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Calpain-catalyzed cleavage and subcellular relocation of protein phosphotyrosine phosphatase 1B (PTP-1B) in human platelets

John V.Frangioni, Atsushi Oda[2,3], Marianne Smith[2], Edwin W.Salzman[2] and Benjamin G.Neel[1]

Molecular Medicine Unit and [2]Department of Surgery, Beth Israel Hospital, Boston, MA 02215, USA
[3]Present address: Department of Internal Medicine, School of Medicine, Keio University, 35 Shinanomachi, Sinjukuku, Tokyo, 160, Japan
[1]Corresponding author

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The non-transmembrane phosphotyrosine phosphatase 1B (PTP-1B) is an abundant enzyme, normally localized to the cytosolic face of the endoplasmic reticulum via a C-terminal targeting sequence. We have found that agonist-induced platelet activation results in proteolytic cleavage of PTP-1B at a site upstream from this targeting sequence, causing subcellular relocation of its catalytic domain from membranes to the cytosol. PTP-1B cleavage is catalyzed by the calcium-dependent neutral protease calpain and is a general feature of platelet agonist-induced aggregation. Moreover, PTP-1B cleavage correlates with the transition from reversible to irreversible platelet aggregation in platelet-rich plasma. Engagement of gpIIb-IIIa is necessary for inducing PTP-1B cleavage, suggesting that integrins regulate tyrosine phosphatases as well as tyrosine kinases. PTP-1B cleavage is accompanied by a 2-fold stimulation of its enzymatic activity, as measured by immune complex phosphatase assay, and correlates with discrete changes in the pattern of tyrosyl phosphorylation. Cleavage and subcellular relocation of PTP-1B represents a novel mechanism for altering tyrosyl phosphorylation that may have important physiological implications in cell types other than platelets.

Key words: calpain/cell death/integrins/protein tyrosine phosphatase/tyrosyl phosphorylation

Introduction

The non-transmembrane protein phosphotyrosine phosphatase 1B (PTP-1B) was originally identified as the major tyrosine phosphatase activity in human placenta (Tonks *et al.*, 1988a,b) and purified as a soluble, 39 kDa protein (321 amino acids; Charbonneau *et al.*, 1989). Subsequent cloning of cDNAs for rat (Guan *et al.*, 1990) and human (Brown-Shimer *et al.*, 1990; Chernoff *et al.*, 1990) PTP-1B predicted that the full-length protein contained 432 (rat) or 435 (human) amino acids (M_r 50 kDa) and was ubiquitously expressed (Brown-Shimer *et al.*, 1990; Chernoff *et al.*, 1990; Guan *et al.*, 1990). In exponentially growing cells, only the full-length, 50 kDa form of PTP-1B protein is found (Frangioni *et al.*, 1992; Woodford-Thomas *et al.*, 1992). Under basal conditions and in all cycling cells tested, full-length PTP-1B associates tightly with the endoplasmic reticulum (ER) membrane such that its catalytic domain is oriented towards the cytosol. The C-terminal 35 amino acids of PTP-1B are necessary and sufficient for directing subcellular localization of PTP-1B to the ER (Frangioni *et al.*, 1992; Woodford-Thomas *et al.*, 1992).

Little is known about PTP-1B regulation. In randomly growing cells, PTP-1B is a seryl phosphoprotein (Frangioni *et al.*, 1992). PTP-1B undergoes cell cycle-dependent (Flint *et al.*, 1993; Schievella *et al.*, 1993; V.Shifrin, J.V.Frangioni and B.G.Neel, unpublished observations) and PKC-dependent (Flint *et al.*, 1993; V.Shifrin, J.V.Frangioni and B.G.Neel, unpublished observations) seryl phosphorylation and is also controlled at the post-transcriptional level. A novel PTP-1B mRNA isoform is generated by alternative splicing during the G_0 to G_1 transition of human diploid fibroblasts and this isoform encodes a protein with an altered C-terminus (Shifrin and Neel, 1993). Although the biochemical and physiological consequences of these modifications are as yet unclear, its seems likely that PTP-1B is involved in some aspect of cell cycle regulation. Since PTP-1B is also highly expressed in many non-cycling cells, it seemed reasonable that it might be regulated differently and/or play different roles in post-mitotic cells.

Human platelets contain several abundant, highly active protein tyrosine kinases (PTKs) such as c-Src (Ferrell and Martin, 1988; Golden and Brugge, 1989; reviewed in Shattil and Brugge, 1991). Under basal conditions, platelets contain relatively low levels of tyrosyl phosphoproteins and treatment with the general tyrosine phosphatase inhibitor vanadate results in a marked increase in tyrosyl phosphorylation (Lerea *et al.*, 1989; Inazu *et al.*, 1990; Pumiglia *et al.*, 1992). Hence, phosphotyrosine phosphatases (PTPs) probably play an important role in determining the steady state level of platelet tyrosyl phosphorylation. Preliminary studies indicated that PTP-1B constituted ~0.2% of total detergent soluble platelet protein (see below), a level comparable with that of c-Src (Golden and Brugge, 1989). These data suggested that PTP-1B might play an important role in platelet physiology.

In vivo, at sites of micro- and macroscopic injury to the endothelium, platelets adhere to basement membrane proteins such as collagen. Adhesion evokes a complex series of events including shape change, alpha and dense granule secretion, and platelet aggregation. Aggregation, in turn, leads to events such as the shedding of procoagulant-rich microvesicles (Fox *et al.*, 1990). In concert with the coagulation cascade, aggregated platelets and fibrin form a clot, which then retracts, sealing the site of injury. In addition to collagen, several physiological (e.g. thrombin, ADP/fibrinogen, thromboxane A_2) and pharmacological (e.g. calcium ionophores, phorbol ester) agents can induce some, or all, of these events. *In vitro*, platelet aggregation in response to these agonists requires the application of a shear force (i.e. stirring). Thus, by either stirring or not stirring platelet

suspensions, molecular events that are the direct consequence of agonist treatment can be experimentally dissociated from events secondary to aggregation.

Although many extracellular and cell surface molecules have been implicated in platelet aggregation, the cell surface glycoprotein gpIIb-IIIa is clearly required. GpIIb-IIIa is a member of the integrin family of adhesion receptors (reviewed in Hynes, 1992). One of its physiological ligands is fibrinogen, which forms a physical 'bridge' between adjoining platelets. Binding of fibrinogen to gpIIb-IIIa is mediated through Arg-Gly-Asp (RGD) repeats (Gartner and Bennett, 1985; Plow *et al.*, 1985), as well as other recently appreciated sequences (Farrell *et al.*, 1992), within the fibrinogen molecule. In response to weak agonists such as ADP (in the presence of fibrinogen), aggregation can be separated into two stages. At low agonist concentrations, platelets undergo primary (reversible) aggregation, during which they loosely adhere and then dissociate, returning to the resting state. At high agonist concentrations, however, the primary phase is augmented by a secondary phase, during which aggregation becomes irreversible. Irreversible aggregation is the end result of a positive feedback loop, which includes secretion of granular contents and recruitment of additional platelets.

Agonist treatment of platelets causes rapid changes in tyrosyl phosphorylation of multiple platelet proteins (Ferrell and Martin, 1988; Golden and Brugge, 1989). Some of these phosphorylation events require aggregation and are mediated through engagement of gpIIb-IIIa (Ferrell and Martin, 1989; Bachelot *et al.*, 1990; Golden *et al.*, 1990; Oda *et al.*, 1992; Clark and Brugge, 1993). Until recently, nearly all studies of platelet tyrosyl phosphorylation have focused on how PTKs are controlled by agonists and aggregation (reviewed in Shattil and Brugge, 1991). Given PTP-1B's abundance, we chose to investigate its regulation in human platelets.

In the course of studying PTP-1B phosphorylation in response to platelet activation, we found that under conditions resulting in platelet aggregation, PTP-1B undergoes proteolytic cleavage in a region between its catalytic domain and its membrane-anchoring C-terminal targeting sequence. Further studies indicated that the calcium-dependent protease calpain (EC 3.4.22.17) is responsible for PTP-1B cleavage. Calpain cleavage results in subcellular relocation of PTP-1B's catalytic domain from membranes to the cytosol, enzymatic activation and concomitant changes in the pattern of protein tyrosyl phosphorylation. Furthermore, engagement of gpIIb-IIIa is necessary and sufficient for inducing the activation of calpain. PTP-1B cleavage is a general feature of aggregation in gel-filtered platelets and occurs at the transition from primary (reversible) to secondary (irreversible) platelet aggregation in platelet-rich plasma (PRP). PTP-1B cleavage and subcellular relocation may have important physiological consequences in platelets as well as in other cell types.

Results

Abundance of PTP-1B in human cells

Although mRNA expression levels suggested that PTP-1B was abundant in tissues (Brown-Shimer *et al.*, 1990; Chernoff *et al.*, 1990; Guan *et al.*, 1990), an estimation of PTP-1B protein levels had not been reported. Using a recombinant glutathione S-transferase (GST) fusion protein (GST−PTP-1B-N; Frangioni *et al.*, 1992) as a mass

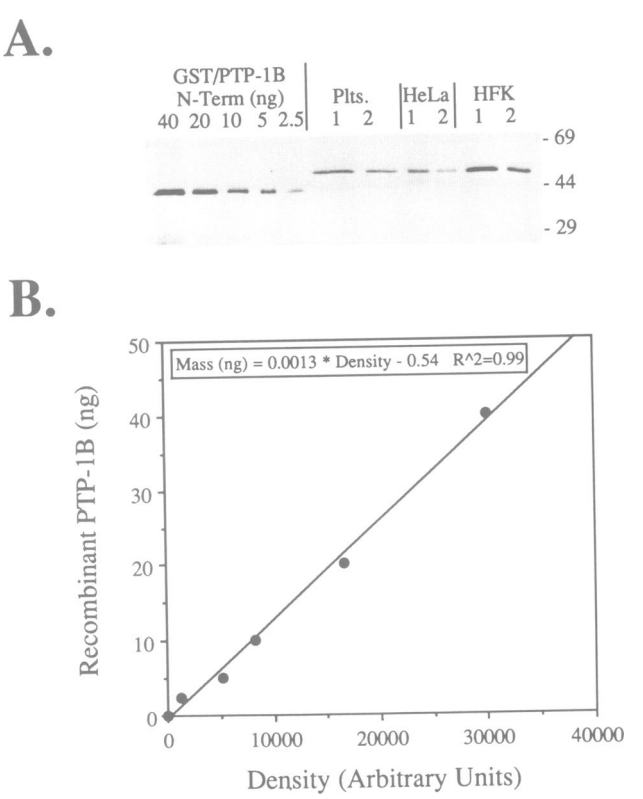

Fig. 1. Abundance of PTP-1B protein in human cells. (**A**) GST/PTP-1B N-Term, a fusion protein of GST with amino acids 1−158 of PTP-1B, was purified and quantitated as described in Materials and methods. Various amounts of GST/PTP-1B N-Term (2.5−40 ng) or NP-40 lysates of human platelets (Plts.), HeLa cells (HeLa) and human foreskin epidermal keratinocytes (HFK) were resolved by SDS−PAGE and immunoblotted with antibody #583N, specific for the N-terminus (amino acids 1−158) of PTP-1B. Cellular lysates were used at 5 μg (lanes 1) or 2.5 μg (lanes 2) of total protein. The positions (in kDa) of prestained molecular weight markers are shown at the right of the figure. The major bands represent GST/PTP-1B N-Term (44 kDa) and cellular PTP-1B (50 kDa). (**B**) The immunoblot shown in panel A was analyzed with a Silver Scan Scanner controlled by Photoshop (Adobe) software. Scan Analysis (BioSoft) software was used to determine the density of detected bands. The graph displays the density of GST/PTP-1B N-Term (abscissa) plotted against the mass of recombinant protein loaded (ordinate). A first order equation fitting the data (boxed inset) was then used to estimate the amount of PTP-1B in each cellular lysate shown in panel A.

standard, detergent soluble total cellular lysates were resolved by SDS−PAGE and immunoblotted with an antibody (Frangioni *et al.*, 1992) specific for amino acids 1−158 of PTP-1B (Figure 1A). Densitometric analysis of the immunoblot allowed the level of PTP-1B from total cellular lysates to be compared with a standard curve (Figure 1B). It was estimated that PTP-1B accounts for 0.2% of total detergent soluble protein in platelets, 0.1% in HeLa cells and 0.6% in human foreskin epidermal keratinocytes.

PTP-1B is cleaved in vivo near its C-terminus by the calcium-dependent, neutral protease calpain

In unstirred, gel-filtered platelets, PTP-1B becomes rapidly phosphorylated on serine after treatment with thrombin and other agonists (A.Oda, J.V.Frangioni, V.Shifrin and B.G.Neel, unpublished observations). In attempting to determine the intervening kinase, we tested several kinase agonists, including the calcium ionophore A23187. As a control to normalize for protein mass, immunoblotting was

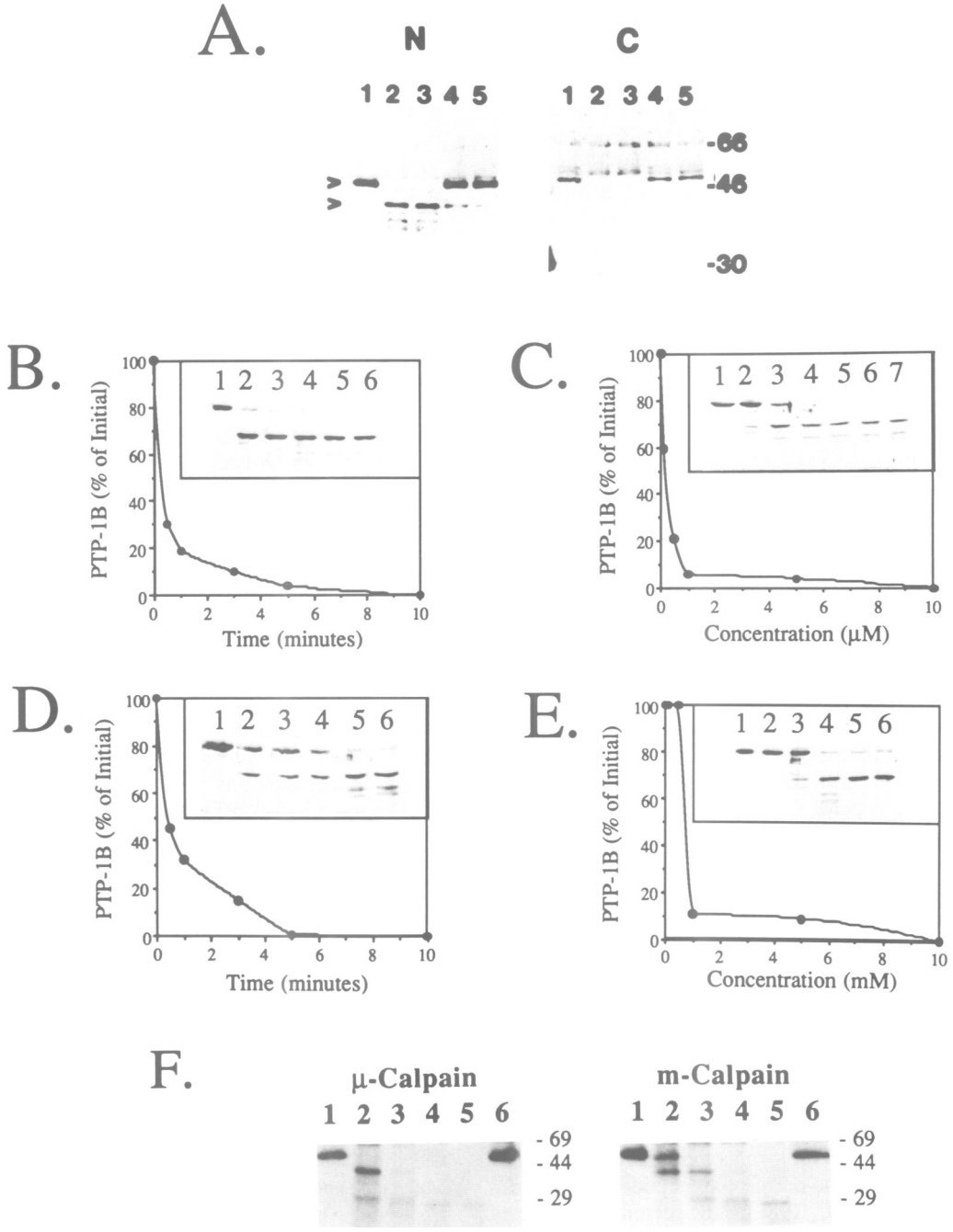

Fig. 2. PTP-1B is proteolytically cleaved *in vivo* and *in vitro* by calpain. (**A**) Unstirred, gel-filtered platelets in modified HEPES−Tyrode buffer supplemented with 1 mM $CaCl_2$ (lanes 1) were stimulated with calcium ionophore A23187 (1 μM, 10 min; lanes 2 and 4) or dibucaine (1 mM, 10 min; lanes 3 and 5), with (lanes 4 and 5) or without (lanes 2 and 3) 5 min pretreatment with calpeptin (30 μM). For each condition, 10 μg of total platelet protein were resolved by SDS−PAGE and immunoblotted with affinity-purified anti-PTP-1B rabbit polyclonal antibodies specific for the N-terminus (N; antibody #932N) or C-terminus (C; antibody #300C; Frangioni *et al.*, 1992). Arrowheads mark the positions of the 50 and 42 kDa forms of PTP-1B. The positions (in kDa) of prestained molecular weight markers are shown at the right of the figure. (**B**) Kinetics of PTP-1B cleavage in gel-filtered platelets after treatment with 1 μM A23187 for 0 (lane 1), 30 (2), 1 (3), 3 (4), 5 (5), 10 (6) or 15 min (7). 10 μg of total platelet protein were resolved by SDS−PAGE and immunoblotted with antibody #583N (inset). Immunoblots were scanned using a Silver Scan Scanner and Scan Analysis (Biosoft) software and the data plotted as percent of full-length PTP-1B present in unstimulated platelets. Half-maximal cleavage occurred at ~20 s. (**C**) Dose−response curve of PTP-1B cleavage in gel-filtered platelets 2 min after treatment with increasing doses of the calcium ionophore A23187: 0 (lane 1), 0.05 (2), 0.1 (3), 0.5 (4), 1 (5), 5 (6) or 10 μM (7). Data presented as described for panel B. The ED_{50} for cleavage was ~0.075 μM. (**D**) Kinetics of PTP-1B cleavage in gel-filtered platelets after treatment with 1 mM dibucaine for 0 (lane 1), 30 (2), 1 (3), 3 (4), 5 (5), 10 (6) or 15 min (7). Data presented as described for panel B. Half-maximal cleavage occurred at ~30 s. (**E**) Dose−response curve of PTP-1B cleavage in gel-filtered platelets 10 min after treatment with increasing doses of dibucaine: 0 (lane 1), 0.1 (2), 0.25 (3), 1 (4), 5 (5) or 10 mM (6). Data presented as described for (C). The ED_{50} for cleavage was ~0.75 mM. (**F**) *In vitro* cleavage of PTP-1B by μ- and m-calpains purified from bovine heart. Full length PTP-1B, immunoprecipitated from [^{35}S]methionine-labeled HeLa cells, was incubated for 20 min at 30°C in calpain protease buffer (see Materials and methods) with 10 mM $CaCl_2$ (lanes 1) to which was added 0.5 (lanes 2), 2.5 (lanes 3), 10 (lanes 4) or 50 μg/ml calpain (lanes 5). Lanes 6 display incubation with 50 μg/ml calpain in protease buffer, without $CaCl_2$, to which 2 mM EDTA was added. The positions (in kDa) of prestained molecular weight markers (different from those in panel A) are shown on the right of the figure.

performed on these lysates. To our surprise, although there was no change in its phosphorylation, the electrophoretic mobility of PTP-1B was found to increase after calcium ionophore treatment. In unstirred, unstimulated gel-filtered platelets, PTP-1B was present as a 50 kDa form, which, as expected, was recognized by antibodies directed against either the N- or C-terminus of the molecule (Figure 2A, lanes 1, upper arrowhead). On SDS−PAGE gels, the 50 kDa form of PTP-1B from platelets co-migrated with that from HeLa cells (Figure 1). After treatment with the calcium ionophore A23187 in the presence of 1 mM extracellular calcium (lanes 2), or the local anesthetic dibucaine, which causes release of calcium from intracellular stores (lanes 3), the electrophoretic mobility of PTP-1B increased significantly, with the major band running at ~42 kDa (lower arrowhead). Treatment of gel-filtered platelets with A23187 in the presence of 1 mM extracellular EDTA completely inhibited PTP-1B cleavage (data not shown). Cleavage of PTP-1B by calcium ionophore A23187 and dibucaine was rapid and occurred at low doses of both agents: 50% cleavage of full-length PTP-1B was seen at 0.075 μM A23187 (Figure 2C) and a time of 20 s after treatment with 1 μM A23187 (Figure 2B), whereas 50% cleavage of PTP-1B was seen at 0.75 mM dibucaine (Figure 2E) and a time of 30 s after treatment with 1 mM dibucaine (Figure 2D). The altered form of PTP-1B was recognized by antibodies (Frangioni *et al.*, 1992) directed against amino acids 1−158 (N-terminal antibodies) of PTP-1B (Figure 2A, panel N), but not by antibodies directed against amino acids 329−435 (C-terminal antibodies) of PTP-1B (Figure 2A, panel C), indicating that it arose as a result of C-terminal truncation. The 42 kDa, C-terminally truncated form of PTP-1B was stable in platelets for at least 30 min after treatment (Figure 2B and D and data not shown), suggesting that is has a discrete signaling function and is not merely a degradation intermediate.

Since agents that raise intracellular calcium promoted PTP-1B cleavage, it seemed likely that calcium-activated proteases were involved. Calpains are calcium-activated cysteine-proteases whose optimal activity is at neutral pH (reviewed in Croall and DeMartino, 1991). μ-calpain (calpain I) requires micromolar calcium concentrations for activation, whereas m-calpain (calpain II) requires millimolar calcium concentrations. Both forms of calpain are inhibited by a specific, membrane-permeable peptide derivative, calpeptin (Tsujinaka *et al.*, 1988), thus permitting a direct test of the possible involvement of calpain in PTP-1B cleavage in platelets. Pre-treatment of gel-filtered platelets for 5 min with calpeptin (30 μM) blocked >90% of PTP-1B cleavage induced by A23187 or dibucaine (Figure 2A, lanes 4 and 5), suggesting that calpain catalyzes PTP-1B cleavage *in vivo*. Treatment with calcium ionophore, followed by addition of a saturating amount of calpeptin before cell lysis, did not prevent PTP-1B cleavage. Moreover, the addition of EGTA after calcium ionophore treatment, but before lysis, had no effect on the observed results (data not shown). These data strongly suggest that PTP-1B cleavage occurred prior to and was not the result of cell lysis. It does not seem likely that PTP-1B cleavage *in vivo* is a gratuitous consequence of generalized proteolysis catalyzed by calpain; except for the cleavage of other known calpain substrates, total protein (as assayed by Coomassie blue staining) was virtually unchanged after treatment with these agents (data not shown; see also Figure 7C). Moreover, T cell phosphatase, a non-transmembrane PTP with >50% sequence identity to PTP-1B, remained uncleaved following calcium ionophore treatment (data not shown).

We further asked whether PTP-1B was a substrate for calpain *in vitro*. Full-length (50 kDa) PTP-1B was immunoprecipitated from HeLa cells metabolically labeled with [^{35}S]methionine (Frangioni *et al.*, 1992) and used as substrate for μ- and m-calpains (Croall and DeMartino, 1984). As shown in Figure 2, either calpain catalyzed PTP-1B cleavage. At intermediate concentrations of enzyme, a 42 kDa form of PTP-1B was generated (Figure 2F, lanes 2), which co-migrated with PTP-1B cleaved *in vivo* (data not shown). Cleavage *in vitro* was inhibited completely by replacing calcium with EDTA (lanes 6), indicating that cleavage was due to calpain and not a contaminating, non-calcium-dependent protease. Interestingly, PTP-1B was cleaved to completion by continued calpain treatment *in vitro* (Figure 2F), yet remained as a stable 42 kDa protein *in vivo*. This difference may reflect additional levels of calpain regulation or inaccessibility of PTP-1B after initial cleavage *in vivo* (see Discussion). Since PTP-1B cleavage in human platelets occurs after activation with agonists known to activate calpain (A23187 and dibucaine), is inhibited by a calpain-specific inhibitor calpeptin, requires calcium and occurs *in vitro* using μ- or m-calpain, we conclude that calpain is responsible for cleavage within the C-terminus of PTP-1B *in vivo*.

Calpain cleavage releases and activates PTP-1B

Based on comparisons with prestained markers and the relative mobilities of PTP-1B truncation mutants transiently overexpressed in COS cells (Frangioni *et al.*, 1992), cleavage of PTP-1B by calpain *in vivo* removes its C-terminal 60−70 amino acids. We have shown previously that the C-terminal 35 amino acids of PTP-1B are both necessary and sufficient for targeting to the cytoplasmic face of the ER membrane (Frangioni *et al.*, 1992). Therefore, we expected that calpain cleavage of PTP-1B would remove the ER localization signal and relocate PTP-1B to the cytosol. We tested this hypothesis by subjecting unstirred, gel-filtered platelets treated with A23187 or dibucaine to hypotonic lysis and subcellular fractionation (Figure 3A). The total lysate (T) was fractionated into a 100 000 *g* soluble fraction (S) and a 100 000 *g* particulate fraction (P), as described in Materials and methods. Equal volumes of each of these fractions were then analyzed by immunoblotting. In unstimulated platelets (Control), the full-length, 50 kDa form of PTP-1B was seen only in the particulate (membrane) fraction. However, after treatment with either A23187 (+A2) or dibucaine (+Db), the truncated form of PTP-1B was generated (major band at 42 kDa); this form was only found in the soluble (cytosolic) fraction.

We next asked whether the cleaved, relocated 42 kDa form of PTP-1B retained tyrosine phosphatase activity. PTP-1B was immunoprecipitated from platelet lysates and subjected to immune complex phosphatase assay using the ^{32}P-phosphorylated peptide substrate Raytide (Frangioni *et al.*, 1992). Full-length PTP-1B immunoprecipitated from unstimulated platelets (Figure 3B, open squares) showed a basal level of tyrosine phosphatase activity. PTP-1B immunoprecipitated from calcium ionophore-treated platelets (open circles) showed 2-fold higher enzymatic activity; immunoblotting of these lysates (inset) confirmed that complete cleavage of PTP-1B had occurred. Increased

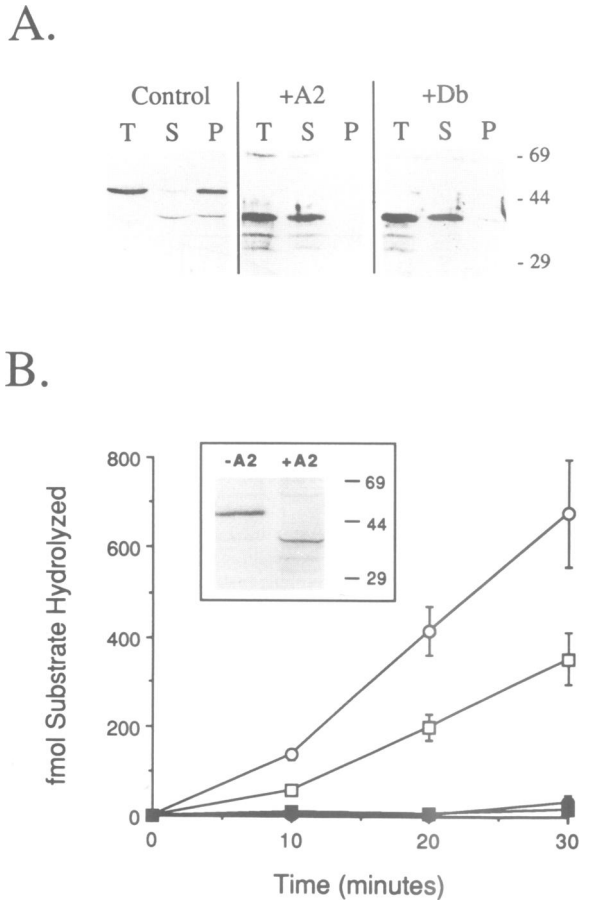

Fig. 3. Calpain-catalyzed cleavage renders PTP-1B freely soluble and enzymatically activated. (**A**) Unstirred gel-filtered platelets in modified HEPES−Tyrode buffer supplemented with 1 mM $CaCl_2$ (Control) were stimulated with 1 μM A23187 for 2 min (+A2) or 1 mM dibucaine for 5 min (+Db) and subjected to hypotonic lysis and subcellular fractionation as described in Materials and methods. Centrifugation of the total lysate (T) at 100 000 *g* yielded soluble (S) and particulate (P) fractions. Equal volumes of each fraction (representing 10 μg of total lysate) were resolved by SDS−PAGE and immunoblotted with antibody #583N, specific for the N-terminus of PTP-1B. The positions (in kDa) of prestained molecular weight markers are shown at the right of the figure. (**B**) Unstirred gel-filtered platelets in modified HEPES−Tyrode buffer supplemented with 1 mM $CaCl_2$ (squares) were stimulated with 1 μM A23187 for 2 min (circles) and lysed with 2 × NP-40 buffer. After immunoprecipitation with anti-PTP-1B mAb FG6 (open circles, open squares) or *S.aureus* alone (closed circles, closed squares), immune complexes were assayed for tyrosine phosphatase activity using ^{32}P-labeled Raytide, as described in Materials and methods. Each point displays the mean and standard deviation (error bar) of three separate immunoprecipitations from 500 ng of total platelet protein. Hydrolyzed substrate was assayed at 10, 20 and 30 min after addition of immune complexes. Inset shows 10 μg of the same lysates immunoblotted with mAb FG6. The data presented are from a single platelet donor. Comparable results were obtained with platelets from five different donors.

enzymatic activity was seen when PTP-1B was immunoprecipitated with either monoclonal antibody FG6 (Figure 3B) or polyclonal antibodies #300F (Frangioni *et al.*, 1992; data not shown). The apparent lag in phosphatase activity seen at the earliest time point could be eliminated by preincubation with DTT and was probably due to oxidation of PTP-1B's essential cysteine residue during immunoprecipitation. Calpain-induced cleavage within PTP-1B's C-terminus thus has two important biochemical consequences: relocation of the enzyme from membranes to cytosol and activation of its tyrosine phosphatase activity.

PTP-1B cleavage is a general feature of platelet agonist-induced aggregation

Our data suggested that conditions that increase intracellular calcium lead to activation of calpain and cleavage of PTP-1B. Increases in intracellular calcium occur during platelet aggregation (reviewed in Ashby *et al.*, 1990). We therefore asked whether PTP-1B cleavage occurred in response to physiological platelet agonists and what role aggregation might play in this process. In unstirred or stirred control platelets, only the full-length, membrane-associated form of PTP-1B was observed (Figure 4A, lanes 1 and 2). Thrombin (1 U/ml) stimulation, in the presence of stirring, resulted in platelet aggregation and cleavage of PTP-1B to a 42 kDa species (lane 4) that co-migrated with the form generated by calcium ionophore treatment (data not shown). In the example shown, ~70% of the PTP-1B mass was converted to the 42 kDa, soluble form after 2 min of thrombin treatment; depending on the particular platelet donor, this value ranged from 40 to 100%. However, thrombin treatment under conditions where aggregation does not occur (no stirring) did not lead to cleavage of PTP-1B (lane 3).

We asked whether PTP-1B cleavage would occur in response to other platelet agonists (Figure 4B). Control platelets with (+) or without (−) stirring (lanes 1) contained only full-length, 50 kDa PTP-1B. Aggregation induced by either the thromboxane A2 analogue U46619 (lanes 2) or the tumor promoter phorbol 12-myristate 13-acetate (PMA; lanes 3) resulted in cleavage of >70% of PTP-1B to the same 42 kDa, soluble form. *In vivo*, platelet aggregation is induced when endothelium is denuded and basement membrane collagen is exposed. Collagen also induced PTP-1B cleavage (lanes 4). PTP-1B cleavage also was observed upon treatment of gel-filtered platelets with ADP (in the presence of fibrinogen), a weak physiological agonist for platelet aggregation (lanes 5). Unlike aggregation induced by stronger agonists, the corresponding cleavage of PTP-1B in response to ADP (and fibrinogen) never totaled >50−60% (see below). As shown in Figure 4C, PTP-1B cleavage in response to thrombin correlated closely with the extent of platelet aggregation (Figure 4D). These results were confirmed in at least three separate experiments utilizing platelets from different donors. Indeed, PTP-1B cleavage appears to be a general feature of pharmacological and physiological platelet aggregation, induced by both strong and weak agonists. Notably, in all cases, agonist treatment alone was insufficient to evoke PTP-1B cleavage. Cleavage was only observed under conditions that allowed platelet aggregation.

PTP-1B cleavage correlates with the transition from reversible to irreversible platelet aggregation

Since platelets have little or no capacity to synthesize protein, cleavage and relocation of a major tyrosine phosphatase might be expected to act as an irreversible 'trigger' for one or more physiological processes. When platelets are stimulated with weak agonists such as ADP (in the presence of fibrinogen), they exhibit a transient, reversible response seen in an aggregometer as a rise and fall in light transmission. This response, termed primary aggregation, increases in amplitude with the dose of ADP until a critical threshold is reached. Thereafter, platelet aggregation initially becomes bi-phasic and ultimately, irreversible (secondary aggregation). At higher concentrations of ADP, the slope

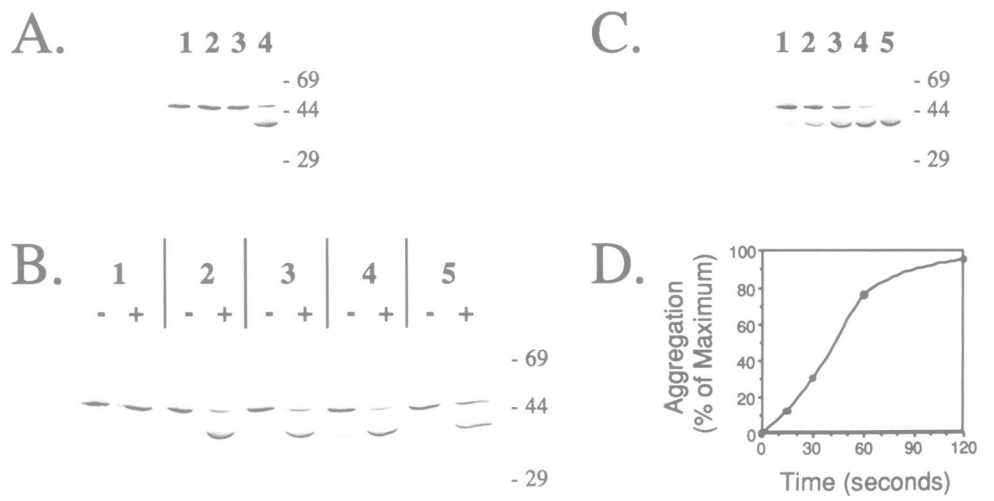

Fig. 4. PTP-1B cleavage is a general feature of agonist-induced platelet aggregation. (**A**) Gel-filtered platelets in modified HEPES−Tyrode buffer supplemented with 1 mM $CaCl_2$ were treated with 1 U/ml of thrombin, for 2 min, in the presence (promoting aggregation), or absence (preventing aggregation), for 2 min, of stirring: lane 1, −thrombin, −stirring; lane 2, −thrombin, +stirring; lane 3, +thrombin, −stirring; lane 4, +thrombin, +stirring. Total platelet lysate (10 μg) from each condition was resolved by SDS−PAGE and immunoblotted with anti-PTP-1B antibody #583N as described in Materials and methods. The positions (in kDa) of prestained molecular weight markers are shown at the right of the figure. (**B**) Gel-filtered platelets in modified HEPES−Tyrode buffer supplemented with 1 mM $CaCl_2$ (lanes 1) were treated with 1 μM U46619 (lanes 2), 100 nM PMA (lanes 3), 20 μg/ml collagen (lanes 4), or 20 μM ADP + 0.3 mg/ml fibrinogen (lanes 5), either without (−) or with (+) stirring. Platelets were lysed after 2 min in 2 × SDS−PAGE sample buffer and 10 μg of each lysate were resolved by SDS−PAGE and immunoblotted with anti-PTP-1B antibody #583N as described in Materials and methods. (**C**) Stirred gel-filtered platelets in modified HEPES−Tyrode buffer supplemented with 1 mM $CaCl_2$ were stimulated with 1 U/ml thrombin and lysed after various times: 0 (lane 1), 15 (lane 2), 30 (lane 3), 60 (lane 4) or 120 s (lane 5). 10 μg of total platelet lysate at each time point were resolved by SDS−PAGE and immunoblotted as above. (**D**) Aggregometer tracings from lysates in panel C plotted as the percentage of maximum aggregation. Maximum aggregation is defined as aggregation seen 5 min after thrombin stimulation.

of the aggregometer tracing is even steeper and the curve becomes more logarithmic in shape. Preliminary experiments with gel-filtered platelets indicated that PTP-1B cleavage correlated with the transition from reversible (primary only) to irreversible (primary and secondary) aggregation and occurred only after a threshold ADP concentration had been reached. However, it was difficult to obtain reproducible reversibility of aggregation using gel-filtered platelets from different donors.

PRP exhibits reproducible reversible aggregation in response to low doses of ADP. However, PRP poses several unique technical problems since albumin and immunoglobulin concentrations are so high as to make immunoblotting and immunoprecipitation difficult. We therefore developed a method to separate and instantaneously lyse platelets from PRP (see Materials and methods). This method proved to be fast and reliable, and did not subject the platelets to the shock of pelleting before lysis. As shown in Figure 5A, treatment of PRP with increasing doses of ADP led to aggregation, which was first reversible (0.05−0.2 μM), then bi-phasic (0.25 μM), then irreversible (0.5 μM and above). Figure 5B displays an immunoblot of PTP-1B from the platelets shown in Figure 5A. As can be seen, PTP-1B cleavage did not occur in control platelets, or in platelets that exhibited only primary aggregation. However, when secondary aggregation occurred (bi-phasic curve for 0.25 μM ADP), PTP-1B cleavage was observed. We conclude that PTP-1B cleavage correlates with the transition from reversible to irreversible aggregation of human platelets in PRP.

Integrin signaling is necessary and sufficient for inducing PTP-1B cleavage

Since PTP-1B cleavage was a general feature of platelet aggregation and aggregation requires engagement of integrins

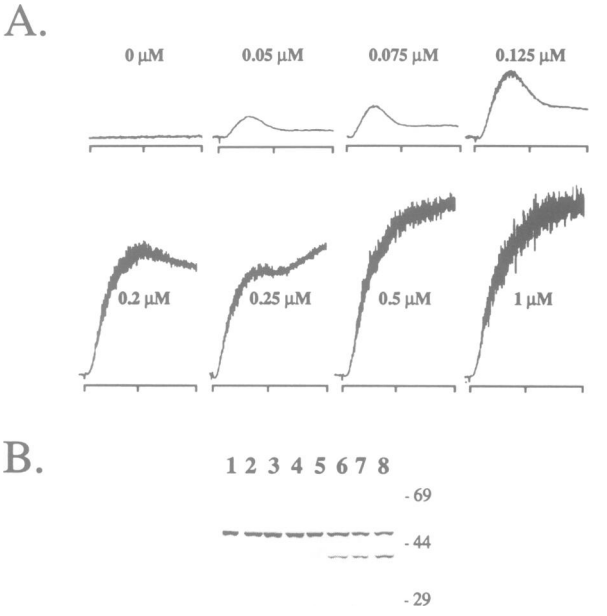

Fig. 5. PTP-1B cleavage accompanies the transition from primary to secondary platelet aggregation. (**A**) Aggregometer tracings from stirred PRP treated with increasing concentrations of ADP (in μM). Ordinate displays light transmission, abscissa displays time. Marks on horizontal bar below tracings indicate 1.5 min intervals. (**B**) Platelets from PRP displayed in panel A were separated from plasma proteins and directly lysed after 3 min of ADP treatment as described in Materials and methods. Approximately 10 μg of each lysate were resolved by SDS−PAGE and immunoblotted with anti-PTP-1B antibody #583N as described in Materials and methods. Lysates are from stirred PRP stimulated with 0 (lane 1), 0.05 (lane 2), 0.075 (lane 3), 0.125 (lane 4), 0.2 (lane 5), 0.25 (lane 6), 0.5 (lane 7) or 1 μM ADP (lane 8). The positions (in kDa) of prestained molecular weight markers are shown on the right of the figure.

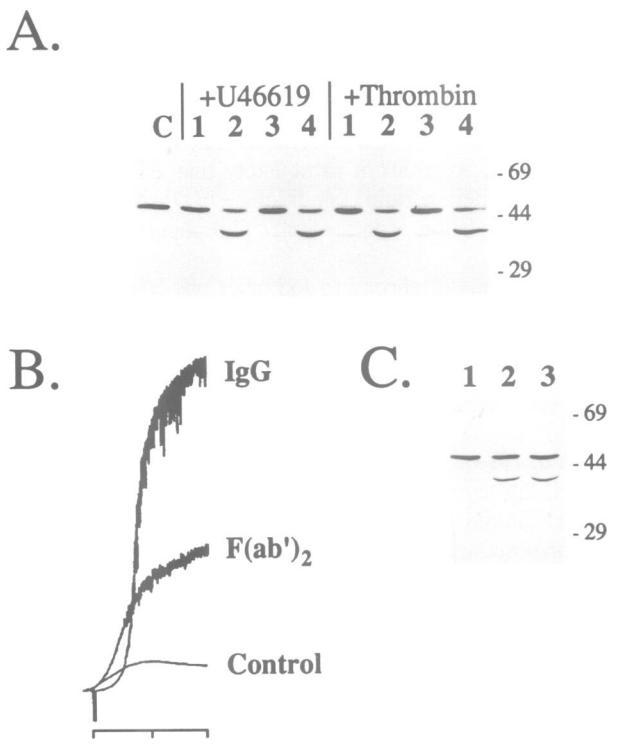

Fig. 6. Integrins are necessary and sufficient for inducing PTP-1B cleavage. (**A**) Control stirred, gel-filtered platelets in modified HEPES−Tyrode buffer supplemented with 1 mM $CaCl_2$ (lane C) were treated with 1 μM U46619 (+U46619) or 1 U/ml thrombin (+Thrombin), either while unstirred (lanes 1), stirred (lanes 2), pretreated with 200 μg/ml RGDS peptide for 5 min at 37°C and stirred (lanes 3), or pretreated with 200 μg/ml RGES peptide for 5 min at 37°C and stirred (lanes 4). Under these conditions, RGD-containing peptides will compete for fibrinogen binding to gpIIb-IIIa and prevent aggregation, whereas RGE-containing peptides will not. After 2 min of agonist treatment, platelets were lysed in 2 × SDS−PAGE sample buffer, resolved by SDS−PAGE and immunoblotted with anti-PTP-1B antibody #583N as described in Materials and methods. The positions (in kDa) of prestained molecular weight markers are shown at the right of the figure. (**B**) Stirred, gel-filtered platelets in HEPES−Tyrode buffer supplemented with 1 mM $CaCl_2$ and 0.3 mg/ml fibrinogen (Control) were treated with P256 anti-gpIIb-IIIa mAb either as intact IgG (IgG; 4 μg/ml) or as an $F(ab')_2$ fragment (8 μg/ml). Aggregometer tracings are shown. Ordinate displays light transmission, abscissa displays time. Marks on horizontal bar below tracings indicate 1 min intervals. (**C**) Stirred, gel-filtered platelets shown in panel B treated with PBS (Control; lane 1), 4 μg/ml P256 mAb IgG (lane 2) or 8 μg/ml P256 mAb $F(ab')_2$ fragments (lane 3). After 2 min of treatment, platelets were lysed in 2 × SDS−PAGE sample buffer, resolved by SDS−PAGE and immunoblotted with anti-PTP-1B antibody #583N as described in Materials and methods. The positions (in kDa) of prestained molecular weight markers are shown at the right of the figure.

on the platelet surface, we asked whether PTP-1B cleavage involved integrin signaling. In Figure 6A (lane C) stirred control platelets lysed after 2 min contained only the full-length, 50 kDa form of PTP-1B. In agreement with Figure 4, treatment of unstirred platelets with either U46619 or thrombin (lanes 1) did not trigger PTP-1B cleavage, whereas agonist treatment and stirring (lanes 2) resulted in cleavage of PTP-1B. Pretreatment of platelets with an excess of the peptide Arg-Gly-Asp-Ser (RGDS), which blocks gpIIb-IIIa engagement, completely blocked aggregation (data not shown) and PTP-1B cleavage (lanes 3), whereas pretreatment with an excess of the peptide RGES (which has no effect on integrin interactions) did not block either aggregation (data not shown) or PTP-1B cleavage (lanes 4). GpIIb-IIIa engagement thus appears to be necessary for inducing PTP-1B cleavage.

We next asked whether aggregation induced by gpIIb-IIIa engagement was sufficient for inducing PTP-1B cleavage. To address this, we treated gel-filtered platelets with a monoclonal antibody (mAb), P256, specific for gpIIb-IIIa; this mAb has been shown to induce platelet aggregation (Bachelot *et al.*, 1990). As shown by the aggregometer tracings in Figure 6B, mAb P256, added either as whole IgG or as $F(ab')_2$ fragments, induced platelet aggregation in the presence of fibrinogen and stirring. The observed magnitude and timing of aggregation in response to these antibodies were exactly as had been reported previously (Bachelot *et al.*, 1990; C.Bachelot, personal communication). Intact IgG could potentially activate the platelet Fc receptor, whereas the $F(ab')_2$ fragment of mAb P256 should only activate gpIIb-IIIa. As shown in Figure 6C, $F(ab')_2$ fragments of mAb P256 promote PTP-1B cleavage to the same extent as IgG, despite the fact that engagement of Fc receptors by IgG evokes a number of additional secondary events (Bachelot *et al.*, 1990). We conclude that engagement of the integrin gpIIb-IIIa is both necessary and (in the presence of stirring) sufficient for inducing cleavage of PTP-1B.

Changes in protein tyrosyl phosphorylation accompany PTP-1B cleavage

We next asked whether cleavage, relocation and activation of PTP-1B correlated with changes in platelet protein tyrosyl phosphorylation. After subcellular relocation to the cytosol, protein substrates on intracellular membranes could conceivably become either hyperphosphorylated or dephosphorylated, depending on the accessibility of membrane-bound PTP-1B to these proteins, whereas cytosolic and plasma membrane proteins, normally inaccessible to PTP-1B, might become dephosphorylated. Although we wished to determine the subcellular localization of phosphotyrosyl proteins in response to thrombin and aggregation, we found it technically impossible to achieve reproducibly cell fractionation of aggregated platelets. Treatment with the calcium ionophore A23187, in the absence of stirring, however, allowed us to analyze the status of tyrosyl phosphorylation in different subcellular fractions and to enquire how phosphorylation correlated with PTP-1B relocation and activation.

As shown in Figure 7A (Control), only the full-length, membrane-associated form of PTP-1B was found in unstimulated platelets. After stimulation with A23187 (+A23187), complete cleavage of PTP-1B to its 42 kDa form occurred and this form no longer associated with membranes. Pretreatment of platelets with calpeptin (+Calpeptin +A23187) resulted in inhibition of >90% of PTP-1B cleavage in response to A23187. In accordance with previously published results (reviewed in Shattil and Brugge, 1991), immunoblotting of unstimulated platelet lysates with an anti-phosphotyrosine mAb revealed several tyrosyl phosphorylated proteins with predominant bands at 60, 62, 64 and 74 kDa (Figure 7B, Control). Virtually all of the major tyrosyl phosphorylated bands were in the membrane (particulate) fraction. As shown in Figure 7B, treatment with calcium ionophore for 2 min (+A23187) resulted in global dephosphorylation of tyrosyl phosphorylated bands in the membrane fraction and the appearance of two new phosphotyrosyl protein bands (at ~52 and 54 kDa) in the

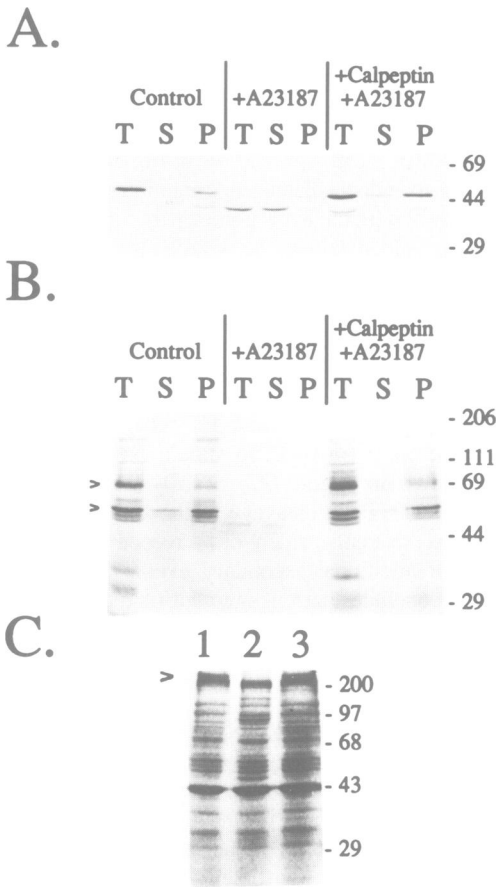

Fig. 7. Subcellular relocation and activation of PTP-1B by calcium ionophore treatment correlates with global dephosphorylation of phosphotyrosyl proteins. (**A**) Unstirred, gel-filtered platelets in modified HEPES−Tyrode buffer supplemented with 1 mM $CaCl_2$ (Control) were stimulated with 1 μM A23187 for 2 min with (+Calpeptin/+A23187) or without (+A23187) pretreatment with 40 μM calpeptin for 10 min. Platelets were then subjected to hypotonic lysis and subcellular fractionation as described in Materials and methods. Centrifugation of the total lysate (T) at 100 000 *g* yielded soluble (S) and particulate (P) fractions. Equal volumes of each fraction (10 μg of total lysate) were resolved by SDS−PAGE and immunoblotted with antibody #583N, specific for the N-terminus of PTP-1B, as described in Materials and methods. The positions (in kDa) of prestained molecular weight markers are shown on the right of the figure. (**B**) 20 μg of the platelet lysates described in panel A were resolved by SDS−PAGE and immunoblotted with anti-phosphotyrosine mAb 4G10 as described in Materials and methods. (**C**) 10 μg of total lysates from control (lane 1), A23187-treated (lane 2) or calpeptin pretreated/A23187-treated (lane 3) platelets described in panel A were resolved by SDS−PAGE and stained with Coomassie blue. Arrowhead marks position of known high molecular weight calpain substrates (see Discussion) not individually resolved on this 10% polyacrylamide gel. The positions (in kDa) of unstained molecular weight markers are shown at the right of the figure.

soluble fraction. Tyrosyl dephosphorylation not only correlated with PTP-1B cleavage (Figure 7A), but was completely inhibited when A23187-induced cleavage of PTP-1B was blocked by pretreatment with calpeptin (Figure 7A and B, +Calpeptin +A23187). Figure 7C displays Coomassie blue-stained total protein from these lysates to verify that A23187 treatment did not lead to global protein cleavage. Indeed, the only bands visibly cleaved in the presence of A23187 (arrowhead) have been previously identified as calpain substrates (e.g. p235 talin, p270 actin binding protein, etc.; see Discussion). Although our data cannot rigorously establish that PTP-1B is the phosphatase responsible for the observed dephosphorylation, the inhibition of dephosphorylation by calpeptin indicates that the responsible PTP(s) must be activated by calpain-induced proteolysis. Given its abundance and its demonstrable cleavage by calpain in platelets, we think it most likely that PTP-1B cleavage results, under certain conditions, in the nearly global dephosphorylation of intracellular phosphotyrosyl-containing proteins.

Treatment with thrombin and other physiological agonists is known to cause dramatic changes in the pattern of protein tyrosyl phosphorylation in platelets (reviewed in Shattil and Brugge, 1991). Tyrosyl phosphorylation of platelet proteins has been reported to occur in three 'waves' peaking at 5−20 s, 1−3 min and 3−5 min, respectively (Ferrell and Martin, 1989; Golden and Brugge, 1989). Figure 8A (bottom panel) displays the pattern of protein tyrosyl phosphorylation in gel-filtered platelets treated with 1 U/ml of thrombin in the presence and absence of stirring. In the absence of stirring, changes in the extent of tyrosyl phosphorylation of several proteins was observed. For example, the 140 kDa band indicated by arrowhead 1 showed a detectable increase within 1 min, which peaked at 3 min and diminished by 10 min. The 125 kDa band indicated by arrowhead 2 exhibited a markedly different temporal pattern, with its phosphorylation lagging behind that of the 140 kDa protein and then remaining constant for the rest of the experiment. Tyrosyl phosphorylation of the 112 kDa protein (arrowhead 3) lagged behind bands 1 and 2, and did not peak until 5 min after thrombin addition. The 75 kDa band indicated by arrowhead 4 actually showed two separate 'waves' of tyrosyl phosphorylation, one which peaked at 30 s and another which peaked at 10 min. In the presence of stirring, however, the pattern of tyrosyl phosphorylation was greatly altered. Tyrosyl phosphorylation of band 1 began to rise at 1 min, but by 3 min was undetectable. Band 2 displayed a characteristic increase, but was dephosphorylated rapidly by 5 min. Band 3 also showed a characteristic rise in tyrosyl phosphorylation but instead of increasing steadily over time, began to decrease at 5 min. Interestingly, band 4 retained its first peak of tyrosyl phosphorylation at 30 s (preceding significant PTP-1B cleavage), but not its second peak seen at 10 min (following PTP-1B cleavage). As shown in Figure 8A (top panel) and confirming data shown in Figure 4, thrombin in the presence of stirring caused time-dependent cleavage of PTP-1B. Approximately 50% of full-length PTP-1B was converted to the 42 kDa form within 3−5 min after thrombin addition. In the absence of stirring, only a trace amount of cleaved PTP-1B was seen 10 min after thrombin addition. Comparison of the top panel of Figure 8A with the bottom panel reveals that the blunted increases in tyrosyl phosphorylation seen with aggregation (thrombin and stirring) correlate in time with cleavage, subcellular relocation and activation of PTP-1B.

Similar and complementary results were obtained in experiments using an RGD-containing peptide. As discussed above, this peptide blocks integrin-mediated platelet aggregation by competitive binding to the fibrinogen receptor gpIIb-IIIa. Figure 8B (bottom panel) displays the pattern of protein tyrosyl phosphorylation in stirred, gel-filtered platelets treated with 1 U/ml of thrombin after pretreatment with either RGES (not blocking) or RGDS (blocking) peptide. Pretreatment with RGES peptide yielded a pattern of tyrosyl phosphorylation with kinetics similar to that shown in

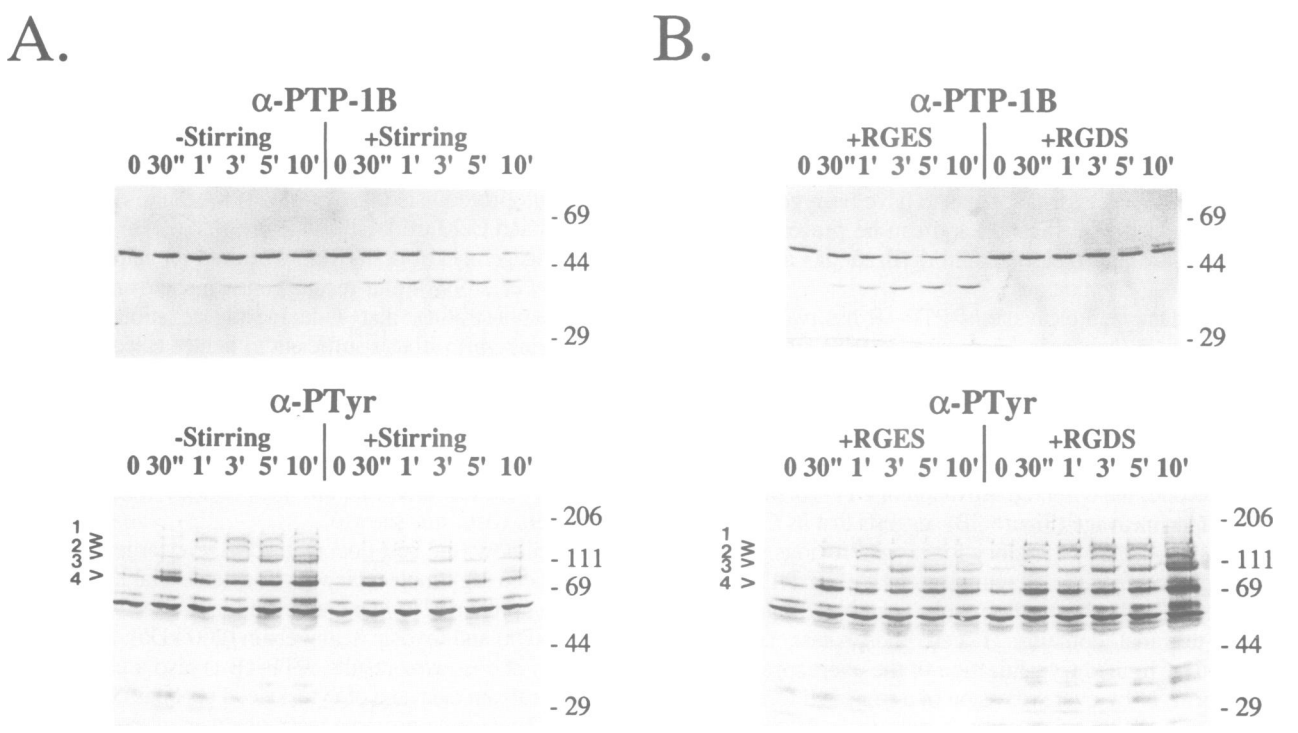

Fig. 8. Subcellular relocation and activation of PTP-1B by physiological agonists correlates with dephosphorylation of a subset of phosphotyrosyl proteins. (**A**) Gel-filtered platelets in modified HEPES−Tyrode buffer supplemented with 1 mM $CaCl_2$ were treated with 1 U/ml thrombin in the absence (−Stirring) or presence (+Stirring) of stirring. Platelets were lysed at the indicated times after thrombin treatment by the addition of 5 × SDS−PAGE sample buffer supplemented with 5 mM sodium orthovanadate and 1 mM phenylarsine oxide. Total protein from the equivalent of 1.3 × 10^7 platelets was resolved by SDS−PAGE and immunoblotted with anti-PTP-1B antibody #583N (α-PTP-1B) or anti-phosphotyrosine mAb 4G10 (α-PTyr), as described in Materials and methods. The positions (in kDa) of prestained molecular weight markers are shown on the right of each panel. Arrowheads (numbered 1−4) at the left of the α-PTyr immunoblot indicate representative proteins whose phosphotyrosyl content changes over the course of the experiment. (**B**) Gel-filtered platelets in modified HEPES−Tyrode buffer supplemented with 1 mM $CaCl_2$ were pretreated for 5 min at 37°C with either 200 μg/ml RGES peptide or 200 μg/ml RGDS peptide, stirred and treated with 1 U/ml thrombin. Platelets were lysed at the indicated times, as described in panel A. Total protein from the equivalent of 1.3 × 10^7 platelets was resolved by SDS−PAGE and immunoblotted with anti-PTP-1B antibody #583N (α-PTP-1B) or anti-phosphotyrosine mAb 4G10 (α-PTyr), as described in Materials and methods. The positions (in kDa) of prestained molecular weight markers are shown at the right of each panel. Arrowheads (numbered 1−4) at the left of the α-PTyr immunoblot indicate representative proteins whose phosphotyrosyl content changes over the course of the experiment.

Figure 8A (bottom panel) for stirred platelets treated with thrombin. Pretreatment with RGDS peptide, however, completely blocked both aggregation (data not shown) and the dephosphorylation seen in the presence of RGES peptide. Moreover, a unique temporal pattern of tyrosyl phosphorylation was observed. Tyrosyl phosphorylation of bands 2 and 3 reached steady state before bands 1 and 4 did not show a dramatic rise and fall between 30 s and 5 min. In addition, a 145 kDa band showed increased tyrosyl phosphorylation that reached steady state by 30 s and tyrosyl phosphorylation of a cluster of bands at 36−40 kDa was seen to rise and fall. Although not visible in Figure 8B, tyrosyl phosphorylation of proteins at 130 and 110 kDa was blocked by RGDS peptide when resolved on a larger, lower percentage gel (data not shown; see also Oda *et al.*, 1992). As shown in Figure 8B (top panel) and confirming data shown in Figure 6, PTP-1B cleavage only occurred in the presence of integrin-induced aggregation. Once again, PTP-1B cleavage, subcellular relocation and enzymatic activation correlated with an attenuated increase in thrombin-induced tyrosyl phosphorylation.

Discussion

PTP-1B is a ubiquitous and abundant enzyme, which undergoes multiple post-transcriptional and post-translational cell cycle-dependent modifications. In this paper, we describe a distinct and novel mechanism for regulation of PTP-1B in a non-cycling cell, the platelet. In human platelets activated by either pharmacological or physiological agonists, PTP-1B is proteolytically cleaved within its C-terminus by calpain. Calpain-induced cleavage causes subcellular relocation of PTP-1B from membranes to the cytosol and activation of its enzymatic activity. PTP-1B cleavage is a general feature of agonist-induced platelet aggregation, occurring in PRP, the normal, physiological medium for platelet aggregation and signaling through the integrin gpIIb-IIIa is necessary for inducing cleavage. Moreover, PTP-1B cleavage occurs during the transition from reversible to irreversible aggregation and correlates with specific changes in the pattern of protein tyrosyl phosphorylation.

PTP-1B normally associates with intracellular membranes via an ER-targeting sequence encoded by its C-terminal 35 amino acids (amino acids 400−435; Frangioni *et al.*, 1992; Woodford-Thomas *et al.*, 1992), whereas its catalytic domain is confined to the N-terminus (amino acids ~40−276; Charbonneau *et al.*, 1989). It also contains an intervening stretch of ~120 amino acids of unknown function. Our immunological and biochemical analyses establish that calpain-induced cleavage of PTP-1B occurs within this region. However, our immunological reagents do not allow us to exclude a small change at the N-terminus as well. Experiments

to determine the precise structure of the cleaved form of PTP-1B in platelets are in progress. It should be emphasized that the cleaved (42 kDa) form of PTP-1B described in this paper, which results from calpain-catalyzed cleavage *in vivo*, is distinct from the shorter (39 kDa) form of PTP-1B originally isolated from human placenta (Tonks *et al.*, 1988a,b). The latter form appears to have been generated by proteolysis during purification and can be prevented by the addition of strong protease inhibitors (Brautigan and Pinault, 1991a).

Calpain-catalyzed cleavage of PTP-1B has two important biochemical consequences. First, cleaved PTP-1B is rendered freely soluble (Figure 3A). This represents *in vivo* confirmation of previously published work demonstrating that deletion of PTP-1B's C-terminus prevents membrane association (Frangioni *et al.*, 1992; Woodford-Thomas *et al.*, 1992). Second, the observed activation of PTP-1B's enzymatic activity upon cleavage (Figure 3B) suggests that its C-terminus performs a negative regulatory function. Previous work had also suggested that the enzymatic activities of PTP-1B and the related T cell phosphatase were negatively regulated by their C-terminal domains. T cell phosphatase activity is increased by limited trypsinization of the overexpressed full-length form, or by overexpression of a truncated form lacking the C-terminal 98 amino acids (Cool *et al.*, 1990); similar results were obtained with purified full-length and C-terminally truncated T cell phosphatase produced in a baculovirus expression system (Zander *et al.*, 1991). Likewise, limited trypsinization of purified full-length PTP-1B results in a 35 kDa molecule with increased activity (Roome *et al.*, 1988; Brautigan and Pinault, 1991b). Data presented herein provide the first direct evidence that the C-terminus (C-terminal amino acids 65–75) of PTP-1B has a negative regulatory function *in vivo*.

Seryl phosphorylation of PTP-1B at mitosis (Flint *et al.*, 1993; Schievella *et al.*, 1993; V.Shifrin, J.V.Frangioni and B.G.Neel, unpublished observations) and phosphorylation in response to activation of protein kinase C (Flint *et al.*, 1993; J.V.Frangioni, V.Shifrin and B.G.Neel, unpublished observations) occur near the calpain cleavage site (Flint *et al.*, 1993; J.V.Frangioni and B.G.Neel, unpublished observations). The effects of seryl phosphorylation on susceptibility to calpain cleavage and vice versa, are under investigation. Since membrane-bound, full-length PTP-1B is part of a high molecular weight complex (Brautigan and Pinault, 1991a; P.H.Beahm and B.G.Neel, unpublished observations), part of the mechanism by which calpain cleavage increases PTP-1B's enzymatic activity might be to dissociate PTP-1B from proteins that would otherwise suppress its activity.

We have established the outlines of a signal transduction pathway for inducing PTP-1B cleavage (Figure 9). Engagement of the platelet integrin gpIIb-IIIa is a necessary and sufficient first step in this pathway. Several recent reports have implicated integrins in the regulation of tyrosine kinases in platelets (Oda *et al.*, 1992; Clark and Brugge, 1993) and other cell types (Kornberg *et al.*, 1991). With the demonstration that gpIIb-IIIa is required for inducing calpain cleavage of PTP-1B, it is apparent that integrins can regulate tyrosine phosphatases as well as tyrosine kinases. Cleavage of PTP-1B in human platelets is catalyzed by the calcium-dependent, neutral protease calpain. Calpains are a family of cysteine proteases, which have been implicated in a variety of processes (for a review see Croall and DeMartino, 1991); however, their precise physiological function is unknown. μ-calpain is the predominant calpain present in human platelets (Kambayashi and Sakon, 1989) and thus is the most likely candidate for the enzyme that catalyzes the cleavage of PTP-1B. Calpain is activated through a complex series of steps which include relocation from soluble to particulate fractions and autoproteolysis (Fox *et al.*, 1983; Samis *et al.*, 1987; Croall and DeMartino, 1991). Previous workers have shown that engagement of gpIIb-IIIa is required for calpain activation (Fox *et al.*, 1993). Our results confirm and extend this work by demonstrating that inducing aggregation, simply by clustering gpIIb-IIIa, is sufficient to induce calpain activation. The detailed mechanism by which integrin engagement leads to calpain activation is unclear. An increase in intracellular calcium appears to be required, however, since only trace cleavage of PTP-1B was seen in response to thrombin when 1 mM extracellular calcium was omitted from gel-filtered platelets (data not shown).

Until now, the best documented *in vivo* targets for calpain have been high molecular weight cytoskeletal proteins: actin binding protein (270 kDa), spectrin (doublet at 240 kDa), talin (235 kDa) and myosin heavy chain (200 kDa). Based on our *in vivo* and *in vitro* results, PTP-1B is also a calpain target. Since calpain cleavage of cytoskeletal proteins correlates with and is believed to promote rearrangement of the cytoskeleton, these data suggest that cytoskeletal rearrangement and protein tyrosyl dephosphorylation may be coordinately regulated in platelets.

Although calpain is capable of cleaving PTP-1B to completion *in vitro* (Figure 2F) proteolysis *in vivo* is limited, such that PTP-1B persists as a stable, 42 kDa molecule (e.g. Figure 2A–D). Calpain's relocation to intracellular membranes could prevent it from interacting further with cleaved PTP-1B, which has relocated to the cytosol. Alternatively, calpain may be rapidly inactivated *in vivo* either by autoproteolysis, by another modification (e.g. phosphorylation) or by binding to an endogenous inhibitor(s) such as calpastatin (reviewed in Croall and DeMartino, 1991). A final possibility is that the high enzyme concentrations utilized in the *in vitro* calpain reactions may have promoted proteolysis at less favored sites, thus leading to complete hydrolysis of PTP-1B.

Since PTP-1B accounts for ~0.2% of total detergent soluble platelet protein and calpain-catalyzed cleavage results in relocation and enzymatic activation of between 40 and 100% of PTP-1B's mass to the cytosol, one might expect dramatic changes in platelet protein tyrosyl phosphorylation. Indeed, in response to calcium ionophore, there is global dephosphorylation of tyrosyl phosphoproteins (Figure 7B). The most economical explanation of these results is that calcium ionophore induces PTP-1B cleavage and that PTP-1B dephosphorylates phosphotyrosyl proteins. The net effect, global dephosphorylation, actually represents a 'balance' between weak activation of tyrosine kinases and strong activation of PTP-1B. Consistent with this hypothesis, low dose A23187 (0.1 μM) leads to minimal PTP-1B cleavage and a time-dependent net increase in tyrosyl phosphorylation (data not shown; see below). Since cleaved PTP-1B shows only 2-fold activation of its enzymatic activity, yet calcium ionophore treatment leads to complete dephosphorylation, it is likely that increased access to substrates plays a major role in the mechanism of PTP-1B's actions. Alternatively, the immune complex assay may underestimate the extent of activation of PTP-1B by calpain cleavage *in vivo*. It should

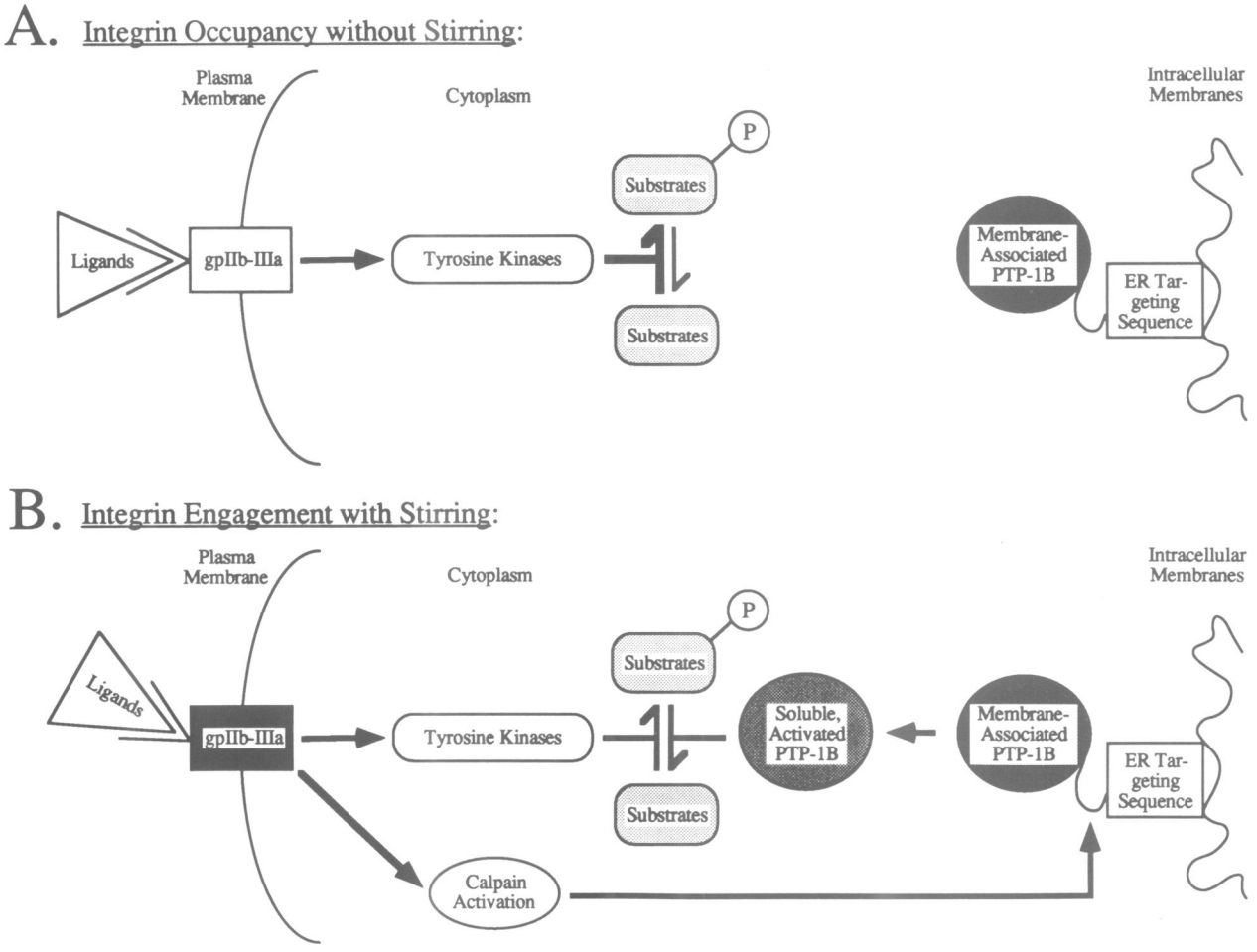

Fig. 9. Model for integrin-dependent control of PTP-1B cleavage and protein tyrosyl phosphorylation. (**A**) In the absence of stirring, binding of ligands to the integrin gpIIb-IIIa leads to activation of one or more tyrosine kinases and subsequent increased phosphorylation of cellular protein substrates on tyrosyl residues. Under these conditions, calpain is not activated, PTP-1B remains tethered to intracellular membranes, and there is relative hyperphosphorylation of certain protein substrates. (**B**) Ligand binding in the presence of stirring activates gpIIb-IIIa such that subsequent activation of both tyrosine kinases and calpain occur. Activated calpain catalyzes the cleavage of PTP-1B within its C-terminus. Calpain-catalyzed cleavage renders PTP-1B's catalytic domain freely soluble and enzymatically activated. Substrates phosphorylated by activated tyrosine kinases can be dephosphorylated by PTP-1B. The overall level of tyrosyl phosphorylation is thus attenuated under conditions that result in cleavage of PTP-1B.

be noted that we only measured PTP-1B enzymatic activation following ionophore-induced cleavage, although we think it likely that similar activation occurs following cleavage induced by other treatments.

Previous workers have reported that following treatment with agonists such as thrombin, platelet tyrosyl phosphorylation occurs in temporal 'waves' representing protein phosphorylation and dephosphorylation (reviewed in Shattil and Brugge, 1991). Figure 8 shows the correlation between thrombin-induced tyrosyl phosphorylation and calpain-catalyzed cleavage of PTP-1B. Treatment with thrombin, in the absence of stirring or with occupancy of gpIIb-IIIa by RGDS peptide, leads to a time-dependent increase in several proteins including those at 140, 125, 112 and 75 kDa. Under these conditions, little or no PTP-1B cleavage occurs. In the presence of stirring, during which aggregation occurs, PTP-1B is cleaved and proteins that would have otherwise shown a dramatic increase in tyrosyl phosphorylation instead show an attenuated increase. Several workers have reported that the tyrosyl phosphorylation of certain proteins is dependent on aggregation (Ferrell and Martin, 1989; Golden *et al.*, 1990; Bachelot *et al.*, 1992; Oda *et al.*, 1992); we also found that the phosphorylation of proteins at 130 and 110 kDa was aggregation-dependent. However, we have found that after thrombin stimulation, the tyrosyl phosphorylation of several proteins is enhanced in the absence of stirring, relative to the level attained in the presence of stirring (Figure 8). We propose that PTP-1B cleavage, subcellular relocation and enzymatic activation account for at least part of the blunted increase seen with aggregation due to dephosphorylation of substrates shared with the activated kinases (Figure 9). The precise level of tyrosyl phosphorylation of any given protein following platelet activation probably depends on a delicate balance in space and time between the opposing actions of several tyrosyl kinases and at least one tyrosine phosphatase, cleaved PTP-1B.

PTP-1B cleavage could have several possible physiological roles. Circulating platelets probably encounter weak stimuli frequently. Our finding that PTP-1B cleavage correlates with the transition from reversible, primary responses to irreversible, secondary responses in PRP (Figure 5) suggests that PTP-1B cleavage, subcellular relocation and activation may be part of the mechanism by which platelets become committed to aggregate irreversibly in response to an *in vivo* stimulus of sufficient strength. Previous studies on the role of calpain activation in platelet aggregation have been

inconclusive and/or contradictory (reviewed in Croall and DeMartino, 1991). Although aggregation induced by thrombin and stirring can clearly occur under conditions where PTP-1B is not cleaved (see above), it is still possible that PTP-1B cleavage plays a role in controlling aggregation induced by weak agonists such as ADP (in the presence of fibrinogen). Unfortunately, although calpeptin is effective in inhibiting PTP-1B cleavage in unstirred platelets in response to calcium ionophores, in our hands, it does not completely inhibit PTP-1B cleavage after agonist-induced aggregation. The reason for this is unclear, but these agonists may activate calpain in such a way (e.g. post-translational modification, relocalization) that it becomes refractory to calpeptin inhibition. We have thus been unable to use calpeptin to determine whether PTP-1B cleavage is causally involved in aggregation. Alternatively, the correlation between PTP-1B cleavage and irreversible aggregation may indicate that cleaved PTP-1B regulates an event distal to irreversible aggregation. One possibility is microvesicle shedding, which has been previously shown to be controlled by calpain (Fox *et al.*, 1990, 1991). Other possibilities include cytoskeletal rearrangement, secretion and/or clot retraction. In this regard, introduction of a truncated form of PTP-1B into pancreatic acinar cells has been shown to promote secretion (Jena *et al.*, 1991).

Finally, it should be noted that the generation of a soluble, activated PTP during physiological platelet activation is reminiscent of *Yersinia* virulence mechanisms. Recently, a family of essential *Yersinia* virulence determinants (the products of the *yop*H gene in *Yersinia pseudotuberculosis* and of the *yop*51 gene in *Yersinia enterocolitica*) have been found to be protein phosphotyrosine phosphatases (Guan and Dixon, 1990). By mechanisms that are not well understood, *Yersinia* phosphatases enter the cytosol of target mammalian cells as soluble, active enzymes, promiscuously dephosphorylate host cellular phosphotyrosyl proteins and kill the cell (Bliska *et al.*, 1991, 1992). Elimination of tyrosine phosphatase activity by mutagenesis of the essential catalytic cysteine residue completely eliminates virulence (Guan and Dixon, 1990; Bliska *et al.*, 1991). Viewed in this context, PTP-1B subcellular relocation and activation might be part of an endogenous pathway for mammalian cell death, which has been usurped by *Yersinia* and perhaps other pathogenic organisms (Guan *et al.*, 1991). There is precedence for a cleaved, activated non-transmembrane tyrosine phosphatase being toxic to cells when overexpressed heterologously: substantially fewer clones were obtained upon transfection of BHK cells with vectors directing the synthesis of C-terminally truncated T cell phosphatase compared with control cells transfected with the parent vector alone (Cool *et al.*, 1992). Our hypothesis predicts that PTP-1B cleavage may occur in other cell systems, under conditions that trigger cell death. Indeed, we have recently found that PTP-1B cleavage, relocation and activation occur in human foreskin epidermal keratinocytes in response to calcium ionophore-induced differentiation/cell death (J.V.Frangioni, Y.Zhao, J.Krueger, H.Hanafusa and B.G.Neel, unpublished observations). Further studies will be required to determine whether cleavage of PTP-1B plays a general role in calcium-mediated cell death pathways.

Materials and methods

Chemicals and reagents

Calcium ionophore A23187 (free acid) was purchased from Calbiochem. Thrombin was purchased from Armour Pharmaceuticals. U46619 was purchased from Biomol (Plymouth Meeting, PA). Human fibrinogen (Grade L) was purchased from Kabi and purified by a previously published method (Shiba *et al.*, 1991). Collagen was purchased from Hormon-Chemie. Calpeptin was the generous gift of Dr Junichi Kambayashi (Osaka University) and was also purchased from Nova Biochem. Phenylarsine oxide (PAO) was purchased from Aldrich. Adenosine 5'-diphosphate (ADP), apyrase (type VIII), dibucaine hydrochloride, phorbol 12-myristate 13-acetate (PMA), RGDS peptide, RGES peptide, sodium orthovanadate and all other chemicals were purchased from Sigma.

Preparation of human platelets

Human blood was drawn by venipuncture into 0.1 vol of 3.8% (w/v) trisodium citrate and gently mixed. PRP was prepared by centrifuging whole blood at 200 *g* for 20 min at room temperature (RT) and aspirating to no less than 2 cm above the buffy coat. For experiments using calcium ionophore or dibucaine, PRP was incubated with aspirin (1 mM) for 30 min at RT. PGE_1 was added (from a 1000× stock in absolute ethanol) to a final concentration of 1 μM and the PRP was centrifuged at 1200 *g* for 15 min at RT to form a soft platelet pellet. The pellet was resuspended in 1 ml of a modified HEPES–Tyrode buffer (10 mM HEPES, pH 7.4, 130 mM NaCl, 8.9 mM $NaHCO_3$, 0.8 mM KH_2PO_4, 0.8 mM $MgCl_2$ and 5.6 mM dextrose) containing 1/10 vol of 3.8% sodium citrate and apyrase (2 U/ml). To prepare gel-filtered platelets, PGE_1 (1 μM) was again added and the platelet suspension was layered onto a Sepharose-2B gel filtration column (10 ml bed volume; Pharmacia LKB) preequilibrated with modified HEPES–Tyrode buffer.

Platelet assays

Gel-filtered platelets were adjusted to a concentration of $3-6 \times 10^8$/ml in modified HEPES–Tyrode buffer containing 1 mM $CaCl_2$. Aggregation and shape change were monitored on a lumi-aggregometer (Chronolog, Model PICA) at 37°C. Where indicated, platelets were stirred at a rate of 1000 r.p.m.

Polyclonal and monoclonal antibodies

Affinity-purified antibodies #300F, #300C and #932N have been described previously (Frangioni *et al.*, 1992; Frangioni and Neel, 1993). Affinity-purified rabbit anti-PTP-1B N-terminus antibody #583N was prepared and characterized exactly as described for antibody #932N. Anti-PTP-1B monoclonal antibody FG6 was the generous gift of Drs David E.Hill and Karen A.Johnson (Oncogene Science). Anti-phosphotyrosine antibody 4G10 (Druker *et al.*, 1989) was the generous gift of Dr Brian J.Druker (Dana Farber Cancer Institute). Anti-gpIIb-IIIa mAb P256 and its $F(ab')_2$ fragment were the generous gifts of Dr Christilla Bachelot (Harvard Medical School).

In vitro calpain proteolysis

μ- and m-calpains, purified from bovine heart by a modification of a previously published method (Croall and DeMartino, 1984) were the generous gifts of Dr Dorothy Croall (University of Maine). Full-length PTP-1B was immunoprecipitated from [^{35}S]methionine-labeled, randomly growing HeLa cells using antibody #300F as described previously (Frangioni *et al.*, 1992), washed in 20 mM HEPES, pH 7.0 and 150 mM NaCl, and resuspended in the same buffer. Each 25 μl reaction was performed in 50 mM HEPES, pH 7.4, 30 mM NaCl, 1 mM DTT, 0.1 mg/ml BSA and either 10 mM $CaCl_2$ or 2 mM EDTA, using 2.5 μl of immunoprecipitate and 2.5 μl of calpain (diluted in 50 mM HEPES, pH 7.4, 1 mM DTT, 0.1 mg/ml BSA). The reactions were incubated at 30°C, 20 min, terminated by the addition of 6.3 μl of 5 × SDS–PAGE sample buffer (350 mM Tris, pH 6.8, 15% SDS, 10% glycerol, 3.6 M β-mercaptoethanol, 0.01% bromophenol blue), resolved on a 10% SDS–polyacrylamide gel, soaked for 20 min in 1 M sodium salicylate, dried and autoradiographed using Kodak XAR film.

Immune complex phosphatase assay

^{32}P-labeled Raytide (Oncogene Science) was prepared to a specific activity of ~25 μCi/nmol as described previously by Frangioni *et al.* (1992). Gel-filtered platelets in modified HEPES–Tyrode buffer were stimulated with agonist for the indicated times and lysed by the addition of 2 × NP-40 lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 2% NP-40, 100 mM sodium fluoride, 2 mM EDTA, 200 μM leupeptin and 4 mM PMSF). After clarification at 15 000 *g* (15 min at 4°C), the supernatant was transferred to a new tube and protein was measured by the BCA method using a commercially available kit (Pierce). Lysates were adjusted to 200 μg/ml and 50 μl were immunoprecipitated for 1 h on ice using mAb FG6 at a final concentration of 10 μg/ml. Immune complexes were collected by incubating with 5 μl of fixed *Staphylococcus aureus* (Pansorbin, Calbiochem) for 20 min, 4°C, followed by centrifugation, three washes in 1 × NP-40 lysis buffer (adjusted to 150 mM NaCl) and resuspension in 50 μl of the same buffer. Five microliters of this suspension were incubated in a 50 μl reaction containing 25 mM HEPES, pH 7.0, 10 mM DTT, 100 nM [^{32}P]Raytide at 30°C for

various times. The concentration of phosphorylated Raytide used in these reactions was less than saturating for the enzyme. The reaction was terminated by the addition of 800 μl of acid-charcoal [0.9 M HCl, 90 mM Na pyrophosphate, 2 mM NaH_2PO_4 and 4% (v/v) Norit A], vortexed, centrifuged for 15 000 *g* for 5 min at room temperature and 400 μl of supernatant were counted as described by Streuli *et al.* (1989).

Subcellular fractionation of platelets

After agonist treatment, 500 μl of gel-filtered platelets were lysed by the addition of an equal volume of ice-cold hypotonic lysis buffer (20 mM Tris, pH 7.4, 1 mM EGTA, 20 μg/ml leupeptin, 1 mM sodium orthovanadate, 100 μM phenylarsine oxide) followed by vortexing. 250 μl of total lysate were diluted with 250 μl of hypotonic buffer and 500 μl of 2 × SDS−PAGE sample buffer yielding the 'total' fraction. The remaining 250 μl of lysate were centrifuged at 100 000 *g* (4°C for 30 min). The supernatant was transferred to a new tube and the pellet was resuspended in 250 μl of hypotonic lysis buffer and centrifuged at 100 000 *g* (4°C for 30 min). The resultant supernatant was pooled with the first and 500 μl of 2 × SDS−PAGE sample buffer were added to yield the 'supernatant' fraction. The 'pellet' fraction was obtained by resuspending the membrane pellet in 1 ml of 1 × SDS−PAGE sample buffer.

Isolation of platelet proteins from PRP

For experiments using PRP, platelets were instantaneously separated from plasma proteins and lysed. This was accomplished by adding 250 μl of non-reducing 1 × SDS−PAGE sample buffer supplemented with 100 μM PAO and 1 mM sodium orthovanadate to a 1 ml polystyrene centrifuge tube (Fisher #4-978-145) and overlaying with 180 μl of a 7:2.4 mixture of silicon oils #550 and #556 (Dow Chemical Co.). After agonist treatment, EGTA was added to the PRP to a final concentration of 10 mM and 500 μl of PRP were layered onto the silicon oil and centrifuged at RT for 1 min at 7000 *g*. Under these conditions, plasma proteins remain above the silicon oil mixture, while platelets spin through the silicon oil mixture and are lysed. Plasma and silicon oils were then aspirated and an ultra-thin pipette tip was used to recover the bottom 200 μl of the non-reduced SDS−PAGE lysate. For immunoblotting of PRP lysates, an aliquot was adjusted to 0.7 M β-mercaptoethanol and boiled for 3 min. In order to load equal amounts of platelet protein, without artifacts from trapped plasma proteins, platelet protein was measured by quantitative immunoblotting with an anti-α-tubulin mAb (Sigma).

PTP-1B fusion proteins, SDS−PAGE and immunoblotting

GST-PTP-N, a fusion protein of glutathione S-transferase (GST) with amino acids 1−158 of PTP-1B, was purified from bacteria as described previously by Frangioni and Neel (1993) and quantified by Coomassie blue staining of SDS−PAGE gels.

Protein concentrations of cellular lysates were determined by the BCA method. Where indicated, gel-filtered platelets were lysed by the direct addition of 2 × or 5 × SDS−PAGE sample buffer supplemented with sodium orthovanadate and/or phenylarsine oxide. After boiling for 3 min, protein concentrations were measured by a Coomassie blue dot blot assay (Harlow and Lane, 1988), cellular lysates were resolved on 10% SDS−polyacrylamide gels (Harlow and Lane, 1988) and electroblotted onto Immobilon P (Millipore) using a semi-dry transfer blotter (Owl Scientific Model HEP-1). For PTP-1B immunoblots, 10 μg of total cellular protein were loaded in each lane and transfer was for 2 h at 400 mA. For anti-phosphotyrosine immunoblots, 20 μg of total cellular protein were loaded and transfer was for 16 h at 200 mA. Prestained protein molecular weight markers were purchased from Gibco-BRL. PTP-1B immunoblots were blocked at RT for 30 min with 3% BSA in TBST (10 mM Tris, pH 8, 150 mM NaCl and 0.05% Tween-20). Anti-phosphotyrosine immunoblots were blocked at RT for 30 min with 3% BSA in PBS (pH 7.4). 4G10 anti-phosphotyrosine antibody was diluted in 3% non-fat dry milk in PBS. All other primary antibodies were diluted in TBST. Primary antibodies were incubated with immunoblots for 1 h at RT at the following dilutions: #300C (0.5 μg/ml), #932N (0.5 μg/ml), #583N (0.5 μg/ml), FG6 (5 μg/ml) and 4G10 (1 μg/ml). Immunoblots were incubated with secondary antibody (alkaline phosphatase-conjugated goat anti-rabbit or goat anti-mouse, Promega, 1:5000 in TBST) for 30 min at RT, washed four times, 5 min each and stained using an NBT/BCIP tablet (Sigma) dissolved in distilled water.

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