Research paper

Characterization of nuclear foci-targeting of Luman/CREB3 recruitment factor (LRF/CREBRF) and its potential role in inhibition of herpes simplex virus-1 replication

Timothy E. Audas a,b, Philip W. Hardy-Smith a, Jenna Penney a, Tiegh Taylor a, Ray Lu a,*

a Department of Molecular and Cellular Biology, University of Guelph, Guelph, ON, N1G 2W1, Canada
b Department of Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, B.C., V5A 1S6, Canada

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ABSTRACT

The recently identified Luman/CREB3-binding partner LRF (Luman/CREB3 recruitment factor) was shown to localize to discrete sub-nuclear foci. Luman is implicated in herpes simplex virus-1 (HSV-1) latency/reactivation and the unfolded protein response (UPR) pathway; therefore, we sought to characterize the formation of the LRF nuclear foci in the context of cellular signaling and HSV-1 replication. Here, we mapped the nuclear foci-targeting sequence to the central region containing the first leucine zipper (a.a.415-519), and found that the integrity of the whole region appears essential for LRF foci formation. LRF foci integrity was unaffected by inhibition of cellular DNA replication and translation, however, disruption of transcription resulted in altered LRF localization. When compared to other cellular and viral foci LRF co-localized with the nuclear co-activator GRIP1, while the HSV-1 gene products ICP4, ICP27 and VP13/14 disrupted foci formation to varying degrees. Interestingly, cells over-expressing LRF were resistant to productive HSV-1 infection and this resistance was dependent upon protein targeting and an N-terminal transactivation domain. When LRF knockdown cells were subjected to primary infection, HSV-1 gene expression and progeny virus yield were enhanced by ~3 fold compared to wildtype cells. Taken together, these results indicate that LRF is a key regulator that may act direct or indirectly as a repressor of essential genes required for productive viral infection.

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1. Introduction

It has been increasingly accepted that the cellular nucleus is functionally compartmentalized and that proteins are targeted to discrete subdomains within the nucleus to perform specialized functions. Contrary to cytoplasmic organelles sub-nuclear domains exist without the boundary of lipid bilayers. These regions appear to be enriched in specific nuclear proteins and RNAs that are thought to be essential for coordinated complex assembly and specific biological functions. Many of these sub-nuclear domains have been identified including; the nucleolus, RNA splicing speckles (reviewed (Lamond and Spector, 2003)), DNA repair domains (Zou and Elledge, 2003), PML nuclear bodies (Ascoli and Maul, 1991) and regions enriched in histone modifying proteins (Downes et al., 2000) or nuclear receptors (Baumann et al., 2001; Tazawa et al., 2003).

In recent years, the role of nuclear bodies during viral DNA replication has received considerable scrutiny, with replication of numerous DNA viruses occurring juxtaposed to these structures (reviewed in ref. Doucas, 2006). Much focus has been given to HSV-1 and its gene products in relation to PML nuclear bodies with active replication compartments forming juxtaposed to these nuclear domains (Maul, 1998; Maul et al., 1996). While the immediate-early gene product ICP4 co-localize with the viral genome (Everett et al., 2003) ICP0 is targeted to PML nuclear bodies at the early stages of infection (Everett and Maul, 1994; Maul and Everett, 1994). This co-localization between ICP0 and PML triggers the dismantling of the nuclear bodies (Everett and Maul, 1994) through the degradation of PML (Everett et al., 1998). The significance of ICP0-mediated disruption of PML remains unclear, however, it has been shown to correlate with viral infection efficiency (Everett, 2001; Maul, 1998; Maul et al., 1996). This observation has led to the hypothesis that these nuclear bodies play a role in the cellular antiviral response limiting viral DNA replication.

The novel protein Luman/CREB3 recruitment factor (LRF) (Audas et al., 2008) was identified through its binding to Luman (also called

* Corresponding author.
E-mail address: rlu@uoguelph.ca (R. Lu).

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LZIP or CREB3) (Freeman and Herr, 1997; Lu et al., 1997, 1998), a cellular transcription factor known to interact with the HSV-1-related cell-cycle regulator Host Cellular Factor-1 (HCF-1) (Kristie et al., 1989; Tyagi et al., 2007; Wilson et al., 1993). The binding of Luman/CREB3 to HCF-1 is mediated by the presence of the consensus HSV-binding motif [D/E]XXY (where X represents any amino acid), a sequence shared by the viral transcription factor VP16 (also known as Vmw65, atf) (Freeman and Herr, 1997; Lu et al., 1998). VP16 utilizes HCF-1 to initiate the HSV-1 gene expression cascade by forming a multiprotein-DNA complex on viral immediate-early (IE or a) gene promoters [reviewed in reference (Roizman and Knipe, 2001; Wysocka and Herr, 2003)]. Recently, other cellular proteins have been shown to bind HCF-1 in a similar fashion including: Zhangfei (Lu and Misra, 2000b), Irox20 (Luciano and Wilson, 2003), E2F4 (Luciano and Wilson, 2003) and HIP1 (Mahajan et al., 2002). The basic region leucine zipper (bZIP) proteins Luman and Zhangfei (Jin et al., 2000; Lu and Misra, 2000b; Lu et al., 1997) have been shown to inhibit HSV-1 gene expression when ectopically expressed. This effect is dependent on their ability to bind HCF-1 (Akhova et al., 2005; Lu and Misra, 2000a,b), suggesting that herpes viruses have co-evolved with their host for 100–200 million years (McGeoch et al., 1995). They mimic important cellular signaling pathways giving the virus a considerable advantage for replication, latency and reactivation.

In the present study we explore the ability of LRF to localize to unique nuclear bodies (Audas et al., 2008). Exogenously expressed LRF localized to foci in a number of different cell types. These foci were not affected by cellular DNA replication or translation inhibition, however, disruption of transcription triggered LRF re-localization to the cytoplasm. Here, we mapped the sub-nuclear targeting signal of LRF to the central region of the protein. Although the exogenous LRF foci were disturbed during HSV-1 infection, these cells were resistant to productive infection by HSV-1 and this resistance was dependent upon both 1) the amino-terminal region containing an activation domain and 2) the ability of LRF to target to sub-nuclear compartments. Analysis of the LRF deficient cells revealed that the overexpression of wildtype LRF, considerably suppressed HSV-1 gene expression and viral yield during lytic infection. We hypothesize that LRF may provide a protective effect to the cell by sequestering important co-factors or acting as a direct effector of essential genes required for productive infection.

2. Materials and methods

2.1. Materials

All restriction endonucleases and modifying enzymes were purchased from New England Biolabs, oligonucleotides from Qiagen Operon, and Expand High Fidelity PCR DNA polymerase from Roche. All other reagents were obtained from Fisher Scientific unless otherwise noted.

2.2. Plasmids

The construction of pFLAG-LRF, pEGFP-LRF were described previously (Audas et al., 2008). The full length LRF(1-639) cDNA was cloned into the pTK-GFP-C expression vector, containing the GFP fusion protein regulated by the HSV thymidine kinase promoter. The cDNA encoding N-terminal deletion mutants LRF(26-639), LRF(214-639), LRF(324-639), LRF(349-639), LRF(451-639), LRF(519-639) and C-terminal mutants LRF(1-525), LRF(1-347), LRF(1-193), LRF(1-101), LRF(1-50) (where the numbers in parenthesis represent the amino acid position in the assumed protein), were generated by PCR and cloned into the EcoRI/Sall site of the mammalian expression vectors pM3 (containing the Gal4 DNA-binding domain; Ivan Sadowski, University of British Columbia, Vancouver, Canada), pEGFP-C2 (for green fluorescent protein fusion proteins; Clontech), phA-C and pFLAG-C (for adding the hemagglutinin [HA] or Flag epitope tag at the N-terminus; modified from pcDNA3.1/myc-His C from Invitrogen). Mutant LRF Δ(415-519), LRF(427R), LRF(467R), LRF Δ(415-429), LRF Δ(428-444), LRF Δ(443-459), LRF Δ(458-474), LRF Δ(473-489), LRF Δ(488-505), LRF Δ(503-519) and LRF Δ(428-444 & 473-489) were generated through site-directed mutagenesis using QuickChange II system (Stratagene) from the full length pEGFP-LRF cDNA. The reporter plasmids pGL3-gc-Luciferase was cloned with the promoter of the gc gene in the HSV-1 genome to include the first 1000 bases upstream of the ORF start codon, while p5× GAL4 Luciferase (Ivan Sadowski, University of British Columbia) contains five repeats of the GAL4 upstream activation sequences linked to the coding sequence for firefly (Photinus pyralis) luciferase. The pRL-SV40 plasmid (Promega) contains the Renilla (Renilla reniformis) luciferase gene under the control of the SV40 immediate-early promoter. The plasmids expressing ICP0, ICP22, ICP4, ICP27, VP16, vhs, VP13/14, and VP22 were PCR amplified from HSV-1 genomic DNA and inserted between the EcoRI and Xhol sites of the pFLAG-C vector.

2.3. Cell culture and transfection

Human embryonic kidney (HEK) 293, NIH 3T3, COS7 HeLa, Vero, LRF+/− and LRF−/− (Audas et al., 2008) cells were grown in monolayer culture in Dulbecco’s modified Eagle’s medium (high glucose) supplemented with 10% (v/v) fetal bovine serum (Invitrogen), 100 IU/ml penicillin and 100 μg/ml streptomycin. All cultures were maintained in a 5% CO2 humidified atmosphere at 37 °C and passaged every 2–3 days. Cells were plated 24 h prior to transfection and allowed to grow to 60–70% confluency. All cell lines were transfected by Dreamfect (OZ Biosciences) as per the manufacturer’s instruction. Treatments were performed for the indicated times using 300 nM thapsigargin (DMSO-soluble), 10 μg/ml cycloheximide (water-soluble), 10 μM nocodazole (DMSO-soluble) or 50 μM 5,6-dichloro-1-beta-d-ribofuranosylbenzimidazole (DRB) (DMSO-soluble).

2.4. Viruses

The wildtype HSV-1 strains KOS was obtained from Karen Mossman at McMaster University. The YFP-ICP27 expressing virus was graciously provided by Roger Everett at the MRC Virology Institute and was described previously (Everett et al., 2004). Viral stocks were propagated in Vero cells and virus titers were determined. Infections were carried out at a multiplicity of infection of 10 PFU/cell, incubating the cells and virus together for 1 h, with gentle rocking every 10 min. After 1 h media was replaced and cells were later harvested at indicated times post-infection.

2.5. Immunofluorescence microscopy

Cells were fixed for 5 min in ice-cold methanol and blocked for 60 min in 10% goat serum at room temperature. Antibody incubations (M2 1:200, Sigma; Alexa594-anti-mouse IgG 1:400, Molecular Probes) were for 30 min at 37 °C with 5% CO2. Glass cover slips were mounted in 50% glycerol/500 pmol DAPI solution and sealed with nail polish. The slides were examined on a Leica TCS SP2 confocal imaging station equipped with a Leica DMIRE2 inverted microscope and images were captured with Hamamatsu ORCA-ER Digital Camera. Figures were prepared using Adobe Photoshop CS2 and Illustrator CS2. Quantification of the proportion of LRF transfected cell displaying a punctated nuclear pattern were analyzed from two.
independent repeats counting greater than 100 transfected cell, noting either foci or other cellular localization patterns.

2.6. Luciferase assays

Cells were transfected as described above. At 16 h post-transfection, media were changed and the cells were allowed to recover for 4 h. For luciferase assays involving viral infection cells were infected 24 h post-transfection and harvested 14 h post-infection. Cellular lysates were extracted and dual luciferase assays were performed according to the manufacturer’s protocols (Promega). Luciferase activity was measured using a Turner TD-20e Luminometer and calculated as relative luciferase activity (firefly luciferase/Renilla luciferase) to correct for transfection efficiency. Assays were independently repeated at least 3 times. Data are shown with standard error.

2.7. RNA analysis

Total RNA was isolated with Trizol (Invitrogen) from cell cultures. Synthesis of cDNA was performed using Superscript II reverse transcriptase (Invitrogen) and oligo(dT) (Roche). Quantitative real-time PCR analysis used SYBR Green PCR Master Mix (Invitrogen) with primers for human LRF (5′-GCCATCTGAGTAGATCCGGA and 5′-CGTTGACTCTTAAGTGTATT), human Luman (5′-CCAGGCGCATGTTAGAG and 5′-GCCGAATTACAGAGGAGGCA), human GAPD (5′-GCCCTAAACCTCATGTA and 5′-CAGCGAGGCAACATGTA), human β-actin (5′-CCAGCCATGATTGAG and 5′-GGCGTAGGAGGAGGCA), HSV-1 ICP27 (5′-CTCCATGCGCTTTGCCCG and 5′-CAGCGGTCTGATGAAATT), HSV-1 ICP0 (5′-TGTGAGCCTACGTGACCGA and 5′-ATGTITCCCGTCCGACGCC), HSV-1 ICP8 (5′-GATGACGCCCTAGGGGTG and 5′-GACGACCTGGCATGTACTA), HSV-1 gC (5′-ACCCTGCGCTTCTGGTACTA and GGTGACTCGCGCTGCTTCTA) and mouse β-actin (5′-CCTGACCTATGGCAACC and 5′-CACGCGCTGATGGTCTAG). Samples were run on an ABI 7300 System and analyzed using the ABI 7300 System Sequence Detection Software v1.2.2. Data is presented as the average of three independent repeats.

2.8. Single-step virus replication curve

5 × 10^3 LRF+/+ or LRF−/− cells were seeded in six-well plates one day prior to infection. Cells were infected as described above at a multiplicity of infection of 10 PFU/cell and allowed to recover for 4, 8, 16 or 24 h post-infection, at which time media was harvested. Viral yields from each sample were titrated on Vero cell monolayers. Data is presented as the average of three independent repeats.

3. Results

3.1. LRF nuclear foci are not cell line-specific

Recently we identified a novel Luman-binding protein, LRF. One key property of LRF is its localization to discrete nuclear foci (Audas et al., 2008). To further verify the authenticity of this localization, we studied the formation of LRF nuclear foci under various conditions such as: different expression levels, epitope tags, and mammalian cell lines. A pTK-GFP-LRF construct was used in cellular transfections; this placed the GFP-LRF fusion protein expression under the control of a weak HSV-1 thymidine kinase (TK) promoter instead of the commonly used strong cytomegalovirus (CMV) immediate-early gene promoter. To rule out the possibility that foci formation was a result of the GFP tag, a shorter FLAG epitope instead of GFP was also included. Both constructs displayed the same punctate nuclear staining when transfected into HEK293 cells (Fig. 1A). However, as previously described (Audas et al., 2008) the pFLAG-LRF appeared to be highly unstable, and treatment with the proteasome inhibitor MG132 was essential for its visualization. To confirm that LRF nuclear foci were not specific to HEK293 cell line, the pEGFP-LRF expression plasmid was also transfected into other cell lines, including: NIH 3T3, COS7 and HeLa cells. Microscopic analyses of all these transfected cell lines showed similar formation of discrete nuclear foci (Fig. 1B). These observations therefore suggest that the formation of LRF foci occurs in all cell types.

3.2. The LRF region of amino acid 415-519 is required for nuclear foci targeting

To map the nuclear focus-targeting domain of LRF, different mutants were made and introduced into HEK293 cells (Fig. 2A). As summarized in Fig. 2B, the region between a.a. 415-519 appeared to be essential for targeting LRF to the nuclear foci. Deletion of the central portion of LRF altered its localization to an exclusively cytoplasmic pattern (Fig. 2A). It is interesting to note that the staining patterns of LRF (1-419) differed from the diffused N-terminal fragment LRF (1-347), suggesting a.a. 347-419 may contain a nuclear...
export signal (NES). All proteins containing the a.a. 451-525 region were located to the nucleus, suggesting it contains a nuclear localization signal (NLS). It is also worth noting that, although LRF (1-525) could form larger nuclear foci, these cells also demonstrated a diffuse nuclear distribution. The integrity of each of the LRF mutants could be detected by western blot (Fig. 2C), and to rule out the possibility that the differential distribution of LRF was a result of cytotoxicity of overexpressed proteins, the cells were assessed for apoptosis by the caspase-3 assay and by cell morphology (unpublished observations), and no signs of apoptosis were observed.

To more finely map the nuclear foci localization signal, we made 14-16 a.a. scanning deletion mutants of the a.a. 419-519 region. HEK293 cells were transfected with these LRF mutants fused to GFP and analyzed by fluorescence microscopy. At least ten fields of view and at least 100 cells were scored for GFP subcellular localization (Fig. 2D). For wildtype LRF, approximately 83% of transfected cells had LRF recruited to discrete nuclear foci. In comparison, removal of the central foci-targeting region (a.a. 415-519) caused complete loss of the nuclear foci (Fig. 2D, bar 2). Removal of a.a. 428-444 or 473-489 showed an apparent reduction (71%) in foci localization, with the co-deletion of both of these regions significantly impairing targeting, as LRF nuclear foci was observed only in 27% of cells (Fig. 2D). Surprisingly, it was not possible to create a mutant variant of LRF that localizes to the nucleus without foci formation, suggesting that sub-nuclear targeting of this protein is linked to the nuclear localization signal. In confirmation with the sequence prediction, our results here indicate that the region a.a. 451-525 may constitute a NLS, while region a.a. 415-525 is essential for foci formation (Fig. 2), likely through interactions among various components of the LRF nuclear foci. We reason that a.a. 473-489 might be the core sequence of the NLS and a.a. 428-444 might be more specifically required for foci targeting.

3.3. Biochemical nature of LRF foci

To determine the biochemical nature of the LRF nuclear bodies and its possible dependency on particular cellular processes, we treated GFP-LRF transfected HEK293 cells with different drugs and examined their effect on the LRF foci. Cells were treated with the translation inhibitor cycloheximide for 1 h, considering the short half-life of LRF, or for 8 h with nocodazole, which stalls the cell cycle at prometaphase by depolymerizing microtubules, or with the transcription inhibitor 5,6-dichloro-1-beta-d-ribofuranosylbenzimidazole (DRB). Neither cycloheximide nor nocodazole had a significant effect on the formation of LRF nuclear bodies; DRB, however, disrupted the LRF bodies completely (Fig. 3A). In most DRB-treated cells, a substantial amount of LRF appeared to be localized in the cytoplasm or on the nuclear envelope, suggesting that the nuclear translocation of LRF may be dependent on active transcription in the cell. This suggests that the LRF nuclear bodies resemble the activity dependent nuclear bodies, which form at sites of particular nuclear function and hence their formation is strictly dependent on this activity (Dundr and Misteli, 2010), which in case of LRF appears to be transcription. However, this defect in nuclear translocation cannot be entirely attributed
Fig. 3. (A) Biochemical nature of the LRF foci. HEK293 cells were transiently transfected with 2 μg pEGFP-LRF and treated with cycloheximide (10 μg/mL) for 1 h, or for 8 h with Nocodazole (10 μM) or DRB (50 μM). Images of the same optical field were captured (B) LRF has an activation domain at the N-terminus. HEK293 cells were transfected with 2.5 μg of each pGAL DBD-LRF plasmid along with the reporter plasmid p5× GAL4 luciferase (1 μg). The parental GAL4 DBD vector pM1 was used as the blank control. Dual luciferase activities were measured 24 h post-transfection. Values are normalized to Renilla luciferase before being referenced to the control. Scale bar; 10 μm.
to transcriptional block as DRB is also known for its diverse cellular effects such as protein kinase inhibition (Zandomeni and Wehmann, 1984), transcriptional activation (Hensold et al., 1996) and apoptosis induction (Turinotto et al., 2009).

3.4. LRF contains an amino-terminal activation domain

Given the disruptive effect observed during DRB treatment (Fig. 3A) and the presence of a putative basic leucine zipper region, a known protein dimerization and DNA binding domain, we sought to determine whether LRF possessed a transcriptional activation domain. After initial testing which showed activation potential for the full-length LRF protein, various deletion constructs of LRF were fused to GAL4 DNA binding domain, and their activation potential was studied with the luciferase reporter, p5×GAL4-Luciferase (Fig. 3B). The N-terminus of the LRF protein was seen to be critical for its activation ability. Deletion of the first 26 a.a. reduced its activity by 7 fold, although further deletions towards the C-terminus did not have additional effect. However, removal of the C-terminal region (194-639) drastically augmented the activation potential by nearly 30 fold as compared to the full-length LRF protein. Together this data suggests that LRF contains a potent transcriptional activation domain, making it a putative transcription factor.

3.5. LRF nuclear foci colocalize with other nuclear domains

Next we sought to compare LRF nuclear bodies to other known nuclear nodules/spots in the literature. In terms of morphology, these LRF nuclear foci appeared to have well defined and smooth edges. They are also much more spot-like when compared to nuclear speckles, which are RNA processing centers (Lamond and Spector, 2003). The number of LRF bodies per cell ranges from 20 to 200, depending on the cell type (Fig. 1), and resembles the well documented promyelocytic leukemia (PML) or ND10 bodies (Borden, 2002; Eskiw and Bazett-Jones, 2002; Negorev and Maul, 2001), RIP140 (nuclear receptor interacting protein 140) bodies (Zilliacus et al., 2001) and GRIP1 (glucocorticoid receptor interacting protein 1)/TIIF2 (transcriptional intermediary factor 2) bodies (Kim et al., 2003). Our results showed that LRF bodies did not colocalize with nuclear speckles, but were juxtaposed with PML bodies and RIP140 bodies (Fig. 4). Strikingly LRF and GRIP1 foci appeared to overlap perfectly (Fig. 4). These observations led us to conclude that the nuclear foci of LRF are a bona fide sub-nuclear domains shared with GRIP1, although the significance of this colocalization requires further study.

3.6. Exogenous HSV-1 gene expression is capable of disrupting LRF foci

As LRF was identified through its interaction with Luman, a cellular factor believed to be involved in HSV-1 latency and reactivation (Lu and Misra, 2000a,b), we sought to determine whether LRF foci co-localized with any HSV-1 gene products. Immediately following viral envelope fusion, the tegument proteins are released into the intracellular environment. These are the first viral proteins found in infected cells, and they play a role in inhibiting cellular gene synthesis, silencing the host cell response and activating the cascade of viral gene expression. To assay the effects of these genes on the sub-nuclear localization of LRF, COS7 cells were transiently co-transfected with pEGFP-LRF and plasmids expressing FLAG tagged VP16 or VP13/14 proteins. Although both major tegument proteins appeared to be expressed primarily in the cytoplasm, VP13/14 triggered a nuclear diffusion of the LRF protein, while VP16 showed no effect on LRF targeting (Fig. 5A).

The HSV-1 E genes are expressed after the viral genome reaches the nucleus, and they disrupt the normal cellular machinery to provide an environment conducive for viral genome synthesis and particle production. Therefore, FLAG tagged ICPO, ICP22, ICP27 and ICP4 were co-transfected with pEGFP-LRF and visualized by fluorescence microscopy. The viral genes ICPO and ICP22 are known to localize to and disrupt PML bodies during viral infection (Everett and Maul, 1994). Co-expression of LRF and ICPO/ICP22 did not disrupt LRF bodies but showed LRF foci that were distinct from those possessing ICPO/ICP22 alone (Fig. 5B). Expression of ICP4, which localizes to nuclear foci during productive viral infection, showed some overlap with LRF foci. Intriguingly, ICP4 expression caused a diffused nuclear pattern of LRF foci (Fig. 5B), suggesting ICP4 may disrupt LRF targeting. The most notable alteration in LRF localization was caused by co-expression of LRF and ICP27. Cells expressing both proteins demonstrated a shift to cytoplasmic LRF expression (Fig. 5B). As LRF foci resemble activity dependent nuclear bodies, it is possible that mRNA transcripts may serve as templates for LRF foci formation (Dundr and Misteli, 2010). Since, ICP27 can export viral mRNAs; it is possible that ICP27 also exports some of the RNA which serves as templates for LRF foci and as result mediate disruption of LRF foci.

3.7. LRF over-expression blocks HSV-1 gene expression, through the transcriptional activation domain.

Over-expression of Luman has been shown to protect cells from productive infection through cytoplasmic sequestration of HCF-1, which is essential for viral IE gene expression (Lu and Misra, 2000a). To assay the effects of LRF on viral gene expression, we infected Vero cells expressing FLAG-LRF with a HSV-1 virus expressing the YFP-ICP27 fusion protein as an indicator, and monitored ICP27 expression. Fluorescence microscopy revealed that cells containing detectable levels of FLAG-LRF showed significantly reduced levels of YFP-tagged ICP27, indicating viral gene expression was inhibited (Fig. 6A). Interestingly, LRF localization also appeared to be altered in these cells, suggesting that there might be certain viral protein activities interfering with LRF foci targeting as explained in Section 3.6. These findings complement our observations in Fig. 5 which shows that several viral proteins found during the early stages of infection disrupt LRF foci.

As ICP27 is required for HSV-1 to transit from the early to the late phase of infection (Rice and Lam, 1994), we wanted to confirm that LRF overexpression also repressed late gene expression. To this end, the first 1000 bp upstream of the translational start site of the HSV-1 late gene glycoprotein C (gC) was cloned in front of the luciferase gene and used as a reporter. This construct was co-transfected with the blank vector pcDNA3.1, or plasmids encoding LRF, LRF (214-639) or LRFΔ415-519. In mock-infected cells, none of these plasmids significantly altered reporter expression driven by the gC promoter. Upon infection with the wildtype HSV-1 (KOS) virus there was a 100-fold increase in the gC promoter activity (Fig. 6B), highlighting the effectiveness of the gC promoter construct. LRF expression repressed the HSV-1–mediated activation of the gC promoter by approximately 60%, while mutant LRF (214-639) showed no significant effect (Fig. 6B). Interestingly, LRFΔ415-519, the construct that was unable to form discrete nuclear foci, augmented HSV-1 infection. Based on the information from PML NB’s (Zhong et al., 2000b), it is possible that the mutant LRF may not be able to interact with its partners, which possess a repressor activity, and thereby inhibit transcription or replication of HSV-1. As a result, HSV-1 replication is favored in the presence of mutant LRF. This may also be a consequence of a dominant negative effect exhibited by the mutant LRF over the endogenous LRF. Analysis of LRF protein levels in these cells was impossible due to the instability of the LRF protein as documented in our previously published results (Audas et al., 2008). Together these results suggest that LRF-mediated repression of the HSV gene cascade is dependent upon
both the ability of LRF to form sub-nuclear foci and the transactivation potential of the N-terminal region of LRF.

3.8. HSV-1 gene expression and viral particle production are enhanced in LRF-deficient cells

To better understand the role of endogenous LRF during HSV-1 infection a time-course infection was carried out, and the mRNA harvested from the infected cells were analyzed for the levels of Luman and LRF by real-time PCR. GADD34 was used as a positive control as it is upregulated in response to HSV-1 infection (Fig. 7A) (Cheng et al., 2005). Interestingly, we found that Luman and LRF showed different response to HSV-1 infection. LRF mRNA levels showed a modest (∼3-fold) increase immediately following HSV-1 infection (2 hpi) and a substantially upregulation of the transcript levels 12 (∼10-fold) & 24 (∼30 fold) hpi (Fig. 7A). Conversely, the overall expression of Luman never surpassed its basal level during HSV-1 infection, with a significantly decrease (60%) observed at 8 hpi time point. This different response seen with Luman and LRF could be because they differ in their modes of viral repression. As seen in Fig. 6, over-expression of LRF caused repression of viral gene expression. To assay for the effects of HSV–1 on wildtype and LRF-deficient cells, LRF+/+ and LRF−/− mouse embryonic fibroblast cell line were examined by real-time PCR for viral transcript levels. In agreement with Fig. 6, both the immediate-early ICP27 and the late gC mRNA levels were significantly upregulated in LRF−/− cells (Fig. 7B). The immediate-early gene ICP0 and the early gene ICP8 were also expressed at higher levels in the null cell line (Fig. 7B).
but due to large standard errors, these differences were not deemed statistically significant.

To further evaluate the importance of LRF in viral particle production, a single-step virus replication curve was carried out in both LRF+/+ and LRF−/− cells. The results indicate that LRF deficient cells produced significantly (3 fold) more viral particles than their wildtype counterparts (Fig. 8), thereby substantiating that LRF is a potent inhibitor of HSV-1 expression, and the absence of LRF expression significantly augments the lytic cycle.

4. Discussion

The activity and function of cellular factors can be regulated at many levels. For the first time we demonstrate that LRF contains a potent transcription activation domain at the N-terminus, making it a putative transcription factor. At the level of localization, further evidence has been provided to show that LRF is recruited to distinct sub nuclear compartments, at varying expression levels and in numerous cell lines. These distinct foci are dependent on the
transcriptional integrity of the cell, and are reminiscent of activity dependent nuclear bodies (Dundr and Misteli, 2010).

We examined nuclear subdomains described in the literature for potential colocalization with LRF foci, including PML/ND10 bodies (Borden, 2002; Eskiw and Bazett-Jones, 2002), nuclear speckles (Lamond and Spector, 2003), nuclear receptor-interacting 140 (RIP140) (Tazawa et al., 2003) and glucocorticoid receptor (GR) interacting protein 1 (GRIPI, also called NCoA2 and TIF2) (Baumann et al., 2001; Hoang et al., 2004). We found that LRF colocalized with GRIPI foci. GRIPI belongs to the SRC/p160 nuclear receptor (NR) coactivator gene family. It interacts with ligand-activated NRs (e.g. glucocorticoid and estrogen receptors) through the conserved LxxLXXL sequence, or the NR box. GRIPI can also function as a corepressor; for instance, it represses estrogen receptor α in TNFα-mediated transcription (Cyoro et al., 2006). Its coactivator/corepressor function can also be determined by the allosteric effects of the hormone response element (Rogatsky et al., 2002). It is known that glucocorticoid-mediated stress response can induce HSV reactivation from latency (Aiello et al., 2010; Glaser et al., 1999; Glaser and Kiecolt-Glaser, 1997; Uchakian et al., 2011). LRF, as well as Luman, may cooperatively work in a similar fashion as GRIP1. Indeed, we have recently found in gene knockout mouse models that both LRF (Martyn et al., 2012) and Luman (unpublished data) play a critical role in modulating glucocorticoid signaling. The LRF nuclear foci may be common subnuclear “depots” that are designated for cellular processes, such as transcription activation/repression or protein refolding/degradation. Promotion of protein turnover by LRF, and Luman being implicated in the unfolded protein response may suggest a link of LRF nuclear bodies to protein refolding and degradation (Audas et al., 2008; Martyn et al., 2012).

The sub-nuclear targeting signal of LRF appeared to be within the region containing the first leucine zipper (aa. 415-519). Surprisingly, data indicates that LRF may also contain a nuclear export signal, as the LRF construct lacking the foci-targeting region was exclusively cytoplasmic, while the truncated N-terminal mutant was present throughout the cell. These results suggest that LRF may be shuttled in and out of the nucleus, a not entirely unprecedented finding as HCF-1 has similarly been shown to cycle between the nucleus and cytoplasm (Mahajan et al., 2002). This trafficking may represent another level of LRF regulation. Finer mapping within the central region of LRF was unable to completely eliminate foci targeting, suggesting multiple localization signals may be present within this region. Two mutants, LRFΔ428-444 and LRFΔ473-489 showed a reduction in the number of foci-targeted cells, and when a double mutant was created, the reduction was augmented. At the amino acid level these regions do not appear to show any sequence similarities. Since the double mutant was still capable of localizing to foci in ~27% of transfected cells (Fig. 3B), it is possible that some of the sub nuclear targeting domain must be still present.

In the light of the viral mimicry of Luman by the HSV-1 protein VP16 and the implication of Luman in HSV reactivation from latency (Lu and Misra, 2000a), the effect of ectopic LRF expression was examined on productive viral infection. Similar to the results observed during Luman overexpression (Lu and Misra, 2000a) exogenous LRF impaired viral gene expression. This result was visualized in transfected/infected cells (Fig. 6A) and quantified by a reporter assay (Fig. 6B). Concurrently, HSV-1 infection appears to inhibit/disrupt foci formation, suggesting that LRF does not impair entry and infection, but rather inhibits only viral gene expression. These results are reminiscent to that observed with silencing effects of PML NB’s which inhibit viral transcription and replication (Garrick et al., 2004; Zhong et al., 2000a), and support the notion that nuclear domains directly influence the latent viral genomes and their transcription (Cávez et al., 2012). The divergent response observed with Luman and LRF in response to HSV-1 could be explained based on the fact that the Luman-mediated repression is based on sequestration of HCF-1 in the cytoplasm (Lu and Misra, 2000a), while that of LRF requires a functional activation domain in the N-terminus.

HSV-1 genes have been shown to have a tight association with numerous sub-nuclear structures. Viral genome replication and/or gene expression events during viral infection have been linked to specific nuclear domains including the ND10 or promyelocytic leukemia (PML) bodies (reviewed (Everett, 2001; Maul, 1998)). At early stages of infection only tegument and immediate early proteins are found within the infected cell. Study of the cellular localization of these genes revealed that 4 of the 5 HSV immediate early proteins: ICP0 (Everett and Maul, 1994), ICP4 (Everett et al., 2004, 2003), ICP22 (Bruni and Roizman, 1998), and ICP27 (Everett et al., 2010) are all expressed within the nucleus (Fig. 6C). The fact that HSV-1 infection can recruit nuclear domains to transcriptionally active foci may provide a mechanism by which viral gene expression is regulated.
et al., 2004; Phelan and Clements, 1997); and several tegument proteins: VP16 (La Boissiere et al., 2004), VP22 (Hutchinson et al., 2002; Negorev and Maul, 2001), VP13/14 (Donnelly and Elliott, 2001a,b) are all capable of localizing to nuclear foci during lytic viral infection. Among them, ICP4 is essential for transcriptional activation of most early and late gene expression. ICP27 is essential for some early and all late gene to be expressed, while playing a role in the nuclear export of viral mRNA. ICP0, although not an essential gene, is required for optimal viral replication and is thought to play a key role in the regulation of HSV latency and reactivation cycle (reviewed in (Everett, 2000; Hagglund and Roizman, 2004; Roizman and Knipe, 2001)). These three HSV proteins all form their own punctate foci in the nucleus at some stage of viral infection. Studies by Everett et al. (2004) indicate that the nuclear foci represented by these three proteins may not be entirely independent. ICP0 is known to colocalize with PML bodies during early infections and causes the degradation of PML, releasing the component proteins. This event is believed to facilitate HSV replication (Everett, 2000; Hagglund and Roizman, 2004; Roizman and Knipe, 2001). At early stages of HSV infection ICP4 forms nuclear foci which do

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not colocalize with, but are often juxtaposed, to PML bodies. Some of the ICP4 foci contain parental genomes, and develop into replication compartments later in viral infection (Everett et al., 2003). ICP27 colocalizes with ICP4 during early stages of infection, but later localizes in nuclear foci that are separate from ICP4 (Everett et al., 2004). From our transient transfections data, we showed that the foci occupied by the immediate early genes were not the same as LRF foci. Two of these genes did manage to disrupt the LRF foci formation entirely or in part (Fig. 5). The most striking similarity to the results observed in the transfected LRF cells infected with HSV-1 was that observed in ICP4 co-transfected cells which caused nuclear diffusion of LRF. When analyzing the transiently transfected tegument genes only VP16 left LRF foci intact (Fig. 8). The viral proteins mediated disruption of LRF foci could be either due to the export of the mRNA which may serve as templates for foci formation as observed in case of PMLNB’s, or could be due to impaired interaction with other proteins which constitute the LRF foci.

In all, these results suggest that the formation and distribution of these sub-nuclear structures is a dynamic response by the cell to the infection, which is potentially modulated by a variety of viral proteins. Whether LRF nuclear bodies are related to any of the nuclear structures represented by these HSV-1 proteins and the functional significance of these interactions in an infected cell environment is currently under investigation.

Conflict of interests

The authors declare that they have no conflicts of interest with the contents of this article.

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Fig. 8. LRF+/+ and LRF−/− mouse embryonic fibroblast cells were infected with 10 MOI of HSV-1 (Kos). Media was harvested at 0 (inoculum), 4, 8, 16 and 24 h post-infection. Viral yields from each sample were titrated in Vero cells, and a single-step virus replication curve was constructed. (•) p<0.005.


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