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Identification of Protein Products Encoded by the Proto-Oncogene *int*-1

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The proto-oncogene *int*-1 is activated by adjacent insertions of proviral DNA in mouse mammary tumor virus-induced tumors and has transforming activity in certain mammary epithelial cell lines. The gene is normally expressed in the central nervous system of mid-gestational embryos and in the adult testis. We raised antibodies against synthetic *int*-1 peptides and used these to identify protein products of the gene in cells transfected or infected with retroviral vectors expressing *int*-1. Four protein species of 36,000, 38,000, 40,000, and 42,000 M_r were immunoprecipitated by antibodies against two different *int*-1 peptides and were not present in control cells. Partial degradation with V8 protease showed the four species to be structurally related to each other and to *int*-1 polypeptide synthesized in vitro. Treatment of the cells with tunicamycin prevented the appearance of all but the 36,000-M_r species, suggesting that the slower-migrating forms are glycosylated derivatives. The unglycosylated 36,000-M_r species migrated faster in polyacrylamide gels than the in vitro translation product of *int*-1 and has probably undergone cleavage of an amino-terminal signal peptide.

The proto-oncogene *int*-1 was first identified as a gene activated by proviral insertions of the mouse mammary tumor virus in the majority of mouse mammary tumor virus-induced carcinomas in C3H mice (11). While no expression of the gene is seen in normal mammary glands, a low level of *int*-1 RNA is detected in the tumors; since the proviral insertions always leave the major open reading frame for *int*-1 intact (17), expression of the protein product of *int*-1 is strongly implicated in tumorigenesis. Direct evidence suggesting that *int*-1 contributes to neoplasia has come from studies of the phenotypic consequences of expressing exogenous *int*-1 alleles in cultured cells. We have shown that a retroviral vector expressing *int*-1 causes morphological transformation and altered growth properties of a mammary epithelial cell line derived from normal mouse mammary tissue (2). It has also been shown that nontumorigenic revertants of a tumor-derived mammary cell line can be converted to a tumorigenic phenotype by the introduction of an active *int*-1 gene (13). It is thus firmly established that *int*-1 has transforming ability in appropriate target cells, although so far these effects have been demonstrated only in certain epithelial cells of mammary origin.

While mammary tumors are the only neoplasms in which *int*-1 has so far been implicated, studies of the normal expression of the gene suggest that it plays important roles in the development of other tissues. No *int*-1 RNA is detected in most adult tissues examined, but the gene is expressed in the testes of sexually mature mice in postmeiotic cells (5, 14). In addition, *int*-1 RNA is expressed during embryogenesis between days 8.5 and 13.5 of gestation (5), predominantly in the developing neural tube (14, 21). This strict spatial and temporal regulation of expression makes *int*-1 a strong candidate for a gene that may be functionally involved in differentiation or development. *int*-1 also displays a remarkable degree of evolutionary conservation: the predicted amino acid sequences of the mouse and human *int*-1 gene products are 99% identical (16), and the *Drosophila* homolog of *int*-1, recently identified as the segmental polarity gene wingless, encodes a protein with over 50% of its amino acid residues identical to those in mouse *int*-1 protein (13a).

As a preliminary step toward elucidating the role of *int*-1 in mammary tumorigenesis and the function of the gene in normal mammalian tissues, we raised antibodies against synthetic *int*-1 peptides and here report the identification of four *int*-1 protein products, ranging in apparent molecular weight (M_r) from 36,000 to 42,000. Three of the proteins represent different glycosylated forms of the 36,000-M_r precursor, and all four appear to have undergone cleavage of an N-terminal signal peptide from the 370-amino-acid translation product. The accompanying paper (12) describes further characterization of the *int*-1 glycoproteins and their subcellular location, which indicates that they enter the secretory pathway.

MATERIALS AND METHODS

Peptides and antibodies. Peptides were synthesized and kindly provided by R. Lerner and his colleagues (Scripps Clinic, La Jolla, Calif.). A cysteine residue was added to the carboxy terminus of each peptide. The peptides were coupled to keyhole limpet hemocyanin with *m*-maleimidbenzoyl *N*-hydroxysuccinimide ester as described by Lerner et al. (8) and then injected into rabbits in an emulsion of Freund adjuvant. A mouse hybridoma producing monoclonal antibodies against *int*-1 peptide A (residues 200 to 212) was generously supplied by H. Niman (Scripps Clinic). Ascites fluid containing the antibody was diluted 1:1 with glycerol and stored at −20°C.

Retroviral vectors and cell culture. Construction of the murine leukemia virus-based retroviral vector MXIN, containing *int*-1 and *neo*, and of MXfsIN, which carries a frameshift mutant *int*-1 allele, has been described previously

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(2). pMV7*int*-1 (P. Pryciak and A. M. C. Brown, unpublished data) was constructed by inserting *int*-1 cDNA from clone 26 (4) into the retroviral vector pMV-7 (P. T. Kirschmeier, personal communication).

7dT cells were derived from an experiment in which primary Fisher rat embryo fibroblasts prepared as described by Land et al. (7) were cotransfected with DNA of the murine leukemia virus-based retroviral construct pMX*int*-1.*neo* (2) together with the mutant c-Ha-*ras* gene from the human bladder carcinoma line EJ (EJ*ras*). Although the experiment yielded no evidence that *int*-1 could augment a low frequency of transformation by EJ*ras* alone in the cotransformation assay, one of the few transformed clones, designated 7dT, was found to express *int*-1-specific RNA approximately 20 times more abundantly than mouse mammary tumors with proviral insertions at *int*-1 (G. M. Shackleford, unpublished data).

To make MV7*int*-1/3T3 cells, we inserted full-length *int*-1 cDNA (clone 26; 4) into the murine sarcoma virus-based retroviral vector pMV7 (P. T. Kirschmeier, personal communication), which carries a bacterial neomycin phosphotransferase gene driven from a herpesvirus *tk* promoter. The resulting construct was introduced into the retrovirus packaging cell line ψ2 (9) by $CaPO_4$ transfection, and helper-free virus stocks were harvested as described by Brown and Scott (1). NIH 3T3 cells were then infected with the MV7*int*-1 virus in the presence of 8 μg of Polybrene per ml, and individual colonies were selected in 400 μg of G418 (Geneticin; GIBCO Laboratories, Grand Island, N.Y.) per ml.

All cell lines were cultured in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. Dexamethasone (10^{-7} M) was added to the culture medium of 7dT cells, and 10 μg of insulin per ml was added to that of C57MG cells (15).

Metabolic labeling, in vitro translation, and immunoprecipitations. Subconfluent 60-mm dishes of cells were washed with phosphate-buffered saline and treated for 30 min with serum-free Dulbecco modified Eagle medium lacking cysteine. The medium was then replaced with 1.5 ml of serum-free Dulbecco modified Eagle medium containing 500 μCi of [^{35}S]cysteine (600 Ci/mmol; Amersham Corp., Arlington Heights, Ill.), and the dishes were incubated for a further 4 h. After two rinses in phosphate-buffered saline, the cells were lysed at 4°C in 1 ml of RIPA buffer (50 mM NaCl, 25 mM Tris hydrochloride [pH 7.5], 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 1% aprotinin [Sigma Chemical Co., St. Louis, Mo.]) containing 10 μg of bovine serum albumin per ml. The lysate was centrifuged at 12,000 × *g* for 10 min at 4°C, and the supernatant was either used directly for immunoprecipitation or frozen at −70°C. Tunicamycin, when used, was added at a final concentration of 5 μg/ml 30 min before the addition of ^{35}S and was present throughout the labeling period. In vitro translation of *int*-1 protein in the presence of [^{35}S]cysteine was performed as described by Fung et al. (4) in a rabbit reticulocyte lysate system, using an *int*-1 RNA template synthesized in vitro from *int*-1 cDNA clone 26 (4) with SP6 polymerase (6).

Immunoprecipitations were performed in 500-μl volumes of RIPA buffer. A 2- to 5-μl sample of rabbit serum or ascites fluid was added to the labeled cell extracts or in vitro translation reaction and incubated at 0°C for 45 min. (Where described, the antibodies were blocked by preincubation for 30 min with 500 ng of peptide per reaction.) A 25-μl portion of a 50% slurry of protein A-Sepharose (Pharmacia, Inc., Piscataway, N.J.) was next added, and the reaction mixtures were mixed periodically at 0 to 4°C for 45 min. Protein A-Sepharose-antibody complexes were pelleted by brief centrifugation and washed three times in RIPA buffer without bovine serum albumin. The final pellets were then suspended in 2× sample buffer (125 mM Tris hydrochloride [pH 6.8], 4% SDS, 10% β-mercaptoethanol, 10% glycerol), boiled for 2 min, and loaded onto SDS-12% polyacrylamide gels. After electrophoresis, the gels were fixed in 10% acetic acid–25% methanol, treated with Amplify (Amersham), dried, and exposed for fluorography.

V8 protease mapping. A 60-mm dish of confluent 7dT cells was labeled for 4.5 h with 800 μCi of [^{35}S]cysteine in 1.5 ml of Dulbecco modified Eagle medium lacking cystine and methionine. A lysate of the cells in RIPA buffer was immunoprecipitated with 20 μl of monoclonal antibody against peptide A plus 4 μl of goat anti-mouse immunoglobulin G as described (12). The final washed immunoprecipitate, as well as 4 μl of an *int*-1 in vitro translation reaction mixture, were subjected to electrophoresis in a preparative SDS-15% polyacrylamide gel which was dried directly upon completion. Slices of gel containing each of the *int*-1 products, as well as the cross-reactive protein X, were excised and divided into three pieces (1 by 2.5 mm). These were then used for proteolysis with three concentrations of *Staphylococcus aureus* V8 protease (0, 0.025, and 0.2 μg per gel fragment) as described by Cleveland et al. (3) and were analyzed on an SDS-17.5% polyacrylamide gel.

RESULTS

The previously determined nucleotide sequence of the major open reading frame in *int*-1 cDNA clones indicates that the primary tran ιtion product is a polypeptide of 370 amino acids (4). Thι principal features of this predicted polypeptide are shown in Fig. 1A. The first 28 residues at the amino terminus form a strongly hydrophobic domain, suggesting that this represents a signal peptide, while the carboxy-terminal region of the molecule is unusually rich in cysteine residues. The amino acid sequence includes four potential sites for N-linked glycosylation, three of which are close to the carboxy terminus (Fig. 1A), and there are also four potential sites for cleavage by serine proteases (Fig. 1A).

Antipeptide antibodies precipitate *int*-1 proteins synthesized in vitro. To identify the translation products of *int*-1, we raised antisera in rabbits against two synthetic oligopeptides, representing amino acid residues 200 to 212 and 275 to 289 and designated A and B in Fig. 1A. In addition, we made use of a mouse monoclonal antibody raised against peptide A (H. Niman and R. Lerner, unpublished data).

We have previously shown that when *int*-1 cDNA clones are transcribed and translated in vitro, they direct synthesis of a single major polypeptide species of 37,000 M_r (4). The apparent molecular weight of this species is close to the theoretical value of 41,000 estimated from the predicted amino acid sequence. Enzyme-linked immunosorption assays showed that the antipeptide sera were reactive against their respective peptides (data not shown), and the availability of the in vitro translation product allowed us to test as well whether the antibodies could immunoprecipitate a known *int*-1 polypeptide. While preimmune rabbit sera did not precipitate the 37,000-M_r *int*-1 translation product (Fig. 1B, lanes 3 and 8), the polypeptide was precipitated by rabbit sera raised against each of the two synthetic peptides (lanes 4 and 9). The mouse monoclonal antibody raised against

peptide A also precipitated the in vitro translation product (lane 6). In contrast, when the immune rabbit sera and mouse monoclonal antibody were preincubated with the respective immunizing peptides, their abilities to precipitate the 37,000-M_r *int*-1 polypeptide were blocked (Fig. 1B, lanes 5, 7, and 10). These results imply a specific recognition of the *int*-1 translation product by the antipeptide antibodies. Since the sample of unprecipitated translation product shown in lane 2 represented only one-fifth of the quantity used in each immunoprecipitation, the intensity of protein bands in the other lanes of Fig. 1B indicates that the efficiency of precipitation of the *int*-1 polypeptide was less than 10%. Nevertheless, the specific precipitation of the in vitro translation product in these experiments encouraged us to test whether these antibodies would detect authentic *int*-1 proteins in cell extracts.

Detection of multiple forms of *int*-1 protein in transfected and infected cells by antipeptide antibodies. To facilitate the initial identification of *int*-1 protein in cells, we made use of a rat fibroblast cell line designated 7dT (see Materials and Methods) which expresses high levels of *int*-1-specific RNA. 7dT cells are stably transfected with DNA of the retroviral expression construct pMX*int*-1.*neo* (2) and contain approximately 20-fold more *int*-1 RNA than mammary tumors bearing mouse mammary tumor virus proviral insertions near *int*-1 (G. M. Shackleford, unpublished data). The cells were labeled for 4 h with [^{35}S]cysteine and lysed in RIPA buffer, and *int*-1 proteins were immunoprecipitated from the cell extract with either polyclonal rabbit serum against peptide B or ascites fluid containing the mouse monoclonal antibody against peptide A. Each of these antibodies precipitated two major protein species of 42,000 and 40,000 M_r, as well as two minor species of 38,000 and 36,000 M_r (hereafter referred to as p42, p40, p38, and p36, respectively) (Fig. 2A, lanes 2 and 3). These proteins were not precipitated by preimmune rabbit serum (lane 1) or when the monoclonal antibody was blocked by preincubation with the appropriate peptide (lane 4). A 42,000-M_r species in lane 1 is a background band which can be resolved on other gels from the 42,000-M_r protein precipitated by the antipeptide sera (data not shown). The monoclonal antibody against peptide A also recognized a protein of around 55,000 M_r (marked X in lane 3) whose precipitation is blocked by incubation with peptide. This species is not specifically precipitated by antisera against peptide B, however, and we provide further evidence below that the 55,000-M_r species is not a product of *int*-1.

Identification of *int*-1 proteins in several cell lines. Since the antibodies used in these precipitations of *int*-1-specific proteins from 7dT extracts were raised against two different *int*-1 peptides, it is likely that the four protein bands detected by both antibodies represent translation products of *int*-1. To obtain further evidence in support of this, we determined whether these proteins are found specifically in other cell lines expressing *int*-1. Since the 7dT cell line was derived from a mixed primary culture of rat embryo cells, it was not possible to obtain an entirely appropriate control cell line for 7dT. We therefore examined *int*-1 proteins in NIH 3T3 cells before and after infection with a recombinant retrovirus, designated MV7*int*-1, which expresses full-length *int*-1 cDNA (P. Pryciak and A. M. C. Brown, unpublished data).The cells were labeled with [^{35}S]cysteine as before, and the lysates were precipitated with antibodies against peptides A and B. As with 7dT cell extracts, both antibodies precipitated p36, p38, p40, and p42 from extracts of MV7*int*-1/3T3 cells, and precipitation was blocked by preincubation with the relevant peptides (Fig. 2B, lanes 2 to 5). In contrast,

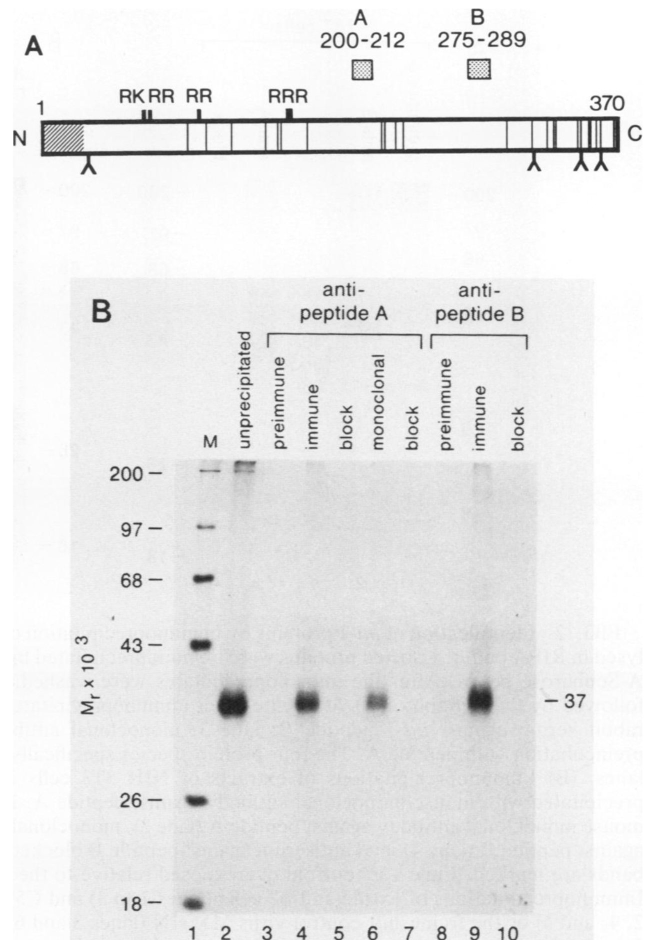

FIG. 1. Immunoprecipitation of in vitro-synthesized *int*-1 protein with antipeptide antibodies. (A) Predicted structure of *int*-1 protein based on the deduced amino acid sequence (4). The primary translation product is 370 amino acids long, with a hydrophobic amino-terminal domain (marked by the stippled box). The protein is rich in cysteine residues (shown by vertical bars), especially in the carboxy-terminal region. Inverted Ys indicate the four potential sites for N-linked glycosylation (at residues 29, 316, 346, and 359), and the positions of double or triple basic residues that are potential sites for cleavage by serine proteases are indicated by R (arginine) and K (lysine). The locations of the synthetic peptides A and B, used to raise antibodies against *int*-1 protein, are shown above the map. (B) Test of antipeptide antibodies by immunoprecipitation of *int*-1 protein synthesized in vitro. A plasmid vector containing *int*-1 cDNA downstream from an SP6 promoter was transcribed in vitro with SP6 RNA polymerase, and the resulting RNA was translated in vitro in a rabbit reticulocyte lysate system in the presence of [^{35}S]cysteine. After immunoprecipitation (under conditions of antibody excess), the protein was loaded onto an SDS-polyacrylamide gel for electrophoresis and subsequently revealed by fluorography. Lane 1, Molecular weight markers. Lane 2, Unprecipitated in vitro translation product. Five times more in vitro product was used for each immunoprecipitation (lanes 3 to 10) than the quantity loaded in lane 2. Immunoprecipitation was performed with the following: lane 3, preimmune serum from rabbit immunized with peptide A; lane 4, serum from rabbit immunized against peptide A; lane 5, rabbit serum against peptide A blocked by preincubation with peptide; lane 6, ascites fluid containing mouse monoclonal antibody against peptide A; lane 7, monoclonal antibody blocked by preincubation with peptide A; lane 8, preimmune serum from rabbit immunized with peptide B; lane 9, serum from rabbit immunized against peptide B; lane 10, rabbit serum against peptide B blocked by preincubation with peptide. The position of the 37,000-M_r *int*-1 translation product is indicated.

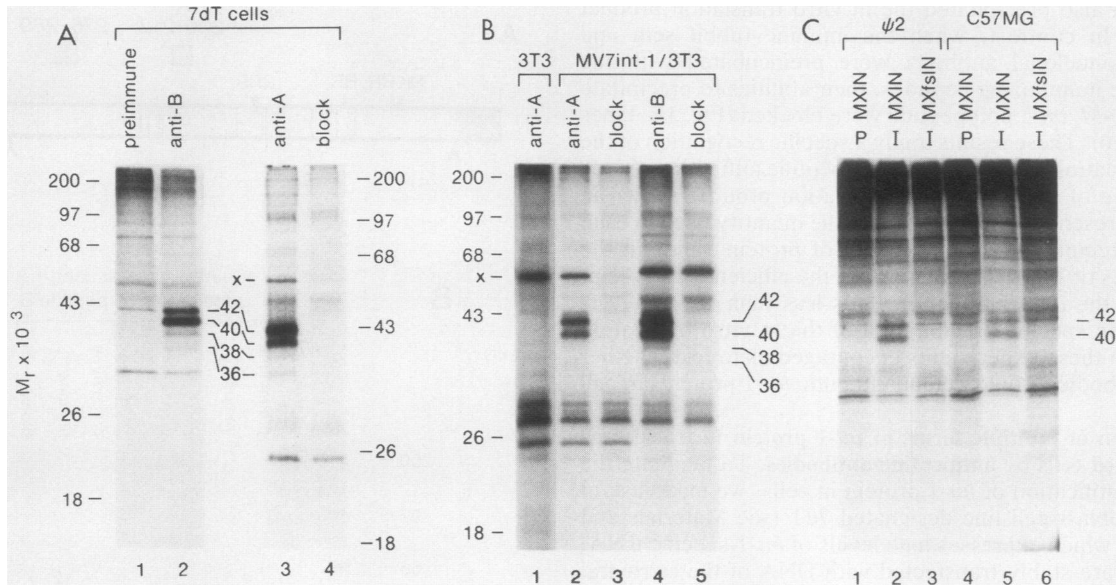

FIG. 2. Identification of *int*-1 proteins by immunoprecipitation of ^{35}S-labeled cell extracts. Cells were labeled for 4 h with [^{35}S]cysteine and lysed in RIPA buffer. Labeled proteins were immunoprecipitated by incubation with antipeptide antibodies and subsequent addition of protein A-Sepharose beads. After the immunoprecipitates were washed, the proteins were analyzed by SDS-polyacrylamide gel electrophoresis followed by fluorography. (A) 7dT cell extract immunoprecipitated with the following antibodies: lane 1, preimmune rabbit serum; lane 2, rabbit serum against *int*-1 peptide B; lane 3, monoclonal antibody against *int*-1 peptide A; lane 4, monoclonal antibody blocked by preincubation with peptide A. The four protein species specifically detected with antibodies against both peptides are indicated between the lanes. (B) Immunoprecipitations of extracts of NIH 3T3 cells infected with MV7*int*-1 retrovirus. Lane 1, Extract of uninfected cells precipitated with mouse monoclonal antibody against peptide A. Lanes 2 to 5, Extract of MV7*int*-1-infected cells immunoprecipitated with mouse monoclonal antibody against peptide A (lane 2), monoclonal antibody blocked by preincubation with peptide (lane 3), rabbit antiserum against peptide B (lane 4), and antiserum against peptide B blocked by preincubation with peptide (lane 5). Positions of the four *int*-1 specific bands are marked. Lane 1 is fourfold overexposed relative to the other lanes to compensate for less efficient labeling of the cells used. (C) Immunoprecipitations of extracts of ψ2 cells (lanes 1 to 3) and C57MG cells (lanes 4 to 6) infected with the *int*-1 retrovirus MXIN (lanes 1, 2, 4, and 5) or the frameshift control virus MXfsIN (lanes 3 and 6). Precipitations were with rabbit antiserum against peptide B (lanes 2, 3, 5, and 6) or with preimmune serum (lanes 1 and 4). The positions of the two major *int*-1-specific lbands at 40,000 and 42,000 M_r are indicated.

none of these proteins was detected in extracts of uninfected NIH 3T3 cells (Fig. 2B, lane 1), indicating that they are only produced in cells expressing *int*-1. A 55,000-M_r protein species (marked X in Fig. 2B) was precipitated from MV7*int*-1/3T3 cell extracts with the antibody against peptide A, but this band was also detected in uninfected 3T3 cells and was again not recognized by the antibody against peptide B.

We also examined expression of these proteins in cell lines infected with the retroviral vectors MXIN and MXfsIN, which we have previously used to show that expression of *int*-1 causes partial transformation of the mammary epithelial cell line C57MG (2). The MXIN virus contains both *int*-1 and *neo*, and MXfsIN is identical to MXIN except for a frameshift mutation near the start of the *int*-1 open reading frame (2). The levels of *int*-1-related proteins in cells infected with MXIN appeared to be lower than those in similar cell types infected with MV7*int*-1. Immunoprecipitation of MXIN-infected ψ2 cells, for example, revealed the p42 and p40 proteins detected above, but the p38 and p36 species were not detectable above background levels (Fig. 2C, lanes 1 and 2). To confirm that the putative *int*-1 proteins are expressed in MXIN-infected C57MG cells, we performed immunoprecipitations of the transformed cells using the rabbit serum against *int*-1 peptide B. Again the abundance of the *int*-1-related proteins was much lower than in 7dT or MV7*int*-1/3T3 cells, and a strong background band of 42,000 M_r in C57MG cells precluded unequivocal detection of p42. However, the p40 protein species was clearly detected in morphologically transformed MXIN/C57MG cells (Fig. 2C, lane 5) and was not seen in C57MG cells infected with the frameshift control virus MXfsIN (Fig. 2C, lane 6).

Confirmation of *int*-1-specific proteins by digestion with V8 protease. While the above data indicate that the protein bands detected by the antipeptide sera are specific to cells expressing an intact *int*-1 allele, we wished to confirm unequivocally the identity of all four of the species detected. All of these protein species, together with the 55,000-M_r protein (X) precipitated by the monoclonal antibody against peptide A, were therefore gel purified after immunoprecipitation of labeled 7dT cell extracts and subjected to partial degradation with *S. aureus* V8 protease. The digestion products of the various proteins were then compared on SDS-polyacrylamide gels, along with the V8 protease digestion products of *int*-1 protein synthesized in vitro. All four of the protein species detected in 7dT cells exhibited related patterns of degradation products (Fig. 3), and moreover, many of the proteolytic fragments were common to the *int*-1 polypeptide translated in vitro. These data demonstrate that all four proteins are structurally related to one another and are indeed translation products of the *int*-1 gene. In contrast, the 55,000-M_r X protein, which was recognized only by the antibody against peptide A (see above), is unrelated to any of the *int*-1 proteins by these criteria.

Tunicamycin inhibits synthesis of multiple species of *int*-1 proteins. The presence of potential sites for N-linked glycosylation in the predicted sequence of *int*-1 protein suggested that the multiple protein species detected in cells might represent different glycosylated forms of a common precur-

sor. To test this, we treated 7dT cells with tunicamycin, an inhibitor of N-linked glycosylation, before and during metabolic labeling with [^{35}S]cysteine. The cell lysates were then immunoprecipitated with antiserum against peptide B as before. Instead of the four proteins of M_r 36,000 to 42,000 normally detected (Fig. 4, lanes 3 and 5), only p36 was seen in the tunicamycin-treated cells (Fig. 4, lanes 4 and 6). This strongly suggests that the three slower-migrating *int*-1 proteins seen in untreated cells are glycosylated forms of a 36,000-M_r precursor.

Figure 4 also shows that the unglycosylated form of *int*-1 protein in 7dT cells migrates faster than the in vitro translation product of *int*-1 in adjacent lanes of the same gel (lanes 6 and 7). In view of the hydrophobic nature of the amino-terminal domain of *int*-1 protein, a possible explanation of this mobility difference is that the *int*-1 protein in vivo undergoes cleavage of a signal peptide during membrane translocation, a process that would not occur in our in vitro translation reactions. Further support for this interpretation is presented below.

DISCUSSION

With the aid of antibodies against synthetic oligopeptides, we have identified a set of four related proteins in cells expressing the *int*-1 gene. By several criteria we have demonstrated that these proteins are products of *int*-1: the same four species are immunoprecipitated by antibodies raised against peptides from two nonoverlapping regions of the predicted *int*-1 sequence but not by control sera; the proteins are detected in more than one cell line expressing an intact *int*-1 allele but not in control cells expressing a mutant *int*-1 gene bearing an amino-terminal frameshift; and all four proteins share proteolytic fragments in common with the in vitro translation product of *int*-1 cDNA.

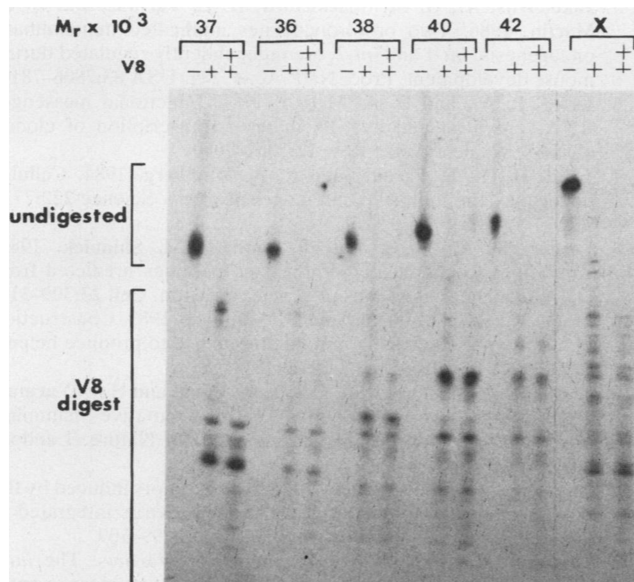

FIG. 3. V8 protease cleavage analysis of *int*-1 proteins. The four *int*-1-related proteins, p36, p38, p40, and p42, together with the cross-reactive protein X, were purified by preparative gel electrophoresis after immunoprecipitation of a 7dT cell lysate. A sample of the p37 in vitro translation product of *int*-1 was also purified from the same gel. Slices of acrylamide gel containing the various individual proteins were then subjected to V8 proteolysis, and the products were analyzed on a second SDS-polyacrylamide gel. Lanes marked − contain undigested samples; those marked + and ++ contain the products of digestion with 25 and 200 ng of *S. aureus* V8 protease, respectively. The particular protein species used in each set of three digests is indicated above the gel by its apparent molecular weight or by X for the cross-reactive 55,000-M_r protein. 37 indicates the lanes containing digestion products of the 37,000-M_r *int*-1 polypeptide synthesized in vitro. Regions of the gel containing undigested samples or proteolysis products are marked alongside.

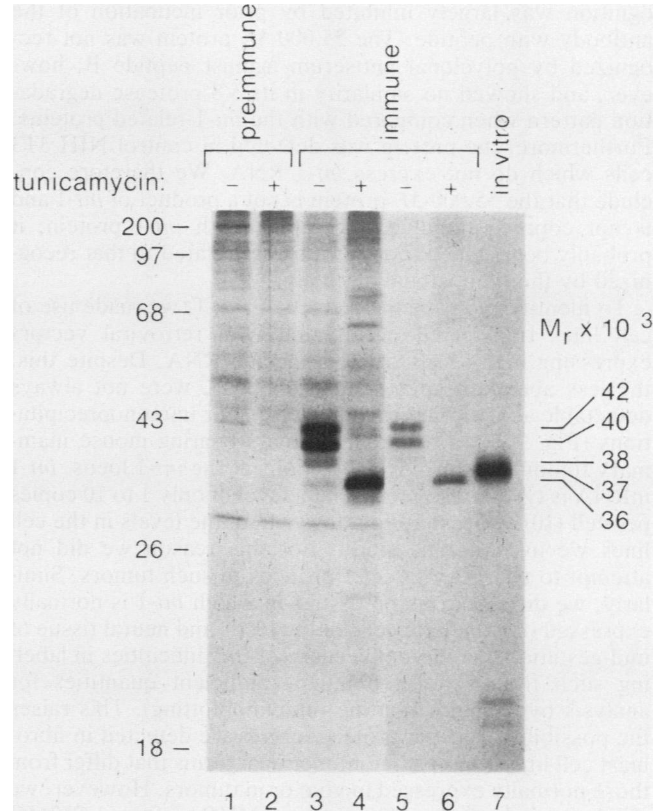

FIG. 4. Immunoprecipitations of 7dT cell extracts showing effects of tunicamycin on *int*-1 proteins. 7dT cells were labeled with [^{35}S]cysteine for 4 h in the presence or absence of 5 μg of tunicamycin per ml, and cell extracts were then precipitated with preimmune rabbit serum (lanes 1 and 2) or with rabbit antiserum against peptide B (lanes 3 to 6). Lanes 5 and 6 represent a shorter exposure of lanes 3 and 4. Of the four *int*-1 protein species normally detected in 7dT cells, only the 36,000-M_r form was seen in cells treated with tunicamycin. Lane 7, In vitro translation product of *int*-1 immunoprecipitated with rabbit serum against peptide B. Comparison of lanes 6 and 7 shows that the 36,000-M_r *int*-1 species detected in tunicamycin-treated cells migrates faster in the gel than the 37,000-M_r in vitro product. Apparent molecular weights are marked alongside the gel.

The four *int*-1 protein products that we identified here have apparent molecular weights of 36,000, 38,000, 40,000, and 42,000 M_r. In the presence of tunicamycin, only the 36,000-M_r species is detected, suggesting that the three larger protein species are glycosylated derivatives. We provide additional evidence for this in the accompanying paper (12). In further experiments we sometimes detected a fifth *int*-1 protein species, with an apparent molecular weight of 44,000 M_r, which was also glycosylated (12). Naturally, it remains a possibility that there are additional forms of *int*-1 protein present at lower abundance than those we detected or which are not recognized by our antipeptide antibodies.

An additional protein species of approximately 55,000 M_r was precipitated from rat and mouse cell lysates by the monoclonal antibody against *int*-1 peptide A, and this rec-

ognition was largely inhibited by prior incubation of the antibody with peptide. The 55,000-M_r protein was not recognized by polyclonal antiserum against peptide B, however, and showed no similarity in its V8 protease degradation pattern when compared with the *int*-1-related proteins. Furthermore, the protein was detected in control NIH 3T3 cells which do not express *int*-1 RNA. We therefore conclude that the 55,000-M_r protein is not a product of *int*-1 and is not coprecipitated as a complex with *int*-1 protein; it probably bears an epitope fortuitously related to that recognized by the monoclonal antibody.

To identify the protein products of *int*-1, we made use of cell lines transfected or infected with retroviral vectors expressing high levels of *int*-1-specific RNA. Despite this, the less abundant species, p36 and p38, were not always detectable above background levels in our immunoprecipitations (Fig. 2C). In mammary tumors bearing mouse mammary tumor virus proviral insertions at the *int*-1 locus, *int*-1 mRNA is typically expressed at a level of only 1 to 10 copies per cell (10), at least 10-fold lower than the levels in the cell lines we used in this study. For this reason we did not attempt to characterize *int*-1 proteins in such tumors. Similarly, we did not examine tissues in which *int*-1 is normally expressed (postmeiotic cells of the testis and neural tissue of mid-gestational embryos) because of the difficulties in labeling such tissues or in obtaining sufficient quantities for analysis by Western blotting (immunoblotting). This raises the possibility that the protein species we detected in fibroblast cell lines represent nonfunctional forms that differ from those normally expressed in vivo or in tumors. However, we did detect at least the p40 species in MXIN-infected C57MG mammary epithelial cells, a line in which *int*-1 is known to be biologically active (2).

Of the four *int*-1 protein species that we detected in fibroblast cell lines expressing the gene, none comigrated in polyacrylamide gels with *int*-1 polypeptide synthesized in vitro. Moreover, the unglycosylated species from cell extracts, p36, migrated faster in the gels than the 37,000-M_r in vitro translation product. While there are several possible explanations of this discrepancy in apparent molecular weight, based on posttranslational modifications, the most likely explanation seems to be that p36 has undergone cleavage of an amino-terminal signal peptide. The first 48 amino acids in the predicted *int*-1 protein sequence are predominantly hydrophobic (4, 17), but there are polar amino acids at positions 29, 30, and 31 and a charged residue at position 33. Together these define the end of a shorter uninterrupted hydrophobic domain at the amino terminus. The amino acid sequence and characteristics of this domain are eminently compatible with the consensus rules for signal sequences governing membrane translocation (18–20), and there is a potential signal peptidase cleavage site after amino acid 27 (Ala-Leu-Ala ↓ Ala) at the end of the hydrophobic region. Von Heijne (20) has devised an algorithm to identify signal peptides and predict probable signal cleavage sites which has a predictive accuracy of at least 75%. In the case of *int*-1, the algorithm predicts a signal cleavage site between residues 27 and 28 with a score of 13.7 (R. Colgrove, personal communication). Since the scores calculated for known signal peptide cleavage sites have a 95% range from 4 to 14, with a modal score around 9 (20), this result indicates a very high probability that the amino-terminal domain of *int*-1 protein is indeed a signal peptide that undergoes cleavage at this site. Final proof of this, however, will require amino-terminal sequence analysis of the intracellular *int*-1 proteins.

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