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HLA Class I Molecules Reflect an Altered Host Proteome Following Influenza Virus Infection

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Abstract

Class I HLA sample and display peptides from thousands of endogenous proteins at the cell surface. During infection, the influenza virus modifies the host cell proteome by (i) triggering host anti-viral responses, (ii) hijacking host processes, and (iii) inhibiting host mRNA processing. In turn, the catalog of HLA class I peptides that decorate the surface of an infected cell is positioned to reflect an altered host cell proteome. To understand the host-encoded peptides presented by class I molecules following influenza infection, we compared by mass spectrometry (MS) peptides eluted from the HLA of naïve and infected cells. We identified 20 peptide ligands unique to infected cells and 347 peptides with increased presentation following infection. Infection with different influenza strains demonstrated that proteome changes are predominantly strain-specific, with few individual cellular interactions observed for multiple viral strains. Modeling by pathway analysis, however, revealed that strain specific host peptide changes represent different routes to the same destination; host changes mediated by influenza are found predominantly clustered around HLA-B, ACTB, HSP90AB1, CDK2, and ANXA2. The class I HLA proteome scanning of influenza infected cells therefore indicates how divergent strains of influenza pursue alternate routes in order to access the same host cell processes.

Keywords

HLA class I; MHC class I; influenza virus; host; mass spectrometry

Introduction

Class I HLA molecules are nature's proteome scanning chip. Thousands of peptides derived from proteins in multiple cellular compartments, including mitochondrial, secreted, and nuclear proteins are sampled by the class I HLA molecule and presented at the cell surface (1). Found on all nucleated cells, class I HLA proteome scanning chips sample and reflect the protein contents of a cell.

Traditionally, interest has focused upon the discovery of virus-derived peptides presented by class I HLA; a virus infects a cell, class I HLA binds a viral peptide for display at the cell

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surface, immune surveillance mechanisms discern the viral peptide/HLA complex, and the virus infected cell is destroyed (2). However, viral peptide presentation by the class I of infected cells represents a small, albeit significant, chapter of a much larger story. Following their entry into a host cell, viruses activate host gene transcription, hijack host proteins for viral replication, and degrade host proteins that impede virion production and assembly (3–5). As class I molecules reflect the proteome and changes therein, and as viruses markedly reorganize the infected host cell, we hypothesize that influenza A virus infection will evoke changes in the host class I HLA peptide repertoire.

Analogous to studies of changes in gene expression following infection (6–11), we posit that changes in the presentation of self epitopes following infection will unveil viral interactions within the host cell and imply points of therapeutic intervention. For example, self peptides unique to unhealthy cells may serve as diagnostic and/or therapeutic immune targets, as is the case for putative cancer cytotoxic T cell (CTL) epitopes (12–14). In addition, self peptides unique to infected cells reveal cellular proteins that have a role in virus replication. For example, increased class I HLA presentation of eIF4G peptide fragments following HIV infection coincides with degradation of eIF4G by the viral protease (15). Immunologically, CTL may target host-encoded class I HLA peptides that are unique to infected cells. During acute measles virus infection in humans, CTL target two HLA-A*0201 host encoded peptides (16). Realization of host ligands unique to infected cells is also positioned to help unravel the autoimmune responses associated with viral infection (17–19). Understanding changes in the host proteome following infection is poised to reveal targets for both small molecules and immunotherapy.

In this study we characterize the host peptides revealed by class I HLA following influenza A virus infection. Class I HLA-peptide complexes were affinity purified from cells expressing the soluble HLA (sHLA) class I molecule A*0201 (sHLA-A*0201). Peptides eluted from the sHLA-A*0201 of naïve cells and from cells infected with three different influenza A virus strains were separated by reverse-phase (RP) HPLC, comparatively mapped by mass spectrometry (MS), and amino acid sequenced by MS/MS fragmentation. Comparative MS analysis of HLA-A*0201 presented peptides identified 20 host-encoded ligands unique to influenza-infected cells and 347 self ligands upregulated during virus infection. Several of the class I HLA-presented host peptides are derived from proteins reported to directly interact with influenza such that numerous points of host-virus interaction, both direct and indirect, are discovered herein by scanning influenza induced peptide changes. The immune targeting and potential therapeutic impact of the changes in the class I HLA peptide repertoire following influenza infection are discussed.

MATERIALS AND METHODS

Cell Lines and Transfectants

HeLa cells (ATCC CCL-2) cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin were electroporated with a pcDNA 3.1- expression vector (Invitrogen) encoding secreted HLA-A*0201. The cytoplasmic and transmembrane domains of HLA-A*0201 were removed by PCR mutagenesis. The production of sHLA-A*0201 was measured by ELISA using anti-W6/32 (20) capture and β 2-microglobulin primary antibodies (21). The peptide repertoire of HLA-A*0201 was examined as it is the most common HLA-A allele in North America (22).

Virus Production

Influenza viruses A/Puerto Rico/8/34 (PR8), A/Oklahoma/7485/01 (7485), and A/Oklahoma/309/06 (309) were propagated in Madin-Darby canine kidney (MDCK) cells. Roller bottles

were seeded with MDCK cells at a density of 20,000 cells/cm², cultured for 2 days, washed with PBS, and inoculated with virus in 10 ml CaMg PBS at a MOI of 0.05. Infection medium (DMEM/F12K supplemented with 6% ITS, 1% penicillin/streptomycin, 1% non-essential amino acids, and 1% sodium pyruvate) was added to the cell culture after 2 h incubation at 37°C. Virus production was monitored daily by HA titration on human red blood cells. Cell supernatant containing virus was centrifuged at 3,000 rpm for 30 min to remove cell debris and stored in 200 ml aliquots at -80°C.

Cell Pharm Infection

7.5×10^9 HeLa cells were pelleted, washed 3 times with CaMg PBS, and incubated with virus at a MOI of 200 for 2 h at 37°C. Influenza-infected HeLa cells were inoculated into a cell pharm hollow fiber bioreactor (Biovest International) and cultured in DMEM supplemented with 6% ITS. Cellular glucose consumption, pH, and sHLA production were monitored daily. The percentage of HeLa cells infected with influenza was measured by intracellular staining with an anti-nucleoprotein antibody (Meridian Life Science Inc.) and anti-serum targeting the influenza core (23) followed by flow cytometric analysis.

Peptide Isolation and Purification

Approximately 25 mg of sHLA-A*0201 peptide complex was affinity purified with W6/32 antibody from the supernatant of naïve and influenza infected HeLa cells. Peptide was eluted from the HLA class I binding groove with a 10% acetic acid boil and separated from class I HLA by passage through a 3 kDa membrane (Millipore). Naïve and infected peptide pools were separated by RP-HPLC into 40 peptide containing fractions. Prior to mass spectrometric analysis, 10% of the naïve and infected peptide pools underwent 14 cycles of N-terminal Edman degradation to confirm the HLA-A*0201 origin of the eluted peptide (data not shown) (24).

Mass Spectrometric Analysis

Peptides in naïve and influenza-infected RP-HPLC fractions were sprayed 3 times via nanospray into a Qstar Elite quadrupole time-of-flight (Q-TOF) mass spectrometer to create a MS ion map for each fraction. The MS ion maps for corresponding naïve and infected fractions were aligned at 20 amu increments to identify ions (putative peptides) unique to influenza infected cells. Peptides exhibiting a fold increase of at least 1.5-fold following infection were identified by summing the intensity of each ion in 3 naïve and infected MS ion maps and calculating the normalized fold increase in the intensity of the ion in the infected versus the naïve MS ion maps (24). A threshold of 1.5-fold or more was established after mass spectrometric analysis of the HLA class I peptide pools isolated from repeat naïve cell pharm runs revealed the extent of variability in peptide presentation between cell pharm runs (unpublished). This threshold limits the impact that stochastic or random changes in protein processing have upon the data presented herein. The identity of peptides in infected MS ion maps was determined by MS/MS fragmentation followed by amino acid sequencing de novo and/or with MASCOT (25). MS/MS fragmentation of peptides in pre, post, and corresponding RP-HPLC fractions of three separate uninfected cell pharm runs and affinity purifications confirmed the absence of unique peptides in the naïve class I HLA-A*0201 peptide pool.

Predicted Binding Affinity

The binding affinity of unique and increased peptides (8-, 9-, 10-, and 11-mers) to HLA-A*0201 was predicted with the SMM binding algorithm accessed on the Immune Epitope Database (IEDB) and Analysis Resource website (26).

Pathway Analysis

Functional analysis of host proteins encoding unique or increased HLA-A*0201 peptides following influenza infection was performed with Ingenuity Pathways Analysis software (Ingenuity Systems). Analysis was limited to direct protein interactions.

RESULTS

Influenza Strain Specificity Among Class I HLA-A*0201 Self Peptides

In this study we assessed the impact of influenza A virus infection upon class I HLA presentation of host encoded peptides. We initially gathered HLA/peptide complexes from naïve and influenza infected cells. A catalog of self peptides eluted from naïve cells was utilized for comparison to the class I repertoire of cells infected with multiple strains of influenza: PR8, a well characterized H1N1 influenza A virus laboratory strain, and two recent influenza A (H1N1 7485 and H3N2 309) circulating strains. Comparative mass spectrometry of HLA-A*0201 peptides gathered from naïve and infected cells identified 20 host-encoded peptides unique to infected MS ion maps (Fig. 1A–C) and 347 different self peptides with presentation increased 1.5 fold or greater following infection (Fig. 1D). Upregulated host peptides were increased by 1.5 to 33.09 fold following influenza infection with an average increase of 4-fold.

Many host peptides demonstrated increased presentation following infection, yet only a handful were increased by multiple strains of the influenza A virus (Fig. 2). With 347 upregulated host ligands characterized following influenza infection, 39 were increased by both strains of H1N1 influenza A (PR8 and 7485) and 6 were shared by recent H1N1 and H3N2 influenza A virus isolates 7485 and 309 (Fig. 2). Only five host-derived peptides (GVYDGEHHSV-MAGEB2, KLDQDLNEV-SMC3, TLYEHNNEL-AAAS, LLDVVHPA-CCT7, and SLFEGTWYL-HMGCS1) exhibited increased presentation following infection with all three influenza strains studied (Fig. 1D and 2, Table 2). These data show that each viral strain has a unique signature of altered self ligands, that influenza A H1N1 strains overlap by approximately 10% of their upregulated ligands (39/347), and that a negligible number of upregulated ligands (6/347) overlap between the H3N2 and H1N1 isolates circulating in the human population.

Although overlap in the presentation of unique and increased host ligands between virus strains was minimal, it is possible that the class I HLA of influenza infected cells sample different peptides of the same host protein. Therefore, we determined the overlap in source proteins revealed by HLA-A*0201 following influenza infection. The 20 unique and 347 increased host HLA-A*0201 peptides are encoded by 297 different proteins. Figure 3 confirms the minimal overlap between the three influenza strains in the source proteins sampled by class I HLA, reflecting the strain specificity of unique/increased self HLA-A*0201 peptides following PR8, 7485, and 309 influenza infection. Approximately 3% of the host proteins (9/297) encoding unique/increased ligands were revealed by class I HLA following infection with all three influenza A virus strains (Fig. 3). The two H1N1 influenza virus strains PR8 and 7485 share 15% (44/297) of host proteins, the recent H1N1 and H3N2 influenza isolates (7485 and 309) overlap in 2% (6/297) of the host proteins exposed by HLA-A*0201, and class I HLA sampled only one protein exclusive to PR8 and 309 influenza strains (Fig. 3). As influenza viruses diverge, so does the signature of class I HLA presented self ligands associated with each viral strain: Unique patterns of self-ligand presentation and host proteins sampled distinguish the various strains of influenza.

Physical Characteristics Distinguish Upregulated and Unique Ligands

Nineteen of 20 unique self peptides resulting from influenza infection are not nonamers and vary in length from 7 to 13 amino acids (Table 1). In contrast, 78% of host peptides with

increased presentation in multiple influenza strains are nine amino acids in length (Table 1 and 2). Unique and upregulated ligands were further distinguished by their HLA-A*0201 binding affinity (Table 1 and 2). The average HLA-A*0201 peptide unique to infected cells has a *lower* (IC50 7570 nM) binding affinity compared to self peptides with increased presentation (IC50 971 nM) (Table 1 and 2). These data indicate that class I HLA presented self peptides unique to influenza infected cells vary in size and have a substantially lower HLA-A*0201 binding affinity when compared to ligands with increased presentation which are predominantly nonamers and exhibit a much higher predicted HLA-A*0201 binding affinity.

Different Influenza Strains are Related in Their Host Interaction

The biologic function of the host proteins most frequently sampled by HLA class I molecules following influenza infection is poised to reveal host cell functions disrupted during viral replication. The functions of source proteins encoding multiple unique and/or increased HLA-A*0201 self peptides were compared to identify common cellular functions altered by viral replication. Table 3 illustrates that infection with H1N1 influenza virus strains PR8 and 7485 triggered the class I presentation of multiple peptides encoded by proteins involved in cellular metabolism. In particular, proteins involved in aerobic and anaerobic glycolysis (i.e. GAPDH, LDHA, LDB, and PKM2) were frequently sampled by class I HLA. Numerous peptides derived from several proteins that function in protein translation elongation such as RPL10A and RPLP0 were presented by HLA-A*0201 following 7485 H1N1 and 309 H3N2 influenza infection respectively indicating a disturbance in protein synthesis (Table 3). In addition, PR8 infection displayed several unique/increased class I peptides encoded by proteins that process RNA (CPSF1, IMP3, and SNRNPG) and chaperone proteins (HSPA2 and CCT7) in the host cell nucleus (Table 3).

To determine associations among influenza mediated changes in the infected cell, host proteins encoding HLA-A*0201 unique/increased self peptides were mapped by Ingenuity Pathway Analysis software. Pathway analysis revealed that 45% (134/297) of the host proteins encoding unique/increased HLA-A*0201 ligands directly interact in the host cell (Fig. 4). Five prominent hubs of influenza/host protein interaction were revealed by these direct interactions: HLA-B, beta-actin (ACTB), heat shock protein 90 (HSP90AB1), cyclin-dependent kinase 2 (CDK2), and annexin A2 (ANXA2). All three influenza A virus isolates influenced related source proteins in these five hubs as detected by HLA-A*0201 peptide sampling (Fig. 4).

Almost half of the proteins in the network directly interact with HLA-B (31/134) and/or beta-actin (31/134), hinting at a major reorganization in the HLA-B class I presentation pathway and cytoskeleton during influenza virus infection (Fig. 4). Approximately 30% of network source proteins directly interact with HSP90AB1 (18/134), CDK2 (11/134), and/or ANXA2 (10/134) molecules which function in protein folding, cell cycle, and calcium-ion dependent phospholipid binding respectively, indicating additional host pathways likely modified by influenza replication (Fig. 4). Moreover, host changes that did not directly interact with one another in these pathways were nonetheless clustered in the same regions. For example, several ribosomal proteins that do not directly interact with one another in the host cell circle around ACTB (RPS3A, RPS8, RPS12, RPS18, RPS19, RPLP0 and RPL7A) and ANXA2 (RPS3A, RPS18, RPS19, RPL19, and RPL35). Collectively, these data reveal that despite minimal overlap in peptide presentation, the host proteins sampled by class I HLA reveal common cellular functions altered by influenza virus infection. Source proteins encoding host HLA class I peptides with unique or increased presentation following influenza infection form a highly integrated web of functional and physical interaction.

The Influenza A Virus *Directly* Interacts with Unique and Increased Self Peptide Source Proteins

Literature reveals that seven HLA-A*0201 host peptides with increased or unique presentation following influenza A virus infection are derived from five proteins that are directly involved in viral propagation (Table 4). The presentation of a nine amino acid peptide derived from actin (FAGDDAPRA) was upregulated following infection with 7485 and 309 influenza virus strains (Table 1). The binding of influenza nucleoprotein (NP) molecules to the actin cytoskeleton helps maintain cytoplasmic retention of vRNPs following nuclear export (27). Infection with the two H1N1 strains resulted in increased presentation of two nonamers (IINEPTAAA and LLDVTPLSL) derived from heat shock protein 70 (HSP70) (Table 1). During influenza infection, HSP70 facilitates nuclear export of vRNPs by binding influenza matrix 1 (M1) molecules attached to vRNPs (28,29). Infection with the H1N1 strains also resulted in the increased presentation of two nonamers (ILDKKVEKV-PR8 and ALLSSGFSL-7485) derived from heat shock protein 90 (HSP90) (Table 4). During influenza infection, the inducible (HSP90AA1) and constitutively (HSP90AB1) expressed forms of HSP90 promote viral replication by facilitating the nuclear import and assembly of influenza polymerase proteins (30–32). PR8 infection revealed the unique presentation of an undecamer peptide derived from nucleolin (TLQEVFEKATF) (Table 2). During influenza infection the non-structural protein 1 (NS1) protein colocalizes with nucleolin in the nucleoli of the host cell, although the significance of this interaction is yet unknown (33). Influenza 309 infection identified a 12 amino acid peptide encoded by annexin A2 (SVIDYELIDQDA) unique to HLA-A*0201 of infected cells (Fig. 1, Table 2), and annexin A2 is positioned to impact viral maturation by activating plasmin which cleaves the HA0 molecule into the HA1 and HA2 subunits (34–36). Thus, within the host cell the influenza virus hijacks actin, HSP70, HSP90, and annexin A2 to carry various stages of viral replication. Collectively, these data indicate that five proteins encoding HLA-A*0201 ligands unique (nucleolin and annexin A2) or increased (actin, HSP70, and HSP90) following influenza infection directly interact with the influenza A virus during various stages of viral replication.

Discussion

Systematic characterization of the HLA class I peptide repertoire is positioned to provide a unique view into the influenza infected cell. Here, we found that infection with 3 different influenza A virus strains identified 20 unique and over 345 increased class I peptides. We were initially surprised at the minimal overlap in peptide presentation and source protein origin between the three strains of influenza A virus. Overlap was limited to upregulated class I peptides; 5 ligands were increased during infection with all strains, 39 upregulated peptides were shared amongst both H1N1 influenza strains, and 6 class I ligands were increased during infection with the recent H1N1 and H3N2 strains. Substantial variation was also observed between the three influenza strains in regards to the source proteins sampled by HLA-A*0201 following infection. There was little overlap in specific peptide presentation or in source protein origin between strains of influenza.

This minimal overlap among individual peptide ligands and source proteins sampled led to the rather surprising preliminary interpretation that these three strains were markedly different in their viral-host interactions. However, a picture of host-influenza interactions began to emerge when the cell biology of these individual ligands was considered. For example, source proteins for the ligands frequently sampled following influenza infection revealed proteins involved in RNA processing, cell metabolism, and protein synthesis; functions required for host cell survival and virus propagation. In corroboration of these results, microarray studies following *in vivo* and *in vitro* influenza infection indicate an increase in the expression of genes encoding proteins involved in growth and proliferation (7,9). The dominance of source proteins

associated with cell metabolism (especially glycolysis following 7485 infection) may reflect the rapid production of viral proteins within the host cell. Most significant was that pathway modeling of source proteins for the unique and increased peptides revealed that the three different strains of influenza substantially overlap in their interaction with the infected host cell. Each of the three viruses mediated changes in the same five prominent hubs: HLA-B, ACTB, HSP90AB1, CDK2, and ANXA2 molecules were at the core of these virus-host interaction hubs. Thus, differences in peptide presentation indicate alternate routes taken by each strain of influenza A strain towards the same five cellular destinations.

It is intriguing to consider the five host destinations at which influenza arrives within the infected cell. Protein interactions revolving around HLA-B may reflect an alteration in the HLA class I presentation pathway. We have recently reported that class I molecules encoded at the HLA-B locus present immune dominant influenza peptide epitopes to CTL, and these data are corroborated by others (37–39). Moreover, it has been reported that HLA-B molecules are vital to the targeting of viruses such as HIV and EBV (40–42). Furthermore, while it is clear that influenza peptide epitopes are presented by class I HLA to CTL, the level of presentation often falls at or below the peptide presentation of low-abundance self peptide ligands (37). It is therefore possible that influenza moderates (but does not abrogate) the presentation of viral peptide ligands to immune surveillance mechanisms, especially by class I molecules encoded at the HLA-B gene locus.

Understanding how the influenza virus interacts with the host cell following infection is positioned to aid the development of anti-viral drugs and therapeutics. Realization of virus-host interactions has led to the development of the anti-viral drugs amantadine and rimantadine that target the influenza matrix protein 2 (M2) ion channel. The anti-viral drugs Tamiflu and Relenza target neuraminidase (NA) molecules at early and late stages of viral replication. Although these current influenza anti-viral drugs inhibit host-virus interactions by targeting viral proteins, the identification of host proteins that interact with viral proteins during viral replication may also serve as potent anti-viral targets. In our study we identified several host HLA class I peptides with increased or unique presentation that are derived from proteins that directly interact with influenza viral proteins in the host cell, including actin and HSP90. Other laboratories have demonstrated that drug inhibitors of actin and HSP90 reduce influenza replication *in vitro*. An intact actin cytoskeleton is required for early (virus internalization) and late (nuclear export of vRNA and budding of progeny virions) stages of influenza replication (27,43,44). Disruption of the actin cytoskeleton can drastically reduce clathrin-mediated internalization of influenza virus particles to near undetectable levels (44). Treatment of polarized epithelial cells with cytochalasin D, an actin inhibitor, drastically impedes the internalization of influenza virions to near undetectable levels (44) but increases viral infectivity of LLC-MK2 cells (45). Inhibition of HSP90 activity *in vitro* with HSP90 specific inhibitors results in decreased influenza replication (31,32). Thus, virus-host dynamics can be disrupted during influenza infection by targeting viral proteins and host molecules necessary for viral replication.

Host HLA class I peptides with unique or increased presentation following influenza infection may also serve as primary targets for CTL eliciting vaccines and T cell receptor mimic (TCRm) antibodies which recognize specific HLA class I-peptide complexes. CTL reactive host HLA class I peptides have been demonstrated during HIV-1 and measles virus infection and in the case of HIV-1 these virus-associated host ligands are being explored as vaccine candidates (16,46). Targeting host HLA class I peptides is not a novel concept as several tumor vaccines have been generated against endogenous class I peptides that mark the surface of tumor tissue (47,48). Recently, antibodies that mimic the T Cell Receptor (TCRm) and recognize tumor associated HLA class I peptides have also been developed, and these TCRm drastically reduce tumor burden *in vivo* in a tumor mouse model (14). TCRm antibodies specific for host class I

HLA peptides that distinguish influenza infected cells from healthy cells may also serve as novel therapeutics, especially against anti-viral resistant and recombinant influenza virus strains.

As nature's proteome scanning chip, HLA class I molecules are poised to reflect alterations in the proteome of virus infected cells. Characterization of the class I HLA peptide repertoire of infected cells acts as a compliment to gene and protein expression arrays, highlighting virus-induced changes in the protein composition of host cells. The host class I peptide profile presented at the cell surface not only reflects alterations in host gene and protein expression, but it also reveals changes in the post-translational modification of proteins, the subcellular location of proteins, protein degradation, and protein-protein interactions. Host proteins that are dysregulated following viral infection appear to function in viral propagation and in the anti-viral host cell response, and the identification of such virus-associated changes is positioned to provide targets for therapeutics and diagnostics. How CTL target unique/increased host HLA class I peptides on the surface of infected cells, as is the case during HIV-1 and measles virus infection, must also be explored to determine the immunogenic nature of unique/upregulated host ligands. Discovery of host ligands with altered class I HLA presentation following infection provides potential immune targets at the cell surface as well as possible points of intervention within the influenza infected cell.

Abbreviations

HLA	human leukocyte antigen
RNA	ribonucleic acid
DNA	deoxyribonucleic acid
MS	mass spectrometry
CTL	cytotoxic T lymphocyte
HIV	human immunodeficiency virus
sHLA	soluble human leukocyte antigen
HPLC	high pressure liquid chromatography
RP-HPLC	reverse phase high pressure liquid chromatography
DMEM	Dulbecco's modification of Eagle's medium
FBS	fetal bovine serum
PCR	polymerase chain reaction
ELISA	enzyme-linked immunosorbent assay
MDCK	Madin-Darby canine kidney cell
MOI	multiplicity of infection
PBS	phosphate buffered saline
ITS	insulin-transferrin-selenium
SMM	stabilized matrix method
IEDB	Immune Epitope Database
PR8	Influenza A/Puerto Rico/8/34
7485	Influenza A/Oklahoma/7485/01
309	Influenza A/Oklahoma/309/06

IC50	inhibitory concentration 50 percent
HA	hemagglutinin
vRNP	viral ribonucleoprotein
NP	nucleoprotein
M1	matrix protein 1
M2	matrix protein 2
NS1	non-structural protein 1
NA	neuraminidase
EBV	Epstein-Barr Virus
HSP70	heat shock protein 70
HSP90	heat shock protein 90
TCRm	T cell receptor mimic
Q-TOF	quadrupole-time of flight

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Fig. 1. Identification of unique and increased peptides by mass spectrometry following influenza A virus infection. The annexin A2_{202–213} (SVIDYELIDQDA) ligand was unique to influenza 309 infected cells. An ion with the mass of 690.87 atomic mass units (amu) was identified as unique to infected cells by aligning the MS spectra of corresponding infected (A) and naïve (B) RP-HPLC fractions at 20 amu increments. Amino acid sequencing of the unique ion by MS/MS fragmentation identified a peptide derived from annexin A2 (SVIDYELIDQDA) (C). TLYEHNNEL, a nine amino acid peptide derived from the AAAS protein, was increased following infection with all three influenza A virus strains (fold increase: PR8-2.49, 7485-2.84, and 309-2.2). The amino acid sequence was determined by MS/MS fragmentation (D).

Unique HLA-A*0201 Host Peptides

Increased HLA-A*0201 Host Peptides

Fig. 2.

Host encoded HLA-A*0201 peptides that are unique or increased following influenza A virus infection. Comparative mass spectrometric analysis of class I HLA-A*0201 peptides purified from naïve and influenza infected HeLa cells identified host-encoded peptides with unique or increased presentation following influenza infection. A Venn diagram reveals overlap in the presentation of unique and increased peptides observed between the PR8 (blue), 7485 (red), and 309 (green) influenza strains. The twenty host peptides unique to influenza infection were presented on a strain-by strain basis (PR8-5, 7485-14, and 309-1). While the majority of peptides with upregulated presentation following influenza infection were limited to the infecting strain, 39 host encoded HLA-A*0201 peptides had increased presentation during infection with both H1N1 strains (PR8 and 7485), 6 peptides were upregulated following 7485 and 309 infection, and 5 peptides exhibited increased presentation during infection with all influenza A virus three strains.

Fig. 3.

Source proteins encoding HLA-A*0201 peptides with unique or increased presentation following influenza A virus infection. HLA-A*0201 self peptides with unique or increased presentation following influenza A virus infection were encoded by 297 different proteins. A Venn diagram illustrates the overlap between PR8 (blue), 7485 (red), and 309 (green) influenza strains in regard to the source proteins encoding unique and increased peptides identified during influenza infection. Minimal overlap was observed in the host proteins sampled by class I HLA among the three influenza A virus strains. The most extensive overlap was displayed by the two H1N1 influenza virus strains (44/297). Both PR8 and 7485 H1N1 strains exhibited slight

overlap with the recent H3N2 isolated 309. Only 9 host proteins were shared by the three influenza strains.

Fig. 4.

Direct interactions of proteins encoding unique/increased HLA-A*0201 following PR8, 7485, and 309 influenza infection. Direct interactions between the source proteins of all unique and increased HLA-A*0201 peptides following PR8, 7485, and 309 infection were mapped by Ingenuity Pathway Analysis. Proteins encoding unique and/or increased peptides that were sampled by HLA-A*0201 during infection with all three influenza strains are shown in red. Host proteins shared by H1N1 influenza A virus strains PR8 and 7485 are illustrated in blue and proteins common to the recent H1N1 and H3N2 isolates 7485 and 309 are colored yellow. The remaining source proteins were unique to either PR8 (light blue), 7485 (pink), or 309 (green) influenza strains. The five most prominent hubs of protein interaction were centered around the HLA-B, ACTB, HSP90AB1, CDK2, and ANXA2 molecules which are circled and labeled in accordance to their dominance in the protein network.

Table 1

Unique HLA-A*0201 host peptides following influenza A infection

Fraction ^a	Ion ^b	Peptide	Strain	AA ^c	IC50 ^d	Protein	Gene
61	594.3	KLMELHGEISS	PR8	11	1369.7	Ribosomal protein S3A	RPS3A
66	579.8	LMTTVHAIAT	PR8	11	693.7	Glyceraldehyde-3 phosphate dehydrogenase	GAPDH
73	614.8	TLAEVERLKGL	PR8	11	136.1	Small nuclear ribonucleoprotein polypeptide A	SNRPA1
75	757.8	KLFDDDDTGKISF	PR8	13	NA	Caltractin	CETN2
80	656.7	TLQEVFEKATF	PR8	11	406.6	Nucleolin	NCL
47	618.3	YVPEDEDLKK	7485	10	37536.2	Coiled-coil domain containing 12	CCDC12
53	456.2	GQIGQLPQA	7485	9	1029.2	E1A binding protein p300	EP300
53	563.8	VLPTETEVAPA	7485	11	566.1	Microtubule-associated protein 4	MAP4
59	799.4	LLDVPTAA	7485	8	20959.2	Interferon gamma inducible protein 30kDa	IFI30
66	823.4	GLFDTAIS	7485	8	1209.9	Nucleoporin 160 kDa	NUP160
68	588.3	GLFGGAGVGKTVL	7485	13	NA	Mitochondrial ATPase H+ beta subunit	ATP5B
71	1071.5	FLDLEPIPGA	7485	10	26.5	5-3 nucleotidase	NT5C
73	1002.5	SLFEGTWY	7485	8	4763.9	Hydroxymethylglutaryl coA synthase	HMGCS1
77	931.5	FLLSLLIL	7485	8	820.6	Chemokine-like factor superfamily 6	CMTM6
78	673.3	ILAADFEIGHFL	7485	12	NA	Nucleosome assembly protein	NAP1L1
78	802.4	SLLDELL	7485	7	NA	Sterol O-acyltransferase	SOAT1
79	461.2	FVFPGEEL	7485	8	33102.8	Neutral amino acid transporter B	SLC1A5
82	938.4	YLFDSFF	7485	7	NA	Carbamoylphosphate synthase I	CPS1
84	895.5	YLFLGGIL	7485	8	3353.5	Bax inhibitor 1	TMBIM6
64	690.8	SVIDYELIDQDA	309	12	NA	Annexin A2	ANXA2

^aRP-HPLC fraction containing the endogenous peptide

^bIon mass in atomic mass units corresponding to the endogenous peptide

^cAmino acids

^dIC50=Inhibitory concentration 50 percent

Table 2

Increased HLA-A*0201 host peptides following influenza A infection

Fraction ^a	Ion ^b	Peptide	Protein	AA ^c	IC50 ^d	PR8	7485	309	Gene
45	460.2	FAGDDAPRA	Actin	9	1083.8	NA	5.21	7.48	ACTB
45	500.2	NLAEEKPKTV	Proteasome associated protein ECM29	9	281.2	NA	2.84	2.66	ECM29
46	508.2	ALKDKIEKA	Heat shock protein 94c	9	1392.9	2.8	3.2	NA	GRP94C
46	800.4	IIPASTGAA	Glyceraldehyde-3 phosphate dehydrogenase	9	6817.7	17.7	12.67	NA	GAPDH
47	450.2	IINEPTAAA	Heat shock protein 70	9	1060.7	21.71	4.28	NA	HSPA2/5/6/8/1A/1B/1L
48	546.2	GVYDGEHSV	Melanoma antigen, family B	10	289	9.92	1.97	1.66	MAGEB2
50	464.7	VVQDGIVKA	Peroxiredoxin 5	9	880.2	7.38	10.11	NA	PRDX5
53	516.2	GLIKSGTAEV	Pyruvate kinase	11	1940.7	NA	2.23	1.69	PKM2
53	537.2	KLDQDLNEV	Structural maintenance of chromosome 3	9	22.5	1.98	2.76	1.57	SMC3
53	578.8	FIQTQQLHAA	Pyruvate kinase	10	2280	10.17	24.26	NA	PKM2
53	959.5	SQVPEVTV	Phosphoinositide-3-kinase, regulatory subunit 4	9	335.2	NA	7.52	1.55	PIK3R4
54	497.2	NLDPAVHEV	GTPase 1	9	27.8	5.03	2.88	NA	GPN1
54	499.8	RVPPPPPIA	Heterogeneous nuclear ribonucleoprotein C	9	8604	6.54	4.96	NA	HNRNPC
54	502.7	NMVAKVDEV	Ribosomal protein L10a	9	116.9	2.93	2.12	NA	RPL10A
54	566.7	TLYEHNNEL	Achalasia, adrenocortical insufficiency, alacrimia	9	81.5	2.49	2.84	2.2	AAAS
55	589.7	YAYDGKDYYA	MHC-B antigen	10	413.9	1.6	2.74	NA	HLA-B
56	444.2	AVSDGVIVK	Cofilin 1	9	140.4	8.98	2.02	NA	CFL1
56	530.2	ILGYTEHQV	Glyceraldehyde-3 phosphate dehydrogenase	9	171.1	23.91	4.47	NA	GAPDH
57	495.3	TLAEVSTRL	Salt-inducible protein kinase-like kinase	9	101.6	NA	1.88	2.61	SNF1LK
58	432.2	LLDVVHPA	TCP-1 HIV-1 Nef interacting protein	8	644.4	33.09	1.83	1.7	CCT7
58	467.7	LLDVVHPAA	TCP-1 HIV-1 Nef interacting protein	9	265	3.43	3.82	NA	CCT7
60	474.2	ILSGVVTKM	Ribosomal protein S11	9	384.7	1.95	1.59	NA	RPS11
60	567.3	ILMEHIHKL	Ribosomal protein L19	9	9.8	5.16	3.04	NA	RPL19
62	507.3	ALNEKLVNL	Eukaryotic translation initiation factor 3, subunit F	9	110.7	1.52	2.49	NA	EIF3F
62	528.7	ALNDHFVKL	Glyceraldehyde-3 phosphate dehydrogenase	9	95.4	23.02	8.32	NA	GAPDH
64	455.8	GVLPNIQAV	Histone H2A	9	509.1	NA	8.69	4.16	HIST3H2A
64	466.7	ALDKATVLL	Programmed cell death 4	9	100.7	2.13	3.8	NA	PDCD4
64	514.8	GLIEKNIEL	DNA methyltransferase	9	56.7	1.58	1.54	NA	DNMT1
64	528.8	KVFDPPVPVGV	Deadbox polypeptide 9	10	112.8	1.74	1.97	NA	DXH9

Fraction ^a	Ion ^b	Peptide	Protein	AA ^c	IC50 ^d	PR8	7485	309	Gene
64	630.3	TLWDIQDKLK	Lactate dehydrogenase B	10	17930.7	1.64	4.79	NA	LDHB
65	1014.5	SMTLAIHEI	Degenerative spermatocyte homolog 1	9	143.6	2.11	1.58	NA	DEGS1
66	471.2	GLDPTVQV	Deadbox polypeptide 49	9	193.4	4.78	1.78	NA	DDX49
66	479.2	GLIDGRLLTI	Signal peptidase complex subunit 2	9	201.1	1.67	2.21	NA	SPCS2
67	544.2	TLWGIQKEL	Lactate dehydrogenase A	9	197.4	3.27	1.72	NA	LDHA
67	878.5	AIIGGTFTV	ER-golgi intermediate compartment 1	9	27	4.17	2.79	NA	ERGIC
68	476.2	LLGPRLVLA	Transmembrane trafficking protein 10	9	276.8	2.09	5.6	NA	TMED10
68	480.2	SLLEKSLGL	Eukaryotic translation elongation factor 1	9	46	1.64	3.7	NA	EEF1E1
68	622.8	KLIGDPNLEFV	Ras-related nuclear protein	11	245.3	2.82	7.2	NA	RAN
69	537.2	IMLEALERV	Small nuclear ribonucleoprotein polypeptide G	9	14.1	1.64	1.54	NA	SNRPG
70	537.7	SMPDFDLHL	AHNAK nucleoprotein	9	85.4	1.83	2.78	NA	AHNAK
70	625.8	FLAEEGFYKF	Subunit of the oligosaccharyltransferase complex	10	284.3	2.98	2.86	NA	STT3A
70	1019.5	GIVEGLMTTV	Glyceraldehyde-3 phosphate dehydrogenase	10	205.5	3.1	1.57	NA	GAPDH
73	541.3	TLAKYLMEL	Cyclin B1	9	16.4	6.81	3.95	NA	CCNB1
73	588.3	GLQDFDLRV	Protein kinase C, iota	10	43.7	2.29	2.9	NA	PRKCI
74	485.8	LLDVTPLSL	Heat shock protein 70	9	153.6	9.68	1.92	NA	HSPA2/8/9
75	488.7	GIWGFIKGV	Proline-rich coiled-coil 1	9	116.7	6.1	2.01	NA	PRRC1
75	533.8	FLPSYIIDV	Cleavage and polyadenylation specificity factor 1	9	2.8	2.57	2.97	NA	CPSF1
75	593.8	IIMLEALERV	Small nuclear ribonucleoprotein polypeptide G	10	43.2	13.68	4.89	NA	SNRPG
76	515.3	SLLDIIEKV	Tuberous sclerosis 2	9	9.8	2.56	1.82	NA	TSC2
79	558.2	SLFEGTWYL	Hydroxymethylglutaryl coA synthase	9	2.6	2.36	1.83	2.79	HMGCS1

^aRP-HPLC fraction containing the endogenous peptide

^bIon mass in atomic mass units corresponding to the endogenous peptide

^cAmino acids

^dIC50=Inhibitory concentration 50 percent

Table 3

Most frequently sampled source proteins

Gene	Peptides	Biologic Function
PR8		
GAPDH	9	Metabolism: Glycolysis
CCT7	2	Protein Binding
CPSF1	2	RNA processing: pre-mRNA processing
GILT	2	Oxidation Reduction
HSPA2/8	2	Protein Binding
IMP3	2	RNA processing: rRNA processing
SNRNPG	2	RNA processing: RNA Splicing
VIM	2	Cell Motility
7485		
LDHA	7	Metabolism: Anaerobic Glycolysis
ASS1	6	Metabolism: Arginine Biosynthesis
CPS1	6	Metabolism: Glutamine/Nitrogen Metabolism
GAPDH	6	Metabolism: Glycolysis
LDHB	4	Metabolism: Anaerobic Glycolysis
RPL10A	4	Protein Synthesis: Translation Elongation
ACTB	3	Cell Motility
ATP5B	3	Proton Transport
CCT7	3	Protein Binding
ADSL	2	Metabolism: Purine Ribonucleotide Biosynthesis
AHNAK1	2	Nervous System Development
ECHS1	2	Metabolism: Lipid Metabolism
EEF2	2	Translation Elongation
HMGCS1	2	Metabolism: Lipid Metabolism
KRT18	2	Cell Cycle
MTAP	2	Metabolism: Nucleoside Metabolism
NOMO3	2	Nodal Signaling
NUP160	2	mRNA Export from Nucleus
PKM2	2	Metabolism: Glycolysis
RPL12	2	Protein Synthesis: Translation Elongation
RPL13a	2	Protein Synthesis: Translation Elongation
RPL19	2	Protein Synthesis: Translation Elongation
RPS11	2	Protein Synthesis: Translation Elongation
RPS17	2	Protein Synthesis: Translation Elongation
SERPINH1	2	Protein Binding
SNRPG	2	RNA processing: RNA Splicing
VCP	2	Vesicle Transport

Gene	Peptides	Biologic Function
309		
RPLP0	2	Protein Synthesis: Translation Elongation

Table 4

Host source proteins encoding HLA-A*0201 unique and increased peptides with direct interaction with the influenza A virus following infection.

Source Protein	Peptide Sequence	Function	Influenza Interaction
Actin	FAGDDAPRA	Cytoskeleton Component	Influenza virus NP binds the actin cytoskeleton to promote cytoplasmic retention of vRNPs
		Cell Structure, Integrity, and Mobility	Integrity of cytoskeleton is necessary for virus internalization into epithelial cells
			Maintains organization of lipid rafts during virus budding
HSP70	IINEPTAAA	Correct folding of nascent polypeptides	Colocalizes with nuclear M1 to promote nuclear export of vRNPs
	LLDVTPLSL	Disassembly of clathrin-coated vesicles	Inhibits nuclear export of vRNPs at 41°C
HSP90	ILDKKVEKV	Correct folding of nascent polypeptides	Nuclear import of influenza polymerase proteins
	ALLSSGFSL	Refolding denatured proteins	Assembly of influenza polymerase complex
Nucleolin	TLQEVFEKATF	Transportation of ribosomes and ribosomal proteins between nucleus and cytoplasm	Influenza virus NS1 localizes with nucleolin in host cell nucleoli
		During Cell Stress: Inhibits DNA replication, Stimulates DNA repair, and Down regulates c-myc	
Annexin A2	SVIDYELIDQDA	Calcium dependent phospholipid binding protein	Incorporated into influenza virions
		Regulates cell growth	Converts plasminogen into plasmin. Plasmin can cleave HA into HA1 and HA2
		Involved in signal transduction pathways	