Proteomic approaches to uncovering virus–host protein interactions during the progression of viral infection

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Abstract

The integration of proteomic methods to virology has facilitated a significant breadth of biological insight into mechanisms of virus replication, antiviral host responses and viral subversion of host defenses. Throughout the course of infection, these cellular mechanisms rely heavily on the formation of temporally and spatially regulated virus–host protein–protein interactions. Reviewed here are proteomic-based approaches that have been used to characterize this dynamic virus–host interplay. Specifically discussed are the contribution of integrative mass spectrometry, antibody-based affinity purification of protein complexes, cross-linking and protein array techniques for elucidating complex networks of virus–host protein associations during infection with a diverse range of RNA and DNA viruses. The benefits and limitations of applying proteomic methods to virology are explored, and the contribution of these approaches to important biological discoveries and to inspiring new tractable avenues for the design of antiviral therapeutics is highlighted.

Keywords

virus–host interactions; mass spectrometry; viral proteomics; AP-MS; IP-MS; interactome

Introduction

Viruses are fascinatingly diverse in composition, shape, size, tropism, and pathogenesis. Infectious virus particles can have core capsids that can be structurally helical, while others are icosahedral. Some are enveloped with lipids and proteins, whereas others are not. Viruses bear their genetic information as RNA or DNA, in a single strand or double-stranded helix. For successful propagation, viruses depend on replication of their genomes, which itself may occur within the host cell cytoplasm or nucleus. For example, alphaviruses replicate in the cytoplasm, such as Sindbis virus (SINV, Figure 1, panels 1b–7b). Conversely, herpesviruses, such as herpes simplex virus-1 (HSV-1, Figure 1, panels 1a–7a), and the retrovirus, human immunodeficiency virus (HIV, Figure 1, panels 1c–7c), replicate in the nucleus. Viruses have a distinct tropism for host species and for cell types, although their
continued coevolution with hosts has sometimes facilitated their capacity to infect new cell types and species.

At the core of virus replication and spread is the formation of virus–host protein interactions. One aspect to consider is the stark contrast between the proteomes of host cells and viral pathogens. The total protein complement of the genome in human cells, or proteome, is estimated at approximately 20,000 protein-encoding genes that, when taking into account the copies per cell, can generate up to 3 million proteins per cubic micron [1-2]. Furthermore, the human proteome can be further diversified by regulatory processes, including the presence of multiple transcription start sites [3], alternative splicing [4-7], alternative mRNA polyadenylation [8], pre-mRNA editing [9], and posttranslational modifications [10-11]. Indeed, genomics studies have identified alternative splicing isoforms in up to 60% of human genes [12-13]. Additionally, a large number of posttranslational modifications have been discovered that add complexity and variability to the proteome [14]. The viral proteome can also be expanded by some of these regulatory processes. However, in contrast to the human proteome, virus genomes encode far fewer proteins, ranging from 1 to approximately 2500 that can be present in hundreds to thousands of copies per virion particle [15-16]. Therefore, even when encoding for a small number of viral proteins, viral replication can still progress successfully by making use of dynamic interactions with the host. In fact, all viruses are obligate parasites and undergo evolutionarily conserved life cycles that dutifully depend on virus–host interactions, frequently mediated by protein–protein associations.

A complete infectious life cycle, regardless of virus classification, necessitates the attachment and entry of the virion particle into the host cell, viral translation of mRNA by host ribosomes, viral genome replication, assembly of viral particles enclosing the genome, and release of infectious particles from the cell (Figure 1). As an example, HSV-1 is a DNA virus that employs a temporal cascade of protein expression and interactions with the host (Figure 1, panels 1a–7a). These virus–host protein interactions are aimed both at ensuring successful virus replication and at blocking host defense mechanisms. First, virion particles bind to host cell surface moieties via viral glycoproteins (Figure 1, panel 1a). The fusion of the virion with the cellular plasma membrane leads to the release of the viral capsid containing the genome and matrix proteins into the cytoplasm. Through associations with cellular motor machinery, viral capsids traverse the cytoplasm, anchor at nuclear pores, and extrude the double-stranded viral DNA genome into the nucleus (Figure 1, panel 2a) [17]. Concurrently, several incoming viral proteins are transported to various subcellular locations to regulate viral gene expression, as well as to inhibit cellular intrinsic and innate immune responses [18-19]. Within the nucleus, the expression of a highly regulated viral gene expression cascade is initiated and is organized into immediate-early, early, and late gene expression (Figure 1, panel 3a). The host cell RNA polymerase is co-opted to begin transcribing viral immediate-early mRNAs. Upon cytoplasmic translation, immediate-early proteins are transported to the nucleus where they stimulate the transcription of early gene, and ultimately late gene products that are required for virion assembly. In the nucleus, immature capsids are packed with viral DNA and bud through the nuclear membranes, Golgi compartments, endosomes, and cell membranes to acquire matrix and envelope components that ultimately comprise a new fully infectious particle (Figure 1, panels 4a–7a). Therefore,
the establishment of virus–host and host–host protein interactions that are temporally and spatially regulated is critical for the progression of the virus life cycle, as well as for the modulation of host antiviral defenses.

In addition to providing mechanistic insights into the biology of an infection, knowledge of temporal virus–host interactions can also reveal viral or host factors that can be targeted in antiviral therapeutics. Viral pathogens such as HIV, Ebola, influenza, and hepatitis C virus represent significant threats to human health, yet efficacious antiviral treatments are not readily available for many of them. Vaccines or effective treatments are also lacking against other significant human pathogens that trigger lifelong infections and are global human health issues, such as human cytomegalovirus (HCMV) and Dengue virus. The identification and characterization of virus–host protein interactions can point to essential events needed for viral entry, replication, or spread, which can be leveraged as new avenues for antiviral therapeutics to predict, prevent, or treat virus-induced afflictions [20, 21].

Proteomics approaches to studying virus–host protein interactions

The last decade has witnessed the increasing application of proteomic approaches to virology studies [22]. Technological advances in mass spectrometry (MS)-based proteomics, as well as in experimental workflows for antibody-based immunoaffinity purification (AP) of protein complexes, have significantly accelerated the unbiased characterization of virus–host protein interactions during viral infection. AP-MS has been successfully implemented in virology studies to isolate virus–virus and virus–host multi-protein complexes, allowing the identification of both indirect and direct protein interactions. This approach has been used to either study interactions during the progression of an infection, providing information about the temporality of interaction events, or following the overexpression of individual viral gene products to gain insight into the functions of single proteins (Figure 2A). The experimental considerations and examples of biological findings derived from AP-MS studies are detailed in the next sections.

In an effort to gain information about direct protein interactions during infection, other proteomic approaches have been implemented and are continuing to undergo development. Such methods include yeast-two hybrid (Y2H) and cross-linking (Figure 2B), and we discuss the advantages and limitations of these techniques in the corresponding sections of this review.

Given the relevance of defining virus–host protein interactions, more recently, a significant interest has been placed into developing proteomics approaches that allow high-throughput profiling of interactions. A method proven useful for such studies is based on self-assembling protein array technology, and termed Nucleic Acid Programmable Protein Array (NAPPA) (Figure 2B). In our review, we dedicate one section to discussing this method and the biological findings derived from these studies.

Altogether, these proteomics approaches have led to biological discoveries that have advanced the current knowledge of how virus–host multi-molecular protein assemblies modulate mechanisms of host cellular defense, virus replication, and virus subversion of
host defenses. Importantly, these approaches are broadly applicable to studying the life cycle and pathogenesis of many types of viruses that infect a diverse range of hosts, from human to mosquitoes and plants.

In the following sections, we review the above-mentioned proteomic methodologies that have been successfully used for studying virus–host protein–protein interactions. We explore the strengths and limitations of these approaches, with an emphasis on understanding the spatial and temporal regulation of these protein interactions. Throughout the description of these approaches, we highlight important biological discoveries attributable to these studies, as well as the diverse range of viruses that have been investigated using proteomics. We finish by providing a perspective of the promise that proteomics-based approaches hold for contributing to fundamental findings in virology.

**Affinity purification MS approaches to defining virus–host protein interactions during infection**

The ultimate goal of virus–host protein interaction studies is to discover interaction events that are critical for different stages of a viral infection, and thereby contributes to the progression and spread of an infection. In addition to characterizing virus–virus protein interactions, identifying which host proteins are targeted by viral proteins during infection can lead to an understanding of the mechanisms involved in the progression of the virus life cycle or in the inhibition of host defenses. With this goal in mind, proteomic approaches have been developed for application to studies in virally infected cells. One of the most frequently implemented methods is AP-MS. Upon viral infection of cells, a conventional AP-MS workflow first involves the isolation of virus–host protein complexes from cell lysates using antibodies against the target viral protein of interest. To obtain cell lysates (Figure 3, left panel), cells may be disrupted using several methods, including incubation with optimized lysis buffers that contain detergents, incubation with glass beads, or passage of cells through needles of appropriate gauges [23]. An effective cell disruption method that has seen increased use for virus–host interaction studies in recent years is cryogenic grinding using a ball mill. Cryogenic cell lysis was shown to provide a reproducible and effective cell disruption and can help the access to challenging protein interactions, such as those occurring within virus replication compartments or intermediate virion assemblies. Additionally, as the cells are maintained cryogenically frozen during the disruption process, and then incubated in a lysis buffer only briefly before the immunoaffinity purification, this strategy was shown to help preserve protein complexes and weak protein interactions [24-25]. Although the majority of the published virus–host AP-MS studies have so far used cell systems, these disruption approaches can be used for similar studies in tissues and animal models for studying viral infections. Additionally, should the interaction study be focused on a particular subcellular compartment, such as on associations occurring at the plasma membrane or within nuclei or mitochondria, the above cell lysis approaches can be implemented following an initial subcellular fractionation.

Following the cell disruption, the viral or host proteins of interest are isolated by incubating the clarified cell lysate with antibodies conjugated to a resin, such as magnetic beads,
sepharose, or agarose. The efficiency and specificity of the immunoaffinity isolation can be impacted by the selection of resin. The use of magnetic beads has increased considerably in recent years. By providing surface binding, these beads tend to be versatile for capturing multi-protein complexes of various sizes. Additionally, given their isolation via a magnet, these beads can be readily subjected to washing steps, reducing the presence of nonspecific associations [23].

The isolated protein complexes are subsequently enzymatically digested into peptides, which can be separated by liquid chromatography (LC) and analyzed by tandem MS [26]. Further bioinformatics analyses and interpretation of the datasets allow the assessment of interaction specificity and the generation of networks of virus–virus and virus–host protein interactions. The selection of controls is critical for assessing the specificity of the identified viral–host protein interactions. In fact, significant effort has been placed in recent years to decrease and assess the presence of nonspecific associations in AP-MS studies. A series of improvements have been made for all the different steps of the AP-MS workflows, from experimental design to data analysis and interpretation. These advances have included the use of rapid isolations [25], optimization of lysis buffers [23], generation of control databases [27], and use of computational algorithms and labeling approaches for measuring the specificity of interactions, such as the Significance Analysis of INTeractome [28–30].

To monitor the changes in virus–host protein interactions across different stages of viral infection, relative protein quantification can be performed using label-free or labeling approaches (Figure 3). Label-free quantification can be based on the comparison of number of acquired MS/MS spectra for each protein (i.e. spectral counting), or of the precursor ion intensities as defined by the peak area under the curve of extracted ion chromatograms. A commonly used labeling method is metabolic labeling with stable isotopes, such as $^{15}$N or isotope labeled amino acids (stable isotope labeling by amino acids in cell culture, SILAC) [31–33]. Metabolic labeling offers the opportunity to label samples prior to sample preparation, for example by culturing cells for multiple passages in media containing light or heavy-labeled amino acids. The samples are then combined prior to analysis, and the relative quantification is carried out by comparing ion intensities of light and heavy peptides at the MS analysis level. Although most frequently used in cell culture, these metabolic labeling methods can also be applied to animal studies. Another evolving approach is the labeling of peptides with isobaric tags, such as tandem mass tags (TMT), in which case the relative quantification is performed at the MS/MS analysis level. The advantage provided by this approach is its multiplexing ability, as samples labeled with different isobaric tags can be combined prior to analysis, making it suitable for simultaneously studying different time points of viral infection. Although this method has not been yet implemented in AP-MS analyses of virus–host protein–protein interactions, it promises to offer a valuable tool for relative quantification in the context of infection, as recently demonstrated in a global proteome study of HCMV infected cells [34].

**Isolation of viral proteins during infection**

In early implementations of AP-MS to virology, studies targeted specific viral proteins at single time points of infection. As an example, the Knipe research group analyzed the
interaction partners of the HSV-1 immediate-early protein ICP8 in human epidermoid HEp-2 cells [35]. By isolating ICP8-associated protein complexes at an early time post infection in the life cycle of HSV-1, 6.5 h post infection (hpi), they identified over 50 cellular and viral proteins, including host proteins that may be involved in viral replication, chromatin remodeling, or recombination repair pathways. Using a similar approach, the Knipe group subsequently identified the eukaryotic translation initiation eIF3 subunits p47 and p116, eIF4G, and poly-adenylate binding protein as cellular interactors of the HSV-1 immediate-early protein ICP27 in human epidermoid cells at 6 hpi. These results suggested that ICP27 may interfere with viral or host mRNA translation [36]. These interactions highlight the multiple functions that can be acquired by a protein through the formation of distinct interactions during the course of a productive infection. Despite the utility of antibodies against viral proteins, such reagents are limited by their availability and oftentimes lack of high affinity required for AP-MS studies in infected cells. To circumvent this restraint, AP may be conducted by using epitope-tagged viral strains.

**Identifying protein interactions using epitope-tagged viruses**

If tags are to be employed in the context of an authentic viral infection, it is necessary to generate a recombinant virus strain that is replication-competent and harbors a full-length genome. This strategy has been exercised for a diverse array of RNA and DNA viruses. Furthermore, the use of a fluorescent tag allows tracking the localization of proteins in live cells, in parallel with the temporal regulation of virus–host interactions [25]. This was demonstrated for the first time by Cristea et al. for the study of SINV protein interactions at different time points of infection, in which a virus strain was constructed to express the nonstructural viral protein nsP3 tagged with green fluorescent protein (GFP) [24]. Using antibodies raised against GFP, proteins co-isolating with nsP3 in fibroblasts were purified on magnetic beads and subjected to MS analyses after different times during infection, that is 2, 4, 6, 8, and 10 hpi. This study led to the discovery of time-specific interactions, with the early and stable recruitment of an endoribonuclease (G3BP), identified at all time points assessed, and the later recruitment of signaling proteins (14-3-3), identified only at the late 6, 8, and 10 hpi time points in the SINV life cycle. One hypothesis inspired by this finding and tested in a subsequent study from the same group was that the viral interaction with G3BP may serve to attenuate viral polyprotein expression during early stages of infection, while late 14-3-3 recruitment may be necessary for host translational shutoff [37]. Overall, this highlights the inherently transient nature of protein associations throughout the progression of infection.

The use of GFP-tagged virus strains in conjunction with AP-MS laid the foundation for subsequent studies on other types of viral infections, including HCMV, pseudorabies virus (PRV), and HSV-1 [38–43]. For example, temporal interactions between HCMV virion components and host factors were used to predict that multiple intermediate vesicles are involved in the assembly of infectious particles [43]. Using GFP-AP-MS studies in PRV infected neurons (neuronal growth factor-differentiated PC12 cells), the Enquist research group found that the viral protein Us9 interacts with the cellular kinesin KIF1A. A PRV strain containing GFP-tagged Us9 was used to infect neurons for 20 h, after which neurons were lysed, and Us9 protein complexes were immunopurified using the anti-GFP antibody.
As Us9 was known to be critical for anterograde neuron-to-cell transport of virion particles [44], the authors further investigate this interaction, demonstrating that KIF1A is required for the transport of viral capsid in axons and for the spread of infection [42]. Similarly, GFP-AP-MS and live cell microscopy in HSV-1-infected primary human fibroblasts led to the discovery that the viral E3 ubiquitin ligase protein ICP0 does not only target host defense proteins for proteasome-dependent degradation, but also a viral protein, the outer tegument protein pUL46. For this study, Lin et al. infected human fibroblasts with a strain of HSV-1 that expressed GFP-tagged pUL46, and the cell lysate was collected at an early and a late time of infection (6 and 14 hpi). The interaction between pUL46 and ICP0, observed and validated at both time points, was further functionally characterized and proposed to likely act as an internal regulation of viral protein levels during the virus life cycle [40].

The implementation of FLAG-tagged virus strains was also shown effective for studying protein interactions during infection. Reitsma et al. used an HCMV strain containing FLAG-labeled pUL27 within its genetic locus to uncover virus–host associations that mediate a viral immune evasion strategy [39]. Upon infection of primary human fibroblasts with the labeled HCMV virus strain, pUL27, a protein of previously unknown function, was found to be necessary and sufficient to destabilize and degrade a cellular acetyltransferase, Tip60. Cells were lysed after 24 h of infection, a relatively early time in the HCMV life cycle, and pUL27-containing protein complexes were isolated using anti-FLAG antibody conjugated to magnetic beads. The findings from this study corroborated prior discoveries for the functions of the HIV protein Tat and the human papilloma virus protein E6. Both Tat and E6 had been previously characterized as regulators of cellular Tip60 degradation in a proteasome-dependent fashion [45–46]. As such, the use of an unbiased AP-MS approach facilitated the further delineation of a potentially conserved viral offensive mechanism against host cells.

**Isolation of host proteins during infection**

Studies have also used host protein tagging and AP-MS to identify associated protein partners during infection. For example, Terhune et al. generated primary human fibroblast cell lines stably expressing the cellular histone deacetylase HDAC1 tagged with GFP, and infected the cells with HCMV for 24 h [38]. By AP-MS, it was observed that the HDAC1-containing nucleosome remodeling and deacetylase complex interacted with viral proteins pUL29/28 and pUL38. This association was ultimately implicated in facilitating the production of immediate-early viral RNAs. It is noteworthy that the study of host protein interactions during infection does not always rely on the use of tagged cellular proteins. These experiments can be performed on endogenous proteins, when antibodies are available for efficient affinity purifications. For example, the antiviral immune effector IFI16 was isolated at two early time points, 3 and 8 hpi with HSV-1 by using a combination of monoclonal antibodies against the endogenous protein [18]. Based on spectral counting provided by the MS analysis, the HSV-1 immediate-early transactivating protein ICP0 was determined to be the most abundant viral interaction with IFI16. Further functional analyses [18] provided additional support for the current model that during early stages of HSV-1 infection, ICP0 employs a virus immune evasion strategy mediated by protein associations to target IFI16 for proteasome-dependent degradation. This early association inhibits the induction of IFI16-mediated intrinsic and innate immune responses, thereby allowing viral
replication to progress [19-47]. Highlighting the importance of this host protein in defense, additional AP-MS studies have demonstrated that another herpesvirus has also acquired a mechanism to inhibit IFI16 [48-49]. Specifically, the major viral tegument protein during HCMV infection was shown to bind to IFI16 in the nucleus, thereby blocking its ability to oligomerize and initiate antiviral cytokine response.

Isolations of host proteins using AP-MS have also been used in conjunction with SILAC quantification to gain knowledge into mechanisms of viral entry. For example, Gerold et al. investigated the interactions of the cellular surface protein CD81, a known receptor for HCV entry, to understand the downstream pathways modulated by CD81 upon virus binding [32]. SILAC and AP-MS was used to compare CD81 interactions in uninfected and HCV-infected hepatoma cells (Huh-7). Serum response factor binding protein 1 was identified as a CD81 interaction, and shown to have a critical role in the cell penetration by HCV. This protein was further demonstrated to be a specific virus entry factor for HCV, functioning for all seven HCV genotypes.

Together with the continuous expansion of antibody resources, such as the human protein atlas [50], these studies emphasize the promise of AP-MS approaches for defining critical host factors during the progression of viral infections.

**AP-MS protein interaction studies using overexpression of individual viral gene products**

To study interactions during an infection process, the approach described above that involves a full-length replication-competent virus strain, is optimal. However, the production of viral strains that can be used in cell and animal systems has not always been possible. Therefore, the ectopic cellular overexpression of single epitope-tagged viral proteins provides an alternative. It is important to consider that this approach has a different biological impact on the host cell than an infection with a full-length virus, thereby limiting the data interpretation. In particular, this strategy lacks the accompanying viral cofactors and temporal protein interactions that may influence viral replication and spread. As such, the viral pathogenesis may not be recapitulated in its entirety, and both biologically irrelevant and false-negative associations may arise in a context-dependent manner. However, this approach can also be valuable when trying to decipher the independent functions of a given viral protein. Indeed, several studies have been conducted to explore the interactions of distinct viral proteins [41-51-57]. For example, GFP tagging and AP-MS led to the identification of a novel association between a protein of unknown function in the rhabdovirus bovine ephemeral fever virus, BEFVα1, and the cellular importins β1 and 7, implicating nuclear trafficking in the progression of infection [41]. For this study, BEFVα1 was fused to GFP, transfected into hamster kidney-derived cells (BHK-BSR), and isolated on agarose beads prior to MS analysis. The authors further investigated the impact of this interaction on the subcellular localization of these karyopherins, showing that overexpression of BEFVα1 leads to a slight decrease in the levels of nuclear importin β1, while not affecting the localization of importin 7. The sequestration of importins in the cytoplasm has been reported for other non-nuclear-replicating viruses, such as Ebola [58], as
a means to inhibit interferon signaling. In the case of this study on BEFV, the authors did not observe a BEFVα-mediated alteration in the nuclear deposition of known importin cargos, such as Histone H1 and SV40T antigen. Therefore, the function of this interaction remains to be further investigated in the presence of an infection with a full-length virus strain.

As an alternative approach, cellular interaction partners for a single viral protein of interest have also been investigated by *in vitro* incubation of the viral protein bait with lysates from uninfected permissive cells. As an example, Li et al. employed this approach to identify a functional cellular receptor of the severe acute respiratory syndrome coronavirus (SARS-CoV) [59]. It was previously known that the glycoprotein spike proteins of other coronaviruses associate with cellular receptors, facilitating virion entry [60–63]. To identify potential entry receptors for SARS-CoV, the authors purified a subunit of the SARS-CoV spike protein (S1) fused to the Fc domain of human IgG. The lysate of uninfected, permissive African green monkey kidney cells (Vero E6) were incubated with purified S1-Fc protein, and protein A sepharose was used for affinity purification. MS analysis revealed the cellular metallopeptidase angiotensin-converting enzyme 2 (ACE2) as an S1 interaction partner, which was further functionally characterized as a functional receptor for SARS-CoV. Viral replication was inhibited upon incubation with antibodies against ACE2, while promoted upon ACE2 overexpression in HEK293T cells that are otherwise non-permissive to SARS-CoV. In view of the knowledge that several coronaviruses use another cellular metalloprotease as a receptor for entry, this finding hints at a potentially conserved mechanism through which corona-viruses recognize and associate with cellular receptors.

Studies using overexpression of individual viral proteins were also expanded to other viruses to construct global networks of association between the virus and the host, which were followed by physiologically relevant validations [52–54].

**Global interactome studies**

An ambitious investigation sought to comprehensively characterize HIV–host protein complexes [54]. In human embryonic kidney (HEK293) and immune T lymphocyte (Jurkat) cells, Jager et al. individually expressed all viral proteins encoding genes with a streptavidin or FLAG affinity purification tag. Using AP-MS, nearly 500 specific HIV–host protein interactions were identified, approximately 40% of which were prevalent in both cell types. Although HEK293 cells are not physiologically relevant for HIV-1 infection, prior studies on protein complexes outside the context of infection have shown that information about protein complexes can be gained when using simpler cell model systems. In these cases, the follow-up investigation of these interactions in a relevant cell type is critical for supporting the findings. Therefore, the comparison of HEK293 cells with Jurkat cells, which are immortalized CD4+ T cells commonly used in HIV-1 studies, helped to substantiate the potential biological relevance of the subset of protein associations identified in both human cell types. Given that protein abundances can vary significantly between different types of cells, it is maybe not surprising that the observed overlap in interactions between HEK293 and Jurkat cells was limited. This observation highlights that protein interactions can be cell type specific, as well as the importance of performing such studies in relevant biological systems. Notably, from this study, several new associations emphasized the interplay
between virus mechanisms to stimulate replication, and cellular host defenses to thwart such strategies. A host subunit of eukaryotic translation initiation factor 3, eIF3d, was found to be targeted for cleavage by an HIV protease. When eIF3d was knocked down via RNA interference, an additional 10 host factors were found to hinder HIV replication by means of virus–host protein interactions.

Larger scale proteomic studies using overexpression of individual viral gene products have not only been performed to characterize the interactions of different viral proteins, but also to compare the interactions and functions of the same viral protein from different disease-relevant viral subtypes. Using a similar AP-MS approach, the E6 and E7 oncoproteins of up to 17 different human papillomavirus (HPV) subtypes were hemagglutinin (HA)-tagged and separately introduced by retroviral transduction in immortalized human keratinocyte cells (N/Tert-1) to identify associated host proteins [64–65]. Anti-HA antibodies coupled to agarose beads were used to isolate the E6 or E7 protein complexes. By assessing various HPV subtypes, clinically classified by severity of oncogenesis and disease, these studies were able to identify both strain-specific associations, as well as interactions conserved across HPV strains. This information may be further leveraged to differentiate proteomic characteristics of disease-causing subtypes from largely innocuous subtypes. As an example, the E6 protein of genus alpha HPV binds specifically to the cellular E6AP protein, while that of the genus beta HPV binds specifically to cellular MAML1. Both virus–host interactions are mediated by a similar motif in the cellular protein, and may be responsible for the different cell type-specific tropisms of the two genera [64–66–69].

**Tandem affinity purifications**

Despite the versatility of using a single tag for exploring interactions during infection, studies using tagged proteins in AP-MS workflows are susceptible to the co-isolation of non-specific associations. Additionally, AP-MS analyses frequently lead to the identification of hundreds of proteins that can represent strong and weak, and direct and indirect associations. Sparked by the necessity to reduce the presence of non-specific interactions and to focus on the strong associations, tandem affinity purification (TAP) tagging was developed as a method that could be integrated into AP-MS workflows. The basic principle of tandem isolation involves the tagging of a protein of interest with two different tags, which allow the subsequent purification of the protein in two sequential affinity steps. Although this approach tends not to be useful for studying transient interactions, which can be lost during the two-step isolation, this method can help in obtaining cleaner purifications in comparison to one-step isolations and for identifying strong protein–protein interactions. In its initial form, TAP tagging utilized a bi-partite fusion tag separated by a protease cleavable spacer. TAP tagging was first used to identify multiprotein yeast complexes in 1999 [70], and has since been employed in numerous biological systems, including virus–host interaction studies. For example, upon transfection of human epithelial cells (HeLa) with the TAP-tagged viral protein EBNA1 from the herpesvirus Epstein–Barr virus (EBV), Holowaty et al. identified a stable interaction with the cellular deubiquitinating protease USP7/HAUSP [71]. Based on prior analyses, it had been hypothesized that EBNA1 contained no enzymatic activity and mediated its essential role in maintaining the EBV genome in proliferating cells through interactions with cellular proteins [71–73]. The
discovered EBNA1-USP7 interaction was confirmed by co-immunoprecipitation in insect cells, which additionally demonstrated that the EBNA1-USP7 interaction does not require other human proteins. Subsequent functional assays further suggested that the virus may sequester USP7 indirectly to inhibit host-induced apoptosis and promote cell cycle growth. Based on previous knowledge that the HSV-1 immediate-early protein ICP0 significantly accelerates viral gene expression and also interacts with cellular USP7 during infection [74–76], this TAP tagging technique facilitated the demonstration of a potentially conserved herpesviral strategy to target USP7.

The TAP technology has also been successfully applied to studies of RNA viruses, many of which constitute major threats to human health. To provide insights into the biology of influenza A, TAP-tagged influenza A virus polymerase subunit and streptavidin-tagged viral nucleoprotein were used by Mayer et al. to study interacting proteins [77]. HEK293 cells were transfected with tagged proteins, cell lysates were harvested 24 h later, and TAP purification was conducted followed by MS analysis. Four proteins were discovered using the TAP-tagged bait, including polymerase-associated cellular factors, while 41 proteins were identified using the nucleoprotein bait. As an illustration of viral usurpation of cellular secretory pathways, Yamayoshi et al. overexpressed the Ebola virus major matrix protein VP40 tagged with both FLAG and HA at the N-terminus in HEK293 cells [78]. The authors established that VP40 interacts with a host component of the COPII vesicular transport system. Through sequential affinity tag purification and subsequent MS, cellular Sec24C was distinguished as a binding partner of VP40. This interaction was confirmed by co-immunoprecipitation of VP40 and localization studies upon infection with Ebola virus for three days. These findings corroborated previous observations that VP40 is sufficient to generate Ebola virus-like particles that form by budding off from host plasma membranes [79–80].

In addition to using two tags on a single protein from its two-step isolation, sequential (tandem) affinity purifications can also be used to isolate two associated proteins of interest that are tagged with two different tags. As a protein can be part of multiple protein complexes, this strategy is beneficial for honing in on one given protein complex that specifically contains two proteins of interest. With this in mind, Hrecka et al. used a dual-tag approach to investigate how an accessory factor of HIV-2, Vpx, enabled HIV-1 to transduce immune cells, monocyte-derived macrophages, which are otherwise non-permissive to HIV-1 [81]. It was previously reported that in myeloid cells transduced with HIV-2, Vpx prevents the inhibition of HIV-1 infection by associating with and hijacking a cellular E3 ubiquitin-protein ligase complex, including the proteins cullin 4 and DCAF1 [82–83]. To examine the precise virus–host associations, the authors purified this complex in the presence of Vpx by sequential affinity isolations in cells transfected with FLAG-tagged cullin 4 and HA-tagged DCAF1. Upon MS analysis, the cellular innate immune protein SAMHD1 was the most spectrally abundant association with the Vpx-E3 ubiquitin ligase complex. It was then determined that Vpx may facilitate the association of SAMHD1 with this complex to promote its proteasome-dependent degradation. As SAMHD1 hinders efficient viral cDNA synthesis during HIV infection, this study illustrates a viral usurpation mechanism by which HIV-2 evolved to establish infection in myeloid cells by inhibiting the host innate immune response.
Tagging was also proven useful and amenable for studying viral protein interactions in *in vitro* analyses. Kaul *et al.* used GST-tagged domains of a viral protein, latency-associated nuclear antigen (LANA), of Kaposi’s sarcoma-associated herpesvirus to pull down domain-specific cellular binding partners in the nuclear extract of immune cells latently infected with the virus [84]. As LANA is known to play an essential role in the modulation of latency in the immune cells, the identification of multiple proteins involved in the control of the cell cycle, DNA polymerase, and RNA polymerase support a model in which specific LANA associations may be critical for the development of oncogenesis.

**Experimental considerations when using tagged proteins in AP-MS studies**

As detailed above, the use of tagging approaches, either for studying individually overexpressed proteins or within replication-competent viral strains, can be powerful for deciphering the interplay between viruses and hosts. However, several technical challenges have to be taken into consideration when designing these experiments (as reviewed in [23]:[85]). The location and size of the tag can impact protein function, and it is critical to determine that the use of a tag does not affect virus titers or the subcellular localization of the protein of interest. While some viral proteins can be tagged at their C- or N-termini (e.g. [86]), others may require the inclusion of the tag within an internal loop (e.g. [24]). The available location for tagging is influenced by the protein conformation and by the necessity of certain domains for protein interactions. Therefore, even the inability to tag a protein at a given location can be informative and highlight a functionally important region. Similarly, while some viral proteins allow the inclusion of a relatively large tag (e.g. GFP, protein A), others require the use of a smaller tag (e.g. FLAG, V5). A larger tag tends to offer higher efficiency of isolation, as the use of polyclonal antibodies or multiple monoclonal antibodies is possible. It is conceivable that in some cases a larger tag can fold outside of a protein, while a small tag may be integrated in the protein folding, altering its function. However, the use of a smaller tag tends to be preferred, as a large tag may impact protein size, affecting its localization and interactions.

**Insight into direct protein interactions during viral infections using Y2H and cross-linking**

The use of AP-MS approaches can reveal the formation of virus–host macromolecular complexes during infection, and inform on downstream pathways regulated by certain functional protein complexes. However, these methods tend not to inform whether a protein interaction is direct or indirect. Several methods have been developed to assess direct pairwise protein interactions (Figure 2B, middle panel). As early as 1996, Bartel *et al.* applied a genome-wide Y2H screen on proteins from *Escherichia coli* and bacteriophage T7 to reveal 25 interactions [87]. Of these interactions, six were in complexes involved in DNA replication and packaging of phage particles. In 2007, an unbiased and systematic Y2H screen was implemented for the first time to investigate virus–host protein–protein interactions [88]. The study generated a protein interaction network consisting of 173 unique associations between herpesvirus EBV proteins and human proteins. Out of the 89 known EBV proteins, all or part of 85 were screened against a human spleen cDNA library in haploid yeast cells. The global strategy adopted by this study and others provided a resource.
for further hypothesis-driven investigations into the functions of both characterized and poorly understood proteins during viral infection. Similarly, using a genome-wide Y2H screen, de Chassey et al. discovered host components that critically interacted with viral replication proteins of the single-stranded RNA virus, hepatitis C virus (HCV) [89]. It was determined that the viral nonstructural proteins NS3 and NS5A, with known roles in HCV RNA replication, had an abundance of associations with cellular proteins. Interestingly, NS3 and NS5A were found to deregulate cellular focal adhesion, which could instigate tumorigenesis and cell detachment from extracellular matrix components [89]. These findings suggest that NS3 and NS5A have multifunctional roles that hijack host cellular pathways through virus–host protein binding. Further highlighting the versatility in Y2H applicability to different viruses, Khadka et al. performed a systematic Y2H screen to identify over 130 novel interactions between Dengue virus and human proteins using a human liver cDNA library [90]. By conducting co-localization, split-luciferase, and siRNA assays, the authors were able to confirm a subset of these interactions.

To gain information about both protein complexes and direct interactions in human cells, several studies have integrated Y2H screens with orthogonal AP-MS approaches. Nearly 3800 virus–host protein interactions were identified by Rozenblatt-Rosen et al. in a single study of DNA tumor virus proteins from four viruses, HPV, EBV, adenovirus Ad5, and human polyomavirus [91]. Human diploid fibroblast cells, IMR-90, were transduced with retrovirus containing ORFs from the DNA tumor viruses. Control cell lines consisted of cells transduced with GFP or the SV40 large T antigen. The identified associations stemmed from 54 viral and 1079 host proteins that pointed to viral subtype-specificity in interactions. For example, the cellular transcriptional regulators cAMP-response element binding protein and EP300 were only found to associate with the E6 oncoprotein of HPV from cutaneous subtypes and not mucosal subtypes. These differences highlight how various virus infections markedly alter the cellular proteome through specific protein interactions that may be responsible for their pathogenesis. Similarly, TAP AP-MS and Y2H were used to survey interaction between viral immune modulator proteins and the human proteome [92]. Seventy viral open reading frames representing previously identified viral immune-evasive modulators from 30 viral species were selected for TAP tagging [92–94]. From this study, researchers identified over 1600 virus–host protein associations that highlighted both species-specific and conserved viral immune-evasive strategies [92]. It was determined that 579 unique host proteins were targeted by at least one of the assessed viral open reading frames. Both of these approaches, Y2H and AP-MS, have certain limitations. For example, Y2H is known to generate false positives as proteins that may not be present in the same subcellular compartment can be artificially made to associate. Similarly, the isolation of protein complexes by AP-MS is known to be affected by the generation of non-specific associations during cell lysis, as detailed above. The integration of AP-MS with Y2H can help to filter some of the false positives and nonspecific associations generated by these approaches, and highlight the most prominent interactions for further functional analyses.

A powerful alternative approach to studying direct protein–protein interactions is chemical cross-linking, which may be used to stabilize interactions in cells. Beginning as early as the 1960s, protein cross-linking has been instrumental in uncovering numerous protein–protein interactions [95–96]. This approach has been effective in capturing transient or weak
intermolecular protein complexes, and gaining knowledge of intramolecular surface
topologies, structural conformations, as well as of the interacting amino acid residues [97].
The cross-linker is a chemical reagent that contains at least two reactive groups flanking a
linker region. Depending on the selected reactive groups, these groups will associate with
particular amino acid side chains that lie spatially close to each other. Today, numerous
chemical cross-linkers are commercially available, consisting of variable linker lengths and
chemical specificities to meet unique experimental needs. As an example, formaldehyde
crosslinking has become an integral element in numerous fundamental biochemical,
molecular, and cellular biological techniques, such as chromatin immunoprecipitation of
protein–nucleic acid complexes and the fixing of cells and tissues for immunocytochemical
assessments of protein localization by microscopy [98]. For example, crosslinking was used
to show that a host defense protein oligomerizes in response to viral infection [49]. Coupling
of chemical cross-linking with MS can be used to explore protein interaction topologies
between the virus and the host. Upon adding cross-linking reagents, cells may then be lysed
for subsequent targeted AP-MS analysis, or directly digested for large-scale interaction
studies to identify cross-linked peptides (Figure 3, right panel). This method covalently links
one protein to another in virus-infected cells, thereby preserving weak or dynamic
noncovalent protein interactions. The cross-linked amino acid residues at the interaction
interphase are subsequently identified by MS. In a prime example, Chavez et al. utilized a
remarkable cross-linking strategy, Protein Interaction Reporter technology, to generate
cross-links that are cleaved within a mass spectrometer for the identification of interacting
protein and peptide sequences [99–100]. By these means, the authors cross-linked proteins in
a purified potato leafroll virus sample and revealed topological interaction data in the virus
that are required for virus–plant associations and its transmission across aphid vectors [100].
Advantageously, this technique addresses the challenges of incomplete cross-linking and
overly noisy and complex spectral data that have hindered the broad application of
traditional cross-linking reagents coupled to MS in virology [101–103] (Figure 3, right
panel).

Global profiling of virus–host protein interactions using protein arrays

The value of proteomic approaches for studying virus–virus and virus–host protein
interactions extends beyond the use of MS-based techniques. This was recently
demonstrated by the NAPPA technology [104] (Figure 2B, right panel). Prior to the
development of NAPPA, protein microarrays were not widely adopted as a result of
difficulties in generating purified proteins to spot onto the arrays by linkage chemistry [105–
108]. To circumvent this challenge, the LaBaer lab has developed protein microarrays
containing printed complementary DNA expression vectors, the proteins of which are
expressed de novo on the chip [109–110]. The DNA encodes both the protein of interest and
an epitope tag, such as glutathione S-transferase (GST). Following an in vitro transcription–
translation step that uses a cell lysate, the generated human or viral protein is immobilized to
the array with an adjacently located anti-tag (e.g. anti-GST) antibody (Figure 2B, right
panel). To assess virus–host protein interactions, a second host protein is tagged with an
alternative epitope that is used to probe the array.

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Available as a cloning resource, the LaBaer lab has since released a panviral proteome set of 2035 open reading frame clones generated from 830 viral genes from both DNA and RNA viruses, including HCMV, HSV-1, KSHV, vaccinia virus, SINV, chikungunya virus, and yellow fever virus [104-111]. These arrays are applicable to studying protein interactions from either the virus or host perspective, as demonstrated by case studies on rubella virus and varicella-zoster virus [112]. For example, NAPPA arrays containing 10,000 purified human cDNA plasmids were incubated with rubella virus-infected cell lysates, leading to the identification and then confirmation of interactions between the viral capsid protein and host proteins [104]. Additionally, the technology has been advantageously employed to profile antiviral antibodies produced by infected cells on a high-density NAPPA array of viral antigens [111]. Detection of specific antibodies may foster the diagnosis and treatment of individuals with virus-associated chronic illnesses. Aspects to keep in mind when using this powerful technology are that the identification of an association does not inform if this interaction is direct or indirect, and that interactions dependent on posttranslational modifications may not always be captured. However, NAPPA provides a platform for the high-throughput analysis of the interactions of a particular protein of interest (viral or cellular in origin) conceivably against thousands of target proteins.

**Corroborating protein interaction datasets using validation and functional studies**

The identification of protein–protein interactions using either AP-MS, Y2H, cross-linking or protein array studies can provide critical biological insight into protein function. However, the next essential step is to validate the newly identified protein interactions. Given that these methods tend to lead to the identification of numerous putative interactions, a first validation step is usually performed using controls and computational approaches. For example, control AP-MS isolations are performed in parallel to the isolation of the viral or host protein of interest. The comparison of the proteins identified in the bait and control isolations can be performed using label-free approaches, such as comparing spectral counts or precursor ion intensities obtained from LC–MS analyses [28]. Alternatively, a more precise comparison of the bait and control IPs can be provided by relative quantification using metabolic labeling with stable isotopes [31-32], as shown by the isotopic differentiation of interactions as random or targeted method [29-113]. These comparative analyses can help remove likely non-specific associations and uncover the protein interactions that are specifically enriched with the bait viral or host protein of interest. In an attempt to predict the associations that are likely to be non-specific, several research groups have put together a resource of AP-MS controls, termed the contaminant repository for affinity purification [27]. This resource provides useful information about recurring sticky proteins in different cell types or when using certain tags or resins for the isolations. Therefore, this resource can be used in conjunction with any protein interactions studies, in particular AP-MS analyses, but also for Y2H, cross-linking, and protein array studies, to predict the likely specificity of the observed associations. However, as this resource is still growing and the available number of controls for infected cells is limited, caution has to be taken when using this repository for predicting nonspecific associations during viral
infection. This is critical, as infections can trigger substantial changes in the proteome of a cell, thereby impacting the formation of nonspecific associations.

Once the subset of predicted specific interactions have been identified, it is imperative to confirm the associations of interest by orthogonal experimental methods. A conventional method is provided by reciprocal isolation, in which the newly identified interacting protein is used as the bait. These isolations can be performed by either using antibodies against the endogenous proteins or by tagging the interacting proteins of interest and using antibodies against the tag. The presence of the initial protein of interest in this reciprocal isolation would substantiate the identified interaction. While straightforward in concept, reciprocal IPs can be challenging. For example, the success of the validation relies on the affinity of the antibodies used for reciprocal isolations. Another challenge occurs if the initial bait interacts with a protein that is either abundant or a part of multiple different complexes. This suggests that in a reciprocal isolation experiment, only a small subset of this protein would interact with the original bait, which can interfere with the validation. An alternative approach for validating an interaction is the assessment of the co-localization of the proteins by immunofluorescence microscopy. This approach informs whether the proteins have the opportunity to interact by coexisting in the same subcellular compartment, which is important for all interaction studies, but in particular for validating Y2H and protein array results. Immunofluorescence microscopy can assess the co-localization of proteins within several hundred nanometers. Noteworthy, the reciprocal IPs and the co-localization by immunofluorescence lack the ability to distinguish between indirect and direct interactions. Several optical techniques may serve as complementary or alternative approaches, such as fluorescence recovery after photobleaching, which assesses the diffusion rate of fluorescently-tagged proteins as indications of weak or strong associations with cellular structures [114]. Additionally, Förster resonance energy transfer (FRET) and proximity ligation assay (PLA) can provide insight into direct interactions in situ.

FRET may be employed to identify direct virus–host protein interactions in live cells through the detection of two fluorophores with intra- and inter-molecular distances as far as 10 nm apart [115-116]. This technique relies on fusing each protein of interest to fluorescent tags that have overlapping fluorescence spectra. Upon ectopic expression in cells and viral infection, cells may be excited by a microscopy laser. If the fluorophores of the tagged proteins are within 10 nm, the excitation will induce the lower wavelength fluorophore (donor) to physically transfer nonradiative energy to the close-by neighboring fluorophore (acceptor). FRET has been implemented to demonstrate that an antiviral host protein interacted with a viral nonstructural protein during HCV infection, potentially to limit viral replication at the replication complex [117]. The spatial resolution of FRET makes it an attractive technique to assess virus–host protein interactions, yet in practice, it requires careful optimization for reliable results. For instance, FRET signals may be attenuated despite the close proximity of two interacting proteins, if the fluorescent tags are on opposing sides of the proteins. As such, the tag location relative to the structure of the protein complex must be taken into account if FRET signals are weak or non-existent. Fluorescent tags with different levels of brightness, as well as protein complex stoichiometry outside of a 10:1 range can complicate interpretations of the fluorescent signals during FRET analysis [115].
The more recently established PLA can be used during viral infection to assess direct interactions of proteins within a range of 40 nm [118]. PLA requires the availability of an antibody specific to each protein, and that the antibodies arise from different species. PLA relies on the principle of proximity-dependent DNA ligation, which ultimately gives rise to signals from fluorescent nucleotides, seen as small puncta. Permeabilized cells are incubated with the respective two primary antibodies. Secondary antibodies against two distinct species are added, which have been previously conjugated to relatively short DNA oligonucleotides. If the two proteins are within 40 nm of each other, the subsequent addition of DNA aptamers that connect the two oligonucleotide strands together is added with DNA polymerase and fluorescently labeled nucleotides to promote ligation by rolling circle amplification. The concatameric product gives rise to fluorescent puncta. PLA has been performed to detect both protein–protein and protein–DNA interactions in virus-infected cells [119–120]. Some technical considerations for PLA include the requirement of highly specific antibodies and the incorporation of multiple controls to assess background fluorescence from the nonspecific binding of the antibodies used.

Once the protein interactions of interest are validated by any of these orthogonal methods, the value of the identified protein–protein interaction comes from understanding the biological function of the interaction during viral infection. A series of functional assays (molecular, cellular, and biochemical) can be performed to test the role of an interaction. A commonly employed approach is to assess the impact of the levels of the interacting protein on viral replication and spread. This can be accomplished by either decreasing (via siRNA, shRNA, or CRISPR) or increasing (via transfection or stable lentivirus transduction) protein levels, which can inform on whether a protein is required for viral replication or used in host defense against infection. Other functional assays can be specifically designed, depending on the prior knowledge regarding the biological functions of the identified associated proteins.

Expert commentary & 5-year view

The significant contribution of proteomic-based approaches to discoveries in virology is starting now to be well recognized [85]. With regard to understanding dynamic virus–host interactions during infection, MS, affinity purifications, cross-linking, and protein arrays are experimental platforms that are synergistically evolving and capable of high-resolution evaluations of protein interactions. To date, these advances in proteomic-based approaches have paved the way for the identification of an impressive number of virus–host protein associations that otherwise may not have been uncovered using more traditional approaches. In fact, these studies have provided the foundation for publically accessible databases, which currently contain over 5000 curated and non-redundant protein interactions between components of the virus and of the permissive host cell [121–127]. These valuable repositories include the Database of Interacting Proteins [127], VirHostNet [124–125], VirusMentha [123], IntAct-MINT, and Uniprot [122].

Despite these numerous elegant studies, this may still be considered a beginning stage of the path to fully understanding virus–host interactions during the progression of a viral infection. The knowledge of interactions, and therefore biological functions, still remains
limited for numerous viral proteins for diverse types of viral infections, many of which are threats to human health. This limited knowledge is connected to a series of experimental challenges, including the generation of appropriate virus strains, performing studies in relevant cell-model systems, and identifying interactions in the context of infection rather than following overexpression of individual viral proteins. To address some of these challenges, one avenue that scientists have probed has been the use of alternative model organisms as surrogate hosts. Noteworthy are several studies using yeast to investigate plant RNA viruses, such as tomato bushy stunt virus, Brome mosaic virus, and cucumber necrosis virus [128–131].

Recent years were also marked by substantial advances in quantitative MS-based proteomics. Some of these methods, such as label-free quantification, metabolic labeling, and targeted MS analyses, have already been successfully applied to virus–host protein interaction studies. For example, the targeted MS quantification method based on selected reaction monitoring [132–134] was recently implemented to understanding the transmission of a plant virus. Specifically, Cilia et al. assessed how aphid transmission may indirectly modulate the virus–host interactions between the cereal yellow dwarf virus (CYDV) and plants [135]. Upon feeding of aphids with virus-infected plants, several plant proteins were found enriched specifically during infection, suggesting that CYDV infection induces changes in the plant proteome that enhance virion uptake by aphids. Despite the successful integration of quantitative proteomics with virology, several valuable quantitative MS methods are yet to be applied to studying protein interactions during infection. For example, peptide labeling with isobaric tags (e.g. isobaric tags for relative and absolute quantitation or TMT) provides multiplexing that can be directly applied to studying different time points of infection.

Most importantly, proteomics is well suited to complement orthogonal approaches, including biochemical, optical, and molecular virology methods, in the pursuit of further elucidating virus–host dynamics. An emerging technique is activity-based protein profiling, a chemo-proteomic approach that employs active site-directed probes to profile the functionality of enzymatically active proteins in whole proteomes [136–137]. Additionally, live cell imaging microscopy, tomography, and optogenetics can be further integrated with proteomics to generate biological insights unattainable by individual techniques. In sum, we expect multi-disciplinary studies to have a crucial impact on future discoveries in virology, with proteomics playing an instrumental role.

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Papers of special note have been highlighted as:

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linking and MS approach uncovers topological protein features in the potato leafroll virus that are required for viral transmission and virus–plant associations. [PubMed: 22390342]


Key Issues

- Successful virus replication and pathogenesis depend upon dynamic virus–host protein–protein interactions within a permissive cell.

- The continued development and use of proteomic techniques, including antibody-based affinity purifications, cross-linking, MS, and protein arrays, have afforded novel biological discoveries pertaining to the mechanisms by which viruses progress through their life cycles and hosts mount defenses.

- Epitope tagging within either replication-competent virus strains or single viral proteins has facilitated the identification of temporally and spatially regulated virus–host interactions by immunoadfinity purification coupled to MS.

- Characterizing virus–host protein interactions can uncover new targets for the development of therapies to predict, prevent, or treat virus-induced illnesses.

- Proteomics can be readily integrated with orthogonal approaches, such as biochemical, optical, and molecular virology methods. These multidisciplinary studies are expected to contribute significantly to future discoveries in virology.
Figure 1.
Overview of the productive life cycles of (a) herpesviruses, (b) togaviruses, and (c) retroviruses in a permissive host cell. The schematic life cycles are depicted for HSV-1, SINV, and HIV, respectively. (1) Virion particles associate with the cellular plasma membrane by binding to host surface moieties and receptors. The viral membrane fuses with the plasma membrane or enters through endocytosis, releasing the virion core and matrix proteins into the cytoplasm. (2) (a) For nuclear-replicating DNA viruses, the nucleocapsid docks at the nuclear pore and ejects the double-stranded DNA (dsDNA) genome into the nucleus. (b, c) For cytoplasmic-replicating RNA viruses, the virion vesicle is acidified and releases RNA into the cytosolic milieu. (3) (a) Viral genomes are transcribed and translated. Host RNA polymerase II is co-opted to begin transcribing viral immediate-early mRNAs from the nuclear dsDNA genome, of which the translated proteins stimulate a temporal cascade of early gene and late gene transcription. (b) Viral RNA is directly translated and processed into RNA polymerases. (c) Alternatively, incoming viral reverse transcriptase reverse transcribes the RNA genome into DNA, which translocates to the nucleus and is transcribed by host RNA polymerase II. (4–6) Viral precursor glycoproteins and membrane proteins are inserted into the nuclear inner and outer membranes and endoplasmic reticulum, while others are transported to the Golgi apparatus for additional modifications. Mature glycoproteins are incorporated into the plasma membrane. (a) Immature viral cores are packed with viral DNA in the nucleus (a), or (b, c) RNA in the cytoplasm, and bud through membranes, Golgi compartments, and endosomes to acquire tegument proteins and viral lipid envelope components. (7) Upon budding from the cell surface, virion particles are released.
Figure 2.
Proteomic workflows employed to identify virus–host protein complexes and direct interactions. (A) Cells or tissue models are either infected with authentic virion particles, or manipulated to overexpress single virion proteins. Recombinant viral particles and single proteins may be additionally modified to express epitope tags for subsequent immunoaffinity isolation. To study virus–host protein interactions regulated in temporal and spatial manners, cell lysate is collected at progressive time points throughout the course of infection, and may be subjected to subcellular fractionation. (B) The isolation of virus-virus and virus–host associations has predominantly involved immunoaffinity purifications (AP) of endogenous virus and host proteins, or epitope-tagged proteins, coupled to MS-based analyses. For the identification of direct protein interactions, chemical cross-linking reagents may be applied to samples prior to MS analysis. Alternatively, yeast two-hybrid and nucleic acid programmable protein array (NAPPA) screens may unbiasedly determine associations.
Figure 3.
Frequently used AP-MS techniques used to identify virus–host protein-protein interactions. Advantages and limitations of respective techniques (label-free approaches, metabolic labeling, isobaric tagging, and chemical cross-linking) are outlined below each AP-MS workflow. The common workflow components include immunoaffinity purification of protein complexes, enzymatic digestion of proteins, nano-liquid chromatography coupled to mass spectrometry (nLC-MS/MS), and bioinformatic analysis to identify proteins. Label-free protein quantification may be performed by MS/MS spectral counting or precursor ion area integration. Metabolic labeling can incorporate stable ‘Light’ or ‘Heavy’ amino acids in cell culture (SILAC), which can be used to quantify protein interactions between two time points of infection. Metabolic labeling used within the “isotopic differentiation of interactions as random or targeted (I-DIRT) method can determine specific and low abundance interactions during viral infection. Peptide abundances can be quantified at the MS level, comparing the ion intensities of light and heavy peptides. Isobaric tagging (such as tandem mass tagging, TMT) of samples can be conducted after proteolysis. The digested peptides from each sample are differentially labeled with isobaric tags consisting of unique reporter masses. The samples are mixed together for MS analysis, and peptide quantification is assessed at the MS/MS level using the reporter ion intensities. Peptide quantitative values
derived from sequences assigned to the same protein are used to calculate the overall relative protein abundance. Chemical cross-linking may be incorporated into an AP-MS workflow prior to IP to improve the capture of weak or transient interactions. IP: Immunoaffinity purification.