Expression and Activation of Signal Regulatory Protein α on Astrocytomas

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ABSTRACT

High-grade astrocytomas and glioblastomas are usually unresectable because they extensively invade surrounding brain tissue. Here, we report the expression and function of a receptor on many astrocytomas that may alter both the proliferative and invasive potential of these tumors. Signal regulatory protein (SIRP) α1 is an immunoglobulin superfamily transmembrane glycoprotein that is normally expressed in subsets of myeloid and neuronal cells. Transfection of many cell types with SIRPα1, including glioblastomas, has been shown to inhibit their proliferation in response to a range of growth factors. Furthermore, the expression of a murine SIRPα1 mutant has been shown to enhance cell adhesion and initial cell spreading but to inhibit cell extension and movement. The extracellular portion of SIRPα1 binds CD47 (integrin-associated protein), although this interaction is not required for integrin-mediated activation of SIRPα1. On phosphorylation, SIRPα1 recruits the tyrosine phosphatases SHP-1 and SHP-2, which are important in its functions. Although SHP-1 is uniquely expressed on hematopoietic cells, SHP-2 is ubiquitously expressed, so that SIRPα1 has the potential to function in many cell types, including astrocytomas. Because SIRPα1 regulates cell functions that may contribute to the malignancy of these tumors, we examined the expression of SIRPs in astrocytoma cell lines by flow cytometry using a monoclonal antibody against all SIRPs. Screening of nine cell lines revealed clear cell surface expression of SIRPs on five cell lines, whereas Northern blotting for SIRPs transcripts showed mRNA present in eight of nine cell lines. All nine cell lines expressed the ligand for SIRPα1, CD47. To further examine the expression and function of SIRPs, we studied the SF126 and U373MG astrocytoma cell lines, both of which express SIRPs, in greater detail. SIRP transcripts in these cells are identical in sequence to SIRPα1. The expressed deglycosylated protein is the same size as SIRPα1, but in the astrocytomas, it is underglycosylated compared with SIRPα1 produced in transfected Chinese hamster ovary cells. It is nonetheless still capable of binding soluble CD47. Moreover, SIRPα1 in each of the two cell lines recruited SHP-2 on phosphorylation, and SIRPα1 phosphorylation in cultured cells is CD47 dependent. Finally, examination of frozen sections from 10 primary brain tumor biopsies by immunohistochemistry revealed expression of SIRPs on seven of the specimens, some of which expressed high levels of SIRPs. Most of the tumors also expressed CD47. This is the first demonstration that astrocytomas can express SIRPs. Given the known role of SIRPs in regulating cell adhesion and responses to mitogenic growth factors, the expression of SIRPα1 on astrocytomas may be of considerable importance in brain tumor biology, and it offers the potential of a new avenue for therapeutic intervention.

INTRODUCTION

Signal regulatory proteins (SIRPs) comprise a receptor family whose structure includes three extracellular immunoglobulin loop (immunoglobulin) domains (reviewed in Refs. 1 and 2). Orthologs of human SIRPα1 are expressed in rats (SHPS-1, MFR, and p93), mice (p84 and BIT), and cattle (MyD-1). SIRPα receptors have a cytoplasmic tail that includes several immunoreceptor tyrosine-based inhibitory motifs (ITIMs). Phosphorylation of the tyrosines in these motifs leads to the recruitment and activation of tyrosine phosphatases SHP-1 and SHP-2 (3, 4). SIRPβ receptors have only a short cytoplasmic tail, with no ITIMs. Instead, they express a charged residue in the transmembrane domain, which permits association with the adapter protein DAP12 (5, 6). SIRPβ receptors have been identified only in cells of the monocyte, macrophage, or dendritic lineages. Although initial reports using tissue Northern blotting suggested that SIRPα receptors are expressed ubiquitously (4), studies of protein expression suggest expression primarily in myeloid cells, subsets of brain cells (7, 8), and endothelial cells (9, 10). In the central nervous system, SIRPα receptors are expressed in the granular and molecular layers of the cerebellum as well as in various regions of the hippocampus, retina, and olfactory bulb (11–13). During development, SIRPs is expressed by embryonic day 9 on the floor plate region of the ventral neuraxis, and it is rapidly up-regulated by postnatal days 2–5 (14, 15). Examination of brain cell subtypes has established that SIRPα is expressed on cortical neurons as well as microglia, whereas neonatal astrocytes or cultured oligodendrocytes do not express detectable SIRPα (7).

A ligand for SIRPα is CD47, as demonstrated by the binding of soluble SIRPα fusion proteins to CD47 (10, 16–19) and binding of soluble CD47 to SIRPα expressed on myeloid cells (20, 21). In the mouse retina, the expression of CD47 and SIRPα is colocalized, and CD47-deficient mice show an altered pattern of SIRPα expression, especially in the cellular and plexiform layers of the retina, suggesting a functional association between the two molecules (12, 16). In rats, SIRPα expressed in neuronal cells demonstrates reduced glycosylation compared with SIRPs from myeloid cells, resulting in altered binding affinity to tissue sections or plant lectins (13, 22, 23), and SIRPα on neuronal cells exhibits differential glycosylation during embryonic development (15). Evidence of differential glycosylation is also seen in the MM5/C1 mouse mammary carcinoma and the A431 human epidermal carcinoma cell lines (4), and rat SIRPα expressed on pituitary macrophages exhibits reduced glycosylation compared with SIRPα on alveolar macrophages (24). The functional significance of this altered glycosylation is unknown, but it might alter the avidity to of SIRPα1 for CD47, its isoforms, or other unknown ligands.

The initial characterization of human SIRPα cDNAs revealed several sequences. For most SIRPα cDNAs, these differences clustered in the membrane distal immunoglobulin region (the V region), although for one cDNA (SIRPα3), the sequence differences were distributed throughout the coding sequence (4). It appears likely that at least two cDNA variants for SIRPα (named SIRPα1 and The gene for SIRPα2 in the initial report above) are alleles of the same locus. The gene for SIRPα1 can undergo alternative splicing, producing a low abundance cDNA that excludes the second and third immunoglobulin-like domains, and there are two polyadenylation sites that can yield the two predominant transcript sizes seen on Northern blotting (11, 25).

SIRPα is phosphorylated on its ITIMs in response to a variety of mitogenic growth factors [epidermal growth factor (EGF), platelet-derived growth factor, insulin, neurotrophins, growth hormone], cytokines (interleukin 1β and tumor necrosis factor α), and lysosphos-
that it can associate in these cells with the signaling adapter molecule SHP-2, and that its phosphorylation in cultured cells is CD47 dependent. Finally, we have shown by immunohistochemistry on 10 frozen sections that SIRPs and CD47 are expressed on primary brain tumor biopsies. To our knowledge, this is the first demonstration that SIRPα is expressed on astrocytomas. The demonstration that SIRPα can function in these cells may be important in the biological and malignant properties of astrocytomas.

**MATERIALS AND METHODS**

**Cell Lines and Transfectants.** Human astrocytoma cell lines were obtained courtesy of the Neurosurgery Tissue Bank at University of California San Francisco (UCSF). The cell lines A172 (48), SF126, SF210, SF268, SF295 (49), U87MG, U251, U343MG, and U373MG (50) have been described previously and were established by long-term culture of explants from brain tumor biopsies. CHO cells were obtained from the UCSF Cell Culture Facility. All cell lines were maintained at 37° in 5% CO2 in RPMI 1640 (Mediatech, Herndon, VA) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Plasmids containing the genes for human SIRPα and SIRPβ were kindly provided by Axel Ullrich (4). These genes were modified by addition of a sequence encoding the FLAG epitope (DYKDDDDK) at the 5′ end of each expressed gene product. The genes for these FLAG-tagged proteins were then subcloned into the pMXneo (for SIRPα) or BSRα (for SIRPβ) expression plasmids (obtained courtesy of Andrey Shaw; Washington University, St. Louis, MO), purified on Qiagen columns (Qiagen, Valencia, CA), transfected into CHO cells by using FuGENE (Roche, Indianapolis, IN), and selected with G418 at 1 mg/ml (Mediatech) or puromycin at 10 μg/ml (Sigma, St. Louis, MO), respectively, to create the cell lines CHO-SIRPs and CHO-SIRPβ. Expression was verified by the presence of a FLAG epitope detected with the anti-FLAG M2 antibody (Sigma).

**Antibodies and Flow Cytometry.** The B6H12 anti-CD47 antibody was obtained from PharMingen (San Diego, CA), and our 2D3 anti-CD47 antibody has been described previously (20). As described under “Results,” the 2D3 mAb does not block binding of CD47 to SIRPα but, instead, enhances it. The B-1 mouse mAb against SHP-2 and polyclonal goat antiserum (C-20) against a COOH-terminal peptide of SIRPα were obtained from Santa Cruz Biotechnology Biotech (Santa Cruz, CA). A mAb against SIRPα was raised by immunizing BALB/c mice with CHO-SIRPα constructs as described above. Mice were boosted four times, and spleens were harvested 3 days after the last boost and fused with SP2/0 cells using standard methods. Clones expressing antibodies with reactivity against 293T cells transfected with SIRPα, but not against untransfected cells, were subcloned and expanded. The resulting antibody, named 6.1, binds to both SIRPα and SIRPβ. Flow cytometry was performed by using a FACSScan (BD Biosciences, San Jose, CA). Fluorescein-conjugated antimouse IgG antibodies were obtained from ICN (Aurora, OH). Phycocerythrin-conjugated antihuman IgG Fc-specific antibody was from Jackson ImmunoResearch Laboratories (West Grove, PA).

**Binding of CD47 Fusion Protein to SIRPα.** A fusion protein (CD47Fc) between the large extracellular loop of CD47 (which includes the ligand binding site for SIRPα) and the human IgG1 constant region has been described previously (20). To test for binding of CD47Fc to cells, 1 μg of the fusion protein and 1 μg of the enhancing antibody 2D3 were added to 106 cells. Cells were incubated for 1 h on ice, washed extensively in PBS, and incubated with phycoerythrin-conjugated antibody against human Fc (Jackson ImmunoResearch Laboratories). This antibody showed no reactivity against mouse IgG and thus did not bind appreciably to cells coated with murine anti-CD47 antibodies. Binding was detected by flow cytometry as described above. Where indicated, the binding of CD47Fc to SIRPα was blocked by addition of the 6.1 anti-SIRPα antibody at 5 μg/ml.

**Western Blotting.** Cells were lysed with 1% digitonin, and immunoprecipitation was performed with the indicated mAb linked to protein G-Sepharose (Amersham, Piscataway, NJ), as described previously (51). Lysates were resolved by nonreducing 10% SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Bedford, MA) by semi-dry transfer. Membranes were blocked overnight at 4°C in Tris-buffered saline containing 0.05% Tween 20 (TBST) and 1% BSA, incubated with the appropriate primary antibody diluted...
in the same solution for 1 h at room temperature, washed extensively in TBST, and then incubated with the appropriate second step antibody for 1 h at room temperature. Blots were again extensively washed in TBST, and bound antibody was visualized with SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL) and exposure to film (Hyperfilm, Amersham). Second-step antibodies used included horseradish peroxidase-conjugated sheep antiserum IgG (Amersham) and horseradish peroxidase-conjugated swine antigoat IgG antiserum (Caltag, Burlingame, CA).

**Deglycosylation of Proteins.** After immunoprecipitation with mAb 6.1, lysates were denatured in 0.5% SDS, and phenylmethylsulfonyl fluoride was added to 1 mM. Deglycosylation was performed with PNGase F (New England Biolabs, Beverly, MA) overnight at 37°C per the manufacturer’s directions, after which samples were subjected to SDS-PAGE and Western blotting as described above, using the C-20 goat antiseraum against the SIRPα cytoplasmic tail and part of the coding domain (Santa Cruz Biotechnology Biotech).

**Northern Blotting.** Total RNA was isolated from cells by using TRIzol (Life Technologies, Inc., Gaithersburg, MD), and 50 µg were enriched for polyadenylated RNA by using Oligotex beads (Qiagen). RNA was separated on 1% agarose formaldehyde gels and transferred to Hybond-N+ (Amersham) by capillary transfer in 20× SSC. Membranes were cross-linked by baking at 80°C for 2 h, prehybridized for 1 h at 42°C with UltraHyb (Ambion, Austin, TX), and hybridized overnight to a DNA probe from the 3’ end of the SIRPα gene labeled with 32P (Rediprime II; Amersham). Membranes were washed at medium stringency and exposed to film (BioMax MS; Kodak, Rochester, NY). To standardize loading, after probing for SIRPα, the blot was stripped by incubating membranes for 30 min in 0.1× SSC/1% SDS at 80°C. The membranes were then reprobed with a 32P-labeled probe for β-actin.

**Reverse Transcription, PCR, and Sequencing.** First-strand cDNA was synthesized from RNA, isolated as described above, by using either oligodT12-18 (Roche) or a primer specific to the 3’- untranslated region of SIRPα (5’-GGCGGGAGCCAGTTGCTTCAAAC-3’) together with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer’s directions. First-strand synthesis was complete, RNA was degraded with RNaseH (Roche), and 1 µl was used for subsequent PCR amplification using Taq polymerase (Roche). To clone the bulk of the coding region of SIRPα, the following primer pair was used: 5’-GCGGGCTGCGGGCTGGTCTGAATG-3’ (predicted to hybridize near the 5’ end of the SIRPα gene) and 5’-GCGGGCTGGCGGGGCTCTGAGATG-3’ (predicted to hybridize near the second IITM motif). To clone the cytoplasmic tail and part of the 3’- untranslated region, the following primer pair was used: 5’-GCGCAGAATGCGAGGAGAAATACACA-3’ (predicted to hybridize between the transmembrane domain and the first IITM) and 5’-GGGGAGGCAGCTGGCTTCAAAC-3’ (predicted to hybridize to 320 bp downstream of the stop codon). PCR fragments were resolved on 1.2% agarose gels, purified with the QIAquick gel extraction kit (Qiagen), and cloned using the TOPO PCR cloning system (Invitrogen). All sequencing and oligonucleotide synthesis was performed at the Biomolecular Resource Center at UCSF.

**Pervanadate Stimulation and SHP-2 Association.** Tyrosine phosphorylation of cell proteins was nonspecifically enhanced by incubation in 10 mM sodium orthovanadate and 0.6% hydrogen peroxide (pervanadate) for 5 min as described previously (51). After incubation, cells were rapidly pelleted and lysed in digitonin as described above. Immunoprecipitation with the B-1 anti-SHP-2 antibody linked to protein G-Sepharose and Western blotting with the 6.1 anti-SIRPα antibody were performed as described above. Blots were subsequently stripped under mild conditions (ReBlot Plus; Chemicon, Temecula, CA) and reprobed with anti-SHP-2 antibody (Transduction Laboratories, Lexington, KY).

**Cell Stimulation and Aggregation.** To stimulate cells by aggregation, astrocytoma cell lines were harvested, washed twice in PBS, resuspended in serum-free RPMI 1640 supplemented with 1% low endotoxin Cohn analogue BSA (Sigma), and added to standard tissue culture plates (Corning, NY) at 5×10⁶ cells/plate. When indicated, plates were coated with vitronectin by incubating plates overnight at 4°C with human vitronectin (Sigma) at 10 µg/ml. Appropriate blocking antibodies were added at 5 µg/ml. After overnight incubation at 37°C in 5% CO₂, digitonin cell lysates were prepared and subjected to immunoprecipitation, resolved on SDS-PAGE, transferred to polyvinylidene difluoride, and analyzed for phosphotyrosine by using the mAb 4G10 as described above. To verify the level of SIRPα, blots were reprobed with biotinylated 6.1 anti-SIRP antibody and alkaline phosphatase-conjugated streptavidin (F Pierce), and bound complexes were detected using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate (Roche) per the manufacturer’s directions.

**Immunohistochemistry.** Multiple sections from fresh specimens of brain tumor biopsies were obtained from the Neurosurgery Tissue Bank at UCSF through a protocol approved by the Human Studies Committee. Sections were stored desiccated at −20°C until ready for use. One section was stained with H&E and evaluated by one of us (E. J. H.) to verify the diagnosis of grade 4 astrocytoma and to quantify the percentage of tumor in each sample. To perform immunohistochemistry with anti-SIRP (mAb 6.1) or anti-CD47 (mAb B6H12), slides were fixed for 5 min in acetone, washed twice in PBS, and incubated with serum-free blocking solution (DAKO, Carpinteria, CA) for 1 h at room temperature. Slides were washed twice in PBS and incubated for 1 h at room temperature with the primary antibody diluted to 1 µg/ml in PBS with 1% BSA. Bound antibodies were detected by using the LSA2 anti-rabbit IgG complex system (DAKO) and 3,3-diaminobenzidine per the manufacturer’s directions. After chromogen development, cells were counterstained for 5 min with hematoxylin (Sigma), dehydrated in xylene, and mounted with Permount (Fisher) per standard protocols. Slides were subsequently read in a blinded fashion by E. J. H. for binding of antibody to SIRP α or to CD47. The method of scoring is presented in “Results.”

**RESULTS**

**SIRPα and CD47 Are Expressed on Astrocytoma Cell Lines.** To assess the expression of SIRPs on the cell surface, we generated a mouse mAb against SIRPα by immunizing BALB/c mice with CHO cells transfected with a gene encoding SIRPα. One antibody, 6.1, reacts with SIRPα expressed on either CHO cells or 293T cells, but not untransfected cells. This antibody cross-reacts with SIRPβ1, reflecting the extensive homology between the extracellular domain of these two genes (4). To test for expression of SIRPs on astrocytoma cell lines, the 6.1 antibody was used in fluorescence analysis of a panel of nine cell lines obtained from the Neurosurgery Tissue Bank at UCSF. As shown in Fig. 1, the 6.1 antibody reacted with five of the cell lines tested (SF126, SF210, SF268, U251, and U373MG). Ex-

Fig. 1. Fluorescence-activated cell-sorting analysis of astrocytoma cell lines. Anti-SIRP or anti-CD47 antibodies (filled histograms) or an isotype-matched control antibody (open histograms) was used in flow cytometry on the indicated cell lines as described in “Materials and Methods.” Binding was detected with a fluorescin-conjugated secondary antibody.

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expression of SIRPs on two other cell lines (U87MG and U343MG) was intermittently detectable above background, whereas SIRP expression on the remaining two cell lines (A172 and SF295) was consistently negative. No reaction with any cell line was observed with an isotype-matched control antibody. Of the cell lines tested, U373MG expressed the highest level of SIRPs. We also tested all cell lines for expression of CD47, a known ligand for SIRPα, by using the antibody B6H12. All cell lines expressed high levels of CD47 (Fig. 1).

The SIRP Expressed on Astrocytoma Cell Lines Is SIRPα and Is Underglycosylated. The above-mentioned studies demonstrated that SIRPs are frequently present on astrocytoma cell lines. However, the 6.1 anti-SIRP antibody does not distinguish between SIRPα and SIRPβ. To formally test for the expression of SIRPα, two astrocytoma cell lines demonstrating the highest reactivity to the 6.1 mAb (SF126 and U373MG) were subjected to immunoprecipitation with mAb 6.1 followed by Western blotting with a commercial antiseraum against a peptide in the cytoplasmic tail of SIRPα1. SIRPβ does not contain a homologous region and thus does not react with this antiseraum. As controls, lysates prepared from CHO cells, CHO cells transfected with the SIRPα1 gene (CHO-SIRPα1), and CHO cells transfected with the SIRPβ1 gene (CHO-SIRPβ1) were also analyzed in parallel. As shown in the first five lanes (no PNGase) of Fig. 2, the 6.1 antibody immunoprecipitated a protein that is recognized by the SIRPα-specific antiseraum in the CHO-SIRPα1, SF126, and U373MG cell lines but not the untransfected CHO cells or the CHO-SIRPβ1 line. This band migrates at M, 70,000–90,000 in CHO-SIRPα1 but at M, 65,000–75,000 in the astrocytoma cell lines.

Additional studies demonstrated that the difference in molecular weight between SIRPs from CHO-SIRPα1 and SIRPs astrocytoma cell lines reflects differences in glycosylation. For these studies, cell lysates were immunoprecipitated as described above and then subjected to deglycosylation with PNGaseF (Fig. 2, + PNGase). After treatment, SIRPαs from both CHO-SIRPα and the astrocytoma cell lines bands migrated as a doublet at M, 53,000–57,000. The smaller size is the predicted molecular weight for human SIRPαs (4), and the larger band likely represents incomplete deglycosylation. Thus, the discrepancy in molecular weight between SIRPα expressed on transfected CHO cells versus SIRPα on astrocytomas reflects differences in glycosylation. A similar reduced glycosylation of SIRPα expressed in the rat brain has been noted by others (23).

Astrocytoma Cell Lines Express Transcripts for SIRPα1 as Assessed by PCR. The initial characterization of SIRPα by Khartonov et al. (4) revealed a family of proteins that differ primarily in the first immunoglobulin (V) domain (3), as well as several forms due to alternative splicing (11, 52). To establish the identity of the SIRPα protein expressed on astrocytoma cell lines, PCR primers were designed based on published sequences of SIRPα receptors and used to clone the expressed gene from both the SF126 and U373MG cell lines. These primer sequences are common to each of the three best-characterized subtypes of SIRPα (SIRPα1, SIRPα2, and SIRPα3). The predominant sequences obtained from the SF126 and U373MG cell lines were identical to the published SIRPα1 sequence in the extracellular domain (4), and are represented by the sequence U373-1 in Fig. 3. Also shown in Fig. 3 are the sequences of three other clones obtained from the U373MG cell line, aligned to the published sequences of SIRPα1 and SIRPα2. A single clone, obtained from the U373MG cell line, is identical to the SIRPα2 sequence in the V domain (sequence U373-4 in Fig. 3). We have also detected transcripts that correspond to the previously described splice variant of SIRPα1 missing the second and third immunoglobulin domains (shown as U373-2), and we have also detected transcripts containing previously undescribed splice variants (shown as U373-3 and U373-4). These latter sequences are similar to U373-2 in that the intracellular l玉ou contain only the V domain, but they differ in that they are missing the transmembrane domain and variable amounts of the intracellular tail. Thus, PCR amplification of SIRPα transcripts from astrocytoma cell lines detected primarily SIRPα1, including full-length and alternatively spliced transcripts. One transcript from U373MG cells, however, was SIRPα2.

Astrocytoma Cell Lines Express Primarily Transcripts for Complete (Three Immunoglobulin Domain) SIRPα by Northern Blotting. Having demonstrated that SIRPα1 is expressed in both SF126 and U373MG astrocytoma cells, we next examined expression of SIRPα transcripts on all cell lines by Northern blotting. We used a probe derived from the 3’-untranslated region of the SIRPα1 gene, which is retained in both the full-length and alternatively spliced forms but is not present in SIRPβ. The results, shown in Fig. 4, demonstrate bands at 4.2 and 2.7 kb, which reflect the expected sizes of the alternatively polyadenylated forms of SIRPα described previously (11, 52). The mature mRNA encoding the single-immunoglobulin domain splice variant would be predicted to be approximately 650 bp shorter than the full-length mRNA. Despite overexposure of several Northern blots, no bands corresponding to this predicted splice variant were consistently detected (data not shown). Therefore, although the single immunoglobulin domain splice variant was detectable by PCR, it is likely a low abundance transcript compared with the full-length mRNA.

Surprisingly, all but one of the astrocytoma cell lines expressed SIRPα transcripts as assessed by Northern blotting, despite quite variable cell surface expression as determined by flow cytometry (see Fig. 1). There was no clear correlation between mRNA abundance and cell surface expression, and one cell line with abundant mRNA for SIRPα expressed no detectable SIRPαs on its cell surface (SF295), whereas cell lines with modest levels of mRNA for SIRPα expressed high levels of SIRPαs on the cell surface (SF210 and SF268). Nonetheless, U373MG cells had both the most abundant transcripts (as assayed by Northern blotting) and the highest levels of SIRP expression (as assessed by flow cytometry). These findings suggest that SIRPα protein expression is regulated in part at the posttranscriptional level. Alternatively, posttranslational modification of SIRPα1 may produce isoforms that are not recognized by our antibody.

SIRPα1 on Astrocytoma Cell Lines Binds to CD47. To begin characterizing the functional significance of SIRPα1 expression on astrocytoma cell lines, we next examined the binding of SIRPα1 on astrocytoma cell lines to its known ligand, CD47. Prior studies with rodent and human cells have shown that SIRPα fusion proteins bind CD47 (10, 16, 18, 42). Similarly, a CD47 fusion protein (CD47Fc) is

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Fig. 2. Immunoprecipitation and Western blotting of astrocytoma cell lines detects signal regulatory protein (SIRP) α. Digitonin lysates were prepared from the indicated cell lines and subjected to immunoprecipitation with anti-SIRPα antibody loaded on protein G-Sepharose beads. Half of the immunoprecipitated protein was subjected to enzymatic deglycosylation with PNGaseF as described in "Materials and Methods." Lysates were then resolved under nonreducing conditions on 10% SDS-PAGE and subjected to Western blotting with an antiseraum specific for SIRPαs. Positions of molecular weight markers are indicated on the border (in thousands).
capable of binding to SIRPα expressed on L cells (20). In these studies, we examined binding by the CD47Fc fusion protein in the presence of an antibody to CD47 (2D3) that does not block binding of CD47Fc to SIRPα but, instead, enhances it. Enhanced binding is not due simply to multimerization of CD47 because enhancement is also seen with Fab fragments of 2D3 antibodies.6 In control studies, CD47Fc bound readily to CHO-SIRPα but not to untransfected CHO cells or to CHO-SIRPα1 (Fig. 5A, top row, left three panels).

By using the combination of CD47Fc and the 2D3 enhancing antibody, we found that all astrocytoma cell lines that expressed SIRPα bound to CD47Fc. Binding to most astrocytoma cells was only detectable in the presence of the enhancing antibody 2D3, with the exception of the U373MG cell line, which expressed the highest level of SIRPα (see Fig. 1; data not shown). In general, the level of binding by CD47Fc correlated with intensity of binding by the anti-SIRP antibody 6.1 (Figs. 1 and 5A).

Binding of CD47Fc could be blocked by excess 6.1 antibody in the SF126 and U373MG cell lines (Fig. 5B), providing additional evidence that this interaction is specific. We conclude that SIRPα expressed on these astrocytoma cell lines is capable of binding CD47, despite underglycosylation compared with

6 E. J. Brown, unpublished results.

Fig. 3. Sequence of signal regulatory protein α genes expressed in U373MG cells. Signal regulatory protein α was cloned from U373MG as described in “Materials and Methods” using PCR. Sequences corresponding to the three immunoglobulin domains (IGR1, IGR2, and IGR3) are shaded, sequences corresponding to the transmembrane domain are overscored, and predicted ITIM motifs are boxed.
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Fig. 4. Northern blot of astrocytoma cell lines. Polyadenylated-enriched RNA from the indicated cell lines was separated on a 1% denaturing formaldehyde gel, transferred to a nylon membrane, and hybridized to a signal regulatory protein α-specific probe (top panel) as described in “Materials and Methods.” Positions of molecular weight marker are indicated on the right, and positions of the 28S and 18S rRNA bands are detected by methylene blue staining are indicated on the left. After detection of signal regulatory protein α-specific bands, the membrane was stripped and rehybridized to a probe for β-actin (bottom panel).

Fig. 5. Binding of CD47Fc fusion protein assayed by fluorescence-activated cell-sorting (FACS) analysis. A, indicated cell lines were incubated with the 2D3 enhancing antibody alone (open histograms) or the CD47Fc fusion protein with 2D3 (filled histograms) as described in “Materials and Methods.” Binding was detected with a phycoerythrin-conjugated secondary antibody specific to human Fc and FACS analysis. B, binding of CD47Fc fusion protein to SF126 (left panel) or U373MG cells (right panel) was performed as described above (open and filled histograms), but binding was also blocked with 5 μg/ml anti-signal regulatory protein α antibody (dotted histograms). Subsequent binding was detected with a phycoerythrin-conjugated secondary antibody specific to human Fc and FACS analysis as described above.

SIRPα is phosphorylated in astrocytoma cell lines. Lysates from the SF126 or U373MG cell lines at rest (lanes 1 and 3) or nonspecifically stimulated with pervanadate (lanes 2 and 4) were subjected to immunoprecipitation with a commercial antiseraum against SHP-2 and then subjected to Western blot analysis using the 6.1 anti-SIRPα antibody (IB: SIRPα). Blots with an isotype-matched control antibody did not reveal bands (data not shown). Blots were subsequently stripped and reprobed with a monoclonal anti-SHP-2 antibody (IB: SHP-2).

Phosphorylation of SIRPα on Astrocytoma Cell Lines Leads to Its Association with SHP-2. Phosphorylation of the ITIM motifs in the SIRPα cytoplasmic domain mediates the recruitment of the tyrosine phosphatase SHP-2 (3, 4). To establish whether this association also occurs in astrocytoma cell lines that express SIRPα1, two lines (SF126 and U373MG) were incubated in pervanadate to nonspecifically block phosphatase activity and thus enhance protein tyrosine phosphorylation. Cell lysates were then subjected to immunoprecipitation with anti-SHP-2 antibody and then assayed by Western blotting with the 6.1 anti-SIRPα antibody. As shown in Fig. 6, SIRPα is coimmunoprecipitated with SHP-2 when, and only when, cells are stimulated with sodium orthovanadate. Thus, on phosphorylation, SIRPα is capable of recruiting SHP-2 in astrocytoma cells.

SIRPα Is Phosphorylated in Astrocytoma Cell Lines in Response to Interaction with CD47. To further establish that SIRPα can be activated in the astrocytoma cell lines, we examined the effect of cell adhesion on the phosphorylation of SIRPα. For these studies, astrocytoma cell lines were grown under serum-free conditions to prevent serum stimulation of SIRPα phosphorylation, after which SIRPα was immunoprecipitated from cell lysates and examined for phosphoryrosine by Western blotting. In hematopoietic cells and transfected cell lines, SIRPα is phosphorylated on adhesion to various extracellular matrix proteins, including vitronectin, laminin, and fibronectin (3, 26, 27). Therefore, we examined cells grown in either the absence or presence of extracellular matrix. By observation, under serum-free conditions, astrocytoma cell lines incubated in uncoated plates did not adhere well to the plates but rather tended to aggregate with each other, whereas cell lines incubated on vitronectin-coated plates adhered with normal morphology (data not shown). Because all of the cell lines express CD47, the ligand for SIRPα, cell aggregation could potentially be due to an interaction between CD47 and SIRPα. However, this was evidently not the only factor mediating astrocytoma aggregation because cell lines without detectable SIRPα expression also adhered to each other, and addition of blocking antibodies to either CD47 or SIRPα did not block this aggregation (data not shown).

Having observed the morphological effects of cell adhesion either to an extracellular matrix protein or to each other, we studied the effects of this adhesion on SIRPα phosphorylation by anti-phosphotyrosine Western blotting. SF126 or U373MG cell suspensions were prepared in serum-free media and incubated either on uncoated plates or vitronectin-coated plates, with either an excess of an anti-CD47 antibody or vitronectin-coated plates adhered with normal morphology (data not shown). Because all of the cell lines express CD47, the ligand for SIRPα, cell aggregation could potentially be due to an interaction between CD47 and SIRPα. Because all of the cell lines express CD47, the ligand for SIRPα, cell aggregation could potentially be due to an interaction between CD47 and SIRPα. Therefore, we examined cells grown in either the absence or presence of extracellular matrix. By observation, under serum-free conditions, astrocytoma cell lines incubated in uncoated plates did not adhere well to the plates but rather tended to aggregate with each other, whereas cell lines incubated on vitronectin-coated plates adhered with normal morphology (data not shown). Because all of the cell lines express CD47, the ligand for SIRPα, cell aggregation could potentially be due to an interaction between CD47 and SIRPα. However, this was evidently not the only factor mediating astrocytoma aggregation because cell lines without detectable SIRPα expression also adhered to each other, and addition of blocking antibodies to either CD47 or SIRPα did not block this aggregation (data not shown).

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the 6.1 anti-SIRP antibody (data not shown). Although CD47 is a known ligand for SIRPα/H9251, this is the first demonstration that CD47 induces phosphorylation of SIRPα/H9251. These results indicate that the phosphorylation of SIRPα requires an interaction with CD47; simple aggregation of cells is insufficient to cause SIRPα phosphorylation if the SIRPα-CD47 interaction is blocked. We also observed that simple adhesion to an extracellular matrix, in this case vitronectin, was insufficient to cause phosphorylation of SIRPα when the SIRPα-CD47 interaction is blocked.

**SIRPs and CD47 Are Expressed on Primary Brain Tumor Specimens.** Although SIRPα is known to be expressed in the developing nervous system, in adult animals it is only expressed in limited regions of the central nervous system (11, 12, 14). Having established the expression of SIRPs on astrocytoma cell lines, we next examined the expression of SIRPs on samples from brain biopsies performed on patients with grade 4 astrocytomas. Immunohistochemistry was performed on frozen specimens because the 6.1 anti-SIRP antibody did not react with formalin-fixed, or paraffin-embedded samples. All specimens were verified to be from high-grade astrocytomas by WHO definitions (53). Slides were read in a blinded fashion by a neuropathologist (E. J. H.) who graded the degree of staining on an arbitrary scale from 0 to 4, and estimated the percentage of cells staining positive. Representative immunohistochemical sections are shown in Fig. 8, and complete results for all 10 samples are given in Table 1. In total, 7 of the 10 sections had detectable staining for SIRPs compared with staining by an isotype-matched control antibody. Control staining was never graded above 1+ (Fig. 8, compare middle panels with left panels), and never showed greater than 0–25% of cells positive. Some tumors expressed SIRPs quite strongly on all cells (sample SF3765), whereas others expressed it strongly on a limited numbers of cells (sample SF3911). Others expressed SIRPs weakly (sample SF4192), and others were completely negative (sample SF4393).

The sections of brain tumors were also examined by immunohis-
Frozen sections of 10 glioblastoma biopsies were stained with an isotype-matched control mAb\(^a\) (control), an anti-SIRP mAb (6.1), or an anti-CD47 mAb (B6H12) as described in “Materials and Methods.” Slides were scored for the percentage of positive cells and intensity of staining in a blinded fashion.

<table>
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<th>Sample no.</th>
<th>Antibody</th>
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<th>Intensity</th>
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<tr>
<td>3765</td>
<td>Control</td>
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<td>Anti-SIRP</td>
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<td>4+</td>
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<td>Control</td>
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<td>0–1+</td>
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<tr>
<td></td>
<td>Anti-CD47</td>
<td>0%</td>
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</table>

\(^a\) mAb, Monoclonal antibody; SIRP, signal regulatory protein.

Table 1  Immunohistochemistry results

To the best of our knowledge, this is the first demonstration that SIRP\(\alpha\) phosphorylation can be induced by interaction with CD47, and adhesion to vitronectin is insufficient to induce SIRP\(\alpha\) phosphorylation without the recognition of CD47. We were unable to demonstrate SIRP\(\alpha\) phosphorylation with our CD47Fc fusion protein (data not shown). Similarly, we were unable to observe SIRP\(\alpha\) phosphorylation upon cross-linking with our 6.1 mAb, which instead blocked SIRP\(\alpha\) phosphorylation in our aggregation assay (data not shown). Thus, the requirements for phosphorylation of SIRP\(\alpha\) appear to extend beyond mere cross-linking of SIRP\(\alpha\) but are met when astrocytoma cells expressing both SIRP\(\alpha\) and CD47 aggregate.

Several reports suggest that cell-cell adhesion can be entirely mediated by a SIRP\(\alpha\)-CD47 interaction (10, 17, 42); however, adhesion of monocytes to endothelial cells is not dependent on the SIRP\(\alpha\)-CD47 interaction (37). In our studies, homotypic adhesion of astrocytoma cell lines to each other was not affected by the SIRP\(\alpha\)-CD47 interaction because aggregation was unaffected by blocking the SIRP\(\alpha\)-CD47 interaction, and cell lines aggregated to the same extent regardless of their expression of SIRP\(\alpha\) (data not shown). Therefore, SIRP\(\alpha\) phosphorylation on astrocytoma cell lines is not dependent on cell aggregation per se but only on the interaction of SIRP\(\alpha\) and CD47.

Our findings may be important with regard to several aspects of astrocytoma behavior. SIRP\(\alpha\) is known to be involved in cytokines-mediated reorganization and cytoskeletal motility in transfected cells (27, 38) as well as neurite outgrowth (13, 14, 41). In addition, multiple reports have established an important role for SIRP\(\alpha\) in the migration and adhesion of macrophages to various substrates (10, 37, 42, 55) as well as in neutrophil chemotaxis (21, 28). Astrocytomas are characterized by extensive infiltration and a striking ability to metastasize locally, processes that are known to be integrin-dependent (reviewed in Refs. 56 and 57). Thus, the presence of SIRP\(\alpha\) might influence the ability of tumor cells to migrate and invade the central nervous system. Indeed, prior studies have reported that transfection of SIRP\(\alpha\) into the glioblastoma cell line U87MG results in defective cell spreading and migration (40). However, it is likely that SIRP\(\alpha\) affects cell motility and adhesion in complex and dynamic ways. For example, fibroblasts derived from mice homozygous for expression of a mutant SIRP\(\alpha\), missing most of the cytoplasmic tail, exhibit accelerated initial adhesion to fibronectin but defective subsequent polarized extension and migration (38). In addition, the role of SIRP\(\alpha\) in the regulation of cell biology may be different between transfected cells and cells that express SIRP\(\alpha\) endogenously. We have thus far not observed any clear correlations between growth characteristics of astrocytoma cell lines and SIRP\(\alpha\) expression, and no consistent differences in morphology are evident between cell lines that express SIRP\(\alpha\) and those that do not. Studies on these cell lines are currently under way to examine the effect of endogenous SIRP\(\alpha\) expression on more subtle tumor characteristics, such as cytoskeletal reorganization, cell motility, and adhesion.

SIRP\(\alpha\) may also affect the response of astrocytomas to mitogenic growth signals. Most reports have shown that expression of SIRP\(\alpha\) inhibits signaling through growth factor receptors, including those with intrinsic tyrosine kinase activity (EGF receptor (4, 33), platelet-derived growth factor receptor (4), and insulin receptor (31)), receptors that recruit cytoplasmic tyrosine kinases (growth hormone receptor (29, 30, 58) and IgE Fc receptor (59)), and G protein-coupled receptors (lysophosphatidic acid receptor (36)). However, one group has shown that SIRP\(\alpha\) enhances MAPK phosphorylation through the insulin receptor in NIH3T3 or Rat1 cells (32). Other groups have...
shown no effect of SIRPα expression on specific aspects of the MAPK signaling cascade, such as EGF-induced MAPK phosphorylation (40), lysophosphatidic acid stimulation of MAPK (36), or fibronectin-mediated phosphorylation of MAPK (26). These experiments all examined cells overexpressing wild-type SIRPα or mutant SIRPα, which could potentially complicate interpretation. We are initiating experiments to examine these effects in astrocytoma lines.

It was important for us to verify the identity of the SIRP expressed on these cell lines because isoforms of SIRPα have been described, and they differ predominantly in the membrane-distal immunoglobulin (V) domain (4). Sequencing of the human genome has thus far revealed only two genes for SIRPα receptors. The first is encoded by eight exons on chromosome 20p13, telomeric to genes for SIRPβ receptors (25, 60). An orthologous gene is encoded on mouse chromosome 2 (61). It appears likely that at least two cDNA variants for SIRPα (SIRPα1 and SIRPα2) are alleles of this locus. These two genes vary almost exclusively in their first immunoglobulin-like domain. The sequence in the NCBI database encodes the SIRPα2 sequence, and there is no other gene or alternate exon in the NCBI database that encodes SIRPα1. There is, however, a second SIRPα gene on chromosome 22, which is encoded as a single exon. The open reading frame of this gene is uninterrupted by stop codons, and it appears to encode the SIRPα3 cDNA. In the two cell lines that we examined in detail (SF126 and U373MG), all but one of the sequences recovered by PCR has matched SIRPα1. Only one PCR clone matched SIRPα2, and in no cases have we identified expression of SIRPα3.

Prior groups have reported alternately spliced forms of SIRPα resulting in a single extracellular immunoglobulin V domain (11, 25). In the present study, most cDNAs cloned by PCR included all immunoglobulin domains, but sequences corresponding to the single immunoglobulin domain splice variant were also detected. Northern blotting, however, showed this variant to be a low abundance transcript. These results are similar to those of Comu et al. (11), who described several minor bands that may correspond to alternatively spliced SIRPα transcripts in mouse brain total RNA. Characterization of the murine SIRPα locus has revealed several other potential splice variants as detected by blotting or direct sequencing (11, 25, 52). In our analysis, these alternative forms were not seen, although other potential novel splice variants were detected at low frequency. These truncated sequences are likely splice variants because the deleted regions correspond exactly to the predicted exonic structure of SIRPα (25), and all sequences are in-frame gene products. The products of the splice variants are not abundant by Northern blotting, but additional studies may reveal that they have functional significance.

In analyzing the expression of SIRPα transcripts on astrocytoma cell lines by Northern blotting, we discovered that eight of nine cell lines expressed mRNA for SIRPα, although only five of these expressed detectable levels of cell surface protein by flow cytometry. We have not detected intracellular SIRPα protein by Western blotting in cells that do not express surface SIRPα (data not shown). Thus, regulation of SIRPα expression probably involves not only transcriptional but also posttranscriptional events. Alternatively, some cell lines may express SIRPα mRNA with mutations that render the SIRPα protein nonreactive to our mAb, or posttranslational modifications of SIRPα may render a gene product not detectable by our 6.1 mAb. This latter possibility seems unlikely because our 6.1 mAb recognizes deglycosylated SIRPα protein (Fig. 2), which implies that it recognizes an epitope on the core polypeptide. Also, we have shown that the degree of SIRPα surface expression, as assayed by the 6.1 mAb, corresponds to the degree of binding to the CD47Fc fusion protein (Fig. 5). Interestingly, Machida et al. (45) have reported that transfection of 3T3 fibroblasts with v-src inhibits SIRPα expression at the transcriptional level, and a recent abstract (62) finds that interfering with the EGF receptor signaling pathway in U87MG or U373MG cells by transfection of a dominant negative EGF receptor mutant can up-regulate SIRPα mRNA levels. We are unaware of any other reports that specifically examine regulation of SIRPα expression at the transcriptional level, although some reports hypothesize that expansion of CCA trinucleotide repeats found in the 3′-untranslated region of the SIRPα gene may be responsible for altered expression in disease states (52, 60). Nevertheless, the caveat remains that mRNA levels may not directly correlate with SIRPα cell surface expression. This may have consequences in experiments using quantitative PCR or cDNA microarrays to examine differential gene expression of SIRPα.

In our attempts to identify the SIRPα expressed on astrocytoma cell lines, we discovered that, although the primary DNA sequence was identical to SIRPα1, the apparent molecular weight of the SIRPα1 protein was slightly less than that of SIRPα1 expressed in CHO cells, due to differences in glycosylation. Reduced glycosylation of SIRPα in neuronal cells has been noted previously in rodent tissues (8, 23, 63), although its functional significance is unknown. In one report, a nonglycosylated SIRPα1-gluathione S-transferase fusion protein produced in bacteria was capable of inhibiting SIRPα1-mediated macrophage fusion, implying that at least one activity of SIRPα1 is not dependent on glycosylation (24). In contrast, the binding affinity of a SIRPα1-Fc fusion protein to tissue sections was changed when the fusion protein was produced in cells deficient in galactosylation (23), arguing that SIRPα1 binding to CD47 is altered on undergalactosylation. In our studies, SIRPα1 expressed by astrocytoma cell lines demonstrated this reduced glycosylation, yet it was still capable of binding to a CD47Fc fusion protein. Detection of binding required coincubation with the 2D3 antibody against CD47, which increases the avidity of the CD47-SIRPα interaction. CD47 has several isoforms that are differentially expressed in various tissues (64), and this diversity may contribute to the variable binding by CD47 to SIRPα that has been observed by others (17, 23). In addition, a SIRPα1 fusion protein binds to CD47 on resting CD4+ or CD8+ T cells, but binding decreases on stimulation of the T cells with concanavalinA, despite equivalent expression of CD47 on the T cells (65). Thus, the affinity of the CD47-SIRPα interaction may vary depending on the biological context and may not simply reflect expression of the molecules per se. It is interesting to consider the possibility that SIRPα1 expressed on astrocytomas may interact with CD47 on T cells, altering the immune response to these tumors.

Although SIRPα1 is expressed on astrocytomas and other tumors, its role in oncogenesis is unknown. Our results show that SIRPα1 is expressed in malignant astrocytes, whereas mouse studies have shown that normal astrocytes do not express SIRPα (7). Our studies demonstrate that levels of SIRPα expression vary considerably in different tumors. It will be of interest to test the correlation between SIRPα expression and outcomes in patients with astrocytomas. Furthermore, the ability of SIRPα to modulate multiple pathways critical to the malignant behavior of tumors may offer new targets for therapeutic intervention in the treatment of malignancies in general and malignant astrocytomas in particular.

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We thank Axel Ullrich for kindly providing the cDNAs for both SIRPα1 and SIRPβ1, Mary Nakamura and Erene Niemi for developing the 6.1 mAb and demonstrating its binding to astrocytomas, Bob Rebres for assistance with...
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