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Cutaneous Human Polyomavirus Small T Antigens and 4E-BP1 Targeting

Julie H. Wu[a, b] Rebecca A. Simonette[a] Thomas Hsiao[a] Hung Q. Doan[a]
Peter L. Rady[a] Stephen K. Tyring[a]

[a]Department of Dermatology, University of Texas Medical School at Houston, and [b]Baylor College of Medicine, Houston, Tex., USA

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Human polyomavirus · Small T antigen · 4E-binding protein 1 · Merkel cell polyomavirus · Trichodysplasia spinulosa-associated polyomavirus · Human polyomavirus 6 · Human polyomavirus 7

Abstract
Merkel cell polyomavirus (MCPyV), trichodysplasia spinulosa-associated polyomavirus (TSPyV), human polyomavirus 6 (HPyV6), and human polyomavirus 7 (HPyV7) are implicated in the pathogeneses of distinct hyperproliferative cutaneous growths and encode small tumor (sT) antigens. The current study demonstrates that the four sT antigens differentially regulate 4E-binding protein 1 (4E-BP1) serine 65 hyperphosphorylation. MCPyV and HPyV7 sT antigens were found to promote the presence of the hyperphosphorylated 4E-BP1-δ isoform, while TSPyV and HPyV6 sT antigens had no significant effects. Given that hyperphosphorylated 4E-BP1 is associated with an aggressive cancer phenotype, our findings confirm the previously reported pathogenicity of MCPyV sT and highlight a novel mechanism by which HPyV7 sT may mediate oncogenesis.

Introduction

In 2008, the discovery of the Merkel cell polyomavirus (MCPyV) shed light on the underlying viral pathomechanisms of Merkel cell carcinoma, a highly aggressive neuroendocrine skin cancer [1]. While MCPyV is, to date, the only oncogenic virus of the polyomavirus family, trichodysplasia spinulosa-associated polyomavirus (TSPyV), human polyomavirus 6 (HPyV6), and human polyomavirus 7 (HPyV7) have all been associated with hyperproliferative epithelial growths. Currently, TSPyV, HPyV6, and HPyV7 are implicated in the development and progression of trichodysplasia spinulosa, the neoplastic growth of keratinocytes in response to BRAF inhibitors, and the pathogenesis of human thymic epithelial tumors, respectively [2–5].

As with other polyomaviruses, MCPyV, TSPyV, HPyV6, and HPyV7 are double-stranded DNA viruses that encode regulatory tumor (T) antigens. Notably, polyomavirus small T (sT) antigens are strongly associated with cell proliferation pathways [6–9]. For example, in a previous report, we showed that TSPyV sT antigen activates the MEK-ERK-c-Jun pathway [7].

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Stephen K. Tyring
6431 Fannin Street
Houston, TX 77030 (USA)
E-Mail styring@ccstexas.com

Recently, Shuda et al. [9] demonstrated that MCPyV sT-mediated oncogenesis was largely dependent on its ability to regulate cap-dependent protein translation pathways. In particular, MCPyV sT antigen facilitated stabilization of the hyperphosphorylated δ-isoform 4E-binding protein 1 (4E-BP1), which was essential for the induction of cellular transformation [9]. In the current study, we aim to further explore the emerging roles of 4E-BP1 in sT antigen pathomechanisms of human polyomaviruses. Our findings indicate that 4E-BP1 is differentially regulated by the MCPyV, TSPyV, HPyV6, and HPyV7 sT antigens, which provide novel mechanistic insight into the patho-oncogenic potential of these viruses.

Materials and Methods

Cell Lines and Cell Culture

HPyV7, MCPyV, TSPyV, and HPyV6 sT antigen-coding genes were subcloned individually into lentiviral vectors that contained tetracycline-inducible CMV promoters and blasticidin antibiotic selection markers (GenTarget Inc). HEK293 cells were infected with the lentiviral constructs; expression of the sT antigen was confirmed by Western blotting with the appropriate custom mouse monoclonal antibodies (GenScript Inc). The antibodies were raised against the HPyV7 sT (clone ID 6H2G7, 1:10,000), MCPyV sT (4H5D4, 1:1000), TSPyV sT (2E10B11, 1:750), and HPyV6 sT (10B11F10, 1:250) epitopes. Cellular proteins were extracted with Mammalian Protein Extraction Reagent™ (Pierce) according to the manufacturer's protocol and lysates were prepared with Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific) supplemented with phosphatase inhibitors.

Western Blotting

Cell lysates were clarified on polyacrylamide gels (10–20% Tris-Glycine; Novex, Invitrogen-Life Technologies). Separated proteins were transferred to polyvinylidine difluoride membranes (Invitrogen-Life Technologies) and Western immunoblot was performed against total 4E-BP1 (9452) and phospho-4E-BP1^{S65} (9456) primary antibodies (Cell Signaling Technology). GAPDH was used as the loading control (GeneTex Inc). SuperSignal West Femto chemiluminescent substrate (Thermo Fisher Scientific) was used to visualize protein bands.

Results

To delineate the roles of MCPyV, TSPyV, HPyV6, and HPyV7 sT antigens, inducible cell lines expressing the respective sT antigen proteins were established. The effects of these sT antigens on 4E-BP1 were analyzed by Western blotting with antibodies against total or phospho-site-specific 4E-BP1 (serine 65). Our Western blot experiments confirmed that MCPyV sT antigen markedly enhanced the presence of the hyperphosphorylated 4E-BP1-δ isoform (fig. 1a). We observed that the HPyV7 sT antigen also promoted the 4E-BP1-δ isoform through S65 phosphorylation under the same experimental conditions (fig. 1b). In contrast, neither TSPyV sT nor HPyV6 sT antigen expression was associated with the presence of the hyperphosphorylated 4E-BP1-δ isoform (fig. 1c, d).

Discussion

4E-BP1 plays a central role in protein synthesis by regulating the assembly of the translation initiation machinery at the mRNA 5′ 7-methylguanosine GTP cap [10, 11]. In its unphosphorylated active conformation, 4E-BP1 binds and inhibits eukaryotic initiation factor 4E (eIF4E) [10]. As uninhibited eIF4E is essential for ribosomal recruitment and subsequent translational activation of oncogenic drivers such as cyclin D1, c-myc, VEGF, and survivin, 4E-BP1 sequestration of eIF4E represents an important step that negatively regulates protein synthesis and represses oncogenesis [10–12]. In the current study, we verified that MCPyV sT antigen does indeed induce 4E-BP1 hyperphosphorylation. Additionally, our data implicate HPyV7 (but not HPyV6 nor TSPyV) sT antigen in a similar mechanism involving 4E-BP1 deactivation. These findings are noteworthy, given that hyperphosphorylation of 4E-BP1, especially on the S65 residue, is known to functionally disable its eIF4E-binding ability and thus increase protein synthesis and mitogenesis [11, 13].

There are four isoforms of 4E-BP1 (α, β, γ, and δ), each of which is known to be sequentially phosphorylated [11, 14]. Among these isoforms, 4E-BP1-δ attracts considerable attention, as this isoform is present at its highest level during mitosis [13]. The current study is the first to demonstrate that HPyV7 sT antigen promotes the presence of the hyperphosphorylated 4E-BP1-δ isoform. This finding suggests a novel mechanism of 4E-BP1 in HPyV7 pathogenesis and provides evidence supporting a possible role for the virus in neoplastic cell growth. Although HPyV7 infection has been associated with epithelial thymomas and pruritic dermal rash, its specific roles in cutaneous diseases remain largely undefined [4, 5]. In this context, it is notable that 4E-BP1 hyperphosphorylation correlates with aggressive cancer growth and worse prognosis for metastatic melanoma patients [9, 15, 16]. Additionally, previous studies have shown that 4E-BP1 phosphorylation status can serve as a predictive marker for drug resistance and a proangiogenic phenotype [16, 17]. As 4E-BP1 is a downstream target of the Akt/mTORC1

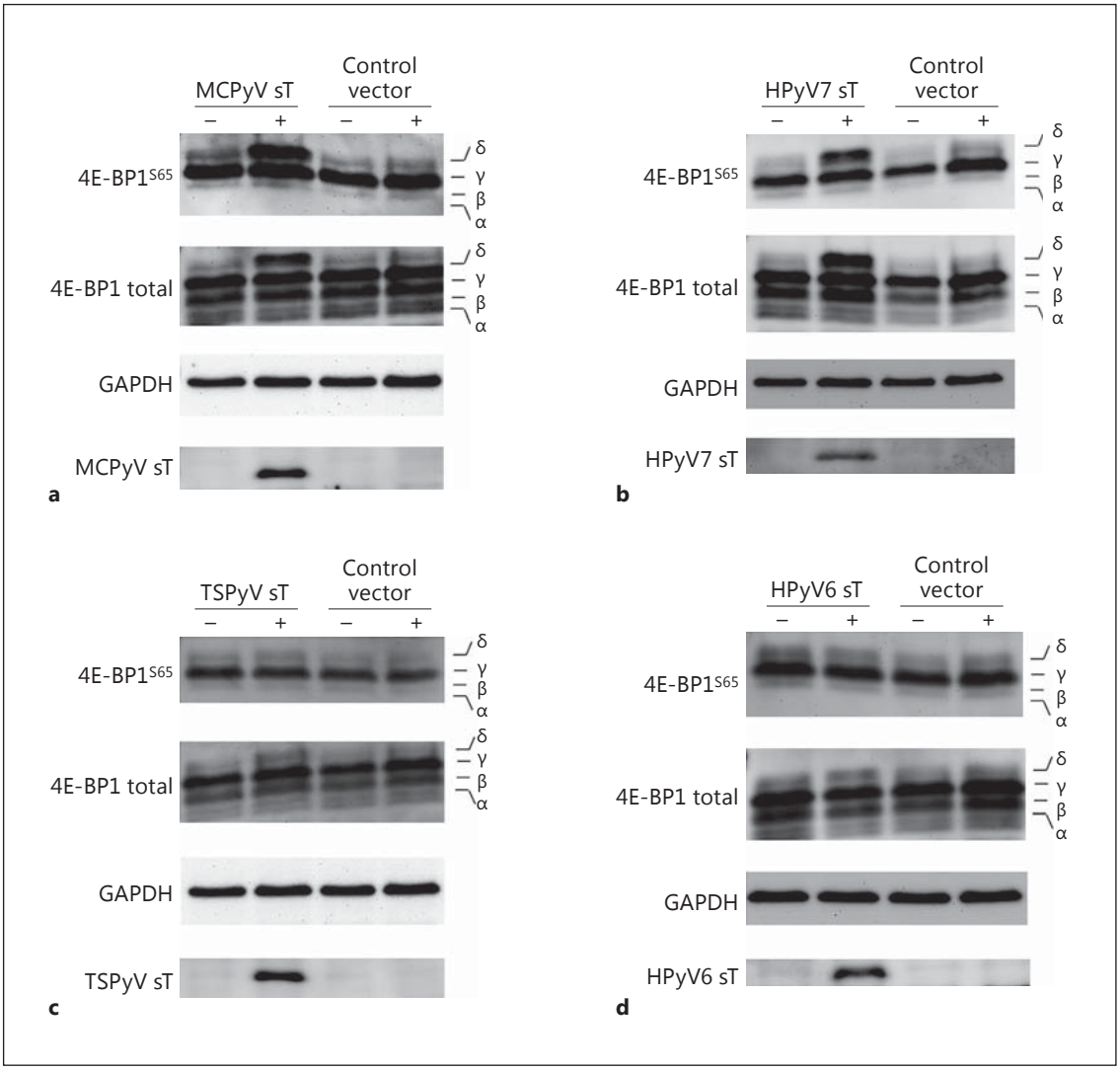

Fig. 1. MCPyV, TSPyV, HPyV6, and HPyV7 differentially target 4E-BP1. Cells were transduced with lentiviral vectors containing specific sT-coding genes or the control empty lentiviral vector. Cellular proteins were subjected to SDS-PAGE and Western blot analysis to evaluate 4E-BP1 phosphorylation status. Lanes designated with a plus sign (+) contained lysates from cells induced to express the appropriate coding gene; lanes designated with a minus sign (–) contained lysates from noninduced cells. Formation of the hyperphosphorylated mitosis-specific δ isoform of 4E-BP1 was markedly elevated by expression of MCPyV sT (**a**) and HPyV7 sT (**b**). In contrast to MCPyV and HPyV7, the presence of TSPyV sT (**c**) and HPyV6 sT (**d**) did not result in significant change of the 4E-BP1 phosphorylation status or the manifestation of the δ isoform.

pathway, it would be logical to consider that polyomavirus sT antigens modulate this pathway due to their interactions with 4E-BP1. However, it has been shown that MCPyV sT antigen-mediated 4E-BP1 hyperphosphorylation is largely independent of this pathway [9]. Interestingly, Shuda et al. [13] reported that 4E-BP1-δ is targeted by the CDK1/CYCB complex, which is activated upon MCPyV sT antigen expression. Given the findings of our current study, further investigation is needed to clarify the role of HPyV7 sT antigen in modulating 4E-BP1 hyperphosphorylation. Whether HPyV7 sT-associated 4E-BP1 deactivation occurs through a similar or differential mechanism compared to MCPyV sT antigen remains unknown. As Merkel cell carcinoma is more aggressive than HPyV7-associated epithelial proliferations, it is possible that these two sT antigens mediate hyperphosphorylation of 4E-BP1 through separate but parallel pathways. Investigating this differential activation of 4E-BP1 hyperphos-

phorylation would shed light on disparate pathway interactions that mediate an aggressive cancer phenotype.

The pathobiological effects and mechanisms of polyomavirus sT antigens are complex and dependent on the characteristics of different viruses as well as the specific tissue/cell context [7, 8, 18]. Computer-assisted genomic analysis of MCPyV, TSPyV, HPyV6, and HPyV7 demonstrated that all four sT antigens share an N-terminal DnaJ domain followed by protein phosphatase 2A-binding sequences and zinc-finger motifs [19, 20]. Further sT truncation studies are required to determine whether these attributes in HPyV7 sT contribute to 4E-BP1 hyperphosphorylation.

In summary, the current study emphasizes the differential effects of MCPyV, TSPyV, HPyV6, and HPyV7 sT antigens on 4E-BP1 phosphorylation. Our results demonstrate that 4E-BP1-δ may represent a common target of both HPyV7 and MCPyV sT antigens; as 4E-BP1 hyperphosphorylation and δ isoform expression are associated with aggressive cutaneous disease, this finding highlights a novel pathway that implicates HPyV7 in oncogenesis. At present, 4E-BP1 phosphorylation has not been implicated in the actions of any other polyomavirus sT antigens. Further studies are warranted to determine whether 4E-BP1 can be targeted for the treatment of HPyV7- and MCPyV-associated diseases.

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