HPV11 E7 Protein Interacts with Nup62 and CRM1 Nuclear Export Receptor

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Abstract

During human papillomavirus (HPV) pathogenesis, the viral E7 protein bypasses the cell cycle control by degradation of the Retinoblastoma protein (pRb), enabling for productive viral DNA replication to occur in differentiated epithelial cells (Longworth and Laimins, 2004). The carboxy terminus of high-risk HPV16 E7 contains a nuclear localization signal (NLS) and a nuclear export signal (NES) within a zinc-binding domain (Knapp, et al., 2009). While most NLSs enable binding to a karyopherin for nuclear import, both high-risk HPV16 E7 and low-risk HPV11 E7 have been shown to enter the nucleus via a Ran-dependent but karyopherin-independent pathway (Angeline et al., 2003; Piccioli et al., 2010). Other studies in the Moroianu lab have also suggested that a leucine-rich export signal in the high-risk HPV16 E7 protein may function in its nuclear export (Knapp et al., 2009).

In this study we investigated the hydrophobic interactions between HPV11 E7 and the FG region of Nup62N through transfection assays with EGFP-11E7 fusion plasmids in HeLa cells and binding assays with GST-Nup62N immobilized on Glutathione-Sepharose beads. We found that EGFP-11cE7 binds to Nup62N. This suggests a possible mechanism for the nuclear import of HPV11 E7 through direct hydrophobic interactions between its carboxy-terminus and the FG region of Nup62. The

interaction between HPV11 E7 and CRM1 nuclear export receptor was also examined using similar methods. Binding between these proteins suggest that nuclear export of 11E7 is mediated by CRM1 binding to its leucine-rich NES. These data stress the importance of HPV11 E7's zinc-binding domain in nuclear import and export.

Abbreviations

HPV11 E7	Human papillomavirus type 11, Early 7 oncoprotein
HPV11cE7	Human papillomavirus type 11, Early 7 oncoprotein, C-terminus
NPC	Nuclear pore complex
Nup62N	Nucleoporin 62, N-terminus
CRM1	Exportin 1
GST	Glutathione-S-transferase
EGFP	Enhanced green fluorescent protein
PBS	Phosphate-buffered saline
IPTG	Isopropyl-1-thio-β-D-galactopyranoside
Kapβ2	Karyopherin β2
NLS	Nuclear localization signal
NES	Nuclear export signal
HRP	Horseradish peroxidase
EDTA	Ethylenediamine tetraacetic acid
DTT	Dithiothreitol

Introduction

I. Human Papillomaviruses (HPVs)

Human papillomaviruses (HPVs) are small, non-enveloped double-stranded DNA viruses. Over two hundred subtypes of HPV have been identified. HPV shows tropism for squamous epithelial cells and causes hyperproliferation of mucosal and cutaneous epithelial tissues. Over 95% of cervical cancers, the second most prominent cancer amongst women worldwide, show infection by high-risk HPVs as a primary cause. HPV infection has also been attributed to anal, oropharyngeal, perianal, penile, vulvar, and non-melanoma skin cancers (Howley, 1996; zur Hausen, 2000).

HPV gains access to the basal lamina of epithelium cells through microabrasions in the tissue. Interactions of the major capsid protein L1 and receptors on the cell then induce viral uptake by the host cell (Doorbar, 2006). Persistent HPV infection is predicted to occur in only 10% of all cases of infection, with the other 90% being cleared naturally by the host immune system within a few years. The persistent infections with high-risk HPVs lead to the successful integration of viral DNA into the host genome (Dunne, et al., 2007).

HPVs are primarily divided into two types: alpha and beta HPVs. Alpha HPVs affect mucosal epithelial tissue and are further divided into low- and high-risk types. Low-risk alpha HPVs lead to genital warts, such as subtypes HPV6 and 11. High-risk alpha HPVs, on the other hand, lead to invasive cervical carcinomas. Beta HPVs lead to cutaneous lesions and non-melanoma skin cancers (Doorbar, 2006).

II. HPV Life Cycle

HPVs are non-enveloped viruses with icosahedral capsids. Their double-stranded DNA genomes are approximately 8 kb long and circular. The HPV life cycle is closely correlated with that of the infected host cell. The virion is initially attached to its host by heparin sulfate when wounds in the epithelium expose basal keratinocytes to infection. Upon and throughout infection, an average copy number of 20 to 100 copies of viral DNA is produced per cell (Longworth and Laimins, 2004).

All HPV subtypes code for eight proteins: E1, E2, E4, E5, E6, E7, and the capsid proteins L1 and L2. E1 and E2 are replication factors that recruit host replication machinery to viral origins of replication. E6, which degrades tumor-suppressor p53, and E7, which targets the retinoblastoma protein (pRB), are the major transforming proteins. L1 and L2 are expressed late in HPV infection before the release of new virions. Interestingly, the E7 protein of low-risk HPVs binds to Rb with much weaker affinity than E7 of high-risk types (Heck et al., 1992).

Many HPV mRNAs are polycistronic and convey three or more ORFs, which sometimes are subject to alternative splicing. HPV's tropism for keratinocytes has been explained by enhancers in upstream regulator region (URR) of the E6 ORF. The URR upstream of E6 acts as the early viral promoter and is expressed before formation of full virions, which require the expression of viral capsid proteins as well as the viral replication proteins, E1 and E2, that are coupled to the late promoter. The HPV life cycle is linked to differentiation of its host keratinocyte, with only suprabasal cells producing complete virions (Longworth et al. 2004). Since productive viral DNA replication occurs in the differentiated epithelial cells, a method to re-enter into S phase is needed. This is

accomplished by the E7 protein, which bypasses cell cycle control by degradation of pRb (Longworth and Laimins, 2004; Piccioli et al, 2010).

III. HPV E7

The HPV E7 protein is a small phosphoprotein composed of 98 amino acids. The amino terminus contains a conserved region (CR1) similar to that of adenovirus E1A and simian vacuolating virus's T antigen, suggesting a significant conserved role in these proteins' transforming abilities. E7's conserved region 2 (CR2) contains a Leu-X-Cys-X-Glu (LXCXE) motif essential for pRb binding and consensus casein kinase II (CKII) phosphorylation site. CR3 at the carboxy terminus contains a zinc-binding domain made up by two Cys-X-X-Cys motifs (Ghittoni et al., 2010). NMR and X-ray crystallography studies show that the amino terminus of the E7 protein is unfolded whilst the carboxy terminus exists in a tight zinc-binding fold (McLaughlin-Drubin and Munger, 2008).

Other targets of E7 include p130 in the nucleus, which aids in inducing re-entry into S phase (Genovese, et al., 2008). HPV 16 and 11 E7 have also been shown to target cytoplasmic ubiquitin ligase p600 (Huh, et al., 2007). E7 also results in the relocation of steroid receptor coactivator (SRC-1) from the nucleus to the cytoplasm (Baldwin et al, 2006). E7 also causes defects in chromosome alignment during prometaphase through associations with the nuclear mitotic apparatus protein (NuMA) (Nguyen and Munger, 2009). Previous studies have shown that HPV 11 and 16 E7 localize predominantly in the nucleus *in situ* and the CaSki cell line, and when expressed in HaCat, and U2O5 cell lines (Guccione et al., 2002). Amino acids 39-98 constitute the C-terminus, which contains a

nuclear localization signal (NLS) and a nuclear export signal (NES) (Angeline, et al., 2003, Knapp, et al., 2009; McLaughlin-Drubin and Munger, 2008).

III. Nuclear Pore Complex (NPC)

The nuclear pore complex (NPC) is one of the largest protein complexes in a cell. Comprised of ring-shaped repeats of thirty nucleoporin proteins (Nups), the vertebrate NPC has a mass of approximately 50 MDa and is 100 nm in diameter (Alber et al., 2007). Electron microscopy of various vertebrate and invertebrate NPCs have shown them to be highly conserved in structure if not in size (Kiseleva, et al., 1998; Hinshaw, wt al., 1992; Yang, et al., 1998).

The central channel is made up of Nup54, Nup58, and Nup62. Nup54 and 58 form a ring that expands from twenty to forty nm, allowing fully folded proteins to pass across the nuclear envelope. Nup62 and 54 form both cytoplasmic and nucleoplasmic sites. They contain phenylalanine and glycine (FG) repeats, which allow binding sites for import proteins. They determine the specificity of the cargo that is imported into the nucleus, thus functioning as a permeability barrier. Import selectivity is determined by weak hydrophobic van der Waals forces between the import receptors and FG nucleoporins (Hoelz et al., 2011).

IV. Nuclear Import and Export Pathways

Karyopherins (Kaps) facilitate import into the nucleus by binding to the cargo protein and interacting with FG nucleoporins (Nigg, 1997). β-karyopherins (Kapβ) can bind to NLS-containing cargo proteins as well as interact with FG nucleoporins. Kapβ

have a high level of sequence conservation in their N-terminal Ran-binding domains (Görlich et al, 1997). Interactions between cargo proteins, karyopherins, and RanGTP enable the nuclear import of proteins larger than 40 kDa through the NPC (Ribbeck and Gorlich, 2002). RanGDP is thought to undergo a conformational change that causes its disassociation from Kapβ1 (Bayliss, et al., 2000). Directionality of import/export into the nucleus is determined by the RanGTP/RanGDP gradient (Hoelz, et al, 2011).

The Ran gradient is maintained by the localization of RanGAP, RanBP1, and RCC1. RanGAP is the GTPase activating protein in the Ran pathway. Ran-binding protein, RanBP1, associates with RanGAP to increase GTPase activity. Both of these proteins are cytoplasmic and ensure a high concentration of RanGDP outside of the nucleus. In contrast, RCC1 is associated with chromatin in the nucleus and acts as a nucleotide exchange factor for Ran, thus keeping a high level of RanGTP inside of the nucleus (Moroianu, 1999).

Association of RanGTP with Kapβ/NLS cargo from nucleoporins causes dissociation of the complex and consequently the release of the cargo (Marelli et al., 1998). The opposite is true for nuclear export and cargo association with CRM1 interactions with the NPC (Nakeilny et al., 2009). CRM1 belongs to a family of proteins related to Kapβ, but is involved in nuclear export rather than import (Görlich et al., 1997). CRM1 has been shown to be an exportin for polypeptides containing a leucine-rich nuclear export signal (NES) (Fornerod et al., 1997). Proteins that are imported into the nucleus contain a nuclear localization signal (NLS). This NLS interacts with importins/karyopherins (Dingwall and Laskey, 1991).

V. Nucleocytoplasmic Transport of HPV11 E7

Previous studies in the Moroianu lab have shown that HPV11 E7 enters the nucleus via a Ran-dependent but nuclear import receptor-independent pathway via a C-terminal NLS (Piccioli et al., 2010). Previous studies of the human T lymphotropic virus type 1 TAX protein have demonstrated that TAX enters the nucleus directly via interactions of its zinc finger domain and FG-nucleoporins (Tsuji et al, 2007). Other studies in the Moroianu lab have also suggested that a leucine-rich export signal in the high-risk HPV16 E7 protein may function as an NES (Knapp et al., 2009).

Materials and Methods

GST Recombinant Protein Expression and Purification

GST, GST-Nup62N, and GST-CRM1 were purified from *E. coli* BL21 CodonPlus previously transformed with the appropriate plasmid. 1 mM IPTG was used to induce the bacterial cultures over three hours. Glutathione-Sepharose beads were then used to purify the GST-fusion proteins using a standard purification procedure.

Transfection Assays

HeLa cells (ATCC HPV18+ cervical carcinoma cell line) were grown overnight in 25 ml flasks in 4 ml DMEM transfection medium with 2.5 μg DNA. DMEM(+) media was aspirated and cells were incubated at 37˚C in 4 ml DMEM(-) media and 250 μl reaction mixture for 5-6 hours. Media was then aspirated and cells were grown overnight in DMEM(+) media to enable expression of the EGFP recombinant plasmid.

HeLa Cell Lysis

HeLa cells previously transfected with EGFP, EGFP-11E7, or EGFP-11cE7 were incubated at 37˚C overnight to allow expression of the appropriate proteins. Cells were then trypsinized, centrifuged, washed with PBS, then incubated for 30 minutes at 4˚C with lysis buffer containing 0.1% Triton X. The lysates were then spun down for 30 minutes (13,300 rpm, 4˚C) to pellet unwanted cell debris. The protein-containing supernatant was removed and subjected to SDS-PAGE and immunoblot analysis with a GFP antibody to analyze the expression of EGFP fusion proteins.

Binding Assays

Glutathione-Sepharose beads were used to immobilize GST, GST-Nup62N, and GST-CRM1 prior to performance of the binding assays. In the binding assays, SDS-PAGE and Coomassie Blue analysis showed both intact and some degraded GST-Nup62N. HeLa cells were transfected with EGFP, EGFP-11E7, and EGFP-11cE7 plasmids for 24 hours then lysed. The lysates were incubated with immobilized GST or GST-Nup62N for one hour at 4˚C. The beads were washed to remove non-specific binding and SDS-PAGE and immunoblotting analysis with a GFP antibody were used to detect the bound proteins. The Kapβ2 nuclear import receptor was used as a positive control for Nup62N binding and was detected with a Kapβ2 antibody.

In the binding assays, the bound proteins were run on an SDS-PAGE gel and visualized with Coomassie staining to confirm the correct molecular weight and to approximate equal quantities of bound protein in following experiments. HeLa cells were transfected with EGFP, EGFP-11cE7, and EGFP-16E7 NES plasmids and then were lysed. The lysates were incubated for one hour at 4˚C with the Glutathione-Sepharose-bound GST or GST-CRM1. The beads were washed three times to remove excess protein and to reduce non-specific binding. SDS sample buffer was used to elute the protein from the beads. Subsequent SDS-PAGE and immunoblotting with a GFP antibody was used to analyze the results of binding.

Results

HPV11 E7 nuclear trafficking

Previous data from the Moroianu lab demonstrated that HPV11 E7 was imported into the nucleus via its zinc-binding domain located in the carboxy-terminus (Piccioli et al., 2010). These sets of experiments were designed to better characterize the specific cellular proteins and mechanisms used by HPV11 E7 for nuclear import and export. To investigate potential interactions between the Nup62 FG domain and CRM1 and the zinc-binding domain of the E7 protein, we used binding assays. Glutathione-Sepharose beads were used to immobilize GST-Nup62 FG domain and GST-CRM1 nuclear export receptor for binding assays with EGFP-tagged HPV11 E7.

Purification and rebinding of GST-tagged proteins

GST-tagged Nup62N and CRM-1 were purified from IPTG-induced *E. coli* BL21 CodonPlus cells. Samples taken from subsequent steps in the purification were run on an SDS-PAGE gel to visualize the efficiency of each purification step (Figures 1 and 4). In some experiments, GST-tagged proteins previously eluted in purification steps were rebound to Glutathione-Sepharose beads. The efficiency of rebinding was visualized on an SDS-PAGE gel (Figure 2).

HPV11 E7 interacts with FG-nucleoporins

Binding assays followed by immunoblotting were performed between EGFP-tagged 11E7 and 11cE7 and GST-tagged Nup62N immobilized on Glutathione-Sepharose beads. The results show binding of 11E7 and 11cE7 to GST-Nup62N at

amounts comparable to that of the known import receptor Kapβ2 (Figure 3, lanes 6, 7, and 8). GST and EGFP used as negative controls did not show any interaction (Figure 3). This data suggests that HPV11 E7 enters the nucleus through direct interactions with FG nucleoporins in the nuclear pore complex. This is consistent with data previously obtained by the Moroianu lab, which shows the zinc-binding domain to be essential in the successful nuclear import of HPV11 E7 and supportive of a nuclear import mechanism dependent on this region of the protein.

HPV11 E7 interacts with the nuclear export receptor CRM1

Binding assays followed by immunoblotting with a GFP antibody were performed with immobilized GST-tagged CRM-1 and EGFP-tagged 11cE7. The results show binding between CRM1 and 11cE7 protein (Figure 5, lane 4). This binding is comparable to that of a fusion protein (EGFP16E7 NES) containing the nuclear export signal of the HIV REV protein (Figure 5, lane 5), which is known to be exported from the nucleus in a CRM-1 dependent manner. EGFP used as negative controls did not show any interaction with CRM1 (Figure 5, lane 6), and EGFP-11cE7 did not interact with GST (Figure 5, lane 7). These data suggest that HPV11cE7 binds directly to CRM1 nuclear export receptor.

Discussion

Previous studies in the Moroianu lab showed that HPV11 E7 enters the nucleus in a Ran GTPase-dependent but Kap β nuclear import receptor-independent pathway and that mutating the zinc-binding domain within the carboxy terminus of E7 impedes its nuclear import (Piccioli et al., 2010). In this study we investigated the relationship between the low risk HPV11 E7 and the Nup62 FG nucleoporin using transfection assays of HeLa cells along with binding assays and immunoblotting of EGFP-tagged fusion proteins. The larger tags used in these experiments ensured that the fusion proteins analyzed were too large for passive nuclear import or export. Binding assays with subsequent immunoblotting showed binding between HPV11cE7 and Nup62N, suggesting that 11 E7 enters the nucleus through direct interactions between its zinc-binding domain and FG nucleoporins.

Binding assays between HPV11cE7 and the nuclear export receptor CRM1 also showed binding of EGFP-11cE7 to the immobilized GST-CRM1. Together, these data suggest that HPV11 E7 requires CRM1 for nuclear export and that this process is dependent on E7's zinc-binding domain.

The data obtained in these and past experiments suggest a potential mechanism of nuclear import and export of HPV11 E7. One possible mechanism for Kap β-independent import is via weak hydrophobic interactions between the zinc-binding domain of E7 and nucleoporins containing phenylalanine-glycine (FG) repeats, thus imitating the interaction of nuclear import receptors and FG nucleoporins at the nuclear pore complex. This mechanism has been shown to be used by the human T lymphotropic virus type 1 TAX protein (Tsuji et al., 2007) and is feasible in the case of HPV11 E7 since the zinc-

binding domain of E7 is rich in nonpolar, hydrophobic residues which could interact with the hydrophobic FG-regions of nucleoporins (Piccioli et al., 2010). Future studies using other FG nucleoporins such as Nup153 and Nup54 could contribute support this mechanism of HPV11 E7's nuclear import.

Studies in the Moroianu lab have suggested that a leucine-rich export signal in the high-risk HPV16 E7 protein may function as an NES (Knapp et al., 2009). The nuclear export receptor CRM1 has been shown to be an exportin that interacts with such leucine-rich NESs, mediating their nuclear export (Fornerod et al., 1997). Consistent with this data and those gathered from these binding assays, a possible mechanism for the nuclear export of HPV11 E7 involves binding of its leucine-rich NES to CRM1.

Figures

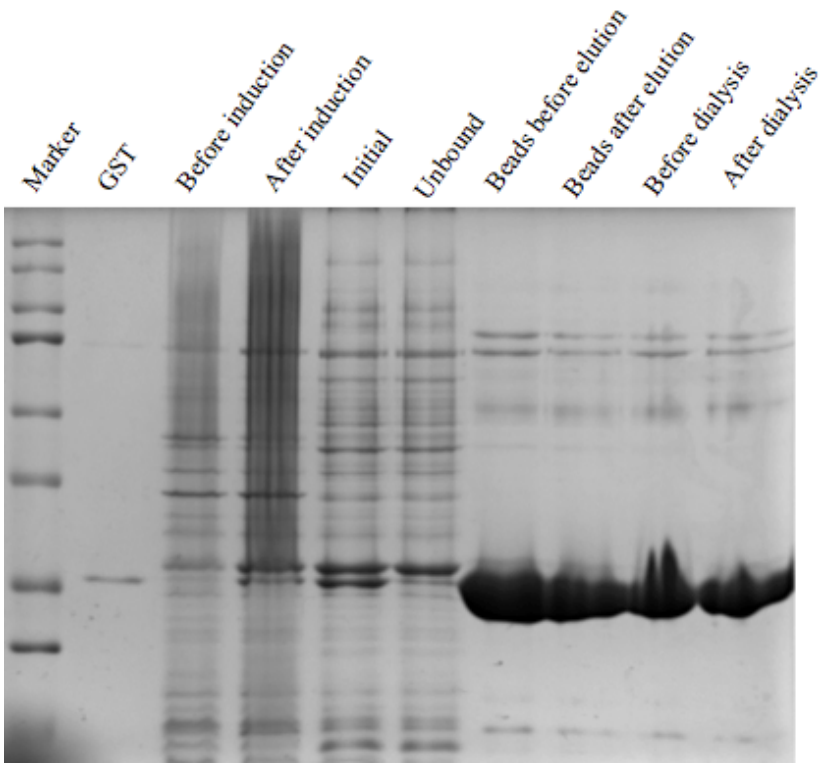

Figure 1. Purification of GST and GST-Nup62N. Proteins were purified by use of Glutathione-Sepharose beads from *E. coli* transformed with the pGEX plasmid and induced with IPTG.

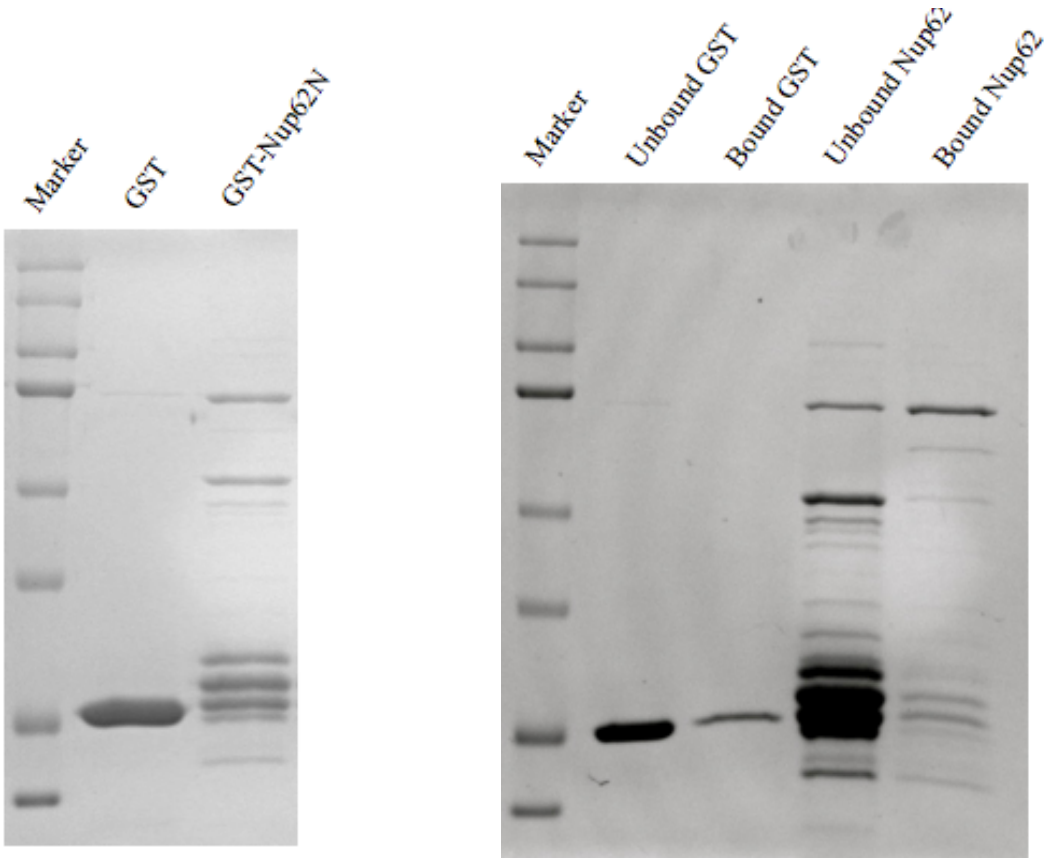

Figure 2. Rebinding of purified GST and GST-Nup62N to Glutathione-Sepharose beads. An SDS-PAGE gel was run of Glutathione-Sepharose beads upon one hour incubation with GST or GST-Nup62N. Pure protein samples were run to visualize the efficiency of rebinding.

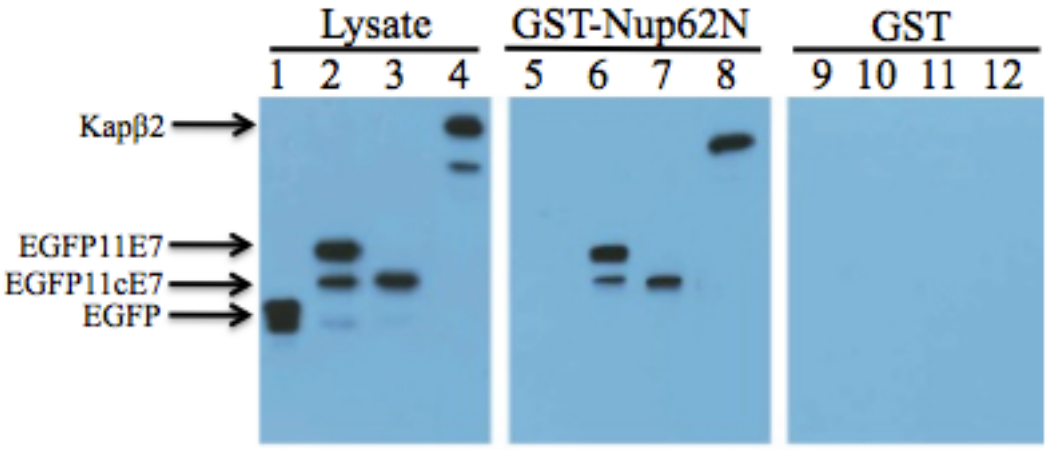

Figure 3. HPV11 E7 interacts through its carboxy-terminus with the amino-terminus of Nup62. HeLa cells were transfected with EGFP, EGFP11E7, and EGFP11cE7. Cell lysates were then incubated with Glutathione-Sepharose beads for one hour. Binding of the EGFP-tagged proteins to Nup62N and GST was visualized through western blotting with an α-GFP antibody. Kapβ2 was used as a positive control.

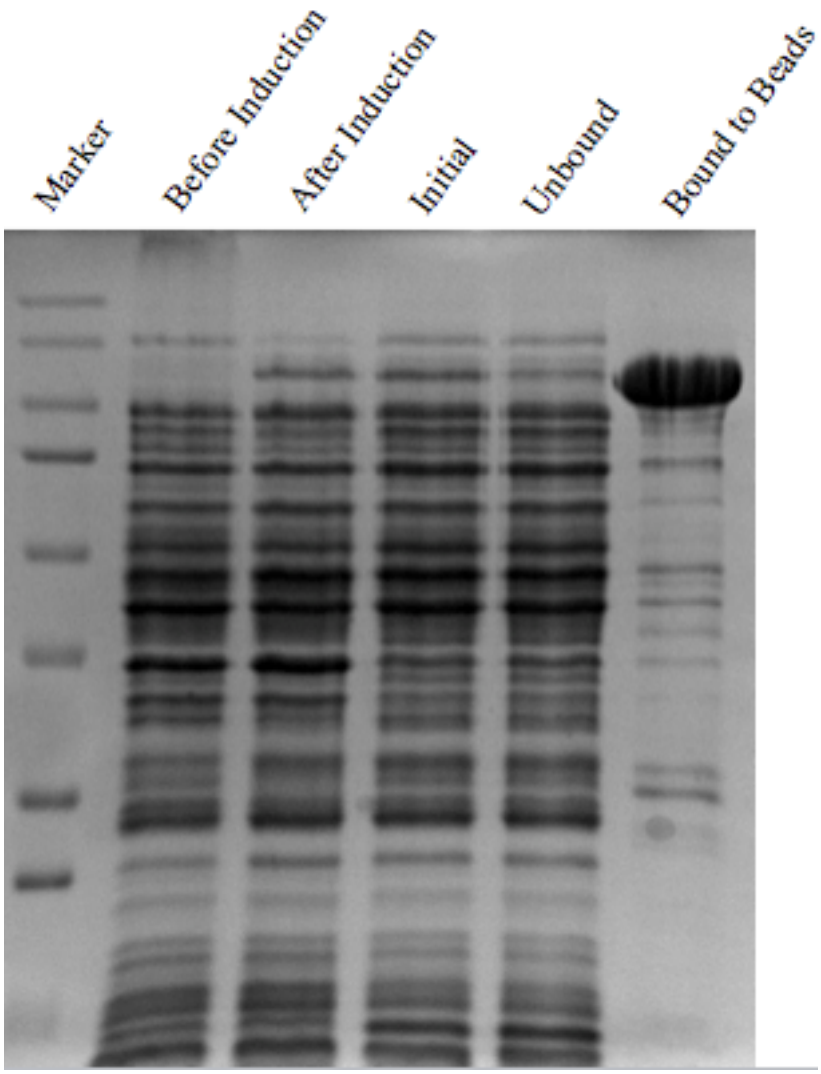

Figure 4. Purification of GST-CRM1. A GST-CRM1 fusion protein was purified with Glutathione-Sepharose beads.

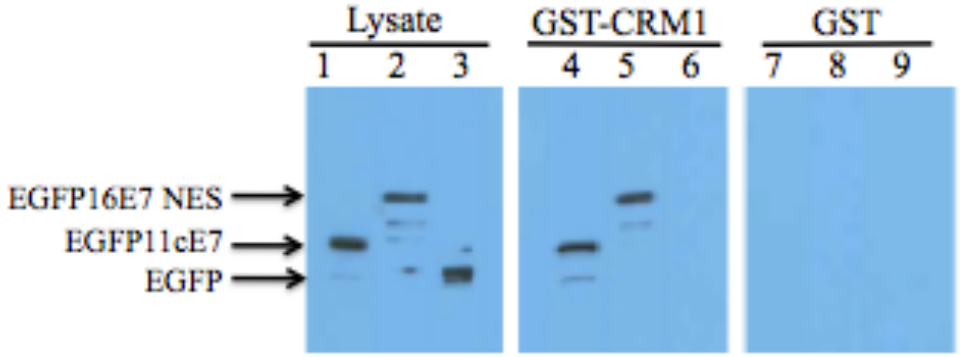

Figure 5. HPV11 E7 interacts with CRM1 through its carboxy-terminus. HeLa cells previously transfected with EGFP, EGFP11cE7, and EGFP16E7 NES were lysed and incubated with Glutathione-Sepharose beads. Binding of the fusion protein of interest to CRM1 and GST was visualized by western blotting with an α-GFP antibody. An HPV16 E7 NES (containing the REV protein nuclear export signal) was used as a positive control.

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