Altered Sialylation of Osteopontin Prevents Its Receptor-Mediated Binding on the Surface of Oncogenically Transformed tsB77 Cells†

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ABSTRACT: It has been reported previously that oncogenically transformed cells secrete different molecular forms of osteopontin (OPN), a sialic acid-rich, adhesive, phosphoglycoprotein, than OPNs secreted by their nontransformed counterparts. However, the origin of the OPN isoform secreted by the transformed cells and whether it has different physiological properties which may serve transformation-specific functions remain poorly understood. Here, we report that Rat-1 cells transformed by a temperature-sensitive mutant of Rous sarcoma virus (tsB77) secrete two discrete molecular forms of OPN, a 69-kDa OPN at the nonpermissive temperature (41 °C) and a 62-kDa form at the permissive temperature (34 °C). However, tsB77 cells at both temperatures transcribe a single 1.6 kb OPN mRNA and contain only the 69-kDa form of OPN intracellularly, suggesting that the 69-kDa OPN is modified to the 62-kDa form prior to or immediately after secretion by cells at 34 °C. We ruled out proteolytic cleavage, differential phosphorylation, or lack of N- or O-linked carbohydrates as the possible mechanism, but found that the 62-kDa OPN contains significantly reduced levels of sialic acid, as compared to its 69-kDa form. The binding assays using ^{32}P-labeled OPN revealed that only the 69-kDa OPN, not its 62-kDa form, undergoes receptor-mediated localization on the cell surface, although tsB77 cells synthesize OPN receptors ($\alpha_v\beta_3$ integrins) at both permissive and nonpermissive temperatures. Furthermore, ^{125}I-labeled purified milk OPN, which is highly sialylated and shows cell surface binding, upon digestion with neuraminidase failed to interact with the cell surface. Taken together, these results suggest that the difference between the 69-kDa and 62-kDa isoforms of OPN resides in their sialic acid content, and sialylation of OPN is crucial for its receptor-mediated binding on tsB77 cells. The data presented here demonstrate for the first time a physiological role of sialic acids in this protein, and raise the possibility that oncogenically transformed tsB77 cells may exploit the lack of OPN−receptor interactions for their invasive behavior.

Osteopontin (OPN)[1] is a secreted, sialic acid-rich, adhesive, glycophosphoprotein with RGDS cell-binding sequence which interacts with several integrins including $\alpha_v\beta_3$ (Oldberg *et al.*, 1986; Reinholt *et al.*, 1990). Recently, it has been demonstrated that OPN also interacts with the CD44 receptor (Weber *et al.*, 1996). Rat OPN is composed of 317 amino acid residues, with a predicted molecular mass of approximately 32 kDa for the peptide backbone (Oldberg *et al.*, 1986). However, various isoforms of OPN exhibit electrophoretic mobilities ranging from 42 to 75 kDa, due to posttranslational modifications. OPN is a substrate of transglutaminase (TG) (Prince *et al.*, 1991), and covalently cross-links to fibronectin (Fn) in a TG-catalyzed reaction (Beninati *et al.*, 1994). This protein has cell-attachment capability (Somerman *et al.*, 1989), exerts a chemotactic effect on arterial smooth muscle cells (Liaw *et al.*, 1994), and binds Ca^{2+} (Chen *et al.*, 1992; Singh *et al.*, 1993). Several studies have shown that transformed fibroblastic and epithelial cells of rodent and human origin, regardless of the transforming agent, overexpress OPN (Senger *et al.*, 1979, 1980, 1983, 1989), and elevated amounts of this protein are present in the blood of patients with disseminated carcinomas (Senger *et al.*, 1988). OPN expression is induced in mouse preneoplastic epidermal cells (JB6) upon treatment with 12-*O*-tetradecanoylphorbol 13-acetate (TPA), which irreversibly transforms these cells (Craig *et al.*, 1989). Furthermore, transfection of oncogenically transformed cell lines with antisense OPN cDNA, which causes drastic reduction in OPN synthesis, also inhibits the malignant potential of the transfected cells (Behrend *et al.*, 1993; Gardner *et al.*, 1994; Su *et al.*, 1995). These observations raise the possibility that OPN has important functional roles in oncogenesis.

Our earlier study showed that retinoic acid (RA)-treated Rous sarcoma virus (RSV)-transformed rat (RR1022) and vole (SR-IT) cells, which show reversible loss of transformed phenotype, secrete a 69-kDa form of OPN instead of the 62-kDa OPN secreted by their untreated counterparts, demonstrating a clear correlation between the release of 69-

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[1] Abbreviations: OPN, osteopontin; Fn, fibronectin; DMEM, Dulbecco's modified Eagle's medium; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; RA, *all-trans-β*-retinoic acid; RIPA, radioimmune precipitation assay; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; KIU, kallikrein inactivating unit(s); TFMS, trifluromethanesulfonic acid; DMSO, dimethyl sulfoxide; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; DSS, disuccinimidyl suberate.

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kDa or 62-kDa OPN and the nontransformed and transformed state of the cell, respectively (Chackalaparampil *et al.*, 1985). Similar observation has been made by Kasugai *et al.* (1991) which shows that normal rat osteoblast cells secrete a 55-kDa OPN, whereas rat osteosarcoma cells release a 44-kDa OPN. To determine a possible role of OPN in cell transformation, we investigated how the two isoforms of OPN secreted by transformed and nontransformed cells originate, and whether the two forms have different physiological properties. Using Rat-1 cells, transformed by a temperature-sensitive mutant of Rous sarcoma virus (tsB77 cells), we show here that these cells grown at permissive (34 °C) and nonpermissive (41 °C) temperatures secrete 62-kDa and 69-kDa OPNs, respectively, and while they are translated from the same size messages and their peptide backbones are identical, the 62-kDa OPN is undersialylated, which affects the mobility of this protein in SDS−PAGE. Most importantly, we present evidence that altered sialylation of the 62-kDa OPN prevents its receptor-mediated localization on the cell surface, raising the possibility that the sialic acid residues of OPN play a critical role in its cell-binding activity, lack of which we speculate may promote the invasive behavior of the oncogenically transformed cells.

MATERIALS AND METHODS

Cell Line and Growth Conditions. tsB77, a Rat-1 cell line, transformed by a temperature-sensitive mutant of the Bratislava 77 variant of Rous sarcoma virus (RSV-B77), was a gift from Dr. Donald R. Senger (Beth Israel Hospital and Harvard Medical School). Cells were maintained at 34 °C (permissive temperature) in DMEM supplemented with 10% newborn calf serum and antibiotics, and subcultured before reaching confluence. All cells were free of mycoplasma contamination.

Soft Agar Growth Assay. The ability of tsB77 cells to form colonies in soft agar at permissive (34 °C) and nonpermissive (41 °C) temperatures was determined by the method described previously (Freedman & Shin, 1974), using a 0.6% agarose base layer and 0.3% agarose as the overlay containing 5×10^3 cells per 60-mm dish.

Metabolic Radiolabeling of Secreted Proteins. Cells were seeded at a density of 2.5×10^5 cells/60-mm tissue culture dish, and incubated at 34 °C for 24 h in a 5% CO_2 environment. Half of the cultures were then maintained at 34 °C, and the remaining cultures were shifted to 41 °C (nonpermissive temperature). Subconfluent cultures were washed twice in serum- and phosphate-free DMEM and incubated for 1 h using the same medium. The cells were labeled for 4 h at either 34 °C or 41 °C in the same medium containing 500 μCi/mL carrier-free [^{32}P]orthophosphate (Amersham). For ^{35}S-labeling, subconfluent cultures were labeled after 1 h of methionine-starvation, with 100 μCi/mL [^{35}S]methionine (1200−1400 Ci/mmol; Amersham) in serum- and methionine-free DMEM for 4 h. Conditioned media of ^{32}P- and ^{35}S-labeled cultures were collected, adjusted to 5 mM PMSF, 100 KIU/mL aprotinin, 2 μg/mL leupeptin, and 1 μg/mL pepstatin, and cleared by centrifugation at 10000*g* for 30 min at 4 °C.

For the preparation of cell lysates, the labeled cell monolayers were washed 3 times with ice-cold PBS and lysed in RIPA buffer [0.05 M Tris-HCl (pH 7.2), 0.15 M NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS] containing 100 KIU of aprotinin/mL, 5 mM PMSF, 5 μg/mL soybean trypsin inhibitor, 2 μg/mL leupeptin, 1 μg/mL pepstatin, 0.2 mM sodium vanadate, and 1 mM sodium molybdate. The lysed cells were scraped using a rubber policeman, pipetted into Eppendorf tubes, vortexed briefly, and centrifuged at 10000*g* for 30 min at 4 °C. Aliquots of supernatants containing 100 μg of total protein were used for immunoprecipitation.

Immunoprecipitation, SDS−PAGE, and Autoradiography. Aliquots of cell lysates and conditioned media containing equal amounts of total protein, estimated by using the Bio-Rad protein assay, were diluted with an equal volume of RIPA buffer and incubated at 4 °C for 2 h with an antiserum raised against a synthetic oligopeptide representing amino acid residues 291−306 of rat OPN (OST-1 serum) (Singh *et al.*, 1992). The resulting immune complexes were incubated with protein A−Sepharose (Pharmacia) at 4 °C for 1 h with gentle agitation. The adsorbed immune complexes were pelleted by centrifugation, washed 3 times with RIPA buffer, and twice with PBS, and finally rinsed with distilled water. The immunoprecipitated proteins were suspended in 50 μL of sample buffer [0.07 M Tris-HCl (pH 6.8), 3% SDS, 5% β-mercaptoethanol, 10% glycerol, and 0.01% bromophenol blue], heated at 95 °C for 5 min, and analyzed by SDS−PAGE using 10% slab gels (Laemmli, 1970). After electrophoresis, gels were dried, and protein bands were visualized by autoradiography or fluorography, as described previously (Singh *et al.*, 1990).

To examine the expression of $\alpha_v\beta_3$ integrins by tsB77 cells at both temperatures, 100 μg of total protein was fractionated on a 7.5% resolving gel. Proteins were electrophoretically transferred to an Immobilon-P membrane (Millipore), rinsed 3 times with PBS, and incubated in PBS containing 3% bovine serum albumin (blocking buffer) at 4 °C. The blot was immunostained with rabbit anti-human vitronectin receptor polyclonal antibody (Chemicon International Inc.) in blocking buffer for 2 h and washed 3 times in PBS containing 0.1% Tween-20. Detection of bound antibodies was carried out by incubation in 1 μCi/mL ^{125}I-labeled protein A solution (ICN) in 3% BSA for 1 h. The blot was washed thoroughly in three changes of PBS and autoradiographed.

RNA Isolation and Northern Blot Analysis. Total RNA was extracted from subconfluent, actively growing cells by the method of Chomczynski and Sacchi (1987). RNA was fractionated on 1.2% agarose gels containing 2.2 M formaldehyde and transferred to Nytran hybridization membranes (Schleicher & Schuell) by vacuum blotting for 2 h in 10 × SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0). Blots were hybridized to radiolabeled probes in the presence of 1 mM NaCl, 1% SDS, and 10% dextran sulfate for 24 h at 60 °C. OPN and β-actin cDNA probes were labeled with ^{32}P-dCTP using a BRL nick translation system. After hybridization, membranes were washed 2 times for 5 min each with 2 × SSC at room temperature, twice for 30 min each with 2 × SSC containing 1% SDS at 60 °C, and twice for 30 min each with 0.05 × SSC at room temperature prior to autoradiography.

V8 Protease and Tryptic Phosphopeptide Mapping. Partial proteolytic mapping using *Staphylococcus aureus* V8 protease was carried out according to the procedure described by Cleveland *et al.* (1977). Immunoprecipitated ^{32}P-labeled proteins from the conditioned medium were separated by

SDS−PAGE, and the OPN bands were excised from the autoradiographed gels and rehydrated in a buffer containing 0.12 M Tris-HCl (pH 6.8), 0.1% SDS, and 1 mM EDTA for 30 min at room temperature. The gel slices were then placed in wells of 15% SDS−PAGE gels containing 1 mM EDTA, allowed to completely rehydrate in the soaking buffer, and overlaid with the same buffer containing 20% glycerol. Incubation buffer containing 0.005% bromophenol blue, 10% glycerol, and variable amounts of V8 protease was layered on top of the wells. Electrophoresis was started at 55 V constant voltage for 1−2 h until the dye front moved through half the length of the stacking gel (5 cm). Power was turned off for 30 min to allow V8 protease digestion, and then electrophoresis was resumed at 90 V constant voltage overnight until the marker dye reached the bottom of the gel. For tryptic phosphopeptide mapping, ^{32}P-labeled OPN from the conditioned medium was immunoprecipitated with anti-OST-1 serum and analyzed by SDS−PAGE as described above. OPN from each band was eluted, precipitated with trichloroacetic acid, and digested with trypsin as described by Kmiecik and Shalloway (1987). The digested products were lyophilized, resuspended in 1 mL of water, relyophilized, dissolved in chromatography solvent (butanol/pyridine/acetic acid/H_2O, 75:50:15:60), spotted onto cellulose thin-layer plates, and separated in two dimensions by electrophoresis at pH 8.9 followed by chromatography, as described by Hunter and Sefton (1980).

Enzymatic Deglycosylation of the 62-kDa and 69-kDa OPNs. ^{32}P-labeled 62-kDa and 69-kDa OPNs in the conditioned media were immunoprecipitated using OST-1 serum, and denatured by boiling in 0.5% SDS and 50 mM β-mercaptoethanol. Samples were then adjusted to a final concentration of 83 mM Tris-HCl (pH 8.0), 1.3% Triton X-100, 0.17% SDS, protease inhibitors (5 mM PMSF, 100 KIU/mL aprotinin, 2 μg/mL leupeptin, 1 μg/mL pepstatin), and 10 units/mL peptide:*N*-glycosidase F (Genzyme, Cambridge, MA). The reaction mixture was then incubated for 12 h at 37 °C, and the digested products were analyzed by SDS−PAGE.

To specifically remove the O-linked oligosaccharides, *O*-sialoglycoprotein endopeptidase, purified from culture supernatants of *Pasteurella haemolytica A1* (gift from Dr. A. Mellors, University of Guelph; Cedarlane), was used (Abdullah *et al.*, 1992). This enzyme has marked specificity for *O*-sialoglycoproteins, especially those with extensive clusters of sialoglycoprotein. The immunoprecipitated 62-kDa and 69-kDa OPNs were resuspended in 30 μL of 50 mM HEPES buffer (pH 7.4) and digested with *O*-sialoglycoprotein endopeptidase for 2 h at 37 °C. The reaction was stopped by boiling in SDS sample buffer, and the proteins were analyzed by SDS−PAGE and autoradiography.

Neuraminidase digestion of OPN was carried out as described (Cunningham *et al.*, 1984). OPNs in the conditioned media of ^{32}P-labeled cell cultures were immunoprecipitated, and the immune complexes were rinsed in distilled water and resuspended in 30 μL of neuraminidase assay buffer (0.1 M sodium citrate, 2 mM $CaCl_2$, and 0.2 mM EDTA, pH 5.5) containing 5 mM PMSF, 100 KIU/mL aprotinin, 2 μg/mL leupeptin, and 1 μg/mL pepstatin. Neuraminidase (purified from *Closteridium perfringens*; ICN) at a concentration of 1.5 units/mL was added to one sample and incubated for 18 h at 37 °C. The control sample was incubated without the enzyme. The digested proteins were then dissolved in sample buffer and analyzed by SDS−PAGE using 10% polyacrylamide gels.

Radiolabeling of Secreted OPN by [^{3}H]Mannose and Neuraminidase Treatment. tsB77 cells were grown at 34 or 41 °C in DMEM supplemented with 10% newborn calf serum and antibiotics. Subconfluent cultures were incubated with the same medium containing 5% serum and grown for an additional 12 h. The cells were labeled with D-[2,6-^{3}H]-mannose (40 μCi/mL) in the same medium at either 34 or 41 °C for 12 h. Conditioned media of ^{3}H-labeled cultures were collected, and the volume was reduced by a Centricon concentrator (Amicon). The concentrated supernatants were immunoprecipitated according to the conditions described above. The immunoprecipitated complex was washed with water and treated with neuraminidase (*Closteridium perfringens*, 1.5 units/mL) containing 30 μL of neuraminidase assay buffer (pH 5.5), as mentioned above. The control samples were incubated without neuraminidase. The samples were then boiled in SDS−PAGE sample buffer and analyzed by SDS−PAGE (4−20%). The gels containing protein bands were incubated with 1 M sodium salicylate at room temperature for 1 h, dried, and autoradiographed.

Determination of Phosphate Contents of the 69-kDa and 62-kDa OPNs. tsB77 cells were labeled with either [^{32}P]-orthophosphate (1 mCi/mL) or [^{35}S]methionine (135 μCi/mL plus 0.5 μg/mL nonradioactive methionine) at 34 or 41 °C for 14 h to ensure incorporation of phosphate or methionine into proteins to near-equilibrium levels (Nagata & Yamada, 1986). Aliquots of the conditioned media from [^{35}S]methionine-labeled cultures, containing equal trichloroacetic acid-precipitable counts, and corresponding volumes of conditioned media from [^{32}P]orthophosphate-labeled cultures at both temperatures, were used for immunoprecipitation using OST-1 serum. Immunoprecipitated OPNs were analyzed by SDS−PAGE and autoradiography. After localization by superimposing the autoradiographed films, the 62-kDa and 69-kDa OPN bands were excised from the gels and incubated in 30% H_2O_2 at 37 °C for 24 h, and the radioactivity in each band was determined by scintillation counting.

Dephosphorylation of the 69-kDa and 62-kDa OPNs. After immunoprecipitation, the protein A−Sepharose-bound immune complexes containing ^{35}S-labeled 69-kDa or 62-kDa OPNs and ^{32}P-labeled 69-kDa or 62-kDa OPNs were resuspended in 250 μL of phosphatase assay buffer [50 mM Tris-HCl (pH 7.4), 50 mM NaCl, 2 mM PMSF, 1 mg/mL trypsin inhibitor, and 100 KIU/mL aprotinin] containing 2 or 4 units of bacterial alkaline phosphatase (Sigma). The reaction mixture was incubated at 25 °C for 16 h with gentle agitation. After dephosphorylation, the beads were collected by centrifugation, and the proteins were eluted and analyzed by SDS−PAGE.

Radioiodination of OPN. Twenty-five micrograms of purified milk OPN was radioiodinated with sodium [^{125}I]-iodide (2 mCi; carrier-free) using the chloramine-T method (Hunter & Greenwood, 1962). The reaction was performed in 50 mM sodium phosphate buffer (pH 7.5) containing 0.1% BSA at 25 °C for 5 min. The ^{125}I-OPN was purified by Sephadex G-25 (coarse) followed by Sephadex G-50 (super fine) column chromatography using 50 mM sodium phosphate buffer (pH 7.5), containing 0.1% BSA and 0.2% sodium azide. Fractions (0.5 mL) were collected, and the sample containing the peak levels of ^{125}I-OPN was measured

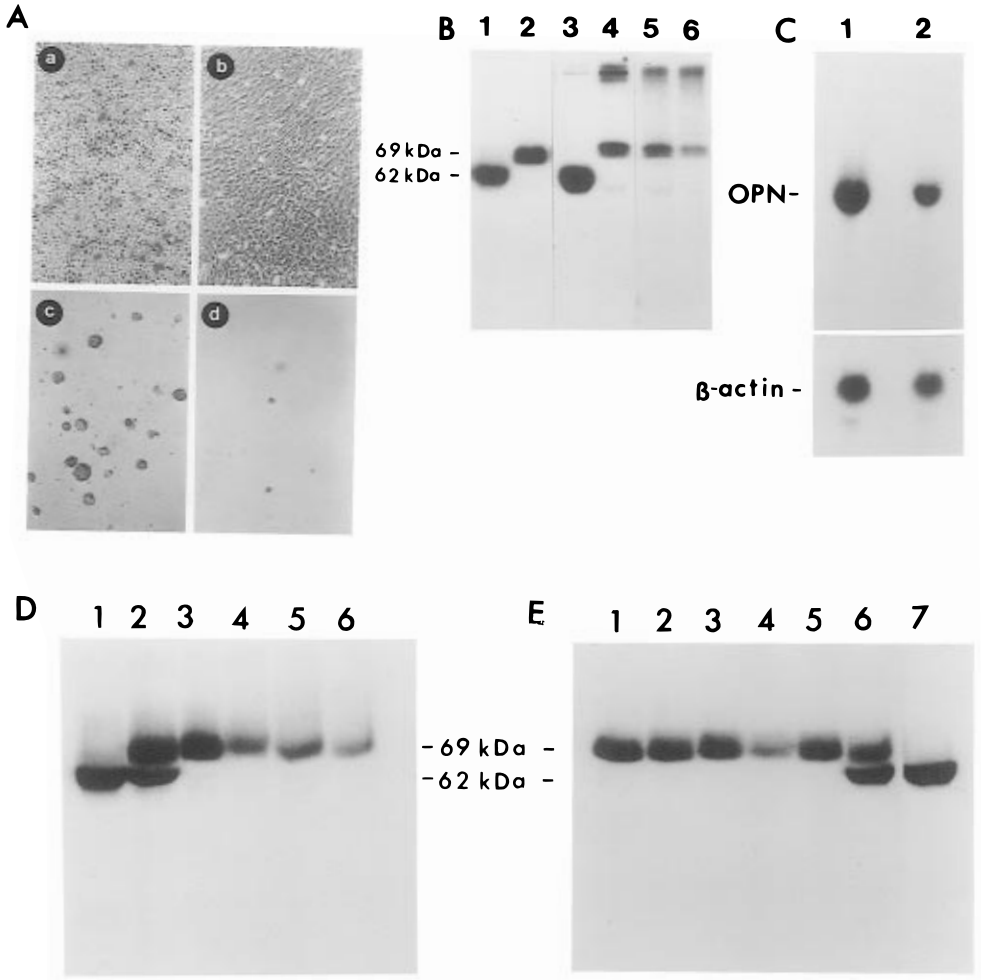

FIGURE 1: (A) Growth of tsB77 cells in monolayer culture and in soft agar medium at 34 and 41 °C. Monolayer culture at 34 °C (panel a) and at 41 °C (panel b). Growth in soft agarose medium at 34 °C (panel c) and at 41 °C (panel d). (B) OPN in the conditioned media and in cell lysates of tsB77 cells at 34 and 41 °C. tsB77 cells were metabolically labeled with [^{32}P]orthophosphate or [^{35}S]methionine; the proteins in the conditioned media or cell lysates were immunoprecipitated with OST-1 serum and analyzed by SDS−PAGE and autoradiography. Conditioned medium from ^{32}P-labeled cells grown at 34 °C (lane 1) and 41 °C (lane 2). ^{35}S-labeled OPN in the conditioned medium of tsB77 cells at 34 °C (lane 3) and at 41 °C (lane 4). Immunoprecipitation of ^{32}P-labeled cell lysates from cells grown at 34 °C (lane 5) and at 41 °C (lane 6) with OST-1 serum. The high molecular mass band in lanes 4, 5, and 6 may represent fibronectin which coprecipitates with OPN. (C) Northern blot analysis of total RNAs. RNAs were isolated from tsB77 cells grown at 34 °C (lane 1) and at 41 °C (lane 2), and subjected to Northern blotting using an OPN cDNA probe. The blot was reprobed with a β-actin probe as indicated in the figure. (D) Time course of the secretion of the 69-kDa OPN by tsB77 cells after shifting from 34 °C to 41 °C. Cells were labeled with [^{32}P]orthophosphate for 3 h after the temperature shift; aliquots of the conditioned media collected at different intervals were immunoprecipitated with OST-1 serum and analyzed by SDS−PAGE. Labeled OPNs in the conditioned media of tsB77 cells following 0 h (lane 1), 12 h (lane 2), 24 h (lane 3), 36 h (lane 4), 48 h (lane 5), and 72 h (lane 6) posttemperature shift. (E) Time course of the secretion of the 62-kDa OPN by tsB77 cells after shifting from 41 °C to 34 °C. 0 h (lane 1), 12 h (lane 2), 24 h (lane 3), 36 h (lane 4), 48 h (lane 5), 72 h (lane 6), and 96 h (lane 7) posttemperature shifts.

by a spectrophotometer (280 nm) and a gamma-counter. The specific activity of purified carrier-free monoiodinated OPN was 86 μCi/μg.

OPN Binding on the Cell Surface. Cell surface OPN-binding assays were performed using cells grown at 34 and 41 °C. Confluent cells grown in 12-well plates were washed once with PBS, pH 7.4, and then incubated with ^{125}I-OPN (3.3×10^5 cpm/well) in the absence or presence of varying concentrations of competing unlabeled OPN in 0.5 mL of Hank's balanced salt solution (HBSS), pH 7.6, containing 0.1% BSA. After incubation at 37 °C for 3 h, the reactions were stopped by rapid removal of the labeling medium followed by three washes in PBS, pH 7.4. The cells were then solubilized by boiling in 2 N NaOH followed by addition of an equal volume of 2 N HCl. The radioactivity was measured using a gamma-counter (ICN Biomedicals, Model 10/600 plus) with a counting efficiency of approximately 80%. The specific binding was calculated by subtracting the nonspecific binding from the total.

Affinity Cross-Linking of OPN to Its Receptor. Confluent tsB77 cells were grown at 34 and 41 °C, washed with PBS (pH 7.4), and incubated with ^{125}I-OPN (6.6×10^5 cpm/well) in 1 mL of HBSS (pH 7.6), containing 0.1% BSA, in the absence or presence of 4 μg of unlabeled OPN at 37 °C for 3 h. After washing twice with PBS, the cells were further incubated with 0.2 mM disuccinimidyl suberate (DSS) in 1 mL of HBSS (pH 7.6), at 37 °C for 30 min. The reaction was terminated by adding Tris-HCl to 50 mM final concentration (pH 7.5); cells were scrapped, collected by centrifugation at 10000*g* for 15 min, and lysed in 40 μL of 1% Triton X-100 solution containing 1 mM PMSF, 20 mg/mL leupeptin, and 2 mM EDTA. Aliquots of the lysate (30 μL) were suspended in SDS−PAGE sample buffer in the presence of 5% 2-mercaptoethanol, boiled for 5 min, and electrophoresed

on a 4−20% gradient SDS−polyacrylamide gel (Bio-Rad). The gels were briefly stained with Coomassie blue, dried in a Bio-Rad gel dryer, and autoradiographed using Kodak X-Omat AR X-ray film. To determine the effects of sialic acid on the cell surface binding of OPN, ^{125}I-OPN was treated with neuraminidase (*Closteridium perfringens*, 1.5 units/mL) in 40 μL of neuraminidase assay buffer (pH 5.5), at 37 °C for 18 h, before carrying out the affinity cross-linking experiment described above.

Metabolic Labeling of OPNs and Their Binding to the Cell Surface. ^{32}P-Labeled 62-kDa and 69-kDa OPNs from tsB77 cells were used for cell-binding studies following the procedure described by Chambers *et al.* (1993). Briefly, the present protocol, like the aforesaid procedure, took advantage of the fact that OPN is the only phosphoprotein secreted by the tsB77 cells at both permissive and nonpermissive temperatures, and analysis of the total proteins in the conditioned medium of ^{32}P-labeled cells by SDS−PAGE indicates that almost all the incorporated radioactivity is associated with the OPN band. Semimonolayers of tsB77 cells grown at 34 and 41 °C were incubated in serum-free and phosphate-free DMEM for 4 h with [^{32}P]orthophosphate (Amersham), the conditioned medium was centrifuged (10000*g* for 10 min), and the labeled OPN was separated from unincorporated [^{32}P]P_i by five cycles of concentration by passing through Centricon-30 columns (Amicon) and resuspending in cold PBS. At the end of each step, a sample of processed media was run on 10% polyacrylamide gels and autoradiographed to detect the presence of any free [^{32}P]-P_i. After this step was repeated 3−4 times, no free [^{32}P]P_i could be detected even when gels were exposed for a prolonged period. ^{32}P-labeled 69-kDa and 62-kDa OPNs were then used in binding experiments using tsB77 cells in suspension. Actively growing cultures of tsB77 cells at 34 and 41 °C were trypsinized and washed twice in serum-free DMEM. The washed cells were resuspended at 5×10^6 cells/mL of serum-free DMEM in 1.5 mL Eppendorf tubes, and 5 μL of ^{32}P-labeled OPN (∼10 000 cpm) was added per tube. Unlabeled milk OPN and bovine serum albumin, when added, were each at a concentration of 6 μg/mL. Our initial experiments indicated that an incubation time of 2 h was sufficient to reach maximum binding. Therefore, in the experiments described here, cells were incubated for 2 h at 34 or 41 °C with gentle agitation, centrifuged at 4000*g* for 5 min, and washed thoroughly in PBS, and the cell surface-bound OPN was assayed by counting the radioactivity present in the total cell lysate in a liquid scintillation counter.

RESULTS AND DISCUSSION

Morphological and Growth Characteristics of tsB77 Cells at Permissive and Nonpermissive Temperatures. Rat-1 cells transformed by a temperature-sensitive mutant of RSV (tsB77 cells) were used for this study because the same cells at permissive temperature (34 °C) show transformed phenotype but at nonpermissive temperature display characteristics of normal cells. As shown in Figure 1A, tsB77 cells grown at 34 °C to confluency exhibit multilayer growth and rounded morphology (panel a) but at 41 °C form a contact-inhibited monolayer, appear fibroblast-like, and show ordered orientation (panel b). When plated onto soft agarose medium, these cells form well-defined colonies at 34 °C (panel c), but at 41 °C fail to form such colonies, even when plated at high cell densities and after prolonged culture (panel d). Since the ability of cells to grow in semisolid medium is the single *in vitro* property that correlates best with *in vivo* tumorigenicity (Freedman & Shin, 1974), these observations show that tsB77 cells are transformed at 34 °C (permissive temperature) and nontransformed at 41 °C (nonpermissive temperature).

Expression of OPN in tsB77 Cells Grown at 34 and 41 °C. tsB77 cells were metabolically labeled with [^{32}P]-orthophosphate or with [^{35}S]methionine for 4 h, and aliquots of the conditioned media containing equal amounts of total protein were immunoprecipitated using OST-1 serum (Singh *et al.*, 1992). Figure 1B shows that [^{32}P]orthophosphate-labeled tsB77 cells grown at 34 °C secrete a 62-kDa OPN (lane 1) but at 41 °C secrete a 69-kDa OPN (lane 2), the same form synthesized by normal rat kidney (NRK) cells (Chackalaparampil *et al.*, 1985). Identical results were also obtained when cells at 34 and 41 °C were labeled with [^{35}S]-methionine (lanes 3 and 4, respectively). However, when lysates of ^{32}P-labeled cells grown at either 34 or 41 °C were immunoprecipitated, and analyzed by SDS−PAGE and autoradiography, only the 69-kDa OPN, and no 62-kDa OPN, could be detected (lane 5, 34 °C; lane 6, 41 °C). Northern blot analysis of total RNAs from cells grown at 34 or 41 °C using a full-length OPN cDNA probe revealed a single 1.6 kb mRNA species at both temperatures (Figure 1C: lane 1, 34 °C; lane 2, 41 °C). The above observations, therefore, clearly demonstrate that tsB77 cells at both temperatures synthesize the 69-kDa OPN and the alteration in molecular mass of OPN from 69 to 62 kDa occurs posttranslationally, prior to or immediately after secretion.

Time Course Analysis of the Appearance of the 62-kDa and 69-kDa OPNs after Temperature Shifts. To determine the length of time required for the appearance of the 69-kDa and 62-kDa OPNs in the conditioned media of tsB77 cells after the shifts in temperature from 34 to 41 °C and from 41 to 34 °C, respectively, a time course study was carried out. tsB77 cells grown at 34 °C were shifted to 41 °C, and 0, 12, 24, 36, 48, and 72 h later, the cells were metabolically labeled with [^{32}P]orthophosphate for 3 h, and proteins in the conditioned media were immunoprecipitated and analyzed by SDS−PAGE. Figure 1D shows that 12 h after the shift from 34 to 41 °C, tsB77 cells begin to secrete the 69-kDa OPN (lane 2) and after 24 h posttemperature shift only the 69-kDa OPN can be detected (lanes 3−6). Figure 1E shows that tsB77 cells at 41 °C secrete the 69-kDa OPN (lane 1) and continue to secrete the 69-kDa OPN until about 48 h of incubation at 34 °C (lanes 2−5). Between 48 and 72 h of incubation, the 62-kDa OPN begins to appear in the conditioned medium (lane 6), and after 96 h posttemperature shift, only the 62-kDa OPN could be detected (lane 7). These results show that the 69-kDa OPN appears in the conditioned medium much faster than the 62-kDa OPN after the respective temperature shifts. The rapid appearance of 69-kDa OPN may be explained by the fact that this form of OPN is synthesized by tsB77 cells at both temperatures and is secreted by cells at the nonpermissive temperature without any modifications. The delayed appearance of the 62-kDa OPN is likely due to the time required for the activation of the mechanism involved in the processing of the 69-kDa OPN to its 62-kDa form at the permissive temperature.

V8 Protease Mapping, Tryptic Phosphopeptide Analyses, and Chemical Deglycosylation of OPN. The question was then addressed whether differential mobilities on SDS−

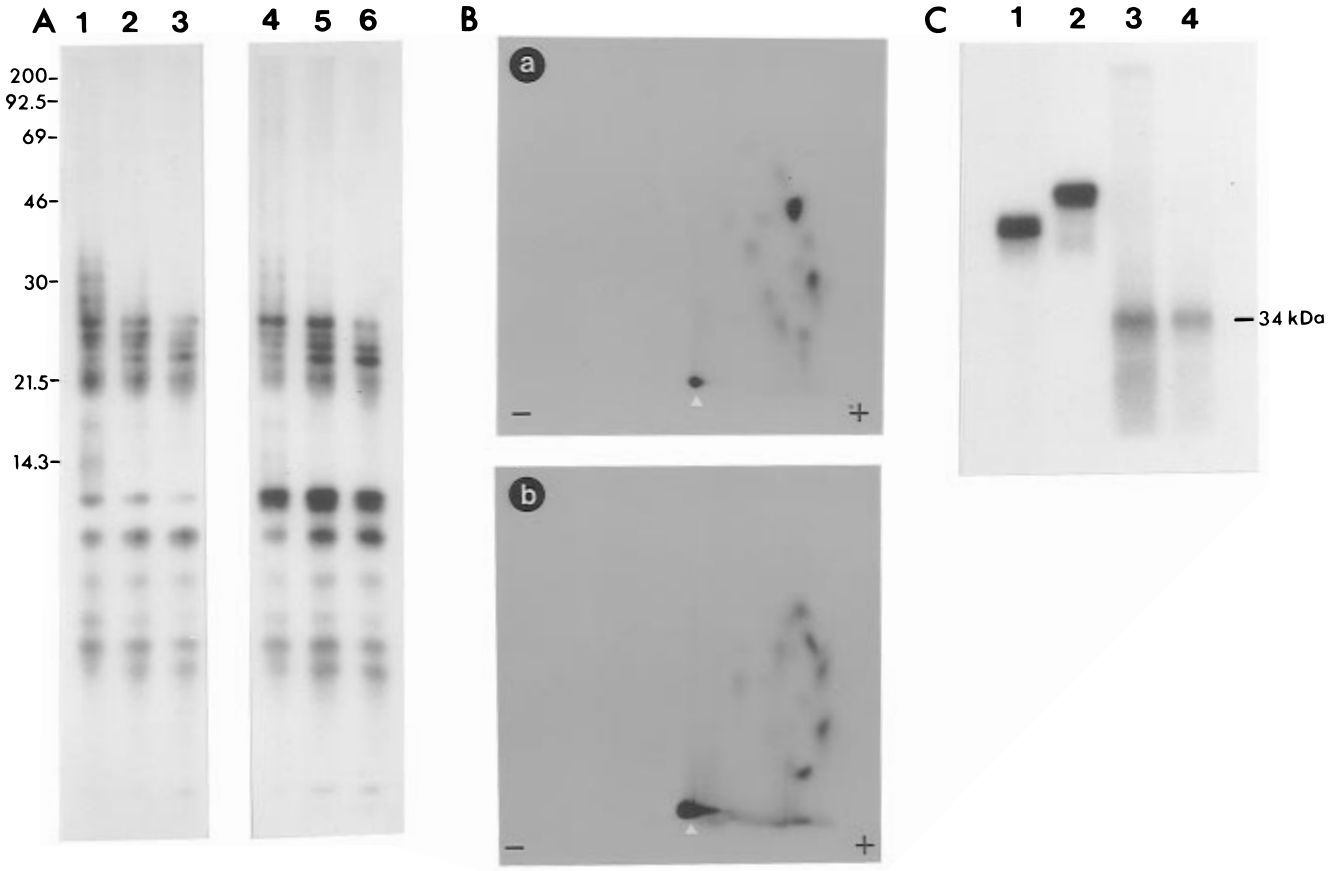

FIGURE 2: (A) V8 protease maps of the 62-kDa and 69-kDa OPNs. 62-kDa OPN digested with 0.1 unit (lane 1), 0.5 unit (lane 2), and 1.0 unit (lane 3) of V8 protease per reaction. 69-kDa OPN digested with 0.1 unit (lane 4), 0.5 unit (lane 5), and 1.0 unit (lane 6) of V8 protease per reaction. (B) Tryptic phosphopeptide maps of the 69-kDa and 62-kDa OPNs. ^{32}P-labeled, gel-eluted OPNs were digested with trypsin, spotted onto cellulose thin-layer plates, and separated into two dimensions by electrophoresis followed by chromatography. 62-kDa (panel a); 69-kDa OPN (panel b). (C) Chemical deglycosylation of the 62-kDa and 69-kDa OPNs. ^{32}P-labeled gel-eluted 62-kDa (lane 3) and 69-kDa OPNs (lane 4) were chemically deglycosylated using the Glycofree Deglycosylation Kit (Oxford Glycosystems), and analyzed by SDS−PAGE. Lanes 1 and 2 show ^{32}P-labeled nondeglycosylated OPNs from tsB77 cells grown at 34 and 41 °C, respectively (controls).

PAGE gels of OPNs secreted by tsB77 cells grown at either 34 or 41 °C are due to differences in the size of the core peptides. The 62-kDa and 69-kDa OPNs were, therefore, digested with V8 protease and analyzed on one-dimensional gels. As shown in Figure 2A, the peptide maps of the 62-kDa (lanes 1−3) and 69−kDa (lanes 4−6) OPNs, digested with either 0.1 unit (lanes 1 and 4), 0.5 unit (lanes 2 and 5), or 1 unit (lanes 3 and 6) of V8 protease, show nearly 100% fragment identity, although the phosphate contents of certain fragments did differ as evidenced by their variable labeling intensities. This suggests that the peptide backbones of both proteins are very similar. To investigate this question further, gel-eluted ^{32}P-labeled 62-kDa and 69-kDa OPNs were digested with trypsin, spotted onto cellulose thin-layer plates, and separated in two dimensions by electrophoresis, followed by chromatography. Figure 2B shows that the tryptic phosphopeptide maps of these two proteins are very similar in that almost all of the radioactive fragments visible in the digest of 69-kDa OPN are also present in the digest of its 62-kDa form (62 kDa, panel a; 69 kDa, panel b). Minor differences that were observed may have resulted from differential glycosylation which may interfere either with the accessibility of the enzyme at certain cleavage sites or with the mobilities of some tryptic fragments. This observation further indicates that the peptide backbones of the 62-kDa and 69-kDa OPNs are very similar, if not identical. Finally, since OPN is heavily glycosylated, we chemically deglycosylated the gel-eluted 62-kDa and 69-kDa OPNs using the Glycofree Deglycosylation Kit (Oxford Glycosystems), and examined their mobilities on SDS−PAGE gels. Figure 2C shows that after deglycosylation, both the 62-kDa (lane 3) and 69-kDa (lane 4) OPNs migrated as a single band of 34-kDa, which approximates the predicted molecular mass of the OPN core peptide (Butler, 1989). Taken together, the above observations demonstrate that the peptide backbones of both proteins are identical in size, and eliminate the possibility that the generation of the 62-kDa OPN results from proteolytic cleavage of the 69-kDa OPN.

Identical Mobilities of OPNs Secreted by tsB77 Cells at 34 and 41 °C after Removal of Their Phosphate Moieties. Rat OPN contains about 13 phosphate groups in the form of 12 phosphoserine and 1 phosphothreonine (Prince *et al.*, 1987). Initial estimation of the ^{32}P/^{35}S ratios carried out as described under Materials and Methods, which reflects the degree of phosphorylation per unit of ^{35}S-labeled protein (Nagata & Yamada, 1986), indicated that the 69-kDa OPN is slightly more phosphorylated than the 62-kDa OPN (data not shown). We, therefore, investigated whether this difference in phosphorylation may contribute to the differential electrophoretic mobilities of OPNs secreted by tsB77 cells at 34 and 41 °C. Immunoprecipitated ^{35}S- and ^{32}P-labeled OPNs, bound to protein A−Sepharose beads, were dephosphorylated using alkaline phosphatase and analyzed by SDS−PAGE, followed by autoradiography. Figure 3A

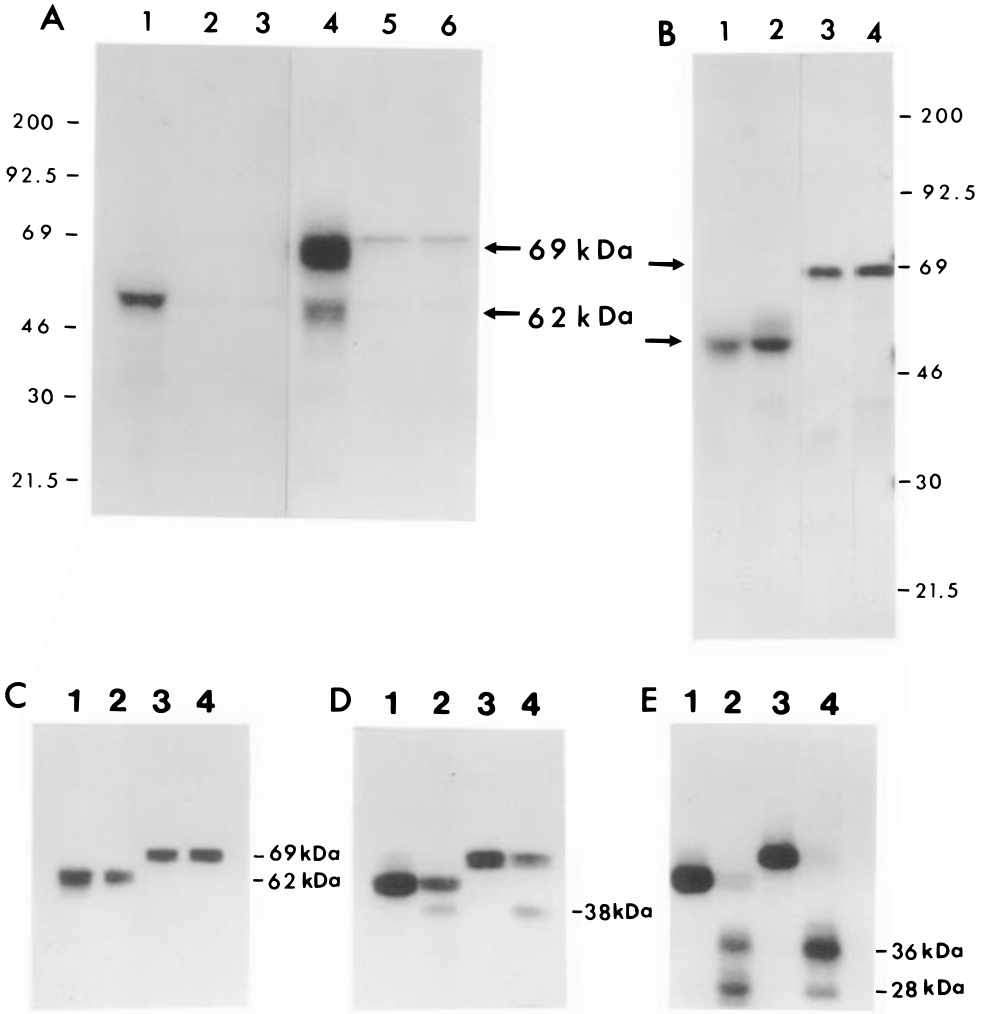

FIGURE 3: (A) Dephosphorylation of 62-kDa and 69-kDa OPNs upon digestion with bacterial alkaline phosphatase. ^{32}P-labeled, 62-kDa (lanes 1−3) and 69-kDa (lanes 4−6) OPNs digested with alkaline phosphatase: lanes 1 and 4, 0 unit; lanes 2 and 5, 2 units; and lanes 3 and 6, 4 units of alkaline phosphatase per reaction. The figure shows that alkaline phosphatase digestion removes almost all the labeled phosphates from both the 62-kDa and 69-kDa OPNs. (B) Migration patterns of dephosphorylated 62-kDa and 69-kDa OPNs on SDS−PAGE gels. ^{35}S-labeled OPNs bound to protein A−Sepharose beads were digested with 2 units of alkaline phosphatase and analyzed by SDS−PAGE as indicated for ^{32}P-labeled OPNs. Undigested 62-kDa OPN (lane 1) and digested (lane 2). Undigested 69-kDa OPN (lane 3) and digested (lane 4). (C) Digestion of ^{32}P-labeled 62-kDa and 69-kDa OPNs with peptide:*N*-glycosidase F. Immunoprecipitated, ^{32}P-labeled 62-kDa and 69-kDa OPNs were digested with peptide:*N*-glycosidase F. Undigested 62-kDa OPN (lane 1) and digested (lane 2). Undigested 69-kDa OPN (lane 3) and digested (lane 4). (D) Digestion of ^{32}P-labeled 62-kDa and 69-kDa OPNs with *O*-sialoglycoprotein endopeptidase. Undigested 62-kDa OPN (lane 1) and digested (lane 2). Undigested 69-kDa OPN (lane 3) and digested (lane 4). Both forms of OPN upon digestion with *O*-sialoglycoprotein endopeptidase migrated as a single 38-kDa band (lanes 2 and 4). (E) SDS−PAGE analysis of ^{32}P-labeled 62-kDa and 69-kDa OPNs after digestion with neuraminidase. Undigested 62-kDa OPN (lane 1) and digested (lane 2). Undigested 69-kDa OPN (lane 3) and digested (lane 4). Both molecular forms of OPN, upon digestion with neuraminidase, migrated identically as two discrete bands with apparent molecular masses of 36 and 28 kDa (lanes 2 and 4).

shows that this enzymatic treatment effectively removes almost all the labeled phosphates from both the 62-kDa (lanes 2 and 3) and 69-kDa (lanes 5 and 6) OPNs. As shown in Figure 3B, dephosphorylation of ^{35}S-labeled OPNs (62 kDa, lane 2; 69 kDa, lane 4) did not result in differential electrophoretic mobilities of these proteins as compared to their phosphorylated counterparts (62 kDa, lane 1; 69 kDa, lane 3), demonstrating that the differences in the migrations of the OPNs secreted by tsB77 cells at 34 and 41 °C cannot be attributed to their phosphate contents. Similar observations have been made by other investigators with phosphorylated and nonphosphorylated OPNs from the conditioned media of TPA-treated and calcitriol-treated mouse JB6 epidermal cells (Chang & Prince, 1991, 1993), and bovine bone-extracted OPN treated with tartrate-resistant acid phosphatase (TRAP) which dephosphorylates the protein (Ek-Rylander *et al.*, 1994). These observations are in agreement with the previous reports that phosphate content only marginally influences the mobility of proteins on SDS−PAGE (Kmiecik & Shalloway, 1987; Hunter & Sefton, 1980).

Sialic Acid Content Differentiates 69-kDa from 62-kDa OPN. Although dephosphorylation of the 62-kDa and 69-kDa OPNs had no effect on their electrophoretic mobilities in SDS−PAGE, upon chemical deglycosylation both proteins migrated as a 34-kDa band (Figure 2C). This was expected since it has been shown that OPN isolated from rat bone contains 1 N-linked and 5 or 6 O-linked oligosaccharides, and 10 out of its 30 monosaccharides are sialic acid residues (Prince *et al.*, 1987). Experiments were, therefore, designed to define the contributions of N-linked, serine/threonine-linked (O-linked) oligosaccharides, and the sialic acid residues in the generation of the two OPN isoforms. First, immunoprecipitated ^{32}P-labeled 62-kDa and 69-kDa OPNs

were digested with peptide:*N*-glycosidase F, and analyzed by SDS−PAGE. Peptide:*N*-glycosidase F treatment of denatured proteins removes N-linked oligosaccharide chains at the asparagine and converts that residue to aspartic acid (Cunningham *et al.*, 1984). Figure 3C shows that digestion with this enzyme did not alter the migration of the two forms of OPNs (lanes 2 and 4), as compared to their undigested counterparts (lanes 1 and 3), suggesting that tsB77 cell-secreted OPN does not contain N-linked oligosaccharides, and therefore may not be responsible for the decrease of the molecular mass of the OPN molecule from 69 kDa to 62 kDa. This conclusion is supported by our previous observations that nonphosphorylated OPN, but not phosphorylated OPN, secreted by NRK cells, contains N-linked carbohydrates. Furthermore, NRK cells treated with tunicamycin secrete a 67-kDa OPN instead of the 69-kDa form, and NRK cell-secreted 69-kDa OPN upon digestion with peptide:*N*-glycosidase F also migrates as a 67-kDa band on SDS−PAGE (Singh *et al.*, 1990). Immunoprecipitated, ^{32}P-labeled 62-kDa and 69-kDa OPNs were then digested with *O*-sialoglycoprotein endopeptidase, and analyzed by SDS−PAGE. This enzyme, isolated from culture supernatants of *Pasteurella haemolytica A1*, specifically cleaves *O*-sialoglycoproteins, but does not cleave N-glycosylated or desialylated proteins (Abdullah *et al.*, 1992). As shown in Figure 3D, both the 62-kDa and 69-kDa OPNs, upon digestion, migrated as a single 38-kDa band (lanes 2 and 4, respectively), suggesting the possibility that differences in O-linked oligosaccharide contents may contribute to the generation of these OPN isoforms. The 62-kDa band in lane 2 and the 69-kDa band in lane 4 represent undigested OPNs, which upon longer treatment with *O*-sialoglycoprotein endopeptidase migrated completely as a 38-kDa band (data not shown).

OPN is rich in sialic acid residues, which are generally found in the terminal position of the carbohydrate chains of glycolipids and glycoproteins. Since *O*-sialoglycoprotein endopeptidase specifically removes the O-linked oligosaccharides along with the associated sialic acid residues, it was difficult to determine from the data in Figure 3D whether variation in the O-linked carbohydrate or sialic acid contents, or both, is responsible for the generation of the 62-kDa OPN. We, therefore, digested immunoprecipitated protein A−Sepharose-bound ^{32}P-labeled 62-kDa and 69-kDa OPNs with neuraminidase from *Closteridium perfringens* (ICN) which removes α-2,3, α-2,6, and α-2,8 sialosides but has a higher preference for α-2,3. The digestion products were then analyzed by SDS−PAGE and autoradiography. Figure 3E shows that both molecular forms of OPNs, after digestion, migrated identically as two discrete bands with apparent molecular masses of 36 and 28 kDa (lanes 2 and 4). As specified by the supplier, we also could not detect any protease activity in this preparation (unpublished data). Even if proteolytic cleavage is considered to be involved in the generation of two fragments upon neuraminidase digestion, such cleavage products of the 69-kDa and 62-kDa OPNs would not have migrated identically as 36-kDa and 28-kDa bands if sialic acid residues did not play a role in the differential mobilities of these two OPN isoforms. We are unable to provide an explanation for the generation of two OPN fragments after neuraminidase treatment of each of the 62-kDa and 69-kDa OPNs, except for the possibility that the removal of sialic acid residues makes the core peptide unstable, resulting in its fragmentation at a specific site. Since

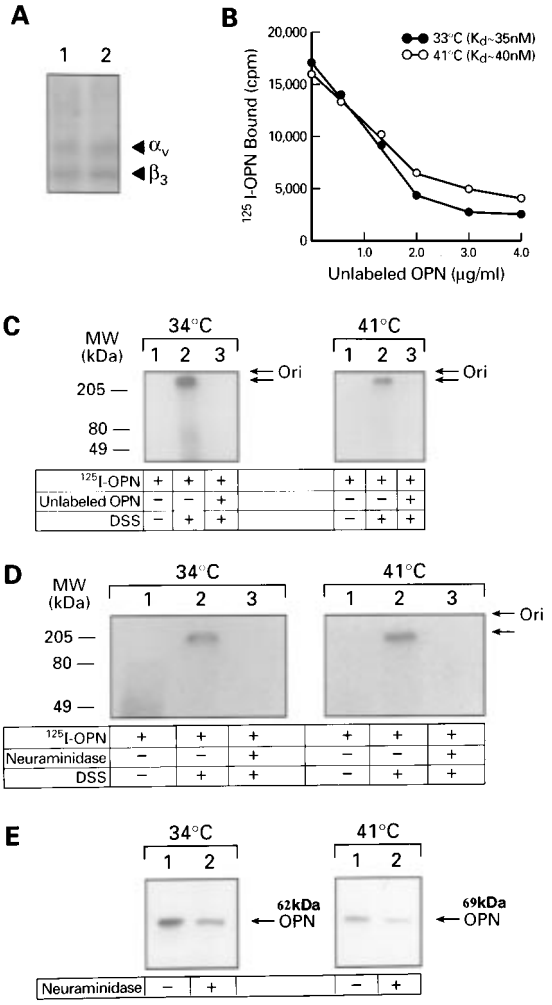

FIGURE 4: (A) Western blot analysis of $\alpha_v\beta_3$ integrins synthesized by tsB77 cells at 34 and 41 °C. Total proteins from the conditioned media of tsB77 cells at 34 and 41 °C were separated by SDS−PAGE, transferred to an Immobilon-P membrane, and immunostained with rabbit anti-human vitronectin receptor polyclonal antibody. Lane 1, lysate from cells at 34 °C; lane 2, at 41 °C. tsB77 cells showed the presence of a 125-kDa α_v subunit and a 105-kDa β_3 subunit under reducing conditions at both temperatures, indicated by arrows. (B) Binding of purified milk OPN on tsB77 cells. ^{125}I-labeled milk OPN was incubated with tsB77 cells using various concentrations of unlabeled OPN at 34 and 41 °C. Graph represents binding of ^{125}I-OPN on the cell surface in the presence of the indicated concentrations of unlabeled OPN. Each point is an average of duplicate determinations. Scatchard plot analysis yielded dissociation constants (K_d) of ~35 and 40 nM at 34 and 41 °C, respectively. (C) Affinity cross-linking of ^{125}I-OPN to its receptor on tsB77 cells. Purified milk OPN labeled with ^{125}I was incubated with tsB77 cells in the presence and absence of unlabeled OPN for 3 h and then cross-linked with a homobifunctional cross-linker, DSS. SDS−PAGE analysis under reducing conditions identified a radiolabeled OPN−receptor complex band with a molecular mass of ~300 kDa in lysates of cells at both 34 and 41 °C. (D) Affinity cross-linking of neuraminidase-treated ^{125}I-OPN to its receptor on tsB77 cells. tsB77 cells at both temperatures were incubated with ^{125}I-labeled milk OPN, digested and undigested with neuraminidase, and then cross-linked with DSS. A ~300 kDa radiolabeled OPN−receptor complex band was identified at both 34 and 41 °C. (E) Neuraminidase digestion of [^{3}H]mannose-labeled 62-kDa and 69-kDa OPNs. The left panel represents 62-kDa OPN (lane 1, undigested; lane 2, digested), and the right panel represents 69-kDa OPN (lane 1, undigested; lane 2, digested).

our present study demonstrates that tsB77 cell-secreted OPN does not contain N-linked oligosaccharides, all sialic acid residues of this OPN must be present in the O- linked

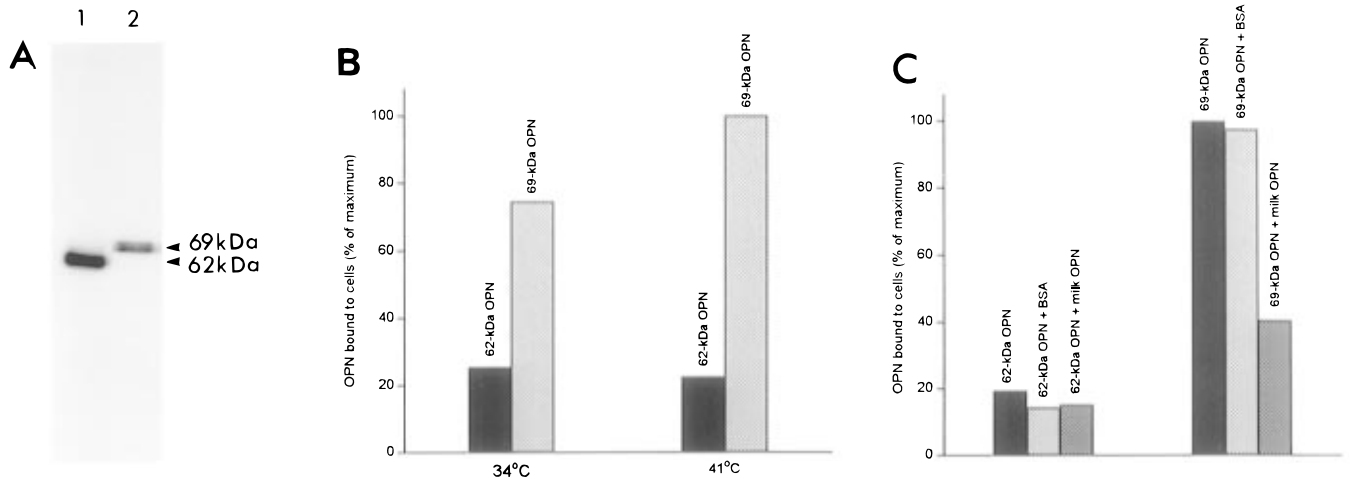

FIGURE 5: Binding of ^{32}P-labeled 62-kDa and 69-kDa OPNs to the surface of tsB77 cells in suspension. (A) SDS−PAGE analysis of ^{32}P-labeled OPNs which were used in the binding experiments described in panels B and C. Lane 1, 62-kDa OPN; lane 2, 69-kDa OPN. (B) Binding of ^{32}P-labeled OPN to tsB77 cells. Significant amounts of 69-kDa OPN were bound to tsB77 cells at both temperatures after 2 h of incubation as compared to 62-kDa OPN, at either temperature. The data represent the average of three independent experiments. (C) Binding of ^{32}P-labeled OPNs in the presence of purified unlabeled milk OPN or BSA at the permissive temperature. Binding of ^{32}P-labeled 69-kDa OPN was less than 50% in the presence of 6 μg/mL unlabeled milk OPN, but remained unchanged in the presence of the same concentration of BSA. The 62-kDa OPN, however, did not show any cell surface binding, above the background level, in the absence or presence of unlabeled OPN or BSA. The data represent the average of three independant experiments.

oligosaccharide chains. Therefore, identical banding patterns observed after digestion of each of the 62-kDa and 69-kDa OPNs with *O*-sialoglycoprotein endopeptidase or neuraminidase suggest that the decrement of 7 kDa in the apparent molecular mass of OPN secreted by tsB77 cells at 34 °C must be attributed to the loss of sialic acid residues. If the two proteins differed in their O-linked oligosaccharides instead of sialic acid contents, the 62-kDa and 69-kDa OPNs would have migrated differentially on SDS−PAGE after treatment with neuraminidase. Furthurmore, confluent cells grown at either 34 or 41 °C were labeled with D-[2,6-^{3}H]-mannose, and the conditioned media containing OPN were immunoprecipitated. The immune complexes were treated with neuraminidase, and the samples were resolved by SDS−PAGE. Figure 4E (lane 2 of both panels) showed that the intensities of the OPN protein band were reduced significantly upon neuraminidase treatment as compared to the untreated samples (lane 1 of both panels) obtained from cells at both 34 and 41 °C, demonstrating that the label is partially removed by neuraminidase treatment. This confirms the conclusion that the difference in the two molecular forms of OPNs is due to their variable sialic acid contents. Differences in molecular weight due to altered sialic acid content between adult and embryonic neural cell adhesion molecules (N-CAM) have also been observed (Rothbard *et al.*, 1982).

Binding of ^{125}I-labeled Purified Human Milk OPN to tsB77 Cells. Before carrying out the ^{125}I-OPN-binding assay, we examined whether tsB77 cells at both 34 and 41 °C express $\alpha_v\beta_3$ integrins on their cell surface. Therefore, cell lysates were separated on 7.5% gels, transferred to an Immobilon-P membrane, and immunostained using rabbit anti-human vitronectin receptor antibody. Figure 4A shows that tsB77 cells at both 34 °C (lane 1) and 41 °C (lane 2) express the $\alpha_v\beta_3$ (osteopontin) receptors. Purified human milk OPN (obtained from Dr. Donald Senger) labeled with ^{125}I was then added to cultures of tsB77 cells at 34 and 41 °C, and incubated for 3 h in the absence or presence of increasing concentrations of unlabeled OPN. Cells were then solubilized, and the radioactivity in aliquots of cell lysates was measured by a gamma-counter. Figure 4B shows that tsB77 cells at both temperatures bind OPN on their cell surface and with equal efficiency. This binding is specific because increasing concentration of unlabeled OPN drastically reduces such binding. Scatchard analysis of the binding data yielded dissociation constants (k_d) of ~35 and 40 nM at 34 and 41 °C, respectively. This observation demonstrates that OPN binds to the cell surface in a receptor-mediated manner. To confirm this observation, tsB77 cells grown at 34 and 41 °C were first incubated with ^{125}I-OPN for 3 h followed by treatment with a homobifunctional cross-linker, DSS, for 30 min, and cell lysates were analyzed by SDS−PAGE. A single radiolabeled protein band with a molecular mass of ~300 kDa (Figure 4C, lane 2 of both left and right panels indicated by an arrow) was identified in lysates of cells at both temperatures. As expected, no protein band was detected in the absence of DSS (lane1 of both panels) or in the presence of unlabeled OPN (lane 3 of both panels). However, when OPN was pretreated with neuraminidase and then incubated with tsB77 cells grown at either temperature, no receptor protein band was visualized (Figure 4D, lane 3 of both panels). Taken together, these observations confirm that tsB77 cells grown both at 34 and at 41 °C synthesize $\alpha_v\beta_3$ integrins with which OPN interacts, and suggest that sialic acid residues of OPN are essential for receptor binding.

Binding of ^{32}P-labeled 62-kDa and 69-kDa OPNs to tsB77 Cells. We then examined the cell-binding abilities of the 62-kDa OPN which contains significantly reduced levels of sialic acid. Therefore, ^{32}P-labeled 62-kDa and 69-kDa OPNs (Figure 5A), prepared as described under Materials and Methods, were added to tsB77 cells in suspension. As shown in Figure 5B, 69-kDa OPN bound to tsB77 cells at both temperatures after 2 h, but no significant binding of 62-kDa OPN was observed at either temperature. To further examine the specificity of binding of the 69-kDa OPN, unlabeled purified milk OPN was added at a concentration of 6 μg/mL along with labeled 69-kDa OPN. Figure 5C shows that in the presence of unlabeled OPN, binding of ^{32}P-labeled

69-kDa OPN was reduced to less than half, whereas the extent of binding remained unaltered in the presence of the same concentration of BSA, indicating that this binding was receptor-mediated. The ^{32}P-labeled 62-kDa OPN did not show cell surface binding above the background level, and such binding remained unaltered when competed with unlabeled purified OPN. Our data, therefore, demonstrate that the 69-kDa OPN, and not its 62-kDa form, interacts with the cell surface. This could be the result of possible conformational changes of the 62-kDa OPN due to altered sialylation, which may prevent its recognition by its receptor ($\alpha_v\beta_3$). The role of sialic acids in influencing the structural diversity of glycoproteins has been reported, and enzymatic removal of sialic acids causes marked differences in the physiological properties of cells and molecules (Schauer, 1985). The aberrant sialylation of 62-kDa OPN may have been caused by a specific sialidase which is expressed by the tsB77 cells at 34 °C, but not at 41 °C. This is supported by the demonstration that neoplastic transformation is accompanied by an increase in activity of specific sialidases (Santer *et al.*, 1989), and the presence of multiple forms of sialidases, including plasma membrane-associated sialidases, and their alterations in carcinogenesis in mammalian cells is well documented (Miagi *et al.*, 1992). The identification and characterization of such a sialidase may provide an important clue for elucidating the putative roles of OPN in oncogenic cell transformation, and the functional significance of sialic acid residues of this protein in that process.

It has been reported recently that the active form of matrix metalloproteinase MMP-2 binds directly to integrin $\alpha_v\beta_3$, and these receptor−ligand complexes are found on the surface of invasive cells. Suggestion has been made that this interaction between an integrin and a protease causes matrix degradation which facilitates cellular invasion (Brooks *et al.*, 1996). In the context of the above observation, the inability of the 62-kDa OPN to interact with the $\alpha_v\beta_3$ integrin, observed in the present study, appears significant. Since tsB77 cells synthesize $\alpha_v\beta_3$ integrins at both permissive and nonpermissive temperatures, in the absence of competing OPN (62-kDa OPN which does not bind to the cell surface), MMP-2 binding to the $\alpha_v\beta_3$ integrins of the tsB77 cells at the permissive temperature may increase significantly, thereby promoting the invasive phenotype of tsB77 cells at the permissive temperature, which is one of the main distinguishing characteristics of transformed cells.

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