

# Differential association of cytoplasmic signalling molecules SHP-1, SHP-2, SHIP and phospholipase C- $\gamma$ 1 with PECAM-1/CD31

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**Abstract** Recent studies have shown that, in addition to its role as an adhesion receptor, platelet endothelial cell adhesion molecule 1/CD31 becomes phosphorylated on tyrosine residues Y<sup>663</sup> and Y<sup>686</sup> and associates with protein tyrosine phosphatases SHP-1 and SHP-2. In this study, we screened for additional proteins which associate with phosphorylated platelet endothelial cell adhesion molecule 1, using surface plasmon resonance. We found that, besides SHP-1 and SHP-2, platelet endothelial cell adhesion molecule 1 binds the cytoplasmic signalling proteins SHIP and PLC- $\gamma$ 1 via their Src homology 2 domains. Using two phosphopeptides, NSDVQpY<sup>663</sup>TEVQV and DTETVpY<sup>686</sup>SEVRK, we demonstrate differential binding of SHP-1, SHP-2, SHIP and PLC- $\gamma$ 1. All four cytoplasmic signalling proteins directly associate with cellular platelet endothelial cell adhesion molecule 1, immunoprecipitated from pervanadate-stimulated THP-1 cells. These results suggest that overlapping immunoreceptor tyrosine-based inhibition motif/immunoreceptor tyrosine-based activation motif-like motifs within platelet endothelial cell adhesion molecule 1 mediate differential interactions between the Src homology 2 containing signalling proteins SHP-1, SHP-2, SHIP and PLC- $\gamma$ 1.

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**Key words:** Platelet endothelial cell adhesion molecule 1; Surface plasmon resonance; Protein-protein interaction; Src homology 2 domain

## 1. Introduction

Platelet endothelial cell adhesion molecule 1 (PECAM-1) (CD31) is a 130 kD glycoprotein that mediates adhesive interactions between vascular cells. While its role as a homophilic adhesion receptor is well-established, recent studies have shown that PECAM-1 engagement is able to transmit signals

into cells leading to a diverse range of functional outcomes [1]. The cytoplasmic domain of PECAM-1 is unusual for an adhesion receptor as it contains a long cytoplasmic tail (118 amino acids) derived from seven separate exons [2]. There is now increasing evidence that the phosphorylation of two tandem tyrosine residues (Y<sup>663</sup> and Y<sup>686</sup>) within the cytoplasmic domain of PECAM-1 is required for the downstream signalling events observed following PECAM-1 ligation [3–12]. Although the cytoplasmic domain of PECAM-1 contains no intrinsic kinase or phosphatase activity, members of the Src- and Csk-related protein tyrosine kinases (PTKs) are able to phosphorylate Y<sup>663</sup> and Y<sup>686</sup> [3,8,9]. When phosphorylated, these residues are responsible for the recruitment and activation of the Src homology 2 (SH2) containing protein tyrosine phosphatases SHP-1 and SHP-2 [3–7,11].

The amino acid residues surrounding PECAM-1 tyrosine residues Y<sup>663</sup> and Y<sup>686</sup> appear to conform to the consensus sequences for both immunoreceptor tyrosine-based activation motifs (ITAMs) and immunoreceptor tyrosine-based inhibition motifs (ITIMs) [4,5,9]. The demonstration that phosphopeptides containing tyrosine residues pY<sup>663</sup> and pY<sup>686</sup> can associate with and activate SHP-1 and SHP-2, suggests that these ITIM-like sequences are functionally important. On the other hand, the engagement of PECAM-1 on T lymphocytes results in proliferation, secretion of several chemokines/cytokines and up-regulation of CD25 in response to sub-optimal concentrations of anti-CD3 antibodies: all characteristic hallmarks of co-stimulatory, ITAM containing receptors [13]. The co-precipitation of several phosphorylated proteins that associate with PECAM-1 suggests that PECAM-1 is likely to regulate intracellular signalling as part of a complex of signalling proteins [4,9].

In this study, we have used surface plasmon resonance to screen a panel of SH2 domains from cytoplasmic signalling proteins, known to become tyrosine-phosphorylated upon cell activation, for their ability to bind a glutathione-S-transferase (GST)-PECAM-1 cytoplasmic domain fusion protein. In addition to SHP-1 and SHP-2, we have found that SHIP and PLC- $\gamma$ 1 can interact specifically with the phosphorylated cytoplasmic PECAM-1 domain. Mapping studies have shown that the SH2 domains of SHP-1, SHP-2, SHIP and PLC- $\gamma$ 1 interact differentially with pY<sup>663</sup> and pY<sup>686</sup> in PECAM-1. Our results suggest that the cytoplasmic domain of PECAM-1 contains overlapping ITIM/ITAM-like motifs which differentially associate with the SH2 containing signalling proteins SHP-1, SHP-2, SHIP and PLC- $\gamma$ 1.

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**Abbreviations:** SH2, Src homology 2; GST, glutathione-S-transferase; ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibition motif; PTK, protein tyrosine kinase; PECAM-1, platelet endothelial cell adhesion molecule 1; N, NH<sub>2</sub>-terminal; C, COOH-terminal; NC, NH<sub>2</sub>- and COOH-terminal; pY, phosphotyrosine; PDGF, platelet derived growth factor

## 2. Materials and methods

### 2.1. Peptides and antibodies

All reagents were purchased from Sigma unless otherwise stated. Anti-phosphotyrosine antibody 4G10 was purchased from Upstate Biotechnology. Biotinylated peptides (NSDVQpY<sup>663</sup>TEVQV and DTETVpY<sup>686</sup>SEVRK) were purchased from Alta Bioscience (Birmingham UK), were HPLC-purified and judged >90% pure by mass spectroscopy.

### 2.2. GST fusion proteins

GST fusion proteins of PLC- $\gamma$ 1, Shc, PI-3 kinase (p85), SHIP and Grb2 were purchased from Santa Cruz (CA, USA). Individual SH2 domains from SHP-1 and SHP-2 and mutant PECAM-1 cytoplasmic domains were produced and purified using Bulk GST purification modules (Pharmacia Biotech, UK) according to the manufacturer's instructions. The fusion proteins used in this study are shown in Fig. 1.

### 2.3. PECAM-1, SHP-1 and SHP-2 GST constructs

The cDNAs for PECAM-1 Y663F, Y686F and Y663/686F constructs in pcDNA3 were kindly provided by Dr. Peter Newman (Blood Research Institute, Milwaukee, WI, USA). Amplified products were cloned into pGEX-4T (Amersham-Pharmacia) using *Bam*HI and *Sal*I sites. The SH2 domains of SHP-1 and SHP-2 were generated by PCR amplification from SHP-1 and SHP-2 cDNA (kindly provided by Dr. Benjamin Neel, Beth Israel Hospital, Boston, MA, USA). Amplified products were cloned into pGEX-4T (Amersham-Pharmacia). All cDNAs were sequenced to exclude PCR-induced errors. Primer sequences are available on request.

### 2.4. In vitro c-Src kinase phosphorylation of PECAM-1 GST proteins

GST-PECAM-1 fusion proteins or GST alone (Santa Cruz) were phosphorylated in vitro by incubating 100  $\mu$ g samples with 18.5 U of c-Src kinase (Upstate biotechnology) in 500  $\mu$ l kinase buffer (50 mM HEPES pH 7.4, 50 mM NaCl, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, 5 mM MgCl<sub>2</sub>, 5 mM MnCl<sub>2</sub> and 5 mM ATP) for 2 h at 20°C. To assess GST fusion proteins in the absence of phosphorylation, ATP and divalent cations were replaced with 10 mM EDTA.

### 2.5. Surface plasmon resonance

Measurements were carried out on a BIAcore 2000 Biosensor (BIAcore AB, Uppsala, Sweden). GST fusion proteins ( $\approx$ 1000 response units (RU)) were immobilised directly onto a CM5 BIAcore sensor chip using the recommended protocol. Biotinylated PECAM-1 phosphopeptides ( $\approx$ 200 RU) were immobilised to streptavidin-coated BIAcore SA biosensor chips using the recommended protocol. In both cases, the presence of accessible phosphotyrosine residues was confirmed using the anti-phosphotyrosine monoclonal antibody 4G10 (data not shown). Data were acquired using the Kinject function of the BIAcore, each injection lasting 120 s with a dissociation period of 200 s and a flow rate of 10  $\mu$ l/min. The response (RU) relative to the pre-injection of analyte was measured 60 s after the end of the injection. Binding to unphosphorylated GST-PECAM-1 was subtracted from phosphorylated GST-PECAM-1 and unphosphorylated GST from phosphorylated GST and in all cases was less than 50 RU.

### 2.6. Immunoprecipitation analysis

Control and stimulated THP-1 cells (typically 10<sup>7</sup>) were lysed in 20mM Tris-HCl, pH8, 150 mM NaCl, 1% Triton X-100, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM AEBSF and 10 mg/ml leupeptin for 20 min at 4°C. Pre-cleared cell lysates were incubated with 2 $\mu$ g anti-PECAM-1 antibody (9G11) or mouse IgG1 control for 1 h and then immunoprecipitated with protein G-sepharose overnight. After three washes with ice-cold lysis buffer, bound proteins were eluted from the sepharose beads by boiling in SDS reducing buffer, resolved on 10% SDS-PAGE, transferred to Hybond-P membranes and probed with the relevant antibodies or GST-SH2 domains. Detection was done with HRP-conjugated secondary antibodies using chemiluminescence. Far Western blots were probed with 2  $\mu$ g of GST fusion protein for 1 h at room temperature. Bound GST proteins were detected using a goat anti-GST polyclonal antibody followed by HRP-conjugated anti-goat antibody.

## 3. Results

### 3.1. PECAM-1 interacts with the cytoplasmic signalling proteins SHP-1, SHP-2, PLC- $\gamma$ 1 and SHIP via their SH2 domains

Previous studies have suggested that in addition to SHP-1 and SHP-2, PECAM-1 can potentially associate with at least three other phosphotyrosine containing proteins [4,8,9]. We therefore screened a panel of SH2 containing signalling proteins for their ability to interact with GST-PECAM-1 in a phosphotyrosine-dependent manner. To validate our screening protocol and confirm that the immobilisation of phosphorylated GST-PECAM-1 fusion protein to the BIAcore sensor chip did not affect the functional ability of PECAM-1 to interact with SH2 domains, we examined the ability of two SH2 containing proteins, SHP-1 and SHP-2, known to interact with PECAM-1. These positive controls bound well to PECAM-1, as did two other signalling proteins, PLC- $\gamma$ 1 and SHIP (Fig. 2). These interactions were specific, titratable over the range 25–400 nM and relied entirely on pY<sup>663</sup> and pY<sup>686</sup> within the cytoplasmic domain of PECAM-1, since there was no significant binding to unphosphorylated PECAM-1 or a Y663/686F mutant (data not shown). There was no binding of p85 PI-3 kinase, Grb2, Shc or GST to PECAM-1 at any of the concentrations used.

We next examined the relative contribution of individual NH<sub>2</sub>- and COOH-terminal (NC) SH domains within SHP-1, SHP-2 and PLC- $\gamma$ 1 to the interaction with PECAM-1 (Fig. 3). As expected, SH3-PLC- $\gamma$ 1 was unable to bind the phosphorylated PECAM-1 cytoplasmic domain. NC-PLC- $\gamma$ 1 fusion protein, containing the two SH2 domains in tandem, bound GST-PECAM-1 as effectively as the NC-SH3-PLC- $\gamma$ 1 fusion protein, confirming that the SH3 domain plays no part in the interaction of PLC- $\gamma$ 1 with PECAM-1. Unlike PLC- $\gamma$ 1 in which both the NH<sub>2</sub> (N) and COOH-terminal (C) SH2 domains were able to bind PECAM-1, both SH2 domains were required for significant binding of SHP-1 to PECAM-1. Only the N-SH2 domain of SHP-2 was able to interact with PECAM-1. In all three cases, the presence of both SH2 domains in tandem led to a significantly enhanced binding compared to either SH2 domain on their own (Fig. 3).

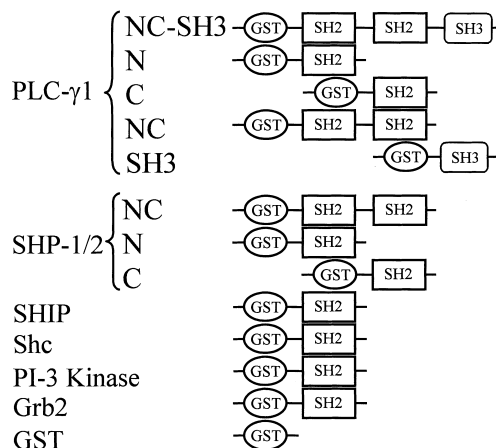


Fig. 1. GST fusion proteins used in this study. Listed are the various GST fusion proteins used in this study. Proteins start with the N-SH2 domains at the left.

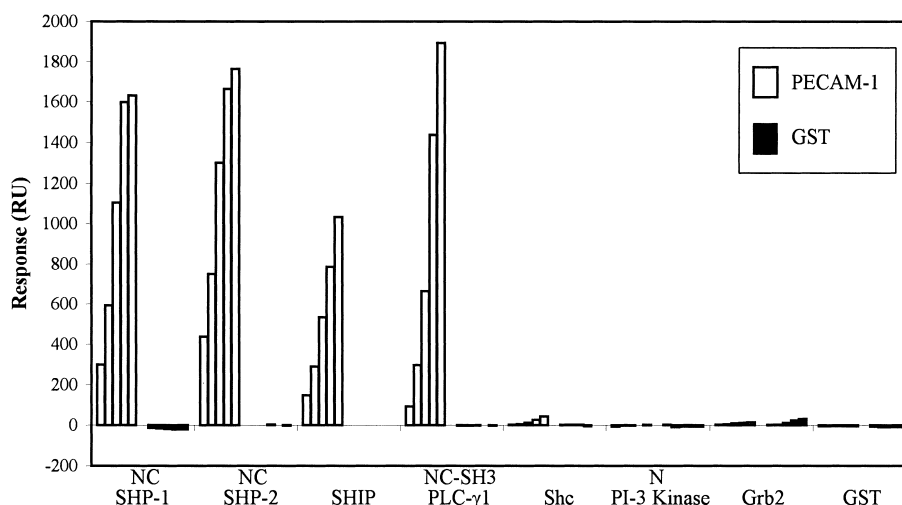


Fig. 2. SH2 domains of SHP-1, SHP-2, SHIP and PLC- $\gamma$ 1 interact specifically with GST-PECAM-1 in a phosphotyrosine and dose-dependent manner. BIAcore sensor chip was immobilised with in vitro kinase-treated GST-PECAM-1 or GST before varying concentrations (25, 50, 100, 200, 400 nM) of GST-SH2 domains from NC-SHP-1, NC-SHP-2, SHIP, NC-SH3-PLC- $\gamma$ 1, Shc, Grb2, N-PI-3 kinase p85, GST were flowed over the chip. Sensorgrams were analysed as described in section 2. Binding to non-phosphorylated PECAM-1 and GST were subtracted from the data and in all cases was less than 50 RU. Binding to in vitro kinase-treated GST was included in each case to exclude any potential interaction between the GST fusion tags. The presence of accessible phosphorylated GST-PECAM-1 was confirmed using 4G10 monoclonal antibody binding.

### 3.2. Differential interaction of the SH2 domains of SHP-1, SHP-2, SHIP and PLC- $\gamma$ 1 with either PECAM-1 pY<sup>663</sup> or pY<sup>686</sup>

In order to determine which of the two phosphotyrosine motifs the individual SH2 domains were binding, we used a pair of biotinylated 11 amino acid phosphopeptides, NSDVQpY<sup>663</sup>TEVQV and DTETVpY<sup>686</sup>SEVRK. We were unable to use GST-PECAM-1 proteins containing mutations at Y<sup>663</sup> and Y<sup>686</sup> as these proteins were not fully phosphorylated by c-Src (data not shown). While NC-SHP-2 interacted predominantly with the pY<sup>663</sup> containing phosphopeptide,

SHIP interacted predominantly with the pY<sup>686</sup> containing peptide. NC-SHP-1 was unable to bind either phosphopeptide (Fig. 4A). In contrast, NC-PLC- $\gamma$ 1 bound both phosphopeptides although the interaction was greater with pY<sup>663</sup> than pY<sup>686</sup> (Fig. 4B). These results suggest that the SH2 domains of these four signalling proteins interact differentially with the two PECAM-1 tyrosine motifs. They also confirm that both phosphopeptides are functionally active when immobilised to the BIAcore chip.

Since SHIP contains a single SH2 domain, we were able to directly map the dominant binding site on PECAM-1 to pY<sup>686</sup>

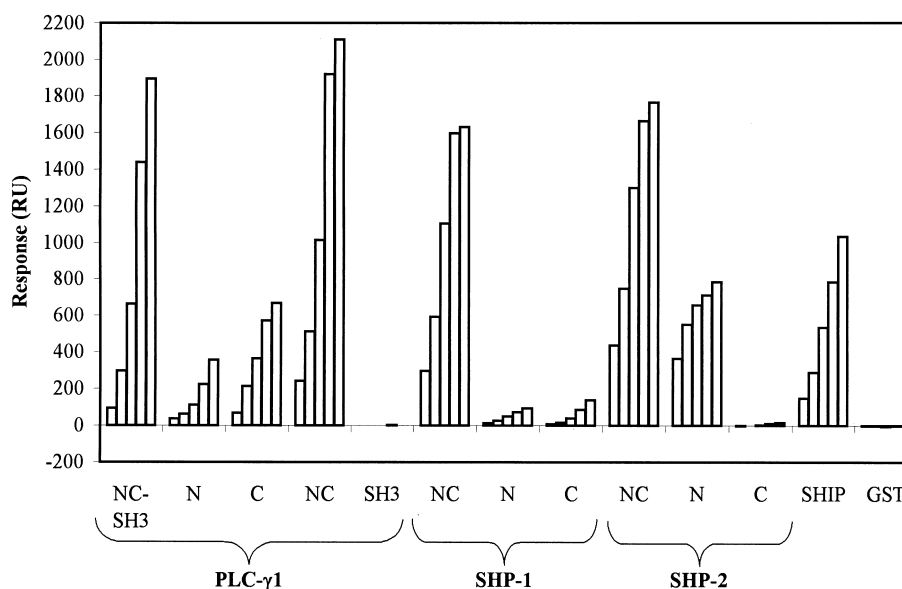


Fig. 3. Interaction of individual NC-SH2 domains from SHP-1, SHP-2 and PLC- $\gamma$ 1 with GST-PECAM-1. BIAcore sensor chip was immobilised with in vitro kinase-treated GST-PECAM-1 or GST before varying concentrations (25, 50, 100, 200, 400 nM) of individual N-SH2, C-SH2 terminal or NC-SH2 domains of SHP-1 and SHP-2 were flowed over the chip. GST is included as a control. For PLC- $\gamma$ 1 binding, individual N-SH2, C-SH2, NC-SH2, NC-SH3 and SH3 domains were used. The presence of accessible phosphorylated GST-PECAM-1 was confirmed using 4G10 monoclonal antibody binding.

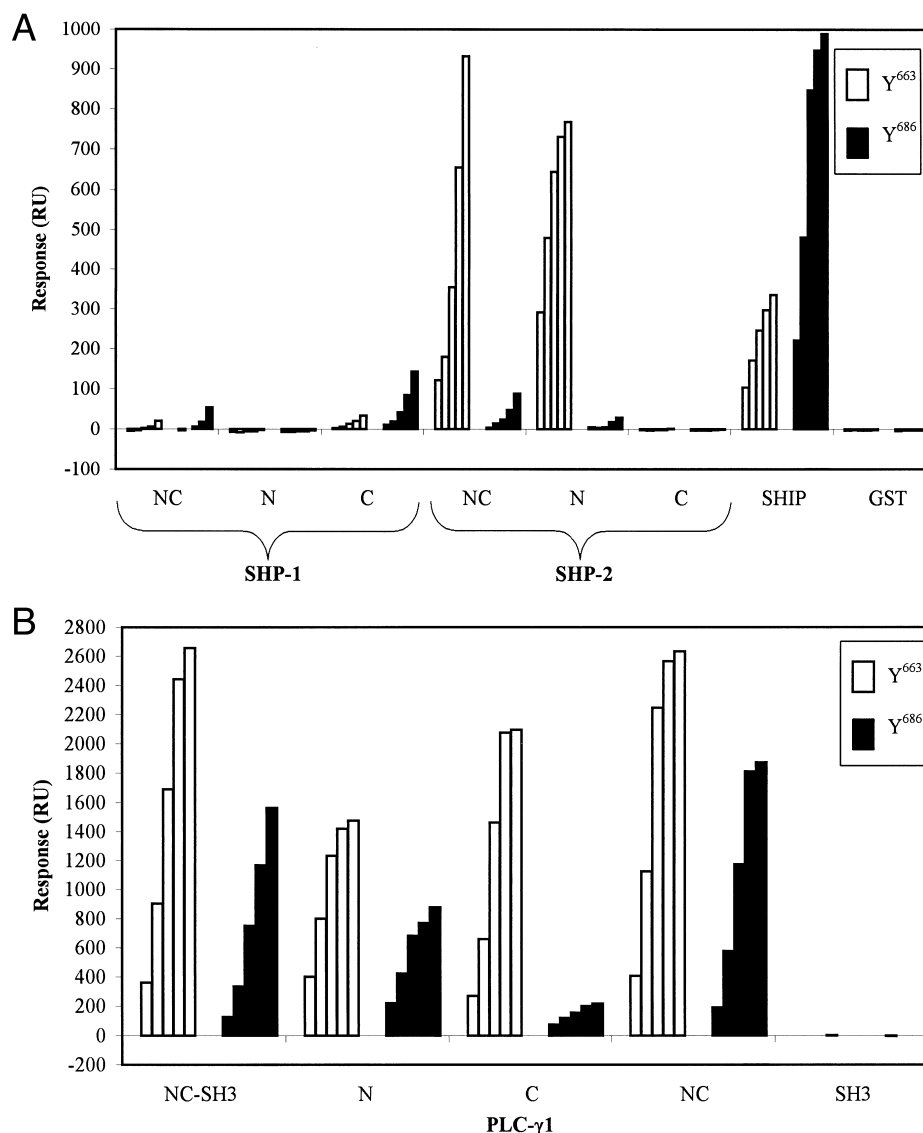


Fig. 4. Interaction of PECAM-1 phosphopeptides with individual SH2 domains from SHP-1, SHP-2 and SHIP and PLC- $\gamma$ 1. (A) Individual N-SH2, C-SH2 terminal or NC-SH2 domains of SHP-1 and SHP-2 and the single SH2 domain of SHIP were allowed to interact at concentrations of 25, 50, 100, 200, 400 nM with the immobilised biotinylated PECAM-1 phosphopeptides NSDVQpY<sup>663</sup>TEVQV and DTETVpY<sup>686</sup>SEVRK. (B) Individual N-SH2, C-SH2, NC-SH2, NC-SH3 and SH3 domains from PLC- $\gamma$ 1 were allowed to interact at concentrations of 25, 50, 100, 200, 400 nM with the immobilised biotinylated PECAM-1 phosphopeptides NSDVQpY<sup>663</sup>TEVQV and DTETVpY<sup>686</sup>SEVRK. In all cases, the presence of accessible phosphorylated peptides was confirmed using 4G10 monoclonal antibody binding. There was no binding to biotinylated non-phosphorylated peptides (data not shown).

(Fig. 4A). SHP-1, SHP-2 and PLC- $\gamma$ 1 all contain a pair of tandem SH2 domains, so to map their relative binding to PECAM-1, we examined the association of individual NC-SH2 domains to the biotinylated phosphopeptides (Fig. 4). N-SHP-2 interacted efficiently with pY<sup>663</sup> containing phosphopeptide whereas C-SHP-2 was unable to bind either phosphopeptide. There was very little interaction between either of the individual SH2 domains of SHP-1 and the PECAM-1 phosphopeptides, suggesting that both SH2 domains of SHP-1 and both tyrosine residues in PECAM-1 are required for efficient SHP-1 binding (Fig. 4A). N-PLC- $\gamma$ 1 bound either phosphopeptide, with a slight preference for Y<sup>663</sup>, whereas C-PLC- $\gamma$ 1 could only bind Y<sup>663</sup> but not Y<sup>686</sup> (Fig. 4B). A model of how these SH2 domains interact with PECAM-1, together with their preferred ligand binding consensus sequences, where known, is shown in Fig. 5.

### 3.3. Direct interaction between tyrosine-phosphorylated cellular PECAM-1 and SHP-1, SHP-2, SHIP and PLC- $\gamma$ 1

To address whether the SH2 domains of SHP-1, SHP-2, SHIP and PLC- $\gamma$ 1 directly associate with PECAM-1 expressed in a cellular context, PECAM-1 was immunoprecipitated from pervanadate-treated THP-1 cells (which express PECAM-1 in a basal unphosphorylated state). Far Western blots using NC-SHP-1, NC-SHP-2, SHIP and NC-PLC- $\gamma$ 1 GST fusion proteins demonstrated that these proteins bound directly to tyrosine-phosphorylated PECAM-1 (Fig. 6). PLC- $\gamma$ 1 also associated with an approximately 80–85 kDa phosphotyrosine containing protein, that co-immunoprecipitated with PECAM-1 in pervanadate-treated cells. The identity of this protein remains to be elucidated. Together, these data provide evidence that the direct association of SHP-1, SHP-2, SHIP and PLC- $\gamma$ 1 with PECAM-1 is not merely an in

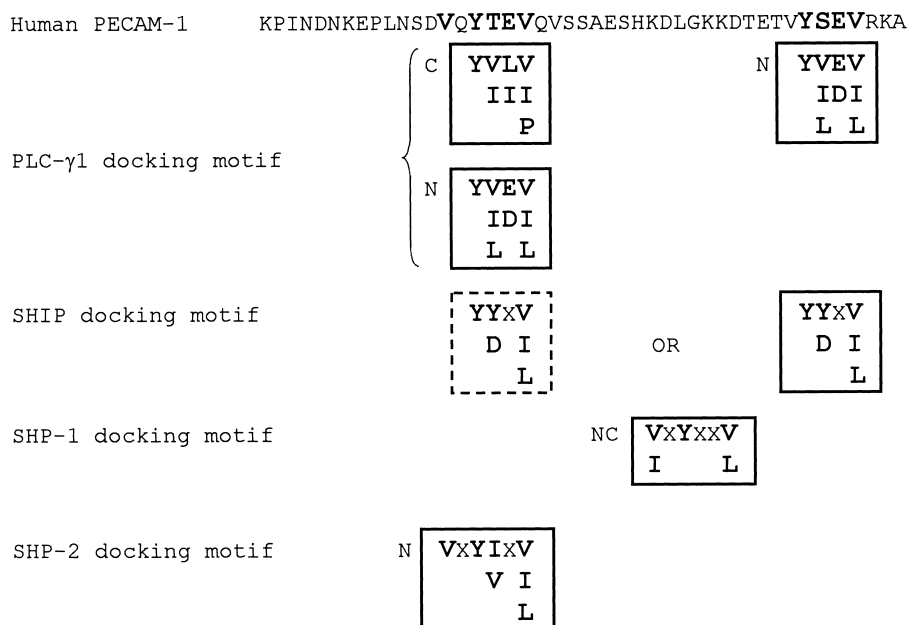


Fig. 5. Comparison of ligand binding motifs within PECAM-1 Y<sup>663</sup> and Y<sup>686</sup> with the predicted consensus sequences for SHP-1, SHP-2 SHIP and PLC- $\gamma$ 1 SH2 domains. Amino acids within the PECAM-1 cytoplasmic domain matching any of the consensus sequences are shown in bold. Individual SH2 domains which interact with PECAM-1 Y<sup>663</sup>TEV or Y<sup>686</sup>SEV are shown boxed. Weak interaction is shown as a hatched box and in the case of SHP-1 where both NC-SH2 domains appear to be required for binding PECAM-1, the interacting box is shown between the two tyrosine residues. The predicted ligand binding motifs for the individual SH2 domains, where known, are shown within the boxes.

vitro phenomenon, but occurs in the context of cellular activated PECAM-1.

#### 4. Discussion

The association of PECAM-1 with SHP-1 and SHP-2 has been previously described and shown to be mediated by the SH2 domains of these two phosphatases [3–7]. We have extended these observations to map the dominant PECAM-1 binding sites for SHP-1 and SHP-2. Both SH2 domains of SHP-1 are required in tandem to bind PECAM-1. Individual phosphopeptides spanning Y<sup>663</sup> or Y<sup>686</sup> in PECAM-1 were unable to support significant binding of SHP-1, suggesting that in addition to tandem SH2 domains, tandem pY<sup>663</sup> and pY<sup>686</sup> residues are also required for optimal SHP-1/PECAM-1 association. A very similar finding has been observed for the

interaction of SHP-1 with CD22 where, in addition to tandem SH2 domains, a minimum of two tyrosine residues in CD22 are required for the association with SHP-1 [14]. In contrast, SHP-2 binds PECAM-1 using predominantly the N-SH2 domain, with the major binding site on PECAM-1 residing within the Y<sup>663</sup> motif, in agreement with other studies [4].

Like PECAM-1, the platelet derived growth factor (PDGF) $\beta$  receptor binds SHP-2 at residues surrounding Y<sup>1009</sup> [15] and shares a significant homology with the phosphotyrosine motif surrounding Y<sup>663</sup> in PECAM-1 (VLY<sup>1009</sup>TAVQ for the PDGF $\beta$  receptor and VQY<sup>663</sup>TEVQ for PECAM-1). The PDGF $\beta$  receptor also associates with PLC- $\gamma$ 1 via tandem tyrosine residues Y<sup>1009</sup> and Y<sup>1021</sup> [16]. Although the binding site for the C-SH2 domain of PLC- $\gamma$ 1 appears to reside predominantly within Y<sup>1021</sup>, Y<sup>1009</sup> also contributes to the activation of PLC- $\gamma$ 1 via the NH-SH2 domain of PLC- $\gamma$ 1, binding the consensus motif Y(L/I/V)(E/D)(L/I/V) [17,18]. Both VQY<sup>663</sup>TEV and TVY<sup>686</sup>SEV in PECAM-1 closely match this consensus sequence, supporting our findings that N-PLC- $\gamma$ 1 can bind both pY<sup>663</sup> and pY<sup>686</sup> containing PECAM-1 peptides equally well (Fig. 4B).

Unlike its N-SH2 domain, the C-SH2 domain of PLC- $\gamma$ 1 can only bind the VQY<sup>663</sup>TEV motif. The consensus sequence for C-PLC- $\gamma$ 1 binding is Y(V/I)(I/L)(P/V/I) which only weakly conforms to the PECAM-1 sequence VQY<sup>663</sup>TEV. However, there appears to be a conflict between the experimentally determined structural requirements for PLC- $\gamma$ 1 binding, using a mutated phosphopeptide binding analysis [17,19] and functional studies in whole cells. Here, additional residues other than the three immediately following the phosphotyrosine residues contribute to the association of PLC- $\gamma$ 1 with the PDGF receptor [20,21].

Our finding that PLC- $\gamma$ 1 can associate with the cytoplasmic tail of PECAM-1 is of interest given the recent findings of

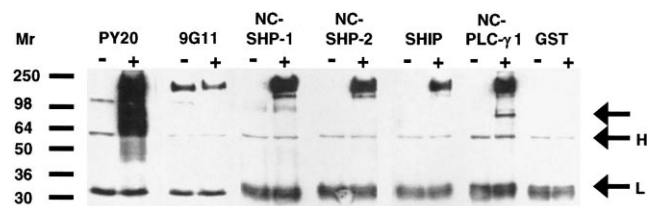


Fig. 6. Direct interaction of tyrosine-phosphorylated cellular PECAM-1 with SH2 domains from SHP-1, SHP-2 SHIP and PLC- $\gamma$ 1. THP-1 cells either stimulated (+) or not (–) with pervanadate were lysed and immunoprecipitated with anti-PECAM-1 antibody, before SDS-PAGE analysis and blotting with either anti-phosphotyrosine antibody, PY20, anti-PECAM-1 antibody 9G11 or GST fusion proteins of NC-SHP-1, NC-SHP-2, SHIP and NC-SH3-PLC- $\gamma$ 1. The positions of the Ig heavy (H) and light chain (L) are indicated as well as a 80–85 kD protein, which associates with PLC- $\gamma$ 1 from stimulated PECAM-1 immunoprecipitates. There was no binding of GST protein.



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