Chapter 6
Proteomic Studies of HIV-1 and Its Posttranslational Modifications

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Introduction: Posttranslational Modifications Important to HIV-1

HIV requires many proteins to complete its life cycle. Many of these proteins are decorated with biologically critical modifications that alter structure and function. In fact, there are many different types of posttranslational modifications (PTMs) involved in the virus life cycle (Fig. 6.1). In this chapter, we will reintroduce the virus life cycle from the perspective of PTMs. While these modifications will be described in detail later in the chapter, we will highlight new modifications in bold as they are introduced.

A mature, budded virion is a 120–150 nm diameter structure composed of a capsid (containing viral RNA, vif, vpr, nef, p7, reverse transcriptase, and integrase) and a p17 viral matrix surrounded by a lipid bilayer of host origin [1]. This lipid bilayer is studded with host proteins and the viral gp120/gp41 heterotrimer glycoprotein complex. **Glycosylation** is widely recognized to participate during the infection of target cells and in particular with interactions between gp120 and CD4. In order to infect the target cell types, $CD4^+$ T cells, macrophages, and microglial cells, the virus initiates binding and entry via gp120 interaction with CD4. This interaction is assisted by *N*-glycans on gp120, particularly at Asn197 [2]. This interaction with CD4 likely stabilizes the protein–protein interaction and results in a conformational change in

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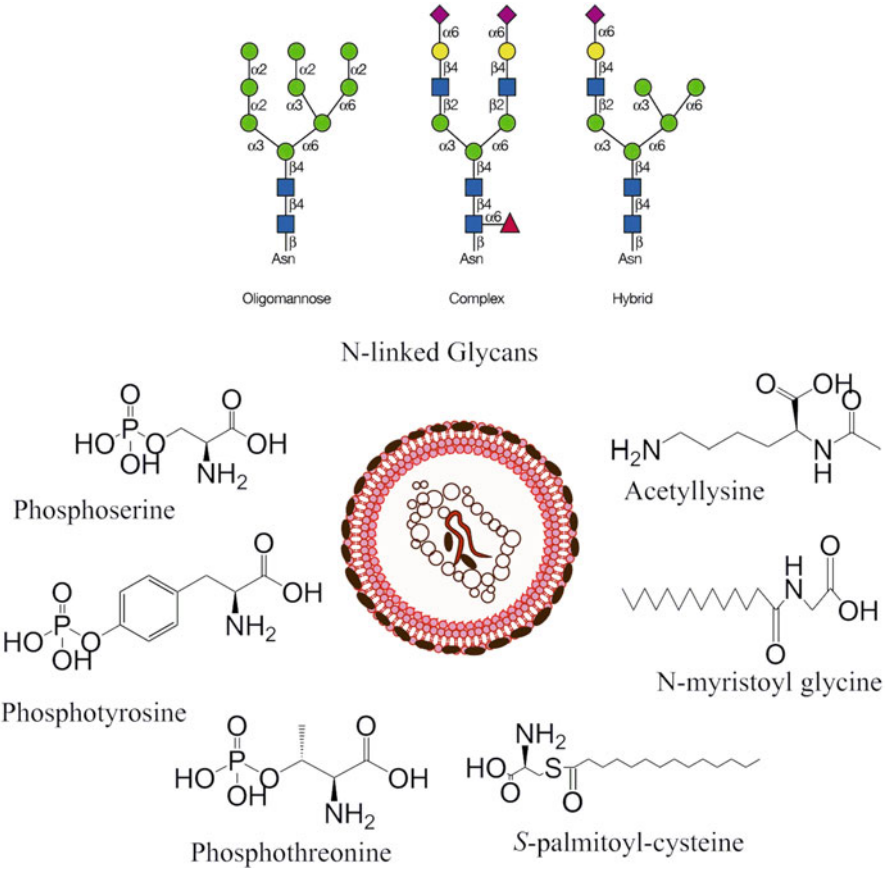

Fig. 6.1 Chemical structures of some of the major protein modifications involved in the HIV-1 life cycle. See Table 6.1 for roles and references. Glycan images modified from *Essentials of Glycobiology*, Chap. 8, Fig. 8.1

gp120 that increases the affinity for coreceptors (CCR5 and CXCR4) and exposes the **palmitoylated** protein gp41 [3]. The interactions with coreceptor further stabilize interactions exposing the fusion peptide of gp41 allowing fusion and entry of the virus [4]. Viral binding and entry can also be enhanced by dendritic cells expressing the C-type lectin (glycan-binding protein) DC-SIGN, which binds gp120 and facilitates interaction of the virus and CD4 and the coreceptor [5]. Once inside the cell, HIV deposits its payload by uncoating and initiating reverse transcription (RT).

This series of events is very poorly understood, as the events occur rapidly and components are hard to isolate and measure [6]; however, Schweitzer and colleagues have recently applied proteomics-based methodologies to understand the early state of virus uncoating and the formation of pre-integration complexes [7]. The mechanisms that drive uncoating and the early steps of infection are unknown at the level of PTMs.

The HIV cDNA generated by the RT is in complex with a number of viral proteins, including matrix and integrase, and a number of host factors related to transcription and chromatin remodeling, RNA binding, and nuclear import factors [8]. Modification of these proteins is critical to nuclear entry and integration; nuclear targeting of the process is thought to be mediated by matrix, which strongly interacts with integrase via tyrosine **phosphorylation** [9]. Conversely, it has also been reported that vpr is responsible for the karyophilic properties of the pre-integration complexes in macrophages, interacting through multiple serine phosphorylations [10]. Nevertheless, the phosphorylation of viral proteins in the pre-integration complex, thought to be carried out by protein kinase A, is critical for nuclear entry. Once in the nucleus, DNA integration must be achieved. Integrase itself is known to be **acetylated** by the host histone acetyl transferase p300, which mediates a stronger interaction between the viral genome and host genetic material and enhances the enzymatic activity of the protein. This mediates integration of the genome to "transcriptionally active regions of chromatin" [11].

Once integrated, the virus enters latency, which can last from months to years. Reactivation occurs when the viral genome is transcribed, which is initiated by activation of T cells and the increased expression of the transcription factor NF-kB. NF-kB has also been implicated in the role of acetylation in reactivation, as it is also involved in the relationship between TNFα and histone deacetylase inhibitors. As the viral mRNA is transcribed, the viral proteins rev and tat are produced, which regulate viral protein (gag and env) and RNA expression and localization [12]. The major PTMs involved in the nuclear stage of HIV infection are phosphorylation, which is active in the signal transduction and complex recruitment, and acetylation, which is a major player in transcriptional regulation. These modifications are thought to have a high frequency of cross talk and related functions [13]. A complex concert of events occurs once viral RNA and protein are produced. The gag polyprotein is co-translationally **myristoylated** in the ER on the matrix subunit, which is sequestered in a hydrophobic pocket as the polyprotein is expressed into the cytoplasm [14]. When gag either interacts with viral genomic RNA or begins to multimerize with other gag proteins, a conformational change occurs and the myristoyl group is exposed, creating a hydrophobic region that preferentially interacts with the plasma membrane [15]. Other viral factors also play a role in gag assembly [16]. Additionally, the localization is coordinated via an interaction involving the lipid tail of phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) [17], which specifically targets the multimers to the lipid raft microdomains where final assembly and budding take place [18]. Meanwhile, the expressed env protein, a precursor to gp120 and gp41, trimerizes and is co-translationally **N-glycosylated** in the Golgi [19]. This gp160 is processed by furin [20] into the mature proteins, which migrate to the membrane to and assemble into a virion in lipid raft microdomains [21]. Budding then occurs at the plasma membrane and is coordinated by a number of host viral proteins, including the nucleocapsid domain of gag [22], ESCRT complexes [23], and the ubiquitin ligase nedd4 [24], which covalently attaches **ubiquitin** to p6 [25]. While the precise mechanisms are not fully understood, a picture of complex protein–protein interactions mediated by a number of critical PTMs is emerging [26].

As the virus buds, the mature virion moves to continue the life cycle by targeting lectins and receptors with the *N*-glycan decorated gp120.

The Increasing Recognition of the Role of Posttranslational Modifications in HIV-1 Proteins

The role of PTMs in HIV has been known for some time. With the advent of more powerful technology, the discoveries in this field have dramatically increased. Since 1990, the number of publications involving HIV and four of the major PTMs has increased approximately fivefold (Fig. 6.2). The areas of greatest interest are phosphorylation and glycosylation, which is logical given the frequency of these modifications (Fig. 6.3) and their important role in HIV infection and pathogenesis (Table 6.1). Other modifications have seen only modest gains in interest, likely due to the inherent challenges in studying these modifications.

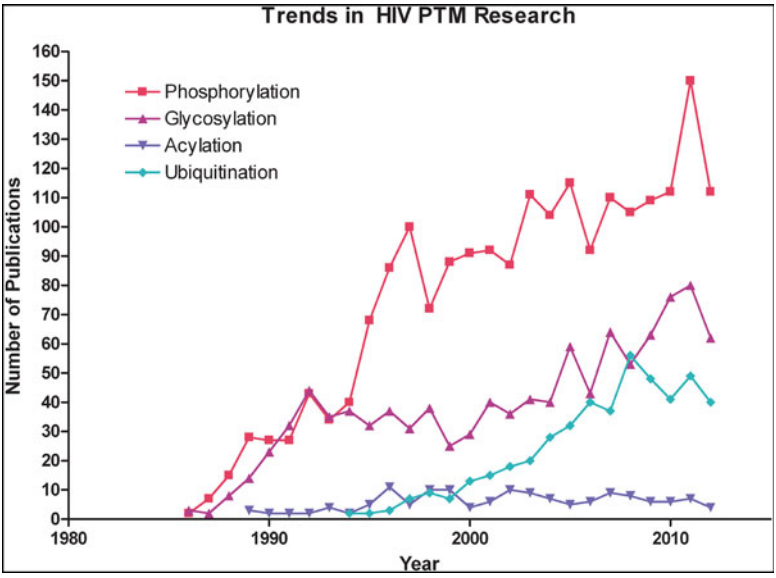

Fig. 6.2 Number of publications in HIV research describing specific posttranslational modifications. A keyword search of Scopus (http://www.scopus.com) for keywords HIV and either phosphorylation, glycosylation, acylation, or ubiquitination in the abstract, title, or keywords was carried out. Results were exported and displayed in prism

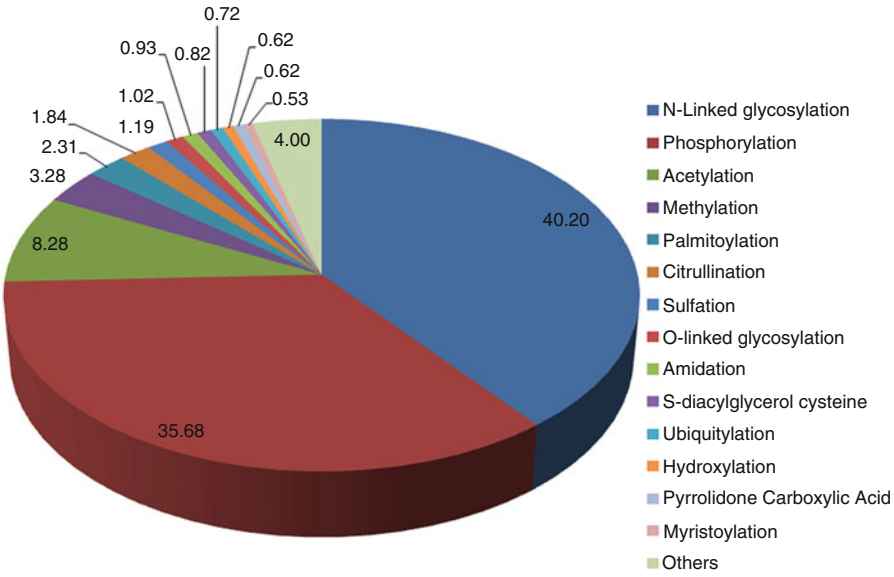

Fig. 6.3 Statistics of major posttranslational modifications on proteins. The Swiss-Prot database was mined for PTM frequency in both experimental and putitative models and the modifications enumerated [30]. Values are represented as a percentage of proteins in the entire database having at least one modification

Table 6.1 A list of some common protein modifications that affect the HIV replication cycle

Modification	Role	Site/motif	References
Phosphorylation	Signaling	S, T, Y	Francis et al. [10]
Acetylation	Transcriptional activation	K	Ott et al. [128]
N-glycosylation	Binding, immune evasion	NxS/T	Raska et al. [129]
O-glycosylation	Not known (in HIV)	S/T	Graham et al. [77]
Myristoylation	Trafficking, localization	N-terminal G	Bentham et al. [130]
Palmitoylation	Cellular localization	C	Rousso et al. [131]
Nitrosylation	Protease inactivation	C	Persichini et al. [63]
Ubiquitination	Release, degradation	K	Strack et al. [132]

Posttranslational Modifications of Host Proteins

The human genome contains somewhere in the region of 21,000 genes [27]. While alternative mRNA splicing allows for much more protein diversity by creating new protein [28], nature's answer to protein diversity appears to be posttranslational modifications. Posttranslational modifications are covalent modifications of the nascent protein following transcription and translation [29]. Posttranslational modifications

are critical to protein function, localization, and protein–protein interactions. Many proteins are modified by at least one PTM; in a survey of the Swiss-Prot database, over 40 % of the sequences were predicted or experimentally observed to contain an N-glycosylation modification, and nearly as many proteins have the potential to be phosphorylated on at least one residue [30]. Other less common modifications are known to occur as well (Fig. 6.3, see uniprot.org/docs/ptmlist for a full list of PTMs).

While this serves as a good estimator of frequency, this does not account for multiple modifications on the same protein, or PTM cross talk, which is a well-established phenomenon [31, 32]. Nevertheless, it is abundantly clear that protein PTMs are common, abundant, and important regulators of function in biological systems.

Enzymatic conjugation confers some level of specificity for the target amino acid (either specific site or sequence motif), making reactions both predictable and more biologically relevant. These modification sites are determined predominantly by the specificity of the transferase or kinase utilized and may have specific amino acid motifs to specify the target amino acid sequence.

Detailed Description of PTMs Involved in the HIV-1 Life Cycle

Phosphorylation

Phosphorylation was first described by Krebs and Fischer in 1955 [33]. It involves the enzymatic addition of a phosphate (PO_4^{3-}) group to the side chain of serine, threonine, and tyrosine residues. This modification is highly dynamic and is involved in a great deal of biological processes, including tumor suppression, signal transduction, homeostasis such as insulin signaling, and protein recycling [34]. Addition of phosphate groups by kinases can activate enzymes, both by altering the tertiary structure of proteins, often resulting in changes in local hydrophobicity and exposure of active sites in the protein [35]. Regulation of phosphorylation is carried out by phosphatases and kinases, which enzymatically modify phosphorylation sites. Other forms of regulation, including cross talk with *O*-GlcNAc, have been described [31, 32]. The most thoroughly documented role of phosphorylation in HIV biology is in the nuclear host–virus interactions [10].

N-Linked Glycosylation

Attachment of complex glycans by an *N*-glycosidic bond to asparagine residues results in the production of *N*-glycans. These are, along with phosphorylation, one of the most common protein modifications observed in biology. *N*-Glycans exist in three classes: high mannose (oligomannose), complex, and hybrid [36]. They are assembled in a complex process involving many enzymes in the endoplasmic

reticulum (ER) and Golgi. The functions of *N*-glycans are varied and include structural defense, self recognition, cell–cell interactions, host–pathogen interactions, regulation of enzymatic processes, and regulation of receptor-binding affinities [37]. The structures can be extremely complex, and alteration of the *N*-glycan structure can alter the function and location of the protein. It has recently been shown that protein structure can dictate the type of modification [38]. Clearly, this is a highly regulated process that is critical to cell biology; it is no surprise that HIV utilizes many of the characteristics of glycoproteins in a vast array of ways to assist in driving function, defense, and pathogenesis [39]. For example, gp120 glycosylation is proposed to act as a steric barrier or glycan shield [40] that assists the virus in evading antibody responses to gp120 protein. Evolution of the glycans confers an adaptable defense against immune response [41]. Recently, by removing these glycans, Huang and colleagues demonstrated that it is possible to generate effective neutralization using antibodies [42], reinforcing the importance of glycans in antibody evasion.

Palmitoylation and Myristoylation (Fatty Acid Acylation)

The addition of long chain lipids to proteins is termed acylation. Myristoyl group (C_{14}) chains, which are rare saturated fatty acids in cells, are covalently attached to N-terminal glycines via the enzyme *N*-myristoyl transferase (NMT) as a cotranslational modification in the ER [43]. Myristoylation of proteins effects an interaction with hydrophobic regions of the cell, such as plasma membranes, allowing proteins to be localized but not necessarily anchored. Enzymatic addition of a C_{16} saturated palmitoyl group onto cysteines via a thioester linkage by palmitoyltransferases confers additional hydrophobicity. Proteins acylated in both forms will interact strongly with the inner leaflet of the plasma membrane [44]. Palmitoylation of proteins also has been shown to preferentially drive proteins to lipid raft microdomains [45]. Functionally, acylation is important for cell signaling. It is best described in its role as a modulator of G-protein-coupled receptor signaling, where dynamic modulation of palmitoylation is thought to regulate the membrane versus cytosolic location of protein domains. This regulation affects receptor activity and downstream responses in a variety of biological functions such as the endocrine, nervous, and cardiovascular systems and, more importantly in the context of HIV, immune response [46].

Ubiquitination/Ubiquitylation

Ubiquitination is the covalent addition of a small ubiquitin protein to a lysine residue in targeted proteins. The reaction is catalyzed by ubiquitin E1, E2, and E3 ligases [47]. The most well-known role of this modification is the signaling of

proteins for degradation and recycling in the proteasome [48]. However, other roles have been postulated, including stress responses, DNA repair, cell cycle control, as well as viral infectivity [47]. Many of these common modifications are involved in the HIV life cycle, which hijacks and spans many common cellular pathways involved in cell signaling, cell trafficking, binding, and complex assembly. These include, but are not limited to, interactions with gag, p6, tat, and integrase, as well as signaling between viral proteins and host factors [49].

Advantages and Challenges of Studying PTMs

The study of PTMs is a burgeoning field. As our overall understanding, biological processes become more detailed; the roles of PTMs in many diseases, cellular functions, and regulation have been revealed [50]. The role of dynamic modifications, such as phosphorylation and glycosylation, was initially characterized by mutation of specific sites of modification, studied using well-characterized protein models. However, the use of -omics technologies has allowed the high-throughput analysis of entire cell cultures or tissues for specific modifications [51]. In addition, more recent work indicates some of these modifications may act in concert, and the dynamic interplay between modifications can dramatically affect the biological state of a system [31, 32]. Finally, since the occupancy or proportion of modified proteins can play a role in the biological effect, novel analytical techniques are being developed in order to characterize and both absolutely and relatively quantify the amount of modified proteins present in different biological states. With the advancement of PTM studies and techniques, the need for comprehensive, reproducible, and validated techniques has become clearly apparent. As with any scientific experiment, advantages, challenges, and limitations must be understood in order to comprehend and place any findings into a biological context.

The single most important advantage of studying PTMs is that we are able to understand a more detailed biological picture of the system being studied. In the context of HIV, by merely measuring protein levels in a cell, the mechanism for the subcellular localization of viral proteins would not be well understood. In contrast, the study of PTMs allows for better characterization, measurement, or emulation of a particular biological system, which is the goal of basic science experiments. The recent interest in systems, or multi-omic, studies emphasizes the need to place findings in the context of all the components [52]. This in itself presents challenges and limitations, since in many instances the comprehensive measurement of proteins, lipids, nucleic acids, and metabolites is untenable or prohibitively expensive to undertake. However, studies that successfully integrate different disciplines often reveal novel mechanisms and associations that were otherwise unapparent. For example, the role of the inner membrane $PI(4,5)P_2$ in gag multimerization and membrane localization required a multidisciplinary approach to clearly identify biological function and role in the process [53, 54].

Many PTMs, as previously noted, are dynamic processes that change in response to stimulus, such as immune activation, stress responses, or analyte and substrate levels. Developing an understanding of the role of dynamic modulation of proteins can lead to a striking view of cellular regulation. For example, the role of *O*-GlcNAc in a large number of diseases is only starting to be realized, and the cross talk between glycosylation and phosphorylation is revealing novel disease markers and mechanisms [55]. Many of these processes initialize a chain reaction of downstream effects, massively altering gene and protein expression in a cell, such as in the use of *N*-myristoyl transferase by Nef in HIV infection [56]. As diseases progress, the levels of modifications may change dramatically. By quantifying PTM levels, the progress or extent of a disease may be better characterized and measured [57]. Subtle interventions at specific targets may be sufficient to abrogate infectivity of viruses [58], to prevent completion of the viral life cycle [59], or to better elucidate poorly understood phases such as latency [60].

Although there are a number of benefits to studying PTMs, there are also clear challenges. Some of these are presented by limitations in instrumentation, such as preservation of modifications and their subsequent detection, which will be covered in detail later in the chapter. Other limitations are inherent in the biology of PTMs, such as temporal expression and modulation, dynamic range, and more broadly, the a priori biological understanding of how subtly modifying a side chain residue of a single amino acid can affect signal downstream biological effects.

Proteomic Methods for PTM Analysis

In order to measure PTMs from biological samples, specific a priori decisions must be made in order to select the correct technique, sample preparation, and analysis strategies. These decisions typically require an assessment of which modifications are being studied and the specificity and range of the involved proteins. For example, if S-palmitoylation is a target, biochemical membrane isolation should be carried out as the proteins of interest are most likely present in this cellular fraction [45]. Conversely, when studying protein phosphorylation, specific inhibitors should be supplemented in the extraction buffer, and sample perturbation should be minimized [61]. Following sample extraction, the choice of analytical technique is often driven by availability of equipment and samples and the cost of an assay. A number of biochemical, gel-based and MS techniques are available, and these are outlined below.

Biochemical Methods

One of the major challenges of studying some PTMs is the lability of the modification and thus the biological interpretation of any analytical data. As discussed earlier, thoughtful and careful sample preparation with knowledge of the sample

lability and treatment conditions can vastly improve the experimental outcomes. In some circumstances, the relative reactivity of modifications can be used in chemical labeling strategies. One example is the detection and measurement of S-nitrosylation, a modification thought to be important in cell signaling, immune defense, and pathogenesis of some diseases [62]. This modification has been shown to have antiviral activity, in part due to its ability to inactivate the HIV-1 protease [63]. The modification itself is extremely susceptible to oxidation and is light sensitive, making measurement difficult. Using the differential oxidation to their advantage, Jaffrey et al. developed a biotin switch method whereby mild ascorbate treatment released the nitrosylation, and the thiol was biotin labeled and subsequently captured [64]. Further refinements have led to fluorescent labeling techniques [65] and other MS-compatible applications [66, 67]. Other dynamic modifications, such as *O*-GlcNAc, can be similarly modified and subsequently measured [68].

Difference Gel Electrophoresis and 2D Electrophoresis

Two-dimensional gel electrophoresis (2DE) was first described by O'Farrell and Klose [69, 70]. With the advent of fluorescent protein labeling, differential analysis within individual gels became possible [71]. Difference gel electrophoresis (DIGE) and 2DE have been used extensively for many years in HIV research (e.g., [72–75]). However, with the development of robust LC-MS/MS techniques, the application of DIGE and gel-based protocols has declined. This is primarily due to the complexity of data analysis and spot identification and the great deal of time and investment required, compared to an iTRAQ or other quantitative MS approach. Furthermore, protein identifications are made following quantification, so only those proteins deemed of interest are actually identified by mass spectrometry (MS). The amount of protein required to visualize low-abundance proteins can be quite high, and reproducibility can be an issue.

With all that in mind, there are several "niche" applications that 2DE and in particular DIGE are well suited to. One of these is the analysis of PTMs. Using two-dimensional electrophoresis followed by Western blotting, Davis et al. were able to demonstrate that HIV-1 reverse transcriptase is present in a number of protein isoforms within the cell [76]. The isoforms were shown by phosphatase activity to be in part due to phosphorylation, and additional experiments showed that the majority of the protein was in the modified form. The phosphorylation site was not determined, as MS analysis was not carried out. In another study, Graham and coworkers described the use of DIGE to differentially identify PTMs in HIV and SIV [77]. By enzymatically cleaving *N*-glycans from the virus in one of two samples, and differentially labeling the resulting proteins, modified proteins were easily visualized, as spot locations changed for deglycosylated glycoproteins. Taking advantage of the deamidation of N to D in PNGase F treatment, MS was utilized to identify sites of N-glycosylation on gp120 for both HIV and SIV. This approach is advantageous, as modified proteins are easily visualized and can be excised for MS identification.

Although not carried out in this study, 2DE samples are also compatible with glycan isoform analysis (see below). Using this technique, along with well-defined hypotheses, allows proteomic screening for specific PTMs of interest and gives information on relative proportions of modified versus unmodified proteins.

Mass Spectrometry of PTMs

Mass spectrometry has been applied to proteins for several decades [78, 79]. "Bottom-up" MS, where tryptic peptides are analyzed and searched against genomic databases [80], has been used with much success for the last decade [31, 32, 81, 82]. Recently, technological advances have rapidly improved the sensitivity, selectivity, and resolution of instrumentation [83, 84]. Naturally, this improvement in technology has enabled an emphasis on targeting specific peptides, and subsequently PTMs, since one of the overarching goals of many proteomic studies is to apply technology to biological questions and place these studies in a biologically relevant framework [52], in which PTMs play a critical role. Thus, in addition to the technical gains in instrumentation, novel methods have been applied to the fragmentation and detection of biomolecules in MS. These methods include MS^3 and MS^n analysis, as well as alternative fragmentation methods such as electron capture dissociation/electron transfer dissociation (ECD/ETD) and higher-energy collision dissociation (HCD) in the orbitrap [85].

Fragmentation of some modified peptides by collision-induced dissociation (CID), particularly phosphorylation and GlcNAcylation, results in elimination of the modification and poor fragmentation on the peptide backbone, typically reporting a mass of the precursor minus the modification, or a neutral loss of m/z 80 and 97 (phosphorylation of Y and S/T, respectively) or 203 (hexosamine). In trapping instruments such as ion traps (which, as the name implies, can accumulate ions until a specified threshold is attained), this neutral loss event was compensated for in one of two ways: firstly, carry out data-dependent neutral loss (DDNL) analysis, whereby a neutral loss of specific m/z in MS to MS^2 triggered an accumulation of the neutral loss ion and subsequent MS^3 fragmentation [86], or, secondly, trigger a second MS^n event while still accumulating data, which is termed "pseudoMS^n" [87]. This development was particularly important since up to an estimated 80 % of ions from a phosphopeptide enrichment contained neutral loss ions [86].

In addition to MS^3 approaches, alternative fragmentation methods have been developed. To overcome the energy transfer to the PTM and poor fragmentation, ECD was developed for Fourier transform MS [88]. This utilizes a free electron, which reacts with the peptide backbone in an exothermic manner, resulting in fragmentation of the backbone without affecting proximal phospho groups [89]. In ETD, the same principle applies, but the reaction is catalyzed by an anthracene anion in ion trap or orbitrap instruments [90]. The resulting MS/MS spectra are rich in c and z ions and give a much better sequence coverage than CID for modified peptides. One important caveat, however, is that ETD has been shown to require larger, higher

charge state precursors for optimal results. Recent work has developed a more robust workflow using the endoprotease LysC in combination with some mobile phase modifiers to optimize results [91]. To our knowledge, these techniques have not been applied to HIV PTM study, making this a potentially fruitful area of research.

Advanced MS Techniques

The data-dependent acquisition utilized by bottom-up MS creates challenges. While bottom-up MS is suited to proteomic discovery, it is not optimal for PTM analysis. As previously mentioned, the detection of PTMs is reliant on the a priori knowledge of their presence. In addition, searching for multiple PTMs can dramatically increase the time taken for data analysis. One way to overcome those limitations is to utilize data-*independent* acquisition. This technique goes by a number of names: SWATH-MS, MS^E, AIF, or PAcIFIC [92]. In this method, the instrument scans mass ranges in MS/MS without isolating specific precursor ions, essentially capturing all the ions present within a given dynamic range. The advantage of this is that you can screen for PTMs based upon a given mass change; the disadvantage is that these experiments are data independent so any neutral loss ions cannot be further fragmented for structural or compositional information.

Another method that has garnered recent attention is the use of "top-down" MS. In contrast to bottom-up, the intact protein is introduced to the mass spectrometer, and subsequently fragmented into smaller pieces, which are then analyzed for the presence of modifications [93]. While it is extremely powerful and can identify any known modification (complex *N*-glycans and other complex modifications aside), there are limitations. The protein sequence must be known in order to fit the fragments to the sequence, and the protein must be purified and of a size that is currently amenable to analysis (large proteins do not sufficiently ionize). While a relatively robust sample preparation tool exists [94], the challenges of implementing this technology still need to be overcome. Once established, this may change the way PTMs are studied in many research areas. For HIV, the ability to screen viruses from different cell types may help to establish in more detail mechanisms of viral tropism and immune responses to different viruses.

Emerging Technologies

A number of emerging technologies are enabling a deeper and more directed analysis of HIV and host PTMs. Advanced sample preparation and labeling strategies will allow for refined analysis and capture of specifically modified proteins, and instrumental and analytical updates will enable analysis of PTMs and proteins from novel and beneficial angles. The two main areas of these emerging technologies are sample labeling/enrichment and improved analytical technologies such as instrumentation and bioinformatic strategies. From the labeling and enrichment side, the emergence of

click chemistry tools and techniques has the potential to dramatically change the way that we study PTMs, since it removes the barriers and limitations of the aforementioned radiolabeling, and is more specific than chromatographic enrichment and analysis. From an analytical aspect, the explosion of simple, accessible nonprotein MS methods, and the subsequent technological advances, opens the door to analyzing not only the proteome but also the functional modifications attached to the proteins in a site-specific manner. Finally, the utilization of the nascent technology of MS imaging, while currently limited in application and robustness, may provide a tool for the specific localization of proteins and their modified counterparts in tissues and cells.

Click Chemistry

Click chemistry is the use of bio-orthogonal synthetic compounds for the specific labeling and capture of modified residues. These residues can be specifically labeled in order to study a particular PTM on proteins or a specific cellular pathway. The term "click chemistry" was coined by Barry Sharpless, who described it as "spring-loaded" reactions, "destined for a single trajectory" [95]. These reactions typically involve 1,3-dipolar cycloadditions that react an azide to an alkyne functional group either catalyzed by copper [96] or using copper-free chemistry for in vivo studies [97]. There are a number of commercially available azide- and alkyne-containing substrates. A great deal of the seminal work was carried out studying cell surface *O*-glycans by feeding azide-modified precursors to cells and detecting incorporation via Western blotting to a clicked substrate, e.g., FLAG tag [96]. Other substrates for modification include *N*-glycans such as sialic acid and fucose precursors [98, 99]; acylations such as palmitoylation, myristoylation, and prenylation [100, 101]; and newly synthesized proteins using azidohomoalanine [102, 103]. As we understand more about the mechanisms and chemistry of PTM incorporation, improvements are made in the selectivity and sensitivity of chemical analogs. For example, by modifying the permeability of compounds, it is possible to reduce the dose of analog to achieve equal labeling efficiency, reducing potential off-target effects [98]. Furthermore, by altering the initial "click" substrate, one may change the target specificity and further define specific subpopulations of proteins and their modifications.

In HIV research, as discussed above, the role of specific PTMs in the life cycle, both on the host and viral sides, still remains largely unknown. However, the effects of some more studied modifications are beginning to be elucidated. For example, it is well known that surface glycosylation of viral proteins is critical in evading and adapting to the host immune response [42, 104, 105]. Obviously, as a high number of cell surface proteins are glycosylated, glycan–protein interactions are known to be critical for cell–cell communication, as well as HIV–cell interactions [2, 106]. These primary interactions are critical and provide insight into specific viral–host mechanistic relationships; however, by specifically targeting a subpopulation of proteins, we may yield more information regarding more subtle secondary interactions that may be just as critical to the viral life cycle [107].

Non-proteomic Mass Spectrometry

The emergence of proteomic MS in the last 30 years has led to an explosion in discovery at the protein level. The methods of bottom-up MS and data analysis using proteogenomic databases have allowed broad access to understanding the "what," "when," and relatively "how much" questions asked of protein expression in specific systems and disease states. In HIV, this has resulted in a broader understanding of the proteins that play a role in HIV infection and life cycles [31, 32, 81, 82, 108–110], as well as the minimally conserved proteome of HIV itself [107, 111]. These studies have certainly advanced knowledge in the field of HIV biology; however, a challenge associated with this type of research is that more subtle biology may be missed, considering we are only studying proteotypic peptides with defined modifications, that is, by searching against protein and genomic databases, and by selecting certain analysis tools and methods, we choose to ignore a vast wealth of information about protein modifications and diversity. For example, labile modifications such as phosphorylations may be lost during sample preparation or analysis, and while a protein may be defined as critical, it may be the effect of phosphorylation that defines the biology. While methods for phosphoproteomic analysis and other relatively simple modifications (e.g., *O*-GlcNAc) are developing rapidly [51, 112, 113], other modifications are not so straightforwardly analyzed. The use of ECD/ETD fragmentation has allowed site-specific analysis of glycoproteins [114], but the detection of glycan isoforms remains a very specialized technique. As mentioned above, a single protein may be multiply glycosylated throughout the sequence, and each site of glycosylation can contain a large number of glycan isoforms, some of which may be functionally distinct [115, 116]. Methods for glycan isoform identification and site localization may lead to a greater understanding of the roles of oligosaccharides on HIV attachment, virion–cell interaction, infectivity, and the host immune response.

The methods for releasing *N*-glycans are well established, using enzymatic cleavage of the glycan via peptide-N4-(*N*-acetyl-beta-glucosaminyl)asparagine amidase (PNGase F) [117]. However, separation and analysis of the glycan isoforms are complicated by relative homogeneity of the saccharide monomers. Non-LC methods for analysis are primarily driven by MALDI applications, where spotted glycans are identified predominantly by their precursor mass, and some structural information is yielded by MS/MS analysis [118]. This method does limit analysis to higher-abundance glycans, and LC separation methods have been advanced in the last several years to compensate for this. One recent advance is the use of porous graphitized carbon (PGC) HPLC-Chip columns for nano-LC separation of glycans and their subsequent analysis by ESI-MS/MS. In this method, the *N*-glycans are released from the protein backbone, enriched using solid-phase extraction, and separated on a gradient of water/acetonitrile. Glycan structure is assigned based upon the precursor mass and using MS/MS fragmentation information; the numbers of hexose, hexNAc, fucose, NeuAc, and NeuGc monomers and some unambiguous structures are assigned [119]. While powerful, this technique does not allow the connection

between the protein structure and the glycan. In order to achieve this, one must isolate both a proteotypic peptide sequence *and* the modification in the same MS/MS spectrum. This has been successfully applied by using pronase to nonspecifically digest the protein backbone, leaving short amino acid tags on the glycans, which are then subjected to LC-MS/MS. This technique relies on the a priori knowledge of the protein sequence. However, if this is known from a complementary analysis, the site occupancy of glycan isoforms on a specific protein may be elucidated [120, 121]. This structural information could have a myriad of applications to HIV biology, from both the host and virion aspect. In conjunction with non-MS methods (e.g., lectin arrays), more detailed understanding of the gp120 glycan shield may allow better design or specificity for arrays of neutralizing antibodies and knowledge of the specific cell–virus interactions [2, 122]. Glycans may also play a role in viral tropism, and identifying host–virus interactions to specific glycoproteins may lead to novel approaches to clinical diagnosis and interventions.

Mass Spectrometry Imaging

While non-proteomic MS techniques are rapidly advancing, there are additional MS tools that are beginning to emerge as potentially powerful tools for the analysis of biological systems. Mass spectrometry imaging (MSI) is a technique that has shown much promise in the past several years. Although MSI was primarily developed for the analysis of small molecules and lipids in tissues, much time has been invested into developing robust methods for the analysis of proteins and peptides in order to add a spatial dimensionality to proteomics information. Analysis of small proteins [123] and peptides [124, 125] is becoming more routine, and novel methods such as 3D imaging are being proposed [126]. However, the analysis of PTMs is some way off, and to our knowledge there are no publications in this area, suggesting an area for further exploration and expansion. Although some limited work on glycan monomers and simple carbohydrates has demonstrated the potential efficacy of this technique for glycan analysis [127], robust and reproducible methods must be established before any biological studies can be launched. The potential to localize specific modified proteins and peptides to tissue and biological regions could be applied to understanding the role of HIV infection in systemic disorders such as HIV-associated neurocognitive dysfunction.

Summary

The analysis of the role of PTMs in HIV is growing, and our understanding of the basic biological roles of modifications on the life cycle of the virus is increasing. With the development of enhanced analytical techniques and better mechanisms for specific labeling, enrichment, and analysis of modifications, it is anticipated that

further developments will be made in understanding the critical roles of PTMs. In particular, the interplay of lipid modifications for trafficking proteins to the cell membrane coupled with glycosylations critical to cellular communication, binding, and interaction may yield a greater understanding of the roles of host proteins integrated into HIV and reveal potential mechanisms of disrupting the viral life cycle. In addition, a thorough characterization of the modifications of both host and viral proteins present in HIV will allow researchers to develop a better systems biology picture of the virus, and potentially connect viral composition to previously unrelated cellular pathways, revealing novel mechanisms for intervention and drug targeting. Further, by continuing the characterization of the glycome of the virus, and in particular gp120, the enhancement of potentially neutralizing vaccines could be realized. In conclusion, the burgeoning field of posttranslational protein modifications will continue to benefit our understanding of HIV and its clinical effects.

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