

# Soluble CD40 ligand induces $\beta_3$ integrin tyrosine phosphorylation and triggers platelet activation by outside-in signaling

K. S. Srinivasa Prasad, Patrick Andre, Ming He, Ming Bao, Jeanne Manganello, and David R. Phillips\*

Department of Cardiovascular Biology, Millennium Pharmaceuticals, Inc., 256 East Grand Avenue, South San Francisco, CA 94080

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We earlier reported that the soluble form of the CD40 ligand (sCD40L), is involved in thrombosis by stabilizing platelet thrombi. In this article, we have determined the mechanism by which this protein affects platelet biology. Addition of sCD40L to washed platelets was found to activate the receptor function of  $\alpha_{IIb}\beta_3$  as measured by the induction of fibrinogen binding and the formation of platelet microparticles. Mutation in the KGD sequence (D117E) of sCD40L, the  $\alpha_{IIb}\beta_3$ -binding domain in the N terminus of the protein resulted in a loss of the platelet-stimulatory activity of this protein. Integrilin, a  $\alpha_{IIb}\beta_3$  antagonist, but not an antibody to CD40 that blocked the ligand-binding activity, inhibited these platelet-stimulatory events. CD40<sup>-/-</sup> platelets bound fibrinogen and formed microparticles similar to WT platelets, again indicating that CD40 is not involved in sCD40L-induced platelet activation. Exposure of platelets to sCD40L, but not D117E-sCD40L-coated surfaces, induced platelet thrombi formation under arterial shear rate. sCD40L-induced platelet stimulation resulted in the phosphorylation of tyrosine-759 in the cytoplasmic domain of  $\beta_3$ . Platelets from the diYF mouse strain, expressing  $\beta_3$  in which both cytoplasmic tyrosines are mutated to phenylalanine, were defective in sCD40L-induced platelet stimulation. These data indicate that sCD40L is a primary platelet agonist and that platelet stimulation is induced by the binding of the KGD domain of sCD40L to  $\alpha_{IIb}\beta_3$ , triggering outside-in signaling by tyrosine phosphorylation of  $\beta_3$ .

Platelet aggregation is now recognized as a primary reaction in arterial thrombosis and, accordingly, is responsible for the ischemic complications of acute myocardial infarction and stroke.  $\alpha_{IIb}\beta_3$ , the most abundant integrin on platelets, has complex roles in this reaction. On unstimulated platelets,  $\alpha_{IIb}\beta_3$  has low affinity for soluble fibrinogen and von Willebrand factor (vWF), and is only capable of recognizing fibrinogen immobilized on surfaces (1). However, in response to platelet stimulation, induced by agents such as collagen, ADP, or thrombin, acting on distinct receptors, inside-out signaling causes the activation of the receptor function of  $\alpha_{IIb}\beta_3$ , allowing it to bind soluble fibrinogen and vWF. The polyvalent structures of these proteins allow them to crosslink the surfaces of activated platelets to mediate platelet aggregation (2). vWF binding to  $\alpha_{IIb}\beta_3$  occurs through the RGD recognition motif found in this ligand. Fibrinogen binding occurs by the AGDV sequence found in the  $\gamma$ -chain of this protein (3).

Whereas  $\alpha_{IIb}\beta_3$  activation and ligand binding are critical for initiating platelet aggregation, the stability of the aggregate appears to depend on  $\alpha_{IIb}\beta_3$  signaling events induced by platelet-platelet contacts occurring during aggregation. One signaling event includes the phosphorylation of tyrosine residues on the cytoplasmic domain of  $\beta_3$  (4). Platelets from mice harboring  $\beta_3$ , in which both cytoplasmic tyrosines have been mutated to phenylalanine, produce unstable platelet aggregates. Other secondary proteins are also involved, including Gas6 (5), Ephrin (6), and CD40L, a protein we recently found to be important in aggregate stability. CD40L, a tumor necrosis factor (TNF) family member is mainly expressed on activated T cells (7) and platelets (8). It is cryptic in unstimulated platelets, but rapidly becomes

exposed on the platelet surface after stimulation where it is subsequently cleaved, producing a soluble hydrolytic product (9). Using an *in vivo* thrombosis model, we found that CD40L<sup>-/-</sup> mice have a platelet thrombosis defect, with a delayed occlusion time due to frequent embolization of the thrombi, despite the fact that these mice have normal hemostatic function, which also appears to be true for hyper-IgM patients (9, 10). The stability of thrombi was restored when the soluble form of the CD40 ligand (sCD40L) protein was infused into CD40L<sup>-/-</sup> mice. sCD40L was also found to be a  $\alpha_{IIb}\beta_3$  ligand. These activities of sCD40L were shown to depend on its KGD sequence, a known  $\alpha_{IIb}\beta_3$  binding motif, because both the  $\alpha_{IIb}\beta_3$  binding and the thrombotic activities could be disrupted by a D117E mutation in the KGD. Although the thrombosis function of CD40L has been linked to  $\alpha_{IIb}\beta_3$ , the platelet integrin, and not to CD40, the mechanism by which CD40L participates in thrombosis is not known.

We initially envisioned that stabilization of platelet thrombi by sCD40L could occur by two mechanisms. First, because sCD40L is a trimer, this protein could crosslink platelets through interactions with  $\alpha_{IIb}\beta_3$  on adjacent platelets. Second, sCD40L could bind  $\alpha_{IIb}\beta_3$  and induce platelet stimulation directly. The studies reported herein demonstrate that sCD40L is a platelet agonist that activates platelets through  $\alpha_{IIb}\beta_3$ -dependent outside-in signaling.

## Materials and Methods

**Mice.** Breeding pairs of CD40<sup>-/-</sup> mice obtained from The Jackson Laboratory, and C57BL/6 diYF mouse (mice expressing  $\beta_3$  integrin carrying Y747F and Y759F mutations) colonies were maintained, according to the Institutional Animal Care and Use Committees regulations at Millennium Pharmaceuticals.

**Preparation of Washed Platelets.** Human and mouse washed platelets were prepared as described (11–13).

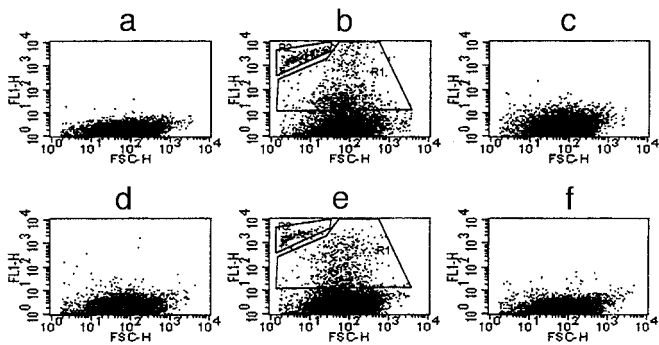
**Flow Cytometry Analysis.** For fibrinogen binding, washed human and mouse platelets ( $1 \times 10^7$  per ml) were incubated for 60 min at 37°C with 2  $\mu$ M sCD40L or (D117E)-sCD40L in the presence or absence of 2.5  $\mu$ M Integrilin or 10  $\mu$ g/ml purified and detoxified anti-human CD40 antibody (ATCC-9110, G28–5). All incubations had 150 nM Alexa-fibrinogen (Molecular Probes). Samples were diluted 10-fold with stain buffer (Pharmingen) after incubation and immediately analyzed by flow cytometry (with a FACSort fluorescence-activated cell sorter, Becton Dickinson). P-selectin expression on the washed platelets were measured with a monoclonal antibody (Becton Dickinson) after stimulating platelets with purified 2  $\mu$ M sCD40L (10) or 5  $\mu$ M thrombin receptor activating peptide (TRAP) (Bachem).

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Abbreviation: sCD40L, the soluble form of the CD40 ligand.

\*To whom correspondence should be addressed. E-mail: david.r.phillips@mpi.com.

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**Fig. 1.** sCD40L induces fibrinogen binding to unactivated human platelets and generation of microparticles. Washed platelets were incubated for 1 h at 37°C with sCD40L (b) or (D117E)-sCD40L (c), sCD40L in the presence of Integrilin (d), or CD40 antibody (e). No treatment (a) and anti-CD40 antibody alone (f) served as controls. Gates R1 and R2 (b and e), respectively, represent platelets and microparticles positive for fibrinogen binding. Flow cytometry data are representative of four experiments performed with four different platelet preparations. FSC-H, forward scatter-height.

Dense granule secretion was measured by activating mepacrine-loaded washed platelets (14) with 2  $\mu$ M sCD40L or 5  $\mu$ M TRAP.

**Tyrosine Phosphorylation of  $\beta_3$  in Spread Platelets, and in Thrombi Formed on sCD40L Surface.** Coverslips were coated with 10  $\mu$ g/ml sCD40L for 2 h at 37°C and blocked with 5% crystalline BSA (Sigma). One-hundred microliters of washed platelets at  $1 \times 10^7$  per ml were allowed to spread for 1 h at 37°C. Nonadhered platelets were washed with PBS (GIBCO/BRL). Adhered platelets were fixed with 4% paraformaldehyde (PFA) and permeabilized with 0.5% Triton X-100 and 0.5% BSA in PBS. Permeabilized platelets were blocked with 3% goat serum and 2% BSA for 30 min, followed by incubation for 2 h at room temperature with purified  $\beta_3$  tyrosine-759 phosphospecific rabbit polyclonal antibody (pY759) raised against a peptide corresponding to the 748–762 amino acid region of the  $\beta_3$  cytoplasmic tail (Research Genetics, Huntsville, AL). The specificity of the staining was determined by incubating the phosphospecific antibody with 250  $\mu$ M of phosphopeptides corresponding to the  $\beta_3$  cytoplasmic 748–762 amino acid sequence containing pY759, or the 740–753 amino acid sequence containing pY747 residues. After 30 min of washing with PBS containing 0.1% Triton X-100 and 0.5% BSA, the coverslips were incubated with Alexa 596 goat anti-rabbit antibody and Alexa 488 phalloidin for 40 min, followed by a brief wash, and were mounted on slides with VECTASHIELD (Vector Laboratories). Images were recorded under oil-immersion  $\times 60$  objective by using a Hamamatsu (Ichinocho, Japan) ORCA-ER digital camera attached to a Nikon eclipse E1000 fluorescence microscope (Technical Instruments, San Francisco).

Platelet thrombi formed on sCD40L-coated Petri plates were rinsed with PBS, fixed with 4% PFA for 20 min, subsequently permeabilized with 0.1% Triton X-100 for 20 min, blocked with normal goat serum for 20 min, stained with purified  $\beta_3$  pY759

**Table 1. The effect of CD40L on washed human platelets**

Treatment	Platelets	Microparticles
Untreated	0.1 $\pm$ 0.1	0.0
sCD40L	15 $\pm$ 5.0	5.0 $\pm$ 3.0
(D117E)-sCD40L	1.0 $\pm$ 0.9	0.1 $\pm$ 0.1
sCD40L plus integrilin	1.5 $\pm$ 1.0	0.2 $\pm$ 0.1
sCD40L plus anti-CD40 antibody	13.0 $\pm$ 7.0	4.0 $\pm$ 3.0
Anti-CD40 antibody	0.5 $\pm$ 0.2	0.0

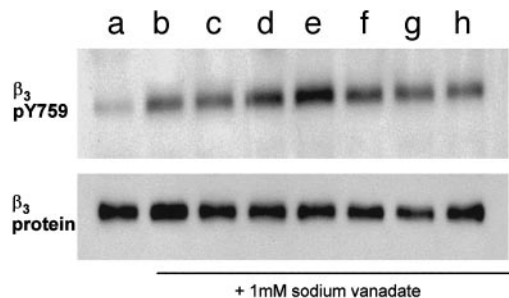
**Table 2. The effect of CD40L on washed mouse platelets**

Treatment	WT		CD40 <sup>-/-</sup>	
	Platelets	Micro-particles	Platelets	Micro-particles
Untreated	0.06 $\pm$ 0.05	0.0	0.02 $\pm$ 0.05	0.0
sCD40L	12.8 $\pm$ 2.2	3.98 $\pm$ 1.2	13.02 $\pm$ 0.6	3.98 $\pm$ 1.2
(D117E)-sCD40L	0.41 $\pm$ 0.28	0.59 $\pm$ 0.36	0.41 $\pm$ 0.10	0.02 $\pm$ 0.01

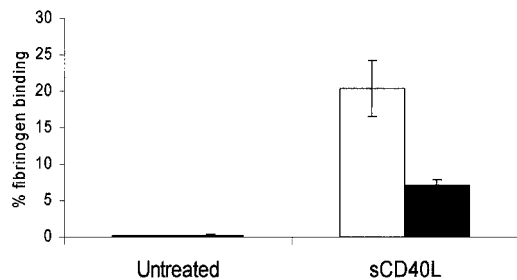
antibody for 50 min, and finally incubated with an FITC-conjugated goat-anti rabbit antibody (1/1,000 dilution) for 30 min. All incubations were performed at 4°C. Between each step, platelet aggregates were rinsed three times with a PBS/0.5% BSA solution. Pictures were taken with an Axiovert 100 (Zeiss) inverted fluorescent microscope.

**Western Blot Analysis of  $\beta_3$  Tyrosine Phosphorylation.** Washed platelets were incubated at  $3 \times 10^8$  per ml at 37°C for 40 min with sCD40L or (D117E)-sCD40L in the presence or absence of 10  $\mu$ M Integrilin, 1  $\mu$ M fibrinogen, and 1 mM sodium vanadate. Tyrosine phosphorylation was analyzed by Western blot analysis on SDS gels by using purified  $\beta_3$  pY759-specific antibody. The blots were stripped and reprobed with C3A, an anti-human  $\alpha_{IIb}\beta_3$  monoclonal antibody to quantitate levels of  $\beta_3$ .

**Perfusion Chamber Assay.** Whole blood, obtained from healthy volunteers who denied having taken aspirin or other platelet function inhibitors in the preceding week, was anticoagulated with 300  $\mu$ mol/liter D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone dihydrochloride (PPACK). A silicone gasket was placed between a flat perfusion chamber (Glycotech, Rockville, MD) and a Petri dish coated with fibrinogen or sCD40L or (D117E)-CD40L. Coating of the proteins was performed by using the spray method, in which 25  $\mu$ l of a 1 mg/ml protein solution is coated onto the plastic with a retouching air brush, resulting in a concentration of 25  $\mu$ g/cm<sup>2</sup> (15). Whole blood with and without Integrilin or 0.1  $\mu$ g/ml aspirin or 100  $\mu$ M 2-methylthio-AMP (2MesAMP) or aspirin and 2MesAMP was perfused for 7 min through flat perfusion chambers at various shear rates. Shear rates of 1,500 and 550 per s were obtained with gasket of a flow path height of 127  $\mu$ m and a flow path width of 2.5 mm at 0.6 and 0.24 ml/min, respectively, and 150 per s with a 254- $\mu$ m thickness, and a 5-mm-width flow path at 0.48 ml/min. Measurement of thrombi (mean thrombus area) was assessed by using SIMPLE PCI software.



**Fig. 2.** sCD40L induces  $\beta_3$  tyrosine-759 phosphorylation. Washed platelets were incubated with sCD40L alone (lane d), fibrinogen alone (lane c), d plus c (lane e), (D117E)-sCD40L alone (lane f), c plus f (lane g), or sCD40L with fibrinogen and Integrilin (lane h) in the presence of sodium vanadate. Unstimulated platelets (lanes a and b) served as controls with and without sodium vanadate, respectively. Platelet samples were lysed and resolved on SDS/10% PAGE and blotted with  $\beta_3$  pY759-specific polyclonal antibody. This blot is representative of eight separate experiments.



**Fig. 3.** The diYF platelets are defective in sCD40L-induced fibrinogen binding. Washed platelets prepared from WT and diYF mice were incubated with Alexa fibrinogen in the presence or absence of sCD40L for 1 h at 37°C, fixed with formaldehyde (0.4% final), diluted, and analyzed by flow cytometry. The percentage of platelets positive for fibrinogen binding was obtained from the total number of gated events represented as a mean value with SD obtained from three separate platelet preparations.

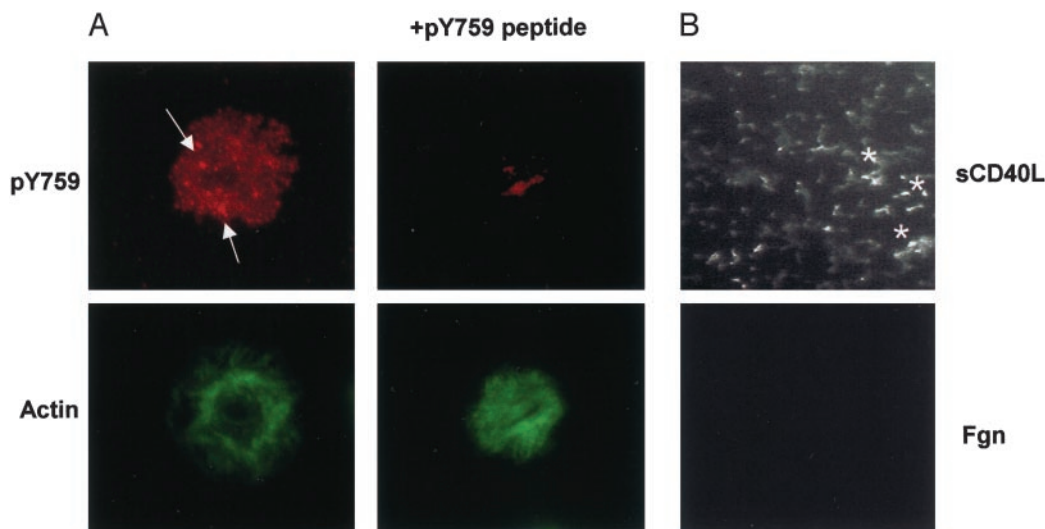
## Results

**sCD40L-Induced Platelet Activation.** We previously showed that sCD40L binds to purified  $\alpha_{IIb}\beta_3$  and that sCD40L stabilizes platelet thrombi and promotes platelet spreading. To determine whether sCD40L has a direct effect on platelet function, we measured markers of platelet stimulation. The addition of sCD40L to washed human (Fig. 1*b* and Table 1) or mouse (Table 2) platelets was found to induce microparticle formation. In addition, sCD40L also allowed for increased fibrinogen binding, to both the sCD40L-stimulated platelets, and to the microparticles formed by this treatment. In contrast, we did not observe any sCD40L-induced expression of P-selectin and dense granule secretion (data not shown). These data indicate that sCD40L induced selective platelet activation.

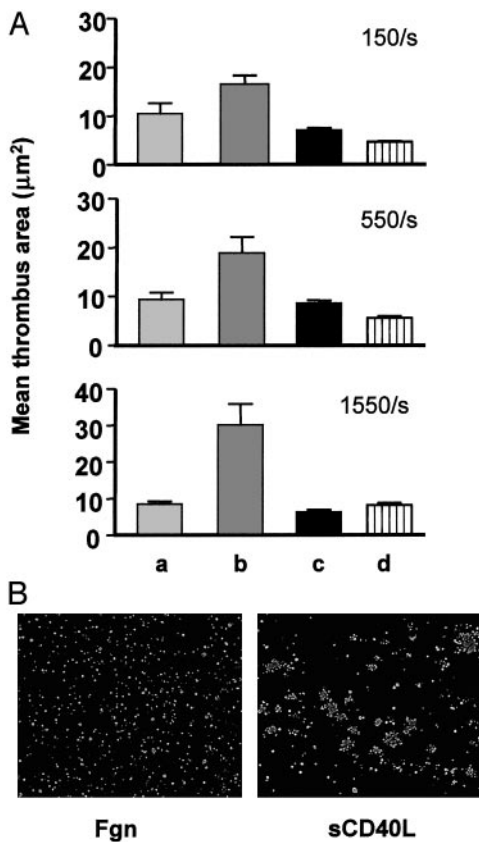
sCD40L has at least two functional domains: a KGD sequence, which binds to  $\alpha_{IIb}\beta_3$  (10), and a TNF homology domain, which binds to CD40 (16, 17). To determine whether the KGD domain is involved in platelet activation, (D117E)-sCD40L was generated and compared with the native sequence for its platelet-activating activity. The induction of fibrinogen binding and the

expression of platelet microparticles was not observed when either human or mouse platelets were incubated with (D117E)-sCD40L (Fig. 1*c* and Tables 1 and 2), suggesting that the KGD domain of sCD40L is involved in the induction of platelet activation. To test the hypothesis that sCD40L induced platelet activation by binding to  $\alpha_{IIb}\beta_3$ , and not to CD40, the effect of specific antagonists on each receptor was examined. sCD40L-induced microparticle formation by human platelets was completely inhibited by Integrilin, a specific  $\alpha_{IIb}\beta_3$  antagonist (Fig. 1*d* and Table 1), but not by a CD40 antibody that blocks the binding of CD40L (Fig. 1*e* and Table 1). The CD40 antibody also had no effect on sCD40L-induced fibrinogen binding (Fig. 1*f*). In addition, platelets from CD40<sup>-/-</sup> mice were activated similarly to those from WT mice, a reaction that was dependent on the KGD sequence of sCD40L (Table 2). These data indicate that sCD40L induces platelet activation by directly binding to  $\alpha_{IIb}\beta_3$  through its KGD sequence.

**sCD40L-Induced  $\alpha_{IIb}\beta_3$  Tyrosine Phosphorylation.** We previously found that outside-in signaling through  $\alpha_{IIb}\beta_3$  is mediated in part by the tyrosine phosphorylation of the cytoplasmic domain of  $\beta_3$ . To determine whether tyrosine phosphorylation of  $\beta_3$  occurs during sCD40L-induced activation of platelets, we blotted gels of lysates with an antibody specific for  $\beta_3$  tyrosine-759 (pY759). We observed that addition of sCD40L to washed platelets caused a >2-fold increase in the tyrosine phosphorylation of  $\beta_3$  (Fig. 2, lane d). A further increase was observed when fibrinogen was included with this incubation (Fig. 2, lane e), but not when fibrinogen was added in the absence of sCD40L (Fig. 2, lane c). Integrilin inhibited sCD40L-induced  $\beta_3$  tyrosine phosphorylation (Fig. 2, lane h). Tyrosine phosphorylation of  $\beta_3$  remained at basal levels when incubated with (D117E)-sCD40L in the absence (Fig. 2, lane f) or in presence of fibrinogen (Fig. 2, lane g). To determine whether tyrosine phosphorylation of  $\beta_3$  is necessary for sCD40L-induced activation of platelets, we next measured the sCD40L-induced fibrinogen binding to platelets from the diYF mouse, a strain expressing  $\beta_3$ , in which both cytoplasmic tyrosines are mutated to phenylalanine. As seen in Fig. 3, whereas  $20 \pm 3.9\%$  of WT platelets bound fibrinogen when



**Fig. 4.** Platelet  $\beta_3$  cytoplasmic tyrosine-759 is phosphorylated when spread on the sCD40L surface under static and shear conditions. (A) Washed platelets spread on sCD40L-coated glass surface were stained with pY759 antibody. Specific staining was blocked when 250  $\mu$ M Y759 phosphopeptide was incubated with the phosphoantibody before adding to spread platelets. Arrows indicate intense stained areas, and they appear to be in focal contacts. Platelets shown here represent a field of spread platelets stained positively for pY759 antibody. (B) Anticoagulated whole blood was perfused over different coated surfaces at arterial shear rate. Adhered platelets were fixed and stained with pY759 antibody. \*, Platelets in the thrombi stained positive for pY759 antibody on the fluorescence image.



**Fig. 5.** The sCD40L-coated surface is prothrombotic. (A) Anticoagulated whole blood was perfused at different shear rates over the surface coated with sCD40L (lane b), or fibrinogen (lane a), or (D117E)-sCD40L (lane c). Whole blood with Integrilin was perfused over the sCD40L-coated surface (lane d). (B) Fibrinogen (Fgn)- and sCD40L-coated chambers after perfusion were fixed and stained for P-selectin expression. Whereas the sCD40L-coated surface contained platelet aggregates, the fibrinogen-coated surface had single platelets.

incubated with sCD40L, only  $7.2 \pm 0.54\%$  of diYF platelets bound fibrinogen.

Fig. 4 shows that tyrosine phosphorylation of  $\beta_3$  also occurs during spreading of platelets and in platelet thrombi on immobilized sCD40L. Tyrosine phosphorylation of  $\beta_3$  in spread platelets was detected by staining with the  $\beta_3$  pY759 antibody, the staining of which appeared to be in focal contacts (Fig. 4A Left Upper). Staining with this antibody appeared to be specific, because it was blocked with 250  $\mu$ M peptide containing the  $\beta_3$  cytoplasmic 748–762 amino acid region with pY759 (Fig. 4A Right Upper), but not by a peptide corresponding to the 740–753 amino acid region containing pY747, a site that also becomes phosphorylated on platelet aggregation (data not shown).

CD40L has been shown to be a prominent component of atherosclerotic lesions (18). Because ruptured lesions initiate thrombosis and sCD40L induces platelet activation, experiments were performed to determine whether immobilized sCD40L could initiate thrombosis. PPACK-anticoagulated human blood was perfused over surfaces coated with sCD40L or fibrinogen at shear rates designed to include those anticipated within the coronary circulation. Platelets are known to form a monolayer on fibrinogen surfaces at low (150 per s) to high (1,550 per s) shear rates, apparently because the adherent platelets are insufficiently activated to recruit and/or retain additional platelets (ref. 19 and Fig. 5A and B Left). However, the sCD40L-coated surface supported thrombus formation in a shear rate-

dependent manner (Fig. 5A and B Right). Two studies indicated that the thrombogenic activity of immobilized sCD40L involved a direct interaction with  $\alpha_{IIb}\beta_3$ . First, (D117E)-sCD40L lacked thrombogenic activity. Second, thrombosis on sCD40L surfaces was completely inhibited by 2  $\mu$ M Integrilin. These data indicate that immobilized sCD40L is capable not only of recruiting platelets at arterial shear rates but also of inducing sufficient platelet activation to cause the recruitment of platelets. This activity of sCD40L is caused by the KGD domain of sCD40L binding to  $\alpha_{IIb}\beta_3$  on platelets. To determine whether platelet secretion is required for thrombus formation on the sCD40L surface, PPACK-anticoagulated whole blood was perfused in the presence or absence of aspirin and 2MesAMP, a P2Y12 antagonist. Our result demonstrated that immobilized sCD40L-mediated thrombosis is secretion independent, which is consistent with our findings in the static assays (data not shown).

Thrombi formed on sCD40L surfaces at arterial shear rates also contained robust staining for the  $\beta_3$  pY759 antibody (Fig. 4B Upper). In contrast, platelets adherent to fibrinogen surfaces only expressed minimal staining for the tyrosine phosphorylated Integrin (Fig. 4B Lower).

## Discussion

The concept of autocrine loops by secreted factors from platelets is well established. For example, ADP, which is secreted from platelet-dense bodies in response to platelet stimulation, induces additional platelet activation to promote thrombotic and hemostatic reactions by binding to its receptors, P<sub>2</sub>Y<sub>1</sub> (20) and P<sub>2</sub>Y<sub>12</sub> (21). Gas6, an  $\alpha$ -granule protein, has similar activity, inducing platelet stimulation by binding to its receptors Sky, mer, and axl. The importance of these events to platelet biology has been demonstrated by the clinical antithrombotic benefit of P2Y<sub>12</sub> antagonists (22, 23), and by the thrombosis defect in Gas6<sup>-/-</sup> mice (6). We previously showed that CD40L<sup>-/-</sup> mice have thrombosis defect, that this thrombosis defect could be corrected by sCD40L (10), and that sCD40L is a  $\alpha_{IIb}\beta_3$  ligand. This article shows that sCD40L induces platelet stimulation, and that this process is mediated by the binding of the KGD domain of sCD40L to  $\alpha_{IIb}\beta_3$ . Biochemical and genetic data demonstrate that this outside-in integrin signaling activity of sCD40L occurs because of the tyrosine phosphorylation of  $\beta_3$ . We conclude that sCD40L functions as a primary platelet agonist, that  $\alpha_{IIb}\beta_3$  is a primary agonist receptor, and that sCD40L functions in auto-crine loop to regulate platelet biology.

Although platelet stimulation by outside-in signaling through  $\alpha_{IIb}\beta_3$  is well known, the activity of sCD40L is, to our knowledge, unique. Typically, the receptor function of  $\alpha_{IIb}\beta_3$  for soluble adhesive protein ligands is expressed by inside-out signaling in response to primary platelet agonists such as collagen, thrombin, and ADP (3, 24). Outside-in  $\alpha_{IIb}\beta_3$  signaling and subsequent platelet stimulation is mediated by adhesive protein binding to  $\alpha_{IIb}\beta_3$  during platelet aggregation and/or during platelet adhesion, with soluble  $\alpha_{IIb}\beta_3$  ligands having minimal platelet-stimulatory activity. We have previously established that outside-in  $\alpha_{IIb}\beta_3$  signaling is mediated, in part, by tyrosine phosphorylation of the cytoplasmic domain of  $\beta_3$  (25–28). This article, however, demonstrates that sCD40L, a soluble  $\alpha_{IIb}\beta_3$  ligand, induces platelet stimulation, as evidenced by the generation of platelet microparticles, and by the activation of the receptor function of  $\alpha_{IIb}\beta_3$  for soluble fibrinogen. This outside-in signaling event appeared to be mediated by tyrosine phosphorylation of  $\beta_3$ .  $\beta_3$  tyrosine phosphorylation occurred both on addition of sCD40L to platelets in suspension, and on the adhesion of platelets to immobilized sCD40L; platelets from the diYF mouse were defective in sCD40L platelet-stimulatory activity. Whereas this might seem a discrepancy, we have found that the sCD40L released from platelets, as well as the recom-

binant sCD40L, is trimeric (data not shown), as is the native sCD40L shed from human T cells (29).

sCD40L is generated *in vivo* in response to thrombotic events, generating concentrations approaching 5–10 ng/ml within the systemic circulation. The concentration of sCD40L required to induce platelet stimulation approaches 50–100 times these values. However, sCD40L is generated at sites of thrombosis during acute coronary syndromes or percutaneous interventions that are 1/5,000 to 1/10,000 of the volume of blood (assuming a thrombus volume of <1 ml and a blood volume of 5 liters). In addition, the interplatelet space available for the generation of sCD40L within a platelet aggregate is further restricted, concentrating the released protein. These considerations indicate that the concentrations of sCD40L developed within a growing thrombus would be sufficient to induce platelet stimulation, and sufficient to account for the thrombosis defect in the CD40L<sup>-/-</sup> mouse.

While this manuscript was in preparation, a publication by Inwald *et al.* (30) appeared, suggesting that sCD40L induces platelet stimulation by binding to CD40 and not to  $\alpha_{IIb}\beta_3$ . These studies, however, were performed with a sCD40L tagged at its N terminus with a isoleucine zipper motif, which is known to create oligomerization of the protein, thereby enhancing and sustaining signaling through CD40 (31). Although it is not known why the  $\alpha_{IIb}\beta_3$ -binding activity of this protein is lost, it may be that exposure of the KGD sequence was lost by the construction of the chimeric protein. Whereas the observations

of Inwald *et al.* (30) might suggest that CD40 signaling may occur during aggregation, the absence of a thrombosis defect in CD40<sup>-/-</sup> mice indicates that such signaling, if present, is not required for platelet aggregate stability.

Patients with acute coronary syndromes, peripheral arterial occlusive disease and other thrombotic disorders, angioplasty, and postcardiopulmonary bypass, have increased concentrations of sCD40L in circulation, most of it coming from the platelet pool (13, 32–34). Plasma sCD40L is now considered as a risk factor for cardiovascular disease in women (35). Increased CD40L expression has been localized to the human atherosclerotic lesions (18), which could be contributed by platelets in the growing thrombi at the lesion, T cells, and vascular cells. Activated platelets are now known to deliver regulated on activation, normal T cell expressed and secreted (RANTES) to the endothelium in lesions (36). It is tempting to speculate that activated platelets could deliver sCD40L to these lesions as well. Although CD40L has a proven role in inflammation (9) and atherosclerotic lesion progression (37, 38), this article suggests that exposure of CD40L after plaque rupture may contribute to the initiation of thrombosis.

Patients with thrombotic disorders (39), infectious diseases like malaria, and who have undergone cardiopulmonary bypass (40), have increased microparticles in circulation (41, 42), which are procoagulant and prothrombotic. sCD40L- $\alpha_{IIb}\beta_3$ -mediated platelet microparticle formation could potentially contribute to these pathologies.

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