Thrombin-induced association of SHP-2 with multiple tyrosine-phosphorylated proteins in human platelets

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Abstract SH2 domain containing phosphatase-2 (SHP-2) has an important regulatory role in a variety of cell types. However, little is known concerning its function in platelets. We show here that, in thrombin-stimulated human platelets, SHP-2 undergoes a time-dependent association with platelet endothelial cell adhesion molecule-1 (PECAM-1) and four low molecular weight phosphoproteins which are attenuated by the Src kinase inhibitor PP1. The low molecular weight proteins, which may be transmembrane proteins, are shown to bind exclusively to the N-terminal SH2 domain of SHP-2 and are therefore possible activators of the phosphatase. In addition, SHP-2 phosphatase activity is shown to be increased following thrombin stimulation or cross-linking of PECAM.

Key words: SH2 domain containing phosphatase-2; Platelet endothelial cell adhesion molecule-1; Platelet; Tyrosine phosphatase

1. Introduction

SH2 domain containing phosphatase-2 (SHP-2) (also known as SH-PTP2, PTP1D, Syp or PTP2C) is a ubiquitously expressed 68 kDa non-receptor protein tyrosine phosphatase comprising two N-terminal tandem SH2 domains linked to a C-terminal catalytic phosphatase domain with a C-terminal tail [1,2]. It belongs to a family of SH2 domain containing protein tyrosine phosphatases which includes SHP-1 and the *Drosophila* homologue of SHP-2, Corkscrew [3,4]. However, unlike SHP-1, which is largely restricted to haematopoietic cells and appears to have a defined negative role in cellular signalling [5,6], the expression of SHP-2 is ubiquitous and its function remains unclear. Attempts to produce SHP-2 knockout mice strains resulted in day 9.5 embryonic lethality due to severe suppression of haematopoietic cell development [7,8]. In vitro studies using both overexpression and dominant negative techniques, however, have indicated both positive and negative regulatory roles for SHP-2. In support of a positive signalling role, SHP-2 has been shown to associate with growth factor receptors [9], cytokine receptors [10] and adhesion molecules [11], ligation of which can lead to MAP kinase activation, cellular growth and differentiation [12,13]. A negative regulatory role for SHP-2 was proposed following the observation by Myers that insulin receptor signalling was enhanced following inhibition of SHP-2 association with the insulin receptor substrate, implying that SHP-2 normally attenuates this pathway [14]. In addition, SHP-2 has recently been shown to attenuate T-cell activation by dephosphorylating the ζ-chain of the T-cell receptor [15]. Thus, it appears that the role of SHP-2 may alter depending upon the cell type and the nature of the associating proteins.

Recent structural analysis of SHP-2 [16] showed it to be activated by binding of tyrosine-phosphorylated proteins to the N-terminal SH2 domain, causing exposure of the active catalytic phosphatase site. It was proposed that the C-terminal SH2 domain plays a role to stabilise associations made by the N-terminal SH2 domain, since the affinity of interaction between two SH2 domains and a binding partner has been shown to be much greater than the sum of the affinities of the individual SH2 domains for their binding partners [17]. Identification of tyrosine phosphoproteins which bind SHP-2 is therefore important since they may represent activators or substrates of the phosphatase. Recent studies have shown that a number of adaptor proteins associate with SHP-2 after receptor activation. These include Grb-2 [18–20], SHPS-1 [21], SIT [22], PZR [23] and the FcεR β-chain [24]. Whilst these adaptor proteins have no enzymatic function, they are believed to link surface receptors to intracellular signalling pathways. Thus, the association of SHP-2 with the β-subunit of the interleukin-3 receptor [10] and the insulin receptor [25] may involve the formation of a protein complex with several tyrosine-phosphorylated adaptor proteins. However, whether association with these adaptor proteins can activate SHP-2 has not yet been demonstrated.

The only SHP-2 binding protein reported to date in human platelets is platelet endothelial cell adhesion molecule-1 (PECAM-1) [26], a 130 kDa glycoprotein expressed on the surface of platelets and endothelial cells where it localises to cell-cell borders and mediates leukocyte transmigration and platelet adhesion [27]. PECAM-1 is a member of the immunoglobulin (Ig) superfamily comprising six extracellular Ig like domains, a single transmembrane region and a long cytoplasmic tail [28]. Studies in platelets have shown that following aggregation, two of the five tyrosine residues in the cytoplasmic tail of PECAM-1 become phosphorylated, facilitating an association between PECAM-1 and the SH2 domains of SHP-2 [29]. It has not been shown, however, whether this association causes the activation of SHP-2 or the triggering of a signal transduction pathway leading to a functional response.

To further understand the role of SHP-2 in platelets, we have sought to identify and characterise tyrosine-phosphorylated proteins which associate with SHP-2 under a variety of activating conditions. Using thrombin-stimulated human platelets, we have identified a novel set of tyrosine-phosphorylated

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Abbreviations: SHP-2, SH2 domain containing phosphatase-2; PECAM-1, platelet endothelial cell adhesion molecule-1; WGA, wheat germ agglutinin

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SHP-2 associating proteins, at least two of which bind the N-terminal SH2 domain of SHP-2 and can therefore be considered as putative activators of SHP-2. In addition, we have shown that both thrombin and PECAM-1 can stimulate SHP-2 phosphatase activity in the absence of SHP-2 phosphorylation.

2. Materials and methods

2.1. Materials

Antibodies were purchased from Santa Cruz (Autogen Bioclear, Devizes, Wiltshire, UK) with the exception of anti-phosphotyrosine monoclonal antibody 4G10 which was obtained from Upstate Biotechnology (TCS Biologicals, Bucks, UK). AB468, a mouse monoclonal anti-PECAM-1 antibody directed against the extracellular portion of the molecule, was used for cross-linking and immunoprecipitation of PECAM whilst a goat polyclonal anti-PECAM antibody (sc1505), directed against the cytoplasmic tail, was used for blotting. The full length SHP-2 and GST-N+C-SH2 constructs were kindly donated by Dr. Steven Shoelson (Harvard Med. School, Boston, USA). The Src family kinase inhibitor PP1 was from Alexis Corporation (Nottingham, UK). The in vitro tyrosine phosphatase assay kit was from Promega (Southampton, UK). Acrylamide/bisacrylamide solution was from National Diagnostics (Hull, UK). All other reagents were purchased from Sigma (Poole, Dorset, UK).

2.2. Preparation of human platelets

Citrated venous blood samples were collected from healthy volunteers not currently taking medication. Platelet-rich plasma was separated by centrifugation ($180\times g$, 20 min) and PGE_1 (40 ng/ml) was added to prevent activation of the platelets during preparation. A platelet pellet was obtained from a second centrifugation ($800\times g$, 10 min) and resuspended in modified tyrodes-HEPES solution (145 mM NaCl, 2.9 mM KCl, 10 mM HEPES, 1 mM $MgCl_2$, 5 mM glucose, pH 7.3) to a density of 1×10^9 platelets/ml with indomethacin (10 μM) to block eicosanoid production. Platelets were rested for 30 min at 30°C prior to use.

2.3. Construction of GST fusion proteins

PCR primers containing *Eco*RI and *Not*I restriction sites were designed and used to amplify the single SH2 domains from a full length SHP-2 template. Following purification and digestion, the SH2 fragments were re-ligated into pGEX-4T-2 and transformed into *Escherichia coli* bacteria. All PCR product sequences were checked before use and primer sequences are available on request. The GST fusion proteins were induced with IPTG and bound to glutathione beads for use in immunoprecipitation assays. For dephosphorylation assays, the purified fusion protein was eluted from the beads with excess glutathione (5 mM).

2.4. Platelet activation, immunoprecipitation and Western blotting

350 μl aliquots of PRP were placed in an aggregometer tube and stirred (800 rpm) at 37°C. Appropriate agonists or inhibitors were added at the concentrations and for the duration stated in Section 3. Stimulation was always carried out in the presence of EGTA (1 mM) and was terminated by the addition of an equal volume of 2×NP-40 lysis buffer (2% (v/v) NP-40, 300 mM NaCl, 20 mM Tris, 1 mM PMSF, 10 mM EDTA, 2 mM Na_3VO_4, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μg/ml pepstatin A, pH 7.3). The tubes were vortexed to ensure complete solubilisation of the platelet proteins prior to centrifugation to pellet insoluble material. For immunoprecipitations, the supernatant was pre-cleared with protein A-Sepharose for 1 h at 4°C prior to the addition of primary antibody. Immunoprecipitations and GST fusion protein precipitations were carried out for 2 h at 4°C after which time the beads were pelleted by centrifugation, washed three times in TBS-T buffer and resuspended in an equal volume of 2×Laemmli sample buffer. Samples were separated on 5–15% polyacrylamide slab gels under reducing conditions, transferred to PVDF membranes and blotted with appropriate antibodies. Membranes were incubated for 60 min at room temperature with primary antibodies, followed by secondary antibodies and bands were detected using ECL reagent (Amersham) and exposure of the blot to photographic film.

2.5. Digestion of transmembrane proteins

Following thrombin stimulation, platelets were incubated at 37°C for 10 min in the presence or absence of trypsin (0.6 mg/ml) and chymotrypsin (25 μg/ml). The digestion was terminated by the addition of PMSF (2.5 mM) prior to lysis of the platelets.

2.6. In vitro phosphatase assay

SHP-2 immunoprecipitates were prepared from resting and stimulated platelets, washed three times in TBS-T and once in phosphate-free water before resuspension in assay buffer (25 mM Na-acetate pH 5, 20% glycerol, 1 mM DTT, 1 mM EDTA). The samples were then added to a 96 well plate containing tyrosine-phosphorylated peptide substrate. Following a 30 min incubation at 30°C, the reaction was terminated by the addition of molybdenate dye solution. Phosphatase activity was assessed by a spectrophotometric assay of released free phosphate.

2.7. Dephosphorylation assays

The ability of SHP-2 to specifically dephosphorylate endogenous substrates was tested by incubating SHP-2 immunoprecipitates in dephosphorylation buffer alone (150 mM NaCl, 50 mM Tris pH 7.4, 5 mM DTT) or in the presence of 10 μg of purified GST-SHP-2 proteins at 37°C for the specified time. To control for dissociation, the assay was repeated in the presence of vanadate to inhibit phosphatase activity. Samples were then pelleted, resuspended in sample buffer and analysed by gel electrophoresis.

3. Results

3.1. SHP-2 associates with tyrosine-phosphorylated proteins in thrombin-activated platelets

To identify possible tyrosine-phosphorylated proteins which associate with SHP-2, we immunoprecipitated SHP-2 from resting platelets and from platelets stimulated with thrombin (1 U/ml) for various time periods (Fig. 1). In resting platelets, a low level of constitutive association of three tyrosine-phosphorylated proteins of approximately 127, 33 and 29 kDa with SHP-2 was observed. Following thrombin stimulation for 15 s, however, a significant increase in the association of all three proteins was demonstrated. In addition, a further two

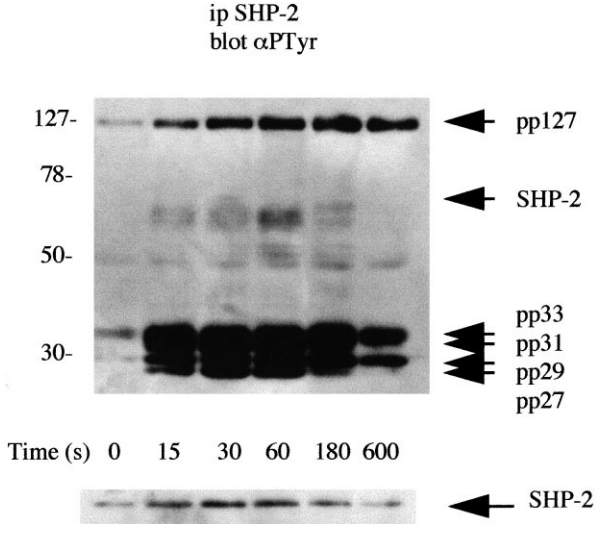

Fig. 1. SHP-2 associates with several tyrosine-phosphorylated proteins in human platelets. SHP-2 immunoprecipitations were carried out on platelets stimulated with thrombin for the time shown before sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis and immunoblotting with anti-phosphotyrosine antibody, 4G10. The positions of PECAM-1, SHP-2 and the four low molecular weight associating proteins are shown.

tyrosine-phosphorylated proteins were seen at 31 and 27 kDa. Under these conditions, SHP-2 itself does not become tyrosine-phosphorylated. The association of all five proteins was maximal by 60 s with a decline in the intensity of the lower bands after this timepoint. By 10 min, the 31 and 27 kDa bands were no longer detectable and by 30 min, all associations had returned to basal levels (data not shown).

3.2. Tyrosine-phosphorylated PECAM-1 associates with SHP-2

In accordance with previous reports, PECAM-1 was detected in SHP-2 immunoprecipitates from platelets stimulated with thrombin (1 U/ml, 1 min) (Fig. 2A) and coincided with the tyrosine-phosphorylated 127 kDa band seen on 4G10 blots. However, the amount of PECAM detected in SHP-2 immunoprecipitations did not correlate with the amount of tyrosine-phosphorylated protein observed on 4G10 blots. To confirm whether PECAM was the sole constituent of pp127, platelets were incubated with trypsin and chymotrypsin following thrombin stimulation to digest extracellular proteins. Under these conditions, PECAM-1 was almost completely digested and most prominently to a protein of 90 kDa. Comparison of the digestion pattern of PECAM in whole cell lysates (Fig. 2B (i)) and pp127 in SHP-2 immunoprecipitates (Fig. 2B (ii) overexposed so as to show all digestion products) showed that whilst a small component of pp127 digested similarly to PECAM, the appearance of a digestion product at 65 kDa suggested that SHP-2 also associates with another protein or proteins in thrombin-stimulated platelets which co-migrate with PECAM-1 at 127 kDa. In addition, there appeared to be a decrease in the intensity of the low molecular weight associating proteins following trypsin digestion, indicating that they also may be transmembrane proteins. This was particularly marked for pp33 and pp31, although the effect on pp29 and pp27 was difficult to assess due to possible overlap with a PECAM digestion fragment.

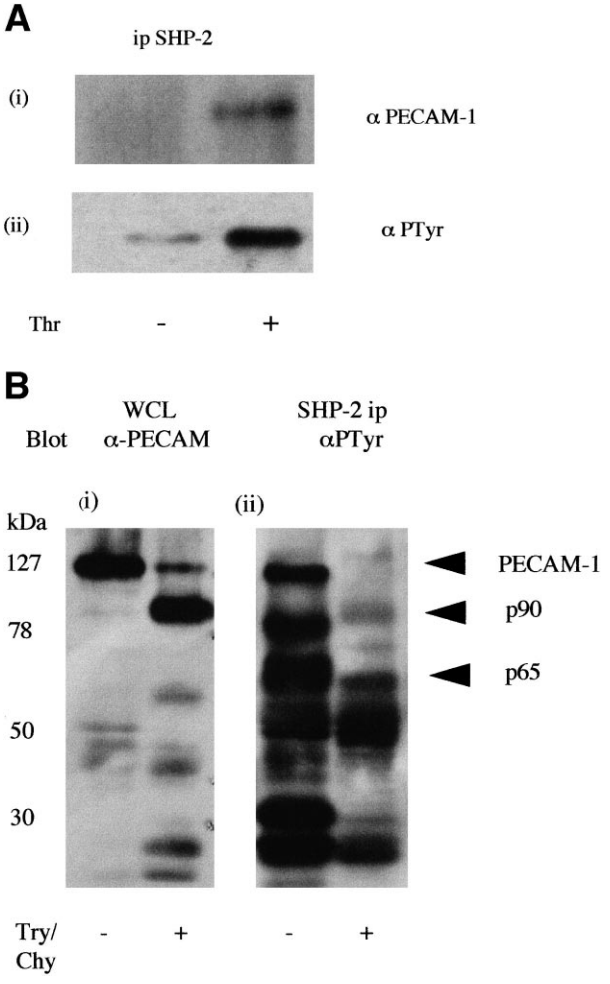

Fig. 2. (A) PECAM-1 becomes phosphorylated and associates with SHP-2 in thrombin-stimulated platelets. SHP-2 immunoprecipitations were carried out on platelets stimulated with thrombin before SDS-PAGE analysis and immunoblotting with anti-PECAM-1 (i). Blots were stripped and re-probed with 4G10 (ii). (B) pp127 consists of more than one protein. Thrombin- stimulated platelets were incubated with trypsin and chymotrypsin for 10 min prior to SDS-PAGE analysis. (i) Whole cell lysates immunoblotted for PECAM (ii) SHP-2 immunoprecipitates immunoblotted for 4G10.

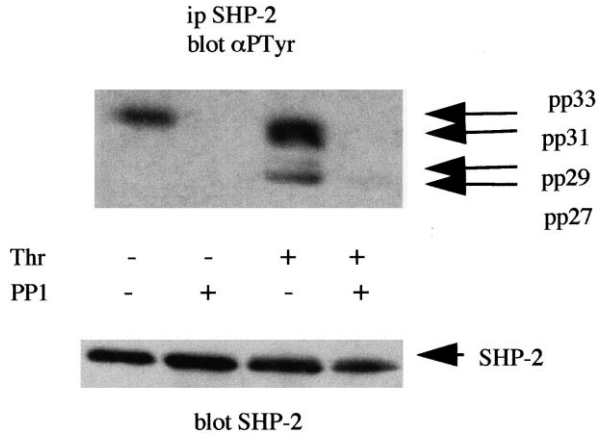

Fig. 3. Phosphorylation of the low molecular weight proteins is dependent upon Src family kinases. Platelets were incubated with tyrodes or PP1 (10 μM) as indicated for 15 min prior to stimulation with thrombin followed by SDS-PAGE analysis and immunoblotting with 4G10.

3.3. Phosphorylation of the low molecular weight proteins is dependent upon Src family kinases

Cao [30] has previously reported that the kinase responsible for the phosphorylation of PECAM-1 on tyrosine residues 663 and 686 may be a member of the Csk or Src family of protein tyrosine kinases. We decided to investigate the role of Src kinases in the phosphorylation of the low molecular weight proteins using the Src family kinase inhibitor PP1. Pre-incubation of platelets with PP1 (10 μM) prior to thrombin stimulation resulted in the disappearance of pp33, 31, 29 and 27 in association with SHP-2, indicating that their phosphorylation and/or association with SHP-2 is downstream of Src kinases (Fig. 3).

3.4. The associating proteins bind differentially to the SH2 domains of SHP-2

To establish the SHP-2 binding sites of the low molecular weight proteins (pp33, 31, 29 and 27), we used GST fusion proteins of the tandem (GST-NC-SH2) and single SH2 domains (GST-N-SH2, GST-C-SH2) of SHP-2 in precipitation experiments. Fig. 4 shows that whilst GST alone failed to precipitate any tyrosine-phosphorylated proteins from thrombin-stimulated platelet lysates, GST-NC-SH2 precipitated all

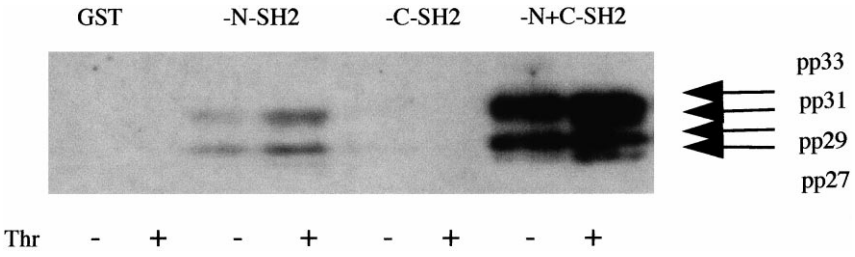

Fig. 4. pp29 and pp33 associate exclusively with the N-SH2 domain of SHP-2. GST fusion proteins were used to immunoprecipitate lysates from thrombin-stimulated platelets prior to SDS-PAGE analysis and immunoblotting with 4G10.

four of the low molecular weight phosphoproteins, indicating that they all associate with SHP-2 via its SH2 domains. Immunoprecipitations using the single SH2 domain GST fusion constructs revealed that pp29 and pp33 associated exclusively with the N-terminal SH2 domain but that pp27 and pp31 did not appear to bind to either of the single SH2 domains.

3.5. Cross-linking of PECAM-1 induces its phosphorylation

We assessed the ability of cross-linking PECAM-1, using specific antibodies, to induce phosphorylation of PECAM-1. As can be seen in Fig. 5, whilst anti-PECAM-1 antibody alone (1 μg/ml, 5 min) had no effect, after cross-linking using a rabbit anti-mouse antibody (30 μg/ml, 5 min) PECAM-1 became strongly phosphorylated. However, this did not appear to result in the induction or specific interaction with any other tyrosine-phosphorylated proteins (data not shown). Neither were we able to detect SHP-2 in PECAM-1 immunoprecipitations by immunoblotting, probably due to the small fraction of total PECAM-1 which associates with SHP-2 (data not shown).

3.6. Thrombin and cross-linked PECAM-1 can activate SHP-2

Despite the failure of SHP-2 to become phosphorylated following thrombin stimulation or PECAM-1 cross-linking, the presence of associating proteins binding to the N-terminal SH2 domain suggested that SHP-2 may be activated under these conditions. We therefore carried out an in vitro phosphatase assay to assess SHP-2 activity in resting and stimulated platelets. As shown in Fig. 6A, both thrombin and PECAM-1 cross-linking caused a significant increase in SHP-2 phosphatase activity, as measured by release of free phosphate from an exogenous tyrosine-phosphorylated peptide substrate.

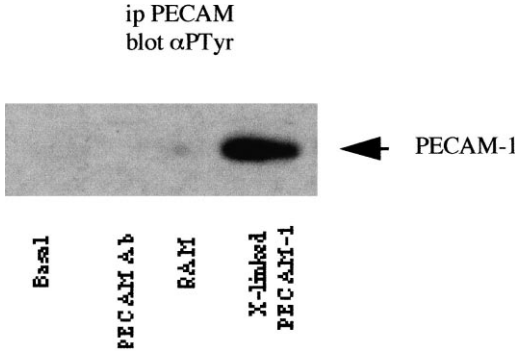

Fig. 5. Cross-linking of PECAM-1 causes its phosphorylation. Platelets were stimulated with anti-PECAM or rabbit anti-mouse antibodies alone or together for 5 min prior to SDS-PAGE analysis and immunoblotting with 4G10.

3.7. The associating proteins are not substrates of SHP-2

Since we have demonstrated that thrombin could activate SHP-2 in vitro, we decided to investigate whether the low molecular weight proteins were also substrates of SHP-2. Incubation of SHP-2 immunoprecipitates in dephosphorylation buffer resulted in a marked decrease in the intensity of the low molecular weight bands. However, no further reduction was observed in the presence of purified GST-SHP-2, neither was the decrease inhibited by vanadate. This indicates that the associating proteins are not SHP-2 substrates and that the observed decrease in the intensity of the bands is due to dissociation of the proteins during the time period of the experiment.

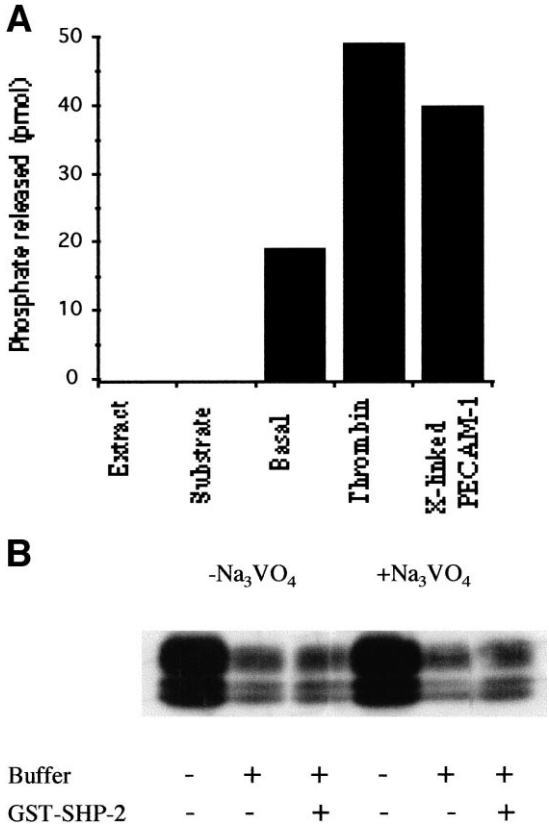

Fig. 6. (A) Thrombin and cross-linked PECAM-1 can activate SHP-2. SHP-2 immunoprecipitates from resting and stimulated platelets were mixed with tyrosine-phosphorylated peptide substrate. Phosphatase activity was assessed by a spectrophotometric assay of released free phosphate. (B) The associating proteins are not substrates of SHP-2. After washing, SHP-2 immunoprecipitates were incubated with dephosphorylation buffer alone or in the presence of GST-SHP-2 fusion protein for 1 h prior to SDS-PAGE analysis and immunoblotting with 4G10.

4. Discussion

The only SHP-2 associating phosphoprotein reported to date in platelets is PECAM. We have further investigated SHP-2 signalling in thrombin-stimulated platelets and demonstrated an additional four low molecular weight proteins which associate with SHP-2 in a time-dependent manner. The trypsin digestion experiments indicated that these low molecular weight proteins may be transmembrane proteins and may therefore have a function either as receptors or as adaptor molecules linking SHP-2 to surface receptors. Of the known SHP-2 associating adaptor proteins, immunoblotting has shown that they are not forms of Grb-2, whilst the molecular weight of SHPS-1 and PZR is too high. The recently cloned SHP-2 binding protein SIT was a possible candidate, but unlike SIT, the molecular weights of the proteins were unaltered under non-reducing conditions (data not shown), implying that they do not normally exist as dimers. Another possible candidate was FcεR β-chain, which is known to be present in platelets and exists in multiple forms due to differential splicing and phosphorylation states and is a substrate of the Src kinase Lyn [31]. However, FcεR β-chain immunoblotting failed to detect proteins in the SHP-2 immunoprecipitates (data not shown).

Further investigations revealed that the phosphorylation of pp27, 29, 31 and 33 is attenuated by PP1, indicating that the phosphorylation step occurs downstream of Src family kinases. PECAM-1 has previously been reported to bind to and to be phosphorylated by a Src kinase [30,32] and Walter has shown that SHP-2 can activate Src by a non-enzymatic mechanism involving binding to the SH3 domain of the kinase [33]. Furthermore, since it was shown that pp33 and pp29 bound exclusively to the N-terminal SH2 domain of SHP-2, which is known to be an important regulatory site for SHP-2 activity, SHP-2 may recruit a Src family kinase to phosphorylate these proteins, which in turn associate with and activate SHP-2. Platelets contain several different Src family kinases and further studies will be required to establish the identity of the specific kinase responsible and its role in regulating the phosphorylation and binding of the low molecular weight proteins to SHP-2.

In agreement with previous studies by Jackson, we have also demonstrated an association between tyrosine-phosphorylated PECAM-1 and SHP-2 in human platelets, which, due to the absence of any other tyrosine-phosphorylated proteins in PECAM-1 immunoprecipitations, appears to be a direct interaction. However, in contrast to Jackson, we found that this association was not dependent upon aggregation. The difference may be accounted for by the use of EGTA instead of RGDS to dissociate the αIIbβ3 receptor or by the use of thrombin instead of TRAP to stimulate the platelets. Indeed, we found that whilst stimulation of the platelets with collagen also induced an association of PECAM-1 with SHP-2, this association could be effectively inhibited by the addition of EGTA (data not shown), indicating that at least some of the signalling pathways regulating the association of PECAM with SHP-2 are aggregation-dependent. Furthermore, by comparison with thrombin, collagen (100 μg/ml, 2 min) and WGA (10 μg/ml, 2 min) also enhanced the association of pp29 and pp33 although neither agonist induced the association of pp27 or pp31 (data not shown). Thus, the association of some of the low molecular weight proteins with SHP-2 is agonist-specific.

Despite the high copy number of PECAM-1 in human platelets, we could only detect small amounts of PECAM-1 associated with SHP-2. Furthermore, we were unable to detect SHP-2 in PECAM immunoprecipitates, implying that only a small proportion of available PECAM-1 associates with SHP-2. This appears to contradict the appearance of a highly phosphorylated band at 127 kDa in SHP-2 immunoprecipitates and whilst PECAM does become highly phosphorylated, we provide evidence here that pp127 consists of more than one protein. The studies involving trypsin/chymotrypsin digestion of PECAM indicated that whilst pp127 contains PECAM-1, the appearance of a digestion product at 65 kDa and the poor appearance of the pp90 digestion product of PECAM-1 imply the presence of a second 127 kDa transmembrane phosphoprotein which associates with SHP-2 in thrombin-stimulated platelets.

Interestingly, despite activation of SHP-2 by thrombin and cross-linking of PECAM-1, SHP-2 is not tyrosine-phosphorylated itself as in other cell types. This demonstrates that phosphorylation is not a requirement of SHP-2 activation, but instead may enable interaction of SHP-2 with other SH2 containing proteins to form signalling complexes.

In conclusion, we have reported and partially characterised four novel low molecular weight phosphoproteins and suggest the existence of a fifth which co-migrates with PECAM-1, all of which, in addition to PECAM, associate with SHP-2 in thrombin-stimulated platelets. Phosphorylation and association of the low molecular weight proteins with SHP-2 is dependent upon Src family kinase activity and the association is mediated through the SH2 domains of SHP-2. Thrombin and PECAM-1 can couple to activation of SHP-2 although the low molecular weight associating proteins are not substrates of the phosphatase. It will be important to identify the associating proteins and to determine whether they mediate the activation of SHP-2.

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