Tyrosine Dephosphorylation, but Not Phosphorylation, of p130^{Cas} Is Dependent on Integrin αIIbβ3-Mediated Aggregation in Platelets: Implication of p130^{Cas} Involvement in Pathways Unrelated to Cytoskeletal Reorganization†

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ABSTRACT: The newly described adapter molecule p130 Crk-associated substrate (Cas) has been reported to contribute to cytoskeletal organization through assembly of actin filaments and to be pivotal in embryonic development and in oncogene-mediated transformation. We characterized the regulation of Cas tyrosine phosphorylation in highly differentiated, anucleate platelets. Phospholipase C-activating receptor agonists, including collagen, thrombin receptor-activating peptide (TRAP), and U46619 (a thromboxane A_2 analogue), and A23187 (a Ca^{2+} ionophore) induced rapid Cas tyrosine phosphorylation in platelets. 12-*O*-Tetradecanoylphorbol 13-acetate and 1-oleoyl-2-acetyl-*sn*-glycerol, protein kinase C (PKC) activators, also induced Cas tyrosine phosphorylation, albeit sluggishly. Cas tyrosine phosphorylation induced by collagen or TRAP was transient in aggregating platelets; Cas became dephosphorylated in a manner dependent on integrin αIIbβ3-mediated aggregation. While BAPTA-AM (an intracellular Ca^{2+} chelator) inhibited Cas phosphorylation induced by collagen or TRAP, Ro31-8220 (a PKC inhibitor) rather prolonged it. Under the conditions, this PKC inhibitor suppressed platelet aggregation but not intracellular Ca^{2+} mobilization. In contrast to Cas involvement in focal adhesions in other cells, platelet Cas phosphorylation preceded the activation of focal adhesion kinase (FAK), and blockage of αIIbβ3-mediated platelet aggregation with a GRGDS peptide resulted in prolongation of stimulation-dependent Cas tyrosine phosphorylation but in suppression of FAK tyrosine phosphorylation. Furthermore, TRAP-induced Cas phosphorylation was insensitive to cytochalasin D, an actin polymerization inhibitor. The failure of FAK to associate with Cas in immunoprecipitation studies also suggests that Cas tyrosine phosphorylation is independent of FAK activation. Of the signaling molecules investigated in this study, Src seemed to associate with Cas. Finally, Cas existed mainly in cytosol and membrane cytoskeleton fractions in the resting state, and remained unchanged during platelet aggregation, when FAK translocated to the cytoskeletal fraction. Our findings on platelet Cas suggest that (i) rapid Cas tyrosine phosphorylation occurs following phosphoinositide turnover by receptor-mediated agonists and may be mediated by intracellular Ca^{2+} mobilization; (ii) PKC activation, by itself, may elicit sluggish Cas phosphorylation; (iii) Cas tyrosine dephosphorylation, but not phosphorylation, is dependent on integrin αIIbβ3-mediated aggregation; and (iv) Cas is not involved in cytoskeletal reorganization. Anucleate platelets seem to provide a unique model system to fully elucidate the functional role(s) of Cas.

p130 Crk-associated substrate (Cas)[1] was originally identified as a major tyrosine-phosphorylated protein of 130 kDa in cells transformed by v-Src or v-Crk (*1*) and is now considered to be a novel adapter protein which is localized at focal adhesions (*2*, *3*). The recent molecular cloning of Cas has revealed that this molecule contains an NH_2-terminal SH3 domain, a proline-rich domain, a cluster of 15 putative SH2-binding motifs, and a COOH-terminal domain which contains Src SH3- and SH2-binding regions (*1*). The unique structure of Cas suggests that this protein plays a role in assembling multiprotein signaling complexes (*1*). Cas is phosphorylated through a variety of pathways, including integrin, G protein-coupled receptors, and the receptor-type tyrosine kinases (*4*−*9*). Following its tyrosine phosphorylation, Cas interacts with a number of SH2-containing signaling molecules, including Crk, Crk-L, protein−tyrosine phosphatase (PTP)-1D, Lyn, Fyn, and Src (*1*−*3*, *6*−*8*, *10*−*13*). In addition, Cas, through its SH3 domain, can constitutively associate with proline-rich domain-containing proteins such as focal adhesion kinase (FAK), proline-rich tyrosine kinase 2 (Pyk2), PTP-1B, and PTP-PEST (*2*, *3*, *12*, *14*−*17*).

Although the exact role of Cas has not been identified, it is now established that this molecule is closely related to the integrin function. Cas becomes tyrosine-phosphorylated

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[1] Abbreviations: Cas, p130 Crk-associated substrate; PTP, protein−tyrosine phosphatase; FAK, focal adhesion kinase; Pyk2, proline-rich tyrosine kinase 2; TxA_2, thromboxane A_2; vWF, von Willebrand factor; CRP, collagen-related peptide; TRAP, thrombin receptor-activating peptide; MoAb, monoclonal antibody; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol; PKC, protein kinase C.

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with kinetics similar to that of FAK in response to integrin-mediated cell adhesion (*18*, *19*) and, in some cells, is found to be actually phosphorylated by FAK (*19*, *20*). Cas is localized to focal adhesions, and the interaction of Cas with FAK (and Src) is functionally important in integrin-mediated signal transduction, including cell shape change and migration (*18*). In addition, Cas is a mediator for FAK-promoted cell migration (*18*, *21*), and formation of a Cas/Crk adapter protein complex serves as a molecular switch facilitating a Rac-dependent cell migration response in the extracellular matrix (*22*). These findings suggest that Cas, an important component of focal adhesions, plays a pivotal role in integrin-related responses (*18*, *19*).

Highly differentiated, anucleate platelets were unexpectedly found to possess abundant tyrosine kinases (*23*, *24*). The majority of tyrosine kinases in platelets reported to date are of the nonreceptor type, including the Src family tyrosine kinases, Syk, and FAK (*24*). It is well established that agonist-induced protein−tyrosine phosphorylation in platelets is divided into integrin-dependent and -independent processes (*24*, *25*). Integrin αIIbβ3 (platelet glycoprotein IIb/IIIa) is the most abundant platelet integrin and acts as a receptor for RGD-containing macromolecules, including fibrinogen (*26*, *27*). Although integrin αIIbβ3 has a low affinity for its ligands under resting conditions, it undergoes a conformational change and acquires a high-affinity binding for its ligands, principally fibrinogen, when platelets are stimulated with agonists such as collagen, thrombin, and thromboxane A_2 (TxA_2) (*26*, *27*). Platelet aggregation is the result of this fibrinogen binding to integrin αIIbβ3 (*26*, *27*). While the early phase of platelet protein−tyrosine phosphorylation, including that of the Src family tyrosine kinases and Syk, is independent of fibrinogen binding to the activated integrin αIIbβ3 and the subsequent platelet aggregation (*24*, *25*), some of the tyrosine-phosphorylated proteins which appear in the later phase are closely related to the integrin-mediated response (*24*, *26*). Platelet aggregation is associated with tyrosine phosphorylation and activation of FAK and tyrosine phosphorylation of additional substrates, including Tec (a tyrosine kinase that contains a PH domain) and SHIP (an SH2 domain-containing inositol 5-phosphatase); none of these changes occur if aggregation is blocked (*26*, *28*−*30*). Although (i) Cas, as well as FAK, is an important cytoskeletal signaling molecule which constitutes the focal adhesion (*2*, *18*, *19*) and (ii) changes in the organization of the actin cytoskeleton (accompanying platelet aggregation) lead to striking changes in the tyrosine phosphorylation of several signaling proteins localized at the focal adhesion plaques (*24*−*26*), Cas phosphorylation and involvement in activated platelets have not been reported.

We recently identified Cas in platelets for the first time (*31*) and, in the present study, report that collagen and ligands for Gq-coupled receptors induce a rapid and transient increase in the tyrosine phosphorylation of Cas in aggregating platelets. Intracellular Ca^{2+} mobilization seems important in this Cas phosphorylation. Unexpectedly, our results also show that Cas tyrosine dephosphorylation, but not phosphorylation, is dependent on integrin αIIbβ3-mediated platelet aggregation. Furthermore, platelet Cas tyrosine phosphorylation is independent of the integrity of the actin cytoskeleton. Platelets seem to provide a unique model system to elucidate the functional role(s) of Cas.

EXPERIMENTAL PROCEDURES

Materials. Botrocetin and von Willebrand factor (vWF) were kindly provided by Dr. Y. Fujimura (Nara Medical University, Nara, Japan), and PT25-2 Fab was kindly provided by Dr. M. Handa (Keio University, Tokyo, Japan). Collagen-related peptide (CRP) was a gift from Dr. M. Moroi (Kurume University, Fukuoka, Japan). Ro31-8220, a specific protein kinase C (PKC) inhibitor (*32*), was a donation from Rosche Products (Welwyn Garden City, Herts, U.K.).

The following materials were obtained from the indicated suppliers: collagen (Hormon-Chemie, Munich, Germany); thrombin receptor-activating peptide (TRAP) (Sawaday Technology, Tokyo, Japan); GRGDS peptide (Peptide Institute, Osaka, Japan); anti-Pyk2 polyclonal antibody, anti-FAK monoclonal antibody (MoAb), anti-Src MoAb, anti-phosphotyrosine MoAb (4G10), anti-PTP-1B polyclonal antibody, and anti-SHP-1 polyclonal antibody (Upstate Biotechnology, Lake Placid, NY); anti-Fyn MoAb, anti-FAK MoAb, anti-Cas MoAb, anti-Crk MoAb, anti-phosphotyrosine MoAb (PY20), anti-SLP-76 MoAb, and anti-PTP-1D MoAb (Transduction Laboratories, Lexington, KY); control mouse IgG (Zymed Laboratories, San Francisco, CA); U46619, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), 1-oleoyl-2-acetyl-*sn*-glycerol (OAG), cytochalasin D, fibrinogen, and enolase (Sigma Chemical Co., St. Louis, MO); anti-Syk MoAb (Wako Pure Chemical Industries, Tokyo, Japan); A23187 (Research Biochemicals Incorporated, Natick, MA); protein A−Sepharose CL-4B (Amersham Pharmacia Biotech, Buckinghamshire, U.K.); [γ-^{32}P]ATP (111 TBq/mmol) (Du Pont-New England Nuclear, Boston, MA); fura2-AM and BAPTA-AM (Dojindo Laboratories, Kumamoto, Japan).

Platelet Preparation and Aggregation Monitoring. Platelet-rich plasma was prepared as previously described (*33*) and incubated with 1 mM acetylsalicylic acid for 30 min to exclude secondary effects of TxA_2. The platelets were washed and resuspended in a buffer containing 138 mM NaCl, 3.3 mM NaH_2PO_4, 2.9 mM KCl, 1 mM $MgCl_2$, 1 mg/mL glucose, and 20 mM Hepes (pH 7.4). Just before centrifugation of the platelet suspensions, a 15% volume of acid−citrate−dextrose A solution and 0.1 μM prostaglandin I_2 were added to inhibit platelet activation. The final platelet suspensions were adjusted to 1.0×10^9 cells/mL and supplemented with 1 mM $CaCl_2$, unless otherwise stated. Platelet aggregation was monitored by measuring light transmission with the use of an AA-100 platelet aggregation analyzer (Sysmex, Kobe, Japan). The instrument was calibrated with a platelet suspension for zero light transmission and with a buffer for 100% transmission.

Unless otherwise stated, platelets were stimulated at 37 °C under continuous stirring at 1000 rpm. Under these conditions, integrin αIIbβ3-mediated platelet aggregation was elicited by stimulants capable of inducing a release reaction; fibrinogen, released from platelet α granules, binds to activated αIIbβ3, leading to platelet aggregation (*27*, *34*). In fact, when platelets were stimulated with collagen, U46619, TRAP, or CRP and transient Cas tyrosine phosphorylation was observed (Figure 1), marked platelet aggregation was observed without the addition of fibrinogen (data not shown). To examine Cas phosphorylation in platelets stimulated with collagen or TRAP, but not aggregated, platelets were preincubated with 500 μM GRGDS (a competitive antagonist to inhibit fibrinogen−αIIbβ3

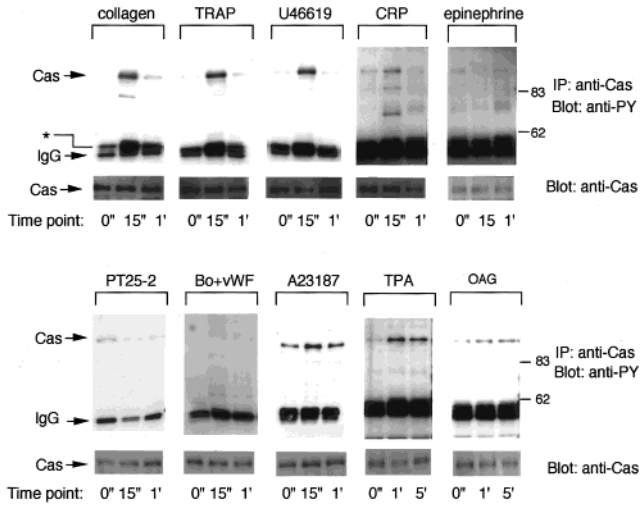

FIGURE 1: Cas tyrosine phosphorylation in human platelets. Platelets were stimulated with 50 μg/mL collagen, 20 μM TRAP, 1 μM U46619, 1 μg/mL CRP, 50 μM epinephrine, 20 μg/mL PT25-2 Fab fragments, and 3 μg/mL botrocetin plus 10 μg/mL vWF (Bo + vWF), 300 nM A23187, 1 μM TPA, or 20 μM OAG for the indicated durations. The platelet protein lysates were immunoprecipitated with anti-Cas MoAb, resolved on an 8% SDS−PAGE, and then immunoblotted with anti-phosphotyrosine (PY) (upper panel) or anti-Cas MoAb (lower panel). Arrows indicate the locations of Cas and IgG (heavy chain). Molecular weight markers are indicated on the right. Unidentified tyrosine-phosphorylated protein (∗), the molecular weight of which was slightly larger than that of IgG, was observed in our Western blots. This band is most probably Src, since its location corresponded to that of Src in reprobing studies; the stimulation-dependent Cas−Src interaction occurs in platelets (see Figure 11).

interaction) (*26*, *27*) for 5 min and not stirred; platelet aggregates were not formed under these conditions (data not shown). On the other hand, when platelets were stimulated with TPA or OAG, only very weak platelet aggregation was observed, despite marked protein kinase C activation, which was monitored by pleckstrin phosphorylation (see Platelet PKC Activation below). This may be due to the fact that TPA is not a potent stimulant for release reactions and enough fibrinogen (for platelet aggregation) could not be released from the platelet granules (*35*). Accordingly, 400 μg/mL fibrinogen was added 5 min prior to stimulation to observe TPA-induced platelet aggregation, while 200 μM GRGDS was used to completely block platelet aggregation.

Immunoprecipitation and in Vitro Kinase Assay. Washed platelet reactions were terminated with an equal volume of 2 × ice-cold lysis buffer [2% Triton X-100, 100 mM Tris-HCl (pH 7.4), 1 mM EGTA, 1 mM EDTA, 2 mM Na_3VO_4, 1 mM PMSF, and 100 μg/mL leupeptin]. For Cas immunoprecipitation, 2% Nonidet P-40 (instead of Triton X-100) was used, as described previously (*36*). All subsequent immunoprecipitation steps were carried out at 4 °C, unless otherwise stated. The lysates were centrifuged at 15000*g* for 5 min and then precleared with protein A−Sepharose CL-4B. The resultant supernatants were incubated for 2 h with the antibody indicated. Protein A−Sepharose CL-4B was then added, and the solution was further incubated for 1 h. The Sepharose beads were washed with a 1 × lysis buffer three times. The samples were then separated into two aliquots. One was solubilized with an SDS sample buffer and used for immunoblotting. When indicated, the other was processed further for in vitro kinase assay, as follows. The beads were washed once with 10 mM Hepes (pH 8.0) containing 1 mM Na_3VO_4 and then incubated with 35 μL of the Hepes buffer containing 12.5 μg of acid-treated enolase. The reaction was initiated by the addition of 15 μL of kinase reaction buffer [300 mM Hepes/NaOH, pH 8.0, 30 μM Na_3VO_4, 150 mM $MgCl_2$, 15 mM $MnCl_2$, and 5 μM [γ-^{32}P]-ATP (0.75 μCi)]. After 15 min at room temperature, the reaction was terminated by the addition of 25 μL of 3 × SDS sample buffer, and the solution was then boiled for 3 min. The proteins were separated by SDS−PAGE and electroblotted onto a PVDF membrane. The membrane was treated with 1 M KOH at 60 °C for 30 min and 10% acetic acid at 20 °C for 15 min and then dried. The phosphorylated protein was quantified using a BAS-2000 Phosphor-image analyzer (Fuji Film, Tokyo, Japan).

Immunoblotting. The proteins were resolved on an SDS−PAGE and then electrophoretically transferred to the PVDF membrane. The membranes were blocked with 1% BSA in phosphate-buffered saline (PBS). After extensive washing with PBS containing 0.1% Tween-80, the immunoblots were incubated with anti-phosphotyrosine MoAb (1 μg/mL PY20 plus 1 μg/mL 4G10), anti-Src MoAb (0.16 μg/mL), anti-Fyn MoAb (1 μg/mL), anti-Syk MoAb (0.2 μg/mL), anti-SLP-76 MoAb (1 μg/mL), or anti-Cas MoAb (0.25 μg/mL) for 2 h. Antibody binding was detected using peroxidase-conjugated goat anti-mouse IgG and visualized with ECL chemiluminescence reaction reagents (Amersham Pharmacia Biotech, Buckinghamshire, U.K.). For reprobing with other antibodies, the antibody binding on the PVDF membrane was removed with a stripping buffer (2% SDS, 62.5 mM Tris-HCl, pH 6.8, 100 μM 2-mercaptoethanol) at 60 °C for 30 min. After being washed twice with PBS containing 0.1% Tween-80, the membranes were blocked with 1% BSA and reprobed with the antibody indicated. When indicated, tyrosine phosphorylation of Cas was quantified using a PDI420oe scanner and Quantity one 2.5a for Macintosh.

Measurement of the Intracellular Ca^{2+} Concentration ($[Ca^{2+}]_i$). The $[Ca^{2+}]_i$ measurement was performed with the use of Ca^{2+}-sensitive fluorophore fura2 as described previously (*37*). The fura2-loaded platelets were adjusted to (1−2) × 10^8/mL, and the fluorescence measurements were made using a FS100 (Kowa, Tokyo, Japan). The $[Ca^{2+}]_i$ values were determined from the ratios of fura2 fluorescence intensity at 340 and 380 nm excitation.

Fractionation of Platelets. Subcellular fractionation of platelets was carried out essentially as described (*38*). Washed platelet reactions were terminated with an equal volume of 2 × lysis buffer [2% Triton X-100, 100 mM Tris-HCl (pH 7.2), 2 mM EGTA, 2 mM Na_3VO_4, 1 mM PMSF, and 100 μg/mL leupeptin]. All subsequent steps were carried out at 4 °C. The cytoskeleton fraction (CS) was isolated by centrifugation of the lysate at 15000*g* for 5 min. The membrane skeleton fraction (MS) was separated from the resultant supernatant by centrifugation at 100000*g* for 3 h with himac CS 100FX (Hitachi Koki Co., Tokyo, Japan). The pellets (CS and MS) were washed three times with a 1 × lysis buffer and solubilized with a 1 × SDS sample buffer. For analysis of the cytosol fraction (CY), the final supernatant was diluted with one-third volume of 3 × SDS sample buffer. The proteins in these fractions, after boiling for 3 min, were separated on an SDS−PAGE and detected by immunoblotting, as described above.

Platelet PKC Activation. PKC activation in intact platelets was evaluated by pleckstrin (p47) phosphorylation as described previously (*39*).

Presentation of Data. Unless stated otherwise, the results shown are from a single experiment representative of at least four separate experiments.

RESULTS

Cas Tyrosine Phosphorylation in Activated Platelets. A variety of stimuli, including integrin activators, ligands for G protein-coupled receptors, and direct protein kinase C activators, have been reported to induce Cas tyrosine phosphorylation (*2*, *4*−*9*, *11*). However, no such information has been reported for platelets. We first examined Cas tyrosine phosphorylation in human platelets stimulated with several activators by Cas immunoprecipitation studies (Figure 1). Collagen is a potent and physiological platelet activator; this extracellular matrix acts on platelets via the integrin $\alpha 2\beta 1$ and glycoprotein VI, leading to phospholipase C-$\gamma 2$ activation and the resultant intracellular Ca^{2+} mobilization (*40*, *41*). This macromolecule was found to induce a robust, but transient Cas tyrosine phosphorylation (Figure 1). CRP, which selectively activates glycoprotein VI (one of the collagen receptors) (*41*), mimicked the collagen action, but in a weaker manner (Figure 1). It is well established that soluble agonists such as TxA_2 and thrombin act on the G protein-coupled receptors expressed on platelets and stimulate most platelet signaling pathways, including phosphoinositide turnover and protein−tyrosine kinase activation (*42*). When platelets were challenged with U46619 (a stable TxA_2 analogue) or TRAP (thrombin receptor-activating peptide), a marked (but transient) Cas tyrosine phosphorylation was again observed (Figure 1). In Figure 2, the concentration dependency and time dependency of platelet Cas tyrosine phosphorylation in response to collagen or TRAP were examined. Cas tyrosine phosphorylation was induced as early as 5 s after stimulation, when only a weak degree of aggregation was observed (data not shown). Cas tyrosine phosphorylation was transient, and its dephosphorylation occurred as platelet aggregation proceeded (Figure 2). Under the conditions described above, platelets were continuously stirred and hence underwent aggregation (see Platelet Preparation and Aggregation Monitoring in Experimental Procedures).

On the other hand, epinephrine, which acts on its G protein-coupled receptor on platelets but just activates the inhibitory G protein (*42*), did not induce Cas phosphorylation (Figure 1). PT25-2 is a murine MoAb, which recognizes a regulatory epitope expressed by the $\alpha IIb\beta 3$ integrin; the Fab fragments of this antibody are reportedly able to induce platelet aggregation (*43*). PT25-2 failed to induce Cas phosphorylation (Figure 1). We recently reported that glycoprotein Ib−vWF interactions activate tyrosine kinase in platelets (*44*). However, in this study, it was found that this mode of platelet activation, elicited by the combined addition of botrocetin plus vWF (*44*), failed to induce Cas tyrosine phosphorylation (Figure 1).

Intracellular Ca^{2+} mobilization and PKC activation are important signaling pathways in activated platelets and have been reported to be involved in various platelet functional responses (*35*, *42*). The Ca^{2+} ionophore A23187 induced transient Cas tyrosine phosphorylation (Figure 1), suggesting that Cas may be phosphorylated in response to enhanced

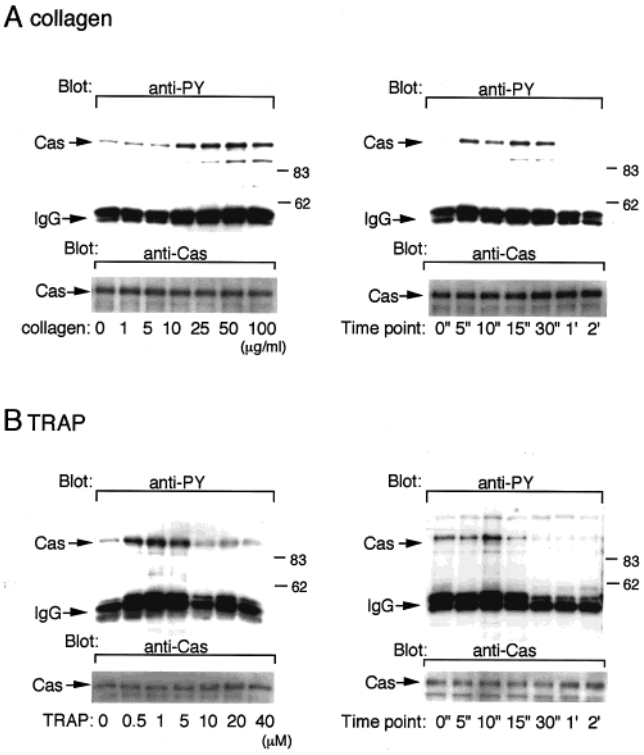

FIGURE 2: Cas tyrosine phosphorylation in platelets stimulated with collagen or TRAP. In (A), platelets were challenged with various concentrations of collagen for 15 s (left panel) or 50 μg/mL collagen for various durations (right panel). In (B), platelets were stimulated with various concentrations of TRAP for 15 s (left panel) or 20 μM TRAP for various durations (right panel). Platelet Cas tyrosine phosphorylation was examined as described in the legend for Figure 1.

levels of cytosolic Ca^{2+} in platelets. Furthermore, when platelets were stimulated with TPA or OAG, which directly activates PKC by acting as a substitute for diacylglycerol (*35*, *45*), a gradual and sustained Cas tyrosine phosphorylation was observed (Figure 1). Under the same conditions, only marginal platelet aggregation was induced (see Platelet Preparation and Aggregation Monitoring in Experimental Procedures).

Dephosphorylation of Cas by $\alpha IIb\beta 3$ Integrin-Mediated Platelet Aggregation. As described above, Cas tyrosine phosphorylation was transient and attenuated as platelet aggregation proceeded upon stimulation with potent agonists. We hence evaluated the involvement of platelet aggregation in Cas tyrosine phosphorylation (with the use of the GRGDS peptide) (see Platelet Preparation and Aggregation Monitoring in Experimental Procedures). As already shown in Figures 1 and 2, Cas tyrosine phosphorylation was transient in collagen- or TRAP-stimulated platelets which were allowed to aggregate (Figure 3A,B, left). On the other hand, sustained Cas tyrosine phosphorylation was observed when platelet aggregation was blocked (Figure 3A,B, right); we confirmed that not only platelet aggregation but also FAK phosphorylation were eliminated under the given conditions (see Comparison between Cas and FAK Tyrosine Phosphorylation; described below). As already shown, TPA or OAG induced weak aggregation but marked Cas tyrosine phosphorylation. When platelets were pretreated with the GRGDS peptide to completely block aggregation, Cas was gradually phosphorylated in a similar way (Figure 3C, left). In contrast, Cas tyrosine phosphorylation was eliminated when TPA-

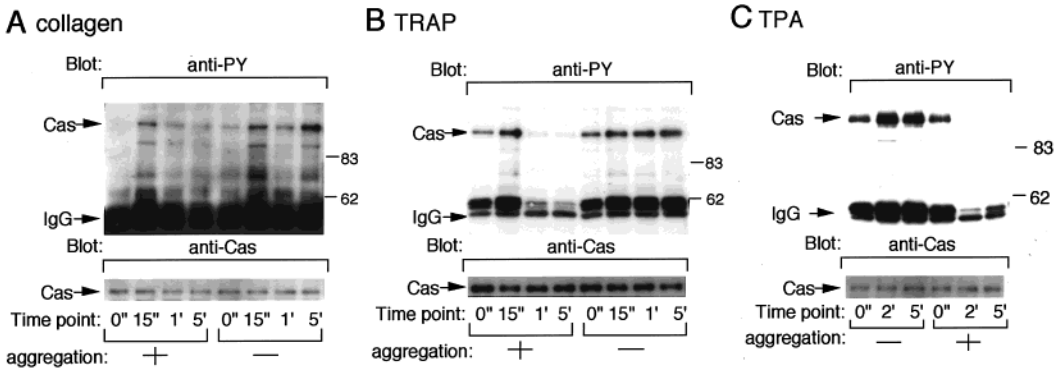

FIGURE 3: Aggregation-dependent Cas dephosphorylation in platelets. Platelets were stimulated with 50 μg/mL collagen (A), 5 μM TRAP (B), or 1 μM TPA (C) for various durations under conditions in which platelets formed aggregates (+) or not (−), as described in Experimental Procedures. Platelet Cas tyrosine phosphorylation was examined as described in the legend for Figure 1.

stimulated platelets were fully aggregated with the addition of fibrinogen (Figure 3C, right) (see Platelet Preparation and Aggregation Monitoring in Experimental Procedures).

All the data described above indicate that Cas dephosphorylation, but not phosphorylation, is related to integrin αIIbβ3-mediated platelet aggregation. We hence examined the effects of pervanadate, which is widely used as a PTP inhibitor (*46*), on aggregation-dependent Cas dephosphorylation. However, pervanadate, by itself, induced Cas tyrosine phosphorylation which was attenuated in an aggregation-dependent manner (data not shown). Accordingly, it was difficult to observe the effects of pervanadate on Cas tyrosine phosphorylation induced by receptor-mediated agonists. The results, from a different angle, may support our hypotheses that Cas tyrosine phosphorylation occurs following phosphoinositide turnover by receptor-mediated agonists and may be mediated by intracellular Ca^{2+} mobilization and that Cas tyrosine dephosphorylation is dependent on integrin αIIbβ3-mediated aggregation (see Discussion); pervanadate induces PLC activation and the resultant Ca^{2+} mobilization, leading to αIIbβ3-dependent platelet aggregation (*47*).

Involvement of Intracellular Ca^{2+} Mobilization, but Not Protein Kinase C Activation, in Platelet Cas Tyrosine Phosphorylation Induced by Receptor Agonists. As described above, collagen, CRP, U46619, and TRAP were found to induce platelet Cas tyrosine phosphorylation. It is established that these activators elicit phospholipase C-catalyzed phosphoinositide turnover, leading to intracellular Ca^{2+} mobilization (following inositol 1,4,5-trisphosphate formation) and PKC activation (following diacylglycerol formation) (*40*−*42*). Accordingly, we examined the involvement of these intracellular signaling pathways in Cas tyrosine phosphorylation.

Platelet Cas tyrosine phosphorylation induced by collagen (Figure 4A) or TRAP (Figure 4B) was not inhibited by pretreatment with EGTA and hence was considered to be independent of the influx of extracellular Ca^{2+}. On the other hand, when platelets were treated with EGTA plus BAPTA-AM (intracellular Ca^{2+} chelator) to block not only Ca^{2+} influx but also Ca^{2+} mobilization from the internal store, the Cas tyrosine phosphorylation was markedly inhibited (Figure 4).

We next employed Ro31-8220, a selective PKC inhibitor (*32*), to examine the involvement of this kinase. We confirmed that Ro31-8220 suppressed platelet aggregation (Figure 5B), a PKC-dependent process (*48*), but not the Ca^{2+}

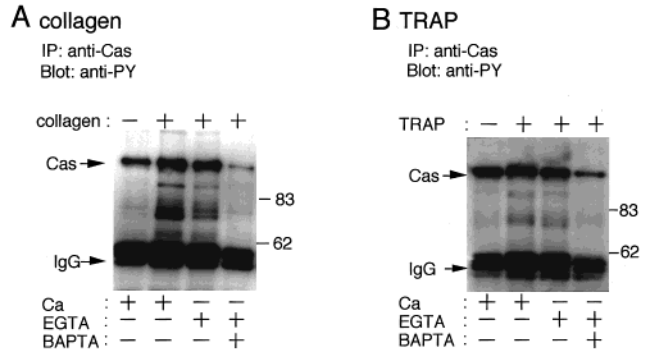

FIGURE 4: Effects of manipulation of intracellular Ca^{2+} levels on platelet Cas tyrosine phosphorylation. Platelets preincubated without or with 1 mM EGTA (instead of $CaCl_2$) or 1 mM EGTA plus 50 μM BAPTA-AM (instead of $CaCl_2$) for 15 min were stimulated with 50 μg/mL collagen (A) or 5 μM TRAP (B), and Cas tyrosine phosphorylation was examined.

mobilization (Figure 5C). This PKC inhibitor failed to inhibit Cas phosphorylation induced by TRAP; TRAP-induced Cas phosphorylation was rather prolonged by Ro31-8220 (Figure 5A). This may be due to the inhibitory effect of Ro31-8220 on platelet aggregation, which is involved in the dephosphorylation of Cas (see Figure 3). Similar results were obtained using collagen-stimulated platelets (data not shown). Of the signaling pathways following phosphoinositide turnover in platelets stimulated with receptor-medicated agonists, intracellular Ca^{2+} mobilization from the internal store seems important for rapid Cas tyrosine phosphorylation.

As expected, TPA-induced sluggish Cas tyrosine phosphorylation was markedly reduced by Ro31-8220 (data not shown). Consistent with the previous report (*35*), TPA did not induce Ca^{2+} mobilization (data not shown). These findings suggest that PKC activation, by itself, may elicit sluggish Cas tyrosine phosphorylation, independently of Ca^{2+} mobilization.

Comparison between Cas and FAK Tyrosine Phosphorylation. We next compared Cas and FAK tyrosine phosphorylation in platelets, because FAK tyrosine phosphorylation (and activation) is dependent on integrin αIIbβ3-mediated platelet aggregation (*28*, *29*) and because a close functional association between FAK and Cas in the focal adhesions is established (*2*, *3*, *18*, *19*). Cas tyrosine phosphorylation was observed at a very early stage after collagen or TRAP stimulation, in contrast to FAK tyrosine phosphorylation, which is aggregation dependent (*28*, *29*) (Figure 6). We next

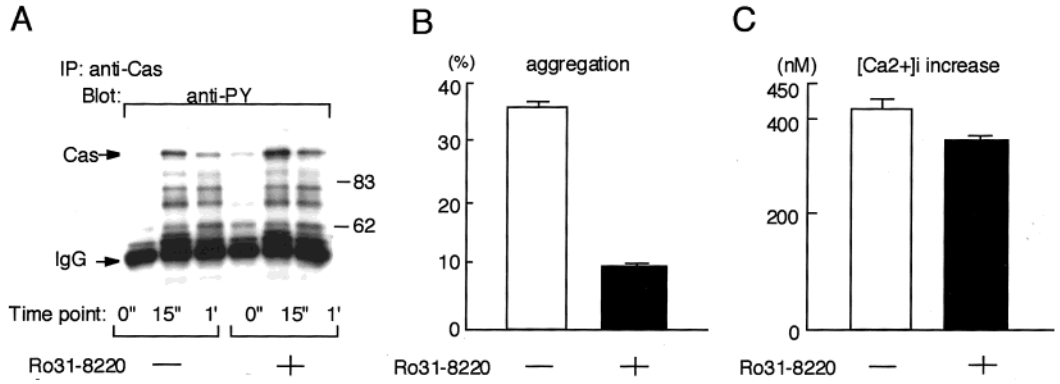

FIGURE 5: Effects of Ro31-8220 on Cas tyrosine phosphorylation, platelet aggregation, and intracellular Ca^{2+} mobilization induced by TRAP. Platelets were pretreated without or with 10 μM Ro31-8220 for 5 min and then stimulated with 5 μM TRAP. In (A), Cas tyrosine phosphorylation was examined. In (B), platelet aggregation was measured at 2 min after TRAP challenge. In (C), peak $[Ca^{2+}]_i$ increases after TRAP challenge were calculated. Columns and error bars represent the mean ± SD ($n = 3$).

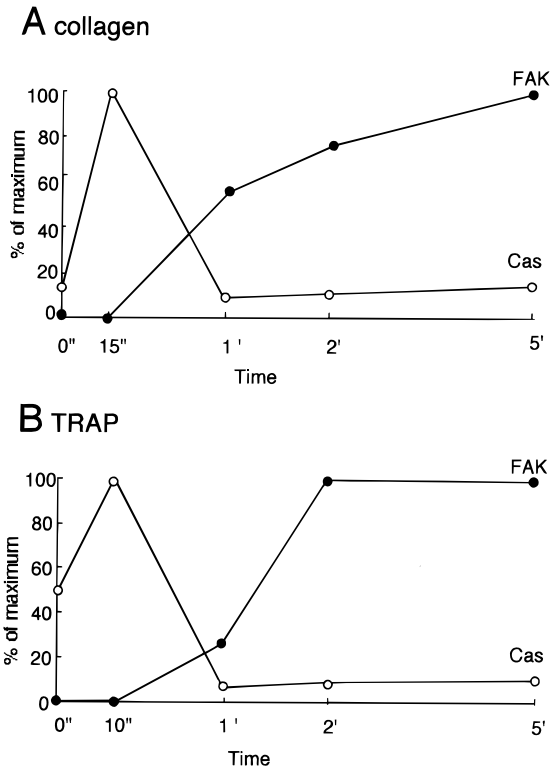

FIGURE 6: Time courses for Cas and FAK tyrosine phosphorylation in platelets stimulated with collagen or TRAP. Platelets were challenged with 50 μg/mL collagen (A) or 20 μM TRAP (B) for various durations. Platelet Cas (open circles) and FAK (solid circles) tyrosine phosphorylations were quantified as described in Experimental Procedures. The results were expressed as a percentage of the maximum.

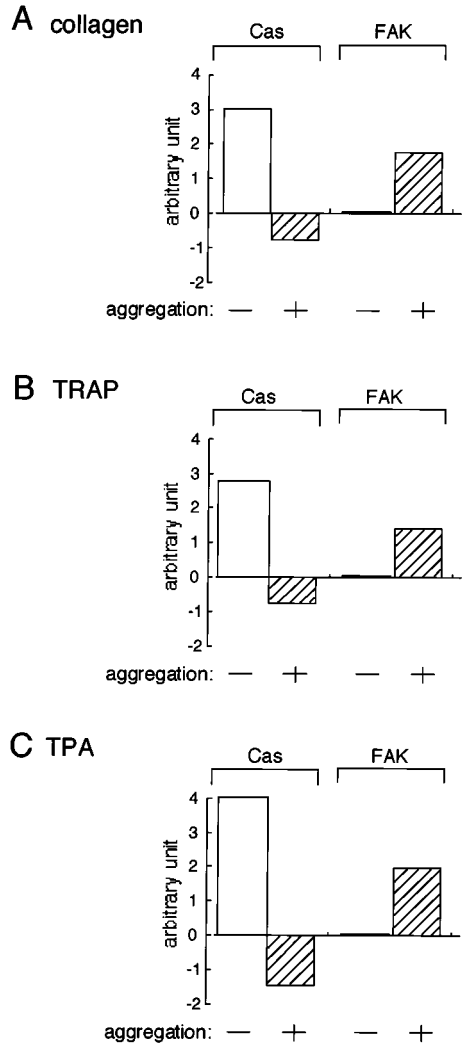

FIGURE 7: Comparison between Cas and FAK tyrosine phosphorylation in platelets stimulated with collagen, TRAP, or TPA. Platelets were challenged with 50 μg/mL collagen (A), 20 μM TRAP (B), or 1 μM TPA (C) for 5 min under conditions where platelets formed aggregates (+) or not (−). The levels of Cas and FAK tyrosine phosphorylation were quantified as described in Experimental Procedures.

evaluated the involvement of αIIbβ3 integrin-mediated aggregation in Cas and FAK tyrosine phosphorylation. As described above, Cas phosphorylation induced by collagen, TRAP, or TPA was prolonged or enhanced when platelet aggregation was abolished (Figure 7, left). Under identical conditions, FAK phosphorylation, which is aggregation dependent, was suppressed (Figure 7, right). These unexpected results suggest that Cas not only fails to serve as a substrate for FAK but also behaves independently of FAK in platelets.

Effects of Cytochalasin D on Platelet Cas Tyrosine Phosphorylation. In a variety of systems, Cas tyrosine phosphorylation has been reported to be dependent on the integrity of the actin cytoskeleton and hence abrogated by cytochalasins, which disrupt the network of actin microfilaments (*4*, *7*, *9*, *11*). In platelets stimulated with TRAP, however, disruption of actin polymerization with cytochalasin

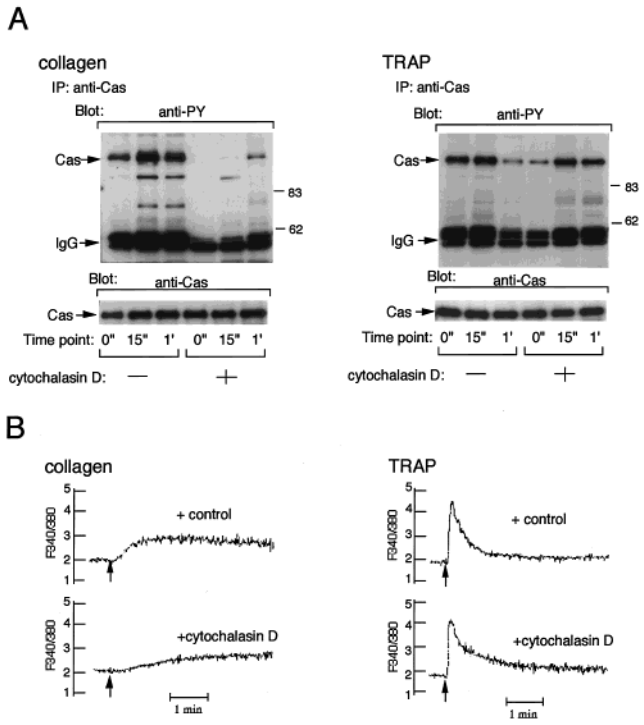

FIGURE 8: Effects of cytochalasin D on Cas tyrosine phosphorylation and intracellular Ca^{2+} mobilization induced by collagen or TRAP. Platelets pretreated without or with 10 μg/mL cytochalasin D for 5 min were stimulated with 50 μg/mL collagen (left panel) or 5 μM TRAP (right panel). In (A), Cas tyrosine phosphorylation was examined. In (B), intracellular Ca^{2+} changes were monitored by the ratio of fura2 fluorescence (340/380 nm).

D failed to block Cas tyrosine phosphorylation (Figure 8A). Hence, the integrity of the actin cytoskeleton is not essential for signal transduction pathways leading to Cas tyrosine phosphorylation, at least in platelets stimulated with TRAP. On the other hand, cytochalasin D did inhibit Cas tyrosine phosphorylation induced by collagen (Figure 8A). This is possibly due to the fact that the interaction of collagen receptors with actin filaments plays a critical role in the early phase of platelet activation induced by this macromolecule (*40*, *49*). In fact, we confirmed that cytochalasin D inhibits Ca^{2+} mobilization induced by collagen, as we showed previously (*49*), but not by TRAP (Figure 8B).

Failure of Cas To Interact with FAK and Pyk2. It is well-known that the SH3 domain of Cas can bind the focal adhesion tyrosine kinase FAK (*2*, *18*, *19*). Pyk2 is a nonreceptor protein tyrosine kinase, which belongs to the FAK subfamily (*50*), and contributes to integrin-mediated signal transduction in place of FAK in FAK-deficient cells (*51*). We thus examined whether Cas interacts with FAK or Pyk2 in platelets. Cas was not present in the immunoprecipitates with anti-FAK (Figure 9A) or anti-Pyk2 (Figure 9B) antibody, in collagen-stimulated platelets. The failure of Cas to interact with FAK or Pyk2 was also confirmed by immunoblotting with anti-Pyk2 or anti-FAK antibody of the immunoprecipitates with anti-Cas antibody (data not shown). Similar results were obtained with TRAP-activated platelets (data not shown). These data also suggest that Cas action is independent of FAK family tyrosine kinases in platelets.

Subcellular Distribution of Cas and FAK in Platelets. We next examined the subcellular Cas distribution in relevance to the cytoskeleton; the FAK−Cas complex reportedly shifts

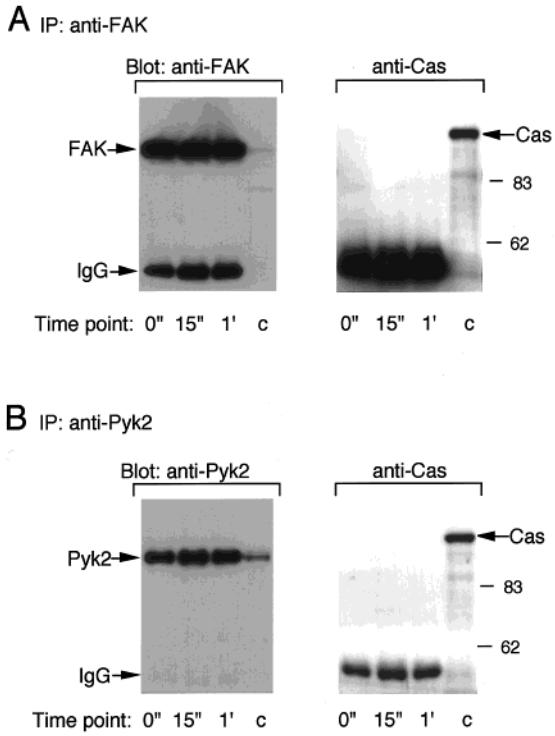

FIGURE 9: Failure of FAK and Pyk2 to interact with Cas. Platelets were stimulated with 50 μg/mL collagen for the indicated durations, and the platelet lysates were immunoprecipitated with anti-FAK MoAb (A) or anti-Pyk2 (B) polyclonal antibody. The immunoprecipitates, along with whole platelet lysates (c), were resolved on an 8% SDS−PAGE and immunoblotted with anti-FAK, anti-Pyk2, or anti-Cas MoAb. Arrows indicate the locations of FAK, Pyk2, Cas, and IgG.

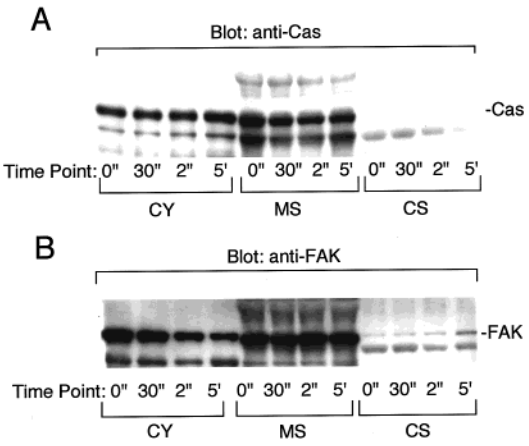

FIGURE 10: Distribution of Cas and FAK among the subcellular fractions in collagen-activated platelets. Platelets were stimulated with 50 μg/mL collagen for various durations. The platelet lysates were fractionated into the cytoskeleton (CY), the membrane skeleton (MS), and the cytosol fraction (CS) and finally immunoblotted with anti-Cas MoAb (A) or anti-FAK MoAb (B).

to a cytoskeleton-associated cell fraction upon transformation by Src in fibroblasts (*36*). In platelets, tyrosine kinases, including FAK, Src, and Yes, translocate to the cytoskeleton upon platelet activation (*52*). Most of the Cas and FAK molecules in platelets were found to exist in the cytosol and membrane skeleton fractions in the resting state (Figure 10). Upon stimulation with collagen, FAK, as previously reported (*53*), translocated to the cytoskeletal fraction (Figure 10B). However, Cas translocation was not observed under the same

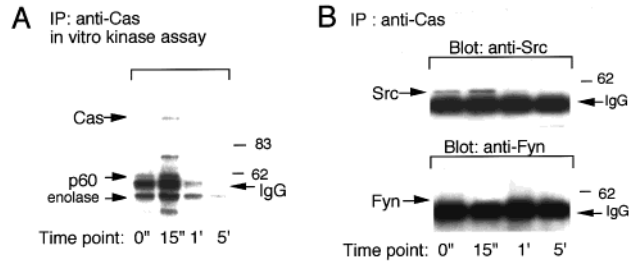

FIGURE 11: Cas-associated kinase activity and Cas−Src interaction in platelets stimulated with collagen. Platelets were stimulated with 50 μg/mL collagen for the indicated durations, and Cas was isolated by immunoprecipitation with anti-Cas MoAb. In (A), an in vitro kinase assay was performed using acid-treated enolase as an exogenous substrate. Arrows indicate locations of Cas, enolase, and IgG. A clear band with a molecular mass of about 60 kDa was also observed (p60). In (B), immunoprecipitates obtained with anti-Cas MoAb were resolved on an SDS−PAGE and then immunoblotted with anti-Src or anti-Fyn MoAb.

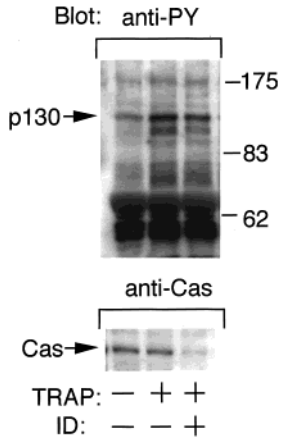

FIGURE 12: Discrimination of Cas from p130. Platelets pretreated with 5 mM EGTA plus 500 μM GRGDS for 5 min were stimulated without (−) or with (+) 20 μM TRAP for 30 s. For Cas immunodepletion, the platelet lysates were immunoprecipitated with anti-Cas MoAb. The platelet lysates without (ID −) or with (ID +) Cas immunodepletion were subjected to 8% SDS−PAGE and immunoblotted with anti-phosphotyrosine (PY) (upper panel) or anti-Cas MoAb (lower panel).

conditions (Figure 10A). Failure of Cas to translocate to the cytoskeleton was also confirmed with TRAP-stimulated platelets (data not shown).

Association of Cas with Src upon Platelet Activation. As described above, the possibility of Cas being the substrate of or interacting with FAK in platelets is remote. To explore the kinase(s) associated with Cas, collagen-stimulated platelets were immunoprecipitated with anti-Cas antibody, and the resultant immune complexes were subjected to in vitro kinase assay. As shown in Figure 11A, stimulation-dependent $^{32}P_i$ incorporation into enolase (added as exogenous substrate), 130 kDa protein (confirmed as Cas with immunoblotting), 70 kDa protein, and 60 kDa protein was observed. The levels of phosphorylation reached their peak 15 s after activation and then rapidly decreased within 1 min (Figure 11A). Since Cas lacks intrinsic kinase activity, the increased $^{32}P_i$ incorporation must be the result of Cas association with catalytically active kinase(s) upon stimulation. Since the Src family tyrosine kinase(s) reportedly phosphorylate Cas in several systems (*9*, *10*, *18*, *36*), we tested the possibility that the 60 kDa tyrosine-phosphorylated protein may be one of the Src family tyrosine kinases. Immunoblotting of Cas immunoprecipitates revealed that Cas interacted with Src (Figure 11B, upper panel) but not Fyn (Figure 11B, lower panel); Src weakly associated with Cas in resting platelets, and the Src−Cas interaction was augmented upon collagen activation. The Src−Cas interaction was also detected under the conditions where maximal Cas tyrosine phosphorylation was induced by TRAP (data not shown). In the Cas immunoprecipitates, a tyrosine-phosphorylated protein with a molecular mass of about 70 kDa was detected (Figure 11A; see also Figures 3−5). We examined the possibility of this being Syk or SLP-76, which has been shown to be phosphorylated upon stimulation with collagen or CRP (*40*, *54*). However, Syk or SLP-76 was not detected in the immunoprecipitates obtained with anti-Cas MoAb (data not shown).

Discrimination of Cas from p130, a Tyrosine-Phosphorylated Platelet Protein Involved in Shape Change. Tyrosine phosphorylation of Cas is an early event in platelet activation and may be involved in some platelet functional responses. Negrescu et al. previously reported a 130 kDa protein (p130), tyrosine phosphorylation of which may be involved in the shape change reaction induced by YFLLRNP (a partial thrombin receptor agonist) (*55*), although the observation against this was reported afterward (*56*). We finally checked the possibility of p130 being Cas. To allow shape change and prevent aggregation, platelets were pretreated with 5 mM EGTA plus 500 μM GRGDS peptide for 5 min. Under the conditions, TRAP (20 μM), as described previously (*55*), rapidly induced p130 tyrosine phosphorylation (Figure 12, upper panel) as well as platelet shape change (data not shown). The cell lysates were prepared from the TRAP-stimulated platelets and immunodepleted with anti-Cas MoAb. This immunodepletion resulted in removal of most Cas from the lysates (Figure 12, lower panel) but not of tyrosine-phosphorylated p130 (Figure 12, upper panel), indicating that Cas is not identical to p130.

DISCUSSION

Stimuli Able To Induce Platelet Cas Tyrosine Phosphorylation. In this report, we observed Cas tyrosine phosphorylation in platelets. The rapidity of Cas tyrosine phosphorylation suggests that this event may be functionally important in platelet activation. While a great deal of observation has been reported for protein−tyrosine phosphorylation in platelets, little is known about its roles in platelet functional responses; Cas is no exception. Tyrosine phosphorylation of Cas (as well as the Src family tyrosine kinases and Syk) is an early event in platelet activation, compared with integrin-dependent responses (including FAK phosphorylation) which appear in the later phase. Accordingly, Cas tyrosine phosphorylation may be involved in initiating platelet functional responses but not the consequences of the platelet activation.

We found that only selective stimuli induce platelet Cas phosphorylation; signals originating from collagen receptors and Gq-coupled receptors are related to Cas tyrosine phosphorylation. Activation of Gi (by epinephrine), signaling pathways mediated by glycoprotein Ib/IX (adhesion proteins belonging to the leucine-rich motif family) (*57*), and a direct activation of the integrin αIIbβ3 failed to induce Cas phosphorylation.

Collagen activates γ-type phospholipase C (*40*, *41*), while Gq-coupled seven-transmembrane receptors (for thrombin

and TxA_2) are linked to the β-type (*42*). Accordingly, phosphoinositide turnover may be essential for Cas tyrosine phosphorylation in platelets. Although Cas phosphorylation following phospholipase C activation has been reported in several systems, little is established at present about the signaling pathways that link phosphoinositide turnover to Cas tyrosine phosphorylation. Protein kinase C-dependent tyrosine phosphorylation of Cas was reported in differentiating neuroblastoma cells, where TPA-induced Cas phosphorylation and its inhibition by the specific protein kinase C inhibitor were observed (*5*). In smooth muscle cells stimulated with angiotensin II, the independence of Cas phosphorylation from protein kinase C and intracellular Ca^{2+} mobilization was reported in one study (*8*), while its dependence on both pathways was reported in another (*9*). In Swiss 3T3 cells, bombesin, which is known to induce rapid and strong hydrolysis of phosphoinositide turnover, induces marked Cas tyrosine phosphorylation (*4*). However, bombesin-induced Cas tyrosine phosphorylation can be dissociated from both protein kinase C activation and Ca^{2+} mobilization from internal stores (*4*).

In platelets, of the signaling pathways originating from phosphoinositide hydrolysis, Ca^{2+} mobilization from the intracellular store seemed essential; Cas tyrosine phosphorylation was suppressed only when platelets were preincubated with EGTA plus BAPTA-AM before stimulation with the phospholipase C-activating agent. In support of this idea, a Ca^{2+} ionophore A23187 by itself was able to stimulate Cas tyrosine phosphorylation. This is also consistent with the fact that epinephrine, which activates Gi protein but is unable to induce Ca^{2+} mobilization (*42*), and PT25-2, which activates the integrin αIIbβ3 independently of intracellular Ca^{2+} mobilization (*43*), failed to induce Cas phosphorylation. Furthermore, platelet glycoprotein Ib−vWF interactions, which elicit an influx of extracellular Ca^{2+}, but not Ca^{2+} mobilization from the internal stores (*58*), also failed to induce Cas phosphorylation.

Although our data suggest that Ca^{2+} mobilization from the internal stores is essential for Cas tyrosine phosphorylation following phosphoinositide turnover, it is also possible that some factors other than Ca^{2+} may contribute to Cas phosphorylation in platelets. When fura2-loaded platelets were stimulated with collagen, only a weak intracellular Ca^{2+} mobilization was observed; $[Ca^{2+}]_i$ increases induced by collagen (50 μg/mL) were 164 ± 35 nM, while those induced by TRAP (20 μM) and U46619 (1 μM) were 539 ± 17 nM and 594 ± 76 nM (mean ± SD, $n = 3$), respectively. In contrast, Cas phosphorylation induced by collagen was of great magnitude, compared with that induced by TRAP or U46619. We postulate that an unknown factor(s), other than Ca^{2+} mobilization, may be present at least in collagen-induced Cas phosphorylation.[2]

The role of protein kinase C in Cas phosphorylation also remains to be elucidated. Although Cas tyrosine phosphorylation (following phosphoinositide hydrolysis) was not inhibited by the protein kinase C inhibitor Ro31-8220, TPA or OAG, which directly activates protein kinase C, induced platelet Cas tyrosine phosphorylation, albeit sluggishly. A similar observation was reported in Swiss 3T3 cells; bombesin induces Cas tyrosine phosphorylation without involvement of protein kinase C, despite the fact that direct activation of protein kinase C by addition of phorbol 12,-13-dibutyrate elicits this phosphorylation (*4*). There may be another pathway involved in Cas phosphorylation, which is dependent on protein kinase C but not on Ca^{2+}. In this context, it should be noted that PKC-dependent TPA-induced Cas phosphorylation occurs gradually, while Ca^{2+}-dependent phosphorylation following receptor-mediated signaling occurs rapidly.

To examine the downstream partners of Cas in platelets, Cas immunoprecipitates were immunoblotted with anti-Src, Syk, SLP-76, Crk, Crk-l, Grb-2, PI 3-kinase, Cbl, Lyn, and Fyn (data not shown). However, only Src was found to associate with Cas, as discussed later.

Involvement of Integrin αIIbβ3-Mediated Platelet Aggregation in Cas Tyrosine Dephosphorylation, but Not Phosphorylation. Aggregation is one of the most important platelet functional responses and is the result of fibrinogen binding to αIIbβ3 integrin (*26*, *27*). Furthermore, many platelet intracellular signals are regulated by this response; activation of protein−tyrosine kinase is no exception (*24*−*26*). Induction of FAK tyrosine phosphorylation requires both fibrinogen binding to this integrin and postoccupancy events during agonist-induced platelet aggregation (*26*, *28*, *29*).

Cas is a member of the focal adhesion complex, is associated with FAK in many cell types, and becomes tyrosine phosphorylated with kinetics similar to that of FAK in response to integrin-mediated cell adhesion in other cells, suggesting that the function of FAK and Cas is regulated in a common signaling pathway (*18*−*20*). However, the mode of Cas tyrosine phosphorylation was different from that of FAK phosphorylation in our study using platelets. As previously reported (*28*, *29*), we confirmed that FAK phosphorylation occurs during a late stage of platelet activation and is dependent on platelet aggregation mediated by fibrinogen binding to αIIbβ3. In contrast, Cas tyrosine phosphorylation was found to occur during the early phase of platelet activation, by an αIIbβ3 integrin-independent mechanism. Independence of Cas from FAK was also supported by the failure of Cas to associate with FAK in immunoprecipitation studies. In platelets, Src, but not FAK, may be responsible for Cas phosphorylation, since Src was coprecipitated with Cas and $^{32}P_i$ was incorporated into the protein of 60 kDa (which corresponds to the molecular weight of Src) in in vitro kinase assay of the Cas immune complex.

Contrary to our initial expectations, Cas was dephosphorylated, but not phosphorylated, in an aggregation-dependent manner. It has been reported that Cas associates with PTPs in several cells, and this may be responsible for the dephosphorylation of Cas, resulting in the termination of tyrosine phosphorylation signaling events (*14*−*18*). In platelets, it has been reported that fibrinogen binding to αIIbβ3 may be involved in the regulation of phosphotyrosine-specific dephosphorylation, suggesting the existence of αIIbβ3-dependent PTPs (*59*). To date, three nonreceptor PTPs, i.e., PTP-1B, SHP-1, and PTP-1D, have been reported to be expressed in platelets (*24*, *30*). Of these, Ezumi et al. reported aggregation-dependent SHP-1 tyrosine phosphorylation (*30*). In contrast, Li et al. reported that SHP-1 phosphorylation is independent of platelet aggregation, but serine phosphoryl-

[2] K. Inoue, Y. Ozaki, T. Ohmori, Y. Wu, K. Satoh, Y. Yatomi, Y. Shin, and T. Morita, manuscript submitted for publication.

ation was mainly observed in this study (*60*). In our case, aggregation-dependent tyrosine phosphorylation of SHP-1, but not PTP-1B or PTP-1D, was observed in platelets stimulated with collagen or TRAP (data not shown). However, none of these PTPs associated with Cas in immunoprecipitation studies (data not shown). Although SHP-1 may be one of the candidates that is responsible for Cas dephosphorylation in platelets, further studies are needed to clarify the mechanism by which Cas is dephosphorylated in a manner dependent on αIIbβ3-mediated platelet aggregation.

Independence of Cas Tyrosine Phosphorylation from Cytoskeletal Reorganization in Activated Platelets. Our present study strongly suggests that Cas tyrosine phosphorylation is independent of αIIbβ3-mediated platelet aggregation and the resultant actin cytoskeletal reorganization. We found that Cas tyrosine phosphorylation occurs during the early phase of platelet activation by a mechanism independent of integrin or FAK, that cytochalasin D, an inhibitor of actin polymerization, fails to block Cas tyrosine phosphorylation, and that Cas is not recovered in the cytoskeletal fraction in activated platelets. To our knowledge, this is the first report on the independence of Cas tyrosine phosphorylation from a cytoskeletal network.

An in vivo experiment using gene-knock out mice (*61*) revealed that (i) Cas contributes to cytoskeletal organization through assembly of actin filaments; (ii) Cas is essential in organization of myofibrils and formation of Z-disks, which are required for normal heart development and maintenance of blood vessel integrity during embryogenesis; and (iii) Cas is required for Src-induced transformation. Since Cas-deficient mice died in utero, the function of Cas-deficient platelets is not examined. Although Cas may maintain cytoskeletal organization and be pivotal in embryonic development and in oncogene-mediated transformation, this adapter molecule may also play an important role in processes unrelated to the cytoskeleton in highly differentiated, anucleate platelets.

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