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INVOLVEMENT OF GPIIb-IIIa ON HUMAN PLATELETS IN PHOSPHOTYROSINE-SPECIFIC DEPHOSPHORYLATION

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Washed platelets from either normal donors or patients with thrombasthenia lacking in integrin GPIIb-IIIa were stimulated by thrombin or STA2 with stirring and their kinetics of protein-tyrosine phosphorylation were compared. The early increase in protein-tyrosine phosphorylation on 115 and 75 kDa protein bands was observed within 10 s after stimulation in both normal and thrombasthenic platelets. While both 115 and 75 kDa tyrosine-phosphorylated protein bands were quickly dephosphorylated in normal platelets, thrombin-induced 115 kDa or STA2-induced 115 and 75 kDa protein bands were not dephosphorylated in thrombasthenic platelets. The delay of phosphotyrosine-specific dephosphorylation on those protein bands was observed when thrombin- or STA2-induced aggregation of normal platelets was inhibited by RGDS, an inhibitor of fibrinogen binding to GPIIb-IIIa. These data indicate that fibrinogen binding to GPIIb-IIIa is involved in the regulation of phosphotyrosine-specific dephosphorylation on certain protein bands.

When platelets are activated by certain agonists such as thrombin and collagen, platelets release granule constituents and aggregate with concomitant biochemical changes including phosphoinositide turnover and activation of protein kinase C (1). Recently, particular attention has been focused on protein-tyrosine phosphorylation and its relation to physiological functions in platelets. Protein-tyrosine phosphorylation is regulated by the balance between tyrosine kinase and protein tyrosine phosphatase activities. Platelets have been known to possess several protein tyrosine kinases among which $pp60^{c\text{-}src}$ is most abundant (2, 3).

There are a few studies which have investigated the regulatory mechanisms of protein-tyrosine phosphorylation and its functional role in platelets. Some have reported the possible regulation of protein-tyrosine phosphorylation by intra- and extra-cellular Ca^{2+} and activation of protein kinase C (4, 5). Others have described that thrombin-induced tyrosine phosphorylation of several proteins is dependent on platelet aggregation mediated by fibrinogen binding to the membrane glycoprotein (GP) IIb-IIIa (6, 7), a member of the integrin family of adhesion receptors (8, 9). Recent studies have shown that protein tyrosine kinase $pp125^{FAK}$ is tyrosine phosphorylated by the integrin-mediated events and that $pp125^{FAK}$ phosphorylating activity in vitro strongly correlates with

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The abbreviations used are: PBS, phosphate-buffered saline; ECL, Enhanced Chemiluminescence.

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tyrosine phosphorylation of pp125^{FAK} in vivo (10). Thus, GPIIb-IIIa is supposed to play a functional role not only in the aggregatory mechanism but also in signal transduction by inducing tyrosine phosphorylation of several proteins.

Here, we report another aspect of relationship between GPIIb-IIIa and protein-tyrosine phosphorylation, that is, the involvement of GPIIb-IIIa in dephosphorylation of certain tyrosine phosphorylated proteins.

MATERIALS AND METHODS

Materials. Tetrapeptide RGDS (Arg-Gly-Asp-Ser) was purchased from Sigma. STA2, an analogue of thromboxane A2(11), was provided by Ono Pharmaceutical Co. All other materials were obtained as described previously (5). Polyclonal anti-phosphotyrosine antibodies were prepared by the method of Ek and Heldin (12). The reactivity of those antibodies to phosphotyrosine was checked as described by Nakamura and Yamamura (13). Monoclonal anti-phosphotyrosine antibody PY20 was purchased from ICN Biomedicals, Inc, Costa Mesa, CA.

Platelet preparation and stimulation. Blood was obtained from healthy donors and patients with Glanzmann's thrombasthenia who had not been on any drug for at least 1 week before venipuncture. Anti-coagulation of blood and preparation of washed platelets were performed as described previously (5). Washed platelets (0.5-1.0x10^9/ml) were stimulated by either 0.5u/ml thrombin or 1μM STA2 with stirring in the aggregometer for appropriate intervals. Just prior to addition of those agonists, 1mM CaCl2 was also added to platelet suspensions. In some experiments to inhibit fibrinogen binding to GPIIb-IIIa, 0.4 or 1.0 mM RGDS was present in platelet suspensions 3 min before addition of stimulants.

Deficient levels of GPIIb-IIIa of platelets from patients with Glanzmann's thrombasthenia were confirmed by flow cytometry according to standard techniques (14) using monoclonal antibody CD41a against GPIIb-IIIa purchased from Immunotech, Marseille Cedex, France. Informed consent was obtained from each patient.

Immunoblot procedure. After washed platelets were activated, the reactions were terminated by boiling for 3 min with Laemmli sample buffer, and proteins were separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (15). Subsequently, the gel was electrophoretically transferred onto a nitrocellulose membrane. The membranes were blocked overnight with 1% bovine serum albumin or 5% nonfat dried milk in phosphate-buffered saline (PBS), washed in PBS containing 0.1% Tween 20 (T-PBS) and incubated at room temperature for 1 hr in 2μg/ml affinity-purified polyclonal rabbit anti-phosphotyrosine antibody or monoclonal antibody PY20. The membranes were washed again in T-PBS and incubated for 30 min with 500 to 1000 times diluted horseradish peroxidase conjugated goat anti-rabbit or -mouse IgG in PBS. Immuno-reactivity was determined using the Enhanced Chemiluminescence (ECL) system (Amersham Corp., Arlington Heights, IL). After ECL reagents were mixed 1:1, the mixture was applied to the membrane which was subsequently exposed to film for 5 to 30 s, and then the film was processed.

RESULTS AND DISCUSSION

Washed platelets from either a normal donor or a patient with Glanzmann's thrombasthenia were stimulated by 0.5u/ml thrombin or 1 μM STA2 with stirring in the aggregometer and the reactions were stopped by the addition of Laemmli sample buffer at various time points. The samples were analyzed by anti-phosphotyrosine immunoblot using either monoclonal antibody PY20 or polyclonal antibody prepared in our laboratory. Fig. 1 shows comparison of kinetics of thrombin-induced protein tyrosine phosphorylation between thrombasthenic platelets and normal ones by the analysis of immunoblot using monoclonal antibody PY20. In normal platelets which showed vigorous aggregation, the increase in tyrosine phosphorylation in proteins with molecular masses of 115 and 75 kDa occurred and reached maximum at 10 s. Thereafter, the maximal tyrosine phosphorylation on those protein bands quickly declined to basal level by 1 min. Another tyrosine-phosphorylated protein band with molecular mass of 95 kDa appeared at 1 min and did not disappear for up to 5 min

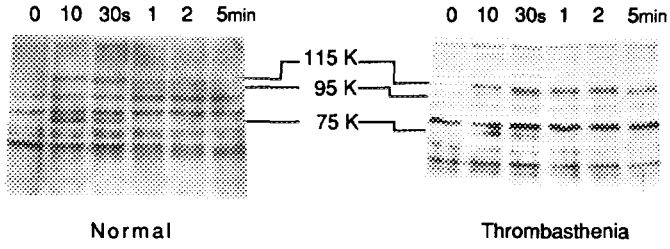

Fig. 1. Time course of protein-tyrosine phosphorylation in thrombin-stimulated platelets. Washed platelets from a normal donor or from a donor with Glanzmann's thrombasthenia were stimulated by 0.5u/ml thrombin with stirring for up to 5 min. The samples were then processed for immunoblot with monoclonal anti-phosphotyrosine antibody PY20 as described under "Materials and Methods".

tested. In thrombasthenic platelets which showed no aggregation, the increase in tyrosine phosphorylation in proteins with molecular masses of 115 and 75 kDa was also observed within 10 s. Although the maximal tyrosine phosphorylation on 75 kDa protein band was quickly dephosphorylated by 1 min as was observed in normal platelets, the dephosphorylation of 115 kDa tyrosine phosphorylated protein band was not observed for up to 5 min tested. Furthermore, the appearance of another 95 kDa tyrosine phosphorylated protein band which was found in normal platelets did not occur. The same samples were also examined using polyclonal anti-phosphotyrosine antibodies prepared in our laboratory. The kinetic profiles of protein tyrosine phosphorylation detected by use of our anti-phosphotyrosine antibodies were quite identical to those by use of monoclonal antibody PY20 (Data not shown).

Fig. 2 shows STA2-induced protein-tyrosine phosphorylation of either thrombasthenic platelets or normal ones which was analyzed by use of our polyclonal antibodies. It was clearly observed that changes in protein-tyrosine phosphorylation of normal platelets stimulated by 1μM STA2 are quite similar to those by 0.5u/ml thrombin. Again, 115 kDa tyrosine phosphorylated protein band was transient in normal platelets but persistent in thrombasthenic ones. In contrast to the result of thrombin stimulation, STA2-induced tyrosine phosphorylation of the 75 kDa protein also persisted for 5 min tested in thrombasthenic platelets but did not in normal platelets. Essentially same results as described above were obtained with immunoblot analysis by use of monoclonal antibody PY20. Although it is not clearly shown in Fig.2, the appearance of 95 kDa tyrosine phosphorylated protein band roughly 1 min after addition of STA2 was observed in normal platelets by repeated immunoblotting of the same samples. This STA2-induced 95 kDa tyrosine phosphorylated protein band did not disappear up to 5 min tested in normal platelets but was never detected in thrombasthenic platelets as was the case with thrombin stimulation. Since thrombasthenic platelets are lacking in membrane GPIIb-IIIa, a fibrinogen receptor, we concluded that the late appearance of tyrosine phosphorylated 95 kDa protein band is regulated by the binding of fibrinogen to GPIIb-IIIa as previously reported by Ferrel and Martin (6). However, the kinetic difference of tyrosine phosphorylated 115 and 75 kDa protein bands between normal and thrombasthenic platelets has not been recognized by any laboratory as yet. In view of the findings that tyrosine phosphorylated 115 kDa protein band is dephosphorylated in normal platelets but not in thrombasthenic platelets, it appears that the disappearance of tyrosine phosphorylated 115 kDa protein band is also regulated by the binding of fibrinogen to GPIIb-IIIa. On the other hand, while tyrosine phosphorylated 75 kDa

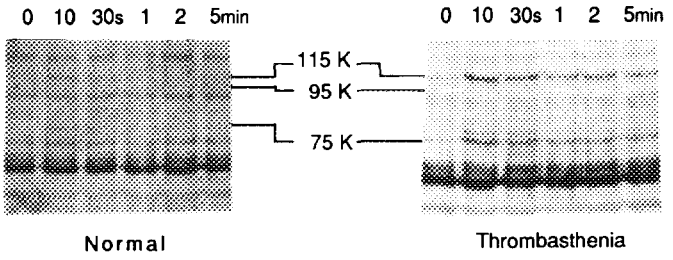

Fig. 2. Time course of protein-tyrosine phosphorylation in STA2-stimulated platelets. Washed platelets from a normal donor or from a donor with Glanzmann's thrombasthenia were stimulated by 1μM STA2 with stirring for up to 5 min. The samples were then processed for immunoblot with polyclonal anti-phosphotyrosine antibodies as described under "Materials and Methods".

protein band induced by thrombin stimulation is dephosphorylated in both normal and thrombasthenic platelets, the one induced by STA2 stimulation is not dephosphorylated in thrombasthenic platelets. This suggests that the mechanism of dephosphorylation regulated by fibrinogen binding to GPIIb-IIIa acts on STA2-induced 75 kDa tyrosine phosphorylated protein but does not on thrombin-induced one.

To determine whether the dephosphorylation of those tyrosine phosphorylated protein bands is actually regulated by the degree of fibrinogen binding to GPIIb-IIIa, we used various concentrations of tetrapeptide RGDS, a specific inhibitor of fibrinogen binding to GPIIb-IIIa, to inhibit platelet aggregation to various degrees. Washed normal platelets in the aggregometer were stimulated by either 0.5 u/ml thrombin or 1μM STA2 in the presence of various concentrations of RGDS and the reactions were stopped by the addition of Laemmli sample buffer at the indicated times for the analysis of anti-phosphotyrosine immunoblot. As shown in Fig.3, the addition of 0.4 or 1mM RGDS to platelet suspension elicited partial or almost complete inhibition of platelet aggregation, respectively, irrespective of stimulants used. As we expected, the disappearance of 115 kDa tyrosine phosphorylated protein was delayed depending upon the degree of inhibition of platelet aggregation. Furthermore, in case of STA2 stimulation, the inhibition of platelet aggregation resulted in the delay of dephosphorylation of both 115 and 75 kDa tyrosine phosphorylated proteins in parallel. On the contrary, in case of thrombin stimulation, the dephosphorylation of 75 kDa tyrosine phosphorylated protein appeared not to be affected by the degree of platelet aggregation. It should be noted that the kinetic patterns of protein tyrosine phosphorylation of 1mM RGDS-treated normal platelets are almost comparable to those of thrombasthenic platelets. From these data, it was concluded that the fibrinogen binding to GPIIb-IIIa is a causal step leading to dephosphorylation of 115 kDa tyrosine phosphorylated protein in thrombin- or STA2-stimulated platelets and of 75 kDa protein in STA2-stimulated ones.

It has been previously shown that thrombin-induced tyrosine phosphorylation of several platelet proteins appears to require the fibrinogen binding to GPIIb-IIIa (6). Golden et al have studied this observation by Ferrell and Martin more in details and demonstrated that agonist-induced tyrosine phosphorylation of several specific platelet proteins is linked to the process of platelet aggregation mediated by the fibrinogen binding to GPIIb-IIIa(7). However, none of those previous reports have pointed out the involvement of GPIIb-IIIa in the regulation of dephosphorylation of certain platelet

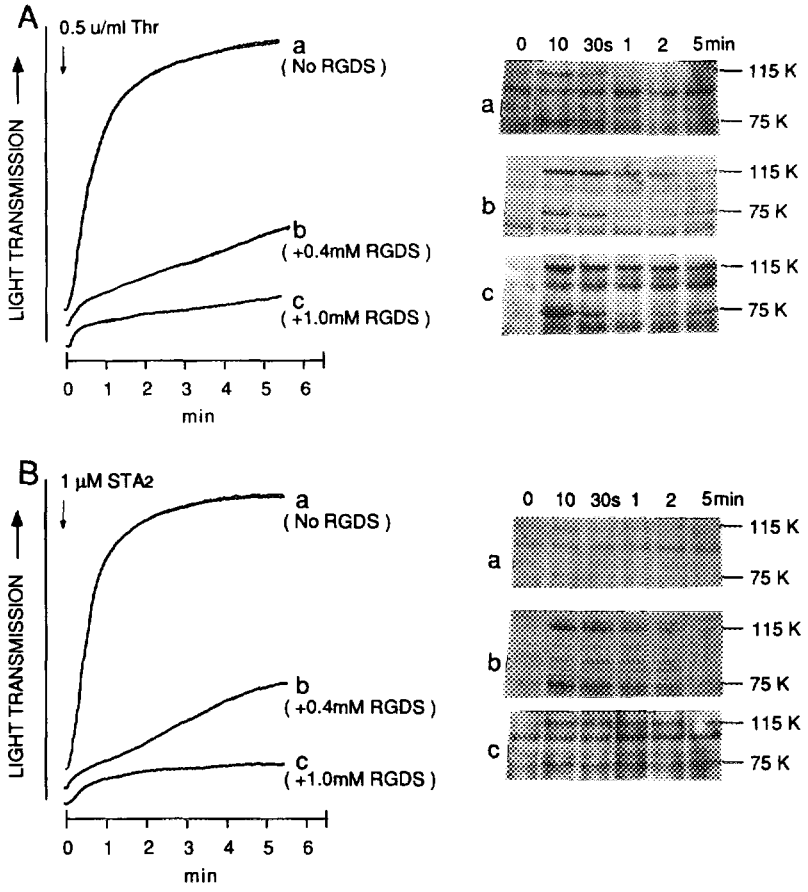

Fig. 3. Effects of RGDS both on dephosphorylation of tyrosine-phosphorylated protein bands and on aggregation in activated normal platelets. Washed platelets from a normal donor were stimulated either by 0.5 u/ml thrombin (A) or by 1μM STA2(B) with stirring in the presence of various concentrations of RGDS for up to 5 min. At the indicated time points, the reactions were stopped by the addition of Laemmli sample buffer and the samples were processed for immunoblot with polyclonal anti-phosphotyrosine antibodies as described under "Materials and Methods".

proteins phosphorylated on tyrosine as reported in this work. It is clear that our findings are not specific to the polyclonal anti-phosphotyrosine antibody prepared in our laboratory, since the same findings were confirmed by monoclonal antibody PY20 which is commercially available and most widely used. It is important to note that the presence or absence of dephosphorylation of certain tyrosine-phosphorylated proteins was dramatically observed by comparing the kinetics of protein-tyrosine phosphorylation of activated normal platelets with that of activated thrombasthenic ones under vigorous stirring rather than shaking. In fact, although Ferrell and Martin compared tyrosine phosphorylation of thrombasthenic platelets with that of normal ones, they did it under shaking rather than vigorous stirring(6). On the other hand, Golden et al have realized the importance of vigorous stirring in the study of protein tyrosine phosphorylation of platelets, but they did not study the comparison of detailed kinetics of protein tyrosine phosphorylation of thrombasthenic platelets with that of normal ones(7). In this regard, it is likely that the subsequent process to platelet aggregation mediated by the fibrinogen binding to GPIIb-IIIa which is caused vigorously by stirring

could be required for phosphotyrosine-specific dephosphorylation. Further studies are in progress in our laboratory to elucidate the mechanisms by which GPIIb-IIIa is involved in the regulation of phosphotyrosine-specific dephosphorylation.

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