Effect of Tryptophan Insertions on the Properties of the Human Group IIA Phospholipase A_2: Mutagenesis Produces an Enzyme with Characteristics Similar to Those of the Human Group V Phospholipase A_2[†]

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ABSTRACT: An important characteristic of the human group IIA secreted phospholipase A_2 (IIA PLA_2) is the extremely low activity of this enzyme with phosphatidylcholine (PC) vesicles, mammalian cell membranes, and serum lipoproteins. This characteristic is reflected in the lack of ability of this enzyme to bind productively to zwitterionic interfaces. Part of the molecular basis for this lack of activity is an absence of tryptophan, a residue with a known preference for residing in the interfacial region of zwitterionic phospholipid bilayers. In this paper we have replaced the eight residues that make up the hydrophobic collar on the interfacial binding surface of the enzyme with tryptophan. The catalytic and interfacial binding properties of these mutants have been investigated, particularly those properties associated with binding to and hydrolysis of zwitterionic interfaces. Only the insertion of a tryptophan at position 3 or 31 produces mutants that significantly enhance the activity of the human IIA enzyme against zwitterionic interfaces and intact cell membranes. Importantly, the ability of the enzyme mutants to hydrolyze PC-rich interfaces such as the outer plasma membrane of mammalian cells was paralleled by enhanced interfacial binding to zwitterionic interfaces. The corresponding double tryptophan mutant (V3,31W) displays a specific activity on PC vesicles comparable to that of the human group V sPLA2. This enhanced activity includes the ability to interact with human embryonic kidney HEK293 cells, previously reported for the group V enzyme [Kim, Y. J., Kim, K. P., Rhee, H. J., Das, S., Rafter, J. D., Oh, Y. S., and Cho, W. (2002) *J. Biol. Chem. 277*, 9358−9365].

An increasing number of 14 kDa human secreted phospholipases A_2 ($sPLA_2$)[1] have been discovered (*1*), but as yet no clear understanding of the physiological roles of these enzymes has emerged (*2*). The most widely studied member of this human family is the group IIA enzyme where at least part of the function of this mammalian enzyme is that of an acute phase protein with antimicrobial properties, particularly against Gram-positive bacteria (*3*, *4*; reviewed in ref *5*). A direct role in the inflammatory response linked to longer term arachidonic release is also indicated (*6*, *7*). However, transgenic studies in mice do not appear to support a major role for this enzyme in the inflammatory response linked to arachidonic release and eicosanoid formation (*8*, *9*).

One major problem in providing a molecular connection between membrane hydrolysis by this enzyme and arachidonic release is that the human group IIA enzyme is almost inactive against the external surface of the plasma membrane (*10*−*13*), the anticipated site of action of this secreted enzyme. This lack of activity is paralleled in vitro using PC substrates. In contrast, the enzyme expresses high activity against anionic vesicles such as PG and, more importantly, against bacterial membranes, where labeled *Escherichia coli* membranes provide the best radioactive substrate for the in vitro assay of this enzyme.

There are two features of this enzyme that appear to reflect its antimicrobial selectivity. First, the highly cationic nature of the enzyme (p*I* 9.4) appears to be essential for penetrating the anionic cell wall of Gram-positive bacteria (*14*). Second, the lack of an interfacial tryptophan (the enzyme does not contain any tryptophan residues) appears to limit the ability of this enzyme to hydrolyze the more condensed zwitterionic interfaces seen in the external leaflet of the human cell membrane. We have previously shown that the insertion of a tryptophan at position 3 of this enzyme (V3W) dramatically enhances the otherwise negligible ability of the enzyme to hydrolyze PC vesicles and cell membranes (*12*). A single tryptophan in this position is already seen with the pancreatic

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[1] Abbreviations: CD, circular dichroism; CMC, critical micelle concentration; DAUDA, 11-(dansylamino)undecanoic acid; DMEM, Dulbecco's modified Eagle's medium; DMPC, dimyristoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; $DO_{et}PC$, dioleylphosphatidylcholine; DOPG, dioleoylphosphatidylglycerol; $DO_{et}PS$, dioleylphosphatidylserine; ESI-MS, electrospray ionization mass spectrometry; FABP, fatty acid binding protein; FCS, fetal calf serum; HBSS, Hanks' balanced salts solution; HEK, human embryonic kidney; HSPG, heparan sulfate proteoglycan; $sPLA_2$, secreted phospholipase A_2; PC, phosphatidylcholine; PG, phosphatidylglycerol; PM, phosphatidylmethanol; PS, phosphatidylserine.

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group IB enzyme, an enzyme that is able to express significant activity against such zwitterionic interfaces when compared with the human group IIA enzyme (*12*).

The ability of tryptophan residues to partition into the interfacial region of zwitterionic PC interfaces has been highlighted (*15*, *16*), and there are numerous examples of tryptophan residues in membrane proteins that are located in the interfacial region. In a quantitative study involving model peptides, the free energy of transfer into PC vesicles for individual amino acids was 1.85 kcal mol^{-1} for tryptophan, 1.13 kcal mol^{-1} for phenylalanine, and 0.94 kcal mol^{-1} for tyrosine. The most effective aliphatic amino acid was leucine at only 0.56 kcal mol^{-1} (*17*).

The presumptive interfacial binding surface of the human group IIA enzyme contains a collar of eight hydrophobic residues (*18*) that collectively are involved in hydrophobic interactions with the phospholipid surface. The residues are Leu-2, Val-3, Ala-19, Leu-20, Phe-24, Val-31, Phe-70, and Tyr-119 (see Figure 7A). In theory, the mutation of any one of these residues to tryptophan could enhance the binding of the IIA enzyme to zwitterionic interfaces and cell membrane hydrolysis. Sites of tryptophan introduction can be identified by comparison with other sPLA2s that have a tryptophan located within this hydrophobic collar and are discussed below.

A human $sPLA_2$ that has received much attention at this time and, like the group IIA enzyme, is located on chromosome 1 is the human group V enzyme (reviewed in ref *19*). The group V enzyme has a more apparent pro-inflammatory role including an ability to hydrolyze human cell membranes. This feature is in part due to the presence of a tryptophan at position 31 whereas a second surface tryptophan, W79, which is not part of the interfacial surface, is not required for this enhanced membrane hydrolysis (*20*). An important similarity between the human group V and IIA enzymes is that both enzymes have a high affinity for heparin, and recently heparin binding has been implicated in the internalization of these enzymes into some cell types (*6*, *21*−*24*). In particular, the group V $sPLA_2$ has recently been reported to be internalized into HEK293 cells and locate to the perinuclear membrane (*25*).

One of the most active enzymes in terms of its ability to hydrolyze zwitterionic vesicles and cell membranes is that from cobra venom, *Naja naja*. This enzyme has at least one tryptophan, W19, which has been implicated in interfacial binding (*26*) and mutagenesis to W19A, has highlighted the significant role of this residue binding to zwitterionic interfaces (*27*). The adjacent tryptophan (W18) does not appear to make a significant contribution to interfacial catalysis (*27*).

The human $sPLA_2$ with the highest activity against zwitterionic interfaces and cell membranes is the group X enzyme located on chromosome 16. Recently, a detailed comparison of the properties of the human group IIA and X enzymes has confirmed the importance of tryptophan residues in this binding and, in particular, the role of tryptophan 67 on the interfacial binding surface of the group X $sPLA_2$ (*28*). There is not a residue equivalent to tryptophan 67 within the hydrophobic collar of the IIA enzyme.

In this paper the properties of the tryptophan mutants of the human group IIA enzyme, L2W, V3W, A19W, L20W, F24W, V31W, F70W, and Y119W, are described [using the homologous core numbering system for $sPLA_2$s (*29*)], together with the double tryptophan mutant (V3,31W). Large increases in catalytic activity were *only* seen with the V3W and V31W mutants, and enhanced binding to zwitterionic vesicles paralleled this activity. Moreover, the double mutant, V3,31W, had properties that combined those of the two single mutants. The double mutant now had biological properties that were very similar to those of the group V enzyme, including the ability to interact with HEK293 cells in a manner similar to that seen with the group V enzyme (*25*), and represents a remarkable gain of physiological activity as a result of mutagenesis. Such a simple mutagenic change in evolutionary terms (two residues) supports the proposal that the properties of these enzymes have evolved for specific physiological functions.

EXPERIMENTAL PROCEDURES

Materials. Oleic acid and *N. naja* venom group IA $sPLA_2$ were obtained from Sigma, Poole, Dorset, U.K. The human group IIA $sPLA_2$ was prepared from a synthetic gene in *E. coli* as a 1-ala (N1A) mutant and is essentially identical to the wild-type enzyme (*30*); it will be referred to as such (*31*). The preparation of the recombinant human group V $sPLA_2$ has been described (*32*). Expression plasmid pET11A and *E. coli* BL21(DE3) were from Novagen (Madison, WI). DAUDA was obtained from Molecular Probes (Eugene, OR). DOPG and DOPC were purchased from Avanti Polar Lipids (Alabaster, AL). Rat liver FABP was prepared as described previously (*33*). THP-1 and HEK293 cells were obtained from the European Collection of Cell Cultures (Porton, U.K.). THP-1 cells were cultured in RPMI 1640 supplemented with 2 mM glutamine, 10% heat-inactivated myoclone FCS and 1% Pen-Strep mix penicillin/streptomycin (10000 units/10000 μg/mL), 2.92 g of L-glutamine, and 10% β-mercaptoethanol from Life Sciences Gibco BRL. HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine, 1% nonessential amino acids (Life Sciences Gibco BRL), and 10% FCS.

Preparation of Tryptophan Mutants. The preparation of the V3W mutant has been described previously (*31*). The other mutants and the V3,31W double mutant were prepared using the two-step PCR method described by Higuchi et al. (*34*). Oligonucleotide primers used for the construction of the mutants were as follows: forward 5′ end, G GAT ATA CAT ATG GCC CTG GTA AAC; reverse 3′ end, CGA TAA GCT TCA CTA TTA GCA ACG; L2W forward, A AAA GGA GAT ATA CAT ATG GCC TGG GTA AAC TTC; L2W reverse, GAA GTT TAC CCA GGC CAT ATG TAT ATC TCC; A19W forward, CC GGT AAA GAA GCC TGG CTG TCT TAC GGT TTC; A19W reverse, GAA ACC GTA AGA CAG CCA GGC TTC TTT ACC GG; L20W forward, GAA GAA GCT GCT TGG TCT TAC GGT TTG; L20W reverse, CA ACC GTA AGA CCA AGC AGC TTC TTC; F24W forward, GCT CTG TCT TAC GGT TGG TAC GGT TGC CAC; F24W reverse, GTG GCA ACC GTA CCA ACC GTA AGA CAG; V31W forward, GT TGC CAC TGC GGT TGG GGC GGC CGC GGG TC; V31W reverse, GA CCC GCG GCC GCC CCA ACC GCA GTG GCA AC; F70W forward, GC GGT ACC AAA TGG CTG TCT TAC AAA TTC TC; F70W reverse, GA GAA TTT GTA AGA CAG CCA TTT GGT ACC GC; Y119W forward, C AAC AAA AAA TAC CAG TGG TAC TCT AAC AAA CAC; Y119W

reverse, GTG TTT GTT AGA GTA CCA CTG GTA TTT TTT GTT G; forward[2] 5′ end, GTT TAA CTT TAA GAA GGA GAT ATA CAT ATG GCC. The underlined bases indicate the location of the tryptophan mutations in the mismatch primers. The V3,31W mutant was created using the V3W mutant DNA created previously (*31*) with the standard reverse 3′ end primer, V31W primers, and the forward[2] 5′ end primer so as to allow the retention of the V3W mutation. The PCR product (mutated gene) was inserted into target plasmid (pET11A) using *Nde*I and *Hin*dIII restriction sites at the 5′ and 3′ end of the gene, respectively. Bacterial protein expression and purification were as described previously (*31*), and the purity of all proteins was confirmed by SDS−PAGE.

Circular Dichroism (CD) and Electrospray Ionization Mass Spectrometry (ESI-MS) Measurements. The structural integrity of the V3W mutant has been reported previously (*31*). Comparative secondary structure CD was performed on the wild type and the other tryptophan mutant proteins using a Jasco J-720 spectropolarimeter. Protein samples (200 μg/mL) were in 10 mM KH_2PO_4, pH 8.0, and measurements were taken between 190 and 250 nm. Spectra were averaged from three accumulations. Mass spectrometry measurements were performed using a Fisons VG QUATTRO II mass spectrometer in electrospray mode and a Micromass LCT orthogonal acceleration time-of-flight mass spectrometer fitted with a nanoelectrospray source.

Fluorescence Displacement PLA_2 Assays. The fluorescence displacement assay has been described previously (*14*, *35*). Briefly, stock assay buffer was prepared containing 0.1 M Tris·HCl, pH 8.0, and 0.1 M NaCl for vesicle assays and HBSS for cell assays together with 1 mM $CaCl_2$ and 1 μM DAUDA. Cell assays were performed with THP-1 or HEK293 cells at a concentration of $\sim 4 \times 10^5$ cells/mL and FABP ($\sim$10 μg), at a final volume of 1 mL. Assays using DOPG or DOPC vesicles (63 μM) have been described (*12*, *14*). Assays were performed in plastic fluorometric cuvettes using a Hitachi F2500 fluorometer coupled to a computer for data recording. All assays were calibrated by the addition of known amounts of a methanol solution of oleic acid to a control assay containing all components except enzyme. All assays were performed at 37 °C.

Fluorescence Spectra Binding Assays. The tryptophan mutants of human group IIA $sPLA_2$ were prepared in 20 mM Tris·HCl and 1 mM EGTA, pH 7.5. All experiments were carried out on a Hitachi F2500 fluorescence spectrophotometer at 25 °C, with excitation carried out at 290 nm and the resultant spectra recorded between 300 and 500 nm. Enzyme concentrations of 0.2 μM were used, and phospholipid SUVs were prepared by methanol injection to concentrations of 180 μM and 1 mM. SUVs were used to minimize light scattering, and all measurements were corrected for blank suspensions of wild-type human group IIA enzyme.

Fluorescence Quenching. All quenching experiments were carried out in 20 mM Tris·HCl and 1 mM EGTA, pH 7.5 at 25 °C. Aliquots of 5 μL of freshly prepared 3 M acrylamide were titrated into 0.2 μM protein to a final concentration of 143 mM, in the presence or absence of 180 μM and 1 mM phospholipid SUVs prepared by methanol injection. All experiments were performed on a Hitachi F2500 fluorescence spectrophotometer. The excitation wavelength was set at 290 nm, and emissions were recorded between 300 and 500 nm or at set wavelengths corresponding to emission wavelength maxima, for wild-type blanks and tryptophan mutants, respectively.

Interfacial Binding of $sPLA_2$s to Sucrose-Loaded Vesicles. The preparation and analysis of 100 nm diameter sucrose-loaded vesicles of 18% $DO_{et}PS$ in $DO_{et}PC$ were carried out according to the method described in ref *28*. Briefly, the binding affinities of human group IIA $sPLA_2$ and tryptophan mutants were determined using a method based on vesicle sedimentation using ultracentrifugation (*36*). Different concentrations of sucrose-loaded vesicles and a constant concentration of $sPLA_2$ were equilibrated, and then the vesicles were pelleted by ultracentrifugation. The enzyme remaining in the supernatant was measured using the fluorescence assay of Radvanyi (*37*), and the K_d values were calculated (*28*). The calculation utilized the standard equation for equilibrium dissociation: $100(E_F/E_T) = K_d/(L + K_d)$, where E_F is the concentration of $sPLA_2$ in the aqueous phase (free), E_T is the concentration of total enzyme (free and vesicle bound) in the binding reaction, L is the total phospholipid concentration in the binding reaction (expressed as total moles of phospholipid divided by the volume of the reaction sample), and K_d is the equilibrium constant for the dissociation of vesicle-bound enzyme into the aqueous phase. This equation assumes that the number of enzyme binding sites on vesicles is large compared to the total number of enzymes so that the total phospholipid concentration can be used without correction for depletion of L due to enzyme binding. This assumption is true for the assay conditions described above.

Interaction of $sPLA_2$s with HEK293 Cells. Interaction of the human group V enzyme with HEK293 cells has been demonstrated using western blotting, and this protocol was utilized (*25*). HEK293 cells cultured in DMEM supplemented with 10% heat-inactivated FCS were treated with 100 nM wild type, V3W, L20W, V31W, and V3,31W mutants of human group IIA $sPLA_2$ for the times indicated. Incubations were quenched by the addition of a solution of ice-cold 0.6 M NaCl supplemented DMEM and then washed in the same solution. The cells were collected by scraping and centrifugation; then the resultant cell pellets were lysed. After centrifugation the supernatants were subjected to SDS−PAGE under reducing conditions using 15% acrylamide gels. The samples were electrotransferred onto a polyvinylidene fluoride membrane using a semidry system (45 mA for 1 h). The membrane was treated with phosphate-buffered saline containing 0.1% Tween 20 (PBS−Tween) and 3% nonfat milk protein, pH 7.4, for 20 min at room temperature. The membrane was then incubated with 5 μg/mL mouse anti-human secretory PLA_2 (Upstate Biotechnology) diluted in 3% milk−PBS−Tween overnight at 4 °C. The membrane was washed three times with PBS−Tween before incubation with a 1:10000 dilution of goat anti-mouse IgG conjugated with horseradish peroxidase (Sigma) diluted in 5% milk−PBS−Tween for 1 h at room temperature. The membrane was then washed three times for 5 min with PBS−Tween and once with water. Visualization of the membrane was performed using Supersignal (Pierce) and the film analyzed.

RESULTS

General Properties of the L2W, V3W, A19W, L20W, F24W, V31W, F70W, Y119W, and V3,31W Mutants of the Human Group IIA $sPLA_2$. The mutant proteins were ex-

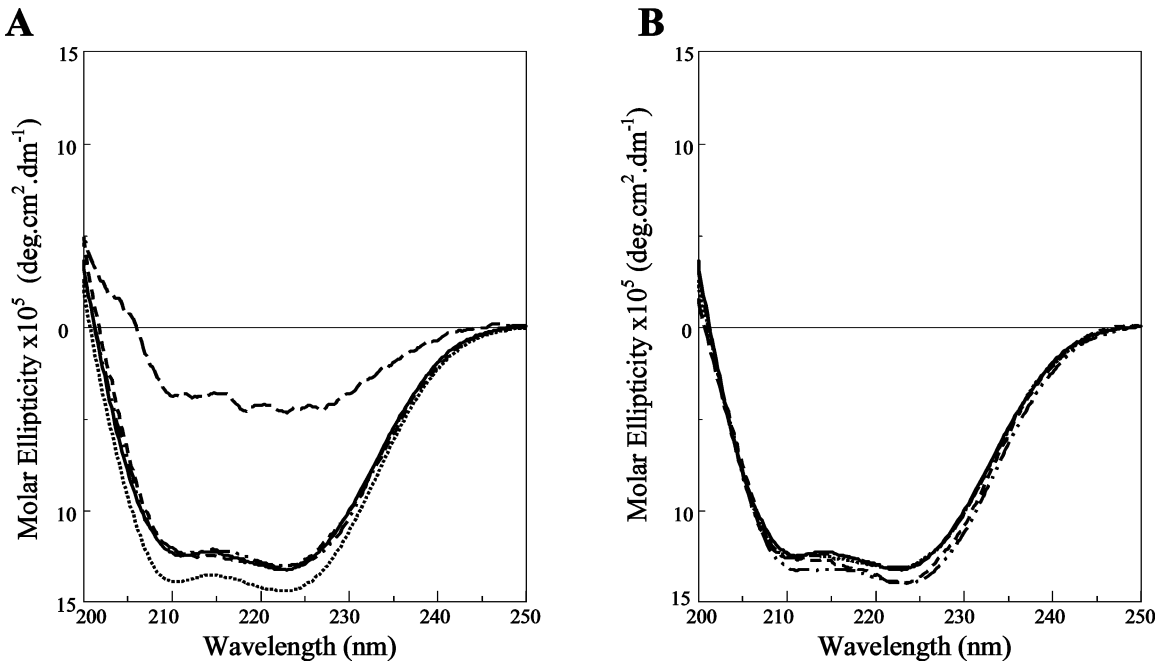

FIGURE 1: CD spectra. Comparative secondary structure CD was performed on (A) the wild type (solid line) and tryptophan mutants L2W (long dashed line), A19W (short dashed line), L20W (dotted line), and F24W (dashed and dotted line) and (B) the wild type (solid line) and tryptophan mutants V31W (long dashed line), F70W (short dashed line), Y119W (dotted line), and V3,31W (dashed and dotted line) of human group IIA sPLA$_2$ as described in Experimental Procedures. Spectra shown were averaged from three accumulations.

pressed and purified in the same manner as described previously for the wild-type IIA and V3W enzymes (*12*, *31*), and the primary structure was confirmed by ESI-MS. The mass values obtained (with the predicted mass in each case shown in parentheses) were as follows: L2W, 13934.3 (13933.9); A19W, 13977.0 (13976.0); L20W, 13933.2 (13933.9); F24W, 13900.6 (13899.9); V31W, 13947.5 (13947.9); F70W, 13899.6 (13899.9); Y119W, 13883.7 (13883.9); V3,31W, 14034.0 (14035.0). The CD spectra (Figure 1) show that wild type and all mutants with the exception of the L2W are virtually indistinguishable, indicating no obvious secondary structure perturbations resulting from mutagenesis. This lack of significant secondary structure perturbation is consistent with the high catalytic activity of all mutants (see below). The L2W was produced in very low yield that limited further analysis while the CD spectrum indicated some incorrect folding.

Anionic Phospholipid Hydrolysis by the Tryptophan Mutants of the Human Group IIA sPLA$_2$. It is well-known that sPLA$_2$s bind with high affinity to anionic phospholipid vesicles and can show scooting kinetics (*38*). Under these conditions the enzyme remains attached to the phospholipid surface during many catalytic turnovers that, in the case of the pancreatic enzyme, can result in complete hydrolysis of the outer monolayer of the vesicle (*38*). The use of scooting conditions in which the enzyme remains attached to the interface eliminates factors affecting interfacial binding and permits an analysis of the catalytic events. Under such conditions all mutants had specific activities similar to that of the wild type (Table 1) with the V31W displaying the largest difference at 151% of wild-type activity. The low activity seen with the L2W mutant may reflect incorrect folding of this poorly expressed mutant, and no further studies were performed with this mutant.

The fact that no large effects are seen as a result of tryptophan insertion is consistent with the binding affinity

Table 1: Relative Activities of sPLA$_2$ on Phospholipid Vesicles[a]

	DOPG SUVs		DOPG SUVs		DOPC SUVs	
sPLA$_2$	activity as % hgIIA	SD	activity as % hgIIA (2 M NaCl)	SD	activity as % V3W	SD
hgIIA	100[b]	31	100[c]	4.4	3	0.2
L2W	21	1.3			2.6	0.1
V3W	116	2.5	584	8.4	100[d]	2.9
A19W	85	9.6			2.9	0.1
L20W	95	18.1	116	7.6	2.5	0.4
F24W	103	5.2			4.1	0.1
V31W	151	12.8	577	18	20	1.2
F70W	103	5.6			5	0.7
Y119W	119	22			5.3	0.2
V3,31W	126	16.5	600	14	206	6.4
hgV	25	4.7			259	14
N. naja	108	9.6			3559	636

[a] Specific activities were determined using a fluorescence displacement assay described in Experimental Procedures, and data are means ± SD ($n = 3$). For all enzyme assays on DOPG the values for specific activity are shown as a percentage of the value for the human group IIA (hgIIA) enzyme run under identical assay conditions. Some assays were also run in the presence of 2 M NaCl. In the case of assays using DOPC, the specific activities are shown as a percentage for the V3W mutant, again run under identical conditions. [b] The absolute specific activity value for hgIIA was 96 ± 30 nmol min^{-1} μg^{-1}. [c] The absolute specific activity value for hgIIA was 43 ± 2 nmol min^{-1} μg^{-1}. [d] The absolute specific activity value for V3W was 17 ± 8 nmol min^{-1} μg^{-1}.

to such anionic vesicles, which is already very high (K_d $<10^{-9}$ M), with a major contribution coming from electrostatic interactions under these assay conditions (*13*, *18*, *39*). The human group V activity was only 25% of wild-type IIA enzyme, consistent with previous reports (*20*). Overall, the results provide further confirmation of the structural integrity of the mutants.

The partial role of electrostatics in supporting interfacial binding can be demonstrated by performing assays under conditions of high salt (Table 1), and this was done for

Table 2: Spectral Characteristics of Tryptophan Mutants of hgIIA $sPLA_2$ in the Presence of 180 μM DOPG and DOPC Phospholipid Vesicles[a]

protein	buffer λ_{max} (nm)	DOPG (180 μM) λ_{max} (nm)	λ_{max} shift (nm)	increase in quantum yield (%)	DOPC (180 μM) λ_{max} (nm)	λ_{max} shift (nm)	increase in quantum yield (%)
V3W	346.3 ± 1.2	339.3 ± 0.6	7 ± 1	28.9 ± 6	345.2 ± 1.9	1.2 ± 2.6	−0.1 ± 2.5
A19W	346.7 ± 0.6	330.5 ± 0.5	16.2 ± 1	5.3 ± 3.6	346.5 ± 0.5	0.2 ± 0.3	−3.3 ± 5.2
L20W	352.3 ± 0.8	332 ± 2.3	20.3 ± 2.3	62.7 ± 6.6	352.5 ± 0.5	−0.2 ± 1.3	−0.7 ± 2.2
F24W	350.8 ± 0.3	339 ± 0.5	11.8 ± 0.8	147.1 ± 4	349.2 ± 0.6	1.67 ± 0.3	7.7 ± 4.3
V31W	353.8 ± 0.8	342.7 ± 0.6	11.2 ± 1.3	144.6 ± 18.8	353.5 ± 0.5	0.3 ± 0.3	1.8 ± 3.5
F70W	350.8 ± 0.3	338.3 ± 0.3	12.5 ± 0.5	134.9 ± 6.9	349.3 ± 0.6	1.5 ± 0.9	1.3 ± 2.1
Y119W	352.3 ± 0.3	335.3 ± 0.3	17 ± 0.5	107.5 ± 16.6	352 ± 0	0.3 ± 0.3	5.9 ± 10.8
V3,31W	349.2 ± 0.3	341.5 ± 0	7.7 ± 0.3	86.6 ± 13.7	341.5 ± 0	7.7 ± 0.3	88.4 ± 10

[a] Tryptophan fluorescence spectra were recorded for the enzymes (0.2 μM) in 20 mM Tris−EGTA or in the presence of DOPG and DOPC SUVs. The phospholipid concentrations were 180 μM, and the spectra were recorded as detailed in Experimental Procedures and are shown in Figures 2 and 3. The data shown are means ± SD ($n = 3$).

certain mutants. In fact, a NaCl concentration of 2 M was required to discriminate between the mutants in terms of potential hydrophobic interactions. Under these conditions a 5−6-fold higher specific activity for hydrolysis of DOPG vesicles was seen with the V3W and V31W mutants compared with wild type and L20W mutant (Table 1), highlighting an enhanced hydrophobic contribution to interfacial binding with these former mutants.

Zwitterionic Phospholipid Hydrolysis by Tryptophan Mutants of the Human Group IIA $sPLA_2$. The human group IIA enzyme is characterized by its extremely low activity with zwitterionic substrate aggregates, an activity that is reflected in the lack of binding to such interfaces. The presence of a tryptophan residue on the interfacial binding surface of the enzyme, in a position to interact productively with the phospholipid interface, should have a significant effect on the ability of that mutant to bind to and hence hydrolyze such phospholipid aggregates. The results of DOPC hydrolysis by these mutants are presented in Table 1, where the activity of the native enzyme and the various mutants is normalized against the activity of the V3W mutant. There is a major difference between the V31W mutant and the other tryptophan mutants. The A19W, L20W, F24W, F70W, and Y119W mutants showed activity similar to that of wild-type enzyme whereas the V31W mutant demonstrated a considerable enhancement of activity. This enhancement (20-fold) under these assay conditions can be compared with an enhancement of about 30-fold for the V3W mutant under the same assay conditions (Table 1). The enhancement figure for the V3W mutant is less than was previously reported (*12*) and reflects the difficulty of obtaining an accurate value for the specific activity of the wild-type (N1A) enzyme due to the very low catalytic rates that are observed.

The V3,31W double mutant shows a larger enhancement of activity on PC vesicles compared with wild type than does the individual V3W and V31W mutants. It is of particular interest that this double mutant now expresses activity similar to that of the human group V enzyme on DOPC vesicles and suggests that this enzyme may now function in a manner similar to that of the group V enzyme under physiological conditions. The very high activity of the *N. naja* enzyme is confirmed under these assay conditions.

Interfacial Binding Properties of the Tryptophan Mutants of Human Group IIA $sPLA_2$. We have demonstrated (Table 1) that there was a considerable difference in ability of the tryptophan-containing mutants to hydrolyze DOPC vesicles. Since expressed activity on DOPG vesicles, where scooting conditions apply, was very similar for all mutants compared to wild type, mutagenesis has not adversely affected the catalytic potential of the enzymes. Therefore, a change in binding affinity to the DOPC interfaces would be anticipated. This change should reflect enhanced binding of the V3W, V31W, and V3,31W compared to the other tryptophan mutants. Tryptophan is an effective fluorescent probe, and its fluorescence characteristics can be used to provide information about the environment of this residue in the free enzyme and to monitor a change in environment on interfacial binding to phospholipid vesicles.

Figures 2 and 3 show the tryptophan fluorescence emission spectra of the tryptophan mutants in buffer and in the presence of 180 μM DOPG or DOPC vesicles. A fluorescence enhancement and blue shift are seen for all tryptophan mutants in the presence of DOPG compared to in buffer, indicating interfacial binding. The changes are tabulated in Table 2. The largest spectral shift (20 nm) was seen with the L20W mutant whereas the largest increase in fluorescence intensity is observed with the F24W and V31W mutants. These changes indicate very different interfacial environments for these tryptophans but confirm that all seven tryptophan insertions are affected by interfacial binding to DOPG.

In contrast, no change in the fluorescence characteristics can be seen for the single mutants in the presence of 180 μM DOPC, but significant binding is seen for the V3,31W double mutant (Figure 2 and Table 2). When the DOPC concentration was increased to 1 mM (Figures 2 and 3 and Table 3), a significant shift of over 5 nm in the fluorescence emission wavelength maximum was seen for both the V3W and the V31W, whereas the other mutants showed at best a wavelength shift of less than 2 nm. Only the V3W, L20W, and V31W showed a significant increase in fluorescence quantum yield. Under these conditions the fluorescence characteristics of free tryptophan were not changed (data not shown).

The fluorescent properties of the V3,31W double mutant in the presence of 1 mM DOPC were very similar to those seen with 180 μM DOPC, suggesting that binding to DOPC was already maximal at the lower DOPC concentration. Enzyme activity assays were performed at 63 μM DOPC, conditions approaching that required for maximal interfacial binding, and produced only about a 3-fold lower expressed activity than with DOPG vesicles (Table 1). This result further emphasizes the remarkable change in catalytic properties of the double mutant.

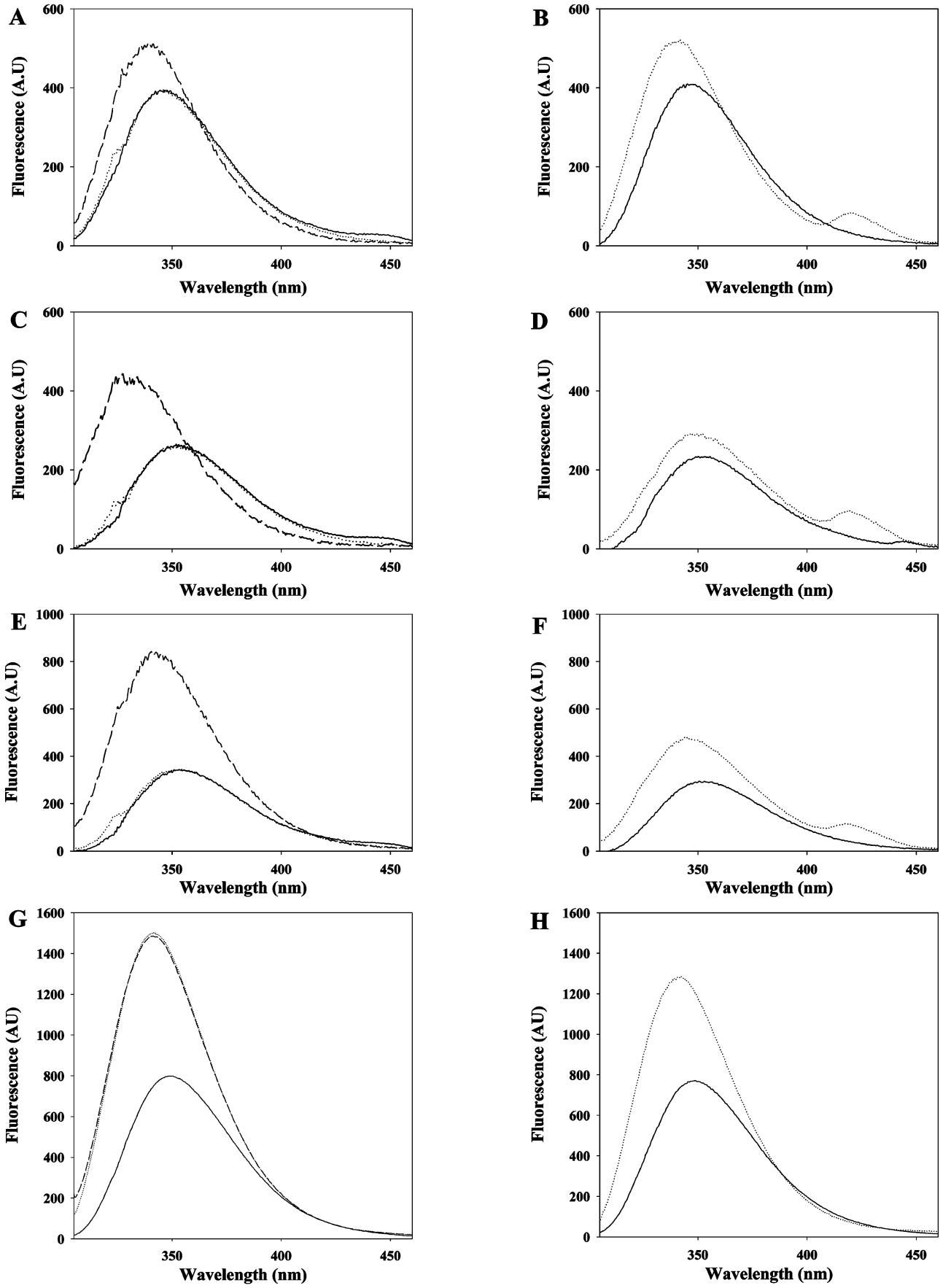

FIGURE 2: Fluorescence emission spectra of V3W (A and B), L20W (C and D), V31W (E and F), and double mutant V3,31W (G and H). The enzymes (0.2 μM) are shown in 20 mM Tris and 1 mM EGTA (solid line), DOPG (dashed lines), and DOPC (dotted lines). The phospholipid concentrations were 180 μM (A, C, E, and G) and 1 mM (B, D, F, and H). Spectra were recorded as detailed in Experimental Procedures, and the results are also tabulated in Tables 2 and 3. The data shown are means $\pm$ SD ($n = 3$).

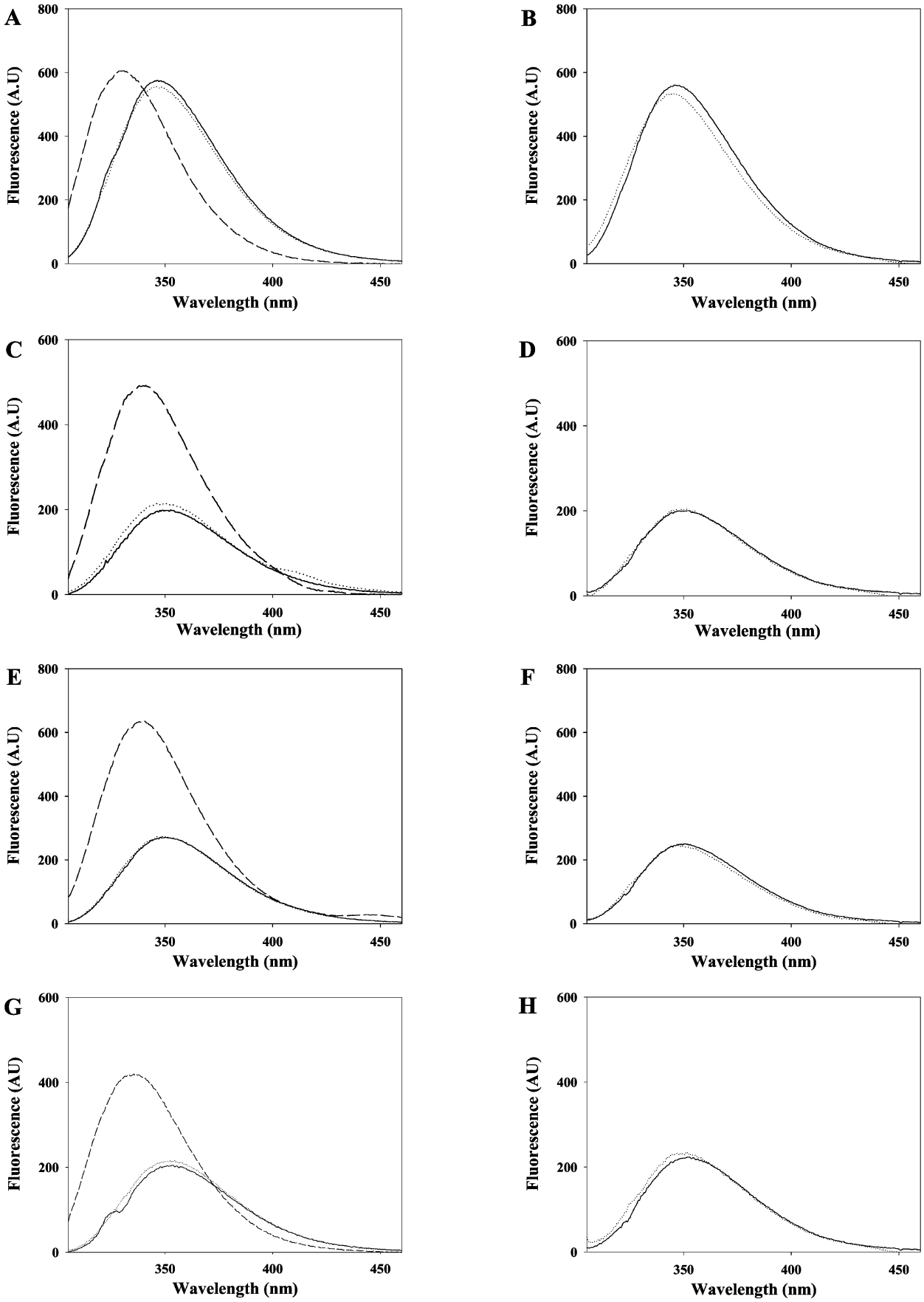

FIGURE 3: Fluorescence emission spectra of A19W (A and B), F24W (C and D), F70W (E and F), and Y119W (G and H). The enzymes (0.2 μM) are shown in 20 mM Tris and 1 mM EGTA (solid line), DOPG (dashed lines), and DOPC (dotted lines). The phospholipid concentrations were 180 μM for both DOPG and DOPC (A, C, E, and G) and 1 mM for DOPC only (B, D, F, and H). Spectra were recorded as detailed in Experimental Procedures, and the results are also tabulated in Tables 2 and 3. The data shown are means $\pm$ SD ($n = 3$).

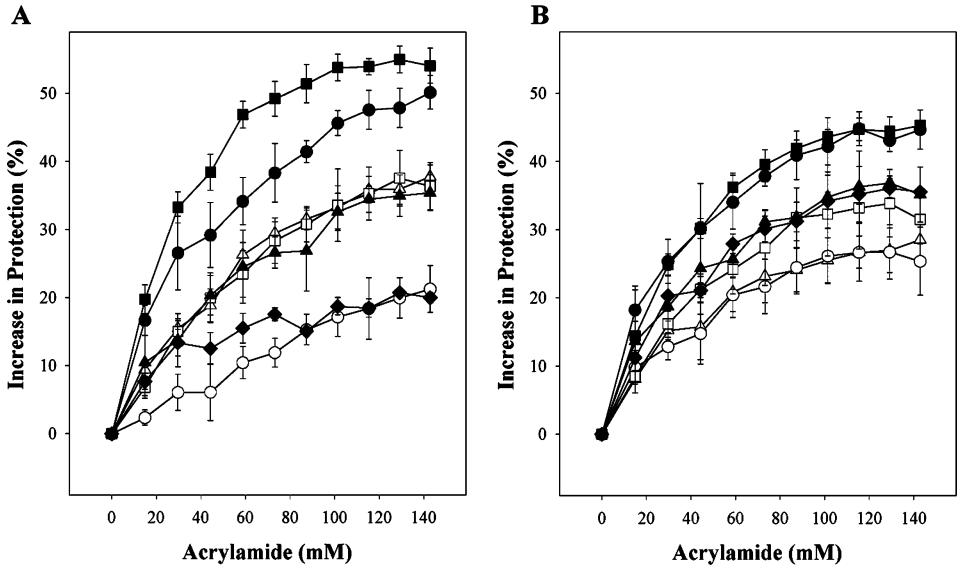

FIGURE 4: Acrylamide protection of tryptophan residues in mutant human group IIA $sPLA_2$ with interfacial binding. Quenching was carried out with 0.2 μM protein in 20 mM Tris, 1 mM EGTA, and 180 μM phospholipid using either 100% DOPG (A) or 20 mol % DOPG/DOPC (B). Excitation was at 290 nm, and the difference in fluorescence intensity when bound to vesicles and in buffer at the wavelength maxima for each mutant is plotted against the amount of acrylamide added. Key: ●, V3W; ■, A19W; ▲, L20W; ◆, F24W; ○, V31W; □, F70W; △, Y119W. Data shown are means ± SD ($n = 3$).

Table 3: Spectral Characteristics of Tryptophan Mutants of hgIIA $sPLA_2$ in the Presence of 1 mM DOPC Phospholipid Vesicles[a]

protein	buffer λ_{max} (nm)	DOPC (1 mM) λ_{max} (nm)	λ_{max} shift (nm)	increase in quantum yield (%)
V3W	346.7 ± 0.3	340.5 ± 0.5	6.2 ± 0.3	27.2 ± 10.5
A19W	346.8 ± 0.3	345 ± 0.5	1.8 ± 0.3	−4.5 ± 6.5
L20W	352.2 ± 0.3	350.3 ± 0.3	1.8 ± 0.3	24.6 ± 10.8
F24W	350 ± 0.5	349.8 ± 0.3	0.2 ± 0.3	1.4 ± 3.3
V31W	352.3 ± 0.8	346 ± 0.9	6.3 ± 1.6	63.6 ± 10.7
F70W	350 ± 0	349 ± 0.5	1 ± 0.5	−2.5 ± 7.8
Y119W	351.8 ±0.3	350 ± 0	1.8 ± 0.3	4 ± 12.5
V3,31W	348.3 ± 0.6	341.2 ± 0.3	7.2 ± 0.3	66.3 ± 7.6

[a] Tryptophan fluorescence spectra were recorded for the enzymes (0.2 μM) in 20 mM Tris−EGTA or in the presence of 1 mM DOPC SUVs. The spectra were recorded as detailed in Experimental Procedures and are shown in Figures 2 and 3. The data shown are means ± SD ($n = 3$).

Effect of Interfacial Binding on the Quenching of Tryptophan Fluorescence by Acrylamide. Acrylamide is an electron-deficient molecule that is believed to quench tryptophan fluorescence in the excited state by electron transfer from the indole ring of tryptophan (*40*). Acrylamide was chosen for this investigation, as it is a neutral quencher that has limited accessibility to phospholipid bilayers. With the exception of the V3W mutant, no significant protection from acrylamide quenching was seen with any tryptophan mutant in the presence of 1 mM DOPC vesicles, again highlighting the apparent low affinity of such mutants for zwitterionic vesicles (data not shown). In contrast, major differences were seen in terms of protection from acrylamide when the tryptophan mutants were bound to DOPG vesicles compared to the enzyme in buffer.

The results are shown in Figure 4A and, for clarity, are plotted as the percent increase in protection from acrylamide quenching with increasing acrylamide concentration for the enzyme bound to 180 μM DOPG compared with the enzyme in buffer. In Figure 4A the result using 100% DOPG highlights the increased protection of W3 compared with W31. However, this difference diminishes using vesicles composed of 20 mol % DOPG in DOPC (Figure 4B). It would appear that the precise topological relationship between the interfacial binding surface of the protein and the phospholipid interface differs, depending on the nature of the interface. In particular, W31 becomes relatively less accessible to acrylamide quenching with a more zwitterionic interface, possibly because this residue plays a more significant role in binding to this interface and is discussed below. In Figure 4A,B the greatest protection is seen for the A19W mutant, indicating significant penetration into the phospholipid interface (see Sucrose-Loaded Vesicles Studies).

Binding of Human Group IIA $sPLA_2$ and Tryptophan Mutants to Sucrose-Loaded Vesicles. Sucrose-loaded large unilamellar vesicles can be sedimented by ultracentrifugation, and the binding of $sPLA_2$s can be monitored by measuring free enzyme in the supernatant relative to total enzyme according to methods described by Bezzine et al. (*28*). To maximize the resolution of this system, vesicles have to be prepared using an optimum percentage of phosphatidylserine in the PC vesicles, and by trial and error this was found to be 18 mol % for these assays. The binding curves are shown in Figure 5 while the calculated K_d values are tabulated in Table 4. The results dramatically highlight the enhanced binding of both the V3W and V31W mutants to such vesicles and the higher affinity of the V3,31W double mutant for this phospholipid interface. As with the activity studies (Table 1), the effect of the double tryptophan mutation is a further enhancement on interfacial binding and provides direct confirmation of how the activity of these enzymes on PC-containing interfaces directly correlates with the ability of the enzyme to bind productively to such interfaces. A measurable increase in interfacial binding was detected with the A19W mutant, consistent with the acrylamide quenching data, probably reflecting the increase in hydrophobic area of this mutant. However, no significant increase in catalysis was seen (Table 1), and this may reflect the location of this

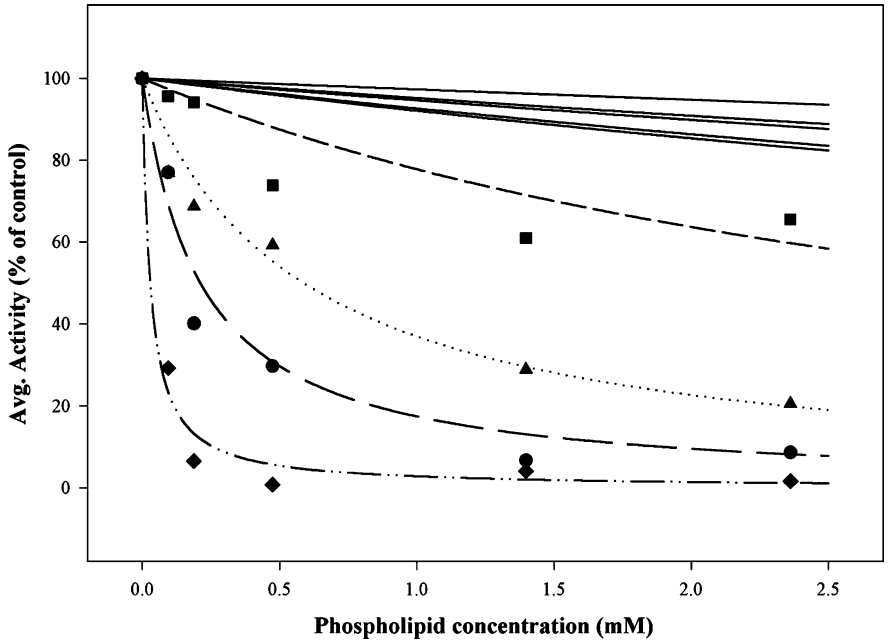

FIGURE 5: Interfacial binding of wild type and tryptophan mutants of hgIIA $sPLA_2$ to phospholipid vesicles. Sucrose-loaded vesicles of 18 mol % $DO_{et}PS$ in $DO_{et}PC$ were pelleted by ultracentrifugation, and the percentage of $sPLA_2$ remaining in the supernatant is plotted as a function of the concentration of total phospholipid in the binding mixture according to Bezzine et al. (*28*). The wild-type and nonbinding tryptophan mutants are indicated by solid lines. The tryptophan mutants that demonstrated detectable binding are V3W (long dashed line, ●), A19W (short dashed line, ■), V31W (dotted line, ▲), and V3,31W (dashed and dotted line, ◆). Independent binding studies were carried out at least three times for each enzyme.

Table 4: Binding Data of Wild-Type and Tryptophan Mutants of hgIIA $sPLA_2$ on 18% $DO_{et}PS$ in $DO_{et}PC$ Sucrose-Loaded Phospholipid Vesicles[a]

	K_d (mM)	SD
hgIIA	no binding	
V3W	0.21	0.06
A19W	3.5	1.7
L20W	no binding	
F24W	no binding	
V31W	0.59	0.1
F70W	no binding	
Y119W	no binding	
V3,31W	0.029	0.004

[a] Apparent K_d values were determined using sucrose-loaded vesicles according to a method described in ref *28*, and data are means ± SD ($n = 3$). No binding means that no significant binding could be detected at 2.4 mM phospholipid.

Table 5: Relative Activities of $sPLA_2$ on Mammalian Cell Membranes[a]

	THP-1 cells		HEK293 cells	
	% (V3W)	SD	% (V3W)	SD
hgIIA	9.2	1.1	6.5	3.0
V3W	100[b]	33	100[c]	24
A19W	8	2.1		
L20W	16	2.6	4.4	4.5
F24W	8.5	2.2		
V31W	73	5.1	60	11
F70W	21.1	2.2		
Y119W	6.8	1.4		
V3,31W	182	21	273	34
hgV	140	9.5	323	49
N. naja	1085	119	2113	293

[a] Specific activities were determined using a fluorescence displacement assay described in Experimental Procedures, and data are means ± SD ($n = 3$). For all enzyme assays using cell suspensions the values for specific activity are shown as a percentage of the value for the V3W mutant run under identical conditions. [b] The absolute specific activity value for V3W was 0.5 ± 0.2 nmol min^{-1} μg^{-1}. [c] The absolute specific activity value for V3W was 1 ± 0.2 nmol min^{-1} μg^{-1}.

residue in the wall of the active site slot such that a bulky tryptophan may restrict access of a PC substrate.

Hydrolysis of Cell Membranes by Tryptophan Mutants. The ability of human $sPLA_2$s to hydrolyze cell membranes is a major factor in determining the physiological roles of these enzymes. These secreted enzymes normally operate in an extracellular environment exposed to millimolar Ca^{2+} concentrations where they would interact with the external monolayer of the plasma membrane and the monolayer coat of lipoproteins. Therefore, an important measure of enzyme activity is that expressed against such physiological phospholipid interfaces. A continuous fluorescence displacement assay (*35*) has the advantage of using normal phospholipid substrates including cell suspensions and lipoproteins. The ability of human group IIA $sPLA_2$ and tryptophan mutants to hydrolyze suspensions of THP-1 and HEK293 cells is shown in Table 5. The activities are expressed as a percent of the V3W mutant rather than the wild-type enzyme because of the inherent difficulty of obtaining accurate values for the negligible activity expressed by the wild-type enzyme. The results parallel data using DOPC vesicles, and it is clear that the V3W is more effective than the V31W. Of the other tryptophan mutants only the L20W and F70W show a detectable increase in activity compared to the wild-type enzyme under these assay conditions. The V3,31W double mutant shows enhanced activity compared with the individual tryptophan mutants, consistent with the phospholipid vesicle studies (Table 1).

The result with *N. naja* for comparison highlights the potential of this type of venom enzyme to hydrolyze cell membranes. This is the physiological role of the enzyme, and the expressed specific activity is over 10-fold that of

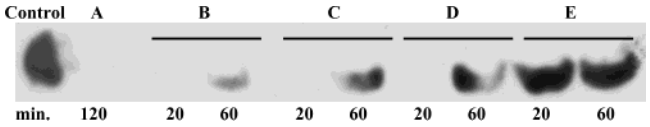

FIGURE 6: Comparison of the interaction of wild type and tryptophan mutants of human group IIA $sPLA_2$ with HEK293 cells detected by Western blotting analysis. HEK293 cells in DMEM were incubated with 100 nM wild-type (A), L20W (B), V31W (C), V3W (D), and V3,31W (E) human group IIA $sPLA_2$ for 20 and 60 min. The wild-type enzyme incubation was extended to 120 min. A control lane was run with 20 ng of wild-type enzyme. Essentially the same results were obtained from duplicate experiments described in Experimental Procedures.

the V3W mutant and 6-fold that of the double mutant. In this context, it should be noted that the human group X enzyme expresses activity similar to that of the cobra venom enzyme on cell membranes (*41*), consistent with human cell membrane hydrolysis being a physiological role of this group X enzyme. Of particular interest is the fact that the V3,31W mutant now expresses activity very similar to that of the group V enzyme under these physiological assay conditions. Thus it would appear that a double mutation has converted the IIA enzyme into one that could show physiological properties similar to those of the group V enzyme, especially as both the IIA and V also bind to heparin and to cell surface HSPG.

Effect of Tryptophan Mutagenesis on the Interaction of the Enzyme with HEK293 Cells. It has been reported, using western blot analysis, that the group V $sPLA_2$ associates with unstimulated HEK293 cells in a process requiring both binding to HSPG and also plasma membrane hydrolysis (*25*). In contrast, the wild-type IIA enzyme does not associate with these cells unless prior plasma membrane hydrolysis was achieved by previous treatment with *N. naja* venom $sPLA_2$ (*25*). The ability of the V3,31W double mutant to hydrolyze cell membranes and PC vesicles at a rate very similar to that of the group V enzyme would suggest that this mutant enzyme should be able to associate with HEK293 cells.

HEK293 cells that were freshly grown from frozen stock samples were incubated with wild-type human group IIA and tryptophan mutants for up to 120 min. After being washed with medium containing high salt to remove any absorbed enzyme, the cells were isolated and disrupted, and the resulting supernatant was analyzed by SDS−PAGE followed by western blotting. The results are visualized in Figure 6. It can be clearly seen that there is a complete failure of the wild-type enzyme to interact with the HEK293 cells even after the cells are incubated for 120 min. In contrast, maximum interaction of the V3,31W double mutant is clearly seen after 20 min. Some interaction of all single mutants is seen after 60 min, being most apparent with the V3W and just visible with the L20W mutant. The L20W mutant was included because it showed no significant increase in cell membrane-hydrolyzing ability of HEK293 cells compared with the wild-type group IIA enzyme although a small but significant increase in hydrolysis was seen with THP-1 cells (Table 5).

These results correlate with the membrane hydrolysis activity shown in Table 5 and appear to provide a more sensitive measure of the membrane hydrolysis that is required for enzyme−cell interaction. Some interaction is seen for the L20W mutant although the rate of actual membrane hydrolysis (Table 5) is indistinguishable from that seen with the wild-type enzyme. This probably reflects the much longer time of cell membrane exposure to enzyme with the cell binding studies compared with initial rates that are measured in the enzyme assays.

DISCUSSION

Interfacial binding is crucial for PLA_2s acting on physiological phospholipid substrates that contain long-chain fatty acids because of the very low monomeric concentration of phospholipid in the aqueous phase (CMC $<10^{-9}$ M). As a result, the enzyme can only access individual substrate molecules and carry out catalysis if it binds productively to the surface of the phospholipid aggregate. Thus interfacial binding can play a very important role in regulating enzyme activity within or outside the cell.

Interfacial discrimination is an important property of the human group IIA $sPLA_2$. This enzyme expresses high activity on anionic interfaces such as those presented by PG but demonstrates negligible activity on the zwitterionic interfaces present as the external monolayer of the plasma membrane or the monolayer coat of lipoproteins. Such discrimination is consistent with the antimicrobial activity of this enzyme in that the bacterial membrane is highly anionic, being rich in PG, and this allows high-affinity binding and phospholipid hydrolysis resulting in bacterial killing (*5*). The extracellular level of the IIA enzyme can rise over 1000-fold under conditions of acute infection while such high concentrations ($\sim$10 μg/mL) are normally present in human tears (*42*) and semen (*43*). Therefore, the inability of this enzyme to hydrolyze the normal host cell membrane or lipoprotein is very important. The enzyme is now regarded as an acute phase protein (*44*).

Many studies of protein−membrane interactions have now highlighted a major role for tryptophan in these interactions as this residue has a propensity to partition into the interfacial region of the phospholipid bilayer (or monolayer) of PC vesicles (*15*, *17*). Such partitioning will facilitate the interfacial binding to PC interfaces of enzymes that present tryptophan on their interfacial binding surface, and this has been reviewed in the case of the $sPLA_2$s (*16*) and examined in some detail in the case of the group IIA and X enzymes (*28*). Moreover, such a preference for PC interfaces may provide a membrane-targeting mechanism for proteins within the mammalian cell to the PC-rich perinuclear membrane (*45*, *46*).

The group IIA enzyme does not contain tryptophan, and this is consistent with the fact that the enzyme is inactive against zwitterionic mammalian cell membranes. If tryptophan residues play a significant role in defining PLA_2 function, then the tryptophan profiles of other PLA_2s together with other aromatic residues in the interfacial region may be crucial to understanding function. Moreover, tryptophan insertion or removal by mutagenesis could change the physiological properties of the particular phospholipase.

The overall aim of this paper was to replace nonpolar residues on the interfacial binding surface of the group IIA $sPLA_2$ (Figure 7A) with tryptophan. Such a strategy should highlight the possible contribution played by individual residues in interfacial binding and catalysis and provide information about the topological relationship between the

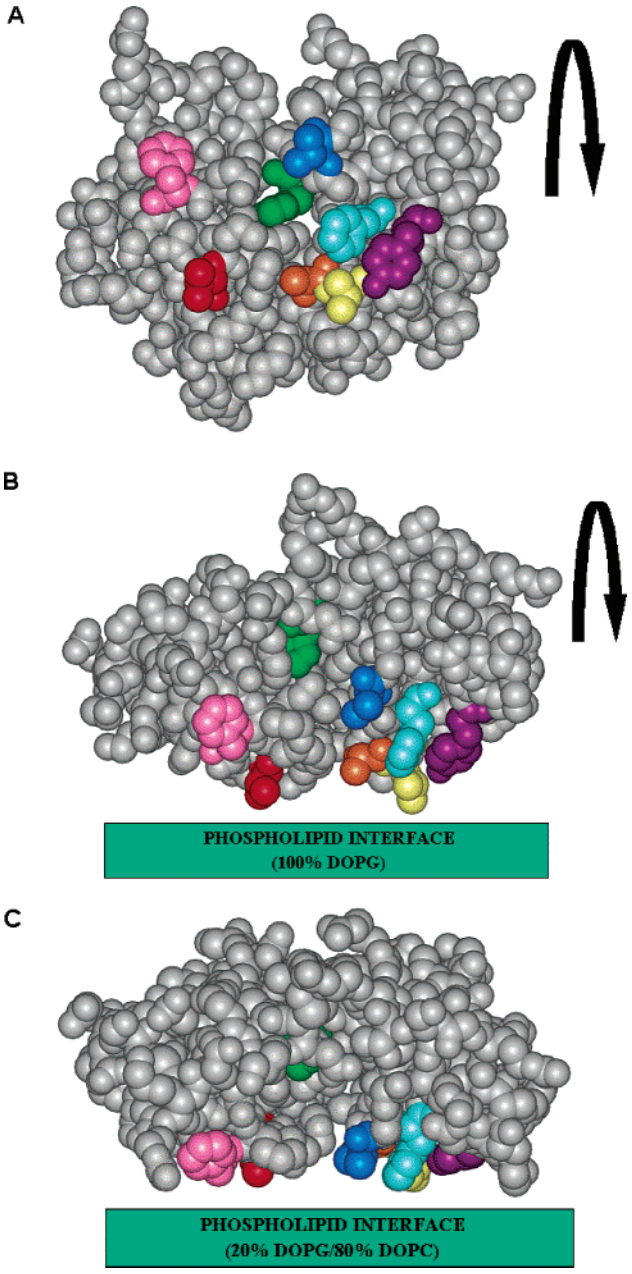

FIGURE 7: Hydrophobic collar of the interfacial binding surface. (A) Hydrophobic residues: brown, L2; red, V3; orange, A19; yellow, L20; cyan, F24; blue, V31; pink, F70; purple, Y119. The numbering system is based on the homologous core developed by Renetseder et al. (*29*). The image is shown in space-filling representation with the interfacial binding surface pointed toward the viewer and the catalytic site histidine shown in green. (B) The image has been rotated toward the viewer in order to dock with a 100% DOPG vesicle surface consistent with the acrylamide quenching data (Figure 4A). (C) Further rotation of the image (about 15°) toward the viewer in order to dock with 20% DOPG in DOPC vesicles consistent with the acrylamide quenching data (Figure 5B). In particular, enhanced protection of positions 24 (cyan) and 31 (blue) is observed when these are mutated to tryptophan.

protein surface and the phospholipid interface when membrane docking has occurred. Moreover, enhanced membrane-hydrolyzing activity could produce a mutant enzyme with physiological characteristics similar to those of the human group V enzyme; both enzymes also bind to heparin and HSPG. This human group V enzyme contains two conserved surface tryptophans at positions 31 and 79, one of which (W31) is on the putative interfacial surface. Mutation of this residue to alanine (W31A) but not W79 resulted in an enzyme with 40-fold reduced activity with DMPC vesicles and also reduced activity on cell membranes (*20*). Thus the presence of tryptophan in the group V enzyme does allow this extracellular enzyme to hydrolyze cell membrane phospholipid, resulting in further interactions and inflammatory effects in the case of neutrophils (*20*) and HEK293 cells (*25*).

The results described in this paper highlight that two positions with the hydrophobic collar, V3 and V31, are particularly sensitive to tryptophan insertion. The V3W and V31W mutants, and especially the V3,31W double mutant, produce enzymes with considerably enhanced abilities to hydrolyze zwitterionic vesicles and mammalian cell membranes. Moreover, this catalytic activity parallels the ability of these mutants to bind to zwitterionic vesicles, monitored either indirectly by changes in tryptophan fluorescence or directly using vesicle sedimentation assays.

Using acrylamide quenching to evaluate tryptophan exposure in the absence or presence of phospholipid vesicles highlighted changes in the exposure of the tryptophans at positions 3 and 31. This exposure of W3 and W31 was very different when 100% DOPG was used in vesicle protection assays but was much more similar with 20% DOPG in DOPC. It was not possible to use 100% DOPC in these studies due to lack of significant binding. The results for DOPG are broadly in line with the proposed interfacial orientation that has been proposed on the basis of spin label quenching studies of the labeled IIA enzyme (*47*). In that study a number of residues including V3, A19, L20, F24, F70, and Y119 were all predicted to be in contact with the interface, and the model proposed (Figure 7B) is consistent with our data for protection against acrylamide quenching in the presence of 100% DOPG. Residues V3 and L20 appear to penetrate into the interfacial region to the greatest extent while V31 is not predicted to be in contact with the interface (*47*), again consistent with our DOPG data.

This difference in protection from acrylamide quenching between DOPG and 20% DOPG in DOPC (Figure 4) suggests that the orientation of the protein at the phospholipid surface is subtly different for the two types of interface. If the orientation of the enzyme at the phospholipid interface undergoes about a 15° tilt when binding to the 80% DOPC vesicles (Figure 7C) relative to the 100% DOPG vesicles, this will bring W31 in closer contact with the phospholipid interface. A difference in alignment of the enzyme with the phospholipid interface dependent on the type of vesicle would suggest that such alignment between protein and the membrane surface is not optimum for one type of interface (or both). Interestingly, multiple charge reversal mutations result in enhanced activity on DOPG vesicles and lysozyme-permeabilized *Micrococcus luteus* (*14*). It is possible that the highly cationic nature of the IIA enzyme required to allow passage through the highly anionic bacterial cell wall results in a less than optimum alignment of the enzyme on a highly anionic membrane interface due to unfavorable electrostatic interactions, resulting in reduced specific activity. The interaction of the IIA enzyme with anionic vesicles is complex with the formation of supramolecular structures (*28*, *47*). However, the fact that enhanced catalytic activity is also seen with lysozyme-treated *M. luteus*, which presumably cannot form a supramolecular structure, suggests that the

enhanced activity with charge reversal mutants may not be a consequence of vesicle aggregation but may result from improved interfacial alignment.

The insertion of tryptophan residues at position 3 or 31 results in a 30-fold or 20-fold enhancement of activity against PC vesicles, respectively. Of particular note is the fact that the double mutant, V3,31W is over 60-fold more active than the native enzyme. The mutation of all the other nonpolar residues in the hydrophobic interfacial collar of the IIA enzyme failed to produce the dramatic rate enhancements seen with the V3W and V31W mutants. It is probable that factors such as residue exposure, orientation, and an increase in exposed hydrophobic surface area as a result of the mutagenesis to tryptophan (*48*) are important in producing enhanced binding to zwitterionic interfaces. To demonstrate unambiguously a unique role for tryptophan, it will be necessary to mutate positions 3 and 31 to other aromatic and aliphatic amino acids.

We were able to indirectly demonstrate increased binding of the V3,31W double mutant to 180 μM DOPC vesicles as a result of fluorescence changes (Table 2) while such binding could be detected for the V3W and V31W mutants when the DOPC concentration was raised to 1 mM (Table 3). The enhanced interaction of the tryptophan-containing mutants with PC vesicles was confirmed by vesicle binding studies using 100 nm sucrose-loaded vesicles containing 18 mol % PS (Table 4). It was necessary to incorporate a small percentage of PS into these vesicles to stabilize them and to enhance the relative binding of mutants to allow significant binding and hence the quantification of the relative binding affinities for the vesicle. The results demonstrate that the enhanced binding of the tryptophan mutants to such vesicles *parallels* the enhanced catalytic activity of such mutants against PC vesicles and cell membranes. These data are a clear illustration of the importance of interfacial binding of the tryptophan mutants of the IIA enzyme to phospholipid interfaces as a prerequisite for membrane hydrolysis.

The contribution of tryptophan residues to interfacial binding is more difficult to determine when the phospholipid aggregate contains anionic phospholipid, such as the sucrose-loaded vesicles described above. The overall contribution of electrostatic interactions to such interfacial binding will depend on the nature of the interfacial binding surface of the enzyme. In the case of the bee venom enzyme, charge reversal mutagenesis of five of the six cationic residues on the interfacial binding surface produced only a modest effect on interfacial binding and highlighted the importance of nonelectrostatic interactions (*49*). The present work involved the highly cationic human group IIA enzyme which has 14 cationic residues on the interfacial binding surface (*18*) and where electrostatic interactions would be expected to contribute a significant fraction of the total binding energy (the precise fraction cannot be determined from the present results) when binding to anionic vesicles. An apparently enhanced nonelectrostatic contribution to overall catalysis was seen for the V3W and V31 tryptophan mutants when they bind to anionic vesicles, but only under conditions of very high salt (2 M NaCl) where electrostatic effects are minimal (Table 1). It is with zwitterionic interfaces where the presence of an interfacial tryptophan will have the most dramatic effect on membrane binding and hence catalysis.

The comparison of the ability of the human group IIA and group V enzymes to hydrolyze cell membranes highlights that, in the case of the V3,31W double mutant, this enzyme shows catalytic properties very similar to those of the group V enzyme. The other important property of both enzymes is their ability to bind to cell surface HSPG. The linkage of such binding to enzyme internalization has been reported in the case of the IIA enzyme (*6*, *21*−*23*) and most recently with the group V enzyme (*24*). In the case of the group V enzyme, this interaction with unstimulated HEK293 cells was linked with the ability to hydrolyze cell membrane phospholipid (*25*).

If the physiological effect on cells of the group V enzyme is simply a reflection of its plasma membrane-hydrolyzing and HSPG-binding properties, then we hypothesized that the V3,31W double mutant of the IIA enzyme should have the same physiological effects. We have been able to show that, unlike the wild-type IIA enzyme, the double mutant is able to hydrolyze mammalian cell membranes (Table 5) and interacts with unstimulated HEK293 cells (Figure 6), consistent with this hypothesis. This is a dramatic gain of activity as a result of mutagenesis and highlights the importance of interfacial binding and catalysis in determining physiological function. The nature of the interactions and processing of the double mutant by these cells will be of interest.

Overall, our understanding of the molecular interactions that govern interfacial binding and activation is not complete, nor is the topological relationship between the protein and the interface although there have been important developments in this area for the group IIA enzyme (*47*). The present study highlights the fact that certain hydrophobic residues within the interfacial binding surface facilitate productive binding to zwitterionic interfaces when converted to tryptophan and provide information about the spatial alignment of the enzyme with the phospholipid interface. The ability to enhance the activity of the human group IIA enzyme by minimal (single or double) mutations suggests in evolutionary terms that the selective hydrolytic properties of the IIA enzyme have evolved to reflect physiological function. By the same argument, the properties of other mammalian enzymes such as the group V and group X enzymes must reflect a different physiological role for these enzymes. It is the interfacial binding step (and other surface properties of the enzyme) that must be dictating physiological function.

For future work it remains to be established using fluorescence microscopy if the V3,31W double mutant of the IIA enzyme targets the PC-rich perinuclear membrane region of HEK293 cells as reported for the group V enzyme (*25*) and if such targeting is linked to arachidonic release and prostaglandin synthesis. In contrast to HEK293 cells, a different physiological response to the V3,31W double mutant would be predicted for human neutrophils where the exogenously added group V enzyme acts on the outer leaflet of the cell to release fatty acids and lysophospholipid leading to group IVA (cytosolic) PLA_2 activation (*50*). However, in this case the group V enzyme is eventually degraded by these cells (*24*).

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