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The interactome of EBV LMP1 evaluated by proximity-based BioID approach

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ABSTRACT

Epstein-Barr virus LMP1 is an oncoprotein required for immortalizing B lymphocytes and also plays important roles in transforming non-lymphoid tissue. The discovery of LMP1 protein interactions will likely generate targets to treat EBV-associated cancers. Here, we define the broader LMP1 interactome using the recently developed BioID method. Combined with mass spectrometry, we identified over 1000 proteins across seven independent experiments with direct or indirect relationships to LMP1. Pathway analysis suggests that a significant number of the proteins identified are involved in signal transduction and protein or vesicle trafficking. Interestingly, a large number of proteins thought to be important in the formation of exosomes and protein targeting were recognized as probable LMP1 interacting partners, including CD63, syntenin-1, ALIX, TSG101, HRS, CHMPs, and sorting nexins. Therefore, it is likely that LMP1 modifies protein trafficking and exosome biogenesis pathways. In support of this, knock-down of syntenin-1 and ALIX resulted in reduced exosomal LMP1.

1. Introduction

Epstein-Barr-virus (EBV) is a member of the gamma herpesvirus family that has established a persistent infection in approximately 90% of the world's population (Cohen, 2000). In immunocompromised or genetically susceptible individuals, EBV infection can contribute to cancer development and has been linked to nasopharyngeal carcinoma, Burkitt lymphoma, Hodgkin lymphoma, post-transplant lymphomas, and a subset of gastric carcinomas (Raab-Traub, 2012). It has been estimated that EBV accounts for roughly 200,000 new cancers each year and nearly 2% of the total global cancer burden (Cohen et al., 2013). The latent membrane protein 1 (LMP1) is expressed in most EBV-associated cancers and it is well established that LMP1 is a viral oncogene (Kempkes and Robertson, 2015; Kieser and Sterz, 2015; Ribeiro et al., 2017; Yoshizaki et al., 2013). Expression of LMP1 alone is sufficient to transform cells, and recombinant EBV lacking LMP1 is incapable of immortalizing B-cells in vitro (Kaye et al., 1993; Wang et al., 1985). Moreover, expression of LMP1 in animal models results in the development of lymphomas and carcinomas (Kulwichit et al., 1998; Minamitani et al., 2017; Shair et al., 2007, 2012; Zhang et al., 2012).

The oncogenic properties of LMP1 are due to its ability to mimic CD40 receptor signaling in the absence of an external ligand through the recruitment of tumor necrosis factor-associated factors (TRAFs) and other effector molecules to signaling domains (CTARs) in its C-terminal cytoplasmic tail (Miller et al., 1998; Mosialos et al., 1995). The molecular events orchestrated by LMP1 result in the activation of a plethora of signaling pathways, including mitogen-activated protein kinase (MAPK/ERK), phosphatidylinositol 3-kinase (PI3K)/AKT, NF-kB, mammalian target of rapamycin (mTOR), and c-Jun N-terminal kinase (JNK). The activation of these pathways results in transcriptional up-regulation of a multitude of genes that are involved with the regulation of apoptosis, cell cycle progression, cell proliferation, migration, and invasion (Cahir-McFarland et al., 2004; Dawson et al., 2003; Eliopoulos and Young, 1998; Everly et al., 2004; Fries et al., 1996; Kung et al., 2011; Laherty et al., 1992; Mainou et al., 2005; Meckes et al., 2013b; Shair et al., 2008). Despite the importance of LMP1 in EBV-associated tumorigenesis there is still an incomplete understanding of the spatial and temporal molecular events that orchestrate downstream LMP1 signaling and transformation.

In addition to its effects within infected cells, LMP1 has been found to be secreted from cells in extracellular vesicles (EVs) called exosomes (Dukers et al., 2000; Keryer-Bibens et al., 2006; Meckes et al., 2010). These 30–150 nm membrane-bound sacs are formed in the host cell endo-lysosomal pathway following budding and fusion events on the limiting membrane of multivesicular bodies (MVBs) (Meckes and Raab-Traub, 2011). Fusion of the MVB membrane with

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the plasma membrane releases the intraluminal vesicles into the extracellular space where they can be transported to distal sites and taken up by recipient cells. We discovered that LMP1-modified exosomes can exert oncogenic signaling functions on neighboring uninfected cells (Meckes et al., 2010). More recently, we demonstrated that EBV dramatically alters the protein content of exosomes released from latently infected B-cells, and that most of the significant changes correlate with LMP1 expression (Meckes et al., 2013a). Others have now established that EBV-modified exosomes enhance proliferation, migration, invasion, and B cell differentiation toward a plasmablast-like phenotype (Aga et al., 2014; Gutzeit et al., 2014; Nanbo et al., 2013). Together, these data suggest that EBV-infected cells can manipulate the tumor microenvironment through the transfer of virally-modified exosomes and may contribute to cancer development and progression (Meckes, 2015). Based on our findings and others, it is likely that LMP1 is an important viral factor contributing to exosome content and function during EBV infection.

In spite of recent advances in our understanding of LMP1-modified exosomes, very little is known about how this viral protein actually enters or manipulates the host exosome pathway. Recent evidence from our lab demonstrates the importance of the tetraspanin protein CD63 in LMP1-mediated enhancement of vesicle production, exosomal trafficking, and signaling (Hurwitz et al., 2017a). Furthermore, it was found that LMP1 still localizes to lipid raft microdomains, activates PI3K/AKT and canonical NF-κB pathways, and transforms cells in the absence of CD63. Interestingly, we observed an increase in LMP1-activated non-canonical NF-κB and MAPK/ERK signal transduction in cells lacking CD63 expression. These data suggest that CD63 may act as a negative regulator of LMP1 signaling and we proposed that the exosomal trafficking of LMP1 is tightly linked to its signaling properties.

Here, using a recently developed proximity-dependent biotin identification (BioID) approach (Roux et al., 2012), we sought to define the wider interactome of LMP1 to gain insights into LMP1 trafficking and signal transduction. The BioID method allows for the identification of proteins that come within close proximity of a protein of interest in live cells by taking advantage of a mutant form of the *Escherichia coli* biotin ligase (BirA). The mutated biotin ligase (BirA [R118A] or BirA*) has lost specificity for its natural target and now promiscuously biotinylates proximal proteins. Compared to traditional co-immunoprecipitation or pull-down methods, the BioID technique is particularly useful in the study of insoluble or inaccessible structures and weak or transient interactions (Varnaitė and MacNeill, 2016). As LMP1 is a large multi-pass transmembrane protein that interacts with the cytoskeleton and localizes to detergent-insoluble membrane microdomains (e.g., lipid rafts and tetraspanin enriched microdomains [TEMs]) (Ardila-Osorio et al., 2005; Meckes et al., 2013b; Yasui et al., 2004), BioID combined with mass spectrometry is ideal approach to identify and study LMP1 interacting proteins and proximal complexes.

Combining BioID with traditional affinity purification methods, we identified over 1000 proteins across seven independent experiments that have direct or indirect associations with LMP1, including previously described LMP1-interacting proteins. Newly identified proteins were enriched in endosomal, signal transduction, metabolic, and transport processes. Interestingly, more than seventy five percent of the proteins identified have been found in extracellular vesicles. Some of these interacting molecules are important for exosome targeting and formation including CD63, syntenin-1, ALIX, TSG101, HRS, charged multivesicular body proteins (CHMP, and sorting nexins. Overall, the findings described in this study provide new insights into LMP1 oncogenic signaling properties and manipulation of vesicular trafficking pathways that may result in altered EV cargo. Future mechanistic studies aimed at specific protein-protein interactions will be critical for understanding these important cellular processes and may offer new therapeutic targets to combat EBV-associated cancers.

2. Methods

2.1. Cell lines and transfection

Human Embryonic Kidney 293 (HEK293) cells were cultured in media composed of DMEM (Dulbecco's Modified Eagles medium, Lonza; 12-604Q) supplemented with 10% fetal bovine serum (FBS; Seradigm; 1400-500), 2 mM L-glutamine (Corning; 25-005-CI), 100 IU of penicillin-streptomycin (Corning; 30-002-CI), and 100 μg/mL:0.25 μg/mL antibiotic/antimycotic (Corning; 30-002-CI). Cells were transfected using JetPrime (Polyplus, 114-15) transfection reagent according to the manufacturer's protocol. Cells were incubated overnight, and the following day (after 12–16 h), biotin was added to each dish to a final concentration of 50 μM and incubated for an additional 24 h.

2.2. DNA constructs

Myc-BioID-LMP1 was constructed by PCR amplification from pBabe-HA-LMP1 (a gift from Nancy Raab-Traub) with primers containing BamHI (Forward primer - AAAAAAGGATCCAATGGAACA CGACCTTGA) and HindIII (Reverse primer - CCCCCCAAGCTTTTA GTCATAGTAGCT) restriction sites using Platinum Taq High Fidelity (Invitrogen) according to the manufacturer's instructions. The resulting PCR product was digested and ligated in frame into pcDNA3.1 mycBioID (Addgene #35700) cut with the same restriction enzymes (Roux et al., 2012).

LMP1-BioID-HA was generated by PCR amplification of LMP1 with primers containing NheI (Forward primer- AACGCTAGCATGGAAC ACGACCTTGAG) and BamHI (Reverse primer - CTTGGATCCAACGTCA TAGTAGCTTAGC). The stop codon of LMP1 was omitted from the reverse primer to allow for complete translation of the C-terminal fusion protein. The resulting PCR product and pcDNA3.1 MCS-BirA*(R118G)-HA (Addgene #36047) vector DNA were cut with BamHI and NheI restriction enzymes (NEB), ligated using T4 DNA ligase according to the manufacturer's instructions (NEB), and propagated in DH5α *E. coli* following DNA transformation. The pcDNA3.1 backbone vectors contain CMV promotors that drive high physiological levels of LMP1 expression and were chosen to better identify low abundant, weak, or transient LMP1 interacting proteins. The pcDNA3.1-based vectors have been used throughout the literature to study LMP1 signaling, trafficking, and protein-protein interactions (Devergne et al., 1998; Miller et al., 1998; Kim et al., 2000; Li et al., 2004; Verweij et al., 2015).

pBabe LMP1-BirA*(R118G)-HA and pBabe myc-BirA*(R118G)-LMP1 were constructed by cloning LMP1 sequence containing the BioID tags into pENTR1A no ccDB (w48-1) vector (Addgene #17424, gift from Eric Campeau) followed by LR recombination into pBabe-puro-gateway (Addgene #51070, gift from Mathew Meyerson) (Campeau et al., 2009; Greulich et al., 2012). The construction of GFP-LMP1 used in this study was described previously (Hurwitz et al., 2017a). CD63 BirA* was generated by PCR amplifying CD63 from the vector RFP-CD63 pQCXP CMV/TO. Gel purified PCR products and pcDNA3.1-MCS Bio-ID were cut using Nhe and EcoRI restriction enzymes. The purified products ligated overnight at 16 °C using T4 DNA ligase. The ligation then used for transforming DH5α and plated on ampicillin-resistant LB-agar plates and grown overnight at 37 °C for transformed colonies. After miniprep isolation of plasmid DNA, the inserts were confirmed by diagnostic digesting and DNA sequencing. BirA*CD63 was generated in a similar way, but PCR amplified from pCT-CD63-GFP (SBI, CYTO120-VA-1) and EcoRI and HindIII were used for digesting the purified PCR products and the vector pcDNA3.1-myc-BioID. Both CD63 BirA* and BirA* CD63 were subjected to point mutations on the CRISPR guide DNA sequences using GENEART mutagenesis kit to mutate the human sequence into mouse. As a result, the CRISPR/Cas9 utilized no longer targeted the mutated DNA sequence, and allowed re-introduction of CD63 constructs under CD63 CRISPR background (Hurwitz et al., 2017a).