Computational modeling to provide insight into KSHV's immune evasion mechanism

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Abstract

Kaposi's Sarcoma-associated Herpesvirus(KSHV) is an oncogenic virus that can lead to the development of skin cancer, particularly Kaposi's Sarcoma (KS). It does so by hijacking the host's immune system and over activating the RAS/ERK pathway, associated with cell proliferation. Activation of the RAS/ERK pathway, particularly with the RSK2 protein, leads to transformation of epithelial cells to more motile forms, such as metastasized cancerous cells. In the case of KSHV, this leads to the development of KS. The purpose of this paper is to discern which particular step of the RAS/ERK pathway the viral protein ORF45 hijacks and what potential mutations within ORF45 could prevent this. Herein we report that ORF45 directly interacts with the ERK1 protein and likely does not interact with the other steps of the RAS/ERK pathway. Furthermore, we report a proposed catalytic triad for ERK1 substrate phosphorylation function, which has yet to be characterized in current literature.

Introduction

Kaposi's Sarcoma Associated Herpesvirus (KSHV), also known as human herpesvirus-8 (HHV-8) is an oncogenic virus that given the right circumstances, can develop into skin cancer, specifically Kaposi's Sarcoma (KS). Kaposi's Sarcoma is associated with high level of inflammation and can lead to the formation of new blood vessels (angiogenesis). KSHV often does not result in KS, but when it does, the patient often has a weakened immune system already, perhaps because of concurrent HIV infection or old age. (Brown et al 2006, Engels et al 2007, Iscovich et al 2000, Minhas et al 2014, Yarchoan et al 2015) Kaposi's Sarcoma develops along lymph or blood vessel cells and often expresses in the form of skin lesions, but can be increasingly more painful when lesions form in the mouth or vital organs such as the lungs. There are four main types of Kaposi Sarcoma: epidemic (AIDS-associated), classic (Mediterranean), endemic (African), and iatrogenic (transplant-related). Rapid development of KS has become less common in the US due to better quality treatment for HIV-infection, with very few people ever progressing to the AIDS phase of the infection. However, the disease is much more devastating in other 3rd-world countries where high quality AIDS treatment, specifically HAART (highly active antiretroviral therapy), is not available (National Cancer Institute, National Comprehensive Cancer Network, Noy et al 2014, Radu et Pantanowitz 2013, Yarchoan et al 2015).

Kaposi's Sarcoma is classified as an AIDS-defining cancer and is one of the most common cancers in many countries (Yarchoan 2015). Initial invasion, where the virus is in the lytic phase, increases the amount of virus actively replicating in the host, but it is latent phase the virus

integrates into the host genome, which allows for hijacking of the host long-term through initiation of sporadic lytic phases which can lead to mutant cell proliferation, leading to the growth of cancer (Mesri 2010). The virus enters the cell primarily through macropinocytosis and clathrin-mediated endocytosis. KSHV interaction with cell membrane receptors quickly induces the transcription factors, such as extracellular signal-regulated kinases (ERK1/2), nuclear factor kappa B (NF-kB), and nuclear factor erythroid 2-related factor 2 (NrF2) to aid in expression of KSHV genes (Kumar 2016). While ERK1/2 is one of the host proteins that are signaled to upregulate transcription upon KSHV infection, the role of ERK1/2 and in general the RAS/ERK pathway is far more complex than just stimulating replication of KSHV genes upon infection.

The RAS/ERK pathway, a vital signaling cascade regulating cell growth and proliferation, involves a series of sequential activations of key proteins (Figure 1). The cascade is initiated by the activation of RAS, a GTPase, usually located at the cell membrane. Upon receiving extracellular signals, RAS becomes activated and triggers downstream signaling events. Subsequently, activated RAS stimulates RAF (Rapidly Accelerated Fibrosarcoma) kinases, notably RAF1, initiating the MAP kinase kinase (MEK) pathway. MEK, in turn, phosphorylates and activates extracellular signal-regulated kinases (ERK), specifically ERK1 and ERK2. Activated ERK translocates into the nucleus, where it phosphorylates various transcription factors, ultimately regulating gene expression related to cell proliferation, differentiation, and survival. This orchestrated cascade, involving RAS, RAF, MEK, and ERK, plays a crucial role in cellular responses to external stimuli, and dysregulation of this pathway is associated with various diseases, including cancer.

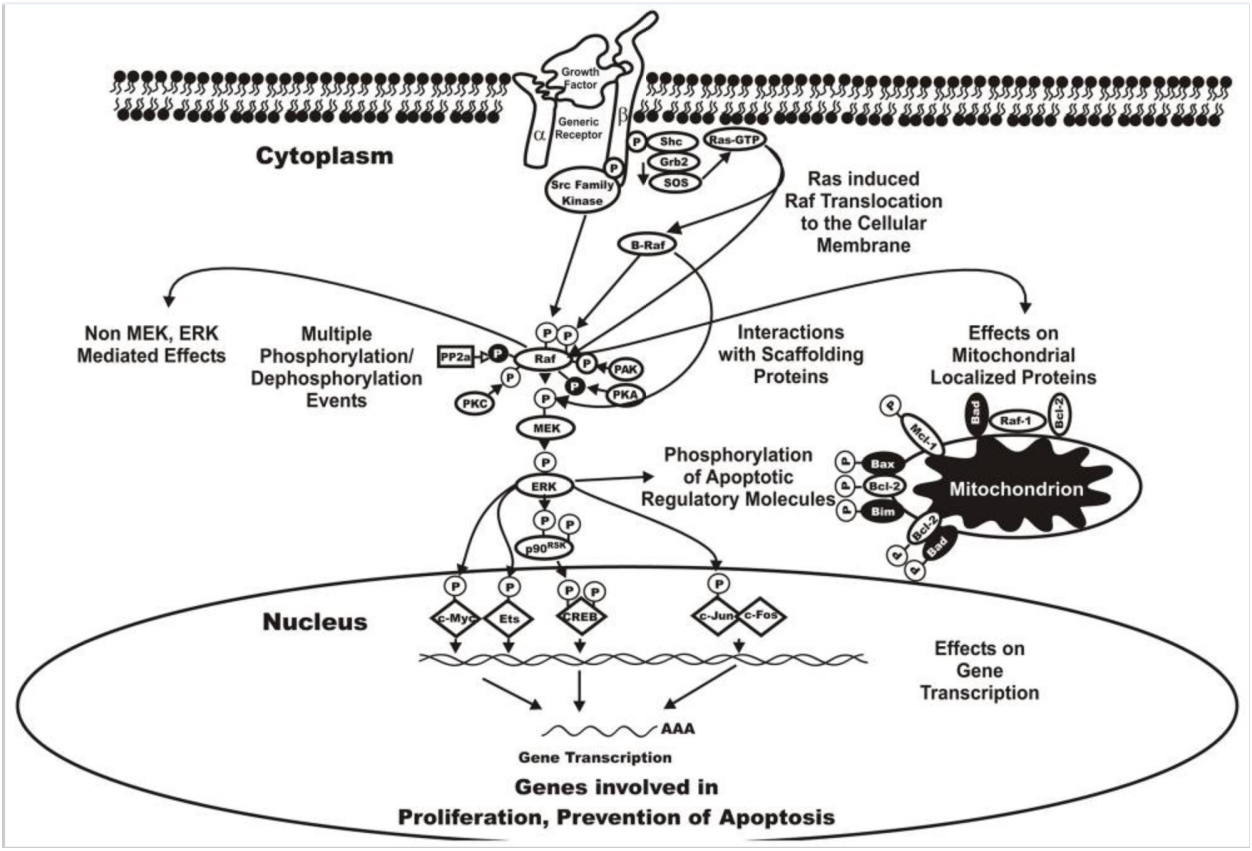


Figure 1. Molecular Mechanism Map of Ras/Raf/MEK/ERK Molecular Pathway. This pathway is associated with cell proliferation and its regulation. These proteins are directly involved in communicating with cancer regulation pathways. (McCubrey et al 2006)

A common phenotype of cancer cells is a malfunctioning RAS/ERK pathway, a phenotype we also see in KS. This pathway is essential for triggering mitosis and signaling cell proliferation. It is also associated with “degradation of the tumor extracellular matrix” and “tumor cell migration” (Guo 2020). Therefore, the RAS/ERK pathway is crucial for the virus’ proliferation in the lytic phase. However, KSHV can stimulate the over action of ribosomal s6 kinase, a key player in the RAS/ERK pathway, which allows for the RAS/ERK pathway to signal epithelial-mesenchymal transition (EMT). This is where the surrounding extracellular matrix is modified to allow the affected cell to develop both motile and invasive characteristics. As RSK is a secondary messenger of ERK, ERK is therefore also highly involved with EMT. When fluoromethyl ketone (FMK) was used to inhibit RSK, multilayering of the cells was blocked, and scattering and migration of the affected cells was reversed (Doehn 2009). Therefore, hijacking of this RAS/ERK pathway can aid the virus in its latent oncogenic phase, but also be the key to inhibiting cancerous progression of KSHV as well.

ORF45 is associated with purified virions and early-stage KSHV lytic activation, however, it is only active when in its phosphorylated state. This implies that ORF45 must interact with a host cell kinase in order to activate its function demonstrating its likelihood that ORF45 is involved in KSHV primary infection (Zhu 2003). According to experimental data, ORF45 has been shown to prolong activation of both ERK and RSK by forming a ternary complex. Essentially, ORF45

associates with ERK/RSK and is phosphorylated by them, which activates ORF45 to modify the host kinase docking systems to prolong activation of pathways key to cell proliferation. The formation of such a complex prevents phosphatase removal of phosphates from both the ERK and RSK2 proteins. This leads to prolonged proliferation and prevents an immune response by blocking the signal for apoptosis. (Alexa 2022) However, it is still unclear by what mechanism this ternary complex is formed to inhibit ERK/RSK function. Therefore, the scope of this paper is to examine which particular step of the RAS/ERK pathway does the viral ORF45 protein hijack through a computational complex predictive structural analysis by which we can answer not only which protein initiates the formation of the complex, aka which protein directly binds to ORF45, but also, we can determine what potential mutations/inhibitors can prevent the formation of the ternary complex, thus inhibiting host hijacking.

Methods

Literature Curation

Literature curation was conducted and presented in the introduction section to discuss what is already known about KSHV and its protein ORF45 in particular. Analysis of literature curation determined which putative protein complexes could be made in the interaction of the host cell factors with ORF45. Five proteins– ERK1, ERK2, RSK2, Raf1, and MEK1– were selected for model predictions of their putative interactions with ORF45. These proteins were selected because of their important roles in the RAS/ERK pathway and cell proliferation. Therefore, these would be the most likely targets of the early-stage viral protein ORF45's lytic pathway as hijacking the means to continue or halt the cell cycle to increase viral proliferation.

Protein Complex Predictive Computational Modeling

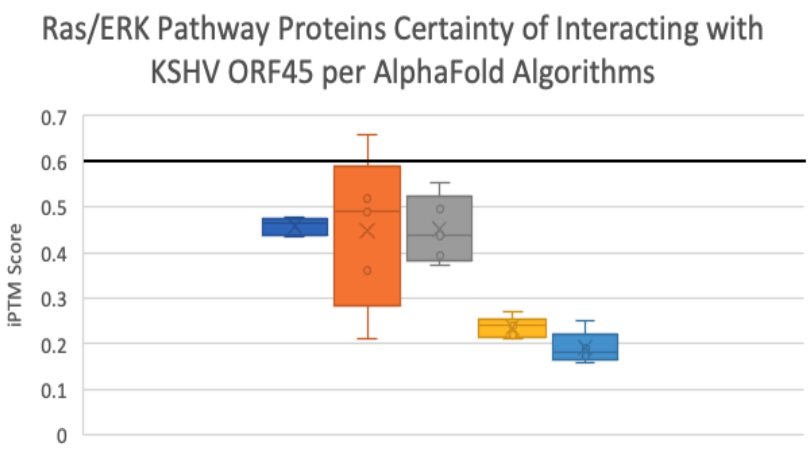
The aforementioned selected candidate proteins were modeled in complex with ORF45 utilizing AlphaFold algorithms, with the models visualized and analyzed utilizing Chimera. (Mirdita et al 2022) Unlike previous studies, these models will be predicted using the full protein sequences rather than just certain docking sites. Each complex pair predicts five models, however, the only models that were analyzed in Chimera are those with an iPTM score of 0.6 or greater, as the iPTM value is the certainty score of how likely this interaction is to occur in vivo. A score of <0.2 infers that the interaction does not occur, a score of 0.2-0.4 infers an interaction likely does not occur, a score of 0.4-0.6 infers an interaction may occur, a score of 0.6-0.8 infers an interaction likely occurs, and a score of >0.8 infers an interaction certainly occurs.

Results

AlphaFold Results

Protein 1	Protein 2	Model #	iPTM score	pTM score
ORF45	RSK2	1	0.436	0.407
ORF45	RSK2	2	0.465	0.427
ORF45	RSK2	3	0.443	0.407
ORF45	RSK2	4	0.474	0.424
ORF45	RSK2	5	0.476	0.417
ORF45	MAPK3-ERK1	1	0.52	0.52
ORF45	MAPK3-ERK1	2	0.21	0.5
ORF45	MAPK3-ERK1	3	0.36	0.52
ORF45	MAPK3-ERK1	4	0.66	0.53
ORF45	MAPK3-ERK1	5	0.49	0.52
ORF45	MAPK1-ERK2	1	0.497	0.527
ORF45	MAPK1-ERK2	2	0.372	0.523
ORF45	MAPK1-ERK2	3	0.395	0.515
ORF45	MAPK1-ERK2	4	0.552	0.534
ORF45	MAPK1-ERK2	5	0.438	0.526
ORF45	MEK1	1	0.22	0.33
ORF45	MEK1	2	0.24	0.33
ORF45	MEK1	3	0.21	0.32
ORF45	MEK1	4	0.24	0.33
ORF45	MEK1	5	0.27	0.33
ORF45	RAF1	1	0.181	0.362
ORF45	RAF1	2	0.16	0.352
ORF45	RAF1	3	0.191	0.359
ORF45	RAF1	4	0.173	0.354
ORF45	RAF1	5	0.252	0.37

Figure 2. Model Prediction Scores from AlphaFold. iPTM score = interaction predictive template model score, indicates the certainty of the interactions between the two proteins in question. pTM score = predictive template model score, indicates the certainty of the individual structures of each protein being modeled. A certain pTM score will be >0.6.



Out of the five proteins modeled to interact with ORF45 using AlphaFold protein-protein interaction predictive algorithms, one ERK1 model had the most successful iPTM score of 0.66.

The other iPTM scores for ERK1 and the other proteins modeled were below the 0.6 threshold, and therefore were not analyzed using Chimera (Figures 2-3). The variation in model certainty of the five ERK1 models could be due to the fact that the AlphaFold algorithm displays a high uncertainty in the structure of ORF45, indicated by the low predictive template modeling scores (pTM), indicating in all the models that ORF45 is an intrinsically disordered protein (Figure 4). However, there has been no literature to support this notion that ORF45 is an intrinsically disordered protein; and conversely there is no literature providing the structure of ORF45 either. This notion that neither the AlphaFold algorithm nor experimental structural biologists have affirmed the structure of ORF45 requires the authors to take these results given by AlphaFold with a grain of salt, noting that these results would be more convincing if the algorithm was more certain on the structure of ORF45 itself. However, because AlphaFold has been demonstrated to be highly accurate, it is likely that ORF45 is an intrinsically disordered protein like many other viral proteins. (Mishra et al 2020) This idea that ORF45 is an intrinsically disordered protein, while novel, is a rational conclusion as scientists have yet to uncover the structure of this protein, likely due to its unstructured nature. This is further supported by AlphaFold's robust accuracy. (Mirdita et al 2022)

Identification of Inhibitory Dephosphorylation Interactions of ERK1 by ORF45

ERK1 is activated through phosphorylation of threonine and tyrosine residues in the 'TEY' motif, which was identified through mutation-based experimentation, by preceding proteins in the RAS/ERK pathway. (Butch, Guan 1996) It is hypothesized in literature that ORF45 prolongs activation of RAS/ERK pathway by inhibiting dephosphorylation of ERK1. (Alexa et al 2022). However, the precise mechanism by which this ORF45-mediated ERK1 inactivation inhibition occurs is largely unknown. Our AlphaFold findings propose a putative mechanism that can explain how ORF45 inhibits phosphatases from accessing the ERK1 phosphorylation site by creating a stabilizing and electrostatic dual interaction between ERK1 and ORF45.

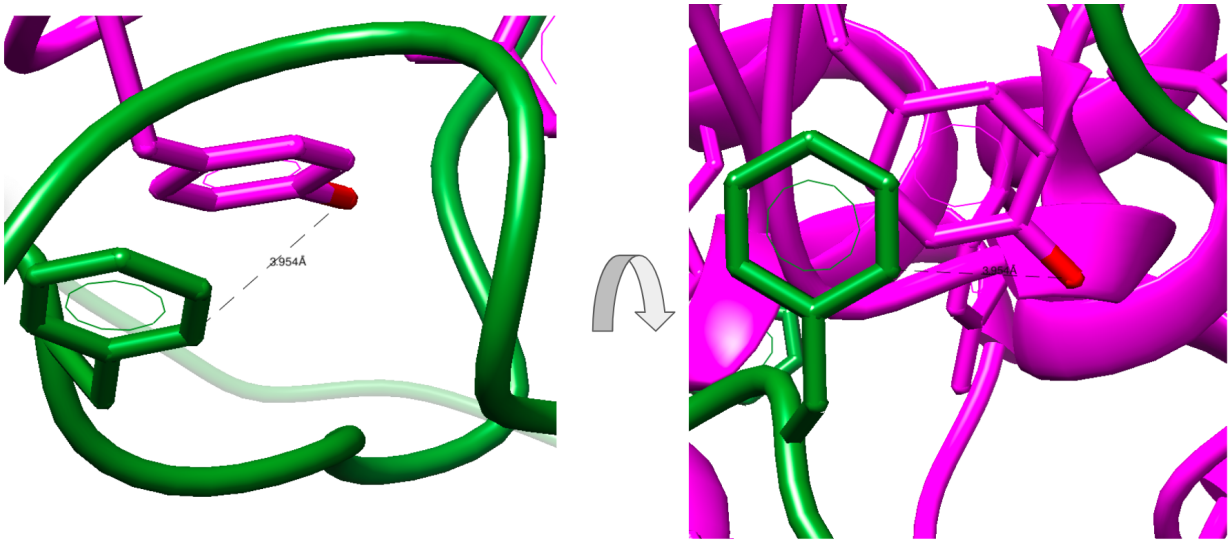


Figure 5. Pi-pi stacking between ERK1 and ORF45 on the TEY phosphorylation motif of ERK1.

Figure 5 demonstrates pi-pi stacking between a tyrosine on ERK1 (green) and a phenylalanine on ORF45 (magenta), and Figure 6 demonstrates an electrostatic interaction between a threonine on ERK1 and an asparagine on ORF45. These are both examples of the inhibitory interaction of ORF45 and ERK1, as the phosphates located on the proximal end of the R groups of Thr and Tyr would be occupied by said ORF45 interaction, blocking ERK1 from being dephosphorylated, maintaining the activity of the RAS/ERK pathway, thereby prolonging cell proliferation, and therefore viral proliferation. Through both pi-pi stacking and electrostatic interactions, both phosphorylated residues become occupied, inhibiting ERK1 access to phosphatases, while these interactions maintain stabilization due to the strong stabilizing forces pi-pi stacking interactions possess. In essence, figures 5 and 6 from the predictive model demonstrate that those key residues, threonine (T) and tyrosine (Y) are essentially protected by ORF45, confirming findings of previous literature that ORF45 prolongs activation of the RAS/ERK pathway through inhibitory interactions. (Alexa et al 2022).

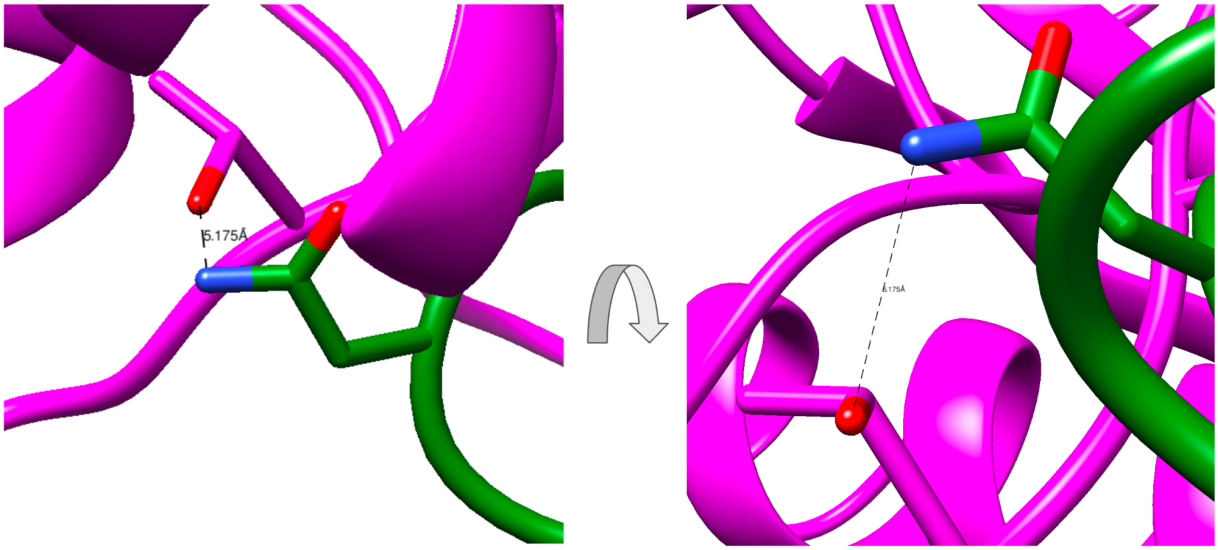


Figure 6. Electrostatic attraction between ERK1 and ORF45 on the TEY Phosphorylation Motif of ERK1.

While we have identified that the phosphorylation site of ERK1 is inhibited by ORF45 to prevent dephosphorylation of ERK1, maintaining ERK1 in its active state prolonging cell proliferation, which was proposed to occur by Alexa et al 2022. However, in order for ERK1 to continue prolonging cell proliferation, its kinase catalytic triad must be uninhibited by ORF45 in order for its kinase action to continue. Since the catalytic triad for ERK1 kinase function has yet to be identified, we aimed to determine putative ERK1 kinase catalytic triads utilizing the predicted model as those catalytic triads that are not inhibited by ORF45 interactions. Figure 7a-b demonstrates our proposed kinase domain for ERK1. This is the only site in the model exposed to the exterior that is not likely to interact with the ORF45. Thus, this structure likely allows for ORF45 to prevent ERK1 from being dephosphorylated at the 'TEY' motif, while not inhibiting the kinase site of ERK1 allowing ERK1 to continue phosphorylating substrates, thus prolonging cell proliferation. The proposed kinase domain shown in Figure 7 contains two potential catalytic triads.

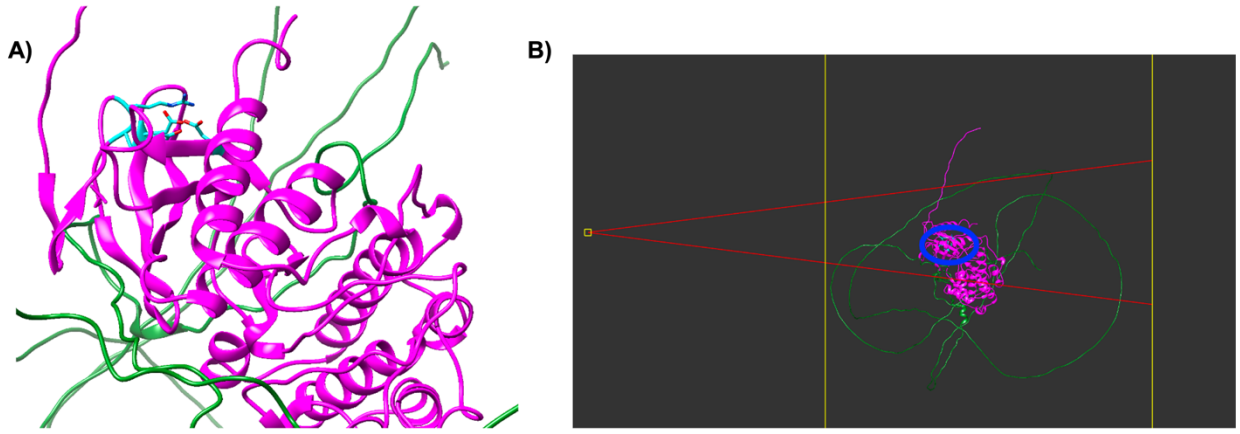


Figure 7. Putative Kinase Site for ERK1. A) Close view of putative kinase domain containing two putative catalytic sites. **B)** Zoomed out view of putative kinase domain circled in blue, demonstrates that this site is external to ERK1 and contains no contact sites with ORF45.

Figure 8 demonstrates that the distances between the residues for proposed catalytic triad A (Figure 8a) are closer but the arginine shares several hydrogen bonds and could potentially not be reactive enough to be an active site. The second arginine for proposed catalytic triad B (Figure 8b) catalytic triad (other two residues are shared) is a little further away but does not share hydrogen bonds so will be more likely to be free to form a catalytic triad. However, this “free” arginine is more close to ORF45, which may be an artifact of the structure of ORF45 being uncertain in majority of the protein, which means it may not be the arginine involved in the ERK1 kinase catalytic triad.

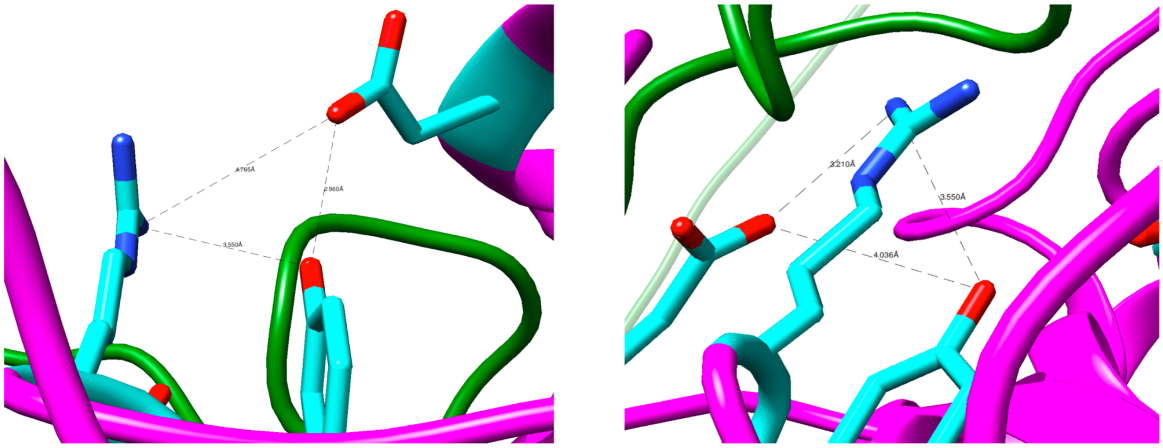


Figure 8. Two Putative Catalytic Triads for the ERK1 Kinase Domain. A) Proposed catalytic triad A, which shares the nucleophilic and acidic residues with proposed catalytic triad **B. B)** Proposed catalytic triad B, the only difference from catalytic triad A is a different nearby arginine fulfilling the basic residue requirement of the catalytic triad.

Discussion

The results of this study shed light on the intricate interactions between Kaposi’s Sarcoma-associated Herpesvirus (KSHV) and the host cell, particularly focusing on the viral protein ORF45 and its role in manipulating the RAS/ERK pathway. The primary finding of this research is the direct interaction between ORF45 and the ERK1 protein, indicating a specific point of hijacking within the RAS/ERK pathway. This interaction is crucial for understanding how KSHV modulates host cell signaling to promote its lytic phase and contribute to the development of Kaposi’s Sarcoma (KS).

The AlphaFold computational models have provided insights into the structural aspects of the ORF45-ERK1 interaction. Despite the uncertainty in the structure of ORF45, the models suggest a potential mechanism by which ORF45 inhibits the dephosphorylation of ERK1, thereby prolonging the activation of the RAS/ERK pathway. The inhibitory interactions involve pi-pi stacking and electrostatic interactions, providing a structural basis for the observed effects of ORF45 on ERK1 phosphorylation. The proposed model suggests that ORF45 protects critical residues on ERK1, preventing dephosphorylation and maintaining the active state of ERK1.

Furthermore, the identification of potential catalytic triads in the ERK1 kinase domain, uninhibited by ORF45 interactions, raises important questions about the regulation of ERK1 activity during KSHV infection. The proposed active sites need experimental validation to confirm their significance and functionality. Future studies could involve mutagenesis experiments targeting these proposed active sites to assess their impact on ERK1 function and the overall viral life cycle.

One notable limitation of this study is the uncertainty in the structural prediction of ORF45, highlighted by the low predictive template modeling scores. While AlphaFold has demonstrated high accuracy in predicting protein structures, experimental validation of ORF45's structure would enhance the credibility of the proposed models. Additionally, the interaction between ORF45 and other proteins in the RAS/ERK pathway, such as ERK2, RSK2, Raf1, and MEK1, was not thoroughly explored in this study. Investigating these interactions could provide a comprehensive understanding of how KSHV manipulates the entire RAS/ERK signaling cascade.

The implications of these results for future studies are significant. Conducting mutagenesis studies on the identified residues involved in the ORF45-ERK1 interaction can elucidate the specific amino acids critical for the viral protein's function. This information is invaluable for designing targeted inhibitors that disrupt the ORF45-ERK1 interaction, potentially inhibiting KSHV's ability to manipulate host cell signaling. Moreover, monitoring the lytic invasion of KSHV in mutants with altered ORF45 can reveal the impact of these mutations on the virus's ability to evade the host immune response and promote cell proliferation.

In conclusion, this research contributes to our understanding of the molecular mechanisms underlying KSHV-induced oncogenesis, specifically focusing on the interaction between ORF45 and ERK1 within the RAS/ERK pathway. The proposed models provide a basis for further experimental validations and open avenues for the development of therapeutic strategies targeting these interactions to combat Kaposi’s Sarcoma and related cancers. Future studies should aim to validate the proposed active sites, explore additional protein interactions, and investigate the therapeutic potential of disrupting the ORF45-ERK1 interaction for clinical applications.

Conclusion

In conclusion, KSHV’s ORF45 protein helps initiate the attack on the host’s immune system by prolonging the activation of the RAS/ERK host cellular pathway. This prolongs signals for cell proliferation, leading to infection by KSHV and an increased risk of cancer. The purpose of this paper was to determine which step of the pathway and how the ORF45 viral protein hijacks the host cell. Through protein-protein interaction predictions and protein complex visualization software, it is likely ERK1 is the primary target of ORF45. Through pi-pi stacking and electrostatic interactions with the TEY phosphorylation site, ORF45 is able to prevent ERK1 from dephosphorylation. Furthermore, the presence of two strong catalytic triads uninhibited by ORF45’s presence suggests a potential active site that allows for ERK1 to continue the cascade

cycle of the RAS/ERK pathway and lead to prolonged cell proliferation. Indeed, ERK1 is shown to directly interact with ORF45. However, RSK2 likely does not, possibly because of its secondary relationship to ERK1. As Kaposi's Sarcoma (KS) is one of the most common cancers among patients with untreated HIV. The results from this project can be used to better develop treatments to fight Kaposi's Sarcoma and other similar viruses that attack the RAS/ERK pathway. ERK1, in particular, is an essential protein because of its importance in relaying messages to both transcription factors and regulators of apoptosis. The proposed active sites of ERK1 should undergo experimental testing to better understand their significance and credibility as active sites. Some potential experiments would be to determine if the virus can still evade the host cell immune response while either of these proposed active sites is mutated.

Acknowledgments

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