Ca²⁺-dependent and phospholipid-independent binding of annexin 2 and annexin 5

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Annexins are a family of homologous proteins that associate with anionic phospholipid (aPL) in the presence of Ca²⁺. Evidence that the function of one annexin type may be regulated by another was recently reported in studies investigating cytomegalovirus–aPL interactions, where the fusogenic function of annexin 2 (A2) was attenuated by annexin 5 (A5). This observation suggested that A2 may bind directly to A5. In the present study, we demonstrated this interaction. The A2–A5 complex was first detected utilizing (covalently linked) fluorescein-labelled A5 (F-A5) as a reporter group. The interaction required concentrations of Ca²⁺ in the millimolar range, had an apparent dissociation constant [*K_d*(app)] of 1 nM at 2 mM Ca²⁺ and was independent of aPL. A2 bound comparably with F-A5 pre-equilibrated with

an amount of aPL that could bind just the F-A5 or to an excess amount of aPL providing sufficient binding sites for all of F-A5 and A2. A2–A5 complex formation was corroborated in an experiment, where [¹²⁵I]A2 associated in a Ca²⁺-dependent manner with A5 coated on to polystyrene. Surface plasmon resonance was used as a third independent method to demonstrate the binding of A2 and A5 and, furthermore, supported the conclusion that the monomeric and tetrameric forms of A2 bind equivalently to A5. Together these results demonstrate an A2–A5 interaction and provide an explanation as to how A5 inhibits the previously reported A2-dependent enhancement of virus–aPL fusion.

Key words: calcium, cytomegalovirus, phospholipid.

INTRODUCTION

Annexin 2 (A2) is a member of the annexin–lipocortin family of homologous proteins [1,2]. Similar to all annexins, A2 associates with anionic phospholipid (aPL; e.g. phosphatidylserine) in a Ca²⁺-dependent manner. Most annexins, including A2, have four internal homology repeats of approx. 70 amino acids, giving a full-length protein of approx. 36 kDa [3,4]. A2 exists in either a monomeric (A2m) or tetrameric (A2t) form. The latter consists of two molecules of A2m non-covalently bridged together through the N-termini by a dimer of a member of the S100 family of proteins, p11 [5–9]. In addition to its quaternary structural properties, A2 differs from most annexins in that both A2m and A2t can facilitate aggregation of aPL-containing membranes, with A2t suggested to be capable of participating in membrane fusion [10]. Whereas the physiological function of A2 is not well understood, the known *in vitro* interactions and effects on membranes suggest strongly a role in membrane trafficking.

A previous study [11] from our laboratory demonstrated that cytomegalovirus (CMV) can exploit the known biochemical functions of A2 to enhance binding and fusion with target aPL-containing membranes. In the absence of A2, the homologous protein annexin 5 (A5) had no effect on these parameters, although it contains a Ca²⁺-dependent high-affinity-binding site for aPL. Since A5 can mediate neither aggregation nor fusion of membranes, we anticipated that the effects of A2 on CMV–membrane interactions would be inhibited by A5 through competition for aPL-binding sites. Interestingly, we observed that A2-dependent fusion, but not binding of CMV to model aPL-containing membranes, was inhibited by A5. The present

study provides an explanation for these observations by showing that A5 and A2 interact directly.

EXPERIMENTAL

Reagents

A previous study [11] showed that A5 attenuates the A2-mediated enhancement of CMV–aPL fusion. To reproduce the experimental conditions of that study, one of the sources of purified A2 used in the present study was from fresh human placenta, based on a procedure described in [12] and later modified in [11]. This source of A2 consisted of a purified mixture of A2m and A2t (A2tm) and was used originally to approximate the proportion that exists in the total cellular pool [11]. Evaluation of the final product by two-dimensional electrophoresis [13,14] and Western-blot analyses showed that A2tm contained A2m and p11 antigen. No other species were detected by silver staining the two-dimensional gels (results not shown), which have been used previously to distinguish the various types of annexins [6]. A2tm concentration was determined by a colorimetric assay (bicinchoninic acid; Pierce, Rockford, IL, U.S.A.) and the molecular mass of A2m (38.5 kDa) was used to estimate the molar concentration, as described previously [11]. In some experiments, the function of A2tm was either compared with purified human recombinant A2m (rA2m) [15] or purified bovine lung-derived A2t [16], which were prepared as described previously. Purified recombinant A5 (known to contain no other detectable type of annexin) was obtained commercially (Pharmingen, San Diego,

Abbreviations used: A2, annexin 2; A5, annexin 5; aPL, anionic phospholipid; CMV, cytomegalovirus; F-A5, fluorescein-labelled A5; LMMH, low-molecular-mass heparin; RU, response unit; SPR, surface plasmon resonance.

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CA, U.S.A.). Fluorescein-labelled A5 (F-A5) was purified to a A5/fluorescein molar ratio of 0.95:1 by Mono Q FPLC (Pharmacia, Baie d'Urfé, Quebec, Canada), as described previously [17]. Small unilamellar vesicles were produced and fractionated by ultracentrifugation as described previously [18]. A2 was iodinated ($[^{125}\text{I}]\text{A2}$) to yield a relatively low specific radioactivity of $< 70\,000$ d.p.m./ μg using the Iodogen method (Pierce) at 4°C .

Fluorescence spectroscopy

Interactions among F-A5, A2tm and aPL at 25°C were followed using an SLM 8000c fluorimeter. For binding studies, excitation was at 495 ± 8 nm with emission monitored at 520 ± 16 nm in a 1 cm path-length quartz cuvette, using a 500 nm cut-off filter, as described previously [17]. Fluorescence data were accumulated in ratio mode and the excitation shutter was kept closed during incubation periods to minimize photobleaching.

All fluorescence experiments were performed at 25°C in 50 mM Hepes (pH 7.4), 0.1 M NaCl and 0.1 mg/ml ovalbumin (HBSO; Grade VI, Sigma). To account for possible changes in light scattering due to the titration of either vesicles or A2tm, fluorescence intensity measurements were corrected for effects on the fluorescence signal observed in the absence of F-A5. Fluorescence measurements were further corrected for dilution due to continuous titration of ligand by addition of an identical volume of HBSO. Ca^{2+} -independent binding of F-A5 to aPL was defined as non-specific and was subtracted from the total signal change, as described previously [17].

The degree to which the fluorescence signal was quenched was determined from the equation:

$$\% \text{ quenched} = [(F_0 - F)/F_0] \times 100$$

where F_0 and F represent the fluorescence intensity of F-A5 before and after titration of either aPL or A2tm. In experiments where F-A5 was pre-equilibrated with aPL, F_0 is the fluorescence intensity corresponding to the F-A5–aPL complex before titration with A2tm. Corrected binding curves were fitted to a simple model that assumed a single class of binding to derive apparent dissociation constants $K_d(\text{app})$, as described in [19].

Solid-phase affinity assay

Break-away microtitre wells (Immulon II, Dynatech) were coated overnight at 4°C with $10 \mu\text{g}/\text{well}$ A5 in HBS [20 mM Hepes (pH 7.4)/150 mM NaCl] and blocked with 50 mg/ml BSA (Sigma) for 3 h at room temperature. $[^{125}\text{I}]\text{A2tm}$ was added to a final concentration of $0.3 \mu\text{M}$ in HBS, containing 0.1 mg/ml BSA in either 2 mM Ca^{2+} or 5 mM EDTA (total volume, $50 \mu\text{l}$), and allowed to equilibrate for 1.5 h at 25°C . Wells were washed three times in HBS containing either 2 mM Ca^{2+} or 5 mM EDTA and the amount of $[^{125}\text{I}]\text{A2tm}$ remaining bound was quantified.

Surface plasmon resonance (SPR)

The interaction of A5 with A2m or A2t was studied using a Biacore 1000TM biosensor system [20] (Biacore, Piscataway, NJ, U.S.A.). Between 1100 and 6000 RU (response units; $0.03\text{--}0.17$ pmol/ cm^2) of A5 were immobilized on flow cells of research-grade CM5 sensor chips (Biacore) by amine coupling at $5 \mu\text{l}/\text{min}$ (pH 5.2), as described previously [21], with some modifications. Briefly, the flow-cell surface was activated for 7 min and pre-blocked for 8 min with ethanolamine before re-activation for 8 min and binding of A5. The introduction of this preblocking step reduced the potential interference of charged groups in the carboxymethyl dextran sensor chip matrix binding to A2 or A5,

which are both known to bind anionic polysaccharides [22]. To reduce further the possibility of A5–dextran interactions, immobilization on the sensor chip was performed in the presence of low-molecular-mass heparin (LMMH, 3 kDa; Sigma) at pH 5.2 [23], which was present in the immobilization running buffer ($1 \mu\text{M}$) and was preincubated ($15 \mu\text{M}$) with A5 for 1 h at room temperature before immobilization. Running buffer and injections of A5 also contained 2 mM Ca^{2+} . To remove the heparin before injection of solution-phase A2m or A2t (analyte), the sensor chip was washed with HBS, containing 2 mM Ca^{2+} and 0.01 % (w/v) ovalbumin until no change in RU occurred due to dissociation of heparin and possibly of non-covalently bound A5. To measure A5–A2 interactions, analyte was flowed over the A5-coated (ligand) sensor chip at $20 \mu\text{l}/\text{min}$ and compared with the response obtained for identical injections over a negative-control ovalbumin-coated sensor chip. Running buffer and injections of analyte contained HBS with 2 mM Ca^{2+} and 0.01 % (w/v) ovalbumin, unless otherwise specified.

RESULTS

Intrinsic association of A5 and A2

The rationale for investigating the possibility of an interaction between A5 and A2 was from a previous study [11], where we showed that A5 attenuated A2tm-mediated enhancement of CMV–membrane fusion, but had no effect on CMV–membrane binding. To reproduce closely the conditions of our previous work, we focused primarily on following interactions of A2tm with A5. To do so, A5 was covalently modified with fluorescein (F-A5), and the fluorescence quenching observed when equilibrated with A2tm was used to follow complex formation. Figure 1(A) shows the spectra, which demonstrate that A2tm has a titratable effect on the fluorescence of F-A5. Further quenching was not observed at concentrations above 20 nM (results not shown), indicating saturation at this concentration. When rA2m was used instead of A2tm (Figure 1B), indistinguishable spectral changes were observed. Since the A2tm used in our experiments consisted of a mixture of A2m and A2t, these results suggested that A5 binds equivalently to either form.

To quantify the solution-phase association of F-A5 with A2tm, the latter was titrated (Figure 1C) and the percentage of

Figure 1 Solution interaction between A2 and F-A5

(A) Emission spectra ($\lambda_{\text{ex}} = 495$ nm) corrected for buffer baseline showing the effects of 10 nM ($\cdots$) and 20 nM ($-$) A2tm on the fluorescence of 0.44 nM F-A5 ($-$) in 2 mM Ca^{2+} . (B) Identical with (A) except that the effects of 10 nM ($\cdots$) and 20 nM ($-$) rA2m on F-A5 ($-$) fluorescence are shown. (C) F-A5 (0.44 nM) was titrated with A2 in the absence ($\bullet$) or presence ($\square$) of a 100-fold excess of unlabelled A5. The fluorescence emission change ($\lambda_{\text{ex}} = 495$ nm, $\lambda_{\text{em}} = 545$ nm) was corrected for the effects of light scattering due to A2, continuous dilution by the titrant and buffer baseline. The data were fitted to a simple rectangular hyperbolic model describing a single class of binding to derive $K_d(\text{app})$ and percentage of Quench_{max}.

Figure 2 Ca^{2+} dependence of the A2–F-A5 interaction

Corrected fluorescence emission changes ($\lambda_{\text{ex}} = 495 \text{ nm}$, $\lambda_{\text{em}} = 545 \text{ nm}$) of constant 0.44 nM F-A5 and 2.0 nM A2 were determined as a function of Ca^{2+} .

Figure 3 Radioassay demonstrating [¹²⁵I]A2–AV binding

Polystyrene microtitre plates were coated with $10 \mu\text{g}$ A5, blocked with BSA and equilibrated with $0.3 \mu\text{M}$ [¹²⁵I]A2tm, in the presence of either 2 mM Ca^{2+} or 5 mM EDTA. After removing unbound ligand by washing with either Ca^{2+} or EDTA, the [¹²⁵I]A2tm remaining bound was quantified. Hatched bars, with A5 coat; filled bars, with BSA coat but without A5 (means $\pm$ S.D.; $n = 3$).

quenching was determined. The fluorescence data were fitted to a rectangular hyperbolic model that implicitly assumed a single class of binding. $K_d(\text{app})$ derived at 2 mM Ca^{2+} and at 25°C was $1.1 \pm 0.2 \text{ nM}$, with maximal fluorescence quenching at $23 \pm 0.9\%$. To rule out the possibility that labelling A5 with FITC facilitates interaction with A2tm, an identical experiment was conducted in the presence of a 100-fold excess of unlabelled A5 over F-A5. As shown in Figure 1(C), unlabelled A5 completely eliminated the observed fluorescence quenching of F-A5, showing that the covalent modification of A5 by fluorescein was not the basis for the interaction.

Since most biochemical functions of A5 and A2 are known to require Ca^{2+} [1], the effect of Ca^{2+} on the observed interaction between F-A5 and A2tm was determined. Results presented in Figure 2 show that millimolar amounts of Ca^{2+} are necessary to facilitate the F-A5–A2 interaction. In the absence of A2tm, a significant fluorescence change of F-A5 due to the effect of Ca^{2+} was not observed (results not shown), indicating that quenching was due to protein–protein and not protein– Ca^{2+} complexes.

To corroborate the fluorescence method used to follow the intrinsic A5–A2tm interaction, a simple solid-phase radioassay was conducted. Consequent to the fluorescence experiment, [¹²⁵I]A2tm bound to A5 coated on to microtitre wells (Figure 3). Binding of [¹²⁵I]A2tm to the wells was insignificant in the absence of A5, indicating the specificity of the interaction. In agreement

Figure 4 Association of F-A5 with A2 in the presence of aPL

(A) Emission spectra showing the effect of a saturating amount of 150 nM aPL alone ($\cdots$) or combined with 20 nM A2 ($—$) on F-A5 fluorescence in 2 mM Ca^{2+} . (B) Binding of 0.44 nM F-A5 to aPL in 2 mM Ca^{2+} was followed to establish quantitatively the concentration of aPL required for saturation. (C) F-A5 was pre-equilibrated in 2 mM Ca^{2+} with an excess of aPL ($1.0 \mu\text{M}$). (D) As in (C), except that the amount of aPL used ($0.15 \mu\text{M}$) was sufficient to provide binding sites only for all of the F-A5. The fluorescence emission data were corrected and fitted as in Figure 1(C).

with the Ca^{2+} dependence observed in the fluorescence experiments, [¹²⁵I]A2tm did not bind to A5 in the presence of EDTA, indicating that Ca^{2+} was required for the A2tm–A5 interaction. [¹²⁵I]A2tm quenched the fluorescence of F-A5 to the same extent as unlabelled A2tm (results not shown), demonstrating that the iodination procedure, which included chromatographic removal of unbound iodine, did not affect the ability of A2tm to bind A5. Although this radioassay corroborated the observation of A5–A2tm complexes by fluorimetry, it was found to be insufficiently sensitive to follow binding at subsaturating concentrations of [¹²⁵I]A2tm required for derivation of $K_d(\text{app})$.

Effects of aPL on A5–A2 complex formation

Both A5 and A2 interact tightly with aPL. Therefore to determine whether aPL affects the A2–A5 interaction, changes in F-A5 fluorescence due to A2tm were evaluated in the presence of aPL. A possible complicating factor in data analysis was that aPL directly quenched F-A5 fluorescence. Consequently, the ability to distinguish the effect of aPL on F-A5 fluorescence from that of A2tm was investigated. As shown in Figure 4(A), when F-A5 was equilibrated with an excess of aPL, the maximum quenching observed was approx. 45%. A further decrease in fluorescence of approx. 20% was observed upon addition of A2tm. These results suggested that the fluorescence signal change due to the interaction of F-A5 with either aPL or A2tm was separable. Therefore to quantify the F-A5–A2tm association in the presence of aPL, F-A5 was pre-equilibrated with a saturating amount of aPL. The fluorescence intensity of F-A5 completely bound to aPL was used as a baseline for the subsequent A2tm titration. Thus the unquenched fluorescence F_0 was defined as that arising from pre-equilibrated F-A5 and aPL.

Since both A5 and A2 bind to aPL, the effect of aPL on F-A5–A2 interaction was studied under two conditions: (1) sufficient aPL to provide binding sites for both F-A5 and A2tm and (2) enough aPL to bind only the pre-equilibrated F-A5. To determine precisely the minimum concentration of aPL needed to bind all of the F-A5 in our experimental system, the association of F-A5 with aPL was followed. The results shown in Figure 4(B) demonstrate that F-A5 was completely bound when equilibrated with 90 nM aPL [$K_d(\text{app}) = 6 \pm 0.8 \text{ nM}$]. This value was used as a basis for fixing concentrations of aPL for subsequent experiments.

Table 1 Binding of A2 and aPL to F-A5

The binding isotherms presented in Figures 4(C) and 4(D) show the fluorescence signal change of F-A5 due only to addition of A2 and not due to pre-equilibration with aPL. To evaluate the contributions of protein–protein and aPL–protein interactions on the overall magnitude of the observed fluorescence signal changes, the maximal amount of quenching derived by fitting these data was examined relative to F-A5 fluorescence in the absence of ligand (Quench_{max}).

	Pre-equilibrated and titrated				
	F-A5	F-A5 + aPL	F-A5 + A2	F-A5–aPL* + A2 (1 μ M aPL)	F-A5–aPL* + A2 (0.15 μ M aPL)
K_d (app) (nM)	—	6.0 $\pm$ 0.8	1.1 $\pm$ 0.2	2.9 $\pm$ 0.9	2.6 $\pm$ 0.6
Quench _{max} (%)	0	55 $\pm$ 2	23 $\pm$ 1	88 $\pm$ 9	84 $\pm$ 6

* Pre-equilibrated complex.

To measure the interaction of F-A5 with A2tm under conditions that would provide aPL-binding sites for both proteins, an amount of aPL (1.0 μ M) in excess over that required to bind all of the F-A5 was used. When A2tm was titrated into this pre-equilibrated mixture of F-A5 and aPL, the resulting binding curve (Figure 4C) revealed a K_d (app) of 2.9 $\pm$ 0.9 nM and maximal quenching of 73.5 $\pm$ 6.5%. The affinity was nearly identical with that observed in the absence of aPL, suggesting that aPL had a negligible effect on the interaction of F-A5 with A2tm.

Since aPL causes nearly 2.5-fold more quenching of F-A5 than A2tm, we expected to observe de-quenching due to A2tm-mediated displacement of F-A5 from a limiting number of aPL-binding sites. Interestingly, this is not what was observed. When F-A5 was pre-equilibrated with aPL, which was only in slight excess over that required to bind all F-A5 (Figure 4D), a K_d (app) of 2.6 $\pm$ 0.6 nM and maximal quenching (64.5 $\pm$ 3.9%), nearly identical with that derived for the F-A5–A2tm interaction in excess aPL was derived. These results demonstrated that the availability of aPL-binding sites for A2tm is not a prerequisite for the formation of F-A5–A2tm complex. Furthermore, F-A5 is suggested to interact simultaneously with aPL and A2tm, even when insufficient aPL-binding sites are available for A2tm.

Under the various experimental conditions evaluated, the maximal amount of quenching observed cannot be compared, because the starting point of fluorescence intensity was influenced by the quenching effects of aPL. Therefore to determine whether the signal changes associated with the ternary F-A5–aPL–A2tm interaction were represented by the sum of binary interactions, the percentage of quenching was examined in relation to F-A5 fluorescence in the absence of a ligand. The resulting maximum percentage of quenching (Quench_{max}) is presented in Table 1, along with a summary of K_d (app) values derived for each equilibrium-binding isotherm. This analysis suggests that the effects of aPL and A2tm on F-A5 fluorescence are additive.

Interaction of A2t or A2m with A5

As an independent method to demonstrate A5–A2 complex formation and to corroborate further the observation suggested by fluorescence that either A2t or A2m can interact with A5, a procedure was developed using SPR. Members of the annexin family, including A5 and A2, are known to bind polysaccharides [22]. Since this property was observed to mediate direct interactions with the polysaccharide-based dextran sensor chip as anticipated (results not shown), all sensor chips were preblocked and subsequently coupled with A5 in the presence of LMMH to reduce dextran binding effectively. To account for the small

Figure 5 Demonstration of A2 binding to A5 by SPR

A5 or ovalbumin was immobilized on a sensor chip and 0.7 μ M rA2m or A2t was flowed over both surfaces. (A) Spectra showing rA2m binding to the A5 surface (—) or the ovalbumin surface (···). Net binding (—) was determined by subtraction of the data for ovalbumin from the A5-coated surface. (B) As in (A) except that A2t binding was followed. (C) Percentage net binding of rA2m (—) or A2t (···) to A5 in the absence of added Ca^{2+} relative to binding in the presence of 2 mM Ca^{2+} (defined as 100%). (D) Percentage net binding of rA2m (—) or A2t (···) to A5 in the presence of 1 μ M LMMH relative to no LMMH (defined as 100%).

amount of residual binding to dextran in these experiments, specific binding was defined as the difference between A5- and ovalbumin-coated sensor chips. Using this method, Figures 5(A) and 5(B) demonstrate a specific, reversible, interaction between rA2m or A2t with A5-coated sensor chips, corroborating our previous observations. Although an approx. 2-fold greater change in RU was observed for A2t over A2m (Figures 5A and 5B), this is indicative of equivalent molar binding due to the larger mass of A2t, and the proportionality between refractive index and size of a protein. The interaction was inhibited on preincubation of 0.32 μ M rA2m or 0.12 μ M A2t with 20 μ M A5 in solution (results not shown), demonstrating the specificity of the A2m–A5 and A2t–A5 interactions. Furthermore, the very rapid apparent dissociation rates that were observed predict that the use of methods relying on physical separation of bound and free ligand would be qualitative, as observed for our microtitre plate assay.

As demonstrated first for A2tm by the fluorescence and microtitre plate assays, the interaction of rA2m or A2t with A5 followed by SPR was Ca^{2+} -dependent and was inhibited either in the absence of Ca^{2+} addition or in the presence of 5 mM EDTA (Figure 5C). Interestingly, the specific interaction of rA2m or A2t with A5 was inhibited by LMMH addition (Figure 5D), suggesting a regulatory role for physiological heparin-like molecules. This finding also indicated that LMMH was sufficiently depleted before flowing analyte over the A5-coated sensor chip. When A5 was linked to the sensor chip in the absence of LMMH, no significant specific binding with either A2t or rA2m was observed (results not shown). Even though binding to dextran was adequately reduced by our ligand-coupling method in the presence of LMMH to enable the detection of A5-mediated binding, the affinity for residual dextran is the likely basis for sensorgrams not fitting with confidence to simple binding models (results not shown). Regardless, these results qualitatively corroborate the fluorescence and microtitre plate experiments by

providing a third independent method showing an interaction between A5 and A2.

DISCUSSION

Previous study [24] from our laboratory suggested that A2t was capable of enhancing the fusion of aPL-containing vesicles with the membrane envelope of CMV, a highly prevalent member of the herpes virus family. Fusion between the viral envelope and cell membrane is a key event in the complex pathway, leading to entry of the virus into the host cell, and must occur for CMV propagation. Of further relevance to the cell entry mechanism, A2m and A2t functioned equivalently to mediate CMV binding to a target membrane [$K_d(\text{app}) = 40 \text{ nM}$]. Since all annexins bind aPL if adequate Ca^{2+} is available, we postulated that A5, which can neither aggregate nor fuse membranes, would inhibit the CMV fusogenic and receptor functions we observed for A2 by competing for aPL-binding sites. Although we found that A5 attenuated the A2t-dependent fusion of CMV to aPL, A5 had no effect on A2-dependent CMV binding to aPL. The latter observation suggested that fusion was being inhibited by a mechanism other than simple competition for aPL-binding sites and suggested that one type of annexin can regulate the function of another type.

To account for the attenuating effect of A5 on the A2-mediated enhancement of CMV–aPL fusion, we hypothesized that a direct interaction between A2 and A5 may exist. Consistent with this hypothesis, in the present study, we used F-A5, radioiodinated A2 and A5 linked to an SPR sensor chip in three independent methods to demonstrate that A5 and A2 indeed form a specific Ca^{2+} -dependent complex. We observed the same extent of interaction regardless of whether a mixture of A2t and A2m (A2tm), approximating the cellular ratio, or purified A2m or A2t was used. This showed that binding of A5–A2 is probably independent of the p11 moiety contained within A2t.

The fluorescence quenching used in the present study to demonstrate first the association of A2 with A5 originated from a previous study [17], in which quenching of F-A5 fluorescence was used to follow the A5–aPL interaction. In the present study, we found that A2 also resulted in quenching of F-A5 fluorescence, which provided a means for quantifying the F-A5–A2 interaction. Despite the documented effect of aPL on F-A5 fluorescence, the F-A5–A2 interaction was quantifiable by this method even in the presence of aPL. Evaluation of the $\text{Quench}_{\text{max}}$ for each of these interactions (Table 1) shows that the sum of values derived for the binary interactions (F-A5 + aPL and F-A5 + A2) of $78 \pm 3\%$ was equal to those experimentally derived for the ternary F-A5–A2–aPL complex (i.e. 88 ± 9 or $84 \pm 6\%$ for 0.15 or 1.0 μM aPL respectively). Therefore the reason that spectral detection of the F-A5–A2 interaction was not obscured in our experiments by the F-A5–aPL interaction is because the binary equilibria appear to have isolated effects on the fluorescent reporter group. The observation that two distinct events are measurable by changes to the environment of a single probe may suggest that proximal functional regions exist within A5. Although compelling, an unambiguous conclusion cannot be drawn at this time, since it is not known if F-A5 is composed of two (or more) populations of F-A5 differing by the location of the single site modified by fluorescein.

Due to avidity effects, we predicted that the concurrent interactions of A5 and A2 with the same aPL-containing membrane would strengthen the apparent affinity between A5 and A2. However, the $K_d(\text{app}) \sim 2 \text{ nM}$ (at 2 mM Ca^{2+}) was not significantly affected by the presence of excess aPL. To provide an explanation, we followed the binding of A2–F-A5, after the

latter had been equilibrated with a limiting amount of aPL. In this experiment, a sufficient amount of aPL was available to bind only the F-A5. Since aPL-dependent quenching of F-A5 is approx. 2.5 times greater than that of A2, dequenching would be expected at high concentrations of A2 if competitive displacement of F-A5 from aPL-binding sites occurred. As shown in Table 1, the amount of quenching was the same regardless of whether the amount of aPL was sufficient only for A5 binding, or for both A5 and A2 binding. This observation suggests that A2 does not reverse the preformed F-A5–aPL complex. Also summarized in Table 1 is the finding that the $K_d(\text{app})$ value for F-A5 binding to A2 is independent of the amount of aPL. To be consistent with these cumulative data, we favour a model in which A5 functions as a membrane-bound receptor for A2. This model does not exclude the possibility that A2 may still interact with aPL. However, to account for the apparently insignificant reversal of A5 binding to a limiting concentration of aPL by A2 and the lack of predicted avidity effects of aPL on the $K_d(\text{app})$ values of A2–A5 the membrane to which A2 is bound would have to be on a different vesicle than the cognate A5. Furthermore, we cannot exclude the less feasible possibility that A2 promotes the dissociation of F-A5 from aPL thereby bridging the F-A5 to aPL, but somehow simultaneously stabilizes the aPL-induced F-A5 conformation that causes fluorescein quenching. Regardless, aPL does not significantly affect the affinity between A5 and A2.

Bridging of aPL to A2 by A5 provides an explanation for our finding that A5 attenuates the A2-mediated enhancement of fusion, but not the binding of CMV to aPL. In this scenario, A2-mediated enhancement of virus binding to a target membrane would persist in the presence of excess A5 because of the A5–A2 interaction. The inter-annexin complex, however, is envisaged to change the geometry of A2 relative to the aPL surface and consequently affect subsequent participation in fusion between the virus envelope and target membrane. This model suggests the intriguing possibility that A5, which exists on the surface of cells [25], may provide natural host resistance to infection.

In addition to providing corroborating evidence for equivalent Ca^{2+} -dependent binding of A5 to A2m or A2t, the SPR experiments performed in the present study demonstrated that heparin inhibits these interactions. Binding of A2m, A2t and A5 to heparin has been shown previously [22]. Combined with the consensus that interactions with heparin-like structures on the cell surface (heparan sulphate proteoglycan) are essential for CMV host cell entry [26], our findings suggest that the virus may have evolved to infect cells with heparan sulphate proteoglycan because these may intrinsically inhibit the association of A2 with A5. In this way, the enhancing effects of A2 on infection can be exploited by the virus. Direct experiments are yet to be conducted to test this hypothesis.

All annexin family members bind multiple Ca^{2+} ions to express function, including binding to aPL. Crystal structures of A5 [27,28] and A2 [29] have revealed two classes of Ca^{2+} -binding sites in each, type II and type III, which are distinct from EF-hand type I sites. For A2, site-directed mutagenesis has shown that the affinity for type II Ca^{2+} -binding ($K_d \sim 10 \text{ nM}$) is at least an order of magnitude stronger than that of the type III sites [30]. Similarities between the crystal structure of A2 and A5 [27–29] and domain-shuttling experiments [31] have suggested analogous differences between the type II and type III binding sites within A5. In the present study, we found that the A2–A5 interaction required concentrations of Ca^{2+} in excess of 1 mM. These results suggested that at least the low-affinity type III Ca^{2+} sites must be occupied and that intracellular levels of Ca^{2+} are insufficient to favour formation of the interaction. The additional involvement of higher-affinity Ca^{2+} -binding sites cannot be excluded at this

time. Nevertheless, the results suggested that the proposed regulation of A2 function by A5 could only occur in the extracellular milieu, where the concentration of Ca^{2+} is sufficiently high to facilitate the binding of A5 and A2.

Although annexin family members are ubiquitous and highly conserved among evolutionarily distinct species, their specific physiological function(s) have been difficult to define. Part of the reason is that all cell types evaluated until now express several annexin types, and evidence is emerging that these have elaborate interplay. As examples, self-associations of A5 [32], A2 [33] and annexin 12 [34] have been documented. Of these, a functional consequence for multimerization of only annexin 12 in membrane ion-channel formation has been reported [35]. Heterologous annexin interactions have also been shown between A5 and annexin I [36] and indirectly between A2 and annexin 4 [33], and annexin 4 with either annexin 7 or annexin 6 [37]. An effect on membrane aggregation was reported for annexin 4 with either annexin 7 or annexin 6 [37]. The rationale for the present study was based on the observed functional effects of A5 on A2 in aPL-membrane fusion. Our identification of specific high-affinity A5–A2 binding thus adds more complexity to our understanding of the *in vivo* function of annexins by further indicating that heterologous annexin interactions may be regulatory.

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