Knockdown of hDaxx in normally non-permissive undifferentiated cells does not permit human cytomegalovirus immediate-early gene expression

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The cellular protein human Daxx (hDaxx), a component of nuclear domain 10 structures, is known to mediate transcriptional repression of human cytomegalovirus immediate-early (IE) gene expression upon infection of permissive cell types, at least in part, by regulation of chromatin structure around the major IE promoter (MIEP). As it is now clear that differentiation-dependent regulation of the MIEP also plays a pivotal role in the control of latency and reactivation, we asked whether hDaxx-mediated repression is involved in differentiation-dependent MIEP regulation.

We show that downregulation of hDaxx by using small interfering RNA technology in undifferentiated NT2D1 cells does not permit expression of viral IE genes, nor does it result in changes in chromatin structure around the MIEP. Viral IE gene expression is only observed upon cellular differentiation, suggesting little involvement of hDaxx in the regulation of the viral MIEP in undifferentiated cells.

Human cytomegalovirus (HCMV) is the prototypic member of the subfamily Beta herpesvirinae. Primary infection of healthy, immunocompetent individuals, although usually asymptomatic, results in lifelong persistence of the virus (Ho, 1990). However, primary infection or reactivation in immunosuppressed individuals, such as transplant or human immunodeficiency virus/AIDS patients, can lead to serious disease (Drew, 1988; Rubin, 1990).

Like other herpesviruses, early in infection, the genome of HCMV becomes associated with nuclear structures known as nuclear domain 10 (ND10), which later become replication compartments (Ishov & Maul, 1996; Maul, 1998). ND10 are discrete, interchromosomal accumulations of specific cellular proteins, several of which are known transcriptional repressors (Negorev & Maul, 2001). Interestingly, the immediate-early (IE) proteins of several human herpesviruses are known to disrupt these structures (Everett, 2001). Indeed, the HCMV major IE protein IE72 has been found to be both necessary and sufficient for this disruption (Kelly et al., 1995; Korieth et al., 1996; Ahn & Hayward, 1997, 2000; Wilkinson et al., 1998).

Other viral proteins also appear to target these nuclear structures. The HCMV tegument protein pp71 has also been found to associate with ND10 via interaction with hDaxx (Hofmann et al., 2002; Ishov et al., 2002). hDaxx is known to act as a promiscuous transcriptional repressor and probably controls gene expression via recruitment of histone deacetylases (HDACs) (Hollenbach et al., 2002; Michaelson & Leder, 2003). Therefore, it has been suggested that hDaxx may play some role in control of HCMV gene expression upon infection. Consistent with this, overexpression of hDaxx in permissive cells causes the abrogation of virus infection, whereas, conversely, downregulation of hDaxx in permissive cells increases gene expression and virus replication (Cantrell & Bresnahan, 2006; Preston & Nicholl, 2006; Saffert & Kalejta, 2006a; Woodhall et al., 2006).

Other evidence also suggests that hDaxx plays an important role in the repression of infection. The recruitment of pp71 to ND10 has been found to cause the proteosomal degradation of hDaxx 3–12 h post-infection (Saffert & Kalejta, 2006a). In addition, pp71 knockout viruses or a pp71 mutant virus unable to bind to hDaxx have been found to result in low levels of IE gene expression upon infection. This is relieved if hDaxx expression is repressed prior to infection (Cantrell & Bresnahan, 2005, 2006; Preston & Nicholl, 2006).

Furthermore, this repression of viral IE gene expression appears to be regulated through hDaxx-mediated changes to chromatin structure around the viral MIEP, as infection of fibroblasts in which hDaxx expression has been inhibited by small interfering RNA (siRNA) technology results in the association of the viral MIEP with transcriptionally active chromatin (Woodhall et al., 2006). As expected, this regulation appears to involve HDACs, as HDAC inhibitors such as trichostatin-A (TSA) relieve this repression (Saffert & Kalejta, 2006a; Woodhall et al., 2006).

hDaxx, therefore, plays an important regulatory role in the control of HCMV IE gene expression from the MIEP.
during productive infection in permissive cells. However, chromatin structure also appears to play an important role in the control of viral IE gene expression in non-permissive cell types and in the differentiation-dependent control of latency and reactivation of HCMV (Sinclair & Sissons, 2006). Firstly, experimental infection of CD34+ cells and monocytes results in the MIEP becoming associated with markers of transcriptional repression (Reeves et al., 2005a), namely heterochromatin protein-1 (HP1) (Bannister et al., 2001), and is consistent with a lack of IE gene expression in these cells. In contrast, infection of monocyte-derived macrophage or mature dendritic cells (DCs) leads to an accumulation of acetylated histones around the MIEP, a marker of transcriptional activation (Strahl & Allis, 2000), and an increase in IE gene expression (Murphy et al., 2002; Reeves et al., 2005a). This regulation of gene expression from the MIEP also appears to take place in cells from naturally infected seropositive donors (Reeves et al., 2005b). However, it is unknown whether hDaxx has any role in the differentiation-dependent regulation of IE gene expression during virus latency or reactivation.

A number of model cell systems exist that recapitulate the differentiation-dependent regulation of viral IE gene expression observed during latency and reactivation of HCMV. The embryonal carcinoma cell system NT2D1 (T2 cells), which are non-permissive for viral infection due to a block in major IE gene expression, can be differentiated with retinoic acid (RA) to a fully permissive phenotype, T2RA cells (Andrews et al., 1984; Gonczol et al., 1984; Lubon et al., 1989). Differentiation leads to changes in the chromatin structure around the viral MIEP in HCMV-infected cells identical to those observed upon reactivation of endogenous latent virus from CD34+ stem cells differentiated to DCs (Murphy et al., 2002; Reeves et al., 2005b). Indeed, treatment of normally non-permissive T2 cells with TSA has also been found to cause permissiveness of these cells for productive infection, entirely consistent with the involvement of HDACs in the control of MIEP activity (Meier, 2001; Murphy et al., 2002). Although T2 cells have been found to have abnormal ND10, with lower levels of PML protein (Hsu & Everett, 2001), these cells have been used by a number of laboratories to mimic changes in cellular function associated with latency and reactivation of HCMV. Therefore, we have sought to address whether knockdown of hDaxx in T2 cells has any effect on expression of viral IE genes during HCMV infection.

Firstly, we transiently downregulated cellular hDaxx expression in T2 cells through the use of siRNA technology described previously (Michaelson & Leder, 2003), supplemented with simultaneous transfection via Lipofectamine 2000 (Invitrogen) of a second hDaxx-specific siRNA (Dharmacon). Knockdown of hDaxx was confirmed 72 h post-transfection both by analysis of RNA levels by RT-PCR (Fig. 1a), using sense primer 5’-GACGGACATTCTCTTCCA-3’ and antisense primer 5’-TCTCATGCACTGACCTTTGC-3’, and by analysis of protein levels by Western blot (Fig. 1b), using an hDaxx-specific antibody (D7810; Sigma) as described previously (Woodhall et al., 2006). hDaxx expression was reduced substantially in sihDaxx-transfected cells (Fig. 1a, b, lane 3), confirmed by serial dilution of both scramble siRNA-transfected and sihDaxx-transfected T2 protein samples (Fig. 1c, lanes 1–4), whereas hDaxx levels remained unaffected in mock- or scramble siRNA-transfected cells (Fig. 1a and b, lanes 1 and 2). The knockdown of endogenous hDaxx had no effect on the differentiation state of T2 cells on the basis of expression of Oct4, a Pou domain transcription factor used routinely as a marker for undifferentiated cells (Rosner et al., 1990), as assessed by RT-PCR using sense primer 5’-GCATACTGTGGACCTCAGG-3’ and antisense primer 5’-CCAAGGGTGCCTCTCTCTG-3’, and by Western blot using an Oct4-specific antibody (ab19857; Abcam) (Fig. 1a, b, lanes 1–3). As can be seen in Fig. 1, little or no induction of IE RNA (Fig. 1a, lane 3) or IE protein (Fig. 1b, lane 3) occurred in hDaxx knockdown cells 24 h post-infection with HCMV strain Toledo. In contrast, T2RA cells showed both an absence of Oct4 expression due to differentiation and the presence of both IE RNA (Fig. 1a, lane 4) and protein (Fig. 1b, lane 4) upon infection. However, as the Western blot analysis suggested that around 10% of hDaxx protein still remains in sihDaxx-transfected T2 cells (Fig. 1b, c), we wanted to ensure that this residual hDaxx was not sufficient to maintain hDaxx-mediated repression of transcription. To this end, we used a nuclear factor κB (NF-κB) luciferase construct (NF-κB-luc; Stratagene) (0.2 μg per sample) that is known to be repressed by hDaxx and whose repression can be relieved by siRNAs to hDaxx (Michaelson & Leder, 2003). All transfections included a β-actin promoter-driven lacZ plasmid (0.5 μg per sample) to normalize transfection efficiency. Fig. 1(d) shows that, 24 h post-transfection, the activity of the NF-κB-luc reporter in sihDaxx-transfected T2 cells was increased by 4.5-fold compared with scramble siRNA-transfected T2 cells. In contrast, an interferon-responsive luciferase construct that is not NF-κB-dependent (ISRE-luc; Poole et al., 2006) (0.5 μg per sample) showed no increase in activity in sihDaxx-transfected cells (Fig. 1d). Thus, the residual level of hDaxx observed in our transient knockdown analysis is insufficient to maintain specific repression of a known hDaxx-repressible promoter.

As knockdown of hDaxx in T2 cells had no effect on IE gene expression, we hypothesized that the viral MIEP would still be associated with transcriptionally repressive chromatin in these cells. Therefore, we carried out chromatin immunoprecipitation (ChIP) analysis of scramble siRNA- or sihDaxx-transfected T2 cells and T2RA cells infected for 24 h with HCMV exactly as described previously (Reeves et al., 2005a). DNA–protein complexes were incubated with either control (1:200 dilution; Sigma-Aldrich), anti-acetylated histone H4 (1:200 dilution; Upstate Biotech) or anti-H1 (1:200 dilution; Serotec) antibody. The acetylation status of histones associated with the viral MIEP was then analysed by real-time quantitative PCR: the MIEP was
amplified with sense primer 5'-CCAAGTCTCCACCCCATTGAC-3' and antisense primer 5'-GACATTTTGGAAA-GTCCCGTTG-3', complementary to positions -157 and -86 relative to the MIEP start site, and using a Taqman probe (FAM-5'-TGGGAGTTTGTTTTGGCACCAAA-3'-TMR). The MIEP in scramble siRNA-transfected T2 cells showed little association with acetylated histones (Fig. 2a, lane 3), but was associated predominantly with HP1 (Fig. 2a,
lane 4), consistent with a lack of IE gene expression within these undifferentiated cells. T2 cells transfected with sihDaxx also showed the MIEP to be associated with low levels of acetylated histones (Fig. 2b, lane 3), but with high levels of HP1 (Fig. 2b, lane 4), again consistent with a lack of IE gene expression in these cells. In contrast, the viral MIEP in T2RA cells, permissive for IE gene expression, was associated predominantly with acetylated histones (Fig. 2c, lane 3) and little HP1 (Fig. 2c, lane 4), as we have shown previously (Murphy et al., 2002). hDaxx knockdown in undifferentiated T2 cells, therefore, appears to have little effect on the chromatin status of the promoter, whereas equivalent levels of hDaxx knockdown in permissive fibroblasts have profound effects on the chromatin structure of the MIEP (Woodhall et al., 2006).

We also confirmed that knockdown of hDaxx did not result in permissiveness of T2 cells for viral IE gene expression by indirect immunofluorescence (Fig. 3a). T2 cells, transfected with either scramble or hDaxx-specific siRNAs for 72 h, and T2RA cells were infected with HCMV (at an m.o.i. of 0.5). After a further 24 h, cells were fixed (4% paraformaldehyde at room temperature for 10 min), permeabilized (70% ethanol at −20°C for 30 min) and stained for IE protein and Oct4 expression by using a mouse anti-IE72/IE86 antibody (clone E13; Argene) and an antibody specific for Oct4 (Abcam). Antibodies were detected by using an Alexa Fluor 594-labelled anti-mouse antibody (Invitrogen) and a fluorescein isothiocyanate-conjugated anti-rabbit antibody (Sigma). Consistent with our Western blot analysis, neither sihDaxx- nor scramble siRNA-transfected cells showed evidence of viral IE gene expression. In contrast, T2RA cells showed high levels of permissiveness for IE gene expression (Fig. 3a), as expected. Differentiation of cells by RA was confirmed by co-staining for Oct4 (Fig. 3).

**Fig. 3.** Expression of HCMV IE protein is only associated with differentiated T2 cells. (a) Scramble siRNA-transfected (Scr) or sihDaxx-transfected T2 cells and T2RA cells were infected with HCMV (m.o.i., 0.5) and then stained for IE72/IE86 (red) and Oct4 (green) expression 24 h post-infection. (b) Scramble siRNA-transfected (Scr) or siOct4-transfected T2 cells and T2RA cells were infected with HCMV (m.o.i., 0.5) and then stained for IE72/IE86 (red) and Oct4 (green) expression 24 h post-infection. Hoechst 33342 staining (blue) was used throughout to identify cell nuclei.
HCMV can bind to and enter undifferentiated T2 cells, but cannot initiate IE gene expression (Meier, 2001), and this is probably due to the presence of high levels of transcriptional repressors and low levels of transcriptional activators of the MIEP (Sinclair & Sissons, 2006). Activators of the MIEP, such as NF-κB, CREB and Sp1 (Hunninghake et al., 1989; Sambucetti et al., 1989; Lang et al., 1992), are known to be at lower levels in undifferentiated cells than repressors such as YY1 and ERF, which also bind to sites in the MIEP (Liu et al., 1994; Bain et al., 2003; Wright et al., 2005). In addition, some of these repressors are able to interact with specific co-repressors, such as HDACs and histone methyltransferases (Yang et al., 1996; Wright et al., 2005). Consequently, the transcriptional milieu of cells is likely to dictate whether infection with HCMV results in viral IE gene expression and productive infection. Indeed, specific knockdown of Oct4, a master regulator of differentiation, in T2 cells resulted in differentiation of these cells, as expected (Hay et al., 2004; Matin et al., 2004), and increased their permissiveness for HCMV IE gene expression (Fig. 3b). This also confirms that our knockdown technology can indeed result in T2 cells becoming permissive for IE gene expression.

In conclusion, although hDaxx has been implicated in repression of IE gene expression from the MIEP in permissive cell types, our results show that hDaxx does not control differentiation-dependent regulation of the viral MIEP in the conditionally permissive cell line NT2D1. IE gene expression and productive infection could only be seen in cells that were differentiated. We are aware that these results are in contrast to results of Saffert & Kalezja (2006b), who observed increased IE gene expression in undifferentiated cells after hDaxx knockdown; in collaboration, experiments are being carried out to address these discrepancies. However, it is also worth noting that levels of hDaxx protein do not differ significantly between non-permissive, undifferentiated cells and differentiated, permissive cells (data not shown), making it difficult to propose that levels of hDaxx protein alone control permissiveness in this model system. Consequently, our data support the argument that the permissiveness of cells for viral IE gene expression ultimately depends on the differentiation status of the infected cell.

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References


