The *int-1* Proto-Oncogene Products Are Glycoproteins That Appear To Enter the Secretory Pathway

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The *int-1* proto-oncogene encodes a primary product of 370 amino acids, is normally expressed in mid-gestational embryos and adult testis, and is activated by proviral insertions during mammary carcinogenesis. Polyclonal and monoclonal antibodies directed against *int-1*-specific synthetic peptides immunoprecipitate up to five forms of *int-1* protein, ranging in size from 36,000 to 44,000 *M_r*, from cell lines that express cloned *int-1* DNA introduced by transfection or infection with retroviral vectors. Pulse-chase labeling experiments and glycosidase digestions suggested that the smallest of the *int-1* proteins is the primary translation product lacking its signal peptide and that it is modified to produce the larger species of sequential glycosylation. Subcellular fractionations demonstrated that all immunoprecipitable forms of *int-1* are mainly associated with membranes. *int-1* proteins in crude microsomal preparations are resistant to proteolysis and extractable at elevated pH, suggesting that they are sequestered within cytoplasmic vesicles in a manner consistent with the behavior of secretory products. However, we were unable to identify secreted *int-1* products in extracellular fluids.

The *int-1* proto-oncogene was discovered as a target for insertional activation of transcription in mammary carcinomas induced by the mouse mammary tumor virus (18, 19), and it is expressed in normal mice only in the neural tube of the mid-gestational embryo and in postmeiotic cells in the mature testis (12, 28, 37). We are attempting to identify, characterize, and determine the subcellular location of the protein products of *int-1* to understand the function of this unusual proto-oncogene.

In the accompanying report, we describe the identification of the *int-1*-encoded proteins p36, p38, p40, and p42 by immunoprecipitation of radiolabeled proteins from lysates of cells bearing *int-1* expression vectors, using antibodies directed against different *int-1*-specific peptides (2). Only the *int-1* product p36 was immunoprecipitated after cells were labeled in the presence of tunicamycin, suggesting that the three larger forms are glycosylated.

In this report, we further characterize these four and one additional *int-1* protein, p44, and determine their subcellular location. The higher-molecular-weight forms have the sensitivity to glycosidases expected of proteins with N-linked glycosylations and, based on kinetic labeling studies, appear to represent sequentially modified forms of p36, the unglycosylated product. While we did not identify an *int-1* product in the culture medium of these cells, we demonstrated that the intracellular forms of *int-1* protein have the subcellular location expected of a secretory product.

MATERIALS AND METHODS

Cells and antibodies. The derivations of the 7dT and MV7int-1/3T3 cell lines that express *int-1* cDNA have been described previously (3). MV7int-1/3T3 cells were infected with clone 1 murine leukemia virus (MLV) (6) to produce the

env proteins used as internal controls for the fractionation experiments.

The monoclonal and polyclonal antisera directed against *int-1*-specific peptides as well as the control sera have been described previously (2). The polyclonal antisera directed against MLV (clone 1) gp70 and v-Ha-ras proteins were provided by H. Fan and A. Levinson, respectively.

Labeling of cells, immunoprecipitation, and gel electrophoresis. 7dT or MV7int-1/3T3 cells (approximately 5×10^5 to 1×10^6 cells per 60-mm dish) were labeled by incubation with 0.5 mCi of [³⁵S]cysteine (Amersham Corp., Arlington Heights, Ill.) in 1.0 ml of Dulbecco modified Eagle medium lacking methionine and cystine. After the indicated times, the label was removed and the monolayers were rinsed once with phosphate-buffered saline RIPA buffer (800 μ l; 0.15 M NaCl, 0.01 M sodium phosphate [pH 7.0], 1% Nonidet P-40 [NP-40], 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 1% aprotinin [Sigma Chemical Co., St. Louis, Mo.]), a modification of the buffer used in reference 2, was added to each monolayer, and the dishes were kept on ice for 20 min at 4°C; the extracts were centrifuged in a microcentrifuge to remove insoluble components.

In the experiment shown in Fig. 1A, one half of each clarified lysate was immunoprecipitated with 3.5 μ l of rabbit serum raised against peptide B. The remaining half of the lysate was immunoprecipitated with this antiserum preincubated with 1 μ g of peptide B to block the immunoprecipitation specifically. After incubation on ice for 1.5 h, 1 mg of washed Pansorbin (Calbiochem-Behring, La Jolla, Calif.) was added to each and incubated for 1 h. The Pansorbin-antibody complex was pelleted with a microcentrifuge and washed two times with RIPA buffer. Final pellets were suspended and boiled for 2 min in 15 μ l of 1 $\times$ sample buffer and centrifuged quickly; the entire supernatant was then loaded onto an SDS-15% polyacrylamide gel. The gels were fluorographed with Amplify (Amersham) as directed by the manufacturer.

In vitro translation. In vitro translation of SP6-derived *int-1* RNA with [³⁵S]cysteine was done as described previ-

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FIG. 1. (A) Immunoprecipitation of *int-1* proteins after different labeling periods. 7dT cells were labeled with [35 S]cysteine for either 20 min (lanes 2 and 3) or 4 h (lanes 4 and 5), and equivalent amounts of cell lysate were immunoprecipitated with the anti-peptide B serum (lanes 2 and 4) or with the same antiserum preabsorbed with an excess of blocking peptide B (lanes 3 and 5). The apparent molecular weights (M_r) and positions of the five *int-1* proteins are indicated. Lane 1 shows the *int-1* in vitro translation product (37,000 M_r) for size comparison. All tracks are from the same 15% polyacrylamide-SDS gel, but the exposure time for lanes 2 and 3 was 4 days, whereas the others were exposed for 22 h. (B) Pulse-chase analysis of *int-1* products. Five equivalent 60-mm dishes of 7dT cells were pulse-labeled for 4 min with [35 S]cysteine. The dishes were then rinsed, one was harvested (0 min), and the rest were incubated in the presence of medium containing an excess of unlabeled cysteine for 10 min, 40 min, 2 h, and 7 h. Cell lysates were prepared at the indicated times, and *int-1* proteins were immunoprecipitated with the polyclonal serum against peptide B.

ously (2). A 1.0- μ l sample of the final translation reaction was used for the gel shown in Fig. 1A.

Pulse-chase analysis. Five equivalent 60-mm dishes of either MV7int-1/3T3 or 7dT cells were incubated for 10 min at 37°C with 2.5 ml of Dulbecco modified Eagle medium lacking methionine and cystine. Each dish was pulse-labeled with 0.5 mCi of [35 S]cysteine for 4 min in 1 ml of Dulbecco modified Eagle medium lacking methionine and cystine. The labeling medium was removed, and the dishes were rinsed quickly with complete Dulbecco modified Eagle medium plus 10% fetal calf serum and then incubated with 2.5 ml of the same medium for the indicated times. The zero time point was taken after the rinse step. The dishes were harvested for immunoprecipitation as outlined above. The entire 800- μ l sample was immunoprecipitated with 5 μ l of anti-peptide B serum and subjected to electrophoresis.

Endoglycosidase treatment. For *N*-glycanase treatment, one 60-mm dish of 7dT cells was labeled for 4 h and prepared for immunoprecipitation. The RIPA lysate was divided in half, and each half was immunoprecipitated with 8 μ l of anti-peptide A serum plus 1.5 μ l of goat anti-mouse immunoglobulin as described previously (2). The final washed pellets were suspended in 10 μ l of 0.5% SDS-0.1 M β -mercaptoethanol and boiled for 3 min. Then 8.8 μ l of 0.68 M

NaPO₄ (pH 8.6), 6.0 μ l of 30 mM EDTA, and 5.0 μ l of 7.5% NP-40 were added to each tube, and one tube was treated with 2.0 μ l of *N*-glycanase (Genzyme). The samples were incubated at 37°C for 16 h. A second 1.0- μ l portion of *N*-glycanase was added and incubated at 37°C for 2 h. An equal volume of 2 $\times$ Laemmli sample buffer was mixed with each sample, and the samples were boiled for 2 min and centrifuged. The entire supernatant was applied to a 15% gel.

For the endoglycosidase H (Endo H) treatment, one 60-mm dish of MLV-infected MV7 int-1/3T3 cells was labeled for 2 h and prepared for immunoprecipitation. The RIPA lysate was divided in half, and each half was immunoprecipitated with 3.0 μ l of anti-peptide B serum as outlined above. The final washed pellets were suspended in 22 μ l of 0.1 M sodium citrate (pH 5.5)-0.1% SDS and boiled for 3 min. Endo H (2 μ l; Boehringer Mannheim Biochemicals, Indianapolis, Ind.) was added to one sample, and both were incubated at 37°C for 16 h. A second 1.5- μ l portion of Endo H was added for 2 h, and both samples were mixed with an equal volume of 2 $\times$ Laemmli sample buffer, boiled, and analyzed on a 15% polyacrylamide gel.

Cell fractionation. Either 7dT or MLV-infected MV7int-1/3T3 cells were fractionated into crude microsomes (particulate) and soluble components as follows (4, 20). For the experiment shown in Fig. 3, one 60-mm dish of each cell type was labeled for 3 h with [35 S]cysteine, rinsed once with phosphate-buffered saline, and incubated on ice for 10 min with 600 μ l of hypotonic lysis buffer (10 mM NaCl, 20 mM sodium phosphate [pH 7.0], 1 mM EDTA, 1% aprotinin) per dish. The cells were scraped from the dish and homogenized with 18 strokes in a 1.0-ml glass Dounce homogenizer. The homogenate was centrifuged for 2.5 min at 500 rpm (H1000B rotor, Sorvall RT 6000 centrifuge), and the supernatant was centrifuged at top speed in a Beckman airfuge (approximately 170,000 $\times g$). The pellet (crude microsomes) was suspended in 600 μ l of RIPA buffer and centrifuged to remove insoluble components. The supernatant (soluble) fraction was adjusted to the composition of RIPA buffer with concentrated solutions of SDS, NP-40, and sodium deoxycholate. A 450- μ l sample of each fraction was immunoprecipitated with 3 μ l of anti-peptide B serum, and the remaining 150 μ l was immunoprecipitated with 2 μ l of anti-MLV gp70 (MV7int-1/3T3 fractions) or 2 μ l of anti-p21^{v-Ha-ras} (7dT fractions).

Proteolysis of microsomal and soluble fractions. One 60-mm dish of MLV-infected MV7int-1/3T3 cells was labeled for 3 h, homogenized in 400 μ l of hypotonic lysis buffer without aprotinin, and centrifuged at 500 rpm. The homogenate was split into two equal parts, and 2 μ l of 20-mg/ml proteinase K was added to one. Both samples were incubated at 0°C for 45 min, at which time 2 μ l of aprotinin and 15 μ l of 100 mM phenylmethylsulfonyl fluoride were added, and both samples were airfused as described above. The pellets were suspended in 400 μ l of RIPA buffer plus 2 mM phenylmethylsulfonyl fluoride. A 300- μ l sample of each fraction was immunoprecipitated with 3 μ l of anti-peptide B serum, and 100 μ l of each fraction was immunoprecipitated with 2 μ l of anti-MLV gp70 as outlined above.

Carbonate extraction of microsomal preparations. Two 5-cm dishes of MLV-infected MV7int-1/3T3 cells were labeled for 2 h, and each was homogenized in 600 μ l of hypotonic lysis buffer and centrifuged at 500 rpm as described above. One homogenate was incubated at 0°C for 1 h and 10 min with 60 μ l of 1 M sodium carbonate (pH 11.0), and the other was incubated at 0°C with 60 μ l of additional hypotonic lysis buffer as a control. The carbonate-treated

sample was neutralized with acetic acid, both mixtures were centrifuged in an airfuge, and the pellets were suspended in 600 μ l of RIPA buffer. A 500- μ l sample of each suspended pellet was immunoprecipitated with 4 μ l of anti-peptide B serum, and 100 μ l of each pellet was immunoprecipitated with 2 μ l of anti-MLV gp70 serum. The supernatant from the carbonate-treated sample was adjusted to the composition of RIPA buffer, and 500 μ l was immunoprecipitated with anti-peptide B serum.

RESULTS

Precursor-product relationships among *int-1* proteins. To determine the optimal conditions for identification and localization of *int-1* proteins, the rat embryo fibroblast line, 7dT, expressing an *int-1* gene under the control of an MLV promoter (2) was labeled continuously with [35 S]cysteine for variable lengths of time, followed by immunoprecipitation of *int-1*-specific proteins. Immunoprecipitation of cells labeled for 4 h revealed five forms of *int-1*: p36, p38, p40, p42, and p44 (Fig. 1A, lane 4). p44 was observed only after long labeling periods; it was not detected in all experiments (cf. Fig. 2 and 4 in reference 2) and produced a weak signal in others (cf. Fig. 2 below). However, p44 was produced in both 7dT cells and in 3T3 cells infected with an MLV vector carrying *int-1* (MV7*int-1*/3T3) (2), was recognized by the two different antipeptide antibodies (cf. Fig. 2), and was confirmed to be *int-1* encoded by mapping with V8 protease (data not shown). The same five *int-1* species could also be detected after a 16-h label (data not shown). In contrast, only the smaller three of the five *int-1* forms (p36, p38, and p40) could be immunoprecipitated after labeling for 20 min with [35 S]cysteine (Fig. 1A, lane 2).

To test whether the results shown in Fig. 1A imply precursor-product relationships among the five species of *int-1* protein, we performed pulse-chase experiments; a representative result is shown in Fig. 1B. After a 4-min pulse-label with [35 S]cysteine, three species of *int-1* protein—p36, p38, and p40—were already visible. Immunoprecipitates from cells at various times during the subsequent chase period showed that the smaller proteins were processed, perhaps sequentially, to p40 and p42. The two smallest proteins were rapidly processed, whereas the interconversion of the larger forms was slower. In additional experiments, p44 was also observed, but always as the ultimate product during the chase (data not shown). Notably, no protein corresponding in size to the full-length, 37,000- M_r product synthesized in vitro was observed in any of these in vivo experiments, consistent with the idea that amino-terminal cleavage by signal peptidase occurs cotranslationally.

The pulse-chase experiments are also informative with respect to protein turnover rates. From visual inspection of the autoradiograms, it appears that the $t_{1/2}$ of p42 is about 5 to 6 h. A small part of this turnover can be accounted for by conversion into p44, but the remainder of the protein must be degraded, secreted, or processed to a form that cannot be immunoprecipitated with our antibodies.

Most *int-1* proteins contain immature N-linked carbohydrate complexes. In previous experiments, we showed that tunicamycin, an inhibitor of N-linked glycosylation, prevents the appearance of all the *int-1* proteins except p36 (2). To explore this issue in more detail, we treated immunoprecipitates from either 7dT or MV7*int-1*/3T3 cells containing the five forms of [35 S]cysteine-labeled *int-1* protein with either *N*-glycanase or Endo H, two enzymes that are capable

FIG. 2. Glycosidase treatment of *int-1* proteins. Immunoprecipitates of *int-1* proteins were prepared from 7dT cells labeled for 4 h (*N*-glycanase) or from MLV-infected MV7 *int-1*/3T3 cells labeled for 2 h (Endo H) with [35 S]cysteine. Each immunoprecipitate was divided in half and incubated either with (+) or without (-) glycosidase. After incubation, sample buffer was added and the entire mixture was subjected to electrophoresis on a 15% polyacrylamide gel. The panel marked N-GLY shows an experiment with *N*-glycanase, and the panel marked Endo H shows an experiment with Endo H. Molecular weights are shown as in Fig. 1.

of removing N-linked carbohydrates (24, 33) (Fig. 2). Endo H converted most of the larger forms to species that comigrate with p36. The sensitivity of p38, p40, and p42 to digestion with Endo H suggested that they contain immature N-linked carbohydrates, with high mannose rather than complex structures, as is characteristic of proteins within the endoplasmic reticulum (ER) or early Golgi compartment (11). The levels of p44 were too low in these experiments to determine the Endo H sensitivity of this protein. Digestion with *N*-glycanase typically increased the amount of radioactivity in p36 and p38 at the expense of p40, p42, and p44. We have no explanation for the failure to convert all of the larger species to protein that comigrates with p36, since *N*-glycanase is expected to remove all N-linked sugars from the protein backbone (33).

***int-1* proteins are not detectable outside cells.** The predicted structure of *int-1* protein and the observed cleavage and glycosylation(s) suggested that the ultimate glycosylated form(s) of *int-1* protein would be secreted. To test this idea directly, we examined the medium from [35 S]cysteine-labeled 7dT cells and MV7*int-1*/3T3 cells by immunoprecipitation for the presence of *int-1* protein(s). Despite varied lengths of metabolic labeling periods and concentration of secreted proteins by methanol or trichloroacetic acid precipitation, in no case did we convincingly identify *int-1* proteins in the medium by immunoprecipitation with antisera against two *int-1* peptides (data not shown). Furthermore, acid washing of cell monolayers did not remove any recognizable *int-1* proteins which might have been adherent to the cell surface or extracellular matrix. Attempts to localize *int-1* proteins on the surface of 7dT cells by radiolabeling with 125 I or by treatment of intact cells with proteases were also unrevealing (data not shown).

FIG. 3. Localization of *int-1* proteins by cell fractionation. MLV-infected MV7int-1/3T3 cells (left panel) and 7dT cells (right panel) were labeled with [35 S]cysteine for 3.0 h. Each cell type was fractionated into crude microsome (P) and soluble (S) fractions as outlined in Materials and Methods. A 75% portion of each fraction was immunoprecipitated with the polyclonal antiserum against *int-1* peptide B (lanes 2 and 4 in left panel and lanes 1 and 3 in right panel). The remaining 25% of each fraction was immunoprecipitated with an antiserum against either MLV *env* proteins (lanes 1 and 3 in left panel) or p21^{ras} (lanes 2 and 4 in right panel). Arrows indicate the positions of *env* proteins (gPr80^{env}, gp70^{env}, and p15E) and *ras* proteins (p23 and p21). The positions of the four visible *int-1* proteins, p36, p38, p40, and p42, are denoted by dots.

***int-1* proteins are associated with microsomes.** As an alternative approach to the destination of *int-1* proteins, we determined their relationship to cellular membranes, since the location of a transmembrane or secreted protein within subcellular membranes is characteristic of its final disposition. To determine the subcellular distribution of p36–44, we first prepared [35 S]cysteine-labeled lysates of 7dT cells and MLV-infected MV7int-1/3T3 cells by homogenization after swelling in hypotonic buffer. The lysates were cleared of unlysed cells and nuclei, separated into soluble (S170) and particulate, membrane-containing (P170) fractions by sedimentation at $170,000 \times g$ (4, 20), and assayed for *int-1* proteins by immunoprecipitation (Fig. 3). To show that the fractionation method gives the expected distribution of other proteins, the same two fractions were assayed for other proteins synthesized in the two cell types. Fractions from MV7int-1/3T3 cells infected with MLV were examined for the three membrane-associated *env* products, gPr80^{env}, gp70^{env}, and p15E (38). Fractions of 7dT cells originally transfected with DNA containing an activated allele of human c-Ha-ras were examined for *ras* proteins found in an immature soluble form (p23^{ras}) or associated with the inner face of the plasma membrane (p21^{ras}) (29).

Most (over 75%) of all detectable species of *int-1* proteins were immunoprecipitated from the P170 fraction, the expected location of a glycoprotein. The integrity of the P170 and S170 fractions was evidenced by the fractionation pro-

files of *env* and *ras* proteins. gPr80^{env}, gp70^{env}, and p15E as well as p21^{ras} were found in the P170 fractions, whereas p23^{ras} was immunoprecipitated from the S170 fraction. In some experiments (cf. Fig. 4), a greater portion (up to 40%) of the *int-1* proteins was found in the S170 fraction, although the MLV *env* products were always restricted to the P170 fraction. The variable appearance of a minority of *int-1* protein in the soluble fraction may be the consequence of release from membrane vesicles during fractionation, since unlike the MLV *env* protein complex, the *int-1* species do not appear to be integral membrane proteins (see below).

Membrane-associated *int-1* protein is protease resistant. To ask whether *int-1* proteins are completely protected within membrane vesicles, as expected for secretory products, we treated a hypotonic homogenate of MV7int-1/3T3 cells with proteinase K before fractionation and immunoprecipitation (Fig. 4). In this experiment, approximately 60% of *int-1* protein was immunoprecipitated from the P170 fraction, and most or all of it was resistant to digestion with proteinase K. In contrast, the *int-1* proteins in the S170 fraction were completely digested, showing that the proteins are not inherently resistant to the protease. In addition, solubilization of the P170 fraction with NP-40 before protease treatment rendered the *int-1* products fully sensitive to proteinase K (data not shown).

As a control, we also examined the behavior of *env*

FIG. 4. *int-1* proteins in microsomal fractions are resistant to protease. MLV-infected MV7int-1/3T3 cells were labeled for 3.0 h with [35 S]cysteine and homogenized to produce crude microsomes as described in Materials and Methods. The homogenate was divided in half and incubated either with (+) or without (–) proteinase K (200 μ g/ml for 45 min at 0°C). Treated and untreated samples were centrifuged to separate crude membranes (P) and soluble fractions (S). The resulting fractions were immunoprecipitated with either the polyclonal antiserum against *int-1* peptide (lanes 1 and 3) or an antiserum against MLV *env* proteins (lanes 2 and 4) and subjected to electrophoresis in 15% polyacrylamide gels. Dots are explained in the legend to Fig. 3.

proteins. gp70^{env} was detected solely in the P170 fraction in a form completely resistant to proteolysis, as expected for a glycoprotein that is largely or entirely sequestered within cytoplasmic vesicles and disulfide bonded to the small transmembrane protein p15E (38). gPr80^{env} and p15E, also detected solely in the P170 fraction, appeared to be truncated slightly as expected for transmembrane proteins with only relatively short cytoplasmic tails (approximately 20 amino acids) that are accessible to proteinase K (30, 38). The resulting shifts in protein mobility are difficult to see in Fig. 4, but similar analyses with a 20% polyacrylamide gel clearly demonstrated the change in size of p15E after digestion (data not shown). All three *env* proteins, like *int-1* proteins, became susceptible to protease after solubilization with NP-40 (data not shown).

***int-1* proteins are not integral membrane proteins.** Although the above results suggested that *int-1* proteins are mostly sequestered within membrane vesicles, as predicted for a secretory protein, we could not rule out the possibility that these *int-1* proteins are anchored to the membrane via a small hydrophobic domain or via interactions with another integral membrane protein. To distinguish among these possibilities, we treated the crude microsomal homogenate of MLV-infected MV7*int-1*/3T3 cells with carbonate at pH 11. This procedure removes secretory proteins, which are free within the membrane vesicles, but will not extract integral membrane proteins (8, 32). After carbonate extraction, gp70^{env}, covalently linked to the transmembrane protein p15E, and gPr80^{env}, itself a transmembrane protein, were retained within the P170 fraction (Fig. 5, lane 3), but very little of the *int-1* species remained (Fig. 5, lane 4). The *int-1* proteins could be recovered by immunoprecipitation from the carbonate extract (Fig. 5, lane 5). These results indicated that *int-1* proteins are distributed within subcellular membranes in a manner consistent with that expected for secretory proteins.

DISCUSSION

We showed in this and the accompanying report (2) that as many as five species of *int-1* protein, ranging in apparent molecular weight from 36,000 to 44,000, are generated by the sequential addition of N-linked carbohydrates to a primary translation product from which an amino-terminal signal sequence has apparently been removed. The proteins are associated primarily with crude membrane functions, and these membrane-associated components are resistant to digestion with proteases and extractable by treatment with carbonate.

These are the properties expected of proteins that have entered the secretory pathway (11, 27, 36). Entry of *int-1* protein into this pathway is likely to occur by cotranslational insertion of its amino-terminal hydrophobic signal sequence into the membrane of the ER, with cleavage of the nascent chain by signal peptidase after residue 27 (see reference 2). Full translocation of the completed protein into the lumen of the ER presumably follows, since the predicted sequence of the remainder of the polypeptide lacks additional hydrophobic domains which could serve as a membrane anchor (7, 34).

Pulse-chase labeling (Fig. 1B) and glycosidase digestions (Fig. 2) indicated that stepwise glycosylation occurs at some or all of four candidate sites in *int-1* protein while it is transported through the ER lumen. We cannot unambiguously specify which glycosylation sites are used; however, the pattern of glycosidase products (Fig. 2) strongly suggests that at least three and possibly all four sites are modified,

FIG. 5. Carbonate extraction of crude microsomes. Microsomal homogenates of MLV-infected MV7*int-1*/3T3 cells labeled for 2 h with [³⁵S]cysteine were prepared as outlined in Materials and Methods. One sample was adjusted to pH 11 with sodium carbonate (+) and the other was maintained at neutral pH (−). After 70 min of incubation at 0°C and subsequent neutralization, the two samples were centrifuged to produce crude membrane and soluble fractions, and proteins were immunoprecipitated with antiserum against either *int-1* peptide (lanes 2, 4, and 5) or MLV *env* proteins (lanes 1 and 3). Lanes 1 to 4 show immunoprecipitations from untreated and carbonate-treated membrane fractions. Lane 5 shows an immunoprecipitate of *int-1* protein from the soluble fraction after carbonate treatment. The positions of these proteins are denoted by dots as in Fig. 3 and 4.

since *N*-glycanase, which hydrolyzes the bond between the carbohydrate and the asparagine attachment site, converts the three most highly modified forms (p40, p42, and p44) to the three more rapidly migrating species (p36, p38, and p40). Three of the four potential sites for N-linked glycosylation reside near the carboxy terminus of the protein; the conclusion that at least some of these sites must be used conforms to other arguments that the protein is fully translocated across the ER membrane, thereby exposing the carboxy-terminal sites to the enzymes involved in carbohydrate addition.

The nearly complete removal of sugar residues from p38, p40, and p42 with Endo H (Fig. 2) implies no further processing of the carbohydrate chains on these proteins, as typically occurs in the Golgi compartments. It is possible that transport of *int-1* proteins from the ER is inherently inefficient. It is also possible that processing and intracellular transport of *int-1* proteins is impaired in cell types other than those in which the gene is normally expressed (cells in the embryonic nervous system or round spermatids [28, 37]). On the other hand, we observed nearly identical patterns of *int-1* proteins in several cell types in which the gene has been expressed, including mouse and rat fibroblasts (this report), mouse mammary epithelial cells (2), *Xenopus* oocytes (J. Papkoff, unpublished observations), and insect cells infected

with baculovirus vectors (M. Chamorro, personal communication).

The *int-1* products we described here may have a function within the lumen of the ER, Golgi, or transport vesicles. However, *int-1* protein does not contain the consensus sequence found in the few proteins which are known to be specifically sequestered within the ER lumen (17). We favor the suggestion that some form of *int-1* protein, perhaps the most highly modified form, p44, is secreted. This proposal is consistent with all of the described properties of *int-1* proteins and with the predicted behavior of the product of wingless, the *int-1* homolog in *Drosophila melanogaster* (22), since wingless mutants behave in a cell nonautonomous manner (16).

An extracellular location for *int-1* protein is also most easily reconciled with the apparently diverse roles of the mouse *int-1* gene as a stimulus to growth and morphological change in mammary epithelium (3) and as a developmental factor in male germ cells and the neural tube (28, 37). Such complex behavior could be mediated by differential responses to signals delivered via one or more *int-1*-responsive transmembrane receptors, as proposed for other growth and differentiation factors (31). However, we have no direct evidence for such receptors or for secretion of any of the five observed species of *int-1* protein, despite repeated efforts to detect *int-1* proteins in culture medium from cells expressing the gene. The modified *int-1* proteins that we observed in rodent fibroblasts may all be precursors to a functional secreted product(s) that we cannot detect because of low abundance or failure to be recognized by our antisera. Alternatively, secreted products of *int-1* might be associated with cell surfaces or extracellular matrices. In this regard, *int-1* proteins may resemble other growth factors, such as platelet-derived growth factor (23), which are difficult to detect physically in extracellular fluids.

int-1 is but one of several cellular genes that have been implicated in mammary tumorigenesis as a consequence of insertion mutations by murine mammary tumor virus proviruses (R. Nusse, in M. E. Lippman and R. Dickson, ed., *Breast Cancer: Cellular and Molecular Biology*, in press). Thus far the protein products of the other implicated genes have not been extensively characterized, but the predicted amino acid sequence of the *int-2* protein (15), which shows no evident homology to that of *int-1* protein, has homology to fibroblast growth factor (5). In view of evidence that these mammary proto-oncogenes can act cooperatively during tumorigenesis (14, 21), it will be important to look for metabolic interactions among their protein products.

It is apparent from the deduced sequence of *int-1* protein (7, 34) and the results presented in these papers that *int-1* does not belong to proto-oncogene families that encode nuclear proteins, tyrosine or serine-threonine kinases, or signal-transducing GTPases (35), but *int-1* can be grouped with other genes and their relatives that encode secreted growth and differentiation factors, such as TGF- β (25), platelet-derived growth factor (10), fibroblast growth factor (1, 9), transforming growth factor α (26), or granulocyte-macrophage colony-stimulating factor (13). Functional and structural studies of *int-1* proteins, including a search for cell surface receptors specific for *int-1* products, will be required to evaluate this assignment.

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