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A Novel Anti-Platelet Monoclonal Antibody Induces Mouse Platelet Aggregation through an Fc Receptor-Independent Mechanism[1]

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Platelets are nonproliferative and terminally differentiated cells. Platelets offer an attractive model system to study the various biochemical events leading to structural and functional alterations in activated cells. When platelets are exposed to stimuli, they are activated, undergo a dramatic shape change, adhere to each other, and aggregate. Several monoclonal antibodies (mAbs) that recognize CD9, GPIIb/IIIa ($\alpha_{IIb}\beta_3$ integrins), or GPIV are known to stimulate human platelet aggregation. However, no mAbs able to induce aggregation of mouse platelets have been reported. We have established an anti-mouse platelet mAb (AIP21) that can promote mouse platelet aggregation by itself. Because mouse platelets did not express the Fc receptor (FcR, CD32) on their surfaces and because AIP21 is an IgM subclass, AIP21 might promote platelet aggregation through an FcR-independent mechanism. We could not identify the antigen recognized by AIP21, but flow cytometric analysis revealed that it was not identical to CD9, GPIV, or integrins (i.e., α_{IIb}, α_v, α_5, α_6, β_1, and β_3 integrins). During the aggregation of mouse platelets mediated by AIP21, several 50-68-kDa proteins are rapidly phosphorylated at tyrosine residues. This phosphorylation by AIP21 was dose-dependent and did not require plasma components. We identified the 52-kDa phosphorylated protein as Shc. These results indicate that AIP21 could be useful for investigating the mechanisms of mouse platelet aggregation.

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Abbreviations used: mAb, monoclonal antibody; FcR, Fc receptor; NR IgM, normal rat IgM; ADP, adenosine diphosphate; PRP, platelet-rich plasma.

Human platelets are stimulated to aggregate by ligating monoclonal antibodies (mAbs) that recognize platelet surface proteins. However, most mAbs do not exhibit this aggregating ability when the Fc fragment has been removed (1-6). Therefore, the platelet Fc receptor (FcRII, CD32) expressed on human platelets might be involved in the mAb-mediated platelet aggregation.

Some mAbs have been reported to possess the ability to stimulate human platelet aggregation through an FcR-independent mechanism because their $F(ab')_2$ fragment promoted the aggregation (7-10). The antigens of these mAbs were reported to be CD9 (1, 11), GPIIb/IIIa ($\alpha_{IIb}\beta_3$ integrins) (2-4, 7-10, 12), or GPIV (CD36) (5). The ligation of these mAbs on human platelets resulted not only in aggregation but also in phospholipase C activation, inositol 1,4,5-trisphosphate and diacylglycerol generation, intraplatelet Ca^{2+} level rise, and ATP secretion (1, 2, 13).

In contrast, no mAbs were reported to induce aggregation of mouse platelets. Hence, it is still unclear which molecules were associated with the FcR-independent aggregation of mouse platelets. We here report that one of our established anti-mouse platelet mAbs, termed AIP21, could stimulate mouse platelet aggregation in a dose-dependent manner. AIP21 induced platelet aggregation even in washed platelets, indicating that plasma components were not required. AIP21 might stimulate mouse platelets to aggregate through an FcR-independent mechanism because mouse platelets did not express FcR on their surfaces, and AIP21 is an IgM subclass. To our knowledge, AIP21 is an unique mAb that possessed the ability to induce aggregation of mouse platelets. Using the AIP21, we examined the undefined mechanisms of mouse platelet aggregation.

MATERIALS AND METHODS

Preparation of mouse platelet-rich plasma and washed platelets. Platelet-rich plasma (PRP) was prepared from fresh heparinized

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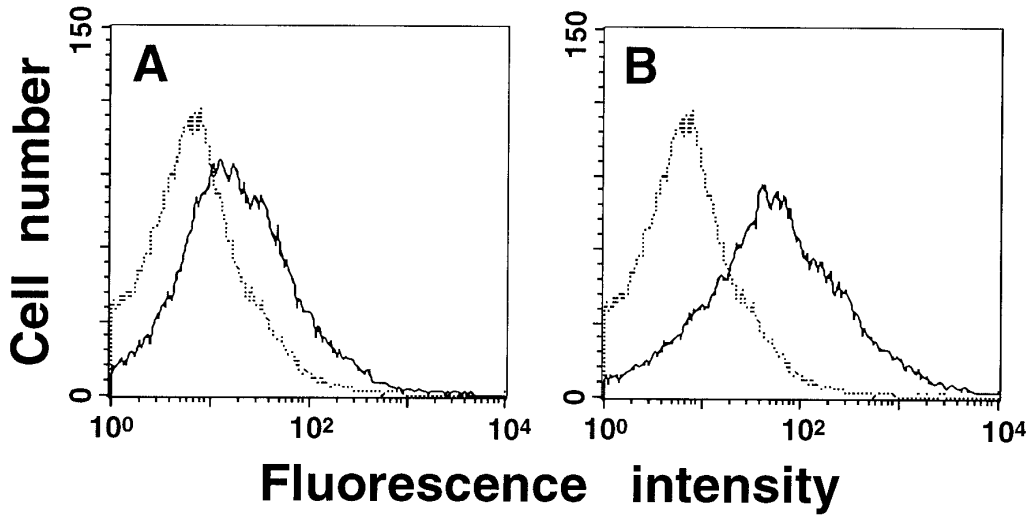

FIG. 1. Recognition of mouse platelet surface protein by AIP21 mAb. Washed mouse platelets were inactivated by freezing for 30 min before ligating mAbs. Then platelets were treated with AIP21, APB2, or NR IgM for 1 h at 4°C and reacted with FITC-conjugated anti-rat IgM. The fluorescence intensity was analyzed using a FACScan. The bold curves indicate platelets treated with AIP21 (A) or APB2 (B). The dotted curves indicate platelets treated with NR IgM (A, B).

blood drawn from 8- to 12-week-old female BALB/c mice (Charles River, Kanagawa, Japan) by cardiac puncture, then centrifuged at 230 × *g* for 7 min at 23°C. When preparing washed platelets, PRP was centrifuged at 400 × *g* for 5 min. Platelets were washed once with PBS and then resuspended in PBS (14).

Cell lines and culture conditions. Mouse melanoma B16F10 cells (15) and mouse colon adenocarcinoma NL-17 cells (16) were cultured in RPMI1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% heat-inactivated FBS (Gibco Laboratories, Grand Island, NY) (RPMI growth medium). The mouse bone-derived endothelial cell line KN-3 (17) was cultured in DMEM (Nissui) supplemented with 10% heat-inactivated FBS (Gibco). These cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Hybridoma production and purification of mAbs. Three SD rats were immunized with 8F11 mAb, which can recognize gp44 antigen and suppress gp44-mediated platelet aggregation (14). The spleen cells were fused with mouse myeloma P3U1 cells, and the culture supernatants of the hybridomas were screened by the MAbs' ability to recognize mouse platelets. The mAbs were purified using an anti-rat IgG column (Sigma Chemical Co., St. Louis, MO).

Flow cytometry. Washed mouse platelets inactivated by freezing for 30 min before ligating mAbs. Thymocytes and splenocytes were prepared from 5-week-old female BALB/c mice (Charles River). Washed mouse platelets, B16F10 cells, KN-3 cells, thymocytes, splenocytes, or peritoneal macrophages were incubated with AIP21, APB2, or control normal rat IgM (NR IgM; Chemicon International Inc., Temecula, CA) or with anti-mouse CD9, α_{IIb} integrin (CD41), α_v integrin (CD51), α_5 integrin (CD49e), α_6 integrin (CDw49f), β_1 integrin (CD29), β_3 integin (CD61), or FcR (CD32) (Pharmingen, San Diego, CA; or Serotec, Oxford, UK) for 1 h at 4°C. After washing with PBS, cells were reacted with FITC-conjugated second antibodies. Fluorescence data were collected using a Becton Dickinson FACScan (San Jose, CA).

Analysis of phosphorylation of the platelet proteins. After incubation of PRP or washed mouse platelets with AIP21, APB2, NR IgM, adenosine diphosphate (ADP), or NL-17 cells at 37°C, platelets were suspended in a lysis buffer, containing 0.5% Triton X-100, 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 100 units of aprotinin, 50 mM sodium fluolide, 50 mM sodium m-vanadate and 30 mM sodium pyrophosphate on ice for 30 min. After centrifugation at 15,000 × *g* for 30 min, the supernatants (20 μg) were subjected to SDS-PAGE in a 10-20% gradient gel. The electrophoresed proteins were transblotted onto a nitrocellulose membrane. The membrane was preincubated in a blocking buffer [4% skim milk in 25 mM Tris-HCl (pH 8.0), 125 mM NaCl, 0.1% Tween 20, 0.1% sodium azide] for 1 h at 23°C. The membrane was then incubated with 1 mg/ml of anti-phosphoserine (PSer) mAb (clone PSR-45; Bio-Makor, Rehovot, Israel), anti-phosphothreonine (PThr) mAb (clone PTR-8; BioMakor), or anti-phosphotyrosine (PTyr) mAb (clone 25.2G4) (18) in a blocking buffer for 2 h. After washing, the membrane was incubated with peroxidase-conjugated anti-mouse IgG (Amersham, Buckinghamshire, UK) for 1 h. The membrane was developed using an enhanced chemiluminescence detection system (Amersham) and Kodak X-Omat AR film.

Immunoprecipitation and Western-blot analysis. Washed mouse platelets were incubated with AIP21 at 37°C for 3 min, then they were suspended in lysis buffer, as described above, for 30 min on ice, followed by centrifugation at 15,000 × *g* for 30 min. The Shc protein was immunoprecipitated from the platelet lysate using protein G-Sepharose 4B gel beads (Zymed, San Francisco, CA) coated with an anti-Shc mAb (Transduction Laboratories, Lexington, KY). After electrophoresing under reducing conditions using a 10-20% gradient gel polyacrylamide, the immunoprecipitated Shc proteins were transblotted onto a nitrocellulose membrane. The membrane was incubated with either 1 mg/ml of anti-Shc mAb or anti-PTyr mAb in a blocking buffer for 1 h. After washing, the membrane was incubated with peroxidase-conjugated anti-mouse IgG. Then the membrane was developed as described above.

Estimation of platelet aggregation. Platelet aggregation was measured turbidometrically using an aggregometer (NKK HEMA Tracer I, Niko Bioscientific Co., Tokyo, Japan). A 200-μl aliquot of PRP or washed platelets was incubated with AIP21 or NR IgM in a cuvette at 37°C under constant stirring in the aggregometer. In some experiments, washed mouse platelets were preincubated with appropriate concentrations of EDTA for 5 min at 37°C before the AIP21 was added. The changes in light transmittance were monitored for 10 min (16).

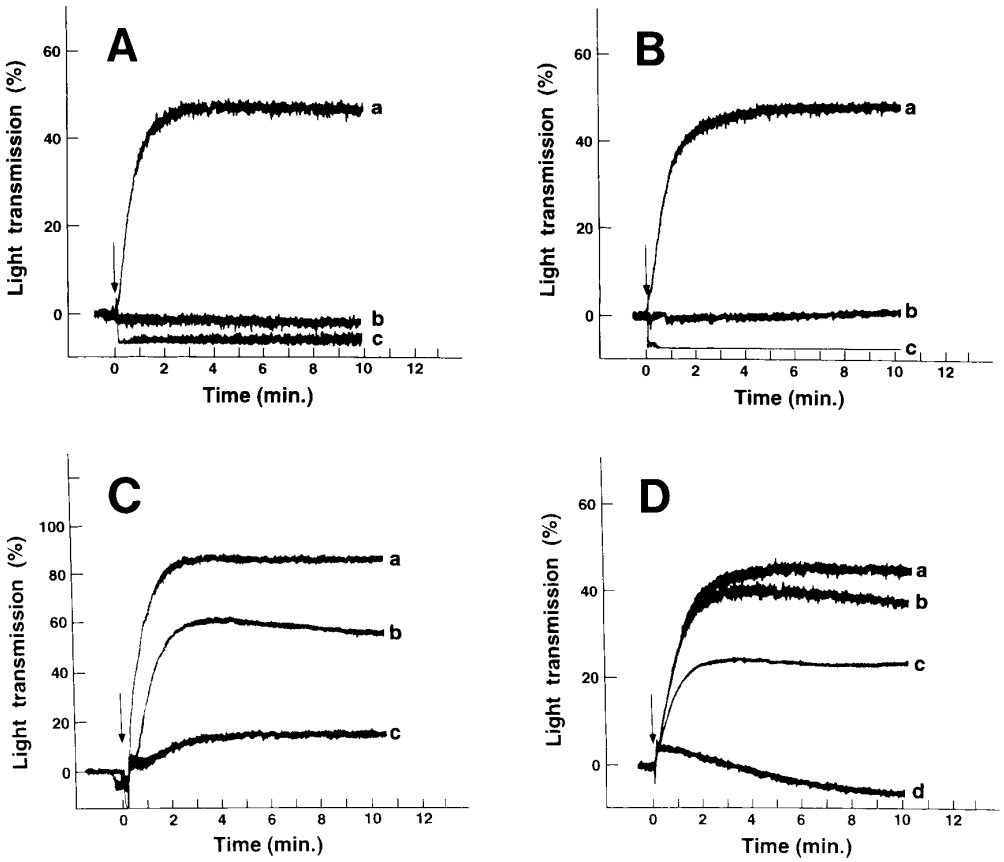

FIG. 2. Mouse platelet aggregation induced by the AIP21 mAb. (A and B), PRP (A) or washed platelets (B) from mice were incubated with 25 μg/ml of AIP21 (a), APB2 (b), or NR IgM (c) in a cuvette at 37°C under constant stirring in an NKK HEMA Tracer I, as described in Materials and Methods. (C), washed mouse platelets were incubated with 50 μg/ml, 5 μg/ml, or 0.5 μg/ml of AIP21 (curves a-c, respectively). (D), washed mouse platelets were preincubated with 0 mM, 5 mM, 50 mM, or 250 mM EDTA for 5 min (curves a-d, respectively). Then the platelets were stimulated to aggregate by adding 25 μg/ml of AIP21. The changes in light transmittance were monitored for 10 min.

RESULTS AND DISCUSSION

Establishment of a mouse platelet aggregation-inducing mAb. We established several anti-mouse mAbs of the IgM subclass (i.e., AIP21 and APB2), during the study of cancer-mediated platelet aggregation (14). As shown in Fig. 1, both AIP21 and APB2 recognized mouse platelet-surface molecules. When AIP21 was added to mouse PRP, platelets were immediately activated to aggregate (Fig. 2A, curve a). Aggregation was also observed when AIP21 was added to washed platelets (Fig. 2B, curve a), indicating that plasma components were not essential for AIP21-mediated platelet aggregation. Fig. 2C shows that this aggregation occurred in a dose-dependent manner. In contrast to AIP21, ADP induced platelet aggregation in PRP but not in washed platelets (data not shown). APB2, the other control anti-mouse platelet mAb of the IgM subclass, could not induce platelet aggregation (Figs. 2A and 2B, curve b), though it did bind a platelet surface molecule (Fig. 1B). Therefore, AIP21 could be a novel mouse platelet aggregation-inducing mAb. The aggregation promoted by AIP21 was inhibited in the presence of EDTA (Fig. 2D). However, flow cytometric analysis revealed chelating Ca^{2+} with EDTA did not reduce the binding activity of AIP21 to mouse platelets (data not shown). Therefore, Ca^{2+} was required for the AIP21-mediated platelet aggregation.

Induction of mouse platelet aggregation by AIP21 through an FcR-independent mechanism. Many mAbs to human platelets have been reported to induce activation (1-5, 7-12). Most of these mAbs, however, stimulate human platelets to aggregate through an FcR-dependent mechanisms because their $F(ab')_2$ fragments cannot promote the aggregation (1-6). It is still unclear whether mouse platelets expressed FcR on their surfaces. We, therefore, examined the expression of FcR on mouse platelets using an anti-FcR mAb. The mAb could not recognize mouse platelets (Fig. 3A), but it could bind mouse macrophages that expressed FcR (Fig. 3B), indicating that mouse platelets did not ex-

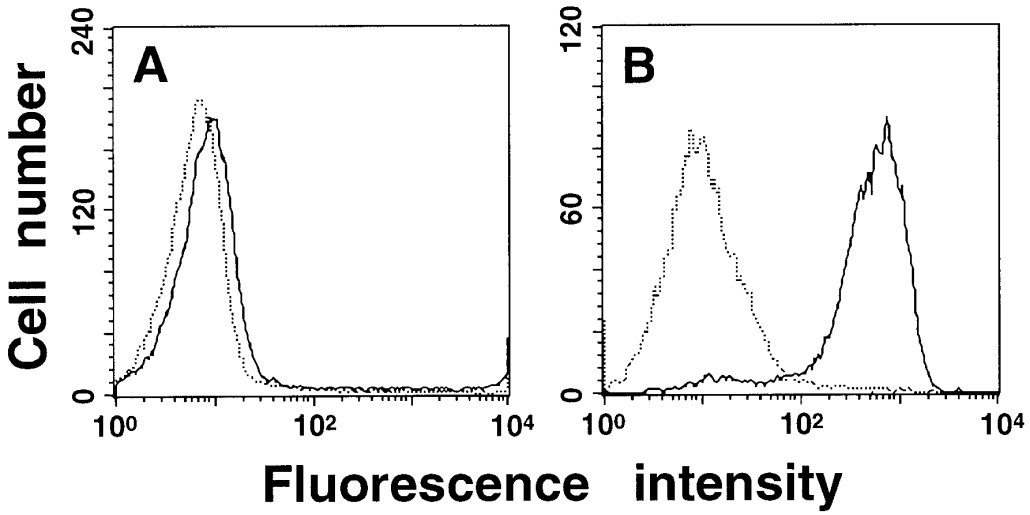

FIG. 3. The absence of FcR expression in mouse platelets. Washed mouse platelets were inactivated by freezing for 30 min before ligating mAbs. Mouse platelets (A) and peritoneal macrophages (B) were treated with an anti-FcR (CD32) mAb (bold curves) or nothing (dotted curves) for 1h at 4°C and then reacted with FITC-conjugated anti-rat IgG. The fluorescence intensity was analyzed using a FACScan.

press FcR on their surfaces. For this reason, no mAbs have been reported that could induce aggregation of mouse platelets.

We have established a mouse platelet aggregation-inducing mAb, AIP21, that is mAb of the IgM subclass (data not shown). Hence, AIP21 induced mouse platelet aggregation through an FcR-independent mechanism. When we made the $F(ab')_2$ fragment of AIP21, it promoted mouse platelets to aggregate (data not shown). This result also suggests that AIP21 stimulates platelet aggregation by directly binding to the platelet surface antigen. However, the aggregation induced by the $F(ab')_2$ fragment of AIP21 was weaker than that induced by intact AIP21. Moreover, Fab fragment of AIP21 could not induce platelet aggregation (data not shown). Therefore, aggregation of the AIP21 antigen or agglutination of platelets is also involved in AIP21-mediated mouse platelet aggregation.

In human platelets, some antigens were known to be associated with the aggregation through an FcR-independent mechanism (i.e., CD9, GPIIb/IIIa ($\alpha_{IIb}\beta_3$ integrins), or GPIV (CD36)) (1-5, 7-12). Platelets were also known to express several integrins, such as α_v, α_5, α_6, and β_1 integrins. When we examined the reactivity of AIP21 by flow cytometry, the AIP21 antigen was expressed on platelets, B16F10 cells, and KN-3 cells but not on thymocytes or splenocytes (Table 1). CD9 was expressed on both thymocytes and splenocytes; integrins, such as α_v, α_5, α_6, β_1, and β_3 integrins, were expressed on either thymocytes or splenocytes. The α_{IIb}

TABLE 1

Distribution of Antigens Recognized by Anti-Mouse Platelet mAbs

	Platelet	Thymocyte	Splenocyte	B16F10	KN-3
AIP21	+	−	−	+	+
Anti-CD9	+	+	+	+	±
Anti-α_{IIb} (CD41)	+	−	−	−	−
Anti-β_3 (CD61)	+	−	+	+	+
Anti-GPIV (CD36)	+	N.D.	±[a]	N.D.	N.D.
Anti-β_1 (CD29)	+	+	−	+	+
Anti-α_v (CD51)	+	−	+	+	±
Anti-α5 (CD49e)	+	+	−	+	±
Anti-α_6 (CD49f)	+	+	−	+	+

Note. Mouse washed platelets were frozen for 30 min before the ligation of mAbs. Mouse platelets, thymocytes, splenocytes, B16F10 cells, or KN-3 cells were incubated with AIP21, anti-mouse CD9, anti-α_{IIb}, anti-mouse β_3, anti-mouse GPIV, anti-β_1, anti-α_v, anti-α_5, or anti-α_6 for 1 h at 4°C. After washing with PBS, platelets were reacted with the appropriate second antibodies. Fluorescence data were collected using a Becton–Dickinson FACScan.

[a] Reference 20.

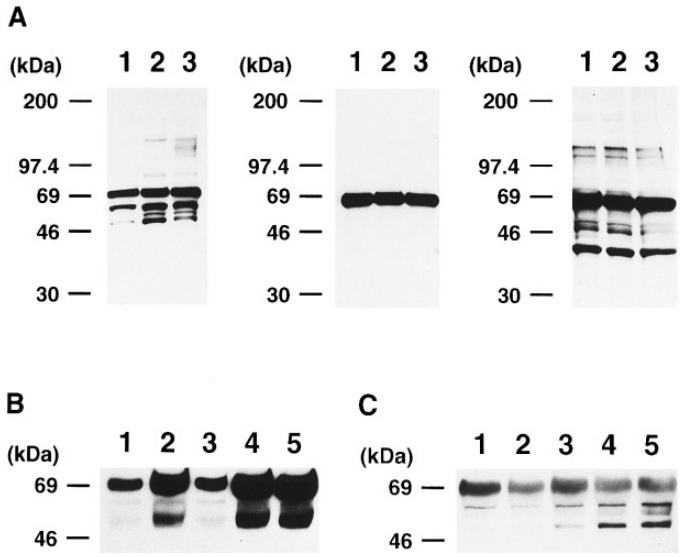

FIG. 4. Phosphorylation of platelet proteins at tyrosine residues during AIP21-mediated platelet aggregation. (A), washed mouse platelets were incubated with vehicle (Lane 1), NL-17 cells (1×10^6 cells/ml; Lane 2), or AIP21 (10 μg/ml; Lane 3) for 5 min at 37°C. The platelet lysates were electrophoresed and transblotted onto a nitrocellulose membrane. The membrane was immunoblotted using an anti-PTyr mAb (left panel), anti-PSer mAb (middle panel), or anti-PThr mAb (right panel). (B), washed mouse platelets were incubated with NR IgM (10 μg/ml; Lane 1), AIP21 (10 μg/ml; Lane 2), or APB2 (10 μg/ml; Lane 3) for 5 min at 37°C. PRP were also incubated with AIP21 (10 μg/ml; Lane 4) or ADP (20 μM; Lane 5) for 5 min at 37°C. The platelet lysates were electrophoresed and transblotted onto a nitrocellulose membrane. The membrane was immunoblotted using anti-PTyr mAb. (C), washed mouse platelets were incubated with 0 μg/ml (Lane 1), 1 μg/ml (Lane 2), 3 μg/ml (Lane 3), 10 μg/ml (Lane 4), or 30 μg/ml (Lane 5) of AIP21 for 1 min at 37°C. The platelet lysates were electrophoresed and transblotted onto a nitrocellulose membrane. The membrane was immunoblotted using the anti-PTyr mAb. After washing, the membrane was incubated with peroxidase-conjugated second antibody and was developed with an enhanced chemiluminescence detection system.

integrin was specifically expressed on platelets. Although we could not obtain the anti-GPIV mAb, GPIV has been reported to express weakly on splenocytes (19). These results indicate that the AIP21 antigen is different from CD9, GPIV, or integrins such as α_{IIb}, β_3, β_1, α_v, α_5, and α_6 integrins and that it may be a novel antigen.

We tried to identify molecules recognized by AIP21 and APB2 by immunoprecipitation and/or Western blot analysis. However, we could not detect the specific band in the lysates from platelets, B16F10 cells, or KN-3 cells (data not shown). Identifying the antigen will be the subject of future study.

Phosphorylation of platelet proteins after ligation of AIP21. Previous studies have shown that several platelet proteins were phosphorylated at tyrosine residues when platelets undergo aggregation induced by some agonists and mAbs (20, 21). Several 50-68-kDa proteins were phosphorylated at tyrosine residues after the coating of washed mouse platelets with AIP21 (Fig. 4A, left panel, Lane 3). Likewise, when the platelets were treated with platelet aggregation-inducing NL-17 cells (Fig. 4A, left panel, Lane 2) (16) and when PRP were treated with a platelet aggregation-inducing factor, ADP (Fig. 4B, Lane 5), these same proteins were tyrosine phosphorylated. However, platelet proteins were not phosphorylated at serine or threonine residues by the ligation of AIP21 (Fig. 4A, middle and right panels, Lane 3). The ligation of APB2 and NR IgM had no effects on the phosphorylation of platelet proteins (Fig. 4B, Lanes 1 and 3, respectively). The tyrosine phosphorylation induced by AIP21 occurred in a dose-dependent manner (Fig. 4C).

Identifying platelet proteins that were tyrosine phosphorylated in response to the ligation of AIP21 would be essential for understanding the mechanisms of signal transduction in mouse platelets. In human platelets, the 52-kDa Shc protein was reported to be tyrosine phosphorylated after thrombopoietin binding to its receptor, c-mpl (21). As the 52-kDa protein was also tyrosine phosphorylated after the ligation of AIP21 to mouse platelets (Fig. 4A), we assumed the 52-kDa protein was Shc protein. Then, the immunoprecipitated Shc protein was electrophoresed and immunoblotted

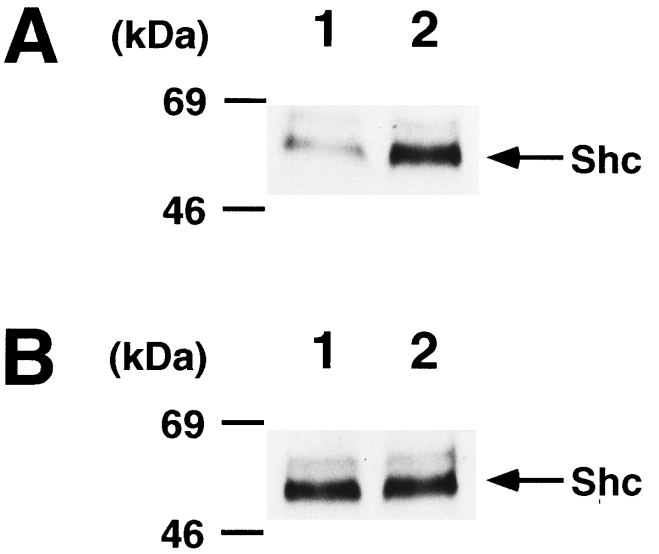

FIG. 5. Phosphorylation of the Shc protein during AIP21-mediated platelet aggregation. Washed mouse platelets were incubated with PBS alone (Lane 1) or AIP21 (50 μg/ml; Lane 2) for 5 min at 37°C. Shc protein was immunoprecipitated from the platelet using a specific anti-Shc mAb. Immunocomplexes were electrophoresed in a 10-20% gradient gel. After blotting onto a nitrocellulose membrane, the membrane was immunoblotted using an anti-PTyr mAb (A) or an anti-Shc mAb (B). After washing, the membrane was incubated with peroxidase-conjugated second antibody and was developed with an enhanced chemiluminescence detection system.

with an anti-PTyr mAb. As shown in Fig. 5A, we found a dramatic increase in tyrosine phosphorylation of the 52-kDa Shc protein in mouse platelets after AIP21 ligation. This result suggests that the Shc protein may be associated with the AIP21-mediated mouse platelet aggregation.

In conclusion, we here report a mouse platelet aggregation-inducing mAb, AIP21. This mAb induced mouse platelet aggregation without the need for plasma components and through an FcR-independent mechanism. We identified that the 52-kDa Shc protein was involved in the signal transduction of the platelet aggregation. Although we could not identify the antigen of AIP21, identification of the antigen, in future, would contribute to the understanding of the undefined mechanisms of mouse platelet aggregation. Because mouse platelets did not express FcR on their surface, analysis of anti-mouse platelet mAbs that induced mouse platelet aggregation would also contribute to the understanding of FcR-independent platelet aggregation.

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