Volume 322, number 3, 285–290 FEBS 12453 May 1993

Anti-CD9 monoclonal antibody elicits staurosporine inhibitable phosphatidylinositol 4,5-bisphosphate hydrolysis, phosphatidylinositol 3,4-bisphosphate synthesis, and protein-tyrosine phosphorylation in human platelets

Yutaka Yatomi, Yukio Ozaki, Kaneo Satoh and Shoji Kume

Department of Laboratory Medicine, Yamanashi Medical College, Yamanashi, Japan

Received 12 March 1993

Phosphoinositide metabolism elicited by anti-CD9 monoclonal antibody, a well-characterized platelet activator, was studied using acetylsalicylic acid-treated human platelets. TP82, which is an anti-CD9 monoclonal antibody, induced classical phosphatidylinositol 4,5-bisphosphate hydrolysis, as monitored by intracellular Ca^{2+} mobilization and phosphatidic acid production, and synthesis of phosphatidylinositol 3,4-bisphosphate, which is a major component of newly-described 3-phosphorylated inositol phospholipids produced during platelet activation. These changes were severely inhibited by 1 μM staurosporine, a potent, though non-selective, protein kinase inhibitor, which also abolished TP82 induction of tyrosine phosphorylation of multiple platelet proteins. Protein-tyrosine phosphorylation appears necessary to initiate both the classical phosphoinositide turnover and synthesis of the newly-described 3-phosphorylated inositol phospholipids in anti-CD9 monoclonal antibody-induced platelet activation.

CD9; Staurosporine; Inositol phospholipid; Phosphatidylinositol 3-kinase; Protein-tyrosine phosphorylation; Human platelet

1. INTRODUCTION

CD9 antigen, one of the leukocyte differentiation antigens, is a 24-kDa surface-membrane glycoprotein present on platelets and a variety of hematopoietic and non-hematopoietic tissues [1]. Recent molecular cloning studies have revealed that it belongs to a new family of membrane-spanning proteins and has extensive similarity with a human melanoma-associated antigen ME491, a *Schistosoma mansoni* membrane protein SM23, and so on [2,3]. Although its physiological role remains to be elucidated, it is well established that anti-CD9 monoclonal antibody triggers platelet activation [4–14] mediated by the immunoglobulin G Fc receptor type II (FcγRII) [12–14]. Anti-CD9 antibody-induced platelet activation involves phospholipase A_2 (PLA_2) [9–11] and phospholipase C (PLC) activation [5–8]. However, the mechanism(s) by which the antibody acts on these effectors is largely unknown. It is established that agonists such as thrombin, the receptor for which is a member of the seven transmembrane domain receptor family [15], induce guanine nucleotide-binding protein (G protein)-mediated PLC activation resulting in phosphatidylinositol 4,5-bisphosphate ($PtdIns(4,5)P_2$) breakdown [16,17]. However, this might not be true for anti-CD9 monoclonal antibody-induced PLC activation since CD9 antigen, with four putative transmembrane domains, belongs to a new family of cell-surface proteins which are completely different from G protein-coupled receptors [2,3].

Recently, a novel pathway of inositol phospholipid metabolism involving phosphatidylinositol (PtdIns) 3-kinase activation and resulting in the formation of 3-phosphorylated phosphoinositides has been discovered [17–20]. Although generation of the newly described 3-phosphorylated inositol lipids was originally shown to be associated with mitogenic responses and oncogenic transformation [18–20], terminally differentiated cells such as platelets have also been reported to generate the lipids upon stimulation with agonists such as thrombin [21–23]. Furthermore, the agonistic stimulation of platelets resulted in enhanced protein-tyrosine phosphorylation [24,25], which is likely to regulate PtdIns 3-kinase activity [18–20]. Hence platelet activation induced by anti-CD9 monoclonal antibody, as well as receptor-mediated agonists, may result in synthesis of 3-phosphorylated phosphoinositides, because the anti-

Correspondence address: Y. Yatomi, Department of Laboratory Medicine, Yamanashi Medical College, Tamaho-cho, Nakakoma-gun, Yamanashi 409-38, Japan. Fax: (81) (552) 73 6713.

Abbreviations: FcγRII, immunoglobulin G Fc receptor type II; PLA_2, phospholipase A_2; PLC, phospholipase C; G protein, guanine nucleotide-binding protein; PtdIns, $PtdIns(4,5)P_2$, and $PtdIns(3,4)P_2$, phosphatidylinositol and its specified monoester phosphate derivatives; PA, phosphatidic acid; ASA, acetylsalicylic acid; HPLC, high-performance liquid chromatography; SAX, strong anion exchange; GroPIns, $Gro(4,5)P_2$, and $Gro(3,4)P_2$, glycerophosphoinositol and its specified monoester phosphate derivatives.

body reportedly elicits protein-tyrosine phosphorylation in human platelets [13].

In the context described above, we show here that TP82, which is an anti-CD9 monoclonal antibody [4,26], induces classical PtdIns turnover, phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P_2) synthesis, and protein-tyrosine phosphorylation. All these changes were inhibited by staurosporine [27,28], a potent, although non-selective, protein kinase inhibitor, which may provide a clue with which to elucidate the mechanism of inositol phospholipid metabolism elicited by anti-CD9 monoclonal antibody.

2. MATERIALS AND METHODS

2.1. *Materials*

The following materials were obtained from the indicated suppliers: [^{32}P]orthophosphate (8,500–9,120 Ci/mmol), [^{14}C]phosphatidic acid (PA) (100–200 mCi/mmol), [1-^{3}H]inositol 1,4,5-trisphosphate (15–30 Ci/mmol), [1-^{3}H]inositol 1,3,4-trisphosphate (200–250 Ci/mmol), and Ptd[2-^{3}H]Ins(4,5)P_2 (2–10 Ci/mmol) (DuPont-New England Nuclear, MA, USA); prostaglandin E_1 (Funakoshi, Tokyo, Japan); acetylsalicylic acid (ASA) and bovine serum albumin (Sigma, MO, USA); thrombin (Green Cross, Osaka, Japan); staurosporine (Kyowa Medex, Tokyo, Japan); genistein (Extrasynthese, Genay, France); herbimycin A and lavendustin A (Life Technologies, Inc., MD, USA); the anti-phosphotyrosine antibody PY20 (ICN, CA, USA); mouse IgG standard (Bethyl Laboratories, Inc., TX, USA); anti-v-*src* antibody (Ab-1) (Oncogene Science, Inc., NY, USA); the anti-FcγRII antibody IV.3 (Medarex, Inc., NH, USA); fura2-AM (Dojindo Laboratories, Kumamoto, Japan); TSK-Gel SAX HPLC column (TOSOH, Tokyo, Japan); methylamine (Aldrich, WI, USA). ST638 was kindly provided by Dr. T. Shiraishi (Biochemical Research Laboratories, Kanegafuchi Chemical Industry, Hyogo, Japan). TP82 was produced and purified as previously described [4].

2.2. *Platelet preparation*

Platelet-rich plasma was prepared as described previously [29]. The platelets obtained were washed and resuspended in the buffer containing 138 mM NaCl, 3.3 mM NaH_2PO_4, 2.9 mM KCl, 1.0 mM $MgCl_2$, 1 mg/ml of glucose, and 20 mM HEPES (pH 7.4). The NaH_2PO_4 was omitted when indicated. During all experiments, just before centrifugation of platelet suspensions, 15% vol. of acid-citrate-dextrose A solution (Terumo, Tokyo, Japan) or 1 μM prostaglandin E_1 was added to inhibit platelet activation. The choice of inhibitor did not affect the results. The final platelet count was adjusted to 3×10^8/ml if not otherwise stated. The suspension was further supplemented with 1 mM $CaCl_2$ 5 min before stimulation of the platelets. All experiments using intact platelet suspensions were performed at 37°C.

2.3. *Treatment with ASA*

The platelet suspension was incubated with 1 mM ASA, a cyclooxygenase inhibitor, for 15 min to exclude the secondary effects of thromboxane A_2. Under these conditions, production of thromboxane A_2 was completely suppressed in cells stimulated with any agonists, which was confirmed by measuring thromboxane B_2 formation using a commercial radioimmunoassay kit (DuPont-NEN, MA, USA).

2.4. *$[Ca^{2+}]_i$ measurement*

Platelet $[Ca^{2+}]_i$ measurements were observed using fura2 as described previously [30]. $[Ca^{2+}]_i$ values were calculated from fura2 ratios (R: the ratio of the fluorescence intensity at 340 nm to that at 380 nm) according to the equation [31]: $[Ca^{2+}]_i = K_d(F_o/F_s)(R - R_{min})/(R_{max} - R)$, where R_{min} and R_{max} are the R-values obtained without Ca^{2+} and with the saturating concentration of calcium, respectively. K_d is the effective dissociation constant (224 nm), F_o is the 380 nm excitation signal in the absence of calcium, and F_s is the same signal with the saturating calcium concentration.

2.5. *Platelet labeling with $^{32}P_i$ and extraction of lipids*

Platelets at a cell density of 3×10^9/ml were resuspended in the buffer without NaH_2PO_4, then [^{32}P]orthophosphate (0.5 mCi/ml) was added, and the platelets were incubated for 120 min at 37°C. The cells were then diluted and washed twice with the buffer (with added phosphate). The ^{32}P-labeled washed platelets (1 ml aliquots) were stimulated as indicated and the reactions were stopped by the addition of 3 ml of $CHCl_3/CH_3OH/HCl$ (20:40:1, v/v) followed by thorough mixing and cooling at 4°C. One milliliter each of $CHCl_3$ and 100 mM EDTA was added into the mixture. After separation of aqueous and organic phases by centrifugation, the $CHCl_3$ layer was removed and another 2 ml of $CHCl_3$ was added to the samples and mixed well. The $CHCl_3$ layer was again removed and mixed with the previous $CHCl_3$ extract. Each sample was dried by evaporation under N_2.

2.6. *Deacylation of lipids*

Methylamine reagent (3 ml), consisting of methylamine/methanol/H_2O/*n*-butyl alcohol (prepared according to the method described by Clarke and Dawson [32]), was added to the dried lipids and incubated at 55°C for 60 min in a tightly stoppered tube. After the incubation, the mixture was cooled on ice, cold 1-propanol (1.5 ml) added, and methylamine was removed in vacuo. The samples were dried under N_2, and the residue was dissolved in 1 ml of H_2O and extracted with 1 ml of *n*-butyl alcohol/petroleum ether/ethyl formate (20:4:1, v/v). The upper phase containing fatty acids was discarded. The lower aqueous phase, containing water-soluble deacylated lipids, was washed twice with 1.0 ml of the above solvent mixture, and filtered. Glycerophosphoinositides in the filtrate were separated on high performance liquid chromatography (HPLC).

2.7. *HPLC of glycerophosphoinositides and inositol phosphates*

Glycerophosphoinositides and inositol phosphates were separated by strong anion exchange (SAX) HPLC as described previously [29]. Authentic ^{14}C-labeled PA and ^{3}H-labeled PtdIns(4,5)P_2 were used to confirm the positive identification of the lipids formed in platelets. We also verified the identification of PA, glycerophosphoinositol 3,4-bisphosphate (GroPIns(3,4)P_2), and glycerophosphoinositol 4,5-bisphosphate (GroPIns(4,5)P_2) by deglyceration of these lipids with $NaIO_4$ and dimethylhydrazine as described by Brown and Stewart [33] and that of the resulting P_i, inositol 1,3,4-trisphosphate, and inositol 1,4,5-trisphosphate, respectively.

2.8. *Identification of phosphotyrosine-containing platelet proteins by immunoblotting*

Protein-tyrosine phosphorylation was analyzed in platelet suspensions at a cell density of 9×10^9/ml. After platelet activation, the reactions were terminated with Laemmli SDS reducing buffer [34] plus 500 mM Na_3VO_4, 10 mM EDTA, and 1 mM phenylmethanesulfonyl fluoride, followed by boiling for 3 min. Platelet proteins were separated by 8% SDS-PAGE [34], and electrophoretically transferred to Clear Blot Membrane-P (Atto, Tokyo, Japan). The membranes were blocked with 1% bovine serum albumin in phosphate-buffered saline. After extensive washing with phosphate-buffered saline containing 0.1% Tween 80, the immunoblots were incubated for 3 h with 1 μg/ml of the monoclonal antibody PY-20, which specifically recognizes phosphotyrosine residues [35], or control mouse IgG or 0.5 μg/ml of anti-v-*src* antibody. Antibody binding was detected using peroxidase conjugated goat anti-mouse IgG (Cappel, PA, USA) and visualized with ECL detection reagents (Amersham, UK).

3. RESULTS

Although anti-CD9 monoclonal antibody-induced platelet stimulation involves activation of both PLC and PLA_2, the secondary effects of thromboxane A_2 initi-

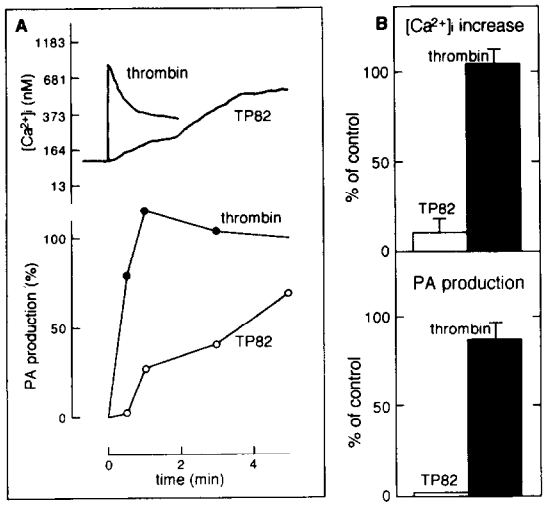

Fig. 1. $[Ca^{2+}]_i$ increase and PA production induced by TP82 or thrombin in ASA-treated human platelets. In A, time courses of $[Ca^{2+}]_i$ transient (upper panel) and PA production (lower panel) induced by 5 μg/ml TP82 or 0.1 U/ml thrombin are shown. In B, the effects of 5 min pretreatment with 1 μM staurosporine on $[Ca^{2+}]_i$ increase (upper panel) and PA production (lower panel) 5 min after the addition of 5 μg/ml TP82 (white columns) or 0.1 U/ml thrombin (black columns) are shown. Each column represents the percent of the control value in the absence of the inhibitor and is the mean ± S.E.M. from 3 determinations.

ated by PLA_2 activation are crucial in platelet activation induced by lower concentrations of the antibody [9–11]. We previously reported that platelet activation, in terms of aggregation, release of intracellular granule contents, production of arachidonic acid metabolites, and elevation of the intracellular Ca^{2+} concentration, induced by lower concentrations of TP82, a monoclonal antibody against CD9 antigen [4], is severely suppressed by ASA treatment [10]. In this study, we used ASA-treated platelets to eliminate the secondary effect of thromboxane A_2 (see section 2). We first observed Ca^{2+} mobilization and PA production, both reflecting PLC-catalyzed PtdIns(4,5)P_2 hydrolysis, induced by TP82. Although PA production can be attributed to hydrolysis of phosphatidylcholine by phospholipase D as well as hydrolysis of PtdIns(4,5)P_2 by a PLC followed by phosphorylation of the diacylglycerol produced, the former reportedly does not represent an important portion in human platelets [36]. Under the conditions employed, TP82, at a concentration as high as 5 μg/ml, induced PtdIns(4,5)P_2 hydrolysis; the time courses of Ca^{2+} mobilization and PA formation under TP82 (5 μg/ml) stimulation, along with those stimulated by thrombin (0.1 U/ml), are shown in Fig. 1A. PtdIns turnover during the antibody-induced activation was delayed, with no apparent peak, but sustained Ca^{2+} mobilization and PA production were observed between 0 and 5 min when compared with those by thrombin, reaching a maximum after 0–1 min (Fig. 1A). Pretreatment with 100 μg/ml of IV.3, an anti-Fcγ RII monoclonal antibody, abolished the TP82-induced PtdIns turnover (data not shown), which is consistent with the previous reports that anti-CD9 monoclonal antibody-triggered platelet activation is mediated by FcγRII [12–14]. The PtdIns(4,5)P_2 hydrolysis induced by TP82 but not thrombin was inhibited by 1 μM staurosporine (Fig. 1B), a potent, although non-selective, protein kinase inhibitor [27,28].

We next studied TP82-induced synthesis of 3-phosphorylated inositol phospholipids produced during platelet activation. The phospholipids extracted from $^{32}P_i$-labeled human platelets were analyzed by SAX HPLC of their glycero-derivatives following deacylation using methylamine. The elution pattern was similar to those previously reported [21,23,29]. Fig. 2A shows

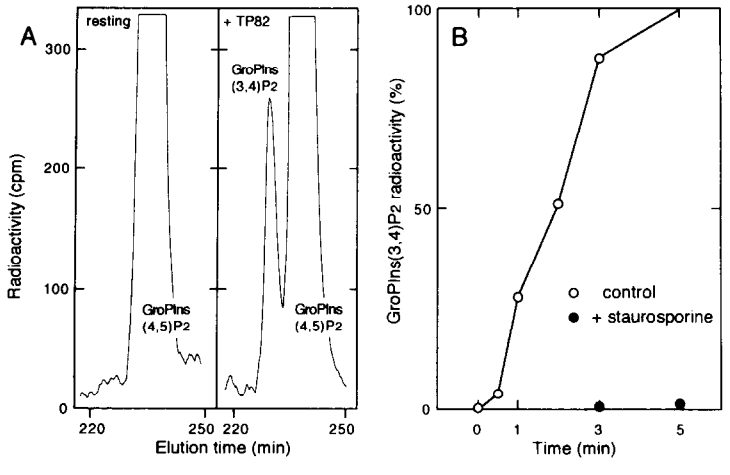

Fig. 2. TP82-induced PtdIns(3,4)P_2 synthesis and its inhibition by staurosporine. GroPIns phosphates extracted and deacylated from platelets labeled with $^{32}P_i$ were separated by SAX HPLC as described in section 2. In A, HPLC analysis of deacylated $^{32}P_i$-labeled lipids from the platelets without (left) or with (right) 5 min treatment with 5 μg/ml TP82 is shown. The peaks of GroPIns(3,4)P_2 and GroPIns(4,5)P_2 are indicated. In B, the time course of TP82 (5 μg/ml)-induced PtdIns(3,4)P_2 production with (•) or without (○) 5 min pretreatment with 1 μM staurosporine are shown. Each point represents the percent of the maximum and is the mean of 2 or 3 experiments.

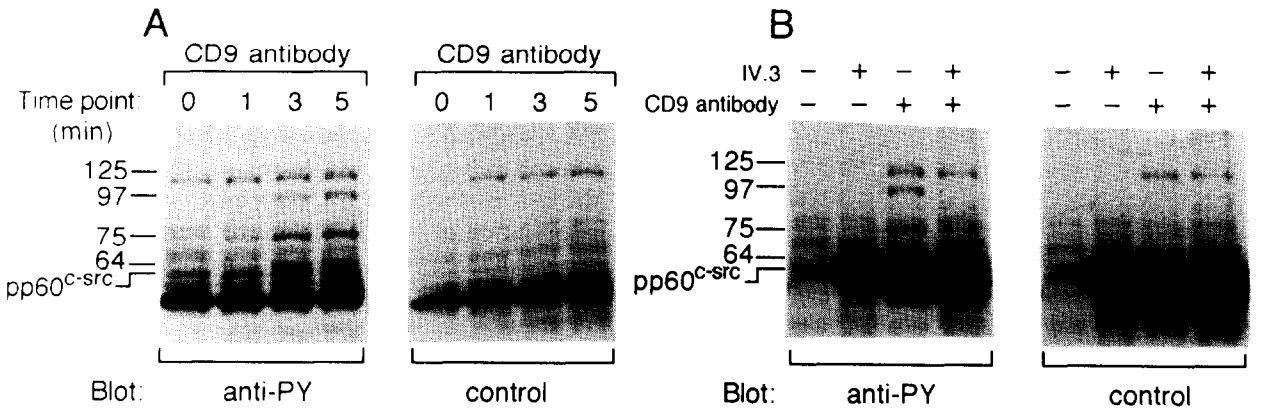

Fig. 3. TP82-induced protein-tyrosine phosphorylation and its inhibition by IV.3. In A, platelets were stimulated with 5 μg/ml TP82 for 0,1, 3 and 5 min, as indicated above lanes. The cells were then immunoblotted with PY20, which specifically recognizes phosphotyrosine residues, (left) or control mouse IgG (right) as described in section 2. The prominent tyrosine-phosphorylated protein bands, upon stimulation, are (in kDa): 64, 75 and 97, and are indicated in the electrophoretogram. Although tyrosine phosphorylation of 125-kDa protein does occur weakly, it is difficult to clearly detect that because of a neighboring non-specific protein band. Other protein bands are also recognized by control mouse IgG as well as by PY20 and are hence considered to be non-specific. The location of $pp60^{c\text{-}src}$ was confirmed by immunoblotting with anti-*v-src* antibody. In B, platelets preincubated without or with 100 μg/ml IV.3 for 5 min were challenged without or with 5 μg/ml TP82 for 5 min, as indicated above lanes. The cells were then immunoblotted with PY20 (left) or control IgG (right) to identify phosphotyrosine-containing proteins.

a typical HPLC profile of deacylated phospholipids extracted from the platelets before and after stimulation of intact cells with 5 μg/ml of TP82; PtdIns(3,4)P_2 was absent from resting platelets, but was produced in the antibody-activated platelets. The TP82-induced synthesis of PtdIns(3,4)P_2 was time-dependent (Fig. 2B). As we had reported previously using thrombin-stimulated platelets [29], PtdIns(3,4)P_2 was the only species of 3-phosphorylated phosphoinositide which was generated upon platelet stimulation with TP82. The stimulated synthesis of PtdIns(3,4)P_2 was completely inhibited by 100 μg/ml of IV.3 (data not shown) or staurosporine (1 μM) (Fig. 2B). Under the conditions employed, we confirmed our previous findings that thrombin (1 U/ml) induces PtdIns(3,4)P_2 synthesis and that it is completely abolished by 1 μM staurosporine [29].

Finally, platelets were incubated with TP82, and protein-tyrosine phosphorylation was assessed by immunoblotting. As shown in Fig. 3A, stimulation of platelets by treatment with 5 μg/ml of TP82 caused an induction of tyrosine phosphorylation of several platelet proteins. The profile of tyrosine phosphorylation induced by TP82 was similar to that induced by thrombin (see Fig. 4B) although in the former, the phosphorylation was delayed and that of 125-kDa protein was not so apparent compared with the latter. The preincubation with 100 μg/ml of IV.3, an anti-Fcγ RII monoclonal antibody, inhibited the anti-CD9 monoclonal antibody-induced tyrosine phosphorylation (Fig. 3B) as previously reported [13]. However, we could not detect phosphorylation of the 40-kDa Fcγ RII itself, which had reportedly been observed in anti-CD9 antibody- but not in thrombin-stimulated platelets [13]. The reason for these discrepancies remains to be clarified. Again the tyrosine phosphorylation elicited by TP82 was completely suppressed by 1 μM staurosporine (Fig. 4A). Under the same conditions, protein-tyrosine phosphorylation induced by thrombin was also inhibited by staurosporine pretreatment (Fig. 4B), as we reported previously [29].

4. DISCUSSION

Although PLA_2 activation with resultant thromboxane A_2 synthesis is crucial in platelet activation induced by anti-CD9 monoclonal antibody, especially at its lower concentrations [9–11], platelets can be fully activated even in the presence of ASA which inhibit cyclooxygenase and resultant thromboxane formation when challenged with high concentrations of TP82, which is an anti-CD9 [4]. The ASA-insensitive pathway is primarily mediated by PLC activation which hydrolyzes PtdIns(4,5)P_2 and leads to a Ca^{2+} signaling pathway [5–8]. We detected delayed PtdIns turnover induced by TP82 (Fig. 1A). The PLC activation induced by TP82 but not thrombin, the receptor for which is a member of the seven transmembrane domain receptor family, was blocked by staurosporine (Fig. 1B). This may provide a clue with which to elucidate the mechanism involving PLC activation induced by anti-CD9 monoclonal antibody, which at present is largely unknown.

Two mechanisms have been identified for receptor-stimulated activation of PLC [17,37,38]. One of these involves the mechanism which is similar to that of receptor-regulated adenylate cyclase or photon-activated cyclic GMP phosphodiesterase and involves G proteins. The other involves protein-tyrosine kinase-mediated phosphorylation of PLC-γ such as that triggered by platelet-derived growth factor receptor. It has been re-

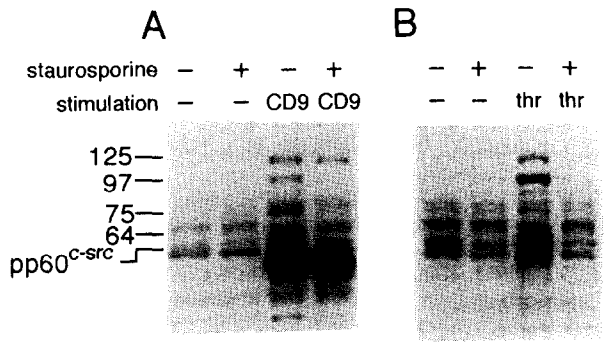

Fig. 4. Inhibition by staurosporine of TP82 (A)- or thrombin (B)-induced protein-tyrosine phosphorylation. Platelet suspensions pretreated with 1 μM staurosporine for 5 min were stimulated with 5 μg/ml TP82 (CD9) or 0.1 U/ml thrombin (thr) for 5 min, as indicated above lanes. The cells were then immunoblotted with PY20 to identify phosphotyrosine-containing platelet proteins.

ported that staurosporine, which non-specifically inhibits several kinases including tyrosine kinase [27,28], clearly discriminates the two mechanisms of PLC activation, dependent upon or independent of a protein-tyrosine kinase. The response through protein-tyrosine phosphorylation is completely suppressed by staurosporine pretreatment [39–41], whereas that triggered by agonists mediated by the G protein-coupled receptor is not inhibited at all [41,42]. In this context, it is most likely that tyrosine phosphorylation plays an important role in PLC activation induced by anti-CD9 monoclonal antibody; PLC activation induced by TP82 but not thrombin, which acts on a G protein-coupled receptor [15], was inhibited by staurosporine (Fig. 1B), which also inhibited TP82-induced protein-tyrosine phosphorylation (Fig. 4A). For further study, we also examined the effect of specific tyrosine-kinase inhibitors such as genistein [43,44], ST638 [45], herbimycin A [46], and lavendustin A [44,47]. However, these agents did not effectively inhibit protein-tyrosine phosphorylation in human platelets (data not shown).

We also showed the TP82-induced stimulation resulted in PtdIns(3,4)P_2 synthesis (Fig. 2), which, to our knowledge, is the first report of stimulated synthesis by anti-CD9 of 3-phosphorylated phosphoinositides. The PtdIns(3,4)P_2 synthesis was also abolished by 1 μM staurosporine. Since generation of 3-phosphorylated inositol phospholipids is supposed to be regulated by events requiring tyrosine kinase activity [18–20], staurosporine inhibition of PtdIns(3,4)P_2 synthesis is most likely due to its inhibition of protein-tyrosine phosphorylation; it did inhibit protein-tyrosine phosphorylation elicited by TP82 (Fig. 4A). It is worth noting that staurosporine suppresses PtdIns(3,4)P_2 synthesis elicited by both TP82 (this study) and thrombin [29] although it exerts discriminating effects on PtdIns(4,5)P_2 hydrolysis induced by these stimulators, as described above.

In conclusion, during anti-CD9 monoclonal antibody-induced platelet activation, both the classical PtdIns turnover and the newly described 3-phosphorylated phosphoinositide synthesis were observed and these were abolished by staurosporine, which was also shown to inhibit protein-tyrosine phosphorylation. It is assumed that tyrosine phosphorylation initiates both the phosphoinositide metabolic pathways in anti-CD9-induced platelet activation.

Acknowledgements: This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

REFERENCES

[1] Horton, M.A. and Hogg, N. (1987) in: Leucocyte Typing III (A.J. McMichael, Ed.) pp. 733–746, Oxford University Press, Oxford.
[2] Boucheix, C., Benoit, P., Frachet, P., Billard, M., Worthington, R.E., Gagnon, J. and Uzan, G. (1991) J. Biol. Chem. 266, 117–122.
[3] Lanza, F., Wolf, D., Fox, C.F., Kieffer, N., Seyer, J.M., Fried, V.A., Coughlin, S.R., Phillips, D.R. and Jennings, L.K. (1991) J. Biol. Chem. 266, 10638–10645.
[4] Higashihara, M., Maeda, H., Shibata, Y., Kume, S. and Ohashi, T. (1985) Blood 65, 382–391.
[5] Higashihara, M., Maeda, H., Yatomi, Y., Takahata, K., Oka, H. and Kume, S. (1985) Biochem. Biophys. Res. Commun. 133, 306–313.
[6] Rendu, F., Boucheix, C., Lebret, M., Bourdeau, N., Benoit, P., Maclouf, J., Soria, C. and Levy-Toledano, S. (1987) Biochem. Biophys. Res. Commun. 146, 1397–1404.
[7] Jennings, L.K., Fox, C.F., Kouns, W.C., McKay, C.P., Ballou, L.R. and Schultz, H.E. (1990) J. Biol. Chem. 265, 3815–3822.
[8] Carroll, R.C., Worthington, R.E. and Boucheix, C. (1990) Biochem. J. 266, 527–535.
[9] Yatomi, Y., Higashihara, M., Ozaki, Y., Kume, S. and Kurokawa, K. (1990) Biochem. Biophys. Res. Commun. 171, 109–115.
[10] Ozaki, Y., Matsumoto, Y., Yatomi, Y., Higashihara, M., Kariya, T. and Kume, S. (1990) Biochem. Pharmacol. 40, 1583–1588.
[11] Ozaki, Y., Matsumoto, Y., Yatomi, Y., Higashihara, M. and Kume, S. (1991) Eur. J. Biochem. 199, 347–354.
[12] Worthington, R.E., Carroll, R.C. and Boucheix, C. (1990) Br. J. Haematol. 74, 216–222.
[13] Huang, M.-M., Indik, Z., Brass, L.F., Hoxie, J.A., Schreiber, A.D. and Brugge, J.S. (1992) J. Biol. Chem. 267, 5467–5473.
[14] Slupsky, J.R., Cawley, J.C., Griffith, L.S., Shaw, A.R.E. and Zuzel, M. (1992) J. Immunol. 148, 3189–3194.
[15] Vu, T.-K.H., Hung, D.T., Wheaton, V.I. and Coughlin, S.R. (1991) Cell 64, 1057–1068.
[16] Siess, W. (1989) Physiol. Rev. 69, 58–178.
[17] Majerus, P.W. (1992) Annu. Rev. Biochem. 61, 225–250.
[18] Downes, C.P. and Carter, A.N. (1991) Cell. Signalling 3, 501–513.
[19] Cantley, L.C., Auger, K.R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R. and Soltoff, S. (1991) Cell 64, 281–302.
[20] Auger, K.R., Serunian, L.A., Soltoff, S.P., Libby, P. and Cantley, L.C. (1989) Cell 57, 167–175.
[21] Sultan, C., Breton, M., Mauco, G., Grondin, P., Plantavid, M. and Chap, H. (1990) Biochem. J. 269, 831–834.
[22] Kucera, G.L. and Rittenhouse, S.E. (1990) J. Biol. Chem. 265, 5345–5348.
[23] Nolan, R.D. and Lapetina, E.G. (1990) J. Biol. Chem. 265, 2441–2445.
[24] Nakamura, S. and Yamamura, H. (1989) J. Biol. Chem. 264, 7089–7091.
[25] Golden, A. and Brugge, J.S. (1989) Proc. Natl. Acad. Sci. USA 86, 901–905.

[26] Ohto, H., Shibata, Y., Takeuchi, A., Chen, R.-F. and Maeda, H. (1985) Scand. J. Haematol. 34, 281–287.
[27] Ruegg, U.T. and Burgess, G.M. (1989) Trends Pharmacol. Sci. 10, 218–220.
[28] Tamaoki, T. (1991) Methods Enzymol. 201, 340–347.
[29] Yatomi, Y., Ozaki, Y. and Kume, S. (1992) Biochem. Biophys. Res. Commun. 186, 1480–1486.
[30] Yatomi, Y., Arata, Y., Tada, S., Kume, S. and Ui, M. (1992) Eur. J. Biochem. 205, 1003–1009.
[31] Cobbold, P.H. and Rink, T.J. (1987) Biochem. J. 248, 313–328.
[32] Clarke, N.G. and Dawson, R.M.C. (1981) Biochem. J. 195, 301–306.
[33] Brown, D.M. and Stewart, J.C. (1966) Biochim. Biophys. Acta 125, 413–421.
[34] Laemmli, U.K. (1970) Nature 227, 680–685.
[35] Glenney, J.R.J., Zokas, L. and Kamps, M.P. (1988) J. Immunol. Methods 109, 277–285.
[36] Huang, R., Kucera, G.L. and Rittenhouse, S.E. (1991) J. Biol. Chem. 266, 1652–1655.
[37] Rhee, S.G. and Choi, K.D. (1992) J. Biol. Chem. 267, 12393–12396.
[38] Michell, R.H. (1992) Trends Biochem. Sci. 17, 274–276.
[39] Olsen, R., Melder, D., Seewald, M., Abraham, R. and Powis, G. (1990) Biochem. Pharmacol. 39, 968–972.
[40] Secrist, J.P., Sehgal, I., Powis, G. and Abraham, R.T. (1990) J. Biol. Chem. 265, 20394–20400.
[41] Yamashita, Y., Hasegawa-Sasaki, H. and Sasaki, T. (1991) FEBS Lett. 288, 46–50.
[42] Watson, S.P. and Hambleton, S. (1989) Biochem. J. 258, 479–485.
[43] Akiyama, T. and Ogawara, H. (1991) Methods Enzymol. 201, 362–370.
[44] O'Dell, T.J., Kandel, E.R. and Grant, S.G.N. (1991) Nature 353, 558–560.
[45] Shiraishi, T., Owada, M.K., Tatsuka, M., Yamashita, T., Watanabe, K. and Kakunaga, T. (1989) Cancer Res. 49, 2374–2378.
[46] Uehara, Y. and Fukazawa, H. (1991) Methods Enzymol. 201, 370–379.
[47] Onoda, T., Iinuma, H., Sasaki, Y., Hamada, M., Isshiki, K., Naganawa, H., Takeuchi, T., Tatsuta, K. and Umezawa, K. (1989) J. Natl. Prod. 52, 1252–1257.