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Tyrosine Kinase and Platelet Functions

Yukio OZAKI

Department of Clinical and Laboratory Medicine,
Yamanashi Medical College, Tamaho, Yamanashi 409–38, Japan

Abstract: Tyrosine protein kinases that phosphorylate substrate proteins at the tyrosine residues are abundant in human platelets. The major component of tyrosine kinase is attributed to $pp60^{c-src}$ which is a protooncogene product. Upon stimulation of platelets, several sets of tyrosine-phosphorylated proteins are expressed, the type and time course of which vary with different agonists employed. The tyrosine kinase activity may be closely associated with the activation of the fibrinogen-binding glycoprotein IIb/IIIa, intracellular Ca^{++} mobilization, and inositol phospholipid metabolism.

Key words: Platelet, Tyrosine kinase, Tyrosine phosphorylation, Genistein

1. Signal transduction pathways in platelet activation:

Platelets play an important role in haemostasis, thrombus formation, and vascular regeneration. In light of these roles, elucidation of the mechanisms underlying platelet activation should contribute to an understanding of the pathophysiological processes involved in various diseases. Numerous studies have examined the possible factors that regulate platelet activation, and one of the factors that has received much attention from the beginning was intracellular Ca^{++}. Subsequently, turnover of inositol phospholipids was focused upon as regulating intracellular Ca^{++} mobilization, and it was not long before inositol trisphosphate (IP_3), a metabolite of phosphatidylinositol bisphosphate (PIP_2), was identified as an intracellular mediator that releases Ca^{++} from intracellular Ca^{++} storage sites. Diacylglycerol, another product of PIP_2 catalyzed by phospholipase C, activates protein kinase C, which, in conjunction with a rise in intracellular Ca^{++}, regulates various aspects of platelet function. Where and how do intracellular Ca^{++} and protein kinase C act to modify cellular processes? In platelets, Ca^{++} has been shown to activate Ca^{++}-calmodulin-dependent protein kinase which results in myosin light

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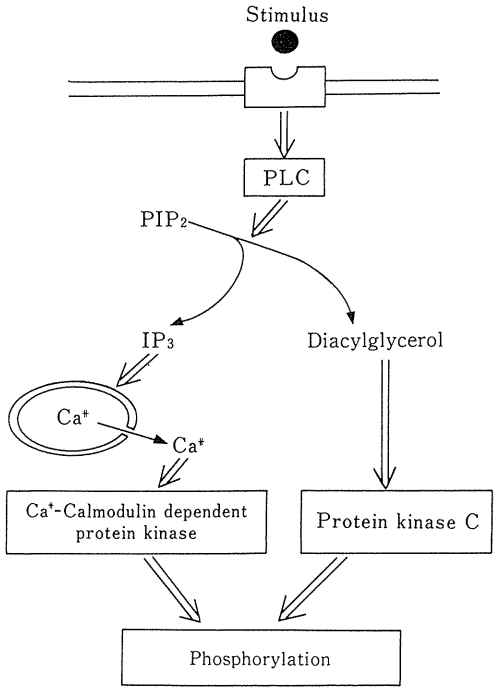

Fig. 1. Postulated signal transduction pathways leading to protein phosphorylation.

chain phosphorylation. This process enhances a series of conformational changes in contractile proteins which result in platelet aggregation. Protein kinase C phosphorylates a 47 kDa protein, and there appears to be a close correlation between the degree of its phosphorylation and platelet activation induced by protein kinase C. While much remains to be elucidated regarding the events which occur after phosphorylation, evidence accumulated thus far suggests that protein phosphorylation plays a key role in platelet activation as well as in a variety of cell types (Fig. 1).

2. Discovery of tyrosine kinases:

Protein kinases catalyze the binding of Pi mainly from ATP to substrate proteins, a process termed phosphorylation. Phosphorylation is believed to induce a conformational change in a substrate enzyme with resultant modification of the enzyme activity. Analysis of phosphorylation sites revealed that phosphorylation occurs mostly at the amino acids that have hydroxyl groups (serine, and threonine), and thus, protein kinases had long been referred to as "serine/threonine kinases". Ca^{++}-calmodulin-dependent protein kinase and protein kinase C found in platelets also fall in this category. In the early 1980's, however, it was demonstrated that a gene product of Rous Sarcoma Virus phosphorylates proteins at tyrosine residues[1]. Tyrosine has a hydroxyl group as serine and threonine do, but the discovery of protein phosphorylation at tyrosine residues was deterred due to its scarcity (only 0.02–0.05% of all phosphorylated amino acids). This report stimulated the pursuit for proteins that phsphorylate substrates at tyrosine residues (tyrosine kinase), and dozens of tyrosine kinases were identified in a short period, most of which turned out to be the products of oncogenes or receptors for growth factors. These findings rendered support for the notion that tyrosine kinase function is closely related to cell growth in general. Tyrosine kinases may also play a major role in signal transduction related to cell-to-cell interaction in light of the facts that tyrosine kinase activities mainly exist in multi-cellular organisms and that they usually reside in the vicinity of cell membranes.

Receptors that express tyrosine kinase activities include those for epidermal growth factor (EGF), platelet-derived growth factor (PDGF), insulin, colony-stimulating factor (CSF). The majority of these factors are linked with cell growth. The binding of specific ligands to these receptors increases the intrinsic tyrosine kinase activity which then phosphorylates substrate proteins at tyrosine residues, and the processes that stimulate cell growth are switched on. Some oncogene products have amino acid sequences identical with those receptors (v-erb products and EGF receptors, and v-fms products and CSF receptors), and are believed to contribute to carcinogenesis by mimicking the activated state of receptors related to cell growth[2),3)]

Platelets and neurons have a comparatively high tyrosine kinase activity. Unlike other types of cells which express tyrosine kinase activities associated with receptors, most of the tyrosine kinase activity in platelets exists in non-receptor type proteins. While a number of proteins appear to possess the activity of tyrosine kinase, $pp60^{c-src}$ is the major and most abundant of all[4]. $pp60^{c-src}$ is a protooncogene product corresponding to $pp60^{v-src}$, a product of the Rous Sarcoma Virus oncogene. It is linked with platelet membranes, but lacks an extracellular domain such as that possessed by EGF receptors. It amounts to 0.2–0.4% of all platelet proteins in weight, and thus platelets are considered to be one of the most suitable sources for $pp60^{c-src}$ purification. Other oncogene products such as $p60^{fyn}$ are also present, but only in 1/10 to 1/20 of the amount[5].

3. Platelet functions and tyrosine kinase:

Thus, the following question arises: why do platelets which lack an ability to proliferate

possess such a high tyrosine kinase activity? We have no clear answer to this issue at present. However, as will be discussed below, an increasing body of evidence suggests tyrosine kinase is somehow related to platelet functions.

The first line of evidence to suggest a close relationship between platelet function and tyrosine kinase is protein tyrosine phosphorylation of platelet proteins upon activation as detected by Western blots. While a few bands of proteins are present in unstimulated platelets whose tyrosine residues are phosphorylated, several new bands of tyrosine-phosphorylated proteins appear upon platelet activation[6)–8)]. The time course of tyrosine phosphorylation correlates well with several parameters of platelet activation such as serotonin release[9)]. A wide variety of agonists have been reported to induce tyrosine phosphorylation, which include thrombin, collagen, platelet-activating factor, vasopressin, phorbol myristate acetate, and A23187. The molecular weights of tyrosine-phosphorylated proteins appear to differ among the agonists described above, suggesting that different types of tyrosine kinases may be involved, depending on the agonists. However, even with the same agonist, there is a great diversity in molecular weights and time courses of tyrosine-phosphorylated proteins among different reports, and no accord has so far been reached on which specific proteins are tyrosine-phosphorylated. This confusion stems from the fact that different antibodies have been used to detect tyrosine phosphorylation in previous reports.

We have also confirmed, using a commercially available monoclonal antibody against phosphotyrosine (PY-20, ICN), that thrombin stimulation induces a new set of tyrosine-phosphorylated proteins (Fig. 2), which closely resemble those reported by Ferrel and Martin[6)].

The second line of evidence for the involve-

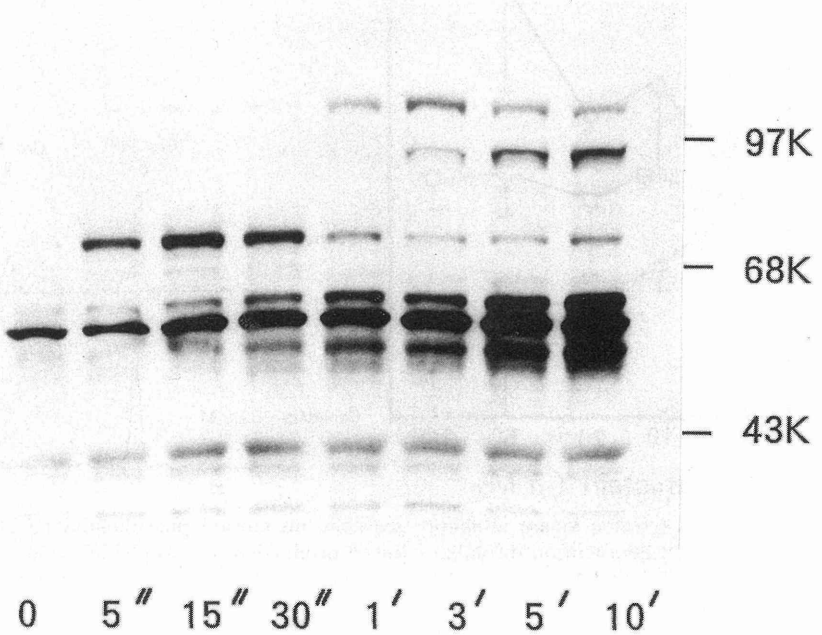

Fig. 2. Time course of tyrosine phosphorylation induced by 1 U/m*l* thrombin. Platelets after thrombin stimulation were solubilized and the samples were applied to SDS-PAGE. Proteins were transferred to a membrane by the Western blot technique, and the tyrosine-phosphorylated proteins were detected using a monoclonal antibody against phosphotyrosine (PY-20).

ment of tyrosine kinase in platelet activation focuses on the effects of various tyrosine kinase inhibitors. Genistein and erbstatin are known to specifically inhibit tyrosine kinase without acting on cAMP-dependent protein kinase or protein kinase C[10),11)]. These agents suppress tyrosine phosphorylation, aggregation, release of granule contents, and phospholipase C activation induced by several agonists[12),13)]. There is also a report that demonstrated an inhibitory effect of genistein on the metabolism of inositol phospholipids induced by U46619, a thromboxane A_2 analogue[14)]. We have also found that inhibition of inositol phospholipid metabolism and Ca^{++} mobilization is a property common to several tyrosine kinase inhibitors (Fig. 3). Although the results obtained with tyrosine inhibitors may not provide direct evidence for the involvement of tyrosine kinase in platelet function, these findings suggest that tyrosine kinase plays a key role in signal transduction pathways regulating Ca^{++} mobilization.

The third line of evidence is related to platelet aggregation. Platelet aggregation appears to have a potentiating effect during platelet activation induced by so called "weak agonists". Since glycoprotein IIb/IIIa which binds fibrinogen and von Willebrand factor is essential for platelet-platelet interactions, it has been suggested that the fibrinogen binding sites of glycoprotein IIb/IIIa are involved in

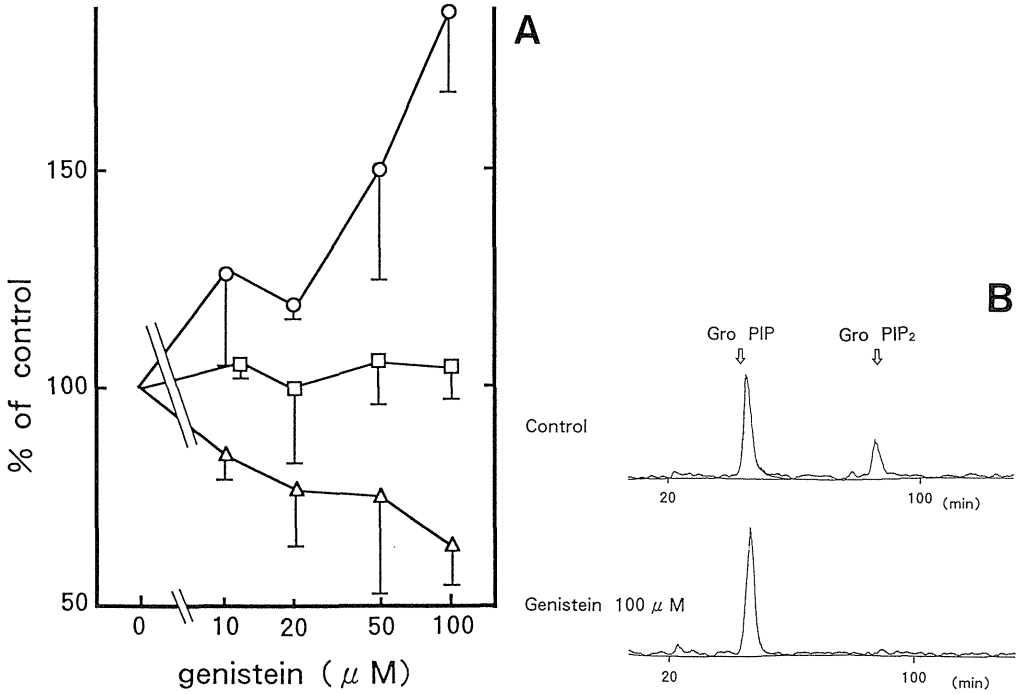

Fig. 3. Effect of a tyrosine kinase inhibitor, genistein, on inositol phospholipid metabolism.
A. Effect of genistein on thrombin-induced production of phosphatidic acid (open boxes), phosphatidylinositol-4-monophosphate (open circles), and phosphatidylinositol-4,5-biphosphate (open triangles). ^{32}P-labeled inositol phospholipids were deacylated by methylamine, and analyzed by strong anion HPLC.
B. Effect of genistein on the production of phosphatidylinositol-4,5-bisphosphate (PIP_2) in platelet homogenates incubated with synthetic H^3-labeled phosphatidylinositol-4-monophosphate (PIP). The lipids extracted from the cell homogenate, and deacylated with methylamine. GroPIP and GroPIP_2 correspond to the deacylated form of PIP and that of PIP_2, respectively.

signal transduction. A recent report has demonstrated that a peptide that inhibits the fibrinogen binding to glycoprotein IIb/IIIa attenuates a subset of the tyrosine phosphorylated proteins formed in response to thrombin, and that the platelets from a thromboathenic patient lacked this set of tyrosine phosphorylation[15]. These findings suggest that glycoprotein IIb/IIIa regulates tyrosine phosphorylation in a certain set of proteins in platelets. In several cell types other than platelets, tyrosine kinase has been shown to copurify with integrin, suggesting that integrin and a protein with tyrosine kinase activity are physically associated in the cytoplasm. Since glycoprotein IIb/IIIa belongs to the integrin superfamily, it is possible that cell-to-cell contact recognized by glycoprotein IIb/IIIa is transmitted via tyrosine kinase which resides in close proximity to glycoprotein IIb/IIIa. On the other hand, there is a report suggesting that glycoprotein IIb/IIIa is phosphorylated by tyrosine kinase which results in modification of its property[16]. The relationship between tyrosine kinase and glycoprotein IIb/IIIa appears to be mutually regulatory.

4. Relationship between tyrosine kinase and other signal transduction pathways:

To date, a number of factors and pathways have been proposed as being involved in signal transduction for cell growth. How is tyrosine kinase related to the factors which have been elucidated so far? What insights can we obtain from the involvement of tyrosine kinase in cell growth on platelet activation? Tyrosine kinase may regulate cell growth in the following modes.

a. activation of serine/threonine protein kinase
b. signal transduction via GTP-binding proteins
c. activation of inositol phospholipid metabolism

Protein kinase C activation may represent the first type of tyrosine kinase involvement. The EGF receptors, which intrinsically has a tyrosine kinase activity, is known to augment protein kinase C by tyrosine-phosphorylating the enzyme[17]. Protein kinase C in turn phosphorylates serine/threonine residues of the EGF receptor, resulting in attenuation of its tyrosine kinase activity[18]. Thus, they seem to form a mutual regulatory relationship. Whether this process also operates in platelet activation remains to be elucidated.

For the second type of tyrosine kinase involvement, we may focus on an oncogene

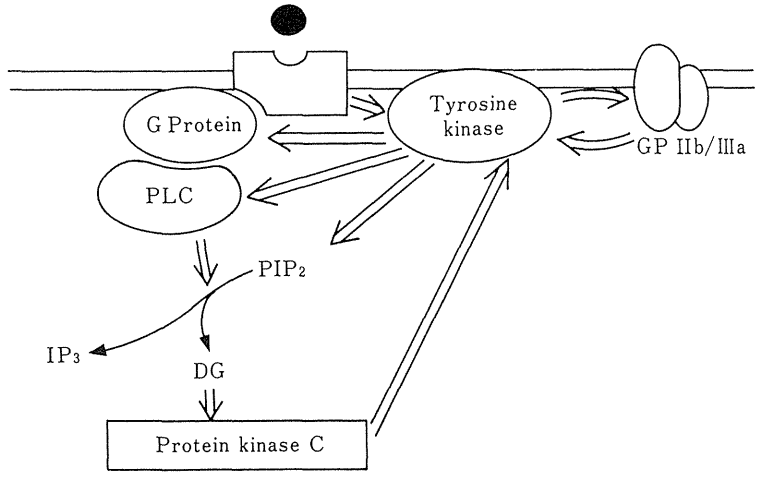

Fig. 4. Cross talk between tyrosine kinase and other signal transduction pathways.

product, ras, which corresponds to a GTP-binding protein. GTP-binding proteins act as "coordinators" between receptors and enzymes involved in signal transduction such as phospholipase A_2, phospholipase C, or adenylate cyclase. Ras appears to accelerate cell growth by acting as an activated form of a GTP-binding protein. There is a report that tyrosine kinase potentiates the activity of ras GTPase-activating protein[3]. Since several ras-related proteins have been found in platelets, cross-talks between tyrosine kinase and these proteins await further evaluations (Fig. 4).

The third mode may be the one that has been drawing the hottest attention of research workers in this field. The most wellknown pathway of inositol phospholipid metabolism is:

1. Phosphatidylinositol (PI) is phosphorylated by PI 4-kinase to form phosphatidylinositol phosphate (PI(4)P).
2. PI(4)P is then phosphorylated by PIP 5-kinase to form $PI(4,5)P_2$.

$PI(4,5)P_2$ is catalyzed by phospholipase C to form inositol trisphosphate (IP_3) and diacylglycerol. Diacylglycerol is eventually phosphorylated by diacylglycerol kinase to form phosphatidic acid, which is then utilized to form PI. A chain of these processes form the "inositol phospholipid cycle". It has been demonstrated that a tyrosine kinase activity is associated with PI kinase in the cytoplasm and works as an activator of PI kinase. To the surprise of many workers, PIP formed by activation of tyrosine kinase was PI(3)P with its inositol position 3 phosphorylated instead of PI(4)P, which is a common precursor of $PI(4,5)P_2$[19]. $PI(3,4)P_2$, formed from PI(3)P, cannot serve as a substrate for phospholipase C, and hence, results in no production of IP_3 that mobilizes intracellular Ca^{++}. While the mechanism by which 3-phosphorylated inositol phospholipids transmit their activation signal remains obscure, there is an accumulating body of evidence to support an important role for $PI(3,4)P_2$ in cell growth regulation. In platelets, thrombin stimulation induces the production of considerable amounts of PI(3)P and $IP_3(1,3,4)$, a metabolite of PI(3)P[20]. $pp60^{c-src}$, the major tyrosine kinase in platelets, is physically associated with PI-kinase[21]. As in other types of cells, there is evidence to implicate that tyrosine kinase contributes to platelet activation via the regulation of PI 3-kinase. Several hypotheses have been proposed on the possible functions of 3-phosphorylated inositol phospholipids, which include their regulatory effects on the metabolism of 5-phosphorylated inositol phospholipids and the stimulatory effect of

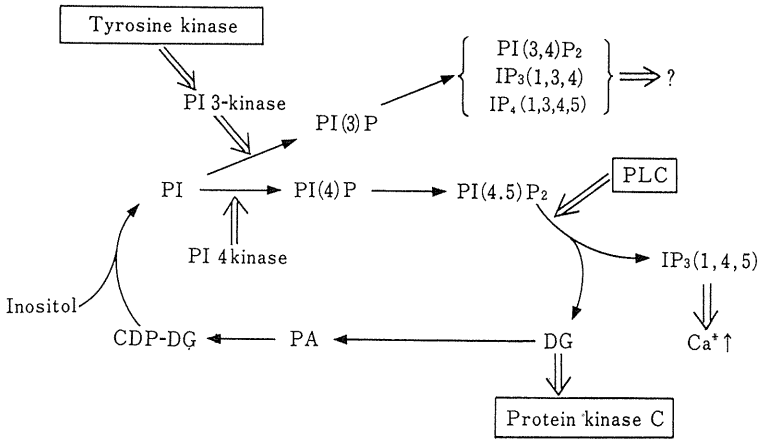

Fig. 5. Relationship between tyrosine kinase and inositol phospholipid metabolism.

$IP_3(1,3,4)$ on $IP_3(1,4,5)$ production (Fig. 5).

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