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Membrane-Bound Tomato Mosaic Virus Replication Proteins Participate in RNA Synthesis and Are Associated with Host Proteins in a Pattern Distinct from Those That Are Not Membrane Bound

Masaki Nishikiori,[1,3] Koji Dohi,[5,6] Masashi Mori,[5,6] Tetsuo Meshi,[2,6] Satoshi Naito,[4] and Masayuki Ishikawa[1,6]*

Plant-Microbe Interactions Research Unit[1] and Division of Plant Sciences,[2] National Institute of Agrobiological Sciences, Tsukuba 305-8602, Graduate School of Agriculture[3] and Graduate School of Life Science,[4] Hokkaido University, Sapporo 060-8589, Research Institute for Bioresources and Biotechnology, Ishikawa Prefectural University, Nonoichi 921-8836,[5] and CREST, Japan Science and Technology Agency, Kawaguchi 322-0012,[6] Japan

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Extracts of vacuole-depleted, tomato mosaic virus (ToMV)-infected plant protoplasts contained an RNA-dependent RNA polymerase (RdRp) that utilized an endogenous template to synthesize ToMV-related positive-strand RNAs in a pattern similar to that observed in vivo. Despite the fact that only minor fractions of the ToMV 130- and 180-kDa replication proteins were associated with membranes, the RdRp activity was exclusively associated with membranes. A genome-sized, negative-strand RNA template was associated with membranes and was resistant to micrococcal nuclease unless treated with detergents. Non-membrane-bound replication proteins did not exhibit RdRp activity, even in the presence of ToMV RNA. While the non-membrane-bound replication proteins remained soluble after treatment with Triton X-100, the same treatment made the membrane-bound replication proteins in a form that precipitated upon low-speed centrifugation. On the other hand, the detergent lysophosphatidylcholine (LPC) efficiently solubilized the membrane-bound replication proteins. Upon LPC treatment, the endogenous template-dependent RdRp activity was reduced and exogenous ToMV RNA template-dependent RdRp activity appeared instead. This activity, as well as the viral 130-kDa protein and the host proteins Hsp70, eukaryotic translation elongation factor 1A (eEF1A), TOM1, and TOM2A copurified with FLAG-tagged viral 180-kDa protein from LPC-solubilized membranes. In contrast, Hsp70 and only small amounts of the 130-kDa protein and eEF1A copurified with FLAG-tagged non-membrane-bound 180-kDa protein. These results suggest that the viral replication proteins are associated with the intracellular membranes harboring TOM1 and TOM2A and that this association is important for RdRp activity. Self-association of the viral replication proteins and their association with other host proteins may also be important for RdRp activity.

The virus particles of positive-strand RNA [(+)RNA] viruses contain single-stranded, messenger-sense genomic RNA, and these viruses replicate via complementary, negative-strand RNA [(−)RNA] templates. Most plant viruses and many animal viruses are (+)RNA viruses. After infection, the genomes of these viruses are translated to produce viral proteins that function in viral RNA replication. The replication of eukaryotic (+)RNA viruses takes place in replication complexes that are formed on the intracellular membranes of infected cells and that contain the viral RNA replication proteins and endogenous (−)RNA templates. The replication complexes synthesize viral (+)RNAs from ribonucleoside triphosphates (rNTPs) and release them into the cytoplasm (4, 24).

The genus *Tobamovirus*, which belongs to the alphavirus-like superfamily of (+)RNA viruses, includes *Tobacco mosaic virus* and *Tomato mosaic virus* (ToMV) and many related viruses. The genome of a tobamovirus is a 6.4-kilobase RNA that encodes a nonstructural protein with an approximate molecular mass of 130 kDa (130,000-molecular-weight [130K] protein), its read-through product of 180 kDa (180K protein), a 30-kDa nonstructural protein that is required for viral cell-to-cell movement, and a coat protein (CP). The 130K and 180K proteins are both involved in viral RNA replication, whereas the 30-kDa protein and CP are dispensable for replication. The 130K protein contains a methyltransferase-like domain that functions in RNA capping and an RNA helicase-like domain. The read-through region of the 180K protein contains an RNA polymerase-like domain. These three domains are conserved among the replication proteins of the members of the alphavirus-like superfamily (for reviews, see references 5 and 13).

Membranes purified from cells infected with tobamoviruses or other (+)RNA viruses contain an RNA-dependent RNA polymerase (RdRp) activity that synthesizes virus-related RNAs (4, 20). Osman and Buck prepared membrane-bound ToMV RdRp from ToMV-infected tomato leaf tissues using sucrose density gradient centrifugation. The RdRp preparation contained endogenous (−)RNA and produced genome-sized double- and single-stranded ToMV RNA as well as a trace amount of a subgenomic RNA (20). Treatment of the membrane-bound ToMV RdRp with micrococcal nuclease (MNase) resulted in a template-dependent RNA polymerase that synthesized a small amount of genome-length (−)RNA using exogenous ToMV genomic (+)RNA as a template and a

* Corresponding author. Mailing address: Plant-Microbe Interactions Research Unit, Division of Plant Sciences, National Institute of Agrobiological Sciences, Tsukuba 305-8602, Japan. Phone and fax: 81 29 838 7009. E-mail: ishika32@affrc.go.jp.

larger amount of ToMV genomic (+)RNA using the synthesized (−)RNA as a template (20). An anionic detergent, taurodeoxycholate (TDC), was found to efficiently solubilize the membrane-bound tobamovirus RdRp (21, 29). In the TDC-solubilized tobamovirus RdRp preparations, the 130K and 180K proteins formed a heterodimer (29) and were associated with eukaryotic translation elongation factor 1A (eEF1A) (31) and with a protein related both to a subunit of wheat eukaryotic translation initiation factor 3 and to *Saccharomyces cerevisiae* GCD10 protein (21). Other experiments have demonstrated that *Escherichia coli*-expressed polypeptides containing the amino acid sequence of the tobacco mosaic virus helicase-like domain form multimers in vitro (9) and that this oligomerization is important for viral RNA replication (10).

In addition to the host proteins mentioned above, host seven-pass and four-pass transmembrane proteins (TOM1 and TOM2A, respectively) have been implicated in tobamovirus multiplication, based on genetic studies (28, 32). In yeast, TOM1 interacts with tobamovirus helicase domain polypeptides and with TOM2A (28, 32). Furthermore, in subcellular fractionation analysis of the lysate of ToMV-infected tobacco protoplasts, TOM1 cofractionates with the membrane-bound viral replication proteins and the viral RdRp activity (11). These observations, together with the apparent lack of transmembrane sequences in the 130K and 180K replication proteins, suggest that TOM1 and TOM2A play direct and important roles in the formation and/or maintenance of tobamovirus RNA replication complexes by tethering the replication proteins to the membranes. However, TOM1 and TOM2A have not yet been shown to associate with tobamovirus replication proteins in infected plant cells.

In the present study, we used an evacuolation technique to prepare cell extracts that can synthesize ToMV-related (+)RNAs, which yield an electrophoretic pattern similar to that observed for ToMV-infected protoplasts in vivo. We examined the viral replication proteins in these extracts for their mode of binding to membranes and other characteristics. Our data show that TDC inactivates the RdRp activity but another detergent, lysophosphatidylcholine (LPC), solubilizes ToMV replication proteins while retaining RdRp activity. In addition, LPC-solubilized 180K protein, which was originally membrane bound, is associated with the 130K, TOM1, TOM2A, and eEF1A proteins.

MATERIALS AND METHODS

Chemicals. Egg yolk-derived LPC was from Wako Pure Chemical Industries (Osaka, Japan). [α-^{32}P]CTP (800 Ci/mmol) was from GE Healthcare Bio-Sciences (Piscataway, NJ).

Transgenic BY-2 cell lines. Transgenic BY-2 cell lines ER20 and ER43, which strongly express the estradiol-inducible transcriptional activator XVE, were established as described previously (7). ER20 and ER43 were further transformed with the Ti plasmid pBICER8-ToMVerG3(SF3)SRz to establish cell lines E113 and E182, respectively. Infection of E113 or E182 with an endoplasmic reticulum (ER)-targeted green fluorescent protein (GFP)-tagged ToMV derivative harboring wild-type replication proteins is inducible by estradiol (7). Data shown in this report were obtained using cell line E182, and the results were confirmed using cell line E113. A plasmid, pTL.180KFSTYG3, which harbors a ToMV-derived sequence encoding FLAG-tagged 180K protein (180K-FLAG), was created from pTL.G3 (17). The nucleotide sequence of pTL.180KFSTYG3, shown between the last codon (underlined) of the 180K protein-coding region and the StuI site (italicized) located in the 30-kDa-protein-coding region, was TGT GGA GAG CTC GGA GGT GAT TAT AAG GAT GAT GAT GAT AAG AAC TGG TCA CAT CCT CAA TTT GAA AAG **TG*A*** *GGC CT* (boldface type indicates the termination codon of 180K-FLAG). The region in the Ti plasmid pBICER8-ToMVerG3(SF3)SRz that encodes a modified ToMV RNA was precisely replaced by the corresponding region of pTL.180KFSTYG3 to create pBICER8-ToMV180KFSTYG3SRz. The BY-2 cell line ER43 was transformed with pBICER8-ToMV180KFSTYG3SRz to establish cell lines RT4 and RT23. Upon induction with estradiol, the ToMV derivative encoding the 180K-FLAG protein replicated and expressed GFP in RT4 and RT23 cells. Data shown in this report were obtained using cell line RT4, and the results were confirmed using cell line RT23.

Preparation of BY-2 cell extracts. Saturated cultures of the transgenic BY-2 cell lines were diluted 1:100 and grown for 2 days. Estradiol was then added to a final concentration of 1 μM, and the induced cultures were grown for an additional 2 days. Protoplasts and evacuolated protoplasts were prepared from the induced cells as described by Ishibashi et al. (12). Both types of protoplasts were then disrupted in 4 volumes of TR buffer (30 mM HEPES-KOH, pH 7.4, 80 mM potassium acetate, 1.8 mM magnesium acetate, 2 mM dithiothreitol [DTT], and one tablet of Complete Mini protease inhibitor mixture [Roche Diagnostics, Penzberg, Germany] per 10 ml of buffer) using about 70 strokes of a tight-fitting Dounce homogenizer. Nuclei and undisrupted cells were removed by centrifugation at 4°C for 10 min at 800 × *g* to obtain cell extracts. The evacuolated protoplast extracts (iBYL) retained RdRp activity for more than 2 years if stored at −80°C. In some experiments, iBYL was further fractionated by centrifugation (15,000 × *g* for 15 min at 4°C) into membrane-containing pellet (P15) and membrane-depleted supernatant (S15) fractions.

Protein analysis. Proteins were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and antigens on Western blots were detected using specific antibodies and the ECL Plus Western blotting detection system (GE Healthcare Bio-Sciences). Rabbit antisera against GFP, ToMV 130K protein, TOM1, and TOM2A have been described previously (7, 11). Rabbit antiserum against a synthetic peptide (LHLVRPFLAFLPEVQSAD RKC) of the N-terminal region of plant Sec61 alpha was a generous gift from Ken Matsuoka (RIKEN, Yokohama, Japan). The anti-Sec61 antibody recognizes tobacco Sec61 as described previously (11, 33). Rabbit antisera against glutathione *S*-transferase-fused *Arabidopsis thaliana* Sar1 (M. Takeuchi and A. Nakano, unpublished) and against eEF1A purified from wheat germ extract (3) were generous gifts from Akihiko Nakano (RIKEN, Wako, Japan) and Karen S. Browning (The University of Texas at Austin), respectively. Mouse monoclonal antibody against Hsp70 and Hsc70 purified from human HeLa cells (SPA-820) was from Stressgen (Victoria, Canada). These antibodies recognized tobacco polypeptides with expected molecular masses of 20 kDa for Sar1, 50 kDa for eEF1A, and 70 kDa for Hsp70 (data not shown).

RNA analysis. RNA was purified by phenol and phenol-chloroform extractions in the presence of 0.2% SDS, followed by ethanol precipitation. Northern hybridization to detect (−)RNA was performed using a ^{32}P-labeled P7P riboprobe (14).

RdRp assay. RdRp reactions were performed by adding 10 μl of 5× RdRp buffer (7.5 mM ATP, 5 mM [each] GTP and UTP, 125 μM CTP, 50 mM DTT, 25 mM magnesium acetate, 10 mM EGTA, 500 μg/ml actinomycin D, 150 mM creatine phosphate, 1 μg/μl creatine kinase [Roche Diagnostics], and 2 U/μl RNasin [Promega, Madison, WI]) containing 20 μCi of [α-^{32}P]CTP to 40 μl of cell extract or other samples. The reaction mixtures were incubated at 25°C for 1 h. RNase protection assays were performed using an RNase protection kit (Roche Diagnostics) according to the manufacturer's instructions. Riboprobes P1M and P7P (14) were used to detect ToMV (+)RNA and (−)RNA, respectively.

Membrane flotation analysis. Membrane flotation analysis (see Fig. 4) was carried out essentially as described by den Boon et al. (6). The P15 membranes of iBYL were washed twice with Mg^{2+}-free TR buffer (30 mM HEPES-KOH, pH 7.4, 80 mM potassium acetate, 1 mM EDTA, 2 mM DTT, 1 tablet of Complete Mini protease inhibitor mixture per 10 ml buffer) to remove host RdRp (20). The washed P15 membranes from 53 μl of iBYL were then suspended in 67 μl of either (i) NTR buffer (TR buffer containing 0.2 mM [each] ATP, GTP, and UTP); (ii) NTR buffer containing 1 M NaCl; (iii) 0.1 M Na_2CO_3 containing 0.2 mM (each) ATP, GTP, and UTP; or (iv) NTR buffer containing 1% Triton X-100. The resuspended membranes were incubated on ice for 30 min with occasional mixing. After incubation, 333 μl of 60% sucrose (wt/wt) in NTR buffer was added to the samples to adjust the final sucrose concentration to 50% (wt/wt). Samples (400 μl) were loaded at the bottom of a Beckman TLS55 ultracentrifuge tube (Beckman Coulter, Fullerton, CA) and overlaid sequentially with 900 μl of 45% (wt/wt) sucrose and 100 μl of 10% (wt/wt) sucrose, both in NTR buffer. The samples were centrifuged at 100,000 × *g* for 12 h at 4°C and then manually fractionated into three gradient fractions of 466 μl each. For

RdRp assay, 20 μl of each fraction was mixed with 20 μl of NTR buffer and 10 μl of 5× RdRp buffer and incubated at 25°C for 1 h. For the analysis with Triton X-100, 0.5% Triton X-100 was included in sucrose solutions for gradients, NTR buffer, and 5× RdRp buffer. Membrane-bound, endogenous template-dependent ToMV RdRp activity was not affected by residual sucrose and NaCl or Na_2CO_3 (final concentrations of 20% [wt/wt] sucrose plus 67 mM NaCl or 6.7 mM Na_2CO_3 in RdRp reaction mixture).

Detergent treatment of membrane-bound ToMV replication proteins. The membrane fractions that were obtained from iBYL by treatment with 1 M NaCl and membrane flotation were diluted and centrifuged to collect purified membranes. Membrane pellets (from 16 μl of iBYL) were then resuspended in 40 μl of TR buffer containing 1% (wt/vol) LPC or other detergents and 0.2 mM (each) ATP, GTP, and UTP and incubated at 15°C for 20 min. Both rNTPs and Mg^{2+} ions were necessary to maintain the stability of LPC-solubilized RdRp (data not shown). For RdRp assay, the detergent-treated iBYL membrane fraction (40 μl) was mixed with 10 μl of 5× RdRp buffer containing the particular detergent (0.5%), divided into halves (25 μl each), and incubated at 25°C for 1 h in the presence or absence of 0.5 μg of ToMV virion RNA. For protein fractionation analysis, the detergent-treated membranes were centrifuged at 15,000 × g for 15 min at 4°C, and recovered supernatants and pellets were used for Western analysis.

Sucrose gradient sedimentation analysis. Continuous sucrose gradients were formed with 1.1 ml (each) of 10% (wt/wt) and 50% (wt/wt) sucrose solutions (see below) using Gradient Mate (BioComp Instruments, Fredericton, NB, Canada) at 50° and 30 rpm for 7 min, followed by 82° and 22 rpm for 22 s. Each sucrose solution contained NTR buffer with or without 0.5% (wt/vol) Triton X-100 or LPC. The S15 fraction from 33 μl iBYL or 1 M NaCl-washed P15 membranes from 80 μl iBYL were incubated at 15°C for 20 min in 200 μl of NTR buffer containing 1% (wt/vol) Triton X-100 or LPC or mock treated. The treated samples (200 μl) were loaded onto sucrose gradients (2.2 ml) and subjected to centrifugation (100,000 × *g* for 1 h at 4°C) in a Beckman TLS-55 rotor. Each detergent-treated sample was analyzed using a sucrose gradient containing that particular detergent. The gradients were manually fractionated into eight fractions of 300 μl each. For RdRp assay, 20 μl of each fraction was mixed with 20 μl of NTR buffer and 10 μl of 5× RdRp buffer containing the particular detergent (0.5%), divided into halves (25 μl each), and incubated at 25°C for 1 h in the presence or absence of 0.5 μg of ToMV virion RNA. The presence of sucrose (final 20% [wt/wt]) in reaction mixtures did not affect the activity of detergent-treated ToMV RdRp.

Affinity purification of the 180K-FLAG protein. The 1 M NaCl-washed P15 membranes from 1.4 ml iBYL were suspended in 1.2 ml of solubilization buffer (30 mM HEPES-KOH, pH 7.4, 150 mM NaCl, 1 mM magnesium acetate, 0.1 mM DTT, 0.2 mM [each] ATP, GTP, and UTP) containing 0.7% LPC and incubated at 15°C for 20 min. The solubilized membrane solution was clarified by centrifugation at 15,000 × *g* for 10 min at 4°C and then used for affinity purification. LPC was added to 0.6-ml aliquots of S15 fractions to a final concentration of 0.2%, and the S15 fractions were then subjected to affinity purification. For affinity purification, samples were mixed with 100 μl of anti-FLAG M2 affinity gel (50% suspension; Sigma, St. Louis, Mo.) for 1 h. The resin was then washed five times with solubilization buffer containing 0.2% LPC and eluted in 250 μl of solubilization buffer containing 0.2% LPC and 100 μg/ml FLAG peptide (Sigma). The RdRp activity of the eluates was assayed as described above in the presence of ToMV virion RNA (1 μg/50-μl reaction mixture). Protein bands in SDS-PAGE gels were visualized with a silver-staining kit (Wako).

RESULTS

Extraction of an RNA polymerase capable of synthesizing ToMV-related (+)RNAs from ToMV-infected, evacuolated BY-2 protoplasts. Most plant cells have vacuoles that contain large amounts of active nucleases and proteases and that occupy a large part of the intracellular space. Breakage of vacuoles usually accompanies disruption of plant cells, resulting in contamination of cell extracts with nucleases and proteases that can interfere with biochemical analyses. We previously found that removal of vacuoles (evacuolation) prior to cell disruption efficiently removes such contaminating enzymes, and cell extracts obtained from evacuolated protoplasts support translation and replication of plant (+)RNA virus genomes (16). In the present study, we used evacuolated protoplasts as starting materials for isolation and characterization of the ToMV RdRp. Although in vitro translation of ToMV RNA in cytoplasmic extracts from uninfected, evacuolated tobacco BY-2 protoplasts has been shown to produce RdRp capable of synthesizing ToMV-related RNAs in a pattern similar to that observed in ToMV-infected cells (16), the amount of RdRp produced in this system was not enough for extensive biochemical analyses. On the other hand, evacuolation of BY-2 protoplasts that had been infected with ToMV RNA by electroporation was possible but inefficient, and reproducibility was poor (data not shown).

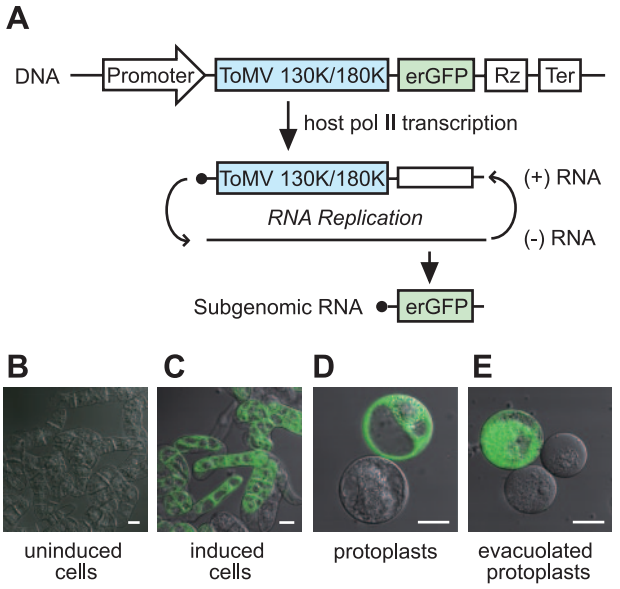

FIG. 1. The estradiol-inducible ToMV infection system. (A) Schematic representation of the estradiol-inducible infection system for a ToMV derivative that carries the ER-targeted GFP (erGFP) gene. A modified ToMV cDNA was placed between an estradiol-inducible promoter and a ribozyme sequence (Rz) followed by a polyadenylation signal (Ter). Upon estradiol induction, a modified ToMV RNA is produced via transcription by cellular RNA polymerase II followed by ribozyme cleavage. This modified ToMV RNA is translated to produce the 130K and 180K replication proteins and subjected to replication. A subgenomic mRNA encoding erGFP is synthesized during replication. (B to E) Estradiol-induced ToMV infection in transgenic BY-2 cells. Nomarski and GFP fluorescence images are superimposed. (B) Uninduced cells; (C) induced cells (2 days after induction); (D) protoplasts prepared from induced cells; (E) evacuolated protoplasts prepared from induced cells. Bars, 25 μm.

We have established transgenic, suspension-cultured BY-2 tobacco cell lines that express a modified ToMV genomic RNA in which the CP-coding region is replaced by the GFP coding sequence (Fig. 1A) and in which expression of the modified genomic RNA is inducible by estradiol. Upon induction, the modified ToMV RNA is generated by transcription, followed by ribozyme cleavage and RNA replication, and GFP is expressed (Fig. 1B to D; see also Fig. 6). We prepared evacuolated protoplasts from induced cells (Fig. 1E), disrupted them with a Dounce homogenizer, and removed the nuclei by low-speed centrifugation to obtain cytoplasmic extracts (iBYL).

When [^{32}P]rNTPs were added to iBYL in the absence of exogenous ToMV RNA template, labeled nucleotides were incorporated into S1 nuclease-sensitive, single-stranded RNAs

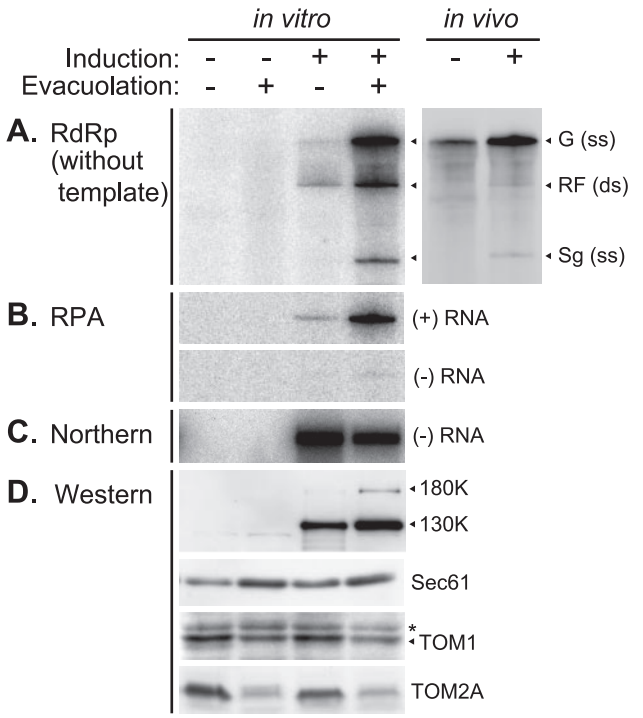

FIG. 2. Effects of evacuolation on ToMV RdRp activity in extracts of infected protoplasts. Equal volumes of transgenic BY-2 protoplast extracts with (+) or without (−) estradiol induction or evacuolation were analyzed. (A) RdRp activity. Cell extracts were assayed for RdRp activity without exogenous RNA template in the presence of [α-^{32}P]CTP. Total RNA was purified from the reaction mixtures and separated by 7 M urea–2.4% polyacrylamide gel electrophoresis, and ^{32}P-labeled bands were visualized using a Fuji BAS 2500 imaging analyzer (left panel). For comparison, transgenic BY-2 cells were cultured in the presence (+) or absence (−) of estradiol for 24 h, and protoplasts were formed and cultured in a medium containing [^{3}H]uridine and actinomycin D (100 μg/ml) for 2 h. Labeled RNA was purified and separated as described above and visualized by fluorography (right panel) (18). The positions corresponding to ToMV genomic ssRNA [G (ss)], RF [RF (ds)], and the GFP subgenomic ssRNA [Sg (ss)] are indicated at right. (B) Strand specificity of RdRp products. ^{32}P-labeled RdRp reaction products from panel A were subjected to RNase protection assays (RPA) using unlabeled P1M and P7P probes (14) to detect ToMV (+)RNAs and (−)RNAs, respectively. (C) Detection of ToMV (−)RNA by Northern hybridization with a ^{32}P-labeled P7P RNA probe (14). (D) Detection of ToMV 130K and 180K proteins and host proteins Sec61, TOM1, and TOM2A by Western blot analysis. The 100,000 × *g* membrane pellet fraction of each extract was used to detect Sec61, TOM1, and TOM2A. The band marked by the asterisk has not been characterized.

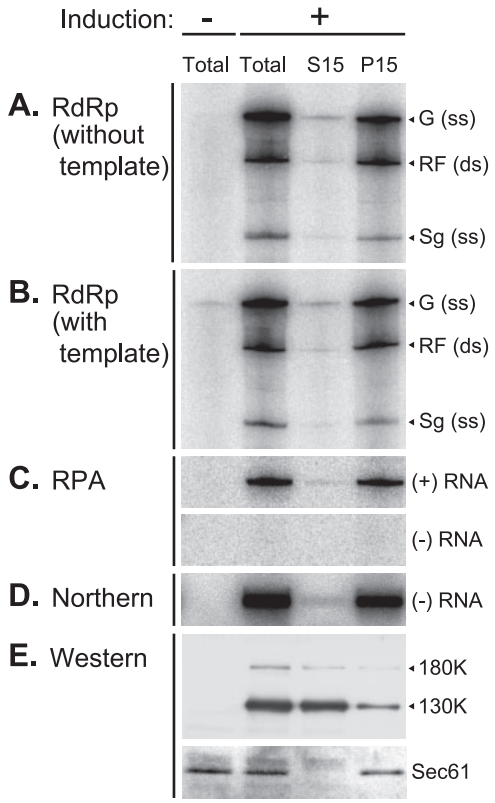

FIG. 3. Fractionation of the ToMV RdRp activity and replication proteins by centrifugation. iBYL was subjected to a centrifugation at 15,000 × *g* for 15 min to obtain the supernatant (S15) and pellet (P15) fractions. Lanes S15, P15, and Total represent equal volumes of iBYL (+). An uninfected, evacuolated BY-2 protoplast extract was concurrently analyzed (−). (A) RdRp activity in the absence of exogenous template. (B) RdRp activity in the presence of exogenous ToMV RNA template (1 μg of ToMV RNA in a 50-μl reaction mixture). The RdRp assay was performed, and the products were analyzed as described in the legend to Fig. 2A. For abbreviations, see the legend to Fig. 2A. (C) Strand specificity of RdRp products. The ^{32}P-labeled RdRp reaction products shown in panel B (reaction in the presence of exogenous ToMV RNA template) were analyzed by RNase protection assays (RPA) as described in the legend to Fig. 2B. (D) Detection of ToMV (−)RNA by Northern hybridization with a ^{32}P-labeled P7P RNA probe (14). (E) Detection of ToMV 130K and 180K proteins and host membrane protein Sec61 by Western blot analysis.

(ssRNAs) of electrophoretic mobilities corresponding to ToMV genomic and subgenomic RNAs and into S1 nuclease-resistant, double-stranded RNA of approximately 6.4 kbp (replicative form [RF]) (Fig. 2A and 3A, and data not shown). The pattern of labeled RNA resulting from iBYL was similar to that resulting from ToMV-infected protoplasts, except that the intensity of the ssRNA bands relative to the RF RNA band was weaker in iBYL (Fig. 2A). The iBYL products were predominantly positive stranded and internally labeled, as determined by RNase protection assays (Fig. 2B). Addition of exogenous ToMV RNA template to the iBYL RdRp reaction mixture did not significantly change the pattern of labeled RNA (Fig. 3B) and failed to induce (−)RNA synthesis (Fig. 3C). These results suggest that the ToMV RdRp activity in iBYL utilizes an endogenous (−)RNA as a template and that the replication proteins in iBYL are incapable of synthesizing (−)RNA using exogenous ToMV RNA as a template.

Relative to iBYL, extracts from nonevacuolated, ToMV-infected BY-2 protoplasts contained higher concentrations of (−)RNA, TOM1, and TOM2A (Fig. 2C and D). This observation is consistent with our previous results that these molecules are partially [for TOM1 and (−)RNA] or predominantly (for TOM2A) localized to tonoplasts, which are removed by evacuolation. Despite the abundance of these molecules in whole-protoplast extracts, labeled nucleotides were incorporated into the genomic RNA only in trace amounts in RdRp assays. Furthermore, the labeled nucleotides were incorporated into RF RNA in smaller amounts for whole protoplast

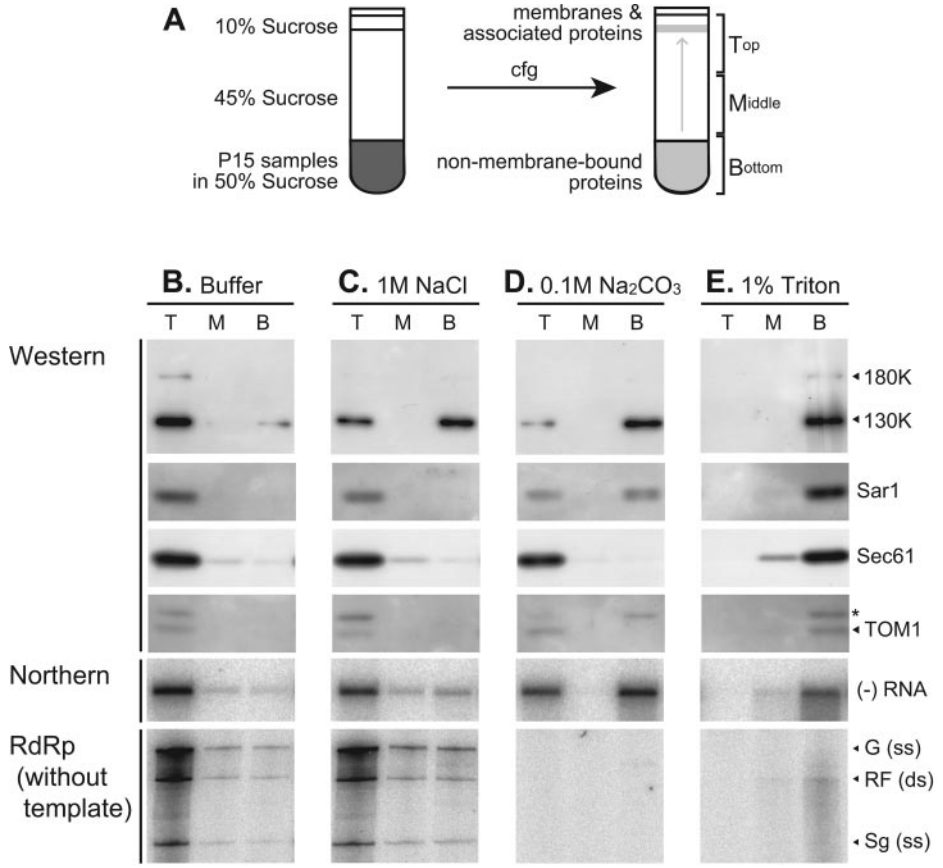

FIG. 4. Membrane flotation analysis of ToMV RdRp activity and replication proteins. (A) Schematic representation of the membrane flotation procedure. The iBYL P15 fraction was treated with either TR buffer (mock treatment) (B), 1 M NaCl (C), 0.1 M Na_2CO_3 (D), or 1% Triton X-100 (E) and then fractionated into top (T), middle (M), and bottom (B) fractions of equal volume. Equal volumes of fractionated samples were then subjected to Western blot analysis for detection of ToMV 130K and 180K proteins and the host proteins Sar1 (a peripheral membrane protein), Sec61 (an integral membrane protein), and TOM1 (an integral membrane protein); Northern hybridization for detection of ToMV (−)RNA; and the RdRp assay. RdRp activity was assayed in the absence of exogenous template RNA. The experimental conditions and abbreviations are described in the legends to Fig. 2D, C, and A for Western, Northern, and RdRp analyses, respectively.

extracts than for iBYL (Fig. 2A). These results suggest that the cell extracts obtained without evacuolation contain nucleases and/or other inhibitors of ToMV RNA synthesis and that these inhibitors are removed by evacuolation.

Membrane association of ToMV replication proteins. Our previous subcellular fractionation analysis of ToMV-infected BY-2 protoplast lysates demonstrated that, although ToMV RdRp activity is present only in fractions that contain membranes, large amounts of the 130K and 180K proteins are also present in fractions that do not contain membranes (11). To confirm that the ToMV RdRp activity in iBYL is membrane associated, we fractionated iBYL into supernatant (S15) and membrane-rich pellet (P15) fractions by centrifugation at 15,000 × *g* for 15 min. The integral ER membrane protein Sec61 (33) was found only in the P15 fraction, confirming that membranes were fractionated into P15 (Fig. 3E). Although approximately 90% of the 130K and 180K proteins were fractionated into S15, these proteins were found also in P15 (Fig. 3E). In contrast, nearly all of the (−)RNA and RdRp activity was fractionated into P15 (Fig. 3A and D). When the S15 fraction was used in the RdRp reaction, faintly labeled, ToMV-related RNAs were detected (Fig. 3A). These bands are probably due to contamination of membranes, since the 100,000 × *g* supernatant of iBYL had <1% of the RNA products synthesized by the 100,000 × *g* pellet (data not shown). These results suggest that only membrane-bound 130K and 180K proteins participate in ToMV-related RNA synthesis, confirming the previously reported results (11).

To further test whether ToMV RdRp activity is membrane associated, we subjected the iBYL P15 fraction to membrane flotation analysis on a sucrose gradient (Fig. 4A). The top fraction was found to contain the 130K and 180K proteins, TOM1, (−)RNA, and RdRp activity, as well as the peripheral and integral ER membrane proteins, Sar1 (1) and Sec61, respectively (Fig. 4B). This result confirms that the ToMV RdRp activity that involves the 130K and 180K proteins and (−)RNA is associated with membranes.

We next examined whether the ToMV RdRp can be dislodged from the membranes by treatment with 1 M NaCl or 0.1 M Na_2CO_3, both of which cause some types of peripheral membrane proteins to be released from membranes (8). After each treatment, membrane association was examined by membrane flotation analysis on a sucrose gradient. After 1 M NaCl treatment, more than half of the 130K and 180K protein was

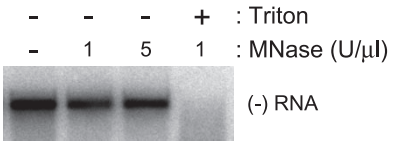

FIG. 5. MNase sensitivity of (−)RNA. The iBYL membranes were suspended in TR buffer containing 2 mM calcium acetate and incubated in the absence (Triton − or MNase−) or presence (Triton + or Mnase 1 or 5) of 1 U/μl or 5 U/μl MNase and 1% Triton X-100 at 30°C for 30 min. After the reaction, total RNA was purified by phenol extraction in the presence of 0.2% SDS. ToMV (−)RNA was detected by Northern hybridization with a ^{32}P-labeled P7P RNA probe (14).

detected in the bottom (loading) fraction (Fig. 4C), indicating that they were partially released from membranes. The ToMV (−)RNA, RdRp activity, the tightly bound peripheral membrane protein Sar1, and the integral membrane proteins Sec61 and TOM1 were enriched in the top fraction (Fig. 4C). These results suggest that only tightly membrane-associated 130K and 180K proteins participate in RNA synthesis.

When P15 membranes were treated with 0.1 M Na_2CO_3, the integral membrane proteins TOM1 and Sec61 remained in the top fraction, and more than half of the 130K and 180K protein, Sar1, and (−)RNA was fractionated into the bottom fraction (Fig. 4D). When the neutral detergent Triton X-100 was added to solubilize membranes, the 130K and 180K proteins and (−)RNA as well as Sar1, Sec61, and TOM1 remained in the bottom fraction. ToMV RdRp activity was inactivated by Na_2CO_3 treatment and greatly reduced by Triton X-100 treatment (Fig. 4D and E). These results suggest that the 130K and 180K proteins and (−)RNA molecules that participate in ToMV RNA replication are tightly and peripherally bound to membranes and that the association of these molecules with membranes is an important factor in RdRp activity.

ToMV (−)RNA was resistant to MNase treatment but became susceptible when treated with Triton X-100 (Fig. 5). A similar result was obtained with another detergent, LPC (data not shown). These results indicate that the (−)RNA template is in an environment inaccessible to MNase, which has an approximate molecular mass of 17 kDa, and that membranes play an important role in the maintenance of the barrier.

Effects of detergents on the state and activity of ToMV replication proteins. Solubilization of ToMV RdRp, which is a critical step in the determination of its constituents, was tried by treating 1 M NaCl-washed membranes from iBYL (iBYL membranes; for preparation, see Materials and Methods) with various detergents at a concentration of 1% (wt/vol). The addition of the neutral detergents Triton X-100, dodecylmaltoside (DoM), and NP-40 or the zwitterionic detergent 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) greatly reduced RdRp activity (Fig. 6A). Addition of exogenous ToMV RNA template to the RdRp reaction mixture with Triton X-100-, DoM-, NP-40-, or CHAPS-treated iBYL membranes slightly increased the incorporation of ^{32}P into the RF and other ssRNA (Fig. 6A). As mentioned earlier, the addition of exogenous ToMV RNA template to non-detergent-treated iBYL membranes caused no detectable change in the pattern of ^{32}P-labeled RNA products in the RdRp reaction (Fig. 3A and B and 6A, lane None). To examine whether the 130K and 180K

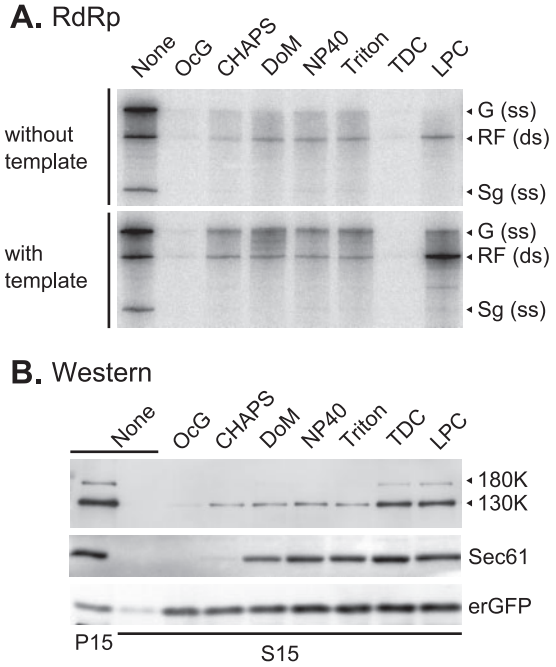

FIG. 6. Effects of detergents on ToMV RdRp activity and the state of the replication proteins. (A) Effect of detergents on ToMV RdRp activity. The iBYL membranes were incubated in the absence or presence of 1% (wt/vol) of the indicated detergents at 15°C for 20 min and then subjected to the RdRp assay. The RdRp assay was performed and the products were analyzed as described in the legend to Fig. 3A and B. For abbreviations, see the legend to Fig. 2A. (B) Western blot analysis of viral and host proteins. The iBYL membranes were treated with the indicated detergents and then subjected to centrifugation at 15,000 × *g* for 15 min. The resulting supernatants (S15) were used for Western blot analysis. As a control, mock-treated iBYL membranes were centrifuged as above, the resulting pellet (P15) was resuspended with TR buffer to the same final volume as the supernatant fraction, and both fractions were analyzed. ToMV replication proteins, an integral membrane protein Sec61, and ER lumen-localized GFP (erGFP) were detected.

replication proteins were solubilized, detergent-treated samples were centrifuged at 15,000 × *g*. For Triton X-100, DoM, NP-40, and CHAPS, only small amounts of the 130K and 180K proteins were detected in the supernatant fractions (where most Sec61 and ER lumen-localized GFP was recovered, with the exception that CHAPS poorly solubilized Sec61) (Fig. 6B). Similar to the above results obtained with the detergents at a concentration of 1%, treatment of iBYL membranes with 0.1% Triton X-100 or DoM also resulted in poor solubilization of the 130K and 180K proteins (data not shown).

Another neutral detergent, octylglucoside (OcG), inactivated RdRp activity and failed to solubilize the 130K, 180K, or Sec61 proteins (Fig. 6, lanes OcG). The anionic detergent TDC solubilized these proteins but inactivated RdRp activity (Fig. 6, lanes TDC). The result obtained with TDC is inconsistent with that reported by Osman and Buck (21), possibly as a consequence of differences in source materials or solubilization conditions. Finally, we found that the zwitterionic detergent LPC solubilized the replication proteins while retaining their RdRp activity (Fig. 6); of all the detergents examined, LPC was the only one to produce this effect. Detailed analyses

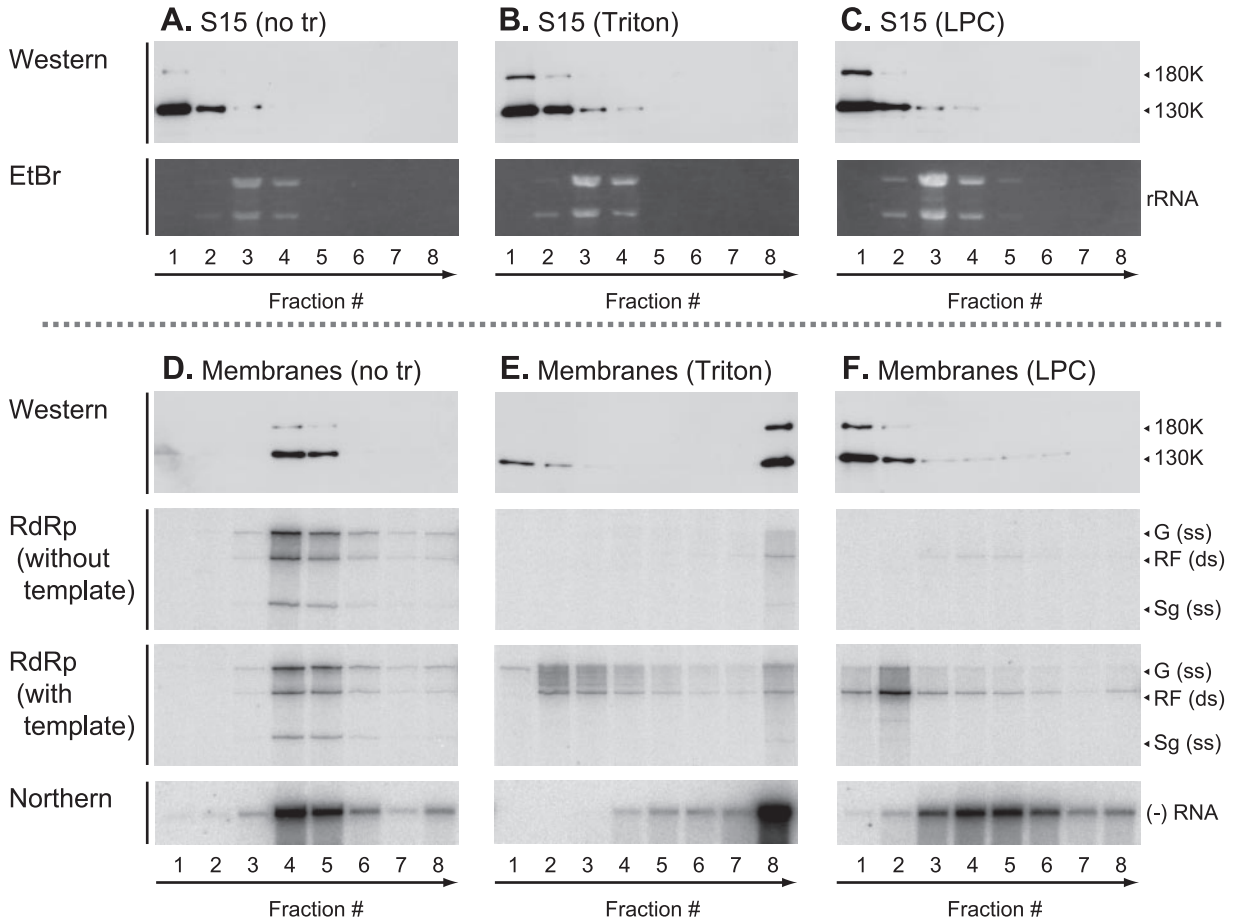

FIG. 7. Sucrose gradient sedimentation analysis of ToMV replication proteins, (−)RNA, and RdRp activity. The iBYL S15 fraction (A to C) or iBYL membranes (D to F) were mock treated (A, D) or treated with 1% Triton X-100 (B, E) or 1% LPC (C, F) at 15°C for 20 min. Samples were then loaded onto a 10 to 50% continuous sucrose gradient and centrifuged. Eight fractions (numbered 1 to 8, from top to bottom of a gradient) were collected and subjected to RdRp assays with or without exogenous ToMV RNA template (0.5 μg ToMV RNA in a 25-μl reaction mixture) and Western blot analysis to detect the 130K and 180K replication proteins. For RdRp product abbreviations, see the legend to Fig. 2A. Total RNA was also extracted from each fraction and subjected to agarose gel electrophoresis with ethidium bromide staining to detect rRNA or subjected to Northern hybridization to detect ToMV (−)RNA with a ^{32}P-labeled P7P RNA probe (14).

of LPC-solubilized ToMV RdRp are described later in this report.

Sucrose-gradient sedimentation analysis was performed to determine the effects of Triton X-100 treatment on iBYL membranes. In the experimental conditions used here, the 80S ribosomes were largely fractionated into fraction 3 (the third centrifugation fraction from the top) (Fig. 7A to C, panels rRNA) and ToMV particles (190S) were fractionated into fraction 4 (data not shown). In the absence of detergent treatment, the 130K and 180K proteins, (−)RNA, and RdRp activity were all found predominantly in fractions 4 and 5, which contained membranes (Fig. 7D). In the Triton X-100-treated iBYL membranes, the 130K and 180K proteins, (−)RNA, and weak, endogenous template-dependent RdRp activity were found predominantly in fraction 8 at the bottom of the gradient (Fig. 7E). Sec61 was found in fraction 1 (data not shown), suggesting that this protein was solubilized. Small amounts of the 130K and 180K proteins were found in fractions 1 and 2 and other upper fractions, and weak, exogenous ToMV RNA template-dependent RdRp activity was found in fractions 2 and 3 (Fig. 7E). One of the possible interpretations of these results would be as follows: upon treatment with Triton X-100, (i) most 130K and 180K proteins and (−)RNA molecules are bound into aggregates or huge oligomers that retain weak, endogenous template-dependent RdRp activity, and (ii) small amounts of the 130K and 180K proteins are solubilized, forming a complex that sediments slightly more slowly than 80S ribosomes and is capable of synthesizing RNA in the presence of exogenous ToMV RNA template.

To test whether non-membrane-bound 130K and 180K proteins present in the S15 fraction of iBYL respond to Triton X-100 treatment in the same way as the membrane-bound 130K and 180K proteins, we performed a similar sucrose gradient sedimentation analysis. Regardless of Triton X-100 treatment, most 130K and 180K proteins in the S15 fraction remained in the loading layer (fraction 1) after centrifugation (Fig. 7A and B). This result suggests that the 130K and 180K proteins in the iBYL membranes differ from those in the S15 fraction in their conformation, association(s) with other molecules, or other properties.

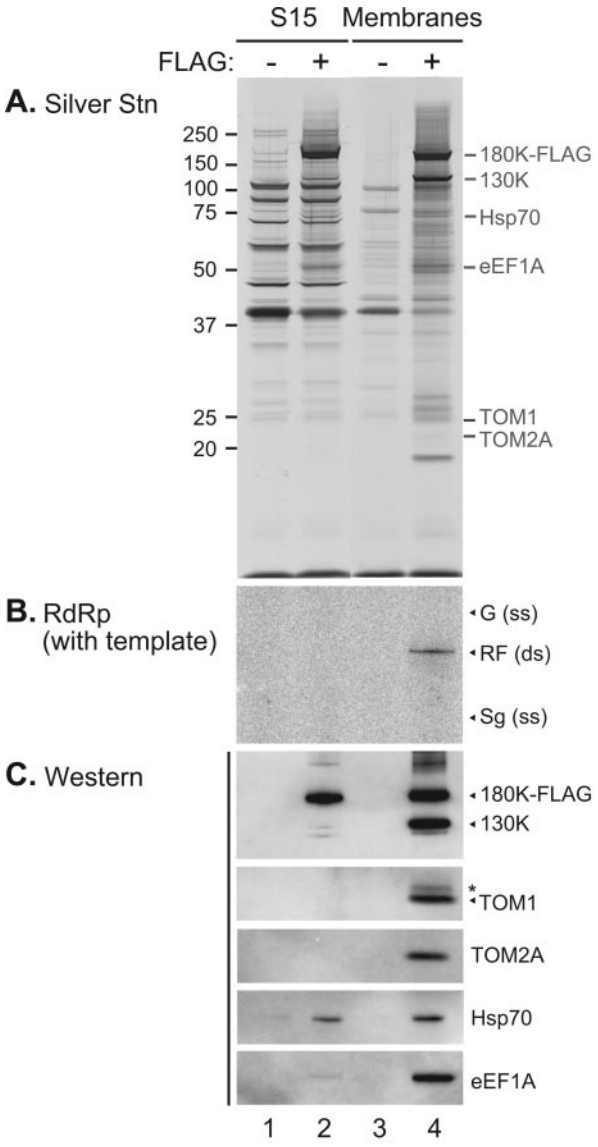

FIG. 8. Affinity purification of FLAG-tagged 180K replication protein. The iBYL S15 fraction (lanes 1, 2) or iBYL membranes (lanes 3, 4) from BY-2 cells were treated with LPC and subjected to affinity purification with anti-FLAG antibody-conjugated agarose beads, as described in Materials and Methods. In the BY-2 cells, replication of a ToMV derivative encoding either 180K-FLAG (lanes 2, 4), or non-tagged 180K proteins (lanes 1, 3) was occurring. (A) Silver staining of affinity-purified proteins separated by SDS–12% PAGE. Equal volumes of purified samples were loaded in each lane. The positions and masses (in kDa) of protein markers are shown on the left. The expected locations of 180K-FLAG, 130 K, TOM1, TOM2A, eEF1A, and Hsp70 are shown on the right. (B) RdRp activity in the presence of exogenous ToMV RNA template. For RdRp product abbreviations, see the legend to Fig. 2A (the single-stranded genomic and subgenomic RNA bands were not detected here). (C) Detection of ToMV replication proteins, the TOM1 and TOM2A proteins, Hsp70, and eEF1A in the purified fractions. In panels B and C, the samples in lanes 1, 2, 3, and 4 were applied in a volume ratio of 1:1:4.3:4.3, respectively. This ratio yielded similar intensities of the 180K-FLAG bands on Western blots in lanes 2 and 4. The abbreviations are described in the legends to Fig. 2A and D for RdRp and Western analyses, respectively.

Solubilization of membrane-bound 130K and 180K proteins with LPC. Of the detergents we tested, LPC had the ability to solubilize the membrane-bound ToMV replication proteins (Fig. 6B). LPC treatment also reduced endogenous template-dependent RdRp activity and activated exogenous template-dependent RdRp activity (Fig. 6A, lanes LPC). In sucrose gradient sedimentation analysis of LPC-treated iBYL membranes, most 130K and 180K proteins were found in the uppermost two fractions (fractions 1 and 2) and the exogenous ToMV-RNA template-dependent RdRp activity was primarily detected in fraction 2 (Fig. 7F). These results suggest that the membrane-bound 130K and 180K proteins are efficiently solubilized by LPC and that the LPC-solubilized, exogenous ToMV RNA template-dependent RdRp activity resides in a complex that sediments slightly more slowly (fraction 2) than do 80S ribosomes (fraction 3) (Fig. 7F).

To purify and characterize the LPC-solubilized RdRp complex that presumably contained the 180K protein, BY-2 cells were transformed with a gene cassette containing an estradiol-inducible promoter-driven, ToMV-derived cDNA encoding 180K-FLAG. The addition of estradiol to the transgenic BY-2 cells successfully induced replication of the ToMV-derived RNA, indicating that tagged 180K protein was functional in supporting ToMV RNA replication. When we prepared iBYL membranes from the induced cells, solubilized them with LPC, and subjected them to affinity purification (Fig. 8A), the FLAG-purified fraction contained exogenous ToMV RNA template-dependent RdRp activity (Fig. 8B) and the 130K protein (Fig. 8C). In addition, the FLAG-purified fraction contained host proteins TOM1, TOM2A, eEF1A, and heat-shock protein Hsp70, but no eIF3 subunits, as detected by Western analysis (Fig. 8C and data not shown).

For comparison, 180K-FLAG was also purified from the iBYL S15 fraction. As expected, membrane proteins TOM1 and TOM2A were not detected in the FLAG-purified fraction. Both the 130K protein and eEF1A copurified with 180K-FLAG, but their amounts relative to 180K-FLAG were smaller than those observed for iBYL membranes (Fig. 8C). In some experiments, the eEF1A concentrations in the FLAG-purified fractions from iBYL S15 fractions with FLAG-tagged and non-tagged 180K proteins were similar. Hsp70 copurified with 180K-FLAG from S15 iBYL, and the amounts of Hsp70 relative to 180K-FLAG in the FLAG-purified fractions from iBYL S15 and iBYL membranes were similar.

DISCUSSION

In the present study, the extracts of ToMV-infected, evacuolated protoplasts (iBYL) were found to be capable of synthesis of ToMV-related (+)RNAs using endogenous (−)RNA as a template. When RNA synthesis was performed in the presence of [^{32}P]rNTPs, both the RF and ssRNAs comigrating with the genomic and subgenomic RNAs were ^{32}P labeled. The electrophoretic pattern of labeled RNA resembled that of [^{3}H]RNAs extracted from ToMV-infected cells that were cultured in the presence of [^{3}H]uridine, suggesting that iBYL contains undamaged ToMV RNA replication complexes. Extracts of ToMV-infected protoplasts prepared without evacuolation contained an RdRp activity that incorporated labeled nucleotides into the RF but not into distinct ssRNA bands.

In previous studies, crude membranes from homogenates of ToMV-infected tomato leaf tissues were found to contain an endogenous template-dependent RdRp activity that incorporated labeled nucleotides into the RF, and purification of the membranes by sucrose density gradient centrifugation caused the RdRp activity to be capable of incorporating labeled nucleotides into both the RF and the genomic ssRNA (20). However, this tomato leaf tissue-derived, sucrose gradient-purified ToMV RdRp synthesized only a trace amount of subgenomic RNA and endogenous (−)RNA was sensitive to MNase, whereas iBYL could synthesize subgenomic RNA and contained a MNase-resistant (−)RNA. These observations suggest that vacuole-derived contaminants not only inhibit the synthesis or accumulation of ssRNA but also irreversibly change the characteristics of the replication complexes in leaf extracts.

Restriction of (−)RNA synthesis and storage in an isolated membranous compartment. Consistent with previous observations regarding the RdRps of tobamoviruses and other eukaryotic (+)RNA viruses, the ToMV RdRp activity and (−)RNA were bound to membranes in iBYL (Fig. 3 and 4). The membrane-associated (−)RNA was resistant to MNase treatment except when treated with detergents, suggesting that the (−)RNA is in a membrane-associated environment inaccessible to MNase (Fig. 5). These features resemble those reported for the replication complexes of eukaryotic (+)RNA viruses including *Brome mosaic virus* (19, 23, 25). Replication complexes of *Brome mosaic virus* are formed in spherular invaginations of the ER membranes or between appressed double membranes (26), which is consistent with the detergent-dependent nuclease sensitivity of the (−)RNA. The morphology of tobamovirus replication complexes remains to be determined.

Although ToMV RdRp and (−)RNA were exclusively associated with membranes, only minor fractions (ca. 10%) of the viral replication proteins were associated with membranes (Fig. 3). The ToMV replication proteins in the iBYL S15 fraction did not show an RdRp activity even when exogenous ToMV RNA was added (Fig. 3B and C). The absence of RdRp activity is not due to the presence of ribonucleases or other inhibitors because unfractionated iBYL was able to synthesize ToMV-related RNAs, and the RdRp activity of the iBYL P15 fraction was not inhibited by the addition of the iBYL S15 fraction (data not shown). The absence of the RdRp activity in the iBYL S15 fraction is consistent with the fact that (−)RNA was virtually undetectable in the fraction. We speculate that the ToMV replication proteins are programmed to acquire RdRp activity capable of (−)RNA synthesis only after they are bound to membranes and perhaps also after the genomic RNA template is sequestered away from the cytoplasm by membranes, so that (−)RNA is never synthesized in the cytoplasm.

The presence of a mechanism to prevent synthesis of (−)RNA in the cytoplasm and to ensure its sequestration in a membranous compartment would be important for successful ToMV RNA multiplication because (−)RNA synthesized in or leaked into the cytoplasm might anneal with the cytoplasmic (+)RNA to form double-stranded RNA that would trigger RNA silencing against ToMV RNA. In contrast, (+)RNA phages, whose hosts do not have an RNA silencing system, would not need to sequester the (−)RNA replication intermediates away from the cytoplasm and, thus, might be able to utilize soluble replicase enzymes (2).

Host proteins associated with ToMV replication proteins. In this study, we found that LPC is able to solubilize membrane-bound 130K and 180K ToMV replication proteins. Although LPC reduced the ability of membrane-bound 130K and 180K ToMV replication proteins to synthesize (+)RNA using the endogenous (−)RNA as a template, the proteins acquired an ability to synthesize (−)RNA using exogenous ToMV RNA as a template (Fig. 6 and 7). This result suggests that intact membranes are important for normal functioning of ToMV replication complexes and that LPC solubilization causes the ToMV RdRp to lose some important protein(s) or factor(s) that are normally present in membrane-bound, native, ToMV replication complexes. The possibility still exists, however, that the LPC-solubilized RdRp retains some of these components.

Affinity purification of LPC-solubilized 180K replication protein from iBYL membranes resulted in specific recovery of the exogenous template-dependent RdRp activity, viral 130K protein, and the host proteins Hsp70, eEF1A, TOM1, and TOM2A (Fig. 8). This result provides indirect evidence for the involvement of TOM1 and TOM2A in tobamovirus RNA replication in plant cells, which is consistent with previously reported genetic and other evidence linking the TOM1 and TOM2A proteins to tobamovirus RNA replication. The eEF1A protein is reported to directly bind to tobamovirus replication proteins (31) as well as to pseudoknot structures in the 3′ untranslated region of tobamovirus RNA (34). Since the tobamovirus replication proteins also bind to tobamovirus RNA (22), the observed association between eEF1A and the 180K protein may occur both through direct physical interaction and through indirect interactions mediated by the ToMV RNA. It is also possible that a fraction of eEF1A is involved in translation.

Treatment of iBYL membranes with 1 M NaCl did not affect ToMV RdRp activity or binding of the (−)RNA to membranes. This treatment caused approximately half of the originally membrane-bound 130K and 180K replication proteins to dissociate from the membranes (Fig. 4C), suggesting that not all of the membrane-bound replication protein molecules reside in active replication complexes. We also do not know the extent of participation of the tightly membrane-bound replication proteins in tobamovirus-related RNA synthesis. In hepatitis C virus RNA replication, only a minor fraction of the membrane-bound replication proteins are participants in viral RNA replication (23). How many of the 180K-associated proteins (Fig. 8A) are directly contributing to RNA synthesis remains to be determined. For the same reason, future work should also focus on minor host proteins that copurify with the 180K protein.

Affinity purification of the 180K protein from the iBYL S15 fraction resulted in copurification of only trace amounts of the 130K protein and eEF1A, although the 130K protein and eEF1A are present in the cytoplasm at high concentrations (Fig. 3 and 8; also data not shown). On the other hand, membrane-bound and non-membrane-bound replication proteins exhibited very different behaviors upon Triton X-100 treatment (Fig. 7). One of the possible interpretations of these observations is that the membrane-bound replication proteins have conformational and other physical properties that are distinct from those of the non-membrane-bound replication proteins. The putative conformational differentiation of ToMV

replication proteins might be achieved through a multistep process involving interactions with the genomic RNA, with host membranes containing TOM1, with themselves, and with other host factors in a fixed order that is as yet unknown. Copurification of Hsp70 with the 180K protein is consistent with results from other viral systems that have also observed that protein chaperones are required for viral RNA replication (15, 26, 27, 30) and suggests that the above process is mediated by chaperones.

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