SIRPβ1 Is Expressed as a Disulfide-linked Homodimer in Leukocytes and Positively Regulates Neutrophil Transepithelial Migration

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Signal regulatory proteins (SIRPs) comprise a family of cell surface signaling receptors differentially expressed in leukocytes and the central nervous system. Although the extracellular domains of SIRPs are highly similar, classical motifs in the cytoplasmic or transmembrane domains distinguish them as either activating or inhibitory isoforms. We reported previously that human neutrophils (polymorphonuclear leukocytes (PMN)) express multiple SIRP isoforms and that SIRPα binding to its ligand CD47 regulates PMN transmigration. Here we further characterized the expression of PMN SIRPs, and we reported that the major SIRPα and SIRPβ isoforms expressed in PMN include Bit/PTPNS-1 and SIRPβ1, respectively. Furthermore, although SIRPα (Bit/PTPNS-1) is expressed as a monomer, we showed that SIRPβ1 is expressed on the cell surface as a disulfide-linked homodimer with bond formation mediated by Cyx-320 in the membrane-proximal Ig loop. Subcellular fractionation studies revealed a major pool of SIRPβ1 within the plasma membrane fractions of PMN. In contrast, the majority of SIRPα (Bit/PTPNS-1) is present in fractions enriched in secondary granules and is translocated to the cell surface after chemotactant (formylmethionylleucylphenylalanine) stimulation. Functional studies revealed that antibody-mediated ligation of SIRPβ1 enhanced formylmethionylleucylphenylalanine-driven PMN transepithelial migration. Co-immunoprecipitation experiments to identify associated adaptor proteins revealed a 10–12-kDa protein associated with SIRPβ1 that was tyrosine-phosphorylated after PMN stimulation and is not DAP10/12 or Fc receptor γ chain. These results provide new insights into the structure and function of SIRPs in leukocytes and their potential role(s) in fine-tuning responses to inflammatory stimuli.

Signal regulatory proteins (SIRPs) are a family of transmembrane receptor-like signaling proteins that are abundantly expressed in hematopoietic cells, including granulocytes, monocytes, dendritic cells, and lymphocytes (1–3). In addition, SIRPs are expressed in neuronal cells (4–6) and certain types of cancer cells (7–10). SIRPs can be divided into two subfamilies, SIRPα and SIRPβ, based on the putative structures of their C-terminal intracellular domains (11). SIRPs share a common immunoglobulin superfamily structures with an N-terminal extracellular domain containing three cysteine-bound Ig-like loops, a single membrane-spanning transmembrane domain, and a C-terminal intracellular domain (11). The C-terminal intracellular domains of the SIRPα subfamily contain a relatively long amino acid sequence (110 amino acids for SIRPα1) that includes four tyrosine residues to form two immunoreceptor tyrosine-based inhibition motifs (ITIM). Conversely, SIRPβ subfamily members have a short intracellular domain containing only a few amino acids (4 amino acids for SIRPβ1). Despite a short cytoplasmic tail, SIRPβ1 contains a positively charged lysine in the transmembrane domain that can mediate interactions with an immunoreceptor tyrosine-based activation motif (ITAM), containing adaptor protein. This type of protein structure and adaptor protein interaction has been shown for other immunoreceptors such as NK cell receptors and Fc receptors (12–14) where the adaptor proteins DAP12 (also termed KARAP (15)), DAP10, and Fc receptor γ chain (FcRγ) interact with the parent receptor through ionic interactions within the transmembrane domain to mediate outside-in signaling (16). However, not all SIRPβ family members share this type of protein structure. SIRPβ2 (also termed SIRPγ) lacks an apparent adaptor-binding element in its transmembrane domain. These different structures of SIRP family members and their associated distinctive signaling elements suggest potential divergent roles of SIRP isoforms in regulating cellular functions.

In a previous study (17), we reported evidence of several SIRP proteins that are expressed in human PMN. In addition, we demonstrated that SIRPα regulates PMN transmigration across epithelial monolayers through interaction with another Ig superfamily cell surface protein CD47. However, given the highly homologous extracellular domain structures of SIRP family members, it was difficult to rigorously define the identities and functional roles of other SIRP proteins in PMN. In the present study, we utilized SIRPα and -β isoform-specific antibodies and RT-PCR to identify the major isoforms of SIRPα and -β in human PMN. From these studies, we discovered important structural differences between SIRPα and -β. In particular, we report for the first time that SIRPβ1 is expressed not as a monomer but as a disulfide-linked homodimer.
disulfide-linked homodimer. Furthermore, our results suggest that, in contrast to SIRPα, antibody-mediated ligation of SIRPβ1 enhances PMN transmigration.

MATERIALS AND METHODS

**Antibodies**—Rabbit anti-SIRPα1.ex antibody that is reactive with multiple SIRP protein species in leukocytes was kindly provided by Dr. Axel Ullrich (11) and was used in immunoblotting as described previously (17). A murine polyclonal antibody specific to SIRPα was generated by immunizing mice with a fusion protein consisting of the extracellular domain of human SIRPα1 (Ig loops 1 + 2 + 3) fused to rabbit Fc (SIRPα1.ex-Fc) (18). Monoclonal antibodies B4B6 and B1D5 that specifically bind to SIRPβ1 were generated as described previously (19). Anti-SIRPα mAbs SE5A5 and SE7C2 were generated and used as described previously (17, 20). Anti-DAP12 mAb D337 (21) was kindly provided by Dr. Lewis Lanier (University of California, San Francisco). In addition, we produced murine polyclonal anti-DAP12 and DAP10 antibodies by immunizing BALB/c mice with a gluthathione S-transferase fusion protein containing the putative intracellular domain of DAP12 and DAP10. Rabbit polyclonal antibody against FcγY was obtained from Upstate Biotechnology, Inc. As a noninhibitory antibody binding control for PMN transmigration assays, we used anti-JAM-A mAb J10.4 (22). As an inhibitory control for PMN transmigration assays, we used anti-CD47 mAb C5D5 as described previously (23).

**PMN, Peripheral Blood Mononuclear Cells (PBMC), and HL60 Cells**—To isolate PMN and PBMC, fresh blood from healthy donors and anticoagulated with 0.38% sodium citrate was centrifuged for 10 min (1000 rpm) at room temperature, and the upper layer of platelet-rich plasma was removed. The lower layer of cells was subjected to dextran (Amersham Biosciences) sedimentation to separate leukocytes from red blood cells. The leukocyte-containing fraction was further separated by Ficoll-Paque (Amersham Biosciences) sedimentation followed by sepa-

**Epithelial Cells**—T84 cells (passages 59–76) were grown in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium supplemented with 6% fetal bovine serum. For transmigration experiments, T84 cells were grown on collagen-coated, permeable polycarbonate filters (5-μm pore size) with a surface area of 0.33 cm² (Costar, Cambridge, MA) as described previously (24).

**Immunoprecipitation and Immunoblotting Experiments**—Cells were lysed with buffer containing 100 mM Tris (pH 7.5), 150 mM NaCl, 2 mM CaCl₂, 2 mM MgCl₂, 1% Triton X-100 or octyl glucoside, 1:100 dilution of proteinase inhibitor mixture (Sigma), and 1 mM phenylmethylsulfonyl fluoride. Cell lysates (200–400 μg of total protein) were pre-cleared with 2–5 μg of control IgG-conjugated Sepharose before incubation with 1–2 μg of specific mAb and protein A-Sepharose (Sigma) for 4 h (4 °C). Washed immunoprecipitates were boiled in SDS-PAGE sample buffer with (reducing condition) or without β-mercaptoethanol (nonreducing condition) and subjected to 6%–15% SDS-PAGE followed by transfer to nitrocellulose under standard conditions. Nonspecific binding was blocked by 5% nonfat dry milk in TBST (2 mM Tris, 50 mM NaCl, 0.5% Tween 20 (pH 7.4)) followed by immunoblotting with the specific antibody. To detect SIRPβ1 in leukocytes, immunoprecipitation was performed using mAb B4B6 or B1D5 followed by immunoblotting with the same mAb or with rabbit anti-SIRPα1.ex. To confirm SIRPβ1 existing as a disulfide-bonded dimer, total cell lysates or SIRPβ1 immunoprecipitates were treated with 30–300 mM iodoacetamide for 30 min at 25 °C before SDS-PAGE under nonreducing conditions and Western blot. To biotinylate SIRPβ1 in PMN, freshly isolated PMN (10⁷) were incubated with N-hydroxysuccinimide-biotin (Pierce) at a final concentration of 1 mg/ml in HBSS for 1 h on ice. Cells were then washed three times with HBSS followed by quenching with 20 mM Tris (pH 7.5) and 100 mM NH₄Cl for 1 h before cell lysis. After immunoprecipitation of SIRPβ1, Western blots of biotin-labeled proteins were probed with streptavidin-peroxidase followed by enhanced chemiluminescence (Amersham Biosciences). SIRPα protein was immunoprecipitated with a murine polyclonal anti-SIRPα antibody. In a subset of experiments, SIRPα was co-precipitated using CD47-AP fusion protein as described below. To chemically cross-link proteins in PMN, unstimulated and fMLP-stimulated PMN (2 × 10⁷) were incubated with 1 mM dithio-bis(succinimidylpropionate) or dithiobis[sulfosuccinimidylpropionate] (Pierce) in HBSS for 1 h. The reaction was terminated by washing and quenching with 0.2 M Tris (pH 7.5) and 0.1 mM NH₄Cl for 30 min. SIRPα, DAP12, and CD11b were detected by immunoblotting of the cell lysates using mAb B4B6, mouse anti-DAP12 antibody, and rabbit anti-CD11b antibody R7928A (23).

**SIRPα Co-precipitation Experiments**—A recombinant fusion protein consisting of the putative extracellular domain of CD47 and alkaline phosphatase (CD47-AP) was generated as described previously (17). A fusion protein containing the extracellular domain of human JAM-A (JAM-AP) was used as a control. Both CD47-AP and JAM-AP (2 μg each) were incubated with PMN cell lysates for 2 h at 4 °C followed by further incubation with 20 μl of anti-AP conjugated agarose (Sigma) for 2 h. The agarose bead–protein complexes were washed three times followed by SDS-PAGE under nonreducing conditions and blot with antibodies against SIRPα and SIRPβ1.

**RT-PCR Amplification of SIRPα and -β Subfamily Isoforms from Leuko-

cytes**—Total RNA was isolated from freshly isolated PMB (5 × 10⁶) and PMN (2 × 10⁶). Because PMN have much less RNA than PBMC and, from our experience, in vitro isolated PMN generally contain 5–10% PMB contamination, we collected PMN after 2 h of migration across collagen-coated filters toward fMLP to greatly enrich preparations in PMN and avoid contamination with PBMC. We found that the vast majority of cells that migrated across in 2 h are PMN (>99.5%) and thus used these cells to isolate RNA. To amplify SIRPα isoforms, RT-PCR was performed, and multiple sense and antisense primers were used as follows: SP1 (5’-ccgggccccatggagcgcggcc); SP2 (5’-ttggttctccagcggccggtatt); SP3 (5’-ggtcagacattcaccggtc); SP4 (5’-gggaagacgctacccgtgct); SP5 (5’-gctgtctggctgctgctgctgct); SP6 (5’-aggttctggctgctgctgctgct); SP7 (5’-ccgccccagctggtctgctgct); SP8 (5’-cagccacggtacgctgctgct); SP9 (5’-ggttctggctgctgctgctgct); bcsirp (5’-cctgattagcgccgctc); xcsirp (5’-ggtcagacattcaccggtc); CS1 (5’-gttctggctgctgctgctgct); CS1 (5’-gttctggctgctgctgctgct); CS2 (5’-ctccaaaatgccgctgctgct); CS2 (5’-ctccaaaatgccgctgctgct); and SPB5 (5’-atatctcgagcagtcaggccttctgtttccagc); SPB2 (5’-gacccacggtacgctgctgct); SPB3 (5’-cctgattagcgccgctc); and SPB4 (5’-ggtcagacattcaccggtc).

**PCRs were also performed using a human leukocyte cDNA library (BD Biosciences, marathon-ready cDNA) as the template, and amplified DNA fragments were sequenced.**

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Site-directed Mutagenesis of Cysteine Residues in SIRPβ1—Full-length, wild-type SIRPβ1 was cloned into pCDNA3.1 and used as a template for mutagenesis. Cys-320 was mutated to serine and alanine using forward primers 5′-agtgtctgcttgtaaacacactgtgcccagg and 5′-agtgtctgcttgtaaacacactgtgcccagg, respectively. The reverse primer for constructing both C320S and C320A was 5′-agtgtctgcttgtaaacacactgtgcccagg and reverse primer 5′-gatgtagatg-ggagacacaccacag. Mutations of Cys-320 and Cys-393 were performed using the GeneTAi1or site-directed mutagenesis system (Invitrogen). After confirmation by DNA sequencing, at least two clones of each mutant were selected and transiently transfected into COS cells using the DEAE-dextran method. COS cells were then lysed 48–72 h after transfection, and SIRPβ1 expression was analyzed by SDS-PAGE and immunoblotting. In addition, expression of SIRPβ1 was also assessed by immunofluorescence staining and flow cytometry analysis (fluorescence-activated cell sorter).

Subcellular Fractionation Experiments—Stimulation of PMN was performed in Petri dishes using 1 μM MLP in HBSS and incubation at 37 °C (30 min). Unstimulated and stimulated PMN (2 × 10⁶ cells per condition) were then resuspended in 5 ml of cavitation buffer (0.34 M sucrose, 10 mM HEPES, 1 mM EDTA, 1 mM MgCl₂, 1 mM Na₂ATP (pH 7.4)) and disrupted by nitrogen cavitation (15 min, 400 p.s.i., 4 °C). The lysates were centrifuged at low speed (1000 rpm), and the supernatants were subjected to isopycnic sucrose density gradient fractionation on linear 20–55% sucrose gradients in a Beckman SW-28 swinging bucket rotor (100,000 × g, 3 h, 4 °C) as described previously (23). From each gradient, 1.5-ml fractions were collected and were analyzed for sucrose density and protein. The subcellular localization of plasma membrane and primary and secondary granules was determined by assays of alkaline phosphatase, myeloperoxidase, and lactoferrin, respectively, as described previously (23).

PMN Transepithelial Migration Assay—PMN transepithelial migration in the physiologically relevant basolateral to apical direction was performed using isolated PMN and intestinal epithelial monolayers exactly as described previously (23, 24). Briefly, confluent inverted T84 monolayers were washed twice with HBSS (20 °C), and PMN (10⁶) in 5 ml of HBSS with or without antibody were added to the upper chamber of the monolayer setup. Transmigration was initiated by adding 1 ml of 1 μM MLP (in HBSS) to the lower chamber followed by incubation at 37 °C. PMN migration across epithelial monolayers into the MLP-containing lower chambers was quantified by myeloperoxidase assay (23, 24).

RESULTS

Identification of SIRP Proteins in Leukocytes—In previous experiments, we used a polyclonal rabbit antibody against the SIRPα1 intracellular domain, anti-SIRPα1.1 (11), to study SIRPα expression in PMN (17). We observed that this antibody labels multiple protein bands in Western blots from detergent-solubilized PMN (17). As shown in Fig. 1A, immunoblots of PMN using anti-SIRPα1.1 revealed broad bands with molecular mass values of 110–120, 65–75, and/or 52–58 kDa (Fig. 1A, arrows) after nonreduced SDS-PAGE. Similar immunoblotting patterns of SIRP proteins were also obtained from detergent-solubilized PBMC and PMN-like Me₅SO-induced HL60 cells by anti-SIRPα1.1 under nonreducing conditions. Because of the high degree of homology between the extracellular domains of SIRP family isoforms, we hypothesized that anti-SIRPα1.1 is likely cross-reactive with several SIRP isoforms.

To better characterize the isoforms of SIRPs expressed in PMN and to study their functional roles PMN, we performed experiments using SIRPβ isofrom-specific antibodies. Monoclonal antibodies B4B6 and B1D5 were generated against the SIRPβ1 extracellular domain (19), and binding was confirmed to be noncross-reactive with SIRPs by enzyme-linked immunosorbent assay (data not shown) and cell binding assays (19). These specific mAbs were used to immunoprecipitate and/or immunoblot SIRPβ from detergent-solubilized PMN and other leukocytes. As shown in Fig. 1B, under nonreducing conditions, mAbs B4B6 and B1D5 exclusively immunoprecipitated and immunoblotted a protein band of 110–120 kDa, which correlates with the highest molecular weight SIRP protein species shown in Fig. 1A. These results were surprising given that SIRPβ has a predicted core molecular mass of ~45 kDa (398 amino acids for SIRPβ1). Because these results were obtained after nonreducing SDS-PAGE, we hypothesized that SIRPβ protein may exist as a disulfide-linked homodimer or hetero-oligomer. Indeed, as shown in Fig. 1C, after reducing SDS-PAGE, the anti-SIRPβ1 mAb-reactive band decreased to 55 kDa. To exclude hetero-oligomerization of SIRPβ with other protein(s) through disulfide bonding, PMN were biotinylated with cell-permeable N-hydroxysuccinimide-biotin followed by immunoprecipitation and nonreduced SDS-PAGE. In these experiments, we excised the 110–120-kDa SIRPβ protein band from the nonreduced acrylamide gels and performed a second electrophoresis under reducing conditions. Western blots of the second running were then performed using peroxidase-conjugated streptavidin. No protein band other than the characteristic 55-kDa SIRPβ protein band was detected (results not shown). Finally, to rule out the possibility that the dimerization observed is a gel artifact due to oxidation of unpaired cysteine residues during electrophoresis, we confirmed that iodoacetamide treatment of PMN had no effect on the electrophoretic mobility of SIRPβ (data not shown). These results thus support the notion that SIRPβ likely exists as disulfide-bonded homodimer in leukocytes.

To identify SIRPa in PMN, we generated SIRPa-specific antibodies using fusion proteins containing either SIRPα1 extracellular domain (SIRPα1.ex-Fc) or SIRPα1 intracellular domain (SIRPα1.ct-GST). As shown in Fig. 1D, under nonreducing and reducing conditions, the SIRPα1 antibodies label a protein band of 65–75 kDa from detergent-solubilized PMN and other leukocytes, which corresponds to the middle protein band (arrow) in Fig. 1A, and had no cross-reactivity with the 110–120-kDa SIRPβ band (Fig. 1B). In addition, we also performed immunodepletion experiments using anti-SIRPa mAb B4B6, and the results confirmed these findings (data not shown).

To confirm further that this 65–75-kDa protein is indeed SIRPa, we performed co-precipitation assays with a SIRPa-binding CD47 extracellular domain fusion protein (CD47-AP), which was previously confirmed to bind specifically to SIRPa but not to SIRPβ1 (17). As shown in Fig. 1E, compared with the control, co-precipitation using another abundantly expressed PMN immunoglobulin superfamily member JAM-A (JAM-AP) (17), CD47-AP specifically precipitated a protein of 65–75 kDa that was labeled by anti-SIRPa. This result is consistent with the immunoblotting results (Fig. 1D) and suggest that the protein of 65–75 kDa is SIRPa. In contrast to SIRPβ, the apparent molecular mass of SIRPa is not significantly decreased after reduction and remains at 65–75 kDa (Fig. 1D), indicating that SIRPa most likely exists as a monomer with a molecular mass of 65–75 kDa in human leukocytes.

The Predominant SIRPa and β Subfamily Isoforms in PMN Are Bit/PTPNS-1 and SIRPβ1—Because previous studies suggest that there are multiple isoforms of SIRPs (SIRPα1 and -α2, Bit (25)/PTPNS-1, and MFR (26)) and SIRPβ (SIRPβ1, -β2), we performed RT-PCR to define the specific SIRPa and -β sequences from human PMN. Total RNA
samples were isolated from transmigrated PMN (see "Materials and Methods") to minimize contamination of other leukocytes. Multiple oligonucleotide primers that correspond to multiple conserved and variable regions of SIRPα isoforms were synthesized according to the sequences of SIRPα1 (NCBI accession number Y10375), SIRPα2, Bit (accession number AB023430)/PTPNS-1 (accession number AL117335), and were used in PCRs with different primer annealing temperatures. DNA sequencing revealed one predominantly amplified sequence that matched Bit/PTPNS-1. A similar strategy was designed to define the specific SIRPβ isofrom and revealed that all differentially amplified DNA fragments matched SIRPβ1.

Cysteine 320 Mediates Homodimer Formation in SIRPβ1—Given that SIRPα (Bit/PTPNS-1) appeared to exist as a monomer in PMN, our data suggesting that SIRPβ1 exists as a disulfide-bonded homodimer were intriguing because the primary structure of the extracellular and transmembrane domains of SIRPβ1 are so similar to those of Bit/PTPNS-1 or SIRPα1. As shown in Fig. 3A, alignment of SIRPβ1 and Bit/PTPNS-1 revealed three pairs of cysteine residues that most likely bridge the putative Ig loops given their conserved nature. Two additional cysteine residues, Cys-320 and Cys-393, are unique to SIRPβ1 and are not present in Bit/PTPNS-1 (Fig. 3A). Cys-320 is within the membrane-proximal extracellular Ig loop, whereas Cys-393 is within...
the transmembrane segment. To test if these cysteine residues are involved in intermolecular disulfide bond formation, we performed site-directed mutagenesis and swapped these cysteine residues with the corresponding residues in Bit/PTPNS-1. Specifically, we changed Cys-320 to serine and Cys-393 to valine followed by transient transfection of these mutants into COS cells.

As shown in Fig. 3B, no SIRPβ1 expression was detected in mock-transfected COS cells. Conversely, a prominent SIRPβ1 band of 110–120 kDa under nonreducing conditions was detected in Western blots of cells transfected with a full-length DNA sequence encoding the wild-type SIRPβ1. Similar to that observed in leukocytes, SDS-PAGE under reducing conditions caused a decrease in the apparent molecular mass of the SIRPβ1 band to 55 kDa (Fig. 3B), consistent with SIRPβ1 forming a disulfide-linked homodimer in COS cells. As shown in Fig. 3B, mutation of Cys-393 to valine (C393V) had no effect on SIRPβ1 dimerization. In contrast, mutation of Cys-320 to serine (C320S) resulted in loss of SIRPβ1 dimerization. In particular, the immunoblot of the C320S transfecant did not reveal an apparent 110–120-kDa SIRPβ1 dimer band under nonreducing conditions (Fig. 3B); however, the 55-kDa SIRPβ1 monomer was detected as with wild-type and C393V transfecants in Western blots under reducing conditions (Fig. 3B). These results indicate cysteine 320 as the key residue that mediates SIRPβ1 intermolecular disulfide bond formation and thus protein dimerization. To confirm the role of Cys-320 and rule out individual amino acid effects, we also mutated Cys-320 to alanine. As shown in Fig. 3, no SIRPβ1 dimer was detected in the C320A transfecant, confirming that Cys-320 is the
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FIGURE 4. Subcellular localization of SIRPs in PMN. Unstimulated and fMLP-stimulated PMN were subjected to isopycnic sucrose density gradient centrifugation to separate subcellular organelles. Localization of plasma membrane and primary and secondary granules was determined by assays for alkaline phosphatase, myeloperoxidase, and lactoferrin, respectively. As shown in the figure, fractions 1–10 represent cytosol. Plasma membrane was distributed in fractions 11–17, secondary granules in fractions 17–20, and primary granules in fractions 20–22. To localize SIRP isoforms, gradient fractions were immunoblotted using anti-SIRPα and anti-SIRPβ1 (mAb B4B6) antibodies. For reference, fractions were also immunoblotted for actin using anti-F-actin antibody (BD Transduction Laboratories).

essential residue for SIRPβ1 dimerization. Interestingly, we have consistently observed that the expression levels of C320S/C320A mutants were lower than those of wild-type and C393V SIRPβ1 (Fig. 3, B and C), suggesting that dimerization of the protein may contribute to stabilize the SIRPβ1 structure.

Subcellular Localization of SIRPα and -β1 in PMN—In our previous study, we observed that SIRPα expression on the PMN cell surface is significantly up-regulated after chemoattractant stimulation (17), suggesting that intracellular pools might be exist for SIRPα in PMN. With our current results indicating that under nonreducing conditions the high molecular weight protein species is SIRPβ1, our previous observations also suggested that the cell surface expression of SIRPβ1 on PMN may not be increased by activation. To further characterize the subcellular localization of SIRP proteins in PMN, we performed subcellular fractionation experiments using isopycnic sucrose density gradients (“Materials and Methods”).

As shown in Fig. 4, immunoblotting of sucrose gradient fractions using SIRPα-specific antibody revealed that SIRPα strongly co-sediments with secondary granules (fractions 17–20) with a minor pool co-sedimenting with plasma membrane markers in unstimulated PMN. After stimulation with fMLP, part of SIRPα redistributed from secondary granule-containing fractions to fractions containing plasma membranes (fractions 12–15). This result is consistent with our previous cell surface biotinylation results demonstrating up-regulation of SIRPα after stimulation with fMLP (17). Most interestingly, the pattern of redistribution SIRPα from intracellular granules to plasma membrane is similar to that observed for its counter-receptor CD47 (23). The SIRPβ1 homodimer was also observed to co-sediment with plasma membrane and secondary granule markers. However, in contrast to SIRPα, SIRPβ1 was abundantly present in plasma membrane fractions (fractions 11–20) in unstimulated cells. Furthermore, stimulation with fMLP did not result in a significant increase in SIRPβ1 associated with plasma membrane fractions. Neither SIRPα nor SIRPβ1 was detected in fractions containing cytosolic proteins (Fig. 4, fractions 1).

FIGURE 5. Antibody-mediated ligation of cell surface SIRPβ1 enhances PMN transepithelial migration. Time course PMN transepithelial migration assays were performed using inverted T84 epithelial monolayers (23). In these experiments, migration in the presence of 20 μg/ml of anti-SIRPβ1 mAb B4B6 was compared with migration in the presence of the same concentrations of control murine IgG (control IgG), a noninhibitory binding mAb J10.4, and an inhibitory anti-CD47 mAb C5D5 (23). Results obtained at different time points represent the sum of PMN that had migrated up to that time point.

Anti-SIRPβ1 mAbs Enhance PMN Transepithelial Migration—Because we demonstrated previously a role for SIRPα in regulating PMN transmigration (17), we performed experiments to test SIRPβ1-specific mAbs for inhibitory or stimulatory effects on fMLP-driven PMN transepithelial migration. In these experiments, T84 colonic epithelial cells were grown as inverted monolayers on permeable transwell filters (23). PMN were added to the upper chambers of transwells in the presence or absence of mAbs and were induced to migrate in a basolateral to apical direction toward the chemoattractant fMLP (23). As shown in Fig. 5, treatment with anti-SIRPβ1 mAb B4B6 resulted in enhanced PMN transepithelial migration after 1 h compared with migration in the absence of antibody or in the presence of the binding, noninhibitory antibody J10.4 (22) (52.2 ± 4.2% migration for anti-SIRPβ1 versus...
homodimer (16). To determine whether SIRPβ1 associates with DAP12 in PMN, we performed co-immunoprecipitation assays. To our surprise, no DAP12 was detected in immunoprecipitates using anti-SIRPβ1 antibody from either unstimulated or fMLP-stimulated PMN. As shown in Fig. 6B, under nonreducing conditions, mAb B4B6 immunoprecipitated SIRPβ1 from both unstimulated and fMLP-stimulated PMN. Probing Western blots of the same immunoprecipitates using anti-DAP12 antibodies DX37 and a murine polyclonal anti-DAP12 antibody failed to detect any DAP12 band (Fig. 6B). However, the post-blot cell lysates from such immunoprecipitations contained abundant DAP12 (not shown). To investigate further whether SIRPβ1 associates with DAP12 in PMN, we performed protein cross-linking experiments. We surmised that if DAP12 associates with SIRPβ1, its apparent molecular mass would increase after protein cross-linking. However, neither treatment of PMN with cell membrane-permeable (dithiobis(succinimidylpropionate)) nor cell impermeable (dithiobis(sulfosuccinimidylpropionate)) cross-linkers detected SIRPβ1 and DAP12 association under conditions where controls were successfully cross-linked (results not shown). Although our results did not support SIRPβ1 binding to DAP12 in PMN, probing Western blots of immunoprecipitated SIRPβ1 with the phosphotyrosine-specific antibody PY20 revealed a small protein of ~10–12kDa that demonstrated increased phosphorylation after fMLP stimulation (Fig. 6B). Further Western blots using anti-DAP10 and Fcγ antibodies indicated that the SIRPβ1-associated phosphorylated protein is neither DAP10 nor FcγRy. This result suggests that another, as yet undefined, adaptor protein associates with SIRPβ1 in PMN that is tyrosine-phosphorylated after chemotactic stimulation and may mediate downstream signaling from SIRPβ1.

**DISCUSSION**

The SIRP family proteins are separated into two major groups or isoforms referred to as SIRPα and SIRPβ that are characterized by the presence or absence of a long C-terminal intracellular domain, respectively (11). Despite the striking difference in their C-terminal domains, the extracellular domains of SIRPα and β members share highly homologous primary structures that are predicted to form three Ig-like loops (11). However, despite highly similar extracellular domains, ligand-based interactions of the SIRP family proteins appear to be remarkably different. SIRPα has been clearly shown to be an extracellular ligand for CD47 (4, 17, 20), another cell surface Ig superfamily member that is expressed on nearly all cells and tissues (29). We and others have defined previously that CD47 exclusively binds to SIRPβ1 extracellular domain fusion proteins (SIRPβ1-transfected cells (17, 19)). In this work, we also failed to detect CD47 binding to SIRPβ1 by co-precipitation assays (Fig. 1E). Thus, it remains unknown what the extracellular ligand for SIRPβ1 is and how SIRPβ1 might regulate cellular function.

The highly similar extracellular domains of SIRP family proteins have created significant obstacles in determining the specific role(s) of indi-
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vidual isoforms in regulating cellular function(s). We have observed that mAbs generated against the SIRPα extracellular domain cross-react with SIRPβ isoforms under different conditions, which has complicated interpretation of results. Given that SIRPα and -β are most often co-expressed in leukocytes, studies using cross-reactive anti-SIRPα antibodies have confounded the analysis of SIRPα function because the contribution of SIRPβ isoforms could not be excluded. We observed previously that a rabbit polyclonal antibody against the extracellular domain of SIRPα1 (termed anti-SIRPα1.ex (11)) reacted with two or three SIRP isoforms in PMN (17) (also shown in Fig. 1). Thus, it was uncertain which protein band truly represented SIRPα. These observations, coupled with the increasing appreciation of the importance of this family of proteins in immunology, prompted us to further define the specific SIRP isoforms in PMN and their functions.

In this study, we report for the first time, a clear distinction of SIRPα from SIRPβ in PMN and other leukocytes. We report that SIRPβ1 exists as 110–120-kDa disulfide-linked homodimer in leukocytes including PMN, PBMC, and HL60 cells. Our data suggest that SIRPα, in its functional form, represents a smaller protein of 65–75 kDa due to differences in tertiary structure. In addition, from our results in Fig. 2, we conclude that the major SIRPα subfamily member in PMN includes Bit/PTPNS-1. We also report that the predominant SIRPβ in PMN is SIRPβ1. We show by site-directed mutagenesis that Cys-320, which is in the membrane-proximal Ig loop of SIRPβ1, mediates intermolecular disulfide bond formation in SIRPβ1 dimers. Our finding of the dimeric structure of SIRPβ1 has implications for interpretation of previous studies because some of these reports included experiments with monovalent fusion protein constructs to assay potential SIRPβ1 binding interactions (19). These results provide new rationale for future experimental design aimed at characterizing the role of SIRPβ1 in cellular functions.

Not only are the protein structures of SIRPβ and SIRPα (Bit/PTPNS-1) different, but we observed distinct cellular distributions of each protein in PMN. As highlighted in Fig. 4, SIRPα appears to localize in intracellular storage pools and is redistributed to the cell surface after chemoattractant stimulation. Such cell surface up-regulation after stimulation is similar to that reported for other proteins important in the regulation of PMN transmigration such as CD47 and CD11b/CD18 (23). We speculate that, like CD47 and CD11b/CD18, SIRPα may also regulate transmigration through adhesion-based signaling events. In contrast, we did not observe major redistribution of SIRPβ1 between subcellular organelles and the plasma membrane after stimulation. On the contrary, we observed that SIRPβ1 is constitutively present in the plasma membrane before and after fMLP stimulation. Most interestingly, as seen in Fig. 4, we also observed subtle changes in the profile of SIRPβ1 co-sedimentation with plasma membrane after fMLP stimulation. Although the significance of this observation is unclear, these changes could be seen with activation-dependent redistribution of SIRPβ1 in membrane microdomains that might play a role in regulating SIRP function. Together, our findings of different distributions of SIRPα and -β1 in PMN support distinct functional properties. This is supported by our results demonstrating that ligation of SIRPα inhibited PMN transmepithelial migration (17), whereas ligation of SIRPβ1 in this study with specific mAbs resulted in enhanced PMN transmigration (Fig. 5).

Such opposite effects of SIRPα and -β1 on PMN transmigration, however, might be expected given that the C-terminal structures would be predicted to have negative and positive regulatory roles. SIRPα has two inhibitory signaling motifs (ITIM) in the intracellular domain, whereas SIRPβ1 contains a positive charged Lys in the transmembrane domain that mediates association with an activation motif (ITAM)-containing adaptor protein. These types of divergent regulatory pathways have been observed in natural killer cell receptors (KIRs and KARs) (14), Fc receptors (FcR) (18), novel immune-type receptors (37), and Ig-like transcripts (38, 39). To date, these ITIM and ITAM domain-mediated signaling pathways have been recognized to be characteristic of crucial cell surface receptors involved in regulating innate immune functions such as NK cell cytotoxicity and macrophage phagocytosis (14, 33). Among such cell surface receptors, inhibitory elements contain ITIM structures in the C-terminal intracellular domain, and the activation isoforms associate with ITAM-containing adaptor proteins such as DAP12, CD3ζ, or FcRγ.

Because previous studies suggested that SIRPβ1 associates with DAP12, we investigated whether SIRPβ1 regulates PMN function through DAP12-mediated signaling events. DAP12 is a 12-kDa homodimeric transmembrane protein that signals through tyrosine phosphorylation events in the intracellular ITAM domain. This adaptor protein was first described on the plasma membrane of natural killer cells and was associated with killer cell activatory receptors (15). DAP12 was later reported to be expressed in other leukocytes, including peripheral mononuclear cells and dendritic cells (40). In this study, we demonstrated expression of DAP12 homodimers in PMN (Fig. 6A). To assess potential SIRPβ1 and DAP12 binding interactions, we tried a variety of different detergents, buffers, and immunoprecipitation conditions (e.g. 1% digitonin (41), Brij/Nonidet P-40 lysis buffer (42)). However, none of our co-immunoprecipitation or cross-linking experiments revealed SIRPβ1 association with DAP12 in PMN. Furthermore, we also examined FcRγ as a potential adaptor protein for SIRPβ1. Curiously, we observed that SIRPβ1 from PBMC but not PMN co-immunoprecipitated with FcRγ.

Although SIRPβ1 was not shown to associate with DAP12 in PMN, our data are consistent with the association of SIRPβ1 with an undefined 10-kDa adaptor protein. As shown in Fig. 6, immunoprecipitates of SIRPβ1 contained an ~10-kDa protein that was tyrosine-phosphorylated after chemoattractant stimulation. In parallel Western blotting experiments, we excluded the possibility that this protein is FcRγ or DAP10 (results not shown). Thus, the identity of this associated protein and whether it mediates signaling events downstream of SIRPβ1 in regulating PMN transmigration remain unclear. Because PMN transmigration plays a central role in innate immunity and is vital for defense against invading pathogens, our findings implicating SIRPα and -β1 as inhibitory and stimulatory signaling elements in the regulation of PMN migration provide additional insight into mechanisms that serve to fine-tune the innate immune response. Additional studies in this area will both advance our understanding of PMN transmigration and may provide ideas for new anti-inflammatory therapeutics.

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