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Identification of a Precursor in the Biosynthesis of the p21 Transforming Protein of Harvey Murine Sarcoma Virus

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The p21 transforming protein coded for by the v-*ras* gene of Harvey murine sarcoma virus (Ha-MuSV) migrates as a doublet band between 21,000 and 23,000 daltons during sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The lower band of the doublet is designated p21, and the upper band is designated pp21 since it comigrates with the phosphorylated form of p21. By pulse-labeling with [^{35}S]methionine, we detected a p21 precursor, pro-p21, which migrated as if it was approximately 1,000 daltons larger than p21. The precursor-product relationship was established by pulse-chase experiments with [^{35}S]methionine in the presence of 100 μg of cycloheximide per ml, which inhibited all de novo protein biosynthesis. Within 4 h, pro-p21 was completely chased into p21, and during the next 24 h pp21 accumulated. Thus, formation of pp21 from p21 did not require de novo protein synthesis. By subcellular fractionation into cytosol and membrane fractions, we found that pro-p21 was synthesized in a non-membrane-bound state and that shortly after its complete synthesis, the p21 product was associated with the membrane fraction. By selective cleavage of p21 at a unique aspartic acid-proline residue with 70% formic acid or with *Staphylococcus aureus* V8 protease, we found that the intramolecular site of pro-p21 processing was located in the C-terminal portion of the pro-p21 molecule. The possibilities that the precursor was involved in the assembly of p21 into the plasma membrane and, alternatively, that the processing was a step in the activation of p21 biochemical activities are discussed.

The genome of Harvey murine sarcoma virus (Ha-MuSV) encodes a 21,000-dalton protein designated p21, which has been shown to be the protein that is responsible for the oncogenic properties of Ha-MuSV (1, 3, 13–15). We have been interested in the biochemistry and metabolism of p21 because of its pivotal role in maintaining the transformed phenotype. In studies on fixed cells, more than 95% of the intracellular p21 has been localized to the inner surface of the plasma membrane in a Ha-MuSV-transformed canine kidney cell line by electron microscopic immunocytochemistry (17). In studies in vitro, the partially purified p21 of Ha-MuSV shows a guanine nucleotide-binding activity and an apparent GTP-specific autophosphorylating activity leading to the formation of a phosphothreonine residue on p21 (10, 12).

Previously, we described two forms of Ha-MuSV p21 in cells, which were recognized by their different rates of migration in a sodium dodecyl sulfate (SDS)-polyacrylamide gel (13). One of these forms is the p21 which migrates at an apparent molecular weight of 21,000, and the other is pp21, a phosphorylated form of p21 which migrates more slowly in acrylamide gels, at an apparent molecular weight of approximately 23,000. To study the biosynthetic relationship between p21 and pp21 in cells, we undertook a kinetic study of the metabolism of p21. In this paper we report that p21 is synthesized in a precursor form, designated pro-p21. Shortly after the synthesis of pro-p21 in the free cytosol fraction of cell homogenates, pro-p21 was processed to p21, and p21 became associated with the plasma membrane. The processing of pro-p21 to p21 involved changes in peptides that were derived from the C-terminal portion of pro-p21. After the formation of p21, pp21 accumulated in the plasma membrane fraction without the requirement for de novo protein biosynthesis.

MATERIALS AND METHODS

Cells and viruses. All cells were grown in Dulbecco-Vogt modified Eagle minimal essential medium (DMEM) containing 10% fetal calf serum. The following Ha-MuSV-transformed nonproducer cell lines were used in this study: Ha-C127, a Ha-MuSV-trans-

formed C127 mouse cell line (8); Ha-MDCK, a Ha-MuSV-transformed MDCK canine kidney cell line (13); and Ha-NIH, a Ha-MuSV-transformed NIH3T3 mouse cell line (13). The following uninfected cell lines were used: C127, a contact-inhibited cell line derived from an RIII mouse (8); NIH3T3, a contact-inhibited cell line derived from a Swiss mouse; and MDCK, a canine kidney cell line (12). For many sequence and peptide cleavage experiments, African green monkey kidney (AGMK) cells infected with a Ha-MuSV–simian virus 40 (SV40) recombinant (SVL_3-Ha-Mu_{src}) containing the Ha-MuSV p21 gene were used (4). The recombinant virus was complemented with an SV40 *tsA* mutant, and the infected cells were maintained at 40.5°C.

Cell labeling and immunoprecipitation. Cells grown in 60-mm plastic petri dishes to near confluency were labeled with 2 ml of [^{35}S]methionine (200 to 500 μCi/ml) in methionine-free DMEM supplemented with 2% dialyzed fetal calf serum at 37°C. At the end of the labeling periods, the ^{35}S-labeled media were discarded, and the cells were lysed immediately with 1 ml of lysis buffer containing 1% Triton X-100, 0.05 M Tris-hydrochloride (pH 8.0), 5 mM $MgCl_2$, and 0.1 M NaCl or buffer containing 1% Triton X-100, 20 mM PIPES [piperazine-*N*,*N*'-bis(2-ethanesulfonic acid)] (pH 6.8), 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 M KCl, and 0.3 M sucrose. No significant difference in subsequent analyses was observed in extracts prepared in these two lysis buffers. The lysates were passed three times through a 20-gauge needle and were clarified by centrifugation at 150,000 × *g* for 30 min. For labeling with ^{32}P, cells were labeled with 2 ml of $^{32}P_i$ (500 μCi/ml) in phosphate-free medium supplemented with 2% dialyzed fetal calf serum. Samples of lysates containing 5 × 10^6 to 10 × 10^6 trichloroacetic acid-precipitable proteins were immunoprecipitated with 5- to 10-μl portions of antisera containing antibodies to p21 from Osborne-Mendel rats bearing tumors induced by syngeneic transplantation of Ha-MuSV nonproducer cells. The control normal sera were from rats that were not inoculated. The antigen-antibody complexes were precipitated with formaldehyde-fixed *Staphylococcus aureus* containing protein A. Immunoprecipitation was performed in a buffer containing 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 0.01 M phosphate buffer (pH 7.5), 0.1 M NaCl, 1 mM EDTA, and 200 U of Trasylol (Calbiochem) per ml. The precipitated proteins were analyzed by 10% SDS–polyacrylamide gel electrophoresis and were visualized by fluorography. The details of the procedures have been described previously (13).

Localization of p21 by subcellular fractionation. A simple and quick procedure was used to fractionate cell homogenates into an S100 cytosol fraction and a P100 membrane fraction for the pulse-labeling experiments (2). After pulse-labeling the cells in one or two 60-mm dishes were scraped into 1 ml of buffer containing 0.01 M Tris-hydrochloride (pH 8.0) and 0.1 mM dithiothreitol. The cells were then broken by 20 strokes of a tight-fitting Dounce homogenizer. After the nuclear fraction was removed by centrifugation at 600 × *g* for 15 min, the supernatants were subjected to high-speed centrifugation at 100,000 × *g* for 30 min. The resulting high-speed supernatants were designated the S100 cytosol fractions, and the resulting pellets were designated the P100 membrane fractions. For immunoprecipitation, the membrane pellets were dissolved in 1% Triton X-100, and the residual insoluble material was removed by centrifugation at 2,500 × *g*.

A more rigorous concanavalin A (ConA) procedure was also used to prepare plasma membrane fractions from pulse-labeled cells (9). At the end of labeling, cells in 60-mm dishes were washed once with isotope-free DMEM and then were incubated with 2 ml of DMEM containing 0.25 mg of ConA per ml at 37°C for 15 min. The cells were swollen by two changes of 5 ml of cold 1 mM Tris-hydrochloride (pH 7.5) (15 min each). The buffer was replaced by 0.5 ml of fresh buffer, and the cells were lysed by scraping the dishes with a rubber policeman. The lysates either were directly centrifuged at 100,000 × *g* for 30 min to obtain the S100 supernatants and the P100 pellet fractions or were layered onto a discontinuous sucrose gradient containing 15, 30, 45, 60, and 70% sucrose solutions (2.3 ml each) to obtain the purified membrane fractions. The tubes were centrifuged at 100,000 × *g* in a Beckman SW41 rotor for 1 h. The membranes at the 30% sucrose–45% sucrose or 45% sucrose–60% sucrose interface were collected by centrifugation at 10,000 × *g* for 20 min. After one to four dilutions with 0.01 M Tris-hydrochloride (pH 8.0)–0.1 mM dithiothreitol, the membranes in the pellets were dissolved in 1% Triton X-100, as described above.

Preparation of the p21 precursor and processed product for peptide cleavage. At 96 h postinfection the AGMK cells containing the SV40–Ha-MuSV recombinants were labeled with [^{35}S]methionine in DMEM; we used 1 mCi of [^{35}S]methionine per ml for 15 min at 40.5°C to label the precursor and 0.2 mCi of [^{35}S]methionine per ml for 24 h at 40.5°C to label the processed

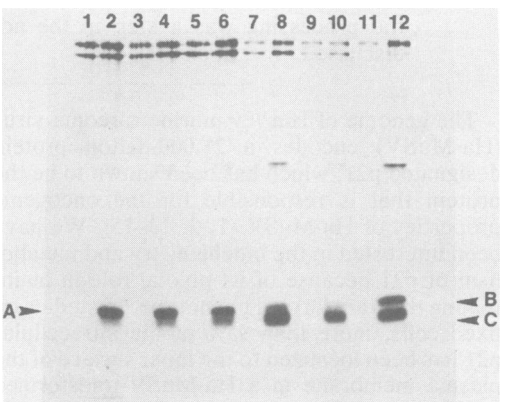

FIG. 1. Pulse-labeling of p21. Ha-MDCK cells in 60-mm petri dishes were labeled in 2 ml of medium containing [^{35}S]methionine (0.2 mCi/ml) for varying times at 37°C. At the end of labeling, the cells were lysed immediately. Lysates containing 5 × 10^6 trichloroacetic acid-precipitable counts per min were immunoprecipitated with 5 μl of antiserum or normal serum. Lanes 1, 3, 5, 7, 9, and 11 contain proteins precipitated with normal serum, and lanes 2, 4, 6, 8, 10, and 12 contain proteins precipitated with antiserum. Lanes 1 and 2, 0.25-h lysate; lanes 3 and 4, 0.5-h lysate; lanes 5 and 6, 1-h lysate; lanes 7 and 8, 3-h lysate; lanes 9 and 10, 4.5-h lysate; lanes 11 and 12, 24-h lysate.

products. p21 was immunoprecipitated with a high-titer anti-p21 monoclonal antibody (generously provided by M. Furth). The proteins which were precipitated were separated by SDS gel electrophoresis. The 15-min pro-p21 precursor bands or the non-phosphorylated p21 doublet bands of the 24-h processed products were cut out of the wet gels and were eluted twice with 2.5 ml of the buffer containing 0.05 M ammonium bicarbonate, 0.1% SDS, 1% β-mercaptoethanol, 100 μg of bovine serum albumin per ml, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium azide. After extensive dialysis against 0.01% SDS, the proteins were lyophilized. The pro-p21 and p21 products were then selectively cleaved with 70% formic acid at the aspartic acid-proline residues (7) or with *S. aureus* V8 protease, as described in the legends to Fig. 7 and 8.

RESULTS

Identification of a p21 precursor (pro-p21) in pulse-labeling experiments. When p21 was pulse-labeled for a very short time, the mobility of p21 on the SDS-polyacrylamide gels during electrophoresis differed from the mobility of p21 labeled for longer times. Figure 1 shows immunoprecipitates of p21 that was labeled for 15 min (Fig. 1, lane 2), 30 min (lane 4), 1 h (lane 6), 3 h (lane 8), 4.5 h (lane 10), and 24 h (lane 12). After the very short pulse of 15 min, p21 appeared mostly as a single band (band A) on SDS gel electrophoresis, but with increasing labeling time band C (which had a slightly faster migration rate) appeared, with a concomitant decrease in the proportion of the slower band A. After 4.5 h of labeling (lane 10), predominantly the faster migrating band C was observed. However, after prolonged labeling (24 h) an additional band appeared (band B), which migrated more slowly than either band A or band C. The p21 from the 24-h labeling experiment was typical of the Ha-MuSV p21 doublet which we described previously (13). The upper band of this doublet (band B) comigrated with the phosphorylated form of p21, and the lower band (band C) was not phosphorylated. The results of this pulse-label-

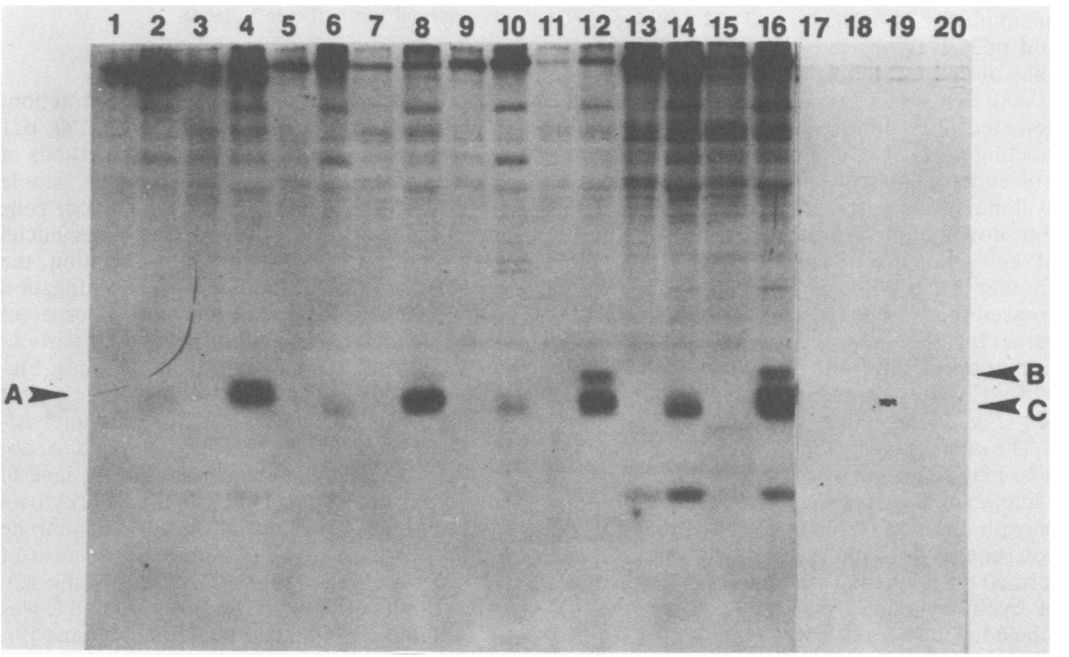

FIG. 2. Pulse-chase of ^{35}S-labeled p21 in the presence of cycloheximide. Ha-MuSV-transformed C127 cells (Ha-C127) and uninfected parental C127 cells were labeled with [^{35}S]methionine for 1 h and then chased for either 4 or 24 h in the presence of 80 μg of cycloheximide per ml. Samples of lysates containing 5×10^6 trichloroacetic acid-precipitable counts per min in proteins were precipitated with 10 μl of normal serum (lanes 1, 3, 5, 7, 9, 11, 13, 15, 17, and 19) or with antiserum (lanes 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20). In lysates prepared from cells treated with cycloheximide before labeling (lanes 17 to 20), the total incorporation of [^{35}S]methionine was between 0.6 and 3% of the incorporation in the control without cycloheximide. In these preparations lysates from five times the equivalent number of cells as in lanes 1 to 4 were used in immunoprecipitations. Lanes 1 and 2, C127, no chase; lanes 3 and 4, Ha-C127, no chase; lanes 5 and 6, C127, 4-h chase; lanes 7 and 8, Ha-C127, 4-h chase; lanes 9 and 10, C127, 24-h chase; lanes 11 and 12, Ha-C127, 24-h chase; lanes 13 and 14, C127, 24-h continuous label and no chase; lanes 15 and 16, Ha-C127, 24-h continuous label and no chase; lanes 17 and 18, C127, 1-h pretreatment with cycloheximide before 18 h of labeling in the presence of cycloheximide; lanes 19 and 20, Ha-C127, 1-h pretreatment with cycloheximide before 18 h of labeling in the presence of cycloheximide.

ing experiment suggested that p21 was synthesized in a precursor form, pro-p21 (band A), and that it was processed into a faster-migrating product, p21 (band C), shortly after synthesis. After a prolonged labeling of up to 24 h, the phosphorylated form of p21, pp21 (band B), began to accumulate. When all of the p21 bands were run on a gel closely side by side, it became obvious that pro-p21 band A exhibited mobility between the mobilities of p21 doublet bands C and B (data not shown). The size difference between pro-p21 and the p21 product was less than 1,000 daltons.

To demonstrate the precursor-product relationship, a pulse-chase experiment was performed (Fig. 2). p21 was labeled for 1 h, and it appeared mostly as the pro-p21 band (Fig. 2, lane 4, band A). The labeled pro-p21 was then chased in the presence of 80 μg of cycloheximide per ml in [^{35}S]methionine-containing DMEM for 4 h, which terminated all de novo protein synthesis (Fig. 2, lane 20). p21 then migrated as the processed product (lane 8, band C). After the preparation was chased with cycloheximide for 24 h, the typical p21 doublet of p21 and pp21 was observed (lane 12). The appearance of the p21 doublet after the 24-h chase was essentially similar to that of the p21 doublet detected after continuous 24-h [^{35}S]methionine labeling without cycloheximide (lane 16). Control experiments revealed that the presence of cycloheximide at this concentration completely shut down de novo protein synthesis. Exposure of cells to cycloheximide for 30 min before another 18 h of [^{35}S]methionine labeling decreased the isotope incorporation to 0.6% of the value for the control without cycloheximide. Immunoprecipitation of the cycloheximide-treated lysates revealed a complete absence of p21 (Fig. 2, lane 20).

The appearance of pp21 in the [^{35}S]methionine-labeled proteins occurred in the presence of cycloheximide. To demonstrate directly that phosphorylation of the upper band (band B) did not require de novo p21 biosynthesis, we performed a ^{32}P-labeling experiment in the presence of cycloheximide. As Fig. 3 shows, the ^{32}P-labeled p21 was observed readily after 24 h of labeling in the presence of cycloheximide. Thus, the pulse-chase studies demonstrated that p21 was synthesized in a precursor form and that shortly after its synthesis, pro-p21 was processed into a smaller p21 product and was phosphorylated to become pp21.

Subcellular localization of p21 synthesis and processing. To demonstrate the subcellular localization of p21 biosynthesis and the processing event, Ha-MuSV-transformed cells were pulse-labeled with [^{35}S]methionine or $^{32}P_i$, and the cell homogenates were then fractionated into a cytosol fraction and a particulate membrane fraction. After solubilization with 1% Triton X-100, p21 was precipitated with antisera. Two methods of subcellular fractionation were used. A simple procedure involving hypotonic swelling of cells and homogenization was used first. After nuclei were removed by low-speed centrifugation, the supernatant was fractionated by centrifugation at 100,000 × *g* for 30 min into the supernatant S100 cytosol fraction and the pellet P100 particulate fraction containing plasma membranes. Figure 4 shows the results of such an experiment with proteins labeled with [^{35}S]methionine. After the 15-min pulse, the pro-p21 band A observed in the total homogenate (Fig. 4, lane 6) was found almost exclusively in the S100 cytosol fraction (lane 2) and not in the P100 membrane fraction (lane 4). After 3 h of labeling, most of the p21 observed was in p21 band C (lane 12). After fractionation, almost all of p21 band C was present in the P100 membrane fraction (lane 10), and a trace amount of pro-p21 band A was observed in the S100 fraction (lane 8). After labeling for 24 h, the typical p21-pp21 doublet observed in the total homogenate (lane 18) was fractionated exclusively into the P100 membrane fraction (lane 16); none of this doublet was in the S100 cytosol fraction (lane 14).

To show more rigorously that the p21 products and the processing event were associated with the plasma membrane, we used a ConA procedure for membrane isolation. ConA treatment of cells before lysis stabilizes the plasma

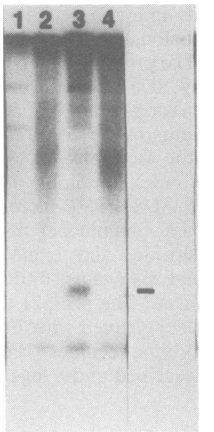

FIG. 3. ^{32}P-labeling of p21 in the presence of cycloheximide. C127 cells (lanes 1 and 2) and Ha-C127 cells (lanes 3 and 4) were pretreated with 100 μg of cycloheximide per ml for 1 h before labeling with 250 μCi of $^{32}P_i$ per ml for 24 h in the continuous presence of cycloheximide. Lysates containing 3 × 10^6 trichloroacetic acid-precipitable counts per min were immunoprecipitated with either antiserum (lanes 1 and 3) or normal serum (lanes 2 and 4).

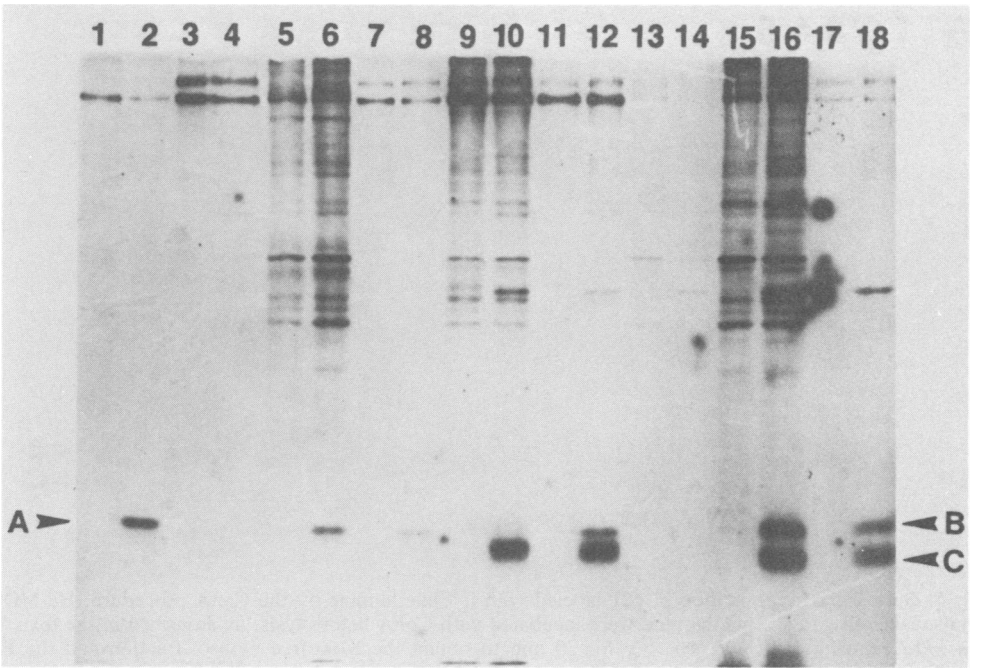

FIG. 4. Subcellular fractionation of p21 pulse-labeled with [^{35}S]methionine. Ha-MDCK cells pulse-labeled with [^{35}S]methionine for 0.25, 3, or 24 h were fractionated into the S100 cytosol fraction and the P100 membrane fraction by centrifugation at 100,000 × *g* for 30 min by the simple method described in the text. After solubilization, samples containing 2 × 10^6 trichloroacetic acid-precipitable counts per min in proteins from the S100 fractions, P100 fractions, and total homogenates were immunoprecipitated with either normal serum (lanes 1, 3, 5, 7, 9, 11, 13, 15, and 17) or antiserum (lanes 2, 4, 6, 8, 10, 12, 14, 16, and 18). Lanes 1 to 6 contained preparations labeled for 0.25 h (lanes 1 and 2, S100; lanes 3 and 4, P100; lanes 5 and 6, total); lanes 7 to 12 contained preparations labeled for 3 h (lanes 7 and 8, S100; lanes 9 and 10, P100; lanes 11 and 12, total); and lanes 13 to 18 contained preparations labeled for 24 h (lanes 13 and 14, S100; lanes 15 and 16, P100; lanes 17 and 18, total).

membrane from fragmentation and vesiculation upon cell lysis, and this procedure has been used successfully to prepare plasma membranes containing adenylate cyclase and β-adrenergic receptors (9). Figure 5 shows the proteins labeled with [^{35}S]methionine. After the 15-min pulse (Fig. 5A), pro-p21 band A was fractionated exclusively into the S100 cytosol (Fig. 5A, lane 4), and the processed p21 band C was found in the P100 pellets (lane 6). The presence of band C in lane 6 but not in the total lysate in lane 2 apparently was due to high enrichment of the processed p21 in the P100 pellet fraction. In the lysate labeled for 24 h (Fig. 5B), the p21-pp21 doublet was found in the P100 pellet (lane 6). Plasma membrane was also purified further by banding the cell lysate in a discontinuous sucrose gradient. The membrane fraction harvested at the 30% sucrose–45% sucrose interface (Fig. 5 lane 8) and that harvested at the 45% sucrose–60% sucrose interface (lane 10) contained the p21 doublet. Again, the heavy doublets in the membrane fractions reflected enrichment of p21 in these fractions. Figure 6 shows the subcellular fractionation of ^{32}P-labeled p21. The phosphorylated pp21 labeled with ^{32}P found in the total homogenate (Fig. 6, lane 2) occurred exclusively in the P100 fraction (lane 6); none occurred in the S100 fraction (lane 4). After further purification of the plasma membranes in the sucrose gradient, the membrane fraction collected at the 30% sucrose–45% sucrose interface (lane 8) and that collected at the 45% sucrose–60% sucrose interface (lane 10) contained phosphorylated p21. In conclusion, these subcellular fractionation experiments indicated that p21 was synthesized in the free cytosol fraction and most likely not on membrane-bound polysomes. Shortly after synthesis, the processed product was detected in the plasma membrane fraction. Only after a much longer elasped time (up to 24 h) did the phosphorylated pp21 begin to accumulate in any significant quantity in the plasma membrane fraction. However, a detailed correlation between the time sequence of the processing event and the product association with plasma membrane was not established in these experiments.

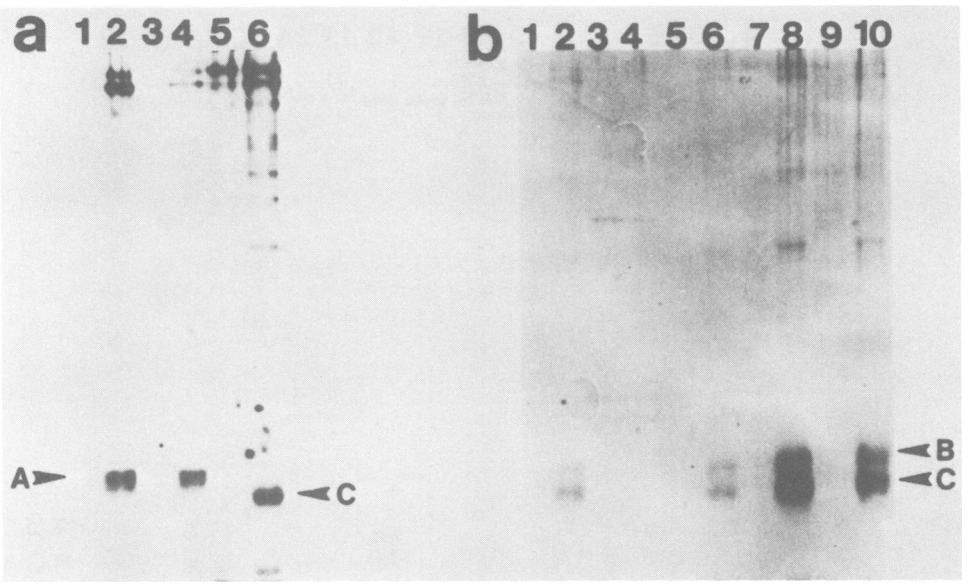

FIG. 5. Subcellular fractionation of p21 labeled with [^{35}S]methionine by the ConA procedure. Ha-MDCK cells pulse-labeled with [^{35}S]methionine were incubated with ConA before lysis, as described in the text. The lysates were centrifuged at 100,000 × *g* for 30 min to obtain the S100 free cytosol fraction and the P100 particulate fraction. Membrane fractions that were further purified were obtained by banding the lysates on a sucrose gradient. After solubilization of the particulate fractions with Triton X-100, p21 was precipitated with antiserum (lanes 2, 4, 6, 8, and 10) or normal serum (lanes 1, 3, 5, 7, and 9) from portions containing 2 × 10^6 trichloroacetic acid-precipitable counts per min in proteins. (a) Preparation pulse-labeled for 15 min. Lanes 1 and 2, Total lysate; lanes 3 and 4, S100; lanes 5 and 6, P100. (b) Preparation pulse-labeled for 24 h. Lanes 1 and 2, Total lysate; lanes 3 and 4, S100; lanes 5 and 6, P100; lanes 7 and 8, membranes at the 30% sucrose–45% sucrose interface; lanes 9 and 10, membranes at the 45% sucrose–60% sucrose interface.

Localization of the processing site within the primary sequence of the p21 molecule. To determine the processing site within the primary sequence for the p21 molecule, we attempted to determine the sequences of the p21 precursor and its processed product and to study the peptides generated by cleavage of the precursor and the products. The results were compared with the amino acid sequences deduced from DNA sequencing experiments. For these studies, we took advantage of the 10- to 20-fold increase in p21 biosynthesis in AGMK cells infected with a SV40–Ha-MuSV recombinant (4). With this system, we obtained an adequate quantity of the purified p21 precursor pulse-labeled with radioactive amino acids. Figure 7 shows that the synthesis of p21 in this SV40–Ha-MuSV recombinant system also involved the precursor. The SV40–Ha-MuSV recombinant-infected AGMK cells were pulse-labeled with [^{35}S]methionine for 0.25, 3, and 24 h. The same mobility shift was observed as in Ha-MuSV-transformed cells, namely, pro-p21 (lane 1), the processed p21 product (lane 3), and pp21 (lane 5). Amino acid sequence determinations by Edman degradation of p21 and its precursor indicated that the N-terminal amino acid residue was blocked by post-translational modification in all three forms of p21 (pro-p21, p21, and pp21) (S. Oroszlan et al., manuscript in preparation).

Alternatively, we compared the peptides derived from known cleavage sites within the p21 molecule. From the DNA sequence of the p21 coding region (R. Dhar et al., manuscript in preparation), we noticed that there is an aspartic acid-proline residue 33 amino acid residues from the N-terminal initiation codon of the p21 coding region. This aspartic acid-proline linkage can be cleaved selectively by acid hydrolysis in 70% formic acid. We predicted that the major large peptide derived from this cleavage would include the C-terminal portion of the p21 molecule. Figure 8 shows a comparison of the cleavage products of the pro-p21 (Fig. 8, lanes 1 and 3) and p21 (lanes 2 and 4) isolated by immunoprecipitation and SDS gel electrophoresis. After formic acid cleavage at 50°C for 4 and 8 h, the peptides were analyzed on a 15% SDS gel. In addition to the original p21 peptides (approximately 21 kilodaltons), there was a large 18-kilodalton cleavage product. This figure shows clearly that the 18-kilodalton peptide derived from pro-p21 (lanes 5 and 7) is larger than the 18-kilodalton peptide of processed p21 (lanes 6 and

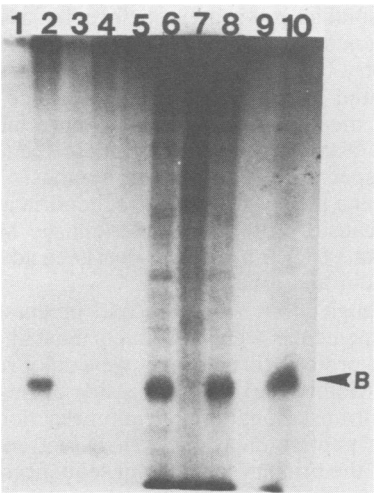

FIG. 6. Subcellular fractionation of ^{32}P-labeled p21 by the ConA procedure. Ha-MCDK cells labeled with $^{32}P_i$ for 24 h were fractionated into the S100 free cytosol fraction and the P100 membrane fraction by centrifugation at 100,000 × *g* for 30 min. Alternatively, the membrane fractions were also prepared by the sucrose gradient procedure described in the text. Samples containing 5 × 10^6 trichloroacetic acid-precipitable counts per min in proteins were immunoprecipitated with 5 μl of either normal serum (lanes 1, 3, 5, 7, and 9) or antiserum (lanes 2, 4, 6, 8, and 10). Lanes 1 and 2, Total homogenate; lanes 3 and 4, S100; lanes 5 and 6, P100; lanes 7 and 8, membranes from the 30% sucrose–45% sucrose interface; lanes 9 and 10, membranes from the 45% sucrose–60% sucrose interface.

8). Thus, the size difference between the p21 precursor and its product is in the C-terminal portion of the p21 molecule.

Cleavage with the *S. aureus* V8 protease generated a large peptide of approximately 13 kilodaltons. The N-terminal amino acid sequence of this peptide has been determined, and a comparison with the DNA sequence showed that this 13-kilodalton peptide was derived from the C-terminal half of the p21 molecule (Oroszlan et al., manuscript in preparation). Figure 9 shows the cleavage products analyzed by SDS gel electrophoresis; lanes 1, 3, and 5 contained pro-p21 and the peptides generated by digestion with 1 and 5 μg of V8 protease, respectively. Lanes 2, 4, and 6 contained similar preparations of p21. This figure shows that the major V8 peptide of the precursor (lanes 3 and 5, band A) was larger than the V8 peptide of p21 (lanes 4 and 6, band B). This study of the peptides derived from known cleavage sites clearly indicated that the precursor-product processing of p21 occurred in the C-terminal region of the molecule and ruled out N-terminal cleavage as the means of processing.

DISCUSSION

In this study we identified a precursor form of the p21 protein which migrated with an apparent molecular size 1,000 daltons larger than the mature non-phosphorylated form of p21. The precursor was detected free in the cytosol fraction, suggesting that the intracellular site of p21 synthesis was on free polysomes rather than on membrane-bound polysomes. Shortly after synthesis, the mature non-phosphorylated form of p21 was associated with the plasma membrane. Only after a much longer time (up to 24 h) did the phosphorylated form accumulate to a significant extent in the p21-pp21 doublet. The phosphorylated form of p21 was associated exclusively with the plasma membrane fraction. However, our kinetic pulse-labeling experiments did not allow us to determine unambiguously whether the precursor was processed into the products before, during, or after association with the plasma membrane. The intramolecular site of p21 processing involved the C-terminal portion of the molecule rather than N-terminal peptide cleavage. It is interesting to speculate that this processing event may activate the biochemical activities of p21 molecules.

Many examples of activation of polypeptides by peptide cleavage have been found in the

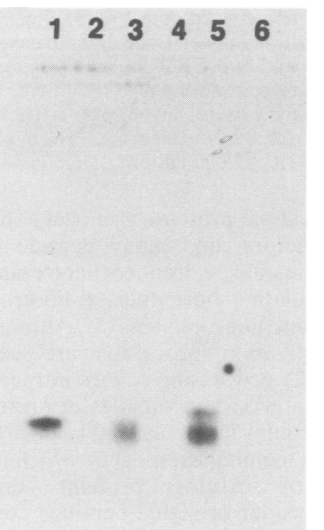

FIG. 7. p21 precursor in AGMK cells infected with an SV40–Ha-MuSV recombinant. At 72 h after infection with the recombinant virus, AGMK cells were labeled with [^{35}S]methionine for 0.25, 3, and 24 h. Lysates containing 5 × 10^6 trichloroacetic acid-precipitable counts per min in proteins were immunoprecipitated with 5 μl of antiserum (lanes 1, 3, and 5) or normal serum (lanes 2, 4, and 6). Lanes 1 and 2, 0.25-h lysate; lanes 3 and 4, 3-h lysate; lanes 5 and 6, 24-h lysate.

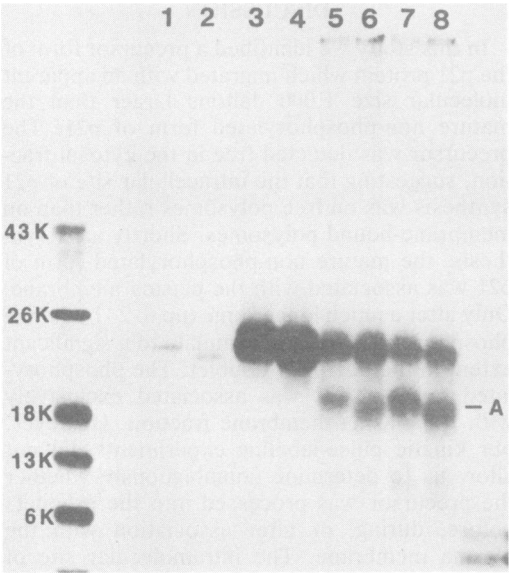

FIG. 8. Formic acid cleavage of the p21 precursor and its processed product. pro-p21 pulse-labeled with [^{35}S]methionine for 15 min and the p21 lower band labeled for 24 h were isolated by immunoprecipitation and SDS gel electrophoresis as described in the text. Samples containing 11,000 cpm of purified p21 and 100 μg of carrier bovine serum albumin were treated with 500 μl of 70% (vol/vol) formic acid at 50°C for 4 or 8 h. After lyophilization to remove the formic acid, the samples were analyzed on a 15% SDS gel. Fluorography was for 1 day (lanes 1 and 2) or 16 days (lanes 3 to 8). Lanes 1 and 3, pro-p21, no acid treatment; lanes 2 and 4, p21 lower band, no acid treatment; lane 5, pro-p21, 4-h acid cleavage; lane 6, p21, 4-h acid cleavage; lane 7, pro-p21, 8-h acid cleavage; lane 8, p21, 8-h acid cleavage. 43K, 43,000 daltons.

gastrointestinal protease zymogens, blood coagulation factors, and many peptide hormones, such as insulin, adrenocorticotropin, melanocyte-stimulating hormones, β-lipotropin, β-endorphin, and neurophysins (5). Although most of these activation processes are extracellular events, p21 processing occurs intracellularly.

Other interesting examples of protein precursors are found in the assembly of proteins into biological membanes, most of which involve the transfer of secretory proteins across membranes. Leader or signal peptides containing in most cases approximately 20 amino acid residues at the N-terminal end are removed by membrane peptidases during these processes (6, 16). p21 is a membrane protein, and it is possible that the precursor may serve a similar function. Most of the secretory proteins are synthesized on membrane-bound polysomes, and transport of the nascent polypeptide chains occurs concurrent with polypeptide synthesis. However, p21 is most likely synthesized on free polysomes as a water-soluble pro-protein before its assembly into the membrane. Electron microscopic cytochemistry has shown that mature p21 seems to be located primarily at the inner surface of the plasma membrane (17), and no appreciable secretion of this protein has been detected (13). In this respect p21 is similar in synthesis to the membrane proteins of the mitochondria and the major capsid protein of bacteriophage M13. A membrane trigger hypothesis has been advanced to explain this process (16).

Although conversion of the p21 precursor into lower-molecular-weight products most likely occurs by proteolytic cleavage, we cannot rule out the possibility that this processing event is due to post-translational modification reactions other than peptide cleavage (18). Direct comparisons of the primary amino acid sequences of the

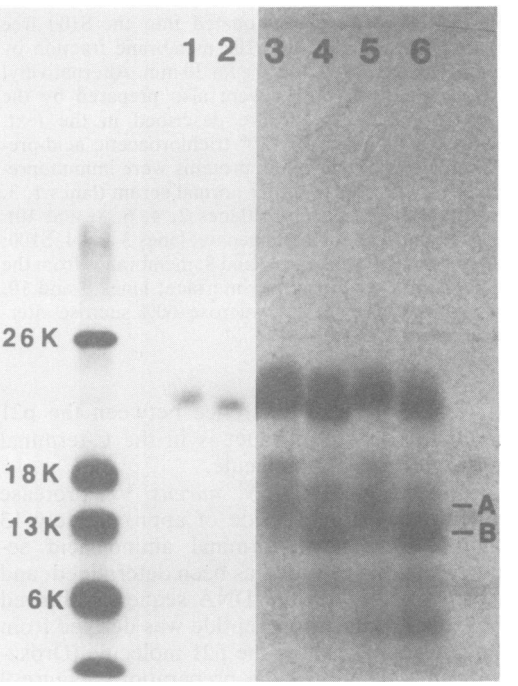

FIG. 9. *S. aureus* V8 protease cleavage of the p21 precursor and its processed product. pro-p21 and the p21 lower band were isolated as described in the legend to Fig. 8. Samples (25 μl) containing 2,500 cpm of purified p21 and 25 μg of carrier bovine serum albumin were digested with 1 or 5 μg of V8 protease at 25°C for 90 min. The cleavage products were analyzed on a 15% SDS gel. Fluorography was for 2 days (lanes 1 and 2) or 15 days (lanes 3 to 6). Lane 1, pro-p21; lane 2, p21 lower band; lane 3, pro-p21, 1 μg of protease; lane 4, p21, 1 μg of protease; lane 5, pro-p21, 5 μg of protease; lane 6, p21, 5 of μg protease. A indicates the location of the major peptide of pro-p21 (lane 5), and B indicates the major peptide of p21 (lane 6). 26K, 26,000 daltons.

precursor and the products, especially at the C termini, will be needed to identify unequivocally the nature of the processing event. Furthermore, the identification of the amino acid sequence and the enzymatic steps involved in the processing event may provide a potential means for intervening in the transforming activity of the p21 protein. The assembly of pro-p21 into the plasma membrane or the activation of the biochemical activities of pro-p21 by the processing event may be interrupted (for example, by a synthetic peptide analog of the signal sequences), thereby reversing the transforming activities of the p21 protein. This model system may provide an approach for a rational basis of chemotherapy for p21-related neoplasia. The p21 biosynthetic pathway described here for Ha-MuSV p21 appeared to be a common pathway for many different forms of other p21-related proteins, such as endogenous cellular p21 homologous to either Ha-MuSV p21 or Kirsten murine sarcoma virus p21 (data not shown). This approach to interrupt the p21 activities may also be useful in attempts to elucidate the natural functions of cellular p21 expressed in a variety of normal uninfected cells (8, 11).

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LITERATURE CITED

1. **Chang, E. H., J. M. Maryak, C.-M. Wei, T. Y. Shih, R. Shober, H. L. Cheung, R. W. Ellis, G. L. Hager, E. M. Scolnick, and D. R. Lowy.** 1980. Functional organization of the Harvey murine sarcoma virus genome. J. Virol. **35**:76–92.
2. **Courtneidge, S. A., A. D. Levinson, and J. M. Bishop.** 1980. The protein encoded by the transforming gene of avian sarcoma virus ($pp60^{src}$) and a homologous protein in normal cells ($pp60^{proto\text{-}src}$) are associated with the plasma membrane. Proc. Natl. Acad. Sci. U.S.A. **77**:3783–3787.
3. **Ellis, R. W., D. DeFeo, T. Y. Shih, M. A. Gonda, H. A. Young, N. Tsuchida, D. R. Lowy, and E. M. Scolnick.** 1981. The p21 *src* genes of Harvey and Kirsten murine sarcoma viruses originate from divergent members of a family of normal vertebrate genes. Nature (London) **292**:506–511.
4. **Gruss, P., R. W. Ellis, T. Y. Shih, M. Konig, E. M. Scolnick, and G. Khoury.** 1981. SV40 recombinant molecules express the gene encoding p21 transforming protein of Harvey murine sarcoma virus. Nature (London) **293**:486–488.
5. **Herbert, E.** 1980. Biosynthesis and processing of cellular and viral polyproteins. Nature (London) **288**:115–116.
6. **Kreil, G.** 1981. Transfer of proteins across membranes. Annu. Rev. Biochem. **50**:317–348.
7. **Landon, M.** 1977. Cleavage at aspartyl-prolyl bonds. Methods Enzymol. **47**:145–149.
8. **Langbeheim, H., T. Y. Shih, and E. M. Scolnick.** 1980. Identification of a normal vertebrate cell protein related to the p21 *src* of Harvey murine sarcoma virus. Virology **106**:292–300.
9. **Lutton, J. K., R. C. Frederich, Jr., and J. P. Perkins.** 1979. Isolation of adenylate cyclase-enriched membranes from mammalian cells using concanavalin A. J. Biol. Chem. **254**:11181–11184.
10. **Scolnick, E. M., A. G. Papageorge, and T. Y. Shih.** 1979. Guanine nucleotide-binding activity as an assay for *src* protein of rat-derived murine sarcoma viruses. Proc. Natl. Acad. Sci. U.S.A. **76**:5355–5359.
11. **Scolnick, E. M., M. O. Weeks, T. Y. Shih, S. K. Ruscetti, and T. M. Dexter.** 1981. Markedly elevated levels of an endogenous *sarc* protein in a hemopoietic precursor cell line. Mol. Cell. Biol. **1**:66–74.
12. **Shih, T. Y., A. G. Papageorge, P. E. Stokes, M. O. Weeks, and E. M. Scolnick.** 1980. Guanine nucleotide-binding and autophosphorylating activities associated with the p21 *src* protein of Harvey murine sarcoma virus. Nature (London) **287**:686–691.
13. **Shih, T. Y., M. O. Weeks, H. A. Young, and E. M. Scolnick.** 1979. Identification of a sarcoma virus-coded phosphoprotein in nonproducer cells transformed by Kirsten or Harvey murine sarcomea virus. Virology **96**:64–79.
14. **Shih, T. Y., M. O. Weeks, H. A. Young, and E. M. Scolnick.** 1980. p21 of Kirsten murine sarcoma virus is thermolabile in a viral mutant temperature sensitive for the maintenance of transformation. J. Virol. **31**:546–556.
15. **Wei, C.-M., D. Lowy, and E. M. Scolnick.** 1980. Mapping of transforming region of the Harvey murine sarcoma virus genome by using insertion-deletion mutants constructed *in vitro*. Proc. Natl. Acad. Sci. U.S.A. **77**:4674–4678.
16. **Wickner, W.** 1979. The assembly of proteins into biological membranes: the membrane trigger hypothesis. Annu. Rev. Biochem. **48**:23–45.
17. **Willingham, M. C., I. Pastan, T. Y. Shih, and E. M. Scolnick.** 1980. Localization of the *src* gene product of the Harvey strain of MSV to plasma membrane of transformed cells by electron microscopic immunocytochemistry. Cell **19**:1005–1014.
18. **Wold, F.** 1981. *In vivo* chemical modification of proteins (post-translational modification). Annu. Rev. Biochem. **50**:783–814.