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Rasgrp2 regulates the permissiveness of NIH3T3 cells to a herpes simplex virus 1 mutant with inactivated ICP34.5 gene

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Summary. – We have previously reported that mtHSV, a herpes simplex virus 1 (HSV-1) mutant with an inactivated gene for β-galactosidase, can efficiently lyse tumor but not normal cells. However, the mechanism of this selective oncolytic activity is so far unclear. In this study, using the phage display screening we identified the cellular protein binding to HSV-1 mutant (mtHSV) as (Ras guanyl releasing protein 2) Rasgrp2 which regulates the Ras signaling pathway. Rasgrp2 was found to bind directly to purified mtHSV as well as to mtHSV present within infected HeLa cells where it aggregated on the cell membrane. NIH3T3 cells were found nonpermissive to mtHSV but they became permissive following transformation with the Rasgrp2 gene. This effect was linked to the activation of the Ras-PKR signaling pathway. These observations indicate a key role of Rasgrp2 in the mtHSV infection of NIH3T3 cells and are important for the potential use of mtHSV in cancer therapy.

Keywords: herpes simplex virus 1 mutant; RAS guanyl releasing protein 2; NIH3T3 cells; permissiveness

Introduction

We previously reported that mutant herpes simplex virus (mtHSV), a conditionally replicating recombinant of herpes simplex virus 1, was constructed by inserting *Escherichia coli* β-gal gene into the loci of the ICP34.5 gene, which is the major determinant of apoptosis-inhibiting and neurovirulence of HSV-1 (Lan *et al*., 2003). mtHSV could replicate selectively in many tumor cells including murine sarcoma cell (S-180) but not in normal cell lines (Xue *et al*., 2005). The data *in vivo* demonstrated that the effect of mtHSV-mediated tumor therapy was efficacious in both nude mice bearing human Hep3B cells and in Balb/c mice loaded with S-180 cells, and no side effect was found in other natural tissues (Zhu *et al*., 2007). Although, even if mtHSV has been proved to be potent therapeutics against tumor and the preclinical studies still continue, its oncolytic mechanism needs further understanding.

The ability of a virus to replicate within specific cell types often depends on viral interactions with, and alterations of, well-regulated cellular pathways. In the case of HSV-1, crucial factor is its ability to regulate the phosphorylation state of the translation initiation factor eIF2α. The activation of the interferon-inducible double-stranded RNA-dependent protein kinase (PKR) during the normal course of infection leads to the phosphorylation of eIF2α and the inhibition of protein synthesis (Gale and Katze, 1998; Roberts *et al*., 1976). Roizman *et al*. proved that the HSV-1 neurovirulence protein ICP34.5 binds to a protein phosphatase and causes dephosphorylation of eIF2α, thereby inhibiting the activity of PKR (Chou and Roizman, 1992; He *et al*., 1997a, b).

Ras signaling pathway is a gateway for HSV-1 infection and oncogenes in Ras signaling pathway regulate host-cell permissiveness to HSV-1 (Farassati and Lee, 2003; Farassati *et al*., 2001). Our previous results show that Ras/RTN3 is important for mtHSV entry to HeLa cells (Su *et al*., 2007).

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Abbreviations: β-gal = β-galactosidase; HSV-1 = herpes simplex virus 1; MBP = maltose-binding protein; mtHSV = HSV-1 mutant; PKR = dsRNA-activated protein kinase R; Rasgrp2 = RAS guanyl releasing protein 2; siFTa = siRNA against farnesyltransferase

Rasgrp2, a long alternatively spliced form of CalDAG-GEFI belonging to the family of RasGEFs, is a plasma membrane-targeted, dual specificity Ras/Rap exchange factor. It switches Ras/Rap from the inactive GDP-bound state to the active GTP-bound state (Caloca *et al.*, 2003; Hogquist, 2001; Roose *et al.*, 2007), that operates as binary switch in key signal transduction pathways regulating cell proliferation and differentiation (Caloca *et al.*, 2004; Clyde-Smith *et al.*, 2000; Dupuy *et al.*, 2001). Rasgrp2 is lipid-modified protein localized in the plasma membrane and expressed preferentially in the brain and blood (Ghandour et al., 2007; Xu et al., 2006b). Recently, Rasgrp2 research is mainly focused on its crucial function for signal integration in platelets (Dower et al., 2000; Ebinu *et al.*, 2000; Eto *et al.*, 2002; Ho-Tin-Noe *et al.*, 2008). In this study, we attempted to disclose the mechanism of the known selective oncolytic activity of mtHSV. The obtained results revealed that, in the mtHSV infection of permissive HeLa cells, mtHSV binds to cellular Rasgrp2, forming aggregates on the cell membrane. Furthermore, NIH3T3 cells, normally nonpermissive to mtHSV became permissive following transformation with the Rasgrp2 gene. These results contribute to the potential application of mtHSV in the cancer therapy.

Materials and Methods

Virus and cells. The mtHSV, which was constructed by inserting *E. coli* β-gal gene into the loci of the apoptosis-inhibiting gene ICP34.5 of HSV-1, was previously described (Lan *et al.*, 2003). mtHSV and HSV-1 wild-type strain F were replicated in Vero cells and purified by cesium chloride (CsCl) ultracentrifugation. The virus titration was measured by plaque assay. HeLa cells and NIH3T3 cells were cultured in DMEM containing 10% calf serum (HyClone, USA) and penicillin-streptomycin (100 U/ml) (Sigma).

Table 1. The primer sequences for real-time PCR

Amplicon	Primer	Sequence (5′-3′)
Human Rasgrp2	hRasgrp2 forward	ACAATCCCGGAAGGACAACTC
	hRasgrp2 reverse	GTCTATGTCGATTAGGCTGCTG
Mouse Rasgrp2	mRasgrp2 forward	GCTCCGTGGTTGCATCGAA
	mRasgrp2 reverse	AGGAAGGTATGTACCAGGGGT
Human GAPDH	hGAPDH forward	CTCACCGGATGCACCAATGTT
	hGAPDH reverse	CGCGTTGCTCACAATGTTCAT
Mouse GAPDH	mGAPDH forward	AGCTTCGGCACATATTTCATCTG
	mGAPDH reverse	CGTTCACTCCCATGACAAACA

Antibodies. Mouse Rasgrp2-specific antibody was purchased from Abnova (Abnova, Taiwan). Rabbit β-actin-specific monoclonal antibody, rabbit Na^+-K^+ ATPase-specific antibody, mouse PKR-specific antibody and mouse eIF2a-specific antibody were purchased from Santa Cruz (Santa Cruz Biotechnology, USA). Rabbit phospho-PKR-specific antibody and rabbit phospho-eIF2a-specific antibody were purchased from Biosource (Life Technologies, USA). HRP-conjugated goat anti-rabbit and goat anti-mouse IgG were purchased from ZhongShan Biotechnology (Beijing, PR China). Rabbit mtHSV-specific antibody was prepared in our laboratory as described previously (Su *et al.*, 2007).

Phage display. The phage-display random 15 aa peptide library was kindly presented from Dr. G. P. Smith (Division of Biological Sciences, University of Missouri, Columbia, Missouri, USA). The panning was carried out as described previously (Yi *et al.*, 2003). Briefly, the immunotubes were coated overnight at 4°C with purified mtHSV (10^{11} PFU) in PBS (pH 7.0), and then washed once with distilled water and blocked with PBS containing 5% skimmed milk powder at 37°C for 1 hr. The blocking solution was removed and the phage-display library (10^{12} PFU) in 4 ml PBS was added and incubated at 37°C for 2 hrs, followed by washing 20 times with PBS containing 0.5% Tween-20 and subsequently 20 times with PBS to remove all non-specifically bound phages. Bound phages were eluted with 0.2 mol/l glycine/HCl buffer (pH 2.2). The elution was neutralized with 0.5 ml of 1 mol/l Tris/HCl (pH 9.1). The eluted phages were used to infect *E. coli* K91 for amplification. Amplified phages were rescued using the same procedure as above and subjected to the next round of panning. After four rounds of panning, the phages were analyzed by ELISA using the vector phage as a negative control. The positive clones were sequenced and identified by BLAST software (www.ncbi.nlm.nih.gov/BLAST).

ELISA. Phage clones amplified by panning were tested by ELISA for their ability to bind specifically to mtHSV. The ELISA was performed as previously described (Krejnusova *et al.*, 2009) using HRP-conjugated mouse anti-E-tag (1:10,000 in BSA; Amersham Pharmacia) and TMB substrate (2 mg/ml TMB (Sigma) in 1 mol/l sodium acetate, 4% H_2O_2). HRP activity was estimated by measuring at A_{405} using a universal microplate reader (EXL-800; Bio-tek). This measurement was repeated twice, using the vector phage as a negative control.

Western blot analysis. Protein extraction and western blot analysis was performed as previously described (Xu *et al.*, 2006a). Primary antibodies included mouse Rasgrp2-specific antibody (1:1,000); rabbit β-actin-specific monoclonal antibody (1:5,000); rabbit Na^+-K^+ ATPase-specific antibody (1:500), mouse PKR-specific antibody (1:1,000); mouse eIF2a-specific antibody (1:500); rabbit phospho-PKR-specific antibody (1:500); rabbit phospho-eIF2a-specific antibody (1:500) and rabbit mtHSV-specific antibody (1:100). Secondary antibodies were used at a dilution of 1:1,000. The signal was developed by the ECL plus Western blotting analysis kit (Boster Biotech, China).

Table 2. Sequenced phage clones

Clone	Nucleotide sequence	Amino acid sequence	Frequency
D6	GCGCCTTTTAGTCGTCTTCTTTTTCCTGATTTTCGTTCTTTTGTT	APFSRLLFPDFRSFV	3
F11	TCTGTTGATTCTCCGTTTGGTTATCTTTTTTCTTTGGCTCCTGCG	SVDSPFGYLFSLAPA	1
E8	CGGGGTGGTTTTTCTGATACTAGTCGTACGGGTTGGGTTTCGGTT	RGGFSDTSRTGWVSV	1
D10	CTTGAGGATAATCGTCCTCCTTGGCCGGTTCTTAGTAGGCGTCTG	LEDNRPPWPVLSRRL	1
A2	TCGAGGGATGGGTTGCATTCTTTTTGTTATGTGGGGTGTCCGCCG	SRDGLHSFCYVGCPP	1
H9	GGTCTTGATCTGCTGGGTGATGTTAGGATTCCTGTGGTTCGTCGG	GLDLLGDVRIPVVRR	1
H8	TGGGCTTCTTTTTATGCTAGTTCTTATAGGGATTCTCGTCTTCTT	WASFYASSYRDSRLL	1
H7	CTTTTTCCTGGTTCTGCTGCGTATGTGGTTAGTTTGAGTCGTTTT	LFPGSAAYVVSLSRF	1

Co-immunoprecipitation assay. To test the *in vivo* interaction of Rasgrp2 and mtHSV, HeLa cells were infected with mtHSV at a MOI of 10 for 6 hrs and then lysed for immunoprecipitation assay using a MPER™ immunoprecipitation kit (Pierce, USA) according to the manufacturer's instructions.

Virus overlay protein binding assay (VOPBA). Recombinant Rasgrp2 protein was expressed and purified using pMAL™ Protein fusion and purification system (NEB Biolabs). Equal amount (20 μg) of MBP-tagged Rasgrp2 fusion protein (Rasgrp2-MBP) or MBP control protein (MBP) was subjected to electrophoresis on 10% native polyacrylamide gel and transferred to nitrocellulose membranes. The blots were blocked with 5% skim milk in TBS overnight, followed by incubation with 10^7 PFU of purified mtHSV in 1% skim milk in TBS for 4 hrs at room temperature and washed three times with TBS. Subsequently, the two blots were incubated respectively with mouse Rasgrp2-specific antibody (1:500) or rabbit mtHSV-specific antibody (1:100) in 5% skim milk in TBS. The blots were visualized by incubation with a secondary goat anti-mouse IgG or goat anti-rabbit IgG respectively and developed as described above.

Cell viability assay. HeLa cells and NIH3T3 cells were grown in 96-well plates infected with mtHSV at MOI of 10. Mock cells were used as negative control. The number of viable cells was determined by the cell viability assay (MTT) at 0, 24, 48, and 72 hrs after infection as previously described (Su *et al.*, 2007). The amount of dead cells at different time was calculated by this formula: dead cells (%) = (1 – average absorbance of experimental group / average absorbance of control group) × 100%.

Immunofluorescence assay. HeLa cell cultures on coverslips were infected with mtHSV at a MOI of 10 for 6 hrs. Immunofluorescence assay was performed as described previously (Xiao *et al.*, 2013).

β-gal assay. NIH3T3 cell monolayers grown in 96-well plates were transfected with pAdtrackCMV-Rasgrp2, after 48 hrs post transfection, the cells were infected with mtHSV at MOI of 1 and incubated at 37°C for 6 hrs. Cells were lysed with 0.5% Nonidet P-40, chlorophenol red-β-D-galactopyranoside (Sigma) was added, and β-gal activity was analyzed at A_{570} with a universal microplate reader. Mean results were calculated for three replicate samples.

Real-time PCR. Total RNA was isolated using RNeasy mini kit (Qiagen) and reverse transcription was done by using PrimeScript RT master mix (TaKaRa). The resulting cDNA samples were amplified by real-time PCR using gene-specific primer sets in conjunction with SYBR Premix Ex Taq (TaKaRa).The primer sequences are listed in Table 1.

Results

Identification of Rasgrp2 as a mtHSV-binding protein using phage display

In an effort to find the proteins involved in the entry of mtHSV, especially the proteins that interact directly with mtHSV, we performed a screening for mtHSV-binding proteins using the phage-display random 15 aa peptide library. After four rounds of screening, mtHSV binding specificity of phage-displayed peptides was analyzed by ELISA with 90 randomly picked clones. Of these, 10 positive clones were selected, amplified and further analyzed by ELISA. After DNA sequencing, the sequences of 10 individual clones were examined and the amino acid sequences were deduced (Table 2). Among them, we found three positive clones encoding the same peptide sequence APFSRLLFPDFRSFV and the BLAST result showed 84% identity with human Rasgrp2.

Confirmation of Rasgrp2-mtHSV interaction

To confirm the interaction between mtHSV and Rasgrp2, we used purified recombinant protein Rasgrp2-MBP in virus overlay protein binding assay (VOPBA). The purified MBP protein was used as a negative control. As shown in Fig. 1a, Rasgrp2-MBP protein (Fig. 1a, line 2) interacted with mtHSV and could be detected by both antibodies of Rasgrp2 and mtHSV, while MBP protein did not (Fig. 1a, line 1). This indicates that Rasgrp2 protein can directly interact with mtHSV *in vitro*. To further analyze Rasgrp2 and mtHSV interaction in human cells, HeLa cells which show high endogenous expression of Rasgrp2 (Fig. 2e) were harvested, gently lysed and used for co-immunoprecipitation assays. In co-immunoprecipitation, we have shown

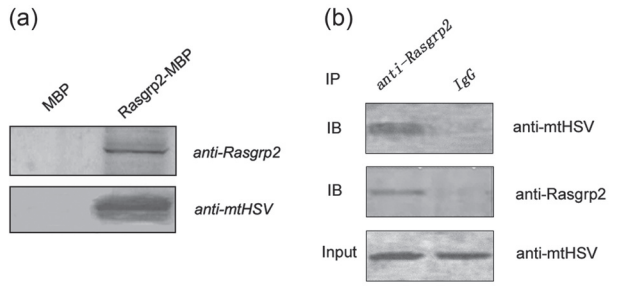

Fig. 1

Rasgrp2 binds to mtHSV

(a) Direct binding of purified Rasgrp2 to mtHSV in VOPBA. (b) Co-immunoprecipitation of Rasgrp2 and mtHSV during infection of HeLa cells. (IP) the antibodies used for immunoprecipitation assay; (IB) western blot analysis; (Input) western blot analysis of the cell lysates served as a input control.

that endogenous Rasgrp2 binds with mtHSV (Fig. 1b). All these results show that Rasgrp2 interacts with mtHSV *in vivo* and *in vitro*.

NIH3T3 cells become permissive to mtHSV following transformation with Rasgrp2 gene

To determine the function of Rasgrp2 in mtHSV infection, we first infected the NIH3T3 and HeLa cells by mtHSV. HeLa cells were used as a positive control. The cell viability assay results demonstrated that in comparison with HeLa cells, the NIH3T3 cells had rarely been killed by mtHSV (Fig. 2a). As mentioned, mtHSV has a *LacZ* gene insertion, so we performed β-gal assay to further confirm the virus replication. The results from β-gal assay showed that mtHSV did not replicate in NIH3T3 cells (Fig. 2b). At 48 hrs after mtHSV infection, HeLa cells showed visible signs of infection, while infected NIH3T3 cells did not (Fig. 2c). All these results indicate that NIH3T3 cells are non-permissive cells of mtHSV.

Interestingly, we found that the expression of Rasgrp2 might have some association with the mtHSV infection. We analyzed level of the endogenous expression of Rasgrp2 in HeLa and NIH3T3 cells respectively. The real-time PCR (Fig. 2d) and western blot (Fig. 2e) results showed that HeLa

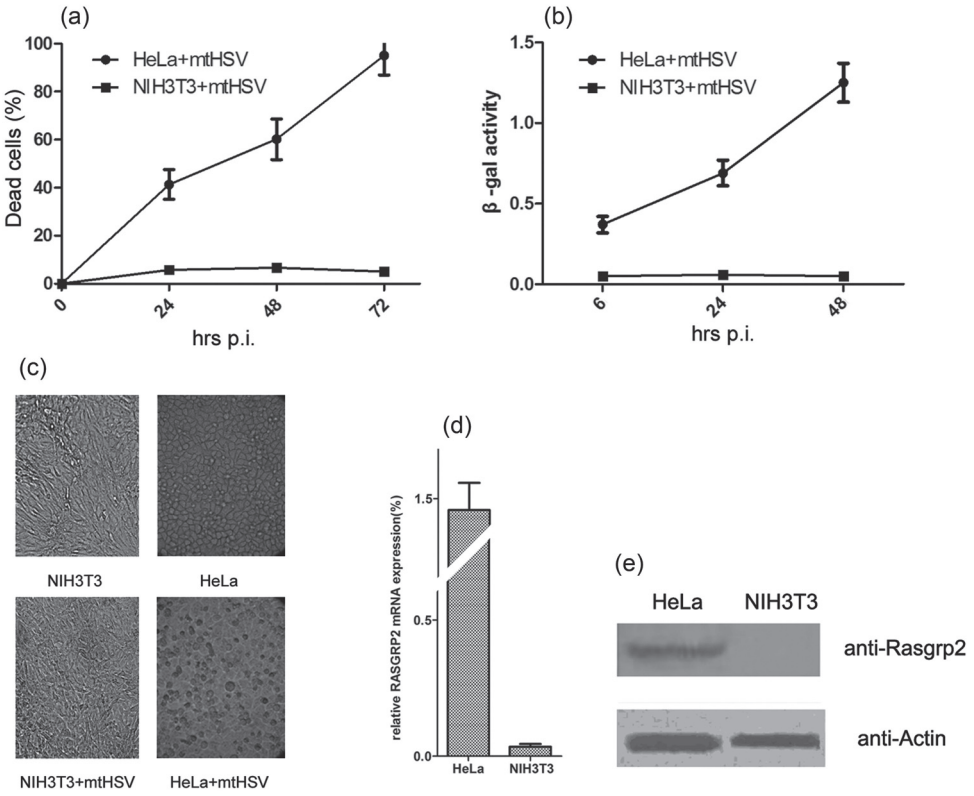

Fig. 2

HeLa, but not NIH3T3 cells, express Rasgrp2 and are permissive to mtHSV

(a) cell viability, (b) β-gal activity, (c) cell morphology, (d) real-time PCR and (e) Western blot analysis of Rasgrp2 expression.

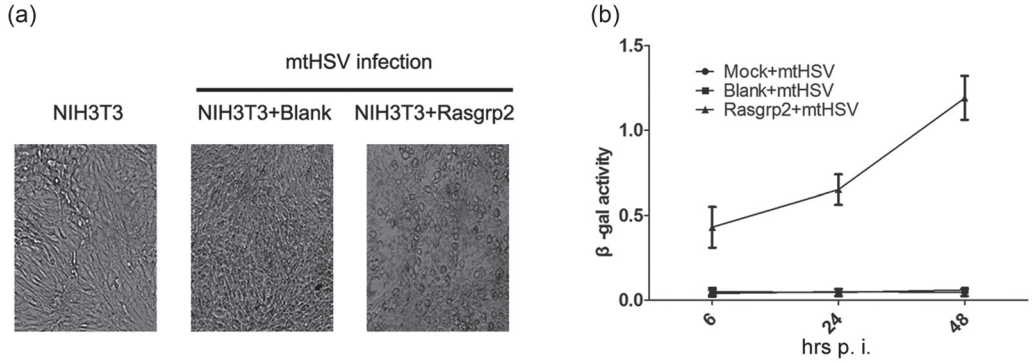

Fig. 3

Transfection with Rasgrp2 gene makes NIH3T3 cells permissive to mtHSV

(a) The cell morphology 48 hrs post infection and (b) β-gal activity of cell lysates.

cells exhibited high levels of Rasgrp2 expression while in NIH3T3 cells the level was undetectable.

So the question, if Rasgrp2 was related to mtHSV infection, arised. Rasgrp2 was transfected into NIH3T3 cells, using normal NIH3T3 cells and cells transfected with empty vector as control. After 48 hrs post transfection, NIH3T3 cells were infected with mtHSV. The transfection experiment showed that at 48 hrs after mtHSV infection, the cells transformed with pAdtrackCMV-Rasgrp2 showed visible signs of viral infection (Fig. 3a), indicating that Rasgrp2 altered NIH3T3 permissiveness to mtHSV. The β-gal assay also showed that mtHSV could normally replicate in pAdtrackCMV-Rasgrp2 transformed NIH3T3 cells, but neither in the mock nor the empty vector transformed cells (Fig. 3b).These results indicated that Rasgrp2 can alter the permissivness of NIH3T3 cells and enables their infection by mtHSV.

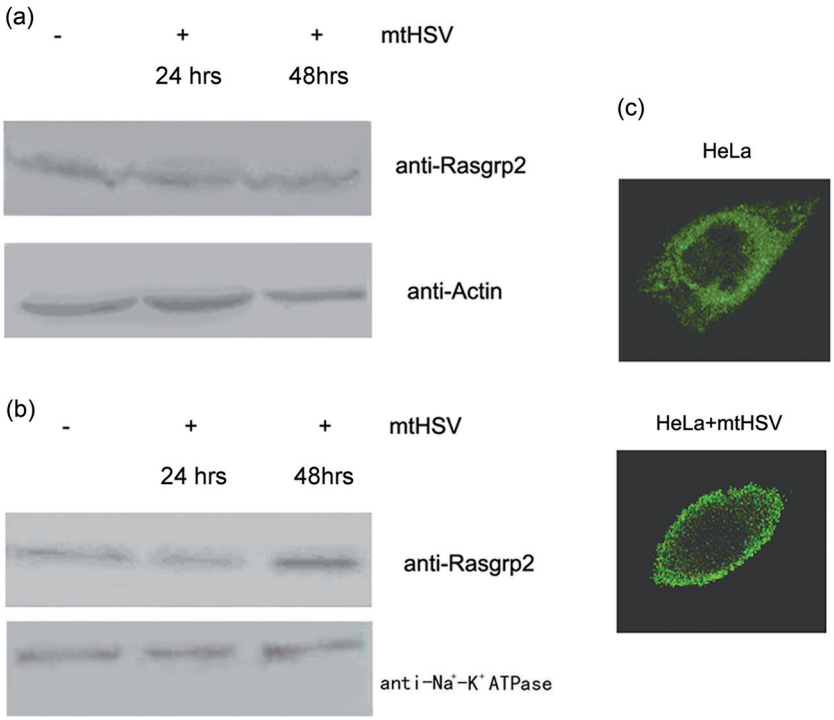

Fig. 4

Rasgrp2 aggregates on the cell membrane of mtHSV-infected HeLa cells

The Rasgrp2 in whole cells (a) and plasma membranes (b) as detected by Western blot analysis and immunofluorescence assay (c).

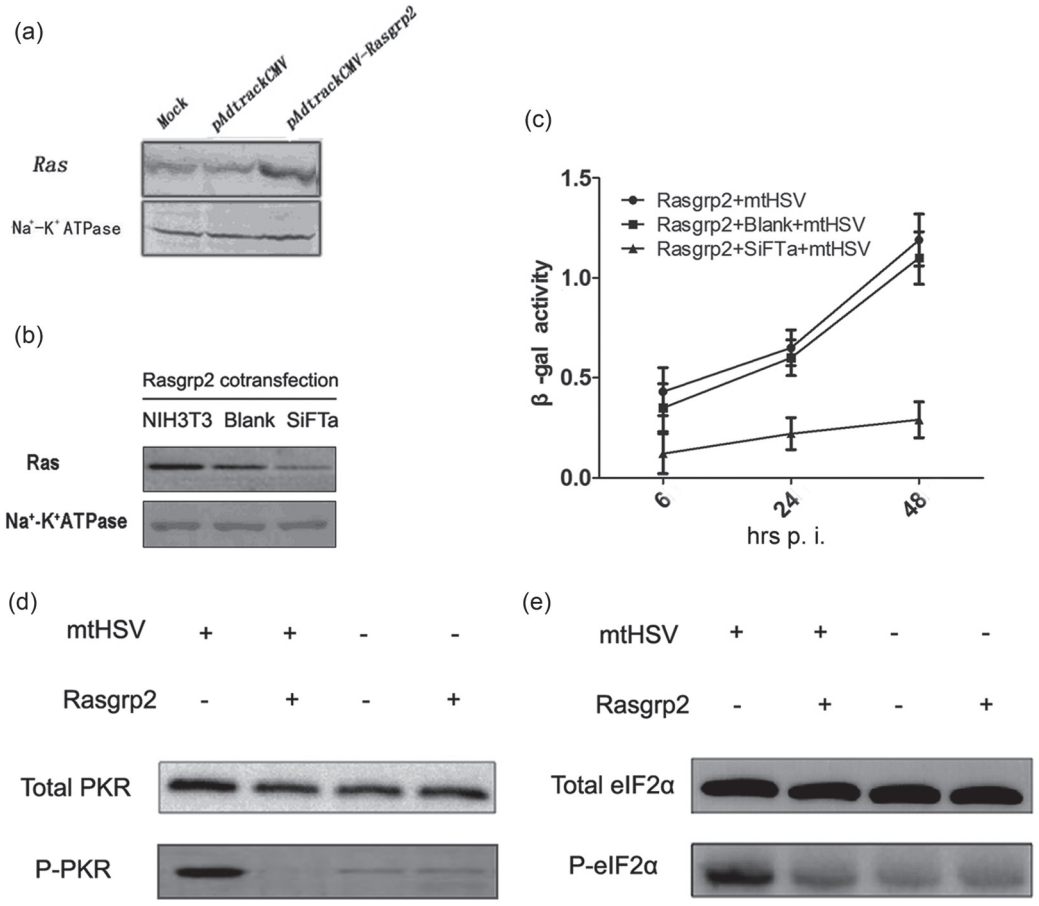

Fig. 5

Upregulation of Rasgrp2 in NIH3T3 cells activates the Ras-PKR signaling pathway

Western blot analysis of plasma membrane Ras in Rasgrp2 transfected cells (a) and siFTA co-transfected cells (b) and cellular β-gal activity (c). Western blot analysis of total PKR (d) and eIF2α (e) and their phosphorylation forms P-PKR and P-eIF2α.

In order to investigate the mechanism that Rasgrp2 uses to regulated mtHSV infection, we analyzed the Rasgrp2 endogenous expression and its cellular location in HeLa cells during mtHSV infection. The results showed that at 24 and 48 hrs after mtHSV infection, the expression of Rasgrp2 in the cytoplasm had no obvious change (Fig. 4a) while the signal of Rasgrp2 on plasma membrane after 48 hrs was evidently stronger (Fig. 4b). The immunofluorescence confirmed these results (Fig. 4c) and showed that mtHSV infection induced Rasgrp2 aggregation on the cell membrane.

Rasgrp2 activates the Ras-PKR signaling pathway in NIH3T3 cells

Our early works showed that the infection of mtHSV was often associated with the host cell′s Ras-PKR signaling activity. Here we detected the difference of Ras expression and the phosphorylation status of PKR and eIF2α in NIH3T3 with/without Rasgrp2 transfection. The western blot results showed that the expression of Ras in NIH3T3 cell membrane obviously increased after Rasgrp2 transfection (Fig. 5a). To efficiently decrease Ras expression on the cell membrane, we used siRNA against farnesyltransferase, named siFTa (Su *et al*., 2007). We found that siFTa significantly inhibited the Ras expression (Fig. 5b) and mtHSV infection (Fig. 5c) induced by Rasgrp2. In the mtHSV infected NIH3T3 cells, the phosphorylated PKR (Fig.5d) and eIF2α (Fig. 5e) significantly increased, but in Rasgrp2 overexpressing NIH3T3 cells, the phosphorylation of PKR and eIF2α, induced by mtHSV infection, was blocked. Intrestingly, this inhibiton was only observed during mtHSV infection, indicating that it might be a stress response of virus infection. These results suggested that Rasgrp2 might regulate mtHSV infection to NIH3T3 through activating Ras-PKR- eIF2α signaling pathway.

Discussion

Oncolytic viruses provide an attractive tool for cancer treatment because of their selective replication within the tumor (McKie *et al.*, 1996; Toyoizumi *et al.*, 1999). It has been recently proposed that Ras/PKR signaling pathway was a gateway for HSV-1 infection (Farassati and Lee, 2003). The gene product of g134.5 (called ICP34.5) presumably forms a complex with protein phosphatase 1, and redirects its activity to dephosphorylate eIF-2α (Wylie *et al.*, 2009). Thus, essentially ICP34.5 has an antagonistic role to that of PKR (Wylie *et al.*, 2009). mtHSV and another famous ICP34.5-deleted HSV-1 strain (R3616), lacking the ICP34.5 function, cannot revert the effects of PKR activation on protein synthesis and therefore its lytic replication might be expected to be limited to tumor cells that display a Ras-activated pathway (Farassati *et al.*, 2001; Garcin *et al.*, 1990; Sarinella *et al.*, 2006). How is this signaling pathway triggered? We suppose, that it is triggered by some protein in Ras signaling pathway that directly interacts with the virus or its receptors.

In this study, we used phage display system and found a potential mtHSV associated protein Rasgrp2. Our further experiments confirmed the direct interaction between mtHSV and Rasgrp2 *in vivo* and *in vitro*. Stone *et al.* reported that Rasgrp2 is a plasma membrane-targeted protein, which lies upstream of Ras in Ras signaling pathway (Coughlin *et al.*, 2006). Our previous results showed that Ras protein in HeLa cells was obviously down regulated on plasma membrane 48 hrs after mtHSV infection (Su *et al.*, 2007). However, here we have found that after mtHSV infection, Rasgrp2 aggregated on the cell membrane. These results imply that besides the function as a Ras/Rap exchange factor, Rasgrp2 might have some other function during the infection. Chinese hamster ovary (CHO) cells and NIH3T3 cells were two well-known HSV-1 non-permissive cell lines (Conner *et al.*, 2005; Farassati *et al.*, 2001), but the non-permissive mechanisms of these two cell lines were different. CHO cells were traditionally regarded as non-permissive cells for HSV-1 infection as they lack the specific entry receptors, and modified CHO cells have been instrumental in the identification of HSV-1 receptors in numerous studies (Conner *et al.*, 2005). In the case of NIH3T3 cells, Farassati *et al.* reported that NIH3T3 cells transformed with the oncogenes *v-erbB*, activated *sos* or *ras* and became significantly more permissive to HSV-1, and the permissiveness mainly relied on the phosphorylation status of PKR and eIF2α (Farassati *et al.*, 2001). In this study, we found that Rasgrp2 is also an oncogene in Ras signal pathway. Rasgrp2 is inactive in normal NIH3T3 cells, while exogenous Rasgrp2 could significantly alter permissiveness of NIH3T3 to mtHSV infection. Further research illustrated that overexpressing Rasgrp2 obviously inhibited the phosphorylation of PKR and eIF2α in the condition of mtHSV infection of NIH3T3. So we could assume that, in mtHSV infected NIH3T3 cells, highly phosphorylated PKR led to the phosphorylation of the translation initiation factor eIF-2α resulting in inhibition of translation of the viral transcripts. In Rasgrp2 transformed cells, mtHSV binds to Rasgrp2, recruits it to the plasma membrane, and Rasgrp2 activates Ras, and then the PKR phosphorylation step is probably prevented or reversed by activated Ras or one of its downstream elements, allowing viral gene translation to proceed.

In conclusion, using the phage display screening we have identified the cellular protein binding to mtHSV as Rasgrp2 protein that regulates the Ras signaling pathway. Rasgrp2 was found to bind directly to purified mtHSV as well as to mtHSV present within infected HeLa cells. In the latter case it aggregated on the cell membrane. NIH3T3 cells were found non-permissive to mtHSV but they became permissive following transfection with the Rasgrp2 gene. This effect was linked to the activation of the Ras-PKR signaling pathway. These observations indicate a key role of Rasgrp2 in the mtHSV infection of NIH3T3 cells and are important for the potential use of mtHSV in cancer therapy.

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References

Caloca MJ, Zugaza JL, Bustelo XR (2003): Exchange factors of the RasGRP family mediate Ras activation in the Golgi. J. Biol. Chem. 278, 33465–33473. http://dx.doi.org/10.1074/jbc.M302807200

Caloca MJ, Zugaza JL, Vicente-Manzanares M, Sanchez-Madrid F, Bustelo XR (2004): F-actin-dependent translocation of the Rap1 GDP/GTP exchange factor RasGRP2. J. Biol. Chem. 279, 20435–20446. http://dx.doi.org/10.1074/jbc.M313013200

Chou J, Roizman B (1992): The gamma 1(34.5) gene of herpes simplex virus 1 precludes neuroblastoma cells from triggering total shutoff of protein synthesis characteristic of programed cell death in neuronal cells. Proc. Natl. Acad. Sci. USA 89, 3266–3270. http://dx.doi.org/10.1073/pnas.89.8.3266

Clyde-Smith J, Silins G, Gartside M, Grimmond S, Etheridge M, Apolloni A, Hayward N, Hancock JF (2000): Characterization of RasGRP2, a plasma membrane-targeted, dual specificity Ras/Rap exchange factor. J. Biol. Chem. 275, 32260–32267. http://dx.doi.org/10.1074/jbc.M006087200

Conner J, Rixon FJ, Brown SM (2005): Herpes simplex virus type 1 strain HSV1716 grown in baby hamster kidney cells has altered tropism for nonpermissive Chinese hamster ovary cells compared to HSV1716 grown in vero cells. J. Virol.

79, 9970–9981. http://dx.doi.org/10.1128/JVI.79.15.9970-9981.2005

Coughlin JJ, Stang SL, Dower NA, Stone JC (2006): The role of RasGRPs in regulation of lymphocyte proliferation. Immunol. Lett. 105, 77–82. http://dx.doi.org/10.1016/j.imlet.2006.01.005

Dower NA, Stang SL, Bottorff DA, Ebinu JO, Dickie P, Ostergaard HL, Stone JC (2000): RasGRP is essential for mouse thymocyte differentiation and TCR signaling. Nat. Immunol. 1, 317–321. http://dx.doi.org/10.1038/80799

Dupuy AJ, Morgan K, von Lintig FC, Shen H, Acar H, Hasz DE, Jenkins NA, Copeland NG, Boss GR, Largaespada DA (2001): Activation of the Rap1 guanine nucleotide exchange gene, CalDAG-GEF I, in BXH-2 murine myeloid leukemia. J. Biol. Chem. 276, 11804–11811. http://dx.doi.org/10.1074/jbc.M008970200

Ebinu JO, Stang SL, Teixeira C, Bottorff DA, Hooton J, Blumberg PM, Barry M, Bleakley RC, Ostergaard HL, Stone, JC (2000): RasGRP links T-cell receptor signaling to Ras. Blood 95, 3199–3203.

Eto K, Murphy R, Kerrigan SW, Bertoni A, Stuhlmann H, Nakano T, Leavitt AD, Shattil SJ (2002): Megakaryocytes derived from embryonic stem cells implicate CalDAG-GEFI in integrin signaling. Proc. Natl. Acad. Sci. USA 99, 12819–12824. http://dx.doi.org/10.1073/pnas.202380099

Farassati F, Lee PW (2003): Ras signalling pathway: a gateway for HSV-1 infection. Sci.Wrld. J. 3, 533–535. http://dx.doi.org/10.1100/tsw.2003.38

Farassati F, Yang AD, Lee PW (2001): Oncogenes in Ras signalling pathway dictate host-cell permissiveness to herpes simplex virus 1. Nat. Cell Biol. 3, 745–750. http://dx.doi.org/10.1038/35087061

Gale M, Jr., Katze MG (1998): Molecular mechanisms of interferon resistance mediated by viral-directed inhibition of PKR, the interferon-induced protein kinase. Pharmacol. Ther. 78, 29–46. http://dx.doi.org/10.1016/S0163-7258(97)00165-4

Garcin D, Michal Y, Jault F, Lyon M, Lenoir G, Jacquemont B (1990): Inhibition of HSV-1 multiplication in rat embryo fibroblasts constitutively expressing the EJ-ras oncogene. Virology 179, 208–216. http://dx.doi.org/10.1016/0042-6822(90)90290-8

Ghandour H, Cullere X, Alvarez,A, Luscinskas FW, Mayadas TN (2007): Essential role for Rap1 GTPase and its guanine exchange factor CalDAG-GEFI in LFA-1 but not VLA-4 integrin mediated human T-cell adhesion. Blood 110, 3682–3690. http://dx.doi.org/10.1182/blood-2007-03-077628

He B, Chou J, Brandimarti R, Mohr I, Gluzman Y, Roizman B (1997a): Suppression of the phenotype of gamma(1)34.5- herpes simplex virus 1: failure of activated RNA-dependent protein kinase to shut off protein synthesis is associated with a deletion in the domain of the alpha47 gene. J. Virol. 71, 6049–6054.

He B, Gross M, Roizman B (1997b): The gamma(1)34.5 protein of herpes simplex virus 1 complexes with protein phosphatase 1alpha to dephosphorylate the alpha subunit of the eukaryotic translation initiation factor 2 and preclude the shutoff of protein synthesis by double-stranded RNA-activated protein kinase. Proc. Natl. Acad. Sci. USA 94, 843–848. http://dx.doi.org/10.1073/pnas.94.3.843

Ho-Tin-Noe B, Goerge T, Cifuni SM, Duerschmied D, Wagner DD (2008): Platelet granule secretion continuously prevents intratumor hemorrhage. Cancer Res. 68, 6851–6858. http://dx.doi.org/10.1158/0008-5472.CAN-08-0718

Hogquist K (2001): RasGRP: the missing link for Ras activation in thymocytes. Trends Immunol. 22, 69. http://dx.doi.org/10.1016/S1471-4906(00)01845-7

Krejnusova I, Gocnikova H, Bystricka M, Blaskovicova H, Polakova K, Yewdell J, Bennink J, Russ G (2009): Antibodies to PB1-F2 protein are induced in response to influenza A virus infection. Arch. Virol. 154, 1599–1604. http://dx.doi.org/10.1007/s00705-009-0479-5

Lan P, Dong C, Qi Y, Xiao G, Xue F (2003): Gene therapy for mice sarcoma with oncolytic herpes simplex virus-1 lacking the apoptosis-inhibiting gene, icp34.5. J. Biochem. Mol. Biol. 36, 379–386. http://dx.doi.org/10.5483/BMBRep.2003.36.4.379

McKie EA, MacLean AR, Lewis AD, Cruickshank G, Rampling R, Barnett SC, Kennedy PG, Brown SM (1996): Selective in vitro replication of herpes simplex virus type 1 (HSV-1) ICP34.5 null mutants in primary human CNS tumours-evaluation of a potentially effective clinical therapy. Br. J. Cancer 74, 745–752. http://dx.doi.org/10.1038/bjc.1996.431

Roberts WK, Hovanessian A, Brown RE, Clemens MJ, Kerr IM (1976): Interferon-mediated protein kinase and low-molecular-weight inhibitor of protein synthesis. Nature 264, 477–480. http://dx.doi.org/10.1038/264477a0

Roose JP, Mollenauer M, Ho M, Kurosaki T, Weiss A (2007): Unusual interplay of two types of Ras activators, RasGRP and SOS, establishes sensitive and robust Ras activation in lymphocytes. Mol. Cell. Biol. 27, 2732–2745. http://dx.doi.org/10.1128/MCB.01882-06

Sarinella F, Calistri A, Sette P, Palu G, Parolin C (2006): Oncolysis of pancreatic tumour cells by a gamma34.5-deleted HSV-1 does not rely upon Ras-activation, but on the PI 3-kinase pathway. Gene Ther. 13, 1080–1087. http://dx.doi.org/10.1038/sj.gt.3302770

Su Y, Zhu H, Xiao W, Xu Q, Zhu L, Zhang X, Wan Q, Zhou S, Du E, Xu H, Yao L, Lv S, Hu B, Liu Y, Qi Y (2007): Selectively oncolytic mutant of HSV-1 lyses HeLa cells mediated by Ras/RTN3. Cancer Biol. Ther. 6, 202–208. http://dx.doi.org/10.4161/cbt.6.2.3628

Toyoizumi T, Mick R, Abbas AE, Kang EH, Kaiser LR, Molnar-KimberKL (1999): Combined therapy with chemotherapeutic agents and herpes simplex virus type 1 ICP34.5 mutant (HSV-1716) in human non-small cell lung cancer. Hum. Gene Ther. 10, 3013–3029. http://dx.doi.org/10.1089/10430349950016410

Wylie KM, Schrimpf JE, Morrison LA (2009): Increased eIF2alpha phosphorylation attenuates replication of herpes simplex virus 2 vhs mutants in mouse embryonic fibroblasts and correlates with reduced accumulation of the PKR antagonist ICP34.5. J. Virol. 83, 9151–9162. http://dx.doi.org/10.1128/JVI.00886-09

Xiao W, Zhou S, Xu H, Li H, He G, Liu Y, Qi Y (2013): Nogo-B promotes the epithelial-mesenchymal transition in HeLa cervical cancer cells via Fibulin-5. Oncol. Rep. 29, 109–116.

Xu H, Xu Q, Zhang P, Yan F, Qi Y (2006a): SLAM expression is not downregulated by measles virus infection. Acta Virol. 50, 235–241.

Xu H, Zhou Q, Liu X, Qi YP (2006b): Co-involvement of the mitochondria and endoplasmic reticulum in cell death induced by the novel ER-targeted protein HAP. Cell. Mol. Biol. Lett. 11, 249–255. http://dx.doi.org/10.2478/s11658-006-0019-1

Xue F, Dong CY, Su Y, Zhu H, Ling W, Liu Y, Xu H, Xiao W, Zhou S, Qi YP (2005): Tumor-targeted therapy with a conditionally replicating mutant of HSV-1 induces regression of xenografted human hepatomas. Cancer Biol. Ther. 4, 1234–1239. http://dx.doi.org/10.4161/cbt.4.11.2094

Yi G, Qian J, Wang Z, Qi Y (2003): A phage-displayed peptide can inhibit infection by white spot syndrome virus of shrimp. J. Gen. Virol. 84, 2545–2553. http://dx.doi.org/10.1099/vir.0.19001-0

Zhu H, Su Y, Zhou S, Xiao W, Ling W, Hu B, Liu Y, Qi Y (2007): Immune analysis on mtHSV mediated tumor therapy in HSV-1 seropositive mice. Cancer Biol. Ther. 6, 724–731. http://dx.doi.org/10.4161/cbt.6.5.3953