mRNAs in eukaryotic cells are presumed to always associate with a set of proteins to form mRNPs. In *Xenopus* oocytes, a large pool of maternal mRNAs is masked from the translational apparatus as storage mRNPs. Here we identified *Xenopus* RAP55 (xRAP55) as a component of RNPs that associate with FRGY2, the principal component of maternal mRNPs. RAP55 is a member of the Scd6 or Lsm14 family. RAP55 localized to cytoplasmic foci in *Xenopus* oocytes and the processing bodies (P-bodies) in cultured human cells: in the latter cells, RAP55 is an essential constituent of the P-bodies. We isolated xRAP55-containing complexes from *Xenopus* oocytes and identified xRAP55-associated proteins, including a DEAD-box protein, Xp54, and a protein arginine methyltransferase, PRMT1. Recombinant xRAP55 repressed translation, together with Xp54, in an *in vitro* translation system. In addition, xRAP55 repressed translation in oocytes when tethered with a reporter mRNA. Domain analyses revealed that the N-terminal region of RAP55, including the Lsm domain, is important for the localization to P-bodies and translational repression. Taken together, our results suggest that xRAP55 is involved in translational repression of mRNA as a component of storage mRNPs.

Translational control of maternal mRNAs is a key step in the regulation of gene expression in animal oocytes. In *Xenopus laevis*, the oocyte genome is active in transcription, providing a large pool of maternal mRNA in the cytoplasm. During oocyte maturation into an egg and early embryogenesis, these mRNAs together with maternal proteins provide the building materials of eggs and embryos (1). mRNAs stored in oocytes are masked from the translational machinery by associating with a set of proteins in storage messenger ribonucleoproteins (mRNPs).2

Growing evidence indicates that mRNAs move between nontranslating (stored) and translating mRNPs states in the cytoplasm not only in germ cells but also in cell types as diverse as neurons and yeast (2–4). Studies on mRNA masking have shed light on several mechanisms by which mRNP components determine whether a given mRNA synthesized *in vivo* is actively translated or stored (5, 6). The most studied are those mRNAs carrying the cytoplasmic polyadenylation element, CPE, in their 3′ untranslated regions (UTRs). The CPE functions with its cognate trans-acting factor CPE-binding protein (CPEB) in RNA masking in immature oocytes (7). CPEB associates with Maskin, which in turn binds to the cap-binding protein eIF4E and precludes the formation of an eIF4E-eIF4G complex that is required for cap-dependent translation (8). During oocyte maturation and early embryogenesis, changes in the length of the poly(A) tail, which occur dependent on or independent of specific cis-elements, have an impact on the translational activity of a mRNA (7, 9–11). Alternatively, the splicing process in the nucleus deposits a protein complex at the exon-exon junction and thereby determines the translational efficiency of an mRNA in the cytoplasm, which demonstrates that a specific gene structure affects translation (12, 13). Furthermore, the amount and activity of the Y-box protein, a core component of cytoplasmic mRNPs, have been shown to significantly affect general translational activity in *Xenopus* oocytes and in somatic cells of different organisms (14, 15).

Y-box proteins FRGY2 and MRNP3 were identified as major RNA-binding components of storage mRNPs in *Xenopus* oocytes (16, 17). Y-box proteins are likely responsible for the packaging of mRNAs into mRNPs (18, 19). The addition of a sufficient excess of Y-box proteins into *in vitro* translation systems leads to a translational repression of reporter mRNAs (20–22). Later studies identified other components of mRNPs, including a DEAD-box ATPase Xp54 and embryonic poly(A)-binding proteins, ePAB and ePABP2 (23–27). Xp54 and its human homologue RCK repress translation *in vitro* and/or in oocytes (28, 29). Xp54 interacts with CPEB in oocytes, depending on the homo-oligomeric formation of Xp54 (30). Xp54 associates with nascent transcripts in the nucleus and, therein, is involved in the assembly of storage mRNPs (31). Furthermore, yeast genetic studies demonstrated that Dhh1p, which is the yeast homologue of Xp54, is involved in general translational repression (29).

To further identify and characterize the components of cytoplasmic mRNPs in *Xenopus* oocytes, we isolated complexes containing FRGY2 from *Xenopus* oocytes. In this report we demonstrate that xRAP55 is communoprecipitated with
FRGY2 in an RNA-dependent manner. We show that xRAP55 and its human homologue localize to cytoplasmic foci in oocytes and cultured cells and that xRAP55 is a component of storage mRNPs and acts as a translational repressor.

**MATERIALS AND METHODS**

**Immunoprecipitation**—50 oocytes expressing FRGY2-FLAG or 3× FLAG-xRAP55 were homogenized in 1 ml of buffer A (90 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM dithiothreitol (DTT), 0.1% Nonidet P-40, and 5% sucrose). The homogenate was centrifuged in a 1.5 ml tube at 15,000 rpm for 10 min at 4 °C to prepare oocyte lysates. To digest RNA, RNase A was added to the oocyte lysates at a final concentration of 50 μg/ml, and the mixture was incubated for 10 min at 37 °C. Oocytes lysates were incubated with 20 μl of anti-FLAG M2 affinity gel (Sigma) for 2 h at 4 °C. The affinity gel was extensively washed with buffer A, and complexes containing FLAG-tagged proteins were eluted with 150 μg/ml of 3× FLAG peptide (Sigma) in 50 mM Tris-HCl (pH 7.5)/150 mM NaCl for 5 min at room temperature. Tandem mass spectrometric analyses of the tryptic peptides were carried out at the Research Resources Center at our institute. RNA was prepared from the lysate treated with or without RNase, analyzed in an agarose gel containing formaldehyde, and visualized by staining with ethidium bromide.

**xRAP55 cDNA Cloning**—A 1444-bp fragment encoding xRAP55 based on the sequence of *Xenopus* expressed sequence tag clones AW200392, BG408666, BG486665, and BE505383 was amplified by PCR from total cDNA of *Xenopus* oocytes using primers, 5′-CGCGGATCCGGCTAGCATGAGCGGG-GGTACTCCATACTAGGTTAG-3′ (KM240) and 5′-CCGAGCTCTGACGAGGCTGCTACCTTTGTTGTTCTTTCT-TATCCA-3′ and was used as a probe to screen a *Xenopus* cDNA library as described previously (32). The nucleotide sequence of the xRAP55 cDNA obtained in this study will appear in the DDBJ/EMBL/GenBank™ nucleotide sequence databases under accession number AB257699.

**Preparation and Microinjection of Xenopus Oocytes**—Ovaries were isolated from *X. laevis* frogs anesthetized in iced water. Defolliculated oocytes were prepared by treating frog ovaries with collagenase. Stage VI oocytes were injected with 5 ng of capped mRNA (unless otherwise specified) and incubated at 18 °C for 16 h in modified Barth’s solution (12). To induce maturation, oocytes were treated with 10 μg/ml progesterone at 20 °C for 8 h. Oocyte lysates were prepared as described previously (32). Where indicated, the lysates from stage VI oocytes and matured oocytes were treated with 0.2–2 units/μl calf intestine alkaline phosphatase (Takara) at 37 °C for 30 min. Immunoblotting was performed as described previously (32).

**Immunocytochemistry of Xenopus Oocytes**—For the immunocytological staining, oocytes and eggs were fixed with 100% methanol or MEMFA (0.1 M MOPS (pH 7.4), 2 mM EGTA, 1 mM MgSO_4_ and 3.7% formaldehyde) and were embedded in Poly-ester Wax (BDH Chemicals, UK) (33). Sectioned specimens were stained with primary and secondary antibodies as described previously (22). The primary antibodies were used at a final concentration of 2 μg/ml. The secondary antibodies were Alexa Fluor 488- or 568-conjugated goat anti-rabbit or anti-mouse IgG antibodies (2 μg/ml, Molecular Probes). The specimens were observed under a laser confocal microscope (Leica TCS-NT).

**Cell Culture**—HeLa S3 cells were maintained as described (34). Cells grown on a glass coverslip in a 24-well plate were transfected with plasmid DNAs using Lipofectamine 2000 (Invitrogen) following the manufacturer’s recommendations. Cells were transfected with small interfering RNA (siRNA) using Oligofectamine following the manufacturer’s instructions, except that the transfection was repeated after 4 h. The sense strands of siRNA used were: RAP55 siRNA, 5′-CCGUG-GGAGUGACAUUAAAGACCUCUAG-3′ and control siRNA, 5′-AAUUCCUCGGAGGUCACGUU-3′. 24 or 48 h after DNA transfection or 3 days after siRNA transfection, cells were fixed for immunocytochemistry or lysed for immunoblotting. Immunocytochemistry was performed as described (34) except that the concentration of bovine serum albumin used for blocking was 1%. The secondary antibodies were Alexa Fluor 488- or 594-conjugated goat anti-rabbit or anti-mouse IgG antibodies (2 μg/ml, Molecular Probes). Nuclei were counterstained with TO-PRO-3 (Molecular Probes). The cells were examined under a laser confocal microscope (Zeiss LSM510).

**Sucrose Gradient Centrifugation**—Sucrose gradient fractionation of oocyte lysates was performed essentially as described previously (35). Fifteen stage VI oocytes were homogenized in 150 μl of buffer B (20 mM Tris–HCl (pH 7.5), 50 mM NaCl, 1 mM DTT, 25 units/ml RNase inhibitor, 2 mM MgCl_2_, and 10 μg/ml cycloheximide). The homogenate was centrifuged in a 1.5 ml tube at 15,000 rpm for 10 min. The supernatant was loaded onto a 15–40% sucrose density gradient prepared in buffer B and centrifuged at 48,000 rpm in a Hitachi P5052 rotor for 2 h at 4 °C. For the EDTA treatment, all the buffers contained 20 μM EDTA instead of MgCl_2_ and cycloheximide. The samples were collected from the top of the gradient in 18 fractions of 280 μl by a piston gradient fractionator (Biocomp). For immunoblotting, a 10-μl aliquot of each fraction was analyzed by SDS-PAGE. RNA was prepared from a 100-μl aliquot of each fraction and analyzed in an agarose gel containing formaldehyde.

**Oligo(dT)-Cellulose Chromatography and UV Cross-linking Assay**—200 oocytes were homogenized in a 500-μl solution of 10 mM Tris–HCl (pH 7.5), 50 mM NaCl, 2 mM MgCl_2_, 8% glycerol, 1 mM DTT, and 100 units/ml RNase inhibitor. The homogenate was centrifuged in a 1.5 ml tube at 15,000 rpm for 10 min at 4 °C. The supernatant was adjusted to 250 mM NaCl, mixed with 200 μl of oligo(dT)-cellulose beads (GE Healthcare), and rotated for 1 h at 4 °C. The cellulose beads were then washed twice with buffer C (10 mM Tris–HCl (pH 7.5), 250 mM NaCl, and 2 mM MgCl_2_), and the bound fraction was eluted with 400 μl of buffer C containing 25% or 50% formamide (36).

**For the UV cross-linking assay,** 200 oocytes were homogenized in 500 μl of protein extraction buffer (90 mM HEPES (pH 7.5), 70 mM KCl, 1 mM DTT, and 5% sucrose). The homogenate was centrifuged in a 1.5 ml tube at 15,000 rpm for 10 min at 4 °C. The supernatant was irradiated with UV light for 5 min on ice, denatured at 65 °C for 5 min, mixed with 250 μl of oligo(dT)–cellulose beads, and rotated for 15 min at room temperature. The cellulose beads were then washed five times with 1 ml of 10 mM Tris–HCl (pH 7.5), 250 mM NaCl, 1 mM EDTA, and 0.5% SDS, and the bound fraction was eluted with elution buffer (10
mm Tris-HCl (pH 7.5), 1 mM EDTA, and 0.05% SDS) at 65 °C for 5 min. The eluates were treated with RNase A (25 μg/ml) for 20 min at 37 °C and analyzed by immunoblotting.

**GST Pulldown Assay**—For GST pull-down assays with purified proteins, 1.5 μg of GST or GST-xRAP55 bound to glutathione-Sepharose was incubated with 2 μg of recombinant Xp54-His6 in buffer D (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM MgCl2, and 0.1% Nonidet P-40) containing 0.4% bovine serum albumin for 1 h at 4 °C and washed twice with buffer D. The bound materials were analyzed by immunoblotting with anti-histidine tag antibody.

**Gel Retardation Assay**—A partial Vg1 3′-UTR fragment (VLE (37)) was prepared by PCR from total cDNA of Xenopus oocytes using 5′-AAATATTTCTATTATTCTCAC-3′ and 5′-CTCAAATCATATGACTATT-3′. The PCR product was cloned into pGEM-Teasy to obtain pGEM-Teasy-VLE. pGEM-Teasy-VLE linearized with Sall was used for in vitro transcription with a MEGAscript T7 kit (Ambion) in the presence of [α-32P]UTP (25 μCi, GE Healthcare). 100 fmol of in vitro synthesized mRNA and recombinant GST-xRAP55 and Xp54-His6 were incubated at 30 °C for 20 min in a 10-μl reaction mixture consisting of 10 mM triethanolamine (pH 7.9), 50 mM KCl, 4 mM MgCl2, 1 mM DTT, and 2 units/ml RNase inhibitor. The reaction mixture was electrophoresed in a 1% agarose gel in 0.5× Tris borate/EDTA buffer (35, 38). The gel was then dried and subjected to autoradiography.

**In Vitro Translation**—β-Actin and chloramphenicol acetyltransferase mRNAs (33P-labeled) were prepared using plasmids pSPcACT1 and pSPCAT, respectively, as described previously (39). 500 fmol of β-actin or chloramphenicol acetyltransferase mRNA was incubated with various amounts of GST-xRAP55 and Xp54-His6 at 30 °C for 20 min in a 10-μl reaction mixture consisting of 10 mM triethanolamine (pH 7.9), 50 mM KCl, 4 mM MgCl2, 1 mM DTT, and 2 units/ml RNase inhibitor. The reaction mixture was electrophoresed in a 1% agarose gel in 0.5× Tris borate/EDTA buffer (35, 38). The gel was then dried and subjected to autoradiography.

**RESULTS**

**Identification of the Components of FRGY2-associated Complexes**—Given that FRGY2 is one of the major components of Xenopus maternal mRNPs, we attempted to search for proteins that interact with FRGY2 in oocytes. To do this, we micro-injected mRNA encoding a FLAG epitope-tagged FRGY2 into oocytes. The FRGY2-FLAG protein was successfully incorporated into mRNPs as judged by fractionation on a sucrose gradient (data not shown, but see Ref. 20). FRGY2-associated complexes were immunoprecipitated from the oocytes with anti-FLAG antibodies. A number of proteins were specifically immunoprecipitated from FRGY2-FLAG-expressing oocytes but not from control oocytes (Fig. 1). RNase treatment prior to immunoprecipitation revealed that the association between FRGY2 and most proteins is likely to be mediated primarily by their binding to the same RNA molecules, with the notable exception of YBA1, a chicken homologue of which has been shown to bind directly to the Y-box protein YB-1 (39). We identified some of the FRGY2-associated proteins by mass spectrometry, and proteins identified are listed on the right. Xp54 in the immunoprecipitates was detected by immunoblotting (bottom).

To prepare RNA, 10 oocytes were lysed in TRI reagent, and RNA was analyzed as described above.
various nuclear RNAs. In addition to these prototypic Lsm rings, the Lsm2–8 ring occurs in the nucleus and is involved in the processing of decapping activator during mRNA degradation. The Lsm1–7 ring occurs in the cytoplasm and functions as a form heteroheptameric rings that bind to single-stranded RNA.

Eukaryotic Lsm domain-containing proteins (Lsm proteins) are putative RNA-binding domains (Fig. 2). The open reading frame encodes a protein of 471 amino acid residues that has 74% identity with Pleurodeles RAP55. xRAP55 possesses an Lsm domain at its N terminus and two RGG-rich domains separated by an FDF domain at its C terminus. The FDF domain is a consensus motif found in Lsm13–16 proteins.

We isolated a cDNA clone encoding the xRAP55 protein from a Xenopus cDNA library. The open reading frame encodes a protein of 471 amino acid residues that has 74% identity with Pleurodeles RAP55. xRAP55 protein possesses an Lsm domain at its N terminus and two RGG-rich domains (RGG1 and RGG2) in its C-terminal region, all of which are putative RNA-binding domains (Fig. 2A). Eukaryotic Lsm domain-containing proteins (Lsm proteins) form heteroheptameric rings that bind to single-stranded RNA and are involved in various steps of RNA metabolism (41, 42). The Lsm1–7 ring occurs in the cytoplasm and functions as a decapping activator during mRNA degradation. The Lsm2–8 ring occurs in the nucleus and is involved in the processing of various nuclear RNAs. In addition to these prototypic Lsm proteins, xRAP55 is a member of the Lsm14 family, or the Scd6 family that was named after the Saccharomyces cerevisiae member Scd6p (43). The C-terminal RGG domains are separated by the FDF domain, which is commonly found in Lsm13–16 proteins.

To facilitate characterization of xRAP55, we have raised a polyclonal antibody against a synthetic peptide corresponding to the C terminus of xRAP55. This antibody reacted with a 60-kDa protein in oocyte lysates, which is most likely xRAP55 (Fig. 2B). Moreover, it detected the recombinant xRAP55 and human RAP55 (hRAP55) proteins (data not shown). Using this antibody, we examined the expression of xRAP55 in oocytes and early embryos. The amount of xRAP55 increased during the oocyte growth, which corresponds with the accumulation of maternal mRNA (Fig. 2C). A similar level of xRAP55 expression was observed throughout early development until the tail bud stage (Fig. 2D). Reprobing with anti-Ela (the Xenopus homologue of HuR) and anti-xCIRP2 served as the loading controls (35, 45).

We noticed a slight decrease in the mobility of xRAP55 in matured oocytes and unfertilized eggs compared with that in immature stage VI oocytes. We therefore tested whether the slow migrating xRAP55 is phosphorylated, and found that, upon treatment with alkaline phosphatase, xRAP55 in matured oocytes migrated as fast as that in stage VI oocytes (Fig. 2E). However, the actual level of xRAP55 in a matured oocyte was not substantially changed when compared with that in a stage VI oocyte after the treatment with alkaline phosphatase (Fig. 2A).

xRAP55 was predominantly located in the cytoplasm, as judged by immunoblotting of the nuclear and cytoplasmic fractions of stage VI oocytes (Fig. 2B). As a control, template-activating factor I (TAF-I), which is involved in chromatin remodeling, was exclusively detected in the nuclear fractions (32). The cytoplasmic localization of xRAP55 was confirmed by the immunostaining of oocytes. In the smallest oocytes in the ovary (stage I), xRAP55 was distributed diffusely in the cytoplasm (Fig. 2F). From stage III onward, immunostaining was...
detected in the perinuclear region and along fibers located perpendicular to the oocyte surface, the latter of which were less orderly organized at the vegetal hemisphere in fully grown stage VI oocytes (Fig. 2F). In addition, cytoplasmic particulate structures were observed at the vegetal hemisphere in stage VI oocytes, which suggests that xRAP55 exists in large particles. These structures were more prominent in a fertilized egg (Fig. 2G). Control rabbit immunoglobulin did not stain the oocytes (data not shown). Further, we found that the immunostaining patterns of FLAG-xRAP55 detected with the anti-FLAG antibody were similar to those obtained with the anti-FRGY2 antibody, although the cytoplasmic particulate structures were more prominent with the anti-FLAG antibody (Fig. S1C).

**Human RAP55 Localizes to the Cytoplasmic Processing Bodies**—xRAP55 was found in RNPs that contain the DEAD-box ATPase Xp54 and eIF4E-T (Fig. 1). Recently, it has been shown that human homologues of Xp54 and eIF4E-T localize to P-bodies, which are cytoplasmic structures detected as discrete foci and considered as sites of either mRNA decay or storage of non-translating mRNAs (46–48). These results together with the observation that xRAP55 or its homologue occurs in cytoplasmic particles in *Xenopus* oocytes and eggs (Fig. 2) and in *Caenorhabditis elegans* embryos (49, 50) prompted us to test whether RAP55 localizes to the P-bodies in mammalian cells. To this end, FLAG-xRAP55 was expressed in human HeLa cells. Immunostaining with anti-FLAG antibody revealed that xRAP55 protein was accumulated in cytoplasmic foci (Fig. 3B). The cells were costained with antibodies raised against the decapping enzyme Dcp1a, which is a representative P-body component (Fig. 3, A and B) (51, 52). FLAG-xRAP55 and Dcp1a colocalized with each other, demonstrating that RAP55 is a constituent of the P-bodies. A fusion protein containing the green fluorescent protein (GFP) attached to the N terminus of hRAP55 also localized to the P-bodies, in addition to diffuse cytoplasmic localization, when expressed at low levels (Fig. 3B).

We next examined whether hRAP55 is essential for the P-body’s formation, given that the human homologue of Xp54 and eIF4E-T are essential constituents of the P-bodies (47, 48). We found that the anti-xRAP55 polyclonal antibody detected hRAP55 as a single band on immunoblotting, although the C-terminal region of hRAP55 contains a single amino acid substitution relative to the epitope peptide (Fig. 3A). In addition, to facilitate double staining we prepared mouse monoclonal antibodies against hRAP55 (Fig. 3A). The specificity of these antibodies was confirmed by pre-adsorption with the recombinant RAP55 proteins, which resulted in elimination of the immunoreactive bands (Fig. S2A). By using siRNAs >90% of the endogenous hRAP55 was depleted, as shown by immunoblotting and immunostaining with one of the monoclonal antibodies (Fig. 3, C and D). In cells depleted of hRAP55, Dcp1a was no longer detected in cytoplasmic foci, although the amount of Dcp1a in the cell was not changed as shown by immunoblotting, indicating that hRAP55 is an essential constituent for the P-body formation. Another xRAP55 siRNA gave similar results (data not shown). When mammalian cells are exposed to stress condition, the...
stress granules (SGs) are formed as sites of storage of nontranslating mRNAs or “triage” of mRNAs where they are sorted for translational repression or degradation (52). SGs contain 40 S ribosomal subunits, translation factors, including phosphorylated eIF2α and poly(A)-binding protein, and the RNA-binding proteins TIA, TIAR, and HuR (52). Recent studies have demonstrated that some constituents of the P-bodies are shuttled into SGs (53, 54). We found that when cells were exposed to heat shock, hRAP55 was detected both in the P-bodies and in the SGs, which was demonstrated by colocalization with Dcp1a in the P-bodies and with HuR in the SGs (Fig. 3, E and F). While this report was in preparation, Yang et al. (55) reported hRAP55 to be one of the autoantigens identified by the serum of patients with autoimmune disease primary biliary cirrhosis. By immunofluorescence using the patient serum, they showed that hRAP55 localizes to the P-bodies and that, when cells are exposed to oxidative stress with arsenite, hRAP55 is detected both in the P-bodies and in the SGs. Collectively, these findings indicate that RAP55 is an essential component of the P-bodies and also localizes to SG upon heat shock or oxidative stress.

To analyze which domains of hRAP55 are required for the accumulation into the P-bodies, we introduced a series of deletion constructs of GFP-hRAP55 into HeLa cells and classified the expression patterns (Fig. S2, B and C). GFP-hRAP55 mutants lacking one or both of the RGG domains were still detected in the cytoplasmic foci, but they also distributed minor portions being in mRNA-free fractions and ribosomal fractions. This distribution of xRAP55 on a sucrose gradient was quite similar to that of FRGY2. To verify this observation, oocyte lysates were treated with EDTA and fractionated on a sucrose gradient in the presence of EDTA (Fig. 4B). The EDTA treatment shifted the distribution of xRAP55 to lighter fractions. Because EDTA releases mRNPs from ribosomes, these results suggest that a portion of xRAP55 in oocytes was associated with the ribosome-bound mRNPs or the ribosomes. We then examined whether xRAP55 is associated with poly(A)+ RNAs. Oocyte lysates were subjected to oligo(dT)-cellulose chromatography under native conditions following a previously described protocol (36). Bound materials were eluted with buffers containing either 25% or 50% formamide. Immunoblotting revealed that xRAP55 was distributed into both eluates as well as FRGY2 (Fig. 4C). Moreover, UV-cross-linking of oocyte lysates prior to oligo(dT) chromatography under denaturing conditions, which allows one to detect proteins covalently cross-linked to mRNAs, confirmed that xRAP55 is associated with poly(A)+ RNA in oocytes (Fig. 4D). Overall, the above findings indicate that xRAP55 is an RNA-binding component of cytoplasmic mRNPs.

Microinjection of mRNA encoding FLAG-tagged xRAP55 into oocytes, followed by the fractionation of the oocyte lysates on a sucrose gradient, revealed that exogenously expressed full-length xRAP55 was incorporated into mRNPs, although to a lesser extent throughout the cytoplasm (Δ1) or in both the cytoplasm and the nucleus (Δ2 and Δ3). Deletion of the N-terminal Lsm domain (Δ4 and Δ5) also significantly reduced the efficiency with which xRAP55 localized to the P-bodies. Further N-terminal deletion of hRAP55 resulted in loss of the ability to localize to the P-bodies (Δ6 and Δ7). Based on these results, we concluded that both the Lsm and RGG domains are required for hRAP55 to accumulate in the P-bodies. Our results also suggest that the FDF domain between the two RGG domains is responsible for the cytoplasmic retention of hRAP55.

xRAP55 Is a Component of Cytoplasmic mRNPs—Given that xRAP55 was communoprecipitated with FRGY2 in an RNase-sensitive manner, we sought to establish that xRAP55 is a component of cytoplasmic mRNPs. Oocyte lysates were fractionated on a sucrose gradient in the presence of cycloheximide and the distribution of xRAP55 was examined in comparison with that of FRGY2 (Fig. 4A). xRAP55 was mainly detected in nontranslating (storage) mRNP fractions with
than the endogenous xRAP55 (Fig. S3A). By microinjecting mRNAs encoding various portions of xRAP55, domains required for incorporation into mRNPs were delineated. The C-terminal deletion analysis revealed the requirement of the C-terminal RGG domain (RGG2). The N-terminal deletion analysis indicated that the N-terminal 272 amino acids were not needed for the incorporation into mRNPs. Furthermore, an internal deletion mutant lacking the FDF domain between two RGG domains was incorporated into mRNPs as efficiently as the full-length xRAP55, whereas another mutant lacking the first RGG domain was not incorporated into mRNPs. Taken together, these analyses show that the two RGG domains of xRAP55 are necessary and sufficient to direct this protein to mRNPs.

**xRAP55 Associates with Xp54**—To identify which set of proteins are associated with xRAP55, a similar approach that had been used for FRGY2-associated complexes was taken, in which a FLAG-tagged xRAP55 was expressed in oocytes and xRAP55-associated complexes were immunoprecipitated from the oocytes with anti-FLAG antibody (Fig. 5A). A number of proteins were specifically coimmunoprecipitated with FLAG-xRAP55, and importantly, the overall patterns of xRAP55- and FRGY2-associated complexes were similar. These results further support the presumption that xRAP55 is associated with maternal mRNP particles, of which FRGY2 is the major component. In addition, a 43-kDa protein was prominent in xRAP55-associated complexes, but not in FRGY2-associated complexes. Mass spectrometry identified it as the protein arginine N-methyltransferase 1 (xPRMT1). PRMT1 preferably binds to and methylates a set of proteins containing RGG repeats, and indeed our previous experiments have shown that xPRMT1 methylates the arginine- and glycine-rich region in another RNA-binding protein xCIRP2 (34).

When lysates were treated with RNase A before the immunoprecipitation with anti-FLAG antibody, most of the proteins that had been identified without prior digestion by RNase were not detected, whereas PRMT1 and Xp54 were still found to be coprecipitated with xRAP55 (Fig. 5A). Immunoblotting of the immunoprecipitates confirmed that Xp54 remained associated with FLAG-xRAP55 but not with FRGY2-FLAG in the RNase-treated lysates (compare Fig. 5A with Fig. 1). To determine whether xRAP55 associates with Xp54 in a purified system, we prepared recombinant GST-xRAP55 and histidine-tagged Xp54 and FRGY2, all of which were overexpressed in *E. coli* and purified (Fig. 5B). GST pull-down assays revealed that GST-xRAP55 interacted with Xp54 but not with FRGY2. The amount of Xp54 interacting with xRAP55 was decreased but still appreciable when the assays were performed after RNase treatment of the recombinant proteins (data not shown), as it was in the oocyte lysates (Fig. 5A).
Domains of xRAP55 that are required for interaction with Xp54 in the oocytes were determined by expressing deletion mutants of FLAG-xRAP55 in oocytes. As shown in Fig. 5C, Xp54 was coimmunoprecipitated with FLAG-tagged xRAP55 by anti-FLAG antibodies, when portions of xRAP55 retained both of the RGG domains. In contrast, in pull-down assays with Xp54-His6 and in vitro translated xRAP55 and its deletion mutants, xRAP55 retaining just one of the RGG domains was able to associate with Xp54 (Fig. 5, B and C). Despite these differences, we conclude that the RGG domains are required for xRAP55 to associate with Xp54 efficiently.

We cannot exclude the possibility that xRAP55 and Xp54 bind to separate sites on the same RNA molecule, and our RNase treatment did not completely digest the RNA between them. However, we prefer the possibility that RNA binding of one of these proteins facilitates direct protein-protein interaction with the other. Therefore, we examined the RNA-binding activity of xRAP55 and Xp54 by conducting a gel-retardation assay (Fig. 5D). Unexpectedly, addition of xRAP55 did not result in the retardation of an RNA probe (lane 2), whereas Xp54 formed a stable complex with the RNA (lanes 3 and 5). However, when both xRAP55 and Xp54 were mixed with the RNA probe, the mobility of the complexes was further decreased in comparison with that of the Xp54–RNA complexes (lanes 4 and 6). Our data suggest that xRAP55 interacts with Xp54 and, consequently, that the association of xRAP55 with RNA is stabilized in the presence of Xp54.

**xRAP55 Represses Translation in Vitro**—The Y-box proteins and the yeast and human homologues of Xp54 have been shown to repress translation in translation systems in vitro using rabbit reticulocyte lysates and wheat germ extracts (20, 21, 29). We then wished to define the role of xRAP55 in translation. When either xRAP55 or Xp54 was added to an in vitro translation system with wheat germ extracts programmed with β-actin mRNA, production of β-actin protein was repressed in a dose-dependent manner (Fig. 6A). This effect is not attributable to stability of the template mRNAs, because they were not degraded when isolated after the translation reactions in the presence of xRAP55 or Xp54. Another RNA-binding protein CiGRP1, which contains a glycine-rich region, and GST itself did not repress translation, demonstrating that the observed translational repression was specifically mediated by xRAP55 or Xp54 (Fig. S4A). Furthermore, we found that translation was severely repressed by the addition of both xRAP55 and Xp54 proteins into the reactions, although with our data we cannot conclude that these two proteins function synergistically (Fig. 6B). This effect is not likely to be specific for the template mRNA used, because similar results were obtained with chloramphenicol acetyltransferase mRNA (Fig. S4B).

**Tethered-xRAP55 Represses Translation of the Reporter mRNAs in Oocytes**—We hypothesized that xRAP55 would affect the fate of mRNAs when forced to bind to them in oocytes. To test this, we tethered xRAP55 to the 3′-UTR of reporter mRNAs (Fig. 7). In vitro synthesized mRNAs encoding either MS2 coat protein or MS2 fused to xRAP55 were microinjected into oocytes (Fig. 7A). After incubation of the oocytes for 6 h to allow protein production, 32P-labeled and capped luciferase reporter mRNAs either containing (Luc–MS2) or not containing (Luc–ΔMS2) MS2-binding sites without poly(A) tails were then injected. The expression of MS2–xRAP55 protein repressed luciferase activity from Luc–MS2 mRNA compared with that of MS2 protein alone (Fig. 7B). Reporter mRNAs were equally stable in oocytes expressing MS2 or MS2–xRAP55, suggesting that the reduced luciferase activity was due to differences in translation but not in reporter mRNA stability (Fig. 7C). Neither the cap at the 5′ terminus nor the poly(A) (at the 3′ terminus of reporter mRNAs substantially affected the translational repression by MS2–xRAP55 (Fig. 7E).

To determine the contribution of separate xRAP55 portions to translational repression, we assayed translation using regions of xRAP55 tethered with MS2 protein (Fig. 7, D and E). A fusion protein containing the N-terminal 272 amino acids repressed translation as efficiently as the full-length xRAP55. In contrast, the C-terminal fragment (amino acids 273–471) did not repress translation significantly. Thus, the N-terminal region of xRAP55 was responsible for translational repression in the tethering assay.

**DISCUSSION**

In this report we described the cDNA cloning and functional characterization of xRAP55. We provided compelling biochemical evidence that xRAP55 is a component of cytoplasmic storage mRNPs in *Xenopus* oocytes. First, xRAP55 was identified in complexes associated with FRGY2, one of the best characterized components of cytoplasmic mRNPs. Second, upon the fractionation of oocyte lysates with a sucrose gradient, xRAP55 distributed mainly in mRNP fractions, which represent stored and translationally repressed maternal mRNPs. Third, when oocyte lysates were subjected to oligo(dT) cellulose chromatography under native conditions as another approach to purifying mRNPs, xRAP55 was detected in the bound fractions. Fourth, xRAP55 covalently bound to poly(A) + RNAs by UV-cross-linking. Thus xRAP55 behaved similarly to FRGY2. Consistent with the conclusion that it is a component of storage mRNPs, xRAP55 repressed mRNA translation in an
FIGURE 7. **xRAP55 represses translation in vivo when tethered to an mRNA.** A–C, oocytes were injected with increasing amounts of mRNAs encoding MS2 or MS2-xRAP55. Expression of MS2 and MS2-xRAP55 protein was analyzed by incubating the oocytes in [35S]methionine for 14 h (A). 6 h after the first injection, 32P-labeled and capped luciferase reporter mRNAs either containing (Luc-MS2) or not containing (Luc-ΔMS2) MS2-binding sites without poly(A) tails, together with β-galactosidase mRNA, were injected. After 14 h, oocytes were harvested, and luciferase and β-galactosidase activities were measured (B). Luciferase activity was normalized with β-galactosidase activity, and luciferase activity in oocytes expressing MS2-xRAP55 was shown relative to the case of MS2 protein, which was set to 100%. The stability of 32P-labeled luciferase mRNAs in oocytes expressing MS2 and MS2-xRAP55 was examined immediately after microinjection (0 h) and after 14 h of incubation (C). Relative count of 32P-labeled mRNA normalized to the value in 0 h, which was 100%, is shown in boxes. Total RNA served as recovery and loading controls. D and E, effect of MS2-xRAP55-(1–272) and MS2-xRAP55-(273–471) on translational activity of capped (E, left) or uncapped (E, right) Luc-MS2 mRNAs with or without a poly(A) tail was examined.
in vitro system. This effect appeared to be mRNA-nonspecific. xRAP55 and FRGY2 bind to the same mRNA molecules in oocytes, because xRAP55 was detected in the FRGY2-associated complexes in an RNase-sensitive manner. We have not obtained any data showing direct protein-protein interaction between xRAP55 and FRGY2. In contrast, xRAP55 is intimately associated with Xp54. Xp54 has been shown to form RNA-dependent homo-oligomers (30). Therefore, the decrease in Xp54 that was coprecipitated with FLAG-RAP55 upon the RNase treatment of the lysates may result from dissolution of the Xp54 oligomers. In addition to these biochemical data we showed that xRAP55 repressed translation in oocytes, when tethered to an mRNA. Thus the results presented in this report establish that xRAP55 is an RNA-binding component of storage mRNPs and acts as a translational repressor both in vivo and in vitro.

RAP55 was originally identified in Pleurodeles oocytes (40). It was shown that the Pleurodeles RAP55 does not bind to mRNA directly using an oligo(dT)-based selection of UV-cross-linked lysates. Via a similar approach, we found that xRAP55 was covalently cross-linked to mRNA. The discrepancy may be due to differences in species, the efficiency of UV-cross-linking, or the efficiency of detection. Recently, diverse functions have been reported for C. elegans and Drosophila homologues of RAP55, CAR-1, and Trailer hitch (Traf), respectively (56). In C. elegans CAR-1 is required for embryonic cytokinesis, regulation of physiological germ line apoptosis, and organization of the endoplasmic reticulum (49, 50, 57). Depletion of CAR-1 leads to a defect in cytokinesis during early embryogenesis resulting from an anaphase spindle defect (57). In Drosophila oocytes and nurse cells, Traf is associated with a subset of endoplasmic reticulum exit sites and is required for the proper secretion of proteins such as Gurken and Yolkless (58). Importantly, interactions between the RAP55 homologues, the Xp54 homologues, and the Y-box proteins are conserved in these organisms, indicating that RAP55 and its homologues constitute core components of mRNPs together with the Xp54 homologues and the Y-box proteins and execute various functions (49, 50, 56, 58). In previous studies with somatic cells such as rabbit reticulocytes or mouse L cells, core components of cytoplasmic mRNPs consisted of the Y-box proteins and the poly(A)-binding protein (59, 60). Therefore, the Xp54 and RAP55 homologues are perhaps either less abundant in mRNPs or less stably associated with them.

xRAP55 contains an Lsm domain at its N terminus and indeed belongs to the Lsm14 protein family (43). It was revealed that the C-terminal RGG domains are required for xRAP55 to be incorporated into mRNPs (Fig. S3). Our data showed that xRAP55 interacts with Xp54 also through the RGG domains (Figs. 5 and S3). On the other hand, when tethered with MS2, the N-terminal 272 amino acids, encompassing the Lsm domain and the serine/threonine-rich domain, are responsible for the translational repression of reporter mRNAs that contain the MS2-binding sites (Fig. 7). Therefore, two separate domains would be required for xRAP55 to repress translation as a component of mRNPs.

By immunostaining we found that xRAP55 localizes to cytoplasmic particles in the vegetal hemisphere in stage VI oocytes and unfertilized eggs. In C. elegans during embryonic division, CAR-1 localizes to P granules, which are cytoplasmic particles enriched in the germ line precursors and contain poly(A) RNAs (49, 50). In Xenopus, germ granules, which are also cytoplasmic granules in oocytes and eggs, are essential for germ line development (61). The punctuated structures that xRAP55 localizes to are, however, unlikely to be the germ granules, because the germ granules move to the vegetal cortex region during the early stages of Xenopus oogenesis. We also found that Xenopus and human RAP55 localize to P-bodies in human cells. RNA interference-mediated knockdown revealed that hRAP55 is an essential constituent of the P-bodies. Thus far, an ever-increasing number of proteins involved in translational control, mRNA decay, and microRNA-mediated gene silencing have been identified as constituents of P-bodies (52). P-bodies contain proteins that are functional in mRNA decay such as the decapping enzyme and associated proteins, including the Xp54 homologue and Edc3, a deadenylase CCR4, and Lsm1–7 complex. Edc3, a member of the Lsm16 family, is known as a decapping activator in yeast and is indeed complexed with the decapping enzyme in human cells (62, 63). P-bodies were identified as active sites for mRNA degradation in yeast and mammalian cells; recent findings in yeast extended the role of the P-bodies as sites for the storage or sorting of nontranslating mRNAs (3, 29, 64). Yang et al. (55) and ourselves (this report) found that hRAP55 localizes to P-bodies and, upon heat shock or oxidative stress, to SGs in addition to the P-bodies. SGs are dynamic structures where untranslated mRNPs are sorted for storage, translational reinitiation, or decay (52). These observations are therefore consistent with our finding that RAP55 acts as a translational repressor. We found that the Lsm domain and the RGG domains are required for the accumulation of xRAP55 to the P-bodies; mutant proteins lacking either of these domains distributed diffusely in the cytoplasm in addition to localizing to the P-bodies. Yang et al. (55) have reported that the N-terminal region containing the Lsm domain was not required to direct hRAP55 to P-bodies. This apparent discrepancy could be due to the differences in the method of detection of GFP-hRAP55 or the cell types used. It can be speculated that the RAP55 Lsm domain interacts with other Lsm proteins that are localized to the P-bodies and help RAP55 concentrate at the P-bodies.

xRAP55, Xp54, or its homologues and Y-box proteins all repress translation when added into in vitro translation systems independently (20, 29, 65). Thus at least three components of storage mRNPs identified so far function as translational repressors. When xRAP55 and Xp54 were added together into the in vitro system, translation was further repressed. This likely reflects the physical interaction between them. Regarding the molecular mechanism with which Xp54 represses translation, a model has been proposed, in which Xp54 sequesters the cap-binding protein eIF4E from the translational machinery in oocytes (30). In this model, Xp54 oligomerized in an RNA-dependent manner is tethered to the 3’-UTR of an mRNA via interaction with CPEB. Because we observed the translational repression activity of xRAP55 only when it was tethered to reporter mRNAs, it is possible that xRAP55 could associate with a specific set of mRNAs in cells directly or through other factors. Further study, such as SELEX or RNA immunoprecipi-
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tation followed by microarray analysis, is required to address these questions. Our results showed that the N-terminal 272 amino acids of xRAP55, including the Lsm domain, play a role in translational repression in oocytes. As discussed above, because the Lsm domain helps RAP55 to localize to the P-bodies, one possible mechanism by which RAP55 mediates translational repression is that this domain recruits the RAP55-bound mRNA to specific sites for translational repression in oocytes, by interacting with other components of such sites. Candidates for such components would be Lsm1–7 and Edc3/Edc16, because they localize to the P-bodies. It should be determined whether one or more as-yet-uncharacterized Lsm complexes exist at such sites.

Acknowledgments—We thank Drs. M. Wickens, J. Lykke-Andersen, and K. Nagata for providing plasmids and antibodies. We also thank S. Tashiro for technical assistance and M. Usui and K. Otsuki for conducting the mass spectrometric analysis.

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