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Cloning and Characterization of Mouse UBPy, a Deubiquitinating Enzyme That Interacts with the Ras Guanine Nucleotide Exchange Factor CDC25^{Mm}/Ras-GRF1*

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Nerina Gnesutta‡§, Michela Ceriani¶§, Metello Innocenti‡, Isabella Mauri‡, Renata Zippel‡, Emmapaola Sturani‡, Barbara Borgonovo‖, Giovanna Berruti‖, and Enzo Martegani¶**

From the ‡Dipartimento di Fisiologia e Biochimica Generali, Università di Milano, Via Celoria 26, Milano, Italy 20133, ¶Dipartimento di Biotecnologie e Bioscienze, Università di Milano-Bicocca, Piazza della Scienza 2, Milano, Italy 20126, and ‖Dipartimento di Biologia, Università degli Studi di Milano, Milano, Italy 20133

We used yeast "two-hybrid" screening to isolate cDNA-encoding proteins interacting with the N-terminal domain of the Ras nucleotide exchange factor CDC25^{Mm}. Three independent overlapping clones were isolated from a mouse embryo cDNA library. The full-length cDNA was cloned by RACE-polymerase chain reaction. It encodes a large protein (1080 amino acids) highly homologous to the human deubiquitinating enzyme hUBPy and contains a well conserved domain typical of ubiquitin isopeptidases. Therefore we called this new protein mouse UBPy (mUBPy). Northern blot analysis revealed a 4-kilobase mRNA present in several mouse tissues and highly expressed in testis; a good level of expression was also found in brain, where CDC25^{Mm} is exclusively expressed. Using a glutathione *S*-transferase fusion protein, we demonstrated an "*in vitro*" interaction between mUBPy and the N-terminal half (amino acids 1–625) of CDC25^{Mm}. In addition "*in vivo*" interaction was demonstrated after cotransfection in mammalian cells. We also showed that CDC25^{Mm}, expressed in HEK293 cells, is ubiquitinated and that the coexpression of mUBPy decreases its ubiquitination. In addition the half-life of CDC25Mm protein was considerably increased in the presence of mUBPy. The specific function of the human homolog hUBPy is not defined, although its expression was correlated with cell proliferation. Our results suggest that mUBPy may play a role in controlling degradation of CDC25^{Mm}, thus regulating the level of this Ras-guanine nucleotide exchange factor.

Ras-guanine nucleotide exchange factors (GEFs)[1] are proteins that stimulate the exchange of guanine nucleotides (GDP/GTP) on Ras proteins. We have previously cloned a mouse brain-specific Ras-GEF, called CDC25^{Mm} (1–2) or Ras-GRF1. CDC25^{Mm} is a large protein (140 kDa) that contains a Ras-exchange domain in the C-terminal region and several different domains in the large N-terminal region, namely two PH (pleckstrin homology) domains, one DH (Dbl homology) domain, and an illimaquinone (IQ) domain (2–3). In addition a coiled-coil region and a PEST sequence were identified (4).

Several evidences indicate that the large N-terminal region of CDC25^{Mm} has a regulatory function and may interact with other cellular components. We have previously shown that the expression in mouse fibroblasts of a truncated form of CDC25^{Mm}, lacking the Ras exchange domain, behaves as a dominant negative protein (5). In addition PH domains could interact with phospholipids (6) and with the $\beta\gamma$ subunits of heterotrimeric G proteins (7), whereas the IQ domain binds calmodulin (3, 8) and is thought to be responsible for the activation of GEF activity by calcium (3). Further evidence that the N-terminal region of CDC25^{Mm} protein is involved in specific protein interaction(s) also comes from the work of Kiyono *et al.* (9) in which it was shown that CDC25^{Mm}/Ras-GRF1 was able to activate Rac1 and that for this activity a functional DH domain is required. Moreover the DH domain was required for homodimerization of Ras-GRF1 or for heterodimerization with Ras-GRF2 (10)

However, so far, only the specific interaction CDC25^{Mm}/calmodulin has been demonstrated *in vivo* (3, 8). In an effort to identify mammalian proteins that could interact with the large N-terminal region of CDC25^{Mm}, we used a yeast "two-hybrid" system for the screening of mouse embryo cDNA libraries (11). Here we report that the cloning and characterization of a cDNA that was positive in this screening. This cDNA was found to encode a new deubiquitinating enzyme belonging to the ubiquitin isopeptidase family (UBPs) and was highly homologous to the recently identified human enzyme hUBPy (12); therefore we called this new cDNA mouse UBPy (mUBPy).

EXPERIMENTAL PROCEDURES

Two-hybrid Screening—The system developed by Vojtek *et al.* (11) was used for the two-hybrid screening. Two mouse embryo cDNA libraries (9.5 and 10.5 dpc) constructed in pVP16 vector (a gift of Stan Hollenberg) were used. Three bait plasmids were prepared by cloning fragments of the N-terminal region of CDC25^{Mm} in the pBTM116 vector, which contained the Lex-A DNA binding domain (11).

The first bait plasmid (pBTEE) contains 1877 bp of CDC25^{Mm} IV (2), which corresponds to the first 625 amino acids; this region includes the first PH domain, the IQ motif, the Dbl homology (DH) domain, and the second PH domain. pBTES contains 1519 bp of CDC25^{Mm} IV, corresponding to the first 506 amino acids, and lacks the second PH domain, whereas pBTEP contains 436 bp, corresponding to the first 145 amino acids, and contains only the first PH domain.

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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) AF057146.

§ These authors contributed equally to the work.

** To whom correspondence should be addressed: Dipartimento di Biotecnologie e Bioscienze, Piazza della Scienza 2, 20126 Milano, Italy. Tel.: 39-02-64483533; Fax: 39-02-64483565; E-mail: enzo.martegani@unimib.it.

[1] The abbreviations used are: Ras-GEF, guanine nucleotide exchange factor for Ras; UBP, ubiquitin isopeptidase; DH, Dbl homolog; PH, pleckstrin homolog; IQ, illimaquinone; bp, base pair(s); aa, amino acid(s); GST, glutathione *S*-transferase; HA, hemagglutinin; RIPA, radioimmune precipitation; dpc, days post-coitus.

The yeast strain L40 (*MAT*a, *his*3, *trp*1, *leu*2, *ade*2, *LYS*2::(lexAop)$_4$-*HIS*3, *URA*3::(lexAop)$_8$-lacZ) was transformed with the bait vector (initially pBTEE) and with the mouse embryo cDNA libraries. Selection was performed as described by Vojtek *et al.* (11) in selective minimal plates containing 5 mM 3-aminotriazole. Positive clones, obtained only by the 10.5-dpc library, were also tested for β-galactosidase activity. Plasmids were recovered from positive clones and used for a new round of screening to test their positivity. Positive clones were sequenced and then tested with the other plasmids containing reduced regions of CDC25^{Mm} (pBTES and pBTEP).

RACE-PCR, Cloning, Plasmids, Sequencing, and Northern Blot Hybridization—RACE reactions were done using Marathon-Ready cDNA (CLONTECH) from 11.5-dpc mouse embryo and a mix of polymerases (Advantage Klen-*Taq* Mix, CLONTECH) suitable for amplification of long regions of DNA with high fidelity. The PCR conditions were set and performed according to the protocols given by CLONTECH. Four oligonucleotides were used, two for 3′ RACE and two for 5′ RACE using a nested PCR procedure to obtain an increased specificity. For 3′ RACE, the external primer was GSS1 (5′-GCTCAGCGAGAACCTTTGACGAGAGC-3′), and the internal one was GSS2 (5′-CCTGAAATGGCTCCTCGTCTGCACC-3′). For 5′ RACE, the external primer was GSA1 (5′-GGTGCAGACGAAGGAGCCATTTCAGG-3′), and the internal one was GSA2 (5′-GCTCTCGTCAAAGGTTCTCGCTGAGC-3′).

Two oligonucleotides (*i.e.* GSS1 and GSA1) were also used in a control reaction to verify the presence of the specific cDNA in the pool. Positivity was revealed by a 176-bp fragment.

The amplification products of 3′ and 5′ RACE were cloned in pMOS-Blue (Amersham Pharmacia Biotech) and sequenced. Three clones were sequenced both for 3′ and 5′ amplification products.

Sequencing was performed on both strands using the *Taq* Dye Terminator Cycle Sequencing Kit (Applied Biosystems) with universal and walking primers and using an automated DNA sequence analyzer (Applied Biosystems 373A). The full-length cDNA was obtained by using the unique *Nde*I site present in the overlapping region and was subcloned in pCDNA3 vector (Invitrogen).

pCDNA3-mUBPy-HA vector was prepared by ligation of a cDNA fragment of 2.5 kilobase pairs comprising the coding sequence of aa 542–1080 of mUBPy in pBSKSII-HA vector (Stratagene), and then a *Sph*I-*Eco*RI fragment coding for a fusion between HA tag and the aa 542–1080 of mUBPy was subcloned in pCDNA3 plasmid. HA-ubiquitin vector and pCDNA3-hUBPy plasmid were obtained by G. Draetta (12).

For Northern blots, polyadenylated RNAs from mouse tissues (total mouse RNA, Ambion) were separated on agarose-formaldehyde gels (5 μg/lane), blotted to a Hybond-N nylon membrane (Amersham Pharmacia Biotech), and hybridized with a digoxygenin-labeled riboprobe. Signals were detected with the Nucleic Acid Detection kit (Roche Molecular Biochemicals). The probe was prepared by subcloning a fragment of mUBPy cDNA in a pGEM3z vector (Promega) and *in vitro* transcription with T7 RNA polymerase and digoxygenin-labeled UTP (Roche Molecular Biochemicals) according to the manufacturer's instructions.

Sequence Analysis—The searches for open reading frames and their conceptual translations were performed with DM software (13). The homology search was done at the BLAST (14) server at NCBI (www.ncbi.nlm.nih.gov/BLAST/). Protein alignments were done with MACAW (15), while the search for motifs in protein was done with Profilescan at the Expasy server (www.expasy.ch/).

Recombinant Protein Production and Preparation of Polyclonal Antibodies—The cDNA contained in one of the positive clones (6/12) was subcloned in the *Sma*I site of the *Escherichia coli* expression vector pGEX-2T (Amersham Pharmacia Biotech) to obtain a fusion protein between GST and 119 amino acids of mUBPy corresponding to position 542 to 660 of the whole protein. *E. coli* DH5α cells bearing the vector for fusion protein were grown in LB broth, induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside at 28 °C for 3 h, and collected for protein extraction. Fusion protein was purified with glutathione-Sepharose resin as described (16). About 1 mg of purified fusion protein (GST-mUBPy$_{542–660}$) was used to immunize New Zealand rabbits. Polyclonal antibodies were then affinity-purified using a GST-coupled Affi-Gel-10 column (Bio-Rad) as described (17). For pull-down experiments, the GST-mUBPy$_{542–660}$ fusion protein was used, coupled to glutathione-Sepharose beads.

Western Blotting—Tissues from adult CD1 mice (Charles River, Calco (LC), Italy) were mechanically homogenized with 4 volumes of Tris (0.125 M, pH 6.8) and SDS (7% w/v). The homogenate was further sonicated in ice and centrifuged (20,000 × *g* for 10 min); the solubilized proteins were separated on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose. Membranes were probed with affinity-purified anti-mUBPy polyclonal antibodies (diluted 1:1000). Bound antibodies were detected with peroxidase-conjugated goat anti-rabbit immunoglobulins and revealed by the ECL detection system (Amersham Pharmacia Biotech).

Cells cultures were harvested, washed with phosphate-buffered saline, and lysed in RIPA buffer (Tris-HCl, 50 mM; NaCl, 150 mM; sodium deoxycholate, 0.5% w/v; SDS, 0.1% w/v; Triton X-100, 1% w/v) containing protease inhibitors (leupeptin, 1 μM; aprotinin, 0.1 μM; sodium vanadate, 0.1 mM; phenylmethylsulfonyl fluoride, 0.1 mM). Aliquots of cleared cell lysate were separated on SDS-polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with anti-mUBPy antibodies.

Cell Culture and Transfection—NIH-3T3, COS7, and Hek-293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Subconfluent cells were transfected using the LipofectAMINE (Life Technologies, Inc.) method according to the protocol of the manufacturer.

Pull-down Experiments—48 h after transfection, COS7 cells were harvested, washed with phosphate-buffered saline, and lysed in RIPA buffer or in lysis buffer (HNGT) (Hepes, 25 mM; NaCl, 50 mM; glycerol, 10% w/v; Triton X-100, 1% w/v). The buffers contained protease inhibitors.

Aliquots of lysates (450 μl) were then incubated with 50 μl of glutathione-Sepharose resin loaded either with GST-mUBPy$_{525–660}$ fusion protein or with GST alone for 2 h at 4 °C. After incubation, the resin was recovered by gentle centrifugation and washed five times with the incubation buffer (RIPA or HGNT). At the end, the resin was treated with 30 μl of 2× SDS sample buffer and boiled for 10 min, and the recovered proteins were used for Western blot analysis.

Coimmunoprecipitation and Immunoblotting—48 h after transfection, cells (COS7 or Hek-293) were harvested and lysed in 500 μl/plate (100 mm) of an ice-cold HNGT buffer as described (18). Immunoprecipitation from cleared lysates was performed with monoclonal anti-HA-11 antibody (BAbCO) or with anti-mUBPy polyclonal antibodies for 1 h at 4 °C. Immunocomplexes were recovered with protein G-Sepharose or with protein A-Sepharose (Sigma) for 1 h at 4 °C and then resuspended in SDS sample buffer and analyzed by immunoblot analysis. Briefly 1/50 of cleared cell lysates and the immunoprecipitates were resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed with anti Ras-GRF1 (C-20, Santa Cruz, CA) or with polyclonal anti-mUBPy raised in our laboratory. Immunocomplexes were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech) using goat anti-rabbit antiserum coupled to horseradish peroxidase as secondary antibodies (Cappel).

Deubiquitination Assays—We used a mouse testis extract as a source of ubiquitinated protein as described in Ref. 19 with minor modifications. The cleared extracts were dialyzed against Tris-HCl 50 mM, $MgCl_2$ 5 mM, and dithiothreitol 2 mM. The sample was then centrifuged and washed several times in a Centricon-3 concentrator (Amicon) to remove residual *N*-ethylmaleimide (12). Aliquots (50 μg) of the proteins were then incubated with immunoprecipitates (anti-HA antibodies) from COS7 cells transfected with pCDNA3-mUBPy-HA vector at 37 °C and processed as described in Ref. 19 using anti-ubiquitin antibodies (Sigma).

In Vivo Ubiquitination of CDC25^{Mm}—Hek-293 cells transfected with the different plasmids (as reported in the figure legends) were lysed in RIPA buffer. Immunoprecipitation from the same amount of total protein was performed with antibodies against Ras-GRF1 or anti-HA (BAbCO). Immunocomplexes were recovered with protein G-Sepharose (for anti-HA monoclonal antibodies) or protein A-Sepharose (for Ras-GRF1 antibodies) and analyzed in Western blotting with the different primary antibodies and then revealed with the corresponding secondary antibody and the ECL system.

Determination of Stability of CDC25Mm Protein—Hek-293 cells were transiently transfected with 1 μg of pCDNA3-CDC25^{Mm} alone and then cotransfected with 1.5 μg of pCDNA3-mUBPy or with 1.5 μg of pCDNA3-human UBPy antisense (12). After 24 h, cells were washed two times with minimum Eagle's medium without methionine. Cells were then incubated for 2 h with minimum Eagle's medium without methionine with [^{35}S]methionine (100 μC/60-mm dish), Hepes 25 mM, and fetal calf serum 0.1%. After the incubation, cells were washed two times with Dulbecco's modified Eagle's medium and further incubated with Dulbecco's modified Eagle's medium supplemented with fetal bovine serum 10%.

At the appropriate time, cells were rinsed in phosphate-buffered saline and lysed in RIPA buffer, and the lysates were clarified. Immunoprecipitation from lysates was performed in the presence of Ras-GRF1 antibody (Sigma) for 2 h at 4 °C, and the immunocomplexes were recovered with protein A (Sigma) for 1 h at 4 °C with gentle rotation.

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hUBPy          MPAVASVPKELYLSSSLKDLNKKTEVKPEKISTKSYVHSALKIFKTAEECRLDRDEERAY    60
mUbpY          MPAVASVPKELYLSSSLKDLNKKTEVKPEKTSTKNYIHSAQKIFKTAEECRLDRDEERAY    60

hUBPy          VLYMKYVTVYNLIKKRPDFKQQQDYFHSILGPGNIKKAVEEAERLSESLKLRYEEAEVRK   120
mUbpY          VLYMKYVAVYNLIKKRPDFKQQQDYYLSILGPANIKKAIEEAERLSESLKLRYEEAEVRK   120

hUBPy.         KLEEKDRQEEAQRLQQKRQETGREDGGTLAKGSLENVLDSKDKTQKSNGEKNEKCETKEK   180
mUbpY          QLEEKDRREEEQLQQQKRPEMGREDSGAAAKRSVENLLDSKTKTQRINGEKSEGAAAAER   180

hUBPy          GAITAKELYTMMTDKNISLIIMDARRMQDYQDSCILHSLSVPEEAISPGVTASWIEAHLP   240
mUbpY          GAITAKELYTMMMDKNTSLIIMDARKIQDYQHSCILDSLSVPEEAISPGVTASWIEANLS   240
                                              ----------------------------------------

hUBPy          DDSKDTWKKRGNVEYVVLLDWFSSAKDLQIGTTLRSLKDALFKWESKTVLRNEPLVLEGG   300
mUbpY          DDSKDTWKKRGSVDYVVLLDWFSSAKDLLLGTTLRSLKDALFKWESKTVLRHEPLVLEGG   300
               ------------------------------------------------------------

hUBPy          YENWLLCYPQYTTNAKVTPPPRRQNEEVSISLDFTYPSLEESIPSKPAAQTPPASIEVDE   360
mUbpY          YENWLLCYPQFTTNAKVTPPPRSRAEEVSVSLDFTYPSLEEPVPSKLPTQMPPPPIETNE   360
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hUBPy          NIELISGQNERMGPLNISTPVEPVAASKSDVSPIIQPVPSIKNVPQIDRTKKPAVKLPEE   420
mUbpY          KALLVTDQDEKLRLSTQPALAGPGAAPRAEASPIIQPAPATKSVPQVDRTKKPSVKLPED   420

hUBPy          HRIKSESTnhEQqspqSGKVIPDRSTKPVVFSPTLMLTDEEKARIHAETALLMEKNKQEK   480
mUbpY          HRIKSENT--DQ----SGRVLSDRSTKPVFPSPTTMLTDEEKARIHQETALLMEKNKQEK   474

hUBPy          ELRERQQEEQKEKLRKEEQEQKAKKKQEAEENEITEKQQKAKEEMEKKESEQAKKEDKET   540
mUbpY          ELWDKQQKEQKEKLRREEQERKAGKTQDADERDSTENQHKAKDGQEKKDSKQTKTEDREL   534

hUBPy          SAKRGKEITGVKRQSKSEHETSDAKKSVEdrGKRCPTPEIQKKSTgDVPHTSVtgdsgsg   600
mUbpY          SADGAQEATGTQRQSKSEHEASDAKVPVE--GKRCPTSEAQKRPA-DVSPASV-------   584

hUBPy          kpfkikgqpeSGILRTGtfredtddternKAQREPLTRARSEEMGRIVPGLPSGWAKFLD   660
mUbpY          ----------SGELNAG------------KAQREPLTRARSEEMGRIVPGLPLGWAKFLD   622

hUBPy          PITGTFRYYHSPTNTVHMYPPEMAPSSAPPSTPPTHKAKPQIPAERDREPSKLKRSYSSP   720
mUbpY          PITGTFRYYHSPTNTVHMYPPEMAPSSAPPSTPPTHKVKPQVPAERDREPSKLKRSYSSP   682

hUBPy          DITQAIQEEEKRKPTVTPTVNRENKPTCYPKAEISRLSASQIRNLNPVFGGSGPALTGLR   780
mUbpY          DITQALQEEEKRRPAVTPMVNRENKPPCYPKAEISRLSASQIRNLNPVFGGSGPALTGLR   742

hUBPy          NLGNTCYMNSILQCLCNAPHLADYFNRNCYQDDINRSNLLGHKGEVAEEFGIIMKALWTG   840
mUbpY          NLGNTCYMNSILQCLCNAPHLADYFNRNCYQDDINRSNLLGHKGEVAEEFGIIMKALWTG   802

hUBPy          QYRYISPKDFKITIGKINDQFAGYSQQDSQELLLFLMDGLHEDLNKADNRKRYKEENNDH   900
mUbpY          QYRYISPKDFKVTIGKINDQFAGSSQQDSQELLLFLMDGLHEDLNKADNRKRHKEENNEH   862

hUBPy          LDDFKAAEHAWQKHKQLNESIIVALFQGQFKSTVQCLTCHKKSRTFEAFMYLSLPLASTS   960
mUbpY          LDDLQAAEHAWQKHKQLNESIIVALFQGQFKSTVQCLTCRRRSRTFEAFMYLSLPLASTS   922

hUBPy          KCTLQDCLRLFSKEEKLTDNNRFYCSHCRARRDSLKKIEIWKLPPVLLVHLKRFSYDGRW  1020
mUbpY          KCTLQDCLRLFSKEEKLTDNNRFYCSHCRARRDSLKKIEIWKLPPVLLVHLKRFSYDGRW   982

hUBPy          KQKLQTSVDFPLENLDLSQYVIGPKNNLKKYNLFSVSNHYGGLDGGHYTAYCKNAARQRW  1080
mUbpY          KQKLQTSVDFPLENLDLSQYVIGPKNSLKKYNLFSVSNHYGGLDGGHYTAYCKNAARQRW  1042

hUBPy          FKFDDHEVSDISVSSVKSSAAYILFYTSLGPRVTDVAT                        1118
mUbpY          FKFDDHEVSDISVSSVRSSAAYILFYTSLGPRITDVAT                        1080
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FIG. 1. **Amino acid sequence of mUBPy predicted open reading frame and comparison with hUBPy.** The sequence in *bold* is that of the 6/12 clone isolated from two-hybrid screening. The *underlined* region identifies Cys and His boxes typical of deubiquitinating enzymes (21, 22); the *dotted underlined region* (aa 198–311) represents the rhodanese/cdc25 fold homology (23). The sequence was deposited in GenBank™, accession number AF057146. The alignment between hUBPy (12) and mUBPy was done with Macaw (15).

Equal numbers of trichloroacetic acid-precipitable counts from cell extracts were immunoprecipitated.

The immunoprecipitates were washed three times with RIPA buffer solution and then resuspended in 50 μl of SDS-sample buffer and separated by SDS-PAGE. The dry gels were exposed to x-ray films (Amersham Pharmacia Biotech), and the intensity of specific bands was analyzed by densitometry with NIH-Image software.

RESULTS

Screening for Proteins Interacting with the N-terminal Region of CDC25^{Mm}, Identification and Cloning of Mouse UBPy—We used the N-terminal region (first 625 aa) of CDC25^{Mm} as a bait for a yeast two-hybrid screening. This fragment comprises all the identified motifs except the catalytic one, *i.e.* the first PH domain, the IQ domain, and the DH-PH module, and it is the same region that, when expressed in mammalian cells, behaves as a dominant negative protein (5). For the screening, we used two different mouse embryo cDNA libraries in pVP16 vector given by S. Hollenberg (11)

After two rounds of selection, we identified three "true" positive clones, all derived from the 10.5-dpc cDNA library. These clones contained small cDNA inserts (about 400 bp), which shared a common overlapping region of ~300 bp. The largest clone (called 6/12) was further characterized for its ability to interact in yeast with different shorter regions of CDC25^{Mm}. A positive interaction was obtained with pBTES plasmid containing the first 506 aa of CDC25^{Mm} and thus lacking the second PH domain, whereas no interaction was observed with pBTEP plasmid that contains only the first PH domain of CDC25^{Mm} (not shown). On the basis of these results, we can conclude that the region of CDC25^{Mm} between aa 145 and aa 506, containing the IQ motif and the DH domain, is required for this *in vivo* interaction. The sequence of the 6/12 clone revealed an open reading frame (3′-5′ open) encoding a short proline-rich polypeptide (119 aa) homologous to a small central region of a human gene product called hUBPy, originally reported to encode a putative deubiquitinating enzyme (20) and recently demonstrated to be actually a deubiquitinase (12).

The full-length mouse cDNA was cloned by RACE-PCR using a mouse embryo-cDNA (Marathon-Ready cDNA, CLONTECH) starting from this central region. Several clones were obtained both for the 3′ region and the 5′ region and were completely sequenced. The 3970-bp cDNA contains a complete open reading frame of 3240 nucleotides encoding a 1080 aa protein, 18 bp upstream the putative AUG start codon and 713 bp downstream the stop codon (Fig. 1). Because the open reading frame shared a great homology (higher than 90%) with the hUBPy, we called this new mouse protein mouse UBPy (mUBPy). The homology is very high in the C-terminal region (97% identity between aa 591–1080) that contains the typical hallmarks of the UBP family of deubiquitinating enzymes, *i.e.* a well conserved UCH-2 domain (aa 739–1072, Profilescan) with the characteristic cysteine and histidine boxes (21, 22). The N-terminal part of mUBPy also contains a highly homologous region (88% identity aa 1–346) followed by a region of lower homology (68% identity aa 346–590). A search for motifs in this

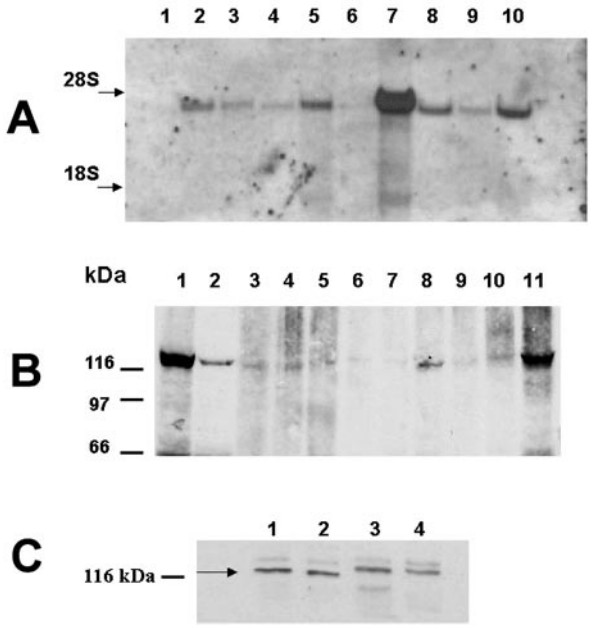

FIG. 2. **Expression of mUBPy in mouse tissues and cell lines.** A, Northern blot analysis. 5 μg of total RNA extracted from various mouse tissues were separated on agarose-formaldehyde gels, blotted to a nylon membrane, and hybridized with a digoxygenin-labeled mUBPy riboprobe. Signals were detected with the Nucleic Acid Detection Kit (Roche Molecular Biochemicals). *Lane 1*, liver; *lane 2*, lung; *lane 3*, thymus; *lane 4*, heart; *lane 5*, 11.5-dpc embryo; *lane 6*, spleen; *lane 7*, testis; *lane 8*, ovary; *lane 9*, kidney; *lane 10*, brain. The amount of RNA loaded in each lane was comparable, as indicated by staining of the gel with ethidium bromide (not shown). *B*, total proteins extracted from adult mouse tissues, separated on SDS-PAGE 7% gels, blotted to nitrocellulose, and probed with anti-mUBPy antibodies. A strong 120-kDa band was observed in brain and testis. About 50 μg of protein were loaded in each lane. *Lane 1*, brain; *lane 2*, lung; *lane 3*, heart; *lane 4*, liver; *lane 5*, spleen; *lane 6*, gut; *lane 7*, kidney; *lane 8*, pancreas; *lane 9*, ovary; *lane 10*, placenta; *lane 11*, testis. *C*, proteins extracted from cultured cells, separated on SDS-PAGE gels, blotted to nitrocellulose, and probed with anti-mUBPy antibodies. *Lane 1*, growing NIH-3T3; *lane 2*, confluent NIH-3T3; *lane 3*, human neuroblastoma cells SK-N-BE; *lane 4*, human Hek-297 cells. About 50 μg of protein were loaded in each lane.

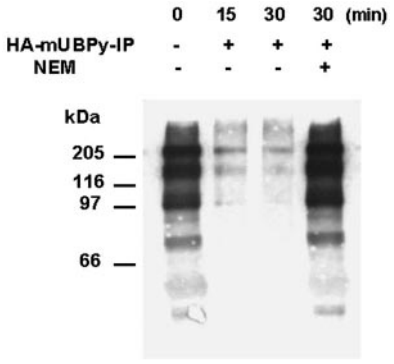

FIG. 3. **mUBPy (aa 542–1080), expressed in COS7 cells, can remove ubiquitin from endogenous ubiquitinated proteins.** Mouse testis extracts were prepared by homogenizing testes in the presence of 20 mM *N*-ethylmaleimide to inactivate endogenous deubiquitinating enzymes (19). Soluble fractions were dialyzed against 50 mM Tris (pH 8), 5 mM $MgCl_2$, and 2 mM dithiothreitol and then centrifuged and washed four times in a Centricon-3 concentrator to remove residual *N*-ethylmaleimide. Aliquots containing 50 μg of total proteins were incubated for 15 or 30 min with an HA-immunoprecipitate obtained from COS7 cells transfected with pCDNA3-mUBPy-HA vector (4 μg/100-mm plate) expressing mUBPy $_{542–1080}$ tagged with HA epitope. The immunoprecipitates of cleared lysate obtained from two dishes were washed three times in HNGT buffer, and the equal aliquots (about ¼ of the total) were used for each assay. As a control, an immunoprecipitate was washed in the presence of 20 mM *N*-ethylmaleimide (+ *NEM*). At the indicated times, samples were quenched with SDS-sample buffer and analyzed by immunoblotting with anti-ubiquitin antibodies.

N-terminal region revealed only a highly conserved Rhodanese/cdc25 fold (aa 198–311) (23). Interestingly the Rhodanese/cdc25 fold has been found in the noncatalytic region of several yeast ubiquitin hydrolases (yeast Ubp4, 5 and 7), in the noncatalytic domain of dual specificity MAPK-phosphatases, and in the yeast phosphotyrosine phosphate-phosphatase (23). Other interesting features of mUBPy protein are the presence of two long proline-rich regions (aa 300–450 and aa 560–740, respectively), which contain several PEST-like motifs, and two coiled-coil regions (aa 95–140 and 460–500).

Expression of mUBPy in Mouse Tissues—The human homolog hUBPy was initially identified as the predicted product of a cDNA found in myeloblasts (20). Successively, Naviglio *et al.* (12) found the protein in several human cell lines (human fibroblasts WI-38, osteosarcoma U2OS, HeLa cells, etc.).

Northern blot analysis performed on RNA extracted from several mouse tissues evidenced the presence of a 4-kilobase mUBPy mRNA in most of the probed tissues (Fig. 2*A*). As expected, this mRNA was also present in mouse embryo and in adult mouse brain; however, mUBPy mRNA was found to be particularly abundant in testis.

Rabbit polyclonal antibodies were raised against a GST fusion protein (containing 119 aa of mUBPy) expressed in *E. coli*. The antiserum was treated with immobilized GST to remove the anti-GST component and then used for Western blot experiments.

An immunoscreening of mouse tissues revealed the presence of a strong specific immunoreactive band of 120 kDa (in agreement with the expected molecular size of the mUBPy open reading frame: 122,579 Da) in mouse brain and testis homogenates (Fig. 2*B*). A lower signal was observed in pancreas, lung, small intestine, and placenta homogenates, whereas a barely detectable signal was present in liver, spleen, kidney, and heart (Fig. 2*B*). These results are generally in agreement with the results obtained with Northern blot experiments. Therefore the mUBPy protein is expressed at a very low level in most of the adult mouse tissues with a strong expression in brain and testis only. Moreover, mUBPy is not an easily soluble protein because to detect it in most tissues, we needed strong detergent conditions (*i.e.* 7% SDS). When a mild extraction procedure (1% Triton X-100) was used, mUBPy could be detected only in brain and testis (not shown). Since the human homolog hUBPy is expressed in several cell lines (12), we also looked for the expression of the mUBPy protein in mouse fibroblasts; moreover we also probed two human cell lines with our antibodies.

A 120-kDa band was detected in the mouse NIH-3T3 fibroblasts extract (Fig. 2*C*, *lanes 1* and *2*); however, no difference was observed between proliferating and density-arrested NIH-3T3 fibroblasts. In addition a sharp 130-kDa band was detected in both Hek-293 cells and SN-K-BE neuroblastoma cells, indicating that our antibodies also recognize the human hUBPy protein. This was not unexpected because the protein region we used for the preparation of anti-mUBPy polyclonal antibodies contains a large amino acid sequence (aa 592–657 of mUBPy, see Fig. 1), which is identical in the two proteins.

mUBPy Is a Deubiquitinating Enzyme—Since mUBPy has a high homology with hUBPy that has been demonstrated to have a UBP (ubiquitin-isopeptidase) activity (12), we expected that also mUBPy has an UBP activity. To verify this, we used a mouse testis protein extract, pretreated with *N*-ethylmaleimide, as a source of ubiquitinated proteins as reported by Lin *et al.* (19). As shown in Fig. 3, the addition of HA-immunoprecipitates from COS7 cells transfected with HA-tagged mUBPy (aa 542–1080) expressing vector greatly reduced the amount of ubiquitinated protein recognized by anti-ubiquitin antibodies,

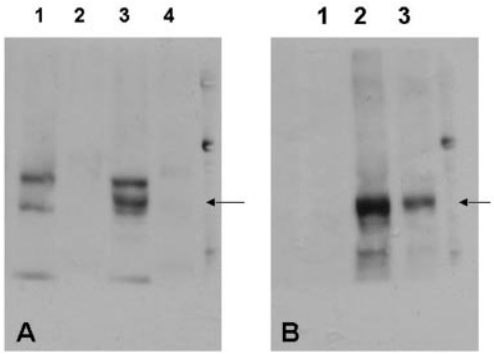

FIG. 4. ***In vitro* interaction of mUBPy (aa 542–660) with the N-terminal domain of CDC25^{Mm} (aa 1–625).** COS7 cells transfected with pHC21 vector, expressing the N-terminal region of CDC25^{Mm} (aa 1–625) (5),(5 μg/100-mm plate), or untransfected COS7 cells were lysed with HGNT buffer. Lysates were incubated with glutathione-Sepharose resin loaded with either GST-mUBPy$_{542–660}$ or GST alone as described (see "Experimental Procedures"). Protein bound to the resin was recovered and processed for Western blot analysis with anti Ras-GRF1 antibodies. *A*, Western blot analysis of protein bound to glutathione-Sepharose beads coupled to GST-mUBPy$_{542–660}$ (*lanes 1* and *3*) or GST alone (*lanes 2* and *4*). *Lanes 1* and *2*, control COS7 cells; *lanes 3* and *4*, extracts of COS7 cells expressing CDC25$^{Mm}_{1–625}$. *B*, immunodetection of CDC25$^{Mm}_{1–625}$ on extracts of COS7 cells. *Lane 1*, total cell protein (lysis in RIPA buffer) of control COS7 cells; *lane 2*, total cell protein of COS7 cells expressing CDC25$^{Mm}_{1–625}$; *lane 3*, lysis buffer (HGNT) extract of COS7 cells expressing CDC25$^{Mm}_{1–625}$. The *arrows* indicate the 70-kDa band expected for CDC25$^{Mm}_{1–625}$. *IP*, immunoprecipitate.

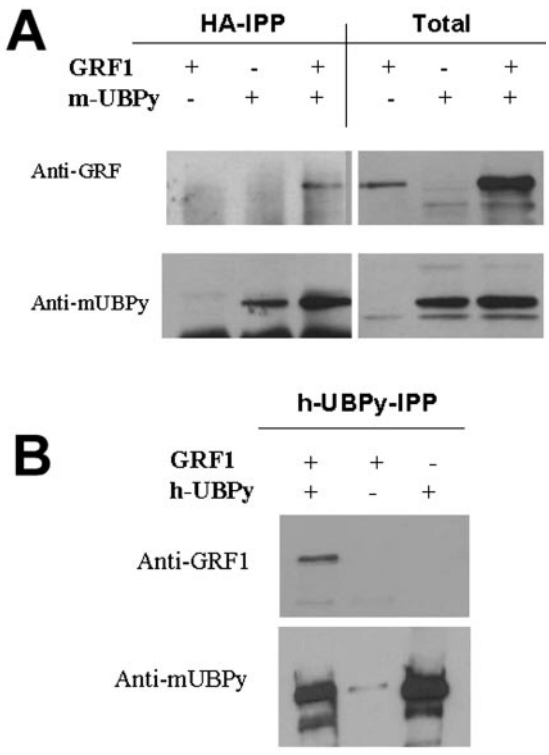

FIG. 5. ***In vivo* interaction between CDC25^{Mm} and mUBPy and hUBPy.** *A*, COS7 cells were transfected with 4 μg/100 mm plate of the indicated vectors (*GRF1*, pCDNA3-CDC25^{Mm} expressing the full-length CDC25^{Mm} protein; *mUBPy*, pCDNA3-mUBPy-HA expressing mUBPy$_{542–1080}$ tagged with HA epitope) using pCDNA3 when required to keep the total DNA to 8 μg/plate. After 48 h, cells were harvested and lysed in HGNT buffer, and lysates were immunoprecipitated with anti-HA antibodies. Immunoprecipitated proteins (*IPP*) and equal aliquots of cleared total lysates (30 μl) were separated by SDS-PAGE, blotted, and probed with either anti-GRF1 antibodies or anti-mUBPy antibodies. *B*, Hek-293 cells were transfected with 4 μg/100-mm plate of the indicated vectors (*GRF1*, pCDNA3-CDC25^{Mm} expressing the full-length CDC25^{Mm} protein; *hUBPy*, pCDNA3-hUBPy expressing hUBPy (12)) using pCDNA3 when required to keep the total DNA to 8 μg/plate. After 48 h, cells were harvested and lysed in HGNT buffer, and lysates were immunoprecipitated with anti-mUBPy antibodies. Immunoprecipitated proteins were separated by SDS-PAGE, blotted, and probed with either anti-GRF1 antibodies or anti-mUBPy antibodies.

indicating that mUBPy can deubiquitinate ubiquitin from high molecular weight ubiquitinated proteins.

Interaction of mUBPy with CDC25^{Mm}—We have previously identified mUBPy in a two-hybrid screening for protein interaction with the Ras-GEF CDC25^{Mm}. It was, however, important to confirm this interaction directly *in vitro* and successively in an *in vivo* assay using mammalian cells.

A GST fusion protein containing the 119 aa of the clone 6/12 found to be positive in the yeast two-hybrid screening was tested for *in vitro* interaction with the N-terminal region of CDC25^{Mm} by pull-down experiments. As shown in Fig. 4, a specific interaction with the CDC25^{Mm} N-terminal fragment can be revealed. This result is in agreement with that obtained by the yeast two-hybrid system.

To validate the above results, we tested the *in vivo* interaction between CDC25^{Mm} and mUBPy by co-expressing the two proteins in mammalian cells. CDC25^{Mm} and a HA-tagged mUBPy (aa 542–1080) were transfected in COS7 cells either separately or together. 48 h after transfection, lysates were prepared and processed for immunoprecipitation with anti-HA antibodies. The recovered immunocomplexes were separated by SDS-PAGE and blotted. The blots were probed with anti-Ras-GRF1 antibodies and with polyclonal antibodies raised against mUBPy. Fig. 5*A* shows that CDC25^{Mm} was coimmunoprecipitated only from extracts obtained by cells expressing both CDC25^{Mm} and HA-mUBPy (*lane 3*) but not from extracts of cells transfected with CDC25^{Mm} alone or with HA-mUBPy alone (*lanes 1–2*). Aliquots of the same total cell lysates were loaded in the same gel to check the expression of CDC25^{Mm} (*lane 4*), HA-mUBPy (*lane 5*), and both proteins in cotransfected cells (*lane 6*). This experiment clearly demonstrates an *in vivo* interaction between CDC25^{Mm} and mUBPy. Interestingly the amount of total CDC25^{Mm} protein found in COS7 cells lysates increased when the mUBPy was co-expressed, suggesting a potential ability of mUBPy to regulate the levels of CDC25^{Mm}.

To test if the human homolog hUBPy is also able to interact *in vivo* with CDC25^{Mm}, we performed similar cotransfection experiments. Briefly Hek-293 cells were cotransfected with pCDN3-RasGRF1 and pCDNA3-hUBPy, and then the cell lysates were immunoprecipitated with anti-mUPBy antibodies and immunoblotted with anti-GRF1 antibodies. As shown in Fig. 5*B*, CDC25^{Mm} was coimmunoprecipitated only if hUBPy was expressed; this indicates that the human homolog is also able to interact with CDC25^{Mm}. When the same filter was probed with anti-mUBPy antibodies, a strong band was detected in extracts of cells transfected with hUBPy, whereas a weak immunoreactive band was also detected in cells not treated with pCDNA3-hUBPy, which is likely due to endogenous hUBPy protein.

CDC25^{Mm} Is Ubiquitinated in Vivo and mUBPy Decreases Its Level of Ubiquitination—As a general rule, regulatory proteins are subjected to a fast turnover, and this is expected to be the case also for the Ras exchange factors. At our knowledge, no data regarding the turnover of CDC25^{Mm}/RasGRF1 have been so far reported in the literature, although RasGRF1 is known to contain a PEST motif (4). However, it has been recently shown that RasGRF2, a protein closely related to RasGRF1, is ubiquitinated and is likely degraded by the 26 S proteasome (24); in addition the ubiquitous mammalian Ras GEF, hSos2, is also rapidly degraded through an ubiquitination step (25). Since we found an interaction between CDC25^{Mm} and a deubiquitinating enzyme (mUBPy), it is reasonable to suppose that

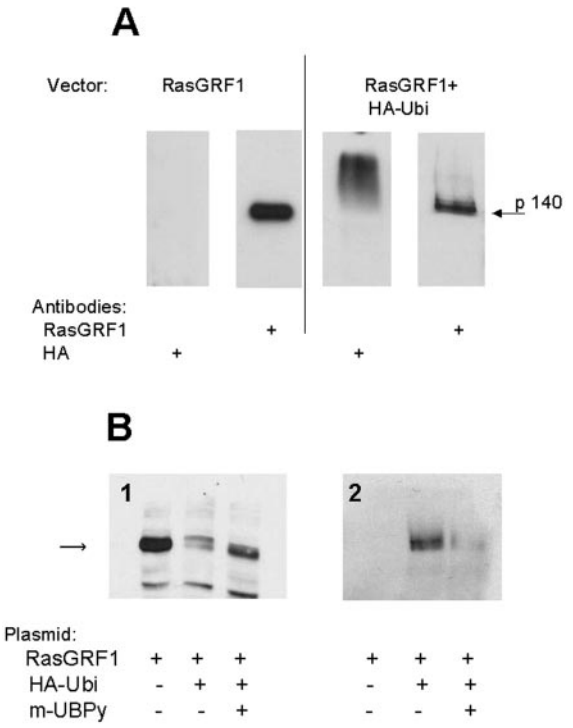

FIG. 6. ***In vivo* ubiquitination of CDC25^{Mm}.** Hek-293 cells were cotransfected with the different plasmids (*RasGRF1*, pCDNA3-CDC25^{Mm} expressing the full-length CDC25Mm; *HA-Ubi*, a pCDNA3 vector expressing HA-tagged ubiquitin; *mUBPy*, pCDNA3-mUBPy expressing the full-length mUPBy). 2 μg of each plasmid were used, keeping a total of 6 μg of DNA/60-mm dish with the empty pCDNA3 vector if required. Cells were lysed in RIPA buffer after 48 h. *A*, equal amounts of protein lysates immunoprecipitated with anti GRF1 antibodies, separated by SDS-PAGE, blotted, and probed with anti-GRF1 or anti-Ha antibodies. *B*, *panel 1*, equal amounts of total proteins (30 μg) separated by SDS-PAGE, blotted, and probed with anti-GRF1 antibodies. The *arrow* indicates the 140-kDa CDC25^{Mm} protein. *Panel 2*, equal amounts of the same lysates used for *panel 1* immunoprecipitated with anti-HA antibodies and probed with anti-GRF1 antibodies. A diffuse band corresponding to ubiquitinated CDC25^{Mm} species with an apparent molecular size of 150–160 kDa is evident in the *second lane* and barely detectable in the *third lane*.

CDC25^{Mm} could be ubiquitinated and then rapidly degraded.

To assay *in vivo* the ubiquitination of CDC25^{Mm}, we cotransfected Hek-293 cells with pCDNA3-RasGRF1 and HA-ubiquitin vector (HA-Ub) (26). The epitope-tagged ubiquitin can be correctly conjugated *in vivo* to cellular proteins, which then become targets for proteolytic cleavage (26). The HA-ubiquitin allows a good quantitative and sensitive detection of ubiquitination of proteins either by immunoblotting or immunoprecipitation with anti-HA monoclonal antibodies (12, 26).

As shown in Fig. 6*A*, immunoprecipitation with anti-RasGRF1 antibodies and immunoblotting with anti-HA allows the detection of high molecular weight HA-immunoreactive species, indicating that CDC25^{Mm}/Ras-GRF1 is ubiquitinated under our experimental conditions.

In other experiments, we cotransfected Hek-293 cells with pCDNA3-RasGRF, HA-Ub, and also with a plasmid expressing mUBPy (Fig. 6*B*). The amount of CDC25^{Mm} present in total extracts clearly decreased in the presence of HA-Ub, suggesting that an increased availability of ubiquitin might induce a faster turnover of CDC25^{Mm} protein, whereas a partial recovery was observed in cotransfection with mUBPy (Fig. 6*B*, *panel 1*). More interestingly, the amount of ubiquitinated CDC25^{Mm} forms detected with anti-RasGRF1 antibodies on HA-immunoprecipitates clearly decreased in the presence of mUBPy (Fig. 6*B*, *panel 2*, *third lane*; compare *third lane* with *second lane*).

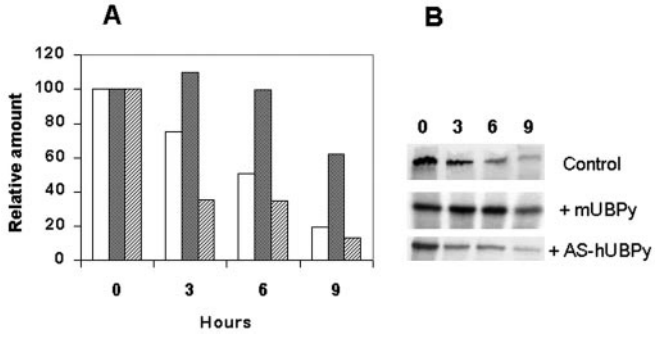

FIG. 7. **Stability of CDC25^{Mm} protein expressed in Hek-293 cells.** Hek-293 cells were transfected with pCDNA3-CDC25^{Mm} (*Control*) or cotransfected with pCDNA3-CDC25^{Mm} and pCDNA3-mUBPy (*+mUBPy*) or pCDNA3-CDC25^{Mm} and pCDNA3-hUBPy-Antisense (*+AS-hUBPy*). For transfection, we used 1 μg of pCDNA3-CDC25^{Mm} and 1.5 μg of the other plasmids/60-mm dish with the empty pCDNA3 vector if required. After 24 h, cells were labeled for 2 h with [^{35}S]methionine (100 μC/dish) and then chased with nonradiolabeled methionine for the number of hours indicated, immunoprecipitated with anti-RasGRF1, and processed for SDS-PAGE. The gels were autoradiographed, and the intensity of the specific bands was analyzed by densitometry. *A*, relative amount of labeled CDC25^{Mm} immunoprecipitated after the chase. *Open bar*, control; *black bar*, +mUBPy; *gray bar*, + AS-hUBPy. *B*, autoradiography of the immunoprecipitated labeled CDC25^{Mm} after 0, 3, 6, and 9 h of chase.

mUBPy Stabilizes the CDC25^{Mm} Protein Expressed in Hek-293 Cells—The finding that Ras-GRF1/CDC25^{Mm} is ubiquitinated *in vivo* strongly suggests that this protein may be rapidly degraded. To measure the stability of the protein, Hek-293 cells transfected with a vector expressing CDC25^{Mm} were metabolically labeled and subjected to a pulse-chase analysis (Fig. 7). The apparent half-life of CDC25^{Mm} was about 5–6 h, thus confirming that this protein is unstable, at least in our experimental conditions.

In parallel experiments, we cotransfected Hek-293 cells with plasmids expressing CDC25^{Mm} and mUBPy or with a vector expressing the antisense of hUBPy (12). The latter was used because Naviglio *et al.* (12) showed that the cDNA of hUBPy cloned in the antisense orientation increased protein ubiquitination, likely by interfering with the expression of endogenous hUBPy that it is present in most human cell lines and also in Hek-293 (Fig. 2).

Clearly the expression of mUBPy stabilized the CDC25^{Mm} protein (Fig. 7), the half-life increases to more than 9 h, whereas the expression of antisense of hUBPy further destabilized the protein (Fig. 7). These data strongly support the hypothesis that mUBPy (and its homolog hUBPy) can regulate the turnover of CDC25^{Mm}.

DISCUSSION

We started this work with the aim to isolate and identify regulatory proteins able to interact with the large N-terminal part of the brain-specific Ras exchange factor CDC25^{Mm} (RasGRF1). At the moment our results partially fulfill this purpose because we have identified a CDC25^{Mm} interacting protein, a deubiquitinating enzyme called mUBPy, highly homologous to the human hUBPy as described by Naviglio *et al.* (12).

At first glance, a deubiquitinating enzyme should not be directly involved in signal transduction mechanisms, but actually there is increasing evidence in the literature that deubiquitinases may play relevant roles in several pathways controlling growth and/or differentiation. In fact the deubiquitinating enzymes are thought to act also through the stabilization of some key components of the pathway, counterbalancing the activity of specific ubiquitinating factor(s) (27). It is worthwhile to mention that the *faf* gene product, involved in eye develop-

ment in *Drosophila* (28). In yeast the *UBP*3 and *UBP*4 (named also *DOA*4) gene products, are involved in transcriptional regulation (21, 29), whereas DUB1 protein is required for cell cycle progression in mouse lymphocytes (30), and UNP deubiquitinase causes tumors in nude mice (31) and is overexpressed in human small cell lung primary carcinoma (32). These few examples indicate that deubiquitinating enzymes could interact in a specific way with a limited variety of substrates. Indeed, more than 90 deubiquitinating enzymes have been so far identified (27), and this may explain the marked heterogeneity in their sequence, apart from the catalytic domain. This peculiarity is present also in mUBPy; in fact its large N-terminal region (aa 1–700) shows no homology with any other known deubiquitinase with the exception of hUBPy, clearly a human homolog of the mouse protein.

Our results indicate that mUBPy interacts both *in vitro* and *in vivo* with CDC25^{Mm}. The region of interaction of mUBPy (aa 540–660) is proline-rich and shows no homology with any other motif known to mediate protein-protein interactions. A similar interaction is here shown to occur also between hUBPy and CDC25^{Mm}. The physiological meaning of this novel interaction is not yet known; however, here we show that CDC25^{Mm} is ubiquitinated and that its ubiquitination level can be reduced by coexpression of mUBPy.

Furthermore the expression of mUBPy stabilizes the CDC25^{Mm} protein expressed in Hek-293 increasing its half-life. Therefore we could hypothesize that mUBPy can take part in the regulation of CDC25^{Mm} turnover in neurons or alternatively play a more general role in the control of proteasome function as previously suggested (12). Further work is needed to clarify its specific role.

The *mUBPy* gene is expressed in several mouse tissues although at a different extent, with a marked expression in the adult testis. At the protein level, we detected a preferential expression in the brain and in the testis. The mUBPy protein was also detected in several other tissues (pancreas, placenta, etc.) were it was expressed at a much lower level. In addition we found a good expression of this protein also in mouse NIH3T3 fibroblasts, in agreement with the data of Naviglio *et al.* (12), who found the human hUBPy in most human cell lines. The pattern of mUBPy expression in mouse tissues is not unusual because several gene products have been found to be specifically or predominantly expressed in brain and testis (33), including another deubiquitinating enzyme belonging to the family of ubiquitin C-terminal hydrolases, UCH-L1 (34). Preliminary results[2] indicate that mUBPy is expressed in the germ cell component of the testis and that it is still present in mature spermatozoa.

On the whole, our results suggest that mUBPy may be involved in different deubiquitinating processes in the different tissues. For example, in the tissues and cell lines where mUBPy is expressed at a low level, the protein could participate in the deubiquitination processes of proteasome substrates (12), whereas in the tissues where mUBPy is strongly expressed (brain and testis), it could exert a more specialized function through its interaction with specific substrates. One of these substrates can reasonably be the brain-specific Ras exchange factor CDC25^{Mm}/Ras-GRF1.

[2] G. Berruti and E. Martegani, unpublished results.

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Cloning and Characterization of Mouse UBPy, a Deubiquitinating Enzyme That Interacts with the Ras Guanine Nucleotide Exchange Factor CDC25 ^{Mm}/Ras-GRF1

Nerina Gnesutta, Michela Ceriani, Metello Innocenti, Isabella Mauri, Renata Zippel, Emmapaola Sturani, Barbara Borgonovo, Giovanna Berruti and Enzo Martegani

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