Chapter 2
HIV-1 Biology at the Protein Level

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The Benefits of HIV-1 Protein Study

To understand a biological entity, one primarily needs to understand its nature and composition. Thus, one of the first experiments carried out after identifying a new virus is to examine its protein content because that is the critical part of the virus that will lead to an understanding of its biology, open up ways for diagnosis, and guide the development of antiviral strategies. Yes, we rightly classify viruses based on their genome: DNA, RNA, double strand, single strand, plus, or minus, and these nucleic acids encode proteins as well as other genetic elements such as microRNAs which, along with proteins, regulate gene expression. Yet, it is the proteins in the virions that catalytically carry out the replication cycle, induce immune responses that can be helpful or detrimental to the host, cause pathogenic effects in the host, and provide for vaccine and drug therapeutic targets. One of the reasons why HIV-1 antivirals were produced so rapidly after the discovery of HIV/AIDS was that we already had an extensive understanding of retroviruses from data gathered from intensive protein analysis of avian, murine, equine, feline, and other viruses, which provided the basic understanding of retroviral biology, from how they replicate and their essential enzymatic reactions to the serological responses against them that are induced in the host. It is important to note that retroviruses led to the discovery of oncogenes since they can recombine with and, thus, transduce cells with cellular regulatory genes that were acquired by the virus during reverse transcription [1, 2]. In fact, before the term retroviruses was coined, most were isolated from tumors or leukemias, e.g., Rous sarcoma virus and murine leukemia virus, and placed under the umbrella term RNA tumor viruses. Thus, retroviral protein studies also greatly

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contributed to the genetics of cancer, signal transduction, differentiation, and cell cycle control [1, 2]. The previous groundwork with retroviruses also saved many lives during the early stages of the AIDS crisis by accelerating the design and production of the HIV-1-induced antibody screening test [3]. The rapid development of this test, from virus discovery in 1983 [4] to commercial test in 1985, reduced an immeasurably large number of new infections by protecting the donated blood supply and providing diagnostic screening for at-risk people to identify AIDS carriers. Additionally, biochemical understanding of the reverse transcriptase process resulted in the use of the first antiviral, AZT/zidovudine, in 1987 [5, 6] only 4 years post discovery, a remarkably rapid drug development for a new pathogen. Later, more effective antiviral therapies targeting protease [7] were approved in 1995 [8], and the early forms of the current clinical antiviral therapy, highly active antiviral therapy (HAART aka CART) [9], were developed soon after in 1996. HAART uses combinations of multiple classes of drugs that initially targeted reverse transcriptase and protease by different mechanisms to combat the mutation-driven HIV resistance to single-drug therapy. Viruses developing resistance to one class have to also have resistance to the others to replicate efficiently. Antivirals against other targets including integrase [10] and Env [11] have been developed and are now used in HAART/CART regimens. The foundation of all of these advances is based on an impressive understanding of the basic biochemistry and structure of the viral proteins.

Evolution of Retroviral Biochemistry

The biochemical study of retroviruses began during the late 1960s and evolved through the 1990s [12–16]. Please note that in this book, the term retroviruses will refer to only orthoretroviruses which include HIV-1. The spumaretroviruses/foamy viruses [17], which are similar yet have several important replication differences, are not discussed in this book. Initially, proteins were studied by rudimentary techniques. Indeed, the essential retroviral biochemistry study that uncovered the basis for the paradigm-shifting discovery of a polymerase, retroviral reverse transcriptase, that reverses the "central dogma" of molecular biology was achieved by classical biochemical enzymatic assays on extracts of partially purified murine and avian retroviral particles [18, 19]. The advent of SDS-polyacrylamide gel electrophoresis in 1965 revolutionized protein analysis, greatly improving separation and resolution by molecular mass [20]. Applying this to retroviral virions provided the first looks into its proteome. In fact, the retroviral proteins are still referred to by some by their molecular mass, e.g., p24 or gp120. Subsequent advancement in technology such as large-scale virus purification combined with immunoblotting, metabolic radiolabeling, Edman degradation sequencing, and amino acid analysis allowed for a remarkable characterization of the proteins in retroviral particles. Contrary to the ease at which nucleic acids are sequenced to deduce protein sequence today, many of the first full sequences of retroviral proteins were produced by painstaking protein sequencing methods. Several protein modifications of viral proteins

by the cell as well as the viral protease processing sites were also uncovered by these protein sequencing analyses, information that cannot be revealed by sequencing DNA. Therefore, even though amazing technical nucleic acid efforts, such as the human genome project, have provided a wealth of information, studying the proteins themselves is still vital as the virus has many tricks that it plays with its genome. So although nucleic acid-based methods reigned in biology in the late 1980s through the mid-2000s, the analysis of the proteins, the active agents in the cell, in the form of high-power mass spectrometry to carry out "proteomic" analysis, i.e., a detailed and refined analysis of each individual protein in a complex mixture, ushered in new appreciation for protein study.

The analysis of HIV-1-associated proteins has been a crucial part of studying human immunodeficiency virus type 1 from the beginning. At the time of the discovery of HIV-1 in 1983, it was fortunate that the methods and ability to analyze retroviral proteins were already in place. These consisted of what would now be considered classical biochemistry: immunoblots, immunoprecipitation, column chromatography, amino acid analysis, and degradative protein sequencing (presented in this chapter). In contrast, mass spectrometry was mostly confined to relatively small molecules. Unlike today, the rather primitive computers of the day also played a role in limiting its technical abilities. Rapidly applying biochemistry steered by the prior knowledge of retrovirology to this "new" virus that caused AIDS brought forth a fountain of basic information about HIV-1: protein makeup of the virion, protein sequence, identification and characterization of critical of enzymes, and detection of new retroviral proteins. In turn, this information along with contributions from other fields generated the AIDS test and drugs that have saved so many lives. While classical biochemistry remains a powerful set of techniques that are still invaluable to protein analysis, mass spectrometry techniques have breathtakingly evolved (presented in Part II), emerging as a dynamic, multifaceted tool that allows for highly sensitive, high-throughput analyses of proteins present in complex mixtures, approaches often placed under the banner proteomics. Yet despite these new courses of study made possible by advanced mass spectrometry, the classical approaches either by themselves or in conjunction with mass spectrometry still remain vital tools. There are experiments that mass spectrometry still cannot carry out. Thus the classical and the new are more complementary rather than redundant.

HIV-1 Virus Genome and Its Proteins: Basics

Retroviral particles are composed of proteins, RNA, and lipids [21]. While RNA, both viral genomic RNA and a host primer tRNA, and lipids are required for infectious particle formation, the viral proteins in the virion do the critical work in replication and, thus, are the most studied components of the virion. As with most RNA viruses, genome coding capacity is at a premium due to the instability of RNA; therefore, HIV-1 produces these proteins in an efficient, temporally managed fashion that is a tour de force in economy and design, using nearly all of the tricks

available in mammalian biology that provide for the parsimonious production of protein activities: protease processing, frameshifting, differential RNA splicing, regulated mRNA nuclear export, overlapping ORFs, alternative translation initiation, and internal ribosomal entry sites [22–24]. HIV-1 produces one unspliced RNA encoding multiple proteins in all three reading frames (Fig. 2.1) and six spliced RNAs. Using several approaches, these RNAs produce three polyproteins, each essential for infectivity, as well as six additional proteins that assist in HIV-1 replication. Thus, the virus-encoded proteome of HIV-1 appears to be relatively small, nine proteins (Fig. 2.1) that include polyproteins which are further processed

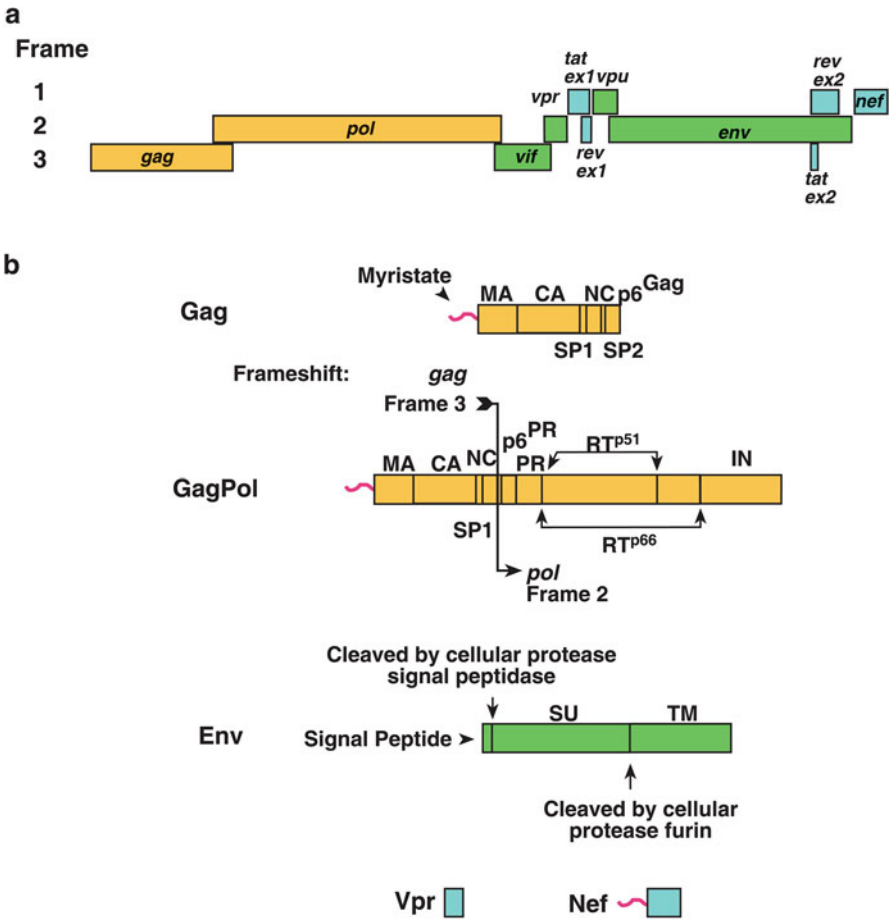

Fig. 2.1 Organization of the HIV-1 genome and HIV-1 virion proteins. (**a**) The organization of the HIV-1 genomic open reading frames is presented with proteins expressed from unspliced genomic RNA in *orange*, single-spliced RNA in *green*, and multiply spliced RNA in *blue*. (**b**) Diagram of the viral proteins incorporated into virions. Unless indicated otherwise, all internal *vertical lines* in protein diagrams denote HIV-1 protease cleavage sites. The GagPol frame shift site is denoted by an *arrow* and labeling. Color scheme for RNA splicing is as above. Myristyl modification is denoted in red

by proteases to provide for the full major complement of 22 distinct peptides from which 15 functional proteins are made [23, 24]. All of the essential structural proteins, i.e., those minimally required within an infectious particle, are produced first as polyproteins, which are cleaved into several smaller mature proteins by a coordinated process that ensures proper folding to carry out replication. These critical proteins, Gag, GagPol, and Env, are expressed, processed, and incorporated into virions by all retroviruses. In addition to the Gag, GagPol, and Env polyproteins and their mature processed forms, two proteins, Vpr and Nef, that are unique to HIV-1 and the closely related HIV-2 and simian immunodeficiency virus (SIV) are also incorporated into virions. The other four initially expressed proteins act in the infected cell, enhancing and regulating transcription/splicing [22] as well as altering cellular processes and defeating innate defenses [25, 26]. The remainder of this chapter will focus on those proteins that are found in the virion, thus readily studied by protein analysis of virus particles.

Viral Proteins in HIV-1 Virions

The most abundant protein in the virion is Gag and its mature protein products [15, 16, 27]. Gag is the only retroviral protein that is strictly required to produce "virus-like" particles; Gag drives particle formation through strong intermolecular interactions with other Gag molecules, with RNA, and with plasma membrane lipids through which the particle buds acquire a host-derived membrane envelope. During this budding process, the viral surface glycoprotein, Env, which provides for target cell binding and entry into the host cell, is also brought into the virion with the plasma membrane [15, 16, 27]. The Env precursor is incorporated into the virion at lower levels than Gag [28], cleaved in the late Golgi apparatus, forming a surface glycoprotein (SU) that is noncovalently attached to a transmembrane (TM) protein that trimerizes to form Env complexes. Env trimers on the surface of the virion bind receptors (CD4 and CXCR4 or CCR5) on host cells and induce a fusion event, which empties the infection machinery into the cell (Fig. 2.2).

Unlike the Gag proteins, only small amounts of the enzymatic proteins of the virus are needed [15, 16, 27], protease (which processes Gag and GagPol during virion maturation), reverse transcriptase (which converts the genomic RNA into viral DNA using a complex series of DNA polymerization steps), and integrase (which places the viral DNA into the host chromosomal DNA to form the provirus that expresses the viral RNAs for another round of replication). To express the small amounts of these Pol proteins required, about 5 % of the Gag translations undergo a -1 frameshift that redirects the ribosome from the *gag* gene reading frame to that of the *pol* gene (Fig. 2.1). This results in a GagPol polyprotein that can join the forming particles with Gag due to intermolecular interactions between Gag. Because both Gag and GagPol are expressed polyproteins that assemble into the virion, the correct ratio of the structural and enzymatic proteins is incorporated into the particle. Both of these polyproteins are subsequently processed by

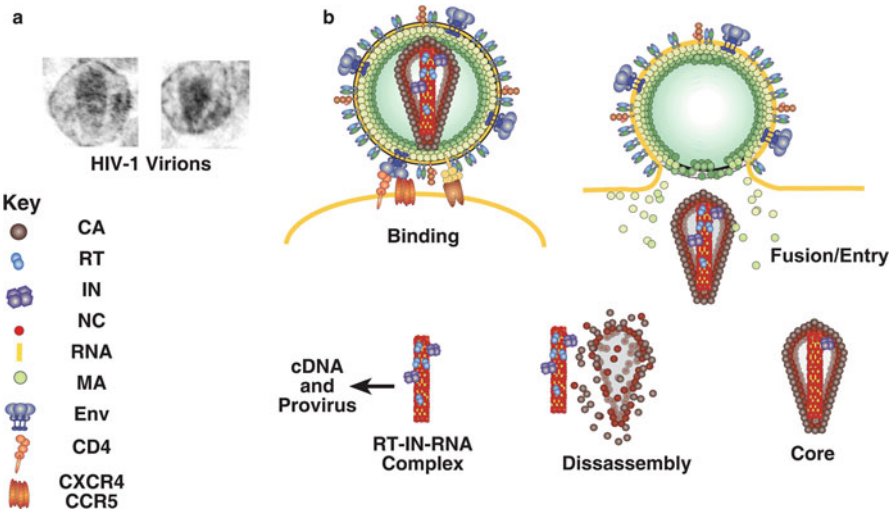

Fig. 2.2 HIV-1 entry and uncoating. (**a**) Electron micrographs of individual HIV-1 virions showing the conical core structure are presented. (**b**) A diagram of HIV-1 host binding through uncoating is presented. A color key identifies the virion proteins, and the HIV-1 receptor, CD4, and coreceptor, CXCR4/CCR5 are presented at left

protease during and after assembly [15, 16, 27]. Processing is a sequential reaction liberating the mature proteins in a coordinated fashion that results in a structural reorganization of the virion interior, forming a mature, fully infectious virion with a conical core [29].

In addition to the essential Gag, GagPol, and Env proteins, the other virally encoded proteins incorporated into virus particles, Vpr and Nef, are referred to, as accessory proteins. Despite the accessory name, these proteins, Vpr, Vif, Vpu, and Nef, are nonetheless essential for robust in vivo infection and pathogenesis [30]. They are only "nonessential" when examined in artificial cell culture-based assays that do not accurately reflect in vivo conditions. The strong selective pressure to economize the relatively low coding capacity genome of RNA viruses, especially retroviruses, ensures that all of the viral proteins are "essential" for the virus in vivo; otherwise, they would not have been maintained in the viral genome. Thus, the nonessential label arises from the artificial perspective of in vitro reductionist studies and in comparison with simpler retroviruses that express the three universal retroviral proteins, such as the prototypic avian retroviruses. Of the four proteins, Vpr, which is thought to assist nuclear entry of the PIC during infection (see below), is incorporated into virions at the greatest extent, ~ 14 % of Gag [31], as fitting for a protein that functions in the target cell. Vpr is brought into the virion through an interaction with the C-terminal region of the Gag polyprotein, sequences in $p6^{Gag}$ [32]. In contrast, Nef, which is mostly believed to act at the productively infected stage of replication, is also incorporated into the virion but in relatively small amounts, consistent with its role in virus production. Nef seems to be incorporated due to its strong binding to the plasma membrane, being a passive bystander caught

up in the budding process. The necessity of incorporating the other proteins into virions is less clear. From biochemical analyses, Tat, Rev, Vif, and Vpu are typically thought to be excluded for the virus particle. Moreover, any reported HIV-1 packaging of these proteins did not appear to be the result of a specific incorporation [33] or have any functional consequences.

HIV-1 Assembly: Why Gag Polyproteins?

Particle assembly relies on getting the right proteins in the right place with the right structure. Due to the large size of the cell and the complexity of the cytoplasm, proteins that are destined to assemble into the virus particle need to have both temporal-spatial and structural coordination. HIV-1 has eight proteins that are required for assembly of an infectious virion: MA, CA, NC, $p6^{Gag}$, PR, RT, IN, and Env, SU, and TM (these proteins are discussed below). If these many parts are expressed individually, then virion assembly would face daunting problems of specific transport, coordinated expression, and proper folding and oligomerization in the chaotic environment of the cell. Combining expression of these proteins into units that blend their properties together in a polyprotein overcomes these issues. Proteins with related roles in replication are linked together for transport so only one protein needs to find its way to the plasma membrane. Polyprotein expression ensures that these proteins are present at the proper levels and in an equal stoichiometry, something especially important for building complex structures. Finally, putting like proteins together allows them to fold coordinately, each making its own intra-subunit interaction with inter-subunit interactions being made easier by cooperative binding between all the subunits in the polyprotein. The polyprotein is an efficient strategy to solve the temporal-spatial and structural demands of assembly.

Thus, HIV-1 Gag as the primary driver of assembly contains all of the proteins, MA, CA, and NC, that provide for the structure of the virion [15, 16, 27]. These subunits, as domains in the Gag polyprotein, interact with the same subunits in other Gag molecules or cellular membranes and RNAs to make it fold into a rodlike structure that possesses both strong plasma membrane binding and oligomerization properties [29]. Upon binding of Gag to the plasma membrane, the strong multimerization properties of Gag organize into larger complexes of Gag hexamers that structurally tie themselves to neighboring hexamers to produce sheets that begin to curve, ultimately producing a spherical particle that buds from the plasma membrane [29].

The virion must have its enzymes for replication, protease, reverse transcriptase, and integrase, but the amount needed is catalytic not structural. In fact, overexpression of GagPol results in premature processing before assembly [34]. Thus, the Pol region is expressed at a much lower level (5 %) than full-length Gag [15, 16, 27]. The strategy of expressing Pol as a frameshift of Gag achieves two important functions; Pol is produced at an appropriately low level and the N-terminus of Pol is fused to Gag allowing for its incorporation into the particle. Also, because Gag and GagPol are produced for the same polysomes, they can interact and assemble together at the same place in the same time in the right amounts, solving the

temporal-spatial and stoichiometric problems. Overall the Gag and GagPol polyproteins are an ingenious strategy used by retroviruses to provide for the incorporation of the essential mature proteins at the correct ratios in the correct structures, rather than doing these functions for each mature protein independently.

Gag and GagPol are mostly assembly machines, possessing strong intermolecular interactions that are very stable [15, 16, 27]. However, infectivity requires the virion proteins to disassociate in order to carry out reverse transcription and integration. So this assembly machine needs to switch to an infection machine. To do this both Gag and GagPol subunits are processed into mature proteins by the Pol-encoded protease both during and after virus release from the cell [35]. Protease processing unleashes the poised subunit structures that, when together in the polyprotein make Gag strongly multimeric, once liberated from each other by protease go their own way forming homotypic interactions and structures that completely restructure the virion and transform it into an infection machine. The mature structure is a particle with a HIV-1 trademark conical capsid core inside the virion [29] (Fig. 2.1). This maturation process is not random as processing occurs in a coordinated fashion that regulates the formation of the different structures, performing a molecular ballet with everything occuring at its proper time. Coordination of processing is critical as alteration of the normal order of stepwise processing results in a noninfectious particle [35]. From assembly through infection, the polyprotein strategy is a sophisticated, elegant, and even beautiful dynamic process used by retroviruses to solve the logistical problems of many proteins, a small genome, and a big cell.

As a polyprotein, Env transverses the secretory system as any transmembrane protein does where it is processed in the Golgi apparatus into the Env molecule (see below). The expression of Env as a polyprotein is most likely due to the need for both subunits to assemble together. The mechanism for Env incorporation into the virion is currently not clear, though it seems to be passively incorporated [15, 16, 27].

The HIV-1 Proteins

Below are brief summaries of the proteins produced by HIV-1 with an emphasis on those incorporated into the virion. Accompanying each entry is the web address for its UNIPROT database reference which contains valuable information on function, modifications, processing, sequences (protein and data), and helpful references.

Gag (http://www.uniprot.org/uniprot/P12493)

The Gag polyprotein is produced from the unspliced full-length RNA species that serves as both an mRNA and the viral genome (reviewed in [36]). As discussed above, Gag produces all of the proteins sufficient for the production of particles and drives particle formation, though expression of Gag only produces particles, viruslike

particles or VLPs, that are not infectious [15]. The bulk of the protein within virions is Gag, each virion containing approximately 2500 copies of Gag protein [37], consistent with its role as the major structural component of the virion. Gag is cleaved by the GagPol-encoded protease, reviewed below, into six mature proteins, MA ($p15^{MA}$), CA ($p24^{CA}$), SP1 (also known as p2), NC ($p7^{NC}$), SP2 (also known as p1), and $p6^{Gag}$, in a process initiated at the start of viral assembly and completed after particle release from the cell (Fig. 2.1). The Gag proteins fall into three categories: (1) those that have a function in the mature virion and infection, MA, CA, and NC; (2) those that act in Gag only, regulating the coordination of Gag processing, SP1and SP2; and (3) $p6^{Gag}$ which interacts with cellular proteins to assist virion release from the cell [15, 16, 27]. Stepwise processing of both the Gag polyprotein and the GagPol protein (discussed below) is strictly required for infectivity of the virus particles release from the cell [35]. In addition to these major proteins, there are also minor proteins that are produced from low levels of internal cleavage of several mature Gag proteins, forming fragments of SP1, SP2, and $p6^{Gag}$ [38]. Their importance in viral biology is currently unclear. As presented above, the individual mature Gag proteins play different, yet similar roles as both subunits in Gag and protein in the mature virion. Below is a brief description of the mature Gag proteins (note: UNIPROT does not contain individual entries for the mature Gag proteins. Please use the Gag entry for them).

MA, Matrix, $p17^{MA}$

This N-terminal protein in Gag is N-terminally myristylated cotranslationally during Gag synthesis. The addition of myristate to the MA subunit in Gag is essential for Gag targeting to the plasma membrane and particle formation [15, 16, 27]. Matrix functions primarily at the assembly stage, directing the localization and membrane binding of the Gag polyprotein by the N-terminal myristyl fatty acid modification, a positive stretch of amino acids, and a phosphatidylinositol-4,5-bisphosphate [PI(4,5)P_2] binding site [39]. These features coordinately cause Gag to be strongly attached to the membrane with a hexameric arrangement of MA trimers [29]. The interactions between the different regions of Gag are complex and beyond the scope of this review. Informative reviews that detail these Gag-Gag assembly interactions and the transition of Gag in particles from its "immature" polyprotein state to its mature infections structure are available [27, 29, 40]. MA in mature virions is loosely associated with the virion membrane. It is not part of the core structure that contains the genome and the essential enzymes for reverse transcription and integration. In addition to myristylation, MA is phosphorylated near a nuclear localization site. Though this was once thought to be important for infection in nondividing cells, phosphorylation of MA does not seem to play an obvious role in replication [41]. The structural organization of the membrane-associated MA hexamers, presumably in the context of Gag, appears to limit the incorporation of Env trimers into the virion by steric hindrance: the long cytoplasmic tails of Env can only fit through the holes in the hexameric lattice made up of MA trimers, rather than a direct and positive MA-Env interaction that drives Env incorporation [42, 43].

CA, Capsid, or p24^{CA}

CA as a region in Gag provides a strong protein-protein interaction force that is critical to particle assembly. Similar to MA, the N-terminus of the CA subunit interacts to produce hexamers, while the C-terminus forms dimers, apparently both within and between Gag hexamer arrays knitting the Gag proteins together [29]. During the proteolytic processing of Gag, sequentially liberated forms of CA rearrange stepwise [35] to gradually produce a cone-shaped core that consists of ~1500 CA proteins, mostly formed as an array of about 250 hexamers with 12 pentamer contacts [29, 44]. This conical structure within HIV-1 particles contains both the dimeric RNA genome and the enzymes required for establishing an infection/provirus, reverse transcriptase, and integrase [45, 46]. Upon fusion of the viral and cellular membranes, the CA cores enter into the cell and later uncoat and disassemble some of the capsid structure, allowing reverse transcription to begin (Fig. 2.2) [46]. Proper formation of the core is critical for the production of an infectious virus [47]. The requirements for CA-CA interactions are relatively strict such that many CA mutations drastically alter the formation of the core. The impact of CA is such that small molecules that interrupt CA processing are being evaluated as anti-HIV-1 therapies [48]. Some cells appear to have an innate immunity that targets CA. There are several cellular proteins, APOBEC3G, Tetherin, and TRIM5α, collectively termed restriction factors, which inhibit HIV-1 replication [49, 50]. Of these, TRIM5α from other species, e.g., rhesus macaque, restricts HIV-1 infection by binding to CA cores. The exact mechanism for this restriction is not known, but it appears that TRIM5α binding advances the timing of core uncoating either directly or through degradation of CA by the ubiquitin proteasome pathway [51, 52], interfering with reverse transcription and integration. CA interacts with cyclophilin A in the infecting cells, and interrupting this interaction can reduce HIV-1 infectivity, but the mechanism is unclear and complicated [53]. CA is phosphorylated on several serines. Although some mutagenic data suggest somewhat that some of these sites are important in CA function, the general intolerance of CA to substitutions makes this finding only suggestive [54, 55]. Thus, the importance of this posttranslational modification is not clear.

SP1, Spacer Protein 1, p2^{Gag}

SP1, which was previously known as p2 due to its apparent molecular mass, appears to play a role in both the assembly of the spherical immature particle and the proper formation of the mature conical core. Mutations in the first four amino acids in SP1 within Gag drastically alter the formation of normal particles in the absence of protease, implying that SP1 acts as a molecular switch to provide an organizing function to immature virion assembly [56]. On the maturation side, the CA-SP1 protease cleavage site is the last to be cleaved by the HIV-1 protease in the normal sequence of

processing [35], releasing SP1 from the C-terminus of CA. Removing SP1 from CA allows it to complete the final stages of core formation [47]; thus this processing controls the timing of the CA-CA interactions that allows for the appropriate conical core formation. Mutations that interfere with the timing of cleavage, either accelerating or preventing cleavage, and small-molecule compounds that block processing at this site form misshapen cores due to the retention of the CA-SP1 structure, yielding virions that are noninfectious [35]. Thus, the primary function of SP1 is to coordinate and regulate the intricate transition of CA from a region that knits Gag together in the spherical immature particle to one that produces the highly ordered CA shell that produces the conical core. SP1 and its cleavage are the target of the core interrupting compounds [48].

Nucleocapsid, NC, $p7^{NC}$

NC is a highly basic protein that binds both RNA and DNA and which plays several different roles in HIV-1 replication [57, 58]. NC as a portion of Gag assists in assembly by binding RNA to provide a scaffold that constrains Gag to bring the monomers into close contact to promote the CA-CA and MA-membrane interactions that drive assembly [59]. The NC region of Gag also selectively binds unspliced HIV-1 RNA to package it as dimeric genomic RNA into the assembling virion [15, 16, 27, 57]. NC is present in the viral core, binding the genomic RNAs. NC also plays a role in reverse transcription, assisting the formation of the viral cDNA from the genomic RNA by acting as an RNA chaperone that alters the genomic RNAs. By melting RNA secondary structure and enhancing formation of alternate structures, NC provides valuable assistance to the complex synthetic molecular gymnastics that occur during the reverse transcription of the genomic RNA into a full-length viral cDNA [46, 60]. Even after cDNA formation, NC still plays an as yet undefined role in integration [58], the formation of a provirus in the host chromosomal DNA (see Integrase section below). NC has not been found to have significant posttranslational modifications.

$p6^{Gag}$

As the C-terminal portion of Gag, $p6^{Gag}$ plays two main roles in assembly. (1) $p6^{Gag}$ binds several cellular proteins, notably Tsg101 and ALIX, that, in turn, stimulate the release of the budding particle from the cell surface through the use of the cellular budding machinery [27, 61]. The $p6^{Gag}$ protein does not seem to have any post-assembly function and is not found in the capsid core [62]. Additionally, the $p6^{Gag}$ in the Gag polyprotein binds the viral Vpr protein causing it to be packaged into the assembling virion [32, 63]. A fraction of the mature $p6^{Gag}$ is ubiquitinated, sumoylated, or phosphorylated. The role of the small amount of ubiquitin attached

to Gag (~2 %) in the virus release remains controversial [64, 65] with some studies favoring an active role of ubiquitinated Gag in release while others support ubiquitination as a by-product, being more of a consequence of an interaction with a ubiquitinating activity as it uses the cellular release machinery [65]. Similar to ubiquitin a small amount of Gag is covalently modified with Sumo-1, though this seems to have a potentially negative effect in infection rather than assembly [66]. The $p6^{Gag}$ protein is phosphorylated on several serine and threonine amino acids; however, the importance of this modification is currently unclear [67, 68].

GagPol (http://www.uniprot.org/uniprot/P12497)

The GagPol polyprotein is produced from the same unspliced RNA as Gag by a -1 ribosomal frameshift that occurs in ~5 % of the Gag translations just after NC (reviewed in [36]). This results in a shift from the *gag* to the *pol* open reading frame, replacing the *gag*-encoded SP2 and $p6^{Gag}$ proteins with the *pol*-encoded $p6^{Pol}$ (also known as the transframe protein or preprotease), protease (PR), reverse transcriptase (RT), and integrase (IN) proteins (Fig. 2.1). GagPol is brought into the assembling virion by Gag interacting with Gag portion of GagPol. There are approximately 125 copies of GagPol proteins in the mature virion, consistent with its role providing the enzymatic component of the virion. Similar to Gag, GagPol is cleaved by the HIV-1 protease, initially with protease as a subunit with low activity that, upon cleavage, liberates highly active mature PR that then finishes GagPol processing concurrently with Gag to produce eight mature proteins, MA, CA, SP1, NC, $p6^{Pol}$, PR, RT, and IN (Fig. 2.1) [35]. Similar to Gag, the Pol portion of GagPol also undergoes sequential processing [69]. An overview of the proteins encoded by the Pol ORF is presented below.

$p6^{Pol}$, Preprotease, Transframe Protein

The $p6^{Pol}$ protein appears to function simply as a regulatory sequence for PR since mutations in this region alter the initial processing of PR from the GagPol polyprotein [70].

PR, Protease

PR is the viral aspartic protease that, acting as a dimer, processes both the Gag and GagPol polyproteins [15, 16, 27]. The sequence requirements for the PR recognition site are fairly promiscuous [71] with optimal sites generally being cleaved before those with less optimal characteristics. This property, along with steric hindrance, causes PR to process the polyproteins in an ordered fashion which, in turn,

induces the proper folding of the mature Gag and Pol proteins into the functional virion as present above [35]. PR processing might also be regulated by reversible oxidation (inactive) and reduction (active) of cysteines in PR [72]. PR is a main target for anti-HIV-1 therapies such as HAART/CART.

RT, Reverse Transcriptase

The RT enzyme provides the RNA-dependant DNA polymerase that converts the dimeric HIV-1 RNA genome into the substrate for integration: a linear cDNA structure with two long terminal repeats (LTRs) at each end [46]. RT is a heterodimer of a p66 subunit and a p51 subunit, the latter produced by a differential cleavage of p66 (Fig. 2.1). The reverse transcription of the genomic RNA carried out by RT involves a complex process that generates a full-length cDNA with long terminal repeats, LTRs, on each end from the mRNA-like structure of the genomic RNA [46]. RT is found associated with NC and the genomic RNAs in the virion core structure (Fig. 2.2) [27]. After reverse transcription, the remnants of the core organize into the preintegration complex, also referred to as a PIC which contains the viral cDNA genome, IN, and Vpr, that carries out the final infection step, integration of the provirus into the host genome [73].

IN, Integrase

The IN protein is found in the core as a tetramer (Fig. 2.2). The IN binds to the LTR ends and integrates the viral cDNA into the host genomic DNA through a mechanism of staggered cuts, ligating one strand of the cDNA to the chromosomal DNA and relying on cellular DNA repair to ligate the second strand [45]. Integration also relies on cellular proteins to allow the preintegration complex to bind to and access the chromatin structure to produce the provirus stage of the replication cycle [45, 73]. In nondividing cells, the IN within the preintegration complex can interact with several cellular proteins allowing it to transverse the nuclear pore and enter the nucleus to access the host chromosomal DNA [45, 73].

Env (http://www.uniprot.org/uniprot/P03377)

The Envelope protein (Env) is the surface glycoprotein protein complex which resides on the surface of HIV-1 virions that allows the core to enter the host cell (Fig. 2.2) and begin the infection process [42, 74]. The Env glycoprotein complex consists of a trimer of $gp120^{SU}$ (SU)/$gp41^{TM}$ (TM) dimers (Fig. 2.2). Env is translated as a gp160 complex that is processed in the Golgi compartment by furin or a furin-like protease

(Fig. 2.2) [42]. The trimeric SU/TM complex, referred to as Env, binds CD4 on HIV-1's target cells which induces a conformational change that opens up a coreceptor-binding site in SU that, in turn, allows Env to bind a host surface protein, a chemokine receptor CXCR4 or CCR5 [42, 74]. Coreceptor binding exposes the fusogenic regions of the TM protein that fuses the virion membrane with that of the target cell, spilling out the contents of the virion allowing the HIV-1 core to enter and start the infection process [42, 74]. SU is heavily glycosylated, a feature that shields it from host antibodies [15, 74]. TM is also glycosylated, though less so than SU, and has two hydrophobic alpha helices arranged in helical bundles, one in the ectodomain, located in the extracellular space that interacts with SU, and the other in the membrane-spanning region [42, 74]. Upon coreceptor activation, the helices in the ectodomain penetrate the target cell plasma membrane. TM draws the two sets of helices together causing virion and target cell membrane fusion [42, 74, 75]. Slightly less than half of TM is a cytoplasmic tail which has several cellular protein binding functions [76] and is palmitoylated at two sites [77], though their significance is not clear.

Accessory Proteins

In addition to the standard Gag, Pol, and Env proteins present in all orthoretroviruses, HIV-1 has several proteins typically described as accessory proteins that, while not strictly required for replication in vitro, are nevertheless required for efficient transmission, replication, and pathogenesis in vivo [25]. Since the focus of this book is the proteomic analysis of HIV-1 virions, only the accessory proteins significantly incorporated into virions, Vpr and Nef, will be discussed at any length. However, Vif (http://www.uniprot.org/uniprot/P12504) and Vpu (http://www.uniprot.org/uniprot/P05923) play important roles in HIV-1 biology, especially in counteracting intrinsic host antiviral defenses, and should be studied by those wishing to understand HIV-1 biology [78].

Vpr (http://www.uniprot.org/uniprot/P12520)

The 11 kDa Vpr protein provides several important functions in HIV-1 replication [79]. About 250 copies of Vpr are packaged per virion through a specific interaction with $p6^{Gag}$ [31] and Vpr is found in the core [62]. One of the most important functions is its ability to bind to and assist the import of the preintegration complex into the nucleus of nondividing and cell-cell cycle arrested cells. Integration of the viral cDNA into the host chromosomal DNA is required, yet is normally prevented by the nuclear membrane. Two ways in which the HIV-1 can access the host genome are (1) during cell division when the nuclear membrane is broken down and (2) through nuclear import of the preintegration complex by interactions of IN and Vpr with the nuclear pore [73, 80]. Vpr is required for the infection of cells that although active

do not divide such as macrophages, which are important in vivo targets for HIV-1 infection and pathogenesis [81]. Vpr also arrests dividing cells at the G_2 stage of the cell cycle, induces apoptosis, and produces other pathogenic effects [82]. The functional implications of these properties are not clear, but likely increase replication, transmission, and possible immune evasion.

Nef (http://www.uniprot.org/uniprot/P03406)

The Nef protein is packaged in only low amounts [83], and most of its properties would not mechanistically require its incorporation: CD4, MHC I, and MHCII downregulation as well as altering cell signaling by binding PAK2 [84]. Nef is also myristylated and binds the plasma membrane so Nef incorporation may be simply due to passive packaging during budding. Nef expression in the producer cell increases the infectivity of the resultant virions, though whether this requires Nef incorporation into the virion remains controversial [85].

Regulatory Proteins

Finally, there are two transcriptional regulatory proteins expressed by HIV-1 that are critical for replication, Tat (http://www.uniprot.org/uniprot/P04610) which transactivates transcription from the HIV-1 promoter and Rev (http://www.uniprot.org/uniprot/P04618) which exports the unspliced, full-length mRNA genome and singly spliced mRNA from the nucleus into the cytoplasm to provide both genomic RNA and Gag and GagPol as well as Env and Vpu expression [86]. While the biology of these proteins is very interesting and important, neither of these appears to be incorporated into the virion, thus beyond the focus of this book. Please see these excellent reviews [22, 87, 88].

Incorporation of Cellular Proteins into HIV-1: Bystanders, Partners, Captives, and Assassins

Viruses by definition heavily depend on host proteins and systems for replication. Unlike DNA viruses which can be quite large and have the genomic capacity to encode a significant amount of synthetic machinery, RNA virus genomes are typically quite small and focused on manipulating the cell with a few proteins to replicate. Examples of this strategy are retroviruses which integrate into the host genome and mimic host genes and picornaviruses which alter the transcriptional machinery to replicate. As discussed above, retroviruses employ many creative expression strategies to maximize viral coding capacity from a small genome. Yet they still rely on host proteins for assistance for all parts of the assembly process.

Cellular proteins are incorporated into and onto HIV-1 virions during assembly. While the functions that cellular proteins provide for the virus inside the cells range from the well studied, e.g., translation, to the less understood, e.g., budding and release, the role, if any for the cellular proteins incorporated into the virion, is less clear. Cellular proteins can associate with the particle in one of four ways: as a consequence of their presence as bystanders present at the site of budding; as partners that assist the viral protein assembly; as captives that are hijacked by specific packaging into HIV to provide a post-assembly function for the virus, e.g., immune evasion or infection; or as assassins, host restriction factors, that have attributes which get them packaged into the virion and inhibit infection. This is a quite large topic and thus is beyond the scope of this review. Interested readers should consult some of the reviews on this subject for details [89, 90]. Each class of incorporated cellular protein provides a helpful clue to the HIV-1 biology. Bystanders, experimentally those proteins that do not require any specific virion protein for incorporation and whose absence does not affect viral replication, mostly implicate the region and nature of the budding site. In contrast, partners, proteins that are specifically incorporated by a virion protein and when absent incorporation the virion fails to assemble, provide mechanistic hints for the cellular side of the assembly process. Captives, experimentally those specifically incorporated proteins in whose absence virions assemble, have reduced infectivity or sensitivity to host antiviral defenses. Unlike the other types, assassins do not play a role in assisting of HIV-1 replication; rather they carryout host suppressive mechanisms; in their absence infectivity and replication are increased, which, in turn, suggest potential approaches for antiviral therapies. Thus, the study of the cellular proteins incorporated into HIV promises to provide clues to the site of budding, assembly, infection and jamming cell defenses, and host restriction factors.

Historically, these proteins were studied using traditional protein chemistry methods. These studies yielded an important but limited set of proteins due to limitations of their sensitivity and ability to examine complex mixtures. Also, some techniques, such as immunoblots, require one to specifically query the sample for a protein of interest rather than identify an unknown protein(s). The evolution of mass spectrometry to provide high sensitivity, high-throughput analysis, and amino acid sequencing of very complex mixtures of proteins has provided for an explosion of proteins associated with HIV-1 virions and provided several important leads for understanding HIV-1 biology [91, 92].

The Purity Problem

In biochemistry, sample purity is paramount to produce unambiguous results. Even though the study of proteins that are incorporated into virions appears to be straightforward, there are several aspects that complicate these studies. When viral proteins are detected in virus particle preparations, it is clear that they are in the virus and not simple contaminants, not so when cellular proteins are found in

virion preparations because large amounts of cellular proteins are released into culture media and biological fluids that can contaminate the sample. These proteins are secreted from the cell by normal cellular processes, released by cell death induced by HIV-1, and present in cell culture medium supplements. Therefore, one critical issue is whether a cellular protein detected is truly in/on the particle or simply present as a contaminant in the sample. Retroviral isolation procedures typically use ultracentrifugation to isolate the virions from the extracellular material. Even though it is possible to remove these proteins by biophysical means, by either density or velocity centrifugation, care must be taken to achieve highly effective removal of soluble proteins. In addition to soluble proteins, even uninfected cells release protein-laden vesicles, either microvesicles (particles that bud from the plasma membrane) or exosomes (particles that first bud into late endosomal compartments that, in turn, fuse to the plasma membrane to be released from the cell) [90, 93]. While these vesicles are fairly heterogeneous, a significant subset of these vesicles has the same density and size as HIV-1 particles. Therefore, while biophysical methods can remove a majority of vesicular contamination, they are unable to remove this fraction which contains a large amount of protein. Even more confounding is that the cellular protein makeup of these vesicles roughly mirrors that of virions. This problem is greatest for virus preparations produced from lymphoid cell lines, which produce a large amount of vesicles. But even preparations produced from transfected epithelial cells contain significant quantities of contaminating vesicles. Effective removal can be achieved by supplementing biophysical methods with other approaches.

Two methods currently used to produce highly pure virion preparations exploit differences between the virus and vesicles, protease digestion, and vesicular immunoaffinity depletion. The digestion approach takes advantage of the fact that virion preparations can be digested with high levels of a nonspecific protease, commonly subtilisin or protease K [90, 93]. These proteases remove the proteins on the outside of the virion, but cannot cross the membrane envelope of the virions, leaving the interior proteins intact. In contrast, the protease digests most of the proteins in the contaminating vesicles, making them lighter. Repurifying the digested virions by density allows for effective removal of these particles, leaving highly pure HIV-1 particles with interior proteins intact and ready for study. Another method exploits the incorporation of CD45 into vesicles produced from hematopoietic cells [90, 93]. This highly abundant surface protein is excluded from virions, presumably due to the inability of the large CD45 cytoplasmic tail to fit into the constrained Gag lattice during assembly and budding. Removal of vesicles, which contain CD45, can be accomplished by immunoaffinity depletion with anti-CD45 microbeads. Despite these powerful tools, no method can remove all of the contaminating particles. Therefore, it is important to establish uninfected controls to monitor the efficiency of the removal of potentially contaminating proteins by using parallel-treated mock virus preparations. Even with the most careful purification, it is unreasonable to expect that contaminating proteins in a complex biological sample can be reduced to zero; there will always be a possibility that a protein is not truly on or in the virus. With increased sensitivity, there is increased

noise from contaminating particles, potentially leading to false positives. While this is a problem with classical biochemistry methods, it is magnified as greater sensitivity mass spectrometry methods are used. Therefore, conformational assays using other often less sensitive methods and direct methods, e.g., confirming mass spectrometry data with immunoblot or Edman protein sequencing, greatly assist in concluding that a particular protein is inside HIV-1 particles.

One occasionally mentioned effort to support specific incorporation is whether a particular protein is at an "enriched" level in the virus versus that found in the cell. On the surface this seems to be logical; however, the great assumption with this line of reasoning is that proteins are uniformly distributed in a cell, which is clearly not true. In fact, proteins are localized to different regions of the plasma membrane. For instance, one would not expect histones to be present in a virus that buds from the plasma membrane, while finding β-actin would be highly likely. Therefore, enrichment over the total cell or even the plasma membrane does not prove specific incorporation as it is the protein composition at the site of budding that is pertinent. Conversely, the array of bystander proteins incorporated into the virions does provide for sort of fingerprint of where the virus assembled and budded from. Taken one step further, it could be possible to determine the source of a virion by its composition, whether it came from a T cell or macrophage and what type could be inferred by the spectrum of proteins found in it.

HIV-1 Proteome Summary

HIV-1 virions contain a variety of proteins, both viral and cellular. Although there has been much progress made in examining these proteins in the virion, it is important to appreciate that there are many more questions still to be answered: what is the comprehensive picture of the extent and type of HIV-1 protein posttranscriptional modifications? To what extent are virion proteins processed further into minor cleavage sites and do they have any regulatory or replication function for HIV-1? What are the differences in the constellation of cellular proteins in/on HIV-1 particles produced from, e.g., macrophages and subtypes of T cells? What immune regulatory molecules on the surface of HIV-1 might impact in vivo replication and pathogenesis? Finally, to what extent are cellular proteins in the virion contributing to HIV-1 replication and immune evasion? These and many more make for a fruitful playground for the HIV-1 biochemist and mass spectrometrists.

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