Localized Domains of G9a-Mediated Histone Methylation Are Required for Silencing of Neuronal Genes

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Summary
Negative regulation of transcription is an important strategy in establishing and maintaining cell-specific gene expression patterns. Many neuronal genes are subject to active transcriptional repression outside the nervous system to establish neuronal specificity. NRSF/REST has been demonstrated to regulate at least 30 genes and contribute to their neuronal targeting by repressing transcription outside the nervous system. Further, human genome database searches reveal that over 800 genes contain an NRSE. Here we report that NRSF recruits the histone dimethylase G9a to silence NRSF target genes in nonneuronal cells. We show that G9a generates a highly localized domain of dimethylated histone H3-K9 around NRSEs, but H3-K27 remains unmethylated. The NRSEs are also associated with HP1. Finally, we demonstrate that dominant-negative G9a abrogates silencing of chromosomal neuronal genes. These findings implicate a role for histone methylation in targeting neuronal gene expression to the nervous system.

Introduction
Negative regulation of gene transcription is an important strategy in establishing and maintaining cell-specific gene expression patterns. The transcriptional repressor neuron restrictive silencing factor (NRSF) (also known as REST) (Chong et al., 1995) has been shown to repress expression of at least 30 neuronal genes outside the nervous system and thus contribute to their neuron-specific transcription pattern (Schoenherr et al., 1996). NRSF was originally identified as a regulator of the sodium channel type 2 (Nall) and SCG10 genes (Chong et al., 1995; Schoenherr and Anderson 1995) by its ability to bind a 23 bp neuron restrictive silencing element (NRSE) (also termed an RE1 site) found in the promoter regions of these genes (Maue et al., 1990; Mori et al., 1990). The NRSE has been identified in over 800 genes in the human genome (Belyaev et al., 2003), underscoring the importance of NRSF in contributing to the control of large programs of transcriptional regulation.

NRSF is expressed in most embryonic and adult nonneuronal tissue. In the nervous system, expression is largely restricted to undifferentiated neuroepithelium although differentiated neurons express low levels of REST/NRSF splice variants (Palm et al., 1998). The implication of aberrant NRSF-mediated repression in models of various syndromes such as Huntington's disease, epilepsy, ischemia, and dilated cardiomyopathy emphasizes the importance of understanding the mechanisms utilized by NRSF in regulating gene expression (Palm et al., 1998; Timmusk et al., 1999; Grooms et al., 2000; Calderone et al., 2003; Kuwahara et al., 2003; Zuccato et al., 2003).

REST/NRSF has been shown to possess at least two independent repression domains, one encompassed by the N-terminal 83 residues and the other by the C-terminal zinc finger (Tapia-Ramirez et al., 1997). The N-terminus of NRSF recruits the SIN3A/B HDAC complex (Huang et al., 1999; Grimes et al., 2000; Roopra et al., 2000). SIN3 is highly conserved in eukaryotes and widely used by many repressors involved in diverse aspects of physiology (reviewed in Pazin and Kadonaga, 1997). The C-terminal repression domain of NRSF has been shown to recruit a distinct HDAC complex via its interaction with CoREST (Andres et al., 1999; Ballas et al., 2001).

Eukaryotic genomes are wrapped around histones and other nuclear proteins into a complex termed chromatin. The N-terminal tails of core histones are prone to a battery of modifications and certain histone modifications or constellations of modifications are associated with active gene expression whereas others are associated with repressed or silent genes (Strahl and Allis, 2000). The discovery that transcription factors are able to recruit chromatin-modifying enzymes to promoters has resulted in a unification of findings regarding chromatin structure and mechanisms of gene regulation. Unlike acetylation, lysine methylation is thought to be a stable and heritable mark. Methylation of H3-K4 is found at active genes and methylation of H3-K9 or H3-K27 at repressed loci (Strahl and Allis, 2000, and references therein).

The demonstration that methylated H3-K9 and H3-K27 are ligands for the heterochromatic protein HP1 (Bannister et al., 2001; Lachner et al., 2001) and polycomb proteins (Cao et al., 2002; Csermin et al., 2002; Kuzmichev et al., 2002; Muller et al., 2002), respectively, via their chromodomains and that acetylated lysines can be bound by bromodomain-containing proteins suggest that histone modifications may represent a “signaling network” (Schreiber and Bernstein, 2002) or “histone code” (Strahl and Allis, 2000) that is interpreted by nuclear proteins to affect programs of gene expression. A number of histone methylases have been described, and each shows substrate specificity in that distinct enzymes methylate specific lysine residues. Further, it is now clear that certain methylases catalyze mono-, di- or trimethylation of lysines. The SUV39 class of methylases trimethylate H3-K9 (Xiao et al., 2003) and mammalian SUV39H colocalises with dense heterochromatic regions (Bannister et al., 2001; Lachner et al., 2001). The euchromatic methylase G9a utilizes H3-K9 and H3-K27 as substrate (Tachibana et al., 2001) and catalyzes primarily mono- and dimethylation reactions (Xiao et al., 2003).

Here we report that NRSF recruits the histone dimethylase G9a to repress NRSE bearing neuronal genes in...
Figure 1. NRSE Elements Associate with Localized Dimethylated H3-K9

(A) ChIPs with anti-HA (sham), anti-K9 (OAc), or anti-H3-K9(Me), performed on JTC-19 chromatin were amplified with primers flanking the NRSEs of the SCG10, Nall, and M4 genes or the intron/exon junction of the first coding exon of the NRSF gene. Nonneuronal cells. Further, we show that NRSF-recruited G9a generates a highly localized domain of H3-K9 methylated nucleosomes around the NRSE and that the majority of histones are dimethylated. Interestingly, H3-K27 remains unmethylated despite recruitment of G9a. The NRSEs are also enriched for HP1α and HP1γ that may be recruited via G9a.

(B) ChIPs with antibodies to the indicated H3-K9 isoforms were quantified by real-time PCR (Q-PCR) with primers flanking or 2 or 6 kb up or downstream of the NaII NRSE. The values shown are the specific binding (total nanogram quantity detected with the indicated antibody and primer pair minus the nanogram quantity detected with sham antibody and the indicated primer pair) as a fraction of total DNA input material for the ChIP.

Results

The NRSE Element Is Associated with a Localized Region of Dimethylated Histone H3

We wished to determine whether histone methylation contributes to NRSF-mediated silencing of neuronal genes in nonneuronal cells. Antibodies to H3-K9(Me), or acetylated lysine-9 (H3-K9(OAc)) were used in a chromatin immunoprecipitation (ChIP) assay with chromatin extracted from the NRSF-expressing rat lung fibroblast cell line JTC-19. The precipitates were interrogated with primers flanking the NRSEs of the repressed SCG10, Nall and M4 genes or the first intron/exon boundary of the active NRSF gene. Figure 1A shows that the SCG10, Nall, and M4 NRSE sequences are all efficiently precipitated with anti H3-K9(Me), antibody but only weakly with the anti-H3-K9(OAc) antibody. The NRSF gene, which is expressed to high levels in this cell line, was robustly associated with H3-K9(OAc). To determine the extent to which methylation of H3-K9 is spread along the chromosome from an NRSE, real-time quantitative PCR (Q-PCR) was performed on chromatin precipitated from JTC-19 cells with antibodies to H3-K9(Me), H3-K9(Me), H3-K9(Me), deacetylated H3-K9 (H3-K9(NH2)), H3-K9(OAc), or sham IgG. The precipitates were interrogated with primer pairs either 6 or 2 kb upstream, directly over the Nall NRSE, or 2 or 6 kb downstream of the NRSE. Figure 1B shows that the predominant H3-K9 isoforms over the Nall NRSE are H3-K9(Me) and H3-K9(NH2). However, as little as 2 kb up or downstream of the NRSE, the H3-K9(Me) signal drops to background levels, and H3-K9(OAc) becomes predominant. For comparison, the equivalently expressed ChIP signal with the H3-K9(Me) antibody and primers flanking the first intron/exon boundary of the active NRSF gene is 0.8 × 10^-4 units. These data demonstrate that the Nall gene is associated with a highly
localized domain of dimethylated H3-K9 around the NRSE which is embedded in a chromatin environment whose overall dimethylation level is no higher than that found at an active locus elsewhere in the genome.

**NRSF Associates with Histone Methylase Activity**

The presence of H3-K9(Me)2 at the NRSEs of neuronal genes suggested that NRSF might associate with HMT activity. To test this possibility, NRSF was immunoprecipitated from HeLa nuclear extract and subjected to a histone methylase assay with core histones as substrate. Figure 2A shows that the NRSF immune complexes associated with 14-fold more HMT activity than sham. We asked which of the two repression domains of NRSF (Tapia-Ramírez et al., 1997) recruits the HMT. Plasmids expressing gal4 DBD fusion to NRSF residues 1008–1097 cells precipitates 21-fold the methylase activity of gal4 alone. NRSF residues 1–138 precipitate 2-fold above gal4 alone (compare lanes 1 and 2). The C-terminal repression domain therefore recruits the bulk of HMT activity (compare lanes 1 and 3). Separation of core histones from the remaining half of the HMT reactions by SDS-PAGE followed by autoradiography clearly shows that histone H3 is the preferred target of this HMT activity (Figure 2C). In summary, these results suggest that the C terminus of NRSF associates with a histone methylase in vivo.

**The NRSF-Associated HMT Activity Does Not Require CoREST**

Given that the C-terminal repression domain interacts with a complex containing CoREST (Andres et al., 1999), we asked whether the C terminus-associated HMT activity was recruited as part of the CoREST complex. HEK cells were transfected with a plasmid expressing gal4 DBD or gal4 fused to CoREST, and the fusion proteins were immunoprecipitated for HMT assays. Surprisingly, no HMT activity was precipitated with the gal4-CoREST fusion protein (Figures 2B and 2C) (compare lanes 1 and 4 of Figure 2B and lanes 2 and 5 of Figure 2C). To verify that the lack of HMT activity in the gal4-CoREST immune complexes was not an artifact of using fusion proteins, we immunoprecipitated CoREST from three different cell lines (HeLa, HEK, and JTC-19) using two different antibodies. One of the antibodies was raised to a region adjacent and N-terminal to the first SANT domain in CoREST (labeled “BD”). The second antibody was raised to the C-terminal 16 residues of CoREST (labeled “CT”) (Humphrey et al., 2001). Figure 2D shows that neither antibody precipitated HMT activity in either HEK (compare lanes 1, 3, and 4) or JTC-19 cells (compare lanes 5, 7, and 8). However, robust HMT activity was precipitated from HeLa extract with the antibody raised to CoREST’s C terminus (compare lanes 9, 11, and 12). The lack of HMT activity in immunoprecipitates from HEK and JTC-19 cells was not due to lack of CoREST expression as shown in Figure 2E. As expected, NRSF immune complexes from all three cell lines yielded robust HMT activity (compare lanes 1 and 2, 5 and 6, 9 and 10). These results suggest that CoREST exists in at least two distinct cell type-specific complexes: an HMT-containing complex (such as in HEK cells) and HMT-lacking complex (such as in JTC-19 and HEK cells). The presence of HMT activity in NRSF immune complexes from cell extracts that show no HMT activity in CoREST immune complexes suggests that NRSF does not require CoREST to recruit histone methylases.

**The NRSF-Associated HMT Activity Targets Lysine 9 and Lysine 27 of Histone H3 In Vitro**

To characterize the NRSF-associated HMT activity further, we determined its substrate specificity. GST fused to the C terminus of NRSF was incubated with HeLa nuclear extract, washed, and incubated with radioactive S.A.M. and biotinylated peptides corresponding to residues 1 to 21 (H3(1–21)) of histone H3 either unmodified, dimethylated at K-4, dimethylated at K-9, dimethylated at both K4 and K9 or biotinylated peptide corresponding to residues 21 to 44 (H3(21–44)) of H3 either unmodified or dimethylated at K-27. After incubation, peptides were captured, washed, and subjected to scintillation counting. Figure 2F shows that H3(1–21) and H3(1–21)K4-Me2 were substrates for NRSF-associated HMT activity (lanes 1 and 2) whereas H3(1–21)K9-Me2 and H3(1–21)K4,K9-Me2 were not (lanes 3 and 4). Surprisingly, unmodified H3(21–44) was also a robust substrate, but H3(21–44)K27-Me2 was not (lanes 5 and 6). The failure of the HMT to add a third methyl group to dimethylated H3-K9 suggests that NRSF recruits a mono- or dimethylase rather than a trimethylyase. These results suggest that NRSF associates with a histone mono- or dimethylase that targets H3-K9 and H3-K27 in peptide assays.

We asked whether the C-terminal domain could recruit both HDAC and methylase activity to mediate the sequential removal of an acetyl group followed by methylation of H3-K9. Figure 2G shows that GST-C could utilize a peptide corresponding to H3(1–21) acetylated at K9 as a substrate in a HMT reaction and that the HDAC inhibitor TSA could completely inhibit this reaction. This result suggests that the C-terminal repression domain can mediate both the deacetylation and methylation of H3-K9.

**NRSF Associates with the Histone Methylase G9a In Vivo**

Tachibana et al. (2001) have demonstrated that the histone methylase G9a targets H3-K9 and H3-K27 in in vitro assays. Further, SUV39H targets H3-K9 only in the absence of prior H3-K4 methylation (Nishioka et al., 2002). Given the H3-K9 and H3-K27 specificity of the NRSF-associated HMT and its ability to methylate H3(1–21) K4(CH3), peptide, we asked whether G9a is the NRSF-associated HMT. HEK cells were transfected with plasmids expressing MYC-tagged NRSF and FLAG-tagged G9a along with HA-tagged SUV39H, lyased, and NRSF immunoprecipitated with anti-MYC antibody or sham antibody. Figure 3A shows that FLAG-tagged G9a was present in MYC-NRSF immune complexes after washing with RIPA buffer but failed to precipitate using sham antibodies (Figure 3A, top panel). Importantly, though
Figure 2. The C Terminus of NRSF Associates with a H3-K9/K27 Targeting HMT in the Absence of CoREST

(A) NRSF was immunoprecipitated from HeLa nuclear extract and subjected to a histone methylase assay using bulk histones as substrate. Anti-HA antibody was used in a sham immunoprecipitation.
CoREST was present in MYC-NRSF immune complexes washed with low stringency buffer (Figure 3A, second panel), we failed to detect CoREST in immune complexes washed in RIPA buffer despite detecting G9a under these conditions (Figure 3A, third panel). In a reciprocal experiment, MYC-NRSF was found in FLAG-G9a immune complexes but failed to precipitate when using sham antibody (Figure 3B). HA-SUV39H failed to precipitate with NRSF when using low stringency buffer (Figure 3A, bottom panel). These results suggest that NRSF and G9a are able to interact in living cells. To ensure that we were not seeing interactions between NRSF and G9a due to overexpression of the proteins, we precipitated endogenous NRSF from HeLa nuclei with anti-NRSF antibody. Figure 3C shows that endogenous G9a could be precipitated with anti-NRSF antibody but not sham antibody. These results show that G9a and NRSF form a complex in vivo and do not require CoREST for this interaction.

The C-Terminal Repression Domain of NRSF Interacts with a RING Finger-like Motif in G9a

Given that NRSF recruits HMT activity via its C terminus and that the activity does not associate with CoREST in HEK cells, we reasoned that G9a should also interact with the C terminus of NRSF and fail to associate with CoREST in these cells. To test this, HEK cells were transfected with plasmids expressing gal4 DBD fusions to the C terminus of NRSF along with FLAG-G9a. Figure 4A shows that gal4 antibody precipitated FLAG-G9a with gal4 fusions to the C terminus of NRSF under conditions where it failed to precipitate with gal4 alone, gal4 fused to CoREST or the N terminus of NRSF along with FLAG-G9a. The concordance of G9a binding with NRSF-associated HMT activity is consistent with G9a being the NRSF-associated methylase. G9a is a modular protein with an N-terminal region displaying low homology to a RING finger motif, six Ankyrin motifs, and a C-terminal Pre-SET, SET, and post-SET tripartite.
SUV39H showed a more punctate nuclear stain and failed to show any colocalization with MYC-NRSF in any nuclei tested (compare panels 4, 5, and 6). These results suggest that NRSF is found in the same nuclear compartments as G9a but not SUV39H.

G9a Is Found at NRSEs

We asked whether G9a was found at the endogenous promoters of NRSF-regulated genes in living cells. ChIP assays were performed on JTC-19 cells with antibodies to NRSF or G9a. Figure 6A shows that both NRSF and G9a are found at the NRSEs of the SCG10, Nall, and M4 genes in JTC-19 cells. We wished to determine whether G9a recruitment to NRSEs allowed spreading of G9a along the chromosome. PCR of G9a ChIP material with primers to flanking regions of the NaII NRSE show that G9a recruitment is highly localized with no signal above background being detected as little as 2 kb up or downstream of the NRSE (Figure 6B, lanes 2, 4, and 6). For comparison, the equivalently plotted values obtained with the G9a and NRSF antibodies at the first intron/exon boundary of the active NRSF gene in these cells (where we would not expect to see any bound G9a or NRSF protein) are 2.4 × 10^{-4} and 1.7 × 10^{-4}, respectively. To assess whether G9a is found at NRSEs in vivo, we performed G9a and H3-K9(Me 2) ChIP on rat liver tissue and interrogated with primers to the NaII NRSE. Figure 6E shows robust association of both G9a and dimethylated H3-K9 at the NaII NRSE. These results show that the histone methylase G9a is found localized at NRSEs in nonneuronal cells.

HP1 Isolforms Are Found at NRSEs

Methylated H3-K9 has been shown to be a ligand for binding of HP1 via its chromodomains (Bannister et al., 2001; Lachner et al., 2001). Using ChIP, we asked whether the H3-K9(Me 2) mark deposited at the NaII NRSE was associated with HP1. ChIP with isoform-specific anti-HP1 antibodies followed by Q-PCR revealed HP1-α, -β, and -γ occupancy at the Nall NRSE (Figure 6C). The α and γ isoforms were more abundant than β. HP1 recruitment was highly localized with little enrichment seen as little as 2 kb upstream of the NRSE. There was much reduced but still detectable HP1 signal 2 kb downstream of the NRSE and lower levels again 6 kb downstream. These results demonstrate that histone modifications around the Nall NRSE are conducive to HP1 binding in an isoform-selective manner.

NRSF and G9a Colocalize to the Same Nuclear Domains

To further characterize the interaction between NRSF and G9a in intact cells, epitope-tagged NRSF and G9a were localized in interphase nuclei of HEK cells using immunocytochemistry and confocal microscopy. Figure 5 shows that in HEK cells both MYC-tagged NRSF and FLAG-tagged G9a localizes primarily to the nuclear periphery with fainter diffuse staining throughout the nucleus (compare panels 1, 2, and 3). One hundred percent of cells (n = 25 cells) that stained for both MYC-NRSF and FLAG-G9a showed robust colocalization with enrichment at the nuclear periphery (data not shown). HA-G9a Associates with HP1α and HP1γ

The similarity in distribution between HP1 and G9a over the Nall NRSE prompted us to ask whether G9a physically interacts with any of the HP1 isoforms. HEK cells were transfected with plasmids expressing MYC-tagged HP1α, β, or γ and FLAG-tagged G9a. Protein was harvested, immunoprecipitated with anti-FLAG antibody, washed in PBS + 0.5% NP40, and Western blotted with anti-MYC antibody. Figure 6D shows that MYC-tagged HP1α and HP1γ interact with FLAG-tagged G9a when expressed in HEK cells. No binding with any HP1 isoform was seen after washing immune complexes in RIPA buffer (data not shown). These results suggest that G9a...
Figure 5. NRSF Colocalizes with G9a but Not with SUV39H

HEK cells were transfected with plasmids expressing MYC-tagged NRSF and FLAG-tagged G9a or MYC-tagged NRSF and HA-tagged SUV39H. After fixation and double label immunostaining, cells were subjected to confocal immunofluorescence microscopy with MYC-TRITC (red) and FLAG-FITC (green) (1, 2, and 3) or MYC-TRITC and HA-FITC (4, 5, and 6).

interacts preferentially with HP1\textsubscript{γ} followed by HP1\textsubscript{α} and very poorly with HP1\textsubscript{β}.

G9a Is Required for NRSF-Mediated Repression of Transcription

To determine whether G9a is required for NRSF-mediated repression, we performed a transient transfection assay in JTC-19 cells with a plasmid driving luciferase reporter gene expression from a E1b TATA box under the control of 5 gal4 binding sites (G5-TATA) (Roopra et al., 2000). G5-TATA was cotransfected with a plasmid driving expression of gal4 DBD or gal4 DBD fused to the C-terminal repression domain of NRSF along with plasmids expressing full-length G9a, G9a(6–618) (as a G9a dominant-negative molecule), SUV39H, or SUV39H-ΔSET in the presence or absence of the histone deacetylase inhibitor trichostatin A (TSA). Figure 7A shows that gal4-C is a potent repressor of reporter gene expression in JTC-19 cells (lane 1) and that this repression is sensitive to TSA (compare lanes 1 and 2). Overexpression of G9a(6–618) but not full-length G9a resulted in a dramatic loss of repression (compare lanes 1, 3, and 5), suggesting that the NRSF-interacting region of G9a acts as a dominant-negative molecule for NRSF C-terminal-mediated repression and that endogenous G9a expression levels are not limiting for this assay in these cells. Interestingly, there was no change in reporter activity in the presence of overexpressed SUV39H or SUV39H-ΔSET (compare lanes 1, 7, and 9), suggesting that this methylase is not necessary for repression by the C terminus of NRSF. These results suggest that on transient templates, the C terminus of NRSF requires the methylase G9a but not SUV39H for HDAC-dependent transcriptional repression. To determine the role of G9a in repression of endogenous NRSF target genes, we transfected JTC-19 cells with plasmids expressing either the DNA binding domain of NRSF, the RING finger homology region of G9a (dominant-negative mutants of NRSF or G9a, respectively), or empty expression plasmid along with a plasmid expressing the cell surface protein CD4. Transfected cells were magnetically sorted with anti-CD4 antibody and RNA harvested, reverse transcribed, and subjected to real-time PCR with primers to the coding region of the Nall gene. Figure 7B shows induction of Nall RNA levels in the presence of both NRSF and G9a dominant-negative constructs compared to control transfection (9.8- and 12.3-fold, respectively) when normalized to actin expression (compare lanes 7, 8, and 9). These results suggest that G9a is required for silencing of NRSF-regulated genes in nonneuronal cells.

Discussion

In this study we demonstrate that NRSF recruits the histone methylase G9a to neuronal genes in nonneuronal cells to generate a localized chromatin domain around NRSE elements that is enriched in dimethylated histone H3 lysine-9 and HP1. These results suggest that NRSF utilizes histone methylation to silence transcription.

NRSEs Associate with a Highly Localized Domain of H3-K9 Methylation

It has been suggested that recruitment of the histone methylase SUV39H and associated HP1 to genomic loci results in spreading of these protein and H3-K9 methylation along the chromatin fiber with the limits of spreading being defined by boundary elements perhaps comprised of highly acetylated histones. We wished to determine: (1) whether NRSEs recruited HMTs, (2) whether NRSEs were associated with H3-K9 methylation, and (3) to what extent this mark was spread along the chromatin fiber. Unlike the case for Swi6/Clr4 at the mat locus (Noma et al., 2001), G9a recruitment to NRSEs and associated H3-K9(\textsubscript{Me2}) is highly localized with no enrichment for G9a seen as little as 2 kb upstream or downstream of the Nall NRSE. The same is true for the HP1 isoforms. Highly localized recruitment of histone methylase has been described at the cyclin E promoter. In this case, E2F/Rb recruited SUV39H and HP1 were shown to mediate methylation and binding of a single nucleosome over the promoter (Nielsen et al., 2001). Also, recruitment of SETDB1 by the KRAB domain to a chromosomal reporter gene in mammalian cells results in localized enrichment of methylase and HP1 (Ayyanathan et al., 2003). These results suggest therefore that recruitment of H3-K9 HMT activity is not sufficient for spreading of
Figure 6. G9a and HP1 Occupy Limited Regions around NRSEs In Vivo
(A) ChIPs with anti-HA (sham), anti-NRSF, or anti-G9a antibodies performed on JTC-19 chromatin were interrogated with primers flanking the NRSEs of the SCG10, NaII, and M4 genes or the intron/exon junction of the first coding exon of the NRSF gene.

(B) ChIPs with antibodies to NRSF or G9a were quantified by Q-PCR as described in Figure 1B.

(C) ChIPs performed with isoform-specific anti-HP1 antibodies were quantified by Q-PCR as described in Figure 1B.

(D) HEK cells transfected with plasmids expressing FLAG-tagged G9a or MYC-tagged HP1α, β, or γ. Cells were lysed, immunoprecipitated with anti-FLAG antibody, washed under mild conditions, and subjected to Western blot with anti-MYC antibody. WCE represents 5% of the whole-cell extract used in the immunoprecipitations.

(E) ChIPs with anti-HA (sham), anti-G9a, or anti-H3-K9(Me)2 antibodies performed on adult rat liver chromatin were interrogated with primers flanking the NRSE of NaII gene.

repressive chromatin along the fiber. Models of spreading of silent methylated chromatin invoke the ordered methylation and binding of H3-K9 by the methylase/HP1 complex. We note that the G9a/HP1αγ interaction was observed only under mild wash conditions (PBS + 0.5% NP40) and was lost by RIPA buffer washes. It is possible...
G9a Recruitment by NRSF

735

Figure 7. G9a Is Required for NRSF-Mediated Transcriptional Repression of Chromosomal Genes In Vivo

(A) JTC-19 cells were transfected with G5-TATA-Luc reporter plasmid along with plasmids expressing the gal4 DBD or gal4-C and plasmids expressing the indicated methylase fragments in the presence or absence of TSA. Firefly luciferase measurements were normalized to cotransfected pRL-TK and expressed as fold gal4 DBD in the presence of the methylase fragments and/or TSA. Values are the average of three experiments performed in triplicate.

(B) JTC-19 cells were transfected with plasmids expressing the NRSF DBD or the RING finger-like NRSF interaction domain of G9a or just a FLAG epitope (pTAG2A) along with a plasmid expressing the CD4 cell surface marker. Cells were harvested and magnetically sorted through magnetized iron columns. After recovery and expansion, RNA was harvested and subjected to RT-Q-PCR with primers to the coding regions of actin or NaII. Lanes 1, 2, and 3 represent absolute picograms of actin cDNA. Lanes 4, 5, and 6 represent absolute picograms of NaII cDNA. Lanes 7, 8, and 9 represent NaII values divided by actin values.

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that the lack of a tight interaction between G9a and HP1 contributes to the lack of spreading of G9a, HP1, and H3-K9 methylation from NRSEs.

**G9a Recruitment by NRSF**

The HMT G9a has a number of functional signatures that distinguish it from the SUV39 class of enzymes. (1) G9a can target H3-K9 and H3-K27 in vitro (Tachibana et al., 2001) whereas SUV39H only targets H3-K9 (Rea et al., 2000). (2) G9a is a dimethylase and SUV39H is a trimethylase (Xiao et al., 2003). (3) G9a can target H3-K9 for methylation in the presence of H3-K4(Me) whereas SUV39H only utilizes H3-K9 in the absence of prior H3-K4 methylation (Nishioka et al., 2002). The observation that NRSF recruits a methylase that (1) can utilize H3-K9 as well as H3-K27 as substrate in vitro, (2) methylates H3-K9 in the presence of H3-K4(Me), and (3) fails to add a third methyl group onto dimethylated H3-K9 (Figure 2E) is consistent with NRSF recruiting G9a. Also, (1) the presence of G9a in NRSF immune complexes, (2) colocalization of NRSF and G9a in nuclei as judged by immunostaining, (3) enrichment of H3-K9(Me) as compared to H3-K9(Me) at NRSF-regulated genes, (4) the presence of G9a at NRSF occupied NRSEs as judged
by ChIP, and (5) the ability of dominant-negative G9a to alleviate repression of NRSF target genes further substantiate the notion that NRSF recruits the H3-K9 targeting/H3-K4 methylation status-independent dimethylase G9a. Despite considerable effort, we failed to demonstrate a functional interaction between NRSF and the SUV39H methylase. Although the ability of NRSF-associated HMT activity to target H3-K27 in vitro and also target H3-K9 in the presence of H3-K4(Me) does not rule out a role for SUV39H since both G9a and SUV39H could be associated with NRSF, the inability to add a third methyl group to H3-K9(Me) and failure to find SUV39H in NRSF immune complexes or observe colocalization of NRSF with SUV39H or detect SUV39H at NRSEs in vivo (data not shown) is inconsistent with SUV39H associating with NRSF.

G9a Generates Dimethylated H3-K9 but Not Methyl H3-K27 around NRSEs

It has been shown that G9a methylase activity targets both H3-K9 and H3-K27 in vitro (Tachibana et al., 2001 and Figure 4). However our ChIP analysis of NRSF-regulated genes in JTC-19 cells showed only dimethylation of H3-K9 (Figures 1 and 6) with no detectable methylation at H3-K27 (data not shown) despite G9a being found at all NRSEs tested (Figure 6). The reason for the lack of methylation at H3-K27 is not clear. We explored the possibility that H3-K27 is acetylated at NRSEs and therefore blocked from methylation but failed to detect the presence of H3-K27(OAc) at the M4, SCG10, or Nall promoters in JTC-19 cells (data not shown). Given that G9a knockout cells do not show a bulk reduction in H3-K27 methylation (Tachibana et al., 2002; Peters et al., 2003; Rice et al., 2003) and the lack of K27 methylation at NRSEs, it is likely that K27 is not a relevant substrate for G9a in vivo and that free histones or histone tail peptides allow a less stringent substrate specificity for this methylase.

NRSF Recruits the CoREST HDAC Complex and G9a Independently

The reported association between the C terminus of NRSF and CoREST (Andres et al., 1999) prompted us to ask whether G9a was recruited as part of a CoREST complex. Two lines of investigation suggest that this is not the case. First, in HEK and JTC-19 cells NRSF associates with robust HMT activity whereas CoREST does not (Figure 2). Second, NRSF immune complexes retain G9a but not CoREST after washing in RIPA buffer suggesting that G9a can bind NRSF in the absence of CoREST (Figure 3). However Shi et al. described a CtBP complex containing both G9a and CoREST (Shi et al., 2003) suggesting that at least in HeLa cells, these two proteins do interact, and indeed we find that CoREST does associate with histone methylase activity in HeLa cells. Nevertheless, the data presented here suggests a CoREST-independent recruitment of G9a by NRSF. Given that the Ct antisemur is able to precipitate HMT activity from HeLa nuclear extract whereas the BD antibody in the region of aa 78 to aa 192, i.e., the BD antibody epitope.

G9a Is Important for NRSF-Mediated Repression of Neuronal Genes

In transient transfection assays using ga4 DBD fusions to the C terminus of NRSF, expression of the G9a RING domain as a dominan-negative molecule markedly reduced repression by the C-terminal repression domain. Importantly, the same G9a domain expressed in JTC-19 cells resulted in induction of both the endogenous Nall and SCG10 genes (Figure 7B and data not shown). Interestingly, not all NRSF-regulated genes were upregulated by dominant-negative NRSF or G9a constructs (data not shown), suggesting that multiple mechanisms act to maintain silencing of neuronal genes by NRSF.

It has been reported that NRSF recruits the histone methylase SUV39H via a CoREST/MeCP2 complex to mediate long range (in excess of 23 megabases) silencing of a “genomic interval” (Lunyak et al., 2002). Lunyak et al. argue that three NRSF-containing genes control the expression of all the genes within a specific chromosomal 3 interval q22-q23 by imposing “silencing across a chromosomal interval, including transcriptional units that do not themselves contain REST/NRSF response elements.” We have been unable to demonstrate any interaction between NRSF and SUV39H. Lunyak et al. suggest that the predominantly heterochromatic SUV39H mediates methylation over the interval and show that the q22-q23 region is associated with dimethylated H3-K9. Our data suggest that NRSEs recruit the euchromatic G9a to form a localized zone of H3-K9 dimethylation no larger than 2–6 kb on either side of the NRSE. Also, it is not clear how the trimethylase SUV39H would generate dimethylated H3-K9 over this region. Indeed, two recent reports suggest that SUV39H regulates H3-K9 trimethylation at centromeric heterochromatin whereas G9a is responsible for regulating levels of euchromatic H3-K9 dimethylation.

During preparation of this manuscript it was reported that NRSF is required to repress expression of the Nall gene in JTC-19 cells but not the M4 gene due to the stable repression of M4 expression by “heterochromatic markers” such as methylated H3-K9 and HP1 isoforms (Belyaev et al., 2003). There is some concordance between our findings and those reported by Baleaev et al. in that dominant-negative NRSF induced Nall but not m4 expression (Figure 7B and data not shown). However, we find that the NRSEs of both the Nall and m4 gene are occupied by NRSF and that the NRSEs of both genes are associated with dimethylated H3-K9 (Figures 1A, 1B, 6A, 6B, and data not shown). We believe that their failure to detect H3-K9(Me) at the Nall locus and NRSF at the M4 locus is due to a lower signal to noise ratio in the ChIP protocol followed by Baleaev et al. Real-time PCR allowed us to determine that (1) NRSF is bound to the M4 NRSE but at one-third the occupancy of Nall (data not shown) and (2) the Nall NRSE is associated with dimethylated H3-K9 at approximately half the enrichment observed at the M4 element. The differences between NRSF and H3-K9(Me) occupancy between M4 and Nall are therefore quantitative and not qualitative.

In aggregate, our data presented here lead us to pro-
pose that neuronal genes regulated by NRSF are repressed in nonneuronal cells by highly localized histone deacetylation and H3-K9 dimethylation mediated by G9a and not SUV39H. The work presented here strongly argues that histone methylation by G9a is an important component of the molecular machinery utilized by NRSF to silence neuronal genes outside the nervous system and thus confer appropriate tissue type specificity of expression.

Experimental Procedures

Chromatin Immunoprecipitations

Cells were crosslinked in 1% formaldehyde in growth media for 15 min at 37°C, washed twice with cold PBS, and harvested into IP buffer (50 mM Tris 8.0, 0.3% Triton X-100, 150 mM NaCl, 10% glycerol, protease inhibitors). Cells were pelleted and resuspended in IP buffer (2 ml/10 cm dish), chromatin solubilized, and sheared by pulsed sonication (the bulk of the DNA was reduced to less than 700 bp) and clarified by centrifugation at 21,000 g for 30 min. Material equivalent to 2 x 10^6 cells was used per IP with 2 μg of purified IgG or 2 μl of crude serum. Immune complexes were harvested with blocked sepharose G beads. Complexes were rinsed once with 1 ml of TSE (20 mM Tris-HCl [pH 8.0], 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl), followed by 1 ml washes for 5 min while rotating at 4°C with TSE, TSE 500 mM NaCl, and four times with LiCl buffer (10 mM Tris-HCl [pH 8.0], 250 mM LiCl, 1% NP40, 1% deoxycholate, 1 mM EDTA). Complexes were decrosslinked and purified by organic extraction and isoopropanol precipitation. For real-time PCR, DNA was dissolved in 100 μl water and 4 μl used per reaction with Takara Ex Taq and SYBR-Green on a MJR Opticon II real-time machine. All ChIPs analyzed by real-time PCR were performed at least in duplicate, and the difference from the mean was less than 20%. For semiquantitative PCR using ethidium bromide staining, DNA was dissolved in 50 μl water and 10 μl used in a 50 μl PCR reaction. Five microelctors was performed at 25, 28, 31, and 34 cycles for visualization (for primer sequences and amplicon sizes see the Supplemental Data at http://www.molecule.org/cgi/content/full/14/6/727/DC1).

Antibodies, Immunoprecipitations, and GST Pull-Downs

IPs and GST pull-downs were performed as described on material extracted in IP buffer (Roopra et al., 2000). Low and high stringency washes were performed with PBS + 0.5% NP40 or RIPA buffer, respectively. The following antibodies were used for IPs, ChIPs, immunofluorescence, and Western blots: anti-H3-K9(Qac) and anti-H3-K9(Me3) from Upstate Biotech, anti-H3-K9(Me3) from Abcam, anti-H3, anti-NRFS(P18), anti-Gal4(sc-577), anti-MYC(E11), and anti-HA from Santa Cruz, anti-FLAG from Stratagene, anti-CoREST(BD) from BD Pharmingen. Anti-CoREST(Ct) (Humphrey et al., 2001) and anti-G9a(Shi et al., 2003) have been described.

Indirect Immunofluorescence and Confocal Microscopy

HEK cells were transfected with FLAG, HA, and Myc epitope-tagged expression constructs. Twenty-four hours posttransfection, cells were washed in PBS, fixed and treated as described (Grimes et al., 2000). Cells were viewed using a 60× objective with a Bio-Rad MRC-1024 Laser Scanning Confocal Microscope. Z sections were acquired at 0.5 microns and analyzed using Graphics Converter.

Histone Methylase Assays

Immune complexes or GST pull-downs were subjected to HMT assays as described (Nielsen et al., 2001).

Cell Line Maintenance and Transfections

HEK293 and JTC-19 cells were maintained in DMEM supplemented with 10% fetal calf serum, 2 mM glutamine, 10 g/l streptomycin, and penicillin. HEK cells were transfected using lipofectamine-2000 (Invitrogen) according to the manufacturer’s instructions. JTC-19 cells were transfected using transfx-50 (Promega). Where required, JTC-19 cells transfected with CD4 expression plasmids were magnetically sorted with anti-CD4 antibody conjugated to paramagnetic microbeads and sorted through magnetized iron columns using the MACSelect kit (Miltenyi Biotech), replated, and allowed to recover for 48 hr before harvesting for RNA analysis. Transient transfection of reporter assays were performed in 24-well dishes as described (Roopra et al., 2000).

Plasmids

PMT-gal4, pMT-gal4-NRSF(1-138) (Roopra et al., 2000), pMT-NRSF (Chen et al., 1998), Gal4-NRSF-[1008-1097] (Tapia-Ramirez et al., 1997), Gal4CoREST (Andres et al., 1999), MYC-H3-K9(Me3),δγ, (Lachner et al., 2001), HA-SUVA39H, and HA-SUV39H1SET (Nielsen et al., 2001) have been described elsewhere. FLAG-G9a expression plasmid and deletion constructs were generated by PCR of cDNA from HEK cells and cloning into pTA2G2a (Stratagene). Details are available upon request.

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