Annexin 2-mediated enhancement of cytomegalovirus infection opposes inhibition by annexin 1 or annexin 5

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Biochemical studies have suggested that annexin 2 (A2) may participate in cytomegalovirus (CMV) infection. In the current work, effects of A2 monomer (p36) and heterotetramer (A2t; p36 2 p11 2 ) were investigated. Demonstrating a role for endogenous A2, the four stages of infection that were followed were each inhibited by anti-p36 or anti-p11 at 37 °C. Immuno-inhibition was attenuated when the virus and cells were pre-incubated at 4 °C to coordinate virus entry initiated afterwards at 37 °C, reconciling controversy in the literature. As an explanation, CMV-induced phosphorylation of p36 was prevented by the 4 °C treatment. Supporting these immuno-inhibition data, purified A2t or p11 increased CMV infectious-progeny generation and CMV gene expression. A specific role for A2t was indicated by purified p36 having no effect. Unlike other steps, primary plaque formation was not enhanced by purified A2t or p11, possibly because of undetectable phosphorylation. As annexins 1 (A1) and 5 (A5) interact with A2, their effect on CMV was also tested. Both purified proteins inhibited CMV infection. In each experiment, the concentration of A1 required for half-maximal inhibition was five- to 10-fold lower than that of A5. Addition of A2 opposed A1- or A5-mediated inhibition of CMV, as did certain A2-specific antibodies that had no effect in the absence of added A1 or A5. Transfection of the p36-deficient cell line HepG2 increased CMV infection and was required for inhibition by the other annexins. These data suggest that CMV exploits A2t at physiological temperature to oppose the protection of cells conferred by A1 or A5.

INTRODUCTION

The current model for cytomegalovirus (CMV) entry into the host cell is a sequential, multistep mechanism (Compton et al., 1993; Kari & Gehrz, 1992, 1993; Nowlin et al., 1991). Formation of the initial weak interaction has been suggested to prime the cell for high-affinity attachment required for subsequent fusion of the virus envelope with the cell membrane (Compton et al., 1993). Only recently have the well-known transmembrane cell-signalling mediators epidermal growth factor receptor (Wang et al., 2003) and several integrin family members (Feire et al., 2004) been implicated in the priming step. In addition to these, a controversial role has emerged for annexin 2 (A2) (Esclatine et al., 2001; Pietropaolo & Compton, 1999; Raynor et al., 1999; Wright et al., 1994, 1995).

A2 belongs to the ubiquitous annexin family of proteins, characterized functionally by calcium (Ca2+) -dependent reversible binding to membranes containing anionic phospholipid (aPL). A2 exists in at least two configurations: a 36 kDa monomer (p36), the structure typical of annexins, and a heterotetramer (A2t), which consists of two p36 subunits bridged non-covalently by a dimer of p11, an S100 protein family member (S100A10) (Glenney, 1986). Both p36 and A2t can aggregate aPL-containing membranes (Blackwood & Ernst, 1990; Liu et al., 1997), but A2t has also been implicated in membrane fusion (Chattopadhyay et al., 2003; Harder & Gerke, 1993; König et al., 1998), providing a biochemical rationale for the latter in CMV infection.

A2 was first implicated in CMV infection when p36 derived from the host-cell surface was shown to associate with intact CMV (Wright et al., 1994). Soon after, a polyclonal anti-A2 serum was shown to inhibit CMV plaque formation partially (Wright et al., 1995). Further studies demonstrated that purified p36 interacts specifically with CMV (Wright et al.,
and that gB on the surface of CMV may have evolved to bind host cell-derived p36 on the purified virus surface (Bold et al., 1996; Pietropaolo & Compton, 1997). Pointing to A2t as being the likely form involved, Raynor et al. (1999) showed that purified A2t accelerated the fusion of well-characterized model membranes with the CMV envelope at concentrations several orders of magnitude lower than that of p36. The finding of both p36 and p11 on the host-cell infection, a later study using a polyclonal antibody specific for p36, a commercial mAb for p11 and purified recombinant p36 concluded that A2 has no direct effect on plaque assays that first involved a 4°C binding step to coordinate subsequent virus cell entry at 37°C (Pietropaolo & Compton, 1999). Using an in-house polyclonal anti-p36 and the same monoclonal anti-p11 antibodies, a subsequent study also concluded that A2 is not essential for entry of CMV into an intestinal epithelial-cell line (Esclatine et al., 2001), further swaying the controversy toward an insignificant effect of A2 on CMV infection.

The physiological function of A2 is still not resolved, possibly because the mechanisms implied by in vitro experiments are more complicated than postulated originally. Rather than A2 location within the cell being static, vascular cell modulators have been shown to initiate the translocation of intracellular A2 to the cell surface (Peterson et al., 2003). This event is preceded by stimulus-induced phosphorylation of p36 (Deora et al., 2004). When accessible on the cell, A2 can furthermore participate directly in cell signalling (Deora et al., 2004; Laumonnier et al., 2006). Thus, the postulated physiological involvement of A2 in signal-mediated membrane trafficking and secretion is a dynamic process that depends on membrane fluidity. A role for A2 in CMV infection must therefore be investigated entirely at physiological temperature, which may help to explain the discrepant literature (Pietropaolo & Compton, 1999).

Opposite to A2, annexin 5 (A5) has been observed to inhibit membrane aggregation and fusion (Andree et al., 1993; Köhler et al., 1997; Oshry et al., 1991; Raynor et al., 1999; Rosales & Ernst, 1997). Suggesting a possible role in host resistance to CMV infection, A5 was shown to inhibit A2-dependent fusion of the CMV envelope with model phospholipid membranes (Raynor et al., 1999). This observation implied an A2–A5 interaction, which was later demonstrated by direct physical methods (Brooks et al., 2002). Similar to A5, annexin 1 (A1) can associate with A2 (Lee et al., 1999) and may have a comparable effect to A5 on A2 in CMV infection events, although no connection to CMV infection has been reported.

In view of the recent literature, the current study was conducted entirely at 37°C to re-evaluate the role of A2t, p11 and p36 at several steps within the CMV infection cycle.

Immuno-inhibition, purified proteins and cell-transfection experiments support the conclusions that A2 indeed enhances CMV production and that A1 and A5 attenuate this effect directly.

**METHODS**

**Materials.** Human foreskin fibroblasts (HFFs) were obtained from the ATCC (CRL-2056) and grown in basal medium Eagle containing 5% bovine calf serum, 14 μM L-glutamine and 1 U gentamicin ml⁻¹ (BME). Native HepG2 cells were obtained from the ATCC (HB-8065) and grown in minimum essential medium containing 10% fetal calf serum, 14 μM L-glutamine and 1 U gentamicin ml⁻¹ (MEM). HepG2 cells transfected stably with p36, a kind gift from Dr Alain Puisieux (Université Claude Bernard, Lyon, France), were grown and maintained in MEM containing 0.4 mg geneticin ml⁻¹. CMV (AD169 strain; Advanced Biotechnologies Inc.) was propagated in HFFs, purified by tartrate/glycerol ultracentrifugation and quantified by electron microscopy (Sutherland et al., 1997) and plaque assay (Wright et al., 1995) as described previously.

Purified A2t and p11, derived from bovine lung, and recombinant human p36 were prepared and characterized by well-established methods (Kang et al., 1997; Khanna et al., 1999). Purified recombinant A5 was obtained from Pharmingen. Purified A1 was prepared from human placenta as described previously for A2 (Raynor et al., 1999) with an additional immuno-affinity purification step. A1 was eluted from an in-house anti-A1 column by using 3 M KSCN and exchanged into HBS (20 mM HEPES, 150 mM NaCl, pH 7.4) by using a 10 kDa cutoff Centricon filter. The resulting A1 and all annexins were >95% pure, evaluated by Coomassie blue staining following SDS-PAGE. The A1 had no reactivity against anti-A2. The function of A2t, p36, A1 and A5 was evaluated by determining their Ca²⁺-dependent binding to sucrose-dense aPL vesicles as described previously (Zeibdawi et al., 2004). Each purified annexin bound completely to an excess of vesicles in the presence of Ca²⁺ (not shown).

**Antibodies.** Rabbit polyclonal anti-p11, directed against residues 21–38, was purified and produced as reported previously (Choi et al., 1998; Peterson et al., 2003). Rabbit polyclonal anti-p36, specific to a peptide corresponding to residues 9–39, was obtained from BioDesign. Mouse mAbs directed against the following antigens were obtained commercially: p36 (Transduction Laboratories; Oncogene or Zymed); p11 (Transduction Laboratories); CMV immediate-early 72 (IE72) (Accurate Scientific); anti-f-actin, anti-phosphoserine, anti-phosphothreonine, anti-phosphotyrosine and isotype-control non-immune mouse IgG and rabbit IgG (Sigma). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG was obtained from Jackson Immunologicals.

**Inoculations.** For all infection assays, HFFs were grown to approximately 80% confluence. In antibody-inhibition assays, cells were pre-washed twice in serum-free BME supplemented with 1 mg BSA ml⁻¹ (BME/BSA) and 20 mM EGTA (Hajjar et al., 1996) and washed twice in BME/BSA alone. In purified protein assays, cells were pre-washed twice in BME/BSA containing 2 mM Ca²⁺ followed by BME/BSA alone. Both cells and virus were pre-incubated separately with antibody or protein for 60 min at 37 or 4°C. Cells were then inoculated with CMV in the presence of antibody or purified protein for 90 min at 37°C or, in some cases, at 4°C as indicated; they were then washed and BME was added. In HepG2 experiments, inoculations were performed in MEM containing 1 mg BSA ml⁻¹ and 2 mM Ca²⁺, using CMV-infected HFF supernatant (Pryzdial & Wright, 1994) as the inoculum.

**Primary infection measured by IE72 assays.** Early infection events leading up to and including production of the first CMV
gene product were evaluated by Western blotting for IE72 antigen (an m.o.i. of 0.003 was selected to be within a range that we determined to be proportional to virus titre; data not shown). At 20 h post-infection, the cells were lysed in Laemmli sample buffer [0.5 M Tris/HCl (pH 6.8), 2 % SDS, 10 % glycerol, 12.5 mg dithiothreitol ml⁻¹], boiled for 10 min and resolved by SDS-PAGE for Western blot analysis.

**Primary infection measured by plaque assays.** Infection events up to CMV intracellular virion assembly and spread were measured by changes in plaque number (Wright et al., 1995). Following inoculation (0.0003 m.o.i.), infection was allowed to proceed, with medium replacement on days 3 and 5 post-infection. Plaques were counted on day 7.

**Secondary infection measured by IE72 assays.** CMV entry, replication, egress and progeny viability up to synthesis of IE72 were measured by inoculating fresh cells with the medium of primary infected cells. Following primary inoculation, infection was allowed to proceed, with medium replacement on days 3 and 5. On day 10 post-infection, supernatants from infected cells were collected and used for secondary inoculation of fresh HFF monolayers and then assayed for IE72 antigen, as above.

**Secondary infection measured by plaque assays.** CMV progeny release into infected-cell supernatants was also measured by plaque assay as above.

**Western blot analysis.** Following SDS-PAGE, proteins were electrotransferred to a PVDF membrane and blocked with 5 % skimmed milk powder in Tris-buffered saline [0.05 M Tris/HCl, 150 mM NaCl (pH 8)] containing 0.05 % (v/v) Tween 20 (TBST) for 60 min. Membranes were incubated with primary antibodies in TBST containing 5 % skimmed milk for an additional 60 min, washed in TBST and incubated with HRP-conjugated goat anti-mouse antibody in TBST for 30 min. After further washing, IE72 antigen was detected by using the enhanced chemiluminescence method (Amersham Biosciences). PVDF membranes were subsequently reprobed for β-actin as a sample-loading control. IE72 band intensity was quantified and normalized to β-actin intensity by using Northern Eclipse imaging software (Empix).

**Immunoprecipitation.** Immunoprecipitation was used to identify changes in phosphorylation of p36 during CMV inoculation. Immediately prior to infection, HFFs grown in six-well plates were fed with fresh BME at either 37 or 4 °C for 5 min, followed by inoculation with CMV or mock treatment for 90 min at the same temperature. Cells were then washed and replaced with fresh BME/BSA at 37 or 4 °C for 30 min. After removing the medium, the cells were lysed with 500 μl RIPA buffer (10 mM Tris, 100 mM NaCl, 1 mM EDTA, 0.5 % sodium deoxycholate, 10 % SDS and 1 % Triton X) and centrifuged at 12,000 g for 5 min. The supernatant was precleared by incubation with protein G-Sepharose (20 μl; Invitrogen) and non-immune mouse IgG (2 μg) for 60 min at room temperature with constant agitation. Anti-p36 mAb (1 μg; Zymed) was preadsorbed onto protein G-Sepharose (20 μl) in RIPA buffer (100 μl) for 60 min at room temperature with constant agitation and combined with pre-cleared supernatant (150 μl) at 4 °C overnight. Following incubation, the beads were washed twice with RIPA buffer and PBS, and subjected to SDS-PAGE under reducing conditions. Western blot analysis was conducted to evaluate phosphorylation of p36 in immunoprecipitated lysates and purified A2t and p36.

**RESULTS**

### Endogenous A2 enhances CMV infection

To investigate the role of endogenous A2, the effect of anti-p36 or anti-p11 on CMV primary infection and generation of secondary infectious progeny was evaluated by IE72 and plaque assays. Fig. 1(a) shows that generation of IE72 during experiments evaluating primary or secondary infection is inhibited by approximately 95 % by antibodies to either p36 or p11. Non-immune rabbit IgG, as well as anti-p36 and anti-p11 that had been pre-adsorbed with A2t, had no significant effect on IE72 expression (Fig. 1b), indicating that the inhibition by anti-p36 and anti-p11 antibodies was specific. The IE72 Western blot band-intensity differences that were quantified are illustrated in Fig. 1(c), corresponding to one of the replicates used to generate the IE72 band intensities shown in Fig. 1(b).

Corroborating the IE72 expression data, Fig. 1(d) shows that primary and secondary plaque formation was also inhibited by anti-p36 or anti-p11. At a maximum of 70 %, the extent of plaque inhibition was somewhat lower than that of inhibition of IE72 expression. As in the IE72 assay, none of non-immune rabbit IgG, A2t-adsorbed anti-p36 or anti-p11 had a significant effect on plaque generation (Fig. 1e), demonstrating the antigenic specificity of inhibition. Combined with the IE72 data, these immunoinhibition experiments suggest that endogenous p36 and p11, presumably associated as A2t, enhance productive cell infection by CMV.

Consistent with the hypothesis that A2 function in CMV infection may be dependent on physiological membrane fluidity, all of our experiments were conducted at 37 °C. To help to reconcile discrepancies between our current findings and a previous report (Pietropaolo & Compton, 1999), we determined whether a 4 °C step during inoculation would affect our immuno-inhibition observations. Fig. 1(d) (inset) shows that lowering the initial inoculation temperature to 4 °C attenuated the inhibitory effects of anti-p36 or anti-p11 that were observed when the entire experiment was carried out at physiological temperature.

### Purified A2t and p11 enhance CMV infection

To confirm the immuno-inhibition data showing that endogenous A2 enhances CMV infection and to clarify which form(s) of A2 may be involved, purified A2t, p11 or p36 was added. Supporting the immuno-inhibition experiments, either purified A2t or p11 enhanced either primary or secondary IE72 expression by approximately three- to fivefold (Fig. 2a), presumably by filling unoccupied cellular binding sites. In contrast, p36 had no significant enhancing effect, although dose-dependent binding of [³²P]p36 to HFFs was observed in separate experiments (data not shown) and has been reported previously (Hajjar et al., 1996).
The effect of purified proteins on IE72 expression was corroborated by following plaques formed by CMV released into the medium after primary infection of cells. Purified A2t and p11 were both observed to enhance the number of two-step plaques by approximately sevenfold (Fig. 2b, right panel). Again, added p36 had no significant enhancing effect, demonstrating the specificity of the enhancement by A2t or p11. In striking contrast to the three other stages of infection that we assayed, purified A2t and p11 did not affect primary plaque formation (Fig. 2b, left panel). The fact that this step of infection was immuno-inhibited by anti-p36 or anti-p11 suggests a functional difference between purified and endogenous A2.

Phosphorylation has been shown previously to modulate the function of A2, including its ability to mediate phospholipid fusion (Chattopadhyay et al., 2003; Harder & Gerke, 1993; König et al., 1998). To provide an explanation for primary plaque formation not being enhanced by purified A2t or p11, we compared the extent of phosphorylation of purified A2 and cellular A2. Fig. 2(b) (inset) shows that neither the purified p36 (lane 1) nor A2t (lane 2) used in our experiments had detectable phospho-Tyr, -Ser or -Thr. However, A2 immunoprecipitated from cells inoculated with CMV at 37°C (lane 6) was found by Western blotting to be phosphorylated extensively, predominantly at Ser and Thr and to a lesser extent at Tyr. This difference provides a possible explanation for the lack of effect of purified A2 on plaque formation. Interestingly, when A2 phosphorylation was evaluated in mock-inoculated cells at 37°C (lane 4), phosphorylated A2 could not be detected. As this finding implicates virus-mediated signalling in A2 phosphorylation, we investigated whether the phosphorylation of A2 was altered when virus infection was preceded by a 4°C binding step. The data showed that A2 phosphorylation is attenuated significantly by low temperature (lane 5), correlating to our finding that cellular A2 had no effect on infection under
these conditions. At this time, we do not know whether monomeric or tetrameric A2 is the predominant form of p36 being phosphorylated.

**A1 or A5 inhibits CMV infection**

To determine whether A1 or A5 has an effect on overall CMV replication, IE72 expression and plaque formation were followed after primary and secondary CMV infection of HFFs. Fig. 3 shows that each step of infection that we assayed was inhibited by approximately 50% by either purified A1 or A5. In each case, A1 was effective at lower concentrations than was A5. No additional inhibition was observed when saturating concentrations of A1 and A5 were combined (not shown). Taken together, these results demonstrate that the addition of either purified A1 or A5 partially inhibits CMV infection of host HFFs and suggest a common point in the infection mechanism.

**Purified A2 opposes A5- or A1-mediated CMV inhibition**

Because A1 and A5 inhibit the same step(s) of CMV infection enhanced by endogenous A2t, we investigated whether A5- and A1-mediated inhibition of CMV infection involved A2. The first approach was to pre-incubate purified A1 or A5 with A2t, p11 or p36 prior to inoculation. As shown in Fig. 2(b) (left panel), purified p36 (1) and A2t (2) or p36 immunoprecipitated from mock-inoculated HFFs (3 and 4) or HFFs inoculated with CMV (5 and 6) at either 4°C (3 and 5) or 37°C (4 and 6) was evaluated for phosphotyrosine (Tyr), phosphoserine (Ser) or phosphothreonine (Thr) by Western analysis. The location of purified p36 determined by Coomassie blue staining (not shown) is indicated by arrows.

**Fig. 2.** Purified A2t or p11 enhances CMV infection. HFFs were inoculated with CMV in the presence or absence of purified A2t (○), p11 (■) or p36 (□). (a) CMV IE72 expression after primary and secondary inoculation was measured by Western blotting as described for Fig. 1(a–c). Each point is the mean±SD of two individual experiments, each consisting of two points; P<0.05 (compared with mock-treated sample). (b) As described for (a), except that plaque assays after primary and secondary inoculation were conducted according to Fig. 1(d, e). Each point is the mean±SD of two individual experiments, each comprising two separate points; P<0.05 (compared with mock-treated sample).

**Fig. 3.** Purified A1 or A5 inhibits CMV infection. HFFs were inoculated with CMV as described for Fig. 2, except in the presence of purified A5 (○) or A1 (●) rather than A2. Primary and secondary (a) IE72 expression and (b) plaque formation were measured as described for Fig. 2. Each point is the mean±SD of two individual experiments, each comprising two separate points; P<0.05 (compared with mock-treated sample).
of A1 (100 nM) was sufficient to restore the expected extent of inhibition fully. These data suggest that inhibition of CMV infection by A1 or A5 is a consequence of reducing the availability of accessible A2 and that interaction between A1 or A5 and A2 has similar functional effects.

**Inhibitory anti-A2 combines with A1 or A5 to attenuate CMV infection partially**

To evaluate further the role of A2 in A5- and A1-mediated inhibition of CMV infection, A1 or A5 was combined with inhibitory anti-p36 or anti-p11. Fig. 5 shows that the presence of these anti-A2 antibodies did not further the extent of inhibition at saturation. At non-saturating concentrations of antibody, their inhibitory effects were combined with that of the purified A1 or A5 added. Non-immune rabbit IgG alone had no effect on infection, nor did it alter A1 or A5 activity (data not shown). These observations suggest that A5, A1 and inhibitory anti-A2 each target a similar CMV infection pathway for inhibition, further supporting the possibility that purified A1 and A5 inhibit infection by opposing endogenous A2 function.

**Non-inhibitory A2-specific antibodies oppose A5- or A1-mediated CMV attenuation**

While evaluating antibodies to p36 or p11 for effects on CMV infection, in addition to the two that we observed to be inhibitory (Fig. 1), four were identified that had no significant effect when added alone during inoculation (Fig. 6a–d). We investigated whether, in the presence of purified A1 or A5, the addition of these ‘non-inhibitory’ A2 antibodies may influence infection. Interestingly, A5-mediated inhibition was found to be attenuated by Transduction Laboratories anti-p36 (Fig. 6a), Zymed anti-p11 (Fig. 6b), Oncogene anti-p36 (Fig. 6c), and Transduction Laboratories anti-p11 (Fig. 6d).
anti-p36 (Fig. 6b) and Oncogene anti-p36 (Fig. 6c), but was unaffected by Transduction Laboratories anti-p11 (Fig. 6d). A1-mediated inhibition was altered by a different profile of anti-A2 antibodies: neither Transduction Laboratories anti-p36 (Fig. 6a) nor Oncogen anti-p36 (Fig. 6c) had a significant effect, whereas both Zymed anti-p36 (Fig. 6b) and Transduction Laboratories anti-p11 (Fig. 6d) reversed A1-mediated inhibition. Non-immune mouse IgG isotype-matched controls had an insignificant effect alone or in the presence of A1 or A5 (data not shown), indicating that the reversing effect of these anti-A2 mAbs was specific. As the A5- and A1-mediated inhibition could be abrogated by the addition of select anti-A2 antibodies, which alone had no effect on CMV plaque formation, this suggests further that A1 and A5 inhibition is dependent upon accessibility of endogenous A2.

**p36 transfection of HepG2 cells enhances infection of CMV and inhibition by A1 or A5**

HepG2 cells are the only known cell line that does not express measurable p36 mRNA or p36 antigen (Puisieux et al., 1996). Although neither native nor p36-transfected HepG2 cells enabled CMV plaque formation (data not shown), both were permissive to CMV entry, leading to IE72 expression. Therefore, IE72 assays were conducted after primary inoculation to determine whether the availability of cellular p36 enhances early events in CMV infection. To re-establish that the transfected HepG2 cells (+) obtained as a generous gift from Dr Alain Puisieux (Université Claude Bernard, Lyon, France) produced p36, Fig. 7(a) shows that p36 antigen was present in total extracts of only the transfected line (B) was not detected in the eluate, indicating that the cells were not permeabilized. Both transfected and parental HepG2 cells were observed to have an abundant source of A5 and neither expressed detectable A1 (Fig. 7a). p36, A1 and A5 antigens were all eluted from the surface of HFFs by chelation (not shown).

Consistent with the immuno-inhibition and purified annexin experiments, expression of p36 enhanced CMV IE72 production, especially at low m.o.i. (Fig. 7b). Furthermore, p36 expression was required for A5- and A1-mediated inhibition of CMV IE72 production by HepG2 cells (Fig. 7c). These observations support the conclusion that endogenous cellular A2 enhances an early step in the CMV infection mechanism and that A1 and A5 protect cells from CMV infection by effects on A2 function.

**DISCUSSION**

In the current study, we hypothesized that predominantly A2t is involved in CMV infection and that cell modulation facilitated at physiological temperature is required. This premise was based on fusogenic properties of A2t that are not observed for p36 (Blackwood & Ernst, 1990), translocation of intracellular A2t to the outer surface of the cell by stimulus induction (Jacovina et al., 2001; Peterson et al., 2003) and phosphorylation of A2 being required for translocation (Deora et al., 2004). Each factor is strictly dependent on membrane fluidity. Our experiments were consequently designed to follow the effects of A2t, p11 and p36 exclusively at 37 °C, differing from the earlier work that focused primarily on p36 and included a pre-incubation of CMV with cells at 4 °C to coordinate subsequent virus entry at 37 °C (Pietropaolo & Compton, 1999).

To investigate the involvement of endogenous cellular A2, immuno-inhibition experiments were conducted by using anti-p36 that recognizes both A2t and monomeric p36, and anti-p11 that recognizes A2t, but not p36. We also followed
the effects of adding purified A2t, p11 and p36. Previous work has demonstrated that A2-binding sites on cells are not saturated (Hajjar et al., 1996) and that adding p11 may form A2t with existing p36. In support of the hypothesis, anti-p36 or anti-p11 antibodies were identified that inhibited each step of infection that we followed, and purified A2t and p11 enhanced progeny formation. Like the earlier work (Pietropaolo & Compton, 1999), we found that purified p36 was insignificant at any stage of infection and the inhibitory anti-A2 antibodies that we used were attenuated if the 4°C coordinating step was included in primary plaque-formation assays. Also consistent with the earlier studies (Esclatine et al., 2001; Pietropaolo & Compton, 1999), we observed no effect using the same source of commercial anti-p11 (Transduction Laboratories), even at 37 °C. To begin to address the possibility that altered cell signalling occurs when virus entry is coordinated at 4 °C, we followed A2 phosphorylation. We observed that CMV induces the rapid phosphorylation of cellular A2 at 37 °C, but not if the 4 °C step is included. These findings reconcile the literature discrepancy by suggesting that predominantly A2t participates in CMV infection through discrete epitopes and that physiological temperature facilitates the functional effects of phosphorylation.

In the current work, all stages of infection that we measured were inhibited specifically by antibodies against p36 or p11, supporting a role for endogenous cellular or viral A2t. Similarly, three of four stages that we measured were enhanced by purified A2t or p11. However, purified A2t or p11 did not affect primary plaque formation, suggesting a functional distinction between purified and endogenous sources of A2. One possible explanation may be differences in phosphorylation, supported by finding that the purified protein was not phosphorylated. Thus, the functional distinction that we noted between purified and endogenous A2 only for primary plaque formation suggests that A2 may participate at more than one step of the infection pathway.

Several lines of evidence presented here indicate that A1 and A5 inhibit CMV infection by interfering with A2-enhanced events. (i) The individual effects of A1 and A5 could be prevented by purified A2t. (ii) In the presence of anti-p36 or anti-p11 antibodies, which alone were inhibitory, no additional inhibition by A1 or A5 was observed at saturation, implying a common target in the infection pathway. (iii) Several anti-p36 or anti-p11 antibodies, which alone had no effect, blocked the inhibition of CMV infection by either A1 or A5, implying that the antibodies prevented A1 and A5 interaction with A2. (iv) A1 and A5 had no effect on CMV IE72 production in HepG2, a p36-deficient cell line that expresses p11. However, when HepG2 cells were transfected with p36, not only was CMV IE72 expression enhanced, but both A1 and A5 became inhibitory. Together, these observations indicate that A1 and A5 inhibit infection by interfering with cellular A2t function.

Notable differences between the protective effects of A1 and A5 on CMV infection were found. (i) Five- to 10-fold lower concentrations of A1 than A5 were required for CMV inhibition. (ii) Whilst A5- or A1-mediated CMV inhibition was inhibited by purified A2t, only A1 was also affected by purified p11 or p36. (iii) A several-fold lower molar ratio of A2t was required to restore infection in the presence of A5 than in the presence of A1. (iv) Different mAbs to p36 uniquely inhibited either A1 or A5, but not both. Despite similar consequences on infection, these observations suggest that the contact sites on A2t for A1 and A5 are not the same.

The opposing role of A1 and A5 on A2 function shown here further complicates our understanding of individual annexins by implying that networking of annexins may regulate overall function. Combined with prior reports that A2t can be induced to the cell surface (Deora et al., 2004; Jacovina et al., 2001; Peterson et al., 2003), these data suggest that the susceptibility to CMV infection may depend largely on host-cell modulation. Whilst A2 does not appear obligate for CMV infection of cultured cells, the several-fold advantage that is conferred may be critical in vivo to oppose resistance to infection by A5, A1 and other mechanisms.

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