^{Lys}$ (Jiang *et al.,* 1993) that is specifically incorporated into virions during assembly and binds to a sequence known as the primer binding site (PBS) located near the N terminus of the genome. NC stimulates the binding of $tRNA^{Lys}$ to the PBS, specific initiation of reverse transcription from the bound $tRNA^{Lys}$, and efficient strand transfer during reverse transcription (De Rocquigny *et al.,* 1992; Peliska *et al.,* 1994; Tsuchihashi and Brown, 1994; You and McHenry, 1994; Darlix *et al.,* 1995; Rodriguez-Rodriguez *et al.,* 1995; Li *et al.,* 1996; Guo *et al.,* 1997). In addition, the selective incorporation of $tRNA^{Lys}$ was re-

portedly blocked by mutations in HIV-1 NC. Since $Pr160^{Gag\text{-}pol}$, rather than $Pr55^{Gag}$, influences the specificity of tRNA incorporation (Mak *et al.,* 1994), it was proposed that the mutations affected binding of $tRNA^{Lys}$ to a $Pr55^{Gag}/Pr160^{Gag\text{-}pol}$ complex (Huang *et al.,* 1997). Detailed analyses of the basic residues in HIV-1 NC revealed that some mutations reduced infectivity to a much greater extent than RNA encapsidation (Ottmann *et al.,* 1995; Poon *et al.,* 1996; Berthoux *et al.,* 1997), and in murine leukemia virus (MuLV), changing zinc-coordinating residues from Cys to His or His to Cys in the (single) CCHC zinc finger led to the production of noninfectious virions containing the full complement of viral RNA. These virions were blocked at an early step in reverse transcription (Gorelick *et al.,* 1996). NC may also function in stabilizing viral DNA early postsynthesis; HIV-1 NC can protect viral DNA fragments from nuclease digestion *in vitro* (Lapadat-Tapolsky *et al.,* 1993), and NC mutations were described that apparently caused degradation of viral DNA synthesized postinfection (Berthoux *et al.,* 1997).

Antivirals

Several considerations have made NC an attractive target for the design of anti-HIV compounds: (i) it functions both early and late in the virus life cycle, (ii) it is highly conserved among virus isolates (Myers *et al.,* 1993), and (iii) the CCHC type of zinc-finger motif that is critical for retroviral NC function is relatively rare among cellular proteins, which typically contain CCHH or CCCC motifs (Berg and Shi, 1996). This latter observation raises the possibility that compounds could be developed which specifically target NC zinc fingers without blocking the function of their cellular counterparts. A number of compounds were shown to react with retroviral zinc fingers, causing zinc to be ejected and NC function to be destroyed (Rice *et al.,* 1995; Tummino *et al.,* 1996; Turpin *et al.,* 1996). This inactivation is irreversible due to the formation of inter-and intramolecular disulfide crosslinks between zinc-finger Cys residues following zinc ejection. As a result, the ability of PR to process $Pr55^{Gag}$ is disrupted (Turpin *et al.,* 1996). The zinc-ejecting compounds block zinc-dependent binding of HIV-1 NC to RNAs containing the ψ site (Tummino *et al.,* 1996) and inhibit HIV-1 infectivity in culture (Rice *et al.,* 1993). A recent study analyzing the effect of zinc-ejecting compounds on MuLV replication and disease induction in mice demonstrated that one compound, Aldrithiol-2, displayed antiviral effects *in vivo* (Ott *et al.,* 1998).

p6

In addition to the MA, CA, and NC domains, whose presence is highly conserved among retroviruses, the *gag* genes of individual retrovirus genera often encode additional open reading frames whose functions have been less intensively investigated. In the case of HIV-1, a small Pro-rich protein, p6, is located at the C terminus of Gag (Fig. 1). An initial study demonstrated that truncation of p6 caused severely impaired virus release; this defect was characterized by an apparent accumulation of virus particles at the plasma membrane (Gottlinger *et al.,* 1991). Many of these particles were tethered to the membrane, suggesting a block at a very late step in budding. Several subsequent studies, mostly using Gag-only expression systems, failed to observe a role for p6 in virus release (Hoshikawa *et al.,* 1991; Royer *et al.,* 1991; Jowett *et al.,* 1992; Paxton *et al.,* 1993). However, using a full-length HIV-1 molecular clone, a requirement for p6 in virus release was confirmed (Huang *et al.,* 1995). By evaluating a number of truncation and single amino acid substitution mutants, the domain responsible for the budding defect was mapped to a Pro-Thr-Ala-Pro-Pro (PTAP) sequence near the N terminus of the protein; sequences downstream in p6 were largely dispensable for virus release (Gottlinger *et al.,* 1991; Huang *et al.,* 1995; Fig. 3D). The result that inactivation of PR partially suppressed the p6 virus release defect (Huang *et al.,* 1995) helped reconcile studies reporting a requirement for p6 in virus release with those not seeing an effect of p6 deletion, since most of the latter studies used systems in which only Gag was expressed. However, the severity of the p6 virus release defect varies between studies and appears to be at least partly dependent on the cell type in which Gag is expressed (Yu *et al.,* 1995; Schwartz *et al.,* 1996). It has also been reported that p6 mutations reduce the level of pol-derived proteins in virions; again, this defect was partially suppressed by inactivation of PR (Yu *et al.,* 1998).

A similar late release function for Gag has been described for other retroviruses, prompting these sequences to be collectively called "late" or "L" domains (Parent *et al.,* 1995). In RSV, this domain maps to a small Pro-rich sequence, p2b, located between MA and Gag p10 (Wills *et al.,* 1994; Xiang *et al.,* 1996). In the lentivirus equine infectious anemia virus (EIAV), a protein known as p9 serves this function (Puffer *et al.,* 1997). Intriguingly, HIV-1 p6 and EIAV p9 can replace the p2b function in RSV, in a manner independent of the location of the late domain in the Gag precursor (Parent *et al.,* 1995). Although it is currently not understood by what mechanism retroviral late domains function, each of these proteins contains a motif (PTAP for HIV-1 p6, PPPY for RSV p2b, and YXXL for EIAV) known to mediate cellular protein–protein interactions. It has been suggested that these domains engage in interactions with host proteins at the plasma membrane which facilitate the final step of virus release (Garnier *et al.,* 1996).

An additional role for HIV-1 p6 has been described. Truncations in p6 result in the production of virus particles lacking the Vpr protein; this function maps to a domain near the C terminus of p6 (Paxton *et al.,* 1993; Lu

et al., 1995; Kondo and Gottlinger, 1996). It is not clear where in the virion p6 is localized or whether p6/Vpr interactions persist following virus release.

CONCLUDING REMARKS

A large number of studies, many of which are discussed above, have contributed significantly to our knowledge of how retroviral Gag proteins function in virus replication. Despite this wealth of knowledge, many questions remain unanswered. For example, during virus assembly and release, it is not clear how HIV-1 Gag specifically traffics to the plasma membrane, how and where Gag multimerization is accomplished, how Gag influences the recruitment of Env glycoproteins into virions, what drives the final steps in virus release, and what rearrangements in protein conformation and interactions take place during PR-mediated virion maturation. Even more mysteries surround the early steps in the virus life cycle. These events are particularly difficult to study because of the high ratio of defective to infectious particles characteristic of retroviral preparations and the large amount of viral protein uptake into target cells even in the absence of a productive infection (Marechal *et al.,* 1998). Details of how uncoating and nuclear import take place are particularly poorly understood. It must be assumed that throughout the life cycle host cell proteins actively participate in Gag function, yet little is known about which cellular proteins are involved and what roles they might play. The answers to these and related questions await further investigation.

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