

# Dual Regulation of Glycogen Synthase Kinase 3 (GSK3) $\alpha/\beta$ by Protein Kinase C (PKC) $\alpha$ and Akt Promotes Thrombin-mediated Integrin $\alpha_{IIb}\beta_3$ Activation and Granule Secretion in Platelets\*

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**Background:** The constitutively active kinase GSK3 $\beta$  is a negative regulator of thrombin-stimulated platelet function.

**Results:** Interfering with PKC $\alpha$  and Akt blocked thrombin-mediated GSK3 $\alpha/\beta$  phosphorylation and reduced platelet function.

**Conclusion:** PKC $\alpha$  and Akt phosphorylate GSK3 $\alpha/\beta$  resulting in reduced GSK3 $\alpha/\beta$  activity and increased thrombin-mediated platelet function.

**Significance:** This study shows a novel mechanism by which GSK3 $\alpha/\beta$  is regulated and contributes to platelet function.

Glycogen synthase kinase-3 is a Ser/Thr kinase, tonically active in resting cells but inhibited by phosphorylation of an N-terminal Ser residue (Ser<sup>21</sup> in GSK3 $\alpha$  and Ser<sup>9</sup> in GSK3 $\beta$ ) in response to varied external stimuli. Recent work suggests that GSK3 functions as a negative regulator of platelet function, but how GSK3 is regulated in platelets has not been examined in detail. Here, we show that early thrombin-mediated GSK3 phosphorylation (0–30 s) was blocked by PKC inhibitors and largely absent in platelets from PKC $\alpha$  knock-out mice. In contrast, late (2–5 min) GSK3 phosphorylation was dependent on the PI3K/Akt pathway. Similarly, early thrombin-mediated inhibition of GSK3 activity was blocked in PKC $\alpha$  knock-out platelets, whereas the Akt inhibitor MK2206 reduced late thrombin-mediated GSK3 inhibition and largely prevented GSK3 inhibition in PKC $\alpha$  knock-out platelets. More importantly, GSK3 phosphorylation contributes to platelet function as knock-in mice where GSK3 $\alpha$  Ser<sup>21</sup> and GSK3 $\beta$  Ser<sup>9</sup> were mutated to Ala showed a significant reduction in PAR4-mediated platelet aggregation, fibrinogen binding, and P-selectin expression, whereas the GSK3 inhibitor CHIR99021 enhanced these responses. Together, these results demonstrate that PKC $\alpha$  and Akt modulate platelet function by phosphorylating and inhibiting GSK3 $\alpha/\beta$ , thereby relieving the negative effect of GSK3 $\alpha/\beta$  on thrombin-mediated platelet activation.

When a blood vessel becomes damaged, platelets quickly adhere to the newly exposed extracellular matrix and become

activated through GPVI signaling, resulting in the exposure of fibrinogen binding sites on integrin  $\alpha_{IIb}\beta_3$  and granule secretion. A subset of platelets will also expose phosphatidylserine on the outer membrane, nucleating the formation of the prothrombinase complex and subsequent thrombin generation. Thrombin is a potent platelet agonist that cleaves the protease-activated receptors (PARs)<sup>3</sup> PAR1 and PAR4 on the platelet surface thereby further activating and recruiting platelets to the site of injury. The PAR receptors are G protein-coupled receptors, which, upon cleavage of the receptor, activate the phospholipase C pathway, leading to an increase in intracellular Ca<sup>2+</sup> and activation of conventional protein kinase C (PKC) isoforms. Of these, the PKC isoforms PKC $\alpha$  and PKC $\beta$  play nonredundant roles in granule secretion and thrombus formation (1–3).

In addition to activation of the phospholipase C pathway, many platelet agonists also activate class I phosphatidylinositol 3 (PI3)-kinases, which phosphorylate PI(4,5)P<sub>2</sub> in the membrane leading to an increase in the signaling molecule PI(3,4,5)P<sub>3</sub>. Human and mouse platelets express all class I PI3K isoforms (PI3K $\alpha$ , PI3K $\beta$ , PI3K $\delta$ , and PI3K $\gamma$ ), and the use of genetic models and isoform-specific inhibitors has revealed that PI3K $\beta$  plays a predominant role in platelet function, in particular, downstream of GPVI and integrin  $\alpha_{IIb}\beta_3$  (4–6). In addition to a role for PI3K in agonist-stimulated platelet function, it has recently become apparent that several platelet primers, such as TPO, IGF-1, and GAS6, also mediate their potentiating effect on platelet function through a PI3K-dependent pathway (7–9). One of the most studied pathways downstream of PI3K is Akt (aka protein kinase B), a Ser/Thr kinase involved in cell proliferation, survival, and metabolism (10). Activation of PI3K results in an increase in PI(3,4,5)P<sub>3</sub>, which recruits the

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<sup>3</sup> The abbreviations used are: PAR, protease-activated receptor; bis-tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; GSK3, glycogen synthase kinase 3; PE, phycoerythrin; PMA, phorbol 12-myristate-13-acetate.

PH domain-containing kinases PDK1 and Akt to the membrane where PDK1 phosphorylates Akt on Thr<sup>308</sup> in the T-loop. Akt is subsequently phosphorylated by mTORC2 on Ser<sup>473</sup>, resulting in maximal Akt activity (11). Studies on various Akt transgenic mice suggest that Akt activation contributes to integrin activation, granule secretion, and thrombosis (12–14). How Akt regulates platelet function is still an active area of research.

Although numerous substrates for Akt have been described, only a few have definitively been identified in human platelets so far; the proline-rich Akt substrate of 40 kDa (PRAS40) (11) and the Ser/Thr kinase glycogen synthase kinase 3 (GSK3) (11, 12, 15). Although PDE3A was initially reported to be an Akt substrate (16), the availability of improved reagents allowed us to demonstrate that PDE3A activity is regulated mainly by PKC in human platelets (17). In addition, eNOS has been proposed to be a direct Akt substrate (18), although whether eNOS is even present in platelets is a matter of considerable debate (19, 20). GSK3 was the first Akt substrate to be identified by its ability to phosphorylate and inhibit the activity of glycogen synthase (21). GSK3 exists in two isoforms  $\alpha$  and  $\beta$ , of which GSK3 $\beta$  is likely to be expressed predominantly in platelets (15, 22). A recent study suggested that GSK3 $\beta$  negatively regulates platelet function as the small molecule GSK3 inhibitor SB216763 potently enhanced PAR1-mediated aggregation, whereas GSK3 $\beta$ <sup>+/-</sup> mice showed increased PAR4-mediated aggregation and *in vivo* thrombosis (22). It has been challenging, however, to demonstrate a direct link among Akt, GSK3 $\alpha/\beta$  phosphorylation, and changes in platelet function. Indeed, GSK3 is a promiscuous substrate, which can be phosphorylated by several kinases including PKC (23, 24), PKA (25), p90RSK (26, 27), and Akt (10). The aim of the present study was therefore to elucidate the role of GSK3 $\alpha$  and GSK3 $\beta$  phosphorylation in platelet function and identify the signaling pathways involved. Using genetic and pharmacological approaches, we present intriguing evidence that both PKC $\alpha$  and Akt phosphorylate and inhibit GSK3, thereby promoting thrombin-mediated integrin  $\alpha_{IIb}\beta_3$  activation and granule secretion.

## EXPERIMENTAL PROCEDURES

**Mice**—All animal studies were approved by local research ethics, and mice were bred for this purpose under a UK Home Office project license. GSK3 $\alpha$ <sup>S21A/S21A</sup>/ $\beta$ <sup>S9A/S9A</sup> (GSK3 KI), control wild-type GSK3 $\alpha/\beta$ <sup>+/+</sup> (GSK3 WT), PKC $\alpha$ <sup>-/-</sup> (PKC $\alpha$  KO), and control wild-type PKC $\alpha$ <sup>+/+</sup> (PKC $\alpha$  WT) animals were generated, bred, and genotyped as described previously (28–30). GSK3 $\alpha$ <sup>S21A/S21A</sup>/ $\beta$ <sup>S9A/S9A</sup> (GSK3 KI) were kindly provided by Dario Alessi, MRC Phosphorylation Unit, Dundee and the PKC $\alpha$ <sup>-/-</sup> (PKC $\alpha$  KO) by Professor Jeff Molkentin, Cincinnati Children's Hospital, Cincinnati, OH.

**Reagents**—pSer<sup>473</sup> Akt, pThr<sup>308</sup> Akt, Akt2 (L79BZ), Akt3(62A8), Akt3 (L47B1), Akt3 (pAb), pSer<sup>9</sup> GSK3 $\beta$ , pSer<sup>21/9</sup> GSK3 $\alpha/\beta$ , GSK3 $\beta$ , pThr<sup>246</sup> PRAS40, PKC phospho-motif (used for analysis of pleckstrin phosphorylation), pThr<sup>202</sup>/Tyr<sup>204</sup> ERK, pThr<sup>180</sup>/Tyr<sup>182</sup> p38, integrin  $\beta_3$  and PKC $\alpha$  antibodies were from Cell Signaling Technologies (New England Biolabs). Akt1 (B-1), P-Selectin (M-20) and PAR4 (C-20) antibodies were from Santa Cruz (Insight Biotechnology, Wembley, UK). The

Akt1 rabbit mAb (AW24) was from Millipore. Akt2 antiserum 1.1 raised against amino acids 453–470 of murine Akt2 in rabbits was kindly provided by Dick Denton and Kelly Moule (School of Biochemistry, University of Bristol). PE-labeled anti-mouse P-selectin was from Emfret Analytics (Wurzberg, Germany). Human brain tissue lysate was from Abcam (Cambridge, UK). PAR1-activating peptide (SFLLRN-NH<sub>2</sub>) was from Bachem (Weil am Rhein, Germany). RPRAATF, RRAAEELD-SRAGS(P)PQL, and PAR4-activating peptide (AYPGKF-NH<sub>2</sub>) were synthesized by Graham Bloomberg (University of Bristol). CHIR99021 was from Merck Chemicals. MK2206 was from Selleck Chemicals (Strattech, Newmarket, UK). Bisindolylmaleimide IX (BIM) was from Tocris (Bristol, UK). Chronolume was from Chrono-log Corporation (Labmedics, Manchester, UK). Microcystin-LR was from Axxora (Nottingham, UK). [ $\gamma$ -<sup>32</sup>P]ATP was from PerkinElmer Life Sciences. Enhanced chemiluminescent detection reagents were from GE Healthcare. Peroxidase-conjugated secondary antibodies were from Jackson ImmunoResearch. NuPAGE SDS-PAGE sample buffer was from Invitrogen. All other reagents were from Sigma unless otherwise indicated.

**Platelet Isolation**—Blood was obtained with approval from the local Research Ethics Committee of the University of Bristol from healthy drug-free volunteers, who gave full informed consent in accordance with the Declaration of Helsinki. Mouse blood was drawn by cardiac puncture under terminal anesthesia. Washed human and mouse platelets were isolated as described previously (31). Platelets were resuspended at  $4 \times 10^8$ /ml in modified HEPES-Tyrode buffer (145 mM NaCl, 3 mM KCl, 0.5 mM Na<sub>2</sub>HPO<sub>4</sub>, 1 mM MgSO<sub>4</sub>, 10 mM HEPES, pH 7.2, 0.1% (w/v) D-glucose, 0.02 unit/ml apyrase, and 10  $\mu$ M indomethacin).

**Platelet Extraction**—Platelets were treated with vehicle (0.2% dimethyl sulfoxide) or compound for 15 min, stimulated as indicated, and lysed directly in 4 $\times$  NuPAGE sample buffer (whole cell lysate). Alternatively, platelets were extracted with an equal volume of ice-cold (i) radioimmunoprecipitation assay buffer (50 mM HEPES, pH 7.4, 400 mM NaCl, 2 mM EDTA, 2% (v/v) IGEPAL CA-630, 1% (w/v) sodium deoxycholate, 0.2% (w/v) SDS, 40 mM sodium  $\beta$ -glycerophosphate, 20 mM sodium pyrophosphate, 2 mM benzamidine, 2  $\mu$ M microcystin-LR, 10 mM sodium orthovanadate, and 2  $\mu$ g/ml each pepstatin, antipain, and leupeptin) for immunoprecipitation of Akt or (ii) Triton X-100 extraction buffer (50 mM HEPES, pH 7.4, 2% (v/v) Triton X-100, 2 mM EDTA, 40 mM sodium  $\beta$ -glycerophosphate, 20 mM sodium pyrophosphate, 2 mM benzamidine, 20% (v/v) glycerol, 10 mM sodium orthovanadate, 2  $\mu$ M microcystin-LR, and 2  $\mu$ g/ml each pepstatin, antipain, and leupeptin) for activity assays.

**Immunoprecipitation of Akt**—Akt1, Akt2, or Akt3 was immunoprecipitated from radioimmunoprecipitation assay lysates by incubation with anti-Akt1 (B1), anti-Akt2 (antiserum 1.1), or anti-Akt3 (L47B1) for 2 h at 4  $^{\circ}$ C. Immune complexes were washed with extraction buffer prior to elution with 2 $\times$  NuPAGE sample buffer.

**Immunoblotting**—Proteins were resolved by electrophoresis on 8% bis-tris gels as described previously (17). Samples were

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transferred to PVDF membrane, blocked with blocking buffer (Sigma), and subjected to immunoblotting.

**Akt and GSK3 Activity Assay**—Akt and GSK3 activity were measured as described previously (33). Briefly, Akt1, Akt2, or Akt3 was immunoprecipitated from platelet lysates by incubation with anti-Akt1 (B1), anti-Akt2 (antiserum 1.1), or anti-Akt3 (L47B1). Immune complexes were washed and resuspended in assay buffer (20 mM HEPES, pH 7.4, 10 mM MgCl<sub>2</sub>, 20 mM sodium  $\beta$ -glycerophosphate, 1 mM EGTA, and 1 mM DTT) in the presence of 50  $\mu$ M ATP (2  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP), 0.5 mg/ml RPRAAF peptide, and 2.5  $\mu$ M PKA inhibitor (IP-20). GSK3 activity in cell lysates was measured in assay buffer in the presence of 50  $\mu$ M ATP (2  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP), 0.71 mg/ml RRAAEELDSRAGS(P)PQL, and 2.5  $\mu$ M PKA inhibitor (IP-20). Assays were terminated after a 30-min incubation at 30 °C by spotting onto P81 phosphocellulose filter paper. Filters were washed with 0.75% orthophosphoric acid before measuring the incorporation of [ $\gamma$ -<sup>32</sup>P]ATP by Cherenkov counting. Akt activity results are expressed as pmol of phosphate incorporated per 10<sup>8</sup> platelets. For GSK3 activity LiCl (50 mM) was used to distinguish and correct for nonspecific activity; therefore, activity is expressed as a percentage of basal LiCl inhibitable activity.

**Platelet Aggregation and Dense Granule Secretion**—Washed platelets (2  $\times$  10<sup>8</sup>/ml) were incubated with vehicle (0.2% dimethyl sulfoxide) or the indicated compounds for 15 min. Agonist-stimulated aggregation and dense granule secretion were simultaneously monitored using a luciferin/luciferase reagent (Chronolume) in a Chronolog 590–2A aggregometer 37 °C under stirring conditions.

**Integrin  $\alpha_{IIb}\beta_3$  Activation and  $\alpha$ -Granule Secretion**—Integrin  $\alpha_{IIb}\beta_3$  activation was assessed using fibrinogen labeled with fluorescein as described previously (34).  $\alpha$ -Granule secretion was assessed as P-selectin exposure with PE-labeled anti-P-selectin. Platelets (2  $\times$  10<sup>7</sup>/ml) were incubated with vehicle (0.2% dimethyl sulfoxide) or the indicated compounds for 15 min before stimulation in the presence of 0.2 mg/ml FITC-fibrinogen and PE-labeled anti-P-selectin for 10 min. Nonspecific binding of FITC-fibrinogen was determined in the presence of 1 mM EDTA. Samples were fixed with 1% paraformaldehyde for 30 min and analyzed by flow cytometry on a BD LSR II (BD Biosciences).

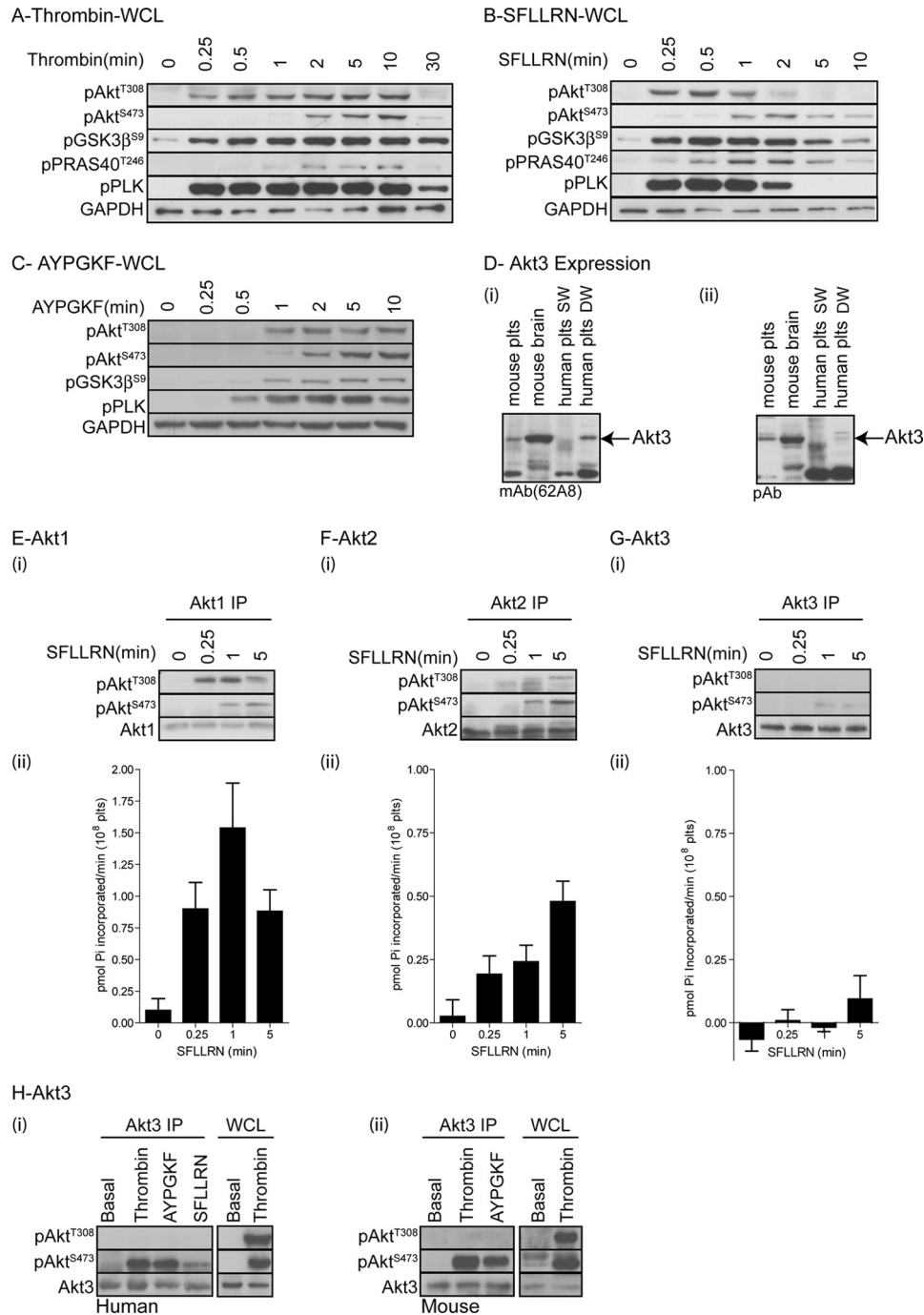
**Statistics**—Data were analyzed and fitted using GraphPad Prism 4.02 software. All data are presented as the mean  $\pm$  S.E. of at least three independent observations. Concentration-response curves were fitted with a four-parameter logistic equation. Differences in best-fit parameters between data sets were determined by F test where the null hypothesis states that both data sets can be fitted using the same parameters. Data presented with statistical analysis were tested using either a paired Student's *t* test (aggregation/ATP secretion data) or two-way analysis of variance with Bonferroni multiple comparison post hoc test (quantification blots/GSK3 activity data).

## RESULTS

**Temporal Correlation among PAR1-mediated GSK3, Akt Thr<sup>308</sup>, and Pleckstrin Phosphorylation**—To obtain insight into the phosphorylation and regulation of GSK3 downstream of

PAR receptors, we initially performed time course studies with thrombin, the PAR1 agonist peptide SFLLRN, and the PAR4 agonist peptide AYPGKF. Fig. 1, *A* and *B*, demonstrates that GSK3 $\beta$  becomes rapidly phosphorylated upon stimulation with thrombin and SFLLRN, respectively, whereas it is more delayed in response to AYPGKF (Fig. 1*C*). Interestingly, thrombin/PAR1-mediated GSK3 $\beta$  phosphorylation correlated with the onset of phosphorylation of Akt Thr<sup>308</sup> and the PKC substrate pleckstrin. In contrast, Akt Ser<sup>473</sup> phosphorylation was more delayed (Fig. 1, *A* and *B*) and correlated closely with phosphorylation of the Akt substrate PRAS40. These data are in agreement with our previous findings that Akt Ser<sup>473</sup> phosphorylation is essential for Akt2 activity and phosphorylation of PRAS40 but dispensable for Akt1 activity and phosphorylation of GSK3 $\beta$  (11). Interestingly, the kinetic differences in Akt, GSK3, and pleckstrin phosphorylation between PAR1/thrombin and PAR4 suggests that PAR1 is responsible for the rapid Akt Thr<sup>308</sup>, GSK3, and pleckstrin phosphorylation by thrombin in human platelets. This is supported by the finding that the PAR1 antagonist SCH79797, prevented early thrombin-mediated Akt Thr<sup>308</sup>, GSK3, and pleckstrin phosphorylation (data not shown).

In addition to expressing Akt1 and Akt2, a recent study suggested that human and mouse platelets also express Akt3 (14). Here, we confirm these findings and show expression of Akt3 in both human and mouse platelets using two different anti-Akt3 antibodies (Fig. 1*D*). Note that Akt3 is picked up in double washed, but not single washed, human platelets, suggesting that residual albumin may interfere with antibody recognition. Mouse brain extract has been used as a positive control (Fig. 1*D*). To directly correlate Akt isoform phosphorylation status with kinase activity, we subsequently immunoprecipitated Akt1, Akt2, and Akt3 isoforms followed by immunoblotting and *in vitro* kinase activity assays. Fig. 1*E* shows that Akt1 is rapidly and transiently phosphorylated on Thr<sup>308</sup> in response to SFLLRN, whereas Ser<sup>473</sup> phosphorylation is more delayed. *In vitro* kinase studies demonstrated that Akt1 activity correlated with the rapid phosphorylation of Thr<sup>308</sup>. In contrast, Akt2 activation was slower, correlating with concomitant phosphorylation of Thr<sup>308</sup> and Ser<sup>473</sup> (Fig. 1*F*, numbering of Akt1 phosphorylation sites is used for simplicity; these correspond to Thr<sup>309</sup> and Ser<sup>474</sup> in Akt2). Note that Akt2 Ser<sup>473</sup> phosphorylation results in a band shift, demonstrating that maximal phosphorylation of Akt2 occurs at the later time point (5 min), where a single band was detected in the pThr<sup>308</sup> blot. In contrast to Akt1 and Akt2, we were unable to detect Akt3 Thr<sup>308</sup> phosphorylation and only weak Akt3 Ser<sup>473</sup> phosphorylation in response to the PAR1 peptide, with no concurrent increase in Akt3 activity (Fig. 1*G*). Similar results were found using an alternative Akt3 antibody (62A8) for immunoprecipitation (data not shown), demonstrating that Akt3 is not activated under these conditions. As Akt3 has previously been implicated in thrombin-stimulated GSK3 $\beta$  phosphorylation in mouse platelets (14), we performed similar experiments using thrombin and the PAR4 peptide AYPGKF on human and mouse platelets. Although thrombin and AYPGKF induced Ser<sup>473</sup> phosphorylation in both human and mouse platelets, we were unable to detect any Akt3 Thr<sup>308</sup> phosphorylation, suggesting



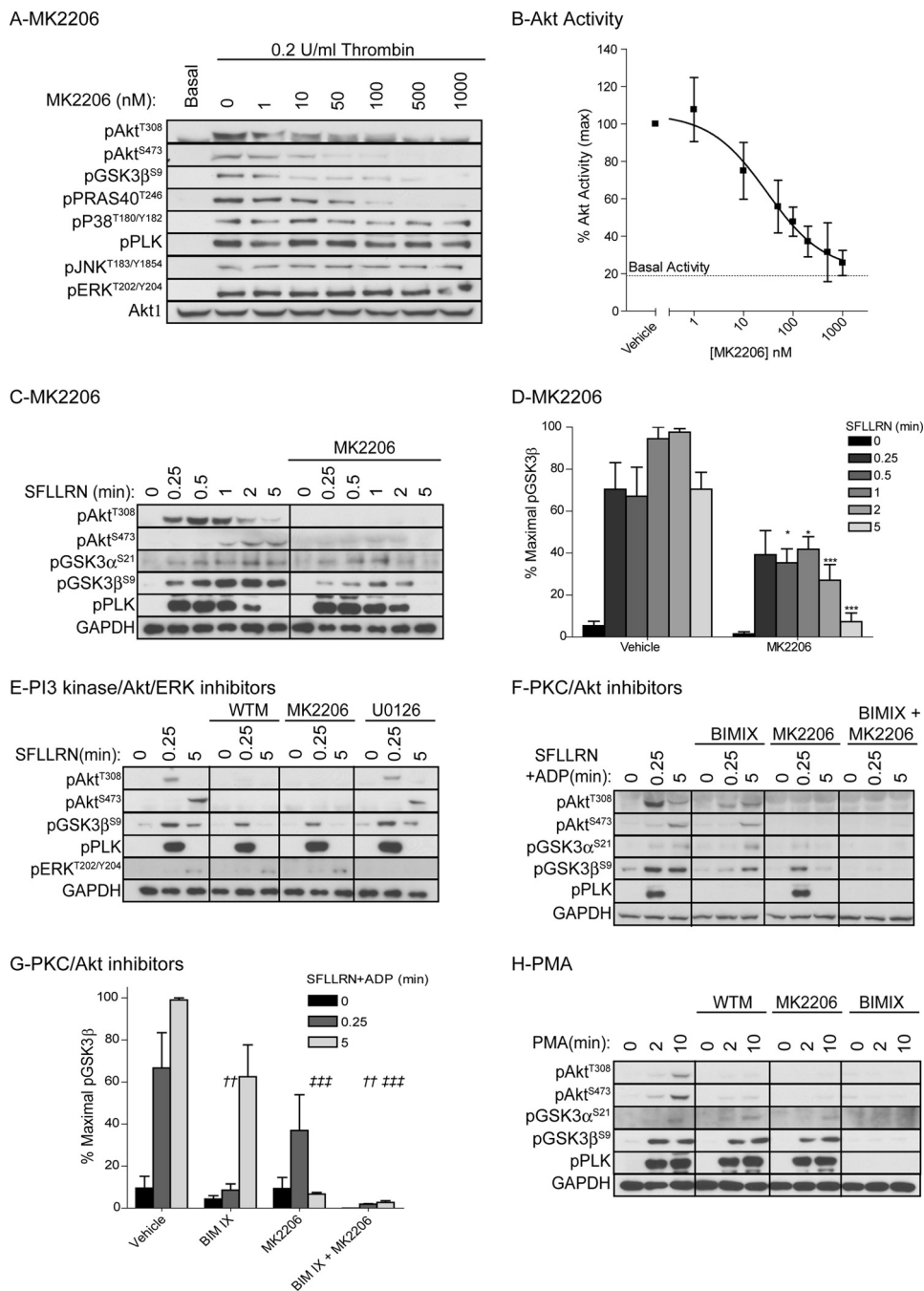
**FIGURE 1. Temporal phosphorylation of different Akt isoforms, GSK3, PRAS40, and pleckstrin in human platelets.** A–D, washed human platelets were stimulated with 0.2 unit/ml thrombin (A), 5  $\mu$ M SFLLRN (B), or 100  $\mu$ M AYPGKF (C) for the indicated time period before extraction in NuPAGE sample buffer and immune blotting with the indicated antibodies (A–C). In addition, mouse brain lysate (positive control) and mouse and human platelet (SW, single washed; DW, double washed) lysates were immunoblotted for Akt3 using two different antibodies (mAb62A8 (i) and pAb (ii) (D)). E–G, alternatively, platelets were stimulated with 5  $\mu$ M SFLLRN for the indicated time period and extracted in Nonidet P-40 extraction buffer followed by immunoprecipitation (IP) of Akt1 (E), Akt2 (F), and Akt3 (G). Akt phosphorylation was analyzed by immunoblotting with the indicated antibodies (i), and *in vitro* Akt activity was analyzed using the peptide substrate RPRAATF (ii). Bar graphs (ii) show activity expressed as pmol of P<sub>i</sub> incorporated per minute for 10<sup>8</sup> platelets (mean  $\pm$  S.E. (error bars), n = 3). H, human (i) and mouse (ii) platelets were stimulated with thrombin (0.2 unit/ml), AYPGKF (100 nM), and SFLLRN (5  $\mu$ M) for 5 min and extracted in Nonidet P-40 followed by immunoprecipitation of Akt3. Akt3 immunoprecipitates (Akt3 IP) and whole cell lysates (WCL) were immunoblotting with the indicated antibodies. Results (A–H) are representative of at least three independent experiments.

that Akt3 is unlikely to be involved in thrombin-mediated GSK3 $\alpha/\beta$  phosphorylation.

*Akt-dependent and Akt-independent Phosphorylation of GSK3 $\beta$* —The SFLLRN time course studies suggested that Akt1 might be involved in GSK3 phosphorylation at early time

points, whereas both Akt1 and Akt2 may be involved at later time points. Because Akt isoform-specific inhibitors are not available, we investigated the role of Akt in GSK3 phosphorylation by incubating platelets with the allosteric Akt inhibitor MK2206. This compound dose-dependently inhibited throm-

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**FIGURE 2. Role for PKC and Akt in PAR1-mediated GSK3 phosphorylation in human platelets.** *A*, washed human platelets were incubated with the indicated concentration of MK2206 for 10 min followed by stimulation with 0.2 unit/ml thrombin for 5 min. Platelets were extracted in NuPAGE sample buffer and immunoblotted with the indicated antibodies. *B*, alternatively, Akt1 was immunoprecipitated and *in vitro* kinase activity determined using the peptide substrate RPRAATF. *In vitro* kinase activity is expressed as a percentage of Akt activity in the presence of vehicle (*B*, mean  $\pm$  S.E. (error bars),  $n = 3$ ). *C–H*, washed human platelets were incubated with vehicle, 1  $\mu$ M MK2206 (*C–H*), 100 nM wortmannin (WT; *E* and *H*), 10  $\mu$ M U0126 (*E*) or 1  $\mu$ M BIMIX (*F–H*) for 10 min before stimulation with 5  $\mu$ M SFLLRN (*C–E*), 5  $\mu$ M SFLLRN + 10  $\mu$ M ADP (*F* and *G*), or 100 nM PMA (*H*) for the indicated time period. Platelets were extracted in NuPAGE sample buffer followed by immunoblotting with the indicated antibodies (*C*, *E*, *F*, and *H*). Results (*A*, *C*, *E*, *F*, and *H*) are representative of at least three independent experiments. The bar graphs (*D* and *G*) depict densitometric analysis of GSK3 $\beta$  phosphorylation of the data shown in *C* and *F*, respectively. Data (mean  $\pm$  S.E.) are expressed as the percentage of maximal GSK3 phosphorylation induced by SFLLRN (*D*,  $n = 5$ ) and SFLLRN + ADP (*G*,  $n = 3$ ). *Star notation* (*D*: \*,  $p < 0.05$ ; \*\*\*,  $p < 0.001$ ) indicates a significant difference between vehicle and MK2206-treated samples at the matching time point (two-way analysis of variance with Bonferroni post test). *Dagger notation* (*G*: ††,  $p < 0.01$ ) indicates a significant difference compared with 0.25-min SFLLRN + ADP treatment in the presence of vehicle, whereas *double dagger notation* (†††:  $p < 0.001$ ) indicates a significant difference compared with 5-min SFLLRN + ADP treatment in the presence of vehicle (two-way analysis of variance with Bonferroni post test).

bin-stimulated Akt phosphorylation and activity with maximal inhibition at 500 nM (Fig. 2, *A* and *B*). MK2206 completely blocked GSK3 $\alpha$ / $\beta$  phosphorylation after a 5-min stimulation with SFLLRN (Fig. 2, *C* and *D*), which is in agreement with our

previous studies using the related compound Akti-1/2 (35, 36). Interestingly, GSK3 $\alpha$ / $\beta$  phosphorylation was only partially reduced 15 s to 2 min after SFLLRN stimulation (Fig. 2, *C* and *D*), under conditions where Akt phosphorylation was absent

(Fig. 2C). Similar results were found using the PI3K inhibitor wortmannin (Fig. 2E, 15 s), showing that early SFLLRN-mediated GSK3 $\alpha/\beta$  phosphorylation is partially independent of the PI3K/Akt pathway.

**Role of PKC $\alpha$  in Early GSK3 $\alpha/\beta$  Phosphorylation**—Alternative candidates to phosphorylate GSK3 independently of PI3K/Akt are PKC (23, 24), PKA (25), and p90RSK (26, 27). Of these, PKA and p90RSK are unlikely to be involved because PKA is not activated in platelets under these conditions, and blocking p90RSK activity using an inhibitor of the upstream kinase MEK (U0126) had no significant effect on GSK3 $\beta$  phosphorylation, despite blocking ERK phosphorylation (Fig. 2E). To examine whether PKC is involved in early GSK3 $\alpha$  and GSK3 $\beta$  phosphorylation, we treated platelets with the PKC inhibitor bisindolylmaleimide IX (BIMIX). Because PKC inhibition blocks secretion of ADP, an important co-factor in PAR-mediated Akt activation (37), platelets were stimulated with SFLLRN in the presence of ADP. BIMIX blocked SFLLRN-mediated GSK3 $\alpha/\beta$  phosphorylation 15 s after stimulation and blocked residual phosphorylation in the presence of MK2206 (Fig. 2, F and G), suggesting that PKC contributes to early GSK3 $\alpha/\beta$  phosphorylation.

The ability of PKC to phosphorylate GSK3 $\alpha/\beta$  was further confirmed in experiments using the PKC activator phorbol 12-myristate-13-acetate (PMA). PMA robustly stimulated pleckstrin and GSK3 $\alpha/\beta$  phosphorylation in a PKC-dependent, PI3K/Akt-independent, manner (Fig. 2H). One of the candidate PKC isoforms that plays an important role in platelet activation and granule secretion is PKC $\alpha$  (1). We next evaluated whether GSK3 $\alpha/\beta$  phosphorylation is affected in platelets from PKC $\alpha$  knock-out mice. Fig. 3A shows the phosphorylation events in mouse platelets in response to thrombin. Interestingly, GSK3 $\alpha/\beta$  phosphorylation is clearly detectable within 15 s of thrombin stimulation, whereas Akt Thr<sup>308</sup> and Ser<sup>473</sup> phosphorylation are more delayed. PKC $\alpha$ -deficient platelets (Fig. 3B) show a significant delay in GSK3 $\alpha/\beta$  phosphorylation, demonstrating that PKC $\alpha$  contributes to early GSK3 $\alpha/\beta$  phosphorylation. In contrast, wortmannin (Fig. 3C) and MK2206 (Fig. 3, D and E) have no effect on early GSK3 $\alpha/\beta$  phosphorylation, but strongly reduce later GSK3 $\alpha/\beta$  phosphorylation in mouse platelets. The residual GSK3 $\alpha/\beta$  phosphorylation present in platelets from PKC $\alpha$  KO animals is blocked by wortmannin and MK2206, confirming that both PKC $\alpha$  and PI3K/Akt contribute to thrombin-stimulated GSK3 $\alpha/\beta$  phosphorylation (Fig. 3, C–E).

**Thrombin-mediated Inhibition of GSK3 $\alpha/\beta$  Activity Is Dependent on PKC $\alpha$  and Akt**—To evaluate the effect of phosphorylation on GSK3 $\alpha/\beta$  activity; we performed *in vitro* GSK3 $\alpha/\beta$  activity assays on platelet extracts. Thrombin stimulation resulted in a time-dependent reduction in GSK3 $\alpha/\beta$  activity (Fig. 3F). The Akt inhibitor MK2206 strongly reduced thrombin-mediated GSK3 $\alpha/\beta$  inhibition at the later (5 min) time point, whereas PKC $\alpha$  KO platelets showed impaired thrombin-mediated GSK3 $\alpha/\beta$  inhibition at the early (15 s) time point. Furthermore, MK2206 largely prevented thrombin-mediated inhibition of GSK3 $\alpha/\beta$  activity in PKC $\alpha$  KO platelets. Together these results demonstrate that phosphorylation and

inhibition of GSK3 $\alpha/\beta$  in platelets are dependent mainly on PKC $\alpha$  and Akt.

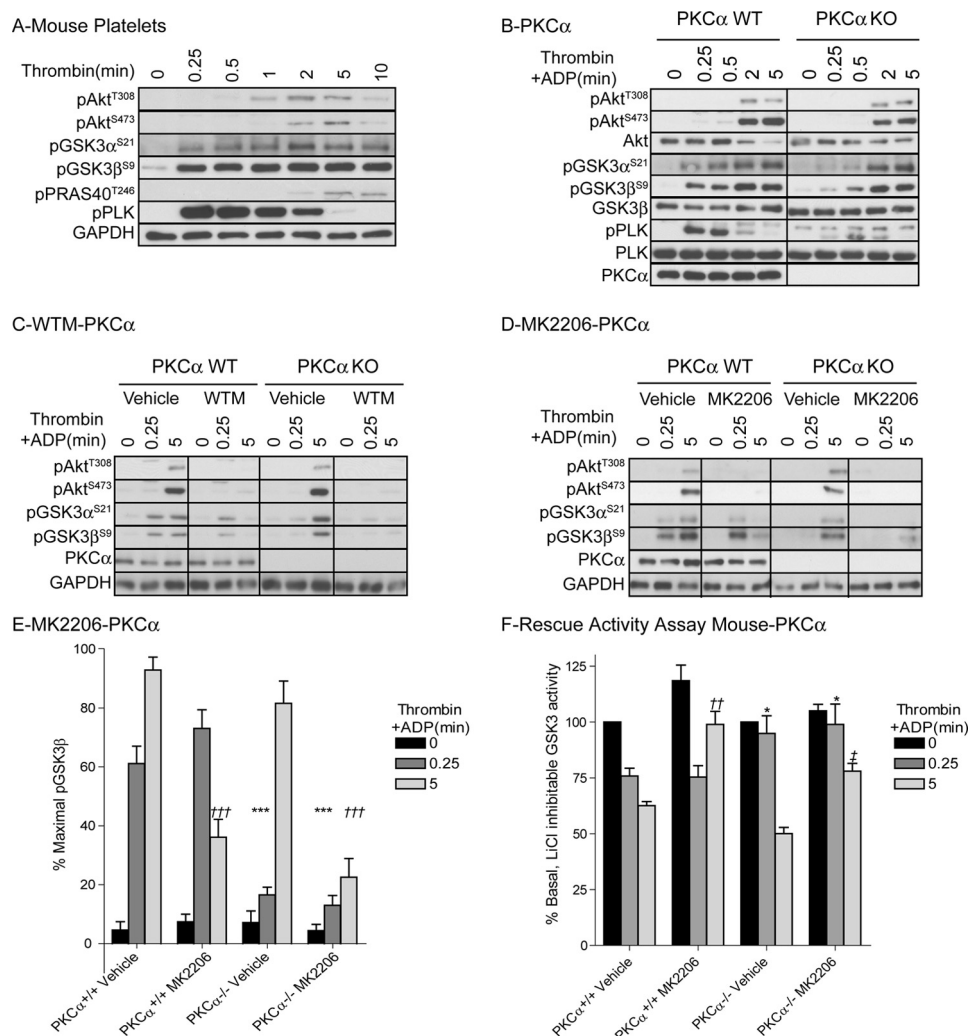
**Phosphorylation of GSK3 $\alpha/\beta$  by Akt and PKC $\alpha$  Contributes to Platelet Function**—To directly link phosphorylation of GSK3 to platelet function, we performed functional studies on platelets from knock-in (KI) mice where the inhibitory Ser phosphorylation site on GSK3 $\alpha$  (Ser<sup>21</sup>) and GSK3 $\beta$  (Ser<sup>9</sup>) were mutated to Ala. Western blotting confirmed that thrombin-stimulated phosphorylation of GSK3 $\alpha$  and GSK3 $\beta$  is absent in platelets derived from GSK3 KI mice (Fig. 4A). Thrombin-stimulated aggregation of GSK3 KI platelets was significantly reduced compared with wild type (Fig. 4B). Furthermore, the concentration-response curve for PAR4-mediated fibrinogen binding was shifted to the right (Fig. 4C). Similar results were found for P-selectin expression, a marker for  $\alpha$ -granule secretion (Fig. 4D). The reduction in functional responses was not due to reduced expression of key pathway components, as levels of integrin  $\beta_3$ , P-selectin and PAR4 were normal in GSK3 KI mice (Fig. 4E). These results together demonstrate that phosphorylation of GSK3 contributes to platelet activation by thrombin. To confirm that inhibition of GSK3 $\alpha/\beta$  indeed results in increased platelet function, we performed additional experiments with the GSK3 $\alpha/\beta$  inhibitor CHIR99021 (also named CT-99021), which has improved specificity over compounds used previously in platelets (36, 38). CHIR99021 dose-dependently inhibited GSK3 $\alpha/\beta$  activity platelets (Fig. 5A), which was associated with significantly increased aggregation (Fig. 5, B and D) and ATP secretion (Fig. 5, C and E) of murine and human platelets, respectively, confirming a negative role for GSK3 $\alpha/\beta$  in thrombin-mediated platelet function. Interestingly, platelet aggregation in response to collagen-related peptide was unchanged in the presence of CHIR99021 (Fig. 5, F and G).

## DISCUSSION

In this study we investigated the role and regulation of GSK3 $\alpha/\beta$  phosphorylation in platelet function by using a combination of genetic and pharmacological approaches. We demonstrate for the first time that GSK3 $\alpha/\beta$  is phosphorylated and regulated by two distinct kinases, PKC $\alpha$  and Akt, and that this phosphorylation event contributes to PAR-mediated platelet activation. The role of the kinases shows differential temporal kinetics with PKC $\alpha$  supporting early, rapid GSK3 $\alpha/\beta$  phosphorylation and Akt taking over at later time points.

Pharmacological inhibitor studies showed that early PAR1-mediated GSK3 $\alpha/\beta$  phosphorylation was only mildly affected by the PI3K inhibitor wortmannin and the Akt inhibitor MK2206, suggesting that another kinase may be involved. Previous literature suggests that PKC (23, 24), PKA (25), and p90RSK (26, 27), of which PKC and p90RSK are activated upon platelet activation, can also contribute to GSK3 phosphorylation under certain conditions. Blocking p90RSK activation using a MEK inhibitor, however, had no effect on GSK3 $\alpha/\beta$  phosphorylation, ruling out a role for p90RSK. In contrast, early GSK3 $\alpha/\beta$  phosphorylation was blocked by the PKC inhibitor BIMIX, suggesting that PKC, rather than Akt, is involved in GSK3 $\alpha/\beta$  phosphorylation. This was furthermore confirmed

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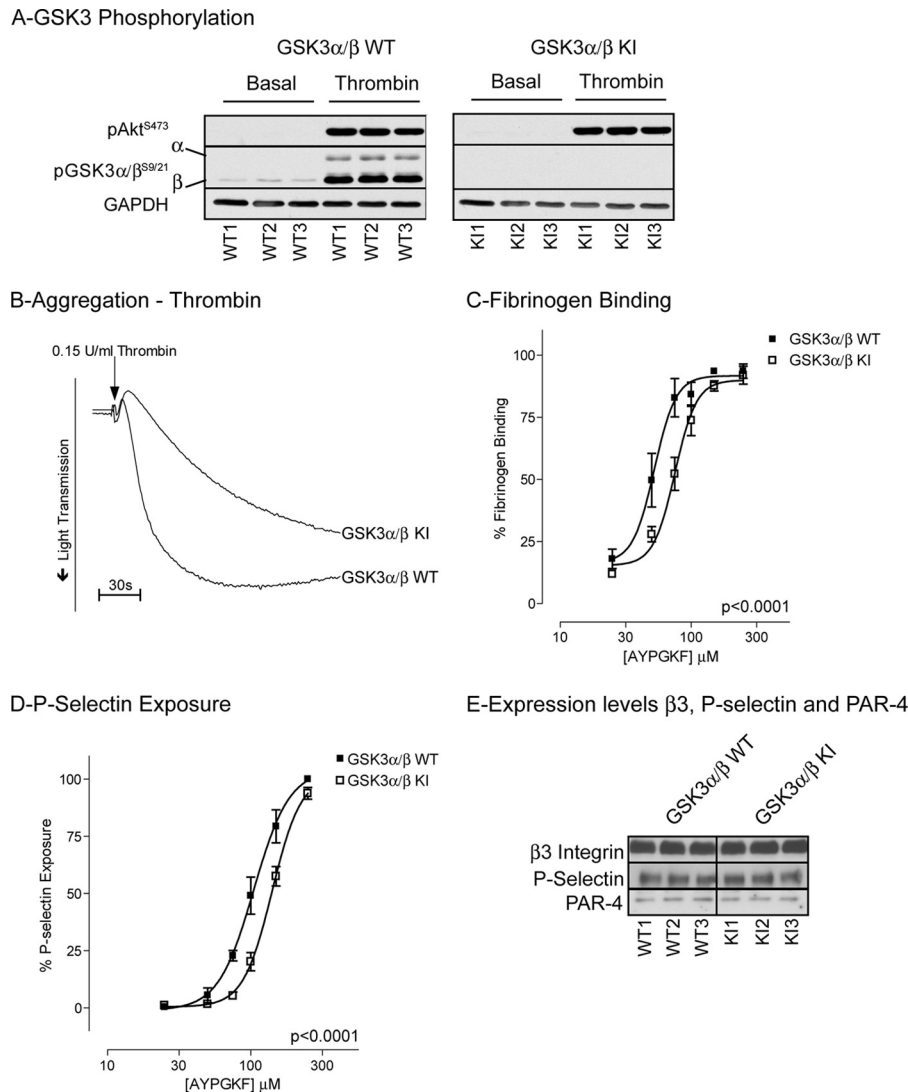


**FIGURE 3. Thrombin-stimulated GSK3 phosphorylation and inhibition of GSK3 $\alpha/\beta$  activity is delayed in platelets from PKC $\alpha$  KO mice.** Washed PKC $\alpha$ <sup>+/+</sup> (PKC $\alpha$  WT, A–F) and PKC $\alpha$ <sup>-/-</sup> (PKC $\alpha$  KO, B–F) mouse platelets were stimulated with 0.2 unit/ml thrombin (A) or 0.2 unit/ml thrombin + 10  $\mu$ M ADP (B–F) for the indicated times. Platelets were extracted in NuPAGE sample buffer followed by immunoblotting with the indicated antibodies (A–D). Results (A–D) are representative of at least three independent experiments. The bar graph (E) depicts densitometric analysis of GSK3 $\beta$  phosphorylation of the data shown in D. Data (mean  $\pm$  S.E. (error bars),  $n = 5$ ) are expressed as the percentage of maximal GSK3 phosphorylation induced by thrombin+ADP in PKC $\alpha$  WT mouse platelets. Star notation (E: \*\*\*,  $p < 0.001$ ) indicates a significant difference compared with 0.25-min thrombin+ADP treatment in vehicle-treated PKC $\alpha$  WT platelets whereas dagger notation (E: †††,  $p < 0.001$ ) indicates a significant difference compared with 5-min thrombin+ADP treatment in vehicle-treated PKC $\alpha$  WT platelets. Alternatively, platelets were extracted in Nonidet P-40 (F) followed by analysis of *in vitro* GSK3 activity using the substrate peptide RRAEELD-SRAGS(P)PQL. *In vitro* kinase activity is expressed as a percentage of GSK3 activity obtained in the absence of thrombin and ADP (mean  $\pm$  S.E.,  $n = 5$ ). Star notation (F: \*,  $p < 0.05$ ) indicates a significant difference compared with 0.25-min thrombin+ADP treatment in vehicle-treated PKC $\alpha$  WT platelets; dagger notation (††,  $p < 0.01$ ) indicates a significant difference compared with 5-min thrombin+ADP treatment in vehicle-treated PKC $\alpha$  WT platelets, whereas double dagger notation (‡,  $p < 0.01$ ) indicates a significant difference compared with 5-min thrombin+ADP treatment in vehicle-treated PKC $\alpha$  KO platelets.

by the ability of the PKC activator PMA to stimulate GSK3 $\alpha/\beta$  phosphorylation in a PKC-dependent, Akt-independent manner. Interestingly, there was a clear temporal discrepancy between GSK3 $\alpha/\beta$  phosphorylation (<15 s) and Akt (Thr<sup>308</sup> and Ser<sup>473</sup>) phosphorylation (1–2 min) in mouse platelets, further supporting the hypothesis that an alternative kinase is involved in early GSK3 $\alpha/\beta$  phosphorylation. Indeed, this kinase is likely to be PKC $\alpha$ , as early thrombin-mediated GSK3 $\alpha/\beta$  phosphorylation was absent in PKC $\alpha$ <sup>-/-</sup> mouse platelets. These findings are supported by a recent study by Li *et al.* (22), reporting that thrombin-mediated GSK3 $\beta$  phosphorylation in platelets is partly PI3K-independent.

The lack of Akt isoform-specific inhibitors makes it challenging to determine which Akt isoforms are involved in GSK3 phosphorylation in human platelets. Previous studies by our

group and others have reported the phosphorylation and activation of Akt1 and Akt2 isoforms in human platelets (11, 12, 39). In addition, a recent study suggested that the Akt3 isoform is also expressed in human and mouse platelets and significantly contributes to GSK3 $\beta$  phosphorylation and thrombin-stimulated platelet function (14). In the present study, we therefore analyzed the temporal activation of all three Akt isoforms to determine their potential contribution to GSK3 $\alpha/\beta$  phosphorylation in human platelets. Although we confirmed that Akt3 was expressed in both human and mouse platelets, we were unable to detect Akt3 Thr<sup>308</sup> phosphorylation and Akt3 activity in human platelets in response to the PAR1 peptide SFLLRN, the PAR4 peptide AYPGKF and thrombin, suggesting that Akt3 is unlikely to contribute to GSK3 $\alpha/\beta$  phosphorylation under these conditions.



**FIGURE 4. Reduced platelet aggregation, fibrinogen binding, and P-selectin exposure in GSK3 $\alpha/\beta$  KI platelets.** *A*, washed GSK3 $\alpha^{+/+}/\beta^{+/+}$  (GSK3 $\alpha/\beta$  WT) and GSK3 $\alpha^{S21A/S21A}/\beta^{S9A/S9A}$  (GSK3 $\alpha/\beta$  KI) platelets were stimulated with 0.15 unit/ml thrombin for 5 min before extraction in NuPAGE sample buffer and immunoblot analysis with the indicated antibodies. *B*, platelets were stimulated with 0.15 unit/ml thrombin under stirring conditions and aggregation monitored for 5 min. *C* and *D*, a representative aggregation curve is presented for three independent experiments. Washed platelets were incubated with the indicated concentrations of PAR4 agonist AYPGKF-NH<sub>2</sub> for 15 min in the presence of FITC-fibrinogen (*C*) or PE-anti-P-selectin Ab (*D*). Samples were fixed in 4% paraformaldehyde and analyzed by flow cytometry (*C* and *D*). Results are expressed as mean  $\pm$  S.E. (*error bars*; *n* = 6) of percentage of maximal fibrinogen binding to GSK3 $\alpha/\beta$  WT platelets. *C* and *D*, significance testing using GraphPad Prism 4.0 determined that different curve fittings were required for GSK3 $\alpha/\beta$  WT and GSK3 $\alpha/\beta$  KI data. *E*, washed GSK3 $\alpha/\beta$  WT and GSK3 $\alpha/\beta$  KI platelets were lysed in NuPAGE sample buffer and receptor expression levels determined by immunoblotting with the indicated antibodies.

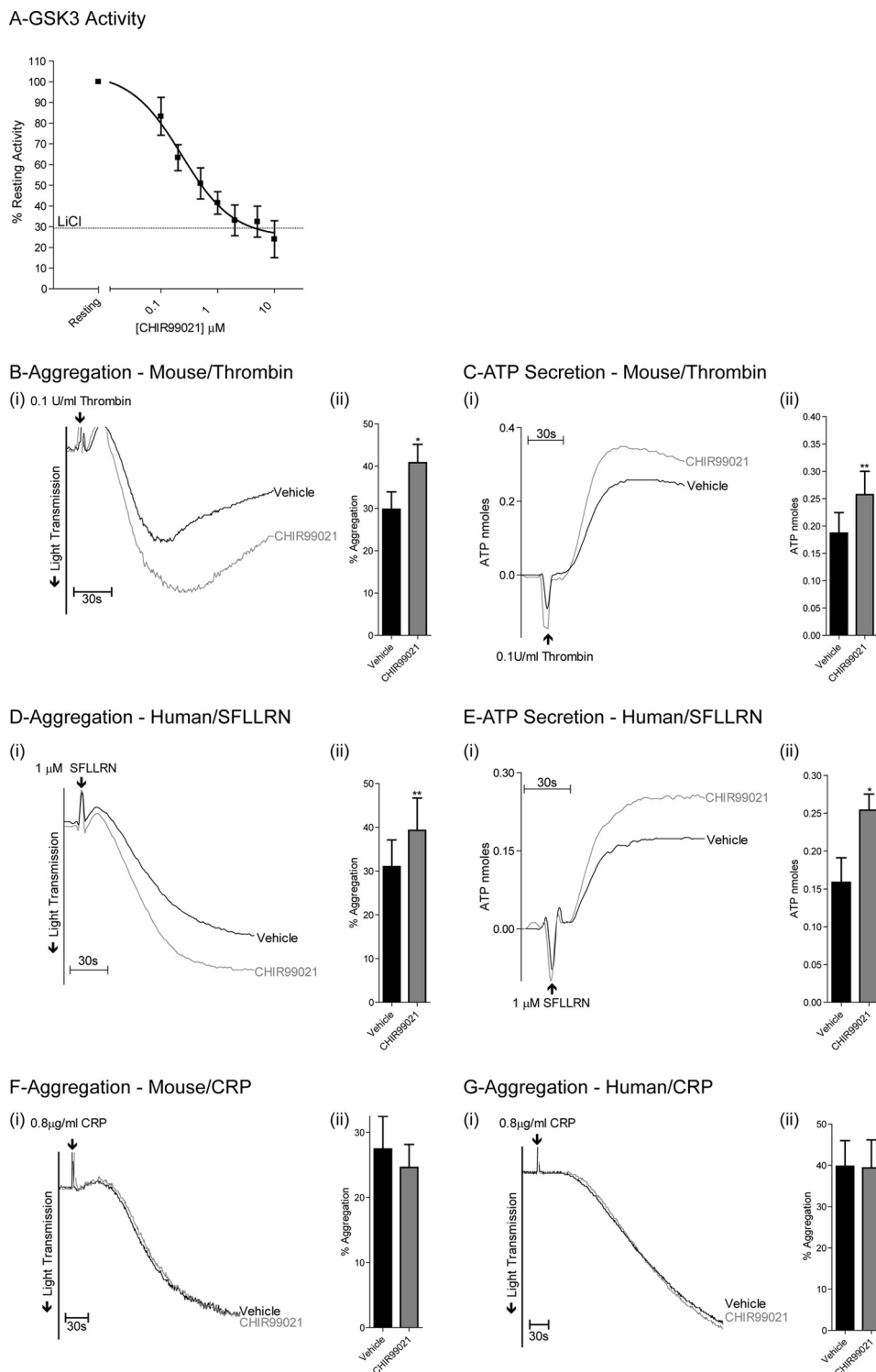
In contrast, Akt1 was very rapidly and transiently activated by SFLLRN, whereas Akt2 activation was more delayed and maximal 5 min after stimulation. These results raised the possibility that Akt1 contributes to early (15 s) GSK3 $\alpha/\beta$  phosphorylation, whereas Akt2 may be involved in later (5 min) GSK3 $\alpha/\beta$  phosphorylation. However, both Akt1 and Akt2 are active at the later time point, and a contribution of either isoform to GSK3 $\alpha/\beta$  phosphorylation can therefore not be ruled out. Indeed, maximal Akt1 activity detected in human platelets was approximately three times higher than maximal Akt2 activity, a finding that corresponds to the relative Akt mRNA expression in human platelets (39). Our results using the PAR1 peptide SFLLRN also revealed that Thr<sup>308</sup> phosphorylation detected in total lysate is very rapid and correlates closely to

Akt1, but not Akt2 phosphorylation, suggesting that Akt1 is the main isoform detected in total lysate.

Interestingly, there was also a clear difference in the kinetics of thrombin-stimulated Akt Thr<sup>308</sup> phosphorylation between mouse and human platelets. In human platelets, Thr<sup>308</sup> was very rapidly phosphorylated (<15 s), whereas in mouse platelets this was much more delayed (>1–2 min). We found that an antagonist of PAR1 (a receptor not expressed on mouse platelets) blocked early thrombin-stimulated Akt Thr<sup>308</sup> and pleckstrin phosphorylation in human platelets (data not shown), suggesting that PAR1 mediates the rapid thrombin-mediated activation of Akt1 and PKC in human platelets. This is in agreement with our finding that Akt Thr<sup>308</sup> and pleckstrin phosphorylation in response to the PAR4 peptide AYPGKF



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**FIGURE 5. Inhibiting GSK3 $\alpha/\beta$  activity in mouse and human platelets increases thrombin-mediated platelet aggregation and ATP secretion.** *A*, washed human platelets were incubated with the indicated concentrations of the GSK3 $\alpha/\beta$  inhibitor CHIR99021 for 10 min, extracted in Nonidet P-40, and *in vitro* GSK3 $\alpha/\beta$  activity was analyzed using the substrate peptide RRAAEELDSRAGS(P)PQL. *In vitro* kinase activity is expressed as a percentage of GSK3 $\alpha/\beta$  activity obtained in the absence of CHIR99021 (*A*, mean  $\pm$  S.E. (error bars)  $n = 3$ ). (i), washed mouse (*B*, *C*, and *F*) and human (*C*, *D*, and *G*) platelets were stimulated with 0.1 unit/ml thrombin (*B* and *C*), 1  $\mu$ M SFLLRN (*D* and *E*), or 0.8  $\mu$ g/ml collagen-related peptide (CRP) (*F* and *G*). Aggregation (*B*, *D*, *F*, and *G*) and ATP secretion (*C* and *E*) were subsequently recorded for 5 min. Traces are representative of four independent experiments. (ii), the bar graphs (*B*–*G*) indicate the average increase in aggregation and ATP secretion (nmoles, mean  $\pm$  S.E.,  $n = 4$ ). \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  (Student's paired *t* test).

was delayed in human platelets. The early PKC-mediated GSK3 phosphorylation in human platelets is therefore dependent on PAR1, whereas in mouse platelets it is dependent on PAR4. This reflects the relative importance of these

receptors in thrombin-mediated platelet activation in human *versus* mouse platelets.

Like human platelets, murine platelets also express all three Akt isoforms. Studies on platelets from Akt1<sup>+/-</sup> Akt2<sup>-/-</sup> mice

and Akt3<sup>-/-</sup> showed a mild reduction in thrombin-stimulated GSK3 $\beta$  phosphorylation in the absence of these isoforms, suggesting any of the isoforms can contribute to GSK3 $\beta$  phosphorylation (14, 22). The relatively small effect of Akt isoform deficiency on GSK3 $\beta$  phosphorylation may therefore be explained by partial redundancy between the Akt isoforms, as well as contribution of PKC $\alpha$  to early GSK3 $\beta$  phosphorylation. Interestingly, a recent study on neutrophils also described a role for phospholipase C/PKC and PI3K/Akt pathway in GSK3 $\alpha/\beta$  phosphorylation (40), suggesting that dual regulation of GSK3 $\alpha/\beta$  by PKC and Akt may be a more general mechanism in hematopoietic cells.

In many cell types, phosphorylation of GSK3 on Ser<sup>21</sup> (GSK3 $\alpha$ ) and Ser<sup>9</sup> (GSK3 $\beta$ ) results in a reduction in GSK3 activity of approximately 20–50%. Here, we confirmed that thrombin stimulation of platelets results in a temporal reduction in GSK3 $\alpha/\beta$  activity in wild type murine platelets. Early (15 s) inhibition, but not late (5 min) inhibition of GSK3 $\alpha/\beta$  activity was completely absent in PKC $\alpha$ -deficient platelets, confirming a role for PKC $\alpha$  in regulating early GSK3 $\alpha/\beta$  phosphorylation and activity. In contrast, late thrombin-mediated inhibition of GSK3 $\alpha/\beta$  was largely prevented by the Akt inhibitor MK2206 in both wild type and PKC $\alpha$ -deficient platelets, supporting a role for Akt in late inhibition of GSK3 $\alpha/\beta$  activity. There was some residual inhibition in GSK3 $\alpha/\beta$  activity in the presence of the Akt inhibitor, suggesting that regulation by an alternative kinase cannot be ruled out completely.

Platelets from GSK3 KI mice bearing nonphosphorylatable alanine at Ser<sup>21</sup>/Ser<sup>9</sup> of GSK3 $\alpha/\beta$  exhibited significantly reduced aggregation, fibrinogen binding, and P-selectin exposure, demonstrating for the first time that GSK3 $\alpha/\beta$  phosphorylation contributes to thrombin-mediated platelet function. As aggregation and secretion are rapid platelet responses that happen in the time frame of PKC-mediated GSK3 phosphorylation, Akt-mediated GSK3 phosphorylation in mouse platelets may be of less functional significance. However, we cannot rule out the possibility that Akt-mediated GSK3 regulation contributes to consolidation of platelet aggregation and thrombus formation.

The GSK3 inhibitor CHIR99021 increased thrombin/PAR-mediated aggregation in mouse and human platelets, showing that a reduction in GSK3 activity supports thrombin-mediated platelet function, a finding in agreement with previous reports (14, 22). Interestingly, we found no effect of CHIR99021 on collagen-related peptide-mediated platelet aggregation, suggesting that the role of GSK3 may be limited to thrombin or G protein-mediated platelet function. In contrast to our results, two reports described an inhibitory effect of GSK3 inhibitors on collagen/collagen-related peptide-mediated aggregation (14, 15). This discrepancy may be explained by the higher specificity of the compound used in our study (36, 38).

It is at present unknown how GSK3 may regulate platelet function in response to thrombin. There are nearly 100 proposed GSK3 substrates described in literature, but for many of these substrates, *in vivo* phosphorylation by GSK3 and its effect on substrate function have not been confirmed (32). Indeed, no GSK3 substrates have thus far been positively identified in platelets. Future identification of GSK3 substrates may provide

more insight in how GSK3 may negatively regulate thrombin-mediated platelet function. In conclusion, our data demonstrate that PKC $\alpha$  and Akt promote thrombin-stimulated platelet function by phosphorylating and inhibiting GSK3 $\alpha/\beta$ , thereby increasing platelet function.

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