Heterogeneity in residual function of MeCP2 carrying missense mutations in the methyl CpG binding domain

S Kudo, Y Nomura, M Segawa, N Fujita, M Nakao, C Schanen, M Tamura

Rett syndrome is a neurodevelopmental disorder with severe mental retardation caused by mutations in the MECP2 gene. Missense mutations identified in patients were analysed by transient expression of the mutant proteins in cultured cells. The effects of mutations were evaluated by analysis of the affinity of MeCP2 to pericentric heterochromatin in mouse L929 cells and on transcriptional repressive activity of MeCP2 in Drosophila SL2 cells. These analyses showed that approximately one-third (9/28) of MBD missense mutations showed strong impairment of MeCP2 function. The mutation of the R111 residue, which directly interacts with the methyl group of methyl cytosine, completely abolished MeCP2 function and mutations affecting β-sheets and a hairpin loop have substantial functional consequences. In contrast, mutations that showed marginal or mild impairment of the function fell in unstructured regions with no DNA interaction. Since each of these mutations is known to be pathogenic, the mutations may indicate residues that are important for specific functions of MeCP2 in neurones.

MATERIALS AND METHODS

Cells and culture conditions

L929 cells (mouse fibroblast cell line, American Tissue Culture Collection) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mmol/l glutamine, 500 U of penicillin per ml, and 100 µg of streptomycin per ml in a humidified 5% CO2 atmosphere. The Drosophila Schneider cell line 2 (SL2) derived from Drosophila embryos (kindly provided by Dr R M Evans) was grown in Schneider’s Drosophila medium (Invitrogen) with the same supplements as described above.
Expression constructs

Expression plasmids encoding MeCP2 mutants bearing MBD missense mutations identified in patients were constructed as follows. MeCP2 mutant cDNAs were generated by site directed mutagenesis using PCR with mismatched primers and a full length MeCP2 cDNA template as described previously. Synthesised mutant DNA was cloned into the enhanced fluorescence vector pEGFP-C1 (Clontech) and the Drosophila expression vector pAc5.1/V5-His (Invitrogen). All constructs were verified by sequencing.

Heterochromatin affinity analysis

GFP fusion expression constructs were introduced into L929 cells using Superfect (Qiagen). Two days later, transfected cells were fixed with 3.7% formaldehyde for 10 minutes, permeabilised with 0.5% Triton X-100 for 20 minutes, and counterstained with 4',6-diamidino-2-phenylindole (DAPI) at a final concentration of 1 µg/ml. The cells were observed under an Olympus fluorescence microscope using the appropriate optical filter. Microscopic images were collected with a CCD camera and a Macintosh G4 computer, and imported into Adobe Photoshop 6.0 for figure presentation.

Luciferase assay

Transient expression analysis using Drosophila SL2 cells was performed as follows. The SNRPN-luciferase reporter construct (pGL-SNRPN) and the leukosialin-luciferase reporter construct (pGL-LS5) were treated with SspI (CpG) methylase (New England Biolabs) in the presence or absence of 5 mmol/l S-adenosylmethionine as described previously. A total of 1.4 x 10^5 SL2 cells were grown in 0.7 ml of Schneider’s Drosophila medium in a 24 well plate. A total of 0.4 µg of the luciferase reporter construct was cotransfected with 0.2 µg of an Sp1 expression plasmid, pPaCSp1, 0.01 µg of pAc5.1-pRL, and 0.1 µg of a Drosophila expression plasmid bearing genes encoding MeCP2 mutants into SL2 cells by the calcium phosphate transfection method.

Figure 1  Effects of MBD missense mutations on the affinity of MeCP2 to mouse pericentromeric heterochromatin. (A) Representative low magnified views of L929 cells transfected with wt or mutant expression plasmids. Localisations of GFP fusion proteins (left panel) were visualised by fluorescence microscopy and the nuclei were counterstained with DAPI (right panel). (B) Higher magnified views of fluorescent staining L929 cells showing the intranuclear localisations of GFP mutant proteins.
Functional analyses of MECP2 mutations

Table 1  Effects of missense mutations in the MBD on transcriptional repressive activity

<table>
<thead>
<tr>
<th>Mutation</th>
<th>pGL-SNFPN</th>
<th>pGL-L55</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Methylated</td>
<td>Unmethylated</td>
</tr>
<tr>
<td>Vector</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>wt</td>
<td>3.2 ± 0.3</td>
<td>56.1 ± 4.3</td>
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<tr>
<td>D97E</td>
<td>11.9 ± 2.4</td>
<td>85.3 ± 5.6</td>
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<tr>
<td>D97Y</td>
<td>15.1 ± 1.9</td>
<td>90.8 ± 10.5</td>
</tr>
<tr>
<td>L100V</td>
<td>3.8 ± 0.5</td>
<td>65.1 ± 3.2</td>
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<tr>
<td>R106Q</td>
<td>2.4 ± 0.2</td>
<td>25.8 ± 3.5</td>
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<tr>
<td>R101L</td>
<td>3.8 ± 0.5</td>
<td>58.7 ± 8.1</td>
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<tr>
<td>R101R</td>
<td>1.9 ± 0.2</td>
<td>39.3 ± 4.9</td>
</tr>
<tr>
<td>P101L</td>
<td>1.9 ± 0.3</td>
<td>28.4 ± 3.6</td>
</tr>
<tr>
<td>P101S</td>
<td>2.2 ± 0.1</td>
<td>29.2 ± 4.1</td>
</tr>
<tr>
<td>R106Q</td>
<td>57.7 ± 4.8</td>
<td>126.9 ± 9.6</td>
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<tr>
<td>R106W</td>
<td>61.2 ± 7.6</td>
<td>137.12 ± 12.5</td>
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<tr>
<td>R111G</td>
<td>127 ± 11.2</td>
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<tr>
<td>R120D</td>
<td>13.7 ± 2.3</td>
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<tr>
<td>L124F</td>
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<td>28.1 ± 2.1</td>
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<td>K135E</td>
<td>12.9 ± 1.8</td>
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<tr>
<td>E137Q</td>
<td>19.2 ± 2.5</td>
<td>96 ± 1.2</td>
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<td>A140V</td>
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<td>P152R</td>
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<td>F155S</td>
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<td>D155E</td>
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<td>D156G</td>
<td>37.1 ± 0.6</td>
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<td>T158A</td>
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<tr>
<td>T158M</td>
<td>17.3 ± 2.1</td>
<td>69.3 ± 4.1</td>
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</table>

*Relative transcriptional activities [%] compared with that of only pAc5.1/V5-His vector are presented.

RESULTS
Effect of MBD missense mutations on affinity for mouse heterochromatin

Accumulating data on MECP2 mutations indicate that missense mutations are present primarily in the MBD. To determine the functional significance of these mutations, we examined the effects of a total of 28 MBD missense mutations on heterochromatin affinity. This assay takes advantage of a unique feature of murine cells, which contain large foci of densely methylated pericentromeric constitutive heterochromatin within the nucleus that attract exogenously expressed MeCP2 protein. Notably, the E137G and A140V mutations indicate that the relative ability of the mutant protein into heterochromatin was minimally differing substitutions at D97, P101, R106, and D156 had more affected focal staining and the diffusion of the GFP signal throughout the nucleus. An intermediate pattern was observed in the Y120D, R133L, K135E, T158A, and T158M mutants, which showed focal staining co-localising with DAPI staining apparent against a background of diffuse nuclear GFP signal, indicating that their affinity for heterochromatin is partially retained. Among the latter mutations, T158A exhibited more distinct foci in the nucleus, whereas the other mutations exhibited faint foci throughout the stained nucleus. Missense mutations at R133 and F155 showed differential effects depending on the amino acid substitution. Substitution of arginine with leucine at residue 133 (R133L) substantially reduced focal staining while changes from arginine to cysteine (R133C) or histidine (R133H) did not visibly affect MeCP2 localisation. Similarly, a change from phenylalanine to serine (F155S) led to diffuse nuclear staining consistent with impaired heterochromatin binding by the mutant protein while the change from phenylalanine to isoleucine (F155I) at F155 did not impair heterochromatin binding. By contrast, differing substitutions at D97, P101, R106, and D156 had more consistent effects on heterochromatin binding patterns. Several mutant GFP fusion proteins accumulated in heterochromatic regions comparable to the wild type protein, producing distinct foci co-localising with strong DAPI staining. Five different mutations at residue P101 showed a clear focal staining in the nucleus, indicating that assembly of the mutant protein into heterochromatin was minimally affected by these mutations at this site. Similarly the L100V, P127L, R133C, R133H, S134C, E137G, A140V, P152R, and F155I mutant proteins produced heterochromatin patterns that were indistinguishable from the wild type protein. Notably, the E137G and A140V are mutations identified in male patients with X linked mental retardation, and...
our previous study indicated that these mutants retain considerable MeCP2 function.16

**Effect of MBD missense mutations on transcriptional repressive activity in Drosophila cells**

To determine the effects of MBD missense mutations on transcriptional repressive activity of MeCP2, we undertook transient expression analysis using *Drosophila* SL2 cells. *Drosophila* cells are suitable for such analysis because they have very low background levels of methyl CpG binding activity and low levels of DNA methyltransferase activity and genomic methylation.16 Our previous studies showing that MeCP2 expressed in SL2 cells substantially repressed Sp1 activated transcription from a methylated promoter indicated that transcriptional repressive activity of MeCP2 could be reconstituted in SL2 cells.17 In this study, we used two reporter constructs containing the luciferase reporter driven by the promoter of the human imprinted *SNRPN* gene (pGL-SNRPN) and the human leukosialin gene (pGL-L55). The SNRPN promoter provides high transcriptional activity and is highly sensitive to MeCP2 transcriptional repression.15 The leukosialin promoter provides high transcriptional activity but is less sensitive to MeCP2 transcriptional repression.15 *Drosophila* expression constructs encoding wild type or mutant MeCP2 were cotransfected into SL2 cells with the luciferase reporter plasmid and an Sp1 expression vector, as well as the pAc5.1-pRL control plasmid for normalisation. Exogenous expression of Sp1 is required for transcriptional activation of a reporter gene since SL2 cells lack Sp1.15 The effects of mutants on the pGL-SNRPN reporter plasmid are summarised in table 1. Wild type MeCP2 strongly repressed Sp1 activated transcription from the methylated SNRPN promoter. Transcriptional activity was reduced approximately 30-fold in assays using 0.1 µg of expression vector. Cells expressing the R106Q, R106W, R111G, F155S, D97E, and D156G mutant proteins, however, showed significant diminution of transcriptional repression, particularly for the R111G mutation, which completely abolished MeCP2 repressive activity on Sp1 activated transcription from both methylated and unmethylated promoters. Intermediate impairment of repressive activity was observed in D97E, D97Y, Y120D, L124F, R133L, K135E, E137G, and T158M mutations, which showed a 5 to 10 fold reduction of transcription from the methylated SNRPN promoter. On the other hand, each of the five P101 mutations had little effect on transcriptional repressive activity of MeCP2, exhibiting substantial reduction of Sp1 activated transcription at levels comparable to wild type protein. Similar and substantial levels of expression were observed in LI00V, P127L, R133C, R133H, S134C, A140V, P152R, F155I, and T158A mutants. When the leukosialin promoter was used as the reporter, the transcriptional repressive level of mutants was lower than with the SNRPN reporter, although similar effects on repressive activity were observed when the promoter was methylated (table 1). Interestingly, the P101H, P101S, P101T, P127L, R133C, R133H, S134C, A140V, P152R, F155I, and T158A mutants. When the leukosialin promoter was used as the reporter, the transcriptional repressive level of mutants was lower than with the SNRPN reporter, although similar effects on repressive activity were observed when the promoter was methylated (table 1). Interestingly, the P101H, P101S, P101T, P127L, R133C, R133H, S134C, A140V, P152R, F155I, and T158A mutants. When the leukosialin promoter was used as the reporter, the transcriptional repressive level of mutants was lower than with the SNRPN reporter, although similar effects on repressive activity were observed when the promoter was methylated (table 1). Interestingly, the P101H, P101S, P101T, P127L, R133C, R133H, S134C, A140V, P152R, F155I, and T158A mutants.
analyses using Drosophila cells and heterochromatin affinity analysis using the mouse cell system. Comparison of the results for both assays for each mutant form indicates that the results of these two methods are largely correlated.

In summary, we introduced a total of 28 mutations at 17 amino acid positions within the MBD. Nine mutations showed strong impairment of function in both assays. For two residues, the effect varied depending on the specific amino acid that was introduced, and at five positions the mutations had minimal to no effect on the function of the protein in these systems.

**DISCUSSION**

The function of MECP2 mutations was analysed by transiently expressing mouse L929 cells and Drosophila SL2 cells with mutant forms of MeCP2 to investigate the effects of mutations on the affinity to pericentromeric heterochromatin and transcriptional repressive activity, respectively. In this study, we also found that the effects of MBD missense mutations on heterochromatin affinity in mouse cells generally correlate with transcriptional repression of Sp1 activated transcription in Drosophila cells (table 2). The effects of these missense mutations can be addressed from a structure-function relationship using the model of the MBD developed by NMR solution analysis of human MB1 bound to methylated DNA.\(^1\)\(^\text{9}\)\(^\text{10}\) This model predicts a novel wedge shaped structure with four antiparallel \(\beta\)-sheets and a helical region. Since the solution structure of the MBD of MeCP2 is almost identical to that of MeCP2,\(^4\)\(^\text{9}\)\(^\text{10}\) we used structural data available for MB1 to infer the location of mutation sites in the secondary structure of the MBD of MeCP2.

The two methyl groups of a methylated CpG base pair are recognised by a hydrophobic surface comprising five residues: three of them (K109, R111, Y123) interact with one methyl group and two of them (R133, S134) interact with the other (fig 2). In this study, we found that R111G produced a complete loss of MeCP2 function. Heterochromatin affinity analysis showed diffuse staining throughout the nucleus with this mutant and transient expression of the mutant in mouse L929 cells produced complete loss of MeCP2 function. Heterochromatin affinity and transcriptional repression with methylated DNA bases as open boxes, phosphates as black circles. The methyl group of 5-methyl cytosine is shown as blue circles. Ionic interaction between residues of the MBD and DNA is indicated by arrows. Hydrophobic interactions are indicated by perpendicular lines, and hydrogen bonds are indicated by dashed lines. Residues seen in missense mutations found in Rett syndrome patients are shown as red letters.

![Mutations of methyl CpG recognition sites. Interaction of methylated DNA with amino acid residues of the MBD are schematically presented on the basis of the NMR analysis of the MBD.](image)

Mutations of residues K135, E137, A140, and T158, which are predicted to interact with the DNA sugar phosphate backbone from NMR studies of the MBD of MB1\(^1\)\(^\text{9}\)\(^\text{10}\) (fig 2), show subtle to intermediate impairment of MBD function. The A140V mutation retained MBD function almost equivalent to wild type, although higher transcriptional repressive activity of Sp1 activated transcription from an unmethylated promoter was observed. Other mutations, K135E, E137G, T158A, and T158M, exhibited intermediate impairment in at least one of the two functional assays. Our functional analyses indicated that T158M had intermediate affinity to heterochromatin and moderate effects on transcriptional repressive activity. These results are consistent with reports that the T158M mutant exhibited only slightly lower binding affinity for methylated DNA compared with that of the wild type protein.\(^1\)\(^\text{9}\)\(^\text{10}\)

Missense mutations in residues found in \(\beta\)-sheets and a hairpin loop strongly affect MBD function. D97E and D97Y in the \(\beta\)-sheet show intermediate impairment of the transcriptional repressive activity and strong impairment of heterochromatin affinity. R106Q and R106W in the \(\beta\)-sheet strongly impair MBD function. Previous NMR structural analysis showed that the R106W mutation leads to misfolding of MBD.\(^1\)\(^\text{9}\)\(^\text{10}\) Furthermore, Y120D and L124F mutations in the \(\beta\)-sheet show intermediate and strong impairment of the MBD function, respectively. Other mutations at sites of residues F155, D156 and T158 in a hairpin loop exhibit variable functional effects. In particular, substitution of phenylalanine with isoleucine at amino acid 155 (F155I) showed a subtle effect, whereas the substitution with serine (F155S) at the same site showed substantial loss of function. A previous structural study also showed that the F155S mutation disrupted proper MBD folding.\(^1\)\(^\text{9}\)\(^\text{10}\) Missense mutations of the D156 residue, which is predicted to form a negatively
charged surface opposite to the DNA interface,11 showed substantial effects on function, possibly resulting from a conformational change in the MBD.

Curiously, several mutant forms, including substitutions of L100, P101, P127, and P152, retained the capacity to bind heterochromatin and repress transcription in these assays. Notably each of these mutations involved residues that are outside the β/α strands and loop structures that are critical for DNA binding. Nonetheless, the retention of function in these assays appears to contradict the fact that they are known to be pathological mutations in humans and underlines the difficulty in developing model systems. In this case, the apparent paradox may result from differences in the chromatin state due to different replicative activity in 1929 cells and postmitotic neurons, both in terms of histone composition and modification (for a recent review, see Ahmad and Henikoff12). It is becoming increasingly evident that histone modifications play critical roles in the transcriptional competency of genomic DNA and, as such, it is likely that MeCP2 binding is directly or indirectly affected by the nucleosomal context of the methylated CpG dinucleotide. These residues may be important for conferring specificity of interaction between MeCP2 and methylated DNA associated with nucleosomal proteins bearing specific modifications, which would not have been discerned by these studies. Interestingly, mutations such as P101H, P101S, P101T, P127L, R133C, R133H, S134C, and A140V exhibited higher transcriptional repressive activities on Sp1 activated transcription from the unmethylated SNRPN promoter compared with that of the wild type protein. These effects may depend on the context of the genes since such effects were not observed using the unmethylated leukosialin promoter reporter. It is possible that higher affinity binding of mutants to unmethylated genes leads to the aberrant gene expression by binding of the mutant protein to inappropriate unmethylated targets.

It is difficult to compare the level of residual function of MeCP2 in these in vitro analyses with phenotypes reported to be associated with specific mutations, in part because the phenotypic outcome is heavily influenced by X chromosome inactivation patterns. In addition, postnatal environment appears to affect the phenotypes of patients even if they have the same mutation. Mutations that showed intermediate to severe impairment have been described in females presenting with classical and atypical Rett syndrome and males presenting with the neonatal onset encephalopathy. For example, a recent report described the T158M mutation in brothers of a patient with classical Rett syndrome.23 One brother with this mutation died within a year of birth with severe encephalopathy. The milder presentations that have been reported including females with atypical Rett syndrome, such as the preserved speech variant, or other forms of X linked mental retardation in males have been associated with mutations that showed preservation of function in these assays. This is illustrated by the R133C and S134C mutations, which displayed preserved heterochromatin binding and transcriptional repressive activities in these assays, and are often associated with relatively mild clinical features in the patients, with no manifestations maintaining the ability to speak.24 The T158M mutation exhibited near normal MBD function in our assays, but this mutation was also identified in a male patient with classical Rett syndrome.24 Since it was reported that this male patient had somatic mosaicism, the influence of this mutation is much stronger than those of A140V and E137G mutations, which were identified in males with mental retardation and showed near normal MBD function.17 This correlation is not straightforward, however, since mutations showing near normal function can also cause typical Rett syndrome. Therefore, it is conceivable that the gene expression is tightly regulated in the neuronal cells and even a subtle change of the gene regulation is observed in transcriptional regulatory potentials of some MBD missense mutants might bring about a significant influence on the neuronal function. Although many factors, such as other methyl CpG binding proteins, skewed X chromosome inactivation, and postnatal environment could be involved in the establishment of patient manifestations, our data provide the basis for understanding the genotype-phenotype correlation of MeCP2 related mental retardation.

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References


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