The EMBO Journal Vol.16 No.9 pp.2342–2351, 1997

Translocation of the Csk homologous kinase (Chk/Hyl) controls activity of CD36-anchored Lyn tyrosine kinase in thrombin-stimulated platelets

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Chk/Hyl is a recently isolated non-receptor tyrosine kinase with greatest homology to a ubiquitous negative regulator of Src family kinases, Csk. To understand the significance of co-expression of Chk and Csk in platelets, we examined the subcellular localization of each protein. Chk, but not Csk, was completely translocated from the Triton X-100-soluble to the Triton X-100-insoluble cytoskeletal fraction within 10 s of thrombin stimulation. Chk and Lyn, but not Csk and c-Src, co-fractionated in the higher density lysate fractions of resting platelets, with Chk being found to localize close to CD36 (membrane glycoprotein IV)-anchored Lyn. The kinase activity of co-fractionated Lyn was suppressed 3-fold. *In vitro* phosphorylation assays showed that Chk suppressed Lyn activity by phosphorylating its C-terminal negative regulatory tyrosine. Upon stimulation of platelets with thrombin, the rapid and complete translocation of Chk away from Lyn caused concomitant activation of Lyn. This activation was accompanied by dephosphorylation of Lyn at its C-terminal negative regulatory tyrosine in cooperation with a protein tyrosine phosphatase. These results suggest that Chk, but not Csk, may function as a translocation-controlled negative regulator of CD36-anchored Lyn in thrombin-induced platelet activation.
Keywords: CD36-anchored Lyn kinase/Csk homologous kinase/platelet activation/translocation/tyrosine phosphorylation

Introduction

Src family protein tyrosine kinases play crucial roles in regulating proliferation and differentiation of multiple cell types, including hematopoietic cells (Bolen *et al.*, 1992; Mustelin and Burn, 1993). The tyrosine kinase activity of Src family kinases is tightly regulated by tyrosine phosphorylation and dephosphorylation events (Cooper, 1990). The non-receptor-type tyrosine kinase C-terminal Src kinase (Csk), has been shown to phosphorylate the C-terminal negative regulatory tyrosine residue of Src family kinases and suppress their kinase activity (Okada and Nakagawa, 1989; Nada *et al.*, 1991, 1993; Okada *et al.*, 1991; Sabe *et al.*, 1992; Bergman *et al.*, 1992; Imamoto and Soriano, 1993).

Hematopoietic consensus tyrosine-lacking kinase (Hyl) was cloned from the human megakaryocytic cell line UT-7 and found to possess greatest homology to Csk (Sakano *et al.*, 1994). Like Csk, Hyl has Src homology 3 (SH3) and SH2 domains and lacks the consensus tyrosine phosphorylation and myristoylation sites found in Src family kinases. The Hyl and Csk genes are also structurally related, having the same intron–exon organization (Hamaguchi *et al.*, 1994). cDNAs encoding proteins closely related to Csk and identical to Hyl, such as Matk, Ctk, Ntk, Lsk and Batk, have been reported (Bennett *et al.*, 1994; Chow *et al.*, 1994a; Klages *et al.*, 1994; Kuo *et al.*, 1994; McVicar *et al.*, 1994). Since these cDNAs represent the mouse, rat and human homologs of the same gene, a new name for these kinases, Csk homologous kinase (Chk), has been proposed (D.W.McVicar, personal communication).

Like Csk, Chk phosphorylates the negative regulatory tyrosine residues of Src family kinases *in vitro* and in a yeast co-expression system, suggesting that Chk may share functional properties with Csk (Chow *et al.*, 1994a; Klages *et al.*, 1994; Avraham *et al.*, 1995). However, Csk is ubiquitously expressed, whereas Chk expression is restricted to hematopoietic cells and neuronal cells in the brain. The expression of both Chk and Csk in these cell types implies either functional redundancy or specific roles for both kinases. While recent studies indicate that Chk and Csk might differentially regulate the functions of Src family kinases (Chow *et al.*, 1994b; Musso *et al.*, 1994; Jhun *et al.*, 1995), the function of Chk is still unknown.

Activation of platelets with thrombin results in shape change, secretion of granular contents and aggregation. Platelets contain a number of non-receptor-type tyrosine kinases, including five Src family kinases (c-Src, c-Yes, Fyn, Lyn and Hck; Horak *et al.*, 1990; Shattil and Brugge, 1991), Syk (Taniguchi *et al.*, 1993) and FAK (Lipfert *et al.*, 1992). Receptor-type tyrosine kinases have not been identified in platelets. During platelet activation, there is a rapid elevation of tyrosine-phosphorylated proteins due to activation of tyrosine kinases (Ferrel and Martin, 1988; Golden and Brugge, 1989; Nakamura and Yamamura, 1989; Dhar and Shukla, 1991; Clark *et al.*, 1994; Schoenwaelder *et al.*, 1994). c-Src and Syk have been shown to translocate from the cell membrane to the cytoskeletal fraction in activated platelets (Horvath *et al.*, 1992; Oda *et al.*, 1992; Clark and Brugge, 1993; Tohyama *et al.*, 1994). Activation of downstream signaling cascades by these tyrosine kinases may require their association with the cytoskeleton.

CD36 (glycoprotein IV, GP IV), one of the major platelet membrane glycoproteins, is physically associated with Lyn, Fyn and c-Yes (Huang *et al.*, 1991). Interaction of CD36 with the fibrinogen-liganded form of integrin αIIbβ3 (GP IIb/IIIa) is hypothesized to stabilize platelet

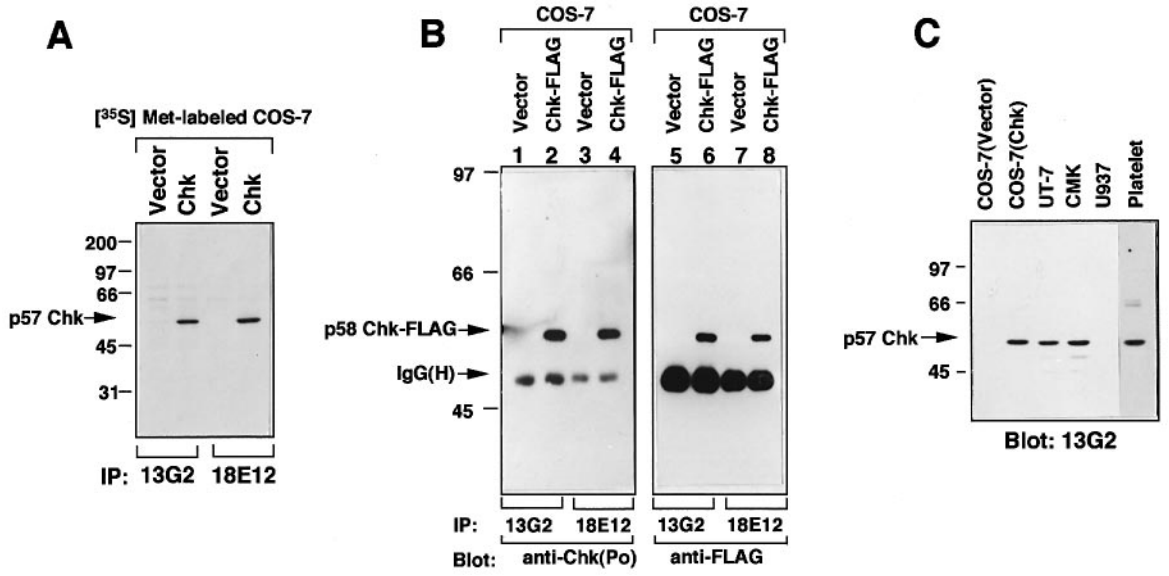

Fig. 1. Detection of Chk protein. (**A**) Fluorogram of SDS–PAGE of immunoprecipitates with two monoclonal antibodies, 13G2 and 18E12, from metabolically labeled COS-7 cells transfected with Chk or vector alone. (**B**) Western blots of immunoprecipitates from COS-7 cells transfected with Chk–FLAG or vector alone probed with anti-Chk (Po: C-2930) or anti-FLAG. IgG(H), heavy chain of immunoglobulin G. (**C**) Western blot of Triton X-100-soluble lysates from various cells, probed with anti-Chk (13G2).

aggregation, leading to completion of the platelet activation process.

In this report we show that Chk, unlike Csk, localizes with CD36-anchored Lyn and negatively regulates its activity in resting platelets. Upon thrombin stimulation the rapid and complete translocation of Chk triggers activation of CD36-anchored Lyn in co-operation with a protein tyrosine phosphatase to dephosphorylate its negative regulatory tyrosine residue. Our findings suggest that Chk, but not Csk, plays an important role in the regulation of kinase activity of CD36-anchored Lyn in thrombin-stimulated platelets.

Results

Detection of Chk in platelets using monoclonal antibodies

Using a GST–Chk fusion protein and a synthetic peptide of the C-terminal region of Chk as antigens, two monoclonal antibodies (13G2 and 18E12) and an affinity-purified polyclonal antibody (C-2930) against Chk were generated. To verify the specificity, expression of Chk and FLAG epitope-tagged Chk (Chk–FLAG) in COS-7 cells was examined. A single protein of 57 kDa was immunoprecipitated with 13G2 or 18E12 from the lysate of [^{35}S]Met-labeled COS-7 cells transfected with Chk (Figure 1A). The immunoprecipitate with 13G2 or 18E12 from the lysate of COS-7 cells transfected with Chk–FLAG was detected by both C-2930 and anti-FLAG (Figure 1B). 13G2 also detected Chk in the lysate of COS-7 cells transfected with Chk by Western blotting (Figure 1C). These results confirm that these monoclonal antibodies specifically recognize Chk.

Next, we examined expression of Chk in several human cell lines by Western blotting. Figure 1C shows that Chk was detected at 57 kDa in UT-7 cells (megakaryocytic) and CMK cells (megakaryoblastic) but not in U937 cells (monocytic), consistent with previous observations of Chk mRNA expression (Bennett *et al.*, 1994; Sakano *et al.*, 1994). Notably, Chk is expressed in human platelets (Figure 1C).

Translocation of Chk to the cytoskeleton in thrombin-activated platelets

Since c-Src and Syk are translocated from the Triton X-100-soluble fraction to the Triton X-100-insoluble fraction (i.e. the cytoskeleton) in activated platelets, we investigated whether Chk was translocated to the cytoskeleton. After stimulation with thrombin for 15 s, Chk was completely translocated to the Triton X-100-insoluble fraction and then gradually degraded (Figure 2A). When platelets were incubated with inactivated thrombin treated with D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK), translocation of Chk to the Triton X-100-insoluble fraction was not observed (data not shown).

On the other hand, c-Src was translocated to the Triton X-100-insoluble fraction at a relatively slow rate during thrombin stimulation and only 20% of c-Src was translocated (Figure 2B), consistent with previous observations (Clark and Brugge, 1993). Csk was also detected in the Triton X-100-soluble fraction (Figure 2C). Interestingly, $<$3% of Csk was translocated to the Triton X-100-insoluble fraction upon thrombin stimulation, with dynamics similar to those of c-Src (Figure 2C), implying that Chk and Csk may play different roles in platelet activation.

Kinase activities of Chk during thrombin stimulation

To examine whether the translocation of Chk affected its kinase activity, immune complex kinase assays were performed. Resting and stimulated platelets were solubilized with RIPA buffer to recover Chk from both the Triton X-100-soluble and Triton X-100-insoluble fractions. Figure 3A shows that Chk immunoprecipitated from resting and stimulated platelets had nearly the same kinase

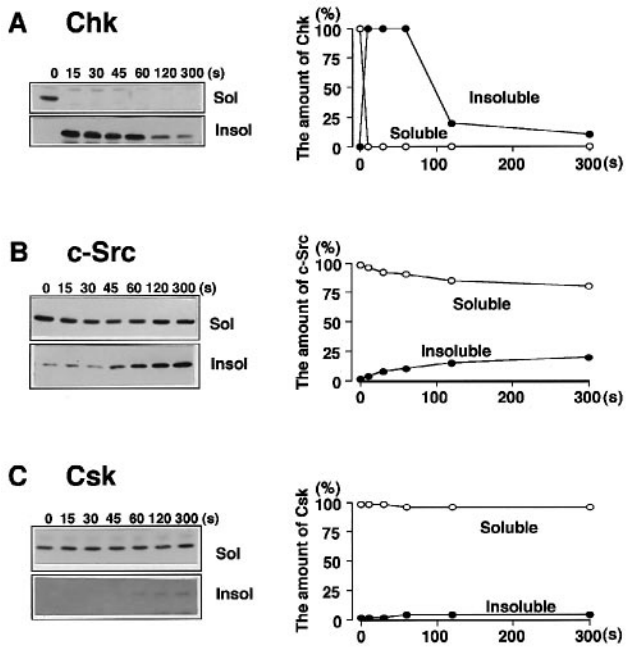

Fig. 2. Distribution of Chk, c-Src and Csk during thrombin stimulation. (Left panels) Western blots of Triton X-100-soluble and Triton X-100-insoluble fractions of platelets stimulated for the indicated times (s) with thrombin probed with anti-Chk (13G2) (**A**), anti-Src (**B**) and anti-Csk (**C**). (Right panels) densitometric quantification of the amounts (%) of kinases in Triton X-100-soluble (open circles) and Triton X-100-insoluble (closed circles) fractions.

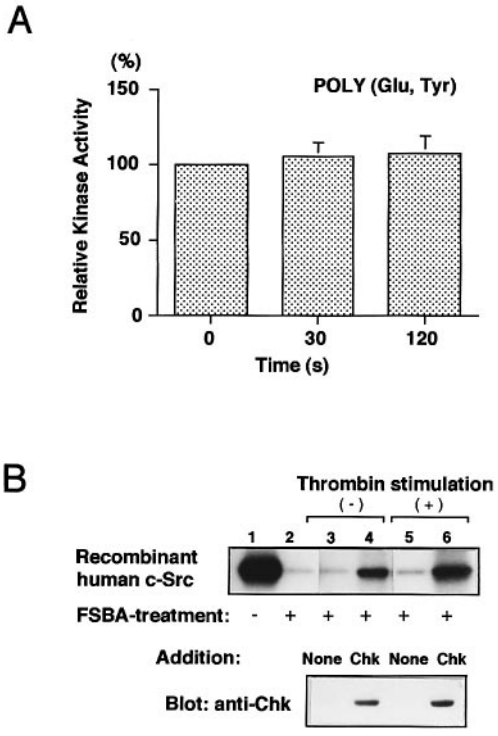

Fig. 3. Kinase activities of Chk during thrombin stimulation. (**A**) *In vitro* kinase assays of immunoprecipitates with anti-Chk (13G2) from platelets stimulated with thrombin for the indicated times. Kinase activities are expressed as the values [means ± SE of three different experiments (%)] relative to the specific activity of Chk in the unstimulated sample after normalizing the incorporation of $^{32}PO_4$ into poly(Glu,Tyr) for each level of Chk protein. (**B**) (Upper panel) autoradiogram of *in vitro* phosphorylation of c-Src (lane 1) and of FSBA-treated c-Src (lanes 2–6) by addition of immunoprecipitates with MOPC21 as a control antibody (lanes 3 and 5) or with anti-Chk (13G2) (lanes 4 and 6) from platelets incubated for 30 s with (lanes 5 and 6) or without (lanes 3 and 4) thrombin. (Lower panel) Western blot of the added immunoprecipitates probed with anti-Chk (13G2).

activity on an exogenous substrate, poly(Glu,Tyr). We next examined the activity of Chk with c-Src as substrate. Autophosphorylation of c-Src was inhibited by treatment with an ATP analog, *p*-fluoro-sulfonylbenzoyl 5′-adenosine (FSBA), which is known to inactivate c-Src by reacting with Lys295 (Figure 3B, lanes 1 and 2; Kamps *et al.*, 1984; Okada and Nakagawa, 1989). The inactivated c-Src was phosphorylated by Chk immunoprecipitated from resting or stimulated platelets (lanes 3–6). The relative specific activities of Chk immunoprecipitated from resting and stimulated platelets were estimated to be nearly the same by normalizing the Chk activity to the amounts of Chk shown in the lower panel. Furthermore, the relative specific activities of Csk immunoprecipitated from resting and stimulated platelets were also estimated to be the same (data not shown). These results suggest that thrombin stimulation does not affect the activities of Chk and Csk.

Plasma membrane anchorage of Chk with a protein complex

Although Chk does not possess any known membrane anchoring motifs, in resting platelets >90% of Chk was localized to the membrane fraction, with the remainder in the cytosol fraction (Figure 4A). In sharp contrast, most Csk was localized to the cytosol fraction. c-Src and Lyn were localized to the membrane fraction, as expected due to their post-translational lipid modification. Figure 4B shows fractionation of a 3-[(3-cholamidopropyl)dimethyl ammonio]-1-propanesulfonate (CHAPS) lysate obtained from resting platelets on density gradients. All Chk and a large amount of Lyn co-fractionated in the higher density lysate fractions (fraction 5 and later fractions, >200 kDa as judged from calibration with molecular markers), whereas all Csk and ~50% of c-Src were detected primarily as monomers. Moreover, cell surface biotinylation of resting platelets revealed 68, 58 and 52 kDa proteins co-immunoprecipitating with Chk, while no biotinylated proteins were co-immunoprecipitated with Csk (Figure 4C). These results suggest that Chk, but not Csk, forms a complex and is physically associated with cell surface membrane proteins.

Association of Chk with a complex of CD36 including Lyn

CD36 is reported to associate with Lyn (Huang *et al.*, 1991). In addition, the distributions of Chk and Lyn overlap with each other (Figure 4B) and the patterns of Chk and CD36 were nearly the same on density gradients (fraction 5 and later fractions; data not shown). We therefore examined whether these kinases physically associated with CD36 in resting platelets. Figure 5A shows that after chemical crosslinking Chk was detected in an immunoprecipitate with anti-CD36. Without chemical crosslinking, an association of Chk with the immune complex of CD36 was not detected (data not shown). The immune complex of CD36 also contained Lyn, but not c-Src (Figure 5A), consistent with previous observations (Huang *et al.*, 1991). In contrast to Chk, Csk was not

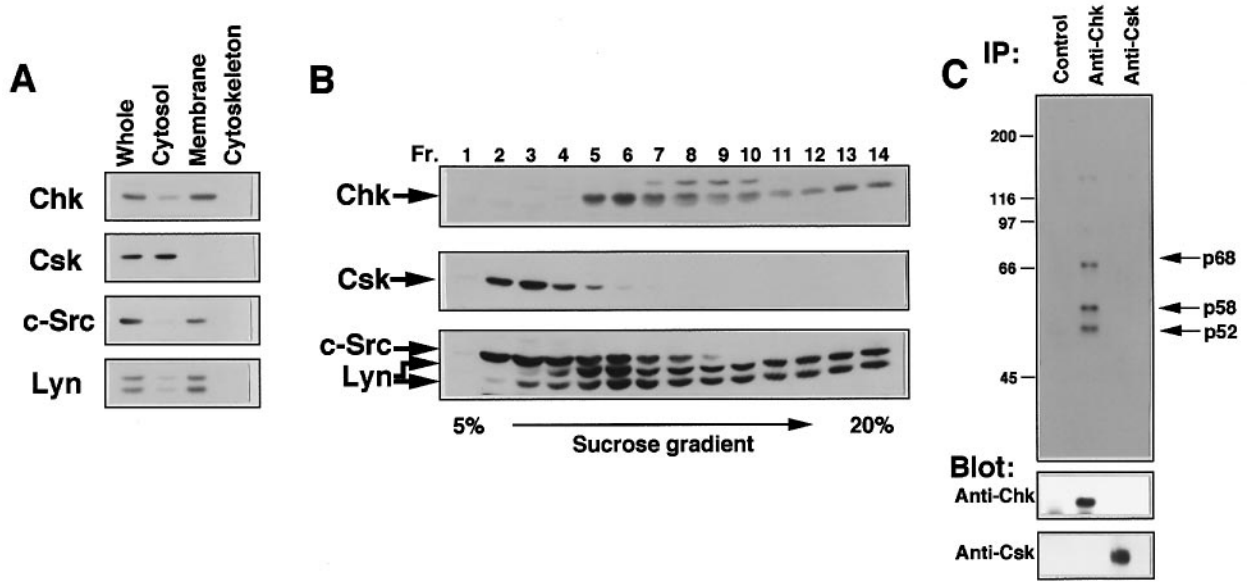

Fig. 4. Localization of Chk in resting platelets. Western blots of subcellular fractions (**A**) and of lysates fractionated on sucrose density gradients (**B**) probed with anti-Chk (13G2), anti-Csk, anti-Src and anti-Lyn. (**C**) Western blots of immunoprecipitates with MOPC21 as a control, anti-Chk (13G2) or anti-Csk from surface-biotinylated platelets probed with HRP-conjugated streptavidin (upper panel) or anti-Chk (13G2) and anti-Csk (lower panels). Chk, 57 kDa; Csk, 50 kDa; c-Src, 60 kDa; Lyn, 53 and 56 kDa.

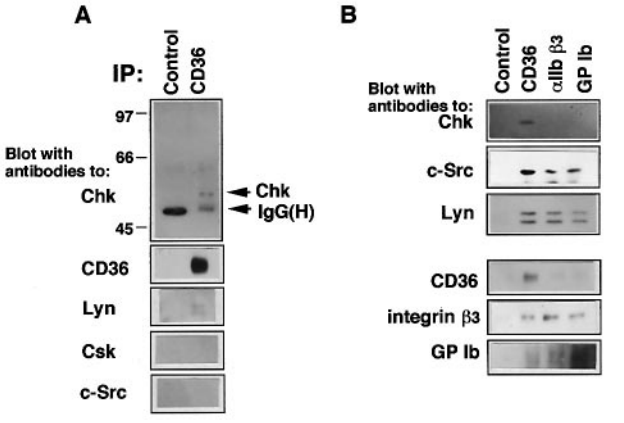

Fig. 5. Association of Chk with a CD36 complex. (**A**) Western blots of immunoprecipitates with MOPC21 as a control and anti-CD36 from platelets chemically crosslinked with DSP and (**B**) Western blots of platelet membrane vesicles purified using anti-CD36, anti-αIIbβ3, anti-GPIb or MOPC21 as a control probed with anti-Chk (13G2), anti-CD36, anti-Lyn, anti-Csk, anti-Src, anti-β3 and anti-GPIb, as indicated.

detected in the immune complex of CD36 with (Figure 5A) or without chemical crosslinking (data not shown).

We then attempted to characterize further the relationship between Chk and CD36 (Figure 5B). Membrane vesicles were prepared from sonicated platelets and affinity purified using anti-CD36, anti-αIIbβ3 or anti-GPIb. Vesicles predominantly expressing CD36 or GPIb were purified. αIIbβ3 molecules were present in the CD36- and GPIb-expressing vesicles as well as in the vesicles purified with anti-αIIbβ3. Chk was detected in the CD36-expressing vesicles but not in the GPIb- or αIIbβ3-expressing vesicles, whereas Lyn and c-Src were found in each membrane vesicle fraction. In contrast to Chk, Csk was not detected in any membrane vesicle fraction (data not shown). These results suggest that Chk, but not Csk, is localized with CD36-anchored Lyn in resting platelets.

Effect of Chk phosphorylation on kinase activity of Lyn

To examine whether Lyn was phosphorylated and negatively regulated by Chk, Lyn immunoprecipitates were subjected to *in vitro* kinase assays. Figure 6A shows that autophosphorylation of Lyn was inhibited by treatment with FSBA (lanes 1 and 2) and that the inactivated Lyn was phosphorylated by Chk (lane 3). Thus, Lyn, as well as c-Src (Figure 3A), was found to be a substrate for Chk. Next, purified Lyn was first phosphorylated by various amounts of purified Chk in the presence of unlabeled ATP and then the activity of Lyn was measured. Figure 6B shows that the extent of phosphorylation of enolase and of autophosphorylation of Lyn was reduced by increasing the amount of Chk added, indicating that phosphorylation of Lyn by Chk results in a decrease in the kinase activity of Lyn.

Since negative regulation of Lyn by Csk is due to phosphorylation at the C-terminal tyrosine residue, Tyr508, of Lyn (Okada *et al.,* 1991), we investigated whether Chk could phosphorylate this negative regulatory tyrosine residue on Lyn. COS-7 cells co-expressing Lyn and Chk were *in vivo* labeled for 3 h with $H_3{}^{32}PO_4$. Phosphorylated Lyn was purified, cleaved with cyanogen bromide (CNBr) and analyzed by SDS–PAGE. Figure 6C shows the major phosphorylated bands of the 8 kDa fragment containing Tyr397 and the 4 kDa fragment containing Tyr508 (left lane in lower panel). Co-expression of Lyn and kinase-inactive Chk resulted in a major phosphorylated band of the 8 kDa fragment containing Tyr397 (right lane in lower panel). This analysis of CNBr cleavage products indicated that Chk-dependent phosphorylation of Lyn occurs on the 4 kDa fragment containing Tyr508. These results suggest that Chk suppresses Lyn kinase activity by phosphorylation of its negative regulatory tyrosine residue.

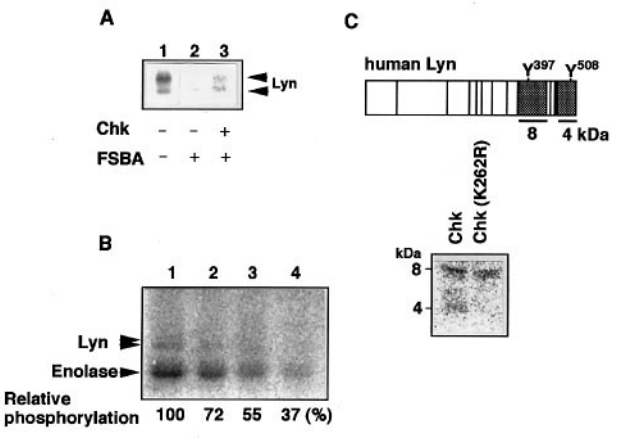

Fig. 6. Effect of Chk phosphorylation on kinase activity of Lyn. (**A**) Autoradiogram of *in vitro* phosphorylation of Lyn immunoprecipitates from platelets. Lane 1, autophosphorylation of Lyn; lane 2, autophosphorylation of FSBA-treated Lyn; lane 3, phosphorylation of FSBA-treated Lyn by addition of purified Chk–FLAG. (**B**) Autoradiogram of *in vitro* Lyn phosphorylation regulated by Chk. The purified Lyn was first incubated with various amounts of purified Chk–FLAG (lane 1, 0 μl; lane 2, 1 μl; lane 3, 5 μl; lane 4, 10 μl) in the presence of unlabeled ATP (2 μM) for 15 min and further incubated with acid-denatured enolase in the presence of 1.5 μM [γ-^{32}P]ATP for 60 min. Lyn kinase activities were determined by measuring the incorporation of $^{32}PO_4$ into enolase. The values are expressed as percentages of the control in the absence of Chk–FLAG. (**C**) Autoradiogram of CNBr-cleaved fragments of Lyn. Upper panel: a schematic representation of the CNBr cleavage sites in Lyn. The 8 and 4 kDa fragments contain the autophosphorylation site (Tyr397) and the negative regulatory site (Tyr508) respectively. Lower panel: Lyn proteins were purified and analyzed by cleavage with CNBr from *in vivo* $^{32}PO_4$-labeled COS-7 cells co-expressing either Lyn and Chk–FLAG (left lane) or Lyn and Chk–FLAG (K262R) (right lane).

Functional association of Chk with CD36-anchored Lyn

To examine the effect of Chk on the kinase activity of Lyn in resting platelets, two Lyn immunoprecipitates were subjected to an *in vitro* autophosphorylation assay, one that co-fractionated with Chk (Figure 4B, fraction 13) and one that did not (fraction 4). Figure 7A shows that autophosphorylation of Lyn immunoprecipitated from the fraction containing Chk was suppressed. The level of activity of this Lyn species was estimated to be ~3-fold lower (right lane) than that of the other species of Lyn in the fraction lacking Chk (left lane) when activity was normalized to the amount of Lyn in each sample. This result suggests that Chk may negatively regulate the kinase activity of Lyn associated with CD36 in resting platelets.

Figure 7B shows that upon thrombin stimulation CD36 was translocated from the Triton X-100-soluble to Triton X-100-insoluble fraction with dynamics similar to those of Lyn, indicating that Lyn continues to anchor CD36 during thrombin stimulation. Although progressive translocation of both CD36 and Lyn from the Triton X-100-soluble to Triton X-100-insoluble fraction was observed, Chk was completely translocated within 10 s of thrombin stimulation (Figure 7B). This result suggests that translocation of Chk to the cytoskeleton may be completed rapidly, before the beginning of translocation of most CD36-anchored Lyn. It is therefore possible that rapid translocation of Chk releases CD36-anchored Lyn from negative regulation.

To examine whether Lyn was activated before its translocation to the cytoskeleton, Lyn was immunoprecipitated from the Triton X-100-soluble fraction and *in vitro* kinase assays were performed with enolase. An increase in the kinase activity of Lyn was detected at 10 s after thrombin stimulation (Figure 7C). The increase was 1.7- to 2.2-fold ($n = 4$), peaked at 30–60 s after thrombin stimulation and was then sustained, with a slight reduction (6–18%) after 120 s stimulation. This result suggests that activation of Lyn occurs before its translocation to the cytoskeleton.

Although Chk was found to suppress Lyn kinase activity by phosphorylation of its C-terminal negative regulatory tyrosine residue (Figure 6), the rapid translocation of Chk away from Lyn may not be sufficient for activation of Lyn. Dephosphorylation of the negative regulatory tyrosine residue of Lyn by a protein tyrosine phosphatase may be also required for activation of Lyn.

We then examined *in vivo* phosphorylation states of the negative regulatory tyrosine residue of Lyn during thrombin stimulation. *In vivo* $^{32}PO_4$-labeled platelets were stimulated with thrombin for 0, 10 or 60 s and phosphorylated Lyn and c-Src were purified from the Triton X-100-soluble fraction before their translocation to the Triton X-100-insoluble fraction. After confirming that each sample contained comparable amounts of purified Lyn or c-Src, the phosphorylation states of Lyn and c-Src were analyzed by CNBr cleavage. Figure 7D shows that a decrease in phosphorylation of the 4 kDa fragment of Lyn containing Tyr508 occurred at 10 s after thrombin stimulation (upper left). The level of phosphorylation was further decreased at 60 s after stimulation.

On the other hand, Figure 7D also shows different phosphorylation states between Lyn and c-Src at the negative regulatory tyrosine residue during thrombin stimulation. A transient and slight decrease in phosphorylation of the 4 kDa fragment containing the negative regulatory tyrosine residue, Tyr530, of c-Src was observed at 10 s after stimulation (upper right), consistent with previous observations (Clark and Brugge, 1993). These results probably reflect an association of Lyn, but not c-Src, with CD36 (Figure 5A). Furthermore, upon thrombin stimulation the extent of decrease in phosphorylation of the negative regulatory tyrosine residue corresponded to the level of kinase activity of Lyn and c-Src (compare upper panels with lower panels in Figure 7D). Therefore, these results suggest that dephosphorylation of the negative regulatory tyrosine residue by a protein tyrosine phosphatase in a Lyn-specific manner gives rise to activation of Lyn before its translocation to the cytoskeleton.

Discussion

In this report we demonstrate co-expression of Chk and Csk in platelets and the significance of the unique subcellular localization of Chk in thrombin-induced platelet activation.

Subcellular localizations of Chk and Csk

We generated monoclonal antibodies against Chk (Figure 1) and found that most of the Chk protein is localized in the Triton X-100-soluble membrane fraction (Figure 4A). Since Chk has been shown to localize mainly to the cytoplasm and appreciably to the detergent-insoluble fraction of several cell lines, but not to the membrane

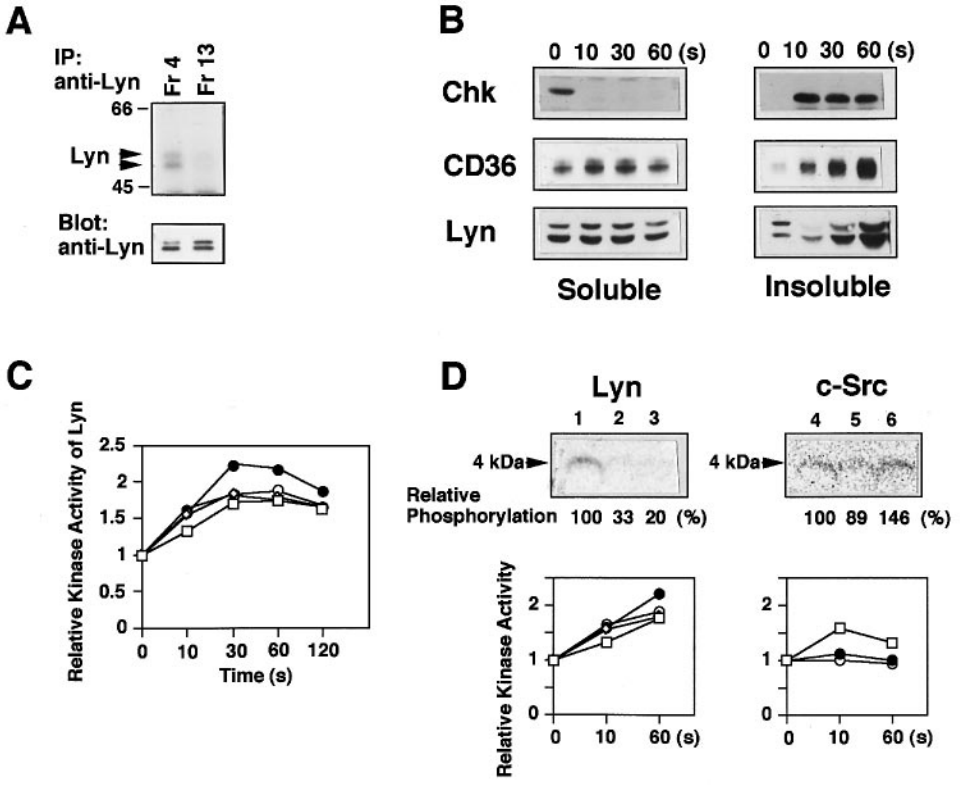

Fig. 7. Functional association of Chk with CD36-anchored Lyn. (**A**) Autoradiogram of *in vitro* autophosphorylation of Lyn immunoprecipitated from density gradient fractions 4 and 13 shown in Figure 4B (upper panel) and the amounts of Lyn immunoprecipitates, blotted with anti-Lyn (lower panel). (**B**) Western blots of the Triton X-100-soluble (left panel) and Triton X-100-insoluble (right panel) fractions of platelets stimulated with thrombin for the indicated times probed with anti-Chk (13G2), anti-CD36 and anti-Lyn. (**C**) *In vitro* kinase assays of Lyn immunoprecipitates from the Triton-soluble fractions of platelets stimulated with thrombin for the indicated times. Kinase activities are expressed as values relative to the specific activity of Lyn in the unstimulated sample after normalizing for incorporation of $^{32}PO_4$ into enolase for each level of Lyn protein. The graph represents results from four different experiments. (**D**) Autoradiograms of *in vivo* phosphorylation of the negative regulatory sites of Lyn and c-Src present in the Triton-soluble fractions upon stimulation. After *in vivo* labeling, stimulation and purification of Lyn and c-Src, the proteins were cleaved with CNBr. Upper panels show phosphorylation states of the resulting 4 kDa fragments of Lyn (left) and c-Src (right) from platelets stimulated with thrombin for 0 (lanes 1 and 4), 10 (lanes 2 and 5) or 60 s (lanes 3 and 6). Incorporation of $^{32}PO_4$ into the 4 kDa fragments was quantitated. *In vitro* kinase activities of Lyn and c-Src immunoprecipitates from the Triton-soluble fractions of stimulated platelets (0, 10 or 60 s) are expressed as described above. The graphs represent results from (C) for Lyn and from three different experiments for c-Src.

fraction (Bennett *et al.*, 1994; Chow *et al.*, 1994b), the membrane localization of Chk in platelets is quite unique. In contrast, the finding that most Csk is present in the cytosol (Figure 4A) agrees with previous data that most Csk protein is localized in the cytoplasm of fibroblastic cells, although a small amount of Csk is concentrated in the membrane fraction, particularly in adhesion plaques (Nada *et al.*, 1991; Sabe *et al.*, 1992; Howell and Cooper, 1994; Bergman *et al.*, 1995). Although Csk and Chk are homologous proteins, Chk contains a unique 41 amino acid N-terminus and the Chk SH3 and SH2 domains show only 30 and 59% amino acid identity respectively to Csk. It is possible that the differential binding to membrane proteins exhibited by Chk and Csk may be the result of these structural differences.

In fact, co-immunoprecipitation analysis showed a physical association of Chk with 68, 58 and 52 kDa biotinylated cell surface proteins in lysis buffer containing 1% Triton X-100 (Figure 4C). This finding indicates an interaction of Chk with the cytoplasmic portion of a surface membrane protein(s). Additionally, density gradient analysis, showing that Chk, unlike Csk, is present in the higher density fractions (>200 kDa; Figure 4B), supports complex formation by Chk and membrane proteins. However, further studies are required to identify the molecules associated with Chk.

Moreover, it is reported that platelets contain five Src family kinases, i.e. c-Src, Lyn, Fyn, c-Yes and Hck, three of which, Lyn, Fyn, and c-Yes, stably interact with one of the major platelet membrane glycoproteins, CD36/GPIV (Huang *et al.*, 1991). In addition to the result that Lyn, unlike Csk and c-Src, is also located in the higher density fractions (Figure 4B), our immunoprecipitation study revealed that a complex of CD36 with Lyn is chemically crosslinked with Chk (Figure 5A). In the absence of a chemical crosslinker, physical association could not be detected between Chk and a complex of CD36 with Lyn. Nonetheless, Chk and CD36–Lyn may be adjacent to and interact with each other because the spacer arm length of dithiobis(succinimidyl propionate) (DSP), the crosslinker used in this study, is only 12 Å. This idea is supported by affinity purification of membrane vesicles without the use of detergents (Figure 5B). As depicted in Figure 8A, our model suggests that Chk is localized close to CD36-anchored Lyn just beneath the plasma membrane in resting platelets, in contrast to Csk, which is present in the cytosol.

Despite co-expression of Chk and Csk in platelets,

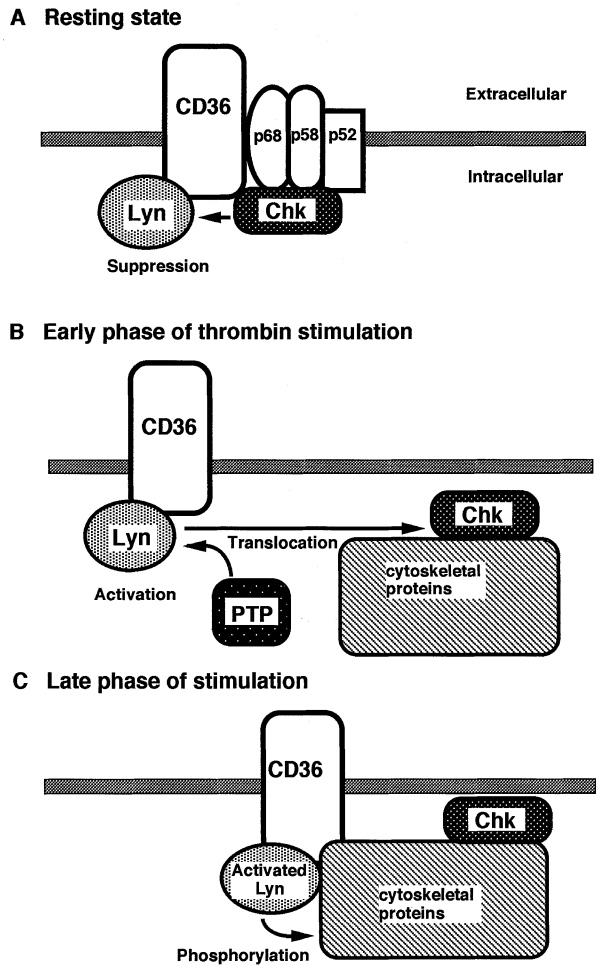

Fig. 8. Proposed mechanism for the activation of CD36-anchored Lyn via Chk translocation. (**A**) In resting platelets, Chk localizes adjacent to CD36-anchored Lyn and suppresses kinase activity. (**B**) Thrombin stimulation releases the CD36-anchored Lyn from suppression through the rapid translocation of Chk. Activation of Lyn requires a protein tyrosine phosphatase (PTP) to dephosphorylate its C-terminal negative regulatory tyrosine residue. (**C**) Subsequently, the activated kinase in turn translocates to the cytoskeleton to phosphorylate its potential substrates.

thrombin stimulation gives rise to translocation of all Chk but not most Csk to the Triton X-100-insoluble fraction (Figure 2). The difference between Chk and Csk with respect to thrombin-induced translocation may result from the observation that Chk is physically associated with surface membrane proteins, while most Csk is freely soluble in the cytosol (Figure 4). Our findings raise the possibility that a complex of Chk with surface membrane proteins is one of the functional units of translocation in response to thrombin.

It should be emphasized that the unique characteristics of the translocation of Chk are exceptional rapidity and completeness. All Chk molecules are capable of translocating to the cytoskeleton within 5–10 s after thrombin stimulation (Figures 2A and 7B). On the other hand, previous reports have shown that the association of c-Src with the cytoskeleton gradually increases upon thrombin stimulation, typically reaching a peak at only 20% of total c-Src within 2–5 min after stimulation (Figure 2B; Horvath *et al.*, 1992; Oda *et al.*, 1992; Clark and Brugge, 1993). The kinetics of translocation of c-Src are also comparable with those of both CD36 and Lyn found in this study (Figure 7B).

Lyn kinase activation through translocation of Chk

Chk, like Csk, has the ability to phosphorylate Lyn *in vitro* at its C-terminal negative regulatory tyrosine residue and suppress Lyn kinase activity (Figure 6). Since Chk is also likely to negatively regulate Lyn activity in platelets *in vivo*, it is necessary to consider the heterogeneity of Lyn in terms of its co-localization with Chk. Density gradient analysis enables us to compare the activity of Lyn minus Chk (Figure 4B, fraction 4) with that of Lyn in complex with Chk (fraction 13). Our observations indicate that the activity of Lyn in complex with Chk is 3-fold lower than that of Lyn minus Chk (Figure 7A). Also, the distributions of CD36 and Chk overlap in fraction 5 and later fractions (Figure 4B; data not shown). These results suggest that Chk may negatively regulate the activity of CD36-anchored Lyn in resting platelets.

The rapid and complete translocation of Chk is likely to influence the activity of CD36-anchored Lyn. Importantly, the kinase activity of Chk is not changed upon stimulation of platelets with thrombin (Figure 3) and translocation of Chk presumably precedes that of CD36-anchored Lyn upon thrombin stimulation (Figure 7B). Translocation of Chk away from CD36-anchored Lyn could allow a protein tyrosine phosphatase to dephosphorylate the C-terminal negative regulatory tyrosine residue of Lyn, resulting in activation. In fact, activation of Lyn can be observed in the Triton X-100-soluble fraction after 10 s stimulation (Figure 7C). Coincidentally, dephosphorylation of the C-terminal negative regulatory tyrosine residue of Lyn occurs with dynamics similar to those of activation of Lyn (Figure 7D). It is likely that dephosphorylation leads to Lyn activation before its translocation to the cytoskeleton. However, dephosphorylation at the C-terminal tyrosine residue and activation of Lyn and c-Src are sustained and transient respectively (Figure 7D). Subcellular localization of Lyn and c-Src (Figures 4B and 5A) may contribute to the differences in phosphorylation states and activities between Lyn and c-Src.

Thus, we propose the following model to describe the role of Chk in activation of Lyn in platelets. In resting platelets (Figure 8A), Chk is localized close to CD36-anchored Lyn and suppresses its activity by phosphorylating its C-terminal negative regulatory tyrosine residue. When platelets are stimulated with thrombin, Chk is rapidly translocated from the vicinity of CD36-anchored Lyn to the cytoskeleton. CD36-anchored Lyn is then released from the negative regulation caused by Chk before its translocation. The activation of Lyn may require a protein tyrosine phosphatase to dephosphorylate its C-terminal negative regulatory tyrosine residue (Figure 8B). The activated Lyn in turn translocates to the cytoskeletal fraction containing its potential substrates, thereby initiating a signaling cascade (Figure 8C).

One potential candidate for a protein tyrosine phosphatase is SHP-1/PTP1C, which is predominantly expressed in hematopoietic cells and functions as a positive or negative regulator (Mourey and Dixon, 1994). SHP-1 contains two SH2 domains potentially involved in its association with signaling molecules (Adachi *et al.*, 1996) and SHP-1 activity in platelets is increased in response to

thrombin (Li *et al.*, 1995). It is possible that SHP-1 might act as a positive regulator in co-operation with translocation of Chk in platelet activation. Further studies are required to elucidate the involvement of SHP-1 in this translocation-controlled negative regulation of CD36-anchored Lyn.

Based on our observations, it is evident that Chk, but not Csk, functions as a translocation-controlled negative regulator of CD36-anchored Lyn in platelet activation. Because CD36 associates with three Src family kinases, Fyn and c-Yes as well as Lyn, in platelets and CD36-expressing cell lines (Huang *et al.*, 1991), it is possible that Chk may regulate CD36-anchored Src family kinases. CD36 is involved in signal transduction and adhesion in platelets and phagocytosis in mature monocytes (Greenwalt *et al.*, 1992; Ren *et al.*, 1995). As Chk is expressed in interleukin 4 (IL4)- or IL13-treated monocytes (Musso *et al.*, 1994), Chk might play a role in the regulation of CD36-anchored Src family kinases in IL4- or IL13-activated monocytes as well as in platelets.

Materials and methods

Antibodies

Monoclonal anti-v-Src (clone 327, Oncogene Science), anti-phosphotyrosine (clone 4G10, Upstate Biotechnology Inc.), anti-Lyn (clone Lyn9, Wako Chemicals, Osaka), anti-FLAG (clone M2, Eastman Kodak), anti-GPIb (clone HPL7, Seikagaku Corp., Tokyo), anti-CD36 (clone FA6–152, Immunotech), anti-αIIbβ3 (clone TP-80, Nichirei, Tokyo) and rabbit anti-integrin β3 (Chemicon) were used. Rabbit anti-Csk and anti-Lyn were purchased from Santa Cruz Biotechnology. Horseradish peroxidase (HRP)-conjugated $F(ab')_2$ fragments of anti-mouse Ig and of anti-rabbit Ig and HRP-conjugated streptavidin were obtained from Amersham. MOPC21 (control myeloma IgG_1) was obtained from Sigma.

Plasmid constructs

Full-length cDNAs for human Chk (Sakano *et al.*, 1994) and human Chk tagged with the FLAG epitope at the C-terminus (Chk–FLAG) were constructed by PCR using the sense primer 5′-AAGAATTCGCGATGGCGGGGCGAGGCTCT-3′ and the antisense primers 5′-AATATCTCGAGTGGGGTCAGGGCTCCTGGC-3′ or 5′-AATATCTCGAGAATCATCATTTATCATCATCATCTTTATAATCGGGCTCCTGGCTTCGGGGCGA-3′ and subcloned into the SRα-driven pMKITneo vector (kindly provided by K.Maruyama and T.Yamamoto; Takebe *et al.*, 1988). The Lys→Arg mutation at position 262 in the ATP binding site of the kinase domain of Chk–FLAG [Chk–FLAG (K262R), kinase-inactive mutant] was generated by site-directed mutagenesis using an Amersham *in vitro* mutagenesis system with the oligonucleotide 5′-GGCCGTGAGAAATATTAAGTGTGA-3′. The Chk–FLAG (K262R) cDNA was subcloned into the SRα-driven pMKITneo vector. A cDNA for Chk fusion protein with glutathione *S*-transferase (GST) was generated by subcloning the full-length cDNA fragment into the vector pGEX-5X-2 (Pharmacia). The inserted cDNA for human Chk was constructed by PCR using the sense primer 5′-ATATCAGGATCCTGATGCCAACGAGGCGCTGG-3′ and the antisense primer 5′-AATATCTCGAGTGGGGTCAGGGCTCCTGGC-3′. The resulting DNA fragments were all confirmed by DNA sequencing. The human Lyn cDNA (Yamanashi *et al.*, 1987) subcloned into the SRα-driven pME18S vector was kindly provided by H.Nishizumi and T.Yamamoto.

Generation of antibodies against human Chk

Monoclonal antibodies. The GST–Chk fusion protein was expressed in the BL21 or AD202 strain of *Escherichia coli* (kindly provided by T.Saito and H.Nakano; Akiyama and Ito, 1990; Nakano *et al.*, 1993) and purified according to established protocols (Pharmacia). Hybridomas were produced by fusion of P3U1 murine myeloma cells with spleen cells from BALB/c mice immunized with the GST–Chk fusion protein. To screen hybridomas, we developed a rapid, sensitive and specific method, i.e. an enzyme-linked immunosorbent assay using enhanced chemiluminescence (ECL, Amersham), combined with the use of non-denatured Chk in the presence of cellular proteins, including Csk. Cytosolic proteins of COS-7 cells transfected with Chk–FLAG or vector alone were recovered by hypotonic treatment. Hybridoma supernatants were incubated in 96-well plates (No. 3192, Falcon) precoated with cytosolic proteins of Chk–FLAG- or vector-expressing COS-7 cells. HRP-conjugated anti-mouse Ig was then added. Positive wells were visualized by ECL on an X-ray film. M2 anti-FLAG was used as a positive control. Two clones which specifically reacted to cytosolic proteins of Chk–FLAG-expressing COS-7 cells were selected (13G2 and 18E12, both of which are IgG_1).

Affinity-purified antibody. Antiserum against Chk was raised in rabbits by immunization with a keyhole limpet hemocyanin-conjugated synthetic polypeptide corresponding to C-terminal amino acid residues 492–506 of human Chk. The antibody (Po: C-2930) was purified on Affi-Gel 102 beads (BioRad) coupled with synthetic polypeptides (residues 499–506 of Chk).

Preparation and stimulation of platelets

Venous blood was obtained from healthy volunteers using 10 mM EDTA, 1 μM prostaglandin E_2 and 1 U/ml apyrase as anticoagulants. Platelets were prepared by gel filtration in Tyrode's buffer lacking calcium with anticoagulants, as previously described (Shattil *et al.*, 1994). Platelets were adjusted to 3×10^8 cells/ml in Tyrode's buffer containing 1 mM $CaCl_2$ and incubated with thrombin (0.5 U/ml) at 37°C under constant stirring. PPACK-treated thrombin was prepared as described (Kettner and Shaw, 1978).

Platelet lysis and subcellular fractionation

Platelets were lysed with an equal volume of 2% Triton lysis buffer [50 mM HEPES, pH 7.4, 2% Triton X-100, 137 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1 mM Na_3VO_4 and protease inhibitors (50 μg/ml aprotinin, 100 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 25 μM pepstatin A)]. The resulting supernatant (Triton-soluble fraction) was collected. After washing, the pellet (Triton-insoluble fraction) was solubilized in SDS sample buffer.

Platelets were lysed with hypotonic lysis buffer (10 mM HEPES, pH 7.4, 10 mM NaCl, 1 mM KH_2PO_4, 5 mM $NaHCO_3$, 5 mM EDTA, 5 mM EGTA, 2 mM Na_3VO_4 and protease inhibitors), followed by sonication (four pulses for 10 s), addition of an equal volume of adjusting buffer (10 mM HEPES, pH 7.4, 290 mM NaCl, 1 mM KH_2PO_4, 5 mM $NaHCO_3$, 5 mM EDTA, 5 mM EGTA, 2 mM Na_3VO_4 and protease inhibitors) and centrifugation at 100 000 *g* for 30 min to collect the cytosol fraction. The pellet was extracted in 1% Triton lysis buffer (Triton-soluble membrane fraction). The remaining Triton-resistant material is referred to as the cytoskeletal fraction. All steps were carried out at 4°C.

Sucrose density gradient sedimentation

Continuous 5–20% sucrose gradient sedimentation analysis was performed essentially as described (Yamaguchi and Fukuda, 1995). Platelets were lysed with 0.6% CHAPS lysis buffer in place of 1% Triton X-100. The resulting supernatant was loaded onto the sucrose gradients and centrifuged at 160 000 *g* for 12 h at 4° C. Fractions were collected from the top.

Affinity purification of membrane vesicles

Platelets were broken in hypotonic lysis buffer by sonication as described above. After centrifugation at 5000 *g* for 5 min and preclearing to remove debris, the resulting supernatant containing membrane fractions was incubated for 4 h at 4°C with protein G–Sepharose 4 FF beads (Pharmacia) precoated with either anti-CD36, anti-αIIbβ3, anti-GPIb or MOPC21. Materials bound to the antibody-coated beads were collected, followed by extensive washing with phosphate-buffered saline.

Protein labeling and chemical crosslinking

COS-7 cells transfected with either Chk, Chk–FLAG or vector alone were metabolically labeled with [^{35}S]methionine (Tran^{35}S-Label, ICN) as described (Yamaguchi and Fukuda, 1995). After labeling for 3 h, cells were lysed with 1% Triton lysis buffer. Cell surface biotinylation was carried out according to the manufacturer's instructions (Amersham). Biotinylated platelets were lysed with 1% Triton lysis buffer. Chemical crosslinking was carried out using DSP at 1 mM, according to the manufacturer's instructions (Pierce). After quenching with a Tris buffer, cells were lysed with 0.6% CHAPS lysis buffer.

Western blotting and immunoprecipitation

Samples were subjected to SDS–PAGE (Laemmli, 1970) and electrotransferred onto polyvinylidene difluoride membranes (PVDF; Millipore).

Immunodetection was performed by ECL as recommended by the manufacturer (Amersham). Precleared Triton-soluble lysates were incubated for 4 h at 4°C with protein G–Sepharose beads precoated with either anti-Chk, anti-Csk, anti-Lyn, anti-Src, anti-CD36 or MOPC21 as a control. The immune complexes were washed with 1% Triton lysis buffer. The samples were subjected to SDS–PAGE, followed by fluorography or Western blotting.

Kinase assays

After washing immune complexes on protein G beads with RIPA buffer (50 mM HEPES, pH 7.4, 10 mM EGTA, 10 mM NaF, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 2 mM Na_3VO_4 and protease inhibitors) and 1% Triton lysis buffer containing 500 mM NaCl, an aliquot of each immunoprecipitate was subjected to an *in vitro* kinase assay and an equal aliquot was applied to a quantitative immunoblot. The immunoprecipitates were suspended in 50 µl kinase buffer (50 mM HEPES, pH 7.4, 10 mM $MnCl_2$, 0.1% Triton X-100) containing 2 µM [γ-^{32}P]ATP. Poly(Glu,Tyr) 4:1 (0.2 mg/ml), purified recombinant c-Src (Upstate Biotechnology Inc.) treated with FSBA or acid-denatured enolase were used as exogenous substrates. After incubation at 30°C for 10 min, reactions were terminated by addition of an equal volume of 2× SDS sample buffer and boiled for 3 min. The samples were separated on SDS–PAGE gels. The gels were treated with 1 M KOH at 56°C for 2 h and subjected to a BAS 2000 BioImage analysis system (FUJIX, Tokyo) or autoradiography. Radioactive bands were quantified with the BAS 2000 system. In some kinase assays of Figures 6 and 7, Chk–FLAG and Lyn proteins were purified from Triton-soluble lysates of COS-7 cells transfected with Chk–FLAG and of intact platelets respectively. Using protein G beads covalently coupled with anti-FLAG or anti-Lyn, purification of both kinases was carried out with an elution buffer (pH 11.0) according to published procedures (Morgan *et al.*, 1989).

^{32}P in vivo labeling and cleavage of Lyn and c-Src with CNBr

Cells were *in vivo* labeled for 3 h with 1 mCi/ml $H_3{}^{32}PO_4$ as described (Clark and Brugge, 1993). COS-7 cells were transfected with a mixture of Lyn and Chk–FLAG cDNAs or of Lyn and Chk–FLAG (K262R) cDNAs. After *in vivo* $^{32}PO_4$-labeling, Lyn proteins were purified from Triton-soluble lysates of the transfected COS-7 cells as described above. *In vivo* $^{32}PO_4$-labeled platelets were stimulated with thrombin and solubilized with Triton lysis buffer to recover Lyn and c-Src from the Triton-soluble fraction. $^{32}PO_4$-labeled Lyn and c-Src were purified with specific antibodies as described above. The purified kinases were subjected to SDS–PAGE and Western blotting to confirm that each sample contained comparable amounts of Lyn or c-Src and did not include any other tyrosine-phosphorylated contaminants (data not shown). The purified kinases were subsequently cleaved with 50 mg/ml CNBr as described (Schuh and Brugge, 1988). The samples were subjected to SDS–PAGE and transferred to PVDF membranes, followed by treatment with 1 M KOH at 56°C for 2 h. Radioactive bands were detected and quantified with the BAS 2000 system.

Acknowledgements

We are grateful to Drs Kazuo Maruyama (Tokyo Medical and Dental University), Tadashi Yamamoto and Hirofumi Nishizumi (The Institute of Medical Science, The University of Tokyo) for generously providing the pMKITneo vector, the pME18S vector and the human Lyn cDNA, to Drs Takashi Saito and Hiroyasu Nakano (Chiba University School of Medicine) for the *E.coli* AD202 strain, to Drs Toshio Ide and Takuma Sasaki (The Chem-Sero-Therapeutic Research Institute, Kumamoto) and Drs Sho Matsushita and Yasuharu Nishimura (Kumamoto University School of Medicine) for kindly synthesizing the peptides and to Dr Michiko N.Fukuda (La Jolla Cancer Research Center, The Burnham Institute, La Jolla, CA) for critical reading of the manuscript. This work was supported in part by Grants-in-aid for Scientific Research from the Ministry of Education, Science and Culture of Japan. A.H. and I.H. are supported by Research Fellowships of the Japan Society for the Promotion of Sciences for Young Scientists.

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Received on June 18, 1996; revised on January 6, 1997