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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.

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),[1] 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 Lys^9 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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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY563621

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[1] The abbreviations used are: MBD, methyl-CpG-binding domain; TRD, transcriptional repression domain; RTT, Rett syndrome; RACE, rapid amplification of cDNA ends; HA, hemagglutinin; GST, glutathione *S*-transferase; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride.

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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 *Xenopus* 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 utilized: for the 5′ RACE, the 3′ p20 (CCGGGGCCTCTTCCACGGGATCC) and for the 3′ RACE the 5′ p20 (ATGTCCCGAGCTATTGGTTCCCTCCTCCTG). The obtained cDNAs were cloned into pCR 2.1 (TA Cloning Kit; Invitrogen) and sequenced. The cDNA sequence of *Xenopus* p20 has been deposited in GenBank™ with the accession number AY563621.

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, GCCTTGCCATGGCCCTGGAGTCTCCAGTGAGATATCATGATGTTTGCCCC; 3′ p20-BamHI, GCCTTGGGATCCTCAGAGGTGCATACATTTC. The amplified product was cloned into NcoI-BamHI-digested pSPUTK (Stratagene). A DNA sequence coding for the FLAG epitope obtained by annealing two complementary oligonucleotides was inserted in between the BglII 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) SmaI-digested.

Bacterial Expression Vectors—The GST-MeCP2 (*Xenopus*) construct has previously been described (12). The p20-NcoI-BamHI amplification product was subcloned into the pGBKT7 vector (Clontech). pGST-p20 was obtained by inserting an NcoI-filled SalI fragment from pGBKT7-p20 into pGEX-4T-3 (Amersham Biosciences) digested with BamHI-filled SalI. pGST-p20ΔWAP1, pGST-p20ΔWAP2, and pGST-p20ΔWAP3 missing the regions including amino acids 22–70, 82–118, and 121–167, respectively, were generated by PCR and cloned into the BamHI site of pGEX-4T-1.

Eukaryotic Expression Vectors—pHA-p20 was cloned by inserting a BamHI-digested PCR fragment, obtained by PCR using the 5′ p20-BamHI (GATCGGATCCCTGGAGTCTCCAGTGAGATATC) and the 3′ p20-BamHI primers, in frame with an N-terminal HA tag in pSG5 (Stratagene). For coupled *in vitro* transcription/translation reactions, hMeCP2 (a kind gift of Dr. Yusufzai (21)) was subcloned by PCR into pSG5. All of the PCR-generated constructs were verified by sequencing.

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 TLA100.4). Approximately 10 mg of extract were loaded on a 5-ml HiTrap chelating column (Amersham Biosciences) charged with Ni^{2+} 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 $H_2(^{18}O)$ to label the C-terminal part of the tryptic peptides. The resulting peptides mixture was desalted on a microcolumn manually packed with 200 nl of POROS R2 material (PerSeptives 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 $MgCl_2$, 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 plus saccharose and fractionated on a saccharose cushion (0.7 ml of buffer A with 0.34 M saccharose and 0.7 ml of buffer A with 2.1 M saccharose) by centrifugation at 15,000 rpm for 15 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 60,000 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 $MgCl_2$, 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 PvuII, 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 mM 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 [^{35}S]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

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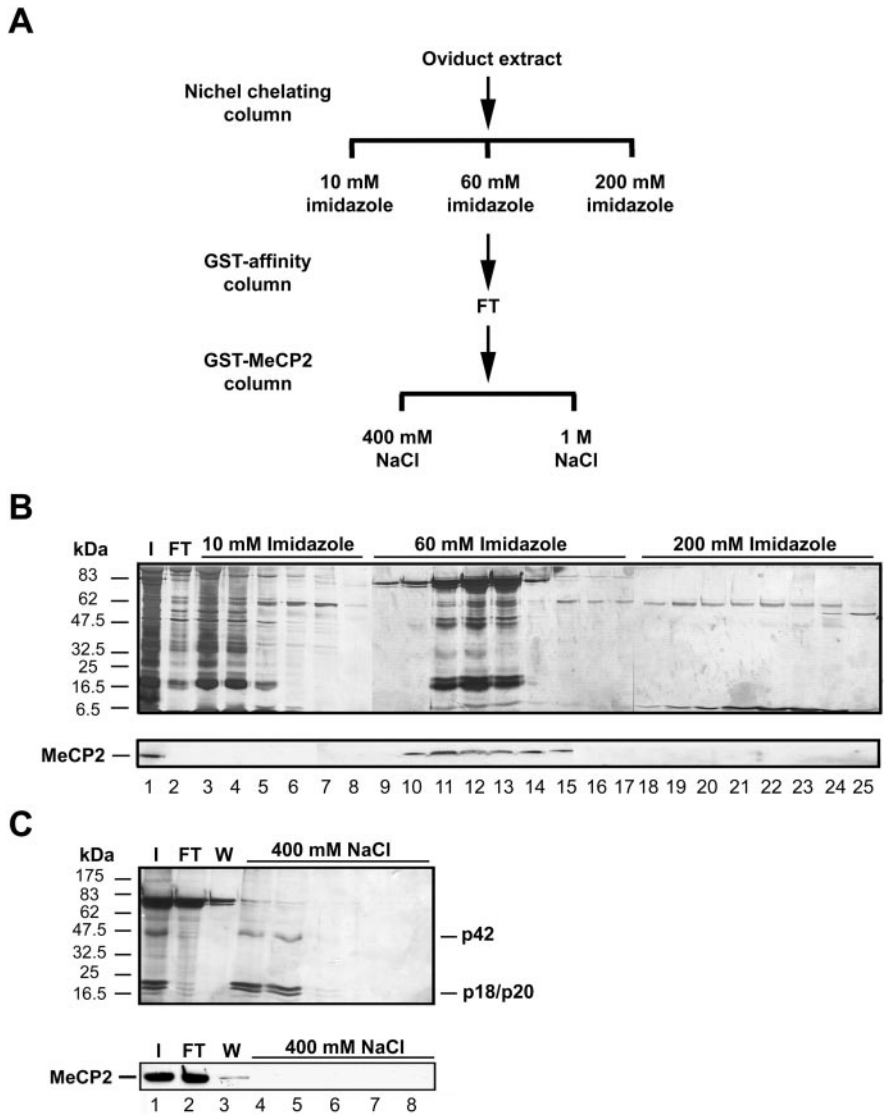

FIG. 1. **Purification of the *Xenopus* p20 protein.** *A*, schematic illustration of the purification protocol. *B*, silver staining (*upper panel*) showing the protein profile of nickel chelating chromatographic steps: Input (*I*, *lane 1*), flow-through (*FT*, *lane 2*), and elutions with 10 mM (*lanes 3–8*), 60 mM (*lanes 9–17*), and 200 mM (*lanes 18–25*) imidazole. MeCP2, as assayed by Western blot (*lower panel*), was found to elute in the 60 mM imidazole fractions. *C*, silver staining, showing the protein profile of 400 mM NaCl elution of the GST-MeCP2 affinity column (*lanes 4–8*). The p42, p20, and p18 proteins obtained in this purification procedure are indicated to the *right*. *I*, input; *FT*, flow-through; *W*, wash.

10% fetal calf serum and antibiotics. Transfections were performed with FuGENE (Roche Applied Science) according to the manufacturer's instructions (PC3) or with $CaPO_4$ precipitation (Phoenix). Cyclohexamide (10 μg/ml) was added 24 h post-transfection, and extracts were prepared at different time points. The cells were lyzed 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 μg of extract were loaded in each lane.

Co-immunoprecipitation Experiments

Xenopus Oviduct—1 ml of extract (2 mg) was incubated with GammaBind™G-Sepharose™ (Amersham Biosciences) for 1 h, after which the cleared extracts were incubated with anti-MeCP2 antibodies or preimmune sera covalently linked to GammaBind™G-Sepharose™ and incubated for 2 h at 4 °C. The washes were performed with 20 mM Hepes, pH 7.5, 100 mM NaCl, 0.01% Nonidet P-40. The bound proteins were resolved by SDS-PAGE and detected with silver staining.

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 GammaBind™G-Sepharose™ and incubated with or without monoclonal anti-HA antibody for 2 h at 4 °C before the addition of GammaBind™G-Sepharose™. 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

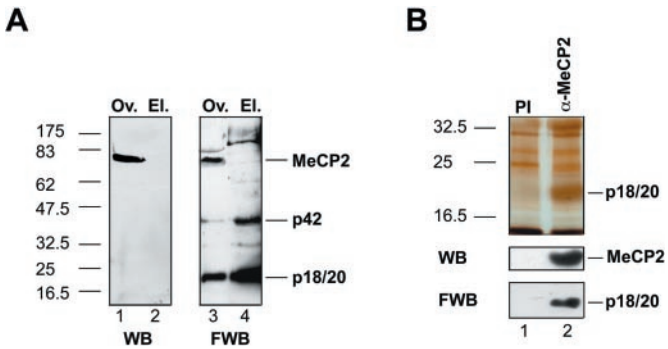

FIG. 2. **Confirmation that *Xenopus* p18/p20 directly interact with MeCP2.** *A*, Western (*WB*) and Far Western (*FWB*) assays were performed with 40 μg of oviduct extract (*lanes 1* and *3*) and 20 μl (almost 1 μg) of the 400 mM NaCl elution product from the GST-MeCP2 column (*lanes 2* and *4*). Proteins transferred to the membrane were probed with recombinant GST-MeCP2 after renaturation (*lanes 3* and *4*). MeCP2 was detected with anti-MeCP2 antibodies. To the *right* are indicated MeCP2 and its interacting proteins. *Ov.*, oviduct extract; *El.*, elution product. *B*, p18/p20 associate with MeCP2 *in vivo*. Co-immunoprecipitation experiment of *Xenopus* oviduct extract (3 mg) using a preimmune serum (*lane 1*) or anti-MeCP2 antibodies (*lane 2*). Half of the precipitated product was used for silver staining (*top panel*), and the other half was used for Western blot (*middle panel*) or Far Western blot using GST-MeCP2 as probe (*bottom panel*). MeCP2 and p18/p20 are indicated to the *right*. *PI*, preimmune serum.

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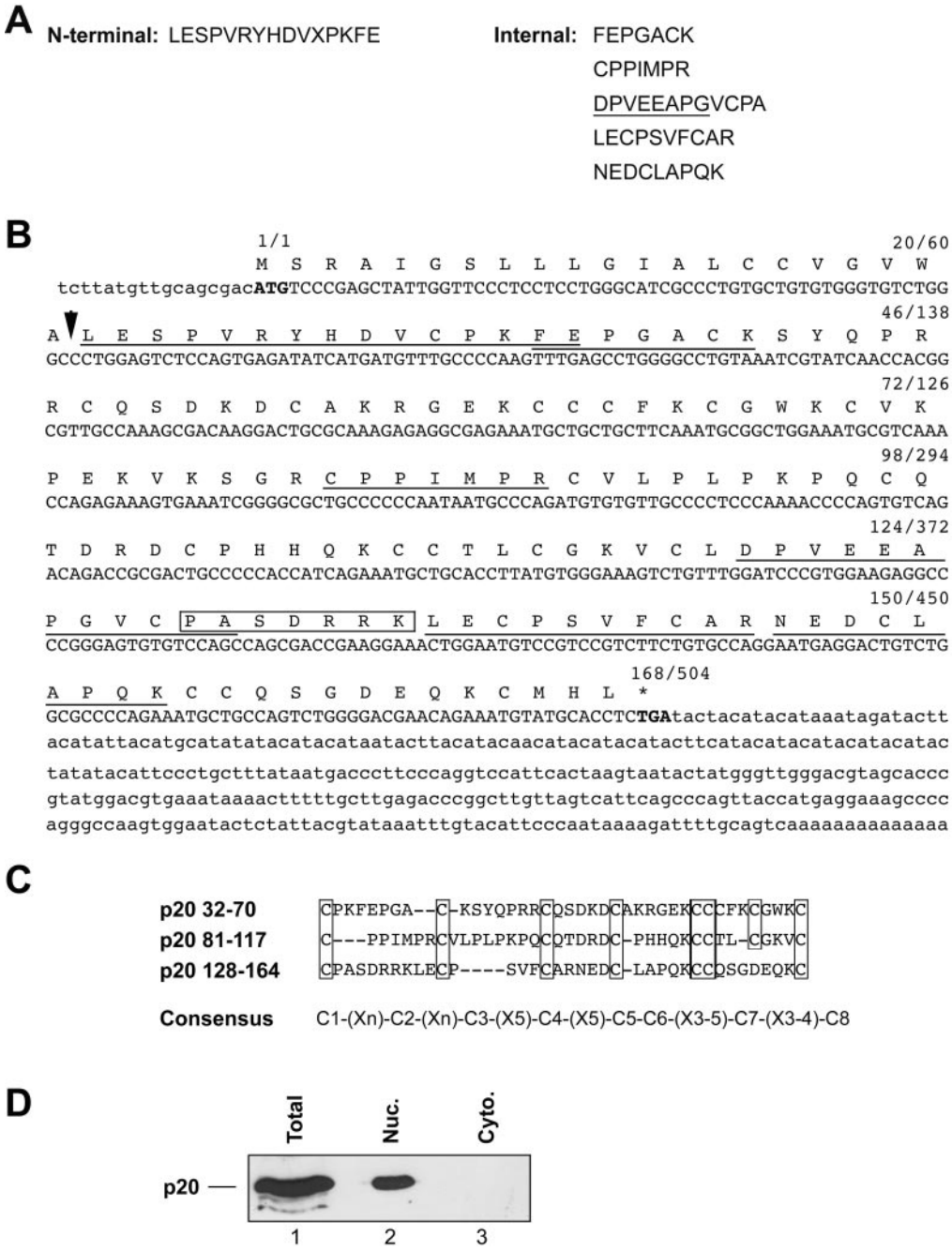

FIG. 3. **A *Xenopus* cDNA encodes the p20 MeCP2-interacting protein.** *A*, the peptides obtained by N-terminal and internal microsequencing. *Underlining* shows the sequence used to design the primer for the 5′ RACE. *B*, the *Xenopus* oviduct cDNA sequence obtained by 5′ and 3′ RACE. The nucleotide sequence written with *uppercase letters* encodes the p20 protein written *above* the nucleotide sequence. The *numbers* correspond to nucleotides and amino acids, respectively, numbered from the start codon. The stop codon is indicated with an *asterisk*. The nucleotide sequence in lowercase shows the 5′-untranslated region (−17 to 1) and the 3′-untranslated region (505–857). The *bars* below the cDNA illustrate the peptides obtained by sequencing. The putative proteolytic cleavage site and nuclear localization signal are indicated with a *triangle* and a *box*, respectively. *C*, alignment of the three presumptive p20 WAP domains. Core cysteines are boxed. The consensus sequence for WAP domains is shown. *D*, p20 is nuclear in *Xenopus* oviducts. Far Western blot was performed on total, nuclear, and cytoplasmic oviduct extracts paying attention in loading comparable amounts of cells. GST-MeCP2 was used as a probe, and interacting MeCP2 was revealed by immunoblotting with an anti-MeCP2 antibody. To the *left* is indicated the p20 protein.

manufacturer's instructions. Immobilized GST proteins (~2 μM) were incubated with 15–20 μl of *in vitro* translated ^{35}S-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, and 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. 1*A*. In this approach, we exploited the natural stretch of 13 histidines

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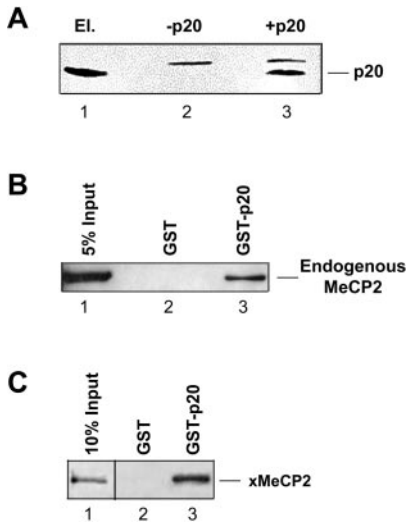

FIG. 4. **p20 encoded by the cloned cDNA interacts with MeCP2.** *A*, the cloned cDNA codes for a protein co-migrating with p20. Far Western assay was performed with 500 ng of the purified p20 (*lane 1*) and the *in vitro* translated p20 (*lane 3*). As control, an equal amount of the *in vitro* translation reaction without the p20 cDNA was included (*lane 2*). Recombinant GST-MeCP2 was hybridized to the renatured proteins and detected with anti-MeCP2 antibodies. To the right is indicated the p20. *El.*, elution product. *B*, the protein encoded by the p20 cDNA interacts with endogenous *Xenopus* MeCP2. Immobilized recombinant GST-p20, or GST, was incubated with 600 μg of *Xenopus* oviduct extract and retained MeCP2 detected by Western blot. Input (*lane 1*) corresponds to 5% of the total protein. *C*, p20-MeCP2 interaction by GST pull-down experiment. *In vitro* translated ^{35}S-labeled *Xenopus* MeCP2 was incubated with immobilized recombinant GST (*lane 2*) or GST-p20 (*lane 3*). Input (*lane 1*) corresponds to 10% of total protein.

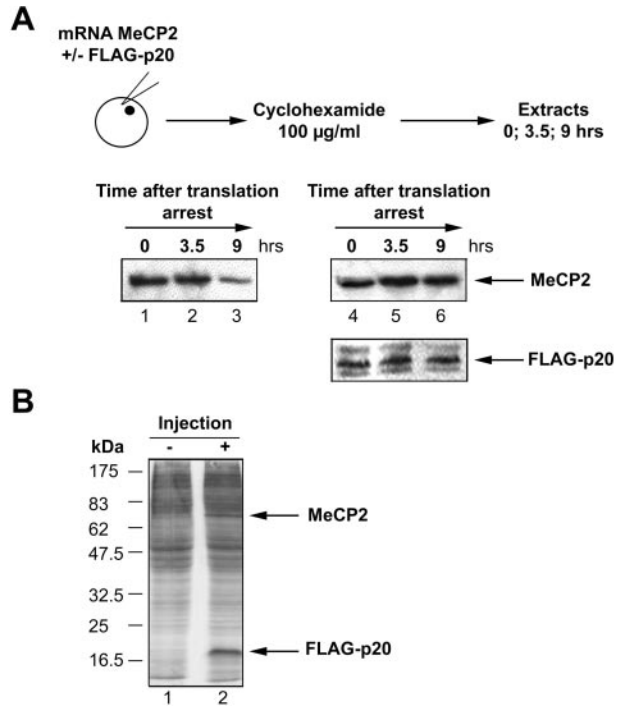

FIG. 5. **p20 increases the stability of *Xenopus* MeCP2.** *A*, stability assay in *Xenopus* oocytes. MeCP2 and FLAG-p20 mRNAs were injected, and oocytes were treated with cyclohexamide according to the diagram. In the absence of FLAG-p20 (*lanes 1–3*) MeCP2 is significantly degraded, whereas in the presence of FLAG-p20 (*lanes 4–6*), MeCP2 results are stable. MeCP2 and FLAG-p20 were detected with anti-MeCP2 and anti-FLAG antibodies (*upper* and *lower panels*, respectively). *B*, autoradiogram showing total extracts prepared from oocytes noninjected (−) or injected with MeCP2 and FLAG-p20 (+) mRNAs and incubated with ^{35}S-labeled methionine and cysteine. MeCP2 and FLAG-p20 are indicated to the *right*.

present in the C-terminal part of xMeCP2, allowing us to perform an affinity purification of the endogenous protein from a *X. laevis* oviduct extract in which MeCP2 is abundantly expressed compared with other tissues (data not shown). As illustrated in Fig. 1*A*, 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. 1*B*, *lower panel*). To further purify MeCP2 interactors, we used a GST-MeCP2 affinity column (Fig. 1*A*). Briefly, the 60 mM imidazole fraction was first passed on a GST-Sepharose column to eliminate proteins aspecifically 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. 1*C* 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. 1*C*, *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 true MeCP2-associated proteins, we wanted to demonstrate the interaction by alternative assays. To this end we performed a Far Western experiment (Fig. 2*A*) in which proteins in the oviduct extract and the 400 mM NaCl fraction (Fig. 1*C*) 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. 2*A*, the antibody recognizes specifically MeCP2 within the oviduct extract and no proteins in the eluted fraction (*lanes 1* and *2*, respectively). On the contrary, several polypeptides are evident in the Far Western experiment; in the oviduct extract (*lane 3*) 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 (*lane 4*). 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 an oviduct extract and analyzed the co-purifying proteins by silver staining (Fig. 2*B*, *top panel*). 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, co-purifying with MeCP2. A Far Western experiment performed on half of the immunoprecipitate unequivocally confirms that the co-purifying protein is a direct MeCP2 interactor (Fig. 2*B*, *bottom panel*). This experiment thus reveals that, *in vivo*, p18/p20 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 we obtained five

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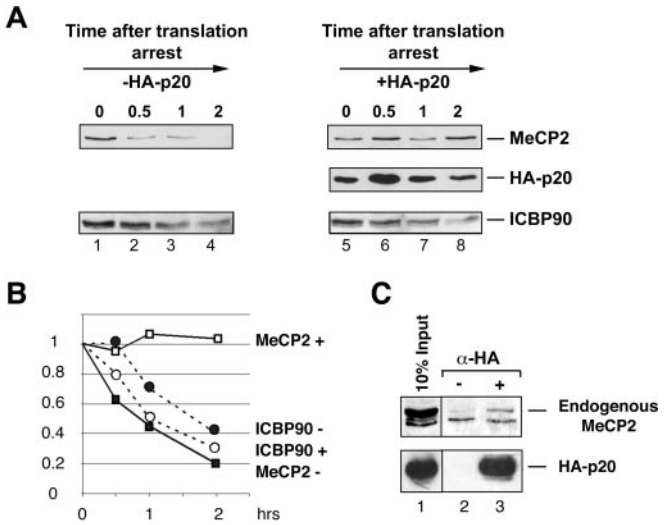

FIG. 6. **p20 interacts with and stabilizes human MeCP2.** *A*, in human PC3 cells overexpression of HA-p20 causes the stabilization of endogenous MeCP2. PC3 cells were transfected with HA-p20 (*lanes 5–8*) or left as nontransfected controls (*lanes 1–4*) and treated with 10 μg/ml of cyclohexamide for the given time points. MeCP2, HA-p20, and ICBP90 were detected with anti-MeCP2, anti-HA, and anti-ICBP90 as indicated. *B*, 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.

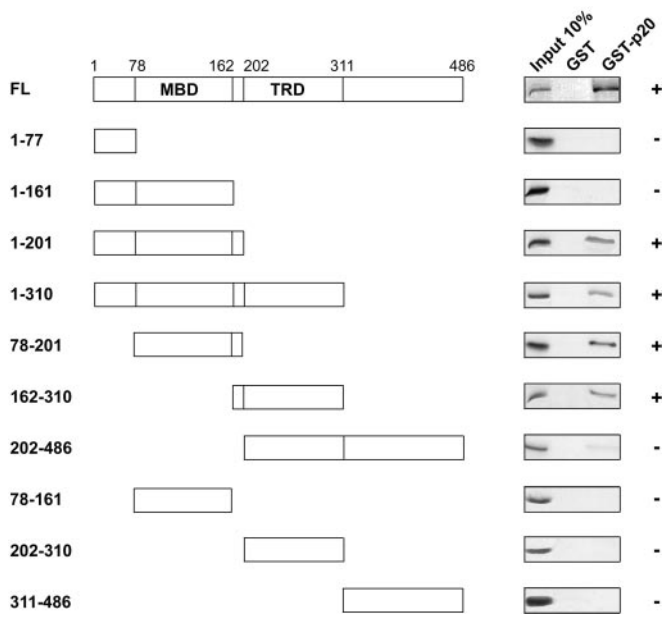

FIG. 7. **The linker region between the MBD and the TRD is required for full interaction with p20.** *In vitro* translated ^{35}S-labeled hMeCP2 and its mutated derivatives were incubated with immobilized recombinant GST and GST-p20. The hMeCP2 derivatives are shown schematically to the *left*, and the corresponding autoradiograms showing the interactions are to the *right*. By Coomassie staining it was verified that comparable amounts of GST and GST-p20 were present in the pellets (data not shown). The numbers above full-length (*FL*) MeCP2 indicate the amino acids included in the MeCP2 derivatives. + and − indicate the presence and absence, respectively, of interaction.

internal peptide sequences and the N-terminal peptide (Fig. 3*A*), which were used to perform a data base search for the corresponding cDNA. A *Xenopus* expressed sequence tag showing high homology with the sequenced peptides was found (accession number AW633466). The nucleotide sequence corresponding to the peptide underlined in Fig. 3*A* was used to perform a 5′ RACE from oviduct RNA leading to the identification of the start codon and a short 5′-untranslated region. This sequence was used to obtain the full-length cDNA of 857 nucleotides characterized by a start and a stop codon, an open reading frame of 167 amino acids, and 336 nucleotides of 3′-untranslated region and a poly(A) tail (Fig. 3*B*).

An analysis of the p20 sequence with the Prosite data base revealed the presence of two WAP (whey acidic protein) domains within the protein (Fig. 3*C*). 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, data base 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. 3*D*, 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 mM NaCl elution fraction; Fig. 1*C*) and to *in vitro* translated p20. As shown in Fig. 4*A* (*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. 4*B* 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. 4*C*, 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 Wolffe (21). As shown in Fig. 5*A*, the oocytes were microinjected with *Xenopus* MeCP2 mRNA, with or without FLAG-p20

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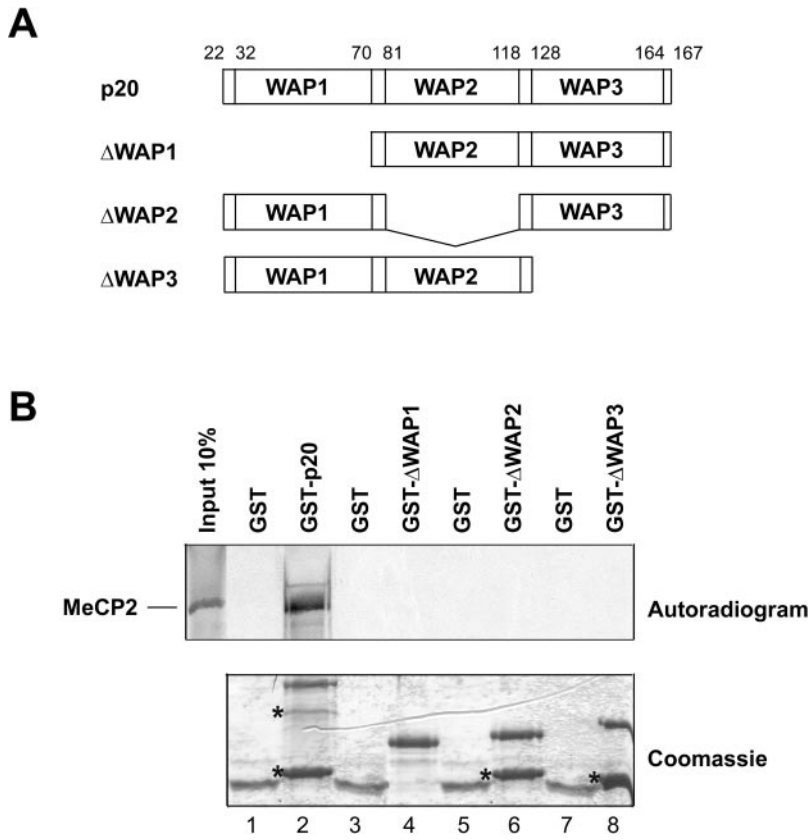

FIG. 8. **Identification of interaction surfaces within p20 by GST pull-down experiments.** *A*, schematic illustration of the p20 derivatives fused to GST. The *numbers* above p20 indicate the amino acids numbering. *B*, all three WAP domains are required for interaction with MeCP2. *In vitro* translated full-length ^{35}S-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.

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. 5*A*, 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 ^{35}S-labeled methionine and cysteine. The autoradiogram of total extracts (Fig. 5*B*) shows the presence of labeled MeCP2 and FLAG-p20 only in the injected oocytes. A densitometer analysis of the bands corresponding to MeCP2 and FLAG-p20 showed a ratio in intensity of ~1:3, and taking into consideration the presence of 27 labeled amino acids in p20 and only 6 in MeCP2, we estimated that the two proteins are close to being equimolar, with a slight excess of p20 (1.5 times), in the stability assays. The stability of MeCP2 seems to be influenced negatively in some of the mutations identified in RTT patients (21), suggesting that an important parameter for regulating MeCP2 activity might be through stabilizing the protein. We therefore wanted to address the question of whether the *Xenopus* p20 might be able to increase the half-life of endogenous MeCP2 in human cells. This was analyzed by transiently overexpressing HA-tagged p20 in human PC3 cells and assaying MeCP2 levels at different time points after the block of protein synthesis. As seen in Fig. 6*A*, in the absence of HA-p20, MeCP2 levels are already dramatically reduced 0.5 h after cyclohexamide treatment and are reduced below detection limits at 2 h. Conversely, in cells overexpressing HA-p20, MeCP2 levels remain constant through the time course of the experiment. The anti-proteolytic activity of p20 seems not to be a general effect because another endogenous protein, ICBP90, does not present an increased stability in the presence of HA-p20 (Fig. 6*A*, *lower panel*). In Fig. 6*B* the mean values of MeCP2 and ICBP90 levels, in the absence or presence of HA-p20, from three independent experiments are illustrated graphically, showing clearly the effect of p20 only on the half-life of MeCP2. Because the hMeCP2 is not completely conserved with respect to the *Xenopus* one (21), we wanted to confirm the capability of the *Xenopus* p20 to interact with the human methyl-binding protein. We therefore analyzed whether endogenous MeCP2 co-immunoprecipitated with transiently overexpressed HA-p20 from human Phoenix cells. As shown in Fig. 6*C*, MeCP2 co-purified with HA-p20 (*lane 3*), whereas no MeCP2 was present in the absence of anti-HA antibodies (*lane 2*). We conclude that the *Xenopus* p20 protein is able to interact with both *Xenopus* and human MeCP2 protecting them against proteolysis.

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. 8*A*) and tested them for their ability to pull down hMeCP2. The autoradiogram in Fig. 8*B* shows that when either of the WAP domains within p20 was deleted, MeCP2 interaction was impaired. The Coomassie staining in Fig. 8*B* reveals that the resins contain equal amounts of GST

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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 TFIIB 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 (Ada2), 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 small, secreted serine protease inhibitors; however, in the literature there are also reports suggesting that WAP domains might not only be secreted factors (34, 35). Data base analysis of the *Xenopus* p20 cDNA showed that the protein might be secreted because of the presence of a putative leader peptide or alternatively might be nuclear because of a nuclear localization signal. Our purification procedure (Fig. 1 and "Experimental Procedures") together with the results obtained from the Far Western assay (Fig. 3) and from co-immunoprecipitation experiments (data not shown) on fractionated oviduct extracts demonstrated that a significant quantity of p20 is localized in the nucleus, the same compartment where MeCP2 accumulates. However, these assays do not exclude the possibility that a fraction of p20 is secreted. Because the purified MeCP2-interacting p20 starts from amino acid 22 immediately following a putative cleavage site, we propose that the nuclear p20 is proteolytically processed.

Even though some reports suggest that WAP domains can have functions other than protease inhibitors (34, 35), we hypothesized that the p20 interaction with MeCP2 might serve to protect the methyl-binding protein against proteolysis. Therefore, we took advantage of the *Xenopus* oocytes to demonstrate that, indeed, the p20 is able to significantly increase the half-life of exogenously expressed MeCP2 (Fig. 5). More importantly, we show that the frog WAP protein is not only able to interact with the human MeCP2 (Figs. 6 and 7) but also to stabilize the endogenous mammalian methyl-binding protein (Fig. 6). This result seems particularly interesting because, as previously discussed, several C-terminal RTT mutations are characterized by an MeCP2 protein with a reduced half-life, indicating that regulation of MeCP2 stability might be a crucial mechanism in humans. Even if we cannot exclude that the activity of p20 in protecting MeCP2 against proteolysis is a specialized oviduct function, we speculate that a human locus encoding a functional homologue of the *Xenopus* p20 might be involved in influencing the RTT phenotype. Because of that we have searched in the data base for a human orthologue of p20. Even though the human genome contains several genes expressing WAP domains (35), so far we have not been able to unambiguously identify the corresponding mammalian gene. This result is not completely unexpected because it is well known that WAP genes are structurally very different. In fact, in addition to the cysteine residues forming the conserved disulfides, WAP proteins are characterized by high sequence diversity. To conclude, these results may reveal the existence of a novel mechanism that, by influencing MeCP2 metabolism, is involved in regulating its activity.

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

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