A Novel Protein, *Xenopus* p20, Influences the Stability of MeCP2 through Direct Interaction*

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MeCP2 is the founder member of a family of methyl-CpG-binding proteins able to repress transcription from methylated DNA. To date, MeCP2 action seems to involve the delivery on modified DNA of histone deacetylase activity, followed by histone methylating activity. It has been recently demonstrated that MECP2 mutations cause Rett syndrome, a childhood neurological disorder that represents one of the most common causes of mental retardation in females. Here we show that a novel *Xenopus laevis* protein of 20 kDa, p20, is able to interact in vivo and in vitro with MeCP2. The p20 sequence revealed that it belongs to the family of the WAP (whey acidic protein) proteins, often functioning as a protease inhibitor. Therefore, we asked whether the p20 can influence the MeCP2 half-life. We demonstrate that, indeed, the xp20 not only can significantly increase the stability of an exogenously expressed MeCP2 in *Xenopus* oocytes but also can stabilize the human endogenous MeCP2. The capability of the mammalian methyl-CpG-binding protein to interact with p20 is confirmed by co-immunoprecipitation experiments performed overexpressing the WAP protein.

Glutathione S-transferase pull-down assays reveal that the MeCP2 residues localized between the methyl-binding domain and the transcriptional repression domain is the primary interaction surface. Our data suggest that regulation of MeCP2 metabolism might be of relevant importance; in accordance with this, previous results have shown that some Rett syndrome mutations are characterized by a decrease in MeCP2 stability.

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DNA methylation at position 5 of cytosine in CpG dinucleotides is the major modification of eukaryotic genomes and is essential for normal mammalian development (1, 2). Accordingly, this epigenetic modification is implicated in tissue-specific gene transcription, X chromosome inactivation, genomic imprinting, senescence, and carcinogenesis (Refs. 3–6 and references therein). DNA methylation is interpreted by specific protein factors that, containing a highly conserved methyl-CpG-binding domain (MBD), specifically bind modified DNA (7). MeCP2 was the first member of this family to be characterized (8). This protein consists of a single polypeptide that contains an MBD and a transcriptional repression domain (TRD) that is able to abrogate gene expression when tethered to DNA (9, 10). Moreover, a new structural domain, common to other regulatory genes significantly expressed in the brain, has recently been identified in the C-terminal part of the protein (11), and the last 63 amino acids have been reported to facilitate binding of MeCP2 to both naked and nucleosomal DNA (12). MeCP2 binds specifically to 5-methyl-cytosine through its MBD, and the TRD recruits a transcriptional silencing complex resulting in chromatin condensation. In particular, MeCP2 interacts with the co-repressor Sin3A to recruit histone deacetylase and 2, which in turn results in deacetylation of core histones and transcriptional silencing (13, 14). Consistent with this model, trichostatin A, a specific inhibitor of histone deacetylases, partially relieves the transcriptional inhibition conferred by MeCP2. This partial relief indicates that additional mechanisms of repression, other than histone deacetylation, contribute to gene silencing mediated by MeCP2. In accordance with this, it has recently been demonstrated that MeCP2 associates with histone methyltransferase activity in vitro and in vivo; moreover, the methyl-binding protein facilitates H3 Lys8 methylation of a *bona fide* MeCP2-regulated gene (15).

An indication of the role of MeCP2 in human development has been provided by the discovery that mutations in the MECP2 gene cause Rett syndrome, a common childhood onset progressive neuro-developmental disorder that causes autism, dementia, ataxia, and loss of speech and hand movements (16–18). Mutations in MECP2 have been identified in 70–90% of sporadic Rett cases and almost 50% of familial cases; these include missense mutations identified in the MBD, in the TRD, or in the C-terminal part of the protein and nonsense and frameshift mutations, affecting for the vast majority the TRD and C terminus (19, 20). It has been demonstrated that many missense mutations within the MBD significantly reduce the affinity of MeCP2 for methylated DNA, whereas proteins truncated within the TRD are impaired in their ability to repress transcription. Importantly, deletions within the C terminus of MeCP2, which are common mutations in classical RTT, significantly decrease protein stability (21–23).

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A Novel Xenopus Protein Stabilizes MeCP2

Considering that the identification of additional MeCP2 interacting factors might help to understand the basis for its selective function, we have made use of conventional biochemical techniques to isolate novel partners. Here, we show that Xenopus laevis MeCP2 associates with a novel 20-kDa protein (p20) both in vitro and in vivo. The analysis of the p20 amino acid sequence revealed that this protein harbors domains involved in the inhibition of specific proteases. By means of a stability assay performed in microinjected Xenopus oocytes, we demonstrate that the MeCP2 turnover is significantly reduced in the presence of p20. More importantly, the p20 interacts with the human methyl-binding protein and stabilizes endogenous MeCP2 in human cultured cells.

**Experimental Procedures**

**Cloning of the Xenopus p20 cDNA**

Total RNA was extracted from Xenopus oviducts with EUROzol (Euroclone) according to the manufacturer’s instructions. 5’ and 3’ RACE were performed with GeneRacer (Invitrogen) according to the provided protocols. The following gene specific oligonucleotides were used for the 5’ RACE, the 3’ p20, and the 3’ RACE the 5’ p20 (ATGTCGCAACTGTGGTCATGTT- CCTCCCTCCTCTG). The obtained cDNAs were cloned into pCR 2.1 (TA Cloning Kit; Invitrogen) and sequenced. The cDNA sequence of Xenopus p20 was deposited in GenBank with the accession number AT563621.

**Plasmid Construction**

Xenopus Expression Vectors—pSPFLAGp20 contains the cDNA corresponding to the purified, sequenced p20 in frame with an N-terminal FLAG epitope. The FLAG-p20 fusion was generated by PCR using the following primers: 5’ p20-NcoI, GCCCTGCACTGGCGCTGATTCTC- CAGTGAGATATCATGATGTTTGCCCC; 3’ p20-BamHI, GCCCTGGG- ATTCAGATATCATGATGTTTGCCCC. The amplified product was cloned into NcoI-BamHI-digested pSPUTK (Stratagene). A DNA sequence coding for the FLAG epitope was obtained by annealing two complementary oligonucleotides was inserted in between the BgIII and the NcoI sites of the pSPUTK vector. Because of the cloning procedure, the alanine codon corresponding to the 21 residue of the full-length cDNA was maintained into the construct. To express xMeCP2 in Xenopus oocytes, the full-length cDNA (14) was cloned into pSP64polyA (Promega; pSPxMeCP2) Smal-digested.

Bacterial Expression Vectors—The GST-MeCP2 (Xenopus) construct has previously been described (12). The p20-NcoI/BamHI amplification product was subcloned into the pGEX-4T-1 vector (Clontech) pGST-p20 was obtained by inserting an NcoI-digested Sall fragment from pGBK7T-p20 into pGEX-4T-3 (Amersham Biosciences) digested with BamHI/Sall. pGST-p20 was digested with HindIII and SalI and cloned into pGEM-T (Stratagene). For coupled in vitro transcription/translation reactions, hMeCP2 (a kind gift of Dr. Yasufaki) was subcloned by PCR into pGEX-4T-1.

Eukaryotic Expression Vectors—pHA-p20 was cloned by inserting a BamHI-digested PCR fragment, obtained by PCR using the 5’ and 3’ p20-BamHI (GATCCGAGATCCACTGGCTCTTCCGATGGAGATGATGATGACCATGCG) and the 3’-p20-BamHI primers, in a frame with an N-terminal HA tag in pSG5 (Stratagene). For coupled in vitro transcription/translation reactions, hMeCP2 (a kind gift of Dr. Yasufaki) was subcloned by PCR into pGEX-4T-1.

**Purification of p20 from X. laevis Oviduct Extracts**

Dissected oviducts, washed extensively with PBS, were homogenized in 5 volumes of extraction buffer (50 mM NaCl, 20 mM Hepes, 3 mM β-mercaptoethanol) containing protease inhibitor mixture (Sigma) and cleared by centrifugation for 2 h at 4 °C at 60,000 rpm (Beckman TL100.4). Approximately 10 mg of extract were loaded on a 5-ml HiTrap chelating column (Amersham Biosciences) charged with Ni²⁺ according to the manufacturer’s instructions. After extensive washes with extraction buffer containing 5 mM imidazole, elutions were performed with 10, 60, and 200 mM imidazole. The fractions containing the peak of MeCP2 were pooled, dialyzed against extraction buffer containing 1 mM EDTA and passed over a GST column prepared by covalently linking GST to a HiTrap affinity column (Amersham Biosciences) according to the manufacturer’s instructions. The flow-through was finally applied to a GST-MeCP2 column prepared as above. After extensive washes with extraction buffer, elutions were performed with 400 mM and 1 M NaCl. The collected fractions from the described columns were subjected to SDS-PAGE and analyzed by silver staining and Western blotting.

**Protein Sequencing and Sequence Analysis**

The peptide sequence of p20 was obtained by N-terminal microsequencing and mass spectrometry. For microsequencing, purified p20 was resolved by SDS-PAGE and electroblotted onto a ProBlott membrane (Applied Biosystems) according to the manufacturer’s instructions. Coomassie-stained membrane was submitted to Edman sequencing (Primm Laboratory). For mass spectrometry, the band of interest was excised, and the protein was digested in gel with trypsin in a buffer containing 33% of H₃[15O]O to label the C-terminal part of the tryptic peptides. The resulting peptides mixture was desalted on a microcolumn manually packed with 200 ml of POROS R2 material (PerSeptive Biosystems) and eluted directly into a nanoelectrospray needle (24). Tandem mass spectrometry experiments were performed on a QSTAR Pulsar quadrupole time-of-flight mass spectrometer (MDS Sciex, Toronto, Canada). Multiply charged peptides were fragmented to deduce the amino acidic sequences using the differential scanning technique (25).

**Fractionated Xenopus Oocyte Extracts**

1.5 grams of dissected oviducts extensively washed with PBS were homogenized in 3 ml of buffer A (10 mM Hepes, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, protease inhibitor mixture) with 0.34 M saccharose. The homogenate was filtered, and the volume was brought to 8 ml with buffer A and centrifuged at 15,000 rpm for 25 min at 4 °C (Beckman SW55Ti). The supernatant was kept as the cytoplasmic fraction. The nuclei were resuspended in 3 ml of buffer A with 2.1 M saccharose, centrifuged through another saccharose cushion (0.25 ml of buffer A with 2.1 M saccharose) at 15,000 rpm for 50 min at 4 °C (Beckman SW55Ti), and washed twice in buffer A with 0.34 M saccharose and finally sonicated in buffer A. The total cell extract was prepared by sonicating homogenized oviducts in buffer A. All three extracts were cleared by centrifugation at 80,000 rpm for 50 min at 4 °C.

**Antibodies**

Polyclonal MeCP2 as described by Jones et al. (14); monoclonal anti-HA was kindly provided by Muzi Falconi. Monoclonal anti-FLAG (M2) was from Sigma. A polyclonal serum against NP95, the murine orthologue of ICBP90, was a kind gift of Ian Marc Bonapace.

**Far Western**

Proteins separated by SDS-PAGE and transferred to nitrocellulose membranes (Hybond; Amersham Biosciences) were renatured by incubation at 4 °C overnight in renaturation buffer (50 mM Tris-HCl, pH 7.5, 100 mM KCl, 5 mM MgCl₂, 0.5% Triton X-100, 10% glycerol) and probed with 3 μg of recombinant GST-MeCP2 in renaturation buffer. MeCP2 was detected with anti-MeCP2 antibodies.

**Microinjection of Xenopus Oocytes and MeCP2 Degradation**

**Assay in Oocytes**

Xenopus oocytes were prepared and injected as previously described (26). FLAG-p20 and MeCP2 mRNAs were obtained by in vitro transcription of linearized pSPFLAGp20 and pSPxMeCP2 (EcoRI and SalI, respectively) using the SP6 mMessageMachine kit (Ambion) according to the manufacturer’s instructions. Respectively 1 and 0.5 ng of MeCP2 and p20 mRNAs were injected into oocytes, and protein synthesis was allowed for 16 h. Cyclohexamide (Sigma; 100 μg/ml) was added, and extracts from eight oocytes were prepared for each time point by homogenizing in oocyte buffer (20 μl/oocytes; 20 μl Hepes, pH 7.5, 70 mM KCl, 1 mM dithiothreitol, 12% glycerol) containing a protease inhibitor mixture (Sigma). The extracts were cleared by centrifugation for 5 min at 14,000 rpm at 4 °C. One oocyte equivalent was loaded in each lane for further analysis. Synthesis of overexpressed proteins was monitored by incubating injected oocytes in [35S]methionine/cysteine (ICN Translabel; 30 μCi/100 μl of buffer) for 16 h.

**Cell Culture, Transfections, Cyclohexamide Treatment, and Extract Preparation**

PC3 (human metastatic prostate carcinoma cells isolated from bone) and Phoenix cells (derived from human embryonic kidney 293 cells) were grown in Dulbecco’s modified Eagle’s medium supplemented with...
10% fetal calf serum and antibiotics. Transfections were performed with FuGENE (Roche Applied Science) according to the manufacturer’s instructions (PC3) or with CaPO4 precipitation (Phoenix). Cyclohexamide (10 \( \mu \text{g/ml} \)) was added 24 h post-transfection, and extracts were prepared at different time points. The cells were lysed by freeze-and-thaw; the proteins were extracted with 10 mM Hepes, pH 7.9, 800 mM NaCl, 0.1 mM EGTA, pH 8.0, 0.5 mM dithiothreitol, 5% glycerol, 1 mM PMSF, protease inhibitors (Sigma); and the extract was cleared by centrifugation at 34,000 rpm at 4°C for 30 min. 50 \( \mu \text{g} \) of extract were loaded in each lane.

Co-immunoprecipitation Experiments

**Xenopus Oviduct**—1 ml of extract (2 mg) was incubated with GammaBindTMG-SepharoseTM (Amersham Biosciences) for 1 h, after which the cleared extracts were incubated with anti-MeCP2 antibodies or preimmune sera covalently linked to GammaBindTMG-SepharoseTM and incubated for 2 h at 4°C. The washes were performed with 20 mM Hepes, pH 7.5, 100 mM NaCl, 0.1 mM EGTA, pH 8.0, 0.5 mM dithiothreitol, 5% glycerol, 1 mM PMSF, protease inhibitors (Sigma); and the extract was cleared by centrifugation at 34,000 rpm at 4°C for 30 min 50 \( \mu \text{g} \) of extract were loaded in each lane.

**Human Cells**—1 mg of extract prepared from Phoenix cells overexpressing HA-p20 was brought to 1 ml with 50 mM Tris, pH 7.5, 50 mM NaCl (final concentration, 150 mM), 1 mM PMSF, and protease inhibitors. The extract was cleared with GammaBindTMG-SepharoseTM and incubated with or without monoclonal anti-HA antibody for 2 h at 4°C before the addition of GammaBindTMG-SepharoseTM. The washes were performed with 150 mM NaCl, 50 mM Tris, pH 7.5, Nonidet P-40 0.1%.

**GST Pull-down and GST-p20 Pull-out**

GST and GST fusion proteins were purified from DH5α cells using glutathione-Sepharose 4B (Amersham Biosciences) according to the instructions (PC3) or with CaPO4 precipitation (Phoenix). Cyclohexamide (10 \( \mu \text{g/ml} \)) was added 24 h post-transfection, and extracts were prepared at different time points. The cells were lysed by freeze-and-thaw; the proteins were extracted with 10 mM Hepes, pH 7.9, 800 mM NaCl, 0.1 mM EGTA, pH 8.0, 0.5 mM dithiothreitol, 5% glycerol, 1 mM PMSF, protease inhibitors (Sigma); and the extract was cleared by centrifugation at 34,000 rpm at 4°C for 30 min 50 \( \mu \text{g} \) of extract were loaded in each lane.

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**Human Cells**—1 mg of extract prepared from Phoenix cells overexpressing HA-p20 was brought to 1 ml with 50 mM Tris, pH 7.5, 50 mM NaCl (final concentration, 150 mM), 1 mM PMSF, and protease inhibitors. The extract was cleared with GammaBindTMG-SepharoseTM and incubated with or without monoclonal anti-HA antibody for 2 h at 4°C before the addition of GammaBindTMG-SepharoseTM. The washes were performed with 150 mM NaCl, 50 mM Tris, pH 7.5, Nonidet P-40 0.1%.

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manufacturer’s instructions. Immobilized GST proteins (−2 μm) were incubated with 15–20 μl of in vitro translated 35S-labeled MeCP2 derivatives for 2 h in 1× PBS, 1 mm PMSF. The washes were performed with PBS, 1 mm PMSF, and 0.1% Nonidet P-40. The retained proteins were resolved by SDS-PAGE and detected by autoradiography. In vitro translated proteins were made using the TNT T7 Quick for PCR DNA and TNT T7 coupled reticulocyte lysate system (Promega) using hMeCP2 as template. For pull-out, GST and GST-p20 proteins bound to the resin were incubated with 600 μg of Xenopus oviduct extract adjusted to 150 mm NaCl in a final volume of 1 ml for 3 h at 4 °C. The washes were performed three times with PBS and 0.05% Nonidet P-40, and the retained proteins were eluted with PBS containing 600 mm NaCl. Eluted proteins and retained GST and GST-p20 were resolved by SDS-PAGE and detected with anti-MeCP2 and anti-GST antibodies, respectively.

**RESULTS**

To identify proteins that, by interacting with MeCP2, could be involved in regulating its activity, we set up a biochemical purification protocol as shown schematically in Fig. 1A. In this approach, we exploited the natural stretch of 13 histidines...
recombinant GST-p20, or GST, was incubated with 600 p20 cDNA interacts with endogenous MeCP2, which was purified by nickel chelating chromatography performed at high ionic strength. Subsequently, the eluted fraction was bound to a GST-MeCP2 affinity column. To further purify MeCP2 interactors, we used a GST-MeCP2 affinity column. Immunoblotting experiments revealed the presence of MeCP2 in the oviduct extract and analyzed the co-purifying proteins. The major polypeptides, named according to their apparent molecular weight, were present in the eluate: p18, p20, and p42. These proteins were also present in the 60 mM imidazole fraction but were absent in the 400 mM NaCl fraction. By microsequencing of the isolated p20 polypeptide, we obtained five peptides. These results are stable. MeCP2 and FLAG-p20 were detected with anti-MeCP2 antibodies in the oviduct extract and no proteins in the eluted fraction. The antibody mainly detects the p42, and a doublet of p18 and p20 also, besides MeCP2. The p42, p20, and p18 are enriched in the eluted fraction. This supports the idea that the three proteins are direct MeCP2 interactors. To demonstrate that the identified proteins are indeed interacting in vivo, we set up a co-immunoprecipitation assay performed with the endogenous proteins. To this purpose, we immunoprecipitated endogenous MeCP2 from the oviduct extract and analyzed the co-purifying proteins by silver staining. The major difference when comparing the immunoprecipitation with the anti-MeCP2 antibody (lane 2) and a preimmune serum (lane 1) is the presence of a protein migrating like the p18/p20 proteins. This protein is present in the C-terminal part of xMeCP2, allowing us to perform an affinity purification of the endogenous protein from an oviduct extract in which MeCP2 is abundantly expressed compared with other tissues (data not shown). As illustrated in Fig. 1A, the oviduct extract was loaded on a nickel chelating column, and the bound proteins were eluted with increasing concentrations of imidazole. Western blot analysis of the eluted fractions revealed that the majority of MeCP2 eluted with 60 mM imidazole (Fig. 1B, lower panel). To further purify MeCP2 interactors, we used a GST-MeCP2 affinity column (Fig. 1A). Briefly, the 60 mM imidazole fraction was first passed on a GST-Sepharose column to eliminate proteins specifically interacting with the GST tag. The flow-through was subsequently loaded on a GST-MeCP2 column. MeCP2-interacting proteins obtained from the previous chromatographic steps were expected to exchange with the immobilized MeCP2 protein. MeCP2-associated proteins were finally eluted with increasing NaCl concentrations. Fig. 1C shows the profile of the proteins eluting from the GST-MeCP2 column at 400 mM NaCl. Immunoblotting experiments revealed the presence of MeCP2 in the input and flow-through fractions but not in the 400 mM NaCl fractions (Fig. 1C, lower panel). Three major polypeptides, named according to their apparent molecular weight, were present in the eluate: p18, p20, and p42. These proteins were also present in the 60 mM imidazole fractions but were evidently further purified by the GST-MeCP2 affinity column. By nickel chelating chromatography performed at high ionic strength (600 mM NaCl), we were able to demonstrate that the identified proteins were retained on the column because of protein-protein interactions and not because of their harboring a histidine tag (data not shown). To unambiguously confirm that the eluted polypeptides were indeed MeCP2-associated proteins, we wanted to demonstrate the interaction by alternative assays. To this end we performed a Far Western experiment (Fig. 2A) in which proteins in the oviduct extract and the 400 mM NaCl fraction (Fig. 1C) were separated by SDS-PAGE, renatured after their transfer to a nitrocellulose membrane, and probed with recombinant MeCP2. A direct interaction with MeCP2 was finally revealed by immunoblotting using anti-MeCP2 antibodies. As seen in the control Western blot in Fig. 2A, the antibody recognizes specifically MeCP2 within the oviduct extract and no proteins in the eluted fraction. On the contrary, several polypeptides are evident in the Far Western experiment; in the oviduct extract (lane 2) the antibody detects the p42, and a doublet of p18 and p20 also, besides MeCP2. The p42, p20, and p18 are enriched in the eluted fraction (lane 4). This experiment thus reveals that, in vivo, p18/p20 proteins are involved in protein-protein interactions with MeCP2. Therefore, we decided to scale up the purification procedure, which lead to the isolation of the p20 polypeptide. By microsequencing of the isolated p20 polypeptide, we obtained five...
internal peptide sequences and the N-terminal peptide (Fig. 3A), which were used to perform a database search for the corresponding cDNA. A Xenopus expressed sequence tag showing high homology with the sequenced peptides was found (accession number GW633466). The nucleotide sequence corresponding to the peptide underlined in Fig. 3A was used to perform a 5’ RACE from oviduct RNA leading to the identification of the start codon and a short 5’ untranslated region. To perform a 5’ analysis, we used HA antibodies (HA-p20 and endogenous MeCP2 detected with anti-MeCP2 and anti-ICBP90 as indicated). The graph represents the mean values of MeCP2 (solid line) and ICBP90 (dotted line) levels in the absence (filled symbols) or presence of HA-p20 (empty symbols) from three independent experiments. + and − to the right indicate the presence and absence, respectively, of HA-p20. C, Xenopus p20 and human MeCP2 interact in vivo. HA-p20 overexpressed in human Phoenix cells was immunoprecipitated with (lane 3) or without (lane 2) anti-HA antibodies and retained HA-p20 and endogenous MeCP2 detected with anti-MeCP2 and anti-HA antibodies (upper and lower panels, respectively). Input (lane 1) corresponds to 10% of total protein.

An analysis of the p20 sequence with the Prosite database revealed the presence of two WAP (whey acidic protein) domains within the protein (Fig. 3C). These are characterized by a disulfide-linked structure called four-disulfide core or WAP motif and are often found in secreted proteins with anti-proteolytic activity (27, 28). A putative third WAP domain is located in the C-terminal part of the protein. Moreover, database searches identified: 1) a sequence showing some homology to leader peptides involved in protein secretion (amino acids 1–21); 2) a putative cleavage site between amino acids 21–22; and 3) a nuclear localization signal in the C-terminal part of the protein.

Even though the sequenced p20 protein was obtained from extensively washed oviducts making unlikely the possibility of purifying a secreted protein, we wanted to confirm the intracellular presence of p20. A co-immunoprecipitation experiment (data not shown) and a Far Western experiment were performed on fractionated oviduct extracts, and as shown in Fig. 3D, we detected significant quantities of p20 in the nucleus without any detectable signal in the cytoplasm. This result rules out the possibility of the identified factor being exclusively secreted and shows a high abundance in the same cell compartment where MeCP2 is localized.

Because the purified p20 was devoid of the 21 N-terminal amino acids, we performed all of the following experiments with a cDNA encoding a p20 identical to the sequenced one. To confirm that this cDNA codes for a protein with the same molecular weight as that of the purified p20, we carried out a Far Western experiment in which recombinant GST-MeCP2 was hybridized to the purified p20 (400 nm NaCl elution fraction; Fig. 1C) and to in vitro translated p20. As shown in Fig. 4A (lanes 1 and 3), the purified p20 co-migrates with the in vitro translated protein. With the purpose of further confirming that the p20 cDNA encoded the original MeCP2 interacting protein, we analyzed whether resin-coupled, recombinant GST-p20 was able to pull out MeCP2 from a Xenopus oviduct extract. Fig. 4B shows that indeed endogenous MeCP2 interacted with the immobilized p20, whereas the GST alone did not retain MeCP2 (compare lanes 2 and 3). Furthermore, MeCP2 interaction of the cloned p20 was verified by a classical GST pull-down experiment, in which a GST-p20 fusion protein, expressed in Escherichia coli, was immobilized on a glutathione-Sepharose resin and challenged with xMeCP2 translated in vitro. As shown in Fig. 4C, MeCP2 is retained on the GST-p20 resin, whereas no MeCP2 is seen on the GST resin. Based on these three different interaction assays, we could thus confirm that the cloned cDNA encodes a Xenopus p20 protein, which is able to establish direct protein-protein interactions with MeCP2.

Because, as previously mentioned, p20 contains WAP domains often associated with anti-proteolytic activity, we hypothesized a role of p20 in protecting MeCP2 against degradation. To this end we analyzed the turnover of exogenous MeCP2 in Xenopus oocytes in the absence or presence of FLAG-p20 by performing a stability assay as described by Yusufzai and Wolfe (21). As shown in Fig. 5A, the oocytes were microinjected with Xenopus MeCP2 mRNA, with or without FLAG-p20
mRNA, and after 16 h, allowing protein translation, protein synthesis was blocked by the addition of cyclohexamide. At the given time points, the oocytes were used for protein extraction, and the amount of MeCP2 was analyzed by Western blotting. As seen in Fig. 5A, in the absence of p20, the quantity of MeCP2 is slightly reduced 3.5 h after block of protein synthesis and is significantly further reduced at 9 h. On the contrary, in the presence of FLAG-p20, MeCP2 levels are fully maintained at 9 h after cyclohexamide treatment, suggesting that p20 is involved in stabilizing MeCP2. In a parallel experiment we evaluated the ratio of the exogenously expressed proteins by incubating injected and noninjected oocytes with 35S-labeled hMeCP2 was incubated with immobilized recombinant GST and GST-p20 derivatives. Retained MeCP2 is visualized in the autoradiogram (upper panel), whereas resin coupled GST and GST-p20 derivatives were detected by Coomassie staining. Input corresponds to 10% of total protein. The asterisks indicate prematurely terminated translation products.

To understand which regions of MeCP2 are engaged in the interaction with p20, we performed classical GST pull-down experiments in which a GST-p20 fusion protein, expressed in E. coli, was immobilized on a glutathione-Sepharose resin and challenged with hMeCP2 and its mutated derivatives (schematically illustrated in Fig. 7, left part). From the deletion analysis it appears that the linker region, containing amino acids 162–201 and separating the MBD and TRD, is required for interaction with p20. The main body of evidence for this is the fact that neither the MBD nor the TRD alone (78–161 and 202–310, respectively) interact with p20, whereas in the presence of the linker each of them is retained on the GST-p20 resin (78–201 and 162–310, respectively). To analyze whether one specific domain within p20 is required for association with MeCP2, we generated fusion proteins containing GST and p20 derivatives lacking each of the three WAP domains (shown schematically in Fig. 8A) and tested them for their ability to pull down hMeCP2. The autoradiogram in Fig. 8B shows that when either of the WAP domains within p20 was deleted, MeCP2 interaction was impaired. The Coomassie staining in Fig. 8B reveals that the resins contain equal amounts of GST.
fusion proteins. We believe that the lack of binding of the p20 derivatives carrying the deletions might be due to a general disruption of the p20 structure or alternatively to the requirement of each WAP domain for the MeCP2 interaction.

**DISCUSSION**

MeCP2, an abundant and ubiquitously expressed transcriptional repressor, is the first reported member of a family of proteins able to specifically recognize methylated CpGs (8). This protein can bind to a single methylated dinucleotide through an N-terminal methyl-CpG-binding domain of 85 amino acids (10) and silences gene expression via a transcriptional repression domain that abrogates transcription when tethered to DNA (9, 10). Because the TRD has been shown to interact with various co-repressor complexes containing histone deacetylase activities (13, 14, 29, 30), it has been suggested that MeCP2 in vivo represses transcription through chromatin modification. In accordance with this, it has recently been demonstrated that MeCP2 also associates with histone H3 methyltransferase activity. Both the MBD and the TRD contribute to the binding of this enzymatic activity (15). However, the capability of the TRD to interact with TFIIIB indicates that MeCP2 may also inhibit gene expression through mechanisms independent of chromatin remodeling (31). Other MeCP2 domains have been described; importantly the last 63 amino acids have been shown to facilitate DNA binding and are also involved in influencing MeCP2 stability (12, 21). Moreover, a new structural domain common with two brain-specific regulatory factors belonging to the Forkhead gene family has been identified in the C-terminal portion of the protein (amino acids 359–430) (11), whereas a SANT (switching-defective protein 3 (Swi3), adaptor 2 (Ad2), nuclear receptor co-repressor (N-CoR), transcription factor (TFIIIB)) domain involved in DNA binding and protein-protein interactions overlaps with the MBD (32, 33).

Data from many laboratories have demonstrated that mutations in the MECP2 gene are the primary cause of Rett's syndrome, a progressive neuro-developmental disorder characterized by mental retardation and autistic features (16–18). Most of the first RTT mutations identified were missense mutations located within the MBD, significantly reducing the affinity of MeCP2 for methylated DNA (19). It was later discovered that RTT patients commonly carry mutations in the TRD or the C terminus. Most of the mutations involving the TRD impair the ability of MeCP2 to inhibit gene expression, whereas deletions within the C terminus significantly decrease the protein stability (21). The absence of a genotype-phenotype correlation suggests that other factors, involved in or contributing to the same system of gene inactivation, have to be identified.

In the present work we report that a novel protein, p20, from *X. laevis* is able to interact in vivo and in vitro with MeCP2 (Figs. 1–4). Sequence analysis of the p20 cDNA revealed the presence of two well conserved WAP domains containing a characteristic disulfide pattern (Fig. 3 and Refs. 27 and 28). Genes encoding WAP domains are generally believed to be involved in influencing MeCP2 stability (12, 21). Moreover, the capability of the TRD to interact with TFIIB indicates that MeCP2 may also inhibit the enzymatic activity (15). However, the capability of the TRD to interact with TFIIIB indicates that MeCP2 may also inhibit gene expression through mechanisms independent of chromatin remodeling (31). Other proteins that might be secreted factors (34, 35) have been demonstrated that MeCP2 also associates with histone H3 methyltransferase activity. Both the MBD and the TRD contribute to the binding of this enzymatic activity (15).

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A Novel Protein, *Xenopus p20*, Influences the Stability of MeCP2 through Direct Interaction

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