Ets-2 repressor factor recruits histone deacetylase to silence human cytomegalovirus immediate-early gene expression in non-permissive cells

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Previous work from this laboratory has shown that expression of human cytomegalovirus (HCMV) immediate-early (IE) genes from the major immediate-early promoter (MIEP) is likely to be regulated by chromatin remodelling around the promoter affecting the acetylation state of core histone tails. The HCMV MIEP contains sequences that bind cellular transcription factors responsible for its negative regulation in undifferentiated, non-permissive cells. Ets-2 repressor factor (ERF) is one such factor that binds to such sequences and represses IE gene expression. Although it is not known how cellular transcription factors such as ERF mediate transcriptional repression of the MIEP, it is likely to involve differentiation-specific co-factors. In this study, the mechanism by which ERF represses HCMV IE gene expression was analysed. ERF physically interacts with the histone deacetylase, HDAC1, both in vitro and in vivo and this physical interaction between ERF and HDAC1 mediates repression of the MIEP. This suggests that silencing of viral IE gene expression, associated with histone deacetylation events around the MIEP, is mediated by differentiation-dependent cellular factors such as ERF, which specifically recruit chromatin remodelers to the MIEP in non-permissive cells.

INTRODUCTION

As with all herpesviruses, human cytomegalovirus (HCMV) is able to maintain a life-long latent infection following primary exposure. In the healthy seropositive, latent virus can frequently reactivate however, this usually results in subclinical symptoms (reviewed by Britt, 1998). Life threatening complications arise if the immune system is compromised (i.e. in AIDS and transplant recipient patients) or if infection occurs in utero (reviewed by Britt, 1998). HCMV productively infects a broad array of cell types during viraemia (Siniger et al., 1995, 1999; Sinzger & Jahn, 1996), but in healthy seropositive individuals virus is maintained latently in the myeloid lineage (Mendelson et al., 1996; Minton et al., 1994; Taylor-Wiedeman et al., 1991). It has also been shown, using peripheral blood monocytes (PBMs) (Ibanez et al., 1991; Lathey & Spector, 1991; Soderberg-Naucler et al., 1997; Taylor-Wiedeman et al., 1994) and model cell systems (Gonzcol et al., 1984; Weinshenker et al., 1988), that there is a clear correlation between permissiveness of cells for viral immediate-early (IE) gene expression and their state of terminal differentiation. Recently, we showed that in such undifferentiated cells, which are non-permissive for HCMV IE gene expression, repression of the viral major IE promoter (MIEP) is correlated with a closed chromatin conformation and hypoacetylation and hypermethylation of histones around the MIEP (Murphy et al., 2002). In contrast, in differentiated permissive cells, the viral MIEP became associated with hyperacetylated histones consistent with its transcriptional activation (Murphy et al., 2002). Consequently, an analysis of the mechanisms which mediate such differentiation-dependent chromatin remodelling of the viral MIEP in undifferentiated and differentiated cells may go some way to help define mechanisms that are involved in the regulation of latency and reactivation of HCMV in vivo.

The HCMV MIEP contains domains that are involved in transcriptional regulation. DNA sequences within many of these domains have been shown to bind cellular transcription factors that can positively or negatively regulate expression from the MIEP (reviewed by Meier & Stinski, 1996). For example, NF-κB (Sambuccetti et al., 1989), cyclic AMP response element-binding protein (CREB) (Hunninghake et al., 1989) and Sp1 (Lang et al., 1992) activate the MIEP by binding to the 18 and 19 bp repeat sequences, respectively. Conversely, cellular proteins such as modulator-binding factors (Kothari et al., 1991; Shelbourn et al., 1989a), Yin Yang 1 (YY1) (Liu et al., 1994), methylated DNA-binding protein (Zhang et al., 1995), modulator recognition factor (Huang et al., 1996),...
growth factor independence-1 (Zweidler-Mckay et al., 1996) and Ets-2 repressor factor (ERF) (Bain et al., 2003) also bind to sequences within the MIEP but repress transcription. Therefore, these repressor factors are good candidates for cellular factors involved in the control of latency and reactivation of HCMV in vivo.

ERF (Bain et al., 2003) and YY1 (Liu et al., 1994) are both able to bind to the 21 bp repeats and modulator sequences in the MIEP to repress IE gene expression. The mechanisms by which YY1 mediates repression of the MIEP may include interactions with the general transcription factor TFIIB (Sinclair & Sissons, 1996) but, intriguingly, may also involve chromatin remodelling as YY1 has been shown previously to interact with histone deacetylases (HDACs; Yang et al., 1996, 1997). The mechanism by which ERF represses MIEP activity is, so far, unknown, but is likely to involve co-factors present in undifferentiated, non-permissive cells (Bain et al., 2003).

It has been known for some time that chromatin-mediated regulation of transcription is regulated by a diverse array of post-translational modifications of histone N-terminal tails, (reviewed by Berger, 2002; Jenuwein & Allis, 2001; Strahl & Allis, 2000). Of these modifications, the effect of acetylation, deacetylation and methylation of core histone tails are by far the best understood (reviewed by Kouzarides, 1999, 2002). Histone acetylation results in chromatin adopting an open structure that enhances access of DNA-binding factors to the DNA template, thus increasing gene expression. Conversely, histone deacetylation, methylation, and recruitment of silencing proteins such as heterochromatin protein 1 (HP1) results in a closed, transcriptionally silenced chromatin state. As the expression of HCMV IE genes from the viral MIEP in undifferentiated and differentiated cells is controlled by such chromatin remodelling (Murphy et al., 2002), and as cellular transcription factors such as ERF are able to bind to and repress the viral MIEP, but in the context of unknown co-factors (Bain et al., 2003), we have analysed whether ERF mediates repression of the MIEP through interaction with factors that modulate chromatin structure.

Here, we show that ERF is able to recruit HDAC1 to the viral MIEP resulting in repression of the MIEP in undifferentiated, non-permissive cells. Therefore, it is likely that changes in chromatin structure around the viral MIEP, previously observed upon differentiation of cells from a non-permissive to permissive phenotype (Murphy et al., 2002), are mediated by differential recruitment of chromatin remodelers such as HDAC1 by factors such as ERF.

**METHODS**

**GST-fusion assays.** GST-fusion protein interaction assays were carried out as described previously (Caswell et al., 1993). All reactions contained equal amounts of GST-fusion protein, Sepharose beads and transcription/translation (TNT; Promega) protein.

**Cell culture.** The NTer-2 cDNA (T2) human teratocarcinoma cell line (Andrews et al., 1984) was cultured in Eagle's minimal essential medium containing 10% (v/v) fetal calf serum (EMEM-10; Gibco-BRL). Differentiation of T2 cells to permissive T2RA cells was induced by the addition of 10⁻⁶ M all-trans-retinoic acid (Sigma) for 5 days. Where necessary, 330 nM Trichostatin A (TSA; Wako Pure Chemical Industries) was added immediately after transfection to inhibit HDAC activity.

**Plasmids.** Chloramphenicol acetyltransferase (CAT) reporter gene constructs pEScat and pIEP1cat have been described previously (Shelbourn et al., 1989b). Briefly, pEScat contains the CAT gene under the control of the HCMV MIEP (the 2100 to +72 region from the AD169 strain of HCMV). The shorter pIEP1cat construct contains CAT under the control of the sequence from −302 to +72 of the MIEP and thus lacks the imperfect dyad symmetry, NF-1 cluster and all of the 21 bp repeat elements of the MIEP enhancer. pHK3 contains the SV40 early promoter upstream of a multiple cloning site and was a kind gift from T. Kouzarides (Cambridge University, UK). pSG5-ERF (a kind gift from G. Mavrothalassitis; University of Crete, Crete) contains the 1-9 kb fragment of ERF cDNA, from nt 113–2039, cloned into the pSG5 plasmid. The SV40 early promoter drives expression in mammalian cells and the T7 promoter drives expression in *in vitro* TNT experiments. The full-length 2-2 kb HDAC1 cDNA was cloned from pEV237 (a kind gift from B. Marshall, University of California, USA) by BamHI/XbaI digest and subcloned into BamHI/XbaI digested pHK3 to create pHK3–HDAC1. pDsRed1-N1 encodes DsRed1, a red fluorescent protein, under the control of a minimal HCMV MIEP (Clontech).

The following plasmids were also used for *in vitro* TNT expression: pBS-IE2 contains IE2–p86 (Caswell et al., 1993), pGelsolin (Kwiatkowski et al., 1986) contains the full-length gelsolin cDNA and was a kind gift from A. Weeds (Cambridge University, UK), pING14A-HDAC1 (Brehm et al., 1998) was a kind gift from A. Cook (Cambridge University, UK), and pBS-HDAC3 contains the 1-45 kb HDAC3 cDNA with a C-terminal FLAG-tag, subcloned from pcDNA3-HDAC3 by a BamHI/XbaI digest into BamHI/XbaI digested PBS (Stratagene).

pGEX-ERF (Bain et al., 2003), pGEX-TBP and pGEX-TFIIB (Caswell et al., 1993), pGEX-P/CAP (Bryant et al., 2000) and pGEX-RB (Kaelin et al., 1992) have previously been described. pGEX-TAF40 was a kind gift from R. Caswell (Cardiff University, UK) and contains the full-length Drosophila TAF40 cDNA cloned as an EcoRI–HindIII fragment into pGEX-2TKP. pGEX-HDAC1 (1–382) and pGEX-HDAC1 (332–482) contain the cDNA encoding aa 1–382 and 332–482 of HDAC1, respectively, and were cloned into EcoRI/BamHI digested pGEX-2TKP (kind gifts from A. Brehm, Cambridge University, UK). pGEX-HDAC1 (1–382) contains the enzymic domain.

**Transient transfections and CAT assays.** These were carried out as described previously (Bain et al., 2003). Typically, 100 ng CAT reporter plasmid and variable amounts of effector plasmids (made up to 15 μg with control vector DNA), were transfected by calcium phosphate co-precipitation by using 2 × 10⁶ T2 cells. Cells were harvested 40 h post-transfection, the protein concentration of each sample was determined, and reactions were performed to establish CAT activity. Because of the differences in transfectability of T2 and T2RA cells, routinely 10-fold less protein was assayed for T2RA samples. Similarly, 10-fold less protein was assayed for pIEP1cat transfected samples compared with pEScat transfections. Results shown are the mean of at least three independent experiments.

**Transient transfection and FACS analysis.** T2RA cells (1 × 10⁶) were transfected using Lipofectamine 2000 as described by the manufacturer (Invitrogen) with 3 μg pHK3 empty vector, 1.5 μg pSG5-ERF and 1.5-5 μg pHK3, 1.5-5 μg pHK3-HDAC1 and 1.5-5 μg pHK3-HDAC3 cDNAs by a BamHI/XbaI digest into BamHI/XbaI digested pHK3.
pHK3 or 1·5 μg pSG3-ERF and 1·5 μg pHK3-HDAC1, using 2·0 μg DsRed1 as a marker for their transfection. After 48 h post-transfection, cells were superinfected with a recombinant HCMV expressing GFP-tagged IE1-p72. Cells were analysed by using a FACSort (Becton Dickinson).

Immunoprecipitation Western blot assays (IP-Westerns). T2 or T2RK (1·2 × 10⁶) cells were lysed in 1 ml EBC buffer [50 mM Tris/Cl pH 8·0, 200 mM NaCl, 200 μM NaVO₄, 10 mM NaF, 0·5% (v/v) NP40 and 50 μM PMSF; supplemented with 1 μl 40 mg BSA ml⁻¹]. Cellular debris was removed by microcentrifugation (13,000 r.p.m. for 15 min at 4°C). Lysates were pre-cleared by addition of control IgG and 50 μl protein A-Sepharose beads (1:1 with PBS) for 3 h at 4°C. For HDAC immunoprecipitation, 5 μg each of three primary anti-HDAC1 antibodies (Santa Cruz Biotechnology and Upstate Biotechnology) or equivalent amounts of isotype matched control antibodies was mixed with 0·2% (w/v) BSA in 500 μl cell lysate. This was rotated for 2 h at 4°C before 50 μl protein A-Sepharose:protein G-Sepharose (1:1) slurry was added. Following further rotation for 1 h at 4°C, the beads were washed three times with 1 ml NETN [20 mM Tris/Cl pH 8·0, 100 mM NaCl, 1 mM EDTA and 0·5% (v/v) NP40], resuspended in 20 μl SDS-loading buffer and proteins separated by SDS-PAGE. Proteins were then transferred overnight to a nitrocellulose filter (Hybond-C; Amersham Biosciences) and Western blotted with an anti-ERF antibody as described below.

Western blot analysis. Primary antibodies were used as described by the manufacturer (ERF: 1:500, Santa Cruz Biotechnology; HDAC1: 1:1000, Upstate Biotechnology) before being incubated with a horseradish peroxidase-conjugated secondary antibody (diluted 1:2000). Antibody binding was detected by using enhanced chemiluminescence (Amersham Biosciences), and then exposing the filter immediately to photographic film. Finally, the nitrocellulose filter was stained with 0·5% (w/v) Ponceau red in 1% (v/v) trichloroacetic acid to ensure equal protein loading and transfer.

Deacetylase assay. These were carried out essentially as described previously (Taunton et al., 1996). Lyophilized peptide corresponding to the first 24 residues of bovine histone H4 (TEA salt; Affiniti Research Products) was chemically acetylated with 3H-NaOAc [5·3 Ci (197·3 GBq) mmol⁻¹; 10 mCi (370 MBq) ml⁻¹ in EtOH; New England Nuclear]. GST-fusion protein (1 μg) attached to Sepharose beads was rotated with 200 μl EBC buffer, supplemented with BSA (40 μg ml⁻¹) for 10 min at room temperature. T2 cell extract (1 ml) was added and tubes rotated for 2 h at room temperature. Samples were microcentrifuged (5 min at 6000 r.p.m.) and the GST pellet was washed four times with 1 ml NETN. At this point, half the bead sample was taken for Western blot analysis (HDAC1: 1:1000; Upstate Biotechnology) and the remainder was resuspended in 200 μl EBC (minus NP40 and PMSF) to which 5 × 10⁴ c.p.m. of acetylated H4 peptide was added before the tubes were incubated at 37°C for 2 h. The reaction was stopped by the addition of 65 μl 1 M HCl/0·16 M acetic acid mix. Released 3H-labelled acetyl was extracted with ethyl acetate and the supernatant transferred to a scintillation vial containing 1 ml scintillation cocktail (PerkinElmer) and the amount of free ³H released from the peptide was counted. It is important to note that all reaction mixes contained equal amounts of GST-fusion protein, Sepharose beads and peptide. Results shown are the mean of at least three independent experiments.

Virus infection. Cells were infected at 10 p.f.u. per well with CR[IE1–EGFP], a recombinant Towne strain that was a kind gift from J. Gawn and R. Greaves (Imperial College Faculty of Medicine, London, UK). This virus expresses an enhanced green fluorescent protein fused to the C terminus of IE1-p72. Virus was left on the cells for 2 h, after which the medium was aspirated and 1 ml fresh EMEM-10 was added to the dishes/slides before the cells were incubated for a further 16 h. Cells were then analysed by using a FACSort (Becton Dickinson).

RESULTS

ERF specifically interacts with the N terminus of HDAC1

Many transcriptional repressors mediate their effects through interactions with basal transcription factors (Pei, 2001; Sinclair & Sissons, 1996; Vassallo & Tanese, 2002; Wong & Privalsky, 1998) and, as ERF-mediated repression is also likely to involve differentiation-specific co-factors (Bain et al., 2003), we looked for cellular proteins that might interact with ERF by using GST-fusion assays (Fig. 1a). The choice of target GST proteins used was based on previous studies showing an interaction between a number of these proteins and putative negative regulators of the MIEP (Sinclair & Sissons, 1996; Usheva & Shenk, 1994, 1996). Other basal transcription factors were also chosen as they represent good candidate targets for transcriptional repression. As we and others have used GST-fusion assays to successfully identify biologically relevant interactions between many of these candidate proteins and the HCMV IE2-p86 protein (Bryant et al., 2000; Caswell et al., 1993; Hagemeier et al., 1993; Lukac et al., 1997), assays were carried out using ³⁵S-methionine-labelled ERF, IE2-p86 as a positive control and gelsolin as a negative control. Interactions between ERF and our panel of candidate factors were judged to be strongly positive if their level of interaction with ERF was as strong as their interaction with IE2-p86. There was no significant interaction between ERF and the general transcription factors: TBP (lane 6), TFII B (lane 9), TAF40 (lane 12) even though, as expected, IE86 interacted with these proteins (lanes 4, 7 and 10, respectively). The interaction of ERF with two chromatin remodelling enzymes was also analysed. Whilst there was no obvious interaction between ERF and the histone acetyl transferase, P/CAF (lane 15), there was a strong interaction between ERF and the N terminus of HDAC1 (lane 21), at least as strong as the interaction between HDAC1 and IE2-p86 (lane 19; J. Murphy and J. Sinclair, unpublished results). However, ERF did not interact with the C terminus of HDAC1 (lane 18). An even stronger interaction was observed between ERF and RB (lane 24).

ERF physically interacts with multiple HDACs in vitro

The observation that ERF, which represses the MIEP (Bain et al., 2003), interacted with HDAC1 is consistent with observations showing that HCMV lytic infection is regulated by histone (de)acetylation events around the MIEP (Murphy et al., 2002). Therefore, to analyse further this ERF/HDAC1 interaction, reciprocal GST-fusion pull-down assays were carried out. Fig. 1(b) shows GST pull-downs using ³⁵S-methionine-labelled gelsolin (lanes —) or HDAC1 on GST control beads or GST–ERF beads. GST–RB beads
were used as a positive control because of RB’s known strong interaction with HDAC1 (Brehm et al., 1998; Luo et al., 1998; Magnaghi-Jaulin et al., 1998). Significant interaction between ERF and HDAC1 was, again, observed (Fig. 1b, lane 8) and these reciprocal assays also confirmed the interaction between HDAC1 and RB (Fig. 1b, lane 5).

As previous work has also shown that some transcription factors may interact with more than one HDAC family member and as we have previously shown that HDAC3 plays a role in regulating MIEP activity (Murphy et al., 2002), we asked whether ERF also bound to HDAC3. As shown in Fig. 1(b), recombinant GST–ERF was able to pull down HDAC3 (lane 9). Consequently, ERF could be recruiting a number of co-repressor/chromatin remodelling proteins in order to inhibit MIEP activity in a manner similar to RB and YY1, which also interact with HDACs 1, 2 and 3 (Lai et al., 1999; Yang et al., 1997). However, although ERF bound equivalently to HDAC1 and HDAC3 in vitro (compare lanes 8 and 9, Fig. 1b), it is known that superexpression of HDAC3 alone is sufficient for inhibition of HCMV infection of differentiated cells (Murphy et al., 2002). In contrast, co-expression of ERF with HDAC1 appeared to be required for HDAC1 to effectively inhibit HCMV infection of differentiated cells (see below). Consequently, we concentrated our analysis of cellular factors required for ERF-mediated repression of the viral MIEP on the ERF-HDAC1 interaction.

**ERF associates with HDAC1 in cell extracts**

To confirm the interaction between ERF and HDAC1, GST-fusion pull-down assays were carried out using T2 cell extracts followed by Western blot analysis (Fig. 2). This clearly showed an interaction between ERF and endogenous cellular HDAC1 (lane 2). When the blot was stripped and probed with an irrelevant control antibody no signal was detected (data not shown), which confirmed the specificity of this interaction.

Thus far, the interactions between ERF and HDACs were detected by using purified GST-fusion proteins. Therefore, to determine if endogenous cellular ERF could interact with

![Fig. 1.](image)

**Fig. 1.** ERF interacts with HDACs in vitro. (a) 35S-methionine-labelled ERF was tested to determine if it interacts with general transcription factors. In vitro TNT proteins used were IE2-p86 (+, positive control), gelsolin (−, negative control) and ERF. (b) Recombinant GST–ERF was tested for its ability to bind HDAC1 and HDAC3. Approximately 200 ng of the fusion proteins indicated were incubated with radiolabelled gelsolin (−, negative control), HDAC1 or HDAC3 to determine any interaction. In both (a) and (b), input proteins shown are one-fifth of proteins used in each pull-down assay. The gel shown is a representative result that was repeated twice, independently.

![Fig. 2.](image)

**Fig. 2.** Recombinant ERF binds endogenous HDAC1 in cell extracts. Recombinant GST proteins were incubated in T2 cell extract before being subjected to Western blot analysis for the presence of HDAC1. An arrow indicates the position of HDAC1. The gel shown is a representative result that was repeated twice, independently.
endogenous HDACs in vivo, immunoprecipitation assays followed by Western blot analysis, in non-permissive (T2) and permissive (T2RA) cell extracts, were carried out. As shown in Fig. 3, ERF is specifically co-precipitated with HDAC1 in T2 cell extracts (lanes 2 and 3).

ERF associates with functional deacetylase activity

Next, deacetylase assays were performed to determine if the HDAC associated with ERF was enzymically active. As shown in Fig. 4, GST–ERF did indeed pull down functional HDAC activity from cellular extracts. Although modest in our hands and much lower than the level of HDAC activity associated with RB, this level of deacetylase activity was similar to that observed using the well-characterized repressor YY1 which is known to bind functional HDACs (Yang et al., 1996, 1997).

ERF and HDAC1 act in concert to repress MIEP-reporter plasmids

To determine if the physical interaction between ERF and HDAC1 was relevant to the regulation of the MIEP, co-transfection assays were performed using ERF and HDAC1 together with MIEP-based reporter plasmids. The T2 human teratocarcinoma cell line was used for these assays as it shows differentiation-dependent permissiveness for MIEP activity and HCMV infection (Gonczol et al., 1984; Lubon et al., 1989). T2RA cells show only low levels of repression of MIEP reporter constructs after co-transfection with ERF suggesting that ERF may require a co-factor present in T2, but not T2RA cells, to repress the viral MIEP (Bain et al., 2003). To determine if HDAC1 was such a co-factor, co-transfection assays were performed using ERF and HDAC1 together with MIEP-based reporter plasmids (Fig. 5a). Co-transfections of ERF or HDAC1 alone routinely repressed pEScat (a CAT reporter construct under the control of the full-length HCMV MIEP) to low levels. However, when ERF and HDAC1 were co-transfected together, there was a more than additive decrease in MIEP activity. pIEP1cat, a CAT reporter construct which lacks any ERF response sequences (Bain et al., 2003), showed little repression by ERF or HDAC1 alone and no such cooperativity of ERF and HDAC1 together (Fig. 5b), consistent with a lack of ERF-binding sites in this promoter construct.

ERF-mediated repression of the MIEP is sensitive to the HDAC inhibitor TSA

Several studies have used TSA, a potent inhibitor of deacetylase activity, to confirm the role of HDACs in repression of gene expression (Murphy et al., 2002; Radkov et al., 1999; Yang et al., 2001). Consequently, we analysed whether TSA could inhibit ERF-mediated repression of the MIEP. For these experiments pEScat, which is known to be repressed to high levels by ERF alone in undifferentiated T2 cells (Bain et al., 2003), was used. Fig. 5(c) shows that addition of TSA to cells co-transfected with ERF and pEScat consistently increased CAT activity from the MIEP two- to threefold arguing that HDAC activity does indeed play a role in ERF-mediated repression of the MIEP. However, TSA did not completely alleviate ERF-mediated repression. Therefore, ERF-mediated repression may function via both histone deacetylase dependent and independent mechanisms as has been proposed for the Epstein–Barr virus (EBV) latency antigen EBNA3C (Radkov et al., 1999).
Overexpression of ERF and HDAC1 decreases IE expression in normally permissive T2RA cells after infection

Differentiation of non-permissive T2 cells with retinoic acid results in T2RA cells that are permissive for HCMV infection (Gonczol et al., 1984). Consequently, if ERF and HDAC1 do interact to mediate repression of the MIEP in normally permissive T2RA cells, it would be predicted that transfection of differentiated cells with ERF and HDAC1 might also result in inhibition of viral IE expression after infection. Therefore, permissive T2RA cells were co-transfected with ERF and HDAC1 and analysed for their permissiveness for HCMV infection. Cells were transfected with combinations of plasmids, using DsRed1 as a marker for their transfection. Transfected cells were then superinfected with a recombinant HCMV expressing GFP-tagged IE1-p72 and the number of transfected cells (red) that were expressing IE antigen (green) was quantified. Fig. 6 shows that transfection of pSG5-ERF alone has little effect on IE expression after their superinfection, which is in good agreement with previous transfection data showing that ERF alone in T2RA cells had only small effects on MIEP activity (Bain et al., 2003). Superexpression of HDAC1 alone had similar effects on levels of IE expression after infection. In contrast, cells transfected with both ERF and HDAC1 showed significantly less numbers of cells expressing GFP-tagged IE1-p72 after infection with GFP-tagged HCMV. This suggests that ERF and HDAC, in concert, play an important role in negatively regulating the viral MIEP in the context of virus infection.

Cellular levels of HDAC1, but not ERF, correlate with permissiveness for HCMV infection

If HDAC proteins are responsible for differentiation-dependent MIEP repression, then it might be expected...
that levels of HDACs would be high in non-permissive T2 cells but low in permissive T2RA cells. Consequently, the levels of ERF and HDAC1 in these cells were determined by Western blot analysis. As shown in Fig. 7(a), the overall level of ERF protein is not significantly affected by differentiation of T2 cells, which is in good agreement with a previous RNA analysis of ERF in these cells (Bain et al., 2003). However, the level of HDAC1 is reduced dramatically upon differentiation of these cells to a permissive phenotype (Fig. 7a), consistent with previous analysis of HDAC3 (Murphy et al., 2002). Similarly, a time-course analysis also showed a steady decrease in HDAC1 expression during retinoic acid (RA)-induced differentiation which coincided with an increase in permissiveness of these cells for IE gene expression (Fig. 7b), as expected.

**DISCUSSION**

Differentiation-dependent repression of HCMV IE gene expression in non-permissive and permissive cell types has been shown to be associated with chromatin remodelling of the viral MIEP (Murphy et al., 2002). However, the mechanism mediating such specific association of the viral MIEP with hypoacetylated histones in undifferentiated cells was unclear. Similarly, a number of cellular transcription factors have been shown to bind and repress the HCMV MIEP (Shelbourn et al., 1989a; Bain et al., 2003; Kothari et al., 1991; Liu et al., 1994) but how such factors mediated this transcriptional repression was also unclear. Our results now suggest that the mechanism by which one of these factors, ERF, mediates repression of the viral MIEP is by direct recruitment of HDAC1 to the major IE promoter.

In vitro, a strong interaction between ERF and HDAC1 through the N terminus of HDAC1 was observed. Like RB and other transcriptional repressors, ERF also interacted with other HDAC family members such as HDAC3. However, unlike HDAC1 which appears to require co-expression of ERF to mediate repression of viral IE expression (this paper), superexpression of HDAC3 alone is sufficient to inhibit viral IE expression after infection of T2RA cells (Murphy et al., 2002). This suggests that HDAC3-mediated repression of the viral MIEP may require other, as-yet-unidentified, co-factors.

The interaction between ERF and HDAC1 was also confirmed in vivo as was an association between ERF and functional deacetylase activity. Consistent with a model where the permissiveness of cells for HCMV infection is controlled by differentiation-specific chromatin remodelling factors, there was no detectable interaction between ERF and HDAC1 in normally permissive T2RA cells; almost certainly as a result of low levels of HDAC1 expression in these T2RA cells (see below).

This interaction was also confirmed functionally in co-transfection assays. Whilst superexpression of ERF or HDAC1 alone had only modest effects on the expression of MIEP-based reporter constructs, there was a substantial augmentation of repression when both ERF and HDAC1 were co-expressed. Interestingly, TSA treatment did not completely alleviate ERF-mediated repression of the MIEP. Consequently, ERF may mediate its repression by both HDAC-dependent and independent mechanisms. Similar observations have been made for numerous other factors (Wotton et al., 1999; Xue et al., 1998; Zhang et al., 1998) including the EBV transcriptional repressor EBNA3C.
(Radkov et al., 1999). Therefore, the known intrinsic repression domain of ERF, located at the C terminus of the protein (Sgouras et al., 1995), may also play a role in repression of the MIEP through a deacetylation-independent mechanism.

As both ERF and HDAC1 independently interacted with RB in our in vitro assays, it is possible that the ERF/HDAC1 interaction observed was indirect, and resulted from bridging by RB itself. To rule this out, an identical series of transient transfection was performed as in Fig. 5(a) using the SAOS-2 cell line that lacks functional RB. This revealed that ERF and HDAC1 still synergistically repress the viral MIEP and suggests that ERF/HDAC1 mediated repression does not involve RB (data not shown).

Interestingly YY1, which like ERF also binds to the 21 bp repeat elements of the enhancer and has been implicated in the repression of the HCMV MIEP (Liu et al., 1994), is also known to interact physically and functionally with HDACs (Yang et al., 1996, 1997). Consequently, YY1-mediated repression of IE gene expression may also be mediated via HDACs. However, the specific sequences in the 21 bp repeat required for YY1 or ERF binding have not yet been mapped and it is not known if binding of YY1 and ERF to the 21 bp repeat elements are mutually exclusive or can occur simultaneously; these experiments are in progress. Whichever is the case, the three copies of the 21 bp repeat elements within the enhancer of the MIEP clearly act as multiple sites through which a number of candidate cellular transcription factors, such as YY1 and ERF, and silencing protein HP1 (Murphy et al., 2002), are likely to be able to prevent expression from the MIEP.

Whilst many groups have consistently shown that the modulator and 21 bp repeats play a major role in inhibiting IE gene expression in transfection assays (Bain et al., 2003; Huang et al., 1996; Liu et al., 1994; Shelbourn et al., 1989a; Zhang et al., 1995; Zweidler-Mckay et al., 1996), studies using large deletion mutants of HCMV lacking the modulator and 21 bp repeat elements of the MIEP have suggested that these domains may not be necessary for the repression of the MIEP (Meier, 2001; Meier & Stinski, 1997). However, the deletion virus used in such studies has also been shown by the same workers to have perturbed IE gene expression even in permissive cells (Meier et al., 2002; Meier & Pruessner, 2000). Such observations may, therefore, cause difficulties in such comparisons of IE gene expression in non-permissive and permissive cell types. Consequently, the role of the modulator and 21 bp repeats in repression of the viral MIEP in the context of the virus await more defined, specific deletions.

Our observation that the level of HDAC1 but not ERF protein appeared to change upon differentiation of non-permissive T2 cells to permissive T2RA cells suggests that the level of chromatin remodelling proteins such as HDAC1, and not the transcription factors which recruit them to the MIEP, play a pivotal role in determining the permissiveness of cells for HCMV infection. However, whilst total levels of ERF may not change upon differentiation of T2 cells to a cell type permissive for HCMV infection, we cannot rule out that differentiation does result in post-translational changes in ERF modifying its effect on the MIEP. For example, the highest levels of repression of the MIEP in T2RA cells occurred upon superexpression of both ERF and HDAC1; not on superexpression of HDAC1 alone. As T2RA cells clearly express as much ERF as undifferentiated T2 cells by Western blot analysis (Fig. 7a), it would appear that the cellular ERF present in T2RA cells is unable to efficiently co-operate with transfected HDAC1 to repress the MIEP.

We have previously shown that the HCMV MIEP in non-permissive cells is associated with hypoacetylated histones and silencing protein HP1 (Murphy et al., 2002). Our present observations now suggest that this chromatin-mediated repression of the MIEP in non-permissive cells, which we believe will also be crucially important for maintaining the latent phenotype, may be due, at least in part, to ERF recruiting HDACs to the viral MIEP. If this is the case, it is likely that reactivation of HCMV from latency in vivo will require similar chromatin remodelling around the MIEP resulting in a more open chromatin architecture; consistent with the known chromatin structure of the MIEP in differentiated permissive cells (Murphy et al., 2002).

Finally, some cellular transcription factors, such as E2F1 and YY1, are themselves acetylated, and this is known to modulate their activity (Martinez-Balbas et al., 2000; Yao et al., 2001). Consequently, we cannot rule out that the ERF/HDAC1 complex not only targets histones for deacetylation but could also target certain other transcriptional activators of the MIEP, resulting in their deacetylation and subsequent inhibition.

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REFERENCES


with strong transcriptional repressor activity, can suppress ETS-associated tumorigenesis and is regulated by phosphorylation during cell cycle and mitogenic stimulation. EMBO J 14, 4781–4793.


