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Inducible Overexpression and Secretion of *int*-1 Protein

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The *int*-1 proto-oncogene is a target for insertional activation of transcription by mouse mammary tumor virus in many murine mammary tumors. Whereas no expression of *int*-1 is seen in normal mammary tissue, *int*-1 RNA can be detected in normal mice in the neural tubes of midgestation embryos and in postmeiotic spermatocytes from adult testes. I report here the results of a study in which several different antibodies against synthetic peptides were produced and used to characterize the processing and secretion of *int*-1 protein. CHO cells were transfected with an inducible *int*-1 expression vector that was subsequently amplified to generate cell lines expressing very high levels of *int*-1 protein. Immunoprecipitation of [^{35}S]cysteine-labeled cell lysates from these CHO cells yielded large amounts of four immature forms of *int*-1 glycoprotein (molecular weights of 36,000, 38,000, 40,000, and 42,000). A significant fraction of these *int*-1 species formed disulfide-linked multimers. Pulse-chase and glycosidase digestion studies demonstrated that some of the immature species of *int*-1 protein move through the secretory pathway and are processed to a mature heterogeneous glycoprotein with a molecular weight of about 44,000. Suramin treatment of the CHO cells during pulse-chase experiments increased the amount of 44,000-molecular-weight *int*-1 protein in the culture medium.

The *int*-1 proto-oncogene is transcriptionally activated in mammary tumors by integration of mouse mammary tumor virus proviral DNA (17, 18), and its protein product is implicated in the formation of these tumors (4, 22, 26, 29). The *int*-1 gene is normally expressed only in round spermatids of adult mouse testes and in the developing central nervous systems of midgestation embryos (11, 25, 30). In addition to a role in murine embryogenesis, the *Drosophila* homolog, wingless, is a segment polarity gene that functions at various stages of development (1, 5, 21, 28).

Relatively low levels of the cysteine-rich protein product of *int*-1 were identified by immunoprecipitation of cell extracts from fibroblasts transfected with *int*-1 cDNA, using antisera directed against synthetic peptides (3). In these cells, the amino-terminal signal peptide of *int*-1 appears to be cleaved, and the protein acquires several N-linked carbohydrate units (3, 19). Biochemical analysis indicated that *int*-1 enters the secretory pathway and is not an integral membrane protein. However, no *int*-1 protein was detected by immunoprecipitation of the culture medium (19). The low abundance of *int*-1 protein together with inefficient secretion by these cell types could explain the lack of a detectable secreted *int*-1 product. Alternatively, a mature *int*-1 product may have not have been recognized by the antipeptide antibodies. Furthermore, a secreted *int*-1 protein might not be found in the culture fluid but instead associate with extracellular matrices or the cell surface.

Since current models for the function of *int*-1 in transformation and differentiation rest on the prediction that *int*-1 protein is secreted (3, 9, 19), it is critical to establish that the biochemical behavior of the *int*-1 protein is consistent with this model. To address these issues and to produce a source of glycosylated *int*-1 protein for purification and biological analysis, I have produced several different antibodies against *int*-1 synthetic peptides and used them to characterize *int*-1 proteins from transfected CHO cells that synthesize very high levels of *int*-1 RNA and protein from an amplified, inducible vector. This inducible expression system was developed by Wurm et al. (32) to produce as much as 1 mg of c-*myc* protein per 10^9 cells. The results presented below show that the abundant quantities of *int*-1 glycoprotein in CHO cells form multimers and move inefficiently through the secretory pathway. A portion of the *int*-1 protein precursors are terminally glycosylated, resulting in a secreted 44,000-molecular-weight (44K) protein. The amount of 44K protein in the culture medium is increased upon suramin treatment of the cells, which might indicate that secreted *int*-1 protein adheres to the cell surface or extracellular matrix.

MATERIALS AND METHODS

Peptides and antibodies. *int*-1 peptides JP1 (residues 104 to 117), JP2 (residues 256 to 271, with an additional amino-terminal cysteine), and JP3 (residues 299 to 314), predicted from the *int*-1 cDNA sequence (9), were synthesized and provided by the group of J. Nestor (Syntex Research). The peptides were coupled via a cysteine residue with *m*-maleimidbenzoyl *N*-hydroxysuccinimide ester to purified protein derivative (Staten Serum Institute) as described elsewhere (13) and injected into *Mycobacterium bovis* BCG vaccine (Glaxo Pharmaceuticals, Ltd.)-primed rabbits (Berkeley Antibody Co.). Positive sera were identified by immunoprecipitation of an *int*-1 in vitro translation product as described previously (3, 9).

Plasmids. Plasmid pCVSVEII-DHFR has been described previously (32) and was provided by R. Kingston (Harvard Medical School). Construction of pSp6HSintR was as follows. An *Eco*RI fragment containing the complete *int*-1 cDNA including 5′ and 3′ untranslated regions was cut out of the vector S621 (9) (provided by T. Brown and H. Varmus, University of California at San Francisco) and subcloned into the *Eco*RI site of pSP6-HS-9 (provided by R. Kingston), an expression vector that consists of a *Drosophila hsp70* promoter inserted into pSP65 (32).

Transfection of CHO cells and vector amplification. Dihydrofolate reductase (DHFR)-deficient CHO cells, CHO-DUKX BII (27), obtained from G. Ringold (Syntex Research), were cotransfected with a 10:1 mixture of pSp6HSintR and pCVSVEII-DHFR, using lipofection as described previously (8). Individual colonies that grew in DHFR selection medium were cloned, expanded into cell lines, and grown in stepwise fourfold-increasing concentra-

tions of methotrexate (MTX), starting with 0.005 μM as described previously (32). All transfected cells were maintained in alpha medium minus nucleosides, supplemented with 10% dialyzed fetal calf serum and with the appropriate MTX concentration. Cells were passaged under MTX selection for at least 4 weeks before analysis of *int*-1 levels.

Heat shock induction of recombinant CHO cell lines and [^{35}S]cysteine labeling. Dishes (60-mm diameter) of recombinant CHO cells at 70 to 90% confluence were induced by feeding with culture medium at 43°C and subsequently incubated in a tissue culture incubator at 42°C for 1.5 h. Dishes were returned to 37°C for 30 min, at which time the culture medium was replaced with 1.5 ml of label medium consisting of 250 to 500 μCi of [^{35}S]cysteine (Amersham Corp.) in modified Eagle medium lacking methionine and cysteine. For the experiments in which the medium was also harvested for immunoprecipitation, 1% fetal calf serum, 1% aprotinin (Sigma Chemical Co.), and, where indicated, 1 mM suramin (Mobay Chemical Co.) were included in the label medium. Optimal times for ^{35}S incorporation into *int*-1 protein were determined; unless indicated otherwise, incubation times were 2.5 h for harvest of cellular *int*-1 forms and 6.0 h for harvest of the secreted form. For the experiment shown in Fig. 2, a 60-mm-diameter dish of MV7*int*-1/3T3 cells (3) was labeled for 3 h without prior heat shock. Except for the experiment shown in Fig. 2, either the 0.05 μM or 1 μM MTX-amplified CHO cell lines (see Fig. 2) were used for the experiments shown.

Preparation and immunoprecipitation of cell extracts and media. After the indicated times, labeled medium was removed, and 1.0 ml of RIPA buffer (0.01 M sodium phosphate [pH 7.0], 0.15 M NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 1% aprotinin) was added directly to the cell monolayer. Dishes were incubated on ice for 20 min, and the cell extracts were clarified by centrifugation for 10 min in a microfuge. For immunoprecipitation, labeled medium was centrifuged in a microfuge to remove cellular debris, and equal portions of concentrated stocks of SDS, Nonidet P-40, and sodium deoxycholate were added to a final concentration equivalent to that of RIPA buffer. Antipeptide antibody (5 μl) was used to immunoprecipitate 250 μl of CHO cell extract. These conditions were determined to be in antibody excess. For the experiment shown in Fig. 2, the amount of lysate for each cell type used was adjusted to equalize the amount of total ^{35}S counts per minute used for immunoprecipitation while maintaining antibody excess conditions. One-half of the total medium harvested from one 60-mm-diameter dish was immunoprecipitated with 5 μl of antibody. Where indicated, the antiserum was preincubated with 1.5 μl of a 10-mg/ml stock of the appropriate peptide for blocking. Immunoprecipitation was performed as previously outlined (19), and each entire immunoprecipitate was solubilized in Laemmli sample buffer and loaded onto an SDS-15% polyacrylamide gel. Gels were fluorographed with Amplify (Amersham) as directed by the manufacturer.

Nonreducing-reducing gels. For the experiment shown in Fig. 3, a standard immunoprecipitate and a peptide-blocked immunoprecipitate of cellular *int*-1 proteins were boiled for 5 min in Laemmli sample buffer lacking β-mercaptoethanol (BME). The samples were divided in half and loaded onto two standard SDS-10% polyacrylamide minigels (CBS Scientific). One gel was fluorographed as usual, and the second gel was dried directly for preparative purposes. Next, gel slices corresponding to the three forms of *int*-1 protein (monomer, dimer, and trimer) were excised from the nonreducing preparative gel, soaked in Laemmli sample buffer containing BME for 5 min at 37°C, and boiled for 1 min. The gel slices were then placed into the wells of another SDS-10% polyacrylamide minigel and subjected to electrophoresis as usual. Amersham ^{14}C-labeled rainbow markers were used to assess approximate molecular weights.

V8 protease mapping. Standard immunoprecipitates of the cellular and secreted *int*-1 proteins were prepared and subjected to electrophoresis in a preparative SDS-10% polyacrylamide gel, which was dried directly upon completion. Individual slices of gel (1 by 3 mm) containing each of the four cellular *int*-1 forms and the larger portion of the secreted *int*-1 species were excised and used for proteolysis with 0.028 μg of *Staphylococcus aureus* V8 protease as described previously (6) and were analyzed on an SDS-15% polyacrylamide gel.

Glycosidase digestions. Standard immunoprecipitates of the cellular and secreted *int*-1 species were prepared, and the final washed immune complex pellets were treated with either endoglycosidase H (endo H; Boehringer Mannheim Biochemicals) or *N*-glycanase (*N*-Gly; Genzyme) exactly as described previously (19).

RESULTS

Strategy for high-level expression of *int*-1 protein. To generate cell lines that express very high levels of *int*-1 protein, I used the system described by Wurm et al. (32) and constructed the pSp6HSintR vector shown in Fig. 1A. This vector utilizes the heat-inducible *Drosophila hsp70* promoter to drive transcription of *int*-1 cDNA. pSp6HSintR was cotransfected with pCVSVEII-DHFR (Fig. 1B; 32), a vector that directs expression of DHFR cDNA, into CHO cells lacking the chromosomal gene for DHFR. Individual clones of cells that grew in DHFR selective medium were expanded into cell lines and screened for *int*-1 protein expression. One of these clones was selected for further study and stepwise amplification with MTX, an antagonist of DHFR. This protocol selects for amplification of the DHFR vector as well as cotransfected sequences such as pSp6HSintR.

Immunoprecipitation of *int*-1 proteins from transfected CHO cells with antibodies against synthetic *int*-1 peptides. To maximize detection of a secreted *int*-1 product, three different rabbit antisera directed against synthetic oligopeptides (residues 104 to 117, 256 to 271, and 299 to 314) predicted from the *int*-1 cDNA sequence (9) were produced. All three antipeptide antibodies recognized the same set of *int*-1 proteins, and the best antiserum, JP2, was used for the experiments described here. Four *int*-1 proteins with apparent molecular weights of 36,000, 38,000, 40,000, and 42,000 (36K, 38K, 40K, and 42K proteins) were immunoprecipitated from *int*-1 cDNA-transfected CHO cells (Fig. 2 and data not shown). Previous studies with rat embryo fibroblasts and NIH 3T3 cells transfected with *int*-1 cDNA (3, 19) indicated that the unglycosylated 36K species has probably undergone cleavage of an amino terminal signal peptide, whereas the 38K, 40K, and 42K forms are N-linked glycosylated derivatives.

Characterization of *int*-1 proteins in overexpressing CHO cells. Two pSp6HSintR-transfected CHO cell lines and five cell lines derived by sequential selection in stepwise increments of MTX were heat shocked at 42°C for 1.5 h, returned to 37°C for 30 min, and labeled for 2 h with [^{35}S]cysteine. The MV7*int*-1/3T3 cell line used for previous studies (3, 19)

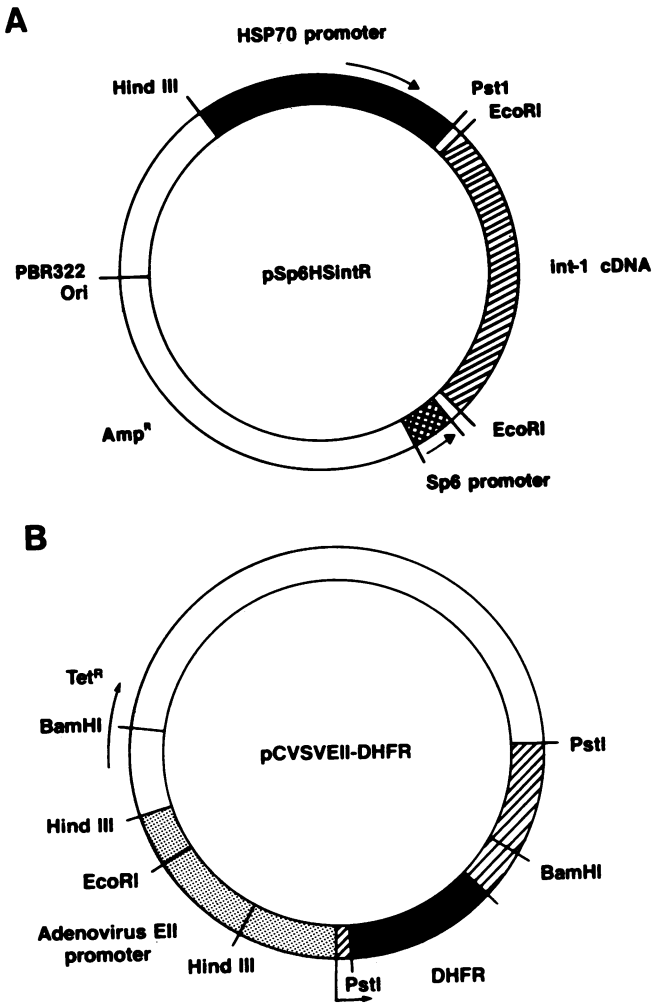

FIG. 1. Plasmids used to establish recombinant CHO cell lines. (A) pSp6HSintR. Positions and orientations of the *Drosophila hsp70* promoter (■), the *int*-1 cDNA insert containing the entire protein coding region as well as the *int*-1 polyadenylation signal (▨), and the Sp6 promoter located in an antisense orientation to the *int*-1 cDNA (▩) are indicated. (B) pCVSVEII-DHFR (32). Positions and orientations of the adenovirus EII promoter (▩), the *dhfr* coding region (■), and processing signals (▨) are indicated, as are positions of genes encoding antibiotic resistance and the location of selected restriction sites.

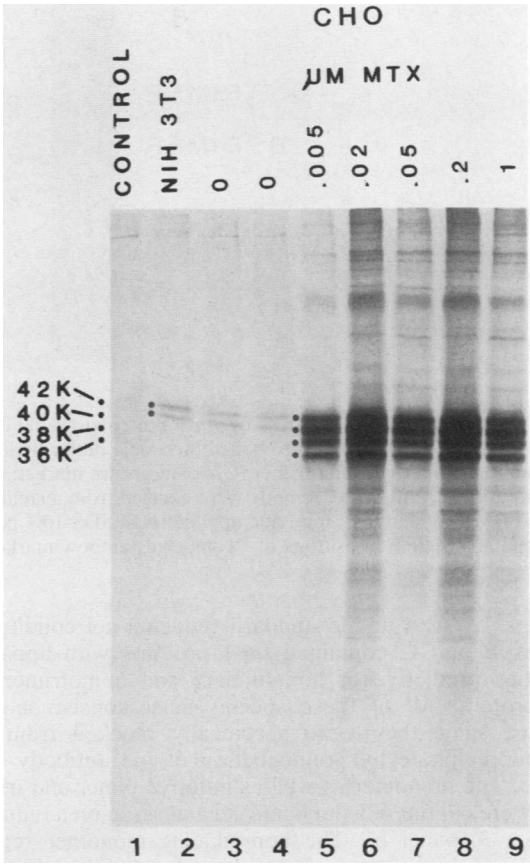

FIG. 2. Comparison of *int*-1 protein levels in transfected and MTX-amplified CHO cell lines. Two pSp6HSintR-transfected CHO cell lines, five cell lines derived by sequential selection in stepwise increments of MTX, and a control pCVSVEII-DHFR CHO transfectant were heat induced and labeled with [^{35}S]cysteine. The MV7*int*-1/3T3 cell line was labeled with [^{35}S]cysteine in parallel. Samples of each cell extract containing equivalent amounts of ^{35}S counts per minute were immunoprecipitated with 5 μl of JP2 antiserum. The final washed immunoprecipitates were analyzed under reducing conditions on an SDS-15% polyacrylamide gel. Lanes: 1, control pCVSVEII-DHFR-transfected CHO cells; 2, MV7*int*-1/3T3 cells; 3 and 4, pSp6HSintR-transfected but unamplified CHO cell lines; 5 to 9, MTX-amplified CHO cell lines grown in 0.005, 0.02, 0.05, 0.2, 1.0 μM MTX, respectively.

was labeled in parallel. Cell extracts were prepared, and equivalent amounts of ^{35}S-labeled protein from each cell line were immunoprecipitated with the JP2 antibody. Immunoprecipitates from the pSp6HSintR-transfected but unamplified CHO cell lines (Fig. 2, lanes 3 and 4) contained low levels of *int*-1 protein, similar to findings for the MV7*int*-1/3T3 cell line (Fig. 2, lane 2). No *int*-1 protein was immunoprecipitated from any of the CHO cell lines without heat induction (data not shown) or from CHO cells transfected with pCVSVEII-DHFR alone (Fig. 2, lane 1). In contrast, immunoprecipitates from the MTX-selected lines contained markedly increased quantities of the four *int*-1 species (Fig. 2, lanes 5 to 9). Densitometric tracing of the data from Fig. 2 demonstrated an approximately 300-fold increase in *int*-1 protein expression in these cells after MTX selection. The elevated *int*-1 protein levels likely resulted from increased pSp6HSintR copy number. Similarly elevated levels of *int*-1 RNA were found in the MTX-selected cells after heat induction (data not shown). It is of note that the four *int*-1 species in the amplified CHO cells were present in equimolar amounts after a 2-h label, whereas in the unamplified CHO lines as well as in the MV7*int*-1/3T3 cells used for previous studies, the 40K and 42K species were the most abundant and the 36K and 38K species were minor components of the *int*-1 immunoprecipitate (Fig. 2; 19). This observation suggests that in cells with high levels of *int*-1, the protein is processed more slowly or is ultimately less extensively glycosylated than in cells that express low levels of *int*-1.

Formation of multimers by *int*-1 protein. To test the possibility that *int*-1 forms disulfide-linked multimers, *int*-1 proteins were examined under nonreducing conditions. Immunoprecipitates were prepared from [^{35}S]cysteine-labeled CHO lysates either without or with blocking peptide and analyzed on a nonreducing gel (Fig. 3, panel a). Under these conditions, three forms of *int*-1 were identified. Region A contained the 36K, 38K, 40K, and 42K monomeric *int*-1

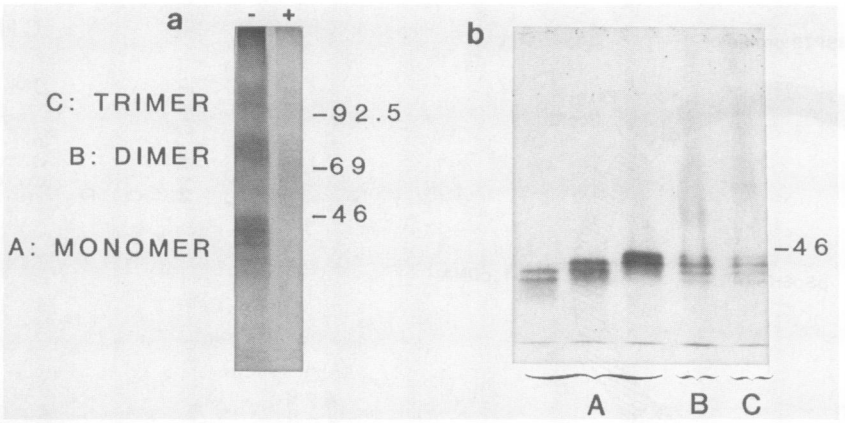

FIG. 3. Formation of multimers by *int*-1 proteins. (Panel a) Standard immunoprecipitates of cellular *int*-1 proteins made with (+) and without (−) blocking peptide were boiled in sample buffer with SDS but without BME and run on an SDS-10% polyacrylamide minigel. *int*-1 monomer (A), dimer (B), and trimer (C) positions are marked. (Panel b) Results of an experiment in which gel pieces corresponding to *int*-1 monomer, dimer, and trimer regions were excised from a similar nonreducing gel. The pieces were soaked in sample buffer containing both SDS and BME, boiled for 1 min, and applied to an SDS-10% polyacrylamide minigel. Lanes were derived from the monomer (A), dimer (B), and trimer (C) regions. Positions of ^{14}C-labeled rainbow markers are indicated (in kilodaltons) to the right of each panel.

products, as seen under standard reducing gel conditions. Regions B and C contained *int*-1 proteins with apparent mobilities predictive of homodimers and homotrimers of *int*-1 protein. All of these species must consist of *int*-1 proteins, since they were specifically blocked from the immunoprecipitate by preincubation of the antibody with peptide. The monomer as well as putative dimer and trimer forms were cut out of a duplicate gel and rerun on a reducing gel (Fig. 3, panel b). The nonreducing monomer region, divided into three portions for technical reasons, yielded the expected 36K, 38K, 40K, and 42K monomer bands. Similarly, the dimer and trimer regions yielded all four monomer forms of *int*-1 upon reduction. These data suggested that approximately 30 to 50% of the *int*-1 species in these CHO cell lines formed disulfide-linked homodimers and homotrimers.

Detection of a glycosylated form of *int*-1 protein in the culture medium. As a first attempt to detect a secreted *int*-1 product from the MTX-amplified CHO cells, one dish of these cells was labeled for 6 h with [^{35}S]cysteine, and both the cells and medium were immunoprecipitated with the JP2 antibody (Fig. 4, lanes A) as well as with JP2 antibody preabsorbed with JP2 peptide (Fig. 4, lanes B). Immunoprecipitates from the cells consisted of the 36K, 38K, 40K, and 42K proteins, with the 40K and 42K products in greater relative abundance. A diffuse protein of approximately 44 to 46K (hereafter referred to as 44K) was immunoprecipitated from the medium (Fig. 4), and it was specifically blocked from the immunoprecipitate with *int*-1 peptide. The quantities of 44K immunoprecipitated from the medium were quite small relative to the levels of intracellular *int*-1 precursors (Fig. 4; compare the levels of four cellular species after a 16-h exposure of gel with the level of the 44K protein from medium after 4 days of exposure). Attempts to increase the level of secretion of the 44K protein by treating the cells with various hormones and secretagogs were unsuccessful (data not shown).

Suramin treatment increases the level of *int*-1 protein detected in the culture medium. CHO cells expressing *int*-1 were treated with suramin, a polyanionic compound that has been used to prevent platelet-derived growth factor and other growth factors from binding to cell surface receptors (2, 31) and to block in vitro infection by duck hepatitis B virus, Rous sarcoma virus, and hepatitis delta virus (20). Considering these previous results, it seemed possible that suramin might be sufficiently nonspecific in its effects to prevent *int*-1 protein from adhering to the CHO cell surface or extracellular matrix. Figure 5 shows a pulse-chase experiment in which the MTX-amplified CHO cells were pulse-labeled for 40 min with [^{35}S]cysteine, followed by incubation in the absence of label (chase) for 4 or 20 h with or without 1 mM suramin in the chase medium. All four species of *int*-1 protein were immunoprecipitated from the CHO cells after a

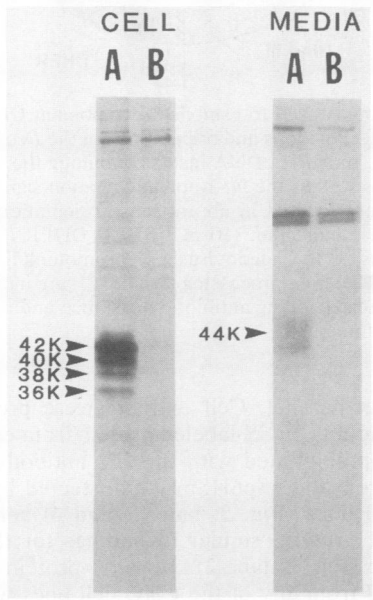

FIG. 4. Immunoprecipitation of a 44K protein from the medium of transfected CHO cells with an antiserum directed against *int*-1. One dish of pSp6HSintR-transfected, MTX-amplified CHO cells was labeled with [^{35}S]cysteine for 6 h, and both cell extract and medium were immunoprecipitated with the JP2 antiserum without (lanes A) and with (lanes B) an appropriate peptide block. Each entire immunoprecipitate was run under reducing conditions on an SDS-15% polyacrylamide gel. The immunoprecipitates of cell extract were exposed to film for 16 h, and the immunoprecipitates of medium were exposed to film for 4 days.

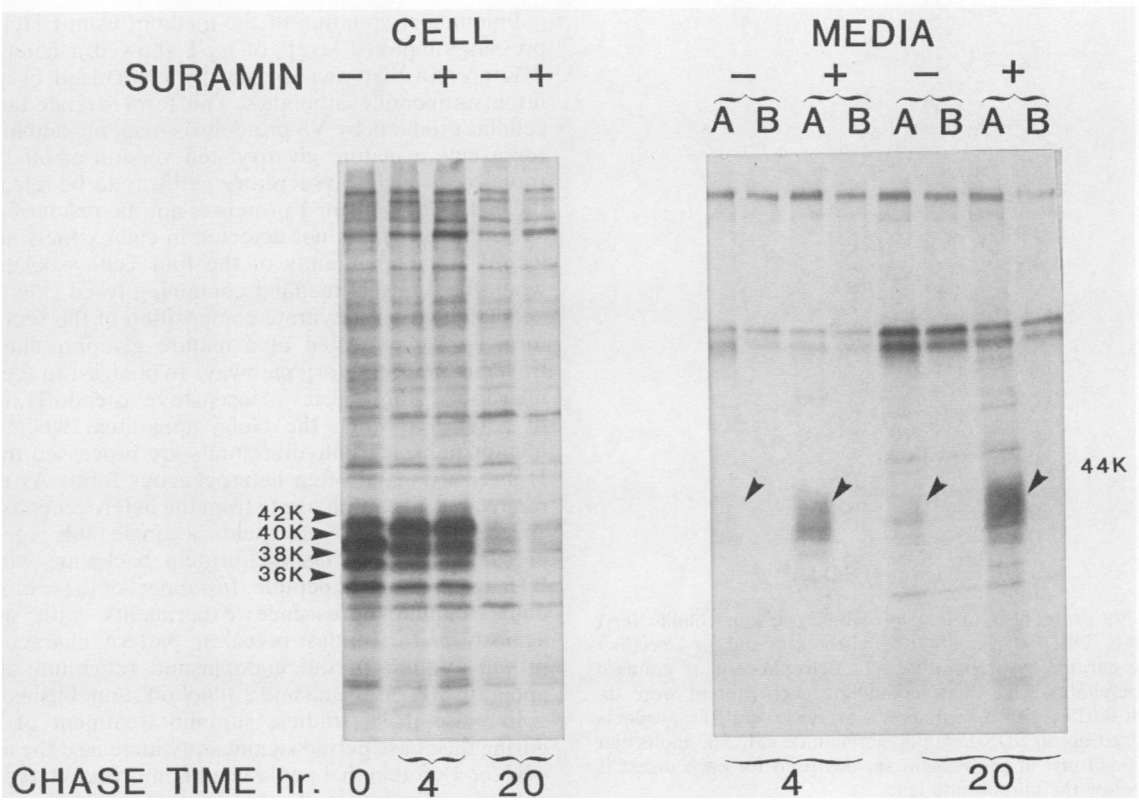

FIG. 5. Pulse-chase analysis of *int*-1 proteins: effect of suramin on the secreted 44K *int*-1 protein. Duplicate dishes of pSp6HSintR-transfected, MTX-amplified CHO cells were pulse labeled for 40 min with [^{35}S]cysteine and subsequently incubated in the absence of label (chase) for either 4 or 20 h. Both cell extracts and media were immunoprecipitated with the JP2 antibody. Medium samples were immunoprecipitated without (lanes A) and with (lanes B) an appropriate peptide block. +, Immunoprecipitates prepared from suramin-treated extracts or media; −, immunoprecipitates prepared from untreated cells or media. Chase time before sample preparation is indicated at the bottom. Each entire immunoprecipitate was solubilized in sample buffer and run under reducing conditions on an SDS-15% polyacrylamide gel.

40-min pulse. The 36K, 38K, and 42K forms were present in equimolar amounts, whereas the 40K form was slightly more abundant. After a 4-h chase, there was some conversion of the 36K, 38K, and 40K forms into the 40K and 42K forms and a slight decrease in the total amount of immunoprecipitable *int*-1 proteins. After a 20-h chase, only a small amount of the 40K and 42K proteins was detected in the cells. The processing and turnover of the cellular *int*-1 proteins during the chase were unaffected by suramin treatment. On the other hand, the 44K species was immunoprecipitated from the medium of these same cells treated with suramin in much greater amounts than was immunoprecipitated from an equivalent volume of medium from the untreated cells. The 44K protein from suramin-treated cells was used for further characterization. This protein was immunoprecipitated with all three antipeptide antibodies, and in all cases the precipitation was blocked by preabsorbing the antiserum with an appropriate peptide (Fig. 4 and 5; data not shown).

Relationship of the 44K secreted protein to the four cellular *int*-1 proteins. To prove that the secreted 44K protein is an *int*-1 product, samples of medium and cells were immunoprecipitated with the JP2 antibody, and V8 proteolysis maps were prepared from the 36K, 38K, 40K, 42K, and 44K species. The V8 proteolysis products from the 44K secreted protein were in common with those from the four cellular forms (Fig. 6), indicating that the 44K species is a form of *int*-1 protein.

The 44K secreted protein is a mature glycosylated form of *int*-1. To test the prediction that the secreted 44K *int*-1 protein is a product of terminal glycosylation of the cellular *int*-1 species, immunoprecipitates of the four cellular *int*-1 forms and of the 44K secreted *int*-1 species were digested with either endo H or *N*-gly. First, digestion of the secreted 44K product with *N*-gly, an enzyme that removes all N-linked carbohydrate, eliminated the 44K form (Fig. 7, lane 5) and yielded a 36K product (Fig. 7, lane 6). *N*-gly treatment of a peptide-blocked immunoprecipitate made from the same culture medium did not produce the 36K species, indicating that this product was derived from *int*-1 and not a background band in the immunoprecipitate (data not shown). *N*-gly treatment of a pool of the four cellular *int*-1 proteins yielded a similar 36K protein (Fig. 7, lanes 1 and 2; 19). Previous results (3, 19) indicated that the 36K *int*-1 form from cells lacks both carbohydrate and probably the NH_2-terminal signal sequence.

In contrast to the results with *N*-gly, endo H, an enzyme that removes only immature high-mannose carbohydrate from proteins, did not cleave the carbohydrate from the 44K secreted *int*-1 form (Fig. 7, lane 7), whereas most of the carbohydrate was removed from the 38K, 40K, and 42K cellular species (Fig. 7, lanes 3 and 4; 19). These results indicated that the carbohydrate moieties of the cellular *int*-1 proteins were immature, whereas the carbohydrates of the 44K form had been processed to a mature endo H-resistant form.

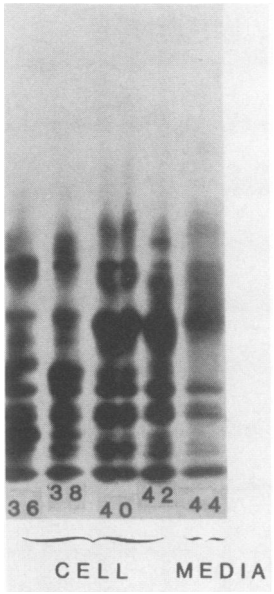

FIG. 6. V8 proteolysis of *int*-1 proteins. The four cellular *int*-1 proteins (36K, 38K, 40K, and 42K) and the secreted *int*-1 product (44K) were purified by preparative gel electrophoresis of standard immunoprecipitates. Gel slices containing each protein were digested with 0.028 μg of *S. aureus* V8 protease, and the products were analyzed on an SDS-15% polyacrylamide gel. The molecular size (in kilodaltons) of the protein species used for each digest is indicated below the appropriate lane.

DISCUSSION

I have used a heat-inducible *int*-1 expression vector, transfected into CHO cells and coamplified with a DHFR expression vector, to produce high levels of *int*-1 protein for studies on *int*-1 processing and secretion and, ultimately, for *int*-1 protein purification. This system yields approximately 300-fold more immunoprecipitable *int*-1 protein than do *int*-1 vector-transfected but unamplified CHO cells or other *int*-1 cDNA-transfected cells that have been studied (3, 19). The level of *int*-1 protein comprises approximately 0.5% of total cell protein in the highest-expressing CHO cells, equivalent to about 1.25 mg/10^9 cells. A mammalian expression system was chosen for *int*-1 because it was expected that this would produce the most accurate posttranslational processing of the *int*-1 glycoprotein. CHO cells were selected since they have been used to secrete efficiently high levels of other proteins such as transforming growth factor beta (10) and tissue-type plasminogen activator (12).

Similar to previous results with NIH 3T3 cells (3, 19), immunoprecipitates from CHO cells overexpressing *int*-1 protein consist of a 36K unglycosylated form and three species of 38K, 40K, and 42K, each of which has N-linked carbohydrate. All four *int*-1 species in CHO cells form disulfide-linked homodimers and homotrimers. The formation of disulfide-linked multimers is not unexpected for a secreted protein such as *int*-1 with 23 cysteine residues (9). Other growth factors such as v-*sis* (23) and members of the transforming growth factor beta family (14) are isolated as dimers. On the other hand, I cannot rule out the possibility that *int*-1 protein normally forms disulfide bonds with a second protein which is not synthesized by CHO cells or that the *int*-1 homomultimers identified in CHO cell lysates are a consequence of overexpression rather than a native configuration.

Immunoprecipitation of the medium from CHO cells expressing amplified levels of *int*-1 showed a heterogeneous 44K protein that was specifically recognized by three different antipeptide antibodies. This form is related to the four cellular products by V8 proteolysis mapping and most likely represents a mature glycosylated version of *int*-1 that has traveled through the secretory pathway to be released from the cells. The 44K *int*-1 protein is not the result of cell lysis, since this protein is not detected in cell extracts and significantly greater amounts of the four cell-associated forms would be found in medium containing lysed cells (data not shown). The carbohydrate composition of the secreted 44K form is that expected of a mature glycoprotein that has traversed the secretory pathway. In contrast to the intracellular forms, this species is insensitive to endo H, indicative of passage through the Golgi apparatus, where primary high-mannose carbohydrate units are processed to an endo H-insensitive and often heterogeneous form. As expected, removal of all carbohydrate from the heterogeneous secreted species by using *N*-gly yields a single 36K species that presumably represents the protein backbone without the NH_2-terminal signal peptide. In support of these biochemical data, immunofluorescence experiments with antibodies against *int*-1 peptides reveal a pattern characteristic of proteins found in the endoplasmic reticulum and Golgi apparatus (R. Medema and J. Papkoff, unpublished results).

In pulse-chase studies, suramin treatment of the cells during the chase period significantly increased the amount of 44K *int*-1 protein that could be immunoprecipitated from the culture medium. On the basis of experiments with other systems (2, 20, 31), this result most likely implies that much

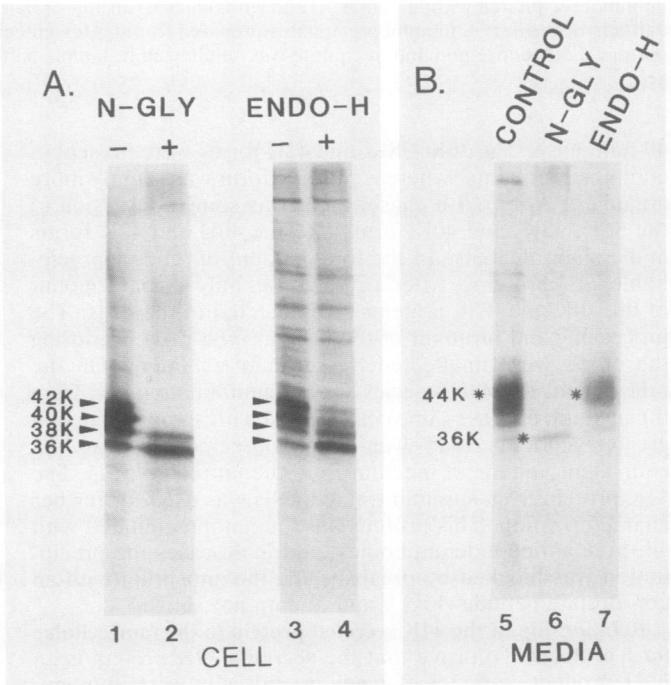

FIG. 7. Comparison of cellular (A) and secreted (B) *int*-1 proteins by glycosidase digestion. Immunoprecipitates of *int*-1 proteins prepared from either ^{35}S-labeled cell extracts or media from pSp6HSintR-transfected, MTX-amplified CHO cells were digested with either endo H (lanes 4 and 7) or *N*-gly (lanes 2 and 6). Each entire digestion product was analyzed under reducing conditions on an SDS-15% polyacrylamide gel. Undigested control samples are shown in lanes 1, 3, and 5.

of the secreted *int*-1 protein is adherent to the cell surface or extracellular matrix and that suramin inhibits this binding. However, it is possible that suramin increases the level of secreted *int*-1 protein by interfering with a normal mechanism that retains *int*-1 in the secretory pathway. It is unclear whether the presumptive association with cell surface or extracellular matrix is a functional characteristic of *int*-1 protein or a nonspecific consequence of the in vitro expression system. Unlike the fibroblast growth factor family, *int*-1 protein does not bind to heparin, an extracellular matrix constituent (J. Papkoff, unpublished results).

Although *int*-1 protein is capable of traversing the secretory pathway to be released from the cell, it is evident from pulse-chase experiments that the majority of cellular *int*-1 precursors turn over slowly and are not terminally processed and secreted. Only approximately 10% of the total *int*-1 protein can be immunoprecipitated from the culture medium. This result is consistent with those of experiments using several other *int*-1 cDNA-transfected cell types in which low levels of the four intracellular int-1 proteins are immunoprecipitated but no secreted *int*-1 protein can be identified (3, 19; B. Schryver and J. Papkoff, unpublished results).

The inefficient secretion of *int*-1 by CHO cells cannot be a consequence of the heat shock protocol, since similar results were obtained when these cells were transfected with a constitutive *int*-1 expression vector and then amplified (data not shown). It is conceivable that *int*-1 protein may not form homodimers or homotrimers when synthesized in the neural tube or spermatocytes but instead participate in a heteromultimeric complex with another protein which is not synthesized in CHO cells. If this was the case, one might expect that *int*-1 protein lacking another subunit would be inefficiently secreted by CHO cells. This situation occurs when the cDNAs for the glycoprotein hormone lutropin (LH) alpha and beta subunits are transfected into c127 cells. When expressed individually, the LH alpha subunit is rapidly and quantitatively secreted, whereas 90% of the LH beta subunit remains in an endo H-sensitive form inside the cell and only 10% traverses the secretory pathway to be released into the medium. Coexpression of LH alpha and beta results in efficient secretion of an endo H-resistant alpha-beta dimer (7).

The studies with *int*-1-overexpressing CHO cells used in this study suggest two possible fates for *int*-1 protein upon entry into the secretory pathway. Both proposed models are consistent with the function of the *Drosophila int*-1 gene, wingless, which is not cell autonomous (15). This indicates that the wingless protein either directly or indirectly influences surrounding cells.

In the first case, *int*-1 glycoprotein would remain associated with membranes of the secretory apparatus, perhaps by virtue of a lipid modification or association with another protein. Under these circumstances, *int*-1 could stimulate secretion of another factor with morphogenetic or growth factor properties. Examples of resident endoplasmic reticulum and Golgi proteins have been identified (24). However, *int*-1 protein lacks the consensus sequence KDEL, which is thought to be responsible for the localization of some of these proteins (16). On the other hand, a fraction of *int*-1 protein could be available on the cell surface to modulate cell-cell interactions. If *int*-1 protein is not normally secreted, then the inefficient release of this protein from overexpressing CHO cells could be a consequence of saturation of a specific retention mechanism.

The alternative model is that *int*-1 protein is normally secreted inefficiently and an extracellular form can be identified only by using high-level expression systems as described here. This would explain the observed lack of secretion from other cell lines studied which express small quantities of intracellular *int*-1 protein, rendering a secreted form undetectable by our methods (3, 19; B. Schryver and J. Papkoff, unpublished results). An inefficient secretion mechanism could serve to regulate the availability of *int*-1 protein to interact with surrounding cells. Both models outlined here predict that at least one other protein interacts with *int*-1 to regulate retention. Proteins that are coimmunoprecipitated with *int*-1 could participate in this retention mechanism and are now under investigation.

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