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Regulation of HSV-1 Thymidine Kinase Gene Expression by Thyroid Hormone Receptor in Cultured Neuronal Cells

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SUMMARY

Herpes simplex virus type-1 (HSV-1) undergoes acute primary infection in epithelial cells followed by establishment of latency in the neurons of trigeminal ganglia. The latent virus maintains a life-long dormant state and can reactivate spontaneously in a small fraction of host cells, suggesting transcriptional silencing occurs in neurons. Computer data mining analyses identified a thyroid hormone response element (TRE), the binding site for the thyroid hormone receptor (TR), in the promoter of HSV-1 thymidine kinase (TK). TRs are transcription factors whose activity is dependent on their ligand thyroid hormone (TH or T₃). We hypothesize that TR and T₃ exert regulation on HSV-1 gene expression in neurons. A neuroblastoma cell line expressing the TR isoform β (N2aTR β) was utilized for initial *in vitro* investigation. Liganded TR repressed TK promoter activity but unliganded TR relieved the inhibition. Chromatin immunoprecipitation (ChIP) assays showed that TRs were recruited to TK TRE regardless of the status of T₃; hyperacetylated histone H3 at lysine 9 was associated with transcriptionally active promoters. In addition, ChIP results indicated that a repressive mono-methylated H3 modified at lysine 9 was enriched in TK promoter in the presence of TR and T₃. T₃-treated N2aTR β cells were suppressive to TK expression and release of infectious viruses at low moi. RT-PCR and plaque assays showed that the TK can be de-repressed and the virus release was increased when the T₃ was removed. These results suggest that T₃ could regulate HSV-1 gene expression through its receptor via histone modification in cultured neuronal cells.

INTRODUCTION

HSV-1 primary infection initiates when the virus invades epithelia and starts active gene expression and replication to produce progeny. The virus may subsequently establish life-long latency in the sensory neurons of trigeminal ganglia (TG). The reactivation may occur spontaneously. The recurrent infections can attack the anterior buccal mucosa, lips, or perioral area of the face and eyes (Fatahzadeh & Schwartz, 2007). The gene expression in the lytic phase follows a cascade and is characterized by a sequential order. However, viral gene expression during latency differs completely from what is observed in acute infection and significant levels of transcription are detected from only one region of the viral genome, the latency-associated transcripts (LAT) (Javier *et al.*, 1988; Jones, 2003; Bloom, 2004). In addition, the profile of viral gene expression during reactivation is likely to be different from

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that observed in primary infection, in which the cascade is disrupted. For example, thymidine kinase (TK), a β gene, could be expressed concurrently or before the expression of α genes (Kosz-Vnenchak *et al.*, 1993; Tal-Singer *et al.*, 1995; Nichol *et al.*, 1996).

A variety of mechanisms have been proposed to describe the establishment of viral latency and reactivation, such as altered immune response (Bystricka & Russ, 2005; Koelle & Corey, 2008), LAT-mediated anti-apoptosis (Perng *et al.*, 2000; Bloom, 2004; Peng *et al.*, 2004; Branco & Fraser, 2005; Carpenter *et al.*, 2007), microRNAs-induced gene silencing (Cui *et al.*, 2006; Umbach *et al.*, 2008) differential neuronal suppression (Block *et al.*, 1994; Su *et al.*, 2000; Moxley *et al.*, 2002; Su *et al.*, 2002), hormonal regulation (Garza & Hill, 1997; Hardwicke & Schaffer, 1997; Noisakran *et al.*, 1998; Marquart *et al.*, 2003), and repressive chromatin (Kubat *et al.*, 2004; Kubat *et al.*, 2004; Amelio *et al.*, 2006; Bedadala *et al.*, 2007; Chen *et al.*, 2007; Pinnoji *et al.*, 2007; Knipe & Cliffe, 2008). In an effort to discover new transcription factor binding sites, our sequence analysis revealed two thyroid hormone responsive elements (TREs) located in the promoter regions of TK, suggesting that thyroid hormone (TH) could have a role in HSV-1 TK regulation and could control viral latency and reactivation.

The thyroid gland produces two hormone products, thyroxine (T_4) and triiodothyronine (T_3), from thyroglobulin protein (Deme *et al.*, 1976). Although triiodothyronine (T_3) is produced less, it is more potent and responsible for the majority of biological effects. TH exerts its action via its nuclear receptors (TRs). They are transcription factors belonging to the superfamily of nuclear hormone receptors (Tsai & O'Malley, 1994). It has been shown that both liganded and unliganded TRs are involved in gene regulation.

The present studies target the interrelationship of T_3 , TRs, and chromatin structure during HSV-1 gene regulation in neuronal cells. The gene regulation of TK was analyzed in neuroblastoma cells N2a and N2aTR β (N2a cell constitutively expressing TR β). The effect of TR and T_3 on histone modification and cofactor recruitment to the TK promoter was analyzed. Our results indicated that TR can control the TK promoter activity and this regulatory effect is determined by its ligand via distinct histone modifications and chromatin remodeling. This is the first *in vitro* evidence that TR exerted epigenetic regulation on HSV-1 gene expression in neuronal cells and could play a role in the complex processes of establishing latency and reactivation of HSV-1.

METHODS

Viruses, cell lines, and culture conditions

The N2a cell, a mouse neuroblastoma cell line, was purchased from the American Type Culture Collection (ATCC) and cultured in DMEM/F12 medium supplemented with 10% charcoal-treated fetal bovine serum (FBS). N2aTR β cell was a gift from Dr. Robert Denver (University of Michigan, Ann Arbor, MI) and was grown under the same conditions. CV-1 cells were cultured in 10% FBS supplemented with DMEM. All cells were maintained in an incubator at 37 ° C with 5% CO₂. The titers of HSV-1 strains 17Syn⁺, 17Syn⁺-EGFP (Foster *et al.*, 1998), and McKrae were determined on CV1 cells. T_3 was purchased from Sigma (St. Louis, MO).

Plasmids

The pRL-TK vector was purchased from Promega (Madison, WI; Cat#: TB240). It contains 722 bp of the HSV-1 TK promoter (Fig. 1). Monster Green[®] Fluorescent Protein pHMGFP Vector (Promega, Cat#: E6421) was used as a transfection efficiency control. Plasmid pICP4-

SEAP contains reporter SEAP driven by ICP4 promoter (Pinnoji *et al.*, 2007) and was used for normalization in Fig. 2.

Transfection

Nucleofector II from Amaxa (Cat#: AAD-1001S, Gaithersburg, MD) was used for high efficiency of transfection essentially described by the manufacturer. The experiments were performed using Kit V (Amaxa, Cat#: VCA-1003) and the protocol number was T-024.

Antibodies

The antibodies used for immunoblot analyses and ChIP are listed in Table 1. The dilution was based on the manufacturer's suggestions.

Reporter assays

The cells were harvested for the luciferase assay after 48 hr of transfection essentially described by the manufacturer. Luciferase activity was measured by a luminometer using the Luciferase reporter assay system (Promega). Luminescence was measured over a 10-sec interval with a 2-sec delay on the 20/20ⁿ Luminometer (Turner Biosystem, Sunnyvale, CA). The results were presented as the fold induction of the reporter plasmid in the presence or absence of T₃ (100 nM). The SEAP assay was described previously (Bedadala *et al.*, 2007).

RT-PCR

Total RNA was isolated by Trizol reagent (Invitrogen, Carlsbad, CA). RT-PCRs were performed using Superscript One-Step RT-PCR (Invitrogen) with 0.5 µg of total RNA and primer set. Their sequences are as follows: *Actin*: 5'-ATT CCT ATG TGG GCG ACG AG-3' and 5'-TGG ATA GCA ACG TAC ATG GC-3'; *TK*: 5'-ATG GCT TCG TAC CCC TGC CAT -3' and 5'-GGT ATC GCG CGG CCG GGT A -3'; *EGFP*: 5'- GCA GAA GAA CGG CAT CAA GGT G-3' and 5'- TGG GTG CTC AGG TAG TGG TTG TC-3'. The RT-PCR reaction was carried out at 45 °C for 20 min followed by 25 cycles of 94 °C for 30 sec, 57 °C for 30 sec, and 68 °C for 30 sec. The RT-PCR products were analyzed using 2% agarose gel electrophoresis. The Kodak Gel-Logic 100 imaging system was used for documentation.

Immunoblot analysis

The protocol was described previously (Pinnoji *et al.*, 2007). Anti-TRβ1 rabbit polyclonal antibody was used at a dilution of 1:1000 and the secondary antibody (anti-rabbit IgG-horseradish peroxidase conjugate) at a dilution of 1:2000 in PBST for 1 h at room temperature. Anti-α-tubulin mouse antibody was added at a dilution of 1:10 000 and the secondary antibody (goat anti mouse IgG – horseradish peroxidase conjugate) was included at a dilution of 1:5000 in PBST for 1 h at room temperature.

ChIP

The protocol was described previously (Pinnoji *et al.*, 2007). PCR primers were: TK 5'-ATG GCT TCG TAC CCC TGC CAT-3' and 5'- GGT ATC GCG CGG CCG GGT A -3'

T₃ removal experiments

N2aTRβ cells were plated on a 6-well plate with 40–50% confluency with the addition of T₃ (100 nM). Media were replaced daily with fresh T₃. On day 6, viral inoculation was performed for 1 h at moi of 1. The inoculum was then removed and the cells were washed twice with 1 ml of PBS followed by the addition of 1 ml of fresh media containing T₃ for 8 h. The T₃ incubation was stopped after 8 h by removing the medium completely and washing the cells

twice with PBS. New medium was added in each well with or without T₃. The media were collected 48 h postinfection (hpi) and subjected to plaque assays.

Plaque assays

Media collected from mock and infected N2aTR β cells at 48 hpi were subjected to plaque assays by infecting CV-1 cells. Monolayers of CV-1 with 90–100% confluency in 24-well plates were incubated with 200 μ l of media for 45 min on a rocking platform at different dilutions followed by addition of 1 ml fresh media to each well for 48 h. At the end of the incubation, the infected cells were washed with PBS and treated with crystal violet (PML Microbiologicals, Wilsonville, OR) for 20 min followed by washing with water. Plaques were counted in each well and the probability was measured by a Student's paired *t*-Test with a two-tailed distribution using Microsoft Excel.

RESULTS

Identification of HSV-1 TK TREs

We identified TREs in the HSV-1 genome located from 47915 to 47932 (5'-tatta **AGGTC**Acgcgtg**TGGCCT**-3', Fig. 1) based on the HSV-1 complete genome sequence (Accession #: X14112). The sequence is represented as inverted repeats with six-nucleotide spacing (IR6). These IR6 TREs are located between the TATA box (47933–47937) and the transcription initiation site (47911). The TREs in this context were suggested to function as negative TREs in neural cells (Park *et al.*, 1993).

HSV-1 TK promoter was repressed by TR in the presence of T₃

To determine the regulatory effect of TR on HSV-1 TK promoter, N2a and N2aTR β cells were cotransfected with pRL-TK as well as pEGFP and pICP4-SEAP in the absence or presence of T₃. Fluorescent microscopy showed that the transfection efficiency was similar between N2a and N2aTR β cells (data not shown). The Luc assays (normalized by SEAP assay) showed that TK promoter activity exhibited a 7-fold decrease by T₃ in N2aTR β cells but exhibited no significant difference in N2a cells (Fig. 2). In addition, the TK promoter activity was four-fold stronger in N2aTR β without T₃ than in N2a cells (Fig. 2, compare lane 2, 3, and 5). Control experiments for normalization purposes using reporter plasmids containing CMV, HSV-1 ICP4, and ICP22 promoters showed no regulatory effect by T₃ and TR (data not shown). These results suggested that HSV-1 TK TREs exert down-regulation on the promoter by TR and T₃. Unliganded TR, in contrast, enhanced TK promoter activity.

Viral infections were done to investigate TR-mediated regulation. N2a and N2aTR β cells were infected with strain 17Syn⁺ at moi=10 with or without T₃. RT-PCR assays revealed no regulatory effect (data not shown), probably due to the α transactivation of TK. To eliminate the effect of transactivation, cycloheximide (CHX) was added to inhibit the synthesis of α proteins and therefore allowed the assessment in the absence of transactivation. Under this condition, TK transcription was inhibited by liganded TR at 8 hpi (Fig. 3A, compare lanes 3 and 4). At moi lower than 1, TK promoter activity was found to be down-regulated by liganded TR at 48 hpi without the effect of CHX (Fig. 3B). Taken together, these results demonstrated that HSV-1 TK was negatively regulated by liganded TR in the neuronal cells.

TR β was recruited to the TK promoter and the repressive histones were enriched at the TK promoter by liganded TR

N2aTR β cells were constructed to express TR β and the overexpression was verified by Northern blot analysis; the T₃ treatment induced N2aTR β cells to differentiate (Lebel *et al.*, 1994). To confirm the protein expression and assess its function, extracts from N2a and

N2aTR β cells were subjected to Western blot analyses using anti-TR β and anti- α -tubulin Ab. The results supported the Northern blot analysis that TR β is overexpressed in N2aTR β but not N2a cells (Fig. 4). Previous studies showed that TR β was bound to TK promoter *in vitro* (Park *et al.*, 1993). The *in vivo* interaction was therefore analyzed by ChIP and the results indicated that TR β was associated with the TK promoter independent of ligand binding (Fig. 5A). The TR β -mediated histone modification was investigated by ChIP using antibodies against acetyl histone H4 and mono-methylated histone H3K9. The results indicated that histone H4 acetylation was significantly reduced (Fig. 5A; lane 4) but mono-methylation of histone H3K9 was enriched at the TK promoter in the presence of T₃ (Fig. 5B; last lane). Altogether, these results demonstrated that TR β was recruited to TK promoter and regulated the gene expression, at least in part, by differential histone modification.

Removal of T₃ activated TK expression and induced the virus release

To study the effect of T₃ on TR-mediated regulation, N2aTR β cells were treated for five days before the infection with or without T₃ using an HSV-1 expressing EGFP at moi = 1. Fluorescent microscopy revealed that the numbers of green fluorescent cells were similar between cells treated with or without T₃, indicating that T₃ treatment did not affect the infection efficiency (data not shown). RT-PCR assays showed that the expression of TK was de-repressed upon washout of T₃ at 48 hpi (Fig. 6A). The plaque assays indicated that the removal of T₃ by washout increased the virus release 6.4-fold more than the cells being treated with T₃ (Fig. 6B). The *P* value measured by a Student's paired t-test with a two-tailed distribution was 0.0015, indicating a significant increase. Given the fact that N2aTR β cells were differentiated after T₃ incubation, these results suggested that subtraction of hormone reversed the TK repression and the enzyme presumably induced viral replication in resting cells and enhanced the replication and virus release.

DISCUSSION

This study focused on the roles of TR and its ligand T₃ in the regulation of HSV-1 TK. Our results demonstrated that the transcription factor TR utilized its ligand to exert a repressive effect on TK in neuronal cells. Unliganded TR interacted with the TK TREs and induced histone H4 hyperacetylation at the TK promoter to promote active transcription. The liganded TR reversed the histone acetylation and recruited repressive mono-methylated H3K9 to the promoter for transcription repression. These results provide evidence suggesting that the novel TR/T₃-mediated regulation could have a role in controlling the establishment of latency and reactivation of HSV-1.

TH participates in development and homeostasis in adults (Evans, 1988). Both sensory neurons and peripheral glial cells in dorsal root ganglia have been shown to possess specific T₃ binding sites (Walter & Droz, 1995). During development, sensory neurons express TR β rather than TR α and the amount of TR β positive neurons increased during the postnatal stages and remained stable in adults. The presence of TR in sensory neurons indicates that the feedback regulation of circulating TH could occur by binding to the receptors (Glauser & Barakat Walter, 1997). T₃ can affect different processes involved in the survival, differentiation, and maturation of neurons (Walter, 1996). In a physiological concentration, T₃ enhances neurite outgrowth of primary sensory neurons in cultures, possibly in combination with nerve growth factor, to regulate the expression of cytoplasmic dynein, a protein that is involved in retrograde axonal transport (Barakat-Walter & Riederer, 1996). In addition, whole body hyperthermia was reported to reduce the level of serum T₃ by 50%, probably due to the suppression of thyroid stimulating hormone release, monodeiodination alteration of T₄ from T₃ to reverse T₄, or enhanced T₃ clearance (Shafer *et al.*, 1980). Hyperthermia can be used to trigger HSV-1

reactivation in the mouse latency model (Sawtell & Thompson, 1992). Therefore, T₃ is likely to participate in the regulation and maintenance of viral latency and reactivation.

The role of T₃ in HSV-1 gene regulation has not been extensively investigated. Park et al reported that the addition of T₃ induced the HSV-1 TK promoter by 4- to 5-fold in pituitary rat cells by transient transfection (Park *et al.*, 1993). However, the authors indicated that similar TREs are found in several genes that were characterized to mediate negative regulation in brain. Our transfection assays indicated that HSV-1 TK was repressed by liganded TRs in neuroblastoma cells. This result was further supported by the infection experiments showing that TK, a β gene, was inhibited by liganded TR at moi of 1 or high moi without α proteins. The role of TK in brain is clear for HSV-1 infection. TK is required to provide dNTP for viral replication in resting cells such as neurons. Furthermore, viral replication is required for efficient α and β expressions in neurons during reactivation (Nichol *et al.*, 1996). Based on these observations, TK was suggested to initiate α transcription and subsequent replication during reactivation (Tal-Singer *et al.*, 1995). Moreover, Tal-Singer *et al.* (1997) reported that during *in vivo* reactivation in TG, TK was one of the first genes to be expressed. Another report showed that a TK-mutant exhibited greatly reduced α and β expression during reactivation (Kosz-Vnenchak *et al.*, 1993). Based on these results, TK was suggested to play a critical role during viral reactivation. However, another report indicated that TK as well as α genes were expressed at the same time (Pesola *et al.*, 2005). Although the role of TK is ambiguous, it is likely that T₃ has the potential to control TK expression and control viral transcription. To test the hypothesis, we utilized a unique cell culture model, N2aTR β . T₃ treatment has been shown to induce these cells to differentiate, mimicking the state of neurons in brain (Lebel *et al.*, 1994). Therefore, we treated N2aTR β cells with T₃ for 5 days to induce differentiation and then infected the treated cells with HSV-1. The results supported our hypothesis that removal of T₃ by washout de-repressed the TK expression and significantly increased virus release, presumably by producing dNTP and inducing viral replication. We determined that viruses were still released from the T₃-treated cells. The release was probably due to the fact that α proteins, transactivated by VP16, induced the expression of TK but the production was remarkably reduced by liganded TR. Further infection experiments with appropriate mutations are underway to investigate the T₃-mediated regulation of viral replication/transcription using this model.

Our results indicated that TK is negatively regulated by liganded TR. TR-mediated negative regulation was not as clear as the positive regulation. Many hypotheses have been proposed such as the binding to TFIID (Crone *et al.*, 1990), inhibition of SP1 stimulation (Xu *et al.*, 1993), hetero- to homo-dimer conformational change (Bendik & Pfahl, 1995), recruitment of HDAC (Sasaki *et al.*, 1999), recruitment of chromatin insulator protein (Burke *et al.*, 2002), CREB competition (Mendez-Pertuz, *et al.*, 2003), TBP recruitment (Sanchez-Pacheco & Aranda, 2003), repression of S-phase genes (Nygard *et al.*, 2003), Sp1 competition (Villa *et al.*, 2004), interaction of GATA2, and TRAP220 dissociation (Matsushita *et al.*, 2007). We tested these hypotheses and showed that the binding of Sp1, TFIID, CREB, CREB binding protein, and TBP to TK promoter was not involved in this regulation (data not shown). Our results further indicated that hypoacetylated histone H4 and mono-methylated H3K9 were enriched at TK by liganded TR. Mono-methylated H3K9 was shown to associate with the inactive promoter and behaved as repressive chromatin (Vakoc *et al.*, 2005). Besides, our transfection experiments showed that TK promoter activity was activated by TR in N2aTR β 4 times more strongly than in N2a cells (Fig. 2). This activation was not seen during infection (Fig. 3A). The mechanism is unknown but is probably due to VP16 transactivation and/or the presence of other virion proteins. For example, virion host shutoff protein (VHS) could inhibit or reduce the expression of important cellular transcription factors for transcription regulation. The underlying mechanisms of TR-mediated TK regulation are being investigated.

The current studies provide evidence that strongly implicates the roles of T₃ and TR in the regulation of HSV-1 gene expression in cultured neurons. The current hypothesis is that the liganded TR inhibited viral replication by repressing TK expression. These outcomes led to the maintenance of latency. Nonetheless while the T₃ concentrations were low, unliganded TR relieved the repression of TK and promoted the viral replication. The active replication and increased transcription of α genes would lead to the subsequent reactivation. More experiments especially using animal models are necessary to identify key mechanisms involved in the establishment of latency and reactivation of HSV-1.

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Fig. 1. HSV-1 TREs

1A: Negative TREs were identified in the HSV-1 TK promoter. The TREs were organized as inverted repeats with six nucleotides spacing (IR6) and located immediately after the TATA box. Transcription initiation site, Sp1 elements and CAAT box were shown.

Fig. 2. HSV-1 TK transcription was negatively regulated by TR and T₃

N2a and N2aTR β cells were cotransfected with various plasmids by Nucleofector II with duplication to analyze the regulatory effects of TR and T₃ on TK promoter. Plasmid pEGFP was used as a reference to monitor the transfection efficiency. Plasmid pICP4 was used as normalization control. Fluorescent microscopy was performed using Nikon Eclipse TS100 microscope. Cells were subjected to luciferase assay using Promega Luciferase Assay Kit at 48-hrs post-transfection. The signal was measured by luminometer. The results were normalized by SEAP assay driven by ICP4 promoter. Error bars represent the mean plus or minus the standard deviation.

Fig. 3A.

Fig. 3B.

Fig. 3. HSV-1 TK was repressed by liganded TR during infections

3A: N2a and N2aTR β cells were infected with wild-type virus 17Syn⁺ at moi=10 "in the absence or presence of T₃ with 50 μ g/ml cycloheximide to block the protein synthesis of HSV-1 α genes. The total RNA was purified and subjected to RT-PCR by primers against TK. Actin primers were used as controls.

3B: N2aTR β cells were infected with wild-type virus 17Syn⁺ at moi=1. The total RNA was purified at 8 hpi and 48 hpi then subjected to RT-PCR using primers against TK and actin. The gel showed that TK transcription was delayed until 48 hpi and was repressed by the addition of T₃ (compare lanes 3 and 4).

Fig. 4. TRβ is overexpressed in N2aTRβ cells

Protein extracts were purified from N2a and N2aTRβ cells and subjected to gel electrophoresis followed by immunoblot analysis using Abs against TRβ1 and α-tubulin.

Fig. 5A.

Fig. 5B.

Fig. 5. TR β bound to the TK promoter and induced T₃-mediated histone modification

5A: Cells were transfected with pRL-TK and treated as noted. The transfected cells were subjected to ChIP after 48 h of treatment and immunoprecipitated by Abs against TR β or acetylated histone H4.

5B: ChIP was performed using the conditions of 5A and immunoprecipitated by anti-mono methylated H3K9 Ab.

Fig. 6A.

Fig. 6B.

Fig. 6. Long-term T₃ treatment prevented the TK expression and the washout of T₃ induced the TK expression and virus release

6A: Cells were pre-treated with T₃ for five days and subsequently infected with 17Syn⁺-EGFP viruses at moi of 1. Total RNA was isolated at 48 hpi and subjected to RT-PCR assays using TK primers to study TR-mediated regulation. RT-PCR assays using EGFP primers were performed as internal viral infection control.

6B: The media of infected cells were collected 48 hpi and subjected to plaque assays using CV1 cells to investigate the release of infectious viruses.

Table 1

The sources of antibodies

Antigen	Species	Clonality	Source
TR β 1	Rabbit	Polyclonal	Upstate (Lake Placid, NY, Cat#: 06-539)
Mono-methyl-histoneH3 (Lys9)	Rabbit	Polyclonal	Upstate (Cat#: 07-395)
Anti acetyl-histone H4	Rabbit	Polyclonal	Upstate (Cat#: 06-866)
Anti- α -tubulin	Mouse	Monoclonal	Calbiochem (Cat#: CP06)
Secondary Ab for Western	Rabbit		Perkin Elmer Lifesciences (Cat#: NEF812)
Secondary Ab for Western	Mouse		Perkin Elmer Lifesciences (Cat#: NEF822)