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Signal-Regulatory Protein Is Selectively Expressed by Myeloid and Neuronal Cells

Susan Adams,* Luc J. W. van der Laan,† Elizabeth Vernon-Wilson,* Chantal Renardel de Lavalette,† Ed A. Döpp,† Christine D. Dijkstra,† David L. Simmons,‡ and Timo K. van den Berg[1†]

Signal-regulatory proteins (SIRP) are transmembrane glycoproteins with three extracellular Ig-like domains, closely related to Ag receptors Ig, TCR, and MHC, and a cytoplasmic domain with two immunoreceptor with tyrosine-based inhibition motifs that can interact with src homology 2 domain-containing phosphatases. SIRP have previously been shown to inhibit signaling through receptor tyrosine kinases, but their physiologic function is unknown. Here we demonstrate by expression cloning that the mAbs ED9, ED17, and MRC-OX41 recognize rat SIRP. In addition, we show for the first time that rat SIRP is selectively expressed by myeloid cells (macrophages, monocytes, granulocytes, dendritic cells) and neurons. Moreover, SIRP ligation induces nitric oxide production by macrophages. This implicates SIRP as a putative recognition/signaling receptor in both immune and nervous systems. ***The Journal of Immunology*****, 1998, 161: 1853–1859.**

The Ig superfamily constitutes a major group of cell surface receptors involved in recognition and/or communication (1). The recently identified family of signal-regulatory proteins (SIRP)[2] constitute a subfamily within the Ig superfamily, closely related to the Ag receptors Ig, TCR, and MHC (2-6). In general, SIRP family members are transmembrane glycoproteins with three Ig-like extracellular domains: a N-terminal V-set domain and two C1-set domains. In humans, at least 15 different SIRP members have been identified. The SIRP cytoplasmic tail contains two immunoreceptor with tyrosine-based inhibition motifs (ITIM), similar to those found in a number of receptor molecules, including FcγRIIB on B cells and killer cell-inhibitory receptors on NK cells (reviewed in Ref. 7). SIRP can negatively regulate signaling through receptor tyrosine kinases (RTK), such as the epidermal growth factor receptor, platelet-derived growth factor receptor, or insulin receptor. The cytoplasmic tyrosines of the SIRP ITIMs are phosphorylated after RTK ligation with growth factors and act as docking sites for, and activate, src homology 2 (SH2) domain-containing phosphatases (SH2-PTP), like SHP-1 and SHP-2. These SH2-PTP mediate activation of the Ras-mitogen-activated protein kinase cascade and are required for RTK signaling (8). Although it is not known how SIRP exert their negative effects on RTK signaling, the apparently stable interaction between SIRP and SH2-PTP may sequester SH2-PTP away from RTK, thereby preventing their positive effects on signaling (2, 3).

The pattern of expression and therefore the physiologic function(s) of SIRPs are unknown. Here we show by expression cloning and immunocytochemistry that the mAbs ED9, ED17, and MRC-OX41 recognize a rat SIRP and that rat SIRP is selectively expressed by myeloid cells (monocytes, Mϕ, granulocytes, dendritic cells (DC)) and neurons. We also demonstrate that anti-SIRP can induce nitric oxide (NO) production in Mϕ.

Materials and Methods

Animals

Male WAG/Rij rats, between 6 and 12 wk of age, were obtained from the breeding facility of the Dutch Cancer Institute (Amsterdam, The Netherlands). Animals were maintained under conventional laboratory conditions and were allowed free access to food and water. Regular serologic testing according to the Federation of European Laboratory Animal Science Associations (FELASA) recommendations showed no sign of viral infections.

Monoclonal antibodies

The mAb ED9 (mouse IgG1) and ED17 (mouse IgG1), both obtained from mice immunized with a rat spleen homogenate, have been described (9, 10). The mAb MRC-OX41 (mouse IgG1) has also been described previously (11). Other mAb used in this study are: ED1 (mouse IgG1 against rat monocytes/Mϕ (12, 13)); ED8 (mouse IgG1 directed against rat CD11b/CD18 (9)); MRC-OX6 (mouse IgG1 against rat MHC class II (14); W3/25 (mouse IgG1 against rat CD4 (15)); and BF5 (mouse IgG1 against human CD4: a generous gift of Dr. J. Wijdenes (Diaclone Laboratories, Besançon, France). All mAbs were purified from supernatants from hybridoma cells cultured in RPMI 1640 containing 5% low IgG FCS (Life Technologies, Gaithersberg, MD) on protein A-Sepharose (Pharmacia, Uppsala, Sweden). Biotinylation was performed using D-biotinoyl-ε-aminocaproic acid *N*-hydroxysuccinimide ester (Boehringer Mannheim, Mannheim, Germany). To prepare endotoxin-free Ab preparations, Abs were run over a polymyxin B column (Pierce, Rockford, IL), which, when tested by the *Limulus* assay, yielded final LPS concentrations in culture of <5 pg/ml.

Cells, culture conditions, and NO synthesis

The rat pleural Mϕ cell line R2 (13, 16) and the NR8383 rat alveolar Mϕ cell line (generously provided by Dr. R. J. Helmke, University of Texas, San Antonio, TX (17)) were grown in RPMI 1640 containing 10% FCS (Life Technologies, Breda, The Netherlands), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Peritoneal and alveolar Mϕ and thioglycolate-elicited peritoneal exudate cells were obtained as described previously (16). For thioglycolate-elicited peritoneal cells (Mϕ, granulocytes), rats were injected i.p. with 8 to 10 ml of thioglycolate broth, and peritoneal cells were harvested after 12 or 48 h. PBMC were isolated using

*Cell Adhesion Laboratory, Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Oxford, United Kingdom; †Department of Cell Biology and Immunology, Faculty of Medicine, Vrije University, Amsterdam, The Netherlands; and ‡Department of Neuroscience, SmithKline Beecham, Harlow, United Kingdom

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[1] Address correspondence and reprint requests to Dr. Timo K. van den Berg, Department of Cell Biology and Immunology, Faculty of Medicine, Vrije University, van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands. E-mail address: t.van_den_berg.cell@med.vu.nl

[2] Abbreviations used in this paper: SIRP, signal-regulatory proteins; ITIM, immunoreceptor with tyrosine-based inhibition motifs; RTK, receptor tyrosine kinases; SH2, src homology 2; SH2-PTP, src homology 2 domain-containing protein tyrosine phosphatases; DC, dendritic cells; Mϕ, macrophage.

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Lymphoprep (Nycomed, Oslo, Norway) as described (18). Suspensions of thymocytes and lymphocytes were obtained by cutting thymus and mesenteric lymph nodes into small fragments and flushing them through a nylon gauze. Embryonic (E17) cortical neurons were isolated as described and cultured for 24 h (19). Dopaminergic neurons were obtained and cultured for 7 days as described (19). Cultures of neonatal microglial cells and astrocytes were established from day 2 neonatal brains. Cells from 6- to 8-day-old mixed cultures were separated and directly used (microglia) or subcultured (astrocytes) for 8 days (19). Oligodendrocytes precursor cells (>80% A2B5+O4+/−) and mature oligodendrocytes (>80% GalC+MBP+/−) derived from day 2 neonatal rat brains were obtained as described (20). Where necessary, cells were detached from the substrate using cold 5 mM EDTA in PBS or by scraping.

For analysis of nitric oxide production, NR8383 cells (1.5×10^6/1.5 ml/well) were grown for in RPMI containing 2% FCS and antibiotics. Purified endotoxin-free Abs (25 μg/ml) or LPS (100 ng/ml) were added, and nitrite production was analyzed after 20 h using Griess reagents as described (21).

Affinity purification and Western blotting

Abs were coupled to CNBr-Sepharose (Pharmacia) according to the manufacturer's instructions. Spleens from 80 rats (43 g) were homogenized in 2% Triton X-100, 50 mM Tris-HCl pH (7.4), 150 mM NaCl, 1 mM EDTA containing protease inhibitors (trypsin inhibitor, leupeptin, pepstatin, antipain, PMSF). To remove CD11b/CD18 and other nonspecific proteins, the lysate was precleared over an ED8 (3 mg Ig/ml matrix) column (4 ml). The lysate was then run over an ED9 (3 mg/ml matrix) column (4 ml), and bound material (~70 μg total) was eluted with 50 mM glycine-HCl, pH 2.5, containing 0.1% octylglucoside, dialyzed against PBS, and concentrated. Samples of eluate (~3 μg of protein) were subjected to SDS-PAGE (10%) under reducing conditions, blotted to polyvinylidene difluoride. Strips of filters were incubated with the indicated biotinylated Abs and streptavidin-peroxidase, and staining was visualized with diaminobenzidine and H_2O_2.

Expression cloning and transfection

An oligo(dT)-primed rat alveolar Mϕ cDNA library was constructed in pCDM8 as described before (22). As a source, Lewis rat alveolar Mϕ were used that had been cultured for 12 h in medium containing 100 IU/ml rat recombinant IFN-γ. After transfection into COS-7 cells, the library was screened using plate-coated ED9, ED17, or MRC-OX41. Appropriate clones were isolated in several rounds of transfection and screening, and recovered plasmids were sequenced using the Sequenase V7 kit using [^{35}S]dATP (Amersham, Arlington Heights, IL). For staining, COS-7 cells were transfected using the DEAE-dextran method (22), and 3 days later they were stained for FACS analysis as described below using biotinylated primary Abs. Human ICAM-1 cDNA (23) was used as a control.

Northern and Southern blotting

Rat SIRP cDNA was digested using *Hin*dIII and *Pst*I, and the insert was purified from a 1% agarose gel using the QIAquick gel extraction kit (Qiagen, Chatsworth, CA). Approximately 25 ng of SIRP probe or rat β-actin probe (Clontech Laboratories, Palo Alto, CA) were labeled with [^{32}P]dCTP using the Multiprime DNA labeling system (Amersham) and purified using Quick Spin Columns (Boehringer Mannheim, Mannheim, Germany) both according to the manufacturer's instructions.

The rat multiple tissue Northern blot (Clontech) was obtained by running 4 μg of poly(A)-containing RNA run on a denaturing formaldehyde, 1.2% agarose gel and transferring it to a charge-modified nylon membrane. The membrane was prehybridized for 1 h at 68°C with ExpressHyb solution (Clontech), containing 0.1 mg/ml denatured salmon sperm DNA (Sigma, St. Louis, MO). Hybridization was conducted for 1 h at 68°C. After a few quick rinses in 2× SSC containing 0.05% SDS, the blots were washed for 30 min at room temperature and at least 40 min at 50°C with several rinses of the same buffer and exposed to ECL Hyperfilm (Amersham) for 1 wk at −80°C (SIRP probe) or 1 h at room temperature (β-actin). Individual bands were quantified using a Molecular Imager (Bio-Rad, Richmond, CA) and analyzed using the Molecular Analist software. For each tissue, the results (SIRP/actin ratio) were calculated and normalized to the highest value.

The rat Southern blot was prepared by running 4 μg of digested rat DNA on a 0.7% agarose gel and transferring it to a charge-modified nylon membrane. The blot was then prehybridized, hybridized with the SIRP probe, washed, and visualized as described above with the exception that prehybridization and hybridization were performed at 60°C.

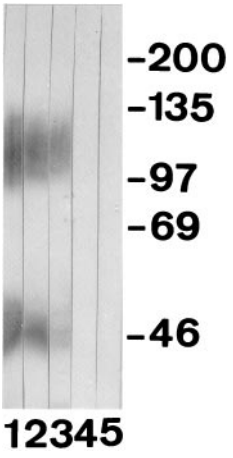

FIGURE 1. Western blotting of affinity-purified ED9 Ag and reactivity with ED9 (*lane 1*), ED17 (*lane 2*), OX41 (*lane 3*), control mouse IgG1 (*lane 4*), and rabbit anti-mouse Ig-peroxidase control (*lane 5*). Samples were run on 10% SDS-PAGE under reducing conditions.

Immunocytochemistry and flow cytometry

Tissues (indicated in Table II) were excised and immediately frozen in liquid nitrogen. Immunocytochemistry of tissues was performed on acetone-fixed cryostat sections (8 μm) as described in detail before (24) using optimal concentrations of unconjugated or biotinylated primary Abs and peroxidase-conjugated rabbit anti-mouse Ig (Dakopatts, Copenhagen, Denmark) or streptavidin-peroxidase (Vector, Burlingame, CA), respectively. Peroxidase activity was visualized using diaminobenzidine and H_2O_2.

FACS analysis was performed as follows. Unfixed cells, which were kept on ice throughout the procedure, were stained for 30 min with optimal concentrations of biotinylated primary Abs (~10 μg/ml) in PBS containing 0.25% BSA (PBS/BSA). After a washing in PBS/BSA, they were incubated for 30 min with phycoerythrin-conjugated streptavidin (STAR 4B, Serotec, Oxford, U.K.) and washed thoughly. When double labeling for the intracellular ED1 Ag was performed, an incubation with ED1-FITC (10 μg/ml) in PBS/BSA containing 0.02% saponin was also performed. Controls included unstained cells (cell control), samples in which the first step was either omitted (conjugate control), or replaced by an isotype matched control mAb (mAb control). The cells were analyzed using a FACScan (Becton Dickinson, Mountain View, CA) equipped with an argon laser with excitation at 488 nm and calibrated using Calibrite beads (Becton Dickinson). Data were collected for 10,000 events and analyzed using the CellQuest program (Becton Dickinson). Where indicated, Mϕ and granulocytes were distinguished by electronic gating.

For cross-blocking studies, R2 Mϕ (kept on ice throughout the procedure) were incubated with saturating concentrations of unlabeled mAb ED9, ED17, MRC-OX41, or ED8 and incubated for 20 min. Subsequently, biotinylated ED9 or ED17 were added and incubated for 30 min. The cells were then washed and stained with phycoerythrin-conjugated streptavidin. Binding was expressed as the mean fluorescence intensity after substraction of control values, as determined from samples stained without biotinylated mAb.

Results

The mAbs ED9, ED17, and MRC-OX41 directed against rat Mϕ have been described previously, and their reactivity with isolated and cultured Mϕ and DC has been established (9–11). Immunohistochemistry demonstrated that all three mAb produce identical staining patterns in a variety of rat tissues (see for details below), and we hypothesized that the mAb were recognizing the same Ag. To test this and to characterize this molecule, it was purified from a rat spleen homogenate using an ED9 affinity column. SDS-PAGE and Coomassie staining showed that the eluted material consisted of two major polypeptides migrating as diffuse bands at 110 and 55 kDa (not shown), which were both recognized by ED9, ED17, and OX41 when subjected to Western blotting (Fig. 1). Cross-blocking experiments demonstrated that ED9 and ED17

Table I. *Cross-blocking with anti-rat SIRP mAb*[a]

	Mean Fluorescence Intensity	
	ED9-bio	ED17-bio
Nothing	42.61	32.74
ED9	15.38	3.98
ED17	13.20	5.84
OX41	44.17	27.84
Control IgG1 (ED8)	39.15	27.79

[a] As determined by immunofluorescence staining of rat R2 macrophages (see *Materials and Methods*).

were recognizing an identical/overlapping epitope, whereas OX41 was recognizing a different epitope and does not interfere with the binding of the other mAb (Table I). That ED9 and OX41 mAb were recognizing different epitopes on the same Ag was confirmed by using them in a sandwich ELISA with the purified Ag (not shown).

ED9, ED17, and OX41 mAb were used to screen, by panning, a library of cDNA from IFN-γ-stimulated rat alveolar Mϕ expressed in COS cells. Panning with each mAb resulted in the isolation of several clones (a total of 24) with identical insert sizes (1.35 kb) and nucleotide sequences. Sequencing revealed a single open reading frame of 1254 bp as shown in Figure 2, and a 5′-untranslated region of 100 bp. The amino acid sequence predicts a type 1 transmembrane glycoprotein of 418 amino acids, beginning with a hydrophobic leader of 22 amino acids, followed by an extracellular domain of 381 amino acids, a hydrophobic transmembrane region of 15 amino acids and a hydrophilic intracellular region of 22 amino acids. The extracellular region apparently contains three Ig-like domains: an N-terminal V-set domain and two C1-set domains (1). Database (BLAST) searches revealed a high homology to members of the

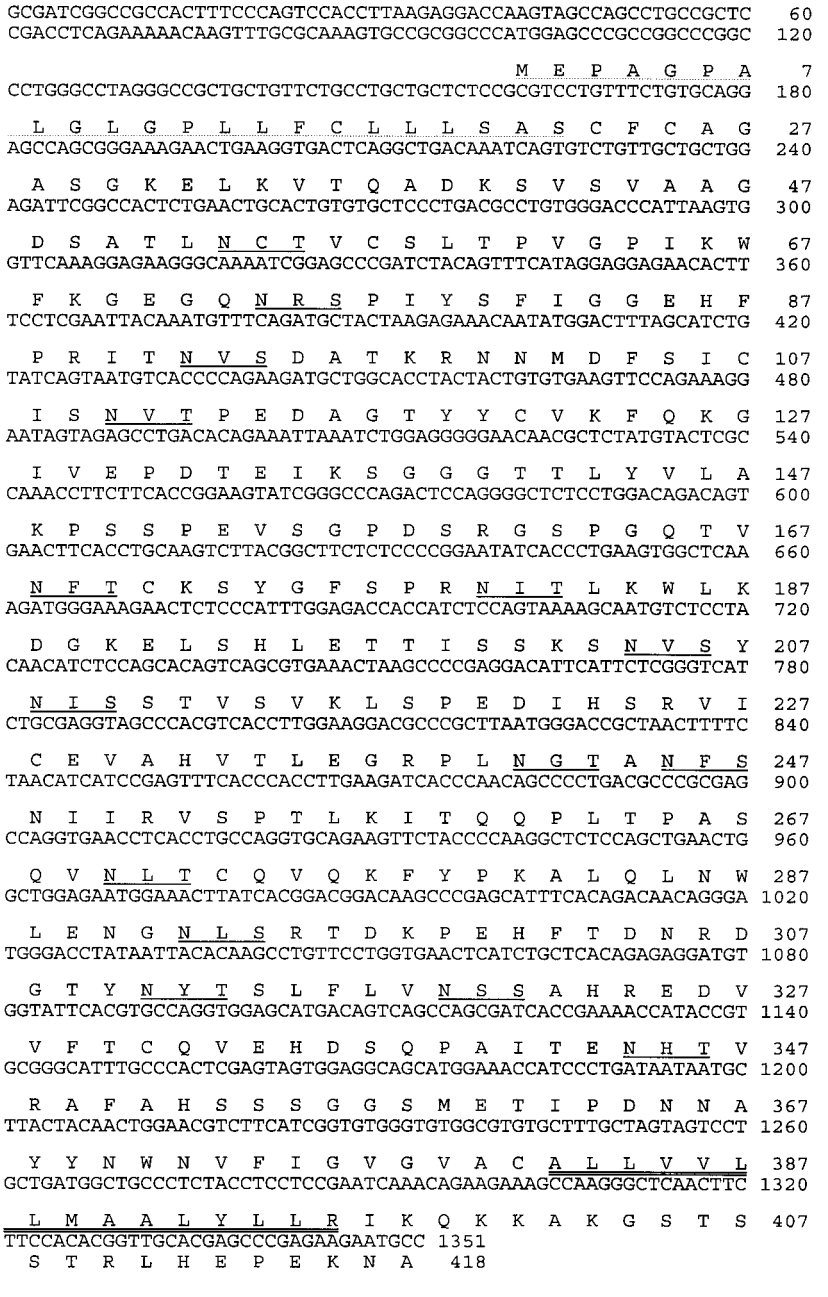

FIGURE 2. cDNA and deduced amino acid sequence of rat SIRP. Underlined are the putative hydrophobic leader (dots), potential *N*-glycosylation sites (single line), and the transmembrane region (double line). Cysteine residues are circled. The predicted domain boundaries are indicated. The rat SIRPβ cDNA sequence has been assigned the EMBL/GenBank accession number AF055065.

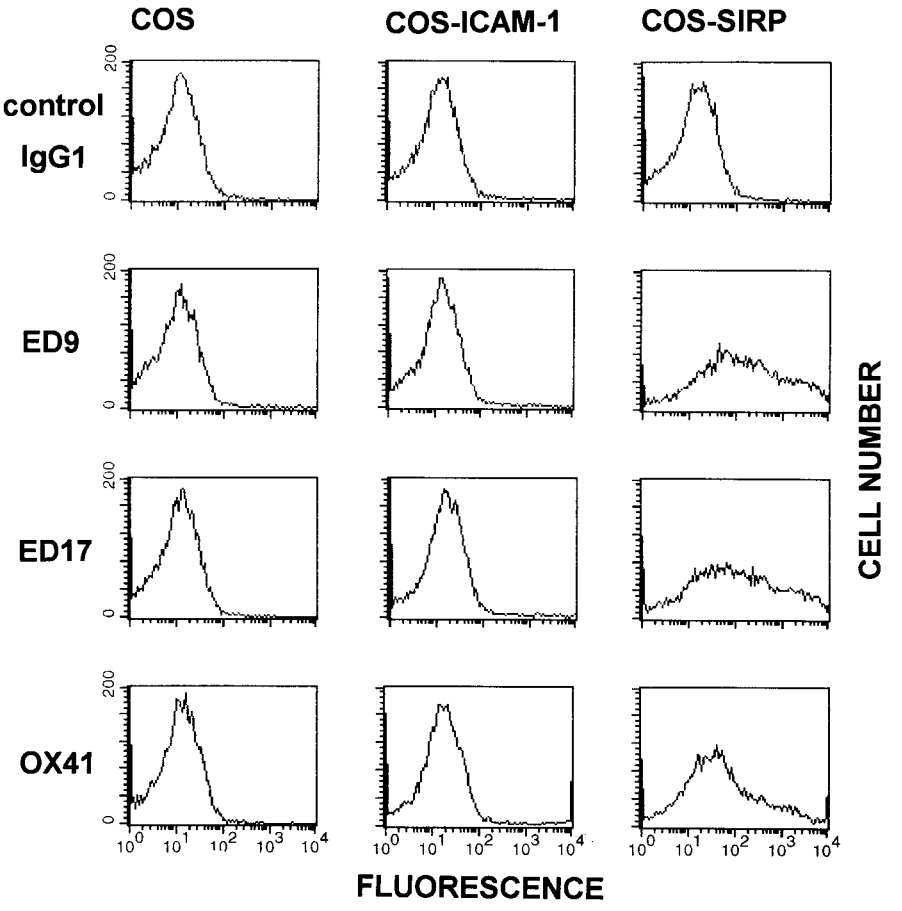

FIGURE 3. Reactivity of ED9, ED17, and OX41 with COS cells transfected with rat SIRP cDNA. COS cells were transiently transfected with SIRP cDNA or human ICAM-1 cDNA or without DNA, and 3 days later cells were stained with biotinylated ED9, ED17, OX41, isotype control mAb, or nothing followed by streptavidin-phycoerythrin. SIRP transfectants are selectively recognized by ED9, ED17, and OX41.

recently recognized family of SIRPs. In fact, our sequence is almost identical with those of two SIRPα cDNA sequences that were cloned recently from rat brain (3, 4). The major difference is that the reported sequences contained a longer cytoplasmic domain with two ITIMs, but the cDNA that we isolated most probably represents a partial clone, which at the 3′ end runs straight into the *Bst*XI linker. Comparison with the published rat sequence also shows two amino acid differences in the leader peptide and a substitution (S57 → C) in the first Ig domain. The extracellular portion of rat SIRP contains a total of 15 potential *N*-linked glycosylation sites and a large number of serines and threonines, which may contain *O*-linked glycans. Considering the difference in size between the nonglycosylated full length rat SIRPα polypeptide (481 amino acids, calculated molecular mass, ~55 kDa) and the mature protein (~110 kDa (Fig. 1 and Refs. 2 and 3)), it seems reasonable to assume that the molecule is strongly glycosylated. Likewise, the difference in molecular mass between SIRP in spleen (110 kDa) and brain (85 kDa (4, 5)) is due to differential glycosylation. Apart from SIRP members, close homologies were also found for a large number of Ig, TCR, and MHC domains (highest identities, ~30%; not shown, discussed in detail by Sano et al. (4). Transient transfection into COS cells confirmed that ED9, ED17, and OX41 were all recognizing the rat SIRP (Fig. 3). Southern blotting of rat genomic DNA digested with several restriction enzymes yielded a number (three to five) of reactive bands in each instance (Fig. 4), compatible with a (relatively large) single copy gene. Northern blotting demonstrated a single transcript of 3.8 to 4.0 kb, which was highly expressed in spleen, and at lower levels in lung, brain, liver, and kidney (Fig. 5).

Although SIRP is known to be expressed by fibroblast cell lines (2, 3), the actual tissue distribution is not known. To examine this, a number of rat tissues were subjected to immunohistochemistry

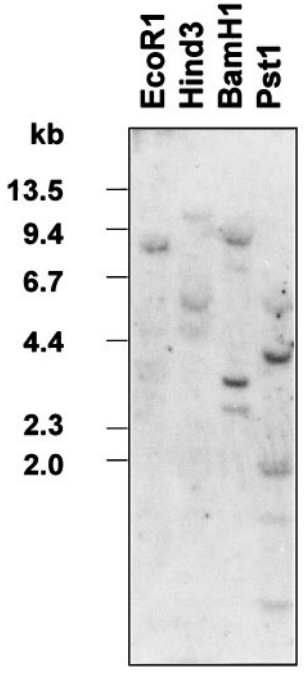

FIGURE 4. Southern blot analysis of rat SIRP genes. Rat genomic DNA (4 μg) was digested with the indicated restriction endonucleases and loaded into each lane. The blot was probed with the entire rat SIRP cDNA.

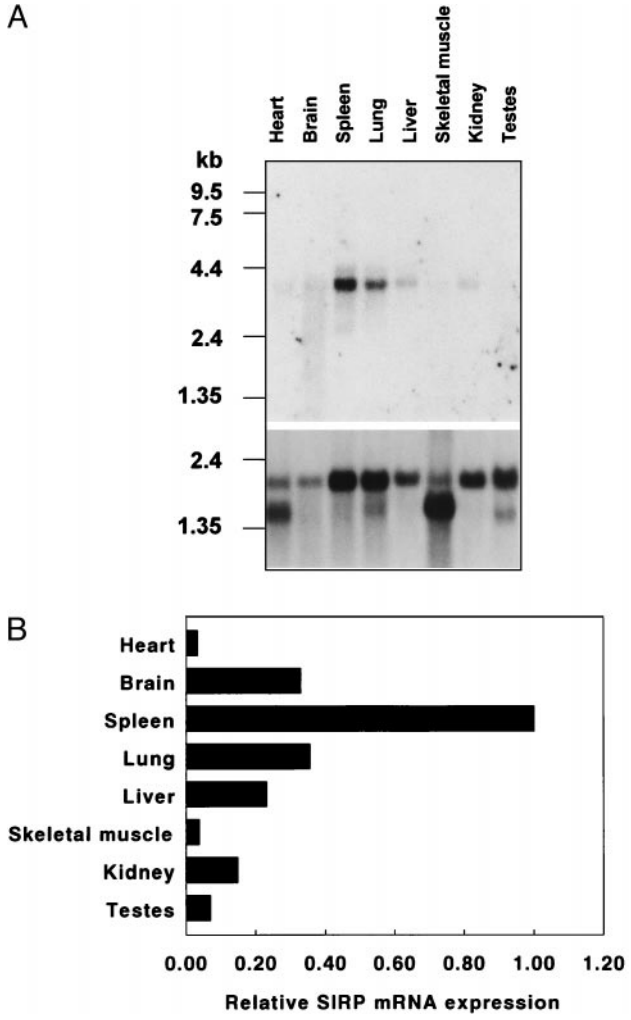

FIGURE 5. Northern blot analysis of rat SIRP mRNA in rat tissues. *A*, Poly(A)-containing RNA (4 μg) was loaded in each lane, separated, and transferred to Hybond N^+. The blot was probed with the entire rat SIRP cDNA (top) or with a β-actin cDNA to control for loading (bottom). *B*, Quantification of relative SIRP expression. The SIRP/β-actin ratio was determined for each tissue, and spleen values were normalized to 1.0.

Table II. *Tissue distribution of rat SIRP*

Tissue	Cell	Staining Intensity
Spleen	Red pulp Mϕ	+++[a]
	Marginal zone Mϕ	+
	Methalophilic Mϕ	+
	Tingible body Mϕ	−
	White pulp Mϕ/DC[b]	+
	Isolated DC	+[c]
Lymph node	Medullary Mϕ	++
	Subcapsular sinus Mϕ	++
	Tingible body Mϕ	−
	Paracortical Mϕ/DC	+
Peyer's patches	Interfollicular Mϕ/DC	++
	Tingible body Mϕ	−
Thymus	Cortical Mϕ	+
	Corticomedullary Mϕ/DC	+
	Medullary Mϕ	−
Bone marrow	Bone marrow Mϕ	++
	Hemopoietic cells	+[d]
Liver	Kupffer cells	+
Lung	Alveolar Mϕ	+++
	Interstitial Mϕ	−
Kidney	Glomerular Mϕ	++
	Interstitial Mϕ	+
Skin	Langerhans cells	++[e]
Brain		Diffuse staining[f]

[a] +++, Strong staining; ++, moderate staining; +, weak staining; −, no staining.
[b] Indicating dendritic cells and/or macrophages.
[c] As determined after isolation of cells.
[d] As determined in bone marrow suspensions (50–70% positive).
[e] As confirmed by staining of epidermal sheets.
[f] See text for details on cultured neurons and glial cells.

using the mAb ED9, ED17, and OX41. Identical results were obtained for each mAb (summarized in Table II, illustrated for spleen and lung in Figure 6). In all tissues, Mϕ were clearly and selectively labeled, as was confirmed by staining of serial sections for (the lysosomal enzyme) acid phosphatase and the pan-monocyte/mϕ marker ED1 (not shown). Although most subpopulations of Mϕ in spleen (Fig. 6*a*), lymph nodes, and Peyer's patches stained SIRP positive, tingible body Mϕ in the B cell follicles showed no detectable reactivity. To investigate whether DC were among the labeled cells within the T cell areas of the lymphoid tissues (i.e., the splenic white pulp, the lymph node paracortex, and the Peyer's patch interfollicular area), DC were isolated from spleen, and cytofuge preparations were analyzed. Most if not all isolated DC (85–90% OX62+) were weakly but clearly SIRP positive. Langerhans cells, the DC of the skin, were also SIRP positive as found in skin sections and epidermal sheets. In the thymus, Mϕ and/or DC in the corticomedullary region and cortex were positive. SIRP was also clearly expressed by resident bone marrow Mϕ and hemopoietic cells and by resident Mϕ in other tissues like liver (i.e., Kupffer cells) and kidney. In the lung, staining was confined to alveolar Mϕ (Fig. 6*b*), and was not detected on interstitial Mϕ. Expression on alveolar Mϕ was confirmed by FACS analysis of bronchoalveolar lavage cells (Fig. 7). A similar level of surface expression was found on peritoneal Mϕ and granulocytes (Fig. 7). Compared with the resident peritoneal cells, a moderate (two- to threefold) increase of surface SIRP expression was detected on Mϕ and granulocytes at 12 h (Fig. 7) and 48 h (not shown) after i.p. thioglycolate injection. Monocytes, as identified as the ED1-positive fraction of PBMC, were strongly SIRP positive, whereas lymph node lymphocytes or thymocytes (not shown) showed no sign of SIRP staining. Staining for SIRP was also observed on the rat Mϕ cell lines R2 (pleural Mϕ) and NR8383 (alveolar Mϕ) (not shown). In brain sections, a diffuse staining was observed throughout the white and gray matter. FACS analysis of postnatal (E17) cortical neurons (Fig. 7) and dopaminergic neurons from adult animals showed that these neurons were clearly expressing SIRP on their surface. Cultured neonatal microglial cells (>97% ED1+, ED8+, W3/25+, MHCII−) were clearly SIRP positive, while neonatal astrocytes (>95% GFAP+) and oligodendrocyte precursors (>80% A2B5+O4+/−) and in vitro matured oligodendrocytes (GalC+MBP+/−) did not express detectable levels of SIRP (not shown).

To investigate the functional significance of SIRP on Mϕ, NR8383 cells were cultured in the presence of anti-SIRP (ED9 (no cross-linking agent added)), and the supernatants were assayed for NO. As can be seen in Figure 8, anti-SIRP induced the synthesis and secretion of NO (~25 μM), whereas control mAb or MRC OX41 (not shown) did not result in NO synthesis. The levels of NO induced by anti-SIRP were ~50% of that induced by an optimal concentration of LPS (100 ng/ml).

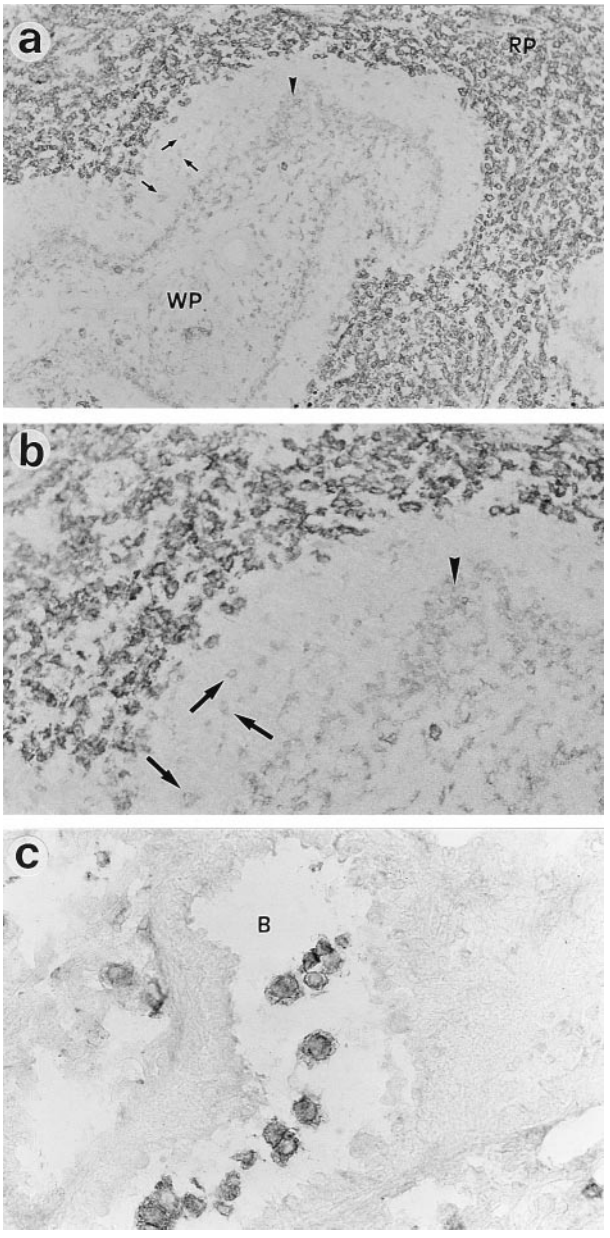

FIGURE 6. Immunohistochemical localization of SIRP in rat spleen at low (*a*) (×50), or high (*b*) (×200) magnification, and lung (*c*) (×400). Frozen sections stained with ED9 using rabbit anti-mouse Ig-peroxidase and DAB/H_2O_2. Note staining of the various Mϕ populations in the spleen, including red pulp Mϕ (RP), marginal zone Mϕ (arrows), the rim of metallophilic Mϕ (arrowhead), and Mϕ/DC in the white pulp (WP). Also note strong staining of alveolar Mϕ in a lung bronchiole (B).

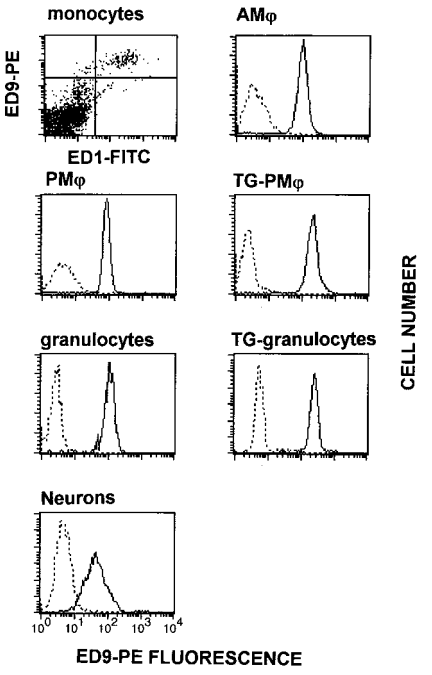

FIGURE 7. FACS analysis of rat monocytes (i.e., the ED1-positive fraction of PBMC (5%); MFI (mean fluorescence intensity)$_{ED9}$ = 1164), alveolar Mϕ (AMϕ; MFI = 96), peritoneal Mϕ (PMϕ; MFI = 81), PMϕ 12 h after i.p. thioglycolate (TG-PMϕ; MFI = 210), peritoneal granulocytes (MFI = 108), peritoneal granulocytes 12 h after i.p. thioglycolate (TG-granulocytes; MFI = 241), and embryonic cortical neurons (MFI = 55). All cells were stained using biotinylated ED9 and streptavidin-phycoerythrin. Monocytes were, for double labeling, also stained with ED1-FITC.

Discussion

In this study, we have identified and characterized mAbs against rat SIRP and have studied its tissue distribution and role in Mϕ activation. Expression cloning and staining of transfected cells demonstrated that the previously described mAb ED9, ED17, and MRC-OX41 recognized a rat SIRP. Our cDNA sequence is essentially identical with two rat sequences published before, which were called SHP substrate-1 (SHPS-1) (3), or brain Ig-like molecule with tyrosine-based activation motifs (BIT) (4), except that our clones apparently lacked the majority of the cytoplasmic domain, probably as a result of a cloning artifact. Thus far, SIRP family members have been identified in rat, mouse, and human (2–4, 6). In general, SIRP molecules are transmembrane glycoproteins with three extracellular Ig-like domains: a N-terminal V-set domain and two C1 domains. These SIRP Ig domains are closely related to those of the Ag receptors, Ig, TCR, and MHC. In the human, 15 different sequences of the first V-set Ig domain have been reported as well as a SIRP member with a truncated cytoplasmic domain, called SIRPβ (2). The former suggests that there may be some extent of diversity comparable with those of the Ag receptors, but this clearly needs further investigation. Thus far, it seems that the three rat sequences that have been cloned, including the one we describe here, are identical. Moreover, the results of our Southern blotting data are compatible with a single copy gene.

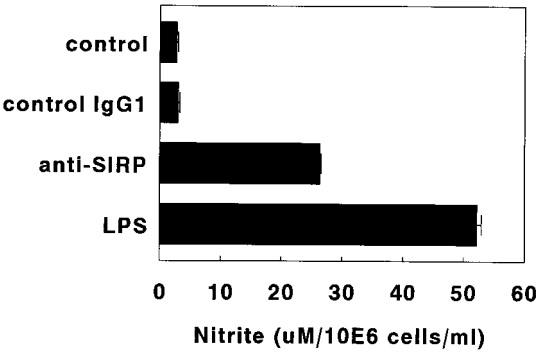

FIGURE 8. Ligation of SIRP on Mϕ induces NO production. NR8383 Mϕ (10^6 cells/ml) were cultured for in the absence or presence of control mAb, 25 μg/ml anti-SIRP mAb (ED9), or 100 ng/ml LPS. Nitrite levels in culture supernatants were determined after 20 h and expressed as mean ± SD.

SIRPα molecules have a cytoplasmic tail with two ITIMs, similar to those found in a number of receptor molecules, including FcγRIIB on B cells and killer cell-inhibitory receptors on NK cells (reviewed in Ref. 7). The tyrosine residues in the ITIMs can be phosphorylated by various stimuli, including the ligation of RTK (e.g., epidermal growth factor receptor, platelet-derived growth factor receptor, and insulin receptor) (2, 3). The resultant phosphotyrosines can serve as docking sites and activators of SH2 domain-containing phosphatases, like SHP-1 and SHP-2 (2–4). These SH2-PTP mediate activation of the Ras-Mϕ-activating protein kinase cascade and are required for RTK signaling (2, 8). Although it is not known how SIRP exert their negative effects on RTK signaling, the apparently stable interaction between SIRP and SH2-PTP may sequester SH2-PTP away from RTK, thereby preventing their positive effects on signaling.

The role of SIRP molecules in cellular signaling has been established using both human and rat fibroblast cell lines and transfectants (2, 3), but the actual tissue distribution of SIRP family members was not known until now. The availability of mAbs against rat SIRP enabled us to study this. Our results demonstrate that rat SIRP is restricted to myeloid cells (monocytes, Mϕ, granulocytes, and DC) and neuronal cells. The distribution of SIRP protein, as seen in immunohistochemistry, corresponds very well with that of SIRP mRNA, as analyzed by Northern blotting, and this suggests that expression regulation predominantly occurs at the mRNA level. The selective expression of SIRP by myeloid and neuronal cells suggests that SIRP is involved in the modulation of myeloid and neuronal cell functions. Our data showing that binding of anti-SIRP mAb to Mϕ induces NO synthesis. Preliminary results also show that SIRP ligation also induces the oxidative burst in Mϕ and homotypic aggregation in Mϕ and granulocytes (T. K. van den Berg, manuscript in preparation). Based on the established regulatory role of SIRP in the signaling of RTK modulation of signaling of Mϕ and neuronal RTK, such as the Mϕ-CSF receptor and the nerve growth factor receptor can be postulated, and we are currently investigating this. Interestingly, it has already been shown that transformation of fibroblasts by v-*fms*, a viral Mϕ-CSF receptor oncogene, can be suppressed by SIRP (2). In addition to evaluating the functions of SIRP in myeloid and neuronal cells, it will be important to dissect the modulation by SIRP of the actual signaling pathways and interactions with other signaling molecules in these cells. For instance, the differential expression of SHP-1 and SHP-2 in hemopoietic cells and brain, respectively (25, 26), suggest that they may serve as cell-specific targets for SIRP action.

The fact that SIRP contains three extracellular Ig-like domains strongly suggests interactions with extracellular ligands, and thus far these have not been described. Very recently, it was shown that purified SIRP can promote the outgrowth of neurons in vitro (4), demonstrating that neurons express both SIRP as well as a putative SIRP ligand(s) and suggesting a possible role of SIRP in neuron-neuron contact.

Taken together, we have identified rat SIRP by expression cloning using mAbs and have demonstrated that rat SIRP is expressed by myeloid cells and neurons. We also provide evidence that SIRP is involved in Mϕ activation. It will be important to identify the putative ligands for SIRPs and to further reveal the physiologic significance of SIRP molecules in immune and nervous systems.

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References

1. Williams, A. F., and A. N. Barclay. 1988. The immunoglobulin superfamily domains for cell surface receognition. *Annu. Rev. Immunol. 6:381.*
2. Kharitonenkov, A., Z. Chen, I. Sures, H. Wang, J. Schilling, and A. Ullrich. 1997. A family of proteins that inhibit signalling through tyrosine kinase receptors. *Nature 386:181.*
3. Fujioka, Y., T. Matozaki, T. Noguchi, A. Iwamatsu, T. Yamao, N. Takahashi, M. Tsuda, T. Takada, and M. Kasuga. 1996. A novel membrane glycoprotein, SHPS-1, that binds the SH2-domain-containing protein tyrosine phosphatase SHP-2 in response to mitogens and cell adhesion. *Mol. Cell. Biol. 16:6887.*
4. Sano, S., H. Ohnishi, A. Omori, J. Hasegawa, and M. Kubota. 1997. BIT, an immune antigen receptor-like molecule in the brain. *FEBS Lett. 411:327.*
5. Ohnishi, H., M. Kubota, A. Ohtake, K. Sato, and S. Sano. 1996. Activation of protein tyrosine phosphatase SH-PTP2 by a tyrosine-based activation motif of a novel brain molecule. *J. Biol. Chem. 271:25569.*
6. Yamao, T., T. Matozaki, K. Amano, Y. Matsuda, N. Takahashi, F. Ochi, Y. Fujioka, and M. Kasuga. 1997. Mouse and human SHPS-1: molecular cloning of cDNAs and localization of genes. *Biochem. Biophys. Res. Commun. 231:61.*
7. Vivier, E., and M. Daëron. 1997. Immunoreceptor tyrosine-based inhibition motifs. *Immunol. Today 18:286*
8. Adachi, M., E. H. Fisher, J. Ihle, K. Imai, F. Jirik, B. Neel, T. Pawson, S. H. Shen, M. Thomas, A. Ullrich, and Z. Zhao. 1996. Mammalian SH2-containing protein tyrosine phosphatases. *Cell 85:15.*
9. Damoiseaux, J. G. M. C., E. A. Döpp, J. J. Neefjes, R. H. J. Beelen, and C. D. Dijkstra. 1989. Heterogeneity of macrophages in the rat evidenced by variability in determinants: two new anti-rat macrophage antibodies against a heterodimer of 160 and 95 kd CD11b/CD18. *J. Leukocyte Biol. 46:556.*
10. Damoiseaux, J. G. M. C., E. A. Döpp, and C. D. Dijkstra. 1991. Cellular binding mechanism on rat macrophages for sialylated glycoconjugates inhibited by the mAb ED3. *J. Leukocyte Biol. 49:434.*
11. Robinson, A. P., T. M. White, and D. W. Mason. 1986. Macrophage heterogeneity in the rat as delineated by two monoclonal antibodies MRC OX-41 and MRC OX-42. *Immunology 57:239.*
12. Dijkstra, C. D., E. A. Döpp, P. Joling, and G. Kraal. 1985. The heterogeneity of mononuclear phagocytes in lymphoid organs: distinct macrophage subpopulations in the rat recognized by monoclonal antibodies. *Immunology 54:589.*
13. Damoiseaux, J. G. M. C., E. A. Döpp, W. Calame, D. Chao, G. G. MacPherson, and C. D. Dijkstra. 1994. Rat macrophage lysosomal antigen recognized by mAb ED1. *Immunol. 83:140.*
14. McMaster, W. R., and A. F. Williams. 1979. Identification of Ia glycoproteins in rat thymus and purification from rat spleen. *Eur. J. Immunol. 9:426.*
15. Williams, A. F., G. Galfre, and C. Milstein. 1977. Analysis of cell surface by xenogenic myeloma-hybrid antibodies: differentiation antigens of rat lymphocytes. *Cell 12:663.*
16. Van den Berg, T. K., I. Van Die, C. Renardel de Lavalette, E. A. Döpp, L. D. Smit, P. H. Van der Meide, F. J. H. Tilders, P. R. Crocker, and C. D. Dijkstra. 1996. Regulation of sialoadhesin expression on rat macrophages: induction by glucocorticoids and enhancement by IFN-β, IFN-γ, IL-4 and lipopolysaccharide. *J. Immunol. 157:3130.*
17. Helmke, R. J., R. L. Boyd, V. F. German, and J. A. Mangos. 1987. From growth factor dependence to growth factor responsiveness: the genesis of an alveolar macrophage cell line. *In Vitro Cell. Dev. Biol. 23:567.*
18. Damoiseaux, J. G. M. C., E. A. Döpp, R. H. J. Beelen, and C. D. Dijkstra. 1989. Rat bone marrow and monocyte cultures: influence of culture time and lymphokines in the expression of differentiation antigens. *J. Leukocyte Biol. 46:246.*
19. Langeveld, C. H., C. A. Jongenelen, E. Schepens, J. C. Stoof, A. Bast, and B. Drukarch. 1995. Cultured rat striatal and cortical astrocytes protect dopaminergic neurons against hydrogen peroxide toxicity independent of their effect on neuronal development. *Neurosci. Lett. 192:13.*
20. Baron, W., J. C. De Jonge, H. De Vries, and D. Hoekstra. 1998. Regulation of oligodendrocyte differentiation: protein kinase C activation prevents differentiation of O_2A progenitor cells towards oligodendrocytes. *Glia. 22:121.*
21. Ding, A. H., C. F. Nathan, and D. J. Stuehr. 1988. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages: comparison with activating cytokines and evidence for independent production. *J. Immunol. 141:2407.*
22. Simmons, D. L. 1993. Cloning cell surface molecules by transient expression in mammalian cells. In: *Cellular Interactions in Development: A Practical Approach.* D. A. Hartley, ed. IRL Press, Oxford, p. 93.
23. Simmons, D., W. Makgoba, and B. Seed. 1988. ICAM-1: an adhesion ligand of LFA-1 is homologous to the neural cell adhesion molecule. *Nature 331:624.*
24. Van den Berg, T. K., E. A. Döpp, J. J. P. Breve, G. Kraal, and C. D. Dijkstra. 1989. The heterogeneity of the reticulum of rat peripheral lymphoid tissues identified by monoclonal antibodies. *Eur. J. Immunol. 19:1747.*
25. Matozaki, T., and M. Kasuga. 1996. Roles of protein tyrosine phosphatases in growth factor signaling. *Cell. Signalling 8:13.*
26. Yi, T., J. L. Cleveland, and J. N. Ihle. 1992. Protein tyrosine phosphatase containing SH2 domains: characterization, preferential expression in hematopoietic cells and localization to human chromosome 12p12–13. *Mol. Cell. Biol. 13:7577.*