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The Ribosomal L5 Protein Is Associated with mdm-2 and mdm-2–p53 Complexes

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Throughout the purification of the mdm-2 or mdm-2–p53 protein complexes, a protein with a molecular weight of 34,000 was observed to copurify with these proteins. Several monoclonal antibodies directed against distinct epitopes in the mdm-2 or p53 protein coimmunoprecipitated this 34,000-molecular-weight protein, which did not react to p53 or mdm-2 polyclonal antisera in a Western immunoblot. The N-terminal amino acid sequence of this 34,000-molecular-weight protein demonstrated that the first 40 amino acids were identical to the ribosomal L5 protein, found in the large rRNA subunit and bound to 5S RNA. Partial peptide maps of the authentic L5 protein and the 34,000-molecular-weight protein were identical. mdm-2–L5 and mdm-2–L5–p53 complexes were shown to bind 5S RNA specifically, presumably through the known specificity of L5 protein for 5S RNA. In 5S RNA–L5–mdm-2–p53 ribonucleoprotein complexes, it was also possible to detect the 5.8S RNA which has been suggested to be covalently linked to a percentage of the p53 protein in a cell. These experiments have identified a unique ribonucleoprotein complex composed of 5S RNA, L5 protein, mdm-2 proteins, p53 protein, and possibly the 5.8S RNA. While the function of such a ribonucleoprotein complex is not yet clear, the identity of its component parts suggests a role for these proteins and RNA species in ribosomal biogenesis, ribosomal transport from the nucleus to the cytoplasm, or translational regulation in the cell.

The mdm-2 gene was originally detected as an amplified DNA sequence on double minute chromosomes in the 3T3DM cell line, which was derived from spontaneously transformed BALB/c 3T3 cells (1). Subsequently, it was shown that overexpression of the mdm-2 gene can increase the tumorigenic potential of cells (5), thus qualifying it as an oncogene. Indeed, the mdm-2 oncogene is amplified in a variety of osteogenic sarcomas and soft tissue sarcomas of humans (4, 14). The mdm-2 gene encodes several proteins with molecular weights that vary between 90,000 and 57,000 (15), and these proteins express distinct epitopes on different mdm-2 proteins, as characterized by using a variety of mdm-2-specific monoclonal antibodies (2).

The only known function of these mdm-2 proteins is that some subset of them bind to the p53 protein and block its ability to act as a transcription factor (12). Amino acid residues 19 to 102 from the mdm-2 protein (of a total of 491) and amino acid residues 1 to 52 of the p53 protein (of a total of 393) are required to form p53–mdm-2 complexes (2), so the N termini of both proteins make these protein contacts. The N-terminal 42 amino acids of p53 constitute the transactivation domain of p53 (6, 17), which presumably makes contact with the transcriptional machinery of the cell (20), resulting in enhanced mRNA synthesis. Indeed, the same two amino acids (Leu-22 and Trp-23) required for transcriptional activation in the N terminus of the p53 protein have also been shown to be critical for mdm-2 binding to the p53 protein (9).

Several forms of the mdm-2 protein lack these critical N-terminal sequences needed for p53 binding and so do not make p53–mdm-2 complexes (2, 15). These observations suggest that some mdm-2 isoforms have unique functions not related to blocking the transcriptional activity of p53. During the purification of mdm-2 and mdm-2–p53 complexes, an unusual protein with a molecular weight of 34,000 (p34) was observed to copurify with these proteins. The p34 protein coimmunoprecipitated with p53–mdm-2 complexes or free mdm-2 protein. This association could be demonstrated with several distinct monoclonal antibodies directed against mdm-2 or p53. The 34,000-molecular-weight protein did not react in a Western immunoblot with either p53 or mdm-2 polyclonal antibodies, suggesting that a distinct or unique protein copurified with and was associated with the mdm-2 and mdm-2–p53 protein complexes.

The 34,000-molecular-weight protein was subjected to N-terminal amino acid analysis and shown to have its first 40 amino acids identical to the L5 rRNA protein. Partial peptide maps of authentic L5 protein and the 34,000-molecular-weight protein were identical. Interestingly, purified mdm-2 or p53–mdm-2 complexes were shown to specifically bind 5S RNA, presumably via the specific L5-5S RNA interactions known to occur (21). Curiously, a small percentage of the p53 protein is thought to be covalently bound to 5.8S rRNA (8, 18, 19). Previous experiments have demonstrated that the L5 protein, the 5S RNA, and 5.8S RNA can readily form a ternary complex in solution (11). Thus, these results suggest that a novel ribonucleoprotein particle, composed of p53–mdm-2–L5 proteins and 5S and 5.8S RNAs, may exist in the cell. While the function of such a complex is unclear, the nature of its components suggests a role for p53 and mdm-2 in ribosome biogenesis, ribosomal transport from the nucleus, or translational regulation in the cell.

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MATERIALS AND METHODS

Cell culture. 3T3DM, A-1 (15), and Sp2 cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (2). Cells were grown at 37°C in a humidified 5% CO_2 atmosphere unless otherwise indicated. For protein labeling, cells were incubated for 30 min in methionine-free Dulbecco's modified Eagle's medium supplemented with 2% dialyzed fetal bovine serum and labeled with 50 μCi of [^{35}S]methionine EXPRESS (Dupont) per ml for 2 h. For RNA detection, cells were incubated for 30 min in phosphate-free Dulbecco's modified Eagle's medium supplemented with 2% dialyzed fetal bovine serum and labeled with 250 μCi of $^{32}P_i$ (Amersham) per ml in the same medium. Cells were rinsed in ice-cold phosphate-buffered saline, scraped from the plate with a rubber policeman, and centrifuged. Cell pellets were stored at −80°C.

Immunoprecipitations. Cells were lysed in lysis buffer (50 mM Tris-Cl [pH 8.0], 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) for 30 min on ice, and lysates were centrifuged at 15,000 × *g* for 10 min. Approximately 10^7 cells from the extract were used to prepare supernatant, which was then incubated with 200 μl of antibody supernatant and 30 μl of 50% protein G-Sepharose. The immunoprecipitates were washed three times in SNNTE buffer (5% sucrose, 1% Nonidet P-40, 0.5 M NaCl, 50 mM Tris-Cl [pH 7.5], 5 mM EDTA) and three times in RIPA buffer (50 mM Tris-Cl [pH 7.5], 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], 1% sodium deoxycholate). Immunoprecipitates were then boiled in sample buffer and fractionated by SDS-polyacrylamide gel electrophoresis (PAGE), and the gel was exposed to fluorography and dried as previously described (2). Immunoprecipitation of p53 or mdm-2, carried out as described above, was also followed by a Western blotting procedure with p53 or mdm-2 monoclonal or polyclonal antisera as described previously (15). This is termed the IP-Western procedure.

mdm-2–p53–L5 copurification. Approximately 10^8 cpm from a ^{35}S-labeled A-1 cellular extract was incubated with a protein A-Sepharose column for 1 h. After centrifugation, the extract was passed over 100 μl of a PAb421 protein A-Sepharose column for 4 h at 4°C. The column was washed with SNNTE buffer, then with RIPA buffer, and finally with lysis buffer. The beads were incubated for 1 h at 4°C in 600 μl of lysis buffer containing 0.25 mg of PAb421-epitope peptide per ml (3). The eluate was dilute lysis buffer (with an NaCl concentration of 50 mM) and was passed twice over a 1-ml Q-Sepharose fast-flow column (Pharmacia). The elution was performed at each salt concentration until no more labeled protein was eluted. Immunoprecipitation with the 2A10 monoclonal antibody was performed on the initial cellular extract, as well as the PAb421 antibody-treated eluate and the first and last fractions at each step of the increasing NaCl elutions from the anion-exchange column.

Partial proteolysis of p34 and L5 proteins. Following immunoprecipitations with PAb2A10 and PAb4B11 from the A1-5 and Sp2 cells and separation of proteins by SDS-PAGE, the gel was rinsed briefly in water, dried, and autoradiographed. The mdm-2 and p34 bands were excised and placed in the well of another SDS-polyacrylamide gel. The L5 protein synthesized in a programmed reticulocyte lysate was also placed in a well of the same gel. The gel slices were overlaid with 10 μl of digestion buffer (0.125 M Tris-Cl [pH 6.8], 0.1% SDS, 1 mM EDTA, 0.002% bromophenol blue, 0.01 mg of bovine serum albumin [BSA] per ml, 20% glycerol) followed by 10 μl of digestion buffer without BSA. A 2.5-μl portion of V8 protease (Boehringer Mannheim) stock (0.25 mg/ml) was added at a 1:100 or 1:10 dilution. Running buffer was slowly added to the wells and then to the upper reservoir. The gel was run at 25 mA (constant amperage). Just before the dye front entered the resolving gel, the gel was turned off for 30 min to allow for protease digestion and then the process was continued at 25 mA. The gel was fixed, exposed to fluorography, dried, and exposed to autoradiography (2).

Analysis of coimmunoprecipitated RNA. A $^{32}P_i$-labeled cellular extract containing RNasin was precleared with protein G-Sepharose and subjected to immunoprecipitation for 2 h. Immunoprecipitates were washed three times each in SNNTE and RIPA buffer. For each immunoprecipitation, 0.1 mg of yeast tRNA and 0.06 mg of proteinase K were added and the mixture was incubated at 56°C for 15 min. Proteinase K was inactivated at 94°C for 5 min, and the beads were incubated on ice. RNA to be extracted was placed in 100 μl of guanidium thiocyanate denaturing solution, phenol-chloroform extracted, and ethanol precipitated.

For each immunoprecipitation, 1 μg of total RNA and 60 μg of yeast tRNA were subjected to a 2% agarose denaturing gel containing 1% formaldehyde and transferred to a nylon membrane. The membrane was exposed to autoradiography (see Fig. 5) or hybridized with 5S and 5.8S DNA probes (see Fig. 6). A 5S probe was generated by PCR of Sp2 cell DNA with primers 5′-GGGACGAGCTCGTCTACGGCCATACC-3′ and 5′-GGGAGCTCGAGAAAGCCTACAGCACC-3′, and a 5.8S probe was generated by using 5′-CGACTCTTAGCGG TGGAT-3′ and 5′-AGCGACGCTCAGACAGGC-3′. PCR products were sequenced, and the sequences match the previously published 5S and 5.8S sequences identically.

PCRs were performed in the presence of 0.2 mM each deoxynucleoside triphosphate except dTTP (0.13 mM) and digoxigenin-labeled dUTP (0.07 mM). All probes were gel purified and then stored at −20°C.

RESULTS

A 34-kDa protein is associated with p53–mdm-2 protein complexes. 3T3DM cells contain multiple copies of the mdm-2 gene and overproduce mdm-2 proteins (15). These cells were labeled with [^{35}S]methionine for 2 h at 37°C, and the soluble proteins from an extract of these cells were immunoprecipitated by using two distinct mdm-2-specific monoclonal antibodies (PAb2A10 and PAb4B11), as well as a rabbit polyclonal anti-mdm-2 antibody (α-mdm-2). The immunoprecipitates were analyzed by SDS-PAGE, and an autoradiogram of that gel is presented in Fig. 1. A major band at a molecular weight of 90,000 and a minor one at 85,000 are mdm-2 proteins. The bands at 57,000 (2A10), 45,000 (4B11) and 67,000 (α-mdm-2) represent serum-specific cross-reactions and not mdm-2 proteins (15). The p53 protein coimmunoprecipitates with these mdm-2 proteins. Consistently, a protein with a molecular weight of 34,000 was observed in these experiments (Fig. 1) and termed p34; it did not react with either p53 polyclonal sera or mdm-2 polyclonal sera in Western blots.

p34 could be detected by immunoprecipitation when a number of distinct mdm-2 monoclonal antibodies (2A10 and 4B11 in Fig. 1 or 4B2 and 3G5 [2, 15]), a polyclonal mdm-2 serum, or the p53 monoclonal antibody PAb421 was used. The 3G5 monoclonal antibody detects only free mdm-2 protein, not mdm-2 complexed with the p53 protein, and it also immunoselected the p34 protein. This means that p34 is related to mdm-2, or associated with mdm-2, as well as being observed in an mdm-2–p53 complex. Western blots of these mdm-2 and p53 proteins, using anti-mdm-2 or anti-p53 monoclonal or

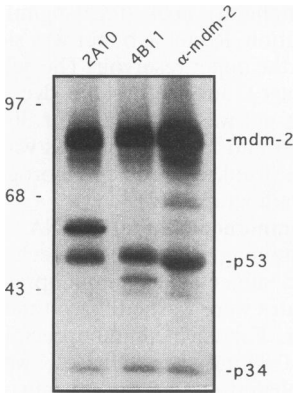

FIG. 1. Coimmunoprecipitation of a 34-kDa protein with mdm-2. 3T3DM cells were labeled with [^{35}S]methionine for 2 h, and the soluble lysate was immunoprecipitated with the PAb2A10 and PAb4B11 mdm-2 monoclonal antibodies and rabbit mdm-2 polyclonal sera. Immunoprecipitates were analyzed by SDS-PAGE (9.5% polyacrylamide), and the gel was exposed to autoradiography.

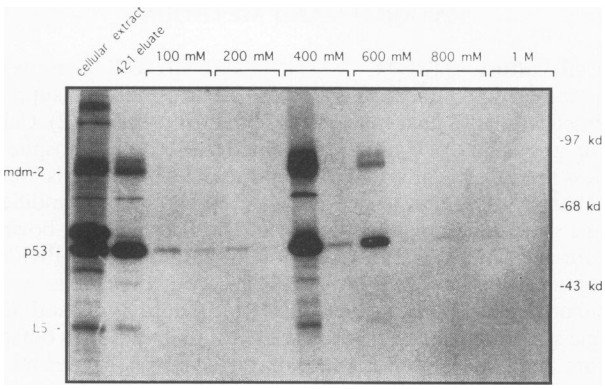

FIG. 2. Copurification of mdm-2, p53, and L5 (p34) proteins. A-1 cells, which contain a temperature-sensitive p53 protein, were incubated at 32°C and labeled with [^{35}S]methionine, and a cellular extract was prepared. p53 was purified by antibody affinity (PAb421) chromatography and elution with a peptide corresponding to the PAb421 epitope (3). The eluate was then passed over a Q-Sepharose anion-exchange column, and the protein was eluted with an increasing NaCl step gradient at the indicated NaCl concentrations. PAb2A10 (mdm-2-specific antibody) was used to immunoprecipitate the initial cellular extract, the PAb421 eluate, and the first and last fractions of step gradient elutions from the anion-exchange column.

polyclonal sera, detect the 90,000- to 85,000-molecular-weight mdm-2 proteins or p53 protein but fail to react with the p34 protein in an immunoprecipitation followed by a Western blotting procedure (see Materials and Methods).

Copurification of mdm-2–p53 and L5 (p34) proteins. The above results suggested the possibility that p34 was not related to p53 or mdm-2 but was associated with mdm-2 in a complex and was coimmunoprecipitated with anti-mdm-2 antibody. To test this, a three-step purification was devised to purify p53–mdm-2 complexes. A-1 cells, which contain a temperature-sensitive p53 protein (7) that stimulates the transcription of the mdm-2 gene at 32°C (but not 39°C) (22), were incubated at 32°C for 18 h and labeled with [^{35}S]methionine for 3 h. Soluble cell extracts were prepared and, prior to a first step in the purification, passed over an antibody affinity column containing antibodies directed against an irrelevant antigen (PAb416, anti-simian virus 40 T antigen) to remove proteins that bind nonspecifically to antibodies on the column matrix. The eluate was next passed over an antibody affinity column containing PAb421, a p53-specific monoclonal antibody. The p53 protein was then specifically eluted with an epitope peptide which reacts in excess with the antibody and releases the protein (12). This eluate was then chromatographed over an anion-exchange column (Q Sepharose; fast flow), and the fractions were eluted with increasing, discontinuous salt concentrations. Finally, each fraction from this column, including the starting cellular extract and the eluate from the PAb421 antibody column, was immunoprecipitated with PAb2A10, an mdm-2-specific monoclonal antibody. This purification procedure, used previously to demonstrate that mdm-2 copurifies with p53 (12), purifies the mdm-2–p53 complex, with little or no other protein contaminants unaccounted for in these final fractions (12). The p53–mdm-2 complex elutes from the anion-exchange column at 400 mM NaCl and to a lesser extent at 600 mM NaCl (Fig. 2). At molecular weights of 90,000 to 85,000 are mdm-2 proteins, confirmed by Western blot analysis. At a molecular weight of 70,000 is the heat shock protein which commonly copurifies with p53 in this step (12). At a molecular weight of 53,000 and in three discrete bands between molecular weights of 43,000 and 53,000 are the p53 protein and its first proteolytic breakdown products, as determined by Western blot procedures (12). Finally, the p34 protein (labeled L5 in Fig. 2) has copurified through two antibody immunoselections (for p53 and mdm-2) and the anion-exchange column (an estimated 5,000-fold purification [12]). Clearly, then, a p34 protein is tightly associated with a p53–mdm-2 complex.

The p34 protein was shown to be associated with mdm-2 in a wide variety of cells: 3T3DM, A-1, and the Sp2 murine B-cell lymphoma cells from both mouse and rat origins. Thus, the p34–mdm-2 protein association is common to a number of cell lines.

Amino acid sequence of p34. The p34 protein was purified from the p53–mdm-2 complex and obtained by being cut out of an SDS-polyacrylamide gel. An N-terminal amino acid sequence was attempted for both the mdm-2 and p34 bands. The N terminus of the mdm-2 proteins was blocked, but a 40-amino-acid sequence of p34 was obtained (Fig. 3). When compared with known amino acid sequences from GenBank, the first 40 amino acids of p34 match the large ribosome-associated L5 protein (Fig. 3).

Partial peptide maps of p34 and L5 proteins. The unexpected result described above raised a significant complication. The L5 protein is one of the more abundant proteins in a cell, and it could have contaminated the radiolabeled p34 protein in the gel. In that case, the labeled protein detected at a molecular weight of 34,000 in a gel would contribute very little to the sequence derived from the unlabeled amino acids from a protein in this fraction. The question then became whether

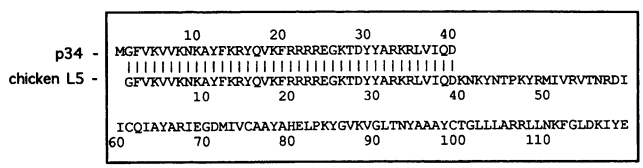

FIG. 3. Amino acid sequence comparison of the N-terminal 40 amino acids of p34 with the first 119 amino acids of the chicken L5 protein as obtained from GenBank.

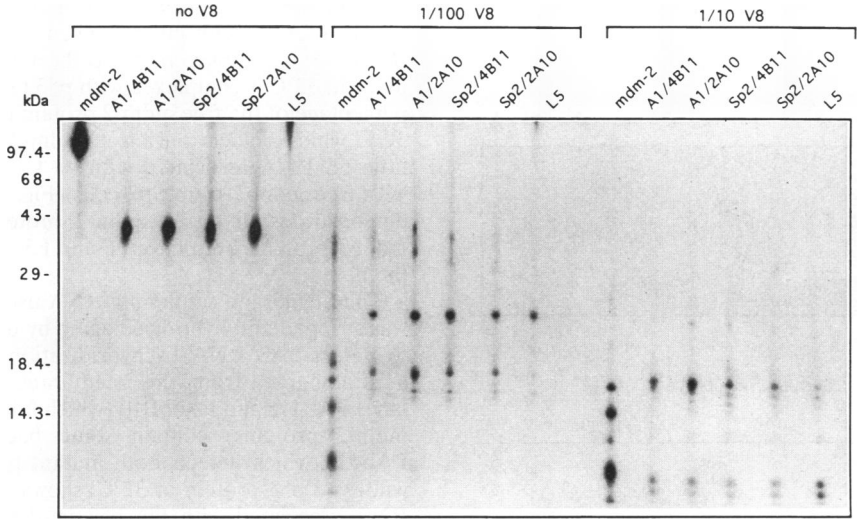

FIG. 4. Partial proteolysis of in vitro translated mdm-2 protein, immunoprecipitated p34 protein from A-1 and Sp2 cells with the PAb4B11 and PAb2A10 antibodies, and the L5 protein translated in vitro. These [^{35}S]methionine-labeled proteins were analyzed by SDS-PAGE with no V8 protease treatment, a 1/10 dilution of a 0.25-mg/ml V8 protease stock, or a 1/100 dilution of this stock as indicated.

the L5 protein was identical to the labeled p34 protein that coimmunoprecipitated with the p53–mdm-2 complex.

To test this, [^{35}S]methionine-labeled mdm-2 protein and p34 protein from two cellular sources (A-1 or Sp2 cells) were immunoselected with either monoclonal antibody PAb2A10 or PAb4B11 and compared by using a partial peptide map analysis with the authentic L5 protein. The authentic L5 protein was obtained by transcribing and translating an L5 murine cDNA clone in vitro in a rabbit reticulocyte system which produced a source of [^{35}S]methionine-labeled authentic L5 protein. The mdm-2 protein, the p34 protein from A-1 cells selected by the PAb2A10 antibody, the p34 protein from A-1 cells selected by the PAb4B11 antibody, the p34 protein from Sp2 cells selected by the PAb4B11 antibody, the p34 protein from Sp2 cells selected by the PAb2A10 antibody, and the authentic L5 protein were all compared by partial proteolysis with the V8 protease at two different concentrations (1/10 and 1/100 dilutions). Figure 4 presents these partial peptide maps. The peptides derived from p34 (two cell sources, two monoclonal antibodies) were identical to the authentic L5 protein and distinct from the mdm-2 protein. The labeled p34 protein that copurifies with mdm-2 protein is the L5 protein.

The p53–mdm-2 complex is associated with 5S RNA. The L5 protein binds specifically to 5S RNA, and this complex is a precursor to ribosome assembly in mammalian cells (21), as well as being present on the ribosome. This raised the question whether the p53–mdm-2–L5 complex was associated with 5S RNA. To test this, Sp2 cells were labeled with $^{32}P_i$ to label the RNA as well as the phosphoproteins. These cells were lysed, and a variety of mdm-2- and p53-specific monoclonal antibodies were used to immunoselect RNA complexes. The RNA was extracted from the immunoprecipitates and analyzed on 8% urea–acrylamide gels by electrophoresis. A tRNA, 5S, 5.8S, and rRNA marker was run (total RNA) to size the RNA detected in this gel. Figure 5 presents an autoradiogram of this gel. Several monoclonal antibodies used for immunoselection in this experiment were used as negative controls. PAb419 and PAb416 detect the simian virus 40 large T antigen not present in Sp2 cells. PAb2A9 is a monoclonal antibody that detects human mdm-2 proteins but does not detect mouse mdm-2 proteins from Sp2 cells, which are murine B-cell lymphoma cells. PAb421 is a p53-specific monoclonal antibody, while PAb4B2, 2A10, 4B11 and 3G5 all recognize murine mdm-2 proteins. 3G5 detects murine mdm-2 proteins only if they are not in a complex with the p53 protein (2). The two rRNAs (28S and 18S) contaminate every sample. The 5S RNA is detected only when mdm-2 (and its associated L5 protein) is present in the immunoprecipitate. A low level of 5.8S RNA is present in all samples (i.e., it is a nonspecific contaminant). The 5.8S RNA previously reported to be associated with p53 protein (8, 18, 19) should not be detected by this assay because it has been reported to be covalently bound to p53. In this experiment, the RNA was extracted from the immunoselected samples without proteinase K treatment (protease), which would be needed to release the 5.8S RNA from its covalent linkage to p53.

5S and 5.8S RNAs in association with mdm-2, p53, and p53–mdm-2 complexes. The previous experiment relied on the size of 5S RNA to look for labeled RNA in an mdm-2 or

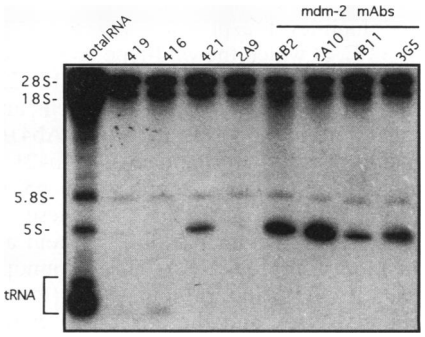

FIG. 5. Immunoselection of 5S RNA associated with mdm-2 and the p53–mdm-2 complex. A $^{32}P_i$-labeled Sp2 cell extract was immunoprecipitated by using antibodies that fail to detect mdm-2 protein (PAb419, PAb416, and PAb2A9), the p53-specific PAb421 antibody, and mdm-2-specific monoclonal antibodies (mAbs) (PAb4B2, PAb2A10, PAb4B11, and 3G5). The total Sp2 cellular RNA and the RNA associated with each immunoprecipitate were extracted from the immunoprecipitates, analyzed on an 8% urea–acrylamide gel, and exposed to autoradiography.

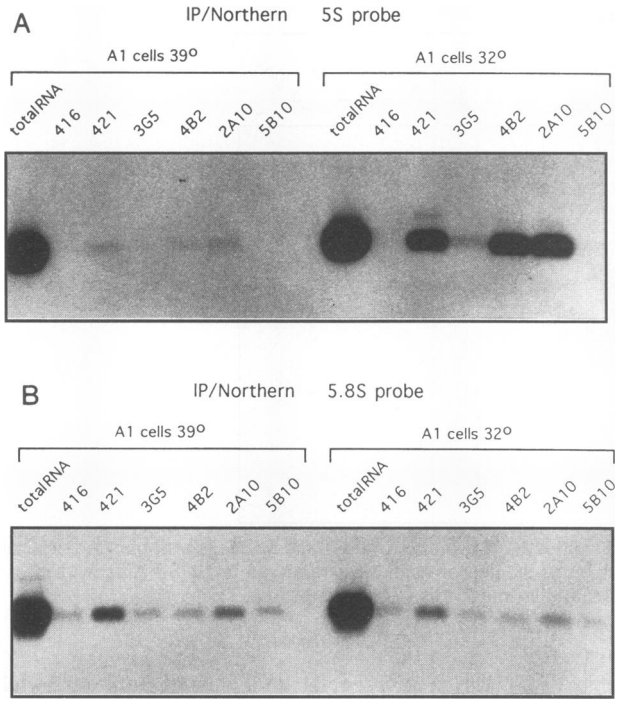

FIG. 6. Northern blot analysis of 5S (A) and 5.8S (B) RNA immunoselected from A-1 cells. Cellular extracts of A-1 cells cultured at 39 and 32°C were immunoprecipitated with PAb416 and PAb5B10 (negative controls that do not detect p53 or mdm-2), PAb421 (a p53-specific antibody), the mdm-2 specific antibodies PAb4B2 and PAb2A10, and antibody PAb3G5 (which recognizes free mdm-2 not associated with p53). The A-1 cell total RNA and the RNA from each immunoprecipitate were separated on a 2% agarose denaturing gel and transferred to a nylon membrane. The membrane was probed with a 5S DNA probe, stripped, and reprobed with a 5.8S DNA probe.

mdm-2–p53 complex. To prove that the RNA detected in these complexes was indeed 5S RNA, a 5S DNA probe was used to detect 5S RNA by using hybridization techniques that detected RNA associated with mdm-2 or p53–mdm-2. A-1 cells were grown at 39°C, a temperature at which mutant p53 protein fails to induce the high-level expression of mdm-2 mRNA or protein, or at 32°C, at which the wild-type p53 protein induces high levels of mdm-2 mRNA and protein (12, 22). Soluble protein extracts were prepared from these cells and p53 and mdm-2 proteins were immunoselected with PAb416 (negative control, anti-simian virus 40 T antigen), PAb421 (anti-p53), PAb3G5 (anti-mdm-2 not in a complex with p53), PAb4B2, PAb2A10 (both directed at mdm-2 in these cells), and PAb5B10 (detects only a human mdm-2 protein and not the mouse or rat mdm-2 proteins) (2). The immunoprecipitates were treated with SDS and proteinase K (to release any covalently bound RNA), and the RNA was extracted from these precipitates. The RNA was analyzed on a sizing gel and transferred to a nylon membrane for Northern (RNA) blot analysis. Figure 6A presents the autoradiogram of a Northern blot obtained by using a 5S RNA probe to detect this RNA associated with mdm-2. A-1 cells at 39°C synthesize very low levels of mdm-2 proteins (12, 22), and so only background levels of 5S RNA are detected by these experiments (Fig. 6, A-1 cells at 39°C). At 32°C, PAb416 and PAb5B10 (negative controls that do not detect mouse or rat mdm-2 proteins) fail to detect 5S RNA, while PAb421 (p53 specific) and PAb4B2 and PAb2A10 (both mdm-2 specific) detect high levels of 5S RNA in this complex. In A-1 cells, most of the mdm-2 protein made at 32°C is complexed with p53 (12, 22), with only a small percentage of the free mdm-2 protein present. For this reason, 3G5, which detects only free mdm-2 protein, has relatively little 5S RNA associated with its L5 protein. A comparison with free mdm-2 from Sp2 cells (Fig. 5, 3G5 antibody) again demonstrates that free mdm-2 protein and p53-complexed mdm-2 proteins both have L5 and L5-5S RNA associated with them.

The membrane containing RNA used for this study (Fig. 6) was stripped and hybridized again by using a 5.8S DNA probe to detect 5.8S RNA by hybridization. Figure 6B shows the autoradiogram from this membrane. The negative control lanes PAb416 and PAb5B10 (which fail to detect murine or rat mdm-2 proteins) contain some background RNA levels. PAb421, which detects both mutant p53 protein at 39°C and wild-type p53 protein at 32°C, shows enhanced levels of 5.8S RNA associated with the p53 in these immunoprecipitates. The best (highest affinity) of the mdm-2 monoclonal antibodies, 2A10, which is the most efficient antibody at detecting low levels of mdm-2 (2), also detects low levels of associated 5.8S RNA (above the background) in cells at 39 and 32°C. With the other mdm-2 monoclonal antibodies used in this study, detection of a p53-5.8S RNA in a complex with mdm-2 is a borderline result, comparable to the background levels of 5.8S contaminating these immunoprecipitates (Fig. 6B). Because of this, it is not possible from these results to be sure that the 5.8S RNA is indeed associated with the 5S–L5–mdm-2–p53 ribonucleoprotein complex.

DISCUSSION

Some forms of the mdm-2 protein, from a variety of cells, are complexed and copurify with a distinct protein with a molecular weight of 34,000. On the basis of both the amino-terminal sequence of this protein (40 amino acids) and a partial peptide map of this protein, it has been shown to be the large ribosomal L5 protein. The L5 protein thus appears to be a subunit of one or more isoforms of the mdm-2 protein. The L5 protein is associated with both free mdm-2 proteins in solution and p53–mdm-2 complexes in solution.

The L5 protein has been shown to bind specifically to 5S RNA during ribosomal biogenesis (21), first in the nucleus and then in the nucleolus, as well as on the large ribosomal subunit of the ribosomes. mdm-2–L5 protein complexes and mdm-2–L5–p53 protein complexes have been shown here to also be associated with 5S RNA. The stoichiometry of this protein-RNA association remains to be determined, but the half-lives of both the p53 and mdm-2 proteins in a cell are very short (20 min) (15, 16) and the molar levels of these proteins are far below the number of L5 protein and 5S RNA molecules in a cell. Previous studies (21) have used anti-lupus antisera to detect L5-RNA ribonuclear protein complexes. These polyclonal antisera detect many additional proteins. The large excess of L5 protein in the cell, compared with mdm-2 or p53 proteins, makes it unlikely that this approach would have detected associated mdm-2 or p53.

The existence of a ribonucleoprotein particle with p53–mdm-2–L5 and 5S RNA is intriguing because of the previous reports by Fontoura et al. showing that a proportion of p53 molecules have a covalent addition of 5.8S RNA linked to the penultimate serine residue of the p53 protein (8). It has been shown that the L5 protein with its associated 5S RNA will form a ternary complex with 5.8S RNA in solution (11). That leads

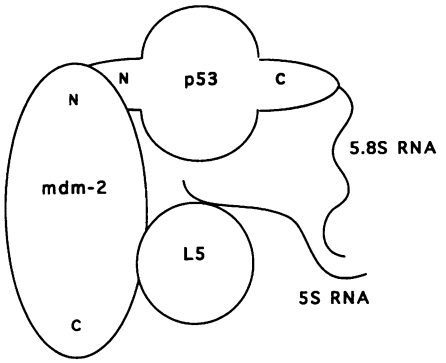

FIG. 7. Schematic diagram of the putative p53–mdm-2–L5–5S RNA–5.8S RNA ribonucleoprotein complex. p53 and mdm-2 interact though their N termini, and the 5.8S RNA is bound to the penultimate serine of p53 (18, 19). The L5 protein forms a tight complex with mdm-2 and is bound to the 5S RNA and 5.8S RNA species in a ternary complex observed previously (11). The experiments presented here do not rule out the possibility that 5S RNA binds to mdm-2 and the L5 protein binds to the 5S RNA.

to the possibility that p53 and mdm-2 are held together by protein-protein interactions at their N-terminal ends (2) while at the same time the mdm-2–L5–5S RNA and p53–5.8S RNA are held together in a ribonuclear protein complex by protein-RNA and RNA-RNA interactions at their C-terminal ends (Fig. 7). While this communication presents some evidence for the existence of such a complex, it is clear that additional proof is required to demonstrate its reality as well as the functional meaning for such a ribonuclear protein in the cell. It should be pointed out that the mdm-2–L5–5S RNA complexes were detected in cells that overproduce mdm-2 proteins (1, 15). In addition, the existence of 5.8S RNA in this complex is not proven by the results presented here.

Despite these reservations, several possible functions for a p53–5.8S–mdm-2–L5–5S RNA complex of this type could be envisioned and tested. While the majority of p53 and mdm-2 proteins in a cell appear to reside in the nucleus (15), some may well be associated with the ribosomes. The p53 protein has been shown to have an antihelicase activity (13), and if the zinc finger mdm-2 proteins provided RNA sequence or structure recognition, such an RNA could act to selectively modulate translation of a species of mRNA. The function of the p53 protein is to respond to DNA damage, giving rise to G_1 arrest (10) or cellular apoptosis (23). It could therefore be useful to block translation of selected mRNAs, preventing the cell from moving from the G_1 to the S phase by selectively inhibiting the translation of cyclins or cyclin-dependent kinase mRNAs. Alternatively, the hypothetical p53–5.8S–mdm-2–L5–5S RNA particle (Fig. 7) might be involved in ribosomal biogenesis (21), in which a p53 antihelicase activity could play a role. To date, all the available evidence supports detection of mdm-2 and p53 (15) in the nucleus of the cell and not in the nucleolus. It is of some interest, however, that DNA damage often results in a decline in translational efficiency so as to allow the cell to repair its damage prior to division.

Although these ideas remain speculative, it is a striking set of coincidences that the p53–mdm-2 protein complex, or mdm-2 itself, appears to be associated with three ribosomal elements: the L5 protein, the 5S RNA, and possibly the 5.8S RNA. It seems likely that this confluence of events has a functional significance that should be elucidated.

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