Novel mesenchymal and haematopoietic cell isoforms of the SHP-2 docking receptor, PZR: identification, molecular cloning and effects on cell migration

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SHP-2 (Src homology phosphatase type-2) is essential for haematopoietic skeletal and vascular development. Thus the identification of its binding partners is critically important. In the present study, we describe a unique monoclonal antibody, WM78, which interacts with PZR, a SHP-2 binding partner. Furthermore, we identify two novel isoforms of PZR, PZR_a and PZR_b, derived by differential splicing from a single gene transcription unit on human chromosome 1q24. All are type 1 transmembrane glycoproteins with identical extracellular and transmembrane domains, but differ in their cytoplasmic tails. The PZR intracellular domain contains two SHP-2 binding immunoreceptor tyrosine-based inhibitory motifs (VIY²⁴⁶AQL and VVY²⁶³ADI) which are not present in PZR_a and PZR_b. Using the WM78 monoclonal antibody, which recognizes the common extracellular domain of the PZR isoforms, we demonstrate that the PZR molecules are expressed on mesenchymal and haematopoietic cells, being present on the majority of CD34⁺CD38⁺ and early clonogenic progenitors, and at lower

levels on CD34⁺CD38[−] cells and the hierarchically more primitive pre-colony forming units. Interestingly, we show by reverse transcriptase-PCR that the PZR isoforms are differentially expressed in haematopoietic, endothelial and mesenchymal cells. Both PZR and PZR_b are present in CD133⁺ precursors and endothelial cells, PZR_b predominates in mesenchymal and committed myelomonocytic progenitor cells, and all three isoforms occur in erythroid precursor cell lines. Importantly, using SHP-2 mutant (Δ46–110) and SHP-2 rescue of embryonic fibroblasts stably expressing the PZR isoforms, we demonstrate for the first time that PZR, but not PZR_a or PZR_b, facilitates fibronectin-dependent migration of cells expressing a competent SHP-2 molecule. These observations will be instrumental in determining the mechanisms whereby PZR isoforms regulate cell motility.

Key words: CD34⁺, genomic structure, immunoglobulin superfamily, Src homology phosphatase (SHP), stem cells.

INTRODUCTION

Complex processes, such as cell growth, differentiation and migration, require the integration of multiple types of extracellular signals, including those delivered by growth factors, cytokines, cell adhesion molecules and extracellular matrix proteins [1]. Most signalling pathways involve changes in cellular tyrosyl phosphorylation which are regulated by a diverse family of protein tyrosine kinases and an equally diverse family of protein tyrosine phosphatases [2]. SHP (Src homology phosphatase)-1 and SHP-2 comprise a subfamily of tyrosine-specific cytoplasmic protein tyrosine phosphatases, with roles in haematopoiesis, bone formation and vasculogenesis [3–6]. Although SHP-1 and SHP-2 share approx. 60% overall sequence identity, contain two SH2 domains and a catalytic domain, they exhibit distinct physiological functions. SHP-1 is predominantly expressed in haematopoietic cells and is a negative regulator of

phosphotyrosine signalling [7–9], whereas SHP-2 is ubiquitously expressed and is considered to be a positive mediator of haematopoiesis [4,8,10,11]. These molecules are cytoplasmic tyrosine phosphatases, each containing two unique SH2 domains which bind to a distinct amino acid sequence surrounding a phosphotyrosine residue.

Evidence for the functional importance of SHP-1 and SHP-2 comes from the analyses of *shp-1* and *shp-2* mutant knock-out organisms. SHP-1 motheaten (me) and motheaten viable (me^v) [7] mice suffer from immune deficiency and autoimmune diseases and exhibit haematopoietic abnormalities, e.g. excessive erythropoiesis, augmented granulocyte and monocyte production and tissue accumulation, over-expansion of CD5⁺ B cells, leucocyte hypersensitivity and de-regulated mast cell and defective T and natural killer cell functions [7,10,12]. In contrast, disruption of the mouse SHP-2 gene causes death of the embryos at mid-gestation, highlighting its importance in development. Moreover,

Abbreviations used: BFU-E, blast-forming unit-erythroid; BMMNC, bone marrow mononuclear cell; ConA, concanavalin A; ES, embryonic stem; FDCP-1, factor-dependent cell Paterson-1; HBMSC, human bone marrow stromal cells; HUVEC, human umbilical vein endothelial cells; IgSF, Ig superfamily; ITIM, immunoreceptor tyrosine-based inhibitory motif; mAb, monoclonal antibody; MAPK, mitogen-activated protein kinase; MEF, mouse embryonic fibroblast; MFI, median fluorescence intensity; mIgG1, mouse IgG1; PB, peripheral blood; PE, phycoerythrin; PZR, protein zero related; RT, reverse transcriptase; SCF, stem cell factor; SH, Src homology; SHPS-1, SHP-substrate 1; SHP-1/2, SH phosphatase type 1/2; SOB, stromal/osteoblast; UCB, umbilical cord blood.

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The nucleotide sequence data reported will appear in DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession numbers AF181660, AF478447 and AF478448.

fibroblasts from mice harbouring a deletion of SHP-2 exon 3, which express low levels of a defective SHP-2 protein that lacks its N-terminal SH2 domain [13], exhibit impaired mitogen-activated protein kinase (MAPK) activation in response to fibroblast growth factor, epidermal growth factor, and insulin-like growth factor I [13,14]. In other pathways, however, SHP-2 binds to distinct signalling intermediates. One class of SHP-2 binding proteins, exemplified by the *Drosophila* daughter of sevenless (*dos*) gene product, consists of an N-terminal pleckstrin homology domain, multiple proline-rich stretches and potential tyrosyl phosphorylation sites [15]. A second class of molecules belonging to the Ig immunoreceptor tyrosine-based inhibitory motif (ITIM) family has been identified as SHP-2-binding proteins. SHP-substrate 1 (SHPS-1) is a prototypical member of this class of receptors, of which there are now more than 30 proteins. This family is characterized by the presence of one or more ITIMs [comprising of 6 amino acids (I/V/L/S)-XYXX(L/V)] in their cytoplasmic domain. The consensus motif is nearly always associated with a second tandem ITIM, separated from the first by a canonical 25–31 amino-acid-long spacer sequence [16]. SHPS-1 was initially identified as a SHP-2-binding protein, the tyrosyl phosphorylation of which is increased in cells expressing catalytically impaired SHP-2 mutants.

In an attempt to identify additional substrates for SHP-2, Zhao and Zhao [17] overexpressed a catalytically inactive mutant of SHP-2 in HEK-293 cells. Following pervanadate stimulation, several hyper-tyrosine phosphorylated glycoproteins associated with SHP-2 were identified, including a 43 kDa membrane protein [17]. The protein was subsequently purified to homogeneity and amino acid sequence derived. Based on this sequence, a full-length cDNA was isolated and found to encode a novel transmembrane protein, which they termed PZR (Protein Zero Related) due to its significant homology to the major structural protein of peripheral myelin, myelin P0. The extracellular domain of this protein contains a single extracellular Ig domain and two intracellular ITIMs (VIY²⁴¹AQL and VVY²⁶³ADI). Using site-directed mutagenesis, these workers demonstrated that these two ITIMs accounted for the entire tyrosine phosphorylation of PZR and were essential for SHP-2 recruitment and activation [18]. Like SHPS-1, PZR was found to be widely expressed. However, it specifically interacts with SHP-2 but not with SHP-1 [18].

In the present study, we describe a unique monoclonal antibody (mAb), WM78, that reacts with the extracellular domain of human PZR. Expression cloning using this mAb and reverse transcriptase (RT)-PCR analysis have identified two novel PZR isoforms, PZR α and PZR β , both lacking ITIM motifs. We further demonstrate the differential expression of these isoforms in cells of haematopoietic and mesenchymal origin. Genomic analysis and *in silico* cloning demonstrate that these three isoforms are transcribed from a single gene transcription unit on human chromosome 1q24. Interestingly, these novel isoforms differentially regulate fibronectin-mediated cell motility in a SHP-2-dependent manner.

EXPERIMENTAL

Cells and WM78 mAb generation

Human haematopoietic cell lines have been described previously [19–21]. Human umbilical vein endothelial cells (HUVEC) cells were provided by Dr I. Rappold and Dr M. Raida. Peripheral blood (PB), posterior iliac crest bone marrow and umbilical cord blood (UCB) mononuclear cells were collected with informed consent and ethical approval [19–21]. U937 cells were cultured in 10% FCS RPMI-1640 medium containing 0.1 μ M PMA (Sigma)

as a differentiating agent for 4 days to induce monocytic differentiation characterized using α -naphthyl acetate non-specific esterase staining [22]. The WM78 mAb was derived after immunization of BALB/c mice with the human T-cell line, 8402, and fusion of spleen cells with the SP2/0 myeloma cell line [23].

Human bone marrow stromal cell (HBMSC) and human bone marrow stromal/osteoblast (SOB) cell cultures and magnetic bead isolation

Stromal cultures were established from bone marrow mononuclear cells (BMMNCs) [24]. Trabecular bone specimens were obtained from the Department of Orthopaedic and Trauma, Adelaide Hospital, Australia, with informed consent and institutional ethical approval. Single cells were prepared by collagenase (3 mg/ml; collagenase Type I; Worthington Biochemical Co., Freehold, NJ, U.S.A.) and dispase (4 mg/ml; neutral protease grade II; Boehringer-Mannheim) treatment. Cell suspensions were analysed by CD14 and CD45 staining (FACS or immunofluorescence) and showed no contamination with macrophages or haematopoietic cells [19–21]. BMMNCs were incubated with CD34 (561) Dynabeads (Dynal, Oslo, Finland), and CD34⁺ cells captured magnetically and recovered with DETACHaBEADTM reagent (Dynal). CD34⁺ and CD133⁺ umbilical cord blood and PB monocytes were also isolated with CD34⁺, CD133⁺ or CD14⁺ magnetic beads respectively (Miltenyi-Biotech, Bergish, Gladbach, Germany) [20,21]. Isolates were labelled with CD34-phycoerythrin (PE), AC133/2-PE or CD14-PE mAbs and were routinely > 95% pure.

In situ immunofluorescence staining

HBMSC cultured in 8-chamber slide flasks (Nunc, Naperville, IL, U.S.A.), were fixed in acetone/methanol (1:1, v/v), blocked with 5% (v/v) normal goat serum and stained with the WM78 mAb or an isotype-matched mouse (m)IgG1 control followed by FITC-conjugated goat anti-mouse F(ab)₂ Ig (Silenius, Hawthorn, Victoria, Australia) [20]. Cells were examined under an Olympus BH2-RFCA fluorescence microscope (Olympus Microsystems, London, U.K.).

Flow cytometry and sorting

For single colour analyses, cells were stained as described previously [21]. For dual colour analyses, cells were labelled with CD4-PE, CD8-PE, CD56-PE or CD19-PE plus biotinylated WM78 mAb and streptavidin-FITC, or with CD14-FITC, CD45-FITC, CD19-FITC, CD15-FITC or glycophorin A-FITC, followed by WM78-biotin plus streptavidin-PE or CD3-PE followed by WM78-biotin plus streptavidin-FITC [21]. For triple colour labelling, cells were stained with CD34-FITC (clone 8G12) and CD38-PE (clone HB-7), plus WM78-biotin and streptavidin-Tricolor (Caltag Laboratories, Burlingame, CA, U.S.A.) [20]. BMMNCs were Fc receptor blocked and stained with CD34-PE (clone 8G12) and WM78-biotin followed by streptavidin-Tricolor and sorted on the FACStar^{PLUS} (Becton-Dickinson, Sunnyvale, CA, U.S.A.) [23].

Clonogenic assays and pre-progenitor cell (pre-CFU) culture

Cells were assayed for day-14 CFU-granulocyte-macrophage (GM), and/or CFU-granulocyte (G) and CFU-macrophage (M) colony forming cells, early blast-forming unit-erythroid (BFU-E) progenitors and multipotential-CFU cells (CFU-Mix) in semi-solid methylcellulose cultures [19,23]. All colonies were scored according to standard criteria after 14 days of incubation. Sorted

cells were cultured in pre-CFU medium containing a cocktail of six haematopoietic growth factors, recombinant human interleukin (IL)-1, IL-3, IL-6, granulocyte colony-stimulating factor, granulocyte/macrophage colony-stimulating factor and stem cell factor (SCF), all at 10 ng/ml, for 28 days [25].

Immunoprecipitation and SDS/PAGE

Goat anti-mouse Ig-coupled Dynabeads (Dyna) were pre-armed with purified Ig (WM78 and isotype matched non-binding controls) as described previously [26]. Samples were run on SDS/PAGE (10% gels) [27], transferred to PVDF membranes (MSI Membranes, Bresatec, Adelaide, Australia) and developed with streptavidin-alkaline phosphatase (Amersham International, Little Chalfont, Bucks., U.K.). Immunoreactive proteins were resolved using a FluorImager and ImageQuant software (both from Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Expression cloning and sequencing of the WM78 cDNA clone

The PZR cDNA was isolated from a HBMSC cDNA library in the retroviral vector pRUFneo [23] using the WM78 mAb. Following PCR recovery of pro-viral cDNA inserts from genomic DNA, unique *Bam*HI and *Xho*I restriction sites present in the 5' and 3' flanking regions respectively were used to re-clone the cDNA into pRUFneo. Stable G418-resistant Ψ_2 virus-producing cell lines were produced by calcium phosphate transfection and used to infect factor-dependent cell Paterson-1 (FDCP-1) cells by co-cultivation [28]. G418-resistant FDCP-1 cells were analysed for antigen expression by indirect immunofluorescence, flow cytometry and immunoprecipitation as described above.

Identification of full-length PZR, PZR α and PZR β cDNAs and real time quantitative PCR

Marathon-ready human Burkitt's lymphoma (Raji cell line) cDNA was purchased from Clontech Laboratories (Palo Alto, CA, U.S.A.). Total RNAs from cell lines described above, and from normal SOB cells, CD133⁺, CD34⁺, CD14⁺ and HUVEC cells, extracted using the RNeasy (Qiagen, Crawley, West Sussex, U.K.) and the RNeasy (Qiagen, Crawley, West Sussex, U.K.) reagents, were reverse transcribed using Sensiscript Reverse Transcriptase system (Qiagen). Human PZR, PZR α and PZR β were generated by PCR amplification of the cDNAs described above using appropriate forward and reverse primers (Sigma) and the ExpandTM Long PCR system (Boehringer-Mannheim) [19]. The cDNAs were ligated into the pGEM-T easy vector (Promega) and PCR amplified using the ExpandTM Long Template System (Boehringer-Mannheim) and pUC M13 forward and reverse primers [19]. The PZR, PZR α and PZR β PCR products were sequenced automatically on an ABI 377 Automatic Sequencer (Perkin-Elmer Applied Biosystems) [19]. Peptide motifs and structural characteristics were predicted using TopPred 2, Das, Signal P, TMHMM, O-Glybase, Net Phos 2.0 Server and MacVector software packages [19]. The PZR and PZR β TaqManTM (Applied Biosystems) systems consist of the following pair of primers (numbers in the brackets indicate the position of the starting base of the primer in the sequence): FPZR (635), 5'-TTAAGCAGGCTCCTCGGAGT-3', RPZR (742), 5'-CGGAGTGGTCTAACTGTGCATATATG-3' and FPZR β (574) 5'-GAAGGAAAACTCTAAACGGGATT-3', RPZR β (688) 5'-GCATACACCACAGACTCTGACTTGT-3' respectively, as well as dual fluorescent probes PZR (5'-CCCTCCGACACTG-AGGGTCTTGTAAGAGTC-3') and PZR β (5'-AACTGTG-

CATATATGACTGGGCCCCAGT-3'). PZR α was not quantified by this method because it was not found in high levels in the majority of cells tested and because the sets of primers and fluorescent probes that can be designed in the region that distinguish PZR α from the other two isoforms (the 3' part of exon 4) can amplify genomic DNA. The reaction was performed as described by Chiu et al. [29]. The difference in the levels of expression between PZR and PZR β in different haematopoietic cells was given by the Δ Ct value (where Ct is the threshold cycle for target amplification)

$$\Delta Ct = |Ct_{PZR} - Ct_{PZR\beta}|$$

The relative expression ratio between the two molecules was calculated using the following formula: $2^{\Delta Ct}$. All the experiments were carried out in duplicate on four independent occasions.

PZR genomic PAC and cosmid DNA clones and Southern blots

Three PZR cDNA probes were used for screening human genomic libraries and for Southern blotting (see Figure 7). Probe A used for screening a human leucocyte PAC library was derived from the *Bam*HI/*Xho*I fragment of PZR cDNA in the pGEM-T vector. Probe B (366 bp), used to probe the human chromosome 1-specific cosmid library, was generated by *Bam*HI and *Hind*III digestion of the PZR cDNA, and probe C was a PCR product generated from PZR α . Each cDNA probe was labelled with 30 μ Ci [α -³²P]dCTP (Amersham International) using the T7 Quickprime kit (Pharmacia) or with the DIG labelling kit (Roche Diagnostics, Mannheim, Germany) [19,20]. The human PAC library was hybridized with α -³²P-labelled probe A. Nine positive PAC clones were identified from these filters or by using the Human Genome Blast program (NCBI Genome Sequencing Centre) for PAC clones. PAC clone 313-L4 was selected for further analysis. A human chromosome 1-specific cosmid library, LLNL Human (HGMP Resource Centre), was probed with PZR α probe B. Seven cosmid DNA clones were isolated and then analysed using the PZR α probe B, or PCR-amplified using a range of primer pairs, and the ah95b1 cosmid clone was selected for detailed analysis. Human PAC, cosmid and human leucocyte genomic DNA (Promega) samples were further analysed using PZR α probes A, B or C (see Figure 7) labelled with [α -³²P]dCTP or with DIG (Roche Diagnostics) [19]. PAC and cosmid DNAs were analysed by PCR amplification using appropriate primers, the ExpandTM Long PCR System (Boehringer Mannheim) and the PCR program described above. Human genomic DNA was PCR amplified as above in order to verify the PZR genomic sequence in the PAC and cosmid clones. The GC 5'-rich region of the gene was amplified by PCR using the Advantage-GC Genomic Polymerase mix (Clontech, Basingstoke, U.K.) with the following conditions: 95 °C for 1 min, 25 cycles of 94 °C for 30 s and 68 °C for 12 min, and 1 cycle of 68 °C for 12 min. The PCR products were isolated by agarose gel electrophoresis and ligated into the pGEM-T vector for sequencing.

Fluorescence *in situ* hybridization and *in silico* cloning

Metaphase spreads were prepared from phytohaemagglutinin-stimulated normal human lymphocytes probed with the biotinylated ah95b1 cosmid clone as described previously [21]. Neither exon 1 nor the first 1.5 kb of intron 1 were present in the 313-L4 PAC or ah95-b1 cosmid clone sequences. Since we located the PZR gene on human chromosome 1q24, and the human chromosome 1 working draft sequence had been determined by the Sanger Centre and provided by the NCBI, the

Figure 1 The WM78 mAb identifies an antigen on cultured human bone marrow SOB and PB cells

(A) Single-colour flow cytometry showing binding of the mAb WM78 (bold histograms) to cultured human bone marrow SOB cells. The anti-HLA Class I mAb was used as the positive control. The non-binding, isotype-matched mIgG1 negative control mAb was 1B5 (non-bold histograms). (B) Representative histograms of PB myeloid subsets gated for CD14, CD15, CD41b or anti-glycophorin positive cells prior to analysis with the isotype matched negative control (non-bold histograms) or the WM78 mAb (bold histograms). M1 marks the position of the positive gate used to determine the percent positive cells in each subset. (C) The table shows the percent of each PB cell subset that stained with WM78 after gating against a negative isotype control. Values are mean $\pm$ S.E.M. for three independent experiments.

sequence of exon 1 and intron 1 was identified *in silico* (GenBank[®] database accession number NT004668).

Production of mouse embryonic fibroblast (MEF) cell lines stably expressing PZR isoforms

MEFs were used as SHP-2^{-/-} MEF cells lacking the intact N-SH2 domain (Δ 46–110) or as 'wild-type' (SHP-2 rescue) MEF cells in which the (Δ 46–110) mutant MEF cell line was stably transfected with a competent SHP-2 gene. PZR, PZR α and PZR β were RT-PCR amplified from KG1A and HUVEC cells. The cDNAs were digested with *Bam*HI and *Xho*I and subcloned into the multiple cloning site of the pRUF. Neo retroviral vector and transfected into the ψ ₂ [23]. Cells were selected in 400 μ g/ml G418 prior to transfer into G418-free Dulbecco's modified Eagle's medium for virus collection and retroviral infection of the MEF cells [23]. Flow cytometry and immunoprecipitation identified PZR, PZR β and PZR α proteins of 48, 36 and 34 kDa using the WM78 mAb (results not shown). The top 50% of the

cells expressing PZR were isolated by flow sorting as above, cultured in 400 μ g/ml G418 and used in the migration assay [23].

Migration assay

The underside of transwell 8 μ m pore filter was coated in triplicate with either 1 ml of 10 μ g/ml fibronectin (Boehringer Mannheim) or 10 μ g/ml BSA as a control, for 2 h at 37 °C or overnight at 4 °C. Prior to seeding the cells (24 h) on to the transwell filter (Costar[®] Corning, NY, U.S.A.) the cell lines were diluted 1 in 2 to ensure log phase growth and 5×10^4 cells in serum-free Dulbecco's modified Eagle's medium added to the upper chamber of the transwells. Serum-free medium (500 μ l) containing 4 μ g/ml of fibronectin was added to the lower chamber and incubated at 37 °C for 5 h. Cells which migrated through the filter were fixed in 3.7% (w/v) paraformaldehyde for 10 min and stained with 0.2% (w/v) Crystal Violet in ethanol (ICN Biomedicals, Costa Mesa, CA, U.S.A.) for 20 min at 25 °C. Crystal Violet was

extracted using 300 μ l of 10 % (v/v) acetic acid for 5 min at 25 °C on a shaker. The results were quantified using a Beckman D4-65 spectrophotometer at 600 nm wavelength. Data points derived from three independent experiments are reported as the mean $\pm$ S.E.M. Analysis of the variance to determine significant differences between treatments was performed using ANOVA factorial analyses.

RESULTS

The mAb WM78 identifies an antigen expressed by bone marrow mesenchymal and PB mononuclear cells

The WM78 mAb was selected for its reactivity with bone marrow-derived mesenchymal or cultured SOB cells (Figure 1A). However, its reactivity was not restricted to these cells. Figure 1(B) illustrates that WM78 binds more strongly to PB monocytes than to granulocytes, platelets and erythrocytes. Indeed, in three independent experiments, median fluorescence intensities (MFIs) of 395.3 ± 55.5 (mean $\pm$ S.E.M.) for CD14⁺ monocytes, 19.8 ± 5.4 for CD15⁺ granulocytes, 16.4 ± 5.4 for CD41b⁺ platelets and 12.7 ± 4.2 for glycophorin A⁺ erythrocytes were obtained, com-

pared with negative isotype control MFIs of 2.9 ± 0.5 , 2.9 ± 0.5 , 2.5 ± 0.5 and 3.4 ± 0.5 respectively. Within the lymphoid compartment, the expression of the WM78-defined antigen was variable, ranging from negative or low to strongly positive. Figure 1(C) illustrates the percentage of CD14⁺ monocytes, CD19⁺ B cells, CD4⁺ and CD8⁺ T cells, and CD56⁺ natural killer cells staining with WM78. In adult human bone marrow, a similar phenomenon was observed, as illustrated in Figure 2.

WM78 differentially binds to CD34⁺ cell subsets and haematopoietic precursors

A uniform level of WM78 mAb binding was found on most CD34⁺ bone marrow precursor cells, with 91.9 ± 3.2 % (mean $\pm$ S.E.M., $n = 3$) of these cells occurring in the CD34⁺WM78⁺ gate (Figure 3A). Within this CD34⁺ population, the CD34⁺CD38⁺ cells expressed higher levels of the WM78-defined antigen than did the more primitive CD34⁺CD38^{low} subset, as determined by three colour flow cytometric staining (Figures 3B and 3C). For example, WM78 MFIs of 54.7 ± 3.5 , 22.4 ± 1.5 and 2.3 ± 0.5 (means $\pm$ S.E.M., $n = 3$) were obtained

B

Percentage WM78 Positive Cells in each Subset after gating against a Negative Isotype Control	
Cellular subset	Percentage Positive Mean $\pm$ SEM (n=3)
CD14 ⁺ monocytes	54.9 $\pm$ 18.5
CD15 ⁺ granulocytes	23.3 $\pm$ 3.2
CD19 ⁺ B cells	69.6 $\pm$ 3.3
Glycophorin A ⁺ erythrocytes	12.7 $\pm$ 4.0
CD3 ⁺ T cells	14.9 $\pm$ 5.9
CD45 ⁺ leucocytes	14.1 $\pm$ 5.5

Figure 2 Differential binding of the WM78 mAb to human bone marrow haematopoietic cell subsets

(A) Representative two-colour dot plots demonstrating the differential binding of CD markers and the WM78 mAb to BMMNCs. The cells were labelled with FITC-conjugated lineage specific markers and biotin-conjugated WM78 mAb plus PE-streptavidin. Mouse IgG1 mAbs conjugated with PE, FITC or biotin were used as negative controls. The percent in each quadrant represents the percentage of BMMNC staining with the mAbs indicated. (B) Cells were gated for each of the positive CD markers separately and the relative percentage of CD14⁺, CD45⁺, CD3⁺, CD19⁺, CD15⁺ and glycophorin A⁺(Gly-A) cells staining with WM78 determined. Values are means $\pm$ S.E.M. for three independent experiments.

Figure 3 The WM78 mAb binds differentially to CD34⁺CD38⁺ and CD34⁺CD38^{low/-} subsets and to haematopoietic progenitor cells

A representative two-colour dot plot of BMMNCs labelled with WM78-biotin plus streptavidin-Tricolour, CD34-FITC and CD38-PE (A), CD34-PE, CD38-FITC and WM78-biotin plus streptavidin-Tricolour (B) and WM78-biotin plus streptavidin-Tricolour, CD38-FITC and CD34-PE (C). The relative CD38-staining of cells in the CD34⁺WM78⁺ and CD34⁺WM78^{low/-} (A) and the relative WM78 staining of cells in CD34⁺CD38⁺ and CD34⁺CD38^{low/-} fractions (B) were determined by flow cytometry as MFI for 3 independent samples. (D) A representative dot plot of BMMNCs labelled with WM78-biotin plus streptavidin-PE and CD34-FITC and flow sorted into the CD34⁺ (Region R1), CD34⁺WM78⁺ (Region R2) and CD34⁺WM78⁻ (Region R3) subsets, showing the percentages in each quadrant. (E) Clonogenic cells were measured from sorted fractions, R1, R2 and R3, and analysed for the presence of day 14 multipotential, erythroid and myeloid progenitor cells. Means $\pm$ S.E.M. from three donors (R, B and Z) are expressed as the recovered clonogenic cells in fractions R2 and R3 on the basis of the percentage of WM78⁺ or ^{low/-} cells in the CD34⁺ fraction R1 and after normalization of values for clonogenic cell output to the CD34⁺ subset to 100%. (F) Cells from two donors (B and Z) sorted into fractions R1, R2 and R3 were also assayed for their content of pre-CFU, as described in the Experimental section. Data represent the fold expansion of nucleated cells after 1000 cells from each fraction were cultured in a cocktail of six haematopoietic growth factors for up to 35 days.

for the CD34⁺CD38⁺, CD34⁺CD38^{low/-} and isotype-matched negative control subsets respectively. To confirm that the WM78 mAb bound to committed haematopoietic progenitor cells, *in vitro* clonogenic and pre-CFU assays were performed on BMMNC-derived FACS isolated CD34⁺ (Region R1), CD34⁺WM78⁺ (Region R2) and CD34⁺WM78⁻ (Region R3) cells (Figure 3, panel D). The majority of day 14 BFU-E and CFU-E, were recovered in the WM78⁺CD34⁺ fraction

Figure 4 Identification of PZRb

(A) After expression cloning, PZRc cDNA was transfected into FDCP-1 cells and analysed by flow cytometry for cell surface binding of the WM78 mAb (upper panel) or a mlgG1 isotype matched negative control (lower panel). The histograms represent sham-transfected FDCP-1 cells labelled with mlgG1 or WM78 mAbs. (B) Immunoprecipitation of the PZRb molecule using mlgG1 (lanes 1 and 3) or the WM78 mAb (lanes 2 and 4) from FDCP-1 cells expressing PZRb (lanes 1 and 2) or in non-expressing FDCP-1 cells (lanes 3 and 4) and analysis by SDS/10% PAGE.

(Figures 3D and 3E, Region R2). FACS isolated (Figure 3D) CD34⁺WM78⁺ (Region R3) and CD34⁺WM78⁻ (Region R2) or CD34⁺ (Region R1) fractions were also assayed for *de novo* generation of nucleated cells in the pre-CFU assay (Figure 3F). When 1000 cells were cultured, all the three CD34⁺WM78⁺ and CD34⁺WM78⁻ or CD34⁺ cell subsets were able to generate nucleated cells.

WM78 identifies PZRb, a novel isoform of the SHP-2 binding partner PZR that does not contain ITIM

Given its reactivity with a cell surface molecule on cultured human BM stromal cells (Figure 1A), the WM78 mAb was used to screen a HBMSC cDNA expression library in the retroviral vector pRUFneo. Automatic sequencing of the resultant cDNA and GenBank®/EMBL database analyses revealed substantial nucleotide identity with the previously cloned PZR molecule that is expressed in epithelial cells [17]. We named this molecule PZRb (GenBank® accession number AF181660). Expression of PZRb cDNA in the murine haematopoietic progenitor cell line FDCP-1, (Figure 4A, upper panel) and immunoprecipitation using the WM78 mAb identified a protein with an apparent molecular mass of approx. 30 kDa (Figure 4B, lane 2). Comparative nucleotide and predicted amino acid sequences of PZRb with PZR are shown in Figure 5 and demonstrate type 1

Figure 5 Comparative cDNA and amino acid sequences of PZR isoforms

The PZR cDNA sequence was obtained by RT-PCR analyses using the primers indicated. The extracellular, the transmembrane and the intracellular domains are indicated by bold italics, underlined, and bold respectively. Cysteines are circled and tyrosine residues are boxed. Potential N-linked (*) and O-linked (#) glycosylation sites are indicated. Differences between the cytoplasmic domains of PZR, PZRa and PZRb isoforms are underlined with a dashed line and identities in the 3' region between PZR and PZRb are underlined twice with a solid line. The positions of FPZR, R904 and R974 primers are highlighted. The additional 5' untranslated region (UTR) and 3' UTR sequences of PZRa were identified from the PZRa cDNA sequence. The 3' UTR sequence of PZR was obtained from the published sequence (GenBank® accession number AF087020).

Figure 6 Distribution of haematopoietic endothelial and mesenchymal derived cells for PZR isoforms

(A) The distribution of PZR isoforms was examined by RT-PCR analysis in KG1A, KG1, HL60, U937, K562, RPMI-1788, as well as HUVEC, normal CD133⁺ progenitor cells (95% purity), CD14⁺ PB monocytes (86% purity) and cultured bone marrow SOB cells (lane 4), CD34⁺ cells (lane 5) and the erythroid precursor cell line K562 (lane 6) with the WM78 mAb and the respective negative controls (lanes 1, 2 and 3) and electrophoresis on SDS/10%-PAGE. Arrows indicate the positions of the predicted PZR and PZRb isoforms. Seablue II markers (Navex, Invitrogen, CA, U.S.A.) were used. (D) A schematic representation of PZR isoforms. (E) The percent amino acid identities and similarities between PZR and PZR_a, PZR_b, myelin P0 and epithelial V-like antigen.

Figure 7 PZR genomic structure

A schematic representation of the genomic structure of PZR gene showing exons 1 to 6 (boxed) and introns 1 to 5 (black line). Sequences encoding the different domains for PZR, PZRα and PZRβ mRNAs are shown. Inset: PZRα probes used for screening PAC and cosmid libraries.

transmembrane molecules with identical extracellular and transmembrane domains but different cytoplasmic tails.

Identification of PZR isoforms in haematopoietic, endothelial and mesenchymal cells

Since our studies revealed that PZR and PZRα contained different cytoplasmic sequences but identical extracellular and transmembrane domain sequences, we amplified cDNAs by PCR using an identical forward primer (FPZR) positioned at -17 from the ATG) but different reverse primers to sequences in the 3' UTRs that would specifically distinguish between PZR and PZRα gene products, namely R904 and R974 respectively (Figure 5). Initial PCR analyses, using these primer pairs with the Raji and KG1A cDNAs, generated three, rather than two, different products. One product of approx. 650 bp produced with the primer pair FPZR and R904 was detected only after 60 cycles of PCR amplification and was identical to the PZRα sequence (Figure 5). The other two products that were generated using the primer pair FPZR and R974 were approx. 900 and 700 bp in size and were generated with 30 cycles of PCR amplification (Figure 6A). Sequence analysis demonstrated that the larger product was identical to PZR (Figure 5) whereas the smaller product was identical to PZR in its extracellular and transmembrane domains, but not in its cytoplasmic tail. We have termed this product, PZRβ (Figure 5). Further RT-PCR analysis revealed that the PZR and PZRβ isoforms are widely expressed in the haematopoietic progenitor cells and cell lines examined (Figure 6A). Using real-time quantitative PCR, it was found that PZR and PZRβ were present in almost equivalent amounts in CD14⁺ PB monocytes and in cell lines representing pre-B cell progenitors (RPMI-1788). However, PZRβ appeared to be the major isoform in CD34⁺ promyelocytic (HL60) and promonocytic (U937) cell

lines (7.31- and 7.11-fold higher expression respectively than PZR) and in multipotent progenitor cells (KG1A). Differentiation of U937 cells along the monocytic lineage revealed a decrease in PZRβ and an increase in PZR expression. Interestingly, PZR was prominent in stromal cells (23.1-fold higher than PZRβ) and to a lesser extent in HUVEC and in erythroid progenitors (K562) (Figure 6B). The PZRα isoform was expressed at significant levels (i.e. using 30 cycles of RT-PCR amplification) in the erythroid progenitor cell line K562, but was detected only after 60 PCR cycles and therefore at putatively low levels in KG1A and Raji cells (Figure 6A and results not shown). The differential distribution of the PZR isoforms in different cell types suggests that a mechanism exists for controlling PZR isoform expression and hence function during lineage development or commitment within the haematopoietic, mesenchymal and vascular systems. Since FACS analysis (see Figures 1-3) revealed that WM78 binds strongly to mesenchymal-derived cells and to a variety of haematopoietic cells and their progenitors, we immunoprecipitated the WM78-defined molecule from human bone marrow SOB cells (Figure 6C, lane D), bone marrow CD34⁺ cells (Figure 6C, lane E) and the erythroid progenitor cell line K562 (Figure 6C, lane F). The results demonstrated the presence of multiple protein bands on SDS/PAGE gels under reducing conditions. A major band with an apparent molecular mass of approx. 32-36 kDa was found in the SOB cell lysate and was weakly detectable in K562 cells, which like bone marrow CD34⁺ progenitor cell lysates, produced a main band at approx. 36-48 kDa. The latter is identical to the molecular mass previously identified for PZR in transfected cells [17], while the former 32-36 kDa band in SOB cells is likely to represent PZRβ. These studies suggest that the different PZR isoforms are differentially expressed in haematopoietic and mesenchymal derived cells.

Amino acid sequence identity and potentially important motifs for the PZR isoforms

The amino acid sequence similarities and predicted structures of the three PZR-like peptides are shown in Figures 5, 6(D) and 6(E) respectively. PZR α , PZR β and PZR cDNAs are predicted to encode type 1 transmembrane proteins of 202, 209 and 269 amino acids respectively. In all three isoforms, the predicted signal peptide cleavage site is positioned between Arg-37 and Leu-38 (VSA↓LE). An IgV-set domain motif linked by disulphide bridges is predicted to occur between amino acids 58 and 135, placing these molecules in the Ig superfamily (IgSF). Each PZR isoform contains two potential N-linked glycosylation sites (at Asp-50 and Asp-130) and three potential O-linked glycosylation sites at Thr-70 and on Ser residues 70 and 75. A 27-amino-acid transmembrane hydrophobic sequence lies between amino acids 162–189. PZR possesses the longest intracellular domain of 80 amino acids, and contains a classical ITIM motif of the type (V/I)XYXX(L/V) and more specifically VIY²⁴⁶AQL and VVY²⁶³ADI (246 and 263 tyrosine phosphorylation sites) that has previously been shown to interact with the SH2 domains of SHP-2 [17,18]. The cytoplasmic domains of PZR α and PZR β are truncated to 13 and 20 amino acids respectively, with both lacking the classical ITIM motif and SHP-2 binding sites. Database analyses revealed one potential tyrosine phosphorylation site at position 200 of the amino acid sequences in both PZR α and PZR β molecules. PZR isoforms show significant amino acid similarity with myelin P0 protein and epithelial V-like antigen 1 (Figure 6E).

Genomic structural analyses reveal that the PZR isoforms are derived by differential splicing of a single gene transcription unit and are located on human chromosome 1q24

To determine if the PZR isoforms are products of one or more genes, we analysed the genomic structure. The 313-L4 PAC clone (GenBank® accession number Z99943) provided a partial sequence for the PZR gene that we predict encompasses part of intron 1 and exons 2 to 6, but lacks the 5' region encompassing the signal sequence and 5' that exon 1 would possess. The exon 1 and intron 1 sequences (GenBank® accession number NT004668) were identified using an *in silico* cloning strategy. Comparative analyses of the genomic sequence with PZR, PZR α and PZR β cDNAs indicated that these isoforms are derived by differential splicing of exons from a single gene transcription unit (Figure 7). The region spanning the coding exons 1 to 6 covers at least 66 kb of genomic DNA. While the PZR isoforms are identical from exon 1 to 4, the sequences of the isoforms diverge in subsequent exons. The signal peptide is encoded by exon 1 and part of exon 2, while the extracellular region is encoded by exons 2 and 3 and part of exon 4 (111–482 nt). The transmembrane sequence is encoded by exon 4 (483–567 nt). For the PZR α transcript, exon 4 begins at +473 bp from the ATG of the translational start site with a TAA stop codon at bp +607 to +609. This is followed by a 3' UTR sequence of at least 541 bp. Similarly, for both the PZR and PZR β transcripts, exon 4 starts at +473 bp and reads through to +605 bp. However, unlike PZR α , both PZR and PZR β then use a dinucleotide GT splice site one base before the stop codon of PZR α to splice on to additional exons. PZR splices on to exon 5, which comprises 103 bp and covers +606 bp to +705 bp of the mRNA sequence, which then splices on to exon 6, which encompasses +706 to +1019 bp, with a TAA stop codon located at +808 to +810 bp. PZR β splices from exon 4 on to exon 6 at +606 bp but reads through to a TAG stop codon at +628 bp. From the cDNA sequence, we would predict a minimum 3' UTR sequence of

Figure 8 The PZR gene is located on human chromosome 1q24

Fluorescence *in situ* hybridization analysis using the ah95b1 cosmid clone as a probe shows that PZR α , PZR β and PZR isoforms are located on human chromosome 1q24. Inset: an ideogram of human chromosome 1 showing the localization of the PZR gene to band q24.

123 bp. Metaphase spreads were examined using the 95b1 cosmid clone containing the PZR gene as a probe. Positive signals were observed on both chromatids of human chromosome 1 at band 1q24 (Figure 8).

PZR, but not PZR α or PZR β , regulates integrin-mediated cell motility

Stable transfectants were analysed for their relative expression levels of the PZR isoforms using flow cytometry with the WM78 mAb. A representative set of histograms (Figure 9A) shows a relatively uniform mean fluorescence intensity for each of the transfectants. Using these cells, we investigated the effects of SHP-2 depletion in relation to PZR and its various isoforms on migration over the extracellular matrix protein, fibronectin. Previous studies have indicated that the SHP-2 mutant cells ($\Delta 46$ –110) migrate at a lower rate than the SHP-2 rescue cells [30], and that the mutant SHP-2 protein without the intact N-SH2 domain ($\Delta 46$ –110) does not function in a dominant negative manner, but rather as a loss-of-function molecule [14]. Thus, the availability of the SHP-2-rescue 'wild-type' fibroblast cell line, where SHP-2 had been re-introduced into SHP-2^{-/-} cells together with the homozygous SHP-2^{-/-} mutant ($\Delta 46$ –110) fibroblast cell line, provided us with a unique opportunity to study PZR-mediated effects in a SHP-2 positive or negative background. Using a transwell migration chamber with 8 μ m pores, the migration of uninfected wild-type and SHP-2^{-/-} ($\Delta 46$ –110) MEF

Figure 9 Cell motility regulated by PZR isoforms

(A) Surface expression of PZR isoform-expressing SHP-2 rescue ('wild-type') and SHP-2 mutant ($\Delta 46-110$) MEF cell lines used in the assays. Cells were stained with 10 $\mu\text{g}/\text{ml}$ of the PZR-specific mAb WM78 (no shading) or isotype control 1B5 (grey shading). Specific staining was detected after incubation with saturating levels of FITC-conjugated sheep anti-mouse Ig. These profiles show a comparable level of PZR expression for each of the different isoforms. (B) The effect of PZR isoforms on migration of SHP-2 rescue 'wild-type' (Res) and SHP-2 mutant ($\Delta 46-110$) (Mut) cell lines was assessed by the Boyden transwell assay. The cells stably transduced with various isoforms of PZR specified or non-transfected cells (UT) were seeded on 8 μm porous membranes coated with either BSA or fibronectin (FN) in Boyden multiwell chambers. Following incubation for 5 h at 37 $^{\circ}\text{C}$, cells which had migrated through the membrane were quantified by Crystal Violet staining and spectrophotometry, using a wavelength of 600 nm. A significant difference ($P < 0.005$, Anova) in the migration potential was conferred to cells expressing the ITIM-containing isoform of PZR. The results represent the mean $\pm$ S.E.M. of three independent experiments.

cells was compared with the migration of the PZR isoform expressing MEF cell lines on BSA or fibronectin. As shown in Figure 9(B), no significant migration was observed using BSA as the chemotactic stimulus. In contrast, in three independent

experiments, migration was increased to variable levels with all cell lines using fibronectin as the integrin-mediated stimulus. Importantly, a 2- to 5-fold enhancement in migration was observed in the wild-type PZR-rescue MEF cell line compared with other PZR isoforms in SHP-2-rescue and mutant MEF cell lines. These results demonstrate that the intact PZR cytoplasmic domain containing the ITIM-motifs is important in modulating fibronectin-mediated cell migration rates, in these murine mesenchymal derived MEF cells.

DISCUSSION

PZR has previously been identified as a SHP-2 binding partner in epithelial cells [17]. In this paper, we report the identification and cloning of two novel isoforms of PZR, PZR α and PZR β , and demonstrate that these three isoforms are differentially expressed in the haematopoietic lineage and in stromal/mesenchymal cells. Of particular interest is the observation that the PZR isoforms are more strongly expressed on CD34⁺CD38⁺ clonogenic haematopoietic precursors than in more primitive CD34⁺CD38^{low} pre-CFU precursors. Furthermore, PZR predominates over PZR β in endothelial cells. In addition, a switch occurs during monocytic differentiation when the ratio of PZR increases relative to PZR β . In mesenchymal cells and osteoblasts, PZR is the major isoform. The significance of the alteration in expression of the PZR isoforms during monocytic differentiation appears to be related to the structural differences between the different isoforms. While all three isoforms possess identical extracellular and transmembrane sequences, only the PZR isoform contains a cytoplasmic tail with two ITIM motifs (VIY²⁴⁶AQL and VVY²⁶³ADI) that can specifically interact with the SH2 domains of SHP-2 [17]. This in turn may endow PZR, but not PZR α or PZR β , with a negative SHP-2 regulatory role in these cells. It further implies that the differential expression of PZR isoforms may differentially modulate signalling pathways that involve SHP-2 in different cell types. During the preparation of this manuscript, Zhao et al. [31] demonstrated that truncation of the intracellular domain of PZR blocks concanavalin A (ConA)-induced PZR tyrosine phosphorylation in epithelial cells. A similar phenomenon may occur during myeloid differentiation from multipotent progenitors when a switch from PZR to PZR β occurs. We have demonstrated that PZR isoforms are generated by differential splicing of a single gene transcription unit on human chromosome 1q24. Other ITIM-containing IgSF members (e.g. killer Ig-like receptors, Ig-like transcripts, leucocyte Ig-like receptors/macrophage Ig-like receptors, leucocyte-associated Ig-like receptor-1 and natural killer p46, carcino-embryonic cell adhesion molecules and sialic acid-binding Ig-like lectins) with both IgV and Ig-C2 domains are concentrated on human chromosome 19, while those with a single IgV domain (e.g. myelin P0, programmed death-1, CMRF-35 and natural killer p44) like PZR are distributed across chromosomes 1, 2, 6 and 7 [32]. Interestingly, the PZR gene is closely linked to molecules on chromosome 1 (e.g. myelin P0) with which it shares similarity, suggesting that these molecules may have similar or related functions. It has been suggested recently that myelin P0 has a crucial role in myelin production, with mutations in the myelin P0 extracellular, transmembrane or ITIM regions leading to inherited neuropathies (e.g. Dejerine-Sottas syndrome and Charcot-Marie-Tooth disease) [33,34]. Similar mutations in PZR resulting in human disease have not been identified.

SHP-2 plays a critical role in haematopoietic and vascular development. Whether SHP-2 expresses its function in stem/progenitor cell or mesenchymal/osteoblast proliferation, survival, migration or differentiation is not fully established. The

identification of its interacting partners and their mechanisms of action is therefore of major importance in determining its function and that of the PZR isoforms. Those partner molecules identified to date include certain cytokine receptors and integrins [8,9,35]. SHP-2 physically interacts with and is tyrosine phosphorylated by the c-Kit receptor tyrosine kinase upon SCF ligand binding to haematopoietic and embryonic stem (ES) cells [36]. Treatment of ES cells with SCF induces Erk kinase activation, but this induction is completely blocked in *shp-2*^{-/-} ES cells. SHP-2 also interacts with the common gp130 subunit of the IL-6, leukaemia inhibitory factor and oncostatin M receptors [8,12]. Thus mutant ES cells lacking the SHP-2 binding site in either SHP-2 or gp130 have impaired abilities to differentiate *in vitro* [37]. It has also been shown that SHP-2 has an important role in cell motility. *shp-2* mutant cells have defects in migration, as well as spreading, and have an increased number of focal adhesions [38]. Dominant negative SHP-2 expression blocks fibroblast growth factor-induced elongation of *Xenopus* animal caps and prevents completion of gastrulation [2,39]. A positive-feedback model is also suggested, in which integrin engagement induces c-Src to catalyse the tyrosyl phosphorylation of SHP-2 substrates, thereby recruiting SHP-2 to the plasma membrane. By further activating Src protein tyrosine kinases, SHP-2 transduces positive signals for downstream events, such as MAPK activation, and this alters cell shape [2].

Whether the ITIM-containing PZR, or PZR α and PZR β (isoforms which do not contain ITIMs), have a function in modulating these cytokine and adhesive responses has been a matter of speculation. We would predict that PZR, because of its distribution pattern, acts as a docking molecule for SHP-2 and prevents, or rapidly down-modulates, cytokine receptor and integrin interactions with SHP-2, following binding of their ligands. PZR α and PZR β , on the other hand, may act as activating molecules for ITAM-containing partners. This would be reminiscent of the SIRP family of molecules, which consist of two subtypes distinguished by the presence (SIRP α) or absence (SIRP β) of a cytoplasmic ITIM domain. Overexpression of SIRP α 1 leads to the inhibition of cell activation induced by insulin, epidermal growth factor and platelet-derived growth factor [40,41]. It has also been shown that SIRP α 1 can positively regulate the MAPK signalling pathway in response to insulin and potentiate integrin-induced MAPK activation [42–44]. In contrast, SIRP β 1 physically associates with the killer cell activating receptor-associated protein (KARAP)/DAP-12 resulting in the functional coupling of SIRP β 1 to the protein tyrosine kinase Syk and to serotonin release and activation of the cells [45]. Alternatively, the PZR α and PZR β isoforms, which do not contain ITIMs, could directly interact with PZR and inhibit the ability of PZR to interact with SHP-2. In the present study, we have clearly demonstrated the importance of SHP-2 in regulating cell motility in our Boyden chamber transwell assays. In general, mutant SHP-2 (Δ 46–110) displayed a decreased capacity to migrate in comparison with the rescue SHP-2 cell lines. Interestingly, the SHP-2 rescue cells, expressing the full-length PZR isoform, exhibited a 5-fold increase in their capacity to migrate on fibronectin. This is of particular interest, since the full length PZR isoform contains tandem ITIMs that act as a docking site for SHP-2. Previous studies show that SHP-2 is involved in the deactivation of focal adhesion kinase which is required for the generation of active, focal adhesion kinase in the turnover of focal adhesions during migration [30]. SHP-2 also negatively regulates signalling pathways that promote migration by reducing integrin-mediated adhesion. Thus integrin engagement by fibronectin may lead to tyrosine phosphorylation of PZR and recruitment and activation of SHP-2, which then

induces dephosphorylation events to induce turnover of focal adhesions, reduced adhesion and increased cell motility. Our studies therefore indicate that the cytoplasmic region of PZR containing ITIMs plays an important role in regulating integrin-mediated cell motility and that truncated forms of PZR lacking ITIMs fail to regulate cell motility. Recent studies [31] also indicate that PZR is a receptor for ConA on epithelial cells and that expression of a truncated form of PZR in an epithelial cell line is able to inhibit ConA-induced tyrosine phosphorylation of PZR. Our unique WM78 mAb has the ability to bind to and tyrosine phosphorylate PZR (unpublished results), and is undoubtedly more specific for PZR than ConA. Such a mAb will be useful for studying PZR specific activation both in haematopoietic and epithelial cells, and may have therapeutic applications. A detailed analysis of the mechanisms of action of the PZR isoforms and of the WM78 mAb will therefore be the subject of further publications and experimentation.

We thank Dr Sharon Horseley for the chromosomal analysis, Guang-qian Zhou and Mrs Shira Gal for assistance with the DIG labelling and TaqMan analysis respectively, Mr Robin Roberts-Gant for help with the figures, and Professor L. B. To and Professor M. Contreras for their continued support. This study was supported by grants from the Anti-Cancer Foundation of the Universities of South Australia, the New South Wales Cancer Council and the National Health and Medical Research Council of Australia (P.J.S., A.C.W.Z. and L.J.B.) and by the National Blood Service, the Leukaemia Research Fund and Medical Research Council, U.K. (S.M.W. and M.R.).

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Received 14 June 2002/28 October 2002; accepted 1 November 2002

Published as BJ Immediate Publication 1 November 2002, DOI 10.1042/BJ20020935