Identification of Galectin-3-binding Protein as a Factor Secreted by Tumor Cells That Stimulates Interleukin-6 Expression in the Bone Marrow Stroma*

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Yasushi Fukaya†, Hiroyuki Shimada‡, Ling-Chi Wang§, Ebrahim Zandi¶ and Yves A. DeClerck§††

From the †Division of Hematology-Oncology and Department of Pediatrics, ‡Department of Pathology, §Department of Molecular Microbiology and Immunology, and ¶Department of Biochemistry and Molecular Biology, Keck School of Medicine of the University of Southern California and The Saban Research Institute of Childrens Hospital Los Angeles, Los Angeles, California 90027

We have previously demonstrated that neuroblastoma cells increase the expression of interleukin-6 by bone marrow stromal cells and that stimulation does not require cell-cell contact. In this study we report the purification and identification of a protein secreted by neuroblastoma cells that stimulates interleukin-6 production by stromal cells. Using a series of chromatographic purification steps including heparin-affinity, ion exchange, and molecular sieve chromatography followed by trypsin digestion and liquid chromatography tandem mass spectrometry, we identified in serum-free conditioned medium of neuroblastoma cells several secreted peptides including galectin-3-binding protein, also known as 90-kDa Mac-2-binding protein. We demonstrated the presence of the galectin-3-binding protein in the conditioned medium of several neuroblastoma cell lines and in chromatographic fractions with interleukin-6 stimulatory activity. Consistently, bone marrow stromal cells express galectin-3, the receptor for galectin-3-binding protein. Supporting a role for galectin-3-binding protein in stimulating interleukin-6 expression in bone marrow stromal cells, we observed that recombinant galectin-3-binding protein stimulated interleukin-6 expression in these cells and that interleukin-6 stimulation by neuroblastoma-conditioned medium was inhibited in the presence of lactose or a neutralizing anti-galectin-3 antibody. Down-regulation of galectin-3-binding protein expression in neuroblastoma cells also decreased the interleukin-6 stimulatory activity of the conditioned medium on bone marrow stromal cells. We also provide evidence that stimulation of interleukin-6 by galectin-3-binding protein involves activation of the Erk1/2 pathway. The data, thus, identifies galectin-3-binding protein as a factor secreted by neuroblastoma cells that stimulates the expression of interleukin-6 in bone marrow stromal cells and provides a novel function for this protein in cancer progression.

It has become increasingly evident that the tumor microenvironment plays an important contributory role to cancer progression (1). Tumor cells closely interact with non-malignant cells present in the tumor stoma through a series of adhesion-dependent and adhesion-independent mechanisms. Among the adhesion-independent interactions, cytokines, chemokines, and growth factors play a critical role (2, 3). In this regard, the bone marrow, which is a frequent site of metastasis, provides a microenvironment that is particularly favorable to cancer progression because it is a source of hematopoietic and mesenchymal cells that produce a large number of cytokines and chemokines (4–6).

Neuroblastoma is a neural crest-derived cancer that is the second most common solid tumor in children (7). At the time it is diagnosed, it has metastasized in 56% of the cases, primarily to the bone marrow, the bone, and the liver (8). The mechanisms by which neuroblastoma cells invade the bone have been recently elucidated by several laboratories, including our group (9–11). We have previously shown that bone metastasis in human neuroblastoma is associated with activation of osteoclasts and an increase in bone resorption (12). We more recently reported (13) that activation of osteoclasts by neuroblastoma cells involves the stimulation of interleukin-6 (IL-6)2 production by bone marrow stromal cells (BMSC), IL-6 being a potent activator of osteoclasts. A similar interaction between tumor cells and BMSC has been previously demonstrated to play a critical role in multiple myeloma, a cancer also characterized by an increase in bone destruction (14, 15). However, in contrast to myeloma, where the production of IL-6 by BMSC is dependent on an adhesion contact between BMSC and tumor cells, in neuroblastoma, stimulation of IL-6 does not involve cell-cell contact and occurs in the presence of serum-free conditioned medium (CM) from neuroblastoma cells. In this manuscript we report the purification and characterization of a protein secreted by human neuroblastoma cells and present in their culture medium that stimulates the expression of IL-6 by human BMSC. This protein was identified as galectin-3-binding protein (Gal-3BP), also known as 90-kDa Mac-2-binding protein, a secreted glycoprotein with a molecular mass of ~90 kDa initially identified and characterized in breast cancer cells and in human milk (16).

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1 To whom correspondence should be addressed: Childrens Hospital Los Angeles, 4650 Sunset Blvd., MailStop 54, Los Angeles, CA 90027. Tel.: 323-361-2150; Fax: 323-361-4902; E-mail: declerck@usc.edu.

2 The abbreviations used are: IL-6, interleukin-6; rh, recombinant human; TNF, tumor necrosis factor-α; BMSC, bone marrow stromal cells; CM, conditioned medium; Gal-3BP, galectin-3-binding protein; Erk, extracellular signal-regulated kinase; MS, mass spectroscopy; siRNA, small interfering RNA.
Gal-3BP Stimulates IL-6 Expression in Bone Marrow Stroma

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Cell Culture**—The human neuroblastoma cell lines CHLA-255 and SK-N-BE(2) were kindly provided by Dr. C. P. Reynolds (Childrens Hospital Los Angeles), and the NB-19 cell line was purchased from RIKEN BioResource Center (Tsukuba, Japan). CHLA-255 cells were cultured in Iscoves modified Dulbecco’s medium containing HEPES (25 mM), penicillin (100 IU/ml), and streptomycin (100 μg/ml) (Cellgro). The medium was supplemented with 10% (v/v) fetal bovine serum, insulin (0.25 μg/ml), transferrin (0.25 μg/ml), selenium (0.25 ng/ml) (Lonza Inc., Basel, Switzerland) and l-glutamine (2 mM). NB-19 cells and SK-N-BE(2) cells were cultured in RPMI1640 (Invitrogen) with penicillin-streptomycin, 10% (v/v) fetal bovine serum, insulin (0.25 μg/ml), transferrin (0.25 μg/ml), selenium (0.25 ng/ml), and l-glutamine. Human BMSC were obtained from ALLCELLS (Emeryville, CA) and maintained in basal medium with stimulatory supplements containing basic fibroblast growth factor (10 ng/ml).

**Reagents and Antibodies**—Trypsin was purchased from Sigma-Aldrich Corp., dissolved in 1 mM HCl, and stored at a concentration of 1 mg/ml at −20 °C. Soybean trypsin inhibitor was purchased from EMD Bioscience, Inc. (San Diego, CA) and stored at −20 °C at a concentration of 10 mg/ml in potassium phosphate (67 mM), pH 7.6. PD98059 was obtained from Calbiochem. Lactose and sucrose were purchased from Sigma-Aldrich. A goat anti-human Gal-3BP antibody was purchased from R&D Systems. A mouse monoclonal antibody against human Gal-3 was kindly provided by Dr. Avraham Raz (Karmanos Cancer Institute, Wayne State University, Detroit, MI).

**Trypsin Digestion**—CM obtained from human neuroblastoma cell lines was incubated with 100 μg/ml trypsin at 37 °C for 1 h. The reaction was stopped by adding 200 μg/ml soybean trypsin inhibitor for 30 min.

**Preparation of CM from Neuroblastoma Cells**—Neuroblastoma cells were plated at 5 × 10⁶ in 150 cm² tissue culture flasks for 72 h in culture medium containing 10% (v/v) fetal bovine serum. The tissue culture flasks were then washed twice with phosphate-buffered saline (4 mM KCl, 0.5 mM Na₂HPO₄, 140 mM NaCl, and 0.15 mM KH₂PO₄), and serum-free medium was added. After 24 h, the CM was collected and centrifuged at 1500 rpm for 10 min at 4 °C before being filtered through a 0.2-μm filter (Corning Inc., Corning, NY) and stored at −20 °C until used for chromatographic separation. Before chromatography, the CM was concentrated 10- and 50-fold by pressure dialysis using a 10,000 nominal molecular weight limit polyethersulfone membrane in a stirred ultra-filtration cell (Millipore, Billerica, MA) at 4 °C.

**Protein Separation by Column Chromatography**—All purification procedures were carried out at 4 °C using an AKTA-prime plus chromatography system (GE Healthcare). Briefly, the concentrated CM was first applied to either a 1- or 5-ml heparin-Sepharose column (HiTrap Heparin, GE Healthcare) equilibrated with 10 mM sodium phosphate buffer, pH 7.0, at a flow rate of 1–2 ml/min. The bound proteins were eluted in one step with 1 M NaCl. The active fractions (see below) were pooled, desalted by passing through a YM-10 membrane, and resuspended in 20 mM Tris-HCl, pH 7.4. The pool was then applied to a 1-ml diethylaminoethyl-Sepharose column (HiTrap DEAE Fast Flow, GE Healthcare) equilibrated with the same buffer at a flow rate of 1 ml/min. Bound proteins were eluted using a linear gradient of NaCl from 0 to 0.5 M. The active fractions were pooled and concentrated by passing through a YM-10 membrane, equilibrated in 20 mM Tris-HCl, pH 7.4, and applied to a molecular sieve column (Superdex 200, 5 mm × 150 μM, GE Healthcare) at a flow rate of 0.2 ml/min. Each fraction eluting from the chromatography was examined for protein concentration using the BCA protein assay reagent kit (Pierce) for IL-6 stimulatory activity (see below) and for protein content by SDS-PAGE (see below).

**Measurement of IL-6 Stimulatory Activity**—Individual fractions separated by chromatography were tested for their ability to simulate the expression of IL-6 in BMSC in culture. In brief, BMSC were seeded in 24-well tissue culture plates at 10⁵ cells/well (1.9 cm², Corning, Inc.) and maintained for 48 h in serum-containing medium. Each well was then washed twice with phosphate-buffered saline and incubated for 24 h with 1 ml of serum-free medium supplemented with an aliquot (50 or 100 μl) of individual chromatographic fractions. The supernatant of the culture medium was then collected, clarified by centrifugation at 12,000 rpm for 5 min at 4 °C, and examined for IL-6 concentration by enzyme-linked immunosorbent assay (ELISA) using a human IL-6 ELISA kit purchased from R&D Systems. The specific activity of each fraction was expressed in pg of IL-6/mg of protein in the fraction tested.

**SDS-PAGE and Immunoblotting**—SDS-PAGE was performed using 4–12% gradient acrylamide gels (Invitrogen). Proteins were visualized by silver staining or transferred onto nitrocellulose membranes by semi-dry blotting (Bio-Rad). After being blocked in 10 mM Tris-HCl, pH 8, 150 mM NaCl, 0.1% Tween 20 containing 5% nonfat dried milk, the membranes were incubated overnight at 4 °C with the primary antibody in blocking buffer. After washing, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (dilution 1:10,000 v/v) for 1 h. The presence of immune complexes was identified by enhanced chemiluminescence (Amersham Biosciences). Gels were scanned, and quantitative analysis was done using the NIH J image software.

**Mass Spectrometry; Trypsin Digestion and Liquid Chromatography Tandem Mass Spectrometry (MS/MS)**—The protein mixture was dissolved in 20 μl of 50 mM ammonium bicarbonate and 4 mM urea and reduced by adding dithiothreitol to a final concentration of 10 mM at 95 °C for 10 min. The sample was immediately alkylated by adding 2 μl of 50 mM iodoacetamide at 37 °C for 30 min. The urea was diluted to 1 M with 72 μl of 100 mM ammonium bicarbonate, and the proteins were digested with 1 μg of sequencing grade modified trypsin (Promega, Madison, WI) at 37 °C overnight. The digestion was continued...
for an additional 24 h by adding 1 µg of trypsin. For MS analysis, 13 µl of 10% (v/v) formic acid was added, and the sample was desalted and concentrated using C18 reverse phase. One-fourth of the sample was loaded on a 10-cm reversed phase chromatography capillary column connected to a spray tip column through a MicroTee with in-unison high voltage contact as previously described (17). Peptides were eluted into the mass spectrometer using the gradients 5–50% acetonitrile + 0.1% formic acid over 75 min and 50–90% acetonitrile + 0.1% formic acid over 40 min. A split tee with a fused silica capillary (25-µm internal diameter) on one end was used to split the high pressure liquid chromatography flow from 50 to 500 µl/min. The other end of the tee was connected to a six-port divert/inject valve, which was used to introduce peptide samples into the column.

A linear ion trap LTQ (Thermo Electron, Inc.) was used to acquire tandem MS/MS spectra with Xcalibur 2.1 software using the following method; a full MS scan was followed by seven consecutive MS2 scans of the top 7 ion peaks from the preceding full scan using dynamic exclusion (4 repetitions in 1.5 min were excluded for 15 min). Data were analyzed using Bioworks 3.2, utilizing the SEQUEST algorithm and Sage-N Sorcerer to determine cross-correlation scores between acquired spectra and NCBI protein FASTA databases. The following parameters were used for the TurboSEQUEST search: molecular weight range, 0–5000; threshold, 1000; monoisotopic; precursor mass, 1.4; group scan, 10; minimum ion count, 20; charge state, auto; peptide, 1.5; fragment ions, 0; static amino acid modifications, Cys 57.05 and Met 15.99. Results were filtered using SEQUEST cross-correlation scores greater than 2.0 for +1 ions, 3.0 for +2 ions, and 3.5 for +3 ions.

**Gal-3BP-blocking Experiment**—Lactose and a blocking antibody against Gal-3 (TIB-166) were used in blocking experiments with BMSC. Lactose (50 to 150 mM) or TIB-166 (diluted 1/1000 to 1/100 v/v) were added for 2 h to the culture medium of BMSC before the addition of neuroblastoma CM or active pooled chromatographic fractions. Sucrose and mouse nonspecific IgG were used as a negative control for lactose and TIB-166, respectively.

**Fluorescence-activated Cell Sorter Analysis**—Cells were washed in phosphate-buffered saline, detached in cell dissociation buffer, and recovered by centrifugation at 4500 rpm for 5 min before being resuspended in phosphate-buffered saline containing 1% (v/v) bovine serum albumin. Aliquots containing 1 × 10⁶ cells were incubated with antibodies at a final concentration of 1 µg/10⁶ cells. After incubation for 15 min, cells were washed and reincubated with a secondary fluorescein isothiocyanate-conjugated anti-mouse IgG antibody (1:1000 v/v). Cells were then analyzed using a FACScan flow cytometer, and the data were analyzed using CellQuest software (BD Bioscience). Nonspecific fluorescence was determined in the presence of an unlabeled anti-mouse IgG antibody.

**Transfection of siRNA**—CHLA-255 neuroblastoma cells were seeded onto 24-well plates and cultured in medium without antibiotics for 24 h before transfection. Two different siRNAs for Gal-3BP were purchased from Qiagen, Inc. (Valencia, CA): Hs_LGALS3BP#1, 5′-CCG UCA UCC UGA CUG CCA A-3′, and Hs_LGALS3BP#5, sense 5′-CCA UGA GUG UGG AUG CUG A-3′. A scrambled control sequence was used as the control. siRNA transfection was done in the presence of Lipofectamine 2000 (Invitrogen), and a fluorescein isothiocyanate-labeled siRNA was used to assess the transfection efficiency.

**Statistical Analysis**—Student’s t test was used for the comparison between two sets of data. The analysis of variance was used for the analysis of trends. A p value of <0.05 was considered statistically significant.

**RESULTS**

**CM of Neuroblastoma Cells Contains a Protein(s) That Stimulates IL-6 Production in BMSC**—In support of the concept that the stimulation of IL-6 by neuroblastoma CM was mediated by a protein(s) produced by neuroblastoma cells, we had previously demonstrated that treatment of neuroblastoma cells with cycloheximide resulted in a loss of IL-6 stimulatory activity in the CM (13). To obtain additional evidence in support of the stimulatory factor being a protein, we demonstrated that treatment of CM from two neuroblastoma cell lines (NB-19 and CHLA-255) with trypsin significantly decreased the amount of IL-6 produced by BMSC cultured in the presence of these CM (Fig. IA). Treatment of CHLA-255 CM with heat (50 and 90 °C) also resulted in a substantial loss of IL-6 stimulatory activity. The data are, thus, consistent with the IL-6 stimulatory factor present in neuroblastoma CM being a (or possibly several) pro-
tein. Interestingly, we also observed an increase in Erk1/2 activation (phosphorylation) when BMSC were cultured in the presence of neuroblastoma CM, raising the possibility that the stimulatory protein could be a ligand signaling via Erk1/2 (Fig. 1B).

**Chromatographic Purification of a Protein with IL-6 Stimulatory Activity**—We, therefore, initiated a series of chromatographic separations in an attempt to isolate and identify the active protein(s). A typical example of the purification protocol used is shown in Fig. 2. Serum-free CM from CHLA-255 cells was concentrated 50-fold by pressure dialysis on a 10,000 nominal molecular weight limit polyethersulfone membrane, applied to a heparin affinity column, and eluted with 1 M NaCl (Fig. 2A). This initial purification step resulted in the identification of 2 sets of fractions (heparin-unbound and heparin-bound) with IL-6 stimulatory activity. The heparin-bound fractions (13 and 14), which had a higher specific activity than the heparin-unbound fractions, were then pooled, desalted, applied to a DEAE anion exchange column, and eluted with a 0–0.5 M NaCl gradient (Fig. 2B). This resulted in the identification of a broad peak of active fractions (20–28) that eluted between 0.15 and 0.35 M NaCl. These fractions (9 ml) were pooled, desalted, concentrated 50× (180 μl), and applied to a gel filtration (Superdex 200) column (Fig. 2C). This chromatographic purification step resulted in the identification of 2 peaks of activity that, however, remained poorly separated. The activity and specific activity recovered during a typical purification procedure of CM from CHLA-255 cells is shown in Table 1. From 6 liters of CM containing 45.26 mg of protein, we obtained at the end 4.6 μg of active material with a specific activity of 203,977 pg of IL-6/mg of protein in the first peak eluting from the molecular sieve column and 7.6 μg of protein with a specific activity of 124,466 pg of IL-6/mg of protein in the second peak. Aliquots of the active fractions identified during these chromatographic steps were examined by SDS-PAGE and silver stain for protein content (Fig. 3). This analysis indicated after chromatographic separation by heparin, ion exchange, and molecular sieve chromatography, the presence of several proteins with M, between 45 and 250 in the last pool of active fractions obtained by molecular sieve chromatography (fractions 7–9 in peak 1) (Fig. 3, lane 6). These pooled fractions were digested in solution with trypsin, and peptides were identified by liquid chromatography-MS/MS. This analysis identified a total of 65 proteins, most of them corresponding to intracellular and membrane-associated proteins (data not shown). However, among these, six proteins corresponded to secreted and extracellular matrix proteins (Table 2). Three peptides were from extracellular matrix proteins including laminin (β and γ chains) and tenasin. One peptide corresponded to the heavy chain of immunoglobulin (a likely contaminant from residual fetal bovine serum) and one to a hypothetical protein T08772. The protein with the highest score (4) was Gal-3BP or Mac-2BP, a 587-amino acid-secreted glycosylated protein with a molecular mass of 65.3 and 90 kDa when glycosylated (16). This latter protein particularly raised our interest as a likely candidate because a literature search revealed that it was the only protein among the six secreted peptides that had been previously reported to stimulate IL-6 expression (18, 19).

**Gal-3BP Is Expressed by Neuroblastoma Cells and Is Present in Active Chromatographic Fractions**—We first confirmed that Gal-3BP was produced by neuroblastoma cells by Western blot analysis using a commercially available anti-human Gal-3BP antibody (Fig. 4). This analysis revealed the presence of 3 broad peptide bands with M, values in the range of 93, 70, and 26 in the CM of 3 neuroblastoma cell lines that stimulated IL-6

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**TABLE 1**

<table>
<thead>
<tr>
<th>Chromatographic purification of conditioned medium from CHLA-255 cells</th>
<th>Volume</th>
<th>Concentration</th>
<th>Protein</th>
<th>Activity</th>
<th>Specific activity</th>
<th>Recovery</th>
<th>-Fold purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>ml</td>
<td>mg/ml</td>
<td>mg</td>
<td>pg/ml</td>
<td>pg/mg</td>
<td>%</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>50× CM</td>
<td>120</td>
<td>0.377</td>
<td>45.26</td>
<td>1,545,924</td>
<td>34,155</td>
<td>100.00</td>
<td>100</td>
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<tr>
<td>Heparin-bound</td>
<td>50</td>
<td>0.125</td>
<td>6.24</td>
<td>621,568</td>
<td>99,570</td>
<td>40.21</td>
<td>291</td>
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<tr>
<td>DEAE-bound</td>
<td>20</td>
<td>0.048</td>
<td>0.96</td>
<td>177,249</td>
<td>184,715</td>
<td>11.47</td>
<td>540</td>
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<tr>
<td>Molecular sieve peak 1</td>
<td>0.8</td>
<td>0.006</td>
<td>0.0046</td>
<td>935</td>
<td>203,977</td>
<td>0.06</td>
<td>597</td>
</tr>
<tr>
<td>Molecular sieve peak 2</td>
<td>0.8</td>
<td>0.010</td>
<td>0.0076</td>
<td>947</td>
<td>124,466</td>
<td>0.06</td>
<td>364</td>
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</table>
expression in BMSC (Fig. 4A). The diffuseness of these bands was consistent with their being glycoproteins, and their Mr values were consistent with previously published data. Gal-3BP has been reported to be cleaved by plasmin and another endogenous protease into a 70-kDa N-terminal fragment with activity and a 26-kDa C-terminal domain (16, 20). A similar Western blot analysis performed on aliquots of pooled active fractions obtained by chromatographic separation as shown in Fig. 2 for the presence of Gal-3BP. Ten μl of each pooled fraction was loaded in each lane. rhGal-3BP (100 ng) was loaded as the control.

**TABLE 2**

Soluble proteins separated by molecular sieve chromatography and identified by liquid chromatography-MS/MS

<table>
<thead>
<tr>
<th>Reference name</th>
<th>P (pro)</th>
<th>Score</th>
<th>Mr</th>
<th>Accession</th>
<th>RSp</th>
<th>Peptide (hits)</th>
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<tr>
<td>Galectin 3-binding protein</td>
<td>2.02E-06</td>
<td>20.18</td>
<td>65,312.6</td>
<td>5,031,863</td>
<td>4</td>
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<tr>
<td>T08772 hypothetical protein</td>
<td>1.18E-10</td>
<td>20.20</td>
<td>104,220.5</td>
<td>7,512,926</td>
<td>2</td>
<td></td>
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<tr>
<td>LGM1 human laminin γ1 chain</td>
<td>3.89E-09</td>
<td>20.29</td>
<td>177,588.0</td>
<td>126,369</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Tenascin C (hexabrachion)</td>
<td>4.25E-06</td>
<td>20.22</td>
<td>240,847.1</td>
<td>4,504,549</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Human laminin, β1 chain</td>
<td>1.20E-07</td>
<td>10.24</td>
<td>198,047.6</td>
<td>4,504,951</td>
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<td>Immunoglobulin λ heavy chain</td>
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<td>2.19</td>
<td>52,709.4</td>
<td>2,765,425</td>
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</table>

**FIGURE 3.** SDS-PAGE of active fractions separated by chromatography. Aliquots of active fractions pooled after each purification steps shown in Fig. 2 were electrophoresed on a 4–12% gradient acrylamide gel under reducing conditions and visualized by silver staining.

**FIGURE 4.** Gal-3BP is expressed by neuroblastoma cells and is present in active chromatographic fractions. A, Western blot analysis on the CM of three human neuroblastoma cell lines for the presence of human Gal-3BP. 20 μg of proteins were loaded in each lane. B, Western blot (IB) analysis of active fractions obtained by chromatographic separation as shown in Fig. 2 for the presence of Gal-3BP. Ten μl of each pooled fraction was loaded in each lane. rhGal-3BP (100 ng) was loaded as the control.

**FIGURE 5.** BMSC express Gal-3. A, Western blot analysis of cell lysate (40 μg/lane) of BMSC cultured in the absence or presence of CM from neuroblastoma cells for the expression of Gal-3. As the positive control (PC) we used a cell lysate from MCF-7 cells (4 μg) (42). B, flow cytometry analysis of Gal-3 expression at the surface of BMSC using an anti-Gal-3 antibody or a neutralizing anti-Gal-3 antibody (TIB-166).
at the cell membrane but also in the cytoplasm and the nucleus of cells (21, 22). We, therefore, determined whether Gal-3 was expressed by BMSC (Fig. 5). A Western blot analysis of whole cell lysates of BMSC indicated the presence of Gal-3 when cells were cultured in serum-free medium or in the presence of CM from CHLA-255 and NB-19 neuroblastoma cells (Fig. 5A). To demonstrate that Gal-3 was also present at the cell surface, we used flow cytometry. This analysis (Fig. 5B) revealed that 22.5–26.5% of BMSC expressed Gal-3 at their surface as determined with two separate anti-Gal-3 antibodies.

Gal-3BP Secreted by Neuroblastoma Cells Stimulates IL-6 Expression in BMSC—To demonstrate that Gal-3BP produced by CHLA-255 cells is an IL-6 stimulatory factor for BMSC, we first tested whether rhGal-3BP stimulated IL-6 expression in BMSC. This experiment (Fig. 6A) demonstrated that rhGal-3BP stimulates IL-6 expression in a dose-dependent manner, increasing IL-6 expression by 3-fold at a concentration of 5 μg/ml, which is consistent with a similar stimulatory activity reported on peripheral blood mononuclear cells (18, 19). To obtain direct evidence that Gal-3BP is the protein responsible for the stimulation of IL-6 in neuroblastoma cells CM, we then tested the effect of lactose, an inhibitor of Gal-3BP interaction with its receptor Gal-3 (23), on the stimulation of IL-6 expression in BMSC cultured in the presence of CM from CHLA-255 cells or heparin-bound active fractions. This experiment revealed a 35.1 and 48.3% inhibition, respectively, at a lactose concentration of 150 mM when compared with sucrose, used as a control (Fig. 6B). Using an anti-Gal-3 neutralizing antibody (TIB-166), we also demonstrated 34.7 and 55.3% inhibition of the stimulatory activity of CHLA-255 CM and heparin-bound fractions, respectively (Fig. 6C). A second line of direct evidence that Gal-3BP produced by neuroblastoma cells is a stimulator of IL-6 expression in BMSC was obtained by down-regulating Gal-3BP expression in CHLA-255 cells transfected with a Gal-3BP siRNA and by testing the effect of the CM from these cells on IL-6 stimulation in BMSC. We selected two specific siRNA sequences (1 and 5) and used a scrambled sequence as a control. Transfection of siRNA 1 and 5 in CHLA-255 cells resulted in a 79 or 65% decrease in protein expression, respectively (Fig. 7A). When tested on BMSC, the IL-6 stimulatory activities in the CM of CHLA-255 cells transfected with siRNA were 53.9 and 64.1% of the values observed in the CM of CHLA-255 transfected with the scrambled siRNA (Fig. 7B). Altogether, these data identify Gal-3BP as being an active factor secreted by neuroblastoma cells that stimulates the expression of IL-6 in BMSC.

Erk1/2 Activation in BMSC for IL-6 Production by Gal-3BP Stimulation—Because we had previously observed an increase in Erk1/2 phosphorylation in BMSC cultured in the
DISCUSSION

Gal-3BP was first characterized as a 90-kDa Mac-binding protein and cloned from the supernatant of the SK-BR-3 human breast cancer cell line and from human milk by Koths et al. in 1993 (16). It was later identified as the ligand for Gal-3 in the search for novel ligands for Gal-3 by Inohara et al. (22). The cDNA for Gal-3BP codes for a 567-amino acid that is preceded by an 18-amino acid leader peptide. It has seven potential N-linked glycosylation sites and is composed of four domains. Domain 1 (N-terminal) is a member of the scavenger receptor cysteine-rich family of proteins and does not induce cell aggregation. Domains 2 and 3 mediate homodimerization and multimerization, which are necessary for binding to the extracellular matrix and for cell attachment. Domain 4 is inactive. Gal-3BP can be proteolytically cleaved by plasmin and another endogenous protease into a 70-kDa N-terminal peptide and a 26-kDa C-terminal domain that contains domain 4 (16, 20). These characteristics likely explain some of the difficulties we encountered in obtaining active chromatographic fractions containing a single protein band. Whereas the CM from neuroblastoma cells contained both the intact and the cleaved proteins, the full-length 93-kDa protein was only found in the active heparin-unbound fractions, whereas the 70- and 26-kDa cleaved peptides were found in the heparin-bound fractions. It is unclear whether both peptides are active at this point since they were not further separated and tested for activity. However, on the basis of published data, the N-terminal (70 kDa) peptide is the only active peptide. Although we did not investigate the mechanism responsible for the cleavage of Gal-3BP in neuroblastoma cells, it is conceivable that it was the result of plasmin, which is present as plasminogen in the culture medium and activated by plasminogen activators that are secreted by neuroblastoma cells (24).

The role of Gal-3BP in cancer is not entirely understood, and Gal-3BP has been shown to have multiple functions. It is expressed in numerous cancer cells including non-small cell lung cancer, astrocytoma, and colon cancer and is a predictor of poor survival (25–27). Gal-3BP binds to multiple proteins of the extracellular matrix like collagens, fibronectin and nidogen (20), and to molecules mediating cell-matrix and cell-cell adhesions that are critical during tumor cell invasion and migration (22). Gal-3BP is also a regulator of the immune system. It has a stimulatory activity on natural killer cells and lymphokine-activated killer cells that is in part mediated through the induction of IL-2 (28). Gal-3BP also stimulates the secretion of many cytokines and interleukins in peripheral blood mononuclear cells, including tumor necrosis factor-α (TNF-α), IL-1, and IL-6 that all play a contributory role in tumor progression and inflammation (18, 29). Our data, thus, identify Gal-3BP as an IL-6 stimulatory factor in BMSC and a critical mediator in bone invasion. To our knowledge this is the first report that Gal-3BP stimulates IL-6 expression in non-inflamatory cells like BMSC. They do not rule out the possibility that Gal-3BP may also stimulate the expression of IL-6 in other cells such as myelomonocytic inflammatory cells.

Gal-3 is the receptor for Gal-3BP, an unusual protein with a pleiotropic function localized to the cytoplasm, cell membrane,
nucleus, and also present in the extracellular milieu. It is expressed by normal and neoplastic cells. It has a pleiotropic function and has been shown to enhance tumorigenicity (30), to stimulate tumor cell migration in vitro and metastasis in vivo (21, 31), and to protect cells from apoptosis (32, 33). In endothelial cells, Gal-3 induces morphogenesis and stimulates angiogenesis (34). It contains the same 4 amino acids (NWGR) conserved in the BH1 domain of Bcl-2 gene family, a domain critical for their anti-apoptotic activity, and has been shown to protect cells from drug-induced apoptosis by inhibiting the mitochondrial apoptotic pathway (33). There is evidence that Gal-3 expressed at the surface of cells can act as a membrane-associated receptor and signal through specific pathways. Gal-3 is a binding partner for K-Ras and activates K-Ras (35, 36). In COS cells it generates a Ras-mediated signal that attenuates Erk but not phosphoinositide 3-kinase activity. Here we demonstrate an increase in Erk1/2 activation in BMSC cultured in the presence of CM from neuroblastoma cells or treated with Gal-3BP that is inhibited by PD98059 or upon Gal-3BP down-regulation in neuroblastoma cells by siRNA. We also show that treatment of BMSC by PD98059 inhibits IL-6 expression when cells are cultured in the presence of CM from neuroblastoma cells. The data support the concept that Gal-3BP signals via Erk1/2 in BMSC. The difference between our data and those of Elad-Sfadia et al. (35) is presently unclear but may reflect differences in cell types. We have examined BMSC, whereas they examined COS cells exposed to epidermal growth factor and Gal-3BP. Consistent with our data, Kobayashi et al. (37) have recently reported that Erk1/2 activation increases IL-6 expression in human keratinocytes.

Our data do not rule out the possibility that other proteins produced by neuroblastoma cells contribute to the stimulatory effect on IL-6 production by BMSC. The absence of complete inhibition of IL-6 stimulatory activity by Gal-3BP inhibitory agents such as lactose or TIB-166 or upon Gal-3BP down-regulation suggests that other proteins than Gal-3BP could be involved. TNF-α and IL-1β are known to stimulate IL-6 (38), and we found that TNF-α is a potent stimulator of IL-6 expression in BMSC that express the TNF receptors 1 and 2 (data not shown). Although TNF-α produced by tumor-associated macrophages may stimulate the expression of IL-6 in stroma cells in the tumor microenvironment, we could not document its presence in the CM of cultured neuroblastoma cells. Vascular endothelial cell growth factor-A, fibroblast growth factor-2, and platelet-derived growth factor have also been shown to stimulate IL-6 (39–41). They are expressed by neuroblastoma cells; however, preliminary experiments in our laboratory in the presence of neutralizing antibody against these factors (data not shown) failed to demonstrate any inhibitory effect on IL-6 stimulation. The identification of other IL-6 stimulatory factors expressed by neuroblastoma cells remains a focus of investigation of our laboratory.

In summary, we have isolated and identified Gal-3BP from the CM of neuroblastoma cells as a secreted protein that stimulates IL-6 expression in BMSC. Our data point to a novel function for this protein in cancer progression and bone metastasis and raise the question of whether Gal-3BP could be a valuable target for therapeutic intervention in metastatic neuroblastoma.

REFERENCES

31. Pienta, K. J., Naik, H., Akhtiar, A., Yamazaki, K., Replage, T. S., Lehr, J.,
Gal-3BP Stimulates IL-6 Expression in Bone Marrow Stroma