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Secreted *int-1* Protein Is Associated with the Cell Surface

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The murine *int-1* proto-oncogene has been implicated in neural development and, when transcriptionally activated by mouse mammary tumor virus, contributes to the genesis of mammary tumors. To understand the function of the *int-1* gene product in these processes, we have characterized the biochemical properties of *int-1* protein expressed in a neuroendocrine cell line transfected with *int-1* cDNA. Here we provide evidence that *int-1* protein is secreted and associates with the cell surface. *int-1* protein was very efficiently processed and secreted through the constitutive secretory pathway, although no *int-1* protein could be immunoprecipitated from the culture medium. Treatment with suramin effectively released mature *int-1* proteins into the culture fluid, which suggests that secreted *int-1* protein associates with the cell surface or extracellular matrix. We have also shown directly, by radioiodination of intact cells and by surface antibody adsorption, that secreted *int-1* proteins can be detected on the cell surface. These data support a model in which *int-1* protein is secreted and functions locally in cell-to-cell signaling.

The *int-1* proto-oncogene is activated for transcription in murine mammary tumors by insertion of proviral DNA (18, 19). Direct evidence that *int-1* activation contributes to the development of mammary neoplasia comes from the observation that expression of an exogenous *int-1* gene in mammary epithelial cell lines leads to a transformed phenotype (4, 24). This conclusion is supported by studies in which mice expressing an activated *int-1* transgene develop mammary hyperplasia and after a period of latency develop mammary tumors (32).

Aside from its role in mammary tumorigenesis, a function for *int-1* during embryogenesis and differentiation has been described for several different organisms. The murine *int-1* gene is expressed in cells of the developing embryonic neural tube and in postmeiotic cells of mature adult testis (6, 11, 28, 35). Transcription of the murine *int-1* gene is also activated when the P19 embryonal carcinoma cell line is stimulated by retinoic acid to differentiate along the neural pathway (26, 31). In *Xenopus* embryos, *int-1* transcription is detected during development of the nervous system (17), and when *int-1* RNA is injected into fertilized oocytes, neural development is perturbed (14). The *Drosophila* homolog of *int-1*, the segment polarity gene wingless, functions at various stages of development and the mutant phenotype is nonautonomous in mosaics, which suggests that the wingless protein functions in cell-to-cell signaling (1, 5, 16, 23, 33). Recently, wingless antigen has been identified both inside and outside of cells in *Drosophila* embryos by immunohistochemical localization (34).

The cysteine-rich glycoprotein product of the murine *int-1* gene was identified in fibroblasts transfected with *int-1* expression vectors (3). Immunoprecipitation with antisera directed against synthetic peptides revealed a primary translation product of 36 kilodaltons (36K protein) which was modified by N-linked glycosylation to 38K, 40K, and 42K species (21). In these cells, *int-1* protein appeared to enter the secretory pathway, yet no *int-1* product could be detected in the culture medium (21). Overexpression of *int-1* protein in CHO cells demonstrated that the 36K, 38K, 40K, and 42K proteins formed disulfide-linked multimers. A small percentage of these precursor forms were inefficiently processed to a mature glycoprotein doublet of about 44 kilodaltons (20) that could be detected in the culture fluid from these cells. Suramin treatment of the CHO cells increased the amount of 44K protein in the culture medium, implying that secreted *int-1* adheres to the cell surface or extracellular matrix (20).

We have extended the analysis of *int-1* protein processing and secretion and show directly that secreted *int-1* protein is associated with the cell surface. For these studies, we used the pituitary tumor cell line AtT-20, which possesses a constitutive secretory pathway, common to all cells, as well as a specialized regulated secretory pathway in which proteins are stored in vesicles until release is triggered by a secretagogue (10, 12). We demonstrate, using AtT-20 cells transfected with an *int-1* expression vector, that *int-1* protein is very efficiently processed through the constitutive secretory pathway. However, the cells will release mature *int-1* protein into the culture medium only when treated with suramin. Furthermore, we have shown directly by radioiodination of intact cells and by surface antibody adsorption that secreted *int-1* protein is associated with the cell surface.

MATERIALS AND METHODS

Antibodies. The polyclonal antiserum JP2, directed against *int-1* peptide residues 256 to 271, has been previously described (20). The polyclonal antiserum JP4, directed against *int-1* peptide residues 275 to 289, was prepared by using the same protocol as for the JP2 antiserum. The JP4 antiserum is essentially the same as polyclonal B (3) except that the JP4 peptide was coupled to purified protein derivative (Staten Serum Institute) instead of keyhole limpet hemocyanin. The monoclonal antibody directed against the v-Ha-*ras* protein, Y13-259, was purchased from Oncogene Science, Inc., and suspended in 1 ml of phosphate-buffered saline at a concentration of 0.2 μg/μl. Goat anti-rat immunoglobulin G (IgG) was purchased from Organon Teknika and suspended in phosphate-buffered saline at a concentration of 1 mg/ml.

Vectors. The pSV2neo expression vector has been described elsewhere (29). The pRSVint-1 expression vector was derived from pRSVCAT (9) essentially by replacement of the chloramphenicol acetyltransferase gene sequences

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with the complete *int-1* cDNA, including 5′ and 3′ untranslated sequences, subcloned from the S621 vector (8). Transcription of the *int-1* cDNA from the pRSVint-1 vector is initiated at the promoter within the Rous sarcoma virus long terminal repeat and is terminated within simian virus 40 sequences 3′ to the *int-1* cDNA.

Cell lines. AtT-20 cells (provided by Regis Kelley, University of California, San Francisco) were cotransfected with the pRSVint-1 and pSV2neo vectors by using lipofection as described previously (7). Transfected cell clones were selected in growth medium with 125 μg of geneticin (G418; GIBCO Laboratories) per ml. Clones expressing *int-1* were screened by Northern (RNA) blotting using an *int-1* probe and by immunoprecipitation with antisera directed against *int-1* peptides. One clone of *int-1*-transfected cells, A37, was selected for the experiments shown. The 7dT cell line, transfected with both *int-1* and v-Ha-*ras* cDNAs, has been described previously (3).

Pulse-chase labeling, immunoprecipitation, and gel electrophoresis. For the pulse-chase experiments shown in Fig. 1A and B and Fig. 2, duplicate 60-mm-diameter dishes of A37 cells were incubated for 20 min at 37°C in 2 ml of Dulbecco modified Eagle medium (DMEM) lacking cysteine and methionine (label medium). The dishes were then incubated for 45 min (pulse) with 1.5 ml of label medium containing 0.35 mCi of [^{35}S]cysteine (Amersham Corp.). At the end of the pulse-label, the dishes were washed with 2 ml of DMEM containing additional cysteine and methionine at 0.3 mM (chase medium) and subsequently incubated for the indicated time periods (chase) with 1.5 ml of chase medium. The experiment shown in Fig. 1C and D was performed exactly as described for Fig. 1A and B except that the pulse-label time was changed to 30 min and 1 mM suramin (Mobay Corp.) was included in the chase medium. For the experiment shown in Fig. 2, a final concentration of 1 mM suramin and, where indicated, a final concentration of 5 mM 8-bromo-cyclic AMP (8Br-cAMP) (Sigma Chemical Co.) was included in the chase medium. At the indicated times, the chase medium was removed and the cell monolayer was extracted with 1 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) for 30 min at 4°C. The chase media and cell extracts were centrifuged in a microfuge for 15 min at 4°C and immunoprecipitated with either the JP2 or the JP4 antiserum (2 μl/300 μl of clarified cell extract or 3 μl/450 μl of chase medium). After 2 h on ice, 1 mg of washed protein A-positive *Staphylococcus aureus* cells (protein A) (Boehringer Mannheim Biochemicals) was added to each sample. The protein A-antibody complexes were pelleted through 300 μl of RIPA buffer plus 10% sucrose in a microfuge and washed once with 500 μl of RIPA buffer. The washed pellets were boiled for 5 min in 1× Laemmli sample buffer and analyzed on an SDS–15% polyacrylamide gel that was fluorographed with Amplify as directed by the manufacturer (Amersham).

Cell surface radioiodination and immunoprecipitation. Dishes (100-mm diameter) of either A37 or 7dT cells at 80% confluence were washed three times with 12 ml of Earle buffered saline solution, pH 7.0 (EBS; GIBCO), after which were added, in order, 1 ml of EBS, 2.5 mCi of Na^{125}I (Amersham) in 35 μl, 125 μl of Enzymobead reagent (Bio-Rad Laboratories), and 120 μl of 1% D-glucose. The iodination reaction was conducted at room temperature for 15 min, the reaction components were removed, and the culture was washed three times with a quenching medium of EBS containing 1 mM NaI and 600 μM L-tyrosine. The iodinated cells were extracted with 3 ml of RIPA buffer and clarified as described above. For Fig. 3A, iodinated A37 cell extract (350 μl) was immunoprecipitated with either 3 μl of JP4 antiserum or 3 μl of JP4 antiserum preabsorbed with 1 μl of 10 mg of JP4 peptide per ml. For Fig. 3B, iodinated 7dt cell extract (400 μl) was immunoprecipitated with 3 μl of JP4 antiserum, 3 μl of preimmune serum, or 5 μl of Y13-259 antibody plus 2 μl of goat anti-rat IgG. Included as a size reference for *int-1* protein in Fig. 3A were immunoprecipitates, made with JP4 antiserum, from extracts of A37 cells labeled with [^{35}S]cysteine for 45 min (lane 1) or pulse-labeled for 45 min, followed by a 3.5-h chase (lane 2). Included for comparison in Fig. 3B were immunoprecipitates of cell extract (lane 1) and chase medium (lane 2) from 7dT cells pulse-labeled with [^{35}S]cysteine for 1 h, followed by a 2-h chase in the presence of suramin. Lane 5 shows an immunoprecipitate with the Y13-259 antibody from an extract of 7dT cells labeled for 45 min with [^{35}S]cysteine (0.3 mCi) plus [^{35}S]methionine (0.15 mCi). The immunoprecipitates shown in Fig. 3A and B were analyzed on an SDS–12.5% polyacrylamide gel and fluorographed as described.

Cell surface adsorption of antibody. For Fig. 4A, 60-mm-diameter dishes of A37 cells were labeled with 0.3 mCi of [^{35}S]cysteine in 1.5 ml of label medium for a total of 3 h. After 2 h of labeling, 167 μl of a 10-mg/ml concentration of bovine serum albumin (fraction V; Boehringer) was added to each dish, followed by the addition of either 15 μl of JP4 antiserum or 15 μl of preimmune serum after 2.5 h of label. The surface adsorption was concluded by washing the dishes four times with 5 ml of DMEM containing 0.1% bovine serum albumin, followed by extraction with 1 ml of RIPA buffer for 15 min on ice. The extract was clarified, and 1 mg of protein A was added. After 30 min at 4°C, the immune complexes were pelleted and washed as described above. For comparison (Fig. 4A, lane 1), a standard immunoprecipitate using 15 μl of JP4 antiserum was prepared from a 1-ml RIPA extract of A37 cells labeled for 3 h with [^{35}S]cysteine. For lane 1, 15% of the total immunoprecipitate and, for lanes 2 and 3, 40% of the total immunoprecipitate were analyzed on an SDS–12.5% polyacrylamide gel with fluorography.

With the 7dT cell line, the method used for surface adsorption of A37 cells was repeated exactly as described above except that the cells were pulse-labeled for 30 min with 1.5 ml of label medium containing 0.3 mCi of [^{35}S]cysteine plus 0.15 mCi of [^{35}S]methionine, followed by a chase for 3 h, during which time the bovine serum albumin and antisera were added as described above. In addition to use of the JP4 and preimmune sera, a surface adsorption was performed with 10 μl of Y13-259 antibody plus 4 μl of goat anti-rat IgG. For comparison (Fig. 4B, lanes 1 and 4), standard immunoprecipitates using either 15 μl of JP4 antiserum or 10 μl of Y13-259 serum plus 4 μl of goat anti-rat IgG were prepared from 1-ml RIPA extracts of 7dT cells pulse-chase labeled in parallel with the surface adsorption. For lanes 1 and 4, 15% of the total immunoprecipitate and, for lanes 2, 3, and 5, 40% of the total immunoprecipitate were analyzed on an SDS–12.5% polyacrylamide gel with fluorography.

For the experiment shown in Fig. 5, two dishes of A37 cells were pulse-labeled for 30 min with [^{35}S]cysteine and then chased for a total of 2.5 h. Suramin was included at 1 mM in the chase medium of one dish. A surface adsorption with JP4 antiserum was performed with both dishes as described above. At the conclusion of the surface adsorption, the label medium containing JP4 antiserum was removed and protein A was added to precipitate *int-1* protein

as described above. The RIPA extracts were processed as described above. Forty percent of each immunoprecipitate was analyzed on an SDS–12.5% polyacrylamide gel with fluorography.

RESULTS

AtT-20 cells efficiently process and secrete *int-1* protein. To generate AtT-20 cells that express *int-1* protein, the pRSV int-1 and pSV2neo expression vectors were cotransfected into this cell line, and individual G418-resistant colonies were screened for expression of *int-1* RNA and protein. One of these clones, A37, was chosen for the studies shown.

Figure 1 shows a pulse-chase experiment in which A37 cells were pulse-labeled for 45 min with [^{35}S]cysteine, followed by a chase, in the absence of label for 0.5, 2.5, 6, or 20 h. Both cell extracts and medium samples were harvested at each time point and immunoprecipitated with an antiserum, JP2, directed against a synthetic *int-1* oligopeptide (20). Immunoprecipitates of cell extracts made from A37 cells after a 45-min pulse-label (Fig. 1A, lane 0) contained the same four species of intracellular *int-1* proteins (36K, 38K, 40K, and 42K) that have been identified in other cell lines transfected with *int-1* cDNA (3, 20, 21). However, in contrast to these other cell types, A37 cells processed *int-1* proteins very rapidly and efficiently. Very little of the 36K and 38K precursor forms could be detected in the pulse-label sample, and virtually all of the smaller *int-1* protein forms were converted to the 42K species by 2.5 h of chase. The 42K protein had a half-life of approximately 3.0 to 3.5 h. We also noticed a small shift in mobility of the 40K and 42K proteins that occurred after approximately 1 h of chase (Fig. 1A; compare mobility of *int-1* proteins after 0.5 and 2.5 h of chase; also see Fig. 3A, lanes 1 and 2). Since the mobility of the protein backbone after enzymatic removal of carbohydrate appears unchanged (J. Papkoff, unpublished results), this size shift most likely indicates processing of the carbohydrates on *int-1* protein as it traverses the secretory pathway. The change in mobility provides a useful marker to distinguish between immature and mature *int-1* protein. No secreted *int-1* protein could be detected in the medium from A37 cells at any point during the chase period (Fig. 1B), which is in agreement with results obtained for other *int-1*-transfected cell lines that express low or moderate levels of *int-1* protein (21; unpublished results).

To identify secreted *int-1* proteins, a pulse-chase experiment similar to the one shown in Fig. 1A and B was performed, but suramin was added to the chase media. Previous studies indicated that suramin was capable of blocking *int-1* adhesion to the extracellular surface or matrix (20). Immunoprecipitation, with JP4 antiserum, of cell extracts made after a 30-min pulse-label and after various times of chase showed that the processing of intracellular *int-1* proteins was unaffected by suramin (Fig. 1C). Immunoprecipitation of the chase medium prepared in the presence of suramin revealed the secreted 42K protein and a smaller amount of 40K *int-1* protein (Fig. 1D). These *int-1* proteins, first detected in the medium after 2.0 h of chase, appeared to accumulate in the chase medium for at least 16 h. In contrast to the results obtained with *int-1*-overexpressing CHO cells (20), in which only a small fraction of total *int-1* protein is secreted, the A37 cells rapidly and efficiently secreted the 40K and 42K *int-1* products (compare Fig. 1C and D).

Detection of efficient secretion of *int-1* protein via a constitutive pathway in the presence of suramin. To determine whether *int-1* protein is secreted via a constitutive or regu-

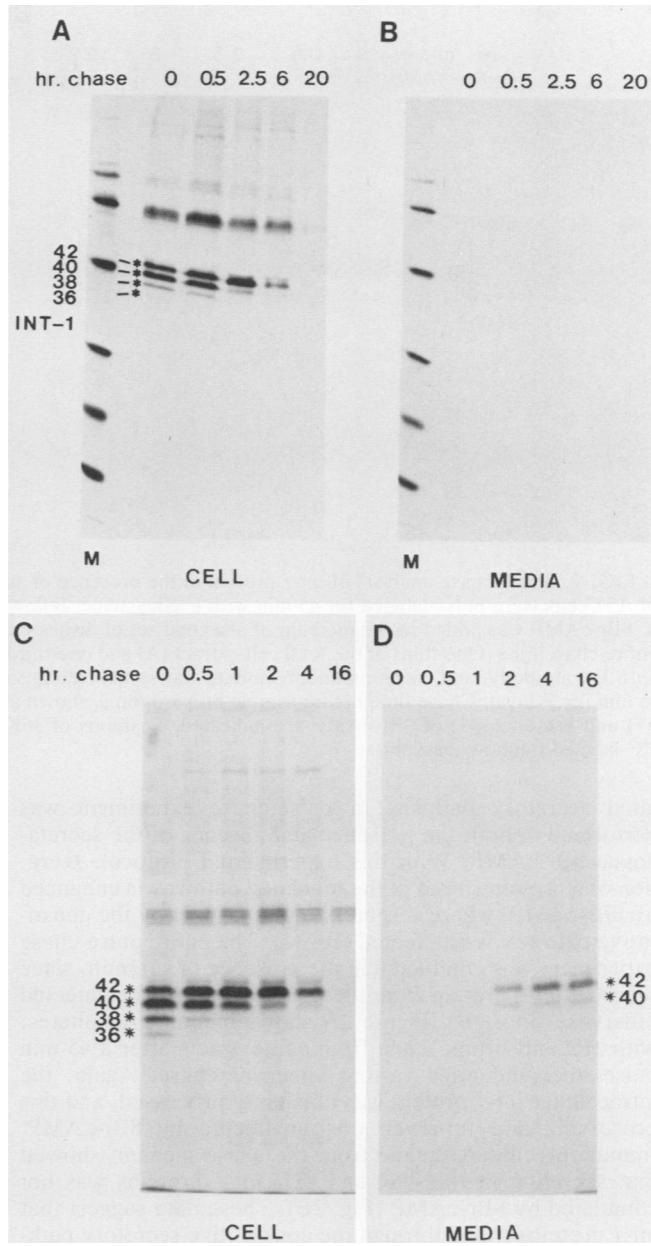

FIG. 1. Pulse-chase analysis of *int-1* protein in transfected AtT-20 cells: effect of suramin on release of secreted *int-1* protein. (A and B) Duplicate dishes of A37 cells were pulse-labeled for 45 min with [^{35}S]cysteine, followed by incubation in the absence of label (chase) for 0, 0.5, 2.5, 6, or 20 h. One-third of the total cell extract (A) and one-third of the total chase medium (B) from each time point were immunoprecipitated with JP2 antiserum. (C and D) Duplicate dishes of A37 cells were pulse-labeled for 30 min with [^{35}S]cysteine, followed by incubation in the absence of label (chase) for 0, 0.5, 1, 2, 4, 6, and 16 h. All dishes were treated with suramin during the entire chase time. One-third of the total cell extract (C) and one-third of the total chase medium (D) from each time point were immunoprecipitated with JP4 antiserum. Each immunoprecipitate was solubilized in sample buffer and analyzed on an SDS–15% polyacrylamide gel that was exposed to film for 2 days (A and B) or an SDS–12.5% polyacrylamide gel that was exposed to film for 3 days (C and D). Chase time before sample preparation is shown above each panel. Positions of 36K, 38K, 40K, and 42K *int-1* proteins from cell extracts and media are indicated. Lanes M show the ^{14}C-labeled rainbow markers.

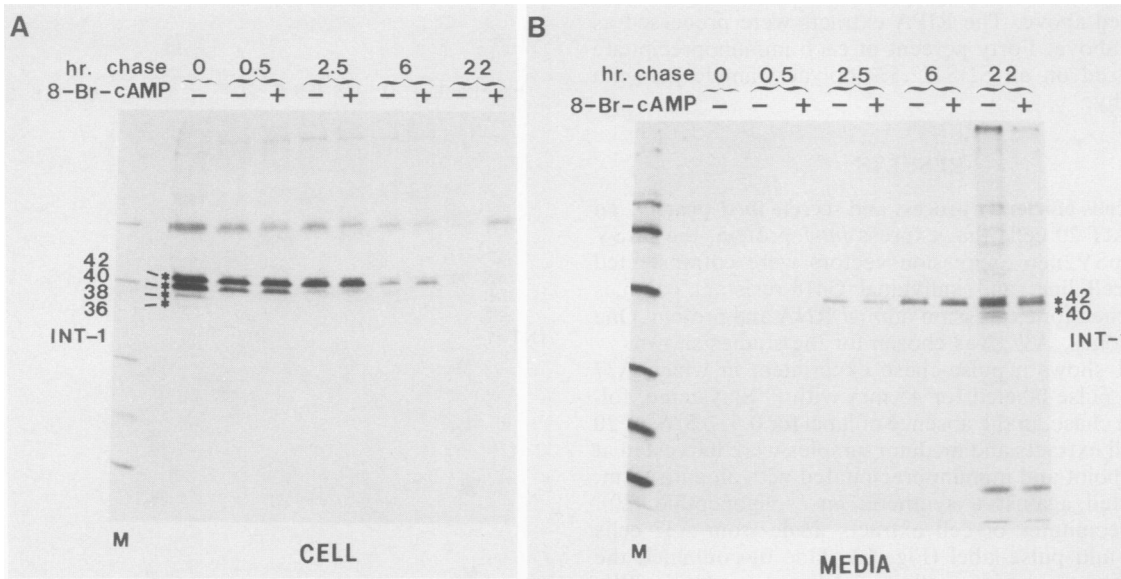

FIG. 2. Pulse-chase analysis of *int-1* protein in the presence of suramin: effect of 8Br-cAMP on *int-1* protein secretion. Duplicate dishes of A37 cells were pulse-labeled for 45 min with [^{35}S]cysteine, followed by incubation in the absence of label (chase) for 0, 0.5, 2.5, 6, or 22 h. 8Br-cAMP was added to the medium of a second set of dishes during the chase periods. All dishes were treated with suramin during the entire chase time. One-third of the total cell extract (A) and one-third of the total medium (B) from each time point were immunoprecipitated with JP2 antibody, and each immunoprecipitate was solubilized in sample buffer and analyzed on an SDS–15% polyacrylamide gel (exposed to film for 3 days). Chase time before sample preparation is shown above each panel. Samples prepared from cells incubated in the absence (–) and presence (+) of 8Br-cAMP are indicated. Positions of 36K, 38K, 40K, and 42K *int-1* proteins are indicated. Lanes M show the ^{14}C-labeled rainbow markers.

lated secretory pathway, a pulse-chase experiment was performed in both the presence and absence of the secretagogue 8Br-cAMP. With this experimental protocol, secretion of a protein stored in the regulated pathway is enhanced by 8Br-cAMP, whereas secretion of a protein by the constitutive pathway is unaffected (10, 12). The entire pulse-chase experiment was conducted in the presence of suramin, since secreted *int-1* protein from these cells could not be detected otherwise (Fig. 1B). Figure 2A shows immunoprecipitates, with JP2 antiserum, made from cell extracts after a 45-min pulse-label and after various times of chase. Again, the intracellular *int-1* protein was efficiently processed, and this processing and turnover was unaffected by 8Br-cAMP. Immunoprecipitates made from the chase medium showed that secretion of the 40K and 42K *int-1* proteins was not stimulated by 8Br-cAMP (Fig. 2B). These data suggest that *int-1* proteins travel through the constitutive secretory pathway and are not stored in regulated secretory vesicles. Control experiments with adrenocorticotropin and viral gp70 proteins, which are naturally secreted by AtT-20 cells via the regulated and constitutive pathways, respectively (10), indicated that these pathways are functional under our experimental conditions (data not shown).

Identification of mature *int-1* protein on the cell surface. The pulse-chase experiments shown in Fig. 1 and 2 demonstrate that *int-1* protein was detected in the culture medium only in the presence of suramin. On the basis of experiments in other systems (2, 22, 36), this result implies that secreted *int-1* protein adheres to the cell surface or extracellular matrix and that suramin blocks this binding.

We sought to demonstrate directly that *int-1* associates with the cell surface. To this end, we made use of two techniques that will identify surface proteins: (i) radioiodination of intact cells and (ii) surface antibody adsorption. We employed a lactoperoxidase method, using ^{125}I, for the surface iodination experiments. Monolayers of A37 cells were iodinated, washed with a quenching medium, and then extracted with RIPA buffer. Immunoprecipitation of the cell extract with JP4 antiserum showed the 42K and a small amount of the 40K form of *int-1* protein (Fig. 3A, lane 3). Immunoprecipitation of these proteins was blocked when JP4 antiserum was preabsorbed with JP4 peptide (lane 4). The surface-iodinated 40K and 42K proteins were also recognized by JP2 antiserum (data not shown). These proteins were not immunoprecipitated with either JP2 or JP4 antiserum from extracts of radioiodinated AtT-20 parent cells (data not shown). The iodinated 42K protein comigrated with the [^{35}S]cysteine-labeled 42K protein that had undergone the small size shift during maturation (Fig. 3A, lane 2). These results demonstrate directly that the mature forms of secreted *int-1* protein are on the cell surface.

To eliminate the possibility that internal cell proteins were labeled during the iodination process or after RIPA solubilization, we also immunoprecipitated the v-Ha-*ras* protein, which is known to be associated with the cytoplasmic face of the plasma membrane and not on the cell surface (37). For this experiment we used 7dT cells, a rat embryo fibroblast cell line that expresses both *int-1* and v-Ha-*ras* proteins from transfected vectors (3). 7dT cell monolayers were iodinated, solubilized, and immunoprecipitated with either JP4 antibody or an antibody, Y13-259, directed against v-Ha-*ras* protein. Similar to results with A37 cells, 40K and 42K *int-1* proteins were immunoprecipitated from extracts of both [^{35}S]cysteine-labeled and surface-iodinated 7dT cells (Fig. 3B, lanes 1 and 3, respectively). The mature *int-1* proteins were slightly larger in 7dT cells than in A37 cells, possibly because of more extensive glycosylation. The 40K and 42K *int-1* proteins from 7dT cells were found in roughly equimolar amounts after cell surface iodination and in medium from suramin-treated cells that were labeled with [^{35}S]cysteine (Fig. 3B lane 2). This is slightly different from the pattern seen with A37 cells, in which the 42K protein was the

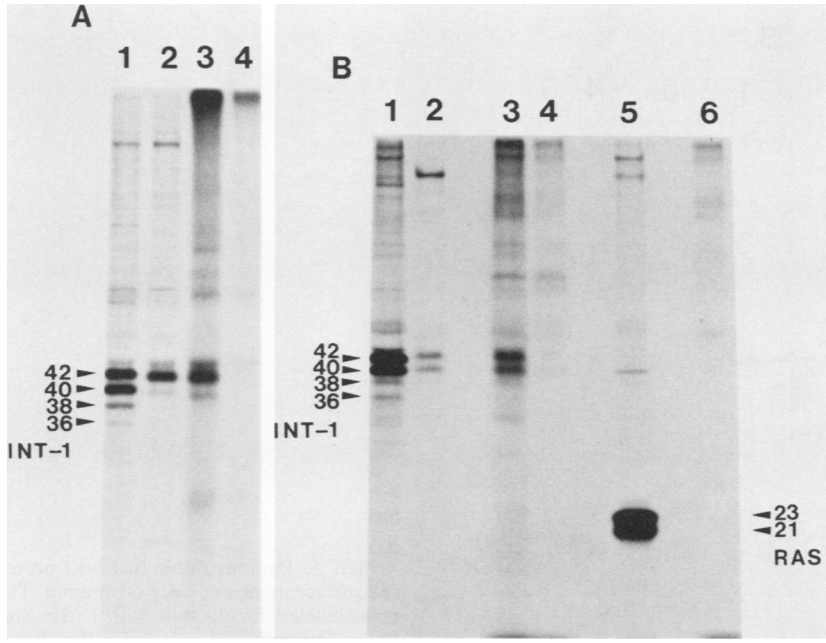

FIG. 3. Cell surface radioiodination and immunoprecipitation of *int-1* proteins. (A) Monolayer cultures of A37 cells were radioiodinated by a lactoperoxidase method. After extensive rinsing and solubilization in RIPA buffer, immunoprecipitations were performed with either JP4 antiserum (lane 3) or JP4 antiserum preabsorbed with JP4 peptide (lane 4). For comparison, RIPA extracts from A37 cells labeled with [^{35}S]cysteine for either 45 min (lane 1) or 45 min followed by a chase for 3.5 h (lane 2) were immunoprecipitated with the JP4 antiserum. Immunoprecipitates were solubilized in sample buffer and analyzed on an SDS–15% polyacrylamide gel (exposed to film for 4 days with an intensifying screen). (B) Monolayer cultures of 7dT cells were radioiodinated by a lactoperoxidase method. After extensive rinsing and solubilization in RIPA buffer, immunoprecipitations were performed with JP4 antiserum (lane 3), preimmune serum (lane 4), or Y13-259 antibody (lane 6). For comparison, lanes 1 and 2 show JP4 immunoprecipitations of cell extracts and culture medium, respectively, from 7dT cells pulse-labeled for 1 h with [^{35}S]cysteine, followed by a chase for 2 h in the presence of suramin. Lane 5 shows an immunoprecipitation using Y13-259 antibody from 7dT cells labeled with [^{35}S]cysteine and [^{35}S]methionine for 45 min. Immunoprecipitates were solubilized in sample buffer and analyzed on an SDS–12.5% polyacrylamide gel (exposed to film for 2 days with an intensifying screen). The positions of 36K, 38K, 40K, and 42K *int-1* proteins and of 21K and 23K v-Ha-*ras* proteins are marked.

predominant form. In contrast to the results with *int-1* protein, the intracellular 21K and 23K v-Ha-*ras* proteins were abundant in immunoprecipitates from [^{35}S]cysteine-labeled 7dT extracts (Fig. 3B, lane 5) but were undetectable in extracts from cells that had been surface labeled with ^{125}I (lane 6). If the ^{125}I reaction was not quenched before RIPA extraction, both the 23K and 21K v-Ha-*ras* proteins were visible in the subsequent immunoprecipitates, indicating that the v-Ha-*ras* proteins can be labeled with ^{125}I (data not shown). Our control experiments with v-Ha-*ras* show that the iodination technique we used did not recognize proteins that are inside the cell.

We used another experimental approach to confirm the results of the surface iodination. For these experiments, the antiserum against *int-1* protein was adsorbed directly to the surface of intact cells that were labeled with [^{35}S]cysteine. A37 cells were labeled with [^{35}S]cysteine for 3 h, and either JP4 antiserum or JP4 antiserum preabsorbed with JP4 peptide was added directly to the medium during the final 30 min of label time. After removal of the label medium and extensive washing of the cell monolayer, the cells were solubilized in RIPA buffer and the antigen-antibody complexes were immediately precipitated. JP4 antiserum (Fig. 4A, lane 2), but not peptide-blocked serum (lane 3), when adsorbed to the cell surface recognized the mature 40K and 42K *int-1* proteins. A similar result was obtained with JP2 antiserum (data not shown).

Again a control experiment was performed with the 7dT cells to show that antiserum adsorbed to the cell surface did not recognize internal proteins (Fig. 4B). Similar to findings with A37 cells, 40K and 42K *int-1* proteins were recognized by JP4 antiserum in a surface adsorption experiment with 7dT cells (lane 2). In contrast, no v-Ha-*ras* protein was immunoprecipitated with Y13-259 antibody from the surface of these cells (lane 5). However, the 21K v-Ha-*ras* protein was efficiently recognized in cells solubilized with RIPA buffer before the addition of antibody (lane 4). In agreement with the iodination experiments, the antibody adsorption data demonstrate that *int-1* protein is localized on the cell surface.

The results presented in the preceding figures predict that suramin treatment of A37 cells before the surface antibody adsorption experiment would result in a loss of detectable *int-1* protein from the cell surface. This was indeed the case (Fig. 5). A37 cells were labeled with [^{35}S]cysteine, and a surface antibody adsorption was performed in the presence of suramin. No *int-1* protein could be detected on the surface of suramin-treated A37 cells (Fig. 5, lane 2). As expected, the 40K and 42K *int-1* proteins were detected in the culture medium from these same cells (lane 4). As a control, lanes 1 and 3 show the cell surface and culture medium immunoprecipitates, respectively, from a surface adsorption experiment performed in the absence of suramin.

DISCUSSION

In an effort to understand the function of the *int-1* gene in embryogenesis and transformation, we have characterized

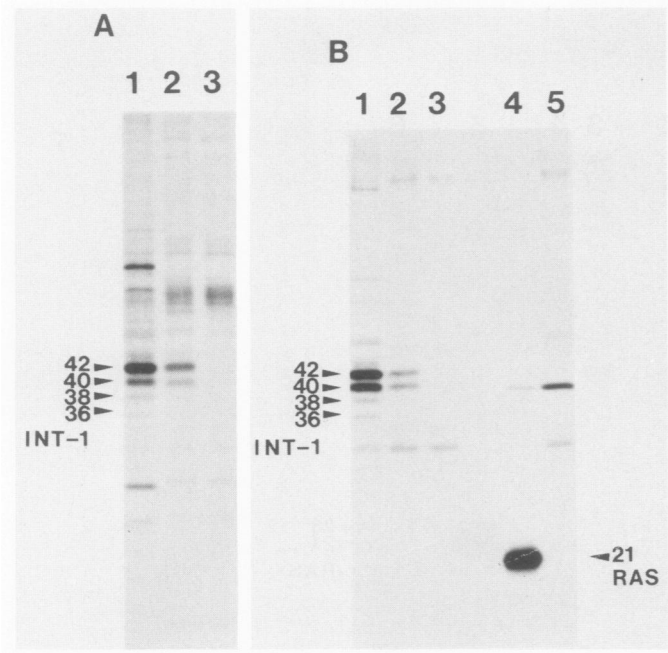

FIG. 4. Cell surface antibody adsorption. (A) Cultures of A37 cells were labeled with [^{35}S]cysteine for 3 h. Either JP4 antiserum or preimmune serum was added to the medium during the last 30 min of label time (surface adsorption). The cells were subsequently washed and solubilized in RIPA buffer, and the antigen-antibody complexes were precipitated as described in Materials and Methods and analyzed on an SDS–12.5% polyacrylamide gel (exposed to film for 2 days). Lanes 1, standard JP4 immunoprecipitate from a RIPA cell extract, made after a 3-h label with [^{35}S]cysteine; 2, surface adsorption made with JP4 antibody; 3, surface adsorption made with preimmune serum. (B) Cultures of 7dT cells were pulse-labeled with [^{35}S]cysteine and [^{35}S]methionine for 30 min, followed by a chase for 3 h in the absence of label. JP4 antiserum, preimmune serum, or Y13-259 antibody was added to the medium during the last 30 min of chase (surface adsorption). The cells were subsequently washed and solubilized in RIPA buffer, and the antigen-antibody complexes were precipitated as described in Materials and Methods and analyzed on an SDS–12.5% polyacrylamide gel (exposed to film for 4 days). Lanes 1, standard JP4 immunoprecipitate from a RIPA extract of 7dT cells pulse-chase labeled in parallel with the surface adsorption experiment; 2, surface adsorption made with JP4 antiserum; 3, surface adsorption made with preimmune serum; 4, standard Y13-259 immunoprecipitate from a RIPA extract of 7dT cells the pulse-chase labeled in parallel with the surface adsorption experiment; 5, surface adsorption made with Y13-259 antibody. The positions of 36K, 38K, 40K, and 42K *int-1* proteins and of 21K v-Ha-*ras* protein are indicated.

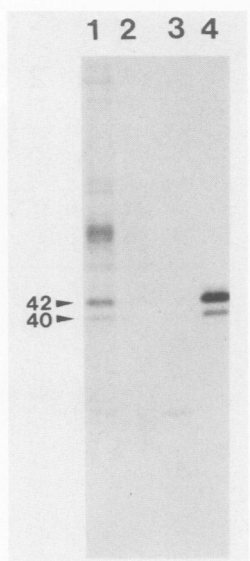

FIG. 5. Demonstration that *int-1* protein is not detectable on the cell surface in the presence of suramin. Two dishes of A37 cells were pulse-labeled for 30 min with [^{35}S]cysteine, followed by a 2.5-h chase. Suramin was included in the chase medium of one dish. JP4 antiserum was added to each dish during the last 30 min of the chase period (surface adsorption). Antigen-antibody complexes were precipitated from the label medium and from RIPA extracts of the washed cell monolayer and analyzed on an SDS–12.5% polyacrylamide gel (exposed to film for 1 week). Lanes 1, JP4 surface adsorption from cells in the absence of suramin; 2, JP4 surface adsorption from suramin-treated cells; 3, immunoprecipitation of *int-1* protein from medium of surface adsorption in the absence of suramin; 4, immunoprecipitation of *int-1* protein from medium of suramin-treated surface adsorption. Positions of 40K and 42K *int-1* proteins are marked.

the biochemical properties of *int-1* protein. For these studies, we have used the neuroendocrine cell line AtT-20 transfected with an *int-1* expression vector. Unlike other *int-1*-transfected cells that we have studied (3, 20, 21), the AtT-20 cell line can efficiently process and secrete *int-1* protein. Pulse-chase studies indicate that the 36K, 38K, and 40K precursor forms of *int-1* protein are rapidly processed to the mature 40K and 42K species that are efficiently secreted. The 42K *int-1* protein from the transfected AtT-20 cells is analogous to the diffuse 44K doublet that is inefficiently secreted from *int-1*-overexpressing CHO cells (20). A likely explanation for the difference in electrophoretic mobility of *int-1* protein from the two cell types is that the CHO *int-1* product is more extensively glycosylated. We have not identified any proteolytic processing products of *int-1* protein in the cell extracts or in the culture medium from *int-1*-transfected AtT-20 cells. This is somewhat unexpected, since the predicted NH_2-terminal sequence of *int-1* protein contains three dibasic and one tribasic amino acid sequence (8) and other proteins with these basic amino acid motifs (e.g., preprosomatostatin or pro-opiomelanocortin) are cleaved by processing proteases in AtT-20 cells (15, 25, 27).

As we have found with several other *int-1*-transfected cell types (20, 21; unpublished data), the *int-1* protein secreted from AtT-20 cells is not free in the culture medium. Treatment of these cells with suramin during a pulse-chase experiment results in efficient release of the 42K and 40K proteins into the culture fluid. This result is similar to a previous observation, made with CHO cells that overexpress *int-1*, that suramin treatment released mature glycosylated *int-1* protein, albeit in small amounts (20). On the basis of results with suramin in other systems (2, 22, 36), these data suggest that secreted *int-1* protein adheres to either the cell surface or the extracellular matrix. The functional importance of secreted, cell surface-associated *int-1* protein can be inferred from the observation that suramin treatment of C57 mammary cells that are partially transformed by a transfected *int-1* gene results in phenotypic reversion (unpublished observation). It is of note that the half-life of *int-1* protein from AtT-20 cells is extended when it is released from the cells by suramin (compare the half-life of *int-1* protein shown in Fig. 1A with that shown in Fig. 1D). One explanation for this observation is that *int-1* proteins are normally removed from the cell surface via internalization and subsequent degradation and that suramin blocks this

process by releasing these proteins into the culture medium, where they are relatively stable to proteolysis.

The data presented above for iodination of intact cells and antibody adsorption experiments provide direct evidence that *int-1* protein is on the cell surface. The predicted amino acid sequence of *int-1* protein does not contain a membrane anchor domain (8), and the protein has biochemical properties that are characteristic of secreted rather than integral membrane protein (20, 21). Furthermore, we are unable to identify any lipid modification of *int-1* protein that might mediate an association with the plasma membrane (unpublished results). One interpretation of these observations is that secreted *int-1* protein adheres to the cell surface through interactions with another protein, such as a receptor. The surface component that associates with *int-1* protein must be present on many different cell types, since in all *int-1*-transfected cell lines that have been examined, *int-1* protein remains completely (21; unpublished results) or mostly (20) cell associated and is not released free into the culture medium. We have been unable to demonstrate an association of *int-1* protein with extracellular matrix by biochemical fractionation, nor can we detect binding of *int-1* proteins to heparin, an extracellular matrix constituent (unpublished results). Experiments are currently under way to identify proteins that associate with *int-1* protein.

The biochemical properties of *int-1* protein described here provide several important clues to the function of *int-1* in embryogenesis and mammary transformation. These and other studies have shown that *int-1* protein has many features in common with secreted factors such as the tumor growth factor β and fibroblast growth factor families of proteins. These proteins are multifunctional and play a role in either growth or differentiation, depending on the cellular context in which they are expressed (13, 30). Unlike many neurotransmitters and hormones (12), *int-1* protein is not stored in secretory vesicles awaiting a stimulus for release. Therefore, the extracellular appearance of *int-1* protein is a constitutive event and likely to be regulated by the availability of *int-1* mRNA for translation. Association of *int-1* protein with the cell surface suggests that secreted *int-1* protein is not free to diffuse beyond the cell that produces it. Thus, the signal transmitted by *int-1* protein must be either autocrine or paracrine, but restricted to adjacent cells. In agreement with our data, immunohistochemical localization of wingless (*int-1*) protein in *Drosophila* embryos shows that this protein is both inside and outside of cells (34). In particular, wingless antigen is concentrated on the cell surface and in the intercellular space. The suggestion that wingless behaves as a paracrine signal comes from the observation that wingless protein is occasionally found inside engrailed-positive cells that are immediately adjacent to the wingless-expressing cells (34). We are currently investigating possible signal transduction pathways that may be utilized by *int-1* protein.

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