Title

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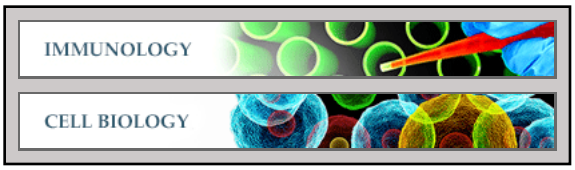

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Pro-inflammatory sPLA-IIA induces integrin activation through direct binding to a newly identified binding-site (site 2) in integrins αvβ3, α4β1, and α5β1.

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Running title: *sPLA2-IIA activates integrins*

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Background: Besides inside-out signaling, integrins are activated by the binding of fractalkine to a newly identified binding site (site 2).
Results: sPLA2-IIA induced integrin activation through site 2. A peptide from site 2 or a small compound that bind to sPLA2-IIA suppressed the activation.
Conclusion: sPLA2-IIA activates integrins through direct binding to site 2.
Significance: Integrin activation through site 2 may be a potential therapeutic target in inflammation.

ABSTRACT

Integrins are activated by signaling from inside the cell (inside-out signaling) through global conformational changes of integrins. We recently discovered that fractalkine activates integrins in the absence of CX3CR1 through the direct binding of fractalkine to a ligand-binding site in the integrin headpiece (site 2) that is distinct from the classical RGD-binding site (site 1). We propose that fractalkine binding to the newly identified site 2 induces activation of site 1 though conformational changes (in an allosteric mechanism). We reasoned that site 2-mediated activation of integrins is not limited to fractalkine. Human secreted phospholipase A2 type IIA (sPLA2-IIA), a pro-inflammatory protein, binds to integrins αvβ3 and α4β1 (site 1) and this interaction initiates a signaling pathway that leads to cell proliferation and inflammation. Human sPLA2-IIA does not bind to M-type receptor very well. Here we describe that sPLA2-IIA directly activated purified soluble integrin αvβ3 and transmembrane αvβ3 on the cell surface. This activation did not require catalytic activity or M-type receptor. Docking simulation predicted that sPLA2-IIA binds to site 2 in the closed-headpiece of αvβ3. A peptide from site 2 of integrin β1 specifically bound to sPLA2-IIA and suppressed sPLA2-IIA-induced integrin activation. This suggests that sPLA2-IIA activates αvβ3 through binding to site 2. sPLA2-IIA also activated integrins α4β1 and α5β1 in a site 2-mediated manner. We recently identified small compounds that bind to sPLA2-IIA and suppress integrin-sPLA2-IIA interaction (e.g., Cmpd21). Cmpd21 effectively suppressed sPLA2-IIA-induced integrin activation. These results define a novel mechanism of pro-inflammatory action of sPLA2-IIA through integrin activation.

Secreted PLA2 type IIA (sPLA2-IIA) was first isolated and purified from rheumatoid synovial fluid (1). sPLA2-IIA is an acute phase reactant and its plasma concentration markedly increases in diseases that involve systemic inflammation such as sepsis, rheumatoid arthritis, and cardiovascular disease (up to 1000-fold and > 1 μg/ml). Inflammatory cytokines such as IL-6, TNF-α, and IL-1β induce synthesis and release of sPLA2-IIA in arterial smooth muscle cells and hepatocytes, which are the major sources of the plasma sPLA2-IIA in these systemic inflammatory conditions (2,3). In addition to being a pro-inflammatory protein, sPLA2-IIA expression is elevated in neoplastic prostatic tissue (4) and dysregulation of sPLA2-IIA may play a role in prostatic carcinogenesis (5), and is a potential therapeutic target in prostate cancer (6).

Notably some biological effects associated with sPLA2-IIA are independent of its catalytic function (7). Catalytically inactive sPLA2-IIA mutants retains the ability to enhance cyclooxygenase-2 expression in connective tissue mast cells (7). Also inactivation of sPLA2-IIA by bromophenacyl bromide does not affect the ability of sPLA2-IIA to induce secretion of β-glucuronidase, IL-6, and IL-8 from human eosinophils (8). It has thus been proposed that sPLA2-IIA action is mediated through interaction with specific receptors. Indeed the enzyme binds to a high affinity receptor of 180 kDa present on rabbit skeletal muscle (9). This so-called M (muscle)-type receptor belongs to the superfamily of C-type lectins and mediates some of the physiological effects of mammalian sPLA2-IIA, and binding of sPLA2-IIA to this receptor induces internalization of sPLA2-IIA (10). However, the interaction between sPLA2-IIA and the M-type receptor is species-specific, and human sPLA2-IIA binds to the human or mouse M-type receptor very weakly (11).

Integrins are a family of cell adhesion receptors that recognize ECM ligands and cell surface ligands (12). Integrins are transmembrane heterodimers, and at least 18 α and 8 β subunits are known (13). Integrins transduce signals to the cell upon ligand binding (12). We previously reported that sPLA2-IIA binds to integrins αvβ3 and α4β1 and induces proliferative signals in an integrin-dependent manner. sPLA2-IIA specifically binds to integrin αvβ3 and α4β1 (14). The integrin-binding site does not include the catalytic center or the M-type receptor-binding site. WT and the catalytically inactive mutant (the H47Q mutant) of sPLA2-IIA induces intracellular signals in monocytic cells, but an integrin-binding defective mutant (the R74E/R100E mutant) does not (14). These results suggest that integrins may serve as receptors for sPLA2-IIA and mediate pro-inflammatory action of sPLA2-IIA in human. We screened small compounds that bind to sPLA-IIA and inhibit integrin binding. We obtained several compounds and compound 21 (**Cmpd21**) suppresses αvβ3-mediated cell adhesion and migration (15). These findings indicate direct binding of sPLA2-IIA to integrins is critical for pro-inflammatory actions of sPLA2-IIA.

It has been proposed that integrin activation is mediated by signaling from inside the cell (inside-out signaling), and that integrin activation is associated with global conformational changes of the integrin molecule (16,17). We recently discovered that the chemokine domain of fractalkine (FKN-CD) directly binds to several integrins and this interaction is critical for fractalkine/CX3CR1 signaling (18). FKN-CD induces ternary complex formation (integrin-FKN-CD-CX3CR1) on the cell surface, suggesting that integrins act as co-receptor for FKN-CD in FKN/CX3CR1 signaling (18). Notably we discovered that FKN-CD can activate integrins in the absence of CX3CR1 through direct binding to integrins probably in an allosteric mechanism (19). We identified a new FKN-CD-binding site in integrins (site 2) that is distinct from the classical RGD-binding site (site 1). The position of site 2 was predicted by docking simulation of interaction between FKN-CD and integrin αvβ3 that has a closed-headpiece conformation. This is based on the premise that site 2 is open in the closed-headpiece αvβ3. A peptide from site 2 (residues 267-286 of β3) directly binds to FKN-CD and suppresses FKN-CD-induced integrin activation (19). We thus propose a model, in which FKN-CD binding to site 2 induces activation of site 1 though conformational changes (in an allosteric mechanism).

The site 2-mediated activation of integrins

may not be limited to FKN-CD. In the present paper we describe that sPLA2-IIA directly activates integrins (αvβ3, α4β1, and α5β1) in cell-free conditions and/or on the cell surface. sPLA2-IIA mutants that are catalytically inactive or defective in binding to M-type receptor still activate integrins, while the integrin-binding defective mutant did not. This suggests that direct integrin binding is required, but catalytic activity or M-type receptor is not. sPLA2-IIA is predicted to bind to site 2 in αvβ3 in a closed-headpiece conformation in docking simulation. Consistently, we obtained evidence that a peptide from site 2 effectively suppressed the sPLA-IIA-induced integrin activation, suggesting that this activation involves the binding of sPLA-IIA to site 2. **Cmpd21** effectively suppressed sPLA2-IIA-induced integrin activation. These results define a novel mechanism of pro-inflammatory action of sPLA2-IIA through integrin activation.

EXPERIMENTAL PROCEDURES

Materials-U937 monocytic cells and Chinese hamster ovary (CHO) cells were obtained from the American Type Culture Collection. K562 erythroleukemia cells that express human integrin αvβ3 (αvβ3-K562) (20) were provided by Eric Brown (University of California, San Francisco, CA). K562 cells that express human integrin α4 (α4-K562), CHO cells that express human integrin β3 (β3-CHO) or integrin α4 (α4-CHO) were described (14). Recombinant soluble αvβ3 was synthesized in CHO-K1 cells using the soluble αv and β3 expression constructs and purified by Ni-NTA affinity chromatography as described (21). Fibrinogen γ-chain C-terminal domain that lacks residues 400–411 (γC399tr) was synthesized as described (22). Fibronectin H120 fragment (FN-H120) (18), Fibronectin type III domains 8–11 (FN8-11) (19), and ADAM15 (23) were synthesized as GST fusion proteins as described in the cited references. Anti-human β3 mAb AV10 was provided by B. Felding-Habermann (The Scripps Research Institute, La Jolla, CA). HRP-conjugated anti-His tag antibody was purchased from Qiagen (Valencia, CA). **Cmpd21** was synthesized as described (15).

Synthesis of sPLA2-IIA-Recombinant sPLA2-IIA proteins (WT and mutants) were synthesized as described (14) using PET28a expression vector. The proteins were synthesized in E. coli BL21 and induced by isopropyl β-D-thiogalactoside as insoluble proteins. The proteins were solubilized in 8 M urea, purified by Ni-NTA affinity chromatography under denatured conditions, and refolded as previously described (14). The refolded proteins were >90% homogeneous upon SDS-PAGE.

Synthesis of site 2 peptides-We introduced 6His tag to the BamHI site of pGEX-2T using 5'-GATCTCATCATCACCATCACCATG-3' and 5'-GATCCATGGTGATGGTGATGATGA-3' (resulting vector is designated pGEX-2T6His). We synthesized GST fusion protein of site 2 peptide (QPNDGQSHVGSDNHYSASTTM, residues 267-287 of β3, C273 is changed to S) and a scrambled site 2 peptide (VHDSHYSGQGAMSDNTNSPQT) by subcloning oligonucleotides that encodes these sequences into the BamHI/EcoRI site of pGEX-2T6His. We synthesized the proteins in E. coli BL21 and purified using glutathione-Sepharose affinity chromatography (18). The corresponding β1, β2, and β4 peptides were generated as described (18).

Binding of soluble αvβ3 to γC399tr-ELISA-type binding assays were performed as described previously (18). Briefly, wells of 96-well Immulon 2 microtiter plates (Dynatech Laboratories, Chantilly, VA) were coated with 100 µl 0.1 M $NaHCO_3$ containing γC399tr or ADAM15 for 2 h at 37°C. Remaining protein binding sites were blocked by incubating with PBS/0.1% BSA for 30 min at room temperature. After washing with PBS, soluble recombinant αvβ3 (5 µg/ml) in the presence or absence of sPLA2-IIA (WT or mutants) was added to the wells and incubated in HEPES-Tyrodes buffer (10 mM HEPES, 150 mM NaCl, 12 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 2.5 mM KCl, 0.1% glucose, 0.1% BSA) with 1 mM $CaCl_2$ for 2 h at room temperature. After unbound αvβ3 was removed by rinsing the wells with binding buffer, bound αvβ3 was measured using anti-integrin β3 mAb (AV-10) followed by HRP-conjugated goat anti-mouse IgG and peroxidase substrates.

Binding of labeled ligands to integrins on the cell surface-The cells were cultured to nearly confluent in RPMI 1640/10% FCS (K562 and U937) or DMEM/10% FCS (CHO cells). The cells were resuspended with RPMI 1640/0.02% BSA or DMEM/0.02% BSA and incubated for 30 min at room temperature to block remaining protein binding sites. The cells were then incubated with WT sPLA2-IIA or mutants for 5 min at room temperature and then incubated with FITC-labeled integrin ligands (γC399tr, FN-H120, FN8-11, and ADAM15) for 15 min at room temperature. For blocking experiments, sPLA-IIA was preincubated with S2-β1 peptide for 30 min at room temperature. The cells were washed with PBS/0.02% BSA and analyzed by FACSCalibur (Becton Dickinson, Mountain View, CA). For inhibition studies using **Cmpd21,** sPLA2-IIA was preincubated with **Cmpd21** for 30min at room temperature.

Binding of S2 peptide to proteins-ELISA-type binding assays were performed as described previously (18). Briefly, wells of 96-well Immulon 2 microtiter plates (Dynatech Laboratories, Chantilly, VA) were coated with 100 µl 0.1 M $NaHCO_3$ containing sPLA2-IIA, γC399tr, FN-H120 for 2 h at 37°C. Remaining protein binding sites were blocked by incubating with PBS/0.1% BSA for 30 min at room temperature. After washing with PBS, S2 peptides were added to the wells and incubated in PBS for 2 h at room temperature. After unbound S2 peptides were removed by rinsing the wells with PBS, bound S2 peptides (GST-tagged) were measured using HRP-conjugated anti-GST antibody and peroxidase substrates.

Adhesion assays-Adhesion assays were performed as described previously (18). Briefly, well of 96-well Immulon 2 microtiter plates were coated with 100 µl 0.1 M $NaHCO_3$ containing sPLA2-IIA (10 µg/ml) and were incubated for 2 h at 37°C. Remaining protein binding sites were blocked by incubating with PBS/0.1% BSA for 30 min at room temperature. After washing with PBS, α4-K562, or K562 cells in 100 µl RPMI 1640 were added to the wells and incubated at 37°C for 1 h in the presence of **Cmpd21** (0-100 µM). After unbound cells were removed by rinsing the wells with RPMI 1640, bound cells were quantified by measuring endogenous phosphatase activity.

Chemotaxis-Chemotaxis was measured in modified Boyden Chambers (Transwell). One µg/ml sPLA2-IIA and 20 µg/ml S2-β1 peptide or control peptides in 600 µl RPMI 1640 medium were placed in the lower chamber, and U937 cells (2×10^5 cells in 100 µl RPMI1640 medium) were placed in the upper chamber. After 5 h incubation at 37°C, cells in the lower chamber was counted.

Docking simulation-Docking simulation of interaction between sPLA2-IIA (1DCY.pdb) and integrin αvβ3 was performed using AutoDock3 as described (24). In the present study we used the headpiece (residues 1-438 of αv and residues 55-432 of β3) of αvβ3 (closed-headpiece form, 1JV2.pdb). Cations were not present in αvβ3 during docking simulation, as in the previous studies using αvβ3 (open-headpiece form, 1L5G.pdb) (14,24).

Other methods-Treatment differences were tested using ANOVA and a Tukey multiple comparison test to control the global type I error using Prism 5.0 (Graphpad Software). Surface plasmon resonance studies were performed as described (18).

RESULTS

sPLA2-IIA activates soluble integrin αvβ3 in cell-free conditions-We recently reported that FKN-CD can activate integrins in the absence of CX3CR1 through direct binding to site 2 of integrins (19). A peptide from site 2 of integrin β3 (S2-β3 peptide) directly binds to FKN-CD and suppresses FKN-CD-induced integrin activation (19). The newly identified site 2 is distinct from the classical RGD-binding site (site 1). We propose that FKN-CD binding to site 2 induces activation of site 1 though conformational changes (in an allosteric mechanism). The site 2-mediated activation of integrins may not be limited to FKN-CD, and we tested if other known integrin ligands activate αvβ3.

We previously reported that sPLA2-IIA binds to integrins αvβ3 and α4β1 and induces signals through integrin pathways (14). We studied if sPLA2-IIA enhances the binding of recombinant

soluble αvβ3 to γC399tr, an αvβ3-specific ligand (22,25) in cell-free conditions. We immobilized γC399tr to wells of microtiter plates and measured the binding of soluble αvβ3 to γC399tr in the presence of sPLA2-IIA. To keep soluble integrin inactive we included 1 mM Ca^{2+} in the assay. WT sPLA2-IIA enhanced the binding of γC399tr to αvβ3 in a concentration-dependent manner (Figs. 1a and 1b). In contrast to WT sPLA2-IIA, the R74E/R100E mutant (integrin-binding defective) (14) was defective in this function (Fig. 1b). H47Q (catalytically inactive) and G29S/D48K (M-type receptor-binding defective) mutants behaved like WT sPLA2-IIA (Fig. 1c). These findings suggest that sPLA2-IIA activates αvβ3 in cell-free conditions and this activation requires the integrin-binding site of sPLA2-IIA but does not require catalytic activity or receptor binding.

We studied if sPLA2-IIA activates integrins on the cell surface by measuring the binding of FITC-labeled γC399tr to cells using flow cytometry. WT sPLA2-IIA activated αvβ3 on U937 (αvβ3+)(Fig. 1d), K562 cells that express recombinant αvβ3 (αvβ3-K562 cells) (Fig. 1e), and CHO cells that express hamster αv/human β3 hybrid (β3-CHO cells) (Fig. 1f). The effects of sPLA2-IIA mutations on sPLA2-IIA-induced αvβ3 activation were similar to those in soluble αvβ3. These findings suggest that sPLA2-IIA activates αvβ3 on the cell surface in a manner similar to that of soluble αvβ3, and that the sPLA2-IIA-induced αvβ3 activation is not cell-type specific.

It is possible that the effect of sPLA2-IIA on αvβ3 may be specific to γC399tr. We thus used the disintegrin domain of human ADAM15, which has an RGD motif and specifically binds to αvβ3 (23). The binding of FITC-labeled ADAM15 disintegrin domain was markedly enhanced by WT sPLA2-IIA, but not by R74E/R100E, in U937 (Fig. 1g), αvβ3-K562 (Fig. 1h) and β3-CHO cells (Fig. 1i), suggesting that the effect of sPLA2-IIA on the ligand binding to αvβ3 is not unique to γC399tr. We confirmed that sPLA2-IIA does not directly interact with the integrin ligands used in this study (Fig. S1). sPLA2-IIA directly binds to integrins.

Docking simulation predicts that sPLA2-IIA binds to site 2 in an inactive form of αvβ3-We studied if sPLA2-IIA-induced activation of αvβ3 involves the binding of sPLA2-IIA to site 2 of αvβ3. Docking simulation of the interaction between sPLA2-IIA and the closed-headpiece form of αvβ3 (PDB code 1JV2) predicts that sPLA2-IIA binds to site 2 with high affinity (docking energy -22.1 kcal/mol) (Fig. 2a), as in the case of FKN-CD (19). Site 2 is located at the opposite side of site 1 (Figs. 2b and 2c). The RGD peptide binds binds to site 1 in the open-headpiece αvβ3 (Fig. 2d). Amino acid residues in sPLA2-IIA and integrin αvβ3 that are involved in sPLA2-IIA-αvβ3 integrin are listed in Table 1. The docking model predicts that Arg74 and Arg100 are within the sPLA2-IIA/αvβ3 interface at site 2 (Fig. 1a), suggesting that the integrin binding interface in sPLA2-IIA at site 2 overlaps with that of site 1. This predicts that sPLA2-IIA may activate integrins through direct binding to site 2, and that the R74E/R100E mutant may be defective in this function.

sPLA2-IIA directly binds to a peptide derived from site 2 of integrin β1-We previously identified a peptide sequence (e.g., residues 256-288 of β3, S2-β3 peptide) from site 2 of αvβ3 that directly interacts with FKN-CD (Fig. 3a). The peptide suppresses FKN-CD-mediated integrin activation, but control scrambled peptide does not (19). We studied if site 2-derived peptides bind to sPLA2-IIA. It was expected that S2-β3 peptide binds to sPLA2-IIA, because the amino acid residues in S2-β3 peptide are located within the integrin-binding interface of sPLA2-IIA in the docking model (Table 1). Interestingly, site 2 peptides from β1 (S2-β1 peptide) bound better to sPLA2-IIA in a concentration-dependent manner than S2-β3 peptide (Fig. 3b and 3c). Control GST or scrambled S2-β3 peptide (S2-β3scr) did not bind to sPLA2-IIA. This suggests that site 2 has different binding specificity to FKN-CD and sPLA2-IIA. S2-β1 peptide suppressed sPLA2-IIA-mediated αvβ3 activation in U937 (Fig. 3d), αvβ3-K562 (Fig. 3e), and β3-CHO (Fig. 3f) cells, while control GST or S2-β3scr peptide did not. These findings suggest that sPLA2-IIA binds specifically to site 2 and that the binding of sPLA2-IIA to site 2 is critical for sPLA2-IIA-mediated αvβ3 activation.

sPLA2-IIA activates α4β1 in a site 2-dependent manner.-We have reported that sPLA2-IIA directly binds to another integrin, α4β1, and induces signals in an α4β1-dependent manner (14). We found that sPLA2-IIA enhanced the binding of FITC-labeled fibronectin fragment specific to α4β1 (H120) to U937 cells (α4+) (Fig. 4a), K562 cells that express recombinant α4 (α4-K562) (Fig. 4c), and CHO cells that express recombinant α4 (α4-CHO) (Fig. 4e). This suggests that sPLA2-IIA activates α4β1. WT sPLA2-IIA markedly increased the binding of H120 to α4β1, while R74E/R100E did not (Fig. 4a, 4c, and 4e). The H47Q or G29S/D48K mutants induced α4β1 activation, like WT sPLA2-IIA, suggesting that catalytic activity or receptor binding of sPLA2-IIA is not important. S2-β1 peptide suppressed the binding of H120 to α4β1 increased by sPLA2-IIA, while control GST or S2-β3scr peptide did not (Fig. 4b, 4d and 4e). These results suggest that sPLA2-IIA activates integrin α4β1 through direct binding to site 2 in a manner similar to that of αvβ3.

Cmpd21 *that binds to sPLA2-IIA Inhibits sPLA2 IIA-mediated αvβ3 and α4β1 activation*-We recently identified small compounds that bind to sPLA2-IIA and suppress sPLA2-IIA binding to αvβ3 (15), including compound 21 (**Cmpd21**) (Fig. 5a). **Cmpd21** was selected because of its ability to bind to WT sPLA2-IIA, but not to R74E/R100E (15). **Cmpd21** binds to the integrin-binding site of sPLA2-IIA and suppresses adhesion of αvβ3-K562 cells to γC399tr (15). Consistently, **Cmpd21** suppressed the binding of sPLA2-IIA to αvβ3 in a concentration-dependent manner in surface plasmon resonance studies (Fig. 5b). We found that **Cmpd21** suppressed the γC399tr binding to αvβ3-K562, U937, and β3-CHO cells in a concentration-dependent manner in three different cell types (Fig. 5c, 5d and 5e). **Cmpd21** also suppressed the adhesion of α4-K562 cells to sPLA2-IIA in a concentration-dependent manner (Fig. 6a), suggesting that the effect of **Cmpd21** is not limited to sPLA2-IIA-αvβ3 interaction. **Cmpd21** suppressed sPLA2-IIA-induced binding of H120 to α4β1 in three different cell types (Fig. 6b-d). These findings suggest that **Cmpd21** suppresses sPLA2-IIA-mediated αvβ3 and α4β1 activation through site 2 by binding to the integrin-binding site of sPLA2-IIA.

sPLA2-IIA enhances the binding of the fibronectin fragment that contains the RGD motif to α5β1.-The interaction between the RGD-containing cell-binding fibronectin type III fragment and integrins has been extensively studied as a prototype cell-extracellular matrix interaction. We decided to study if sPLA2-IIA enhances the binding of this fragment to integrin α5β1 using rat fibronectin domains 8-11 (FN8-11). We thus studied FN8-11 binding to α5β1. We found that sPLA2-IIA bound to integrin α5β1 in K562 cells, in which α5β1 is the only β1 integrin, and that mAb KH72 specific to α5 suppressed the binding, suggesting that sPLA2-IIA is a ligand of α5β1 (Fig. 7a). The binding of sPLA2-IIA to U937 cells was suppressed by KH73 (anti-α5), 7E3 (anti-αvβ3), and SG73 (anti-α4), suggesting αvβ3, and α4β1, in addition to α5β1, are involved in sPLA2-IIA binding to U937 cells (Fig. 7b). Cmpd21 effectively suppressed the adhesion of K562 cells to sPLA2-IIA (Fig. 7c). These findings suggest that sPLA2-IIA interacts with α5β1. We discovered that sPLA2-IIA markedly increased the binding of FITC-labeled FN8-11 to α5β1 on U937, K562, and CHO cells, while R74E/R100E did not (Figs. 7d-f). The H47Q or G29S/D48K mutants induced α5β1 activation, like WT sPLA2-IIA. These findings suggest that sPLA2-IIA activates integrin α5β1, and that catalytic activity or receptor binding of sPLA2-IIA is not required for this process, as in the case of αvβ3 and α4β1. S2-β1 peptide suppressed the binding of FN8-11 to α5β1 increased by sPLA2-IIA, while control GST or scrambled β3 peptide did not (Fig. 7g-i). **Cmpd21** suppressed sPLA2-IIA-induced binding of FN8-11 to α5β1 in three different cell types (Fig. 7j-l), suggesting **Cmpd21** suppresses sPLA2-IIA-induced α5β1 activation through site 2 as well. Taken together, these results suggest that sPLA2-IIA enhances FN8-11 to integrin α5β1 through direct binding of sPLA2-IIA to site 2, as in the case of αvβ3 and α4β1.

sPLA2-IIA suppresses H120 binding to α4β1 at high concentrations.-If sPLA2-IIA binds to site 1 (14) and site 2 (the present study), it is predicted that sPLA2-IIA competes with ligands for binding to site 1. To address this question, we determined the effect of sPLA2-IIA as a function of sPLA2-

IIA concentrations up to 500 μg/ml. The binding of H120 to α4-CHO cells was maximum at 20 μg/ml sPLA2-IIA and then reduced as sPLA2-IIA concentration increases (Fig. 8). This suggests that 1) sPLA2-IIA at low concentrations binds to site 2 of closed α4β1 (site 1 closed, site 2 open) and activates α4β1 (site 1 open). 2) when site 2 is saturated with sPLA2-IIA, sPLA2-IIA competes with H120 for binding to site 1 (open) and reduce the binding of H120.

DISCUSSION

The present study establishes that sPLA2-IIA activates integrins αvβ3, α4β1, and α5β1 through direct binding to site 2. sPLA2-IIA activated recombinant soluble αvβ3 in cell-free conditions, suggesting that inside-out signals or other molecules are not involved. This process does not include catalytic activity or receptor binding of sPLA2-IIA since mutating the catalytic center or receptor-binding site of sPLA2-IIA did not affect sPLA2-IIA-mediated integrin activation. sPLA2-IIA induced integrin activation through binding to site 2 is a novel mechanism of integrin activation and pro-inflammatory action by sPLA2-IIA. sPLA2-IIA may activate other integrins through direct binding.

sPLA2-IIA-mediated integrin activation happens in biological fluids (at least in tears). In the present study, >5 μg/ml sPLA2-IIA was required to detect sPLA2-IIA-induced integrin activation. Notably, the concentration of sPLA2-IIA is exceptionally high in human tears (26-28). In normal subjects, the concentration of sPLA2-IIA in tears is 54.5 +/- 33.9 μg/ml, one of the highest levels of sPLA2-IIA reported in any normal human secretions (29). Therefore integrin activation by sPLA2-IIA happens at least in tears. sPLA2-IIA appears to be secreted by both the lacrimal glands and the goblet cells of conjunctival epithelia (26,30). Since sPLA2-IIA is bacteriocidal and kills Listera at much lower concentrations (<0.1 nM), it is possible that the primary functions of sPLA2-IIA at such high concentrations in tears might be integrin activation. It is likely that sPLA2-IIA in tears may play a role in enhancing immune response to bacterial pathogens through local integrin activation in tears or perhaps in other tissues. Serum levels of sPLA2-IIA are increased only up to 1 μg/ml during systemic inflammation (2,3). sPLA2-IIA may not effectively activate integrins at these concentrations. It is, however, possible that sPLA2-IIA may be highly concentrated in diseased tissues in chronic inflammation or on the cell surface through binding to proteoglycans.

The sPLA2-IIA-induced integrin activation is expected to enhance interaction between cells and extracellular matrix (e.g., fibrinogen and fibronectin) and thereby induce massive proliferative signals. Since integrins are involved in growth factor signaling through crosstalk with growth factor receptors, sPLA2-IIA-induced integrin activation is also expected to enhance cellular responsiveness to growth factors. We have reported that integrins crosstalk with several growth factor receptors through direct binding to growth factors (e.g., fibroblast growth factor-1 (24,31-33), insulin-like growth factor-1 (34-37), neuregulin-1 (38), and fractalkine (18)). We propose that sPLA2-IIA-induced integrin activation indirectly affects intracellular signaling by these growth factors through enhancing integrin binding to growth factors.

We establish that site 2 is involved in integrin activation by sPLA2-IIA (the current study) and FKN-CD (19) (Fig. 9). This is a new mechanism of integrin activation. It has previously been reported that the binding of a RGD-mimetic peptide induces changes in the tertiary structure of αvβ3 (39) and αIIbβ3 (40) in the β3 I-like domain. RGD or ligand-mimetic peptides activate purified, non-activated αIIbβ3 (41) and αvβ3 (42). This process does not require inside-out signal transduction and it appears that RGD or ligand-mimetic peptide triggers conformational changes that lead to full activation of integrins. These findings suggest that these peptides enhance integrin affinity by conformational changes in the headpiece possibly through additional ligand-binding sites in the integrin (41). A previous study suggests that there are two RGD-binding sites in integrin αIIbβ3, and that one binding site acts as an allosteric site based on binding kinetic studies (43). Also, another study suggests that two distinct

cyclic RGD-mimetic peptides can simultaneously bind to distinct sites in αIIbβ3, and the estimated distance between two ligand-binding site is about 6.1 +/- 0.5 nm (44). The possible allosteric ligand-binding site has not been pursued probably because the αvβ3 structure (ligand occupied, open-headpiece) contains only one RGD-binding site (39). In our docking model the distance between site 1 and site 2 is about 6 nm. Thus, the position of site 2 is consistent with the previous report. Based on previous studies it is likely that the newly identified site 2 has ligand specificity that overlaps with that of site 1, interacts with integrin ligands other than FKN-CD (e.g., RGD), and is potentially involved in integrin regulation in an allosteric mechanism. It is reasonable to assume that FKN-CD or sPLA2-IIA binding to site 2 induces conformational changes in integrins. We suspect that other proteins also bind to site 2 and affect integrin functions. Also it is likely that integrins other than αvβ3, α4β1, and α5β1 are activated by site 2-mediated mechanism. It would be interesting to address this question in future studies.

It is unclear if sPLA2-IIA-induced integrin activation requires global conformational changes in integrins. In current models of integrin activation, activation of β1 integrins induces a swing-out movement of the hybrid domain and exposes epitopes recognized by activation-dependent antibodies (anti-human β1 HUTS4 and HUTS21)(45). The HUTS4 and HUTS21 epitopes are located in the hybrid domain of β1 (46,47). In our preliminary experiments, sPLA-IIA did not change reactivity of β1 integrins to HUTS4 and HUTS21 in U937, K562, and α4-K562 cells in RPMI1640 medium under the conditions in which sPLA2-IIA enhanced the binding of β1 integrins to ligands. It is possible that the binding of sPLA2-IIA to site 2 induces only local conformational changes within the headpiece of integrins. Interestingly, the open-headpiece (PDB code 1L5G) and closed-headpiece (PDB code 1JV2) conformations of αvβ3 are very similar (39,48). Surprisingly, the docking simulation distinguished the two conformations and predicted the position of site 2 in the closed-headpiece form. We showed that sPLA2-IIA (the current study) FKN-CD (19) actually bind to site 2 and the binding of sPLA2-IIA and FKN to site 2 is required for integrin activation using the peptides from site 2. It is likely that integrins that are activated by sPLA2-IIA and FKN-CD through site 2 have conformations similar to the open-headpiece αvβ3 that has no global conformational changes compared to the closed-headpiece form. The open- and closed-headpiece conformations of αvβ3 may really reflect the fact that integrins can be activated without global conformational changes through allosteric mechanism.

The site 2-derived integrin peptides were used for establishing that sPLA2-IIA really binds to site 2, and the interaction is involved in integrin activation. We studied if S2-β1 peptide acts as an antagonist for sPLA2-IIA-mediated pro-inflammatory signals (Fig. S2). S2-β1 peptide suppressed chemotaxis induced by sPLA2-IIA. Although it is unclear if S2-β1 peptide suppressed the binding of sPLA2-IIA to site 1 or site 2 at this point, the results suggest that S2-β1 peptide has potential as a therapeutic.

Specific inhibitors of sPLA2-IIA catalytic activity S-5920/LY315920Na and S-3013/LY333013 failed to demonstrate a significant therapeutic effect in rheumatoid arthritis (49) and asthma (50). Our previous study suggests that sPLA2-IIA-integrin interaction may be a potential target for chronic inflammatory diseases (14). **Cmpd21** was screened for its ability to bind to the integrin-binding site of sPLA2-IIA (15). Indeed Cmpd21 suppressed the binding of sPLA2-IIA to integrins, and, in the present study, we demonstrated that **Cmpd21** suppressed the sPLA2-IIA-induced integrin activation via site 2 as well. It would be interesting to study if **Cmpd21** or its variants suppress inflammation in vivo in future studies.

REFERENCES

1. Vadas, P., Stefanski, E., and Pruzanski, W. (1985) Characterization of extracellular phospholipase A2 in rheumatoid synovial fluid. *Life Sci* **36**, 579-587
2. Jaross, W., Eckey, R., and Menschikowski, M. (2002) Biological effects of secretory phospholipase A(2) group IIA on lipoproteins and in atherogenesis. *Eur J Clin Invest* **32**, 383-393
3. Niessen, H. W., Krijnen, P. A., Visser, C. A., Meijer, C. J., and Erik Hack, C. (2003) Type II secretory phospholipase A2 in cardiovascular disease: a mediator in atherosclerosis and ischemic damage to cardiomyocytes? *Cardiovasc Res* **60**, 68-77
4. Jiang, J., Neubauer, B. L., Graff, J. R., Chedid, M., Thomas, J. E., Roehm, N. W., Zhang, S., Eckert, G. J., Koch, M. O., Eble, J. N., and Cheng, L. (2002) Expression of group IIA secretory phospholipase A2 is elevated in prostatic intraepithelial neoplasia and adenocarcinoma. *Am J Pathol* **160**, 667-671
5. Dong, Q., Patel, M., Scott, K. F., Graham, G. G., Russell, P. J., and Sved, P. (2006) Oncogenic action of phospholipase A2 in prostate cancer. *Cancer letters* **240**, 9-16
6. Sved, P., Scott, K. F., McLeod, D., King, N. J., Singh, J., Tsatralis, T., Nikolov, B., Boulas, J., Nallan, L., Gelb, M. H., Sajinovic, M., Graham, G. G., Russell, P. J., and Dong, Q. (2004) Oncogenic action of secreted phospholipase A2 in prostate cancer. *Cancer research* **64**, 6934-6940
7. Tada, K., Murakami, M., Kambe, T., and Kudo, I. (1998) Induction of cyclooxygenase-2 by secretory phospholipases A2 in nerve growth factor-stimulated rat serosal mast cells is facilitated by interaction with fibroblasts and mediated by a mechanism independent of their enzymatic functions. *J Immunol* **161**, 5008-5015
8. Triggiani, M., Granata, F., Balestrieri, B., Petraroli, A., Scalia, G., Del Vecchio, L., and Marone, G. (2003) Secretory phospholipases A2 activate selective functions in human eosinophils. *J Immunol* **170**, 3279-3288
9. Lambeau, G., Ancian, P., Barhanin, J., and Lazdunski, M. (1994) Cloning and expression of a membrane receptor for secretory phospholipases A2. *J Biol Chem* **269**, 1575-1578
10. Nicolas, J. P., Lambeau, G., and Lazdunski, M. (1995) Identification of the binding domain for secretory phospholipases A2 on their M-type 180-kDa membrane receptor. *J Biol Chem* **270**, 28869-28873
11. Cupillard, L., Mulherkar, R., Gomez, N., Kadam, S., Valentin, E., Lazdunski, M., and Lambeau, G. (1999) Both group IB and group IIA secreted phospholipases A2 are natural ligands of the mouse 180-kDa M-type receptor. *J Biol Chem* **274**, 7043-7051
12. Hynes, R. O. (2002) Integrins: bidirectional, allosteric signaling machines. *Cell* **110**, 673-687
13. Takada, Y., Ye, X., and Simon, S. (2007) The integrins. *Genome Biol* **8**, 215
14. Saegusa, J., Akakura, N., Wu, C. Y., Hoogland, C., Ma, Z., Lam, K. S., Liu, F. T., Takada, Y. K., and Takada, Y. (2008) Pro-inflammatory secretory phospholipase A2 type IIA binds to integrins αvβ3 and α4β1 and induces proliferation of monocytic cells in an integrin-dependent manner. *J Biol Chem* **283**, 26107-26115
15. Ye, L., Dickerson, T., Kaur, H., Takada, Y. K., Fujita, M., Liu, R., Knapp, J. M., Lam, K. S., Schore, N. E., Kurth, M. J., and Takada, Y. (2013) Identification of inhibitors against interaction between pro-inflammatory sPLA2-IIA protein and integrin αvβ3. *Bioorg Med Chem Lett* **23**, 340-345
16. Zhu, J., Zhu, J., and Springer, T. A. (2013) Complete integrin headpiece opening in eight steps. *J Cell Biol* **201**, 1053-1068
17. Xiong, J. P., Mahalingham, B., Alonso, J. L., Borrelli, L. A., Rui, X., Anand, S., Hyman, B. T., Rysiok, T., Muller-Pompalla, D., Goodman, S. L., and Arnaout, M. A. (2009) Crystal structure of the complete integrin αVβ3 ectodomain plus an α/β transmembrane fragment. *J Cell Biol* **186**,

589-600
18. Fujita, M., Takada, Y. K., and Takada, Y. (2012) Integrins αvβ3 and α4β1 Act as Coreceptors for Fractalkine, and the Integrin-Binding Defective Mutant of Fractalkine Is an Antagonist of CX3CR1. *J Immunol* **189**, 5809-5819
19. Fujita, M., Takada, Y. K., and Takada, Y. (2014) The Chemokine Fractalkine Can Activate Integrins without CX3CR1 through Direct Binding to a Ligand-Binding Site Distinct from the Classical RGD-Binding Site. *PLoS One* **9**, e96372
20. Blystone, S. D., Graham, I. L., Lindberg, F. P., and Brown, E. J. (1994) Integrin αvβ3 differentially regulates adhesive and phagocytic functions of the fibronectin receptor α5β1. *J Cell Biol* **127**, 1129-1137
21. Takagi, J., Erickson, H. P., and Springer, T. A. (2001) C-terminal opening mimics 'inside-out' activation of integrin α5β1. *Nat Struct Biol* **8**, 412-416
22. Yokoyama, K., Zhang, X. P., Medved, L., and Takada, Y. (1999) Specific binding of integrin αvβ3 to the fibrinogen gamma and αE chain C-terminal domains. *Biochemistry* **38**, 5872-5877
23. Zhang, X. P., Kamata, T., Yokoyama, K., Puzon-McLaughlin, W., and Takada, Y. (1998) Specific interaction of the recombinant disintegrin-like domain of MDC-15 (metargidin, ADAM-15) with integrin αvβ3. *J Biol Chem* **273**, 7345-7350
24. Mori, S., Wu, C. Y., Yamaji, S., Saegusa, J., Shi, B., Ma, Z., Kuwabara, Y., Lam, K. S., Isseroff, R. R., Takada, Y. K., and Takada, Y. (2008) Direct Binding of Integrin αvβ3 to FGF1 Plays a Role in FGF1 Signaling. *J Biol Chem* **283**, 18066-18075
25. Yokoyama, K., Erickson, H. P., Ikeda, Y., and Takada, Y. (2000) Identification of amino acid sequences in fibrinogen gamma -chain and tenascin C C-terminal domains critical for binding to integrin αvβ3. *J Biol Chem* **275**, 16891-16898
26. Nevalainen, T. J., Aho, H. J., and Peuravuori, H. (1994) Secretion of group 2 phospholipase A2 by lacrimal glands. *Investigative ophthalmology & visual science* **35**, 417-421
27. Qu, X. D., and Lehrer, R. I. (1998) Secretory phospholipase A2 is the principal bactericide for staphylococci and other gram-positive bacteria in human tears. *Infection and immunity* **66**, 2791-2797
28. Birts, C. N., Barton, C. H., and Wilton, D. C. (2010) Catalytic and non-catalytic functions of human IIA phospholipase A2. *Trends Biochem Sci* **35**, 28-35
29. Saari, K. M., Aho, V., Paavilainen, V., and Nevalainen, T. J. (2001) Group II PLA(2) content of tears in normal subjects. *Investigative ophthalmology & visual science* **42**, 318-320
30. Aho, H. J., Saari, K. M., Kallajoki, M., and Nevalainen, T. J. (1996) Synthesis of group II phospholipase A2 and lysozyme in lacrimal glands. *Investigative ophthalmology & visual science* **37**, 1826-1832
31. Yamaji, S., Saegusa, J., Ieguchi, K., Fujita, M., Takada, Y. K., and Takada, Y. (2010) A novel fibroblast growth factor-1 (FGF1) mutant that acts as an FGF antagonist. *PLoS One* **5**, e10273
32. Mori, S., and Takada, Y. (2013) Crosstalk between Fibroblast Growth Factor (FGF) Receptor and Integrin through Direct Integrin Binding to FGF and Resulting Integrin-FGF-FGFR Ternary Complex Formation. *Medical Sciences* **1**, 20-36
33. Mori, S., Tran, V., Nishikawa, K., Kaneda, T., Hamada, Y., Kawaguchi, N., Fujita, M., Takada, Y. K., Matsuura, N., Zhao, M., and Takada, Y. (2013) A Dominant-Negative FGF1 Mutant (the R50E Mutant) Suppresses Tumorigenesis and Angiogenesis. *PLoS One* **8**, e57927
34. Saegusa, J., Yamaji, S., Ieguchi, K., Wu, C. Y., Lam, K. S., Liu, F. T., Takada, Y. K., and Takada, Y. (2009) The direct binding of insulin-like growth factor-1 (IGF-1) to integrin αvβ3 is involved in IGF-1 signaling. *J Biol Chem* **284**, 24106-24114
35. Fujita, M., Ieguchi, K., Davari, P., Yamaji, S., Taniguchi, Y., Sekiguchi, K., Takada, Y. K., and Takada, Y. (2012) Cross-talk between integrin α6β4 and insulin-like growth factor-1 receptor (IGF1R) through direct α6β4 binding to IGF1 and subsequent α6β4-IGF1-IGF1R ternary

complex formation in anchorage-independent conditions. *J Biol Chem* **287**, 12491-12500
36. Fujita, M., Ieguchi, K., Cedano-Prieto, D. M., Fong, A., Wilkerson, C., Chen, J. Q., Wu, M., Lo, S. H., Cheung, A. T., Wilson, M. D., Cardiff, R. D., Borowsky, A. D., Takada, Y. K., and Takada, Y. (2013) An Integrin Binding-defective Mutant of Insulin-like Growth Factor-1 (R36E/R37E IGF1) Acts as a Dominant-negative Antagonist of the IGF1 Receptor (IGF1R) and Suppresses Tumorigenesis but Still Binds to IGF1R. *J Biol Chem* **288**, 19593-19603
37. Fujita, M., Takada, Y. K., and Takada, Y. (2013) Insulin-like Growth Factor (IGF) Signaling Requires αvβ3-IGF1-IGF Type 1 Receptor (IGF1R) Ternary Complex Formation in Anchorage Independence, and the Complex Formation Does Not Require IGF1R and Src Activation. *J Biol Chem* **288**, 3059-3069
38. Fujita, M., Ma, Z., Davari, P., Taniguchi, Y., Sekiguchi, K., Wang, B., Takada, Y. K., and Takada, Y. (2010) Direct binding of the EGF-like domain of neuregulin-1 to integrins (αvβ3 and α6β4) is involved in neuregulin-1/ErbB signaling. *J Biol Chem* **285**, 31388-31398
39. Xiong, J. P., Stehle, T., Zhang, R., Joachimiak, A., Frech, M., Goodman, S. L., and Arnaout, M. A. (2002) Crystal structure of the extracellular segment of integrin α Vβ3 in complex with an Arg-Gly-Asp ligand. *Science* **296**, 151-155.
40. Xiao, T., Takagi, J., Coller, B. S., Wang, J. H., and Springer, T. A. (2004) Structural basis for allostery in integrins and binding to fibrinogen-mimetic therapeutics. *Nature* **432**, 59-67
41. Du, X. P., Plow, E. F., Frelinger, A. L., 3rd, O'Toole, T. E., Loftus, J. C., and Ginsberg, M. H. (1991) Ligands "activate" integrin αIIbβ3 (platelet GPIIb-IIIa). *Cell* **65**, 409-416
42. Legler, D. F., Wiedle, G., Ross, F. P., and Imhof, B. A. (2001) Superactivation of integrin αvβ3 by low antagonist concentrations. *J Cell Sci* **114**, 1545-1553
43. Hu, D. D., White, C. A., Panzer-Knodle, S., Page, J. D., Nicholson, N., and Smith, J. W. (1999) A new model of dual interacting ligand binding sites on integrin αIIbβ3. *J Biol Chem* **274**, 4633-4639
44. Cierniewski, C. S., Byzova, T., Papierak, M., Haas, T. A., Niewiarowska, J., Zhang, L., Cieslak, M., and Plow, E. F. (1999) Peptide ligands can bind to distinct sites in integrin αIIbβ3 and elicit different functional responses. *J Biol Chem* **274**, 16923-16932
45. Luo, B. H., Carman, C. V., and Springer, T. A. (2007) Structural Basis of Integrin Regulation and Signaling. *Annu Rev Immunol* **25**, 619-647
46. Luque, A., Gomez, M., Puzon, W., Takada, Y., Sanchez-Madrid, F., and Cabanas, C. (1996) Activated conformations of very late activation integrins detected by a group of antibodies (HUTS) specific for a novel regulatory region (355-425) of the common β 1 chain. *J Biol Chem* **271**, 11067-11075
47. Mould, A. P., Barton, S. J., Askari, J. A., McEwan, P. A., Buckley, P. A., Craig, S. E., and Humphries, M. J. (2003) Conformational changes in the integrin β A domain provide a mechanism for signal transduction via hybrid domain movement. *J Biol Chem* **278**, 17028-17035
48. Xiong, J. P., Stehle, T., Diefenbach, B., Zhang, R., Dunker, R., Scott, D. L., Joachimiak, A., Goodman, S. L., and Arnaout, M. A. (2001) Crystal structure of the extracellular segment of integrin αVβ3. *Science* **294**, 339-345
49. Bradley, J. D., Dmitrienko, A. A., Kivitz, A. J., Gluck, O. S., Weaver, A. L., Wiesenhutter, C., Myers, S. L., and Sides, G. D. (2005) A randomized, double-blinded, placebo-controlled clinical trial of LY333013, a selective inhibitor of group II secretory phospholipase A2, in the treatment of rheumatoid arthritis. *J Rheumatol* **32**, 417-423
50. Bowton, D. L., Dmitrienko, A. A., Israel, E., Zeiher, B. G., and Sides, G. D. (2005) Impact of a soluble phospholipase A2 inhibitor on inhaled allergen challenge in subjects with asthma. *J Asthma* **42**, 65-71

FOOTNOTES

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Abbreviations: FKN-CD, the chemokine domain of fractalkine; FN-H120, fibronectin H120 fragment; γC399tr, Fibrinogen γ-chain C-terminal domain that lacks residues 400–411; sPLA2-IIA, secreted phospholipase 2 type IIA

FIGURE LEGENDS

Figure 1. sPLA2-IIA activates αvβ3 integrin in cell-free conditions (through direct integrin binding). a. Activation of soluble αvβ3 by sPLA2-IIA as a function of γC399tr concentration**.** Binding of soluble αvβ3 (5 μg/ml) to immobilized γC399tr in the presence or absence of WT sPLA2-IIA (50 μg/ml) was performed as described in the methods. Data are shown as means ± SEM of three independent experiments. b. Activation of soluble αvβ3 by sPLA2-IIA as a function of sPLA2-IIA concentration. Wells of 96-well microtiter plates were coated with γC399tr (100 μg/ml) and incubated with soluble αvβ3 (5 μg/ml). Data are shown as means ± SEM of three independent experiments. c. The effects of sPLA2-IIA mutations on integrin αvβ3 activation. Activation of soluble αvβ3 was measured as described above. SPLA2-IIA (50 μg/ml) and γC399tr (100 μg/ml for coating) were used. R74E/R100E, defective in integrin-binding; H74Q, catalytically inactive; G29S/D48K, defective in the binding to M-type receptor. d-f. The effects of sPLA2-IIA mutations on the binding of γC399tr to integrin αvβ3 on the cell surface. The binding of FITC-labeled γC399tr to the cells in the presence of sPLA2-IIA (up to 20 μg/ml in the case of U937 cells (d) and 20 μg/ml in αvβ3-K562 (e) and β3-CHO (f) cells) was measured using flow cytometry as described in the methods. Data are shown as means (median fluorescent intensity, MFI) ± SEM of three independent experiments. g-i, The effects of sPLA2-IIA mutations on the binding of ADAM15 to integrin αvβ3. The binding of FITC-labeled ADAM15 to αvβ3 in the presence of sPLA2-IIA (20 μg/ml) in U937 (g), αvβ3-K562 (h) and β3-CHO (i) cells was measured using flow cytometry. Data are shown as means (MFI) ± SEM of three independent experiments.

Figure 2. Docking simulation predicts that sPLA2-IIA binds to a binding-site that is distinct from the classical RGD-binding site in closed-headpiece αvβ3. a. A docking model of sPLA2-IIA-integrin αvβ3 (inactive) interaction. The headpiece of an inactive form of integrin αvβ3 (PDB code 1JV2) was used as a target. b. A docking model of sPLA2-IIA-integrin αvβ3 (active) interaction (14). The headpiece of ligand-bound form of integrin αvβ3 (PDB code 1L5G) was used as a target. The model predicts that sPLA2-IIA (PDB code 1DCY) binds to the classical RGD-binding site of the integrin αvβ3 headpiece (site 1). The model predicts the position of the second sPLA2-IIA-binding site (site 2). c. Superposition of two models shows that the positions of two predicted sPLA2-IIA binding sites are distinct. d. open-headpiece αvβ3 structure (1L5G) with an RGD-containing peptide (site 1).

Figure 3. Site 2 peptide from β1 binds to sPLA2-IIA. a. Alignment of peptides from site 2 from different integrin β subunits. b. Binding of site 2 peptides from different integrin β subunits (S2-β1, β2, β3, and β4) to immobilized sPLA2-IIA (20 μg/ml). The binding of peptides to immobilized sPLA2-IIA was measured as described in the methods. Data are shown as means ± SEM of three independent experiments. c. Binding of S2-β1 peptide to sPLA2-IIA as a function of sPLA2-II concentrations. The binding of the peptide to immobilized sPLA2-IIA was measured as described in (b). Data are shown as means ± SEM of three independent experiments. d. Suppression of γC399tr to U937 cells by site 2 peptide. The binding of FITC-labeled γC399tr to αvβ3 on U937 was measured in flow cytometry as described in the Methods section. Data are shown as means ± SEM of MFI of three independent experiments. e. Suppression of

γC399tr to αvβ3-K562 cells by site 2 peptide. The binding of FITC-labeled γC399tr to αvβ3 on αvβ3-K562 was measured in flow cytometry as described in the Methods section. Data are shown as means ± SEM of MFI of three independent experiments. sPLA2-IIA (20 μg/ml) and site 2 peptides (200 μg/ml) were used. f. Suppression of γC399tr to β3-CHO cells by site 2 peptide. The binding of FITC-labeled γC399tr to αvβ3 on β3-CHO was measured in flow cytometry as described in the Methods section. Data are shown as means ± SEM of MFI of three independent experiments. sPLA2-IIA (20 μg/ml) and site 2 peptides (200 μg/ml) were used.

Figure 4. sPLA2-IIA enhances the binding of the fibronectin fragment that contains CS-1 (H120) to α4β1 through binding to site 2. a. The binding of FITC-labeled FN H120 fragment (an α4β1-specific ligand) to α4β1 on U937 cells was measured by flow cytometry. WT sPLA2-IIA, catalytically inactive mutant (H47A), and the receptor-binding mutant (G29S/D48K) enhance the binding of H120 to α4β1, but the integrin-binding defective mutant (R74E/R100E) does not. Data are shown as means ± SEM of MFI of three independent experiments. b. Site 2 peptide from β1 (S2-β1) suppressed sPLA2-IIA-induced α4β1 activation in U937 cells (20 μg/ml WT sPLA2-IIA was used). Data are shown as means ± SEM of MFI of three independent experiments. c, d, e. sPLA2-IIA mutations and S2- β1 peptide affect α4β1 activation by sPLA2-IIA in α4-K562 (c and d), and α4-CHO (e) cells. The binding of FITC-labeled FN H120 fragment to α4β1+ cells was measured by flow cytometry. sPLA2-IIA (20 μg/ml) and site 2 peptides (200 μg/ml) were used. Data are shown as means ± SEM of MFI of three independent experiments.

Figure 5. Cmpd21 blocks the binding of γC399tr to αvβ3 on the cell surface through binding to site 2. The effect of Cmpd21 on the binding of γC399tr to αvβ3 and on the sPLA2-IIA-induced integrin activation was studied. a. Structure of **Cmpd21**. Amino acids with asterisk are D isomers. b. **Cmpd21** suppresses the binding of sPLA2-IIA to αvβ3. Soluble αvβ3 was immobilized to a sensor chip and **Cmpd21** was added to the solution phase together with sPLA2-IIA in surface plasmon resonance (SPR) study. c-e. **Cmpd21** suppressed the binding of FITC-labeled γC399tr enhanced by sPLA2-IIA (20 μg/ml) to αvβ3 on U937 (c), αvβ3-K562 (d), and β3-CHO cells (e). The concentration of Cmpd21 in d and e is 50 μM. Data are shown as means ± SEM of MFI of three independent experiments.

Figure 6. Cmpd21 suppresses sPLA2-IIA-induced α4β1 activation in U937, α4-K562, and α4-CHO cells. a. **Cmpd21** suppressed the binding of α4β1 to sPLA2-IIA. Adhesion of α4-K562 cells to sPLA2-IIA (at 10 μg/ml coating concentration) in RPMI1640 medium was measured. Data are shown as means ± SEM of three independent experiments. b-d. **Cmpd21** suppressed the binding of FITC-labeled H120 enhanced by sPLA2-IIA (20 μg/ml) to α4β1 on U937 (b), α4-K562 (c), and α4-CHO cells (d). The concentration of **Cmpd21** in c and d is 50 μM in d. Data are shown as means ± SEM of MFI of three independent experiments.

Figure 7. sPLA2-IIA enhances the binding of the RGD-containing fibronectin fragment (FN8-11) to α5β1

through binding to site 2. sPLA2-IIA at 20 μg/ml and FN8-11 at 20 μg/ml were used if not indicated otherwise. a and b. Specific binding of sPLA2-IIA to integrin α5β1. FITC-labeled sPLA2-IIA (10 μg/ml) was incubated with K562 cells (a) or U937 cells (b) in the presence of mouse IgG, KH72 (anti-α5 mAb), SG73 (anti-α4) or 7E3 (anti-β3 mAb) (10 μg/ml). Bound FITC was measured using flow cytometry. Data are shown as means ± SEM of MFI of three independent experiments. c. Comp21 suppresses sPLA2 binding to α5β1. Adhesion of K562 cells to sPLA2-IIA (coating concentration 10 μg/ml) was measured in the presence of **Cmpd21**. Data are shown as means ± SEM of % adhesion of three independent experiments. d-f. The effect of sPLA2-IIA mutants on the binding of FITC-FN8-11 was measured in U937 cells (d), K562 cells (e), and CHO cells (f). Data are shown as means ± SEM of MFI of three independent experiments. FITC-sPLA2-IIA was used at 20 μg/ml if not indicated otherwise. g-i, The binding of FITC-FN8-11 to U937 cells (g), K562 cells (h), or CHO cells (i) was measured in the presence of S2-β1 peptide. Data are shown as means ± SEM of MFI of three independent experiments. j-l, The binding of FITC-FN8-11 to U937 cells (j), K562 cells (k) or CHO cells (l) was measured in the presence of **Cmpd21**. Data are shown as means ± SEM of MFI of three independent experiments.

Figure 8. sPLA2-IIA suppresses the binding of H120 to site 1 in α4β1.

The binding of H120 to α4-CHO cells as a function of sPLA2-IIA concentrations. The binding of FITC-H120 (10 μg/ml) was measured in flow cytometry (a). Data are shown as means ± SEM of MFI of three independent experiments (b). * $P<0.05$ compared to the MFI at 20 μg/ml sPLA2-IIA (arrow).

Figure 9. A model of sPLA2-IIA-induced integrin activation through site 2. We propose a model, in which sPLA2-IIA binds to site 2 of inactive/closed-headpiece integrins and induces conformational changes and enhance ligand binding to site 1 (the classical RGD-binding site). This activation is blocked by a peptide that is derived from site 2 or a small compound (**Cmpd21**) that binds to the integrin-binding site of sPLA2-IIA.

Table 1. Amino acid residues involved in the interaction between sPLA2-IIA and integrin αvβ3. Amino acid residues within 6 angstrom between sPLA2-IIA and αvβ3 were selected using pdb viewer (version 4.1). Amino acid residues in β3 site 2 peptide (S2-β3) are shown in bold.

αv	β3	sPLA2-IIA
Glu15, Lys42, Asn44, Gly49, Ile50, Val51, Glu52, Asn77, Ser90, His91, Trp93, Arg122, Ala397, Arg398, Ser399	Pro160, Val161, Ser162, Met165, Ser168, Pro169, Pro170, Glu171, Ala172, Leu173, Glu174, Asn175, Leu185, Pro186, Met187, Phe188, His192, Val193, Leu194, Glu206, **Ala263, Gly264, Gln267, Gly276, Ser277, Asp278, Asn279, His280, Ser282, Ala283, Thr285, Thr286**	Lue11, Thr13, Gly14, Lys15 Ser35, Pro36, Lys37, Asp38, Ala39, Arg42, Val45, Thr46, His47, Cy49, Cy50, Arg53, Ser71, Gly72, Ser73, Arg74, Cys97, Arg100, Asn101, Lys102, Thr103, Thr104, Tyr105, Asn106, Lys107, Lys108, Tyr109, Tyr112, Arg118, Ser120, Pro122, Arg123, Cys124

Fig. 1

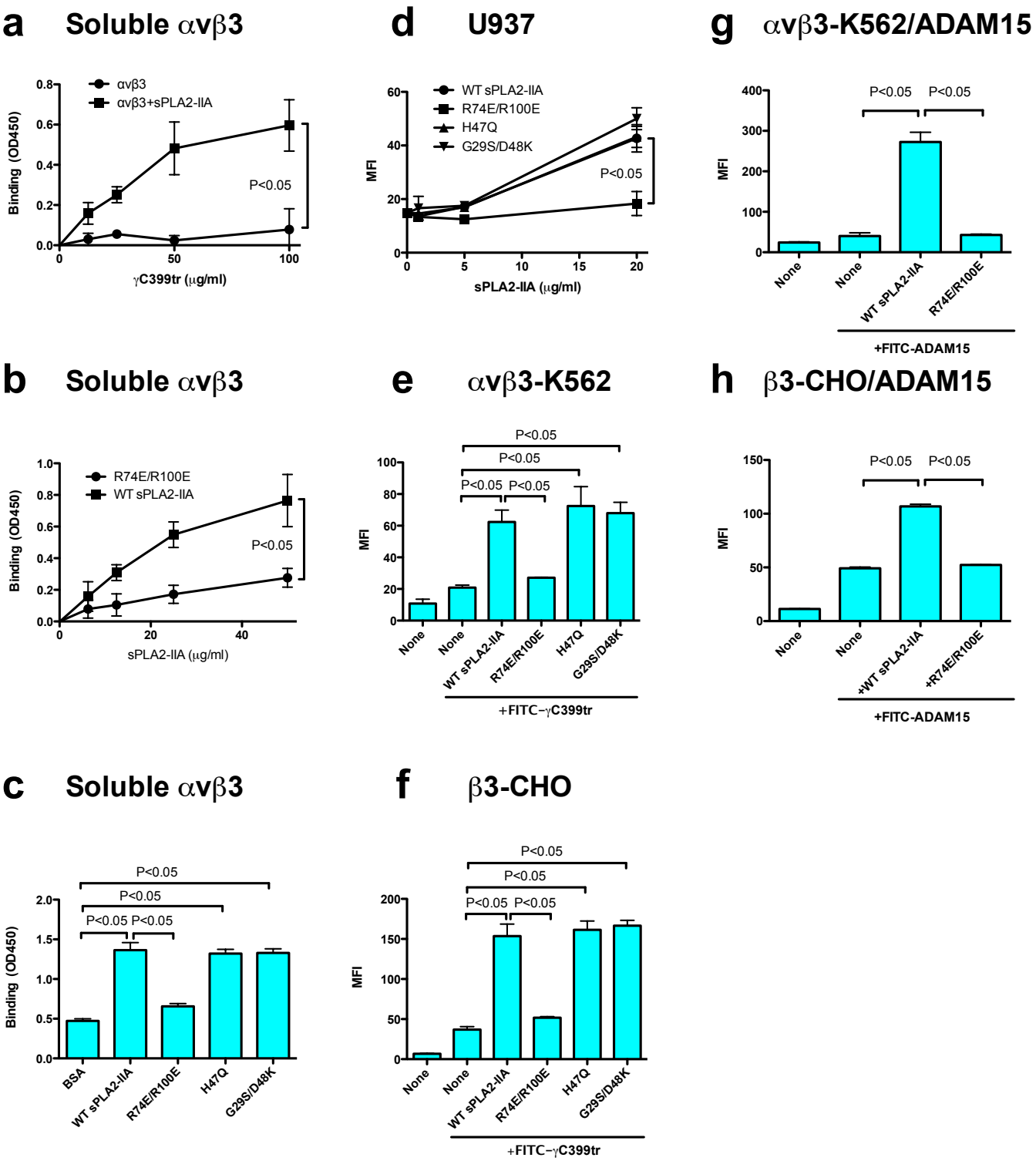

a
Soluble αvβ3
αvβ3
αvβ3+sPLA2-IIA
Binding (OD450)
P<0.05
γC399tr (μg/ml)
d
U937
WT sPLA2-IIA
R74E/R100E
H47Q
G29S/D48K
MFI
P<0.05
sPLA2-IIA (μg/ml)
g
αvβ3-K562/ADAM15
P<0.05 P<0.05
MFI
None
None
WT sPLA2-IIA
R74E/R100E
+FITC-ADAM15
b
Soluble αvβ3
R74E/R100E
WT sPLA2-IIA
Binding (OD450)
P<0.05
sPLA2-IIA (μg/ml)
e
αvβ3-K562
P<0.05
P<0.05
P<0.05 P<0.05
MFI
None
None
WT sPLA2-IIA
R74E/R100E
H47Q
G29S/D48K
+FITC−γC399tr
h
β3-CHO/ADAM15
P<0.05 P<0.05
MFI
None
None
+WT sPLA2-IIA
+R74E/R100E
+FITC-ADAM15
c
Soluble αvβ3
P<0.05
P<0.05
P<0.05 P<0.05
Binding (OD450)
BSA
WT sPLA2-IIA
R74E/R100E
H47Q
G29S/D48K
f
β3-CHO
P<0.05
P<0.05
P<0.05 P<0.05
MFI
None
None
WT sPLA2-IIA
R74E/R100E
H47Q
G29S/D48K
+FITC−γC399tr

Fig. 2

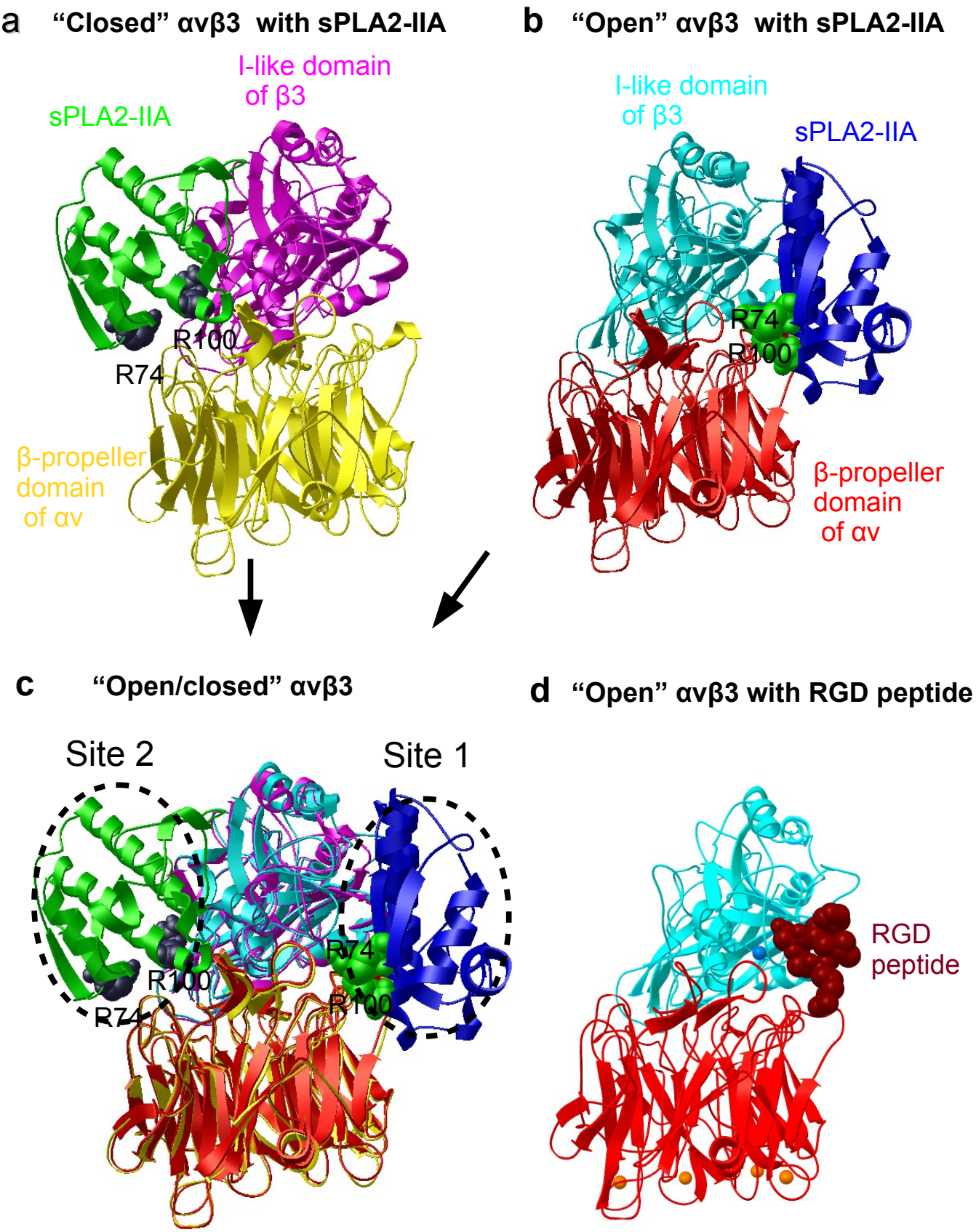
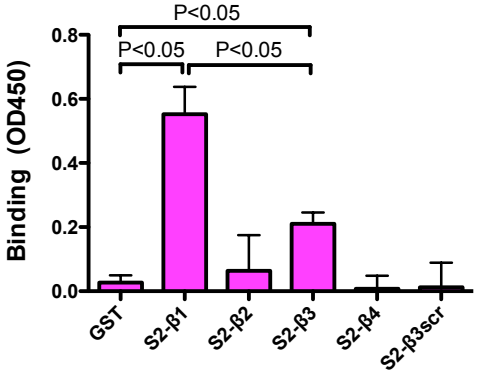


Fig. 3

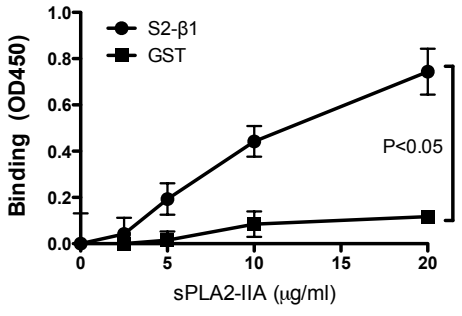
a Alignment of site 2 peptides

```
β1  LPNDGQCHLE-NNMYTMSHYY (275-294)
β2  TPNDGRCHLE-DNLYKRSNEF (258-277)
β3  QPNDGQCHVGSDNHYSASTTM (267-287)
β4  SRNDERCHLDTTGTYTQYRTQ (255-275)
```

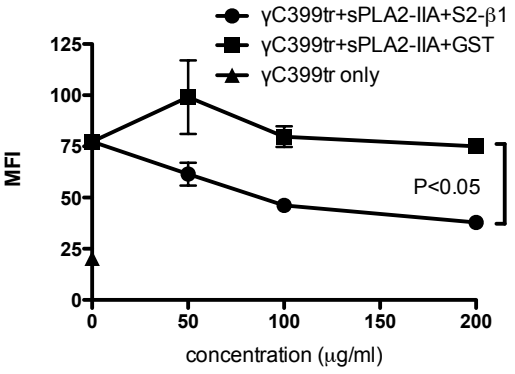
b Site 2 peptide binding to sPLA2-IIA



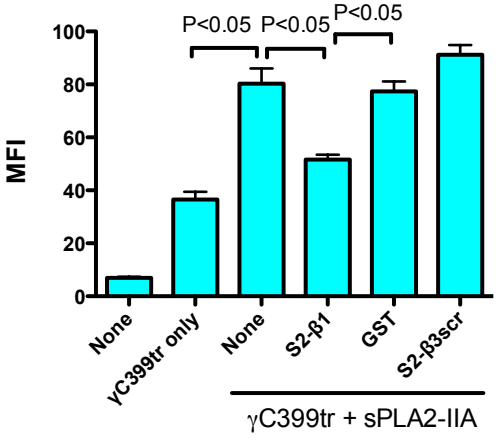
c Site 2 peptide binding to sPLA2-IIA



d U937



e αvβ3-K562



f β3-CHO

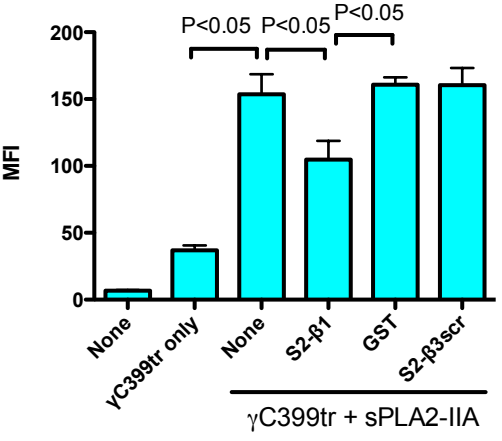
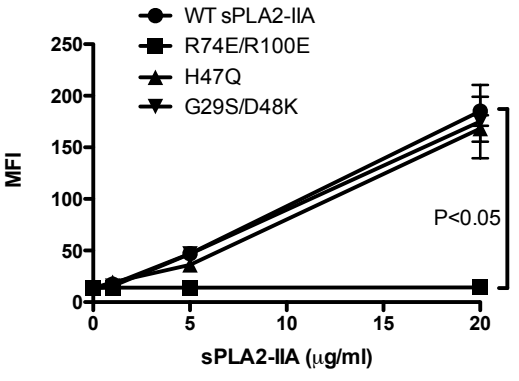
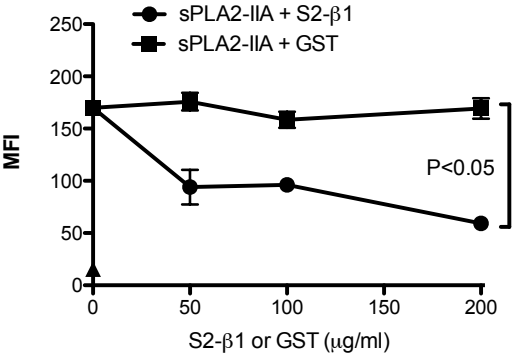


Fig. 4

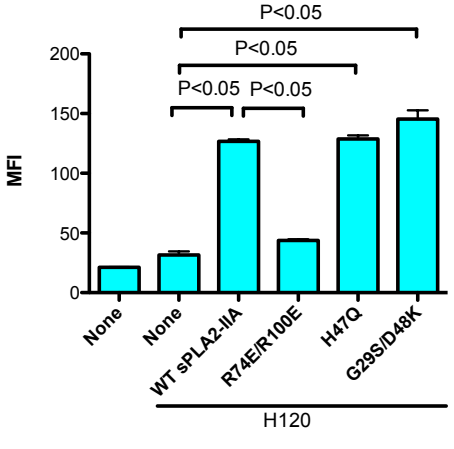
a U937



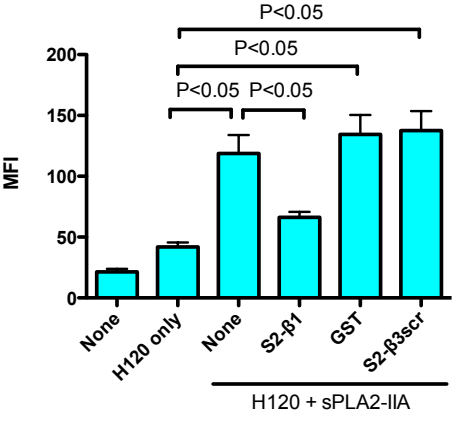
b U937



c α4-K562



d α4-K562



e α4-CHO

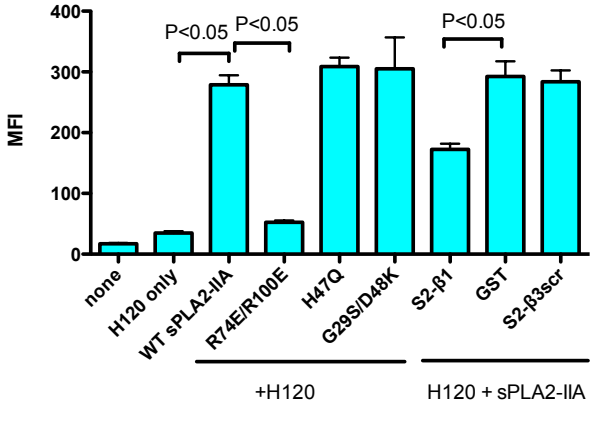


Fig. 5

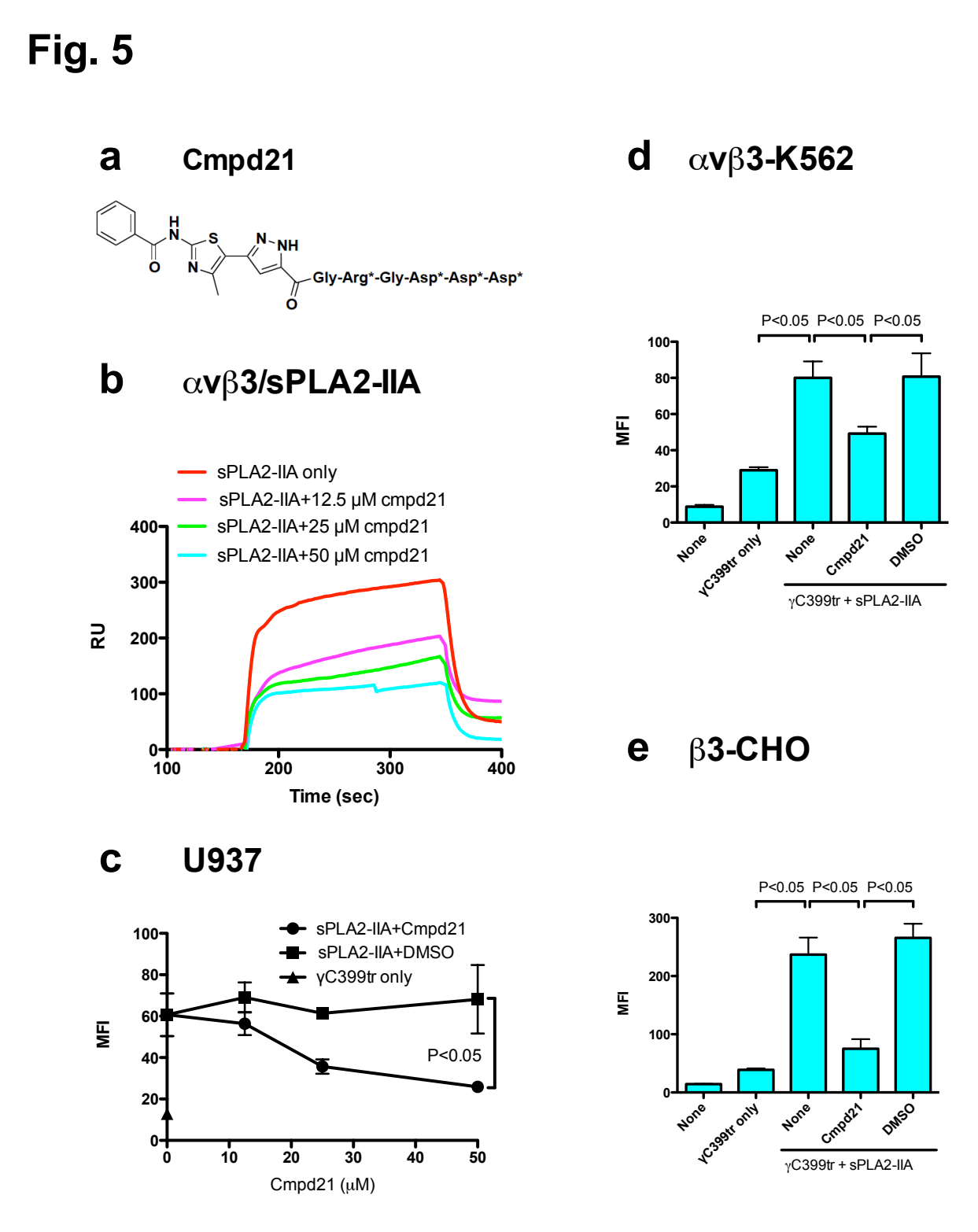

a
Cmpd21
Gly-Arg*-Gly-Asp*-Asp*-Asp*
b
αvβ3/sPLA2-IIA
sPLA2-IIA only
sPLA2-IIA+12.5 μM cmpd21
sPLA2-IIA+25 μM cmpd21
sPLA2-IIA+50 μM cmpd21
RU
Time (sec)
c
U937
sPLA2-IIA+Cmpd21
sPLA2-IIA+DMSO
γC399tr only
MFI
P<0.05
Cmpd21 (μM)
d
αvβ3-K562
P<0.05 P<0.05 P<0.05
MFI
None
γC399tr only
None
Cmpd21
DMSO
γC399tr + sPLA2-IIA
e
β3-CHO
P<0.05 P<0.05 P<0.05
MFI
None
γC399tr only
None
Cmpd21
DMSO
γC399tr + sPLA2-IIA

Fig. 6

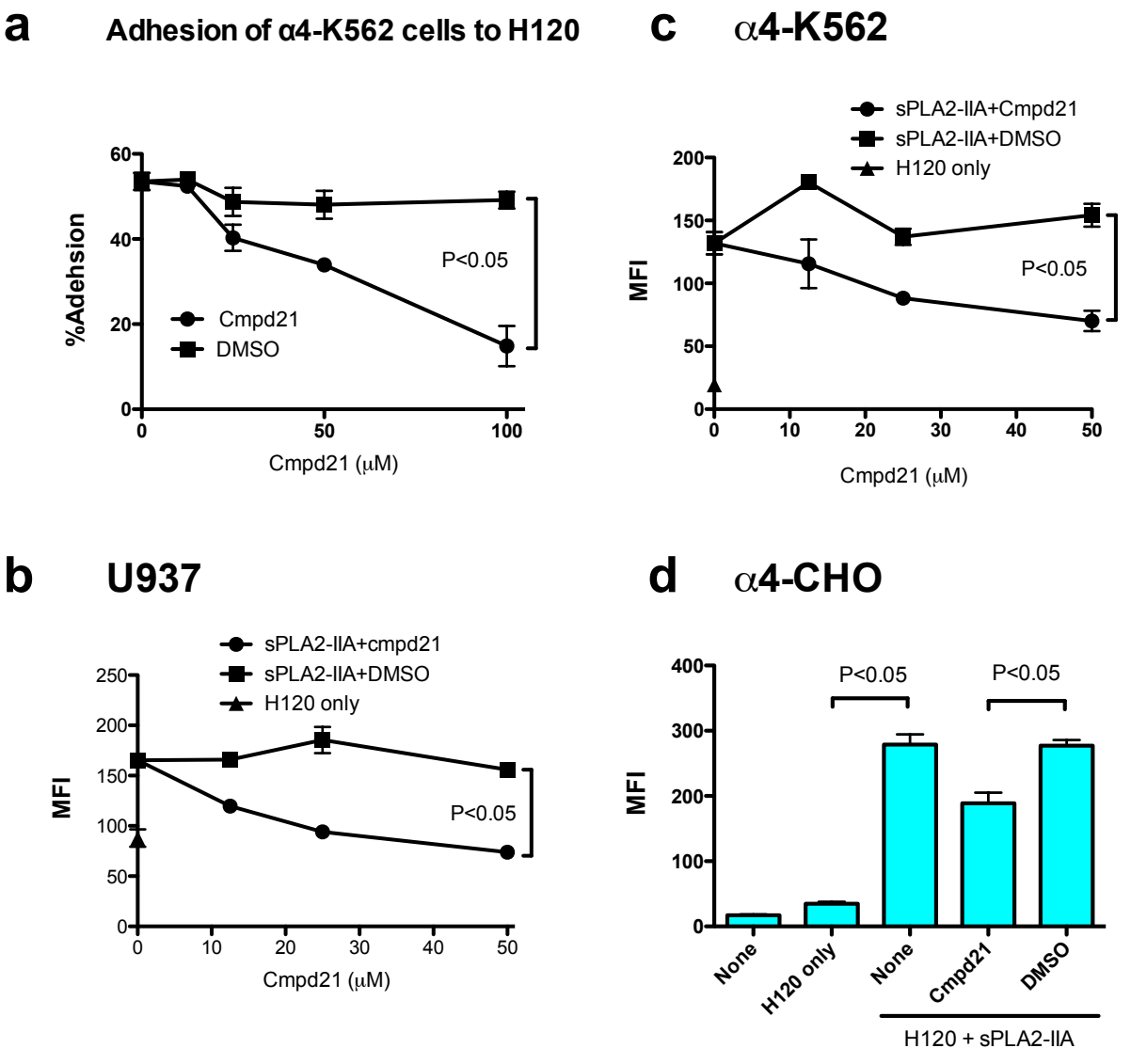

a
Adhesion of α4-K562 cells to H120
%Adehsion
60
40
20
0
0
50
100
Cmpd21 (μM)
P<0.05
Cmpd21
DMSO
c
α4-K562
sPLA2-IIA+Cmpd21
sPLA2-IIA+DMSO
H120 only
MFI
200
150
100
50
0
0
10
20
30
40
50
Cmpd21 (μM)
P<0.05
b
U937
sPLA2-IIA+cmpd21
sPLA2-IIA+DMSO
H120 only
MFI
250
200
150
100
50
0
0
10
20
30
40
50
Cmpd21 (μM)
P<0.05
d
α4-CHO
MFI
400
300
200
100
0
P<0.05
P<0.05
None
H120 only
None
Cmpd21
DMSO
H120 + sPLA2-IIA

Fig. 7

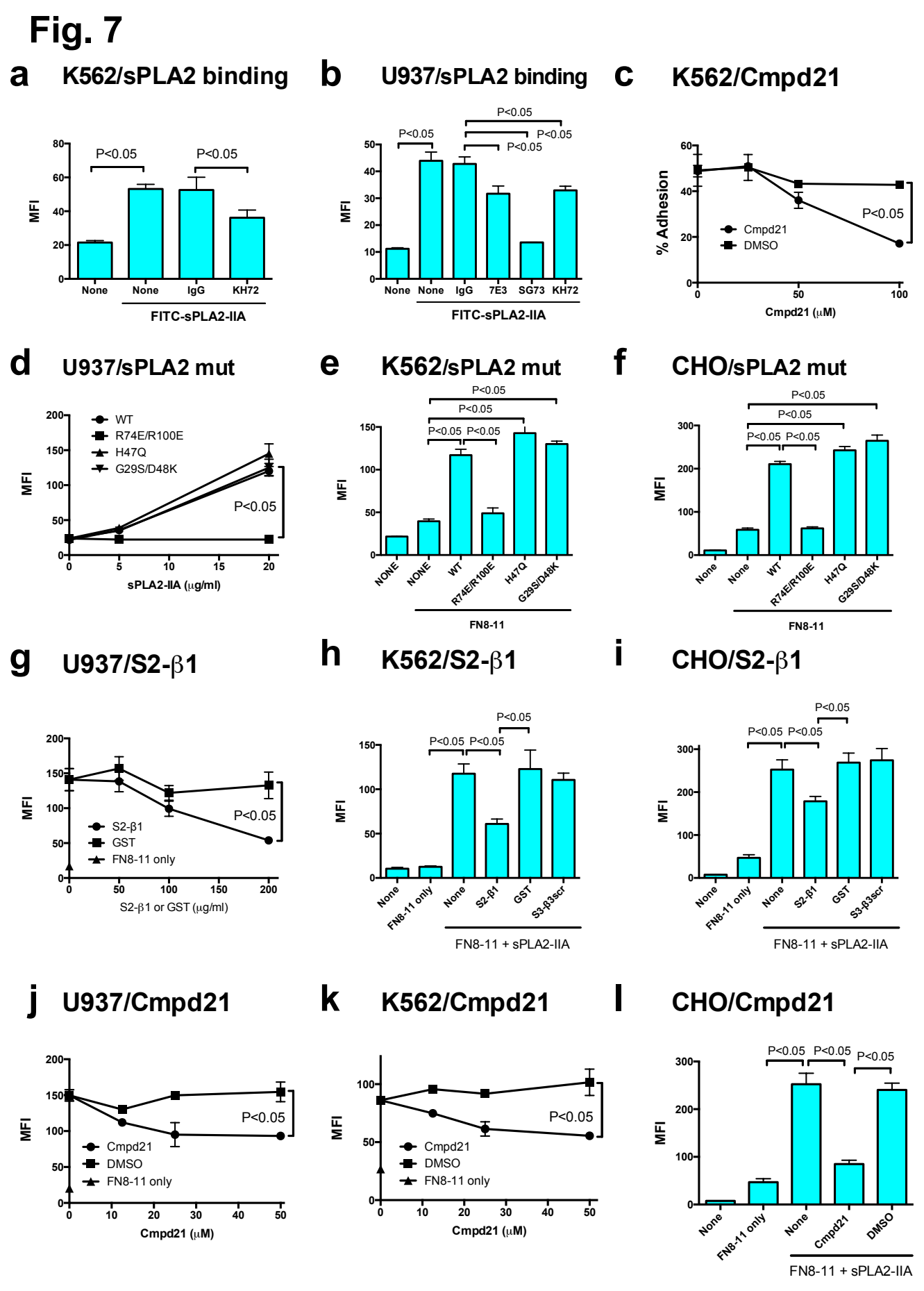
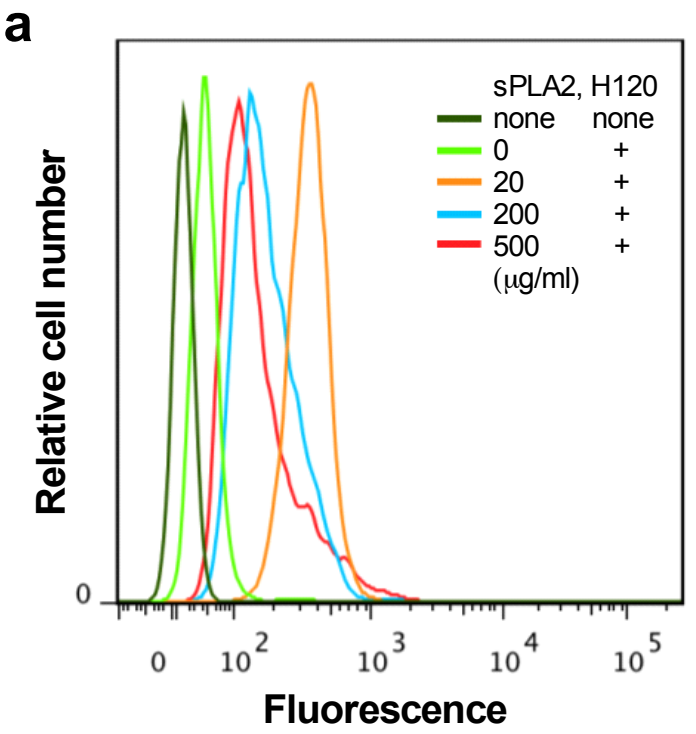

a
K562/sPLA2 binding
MFI
P<0.05
P<0.05
None
None
IgG
KH72
FITC-sPLA2-IIA
b
U937/sPLA2 binding
MFI
P<0.05
P<0.05
P<0.05
P<0.05
None
None
IgG
7E3
SG73
KH72
FITC-sPLA2-IIA
c
K562/Cmpd21
% Adhesion
Cmpd21
DMSO
P<0.05
Cmpd21 (μM)
d
U937/sPLA2 mut
MFI
WT
R74E/R100E
H47Q
G29S/D48K
P<0.05
sPLA2-IIA (μg/ml)
e
K562/sPLA2 mut
MFI
P<0.05
P<0.05
P<0.05
P<0.05
NONE
NONE
WT
R74E/R100E
H47Q
G29S/D48K
FN8-11
f
CHO/sPLA2 mut
MFI
P<0.05
P<0.05
P<0.05
P<0.05
None
None
WT
R74E/R100E
H47Q
G29S/D48K
FN8-11
g
U937/S2-β1
MFI
S2-β1
GST
FN8-11 only
P<0.05
S2-β1 or GST (μg/ml)
h
K562/S2-β1
MFI
P<0.05
P<0.05
P<0.05
None
FN8-11 only
None
S2-β1
GST
S3-β3scr
FN8-11 + sPLA2-IIA
i
CHO/S2-β1
MFI
P<0.05
P<0.05
P<0.05
None
FN8-11 only
None
S2-β1
GST
S3-β3scr
FN8-11 + sPLA2-IIA
j
U937/Cmpd21
MFI
Cmpd21
DMSO
FN8-11 only
P<0.05
Cmpd21 (μM)
k
K562/Cmpd21
MFI
Cmpd21
DMSO
FN8-11 only
P<0.05
Cmpd21 (μM)
l
CHO/Cmpd21
MFI
P<0.05
P<0.05
P<0.05
None
FN8-11 only
None
Cmpd21
DMSO
FN8-11 + sPLA2-IIA

Fig. 8


a
Relative cell number
sPLA2, H120
none none
0 +
20 +
200 +
500 +
(μg/ml)
0
0 10^2 10^3 10^4 10^5
Fluorescence

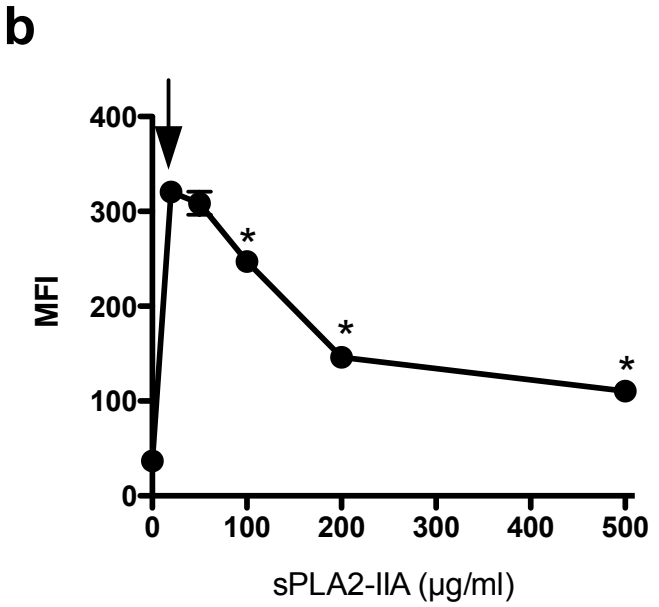

b
MFI
400
300
200
100
0
*
*
*
0 100 200 300 400 500
sPLA2-IIA (μg/ml)

Fig. 9