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Selective inhibition of human group IIA secreted phospholipase A_2 (hGIIA) signaling reveals arachidonic acid metabolism is associated with colocalization of hGIIA to vimentin in rheumatoid synoviocytes *.

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*Running title: *Functionally selective inhibition of human secreted phospholipase A_2.*

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Background: Group IIA secreted phospholipase A_2 (hGIIA[&]) is a bifunctional protein that regulates arachidonic acid metabolism by both catalysis-dependent and catalysis–independent mechanisms.
Results: Selective inhibition of the catalysis-independent signaling function perturbs a hGIIA-vimentin interaction in rheumatoid synoviocytes.
Conclusion: The signaling and catalytic functions of hGIIA are pharmacologically separable.
Significance: Functionally selective inhibitors of hGIIA may provide new avenues for

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investigation and treatment of immune-mediated inflammation.

SUMMARY

Human group IIA secreted phospholipase A_2 (hGIIA) promotes tumor growth and inflammation, and can act independently of its well-described catalytic lipase activity via an alternative poorly understood signaling pathway. With six chemically diverse inhibitors we show that it is possible to selectively inhibit hGIIA signaling over catalysis and X-ray crystal structures illustrate that signaling involves a pharmacologically distinct surface to the catalytic site. We demonstrate in rheumatoid fibroblast-like synoviocytes that non-catalytic signaling is associated with rapid internalization of the enzyme and colocalization with vimentin. Trafficking of exogenous hGIIA was monitored with immunofluorescence studies, which revealed that vimentin localization is disrupted by inhibitors of signaling that belong to a rare class of small molecule inhibitors that modulate protein-protein interactions. This study provides structural and pharmacological evidence for an association between vimentin, hGIIA and arachidonic acid metabolism in synovial inflammation, avenues for selective interrogation of hGIIA signaling and new strategies for therapeutic hGIIA inhibitor design.

Phospholipase A_2 (PLA_2) enzymes (EC 3.1.4.4) catalyze the hydrolysis of the *sn-2* ester bond of glycerophospholipids, thereby regulating the release of biologically active fatty acids and lysophospholipids from membrane phospholipid pools. These lipids are further metabolized within cells to over 100 potent autocrine and paracrine lipid mediators. The eicosanoids, which are products of arachidonic acid metabolism by cyclooxygenase, lipoxygenase and P450 epoxygenase enzymes or by non-enzymatic oxidation, are well-recognized modulators of both cell and organ function (1-3). Cell-type-specific regulation of the production, concentration and relative abundance of individual lipid mediators in response to external stimuli serves as an integrated, self-referent and self-limiting biochemical signaling network in higher organisms. This lipid-signaling network regulates embryonic stem cell differentiation and development and in adult organisms, maintains physiological homeostasis in disparate organs such as the gastrointestinal, cardiovascular, renal, reproductive, nervous and immune systems.

In recent decades, manifold PLA_2 proteins with widely divergent selectivity for phospholipid substrate and diverse mechanisms by which their production and enzyme activity are regulated have been discovered in mammals. There are 22 human PLA_2 enzymes, which are divided into 15 subgroups defined by structural similarity, catalytic mechanism, calcium requirement and whether they are secreted (4). This structural and functional diversity within a single enzyme class suggests that the activation and expression of PLA_2 enzymes may be an important control point at which the responsiveness of lipid mediator biosynthesis pathways to external stimuli is regulated and highlights the need for a more detailed understanding of the function and expression of each enzyme.

Aberrant control of eicosanoid signaling contributes to a wide range of human pathological states from headaches to certain cancers. In this context, PLA_2 enzymes have been targeted in the hope that limiting substrate supply rather than selectively blocking the production of particular mediators may be of therapeutic benefit (4). The secreted enzymes ($sPLA_2$s) (5) have been most aggressively targeted thus far. A pan Group IIA/ Group V / Group X $sPLA_2$ inhibitor has been tested in clinical trials in sepsis (6), rheumatoid arthritis (7) and coronary artery disease (8) with limited success, likely due to the lack of subtype selectivity of this compound and further highlighting a need for a better understanding of the selectivity of these inhibitors and the function of $sPLA_2$ subtypes in eicosanoid metabolism *in vivo*.

The molecular basis for Group IIA $sPLA_2$ (hGIIA) catalysis is well characterized (5) and can be potently inhibited with substrate analogues or by covalent modification of active

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site residues. However, recent studies show that hGIIA is a bifunctional protein contributing to arachidonic acid metabolism in cells derived from pathological states by acting both as an enzyme capable of releasing arachidonic acid from microvesicles shed by activated cells and as a catalytic activity-independent indirect amplifier of intracellular eicosanoid production through upregulation of cytosolic PLA_2-cyclooxygenase-2 (COX-2) pathway (9). The bifunctional nature of hGIIA in regulating arachidonic acid metabolism raises important questions about the relative contribution of each function to the production of inflammatory mediators and the subsequent promotion of inflammation. Further the findings raise the possibility of selective pharmacological inhibition of each function as a means of investigating the importance of these roles.

Neither the mechanism of hGIIA-mediated signaling, nor the relative importance of the signaling function in the promotion of inflammation is known. Here we characterize a chemically diverse set of hGIIA inhibitors in each of two functionally selective assays. We show that there is no correlation between the potency of inhibitors in blocking hGIIA catalysis and their potency in blocking prostaglandin E_2 (PGE_2) production in human rheumatoid fibroblast-like synoviocytes (FLSs), which is a catalytic activity-independent function of hGIIA. Furthermore, we identify three classes of hGIIA inhibitors; those that are (i) selective for catalysis, (ii) non-selective, or (iii) selective for catalysis-independent activity and determine the crystal structures of hGIIA bound to class (i) and (ii) inhibitors. In combination with the functional assays, these structures indicate that inhibitors of hGIIA signaling in rheumatoid FLS cells operate by perturbing a vimentin-binding surface on hGIIA. Finally we demonstrate that the catalysis-independent function of exogenous hGIIA is associated with rapid internalization and colocalization with vimentin in FLSs that is dramatically disrupted by both dual-function and signaling-selective inhibitors but not by catalysis-selective inhibition. Thus, these inhibitors of hGIIA-signaling belong to a rare class of small molecule inhibitors that act by modulating protein-protein interactions. The structures also suggest that the signaling and catalytic actions of hGIIA may be mutually exclusive with transition between the two modes of action being regulated by a conformational switch that is unique to the hGIIA subtype involving displacement of His6. These results provide a solid foundation for the design of further functionally-selective inhibitors that will be useful in assessing how the pathophysiology of $sPLA_2$ segregates into its catalytic or non-catalytic actions *in vivo* and in turn the feasibility of developing efficacious $sPLA_2$ inhibitors.

EXPERIMENTAL PROCEDURES.

Materials. hGIIA protein was expressed using the stably-transfected hGIIA-expressing CHOK1 cell line 5A2 (10) grown on alginate beads and cultivated in a stirred fermenter with 10 liter working volume. hGIIA was purified and quantified as described previously (10). LY311727 was a gift from Eli Lilly and Co (Indianapolis, IN), KH064 was purchased from Sigma (Sydney, Australia), Me-Indoxam, an indole derivative of the indolizine inhibitor Indoxam (11) was synthesized as described (12). The cyclic peptide inhibitors cyclo-FLSYR and cyclo-2-Nal-LS-2-Nal-R were synthesized by Auspep (Melbourne, Australia)

Enzyme activity assay. hGIIA catalytic activity was measured with a colorimetric microtiter-plate, mixed-micelle assay (13) using diheptanoylthiophosphatidylcholine as substrate (Cayman Chemical, Ann Arbor, MI) as described (9) with the following modifications. Absorbance was read at 405 nm over 70-90 minutes on a SPECTRAmax 250 microtiter plate spectrophotometer. Statistical data analysis was performed on Microsoft® Excel and curve fitting and kinetic data analysis was performed using Sigma Plot (SYSTAT, San Jose CA, USA). Purified hGIIA had a specific activity of 27.8 ± 2.3 μmol diheptanoylthiophosphatidyl-choline/min./mg. protein.

hGIIA signaling assay. FLS cells were isolated and cultured from synovial tissue of patients diagnosed with rheumatoid arthritis (14) undergoing joint surgery at St Vincent's Hospital, Sydney, using procedures approved by the St Vincent's Hospital Ethics Committee as described (9,10). Cells (passage three-ten) were

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plated on 96 well microtiter plates (Greiner), grown to 85 - 95% confluence and stimulated for 18 hours in serum free media (DMEM/Ham's F-12 with 0.1% BSA, 200 µL) with 10 ng/mL TNF (20 µL). hGIIA (20 µL, 4 µg/mL) with or without inhibitor was added simultaneously with TNF in inhibitor assays and with various inhibitor concentrations (0.3 - 10 µg/mL) in dose response experiments as indicated. Inhibitors (dissolved in DMSO and diluted in serum free media) were added (20 µL) where appropriate. Culture medium PGE_2 was quantified by enzyme immunoassay (Cayman Chemical). Cells were detached with 0.05% (w/v) trypsin-0.53 mM EDTA and triplicate wells were combined and centrifuged at 8000 x g for 10 minutes at 4°C. Cold lysis solution (1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS and 1 mM ethyleneglycol-bis(*p*-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) in PBS with freshly added protease inhibitors 50 µg/mL aprotonin, 200 µM leupeptin and 1 mM phenylmethylsulphonyl fluoride (PMSF)) was added to the cells and incubated overnight at 4°C. PGE_2 was normalized for cell count, quantified by measuring total protein in lysates with a detergent compatible protein assay (Bio-Rad). Independent experiments were performed using cells derived from separate patients.

Crystallization. Pure concentrated hGIIA (5 - 20 mg/mL) was buffer exchanged into crystallization buffer containing 0.1 M Tris-HCl pH 7.4, 10 mM $CaCl_2$, 0.5 mM β-octyl glucoside (βOG) and 2 M NaCl precipitate, with 5 kDa Ultrafree-MC Centrifugal Filter Units (Millipore, Billerica Massachusetts, USA). Where appropriate, purified hGIIA (1 mg/ml) was chemically modified with *p*-bromophenacyl bromide (BPB) (Sigma, Sydney, Australia) by coincubation at room temperature overnight with 1 mM BPB in 0.1 M Tris-HCl pH 8.0, 0.1 M NaCl and 0.7 mM EDTA. The hGIIA-BPB complex was then exchanged into crystallization buffer containing 0.1 M Tris-HCl pH 7.4, 10 mM $CaCl_2$, 0.5 mM βOG and 1 M NaCl, concentrated 10 fold then flash frozen in liquid nitrogen and stored at -80°C for use in activity assays or crystallization trials.

Crystals were grown by the hanging-drop vapor-diffusion method using 24-well tissue-culture plates (Linbro, ICN Biomedicals, Ohio). Drops were equilibrated with crystallization buffer with higher NaCl concentrations between 3 - 5 M. Cover slips were purchased pre-siliconized from Hampton Research (Laguna Niguel, CA, USA). 2 µL drops were used in the presence of the inhibitor at approximately two to five fold molar excess over protein. Solutions and tissue-culture plates were all stored at crystal growth temperature (293 K) in a Sanyo MIR-2530 incubator (Sanyo Electric, Japan) unless otherwise stated. Macroseeding was performed in some instances by immersing nylon loops or human hair into drops containing crushed native crystals then streaking the hair across fresh drops containing enzyme with inhibitor.

Structure Determination. Crystals were mounted onto a nylon loop and flash frozen in liquid nitrogen without cryoprotectant and stored in liquid nitrogen for data collection. X-ray diffraction data was collected ($\Delta\varphi = 0.5°$) at a wavelength of 0.9002 Å with the crystals under a nitrogen stream at 100 K at beamline 14-BMC at the Advanced Photon Source (APS) in Chicago IL, USA, on a Area Detector Systems Corporation (ADSC) Quantam-315 charged coupled device (CCD) detector. Diffraction indexed and integrated with Denzo and scaled with Scalepack using the HKL-2000 program package (15). Datasets were solved using molecular replacement with either Molrep or Phaser in the CCP4 program suite (16). Coot(17) was used to build and fit models into electron density and refinement was carried out using Refmac 5.0 in the CCP4 program suite. Models were refined to convergence before waters were added and inhibitors were not used in rounds of refinement until all identifiable waters were built. The stereochemistry of the structures were assessed using the Molprobity internet server (18) (http://molprobity.biochem.duke.edu/). Structures were overlayed using least squares refinement using Lsqkab in the CCP4 program suite. Ray traced images were generated using the PyMOL(19). All 1Fo-1Fc electron density difference maps of inhibitors were generated prior to building any atoms into the inhibitor density to minimize phase bias. The protein structure data from this publication have been

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submitted to the Protein Data Bank database and assigned the following accession codes: native hGIIA, 3U8B; hGIIA-BPB complex, 3U8I; hGIIA-KH064 complex, 3U8H; hGIIA-LY311727 complex, 3U8D.

Immunofluorescence and imaging. When indicated, FLSs (RA61 and RA57; passage 8 to 13) were treated with hGIIA (2.5 µg/mL) diluted in PBS/0.1%BSA for 2 minutes on ice. Cells were then fixed for 2.5 minutes in 4% paraformaldehyde/0.1% Triton X-100 for 10 minutes, blocked for 30 minutes in blocking solution (0.1% BSA and 10% donkey serum in PBS) and incubated with 4A1 (20) (6µg/mL), an anti-hGIIA murine monoclonal primary antibody, in blocking solution for 1 hour. Cells were washed in PBS and incubated for 30 minutes in the dark with Alexa Fluor 488 donkey anti-mouse secondary antibody (Invitrogen). After washing, cells were incubated again with 4μg/ml anti-vimentin antibody (Santa Cruz Biotechnology C-20) in blocking solution for 1 hour, washed and incubated in the dark with Alexa Fluor 555 donkey anti-goat secondary antibody for 30 minutes. Cells were then mounted in ProLong Gold antifade medium (Invitrogen). Immunofluorescence was collected using laser scanning confocal microscope (Leica TCS SP upright). Images shown are single sections taken from z-stacks acquired using the manufacturer's software. ImageJ (NIH) was used to invert colors, generate merge pictures and add scale bars. Figures were assembled and levels were adjusted uniformly using PhotoShop CS4 (Adobe).

RESULTS

Functionally selective inhibition of hGIIA. We have previously shown that exogenous addition of hGIIA causes upregulation of COX-2 expression in FLSs that, on costimulation with the cytokine TNF, causes an increase in PGE_2 production (10). Here we show that covalent modification of the active site His 48 residue with the irreversible inhibitor BPB (21), has no effect on dose dependent stimulation of PGE_2 production in rheumatoid FLS cells, yet completely abrogates enzyme-dependent hydrolysis of diheptanoylthiophosphatidylcholine (DTPC) micelles (Fig. 1A and B). Thus, these data, together with our similar observations with the activity impaired hGIIA mutant H48Q (9) establish unequivocally that hGIIA stimulation of PGE_2 production and upregulation of COX-2 occurs independently of its enzyme activity in FLSs. Indeed, two cyclic peptide hGIIA inhibitors (22) appear to selectively inhibit PGE_2 production, while being only very weak inhibitors of DTPC hydrolysis, with maximal inhibition only observed with more than $1x10^6$ fold molar excess of peptide (Fig. 1C and D). These peptides, cyclo-FLSYR (cF) and the analogue cyclo-(2-Nal)-LS-(2-Nal)-R (c2) were derived from residues 70-74 of the primary sequence of hGIIA (22,23). The ability to selectively inhibit the catalytic and non-catalytic actions of hGIIA establishes that these two functions may be pharmacologically separated and that inhibition of the signaling function of hGIIA does not depend on direct perturbation of the well-characterized His48 - Asp99 hGIIA catalytic site(5). Finally, the competitive inhibitors, Me-Indoxam (12), LY311727 (24) and KH064 (25), dose-dependently inhibited both DTPC hydrolysis, as well as PGE_2 production in FLSs (Fig. 1C and D). However, their relative potency in inhibition of catalytic activity (Me-Indoxam > LY311727 > KH064, spanning an 8-fold range in IC_{50}) appears to be unrelated to their relative potency against cell signaling (KH064 > LY311727 ~ Me-Indoxam, spanning a 2-fold range in IC_{50}). The hGIIA used in these experiments was expressed in mammalian cells, however, lipopolysaccharide (LPS) contamination would nicely explain why BPB-inactivated hGIIA and the H48Q mutant (9) elicit PGE_2 production. Importantly, this LPS hypothesis is ruled out by the fact that both cyclic peptides as well as KH064, LY311727 and Me-Indoxam block hGIIA-dependent PGE_2 production.

As hGIIA signaling is likely mediated by protein-protein interactions rather than by catalysis, inhibitors of signaling probably belong to a relatively rare class of small-molecule inhibitors that operate by disrupting surface protein interactions. To understand how inhibition of hGIIA signaling is mediated and to identify the relevant binding surface and in turn

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the relevant interacting protein, we determined the crystal structure of the native enzyme in the presence of the active site calcium and in complex with BPB, KH064 and LY311727.

The crystal structure of the hGIIA with the active site calcium. While the structure of the hGIIA without inhibitor bound has been known for well over two decades (26), remarkably there is no native structure modeled with the active site calcium ion bound, which is required for the catalytic activity of hGIIA. Furthermore, as mutations in the invariant Gly 30, and the conserved Leu 31 of the calcium binding loop affect $sPLA_2$ binding to its M-type receptor (27), the presence of the calcium may affect the non-catalytic function of hGIIA. To address this, we crystallized hGIIA in similar conditions to those used by Scott *et al*. (28), with 10 mM $CaCl_2$. Hexagonal crystals grew overnight (spacegroup $P6_122$) and diffraction data were collected to 2.26 Å resolution. Diffraction data, refinement and model validation statistics are summarized in Table 1 and 2 respectively. There was a strong density peak in the active site calcium-binding site. The center of the peak was approximately 2.3 Å from the carbonyl oxygen atoms of His 28, Gly 30, Gly 32 and the carboxylate moiety of Asp 49 (Fig. 2 A, B). This typifies the expected stereochemistry of a calcium-binding site. A calcium ion was modeled into the peak and its occupancy refined to 63%, which resulted in minimal residual 1Fo-1Fc difference density. Attempts to refine a fully occupied water molecule in the density peak resulted in significant positive 1F-1Fc difference density.

The structure of the hGIIA-BPB complex. hGIIA was chemically modified with BPB and crystallized. The crystallographic structure of BPB-inhibited hGIIA was determined from X-ray diffraction data to 1.1 Å resolution from these crystals and has two molecules in the asymmetric unit of a $P4_1$ unit cell. This is the highest resolution crystal structure of any PLA_2 enzyme and individual atoms are clearly resolvable within the polypeptide chain. Diffraction data, refinement and model validation statistics are summarized in Table 1 and 2 respectively. BPB is covalently bound to the Nδ of the active site His 48 residue and found wholly within the active site cavity (Fig. 2C, D, Supplemental Fig. 1). BPB binds in the same way to bovine pancreatic (29) and snake toxin $sPLA_2$ enzymes (30,31). The phenyl ring on BPB is in contact with Phe 5, Phe 93 and Gly 30 in the hydrophobic active site cavity and the bromide atom appears to cause a subtle movement in the His 6 side chain away from the active site cavity with the $N\delta^1$ deviating by approximately 1.3 Å from the native structure (Supplemental Fig. 1). Interestingly the hGIIA-BPB structure retains the active site calcium. This is unique to the BPB inhibited $sPLA_2$ structures known to date. The calcium ion is coordinated by the carbonyl oxygen atoms of His 28, Gly 30 and Gly 32 on the putative calcium binding loop (28) and a carboxyl oxygen on the Asp 49 side chain. In addition, for the first time, two structural water molecules that coordinate the active-site calcium are clearly resolvable. One of these water molecules is on the surface of the enzyme exposed to bulk solvent, the other (HOH) is in the active site cavity and is sterically clashing (2.4 Å) with the carbonyl carbon of BPB (CR) (Supplemental Fig. 1). Thus, a consequence of the presence of the active site calcium ion in a BPB modified hGIIA structure is that the active site cavity must be co-occupied by a water molecule and BPB, which forces an apparently energetically unfavorable interaction. This explains both the weaker affinity of calcium for the covalently modified enzyme complex and how mM concentrations of calcium can inhibit BPB modification (21,32,33). In addition, the active site calcium shifts away from the inhibitor in the active site cavity by 0.5 Å and subsequently the coordinating calcium binding loop has also moved substantially to compensate with Cα deviations up to 2.6 Å. Further, the Asp 49 bidentate coordination to the calcium found in the native structure is disrupted with only one of the Oδ oxygens coordinating to the calcium and the other hydrogen bonded to HOH.

The crystal structure of the hGIIA-LY311727 complex. The crystals of the hGIIA-LY311727 complex diffracted to 1.8 Å resolution and are primitive hexagonal, belonging to space group $P3_121$. The crystals were twinned with a *pseudo* $P6_122$ symmetry. Diffraction data, refinement and model validation statistics are summarized

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in Table 1 and 2, respectively. As seen in the structure of hGIIA complexed to indole 8, which differs from LY311727 by a single methyl group (24), a phosphate and carbonyl oxygen of LY311727 coordinates to the active site calcium and a primary amine makes polar contacts with His 48 and Asp 49 residues (Fig. 2E, F). In addition the hydrophobic indole and phenyl ring makes several hydrophobic interactions along the active site cavity. The additional methyl group on LY311727, absent in indole 8, is buried deep within the hydrophobic cavity and improves the surface complimentarity between the inhibitor and hGIIA. It makes additional hydrophobic contacts with Phe 5, Phe 98 and Ile 9 explaining the 2-fold higher potency of LY311727 in comparison to indole 8 (24) (Fig. 2F). Despite their similarities, the hGIIA-LY311727 structure crystallized in a different space group, with two monomers in the asymmetric unit (Fig. 2E) suggesting that LY311727 but not indole 8 binding disturbs a crystal contact.

The crystal structure of the hGIIA-KH064 complex. A structure of the hGIIA-KH064 complex was previously reported by Hansford *et al*. (25). However, the inhibitor was modeled in alternate conformations, with steric clashes with the calcium-binding loop (PDB accession code: 1KQU Supplemental Fig. 2A, B). To resolve any ambiguity in the binding mode of KH064, we independently determined the crystal structure of the same complex. As described (25), the hGIIA-KH064 complex crystallized with 2 monomers in the asymmetric unit with inhibitors bound to both monomers (Fig. 2G). The structure of the enzyme in each monomer was well preserved with an overall Cα RMS deviation of 0.16 Å. The inhibitor was clearly seen in the active site cavity of both monomers, coordinated to the active site calcium in unbiased 1Fo-1Fc difference electron density and all regions of both inhibitors were well defined and able to be modeled without ambiguity (Fig. 2H). The inhibitor comprises a charged head group and two hydrophobic tails mimicking the *sn*-1 and *sn*-2 fatty acid in a glycerophospholipid substrate. The head group and the *sn*-2 tail of both inhibitors in the asymmetric unit bound to the active site calcium and the hydrophobic cavity respectively in a similar fashion. In contrast to the published hGIIA-KH064 structure (1KQU) the conformation of the *sn*-1 tail is not in alternate conformations but in distinct complementary conformations in each monomer that do not clash with the calcium-binding loop (Fig. 2H and Supplemental Fig. 2C, D). The phenyl ring extends beyond the active site where it interacts with the equivalent moiety in the second monomer in the asymmetric unit, possibly creating a hydrophobic dimeric interface.

Inhibitors of hGIIA signaling perturb surface properties without disrupting the overall structure. We compared the structure of native hGIIA containing the active site calcium with the structures of the inactivated H48Q mutant (PDB accession code; 1N28 (34)), hGIIA-BPB, hGIIA-LY311727 and hGIIA-KH064. The overall fold of the protein was conserved across all structures indicating that inhibitors of the signaling function of hGIIA do not induce gross conformational changes (Fig. 3). However, inhibitors of hGIIA signaling (KH064 and LY311727), but not the catalytic-selective inhibitor BPB perturb the surface properties at the entrance to the active site in two ways. First, they displace the His 6 side chain resulting in the formation of a new hydrogen bond between the His 6 $N\delta^1$ and the Oδ of Glu 17 (Fig. 4A). Second, the His 6 side chain is replaced by a large hydrophobic surface created by phenyl rings of LY311727 and KH064 (Fig. 4B, C). This exposed hydrophobic surface spans approximately 40 $Å^2$ over the surface of the enzyme for the hGIIA-LY311727 complex and more than 80 $Å^2$ for the hGIIA-KH064 complex.

The surface that is perturbed by inhibitors of hGIIA signaling correlates to the vimentin-binding surface. hGIIA has a large number of solvent exposed lysine and arginine side chains on the enzyme surface, which impart an electropositive coat that is important for heparan sulfate proteoglycan binding (35). Charge reversal of clusters of proximal lysine and arginine residues on the face of hGIIA that includes the entrance to the active site cavity dramatically reduced binding to vimentin, whereas mutations on the opposite side do not (36). In addition, the V3W mutation on the N-terminal helix increased binding to vimentin three fold over wild-type. Thus, the location of

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the vimentin binding face correlates with the displaced His 6 side chain and perturbed surface in signaling-inhibited hGIIA structures (Fig. 4B-D). These data identify vimentin as a potential binding partner for hGIIA in mediating its signaling function. To test this possibility, we examined the localization of hGIIA when it was added exogenously to FLS cells.

hGIIA colocalizes with vimentin. hGIIA was applied to FLS cells grown on cover slips for a short time and its localization was determined by confocal immunofluorescence microscopy of fixed and permeabilized cells using the hGIIA-specific monoclonal antibody 4A1 (20). The antibody showed negligible staining of cells in the absence of added hGIIA (Fig. 5A). On exogenous addition, hGIIA was rapidly internalised by FLS cells and localised in the cytoplasm in filamentous strands with perinuclear staining, with negligible hGIIA detected in the nucleus. (Fig. 5B). Internalization occurred when cells were preincubated at 4ºC with no obvious effect on the time course, indicating that internalization is likely an energy independent event. Cells were also stained with anti-vimentin antibodies which showed the well-known cytoplasmic and perinuclear staining pattern seen in mesenchymal cells (Fig. 5C). In dual immunofluorescence studies, hGIIA colocalized with vimentin (Fig. 5D, Supplemental Figs 3, 4).

Signaling inhibitors perturb the hGIIA:vimentin interaction. To determine the effect of inhibitors on the hGIIA:vimentin interaction, we repeated the co-immunofluorescence microscopy experiments in the presence of catalysis selective (BPB), signaling-selective (c2 and cF) and non-selective (KH064 and LY311727) inhibitors. Apart from BPB, which becomes covalently bound to the enzyme, inhibitors were incubated with hGIIA prior to addition to FLSs and dual immunofluorescence labeling. The inhibitors had no observable effect on rapid internalization of hGIIA (Fig 6, Supplemental Fig. 5), and BPB non-modified and modified enzyme colocalized to vimentin (Fig. 6A-B, Supplemental Fig. 5A-B). In contrast, in the presence of KH064, c2 and cF, hGIIA staining was diffuse in the cytoplasm (Fig. 6 D-F, Supplemental Fig. 5D-F) rather than colocalized with vimentin as seen in the absence of inhibitor (Fig. 6C, Supplemental Fig. 5C). In the presence of LY311727, hGIIA staining was also more diffuse than control, however some colocalization with vimentin was still observable at the concentration used (arrowheads in Fig. 6G and Supplemental Fig. 5G), possibly reflecting differences in the potency of the two compounds in the signaling assay (KH064 > LY311727) relative to the enzyme inhibition assay (Fig. 1).

DISCUSSION

Recent studies have shown that hGIIA catalysis and signaling independently regulate arachidonic acid metabolism in cells depending on the biochemical context of the protein. For example, as shown here, hGIIA-dependent PGE_2 production and induction of COX-2 (9) in rheumatoid synovial cells is dependent solely on the signaling function of hGIIA. In contrast, the upregulation of prostate cancer cell proliferation by exogenous hGIIA requires the catalytic function of hGIIA (37), since abrogation of catalytic activity with the H48Q mutation also abrogates the proliferative capacity of hGIIA. Our observation that these functions may be pharmacologically separated raises the possibility of development of potent and selective inhibitors of either catalysis or signaling that are active *in vivo* that might refine the specificity of the dual-function indole inhibitors tested thus far in the clinic. Current activity-selective inhibitors such as BPB are not subtype-specific in that they covalently modify histidines of several target proteins and the structural studies presented here may aid in the design of more specific catalysis-selective inhibitors. In addition, of the ten human $sPLA_2$ enzymes, six are known to bind to protein partners (38). The $sPLA_2$ enzymes have a highly conserved tertiary structure and catalytic site, however they vary significantly in primary sequence (38) suggesting that development of functionally selective inhibitors may be possible and of benefit in dissecting out the mechanism of action of these enzymes in different biological contexts. Such inhibitors may also be useful in targeting rheumatoid arthritis, since *in vivo* animal model studies indicate that selective inhibition of hGIIA over other $sPLA_2$ enzymes is likely to be advantageous (39).

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His 6 is unique to hGIIA in the family of $sPLA_2$ enzymes. In all structures of signaling active proteins (native, hGIIA-BPB and the H48Q mutant), His 6 blocks substrate access to the active site cavity suggesting that it must be displaced for substrate binding and catalysis to occur. This is indeed the case in the crystal structure of hGIIA bound to a transition state analogue (TSA) (28). Furthermore, in the hGIIA-TSA structure the His 6 is stabilized by a hydrogen bond between His 6 $N\delta^1$ and the $O\delta$ of Glu 17, which appears to be a hallmark of signaling inhibited hGIIA in rheumatoid FLS cells (Fig. 4A). Taken together, these data suggest that catalysis and signaling may be mutually exclusive and that His 6 may uniquely act as a switch in hGIIA between its signaling and catalytic modes of action.

These structures, coupled with the pharmacological data on the inhibitors presented here, raised the possibility that signaling inhibitors perturb the binding surface between vimentin and hGIIA, consistent with the observation that LY311727 is able to inhibit vimentin binding *in vitro* (36). Our immunofluorescence studies establish that exogenous hGIIA does indeed rapidly internalize in FLSs and very rapidly co-localizes with intracellular vimentin filaments. Importantly, abrogation of catalytic activity alone is insufficient to perturb hGIIA:vimentin colocalization. Further, as predicted by the structural analysis, inhibitors that block hGIIA-mediated signaling, either selectively (c2 and cF) or non-selectively (LY311727 and KH064) also block hGIIA colocalization to vimentin.

These studies provide pharmacological and structural evidence for the interaction between vimentin and hGIIA having a role in the catalysis-independent function of hGIIA in rheumatoid FLSs that warrants further investigation. In the presence of a cytokine the intracellular enzyme $cPLA_2$-α is stimulated resulting in arachidonic acid flux to COX-2. This, together with the hGIIA-mediated increase in COX-2 protein, leads to a significant upregulation of prostaglandin production (9). Importantly, the studies imply that perturbation of this interaction is a plausible mechanism by which hGIIA-mediated eicosanoid production is blocked in these cells by both selective and non-selective signaling inhibitors. The structural data presented support such a mechanism, since inhibition of catalysis alone, without perturbing the known vimentin-binding surface of hGIIA is insufficient to block hGIIA-mediated eicosanoid production or to perturb the vimentin:hGIIA interaction. These findings identify the hGIIA-vimentin association as an important target for further study with respect to hGIIA function in regulating eicosanoid biosynthesis and raise important questions about the mechanism by which hGIIA colocalization could mediate upregulation of COX-2.

The ability of these inhibitors to interfere with hGIIA signaling and to disrupt an interaction with vimentin provides the first evidence for a possible link between vimentin binding, hGIIA function and arachidonic acid metabolism in synovial inflammation. Given the importance of hGIIA in mediating inflammation in models of arthritis (39), this mechanism also predicts a role for vimentin in arthritis pathology and identifies it as a potential target for drug development in this disease. In recent years, vimentin has been shown to play a much wider role in cell function than its traditional role as an inert cellular scaffold (40). This study extends vimentin function to a lipid metabolic pathway important in the aberrant inflammatory response induced in immune-mediated pathological states in addition to an emerging functional role for vimentin in cancer progression.

The association between vimentin and hGIIA shown here identifies vimentin as an interesting target for further study in other diseases where a role for hGIIA has been established. This is particularly true in the case of tumorigenesis. Vimentin staining is frequently used as a marker for tumor identification and progression, is a signature marker, along with COX-2, of tumor dedifferentiation through the epithelial-mesenchymal transition that is a feature of cancer (41) and is increasingly being implicated functionally in cancer pathology (42). hGIIA is overexpressed in many cancers and this expression has also been functionally implicated in mediating the pathology of certain types of cancer (43). This study suggests a potential mechanism whereby overexpression of hGIIA

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may potentiate this process that warrants further study.

When compared with small-molecule inhibitors that traditionally target catalytic sites, the manipulation of protein-protein interactions promises more versatility in the control of regulatory mechanisms. However, this approach is relatively un-charted for therapeutic agents. Targeting protein-protein interactions with small molecules is difficult as these interfaces generally lack well-defined binding pockets. Although antibody targeting of specific epitopes may be an effective means to inhibit the formation of protein complexes, these are generally expensive to produce and do not readily cross membrane bilayers. Thus, they are largely restricted to targeting extracellular epitopes (44). Furthermore, as the interface between two proteins is potentially much larger than can be covered by small molecules, an additional challenge is to target surfaces within these interfaces that will abrogate binding (45). In the case of LY311727 and KH064, the location of the active site cavity, central to the vimentin binding surface, provides a convenient and specific tether to target the hGIIA-vimentin interaction.

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FOOTNOTES.

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[&]. The abbreviations used are: hGIIA, human Group IIA secreted phospholipase A_2; PLA_2, phospholipase A_2; $sPLA_2$, secreted phospholipase A_2; COX-2, cyclooxygenase-2; PGE_2, prostaglandin E_2; FLS, fibroblast-like synoviocyte; BSA, bovine serum albumin; TNF, tumor necrosis factor; DMSO, dimethylsulfoxide; DMEM, Dulbecco's modified eagle medium; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; EGTA, ethyleneglycol-bis(*p*-aminoethyl ether)-N,N,N',N'-tetraacetic acid; PMSF, phenylmethylsulphonyl fluoride; β-OG, β-octylglucoside; BPB, *p*-bromophenacyl bromide; APS, Advanced Photon Source; ADSC, Area Detector Systems Corporation; CCD, charge coupled device; DTPC, diheptanoylthiophosphatidylcholine; cF, cyclo-(Phe-Leu-Ser-Tyr-Arg); c2; cyclo-((2-Nal)-Leu-Ser-(2-Nal)-Arg); $cPLA_2$-α; cytosolic phospholipase A_2-α, Group IVA phospholipase A_2; HOH, water.

FIGURE LEGENDS

FIGURE 1. Selective inhibition of hGIIA catalytic activity and signaling. The effect of BPB modification of hGIIA (BPB in A or hGIIA-BPB in B) relative to unmodified hGIIA (native in A or Native hGIIA in B) on catalytic activity (A.) and dose-dependent hGIIA-mediated PGE_2 production (B) is shown. (C and D) show the dose-dependent effect of non-covalent hGIIA inhibitors on native hGIIA catalytic activity and hGIIA-mediated PGE_2 production respectively. Catalytic activity was determined using a commercially available mixed-micelle colorimetric assay as described in Methods. The assay was run for

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70 - 90 minutes and the initial stable gradient was used to calculate reaction rates. Data (A and C) are presented as % of uninhibited native hGIIA activity (specific activity, 27.8 ± 2.3 µmol. DTPC/min./mg. protein) and are means ± SE of 2 - 3 independent experiments each performed in triplicate with data combined for analysis. PGE_2 production was assessed in cultured FLS cells. Triplicate wells with 90% confluent FLS cells (n=5) in DMEM/Ham's F12 containing 0.1% BSA were stimulated with TNF (10 ng/mL) together with indicated concentrations of either BPB-modified hGIIA or native hGIIA (B). In panel D, native hGIIA with inhibitors at the indicated concentrations was added to cells along with TNF. In all cases cells were incubated for 16 - 20 hours. PGE_2 concentrations were determined in triplicate from harvested media using a PGE_2 EIA as described in Experimental Procedures. Cells were then harvested and PGE_2 production was normalized for protein concentration of the total cell lysate of 3 combined wells, as determined by a detergent compatible protein assay. Data are the mean ± SE of 2-5 independent experiments performed in triplicate on primary cells from 2-3 different patients with data combined for analysis.

FIGURE 2. Crystal structures of native and inhibited hGIIA structures. The asymmetric units of native (A), BPB modified (C), LY311727 bound (E), and KH064 bound (G) hGIIA are rendered in blue ribbon format with active site calcium ions shown as green spheres and inhibitors in yellow in stick format. Where applicable, Cα RMSD values from the native structure are shown underneath their corresponding chains. (B) depicts the coordination of the active site calcium in the native hGIIA structure. Unbiased 1fo-1fc density of BPB (D), LY311727 (F) and KH064 (H) calculated before the inclusion of inhibitors in rounds of refinement are displayed in green mesh contoured at 3 σ. Final 2fo-1fc density is also contoured at 1.5 σ around selected residues in (D).

FIGURE 3. Inhibition of hGIIA activity does not alter its overall structure. Backbone traces of native hGIIA (white), the H48Q mutant (green) and when bound to BPB (yellow), LY311727 (blue) and KH064 (red) are overlayed in (A). Calcium ions are rendered as spheres. (B) shows a trace of the Cα RMSD of mutant or inhibited structures from the native enzyme.

FIGURE 4. Structural changes induced by inhibitors of hGIIA signaling. (A) His 6 is displayed in white for signaling active hGIIA structures (native, H48Q mutant and hGIIA-BPB) and in blue for signaling inhibited structures (hGIIA-LY311727 and hGIIA-KH064) The hydrogen bond formed between His 6 and Glu 17 of the hGIIA-LY311727 structures is also labeled. Inhibitors are shown in yellow. Arginine and Lysine mutations that inhibit or alter hGIIA binding to vimentin are colored in green in (B-D) and those that do not inhibit vimentin binding are colored in red. The displaced His 6 side chain and the location of the V3W mutation that increases affinity to vimentin are colored in magenta and blue respectively on the hGIIA-KH064 (B) and the hGIIA-LY311727 (C) complexes. The opposite face of hGIIA-LY311727 is displayed in panel D.

FIGURE 5. hGIIA internalizes rapidly and co-localizes with vimentin. Immunofluorescence microscopy on cells stained with anti-hGIIA antibody (4A1 (20)) and/or anti-vimentin antibody. (A) Cells were stained for hGIIA primary antibody alone and subsequently with both 488 and 555 secondary antibodies in the absence of hGIIA treatment and imaged in both 488nm (hGIIA) and 555nm (vimentin) channels.. Exogenous hGIIA was applied and cells were stained for (B) hGIIA alone, (C) vimentin alone (C) or hGIIA and vimentin in (D) and imaged as in A as described in Experimental Procedures. Cells were RA61 human rheumatoid synovial fibroblast cells (FLSs). Columns 1-3 show vimentin (green), hGIIA (red) and a merge image, respectively. Scale bars represent 20µm.

FIGURE 6. Functionally selective inhibition of vimentin colocalization. Immunofluorescence microscopy on RA61 FLSs treated with (A) exogenous hGIIA alone or (B) hGIIA incubated with BPB. In a separate

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experiment, RA61 FLSs were treated with (C) exogenous hGIIA alone, (D) hGIIA incubated with KH064, (E) hGIIA incubated with c2, (F) hGIIA incubated with cF or (G) hGIIA incubated with LY311727. Staining and imaging of both hGIIA and vimentin was performed in all experiments as in Figure 5 and described in Experimental Procedures. Columns 1-3 show vimentin (green), hGIIA (red) and a merge image, respectively. Scale bars represent 20 μm.

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Table 1 Data Collection and Refinement Statistics*

hGIIA	Native	BPB	KH064	LY311727
Data collection				
Space group	$P6_122$	$P4_1$	$P3_1$	$P3_121$
Cell dimensions				
a, *b*, *c* (Å)	74.7, 74.7, 89.2	70.4, 70.4, 47.3	74.8, 74.8, 49.9	74.4, 74.4, 93.7
α, β, γ (°)	90.0, 90.0,120.0	90.0, 90.0, 90.0	90.0, 90.0, 120.0	90.0, 90.0, 120.0
Resolution (Å)	36.7-2.30(2.42-2.30)	40.0-1.10(1.16-1.10)	29.9-2.30(2.42-2.30)	19.8-1.80(1.90-1.80)
R_{merge}	6.6(67.0)	13.5(56.6)	5.5(24.2)	7.9(48.2)
Mean I / σI	23.0(4.0)	7.5(2.1)	11.8(3.4)	18.3(3.7)
Completeness (%)	99.3(99.3)	99.9(99.9)	97.8(98.5)	99.9(98.4)
Redundancy	10.4(11.1)	4.3(3.4)	2.8(2.8)	4.7(4.5)
Refinement				
Resolution (Å)	24.4-2.3	34.3-1.10	24.8-2.30	19.8-1.80
No. reflections	6909	91537	12907	28010
R_{work} / R_{free}	20.7/25.7	16.5/18.6	19.2/22.5	12.1/18.9
No. atoms (no H)	1023	2430	2093	2358
Average *B*-factor	66.7	16.6	21.9	20.2
R.m.s. deviations				
Bond lengths (Å)	0.018	0.010	0.008	0.016
Bond angles (°)	1.77	1.33	1.10	1.21

*Values in parentheses are for highest-resolution shell.

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Table 2. Molprobity Structure Validation Statistics.

	Native hGIIA	**hGIIA-BPB**	**hGIIA-KH064**	**hGIIA-LY311727**
Clashscore[&], all atoms:	4.32	5.42	3.7	6.2
Rotamer outliers	0.00%	0.00%	0.48	0.48
Ramachandran outliers	0.00%	0.00%	0.00%	0.41
Ramachandran favored	97.5%	97.0%	97.13	96.7%
Cβ deviations >0.25Å	0	0	0	0
Overall molprobity score*	100th n=8821(2.3±0.25Å)	70th n=159 (0.09-1Å)	100th n=8909(2.3±0.25Å)	92nd n=11256(1.8Å±0.25Å)

[&] Clash score is the number of steric overlaps (> 0.4 Å) per 1000 residues
[@] Clash score only of atoms with a temperature factor of less than 40
* Values written as percentile where 100th percentile is the best among n structures of comparable resolution (in parenthesis)

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Figure 1

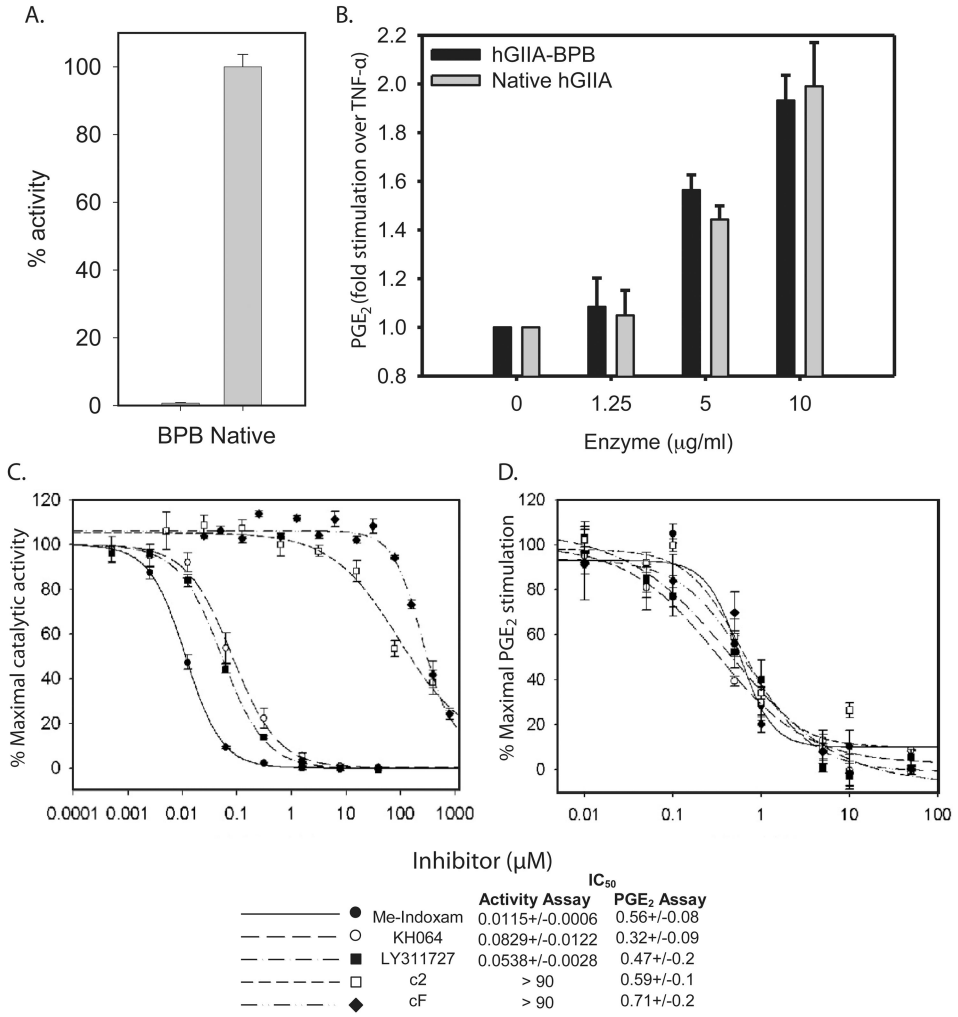

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Figure 2.

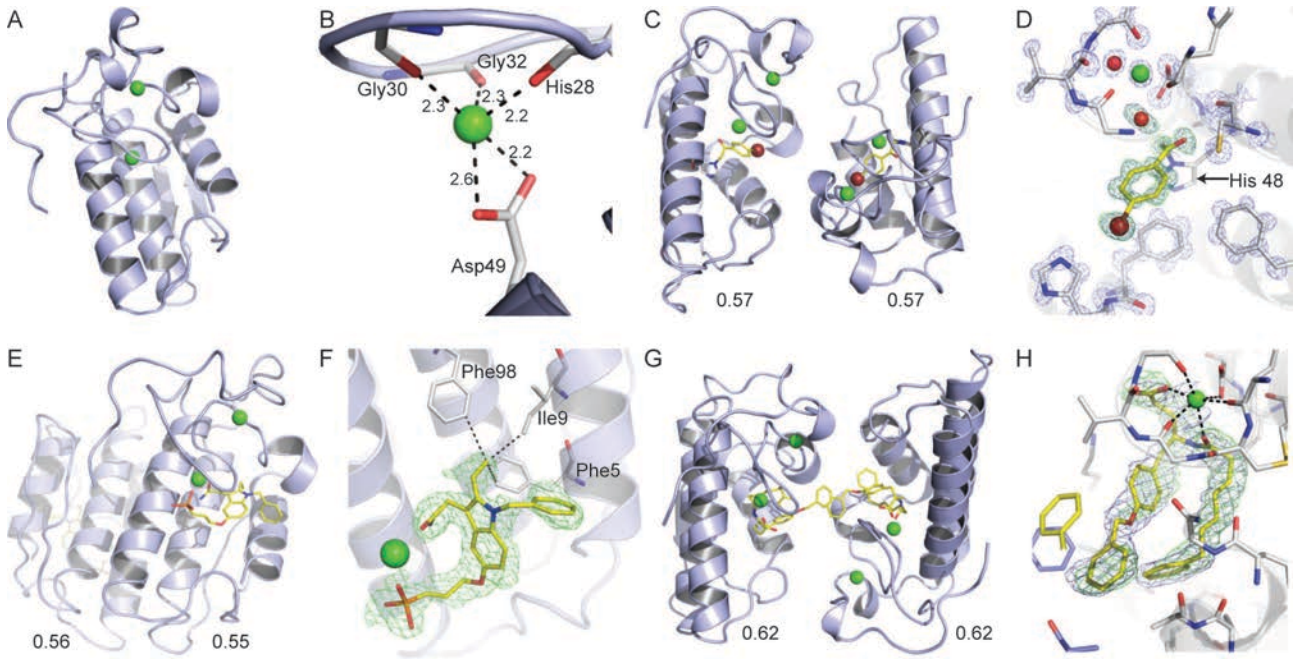

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Figure 3.

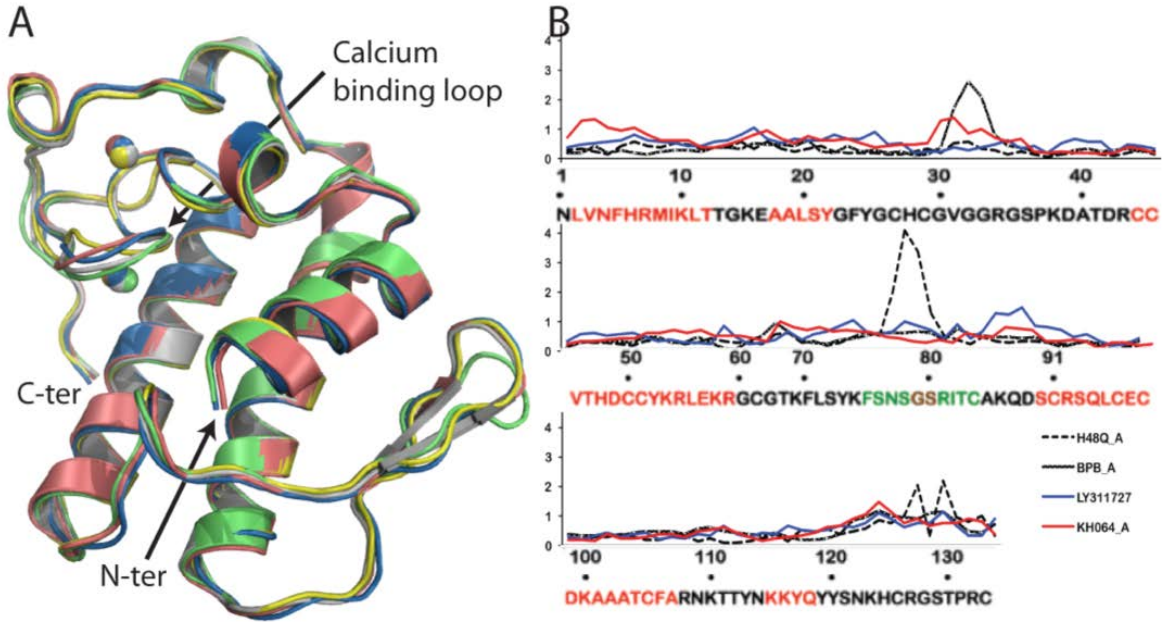

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Figure 4.

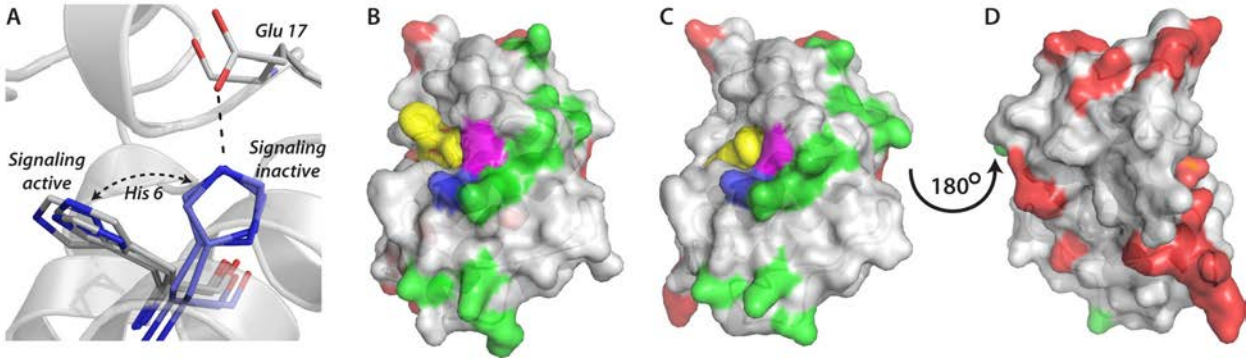

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Figure 5.

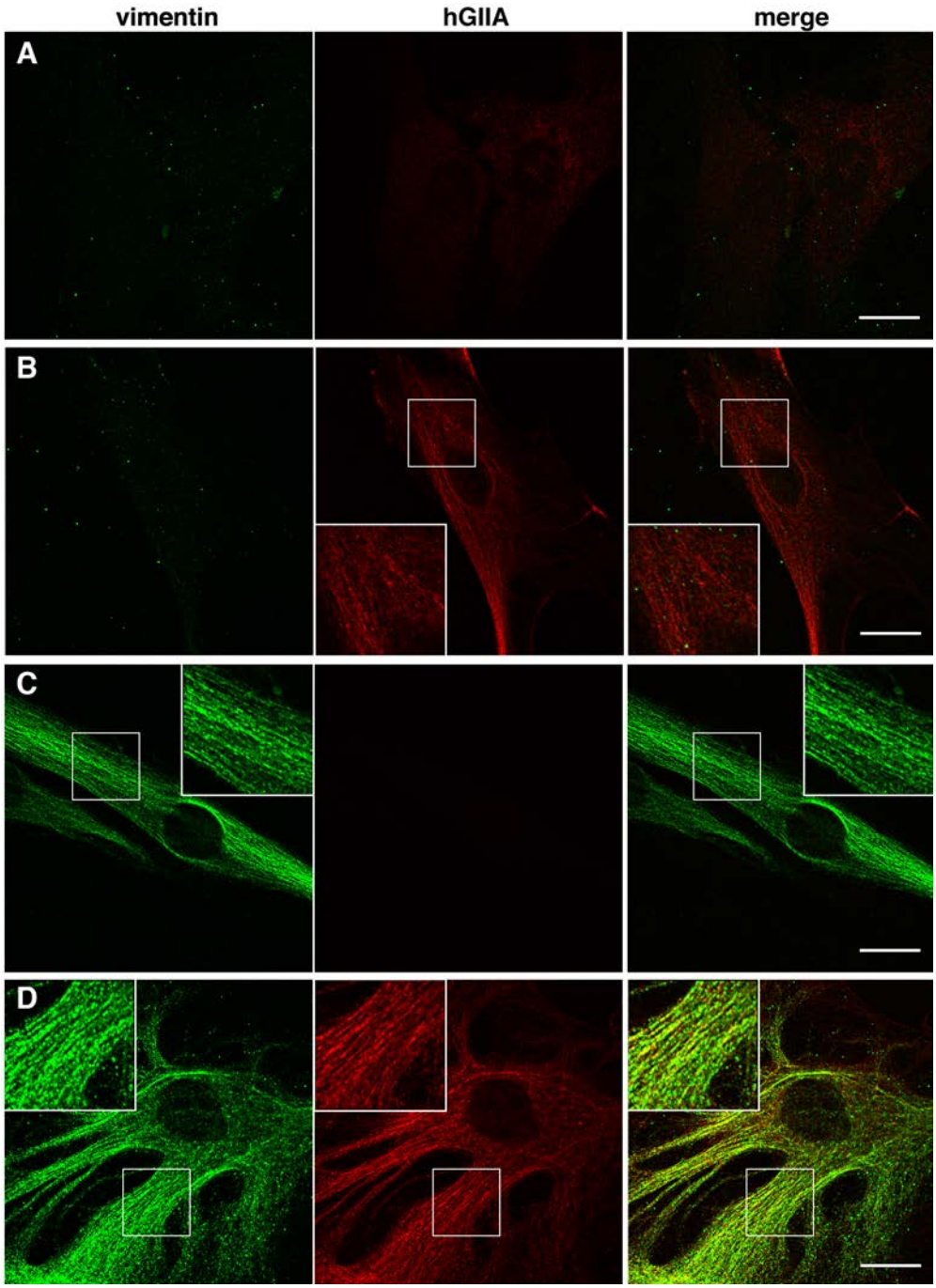

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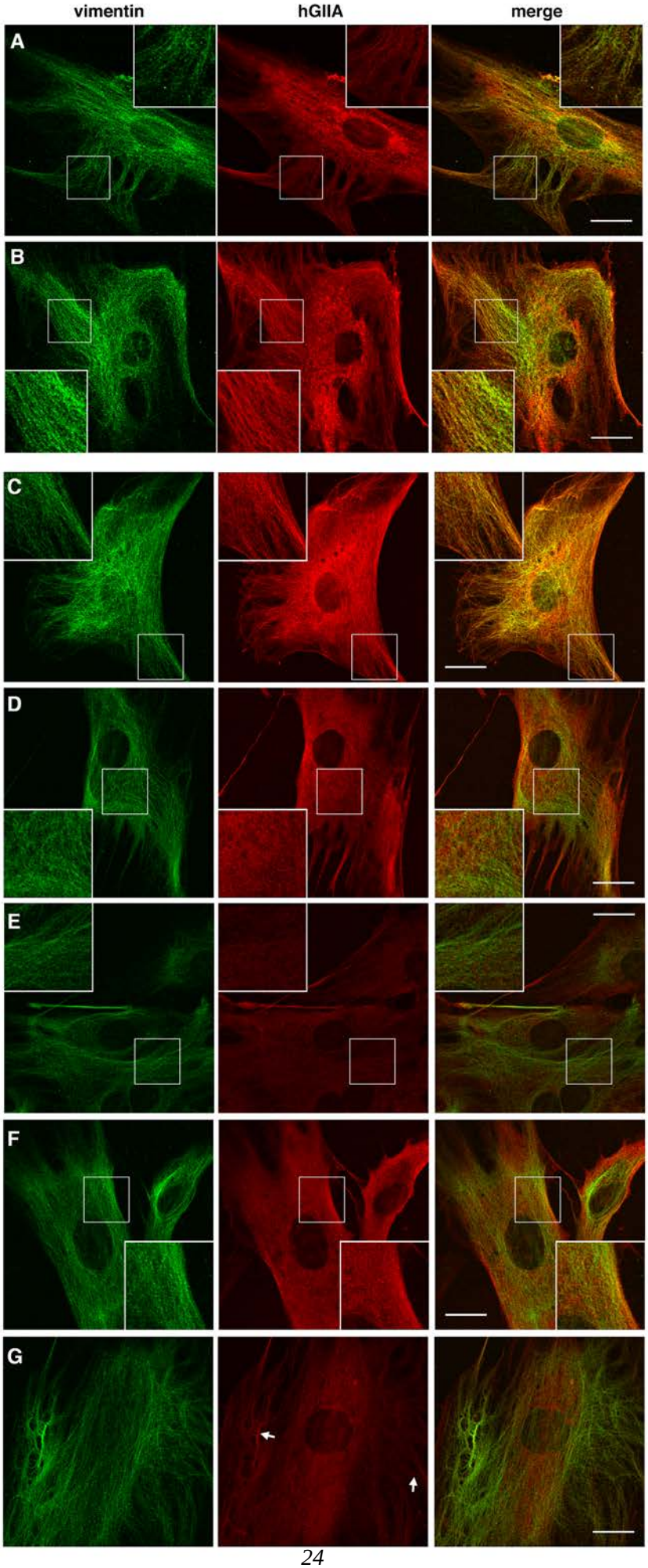
Figure 6.



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