Association of Fyn and Lyn with the Proline-rich Domain of Glycoprotein VI Regulates Intracellular Signaling

Received for publication, January 30, 2002, and in revised form, April 3, 2002
Published, JBC Papers in Press, April 9, 2002, DOI 10.1074/jbc.M201012200

Katsue Suzuki-Inoue‡‡, David Tulasne‡, Yang Shen‡, Teresa Bori-Sanz‡, Osamu Inoue‡‡, Stephanie M. Jung‡, Masaaki Moroi‡‡, Robert K. Andrews‡, Michael C. Berndt‡‡, and Steve P. Watson‡

From the ‡Department of Pharmacology, University of Oxford, Mansfield Road, Oxford OX1 3QT, United Kingdom, the †Hazel and Pip Appel Vascular Biology Laboratory, Baker Medical Research Institute, St. Kilda Rd, Central, Melbourne, Victoria, Australia 8008, the ‡Division of Medical Sciences, The Medical School Edgbaston, Birmingham B15 2TT, United Kingdom, the **Institute of Life Science, Kurume University, 2432-2 Aikawa, Kurume, Fukuoka, 839-0861 Japan, and the ‡‡Department of Biochemistry and Molecular Biology, Monash University, Clayton, Victoria, Australia 3168

The glycoprotein VI (GPVI)-Fc receptor (FcR) γ-chain complex, a key activator receptor for collagen on platelet surface membranes, is constitutively associated with the Src family kinases Fyn and Lyn. Molecular cloning of GPVI has revealed the presence of a proline-rich domain in the sequence of GPVI cytoplasmic tail which has the consensus for interaction with the Src homology 3 (SH3) domains of Fyn and Lyn. A series of in vitro experiments demonstrated the ability of the SH3 domains of both Src kinases to bind the proline-rich domain of GPVI. Furthermore, depletion of the proline-rich domain in GPVI (Pro(−)-GPVI) prevented binding of Fyn and Lyn and markedly reduced phosphorylation of FcR γ-chain in transiently transfected COS-7 cells, but did not affect the association of the γ-chain with GPVI. Jurkat cells stably transfected with wild type GPVI show robust increases in tyrosine phosphorylation and intracellular Ca(2+) in response to the snake venom convulxin that targets GPVI. Importantly, convulxin is not able to activate cells transfected with Pro(−)-GPVI, even though the association with the immunoreceptor tyrosine-based activation motif-containing chains is maintained. These findings demonstrate that the proline-rich domain of GPVI mediates the association with Fyn/Lyn via their SH3 domain and that this interaction initiates activation signals through GPVI.

The adhesion and activation of platelets by subendothelial collagen fibers initiates aggregate formation at sites of vessel damage. Glycoprotein (GP) VI plays a critical role in the activatory events induced by collagen as shown by the lack of response to collagen in human and mice platelets deficient in the glycoprotein (1, 2). A collagen-related peptide and a snake venom toxin, convulxin, interact specifically with GPVI and mimic many of the responses to collagen (3–5).

Because of the physiological importance of GPVI, the mechanism of the GPVI-mediated signaling system has been extensively investigated (6–8). GPVI is present as a complex with Fc receptor (FcR) γ-chain in the platelet membrane (8–10). The Src family kinases, Fyn and Lyn, are associated with GPVI-FcR γ-chain complex in platelets and initiate activation through phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM) in the FcR γ-chain leading to binding and activation of the tyrosine kinase Syk. A series of adapter molecules including LAT and SLAP76 orchestrate a carefully regulated signaling network leading to activation of PLCγ2, phosphoinositol 3-kinase, and small molecular weight G proteins, leading to platelet activation (6, 7).

The cloning of GPVI (11–14) has revealed it to be a member of the immunoglobulin (Ig) superfamily, showing close homology to FcαRI. GPVI has a charged arginine residue in its transmembrane domain. This arginine, together with elements within the cytoplasmic domain, is crucial for association of GPVI with FcR γ-chain and GPVI-mediated signal transduction (15, 16). In addition, the cytoplasmic tail of GPVI has a cluster of 6 proline residues of unknown function (11–14). This sequence of GPVI, RPLPPPLPFP, contains a consensus Src family kinase-SH3 recognition motif (RPLPPLP) (17, 18), and provides a potential site of interaction with Fyn and Lyn via their SH3 domains.

In this study, we demonstrate that depletion of the proline-rich domain in GPVI abolishes the association with Fyn and Lyn and prevents tyrosine phosphorylation of FcR γ-chain and downstream responses. From these findings, we suggest that Fyn/Lyn directly bind the proline-rich domain of GPVI and that this association is necessary for phosphorylation of the ITAM and downstream signals.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Anti-human GPVI monoclonal antibody, MM20411, was generated by S. Jung and M. Moroi. Anti-Src monoclonal antibody was donated by Dr. S. J. Shattil. GST-Fgr-SH3 and GST-Src-SH3 fusion protein constructs were generous gifts from Yamounouchi Research Institute (Oxford, UK). GST-Btk-SH3 fusion protein and GST-Fyn-SH3 fusion protein constructs were kind gifts from Dr. C. Kinnon (Institute of Child Health, University College London, London, UK) and Dr. B. Schraven (Institute for Immunology, Otto-von-Guericke-Universitat Magdeburg, Magdeburg, Germany), respectively. The GST-Lyn-SH3 domain fusion protein construct was provided by Dr. P. Lock (Ludwig Institute for Cancer Research, Parkville, Australia). Collagen-related peptide was generated as described previously (6). The GST-PLCγ2-SH3 domain fusion protein construct were generated as
Src Kinases Associate with the Proline-rich Domain of GPVI

Src Kinases Associate with the Proline-rich Domain of GPVI

Preparation of MBP and GST Fusion Proteins—A maltose-binding protein (MBP) GST fusion protein was purified from cells overexpressing the GPVI cytoplasmic sequence Glu662-Ser316 (11) subcloned into a plasmid vector (New England Biolabs). According to the manufacturer's instructions (New England Biolabs), the correct sequence was verified by sequencing. MBP and MBP-GPVI expressed in E. coli were purified on amylose-Sepharose as described previously (19). Where appropriate, GST-Lyn-SH3 and GST-Src-SH3 were radiiodinated with sodium [125I]iodide using the chloramine-T method, and separated from free label on Sephadex G-25 (Amersham Biosciences) washes (1). Protein was added to 0.1% (v/v) BSA in TS buffer (0.01 M Tris-HCl, 0.15 M NaCl, pH 7.4) as previously described (21, 22).

Glycoprotein Ib (GPIb) and αIIbβ3 were expressed in COS-7 cells. GPIb and αIIbβ3 were purified on Protein A-Sepharose as described previously (21, 22). Briefly, COS-7 cells were grown in Dulbecco's modified Eagle's medium. Both media contain 10% (v/v) fetal bovine serum. Gibco BRL Life Technologies Ltd. (Paisley, UK). Other reagents were purchased from Upstate Biotechnology, Inc. (TCS Biological Ltd., Botolph Claydon, UK). Anti-Fyn polyclonal antibody, anti-Lyn polyclonal antibody, anti-PLCγ polyclonal antibody, and anti-γ-catenin monoclonal antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Dulbecco's modified Eagle's medium and trypsin-EDTA were from Invitrogen-RBL Life Technologies Ltd. (Paisley, UK). Other reagents were from previously described sources (16, 20).

Preparation of MBP and GST Fusion Proteins—A maltose-binding protein (MBP) GST fusion protein was purified from cells overexpressing the GPVI cytoplasmic sequence Glu662-Ser316 (11) subcloned into a plasmid vector (New England Biolabs). According to the manufacturer's instructions (New England Biolabs), the correct sequence was verified by sequencing. MBP and MBP-GPVI expressed in E. coli were purified on amylose-Sepharose as described previously (19). Where appropriate, GST-Lyn-SH3 and GST-Src-SH3 were radiiodinated with sodium [125I]iodide using the chloramine-T method, and separated from free label on Sephadex G-25 (Amersham Biosciences) washes (1). Protein was added to 0.1% (v/v) BSA in TS buffer (0.01 M Tris-HCl, 0.15 M NaCl, pH 7.4) as previously described (21, 22).

Glycoprotein Ib (GPIb) and αIIbβ3 were expressed in COS-7 cells. GPIb and αIIbβ3 were purified on Protein A-Sepharose as described previously (21, 22). Briefly, COS-7 cells were grown in Dulbecco's modified Eagle's medium. Both media contain 10% (v/v) fetal bovine serum. Gibco BRL Life Technologies Ltd. (Paisley, UK). Other reagents were purchased from Upstate Biotechnology, Inc. (TCS Biological Ltd., Botolph Claydon, UK). Anti-Fyn polyclonal antibody, anti-Lyn polyclonal antibody, anti-PLCγ polyclonal antibody, and anti-γ-catenin monoclonal antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Dulbecco's modified Eagle's medium and trypsin-EDTA were from Invitrogen-RBL Life Technologies Ltd. (Paisley, UK). Other reagents were from previously described sources (16, 20).

Preparation of MBP and GST Fusion Proteins—A maltose-binding protein (MBP) GST fusion protein was purified from cells overexpressing the GPVI cytoplasmic sequence Glu662-Ser316 (11) subcloned into a plasmid vector (New England Biolabs). According to the manufacturer's instructions (New England Biolabs), the correct sequence was verified by sequencing. MBP and MBP-GPVI expressed in E. coli were purified on amylose-Sepharose as described previously (19). Where appropriate, GST-Lyn-SH3 and GST-Src-SH3 were radiiodinated with sodium [125I]iodide using the chloramine-T method, and separated from free label on Sephadex G-25 (Amersham Biosciences) washes (1). Protein was added to 0.1% (v/v) BSA in TS buffer (0.01 M Tris-HCl, 0.15 M NaCl, pH 7.4) as previously described (21, 22).

Glycoprotein Ib (GPIb) and αIIbβ3 were expressed in COS-7 cells. GPIb and αIIbβ3 were purified on Protein A-Sepharose as described previously (21, 22). Briefly, COS-7 cells were grown in Dulbecco's modified Eagle's medium. Both media contain 10% (v/v) fetal bovine serum. Gibco BRL Life Technologies Ltd. (Paisley, UK). Other reagents were purchased from Upstate Biotechnology, Inc. (TCS Biological Ltd., Botolph Claydon, UK). Anti-Fyn polyclonal antibody, anti-Lyn polyclonal antibody, anti-PLCγ polyclonal antibody, and anti-γ-catenin monoclonal antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Dulbecco's modified Eagle's medium and trypsin-EDTA were from Invitrogen-RBL Life Technologies Ltd. (Paisley, UK). Other reagents were from previously described sources (16, 20).

Preparation of MBP and GST Fusion Proteins—A maltose-binding protein (MBP) GST fusion protein was purified from cells overexpressing the GPVI cytoplasmic sequence Glu662-Ser316 (11) subcloned into a plasmid vector (New England Biolabs). According to the manufacturer's instructions (New England Biolabs), the correct sequence was verified by sequencing. MBP and MBP-GPVI expressed in E. coli were purified on amylose-Sepharose as described previously (19). Where appropriate, GST-Lyn-SH3 and GST-Src-SH3 were radiiodinated with sodium [125I]iodide using the chloramine-T method, and separated from free label on Sephadex G-25 (Amersham Biosciences) washes (1). Protein was added to 0.1% (v/v) BSA in TS buffer (0.01 M Tris-HCl, 0.15 M NaCl, pH 7.4) as previously described (21, 22).

Glycoprotein Ib (GPIb) and αIIbβ3 were expressed in COS-7 cells. GPIb and αIIbβ3 were purified on Protein A-Sepharose as described previously (21, 22). Briefly, COS-7 cells were grown in Dulbecco's modified Eagle's medium. Both media contain 10% (v/v) fetal bovine serum. Gibco BRL Life Technologies Ltd. (Paisley, UK). Other reagents were purchased from Upstate Biotechnology, Inc. (TCS Biological Ltd., Botolph Claydon, UK). Anti-Fyn polyclonal antibody, anti-Lyn polyclonal antibody, anti-PLCγ polyclonal antibody, and anti-γ-catenin monoclonal antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Dulbecco's modified Eagle's medium and trypsin-EDTA were from Invitrogen-RBL Life Technologies Ltd. (Paisley, UK). Other reagents were from previously described sources (16, 20).

Preparation of MBP and GST Fusion Proteins—A maltose-binding protein (MBP) GST fusion protein was purified from cells overexpressing the GPVI cytoplasmic sequence Glu662-Ser316 (11) subcloned into a plasmid vector (New England Biolabs). According to the manufacturer's instructions (New England Biolabs), the correct sequence was verified by sequencing. MBP and MBP-GPVI expressed in E. coli were purified on amylose-Sepharose as described previously (19). Where appropriate, GST-Lyn-SH3 and GST-Src-SH3 were radiiodinated with sodium [125I]iodide using the chloramine-T method, and separated from free label on Sephadex G-25 (Amersham Biosciences) washes (1). Protein was added to 0.1% (v/v) BSA in TS buffer (0.01 M Tris-HCl, 0.15 M NaCl, pH 7.4) as previously described (21, 22).

Glycoprotein Ib (GPIb) and αIIbβ3 were expressed in COS-7 cells. GPIb and αIIbβ3 were purified on Protein A-Sepharose as described previously (21, 22). Briefly, COS-7 cells were grown in Dulbecco's modified Eagle's medium. Both media contain 10% (v/v) fetal bovine serum. Gibco BRL Life Technologies Ltd. (Paisley, UK). Other reagents were purchased from Upstate Biotechnology, Inc. (TCS Biological Ltd., Botolph Claydon, UK). Anti-Fyn polyclonal antibody, anti-Lyn polyclonal antibody, anti-PLCγ polyclonal antibody, and anti-γ-catenin monoclonal antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Dulbecco's modified Eagle's medium and trypsin-EDTA were from Invitrogen-RBL Life Technologies Ltd. (Paisley, UK). Other reagents were from previously described sources (16, 20).

Preparation of MBP and GST Fusion Proteins—A maltose-binding protein (MBP) GST fusion protein was purified from cells overexpressing the GPVI cytoplasmic sequence Glu662-Ser316 (11) subcloned into a plasmid vector (New England Biolabs). According to the manufacturer's instructions (New England Biolabs), the correct sequence was verified by sequencing. MBP and MBP-GPVI expressed in E. coli were purified on amylose-Sepharose as described previously (19). Where appropriate, GST-Lyn-SH3 and GST-Src-SH3 were radiiodinated with sodium [125I]iodide using the chloramine-T method, and separated from free label on Sephadex G-25 (Amersham Biosciences) washes (1). Protein was added to 0.1% (v/v) BSA in TS buffer (0.01 M Tris-HCl, 0.15 M NaCl, pH 7.4) as previously described (21, 22).

Glycoprotein Ib (GPIb) and αIIbβ3 were expressed in COS-7 cells. GPIb and αIIbβ3 were purified on Protein A-Sepharose as described previously (21, 22). Briefly, COS-7 cells were grown in Dulbecco's modified Eagle's medium. Both media contain 10% (v/v) fetal bovine serum. Gibco BRL Life Technologies Ltd. (Paisley, UK). Other reagents were purchased from Upstate Biotechnology, Inc. (TCS Biological Ltd., Botolph Claydon, UK). Anti-Fyn polyclonal antibody, anti-Lyn polyclonal antibody, anti-PLCγ polyclonal antibody, and anti-γ-catenin monoclonal antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Dulbecco's modified Eagle's medium and trypsin-EDTA were from Invitrogen-RBL Life Technologies Ltd. (Paisley, UK). Other reagents were from previously described sources (16, 20).
Src kinases associate with the proline-rich domain of GPVI in vitro. A, detachable microtiter wells were coated with MBP or MBP-GPVI, followed by blocking with 5% BSA. To each well was added 200 μl of (i) 125I-labeled GST-Lyn-SH3 or (ii) GST-Src-SH3. Parallel assays included a 50-fold excess of (i) unlabeled GST-Lyn-SH3 or (ii) GST-Src-SH3. B, detachable microtiter wells were coated with a synthetic peptide corresponding to the GPVI cytoplasmic sequence His298-Ser316, followed by incubation with 5% BSA. To each well was added 200 μl of 125I-labeled GST-Lyn-SH3. Parallel assays included a 50-fold excess of unlabeled GST-Lyn-SH3 or (i) 50 μM GPVI synthetic peptide Val292-298, Ser298, or (ii) 5-100 μM GPVI synthetic peptide Val292-Ser298. After 30 min at 22 °C, bound radioactivity was counted in a γ-counter. C, washed platelets stimulated with 10 μg/ml convulxin for 20 s were lysed with lysis buffer. Proteins, precipitated with GST fusion proteins containing the SH3 domain of the indicated proteins, were resolved by 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and immunoblotted with antibodies against (A, i) Lyn, (A, ii) Lyn, (A, iii); and (B, ii), recruitment of GPVI was confirmed by convulxin ligand blotting. The data are representative of four experiments.

The ability of the SH3 domains of Src kinases to bind GPVI was further investigated using platelet lysates. GST fusion proteins of the SH3 domains of the Src kinases Lyn, Fyn, Src, and Fgr bound similar levels of GPVI in control and convulxin-stimulated platelets, as measured by ligand blotting using convulxin, whereas there was no specific binding to GST alone or the SH3 domains of PLCγ2 and Btk (Fig. 1C). These observations confirm the ability of the GPVI tail to selectively associate with the SH3 domains of Src kinases in vitro.

**Lyn and Lyn Bind to the GPVI-FcR γ-chain Complex in Platelets**—The association of Src kinases with the GPVI-FcR γ-chain complex in platelets was investigated through immunoprecipitation of the glycoprotein receptor combined with Western blotting for Src kinases. In agreement with the results of Ezumi et al. (8), we observed a specific association of Fyn and Lyn (Fig. 2A), but not Src (Fig. 2B) with this complex in resting platelets. We also confirmed the absence of Lyn or Lyn association with control mouse IgG immunoprecipitates (data not shown). A small increase (<30%) in association with both kinases was observed after a delay of ~20 s was seen in some but not all experiments. These findings demonstrate that Lyn and Lyn associate with GPVI-FcR γ-chain complex in platelets and that an increase in this association is an early response to GPVI stimulation. However, these results do not provide information on the site of association of Src kinases within this receptor complex, bearing in mind that Src kinases have also been reported to bind non-phosphorylated and phosphorylated ITAMs (26–29).

**Lyn and Lyn Bind Directly to the Proline-rich Domain of GPVI in Vivo**—To investigate whether Lyn and Lyn associate directly with the SH3 domain of GPVI in vivo, we used COS-7 cells reconstituted with wild type GPVI (WT-GPVI) and a receptor mutant lacking the proline-rich domain (Pro−-GPVI; see Fig. 3A). Both forms of GPVI were tagged with FLAG at the C terminus to facilitate analysis through precipitation. Western blotting studies using convulxin and an antibody to convulxin demonstrated that COS-7 cells express similar levels of WT- and Pro−-GPVI on their surface despite the absence of the FcR γ-chain (data not shown). Immunoprecipitation of WT-GPVI with an anti-FLAG antibody demonstrated a direct association with co-transfected Lyn and Lyn (Fig. 3, B and C). In sharp contrast, there was only minimal association of the two Src kinases with Pro−-GPVI despite a similar level of expression of the glycoprotein and the two Src kinases in the transfected cells (Fig. 3, B and C). These findings demonstrate a direct association of Lyn and Lyn with GPVI in vivo and confirm that the proline-rich domain is critical for this interaction.

**Fig. 1.** Src kinases bind to the proline-rich domain of GPVI in vitro. A, detachable microtiter wells were coated with MBP or MBP-GPVI, followed by blocking with 5% BSA. To each well was added 200 μl of (i) 125I-labeled GST-Lyn-SH3 or (ii) GST-Src-SH3. Parallel assays included a 50-fold excess of (i) unlabeled GST-Lyn-SH3 or (ii) GST-Src-SH3. B, detachable microtiter wells were coated with a synthetic peptide corresponding to the GPVI cytoplasmic sequence His298-Ser316, followed by incubation with 5% BSA. To each well was added 200 μl of 125I-labeled GST-Lyn-SH3. Parallel assays included a 50-fold excess of unlabeled GST-Lyn-SH3 or (i) 50 μM GPVI synthetic peptide Val292-298, Ser298, or (ii) 5–100 μM GPVI synthetic peptide Val292-Ser298. After 30 min at 22 °C, bound radioactivity was counted in a γ-counter. C, washed platelets stimulated with 10 μg/ml convulxin for 20 s were lysed with lysis buffer. Proteins, precipitated with GST fusion proteins containing the SH3 domain of the indicated proteins, were resolved by 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and immunoblotted with antibodies against (A, i) Lyn, (A, ii) Lyn, (A, iii); and (B, ii), recruitment of GPVI was confirmed by convulxin ligand blotting. The data are representative of four experiments.

**Fig. 2.** Lyn and Lyn bind to GPVI in platelets. Washed platelets stimulated with 10 μg/ml convulxin for the indicated times were lysed with lysis buffer. Proteins precipitated with anti-GPVI antibody were dissolved in 10% SDS-PAGE, and transferred to polyvinylidene difluoride membranes, and immunoblotted with antibodies against (A, i) Lyn or (A, ii) Lyn, A, iii; and (B, ii), recruitment of GPVI was confirmed by convulxin ligand blotting. The data are representative of four experiments.
Inhibition of FcR γ-Chain Phosphorylation by Deletion of GPVI Proline-rich Domain—We then used COS-7 cells expressing WT or Pro(−)-GPVI and co-transfected FcR γ-chain to investigate whether the association with Lyn and Fyn is necessary for tyrosine phosphorylation of the γ-chain ITAM. Tyrosine phosphorylation of γ-chain was measured by Western blotting following precipitation with a GST fusion protein of the tandem SH2 domains of Syk which selectively binds tyrosine-phosphorylated ITAMs (30). There was no phosphorylation of the FcR γ-chain in the absence of Fyn or Lyn in either WT- or Pro(−)-GPVI expressing cells (not shown). In contrast, the FcR γ-chain was tyrosine phosphorylated under non-stimulated conditions in COS-7 cells transfected with WT-GPVI and Lyn or Fyn but not in cells transfected with Pro(−)-GPVI and either Src kinase (Fig. 4, A and B). Furthermore, the level of tyrosine phosphorylation of the FcR γ-chain was increased in WT-GPVI expressing cells stimulated by convulxin, whereas there was only a marginal change in cells expressing the Pro(−) mutant (Fig. 4, A and B). Importantly, the association of the FcR γ-chain with GPVI was similar in both the WT- and Pro(−)-expressing cells as revealed by FLAG precipitation and convulxin blotting for the FcR γ-chain (Fig. 4C). This demonstrates that the reduced level of tyrosine phosphorylation of the FcR γ-chain in Pro(−)-GPVI-transfected cells is not due to disruption of the GPVI-FcR γ-chain complex. These results demonstrate that the proline-rich domain of GPVI is necessary for interaction with Fyn and Lyn and for subsequent tyrosine phosphorylation of FcR γ-chain upon activation by convulxin.

Deletion of the Proline-rich Domain Abolishes Responses to GPVI following Stable Transfection into Jurkat Cells—Many of the proteins present in the GPVI signaling cascade are restricted to the hematopoietic cell lineage and are therefore absent in COS-7 cells. Because of this, it was important to extend these studies to cells of an hematopoietic background. Available immortalized megakaryocytic-like cells, however, express low levels of endogenous GPVI and/or are not readily susceptible to transfection using standardized methodology. For these reasons, we chose to express WT-GPVI and Pro(−)-GPVI in human Jurkat T cells. It is not necessary to co-transfect the FcR γ-chain to obtain functional responses as GPVI associates with the ITAM-containing γ-chains in this cell (16, 32). In addition, Jurkat cells express the Src kinases Lck and FynT (31).

The snake toxin convulxin stimulated a rapid and sustained increase in intracellular Ca2+ and whole tyrosine phosphorylation in Jurkat cells stably transfected with WT-GPVI but not with Pro(−)-GPVI (Fig. 5A), despite similar levels of receptor expression at the cell surfaces as demonstrated by flow cytometry (Fig. 5B). The G protein-coupled agonist thrombin stimulated a brisk increase of intracellular calcium in Jurkat cells transfected with Pro(−)-GPVI (Fig. 5B, ii), demonstrating that
Src Kinases Associate with the Proline-rich Domain of GPVI

We have demonstrated that the proline-rich region in the GPVI tail associates with the SH3 domains of Fyn and Lyn and have presented evidence in support of a critical role for this interaction in mediating signaling by the receptor. These observations extend a number of previous studies proposing a molecular basis for this interaction (12, 25, 33). The results suggest a model in which cross-linking of the glycoprotein receptor brings Fyn and Lyn to the FeR γ-chain ITAM leading to tyrosine phosphorylation and initiation of downstream events through the tyrosine kinase Syk.

Several lines of evidence support a direct association of Src kinases with the proline-rich region in GPVI. Src kinases were found to bind to a chimera of MBP and the GPVI tail but not to MBP alone, and to a peptide containing the proline-rich region of GPVI. In addition, the SH3 domains of several Src kinases, but not those of Btk and PLCγ2, precipitated GPVI from control and stimulated platelets. Furthermore, the proline-rich domain of GPVI (RPLPLPLL) has the consensus sequence for binding to SH3 domains of the Src kinases (17, 18). A direct association of the Lyn and Fyn kinases in a cellular environment with GPVI was confirmed by transient transfection studies in COS-7 cells, which lack the FcR γ-chain. The functional significance of this interaction was demonstrated using COS-7 and Jurkat cells transfected with WT- and Pro(−)-GPVI. Cells transfected with WT-GPVI, but not Pro(−)-GPVI, exhibited a marked increase in tyrosine phosphorylation of the FcR γ-chain ITAM and intracellular calcium mobilization. Importantly, the association of WT- and Pro(−)-GPVI with FcR γ-chain or ζ-chain was similar in COS-7 and Jurkat cells, respectively.

Src kinases are known to associate with a number of immune receptors and to play an important role in initiating signals. In Jurkat cells, Fyn and Lck associate with the T cell receptor (TCR)–CD3 complex and CD4, respectively, and it has been proposed that these associations mediate phosphorylation of the CD3- and ζ-chain ITAMs (34–36). Fyn and Lck interact directly with CD3 and CD4 complex via their N-terminal unique domains, respectively (34–36). In addition, the N-terminal unique domains of the two Src kinases provide a signal for fatty acid acylation and specific plasma membrane localization, which may also serve to stabilize the interactions between the Fyn SH2 domain and phosphorytrosines in TCR ζ-chain ITAMs (35). Interestingly, the CD3 ε-chain has a cluster of proline residues although, as yet, it is not known whether Fyn or Lck associates with this region via their SH3 domain. However, Denny et al. (37) reported that expression of SH3 domain-deleted Lck in an Lck-deficient T cell line inhibited activation of the mitogen-activated protein kinase pathway but not tyrosine phosphorylation of ζ-chain. This demonstrates that the SH3 domain-proline-rich domain interaction is dispensable for T cell receptor signaling. In B cells, Lyn and Fyn are reported to associate with a short sequence, Asp-Cys-Ser-Met, within the Ig-α chain of the B cell antigen complex via their unique N terminals (26).

We have proposed a model in which the association of Src kinases with the proline-rich region of GPVI is necessary for ITAM phosphorylation. The SH3 domains of Src family kinases have been proposed to be autoinhibitory. X-ray crystallographic studies of Src and Hck revealed that the SH3 domain mediates an intramolecular interaction with an atypical binding site in the region linking the SH2 and kinase domains (38, 39). Erpel et al. (40) have reported that inactivating the SH3 domain of Src induces an 8–10-fold elevation of its kinase activity. Based on these findings, it seems likely that binding of the SH3 domain of Fyn and Lyn to the GPVI tail will increase their intrinsic activity. Since platelets need to be activated promptly upon platelet adhesion to collagen in subendothelium at the site of vesel damage, Src kinases may need to be “ready-to-go” even before stimulation. On the other hand, immune cells do not need to react so promptly to external stimuli, and, moreover, it may be favorable to have Src kinases in a “low” state of reactivity to avoid unwanted activation. Nevertheless, it is
important to investigate whether proline-rich regions in other immune receptors are important for mediating activation signals.

In conclusion, we have shown that the proline-rich domain of the GPVI tail is necessary for the association with Src kinases via their SH3 domain and for mediating activation. This study therefore demonstrates a novel pathway of regulation of ITAM phosphorylation by an Ig domain containing receptor.

Acknowledgments—We are grateful to Drs. S. J. Shattil, C. Kinnon, B. Schraven, P. Lock, M. Leduc, and C. Bon for donating reagents.

REFERENCES

1. Moroi, M., Jung, S. M., Okuma, M., and Shimmyo, K. (1989) J. Clin. Invest. 84, 1440–1445
2. Ryo, R., Yoshida, A., Sugano, W., Yasunaga, M., Nakayama, K., Saijo, K., Adachi, M., Yamaguchi, N., and Okuma, M. (1992) Am. J. Hematol. 39, 25–31
3. Kehrel, B., Wierwille, S., Clemetson, K. J., Anders, O., Steiner, M., Knight, C. G., Farndale, R. W., Okuma, M., and Barnes, M. J. (1998) Blood 91, 491–499
4. Asselin, J., Gibbins, J. M., Achison, M., Lee, Y. H., Morton, L. F., Farndale, R. W., Barnes, M. J., and Watson, S. P. (1997) Blood 89, 1235–1242
5. Polgar, J., Clemetson, J. M., Kehrel, B. E., Wiedemann, M. E., Magenat, M., Wells, T. N., and Clemetson, K. J. (1997) J. Biol. Chem. 272, 13576–13583
6. Watson, S. P. (1999) Thromb. Haemostasis 82, 365–376
7. Pasquet, J. M., Gross, B., Quek, L., Asazuma, N., Zhang, W., Sommers, C. L., Schweighoffer, E., Tybulewicz, V., Judd, B., Lee, J. R., Koretzky, G., Love, P. E., Samelson, L. E., and Watson, S. P. (1999) Mol. Cell. Biol. 19, 8326–8334
8. Ezumi, Y., Shindoh, K., Tsuji, M., and Takayama, H. (1998) J. Exp. Med. 188, 267–276
9. Poole, A., Gibbins, J. M., Turner, M., van Voet, M. J., van de Winkel, G. J. G., Saito, T., Tybulewicz, V. L., and Watson, S. P. (1997) EMBO J. 16, 2333–2341
10. Gibbins, J. M., Okuma, M., Farndale, R., Barnes, M., and Watson, S. P. (1997) FEBS Lett. 413, 255–259
11. Clemetson, J. M., Polgar, J., Magenat, M., Wells, T. N., and Clemetson, K. J. (1999) J. Biol. Chem. 274, 29019–29024
12. Jandrot-Perrus, M., Busfield, S., Lagrue, A. H., Xiong, X., Debili, N., Chickering, T., LeCouedic, J. P., Goodearl, A., Dussault, B., Fraser, C., Vainchenker, W., and Villeval, J. L. (2000) Blood 96, 1788–1807
13. Ezumi, Y., Uchiyama, T., and Takayama, H. (2000) Biochem. Biophys. Res. Commun. 277, 27–36
14. Miura, Y., Ohnuma, M., Jung, S. M., and Moroi, M. (2000) Thromb. Res. 98, 301–309
15. Zheng, Y. M., Liu, C., Chen, H., Locke, D., Ryan, J. C., and Kahn, M. L. (2001) J. Biol. Chem. 276, 12999–13006
16. Berlanga, O., Tulasne, D., Bori-Sanz, T., Snell, D. C., Miura, Y., Jung, S., Moroi, M., Frampton, J., and Watson, S. P. (2002) Eur. J. Biochem., in press
17. Rickles, R. J., Botfield, M. C., Weng, Z., Taylor, J. A., Green, O. M., Brugge, J. S., and Zoller, M. J. (1994) EMBO J. 13, 5098–5104
18. Kay, B. K., Williamson, M. P., and Sudol, M. (2000) FASEB J. 14, 231–241
19. Gross, B. S., Melford, S. K., and Watson, S. P. (1999) Eur. J. Biochem. 263, 612–623
20. Asazuma, N., Wilde, J. I., Berlanga, O., Leduc, M., Lee, A., Schweighoffer, E., Tybulewicz, V., Bon, C., Liu, S. K., McGlade, C. J., Schraven, B., and Watson, S. P. (2000) J. Biol. Chem. 275, 33427–33434
21. Shen, Y., Romo, G. M., Dong, J. F., Schade, A., McIntire, L. V., Kenny, D., Whistock, J. C., Berndt, M. C., Lopez, J. A., and Andrews, R. K. (2000) Blood 95, 903–910
22. Du, X., Fox, J. E., and Pei, S. (1999) J. Biol. Chem. 274, 7362–7367
23. Gu, M., Xi, X., Englund, G. D., Berndt, M. C., and Du, X. (1999) J. Cell Biol. 147, 1985–1996
24. Berlanga, O., Bube, R., Becker, M., Murphy, G., Leduc, M., Bon, C., Barry, F. A., Gibbins, J. M., Garcia, P., Frampton, J., and Watson, S. P. (2000) Blood 96, 2740–2745
25. Quek, L. S., Pasquet, J. M., Hers, I., Cornall, R., Knight, G., Barnes, M., Hibbs, M. L., Dunn, A. R., Lowel, C. A., and Watson, S. P. (2000) Blood 96, 4246–4253
26. Clark, M. R., Johnson, S. A., and Cambier, J. C. (1994) EMBO J. 13, 1911–1919
27. Cambier, J. C. (1995) J. Immunol. 155, 3281–3285
28. Osman, N., Lucas, S., and Cantrell, D. (1995) Eur. J. Immunol. 25, 2863–2869
29. van’t Hof, W., and Reis, M. D. (1999) J. Cell Biol. 145, 377–389
30. Gibbins, J., Asselin, J., Farndale, R., Barnes, M., Law, C. L., and Watson, S. P. (1999) J. Biol. Chem. 274, 18095–18099
31. Wilde, J. I., and Watson, S. P. (2001) Cell. Signal. 13, 691–701
32. Andrews, R. K., Gardiner, E. E., Asazuma, N., Berlanga, O., Tulasne, D., Niewandt, B., Smith, A. I., Berndt, M. C., and Watson, S. P. (2001) J. Biol. Chem. 276, 28092–28097
33. Briddon, S. J., and Watson, S. P. (1999) Biochem. J. 338, 203–209
34. Ledbetter, J. A., Deans, J. P., Arrufo, A., Grosmaire, L. S., Kanner, S. B., Bolen, J. B., and Schieve, G. L. (1993) Curr. Opin. Immunol. 5, 334–340
35. zur Hausen, J. D., Burn, P., and Amrein, K. E. (1997) Eur. J. Immunol. 27, 2643–2649
36. Timson D., Gauen, L. K., Linder, M. E., and Shaw, A. S. (1996) J. Cell Biol. 133, 1007–1015
37. Denny, M. F., Kaufman, H. C., Chan, A. C., and Straus, D. B. (1999) J. Biol. Chem. 274, 5146–5152
38. Sicheri, F., Mearelli, I., and Kuriyan, J. (1997) Nature 385, 602–609
39. Xu, W., Harrison, S. C., and Eck, M. J. (1997) Nature 385, 595–602
40. Erpel, T., Superti-Furga, G., and Courtneidge, S. A. (1995) EMBO J. 14, 963–975
Association of Fyn and Lyn with the Proline-rich Domain of Glycoprotein VI Regulates Intracellular Signaling

Katsue Suzuki-Inoue, David Tulasne, Yang Shen, Teresa Bori-Sanz, Osamu Inoue, Stephanie M. Jung, Masaaki Moroi, Robert K. Andrews, Michael C. Berndt and Steve P. Watson

J. Biol. Chem. 2002, 277:21561-21566.
doi: 10.1074/jbc.M201012200 originally published online April 9, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M201012200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 39 references, 21 of which can be accessed free at http://www.jbc.org/content/277/24/21561.full.html#ref-list-1