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13. ABSTRACT (Maximum 200 Words)

Signal regulatory proteins (SIRPs include SIRPβ1, which activates cells, and SIRPα1, which inhibits the cellular response to several growth factors, and which regulates cell adhesion and spreading.

We demonstrated by PCR that 3 of 3 prostate cancer cell lines (PC-3, DU-145 and LNCaP) express transcripts for SIRPs. Under this contract, we generated a monoclonal antibody that recognizes both SIRPβ1 and SIRPα1, thereby confirming the expression of SIRPs on PC-3 cells and, to a lesser extent on DU-145 cells. The receptor could not be detected on LNCaP cells. We have since shown by PCR, Western blotting, and by surface staining that PC-3 and DU-145 cells express SIRPα1 but not SIRPβ. We find that they also express the tyrosine phosphatase, SHP-2, and that SHP-2 binds to SIRPα1 when it is phosphorylated, demonstrating that this pathway for the function of SIRPα1 is intact. We have created constructs of epitope-tagged SIRPα1, either intact or with mutations that would alter SHP-2 binding, in order to study its function in PC-3 cells.

We have also worked in particular on the characterization of the SIRPα1 protein in prostate cancer cells. Is there more than one form, due either to alternate splicing or to post-translational modification? These studies have proved challenging, but we expect to complete them, along with all of the objectives of the contract, over the coming year (no-cost extension).

14. SUBJECT TERMS

Signaling, Prostate Cancer, Growth Factor, signal Regulatory Protein SHPS-1

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Table of Contents

INTRODUCTION

Signal regulatory proteins (SIRPs, also known as SHPS-1, BIT, p84, and Myd-1) are normally expressed on certain hematopoietic cells and some brain cells (1-3). SIRPβ1 activates cells and is expressed on cells of monocyte/macrophage lineage. Its ligand is unknown. SIRPα1 inhibits the response of several cell types to growth factors (1), and it regulates integrin-mediated cell adhesion and spreading (4,5). Its ligand is CD47 (integrin associated protein)(6,7). SIRPβ1 and SIRPα1 are highly homologous in their extracellular portions, which include three immunoglobulin (Ig)-like domains (one V and two C domains). By alternative splicing, SIRPα1 can also be expressed with a single Ig-like (V) domain (8). Phosphorylated SIRPα1 binds SHP-2, a tyrosine phosphatase that is widely distributed (1). Thus, expression of SIRPs on tumor cells might be functional and could regulate the response to growth factors and/or the capacity of tumors to invade.

Our Studies were based on our identification by PCR of transcripts for signal regulator protein a1 (SIRPα1) in three prostate cancer cell lines, PC-3, LNCaP, and DU-145. Because of the role of SIRPα1 in regulating the response to growth factors and of other functions that may affect the malignant potential of prostate cancer, we proposed to further examine the expression and function of SIRPs in these prostate cancer cells. To this end, we proposed six objectives:

1. Examine SIRP transcript size and expression level in different prostate cancer cell lines by Northern blotting.
2. Use RT-PCR to obtain and to sequence full-length SIRP transcripts from the prostate cancer cell lines PC-3, DU-145, and LNCaP.
3. Use hybridization to screen cDNA libraries from these prostate cancer cell lines to identify additional SIRP cDNA clones.
4. Overexpress wild-type and mutated SIRPs in prostate cancer lines and, as a control, in NIH3T3 fibroblasts, to assess their effect on the cellular growth response to EGF. These will include studies of the effect of cross-linking SIRPs in cell growth. Mutational studies will include: for alpha SIRPs, mutate the cytoplasmic tyrosines to phenylalanine, for beta SIRPs, mutate the transmembrane lysine to alanine.
5. Assess the effect of EGF on the phosphorylation of SIRPs in prostate cancer cells and of associated proteins obtained by co-immunoprecipitation.
6. Produce monoclonal antibodies (mAbs) against SIRPs.

BODY

This is a revised final report. The prior review of this report requested resubmission with additional data to demonstrate that we had adequately tested the hypotheses. In discussion of the report with Dr. Nrusingha Mishra, it was pointed out that this was in

part necessary because our studies had not produced publishable results. The reviewer noted that our basic hypothesis has proven to be wrong, but the results are again briefly summarized below in the event that there are new reviewers, and to provide some additional comments.

Our studies confirmed the expression of SIRPα in prostate cancer cell lines, particularly PC-3 cells, and they demonstrated that SIRPα in these cells is active in that it binds the tyrosine phosphatase SHP-2. Heartened by these results, we extended our studies beyond the proposed work in cancer cell lines, to define the expression of SIRPs in prostate cancer biopsies. These studies were done in collaboration with Dan Sudilovsky, M.D., a pathologist at UCSF who specializes in the pathology of the prostate. Using our monoclonal antibody to SIRPs (derived as part of this grant) in immunohistochemical studies, we unfortunately could not confirm the expression of SIRPs in freshly obtained biopsies of prostate cancer. In light of these negative findings, we have not yet considered our studies of prostate cancer cell lines worthy of publication beyond the AACR abstract in which they were initially announced.

We also, however, gave the cDNAs for both SIRPα1 and for SIRPβ1 to Chris Haqq, M.D., at UCSF, and the expression of SIRPs has been included in the ongoing studies at UCSF of gene expression in prostate cancer. Per Dr. Haqq, the signal strength from SIRPs is low in biopsies of both prostate and prostate cancer, and if there is any difference between them (which is not yet clear), it is lower in cancer. The UCSF group, however, is also comparing primary prostate cancer to metastatic prostate cancer. These studies are at an early stage (9). It may be of note that the PC-3 prostate cancer line, in which SIRPα1 is most prominently expressed, was derived from a prostate adenocarcinoma that was metastatic to bone (10). Also, as noted in our original application, a sequence segment with homology to SIRPα1 has been identified in a specimen of prostate cancer that was metastatic to bone (GenBank accession AA937670). Thus, we are following with interest the possibility that SIRPs might be selectively expressed in some metastases of prostate cancers or in invasive cancer. If this proves to be the case, it will give new importance to our studies of SIRPs in cell prostate cancer cell lines.

In the prior report, I failed to include a publication from my lab regarding the expression of SIRPs in glial tumors (11). These studies made use of our monoclonal antibody to SIRPs, and the approach to these studies derived from our studies of SIRP expression in prostate cancer cell lines. The publication therefore made appropriate reference to partial support from our DoD grant, although the primary support was from a grant from UCSF. I have appended a copy of this publication. In these studies, we were able to demonstrate SIRP expression not only on astrocytoma cell lines but also, by immunohistochemistry, in fresh specimens of glial tumors. These findings gave us additional confidence that our inability to detect SIRP expression in biopsies of prostate cancer was because SIRPs were not expressed at appreciable levels in these specimens. These studies also demonstrate the potential usefulness of our antibody, and we have made it freely available.

In the prior report, the results of our studies were presented chronologically. We have here presented them instead in the order of the specific aims, in order to facilitate review of each, though we have retained the list of accomplishment by the year in which they were obtained. I have included figures in particular for recent work. I have not included the immunohistochemical stains of prostate cancer biopsies because these were negative.

Specific Aim 1. Examine SIRP transcript size and expression level in different prostate cancer cell lines by Northern blotting. Completed. Although transcripts for SIRPs could be detected in PC-3, DU-145, and LNCaP cells by RT-PCR, the expression in LNCaP cells was very weak, and we could not detect it by Northern blotting. These results were confirmed by flow cytometric analysis of SIRP expression using our monoclonal antibody, produced as part of Specific Aim 6; SIRPS could be detected on PC-3 cells, to a lesser extent on DU-145 cells and not on LNCaP cells, as shown in Fig. 1.

Specific Aim 2. Use RT-PCR to obtain and to sequence full-length SIRP transcripts from the prostate cancer cell lines PC-3, DU-145, and LNCaP. Largely completed. Because partial sequences from the three cell lines were identical, we focused these studies on PC-3 cells. We obtained sequence from multiple PCR clones derived by using different PCR primers. All of these sequences indicate that the expressed transcript is encoded by SIRPα1, not SIRPβ, but many sequences were fragmented and some had alternate starts. In none have we been able to sequence in a single run from the 3' end to the 5' end, because there is a block to sequencing at the 5' end that we have not been able to overcome. Therefore sequences are derived from overlapping RT-PCRs from the same clone. Some of the derived protein sequences are shown in Fig. 2, where they are compared to the sequences for SIRPα1 and SIRPβ1. The most complete clone (hcp1607188b) is almost identical to SIRPα1 except that it has an alternate start site, adding 22 resides, but many other partial clones use the published start site. We conclude that PC-3 cells express SIRPα1 and not SIRPβ1, but that alternate forms of SIRPα1 may be expressed.

Specific Aim 3. Use hybridization to screen cDNA libraries from these prostate cancer cell lines to identify additional SIRP cDNA clones. Completed through alternate approaches. In collaboration with John Kokontis at the University of Chicago, we screened his cDNA library from PC-3 cells. We obtained no clones and we subsequently confirmed by both RT-PCR and flow cytometry that his subclone of PC-3 cells has lost the expression of SIRPs. With the publication of the human genome, it was revealed that there is only one SIRPα1 gene within the SIRP family locus on chromosome 20. As discussed in our attached paper, there is also a second SIRPα gene on chromosome 22 that is encoded as a single exon, but it has not yet been demonstrated that this gene is expressed. We have therefore not further pursued the use of libraries to identify alternate forms of SIRPα1 in addition to those identified by PCR, using primers from different areas of the gene.

Specific Aim 4. Overexpress wild-type and mutated SIRPs in prostate cancer lines and, as a control, in NIH3T3 fibroblasts, to assess their effect on the cellular growth response to EGF. These will include studies of the effect of cross-linking SIRPs in cell growth. Mutational studies will include: for alpha SIRPs, mutate the cytoplasmic tyrosines to phenylalanine, for beta SIRPs, mutate the transmembrane lysine to alanine. Completed, but unsuccessfully. For these studies, we mutated the cDNA for SIRPα1, changing the tyrosines in both ITIMs (immunoreceptor tyrosine-based inhibitory motifs) to phenylalanines (residues 465 and 495). Both the intact and mutated receptor were expressed in PC-3 cells, appending a FLAG epitope to the 5' end to allow their identification and their ligation by anti-FLAG antibody without ligating the endogenous SIRPα1 receptors. The wild type and mutated receptor were expressed at comparable levels. We could not, however, express the effect of these receptors on the response of the PC-3 cells to EGF because we could not demonstrate a change in the growth of PC-3 cells in response to EGF. These studies included varying periods of resting cells in serum-free medium prior to stimulation. There have been conflicting reports on the capacity of EGF to stimulate growth of PC-3 cell, but there is increasing evidence against responsiveness to EGF in the absence of androgens (12-15). We find the same. An example is shown in Fig. 3. Overexpression of intact SIRPα1 reduced the basal level of proliferation of PC-3 cells, although inconsistently, and the cells remained unresponsive to EGF. Overexpression of mutated SIRPα1 affected neither the basal level of PC-3 proliferation nor its response to EGF. Ligation of the receptor with antibodies to FLAG had no effect on the growth response of PC-3 cells to EGF. Because of these results, we have not further pursed this specific aim.

Specific Aim 5. Assess the effect of EGF on the phosphorylation of SIRPs in prostate cancer cells and of associated proteins obtained by co-immunoprecipitation. Alternate studies pursed and completed. Because our PC-3 cells proved unresponsive to EGF, we added an alternative approach to demonstrate the functional association of SIRPα1 with signaling molecules. In hematopoietic cells ITIMs recruit the tyrosine phosphatases SHP-1 and SHP-2, and SIRPα1 has been shown to associate with SHP-1, which is the more abundant. The expression of SHP-1, however, is restricted to hematopoietic cells, while that of SHP-2 is not. We were therefore interested to determine if SIRPα1 in PC-3 cells could associate with SHP-2. To test this, PC-3 cells were exposed to pervanadate, an inhibitor of tyrosine phosphatase, in order to increase phosphorylation of SIRPα1. Cell lysates were then immunoprecipitated with an antibody to SHP-2 (transduction laboratories) under conditions that favor co-immunoprecipitation of associated molecules. The immunoprecipitate was resolved by SDS-PAGE and examined for SIRPα1 by Western blotting with our mAb to SIRPα1. As controls, we examined CHO cells transfected with the cDNA for SIRPα1. As shown in Fig. 4, immunoprecipitation of SHP-2 from PC-3 also precipitates SIRPα1. From these results, we conclude that SIRPα1 in PC-3 cells is functional to the extent that it can recruit SHP-2.

Specific Aim 6. Produce monoclonal antibodies (mAbs) against SIRPs. Completed. We found that this antibody recognizes both SIRPα and SIRPβ. The use of this

antibody is demonstrated in our Western blots and in flow cytometry. It is also used in the attached paper.

Additional studies. Some of our additional studies have been noted above, including the use of our mAb to demonstrate the expression of SIRPα1 on PC-3 cells. We considered the possibility, however, that our antibody was recognizing something other than SIRPα1 on the PC-3 cells, and so we also used immunoprecipitation followed by Western blotting to assess the expression of SIRPα1 on PC-3 cells, with comparison to CHO cells that had been transfected with SIRPα1 (CHO- SIRPα1). Our mAb recognizes SIRPs broadly, and we wanted specifically to exclude recognition of SIRPβ (even though we could not find message for SIRPβ). We therefore used a commercial antiserum to the cytoplasmic domain of SIRPα1, so that we could immunoprecipitate with this and then blot with our mAb to SIRPs. (SIRPβ1 has almost no cytoplasmic domain.) This proved to be a very challenging task, and it was complicated by the fact that SIRPs in both PC-3 cells and CHO- SIRPα1 cells are variably glycosylated, yielding isoforms that were not distinctly resolved and that differed in size between the two lines. We therefore added a step in which the immunoprecipitates were deglycosylated before electrophoresis and Western blotting. The SIRPα1 in CHO- SIRPα1 cells was partially resistant to this step, perhaps because of its abundance, but eventually we were able to confirm that both PC-3 cells and CHO- SIRPα1 cells expressed a protein of the same size (and the appropriate size for SIRPα1), as shown in Fig. 5.

We hope that this revised report makes evident the extensive amount of work that we have put into this project, extending beyond our original specific aims.

KEY RESEARCH ACCOMPLISHMENTS

Year 1

1. The production of monoclonal antibodies to SIRPs
2. The use of monoclonal antibodies to confirm the expression of SIRPs on prostate cancer cells.
3. Stable overexpression of SIRPα1 and of SIRPβ1 in PC-3 cells.

Year 2

1. Confirmation of SIRP transcripts in prostate cancer cells by Northern blotting.
2. Confirmation of SIRPα1 transcripts in PC-3 cells by PCR (no evidence for SIRPβ).
3. Conformation by Western blotting that SIRPα is expressed in PC-3 cells
4. Demonstration that PC-3 cells express the SIRP substrate SHP-2
5. Demonstration in PC-3 cells of the interaction of SIRP with SHP-2.

Year 3

1. Resolution of SIRPα protein size, expressed in prostate cancer cells in both glycosylated and deglycosylated forms.
2. Demonstration by flow cytometry that prostate cancer cells do not express SIRPβ.
3. Construction of mutant SIRPα1, lacking the cytoplasmic tyrosine required for the recruitment of SHP-2.

Year 4

1. Mutation of SIRPα1 at tyrosine residues that are required for the recruitment of the tyrosine phosphatase SHP-2.
2. Expression of intact and mutated SIRPα1 in PC-3 cells
3. Studies (negative) of the effects of SIRPα1 on the growth response to EGF, whether SIRPα1 is ligated or not.

REPORTABLE OUTCOMES

See Conclusions.

CONCLUSIONS

Our studies confirm that PC-3 cells express SIRPα1, and that phosphorylation of this receptor leads to its association with the tyrosine phosphatase, SHP-2. We have not, however, been able to discern a functional effect of SIRPα1 on the response of these cells to EGF, and the importance of our finding in prostate cancer cells is diminished by the growing evidence that expression of SIRPα1 is not increased in fresh specimens of prostate cancers. Thus, while we could report our findings in PC-3 cells, we are not, in the end, inclined to assert that this has importance in prostate cancer, unless it proves that SIRPα1 is differentially expressed in metastases of prostate cancer or in invasive tumor specimens.

On the plus side, our work has provided a series of reagents that are available to the scientific community, and our work led a young investigator in our lab, Tom Chen, to study the expression of SIRPS in brain cancer. Here the evidence supports a role for SIRPs in cancer (11). Dr. Chen has received a Career Development Award from the Veterans Administration to pursue this work. Thus, some good has come of our studies, though not the good that we expected.

REAGENTS DEVELOPED

Notable among these are:

1. Mouse monoclonal antibody to human SIRPs, cross-reactive with both SIRPα and SIRPβ.

2. cDNA for SIRPα1, mutated to inactivate both ITIMs.

3. Cell lines (PC-3, CHO) expressing transfected SIRPα1 or SIRPβ1, both FLAG-tagged.

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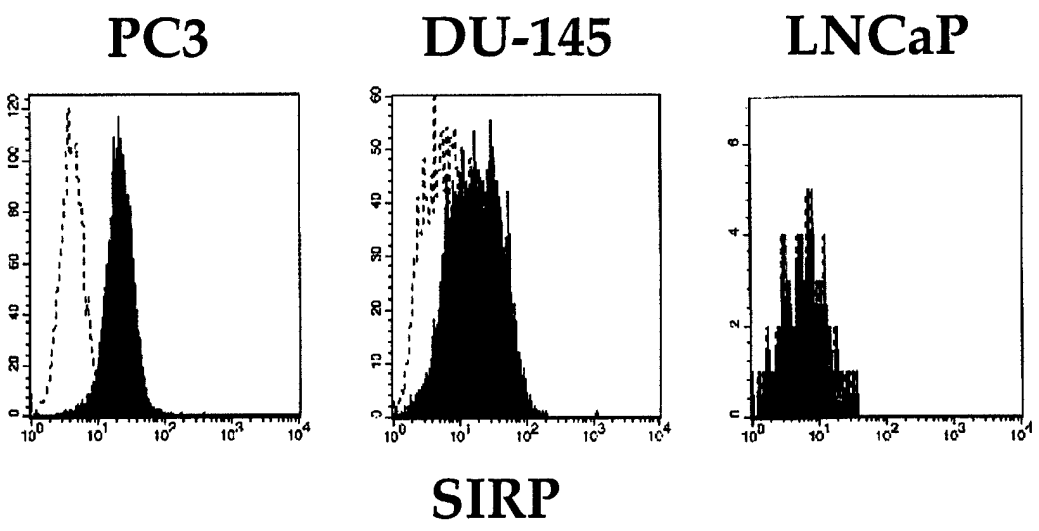

Figure 1. SIRP expression on prostate carcinoma cell lines as assessed by flow cytometry with our anti-SIRP mAb (followed by a second-step anti-mouse antiserum). The open line shows staining with second-step antiserum alone. The filled line shows staining with anti-SIRP. The results demonstrate that SIRPs are expressed on the surface of PC-3 cells. They are also expressed on the surface of DU-145 cells, although the staining is broader in distribution compared to PC-3 cells, and the background for DU-145 cells is higher, reducing discrimination between stained and unstained cells, SIRPs could not be detected on LNCaP cells. These results correlate with levels of message for SIRPs, as assessed either by RT-PCR or by Northern blotting.

Figure 2. Predicted peptide sequences from 5 independently-derived cDNAs, obtained by RT-PCR from PC-3 cells, using primers derived from SIRPs. The sequences are compared to the predicted sequence for (top row) and for SIRPα1 (bottom row).

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                                                                     1                                                                   60
SIRPA1-Ullrich  ---------- ---------- ---------- ---------- ---------- ---------- ---------- --MEPAGPAP GRLGPLLCLL LAASCAWSGV
    hcp1607188b ---------- ---------- ---------- ---------- MWGQGGALFS ALIFILRKPS GQVSLAGGVA AAHGARGPVP GRLGPLLCLL LPASCAWSGV
     hcp1628334 ---------- ---------- ---------- ---------- ---------M SAPTCLAHLP PCFLLLALVL VPSDASGQSS RNDWQVLQPE GPMLVAEGAG
    hcp1628344b ---------- ---------- ---------- ---------- ---------M SAPTCLAHLP PCFLLLALVL VPSDASGQSS RNDWQVLQPE GPMLVAEGAG
       hcp49210 ---------- ---------- ---------- ---------- ---------- ---------- ---------- --MEPAGPAP GRLGPLLCLL LAASCAWSGV
       hcp49218 ---------- ---------- ---------- ---------- ---------- ---------T DVWTEQAPKV SRMPVPASWP HLPSPFLLMT LLLG.RLTGV
SIRPB1-Ullrich  ---------- ---------- ---------- ---------- ---------- ---------- ---------- --MPVPASWP HLPSPFLLMT LLLG.RLTGV

                61                                                                                                      160
SIRPA1-Ullrich  AGEEELQVIQ PDKSVSVAAG ESAILHCTVT SLIPVGPIQW FRGAGPAREL IYNQKEGHFP RVTTVSESTK RENMDFSISI SNITPADAGT YYCVKFRKGS
    hcp1607188b AGEEELQVIQ PEKSVSVAAG ESAALQCTVT SLNPVGPIQR FRGAGPGRKL IYHQKEGHFP RVTTVSDLTK RTNMDFSICI SNITPADAGT YYCVKFQKGS
     hcp1628334 DPEPDLWIIQ PQELVLGTTG DTVFLNCTVL GDGPPGPIRW FQGAGLSREA IYNFGGISHP KATAV....Q ASNNDFSILL QNVSSEDAGT YYCVKFQR..
    hcp1628344b DPEPDLWIIQ PQELVLGTTG DTVFLNCTVL GDGPPGPIRW FQGAGLSREA IYNFGGISHP KATAV....Q ASNNDFSILL QNVSSEDAGT YYCVKFQR..
       hcp49210 AGEEELQVIQ PDKSVLVAAG ETATLRCTAT SLIPVGPIQW FRGAGPGREL IYNQKEGHFP RVTTVSDLTK RNNMDFSIRI GNITPADAGT YYCVKFRKGS
       hcp49218 AGEDELQVIQ PEKSVSVAAG ESATLRCAMT SLIPVGPIMW FRGAGAGREL IYNQKEGHFP RVTTVSELTK RNNLDFSISI SNITPADAGT YYCVKFRKGS
SIRPB1-Ullrich  AGEDELQVIQ PEKSVSVAAG ESATLRCAMT SLIPVGPIMW FRGAGAGREL IYNQKEGHFP RVTTVSELTK RNNLNFSISI SNITPADAGT YYCVKFRKGS

                161                                                                                                     260
SIRPA1-Ullrich  P.DTEFKSGA GTELSVRAKP SAPVVSGPAA RA.TPQHTVS FTCESHGFSP RDITLKWFKN GNELSDFQTN .VDPVGESVS YSIHSTAKVV LTREDVHSQV
    hcp1607188b P.DVELKSGA GTELSVRAKP SAPVVSGPAA RA.TPDHTVS FTCESHGFSP RDISLKWFKN GNQLSDFQTN .VDPARESVS YSIHSTANVV LTRGDIHSQV
     hcp1628334 KPNRQYLSGQ GTSLKVKAKS TS.. SKEAE FTSEPATEMS PTGLLVVFAP VVLGLKAITL AALLLALATS RRSPGQEDVK TTGPAGAMNT LAWSKGQE--
    hcp1628344b KPNRQYLSGQ GTSLKVKAKS TS.. SKEAE FTSEPATEMS PTGLLVVFAP VVLGLKAITL AALLLALATS RRSPGQEDVK TTGPAGAMNT LAWSKGQE--
       hcp49210 PDDVEFKSGA GTELSVRAKP SAPVVSGPAA RA.TPQHTVS FTCESHGFSP RDITLKWFKN GNELSDFQTN .VDPVGDSVS YSIHSTAKVV LTREDVHSQV
       hcp49218 PDDVEFKSGA GTELSVRAKP SAPVVSGPAV RA.TPEHTVS FTCESHGFSP RDITLKWFKN GNELSDFQTN .VDPAGDSVS YSIHSTARVV LTRGDVHSQV
SIRPB1-Ullrich  PDDVEFKSGA GTELSVRAKP SAPVVSGPAV RA.TPEHTVS FTCESHGFSP RDITLKWFKN GNELSDFQTN .VDPAGDSVS YSIHSTARVV LTRGDVHSQV

                261                                                                                                     360
SIRPA1-Ullrich  ICEVAHVTLQ GDPLRGTANL SETIRVPPTL EVTQQPVRAE NQVNVTCQVR KFYPQRLQLT WLENGNVSRT ETASTVTENK DGTYNWMSWL LVNVSAHRDD
    hcp1607188b ICEVAHVTLR GDSFRGTANL SETIQVPPTL EVTQQPMRAE NQVNITCQVT KFYPQRLQLT WLENGNVSRT ETASTLTENK DGTYNWMSWL LVNVSAHRDD
     hcp1628334 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
    hcp1628344b ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
       hcp49210 ICEVAHVTLQ GDPLRGTANL SETIR..... .......... .......... .......... .......... .......... .......... .......GDD
       hcp49218 ICEIAHITLQ GDPLRGTANL SEAIRVPPTL EVTQQPMRAE NQANVTCQVS NFYPRGTTAD LV..GEWKCV PEQKQLRPS- ---------- ----------
SIRPB1-Ullrich  ICEMAHITLQ GDPLRGTANL SEAIRVPPTL EVTQQPMRAE NQANVTCQVS NFYPRGLQLT WLENGNVSRT ETASTLIENK DGTYNWMSWL VKLTCQVEHD
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                361                                                                                                  460
SIRPA1-Ullrich  VKLTCQVEHD GQPAVSKSHD LKVSAHPKEQ GSNTAAENTG SNERNIYI.. VVGVVCTLLV ALLMAALYLV RIRQKKAQGS TSSTRLHEPE KNAREITQDT
   hcp1607188b  VKLTCQVEHD GQSAVSKSHD LKVSAHLKEQ SSNTAAENTG PNEQNIYI.. VVGVVCTLLV ALLMEALYLV RIRQKKAQGS TSSTRLHEPE KNARKITQDT
    hcp1628334  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
   hcp1628344b  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
      hcp49210  GQPAVSKSHD LKVSAHPKEQ GSNTAAENTG SNERNIYI.. VVGVVCTLLV ALLMAALYLV RIRQKK.... .......... .......... ..........
      hcp49218  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
SIRPB1-Ullrich  LVNTCAHRDD VVLTCQVEHD GQQAVSKSYA LEISAHQKEH GSDITHEPAL APTAPLLV.. ALLLGPKLLL VVGVSAIYIC WKQKA------ ----------

                461                                                                                  540
SIRPA1-Ullrich  NDITYADLNL PKGKKPAPQA AEPNNHTEYA SIQTSPQPAS EDTLTYADLD MVHLNRTPKQ PAPKPEPSFS EYASVQVPRK ----------
   hcp1607188b  NDITYADLNL PKGKKPAPRA AEPNNHTEYA SIQTSLQPAS EDTLTYADLD MVHLNRTPKQ LAPKPELSFS EYASIQVPRK ----------
    hcp1628334  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
   hcp1628344b  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
      hcp49210  ......DLNL PKGKKPAPQA AEPNNHTEYA SIQTSPQPAS EDTLTYADLD MVHLNRTPKQ PAPKPEPSFS EYASVQVPRK ----------
      hcp49218  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
SIRPB1-Ullrich  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
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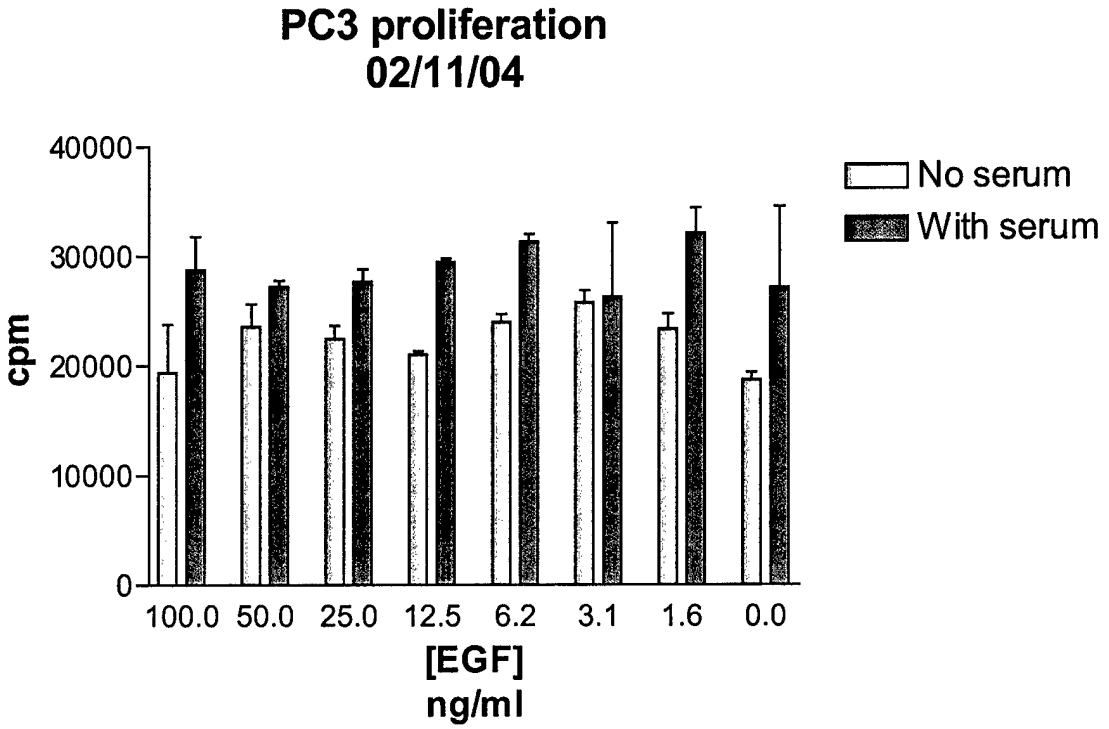

Figure 3. EGF does not alter the proliferation of PC-3 cells grown either in serum or without serum (after serum-starvation for 24 hrs. PC-3 cells were cultured with or without serum in the presence of EGF at the concentrations shown. In this experiment, ^{3}H-thymidine was added at the onset, and uptake into cells was assessed 18 hr later.

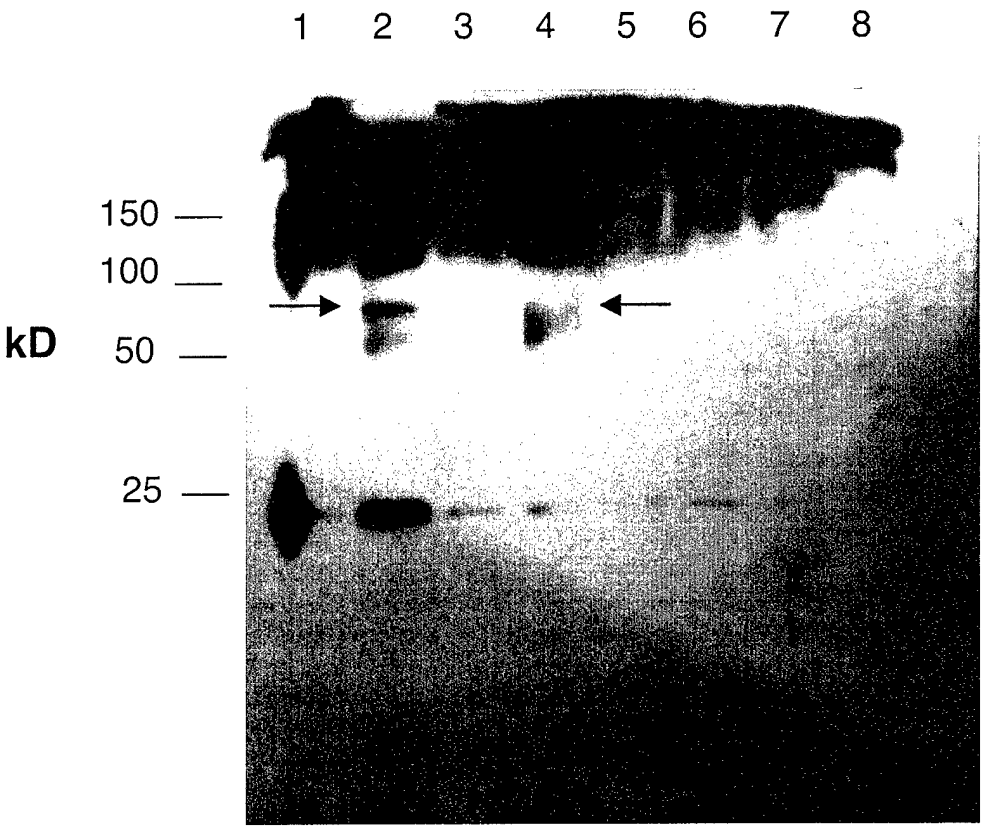

Figure 4. Co-immunoprecipitation of SIRP from PC-3 cells by antibody to SHP-2. SIRP is identified after SDS-PAGE by Western blotting with monoclonal antibody to SIRP. Lane1 = unstimulated PC-3 cells. Lane 2 = pervanadate-stimulated PC-3 cells. Lane 3 = unstimulated CHO-SIRPα1 cells. Lane 4 = pervanadate-stimulated CHO-SIRPα1 cells. Lane 5 = unstimulated CHO-SIRPβ1 cells. Lane 6 = pervanadate-stimulated CHO-SIRPβ1 cells. Lane 7 = unstimulated CHO cells (untransfected). Lane 8 = pervanadate-stimulated CHO cells (untransfected). Right arrow indicates blotting of SIRP in SHP-2 precipitates from pervanadate-treated PC-3 cells. Left arrow indicates blotting of SIRP in SHP-2 precipitates from pervanadate-treated CHO-SIRPα1 cells. No blotting for SIRP was seen in pervanadate-treated CHO-SIRPβ1 cells, in pervanadate-treated CHO cells (untransfected), or in any cells that were unstimulated by pervanadate. The identity of the band below 25 kD, found in all cells, is unknown.

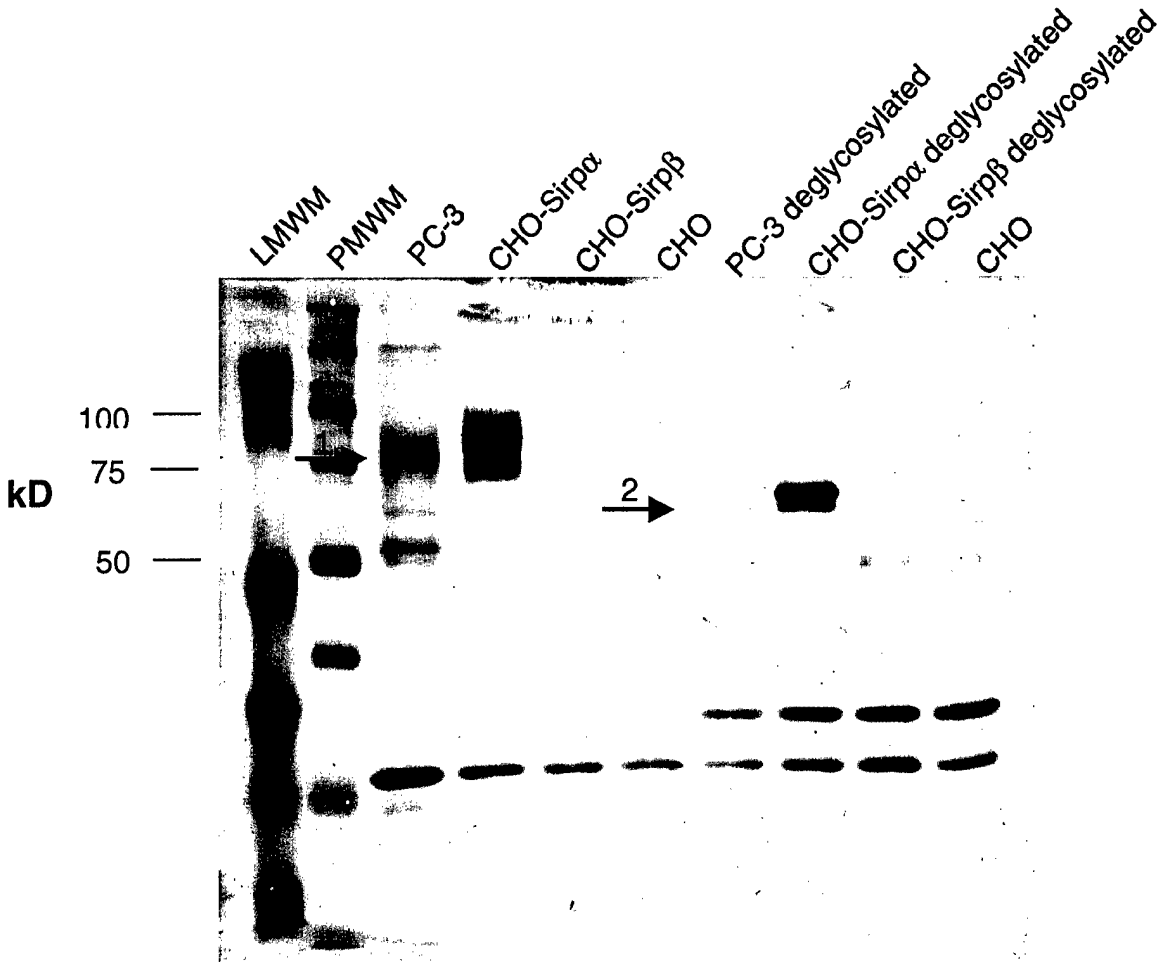

Figure 5. Immunoprecipitation of glycosylated and deglycosylated SIRPa from PC-3 cells, compared to CHO cells transfected with SIRPα1 (or with SIRPβ1 and with untransfected CHO cells). Cell lysates were first immunoprecipitated with a commercial antiserum to the cytoplasmic domain of SIRPα1. Cell lysates were resolved by SDS-PAGE and subjected to Western blotting with our mAb to SIRPs. Arrow 1 shows glycosylated SIRP precipitated from PC-3 cells, compared to CHO-SIRPα1 cells in the next lane. Although both bands are broadly distributed, the SIRP from PC-3 cells runs below that from CHO- SIRPα1. Arrow 2 shows degylcosylated SIRP from PC-3 cells. Although it's intensity is not well seen on this print, it is more clearly seen on the original blot. It runs level with a band of deglycosylated SIRPα1 from CHO- SIRPα1 cells. There is also a more intense band just above this. We suspect, but cannot prove, that this is SIRPα1 that has not been completely deglycosylated.

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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 SIRPα 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 astrocytoma cells, 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 SIRPα. Given the known role of SIRPα 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, SIRPα 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 SIRPα 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 peritoneal 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 different 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 lysophos-

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phatidic acid or after integrin-mediated adhesion to extracellular matrix components (reviewed in Refs. 1 and 2). In the case of adhesion, this phosphorylation is dependent on Src family kinases (26–28), but the kinases responsible for mitogen-stimulated phosphorylation of SIRPα remain unclear. In addition to being phosphorylated by these stimuli, SIRPα down-regulates responsiveness to EGF (4), growth hormone (29–31), insulin-like growth factor (4, 32), serum (3, 4, 33), neurotrophins (34, 35), and lysophosphatidic acid (36). Furthermore, perhaps through its influence on integrin-mediated responses, SIRPα regulates cellular adhesion and/or motility. For example, migration of monocytes or neutrophils is reduced by blocking the interaction of SIRPα with CD47 (21, 37), and the migration of fibroblasts derived from SIRPα mutant mice reveals defective cell spreading on fibronectin, although adhesion is enhanced (38). Furthermore, the process of giant cell formation by macrophage fusion involves phosphorylation of SIRPα, and blockade of the SIRPα-CD47 interaction is sufficient to inhibit fusion (24, 39). Transfection of SIRPα into the glioblastoma cell line U87MG similarly inhibits cell migration and spreading on fibronectin (40). Direct aggregation of Ba/F3 B cells to each other can be mediated by SIRPα-CD47 interactions (17), and neurite outgrowth and adhesion are augmented on surfaces coated with affinity-purified mouse SIRPα (14, 41).

Given the involvement of SIRPα in regulating mitogenesis, cell motility, and adhesion, its expression on tumor cells might be expected to alter their malignant properties. In support of this, SIRPα expression is reduced in blasts from both chronic and acute myeloid leukemias (42). By Northern blotting, SIRPα expression in hepatocellular carcinomas is inversely proportional to tumor size and/or metastasis (43, 44). Expression of transforming *v-src* in fibroblasts down-regulates SIRPα, whereas overexpression of SIRPα reduces anchorage-independent cell growth (45). Although SIRPα is not normally expressed on B cells, representational difference analysis of a Burkitt's lymphoma B-cell line has demonstrated up-regulation of SIRPα on immunoglobulin cross-linking (46). Recent reports have also shown reduced expression of SIRPα in breast cancer relative to paired normal breast tissues, as examined by Western blotting, although the cell types that expressed SIRPα were not identified (47). Finally, transfection of SIRPα into the glioblastoma cell line U87MG affects a number of tumor-related characteristics, including reduced focus formation, increased susceptibility to radiation-induced apoptosis, reduced cell migration and spreading, and reduced EGF-induced phosphatidylinositol 3′-kinase activation (40). Interestingly, in U87MG cells, SIRPα has no apparent effect on cell proliferation or mitogen-activated protein kinase (MAPK) activity as measured by extracellular signal-regulated kinase (ERK) 1, ERK2, and c-Jun NH_2-terminal kinase phosphorylation of *in vitro* substrates. This latter finding is similar to results in Chinese hamster ovary (CHO) cells, where expression of SIRPα does not affect phosphorylation of ERK1 and ERK2 in response to EGF (33). In contrast, SIRPα transfected into NIH3T3 cells inhibits EGF-stimulated phosphorylation of ERK1 and ERK2 (4), and SIRPα transfected into either NIH3T3 or Rat1 cells expressing insulin receptor augments insulin-stimulated phosphorylation of MAPK (32). Thus, the ultimate effects of SIRPα expression may depend on the cell in which it is expressed as well as the signaling complexes in question.

To address the role of SIRPα in the biology of human tumors, we generated a monoclonal antibody (mAb) against SIRPs and used this to survey a panel of cell lines derived from malignant brain tumors for SIRP expression. We found that five of nine cell lines expressed SIRPα, and all cell lines expressed CD47, the ligand for SIRPα1. The SIRPα expressed on all astrocytoma cell lines bound to a soluble CD47 fusion protein. Additional studies of the astrocytoma cell lines SF126 and U373MG confirmed that the expressed SIRP is SIRPα1, 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 SIRPα1 is expressed on astrocytomas. The demonstration that SIRPα1 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 cells were maintained at 37° in 5% CO_2 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 SIRPα1 and SIRPβ1 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 SIRPα1) or BSRα (for SIRPβ1) 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-SIRPα 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 SIRPα1 but, instead, enhances it. The B-1 mouse mAb against SHP-2 and polyclonal goat antiserum (C-20) against a COOH-terminal peptide of SIRPα1 were obtained from Santa Cruz Biotechnology Biotech (Santa Cruz, CA). A mAb against SIRPα was raised by immunizing BALB/c mice with CHO-SIRPα, constructed 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 SIRPα1, but not against untransfected cells, were subcloned and expanded. The resulting antibody, named 6.1, binds to both SIRPα1 and SIRPβ1. Flow cytometry was performed by using a FACScan (BD Biosciences, San Jose, CA). Fluorescein-conjugated antimouse IgG antibodies were obtained from ICN (Aurora, OH). Phycoerythrin-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 10^6 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-SIRP 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 anti-mouse IgG antiserum (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 phenylmethanesulfonyl fluoride was added to 1 mM. Deglycosylation was performed with PNGaseF (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 antiserum against the SIRPα cytoplasmic 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 ^{32}P (Rediprime II; Amersham). Membranes were washed at medium stringency and exposed to film (BioMax MS; Kodak, Rochester, NY). To standardize loading, after probing for SIRPα1, 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 ^{32}P-labeled probe for β-actin.

Reverse Transcription, PCR, and Sequencing. First-strand cDNA was synthesized from RNA, isolated as described above, by using either oligo(dT)$_{12-18}$ (Roche) or a primer specific to the 3′-untranslated region of SIRPα (5′-CGGGGAGGCAGTCGAGGGTCTTCAAAAC-3′) together with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's directions. After 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′-GGCGGGTGAGGAGGAGCTGCAGGTGAT-3′ (predicted to hybridize upstream from the V region) and 5′-GCGGGCTGCGGGCTGGTCTGAATG-3′ (predicted to hybridize near the second ITIM motif). To clone the cytoplasmic tail and part of the 3′-untranslated region, the following primer pair was used: 5′-GCCCGAGAAGAATGCCAGAGAAATAACACA-3′ (predicted to hybridize between the transmembrane domain and the first ITIM) and 5′-CGGGGAGGCAGTCGAGGGTCTTCAAAA-3′ (predicted to hybridize 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 by 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 × 10^6 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% CO_2, 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 (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 frozen 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 LSAB2 avidin-biotin 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 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 SIRPs by immunizing BALB/c mice with CHO cells transfected with a gene encoding SIRPα1. One antibody, 6.1, reacts with SIRPα1 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-

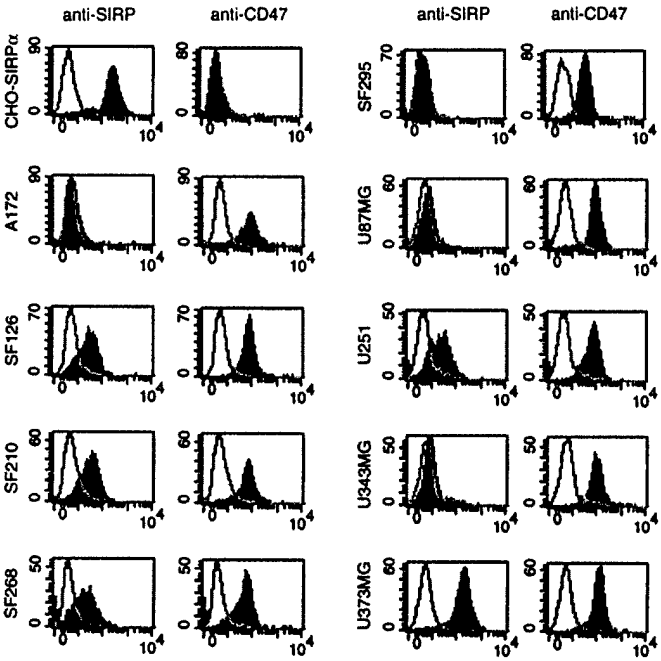

Fig. 1. Fluorescence-activated cell-sorting analysis of astrocytoma cell lines. Anti-signal regulatory protein or anti-CD47 antibodies (*filled histograms*) or an isotype-matched control antibody (*open histograms*) was used in flow cytometry on the indicated cell lines as described in "Materials and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

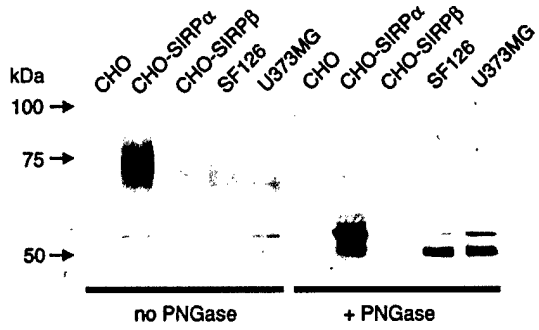

Fig. 2. Immunoprecipitation and Western blotting of astrocytoma cell lines detects signal regulatory protein (SIRP) α. Digitonin lysates were prepared from the indicated cell lines and subjected to immunoprecipitation with anti-SIRPα antibody loaded on protein G-Sepharose beads. Half of the immunoprecipitated 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 antiserum specific for SIRPα. Positions of molecular weight markers are indicated on the border (in thousands).

pression 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 antiserum against a peptide in the cytoplasmic tail of SIRPα1. SIRPβ does not contain a homologous region and thus does not react with this antiserum. 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 antiserum 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_r 70,000–90,000 in CHO-SIRPα1 but at M_r 65,000–75,000 in the astrocytoma cell lines.

Additional studies demonstrated that the difference in molecular weight between SIRPα from CHO-SIRPα1 and SIRPα 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, SIRPα from both CHO-SIRPα and the astrocytoma cell lines bands migrated as a doublet at M_r 53,000–57,000. The smaller size is the predicted molecular weight for human SIRPα (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 Kharitonenkov *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 extracellular sequences 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 SIRP 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 SIRPα on its cell surface (SF295), whereas cell lines with modest levels of mRNA for SIRPα expressed high levels of SIRPα 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

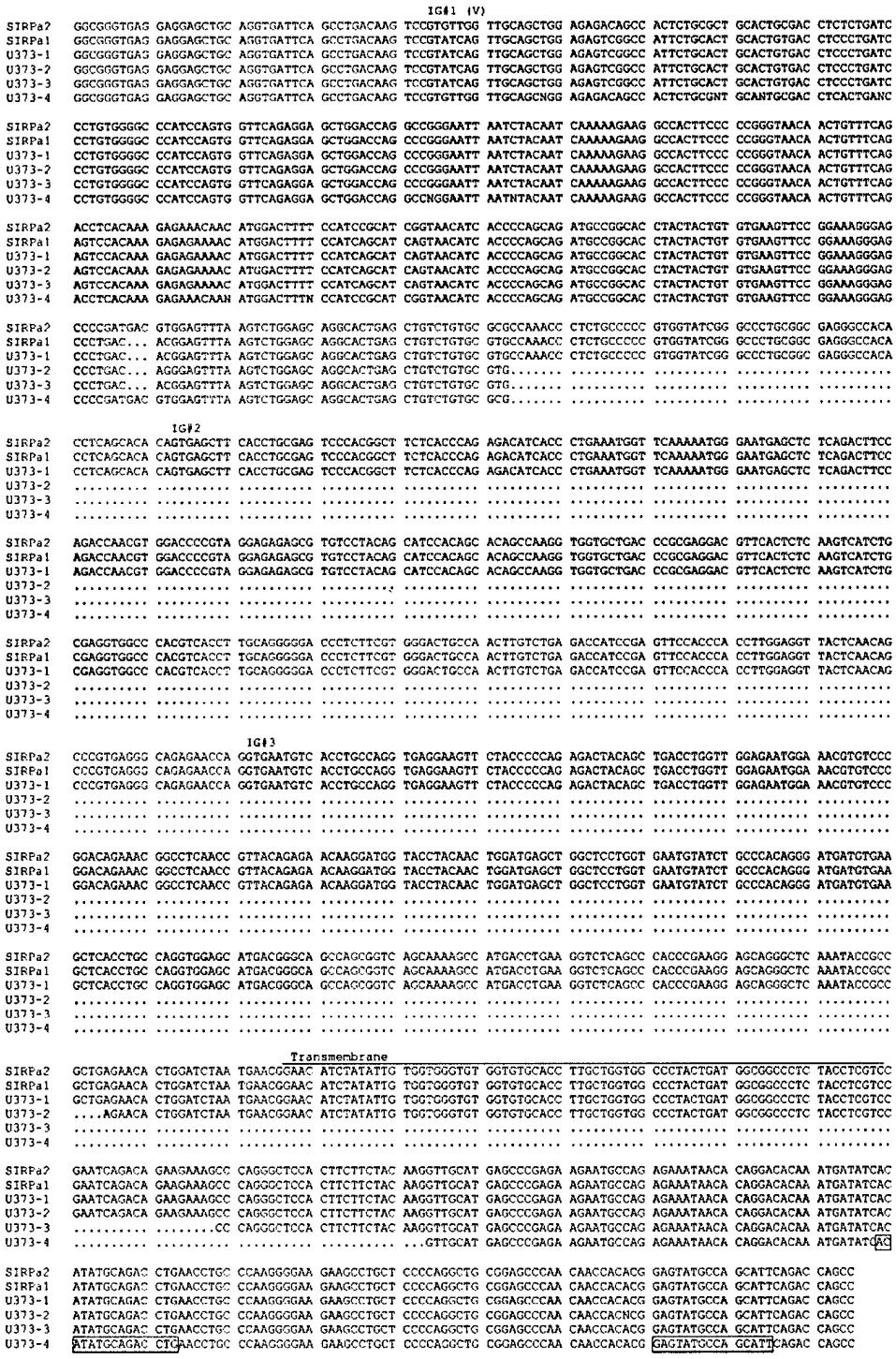

Fig. 3. Sequence of signal regulatory protein α genes expressed in U373MG cells. Signal regulatory protein α was cloned from U373MG as described in "Materials and Methods" using PCR. Sequences corresponding to the three immunoglobulin domains (*IG#1*, *IG#2*, and *IG#3*) are *shaded*, sequences corresponding to the transmembrane domain are *overscored*, and predicted ITIM motifs are *boxed*.

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 SIRPα1 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-SIRPβ1 (Fig. 5*A*, *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 5*A*). Binding of CD47Fc could be blocked by excess 6.1 antibody in the SF126 and U373MG cell lines (Fig. 5*B*), 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.

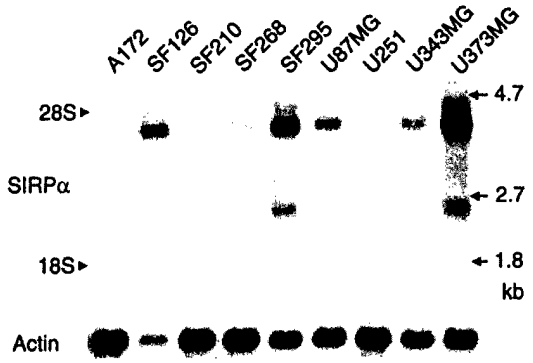

Fig. 4. Northern blot of astrocytoma cell lines. Polyadenylated-enriched RNA from the indicated cell lines was separated on a 1% denaturing formaldehyde gel, transferred to a nylon membrane, and hybridized to a signal regulatory protein α-specific probe (*top panel*) as described in "Materials and Methods." Positions of molecular weight marker are indicated on the *right*, and positions of the 28S and 18S rRNA bands as 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*).

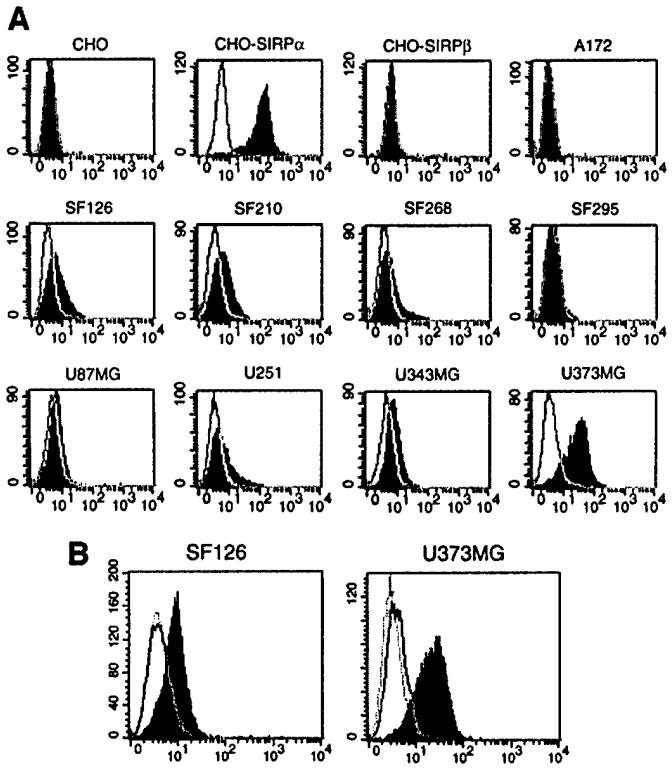

Fig. 5. Binding of CD47Fc fusion protein assayed by fluorescence-activated cell-sorting (FACS) analysis. *A*, indicated cell lines were incubated with the 2D3 enhancing antibody alone (*open histograms*) or the CD47Fc fusion protein with 2D3 (*filled histograms*) as described in "Materials and Methods." 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 expressed on CHO cells. These results also indicate that our mAb to SIRPα1 detects all forms of SIRPα1 that are capable of binding CD47.

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-SIRP 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 phosphotyrosine 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 that blocked its interaction with SIRPα (B6H12), a non-blocking anti-CD47 antibody (2D3), or an isotype control antibody. Cell viability or the extent of aggregation was unaffected by the addition of either blocking antibody (data not shown). SIRPα was heavily phosphorylated on cell aggregation, and this phosphorylation was inhibited when the SIRPα-CD47 interaction was blocked by B6H12 (Fig. 7). Phosphorylation of SIRPα was similarly blocked by

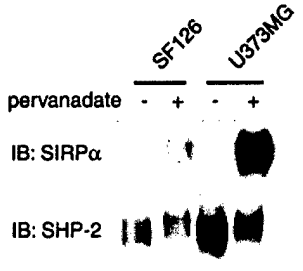

Fig. 6. Association of SHP-2 and signal regulatory protein (SIRP) α in astrocytoma cell lines. Lysates from the SF126 or U373MG cell lines at rest (*Lanes 1* and *3*) or nonspecifically stimulated with pervanadate (*Lanes 2* and *4*) were subjected to immunoprecipitation with a commercial antiserum against 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*).

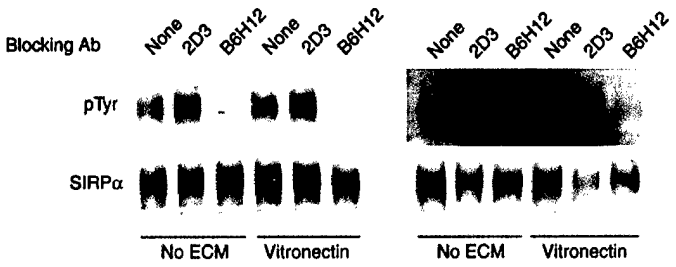

Fig. 7. Phosphorylation of signal regulatory protein (SIRP) α on cell aggregation or adhesion. Experiments were performed using 5×10^6 SF126 (*left panels*) or U373MG (*right panels*) cells resuspended in serum-free media and plated on standard uncoated tissue culture plates (*No ECM*) or vitronectin-coated plates (*Vitronectin*). Appropriate blocking antibody was added [either isotype-matched control (*None*), nonblocking anti-CD47 (*2D3*), or blocking anti-CD47 (*B6H12*)]. Cells were incubated overnight, and cell lysates were prepared and analyzed for SIRPα phosphorylation by immunoprecipitation and Western blotting as described in "Materials and Methods." The blot was then reprobed with a biotinylated anti-SIRP antibody and detected as described in "Materials and Methods." *pTyr*, phosphotyrosine.

the 6.1 anti-SIRP antibody (data not shown). Although CD47 is a known ligand for SIRPα, this is the first demonstration that CD47 induces phosphorylation of SIRPα. 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-

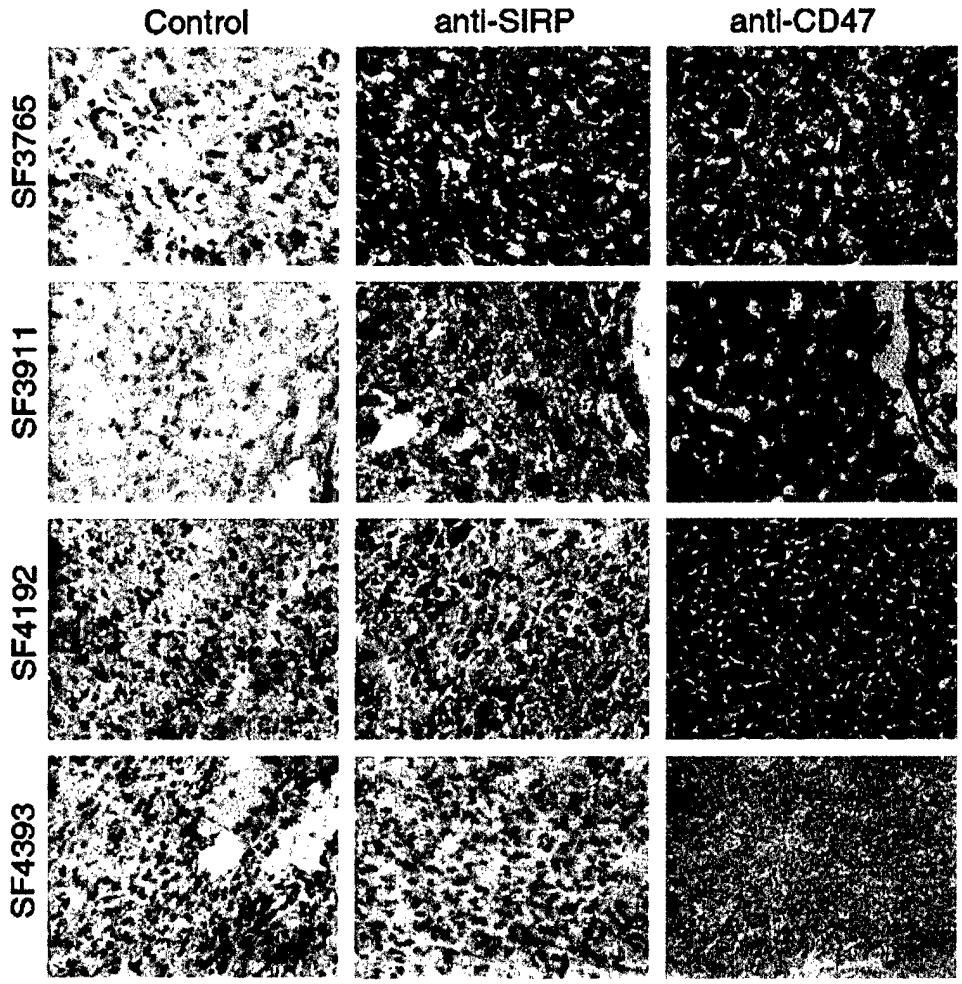

Fig. 8. Immunohistochemistry of astrocytoma specimens. Frozen sections from primary brain biopsies were analyzed by immunohistochemistry against signal regulatory protein α or CD47 and counterstained with hematoxylin. Four representative samples are shown. Staining with an isotype-matched control antibody is shown on the *left panels*, staining with anti-signal regulatory protein is shown on the *middle panels*, and staining for CD47 is shown on the *right panels*.

Table 1 *Immunohistochemistry results*

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.

Sample no.	Antibody	% positive	Intensity
	Control	0–25%	1+
3765	Anti-SIRP	75–100%	4+
	Anti-CD47	100%	2+
	Control	n0–25%	1+
3910	Anti-SIRP	25–50%	2+
	Anti-CD47	75–100%	1+
	Control	0–25%	0–1+
3911	Anti-SIRP	25–50%	2+
	Anti-CD47	75–100%	1+
	Control	0–25%	1+
3932	Anti-SIRP	25–50%	1+
	Anti-CD47	0%	0+
	Control	0–25%	1+
4127	Anti-SIRP	75–100%	2+
	Anti-CD47	75–100%	2–3+
	Control	0%	0+
4192	Anti-SIRP	50–75%	1+
	Anti-CD47	75–100%	1+
	Control	0–25%	1+
4196	Anti-SIRP	50–75%	2+
	Anti-CD47	75–100%	2+
	Control	0%	0+
4209	Anti-SIRP	0–25%	1+
	Anti-CD47	75–100%	1+
	Control	0–25%	1+
4237	Anti-SIRP	0–25%	1+
	Anti-CD47	100%	3–4+
	Control	0%	0+
4393	Anti-SIRP	0%	0+
	Anti-CD47	0%	0+

[a] mAb, Monoclonal antibody; SIRP, signal regulatory protein.

tochemistry for expression of CD47. Although all sections stained strongly with the anti-CD47 mAb and not with control mAb, there was occasionally background staining of areas without viable cells so that we were confident of CD47 expression on tumor cells in only 8 of the 10 specimens. In summary, both primary brain tumors and glioblastoma-derived cell lines frequently express SIRPs, and most, if not all, cell lines and frozen tumors express the SIRPα ligand CD47.

DISCUSSION

In this report, we first provide evidence for expression of SIRPα on both astrocytoma cell lines and brain tumor biopsies. The majority of cell lines and tumors express SIRPα, although levels of expression vary considerably. As assessed by studies of tumor cell lines, most, if not all, of the SIRPα expressed on astrocytomas is identical in sequence to SIRPα1 (3, 4). Furthermore, Northern blotting demonstrates the characteristic pattern for SIRPα1 in all cell lines that are positive for SIRPα expression, although transcripts are also expressed in cell lines without detectable SIRPα1 on the cell surface. Second, we have shown that SIRPα in these astrocytoma cell lines is functional in that it can bind its ligand CD47, is phosphorylated in a CD47-dependent manner, and associates with SHP-2 on phosphorylation. To the best of our knowledge, this is the first demonstration that SIRPα is expressed on astrocytoma cell lines or on primary brain tumors.

Proximal signaling events in SIRPα activation include phosphorylation on tyrosine residues on ITIMs. This phosphorylation can be induced by several stimuli, although to our knowledge phosphorylation of SIRPα by CD47 fusion proteins has not been directly examined (7, 20, 21, 54). Our studies indicate that SIRPα phosphorylation can be induced by interaction with CD47, and adhesion to vitronectin is insufficient to induce SIRPα phosphorylation without the recognition of CD47. We were unable to demonstrate SIRPα phosphorylation with our CD47Fc fusion protein (data not shown). Similarly, we were unable to observe SIRPα phosphorylation upon cross-linking with our 6.1 mAb, which instead blocked SIRPα phosphorylation in our aggregation assay (data not shown). Thus, the requirements for phosphorylation of SIRPα appear to extend beyond mere cross-linking of SIRPα but are met when astrocytoma cells expressing both SIRPα and CD47 aggregate.

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

Our findings may be important with regard to several aspects of astrocytoma behavior. SIRPα is known to be involved in cytoskeletal reorganization and cell motility in transfected cells (27, 38) as well as neurite outgrowth (13, 14, 41). In addition, multiple reports have established an important role for SIRPα 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 SIRPα might influence the ability of tumor cells to migrate and invade the central nervous system. Indeed, prior studies have reported that transfection of SIRPα into the glioblastoma cell line U87MG results in defective cell spreading and migration (40). However, it is likely that SIRPα affects cell motility and adhesion in complex and dynamic ways. For example, fibroblasts derived from mice homozygous for expression of a mutant SIRPα, 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 SIRPα in the regulation of cell biology may be different between transfected cells and cells that express SIRPα endogenously. We have thus far not observed any clear correlations between growth characteristics of astrocytoma cell lines and SIRPα expression, and no consistent differences in morphology are evident between cell lines that express SIRPα and those that do not. Studies on these cell lines are currently under way to examine the effect of endogenous SIRPα expression on more subtle tumor characteristics, such as cytoskeletal reorganization, cell motility, and adhesion.

SIRPα may also affect the response of astrocytomas to mitogenic growth signals. Most reports have shown that expression of SIRPα 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 SIRPα 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*.[7] There is, however, a second SIRPα gene on chromosome 22, which is encoded as a single exon.[7] 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 SIRPs 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.[7] 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 SIRPα 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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[7] T. Chen and W. Seaman, unpublished observations.

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