Determining the Function of ALTO, a Novel Protein of Merkel Cell Polyomavirus

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Abstract

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Merkel Cell Polyomavirus (MCPyV) was discovered clonally integrated in a rare skin cancer called Merkel Cell Carcinoma (MCC). Our group recently showed that MCPyV has a novel gene named Alternative to Large T Open reading frame (ALTO). ALTO's function is unknown. ALTO is not an oncogene but it is related to Middle T antigen (MT), the main oncogene of the well-studied Murine Polyomavirus (MuPyV). ALTO and MT have a conserved membrane anchor and an adjacent basic amino acid motif. These domains allow MT to associate with membrane cytoskeleton and to localize with cellular interactors. ALTO also has tyrosine motifs that resemble those of MT. For MT these tyrosines are docking sites for cellular interactors. However, ALTO and MT are very divergent. We used affinity-purification mass spectrometry to identify ALTO's binding partners. We tested the effect of ALTO on the activities its interactors so as to gain insight into ALTO's function.

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1. Introduction and background

I. Polyomavirus genome structure

Polyomaviruses are non-enveloped viruses with circular dsDNA genomes of about 5kb [1, 2]. The genome is divided into two temporally regulated transcriptional units—Early Region (ER) and Late Region (LR)—and a non-coding control region which has the origin of replication as well as enhancer and promoter elements [1, 2]. The ER encodes T antigens expressed during viral genome replication, and the LR encodes structural proteins expressed late in the lifecycle. Large T antigen (LT) and Small T antigen are expressed from the early region and they both interact with chaperones through an N-terminal J domain but they have different C-termini [3, 4]. LT has an origin binding domain (OBD) and a helicase domain to unwind the viral genome for replication and an LXCXE motif that binds to RB-family proteins to induce cell-cycling [5, 6]. The ST C-terminus binds to and deregulates the phosphatase, PP2A [2]. An additional T antigen, Middle T (MT) is found in rodent polyomaviruses; MT's first exon is similar to ST, but MT is spliced so its second exon is in the +1 frame [2]. MCPyV encodes a novel gene, ALTO, also found in the +1 frame; its coding sequence overlaps with LT's LXCXE motif and also partly with the OBD [7]. ALTO can theoretically be expressed from any of the alternatively spliced transcripts of the MCPyV early region [7].

II. Comparison of MCPyV to well-known polyomavirus models of oncogenesis

There are two well-described polyomavirus models of oncogenesis; MuPyV and simian polyomavirus SV40. SV40 LT is necessary to transform cells, and this is a result of direct binding and inactivation of the tumor suppressors RB and p53 [1]. RB inactivation

allows cell-cycle entry, and tumor viruses often inactivate both RB and p53 because ectopic inactivation of RB triggers p53 mediated apoptosis [8]. MuPyV LT, on the other hand, binds and inactivates RB but not p53 [2]. MuPyV MT is the oncogene necessary for cell-transformation, it activates tyrosine signaling leading to cell growth [9, 10]. In a limited manner MCPyV resembles the MuPyV model because its LT protein binds and inactivates RB but not p53 [11]. Further, MCPyV LT has not been clearly linked to cell transformation [11, 12].

MCPyV ST binds and deregulates PP2A, and this is the case for SV40 and MuPyV as well [1, 2, 16]. This PP2A deregulation has several effects that can enhance transformation by SV40 LT and MuPyV MT, but in either case ST is not sufficient for transformation [1, 2]. However, MCPyV STs role in oncogenesis is an area of debate; in one report it drove cell transformation by increasing the levels of phosphorylated 4EBP1 and cap dependent translation, but another group reported that MCPyV ST is dispensable for MCC growth [13-15]. Moreover, the mechanism of MCPyV ST's influence on 4EBP1 is unclear since binding to PP2A was not necessary for transformation [14, 16]. Finally, ALTO is not a transforming protein [J Carter (FHCRC), unpublished]. Therefore, the mechanism by which MCPyV causes tumorigenesis is largely unresolved but MCPyV has some resemblance to the MuPyV model of oncogenesis.

III. MCPyV association with Merkel Cell Carcinoma (MCC)

MCC is a highly aggressive skin cancer that affects about 1500 people in the U.S each year [17, 18]. Merkel cells are skin mechanoreceptor cells responsible for the sense of

touch [17]. MCC presents as a red dome-like lesion usually in the head and neck area, and it predominantly affects immunocompromised individuals [17, 18]. MCPyV is strongly associated with MCC: The viral genome is clonally integrated in 80% of cases [19]. Expression of MCPyV proteins is important for MCC growth because shRNA-knockdown of the viral early transcript in an MCPyV-positive MCC cell line reduced cell proliferation while a similar knockdown in an MCPyV-negative MCC cell line did not affect proliferation [20]. But for the virus, causing cancer appears to be an unfortunate accident that is not part of the normal lifecycle. In almost all instances of MCPyV integration in MCC there are characteristic mutations in the early region of the virus that preclude replication [21, 22]. These mutations cause truncations of the LT protein such that its OBD and helicase domain are lost. The ALTO gene partially overlaps with the coding sequence for the OBD, and in many MCC tumors ALTO is also predicted to be truncated or lost [7]. Furthermore, using western blot and immunohistochemistry ALTO expression was not detected in MCC tumors, not even in those tumors that have the full-length ALTO reading frame [J. Carter (FHCRC), unpublished]. This is in contrast to truncated LT and full-length ST which are readily detected in MCPyV-positive MCC tumors [14, 23]. It is therefore unlikely that ALTO plays a role in MCC.

IV Similarities between ALTO and MT provide clues about ALTO's binding partners

ALTO is related to MT, and since MT is well-studied it is useful to compare the two proteins. MT is a membrane-anchored protein that inserts into membranes in the endoplasmic reticulum and traffics to the plasma membrane through vesicles [24]. MT's hydrophobic domain is necessary for localization, for interaction with signaling proteins and for function [24]. MT binds to Src Family Kinases (SFK) through protein sequence

encoded by both MT exons [Fig.1]. MT activates SFK's and induces tyrosine signaling that promotes cell-growth creating an environment conducive for viral replication, and thus a knockout of MT significantly reduces viral replication [1, 25]. ALTO knockout did not affect replication of the viral genome, but the effects of ALTO on other phases of the lifecycle have not been tested [7]. Like MT exon two, ALTO is in the +1 reading frame but ALTO does not have any sequence corresponding to MT exon one [Fig. 1]. Additionally, ALTO and MT are very divergent and the only conserved feature between these two proteins is a C-terminal hydrophobic domain and an adjacent basic amino acid motif [7]. The conserved hydrophobic domain serves as the membrane-anchor for MT while the basic amino acids play a role in localizing MT with membrane cytoskeleton [24, 26]. Therefore, it is plausible that ALTO has similar localization to MT as a result of having the basic amino acid motif and the hydrophobic domain.

ALTO has tyrosine motifs that resemble tyrosine motifs found on MT. When tyrosine's 250, 315 and 322 on MT are phosphorylated by src they serve as respective docking sites for Shc, PI3K and PLCγ [Fig. 1]. These cellular proteins that interact with MT have Src homology 2 (SH2) domains that recognize phosphorylated tyrosine's in specific conserved motifs [27]. All SFKs have a similar organization with an N-terminal myristoylated region, SH3 and SH2 domains, followed by a kinase domain and a C-terminal-regulatory region [28]. The SH3 and SH2 domains function in regulating kinase activity. The SH3 domain recognizes a specific proline motif in the linker region between the SH2 domain and the kinase domain, while the SH2 domain recognizes specific tyrosine motifs in the regulatory C-terminal region [27-29]. The intramolecular interactions between SH domains and their corresponding recognition motifs cause the

protein to adopt a closed conformation that reduces kinase activity [27-29]. On the other hand, cellular proteins containing specific tyrosine motifs and/or proline motifs can bind to SFKs, prevent the intramolecular binding of the SH domains, and thereby, relax the SFK's conformation and increase kinase activity [27-29]. It is important to note that SFKs bind to MT through protein sequence encoded by both MT exons and ALTO does not have this sequence**.** Nonetheless**,** ALTO's two tyrosines can potentially be recognized by SH2 domains of SFKs and other SH2 domain-containing cellular proteins [Fig.1]. ALTO is also proline-rich and has multiple motifs that could be recognized by SH3 domains [7].Therefore, we hypothesized that ALTO interacts with proteins that have SH2 and/or SH3 domains.

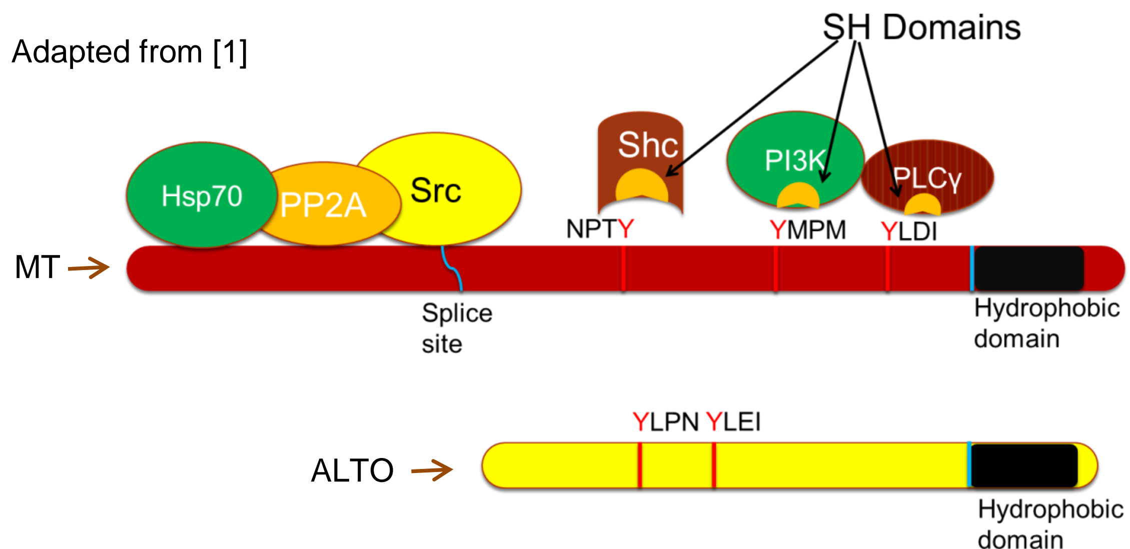


FIG. 1. ALTO and MT have limited similarities. (Top) Middle T antigen (MT) is shown bound to its interactors. The conserved hydrophobic domain is shown in black. Src binds MT via sites encoded on both MT exons. Shc, PI3K and PLCγ bind to MT tyrosines. (Bottom). ALTO has similar tyrosine motifs to MT but ALTO does not have sequence corresponding to the MT first exon.

2. Specific aims

The identification of a novel gene in MCPyV raises questions about its function. Preliminary evidence indicates that ALTO is not an oncogene and it likely does not play a role in MCC. ALTO does not have enzymatic activity and the most plausible mechanism by which it could function is through direct protein-protein interactions. As mentioned above ALTO has tyrosine and proline motifs that could be recognized by SH domain-containing proteins. We therefore sought to identify ALTO's binding partners as a way to investigate its function. The specific aims of this study are as follows;

I. AIM 1: Identify cellular proteins that interact with ALTO. We used Affinity Purification-Mass Spectrometry (AP-MS) to identify ALTO interactors. We confirmed AP-MS hits with co-immunoprecipitation and co-localization experiments.

II. AIM 2: Determine ALTO's effects on SFK signaling. AP-MS results indicated that ALTO binds to Src Family kinases (SFKs). SFKs can be activated by ligand binding so we tested the effect of ALTO on SFK kinase activity. A known SFK substrate, Beta-catenin, was identified as an ALTO interactor. Therefore, we investigated whether SFK phosphorylation of Beta-catenin is affected by ALTO.

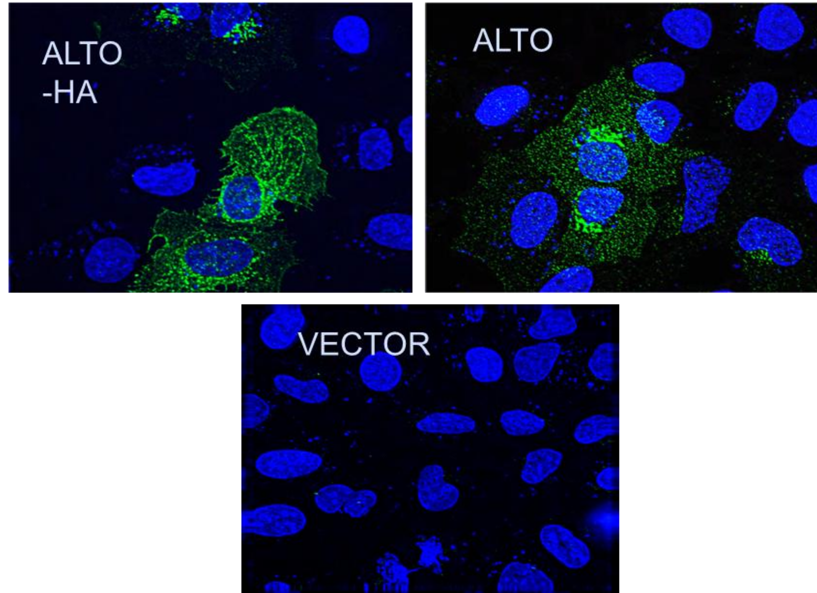
3. Methods

I. Co-immunoprecipitaton and sample preparation for Affinity-Purification-Mass Spectrometry.

ALTO was tagged with the HA epitope at the C-terminus [Fig. 2A]. The decision to tag the C-terminus was based on data showing that tagging MT at the N-terminus abrogates function while tagging it at the C-terminus does not [S. Dilworth (Imperial College), unpublished]. Immunofluorescence was used to check that the HA tag did not change ALTO's localization: The ALTO-HA fusion protein was localized in punctate structures similar to wild type ALTO [Fig. 2B]. Next, ALTO-HA and ALTO were stably transfected into U2-OS cells. The ALTO-HA stable transfection was repeated on a different day to make biological replicates. Lysates of ALTO-HA and ALTO stable cells were immunoprecipitated with HA-coupled magnetic beads [Pierce, Rockford, IL], the beads were washed with 0.5% NP40, a low stringency buffer so as to preserve transient interactions, and protein was eluted from the beads using HA-peptide (Sigma Aldrich, St Louis, Mo). ALTO was detected in IPs from ALTO-HA cells but not from ALTO cells [Fig. 2C]. This confirmed that ALTO-HA was specifically immunoprecipitated using HA antibody. Eluted proteins were separated by poly-acrylamide gel electrophoresis, and whole-lanes corresponding to ALTO-HA sample or ALTO control were excised from the gel. The gel slices were subjected to in-gel trypsin digestion and mass-spectrometry (FHCRC proteomics). The AP-MS experiment was repeated on a different day using the biological replicate stable cells.

A.

B.



C.

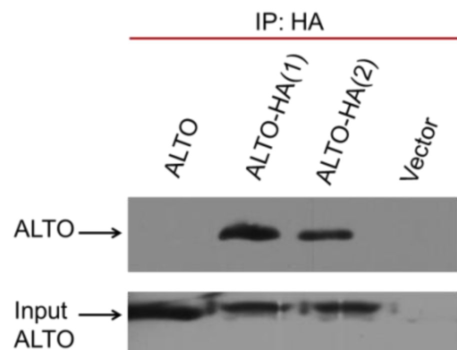


FIG. 2. Affinity purification protocol. (A) The HA epitope was cloned onto the C-terminus of ALTO. (B) Immunofluorescence of U2-OS cells transiently expressing ALTO-HA, ALTO or vector. Staining with ALTO-specific antibody shown in green and DAPI staining of nuclei is in blue. (C) Cell Lysates of U2-OS cells stably expressing ALTO-HA, ALTO or vector were Immunoprecipitated with HA-coupled magnetic beads and western blotted with an ALTO-specific antibody. ALTO-HA (1) and ALTO-HA (2) are biological replicates.

II. Mass-spectrometry data analysis

Proteins identified in the AP-MS experiments were compared to contaminants identified in published AP-MS experiments so as to identify hits most likely to be true ALTO interactors. The database used was the Contaminant Repository for Affinity Purification-mass-spectrometry experiments (CRAPome) [30]. The CRAPome software was used to score potential interactors: Hits least-often detected in negative controls of published AP-MS experiments get the highest scores while hits that are more commonly detected in negative controls are given lower scores.

4. Results

I. Hits identified by AP-MS

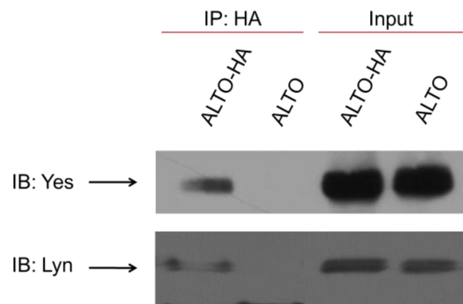
Biological replicate AP-MS were performed to identify ALTO's binding partners, and as described in the methods, hits were analyzed using CRAPome software. After this analysis the number of hits was 300 for the first experiment and 175 for the second experiment with 75 proteins being found in both experiments. Table 1 lists hits identified in both biological replicate experiments. They are grouped into cytoskeletal proteins, proteins that have SH3 and SH2 domains, and known substrates of SFKs. All of the top 5 potential interactors were cytoskeletal proteins, mainly actin binding proteins and actin filaments. The highest scored interactor in both experiments was the actin-binding protein, plectin. Among the proteins that have SH3 and SH2 domains were SFKs; yes and lyn. Also, Beta-catenin, a known substrate of SFKs was detected in both biological replicate AP-MS experiments.

Table.1. Hits identified by Affinity Purification-Mass-Spectrometry

Cytoskeletal proteins	Rank(/75)
Plectin	1
Tropomyosin beta chain	2
Unconventional myosin	3
Tropomyosin Alpha	4
Drebrin	5
SH2 and SH3 containing proteins	
Src family kinase, Yes	22
Src family kinase, Lyn	37
Known substrates of Src family kinases	
Beta-catenin	55

II. Confirmation of AP-MS Hits

Co-immunoprecipitation (co-IP) and co-localization experiments were then used to confirm AP-MS hits. ALTO co-immunoprecitated with yes and with lyn [Fig. 3A]. ALTO and yes also partially co-localized [Fig. 3B]. This confirmed that ALTO interacts with SFKs. Given the fact that cytoskeletal proteins were identified as potential ALTO interactors we decided to test whether ALTO co-localizes with filamentous actin which is known to interact with MT [26]. Immunofluorescence performed on ALTO expressing cells revealed partial co-localization between ALTO and filamentous actin [Fig. 4]



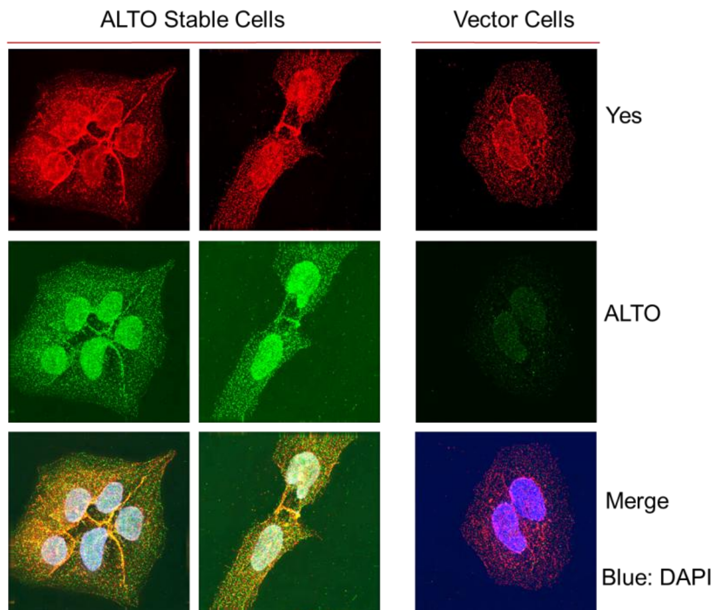


FIG. 3. ALTO interacts with src family kinases. (A) Cell lysates from U2-OS cells stably expressing ALTO-HA or ALTO were immunoprecipitated with HA-coupled magnetic beads and blotted with antibodies specific for yes or lyn. (B) Immunofluorescence of U2-OS cells stably expressing ALTO or vector; staining with yes antibody in red, ALTO-specific antibody shown in green, and DAPI staining of nuclei is in blue.

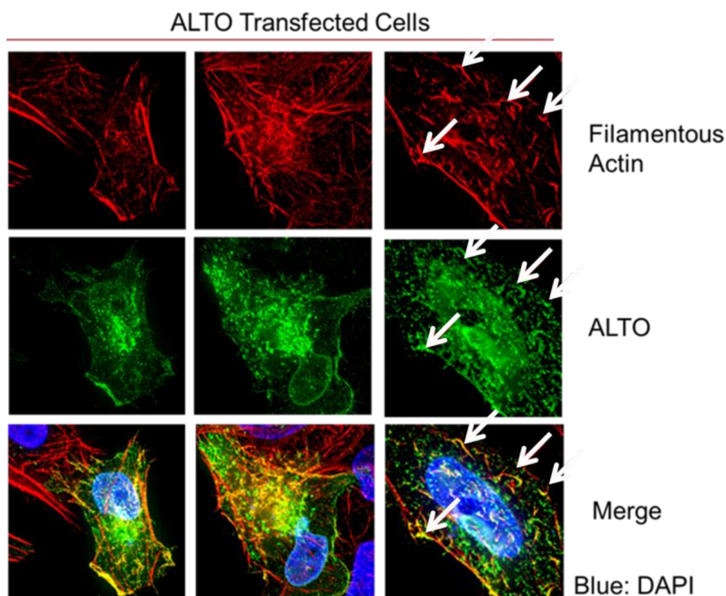


FIG. 4. ALTO partially co-localizes with actin. Immunofluorescence of U2-OS cells transiently transfected with an ALTO expression construct; staining for filamentous actin in red, ALTO-specific antibody shown in green, and DAPI staining of nuclei is in blue. Arrows indicate regions of overlap between ALTO and filamentous actin

III. ALTO effect on SFK signaling

The protein conformation of SFKs changes upon ligand binding to their SH2 and SH3 domains and the kinases become more active [28]. ALTO bound to the SH domains of SFKs (src, fyn and yes) and increased tyrosine phosphorylation of cellular proteins by these kinases [J. Carter (FHCRC), unpublished].

In light of evidence that ALTO activates SFKs we hypothesized that ALTO forms complexes with SFKs and specific substrates and this leads to increased tyrosine phosphorylation. Since we had identified Beta-catenin, a known src substrate as a potential ALTO interactor we decided to test whether its phosphorylation by src is affected by ALTO. Beta-catenin was phosphorylated at higher levels in 293 cells expressing ALTO plus src compared to src alone, and mutation of ALTO's two tyrosines abrogated this increase [Fig. 6]. Therefore, our preliminary data suggests that ALTO activation of SFKs influences the Beta-catenin pathway.

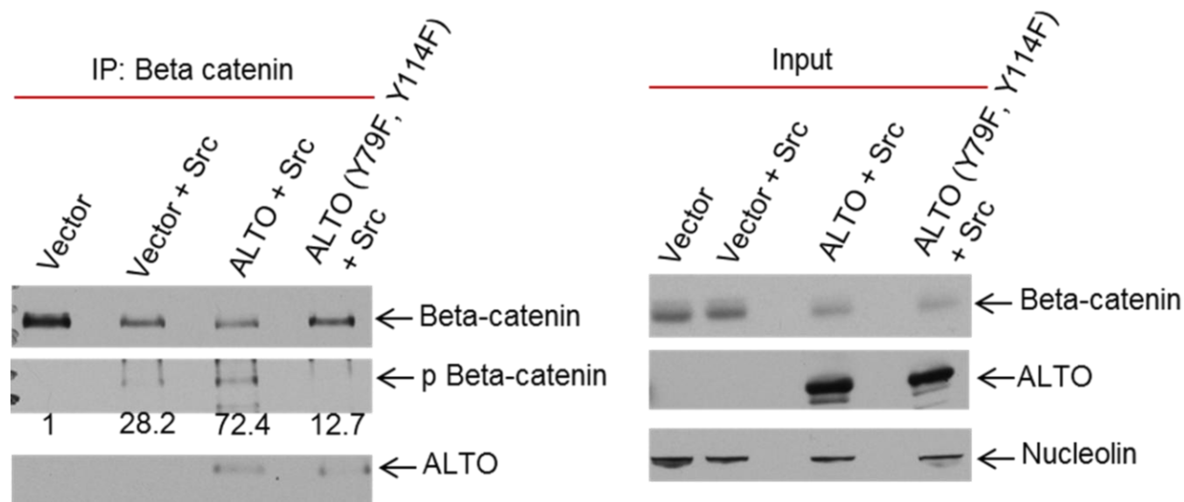


FIG. 6. ALTO increases phosphorylation of Beta-catenin by src. Beta-catenin was immunoprecipitated from lysates of 293 cells transiently transfected with expression constructs for src, ALTO, and ALTO (Y79F, Y114F). Equal amount of vector DNA was used as a negative control. Immunoprecipitated protein was blotted with a Beta-catenin antibody, a phosphotyrosine antibody or an ALTO antibody. Numbers indicate Image J quantification of phosphorylated-Beta-catenin normalized to total Beta-catenin. Input from each sample was blotted with Beta-catenin and ALTO antibodies. Nucleolin was used as a loading control

5. Conclusion

ALTO associated with specific cytoskeletal proteins, SFKs and Beta-catenin, a SFK substrate. ALTO activated SFKs leading to increased tyrosine phosphorylation of Beta-catenin. Therefore, we were successful in identifying ALTO binding partners but we acknowledge that this study is not comprehensive in part because of the limitations of AP-MS [31]. On the other hand, we took care to use low stringency in the affinity purification so as to identify transient interactors but this may have increased the chances of false positives. Nonetheless, because we used CRAPome analysis and verified hits by co-IP and co-localization we can argue that the risk of false positives was greatly reduced.

A different study showed that ALTO binds to SFKs through their SH domains, and that ALTO activates SFKs [J. Carter (FHCRC), unpublished]. This is in agreement with our finding that ALTO increased src-mediated phosphorylation of Beta-catenin, and taken together the data supports our hypothesis that ALTO increases src-mediated phosphorylation of its interactors.

Association of ALTO with cytoskeletal proteins was expected because ALTO has basic amino acids homologous to those of MT that associate with cytoskeleton. Specifically, we found co-localization between ALTO and filamentous actin, which is known to associate with MT. Further, the highest scored interactor in the AP-MS experiments was plectin, an actin binding protein. We can thus conclude that ALTO interacts with components of the cytoskeleton.

6. Future directions

I. Describing the mechanism of ALTO's interactions.

Defining the sub-cellular loci where ALTO interacts with cellular proteins could provide important clues. For instance, SFKs associate with and regulate integrins at cell-matrix interfaces which can also contain plectin [32]. We suspect that ALTO could localize at cell-matrix contacts. Additionally, Beta-catenin associates with transmembrane proteins at cell-cell contacts and it also localizes in the cytoplasm and the nucleus [33]. Because ALTO is predicted to be a membrane-anchored protein we would expect it to interact with the fraction of Beta-catenin that is associated with membrane proteins at cell-cell junctions.

ALTO binds to SFKs using its tyrosine and proline motifs, and similarly we hypothesize that ALTO's tyrosines and/or proline motifs play a role in binding to other interactors such as Beta-catenin. Further, the basic amino acid motif likely facilitates interactions with the cytoskeleton. Therefore, follow up is needed to investigate the mechanisms by which ALTO binds to Beta-catenin and to cytoskeletal proteins. This can be done by mutating ALTO's tyrosines, prolines, basic amino acid motif or hydrophobic domain and observing if they affect ALTO's interactions.

II. Determining the effect of ALTO on SFK signaling

Beta-catenin is a member of the catenin family of proteins that includes Alpha-catenin and p-120 [34]. Catenins are part of a protein complex that establishes cell-cell adhesion. In this adhesion complex catenins link the cytoskeleton to transmembrane proteins called cadherins [34]. The cadherin extracellular domains form homotypic bonds with cadherins of adjacent cells while their intracellular domains specifically bind to Beta-catenin [34]. Beta-catenin also functions as a transactivator of genes such as c-myc and cyclin D which regulate cell survival and growth [35]. Tyrosine phosphorylation works as a switch to shift Beta-catenin from its functions at the adhesion complex to its transcriptional functions [35]. We hypothesize that ALTO increases phosphorylation of Beta-catenin, and thereby, reduces Beta-catenin interaction with cadherin and also reduces cell-cell adhesion. ALTO may also have an effect on Beta-catenin transactivation, and in this case our hypothesis is that ALTO increases tyrosine phosphorylation of Beta-catenin leading to increased transactivation of Beta-catenin responsive genes. The effect of ALTO on the Beta-catenin pathway can be tested using

a Beta-catenin-responsive luciferase reporter which measures transactivation, and also through testing the effect of ALTO on markers of cell-cell adhesion using immunofluorescence and western blot.

7. Summary

In summary our data identifies ALTO interactors and provides a basis for investigating the effect of ALTO on cellular proteins. The signaling that results from ALTO activation of SFKs could have various uses for the virus, for instance, if ALTO influences Beta-catenin transactivation of genes involved in cell growth then it may create an environment conducive for viral replication.

In preliminary experiments ALTO knockout did not affect the ability of the MCPyV genome to replicate, but other possibilities need to be tested including ALTO's effect on infectivity and viral gene expression [7]. The overprinting genes of other viruses often have accessory functions that are not always readily discernible [36]. This may explain why a phenotype for ALTO has not been found but more investigation is warranted.

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